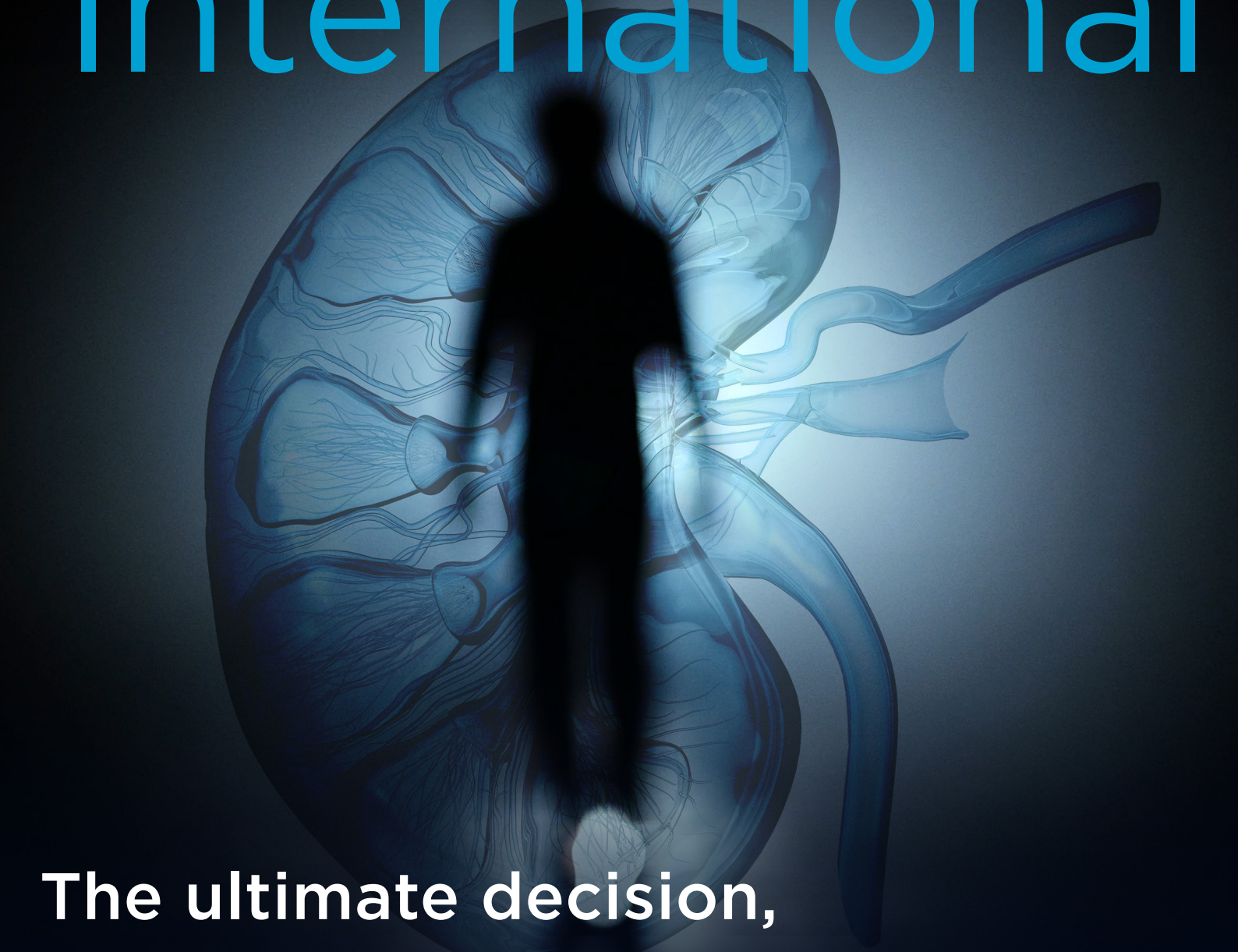
ESOT

Volume 37 | Issue 10
October 2024

Transplant International



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ISSN 1432-2277
ISBN 978-2-8325-5717-4
DOI 10.3389/978-2-8325-5717-4

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TRANSPLANT TRIAL WATCH
published: 14 October 2024
doi: 10.3389/ti.2024.13860

Transplant Trial Watch

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Keywords: randomised controlled trial, heart transplantation, everolimus, Bk virus, monoclonal antibody

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

RANDOMISED CONTROLLED TRIAL 1

Long-Term Follow-Up of the Randomized, Prospective Scandinavian Heart Transplant Everolimus *De Novo* Study With Early Calcineurin Inhibitors Avoidance (SCHEDULE) Trial.

by Bollano, E., et al. Journal of Heart and Lung Transplantation 2024 [record in progress].

Aims

The aim of this study was to report the long term outcomes of calcineurin inhibitor (CNI) discontinuation and early initiation of everolimus in comparison to receiving a standard CNI-based regimen, in heart transplant recipients.

Interventions

Participants were randomised to either the everolimus group or the CNI-group.

Participants

115 adult *de novo* heart transplant recipients.

Outcomes

The primary outcome was renal function. The secondary outcomes included time to death of any cause; a composite endpoint of death, myocardial infarction, re-transplantation, percutaneous coronary intervention (PCI), cancer, dialysis or kidney transplantation; myocardial structure and function; quality of life; and number of adverse events or serious adverse events.

Follow-Up

12 years.

CET Conclusion

by John O'Callaghan

This is an interesting study in heart transplantation with long follow up (11 years) after transplantation. It is the latest publication following the SCHEDULE trial in which heart transplant recipients were randomised to everolimus with reduced CNI exposure, followed by

Transplant Trial Watch

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Received: *27 September 2024*
Accepted: *01 October 2024*
Published: *14 October 2024*

Citation:
Knight SR and O'Callaghan JM (2024) Transplant Trial Watch. Transpl Int 37:13860. doi: 10.3389/ti.2024.13860

CNI withdrawal at week 7–11 post-transplant. This was compared to standard dose and continuation of CNI. Both these regimens were given alongside mycophenolate mofetil and corticosteroids. 78 of the 115 patients from the initial study were included at this follow up stage, with a very similar number from each arm of the study (40 versus 38). Analysis was by intention to treat, even at 11 years after randomisation, this is excellent allocation maintenance. Approximately 87% in each group were still on the allocated treatment. There was still a large statistically significant and clinically significant benefit for the everolimus group in terms of renal function (mean 83 mL/min versus 61 mL/min). There was no significant difference in heart transplant function, rejection or mortality. The earlier reports from this trial had raised concerns about increased risk of rejection in the everolimus group; however, this did not translate to later adverse outcomes in longer follow-up.

Jadad Score
2.

Data Analysis
Per protocol analysis.

Allocation Concealment
Yes.

Trial Registration
N/A.

Funding Source
Industry and non-industry funded.

RANDOMISED CONTROLLED TRIAL 2

Nonclinical and Clinical Characterization of MAU868, A Novel Human-Derived Monoclonal Neutralizing Antibody Targeting BK Polyomavirus VP1.

by Abend, J. R., et al. American Journal of Transplantation 2024 [record in progress].

Aims
The aim of this study was to report *in vitro* and first in-human studies assessing the safety, tolerability and pharmacokinetics of MAU868, a novel human immunoglobulin (Ig) G1 monoclonal antibody, against BK polyomavirus VP1 in healthy adults.

Interventions
Participants were randomised to receive either MAU868 or placebo.

Participants
33 healthy adults (aged 18–60 years) weighing between 40 and 120 kg.

Outcomes
The primary outcomes included safety and tolerability outcomes (adverse events and serious adverse events). Secondary outcomes were assessment of pharmacokinetics and the potential immunogenicity of MAU868.

Follow-Up
182 days.

CET Conclusion
by Simon Knight

This interesting paper reports *in vitro* and first-in-human studies of a novel human IgG1 monoclonal antibody against BK virus. *In vitro* studies demonstrate binding and neutralisation of infection with no evidence of resistance in long-term selection studies. The first-in-human clinical study in healthy volunteers demonstrated the pharmacokinetic profile of the drug, and the treatment was well tolerated with no major side effects at all doses. The clinical study was well designed, with randomisation and placebo control, although the exact method of randomisation and allocation concealment is not described. Given the lack of existing treatments for BK virus, this is a very promising initial study.

Jadad Score
4.

Data Analysis
Per protocol analysis.

Allocation Concealment
No.

Trial Registration
N/A.

Funding Source
Industry funded.

CLINICAL IMPACT SUMMARY

by Simon Knight

BK virus infection, leading to viraemia and nephropathy, remains a significant issue in renal transplantation. Viraemia develops in up to 30% of kidney recipients, and despite improvements in care, infection still results in graft dysfunction and graft loss in around 15% affected patients [1, 2]. Most transplant centres monitor for presence of virus, either in the urine or blood, and respond to increasing levels of viraemia. A number of different approaches to management have been trialled, but mainstay of treatment remains immunosuppression reduction, sometimes with IvIG in patients who do not respond [3]. Antiviral therapy with leflunomide or cidofovir has not shown consistent benefit in randomised trials.

A lack of effective treatment for BK virus has led to two recent early-phase studies of novel therapies. Earlier this year, Chandraker and colleagues published a randomised, double blind safety study of Posoleucel, an allogeneic, multivirus specific T-cell therapy with activity against BK virus,

adenovirus, cytomegalovirus, Epstein–Barr virus, human herpesvirus 6, and John Cunningham virus [4]. The treatment was shown to be safe and well-tolerated, and Posoleucel resulted in a reduction in BK viraemia compared to placebo with an increase in circulating BK-virus specific T-cells.

In the July issue of the American Journal of Transplantation, Abend and colleagues report a phase 1 study of a novel monoclonal antibody, MAU868, which targets the major capsid protein VP1 [5]. *In vitro* studies demonstrated binding and neutralisation of the 4 major BK virus genotypes, with no evidence of resistance in long-term selection studies. A first-in-human clinical study in healthy volunteers demonstrated the pharmacokinetic profile of the drug, and the treatment was well tolerated with no major side effects at all doses tested.

Clinical studies of both of these drugs are in early stages, and further clinical trials will be needed to demonstrate whether the activity against BV viraemia results in sustained viral reduction and improved clinical outcomes. Nonetheless, it is encouraging to see the development of new agents against this problematic virus.

Clinical Impact

3/5.

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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.

GENERATIVE AI STATEMENT

The author(s) declare that no Generative AI was used in the creation of this manuscript.

ACKNOWLEDGMENTS

Edited by Reshma Rana Magar.

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NEWS AND VIEWS
published: 31 October 2024
doi: 10.3389/ti.2024.13873

A Hitchhiker's Guide to the BK Galaxy

Hans H. Hirsch[1*†] *and Camille N. Kotton*[2†]

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Keywords: kidney transplantation, guidelines, BK virus, polyoma, nephropathy, BK polyomavirus

For the last two decades, patients and transplant clinicians have found themselves being suddenly confronted with the hostile galaxy of BK polyomavirus (BKPyV) while surfing through the kidney transplant universe. Deep thought consultation then revealed the existence of underappreciated worlds full of challenging experiences and poor outcomes as well as daring suggestions on how to rescue the journey and to reduce short- and longer-term damage. This seemingly endless odyssey has been accompanied by an expanding and contracting information space, occasionally brightened by short-lived shooting stars, most of them with limited impact for down-to-earth practice. What is more, the mere existence of the BK galaxy, its focal impact and dire costs eventually needed to be communicated to the key passengers of this journey, patients and their relatives, most of whom had possibly never heard of this nebulous conglomeration before. Two years ago, however, a brave mission was concluded by 55 people who had accepted the challenging invitation by The Transplantation Society (TTS) to embark on six working groups with the task to better chart and tackle this not so remote galaxy centering around BK polyomavirus. The *TTS International BK Polyomavirus Consensus Group* safely now returned and published together one of the most updated and comprehensive reports, *The Second International Consensus Guidelines on the Management of BK Polyomavirus in Kidney Transplantation* [1].

Given its significant content and claim, what is a reasonable and lean approach to the BK galaxy, a hitchhiker's guide facilitating clinical translation and implementation of the new TTS BKPyV guidelines? While the underlying mantra remains regular screening and prompt response to BK polyomavirus-DNAemia by reducing immunosuppression, there are three fixed stars with their own gravity fields, nevertheless clearly interconnected in this travel guide: the *infographic*, the *timeline*, and the *flow-chart*. Rather than being stunned or scared by the collection of tables and their detailed Swiss army knife-like content for every eventuality, we suggest the following approach:

First, consider the infographic, which is miraculously concise given the encyclopedic character of the updated TTS BK polyomavirus guidelines (**Supplementary Figure S1**). There, the main recommendations are summarized in their proactive character and directly prepare the quest for more professional and detailed information.

Second, review the conceptual timeline after kidney transplantation, which paradigmatically leads through the relevant sequence of virology, immunity and pathology, integrates diagnostic measures and management considerations, and allows for cross-comparison at a given time point (**Supplementary Figure S2**).

Third, walk through the flowchart and explore the suggested decision tree, which gives specific reference to the respective recommendations elaborated in tables of the TTS BK polyomavirus guidelines (**Supplementary Figure S3**).

These three steps allow to obtain overview, concepts and a first sense of detail - but being primed for now reviewing the current practice in your center is perhaps the most valuable item:

On the positive side, this helps to identify the "*have*" of tools, procedures and staff that are already existing in current center practice as well as those "*must haves*" that are not optimally used or clearly missing.

News & Views

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Received: *01 October 2024*
Accepted: *24 October 2024*
Published: *31 October 2024*

Citation:
Hirsch HH and Kotton CN (2024) A Hitchhiker's Guide to the BK Galaxy. Transpl Int 37:13873. doi: 10.3389/ti.2024.13873

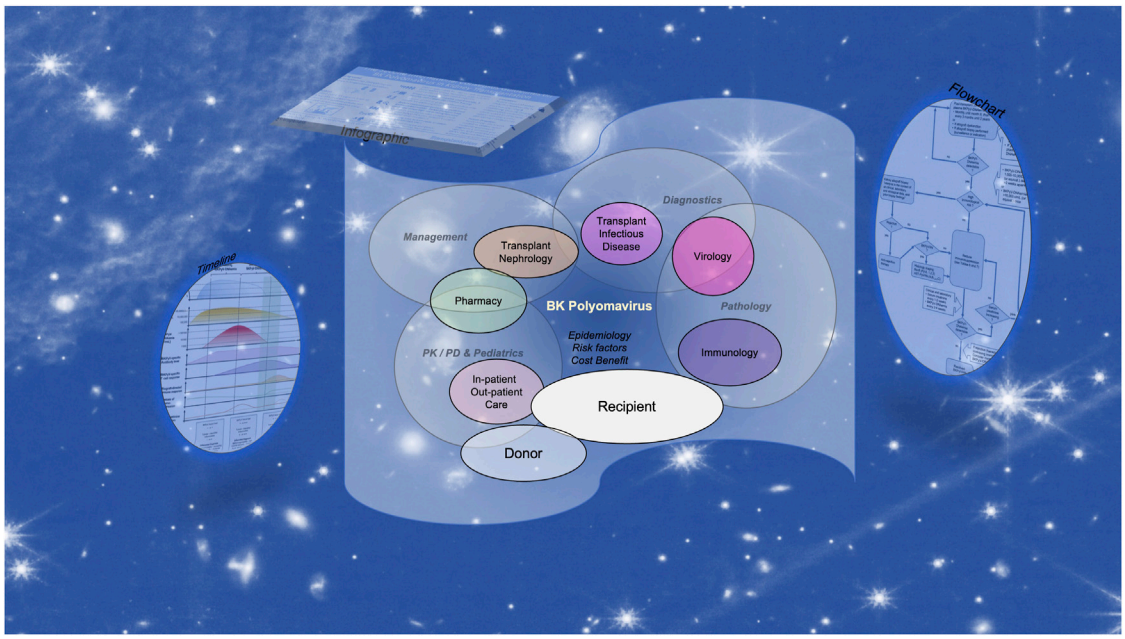

FIGURE 1 | The multidisciplinary challenge of implementing the updated TTS BK polyomavirus guidelines for developing and updating standard operating procedures.

Given the heavy load and the multidisciplinary character, we presume that the task of harmonizing and successfully translating the updated TTS BK polyomavirus guidelines is best accomplished by a team approach (**Figure 1**). Perhaps a tabular comparison of "is" *versus* "suggested" or "have" *versus* "must have" provides a first overview and can be complemented by "priorities" and "timelines" to realization in order to create a helpful and traceable planning tool.

One of the key deliverables is the timely reduction of maintenance immunosuppression guided by a significant plasma BK polyomavirus DNA load. For standard immunological risk and patients with baseline renal function, there is no biopsy required. There is currently no data from randomized clinical trials supporting superiority of either management *strategy 1* (first step reducing mycophenolate) or of *strategy 2* (first reducing tacrolimus). Though frequently mentioned or considered for other reasons, we like to emphasize that there currently is, for no other opportunistic complication posttransplant, more consistent and better documented evidence of feasibility, for rates of success or harm than for the deliberate reduction of immunosuppression for BK polyomavirus. Clearly, trigger and timing remain key determinants [2].

To develop the local *standard operating procedure*, the active participation of all different experts and providers is expected to not only build and expand a broad foundation of knowledge, but also competence for critical (re-)evaluation. Deviation from the current recommendations always remains an option, but then they are the result of active informed decision instead of ignorance. Broad foundation of knowledge also prepares the transplantation team for participation in randomized clinical trials, which are particularly lacking for management decisions.

The new TTS BK polyomavirus guidelines also identify areas of uncertainty and unmet clinical need, where more excellent research is needed and expected to make a difference for patients on their hopefully timeless journey of kidney transplantation. This starts at transplantation with the investigations addressing the value of donor urine virus loads, donor and recipient BK polyomavirus-specific antibodies, virus-specific cell mediated immunity, biomarkers of allograft damage and differentials of T cell mediated rejection or antibody-mediated rejection, as well as therapeutic, preemptive or prophylactic transfer of humoral and cellular immune effectors. But even for the lower hanging fruits, more conclusive data from randomized clinical trials must be considered valuable. These include evaluation of other tantalizing forces in our management universe such as switching to mTOR inhibitors, perhaps combined with low-dose cyclosporine instead of tacrolimus [3], or for patients with persisting BK polyomavirus-DNAemia on tacrolimus monotherapy to switch to belatacept for maintenance.

Importantly, all of the TTS working group members and their leaders are committed to assist transplant clinicians with their expertise in the management of difficult cases as well as in establishing the best local standard operating procedures. Indeed, the challenges of BK polyomavirus and how it affects a significant part of kidney transplant recipients should be explained to the patients and relatives pre-transplant when preparing for one of the otherwise most successful journeys in modern medicine. As a disclaimer known from others, it remains to conclude that "The *Guide* is definitive. *Reality* is frequently inaccurate" [4].

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

HH and CK identified the need for this communication and designed project, HH wrote the first draft of the manuscript and CK and HH finalized the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was funded by the personal appointment grant MM2109 to HH by the University of Basel, Basel, Switzerland and an SNF grant 310030_212589 to HH. The TTS BK polyomavirus guidelines were supported by independent funding of Roche diagnostics provided to The Transplantation Society for the realizing the BK Polyomavirus guidelines hybrid meeting in Basel, April 7–8, 2022.

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.

GENERATIVE AI STATEMENT

The author(s) declare that no Generative AI was used in the creation of this manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2024.13873/full#supplementary-material

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FORUM
published: 18 October 2024
doi: 10.3389/ti.2024.13806

Results of Kidney Transplantation from Donors Following Euthanasia in the Netherlands: Benchmarking Science and Ethical Challenge

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Keywords: transplantation, organ donation, euthanasia, ethical challenges, DCD-V

A Forum discussing:

Promising Results of Kidney Transplantation From Donors Following Euthanasia During 10-Year Follow-Up: A Nationwide Cohort Study
by Susanna C, Van Dijk N, de Jongh W, Verberght H, Van Mook WNKA, Bollen J and van Bussel B (2024). Transpl Int 37:13142. doi: 10.3389/ti.2024.13142

INTRODUCTION

Susanna et al. report the 10 years follow-up results of kidney transplantation in the Netherlands from donors following euthanasia [1]. These are patients who following the legal administration of euthanasia under Dutch law, proceed to donation after circulatory death (DCD-V). They compared this group to the outcomes over the same period in controlled DCD (DCD-III) and donation after brain death (DBD). Uncontrolled DCD (DCD-II), donation after cardiac arrest in a brain dead patient (DCD-IV) and living kidney donation were excluded. The authors found from their study that the graft results of these kidneys have less delayed graft function than DCD-III donors and at least comparable longitudinal eGFR and graft function over 10 years as compared to kidneys from DCD-III and DBD. Their conclusion was that "...these results support the concept that ODE kidneys are a promising contribution to the donor pool, *and organ donation after euthanasia (ODE) should be continued*".

Their paper raises two aspects of particular interest to the international donation and transplantation community – benchmarking science and ethical challenge.

BENCHMARKING SCIENCE

The publication of outcome data and process metrics by countries is helpful because it allows other countries who do similar, or wish to do, to have a benchmark to compare against. The finding by Susanna et al. that delayed graft function was less in their DCD-V cohort than their DCD-III seems at face value, physiologically self-evident. DCD-III patients typically have had a devastating brain injury and a prolonged ICU stay before donation occurs. While the reasons for euthanasia in the DCD-V cohort were not provided in the paper, they are typically related to either neurodegenerative or psychiatric disorder in the Netherlands. Another expectation might be that warm ischemia time would be longer in DCD-III. This however was not the case and no difference was seen.

Forum

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Received: 15 September 2024
Accepted: 23 September 2024
Published: 18 October 2024

Citation:
Jansen NE and Gardiner D (2024) Results of Kidney Transplantation from Donors Following Euthanasia in the Netherlands: Benchmarking Science and Ethical Challenge. Transpl Int 37:13806. doi: 10.3389/ti.2024.13806

The comparable longitudinal eGFR and graft function over 10 years compared to both DCD-III and DBD is very reassuring and certainly gives credence to the authors' claim that – medically at least – DCD-V kidneys are a safe extension of the donor pool.

ETHICAL CHALLENGE

Just because you can does not mean you should. For many countries in the world, even those with long established deceased donation programs, DCD-V is a step too far. And yet, there is a growing number of advanced economy countries who have, in the last decade, legalized euthanasia. Once they do, whether intended or not, DCD-V follows soon after. Belgium introduced a euthanasia law in 2002 and performed the world's first DCD-V in 2005. The Netherlands legalized euthanasia in 2001, followed by the first DCD-V in 2012. In Spain medical assistance in dying became legal in June 2021, and by 2022, 4.6% of their DCD cases were DCD-V; which exceeds their long established DCD-II program (2.6%) [2]. The State of Victoria, Australia, introduced voluntary assistance in dying in 2019, they had their first DCD-V case in 2023 [3]. Other States in Australia have followed. In Canada, medical assistance in dying became legal in 2015, and Quebec carried out its first DCD-V in 2017. By 2022 DCD-V represented 14% of Quebec's total deceased donation activity [4].

In the Netherlands, 15% of all DCD donors were DCD-V donors in 2023, which, like Quebec, is a substantial contribution to the donor pool. However, that does not mean that in all hospitals in the Netherlands DCD-V is facilitated. A small number of hospitals remain against euthanasia, for example, due to religious beliefs. Also, not all general practitioners are willing to grant their patient's request for euthanasia. These patients have the option to apply to the "Expertise Centre Euthanasia," where independent doctors assess whether euthanasia can be granted, and if so, they are involved in carrying out this request (sometimes including organ donation). Another issue, that still requires attention, is in cases where the request for euthanasia is based on psychiatric suffering. For these patients it is even harder to find a physician (psychiatrist) willing to facilitate euthanasia and a donor hospital where organ donation can take place. Although DCD-V fulfils the patient's explicit wish and has a promising contribution to the donor pool, it is wrong to assume that the practice is universally supported in the Netherlands, especially for the condition of psychiatric suffering.

In the UK euthanasia is illegal, though this does not mean individuals are necessarily prosecuted if they provide assistance to their loved one's death. For those in the UK who strongly wish for assisted dying but with clearer legal safeguards for their family and friends, they often make the journey to Dignitas in Switzerland [5]. In the UK the Scottish Parliament [6] and the Channel Island of Jersey [7] are both considering the introduction of medical assistance in dying. If it comes, the example from other nations, is that the donation and transplantation community will need to be ready for patients also making requests for DCD-V.

Susanna et al. paper may provide us with the benchmarking science, the ethical challenge is harder to resolve.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

NJ and DG wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

The authors declare that no financial support was received for the research, authorship, and/or publication of this article.

CONFLICT OF INTEREST

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

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ORIGINAL RESEARCH
published: 18 October 2024
doi: 10.3389/ti.2024.13142

Promising Results of Kidney Transplantation From Donors Following Euthanasia During 10-Year Follow-Up: A Nationwide Cohort Study

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The outcome of kidneys transplanted following organ donation after euthanasia (ODE) remains unclear. This study analyzed all kidney transplantations in the Netherlands from January 2012 to December 2021, comparing the outcomes following ODE, donation after circulatory death (DCD-III), and donation after brain death (DBD). 9,208 kidney transplantations were performed: 148 ODE, 2118 DCD-III, and 1845 DBD. Initial graft function was compared between these categories. Immediate graft function, delayed graft function and primary non-function in ODE kidney recipients were 76%, 22%, and 2%, respectively, 47%, 50% and 3% in DCD-III kidney recipients and 73%, 25%, and 2% in DBD kidney recipients (overall p-value: $p < 0.001$). The number of kidneys transplanted over a median follow-up period of 4.0 years (IQR 2.0–6.6), was 1810, including 72 ODE, 958 DCD-III and 780 DBD kidneys. In this period, 213 grafts (11.8%) failed [7 grafts (9.7%) from ODE donors, 93 grafts (9.7%) from DCD-III donors, and 113 grafts (14.5%) from DBD donors]. Kidneys transplanted after euthanasia have a good immediate graft function, a comparable longitudinal 10 years eGFR, and similar graft failure hazard to kidneys from DCD-III and DBD. Kidney transplantation following ODE is a valuable and safe contribution to the donor pool.

Keywords: organ donation, euthanasia, donation after circulatory death, donation after brain death, kidney transplantation, organdonation after euthanasia, medical assistance in dying, physician assisted death

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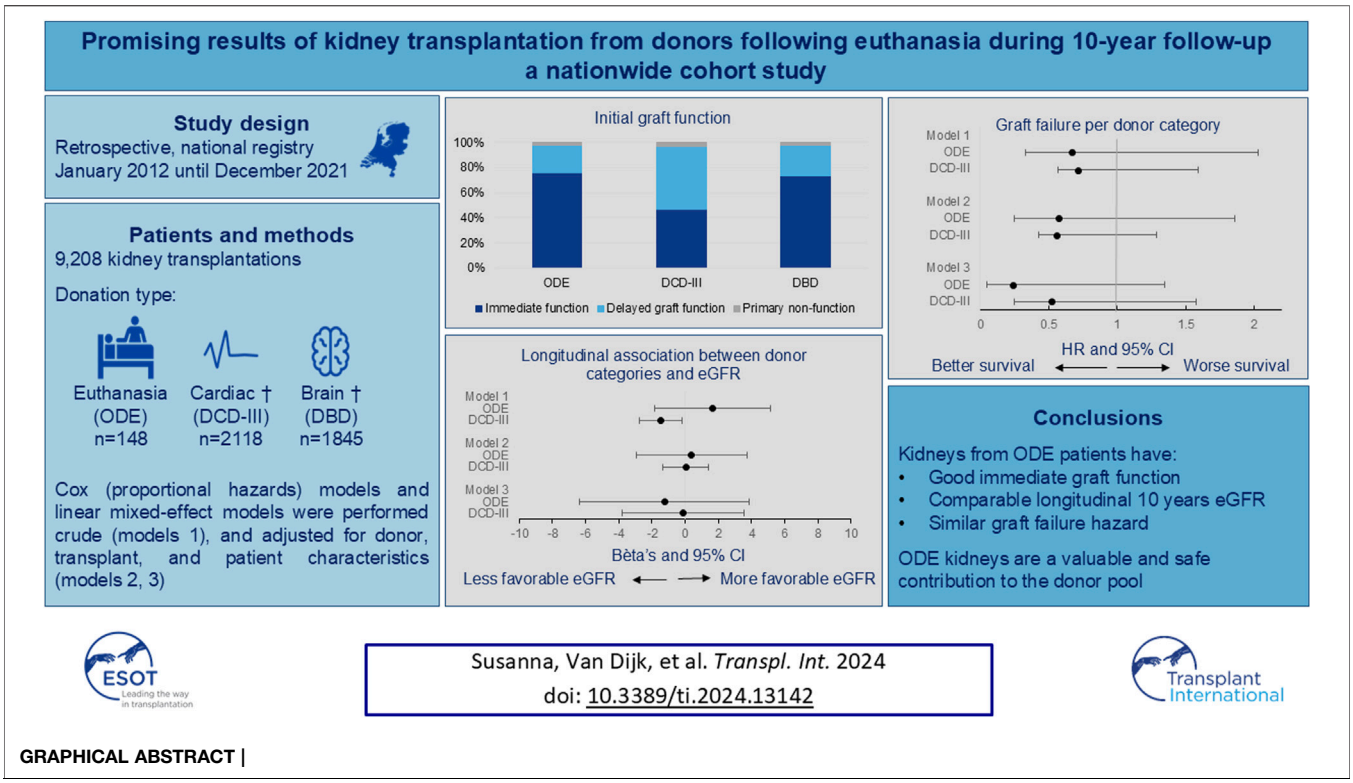
Received: *16 April 2024*
Accepted: *23 September 2024*
Published: *18 October 2024*

Citation:
Susanna C, van Dijk N, de Jongh W, Verberght H, van Mook W, Bollen J and van Bussel B (2024) Promising Results of Kidney Transplantation From Donors Following Euthanasia During 10-Year Follow-Up: A Nationwide Cohort Study. Transpl Int 37:13142. doi: 10.3389/ti.2024.13142

INTRODUCTION

Post-mortem organ donation can be performed after brain death (Donation after Brain Death, DBD) or following circulatory death (Donation after Circulatory Death, DCD). DCD is categorized based on the Maastricht classification into four types, of which DCD-I, DCD-II, and DCD-IV are classified as uncontrolled donations; DCD-III is classified as donation following withdrawal of life-sustaining therapy and is a controlled donation [1]. Since July 2017, DCD-I and DCD-II procedures are no longer performed in the Netherlands.



GRAPHICAL ABSTRACT |

In ODE, a patient dies in a controlled manner, following administration by a physician of euthanasia drugs. However, the dying process of these patients differs from that of patients who donate their organs after circulatory death (i.e., DCD) following withdrawal of life-sustaining treatments in the ICU (i.e., DCD-III) or following brain death (i.e., DBD), as these patients are critically ill [2]. Hence, the DCD classification recently proposed to include ODE patients in a separate category of highly controllable DCD: DCD-V [3, 4].

As the present study describes data from Netherlands, we use organ donation after *euthanasia* (ODE) as terminology rather than MAID (Medical Assistance in Dying), which is used in other countries [5, 6]. The ethical, legal, and logistical implications of ODE, in general, have been extensively discussed in both the scientific literature and public media [7, 8].

The possibility of ODE is expanding to more countries, and the number of procedures is increasing annually in most countries where ODE is already available [9, 10]. Organ donation after euthanasia may increase the number of donor organs and thus aid in narrowing the gap between the demand and availability of organs for transplantation. The next question to consider is whether the outcomes of the transplanted organs after ODE are sufficient to continue the procedure.

Data on the outcome of kidneys transplanted following ODE is scarce [11]. A conference abstract reported graft function of transplanted kidneys following ODE that was comparable to DCD-III and DBD over a 5-year follow-up period [12]. We hypothesized that transplant outcomes after ODE have favorable initial graft function, favorable estimated glomerular filtration rate, and less graft failure compared to transplant outcomes from DCD-III and DBD over a 10-year follow-up period. We investigated whether this was independent of a comprehensive set of donor, recipient, and transplant variables. This investigation provides the results of kidney transplants following ODE compared to kidney transplants from other forms of donation during a 10-year study period.

PATIENTS AND METHODS

Data from the Dutch Transplant Foundation (Nederlandse Transplantatie Stichting, NTS) are recorded in the Netherlands Organ Transplantation Registry (NOTR), which includes all kidney transplantations performed in the Netherlands. The authors requested and obtained data on transplantations between 1st January 2012 (the year of the first ODE retrieval in the Netherlands), and 31st December 2021, from the NTS registry NOTR in accordance with their data registry governance.

These data were used to construct a retrospective cohort of patients who underwent a kidney transplantation, to compare the graft function between ODE, DCD-III, and DBD derived grafts.

Next, we excluded organ transplantations from donors younger than 18 years, and DCD-I, DCD-II, and DCD-IV donations, as defined in the Maastricht Category [1], and living donation retrievals. No donors in the dataset were represented in multiple transplantation categories (e.g., a living

transplantation followed by a post-mortem donation). This resulted in the following categories to be studied: organ donation after euthanasia (ODE, DCD-V); organ donation after circulatory death, Maastricht Category III (DCD-III); and organ donation after brain death (DBD) [1, 3].

Recipients may undergo multiple kidney transplantations during their disease course. For the primary investigation, we included and characterized the most recent transplant (i.e., the latest transplant) in a recipient. In this way any recipient with multiple kidney transplantations was included only once in the analyses.

We described the donors, the recipients, and transplantation and graft characteristics for ODE (DCD-V), DCD-III and DBD categories. Initial graft function, as well as estimated glomerular filtration (eGFR) rate over 10 years and graft failure, were described for ODE, DCD-III and DBD categories.

Donor Characteristics

For donor characteristics, we described age in years, sex, serum creatinine concentrations in μmol/L, medical history of hypertension, diabetes mellitus, and smoking status (dichotomous outcome measures and in pack years), as reported in the NOTR.

Recipient Characteristics

For recipient characteristics, age was defined as the recipient's age at transplantation in years. Furthermore, we described sex, dialysis time and panel reactive antibody (PRA). The PRA test was used to estimate the degree of sensitization in recipients' blood to donor-specific antibodies. Traditionally, the recipient's serum is exposed to a panel of random donor lymphocytes. The PRA test indicates the risk of transplant failure to the host response to transplantation [13, 14]. PRA is classified as low (≤5%), intermediate (6%–84%), and high (≥85%). Dialysis time was measured as the days of dialysis of the recipient before transplantation and presented in years by dividing by 365.25.

Transplantation Characteristics

The warm ischemia time (WIT) is defined as the time between the circulatory arrest (e.g., loss of cardiac output in a DCD-III and ODE (DCD-V), and arterial clamping in DBD until the start of cold aortic flush (*in situ* preservation) or the start of normothermic aortic flush in case of normothermic machine perfusion of the donor [15]. The cold ischemia time (CIT) is defined as the start of cold aortic flush (*in situ* preservation) until cessation of hypothermic machine perfusion respectively taken off ice. The anastomosis time (AT) is defined as the time between the end of the hypothermic state and reperfusion of the kidney in the recipient.

Graft Characteristics

Initial Graft Function

Graft function within the first week post-transplantation was categorized into primary non-function, delayed graft function, and immediate graft function. Kidney transplantations that failed (e.g., non-viable kidneys, or graft loss) in the first week post-transplantation were categorized as primary non-function. Kidney transplantations that required dialysis the first week post-transplantation were categorized as delayed graft function. The remaining kidney transplantations were categorized as immediate graft function.

Graft Failure

Graft failure and its causes were pre-scored in the NOTR and included hyperacute rejection, infection (not graft-related), infection of graft, non-viable kidney, patient dying with a functioning transplant, permanent non-function, recurrent primary renal disease, rejection after stopping all immunosuppressive drugs, rejection while taking immunosuppressive drugs (acute/chronic), removal of functioning graft, technical problems, thrombosis/infarction, vascular or ureteric problems, vascular problems: none-operative or rejection related, other (renal) and unknown (**Supplementary Table S1**).

Estimated Glomerular Filtration Rate

Graft function was studied using 10-year follow-up on serum creatinine. The eGFR was calculated using the re-expressed MDRD-4-formula [16]. The concentration of serum creatinine (in μmol/L) was converted to serum creatinine in mg/dL by using the molecular weight of creatinine (113.12 g/mol). Increased age of recipients for creatinine measurements post-transplant was considered (e.g., for eGFR estimation 2 years post-transplant, the following age was used: age at date of transplantation plus 2 × 365.25). The eGFR was presented as mean and standard error (SE) to indicate that time moments may have more or fewer observations in the ODE, DCD-III, and DBD groups. Invalid (negative) creatinine values were removed from the dataset (n = 1,021). The number of invalid creatinine values at 3 months, 1 year, 2 years, 3 years, 4 years, 5 years, 6 years, 7 years, 8 years, 9 years, and 10 years were, respectively, 42, 36, 110, 178, 167, 140, 111, 102, 67, 43, and 25.

Statistical Analysis

This observational study is reported in accordance with the STROBE guideline [17].

Continuous data was visually inspected for normality and presented as mean ± standard deviation or as median (interquartile range). Categorical variables were presented as percentages. One-way ANOVA, Chi-square test, and Fisher's exact test were used to test overall differences between ODE, DCD-III and DBD categories. Pairwise comparisons were conducted as post-hoc analysis to identify differences between 2 of the 3 categories if an overall test indicated statistical significance.

First, we used linear mixed-effects models to analyze whether longitudinal kidney function over 10 years, based on eGFR, differed between donor categories, with DBD as the reference category. We investigated a model containing donor category and time as independent variables (model 1). Model 1 was subsequently adjusted for donor age, donor sex, donor smoking, recipient age, recipient sex, and transplant ischemic times (CIT and AT) (model 2). Next, this model was further

adjusted for donor hypertension, donor diabetes mellitus, WIT and transplant PRA (model 3). Recipient ID was added as a random effect to the models. The longitudinal mixed-effects models were repeated with time as random slopes. Fixed effects were presented as coefficients (β) and 95% CI, with a negative coefficient indicating a lower eGFR per donor category, as compared to the reference category.

Then, we used Cox proportional hazard models for the main analyses to investigate the association between donor categories and graft failure, with DBD as reference category. Grafts in which primary non-function occurred within the first week post-transplant, and therefore failing grafts, were excluded from the primary Cox analyses, because it is considered a short-term outcome with another presumed mechanism than those involved over the longer periods of time. Death of the recipient was considered a censored event in the main analyses when the recipient died with a functioning graft. Crude (model 1) models were adjusted (models 2 and 3) for the same set of variables in accordance with adjustments for the linear-mixed effects models above. For the Cox models, we report hazard ratios (HR) with their 95% confidence intervals (CI), with an HR higher than 1 indicating an increased hazard per donor category as compared to the reference category. The proportional hazards assumption was checked using the scaled Schoenfeld residuals.

We performed four sensitivity analyses and re-analyzed the above Cox models by first replacing the recipients for organs donated (i.e., including all transplantations of each recipient; sensitivity analysis 1) to determine whether the outcomes remain consistent with the primary models. Next, we also re-analyzed model 1 and model 2, in which recipient death was not censored, but included as an event (sensitivity analysis 2). A third sensitivity analysis was performed to re-analyze model 1 and model 2, in which primary non-function, which was assumed to have a separate etiology from graft failure occurring after a prolonged period, was included (sensitivity analysis 3). Finally, a fourth sensitivity analysis was performed re-analyzing model 1 and model 2, in which only the first transplantation within a recipient was used in the analyses, instead of the last transplantation within a recipient in the primary analyses. Although we assumed that matching a kidney between the donor and recipient is independent, HLA mismatch and antibody production could change due to re-transplantations. The fourth sensitivity analysis excluded such mechanisms by showing similar results (**Supplementary Table S2**) [18]. In addition to regression coefficients, hazard ratios and their 95% confidence intervals, we report p-values, which were considered statistically significant at $p < 0.05$. We analyzed the data using IBM SPSS Statistics 27 and R x64 i4.1.3 and R studio 2023.

RESULTS

Of the 9,208 kidney transplantations performed in the Netherlands between 1st January 2012 and 31st December 2021, 9,070 were from donors aged 18 years and older. After excluding 4,790 transplantations from living donors and 13 transplantations from DCD-I, DCD-II, and DCD-IV donors, and after excluding previous transplantations within the recipients, 4,111 kidney transplant recipients remained, with kidney transplants originating from 2,730 unique donors (**Figure 1**). In total, 148 recipients received a kidney from a donor after ODE, 2,118 from a donor after DCD-III, and 1,845 from a donor after DBD (**Figure 1**).

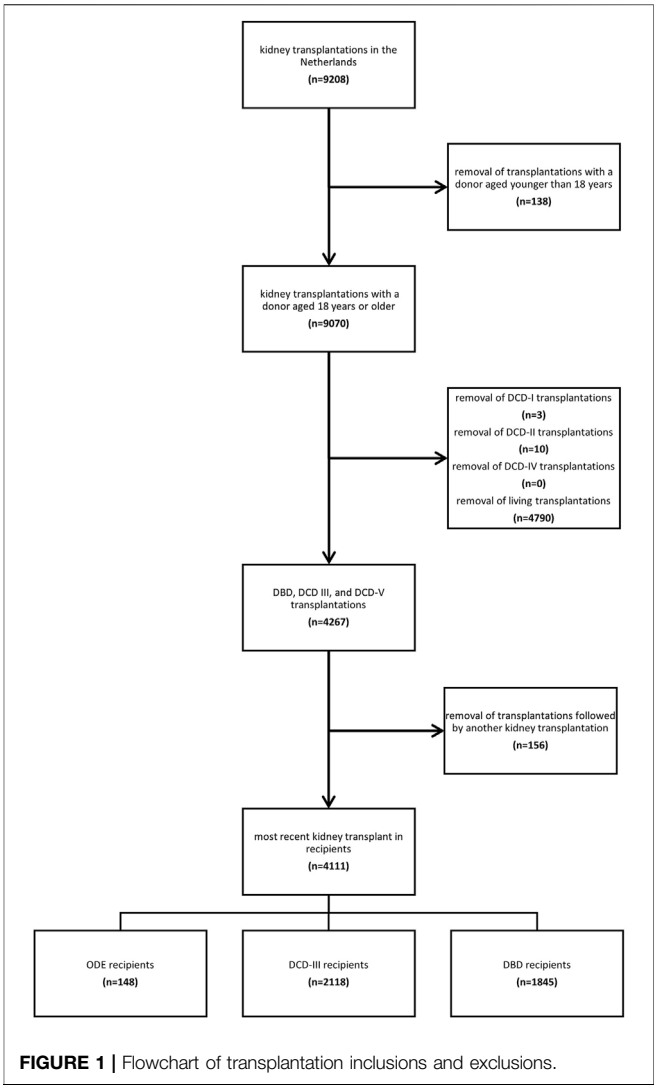


FIGURE 1 | Flowchart of transplantation inclusions and exclusions.

Donor recipient and transplantation baseline characteristics are presented in **Table 1**. ODE donors had lower serum creatinine concentrations ($p = 0.046$) compared to DBD donors. ODE recipients were younger ($p = 0.034$) than DCD-III recipients. A minor, not clinically relevant difference, was found in WIT between ODE transplantations and DCD-III transplantations ($p = 0.022$). As expected, WIT was longer in ODE as compared to DBD transplantations ($p < 0.001$). CIT was shorter in ODE transplantations as compared to DBD ($p < 0.001$), no difference was found for CIT in ODE and DCD-III (**Supplementary Table S3**).

Initial graft function was available for ODE 127 out of 148 (86%), for DCD-III 1940 out of 2,118 (92%), and for DBD

TABLE 1 | Baseline characteristics of donors and recipients, and graft characteristics.

Characteristics	ODE	DCD-III	DBD	p-value
Donor, n	91	1,304	1,335	
Age (yrs)	53 ± 12	55 ± 14	53 ± 14	<0.001[a]
Sex (male, n)	55% (50)	61% (801)	50% (663)	<0.001[b]
Creatinine (μmol/L)	66 ± 17	68 ± 29	77 ± 45	<0.001[a]
Hypertension (n)	14% (11)	27% (301)	29% (228)	0.021[b]
Diabetes (n)	0% (0)	2% (19)	2% (20)	0.512[c]
Smoking (n)	54% (49)	56% (717)	56% (691)	0.896[b]
Smoking pack years	22 ± 15	26 ± 17	25 ± 16	0.197[a]
Recipient, n	148	2,118	1845	
Age (yrs)	55 ± 13	58 ± 13	55 ± 15	<0.001[a]
Sex (male, n)	61% (91)	63% (1,340)	61% (1,129)	0.399[b]
Dialysis time (yrs)	2.7 (1.5–4.6)	2.4 (1.4–3.9)	2.6 (1.4–4.2)	0.001[a]
PRA				<0.001[c]
≤5% (n)	89% (121)	92% (1866)	88% (1,544)	
6%–84% (n)	11% (15)	7% (141)	11% (192)	
≥85% (n)	0% (0)	1% (17)	1% (23)	
Graft, n	148	2,118	1845	
Warm ischemia time (min)[a]	15 (13–18)	16 (13–19)	0 (0–0)	<0.001
Cold ischemia time (hours)[a]	12 ± 5	13 ± 5	15 ± 7	<0.001
Anastomosis time (min)[a]	32 ± 12	33 ± 13	33 ± 15	0.506

[a]*One-Way Anova.*
[b]*Chi square test.*
[c]*Fisher's exact test.*
ODE, organ donation after euthanasia; DCD, donation after circulatory death; DCD-III, donation after circulatory death following withdrawal of life sustaining treatments in the ICU. DBD, donation after brain death; PRA, panel reactive antibody. Negative ischemia periods and negative anastomosis times have been removed from the set. Cold ischemia times and anastomosis times of zero have been removed from the set. Donor sample sizes of ODE, DCD-III and DBD are, respectively, 91, 1304, 1335. Recipient sample sizes of ODE, DCD-III and DBD are, respectively, 148, 2118, 1845. Graft sample sizes of ODE, DCD-III and DBD are, respectively, 148, 2118, 1845. P-values <0.05 indicate a statistical difference in the overall comparison of donor categories.

TABLE 2 | Initial graft function and transplant function using estimated glomerular filtration rate (eGFR) up until 10 years after transplantation.

Variable	ODE N = 148	DCD-III N = 2,118	DBD N = 1845	p-value
Initial graft function				<0.001
Immediate function (n)	76% (96)	47% (904)	73% (1,201)	
Delayed graft function (n)	22% (28)	50% (976)	25% (401)	
Primary non-function (n)	2% (3)	3% (60)	2% (34)	
Transplant function over time				
eGFR at 3 months (mL/min)	46 (1.6)	43 (0.6)	46 (0.6)	<0.001
eGFR at 1 year (mL/min)	48 (1.6)	46 (0.4)	48 (0.5)	0.001
eGFR at 2 years (mL/min)	48 (1.9)	46 (0.5)	48 (0.6)	0.092
eGFR at 3 years (mL/min)	50 (2.5)	46 (0.6)	47 (0.7)	0.089
eGFR at 4 years (mL/min)	48 (2.5)	46 (1.0)	47 (0.7)	0.948
eGFR at 5 years (mL/min)	48 (2.4)	46 (0.9)	46 (0.7)	0.791
eGFR at 6 years (mL/min)	50 (3.1)	47 (0.9)	47 (1.0)	0.631
eGFR at 7 years (mL/min)	57 (4.4)	47 (1.0)	46 (1.1)	0.163
eGFR at 8 years (mL/min)	52 (5.4)	48 (1.4)	45 (1.3)	0.305
eGFR at 9 years (mL/min)	59 (10.3)	50 (1.5)	43 (1.8)	0.006
eGFR at 10 years (mL/min)	58 (13.4)	46 (2.1)	46 (3.1)	0.718

Sample sizes of ODE, DCD-III, and DBD, grafts are, respectively, 148, 2118, 1845. Transplant function over time is presented as mean (SE). The number of observations for creatinine of ODE recipients after 3 months, 1 year, 2 years, 3 years, 4 years, 5 years, 6 years, 7 years, 8 years, 9 years, and 10 years were 129, 124, 97, 71, 60, 35, 29, 14, 10, 5, 2, respectively. The number of observations for creatinine of DCD-III recipients after 3 months, 1 year, 2 years, 3 years, 4 years, 5 years, 6 years, 7 years, 8 years, 9 years, and 10 years were 1933, 1823, 1,497, 1,157, 923, 732, 524, 368, 248, 159, 67, respectively. The number of observations for creatinine of DBD, recipients after 3 months, 1 year, 2 years, 3 years, 4 years, 5 years, 6 years, 7 years, 8 years, 9 years, and 10 years were 1,647, 1,500, 1,268, 1,054, 835, 657, 496, 368, 242, 148, 66, respectively. P-values <0.05 indicate a statistical difference in the overall comparison of donor categories.

1636 out of 1845 kidneys (89%) (**Table 2**). First-week post-transplantation outcomes showed that immediate graft function was higher in ODE, which was similar to DBD, when compared to DCD-III (overall p-value: $p < 0.001$) (**Table 2**). DCD-III showed more delayed graft function as compared to ODE and DBD, whereas primary non-function was similar (overall p-value: $p < 0.001$) (**Table 2**; **Supplementary Table S1**).

TABLE 3 | Longitudinal association between donor categories and estimated glomerular filtration rate over 10 years.

Variable	eGFR β (95% CI)	p-value	p-value ODE vs. DCD-III
Model 1, multivariable			
Donor category			
DBD	Reference	0.356	0.078
ODE	1.64 (−1.84; 5.13)	0.023	
DCD-III	−1.47 (−2.74;-0.20)		
Model 2, multivariable			
Donor category			
DBD	Reference	0.821	0.833
ODE	0.39 (−2.95; 3.72)	0.969	
DCD-III	0.03 (−1.33; 1.38)		
Model 3, multivariable			
Donor category			
DBD	Reference	0.634	0.592
ODE	−1.25 (−6.38; 3.88)	0.939	
DCD-III	−0.14 (−3.82; 3.53)		

Data are regression coefficients of fixed effects (β) with their 95%CI that indicate the longitudinal association between donor category, and eGFR over a 10-year period, with DBD as reference category. Estimated glomerular filtration rate (eGFR) is the dependent variable in all models. Random intercepts were used for recipient ID. Model 1, with 17,799 observations and 3,599 recipient IDs, includes fixed effects of donor category and time. Model 2, with 14,135 observations and 2,946 recipient IDs, is model 1 additionally adjusted for donor sex, donor age, donor smoking, recipient age, recipient sex, CIT, AT, and initial graft function. Model 3, with 9,385 observations and 1857 recipient IDs, is model 2 additionally adjusted for donor hypertension, donor diabetes, WIT and transplant PRA. Negative coefficients of fixed effects indicate lower eGFR per donor category, as compared to DBD. P-values <0.05 indicate a statistical significant regression coefficient.

TABLE 4 | Association between ODE, DCD-III and DBD, and graft failure.

Variable	Graft failure Hazard ratio (95% CI)	p-value	p-value ODE vs. DCD-III
Model 1, crude			
Donor category			
DBD	Reference	0.266	0.873
ODE	0.67 (0.33–1.36)	0.001	
DCD-III	0.71 (0.57–0.88)		
Model 2, multivariable			
Donor category			
DBD	Reference	0.179	0.969
ODE	0.57 (0.25–1.29)	<0.001	
DCD-III	0.56 (0.43–0.73)		
Model 3, multivariable			
Donor category			
DBD	Reference	0.068	0.287
ODE	0.24 (0.05–1.11)	0.074	
DCD-III	0.52 (0.25–1.06)		

Data are HR, with their 95%CI that indicate the association between donor category and graft failure, with DBD as reference category. Model 1 crude with 3606 observations and 360 events. Model 2, with 2953 observations and 281 events, adjusted for donor sex, donor age, donor smoking, recipient age, recipient sex, CIT, AT, and initial graft function. Proportional hazard assumption model 2 was met (p 0.221). Model 3, with 1860 observations and 176 events, additionally adjusted for donor hypertension, donor diabetes, WIT, and transplant PRA., Proportional hazard assumption model 3 was met (p = 0.285). HR higher than 1 indicating a higher hazard per donor category, as compared to DBD. P-values <0.05 indicate a statistical significant regression coefficient.

Mean graft function over time by eGFR is shown in **Table 2**. Longitudinal mixed-effects regression analyses adjusted for donor category and time (model 1, **Table 3**) showed that, compared to DBD, longitudinal eGFR for ODE was (β: 95% CI) 1.64 mL/min/1.73 m2 (−1.84; 5.13) and for DCD-III was −1.47 mL/min/1.73m2 (−2.74;-0.20) over the 10-year period. After additional adjustments for donor sex, donor age, donor smoking, recipient age, recipient sex, cold ischemic period, anastomosis time, and initial graft function (model 2, **Table 3**), and further adjustment for donor hypertension, donor diabetes mellitus, WIT and transplant PRA (model 3, **Table 3**) this association disappeared. Mixed-effects analyses with random slopes for time showed similar results (model 1–3, **Supplementary Table S4**).

After exclusion of primary non-functioning grafts, over a median follow-up period of 4.0 years (IQR 2.0–6.6), 1810 grafts were transplanted, which included 72 ODE, 958 DCD-III, and 780 DBD grafts. Over the median follow-up period, 213 grafts (11.8%) failed. This included 7 grafts (9.7%) for ODE, 93 grafts (9.7%) for DCD-III, and 113 grafts (14.5%) for DBD. Median follow-up periods of each donor category were 3.7 years (IQR 2.0–5.8) for ODE, 4.0 years (IQR 2.0–6.3) for DCD-III and 4.1 years (IQR 2.1–7.0) for DBD grafts.

When studying the association between graft failure and donor category, compared to DBD, the hazard ratio for ODE was (HR: 95% CI) 0.67 (0.33–1.36) and for DCD-III was 0.71 (0.57–0.88) using crude Cox regression analysis (model 1, **Table 4**). After adjustment for donor sex, donor age, donor smoking, recipient age, recipient sex, cold ischemic period, anastomosis time and initial graft function, the hazard ratio was, compared to DBD, 0.57 (0.25–1.29) for ODE and 0.56 (0.43–0.73) for DCD-III. After additional adjustments for donor hypertension, donor diabetes mellitus, WIT, and transplant PRA, the statistically significant difference between DBD and DCD-III grafts disappeared. The proportional hazards assumption was met (**Supplementary Figures S1–S3**).

The four sensitivity analyses, re-analyzing the above models by replacing the recipients for organs donated (i.e., including all kidneys transplanted in each recipient; sensitivity analysis 1, **Supplementary Table S2**); in which recipient death was not censored, but included as an event; (sensitivity analysis 2, **Supplementary Table S2**); in which primary non-function was included (sensitivity analysis 3, **Supplementary Table S2**); and in which only the first transplantation of each recipient was included (sensitivity analysis 4, **Supplementary Table S2**), all showed similar results as the primary analyses.

DISCUSSION

This study addresses the transplant outcomes of kidneys donated after euthanasia over a 10-year study period, compared to DCD-III and DBD, and has three main findings. First, immediate graft function was higher in ODE, when compared to DCD-III, and similar to DBD. Second, longitudinally, eGFR for ODE did not differ from eGFR for DBD and eGFR for DCD-III over 10 years, after adjustment for donor sex, donor age, donor smoking, recipient age, recipient sex, CIT, AT, initial graft function and donor hypertension, donor diabetes mellitus, WIT and transplant

PRA. Third, graft failure for ODE did not differ from graft failure for DBD and graft failure for DCD-III, after adjustment for donor sex, donor age, donor smoking, recipient age, recipient sex, CIT, AT, and initial graft function, donor hypertension, donor diabetes mellitus, WIT and transplant PRA.

Human kidney transplantation remains the treatment of choice for the majority of patients with end-stage renal failure [19–21]. Despite increased numbers of donor organs due to expanded donor criteria, organs from living donors, and donation after circulatory death, the gap between the demand and availability of kidneys for transplantation remains substantial [22–25]. Although the results for ODE kidneys regarding longitudinal eGFR and graft failure were not statistically significantly different compared to those for DBD or DCD-III kidneys, the overall results for ODE support the concept that ODE kidneys are a promising extension of the donor pool. Notably, extension of the donor pool is not the primary goal of the procedure, because ODE is the patient's final altruistic wish.

Previous research on the outcomes of kidney transplantations following ODE was done in smaller cohorts and case series [11, 12], while no study has assessed longitudinal eGFR and graft function over 10 years. In contrast to others who included data that did not measure an extensive set of potential confounders [18] or only studied ODE compared to DCD-III [14], we focused on DBD, DCD-III, and ODE, using comprehensive data from a nationwide registry.

With regard to other organs donated following ODE, preliminary studies on graft function of transplanted lungs after ODE [26–28] yielded outcome results comparable to DCD-III grafts, and similar results were reported for transplanted livers [29–31]. Recently, the first successful heart transplantation after donation after euthanasia was published [32]. The current study thus found comparable transplant outcomes between ODE and DCD-III [29] and between ODE, DCD-III, and DBD [26] regarding graft failure for kidneys transplanted.

In the future, more patients will request to donate their organs after euthanasia, and it is expected that an increasing number of countries will allow this procedure. Observational data showed that approximately 10% of all patients undergoing euthanasia might be medically eligible to donate at least one organ [33].

However, the vast majority of patients who undergo euthanasia are suffering from end-stage malignancy, which makes them unsuitable as a donor. "Euthanasia donors" often suffer from neurodegenerative or psychiatric disorders, which are not primarily associated with deterioration of organ function of potentially transplantable organs, such as the heart, lungs, liver, kidneys and pancreas [4, 34]. DCD-III donors, who die after withdrawal from life-sustaining therapy, inevitably suffer from hypoxia, hypotension, and inadequate organ perfusion during the progression to circulatory arrest (agonal phase) and the mandatory 5-min period of warm, pulseless ischemia [35, 36]. Donors after brain death suffer from a systemic inflammatory response with a potentially negative impact on graft outcomes [37].

