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Transplant International

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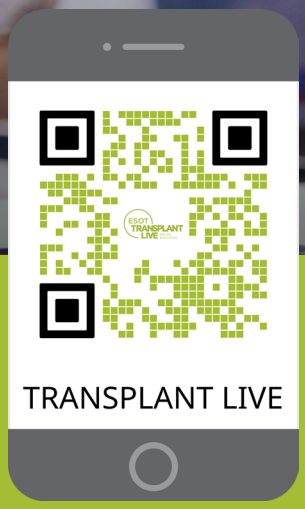
EDUCATION COMMITTEE ESOT

TRANSPLANT LIVE

ONLINE EDUCATION



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ESOT TLJ3.0
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PRAGUE, 13-15 NOV 2022

Consensus Conference

At TLJ 3.0, participants will be introduced to nine key transplantation topics following a systematic review. Delegates will convene for three days of high-quality debate, discussion and exploration in order to finalise a series of consensus reports that will be submitted for publication.

MACHINE PERFUSION IN CARDIOTHORACIC TRANSPLANTATION

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PREHABILITATION FOR SOLID ORGAN TRANSPLANT CANDIDATES

MOLECULAR BIOLOGY TESTING FOR NON-INVASIVE DIAGNOSIS OF ALLOGRAFT RE

and

EDUCATIONAL WORKSHOP: GUIDELINES DEVELOPMENT

#ESOTTLJ

ECTORS
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ROTTERDAM

The Netherlands

3rd ECTORS meeting

29-30 September 2022

#ESOT_ECTORS #ECTORSmeeting

learn more

The main topics for 3rd ECTORS meeting will be:

- Stem cells
- organoids
- machine perfusion
- regeneration

Learning Objectives:

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

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

ESOT | Transplant International | THE PETER MORRIS CENTRE FOR EVIDENCE IN TRANSPLANTATION

TRANSPLANT TRIAL WATCH
published: 01 June 2022
doi: 10.3389/ti.2022.10580

Transplant Trial Watch

Simon R. Knight[1,2]*

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Keywords: heart transplantation, perfusion, attitudes, HCV, donor

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

Clinician and Patient Attitudes Toward Use of Organs From Hepatitis C Viremic Donors and Their Impact on Acceptance: A Contemporary Review.

by Fleetwood V. A., et al. Clinical Transplantation. 2021; 35 (12):e14519.

Aims

This study aimed to evaluate the attitudes of clinicians and patients regarding the use of organs from hepatitis C viremic donors and their impact on acceptance.

Interventions

A literature search was conducted on PubMed, MEDLINE, and SCOPUS. Studies were selected for inclusion by two independent reviewers. Data were extracted by the primary author.

Participants

8 studies were included in the review.

Outcomes

The outcomes of interest included knowledge of HCV-specific outcomes, HCV-specific concerns, willingness to accept viremic organs and factors that contributed to acceptance or non-acceptance.

Follow-up

Not applicable.

CET Conclusion

This is an interesting review concerning patient attitudes towards receiving organs from Hepatitis C positive donors. Multiple databases were searched, and papers were assessed in

Transplant Trial Watch

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duplicate, although one author conducted the data extraction. Eight articles were included (6 survey questionnaires, 1 semi-structured interview and 1 conjoint analysis). The paper provides a narrative review of the included articles, summarised in key themes. The authors have done well to summarise a difficult topic and provide a synthesis of the included studies. There is however no assessment of the quality of the included papers.

Funding Source
No funding was received for this study.

RANDOMISED CONTROLLED TRIAL 2

Long-Term Outcomes After Heart Transplantation Using *Ex Vivo* Allograft Perfusion in Standard Risk Donors: A Single-Center Experience.

by Chen Q., et al. Clinical Transplantation 2022; e14591.

Aims
This study aimed to assess the long-term outcomes of heart transplant patients that received allografts preserved using the Organ Care System (OCS) versus standard cold storage (CS).

Interventions
Participants were randomised to receive allografts preserved with either CS or OCS.

Participants
38 heart transplant candidates.

Outcomes
The primary outcomes were 8-year overall survival and freedom from cardiac allograft-related death up to 8 years. Secondary outcomes were 8-year freedom from cardiac allograft vasculopathy (CAV), freedom from non-fatal major adverse cardiac events and freedom from rejections.

Follow-up
8 years.

CET Conclusions
This paper reports the long-term outcome from hearts randomised in the randomised PROCEED II study at a single centre. Previous publications of the PROCEED II study have already shown non-inferior short-term outcomes comparing perfusion on the OCS device to cold storage on ice. The study as a whole included 130 patients randomised in a 1:1 fashion, and this single-centre follow up reports on only 38. As such, this latest report is underpowered to identify all but the most obvious of clinical differences and the authors acknowledge this limitation. Follow-up in this cohort of 38 was acceptable, at 92%, which equates to 3 lost-to follow up. Recipients in the cold-storage arm were significantly older, by 8 years. There was no significant difference in overall survival at median follow up of 8.4 years and no difference in cardiac allograft vasculopathy. The study outcomes should be viewed in the context of a highly selected donor and recipient population, with any potential benefits more likely to show themselves when using extended criteria donors.

Trial Registration
ClinicalTrials.gov—NCT00855712.

Funding Source
Non-industry funding.

CLINICAL IMPACT SUMMARY

Utilisation of deceased donor cardiothoracic organs is typically lower than those of abdominal organs (1). This has led to interest in methods for *ex-vivo* preservation and viability assessment, which have the potential to prolong preservation times, recondition organs and improve outcomes by allowing assessment prior to transplantation.

The first randomised controlled trial of normothermic *ex-vivo* cardiac preservation (PROCEED II) was reported in the Lancet in 2015 (2). The study randomised 130 transplant recipients to receive a heart either stored using conventional static cold storage (SCS) or preserved using the Organ Care System (OCS) perfusion device. The authors reported non-inferiority of perfused hearts, with no measurable difference in patient or graft survival despite longer overall preservation times in the OCS group. Of note, 5 hearts were discarded due to preservation parameters in the OCS group, but despite the potential advantages of discarding suboptimal organs, there was no measured clinical benefit (3). All hearts in the study had to be suitable for either arm and were relatively low-risk, meaning that any impact on organ utilisation cannot be assessed.

In a recent paper published in Clinical Transplantation, Chen et al. report long-term outcomes in 38 patients from a single participating centre from the trial (4). Eight-year survival was numerically lower in the OCS group (57.9% vs. 73.7%, $p = 0.24$) but not meeting statistical significance in this small sample. The apparent excess mortality in the OCS group seemed mainly related to events that are difficult to attribute to the preservation method (e.g., CMV infection or malignancy), supported by a lack of difference in the rate of graft-related mortality (84.2% in both groups).

In contrast to the survival data, there was numerically higher freedom from coronary allograft vasculopathy (CAV; 89.5% vs. 67.8%) and non-fatal major cardiac events (89.5% vs. 67.5%) in the OCS group. Differences in CAV rate may relate to the shorter cold-ischaemic times in the OCS group, reducing ischaemia reperfusion injury.

Overall, the small sample size means that firm conclusions are difficult to draw and this study is unlikely to have a

significant impact on clinical practice. It would perhaps have been more useful to compile long-term outcomes from all patients in the original study to increase statistical power and see if the trends seen here were borne out in other centre's data.

Use of the OCS device is feasible and likely safe, but there is limited evidence of clinical benefit in standard-risk hearts. Whether *ex-vivo* perfusion will have a greater utility in preservation and viability assessment of hearts from more marginal donors remains to be seen.

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2. Ardehali A, Esmailian F, Deng M, Soltesz E, Hsich E, Naka Y, et al. *Ex-Vivo* Perfusion of Donor Hearts for Human Heart Transplantation (PROCEED II): A Prospective, Open-Label, Multicentre, Randomised Non-Inferiority Trial. *The Lancet* (2015) 385:2577–84. doi:10.1016/s0140-6736(15)60261-6
3. Freed DH, White CW. Donor Heart Preservation: Straight Up, or on the Rocks? *The Lancet* (2015) 385:2552–4. doi:10.1016/s0140-6736(15)60614-6

AUTHOR CONTRIBUTIONS

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

CONFLICT OF INTEREST

SK has received consultancy fees from OrganOx Ltd for research design in the past.

4. Chen Q, Singer-Englar T, Kobashigawa JA. Long-Term Outcomes After Heart Transplantation Using *Ex Vivo* Allograft Perfusion in Standard Risk Donors: A Single-Center Experience. *Clin Transplant* (2022) 36: e14591. doi:10.1111/ctr.14591

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ORIGINAL RESEARCH
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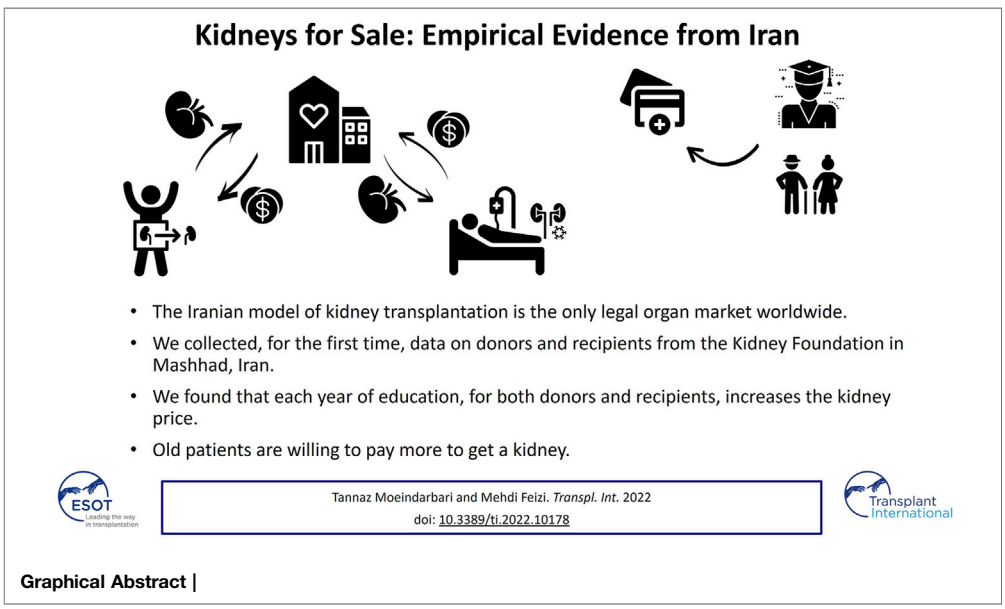
Kidneys for Sale: Empirical Evidence From Iran

*Tannaz Moeindarbari and Mehdi Feizi**

Department of Economics, Faculty of Economics and Administrative Sciences, Ferdowsi University of Mashhad, Mashhad, Iran

The kidney market in Iran is the only legal market of this sort globally. Yet, it has not been empirically studied based on real data. For the first time, we obtained data on donors and recipients from the Kidney Foundation in Mashhad, April 2011 up to March 2018, and assessed which individualistic characteristics contribute to a kidney's price. Our findings indicate that each year of education for both donors and recipients increases the kidney price. Moreover, old patients are willing to make a higher payment to young vendors. We have also provided some policy implications to improve the efficiency of kidney allocations.

Keywords: live kidney transplantation, kidney market, organ sales, compensated donation, Iranian model of kidney transplantation



Graphical Abstract |

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Abbreviations: DoI, Declaration of Istanbul; ESRD, End Stage Renal Disease; IMKT, Iranian Model of Kidney Transplantation; KF, Kidney Foundation.

INTRODUCTION

The insufficient philanthropic supply of organs has led to a significant organ shortage, mounting transplant waiting lists, and many renal patients losing their lives throughout the world. None of the new approaches to increasing the kidney donor pool in developed countries, such as developing deceased donation, introducing kidney exchange programs, and optimizing the allocation algorithms, have been successful in eliminating the drastic shortage of transplantable kidneys. Nevertheless, market-based arrangements to increase donations of human organs are broadly considered unacceptable from ethical perspectives and are therefore not relevant in almost all countries (1).

Since kidney markets are illegal everywhere, except Iran, there is very little known about the consequences of such a market. This paper studies the monetary market for kidneys in Mashhad, the second-largest kidney market in Iran, after the market in Tehran. Our analysis is based on a unique inclusive dataset of this market for about 7 years. For the very first time to the best of our knowledge, we assess which individualistic characteristics and institutional factors could explain a realized price of a kidney. We shed light on its several socio-economic aspects and provide evidence that gives readers a better understanding of how a monetary market for organs could work and its pros and cons.

A kidney market can considerably release patients from suffering under dialysis, increase their lifetimes, and cut healthcare costs. Nevertheless, such a market creates some ethical concerns, and our analysis should not be seen as an authorization for it. Many opponents of a market for kidneys are concerned that the two sides of the market are divided by wealth, where the majority of buyers are the rich, and most sellers are the poor who sell their kidneys because they desperately and sometimes urgently need money. That is why some opponents argue that a market for organs can be coercive (2).

However, we should note that as a kidney market had not made potential donors poor, it should not be blamed for that. Such a market provides a costly signal, i.e., selling one's kidney, that make desperate poor people visible. Therefore, a kidney market could even provide a truthful mechanism to distinguish poor people and do something for them. After all, we do not expect that a wealthy individual sells his/her kidney just to get financial support from the government.

Nevertheless, a market for organs can have a crowding-out effect on intrinsic motivations for an altruistic kidney donation. Our data from the Kidney Foundation, KF hereafter, in Mashhad confirm this concern as very few kidneys have been donated altruistically. The KF is a non-profit, volunteer-run charitable organization that mediates between recipients and donors to assist both and further applies for related government and charitable benefits with no incentives for making the pairs.

Another concern about the kidney market is that low-income patients might not be able to afford live kidneys. However, as the KF in Iran is a charity in the first place, it subsides poor patients to get a kidney. Moreover, we could design a market where the government is the only authority that could legally purchase kidneys and then allocate them similar to how cadaver kidneys are allocated. Our collective responsibilities for people who suffer from kidney failure are best accomplished through a government-monopsony market in kidneys where the government is the only buyer who distributes kidneys based on need, but not ability to pay (3). In this way, we treat all patients equally, and they all have equal access to kidneys, disrespectful of their wealth level.

Notably, this is a self-financing scheme since savings from dismissing patients from dialysis and shrinking the waiting list for kidneys are much more than the costs of purchasing live kidneys. Spending even a portion of this saving on improving the living conditions of donors, e.g., post-transplant medical care, and special social services, reduces the long-term adverse effects of kidney transplantation for donors while saves many lives without irreparable damage to others.

The paper is organized as follows. In Section *Related Literature*, reviews the related literature. Section *The Iranian Experience: The Case of Mashhad* explains in short how the Iranian model of the kidney market works. Section *Data Analysis* introduces data and analyzes it descriptively, reports and discusses multivariate regressions, and provided some policy implications. Section *Conclusion* concludes.

RELATED LITERATURE

Several U.S. states have legislated laws providing leave or tax benefits to organ and bone marrow donors and their employers. The passage of tax incentive legislation increased living unrelated kidney donation rates in New York (4). However, this legislation works for moderately invasive procedures such as bone marrow donation, but it cannot increase the quantity of organ donation, which is more hazardous and troublesome (5).

Organ sales ban forces the organ trade underground, strengthens the role of organ brokers, and lessens organ sellers' bargaining power, leaving them exposed to even higher levels of exploitation (6). The urgent monetary destitution for the poor, who commonly do not have appropriate access to the financial market, gives them no other choice than vending their organ. In this regard, it seems impossible to stop the illegal organ trade. Regulating the market minimizes harm by making it possible to scrutinize the market, to enforce compliance with standards that protect both donors and recipients, and to remove greedy dealers, thus enabling the poor to receive transplants on an equal footing with the rich (7).

Regulated and incentivized systems that eliminate impediments to donation and remunerate donors could raise donations and reduce the unregulated markets and their harms. Working Group on Incentives for Living Donation suggest standards and guidelines for such a donation mechanism that would do more good than harm. Its critical components are protection, regulation, oversight, and transparency under the auspices of the appropriate government or government-recognized body (8).

There are some concerns about the long-term well-being of kidney donors. They are at increased risk of long-term risk for end-stage renal disease, ESRD hereafter, cardiovascular, and all-cause mortality compared with a control group of non-donors who were eligible for donation (9, 10). Therefore, prospective

donors must be fully and adequately informed about the consequences of a kidney transplant (11).

The US public is potentially amenable to compensating kidney donors (12). They supports limited incentives for living donation while ethnic minorities and low-income Whites are more accepting of specific monetary incentives. Most of them favored reimbursement of medical costs, paid leave, and priority on the waiting list for living donation (13). Most of the ESRD patients are willing to pay for a kidney while male, ailing and wealthy patients are more willing to pay (14).

However, not all renal patients are willing to accept an altruistic live-donor transplant since they do not perceive an opportunity for direct reciprocity. Some feel either unworthy of an altruistic live-donor transplant or responsible for the risks to an altruistic donor. Therefore, receipt of an altruistic transplant might be an even more complicated decision than a donation (15). Since altruism is significantly related to donor motivation only for donations to direct family members, limited material incentives may be necessary for improving donations among individuals unrelated to kidney transplant recipients (16).

Some studies proposed a monetary incentive for living donors that would increase organs supply, discharge waiting in massive queues, raise the quality of life, and put an end to thousands of needless deaths (17–19). They estimated that a price of $15,000 per living donor would be enough to eliminate the shortage of kidneys and the waiting list in the US. Even paying a more substantial figure of $45,000 for living donors and $10,000 for deceased donors has far more benefits than costs (20), since $5,000 and $10,000 are the Median lowest monetary compensation that would urge to donate for relatives and strangers, respectively, while with ten times more money, one could no longer decline to donate (21). Based on donors' data from the most extensive online kidney matching point in Iran, and naturally around the globe, most kidney donors are male, around 31 years old, having an average willingness to accept of almost 12,400 USD (22).

Based on individual-level data from the United States and the European Union collected in 2001–2002, individuals who were familiar with the organ donation process or even had just some encounter with the health system were more likely to become organ donors, while minorities were less likely to donate (23). Mother's education also had a significant positive effect on organ donation. The decision to be an organ donor is affected by relational ties, religious beliefs, cultural influences, family controls, body integrity, knowledge about the organ donation process, and previous interactions with the health care system, e.g., medical mistrust, and fear of early organ retrieval (24).

THE IRANIAN EXPERIENCE: THE CASE OF MASHHAD

The Iranian model of kidney transplantation, IMKT hereafter, established in 1988, is an example of a compensated and regulated living unrelated renal donation. It is an efficient and ethical model that can be employed by all other countries, which currently lack the necessary regulatory supervision (25). The IMKT has provided a unique opportunity for socio-economic analysis of a market for organs, which has not been fairly addressed.

In line with the Declaration of Istanbul, DoI hereafter, organ trafficking and transplant tourism are prohibited in the IMKT. It authorizes monetary compensation for kidney transplantation but does not tolerate transplant commercialism. Commercialism refers to the possibility within the free-market system to abuse vulnerable people to make a private profit. However, donors in the IMKT are not exploited, but they are supported by law and protected by medical insurance. Therefore, the IMKT adheres to the DoI.

Since April 2000, when the Iranian parliament passed the Organ Transplantation and Brain Death Act that approved deceased organ donations, the share of transplants from deceased donors has firmly risen to more than half of transplants. Nevertheless, there are other legal barriers, e.g., the consent of all close related families for the transplantation right after the death, making the deceased organ donations not enough to eliminate the excess demand for kidneys. Even with a supply of live kidneys from the monetary market, patients in Iran should still wait for months to receive a kidney for transplantation.

The IMKT includes a compensation negotiated directly between the recipient and living donor. In Iran, the word that is used for kidney vendors is donor, though they get paid. We use the same tradition in this paper but have in mind the tautology. Additionally, the government pays a reward to donors, a fixed 10 million Rials, equal to about 1,200 USD at that time and 150 USD at present, called the gift of altruism. Every few years, the Kidney Foundation of Iran announces a new official floor price for a kidney that each of 39 branches of the KF in each province is obligated to follow. This fixed price is independent of individualistic features such as gender and health status. However, the government has allowed an additional payment above this threshold negotiated directly between the patient and living donor.

The legal kidney market in Iran is not working the same in all cities. On the one extreme, it has its remarkable function in Mashhad with transparent side payments (26). In Mashhad, the KF tries to prevent the poor from unadvisedly selling their kidneys by informing them about the consequences of a kidney transplant, fixing their financial needs, and imposing several legal obstacles before a transplant is authorized (27). These measures exclude a majority of potential donors who want to sell their kidneys and address the concern that the poor might sell their kidneys without explicitly knowing the health consequences of their decision. On the other extreme in Shiraz, the prohibition of payment beyond the official national rate has naturally fostered a black market for kidneys. Donors and recipients in such a market surreptitiously exchange money under the table while they had signed an agreement assuring that no payment would be made over the official rate.

TABLE 1 | Descriptive statistics.

Variables	Mean	S. D	Min	Max
Price (million Rials)	134.52	57.29	50	450
Donor Age	29.91	4.78	20	40
Patient Age	37.94	13.46	8	68
Donor Years of Education	8.04	3.71	0	16
Patient Years of Education	9.09	5.07	0	22

Any ESRD patient with no willing related donors is referred by a physician's letter to the corresponding KF in that province where s/he could enter the kidney waiting list. Each potential kidney donor also registers at the KF after undergoing the preliminary medical tests and bringing the notarized consent of him/herself and his/her family. There are four different matching lines for each blood type, and a donor is paired with the first renal patient in the same blood type line, based on the first-come/first-served, who is matched in terms of Human Leukocyte Antigens.

Although this matching mechanism is not the most efficient one, it raises the chance of a successful transplant. Nevertheless, this is not the only way of matching, and both sides could publicly advertise and find each other outside the KF. However, since nephrologists discourage patients from contacting random donors and transplantation centers only accept donors referred by KF, both donors and recipients have to register there and go through the required paperwork and medical tests.

Once any matched pair agrees on a price, payment is made through the KF by sending a letter to the transplantation centers located at university hospitals under the scrutiny of the *Ministry of Health and Medical Education*. The government also pays for all transplant-related expenses and provides donors with medical coverage for 1 year after the nephrectomy and even military service exemption in case it applies. Therefore, in contrast to other organ markets in developing countries, the medical team has no share of the money paid by the recipient to the donor (28). Nevertheless, the recipient bears the main payment burden as the governmental compensation has remained fixed since its initiation in 1998 and is now worth about one-eighth.

As a result of the Iranian system of compensated donation, the number of renal transplants conducted has substantively enhanced such that from about a decade afterward, the renal transplant waiting list has been almost eliminated, (29) and most of the Iranian kidney transplant candidates, irrespective of their socioeconomic class, have access to kidney transplantation (28). The Iranian system, despite its success, has definite defects and shortcomings, such as stigmatization of donors, (30) which deter donors from following up their medical status, crowding out effect which defeats altruistic and prosocial donation, (31, 32) commercialization and commodification, (33) which exploits the poor and disrespect human integrity (34).

DATA ANALYSIS

We collected 436 paired kidney donors and recipients from April 2011 (the beginning of the year 1,390 in Persian Calendar) up to March 2018 (the end of the year 1,396 in Persian Calendar) KF in Mashhad, the second most populated city in Iran. In Mashhad, the realized side payment to donors beyond the official floor price is exchanged through the KF and documented in both donors' and recipients' profile. This procedure makes the kidney market in Mashhad unique, while in other major markets in main cities of Iran such as Tehran, Shiraz, and Kermanshah, there is no such data. **Table 1** shows the descriptive statistics of our data. The average kidney price is about 134.5 million Rials (almost 4,400 USD), significantly higher than the average floor price, about 97.9 million Rials (almost 3200 USD), and less than 2 years of work with the minimum level of wage (35).

A large number of the available studies suggest that most donors are female, while the majority of recipients are male (36–39). Women might perceive organ donation as their motherly responsibility or spousal obligation to save their suffering child or partner. (35) They may be more likely to demonstrate altruistic nurturing behavior, (37, 41) more vulnerable to be influenced by family pressure to donate, and less able to resist this burden (37, 40, 42).

However, kidney vending may secure low-status women in the Middle East from being forced to serve as altruistic family donors (43). The kidney market in Iran is biased and favors women because they are less likely to donate and more likely to receive a kidney. As men are traditionally supposed to be the breadwinner of the family in Iran, they have prevalence among donors. There are more male and married donors in our dataset than recipients (almost 85% male and 79% married in donors, and about 65% male and 74% married in recipients). Donors were also mostly literate, with 8 years of education at secondary school, on average (35).

Donors tend to be poor young married men, who are financially motivated towards donation, but recipients are unfortunately not that wealthy, as 47% of them were unemployed. Interestingly, we had five closely related donors who sold their kidneys, albeit at much lower prices. We made a dummy variable for these cases. These descriptive statistics confirm the similar picture illustrated already in the literature that showed between 84% and 90% of living unrelated renal donors were male, 80% were married, and the majority were at the level of high school education (44, 45).

We found various education levels, e.g., primary, secondary, high school, and Bachelor, for both donors and recipients. In Iran, the education system used to have 5 years of primary school, 3 years of secondary school, 3 years of high school, and 1-year of pre-college. However, we realized in our data that having any education level does not necessarily mean that one has indeed finished that level. Instead, he or she was mostly about to get to that level. We considered the average years of education at each level for those who claimed they educated up to that level. Namely, we considered three, seven, and 10 years of education for primary, secondary, high school levels of education.

TABLE 2 | OLS regressions on the logarithm of inflation-adjusted kidney price.

Variables	Model I	Model II	Model III	Model IV
Constant	4.19*** (0.025)	4.089*** (0.038)	4.074*** (0.039)	4.078*** (0.039)
2012	0.024 (0.035)	0.023 (0.035)	0.03 (0.035)	0.034 (0.035)
2013	–0.045 (0.038)	–0.045 (0.038)	–0.044 (0.038)	–0.04 (0.038)
2014	0.077** (0.036)	0.075** (0.036)	0.077** (0.036)	0.084** (0.036)
2015	0.084** (0.035)	0.077** (0.034)	0.081** (0.034)	0.085** (0.034)
2016	0.095** (0.039)	0.101*** (0.038)	0.103*** (0.038)	0.103*** (0.038)
2017	0.117** (0.045)	0.099** (0.045)	0.1** (0.045)	0.1** (0.044)
Donor Education		0.007*** (0.002)	0.008*** (0.002)	0.008*** (0.002)
Recipient Education		0.004** (0.002)	0.004** (0.002)	0.004* (0.002)
Age Difference			0.001** (0.000)	0.001** (0.000)
Relative Donor				–0.265*** (0.096)
N	432	432	431	431
R_squared	0.052	0.079	0.087	0.104
Adjusted R-squared	0.039	0.062	0.068	0.082
Prob (F-statistic)	0.000	0.000	0.000	0.000

We adjusted the kidney price with the Iranian Central bank's monthly consumer price index to make data from different years comparable in a pooled setting and takes its logarithm as the dependent variable. Our regressions in **Table 2** illustrate that each extra year of education for both donors and recipients, as a proxy for their income level, raises the kidney price, although the intensity of increase varies. Each extra year of education for a donor compared to a patient has double effects on the kidney price and increases it by 0.8 million Rials (almost 26.2 USD).

However, as it is distinct from **Table 1**, donors tend to be relatively less educated than recipients (on average about 1 year, with no degree higher than Bachelor). Therefore, each extra year of additional education has a higher level of marginal effect on their income, especially given that they are relatively more impoverished. This difference in the effect of education on price might also reflect the difference between patients' willingness to pay and donors' willingness to accept. After all, donors should be much more averse to losing their organs than those about to receive ones.

Moreover, donors compared to recipients tend to be relatively younger, about 8 years on average. However, patients have wider variations in their age, as it is not restricted, and after all, the disease could emerge at any age, and it is more probable for elders, while donors' age has much less variance since it is restricted by law to be between 18 and 40 years old. There are different views on the effect of age on graft survival and, consequently, the kidney's price. While kidney allocation mechanisms do not consider factors other than blood type and tissue compatibility, the market mechanism itself considers each pair's age difference. **Table 2** indicates that the age difference between donor and recipient in each pair significantly augments the kidney price. Namely, when a kidney from a young donor is assigned to an old patient, the price is significantly higher compared to another case where the old patient gets a kidney from an old donor. A younger donor can receive a larger payment, up to about 100 thousand Rials (almost 3.25 USD), for each year of the age difference.

According to the estimation results, a family relationship between the donor and the patient reduces the kidney price. A related donor, who decides not to donate his or her organ for free, vends it to his or her relative for about 26.5 million Rials (about 867.15 USD) less than non-related donors. The dummy variables of all years, except 2012 and 2013, raise kidney prices in all models. This robust and positive effect could be because, compared to the official price in 2011, in these 2 years, the official prices increased a little, from 60 million Rials (almost 1963.35 USD) to 70 million Rials (almost 2,290.5 USD) and 90 million Rials (almost 2,945 USD) respectively, while afterward, it increases to 140 million Rials (almost 4,581.15 USD).

CONCLUSION

A market for organs is a typical example of market failure where the market equilibrium does not maximize social welfare. Iran is the only country in the world where it is not illegal to exchange an organ, e.g., a kidney, for money. The only government intervention so far in Iran's kidney market has been setting a minimum price for the whole country. While there is a scoring system for patients with renal disease in Iran that prioritize them getting a kidney from a deceased donor, Iran's kidney market does not prioritize patients and works simply on the first-come-first-serve basis. This paper is the very first attempt to provide a cornerstone to regulate the kidney market more efficiently.

We tried to explain variations in kidney price based on individualistic characteristics such as age and education level. Our findings indicate that related donors, who need to be compensated, vend their kidneys to close relatives for significantly less monetary compensation. We could interpret this impact as the crowding-out effect. Moreover, each year of education for both donors and recipients increases the kidney price. While kidney allocation mechanisms do not consider factors other than blood type and tissue compatibility, the market mechanism itself considers age difference and allows a higher price for assigning a kidney from a young donor to an old patient. These findings call for a revised mechanism for the

Iranian kidney market that should not be merely based on the similarity of blood types, but also it is supposed to consider individual characteristics of donors and recipients such as age.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because access to data is restricted to protect proprietary information. It can be made available upon request with permission of the kidney foundation in Mashhad. Requests to access the datasets should be directed to feizi@um.ac.ir.

AUTHOR CONTRIBUTIONS

TM collected data, analyzed it, wrote the first draft of the manuscript, and proofread it. MF was the initiator who defined the research question, analyzed the data, and finalized the manuscript.

CONFLICT OF INTEREST

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

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Kidneys for Sale? A Commentary on Moeindarbari's and Feizi's Study on the Iranian Model

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Over the last years, efforts by transplant professionals and transplant organizations have resulted in the strengthening of laws and sentences against virtually all forms of organ trade (1–4). The prevailing belief is that organ trade can be prevented by countries becoming "self-sufficient" (4, 5). Iran is the only country that reports to have eliminated its kidney transplant wait list (6, 7). Yet, it is largely condemned for having accomplished this by paying living kidney donors (8–10). Transplant professionals from Iran state that they are often prevented from presenting data about the Iranian model at international transplant conferences and in transplant journals. Furthermore, the regulations that underlie Iran's decentralized, semi-regulated organ payment programs, differ between the country's states, leading to differing outcomes (11–14). These cross-country variations, in conjunction with the limited available data, hampers an in-depth understanding of the Iranian model (10, 15, 16).[1]

Moeindarbari's and Feizi's study contributes to vital knowledge gaps in this regard. Drawing on a unique data-set collected from the Kidney Foundation in Mashhad, Moeindarbari and Feizi present an analysis of price arrangements between 436 donors and recipients. The findings illustrate, amongst other things, the effects of education, gender, age difference and donor-recipient relationships on kidney prices. In addition, the findings suggest that related donors sell their kidneys to close relatives for a significantly lower price. Government payments are additionally made under the scrutiny of the Ministry of Health for all transplant-related expenses. The authors further explain that donors are provided with medical coverage for 1 year after the nephrectomy and that they are exempted from military service (6).

There are however some concerns about the Iranian model. Mashhad's kidney transplant program tolerates side payments between recipients and donors besides the fixed government fee. This is problematic because prices fluctuate according to the bargaining skills and abilities of donors and recipients. These unregulated transactions in turn may cause and exacerbate a variety of issues including inequality and interpersonal exploitation. Furthermore, while donors are provided with medical coverage for 1-year post-donation, it is unclear whether life-long follow up is guaranteed.

[1]In fact this raises the question whether "the Iranian Model" is an appropriate term. The term, "Iranian models," seems more suitable.

Forum

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Moeindarbari and Feizi recognize these concerns and state that a monopsonistic program, where the government pays a fixed sum to donors and where patients do not pay, would allow for more equality and fairness (6). Although a monopsonistic transplant program would not address the conditions of poverty that compel people to sell a kidney, it could reduce the risk of interpersonal exploitation by preventing donors and recipients from negotiating payments (17, 18). While we oppose Iran's tolerance of unregulated organ payments between donors and recipients, removing criminal penalties for selling a kidney at the very least enables kidney sellers to report harm without risking prosecution (19). Previous research from Iran (13, 20), and from Mashhad in particular (11, 16), suggests that the degree of exploitation reported by Iranian kidney donors is less severe than those who sell their kidneys on the black market, because Iranian kidney donors are protected by law (11, 16). Moeindarbari and Feizi corroborate these findings by pointing out that medical teams in Mashhad have no share of the money paid by the recipient to the donor, that prospective donors are informed about the potential health consequences of their donation and that they receive pre –and post-operative care (6). Any examination of the Iranian model should thus compare the well-being of its donors to those who sell their kidneys on the black market (16, 17, 21).

A growing body of empirical evidence from a number of countries reveals that while organ sales are prohibited by law, they are tolerated in practice (19, 22-26). In addition, research assessing the impact of prohibitive measures suggests that organ trade is being pushed further underground, increasing the role of criminal intermediaries, and exposing donors to more violent means of recruitment (19, 27). Studies further indicate that transplant professionals who facilitate illegal transplants can also be complicit in the exploitation of donors and recipients by not providing (adequate) pre –and post-operative care (29–32). There is however a critical lack of attention for the implications of prohibition and a lack of accountability of those who facilitate illegal transplants, including medical institutions and medical staff (19, 28, 29). Although complicit transplant professionals reportedly profit the most from illegal transplants (19, 29, 32), successful convictions of medical institutions and their staff remain virtually absent (22, 29, 32, 33). The reluctance of organ sellers to report harm (because they risk conviction), further inhibits investigation and prosecution of criminal cases (19, 29).

More empirical data is needed to develop workable solutions grounded in the empirical reality of people directly affected by the trade in organs. Dismissing evidence-based studies assessing the impact of regulatory controls in Iran, currently the only country with a semi-regulated organ market, would be counterintuitive. The implications of prohibition and the growing organ scarcity warrant a data-driven exploration of alternative models that move beyond prohibition and that may more effectively reduce the risk of exploitation of vulnerable donors and diminish patient mortality on transplant wait lists (19, 28, 34).

To this end, more rigorous data from Iran is needed that demonstrates how exactly its organ payment schemes reduce the risk of exploitation. It would be particularly helpful to learn more about donors' and recipients' experiences with and attitudes towards Iran's organ payment programs (11). While Moeindarbari's and Feizi's analysis is perhaps more useful for economists who study market designs, studies about Iran's organ payment programs should not be rejected exclusively on moral grounds. Rather, an honest and open dialogue is needed in which data from different countries and models is comparatively discussed. To this end, studies from Iran, even if we disagree with them, should be welcomed.

AUTHOR CONTRIBUTIONS

FA and SC wrote the article. MA and NI provided comments.

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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Criminal, Legal, and Ethical Kidney Donation and Transplantation: A Conceptual Framework to Enable Innovation

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A Forum discussing:

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Criminal, legal, and ethical actions are three very different issues: this applies to all human activities, including living kidney donation and transplantation.

Criminal live donor kidney transplantation happens in countries with illegal black markets for organ transplantation. In these countries, surgeons perform the procedure outside regular medical centers, where donors and recipients receive poor surgical care and no postoperative care. Therefore, patients return to traditional medical institutions with no documentation and often with severe and life-threatening opportunistic infections (1). So far, longstanding efforts to eliminate these markets have failed, despite widespread repugnance to them, and the passage of laws criminalizing payments to donors (2).

Moeindarbari and Feizi (3) discuss the kidney market in Mashad. In Iran, it is legal to pay kidney donors. Transplants and nephrectomies are conducted in well-qualified transplant centers, which are also responsible for postoperative care of donors and recipients. The vast majority of the world transplant community opposes payments to organ donors, whether legal or illegal. Iranians emphasize the difference between criminal and legal live donor kidney transplantation. Many members of the international transplant community have witnessed that the legal live donor kidney transplantation in Iran is conducted with the highest medical and surgical standards.

We strongly believe that international efforts should concentrate on increasing the availability of ethical high-quality live donor kidney transplantation options in all countries.

This is not the same as accepting legalized organ markets, as in Iran. But the present state of the discussion, and its legitimate concern with black markets, has become so dysfunctional that caught in the crossfire of these counterproductive discussions have been other ways of increasing the availability of legal, ethical and safe transplantation and donation. Vigorously opposing criminal black markets should not be conflated with opposing all innovations in living kidney donation that draw closer to the line of valuable consideration. Many recent innovations, such as various forms of kidney exchange, remain inappropriately associated with illegal black markets, when in fact they are opportunities to reduce the demand for illegal black markets.

Kidney exchange has become well established as a standard form of ethical live donor kidney transplantation in several countries, and has led to tens of thousands of additional living donor kidney transplants over the last 2 decades. Yet it is still far from being as available as it could and should be (4).

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In some countries, non-directed donor chains are not allowed (but these account for the majority of kidney exchange transplants in the United States) (5, 6). Initiating such chains with deceased donor kidneys would further expand their scope (7, 8). In India, the range of family members eligible to be the donor in an incompatible patient-donor pair is more restrictive than those authorized to give a transplant directly: e.g., a patient is authorized to receive a kidney from her uncle if he is a compatible donor, but not to enter into kidney exchange with him if he is incompatible. And many countries do not yet have kidney exchange, such as Brazil and Germany, where kidney exchange remains illegal except in exceptional cases. Many other countries, like Switzerland or Denmark, are too small to be able to offer enough matching opportunities for kidney exchange in a self-sufficient environment. Even the United States is too small to have ready exchange opportunities for the most highly sensitized patients. A limited number of successful international exchanges have taken place, overcoming significant obstacles. For example, a first exchange between Israel and the UAE took place last summer (9). In each case, expanding the opportunity for ethical live donor kidney exchange might give someone a safe, legal and ethical kidney transplant. Any obstacle to ethical kidney transplantation activity supports criminals because it creates demand for an illegal, unsafe, and unethical black market transplant.

The 2017 Statement of the Pontifical Academy of Sciences Summit on Organ Trafficking and Transplant Tourism states that "organ trafficking and human trafficking for the purpose of organ removal" are "crimes against humanity" and specifies what should be considered as qualifying for that designation by recommending: "That all nations and all cultures recognize human trafficking for the purpose of organ removal and organ trafficking, which include the use of organs from executed prisoners and payments to donors or the next of kin of deceased donors, as crimes that should be condemned worldwide and legally prosecuted at the national and international level."

Note that "crimes against humanity" entered the legal lexicon in the post-World War II Nuremberg trials of Nazi war criminals. There probably is little controversy about extending that term to murdering prisoners for their organs. But is that equivalent to "payments to donors or the next of kin of deceased donors?" Suppose that one of us is called to judge the Nazi war criminals responsible for the Shoah, the Chinese government formerly tolerating the retrieval of organs from executed prisoners (10), and the Iranian government allowing payments to donors, or payments to the next of kin of deceased donors. Are the Nazi, Chinese, and Iranian governments committing the same crimes against humanity?

These types of overbroad generalizations are unhelpful and we agree with the view taken by the American Society of Transplant Surgeons and the American Society of Transplantation calling for exploration of ways to increase legal and ethical transplantation that could involve an "'Arc of Change' from removing disincentives to testing incentives." While not supporting direct payments to donors they write:

> "*We believe it important not to conflate the illegal market for organs, which we reject in the strongest possible terms, with the potential in the United States for concerted action to remove all remaining financial disincentives for donors and critically consider testing the impact and acceptability of incentives to increase organ availability in the United States*" (11).

Discussions of black markets are often conducted with reference to the 2008 Declaration of Istanbul, which defines:

> "*Organ trafficking is the recruitment, transport, transfer, harboring or receipt of living or deceased persons or their organs by means of the threat or use of force or other forms of coercion, of abduction, of fraud, of deception, of the abuse of power or of a position of vulnerability, or of the giving to, or the receiving by, a third party of payments or benefits to achieve the transfer of control over the potential donor, for the purpose of exploitation by the removal of organs for transplantation*"

and

> "*Transplant commercialism is a policy or practice in which an organ is treated as a commodity, including by being bought or sold or used for material gain*" (12).

Moeindarbari and Feizi write that the Iranian market is "In line with the Declaration of Istanbul" because:

> "*It authorizes monetary compensation for kidney transplantation but does not tolerate transplant commercialism. Commercialism refers to the possibility within the free-market system to abuse vulnerable people to make a private profit. However, donors in the Iranian Model of Kidney Transplantation are not exploited, but they are supported by law and protected by medical insurance. Therefore, the Iranian Model of Kidney Transplantation adheres to the Declaration of Istanbul.*"

We doubt that all the drafters of the Declaration's language would agree with this interpretation. But we welcome and encourage more attention to the dangers of black markets and the ways in which increasing safe, legal and ethical transplant opportunities around the world can put an end to criminal black markets, which remain busy and profitable due to the shortage of legal and ethical alternatives.

AUTHOR CONTRIBUTIONS

AR, IM, KK, and MR contributed to the writing of this commentary.

CONFLICT OF INTEREST

MR is the unpaid CEO of the Alliance for Paired Kidney Donation. MR, AR, and IM own an equity interest in

Rejuvenate Kidney Transplant Solutions that aims to improve the quality of care and reduce the cost of care for patients with kidney disease and their healthcare payers.

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

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Kidneys for Sale: Are We There Yet? (Commentary on Kidneys for Sale: Empirical Evidence From Iran)

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Keywords: kindey, transplant, organ donation, compensation, Iran, debate

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Direct financial compensation through organized markets has been proposed as one strategy to increase the number of potential organ donors. However, this controversial practice is legal in only one country in the world—Iran; thus, there are limited data to demonstrate how this type of system might practically function. In a new report, Moeindarbari and Feizi provide granular real-world data on the demographics of kidney vendors and recipients from 2011 to 2018 in the kidney market in Mashhad, Iran [1]. This study provides valuable insights from a direct financial compensation program for organ donation and helps contextualize the debate surrounding this controversial issue.

Most prior attempts to quantify the potential impact of direct financial compensation for donors have been limited to surveys or structured interviews about hypothetical compensation and willingness to donate, rather than the real-world data presented in this article [2–7]. These have generally found that direct financial compensation to donors would likely increase the number of people who would donate an organ. In one web-based survey of members of the Canadian general public, 54% of people who would not consider donation to a relative without any compensation would actually change to being willing to consider donation for a $10,000 payment [3]. Even among people who would already consider donation to a family member or a friend, a payment of $50,000 would make 60% of people even more likely to donate a kidney in a study from the United States [4].

The data presented by Moeindarbari and Feizi confirm that, even in a partially regulated organ sales market, donors are younger than recipients and have fewer years of education. This potentially validates previous concerns of donor exploitation and socio-economic inequalities that have been shown across many countries [8–10]. For example, the Phillipine Organ Donation Program allowed for direct financial payment to donors from 2002 to 2008, and 78% of donors did not have a single follow-up visit post-donation [10]. Importantly, Moeindarbari and Feizi point out that in addition to the market price set by the government for a kidney, the recipients are allowed to pay donors, which seems to undermine the idea of a "regulated" market and further engenders donor exploitation. The authors outlined policy recommendations and improvements moving forward to more fairly consider the market value of a kidney in Iran. However, we would suggest that we are not there yet: before we go down the road of commercial sales, there are many other methods to improve altruistic organ donation that have been underexplored and underutilized.

An increase in altruistic living and deceased donation could eliminate the need for commercial organ sales entirely. After the Israeli government criminalized organ brokering, altruistic living donation rose by 339% over 10 years [11]. Similar results were seen after the Pakistani government

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banned commercial transplants [12]. Iran has the opportunity to increase deceased donation efforts, given that deceased donors have only increased from 4% to 10% in 30 years [13]. Other ways to improve altruistic donation include the removal of disincentives to donate (such as expenses linked to donation, travel expenses, and lost wages) and removal of HLA, ABO, and other incompatibility barriers to living donation through paired exchange [14]. Other countries have shown these methods can substantially increase the access to living donation without commercial markets.

Even beyond simply removing disincentives to living donation, there are a number of other strategies that have been developed to increase the number of living donor kidney transplants being performed. For example, the Live Donor Champion program trains a friend, family member, or community member to advocate on behalf of a transplant candidate to identify a potential living donor and has been shown to increase the number of potential living donors who come forward to donate [15]. Other focused interventions have been developed to directly increase the number of donors who come forward for people who are racial/ethnic minorities or socioeconomically disadvantaged, although these have not been universally effective [16, 17]. In a randomized controlled trial of 145 African American kidney transplant candidates in the United States, 82% of candidates who received house calls (structured educational sessions delivered to candidates, family, and invited guests in their own home) received at least one donor inquiry compared to 47% of candidates who received traditional individual counseling in the transplant clinic [16]. In another study of 300 African American kidney transplant candidates in the United States, candidates were randomized to receive additional education from a transplant social worker (with or without living donor financial assistance) [TALKS] or to usual care [17]. The TALKS program was designed specifically to address issues precluding living donation raised by African Americans in prior studies. Although the TALKS intervention did not lead to an increase in living kidney donation, 99% of candidates who received the intervention reported a high degree of satisfaction with the intervention. Continued expansion and development of targeted interventions to increase living donation could help avoid the need for direct financial payment of living donors.