This study has strengths and limitations. A major strength is the large nationwide registry including all donations and transplantations in the Netherlands with a comprehensive set of donor, recipient and transplantation variables that created the opportunity to investigate potential confounding in the associations under investigation. Indeed, different studies have shown associations between either prolonged CIT or prolonged AT or both, and both kidney function and post-transplant graft failure [38, 39]. AT has also been associated with delayed graft function [40]. Hence the adjustments for these variables in the models of the present study. Furthermore, the Cox models were adjusted for transplant PRA as it indicates the risk of transplant failure to the host-response to transplantation [13, 14]. In addition, hypertension and diabetes mellitus, and tobacco exposure, have each been associated to worse eGFR [41]. Therefore, models were adjusted for recipient hypertension and diabetes mellitus and donor smoking behavior. As no information on smoking behavior in recipients was available, residual confounding might have caused us to underestimate the present associations, although most likely recipient smoking behavior is not dependent on ODE, DBD or DCD-III donors [42]. The criteria for HLA-mismatch are different for the first transplantation and for later transplantations and we chose to study the population that comprises the most recent transplantation of recipients in the analyses. Therefore, the associations could not be adjusted for HLA mismatch and this could have led to an underestimation of the present results.

Another strength is a total of four sensitivity analyses that were conducted. In sensitivity analysis 1, all kidney transplantations within recipients were included to investigate whether including multiple transplants for the same recipients changed the results. In the second sensitivity analysis, recipient death with functioning transplant, was included as an event, as graft survival and recipient deaths may be related. In sensitivity analysis 3, primary non-function was included as an event, despite it being considered a short-term transplant outcome. The fourth sensitivity analysis, considering only the first kidney transplantation per recipient, was performed as HLA matching is not independent of the number of transplantations within a recipient. The presence of donor-specific HLA antibodies before transplantation is considered a risk factor for graft rejection. Furthermore, waiting time until transplantation increases the risk of higher sensitization levels. Organ transplantation induces HLA alloimmunization, affecting the matching of a re-transplant and waiting time until transplantation [18].

Another strength lies in the approach of investigating the 10-year post-transplantation outcome in two different ways: using Cox proportional hazards analyses for the association between donor categories and graft failure and longitudinal analyses for the association between donor categories and eGFR, which together increase the validity of the results. The study has several limitations as well. In the Netherlands, post-mortem donation allocation is based on blood and tissue match between the donor and recipient of the organ, the medical urgency of the recipient, and other circumstances related to the condition of the organ or the background of the recipient. Neither the donor nor their relatives are allowed to choose a recipient [43]. One donor could have donated two kidneys to two

different recipients. However, we did not take into account the potential dependency between recipients who received a kidney from the same donor, which is a limitation of this study. This dependency between recipients could potentially have affected transplant outcomes, although the direction of its effect is difficult to assess. With regard to sensitivity analysis 1, to investigate whether including multiple transplants for the same recipients changed the results, it needs to be recognized that multiple kidney transplantations within a recipient during the disease course are dependent and this was not accounted for in the Cox models.

Furthermore, the current study's ODE sample size decreased considerably after 6 years of follow-up, potentially compromising the reliability of the longer-term findings. This limitation requires a cautious interpretation of results during the extended follow-up period of ODE grafts, suggesting that conclusions towards 10 years should be less strongly conveyed. Future research should thus focus on larger cohorts to enhance the robustness of long-term conclusions. Given the contemporary annual increase in the number of ODE procedures, it is, however, estimated that an analysis of the first 300 kidney transplantations will take at least 5 additional years.

Another limitation of the study is that recipient ethnicity could not be used in the MDRD4 equation, due to lack of information. This has potentially led to a small underestimation of follow-up eGFR. However, since the same error has been made within each recipient, this will not make major difference in the trend over time.

In conclusion, kidneys transplanted after euthanasia have a good immediate graft function and a comparable longitudinal eGFR over 10 years and comparable hazard for graft failure when compared to kidneys transplanted after brain death or circulatory arrest. Overall, these results support the concept that ODE kidneys are a promising contribution to the donor pool, and ODE should be continued.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: The dataset is available upon specific request to the NTS (Netherlands Transplantation Society). Requests to access these datasets should be directed to Cynthia Konijn-Janssen, dataverzoek@transplantatiestichting.nl.

ETHICS STATEMENT

Ethical approval was not required for the studies involving humans because the data were generated from a national dataset and anonymized beforehand. The data represent routine care. Therefore, ethical approval is not required. The studies were conducted in accordance with the local legislation and institutional requirements. The human samples used in this study were acquired from a by-product of routine care or industry. Written informed consent to participate in this study was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

WJ, JB, and WM established the study setup and design and collected the data. CS and BB analyzed the data and wrote the initial draft of the manuscript, after which subsequent drafts were collaboratively revised with ND, WM, HV, and JB. BB provided statistical support. WM coordinated the team efforts. ND submitted the manuscript. CS and ND contributed equally to this manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

CONFLICT OF INTEREST

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

ACKNOWLEDGMENTS

We thank Dr. Maarten H.L. Christiaans, nephrologist, Maastricht UMC+, for his valuable input during the first revision of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2024.13142/full#supplementary-material

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POSITION PAPER
published: 14 October 2024
doi: 10.3389/ti.2024.13485

ESOT Roadmap for Advanced Therapy Medicinal Products in Transplantation: Navigating Regulatory Challenges to Enhance Access and Care

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The field of organ transplantation is experiencing a transformative shift with the rise of Advanced Therapy Medicinal Products (ATMPs), which include gene therapies, somatic cell therapies, and tissue-engineered products. These therapies offer new, potentially curative treatments for longstanding medical challenges, impacting numerous patients. However, their adoption is hindered by complex regulatory frameworks, high production costs, and inconsistent access across Europe. The ESOT ATMP Task Force's position paper analyzes these challenges from research to clinical application, advocating for a coordinated strategy to position Europe as a leader in ATMP development. It proposes specific actions such as streamlining regulatory pathways to accelerate approvals, boosting funding for ATMP research, and creating specialized facilities for development and implementation. The paper also highlights the critical roles of patient engagement and real-world evidence in optimizing clinical and regulatory practices.

Keywords: organ transplantation, cell transplantation, advanced therapy medicinal products (ATMPs), regulatory challenges, patient access

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Received: *03 July 2024*
Accepted: *19 September 2024*
Published: *14 October 2024*

Citation:
Berishvili E, Piemonti L, de Koning EJP, Lindstedt S, Scholz H, Scott WE 3rd, Auxenfans C, Johnson P, Martin DE, Gunther P, Mey D, Potena L and Thaunat O (2024) ESOT Roadmap for Advanced Therapy Medicinal Products in Transplantation: Navigating Regulatory Challenges to Enhance Access and Care. Transpl Int 37:13485. doi: 10.3389/ti.2024.13485

Abbreviations: ESOT, European Society for Organ Transplantation; ATMPs, Advanced Therapy Medicinal Products; EU, European Union; EMA, European Medicines Agency; HE, Hospital Exemption; RWE, Real-World Evidence; RWD, Real-World Data; EVOP, *Ex Vivo* Organ Perfusion; CAR, Chimeric Antigen Receptor; Treg, Regulatory T-cell; MSCs, Mesenchymal Stromal Cells; TRL, Technology Readiness Level; GMP, Good Manufacturing Practices; FDA, Food and Drug Administration; CAT, Committee for Advanced Therapies; EC, European Commission; ENVI, Committee on the Environment Public Health and Food Safety; EMA/CAT, EMA's Committee for Advanced Therapies; SVF, Stromal Vascular Fraction; EC/1394/2000, Regulation EC/1394/2000; 2001/83/EC, Directive 2001/83/EC; FDA, Food and Drug Administration; GSK, GlaxoSmithKline.

INTRODUCTION

Advanced Therapy Medicinal Products (ATMPs) mark a transformative shift in the field of organ transplantation. These products, also known as "Biologics," are produced through complex biological processes involving living cells, tissues, or genetic materials. The development of ATMPs in organ transplantation aims to address critical issues such as organ scarcity, graft rejection, and the long-term viability of transplant outcomes. In the European Union, ATMPs fall under the regulatory framework for biological products, specifically Directive 2001/83/EC and Regulation EC/1394/2000. Within the EU, ATMPs are categorized into four main groups: gene therapy, somatic cell therapy, tissue-engineered therapies, and combined advanced therapies.

Despite their significant potential, ATMPs face numerous obstacles that hinder their practical application in clinical settings. One of the primary obstacles is the complex and lengthy regulatory approval process for ATMPs. The European Medicines Agency (EMA) and national competent authorities have established stringent guidelines to ensure the safety and efficacy of these innovative therapies. However, navigating these regulatory requirements can be time-consuming and resource-intensive, particularly for academia that lack the necessary expertise and infrastructure. Furthermore, high manufacturing costs associated with ATMP development and production, and significant economic hurdles after product approval, all of which impede the progress of ATMPs from research to clinical application and consequently limit patient access to these innovative therapies.

This position paper by the ESOT ATMP Task Force provides a detailed analysis of the multifaceted challenges that arise from the research phase through to the clinical implementation of ATMPs in organ transplantation across Europe. It identifies key hurdles in the development and deployment of these therapies and suggests strategic recommendations to facilitate their integration into healthcare systems. This would subsequently broaden the accessibility of ATMPs to patients across Europe, significantly enhancing outcomes in transplantation medicine.

PROMISES OF ATMPS IN TRANSPLANTATION

Organ Shortage

The demand for organ transplants far exceeds the available supply, leading to significant morbidity and mortality among patients requiring transplantation. ATMPs, including tissue engineering and xenotransplantation, represent promising solutions in regenerative medicine. These technologies offer potential breakthroughs in addressing significant medical challenges by repairing or replacing damaged tissues and organs. Tissue engineering aims to create functional organ substitutes or enhance the body's innate regenerative capabilities, potentially alleviating the demand for donor organs [1, 2]. Xenotransplantation, involving the transplantation of organs or tissues from one species to another, has been explored to address organ scarcity; notably from genetically modified pigs to humans in recent years [3].

Tissue engineering approaches, such as biodegradable scaffolds loaded with cells to create functional replacements for damaged tissues are being explored to address the limited availability of donor organs and tissues [4–8]. These methods leverage the body's natural regenerative capabilities to provide personalized and biocompatible solutions for patients requiring functional organ or tissue replacement.

Improvement of Graft Quality/Regeneration

Recent advancements in *ex vivo* organ perfusion (EVOP) have positioned it as a promising platform for organ-specific gene and cell therapy in transplantation [9, 10]. EVOP systems allow for precise genetic modifications to organs, delivery of gene therapies to the perfused organ, making it possible to correct genetic defects or enhance the organ's resilience against ischemia-reperfusion injury, a common post-transplant complication. Importantly, adenoviral vectors used during EVOP have been shown to reduce inflammation associated with gene delivery, as demonstrated in pig models of lung transplantation [11, 12]. This finding suggests that gene therapy can be effectively integrated into organ perfusion processes without exacerbating inflammatory responses, thus improving transplant outcomes. Moreover, the controlled environment of EVOP reduces the risk of off-target effects, increasing the safety of gene therapy. While EVOP's ability to assess and preserve organs remains significant, its role in enabling targeted gene delivery is particularly relevant for advancing transplantation outcomes and expanding the available donor pool by potentially rescuing marginal organs [9, 10, 13–16].

Somatic cell therapies involve manipulating cells or tissues to modify their biological characteristics. These therapies span a range of applications, from treating acute injuries to chronic diseases, and are particularly relevant for regenerating or repairing damaged tissues; potentially negating the need for complete organ replacement. Developments in stem cell research enhance the potential of somatic cell therapies, allowing for patient-specific stem cells to be used in various medical applications, including drug testing and regenerative medicine [17, 18].

Graft Rejection and Long-Term Outcomes

The recipient's immune system rejecting the transplanted organ remains a major hurdle in organ transplantation. It largely explains the stagnation of graft half-life over the last decades [19], thereby contributing to organ shortage.

Current prevention strategies predominantly involve immunosuppressive therapy, which poses significant side effects and long-term complications, including increased susceptibility to infections and cancer. ATMPs such as Chimeric Antigen Receptor (CAR) T-cell therapy and regulatory T-cell (Treg) therapy offer innovative approaches to mitigate these risks. CAR T-cell therapy, a type of gene therapy, has proven effective in treating post-transplant lymphoproliferative disorders, a common complication in solid organ transplants, by allowing genetically engineered T-cells to target and eliminate cancerous cells [20]. Treg therapy aims to

induce immune tolerance, potentially reducing the dependency on immunosuppressive treatments [16, 21–23]. Moreover, the development of immune cell phenotype modifications, such as CAR T, CAR Treg, and CAR B cells, is advancing transplant immunology by enhancing the specificity and efficacy of immunosuppression. CAR Treg therapy merges the targeting capabilities of CAR T-cell therapy with the regulatory properties of Tregs, providing a dual benefit in organ rejection prevention. Emerging CAR B-cell therapy may further enhance post-transplant immune regulation. Mesenchymal stromal cells (MSCs) offer an alternative cell-based approach for enhancing transplant outcomes by modulating immune responses and promoting tissue repair. Early-phase clinical trials have demonstrated the safety and feasibility of MSC infusions in kidney and liver transplant recipients. MSCs can potentially reduce the reliance on immunosuppressive medications by secreting anti-inflammatory cytokines and promoting graft tolerance. This approach aims to improve graft survival and decrease the side effects associated with long-term immunosuppression [24–26].

The longevity and functionality of transplanted organs are critical for patient outcomes. Chronic rejection, characterized by the gradual loss of organ function, remains a leading cause of transplant failure. ATMPs, through their ability to engineer organs with enhanced biocompatibility or to modulate the immune response more precisely, hold the promise of extending the life and function of transplanted organs. Tissue-engineered organs using the recipient's own cells combined with approaches that repair, or regenerate organ tissues could decrease rejection risks and enhance both functionality and longevity [1, 2].

Gene therapy could be used to modify the genetic material of donor organs *ex situ* to decrease rejection likelihood or induce recipient tolerance. By producing immunomodulatory proteins within donor grafts, gene therapy can achieve localized immunosuppression or even donor-specific tolerance, thus possibly eliminating the need for general systemic immunosuppression [27].

ACCESSIBILITY TO APPROVED ATMPS ACROSS EU AND REIMBURSEMENT CHALLENGES

The integration of ATMPs into clinical practice heralds a new era in transplantation medicine, where it may be possible to tailor treatments to the individual patient's needs, improving quality of life and longevity. However, this innovation comes with the requirement for rigorous evaluation to ensure safety, efficacy, and ethical considerations; underscoring the importance of robust regulatory frameworks and clinical guidelines to manage their application in transplantation. Due to the broad scope of applications, navigating the journey from research to market for ATMPs presents a complex and formidable challenge. The development and commercialization process is not only lengthy and expensive but also carries a high risk of failure. Following approval, these therapies often encounter what is termed the "economic valley of death," a phase where financial constraints significantly restrict patient access to these treatments. This issue is pervasive across medical fields, signalling a widespread systemic challenge.

Academic institutions and charities, which have been instrumental in pioneering ATMP research, frequently lack the financial capacity to support costly clinical trials. This financial gap often necessitates forming partnerships with corporations or establishing biotech startups to secure the necessary funding for continued development. The production costs associated with ATMPs, such as viral vectors and cell products, are substantial, sometimes reaching several million euros per patient. These costs are driven by stringent safety and quality standards coupled with the treatment of conditions that range from relatively common to extremely rare, which limits the patient base and increases unit costs.

Consequently, the high costs of these therapies result in prolonged or unsuccessful negotiations with health systems, leading to significant variations in access to approved ATMPs across Europe, driven by diverse reimbursement systems and policies [28]. Germany widely reimburses ATMPs through its public health system, whereas Ireland does not cover several EMA-approved ATMPs. The UK, France, and Spain provide more limited reimbursement, restricted to specific clinical indications. Such economic pressures have led to the withdrawal from the European market of several efficacious and approved products; depriving patients of potentially life-saving treatments. Bluebird bio withdrew Skysona and Zynteglo in 2022 despite their efficacy, citing non-viable reimbursement negotiations.[1]

Furthermore, despite considerable industry efforts to broaden access to autologous CAR T-cell therapy, numerous barriers persist, including complex logistics involving intercontinental cell shipments, manufacturing slot reservations, and bureaucratic delays.[2] A 2020 study on the accessibility of CAR T-cell therapy for patients with diffuse large B-cell lymphoma in Germany, France, Italy, and Spain showed that a significant proportion of patients, 58%–83% within the EMA-approved label population and 29%–71% deemed clinically eligible, did not receive commercially approved CAR T-cell products.[3] In Spain, it takes about 18 months from EMA approval to the authorization of price and reimbursement for orphan drugs, which include most ATMPs. Nearly one-third of these approved orphan drugs fail to secure reimbursement, with half being the only available treatment options for their respective diseases.[4] As of May 2023, only 20% of EMA-approved ATMPs were reimbursed by Spain's public national health system.[5] These reimbursement challenges are likely driven by cost implications.

In some cases, companies discontinue an ATMP post-approval if the economic returns do not justify the investment. For instance, UniQure discontinued Glybera in 2017 after

[1]https://joppp.biomedcentral.com/articles/10.1186/s40545-021-00311-0
[2]https://doi.org/10.1007/s40290-022-00428-w
[3]https://doi.org/10.3389/fmed.2023.1128295
[4]https://doi.org/10.1186/s13023-022-02610-4
[5]https://doi.org/10.1016/j.stem.2023.07.004

treating only one patient, as the costs and limited use did not justify the investment: even with a price tag of one million euros per patient. Similarly, Strimvelis®, initially developed by GSK and later acquired by Orchard Therapeutics, was discontinued due to commercial viability issues. Additionally, financial withdrawal by Valline Holding Srl led to the cessation of Holoclar®, the first stem cell derived ATMP in Europe, highlighting the precarious nature of sustaining such innovative treatments in the European market [1].

Systemic challenges in the European sector have led to the discontinuation of ATMPs, thereby jeopardizing access to life-saving treatments for a considerable number of citizens. Reimbursement constraints, insufficient economic returns, and bureaucratic hurdles have impeded the sustained development and commercialization of these therapies. Additionally, the high costs associated with the development and approval process deter many promising treatments from reaching the market. While Europe has historically been a leader in developing ATMPs, it has recently been outpaced by the US and Asia in advancements within this field. From 2014 to 2021, the number of ATMP clinical trials in the US and Asia-Pacific grew by 70% and 67%, respectively, while Europe's growth remained stagnant.[6] The US now hosts twice as many ATMP trials as Europe, and China nearly three times as many. Furthermore, the US dominates in the number of companies developing ATMPs and their contributions to the global new drug pipeline, with China's growth in this area increasing by 456% between 2016 and 2021.

Despite Europe's strong academic output in ATMP research, it falls short in translating this into practical therapies, contrasting sharply with the US and Asia where regulatory approvals are faster and the market pricing for ATMPs tends to be higher. Europe also struggles with a siloed approach to policymaking and has not capitalized on opportunities to foster growth in ATMP clusters, leaving it less competitive in attracting clinical trials and housing only 50% of the world's ATMP manufacturing facilities compared to Asia's rapid rise.[7]

This discrepancy poses a critical decision for the EU: to either remain a consumer of high-cost therapies developed abroad, potentially limiting patient access due to affordability issues, or to actively participate in the development of these therapies. To regain its footing, Europe should enhance funding, streamline regulatory processes, and strategically support ATMP innovation hubs.

ESOT ATMP TASKFORCE POSITION

Europe's ambition to emerge as a leader in the field of ATMPs hinges on its ability to increase funding, streamline regulatory processes, and strategically foster ATMP-innovation hubs to expedite clinical development. Recognizing the challenges associated with ATMPs—from the bench to bedside—the European Society for Organ Transplantation (ESOT) has convened several meetings to underscore the need for efficient procedures that facilitate the assessment, access, and clinical integration of these therapies before they become widely available in the market. This proactive approach aims to eliminate access barriers and streamline the journey from experimental phases to clinical application.

ESOT strongly advocates for refining the regulatory framework to ensure quick access to ATMPs without compromising on their safety and efficacy. The society promotes a holistic strategy addressing regulatory, manufacturing, cost, and healthcare system integration challenges. A collaborative approach involving stakeholders and utilizing evidence-based methods is essential to enhance the regulatory framework, enabling timely access to ATMPs while upholding rigorous safety and efficacy standards.

In support of this initiative, ESOT has established a task force focused on advancing ATMPs, dedicated to improving patient access to these advanced therapies, managing regulatory and ethical changes, and promoting the continuous development of innovative healthcare solutions within the European Union.

The journey of ATMPs from experimental phases to clinical application is intricate and multifaceted. Academic institutions, typically at the forefront of developing these innovative therapies, have been compelled to conform to stricter pharmaceutical manufacturing and clinical trial standards. A critical element of the ATMP regulation is the HE clause, which allows individual Member States to authorize the use of ATMPs without centralized marketing under specific conditions, primarily for patients with unmet medical needs. This exemption not only provides patients with access to potentially life-saving therapies but also opens avenues for collecting real-world evidence (RWE). RWE, derived from real-world data (RWD) collected outside conventional clinical trial settings, provides invaluable insights into the effectiveness, safety, and practical application of ATMPs, including cell and gene therapies, in routine clinical practice. Integrating RWE into ATMP development can significantly streamline the process by providing evidence on long-term outcomes, patient quality-of-life, and comparative effectiveness. This integration facilitates a more informed and nuanced understanding of ATMPs' real-world impact, aiding in the refinement and innovation of clinical trial designs and the development of more targeted and effective therapeutic interventions. However, concerns about the quality, consistency, and standardization of RWD must be addressed to ensure that the evidence generated is reliable and actionable. Efforts by regulatory bodies such as the EMA, particularly through initiatives like DARWIN EU®, are aimed at enhancing RWD quality and fitness for regulatory purposes by establishing standard methodologies and fostering data sharing across borders.[8]

[6]https://www.efpia.eu/news-events/the-efpia-view/efpia-news/europe-s-share-of-global-medicines-rd-shrinks-by-a-quarter-in-20-years-as-sector-s-declining-trends-continue/

[7]https://ecipe.org/publications/strategic-autonomy-competitiveness-europes-innovative-pharmaceutical-sector/

[8]https://www.ema.europa.eu/en/news/use-real-world-evidence-regulatory-decision-making-ema-publishes-review-its-studies

The interpretation and implementation of HE varies widely across the EU, leading to inconsistencies in patient access and potential safety concerns. The issue of non-exportability of ATMPs under the HE poses another significant challenge, particularly since these treatments are intended for patients in therapeutic impasse. To address this, it is necessary to facilitate the exchange of ATMPs under HE between Member States, underscoring the importance of European mutual assistance. Furthermore, the coexistence of HE with the central marketing authorization route often leads to perceptions of unfair competition against commercially marketed medicinal products.[9,10]

The Committee on the Environment, Public Health, and Food Safety (ENVI) addressed some of these concerns by voting on 19 March 2024, on a set of 100 Compromise Amendments. These amendments not only preserve the Hospital Exemption within the Member States unchanged but also strengthen its implementation in the cross-border exchange of ATMPs, aiming to improve access and equity across Europe. In its current version, the Directive proposal does not introduce any change that could have a major impact on the management of manufacturing authorizations for ATMPs under hospital exemption. However, the modifications made by the Directive proposal suggest a strong willingness, through the collection of data, to reinforce the management of these products by the Member States and, through the communication with the EMA, to further harmonize the rules governing ATMPs under hospital exemption.

To this end, leveraging RWE becomes imperative. Recognizing the significance of this approach, the ESOT is well-positioned to lead the establishment and management of ATMP registries in the field of organ, cell, and tissue replacement. By doing so, ESOT can facilitate the systematic collection and analysis of RWD, thereby enriching the RWE pool. These efforts can contribute significantly to shaping the regulatory landscape, informing policy decisions, and guiding clinical practice in organ transplantation. The ESOT intends to require for standardized data collection methods to ensure the reliability and validity of the RWE generated. Furthermore, ESOT can play a pivotal role in fostering collaborations among scientific societies, transplantation centers, regulatory bodies, and the pharmaceutical industry to promote the sharing of RWD and RWE, thus driving forward the field of ATMPs.

The European legislation on ATMPs, which includes cell therapies, gene therapies, and tissue-engineered products, has significantly influenced academic networks involved in their development since its enactment approximately 20 years ago. This legislation, particularly Regulation (EC) No 1394/2007, reclassified cell and gene therapies that underwent "substantial manipulation" or were intended for non-homologous functions, moving them from the organ and tissue transplantation regulatory framework to that of pharmaceuticals. While this shift enhanced regulatory oversight and experience with ATMPs, it has affected approval rates due to the stringent and inflexible regulatory demands, significantly increased costs, and generated market uncertainty, which restrict investment from the pharmaceutical sector. Moreover, some holders of approved products have subsequently withdrawn them, primarily for regulatory and economic reasons, as discussed above.

Recently, the EMA released the second version of the draft guideline on quality, non-clinical, and clinical requirements for investigational ATMPs (EMA/CAT/123573/2024). This release marks a significant step in the review of the EMA's pharmaceutical framework. The feedback from the initial draft underscored the necessity for clearer guidelines on non-viral and genome editing therapies and called for greater regulatory alignment, especially with FDA standards. The revisions improved terminology consistency and introduced a risk-based approach, yet stakeholders indicated that further refinements were needed, especially a clearer separation of guidelines for different ATMP types and more detailed guidance for their development. These ongoing legislative reforms and the discussions taking place across various platforms present an opportunity for ESOT to influence and help shape a regulatory framework that is both robust and conducive to the rapid development and integration of ATMPs and other emerging technologies in clinical practice. This framework should also establish clear guidelines for new and evolving therapeutic approaches, ensuring that such innovations are neither restricted by outdated regulations nor delayed when patient lives are at risk. This approach would enable the safe and effective delivery of promising therapies to patients across the EU, continuing the tradition of innovation in medical treatment.

ESOT RECOMMENDATIONS

To streamline the development and accessibility of ATMPs in the field of transplantation in Europe, ESOT recommends implementing following strategies:

Streamlining Regulatory Processes

The complexity and duration of the regulatory approval process can significantly impact the development and accessibility of ATMPs. It is essential for regulatory bodies to streamline their procedures to expedite the approval timeline and reduce associated costs, while still upholding stringent safety and efficacy standards. In revisiting the EMA/CAT definition of ATMPs, consideration should be given to whether minimally modified cell therapy products like the Stromal Vascular Fraction (SVF) warrant distinct regulatory pathways depending on their use in homologous versus non-homologous therapies. For example, the classification of SVF could potentially vary between applications in plastic surgery and treatments for scleroderma, focusing more on the nuances of the manufacturing process rather than just the clinical application. This approach would allow production facilities to use targeted risk assessments as a measure of manufacturing process quality, ensuring that regulatory standards are met without unnecessarily impeding product development and innovation.

[9]https://www.eahp.eu/sites/default/files/he_atmp_position_eptri_eahp_eueye_july2023_final_c.pdf?utm_medium=email&utm_campaign=website&utm_source=EAHP25 Congress

[10]https://www.bag.admin.ch/dam/bag/de/dokumente/biomed/transplantationsmedizin/studie-hospital-exemptions-atmp-eu-2022.pdf.download.pdf/studie-hospital-exemptions-atmp-eu-2022.pdf

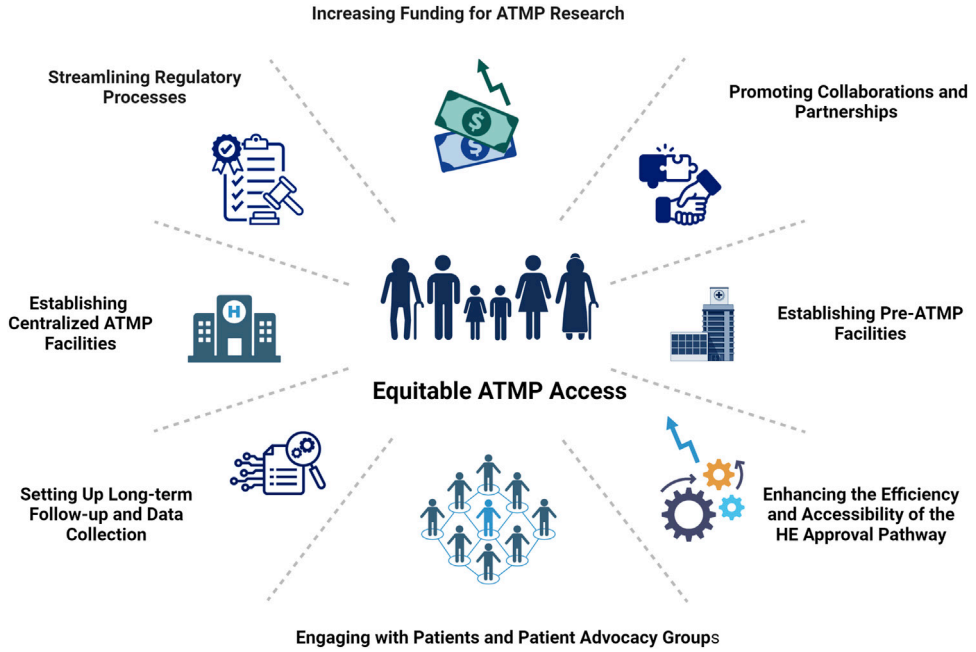

FIGURE 1 | ESOT vision for accelerating equitable access to ATMPs. This figure outlines ESOT's collaborative strategies to address translational barriers and facilitate the safe, rapid integration of ATMPs into clinical practice, promoting sustainable growth and equitable patient access to innovative medicines. Created in BioRender. Berishvili, E. (2024) BioRender.com/j50w273

Increasing Funding for ATMP Research

Significant investment is required to develop ATMPs, yet public funding often overlooks these needs. National science funding should prioritize not only the creation of new knowledge but also the establishment of clinical trials and safety studies, activities typically classified at TRL4 and higher. Funding agencies are encouraged to increase their investments in ATMP research with a focus on academic research and development to facilitate scaling.

Promoting Collaborations and Partnerships

Effective development and translation of ATMPs require collaboration among academic researchers, industry partners, and regulatory agencies. Increased support for partnerships, including funding, infrastructure, and regulatory support, is essential.

Establishing Pre-ATMP Facilities

Developing pre-ATMP facilities can enhance the efficiency and success rate of ATMP projects by providing a platform for extensive testing of products for compatibility, safety, and efficacy before full-scale GMP production and for developing and validating quality control methods, particularly potency assays. This is critical for ensuring that promising therapies are not held back by a lack of foresight and experience by using non-compliant products in the formative research activities. Collaborative efforts to standardize potency assays for ATMPs with similar modes of action would streamline processes, save time, and facilitate easier comparisons between products.

Establishing Centralized ATMP Facilities

Specialized facilities and expertise are crucial for the successful development and production of ATMPs. Centralized ATMP facilities would provide accessible infrastructure and regulatory affairs expertise, which are vital for clinical translation. Experts in these facilities should possess knowledge in establishing quality management systems, training personnel in Good Manufacturing Practices (GMP), defining release criteria, and navigating approval requirements.

Enhancing the Efficiency and Accessibility of the Hospital Exemption (HE) Approval Pathway

HE pathway facilitates the use of ATMPs outside standard marketing authorization for patients with unmet medical needs. However, variations and inconsistent applications across countries reduce its effectiveness. Harmonizing HE rules is crucial to ensure that ATMPs, especially those not commercially viable, can still reach patients, support rapid manufacturing innovations, and provide uninterrupted treatment during clinical development.

Optimizing ATMP Development in Europe Through Comprehensive Data Collection and Analysis

To assess the current status of ATMPs across Europe, it is critical to conduct a comprehensive survey that incorporates RWE

derived from RWD collected beyond conventional clinical trials. RWE is essential for transitioning ATMPs from experimental phases to clinical use, providing deep insights into their effectiveness, safety, and impact on patient quality of life. These insights help improve clinical trial design and treatment efficacy.

To maximize the benefits of HE in ATMP development, it is imperative to establish robust registries. These registries should meticulously record patient outcomes, treatment specifics, and adverse events. The detailed data collected will facilitate the generation of RWE, enhancing the foundation for more informed regulatory and clinical decisions. This structured approach ensures that ATMPs are not only effective but also safe and well-suited to meet patient needs.

Engaging With Patients and Patient Advocacy Groups

Incorporating the perspectives of patients and patient advocacy groups is crucial in the clinical development and translation of ATMPs. Engaging with these stakeholders early in the development process is essential for identifying and understanding the barriers and facilitators that could influence the adoption and impact of ATMPs on patient communities. Conducting empirical research on patient perspectives ensures that the clinical translation of ATMPs is responsibly aligned with the needs and expectations of patients. This collaborative approach can lead to more effective and patient-centered healthcare solutions.

CONCLUSION

The field of organ transplantation, and more broadly, medicine, is undergoing a significant transformation driven by the development and integration of ATMPs. These innovative therapies are poised to revolutionize the treatment landscape by offering new, potentially curative options for conditions that have long challenged medical professionals and affected the lives of countless patients.

The ESOT ATMP task force has meticulously outlined the critical challenges and strategic recommendations in this position paper focused on the European context. As ATMPs continue to evolve, the key to harnessing their full potential lies in the effective collaboration among a diverse array of stakeholders. These include scientific societies such as ESOT, academic researchers who are often at the forefront of ATMP innovation, and pharmaceutical and biotechnological companies that facilitate the scaling and distribution of these therapies. Additionally, regulatory bodies play a pivotal role in ensuring that these therapies are both safe and effective, while patient groups contribute invaluable insights that help tailor ATMPs to meet actual patient needs and expectations.

The collective effort of these stakeholders is fundamental not only in overcoming regulatory, financial, and technical barriers but also in establishing a robust framework that supports the rapid development, approval, and integration of ATMPs into clinical practice (**Figure 1**). This collaborative approach ensures that the revolutionary potential of ATMPs can be realized, ultimately changing the course of diseases and significantly improving patient outcomes.

In essence, the transition of ATMPs from experimental therapies to mainstream treatment options represents a paradigm shift in medicine that holds promise for a future where many diseases may no longer be seen as terminal or incurable. As this field continues to advance, the continued engagement and coordination of all parties involved will be critical in making these life-altering therapies accessible to those who need them most.

DATA AVAILABILITY STATEMENT

This position statement does not present original data. It is informed by a comprehensive review of current regulatory frameworks governing ATMPs in transplantation, relevant policy documents, and expert consensus within the ESOT task force.

AUTHOR CONTRIBUTIONS

All authors contributed to the conception and design of the study. EB wrote the first draft of the manuscript. LP, CA, and OT wrote sections of the manuscript. All authors contributed to the critical editing of the article and approved the submitted version.

FUNDING

The authors declare that no financial support was received for the research, authorship, and/or publication of this article.

CONFLICT OF INTEREST

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

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REVIEW
published: 17 October 2024
doi: 10.3389/ti.2024.11872

Outcome of Solid Organ Transplantation in Patients With Intellectual Disability: A Systematic Literature Review

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Access to solid organ transplantation in patients with intellectual disability is associated with health inequities due to concerns about treatment adherence, survival rates, and post-transplant quality of life. This systematic literature review aims to compare outcomes after organ transplantation in patients with intellectual disability compared to patients without intellectual disability. Embase, Medline Ovid, PsycINFO, Web of Science, Cochrane Central Register of Trials, and Google Scholar databases were systematically searched for studies concerning pediatric or adult solid organ transplantation in recipients with a diagnosis of intellectual disability prior to transplantation. Primary outcomes were patient and graft survival rates. Secondary outcomes were acute rejection rate, adherence rates, and quality of life. Nine studies were included, describing kidney (n = 6), heart (n = 4) and liver (n = 1) transplantation. Reported graft survival rates were non-inferior or better compared to patients without intellectual disability, while patient survival was reportedly slightly lower in two studies reporting on kidney transplantation. Although current evidence has a potential selection bias based on including patients with a sufficient support network, intellectual disability alone should not be regarded a relative or absolute contra-indication for solid organ transplantation.

Keywords: organ transplantation, intellectual disability, graft survival, rejection, adherence

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Received: *30 July 2023*
Accepted: *26 September 2024*
Published: *17 October 2024*

Citation:
de Rover I, Orlandini L, Darwish Murad S, Polak WG, Hartley J, Sharif K, Sneiders D and Hartog H (2024) Outcome of Solid Organ Transplantation in Patients With Intellectual Disability: A Systematic Literature Review. Transpl Int 37:11872. doi: 10.3389/ti.2024.11872

INTRODUCTION

Intellectual disability (ID) as defined by the DSM-5 criteria affects approximately 1% of the general population [1, 2]. ID is associated with increased incidence of concomitant chronic disease and decreased life-expectancy [3]. Additionally, clinicians consider quality of life to be decreased in patients with ID, however when asked, many patients with ID report an acceptable quality of life [4]. Organ transplantation in patients with ID may raise additional concerns, regarding treatment adherence, post-transplant survival benefit, and whether improvement in quality of life after organ transplantation is achievable [5, 6]. Therefore, ID has historically been considered a relative or absolute contraindication for organ transplantation [7, 8].

In the face of organ shortage, transplant benefit and graft utility are important principles guiding access to transplantation and allocation of organs. Along with criteria such as the patients need or urgency and the probability of a successful outcome [9]. However, a report written by the National Council on Disability stated that many transplant centers in North America still have reservations about solid organ transplantation in people with ID: studies from 2006 to 2008 found that 43%–60% of transplant centers considered some degree of ID as an absolute or relative contraindication to transplantation [10]. These assumptions also impacted on a centers' willingness to evaluate a patient with ID and place them on the waiting list. Approximately one-fifth of transplant centers had formal guidelines for listing candidates with ID and half had informal guidelines [11]. To prevent potential discrimination against people with ID in the allocation of donor organs, decision-making should ideally be based on scientific data, and consensus guidelines would be required.

The present systematic literature review aims to provide an evidence-based analysis of the currently available literature concerning the outcomes of solid organ transplantation in patients with ID, while comparing this to patients without a disability.

METHODS

This systematic literature review was written according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) [12]. Additionally, guidelines for synthesis without meta-analysis (SWiM) in systematic reviews were followed [13]. The systematic literature review protocol was PROSPERO registered under registration number CRD42020161607.

Search Strategy

Comprehensive searches were performed by a biomedical information specialist. Six databases were searched for relevant articles: Embase, Medline Ovid, PsycINFO, Web of Science, Cochrane Central Register of Trials, and Google Scholar (**Supplementary Appendix 1**). Duplicate entries were removed. Subsequently, unique records were reviewed based on title and abstract by two independent reviewers (IdR, LO). Records selected based on title and abstract were further reviewed for final selection based on the full text article. Disagreement was resolved by consensus with a third reviewer (DS). Finally, manual cross-referencing was performed to identify potentially relevant studies not included in the initial search.

Study Selection

Original studies were included if they studied pediatric or adult patients with a pre-transplantation diagnosis of ID and compared results to a control group in the setting of solid organ transplantation. Studies were included if they described any of the primary outcomes (graft and patient survival). We excluded case reports and studies discussing ID diagnosed post-transplantation. Studies without an available full text record or written in other languages than English were also excluded.

Data Extraction and Study Outcomes

Data extraction was performed with a standard extraction table and included study design, type of solid organ transplantation, age, sex, ethnicity, average IQ, definition, assessment, and selection of patients with ID for transplantation, diagnosis regarding ID, and indications for transplantation. The primary outcome of this systematic literature review was defined as the patient and graft survival in solid organ transplantation patients with pre-transplantation diagnosed ID. Episodes of rejection, adherence rates and quality of life were secondary outcomes.

Quality Assessment

Quality assessment was conducted by two independent reviewers (IdR, LO). The Robins tool, a standard quality assessment tool for non-interventional and observational studies [14], did not differentiate well between the quality of included studies. Therefore, the quality of methodological steps was assessed and summarized for all studies, including source population, case definition, patient selection bias, definition of outcomes and data collection methods. Overall quality of the individual studies was summarized along principles of scope and purpose, design, sampling of the studied cohort, data collection, analysis, validity, generalizability, and credibility.

Data Synthesis

Outcome data was extracted and grouped per specific organ, and then tabulated or described in the review text. Possible outcomes were described with reference to the accurate definition and classification of the outcome. Survival proportions were given as described by the individual studies or estimated from survival curves as described and validated previously [15].

RESULTS

Literature Search Results

3,690 records (**Figure 1**) were screened based on title and abstract, after removal of duplicates. A total of 142 full texts were assessed and finally nine studies were included for quantitative synthesis [16–24]. Three studies were excluded since a more recent study provided an update of previous data [24–27]. One study presented data on kidney, heart and liver transplantation [24] whereas the other studies presented data on either kidney (n = 5) [16–18, 21, 22] or heart (n = 3) [19, 20, 23] transplantation. All studies included patients with ID and patients without ID. Three studies were single-centered [16–18], whereas the other studies were multicentered. Three studies presenting data on heart transplantation and two studies on kidney transplantation likely used, in part, duplicate data from registries (UNOS/OPTN/Medicare) with overlapping inclusion periods between 2004 and 2017 [20, 21, 23, 24].

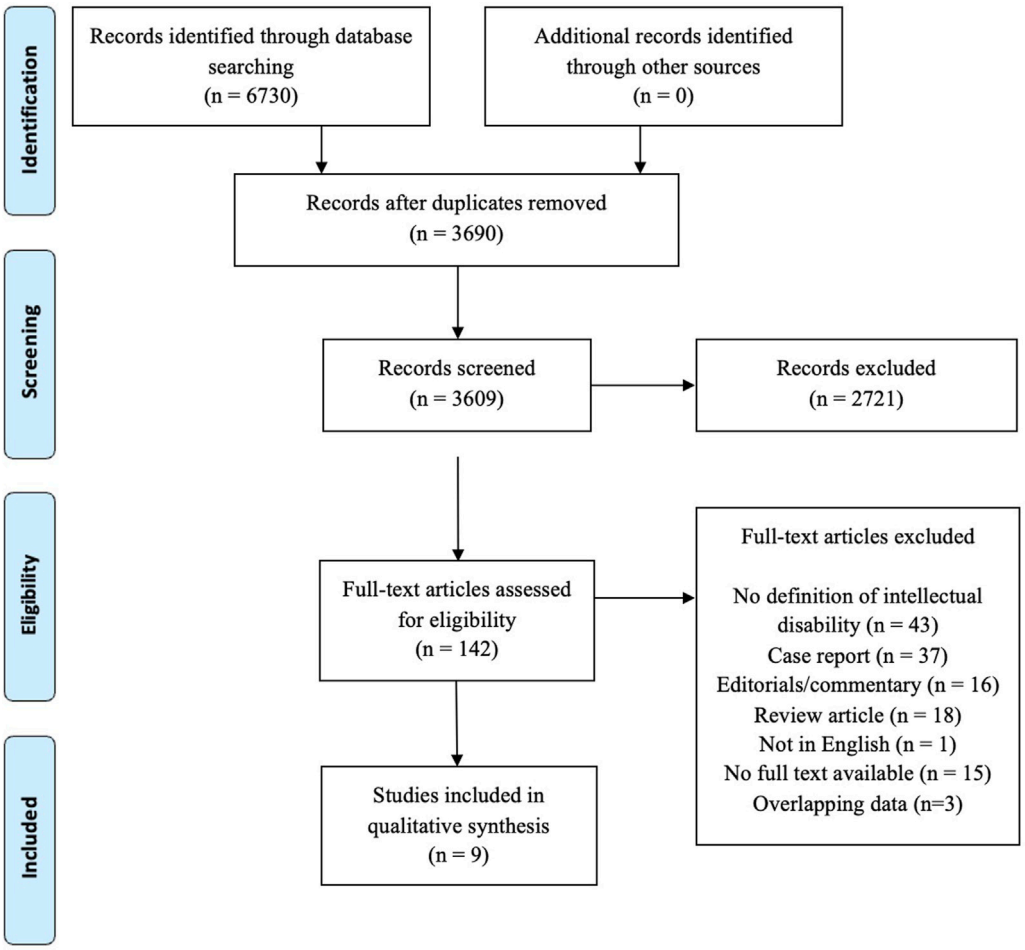

FIGURE 1 | PRISMA flow chart.

Quality Assessment

Quality assessment of individual studies and of the entire review sample is summarized in **Table 1** and **Figure 2**. All studies were observational studies, collecting their data from patient charts or prospectively maintained registry databases. Definition of ID was clearly stated by five studies [16–18, 21, 22], and three of them commented appropriately on the assessment of ID [16, 17, 22]. Eight out of nine studies are at risk for selection bias as the studied populations may not represent the entire source population of patients with ID assessed or waitlisted for transplantation [16–20, 22–24]. Additionally, most studies were at risk of bias related to sampling of the population [16, 19, 20, 22–24]. Adequate follow-up periods (i.e., median follow-up above 36 months) were described by five studies [16, 17, 19, 23, 24]. Definitions of outcomes were infrequently provided. Five studies corrected results for potential confounding factors [16, 19, 21, 23, 24].

Study Characteristics

Supplementary Table A, B summarize the baseline characteristics of included studies. Studies were published between 1968 and 2023 in Japan (n = 1), Brazil (n = 1) or the USA (n = 7). Six out of nine studies (69%) included pediatric patients only [17, 19, 20, 22–24]. The other three studies included pediatric patients, young adults and adults with a maximum age of 49 [16, 18, 21]. Since outcome data for individual patients was not available, it was not possible to perform a sub-analysis on pediatric and adult patients. Various underlying disorders, such as genetic syndromes, congenital disorders, cerebral palsy, and developmental brain anomalies, were registered as cause of ID in all included studies. One study divided the study population in definite ID, probable ID and no-ID [20], whereas one study divided the patients into "within 1 grade level of peers," "delayed grade level" or "in need of special education" [23].

Definition and Assessment of ID

Two studies followed the definition of the American Association on Intellectual and Developmental Disabilities [17, 18] and two others the American Psychiatric Association definition [16, 22] (**Table 2**). Five studies used definitions that were not uniformly based on consensus guidelines or included registry data.

TABLE 1 | Quality assessment of individual studies.

	Definition intellectual disability	Assessment of ID	Prospective data collection	Representative source population	Sampling (potential for selection bias)	Follow-up	Definition of rejection	Definition of compliance	Definition of quality of life	Controls from similar source population	Sampling controls (potential for selection bias)	Correction for confounders
Benedetti et al. [16]	1	1	3	3	3	1	3	1	3	1	3	1
Chen et al. [17]	1	1	3	3	1	1	2	4	4	1	1	2
Godown et al. [19]	3	2	3	3	3	1	1	4	3	3	3	1
Hand et al. [21]	1	2	3	1	1	3	1	4	4	1	1	1
Galante et al. [18]	1	2	3	3	2	3	2	1	4	3	3	3
Otha et al. [22]	1	1	3	3	3	3	1	1	3	3	3	3
Goel et al. [20]	3	3	3	3	3	3	2	4	4	1	3	2
Prendergast et al. [23]	3	3	3	3	3	1	4	4	4	1	1	1
Wightman et al. [24]	3	3	3	3	3	1	3	4	3	1	1	1

ID, intellectual disability, 1 no concerns, 2 not reported, 3 Any concern, 4 Not applicable.

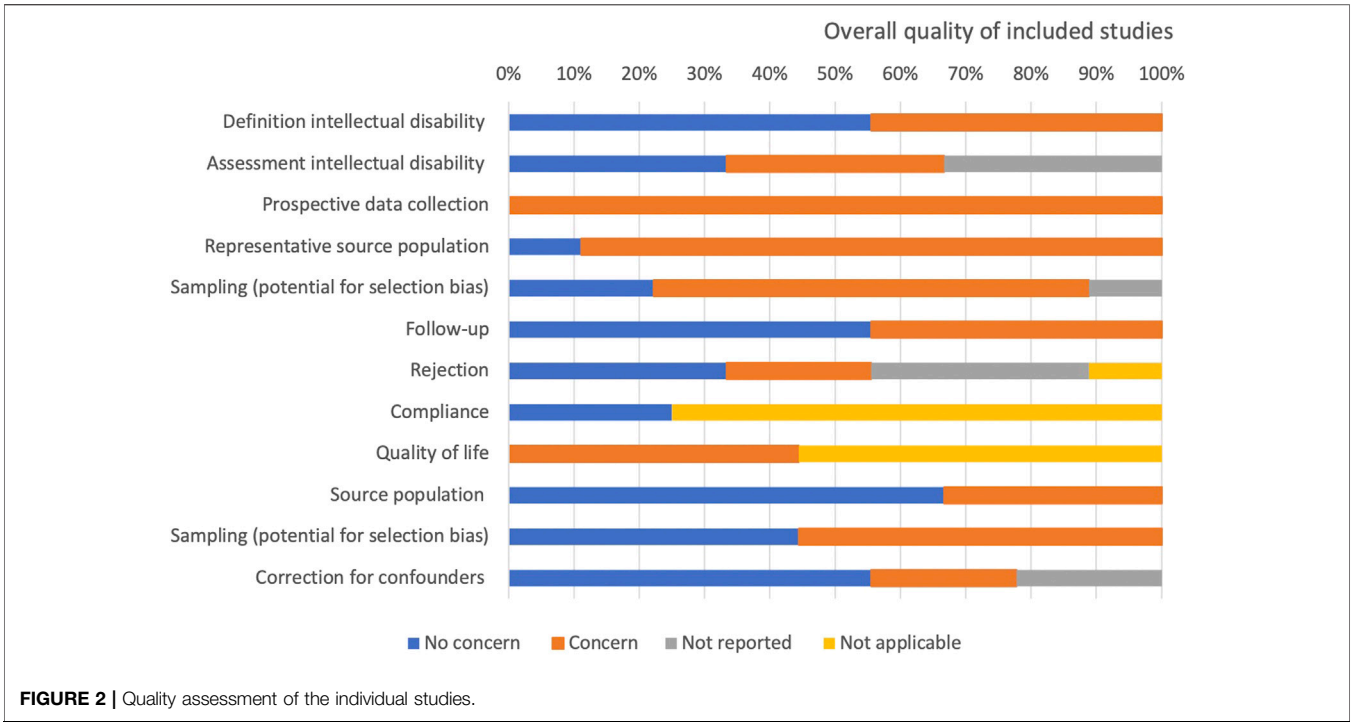

FIGURE 2 | Quality assessment of the individual studies.

TABLE 2 | Definition of intellectual disability by included studies.

Study	Definition of ID	Assessment of ID
Benedetti et al. [16]	A significantly subaverage general intellectual functioning and concurrent deficits in adaptive functioning with onset prior to age 18. (American Psychiatric Association)	Standardized intelligence tests, IQ < 70, administered by a consultant neuropsychologist
Chen et al. [17]	Patient with severe deficits in multiple areas of function (adaptive, language, cognitive, motor, and self-care) who need a full-time caregiver irrespective of age based on definition of ID. (AAIDD)	Criteria from DSM-5 or Bayley II
Galante et al. [18]	Defined as stated by the AAIDD	ND
Goel et al. [20]	Definite ID: definite cognitive delay/impairment Probable ID: patients who met two of the three criteria: "probable" or "questionable" cognitive delay/impairment, "reduced academic load/non-participation," or "delayed grade level/special education"	UNOS registry data was used, therefore assessment may vary
Godown et al. [19]	Patients with Down syndrome	ND
Hand et al. [21]	ICD codes for intellectual disability, pervasive developmental disorders, cerebral palsy or Down syndrome	ND
Prendergast et al. [23]	CD: DGL/need for special education/documented by provider as definite, probable, or questionable CD	OPTN registry data was used, therefore assessment may vary
Ohta et al. [22]	A significantly subaverage general intellectual functioning and concurrent deficits in adaptive functioning with onset prior to age 18 (American Psychiatric Association)	Intelligence quotient (IQ) or/and developmental quotient (DQ) by standardized intelligence tests as Wechsler. Intelligence Scale for Children-Third Edition or tests as Kyoto Scales of Psychological Development and Emoji Developmental Test
Wightman et al. [24]	Likert scales for (definite or probable) cognitive delay/impairment	UNOS registry data was used, therefore assessment may vary

ID, intellectual disability; AAIDD, American association on intellectual and developmental disabilities; CD, cognitive delay; IQ, intelligence quotient; DQ, development quotient; ICD, international classification of disease; OPTN, organ procurement and transplantation network; WGL, within 1 grade level of peers; DGL, delayed grade level; SE, special education; ND, Not described. #Likert scales: 1, definite cognitive delay/impairment; 2, probable cognitive delay/impairment; 3, questionable cognitive delay/impairment; 4, no cognitive delay/impairment; and 5 not assessed.

Assessment of ID differed among the included studies. Two studies based their assessment on IQ, after assessment by a neuropsychologist [16, 22]. Another study assessed ID following the criteria of the DSM-5 or Bayley-II [17]. Three studies did not comment on the exact assessment of ID within the study population or used registry data [20, 23, 24].

TABLE 3 | Graft survival after kidney, heart and liver transplantation in patients with intellectual disability and controls.

Graft survival	Sub group	N (ID)	N (Control)	1 year		3 years		5 years		10 years		P-value
				ID (%)	Control (%)	ID (%)	Control (%)	ID (%)	Control (%)	ID (%)	Control (%)	
Kidney transplantation												
Benedetti et al. [16]		8	100	100	86		ND	100	66		ND	0.04
Chen et al. [17]		10	62	100	88	100	80	100	77		ND	NS
Galante et al. [18]		16	83	88	94	81.2	88	81.2	80.2	73	70	NS
Ohta et al. [22]		25	164	100	95		ND	100	87		ND	NS
Hand et al. [21]		629	629	ND	ND[a]		ND		ND		ND	NS
Wightman et al. [24]^		594	5,643	98	97		ND	93	85	71	64	<0.01
Heart transplantation												
Goel et al. [20]	Def ID	131	1,959	88	91	84	84		ND		ND	NS
	Prob ID	434	1,959	91	91	82	84		ND		ND	NS
Prendergast	DGL	269	1,707	95	97	88	90	77	85		ND	<0.001
et al. [23]	SE	269	1,707	97	97	93	90	89	85		ND	NS
Wightman et al. [24]^		324	2,762	99	85	ND	ND	94	92	75	85	NS
Liver transplantation												
Wightman et al. [24]^		318	3679	93	95	ND	ND	92	92	92	87	NS

ID, intellectual disability; Def, definite; Prob, probable; DGL, delayed grade level; SE, special education; ND, not described; NS, no significant difference.
[a]*Exact numbers for ID and control not provided, overall >98%, ^ = death-censored graft survival.*

TABLE 4 | Patient survival after kidney, heart and liver transplantation in patients with ID and controls.

Patient survival	Sub group	N (ID)	N (Control)	1 year		3 years		5 years		10 years		P-value
				ID (%)	Control (%)	ID (%)	Control (%)	ID (%)	Control (%)	ID (%)	Control (%)	
Kidney transplantation												
Benedetti et al. [16]		8	100	100	97		ND	100	94		ND	NS
Chen et al. [17]		10	62		ND	100	98		ND		ND	NS
Galante et al. [18]		16	83	87	100	81	100	81	97	72	97	<0.05
Ohta et al. [22]		25	164	100	98		ND	100	98		ND	NS
Hand et al. [21]		629	629	ND	ND[a]		ND		ND		ND	NS
Wightman et al. [24]		594	5,643	99	99		ND	96	98	95	96	<0.01
Heart transplantation												
Goel et al. [20]	Def ID	131	1,959	89	92	86	86		ND		ND	NS
	Prob ID	434	1,959	92	92	82	86					NS
Godown et al. [19]		23	ND	100	ND	92	ND	92	ND	92	ND	NS
Wightman et al. [24]		324	2,762	95	92	ND	ND	86	83	73	72	NS
Liver transplantation												
Wightman et al. [24]		318	3,679	96	95	ND	ND	91	92	85	90	NS

*ID, intellectual disability; Def, definite; Prob, probable; *, p < 0.05; n, population; NS, no significant difference; ND, not described.*
[a]*Exact numbers for ID and control not provided, overall >98%.*

Selection of Patients With ID for Transplantation

The selection criteria of patients with ID for organ transplantation varied slightly between the studies. Four studies selected patients based on the reliability of their support network and the ability to take oral medication under supervision in order to minimize risk of rejection [16–18, 22]. Three studies did not specify how patients with ID were selected or excluded from organ transplantation [19, 23, 24]. One of the included studies evaluated a cohort of patients with end stage kidney disease. In this study, patients with ID were less likely to be evaluated for transplantation (OR: 0.46; 95% CI, 0.43–0.50) and less likely to be transplanted (OR: 0.38; 95% CI, 0.34–0.42) compared to propensity score matched patients without ID [21]. However, the latter study was based on registry data therefore criteria on which patients were selected remain unclear.

Graft and Patient Survival

Reported graft and patient survival is summarized in **Table 3**, **4**. Two studies on kidney transplantation and three studies on heart transplantation with potentially overlapping data, are shown in parallel [20, 21, 23, 24]. Reported graft survival was better or equal in patients with ID compared to control patients in seven out of nine studies. A study on heart transplantation reported a significantly lower graft survival in patients with delayed grade

TABLE 5 | Rejection in intellectual disability versus no intellectual disability per organ transplantation.

Study	Definition of rejection	Intellectual disability	Control	P-value
Kidney transplantation				
Chen et al. [17]	Biopsy proven rejection	1/10 (11%)	17/62 (27%)	0.29
Galante et al. [18]	Rejection-free survival	7/16 (75%)	24/83 (67%)	0.79
Ohta et al. [22]	Clinically manifested and treated rejection	7/25 (28%)	61/164 (37%)	0.40
Benedetti et al. [16]	Biopsy proven rejection	4/8 (50%)	46/100 (46%)	0.38
Hand et al. [21]	ICD-10 code T68.11 (since 2015) corresponding to graft rejection	50/629 (8.0%)	47/629 (7.5%)	NS
Wightman et al. [24]	UNOS definition	101/594 (17%)	1,524/5,643 (27%)	NS
Heart transplantation				
Goel et al. [20]	UNOS definition	Def ID: 22/131 (24%) Prob ID: 57/434 (18%)	295/1959 (20%)	Def ID: 0.207 Prob ID: 0.354
Godown et al. [19]	Clinical event, biopsy confirmed or not, that prompted augmentation of immunosuppression regimen	10/23 (43%)	ND	0.77
Wightman et al. [24]	UNOS definition	42/324 (13%)	249/2,762 (9%)	NS
Liver transplantation				
Wightman et al. [24]	UNOS definition	32/318 (10%)	405/3,679 (11%)	NS

Def, definitive; Prob, probable; n, population; p, p*-value; NS, no significant difference; ND, not described.*

level compared to controls, whereas this was not reported for patients with special education [23]. Patient survival was reported to be equal in patients with ID compared to control patients in the majority of studies. Two studies reported significantly lower patient survival in kidney transplant recipients with ID compared to control patients. The study by Galante et al. reported significantly lower patient survival (survival at 5 years: ID 81%, n = 16 versus control: 97%, n = 83, $p < 0.05$). The larger registry based study by Wightman et al. reported significantly lower patient survival as well, although the actual reported survival difference was fairly minimal 95% versus 96% estimated survival at 10 years [18, 24].

Treatment Adherence

Three studies (including 369 patients) presented data on medication adherence [16, 18, 22]. The criteria for non-adherence to the overall treatment process included cyclosporine or tacrolimus levels below 30 ng/mL or 1.5 ng/mL, >20% missed clinical visits and/or a post-transplantation weight gain of more than 20% above ideal body weight. All three studies reported complete treatment adherence (i.e. 100%, n = 49) amongst patients with ID. In two studies including a control group, adherence rates were 94% (n = 83) and 100% (n = 164) in patients without ID [18, 22].

Acute Rejection

Data on acute rejection was reported by 8 out of 9 studies and is summarized in **Table 5**. Definitions of rejection were reported by six studies [16–19, 21, 22] and were defined as biopsy proven or the need to adjust the immunosuppression regimen. Two studies were based on registry data and therefore used the definition as provided by UNOS [20, 24]. None of the included studies reported a significant difference in incidences of acute rejection in patients with and without ID.

Quality of Life

Quality of life was assessed in four studies [16, 19, 22, 24]. Nearly all patients receiving a kidney transplant were on peritoneal dialysis or hemodialysis prior to transplantation. One study described an increase in quality of life in all patients and in 60% of the main caregivers [22]. Another study found that 100% of the main caregivers expressed the opinion that the patients' quality of life had improved compared to dialysis [16]. Both studies used caregiver reported outcome measures rating the patient's quality of life on a five-point Likert scale and comparing potential impact of kidney transplantation. None of these changes in quality of life have been compared to scores in controls. A study concerning heart transplantation scored the functional status post-transplantation of the patients according to the assistance needed in daily activities and found similar values pre- and post-transplantation. These results were not compared to a control group [19]. The study including patients with kidney, liver and heart transplantation presented data on functional status and found an improvement of 90%–100% post-transplantation in all groups [24].

DISCUSSION

This study provides a systematic overview of available literature on the outcomes after solid organ transplantation in patients with ID, compared to patients without ID. Graft and patient survival was not impaired in patients with ID in the majority of reports. Although varying definitions were used, acute rejection rates were not increased in patients with ID. Available studies do not suggest a substantial deficit in treatment adherence in patients with ID. Quality-of-life post-transplantation was studied in nearly half of the included studies. Although using various scoring tools, transplantation appears associated with improved quality of life in patients with ID. Among included studies both the

definition and assessment of ID differed substantially or was not fully described. One study assessed patients with end-stage kidney disease and found the chances to be evaluated for transplantation and to actually receive a transplantation to be significantly lower (54% and 62% respectively) in patients with ID as compared to matched control patients [21]. Also, if pre-transplant selection criteria were reported, it was unclear what the criteria, such as 'sufficient support network' were. More data is required to detail the support network of the patients with ID, the amount of self-support, and their health status before transplantation.

Results of solid organ transplantation in patients with ID appear favorable, reporting adequate survival, adherence, and improved quality of life when an adequate support network is present. This is in accordance with a prior review from Wightman et al. [28], which included in addition disease-specific case studies on disorders variably causing ID. Another report from Thom et al. [29] supports this conclusion and discussed the ethical and legal aspects of the access to organs for patients with impaired decision making capacity. Current perceptions on ID being a relative or absolute contra-indication for organ transplantation are not ethically justifiable. Allocation of organs should be based on outcomes of transplantation in patients with and without ID rather than ethical considerations about benefit, utility, and fairness.

As quality of life is not routinely measured or considered in organ allocation, the relativity and subjectivity of such argumentation in the current context is emphasized. Societal and ethical values or impact are even more complex to quantify. For detailed ethical considerations we would refer to the excellent review written by Wightman et al., who concluded that exclusion based on intellectual disability would not be defensible from a societal and ethical perspective, and the recent recommendations by Thom et al. [6, 29]. In order to prevent discrimination of patients with ID and reach consensus among transplantation centers, it is important to define specific legislation. In North America, this is currently being developed, with the most recent being the introduction of the Charlotte Woodward Organ Transplant Discrimination Prevention Act to the senate of the United States [30], which prohibits to deny or restrict individual access to organ transplants solely on the basis of ID. In Europe, the European Disability Strategy was launched in 2021 by the European Union in order to protect the rights of people with disabilities [31]. The rising number of laws have also evoked criticism because interpretation in practice can still be highly ambiguous [32]. In a survey study from Richards et al. more than half of the included transplant programs report that informal processes guide the use of neurodevelopmental delay in the decision of listing a patient for transplantation and thereby emphasizes the lack of clinical implementation [33]. Some say rather than legislation, the field could benefit from unambiguous definition of the meaning and role of disability for consideration for solid organ transplantation [34]. An interesting approach is the social model of disability, proposed by Sara Goering, that describes how social norms can be disabling, rather than the objective impairment itself [35]. For example, the presumption that disability indicates a decreased quality of life may not be how intellectual disabled patients experience this themselves. Listening to these experiences can challenge how clinicians understand disability and its role considering scarce resources. Additionally, a more pragmatic perspective on this matter was studied by Freiberger et al. [36] at the Boston Children's Hospital Center by assigning an advisory committee to ensure transplant selection criteria were nondiscriminatory. Data showed that amongst race and socioeconomic factors, patients with a severe neurodevelopmental delay had a significantly lower chance of being listed compared to controls. The suggested institutional committees can fill in the gaps between law and practice, and provide solutions were possible. Although more international data on decision making, listing and quality of life after transplantation is needed to ensure fair distribution of transplant organs, regional initiatives, as seen in Boston, show a valuable contribution to this matter.

Limitations

This systematic literature review has several limitations. Included studies focused mainly on post-transplant outcomes, little data is provided on patients with ID on the transplant waitlist or patients with end stage organ failure not considered for transplantation. Therefore, it remains unclear how large the total population of patients with ID and end-stage organ failure is in need for organ transplantation. In addition, a selection bias of patients with an adequate support network and therefore suspected sufficient adherence may have occurred, resulting in favorable outcomes. Nevertheless, it may also be argued that adherence in patients lacking decisional capacity is mostly higher due to engagement of caregivers [29]. Three included studies on heart transplantation and two studies on kidney transplantation used registry databases with overlapping inclusion periods [20, 21, 23, 24]. Unfortunately, varying definitions of ID were used, and severity of ID was usually not considered. Generally, studies were small or presented a low level of detail, used unclear or wide definitions, and assessment methods were often unstandardized or subjective, therefore pooled analysis was not possible.

Conclusion

Based on the current available literature, albeit of suboptimal methodological quality and limited scope, there is no evidence to support views that intellectual disability should in and of itself be considered a contra-indication for solid organ transplantation. Our results support the recommendations stating that specific international guidelines and their translation to clinical practice are necessary to prevent discrimination based on intellectual disability in the allocation of organs. Solid organ transplantation in patients with intellectual disability may have predominantly been performed in patients with a network available to support management and treatments required when living with a donor organ. In these patients, outcomes appear satisfactory and do not suggest lack of adherence or insufficient improvement in quality of life, although more data is needed to validate these conclusions.

AUTHOR CONTRIBUTIONS

IdR: participated in research design, performance of the research, data analysis and writing of the paper. LO: participated in research design, performance of the research, data analysis and writing of the paper. SM: participated in the writing of the paper. WP: provided in the search strategy for the paper, participated in the writing of the paper. JH: participated in the writing of the paper. KS: participated in the writing of the paper. DS: participated in research design, performance of the research, data analysis and writing of the paper. HH: participated in research design, performance of the research, data analysis and writing of the paper. All authors approved the manuscript.

FUNDING

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

CONFLICT OF INTEREST

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

ACKNOWLEDGMENTS

Internal resources were used for the study conduct and manuscript preparation.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2024.11872/full#supplementary-material

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ORIGINAL RESEARCH
published: 31 October 2024
doi: 10.3389/ti.2024.13569

Activation of the Innate Immune System in Brain-Dead Donors Can Be Reduced by Luminal Intestinal Preservation During Organ Procurement Surgery - A Porcine Model

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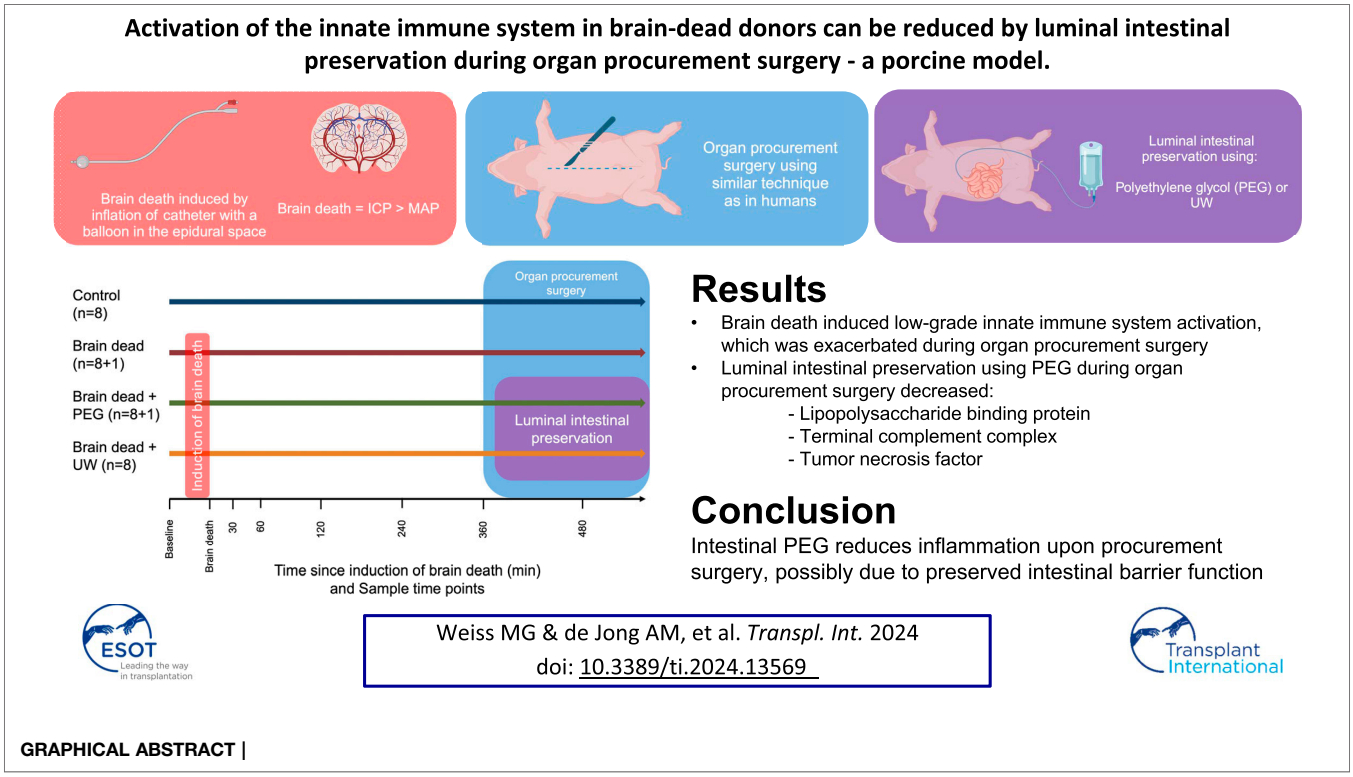
Received: *22 July 2024*
Accepted: *14 October 2024*
Published: *31 October 2024*

Citation:
Weiss MG, de Jong AM, Seegert H, Moeslund N, Maassen H, Schjalm C, de Boer E, Leuvenink H, Mollnes TE, Eijken M, Keller AK, Dijkstra G, Jespersen B and Pischke SE (2024) Activation of the Innate Immune System in Brain-Dead Donors Can Be Reduced by Luminal Intestinal Preservation During Organ Procurement Surgery - A Porcine Model. Transpl Int 37:13569. doi: 10.3389/ti.2024.13569

Organs obtained from brain dead donors can have suboptimal outcomes. Activation of the innate immune system and translocation of intestinal bacteria could be causative. Thirty two pigs were assigned to control, brain death (BD), BD + luminal intestinal polyethylene glycol (PEG), and BD + luminal intestinal University of Wisconsin solution (UW) groups. Animals were observed for 360 min after BD before organ retrieval. 2,000 mL luminal intestinal preservation solution was instilled into the duodenum at the start of organ procurement. Repeated measurements of plasma C3a, Terminal Complement Complex (TCC), IL-8, TNF, and lipopolysaccharide binding protein were analysed by immunoassays. C3a was significantly higher in the BD groups compared to controls at 480 min after brain death. TCC was significantly higher in BD and BD + UW, but not BD + PEG, compared to controls at 480 min. TNF was significantly higher in the BD group compared to all other groups at 480 min. LPS binding protein increased following BD in all groups except BD + PEG, which at 480 min was significantly lower compared with all other groups. Brain death induced innate immune system activation was decreased by luminal preservation using PEG during organ procurement, possibly due to reduced bacterial translocation.

Keywords: brain dead donor, luminal intestinal preservation, C3a, terminal complement complex, lipopolysaccharide binding protein, innate immune system, organ procurement and porcine model



GRAPHICAL ABSTRACT |

INTRODUCTION

Deceased donors for organ transplantation comprise brain-dead donors and circulatory dead donors. Studies have established worse outcomes in donor organs obtained from brain dead donors, compared to healthy living donors [1–3] due to a multitude of factors including activation of the immune system [3–9]. The inflammatory response to brain death includes the release of damage-associated molecular patterns and activation of the innate immune system [4–6, 10]. Activation of the innate immune system in brain dead donors leads to damage to the donor organs, resulting in poorer outcomes in the recipients [3, 5, 7–9, 11, 12].

The trauma inflicted by organ procurement surgery is thought to exacerbate the initial inflammatory response in brain-dead donors, leading to additional activation of the immune system during organ procurement [13]. However, the mechanisms involved are unknown.

Although the effects of brain death on the immune system are already established at the time of organ donation decision-making, improvements can be made to limit the additional impact of organ procurement surgery.

Damage to the intestines upon brain death and possibly during organ procurement surgery leads to translocation of bacteria and bacterial remnants to the systemic circulation, which may activate and prolong activation of the innate immune system and trigger an adaptive immune response [14, 15]. Intestinal leakage can be reduced by preserving the intestinal barrier function [16], which has been demonstrated in several studies during static cold storage of intestinal grafts in rats [17–21], pigs [22] and humans [23] using luminal intestinal preservation. In these studies, luminal intestinal preservation was introduced for the preservation of intestinal grafts and was applied following vascular flush. Several clinically available organ preservation solutions have been tested for luminal intestinal preservation. University of Wisconsin static cold storage solution (UW) has been evaluated in several studies [24–26]. Polyethylene glycol (PEG) has been found to have the same preservation characteristics as UW luminal intestinal preservation [18, 19, 22].

We hypothesized that implementing a luminal intestinal intervention using established intestinal preservation fluids at the onset of organ procurement surgery in brain dead organ donors could decrease intestinal leakage and systemic immune system activation caused by the organ procurement surgery. We utilized an established porcine model of brain death to evaluate the impact of an intervention on the small intestine, consisting of luminal intestinal preservation during organ procurement surgery, using either PEG or UW solution. Our objectives were to characterize the immune response to brain death and to test two commonly used intestinal preservation solutions' effect on this immune response.

MATERIALS AND METHODS

Ethics and Animals

Thirtytwo female laboratory pigs (Danish landrace, Duroc, and Yorkshire crossbreed) from the same breeder, weighing 58–62 kg

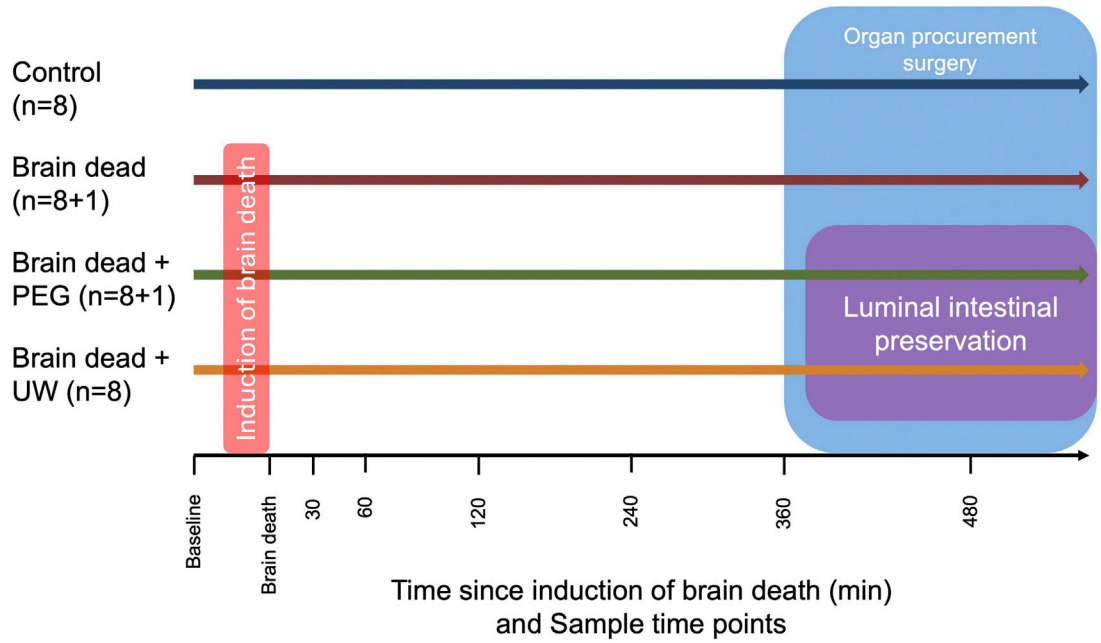


FIGURE 1 | Overview of experimental groups, timeline of sample time points and interventions by groups. Animals in the brain dead, brain dead + PEG and brain dead + UW group had brain death induced while animals in the control group did not. All animals had a intracranial pressure sensor placed. In the control group, the observation period began 20 min after placing the pressure sensor to ensure approximate equal observation time. Organ procurement surgery was caried out in all groups, animals in the brain dead + PEG and brain dead + UW groups had 2,000 mL of the group appropriate solution instilled in the small intestine over approximately 2 h during surgery. Control: control group, Brain dead: brain dead without luminal intestinal intervention, Brain dead + PEG: brain dead with luminal intestinal intervention using polyethylene glycol, Brain dead + UW: brain dead with luminal intestinal intervention using University of Wisconsin solution.

were planned. Two additional pigs were added later in the study due to extra pigs needed in two studies utilizing organs from this study. One pig was added to the brain dead without luminal intestinal preservation group for a project using the kidneys [27] and one pig was added to the brain dead with luminal intestinal preservation using PEG for a project using the small intestine (unpublished). All animal handling was conducted in concordance with the European Union- (directive 2010/63/EU) and local regulations. The project was approved by the Danish Animal Experiments Inspectorate (reference number: 2019-15-0201-00157).

Study Design

Animals were divided into four groups, non-brain-dead control (Control) (n = 8), brain dead without luminal intestinal preservation group (Brain dead) (n = 8 + 1), brain dead with luminal intestinal intervention using PEG (Brain dead + PEG) (n = 8 + 1) and brain dead with luminal intestinal intervention using UW (Brain dead + UW) (n = 8) (**Figure 1**).

Animals were excluded from the study when one of the predefined criteria occurred at any time during the experiment: illness (diarrhea, signs of active pericarditis, peritonitis or tumors), brain death was not possible to establish, SaO_2 < 90% with FiO_2 of 0.3 for more than 15 min, mean arterial blood pressure < 60 mmHg for more than 15 min despite fluid resuscitation and noradrenaline infusion, death before vascular flush.

Anesthesia

The pigs were fasted from midnight with free access to water. Pigs were sedated using "Zoletil-mix" [Zoletil 50 vet. (125 mg Tiletamin, 125 mg Zolazepam), 125 mg Xylazin, 125 mg Ketamin, 25 mg Butorphanol] intramuscularly before transport. Upon arrival at the research facility, two 18G intravenous catheters were placed, one in each ear. If necessary 50–100 mg of Propofol was administered intravenously for intubation. After intubation, anesthesia was maintained using Propofol (8 mg/kg/h) and Fentanyl (10 μg/kg/h). Ventilation was achieved with a tidal volume between 8 and 10 mL/kg and regulated to a $PaCO_2$ between 5.5 and 6.5 kPa. A 14Fr urethral indwelling catheter was placed and connected to a two-chamber collection bag, allowing for precise hourly measurements of urine output.

After infusion of 1 Liter of Ringers acetate, 400 mL of blood was removed from all pigs, regardless of group allocation. This blood was used for normothermic machine perfusion of the kidneys in another study [27].

Induction of Brain Death and Observation

After turning the pig to a prone position, an approximately 10 cm incision was made along the sagittal suture. Intracranial pressure monitoring was established in all animals using a ralk hand drill with a 5 mm drill bit, a CH5 bolt, and a NEUROVENT-PTO catheter (Raumedic, Helmbrechts, Germany). A 20 mm Hudson hand drill and back-biting forceps were used for the brain dead

groups to make a burr hole. Subsequently, a CH22 Foley-type catheter (Unomedical, Lejre, Denmark) was placed in the epidural space through the burr hole.

Induction of brain death was performed according to a previously established model [28]. Briefly, the balloon of the CH22 catheter was inflated with saline, at a rate of 1 mL/min for 10 min, followed by 0.5 mL/min for an additional 10 min, and 0.25 mL/min, until a persistent negative cranial perfusion pressure (mean arterial pressure minus intracranial pressure) confirmed brain death. Following confirmation of brain death, the balloon was inflated with an additional 10 mL of saline to ensure maintenance of brain death during the entire experiment. Induction of brain death was followed by a 30-minute period with no additional interventions.

The control group only had the NEUROVENT-PTO catheter placed, the start of the observation period before organ procurement surgery began 20 min after finishing placement of the catheter, to ensure approximately equal observation time in all groups.

After these 30 min, treatment was administered for hypotension (mean arterial blood pressure < 60 mmHg: fluid bolus, noradrenaline infusion), hypertension (mean arterial blood pressure > 150 mmHg: Propofol, Labetalol), bradycardia (heart rate < 30/min: Atropine), tachycardia (heart rate > 200/min: fluid bolus, Metoprolol, Labetalol) and diabetes insipidus (urine output > 1,000 mL/h: Desmopressin) guided by an anesthesiologist trained in intensive care management and organ donor care.

Surgical Procedure

After 360 min of observation following induction of brain death, the pig was turned back to a supine position and the organ procurement surgery commenced with a midline laparotomy. A feeding tube was inserted into the duodenum through a small incision in the ventricle's anterior surface. The distal ileum and proximal duodenum were ligated in pigs of the brain dead + PEG and brain dead + UW groups. The small bowel was filled with 2 Liters of 4°C group-appropriate preservation fluid from the start of surgery and for the following 2 h. The filling volume was based on a presumed safe filling volume of <2 mL/cm of intestine in a 60 kg pig [22].

The abdominal organs were prepared for explantation in sequential order: kidneys, liver, and pancreas. The aorta and vena cava inferior were dissected free from the surrounding tissue and ligatures were placed around both vessels to allow for distal closure and cannulation of the abdominal aorta for vascular flush. Next, a sternotomy was performed, and the ascending aorta and the pulmonary trunk were separated and prepared for canulation.

A timeout was held, 500 IE/kg of heparin was administered and after 3 min, a St. Thomas cannula was placed in the ascending aorta. The abdominal aorta and vena cava inferior were closed distally and the abdominal aorta was canulated using a 14Fr cannula (Bridge to Life, London, England). The ascending aorta was cross-clamped, 1,000 mL of cold St. Thomas I solution was infused through the St. Thomas cannula, and the inferior vena cava was transected above the diaphragm. The abdominal aorta was cross-clamped above the liver, 4 L of cold Belzer UW Cold Storage Solution (Bridge to Life, London, England) was infused through the cannulation of the abdominal aorta, and the inferior vena cava was transected proximally to the ligature closing of the venous return from the legs. Crushed glucose ice (50 mg/mL glucose saline) was placed around all transplantable organs to provide external cooling and two suction devices were used to remove warm blood from the thorax and abdomen. Upon cardiac arrest, tissue biopsies were taken from the heart and lungs. After the vascular flush of the abdominal organs was completed, the small intestine was ligated in both ends and removed. Next, tissue samples of the liver, pancreas, and psoas muscle were obtained and the kidneys were removed.

Blood Samples

Blood samples were collected at baseline (immediately following intubation and central venous access), time of brain death (T = 0), 30, 60, 120, 240, and 360 min after brain death. Additional blood samples were collected after 2 h of procurement surgery, prior to vascular flush (480 min after brain death). Blood was collected in EDTA vacutainer tubes and stored on ice for 30 min before centrifugation at 2.500G at 4°C for 15 min. Supernatants were aliquoted and stored at −80°C until analysis.

Lipopolysaccharide Binding Protein, Cytokines, and Complement Factors

EDTA Plasma samples were analyzed for lipopolysaccharide binding protein (LBP) (Hycult Biotech, HK503, Uden, Netherlands), IL-8 (Merck & Co., PCYTMAG-23K, Rahway, NJ, United States), and TNF (R&D Systems, PTA00, Minneapolis, MN, United States) according to the manufacturer's instructions. Complement components C3a and terminal complement complex (soluble TCC, sC5b-9) were assessed using highly specific in-house porcine ELISA as described previously [29]. Briefly, neoepitopes on sC5b-9 and C3a were detected by specific monoclonal capture antibodies reacting with the C3 fragment [30] and activated C9 [31], respectively. Activation levels were related to the Internation Complement Standard #2, defined to contain 1,000 complement arbitrary units (CAU) per milliliter [32]. All results are normalized to albumin to account for the differences in volume status between brain dead and non-brain dead animals.

Tissue Samples

Snap frozen punch biopsies were taken from the heart, lungs, liver, small intestine, pancreas, kidneys, and psoas muscle at the time of removal. Frozen punch biopsies were stored at −80°C. The biopsies were homogenized and prepared for analysis for content of TNF, C3a, TCC, and IL-8 as described previously [33]. However, tissue IL-8 was not reliably detectable in >50% of samples and was thus excluded.

Statistics

The sample size of eight animals per group was calculated based on an estimated reduction of innate immune system activation of >20% in the BD groups with luminal intestinal intervention, compared to BD without luminal intestinal intervention.

TABLE 1 | Baseline characteristics. Characteristics of experimental animals after anesthesia and establishing of invasive monitoring.

	Control (n = 7)	Brain dead (n = 8)	Brain dead + PEG (n = 7)	Brain dead + UW (n = 8)	Excluded (n = 4)	*p*-value
Animal weight, kg	63.0 (3.6)	62.5 (4.1)	66.0 (9.6)	60.2 (1.6)	61.8 (2.2)	0.32
Temperature, C°	37.4 (0.6)	37.0 (0.6)	37.0 (0.8)	36.9 (0.5)	37.4 (0.4)	0.38
Circulation						
Heart rate/min	73 (18)	60 (21)	66 (22)	71 (18)	84 (25)	0.41
Systolic blood pressure, mmHg	119 (11)	112 (11)	125 (17)	117 (16)	123 (18)	0.56
Diastolic blood pressure, mmHg	78 (14)	71 (10)	82 (16)	77 (12)	81 (15)	0.63
Mean arterial blood pressure, mmHg	97 (15)	90 (9)	100 (17)	96 (14)	98 (18)	0.76
Continuous cardiac output, L/min	6.5 (2.2)	5.5 (1.4)	4.9 (0.7)	5.0 (0.6)	4.9 (0.1)	0.45
Mixed venous O_2, %	63 (9)	65 (3)	63 (6)	65 (5)	64 (8)	0.98
Respiration						
End tidal CO_2, kPa	6.1 (0.6)	6.1 (0.7)	5.9 (0.8)	5.9 (0.6)	6.1 (1.0)	0.95
Saturation, f	1.00 (0.01)	0.88 (0.32)	0.99 (0.01)	0.99 (0.01)	0.99 (0.02)	0.55
Arterial blood gas						
pH	7.45 (0.03)	7.46 (0.05)	7.44 (0.05)	7.47 (0.06)	7.47 (0.05)	0.76
Lactate, mmol/L	1.3 (0.6)	1.3 (0.3)	1.4 (0.5)	1.3 (0.1)	1.0 (0.2)	0.67
Bicarbonate, mmol/L	31.8 (0.8)	31.4 (2.9)	30.2 (1.4)	31.5 (1.3)	31.8 (1.1)	0.45
Glucose, mmol/L	6.5 (1.0)	6.2 (1.0)	6.0 (0.6)	6.1 (0.8)	5.9 (1.0)	0.86
Potassium, mmol/L	3.72 (0.17)	3.63 (0.13)	3.76 (0.14)	3.73 (0.18)	3.80 (0.08)	0.41
Sodium, mmol/L	141 (2)	141 (1)	141 (1)	140 (2)	140 (1)	0.60
Biochemistry						
Hemoglobin, mmol/L	6.6 (0.4)	6.1 (0.6)	6.2 (0.3)	6.0 (0.5)	6.2 (0.4)	0.19
Leukocytes, $\times 10^9$/L	16.7 (1.9)	14.6 (2.6)	15.2 (4.4)	15.6 (1.6)	19.9 (3.1)	0.085
Thrombocytes, $\times 10^9$/L	311 (85)	272 (67)	321 (105)	284 (74)	305 (46)	0.77
Alanine transferase, mmol/L	70.0 (11.9)	71.6 (27.0)	68.7 (16.0)	77.1 (16.0)	77.3 (22.5)	0.90
Lactate dehydrogenase, U/L	574 (149)	682 (244)	545 (68)	631 (160)	699 (201)	0.68
Creatinine, µmol/L	102 (19)	106 (29)	120 (24)	114 (25)	112 (18)	0.70
Urea, mmol/L	1.87 (0.06)	1.77 (0.29)	2.00 (0.36)	2.06 (0.53)	2.05 (0.64)	0.88
Albumin, g/L	10.7 (1.1)	11.3 (1.3)	10.7 (1.3)	10.8 (1.0)	11.0 (0.0)	0.87

All values are presented as mean (standard deviation). Comparison between groups using one-way analysis of variance.

Additionally, it was estimated that approximately 10% of animals would get excluded due to the above-mentioned criteria. Complete randomization was not possible due to the kidneys and intestines being used in other projects [27], requiring prior planning. Instead, subjects were randomized before each experiment between either control and brain dead or brain dead + PEG and brain dead + UW.

All analyses of blood, and tissue samples were performed blinded to group allocation.

At baseline, continuous variables were presented as mean and standard deviation when normally distributed, as assessed by quantile-quantile plots. Comparison of characteristics at baseline and of experimental characteristics was conducted using one-way analysis of variance with *post hoc* comparison of multiple means using Tukeys test. Repeated measurements of blood samples were described as median and interquartile range (IQR). Comparisons between groups and time points for repeated measurements were made with a multilevel mixed-effects linear regression model with group and time as fixed effects and individual pigs as random for all analysis, except circulating IL-8 and TNF, which were analysed using Wilcox signed-rank test, due to the non-normal distribution of values. To visualize the cytokine development from baseline to end-of-procurement, relative changes of plasma cytokine concentrations were calculated as a X-fold induction at end-of-procurement compared to baseline values. Comparison of tissue C3a, TCC, and TNF was done by Kruskal-Wallis one-way analysis of variance. Statistical significance was defined as a p-value of < 0.05. All statistical analyses were performed using STATA 17.0 (STATA Corp., College Station, Texas, United States) and GraphPad Prism 10.0 (GraphPad Software, Boston, Massachusetts, United States).

RESULTS

Study Characteristics

A total of 30 animals remained in the study: control (n = 7), brain dead (n = 8), brain dead + PEG (n = 7), and brain dead + UW (n = 8). Four animals were excluded due to the predefined exclusion criteria [pig #7 (brain dead + PEG; peritonitis), #8 (control; death before vascular flush), #14 (brain dead + PEG; death before vascular flush) and #30 (brain dead; pericarditis and peritonitis)].

At baseline, there were no significant differences between the four groups and/or the animals excluded (**Table 1**). After induction of brain death, intracranial pressure was consistently above 100 mmHg, ensuring abolished perfusion of the brain (**Figure 2**). Brain death resulted in a significantly increased heart rate ($p \leq 0.001$) and cardiac output ($p = 0.005$), compared to controls. This difference persisted throughout the experiment. Mean arterial pressure was kept within predefined limits using fluid boluses and vasoactive drugs, mainly in the brain dead

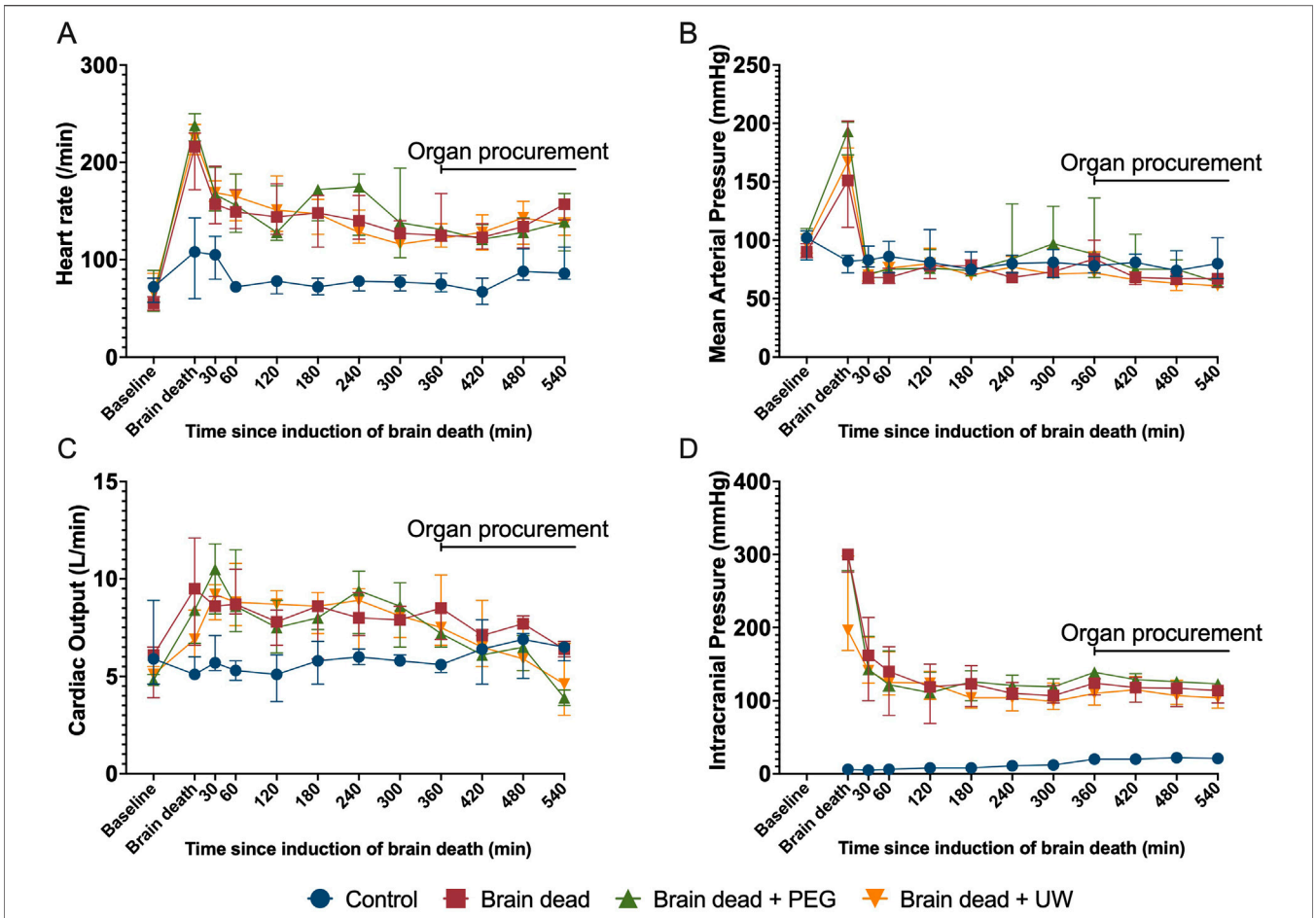


FIGURE 2 | Systemic circulation during brain death, 6 h of observation, and organ procurement surgery. Heart rate **(A)**, mean arterial pressure **(B)**, continuous cardiac output **(C)**, and intracranial pressure **(D)** were kept stable in brain-dead animals after initial typical Cushing-reflex reactions upon cessation of brain perfusion due to high intracranial pressure. Animals received fluids and norepinephrine and a mean arterial pressure of above 65 mmHg was maintained throughout the experiment and organ procurement period. Control: control group, Brain dead: brain dead without luminal intestinal intervention, Brain dead + PEG: brain dead with luminal intestinal intervention using polyethylene glycol, Brain dead + UW: brain dead with luminal intestinal intervention using University of Wisconsin solution. Values presented as median ± Interquartile range.

TABLE 2 | Experimental characteristics. Characteristics of experiments and end of surgery.

	Control (n = 7)	Brain dead (n = 8)	Brain dead + PEG (n = 7)	Brain dead + UW (n = 8)	Control vs. Brain dead (*p*-value)
Total experiment duration, h:m	11:16 (00:19)	11:30 (00:31)	10:53 (00:21)	11:14 (00:27)	0.071
Duration of brain death induction, h:m	N/a	00:21 (00:11)	00:17 (00:08)	00:21 (00:06)	0.781
Duration of organ retrieval surgery, h:m	03:20 (00:16)	03:20 (00:32)	03:04 (00:20)	03:06 (00:19)	0.379
Duration of vascular flush h:m	00:10 (00:02)	00:09 (00:01)	00:10 (00:01)	00:11 (00:03)	0.452
Total amount of fluid given, l	6.7 (1.0)	9.6 (1.7)*	8.7 (1.3)	9.2 (1.6)*	0.003
Total amount of diuresis, l	0.9 (0.2)	3.8 (1.1)*	3.3 (1.4)*	3.6 (1.3)*	<0.001
Potassium, mmol/L	4.1 (0.2)$^{\#}$	3.9 (0.3)$^{\#}$	4.0 (0.3)$^{\#}$	5.5 (0.4)	<0.001
Sodium, mmol/L	136 (1.7)	148 (3.5)*	144 (3.7)*	144 (4.2)*	<0.001

All values are presented as mean (standard deviation). Comparison between groups using one-way analysis of variance and post hoc *comparison of multiple means using Tukey test; N/a, Not available. * Signifies statistical significance of the group compared with the control group only; # Signifies statistical significance of the group compared with the brain dead + UW group only.*

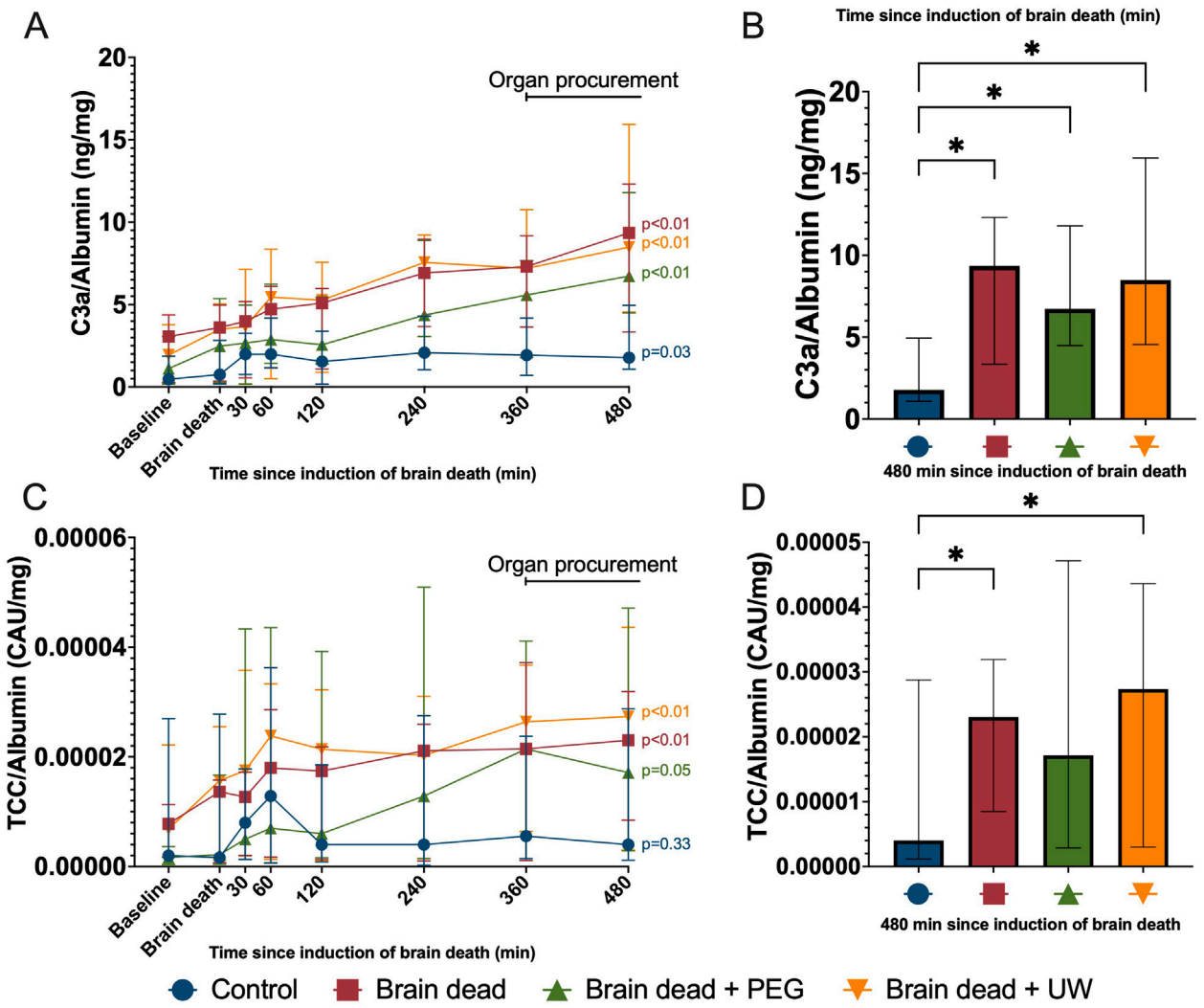


FIGURE 3 | Brain death-induced complement activation irrespective of luminal intervention. Brain death led to a significant increase of plasma C3a **(A)** in all groups and TCC **(C)** in the brain dead and brain dad + UW groups compared to baseline. At the end of organ procurement, C3a was significantly higher in all brain dead groups, compared to control **(B)**. TCC **(D)** was significantly higher in brain dead and brain dead + UWgroups compared to control. Control: control group, Brain dead: brain dead without luminal intestinal intervention, Brain dead + PEG: brain dead with luminal intestinal intervention using polyethylene glycol, Brain dead + UW: brain dead with luminal intestinal intervention using University of Wisconsin solution, C3a: Complement 3a, TCC: Terminal complement complex. Values presented as median ± Interquartile range. Mixed model. *$p < 0.05$.

groups. The animals were kept stable throughout the observation period (**Figure 2**).

There were no significant differences regarding the duration of the experiment, brain death induction, organ retrieval, or vascular flush between the groups (**Table 2**). The total amount of intravenous fluids given and diuresis throughout the experiment were higher in brain dead compared with non-brain dead groups but similar between brain dead groups (**Table 2**). In the brain dead groups, the sodium concentration increased steadily from the time of brain death. In the brain dead + UW group, the serum potassium content increased upon UW luminal instillation from 3.8 (95% CI: 2.7; 4.9) to 5.7 (95% CI: 5.7; 5.8) mmol/L at the end of procurement surgery. Additional blood gas parameters were within acceptable ranges throughout the observation period (**Supplementary Figure S1**).

Plasma Analyses of Complement Activation and Cytokines

Total albumin-corrected complement C3a increased following induction of brain death in all groups, compared to baseline values (**Figure 3A**). At 480 min, significantly higher levels of C3a were observed in the brain dead ($p = 0.0005$), brain dead + PEG ($p = 0.0018$), and brain dead + UW ($p < 0.001$) groups compared with the control group (**Figure 3B**). Total albumin-corrected TCC increased following induction of brain death in the brain death and brain death + UW groups compared to

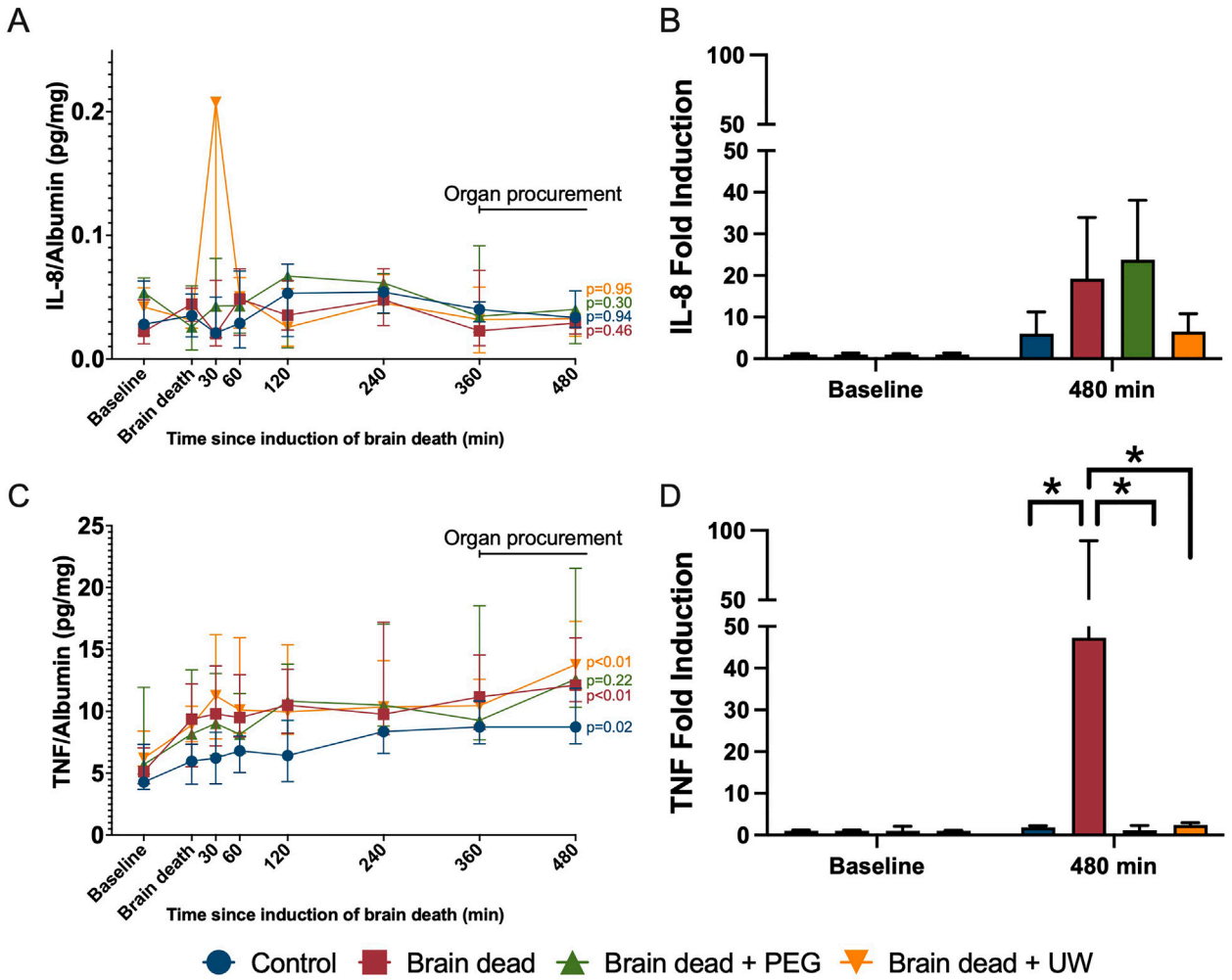


FIGURE 4 | Luminal intervention prevented brain death induced release of cytokines. Brain death did not significantly increase IL-8 in any groups compared to baseline **(A)**. Brain death led to a significant increase of plasma TNF in all groups except the brain dead + PEG group com pared to baseline **(C)**. At the end of organ procurement, IL-8 **(B)** was non-significantly higher in brain-dead animals, while TNF **(D)** was significantly higher in the brain dead group compared to all other groups **(D)**. Control: control group, Brain dead: brain dead without luminal intestinal intervention, Brain dead + PEG: brain dead with luminal intestinal intervention using polyethylene glycol, Brain dead + UW: brain dead with luminal intestinal intervention using University of Wisconsin solution, IL-8: Interleukine 8, TNF: Tumor necrosis factor. Values presented as median ± Interquartile range **(A, C)** and **(B, D)** X-fold induction comparing cytokine plasma values at 480 min to baseline values as mean ± standard error of the mean. Mixed model. *$p < 0.05$.

baseline values (**Figure 3C**). At 480 min, significantly higher levels of TCC were observed in the brain dead ($p = 0.05$) and brain dead + UW ($p = 0.023$) groups compared to the control group (**Figure 3D**). Circulating levels of IL-8 were comparable at baseline between groups and remained comparable throughout the observation period with a modest increase until the end of the experiment (**Figure 4A**). Circulating levels of TNF were comparable at baseline between groups. They increased following induction of brain death in all groups, significantly in the control, brain dead, and brain dead + UW groups, but not in the brain dead + PEG group (**Figure 4C**). Luminal intervention with UW and PEG significantly reduced TNF increase, but not IL-8 at 480 min compared with controls (**Figures 4B, D**).

Tissue Samples

Tissue TCC was significantly higher in kidneys in the brain dead + PEG group compared with the brain dead + UW group. No other differences were found for tissue levels of C3a, TCC, and TNF (**Supplementary Figure S2**).

Lipopolysaccharide Binding Protein

No significant differences were observed at baseline or at the time of induction of brain death for LBP, (**Figure 5A**). Levels of LBP did not increase significantly in the brain dead + PEG group compared to the level at induction of brain death ($p = 0.69$). In contrast, all other groups increased significantly compared to baseline ($p < 0.01$) (**Figure 5A**). At 480 min, levels of LBP in the brain dead + PEG group were significantly lower compared to the

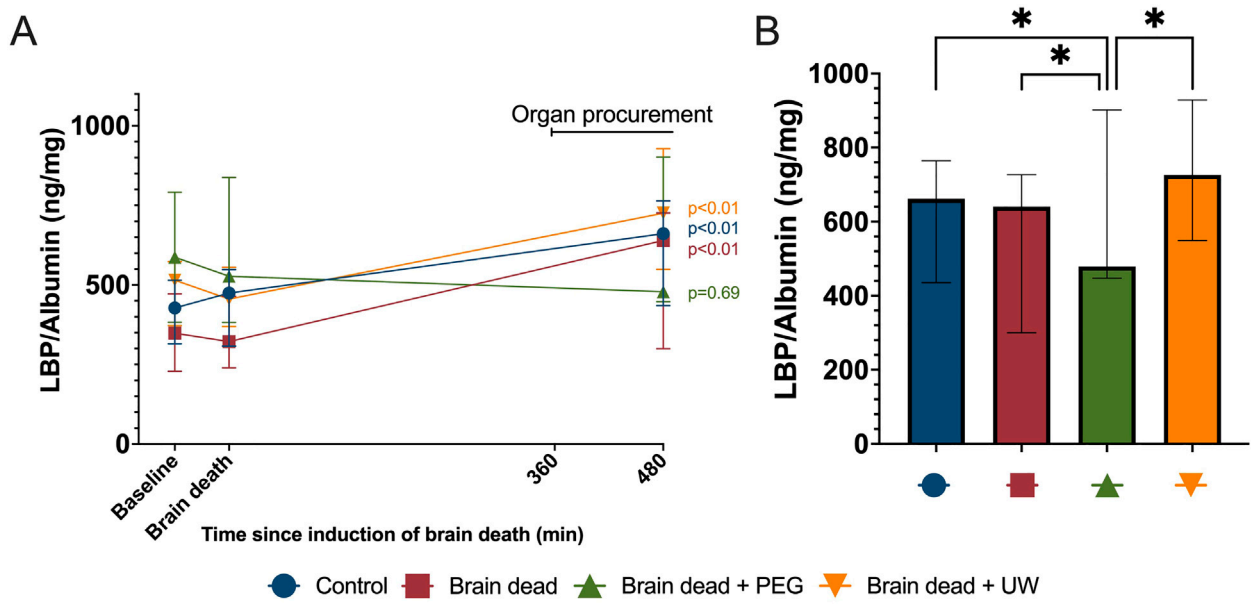


FIGURE 5 | Procurement surgery-induced release of lipopolysaccharide binding protein in plasma was reduced by intestinal PEG intervention. Procurement surgery led to a significant increase in lipopolysaccharide binding protein (LBP) in all groups except the PEG intervention group **(A)**. At the end of organ procurement, lipopolysaccharide binding protein was significantly lower in the brain dead + PEG-group compared to all other groups **(B)**. Control: control group, Brain dead: brain dead without luminal intestinal intervention, Brain dead + PEG: brain dead with luminal intestinal intervention using polyethylene glycol, Brain dead + UW: brain dead with luminal intestinal intervention using University of Wisconsin solution, LBP: Lipopolysachharide binding protein. Values presented as mean ± 95% Confidence Interval. Mixed model. *$p < 0.05$.

control ($p = 0.012$), brain dead ($p = 0.012$), and brain dead + UW ($p = 0.0002$) groups (**Figure 5B**).

DISCUSSION

We showed in a porcine brain death model that intervention on the small intestine using PEG or UW fluid reduced systemic inflammation upon procurement surgery. Brain death itself induced low-grade systemic complement activation in all groups and procurement surgery led to an increase in systemic inflammation. Intestinal treatment upon procurement surgery with PEG reduced systemic LBP, TCC, and TNF. UW treatment reduced TNF but led to a significant rise in serum potassium.

Brain death affects the whole body and leads to hormonal, circulatory, and respiratory disturbances [34]. In this study, brain dead animals were comparable to non-brain dead animals concerning systemic circulation and organ function. This was achieved by medical interventions frequently used in intensive care settings and organ donor care. Brain death as well as intensive care treatment affects the immune response. We observed increasing levels of C3a, TCC, IL-8, and TNF in all animals following anesthesia and instrumentation, which is in line with previous observations [35, 36]. However, this increase was either transient or remained at low levels in the control group, confirming previous findings, that brain death, not the intensive care setting, is the main driving force of systemic immune system activation [5].

Systemic parameters of complement activation increased significantly in the brain dead groups following induction of brain death with a continuing increase of C3a and TCC throughout the observation period. These findings are in line with previous observations of the immunological response to brain death, which includes local and systemic immune responses [3–5, 7–9, 11, 12]. These responses are likely due to a combination of internal factors, such as general immune reaction, and external factors, such as the mechanism of brain death, hospital interventions, and length of stay in an intensive care unit [5, 28, 37–39]. Systemic inflammation with an increase of the pro-inflammatory protein TNF was also observed, but the observed level was low compared to e.g., systemic infection as observed in sepsis [40, 41]. However, even low-grade systemic complement activation and inflammation have been associated with long-term reduced organ function [42]. Most immunological markers increased only in the brain-dead groups after the start of the organ procurement surgery. We speculate that the initial trauma of brain death predisposes the immune system to react to the surgical trauma of organ procurement. This might be due to low-grade complement system activation.

Luminal intestinal instillation of PEG prevented the increase of TCC and TNF. PEG is part of the IGL-1 preservation solution and experimental as well as one retrospective clinical observational study have shown beneficial effects of PEG on intestinal integrity and barrier function [43, 44]. Intestinal PEG has previously been tested during storage of intestinal grafts and has shown improved preservation in animal models

using rats [18–21] and humans [23]. Luminal intestinal instillation of UW only prevented the increase of TNF, while significantly increasing systemic potassium levels due to the high potassium content. High systemic potassium levels could lead to missed donations due to cardiac arrhythmias and potential cardiac arrest.

Luminal PEG significantly decreased systemic LBP, which is generated upon gram-negative and -positive bacteria stimulation [45]. TNF induction is lipopolysaccharide and LBP-dependent [46]. Although a causal link can not be made, the translocation of intestinal bacteria to the systemic circulation during procurement surgery has been hypothesized to induce inflammation and reduce donor-organ quality [47]. A previous study in rats showed that LBP increases upon procurement surgery and liver transplantation and that LBP inhibition reduces TNF levels [48]. PEG installation in the intestine might reduce this translocation by preserving intestinal barrier function [23].

However, both intestinal preservation groups showed reduced levels of TNF to the same levels as non-brain death animals, indicating reduced immune system activation in both luminal intestinal intervention groups. The effect of UW on TNF seems to be intestinal barrier independent, due to the increase in LBP, and might be explained by a rapid increase in serum potassium levels, which has been reported to inhibit inflammatory responses [49]. In addition, UW contains allopurinol, a known inhibitor of xanthine oxidase blocking the production of reactive oxygen species [50] and decreasing the production of TNF and other cytokines [51].