In summary, the data provided by Moeindarbari and Feizi shed both positive and negative light on how a regulated system of direct financial payment to organ donors actually functions. While such a system would likely increase the number of transplants performed, major concerns remain, and we would suggest that expanding systems designed to support and compensate donors for actual incurred expenses could substantially expand the number of donors without needing to directly provide payments for living donors. Additionally, continuing to innovate and expand the utilization of targeted interventions to increase living donation could also help avoid the need for direct financial compensation for living donors. Ultimately, we agree with the authors that careful study of this controversial topic is critical to ensuring protection of living donors.

AUTHOR CONTRIBUTIONS

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

CONFLICT OF INTEREST

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

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Donor Derived Cell Free DNA in Kidney Transplantation: The Circa 2020–2021 Update

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The routine surveillance of kidney transplant allografts has relied on imperfect non-invasive biomarkers such as creatinine and urinary indices, while the gold standard allograft biopsy is associated with risk of bleeding, organ injury and sampling errors. Donor derived cell free DNA (dd-cfDNA) is being employed as a biomarker that addresses limitations of these surveillance methods, albeit has inherent drawbacks. This review provides an update on the enhanced understanding of dd-cfDNA and its expanded use beyond the conventional indication of detecting allograft rejection.

Keywords: kidney, biomarker, rejection, transplant, cell free DNA

INTRODUCTION

In the past 5 decades of the successful practice of kidney transplantation, a biomarker for monitoring of allograft rejection continued to elude the field. Donor derived cell free DNA (dd-cfDNA) has gained widespread utility as that biomarker in the transplant community since its introduction. After the initial demonstration of its use in detecting T-cell mediated and antibody mediated rejection in kidney transplantation (1), multiple studies have looked to further validate it and address challenges in diagnosis and interpretation. In addition, the application of dd-cfDNA is starting to expand beyond the conventional use of rejection. This includes monitoring of the effect of non-HLA antibodies, oncologic therapy, and opportunistic infections (2-4). The objective of this review is to provide an update on these newly elucidated facets of dd-cfDNA.

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ADDING NUANCE TO THE BIOMARKER

A multitude of clinical studies have documented the efficacy of dd-cfDNA in detecting rejection, however, is role in surveillance of kidney allografts in routine clinical practice has not been well elucidated. The ADMIRAL study (Assessing AlloSure Dd-cfDNA, Monitoring Insights of Renal Allografts with Longitudinal Surveillance; NCT04566055) looked to address this aspect through a large, multicenter, observational cohort study of kidney transplant recipients monitored with dd-cfDNA for ≤3 years (5). In addition to assessing the utility of dd-cfDNA in surveillance of allografts to detect rejection, the study also looked to delineate the correlation between dd-cfDNA and estimated glomerular filtration rate.

In a cohort of nearly 1,100 patients from over seven major transplant centers in the United States, dd-cfDNA measurements were done at regular intervals done as part of surveillance and for-cause in the setting of graft dysfunction to examine its "real world" application. Transplant kidney biopsies were performed as a part of the study in the setting of worsening creatinine, proteinuria and/or

development of *de novo* donor specific antibody. One of the salient findings of the study was that a relative change in serial dd-cfDNA, in addition to an isolated absolute measurement, may signal allograft injury and dnDSA formation. An increase in dd-cDNA of ~150% warrants consideration for closer monitoring and/or further investigation of potential graft injury.

Circulating Donor-Derived Cell-Free DNA in Blood for Diagnosing Acute Rejection in Kidney Transplant Recipients (DART) study demonstrated that a dd-cfDNA threshold of >1% aided in discerning presence of rejection (1). Data from the ADMIRAL further adds to understanding the interpretation of dd-cfDNA measurements-values < 0.5% were indicative of absence of allograft injury or presence of allograft quiescence (causes for injury included out-of-range tacrolimus level <4 ng/ml or >12 ng/ml, BK viremia, dnDSA-positive, urinary tract infection, proteinuria, allograft rejection, or recurrent focal segmental glomerulosclerosis). The investigators assessed dd-cfDNA as a marker of graft quiescence with paired biopsies <30 days after dd-cfDNA measurements. This shows that dd-cfDNA could bguide clinicians to avoid unnecessary investigations, including invasive procedures such as kidney transplant biopsies.

A decline in eGFR 1–3 years post kidney transplantation portends an increased risk of graft failure and death (6, 7). The ADMIRAL study demonstrated a correlation between elevated dd-cfDNA and eGFR decline during this period. Continually elevated dd-cfDNA (more than 1 result of >0.5%) was associated with doubling of risk of 25% decline in eGFR. This is the first study to show the correlation between dd-cfDNA and renal function decline, a measure that is pivotal in the real-world scenario. Persistent elevations in dd-cfDNA can signal not only the presence of possible ongoing allograft injury, but also forecast future decline of kidney allograft function.

While the ADMIRAL study expanded the repertoire of dd-cfDNA interpretation, it is important to address its limitations. Many dd-cfDNA and biopsy samples were not truly paired, with the investigators allowing for biopsies to be done within 30 days of dd-cfDNA measurements. It is possible that many early disease processes may have been missed or a new pathology may have arisen in the interim. This confounding cannot be accounted for, and future studies should endeavor to limit the duration elapsed between the dd-cfDNA measurement and subsequent kidney biopsy. There could also be observer bias since all biopsies were read locally and lacked centralized reporting. Given that this study was designed to assess the "real world" utility of dd-cfDNA, the investigators could have also assessed the correlation of dd-cfDNA with proteinuria. The presence of proteinuria is strongly associated with reduced graft survival (8) and since dd-cfDNA could now be a prognosticating tool, it would be important for future studies to examine the existence of a correlation between the two measurements. Lastly, in keeping with previous studies, dd-cfDNA appears to be more sensitive in detecting ABMR compared to TCMR (1, 5).

The Molecular Microscope Diagnostic System (MMDx) is a method of elucidating various pathologies on allograft biopsy sample by utilizing automatic genome-wide microarray measurements and interprets disease states by machine learning–derived classifiers and archetype scores (9). The Banff Molecular Diagnostics Work Group now recommends utilization of the Banff Human Organ Transplant gene expression panel consisting of 770 genes related to rejection, tolerance, and viral infections, and innate and adaptive immune responses (10). The correlation of disease effector gene transcripts, histology and dd-cfDNA has not been well defined until recently. The Trifecta study, an international prospective trial, assessed the relationship of dd-cfDNA done at the time of kidney allograft biopsy with gene transcriptomic signatures on the MMDx. In a cohort of 300 biopsies, the authors report a case representation similar to that of previous studies with 60% demonstrating no rejection, while the rest showing antibody mediated rejection (30%) and T-cell mediated (TCMR)/mixed rejection (10%). The top 20 gene transcripts (of 49,495 total probe sets) that have been previously shown to be highly associated with all types of rejection, correlated positively with dd-cfDNA. These gene transcripts mostly represented natural killer (NK) cells and those induced by interferon gamma.

The correlation of multigene measurement scores (transcript sets) with dd-cfDNA were strongest with ABMR and all-rejection scores, while being moderate with TCMR scores, and weak with recent parenchymal injury, dedifferentiation, and atrophy-fibrosis scores. The investigators performed a principal component analysis (PCA), in which the dd-cfDNA vector highly approximated the peritubular capillaritis molecular classifier vector in all three dimensions (all rejection, ABMR and early stage ABMR). Dd-cfDNA, therefore, correlated with an important component of the Banff classification used for diagnosis of ABMR-peritubular capillaritis.

Active rejection based on molecular measurements had the highest dd-cfDNA levels, while biopsies with no molecular or histologic evidence of rejection has the lowest values. Importantly, the molecular scores predicted dd-cfDNA ≥1.0% better than histologic scores. This finding adds further to accumulating evidence that histology, while regarded as the gold standard for diagnosing a vast array of allograft pathologies, may not correlate with extent of damage.

The Trifecta study findings of lower dd-cfDNA levels in TCMR in comparison to ABMR is in line with that of the DART study (1). The Trifecta investigators present an intriguing hypothesis to explain this phenomenon-the degree of dd-cfDNA released by TCMR reflects the activation state of the effector T cells in those TCMR biopsies. Previous archetypal analyses have established that TCMR has two phenotypes varying in molecular activity- TCMR1 (intense TCMR, sometimes mixed with ABMR) and TCMR2 (less active TCMR). While the TCMR1 phenotype has more intense interferon gamma expression, it can also have some ABMR features, in comparison to TCMR2. Therefore, explaining release of dd-cfDNA, which has strongly correlated with ABMR and interferon gamma activity in this study. However, TCMR with lower dd-cfDNA levels may have T-cells with attenuated activity as corollary of immunosuppression or exhaustion. It is also important to note that all biopsies included in this study were "for cause" and no subclinical features of rejection were investigated. Therefore, it is difficult to assess the correlation of dd-cfDNA with incipient subclinical rejection. Morever, some cases had high levels of dd-cfDNA with absence of biopsy proven rejection.

Histologic lesions of borderline and TCMR 1A can exhibit considerable overlap, with clinical relevance of either lesions and

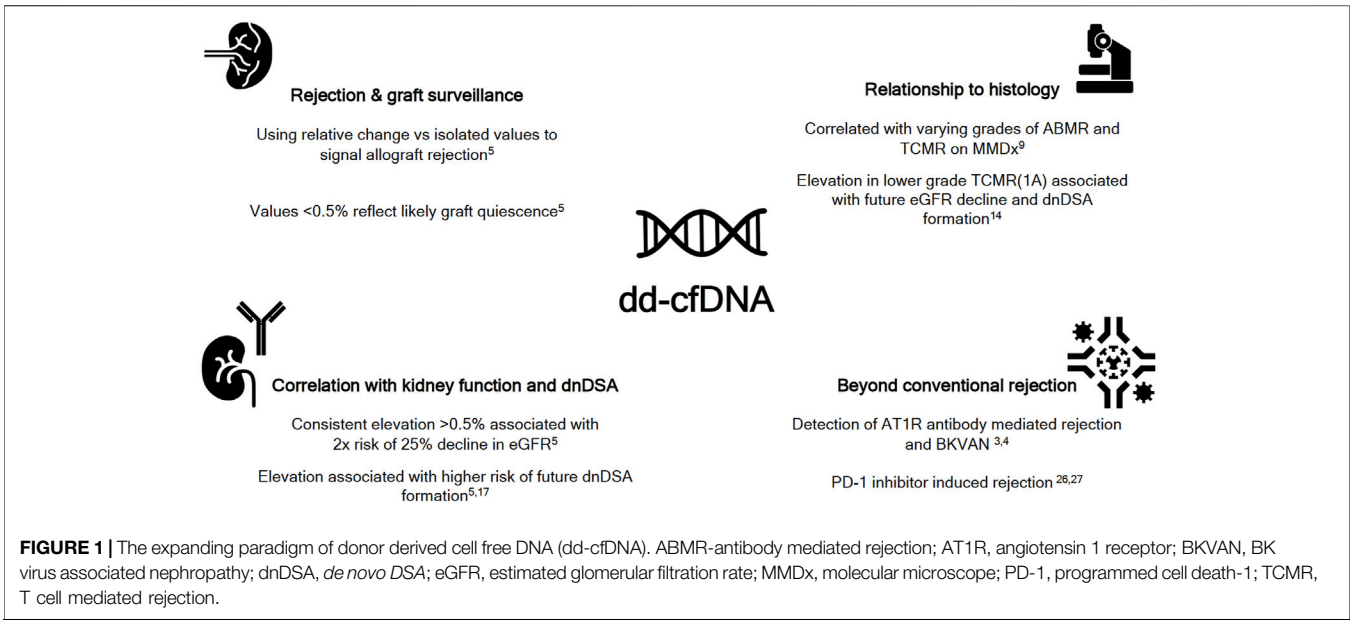

FIGURE 1 | The expanding paradigm of donor derived cell free DNA (dd-cfDNA). ABMR-antibody mediated rejection; AT1R, angiotensin 1 receptor; BKVAN, BK virus associated nephropathy; dnDSA, *de novo DSA*; eGFR, estimated glomerular filtration rate; MMDx, molecular microscope; PD-1, programmed cell death-1; TCMR, T cell mediated rejection.

optimal treatment continues to debated (11). However, it is being increasingly recognized that borderline TCMR portends to inferior graft outcomes even in the event of subsequent resolution of inflammatory infiltrates (12). An objective measure that could aid in discerning actual presence of tissue damage in the presence of these lesions could augment the Banff diagnostic categories.

Previous studies elucidating the use of dd-cfDNA in kidney allograft rejection demonstrated that a proportion of patients with TCMR 1A did not have elevated levels (1, 13). It could be argued that this subset of patients may not actually have a true rejection episode with the infiltrate devoid of any deleterious effects. A multicenter study assessed if elevated dd-cfDNA was associated with adverse outcomes in patients with borderline and TCMR 1A rejection (14). Over a 3-year period, in the cohort with elevated dd-cfDNA (>0.5%) the estimated glomerular filtration rate declined by 8.5% (vs. 0% in those with low dd-cfDNA <0.5%), *de novo* donor specific antibody (dnDSA) was seen in 40% (vs. 2.7%) and future or persistent rejection occurred in 22% (vs. 0%). This study demonstrates that dd-cfDNA could be used to detect early rejection and aid in discerning which lesions are actually associated with injury, which in turn, are associated future adverse consequences. In addition, authors of this study have put forward a recommendation that a threshold of 0.5% be considered for indicating damage/rejection, with interpretation of this test be as a continuous variable.

THE *de novo* DSA LINK AND MEASUREMENT PREDICAMENT

The generation of dnDSA is associated with adverse consequences, including development of antibody mediated rejection and eventual graft loss. Patients with dnDSA have a significant reduction in 10-year graft survival in comparison to those who do not (57% vs. 96%) (15). As with early rejection entities borderline and TCMR 1A, data for risk stratification by type of dnDSA is lacking and there is no established agreement on treatment once dnDSA has been detected. There is emerging evidence that dd-cfDNA may be a potent stimulator of immune mediated inflammation (16). A retrospective cohort study of the Circulating Donor-Derived Cell-Free DNA in Blood for Diagnosing Acute Rejection in Kidney Transplant Recipients (DART) assessed the association of dnDSA and dd-cfDNA (17). Levels of dd-cfDNA were higher in patients with dnDSA compared to those with none. Elevated dd-cfDNA (>1%) in the first-year post transplant year is associated with eGFR decline of >25% in the following year. It is important to note that patients with rejection were excluded in this cohort and the finding of higher dd-cfDNA is likely reflective of ongoing subclinical allograft injury, with demonstration of eventual decline in eGFR. Utilization of dd-cfDNA in concurrence with dnDSA may aid in discerning pathogenic from non-pathogenic antibodies and identifying patients at high risk for future allograft dysfunction, who may benefit from augmentation of immunosuppression.

The ADMIRAL study provided further granularity to relationship between dd-cfDNA and dnDSA (5). Dd-cfDNA levels >0.5% was associated with a 3-fold higher risk of dnDSA production in the future, with persistent elevation of dd-cfDNA in all patients with detectable dnDSA. Additionally, every 1% increase in dd-cfDNA levels was associated with a 20% increase in risk of dnDSA formation, and a median increase of ~120% in dd-cfDNA from previous values occurred at a median of 91 days prior to development of dnDSA.

Another study investigated the diagnostic value of dd-cfDNA when added to DSA in detecting ABMR in two independent cohorts of kidney transplant patients (one cohort with subclinical cases identified with DSA testing >180 days post transplantation and the other with indication biopsies >1 month post transplantation) (18). The addition Dd-cfDNA to DSA or vice-versa significantly improved the diagnostic yield in identifying ABMR in the first cohort. However, the combination of DSA and dd-cfDNA did not translate into a similar diagnostic value given disparate number of biopsy proven diagnosis in the indication biopsy cohort, which included TCMR, glomerulonephritis and BK associated

nephropathy. While this study strengthens the diagnostic axis of dd-cfDNA, DSA and AMBR, the diagnostic accuracy of dd-cfDNA in identifying other pathologies remains suboptimal.

Given dd-cfDNA is calculated as percentage of the total circulating DNA (donor derived and recipient derived cell free DNA), any change in background cell free DNA may result in a false result. High body mass index (BMI) and increasing age may result in higher background cell free DNA given association with increased inflammation and escalated cell senescence respectively (19, 20). A study examined this plausible effect of BMI and age on dd-cfDNA demonstrating a significant negative correlation between increasing BMI and baseline dd-cfDNA levels, with no influence of age on the biomarker (21). This, albeit, being a small study, highlights the need for further studies to assess the influence of BMI on dd-cfDNA and if levels need to adjusted based on body habitus. Clinicians should be mindful of possible falsely low levels in the setting of high BMI, which may in turn, lead to missing evolving rejection.

THE PANDEMIC ANGLE

The COVID-19 pandemic necessitated dramatic changes in delivery of healthcare. From a transplant perspective, measures to reduce exposure to the virus and augmenting vaccine response have been the most essential initiatives to mitigate the viral infection in the vulnerable transplant population. Telemedicine and remote home phlebotomy were employed as methods to minimize healthcare associated exposure the virus.

Two studies demonstrated that dd-cfDNA drawn via remote home phlebotomy could be utilized for surveillance of allografts (22, 23). This aided in identifying patients at risk of rejection and subsequent triage for allograft biopsies. These studies did not identify if this reduced the need or could be a replacement for protocol biopsies, however, they do represent a potential blueprint for allograft monitoring for subsequent waves of COVID-19 and future pandemics.

BEYOND CONVENTIONAL REJECTION

The utilization of dd-cfDNA has been extensively validated in TCMR and HLA antibody induced antibody mediated rejection (ABMR). It is now being employed beyond these conventional indications (**Figure 1**):

(1) Angiotensin-1 receptor (AT1R) antibody mediated rejection: the presence of AT1R antibodies has been demonstrated to be independently associated with high risk for development of ABMR and decreased long term graft survival (24). However, these antibodies can be present prior to transplantation, its levels cannot predict presence of rejection and a proportion of patients with the antibodies do not eventually develop rejection (25). A multicenter study involving with patients with biopsy proven ABMR and pre-existing positive AT1R antibodies, showed that dd-cfDNA correlated well with Banff components of rejection (3). Therefore, dd-cfDNA could be utilized to for surveillance and detection of incipient rejection in this setting.

(2) Anti-programmed cell death-1 (PD-1) inhibitor induced rejection: Immune check point inhibitors are being increasingly used to treated numerous cancers. In addition to being associated with multi-systemic adverse effects, allograft rejection can be a devastating consequence of these agents (26-28). Two case reports demonstrated the use of dd-cfDNA for monitoring for rejection while successfully continuing PD-1 inhibitor therapy. Larger studies are required to validate these preliminary reports (27, 28).

(3) Distinguishing BK virus associated nephropathy (BKVAN) from BK viremia (BKV): it can be challenging to discern progression of BKV to BKVAN- especially with reliance on the often debated cut off viral load of >10,000 copies/mL and eventual allograft biopsy, which itself is associated with discordant reads (29, 30). A retrospective analysis of the DART study demonstrated that dd-cfDNA could distinguish BKV from BKVAN, and that levels of dd-cfDNA correlated with BK viral loads (4).

CONCLUSION

As dd-cfDNA continues to integrate into surveillance regimes of kidney allografts, some aspects continue to remain unanswered. There is yet to be a defined frequency of dd-cfDNA testing substantiated by a robust clinical study (31). The Kidney Allograft Outcomes Registry (KOAR) study (NCT033226076) will look to assess this aspect with planned dd-cfDNA testing at various pre-defined intervals along with planned 12-month allograft biopsies-this will also aid in ascertaining if dd-cfDNA could reduce the need for protocol biopsies. This biomarker is predominantly beneficial in detecting alloimmune damage, however, has no utility in identifying non-immune causes such as acute tubular injury. Further nuance is definitely required to determine the optimal threshold of dd-cfDNA to proceed with allograft biopsy and identify patients that can be safely monitored since high levels can be present in the absence of rejection. Larger studies are also required to elucidate whether absolute graft derived cfDNA or fractionated measurements are more accurate in detection of rejection, along with appropriate context of their application.

AUTHOR CONTRIBUTIONS

Writing–original draft preparation, SK; Writing—review and editing, DB.

CONFLICT OF INTEREST

SK has received a fellowship funded by CareDx. DB has received honoraria and consulting fees from CareDx, and Natera, and Johns Hopkins; has received research support from CareDx and Natera; and funding to support a transplant nephrology fellowship from CareDx.

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SYSTEMATIC REVIEW AND META-ANALYSIS
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Elevated Cardiac Troponin to Detect Acute Cellular Rejection After Cardiac Transplantation: A Systematic Review and Meta-Analysis

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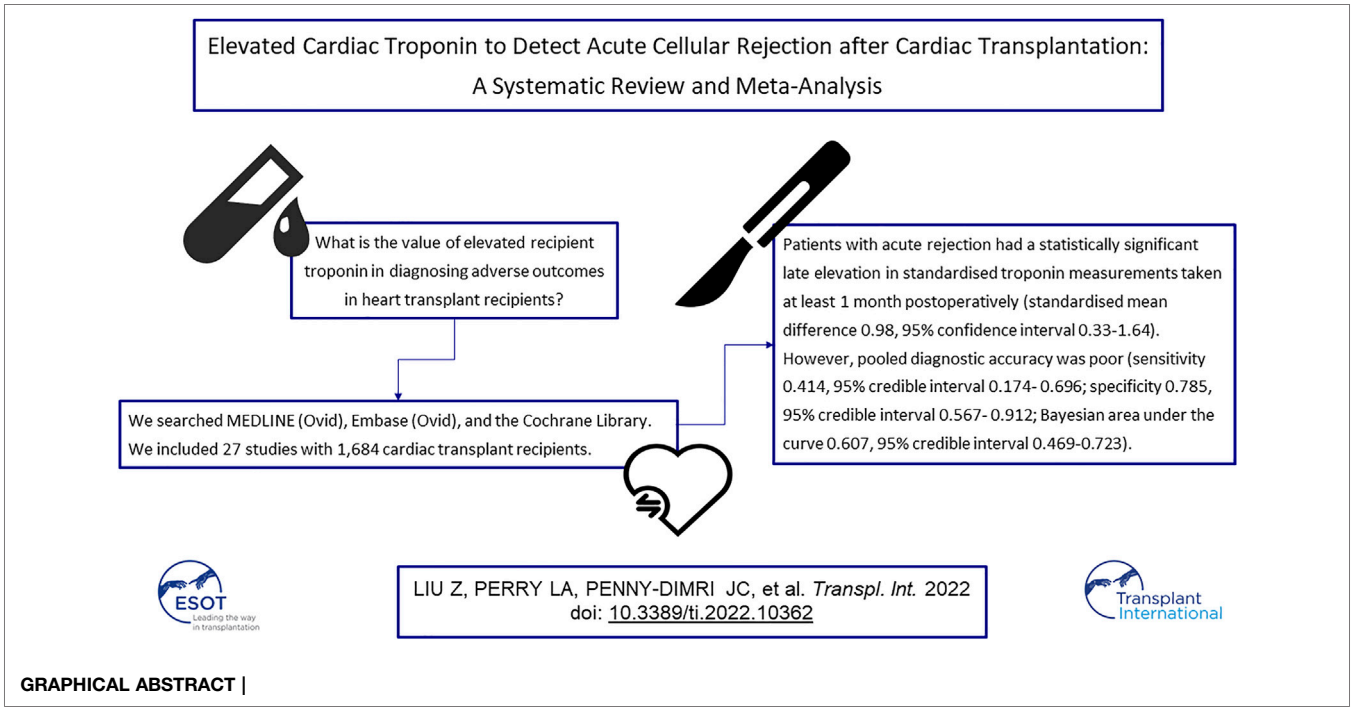
Cardiac troponin is well known as a highly specific marker of cardiomyocyte damage, and has significant diagnostic accuracy in many cardiac conditions. However, the value of elevated recipient troponin in diagnosing adverse outcomes in heart transplant recipients is uncertain. We searched MEDLINE (Ovid), Embase (Ovid), and the Cochrane Library from inception until December 2020. We generated summary sensitivity, specificity, and Bayesian areas under the curve (BAUC) using bivariate Bayesian modelling, and standardised mean differences (SMDs) to quantify the diagnostic relationship of recipient troponin and adverse outcomes following cardiac transplant. We included 27 studies with 1,684 cardiac transplant recipients. Patients with acute rejection had a statistically significant late elevation in standardised troponin measurements taken at least 1 month postoperatively (SMD 0.98, 95% CI 0.33–1.64). However, pooled diagnostic accuracy was poor (sensitivity 0.414, 95% CrI 0.174–0.696; specificity 0.785, 95% CrI 0.567–0.912; BAUC 0.607, 95% CrI 0.469–0.723). In summary, late troponin elevation in heart transplant recipients is associated with acute cellular rejection in adults, but its stand-alone diagnostic accuracy is poor. Further research is needed to assess its performance in predictive modelling of adverse outcomes following cardiac transplant.

Systematic Review Registration: identifier CRD42021227861

Keywords: heart transplantation, meta-analysis, systematic review, cardiac troponin, acute cellular rejection

Abbreviations: ANOVA, analysis of variance; AUC, area under the ROC curve; BAUC, Bayesian AUC; BSROC, Bayesian summary ROC; CI, confidence interval; CrI, credible interval; EMB, endomyocardial biopsy; ISHLT, International Society for Heart and Lung Transplantation; MOOSE, meta-analysis of observational studies in epidemiology; QUADAS-2, quality assessment of diagnostic accuracy studies 2; ROC, receiver operating characteristic; SMD, standardised mean difference.



GRAPHICAL ABSTRACT |

INTRODUCTION

The endomyocardial biopsy (EMB) has remained the gold standard for detecting acute allograft rejection after cardiac transplant since its introduction in the early 1970s (1). However, this diagnostic test is invasive, can be poorly concordant amongst grading pathologists (2), and repeat procedures are associated with small but significant risks of complications including tricuspid regurgitation, cardiac tamponade, arrhythmias, and haemorrhage (3–5).

In light of these challenges, various biomarkers have been explored as diagnostic alternatives to EMB, contributing to an emerging sphere of multidisciplinary interest in the predictive (both diagnostic and prognostic) ability of routine serum biomarkers for adverse outcomes in a variety of conditions (6–13). In particular, cardiac troponin, a sensitive and specific marker of myocardial injury, is of broad prognostic significance across a range of cardiovascular diseases (14, 15). Although most classically elevated in the context of acute coronary syndromes, elevated troponin levels are also associated with a range of other cardiac and non-cardiac conditions including atrial fibrillation, congestive cardiac failure, myocarditis, myocardial contusion, pulmonary embolism, sepsis, renal failure, and hypovolaemia (16). Both donor and recipient troponin have been associated with adverse outcomes following cardiac transplant (17, 18). We have previously found that troponin elevations in cardiac transplant recipients may be prognostic for primary graft failure, adverse cardiac events, coronary artery disease, and long-term mortality, but its prognostic value in the context of acute rejection up to 1 year after transplant was uncertain (19). Donor troponin elevations though, were not associated with increased 30-day, 1-year, or long-term mortality post cardiac transplant despite increasing the risk of graft rejection at 1 year (but not at 30 days) (20).

However, the diagnostic utility of elevated cardiac troponin is controversial, and this biomarker has yet to be routinely integrated into the diagnostic pathway for acute allograft rejection or recommended by international guidelines (21, 22). Hence, we conducted this systematic review and meta-analysis of elevated cardiac troponin in diagnosing acute allograft rejection in heart transplant recipients.

METHODS

Study Design and Registration

This systematic review and meta-analysis evaluated study level data, and was reported in compliance with the Meta-analysis Of Observational Studies in Epidemiology (MOOSE) guidelines (23). Protocol details were prospectively registered on PROSPERO (CRD42021227861) and there were no major protocol deviations.

Eligibility Criteria

We included all original research studies which reported the diagnostic accuracy of elevated recipient troponin to detect adverse outcomes in heart transplant recipients. We excluded non-human studies, abstracts and conference presentations, case reports and series, editorials and expert opinions, review articles, and studies with incompletely reported data.

Search Strategy

We searched MEDLINE (Ovid), Embase (Ovid), and the Cochrane Library from inception to December 2020. Our search strategy included a comprehensive set of search terms for troponin and cardiac transplantation (**Supplementary Material**) (24). We placed no restrictions on language or publication period.

Study Selection

Two authors (ZL and MH) independently screened titles and abstracts of each search result for potentially relevant studies. The same two authors assessed full texts of shortlisted studies against eligibility criteria, with a third author (LAP) adjudicating any disagreements. We reviewed the reference and citation lists of included studies for further potentially relevant studies.

Data Extraction and Management

Two authors (ZL and LAP) independently extracted data from included studies using standardised spreadsheets. We recorded the following, where reported and applicable: study design, population baseline characteristics including comorbidities, operative details, troponin type and measurement details, troponin threshold, definitional threshold of significant rejection by the International Society for Heart and Lung Transplantation (ISHLT) acute cellular rejection grade (25), outcomes, and diagnostic performance measures. Where studies reported dichotomous measures of diagnostic performance, we standardised reported data in confusion matrices and calculated sensitivity and specificity values; where studies reported continuous measures of effect, we standardised data reported as mean and standard deviation and calculated standardised mean differences (SMDs) (26).

Assessment of Methodological Quality and Risk of Bias

Two authors (ZL and LAP) independently assessed the methodological quality of included studies using a modified version of the Quality Assessment of Diagnostic Accuracy Studies 2 (QUADAS-2) tool (27), with discrepancies resolved through discussion with a third author (MH). For this study, we expanded the grading of overall risk of bias to three categories (low, unclear, and high risk) from 2 categories (low risk and at risk), for greater consistency with the domain level risk of bias reporting (also low, unclear, and high risk) (28).

Statistical Analysis and Data Synthesis

A detailed description of the statistical analysis is provided in the **Supplementary Material**. Anticipating significant between study variation in included studies, we pre-specified the use of random-effects models in all meta-analyses performed. Where studies reported continuous effect measures, we tabulated SMDs and associated confidence intervals (CIs) of recipient troponin measurements between acute cellular rejection and non-rejection groups, and used random effects inverse variance modelling to generate pooled SMDs. Where studies reported dichotomous effect measures and used receiver operating characteristic (ROC) analysis we noted optimised cut-off values, areas under the ROC curve (AUCs), sensitivities, specificities, and associated 95% CIs. From these, we calculated true positive, false positive, false negative, and true negative rates, and generated Bayesian Summary ROC (BSROC) curves and summary sensitivity, specificity, and Bayesian AUC (BAUC) statistics with 95% credible intervals (CrI) using a bivariate Bayesian modelling approach (29).

We estimated statistical heterogeneity using the I^2 statistic for each meta-analysis. Where reporting of pre-specified covariates was sufficient across included studies, we used meta-regressions to explore possible sources of heterogeneity.

Where there were more than 10 included studies, we formally assessed publication bias with visual inspection of funnel plot skew and a regression test for funnel plot asymmetry (30). All analyses and figures were generated using Review Manager (RevMan) 5.4 (31) and the R statistical packages "metafor" (32) and "bamdit" (33).

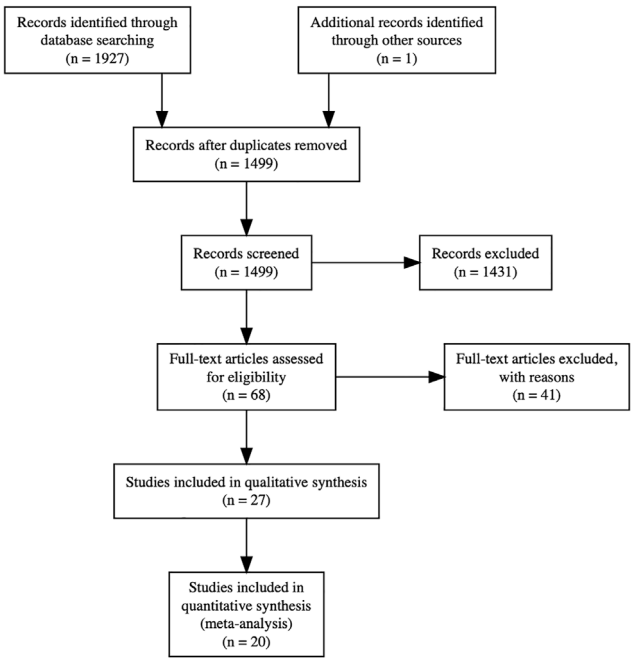


FIGURE 1 | Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram. Full text articles were excluded for the following reasons: 19 due to incorrect exposure measurement (donor troponin rather than recipient troponin), 15 due to incorrect study design (prognostic rather than diagnostic), 4 due to lack of troponin reporting, and 3 due to incorrect outcome measurement. Twenty studies were included in quantitative syntheses: for acute cellular rejection in adults with no exclusion of measurements from the early postoperative period, 8 studies were included in the meta-analysis of dichotomous effect measures and 11 studies were included in the meta-analysis of continuous effect measures; for acute cellular rejection in adults with exclusion of measurements from the early postoperative period, 8 studies were included in the meta-analysis of dichotomous effect measures and 5 studies were included in the meta-analysis of continuous effect measures.

TABLE 1 | Characteristics of included studies.

Study ID	Design	Number of patients, number of samples, and demographic	Age (Mean ± SD, years)	Sex (% male)	Troponin type	Troponin measurement period post transplantation and early measurement exclusions	Troponin measurement method	Troponin threshold (ng/ml) and Selection method	Classification threshold for significant rejection and samples with significant rejection (%)	Outcome(s) and effect measure(s)	Modified QUADAS-2 risk of bias
Ahn (34)	Single Centre Retrospective	47 252 Adult	47.4 ± 15.8	68.1%	TnI, hsTnI Index[a]	2 weeks postoperative onwards Exclusions: none and first 2 months after transplantation	ARCHITECT i2000sr STAT TnI and hsTnI assay (Abbott Diagnostics, Abbott Park, Illinois, USA)	1.17 (hsTnI Index) Receiver operating characteristic analysis	ISHLT 2004, 2R 7%	Acute Cellular Rejection Dichotomous and continuous	high
Alexis (35)	Single Centre Prospective	90 256 Adult	48.0 ± 15.2	74.4%	TnT	1 week to 72 months postoperative Exclusions: none and first 3 months after transplantation	Enzymun-Test TnT enzyme immunometric assay (Boehringer Mannheim Diagnostics GmbH, Mannheim, Germany)	0.1 Manufacturer's recommendation	ISHLT 1990, 3A 5%	Acute Cellular Rejection Dichotomous	high
Balduini (36)	Single Centre Prospective	57 149 Adult	37.5 (SD not reported)	Not reported	TnT	1 month to 12 months Exclusions: first 1 month after transplantation	Elecsys Electrochemiluminescence Immunoassay (Roche Diagnostics, Indianapolis, Indiana, USA)	Not reported Not reported	ISHLT 1990, 1B 23%	Acute Cellular Rejection Continuous	unclear
Cauliez (37)	Single Centre Prospective	56 100 Adult	Not reported	Not reported	TnI	10 to 3,807 days (median 458 days) No exclusions	Stratus Cardiac TnI fluorometric enzyme immunoassay (Dade-Behring, Newark, Delaware, USA)	0.6 Manufacturer's recommendation	ISHLT 1990, 2 9%	Acute Cellular Rejection Continuous	unclear
Chance (38)	Single Centre Prospective	145 704 Adult	Not reported	Not reported	TnT	3 days to 206 months (median 29 months) Exclusions: none and first 1 month after transplantation	Elecsys Electrochemiluminescence Immunoassay (Roche Diagnostics, Indianapolis, Indiana, USA)	0.1 Manufacturer's recommendation	ISHLT 1990, 3A 20%	Acute Cellular Rejection Dichotomous and continuous	unclear
Dengler (39)	Single Centre Retrospective	95 271 Adult	48.2 ± 11.4	82.1%	TnT	3 months–70 months (median 15 months) Exclusions: first 3 months after transplantation	Enzymun-Test TnT enzyme immunometric assay (Boehringer Mannheim Diagnostics GmbH, Mannheim, Germany)	0.015 Lower limit of assay detection	ISHLT 1990, 3A 17%	Acute Cellular Rejection Dichotomous and continuous	unclear
Dyer (40)	Single Centre Prospective	42 53 Paediatric	11.1 (SD not reported)	Not reported	hsTnT	3 months onwards (median 24 months) Exclusions: first 3 months after transplantation	Elecsys Electrochemiluminescence Immunoassay (Roche Diagnostics, Indianapolis, Indiana, USA)	0.014 99th percentile of healthy adult reference population	ISHLT 2004, 2R 13%	Acute Cellular Rejection Dichotomous and continuous	unclear
Faulk (41)	Single Centre Prospective	68 151 Adult	30.3 ± 14.2	60.3%	TnT	6 months onwards Exclusions: first 6 months after transplantation	Enzymun-Test TnT enzyme immunometric assay (Boehringer Mannheim Diagnostics GmbH, Mannheim, Germany)	0.1 Manufacturer's recommendation	ISHLT 1990, 3A 6%	Acute Cellular Rejection Dichotomous	high
Forni (42)	Single Centre Prospective	114 385 Adult	52.0 ± 6.0	86.0%	TnI	15 to 1,740 days (mean 640 ± 95 days) No exclusions	Dimension Rx L clinical chemistry system (Siemens Medical Solutions Diagnostics, Erlangen, Germany)	0.1 Manufacturer's recommendation	ISHLT 1990, 3A 3%	Acute Cellular Rejection Dichotomous and continuous	high

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

Study ID	Design	Number of patients, number of samples, and demographic	Age (Mean ± SD, years)	Sex (% male)	Troponin type	Troponin measurement period post transplantation and early measurement exclusions	Troponin measurement method	Troponin threshold (ng/ml) and Selection method	Classification threshold for significant rejection and samples with significant rejection (%)	Outcome(s) and effect measure(s)	Modified QUADAS-2 risk of bias
Garrido (43)	Single Centre Prospective	21 Not applicable Adult	60.0 ± 10.0	81.0%	TnT	1 year onwards No exclusions	Electrochemiluminescence immunoassays with a Modular Analytics E170 analyzer (Roche Diagnostics GmbH, Mannheim, Germany)	0.026 Receiver operating characteristic analysis	Not applicable	Cardiac allograft vasculopathy Dichotomous and continuous	high
Gleissner (44)	Single Centre Retrospective	132 788 Adult	58.5 ± 9.4	85.6%	TnT	3 months–48 months (mean 13 months) Exclusions: first 3 months after transplantation	Enzymun-Test TnT enzyme immunometric assay (Boehringer Mannheim Diagnostics GmbH, Mannheim, Germany)	0.14 Receiver operating characteristic analysis	ISHLT 1990, 3A 13%	Acute Cellular Rejection Dichotomous and continuous	Low
Halwachs (45)	Single Centre Retrospective	15 183 Adult	49.8 ± 13.6	80.0%	TnT	1 day to 2 years No exclusions	Enzymun-Test TnT enzyme immunometric assay (Boehringer Mannheim Diagnostics GmbH, Mannheim, Germany)	0.2 Manufacturer's recommendation	ISHLT 1990, 3A 1%	Acute Cellular Rejection Continuous	unclear
Hossein-Nia (48)	Single Centre Prospective	15 65 Adult	Not reported	Not reported	TnT	Postoperative onwards No exclusions	Enzymun-Test TnT enzyme immunometric assay (Boehringer Mannheim Diagnostics GmbH, Mannheim, Germany)	0.2 Manufacturer's recommendation	ISHLT 1990, 2 16%	Acute Cellular Rejection Continuous	low
Hossein-Nia (46)	Single Centre Prospective	29 Not reported Adult	48.5 ± 7.8	83.9%	TnT	Postoperative onwards (mean 87 ± 32 weeks) No exclusions	Enzymun-Test TnT enzyme immunometric assay (Boehringer Mannheim Diagnostics GmbH, Mannheim, Germany)	0.2 Manufacturer's recommendation	ISHLT 1990, 2 Not reported	Acute Cellular Rejection Dichotomous	unclear
Hossein-Nia (47)	Single Centre Prospective	17 214 Adult	Not reported	Not reported	TnI	Postoperative onwards (mean 61 ± 16 days) No exclusions	TnI Assay (Sanofi Diagnostic Pasteur Ltd., Guildford, United Kingdom)	Not reported Not reported	ISHLT 1990, 2 4%	Acute Cellular Rejection Continuous	unclear
Hsu (49)	Single Centre Prospective	51 71 Adult	47.8 ± 11.3	80.0%	TnI	1 week to 5 years No exclusions	R&D Systems ELISA (R & D Systems USA, Minneapolis, Minnesota, USA)	Not reported Not reported	ISHLT 1990, 2 23%	Acute Cellular Rejection Continuous	high
Mendez (50)	Multicentre Prospective	73 224 Adult	54.0 ± 14.0	71.2%	hsTnT	Within 3 months of surgery to over 18 months, as needed No exclusions	Elecsys Electrochemiluminescence Immunoassay (Roche Diagnostics, Indianapolis, Indiana, USA)	0.017 Receiver operating characteristic analysis	ISHLT 2004, 2R 7%	Acute Cellular Rejection Dichotomous and continuous	low
Moran (51)	Single Centre Prospective	37 85 Paediatric	Median 12.4, range 1.3–30.0	Not reported	TnI, TnT	2.05 ± 2.43 years (median, 0.9; range, 0.03–9.1) No exclusions	Elecsys Electrochemiluminescence Immunoassay (Roche Diagnostics, Indianapolis, Indiana, USA)	TnI: 0.5 Receiver operating characteristic analysis TnT: Not reported	ISHLT 1990, 3A 15%	Acute Cellular Rejection Dichotomous and continuous	high

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

Study ID	Design	Number of patients, number of samples, and demographic	Age (Mean ± SD, years)	Sex (% male)	Troponin type	Troponin measurement period post transplantation and early measurement exclusions	Troponin measurement method	Troponin threshold (ng/ml) and Selection method	Classification threshold for significant rejection and samples with significant rejection (%)	Outcome(s) and effect measure(s)	Modified QUADAS-2 risk of bias
Mullen (52)	Single Centre Prospective	29 173 Adult	52.0 ± 5.4	79.3%	TnI, TnT[b]	12–564 days (mean 129 ± 9 days) No exclusions	Not reported	Not reported Not reported	ISHLT 1990, 3A 1%	Acute Cellular Rejection Continuous	low
Munoz-Esparza (53)	Single Centre Prospective	72 Not reported Adult	53.0 ± 13.0	75.0%	hsTnT	Within 1 year No exclusions	Elecsys Electrochemiluminescence Immunoassay (Roche Diagnostics, Indianapolis, Indiana, USA)	0.035 Receiver operating characteristic analysis	ISHLT 2004, 2R 43%	Acute Cellular Rejection Dichotomous and continuous	high
Ogawa (54)	Multicentre Prospective	69 683 Adult	50.0 ± 10.0	79.7%	TnT	9–141 weeks (mean 53 ± 26 weeks) No exclusions	Elecsys Electrochemiluminescence Immunoassay (Roche Diagnostics, Indianapolis, Indiana, USA)	Not reported Not reported	ISHLT 1990, 3A 4%	Acute Cellular Rejection Continuous	unclear
Patel (55)	Multicentre Retrospective	98 418 Adult	53.8 ± 12.1	83.0%	hsTnI	1 week—long term (median 1522 (IQR 773–2160) days) No exclusions	ARCHITECT i2000sr STAT high-sensitivity cTnI assay (Abbott Diagnostics, Abbott Park, Illinois, USA)	0.015 Receiver operating characteristic analysis	ISHLT 2004, 2R 5%	Acute Cellular Rejection Dichotomous and continuous	unclear
Siaplaouras (56)	Single Centre Retrospective	25 Not reported Paediatric	Mean 2 months, range 2 weeks–13 years	40.0%	TnI	3 weeks to 4 years No exclusions	Stratus Cardiac TnI fluorometric enzyme immunoassay (Dade-Behring, Newark, Delaware, USA)	0.6 Manufacturer's recommendation	ISHLT 1990, 3A Not reported	Acute Cellular Rejection Dichotomous	high
Vazquez-Rodriguez (57)	Single Centre Prospective	62 259 Adult	Not reported	85.5%	TnT	Postoperative onwards Exclusions: None and first 3 months after transplantation	Enzymun-Test TnT enzyme immunometric assay (Boehringer Mannheim Diagnostics GmbH, Mannheim, Germany)	0.1 Manufacturer's recommendation	ISHLT 1990, 2 25%	Acute Cellular Rejection Dichotomous	low
Wåhlander (58)	Single Centre Prospective	14 78 Paediatric	Not reported	Not reported	TnI	1 month onwards Exclusions: first 1 month after transplantation	Elecsys Electrochemiluminescence Immunoassay (Roche Diagnostics, Indianapolis, Indiana, USA)	0.1 Manufacturer's recommendation	ISHLT 1990, 3A 12%	Acute Cellular Rejection Dichotomous and continuous	unclear
Walpoth (59)	Single Centre Prospective	25 392 Adult	Not reported	Not reported	TnT	Postoperative to 2 years No exclusions	Enzymun-Test TnT enzyme immunometric assay (Boehringer Mannheim Diagnostics GmbH, Mannheim, Germany)	0.2 Manufacturer's recommendation	Texas score, 4 Not reported	Acute Cellular Rejection Continuous	unclear
Wang (60)	Single Centre Prospective	186 358 Adult	Not reported	Not reported	TnI, TnT[b]	Postoperative onwards Exclusions: first 5 weeks after transplantation	TnI: Stratus Cardiac TnI fluorometric enzyme immunoassay (Dade-Behring, Newark, Delaware, USA) TnT: Enzymun-Test TnT enzyme immunometric assay (Boehringer Mannheim Diagnostics GmbH, Mannheim, Germany)	TnI: 1.7 Not reported TnT: 0.07 Not reported	ISHLT 1990, 3A 21%	Acute Cellular Rejection Dichotomous and continuous	high

[a]*Where studies measured both conventional and high sensitivity troponin variants and underwent meta-analysis, high sensitivity troponin was included in quantitative analysis where appropriate.*

[b]*Where studies measured both troponin I and T subtypes and underwent meta-analysis, troponin I measurements was chosen for quantitative synthesis and a sensitivity analysis was performed by including troponin T measurements to determine the impact of this decision. TnT, Troponin T; TnI, Troponin I; hsTnT, High Sensitivity Troponin T; hsTnI, High Sensitivity Troponin I.*

RESULTS

Search Results

We identified 1,927 results through the search, and one additional citation through reference lists. After automatic deduplication, we screened 1,499 titles and abstracts. We reviewed full texts of 68 potentially relevant studies, from which 27 were included in this review, with 20 in quantitative form (**Figure 1**).