Both intestinal preservation groups had fluid instilled at 4°C, which could help protect the intestine from ischaemic damage during the procurement surgery due to a moderate hypothermic effect [52]. However, the intestinal temperature during surgery was not assessed and the comparable levels of LBP in the UW group compared with both control groups suggest that the intestinal barrier function was preserved by a mechanism different from hypothermia.

Luminal intestinal application of PEG is easy to implement through an already available nasogastric tube during organ procurement surgery. PEG is cheap, requires no special equipment, is clinically available as a laxative, and has no serious side effects. Additionally, the potential mechanism of protection, in the form of preserved intestinal barrier function is feasible, and negative affection of transplantable organs is highly unlikely. On the contrary, luminal intestinal application of UW did not offer the same potential benefits compared to PEG, it is expensive and presents a potential risk to the donor due to the increase in plasma potassium caused by the UW solution itself.

Intestinal leakage caused by the trauma of either circulatory- or brain death has been suggested as a possible contributory factor to worse outcomes for recipients of organs from deceased compared to healthy living donors [15, 53]. Several mechanisms have been proposed for intestinal leakage such as prolonged hypotension and hypoperfusion [54–57], mesenteric lymphatic system activation [58], and lymphocyte infiltration into the intestinal wall [15, 57]. Future clinical studies should evaluate the effect of luminal instillation of PEG at the start of organ procurement surgery on graft function and survival to improve long-term recipient survival.

This study has limitations. The withdrawal of blood early in the experiment was necessitated by use in another experiment [27]. It is possible that this exacerbated the catecholamine storm induced by brain death, which may lead to hemodynamic collapse [59]. The blood withdrawal might also have contributed to the hypovolemia induced by brain death, leading to increased use of noradrenaline and fluid to keep the animals hemodynamically stable [28, 38]. However, the blood volume removed was below 15% of total blood volume and a standard protocol of volume resuscitation and vasopressor was used. In addition, the removal of the same amount of blood in the control group did not lead to increased inflammation compared to baseline. Due to logistic reasons, randomization was only partial and the surgeon was not blinded for the intervention, which may introduce bias. However, all analyses were performed blinded to group allocation and by others than the surgeon.

Brain death induces low grade innate immune system activation, which exacerbates during organ procurement surgery. Intestinal preservation reduces systemic inflammation and PEG appears to be a better strategy compared to UW solution. PEG preservation reduces the response to intestinal bacterial translocation, which might be causative for reduced systemic inflammation during organ procurement surgery.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was approved by Danish Animal Experiments Inspectorate. The study was conducted in accordance with the local legislation and institutional requirements.

AUTHOR CONTRIBUTIONS

MW, AdJ, HS, and HM conducted the experiments. MW, BJ, and SP wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This work was funded by: the Novo Nordic Foundation (NNF19OC0056466), Aase and Ejnar Danielsens Foundation (19-10-0392), A.P. Moeller Maersk Foundation for Medical Research (19-L-0132), and the Danish Renal Disease Research Foundation.

CONFLICT OF INTEREST

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

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2024.13569/full#supplementary-material

Supplementary Figure S1 | Arterial bloodgas derived pH, lactate, sodium, and potassium. **(A)** pH remained stable in the upper normal range, with a transient decrease following the induction of brain death due to an increase in lactate. **(B)** Lactate increased upon brain death and remained elevated compared to controls. **(C)** Sodium increased slowly in the brain-dead groups following induction of brain death. Intestinal intervention showed no effect on pH, lactate, and sodium **(A–C)**. **(D)** Potassium remained within normal range in all groups except the brain-dead group receiving luminal intestinal University of Wisconsin solution, which showed a rapid increase of potassium following luminal intestinal intervention. Control: control group, Brain dead: brain dead without luminal intestinal intervention, Brain dead + PEG: brain dead with luminal intestinal intervention using polyethylene glycol, Brain dead + UW: brain dead with luminal intestinal intervention using University of Wisconsin solution, Na: Sodium, K: Potassium. Values presented as mean ± 95% Confidence Interval. Mixed model.

Supplementary Figure S2 | Effect of brain death and luminal intestinal intervention of organ markers of inflammation. Tissue TCC, C3a and TNF concentrations were generally low and only mildly affected by brain death. Significant differences were observed between kidneys in the intestinal intervention groups using PEG and UW for TCC. Control: control group, Brain dead: brain dead without luminal intestinal intervention, Brain dead + PEG: brain dead with luminal intestinal intervention using polyethylene glycol, Brain dead + UW: brain dead with luminal intestinal intervention using University of Wisconsin solution; C3a, Complement 3a; TCC, Terminal complement complex; TNF, Tumor necrosis factor. All values presented as median ± interquartile range. $^{*}p < 0.05$.

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ORIGINAL RESEARCH
published: 14 October 2024
doi: 10.3389/ti.2024.13495

Tacrolimus Dose Requirement in *De Novo* Adult Kidney Transplant Patients Treated With Adoport® Can Be Anticipated

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All the factors potentially influencing tacrolimus dose requirement and combinations thereof have never been thoroughly investigated, precluding accurate prediction of tacrolimus starting dose. This prospective, non-interventional, multicenter study in *de novo* adult kidney transplant recipients over the first year after transplantation aimed to investigate the factors influencing tacrolimus dose-standardized trough blood concentration (C_0/D) over the first week post-transplant (D4-D7, primary objective), D8-M3 and M3-M12 (secondary objectives). Statistical analysis employed mixed linear models with repeated measures. Eighteen sites enrolled 440 patients and followed them up for 9.5 ± 4.1 months. Age at baseline (p = 0.0144), end-stage renal disease (p = 0.0092), CYP3A phenotype ($p < 0.0001$), dyslipidemia at baseline (p = 0.0031), hematocrit (p = 0.0026), total bilirubin (p = 0.0261) and plasma creatinine (p = 0.0484) independently increased with log(C_0/D) over D4-D7, explaining together 72.3% of the interindividual variability, and representing a robust model to estimate tacrolimus initial dose. Donor age and CYP3A phenotype were also influential over D8-M3 and M3-12, in addition to recipient age. Corticosteroids, diabetes at baseline, and ASAT yielded inconstant results between D8-M3 and M3-M12. We found no ethnicity effect when CYP3A phenotype was accounted for, and no food effect. Intra-individual variability over M3-M12 was moderate, and significantly lower in patients with chronic hepatic disorder (p = 0.0196) or cancer (p = 0.0132).

Keywords: tacrolimus, pharmacokinetic variability, diet, ethnicity, genetic polymorphisms

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Received: *04 July 2024*
Accepted: *20 September 2024*
Published: *14 October 2024*

Citation:
Marquet P, Anglicheau D, Humeau A, Adrouche S, Saada L, Bisiaux J, Guillemin S, Lardy-Cléaud A and Rostaing L (2024) Tacrolimus Dose Requirement in De Novo Adult Kidney Transplant Patients Treated With Adoport® Can Be Anticipated. Transpl Int 37:13495. doi: 10.3389/ti.2024.13495

Abbreviations: ASAT, ASpartate Amino Transferase; BMI, Body Mass Index; CI_{95}, 95% Confidence Interval; C_0/D, dose-standardized trough blood concentration; CYP3A, cytochrome P450 family 3 subfamily A; D0, day of transplant; D7, 7 days post-transplant; D8, 8 days post-transplant; FAS, Full Analysis Set; FAS2, Full Analysis Set 2; IIV, Inter-Individual Variability; IOV, Inter-Occasion Variability; MedDRA, Medical Dictionary for Regulatory Activities; M3, 3 Months post-transplant; M12, 12 Months post-transplant; NODAT, New Onset Diabetes After Transplant; P-gp, P-glycoprotein; SAS, Safety Analysis Set; SD, Standard Deviation; TEAE, Treatment-Emergent Adverse Event.

INTRODUCTION

Tacrolimus is an immunosuppressant widely used for the prevention of allograft rejection in solid organ transplantation. However, it is characterized by a narrow therapeutic window and extensive Inter-Individual Variability (IIV) resulting in a challenging determination of the appropriate dose that is both safe and effective at the individual level [1–4]. Rapid achievement of trough blood levels in the desired target range is critical to optimize safety and efficacy during the early post-transplant period [1].

Several clinical studies aimed to identify the variability factors influencing tacrolimus exposure, with the goal of tailoring tacrolimus dose to each patient. The factors known to influence tacrolimus pharmacokinetics include: food-drug interactions, drug-drug interactions and erratic gastrointestinal motility, which all impact tacrolimus absorption velocity or intensity in the gastrointestinal tract; the efflux-pump P-glycoprotein (P-gp) activity, which affects tacrolimus transport back to the digestive lumen; weight and hematocrit that influence tacrolimus distribution; genetic polymorphisms in cytochrome P450 family three subfamily A (CYP3A) enzymes that modulate tacrolimus metabolism and elimination [1, 3, 4].

Ethnicity has also been reported to be a variability factor. In particular, "African-American" transplant patients require higher tacrolimus doses than "Caucasians" to maintain comparable tacrolimus trough concentrations [5–7]. These differences partly arise from variations in intestinal CYP3A or P-gp activities between ethnic groups [8–10]. Dietary habits, which differ between ethnicities, might play a role as well [11].

However, none of these clinical studies extensively evaluated many factors at the same time, including food effect (encompassing the timing of tacrolimus intake and high-fat food consumption), genetic polymorphisms and ethnicity (not only "African-Americans" and "Caucasians", but in general).

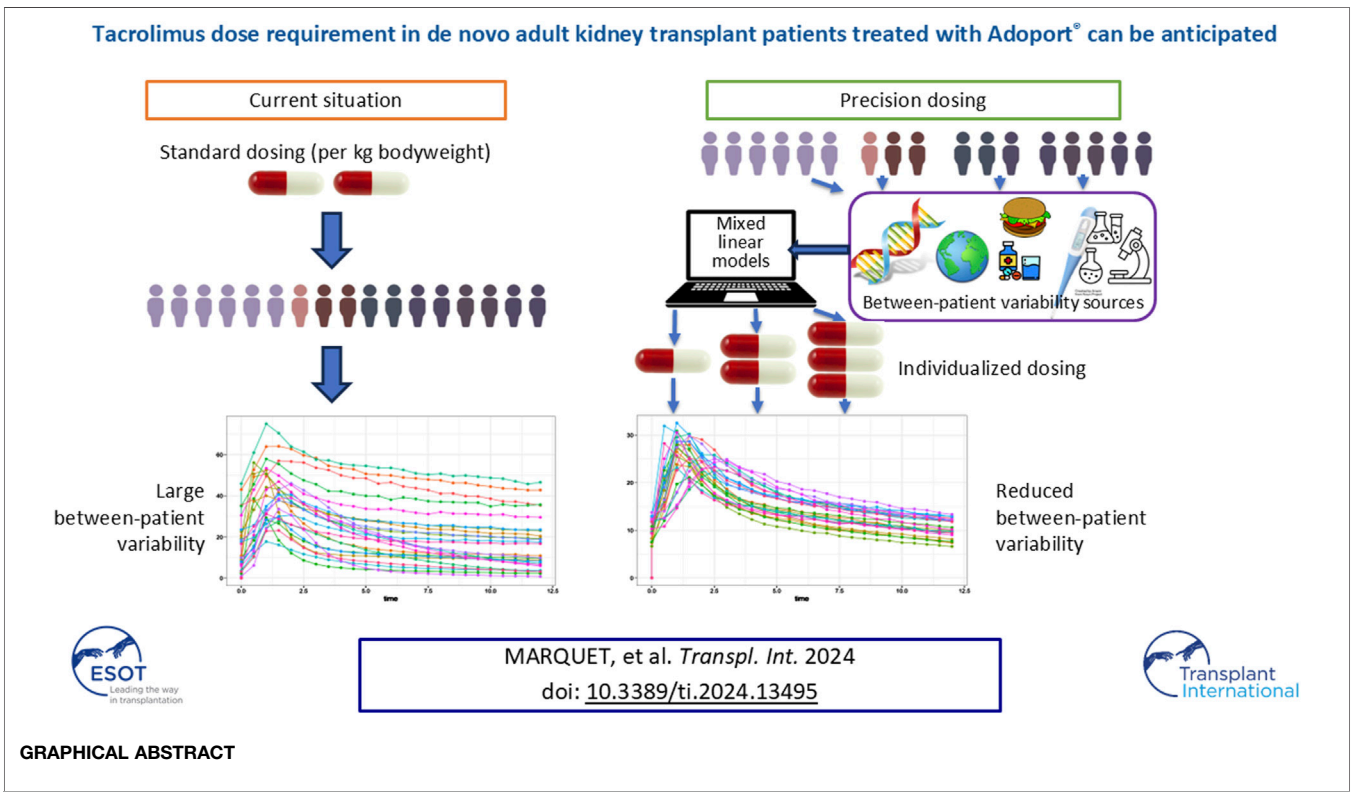
The aim of this study was to thoroughly investigate the different factors influencing tacrolimus exposure over the first week and the first year post-transplant in kidney transplant recipients.

PATIENTS AND METHODS

Study Design and Conduct

This is a prospective, non-interventional, multicenter study conducted at 18 kidney transplant centers in France between April 2018 and October 2020. All the French kidney transplant centers known for using tacrolimus Adoport® (SANDOZ, Levallois-Perret, France) as maintenance therapy for kidney transplant recipients were considered during the site selection process, in order to obtain the most representative population sample.

The primary objective of the study was to investigate the variability factors affecting tacrolimus dose-standardized trough blood concentration (C_0/D) in adult kidney transplant recipients



GRAPHICAL ABSTRACT

over the first week post-transplant. Secondary objectives were to investigate the same variability factors and evaluate tacrolimus safety during the first year post-transplant.

The study protocol and its amendments were approved by an Ethics Committee in France (reference 2-17-47, ID-RCB: 2017-A02512-51) and the study was conducted in accordance with the Good Clinical Practice guidelines, the Declaration of Helsinki, the Declaration of Istanbul and all other applicable regulatory requirements. All the participants provided written informed consent.

It is a non-interventional study, meaning that no specific procedures were required as per the protocol. In particular, the investigating sites were already using Adoport® before they were selected for this study and the decision to initiate tacrolimus treatment had been made by their physician before the patients consented to participate in the study. Treatments, patient follow-up schedule, laboratory tests and data collection were performed according to usual clinical practice. Of note, the Summary of Product Characteristics recommends taking Adoport® twice daily [12]. Patients were only asked to fill in questionnaires related to the tacrolimus intake recommendations they received (and remembered) as well as to the timing of tacrolimus intake and what they usually ate for dinner. Data referring to the following timepoints were collected: day of transplant (D0), 7 days post-transplant (D7) or - at the latest - on the day of hospital discharge (baseline), 3 months post-transplant (M3), and 12 months post-transplant (M12) or premature discontinuation; this schedule was chosen to mirror usual clinical care across investigating sites. Tacrolimus whole-blood C_0 measurements were performed at each site, whereas those to characterize genetic polymorphisms were performed by a central laboratory.

Patient Population

To minimize selection bias, patients were consecutively included by each site.

Adult recipients of a first kidney allograft, treated *de novo* with tacrolimus (Adoport®) for transplant rejection prophylaxis, and for whom the first dose of tacrolimus was taken on the transplantation day or within 24 h post-transplant, were included in the study. The criteria related to first transplant and *de novo* tacrolimus treatment enabled avoiding any previous tacrolimus impregnation. Moreover, the criterion related to tacrolimus initiation on the transplantation day or within 24 h post-transplant ensured timing homogeneity between subjects.

Patients who had a combined transplant, who were taking during the first week post-transplant major enzymatic inhibitors (i.e., azole anti-fungal drugs, protease inhibitors against the human immunodeficiency or the hepatitis C viruses, erythromycin) or major enzymatic inducers (i.e., phenytoin, rifampicin, St John's Wort) – all known to interact with tacrolimus [12] - or who were participating in an interventional study, were excluded.

Sample Size

Assuming a correlation rhô = 0.2 between C_0/D and quantitative factors, and using multiple regression with an alpha risk of 5% and a power of 80%, 400 patients were required to select 10 factors out of the predefined 45 [13]. As it was anticipated that 10% of the patients would not be evaluable (i.e., patients dropping out of the study before M12), at least 440 patients were to be included in the study.

Statistical Analysis

Analyses were performed on $\log(C_0/D)$ to account for non-normality.

The primary endpoint, which relates to IIV of $\log(C_0/D)$ between D4 and D7 and associated variability factors, was evaluated in all the included patients who met all the eligibility criteria and for whom at least one C_0/D value was available over D4-D7 [Full Analysis Set (FAS)].

IIV and variability factors of $\log(C_0/D)$ between D8 and M3 as well as between M3 and M12 were analyzed as secondary endpoints in all the included patients who met all the eligibility criteria and for whom at least one C_0/D value was available over the respective periods [Full Analysis Set 2 (FAS2)].

For the primary objective, we investigated: demographic characteristics and medical history/comorbidities at baseline; the CYP3A phenotype inferred from the CYP3A5*1, *3, *6, *7 and the CYP3A4*22 SNPs, as proposed by Elens et al. [14, 15]; the P-gp phenotype derived from the ABCB1 exons 12, 21 and 26 as proposed by Woillard et al. [16]; transplant characteristics; as well as, over the D0-D7 period, tacrolimus initial dose and number of C_0/D measurements, concomitant treatments with known interactions with tacrolimus, renal function, laboratory test results, diarrhea and New Onset Diabetes After Transplant (NODAT). For the secondary objectives, we investigated the same variables over the D8-M3 and M3-M12 period, as well as: the existence of a therapeutic education program; the recommendations received at hospital discharge regarding tacrolimus intake in accordance with the physician's opinion regarding the patient's understanding of tacrolimus prescription and recommendations at M3 or M12; whether a biopsy was performed before M3; dietary habits and timing of tacrolimus intake as reported by the patient on the questionnaires filled-in at M3 and M12.

It should be noted that the timing of tacrolimus intake over D4-D7 was not evaluated as part of the primary objective because, during this period, patients are still hospitalized, and tacrolimus is presumably administered on an empty stomach by healthcare professionals. CYP3A phenotype was classified as slow, intermediate or rapid based on *CYP3A4* and *CYP3A5* genotypes, as described in **Table 1** and following Elens and Haufroid, and Lloberas et al. [14, 15]. P-gp phenotype was classified as slow, intermediate or rapid based on *ABCB1* genotype (slow for homozygous TTT haplotype, intermediate for heterozygous TTT haplotype and rapid for lack of TTT haplotype), following Woillard et al. [16].

To investigate the univariate effect of factors, we employed ANOVA or *t*-test for categorial factors, and Spearman rank-order correlation coefficient [with its 95% Confidence Interval (CI_{95})] calculated with the Fisher's z transformation for continuous factors. Multivariate analyses were run on all the factors with $p < 0.05$ at the univariate tests. If the CYP3A or P-gp phenotypes

TABLE 1 | Classification of CYP3A phenotypes inferred from the most frequent CYP3A4 and CYP3A5 SNPs following Elens et al. [14].

CYP3A phenotype	*CYP3A5* genotype	*CYP3A4* genotype
Poor	No *CYP3A5*1* allele	Heterozygous or homozygous for *CYP3A4*22*
Intermediate	Homozygous or heterozygous for *CYP3A5*1*	Heterozygous or homozygous for *CYP3A4*22*
	No *CYP3A5*1* allele	No *CYP3A4*22* allele
Extensive	Homozygous or heterozygous for *CYP3A5*1*	No *CYP3A4*22* allele

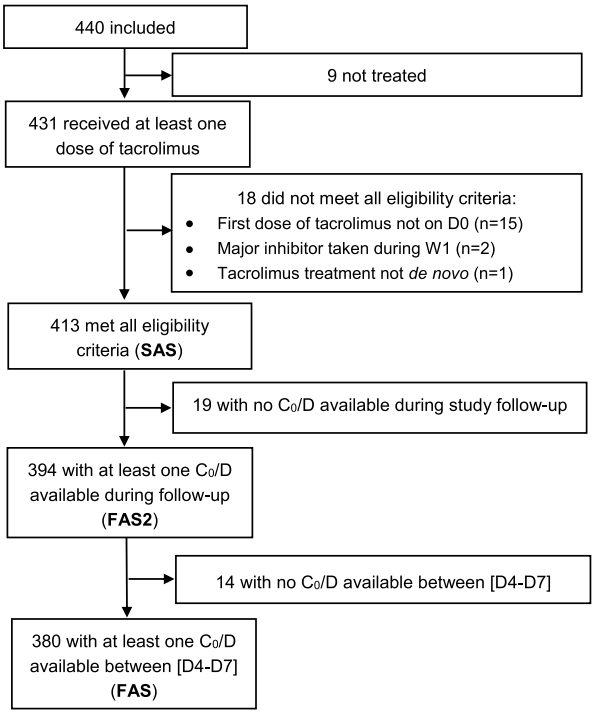


FIGURE 1 | Flowchart of the different analysis populations.

were significant, they were selected for multivariate analysis and the corresponding genotypes were not. These factors were entered, as fixed effects, in a mixed model for repeated measurements. For all the mixed linear models, the subject was considered as a random effect and the number of days since transplant as a fixed effect. For the IIV the same mixed linear model and parameters, without factors, were used. Intra-individual, or Inter-Occasion Variability (IOV), was studied in patients with at least 3 C0 values available in the eCRF over the M3-M12 period. The influence of the same factors on log(C0) IOV was analyzed using univariate and then multivariate multilinear models.

Tacrolimus safety was evaluated in all the included patients who met all the eligibility criteria and received at least one dose of tacrolimus [Safety Analysis Set (SAS)]. Adverse events were coded using the international Medical Dictionary for Regulatory Activities (MedDRA) reference dictionary version 20.1.

Missing data were not replaced. Sample size calculation was performed using SAS software v9.4, and univariate and multivariate statistical analyses using R v4.4.0 (R Foundation) and the R packages lme4 (v1.1-35.3) and lmerTest v3.1-3.

RESULTS

Patient Population

In total, 440 patients were included at 18 investigating sites and followed up over 9.5 ± 4.1 months on average. As illustrated in **Figure 1**, 413 patients constituted the SAS, 394 the FAS2 and 380 the FAS. Regarding follow-up, among the 413 patients of the SAS, 367 (88.9%) completed the M3 visit and 284 (68.8%) completed the M12 visit (**Figure 2**).

Baseline characteristics of the SAS patients are described in **Table 2**. Overall, they were aged 55.2 ± 15.2 years [mean ± Standard Deviation (SD)] and 65.1% of them were males. Their mean Body Mass Index (BMI) ± SD was 25.6 ± 4.3 kg/m^2. Regarding ethnicity, 77.7% of the patients considered themselves as White European, 11.1% as North African or from the Middle East, 9.0% as Black African or Black Caribbean, 1.9% as Asian and 0.2% as Other. These baseline characteristics were similar in FAS and FAS2. At inclusion, 95.7% patients had received an induction treatment and all received immediate-release tacrolimus Adoport®, co-administered with a mycophenolate drug (95.9%) and corticosteroids (98.3%).

Variability of Tacrolimus log(C_0/D) Over D4-D7

The IIV of log(C_0/D) over D4-D7 was 0.32 (CI_{95} [0.28; 0.38]). Univariate analysis results are presented in **Supplementary Table S1**. Among the 21 factors considered in the multivariate model, those that influenced tacrolimus log (C_0/D) in the FAS were: recipient age at baseline (p = 0.0144), the main cause of end-stage renal disease (p = 0.0092); CYP3A phenotype (p = 0.0001); dyslipidemia at baseline (p = 0.0031); and hematocrit (p = 0.0026), total bilirubin (p = 0.0261) and plasma creatinine (p = 0.0484) over the D4-D7 period (**Table 3**). Together, they explain 72.3% of the IIV, while IOV explained another 19.2%.

Variability of Tacrolimus log(C_0/D) Over D8-M3

The IIV of log(C_0/D) over D8-M3 was 0.27 (CI_{95} [0.23; 0.31]). Among the 12 factors (including "timing of tacrolimus intake") selected for the multivariate model, those that affected tacrolimus log (C_0/D) in the FAS2 were: donor age (p = 0.0121), recipient age at baseline (p = 0.0245), CYP3A4 slow or intermediate phenotype

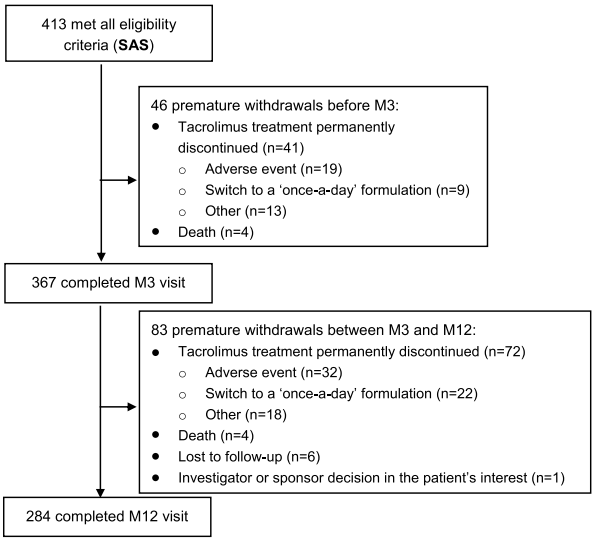


FIGURE 2 | Flowchart of patient follow-up.

($p < 10^{-14}$) and corticosteroid treatment over the time period (p = 0.0470), explaining 33.5% of the total variability. The IOV explained another 40.4%.

Variability of Tacrolimus log(C_0/D) Over M3-M12

The IIV of log(C_0/D) over M3-M12 was 0.33 (CI_{95} [0.28; 0.39]). Among the eight factors selected for the multivariate model, those that affected tacrolimus log (C_0/D) in the FAS2 were: donor age (p = 0.0190), recipient age at baseline (p = 0.0083), CYP3A4 slow or intermediate phenotype ($p < 10^{-20}$), diabetes at baseline (p = 0.0328) and ASAT over the time period (p = 0.0023), explaining 36.4% of the total variability (as compared with 46.9% for the IOV).

In 254 patients with at least 3 C0 values saved in the eCRF over the M3-M12 period, IOV (intra-individual variability) of log(C_0/D) and C_0/D over the same time period was moderate (**Table 4**). However, there were a few outliers with CV > 40%, up to 92.2% (**Supplementary Figure S2**). The IOV was significantly lower in patients with chronic hepatic disorder or cancer (**Supplementary Table S3**).

Safety

Out of the 413 patients in the SAS, 369 (89.3%) experienced at least one Treatment-Emergent Adverse Event (TEAE) (i.e., adverse events that occurred after the first tacrolimus intake), for a total of 2,477 TEAEs. TEAEs reported in more than 5% of the patients are presented in **Table 5**. The most common were anemia (26.9% of the patients, 120 TEAEs), diarrhea (22.8%, 117 TEAEs), tremor (17.2%, 72 TEAEs), hypertension (15.3%, 67 TEAEs) and leukopenia (13.8%, 77 TEAEs). Of note, transplant rejection occurred in 5.1% of the patients (21 TEAEs). Out of these 2,477 TEAEs, 377 (that occurred in 190 patients (46.0%)) were deemed to have a suspected causality to tacrolimus by the investigators.

Among the 2,477 TEAEs, 703 [which occurred in 238 patients (57.6%)], were considered as serious TEAEs. The most common ones were acute kidney injury (9.4% of the patients, 56 TEAEs) and anemia (7.5%, 34 TEAEs). Ninety-two (92) serious TEAEs [that occurred in 74 patients (17.9%)] were deemed to have a suspected causality to tacrolimus by investigators.

Nine patients (2.2%) died during the study following the occurrence of one or several TEAEs, none of which had a suspected causality to tacrolimus.

DISCUSSION

To the best of our knowledge, this study is the first to investigate so extensively the combination of variability factors influencing tacrolimus dose-standardized exposure (also called 'dose requirement'), including food effect (through both timing of tacrolimus intake and high-fat food consumption), CYP3A and P-gp phenotypes, and ethnicity (as mostly represented in France). In their comprehensive review article, Vanhove et al. presented evidence for each of them separately, but not combined [17]. Tacrolimus oral clearance is known to decrease progressively over the first 3–9 months post-transplant [18], explaining a natural decrease in C_0/dose which, together with a decreasing risk of rejection, justifies progressive lowering of tacrolimus doses over the first year post-transplant. For this reason, we considered three time periods in this study, from the first days after surgery up to 1 year.

Multivariate analysis evidenced seven factors significantly influencing tacrolimus log(C_0/D) over D4-D7 (primary endpoint): recipient age at transplantation, the main cause of end-stage renal disease, CYP3A phenotype (encompassing the CYP3A5 *1, *3, *6, *7 the CYP3A4*22 and the POR*28 SNPs to account for ethnic diversity), dyslipidaemia at baseline and hematocrit, total bilirubin and plasma creatinine over the time period. Together, these seven factors explain 72.3% of C_0/D variability, meaning that they may be leveraged to adapt the initial dose of tacrolimus to each patient, probably more effectively than previous attempts limited to the CYP3A5*3 genotype [19, 20].

Multivariate analysis of the same variables plus the timing of tacrolimus intake over the other two time periods (D8-M3 and M3-M12) only confirmed the steady influence of recipient age at baseline and CYP3A phenotype. Donor age only reached significance at these later two periods. In contrast, the main cause of end-stage renal disease lost significance after D7. The other variables (dyslipidemia at baseline, diabetes at baseline, corticosteroid treatment, ASAT, haematocrit, plasma creatinine) were only significant at one period.

Regarding CYP3A, the slow/intermediate metabolizing phenotype was associated with higher log (C_0/D), as expected [4, 14, 15, 21]. Indeed, when CYP3A activity is slow or intermediate, tacrolimus is less metabolized [4], which decreases dose requirement.

TABLE 2 | Baseline characteristics (SAS, n = 413).

Age, mean (SD) (years)	55.2 (15.2)
Male sex, n (%)	269 (65.1)
Height, mean (SD) (cm)	169.8 (10.3)
Weight at D0, mean (SD) (kg)	73.9 (15.1)
BMI, mean (SD) (kg/m^2)	25.6 (4.3)
Ethnicity according to the patient, n (%)	
White European	321 (77.7)
North African or Middle East	46 (11.1)
Black African or black Caribbean	37 (9.0)
Asian	8 (1.9)
Other	1 (0.2)
Diabetes, n (%)	94 (22.8)
Heart failure, n (%)	19 (24.7)
CYP3A phenotype, n (%)	
Poor	27 (7.1)
Intermediate	253 (66.9)
Extensive	98 (25.9)
Missing	35
P-gp phenotype, n (%)	
Slow	51 (13.5)
Intermediate	150 (39.7)
Rapid	177 (46.8)
Missing	35
Induction treatment, n (%)	
Non-depleting antibodies	219 (53.0%)
Depleting antibodies	175 (42.4%)
None except corticosteroid bolus	19 (4.6%)
Adoport® starting dose, mean (SD) (mg)	7.1 (3.5)
Other maintenance immunosuppressive treatment at inclusion	
Mycophenolate mofetil or mycophenolic acid sodium salt, n (%)	396 (95.9%)
Corticosteroids, n (%)	406 (98.3%)

The significant influence of recipient age at baseline on tacrolimus dose-standardized exposure in adults may be related to decreased absorption rate [22] and/or increased volume of distribution due to changing body composition with age. Potential confounders of this age effect, such as concomitant treatments, particularly with CYP34 inhibitors or inducers, did not pass univariate analysis. Corticosteroids are known to affect the oral bioavailability of tacrolimus [23] through P-gp and CYP3A4 induction [24]. An observational study in 83 renal transplant recipients showed that the higher the steroid dosage, the higher the dosage of tacrolimus needed to achieve target trough levels in these patients. The most likely interaction mechanism is specific enzymatic induction of CYP3A and/or P-gp and this interaction is present, even when the steroid dosage is low [25]. With regards to dyslipidemia, the SmPCs of several tacrolimus formulations list hyperlipidemia, hypercholesterolemia and hypertriglyceridemia as frequent adverse effects. In addition, significant associations between Tac C0 and hyperlipidemia were reported by several groups, e.g., in 132 Korean kidney transplant recipients using multivariate analysis [26], or in 63 European kidney transplant patients for hypercholesterolemia and hypertriglyceridemia [27]. However, the causality of this association may go both ways, because *in vitro*, 60% of tritiated ciclosporine or tacrolimus are transported by HDL-cholesterol in normolipemic sera, whereas approx. 50%–60% are transported by LDL-cholesterol in hypertriglycemic sera [28].

Donor age was significant at two different periods, suggesting a "false negative" result at D4-D7 or an interaction with time. However, the underlying mechanism of the influence of donor age on tacrolimus IIV is not obvious, since tacrolimus is not substantially excreted in urine.

The inconstant statistical results across the three time periods may be chance findings, but most of them have already been reported in the literature and have plausible pathophysiological explanations. First of all, the relationship between hematocrit and tacrolimus log(C_0/D) at D4-D7 was expected since tacrolimus in blood is highly bound to red blood cells [29]. This is also consistent with the literature [30–35]. Secondly, the influence of diabetes at baseline on tacrolimus variability in the latest time period may be due to its influence on the interstitial cells of Cajal, the gastric pacemaker cells [36], resulting in delayed gastric emptying [37] and a flatter pharmacokinetic profile, with lower C_{max} and higher C_0. This is contrasted with the absence of association with new-onset diabetes, possibly due to a much shorter exposure to diabetes. The association of C_0/D with dyslipidemia found at D4-D7 was not confirmed at the later time periods, maybe because it is favoured by early post-transplant cholestasis, which disappears rapidly [38, 39]. The (weak) link with plasma creatinine in the same period may be more a consequence than a cause of high C_0/D.

The absence of statistical association between tacrolimus pharmacokinetic IIV and some variables is also interesting. The P-gp phenotype resulting from the combination of the most influential genotypes [40–43] did not show significant effect on log(C_0/D), although tacrolimus is a substrate of P-gp, that tends to oppose its digestive absorption and favor the biliary and renal elimination of its metabolites [44]. However, tacrolimus dose in the gut lumen probably saturates P-gp efflux capacities, and tacrolimus is hardly excreted unchanged in bile or urine.

Also, contrary to what was expected, no association between log(C_0/D) and high-fat food consumption was identified as a result of univariate analysis over D8-M3 and M3-M12 (it was not tested at the first time period since the patients were hospitalized). High-fat meals influence both the rate and intensity of oral tacrolimus absorption [1, 12], which is the reason why the tacrolimus label recommends taking it on an empty stomach, that is, at least 1 h before or 2 h after eating [1]. In the present study, high-fat food consumption was defined as the consumption of at least two types of high-fat food during dinner, at least twice a week. Over D8-M12, no statistical association was observed between log(C0/D) and tacrolimus intake during meals. The causes of this apparent discrepancy with the drug label may be: that the regulator recommends that the food effect is evaluated based on the AUC and Cmax in healthy volunteers receiving a single drug dose [45], as opposed to steady-state C0 in patients here; and that we considered high-fat meals when at least two categories of high-fat food were ingested, which in the absence of quantities may not match the FDA definition. As a reminder, the FDA recommends "a high-fat (approximately 50 percent of total caloric content of the meal) and high-calorie (approximately 800–1,000 calories) meal as a test meal for food-effect bioavailability. This test meal should

TABLE 3 | Multivariate analysis of potential variability factors of tacrolimus log(C_0/D) over D4-D7 (FAS, n = 380), D8-M3 (FAS2, n = 394) and M3-M12 (FAS2, n = 394).

Variable	D4-D7 (primary objective)		D8-M3		M3-M12	
	Beta	*P*-value	Beta	*P*-value	Beta	*P*-value
Donor age	−0.0042 [−0.0162; 0.0078]	0.4976	−0.0084 [−0.0149; −0.0019]	**0.0121**	−0.0081 [−0.0148; −0.0014]	**0.0190**
Recipient age at baseline	0.0193 [0.0043; 0.0343]	**0.0144**	0.0087 [0.0012; 0.0162]	**0.0245**	0.0104 [0.0027; 0.0180]	**0.0083**
Recipient gender (vs. male)	0.1998 [−0.0356; 0.4351]	0.1008				
Ethnicity (vs. White European, n = 302*)						
Asians (6)	−0.0358 [−0.8076; 0.7360]	0.1702	0.6749 [0.1620; 1.1879]	0.0951	0.5527 [0.0386; 1.0668]	0.1437
Black Africans and Caribbeans (34)	0.6975 [0.0388; 1.3561]		−0.0121 [−0.2577; 0.2335]		0.2448 [0.0052; 0.4845]	
North Africans and Middle East (37)	0.0787 [−0.3809; 0.5382]		−0.0691 [−0.2883; 0.1500]		0.1039 [−0.1170; 0.3248]	
Others (1)	NA	NA	0.2167 [−0.5698; 1.0032]		0.1275 [−0.7407; 0.9957]	
CYP3A phenotype: intermediate vs. rapid	0.7814 [0.4042; 1.1585]	**0.0001**	0.6662 [0.4996; 0.8328]	**$<10^{-14}$**	0.8696 [0.6962; 1.0430]	**$<10^{-20}$**
CYP3A phenotype: slow vs. rapid	1.5607 [0.8755; 2.2459]		0.9810 [0.7293; 1.2327]		1.2112 [0.9444; 1.4780]	
P-gp phenotype: intermediate vs. rapid	−0.0037 [−0.2715; 0.2642]	0.6829	−0.0412 [−0.1690; 0.0866]	0.5590		
P-gp phenotype: slow vs. rapid	−0.1281 [−0.4441; 0.1880]		0.0595 [−0.1334; 0.2524]			
Main cause of end-stage renal disease (vs. hypertension, n = 62)						
Chronic interstitial nephropathy and pyelonephritis (24)	−0.4915 [−0.9719; −0.0111]	**0.0092**	−0.1584 [−0.4415; 0.1246]	0.4863		
Diabetes mellitus (39)	0.1143 [−0.3641; 0.5926]		−0.0713 [−0.3320; 0.1894]			
Dysimmune nephropathy including lupus and vascularitis (11)	−0.9491 [−1.7282; −0.1699]		−0.0293 [−0.3896; 0.3310]			
Glomerulopathy including IgA nephropathy (90)	0.4351 [0.0297; 0.8406]		−0.1901 [−0.3935; 0.0132]			
Polycystic kidney disease (73)	0.0880 [−0.2981; 0.4741]		−0.1547 [−0.3513; 0.0419]			
Uropathy including reflux nephropathy (13)	−0.1868 [−0.7753; 0.4016]		0.0355 [−0.3174; 0.3883]			
Undetermined (68)	−0.1384 [−0.5187; 0.2420]		−0.2046 [−0.4012; −0.0080]			
Other (14)	0.5866 [−0.0173; 1.1905]		−0.3229 [−0.7408; 0.0949]			
Cardiovascular disease (Y/N)	0.2285 [−0.1214; 0.5783]	0.2052	0.1528 [−0.0047; 0.3103]	0.0587		
Diabetes at baseline (Y/N)	0.2029 [−0.1981; 0.6039]	0.3253	0.0932 [−0.1025; 0.2889]	0.3517	0.1639 [0.0142; 0.3135]	**0.0328**
Dyslipidemia at baseline (Y/N)	0.3757 [0.1370; 0.6145]	**0.0031**	0.0571 [−0.0706; 0.1847]	0.3820	0.0379 [−0.0880; 0.1639]	0.5555
BMI at D0	−0.0002 [−0.0307; 0.0302]	0.9895				
Requirement for dialysis over the 1st week post-transplant (Y/N)	−0.1052 [−0.5217; 0.3113]	0.6223				
Number of dialyses	0.0105 [−0.0741; 0.0951]	0.8081				
Corticosteroids (Y/N)	NA	NA	−0.1501 [−0.2972; −0.0029]	**0.0470**		
Laboratory test results over the targeted periods						
Total bilirubin	0.0517 [0.0071; 0.0964]	**0.0261**	0.0001 [−0.0080; 0.0083]	0.9756		
ASAT					0.0034 [0.0012; 0.0056]	**0.0023**
Gamma GT	−0.0003 [−0.0013; 0.0007]	0.5840			0.0002 [−0.0002; 0.0005]	0.4329
Hematocrit	0.0398 [0.0147; 0.0649]	**0.0026**				
Plasma creatinine	0.0009 [0.0000; 0.0018]	**0.0484**				
eGFR	0.0006 [−0.0033; 0.0045]	0.7606				
Urine creatinine	−0.0189 [−0.0500; 0.0122]	0.2394				
Number of C_0/dose measurements	0.0596 [−0.0567; 0.1759]	0.3187				
Timing of tacrolimus intake (during meals vs. outside meals)	NA	NA	−0.0741 [−0.2978; 0.1497]	0.5172		

**Patient numbers in brackets are for the period D0-D7.*
NA, not assessed.
Cells are left empty when variables were not significant at the univariate stage.
Significant p-values (<0.05) are in bold characters.

TABLE 4 | Intra-individual variability (IOV) of log(C_0/dose) and C_0/dose over the M3-M12 period.

Metrics	IOV of (C_0/dose)	IOV of log (C_0/dose)
Minimum	0	−23.292
1st quartile	0.149	−0.234
Median	0.212	0.274
Mean	0.241	−0.023
3rd quartile	0.283	0.568
Maximum	0.922	17.857

derive approximately 150, 250, and 500–600 calories from protein, carbohydrate, and fat, respectively" [45].

No association between log(C_0/D) and ethnicity was identified as a result of multivariate analysis at any time period, probably because ethnicity was confounded by the CYP3A and possibly the P-gp phenotypes [46, 47]. Actually, significant association was found between ethnicity and the CYP3A phenotype ($p < 0.001$) when studying the collinearity or covariation of the potential covariates (**Supplementary Figure S1**). For this reason, we re-ran the multivariate analyses without considering the CYP3A phenotype (**Supplementary Table S2**), unveiling a significant influence of ethnicity at all periods ($p = 0.0234$, $<10^{-4}$ and $<10^{-4}$ at D4-D7, D8-M3 and M3-M12, respectively) and confirming confusion between the two groups of variables, but also showing that the models with the CYP3A phenotype account for much more variability than those with ethnicity (72.3% vs. 60.9% at D4-D7, 33.5% vs. 15.9% at D8-M3 and 36.4% vs. 13.1% at M3-M12).

Tacrolimus is most often presented as a drug with large IIV and IOV. This study shows that IOV is actually moderate, from 19.2% over D4-D7 to 24.1% over M3-M12. This is not surprising, since individual dose adjustment would be useless in case of short- or mid-term large IOV. It is most probably larger over the full first year post-transplantation, due to the progressive "maturation" of tacrolimus oral clearance, which is the reason why we focused on the latest period to evaluate the determinants of IOV. Despite being moderate on average, IOV was much larger (ca. between 40% and 92%) in a minority of patients, and many studies showed that it is an independent risk of treatment failure [48]. In contrast, IOV was lower in patients with chronic hepatic

TABLE 5 | TEAEs.

MedDRA System Organ Class - Preferred Term	Number of TEAEs	Number of patients	Percentage of patients
Any TEAEs	**2,477**	**369**	**89.3**
Blood and lymphatic system disorders	**336**	**194**	**47.0**
- Anemia	120	111	26.9
- Leukopenia	77	57	13.8
- Neutropenia	50	43	10.4
- Thrombocytopenia	25	22	5.3
Gastrointestinal disorders	**235**	**148**	**35.8**
- Diarrhea	117	94	22.8
General disorders and administration site conditions	**122**	**90**	**21.8**
- Oedema peripheral	48	41	9.9
Hepatobiliary disorders	**48**	**40**	**9.7**
- Hepatocellular injury	23	22	5.3
Immune system disorders	**36**	**32**	**7.7**
- Transplant rejection	21	21	5.1
Infections and infestations	**291**	**182**	**44.1**
- BK virus infection	29	29	7.0
- Cytomegalovirus infection	41	38	9.2
- Urinary tract infection	38	34	8.2
Injury, poisoning and procedural complications	**146**	**106**	**25.7**
- Delayed graft function	23	23	5.6
- Overdose	31	25	6.1
Metabolism and nutrition disorders	**369**	**167**	**40.4**
- Diabetes mellitus	39	39	9.4
- Hyperkaliemia	34	31	7.5
- Hypokalemia	40	31	7,5
- Hypophosphatemia	37	33	8.0
- Metabolic acidosis	25	23	5.6
Nervous system disorders	**116**	**98**	**23.7**
- Tremor	72	71	17.2
Renal and urinary disorders	**244**	**150**	**36.3**
- Acute kidney injury	69	48	11.6
- Renal impairment	38	35	8.5
Respiratory, thoracic and mediastinal disorders	**59**	**50**	**12.1**
- Dyspnea	21	21	5.1
Vascular disorders	**144**	**111**	**26.9**
- Hypertension	67	63	15.3
- Lymphocele	22	21	5.1

disorder or cancer. Hepatic disorders may result in a lower metabolic capacity, but the link with a lower IOV is not straightforward. We have no explanation to offer either for the impact of cancer on IOV. Both findings should obviously be confirmed in independent patient populations. Another interesting finding is that none of the other factors, including CYP3A or P-gp phenotypes, ethnicity and food effect, had a significant influence on IOV.

Regarding safety, a high number of TEAEs was expected in view of patient conditions and polypharmacy. Indeed, the primary kidney disease, existing comorbidities, surgery itself, and numerous concomitant therapies may result in many adverse events in the early post-transplant phase [12, 49]. The high number of TEAEs with a suspected causality to tacrolimus was also expected, and is in line with tacrolimus safety profile in adult recipients of a first kidney allograft [50]. All reported TEAEs were known and there was no unexpected safety signal.

This study presents several limitations. First, only a few patients declared themselves as Asians (1.9%), resulting in limited representativity of this group. Moreover, Asia is made up of multiple ethnicities with wide variations in the frequency of CYP3A and ABCB1 polymorphisms between them, and for this reason we recommend replicating this study in Asia [46]. More generally, the present results might not be extrapolated to people from origins not or poorly represented in the study. Also, considering that we only enrolled *de novo* adult kidney transplant patients, our results might not be extrapolated to pediatric patients, or patients transplanted with another organ.

In summary, this prospective, non-interventional, multicenter study, conducted in 440 *de novo* adult kidney transplant patients treated with twice daily tacrolimus, evaluated the combined influence of the timing of tacrolimus intake, high-fat food consumption, CYP3A and P-gp phenotypes, ethnicity and many other variability factors on tacrolimus exposure over the first week and up to 1-year post-transplant in a real-life setting. Over D4-D7, recipient age at baseline, the main cause of end-stage renal disease, CYP3A phenotype, dyslipidemia at baseline and hematocrit, total bilirubin and plasma creatinine over the time period influenced tacrolimus exposure. Together with the multivariable model developed, they may be leveraged to determine the initial dose of tacrolimus. Recipient age at baseline and the CYP3A phenotype were also found to be variability factors over D8-M3 and M3-12, whereas the use of corticosteroids, diabetes at baseline, and ASAT yielded inconstant results between D8-M3 and M3-M12. Tacrolimus intake during meals and high-fat food consumption had no significant influence, while ethnicity was confounded by the CYP3A phenotype. Finally, intra-individual variability in the more stable period M3-M12 was mild and was only influenced by hepatic disorder and cancer, not by CYP3A or Pgp phenotypes, nor ethnicity.

DATA AVAILABILITY STATEMENT

The authors will make the study protocol and statistical analysis plan available upon request to interested researchers. The data cannot be shared for legal, ethical and patient privacy restrictions.

ETHICS STATEMENT

The study protocol and its amendments were approved by an Ethics Committee in France (reference 2-17-47, ID-RCB: 2017-A02512-51) and the study was conducted in accordance with the Good Clinical Practice guidelines, the Declaration of Helsinki, the Declaration of Istanbul and all other applicable regulatory requirements. The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

PM, DA, and LR conceived and designed the study with Sandoz S.A.S. JB, SA, and LS managed the conduct of the study. PM, DA, and LR participated in the study as investigators. AH and AL-C performed the statistical analyses. PM, SG, and AL-C wrote the manuscript. All the authors commented and revised the manuscript.

FUNDING

The authors declare that financial support was received for the research, authorship, and/or publication of this article. The authors declare that this study received funding from SANDOZ, S.A.S, Levallois-Perret, France. The funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication.

CONFLICT OF INTEREST

SA and LS are employees of SANDOZ S.A.S, Levallois-Perret, France, which funded the study. JB, SG, and AL-C are employees of RCTs, the contract research organization that conducted the study on behalf of SANDOZ S.A.S. AH is a biostatistician at University Hospital of Limoges, France. PM, DA, and LR received honoraria from SANDOZ S.A.S as members of the study's independent Scientific Committee. PM is a consultant/medical expert for Astellas, BMS, Chiesi, Exeltis, MedIncell, Pfizer, Siemens and Womed. DA is a consultant/medical expert for Astellas, Astra Zeneca, BMS and Chiesi. LR is a consultant/medical expert for BMS and Sanofi.

ACKNOWLEDGMENTS

The authors would like to acknowledge and thank all the participating investigators who collected study data and enrolled and took care of study patients: LR, CHU Grenoble Alpes, Grenoble; Dr. Dominique Bertrand, CH Bois Guillaume, Rouen; Pr. Lionel Couzi, CHU Bordeaux Pellegrin, Bordeaux; Pr. Jacques Dantal, CHU Hotel Dieu, Nantes; Pr. Matthias Buchler, CHU Tours, Tours; DA, Hôpital Necker, Paris; Dr. Michel Delahousse,

Hôpital Foch, Suresnes; Dr. Jean-Philippe Rerolle, CHU Limoges, Limoges; Dr. Valérie Garrigue, CHU Montpellier, Montpellier; Dr. Laetitia Albano, CHU Nice, Hôpital Pasteur 2, Nice; Dr. Nicolas Bouvier, CHU de Caen, Caen; Pr. Benoît Barrou, CH Pitié-Salpêtrière, Paris; Pr. Philippe Grimbert, Hôpital Henri Mondor, Créteil; Pr. Antoine Thierry, CHU Poitiers, Poitiers; Pr. Yann Le Meur, CHU Cavale Blanche, Brest; Pr. Alexandre Hertig, Hôpital Tenon, Paris; Dr. Cyril Garrouste, CHU Clermont-Ferrand, Clermont-Ferrand; Pr. Christophe Mariat, CHU Saint-Etienne, Saint-Etienne; Pr. Nicolas Picard, UMR1248 Pharmacology and Transplantation, Inserm, Université de Limoges.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2024.13495/full#supplementary-material

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ORIGINAL RESEARCH
published: 15 October 2024
doi: 10.3389/ti.2024.13407

C1q Binding Ability for Prior Risk Assessment of Acute Antibody-Mediated Rejection in ABO-Incompatible Kidney Transplantation

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In ABO blood group incompatible kidney transplantation (ABO-I), potential issues on acute antibody-mediated rejection (ABMR) remain to be solved. This study aimed to assess the risk factors of acute ABMR using recipient- or donor-derived specimens. Quantitative analysis of A/B antigen expression was conducted in 104 donor kidney tissues (Kt), platelets (Plt), and red blood cells (RBC) by immunohistochemical staining or flow cytometry (FCM). ABO-I pre-transplant recipient serum samples (ABMR = 12, non-ABMR = 27) were extracted by propensity score matching. Anti-A antibody titers of IgM, IgG and IgG subclasses, and C1q binding ability (%) on antibody were measured using RBC-FCM. No association was observed between ABMR and A/B antigen expression levels in donor's Plt, RBC, or Kt. In recipient's sample, C1q-IgG binding ability was significantly higher in the ABMR group than in the non-ABMR group (C1q–IgG: 9.04% vs. 5.93% $p = 0.049$). Neither the A/B antigen expression level in donors (grafts) nor anti-blood group IgG/IgM antibodies in recipient sera before desensitization seemed to influence ABMR incidence in ABO-I. In contrast, C1q-IgG binding ability could be a potential predictor for ABMR in ABO-I.

Keywords: ABO-incompatible kidney transplantation, acute antibody-mediated rejection, A/B antigen expression levels of donor specimens, IgG subclasses, C1q binding ability

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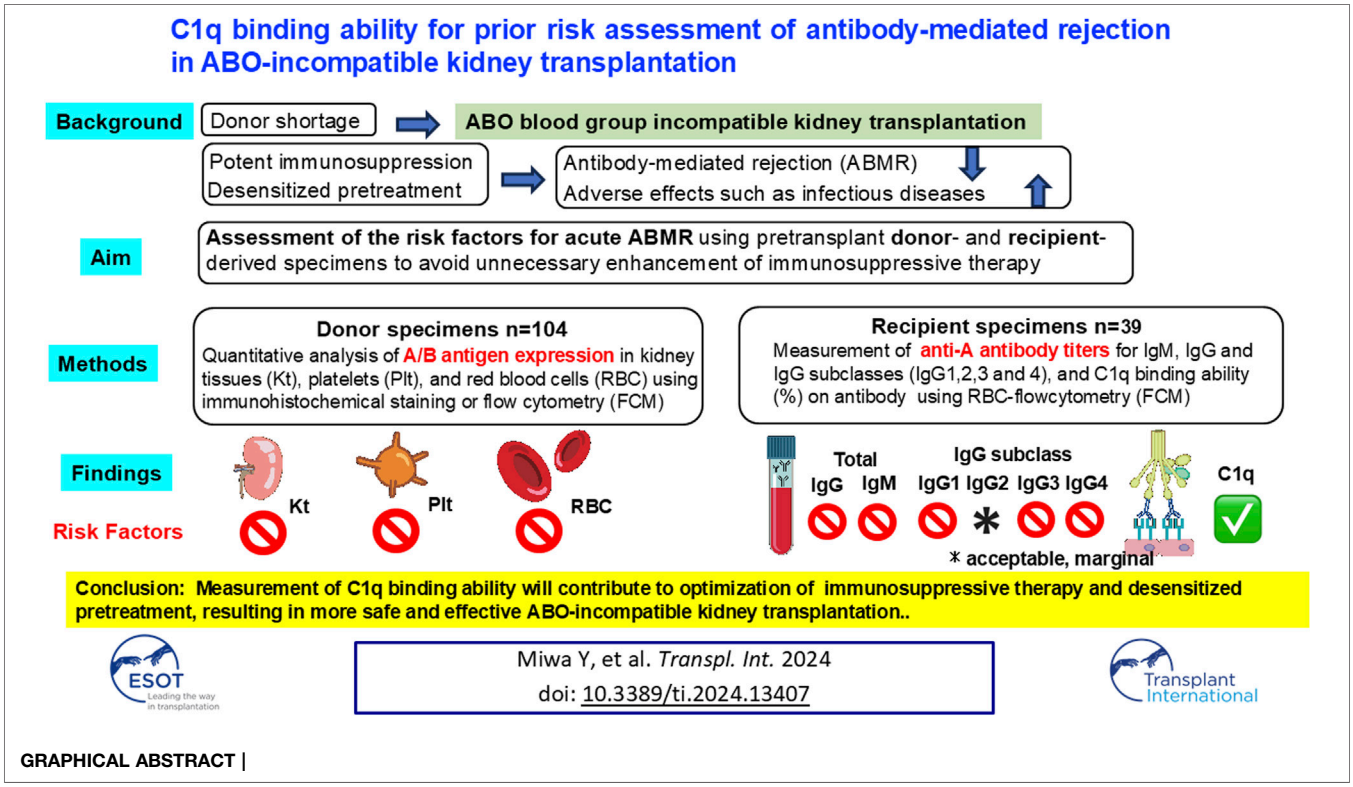
Received: *19 June 2024*
Accepted: *30 September 2024*
Published: *15 October 2024*

Citation:
Miwa Y, Iwasaki K, Murotani K, Okada M, Nagasaka T, Watarai Y, Takeda A, Shizuku M, Ashimine S, Ishiyama K, Maruyama S and Kobayashi T (2024) C1q Binding Ability for Prior Risk Assessment of Acute Antibody-Mediated Rejection in ABO-Incompatible Kidney Transplantation. Transpl Int 37:13407. doi: 10.3389/ti.2024.13407

Abbreviations: ABO-I, ABO blood group incompatible kidney transplantation; ABO-Id/C, ABO blood group identical or compatible kidney transplantation; ABMR, Antibody-mediated rejection; AR, Acute rejection; AUC, Area under the ROC curve; CV, Coefficient of variation; DSA, Donor specific HLA antibody; EC, Endothelial cells; IHT, Isohemagglutinin titer; IST, Immunosuppressive therapy; Kt, Kidney tissues; MFI, Mean fluorescence intensity; NoR/S, Neither rituximab nor splenectomy; Plt, Platelets; RBC, Red blood cells; RIT, Rituximab; ROC, Receiver operating characteristic; ROI, Region of interest; SPX, Splenectomy.



GRAPHICAL ABSTRACT |

INTRODUCTION

Effective desensitization therapy has improved the outcomes of ABO-incompatible (ABO-I) kidney transplantation [1, 2]. However, the graft survival rate of ABO-I is slightly inferior to ABO-Identical/compatible kidney transplantation (ABO-Id/C) [3, 4]. This may be due not to acute antibody-mediated rejection (ABMR) but adverse effects such as infectious diseases [5]. The recent SARS-COV-2 pandemic has caused fear of infection in immunosuppressed transplant patients [6, 7]. Furthermore, patients under rituximab (RIT) treatment showed low vaccine efficacy [8]. Therefore, unnecessary desensitization therapy should be avoided. Optimization of immunosuppressive therapy (IST) including desensitization by risk stratification of acute ABMR (i.e., reduction of desensitization regimen for patients with a low risk) may further improve outcomes in ABO-I.