Description of Included Studies

Twenty-seven studies (34–60) involving 1,684 cardiac transplant recipients were included. Detailed characteristics of included studies are reported in **Table 1**.

Methodological Quality

Methodological quality was variable. Five studies (44, 48, 50, 52, 57) were deemed low risk of bias, 12 studies (36–40, 45–47, 54, 55, 58, 59) unclear risk of bias due to no specific reporting of certain domain characteristics, and 10 studies (34, 35, 41–43, 49, 51, 53, 56, 60) high risk of bias. The full QUADAS-2 assessment can be found in the **Supplementary Material**.

Descriptive Analyses and Meta-Analysis

Acute Cellular Rejection

Adult

No Temporal Exclusion Criteria.

Dichotomous Measure of Diagnostic Accuracy. Eight studies (35, 38, 42, 50, 53, 55, 57, 60) with 840 participants reported sensitivity, specificity, and AUC values regarding the ability of troponin to diagnose acute cellular rejection in heart transplant recipients. We found a pooled sensitivity of 0.479 (95% CrI 0.190–0.783), specificity of 0.702 (95% CrI 0.395–0.910), and BAUC 0.584 (95% CrI 0.377–0.760) (**Figure 2**).

As one included study (60) measured both troponin I and T values, we performed a sensitivity analysis investigating the effects of including troponin T measurements instead of troponin I in quantitative synthesis. The result was not significantly different; pooled sensitivity was 0.498 (95% CrI 0.206–0.788), specificity 0.696 (95% CrI 0.387–0.901), and BAUC 0.591 (95% CrI 0.385–0.758) (**Supplementary Figure S1**).

Hossein-Nia 1995 (46) reported sensitivity of 0.333 but did not report a corresponding specificity.

We investigated potential sources of statistical heterogeneity with a meta-regression, and found that the troponin assay sensitivity and ISHLT rejection criteria, study year, and number of study centres were significant AUC modifiers (**Supplementary Table S1**). In particular, studies which used high sensitivity troponin assays were also those which used the ISHLT 2004 criteria, and this was associated with a 0.210 increased AUC (p = 0.0006) (**Supplementary Figure S2**). A unit increase in study year was associated with an increased AUC of 0.014 (p = 0.0010), and a multicentre study design was associated with an increased AUC of 0.189 (p = 0.0154) compared to a single centre design (**Supplementary Figure S3**). Notably, the following were not significant AUC modifiers: ISHLT cut-off grade for definition of significant rejection (1R vs. 2R in ISHLT 2004; 2 vs. 3A in ISHLT 1990), prevalence of samples with significant rejection per cohort, troponin threshold, and study risk of bias.

Continuous Measure of Diagnostic Accuracy. Eleven studies (34, 37, 42, 45, 47, 49, 50, 52–55) with 641 participants reported troponin mean differences between those with and without acute cellular rejection. We found that the standardised troponin measurements were not significantly different in those with and without acute cellular rejection (SMD 0.49, 95% CI −0.33–1.31) (**Figure 3**).

As one included study (52) measured both troponin I and T values, we performed a sensitivity analysis investigating the effects of including troponin T measurements instead of troponin I in quantitative synthesis. The result was not significantly different (pooled SMD 0.26, 95% CI −0.64–1.16) (**Supplementary Figure S4**).

Wang 1996 (60) reported mean measurements in both troponin I and T between acute cellular rejection vs. non-rejection groups (0.216 vs. 0.707 and 0.134 vs. 0.088 ng/ml respectively); however, neither were statistically significant (p = 0.357 and p = 0.374 respectively). Contrary to this, Walpoth 1998 (59) reported statistically significant elevations (no measure of statistical significance reported) troponin T measurements between acute cellular rejection (0.77 ± 0.80 ng/ml) and non-rejection (0.02 ± 0.05 ng/ml) groups. Hossein-Nia 1993 (48) reported an elevated median troponin T in those with acute cellular rejection compared to without (0.370 vs. 0.300 ng/ml); however, statistical significance was not reported.

Between-study statistical heterogeneity was considerable (I^2 statistic 95%). We investigated potential sources of statistical heterogeneity with a meta-regression, and found that the troponin assay sensitivity and ISHLT rejection criteria (overlapping exactly; all studies using high sensitivity troponin also used ISHLT 2004 criteria), study year, troponin threshold, and standard deviation of age were significant SMD modifiers and accounted for up to 49% of heterogeneity on univariable analysis (**Supplementary Table S2**). Notably, the following were not significant SMD modifiers: ISHLT cut-off grade for definition of significant rejection (1R vs. 2R in ISHLT 2004; 2 vs. 3A in ISHLT 1990), prevalence of samples with significant rejection per cohort, and study risk of bias.

A regression test for funnel plot asymmetry was unable to detect significant publication bias (p = 0.1023) (**Supplementary Figure S5**).

Early Postoperative Exclusion Criteria.

Dichotomous Measure of Diagnostic Accuracy. After exclusion of measurements from the early postoperative period (at least 1 month postoperatively), eight single centre studies (34, 35, 38, 39, 41, 44, 57, 60) with 825 participants reported sensitivity, specificity, and AUC values regarding the ability of troponin to diagnose acute cellular rejection in heart transplant recipients. We found a pooled sensitivity of 0.414 (95% CrI 0.174–0.696), specificity of 0.785 (95% CrI 0.567–0.912), and BAUC 0.607 (95% CrI 0.469–0.723) (**Figure 4**).

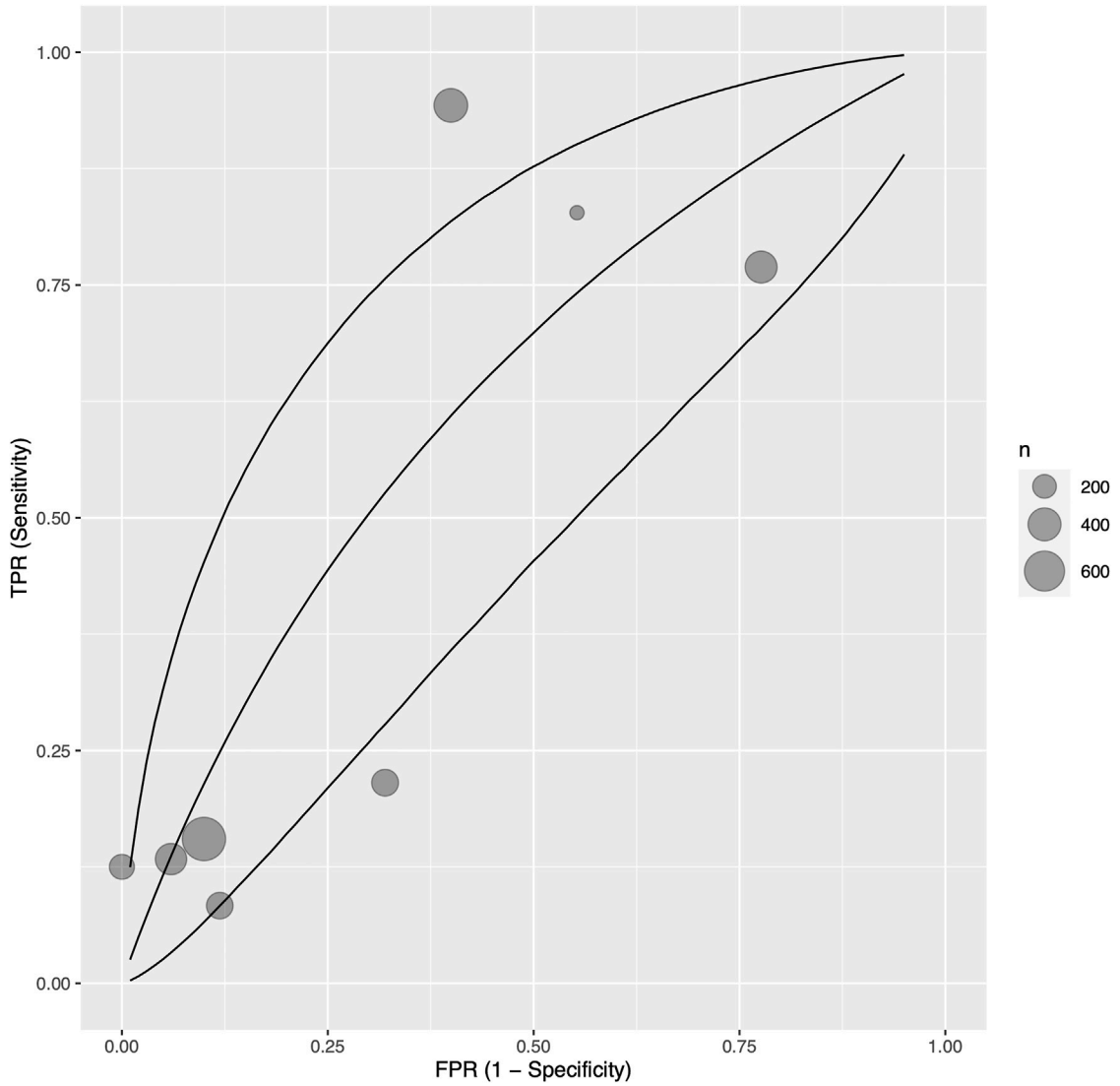

FIGURE 2 | Bayesian summary receiver operating characteristic curve showing summary diagnostic accuracy of recipient troponin in acute rejection with no temporal exclusions, with upper and lower 95% credible bands. Each filled circle represents one included study, the size of which is weighted in proportion to the study's sample size.

We investigated potential sources of statistical heterogeneity with a meta-regression, and found that the troponin assay sensitivity and ISHLT rejection criteria, and troponin type, and study design were significant AUC modifiers (**Supplementary Table S3**). In particular, use of high sensitivity troponin I assays by one study (34) corresponded exactly to use of ISHLT 2004 criteria, and was associated with a 0.257 increase in AUC ($p = 0.0270$) (**Supplementary Figure S6**). Of note, the length of early postoperative exclusion (from 1 month to 6 months) was not associated with significant changes to troponin's diagnostic ability. Additionally, the following were not significant SMD modifiers: ISHLT cut-off grade for definition of significant rejection (1R vs. 2R in ISHLT 2004; 2 vs. 3A in ISHLT 1990), prevalence of samples with significant rejection per cohort, troponin threshold, and study risk of bias.

Continuous Measure of Diagnostic Accuracy. Five studies (34, 36, 38, 39, 44) with 476 participants reported troponin mean differences between those with and without acute cellular rejection. We found that the standardised troponin measurements were higher in those with acute cellular rejection, and that this was a large and statistically significant effect (SMD 0.98, 95% CI 0.33–1.64) (**Figure 5**).

Wang 1996 (60) reported mean measurements in both troponin I and T between acute cellular rejection vs. non-rejection groups (0.059 vs. 0.102 and 0.069 vs. 0.044 ng/ml respectively) after measurements during the first 5 weeks were

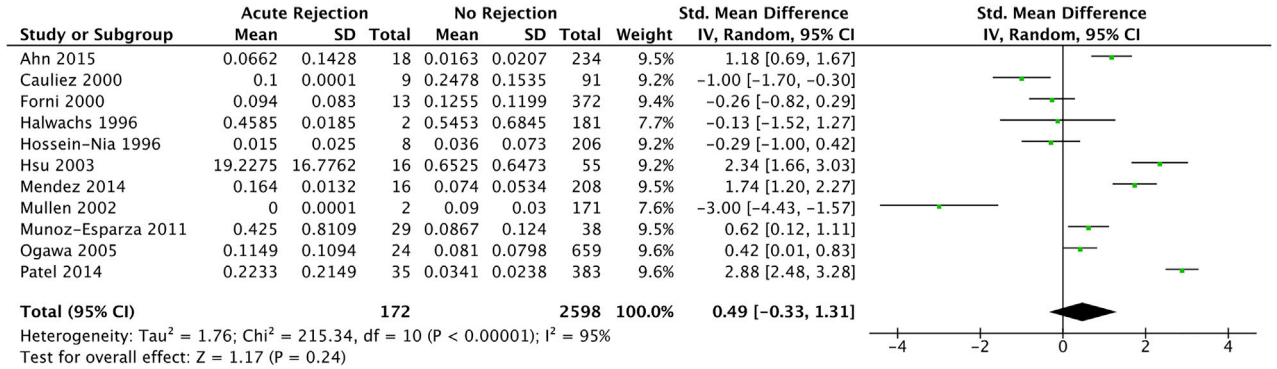

FIGURE 3 | Forest plot of standardised mean differences for elevated recipient troponin in diagnosing acute rejection post cardiac transplantation, with no temporal exclusions.

excluded; however, neither were statistically significant ($p = 0.713$ and $p = 0.382$ respectively).

Statistical heterogeneity was considerable (I^2 statistic 95%); however, meta-regression was not possible due to insufficient study numbers ($n = 5$).

Paediatric

No Temporal Exclusion Criteria. Two studies (51, 56) with 62 participants investigated the association between troponin and adverse outcomes in cardiac transplantation recipients. Moran 2000 (51) found that troponin I values differed significantly across ISHLT 1990 grades 0, 1, 2, and 3 on analysis of variance (ANOVA) ($p = 0.034$), with a diagnostic sensitivity of 0.500 and specificity of 0.776. However, troponin T values were not significantly different across ISHLT 1990 grades 0, 1, 2, and 3 on ANOVA ($p = 0.16$)—sensitivity was 0.421 and specificity was 0.837. Siaplaouras 2003 (56) found a sensitivity of 0.750, but did not report a corresponding specificity.

Early Postoperative Exclusion Criteria. After exclusion of measurements from the early postoperative period (at least 1 month postoperatively), three studies (40, 56, 58) with 81 participants studied the association between troponin and adverse outcomes in cardiac transplantation recipients. Excluding measurements from the first 3 months after transplantation, Dyer 2012 (40) reported a statistically significant elevation in high sensitivity troponin T values in those with acute cellular rejection (SMD 2.44, 95% CI 1.51–3.37), and a sensitivity of 0.857 and specificity of 0.913. Wa˚hlander 2002 (58) found that conventional troponin T values were also elevated in those with acute cellular rejection (SMD 1.43, 95% CI 0.70–2.17), reporting a sensitivity of 0.556 and specificity of 0.768. Siaplaouras 2003 (56) found a sensitivity of 0.591, but did not report a corresponding specificity.

DISCUSSION

In this systematic review and meta-analysis of 27 diagnostic observational studies involving over 1,600 patients, we provide the most up-to-date evidence of the value of troponin in diagnosing adverse outcomes in heart transplant recipients. We found that late troponin levels (measured at least 1 month postoperatively) were significantly elevated in adult recipients with acute cellular rejection. Diagnostic accuracy of plasma troponin was slightly higher for measurements taken after the early postoperative period compared to those taken across all postoperative periods; however, the diagnostic ability of both were poor.

The poor diagnostic utility of troponin in the immediate to early post-operative period in detecting acute cellular rejection is not surprising given the manifold pathologies that can drive elevated plasma troponin in this setting (61). Our results suggest that this “early” post-operative period is confined to 1 month, with no significant difference in diagnostic accuracy of troponins measured after 1 month compared to 6 months post-transplant. However, risk of rejection is also highest in the first months after transplant (62), coinciding with this period of poorer diagnostic utility. Biomarkers capable of distinguishing between early acute rejection and routine perioperative cardiac injury are needed.

Additionally, our meta-regressions suggest that the utility of troponin may be improving over time, with study year being positive effect modifier in multiple analyses. While this is possibly attributable to the transition to high-sensitivity troponin assays, these findings are also confounded by a perfect overlap with a transition to the ISHLT 2004 criteria for acute cellular rejection.

Our search revealed one other systematic review, without meta-analysis, on a similar topic (63). However, this literature search excluded key databases (Embase and the Cochrane Library) and therefore may have lacked sensitivity, with only 88 abstracts identified and 12 studies included in the final analysis; there was no formal assessment of methodological quality; and there was no quantitative meta-analysis or assessment and management of potential sources of heterogeneity. Hence, we believe our study adds to the existing knowledge base, and provides the most recent and high-quality synthesis regarding the diagnostic value of cardiac troponin in heart transplant recipients.

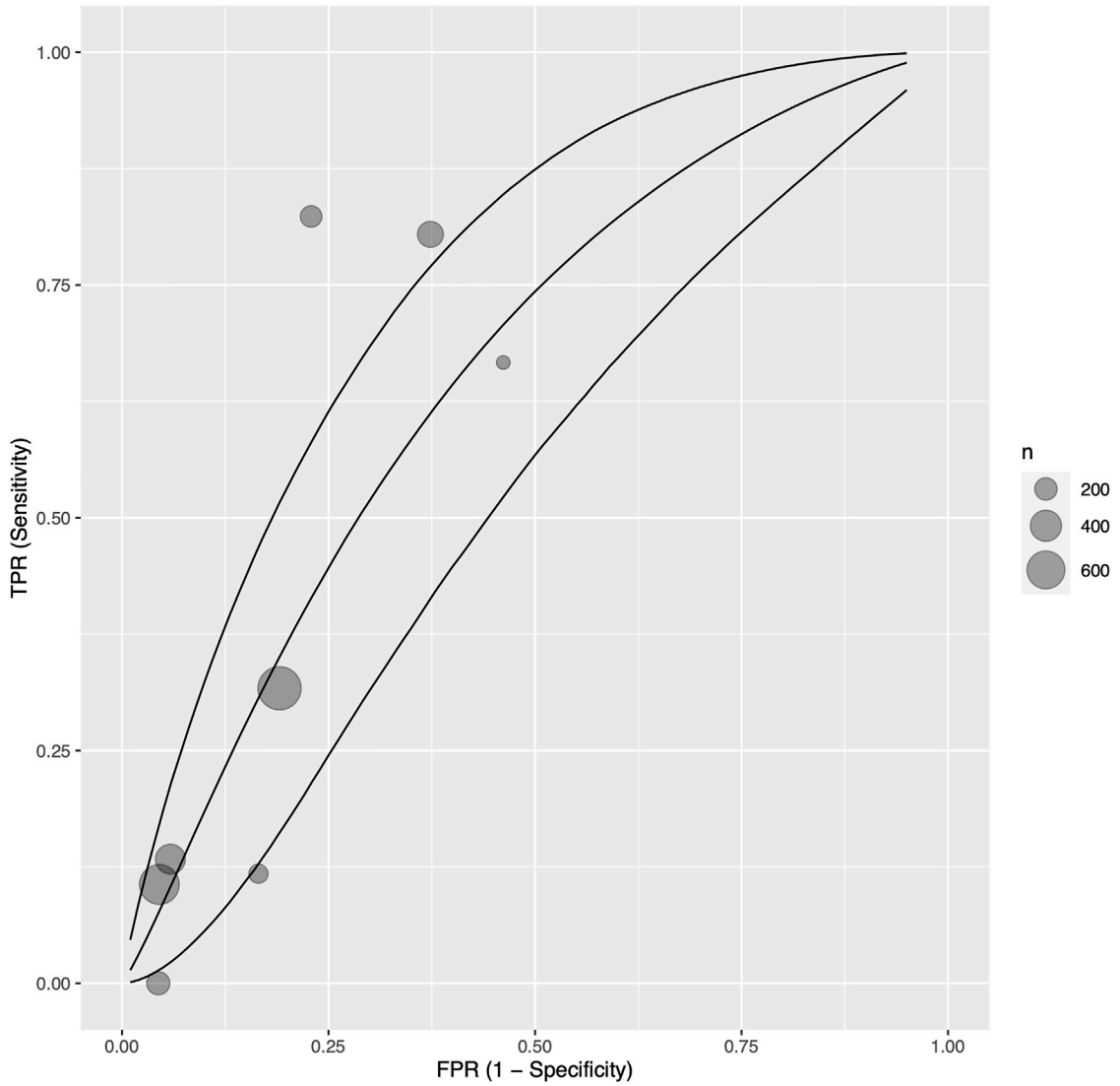

FIGURE 4 | Bayesian summary receiver operating characteristic curve showing summary diagnostic accuracy of recipient troponin in acute rejection with early postoperative measurements (at least 1 month postoperative) excluded, with upper and lower 95% credible bands. Each filled circle represents one included study, the size of which is weighted in proportion to the study's sample size.

	Acute Rejection			No Rejection				Std. Mean Difference
Study or Subgroup	Mean	SD	Total	Mean	SD	Total	Weight	IV, Random, 95% CI
Ahn 2015	0.0593	0.1281	17	0.0096	0.0096	166	18.8%	1.26 [0.75, 1.78]
Balduini 2003	0.1063	0.0958	34	0.06	0.0445	115	19.8%	0.77 [0.38, 1.16]
Chance 2001	0.1061	0.0525	39	0.0578	0.022	624	20.2%	1.95 [1.61, 2.29]
Dengler 1998	0.0601	0.0576	46	0.0276	0.0384	369	20.3%	0.79 [0.48, 1.10]
Gleissner 2003	0.133	0.1566	101	0.1051	0.1406	687	20.9%	0.20 [−0.01, 0.40]
Total (95% CI)			**237**			**1961**	**100.0%**	**0.98 [0.33, 1.64]**

Heterogeneity: $Tau^2 = 0.52$; $Chi^2 = 78.42$, df = 4 ($P < 0.00001$); $I^2 = 95\%$
Test for overall effect: $Z = 2.96$ ($P = 0.003$)

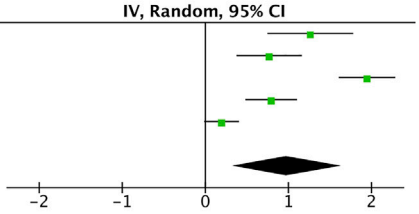

FIGURE 5 | Forest plot of standardised mean differences for elevated recipient troponin in diagnosing acute rejection post cardiac transplantation, with early postoperative measurements (at least 1 month postoperative) excluded.

Our review should be interpreted with the following limitations. While five studies were identified to be at low risk of bias, the 22 remaining studies were at unclear or high risk of bias; reassuringly though, study risk of bias was not found to be a significant effect modifier in all meta-regressions where this was possible. Studies did not report timing of troponin sample

procurement—before vs. after EMB—despite this being a possible confounder as procedure related injury can occur. The majority of studies were single centre, raising potential concerns for external validity. Finally, despite our efforts in determining significant sources of statistical heterogeneity, we were not able to account for all observed statistical heterogeneity. Although our prespecified use of a random-effects model is a strength of our study design, our findings are nonetheless tempered by unaccounted heterogeneity—an inherent part of meta-analysis methodology—which may be attributable to systematic differences in unreported study baseline characteristics as well as other study and patient-level factors. Large, high quality, randomised studies would be needed to control for these unmeasured factors in particular.

In accordance with international guidelines (21, 22), our results do not support the routine use of troponin for surveillance or diagnosis of acute cellular rejection. However, our work identifies many opportunities for future research. The current gold standard diagnostic test for acute cellular rejection involves an invasive EMB which exposes patients to small but significant risks (3–5) and can be associated with poor pathological concordance (2); safer and more effective diagnostic tests are urgently needed. While numerous imaging parameters and biomarkers have been investigated for this purpose, donor-derived cell-free DNA has recently emerged as a promising, non-invasive marker of acute rejection detection (64). Not only is donor-derived cell-free DNA able to detect episodes of rejection with remarkable sensitivity and specificity, but it may also be able to distinguish between acute cellular rejection and antibody mediated rejection, at time points earlier than possible with EMBs (65). As accurate as any one diagnostic marker may be however, experience from multiple disciplines has supported the implementation of well-selected multi-biomarker diagnostic panels over any singular biomarker (66–68). Accordingly, opportunity exists to assess elevated high-sensitivity cardiac troponin—a sensitive and specific marker of the cardiomyocyte death which occurs during acute rejection—in conjunction with emerging biomarkers representing different pathophysiological aspects of acute cellular rejection to optimise the "liquid biopsy" approach and reduce uncertainty and clinical risk of the current EMB approach. While the diagnostic ability of troponin (in the early postoperative month in particular) as a single parameter is insufficient to warrant implementation, whether or not its diagnostic utility can be enriched by integration into sophisticated multivariable diagnostic models with other non-invasive haematological and clinical markers is a field with significant potential. High-sensitivity troponin in particular may possess sufficiently high negative predictive value aid in ruling out acute cellular rejection (55, 63). Additionally, in order to optimise methodological quality and minimise risk of study bias, future researchers should design and report diagnostic test accuracy studies in accordance with QUADAS-2, among other design and reporting guidelines.

CONCLUSION

In this systematic review and meta-analysis, we found a positive association between late troponin elevation and acute cellular rejection in adults, however diagnostic performance was insufficient to support its routine use in the diagnostic pathway. Further research is warranted to assess whether the addition of troponin to emerging biomarkers of acute cellular rejection, such as circulating cell-free DNA, could lead to an enhanced "liquid biopsy" capable of superseding invasive testing.

AUTHOR CONTRIBUTIONS

ZL—Data acquisition, analysis, and interpretation, drafting and critical revisions of manuscript. LP—Study conception, data interpretation, critical revisions of manuscript. JP-D—Study conception, data analysis, critical revisions of manuscript. MH—Data acquisition, analysis, and critical revisions of manuscript. IO—Data acquisition and critical revisions of manuscript. MP—Data interpretation and critical revisions of manuscript. RS—Data interpretation and critical revisions of manuscript. JS—Data interpretation and critical revisions of manuscript. All authors approve the version to be published.

CONFLICT OF INTEREST

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

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2022.10362/full#supplementary-material

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Using Information Available at the Time of Donor Offer to Predict Kidney Transplant Survival Outcomes: A Systematic Review of Prediction Models

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Statistical models that can predict graft and patient survival outcomes following kidney transplantation could be of great clinical utility. We sought to appraise existing clinical prediction models for kidney transplant survival outcomes that could guide kidney donor acceptance decision-making. We searched for clinical prediction models for survival outcomes in adult recipients with single kidney-only transplants. Models that require information anticipated to become available only after the time of transplantation were excluded as, by that time, the kidney donor acceptance decision would have already been made. The outcomes of interest were all-cause and death-censored graft failure, and death. We summarised the methodological characteristics of the prediction models, predictive performance and risk of bias. We retrieved 4,026 citations from which 23 articles describing 74 models met the inclusion criteria. Discrimination was moderate for all-cause graft failure (C-statistic: 0.570–0.652; Harrell's C: 0.580–0.660; AUC: 0.530–0.742), death-censored graft failure (C-statistic: 0.540–0.660; Harrell's C: 0.590–0.700; AUC: 0.450–0.810) and death (C-statistic: 0.637–0.770; Harrell's C: 0.570–0.735). Calibration was seldom reported. Risk of bias was high in 49 of the 74 models, primarily due to methods for handling missing data. The currently available prediction models using pre-transplantation information show moderate discrimination and varied calibration. Further model development is needed to improve predictions for the purpose of clinical decision-making.

Systematic Review Registration: https://osf.io/c3ehp/l.

Keywords: kidney transplantation, prognosis, systematic review, clinical prediction model, decision-making, kidney transplant outcomes, prediction tool

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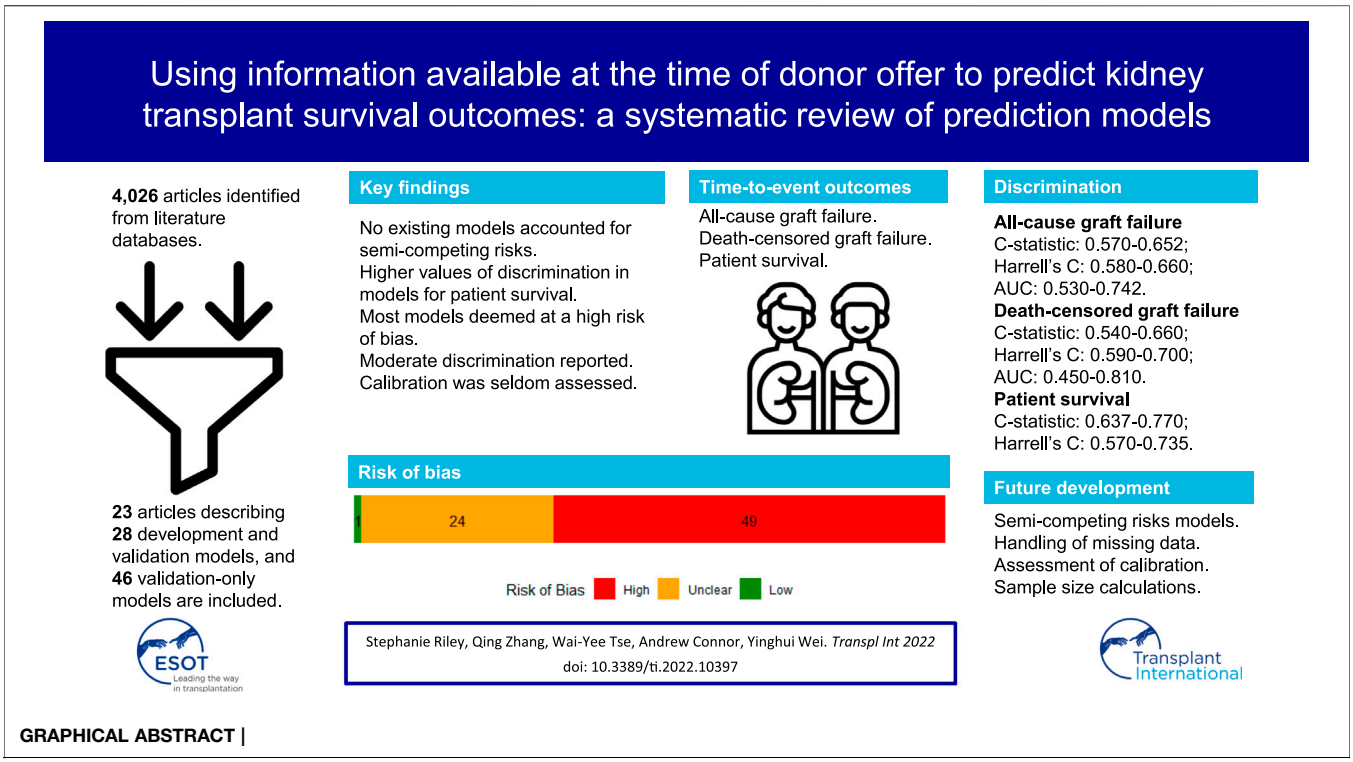
Abbreviations: AUC, area under receiver operating characteristic curve; CHARMS, Critical Appraisal and Data Extraction for Systematic Reviews of Prediction Modelling Studies; DGF, delayed graft function; EPTS, Estimated Post Transplant Survival; ESKD, end-stage kidney disease; KDRI, Kidney Donor Risk Index; LKDPI, Living Kidney Donor Profile Index; MAPI, Maryland Aggregate Pathology Index; NHSBT, National Health Service Blood and Transplant; PITHIA, PreImplantation Trial of Histopathology In renal Allografts; PRISMA, Preferred Reporting Items for Systematic reviews and Meta-Analyses; PROBAST, Prediction Model Risk of Bias Assessment Tool; RoB, risk of bias; UK KDRI, United Kingdom Kidney Donor Risk Index.



GRAPHICAL ABSTRACT |

INTRODUCTION

End-stage kidney disease (ESKD) is the most advanced stage of chronic kidney disease. Kidney transplantation is the optimal treatment for many patients with ESKD. In the UK, approximately 3,000 kidney transplants are performed every year, but the number of patients waiting for a transplant is around 5,000 (1). The success, in terms of efficacy and longevity, of an individual transplant will be influenced by a host of factors, some of which can be determined prior to transplantation. A balance must be struck to ensure maximal organ utilisation without compromising transplant outcomes. This is further complicated by the fact that "one size does not fit all"—the definition of a successful transplant will vary depending on the recipient and their clinical scenario. As such, every potential kidney transplant must be carefully considered in the context of the donor and recipient details.

In the UK donor organs are offered through a national donation system, which utilises an algorithm to balance patient priority and the intent to match immunological and additional parameters. The donor offers are reviewed by clinicians acting on behalf of the recipient and a prompt decision must be made to accept or reject each offer. Whether or not to accept a transplant offer remains a challenging clinical decision. Clinical prediction models that utilise information which would commonly be available to the clinician at the time of the donor kidney offer may help to inform the decision-making process.

The anticipated longevity of a kidney transplant is, of course, an important consideration for a clinician faced with the kidney donor acceptance decision. However, given that donor kidneys are a scarce resource and potential recipients must therefore sit on waiting lists, it is often appropriate to balance the anticipated longevity against the alternative of remaining on dialysis. As such, models that can predict graft survival outcomes would be of great clinical utility.

Prediction models have previously been developed for kidney transplant survival outcomes with the aim of advising clinicians at the time of the offer of a donor kidney. The number of articles related to clinical prediction models for kidney transplant survival outcomes is increasing year on year, suggesting a recognition of the clinical interest. The Kidney Donor Risk Index (KDRI) (2), Estimated Post Transplant Score (EPTS), Maryland Aggregate Pathology Index (MAPI) (3) and Living Kidney Donor Profile Index (LKDPI) (4) are commonly reported risk indices. The KDRI and EPTS are part of the kidney allocation system in the US.

The aforementioned risk indices were developed in the US population. A similar index has been produced in the UK (UK KDRI) (5), though is not widely used in practice. In the UK kidney allocation system NHS Blood and Transplant (NHSBT) use their own risk indices for donors and recipients (6). This is to help ensure that the pool of donor kidneys is utilised to best effect. Through this system, for example, younger recipients will typically receive offers of kidneys from younger donors (in order to optimise the chances of transplant longevity) whilst a greater tolerance of less favourable immunological matches will be accepted for older recipients (in order to maximise offers for a

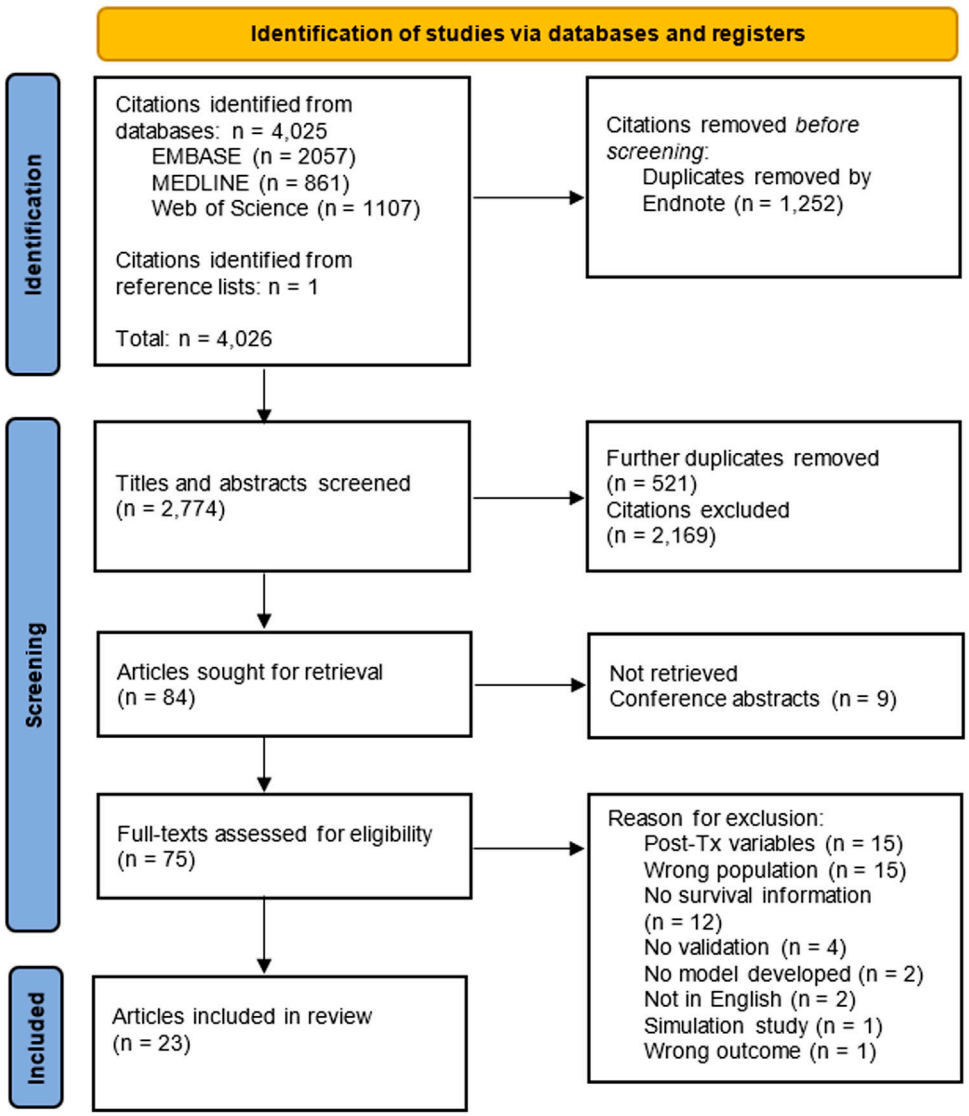


FIGURE 1 | Flowchart of articles eligible for inclusion in the systematic review. Each database was searched from their respective date of inception until April 8th 2021.

cohort in whom immunological matching is of slightly less importance).

We identified two systematic reviews exploring existing prediction models for kidney transplantation. Kaboré et al. (7) reviewed prediction models for graft outcomes published between 2005 and 2015, while Senanayake et al. (8) reviewed machine learning methods to predict graft failure, delayed graft function (DGF) and acute graft rejection. Since only machine learning models were eligible, their review excluded articles that used the Cox model, which is the model most used for time-to-event analyses.

Both reviews allowed the inclusion of predictors that only become available after transplantation, such as whether patients experienced DGF. To our knowledge this is the first review to focus only on models that could aid clinical decision-making at the time of the donor offer.

In this systematic review we aim to identify, appraise and summarise existing clinical prediction models for kidney transplant survival outcomes. Only prediction models that use information available at the time of the single kidney-only offer were included, allowing us to focus on models with the most clinical utility.

METHODS

We prospectively developed a protocol which is publicly available from OSF (9). The findings of this review are reported in

TABLE 1 | Summary of sample size used in models for each outcome and model type.

	Number of models	Range	Median	Mean	SD
All-cause graft failure					
Development and validation	11	785–156,069	39,108	41,127	48,719
Validation only	15	416–69,994	5,042	8,641	17,141
Death-censored graft failure					
Development and validation	5	259–10,086	6,662	5,586	4,811
Validation only	19	56–6,405	1,299	3,017	2,909
Patient survival					
Development and validation	11	837–120,818	47,535	41,319	38,270
Validation only	11	935–5,042	4,983	3,323	2,007

Two models with other outcomes which do not fall into the above definitions have sample size of 20,085 and 2,734, respectively.

accordance with the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) statement (10).

Eligibility Criteria

We included studies with adult recipients (aged 18 years or older) of single, kidney-only transplants. No restrictions were placed on donor type.

No limit was set on publication date. Only full texts published in English were eligible. Conference abstracts without full text were excluded from review.

The outcomes of interest were one or both of the following outcomes, time to graft failure and time to death at any time point following kidney transplantation. Models that did not account for time-to-event information were excluded.

We considered prediction models that make use of information available at the time of a donor kidney offer to inform the acceptance decision. Prediction models developed using predictors that only become available after transplantation were not included, as the decision would have been made by that time.

We included studies which were developed and validated for the outcomes of interest, and validation-only studies which validated existing models developed from independent cohorts. Any measure of predictive performance, such as calibration or discrimination, that was reported alongside a model was considered a form of validation. Validation-only model refers to the case where the current study validates an existing model.

Information Sources and Search Strategy

Electronic databases Embase, MEDLINE and Web of Science were searched from their respective dates of inception up to April 8th, 2021. The search strategy is presented in **Supplementary Table S1**.

All citations from the search results were exported to Endnote, where duplicates were automatically removed from review. Titles and abstracts of all records were independently screened against the above eligibility criteria by two reviewers (SR and QZ) and managed through Rayyan (11). A third reviewer (YW) also independently screened 10% of the titles and abstracts. Two reviewers then independently reviewed full-text reports to assess eligibility (SR and QZ). Any discrepancies were resolved through discussion.

Data Extraction

Data were extracted from eligible articles according to the Critical Appraisal and Data Extraction for Systematic Reviews of Prediction Modelling Studies (CHARMS) checklist (12). The full list of data extracted are given in **Supplementary Table S2**. Data were extracted independently by two reviewers (SR and YW) and any discrepancies were resolved through discussion.

Risk of Bias

We assessed the risk of bias (RoB) in individual models using the Prediction Model Risk of Bias Assessment Tool (PROBAST) (13). Two reviewers (SR and YW) independently determined the RoB of each model and any disagreements were resolved by discussion.

Outcomes

All-Cause Graft Failure

All-cause graft failure, as a composite outcome, is defined as the earliest time to graft failure or death.

Death-Censored Graft Failure

Death-censored graft failure considers the time until graft failure, but patients are censored at the time of death. Graft failure and death are semi-competing events (14). Semi-competing events arise when a terminal event precludes a non-terminal event, but not vice-versa (15).

Death

This measures time to recipient death, of any cause, as the outcome of interest.

Analysis

Study Characteristics

We summarised the year of publication, geographical location, model type, and model being validated. We explored the discrimination measures by sample size and predictor type (donor, recipient, transplant, or combination of these). For each outcome, we summarised the type of predictors, modelling methods, and methods for handling missing data.

Measures of Model Performance

Model performance was evaluated by calibration and discrimination. Calibration assesses the agreement between

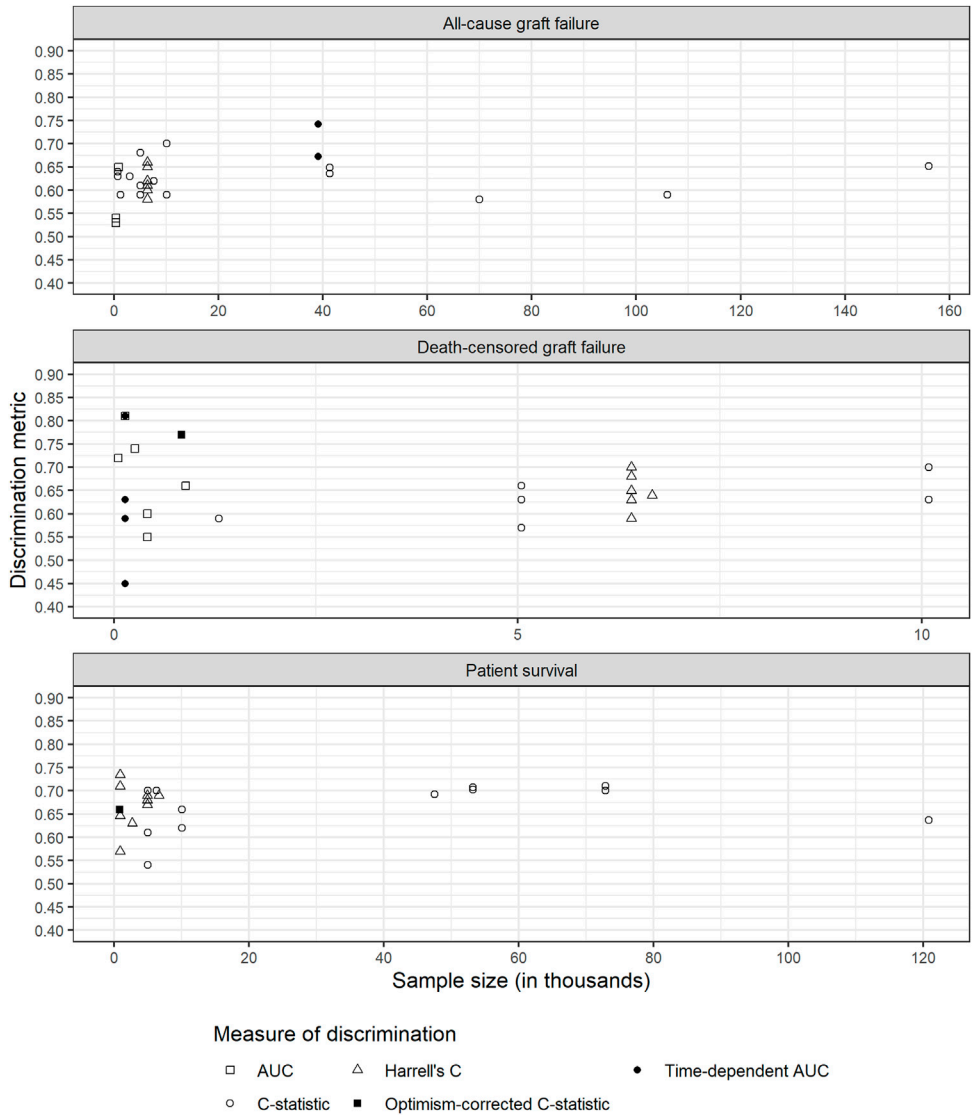


FIGURE 2 | Discrimination metrics against sample size for each outcome. AUC: area under receiver operating characteristic curve; C-statistic: concordance statistic; Harrell's C: adapts the C-statistic to account for censoring; Optimism-corrected C-statistic: measures the C-statistic while accounting for optimism in model performance; Time-dependent AUC: a measure of the AUC at specified timepoints since time origin.

observed and predicted risk and is often reported through a calibration plot. Discrimination measures a model's ability to separate recipients who will experience the outcome event versus those who will not. It is often measured using Harrell's C statistic, area under receiver operating characteristic curve (AUC) or time-dependent AUC, which account for the censoring of the time-to-event outcome. When a model is developed and internally validated in the same dataset it understandably performs well. Methods to correct for this optimism can be administered using bootstrapping, and resulting measures are referred to as optimism-corrected (16). Where studies did not explicitly state that the C-statistic was adapted for censoring, we elected to report the terminology used in the original articles.

RESULTS

We retrieved 4,025 citations from three databases through our search and identified one record related to one of the conference abstracts we screened. After the initial screening of titles and abstracts, 75 articles were retrieved for full-text review. Of these records, 23 articles describing 74 models met the inclusion criteria (3–5, 17-36) (**Figure 1**).

Characteristics of Included Studies

Of the 74 eligible models, 28 developed and validated a clinical prediction model for our outcomes of interest. The remaining 46 models validated the performance of an existing model in an independent cohort. Articles were published between 2005 and

TABLE 2 | Summary of commonly reported risk indices for predicting kidney transplant survival outcomes.

Model	Donor	Recipient	Transplant organ/process	Histopathology	Validation studies
EPTS	NA	Age; Diabetes status; Prior solid organ transplants; Time on dialysis	NA	NA	(22) (23) (28)
LKDPI	Age; eGFR; BMI; Ethnicity; History of cigarette use; Systolic blood pressure; Sex; Weight	Sex (compared to donor); Weight (relative to donor weight)	Number of HLA mismatch at HLA-B and HLA-DR; ABO compatibility	NA	(30)
MAPI	NA	NA	NA	Arteriolar hyalinosis; Glomerulosclerosis; Periglomerular fibrosis; Scar Wall-to-lumen ratio interlobular arteries	(25) (29)
UK KDRI	Age; Days in hospital; History of hypertension; Use of adrenaline; Weight	NA	NA	NA	(21)
US KDRI	Age; Cause of death; DCD; Diabetes status; Ethnicity; HCV status; Height History of hypertension Serum creatinine Weight	NA	Cold ischaemic time; Double kidney transplant; En-bloc transplant; Number of HLA mismatch at HLA-B and HLA-DR	NA	(20) (21) (23) (25) (4) (30) (5) (35)

BMI, body mass index; DCD, deceased cardiac donor; eGFR, estimated glomerular filtration rate; HCV, hepatitis C virus; HLA, human leukocyte antigen.

2020; fifteen of the twenty-three articles (65.22%) were published after 2015. Twelve articles used data of recipients from the United States, four from mainland Europe, three from Canada, two from Australia and New Zealand, and one each from the United Kingdom and Thailand. Characteristics of included studies for each model are available in **Supplementary Tables S3–S6**.

In the 28 development and validation models, 27 used the Cox proportional hazards model, while one (17) used a survival random forest. Only eight of the Cox models assessed the proportional hazards assumption.

There was considerable variability in sample sizes used for models (**Table 1**; **Figure 2**). In general, models performing validation alone tended to have smaller sample size. Models with smaller sample sizes did not have noticeably poorer discrimination for any of the outcomes (**Figure 2**).

We considered three types of predictors, donor characteristics, recipient characteristics and transplant process. We found no clear evidence that the type of predictors was associated with better discrimination for any outcome (**Supplementary Figures S1–S3**). Clayton et al. (21) validated the US and UK KDRI, while also adjusting for recipient characteristics and transplant process. Those with higher values of discrimination (models 9 and 12) were adjusted for other donor, recipient and transplant related predictors. This was also observed by Molnar et al. (28). However, this increase could simply be due to having more variables in the model.

Nine of the 28 development and validation models (4, 24, 27, 28) were available in the form of an online tool or calculator. One of the models (32) was presented in the form of a nomogram and another (17) as a contour plot of survival probability.

Commonly validated risk indices, as described in **Table 2**, included the KDRI, EPTS, UK KDRI, LKDPI, and MAPI. Other models validated included those developed by Kasiske et al. (27), Nyberg et al. (37) and Remuzzi et al. (38).

Risk of Bias

The overall RoB was high in 49 of the 74 models, unclear in 24, and low in only one (**Figure 3**). Of those that were considered a

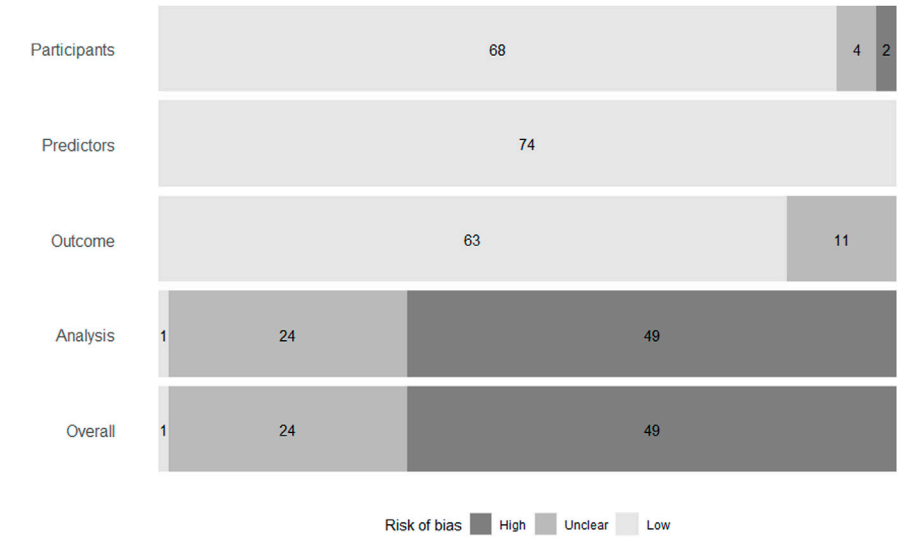


FIGURE 3 | Summary of risk of bias of models in individual domains, and overall. Risk of bias was assessed using the Prediction Model Risk of Bias Assessment Tool (13).

high RoB overall, all of them were at a high RoB in the analysis domain.

Sample size was reported for all models. However, the number of events were not reported for 37 of the models, therefore it was unclear whether there were a reasonable number of participants with the outcome.

Missing data were not discussed for 12 models. For models that did discuss missing data, 20 performed their analysis based only on those patients that did not have any missing data. This is called a complete-case analysis. All included models reported some measure of discrimination, but calibration was only reported for 13 models. Some models reported the C-statistic but did not discuss whether they had adapted it to account for censoring. This also contributed to a lack of clarity on the suitability of performance measures.

Twenty-two of the 28 development and validation models avoided univariable selection, reducing the possibility of bias in the analysis domain. Sixteen models did not account for overfitting or optimism, rendering them a high RoB.

Development and Validation Models

All-Cause Graft Failure

All-cause graft failure was reported in 11 of the 28 development and validation models. Summary data for each model with this outcome are shown in **Supplementary Table S3**.

In eight models, only deceased donor information was used. Three models utilised a combination of living and deceased donors.

Four models performed a complete-case analysis and two models used multiple imputation (39) to handle missing data. Three models imputed values based on mean or median, and two models assigned missing values to a missing category.

All models assessed discrimination. Discrimination measures reported included nine C-statistics (0.59–0.652) and two time-dependent AUC at 20 years (0.673 and 0.752) (**Figure 4**). In four models that also assessed calibration, two did so using a calibration plot and the remaining two reported the calibration slope (1.04 each).

Death-Censored Graft Failure

In one of the five models for death-censored graft failure the eligible population was deceased donor kidney recipients whilst in one model it was living donor recipients (**Supplementary Table S4**). Three models utilised a combination of both living and deceased donors.

For death-censored graft failure, four models used multiple imputation and one failed to report any methods for handling of missing data.

All models included at least one measure of discrimination and four evaluated the calibration. Discrimination measures reported included Harrell's C (0.69), AUC (0.74), C-statistic (0.59, 0.63), and optimism-corrected C-statistic (0.66) (**Figure 5**). Three models graphically assessed calibration and one used the Hosmer-Lemeshow test.

Patient Survival

Only one of the 11 models utilised living donors, whilst six used deceased donor transplant data and four considered a combination of living and deceased donors (**Supplementary Table S5**).

Eight models handled missing data using multiple imputation and one used single imputation. One model undertook a complete-case analysis and handling of missing data was not reported for one model.

The C-statistic was the most used measure of discrimination (9 models) with reported values between 0.637 and 0.71 (**Figure 6**). Other measures included Harrell's C (0.64) and optimism-corrected C-statistic (0.77). Calibration was also assessed in four models, three of which presented a calibration plot while the other performed the Hosmer-Lemeshow test.

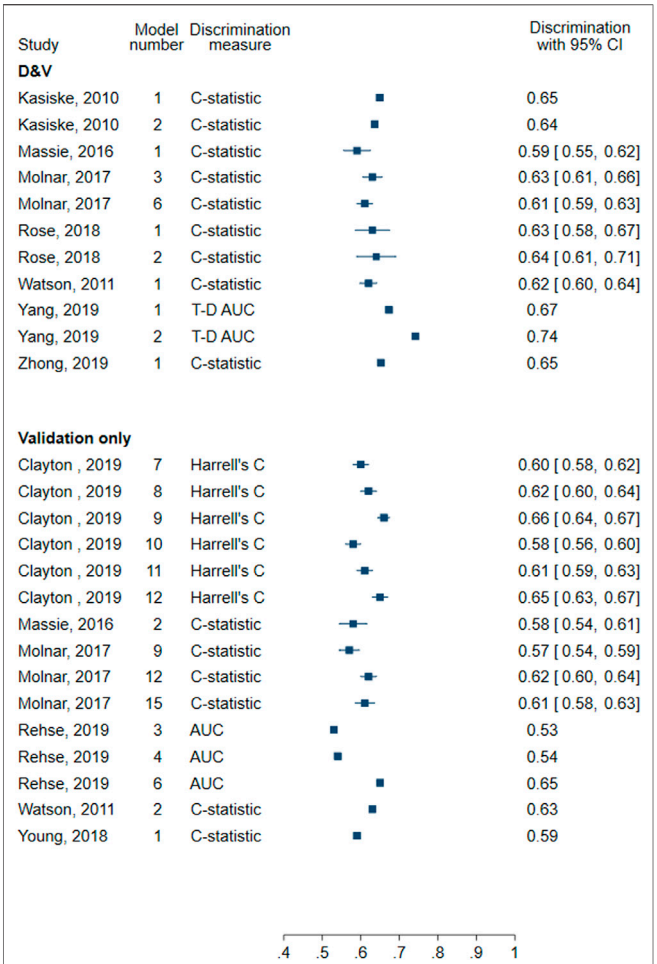


FIGURE 4 | Forest plot of discrimination in models for all-cause graft failure. D&V: Development and validation; AUC: area under receiver operating characteristic curve; C-statistic: concordance statistic; Harrell's C: adapts the C-statistic to account for censoring; T-D AUC: Time-dependent AUC, a measure of the AUC at specified timepoints since time origin.

Validation-Only Models

All-Cause Graft Failure

All-cause graft failure was reported in 15 of the 46 validation-only models (**Supplementary Table S3**).

In 10 models, only deceased donor information was used and only living donor data in two models. The remaining three models utilised a combination of living and deceased donors.

Seven conducted a complete-case analysis. Three models used multiple imputation to handle missing data. Two models imputed values based on mean or median. For two models it was unclear how missing data were handled and one model did not discuss missing data.

All models assessed discrimination. Seven assessed discrimination using Harrell's C (0.55–0.66) and six reported the C-statistic (0.57–0.63) (**Figure 4**). Two models used the AUC (0.53–0.65). No models assessed calibration.

Death-Censored Graft Failure

In 14 of the 19 validation-only models the eligible population was deceased donor kidney recipients whilst in two models it was living donor recipients (**Supplementary Table S4**). Three models utilised a combination of both living and deceased donors.

For death-censored graft failure, six models did a complete-case analysis, three models used multiple imputation and one used median imputation. For three models the methods for handling missing data were unclear, and six did not discuss missing data.

All models evaluated the discrimination, but none assessed the calibration. Four models reported the C-statistic (0.54–0.66) and six reported Harrell's C (0.59–0.70). Five models assessed AUC (0.55–0.81), and four assessed time-dependent AUC evaluated 2 years following transplantation (0.45–0.81) (**Figure 5**).

Patient Survival

Eight out of 11 models used data from deceased donor transplant recipients and three used a combination of living and deceased donors (**Supplementary Table S5**).

Seven models handled missing data using multiple imputation and one conducted a complete-case analysis. Three models failed to discuss missing data.

Eight models assessed discrimination using Harrell's C (0.57–0.735) and three using the C-statistic (0.66–0.70) (**Figure 6**). Calibration was not assessed in any of the validation-only models for patient survival.

DISCUSSION

Principal Findings

Our review focussed on prediction models to inform the kidney donor acceptance decision. Thus, we only included models which used pre-transplantation information. The MAPI (3), for example, utilises histopathological data from pre-transplantation donor kidney biopsies to predict graft survival. However, clinicians in the UK would not typically have access to biopsy results at the time of offer this model has limited utility. The PreImplantation Trial of Histopathology In renal Allografts (PITHIA) (39) is ongoing and assesses whether pre-implantation biopsy analyses improve graft function. As such, there may be scope for the MAPI to be clinically useful.

Discrimination was well reported overall unlike calibration. Existing reviews also observed that calibration is poorly reported (40-43). Without both measures of performance, it is difficult to determine the predictive capability.

Twenty of the 28 development and validation models were developed in the US population, though the discrimination of these models remained similar in external validation in other countries. Overall performance of both development and validation-only models was most determined by measures of discrimination, such as the C-statistic, Harrell's C, or

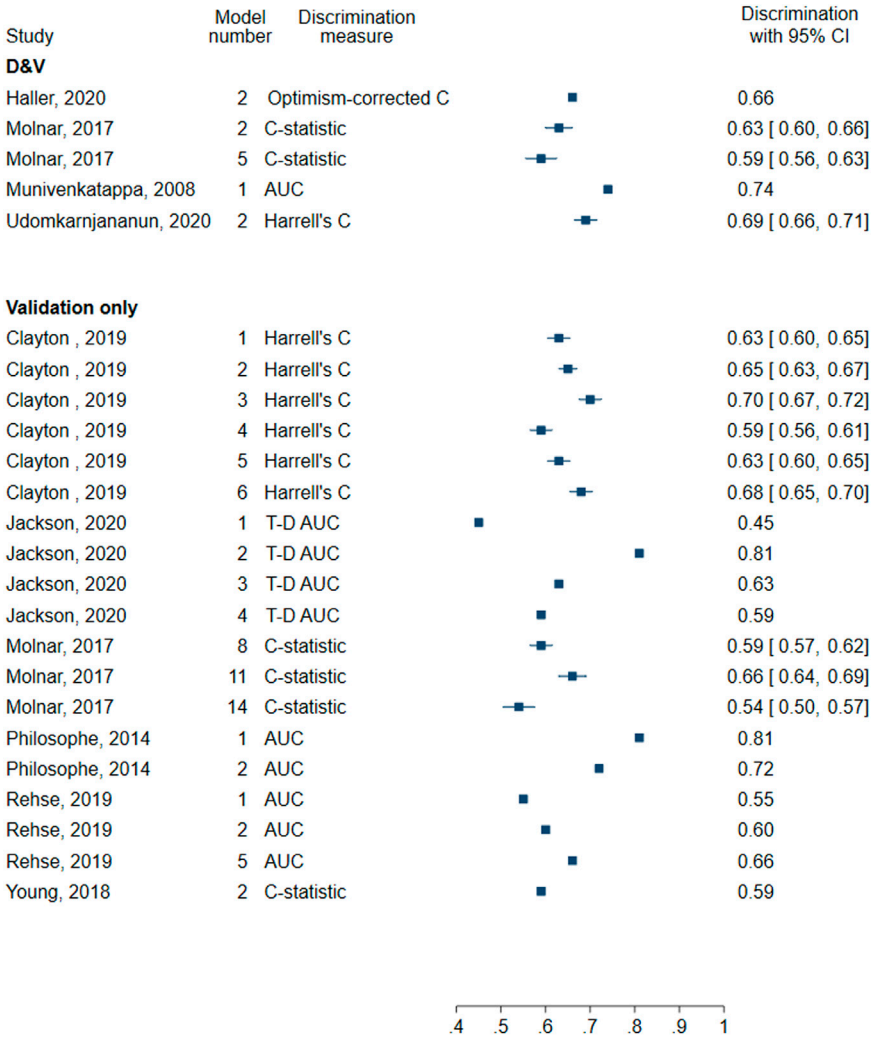


FIGURE 5 | Forest plot of discrimination in models for death-censored graft failure. D&V: Development and validation; AUC: area under receiver operating characteristic curve; C-statistic: concordance statistic; Harrell's C: adapts the C-statistic to account for censoring; Optimism-corrected C-statistic: measures the C-statistic while accounting for optimism in model performance; T-D AUC: Time-dependent AUC, a measure of the AUC at specified timepoints since time origin.

AUC, which ranged between 0.59 and 0.77 for development and validation models, and 0.45 and 0.81 for validation-only models.

All included models considered the censoring of the time-to-event data using either Cox models or survival random forest. However, models for death-censored graft failure should have ideally considered the semi-competing events graft failure and death. Calvillo-Arbizu et al. (20) noted that death with a functioning graft is a competing event for graft failure but used this as part of the exclusion criteria. Methods such as Fine and Gray (44) and multistate models (45) can be used to account for semi-competing events without discarding the data.

The model by Haller et al. (24), reported optimism-corrected C-statistic 0.77 and showed good calibration. It predicted the survival of recipients of a living donor kidney using a combination of donor, recipient and transplant factors as predictors. However, it has not been externally validated, so its generalisability to other populations is not known.

The LKDPI by Massie et al. (4) predicted all-cause graft failure with C-statistic 0.59 (95% CI: 0.55–0.62). In external validation studies (30) conducted in Germany, the model continued to show moderate to poor discrimination. The development and validation model by Molnar et al. (28) predicting death-censored graft failure had similarly poor discrimination reporting a C-statistic 0.59 (95% CI: 0.56–0.63) but showed good calibration. No other article externally validated this model.

For deceased donors the model by Yang et al (34) reported time-dependent AUC equal to 0.742 for graft survival, but has not yet been validated externally.

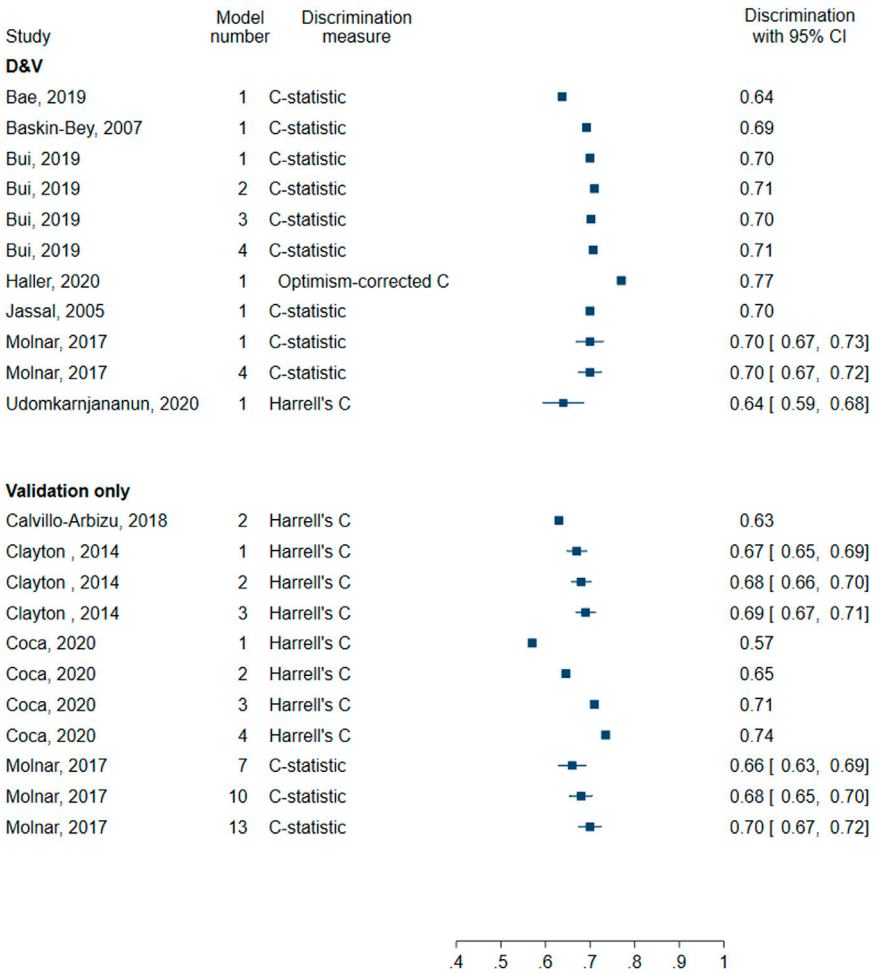


FIGURE 6 | Forest plot of discrimination in models for patient survival. D&V: Development and validation; C-statistic: concordance statistic; Harrell's C: adapts the C-statistic to account for censoring; Optimism-corrected C-statistic: measures the C-statistic while accounting for optimism in model performance.

The UK KDRI (5) for predicting transplant survival had moderate discrimination with a C-statistic of 0.62. External validation in Australia and New Zealand (21) reported Harrell's C equal to 0.59 (95% CI: 0.56–0.61) and 0.58 (95% CI: 0.56–0.60) for predicting death-censored graft failure and all-cause graft failure, respectively.

Overall RoB was high in 49 out of 74 of the included models, largely due to the analysis methods. One such aspect was the handling of missing data. Twelve models did not discuss missing data at all, and twenty models handled missing data using a complete-case analysis. This analysis approach can lead to biased results (39) due to reduced sample size and increased risk of overfitting. Other methods, such as multiple imputation, are preferred over a complete-case analysis (46).

Overall, there was no clear indication whether the type of predictors affected discrimination (**Supplementary Figures S1–S3**). However, in individual articles we saw that models developed using combinations of type of predictors, rather than donor-only, showed better discrimination. Models with a small sample size relative to the number of predictors are more susceptible to overfitting (47), which can result in poorer predictive performance.

Sufficient sample size was rarely considered and was one contributing factor to models being deemed at a high RoB. Methods for calculating the effective sample size for the development of a prediction model for time-to-event outcomes have been proposed by Riley et al. (48). A sample size calculation is standard practice in clinical trials, and we believe this practice should cross over into prediction modelling.

Strengths

To our knowledge this is the first review focusing on prediction models that only use information known prior to transplantation as predictors, and does not restrict to either regression or machine learning methods. Furthermore, we reviewed all articles published from the date of inception of each database, allowing us to maximise the number of articles included.

Limitations

Our review was restricted to articles published in English. We focussed on models that would be of practical use at the time of an offer of a donor kidney. As such notable models including those by Loupy et al. (49) and Foucher et al. (50), which include post-transplantation information, were not eligible for our review. Based on the existing prediction models, we cannot conclude which methods work better than the other. This opens the opportunity for evaluation, application and testing a range of appropriate methods in future research.

CONCLUSION

Development of clinical prediction models to inform organ acceptance decision-making should be driven by the clinical utility of such models. The currently available prediction models using pre-transplantation information provide moderate discrimination and varied calibration for patient and graft survival. Sample size calculations, handling of missing data and assessment of calibration are required, alongside better reporting of methods, to increase the quality of the studies. Opportunities to improve predictive performance include the identification of further important predictors and advancement of the development models by acknowledging the complex data such as semi-competing risks between graft failure and death. Until the predictive tools have the desirable performance, they have limited utility in clinical decision-making.

DATA AVAILABILITY STATEMENT

Data extracted, risk of bias assessment and code are available from Github: https://github.com/Yinghui-Wei-team/methods-review-kidney-transplant-prediction.

AUTHOR CONTRIBUTIONS

SR: Design of study, screening of titles, abstracts and full text of all citations, data extraction, risk of bias assessment, analysis of data, writing—original draft, and reviewing and editing. QZ: Screening of titles, abstracts and full text of all citations. W-YT: Clinical insight, writing—reviewing and editing. AC: Supervision, design of study, clinical insight, writing—reviewing and editing. YW: Supervision, design of study, data extraction, risk of bias assessment, analysis of data, writing—reviewing and editing.

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CONFLICT OF INTEREST

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

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2022.10397/full#supplementary-material

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ORIGINAL RESEARCH
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The Cyclophilin-Dependent Calcineurin Inhibitor Voclosporin Inhibits SARS-CoV-2 Replication in Cell Culture

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Kidney transplant recipients (KTRs) are at increased risk for a more severe course of COVID-19, due to their pre-existing comorbidity and immunosuppression. Consensus protocols recommend lowering immunosuppression in KTRs with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, but the optimal combination remains unclear. Calcineurin inhibitors (CNIs) are cornerstone immunosuppressants used in KTRs and some have been reported to possess antiviral activity against RNA viruses, including coronaviruses. Here, we evaluated the effect of the CNIs tacrolimus, cyclosporin A, and voclosporin (VCS), as well as other immunosuppressants, on SARS-CoV-2 replication in cell-based assays. Unexpected, loss of compound due to plastic binding and interference of excipients in pharmaceutical formulations (false-positive results) complicated the determination of EC50 values of cyclophilin-dependent CNI's in our antiviral assays. Some issues could be circumvented by using exclusively glass lab ware with pure compounds. In these experiments, VCS reduced viral progeny yields in human Calu-3 cells at low micromolar concentrations and did so more effectively than cyclosporin A, tacrolimus or other immunosuppressants. Although, we cannot recommend a particular immunosuppressive regimen in KTRs with COVID-19, our data suggest a potential benefit of cyclophilin-dependent CNIs, in particular VCS in reducing viral progeny, which warrants further clinical evaluation in SARS-CoV-2-infected KTRs.

Keywords: SARS-CoV-2, kidney transplantation, tacrolimus, voclosporin, cyclosporin A, calcineurin inhibitors

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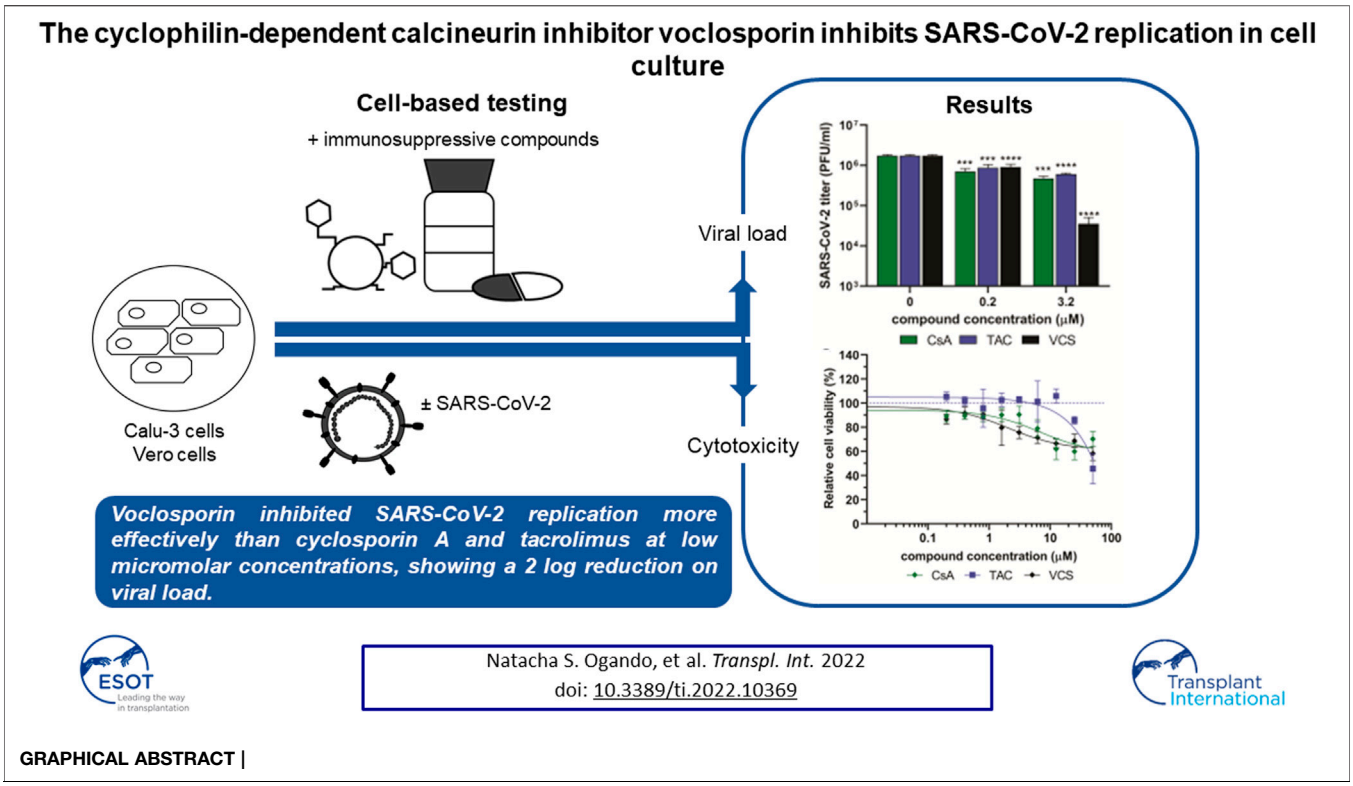
Abbreviations: CNI, calcineurin inhibitors; CoV, coronavirus; COVID-19, coronavirus disease 2019; CPE, cytopathic effect; CsA, cyclosporin A; Cyp, cyclophilin; EVL, everolimus; KTR, kidney transplant recipients; MMF, mycophenolate mofetil; MPA, mycophenolic acid; PFU, plaque-forming unit; RDV, remdesivir; SARS-CoV, severe acute respiratory syndrome coronavirus; TAC, tacrolimus; VCS, voclosporin.



GRAPHICAL ABSTRACT |

INTRODUCTION

Between December 2019 and May 2022, severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), the causative agent of coronavirus disease-2019 (COVID-19), has resulted in over 500 million cases of infection with a reported estimated death toll of 6 million people globally (1). The severity of clinical manifestations of COVID-19 has been correlated to various comorbidities commonly present in transplant recipients (2–4). Moreover, some reports showed that transplant recipients are at increased risk of a more severe course of COVID-19 and related death (2–6).

Finding the right balance between preventing rejection and controlling infections is generally the conundrum when prescribing immunosuppression for transplant recipients (7). The current standard of care, specifically in kidney transplant recipients (KTR) consists of a calcineurin inhibitor (CNI), either tacrolimus (TAC) or cyclosporin A (CsA), an antimetabolite agent such as mycophenolate (MPA/MPS) and most often corticosteroids. An mTOR inhibitor such as everolimus (EVL) as part of the regimen may also be prescribed alternatively (8). So far, the precise impact of immunosuppression on the course of COVID-19 and the excess mortality observed in KTRs (9) is poorly understood. On the one hand, (over)immunosuppression might hamper antiviral responses to control SARS-CoV-2 infection, whereas unopposed (hyper-) inflammation from immune overactivation is thought to result in a more severe disease course. Consequently, consensus protocols recommend to reduce but not completely cede immunosuppression in SARS-CoV-2-infected KTR's, depending on the risk of rejection and disease severity (10, 11).

Previous reports suggested that CNIs have antiviral activity against coronaviruses (12). TAC (which targets FKBP12) was reported to inhibit CoV replication in cell culture (13), and was recently proposed as a potential inhibitor of SARS-CoV-2 replication by computational analysis (14). Next to its immunosuppressive effects (15–20), CsA was reported to inhibit replication of different RNA viruses in cell culture (17, 21, 22), including human and zoonotic CoVs (20, 23–26). Several non-immunosuppressive CsA derivatives, like alisporivir (Debio-025), also inhibit the replication of CoVs in cell culture (15, 24, 27), including SARS-CoV-2 (28, 29). Collectively, these studies established the broad-spectrum antiviral activity of CsA and derivatives in cell culture-based infection models. These studies suggested that cyclophillins (cyps) are involved in CoV replication. However, knock-down of different Cyps in cells lead to variable effects on the replication of different CoVs (20, 24, 25, 30). Thus, the exact role of Cyps host proteins in CoV replication remains elusive (30, 31). Still, CsA has been suggested as the drug-of-choice for KTRs during the COVID-19 pandemic as an alternative to other regimens to prevent rejection (32).

Voclosporin (VCS) is a novel CNI which has been studied in psoriasis and renal organ transplantation. Additionally, VCS was recently FDA-approved for treatment of active lupus nephritis in combination with background immunosuppressive therapy (33–35). Structurally similar to CsA, VCS incorporates a

methyl group at the amino acid residue position 1, which enhances its binding to calcineurin, and confers better metabolic stability (36, 37). (Pre)clinical observations suggested that VCS is more potent and less toxic at therapeutic levels than other immunosuppressants in its class, including CsA (34, 36–40). VCS was shown to inhibit norovirus replication in a CypA-dependent manner and more effectively than CsA (19). Therefore, VCS is an interesting candidate to evaluate for inhibitory activity on SARS-CoV-2 replication.

In this study, we compared the effect of three calcineurin inhibitors (TAC, CsA, VCS) and other immunosuppressants commonly used in transplant medicine on SARS-CoV-2 replication using cell-based assays. Our results showed that out of the three calcineurin inhibitors VCS was the most potent inhibitor of SARS-CoV-2 replication, using cell-based assays. Since VCS is also a more potent immunosuppressant than CsA with comparable potency to TAC, we concluded that VCS might be an interesting CNI to investigate further in KTRs COVID-19 patients.

MATERIAL AND METHODS

Virus and Cell Lines

For all infections, SARS-CoV-2 isolate Leiden-0002 (GenBank MT510999) was used (41). Vero E6 cells and Calu-3 2B4 cells (42), were cultured and infected as described previously (41). All experiments with infectious SARS-CoV-2 were performed in the LUMC biosafety level 3 facilities.

Immunosuppressive Compounds

Voclosporin (Lupkynis™, Aurinia Pharmaceuticals Inc.), cyclosporin A (Neoral®, Novartis), tacrolimus (Prograf®, Astellas), mycophenolate mofetil (CellCept®, Roche) or everolimus (Certican®, Novartis) stock solutions were prepared by dissolving the pharmaceutical formulation of these drugs in dimethyl sulfoxide (DMSO). Placebo capsules and pure VCS powder (Aurinia Pharmaceuticals Inc.), Tacrolimus (PHR1809), cyclosporin A (30024) and mycophenolic acid (M5255) (all from Sigma-Aldrich) and remdesivir (RDV; HY-104077, MedChemExpress) were dissolved in DMSO and stored at −20°C as single-use aliquots. Remdesivir was used as a standard positive control in all experiments.

Measurement of Cyclosporin A, Tacrolimus and Voclosporin Concentrations by LC-MS/MS

Before analysis, samples were diluted in methanol and subsequently in blank whole blood to fall within the calibration line of 0-to 600 µg/L of VCS. Human whole blood was added to a final volume of 200 µL and mixed with 200 µL of 0.1 M zinc-sulphate and 500 uL of internal standard solution (32 ug/L of VCS D_4 in acetonitrile). After centrifugation, supernatant was transferred to an autosampler vial after which 20 µL was injected into LC-MS/MS system. Quantification of VCS was performed with LC-MS/MS using a Thermo Quantiva UPLC-MS/MS system (ThermoFisher Scientific) (43), similarly to the validated protocol for measuring CsA and TAC. The equipment consisting of an Ultimate 3000 series UHPLC system, coupled to a TSQ Quantiva triple stage quadrupole mass spectrometer was used. Chromatographic separation was achieved using an Acquity UPLC BEH C18 1.7 µm; 2.1 × 50 mm column coupled to a VanGuard BEH C18 1.7 µm precolumn. Online solid phase extraction was performed using a Xbridge 10 µm 30 × 2.1 mm column. This protocol was validated according to the EMA bioanalytical method validation guideline (44).

Cytopathic Effect Reduction Assay

CPE reduction assays in Vero E6 cells were performed as previously described (28). Briefly, Vero E6 cells seeded in 96-well cell culture plates were pre-incubated with 2-fold serial dilutions of compounds for 30 min. Subsequently, cells were either mock-infected (to assess cytotoxicity of compounds) or were infected with 300 PFU of SARS-CoV-2 per well. Each well contained a total volume of 150 µL of medium with compound. Plates were incubated for 3 days at 37°C. After, cell viability was determined via a colorimetric method by measuring absorption at 495 nm with an EnVision Multilabel Plate Reader (PerkinElmer). Both EC50 (50% effective concentration, required to inhibit virus-induced cell death by 50%), and CC50 (50% cytotoxic concentration, reduces the viability of uninfected cells to 50% of control) were determined using non-linear regression with GraphPad Prism v8.0. For each compound, at least two independent experiments (each in quadruplicate) were performed.

Virucidal Activity Assay

Compound dilutions were prepared in EMEM-2% FCS to mimic the conditions of cell-based assays. Tween-20 and Tween 40 were diluted in MilliQ water to concentrations lower than 1% as present on the composition of Lupkynis™ capsules (45). Phosphate-buffered saline (PBS) was used as a negative control and 50% ethanol as a positive control. In order to assess its virucidal activity, SARS-CoV-2 (5×10^4 PFU) was incubated with the material for 2 h at 37°C with rocking. Then, serial dilutions (ranging from 10^{-1} to 10^{-6}) of compound mixed with virus were prepared in EMEM-2% FCS and added to Vero E6 monolayers. After 1 h incubation, inoculum was removed and 2 ml/well of overlay containing DMEM, 1.2% Avicel (FMC BioPolymer), 2% FCS, 50 mM HEPES, and antibiotics were added. After a 3-day incubation, monolayers were fixed with 3.7% formaldehyde in PBS, and plaques were visualized using crystal violet staining (41).

Virus Yield Reduction Assays

Calu-3 cells were seeded in 96-well plates (3×10^4 cells per well). The next day, cells were pre-incubated for 60 min with 2-fold serial dilutions of CsA, TAC or VCS. Subsequently, cells were infected with SARS-CoV-2 (MOI of 1, based on titer determined on Vero E6 cells). After a 1 h incubation at 37°C, cells were washed three times with PBS and medium with compound was added. The medium was harvested at 24-h post-infection (h p.i.) and virus titers were determined by plaque assay on Vero E6 cells as described before (46). In parallel, a cytotoxicity assay with mock-infected cells treated

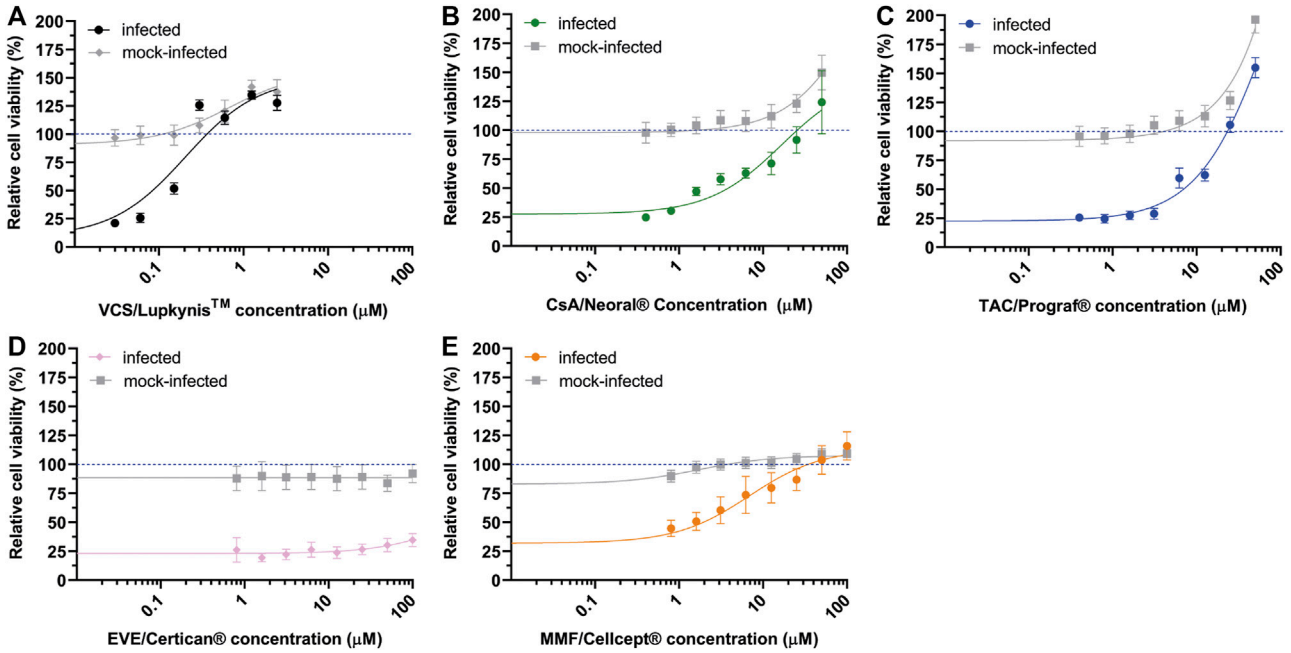


FIGURE 1 | Effect of immunosuppressive drugs on SARS-CoV-2 replication. Inhibition of SARS-CoV-2 replication (colored symbols and curves) in Vero E6 cells by various drugs was determined by CPE reduction assay. For each drug, two-fold serial dilutions of the pharmaceutical formulations were tested. **(A)** VCS/Lupkynis, **(B)** CsA me/Neoral, **(C)** TAC/Prograf, **(D)** EVL/Afinitor, and **(E)** MMF/Cellcept. After preincubation with compound, Vero E6 cells were infected with SARS-CoV-2 and kept in medium containing the drug for 3 days, after which cell viability was measured with a colorimetric assay. Cytotoxicity of the drugs was evaluated in parallel using mock-infected, compound-treated cells (solid grey line). Data points represent the mean ± SD of two independent experiments. The CC50 and EC50 were determined by non-linear regression analysis and the regression curves are plotted in the graphs (solid lines).

using the same concentration of compounds was performed (*Cytopathic Effect Reduction Assay* section). VCS concentrations were measured by validated LC-MS/MS.

Coating of Plastic Materials

The following coating solutions were prepared fresh before each experiment: BSA, 100 mg/ml bovine serum albumin (Sigma) in PBS; PEG, 1% polyethylene glycol 3350 (Sigma) in MilliQ water; Tween-40, 0.2% polysorbate 40 (Fluka) in MilliQ water; and 500 mM VCS in DMSO (Sigma). All plastic labware, including tubes and tips, was filled with each solution and incubated for 2 h at room temperature with rocking to homogenously coat the surfaces. After rinsing twice with MilliQ water, the items were left to dry at room temperature until further use in experiments.

Virus Yield Reduction Assays in Glass Bottles

Borosilicate glass reagent bottles (50-ml) were treated with glacial acetic acid, washed twice with absolute ethanol, dried and UV-sterilized prior to use. Three times concentrated compound solutions were prepared in EMEM-2% FCS using sterile glass culture tubes, a glass syringe (Hamilton) and glass Pasteur pipettes. One ml of each compound dilution was transferred to three different reagent bottles (triplicates). Confluent monolayers of Calu-3 cells grown in culture flasks were infected with SARS-CoV-2/Leiden-002 (MOI of 1). Inoculum was removed after 1 h incubation at 37°C. Cells were washed three times with PBS, trypsinized and resuspended in EMEM-2% FCS. Two ml of this cell suspension (~10^6 cells) was transferred to each reagent bottle, containing compound solution. Medium was collected 24 h p.i. and virus titer was determined by plaque assay. VCS concentrations in the medium were determined by LC-MS/MS.

Determination of Compound Cytotoxicity in Glass Culture Tubes

Calu-3 cells were trypsinized and 1.5 × 10^5 cells in 1 ml of EMEM-2% FCS were divided over glass culture tubes. Two-fold dilutions of VCS, TAC and CsA were prepared in EMEM-2% FCS medium using glass labware, and added to corresponding tubes with cells (three tubes per concentration). After a 24 h incubation, cell viability was determined (see *Cytopathic Effect Reduction Assay* section).

RESULTS

Inhibition of SARS-CoV-2 Replication in Cell Culture by Pharmaceutical Formulations of Immunosuppressive Drugs

To compare the antiviral effect of different immunosuppressive drugs commonly used in KTRs, we performed SARS-CoV-2 CPE

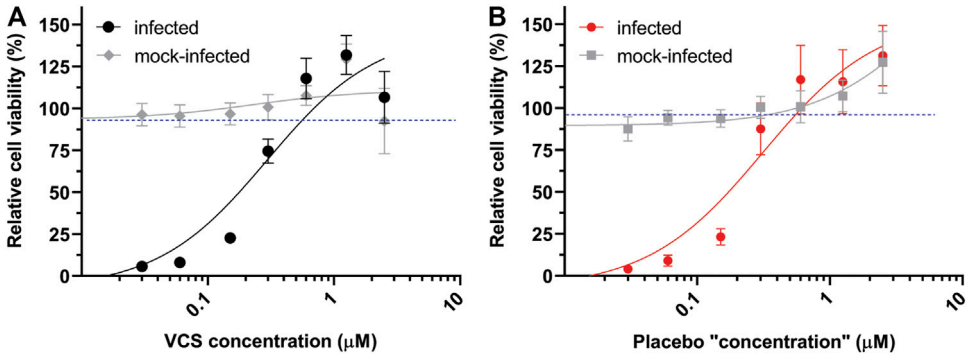


FIGURE 2 | Comparison of the effect of the pharmaceutical formulation of VCS and placebo capsules on SARS-CoV-2 replication. The inhibition of SARS-CoV-2 replication in Vero E6 cells treated with the DMSO-dissolved content of VCS capsules **(A)** or placebo capsules **(B)** was determined by CPE reduction assays as described in the legend of **Figure 1**.