The intensity of the antigen-antibody reaction is defined by the density of antigen expression and the amount of antibody. Determining the antigen expression in vascular endothelial cells (EC) of donor grafts before transplantation could provide important information on donor risk factors. However, since the kidney tissues (Kt) of donors are not commonly available before transplantation, platelets (Plt) and red blood cells (RBC) in the peripheral blood, and which express blood group A/B antigens [9–12], were examined to test whether their expression levels correlate with the amount of A/B antigen in the graft's EC. Although the carbohydrate binding protein [13] or carbohydrate chain of a glycan precursor [14, 15] properties of A/B antigens seem to differ between RBC and EC, it would be important to know whether the A/B antigen expression levels in RBC or Plt can reflect those in EC. Currently, measuring the anti-A/B antibody titer in the recipient serum by hemagglutination is widely used as a main pre-test in ABO-I, but anti-A/B antibody titer alone may be insufficient in clinical settings [16, 17].

Another risk factor, this time in recipients, is related to a difference in complement activation ability between IgG subclasses: both IgG1 and IgG3 have higher ability than IgG2 or IgG4 [18, 19]. Furthermore, the IgG1, IgG2, IgG3, and IgG4 distribution in peripheral blood differs from person to person [14, 20]. However, whether the patterns of IgG subclasses in the recipient's pre-transplant blood can be a risk factor of acute ABMR in ABO-I remains unknown. In contrast, in HLA (Human Leukocyte antigen) -incompatible kidney transplantation (HLA-I), there are reports on the value of IgG subclass post-transplant measurement in recipients as a prognostic marker [21–23]. C1q, the first component of the complement activation through the classical pathway, binding ability to donor specific HLA antibody (DSA) has been associated with ABMR and graft loss [24, 25], whereas the correlation between ABMR incidence in ABO-I and C1q binding ability to anti-A/B antibody has not been reported yet.

In this study, we examined whether A/B antigen expression in the donor (Kt, RBC, and Plt) and C1q binding ability against donor RBC, and anti-A antibodies in recipient sera could predict ABMR in ABO-I.

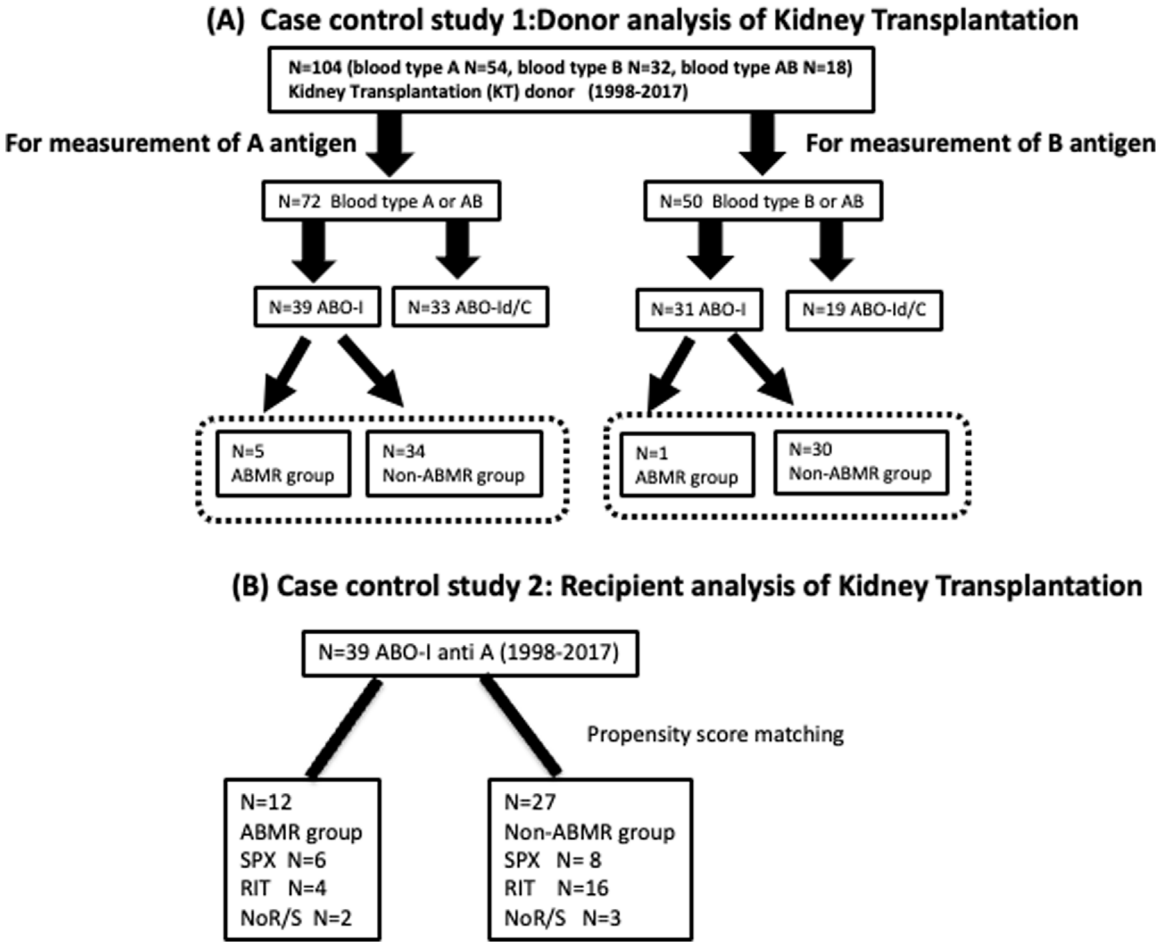


FIGURE 1 | Flow chart of patient selection **(A)** Case control study 1: Donor analysis of kidney transplantation Plt, RBC, and Kt were collected from 104 ABO-I and ABO-Id/C donors to measure A/B antigen expression. Then, in ABO-I, we compared whether there was a difference in A antigen expression between the ABMR and non-ABMR groups. Plt, platelets; RBC, red blood cells; Kt, kidney tissues; ABO-I, ABO-incompatible kidney transplantation; ABO-Id/C, ABO-identical or compatible kidney transplantation. **(B)** Case control study 2: Recipient analysis of kidney transplantation. The background of anti-A 39 patients who underwent ABO-I at the Japanese Red Cross Aichi Medical Center Nagoya Daini Hospital between 1998 and 2017 were compared between ABMR and non-ABMR groups. RIT, rituximab; SPX, splenectomy; NoR/S, neither rituximab nor splenectomy.

MATERIALS AND METHODS

Study Design and Patients

[Donor Patients] Case Control Study 1

Kidney grafts from 104 living donors (A group: n = 54, B group: n = 32, AB group: n = 18) were transplanted at the Japanese Red Cross Aichi Medical Center Nagoya Daini Hospital, between 1998 and 2017. Among 72 patients expressing A antigen in the grafts, 39 patients and 33 patients were ABO-I and ABO-Id/C, respectively. Five of the 39 ABO-I had ABMR. Similarly, only one in 30 ABO-I expressing blood group B in the graft showed acute ABMR (**Figure 1A**).

[Recipient Patients] Case Control Study 2

The backgrounds of 42 patients with blood group A mismatch who underwent ABO-I at the Japanese Red Cross Aichi Medical Center Nagoya Daini Hospital between 1998 and 2017 were compared after classification into the ABMR and non-ABMR groups (**Figure 1B**). Patients in the non-ABMR group were extracted based on propensity score matching; there was no significant difference in age, sex, blood group, desensitization therapy, and maintenance IST of recipient and donor patients. Patient characteristics are summarized in **Table 1**. The study was performed in accordance with the guidelines of the Declaration of Helsinki, after approval from the hospital's institutional ethical committee of Aichi Medical University School of Medicine (authorization number 15-092, 15-H072).

Desensitization Protocol

ABO-I recipients were pretreated with mycophenolate mofetil (MMF) from day −14, double-filtration plasmapheresis (DFPP) and either splenectomy (SPX), rituximab (RIT) (200 mg/body; twice; days −14 and −1, available from 2008), or neither (due to low anti-A/B antibody titers). Preoperative DFPP was routinely performed four times (days −6, −4, −2, and −1) in RIT or SPX and twice (days −2 and −1) in NoR/S.

TABLE 1 | Characteristics of the anti A patients of ABO-I.

	ABO-I ABMR (n = 12)	ABO-I non-ABMR (n = 27)	P-value
Male, n (%)	6 (50.0)	18 (66.7)	0.323
Age, y.o, median (range)	46 (19–76)	52 (22–71)	0.268
Donor age, y.o, median (range)	59 (44–74)	62 (43–82)	0.277
ABO blood type of donor → Recipient			
A→O, n (%)	10 (83.3)	16 (59.3)	0.141
AB→O, n (%)	0	2 (7.4)	0.333
A→B, n (%)	2 (16.7)	6 (22.2)	0.692
AB→B, n (%)	0	3 (11.1)	0.229
Desensitization therapy			
Splenectomy (SPX), n (%)	6 (50.0)	8 (29.6)	0.221
Rituximab (RIT), n (%)	4 (33.3)	17 (59.3)	0.135
SPX (–), RIT (–) (%)	2 (16.7)	3 (11.1)	0.632
HLA antibody			
Anti HLA sesitized recipients (n,%)	0	0	—
de novo DSA (n,%)	0	4 (14.8)	0.159
Maintenance Immunosuppression			
Cyclosporine A, n (%)	10 (83.3)	22 (81.5)	0.889
Tacrolimus, n (%)	2 (16.7)	5 (18.5)	0.889

ABO-I, ABO blood group incompatible kidney transplantation; ABMR, Antibody-mediated rejection.
P < 0.05.

Immunosuppression Protocol

All transplant recipients received 500 mg methylprednisolone intravenously before graft reperfusion and 20 mg of basiliximab intravenously on days 0 and 4. The immunosuppressive regimen consisted of a calcineurin inhibitor (cyclosporine or tacrolimus), an antimetabolite (MMF or mizoribine) or mammalian target of rapamycin inhibitor (everolimus, available from 2008), and prednisolone. The dosage of all oral immunosuppressive medications, except prednisolone, was strictly adjusted according to pharmacokinetics (AUC 0–4 h or trough level). Cyclophosphamide was used as an antimetabolite only in case of SPX.

ABMR Diagnosis

In this study, recipients with preformed DSA were not extracted. Whenever rejection was clinically suspected, an episodic biopsy was performed. The diagnosis of rejection was made by a pathologist at the Japanese Red Cross Aichi Medical Center Nagoya Daini Hospital. If no anti-donor HLA Abs were detected at the time of rejection, the diagnosis of ABMR due to anti-A or anti-B Abs was made using the pathology findings of ABMR (Banff 1997, 2001, 2005, 2007, 2013, 2017) during the study period 1998-2017.

Immunohistochemical Staining of Kt

Donor renal tissue of 1-h biopsy after transplantation was formalin-fixed and embedded in paraffin. Staining for blood group A and B antigen was performed on 1 μm thick paraffin embedded sections. After deparaffinization, sections were incubated with a monoclonal mouse IgM anti-A antibody (clone MH04,3D3; Ortho Clinical Diagnostics, Tokyo, Japan) and a monoclonal mouse IgM anti-B antibody (clone NE11.19,5A5,3D4; Ortho Clinical Diagnostics) as primary antibodies. Next, sections were incubated with Dako Envision detection System (DAKO, Glostrup, Denmark) as second antibody. Peroxidase activity was visualized by staining with a 3,3′-Diaminobenzidine, tetrahydrochloride (DAB) solution. Immunostained slides were scanned in a virtual slide microscopy (VS120, Olympus, Tokyo, Japan). In this study, the DAB stain area of A/B antigens, was measured using the image analysis software Tissuemorph DP (Visiopharm, Hoersholm, Denmark). A/B antigen expression was analyzed in three selected renal glomeruli; Tissuemorph DP shows the area of DAB stain in green, the nuclei in blue, and a region of interest (ROI) around blue dotted line. The index of A/B antigen immunopositivity was the ratio of the total DAB stain area and total ROI area (Max DAB/ROI value; **Supplementary Figure S1**) [26].

Flow Cytometry Analysis of Blood Type A, B Antigen Expression on Plt and RBC

Platelet-rich plasma (PRP) was prepared by centrifugation of anti-coagulated whole blood in acid-citrate-dextrose (ACD) tube at 250 g for 15 min. Then, the PRP was diluted three times with 20% ACD in Plt buffer (0.14 M NaCl, 5 mM KCl, 1 mM MgSO4, and 10 mM HEPES, pH 7.4), and centrifuged at 750 *g* for 2.5 min to form platelet pellets. Plt were stabilized by fixation in paraformaldehyde at a final concentration of 1%. RBC was collected by centrifugation at 1,000 g from citric acid-treated blood and washed twice with PBS (–) containing 0.2% bovine serum albumin and 0.1% NaN_3 (wash buffer). Then, they were incubated with 3 mg/mL dimethyl suberimidate dihydrochroride (DMS) in 0.1 M Na_2CO_3 containing 0.15 M NaCl and 0.1 mM EDTA at 37°C for 20 min to prevent agglutination. DMS-treated RBC were washed with wash buffer twice and suspended in wash buffer at 1% concentration. For the detection of blood group A/B antigen in Plt and RBC using flow cytometry (FACSCanto II, Becton Dickinson, San Jose, CA, United States), 4.0×10^6 Plt and 4.5×10^5 RBC were incubated with monoclonal mouse IgM anti-A or B antibody (Ortho Clinical Diagnostic) for 20 min at room

temperature. Fluorescein (FITC)-labeled goat anti-mouse IgM (American Qualex Antibodies, San Clemente, CA) was used as secondary antibody. A/B antigen expression levels were analyzed by the mean fluorescence intensity (MFI).

Detection of Anti-A IgG, IgM, and IgG Antibody Titers in Patient Serum

For the detection of anti-A antibody titer in patient pre-treatment serum using RBC flow cytometry, 30 μL of 1×10^7/mL DMS-treated RBCs and 15 μL of heat-inactivated patient serum were incubated in 96-well plates for 20 min at room temperature. After three washes with 0.1% BSA in PBS (−), RBC were incubated with a diluted secondary antibody, either FITC-labeled rabbit anti-human IgG, IgM (DAKO) or R-phycoerythrin (R-PE)-labeled mouse IgG1, IgG2, IgG3, IgG4 (SouthernBiotech, Birmingham, AL, United States). The stained RBC were analyzed using high-throughput flow cytometry (FACS Canto II High Throughput Sampler option, Becton Dickinson), which allows simultaneous testing of large patient's samples in 96-well plates. The anti-A antibody isohemagglutinin antibody titers for IgG and IgM were serially measured as previously reported [27].

Detection of Complement C1q (C1q–IgG and C1q–IgG+IgM) Binding Ability in Patient Serum

To degrade IgM antibodies, heat-inactivated patient serum was incubated with 5 mM dithiothreitol (DTT) at 37°C for 30 min. At first 30 μL of 1×10^7/mL DMS-treated RBC and 15 μL of patient serum (DTT treated or non-treated) were incubated for 20 min at room temperature. After three washes with 0.1% BSA in PBS (−), RBC were incubated with 5 μL of complement component C1q from human serum (Sigma-Aldrich, St Louis, MO, United States) in PBS (−) at room temperature for 20 min. Then, after adding 50 μL of ×20 diluted FITC-labeled rabbit polyclonal anti-human C1q antibody (ab4223; Abcam plc, Cambridge, United Kingdom), RBC were incubated at room temperature for 20 min. After washing RBC twice with 0.1% BSA in PBS (−), RBC were measured using flow cytometry (FACS Canto II, Becton Dickinson). To assess C1q binding ability, RBC reacted with C1q; secondary antibody were used only as negative controls and threshold lines were drawn at 3% C1q binding ability of the AB blood type serum and compared in terms of positivity rate (%).

Statistical Analysis

The variability of groups with different units was expressed by the coefficient of variation (CV). The Mann–Whitney U test was used to compare two groups of continuous variables. Medians with a 25th and 75th percentile were calcurated. The cut-off value was determined by receiver operator characteristic curve (ROC) analysis using Youden index. Moreover, Fisher's exact test in a 2 × 2 contingency table was used to compare categorical data between groups. P values < 0.05 were considered statistically significant. Statistical analyses were performed using GraphPad Prism ver 5.03 and JMP ver 13.2.

RESULTS

Individual Differences in Blood Type A/B Antigen Expression in Donors' Plt, RBC, and Kt

We measured A/B antigen expression levels in the Plt, RBC, and Kt of 104 donors (**Figure 1A**), A antigen: n = 72 [ABMR(+) n = 5, ABMR(−) n = 34, ABO-Id/C n = 33], B antigen: n = 50 [ABMR(+) n = 1, ABMR(−) n = 30, ABO-Id/C n = 19] of donor patients (**Figure 2**). The inter-individual differences in both A and B antigen in Plt were larger than those in RBC and Kt [CV; 0.74 (Plt) vs. 0.19 (RBC) and 0.26 (Kt) in A antigen, 2.04 (Plt) vs. 0.23 (RBC) and 0.44 (Kt) in B antigen]. No correlation in A/B antigen expression levels was observed between Plt, RBC, and Kt (**Figure 2**).

Expression Levels of Blood Type A Antigen of Plt, RBC, and Kt in ABMR and Non-ABMR Groups

Next, we compared A antigen expression in the Plt, RBC, and Kt of ABO-I donors between ABMR and non-ABMR groups. No significant difference in A antigen expression levels was observed between groups (**Figure 3**). Regarding B antigen expression, although statistical analysis could not be performed because of the very small number of patients with ABMR, no increasing tendency was observed in B antigen expression levels in the ABMR group (**Figures 2D–F**).

Anti-A Total IgG and IgM Titers in ABMR and Non-ABMR Groups

Anti-A antibody median total IgG titers were higher in the ABMR group than in the non-ABMR [MFI: 6.59×10^4 (25th–75th percentile, 3.08×10^4–11.9×10^4) vs. MFI: 1.53×10^4 (25th–75th percentile, 1.01×10^4–7.13×10^4; $p = 0.110$)], as were anti-A antibody total IgM median titers [MFI: 3.35×10^4 (25th–75th percentile, 1.91×10^4–6.91×10^4) vs. MFI: 1.96×10^4 (25th–75th percentile, 1.15×10^4–3.74×10^4; $p = 0.175$)] (**Figure 4**; **Table 2**). MFI values were normalized to those obtained in normal control serum. The cut-off values were calculated from ROC analysis [anti-A IgG: 2.76×10^4, which is a hemagglutination test equivalent to 64 times, area under the curve (AUC) = 0.664, IgM: 2.89×10^4, which is the hemagglutination test equivalent to 32 times, AUC = 0.639] (**Table 2**; **Supplementary Figure S3**). Statistical analysis of anti-B titers was not possible due to the small number of ABMR patients.

Anti-A IgG Subclass Distribution in ABMR and Non-ABMR Groups

The anti-A antibody IgG1, IgG2, IgG3, and IgG4 levels were not significantly higher in the ABMR group than in the non-ABMR group [MFI: 3.07×10^4 (25th–75th percentile, 0.81×10^4–5.12×10^4) vs. MFI: 0.67×10^4 (25th–75th percentile, 0.24×10^4–2.89×10^4; $p = 0.131$ in IgG1], [MFI: 6.85×10^4 (25th–75th percentile,

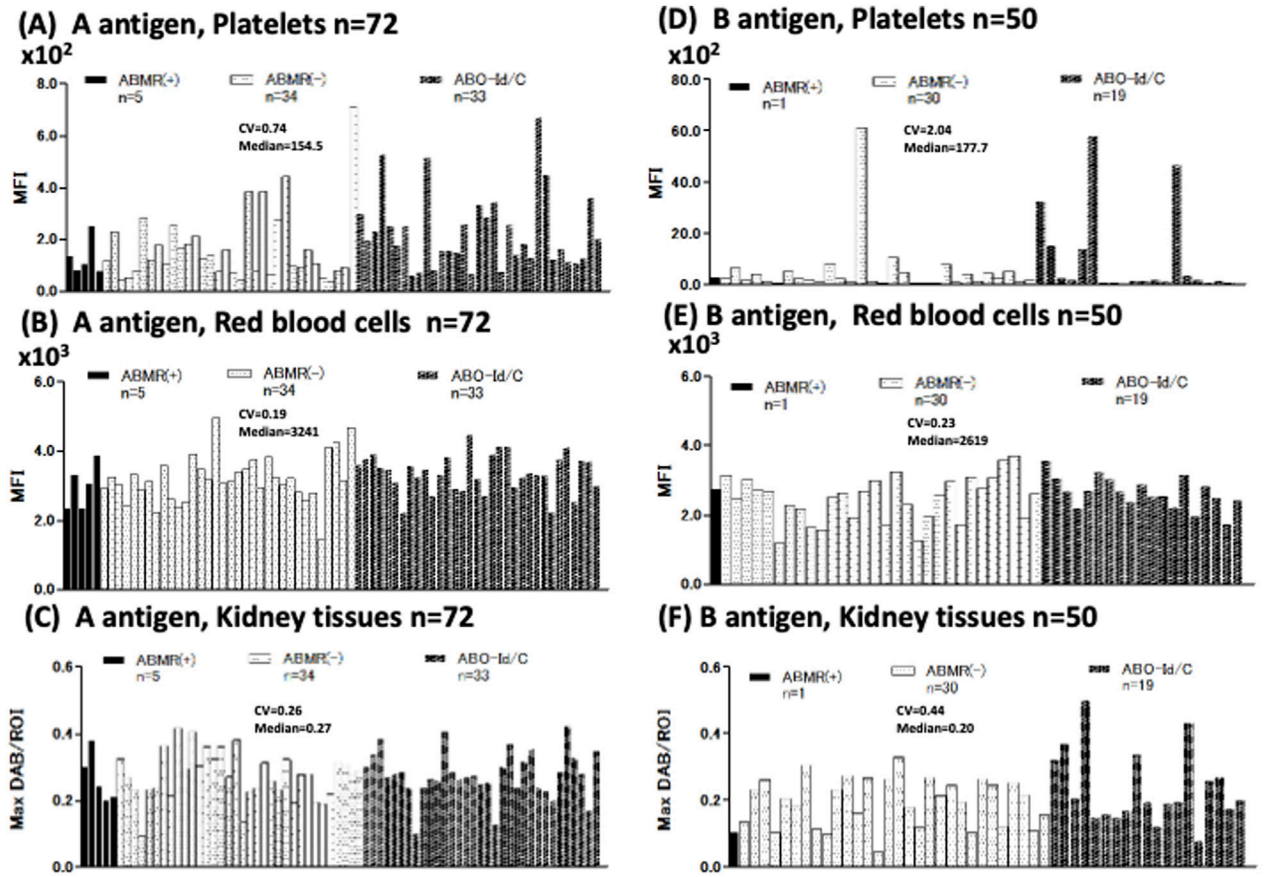


FIGURE 2 | Individual differences in blood group A/B antigen expression in platelets (Plt), red blood cell (RBC), and kidney tissue (Kt). Blood group A antigen expression of Plt, RBC, and Kt **(A–C)** Antigen A expression of 72 donors [ABMR(+) n = 5, ABMR(–) n = 34, ABO-Id/C n = 33]. Coefficient of variation (CV), index for comparing individual differences. Individual differences in Plt's antigen A expression were higher than in RBC and Kt (Plt, RBC, and Kt CV = 0.74, 0.19, and 0.26, respectively). B antigen expression of Plt, RBC, and Kt **(D–F)**. B antigen expression of 50 donors [ABMR(+) n = 1, ABMR(–) n = 30, ABO-Id/C n = 19]. Individual differences in Plt's B antigen expression were strikingly higher than in RBC and Kt (Plt, RBC, and Kt CV were 2.04, 0.23, and 0.44, respectively).

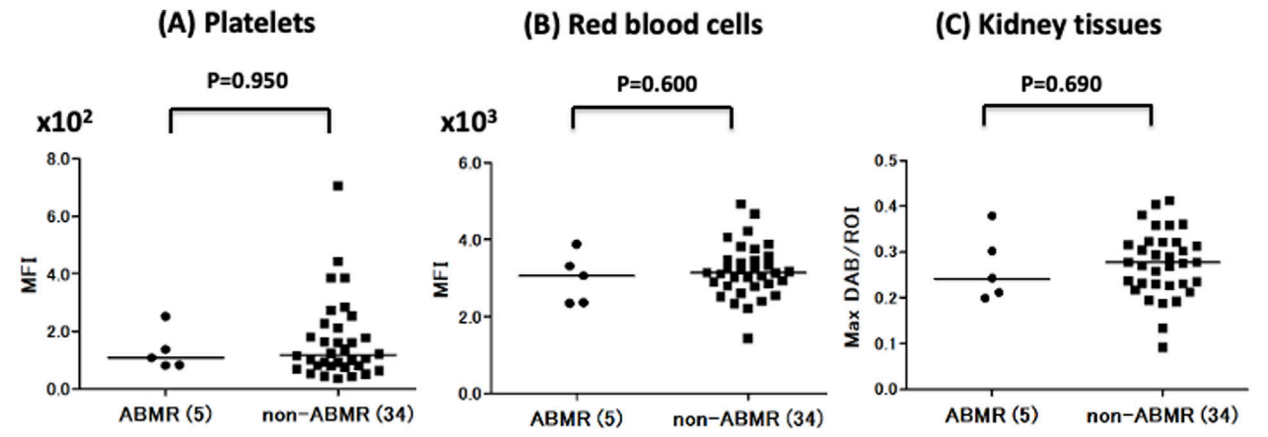


FIGURE 3 | Comparison of Plt, RBC, and Kt's blood group A antigen expression levels in the antibody-mediated rejection (ABMR) and non-ABMR groups. There were no significant differences in antigen A expression in Plt **(A)**, RBC **(B)**, and Kt **(C)** between the ABMR group and non-ABMR group.

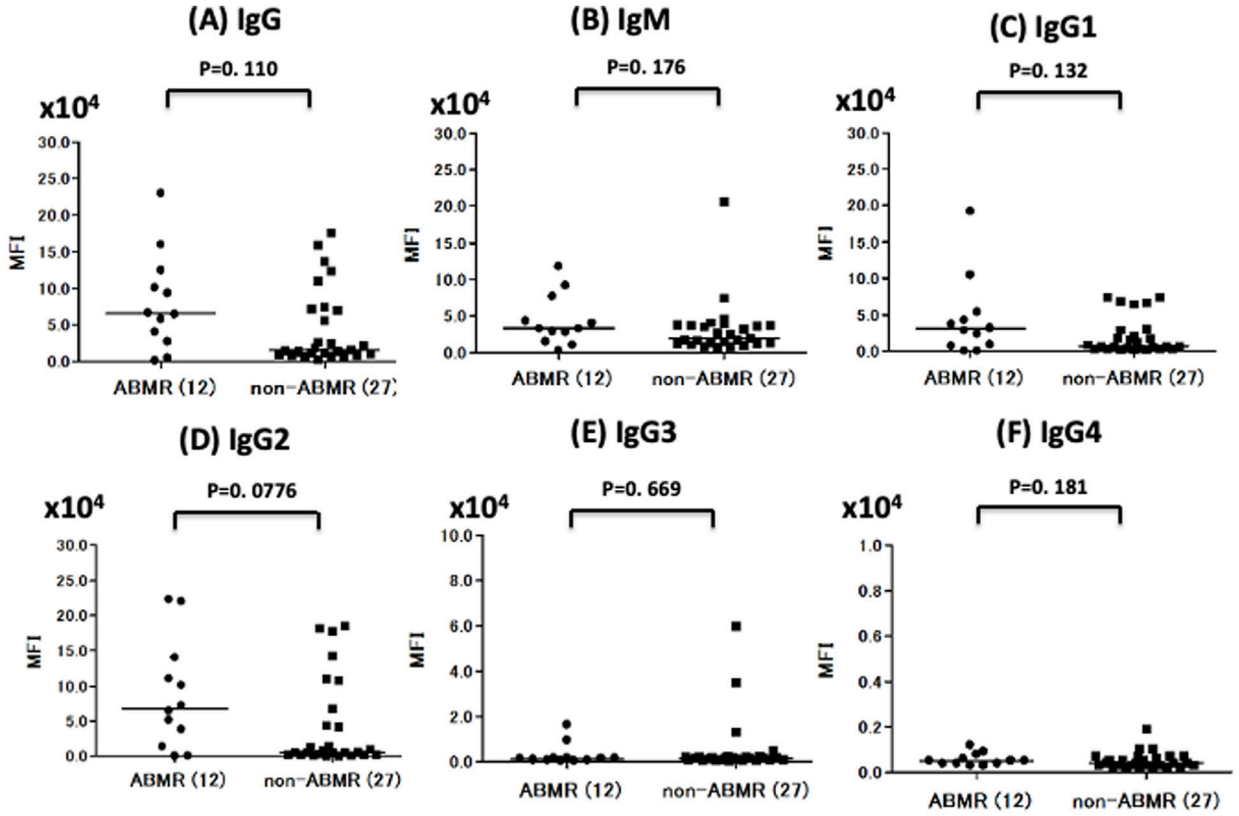


FIGURE 4 | Comparison of anti-A IgG, IgM, and IgG subclass titer in ABMR and non-ABMR groups. The anti-A antibody total IgG median titer (MFI) was higher in the ABMR group than in the non-ABMR ($p = 0.110$). **(A)** The anti-A antibody total IgM MFI was higher in the ABMR group than in the non-ABMR ($p = 0.175$). **(B)** In anti-A, IgG1 and IgG2 had no significant difference between ABMR group and non-ABMR group. [IgG1: $p = 0.131$, IgG2: $p = 0.077$, IgG3: $p = 0.669$, IgG4 = 0.180; **(C–F)**].

TABLE 2 | Comparison of the patients with anti A IgG, IgM, IgG subclass and C1q titer of ABMR and non-ABMR group in ABO-I.

	Median (25th and 75th percentile)		Mann-Whitney U-test	ROC curve (Receiver Operator Characteristic Curve) analysis	
	ABMR group (n=12)	non-ABMR group (n=27)	*P* value	cut off (IHT)	AUC
IgG	6.59×10^4 (3.08×10^4–11.9×10^4)	1.53×10^4 (1.01×10^4–7.13×10^4)	0.110	2.76×10^4 (×64)	0.664
IgM	3.35×10^4 (1.91×10^4–6.91×10^4)	1.96×10^4 (1.15×10^4–3.74×10^4)	0.175	2.89×10^4 (×32)	0.639
IgG1	3.07×10^4 (0.81×10^4–5.12×10^4)	0.67×10^4 (0.24×10^4–2.89×10^4)	0.131	2.40×10^4	0.654
IgG2	6.85×10^4 (2.02×10^4–13.4×10^4)	0.54×10^4 (0.19×10^4–6.77×10^4)	0.077	1.39×10^4	0.679
IgG3	0.13×10^4 (0.07×10^4–0.16×10^4)	0.15×10^4 (0.07×10^4–0.20×10^4)	0.669	0.16×10^4	0.537
IgG4	0.05×10^4 (0.04×10^4–0.08×10^4)	0.04×10^4 (0.03×10^4–0.07×10^4)	0.180	0.03×10^4	0.639
C1q-IgG	9.04% (7.63–26.7)	5.93% (4.48–10.3)	0.049	7.47%	0.701
C1q-IgG+IgM	37.4% (5.48–80.5)	9.70% (2.00–57.1)	0.120	26.6%	0.659

ABMR, Antibody-mediated rejection; IHT, Isohemagglutinin titer; AUC, Aria under the ROC curve.
P < 0.05.

2.02×10^4–13.4×10^4) vs. MFI: 0.54×10^4 (25th–75th percentile, 0.19×10^4–6.77×10^4), $p = 0.077$ in IgG2], [MFI: 0.13×10^4 (25th–75th percentile, 0.07×10^4–0.16×10^4) vs. MFI: 0.15×10^4 (25th–75th percentile, 0.07×10^4–0.20×10^4), $p = 0.669$ in IgG3], [MFI: 0.05×10^4 (25th–75th percentile, 0.04×10^4–0.09×10^4) vs. MFI: 0.04×10^4 (25th–75th percentile, 0.04×10^4–0.08×10^4), $p =$

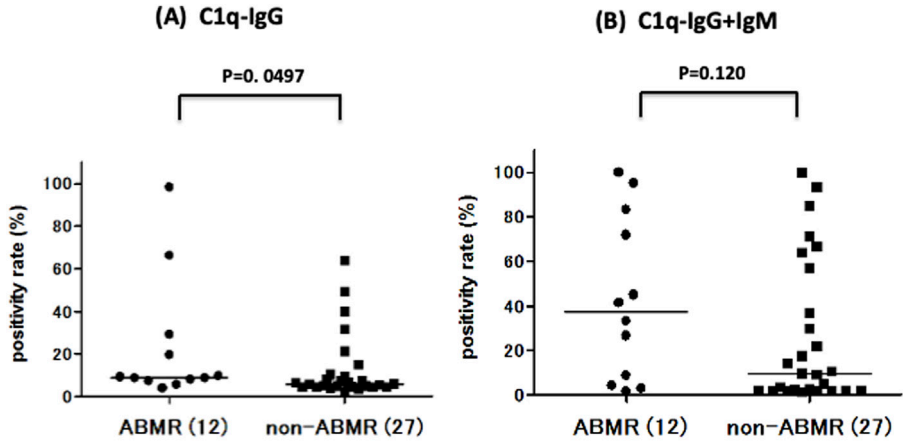


FIGURE 5 | Comparison of anti-A complement C1q titer between ABMR and non-ABMR groups. There was a significant difference between groups in C1q, after removing the influence of IgM binding by DTT, there was a significant difference between groups in anti-A C1q–IgG positive rate (%) [anti-A: $p = 0.049$; **(A)**] in C1q–IgG+IgM positive rate (%) [anti-A: $p = 0.120$; **(B)**].

0.180 in IgG4], (**Figure 4**; **Table 2**). The MFI cut-off values were calculated from ROC analysis (IgG1: 2.40×10^4, AUC = 0.654, IgG2: 1.39×10^4, AUC = 0.679, IgG3: 0.16×10^4, AUC = 0.537, IgG4: 0.03×10^4, AUC = 0.639: **Table 2**).

C1q Binding Ability to Anti-A Antibody in ABMR and Non-ABMR Groups

C1q binding ability was measured under C1q–IgG and C1q–IgG+IgM (**Figure 5**). The positivity rates of C1q binding to anti-A antibody were compared between ABMR and non-ABMR groups. C1q–IgG positivity rates were significantly higher in the ABMR group than in the non-ABMR group [DTT-treated C1q, 9.04% (25th–75th percentile, 7.63–26.7) vs. 5.93 (25th–75th percentile, 4.48–10.3), $p = 0.049$ in anti-A (**Figure 5A**; **Table 2**)], as were C1q–IgG+IgM positivity rates [DTT-non-treated C1q, 37.4% (25th–75th percentile, 5.48–80.5) vs. 9.70 (25th–75th percentile, 2.00–57.1), $p = 0.120$ in anti-A (**Figure 5B**; **Table 2**)]. The MFI cut-off values were calculated from ROC analysis (C1q–IgG: 7.47% AUC = 0.701, C1q: 26.6%, AUC = 0.659; **Table 2**).

DISCUSSION

ABO(H) antigens are oligosaccharides expressed as glycoproteins or glycolipids on cells and tissues, synthesized by glycosyltransferase from different precursor chains based on subtype-1,2,3,4 glycans in humans, depending on the type of cell or tissue [28]. Jeyakatanthan et al. reported differential subtype antigen expression between RBC and tissues or organs [15]. In this study, the quantitative analysis of A/B antigen in Kt, RBC, and Plt demonstrated that neither was associated with ABMR, despite the large inter-individual differences observed in Plt. Ogasawara et al. reported that 7% of Japanese had high A and B antigen expression on Plt [9], and Curtis et al. also found that 7% and 4% of Caucasians showed high A and B antigen expression on Plt, respectively [10]. However, our data did not show a positive correlation between high A/B antigen expression on Plt and ABMR.

The origin of anti-A/B antibodies is still controversial, but the natural antibodies appearing in the neonatal period (3–6 months) are IgMs [29, 30]. Although natural antibodies are usually produced in the absence of exogenous antigens, adult humans have anti-A/B antibodies of the IgG and IgA types produced by sensitization to food, bacteria and viruses which have similar antigens to those of A/B antigens [31]. ABO antigens are glycoprotein antigens, unlike HLA protein antigens. In general, protein antigens promote IgG1 and IgG3 production in B cells, after activation by T cells, whereas glycoprotein antigens mostly promote IgG2 and IgG4 production by B cells in the absence of T cells [32, 33]. The strength of complement activation varies by IgG subclass [18, 19]. IgG1 and IgG3 have a strong affinity for C1q, the first component of the complement pathway, and can thereby activate the complement [34]. Although IgG2 has a weaker complement activation ability than IgG1 and IgG3, the induction of complement activation depends on the density of antigen and antibody [19]. Therefore, high antibody titers of IgG2 can also activate the complement. It is also not yet clear which isotype (IgG or IgM) is more clinically important in ABO-I [35–37]. In the present study, we examined the total IgG/IgM, IgG subclass, and C1q binding ability to IgG/IgG+IgM ABO antibodies in the serum of patients undergoing pre-desensitization therapy. Higher IgG levels were more likely to be a risk factor for acute ABMR than IgM, but there was no significant difference between ABMR and non-ABMR groups. There was also a trend among IgG subclasses toward higher IgG1 and IgG2 levels being risk factors for ABMR, but there was no significant difference among subclasses between ABMR and non-ABMR groups. Comparatively, C1q binding ability (C1q-IgG) is likely to be a marker for ABMR, given the significant

differences between ABMR and non-ABMR groups. The C1q binding ability to anti-A antibodies may reflect the density of IgG1 and IgG2 antibodies bound to ABO antigens. Schaub et al. reported that the C1q binding ability to HLA antibodies only reflects the density of bound antibodies and not the composition of IgG subclasses (IgG1-IgG4) [38].

The slightly worse graft engraftment rate of ABO-I compared to ABO-Id/C might be due to side effects such as infection and malignancy or cardiovascular disease [6]. Moreover, renal transplant recipients receiving RIT therapy are less likely to produce antibodies against SARS-Cov-2 [39]. Therefore, introduction of RIT-avoidance (free) protocol may be preferable and could be considered in a certain group [40, 41]. To safely implement such a protocol, we analyzed the association between the C1q binding ability and ABMR, and showed a possibility that C1q binding ability might be a useful marker for RIT avoidance (reduction).

This study has some limitations, including its cross-sectional design (one-point test) which does not allow analyzing changes over time; in addition, there was heterogeneity in immunosuppressive therapy. Nevertheless, this study has two strengths. First, we conducted analysis of antigen expression levels on donors. Second, a complement binding assay, used for detailed examination of HLA antibodies, could be applied to anti-A/B antibodies as well, even if a DTT treatment was necessary to remove the influence of anti-A/B IgM antibodies.

In conclusion, although the amount of A/B antigen in donors cannot explain ABMR in ABO-I, C1q binding ability could be a risk factor for ABMR. Further prospective studies are needed to justify a reduction in desensitization therapy.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving humans were approved by The hospital's institutional ethical committee of Aichi Medical University School of Medicine (authorization number 15-092, 15-H072). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

YM, Participated in research design and collecting data, performance, data analysis and writing of the article. KI, Participated in data analysis and interpretation. KM, Participated in statistic analyzing the data. MO, Participated in collecting data and review of the article. TN, Participated in data analysis and interpretation. YW, Participated in collecting data and review of the article. AT, Participated in pathological analysis. MS, Participated in data analysis and interpretation. SA, Participated in data analysis and interpretation. KI, Participated in data analysis and review of the article. SM, Participated in pathological analysis. TK, Participated in research design and data interpretation, review of the article and writing of the article. All authors contributed to the article and approved the submitted version.

FUNDING

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was partly supported by a Grant-in-Aid for Scientific Research (No. 16K11063) from the Japan Society for the Promotion of Science (KAKENHI).

CONFLICT OF INTEREST

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

ACKNOWLEDGMENTS

The authors thank Ms. Yuriko Sawa for her excellent pathologic technical assistance. We gratefully thank institute of comprehensive medical research promotion, Aichi Medical University for the flow cytometry measurements. We wish to acknowledge Division for Medical Research Engineering, Nagoya University Graduate School of Medicine, for analysis with FACS Canto II High Throughput Sampler option and Tissuemorph DP. The authors would like to thank Ms. Yuko Matsuzaki for her excellent illustrations. The authors would also like to thank MARUZEN-YUSHODO Co., Ltd. (https://kw.maruzen.co.jp/kousei-honyaku/) for the English language editing.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2024.13407/full#supplementary-material

Supplementary Data 1 | Measuring of the DAB stain area of A/B antigens using the image analysis software Tissuemorph DP. The anti-A/B antibodies were diluted (dilution magnification ranged from ×25 to ×800) and compared in each sample with the highest DAB/ROI value.

Supplementary Data 2 | Calculation of the MFI cut-off values of anti-A IgG, IgM, IgG subclasses antibody and C1q-binding-ability by ROC analysis.

Supplementary Data 3 | Correlation between hemagglutinin titers and MFI of anti-A antibody IgG and IgM.

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ORIGINAL RESEARCH
published: 30 October 2024
doi: 10.3389/ti.2024.13551

Performance of a Global Functional Assay Based on Interferon-γ Release to Predict Infectious Complications and Cancer After Kidney Transplantation

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The QuantiFERON-Monitor assay (QTF-Monitor) is intended to assess innate and adaptive immune responses by quantifying interferon (IFN)-γ release upon whole blood stimulation with a TLR7/8 agonist and an anti-CD3 antibody. We performed the QTF-Monitor in 126 kidney transplant recipients (KTRs) at different points during the first 6 post-transplant months. The primary outcome was overall infection, whereas secondary outcomes included bacterial infection, opportunistic infection and *de novo* cancer. The association between IFN-γ production and outcomes was analyzed as "low" immune responses (<15 IU/mL) and as a continuous variable to explore alternative thresholds. There were no significant differences in the occurrence of overall infection according to the QTF-Monitor at any monitoring point. Regarding secondary outcomes, KTRs with a low response at week 2 experienced a higher incidence of bacterial infection (50.8% versus 24.4%; *P*-value = 0.006). Low response at month 1 was also associated with opportunistic infection (31.6% versus 14.3%; *P*-value = 0.033). The discriminative capacity of IFN-γ levels was poor (areas under the ROC curve: 0.677 and 0.659, respectively). No differences were observed for the remaining points or post-transplant cancer. In conclusion, the QTF-Monitor may have a role to predict bacterial and opportunistic infection in KTRs when performed early after transplantation.

Keywords: kidney transplantation, infection, immune monitoring, functional, prediction

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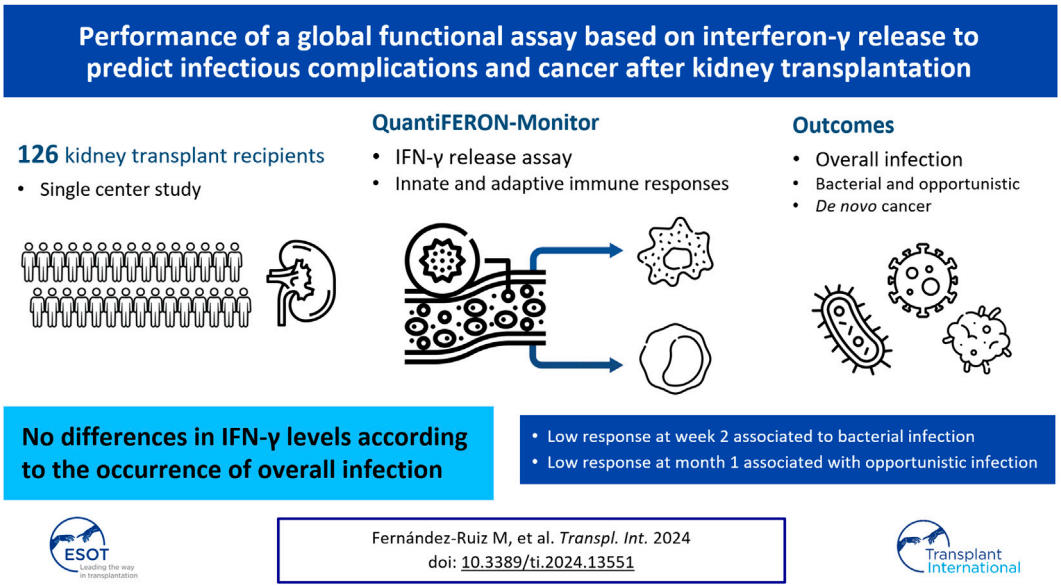
Received: 17 July 2024
Accepted: 22 October 2024
Published: 30 October 2024

Citation:
Fernández-Ruiz M, Ruiz-Merlo T, Rodríguez-Goncer I, Caso JM, López-Medrano F, Parra P, San Juan R, Polanco N, González E, Andrés A, Aguado JM and Redondo N (2024) Performance of a Global Functional Assay Based on Interferon-γ Release to Predict Infectious Complications and Cancer After Kidney Transplantation. Transpl Int 37:13551. doi: 10.3389/ti.2024.13551

Abbreviations: ATG, antithymocyte globulin; auROC, area under the receiving operating characteristic curve; BKPyVAN, BK polyomavirus-associated nephropathy; CI, confidence interval; CMV, cytomegalovirus; ELISA, enzyme-linked immunosorbent assay; IFD, invasive fungal disease; IFN, interferon; IGRA, interferon-γ release assay; IQR, interquartile range; IU, international unit; KT, kidney transplantation; LT, liver transplantation; LuT, lung transplantation; NPV, negative predictive value; PRR, pattern recognition receptor; PPV, positive predictive value; QTF-Monitor, QuantiFERON Monitor; SD, standard deviation; SOT, solid organ transplantation; TLR, toll-like receptor; TTV, Torque Teno virus.



GRAPHICAL ABSTRACT |

INTRODUCTION

The excellent results achieved with current immunosuppressive regimens in terms of graft function and patient survival after solid organ transplantation (SOT) are threatened by the development of complications such as infections or cancer [1, 2]. Therefore, the discovery and validation of biomarkers capable of informing on the net state of immunosuppression constitutes a research priority [3]. Many of these assays are designed to quantify the adaptive response against specific pathogens, typically cytomegalovirus (CMV) [4]. In addition, some non-pathogen-specific parameters have been proven to predict the occurrence of post-transplant infection [5] or cancer [6]. None of these approaches, however, provides a comprehensive assessment of the functionality of the innate and adaptive components of the immune system.

The innate immunity is triggered through various families of pattern recognition receptors (PRRs) that detect distinct evolutionarily conserved structural motifs present in microorganisms. Toll-like receptors (TLRs) are central actors in the orchestration of the innate immunity and its interplay with the adaptive arm [7]. The activation of TLR signaling pathways leads to the transcriptional upregulation of genes involved in inflammatory responses, such as proinflammatory cytokines or type I interferons (IFNs) [8]. Research efforts have been focused on the effect exerted by immunosuppressive agents on T-cell and B-cell responses, given their role in allorecognition and graft rejection. In addition, a renewed interest has emerged on the contribution of innate responses to post-transplant events [9]. Multiple studies have shown that polymorphisms in genes encoding for PRRs modulate individual susceptibility to bacterial, viral and fungal pathogens [10–12]. It may be hypothesized that the relative contribution of innate immunity to the host defense becomes more evident upon abrogation of adaptive responses by long-term immunosuppression.

The QuantiFERON-Monitor (QTF-Monitor) is a commercial enzyme-linked immunosorbent assay (ELISA)-based IFN-γ release assay (IGRA) intended to quantify innate and adaptive immune responses following incubation of heparinized whole blood with an agonist of TLR7/8 (R848 or resiquimod) and an anti-CD3 monoclonal antibody [13]. Despite the advantages of this comprehensive approach, only a few studies have investigated the usefulness of QTF-Monitor to predict infectious complications after KT [14, 15], liver transplantation (LT) [16] or lung transplantation (LuT) [17]. In addition, no previous studies have evaluated the potential application of this assay to evaluate the risk of *de novo* malignancies after transplantation. The pathogenesis of this complication is multifactorial, with the participation of host (older age, sun exposure, pre-transplant history of cancer, smoking and alcohol consumption, latent infection by oncogenic viruses) and transplant-related factors (such as donor-transmitted cancer) [18]. Nevertheless, the deleterious effect of immunosuppressive therapy on cancer immune surveillance and the assumed concept that post-transplant cancer acts as a marker of over-immunosuppression provide the rationale to investigate whether an assay able to interrogate innate and adaptive responses may be also useful to predict the occurrence of malignancy.

With these research gaps in mind, we have assessed the functional immune status of a single-center cohort of KT recipients by means of the QTF-Monitor assay performed at multiple points throughout the first 6 months in order to characterize the dynamics of IFN-γ

levels and their correlation with the development of infection and cancer.

PATIENTS AND METHODS

Study Design and Setting

We included consecutive adult patients that underwent KT at our institution between February 2018 and July 2019. Patients experiencing primary graft non-function or early (first week) graft loss were excluded. All participants provided written informed consent at study entry, which was carried out in accordance with the ethical standards laid down in the Declarations of Helsinki and Istanbul. The study protocol was approved by the local Clinical Research Ethics Committee (reference 14/030).

All the participants were prospectively followed-up for at least 12 months, unless graft loss or death occurred earlier. Immunosuppression and prophylaxis regimens are described in Supplementary Methods. A number of pre-transplant, transplant-related and post-transplant variables were collected by means of a standardized case report form.

The QTF-Monitor assay was performed at week 2 (±4 days) and months 1, 3, and 4 (± 1 week) and 6 (±3 weeks). Peripheral blood lymphocyte subpopulations ($CD3^+$, $CD4^+$, and $CD8^+$ T-cell counts) were assessed at months 1, 3, and 6 with an automated multicolor flow cytometry system (BD Multitest™ six-color TBNK reagent with acquisition on the BD FACSCanto II instrument using BD FACSCanto clinical software, all from BD Biosciences, San Jose, CA).

Study Outcomes

The *primary study outcome* was the incidence of post-transplant infection during the follow-up according to the functional immune competence (low versus moderate or high responses) as assessed by the QTF-Monitor assay. As *secondary outcomes* we separately analyzed the incidence of bacterial and opportunistic infection, as well as post-transplant *de novo* malignancy. For those outcomes for which a significant association with the presence of a low response (as defined by the manufacturer) was observed, alternative cut-off values for IFN-γ levels were explored on the basis of the best combination of sensitivity and specificity, as detailed below. Finally, as an additional secondary outcome we investigated the clinical variables that were associated with a low immune response at the different times after transplantation.

Procedure for the QTF-Monitor Assay

The QTF-Monitor assay (Qiagen GmbH, Hilden, Germany) was performed according to manufacturer's recommendations. Whole blood samples were obtained by venipuncture in lithium heparin vacuum blood collection tubes, stored at room temperature and processed within less than 6 h. 1-mL aliquots were transferred to the QTF-Monitor blood collection tubes for stimulation and incubation. The QTF-Monitor lyophilized stimulants (LyoSpheres) containing the immune ligands anti-CD3 and R848 were equilibrated to room temperature, and one LyoSphere was transferred to the blood collection tube, which was gently shaken 5–10 times to ensure complete dissolution. The QTF-Monitor tubes were immediately placed into a 37°C incubator for 16–24 h. After incubation, plasma was harvested by centrifugation at 2,000 to 3,000 × g for 15 min, and stored at −80°C until analysis. The amount of IFN-γ produced was quantified in undiluted and diluted (1:10 and 1:100) plasma samples by means of the QTF-Monitor ELISA kit and given as international units (IU)/mL by means of the QTF-Monitor Analysis Software (all from Qiagen). The lyophilized IFN-γ standard was reconstituted with distilled water to prepare the standard curve. All these procedures were performed by a single technician that was blind to patient characteristics. Results were interpreted according to the cut-off values for IFN-γ proposed in the package insert: low (<15 IU/mL), moderate (15–1,000 IU/mL) and high (>1,000 IU/mL) immune responses.

Study Definitions

The diagnosis of post-transplant infection was based on microbiological findings in association with a compatible clinical syndrome according to the definitions proposed by the Centers for Disease Control and Prevention's National Healthcare Safety Network (CDC/NHSN) [19]. Febrile episodes with no microbiological documentation that resolved spontaneously without antimicrobial treatment were excluded, as were asymptomatic bacteriuria and lower urinary tract infection. The diagnosis of CMV disease (viral syndrome or end-organ disease) required the demonstration of CMV replication by real-time PCR in the presence of attributable symptoms [20]. Opportunistic infection was operationally defined according to previous studies [21, 22] and included tuberculosis, listeriosis, infections due to facultatively intracellular bacteria (e.g., *Rhodococcus),* herpes simplex virus and varicella-zoster virus (shingles), proven/presumptive BK polyomavirus-associated nephropathy (BKPyVAN) [23], proven/probable invasive fungal disease (IFD) [24], *Pneumocystis jirovecii* pneumonia, toxoplasmosis and visceral leishmaniasis. The diagnosis of *de novo* cancer required histological confirmation and the absence of a pre-transplant history of such malignancy (i.e., type and site). Additional definitions are provided in Supplementary Methods.

Statistical Analysis

Quantitative data were expressed with the mean ± standard deviation (SD) or the median with interquartile range (IQR). Categorical variables were compared with the χ^2 test. Student's t-test or *U* Mann-Whitney test were applied for continuous variables. Repeated QTF-Monitor results within the same patient were compared with the Wilcoxon test, whereas paired proportions were compared with the McNemar test. Correlations were assessed using either Pearson's r or Spearman's rho. The association between the QTF-Monitor assay at each point and subsequent outcomes was explored by stratifying IFN-γ levels as per the interpretative cut-off values offered in the assay package insert (low versus moderate-high responses). Alternative cut-off values were subsequently evaluated for those primary or secondary outcomes depicting significant associations in the previous approach by means of the Youden's J statistic, which

TABLE 1 | Demographics and clinical characteristics of the study cohort (n = 126).

Variable	
Age of recipient, years [mean ± SD]	54.9 ± 15.5
Male gender of recipient [n (%)]	83 (65.9)
Prior or current smoking history [n (%)]	48 (38.1)
BMI at transplantation, Kg/m^2 [mean ± SD][a]	25.4 ± 4.3
Pre-transplant chronic comorbidities [n (%)]	
Hypertension	100 (79.4)
Diabetes mellitus	38 (30.2)
Other chronic heart disease	17 (13.5)
Coronary heart disease	13 (10.3)
Chronic pulmonary disease	11 (8.7)
Cerebrovascular disease	7 (5.6)
Peripheral arterial disease	4 (3.2)
Previous kidney transplantation [n (%)]	27 (21.4)
Underlying end-stage renal disease [n (%)]	
Glomerulonephritis	29 (23.0)
Diabetic nephropathy	32 (25.4)
Polycystic kidney disease	11 (8.7)
Nephroangiosclerosis	9 (7.1)
Chronic interstitial nephropathy	8 (6.3)
Loss of renal mass and hyperfiltration injury	6 (4.8)
Reflux nephropathy	5 (4.0)
Lupus nephropathy	4 (3.2)
Congenital nephropathy	5 (4.0)
Unknown	10 (7.9)
Other	7 (5.6)
CMV serostatus [n (%)]	
D+/R+	74 (58.7)
D−/R+	26 (20.6)
D+/R−	24 (19.0)
D unknown/R+	1 (0.8)
D-/R-	1 (0.8)
Positive EBV serostatus (anti-EBNA IgG) [n (%)][b]	115 (91.3)
Positive HCV serostatus [n (%)]	7 (5.6)
Positive HBsAg status [n (%)]	4 (3.2)
Positive HIV serostatus [n (%)]	3 (2.4)
Pre-transplant renal replacement therapy [n (%)]	110 (87.3)
Hemodialysis	85/110 (67.5)
Continuous ambulatory peritoneal dialysis	25/110 (19.8)
Time on dialysis, months [median (IQR)]	23.1 (12.9–46.8)
Type of transplantation [n (%)]	
Single kidney	118 (93.7)
Double kidney	2 (1.6)
Simultaneous pancreas-kidney	6 (4.8)
Age of donor, years [mean ± SD]	53.4 ± 17.0
Male gender of donor [n (%)]	66 (52.4)
Type of donor [n (%)]	
DBD donor	78 (61.9)
Uncontrolled DCD donor (Maastricht categories 1–2)	11 (8.7)
Controlled DCD donor (Maastricht categories 3–4)	12 (9.5)
Living donor	25 (19.8)
Cold ischemia time, hours [mean ± SD]	
Number of HLA mismatches [median (IQR)]	4 (3–5)
Induction therapy [n (%)]	
Antithymocyte globulin	59 (46.8)
Basiliximab	57 (45.2)
None	10 (7.9)
Primary immunosuppression regimen [n (%)]	
Prednisone, tacrolimus and MMF/MPS	111 (88.1)
Prednisone, tacrolimus and everolimus	10 (7.9)
Prednisone, tacrolimus and azathioprine	5 (4.0)
CMV prevention strategy [n (%)]	
Antiviral prophylaxis with VGCV	75 (59.5)
Duration of prophylaxis, days [median (IQR)]	111 (91–183)
Preemptive therapy	51 (40.5)
Follow-up, days [median (IQR)]	532 (480–727)
Post-transplant complications at 1 year [n (%)]	
Delayed graft function	45 (35.7)
Number of dialysis sessions [median (IQR)]	2 (1–4)
Development of *de novo* DSA	8 (6.3)
Surgical reintervention within the first month	18 (14.3)
Renal artery stenosis	14 (11.1)
New-onset diabetes	11 (8.7)
Atherothrombotic event	2 (1.6)
Biopsy-proven acute graft rejection	12 (9.5)
Time from transplantation, days [median (IQR)]	86 (14.8–154.5)
T-cell-mediated rejection	6 (4.8)
Borderline T-cell-mediated rejection	5 (4.0)
Antibody-mediated rejection	1 (0.8)

(Continued in next column)

TABLE 1 | (*Continued*) Demographics and clinical characteristics of the study cohort (n = 126).