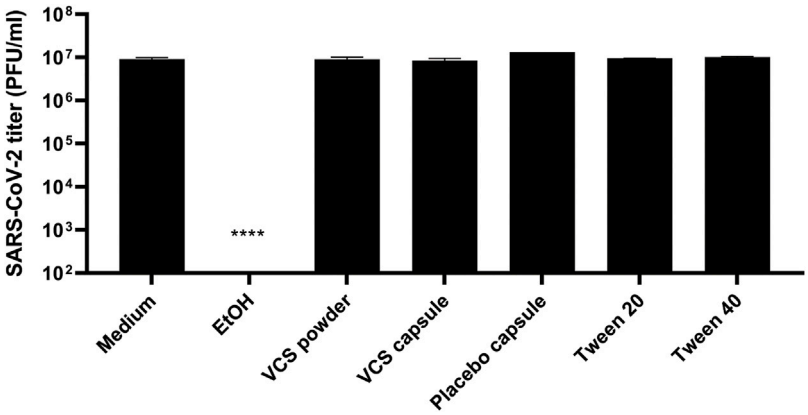


FIGURE 3 | Virucidal activity of VCS and placebo capsules. The drug formulation of VCS (3.2 μM), and content of placebo capsules (corresponding to 3.2 μM VCS), and 50% ethanol were incubated with a SARS-CoV-2 virus stock for 2 h, followed by quantification of the remaining infectious virus titer by plaque assay in Vero E6. Statistical significance was determined by one-way ANOVA. *, $p < 0.1$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

reduction assays with VCS, cyclosporin, microemulsion (CsA_me), TAC, EVL, and MMF. These experiments were performed using the pharmaceutical formulations of the compounds to ensure optimal solubility and stability. At the start of our study only the pharmaceutical formulation of VCS) was available to us from a previous study. In each experiment, drug cytotoxicity was assessed in parallel, in non-infected cells. RDV was included as a standard positive control for inhibition of viral replication [data not shown (28)].

The EC_{50} values of VCS, CsA_me and TAC were measured in the low-micromolar range, respectively: 0.22 ± 0.01 μM, 4.3 ± 0.6 μM and 10 ± 1 μM (**Figures 1A–C**). No inhibitory effect was observed for EVL (**Figure 1D**). The prodrug MMF (**Figure 1E**) was included in our comparison, but was not expected to inhibit virus replication, as it is likely not metabolized into its active form MPA (47) in our assay. Thus, we attributed the apparent antiviral effect of MMF to excipients present in the drug formulation (see *An Excipient in the Pharmaceutical Formulation of Voclosporin Inhibits SARS-CoV-2 Replication in Cytopathic Effect Reduction Assays* section).

Apart from VCS, none of the compounds caused cytotoxicity at tested concentrations (CC_{50} values >100 μM). Although VCS had a CC_{50} around 4 μM, its EC_{50} was also 18–45 times lower than the other compounds tested (**Figure 1**).

An Excipient in the Pharmaceutical Formulation of Voclosporin Inhibits SARS-CoV-2 Replication in Cytopathic Effect Reduction Assays

In order to evaluate whether any excipients in the pharmaceutical formulation of VCS contributed to the observed antiviral effect (**Figure 1A**), VCS capsules and placebo capsules were compared side-by-side using CPE reduction assay. Both capsules provided by Aurinia Pharmaceuticals Inc. had similar composition (45), with exception of the VCS compound. The absence of VCS in placebo capsules was confirmed by LC-MS/MS analysis (not shown). Surprisingly, both the VCS formulation (**Figure 2A**) and the placebo (**Figure 2B**) inhibited SARS-CoV-2 replication in a

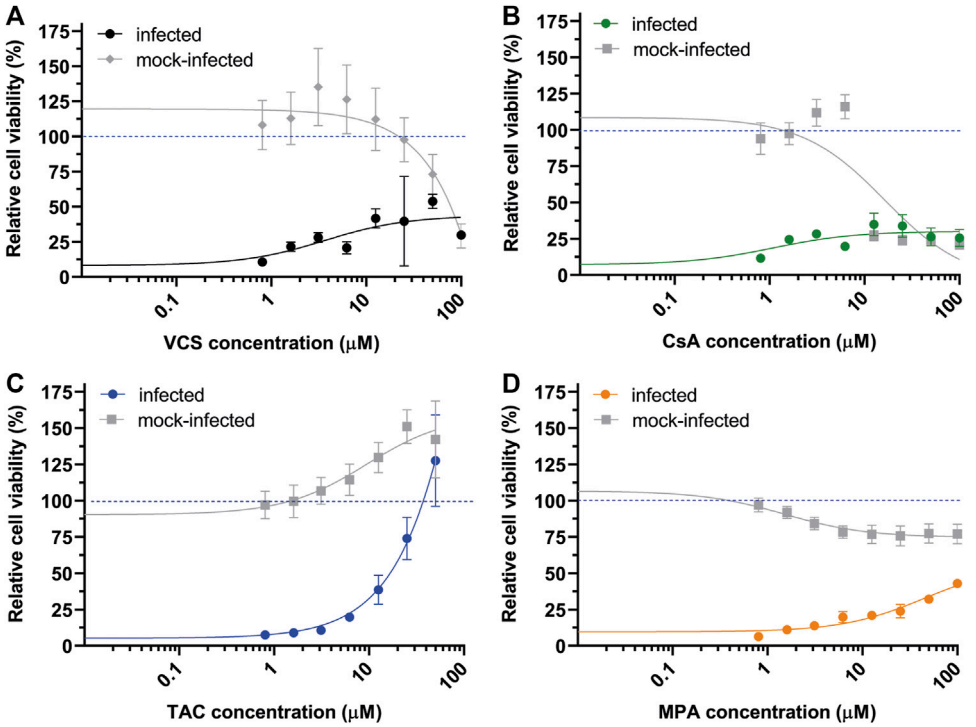


FIGURE 4 | Effect of the pure active ingredients of immunosuppressive drugs on SARS-CoV-2 replication in CPE-reduction assays. **(A)** VCS, **(B)** CsA, **(C)** TAC, and **(D)** MPA. Assay was performed using Vero E6 cells. For details, see the legend to **Figure 1**.

TABLE 1 | VCS concentration in samples incubated in plastic labware with different coatings, measured by LC-MS/MS.

Incubation time	Type of coating applied									
	Uncoated		500 mM VCS		100 mg/ml BSA solution		1% PEG-3350 solution		0.2% Tween-40 solution	
	Conc. (μM)	% remaining	Conc. (μM)	% remaining	Conc. (μM)	% remaining	Conc. (μM)	% remaining	Conc. (μM)	% remaining
0 h	0.56 ± 0.25	**28**	17.21 ± 2.36	**861**	0.55 ± 0.21	**27**	0.51 ± 0.16	**26**	0.56 ± 0.35	**28**
2 h	0.13 ± 0.07	**7**	2.73 ± 1.00	**137**	0.10 ± 0.04	**5**	0.09 ± 0.02	**4**	0.09 ± 0.04	**4**

Conc. means concentration.

Note: The percentages indicate the remaining concentration relative to the concentration of the original 2 μM of VCS stock solution. The bold values indicate the percentages of VCS that remain in solution after treatment or contact.

similar dose-dependent manner. This indicated that one or more excipients in the drug formulation might have an antiviral effect in this experimental setup.

Since the pharmaceutical formulation includes surfactants like Tween-20 and Tween-40 that may destroy the viral envelope, the virucidal activity of these reagents, VCS and placebo capsules were analysed. A control treatment with 50% ethanol reduced SARS-CoV-2 titers to below the limit of detection (<100 PFU/ml), while none of the other treatments significantly reduced the number of infectious particles (**Figure 3**). Therefore, we conclude that excipients in the drug formulation had no virucidal activity or impact on viral infectivity, but that they caused a yet poorly understood antiviral effect in the CPE reduction assays through an unknown mechanism. This invalidated the previously determined EC50 values when calcineurin inhibitors were tested using their pharmaceutical formulations (**Figure 1**).

Evaluation of Antiviral Activity Using Calcineurin Inhibitors in Pure Compounds Form

To avoid interference by excipients, we performed CPE reduction assays with DMSO solutions prepared using high purity powders of the various immunosuppressive drugs. In the case of Neoral (cyclosporin microemulsion), CsA powder was evaluated. VCS solutions prepared from pure powder did not confer the same level of protection to SARS-CoV-2 infected-cells (**Figure 4A**) as the pharmaceutical formulation (**Figure 2A**). Additionally, less cytotoxicity was measured/observed CC50 > 50 μM. Similar

TABLE 2 | Concentration of TAC and CsA in samples incubated in plastic labware, measured by LC-MS/MS.

Incubation time	TAC		CsA	
	Conc. in μM	% remaining	Conc. in μM	% remaining
0 h	0.85		0.76	
2 h	0.65	**76**	0.47	**62**

Note: The percentages indicate the remaining concentration relative to the concentration of the original compound stock solution (0.8 μM). The bold values indicate the percentages of VCS that remain in solution after treatment or contact.

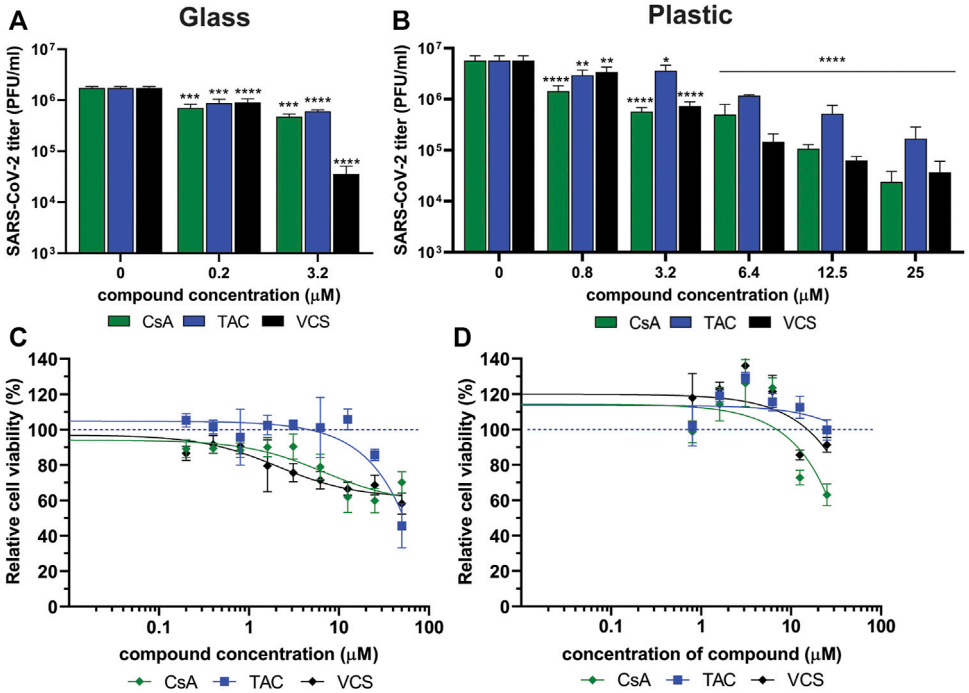


FIGURE 5 | Effect of CsA, TAC and VCS treatment on the production of infectious SARS-CoV-2 progeny by human Calu-3 cells. Experiments were performed using either glass **(A,C)** or plastic labware **(B,D)**. Cells were infected with SARS-CoV-2 in the presence of different concentrations of VCS, CsA and TAC using stock solutions prepared from pure powders dissolved in DMSO. The viral load in the medium of infected cells was determined by plaque assay on Vero E6 cells using supernatant harvested at 24 h p.i. Viability of uninfected Calu-3 cells treated with the same range of compound concentrations was measured in parallel by a colorimetric viability assay (C; $n = 12$; D; $n = 3$). Mean values ±SD are shown and statistical significance of the difference between each concentration and solvent control was assessed by one-way ANOVA. *, $p < 0.1$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

reductions in antiviral potency were observed for CsA and MPA (**Figures 4B,D**), suggesting that -also in cell-based assays-these drugs need excipients to ensure solubility/bioavailability or stability for optimal activity. Interestingly, TAC solutions prepared from pure powder inhibited SARS-CoV-2 with similar efficacy as the drug formulations, i.e., with an EC_{50} of ~15 μM (compare **Figures 1C**, **4C**), suggesting that the pharmaceutical formulation of TAC does not contain excipients contributing to either antiviral or virucidal effects.

Binding of Voclosporin to Plastic Strongly Affects Bioavailability and Efficacy in Antiviral Assays

We searched for potential reasons to understand the lower inhibitory effect of VCS active molecule compared to pharmaceutical formulation. Interactions between plastic and lipophilic or hydrophobic compounds, have been described (48–50). Thus, we hypothesized as VCS is highly lipophilic, it may bind to plastic which could compromise its bioavailability in standard cell-based assays using plastic labware. VCS concentrations were measured in medium using LC-MS/MS. Only 27% of the original VCS concentration could be recovered from plates due to loss of compound by binding to pipette tips and tubes during the preparation of dilutions as soon as t = 0 (**Table 1**).

To test whether we could prevent VCS binding to plastic in our standard antiviral assays, we coated all plastic labware using 3 different agents found in literature: BSA (51), PEG-3350 (52, 53) and Tween 40 (54). Unfortunately, none of the treatments tested was able to reduce binding of VCS to plastic (**Table 1**), as only 5–7% of its original concentration was recovered in solution.

TABLE 3 | VCS concentration in samples from experiments using only glass labware, measured by LC-MS/MS.

Incubation time	Concentration of VCS in supplied solution											
	3.2 μM		3.2 μM		1.6 μM		0.8 μM		0.4 μM		0.2 μM	
	Without cells		With cells									
	Conc. in μM	% remaining	Conc. in μM	% remaining	Conc. in μM	% remaining	Conc. in μM	% remaining	Conc. in μM	% remaining	Conc. in μM	% remaining
0 h	2.91		2.91		1.77		0.99		0.45		0.33	
24 h	2.79	**96**	0.82	**28**	0.35	**20**	0.15	**15**	0.10	**22**	<0.07[a]	ND

[a]*Below detection limit of LC/MS-MS.*

Note: The percentages indicate the ratio of the measured (true) concentration at 24 h and the concentration of the prepared solution administrated to the cells (at 0 h incubation time). The bold values indicate the percentages of VCS that remain in solution after treatment or contact.

Alternatively, we tested if saturation of binding sites on plastic with a highly concentrated VCS solution (500 mM) prevented loss of compound. Leaching of compound from plastic was observed, resulting in unpredictable concentrations of VCS in solution, e.g., we measured a VCS concentration of >15 μM when a 2 μM stock solution was used (**Table 1**).

Similarly, TAC and CsA concentrations were measured using the same setup as for VCS. A 76% of the original TAC concentration and 62% of the initial CsA concentration could be recovered in solution (**Table 2**). This emphasized the need to use different type of materials to perform our experiments to truly evaluate these CNIs antiviral activity.

Inhibition of SARS-CoV-2 Replication by Voclosporin, Cyclosporin A and Tacrolimus in Calu-3 Cells

To evaluate the effect of VCS, CsA and TAC on SARS-CoV-2 replication, viral load reduction assays were performed using human lung epithelial cells (Calu-3). Moreover, we developed custom assays using exclusively glass labware to circumvent the problem of VCS binding to plastic.

Calu-3 cells in glass remained viable and supported SARS-CoV-2 replication as an increase in viral titer was measured at 24 h p.i. (**Figure 5A**). RDV was included as a positive control for inhibition of SARS-CoV-2 replication and assay validation. Treatment of infected cells with 10 μM RDV inhibited viral replication by > 4log(data not shown), which is in agreement with previously reported data (55). Treatment of cells with 3.2 μM VCS (pure compound) caused a more than 1.5 log reduction in SARS-CoV-2 infectious progeny titers, while an ~0.5 log reduction was observed when the same concentration of CsA or TAC was used (**Figure 5A**). However, treatment with 3.2 μM VCS or CsA also caused cytotoxicity, as cell viability dropped to ~75% (**Figure 5C**). Therefore, it cannot be excluded that part of the observed antiviral effect is due to pleiotropic effects (toxicity).

In experiments using plastic materials, a dose-dependent reduction in infectious progeny titers was observed when cells were treated with VCS, leading to a more than 1 log reduction at 6.4 μM (**Figure 5B**). CsA treatment led to a similar reduction at 25 μM. CsA displayed significant cytotoxicity at concentrations of 12.5 μM or above while VCS did not (**Figure 5D**). In contrast, a higher concentration of TAC (25 μM) was required to reduce the viral titer by more than 1 log. Overall, VCS showed a stronger inhibitory effect in experiments performed with glass instead of plastic labware.

Measurement of the VCS concentration in glass containers without cells demonstrated no significant loss of compound from solution (**Table 3**). When VCS solutions of 0.2–3.2 μM were used in glass bottles with Calu-3 cells, a ~75% reduction of the VCS concentration was measured, suggesting the compound was bound or taken up by cells. In contrast, in experiments using standard plastic labware, we measured a 0.68 μM concentration of VCS in medium of cells treated with 25 μM VCS solution. Taking into account a similar reduction in virus titer using 3.2 and 25 μM of VCS in glass and plastic, respectively, this corroborated that when using plastic, the bioavailable amount of VCS is likely only 10% of that in the input solution.

DISCUSSION

Transplant recipients are at increased risk for developing a severe course of COVID-19 owing to their immunocompromised state combined with older age and comorbidities (5, 56, 57). The attributable effect of immunosuppression to a more severe course of COVID-19 and the optimal treatment is yet unclear (7, 12). As the efficacy of approved vaccines is uncertain in KTRs, it is crucial to gain more insight into the effect of immunosuppression. In this study, we evaluated the impact of VCS and different immunosuppressive compounds on the replication of SARS-CoV-2 *in vitro* using cell-based assays (**Figure 1**).

Previous studies demonstrated that CNIs like CsA and TAC inhibit replication of a variety of other CoVs, including SARS-CoV and Middle East respiratory syndrome (MERS) CoV (13, 20, 23–25, 58, 59). As these betacoronaviruses are closely related to SARS-CoV-2 (12, 60, 61), we expected to observe a similar inhibitory effect. In this study, we also evaluated the antiviral activity of a novel CNI, VCS (40, 62). In Calu-3 cells, VCS (pure compound) inhibited SARS-CoV-2 replication with an EC_{50} in the sub-micromolar range (<3. 2 μM), at lower concentrations than CsA or TAC (**Figure 4**). Our findings are in line with recent reports, showing that CsA inhibited SARS-CoV-2 replication in HuH7.5 and Calu-3 cells, but not in Vero cells (63). Notably, Dittmar et al found no activity when using TAC in any of these cell lines (63) In contrast to our finding that TAC showed antiviral activity in Vero E6 cells with an EC_{50} of ~15 μM

(**Figures 1**, **4**). This discrepancy might be explained by use of different Vero cell subclones.

While testing the pharmaceutical formulations of CNIs, we discovered that the excipients in these preparations had (apparent) antiviral effects in our cell-based assays (**Figure 2**). Unexpectedly, this was not due to a virucidal effect of the surfactants in these formulations (**Figure 3**) which could have the potential to damage the viral envelope (64–66). This undesired effect of excipients did not allow us to proceed testing pharmaceutical formulations in our cell-based assays, as it would lead to false positive results for various compounds. This evidences the necessity of proper controls in studies investigating the potential antiviral effect of CNI's.

VCS is a highly lipophilic compound and we observed that binding to plastic surfaces of commonly used labware strongly reduced its bioavailability in assays. We measured losses of >80% of the compound in solution (**Table 1**). This demonstrates that the use of plastic labware can lead to a serious underestimation of the efficacy of compounds in (antiviral) assays, in line with suggestions from previous publications (48–50). Our attempts to prevent binding of VCS to plastic by various (coating) treatments of labware were unsuccessful as none led to a more than ~10% recovery of the initial VCS concentration (**Table 1**). As a solution to circumvent plastic binding, we performed experiments using glass labware, which supported growth of human Calu-3 cells and SARS-CoV-2 replication (**Figure 5**). Measurement of VCS concentrations by LC-MS/MS demonstrated that there was hardly any loss of the compound (**Table 3**). Using this setup, we demonstrated that VCS reduced the production of SARS-CoV-2 infectious progeny in a dose-dependent manner, and more effectively than CsA and TAC.

It is difficult to translate the *in vitro* finding to the clinical context. Ideally, an immunosuppressive regimen should prevent rejection, inhibit viral replication and reduce (over)inflammation, while also allowing the host to still mount an effective antiviral response. Some CNI's are already being evaluated in clinical trials to determine their efficacy in COVID-19 patients [reviewed in (67)]. Interestingly, one study found a clear survival benefit for patients on CsA compared to other experimental anti-inflammatory therapy for COVID-19 (68). In the current study we demonstrate that cyclophilin-dependent CNIs, VCS or CsA, inhibit SARS-CoV-2 replication in cell culture more potently than TAC. VCS inhibited SARS-CoV-2 replication by ~2log at 8-fold lower concentrations than TAC (**Figure 4A**). Of note, TAC concentrations that are required to inhibit SARS-CoV-2 replication likely correlate with intolerable or toxic concentrations in humans (EC_{50} of 0.2 μM equals 160 ng/ml for TAC), without even taking into account that the free fraction in traffic is only ~10%. For CsA and VCS 0.2 μM correspond to a concentration of 241 and 243 ng/ml respectively (40, 69), which may come closer to peak concentrations *in vivo*. Moreover, the distribution of VCS over different organs might also be beneficial as concentrations in the lungs are higher than in blood (40, 45).

In conclusion, VCS reduced viral progeny yields in human Calu-3 cells at low micromolar concentrations and did so more effectively than CsA and TAC. The efficacy to prevent rejection in KTRs of VCS and TAC are considered to be comparable according to a phase 2b study (34). In cell culture, VCS inhibits SARS-CoV-2 replication at concentrations that are considered safe in humans. Therefore, VCS might be an attractive alternative CNI for therapy of patients that need calcineurin-based immunosuppression. Based solely on this study's experimental data, we do not advocate the use of VCS merely for its potential antiviral properties. However, our data suggest a potential benefit of cyclophilin-dependent CNIs, in particular VCS. This warranted further clinical evaluation and VCS is currently under investigation in SARS-CoV-2-infected KTRs [EudraCT 2020–001467-82].

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

Conceptualization, NO, YT, AV, and MH; methodology, NO, EM, DM, and AT; validation, NO, EM, DM, and AT; formal analysis, NO, EM, and DM; resources, JC and ES; writing—original draft preparation, NO, DM, JC, and MH; writing—review and editing, NO, EA, ES, YT, AV, and MH; visualization, NO and MH; supervision, AV and MH; project administration, YT, AV, and MH; funding acquisition, YT, ES, and MH. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST

This is investigator-initiated research. JC is an employee of Aurinia Pharmaceuticals Inc. YT received a grant without restrictions from Aurinia Pharmaceuticals Inc. to support part of this project and is an investigator of Aurinia clinical trials. Aurinia Pharmaceuticals Inc. had no role in the decision (what and when) to publish.

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

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ORIGINAL RESEARCH
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Cytomegalovirus Management in Solid Organ Transplant Recipients: A Pre-COVID-19 Survey From the Working Group of the European Society for Organ Transplantation

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Infections are leading causes of morbidity/mortality following solid organ transplantation (SOT) and cytomegalovirus (CMV) is among the most frequent pathogens, causing a considerable threat to SOT recipients. A survey was conducted 19 July–31 October 2019 to capture clinical practices about CMV in SOT recipients (e.g., how practices aligned with guidelines, how adequately treatments met patients' needs, and respondents' expectations for future developments). Transplant professionals completed a ~30-minute online questionnaire: 224 responses were included, representing 160 hospitals and 197 SOT programs (41 countries; 167[83%] European programs). Findings revealed a heterogenous approach to CMV diagnosis and management and, sometimes, significant divergence from international guidelines. Valganciclovir prophylaxis (of variable duration) was administered by 201/224 (90%) respondents in D+/R– SOT and by 40% in R+ cases, with pre-emptive strategies generally reserved for R+ cases: DNA thresholds to initiate treatment ranged across 10–10,000 copies/ml. Ganciclovir-resistant CMV strains were still perceived as major challenges, and tailored treatment was one of the most important unmet needs for CMV management. These findings may help to design studies to evaluate safety and efficacy of new strategies to prevent CMV disease in SOT recipients, and target specific educational activities to harmonize CMV management in this challenging population.

Keywords: survey, organ transplantation, infection cytomegalovirus, prophylaxis, pre-emptive therapy, cellular immunity, ESOT

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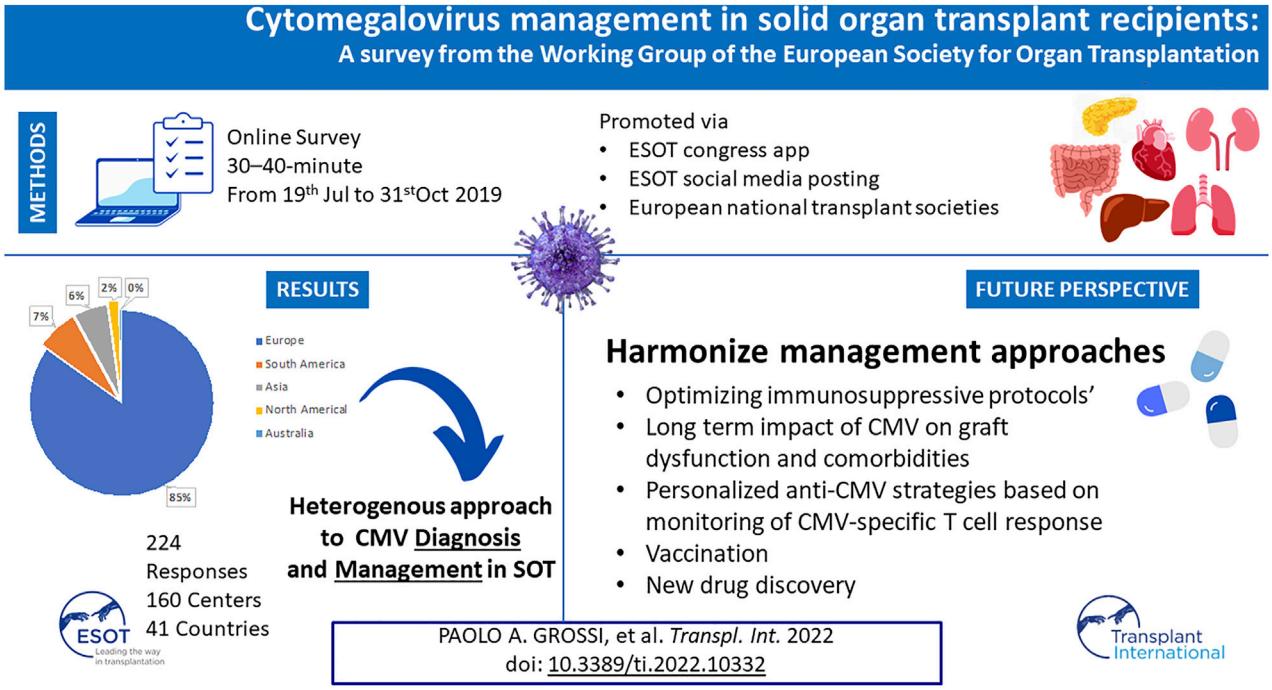

GRAPHICAL ABSTRACT |

INTRODUCTION

Cytomegalovirus (CMV) is one of the most important opportunistic viral pathogens in the solid organ transplant (SOT) setting (1–3); CMV infection and disease (defined as evidence of infection with attributable symptoms (4)) can cause adverse outcomes for allograft and recipient survival, increase the cost of transplantation, and negatively impact health-related quality of life (1–3).

Pre-transplant CMV immunoglobulin (Ig)G serological testing is generally undertaken in both donor and recipient to establish CMV disease risk and guide infection prevention strategies (5–9). CMV-seronegative recipients who receive organs from CMV-seropositive donors (D+/R−) are at highest risk of disease since they lack the ability to mount an effective and timely immune response, because of pharmacological immunosuppression post-transplantation (10). CMV-seropositive recipients may also experience CMV reactivation and/or reinfection in up to 20% of cases, representing an "intermediate risk" subgroup (11, 12).

Two approaches that reduce risk of CMV infection and disease following SOT are universal prophylactic therapy for all "at-risk" patients (excluding D−/R−), and pre-emptive antiviral treatment (PET) for those with evidence of infection but no overt disease (8, 9, 13, 14). Despite universal prophylaxis, CMV disease can arise following discontinuation of antiviral prophylaxis, or because of resistance to antiviral treatment, with breakthrough CMV infections occurring in patients on antiviral prophylaxis (1).

The CMV-DNA polymerase inhibitors ganciclovir and valganciclovir are first-line agents for CMV prevention and treatment; foscarnet and cidofovir are reserved for refractory/resistant infections. Although these therapies are generally efficacious in SOT recipients, their clinical value is limited by their toxicity profiles: adverse events observed include myelosuppression (ganciclovir and valganciclovir), nephrotoxicity (foscarnet and cidofovir), and electrolyte imbalances (foscarnet) (15).

Regular post-transplant monitoring of viral replication helps to predict CMV disease risk and guide decisions relating to treatment duration and efficacy (8, 9, 16–18). Monitoring was traditionally undertaken with the pp65 antigenemia assay and qualitative polymerase chain reaction (PCR) (7, 19), but there has been a shift toward molecular methods such as quantitative nucleic acid testing. In addition, monitoring CMV-specific T cell immunity post-transplantation is an emerging tool for predicting and controlling CMV infection, and for guiding tailored prevention strategies (8, 9, 16, 20). CMV has a broad impact across the immune system (20–22), with the T cell-mediated adaptive immune response being predominant in conferring protection against CMV-related disease.

Despite these apparently successful approaches for managing risk of CMV disease in SOT recipients, a retrospective analysis of French data from 2007 to 2011 involving 20,473 SOT recipients demonstrated that ~12% developed CMV disease within 24 months post transplantation (1). CMV disease was significantly associated with increased risk of allograft rejection and mortality (1). These findings demonstrate the continuing burden of CMV disease in SOT recipients and indicate the ongoing need to improve clinical outcomes for these people.

To better understand current international practices within transplant professionals, the European Society for Organ Transplantation (ESOT) conducted a survey that aimed to characterize strategies used to prevent, diagnose, and treat CMV infection in SOT recipients. The survey also sought to analyze variations in clinical practice by organ type and donor/recipient match, and to investigate drivers for variations in the use of immunosuppressive therapy regimens. Monitoring CMV-specific T cell immunity was also investigated. It was anticipated that the survey findings might influence the design of prospective multicenter studies, and identify educational needs of the transplant professional community, to help improve CMV management in SOT recipients.

MATERIALS AND METHODS

This was a questionnaire-based, cross-sectional online study, devised by ESOT and undertaken among the ESOT transplant professional's community. A Working Group was established in May 2019 to develop, refine, conduct, interpret, and publish this survey: group members were selected by the ESOT executive council, based on specific expertise in the management of CMV in SOT rather than by geographic location or nationality. A key objective was to involve experts from the field of infectious diseases and those with organ-specific expertise, including multiorgan transplantation.

The Working Group developed the questionnaire via several rounds of in-person and virtual discussion/revision, and the content was ratified in a virtual meeting in June 2019. The final survey consisted of 57 questions, including structured (multiple-choice) and open-ended questions (**Supplementary Table S1**), which took ~30–40 min to complete. The survey was hosted on cloud-based software (SurveyMonkey®, San Mateo, CA, United States) between 19 July and 31 October 2019.

The survey was promoted via a targeted online newsletter to all persons in the ESOT contact database via the congress app during the ESOT 2019 congress in Copenhagen, Denmark (September 2019), and via ESOT social media postings. European scientific organizations with an infectious disease focus [e.g., ESCMID (European Society of Clinical Microbiology and Infectious Diseases)] and European national transplant societies were also asked to promote the survey among their members.

The survey could be completed by any respondent, provided they had direct experience with managing CMV infection. Because the focus of the survey was the practice, knowledge, and opinions of the single transplant professional, responses from multiple personnel from the same institute were permitted. Questions 1–4, which focused on the respondent's specialty area and length of active clinical practice, were included to eliminate practitioners who did not declare appropriate experience.

Participants were provided with information outlining the survey objectives prior to their involvement. Those who answered all questions were offered 1 year's free access to the ESOT e-learning platform, *Transplant Live*, *via* a promotional code. No personal details were requested of participants, to maintain confidentiality.

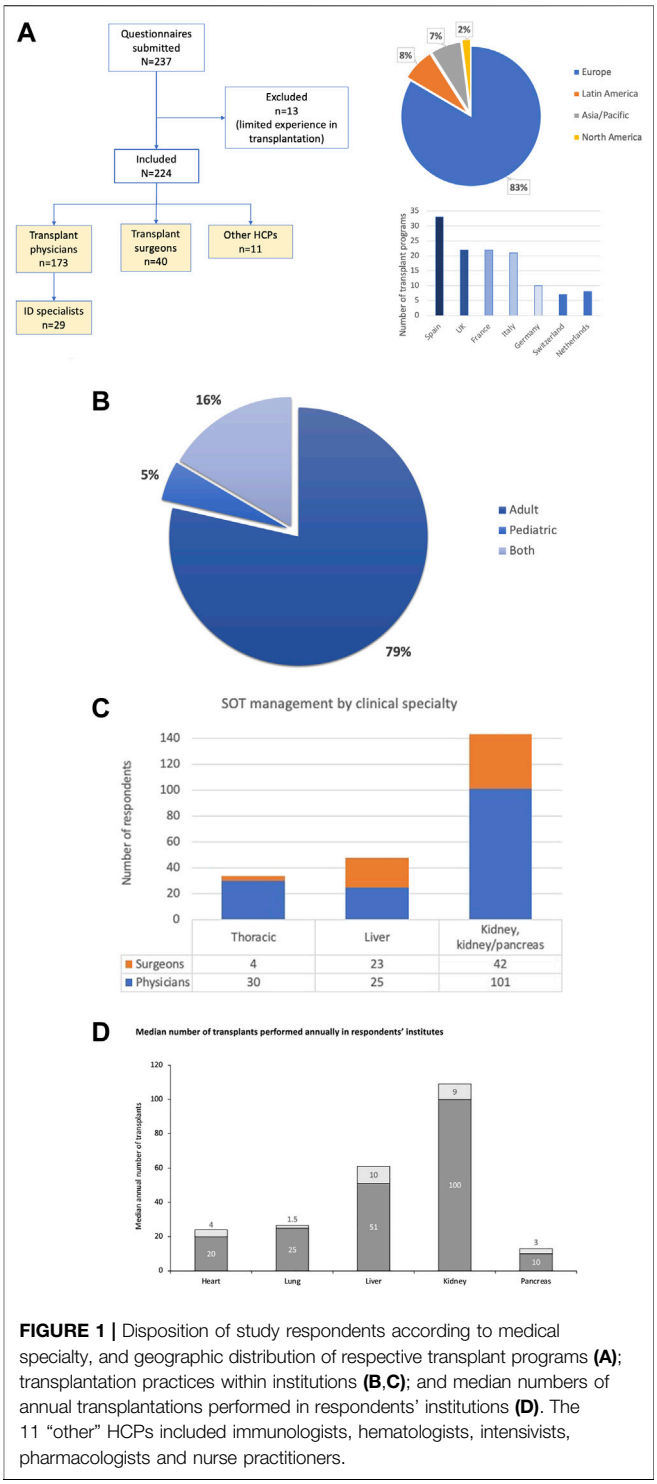

FIGURE 1 | Disposition of study respondents according to medical specialty, and geographic distribution of respective transplant programs **(A)**; transplantation practices within institutions **(B,C)**; and median numbers of annual transplantations performed in respondents' institutions **(D)**. The 11 "other" HCPs included immunologists, hematologists, intensivists, pharmacologists and nurse practitioners.

Request for authorization by the ethics committee at each center was deemed unnecessary: the survey was only intended to collect the personal perceptions/opinions of transplant professionals and neither directly involved patients nor sought patient-specific data.

Statistical Analyses

All fully completed surveys were included in the analysis. Summary statistics were generated from SurveyMonkey®. Raw data were downloaded onto an Excel® (Microsoft Office, Redmond, WA, United States) spreadsheet, for subsequent analysis. Continuous variables are reported as mean ± SD or median; categorical variables are reported as %. Most of the questions returned categorical answers, therefore for the scope of this manuscript, between-group differences were analyzed using the χ^2 test (PRISM 7; GraphPad Software Inc., San Diego, CA, United States). p-values <0.05 were considered statistically significant.

For several questions, respondents were asked to grade their opinions regarding specific statements by using a 1–7 ranking, where 1 indicated maximum disagreement and 7 indicated highest agreement. Responses were analyzed by calculating the weighted average for each item: if the weighted average was >5, we assumed consensus for that statement; if it was <3, we assumed consensus against; scores between 3 and 5 were interpreted as no consensus.

RESULTS

Respondent Demographics

Disposition of study respondents is shown in **Figure 1A**. Of the 160 institutions represented, 128 (80%) were European, and most were responsible for multiple transplant programs. Survey responses represented 41 countries in Europe, South America, Asia, North America, and Australia.

Of 224 responses, 197 (88%) were completed by individuals from different transplant programs, and when analyzing responses by the few individuals from the same center we often found differences in some of the practice areas, with most variability in behaviors such as pre-emptive treatment threshold. The majority of respondents (213; 95%) were involved in adult transplantations. In addition, the majority of clinicians were physicians rather than surgeons (**Figures 1B,C**). Some respondents managed or conducted several types of SOT. Respondents had been in active clinical SOT practice for a mean of 14.1 years. The median number of transplants reported annually by organ in the respondents' institutions is shown in **Figure 1D**: centers volumes indicate that survey participants were mostly practicing in medium to large transplant centers.

When considering European representation, the 128 European hospitals represented in this survey are 40% of the total (316) transplant hospitals active in the 27 European countries included in the survey.

Cytomegalovirus Diagnosis

The majority of respondents (217/224; 97%) indicated blood CMV-DNA PCR as the tool used to diagnose CMV infection: only 7 (3%) centers used antigenemia. However, the types of assay and units of measurement utilized revealed substantial variability: 124 (57%) used whole-blood PCR and 92 (42%) used plasma PCR. Of note, while 162 respondents (72%) declared that their laboratory used World Health Organization (WHO) standard units to measure DNAemia, only 66 (40%) reported using them for clinical decision making (e.g. threshold for PET initiation) instead of the non-standardized DNA copies/ml. In seven institutions, the WHO standard was not used, and 55 respondents (24%) were unaware as to whether their laboratory used the measurement unit.

Prevention of Cytomegalovirus Infection/ Disease

In total, 193/224 (86%) respondents reported having an established protocol for CMV prevention in SOT recipients, modulated according to organ type in 135 (70%), D/R serostatus in 182 (94%), and use of antithymocyte globulin (ATG) in 114 (59%) of centers. In this context, 31 (13%) of respondents never used PET, and only 10 (5%) never used prophylaxis.

Antiviral prophylaxis was administered in D+/R− transplantations by 201 (90%) of respondents (**Figure 2A**). As expected, prophylaxis was less commonly administered in R+ than in D+/R− transplantations (**Figure 2B**), and was most commonly administered by respondents performing lung transplantations (**Figure 2C**).

Prophylaxis use was reported by 99 (44%) of respondents in D+/R+ and 87 (39%) in D−/R+ transplantations. However, 18% and 26% of respondents used neither prophylaxis nor PET in D+/R+ and D−/R+ transplantations, respectively. Conversely, despite D−/R− having the lowest risk of CMV infection, 16% of respondents used prophylaxis in these patients. In this relatively low-risk group, prophylaxis was significantly more commonly utilized in thoracic organ recipients and least utilized in liver transplant recipients ($p < 0.01$) (data not shown).

Prophylaxis duration in R+ recipients was significantly shorter than in D+/R− recipients (**Figure 2B**). Again, lung transplant recipients had the significantly longest treatment period, with prophylaxis lasting ≥6 months in ~60% of responses ($p < 0.01$ when compared with abdominal transplantation programs).

While antiviral prophylaxis was evenly distributed across SOT types, duration varied significantly (**Figure 2B**), with lung transplant specialists using prophylaxis for >12 months in >60% of cases, and liver transplant specialists reporting the shortest duration (all patients <12 months, 49% < 3 months; $p < 0.01$). Of note, 28 (14%) respondents performed CMV surveillance and PET after the end of prophylaxis. Furthermore, there appeared to be no consensus on either the frequency of assessing CMV DNAemia or the duration of PET after prophylaxis.

Valganciclovir was the drug most frequently utilized in prophylaxis regimens. Respondents reported that ~90% of patients received valganciclovir, while ~10% of patients might also require intravenous ganciclovir because they were unable to take the oral formulation in the early postoperative period. According to 183 (80%) of respondents, prophylaxis commenced within the first week after transplantation, and 18 (8%) reported the addition of CMVIg for D+/R− patients or those receiving ATG.

Despite its widespread use, myelotoxicity was considered to have substantial negative impact on valganciclovir administration

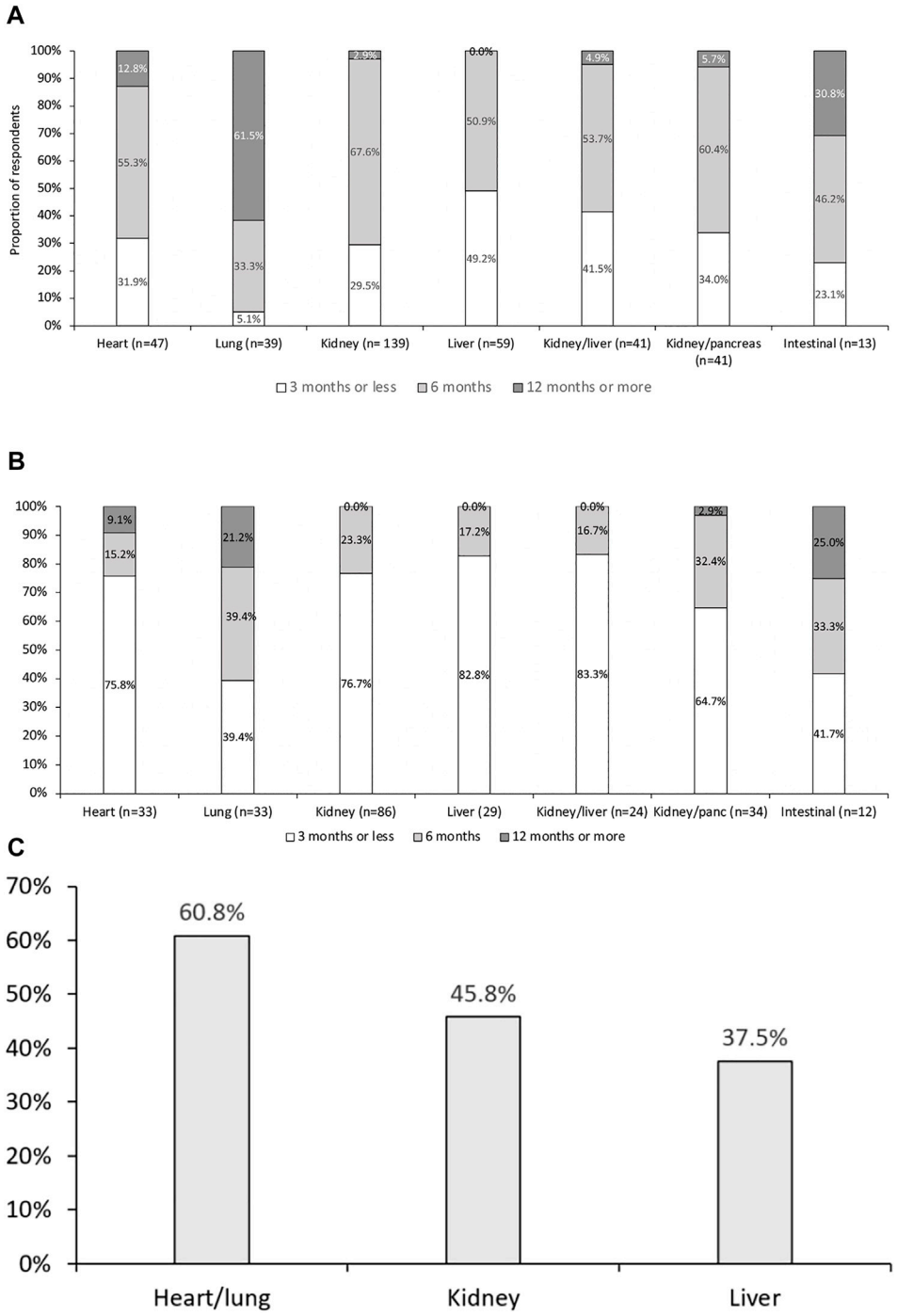

FIGURE 2 | Duration of prophylactic antiviral therapy for cytomegalovirus infection in **(A)** D+/R−, and **(B)** D+/R+. Proportion of respondents using prophylaxis in R+ patients **(C)**. $p < 0.01$ across all groups. After correction for multiple comparisons, duration following lung transplantation was significantly different from all other groups. Antiviral therapy duration following heart transplantation was also significantly different from those following lung and liver transplantation.

by 174 (78%) of respondents (leading to drug discontinuation in 10%–20% of SOT recipients, according to 98 [43%] of respondents) (**Figure 3**). In this regard, treatment of myelotoxicity took a stepwise approach. The most common first step (reported by 100 [44%] respondents) was a reduction in (or withdrawal of) mycophenolic acid derivatives, followed by withdrawal of trimethoprim/sulfamethoxazole. Valganciclovir withdrawal was only considered to be the third step in the approach to myelotoxicity. Of note, 109 (49%) of respondents reported the need for granulocyte colony-stimulating factor in

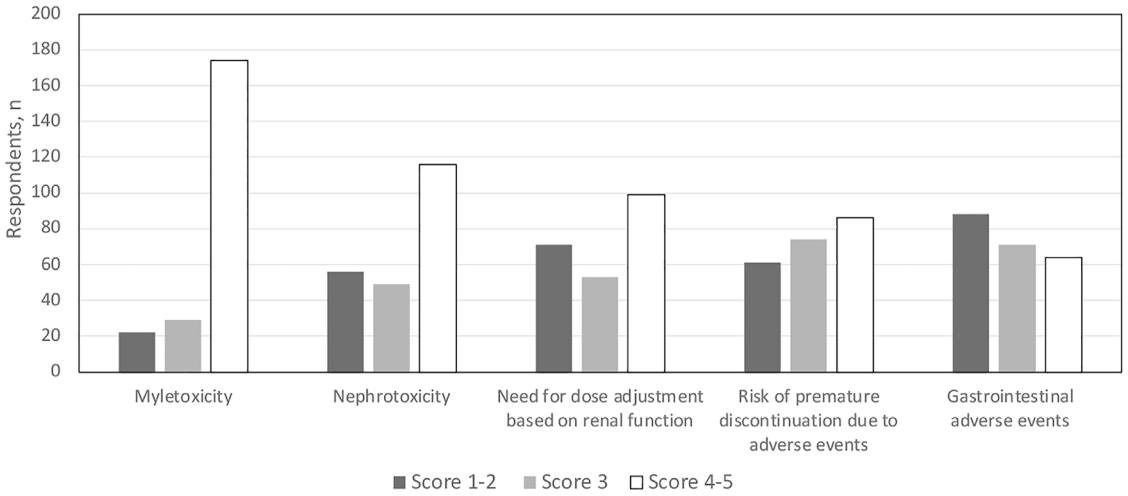

FIGURE 3 | Conditions impacting use of currently approved anti-CMV agents in solid organ transplant recipients. Scores 1–2 do not impact use; score 3 has moderate impact on use; scores 4–5 substantially impact use.

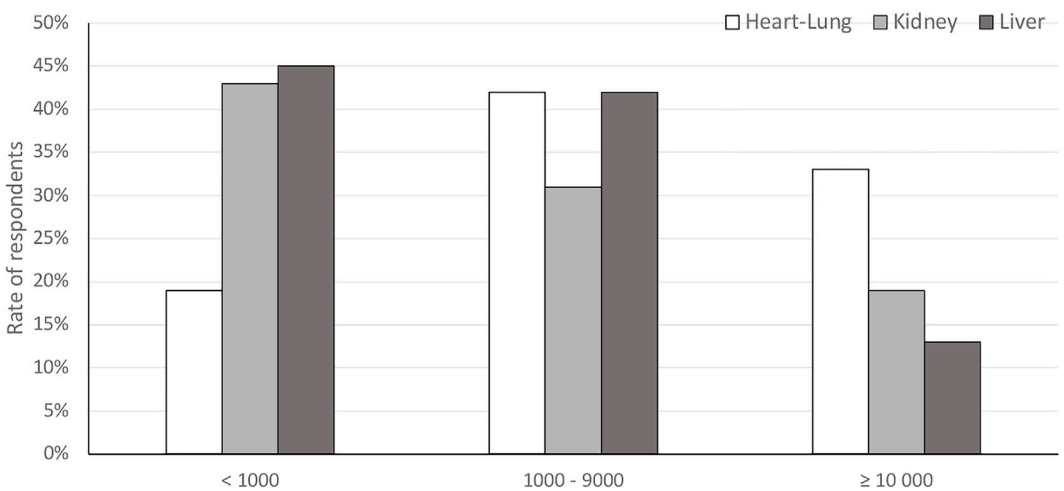

FIGURE 4 | Reported thresholds for pre-emptive antiviral therapy initiation in different organ recipients from respondents using whole-blood polymerase chain reaction assays.

≥10% of patients because of CMV prophylaxis or PET-related myelotoxicity.

Pre-Emptive Antiviral Treatment Initiation

Responses regarding PET management provided a snapshot of the extreme variability and lack of standardization in assay and criteria for treatment initiation. Of 201 respondents indicating DNA thresholds for PET initiation, 58% were based on whole-blood assays; only 66 responses gave thresholds in WHO units. Thresholds were dispersed over a wide range of plasma and whole-blood DNAemia values across all SOT recipients. The median reported plasma DNA threshold value for PET initiation was 1,000 copies/ml (range, 10–10,000 copies/ml), and the median reported whole-blood threshold DNA value was 1,000 copies/ml (range, 5–20,000 copies/ml). **Figure 4** shows the distribution of thresholds for whole-blood PCR, which was the most frequently reported assay.

Most respondents (185; 83%) monitored patients after cessation of prophylaxis or PET, with a wide variability of schedules used within 3–6 months after cessation. Values ranged from testing once- or twice-weekly to every 2–3 weeks, depending on time since transplantation and level of infection risk.

PET was widely used, especially in liver recipients: 48% of centers used PET in R+ patients and 12% in D+/R– transplantations. Valganciclovir was the most common first-line PET strategy (reported by 195 [87%] of respondents), followed by intravenous ganciclovir, which was often

administered in inpatient settings. In 6% of centers, CMVIg was added to valganciclovir or ganciclovir.

Treatment of Cytomegalovirus Disease

Treatment of CMV disease was assessed by a question allowing multiple responses. Most respondents (172/224; 77%) indicated intravenous ganciclovir as treatment of choice for CMV disease, but 140 (63%) also administered valganciclovir, supporting an initial uptake of the evidence deriving from the VICTOR trial (23). This survey did not specifically dissect the reasons for choosing intravenous ganciclovir versus valganciclovir, but according to comments added by some respondents it is likely that intravenous ganciclovir was prescribed as the attack strategy, followed by valganciclovir for maintenance; there is no indication if choice was based on disease severity (i.e., end-organ disease versus CMV syndrome), as recommended by current guidelines. Of note, 31 (14%) used CMVIg (in addition to intravenous ganciclovir or valganciclovir) to treat primary CMV infection in cases that involved D+/R− patients, hypogammaglobulinemia (<500 mg/dl), pneumonia, enteritis, or severe leukopenia.

Treatment Resistance

Molecular diagnostic approaches for detecting CMV resistance were employed by 102/224 (46%) of respondents, while 80 (36%) said that resistance testing was unavailable and 34 (15%) did not know whether testing was available in their institution. Ganciclovir resistance was quite rare, with annual incidence rates of <1% reported by 180 (80%), rates of 1%–5% reported by 39 (17%), and rates of 6%–10% reported by 5 (2%) of respondents.

Infections caused by ganciclovir-resistant CMV strains were treated with high-dose ganciclovir by 109 (49%) of respondents; most of these (157; 70%) used foscarnet, which was usually given following high-dose ganciclovir. A smaller rate of respondents used cidofovir (22%). CMVIg was administered by 69 (31%) of respondents, in combination with antivirals, and 69 (31%) respondents switched patients with infections resultant from ganciclovir-resistant CMV strains to mTOR inhibitors.

Ganciclovir resistance was considered a relevant issue in current CMV management by only 57% of respondents (128 scored ≥5 on a 7-point scale; mean score 4.75). Conversely, when asked about relevant issues for future research, 169 (75%) respondents said that improvement of strategies to manage CMV resistance would be relevant (mean score, 5.29).

Monitoring Cytomegalovirus-Specific T Cell Response

Most respondents (183/224; 82%) said that monitoring of CMV-specific T cell responses was not routinely performed. Centers that offered this facility generally utilized QuantiFERON (26/49; 53%) and/or ELISpot (21/49; 43%). Only 2/49 (4%) utilized other techniques, such as intracellular cytokine staining, MHC multimer, or Viracor. Questions 51 and 52 (**Supplementary Table S1**) investigated current perceptions of the importance of such analyses and the likelihood that monitoring CMV-specific T cell responses could become standard-of-care in the next 1–3 years. Respondents indicated that immunologic monitoring is not of primary importance but is likely to become more important within the next 1–3 years (**Figure 5**).

Key Issues in Cytomegalovirus Management in Solid Organ Transplantation and Future Research

When addressing opinions regarding the major open issues for CMV management, we recorded consensus for "drug toxicities" (5.15), late CMV infection (5.09), and "ease of administration" (5.07). The relevance of "management of CMV resistance" (4.74), "drug cost" (4.57), and "drug interactions" reached lower levels of consensus.

Respondents were asked to indicate what would be relevant for future research and development of CMV in SOT populations (Question 57; **Supplementary Table S1**). Weighted averages for structured answers ranged from 5.41 to 5.66, indicating consensus that all prespecified topics were considered highly important. The five responses with the highest weighted average scores were "optimizing immunosuppressive protocols" (weighted average 5.66), "long term impact of CMV on graft dysfunction and comorbidities" (5.63), "personalized anti-CMV strategies based on monitoring of CMV-specific T cell response" (5.56), "vaccination" (5.47), and "new drug discovery" (5.41).

DISCUSSION

This article presents the key findings of an international survey designed to investigate current practices in the management and prevention of CMV infection among members of the ESOT community. Our aims were to assess the distance between current practice and established guidelines, identify educational gaps, explore the unmet needs of currently available treatments, and anticipate the developments in this field.

The findings provide a real-world snapshot, covering a large proportion of the transplant units in Europe, with a glimpse of extra-European practice. As opposed to another recent survey (24), these data capture mostly the opinions of transplant physicians and surgeons in managing CMV infection, with only a small proportion of respondents being infectious disease specialists. The data are nuanced regarding levels of consistency between daily practices, guideline use, and reliance on scientific evidence. While there is an appropriate trend toward a customized approach (related to the difference in CMV risk across the transplanted organs, and donor/recipient serology match), conversely there remains very wide variability in approaches to specific problems including drug resistance, monitoring for infection, and use of laboratory tools to detect CMV-DNA. In addition, the survey revealed some practices clearly not recommendable, based on current evidence.

Prevention and management of CMV infection had a center-specific approach, with some divergence from current guidelines (8,9) (**Table 1**).

Regarding diagnostic strategies, CMV-DNA was widely used, with a preference for whole blood as a matrix. Antigenemia was

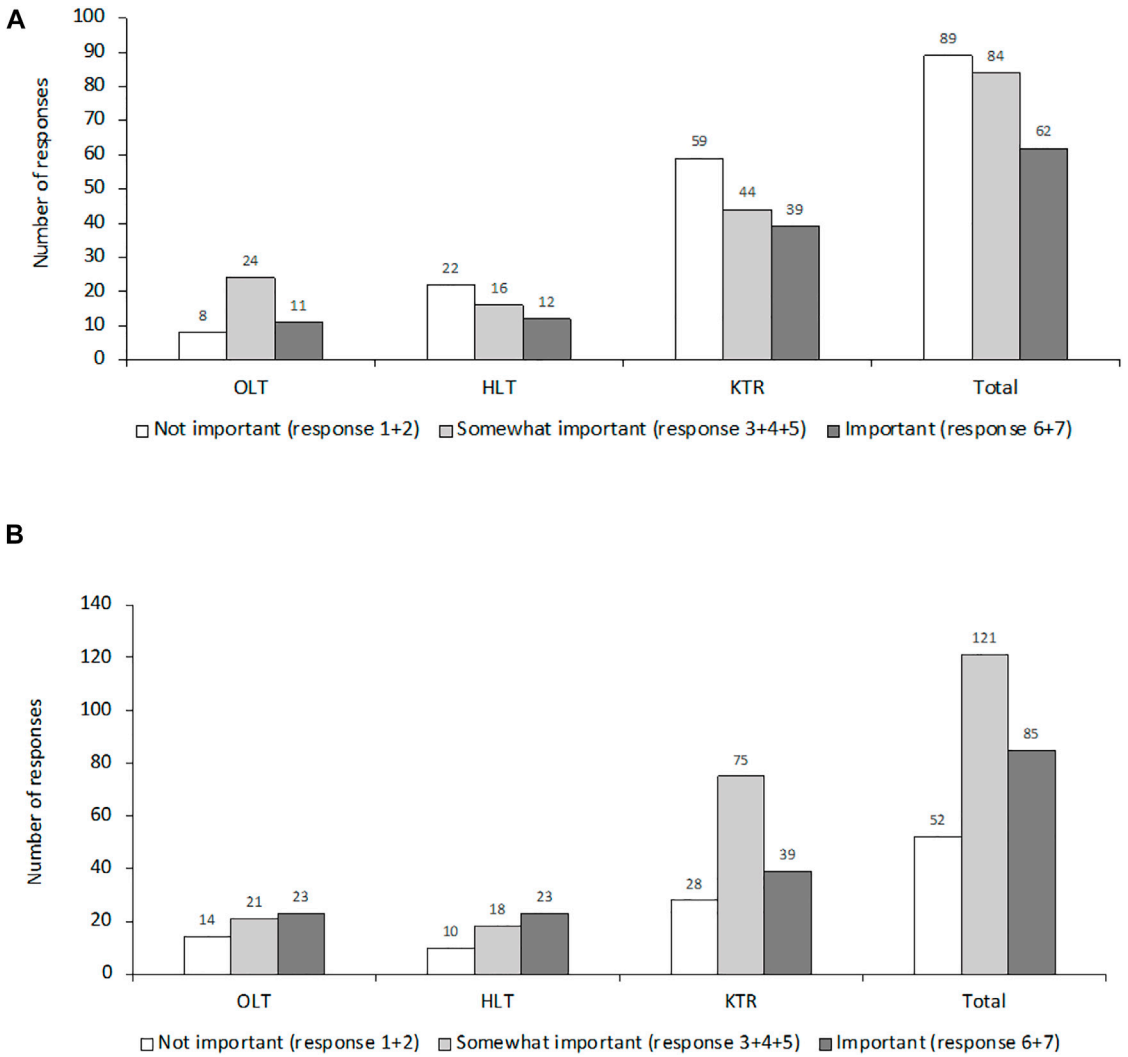

FIGURE 5 | Perception among respondents regarding the importance of CMV-specific T cell response **(A)** at the time of the survey and **(B)** in 1–3 years.

TABLE 1 | Major discrepancies between guideline recommendations and survey-reported practices.

	Guideline recommendation	Survey-reported practice
CMV-DNA assay	• WHO units for DNAemia are recommended	• Only 40% of respondents use them for decision making • 28% don't know what WHO units are
CMV prophylaxis prescription	• Prophylaxis is NOT recommended in D−/R− patients	• 15% of respondents claim to use prophylaxis in D−/R− patients
CMV prophylaxis duration in D+/R−	• 6 months for KTx • 3–6 months for HTx and LTx • 6–12 months for LuTx	• Most respondents are in line with recommendations, but HTx usually seem to receive longer-term prophylaxis than KTx
Post-prophylaxis DNAemia surveillance	• Suggested only in high-risk patients with high immunosuppression burden • Weekly/biweekly frequency is preferred	• 12%–16% of respondents perform post-prophylaxis monitoring, but with frequencies between 1 and 3 months
CMV resistance	• CMV genotyping is recommended if viral load persists over 6 weeks of adequate GCV administration	• CMV genotyping is unavailable or unknown to 54% of respondents

HTx, heart transplant; KTx, kidney transplant; LTx, liver transplant; LuTx lung transplant.

still used by a small minority of centers represented. A previous survey of CMV management in European transplant recipients showed an almost identical proportion of centers using whole blood and plasma (42% vs. 41%, respectively) (24). In our study, a slightly larger proportion of centers utilized whole blood (57% vs. 39% using plasma). In this context, the knowledge and experience in using WHO standard for quantitative DNA PCR was suboptimal (25), identifying an educational gap in awareness and interpretation of laboratory results of viral monitoring.

Current guidelines/recommendations are apparently clear in terms of diagnosis, duration of prophylaxis, and CMV-DNA monitoring, followed by pre-emptive strategies and treatment of CMV disease (8, 9). Nonetheless, few centers strictly followed these guidelines. It is difficult to suggest reasons for this, which in part may be related to budgetary or reimbursement policies in specific countries. Another reason might be the increased usage of mTOR inhibitors and their beneficial effect on CMV replication (26, 27). Finally, there may be some reticence by clinicians to change their usual practice. Nonetheless, these findings clearly outline an educational gap to be filled and provide a foundation on which the limited adherence to management guidelines should be analyzed in greater detail.

Preventive strategies in high-risk (D+/R−) transplantations appeared to rely heavily on valganciclovir prophylaxis. Prophylaxis was used more widely in thoracic organ transplantations than in other SOT procedures, perhaps due to the higher risk of direct and indirect effects of CMV infection in lung recipients, and the fear of indirect CMV effects in heart transplant patients (1, 28).

The observed low rate of prophylaxis in R+ liver transplant recipients is consistent with the perceived lower risk of CMV disease and the reports of lower activity of valganciclovir in these patients (29, 30). Duration of prophylaxis varied across centers, ranging from 3 to 6 months in kidney/liver transplant recipients and from 3 to 12 months in heart/lung recipients. In intermediate- and low-risk groups, the approach to prophylaxis appeared to be heterogeneous in terms of strategy and duration, with most institutes treating prophylactically for up to 6 months. For reasons that are unclear, 25% of respondents reported the absence of a preventive strategy in R+ patients. It was also very surprising that a considerable number of respondents reported using prophylaxis in low-risk patients, an approach not recommended by current guidelines (8, 9).

Although expected, between-center variations in PET cut-off values and sampling schedules were striking. However, most relied on whole-blood (frequently) or plasma (occasionally) CMV-DNA threshold levels in the range of 500–5,000 copies/ml. More than the CMV-DNA value disparity, it is noteworthy that the two matrices were used as synonymous specimens, while it is known that whole blood overall contains 1 $\log_{10}$ more CMV-DNA than plasma. Furthermore, ~40% of centers (those using plasma) were indeed starting PET at a 1 $\log_{10}$ higher CMV-DNA level than those using whole blood. These findings reinforce the need for prospective studies to determine the optimum cut-off, sampling schedule, and standardized assay and units of measurement before recommendations for using PET can be decisively made.

Most respondents reported no ganciclovir resistance in their centers, despite extensive use of prophylaxis and the availability of resistance testing. It is unclear whether this reflects the wider real-world situation or represents underestimation specific to this survey. Although a few participants reported using CMVIg in cases of resistance, it is unclear whether this reflects actual documented resistance or merely refractory or recurrent CMV infection. We believe that these uncertainties highlight another educational gap that should be specifically addressed.

Although monitoring the CMV-specific T cell response was not considered crucial, respondents felt it would soon become more important. There is a strong scientific rationale for monitoring the CMV-specific immune response (8, 9), but there are no sufficiently validated procedures to enable this to be done effectively in routine practice. This is therefore another unmet need in CMV management.

The current study had several potential limitations. The survey was advertised to the broad ESOT community, targeting individual healthcare professionals rather than institutions. This approach may have introduced some degree of interest bias by collecting responses from people interested and educated in CMV, while missing data on practices from institutions where CMV infection in SOT is not considered relevant or is not addressed properly. Consequently, we could have missed some education gaps that need to be addressed specifically. Another potential limitation is that some answers were ambiguous. Finally, this survey was conducted in the pre-COVID-19 era, which might have had an impact on the policies within centers, particularly in those using pre-emptive strategies to prevent CMV disease in order to avoid frequent access to the hospital for CMV testing. Nevertheless, this survey highlights a very wide variability in clinical practice, often in discordance with current evidence, thus prompting us to encourage specific education activities to favor a more homogeneous management approach for CMV infection in SOT patients.

Although the burden of CMV in SOT has been alleviated through advances in diagnosis, prevention, and treatment, this viral infection continues to have substantial impact in this patient population. The present survey adds to the body of evidence demonstrating a heterogenous approach to CMV infection management and a divergence from international guidelines. Myelotoxicity is perceived as the major drawback with current agents, underlying the need for novel therapies for prophylaxis, to facilitate a safer and more effective strategy to prevent CMV infection. In this setting, the potential availability of letermovir in SOT may represent a relevant step forward (31–35).

Managing ganciclovir-resistant CMV strains was perceived as a major challenge for most centers. Results of a phase II and III trial with maribavir in refractory/treatment-resistant CMV infection may contribute to improved management of resistance (32, 33).

In conclusion, this study highlights several education gaps and unmet needs in the context of management of CMV infection in SOT. Toxicities of current first-line therapies are a major drawback in clinical practice, while improving the knowledge of WHO standard units for CMV-DNA assays may help to design studies targeted to identify the most appropriate threshold to initiate PET. In this context, further development of assays for

CMV-specific immunity could represent a key asset to tailor both PET and prophylaxis approaches (36). Finally, these findings will help to guide the development and promotion of targeted educational activities. The ESOT Working Group will continue this project to try to harmonize and improve the management of CMV infection in this challenging population.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

PG, NK, FS, JA, and LP were part of the Working Group and contributed to the development of the survey. All authors helped to analyze the data and reviewed/provided input into the entire manuscript, attending meetings/telephone calls to aid its development; PG, NK, FB, and LP wrote specified sections of the manuscript. PG was also Chair of the Working Group and facilitated development of the manuscript with the medical writer. The first full draft of the article was reviewed by PG and LP; the revised draft was reviewed, finalized, and approved by all co-authors before submission for publication.

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CONFLICT OF INTEREST

PG has been a consultant or member of an advisory committee for Angelini, Becton Dickinson, Biotest, Correvio, Gilead Sciences, Merck Sharpe & Dohme, Nordic Pharma, Paratek Pharmaceuticals, AlloVir, and Shire; and a speakers' bureau member for Becton Dickinson, Biotest, Gilead Sciences, Merck Sharpe & Dohme, Pfizer, Atara, and Vertex. NK has been a speaker or an advisory board member for AbbVie, Amgen, Astellas, Biotest, Chiesi, CSL Behring, Gilead, Fresenius Medical Care, Merck Sharpe & Dohme, Neovii, Novartis Pharma, Sandoz, Sanofi, and Shire. FS has received speaker's honoraria and/or research grants from Novartis, Astellas, Chiesi, Gilead, Merck Sharp & Dohme, Neovii, Sandoz, Pfizer, Biotest, Takeda, and Baxter. FB has received grants/research support from AB Analitica, DiaSorin, ELITechGroup, NTP, and Qiagen; he has also received honoraria or consultation fees from Biotest, DiaSorin, HUMABS, Merck Sharpe & Dohme, Qiagen, and Shire. JA has been a consultant and speakers' bureau member for Astellas Pharma, Biotoscana, Gilead, Merck Sharpe & Dohme, Pfizer, Roche, and United Medical. LP has been a consultant for Novartis and Biotest and received speaker fees from AstraZeneca, Boehringer Ingelheim, Abbott, and Paragonix.

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

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2022.10332/full#supplementary-material

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Post-Transplantation Cytomegalovirus Infection Interplays With the Development of Anastomotic Biliary Strictures After Liver Transplantation

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Background: Anastomotic biliary stricture (ABS) remains the most frequent complication after liver transplantation (LT). This study aimed to identify new anastomotic biliary stricture risk factors, with a specific focus on postoperative events. Additionally, ABS management and impact on patient and graft survival were assessed.

Methods: All consecutive patients who underwent LT with duct-to-duct anastomosis between 2010 and 2019 were included. All patients who died within 90 days after LT due to non-ABS-related causes were excluded.

Results: Among 240 patients, 65 (27.1%) developed ABS after a median time of 142 days (range, 13–1265). Median follow-up was 49 months (7–126). Upon multivariable analysis, donor BMI (OR=0.509, $p = 0.037$), post-LT CMV primoinfection (OR = 5.244, $p < 0.001$) or reactivation (OR = 2.421, $p = 0.015$) and the occurrence of post-LT anastomotic biliary fistula (OR = 2.691, $p = 0.021$) were associated with ABS. Anastomotic technical difficulty did not independently impact the risk of ABS (OR = 1.923, $p = 0.051$). First-line ABS treatment was systematically endoscopic (100%), and required a median of 2 (range, 1–11) procedures per patient. Repeat LT was not required in patients developing ABS. The occurrence of ABS was not associated with overall patient survival ($p = 0.912$) nor graft survival ($p = 0.521$).

Conclusion: The risk of developing ABS after LT seems driven by the occurrence of postoperative events such as CMV infection and anastomotic fistula. In this regard, the role of CMV prophylaxis warrants further investigations.

Keywords: liver transplantation, anastomotic biliary complications, biliary reconstruction, CMV infection, primoinfection

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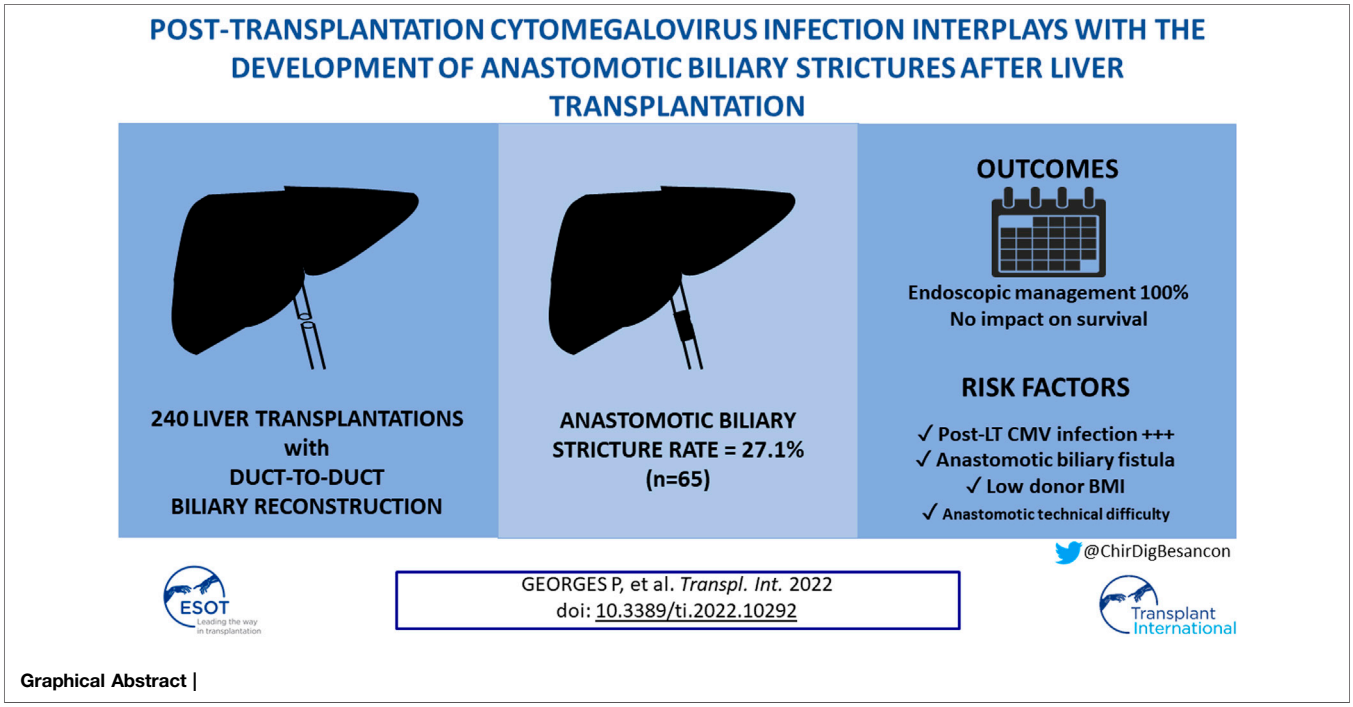
Abbreviations: ABS, anastomotic biliary stricture; BMI, body mass index; CMV, cytomegalovirus; DNA, desoxyribonucleic acid; ECD, extended criteria donor; ERCP, endoscopic retrograde cholangio pancreatography; ISGLS, international study group for liver surgery; LT, liver transplantation; MELD, model for end stage liver disease; PCR, polymerase chain reaction.



Graphical Abstract |

INTRODUCTION

Although advances in organ preservation, immunosuppression, and surgical techniques have improved outcomes after liver transplantation (LT), biliary complications remain the most frequent cause of morbidity after LT (1). Biliary complications are conventionally classified as anastomotic biliary strictures (ABS), non-anastomotic biliary strictures, and anastomotic biliary fistula. Among these, ABS generally occurs within 1 year after transplantation and remains the most frequent biliary complication, accounting for 15.1%–35% of complications (2–4). Yet the physiopathology of ABS remains unclear. Due to the vulnerable vascularization of extrahepatic bile ducts, technical issues and local ischemia are risk factors classically reported in the literature (5, 6). Additionally, the use of the Model for End-stage Liver Disease (MELD) score for organ allocation and the expansion to extended criteria donor (ECD) have been associated with the risk of ABS (7, 8). Overall, risk factors are multiple as they are related to recipient and donor characteristics and transplantation techniques. Consequently, reported risk factors are highly variable and conflict between existing series (9, 7, 10, 11). Such a heterogeneity across the literature is explained in part by the lack of consensus on an ABS definition and the heterogeneity of included patients in terms of recipient severity, graft type, biliary reconstruction techniques, and biliary stricture types (e.g., anastomotic or not), among other potential risk factors. Notably, the increasing use of ECD-focused research on graft optimization to improve outcomes, along with other factors such as postoperative events, might interplay with the occurrence of ABS.

This study aimed to identify preoperative, intraoperative, and postoperative risk factors of ABS after deceased donor liver transplantation. Additionally, ABS management and impact on patient and graft outcomes were evaluated.

METHODS

Study Population

All consecutive patients who underwent LT with duct-to-duct anastomosis between January 2010 and December 2019 were considered for inclusion. All patients who underwent a bilio-enteric reconstruction and those lost in follow-up or requiring early retransplantation after LT were excluded. Additionally, to avoid competing risks of early mortality due to causes other than ABS, patients who died within 90 days post-LT owing to ABS-unrelated causes were excluded. Recipients were divided into two groups based on the occurrence of ABS or not.

Data Collection

Data were retrieved from electronic medical records and from the prospectively maintained CRISTAL on-line data base from the Biomedicine Agency, approved by the French Data Protection Authority (Commission Nationale de l'Informatique et des Libertés) (Decision n°96-025 of March 19 1996). The following data were collected: recipients' characteristics at the time of transplantation, donors' characteristics, intraoperative data, and postoperative outcomes. Extended criteria donors were those older than 75 years, or with confirmed steatosis >30%. All postoperative complications occurring within 90 days after surgery were collected and graded according to the

Dindo–Clavien classification. In patients with multiple complications, the highest grade was retained for analysis. Regarding anastomotic biliary complications other than ABS, anastomotic biliary fistula was defined according to the ISGLS definition (12). Early allograft dysfunction was defined according to the current definition (13). Early rejection corresponded to a histological diagnosis defined upon Banff criteria within the first 3 months after LT (14). Medical complications including extra-abdominal infection, CMV infection, or reactivation and acute kidney injury were also collected.

Liver Graft Procurement and Transplantation

All grafts were procured from a brain-dead donor using the *en bloc* technique (15) unless the pancreas was simultaneously procured for organ transplantation. Full dissection of the graft hepatic pedicle was next carried out on a hypothermic back table using an ice basin filled with cold preservation solution. Care was taken not to dissect above the gastroduodenal artery to prevent any proper hepatic artery lesion. Similarly, cholecystectomy was performed at this stage and the common hepatic duct was bluntly dissected and divided as low as possible after pancreatic head removal to avoid any injury or devascularization.

Regarding biliary reconstruction after vascular implantation, duct-to-duct anastomosis was performed using a 6/0 or 7/0 polyglyconate long-term monofilament absorbable sutures (Maxon™, Covidien, Medtronic, Watford, United Kingdom). Both graft and recipient bile duct were trimmed to length to ensure a tension-free anastomosis between two appropriately vascularized ducts. End-to-end anastomosis was then fashioned using a posterior running suture and anterior interrupted suture or two posterior and anterior running sutures, systematically knotted on the outside. In case of anticipated anastomotic difficulty, an anterior ductoplasty technique or a T-tube insertion could be used at the discretion of the transplant surgeon. Anastomotic difficulty was anticipated when the graft and/or the recipient bile duct diameter was smaller than 5 mm or when a donor-recipient duct size mismatch larger than 4 mm was present.

Postoperative Management

Systematic Doppler ultrasounds were performed daily from postoperative day one to five, then once a week to detect any vascular complication. Pre-transplantation screening of donors and recipients for CMV serological status (IgG) defined the strategy employed for the prevention of CMV reactivation or primary infection. A 6-month CMV prophylaxis was routinely given to "high-risk" recipients defined as seronegative recipients receiving a graft from a seropositive donor. The pre-emptive strategy was applied to other patients with routine determination of CMV viremia by sensitive assay (molecular diagnosis). Of note, in this situation, CMV antigenemia assay and qPCR were weekly checked from LT to patient discharge, then once a month for the first year. In case of positive CMV viremia, whether symptomatic or not, CMV therapy was systematically initiated to prevent progression to clinical disease.

The diagnosis of ABS was suspected on the presence of a size discrepancy at the site of the bilio-biliary anastomosis with or without upstream bile duct dilatation on imaging (ultrasound, cholangiography, CT scan, or MR-cholangiography). This had to be associated with a concomitant cholestasis and/or an elevated serum bilirubin after excluding other cholestasis causes such as graft rejection and viral reactivation. Each patient with suspected ABS underwent an endoscopic retro-grade cholangiopancreatography (ERCP) to confirm ABS. In case of ABS confirmation, a plastic or self-expandable metallic stent was placed at the discretion of the endoscopic team. Endoscopic stent replacement was repeated each 4–6 months until ABS clearance.

Statistical Analysis

The χ^2 test or Fischer exact test was used for analysis of categorical variables, as appropriate. Continuous variables with a normal distribution were presented as mean (standard deviation) and non-normally distributed variables as median (range); t test and Mann–Whitney U test were used for statistical analysis. All perioperative variables associated with the occurrence of ABS in univariable analysis ($p < 0.100$) were included in a binary logistic regression model to identify independent risk factors of ABS. Backward selection was used, with a 0·1 cut-off for entry into the model. In case of collinearity between variables, only the most relevant variable was included in the model. Regarding postoperative variables, given the potential time-dependent relationship between the occurrences of postoperative events, only those occurring before the occurrence of ABS were deemed of interest and were retained in multivariable analysis. Performance of the multivariable model was assessed in terms of discrimination, expressed as the area under the curve (AUC) ± standard error (SE). Additionally, overall and graft survival estimates were calculated using the Kaplan–Meier method. OS and graft survival corresponded to the interval between LT and the date of last follow-up or death and between LT and date of graft failure. Survival differences between patients who did and did not experience ABS were compared using the log rank test. All p values were based on two-tailed statistical analysis and $p < 0{\cdot}050$ was considered to indicate statistical significance. Analyses were performed with SPSS® software, version 27.0 for Windows® (IBM, Armonk, New York, United States). The present study complied with the STROBE Guidelines (16).

RESULTS

Population

Over the study period, 288 LT were performed, of which 48 LT were excluded (ABS-unrelated 90-day mortality in patients with bilio-biliary reconstruction, $n = 25$; bilio-enteric reconstruction, $n = 18$; early retransplantation, $n = 5$) and 240 LT were included (**Figure 1**). Recipients' characteristics are listed in **Table 1**. Mean recipient age and mean MELD score were respectively 55.7 years old and 21.2 at the time of transplantation. Donors' characteristics are listed in **Table 2**. Mean donor age was 57.6 years old. Most grafts (90.4%) were allocated according to the standard national liver score system. ECD was used in 141 LT

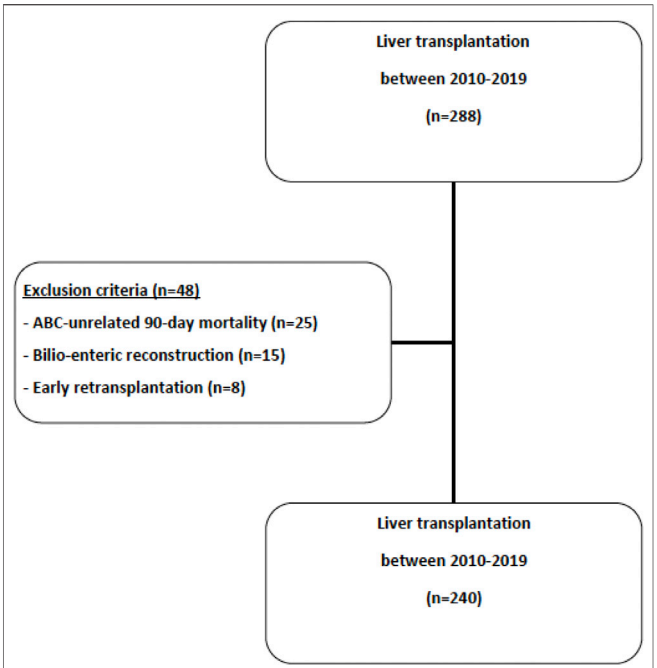


FIGURE 1 | Study flowchart.

(58.8%). Main causes for brain death were stroke (58.2%) and trauma (24.6%) and nearly one third (28.7%) of donors presented cardiac arrest. Pre-transplantation donors' and recipients' serological CMV status are described in **Table 2**.

Intraoperative data are reported in **Table 3**. Mean cold ischemia duration was 533.2 min. Anastomotic technical difficulty as defined above was encountered in 81 LT (33.8%). Both biliary ductoplasty (n = 45; 18.8%) and T-tube placement (n = 46; 19.2%) were not performed routinely. Owing to significant bleeding (n = 4) or failure to fascial closure (n = 3), an open abdomen approach with negative wound therapy was adopted at the end of LT in seven patients (2.9%), of which one had a delayed biliary reconstruction.

Postoperative Outcomes

Among all transplanted patients with bilio-biliary reconstruction over the study period (n = 288), the 90-day mortality rate was 10.4% (n = 25). Causes of 90-day mortality are listed in **Supplementary Table S1**. No patient died due to an anastomotic biliary fistula or stricture after a bilio-biliary reconstruction. Among excluded patients, one patient with

TABLE 1 | Characteristics of recipients at transplantation (n = 240).

	Overall (n = 240)	ABS+ (n = 65)	ABS- (n = 175)	*P*
Age, years	55.7 (10.4)	56.7 (10)	55.1 (10)	0.090
Gender				0.711
Male	173 (72.1%)	48 (73.8%)	125 (71.4%)	
Female	67 (27.9%)	17 (26.2%)	50 (28.6%)	
Body mass index, kg/m^2	26.4 (4.8)	26 (4)	26 (5)	0.605
Preoperative recipient morbidity and acuity				
Hypertension	79 (32.9%)	23 (35.4%)	56 (32%)	0.620
Type 2 diabetes	69 (28.7%)	17 (26.2%)	52 (29.7%)	0.588
Active smoking	67 (27.9%)	16 (24.6%)	51 (29.1%)	0.492
Mechanical ventilation (24 h pre- LT)	9 (3.8%)	2 (3.1%)	7 (4%)	0.738
Preoperative organ failure	7 (2.9%)	—	7 (4%)	0.204
MELD score	21.2 (9.8)	21.7 (10)	21 (10)	0.656
Child-Pugh score				0.170
A	65 (27.1%)	13 (20%)	52 (29.7%)	
B	41 (17.1%)	15 (23.1%)	26 (14.9%)	
C	134 (55.8%)	37 (56.9%)	97 (55.4%)	
Main indication for LT				0.186
Cirrhosis	138 (57.5%)	43 (66.2%)	95 (54.3%)	0.237
Hepatocellular carcinoma	59 (24.6%)	12 (18.5%)	47 (26.9%)	0.108
Acute liver failure	22 (9.2%)	7 (10.8%)	15 (8.6%)	0.600
Other	17 (7.1%)	3 (4.6%)	14 (8%)	0.571
Repeat LT	4 (1.7%)		4 (2.3%)	0.577
Previous upper GI surgery	36 (15%)	12 (18.5%)	24 (13.7%)	0.360
Waiting-list time, months	3 (0–56)	2 (0–38)	3 (0–56)	0.415

Numbers are expressed as mean (standard deviation), unless otherwise specified LT, liver transplantation; MELD, Model for End-stage Liver Disease.

TABLE 2 | Characteristics of donors (*n* = 240).

	Overall (*n* = 240)	ABS+ (*n* = 65)	ABS- (*n* = 175)	*P*
Age, years	57.6 (18.5)	58.8 (20)	57.2 (18)	0.325
Gender				0.827
Male	132 (55%)	35 (53.8%)	97 (55.4%)	
Female	108 (45%)	30 (46.2%)	78 (44.6%)	
Body mass index, kg/m^2	25.6 (4.9)	24.5 (4.3)	26.1 (5.1)	0.015
Graft allocation type				0.796
Acute	15 (6.3%)	5 (7.7%)	10 (5.7%)	
Standard	217 (90.4%)	57 (87.7%)	160 (91.4%)	
Hors-tour	6 (2.5%)	2 (3.1%)	4 (2.3%)	
DCD	2 (0.8%)	1 (1.5%)	1 (0.6%)	
Cardiac arrest	69 (28.7%)	19 (29.2%)	50 (28.6%)	0.920
Down time, minutes	25 (18.9)	30.4 (29.6)	22.9 (12.3)	0.891
Cause of death				0.512
Stroke	137 (58.2%)	37 (56.9%)	100 (57.1%)	
Trauma	59 (24.6%)	18 (27.8%)	41 (23.4%)	
Anoxia	37 (15.4%)	7 (10.8%)	30 (17.1%)	
Other	7 (2.9%)	3 (4.6%)	4 (2.3%)	
Donor morbidity				
Active smoking	94 (39.2%)	25 (38.5%)	69 (39.4%)	0.892
Alcohol intoxication	27 (11.3%)	3 (4.6%)	24 (13.7)	0.064
Hypertension	85 (35.4%)	21 (32.3%)	64 (36.6%)	0.539
Diabetes	34 (14.2%)	9 (13.8%)	25 (14.3%)	0.931
Cardiovascular disease	39 (16.3%)	11 (16.9%)	28 (16%)	0.863
Donor-recipient ABO matching	232 (96.7%)	62 (95.4%)	170 (97.1%)	0.530
Extended criteria donor	141 (58.8%)	103 (58.9%)	38 (58.5%)	0.956
Donor-recipient CMV status				0.032
Donor- Recipient-	50 (20.8%)	7 (10.8%)	43 (24.6%)	Reference
Donor + Recipient-	72 (30%)	24 (36.9%)	48 (27.4%)	0.009
Donor + Recipient+	62 (25.8%)	18 (27.7%)	44 (25.1%)	0.044
Donor- Recipient+	56 (23.3%)	16 (24.6%)	40 (22.9%)	0.046

Numbers are expressed as mean (standard deviation), unless otherwise specified CMV, Cytomegalovirus; DCD, Donor after Circulatory Death; ECD, Extended criteria donor.

TABLE 3 | Intraoperative data (*n* = 240).

	Overall (*n* = 240)	ABS+ (*n* = 65)	ABS- (*n* = 175)	*P*
Operative time, minutes	343 (78.4)	328 (69)	349 (81)	0.185
Estimated blood loss, l	3.4 (3.1)	3.3 (3.3)	3.4 (3)	0.221
Cold ischemia duration, minutes	533 (114)	536 (120)	532 (112)	0.959
Intraoperative red pack transfusion	194 (80.8%)	50 (76.9%)	144 (82.3%)	0.348
Intraoperative fresh frozen plasma	185 (77.1%)	48 (73.8%)	137 (78.3%)	0.467
Intraoperative platelets transfusion	120 (50%)	32 (49.2%)	88 (50.3%)	0.885
Temporary portocaval shunt	67 (27.9%)	20 (30.8%)	47 (27.8%)	0.654
Arterial reconstruction				0.809
One anastomosis	216 (90%)	59 (90.8%)	157 (89.7%)	
Two anastomoses	24 (10%)	6 (9.2%)	18 (10.3%)	
Caval replacement	4 (1.7%)	2 (3.1%)	2 (1.1%)	0.301
Portal thrombectomy	25 (10.4%)	3 (6.7%)	22 (15.4%)	0.206
Anastomotic technical difficulty	81 (33.8%)	31 (47.7%)	50 (28.6%)	0.005
Biliary ductoplasty	45 (18.8%)	11 (25%)	34 (24.8%)	0.981
T-tube use	46 (19.2%)	9 (13.8%)	37 (21.1%)	0.268
Delayed biliary reconstruction	1 (0.4%)	—	1 (0.6%)	>0.999
Open abdomen	7 (2.9%)	1 (1.5%)	6 (3.4%)	0.678

Numbers are expressed as mean (standard deviation), unless otherwise specified.

TABLE 4 | Postoperative complications after LT (n = 240).