BMI, body mass index; CMV, cytomegalovirus; D, donor; DBD, donation after brain death; DCD, donation after circulatory death; DSA, donor-specific antibody; EBV, Epstein-Barr virus; EBNA, EBV nuclear antigen; HLA, human leukocyte antigen; HBsAg, hepatitis B virus surface antigen; HCV, hepatitis C virus; HIV, human immunodeficiency virus; IQR, interquartile range; MMF/MPS, mycophenolate mofetil/enteric-coated mycophenolate sodium; R, recipient; SD, standard deviation; VGCV, valganciclovir.
[a]*Data on BMI not available for 25 patients.*
[b]*Data on EBV serostatus not available for 4 patients.*

combines sensitivity and specificity into a single measure (J = sensitivity + specificity − 1). The discriminative capacity of IFN-γ levels analyzed as a continuous variable was explored with the area under the receiving operating characteristic (auROC) curve. We estimated the diagnostic accuracy (sensitivity, specificity, positive [PPV] and negative predictive values [NPV] with the corresponding 95% confidence intervals [CIs]). Time-to-event curves were plotted by the Kaplan-Meier method and inter-group differences were compared with the log-rank test. IFN-γ levels were $\log_{10}$-transformed for statistical analyses. Statistical analysis was performed with SPSS version 29.0.1.0 (IBM Corp., Armonk, NY).

RESULTS

Clinical Characteristics

The study cohort comprised 126 KT recipients (**Table 1**). The QTF-Monitor assay was performed at 439 different instances, with a median of 4 (IQR: 3–4) measurements per patient. In detail, the assay was available for 112 patients at week 2 (91.1% of those that survived with a functioning graft at that point), 108 patients at month 1 (87.8%), 67 patients at month 3 (54.9%), 52 patients at month 4 (42.9%), and 100 patients at month 6 (82.6%).

Post-Transplant Kinetics and Clinical Determinants of IFN-γ Production

Overall, median IFN-γ levels showed a significant increase from week 2 [0.9 (IQR: 0.1–1.8) $\log_{10}$ IU/mL] to month 1 [1.5 (IQR: 0.9–2.2) $\log_{10}$ IU/mL; *P*-value < 0.0001] and month 3 [1.9 (IQR: 1.4–2.7) $\log_{10}$ IU/mL; *P*-value < 0.0001], to reach a plateau

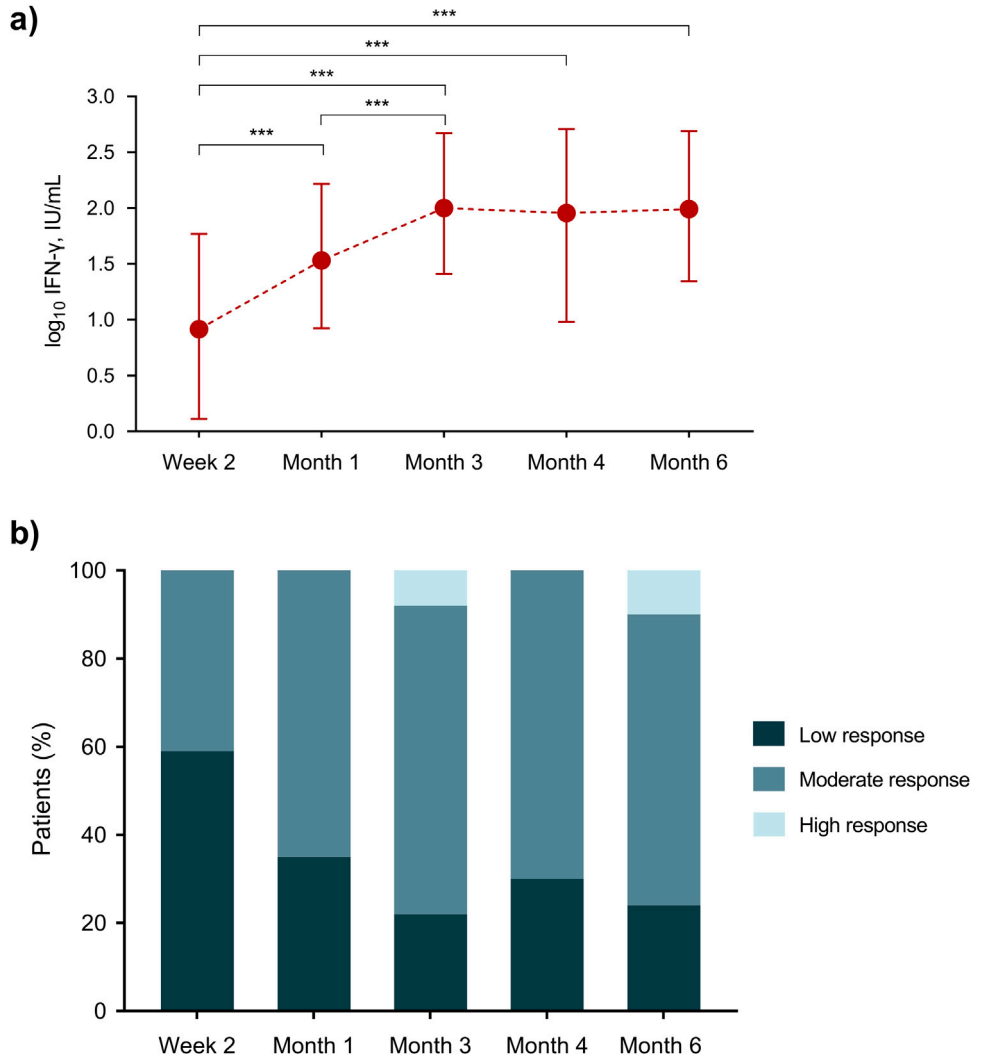


FIGURE 1 | (A) Kinetics of IFN-γ levels measured by the QTF-Monitor assay; points and bars show the median and interquartile range, respectively. **(B)** Proportion of patients with different immune responses according to the cut-off values for IFN-γ production proposed by the manufacturer [low (<15 IU/mL), moderate (15–1,000 IU/mL) and high (>1,000 IU/mL)]. *** *P*-value for repeated measures <0.0001. IFN-γ: interferon-γ; IU: international unit.

beyond that point. In accordance, the proportion of patients with a low immune response (<15 IU/mL) decreased from week 2 [58.9% (66/112)] to month 6 [24.0% (24/100); *P*-value < 0.0001] (**Figure 1**).

We explored the clinical variables predictive of a low immune response. Pre-transplant dialysis [93.9% (62/66) versus 78.3% (36/46); *P*-value = 0.014], induction therapy with antithymocyte globulin (ATG) [59.1% (39/66) versus 28.3% (13/46); *P*-value = 0.001] and delayed graft function [45.5% (30/66) versus 21.7% (10/46); *P*-value = 0.016] were more common in KT recipients exhibiting a low response at week 2 (**Supplementary Table S1**). The associations with pre-transplant dialysis and ATG induction were also observed for the results of the assay at month 1. Living donation was less likely in recipients with low responses at that point [5.3% (2/38) versus 27.1% (19/70); *P*-value = 0.006]. In addition, absolute lymphocyte and $CD3^+$ and $CD4^+$ T-cell counts were lower in this group (**Supplementary Table S2**). No significant associations were found between clinical features or laboratory values and the assay results at month 6 (**Supplementary Table S3**).

To further investigate the effect of induction therapy, we analyzed IFN-γ levels as a continuous variable. Patients treated with ATG showed a significantly lower production of IFN-γ at week 2 and month 1 as compared to those that received basiliximab or no induction (**Figure 2**). In accordance with the lymphocyte-depleting effect of ATG, a significant correlation was observed between IFN-γ levels and $CD3^+$, $CD4^+$ and $CD8^+$ T-cell counts at month 1 (but not at months 3 or 6), with Spearman's Rho coefficients ranging from 0.346 to 0.378 (**Supplementary Figure S1**).

We also investigated whether IFN-γ production was correlated with concurrent measurements of tacrolimus

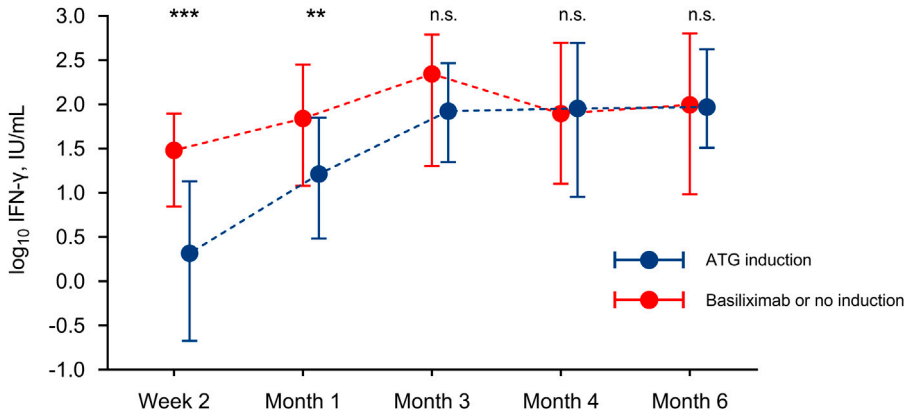


FIGURE 2 | Kinetics of IFN-γ levels according to the administration of induction therapy with ATG; points and bars show the median and interquartile range, respectively. ** *P*-value < 0.001, *** *P*-value < 0.0001. ATG, antithymocyte globulin; IFN-γ, interferon-γ; IU, international unit; ns, not significant.

trough levels. We only found a weak inverse correlation at month 6 after transplantation (Pearson's r: −0.338; *P*-value = 0.010), whereas no correlations were observed for week 2 (r: −0.181; *P*-value = 0.152), month 1 (r: −0.001; *P*-value = 0.993), month 3 (r: −0.049; *P*-value = 0.771) or month 4 (r: 0.033; *P*-value = 0.876).

Post-Transplant Infection and Cancer

Overall, 72 patients (57.1%) experienced 145 episodes of post-transplant infection (primary outcome). The median interval to the first episode was 83.5 days (IQR: 26.5–227.8). Acute graft pyelonephritis [51 episodes (35.2%)] and pneumonia [17 (11.7%)] were the most common types. Enterobacterales accounted for most of the microbiologically documented cases, with predominance of *Escherichia coli* [33 episodes (22.7%)] and *Klebsiella pneumoniae* [24 (16.5%)] (**Supplementary Table S4**).

Regarding secondary outcomes, 50 patients (39.7%) were diagnosed with 105 episodes of bacterial infection [median interval to the first episode of 64.5 days (IQR: 17.8–196)]. On the other hand, 28 episodes in 26 patients (20.6%) met the definition of opportunistic infection [median interval of 167.5 days (IQR: 82.8–295.8)], with CMV disease [12 episodes (42.9%)] and herpes zoster [6 (21.4%)] as the most common forms (**Supplementary Table S5**). Eleven patients (8.7%) developed *de novo* cancer at a median of 364 days (IQR: 169.5–594). In detail, there were six cases of non-melanoma skin cancer and six cases of solid cancer (one patient had both) (**Supplementary Table S6**).

Association Between the Functionality of Immune Response and Overall Post-transplant Infection

There were no significant differences in the cumulative incidence of overall infection between KT recipients exhibiting a low immune response (IFN-γ <15 IU/mL) and those with a moderate or high response at each monitoring point. We only found a non-significant trend towards a higher risk among patients with low responses at month 1 [65.8% (25/38) versus 47.1% (33/70); *P*-value = 0.063] (**Figure 3A**). There were no significant differences in IFN-γ levels (taken as a continuous variable) between patients with or without infection (**Figure 3B**).

As a measure of sustained over-immunosuppression, we compared the incidence of infection between KT recipients with responses categorized as low in all the assays performed throughout the first post-transplant months and the rest of the cohort. There were no significant differences for persistent low responses either during the first 3 [45.5% (10/22) versus 43.0% (43/100); *P*-value = 1.000] or 6 months [33.3% (6/18) versus 35.9% (37/103), respectively; *P*-value = 1.000].

Association Between the Functionality of Immune Response and Secondary Outcomes

Patients with a low response at the early (2-week) assessment had a higher cumulative incidence of bacterial infection than those with an intermediate response [50.8% (33/65) versus 24.4% (11/45), respectively; *P*-value = 0.006] (**Figure 4A**). IFN-γ production at week 2 was accordingly lower among patients developing bacterial infection (**Figure 4B**). One-year bacterial infection-free survival was significantly lower in the presence of a low response (**Figure 4C**). On the contrary, there were no differences for the remaining points in terms of the magnitude of response (low versus intermediate-high) (**Figure 4A**; **Supplementary Table S7**) or the absolute IFN-γ level (**Supplementary Table S8**).

Regarding opportunistic infection, the presence of a low response at month 1 was associated with the subsequent development of this secondary outcome [31.6% (12/38) versus 14.3% (10/70); *P*-value = 0.033] (**Figure 5A**). The IFN-γ level at this point was also lower in patients developing opportunistic infection (**Figure 5B**), as was the 1-year event-free survival in patients with a low response (**Figure 5C**). No differences were observed for the remaining time points (**Supplementary Tables S7 and S8**).

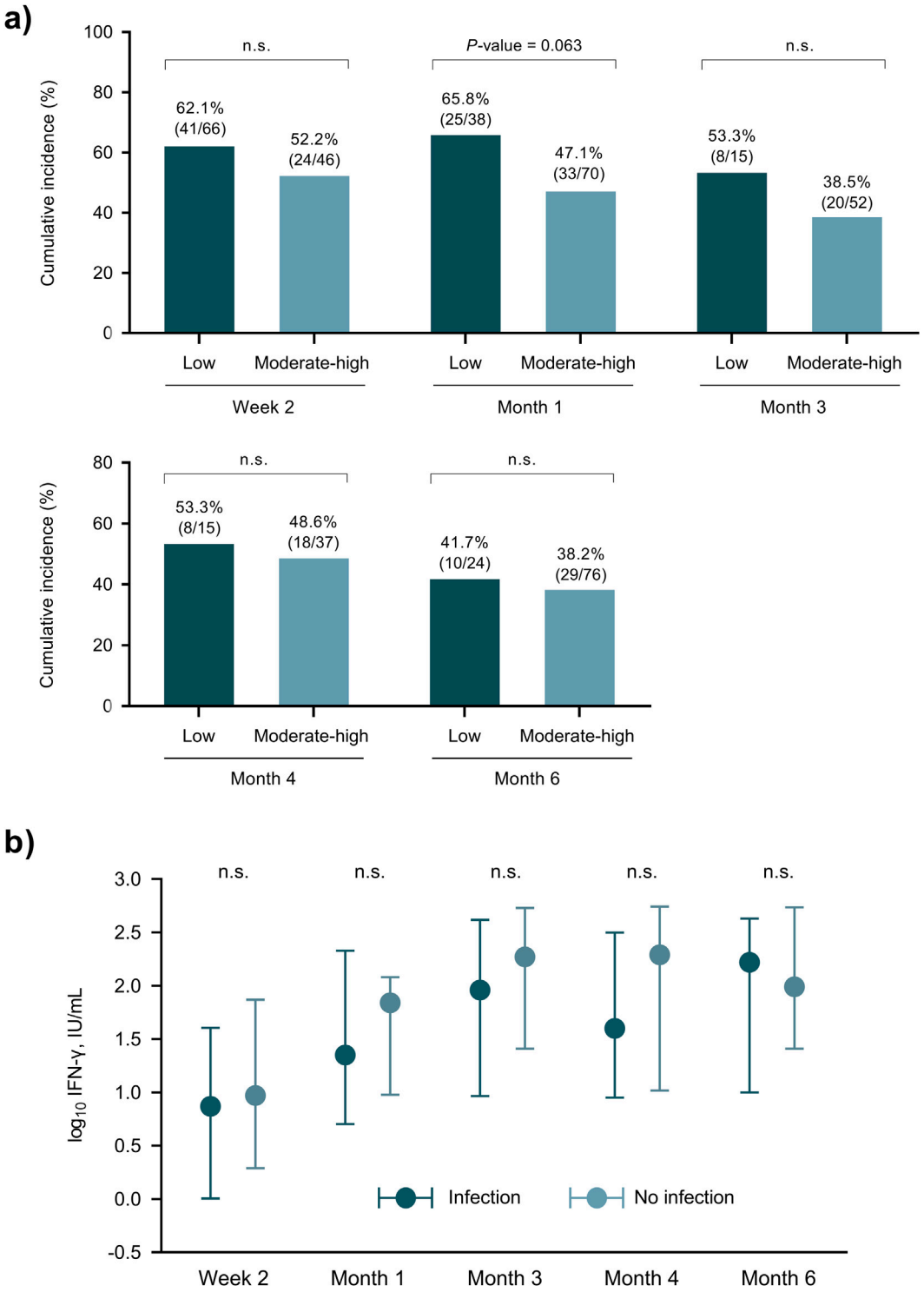


FIGURE 3 | (A) Cumulative incidence of post-transplant infection (primary outcome) according to the immune response in the QTF-Monitor assay at different time points after transplantation. **(B)** IFN-γ levels according to the subsequent occurrence of infection; points and bars show the median and interquartile range, respectively. ns, not significant; IU, international unit.

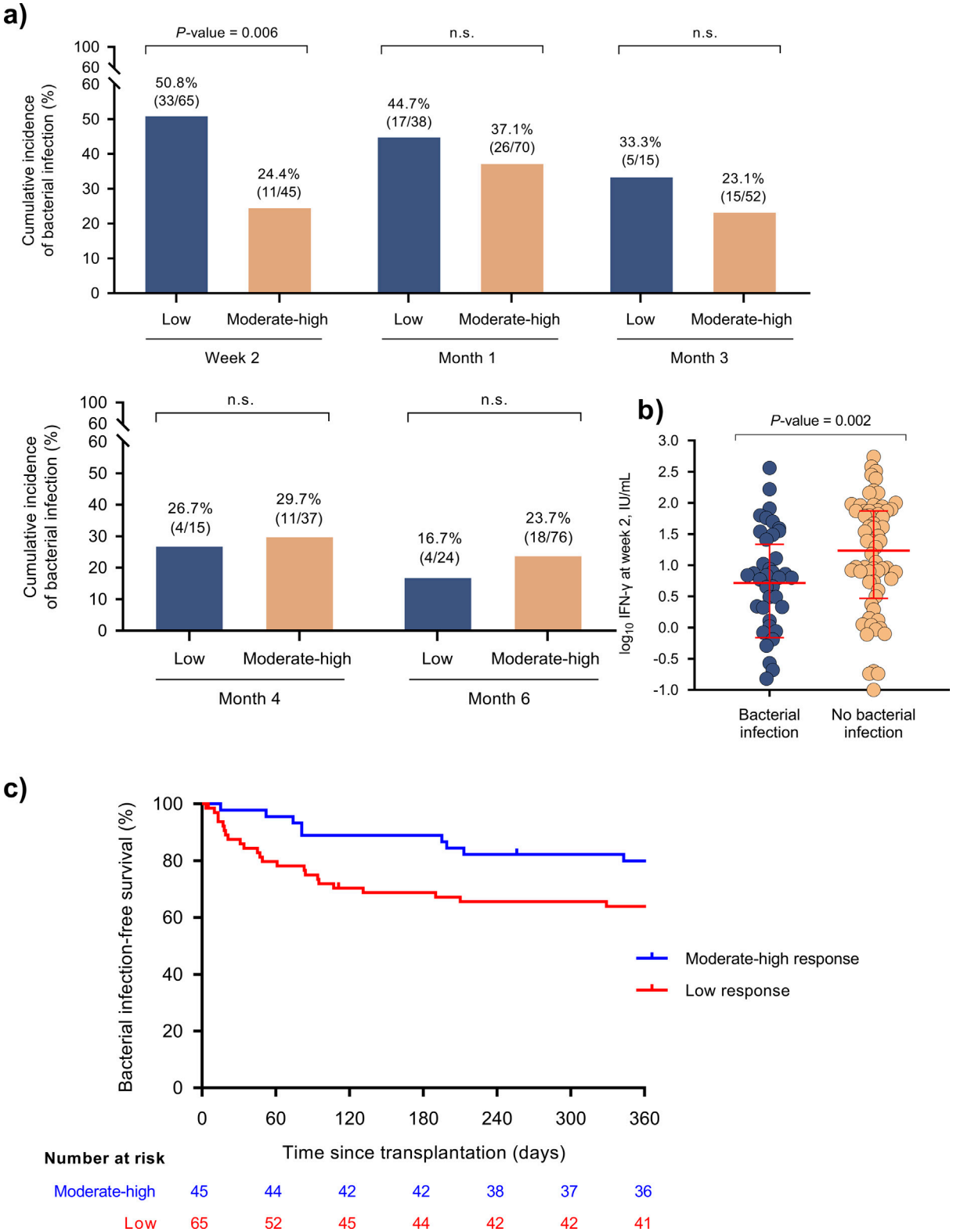


FIGURE 4 | (A) Cumulative incidence of post-transplant bacterial infection (secondary outcome) according to the immune response in the QTF-Monitor assay at different time points after transplantation. **(B)** IFN-γ levels at week 2 according to the subsequent occurrence of bacterial infection; points and bars show the median and interquartile range, respectively. **(C)** Bacterial infection-free survival according to the immune response at week 2 (log-rank test *P*-value = 0.009). IFN-γ, interferon-γ; IU, international unit.

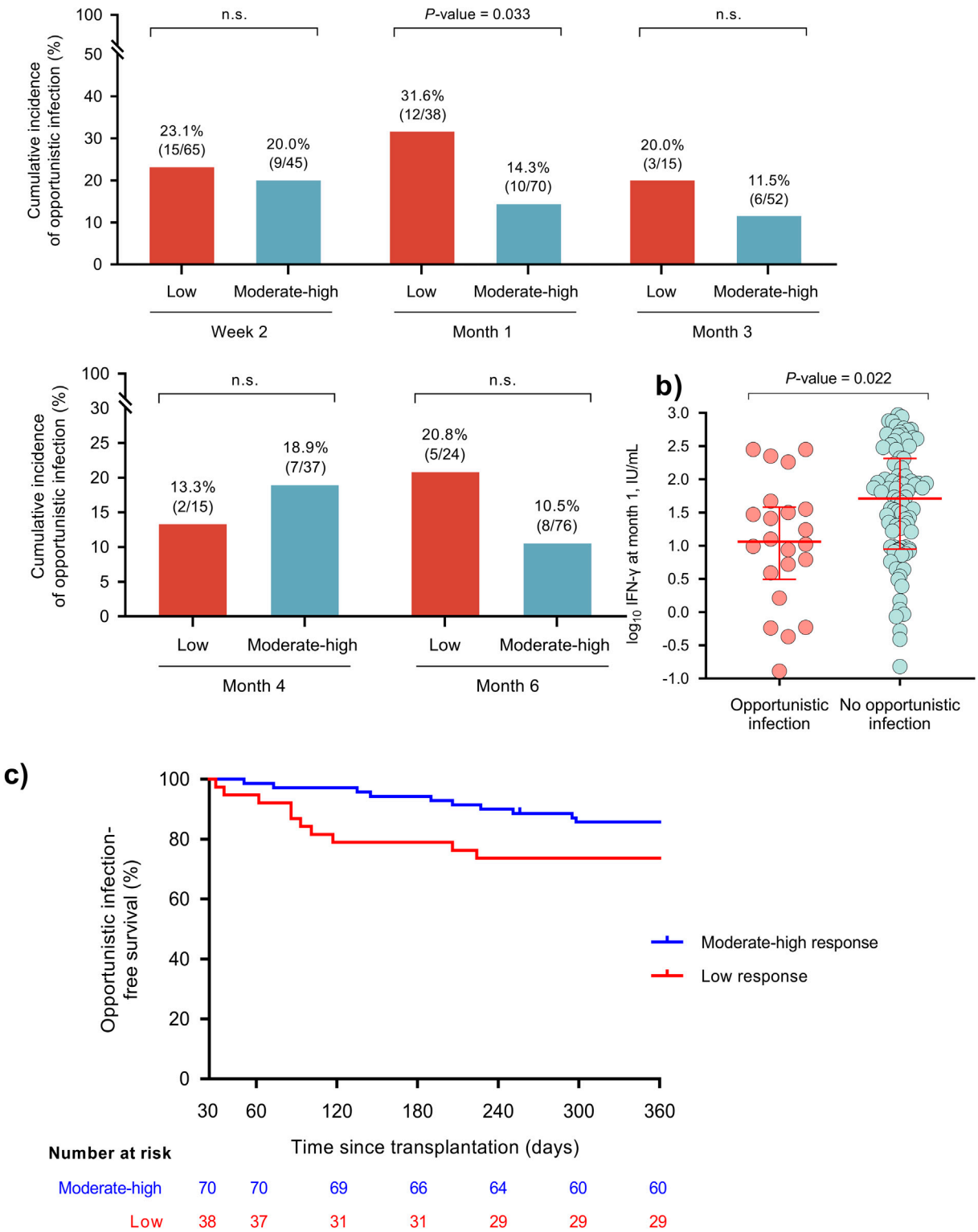


FIGURE 5 | (A) Cumulative incidence of post-transplant opportunistic infection (secondary outcome) according to the immune response in the QTF-Monitor assay at month 1. **(B)** IFN-γ levels at month 1 according to the subsequent occurrence of bacterial infection; points and bars show the median and interquartile range, respectively. **(C)** Opportunistic infection-free survival according to the immune response at week 2 (log-rank test *P*-value = 0.026). IFN-γ, interferon-γ; IU, international unit.

Finally, there were no differences in the incidence of *de novo* malignancy according to the functionality of immune responses (**Figure 5A**; **Supplementary Table S7**) or IFN-γ levels (**Supplementary Table S8**).

Diagnostic Accuracy of the QTF-Monitor Assay to Predict Bacterial and Opportunistic Infection

In view of the associations found at the early assessment, we further explored the diagnostic accuracy for the secondary outcomes of bacterial and opportunistic infection. By applying the cut-off value proposed by the manufacturer (IFN-γ <15 IU/mL), we obtained a sensitivity of 75.0% (95% CI: 59.7–86.8) and specificity of 51.5% (95% CI: 38.9–64.0) to predict bacterial infection beyond week 2. The corresponding values for the development of opportunistic infection beyond month 1 were 54.6% (95% CI: 32.2–75.6) and 69.8% (95% CI: 58.9–79.2), respectively (**Supplementary Table S9**). The discriminative capacity of IFN-γ levels was overall low, with auROCs for predicting bacterial and opportunistic infection of 0.677 (95% CI: 0.576–0.778) and 0.659 (95% CI: 0.539–0.779), respectively.

We also explored alternative cut-off values according to the optimal balance between sensitivity and specificity. By using a threshold at week 2 of 7.9 IFN-γ IU/mL, the 1-year bacterial infection-free survival curve of patients with low response was more clearly separated from those above the cut-off (**Supplementary Figure S2**), yielding improved specificity [66.7% (95% CI: 53.9–77.8)] and PPV [57.7% (95% CI: 47.8–66.9)] at the expense of a loss of sensitivity [68.2% (95% CI: 52.4–81.4)]. On the other hand, the optimal cut-off value to predict opportunistic infection beyond month 1 was set at 47.3 IU/mL, which also resulted in more clearly separated even-free survival curves (**Supplementary Figure S3**). As compared to the manufacturer's criterion, this alternative cut-off resulted in improved sensitivity [81.8% (95% CI: 59.7–94.8)] and NPV [91.7% (95% CI: 81.6–96.5)], but poorer specificity (51.2% 95% CI: 40.1–62.1) (**Supplementary Table S9**).

DISCUSSION

Most of the biomarkers proposed to determine the net state of immunosuppression after SOT share two limitations: the lack of functional measurements —as is the case with immunoglobulin levels or lymphocyte counts [3]— and the sole interrogation of virus-specific adaptive responses [4]. The QTF-Monitor assay offers the possibility of a broader functional assessment by measuring IFN-γ release upon *in vitro* stimulation of the innate and adaptive arms [13]. In the present experience the assay's performance was moderate at best, since no association could be demonstrated between IFN-γ production (either categorized as "low" immune responses or as a continuous variable) at different points during the first 6 months and the primary outcome of overall infection. Nevertheless, we found that the QTF-Monitor results obtained during the first weeks may still be valuable to specifically predict the occurrence of bacterial or opportunistic infection, although this finding should be taken with caution due to the non-negligible false positive risk in the assessment of secondary outcomes. On the other hand, no apparent associations were found for *de novo* cancer.

The performance of the QTF-Monitor assay to predict post-transplant infection has been investigated by a few groups, with variable reported accuracy [14–17]. In a mixed cohort of 137 SOT recipients, Mian et al. observed that IFN-γ levels measured between months 1 and 6 were significantly lower in patients that developed subsequent infection and proposed an optimal threshold of ≤10 IU/mL. Urinary tract infection and pneumonia were the most common syndromes during the early post-transplant period, with a shift to predominance of viral pathogens beyond month 3. No multivariate analysis was performed to confirm the predictive value of IFN-γ production [14]. In contrast, a cross-sectional study at a mean of 2.6 post-transplant years failed to show differences in IFN-γ levels between stable KT recipients and those with infection. A subgroup analysis revealed that patients with bacterial infection had a significantly decreased IFN-γ release. Such an association, however, was not confirmed after adjustment for steroid dose and tacrolimus levels [15]. In a single-center cohort of LT recipients, IFN-γ levels at week 1 exhibited a fairly good capacity to predict infection through the first month, with the majority of the events being classified as opportunistic [16]. Finally, a recent study recruited 80 LuT recipients in which the QTF-Monitor was performed at 2, 6, 12, 24, and 52 weeks. The presence of IFN-γ levels <10 and <60 IU/mL at weeks 12 and 24, respectively, was associated with the diagnosis of opportunistic infection (mainly CMV viremia and IFD). Similar results were not observed for earlier monitoring points [17].

The discordant results from the existing literature, including those reported herein, may be partially attributable to differences across studies in outcomes and definitions, as well as in the timing and frequency of monitoring. Taken together, they would suggest that the QTF-Monitor assay may perform better for predicting some specific types of infection —particularly of bacterial origin [15]— and when performed early after transplantation. Indeed, we have only identified differences in the assay results obtained at week 2 and month 1 according to the subsequent diagnosis of bacterial and opportunistic infection, respectively (with the latter mostly represented by CMV disease and herpes zoster). These results are in line with those previously observed among LT recipients [16]. Of note, the discriminative capacity for both outcomes was low, as indicated by auROC values below 0.700. Sood et al. reported a slightly better accuracy for the results obtained at week 1 after LT (auROC of 0.740) [16]. To put these findings into context, our group has reported higher discriminative capacities for other non-pathogen-specific biomarkers, such as the $CD8^+$ T-cell count at month 1 (auROC of 0.739) or the total lymphocyte count at month 6 (auROC of 0.820) to predict opportunistic infection [25], Torque Teno virus (TTV DNAemia) at month 1 for predicting opportunistic infection and/or cancer (auROC of 0.704) [22], or serum sCD30 at month 1 for predicting bacterial infection (auROC of 0.846) [26]. Therefore, the potential contribution of the assay to the existing prediction models for post-transplant

infection —such as the externally validated SIMPLICITY Score [5]— should be explored in future studies.

Our results align with the cross-sectional study by Margeta et al [15] in that the performance of QTF-Monitor assay decreases at late periods after transplantation, once the amount of immunosuppression has been stabilized in most recipients. No differences in IFN-γ levels beyond month 1 were observed for any of the outcomes analyzed. Interestingly, we found no association between the QTF-Monitor results and the development of post-transplant cancer, a complication that usually results from the long-term effect of sustained over-immunosuppression [27]. No previous studies have investigated the role of QTF-Monitor assay to predict *de novo* malignancy. Although the number of events was low (n = 14), this negative finding would point to a lower relative contribution to the assay results of T-cell responsiveness (as compared to TLR-mediated innate responses), taken into account the pivotal role of cellular immunity in cancer immune surveillance. In contrast, we and others have shown that certain immune biomarkers assessed within the first months are useful to identify SOT recipients at increased risk of developing cancer in the mid- and long-term follow-up, such as $CD4^+$ and $CD8^+$ T-cell counts [6, 28], monocytic myeloid-derived suppressor cells [29] or TTV DNAemia [22].

The kinetics of IFN-γ levels measured by the QTF-Monitor assay was comparable to previous studies, which typically describe a sharp decline from the pre-transplant assessment followed by a progressive recovery through months 3–6 and a plateau thereafter [13, 14, 16, 17]. This pattern is in line with the accepted timing for immune reconstitution after SOT, as validated with other biomarkers such as TTV DNA load [30, 31]. The clinical factors influencing assay results have been only partially investigated. The association between the use of ATG as induction therapy and a lower IFN-γ production has been reported by other authors [14]. In our experience this effect persisted until month 1 and was supported by the inverse correlation observed between IFN-γ levels and T-cell counts. The impact of tacrolimus levels is less consistent, with studies reporting either strong [17] or borderline correlations [15], or even no apparent association [14]. We only found a weak inverse correlation with tacrolimus levels at month 6. Mian et al. also reported an association with daily doses of prednisone and mycophenolate [14], which were not recorded in our database. Although beyond the scope of our research, we found no significant association between the immune status measured by the QTF-Monitor assay at the different monitoring points and the subsequent occurrence of biopsy-proven acute rejection (data not shown). Patients with a low response at week 2 were more likely to have received pre-transplant dialysis and to have experienced delayed graft function (defined by the early requirement of renal replacement therapy). The deleterious effect of dialysis on the T-cell ability to produce IFN-γ after specific stimulation is well established for IGRAs used to detect *Mycobacterium tuberculosis* infection due to insufficient mitogen response and premature immune aging [32, 33]. Inversely, living donation was associated with a more robust immune response, which may be explained by the lower recipient age and the immediate graft function in this subgroup.

What may be the position of the QTF-Monitor assay for immune monitoring in the clinical arena? With the limitations inherent to multiple secondary outcome analyses and the lack of consistent associations at later points, our results would point out to the potential usefulness of the early assessment within the first weeks with the specific aim of predicting bacterial infection. By decreasing the IFN-γ threshold to <7.9 IU/mL we obtained a sensible improvement in specificity without a major impact in sensitivity, although the resulting estimates (66.7% and 68.2%, respectively) were far from excellent. Sood et al. proposed a clinical threshold of <1.30 IU/mL as the most discriminative to predict infection beyond the first week after LT, with a diagnostic accuracy in the line of our results (sensitivity of 71.4% and specificity of 63.0%) [16]. On the other hand, an alternative threshold (<47.3 IU/mL) at month 1 yielded a reasonable sensitivity (81.8%) to predict opportunistic infection, at the expense of a poor specificity (51.2%). Gardiner et al. also found a relatively low discriminative ability for different outcomes (overall infection, severe infection or opportunistic infection) and monitoring points after LuT [17]. In our opinion, any decision regarding the implementation of the QTF-Monitor assay in daily practice must balance diagnostic accuracy (which was found to be suboptimal in our experience), requirement of laboratory resources and economic costs with those of alternative biomarkers [3]. For instance, the observed impact on IFN-γ production of ATG induction and $CD4^+$ and $CD8^+$ T-cell counts would suggest that low responses may ultimately act as a surrogate for the presence of lymphocytopenia, which constitutes a well-established biomarker for opportunistic infection [25, 34–38].

Our study is based on a large cohort of KT recipients with regular monitoring, and it is strengthened by the assessment of immunosuppression-related complications which comprised infections and malignancies. We also provided an insight into the clinical determinants of the IFN-γ kinetics, including peripheral blood lymphocyte subpopulations. Nevertheless, a number of limitations must be noted, such as the relatively low number of some events, which may have limited statistical power. Due to logistical reasons, the assay could not be tested in certain patients at all the scheduled points. Although the minimum follow-up was set at post-transplant month 12, the last monitoring point was performed at month 6. In addition to budgetary considerations, the rationale for such decision was that most events would have occurred within the first 6 months, according to the classical timeline for post-transplant infection [39]. In addition, the overall amount of immunosuppression (i.e., prednisone dose and targeted trough tacrolimus levels) is usually stabilized beyond that point in most KT recipients. Therefore, it is not to be expected major changes in the results of the QTF-Monitor assay beyond month 6, as supported by the plateau between months 3 and 6 observed for IFN-γ levels (**Figure 1**). In addition, any conclusion on the potential usefulness of the QTF-Monitor assay for predicting bacterial or opportunistic infection should take into account that both events were considered as secondary outcomes.

In this cohort of KT recipients we found no significant association between IFN-γ production measured with the QTF-Monitor assay and the primary outcome of overall post-transplant infection. Secondary outcome analysis would suggest

that the usefulness of this assay is presumably limited to the prediction of bacterial and opportunistic infection when performed within the first weeks after transplantation. Further studies are needed to establish the role of this promising method in the available repertoire of non-pathogen-specific immune monitoring biomarkers.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving humans were approved by the Clinical Research Ethics Committee of the University Hospital "12 de Octubre" (reference 14/030). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MF-R, JMA, and NR designed research; TR-M, PP, and NR performed laboratory experiments; MF-R, IR-G, JMC, FL-M, RSJ, NP, EG, and AA performed patient selection and management; MF-R, TR-M, IR-G, JMC, FL-M, RSJ, NP, and EG performed data collection; MF-R and NR performed data analysis; MF-R wrote the paper; and AA, JMA, and NR critically reviewed and completed the final draft of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This work was supported by the Instituto de Salud Carlos III (ISCIII) through the projects PIE13/00045, PI19/01300, and PI22/01062, and co-funded by the European Union. MF-R has hold a contract "Miguel Servet" (CP18/00073) from the ISCIII, Spanish Ministry of Science and Innovation. This study was partially presented at the 32nd European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), which was held in Lisbon, Portugal, from 23 to 26 April 2022 (oral communication O0842).

CONFLICT OF INTEREST

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

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2024.13551/full#supplementary-material

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ORIGINAL RESEARCH
published: 31 October 2024
doi: 10.3389/ti.2024.13641

Liver Transplantation for Intrahepatic Cholangiocarcinoma After Chemotherapy and Radioembolization: An Intention-To-Treat Study

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Liver transplantation (LT) is a potentially curative experimental treatment for unresectable intrahepatic cholangiocarcinoma (iCC). Pre-transplant downstaging may help defining tumor aggressiveness and drive patient selection. We report the preliminary results of LT for liver-limited unresectable iCC after sequential downstaging with systemic chemotherapy and radioembolization (SYS-TARE). In case of sustained disease stability after SYS-TARE, patients underwent surgical nodal sampling and, if negative, were listed for LT. In this study, 13 patients with unresectable iCC underwent downstaging with SYS-TARE. The median age was 70 years and 77% were female. All had single bulky lesions at diagnosis. After SYS-TARE, 9 (69%) dropped out: 3 due to progressive disease after TARE with no response to second-line, 4 due to extrahepatic disease development and 2 due to positive nodal disease at pre-listing abdominal exploration. The median OS after dropout was 11.5 months. Four (31%) were successfully listed and transplanted. At pathology, viable tumor ranged from 30% to less than 5%. All four patients are alive and disease-free at 73, 40, 12, and 8 months from LT. LT for unresectable iCC after downstaging with SYS-TARE appears to select suitable patients for LT, achieving optimal oncological outcomes in case of response to therapy and no lymphnodal spread.

Keywords: biliary tract cancers, downstaging, gemcitabine-cisplatin, Yttrium-90, tare

Abbreviations: LT, liver transplantation; iCC, intrahepatic cholangiocarcinoma; TARE, transarterial radioembolization; MDT, multidisciplinary team; SYS, systemic therapy.

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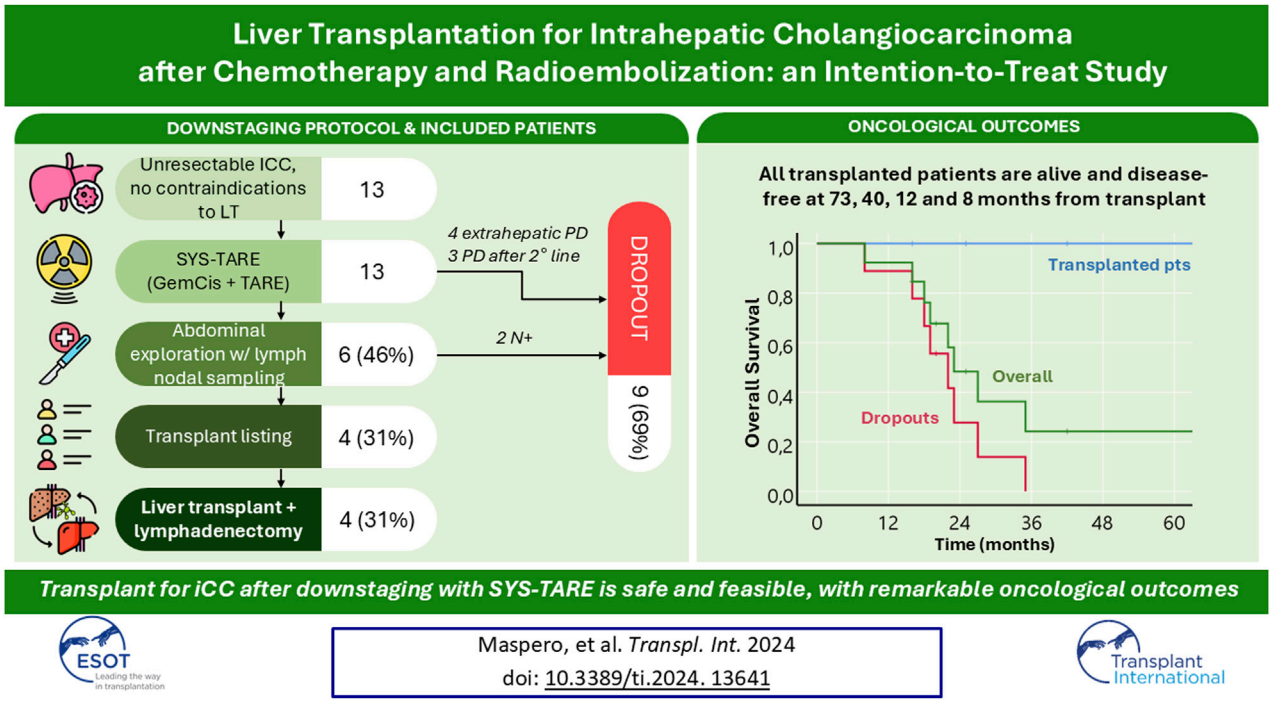
Received: 09 August 2024
Accepted: 18 October 2024
Published: 31 October 2024

Citation:
Maspero M, Sposito C, Bongini MA, Cascella T, Flores M, Maccauro M, Chiesa C, Niger M, Pietrantonio F, Leoncini G, Bellia V, Bhoori S and Mazzaferro V (2024) Liver Transplantation for Intrahepatic Cholangiocarcinoma After Chemotherapy and Radioembolization: An Intention-To-Treat Study. Transpl Int 37:13641. doi: 10.3389/ti.2024.13641



GRAPHICAL ABSTRACT |

INTRODUCTION

Intrahepatic cholangiocarcinoma (iCC) is an aggressive biliary malignancy and surgical tumor removal represents the only curative treatment option [1]. Up to 70%–80% of patients with iCC are however unresectable at diagnosis, and the median overall survival without surgery is around 18 months, with less than 10% of patients being alive at 5-year [2]. The first-line therapeutic option for unresectable iCC is systemic therapy with gemcitabine + cisplatin, in combination with durvalumab as per the recently published TOPAZ-1 trial [3, 4]. Locoregional treatment may be used in combination with systemic therapy to improve response rates and increase conversion to resection. The phase II MISPHEC trial evaluating transarterial radioembolization (TARE) plus chemotherapy as first-line treatment of locally advanced iCC suggested that this was an effective strategy, but survival without surgery remains dismal [5].

Liver transplantation (LT) expands the conventional margins of liver resection and represents an alternative curative-intent option for patients with unresectable disease [6]. However, with the exception of cirrhotic patients with small tumors (≤2 cm), LT alone does not confer a significant survival advantage in iCC [7]. Conversely, patients with unresectable iCC that respond to downstaging seem to be the best candidates for LT. In a recent experience from Houston Methodist, they reported a 5-year survival of 83% for six highly selected cases with locally advanced iCC who were transplanted after intensive neoadjuvant therapy [8]. Their experience was updated in 2022, with the report of 32 listed patients and 18 transplants with a 5-year overall survival of 57% [9].

Since 2018, our Center has implemented an intention-to-treat strategy for unresectable iCC that draws from those experiences and combines them in a multistep sequential protocol of local and systemic treatment to select LT candidates considered suitable candidates after multidisciplinary (MDT) assessment. The protocol takes advantage of a consistent experience with radioembolization as a mean to deliver radiation therapy to liver tumors. Here we report the intention-to-treat outcomes of the first thirteen cases, of which four (31%) were successfully transplanted.

PATIENTS AND METHODS

Combined Systemic Therapy–Radioembolization (SYS-TARE) Protocol

The flowchart of the protocol applied to patients with unresectable iCC with liver-only tumor presentation and no absolute contraindications to LT is reported in **Figure 1**. Inclusion criteria for the protocol were: 1) Histologically proven mass-forming iCC with a single measurable lesion with or without associated peritumoral satellites; macroscopic vascular

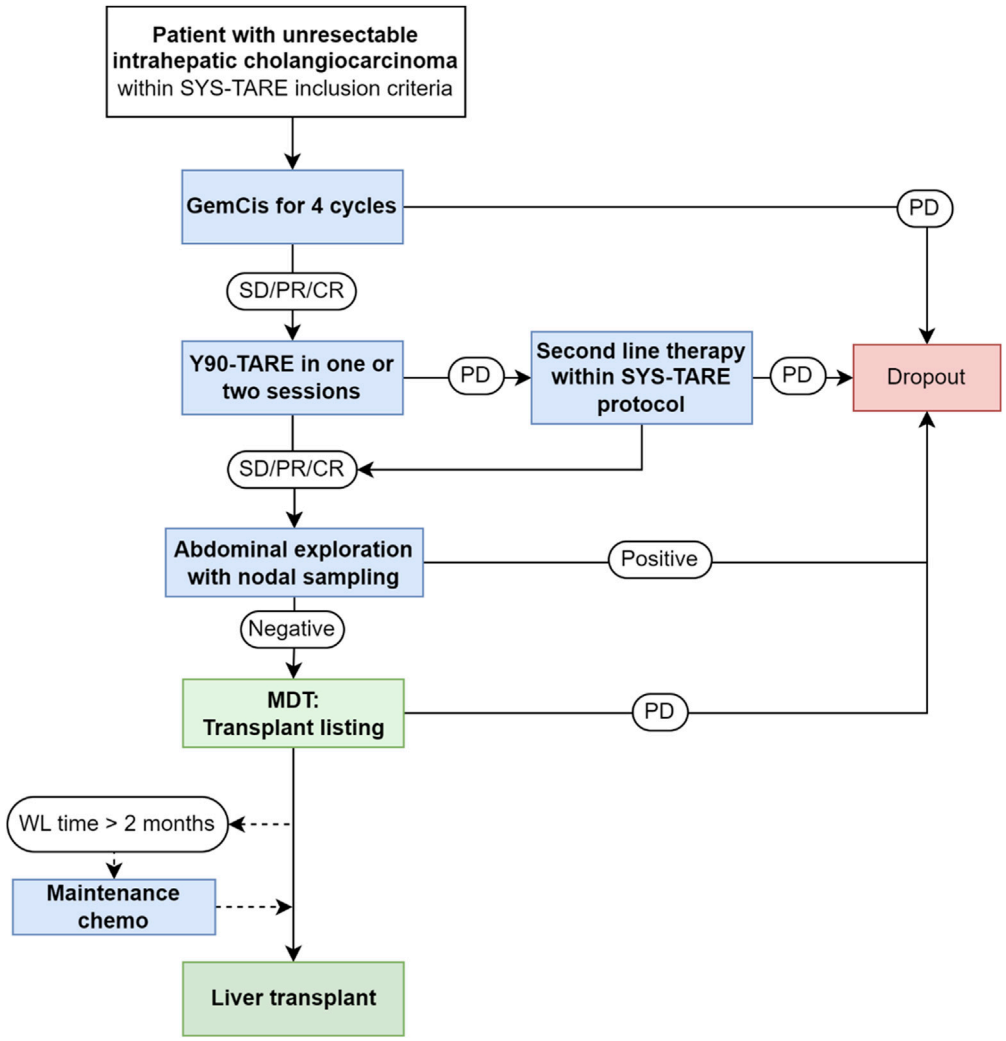


FIGURE 1 | Neoadjuvant combined systemic therapy and radioembolization (SYS-TARE) protocol for unresectable intrahepatic. iCC, intrahepatic cholangiocarcinoma; GemCis, gemcitabine + cisplatin; SD, stable disease; PR, partial response; CR, complete response; PD, progressive disease; MDT, multidisciplinary team; WL, waitlist; CT, chemotherapy. Second-line chemotherapy was indicated according to standard of care, preferably with targeted therapy if actionable mutations were present at next-generation sequencing analysis. Maintenance chemotherapy included additional cycles of GemCis until transplant or disease progression.

encasement was allowed as long as tumor thrombosis was excluded and the extent of tumor vascular encasement was limited to the intrahepatic portion of portal/hepatic veins; 2) Unresectable disease due to tumor location or underlying liver disease; 3) Age between 18 and 70 years; 4) No lymphatic or extrahepatic spread; 5) Performance status 0-1; 7) Written informed consent. Exclusion criteria were: 1) Multifocal iCC involving multiple segments; 2) Macroscopic vascular thrombosis/tumor invasion; 3) Prior resected extrahepatic tumor spread; 4) Concomitant malignancies or history of other malignancies in the previous 5 years; 5) Non-oncological contraindications to LT.

Enrolled patients underwent a sequential downstaging treatment with 4 cycles of gemcitabine + cisplatin, followed by TARE with Yttrium^{90} glass microspheres (Therasphere, Boston Scientific, Marlborough, MA) in one or two sessions.

Chemotherapy, Radioembolization and Evaluation of Response

Chemotherapy was started after pathology confirmation of intrahepatic cholangiocarcinoma and assessment of non-resecability by an experienced hepato-biliary surgical team. Chemotherapy consisted of at least four cycles of gemcitabine (1000 mg/m^2) and cisplatin (25 mg/m^2) administered intravenously on day 1 and day 8 of a 21-day cycle, as per standard of care.

Trans-arterial radioembolization (TARE) was performed, as previously described [10]: a simulation of treatment was

performed by the injection of ^{99}Tc-MAA into the hepatic arterial vasculature reproducing Y^{90} microspheres distribution, in order to estimate the degree of lung shunt and/or extrahepatic deposition and tumor uptake by means of planar and SPECT scintigrams. The dose calculation was individualized according to ^{99}mTc-MAA SPECT voxel dosimetry [11]. Patients were treated on average with 2.8 million of microspheres per GBq. The treatment was performed two to 3 weeks after the simulation, by the injection of glass microspheres loaded with ^{90}Yttrium on the day of admission. Before injection, patients were given 2 g of cefazoline intravenously. After TARE, patients were hospitalized for 48 h for clinical observation.

All patients underwent restaging with CT scan, FDG-PET and tumor markers after four cycles of chemotherapy, then 1 month after TARE, then every 2 months. Follow up continued in the same manner every 2 months while on the transplant waitlist. Response to SYS-TARE was evaluated with CT scan according to RECIST criteria [12] and Choi criteria [13], and FDG-PET. In particular, response according to Choi was calculated by assessing the change in density of the most vascularized and/or representative slice of the entire lesion during an arterial phase at baseline and after treatment. In case of partial response or stable disease according to the previous radiological/metabolic criteria, as well as a comparable CA19-9 decrease/stability for at least 4 months, the patients underwent a surgical exploration of the abdomen (either laparotomic or laparoscopic) to determine disease burden with intraoperative ultrasound, peritoneal exploration and washing and lymph nodal assessment.

Assessment of lymphatic spread involved nodal sampling of stations 8 and 12 in absence of clinically suspicious nodes, aiming for a minimum of 5 lymph nodes for adequate assessment; in patients with suspicious nodes at pre-op imaging, lymphadenectomy of suspicious nodes was performed in addition to stations 8 and 12. In clinically N0 patients, the dissection was carried out until sufficient tissue was retrieved to assess the minimum number of required nodes. In patients with suspicious nodes, the dissection was carried out until all radiologically suspicious nodes were excised. In case of persistence of suspicious lymph nodes at post-surgical exploration imaging, a re-exploration was performed. Cytology from peritoneal washing was also carried out in all cases: 250 mL of saline were injected in the abdominal cavity and retrieved after putting the patient in Trendelenburg position. After having excluded the presence of extrahepatic disease, a formal assessment of transplant eligibility was conducted during the dedicated MDT and in case of no general contraindications the patient was listed for LT. No specific priority was assigned in case of LT listing even though reassessment of priority was planned every 2 months while on active list.

At whichever stage of the process, in case of intrahepatic disease progression second line therapy was allowed preferably with targeted therapy in case of presence of actionable mutations at next-generation sequencing (NGS) analysis and waitlisting reconsidered only in case of partial response for at least 4 months. Disease stability was assessed every 2 months while on the waiting list. In case of waiting time longer than 2 months, additional cycles of gemcitabine and cisplatin were allowed. In case of extrahepatic progression or positive nodal sampling, the patient dropped out of the protocol. LT was carried out with grafts procured from deceased donors according to standard practice. During total hepatectomy, *en bloc* lymphadenectomy of stations 8, 9, 11p, 12a, 12b, 12p, and 13, if not previously removed, was performed in all patients [14].

TABLE 1 | Characteristics of the overall cohort.

Variable	Overall cohort (n = 13)
Age at diagnosis (years)	60 (55–67.5)
Sex	11 (77%)
Female	3 (23%)
Male	
Liver status	4 (31%)
Healthy	7 (56%)
MASLD	1 (7%)
HBV	1 (7%)
Wilson's disease	
Comorbidities	4 (31%)
Hypertension	3 (23%)
Dyslipidemia	3 (23%)
Smoking habit	2 (15%)
Obesity (BMI ≥ 30)	1 (7%)
COPD	
Number of lesions at diagnosis	1 (1)
Size of largest lesion at diagnosis (mm)	100 (62.5–117)
CEA at diagnosis (ng/mL)	3 (1.5–4)
CA19-9 at diagnosis (U/mL)	48 (29–189)
Number of total GemCis cycles	7 (4–11)
Number of TARE	7 (56%)
One	6 (43%)
Two	
Mean dose to the lesion (Gy)	311 (206–629)
First TARE	359 (89–833)
Second TARE	
Dropout	9 (69%)
Time from TARE to dropout (months)	5 (2.5–6.5)
Median follow up (months)	43 (30–81)
Deaths	8 (61.5%)
Median survival after dropout	11.5 (6.5–14.5)

Data is number (percentage) and median (interquartile range).
MASLD, metabolic-associated steatotic liver disease; HBV, hepatitis B virus; BMI, body mass index; COPD, chronic obstructive pulmonary disease; GemCis, gemcitabine + cisplatin; TARE, radioembolization; Gy, gray.

Statistical Analysis

Descriptive variables were calculated for the overall cohort: categorical variables were expressed as number (percentage), while numerical variables as median (interquartile range). Median follow-up was calculated with the inverse Kaplan Meier method. Overall survival (OS) was calculated using the Kaplan Meier method, with censoring at death or last follow up: OS was calculated from diagnosis and from last treatment within the SYS-TARE protocol for the overall cohort; from dropout for patients who exited the SYS-TARE protocol; and from transplant for patients who were successfully transplanted. Disease-free survival, defined as the interval between LT and iCC recurrence, was calculated for transplanted patients using the Kaplan Meier method. All analyses were conducted using IBM SPSS Statistics for Windows, version 26 (IBM Corp., Armonk, N.Y., United States).

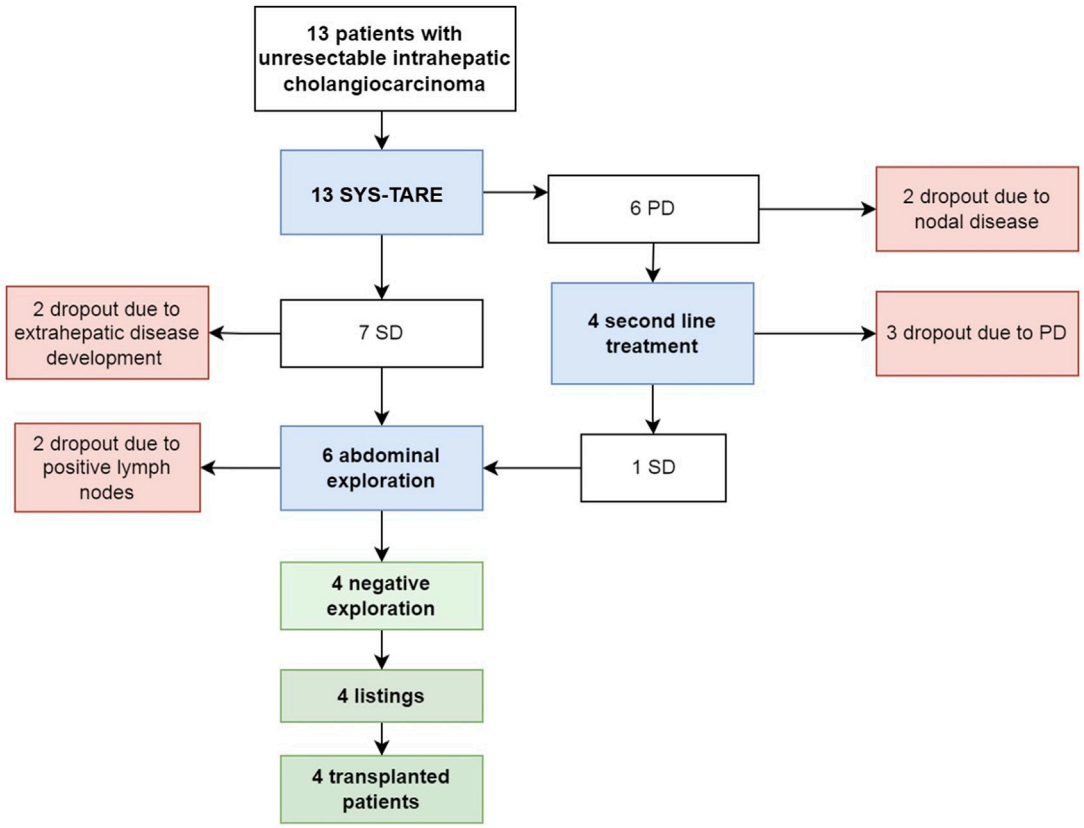


FIGURE 2 | Flowchart summarizing treatment allocation and evolution of 13 unresectable mass-forming iCC, according to tumor response after combined chemotherapy and radioembolization.