	Overall (n = 240)	ABS+ (n = 65)	ABS- (n = 175)	P
Intensive care unit stay, days	8.4 (11)	7.5 (6)	8.7 (12)	0.918
Early allograft dysfunction	77 (33.5%)	18 (27.7%)	59 (33.7%)	0.333
Biliary complications	89 (37.1%)			
Anastomotic stenosis	65 (27.1%)	65 (100%)	—	—
Non anastomotic stenosis	20 (8.3%)	4 (6.2%)	16 (9.1%)	0.603
Anastomotic fistula	31 (12.9%)	16 (24.6%)	15 (8.6%)	0.001
Arterial complications	26 (10.9%)	9 (13.8%)	17 (9.7%)	0.360
Portal vein thrombosis	4 (1.7%)	1 (1.5%)	3 (1.7%)	>0.999
Intraabdominal infection	38 (15.8%)	6 (9.2%)	32 (18.3%)	0.111
CMV infection				<0.001
None	119 (49.6%)	42 (17.5%)	79 (32.9%)	
Primoinfection	42 (17.5%)	20 (30.8%)	22 (12.6%)	
Reactivation	79 (32.9%)	26 (40%)	53 (30.3%)	
Acute kidney injury	164 (68.3%)	46 (70.8%)	118 (67.4%)	0.621
Reoperation	34 (14.2%)	12 (18.5%)	22 (12.6%)	0.245
Early rejection	46 (19.2%)	11 (16.9%)	35 (20%)	0.713

Numbers are expressed as mean (standard deviation), unless otherwise specified CMV, cytomegalovirus.

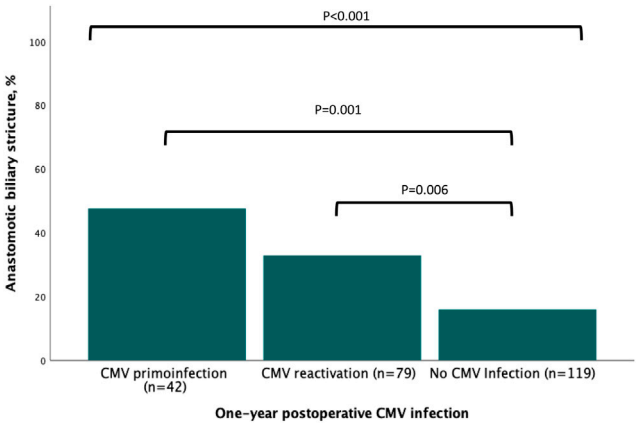


FIGURE 2 | Association between the occurrence of postoperative infection and anastomotic biliary stricture.

TABLE 5 | Risk factors for anastomotic biliary strictures in multivariable analysis (n = 240).

	OR	CI95%	P
Recipient and donor characteristics			
Recipient age	1.002	0.972–1.033	0.906
Donor BMI >25 kg/m^2	0.509	0.270–0.959	0.037
Extended criteria donor	0.972	0.509–1.856	0.932
Intraoperative characteristics			
Anastomotic technical difficulty	1.923	0.996–3.712	0.051
Postoperative complications			
Anastomotic biliary fistula	2.691	1.162–6.233	0.021
CMV infection			<0.001
None	Reference		
Primoinfection	5.244	2.281–12.054	<0.001
Reactivation	2.421	1.192–4.920	0.015

Numbers are expressed as mean (standard deviation), unless otherwise specified BMI, body mass index; CMV, cytomegalovirus.

bilio-enteric reconstruction died of multiorgan failure due to an anastomotic biliary fistula.

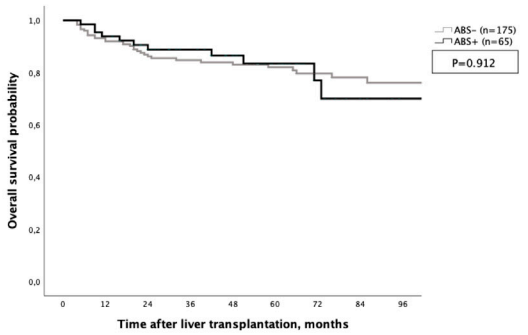
Surgical reintervention within 90 days after LT was needed in 34 patients (14.2%), mostly for hemorrhage (n = 24), anastomotic biliary fistula (n = 8), arterial complication (n = 3), large for size syndrome (n = 2), and portal vein thrombosis (n = 1). Postoperative outcomes are displayed in **Table 4**.

Incidence and Risk Factors of Anastomotic Biliary Strictures

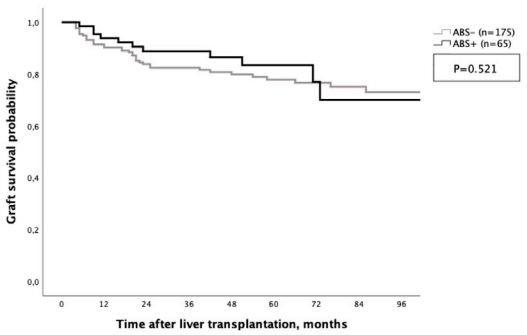
Median follow-up was 49 months (range, 7–126). Overall ABS rate was 27.1% (n = 65), of which 20 (33.8%) developed within 90 days after LT. Median time to ABS diagnosis was 142 days (range, 13–1,265).

Upon univariable analysis, ABS risk factors related to donor, recipient, intraoperative characteristics, and postoperative outcomes are listed in **Tables 1–4**. Recipient age (p = 0.090), donor BMI (p = 0.015), and donors' and recipients' serological CMV status (p = 0.030) were statistically associated with ABS. Intraoperatively, the existence of anastomotic technical difficulty was associated with ABS (p = 0.005). Regarding postoperative events, both the occurrence of anastomotic biliary fistula (p < 0.001) and a CMV infection (p < 0.001, **Figure 2**) were associated with ABS.

Upon multivariable analysis, elevated donor BMI (OR = 0.509, CI95% 0.270–0.959; p = 0.037), postoperative CMV primoinfection (OR = 5.244, CI95% 2.281–12.054; p < 0.001) or CMV reactivation (OR = 2.421, CI95% 1.192–4.920; p = 0.015) and the occurrence of anastomotic biliary fistula (OR = 2.691, CI95% 1.162–6.233; p = 0.021) were independently associated with ABS, although anastomotic technical difficulty did not reach a statistically significant association (**Table 5**). Discrimination of



At risk	240	200	158	136	112	83	58	40	16
AS+	65	56	45	37	28	17	9	5	2
AS-	175	144	113	99	84	65	49	35	14



At risk	240	198	158	135	112	83	58	40	16
AS+	65	56	45	38	28	17	9	5	2
AS-	175	142	113	98	84	65	49	35	14

FIGURE 3 | Overall survival and graft survival according to the development of anastomotic biliary stricture.

the estimated risks from the multivariable analysis was deemed acceptable (AUC, 0.740; SE, 0.035, $p < 0.001$).

Interaction Between Anastomotic Biliary Strictures and Statistically Significant Postoperative Events

Among 65 patients who developed ABS, 46 (70%) experienced postoperative CMV infection, of which eight (17%) were under CMV prophylaxis. Among these 46 patients, 34 patients (75%) developed first postoperative CMV infection before experiencing ABS diagnosed after a median of 172 days after LT (range, 18–1,222). In contrast, 12 patients (25%) developed first ABS with a median interval after LT of 131 days (range, 30–447) before presenting CMV infection.

Similarly, 31 (12.9%) patients experienced anastomotic biliary fistula including three grade A (10%) and 22 grade B (71%) all managed endoscopically, either alone ($n = 15$) or combined with a percutaneous drainage ($n = 7$). The remaining six patients (19%) were deemed grade C as they required reoperation. Of them, 15 (48%) developed subsequent ABS after a median interval between anastomotic biliary fistula and stricture of 41 days (range, 5–239).

Management of ABS and Impact on Long-Term Outcomes

First-line ABS treatment was systematically endoscopic (100%), requiring a median treatment duration of 252 days (range, 133–912) for a median number of two (range, 1–11) procedures per patient. Twelve patients (18.5%) eventually developed ABS recurrence, of which nine were managed endoscopically, two percutaneously, and one surgically. Repeat LT was not required due to ABS but was performed in 11 patients owing to ischemic cholangitis ($n = 5$), acute hepatic artery thrombosis ($n = 3$), and chronic rejection ($n = 3$).

Regarding long-term outcomes, 1-year and 5-year overall survival and graft survival rates were respectively of 93%, 72% and 92%, 71%. The occurrence of ABS was not associated with OS ($p = 0.912$) and graft survival ($p = 0.521$) (**Figure 3**).

DISCUSSION

In the current study, the ABS rate was 27.1% and reported rates classically range from 15 to 35% (2–4). Such variations in the literature are likely related to multiple factors. Mostly, no consensus definition and monitoring guidelines are available. This results in heterogeneous detection rates, causing unbiased comparisons between existing series in the literature. In the current series, 10 patients (15%) underwent ERCP with biliary stenting while no upstream biliary dilatation was found at MR-cholangiography and ERCP (17). While this remains difficult to ascertain that these patients had definitive bona fide ABS, given the absence of consensus definition, such clinical situations might not have been considered as ABS at other institutions.

The occurrence of ABS is often attributed to the use of marginal donors. In the current study, liver graft from ECD was used in nearly 60% of LT and was not associated with the occurrence of ABS. Instead, three independent risk factors have been identified. Although the protective role of high donor BMI on the occurrence of ABS remains difficult to discuss, Jarlot-Gas et al. recently reported the same association (3). One could hypothesize that high donor BMI is related to large graft bile duct, resulting in less difficult biliary reconstruction. More importantly, the predominant role of postoperative events on the development of ABS has to be highlighted. Regarding the association between the occurrence of anastomotic biliary fistula and the development of ABS, this relationship has been previously shown in various series (3, 18, 19). Anastomotic biliary fistula would indeed cause local inflammation, eventually leading to local fibrosis at the site of healing, resulting in ABS.

More interestingly, the occurrence of postoperative CMV infection was independently associated with the risk of ABS. Upon univariable analysis, pre-transplantation donors' and recipients' serological CMV status was associated with an increased risk of ABS. This suggested the propensity of patients

at risk of postoperative CMV infection to develop ABS. This was confirmed upon multivariable analysis as the occurrence of CMV primo-infection or reactivation was independently associated with an increased risk of developing ABS. Overall, the majority of patients who developed ABS presented CMV infection in their postoperative course. In most cases, CMV infection preceded ABS diagnosis and was virally reactivated. While the relevance of CMV detection and prevention after LT has been largely established, the association between CMV infection and ABS development remains unclear (20–22). Among the large existing body of literature focused on ABS risk factors, very few series have found similar findings (23–25). The current study was not designed to investigate mechanisms of CMV on ABS occurrence, but one can hypothesize that destruction of vascular endothelial cells due to CMV infection might lead to arterial thrombosis, resulting in biliary ischemia (26). Additionally, it has been shown that CMV can be latent in epithelial cells and be shed in bodily fluids (27). Notably, CMV DNA has been found to be more prevalent in biliary fluid than in liver biopsy or blood serum after LT (28). Gotthardt et al. reported that the presence of CMV DNA in the biliary tract after LT was significantly associated with the development of biliary stricture (29). However, the presence of CMV DNA in bile was significantly associated with non-anastomotic biliary stricture instead of ABS. Future investigations are consequently needed to further understand mechanism and develop prevention and treatment strategies (30).

In addition to preoperative characteristics and postoperative events, intraoperative data were also investigated. Upon univariable analysis, only the anastomotic technical difficulty defined as bile duct diameter smaller than 5 mm or donor-recipient duct size mismatch larger than 4 mm was found associated with an increased risk of ABS. Yet, in the current study, this failed to reach statistical significance upon multivariable analysis. However, a tiny duct size has been already reported as an ABS risk factor in multiple series including one randomized trial (25, 31). In order to overcome the technical difficulty, using ductoplasty techniques or T-tube insertion was at the discretion of the surgeon. As previously shown, none of these technical refinements were associated with a reduced ABS rate (32–34).

Regarding ABS management and impact on survival, the current study confirmed findings from a large body of the previously published literature. First, ABS endoscopic management was effective in most cases as a first-line approach with a recurrence rate around 20% (3, 35–38). Further, even in case of ABS recurrence, repeat endoscopic treatment allowed a successful treatment in most cases, thereby obviating the need for percutaneous transhepatic or surgical treatment (35, 39). Second, the occurrence of ABS did not negatively impact long-term survival. This observation is in line with other series (3, 40, 41). Yet, a recent large study showed the negative impact of early anastomotic biliary complication occurring within the first 3 months after LT (42). Nevertheless, such contrasting results from this series among others must be interpreted cautiously (43, 44). These series are indeed heterogenous in terms of study period, biliary complications timing, e.g. early or late, types e.g., anastomotic or not, definitions, and management. In contrast, the current single center cohort, despite spanning over 9 years, was focused on ABS after LT with duct-to-duct reconstruction using total liver grafts from brain dead donors and provided a certain homogeneity in terms of management with all patients following a standardized management pathway whether regarding perioperative monitoring or intraoperative techniques.

In addition to its retrospective nature limiting any causality analysis, some limitations of the present study have to be discussed. First, when ABS is suspected, graft rejection is also classically suspected. This might lead to intensified immunosuppression or cessation of CMV prophylaxis, thereby contributing to a higher risk of CMV infection. It may be difficult to disentangle cause and consequence in this setting. Second, the study period may implicate time lead bias, especially considering potential changes in organ preservation protocols and surgical techniques over time. However, there was no significant change within the last decades. Third, intraoperative data such as reperfusion syndrome, arterial ischemia duration, or the use of vasopressive drugs were not available in the data set. Such data might be associated with the development of ABS. Fourth, the occurrence of postoperative CMV infection was independently associated with the risk of ABS. However, quantitative data on CMV viral load in the blood was lacking for some patients. Further, no data was available on the presence of CMV in the bile, whether at the time of LT or later. Finally, external validation would be of value to confirm the impact of postoperative CMV infection as well as performances of the multivariable model.

In conclusion, the risk of developing ABS after LT is multifactorial but seemed mostly driven by the occurrence of postoperative events such as CMV infection, especially primoinfection and anastomotic biliary fistula. Generally managed endoscopically, ABS did not seem to impact survival after LT.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

PG, CC, and JM collected the data. PG, CC, VDM, ML, BH, and AD performed the analyses. PG, VDM, PC, ML, and AD drafted the manuscript. PG, CC, CT, VDM, BP, AM, PC, JM, LV, DW-V, ZL, CV, ML, BH, and AD revised the manuscript in its current version.

CONFLICT OF INTEREST

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

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2022.10292/full#supplementary-material

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ORIGINAL RESEARCH
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Validation of a Simple, Rapid, and Cost-Effective Method for Acute Rejection Monitoring in Lung Transplant Recipients

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Despite advances in immunosuppression therapy, acute rejection remains the leading cause of graft dysfunction in lung transplant recipients. Donor-derived cell-free DNA is increasingly being considered as a valuable biomarker of acute rejection in several solid organ transplants. We present a technically improved molecular method based on digital PCR that targets the mismatch between the recipient and donor at the *HLA-DRB1* locus. Blood samples collected sequentially post-transplantation from a cohort of lung recipients were used to obtain proof-of-principle for the validity of the assay, correlating results with transbronchial biopsies and lung capacity tests. The results revealed an increase in dd-cfDNA during the first 2 weeks after transplantation related to ischemia-reperfusion injury (6.36 ± 5.36%, $p < 0.0001$). In the absence of complications, donor DNA levels stabilized, while increasing again during acute rejection episodes (7.81 ± 12.7%, $p < 0.0001$). Respiratory tract infections were also involved in the release of dd-cfDNA (9.14 ± 15.59%, $p = 0.0004$), with a positive correlation with C-reactive protein levels. Overall, the dd-cfDNA percentages were inversely correlated with the lung function values measured by spirometry. These results confirm the value of dd-cfDNA determination during post-transplant follow-up to monitor acute rejection in lung recipients, achieved using a rapid and inexpensive approach based on the HLA mismatch between donor and recipient.

Keywords: lung transplantation, biomarker, acute rejection, cell free circulating DNA, droplet-digital PCR

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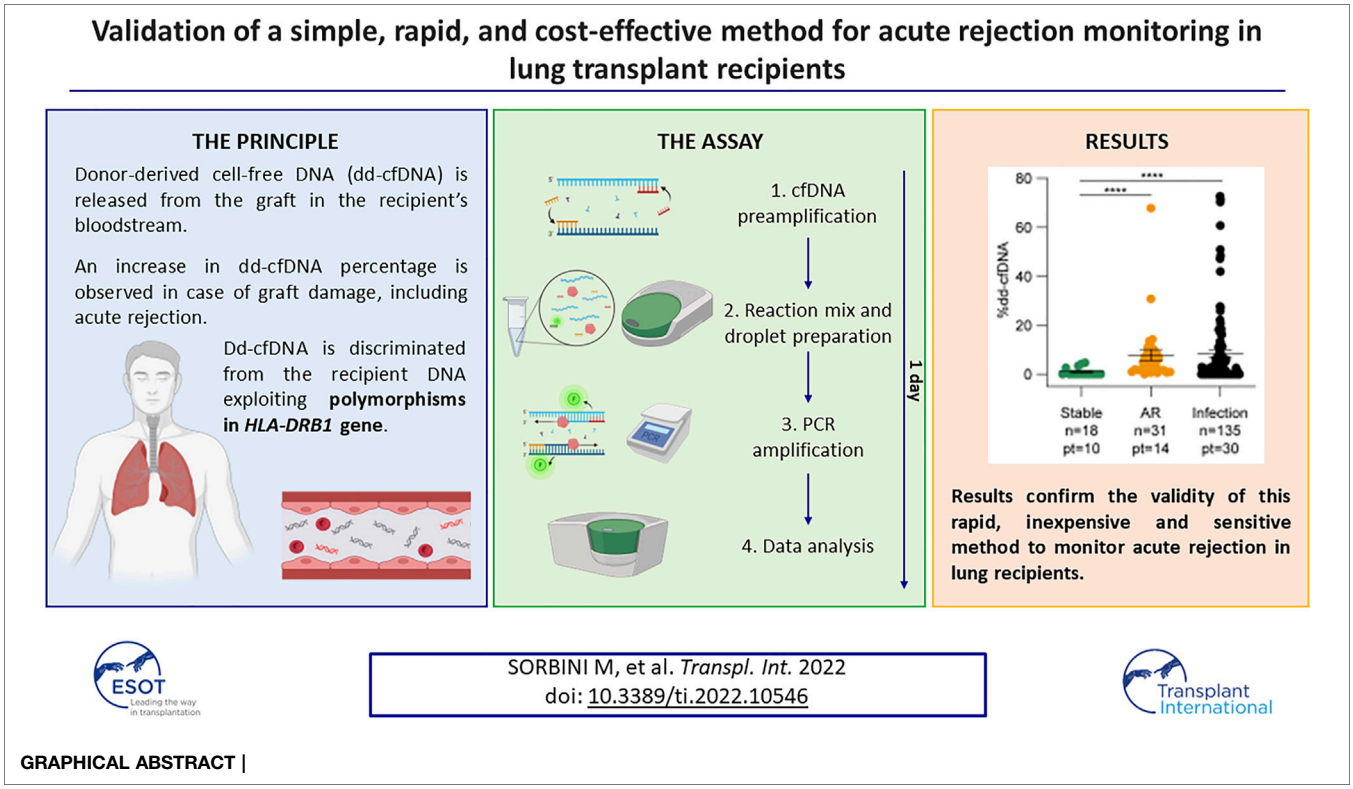
Abbreviations: ACR, acute cellular rejection; AMR, antibody-mediated rejection; AR, acute rejection; AUC, area under the curve; BOS, bronchiolitis obliterans; CLAD, chronic lung allograft dysfunction; CRP, C-reactive protein; DCD, donation after circulatory death; dd-cfDNA, donor-derived cell-free DNA; DSA, donor-specific antibody; ECMO, extracorporeal membrane oxygenation; EVLP, *ex vivo* lung perfusion; FEV1, forced expiratory volume in 1 s; HLA, human leukocyte antigen; ICU, intensive care unit; ISHLT, International Society of Heart and Lung Transplantation; MFI, mean fluorescence intensity; n, number of samples; NGS, next-generation sequencing; PCR, polymerase chain reaction; PGD, primary graft dysfunction; ROC, receiver operating characteristics; SD, standard deviation; SNPs, single nucleotide polymorphisms; TBB, transbronchial lung biopsy.



GRAPHICAL ABSTRACT |

INTRODUCTION

Affecting almost one patient in three between discharge and 1-year follow up, acute rejection (AR) represents one of the most common causes of allograft dysfunction after lung transplantation (1). If not promptly recognized and treated, AR can lead to chronic lung allograft dysfunction (CLAD), significantly reducing patient survival (2-4). In contrast, inappropriate treatment of AR episodes with immunosuppressive drugs to limit organ damage can significantly increase the risk of infections, which can be a potentially lethal complication in lung transplant recipients (5, 6). Overall, while advances in immunosuppression regimens have improved 1-year survival to >90%, 5-year survival remains around 50% (7, 8).

Bronchoscopy with associated transbronchial lung biopsy (TBB) and cytology are typically used to monitor acute cellular rejection (ACR), whereas analysis of donor-specific antibodies (DSA) in recipients' sera detects antibody-mediated rejection (AMR). However, even if both techniques are currently the "gold standard" in rejection monitoring, they can be poorly informative. First, TBB is invasive with possible complications, whereas DSA only detects anti-HLA antibodies, limiting their clinical impact and stressing the need for additional tools in post-transplant monitoring (7, 9-11).

Donor-derived cell-free DNA (dd-cfDNA) has recently been proposed as a biomarker for graft injury (12). DNA is released from donor cells because of allograft damage and can be detected in the recipient bloodstream. Dd-cfDNA levels increase during acute rejection episodes according to the severity of damage in many solid organ transplants (12-16). In lung transplant recipients, donor DNA levels were found to increase during acute rejection episodes (5, 17, 18) and during respiratory tract infections (5, 19, 20).

Donor DNA can be distinguished from the recipient DNA by using single nucleotide polymorphisms (SNPs). The most sensitive techniques are based on the simultaneous evaluation of dozens of SNPs using next-generation sequencing (NGS), guaranteeing high accuracy (13, 14, 21). However, NGS-based techniques are very expensive (13, 21, 22) and usually need to be analyzed in pools, implying that a single sample may be waitlisted until a set number is reached, representing significant limitations to its widespread application.

We have previously optimized a simple method to quantify dd-cfDNA based on genetic differences between the donor and host at the *HLA-DRB1* locus, which is routinely analyzed before transplantation. The assay, which is based on a droplet digital PCR technique, is more rapid, technically easier, and significantly less expensive than NGS-based analysis of dozens of SNPs. The results obtained in a cohort of heart-transplanted patients show that this assay is effective in identifying patients undergoing rejection, with 64.2% sensitivity and 70.8% specificity (23). Here, we present the results of a dd-cfDNA analysis from a cohort of lung transplant recipients performed with an improved version of the test. This now exploits two panels of probes targeting *HLA-DRB1*, labelled with two different fluorophores, with increased sensitivity and lower costs.

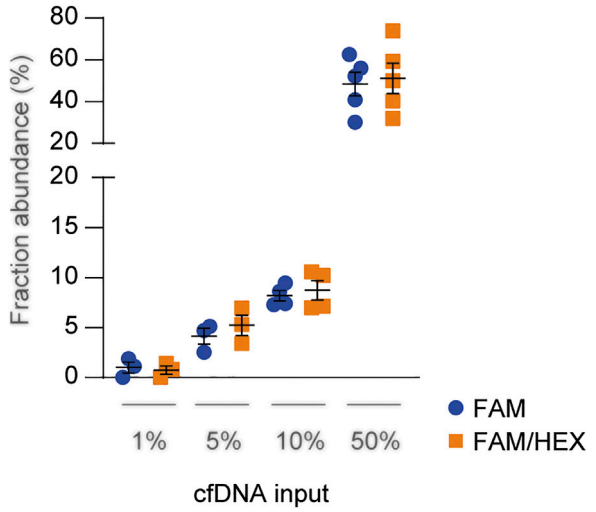


FIGURE 1 | Technical comparison between *HLA-DRB1* FAM and FAM/HEX probe panels. Serially diluted cfDNAs were spiked into a constant level of background cfDNA and quantified through droplet digital PCR assay using both the FAM-only and FAM/HEX methods. The total DNA concentration was 10 ng and the percentage of spiked DNA is shown in the graph. The results were reported as the mean fraction abundance. Error bars represent SEM. *p*-values were obtained using the Mann-Whitney nonparametric test.

MATERIALS AND METHODS

Patient Recruitment

This study was approved by the Ethics Committee of Città della Salute e della Scienza University Hospital of Turin (approval #CS2/1202, 9 April 2019). The enrolled patients underwent lung transplantation from 1 July 2019, to 31 March 2021, and provided written informed consent. Exclusion criteria were refusal or inability to provide informed consent, any form of substance abuse, psychiatric disorders, or conditions that could complicate communication between the investigator and the patient. Patient data were anonymized using an alphanumeric ID and all sensitive information was conserved on the RedCap online platform (https://www.medcap.unito.it/redcap/index.php) and used for analysis.

Donor and Recipient HLA Typing

Donor and recipient HLA typing was performed by the Immunogenetics and Transplant Biology Service, Città della Salute e della Scienza University Hospital of Turin, as routine management. Patients were HLA-typed by Luminex using the LabType SSO and LabType SSO XR kits (One Lambda, Inc., West Hills, CA, United States). Donor HLA loci were assessed by real-time PCR using a LinkSeq HLA typing kit (One Lambda, Inc., West Hills, CA, United States). Donor and recipient pairs sharing the same *HLA-DRB1* allele were excluded from the analysis.

Transplantation

According to a national protocol, urgent lung transplantation was reserved for young patients (age ≤ 50 years) requiring mechanical ventilation and/or extracorporeal lung support with extracorporeal membrane oxygenation (ECMO) (24). The graft was preserved with an anterograde and retrograde flush using Perfadex and stored at 4°C. Grafts considered unsuitable for direct transplantation underwent *ex vivo* lung perfusion (EVLP) prior to transplantation, performed according to the Toronto technique (25). Lungs from two donors recovered after cardiac death (DCD) in the Maastricht III group (26). Lung transplantation was performed according to standard techniques. Cardiopulmonary bypass was used in cases of poor oxygenation on monolateral ventilation, hemodynamic instability after pulmonary artery clamping, or in patients on extracorporeal ventilation before transplant.

Post-Transplant Clinical Management

All patients were admitted to a dedicated intensive care unit ICU, allowing controlled ventilator weaning. Primary graft dysfunction (PGD), defined according to the International Society of Heart and Lung Transplantation (ISHLT) guidelines (27), was evaluated at the time of admission to the ICU and after 24 and 72 h. Immunosuppressive therapy during the induction phase included thymoglobulin (1 mg/kg/day for 5 days) and steroids. Immunosuppression maintenance was based on calcineurin inhibitors (mainly cyclosporine), antimetabolites (mycophenolate), and corticosteroids. After discharge, patients were followed up in our lung transplant day hospital using spirometry, blood gas analysis, and medical and radiologic examinations to assess lung function. Rejection events determined by histological and clinical examination were mainly treated with pulse dose glucocorticoids (methylprednisolone 15/mg/kg/day for 3 days). CLAD was defined as a substantial and persistent decline (≥20%) in the forced expiratory volume in 1 s (FEV1) when compared with the post-transplant baseline (28), and based on its duration classified as possible (<3 weeks), probable (≤3 months) and definite (>3 months). Biochemical and microbiological evaluations on blood and bronchoalveolar lavage samples were performed routinely and in case of suspicion of bacterial and viral infections.

Sample Collection

Plasma samples were collected using PAXgene Blood ccfDNA tubes (#768165; Qiagen, Hilden, Germany). Blood samples were collected weekly during hospitalization following transplantation, and then every time the patients underwent medical examinations or transbronchial biopsies. A total of 372 plasma samples were obtained from 30 patients (average, 12,4/patient). Plasma was separated by centrifugation (2,000 ×g, 15 min, 18°C) and stored at −80°C in the Teseo Biobank of the Department of Medical Sciences of University of Turin (http://www.progettoeccellenzateseo.unito.it/) until further processing. Cell-free DNA (cfDNA) was extracted from 1 ml of plasma using MagMAX Cell free DNA isolation kit (#A29319, Applied Biosystems, Waltham, MA, United States) and stored at −20°C until analysis.

Donor DNA Quantification

Two nanograms of cfDNA was amplified using Sso PreAmp Assays (#1725160, Bio-Rad, Hercules, CA, United States) to

TABLE 1 | Donors and recipients' characteristics. List of main features of donors and recipients included in the study. The number and percentage of subjects in each group are shown.

	Variable	Monopulmonary LTx	Bipulmonary LTx	Combined LTx	Total
		N = 4	*N* = 21	*N* = 5	*N* = 30
Donor	Age (y), mean ± SD	41.1 ± 17.6	42.1 ± 16.2	41.8 ± 16.8	42.5 ± 16.1
	Male sex, n (%)	3 (75.0)	7 (33.3)	3 (60.0)	13 (43.3)
	Cardiac death, n (%)	0 (0)	2 (9.52)	0 (0)	2 (6.70)
	Brain death, n (%)	4 (100)	19 (90.5)	5 (100)	28 (93.3)
	Ischemic time (minutes), mean ± SD	352.0 ± 181.1	331.0 ± 157.5	342.4 ± 170.8	332.9 ± 159.4
Recipient	Age (y), mean ± SD	48.2 ± 17.2	47.0 ± 15.7	46.7 ± 17.1	47.0 ± 15.5
	Male sex, *n* (%)	4 (100)	7 (33.3)	4 (80.0)	15 (50.0)
	Disease, *n* (%)				
	IPF	3 (75.0)	8 (38.1)	0 (0)	11 (36.7)
	CF	0 (0)	4 (19.0)	3 (60.0)	7 (23.3)
	COPD	0 (0)	5 (23.8)	1 (20.0)	6 (20.0)
	BOS	1 (25.0)	1 (4.8)	0 (0)	2 (6.7)
	Ciliary dyskinesia	0 (0)	2 (9.5)	0 (0)	2 (6.7)
	PH	0 (0)	1 (4.8)	1 (20.0)	2 (6.7)
	Total hospital stay (d), mean ± SD	67.8 ± 59.0	67.8 ± 54.8	68.3 ± 57.8	66.7 ± 54.2
	CEC, *n* (%)	0 (0)	9 (42.9)	2 (40.0)	11 (36.7)
	ECMO, *n* (%)	0 (0)	10 (47.6)	2 (40.0)	12 (40.0)
	EVLP, *n* (%)	0 (0)	4 (19.0)	0 (0)	4 (13.3)
	Hemodynamic support, *n* (%)	3 (75.0)	19 (90.5)	5 (100)	27 (90.0)
	Dobutamine	0 (0)	11 (52.4)	5 (100)	13 (43.3)
	Noradrenaline	3 (75.0)	15 (71.4)	1 (20.0)	19 (63.3)
	iNO	1 (25.0)	5 (23.8)	2 (40.0)	8 (26.7)
	Pulmonary infections, *n* (%)	4 (100)	21 (100)	5 (100)	30 (100)
	Bacteria	1 (25.0)	18 (85.7)	5 (100)	24 (80.0)
	Virus	3 (75.0)	18 (85.7)	2 (40.0)	23 (76.7)
	Fungi	1 (25.0)	8 (38.1)	2 (40.0)	11 (36.7)

LTx, lung transplant; SD, standard deviation; IPF, idiopathic pulmonary fibrosis; CF, cystic fibrosis; COPD, chronic obstructive pulmonary disease; BOS, bronchiolitis obliterans; PH, pulmonary hypertension; CEC, extracorporeal circulation; ECMO, extracorporeal membrane oxygenation; EVLP, ex vivo *lung perfusion; iNO, inhaled nitric oxide.*

enrich the number of *HLA-DRB1* gene molecules, and 2 µl of the amplified product was used in the following step. Dd-cfDNA was quantified using an Expert Design assay probe panel (Bio-Rad, Hercules, CA, United States) designed to target the *HLA-DRB1* gene. A list of the available probes is reported in **Supplementary Table S1**. Donor and recipient DNA were amplified using specific primers and probes labelled with FAM and HEX fluorescent dyes, respectively. Droplet digital PCR reaction mix included 11 µl of ×2 droplet digital PCR Supermix for Probes no UTP (#186-3023, Bio-Rad, Hercules, CA, United States), 1.1 µl of Bio-Rad Expert Design assay FAM probe and 1.1 µl of Bio-Rad Expert Design assay HEX probe (final volume 20 µl). Droplet generation and amplification were performed as reported before (19, 23). Donor DNA was quantified as the ratio between donor and total copies and was expressed as a percentage. All measurements were performed in triplicates.

Histopathology

Surveillance lung allograft bronchoscopy and TBBs were performed at 4, 8, 12- and 18-month post-transplant. In addition, bronchoscopy and TBB were performed whenever there was clinical suspicion of rejection or pulmonary infection. The Working Formulation of the ISHLT criteria (29) was applied by experienced transplant pathologists to diagnose and grade all graft TBBs. In particular, the diagnosis of AR is based on the presence of perivascular and interstitial inflammatory cell infiltrates. Subendothelial infiltration/endotheliitis was also considered relevant for the final diagnosis of AR. Based on the histological extent of injury and inflammation, AR was graded as absent (grade A0), minimal (grade A1), mild (grade A2), moderate (grade A3), or severe (grade A4). Grade A2 is generally considered a threshold for therapeutic intervention. Morphological (e.g., neutrophilic margination, neutrophilic capillaritis, and acute lung injury with or without hyaline membrane deposits) and immunohistochemical (i.e., C4d deposition in interstitial alveolar capillaries) features of AMR were assessed and graded according to ISHLT and Banff recommendation (30-32).

DSA Evaluation

Sixty serum samples were collected at the time of liquid biopsy during posttransplant management. Sera were assessed for DSA by Luminex using commercially available SAB kits (LSM12, LS2A01, and LSA104 assays, One Lambda, West Hills, CA, United States), and the results were expressed as mean fluorescence intensity (MFI, cut-off positive value > 1,000). All the patients were DSA-negative at the time of transplantation. In addition, all patients had a negative cross-match, as determined by flow cytometry (FACSLyric, BD Biosciences) testing of sera for the presence of IgG and IgM antibodies against donor T and B lymphocytes isolated from peripheral blood samples.

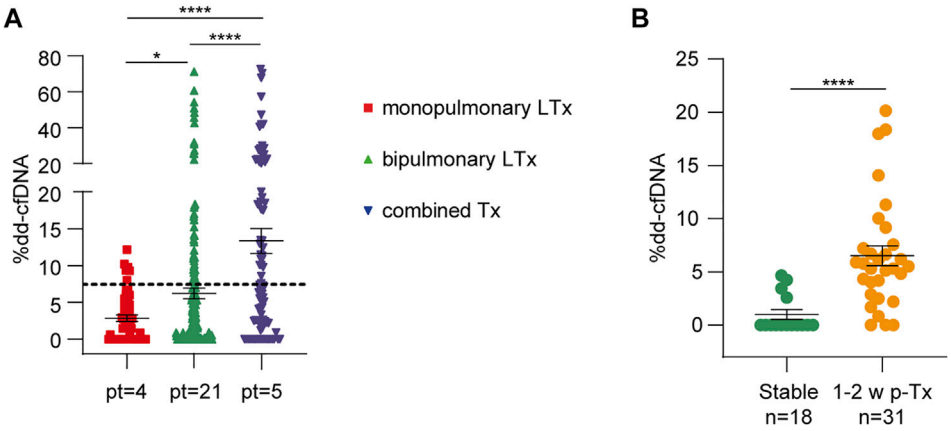


FIGURE 2 | Dd-cfDNA release is influenced by the type of lung transplant and ischemia-reperfusion injury. **(A)** Dd-cfDNA quantification in monopulmonary, bipulmonary, and combined lung transplants (LTx). The number of patients (pt) is reported for each group. The dotted line represents the total average percentage of dd-cfDNA. **(B)** dd-cfDNA levels during the first 2 weeks after transplantation (31 measurements) were compared to stable condition samples (18 measurements from 10 patients). The number of samples (n) in each group is shown below. The results are reported as percentages and shown as dot plots. Error bars represent SEM. *p*-values were obtained using the Mann-Whitney nonparametric test.

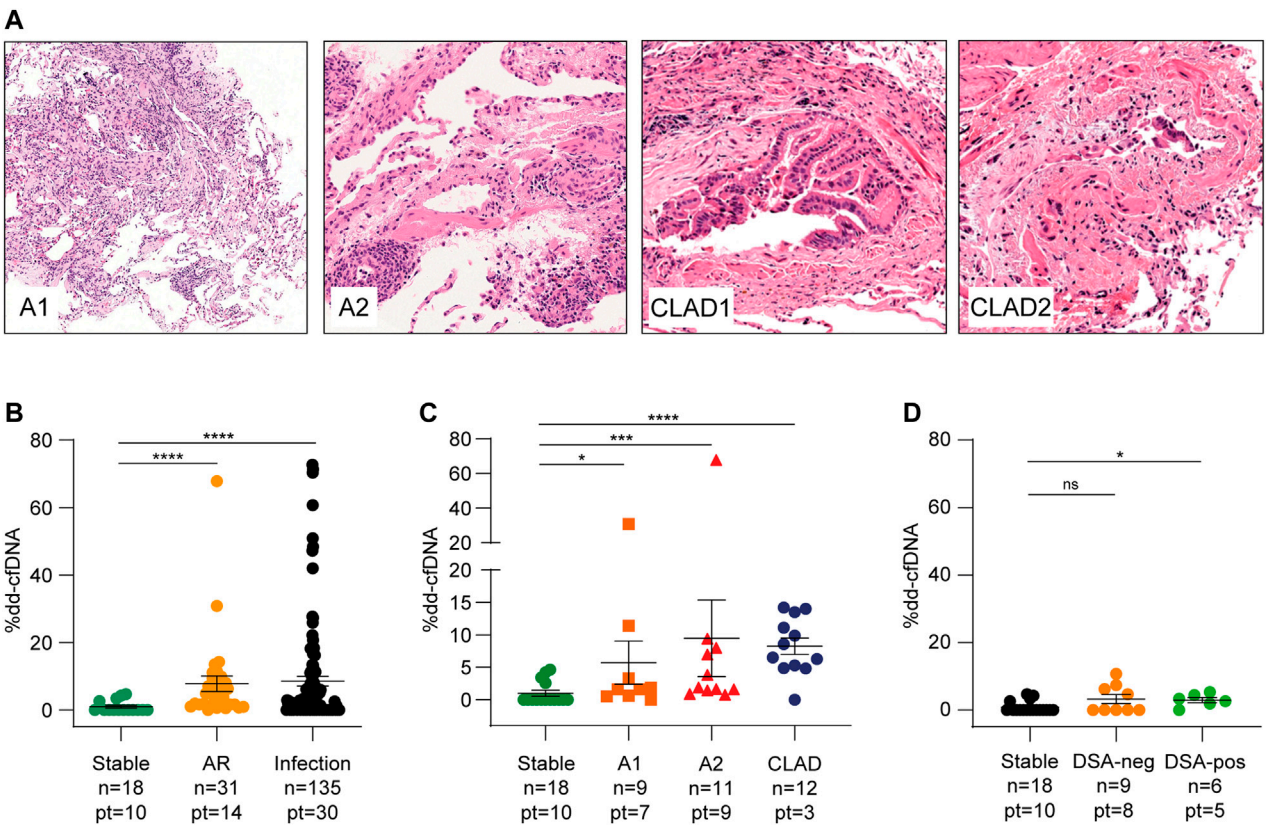


FIGURE 3 | Acute rejection is followed by a significant increase of dd-cfDNA. **(A)** Histopathological features of acute rejection grades A1 and A2 and evidence of bronchiolar wall fibrosis with lumen narrowing (CLAD1) and epithelial damage (CLAD2) in patients with obliterative bronchiolitis syndrome (BOS-CLAD). Hematoxylin and eosin staining, A1 ×100 original magnification, A2 and CLAD ×200 original magnification. **(B)** dd-cfDNA values during acute rejection (AR) and infectious events compared to stable conditions. **(C)** donor DNA levels in minimal (A1) and mild (A2) rejection and in chronic lung allograft dysfunction (CLAD) episodes. **(D)** dd-cfDNA percentages in DSA-negative and DSA-positive samples compared to those under stable conditions. The numbers of samples (n) and patients (pt) in each group are indicated. The results are reported as percentages and shown as dot plots. Error bars represent SEM. *p*-values were calculated using the Mann-Whitney non-parametric test.

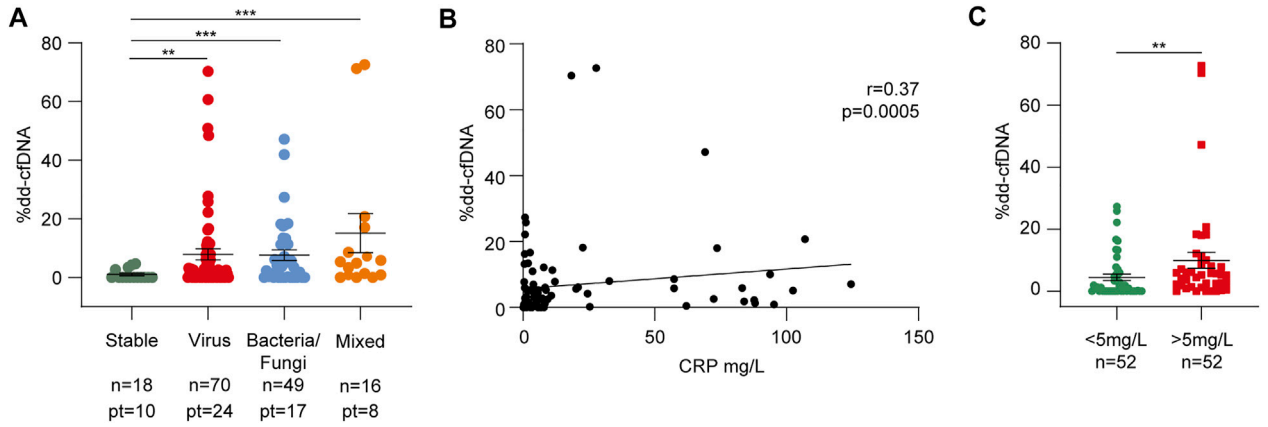


FIGURE 4 | Respiratory tract infections cause dd-cfDNA release in the recipient bloodstream. **(A)** dd-cfDNA quantification related to infections divided into virus, bacteria/fungi, and mixed groups. The number of samples and patients (pt) from whom the samples were collected are shown for each category. **(B)** Linear regression between dd-cfDNA percentage and relative C-reactive protein (CRP) level (n = 104). Correlations were calculated using the nonparametric Spearman's test. **(C)** Differences between %dd-cfDNA in the low (<5 mg/L) and high (>5 mg/L) CRP samples. The results in panels **(A)** and **(C)** are reported as percentages and shown as dot plots. Error bars represent SEM. p-values were obtained using the Mann-Whitney nonparametric test.

Statistical Analysis

Dd-cfDNA quantification is reported as mean ± standard deviation (SD). Differences between mean values in each group were compared using the Mann-Whitney nonparametric test, since data resulted to be not normally distributed using the Shapiro Wilk test. p-values lower than 0.05 were considered as statistically significant. The correlation between two continuous variables was analyzed using the nonparametric Spearman test. Receiver operating characteristic (ROC) curves were calculated using the Wilson-Braun method. All statistical analyses were performed using GraphPad Prism version8.0.2.

RESULTS

Validation of Expert Design Assay Probe Panel

The sensitivity and specificity of *HLA-DRB1* FAM probes have been assessed previously assessed (19, 23). The HEX panel was validated by testing each probe with several cfDNAs to assess specificity, and by performing serial dilution to determine sensitivity (**Supplementary Figure S1**). Combinations of cfDNA (**Figure 1**) or genomic DNA (data not shown) carrying different *HLA-DRB1* alleles were loaded at known concentrations (1%, 5%, 10%, and 50%) and quantified using the FAM-only probe method described before (23), or by combining FAM and HEX probes targeting the two alleles in the same reaction. Since the results were consistent between both methods, we concluded that dd-cfDNA quantification of both the donor and recipient in the same well was feasible, with a reduction in time and costs of analyses while maintaining comparable accuracy.

Patients' Characteristics

Thirty consecutive patients who underwent primary lung transplantation at our institution between 1st July 2019 and 31st March 2021 were recruited for this study (**Table 1**). In 28 out of 30 cases (93.3%), organs were recovered from heart-beating donors (13 males, 43.3%) with a mean age at death of 42.5 ± 16.1. In the two remaining cases, donations occurred after circulatory death in patients aged 58 and 69 years, respectively. The mean waiting list time was 306.0 (range: 3–1,607) days, with a median of 226.5 days. The mean age at transplantation was 47.0 ± 15.5. Twenty-one patients (70.0%) received a double lung transplant, four (13.3%) received a single lung transplant, and five (16.7%) received a bilateral lung transplant associated with another solid organ transplantation (one lung-heart, one lung-kidney, and three lung-liver-pancreas). Idiopathic pulmonary fibrosis (11 cases, 36.7%) was the most common disease, followed by cystic fibrosis (7 patients, 23.3%), and chronic obstructive pulmonary disease (6 cases, 20.0%). Nine patients (30.0%) received a transplant on an urgent basis, four (13.3%) received mechanical ventilation, and 6 (20.0%) received ECMO before transplantation. 22 subjects (73.3%) presented with clinical signs of primary graft dysfunction (PGD) of any grade within the first 72 h after transplantation. Three patients (10.0%) experienced grade 3 PGD 72 h after transplant. Lastly, four recipients (13.3%) received organs that underwent EVLP before transplantation. The mean total organ ischemia was 332.9 ± 159.4 min. The median total hospital stay was 47.5 days, and none of the patients died before discharge.