RESULTS

Since 2018, thirteen patients were enrolled into the protocol. Their characteristics are reported in **Table 1**. The median age was 60 years, and the majority (77%) were female. Four (31%) patients had iCC arising on normal liver, 7 (57%) on metabolic-associated steato-hepatitis, 1 (7%) on Wilson's disease and 1 (7%) on hepatitis B virus-associated chronic liver disease. All except one had a single lesion at diagnosis, with a median diameter of 100 mm. Non-resectability was due to central location with hepatic outflow encasement in 12 patients, and to tumor location and underlying Wilson's disease in one case. Patients received on average 6.5 cycles of chemo for a median time of 4.5 months. All patients underwent TARE with ^{99}mTc-MAA SPECT voxel dosimetry.

The median dose delivered to the lesion during TARE was not significantly higher in patients who subsequently underwent transplant (626 Gy, IQR 427–1462) than patients who dropped (224 Gy, IQR 159–316), p = 0.059. Dropout occurred in 9 (69%) cases after a median of 5 months, while four (31%) patients were listed and transplanted (**Figure 2**). Dropout was due to progression of disease (PD) after TARE in 4 cases, after second line treatment in 3 cases, and due to tumor spread in the hilar lymphnodes at abdominal exploration (N+) in 2 cases.

Transplanted Patients

Four out of 13 (31%) patients were successfully downstaged and transplanted within the protocol. Their neoadjuvant treatment sequences are summarized in **Figure 3**. Two patients underwent the SYS-TARE sequence, had sustained disease stability, were listed for LT and transplanted within 2 months from listing. One patient had sustained disease stability after the SYS-TARE sequence but received 5 additional cycles of gemcitabine + cisplatin because of increased waiting time. One patient had an initial disease stability but developed intrahepatic disease progression after TARE with a single subcentimetric lesion. The patient underwent NGS analysis where an actional FGFR2 mutation was found. The MDT decided to proceed with a second-line treatment with the FGFR2 inhibitor pemigatinib, to which the patient had a partial response, leading to listing after 8 cycles. All patients had an uneventful negative abdominal exploration (laparoscopic in 3 cases, laparotomic in 1). Two out of four patients required two TARE sessions due to large centrohepatic lesions with bilobar feeding arteries. The average dose/sphere delivered of radiotherapy was 626Gy (IQR 427–1462) at first TARE and 495 Gy (40–951) at second TARE. Neither severe adverse event or dose reduction related to chemotherapy or TARE treatment were registered. The best response obtained before

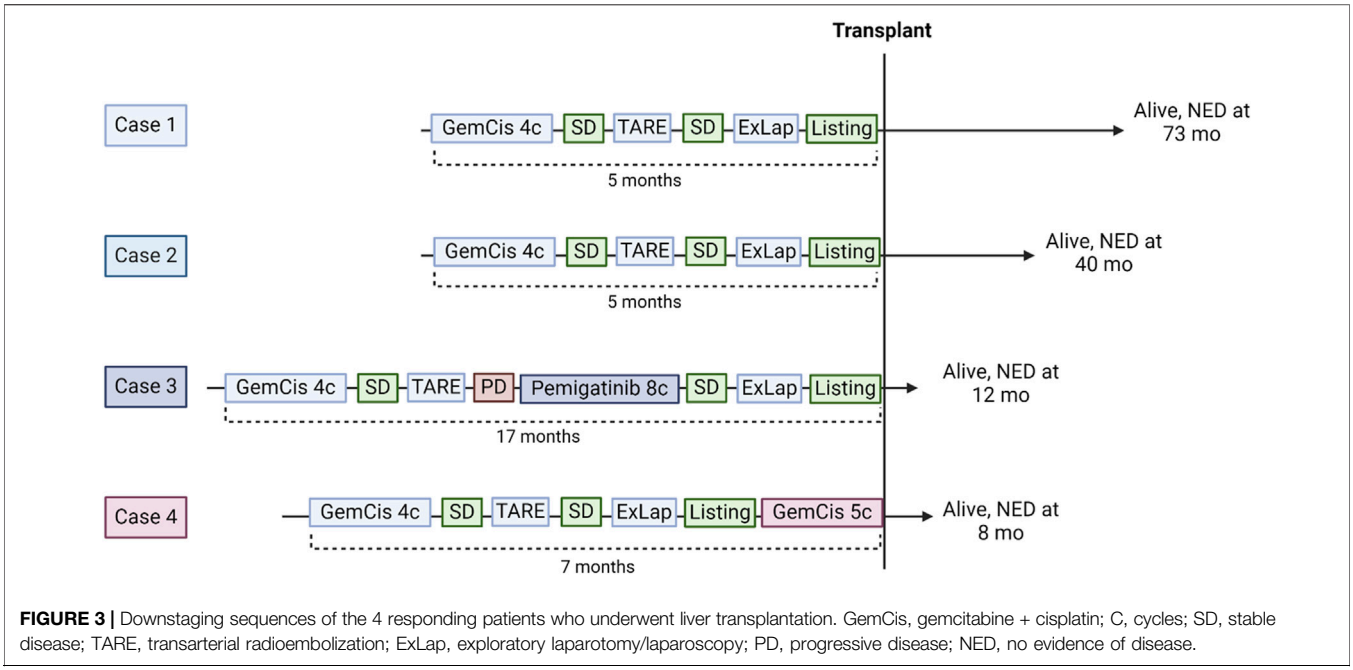


FIGURE 3 | Downstaging sequences of the 4 responding patients who underwent liver transplantation. GemCis, gemcitabine + cisplatin; C, cycles; SD, stable disease; TARE, transarterial radioembolization; ExLap, exploratory laparotomy/laparoscopy; PD, progressive disease; NED, no evidence of disease.

listing was stable disease (SD) in all patients according to RECIST criteria, and partial response (PR) in all patients according to Choi criteria with a median decrease of tumor density of 52.5%.

Peri- and post-transplant characteristics are shown in **Table 2**. The median intervals from diagnosis to listing and transplant were 6 and 9 months, respectively. All patients had good liver function (median MELD 7), CEA <10 ng/mL and CA 19-9 < 100 U/mL both at listing and at transplant. The median time on waitlist was 57 days, while the median time between the last treatment and LT was 36 days. All patients received full grafts from deceased donors (3 after brain death, 1 after cardiac death). The donor-risk index was 1,0 (range 1.1–2.2). All donors were extended criteria donors for at least one characteristic: two were older than 70 years, one had a ICU stay longer than 10 days, and one was a donor after cardiac death (DCD). The graft from the DCD donor underwent hypothermic oxygenated machine perfusion for 2 h. The median OR time was 10 h. Two cases required removal of the native vena cava to ensure oncological radicality due to lesions located near the hepatocaval confluence, while the other two received a piggy-back implantation technique. No intraoperative complications occurred.

The median length of hospital stay was 11.5 days. Two patients experienced major complications within 90 days: one developed partial hepatic artery thrombosis requiring stent placement (comprehensive complication index, CCI 45.4); another developed a kinking and leak of the biliary anastomosis, requiring reoperation and hepatojejunostomy, followed by duodenal leak requiring pancreatoduodenectomy and two further operations for hemoperitoneum and eventration (CCI 73.7).

At final pathology, in all patients the diagnosis of mass-forming iCC was confirmed with various degrees of response to neoadjuvant therapy, with residual viable tissue ranging from 30% to less than 5%. No patient had satellitosis nor invasion of major intrahepatic vessels. In all cases of hepatic vein encasement, histology confirmed that the tumor did not invade the intima (**Figure 4**). With a median of 8 retrieved lymph nodes, all patients were N0. After MDT discussion, no patient underwent post-transplant adjuvant therapy. Given the lack of disease recurrence, no NGS analysis was performed on the other three patients.

The post-transplant immunosuppressive regimen included steroids and tacrolimus. All patients stopped steroids within 1 months from transplant. Currently, two patients are on tacrolimus alone, while two patients are on a combination of tacrolimus and everolimus due to monotherapy intolerance.

Long-Term Follow Up

After a median follow up of 45 (IQR 32–83) months from diagnosis, 8 patients (61.5%) are dead, all belonging to the non-transplanted cohort. Median OS from diagnosis was 33 (IQR 26–42) months overall and 29 (IQR 23–33) months for the non-transplanted cohort (**Figure 5**). The median OS from last treatment within the SYS-TARE combo was 18 (12–35) months overall and 17 (12–19) months for the non-transplanted cohort. The median OS after dropout was 11.5 (6-5–14.5) months with 4 patients surviving for at least 1 year. All deaths were cancer-related due to disease progression. Of the two patients in the non-transplanted cohort who are still alive, one is undergoing hospice care, while the other is in stable disease after a rechallenge with GemCis with the addition of durvalumab. All transplanted patients are alive and disease-free at 73, 40, 12, and 8 months from LT.

DISCUSSION

In the presented case series of 13 patients, the preliminary results of liver transplantation for unresectable intrahepatic

TABLE 2 | Characteristics of transplanted patients at listing, transplant, and follow-up.

	Overall (n = 4)	Case 1	Case 2	Case 3	Case 4
Interval between diagnosis and LT listing (months)	Median 6	5	5	17	7
MELD-NA at listing	Median 7	7	6	8	7
Presence of viable tumor at listing (DWI or PET)		—	Yes	—	Yes
RECIST response at listing	100% SD	SD	SD	SD	SD
Choi response at listing	100% PR	PR	PR	PR	PR
Decrease in tumor density from baseline to listing	Median 52.5%	47%	35%	58%	73%
CEA at listing	Median 3.9	4.05	3.2	6.63	3.8
CA19-9 at listing	Median 40	22	38.4	46.9	42
Time on waitlist (days)	Median 57	49	2	65	127
Interval diagnosis and LT (months)	Median 10.5	10	5.5	19	11
Interval last treatment and LT (days)	Median 36	59	58	5	14
CEA at LT (mg/dL)	Median 3.3	3.35	2.7	8.5	3.4
CA19-9 at LT (mg/dL)	Median 43	17.7	41.5	75.7	44.5
MELD-Na at LT	Median 7	6	7	7	7
LT duration (hours)	Median 10	13	11	9	9
Venovenous bypass	2/4 (50%)	Yes	No	Yes	No
Final pathology	4/4 iCC	iCC	iCC	iCC	iCC
TNM staging T1aN0 T2N0	2/4 (50%) 2/4 (50%)	ypT2N0	ypT1aN0	ypT1aN0	ypT2N0
Number of lesions	Median 1	1	1	2	1
Size of lesions (mm)	Median 74	72	23	76	84
% viable tumor at final pathology	Median 25%	30%	30%	<5%	20%
Grading	3/4 (75%) G3 1/4 (25%) G2	G3	G3	G3	G2
Lymphovascular invasion	2/4 (50%)	Present	Absent	Absent	Present
Perineural invasion	2/4 (50%)	Present	Absent	Absent	Present
Number of metastatic/retrieved lymph nodes	0/8	0/7	0/9	0/10	0/6
LOS (days)	Median 11.5	12	11	44	10
Postoperative complications	Major complications 2/4 (50%)	Pleural effusion CD II	Yes, partial hepatic artery thrombosis CD IIIa CCI 45.4	Yes Biliary stenosis, hemoperitoneum, duodenal fistula CD IIIb CCI 73.7	No
Follow-up (months)	Median 26	73	40	12	8
Recurrence	0/4	No	No	No	No
Alive, NED at latest follow up	4/4	Yes	Yes	Yes	Yes

Data is number (percentage) and median (interquartile range). LT, liver transplant; CRLM, colorectal liver metastases; T-bil, total bilirubin; MELD-Na, model for end stage liver disease–sodium; DBD, donation after brain death; LDLT, living donor liver transplant; CIT, cold ischemia time; WIT, warm ischemia time; LOS, length of stay; CD, clavien dindo grade; CCI: comprehensive complication index; NED, no evidence of disease.

cholangiocarcinoma after sequential downstaging with chemotherapy and radioembolization (SYS-TARE) is reported. Out of thirteen patients, four (31%) were successfully transplanted after neoadjuvant SYS-TARE, with one requiring an additional line with targeted therapy due to progression after TARE. The median intention-to treat survival of the presented consecutive series was 33 (IQR 26–42) months, which compares favourably with the median OS of 12.8 (11.1–14.0) months observed with the current standard of care [3]. All patients who had a sustained response up to transplant are alive with no evidence of recurrence after a median of almost 2 years of follow up.

The rationale for our prospective protocol draws from the pioneering experiences with LT for ICC from several contexts [8, 9, 15], as well as from the advances in the systemic and locoregional treatment of unresectable iCC [16]. It is increasingly evident that LT for iCC can offer a significant survival benefit, differently from what was previously thought, if the key principles residing in pre-transplant tumor response to combined chemo-radiation treatments and consequent patient selection are respected. After all, therapeutic efficacy and depth of response could be considered valid surrogates of tumor biology in iCC, as increasingly demonstrated in other transplant oncology indications, such as LT for colorectal liver metastases, HCC and perihilar cholangiocarcinoma after multimodal downstaging protocols [8, 17].

Our patient population was carefully selected according to pre-determined criteria combined with dynamic assessment of tumor response to treatment. Tumor presentation was also considered, as patients with multifocal disease were excluded

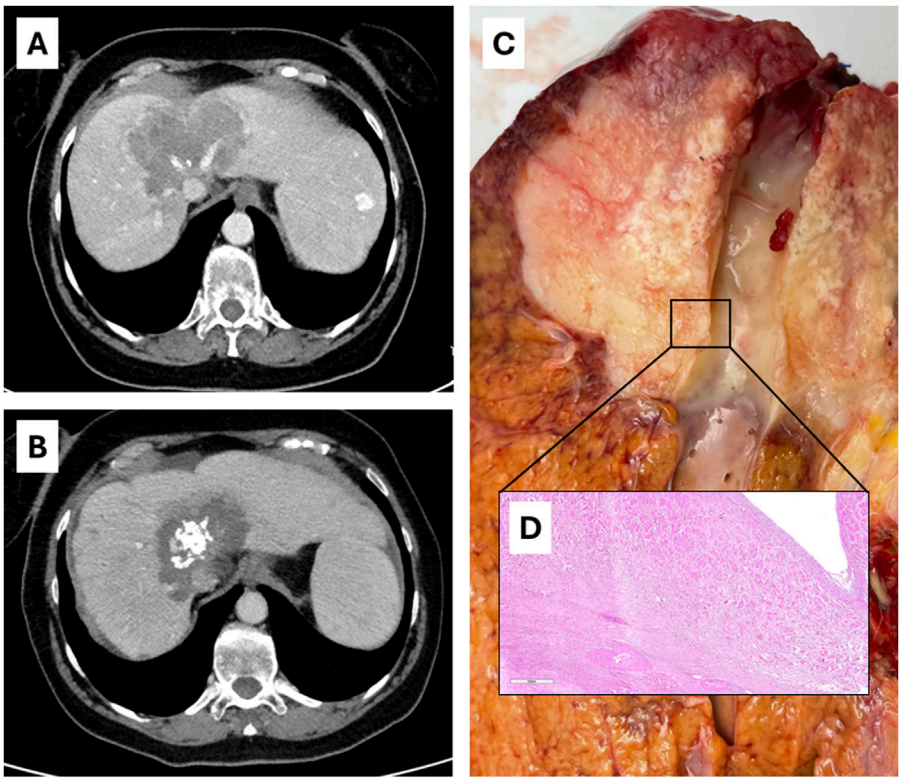
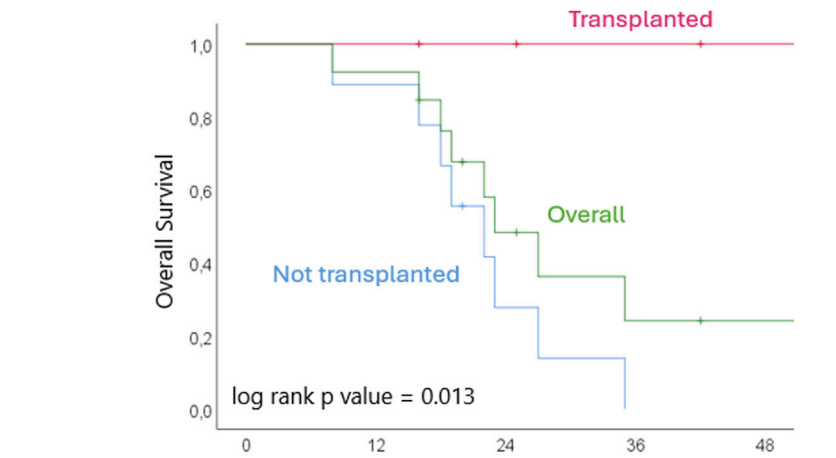


FIGURE 4 | Case 3 of **Table 2**: radiological [**(A)**, before therapy; **(B)**, after SYS-TARE], *ex vivo* **(C)** and histological **(D)** appearance of hepatic vein encasement without intimal penetration.



N. at risk					
Transplanted	4	4	3	2	1
Not transplanted	9	7	1	0	0

FIGURE 5 | Kaplan Meier curves of overall survival from completion of SYS-TARE (i.e., last TARE within protocol) for transplanted and non-transplanted patients.

as associated with unfavorable biology, while vein encasement by single bulky lesions was not considered as a contraindication for LT consideration. As a matter of fact, all but one patient had single lesions at baseline, with no satellitosis and no clinically suspicious lymph nodes. All patients were not jaundiced and, in such condition, CA19-9 had to be below 100 U/mL for LT

consideration. Those characteristics are similar to those reported by McMillan et al. [9] and confirm that tumor size in iCC on non-cirrhotic liver has a relatively negligible impact on prognosis, as suggested by both transplant and non-transplant series [15, 18]. Chronic liver disease in our series was minor, mainly related to metabolic syndrome without untreatable comorbidities, except one patient who had Wilson's disease which was cured with LT.

The presented protocol differs from previously published experiences due to its intention-to-transplant design. In contrast with others [8], in which patients were selected for LT consideration among those responding to non-systematic neoadjuvant treatments, our patients entered the protocol from first referral with a specific downstaging-to-transplant aim. This accounts for a dropout rate of 69% before LT listing, mostly occurring between TARE and abdominal exploration due to intrahepatic progression. The test of time between TARE and listing seems therefore to be important for accurate patient selection. Conversely, the added radioembolization in our and other series seems to guarantee a surprisingly long survival despite progression and dropout from LT consideration. The consequent suggestion to expedite patient listing in case of objective radiological response to TARE in iCC needs further confirmation and more extended follow-up.

The present study is not the first report of LT for iCC after TARE: Gruttadauria et al. [19], in 2021, reported two patients who received TARE as neoadjuvant therapy before LT for iCC (although one had a mixed hepatocellular-cholangiocarcinoma). The presented protocol combined first-line chemotherapy with TARE as a source of radiotherapy in single bulky unresectable iCC and proved to be effective in terms of pathological response and patient outcomes. This combination achieved at least 70% of tumor response in all patients, up to over 95% in one patient. Although the superiority of the combination of radiation therapy + chemotherapy versus chemotherapy alone in unresectable iCC is not supported by randomized controlled trials, we believe that the available retrospective and prospective evidence showing consistent benefits in terms of local disease control [5, 20–22], as well as high tolerability in the non-cirrhotic setting [23], strongly supports the use of this combination as a neoadjuvant strategy before liver transplantation and resection.

The rationale for the combination of systemic therapy and TARE in iCC is even stronger now that the new standard of care for the treatment of unresectable iCC adds to the GemCis scheme the immune checkpoint inhibitor (ICI) durvalumab [3]. The synergistic effect of ICI and radiation is supported by mechanistic notions as well as clinical evidence [24–26], and has been demonstrated also after TARE [27]. The abscopal effect of radiation therapy on the antitumoral immune response is well known, and this effect is even more robust when combined with ICI. For this reason, our protocol has been updated to include GemCis + durvalumab followed by TARE with the aim of achieving even a more profound and sustained local control in downstaging-to-transplant for iCC [25]. For the time being, we have decided to offer chemotherapy, which is the standard of care treatment for advanced iCC, before TARE, however if the rationale of this downstaging strategy is confirmed, it could be considered to offer TARE first to boost the effects of the subsequent chemo-immunotherapy.

Another hint of flexibility in neoadjuvant approach to iCC is targeted therapy allowed as second-line, as per standard of care. The patient who underwent LT after second-line FGFR2 inhibitors demonstrated the most profound pathological tumor response, with less than 5% viable tumor tissue in the hepatectomy specimen. This is in line with previous experiences in the LT setting [9]. Accordingly. more systematic tumor profiling in patients entering protocols of ore-LT downstaging needs to be further investigated.

With respect to tumor response, pre-transplant radiological assessment of response to SYS-TARE was poorly encapsulated by RECIST criteria, that classified all pre-LT observed responses as stable disease, while explant histology demonstrated more relevant effects. Consequently, RECIST criteria may not be appropriate for evaluation of iCC in the setting of neoadjuvant treatments, and similar considerations may be made regarding modified RECIST (mRECIST) criteria, as it is challenging to give an mRECIST evaluation of lesions with an hyperenhancing border as is often the case with iCC. In our experience, Choi criteria appear to have a higher correlation with pathological response [13, 28].

All patients who were made eligible to LT underwent abdominal exploration with nodal sampling before listing. The invasiveness of such surgery, especially in case of hilar lymphadenectomy, may be questioned. However, occult lymph node metastases in iCC occur in 24%–40% of T1-T2 tumors [29], and are a significant prognostic factor after resection [30]. Accordingly, the preliminary assessment of at least stations 8 and 12 are deemed crucial in patient selection as those stations cover >80% of possible lymph nodal metastatic sites in iCC [31]. Two out of six patients (33%) who underwent abdominal exploration were excluded due to positive nodal spread that was not detect with imaging or FDG-PET. Differently from other experiences [9], our protocol did not include adjuvant therapy and no patient was deemed eligible to adjuvant therapy due to unexpected nodal positivity or high-risk features at pathology.

Finally, a mention should be given to the widespread concern that expanding the indications for transplant oncology will result in an unbearable pressure on the donor pool. Our experience, similarly to others before ours, does not seem to support this concern. First, the cohort of patients with iCC who fulfills the criteria for transplantation with acceptable 5-year survival represent a minority of patients for an already rare disease. Secondly, as shown by our median donor risk index, most of these patients were transplanted with marginal grafts with excellent long-term functional results. For these reasons, it seems unlikely that the inclusion of carefully selected patients with iCC into the standard indications for LT will result in an unacceptable increase in transplant candidates in most local scenarios. Median time on waitlist in our cohort was around 2 months, which we recognize may not be as easily achievable in different scenarios. Given the excellent depth of response in the patients who eventually made it to LT and the lack of dropouts during the waiting period, it can however be speculated that carefully selected patients may withstand longer waiting periods, especially if they can continue systemic treatment in the meantime.

This study has several limitations. It is a monocentric experience with a small sample size. There is no control

group, thus the survival benefit of LT in this specific population can only be inferred using comparisons from the literature. Chemotherapy was performed according to the standard of care at the time (i.e., GemCis), which is not the standard anymore. Finally, two of the four patients who underwent transplant have less than 2 years of follow-up from LT.

In conclusion, the intention-to-treat results of this series of 13 patients with unresectable iCC who underwent of neoadjuvant SYS-TARE suggest that this combination may results in sustained response rates that could be considered sufficient to offer LT with excellent survival, if associated to pre-transplant abdominal exploration excluding nodal disease. Post-transplant outcomes in this setting compare favourably with previous reports offering non-transplant options and with the patients who continued follow-up without LT. Further prospective studies with larger sample sizes and longer follow-up are needed to confirm our findings.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving humans were approved by the International Review Board, Fondazione IRCCS Istituto Nazionale dei Tumori. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

Conceptualization: VM, CS, MMs, MB, and SB. Formal analysis: MMs, CS, and MB. Data curation: MMs, CC, TC, MMc, and GL. Writing–original draft: MMs, CS, MB, and VM. Writing–review and editing: VM, CS, MMs, FP, MN, SB, VB, MF, and GL. Supervision: VM and CS. All authors contributed to the article and approved the submitted version.

FUNDING

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

CONFLICT OF INTEREST

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

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ORIGINAL RESEARCH
published: 25 October 2024
doi: 10.3389/ti.2024.13313

Post-Transplant Vitamin D Deficiency in Lung Transplant Recipients: Impact on Outcomes and Prognosis

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Despite the recognized clinical significance of vitamin D deficiency in other solid organ transplant recipients, its specific relevance in lung transplantation remains to be fully understood. In this study, we performed a retrospective observational study on the impact of vitamin D deficiency on clinical outcomes and prognosis in 125 lung transplant recipients (LTRs) from October 2014 to March 2020 at a university hospital in Seoul, South Korea. Among 125 LTRs, 51 patients (40.8%) were vitamin D deficient. LTRs in the vitamin D-deficient group exhibited a higher incidence of post-transplant pneumonia and overall mortality than those with normal vitamin D levels during the follow-up period. This trend persisted when subjects were stratified into vitamin D tertiles. Furthermore, post-transplant vitamin D levels and C-reactive protein (CRP) significantly impacted pneumonia incidence and survival outcomes. Prognosis also varied based on cumulative vitamin D supplementation after transplantation, with patients receiving higher cumulative supplementation demonstrating improved prognosis. Our findings underscore the importance of assessing and maintaining optimal vitamin D levels post-transplantation, suggesting a potential avenue for improving outcomes in lung transplant recipients, especially in mitigating infection risk and enhancing long-term survival. Further research into optimal vitamin D levels and supplementation strategies in this population is warranted.

Keywords: vitamin D deficiency, lung transplantation, survival, prognosis, pneumonia

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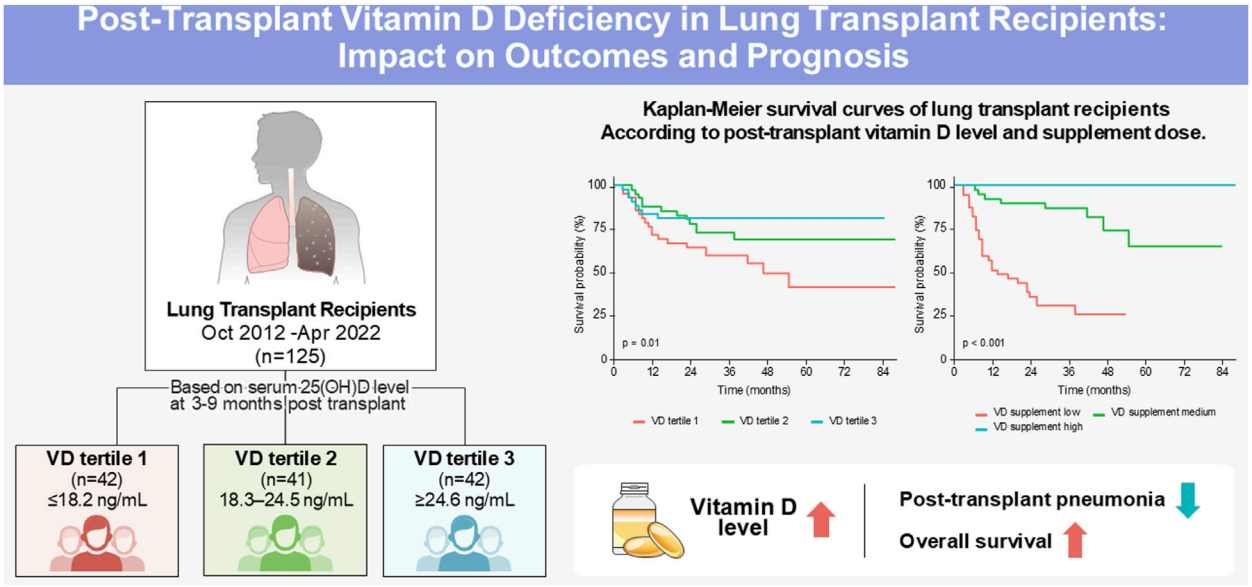
Received: *29 May 2024*
Accepted: *15 October 2024*
Published: *25 October 2024*

Citation:
Ki MS, Kim NE, Woo A, Kim SY, Kim YS, Kim HE, Lee JG, Paik HC and Park MS (2024) Post-Transplant Vitamin D Deficiency in Lung Transplant Recipients: Impact on Outcomes and Prognosis. Transpl Int 37:13313. doi: 10.3389/ti.2024.13313

INTRODUCTION

Beyond calcium homeostasis and bone metabolism, vitamin D deficiency is associated with numerous chronic diseases. Receptors and enzymes involved in vitamin D metabolism are broadly expressed in almost all tissues and cells *in vivo*, thus mediating various extraskeletal effects [1]. These include immunomodulatory and anti-infective properties, so vitamin D has been linked to major lung diseases and lung transplant status.

Abbreviations: 25(OH)D, 25-hydroxyvitamin; 6MWT, six-minute walk test; AKI, acute kidney injury; BOS, bronchiolitis obliterans syndrome; BPF, bronchopleural fistula; ECMO, extracorporeal membrane oxygenation; FEV1, forced expiratory volume in 1 sec; ICU, intensive care unit; LTRs, lung transplant recipients; PFT, pulmonary function test; PGD, primary graft dysfunction; RRT, renal replacement therapy.



GRAPHICAL ABSTRACT

Vitamin D deficiency appears to be associated with the prognosis of various respiratory diseases, including chronic obstructive pulmonary disease (COPD) [2–4], bronchial asthma (BA) [5, 6], respiratory infections [7–9], and interstitial lung disease (ILD) [10, 11]. Vitamin D deficiency is frequently observed in solid organ transplant recipients as well. It has been reported to be related with an increased risk of acute rejection and infection, as well as overall survival in liver, kidney, and lung transplant recipients (LTRs) [12–14]. Studies have shown that solid organ transplant recipients who received vitamin D supplementation had a lower incidence of rejection than those who did not [12, 15].

However, high-dose vitamin D supplementation showed no significant difference from the placebo control group in chronic rejection and overall survival in a randomized controlled trial for LTRs [16]. It is unclear whether low vitamin D status in LTRs merely reflects the patient's severity and poor health condition or is a risk factor independent of morbidity and mortality.

In this context, this study was performed with the aim of elucidating the clinical relevance of post-transplant vitamin D status in lung transplant recipients (LTR), with a specific focus on clinical outcomes and prognosis.

MATERIALS AND METHODS

Study Participants

The study included adult patients who underwent lung transplantation at a tertiary hospital in Seoul, South Korea, between October 2014 and March 2020. Exclusions comprised cases of retransplantation, multi-organ transplantation, and patients with a survival duration of less than 1 month. Post-transplant vitamin D status was determined based on levels measured 3–9 months after lung transplantation. Thirty-six transplant recipients missing vitamin D level data at this specific point were excluded from the analysis (**Figure 1**).

Determination of Vitamin D Status

Vitamin D status was determined by measuring serum 25(OH) D according to the guidelines, and the measurement was performed using the radioimmunoassay instrument in our institution (Dream Gamma-10, Shin Jin Medics Inc., Goyang, South Korea) [17]. In the case of multiple vitamin D values, post-transplant vitamin D was determined as the average. According to the clinical practice guideline of the American Endocrinology Association, patients with serum 25(OH) < 20 ng/mL were assigned as the vitamin D deficient group, and those above that were classified as the vitamin D normal group [17]. Given the lack of an established reference for optimal vitamin D levels in lung transplant recipients, we stratified the study population into tertiles based on their vitamin D levels to examine sequential trends. The cut-off points for the vitamin D tertiles were 18.2 ng/mL and 24.5 ng/mL, with the groups named VD tertile 1, VD tertile 2, and VD tertile 3 in order of increasing levels (**Supplementary Figure S1**).

This research protocol was approved by the Institutional Review Board of Severance Hospital (IRB number: 4-2020-

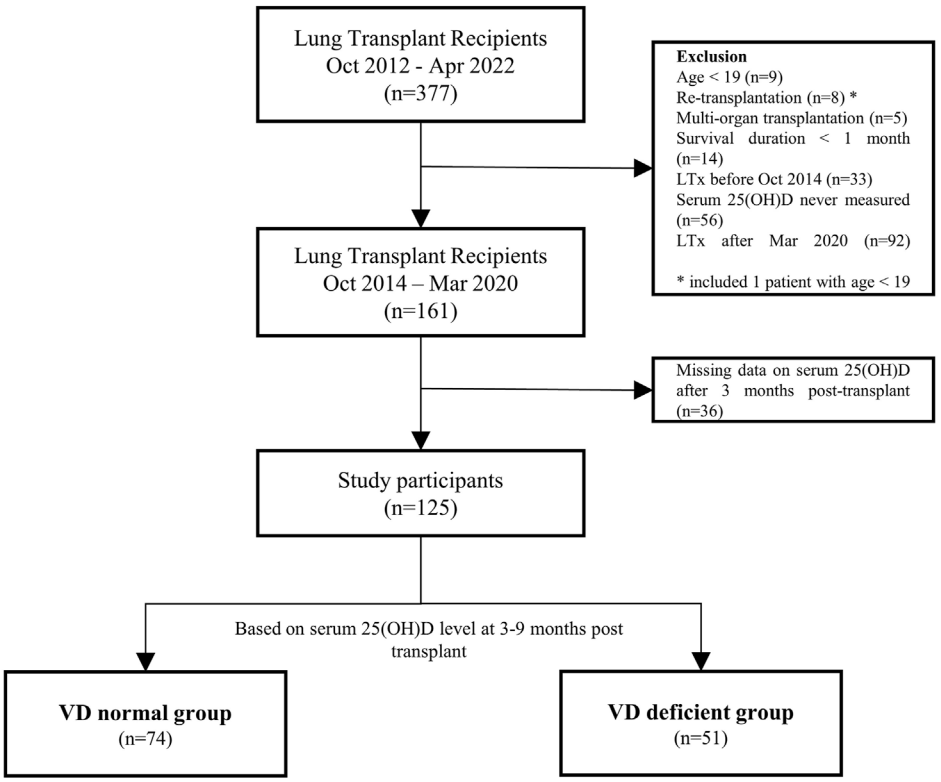


FIGURE 1 | Flowchart of the study participants. LTx, lung transplantation; 25(OH)D, 25-hydroxyvitamin D.

0228). The appropriate ethics review boards approved the study design, and informed consent was waived.

Data Collection

Clinical and demographic data, such as age, sex, preoperative body weight and body mass index (BMI), pre-transplant diagnosis, and comorbidities, were examined from electronic medical records. Transplant waiting time, preoperative intensive care unit (ICU) admission, ventilator care, and extracorporeal membrane oxygenation (ECMO) care were also checked.

Operative findings and postoperative complications that occurred within 1 month after lung transplantation were also investigated. The diagnosis of acute rejection was determined based on the International Society for Heart and Lung Transplant (ISHLT) standard guidelines [18]. As variables for postoperative complications, we investigated whether complications occurred by major organs after lung transplantation: respiratory complication [pneumonia, primary graft dysfunction (PGD), respiratory failure including re-intubation and tracheostomy], postoperative acute kidney injury (AKI), renal replacement therapy (RRT) use, bacteremia, infection (bacterial, viral or fungal), neurologic complication, cardiovascular complication, and gastrointestinal complication were examined. PGD was graded according to the International Society of Heart and Lung Transplant (ISHLT) Working Group criteria. The proposed standardized definition of PGD was based on diffuse pulmonary edema in an allograft on a chest radiograph and a PaO_2/FiO_2 (P/F) ratio [19]. Based on the kidney disease: improving global outcomes (KDIGO) guideline, AKI is defined as any of the following: an increase in serum creatinine by ≥ 0.3 mg/dL (≥26.5 μmol/L) within 48 h or an increase in serum creatinine to ≥1.5 times the baseline value, which is known or presumed to have occurred within the prior 7 days; or urine volume <0.5 mL/kg/h for 6 h) [20]. Neurologic, cardiovascular, gastrointestinal, and wound-related complications were defined as cases with appropriate intervention after discussion with the lung transplant team and in collaboration with relevant specialists.

We examined vitamin D supplementation (Cholecalciferol or Calcitriol) pre- and post-transplant, including cumulative dosages. The estimated daily vitamin D supplementation was calculated by dividing the total cumulative supplementation by the follow-up period post-transplantation. To assess prognosis based on vitamin D supplementation, subjects were divided into tertiles of cumulative supplementation. The cut-off points were 864,666.7 IU and 2,731,666.7 IU, labeled VD supplement tertiles 1, 2, and 3 in ascending order. Post-transplant tests were also reviewed, including serum C-reactive protein, pulmonary function tests (PFTs), and 6-minute walk tests (6MWT) measured 3–9 months after lung transplantation. According to relevant guidelines, PFT and 6MWT were performed if the patient's condition was not limited [21, 22]. Among the study participants, LTRs with a survival period of 1 year or more were

investigated for the development of bronchiolitis obliterans syndrome (BOS) based on the ISHLT diagnostic criteria published in 2019 [23].

Colonization by *Pseudomonas* and *Aspergillus* was determined based on the presence of these microorganisms in bronchial washing and bronchoalveolar lavage cultures. The cumulative incidence of post-transplant pneumonia episodes was established by identifying cases that fulfilled both criteria: 1) pneumonia detection on Chest computed tomography (CT) scan and 2) intravenous antibiotic administration within 1 week before and after the pneumonia-detected CT scan date.

All LTRs were initially given a triple immunosuppressive therapy that included a calcineurin inhibitor, an antimetabolite or purine synthesis inhibitor, and corticosteroids. Follow-up duration was defined as from the date of lung transplantation to the date of death or the last follow-up. The end date of the survival analysis was 1 September 2021.

Statistical Analysis

Continuous variables were summarized using means or medians, while categorical variables were represented by counts and percentages. The Student's t-test or Mann-Whitney test was employed for continuous variables, and the Chi-squared test or Fisher's exact test for categorical variables was used to compare the two groups. The survival was estimated using the Kaplan-Meier method, and the significance of the difference was assessed using a log-rank test. Univariable and multivariable analysis of overall survival was conducted using the Cox proportional hazard model to identify predictors of overall survival. In the multivariate logistic regression model, continuous variables, including age, estimated blood loss, operation time, total hospitalization, 6MWT distance, and FEV_1, were categorized by their median values and incorporated into the analysis. Logistic regression analysis was conducted to determine if vitamin D deficiency significantly contributed to the development of pneumonia post-transplantation. P-values less than 0.05 were considered statistically significant. All statistical analyses were performed using R, version 4.1.1 (R Foundation for Statistical Computing).

RESULTS

Among the 125 LTRs, there were 74 patients (59.2%) in the post-transplant vitamin D normal group (VD normal group) and 51 patients (40.8%) in the vitamin D deficient group (VD deficient group). The VD deficient group exhibited an older average age, a higher rate of male sex, and a higher prevalence of pre-transplant cardiovascular disease compared to the VD normal group. Operative findings revealed a higher proportion of recipients with pleural adhesion in the VD deficient group compared to the VD normal group, along with an increased estimated blood loss during lung transplantation. There was no statistically significant difference in the incidence of postoperative complications between the two groups. Total hospitalization periods for lung transplantation were more extended in the VD deficient group than in the VD normal group (**Table 1**).

The VD normal group had higher post-transplant vitamin D supplementation rates and a greater cumulative dose of vitamin D supplementation. Post-transplant, the VD deficient group showed significantly higher CRP levels and a shorter 6MWT distance than the VD normal group (**Table 2**).

The average follow-up period for the study participants was 35 months. During the follow-up period, the two groups had no statistically significant differences in the incidence of BOS, *Pseudomonas*, and *Aspergillus* colonization. However, the VD deficient group exhibited significantly higher rates of post-transplant pneumonia and a greater cumulative number of post-transplant pneumonia. The VD deficient group experienced a higher overall mortality rate during the follow-up duration compared to the VD normal group (20.3% vs. 51.0%, $p = 0.001$), with infection identified as the primary cause of death in both groups (**Table 3**).

In the survival analysis, the VD deficient group showed a lower survival rate than the VD normal group (log-rank test, $p < 0.001$, **Figure 2A**). The univariate and multivariate Cox proportional hazard analyses were conducted, including post-transplant vitamin D status and covariates that showed statistically significant differences in the two groups. Variables with significant missing values (e.g., post-transplant FEV_1, post-transplant 6MWT; 20 or more missing) or notable correlations (e.g., total hospitalization, estimated blood loss) were selected for inclusion in the Cox proportional hazards regression model. Following the multivariate analysis, post-transplant VD deficiency [adjusted hazard ratio (aHR) 2.22, 95% confidential interval (CI) 1.05–4.69, $p = 0.036$] and higher CRP level (aHR 9.38, 95% CI 3.61–24.4, $p < 0.001$) emerged as factors significantly related with the prognosis of lung transplant recipients (**Table 4**).

Comparisons of the Vitamin D Tertiles

The results of the comparison divided into vitamin D level tertiles also showed that the lower the vitamin D level, the higher the age, the higher the male ratio, and the higher the rate of cardiovascular disease. Otherwise, there were no significant differences between vitamin D tertiles in the remaining baseline characteristics (**Supplementary Table S1**). The differences in vitamin D supplementation and post-transplant test results among the vitamin D tertiles mirrored those observed in the VD deficient/normal group. The estimated daily vitamin D supplementation doses for tertiles 1, 2, and 3 were approximately 871 IU, 1685 IU, and 1884 IU, respectively (**Supplementary Table S2**).

In the vitamin D tertiles, lower vitamin D levels were linked to a higher incidence of post-transplant pneumonia over a shorter follow-up period. Additionally, a significant difference in the overall mortality rate was observed, demonstrating a sequential trend related to vitamin D levels [VD tertile 1: 21/42 (50.0%), VD tertile 2: 12/41 (29.3%), VD tertile 3: 8/42 (19.0%); $p = 0.009$, **Supplementary Table S3**].

TABLE 1 | Basic characteristics of lung transplant recipients according to vitamin D status.

	VD normal group (N = 74)	VD deficient group (N = 51)	*p*-value
Age	52.6 ± 12.2	57.6 ± 11.4	0.021
Male sex, n (%)	42 (56.8%)	43 (84.3%)	0.002
Body weight (kg)	57.0 ± 11.2	59.4 ± 10.5	0.234
BMI (kg/m^2)	21.2 ± 3.8	21.4 ± 3.5	0.697
Pre-transplant diagnosis, n (%)			0.443
COPD and emphysema	5 (6.8%)	4 (7.8%)	
ILD	52 (70.3%)	40 (78.4%)	
Bronchiectasis	9 (12.2%)	2 (3.9%)	
Others	8 (10.8%)	5 (9.8%)	
Comorbidities, n (%)			
DM	10 (14.1%)	13 (26.0%)	0.159
HTN	8 (11.3%)	8 (16.0%)	0.628
CV	10 (14.1%)	20 (40.0%)	0.002
CKD	7 (9.9%)	6 (12.0%)	0.939
Tuberculosis	25 (34.7%)	14 (28.0%)	0.558
Transplant waiting time (days)	121.0 [41.0; 231.0]	103.0 [44.5; 249.5]	0.890
Preoperative status, n (%)			
Preop ICU admission	29 (39.2%)	23 (45.1%)	0.635
Preop ventilator care	26 (35.1%)	23 (45.1%)	0.350
Preop ECMO care	22 (29.7%)	20 (39.2%)	0.362
Operative findings, n (%)			
Intraoperative ECMO weaning	43 (65.2%)	36 (70.6%)	0.672
Transplantation Type, Double	66 (95.7%)	50 (100.0%)	0.368
Size mismatch, Bronchus, or PA	37 (56.1%)	29 (59.2%)	0.885
Status of pleura, Adhesion	39 (55.7%)	37 (74.0%)	0.063
Estimated blood loss (mL)	1800.0 [1050.0; 3000.0]	2300.0 [1600.0; 3600.0]	0.036
ECMO time (min)	300.0 [280.0; 360.0]	300.0 [248.0; 390.0]	0.825
Operation time (min)	380.9 ± 79.6	407.2 ± 71.5	0.090
Anesthesia time (min)	479.0 ± 84.8	496.8 ± 70.9	0.280
Postop complications, n (%)			
Acute rejection	2 (2.7%)	0 (0.0%)	0.647
Respiratory[a]	30 (42.9%)	28 (54.9%)	0.260
BPF	2 (2.7%)	3 (5.9%)	0.669
Pneumothorax, pleural effusion	9 (12.3%)	7 (13.7%)	1.000
Bronchial stenosis	7 (9.5%)	5 (9.8%)	1.000
PA stenosis	3 (4.1%)	3 (5.9%)	0.965
Postop AKI	4 (5.6%)	8 (15.7%)	0.126
Postop RRT use	5 (6.8%)	4 (7.8%)	1.000
Bacteremia	1 (1.4%)	3 (5.9%)	0.393
Infection	5 (7.0%)	7 (13.7%)	0.360
Neurologic	2 (2.8%)	2 (3.9%)	1.000
Cardiovascular	2 (2.9%)	3 (5.9%)	0.717
Gastrointestinal	8 (11.1%)	7 (13.7%)	0.875
Postop ICU stay (days)	7.0 [5.0; 13.0]	7.0 [4.5; 13.0]	0.670
Total hospitalization (days)	53.5 [31.5; 91.0]	86.0 [40.0; 136.0]	0.011

Values are displayed as median (interquartile range), n (%), or mean ± standard error of the mean where appropriate. BMI, body mass index; COPD, chronic obstructive pulmonary disease; ILD, interstitial lung disease; DM, diabetes mellitus; HTN, hypertension; CV, cardiovascular; CKD, chronic kidney disease; ICU, intensive care unit; ECMO, extracorporeal membrane oxygenation; BPF, bronchopleural fistula; PA, pulmonary artery; AKI, acute kidney injury; RRT, renal replacement therapy.

[a]*Respiratory complications: pneumonia, primary graft dysfunction (PGD), respiratory failure including re-intubation and tracheostomy.*

Among vitamin D tertiles 1, 2, and 3, a poorer survival curve was observed at lower vitamin D levels (log-rank test, $p = 0.01$, **Figure 2B**). Multivariate Cox proportional hazards analysis using vitamin D tertiles indicated that VD tertile 1 demonstrated a marginally significant hazard ratio in comparison to VD tertile 3 (aHR 2.45, 95% CI 0.92–6.54, $p = 0.074$, **Supplementary Table S4**). Logistic regression analysis of post-transplant pneumonia occurrence indicated that lower post-transplant vitamin D levels and higher post-transplant CRP levels were significant covariates (**Supplementary Table S5**; **Figure 3**).

Comparisons of the Vitamin D Supplement Tertiles

The 125 lung transplant recipients were categorized into three tertiles of cumulative vitamin D supplementation after lung transplantation (VD supplement tertile 1, VD supplement

TABLE 2 | Vitamin D measurements, supplementation and post-transplant test results according to vitamin D status.

	VD normal group (N = 74)	VD deficient group (N = 51)	*p*-value
Post-transplant 25(OH)D (ng/mL)	26.5 ± 5.1	14.7 ± 3.5	<0.001
Post-transplant 25(OH)D (ng/mL)	25.5 [22.3; 29.3]	15.4 [12.1; 17.8]	<0.001
Number of 25(OH)D measurements	2.7 ± 1.1	2.3 ± 0.8	0.023
Delta 25(OH)D[a]	7.3 ± 7.1	0.5 ± 6.9	<0.001
Delta 25(OH)D[a]	7.6 [2.6; 11.8]	0.4 [−3.4; 6.4]	0.001
Preop VD supplementation[b], n (%)	67 (90.5%)	40 (78.4%)	0.102
Preop VD cumulative dose (IU)	437,800 [181,000; 688,600]	183,000 [94,000; 458,400]	0.008
Postop VD supplementation[b], n (%)	73 (98.6%)	44 (86.3%)	0.016
Postop VD cumulative dose (IU)	2,713,200 [975,000; 3,766,000]	760,800 [212,500; 1,854,500]	<0.001
Post-transplant tests			
CRP (mg/L)	7.9 ± 14.3	22.5 ± 33.2	0.004
CRP (mg/L)	1.9 [0.7; 7.8]	6.7 [1.4; 33.8]	0.003
FEV1, predicted %	69.9 ± 20.0	68.4 ± 15.9	0.690
FEV1, liter	2.0 ± 0.7	2.0 ± 0.5	0.862
FVC, predicted %	62.9 ± 16.0	59.2 ± 16.0	0.262
FVC, liter	2.4 ± 0.8	2.4 ± 0.7	0.953
DLCO, predicted %	66.0 ± 21.4	63.0 ± 18.1	0.526
6MWT distance (m)	384.5 ± 129.0	320.0 ± 149.0	0.027

Values are displayed as median (interquartile range), n (%), or mean ± standard error of the mean where appropriate. CRP, C-reactive protein; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; DLCO, diffusing capacity of the lungs for carbon monoxide; 6MWT, 6-minute walking test.

[a]*Delta 25(OH)D = post-transplant 25(OH)D - pre-transplant 25(OH)D.*

[b]*VD supplementation: Cholecalciferol or Calcitriol.*

TABLE 3 | Overall mortality rate and incidence of infection/rejection of lung transplant recipients according to vitamin D status.

	VD normal group (N = 74)	VD deficient group (N = 51)	*p*-value
Follow-up duration, months	46.1 ± 26.0	33.4 ± 22.9	0.006
Follow-up duration, months	41.5 [28.0; 68.0]	32.0 [13.0; 42.5]	0.008
BOS[a], n (%)	18 (26.1%)	8 (17.0%)	0.356
Pseudomonas colonization, n (%)	20 (27.0%)	14 (27.5%)	1.000
Aspergillus colonization, n (%)	13 (17.6%)	13 (25.5%)	0.396
Post-transplant pneumonia, n (%)	31 (41.9%)	37 (72.5%)	0.001
Cumulative episodes of post-transplant pneumonia	0.0 [0.0; 2.0]	1.0 [0.0; 3.5]	0.001
Cumulative episodes of post-transplant pneumonia	1.1 ± 1.9	2.1 ± 2.6	0.014
Overall mortality, n (%)	15 (20.3%)	26 (51.0%)	0.001
Cause of death, n (%)			0.750
Sepsis/Infection	10 (66.7%)	17 (65.4%)	
Neurologic	0 (0.0%)	1 (3.8%)	
Hematologic	1 (6.7%)	2 (7.7%)	
Cardiac	1 (6.7%)	1 (3.8%)	
GI	1 (6.7%)	0 (0.0%)	
Miscellaneous	2 (13.3%)	5 (19.2%)	

Values are displayed as median (interquartile range), n (%), or mean ± standard error of the mean where appropriate. BOS, bronchiolitis obliterans syndrome; GI, gastrointestinal.

[a]*Investigated among patients with a survival period of more than 1 year.*

tertile 2, and VD supplement tertile 3), with higher cumulative doses resulting in greater vitamin D levels. These tertiles' estimated daily supplementation doses were 747 IU, 1735 IU, and 1934 IU, respectively (**Table 5**). The group receiving higher vitamin D supplementation demonstrated improved post-transplant lung function, a reduced incidence of pneumonia, and lower mortality rates. However, the frequency of BOS exhibited an opposite trend (**Table 5**). The survival curves of lung transplant recipients (LTRs) who received less vitamin D supplementation were inferior to those of LTRs who received higher doses of vitamin D (log-rank test, $p < 0.001$, **Figure 4**).

DISCUSSION

In this study, deficiencies in vitamin D levels were observed in a significant number of lung transplant recipients despite high rates of vitamin D supplementation, with differences in frequency of post-transplant pneumonia, overall mortality and survival rates

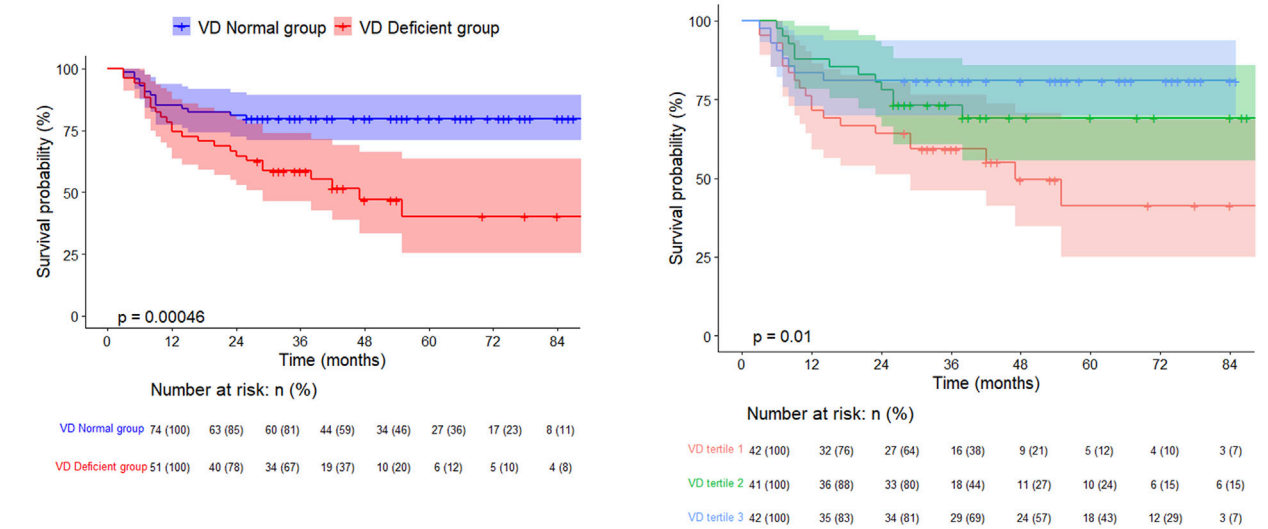


FIGURE 2 | Kaplan-Meier survival curves for lung transplant recipients: **(A)** by post-transplant vitamin D status and **(B)** stratified by Vitamin D tertiles.

TABLE 4 | Cox proportional hazard analysis for lung transplant recipients' survival.

Variables	Univariable			Multivariable		
	HR	95% CI	*p*-value	aHR	95% CI	*p*-value
Age ≥ 58 (vs. < 58)	2.5	1.29–4.85	0.007	2.33	1.13–4.82	0.022
Female (vs. male)	1.09	0.57–2.08	0.793	1.96	0.89–4.30	0.094
BMI (kg/m^2)	1.01	0.93–1.10	0.784			
Cardiovascular disease: Presence (vs. Absence)	1.09	0.53–2.23	0.823			
Status of pleura: Adhesion (vs. Normal)	1.19	0.62–2.29	0.597			
Estimated Blood loss (mL) ≥ 2,000 (vs. < 2,000)	3.58	1.78–7.18	<0.001	1	1.00–1.00	0.002
Operation time (min) ≥ 377 (vs. < 377)	1.97	0.93–4.16	0.077			
Total Hospitalization (days) ≥ 61 (vs. < 61)	4.05	1.95–8.38	<0.001			
Post-transplant VD: Deficient (vs. Normal)	2.96	1.56–5.62	0.001	2.22	1.05–4.69	0.036
CRP (mg/L) > 3.1 (vs. < 3.1)	1.06	1.04–1.07	<0.001	9.38	3.61–24.4	<0.001
6MWT distance (m) < 375 (vs. ≥ 375)	3.77	1.35–10.53	0.011			
FEV1, predicted (%) < 70 (vs. ≥ 70)	3.21	1.26–8.22	0.015			
Post-transplant pneumonia: Presence (vs. Absence)	1.9	0.98–3.67	0.057	0.64	0.29–1.42	0.273
Cumulative episodes of post-transplant pneumonia	1.09	0.98–1.20	0.108			

HR, hazard ratio; aHR, adjusted hazard ratio; CI, confidence interval; BMI, body mass index; CRP, C-reactive protein; 6MWT, 6-minute walk test; FEV_1, forced expiratory volume in 1 s.

based on post-transplant vitamin D status. Stratifying subjects into vitamin D tertiles revealed a sequential trend in outcomes based on vitamin D levels. Post-transplant vitamin D levels and CRP significantly influenced pneumonia incidence and survival. Additionally, the prognosis varied with the cumulative vitamin D supplementation after transplantation.

Vitamin D is a fat-soluble vitamin absorbed by the body through food (20%) or synthesized in the skin (80%) from 7-dihydrocholesterol after ultraviolet B-ray exposure [24]. Vitamin D from food and skin is hydroxylated in the liver and converted to 25-hydroxyvitamin D [25(OH)D], which has a long half-life and is used to measure and evaluate the vitamin D status of patients. 25(OH)D is metabolized once more in the kidneys to the fully active form of 1,25-dihydroxyvitamin D [1,25(OH)D], which is closely controlled by blood parathyroid hormone and calcium/phosphate levels [25, 26]. Physiologically activated vitamin D mediates various physiological functions by acting on bone, immune cells, and target cells of various organs [27].

Low vitamin D levels have been observed in various disease groups, including end-stage lung diseases and LTRs. Vitamin D deficiency has been reported in 20%–50% of patients with advanced lung disease and in up to two-thirds of patients waiting for lung transplantation [28–31]. In these individuals, inadequate vitamin D levels are associated with lower fat mass,

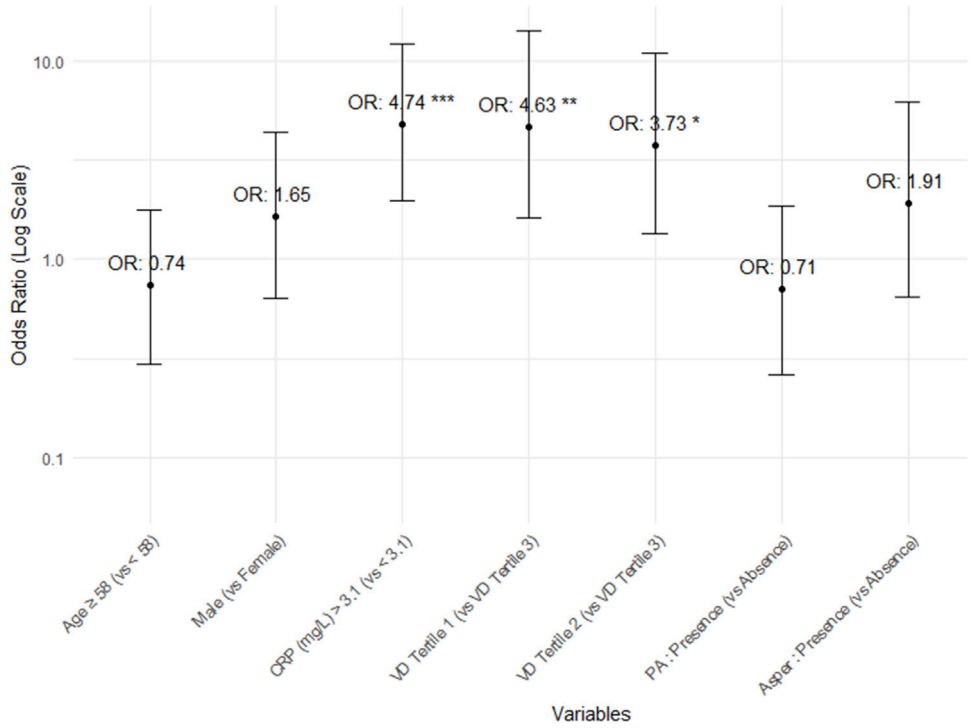


FIGURE 3 | Odds ratios and 95% confidence intervals for the occurrence of post-transplant pneumonia.

TABLE 5 | Clinical outcomes of lung transplant recipients by vitamin D supplementation tertiles.

	VD supplement tertile 1 (N = 39)	VD supplement tertile 2 (N = 39)	VD supplement tertile 3 (N = 39)	*p*-value
Post-transplant 25(OH)D (ng/mL)	18.9 [15.8; 22.6]	21.3 [17.1; 26.6]	25.5 [21.7; 29.0]	<0.001
Number of 25(OH)D measurements	2.3 ± 1.0	2.6 ± 1.0	2.8 ± 1.0	0.101
Postop VD cumulative dose (IU)	269,000 [181,500; 639,000]	1,820,000 [1,394,000; 2,369,200]	3,821,000 [3,179,500; 4,599,600]	<0.001
Estimated daily VD supplement dose (IU)	747.2 [251.6; 982.3]	1,734.5 [1,160.5; 2,120.9]	1,933.7 [1,565.1; 2,643.2]	<0.001
Post-transplant tests				
CRP (mg/L)	14.2 [4.3; 36.2]	2.4 [1.2; 7.3]	1.2 [0.5; 3.8]	<0.001
FEV_1, predicted %	63.2 ± 19.4	64.5 ± 16.9	76.3 ± 18.1	0.006
FEV_1, liter	1.8 ± 0.6	1.9 ± 0.7	2.1 ± 0.7	0.119
FVC, predicted %	56.0 ± 18.2	56.4 ± 15.0	67.8 ± 13.2	0.002
FVC, liter	2.2 ± 0.8	2.2 ± 0.8	2.6 ± 0.7	0.075
DLCO, predicted %	61.5 ± 18.0	54.5 ± 20.9	73.0 ± 18.9	0.001
6MWT distance (m)	327.4 ± 162.8	344.9 ± 139.5	386.7 ± 129.6	0.238
Follow up duration, months	14.0 [7.5; 33.0]	35.0 [29.0; 47.5]	68.0 [54.0; 81.5]	<0.001
BOS, n (%)[a]	5 (13.9%)	5 (13.5%)	16 (44.4%)	0.002
Pseudomonas colonization, n (%)	12 (30.8%)	10 (25.6%)	11 (28.2%)	0.881
Aspergillus colonization, n (%)	12 (30.8%)	6 (15.4%)	7 (17.9%)	0.207
Post-transplant pneumonia, n (%)	28 (71.8%)	20 (51.3%)	16 (41.0%)	0.021
Cumulative episodes of post-transplant pneumonia	2.0 [0.0; 2.0]	1.0 [0.0; 2.0]	0.0 [0.0; 1.0]	0.040
Cumulative episodes of post-transplant pneumonia	1.8 ± 2.1	1.8 ± 2.8	1.1 ± 2.1	0.352
1-year mortality, n (%)	17 (43.6%)	3 (7.7%)	0 (0.0%)	<0.001
3-year mortality, n (%)	27 (69.2%)	5 (12.8%)	0 (0.0%)	<0.001
Overall mortality, n (%)	28 (71.8%)	8 (20.5%)	1 (2.6%)	<0.001

Cut-off points for Vitamin D supplementation were 864,666.7 IU and 2,731,666.7 IU, creating VD supplement tertiles 1 (≤864,666.7 IU), 2 (864,666.8–2,731,666.7 IU), and 3 (≥2,731,666.8 IU). Estimated daily supplementation doses were 747 IU, 1,735 IU, and 1,934 IU for tertiles 1, 2, and 3, respectively.

Values are displayed as median (interquartile range), n (%), or mean ± standard error of the mean where appropriate. CRP, C-reactive protein; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; DLCO, diffusing capacity of the lungs for carbon monoxide; 6MWT, 6-minute walking test; 25(OH)D, 25-hydroxyvitamin D; IU, international unit; BOS, bronchiolitis obliterans syndrome; GI, gastrointestinal.

[a]*Investigated among patients with a survival period of more than 1 year.*

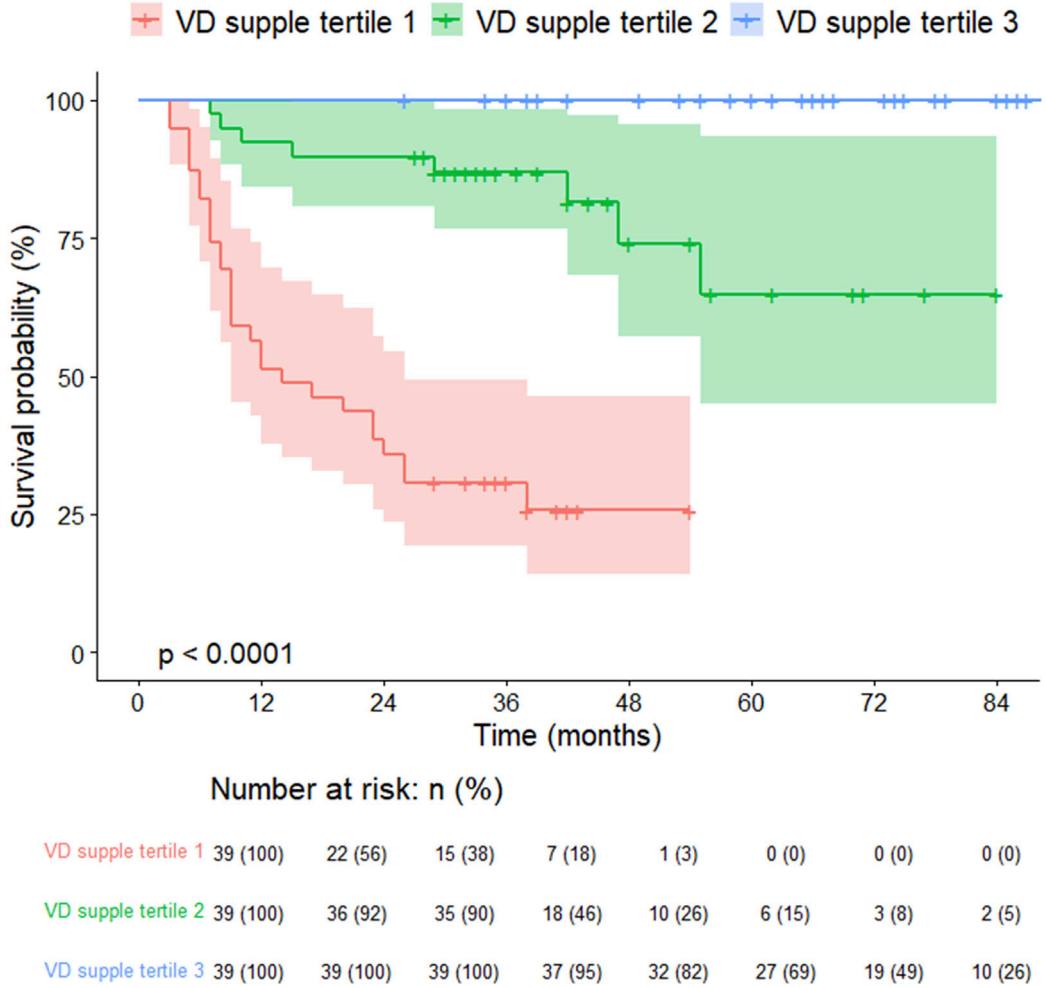


FIGURE 4 | Kaplan-Meier survival curves for lung transplant recipients by vitamin D supplementation tertiles.

obstructive pulmonary disease, insufficient dietary vitamin D intake, and limited sunlight exposure and have been investigated as predictors of reduced walking distance [31, 32]. Poor health conditions after transplantation, changes in vitamin D metabolism due to glucocorticoid use, and limited sun exposure due to increased risk of skin cancer may further lower vitamin D levels in lung transplant recipients [33, 34].

This study identified post-transplant vitamin D status as an independent variable related to survival. Studies of liver and kidney transplant recipients have also identified post-transplant vitamin D status as an independent factor associated with prognosis [12, 35]. Vitamin D levels after solid organ transplantation appear to reflect the patient's clinical course, and more needs to be discovered to determine its relationship to prognosis.

In this study, the LTRs with post-transplant vitamin D deficiency had poorer baseline characteristics, including older age, comorbidities such as cardiovascular disease, and surgical findings like pleural adhesion, longer operation times, and extended hospitalization periods. Additionally, they experienced more pneumonia episodes during follow-up.

Lung transplant prognosis is influenced by pre-transplant characteristics and intraoperative factors, with their impact evolving during the post-transplant period [36]. The key prognostic factors include: recipient factors such as age, sex, BMI, pre-transplant diagnosis, ECMO or ventilator use, hospitalization, pulmonary hypertension, and malnutrition; donor factors, including donor age and donor-recipient weight/height mismatch; procedural factors such as ischemic time, severe bleeding, and pleural adhesions; and post-transplant factors, including ECMO requirement, infection, PGD, BOS/chronic lung allograft dysfunction (CLAD), and immunosuppression levels [37]. Previous studies have also shown that prolonged ischemic time, massive bleeding due to pleural adhesions, and other factors are associated with poor prognosis in lung transplant patients [37]. Despite these factors indicating potentially poorer functional status and lower survival rates, low vitamin D status emerged as a significant prognostic

factor alongside CRP in multivariate analysis, highlighting its independent impact on outcomes.

In this study, the group with higher vitamin D levels exhibited a greater frequency of BOS, though this was not statistically significant. Additionally, a higher frequency of BOS was observed in those receiving greater cumulative supplementation doses. This trend may be attributed to the longer follow-up period, which could lead to increased BOS diagnoses among patients with higher vitamin D levels and supplementation. Our study had a median follow-up period of 35 months, and according to the ISHLT report, about 65% of transplant recipients did not develop BOS at this time [38]. Considering the complex mechanism and diagnostic process of BOS [23, 39], it seems necessary to figure out the link between vitamin D status and the development of BOS through a sufficiently extended follow-up period.

Similar to the study by Lowery et al., the LTRs with low vitamin D had more episodes of pneumonia after lung transplantation in this study [14]. Considering that the majority of patients who died in this study were due to infection, frequent cases of infection may have contributed to the poor prognosis of the LTRs. Infection is the most common cause of death within the first year after lung transplantation and the second most common cause of death between one and 5 years after transplantation [40].

The association between vitamin D levels and prognosis within 5 years after lung transplantation can primarily be attributed to vitamin D's protective effects against infections. The activated form of vitamin D, 1,25(OH)D, produced by CYP27B1 (the 25-hydroxyvitamin D 1α-hydroxylase), in various peripheral tissues, initiates signaling pathways that regulate both innate and adaptive immune responses [41]. This signaling enhances the expression of genes crucial for innate immune defense, including those coding for cytokines, chemokines, antimicrobial peptides, and pattern recognition receptors [41]. Additionally, 1,25(OH)D promotes bacterial killing and viral clearance through autophagy, playing a vital role in human defense mechanisms beyond skeletal health [41].

Epidemiological studies and randomized controlled trials have highlighted vitamin D's protective effects against respiratory infections [42, 43], which may be especially significant for lung transplant recipients immunocompromised due to medications, prolonged hospitalization, and malnutrition. Given that infections are a leading cause of mortality in the years following transplantation [40], vitamin D deficiency could lead to increased infection rates and poorer outcomes in these patients.

This study's logistic regression analysis demonstrated that low vitamin D levels were associated with higher instances of pneumonia post-transplant. Frequent pneumonia hospitalizations correlate with adverse outcomes, including reduced lung function, diminished quality of life, and increased mortality. Thus, vitamin D deficiency likely exacerbates lung transplant recipients' already compromised infection defense mechanisms.

Although vitamin D supplementation has not shown overall health benefits in clinical studies for various chronic diseases, it has been reported to result in some extraskeletal benefits, such as reduced infections and increased lung function, in patients with profound vitamin D deficiency [43, 44]. Several clinical trials have been conducted in solid organ transplant recipients to investigate the clinical benefits of correcting vitamin D deficiency [16, 44, 45]. In a clinical trial targeting LTRs, high-dose vitamin D supplementation failed to prove a clinical benefit in chronic lung allograft dysfunction prevalence, overall survival, pulmonary function, acute rejection, and respiratory infections [16]. In the previous trial, the placebo group also received a standard-dose vitamin D supplementation and maintained serum 25(OH)D levels above 30 ng/mL 1 year after lung transplantation, limiting the interpretation of the clinical significance of the much lower vitamin D levels [16]. Considering vitamin D's impact on the immune system and inflammatory cascade [46, 47], maintaining adequate vitamin D levels after lung transplantation may be necessary in reducing the risk of infection and improving prognosis. Further exploration into how vitamin D deficiency intertwines with infections and prognosis in the intricate immune context of lung transplant recipients is warranted. Additionally, research into optimal vitamin D levels and supplementation dosages in LTRs holds clinical promise.

Most LTRs in this study received vitamin D supplementation, but doses varied widely. Given the lack of clear guidelines on appropriate supplementation doses, we compared prognoses based on these doses. The tertile of LTRs receiving the highest supplementation had an estimated daily intake exceeding the recommended 1,000 IU and achieved serum 25(OH)D levels in the mid-20s ng/mL [48].

A trend of increased pneumonia and poorer prognosis was noted at vitamin D levels below 10–20 ng/mL. Drawing from previous randomized controlled trials [16] and research in other fields [49], further investigation is needed to determine the optimal vitamin D levels for effective infection defense in lung transplant recipients. This research would enable tailored supplementation and management strategies in vitamin D deficiency. Larger studies focusing on the prognosis of lung transplant recipients with vitamin D deficiency could also enhance predictions and outcomes in this population.

This study has several limitations. It examined only 125 lung transplant recipients (LTRs) from a single center, which limits its generalizability and applicability to broader populations. Additionally, the relatively short follow-up period restricts our ability to assess the relationship between low vitamin D levels and chronic rejection. Variability in the timing of vitamin D measurements among patients and the reliance on prescription history rather than actual dosing for vitamin D supplementation further complicate the findings. Despite these limitations, this study highlights the clinical significance of vitamin D deficiency in relation to short-term outcomes after lung transplantation. It also suggests a potential supplementation dose that could serve as a foundation for future large-scale studies to determine optimal vitamin D levels and supplementation strategies.

CONCLUSION

This study highlights the significant impact of vitamin D deficiency on clinical outcomes in lung transplant recipients,

emphasizing the need for further exploration of its role, optimal levels, and supplementation strategies in this population.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving humans were approved by the Institutional Review Board of Severance Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. The ethics committee/institutional review board waived the requirement of written informed consent for participation from the participants or the participants' legal guardians/next of kin because of the nature of a retrospective observational study using electronic medical records.

AUTHOR CONTRIBUTIONS

MK and NK participated in the writing of the manuscript and data analysis; AW, SK, MP, and YK participated in research design, data acquisition, data analysis, and reviewing of the manuscript; HK, JL, and HP participated in data acquisition. All authors contributed to the article and approved the submitted version.

FUNDING

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

CONFLICT OF INTEREST

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

ACKNOWLEDGMENTS

MID (Medical Illustration and Design), as a member of the Medical Research Support Services of Yonsei University College of Medicine, providing excellent support with medical illustration.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2024.13313/full#supplementary-material

Supplementary Figure 1 | Flow diagram of study participants by vitamin D tertiles. Cut-off points for Vitamin D tertiles were 18.2 ng/mL and 24.5 ng/mL, classifying participants into VD tertile 1 (≤18.2 ng/mL), VD tertile 2 (18.3–24.5 ng/mL), and VD tertile 3 (≥24.6 ng/mL).

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ORIGINAL RESEARCH
published: 25 October 2024
doi: 10.3389/ti.2024.13227

Assessment of the Therapeutic Potential of Enhancer of Zeste Homolog 2 Inhibition in a Murine Model of Bronchiolitis Obliterans Syndrome

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Bronchiolitis obliterans syndrome (BOS) is a chronic complication following lung transplantation that limits the long-term survival. Although the enhancer of zeste homolog 2 (EZH2) is involved in post-transplantation rejection, its involvement in BOS pathogenesis remains unclear. We aimed to investigate the therapeutic potential of EZH2 inhibition in BOS. 3-deazaneplanocin A (DZNep) was administered intraperitoneally to heterotopic tracheal transplant recipient model mice. Tracheal allografts were obtained on days 7, 14, 21, and 28 after transplantation. The obstruction ratios of the DZNep and control groups on days 7, 14, 21, and 28 were 15.1% ± 0.8% vs. 20.4% ± 3.6% ($p = 0.996$), 16.9% ± 2.1% vs. 67.7% ± 11.5% ($p < 0.001$), 47.8% ± 7.8% vs. 92.2% ± 5.4% ($p < 0.001$), and 60.0% ± 9.6% vs. 95.0% ± 2.3% ($p < 0.001$), respectively. The levels of interleukin (IL)-6 and interferon-γ on day 7 and those of IL-2, tumor necrosis factor, and IL-17A on days 14, 21, and 28 were significantly reduced following DZNep treatment. DZNep significantly decreased the number of infiltrating T-cells on day 14. In conclusion, DZNep-mediated EZH2 inhibition suppressed the inflammatory reactions driven by pro-inflammatory cytokines and T cell infiltration, thereby alleviating BOS symptoms.

Keywords: bronchiolitis obliterans syndrome, chronic lung allograft dysfunction, 3-deazaneplanocin A, enhancer of zeste homolog 2, lung transplantation

OPEN ACCESS

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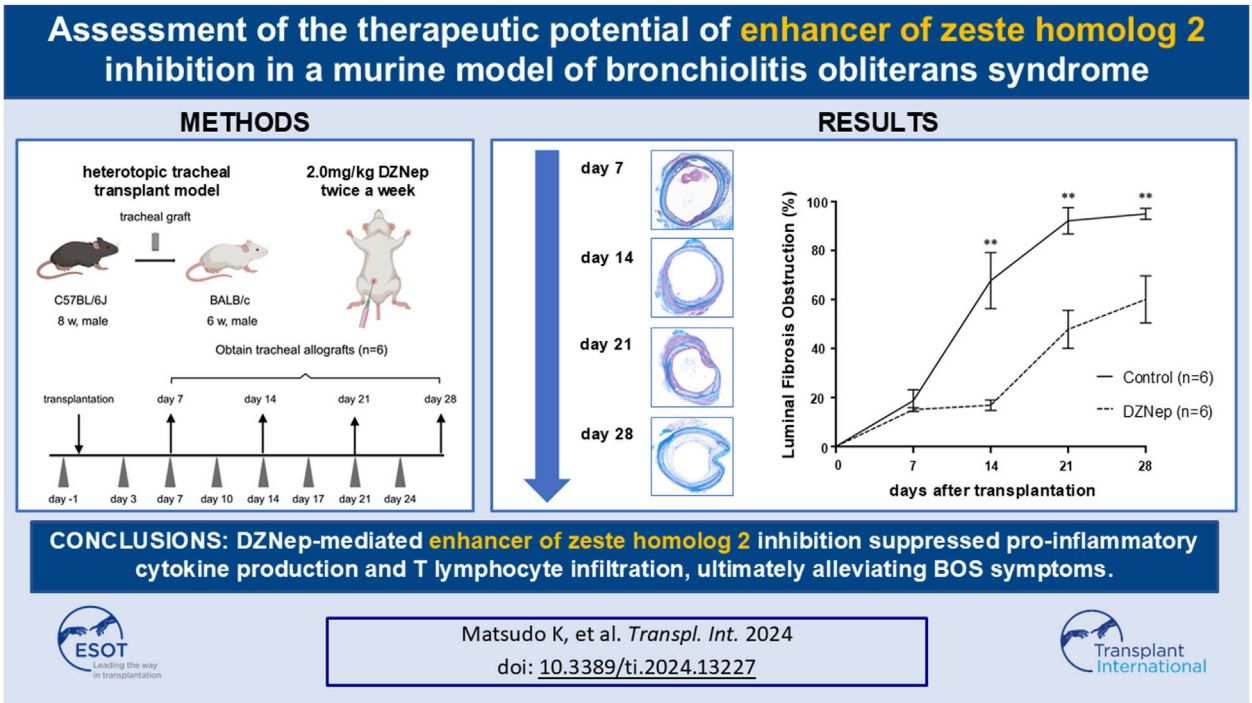
Received: *05 May 2024*
Accepted: *15 October 2024*
Published: *25 October 2024*

Citation:
Matsudo K, Takamori S, Takenaka T, Shimokawa M, Hashinokuchi A, Nagano T, Kinoshita F, Akamine T, Kohno M, Toyokawa G and Yoshizumi T (2024) Assessment of the Therapeutic Potential of Enhancer of Zeste Homolog 2 Inhibition in a Murine Model of Bronchiolitis Obliterans Syndrome. Transpl Int 37:13227. doi: 10.3389/ti.2024.13227

Abbreviations: AMR, Antibody-mediated rejection; BOS, bronchiolitis obliterans syndrome; CLAD, chronic lung allograft dysfunction; DZNep, 3-deazaneplanocin A; EZH2, enhancer of zeste homolog 2; FEV1, expiratory volume in 1 second; HTT, heterotopic tracheal transplant; IFN-γ, interferon-gamma; IL, interleukin; OB, obliterative bronchiolitis; PBS, phosphate buffered saline; STAT3, signal transducer and activator of transcription 3; Th, T helper cell; TNF, tumor necrosis factor.



GRAPHICAL ABSTRACT |

INTRODUCTION

As a last resort for severe respiratory conditions, lung transplantation is the treatment of choice for patients with progressive lung disease and irreversible pulmonary failure when no other effective treatments are available and the patient's life is at risk. However, long-term survival rates remain low owing to chronic lung allograft dysfunction (CLAD) [1]. Chronic lung allograft dysfunction has four subtypes: bronchiolitis obliterans syndrome (BOS), restrictive allograft syndrome, mixed CLAD, and undefined CLAD [2].

Chronic lung allograft dysfunction is defined as a substantial and persistent decline (≥20%) in forced expiratory volume in 1 s (FEV_1) relative to the reference FEV_1 [2]. FEV_1 is the average of the two best postoperative FEV_1 readings, taken at least 3 weeks apart [3], after excluding other pulmonary and extrapulmonary causes for FEV_1 decline. The most common manifestation of CLAD is airflow limitation caused by BOS. Previously, the diagnostic criteria for CLAD were applied to BOS. Now, BOS is defined by a decline of FEV1 (≥20%) from the previous baseline, a ratio of FEV1 to forced vital capacity <0.7, and no opacities on chest imaging [4]. According to a recent report, the median time from BOS onset to death or retransplantation was 500 days [5]. Although BOS was first introduced in 1993 [3] and clinically defined in 2003 [6], the therapeutic options for this condition remain limited and lack a clearly established protocol. A consensus guideline for the diagnosis and treatment of BOS published in 2014 evaluated the existing literature and used the Grading of Recommendations, Assessment, Development, and Evaluation system to demonstrate that most BOS therapies were inadequate [7]. Thus, developing more effective treatment options for patients with BOS is crucial.

The enhancer of zeste homolog 2 (EZH2), which methylates histone H3 on lysine 27 (H3K27me3), regulates cellular differentiation via histone methylation [8]. Different types of EZH2 inhibitors have been developed [9], and some are already used in clinical practice to treat malignancies, including B-cell lymphoma [10]. The importance of EZH2-targeted treatment in managing acute and chronic rejection post-transplantation is increasingly recognized. For instance, EZH2 inhibition has been shown to suppress acute renal allograft rejection in a rat model [11]. Additionally, Zaiken et al. reported that EZH2 inhibition improved pulmonary function in a chronic graft-versus-host disease mouse model with bronchiolitis obliterans [12]. In these studies, inhibiting or deleting EZH2 was shown to suppress the differentiation and functions of immune cells, especially T cells. Given the critical roles of inflammatory reactions in the acute phase and immune rejection in the chronic phase in triggering BOS formation [13], we hypothesized that inhibiting EZH2 could comprehensively control these reactions and suppress BOS.

3-deazaneplanocin A (DZNep) is an inhibitor of S-adenosylhomocysteine hydrolase that inhibits H3K27 methylation and the activity of EZH2 [10]. Although DZNep has not yet been applied clinically in humans, it has garnered attention for its various potential benefits [14]. In this study, we aimed to assess the effects of DZNep-mediated

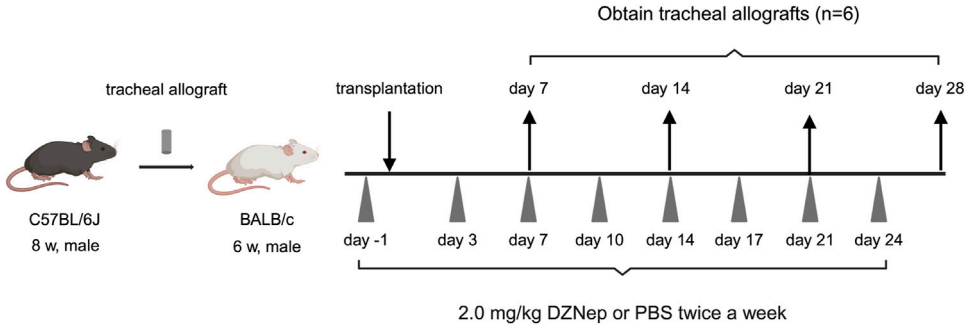


FIGURE 1 | A schematic overview of the murine heterotopic tracheal transplant model experiments. Briefly, recipient mice were intraperitoneally injected with either 2.0 mg/kg DZNep suspended in 400 μL of PBS or 400 μL of PBS alone on days −1, 3, 7, 10, 14, 17, 21, and 24. Tracheal allografts were harvested on the indicated days after transplantation. DZNep, 3-deazaneplanocin A; PBS, phosphate-buffered saline.

EZH2 inhibition on BOS using a murine heterotopic tracheal transplant (HTT) model.

MATERIALS AND METHODS

Induction of the Murine BOS Model

Male 8-week-old C57BL/6 J and 6-week-old BALB/c mice were purchased from Kyudo Ltd. Animals were housed in a specific pathogen-free facility at Kyushu University, Japan. All mice received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health. The Institutional Review Board approved the animal experiments (No. A22-291-0). A well-established HTT model was used in this study (**Figure 1**) [15–17]. Briefly, donor mice were anesthetized with isoflurane, and euthanized by cervical dislocation. After being placed in a supine position, a midline cervical incision was made to expose the entire trachea. Tracheal allografts were taken from the first tracheal ring to the carina, such that each tracheal segment was >8 mm. Tracheal allografts were suspended in phosphate-buffered saline (PBS) and stored on ice until implantation. The recipient mice were anesthetized using isoflurane. Donor tracheal allografts were transplanted into a subcutaneous pouch created on the dorsal site of the recipient mice. The skin was closed with sutures.

DZNep was purchased from Nacalai Tesque (Kyoto, Japan). To evaluate the role of EZH2 inhibition in BOS, recipient mice were intraperitoneally injected with 2.0 mg/kg DZNep suspended in 400 μL of PBS 1 day before transplantation and subsequently on days 3, 7, 10, 14, 17, 21, and 24 post-transplantation (twice a week). The dosing regimen for DZNep was established based on previous studies [18, 19], which confirmed the absence of serious adverse events in the test administrations. Control mice received the same frequency and dose of PBS intraperitoneally as the DZNep mice to ensure comparable conditions.

Grafts were obtained on days 7, 14, 21, and 28 post-transplantation (n = 6 per group). All the grafts were cut in half. Half of the tracheae were fixed in 4% paraformaldehyde for 24 h at room temperature for histopathological assessment, whereas the other half were stored as frozen specimens and used for cytokine expression by flow cytometry.

Histopathologic Evaluation

Formalin-fixed tissues were paraffin-embedded and cut into 5-μm sections. The slides were stained with hematoxylin and eosin and Masson's trichrome. The extent of luminal obstruction in the trachea was calculated using Masson's trichrome staining, according to the following formula: (area obstructed by fibrotic tissue)/(area within the cartilage) × 100%. The obstruction ratio was assessed using ImageJ 1.50 software (National Institutes of Health, Bethesda, MD, United States).

Quantification of Cytokine Production

Frozen tracheal allografts were homogenized in radioimmunoprecipitation assay buffer containing sodium dodecyl sulfate (Nacalai Tesque, Kyoto, Japan). The concentrations of interleukin (IL)-2, IL-4, IL-6, interferon (IFN)-γ, tumor necrosis factor (TNF), IL-17A, and IL-10 in the allografts were simultaneously measured using the BD Cytometric Bead Array Mouse Th1/Th2/Th17 Cytokine Kit (560485; BD Biosciences, San Diego, CA, United States) on a FACSuite flow cytometer (BD Biosciences). The assays were performed and analyzed by a single operator using CBA Analysis Software 1.1.14.

Immunohistochemical Staining

Immunohistochemical staining for EZH2, CD8, and CD4 was performed on 4-μm formalin-fixed and paraffin-embedded tissues according to the manufacturer's instructions. The sections were deparaffinized, blocked with 10% normal goat serum, and incubated with the following primary polyclonal antibodies at 4°C overnight: rabbit monoclonal anti-EZH2 antibody (1:100 dilution, Abcam, Cambridge, United Kingdom), rabbit monoclonal anti-CD8 (1:2000 dilution, Abcam), and rabbit monoclonal anti-CD4 (1:1,000 dilution, Abcam). The immune complexes were detected using the Dako EnVision Detection System (Dako, Glostrup, Denmark). Finally, the sections were

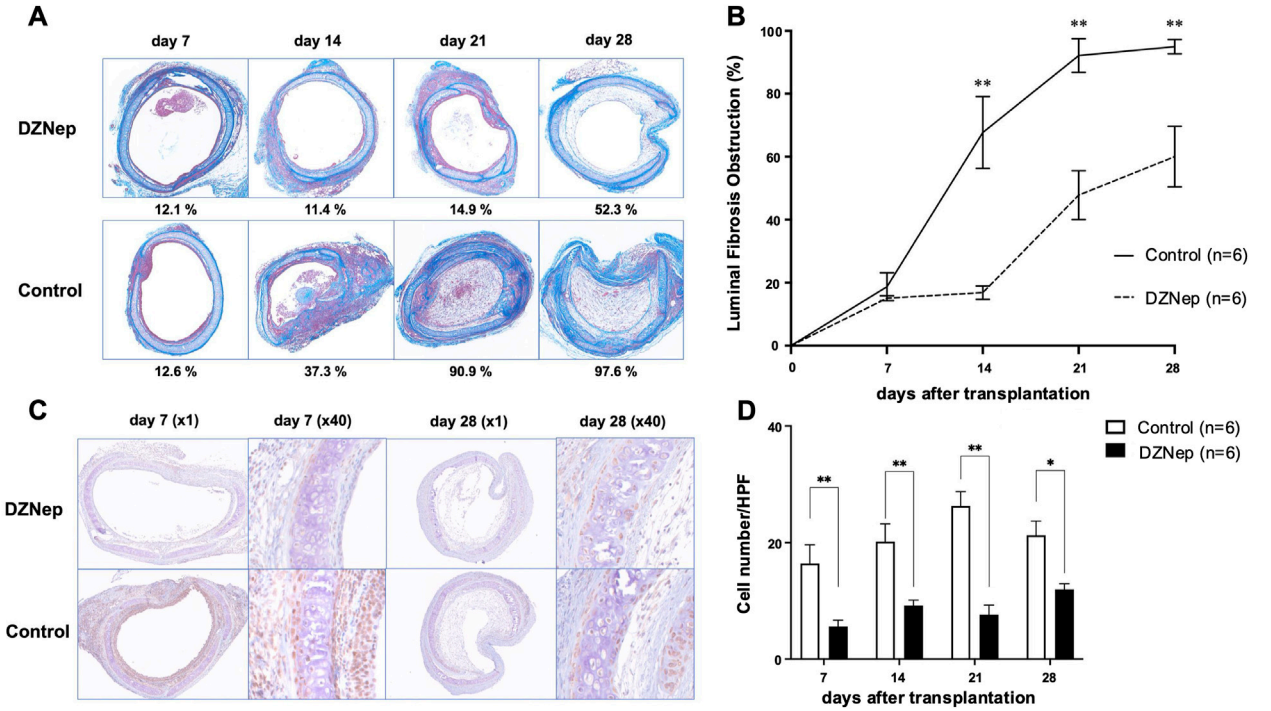


FIGURE 2 | DZNep treatment reduces the extent of tracheal lumen occlusion following transplantation. **(A)** Representative Masson's trichrome staining images of the pathological features of heterotopically transplanted tracheal allografts in the DZNep and control groups on days 7, 14, 21, and 28 after transplantation (original magnification, ×100). **(B)** Analysis of luminal fibrous occlusion in the DZNep and control groups (n = 6 per group) at the indicated timepoints. **(C)** Representative images of EZH2 staining in heterotopically transplanted tracheal allografts from the DZNep and control groups on days 7 and 28. **(D)** The number of EZH2-positive cells per high-powered field (×1,000), averaged across at least five fields and six mice (per group). Data represent the mean ± standard error of the mean. DZNep, 3-deazaneplanocin A; EZH2, enhancer of zeste homolog 2. *$p < 0.05$; **$p < 0.01$.

treated with 3,3-diaminobenzidine, counterstained with hematoxylin, and mounted. EZH2 protein levels were measured by counting the number of EZH2-positive cells in each high-power field (×1,000) and averaging over at least five fields per graft, with six mice per group. The extent of $CD8^+$ and $CD4^+$ T-cell infiltration was evaluated by counting the number of $CD8^+$ and $CD4^+$ cells in each high-power field (×1,000) and averaging over at least five fields per graft and six mice per group.

Statistical Analysis

Data were expressed as mean ± standard error of the mean. One-way analysis of variance was used to compare multiple groups, and Student's t-test was used to compare two groups. Differences were considered statistically significant if the p-value was <0.05. All analyses were conducted using the JMP® 16.0 software (SAS Institute, Cary, NC, United States).

RESULTS

Luminal Fibrous Occlusion in Untreated Allografts After HTT

Initially, we observed the extent of luminal fibrous occlusion in HTT allografts (**Figure 2A**). Infiltration of inflammatory cells into the epithelial layer was observed 7 days after transplantation. By day 14, inflammatory cells had reached the tracheal lumen, leading to fibrosis. On day 21, the lumen was almost completely occluded by fibrous connective tissue and extensive inflammatory cell infiltration was observed. The tracheal lumen was occluded on day 28. The mean obstruction ratios on days 7, 14, 21, and 28 were 20.40% ± 3.62%, 67.68% ± 11.46%, 92.15% ± 5.38%, and 94.97% ± 2.28%, respectively (**Figure 2B**).

DZNep Treatment Reduces EZH2 Protein Levels and Alleviates Tracheal Luminal Fibrous Occlusion

To investigate whether EZH2 is implicated in BOS pathogenesis, we administered DZNep to recipient mice (**Figure 2A**). The obstruction ratios of the DZNep group and the control group on days 7, 14, 21, and 28 were 15.1% ± 0.8% vs. 20.4% ± 3.6% ($p = 0.996$), 16.9% ± 2.1% vs. 67.7% ± 11.5% ($p < 0.001$), 47.8% ± 7.8% vs. 92.2% ± 5.4% ($p < 0.001$), and 60.0% ± 9.6% vs. 95.0% ± 2.3% ($p < 0.001$), respectively (**Figure 2B**). Thus, DZNep significantly reduced the obstruction ratio of the trachea transplanted into HTT model mice.

The protein levels of EZH2 were examined using immunohistochemical staining to confirm the reduced

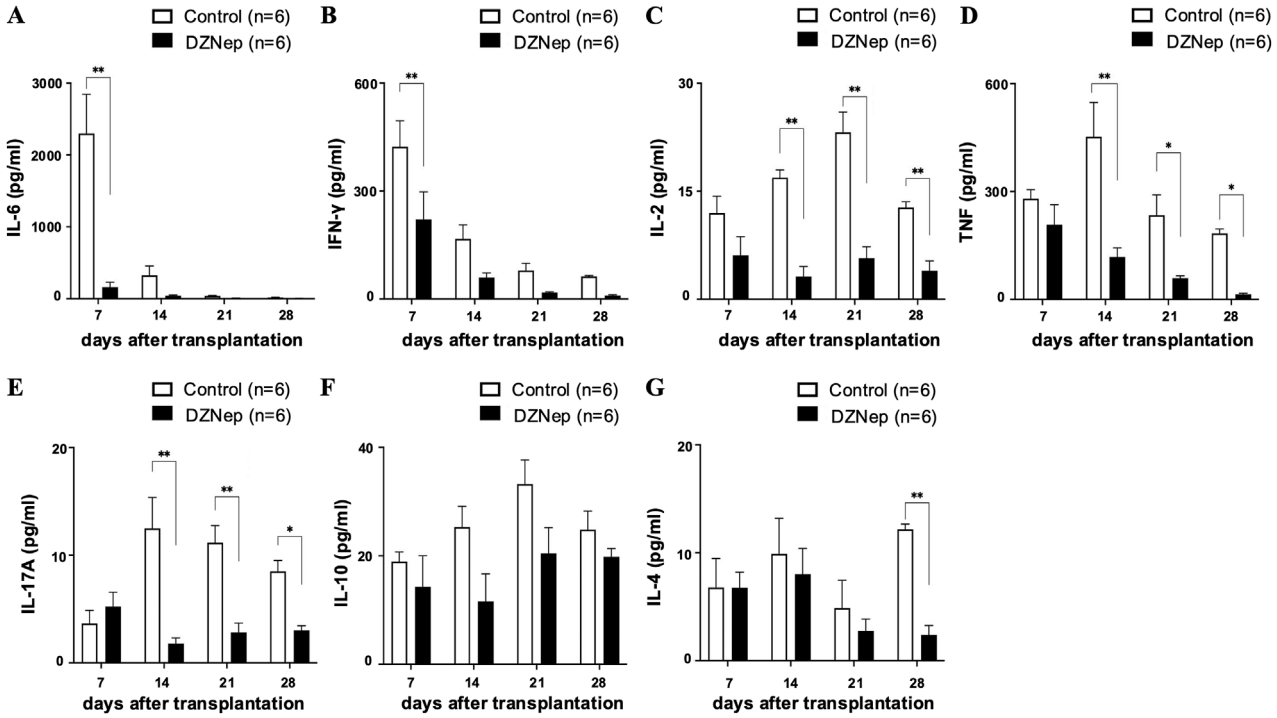


FIGURE 3 | DZNep treatment modifies cytokine levels in tracheal allografts. Analysis of IL-6 **(A)**, IFN-γ **(B)**, IL-2 **(C)**, TNF **(D)**, IL-17A **(E)**, IL-10 **(F)**, and IL-4 **(G)** concentrations in the DZNep and the control groups after heterotopic tracheal transplantation (n = 6 per group). Data represent the mean ± standard error of the mean. DZNep, 3-deazaneplanocin A. IFN-γ, interferon-gamma; IL, interleukin; TNF, tumor necrosis factor. *$p < 0.05$; **$p < 0.01$.

expression of EZH2 in the transplanted trachea following DZNep administration (**Figure 2C**). EZH2 protein levels were significantly lower in the DZNep treatment group than in the control group on days 7 (5.6 ± 1.1/HPF vs. 16.4 ± 3.2/HPF; p = 0.004), 14 (9.2 ± 0.9/HPF vs. 20.2 ± 3.1/HPF; p = 0.004), 21 (7.6 ± 1.6/HPF vs. 26.3 ± 2.5/HPF; $p < 0.001$), and 28 (12.0 ± 1.0/HPF vs. 21.2 ± 2.5/HPF; p = 0.017) (**Figure 2D**).

DZNep Treatment Alters Cytokine Levels in Tracheal Allografts

Since inflammatory reactions in the acute phase and immune rejection in the chronic phase are considered key triggers of BOS formation, we measured cytokine levels in frozen tracheal allografts on days 7, 14, 21, and 28 post-transplantation. The levels of IL-6 and IFN-γ (two cytokines which exert pleiotropic effects on inflammation and immune responses [20, 21]) were significantly lower in the DZNep group than in the control group on day 7 (IL-6: 160.0 ± 67.3 pg/mL vs. 2298.1 ± 546.4 pg/mL; $p < 0.001$, IFN-γ: 221.0 ± 76.6 pg/mL vs. 422.5 ± 72.7 pg/mL; p = 0.005) (**Figures 3A, B**). Meanwhile, the levels of IL-2 (a proinflammatory cytokine which is produced by T helper cell 1 (Th1) lymphocytes and is a potent activator of T cells and natural killer cells [22]) were significantly lower in the DZNep group than in the control group on days 14 (3.2 ± 1.4 pg/mL vs. 16.9 ± 1.1 pg/mL; $p < 0.001$), 21 (5.7 ± 1.6 pg/mL vs. 23.2 ± 2.8 pg/mL; $p < 0.001$), and 28 (4.0 ± 1.3 pg/mL vs. 12.8 ± 0.8 pg/mL; p = 0.008) (**Figure 3C**). The levels of TNF (a pro-inflammatory cytokine that promotes the activation of Th1 lymphocytes, neutrophils, and macrophages [22, 23]) were also significantly reduced in the DZNep group vs. the control group on days 14 (118.2 ± 25.2 pg/mL vs. 452.2 ± 95.6 pg/mL; $p < 0.001$), 21 (59.3 ± 6.6 pg/mL vs. 234.5 ± 56.4 pg/mL; p = 0.040), and 28 (14.0 ± 3.0 pg/mL vs. 183.4 ± 12.7 pg/mL; p = 0.050) (**Figure 3D**). The levels of IL-17A (a pro-inflammatory cytokine produced by T helper 17 (Th17) cells that promotes the migration of inflammatory cells [21]) were also lower in the DZNep group than in the control group on days 14 (1.8 ± 0.5 pg/mL vs. 12.5 ± 2.9 pg/mL; $p < 0.001$), 21 (2.8 ± 0.9 pg/mL vs. 11.2 ± 1.6 pg/mL; $p < 0.001$), and 28 (3.6 ± 0.2 pg/mL vs. 8.5 ± 1.0 pg/mL; p = 0.039) (**Figure 3E**).

We found no significant difference in the levels of IL-10 (an anti-inflammatory cytokine [24]) between the DZNep and control groups at any of the timepoints (**Figure 3F**). However, the production of IL-4 (a cytokine with an important role in T helper 2 (Th2) cell differentiation [21]) was significantly suppressed on day 28 in the DZNep group vs. the control group (2.4 ± 0.9 pg/mL vs. 12.2 ± 0.5 pg/mL; p = 0.008) (**Figure 3G**).

DZNep Reduces T Lymphocyte Infiltration Into the Allograft

Immunohistochemical staining for CD8 and CD4 was performed on the tracheal allografts to determine changes in the distribution of T lymphocytes after transplantation. The numbers of $CD8^+$

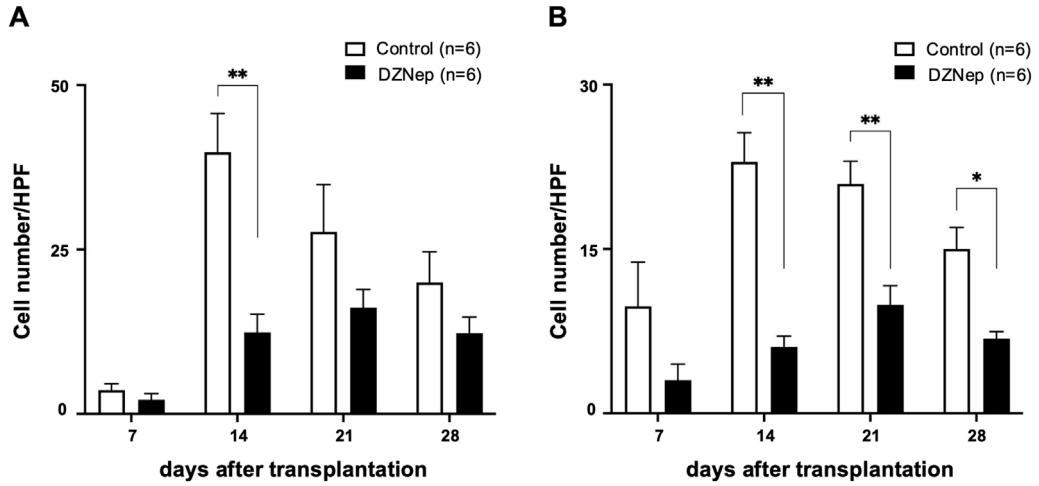


FIGURE 4 | Effect of EZH2 inhibition on T cell infiltration. The number of $CD8^+$ **(A)**, and $CD4^+$ **(B)** T cells per high-powered field (×1,000) averaged across at least five fields and six mice (per group). Data represent the mean ± standard error of the mean. EZH2, enhancer of zeste homolog 2. $^*p < 0.05$; $^{**}p < 0.01$.

and $CD4^+$ T lymphocytes infiltrating the allografts were significantly lower in the DZNep group than in the control group on day 14 (CD8: 12.4 ± 2.8/HPF vs. 39.8 ± 5.9/HPF; $p < 0.001$, CD4: 6.1 ± 1.0/HPF vs. 22.9 ± 2.7/HPF; $p < 0.001$) (**Figures 4A, B**). Although no significant difference was observed in the number of infiltrating $CD8^+$ T lymphocytes between the groups at the later timepoints, the number of $CD4^+$ T cells was significantly lower on days 21 (9.9 ± 1.7/HPF vs. 20.9 ± 2.1/HPF; $p = 0.004$) and 28 (6.8 ± 0.6/HPF vs. 15.0 ± 2.0/HPF; $p = 0.045$) owing to DZNep treatment (**Figure 4B**).

DISCUSSION

Lung transplantation is the most effective treatment for patients with severe or terminal lung diseases. However, despite clinical advances, the outcomes of lung transplantation remain worse than those of other solid organ transplantations. The overall survival of lung transplantation patients at 5 years is only approximately 50%–70% [25, 26]. Obliterative bronchiolitis (OB) is a pathological condition characterized by airflow limitation due to scarring or the filling of the airway lumen with a collagen matrix. BOS is a clinical syndrome with the pathogenesis of OB [27]. For patients with BOS who died or underwent retransplantation, the median time from BOS onset to death or retransplantation was 500 days [5]. This duration varies depending on the time of BOS onset [28, 29]. The pathogenesis of BOS has three stages. In the first phase, immune and/or non-immune factors (such as acute rejection and lymphocytic bronchiolitis) damage the airway epithelium. Subsequently, infiltrating immune cells are stimulated to produce various cytokines and chemokines, initiating an inflammatory cascade. Finally, persistent inflammation drives bronchiolar tissue remodeling, resulting in fibrosis and airway lumen occlusion [13, 30]. Controlling inflammatory reactions during the acute phase is crucial for reducing the pathogenesis of BOS.

In this study, we used an HTT model (which was first developed for the investigation of OB in 1993 [16]) to investigate the effects of EZH2 on BOS. In this model, tracheal allografts follow a process similar to that of OB, with lumen closure. The process initially involves the loss of epithelial cells, which is followed by the induction of inflammatory cells in the grafts [31, 32]. Excessive fibroblast/myofibroblast proliferation results in total occlusion of the intratracheal region [17]. Since the HTT model is suitable for investigating immunological changes, epithelial damage/regeneration, and fibrosis [33], as noted above, we used this model to observe whether EZH2 inhibition could prevent inflammatory cell infiltration and fibrotic obstruction.

EZH2 has important roles in the regulation of various cellular functions, including development and differentiation [8, 34]. One of its functions is to promote the differentiation and infiltration of inflammatory cells by activating the signal transducer and activator of transcription 3 (STAT3) [35]. Zhang et al. reported that inhibiting the STAT3 signaling pathway by blocking EZH2 reduces inflammatory cell infiltration and cytokine release in a cecal ligation and puncture mouse model [36]. Consistent with these findings, our study suggests that EZH2 plays an important role in the pathogenesis of BOS by inducing pro-inflammatory cytokines production and T-lymphocyte infiltration during both the early and late post-transplantation periods (**Figure 5**). Specifically, we showed that DZNep treatment significantly suppressed the release of IL-6 and IFN-γ on day 7 post-transplantation, and the production of other pro-inflammatory cytokines (including IL-2, TNF, and IL-17A) on days 14, 21, and 28. DZNep administration also reduced the infiltration of $CD8^+$ and $CD4^+$ T lymphocytes into the allograft, with peak suppression observed at 14 days post-transplantation. We demonstrated that EZH2 inhibition prevented the inflammatory reactions triggered by the release of pro-

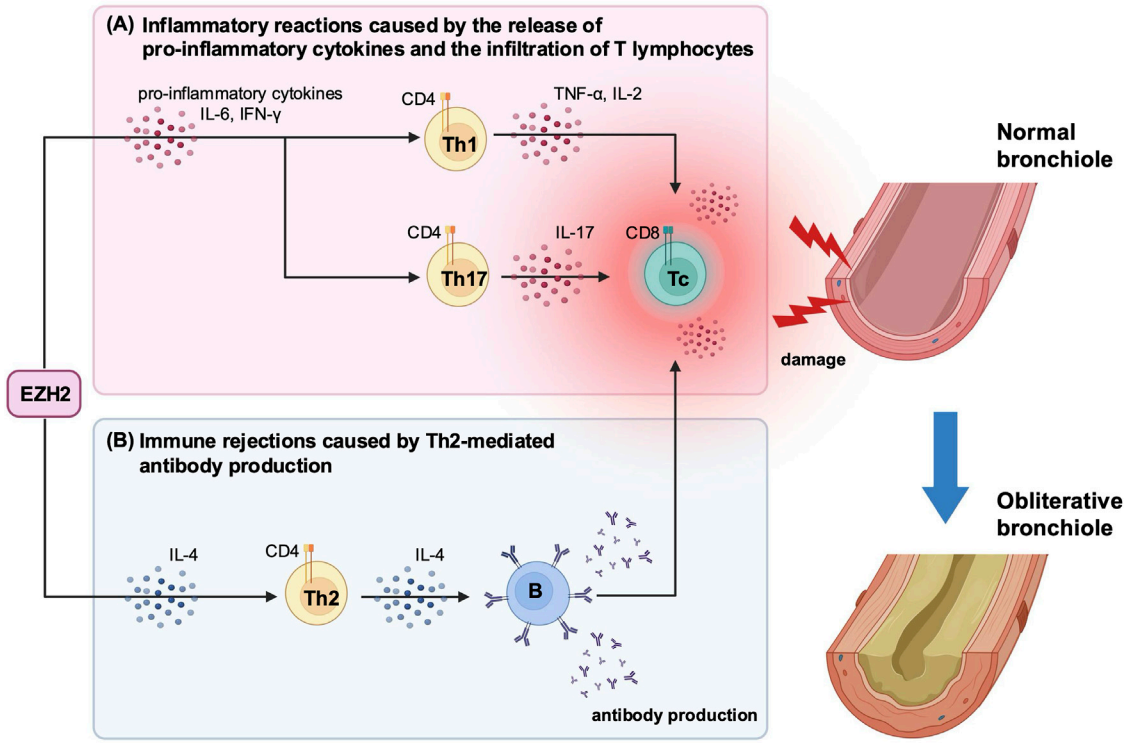


FIGURE 5 | A schematic diagram illustrating the immunomodulatory effects of EZH2-mediated cytokine production following heterotopic trachea transplantation. **(A)** In the early post-transplantation period, EZH2 promotes the production of pro-inflammatory cytokines, including IL-6 and IFN-γ. These cytokines stimulate further release of pro-inflammatory cytokines and the infiltration of T lymphocytes. **(B)** In the late post-transplantation period, EZH2 sustains the production of IL-4, which drives Th2-mediated antibody production. This antibody production induces the recruitment of immune cells, leading to graft dysfunction and tissue damage. These inflammatory reactions and immune rejections are key triggers for BOS development. DZNep-mediated EZH2 inhibition significantly suppresses the production of both pro-inflammatory cytokines and IL-4 post-transplantation, which may reduce the risk of developing BOS and protect the graft from immune-mediated damage. BOS, bronchiolitis obliterans syndrome; DZNep, 3-deazaneplanocin A; EZH2, enhancer of zeste homolog 2, IFN-γ, interferon-gamma; IL, interleukin; Th, T helper cell; TNF, tumor necrosis factor.

inflammatory cytokines and T cell infiltration, potentially protecting the allograft in the early post-transplantation period. Although not significant, $CD8^+$ T lymphocytes were reduced on days 21 and 28, and pro-inflammatory cytokine levels were significantly low after day 21. These findings may contribute to long-term allograft survival.

Multiple cytokines are implicated in OB development, among which the role of IL-17 in BOS has been reported by many studies [37, 38]. For instance, IL-17 participates in the pathogenesis of OB by regulating macrophage polarization in a murine HTT model [37]. Meanwhile, blocking IL-17A reduces the overall IFN-γ-mediated lymphocyte response and decreases the likelihood of OB development [38]. Furthermore, IFN-γ alone appears to be closely associated with airway inflammation and fibrosis following lung transplantation [39, 40]. Elevated IL-6 concentrations are also correlated with BOS [41]. Thus, the therapeutic effect of EZH2 inhibition likely stems from its role as an epigenetic regular, which leads to increase BOS-associated pro-inflammatory cytokine production (**Figure 5**).

In this study, IL-4 levels were significantly suppressed in the DZNep group on day 28. Additionally, the number of $CD4^+$ T lymphocytes was significantly reduced in the DZNep group after day 14. These results suggest that DZNep treatment may suppress Th2-mediated antibody production during the late post-transplantation period. Similar to these findings, several studies have reported that EZH2 both directly and indirectly regulates antibody production from B cells through Th2 cells [42, 43]. Antibody binding triggers both complement-dependent and complement-independent recruitment of immune cells, which can lead to graft dysfunction and tissue damage post-transplantation [44]. Antibody-mediated rejection (AMR) is also recognized as a predictor of CLAD development, with new therapies aimed at reducing AMR risk currently under investigation [4]. The suppression of antibody production through DZNep-mediated EZH2 inhibition could prevent Th2-mediated immune rejection in the chronic phase and enhance long-term allograft survival, although further validation is needed.

To the best of our knowledge, this is the first study to demonstrate the potential of DZNep-mediated EZH2 inhibition in BOS. DZNep has several advantages over traditional immunosuppressive agents such as cyclosporine A

and tacrolimus. First, it exhibits broad-spectrum and potent antiviral activity, including against human cytomegalovirus [45, 46], which causes serious infections in immunocompromised transplant recipients [46]. Second, pharmacological EZH2 inhibition by DZNep is associated with beneficial therapeutic effects in several cancers [9, 47]. Given that the long-term use of immunosuppressive agents after transplantation increases the risk of malignancy, the antitumor effects of DZNep should not be overlooked. Thus, DZNep is a promising therapeutic agent for organ transplantation; nevertheless, its efficacy and safety warrant further investigation in clinical studies.

The murine HTT model used in this study has some shortcomings. Notably, this model lacks blood vessels and an interface with air. Although a single-lung transplant mouse model has been successfully created [48, 49], we used an HTT model in this study due to its superiority in terms of high OB reproducibility. Moreover, another reason for adopting this model was the possibility to observe pathological changes in a short period following a simple and easy procedure [33]. However, the role of EZH2 in BOS pathogenesis requires further validation using an alternative model, such as a murine orthotopic transplant model.

Conclusively, we used a murine HTT model to demonstrate that EZH2 plays an important role in BOS pathogenesis. Our findings demonstrate that DZNep-mediated EZH2 inhibition reduces inflammation by suppressing the release of pro-inflammatory cytokines and T cell infiltration during both early and late post-transplantation periods, ultimately reducing the severity of BOS. Collectively, our preclinical results imply that DZNep holds promise as a therapeutic agent for lung transplantation; however, its efficacy and safety must be further validated through rigorous clinical testing.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was approved by Kyushu University Institutional Review Board concerning laboratory animal use and care (No. A22-291-0). The study was conducted in accordance with the local legislation and institutional requirements.

AUTHOR CONTRIBUTIONS

KM participated in conceptualization, investigation, data curation, formal data analysis, and writing-original draft. ST participated in conceptualization, methodology, data curation, writing-original draft, and writing - review and editing. TT participated in supervision and writing-review and editing. MS participated in formal data analysis. AH, TN, FK, TA, and MK participated in data curation. GT participated in conceptualization. TY participated in project administration, resources, and writing-review and editing. All authors contributed to the article and approved the submitted version.

FUNDING

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

CONFLICT OF INTEREST

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

ACKNOWLEDGMENTS

We would like to thank Editage (www.editage.jp) for English language editing.

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