Donor-Derived Cell-Free DNA Release Is Influenced by Ischemia-Reperfusion Injury

In total, 372 plasma samples were obtained from 30 patients (mean 12, 4 samples/patient). The mean dd-cfDNA percentages obtained at all times differed significantly between monopulmonary, bipulmonary, and combined transplant recipients, reflecting the number of donor cells present in the recipient (**Figure 2A**). In fact, mean donor DNA levels were lower in single-lung recipients (2.8 ± 3.2%) than in double-lung (6.2% ± 10.9%, p = 0.02) or combined transplant recipients (13.3% ±

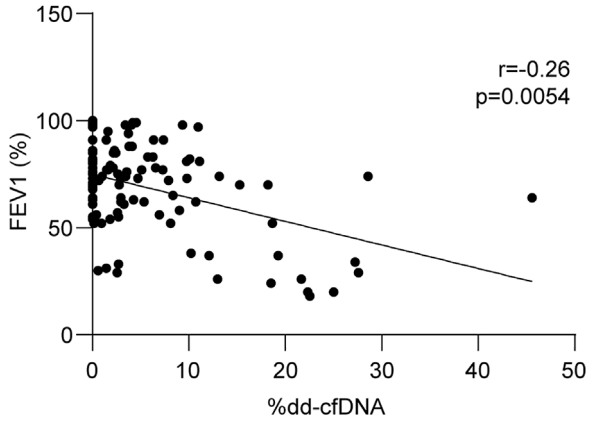


FIGURE 5 | Dd-cfDNA is inversely related to respiratory function. Linear regression between forced expiratory volume in 1 s (FEV1) and related dd-cfDNA levels (n = 114). FEV1 was calculated considering recipient characteristics for normative equations. Correlations were obtained using the nonparametric Spearman test.

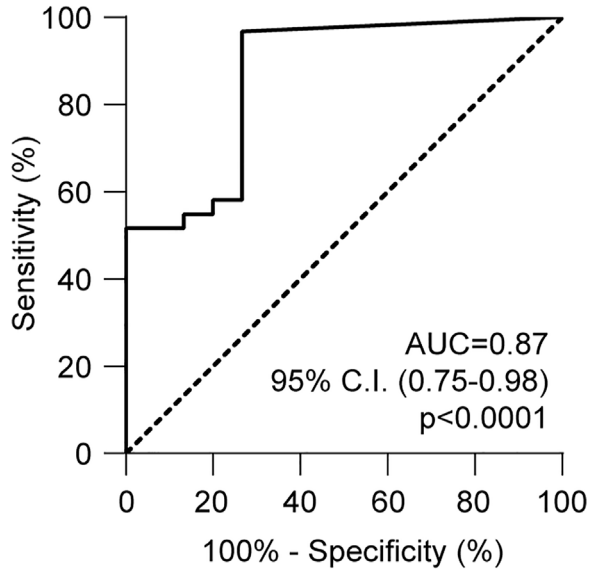


FIGURE 6 | ROC analysis of *HLA-DRB1* droplet digital PCR assay. The ROC curve was obtained considering the dd-cfDNA values associated with rejection and no rejection. The curve was calculated using the Wilson-Braun method. Area under the curve (AUC) = 0.87 (95% C.I., 0.75–0.98).

16.2%, $p < 0.0001$). During the first 2 weeks after transplantation, dd-cfDNA peaked (mean value 6.36 ± 5.36%), in line with previous results, demonstrating organ damage due to ischemia-reperfusion (**Figure 2B**). In patients without complications, the mean donor dd-cfDNA quantification slowly stabilized at 2 weeks after transplantation. To determine the baseline value to be used for comparisons, we selected 18 samples from 10 patients (one monopulmonary, one combined, and eight bipulmonary recipients) at a time when no sign of rejection, infection, or worsening of their clinical condition could be observed. The mean dd-cfDNA calculated from these samples (2.18% ± 3.26%) was considered as the baseline.

Acute Rejection Is Followed by a Significant Increase of Donor-Derived Cell-Free DNA

A total of 20 out of 115 transbronchial biopsies (17.4%) scored positive for cellular rejection. Nine biopsies were classified as minimal grade (indicated as A1) and 11 as mild grade (indicated as A2, **Figure 3A**). No grade ≥ A3 biopsies were observed during the follow-up period. Donor DNA levels were more elevated during AR events than under stable conditions (7.81 ± 12.7%, $p < 0.0001$, **Figure 3B** and **Supplementary Figure S2**). In addition, levels varied significantly according to the severity of rejection; A1 events were related to a modest increase in donor DNA amount (mean value 5.74 ± 10.0%, $p = 0.03$), whereas A2 rejection caused a stronger increase (9.48 ± 19.60%, $p = 0.008$, **Figure 3C**). No biopsy showed morphological or immunohistochemical features of AMR (**Supplementary Figure S3**), even though five patients (16.7%) developed DSA after transplantation and two of them had anti-HLA-DQ antibodies, which are generally associated with AMR. Of the 60 DSA tests performed, 38 (63.3%) were negative in accordance with negative biopsies, 2 (3.3%) were positive and associated with biopsy-proven A2 rejection, and 15 (25.0%) did not agree with the histochemical evaluation. The remaining five (8.4%) samples were not temporally related to graft tissue collection. Donor DNA percentages obtained from seven samples temporally close to DSA-positive sera were higher than those obtained from DSA-negative samples (nine samples). To avoid confounding factors that could affect the analysis, this statistical evaluation was performed considering only serum samples collected in the absence of documented infections and other evidence of graft damage not due to rejection (16 samples). Even if the number of samples included in the statistical analysis was limited, results reached significance when comparing stable conditions vs. DSA-positive ($p = 0.01$, **Figure 3D**). On the contrary, we could not observe significant differences between DSA-positive and DSA-negative samples. Finally, three patients (10.0%) experienced possible CLAD, two of whom showed clinical signs of bronchiolitis obliterans (BOS) and then recovered (**Figure 3A**), whereas the remaining patient developed a mixed form of BOS and restrictive allograft syndrome and died from severe pulmonary insufficiency caused by chronic rejection and pneumonia. All samples collected during these episodes showed elevated levels of dd-cfDNA (8.26 ± 4.41%, $p < 0.0001$, **Figure 3C**).

Respiratory Tract Infections Were Related to Significant Changes in Donor-Derived Cell-Free DNA Levels

During follow-up, every patient experienced respiratory tract infections: bronchoalveolar lavage contained bacteria in 24 (80.0%), viruses in 23 (76.7%), and fungi in 11 (36.7%) cases, with specimens from eight patients (26.7%) showing mixed contamination (**Table 1**). Among the bacteria, the most frequent pathogens were *Pseudomonas aeruginosa* (12

specimens, 40.0%) and *Klebsiella pneumoniae* (5 specimens, 16.7%), while C*ytomegalovirus* (20 specimens, 66.7%) was the most common. Dd-cfDNA levels significantly increased during infectious episodes compared to stable conditions, with a slightly stronger increase observed during viral (7.70 ± 14.20%, $p = 0.004$) and mixed infections (13.7 ± 23.5%, $p = 0.0007$, **Figures 3B**, **4A**; **Supplementary Figure S2**). Consistently, dd-cfDNA levels showed a positive association ($r = 0.37$, $p = 0.0005$) with C-reactive protein (CRP) blood levels, as determined by studying dd-cfDNA levels in 104 samples from 28 patients and collected close to CRP measurements during infection episodes (**Figure 4B**). One time point was excluded from the analysis because its CRP value was >300 mg/L, representing a potential bias in the statistical analysis. Considering 5 mg/L as a clinical cut-off value, the same samples were divided into low and high CRP groups. With this classification, samples collected from patients with CRP levels ≥5 mg/L showed significantly higher mean dd-cfDNA percentages (9.91 ± 16.4%) than low CRP samples (4.44 ± 7.13%, $p = 0.004$, **Figure 4C**).

Donor-Derived Cell-Free DNA Percentages Correlate With Respiratory Function

Lung transplant function was assessed using spirometry. FEV1 was quantified using recipient characteristics for the normative equation and considered a respiratory function measure. A total of 114 liquid biopsies were collected close to the spirometry tests, and dd-cfDNA quantification was correlated with relative FEV1 percentages. As shown in **Figure 5**, there was a statistically significant inverse relationship between the two variables ($r = -0.26$, $p = 0.0054$).

Accuracy of the Test

Receiver operating characteristic (ROC) analysis was performed to assess the performance of this method. The area under the curve (AUC) was 0.87, (95% confidence interval: 0.75–0.98, $p < 0.0001$, **Figure 6**). With a cut-off value of 1.25%, dd-cfDNA had 80.7% sensitivity and 73.3% specificity for distinguishing AR from non-rejection. In particular, the test correctly identified 25 of the 31 biopsies classified as positive for rejection, and by excluding samples in which rejection occurred together with infection, dd-cfDNA quantification was above the cut-off value in 14 of 16 (87.5%) biopsies.

DISCUSSION

Long-term survival in lung-transplanted patients is limited by acute and chronic rejection, which represent the leading cause of graft failure and death, together with non-cytomegalovirus infections (6). Presently considered the gold standard tools for rejection monitoring, TBB and serum DSA evaluation show significant limitations in terms of sensitivity and specificity, thus limiting precise early diagnosis of graft damage and correct modulation of the immunosuppressive regimen (33-35). In this context, dd-cfDNA is emerging as a valuable adjunct and a reliable indicator of acute rejection after transplantation of several solid organs (13, 14, 16, 36). dd-cfDNA increases during rejection episodes based on the severity of the damage, whereas it remains low to undetectable in stable patients. In addition, dd-cfDNA can be easily purified from blood samples, causing minimal discomfort and stress to patients.

Donor DNA can be distinguished from the recipient's exploitation of any type of genetic polymorphism spread across the genetic code. *HLA* genes are among the most polymorphic, and since this genetic diversity can be the basis of rejection, HLA locus is routinely typed before organ transplant. While this procedure is needed to exclude recipients who are already immunized against HLA alleles of the donor, in the case of lung transplantation, it has no impact on donor-recipient selection. It is therefore very infrequent that recipient and donor are matched: in our cohort of 328 lung transplants from 2000 to 2020, no transplants were fully HLA matched, while 29 (8.84%) were fully matched at the *HLA-DRB1* locus. These donor-recipient pairs were excluded from our assay, as there would be no genetic differences to monitor. However, it should be noted that if *HLA-DRB1* is matched, rejection occurs less frequently, and survival rates are higher (37-40)(**Supplementary Figure S4**). We previously applied a dd-cfDNA quantification method based on a probe panel targeting *HLA-DRB1* alleles and showed that it could identify rejection episodes in a cohort of heart-transplanted patients (23). The current technical improvement is that the same probe panel is now bound to two alternative fluorophores, FAM or HEX, and the differently labelled probes are loaded in the same reaction well, allowing the quantification of donor and recipient DNA percentages at the same time, thereby reducing costs by half while maintaining comparable sensitivity and specificity.

Compared to NGS, droplet digital PCR is more rapid, with results available 24 h after blood draw, feasible, and significantly cheaper. Using our optimized method, the cost of reagents for a single reaction is in the range of 80 euros, which is comparable to that of the Luminex assay for DSA monitoring (15, 23, 41).

The mean donor DNA percentages showed a clear correlation with the amount of donor tissue transplanted; bilateral transplant samples showed values approximately double those of single-lung transplants. Moreover, samples from patients who received more than one organ presented a significantly higher amount of dd-cfDNA, reflecting the higher number of donor cells inside the recipient. All samples collected in the first 2 weeks after transplantation demonstrated high levels of dd-cfDNA, consistent with ischemia-reperfusion injury and in line with previous data reported in the literature (5, 17, 33).

Donor DNA kinetics exhibited low percentages in samples collected from patients in stable conditions (**Supplementary Figure S2**), whereas values increased significantly in relation to ACR episodes, with moderate A2 rejection associated with a stronger release than A1 rejection. In addition, samples from patients with clinical signs of possible CLAD showed the highest dd-cfDNA levels. In particular, one of the CLAD patients developed early A2 rejection after 1-month post-transplant, and then suffered from relapsing pneumonia and chronic rejection treated with immunosuppressive boluses and photopheresis. Finally, the patient developed severe pulmonary insufficiency as a consequence of graft failure and died on post-transplant day 343. His dd-cfDNA levels

increased early and did not decrease even after immunosuppressive treatment, consistent with severe rejection. Regarding the other two cases of CLAD, no specimens were collected after the additional immunosuppressive treatment, therefore no information could be obtained about their dd-cfDNA variations.

Morphological and immunohistochemical evaluations did not report evidence of AMR in any TBB collected during the follow-up period, even though seven recipients developed DSA. Although we observed a significant difference between DSA-positive samples and stable conditions, the reduced number of samples do not allow any speculation about the value of dd-cfDNA as a biomarker of AMR.

Remarkably, dd-cfDNA also increased in the presence of infection, in keeping with the notion that it is a marker of graft damage, independent of the cause (6). Therefore, for optimal clinical use, dd-cfDNA quantification should be performed together with a set of biomarkers of infection and radiological examination of the lung. The finding of increased dd-cfDNA in the absence of any sign of infection should prompt biopsy evaluation of the transplanted lung. Thus, dd-cfDNA could reduce the number of biopsies in a population of patients with a high suspicion of rejection.

In conclusion, we present an improved molecular method to monitor lung-transplant outcomes that allows rapid rejection identification through dd-cfDNA quantification at high costs. Larger clinical studies are needed to determine the best way to integrate this biomarker in the routine post-transplant management of lung transplant recipients to improve graft survival and patients' quality of life.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Comitato Etico Interaziendale Città della Salute e della Scienza Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Study design: MS, PS, MB, MR, CA, AA, TV, and SD. Data collection and analysis: MS, GT, ES, MaM, FM, MC, FA, CC, MoM, PM, AG, LD, and MP. Writing of manuscript: MS and SD. Acquisition of funding: AA, TV, and SD.

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CONFLICT OF INTEREST

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

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2022.10546/full#supplementary-material

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Pancreas Transplantation in Black, Asian and Minority Ethnic Patients-Single Centre Experience in the UK

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Ethnic disparities in the outcomes after simultaneous pancreas kidney (SPK) transplantation still exist. The influence of ethnicity on the outcomes of pancreas transplantation in the UK has not been reported and hence we aimed to investigate our cohort. A retrospective analysis of all pancreas transplant recipients (n = 171; Caucasians = 118/Black Asian Ethnic Minorities, BAME = 53) from 2006 to 2020 was done. The median follow-up was 80 months. Patient & pancreas graft survival, rejection rate, steroid free maintenance rate, HbA1c, weight gain, and the incidence of secondary diabetic complications post-transplant were compared between the groups. $p < 0.003$ was considered significant (corrected for multiple hypothesis testing). Immunosuppression consisted of alemtuzumab induction and steroid free maintenance with tacrolimus and mycophenolate mofetil. Pancreas graft & patient survival were equivalent in both the groups. BAME recipients had a higher prevalence of type-2 diabetes mellitus pre-transplant (BAME = 30.19% vs. Caucasians = 0.85%, $p < 0.0001$), and waited for a similar time to transplantation once waitlisted, although pre-emptive SPK transplantation rate was higher for Caucasian recipients (Caucasians = 78.5% vs. BAME = 0.85%, $p < 0.0001$). Despite equivalent rejections & steroid usage, BAME recipients gained more weight (BAME = 7.7% vs. Caucasians = 1.8%, $p = 0.001$), but had similar HbA1c (functioning grafts) at 3-,12-, 36-, and 60-months post-transplant.

Keywords: pancreas transplantation, race, ethnicity, metabolic outcomes, SPK transplantation, Caucasian, BAME

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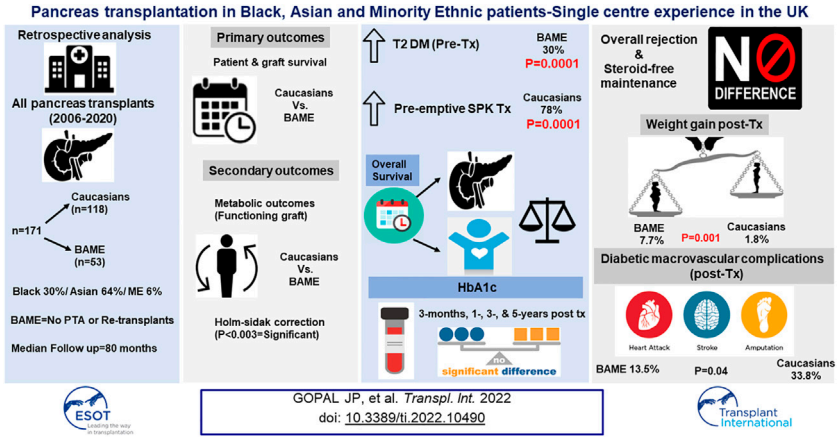

GRAPHICAL ABSTRACT |

INTRODUCTION

Despite increasing interest in equitable healthcare, disparities in access to solid organ transplantation, especially for ethnic minority patients, still exists (1, 2). Most of the literature on ethnicity-based outcomes in pancreas transplantation are from the USA (3–6), and the healthcare delivery in the USA is predominantly through insurance companies. There is no equivalent data from the UK, where the healthcare system is publicly funded. In view of this and in addition, as our centre serves an ethnically diverse patient population (7) (which corresponds to the geographical location and the ethnic spread in the locality), we aimed to investigate the influence of ethnicity on the outcomes of pancreas transplantation in our patient cohort, with a special focus on metabolic outcomes. This represents the first single center experience on ethnicity-based outcomes of pancreas transplantation from the UK.

MATERIALS AND METHODS

Following institutional audit committee approval, a retrospective analysis of all pancreas transplants (including simultaneous pancreas kidney-SPK, solitary pancreas, and re-transplants) performed between January 2006 and March 2020 was done. Data was collected from a prospectively maintained local database and National Health Service Blood and Transplant's centre database.

Donor Selection Criteria

According to our center's organ acceptance policy, all the DBD donors were less than 65 years old and all the DCD donors were less than 55 years old. The donor's body mass index (BMI) cut off was ≤30 kg/m^2. All the DCD donors had a functional warm ischemia time (systolic blood pressure <50 mmHg and/or oxygen saturation of 70%) of less than 60 min and a downtime of less than 30 min.

Immunosuppression

Immunosuppression consisted of induction with intravenous alemtuzumab 30 mg (single dose) and methylprednisolone 500 mg. Maintenance immunosuppression was with tacrolimus, mycophenolate mofetil, and a short course of steroids (7 days). Post-transplant target tacrolimus trough levels were 8–12 ng/dl.

Criteria for Transplanting Type 2 Diabetes Mellitus

Patients were classified as type 2 diabetes mellitus if they have a detectable C-peptide and the classification was predominantly based on the diagnosis made by the referring diabetologist. The following is the criteria for transplanting type 2 diabetic patients: Insulin treated diabetes along with end stage renal failure with a body mass index of ≤30 kg/m^2, glycaemic lability, and insulin requirement of less than 1 Unit/Kg/day.

Outcome Measures Studied

All primary and secondary outcomes were compared between Caucasian and BAME recipients; the primary outcome measures were patient and pancreas graft survival, secondary outcome measures were metabolic outcomes among those with a functioning graft (weight gain, HbA1c, and incidence of secondary diabetic macrovascular complications post-transplant), rejection rate, and steroid usage between the two groups.

Definition of Outcome Parameters

A functioning graft is defined as remaining insulin independent post-transplantation. Secondary diabetic macrovascular complications are defined as any of the following events post-transplant: myocardial infarction, cerebrovascular accident, transient ischemic attack, and/or limb amputations (minor or major). Rejection episodes are either cellular or antibody mediated or mixed, and comprise of either pancreas or kidney rejection (in case of simultaneous pancreas-kidney transplantation). The rejections defined are either biopsy

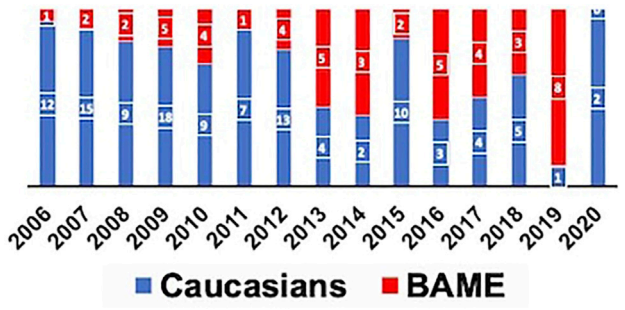

FIGURE 1 | Proportion of Caucasian and BAME recipients transplanted each year.

proven or those episodes that were treated based on clinical suspicion (raising serum amylase, positive circulating donor specific antibody, or delayed pancreatitis on CT scan).

Statistical Analysis

Categorical variables are expressed as frequency (%) and continuous variables as median. Differences between the categorical variables were assessed using Fisher's exact test and differences between the continuous variables were assessed by using Mann Whitney test. Survival analysis was done by using Kaplan-Meir survival plots. Holm-Sidak correction was done for multiple comparisons and a p value of <0.003 was considered significant. All the statistical analyses were performed using Graph Pad Prism software (Version 9.0).

RESULTS

A total of 171 pancreas transplants (SPK-129/PAK-27/PTA-4/Re-transplants-11) were performed during the study period of which 118 recipients were Caucasians and 53 were from the BAME group. Among the BAME group 64% (n = 34) were from Asian communities, 30% (n = 16) from Black communities, and 6% (n = 3) from Minority Ethnicities. The median follow-up period of the study was 80 months. The year-wise distribution of pancreas transplant activity in our center is shown in **Figure 1**. Donor and recipient characteristics is shown in **Table 1**. The HLA mismatch was grouped into 4 levels as per the NHSBT data description. The definition of each level is shown in **Table 1**.

There was no pancreas transplant alone or re-transplants in the BAME cohort. The waiting time defined as the time from activation in the national transplant waiting list to transplantation were similar for both the groups, although the pre-emptive transplantation rate (for SPK transplantation) was significantly higher for Caucasian recipients. Data on workup time, which is the time from referral to our centre to activation in the national transplant waiting list, were available for 45 patients and were similar for both the groups. Data on estimated glomerular filtration rate at the time of referral for SPK transplantation were available for 51 patients and were similar for both the groups. There was a significantly higher prevalence of type 2 diabetes mellitus in the BAME group.

Patient and Pancreas Graft Survival

The 1-, 3-, and 5-year patient survival were comparable between the two groups (**Table 2**). There were 22 early graft losses (within 90 days post-transplant) in total. The following were the causes for early graft loss; thrombosis (n = 6), bleeding (n = 6), sudden cardiac death (n = 3), pancreas failed to perfuse on table (n = 2), severe graft pancreatitis (n = 2), duodenal anastomotic leak (n = 1), Y-graft pseudoaneurysm (n = 1), and unknown (n = 1). The early graft losses were not significantly different between the two groups (Caucasians = 16.10%, n = 19 vs. BAME = 5.6%, n = 3, p = 0.05). The 1-, 3-, and 5-year pancreas graft survival for both SPK and isolated pancreas transplants (PAK/PTA) were comparable between the two groups (**Table 2**).

Steroid-Free Maintenance and Rejection Rate

The overall rejection rates and steroid-free maintenance rates were comparable between the two groups (Caucasians = 18.1% vs. BAME = 22.6%, p = 0.49; Caucasians = 81.8% vs. BAME = 81.1%, p = 0.92, respectively).

Metabolic Outcomes

The metabolic outcomes were compared in those recipients with a functioning graft. There were no significant differences in the HbA1c at 3-month, 1-, 3-, and 5-year post-transplant between the two groups (**Figure 2**). BAME recipients gained significantly more weight post-transplant compared to their Caucasian counterparts (Median percentage weight gain; BAME = 7.7% vs. Caucasians = 1.8%, p = 0.001). The overall incidence of secondary diabetic macrovascular complications post-transplant was not significantly different between the two groups (Caucasians = 33.8% vs. BAME = 13.5%, p = 0.04). There were 10 cardiovascular events (Caucasians = 8 vs. BAME = 2), 9 cerebrovascular events (Caucasians = 8 vs. BAME = 1), and 9 peripheral vascular events (Caucasians = 7 vs. BAME = 2).

Type 1 vs. Type 2 Diabetes-Outcomes

A subgroup analysis was performed to look into whether type 2 diabetes mellitus had an impact on the ethnicity-based outcomes. The overall pancreas graft and patient survival were similar for pre-transplant type-1 and tye-2 diabetic patients (Log rank p = 0.04, and 0.98, respectively). The post-transplant HbA1c among those with a functioning graft were similar for both type-1 and type-2 diabetic patients at 3-month, 1-, 3-, and 5-year (p = 0.02, 0.01, 0.02, and 0.02, respectively). There was no significant difference in the post-transplant median percentage weight gain either (Type-1 diabetes = 3.1% vs. Type-2 diabetes = 7.7%, p = 0.07).

DISCUSSION

A successful pancreas transplantation leads to improvement in quality of life as well as improvement in cardiovascular risk profile, and reduction in macrovascular disease along with survival benefits

TABLE 1 | Donor and recipient characteristics.

Donor and recipient characteristics	Caucasians	BAME	*p* value
Donor age in years (Median)	35	32	0.39
Donor BMI in kg/sq. m (Median)	23.10	24.10	0.12
DCD donors (%)	12.71	16.98	0.48
Recipient age in years (Median)	45	41	0.02
Sensitized recipients % (CRF>5%)	20.34	24.53	0.55
Re-transplants	11	0	—
HbA1c at registration in mmol/mol (Median)	71.8	63.9	0.03
Duration of diabetes in years (Median)	30.50	23	<0.0001
Age at onset of diabetes in years (Median)	13	20	0.01
Pre-transplant insulin use (IU/Day)-Median	44	41.50	0.54
Solitary pancreas transplants-% (PAK/PTA)	27.12	13.21	0.05
Pre-transplant type 2 diabetes (%)	0.85	30.19	0.0001
Pre-transplant secondary diabetic macrovascular complications (%)	12.7	3.7	0.07
Pre-transplant registered blind (%)	10.17	13.21	0.55
eGFR at referral in ml/min (Median)	20	14.5	0.47
Waiting time in days (Median)	232	217	0.96
Time taken for workup in days (Median)	166	122	0.60
Cold ischemia time in mins (Median)	938	799	0.001
Pre-emptive SPK transplantation (%)	78	43	0.0001
HLA group 1 (0) & 2 (0DR+0/1B)	6%	—	0.06
HLA group 3 (0DR+2B) or (1DR+0/1B)	28%	32%	0.55
HLA group 4 (1DR+2B) or (2DR)	66%	68%	0.83

TABLE 2 | Pancreas graft and patient survival by ethnicity.

Survival	Caucasians,%	BAME,%	Log rank p
1-year (SPK)-Pancreas	84.07	88.60	0.47
3-year (SPK)-Pancreas	77.02	85.84	0.36
5-year (SPK)-Pancreas	75.18	85.84	0.29
1-year (PAK)-Pancreas	54.54	100	0.04
3-year (PAK)-Pancreas	41.06	100	0.02
5-year (PAK)-Pancreas	41.06	75	0.03
1-year (Patient)	98.21	96.18	0.41
3-year (Patient)	93.72	84.42	0.08
5-year (Patient)	86.23	80.20	0.25

in diabetic patients (8–12). Hence, the real argument for pancreas transplantation is to achieve optimal metabolic control whereas improving survival should be an added advantage. The studies reporting ethnicity-based outcomes in pancreas transplantation so far have not looked into the metabolic outcomes. As the prevalence of type-1 diabetes is less common in the non-Caucasian population (13), it is vital that metabolic outcomes should be considered alongside survival outcomes in this cohort. A review of data from the United Network for Organ Sharing (UNOS) database/Organ Procurement and Transplantation Network (OPTN) have reported that African-Americans have worse long-term survival rates (both patient and graft) compared to the other ethnic groups (3, 4). Access to pancreas transplantation has also been reported to be limited for African-Americans (5, 6), which could be due to a referral bias because of the presumed inferior outcomes in this patient group. In an era of increasing global immigration, it is crucial to avoid ethnic disparities in access to transplantation.

We noted equivalent patient and graft survival (for both SPK and solitary pancreas transplants) in Caucasian and BAME recipients. This is in contradiction to some of the major studies that have been published before (3, 4). From USA data, it transpires that Asians and Hispanics are reported to have the best survival outcomes and African-Americans have better short-term outcomes compared to Caucasians but poor longer-term outcomes (3). As Asian recipients comprised the majority of the BAME cohort in our centre, our results could be that of an Asian subtype rather than BAME community as a whole. It is also important to note that as the BMI cut off for Type 2 diabetic patients was 30 kg/sq.m, some of them might have non-classical type of diabetes rather than presumed type 2 diabetes mellitus (14). In the UK, minority groups are collectively referred to as BAME. Despite the fact that this terminology has been criticised due to the heterogeneity within the group, it is still widely in use. This heterogeneity might also explain similar survival outcomes observed in our study. Although this has been the case, our study supports the more recent observation based on data from the USA that outcomes are not necessarily inferior for non-Caucasian recipients (15–17).

HbA1c among those with a functioning graft was not significantly different between the two groups until 5 years post-transplant, although there is a trend towards higher HbA1c in BAME recipients at 5 years. Longer term follow-up data might uncover the effect of weight gain on HbA1c and graft survival. As HbA1c is known to be an independent predictor of long term pancreas graft failure (18), other metabolic parameters such as mixed meal tolerance test and C-peptide measurements were performed only selectively in patients with allograft dysfunction with consideration of intervention aimed at optimizing graft function and were not part of the routine follow up protocol.

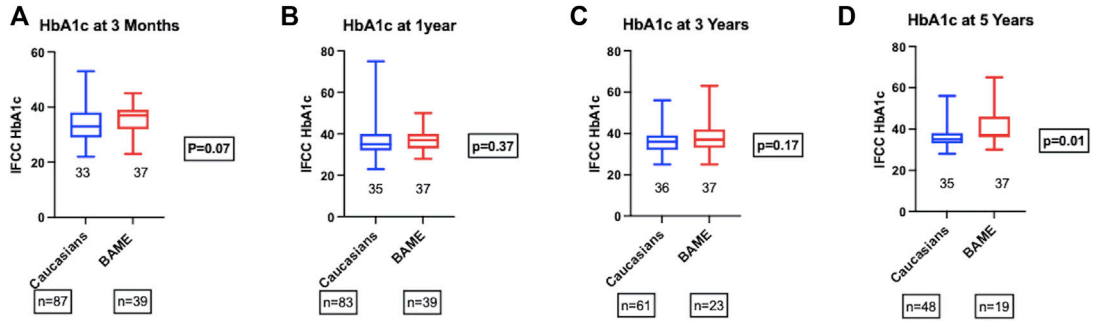

FIGURE 2 | HbA1c at **(A)** 3-month, **(B)** 1-year, **(C)** 3-year, and **(D)** 5-year post-transplant.

Despite similar rejection rates and steroid usage in both the groups, BAME recipients gained significantly more weight post-transplantation. In our study we were unable to characterise whether the weight gain was due to an increase in lean body mass or due to increased adiposity. Peripheral hyperinsulinism resulting from systemic venous drainage has been postulated as a cause for excessive weight gain post-SPK transplantation (19). The explanation for higher weight gain in BAME recipients could only be speculative due to the retrospective nature of the study. Younger recipient age is known to be associated with weight gain post kidney and pancreas-kidney transplantation (20, 21). Weight gain post-transplantation has been observed in patients with a positive C-Peptide at the time of pancreas transplantation (22). The following could be the reasons for excessive weight gain in BAME group; BAME recipients were relatively younger compared to their Caucasian counterparts, and the majority of type-2 diabetics were from the BAME group. Longitudinal data on weight gain would be useful in identifying the time frame where recipients start to gain excess weight. This might help in planning dietary/behavioural modification or metabolic interventions such as the introduction of GLP-1 analogues in those at risk (23, 24). Additionally, the use of calcineurin inhibitors is known to cause insulin resistance thereby leading on to excessive weight gain (25, 26). Furthermore, it is well known that African-Americans need aggressive tacrolimus dosing to achieve optimal trough levels due to the ethnic difference in the prevalence of CYP3A5*3 genotype, which is responsible for the metabolism of tacrolimus (27, 28). Further studies looking at the circulating tacrolimus trough levels and metabolic parameters will provide more insight into strategies for optimal maintenance immunosuppression.

The incidence of pre-, and post-transplant secondary diabetic macrovascular complications were numerically higher in Caucasian recipients, although, statistically insignificant. There are several reasons for this observation. Firstly, Caucasians had an early onset and a significantly longer duration of diabetes compared to the BAME group. Hence Caucasian recipients had more macrovascular complications due to poor metabolic control. BAME recipients might have had a good metabolic control for a longer period than Caucasians before worsening control and hence the lower incidence of pre-transplant macrovascular complications. Secondly, this is also be due to a more conservative approach in listing Type 2 diabetic patients for pancreas transplantation, as is the case in BAME patients in whom Type 2 diabetes mellitus was more prevalent (29–31). Excessive weight gain observed in the BAME group could potentially lead on to post transplant metabolic syndrome and may increase the risk of cardiovascular complications in the longer term.

Prior to 2012, a majority of the patients transplanted were Caucasians and the cold ischemia times were longer. Post 2012, more BAME patients were being transplanted and the cold ischemia time was progressively shorter. Our centre's change in practice reflected the evolving evidence base as convincing literature evidence was generated around the same time supporting pancreas transplantation in Type 2 diabetes mellitus (32–34). Due to the timeline effect there was a significant difference in the cold ischemia time between the two cohorts and there are no other explanations.

The pre-emptive transplantation rate was higher for Caucasian recipients, although, the estimated glomerular filtration rate (eGFR) at the time of referral, time taken for transplant work-up (time from referral to listing), and the waiting times were similar for both the groups. There can be several reasons for this. Most of the studies from around the world have reported that lower socio-economic status is independently associated with reduced access to pre-emptive listing (35–37). Despite a publicly funded healthcare system in the United Kingdom, socio-economic and geographic disparities in the utilisation of live donor kidney transplantation has been reported (38). Due to lack of data on socio-economic status; we are unable to comment further on that. The other reason could be the location of the patient and the proximity to a transplant centre. Being registered with a transplanting centre is known to be associated with higher pre-emptive listing because of the onsite availability of specialist services for assessing suitability. Health literacy is another important factor. Involvement of BAME ambassadors in the discussion about transplantation might reduce the socio-cultural, and language barrier and also may improve the engagement rate of BAME patients to transplantation services. The use of social media and interactive ways of reaching out, rather than traditional written pamphlets about organ donation and transplantation

might also improve the awareness among BAME patients. Further multicentre studies will shed more light on BAME access to pancreas transplantation and outcomes, in addition to centre variation in practice in the UK.

This is the first study from the UK reporting ethnicity-based outcomes in pancreas transplantation and the first study reporting metabolic outcomes in Caucasian and BAME patients. Type-2 diabetes was more prevalent in BAME patients. BAME and Caucasian recipients had similar HbA1c until 5 years post-transplantation. Despite similar rejection rates and steroid usage, BAME recipients gained more weight post-transplantation. BAME patients experience similar survival outcomes (graft and patient) to those of Caucasian recipients. Although the waiting time and work-up time were similar, Caucasians had a higher proportion of pre-emptive SPK transplantation.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Imperial College Healthcare NHS Trust. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

ARM conceptualised the study; JG collected, analysed and interpreted the data, and wrote the manuscript; AM, JC, PH, VP, FD, and ARM contributed to the data generation, made critical revisions to the manuscript and approved the final version of the manuscript.

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CONFLICT OF INTEREST

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

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BRIEF RESEARCH REPORT
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Evaluation of Expanded Criteria Donors Using the Kidney Donor Profile Index and the Preimplantation Renal Biopsy

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The increasing comorbidity of kidney transplant (KT) donors make it necessary to develop scores to correctly assess the quality of kidney grafts. This study analyzes the usefulness of the preimplantation biopsy and the Kidney Donor Profile Index (KDPI) as indicators of KT survival from expanded criteria donors (ECD). Retrospective study of KT in our center between January 2010 to June 2019 who received a kidney from an ECD and underwent a preimplantation biopsy. 266 KT were included. Graft survival was categorized by KDPI quartiles: Q1 = 86%, Q2 = 95%, Q3 = 99% and Q4 = 100%. KT from KDPI Q1 presented better survival ($p = 0.003$) and Q4 donors had worse renal function ($p = 0.018$) and poorer glomerular filtration rate (3rd month; $p = 0.017$, 1st year; $p = 0.010$). KT survival was analyzed according to KDPI quartile and preimplantation biopsy score simultaneously: Q1 donors with biopsy score ≤3 had the best survival, especially comparing against Q3 with a biopsy score >3 and Q4 donors ($p = 0.014$). In multivariable analysis, hyaline arteriopathy, glomerulosclerosis, and KDPI Q4 were predictors for graft survival. High KDPI and a greater histological injury in the preimplantation biopsy, especially glomerular and vascular lesions, were related to a higher rate of KT loss from ECD.

Keywords: kidney transplantation, expanded criteria donors, kidney donor profile index, preimplantation biopsy, graft survival

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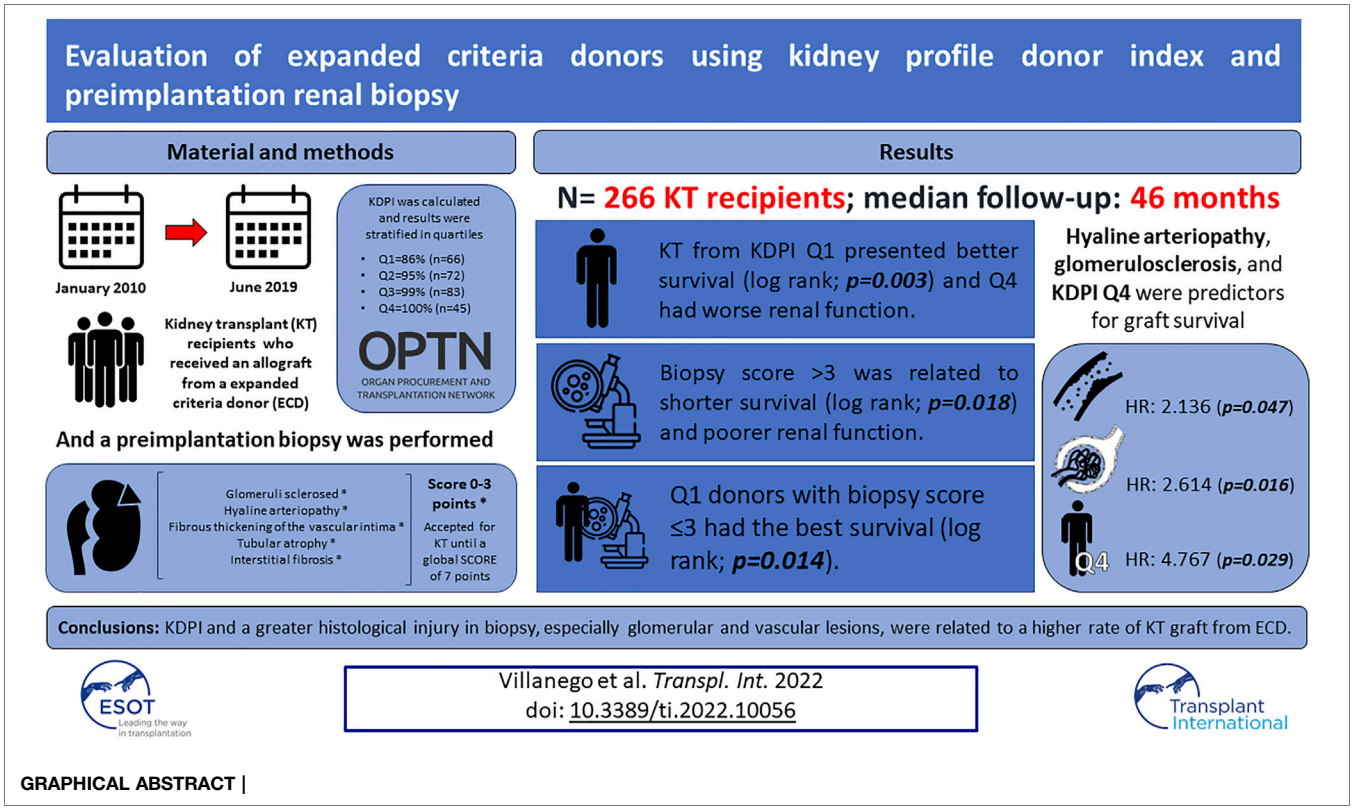
Abbreviations: DGF, delayed graft function; ECD, expanded criteria donors; EPTS, estimated post-transplant survival; KDPI, kidney donor profile index; KT, kidney transplantation; MDRD-4, Modification of Diet in Renal Disease-4; SCD, standard criteria donor; UNOS, United Network for Organ Sharing.



GRAPHICAL ABSTRACT |

INTRODUCTION

The shortage of kidneys for transplantation has led to the increased use of suboptimal donors. These changes in the demographics of kidney transplant (KT) donors make it necessary to develop tools to assess the suitability of the grafts [1].

Usually, KT viability was determined according to the United Network for Organ Sharing (UNOS) criteria and donors were identified as standard criteria donors (SCD) or expanded criteria donors (ECD) [2]. However, this classification does not adequately reflect the kidney donor's quality [3,4].

In the last years, several scales have been developed to measure the prognosis of the KT trying to eliminate the dichotomy of SCD versus ECD. The Kidney Donor Profile Index (KDPI) was developed in 2014 by the American Transplantation Registry. It gives a score from 0% to 100% which summarizes the risk of graft failure and it is calculated using 10 donor factors [5]. KDPI is not validated in Spain, but some publications in our country relate the KDPI to renal graft survival [6-9].

Preimplantation biopsy has been used to evaluate the kidney graft, mostly in ECD [10]. There are several scoring systems, such as the Pirani-Remuzzi score or the Maryland Aggregate Pathology index [11,12]. Spanish guidelines for evaluating KT biopsies have been published previously and to date, the acceptance of a kidney from an ECD has been based almost exclusively on the preimplantation biopsy [13]. However, it still has a controversial role in assessing the viability of the renal graft [14,15].

Our main purpose is to analyze the value of preimplantation biopsy and the KDPI in our setting as indicators of KT graft survival from ECD. As a secondary objective, we analyzed the renal graft function and its relationship to the KDPI score and the histological findings in a preimplantation biopsy.

METHODS

Design and Study Population

We present a retrospective cohort study of KT patients at Puerta del Mar Hospital between 01/01/2010 and 01/06/2019 who received a KT from an ECD (60 years and older and those aged 50–59 years who meet at least two of the following conditions: serum creatinine >1.5 mg/dl, cerebrovascular accident as a cause of death, or hypertension) [3]. We perform a preimplantation biopsy in all kidney grafts from ECD. All patients had a minimum follow-up of 1-year post-KT.

All recipients received immunosuppressive induction with basiliximab or thymoglobuline (5 daily doses of 1 mg/kg, adjusted according to lymphocyte count). Maintenance immunosuppression included tacrolimus (trough level 5–10 ng/ml), mycophenolate mofetil (1,000–2,000 mg/day), and prednisone (5 mg/day).

TABLE 1 | Characteristics of KT donors and recipients included.

Donors	***n* = 161**
Sex female, n (%)	68 (42.2)
Age (years), median [IQR]	66 [60,70]
HBP, n (%)	72 (44.7)
DM, n (%)	27 (16.8)
Brain death donor, n (%)	132 (81.9)
Smoking, n (%)	49 (30.4)
Serum creatinine (mg/dl), mean ± SD	0.8 ± 0.3
Height (cm), mean ± SD	165.2 ± 7.8
Weight (kg), ±SD	79.1 ± 13.5
KDPI quartile	
Q1: 86%, n (%)	66 (24.8)
Q2: 95%, n (%)	72 (27.1)
Q3: 99%, n (%)	83 (31.2)
Q4: 100%, n (%)	45 (16.9)
KDPI (%), median [IQR]	95 [86,99]
Biopsy score, median [IQR]	2 [2,3]
Recipients	***n* = 266**
Sex female, n (%)	97 (36.5)
Age (years), median [IQR]	62 [52.75, 68]
Etiology of CKD	
DM, n (%)	35 (13.1)
HBP, n (%)	18 (6.8)
GN, n (%)	42 (15.8)
Others, n (%)	79 (29.7)
Unknown, n (%)	92 (34.6)
Retransplant, n (%)	22 (8.3)
RRT pre-KT	
HD/PD/preemptive KT, n (%)	192 (72.2)/63 (23.7)/11 (4.1)
RRT time (months), median [IQR]	17 [8,28]
HCV+, n (%)	7 (2.6)
Transplant	
CIT (minutes), median [IQR]	1195 [946,1390]
DGF, n (%)	110 (41.3)
Q1, n (%)	25 (37.8)
Q2, n (%)	30 (41.6)
Q3, n (%)	25 (30.1)
Q4, n (%)	30 (66.6)
Primary graft non-function, n (%)	14 (5.2)
Q1, n (%)	2 (3)
Q2, n (%)	2 (2.7)
Q3, n (%)	4 (4.8)
Q4, n (%)	6 (13)

IQR, interquartile range; HBP, high blood pressure; DM, diabetes mellitus; SD, standard deviation; KDPI, kidney donor profile index; DGF, delayed graft function; Q, quartile; CKD, chronic kidney disease; GN, glomerulonephritis; KT, kidney transplant; HD, hemodialysis; PD, peritoneal dialysis; RRT, renal replacement therapy; HCV, hepatitis C virus.

Biopsies Assessment

Graft biopsies were obtained by renal wedge during the bench surgery from a representative part of the graft, avoiding scars. KT biopsies were analyzed by four expert pathologists. All of these biopsies had more than 25 glomeruli. The ECD KT with biopsies from other centers were excluded. The samples were processed fresh and tissue was frozen immediately using methyl butane which was cooled in liquid nitrogen. Subsequently, the cuts were made in the cryostat, and stained with rapid hematoxylin-eosin staining. The results are obtained in 15 min approximately.

The biopsy score was calculated following the Spanish protocol for preimplantation biopsy, based on the Remuzzi score. Five parameters were evaluated: glomerular sclerosis, myointimal elastosis, hyaline arteriopathy, interstitial fibrosis, and tubular atrophy. They were scored from 0 to 3, depending on the degree of injury. A global score ≥7 or a score of 3 in any of the first 3 histological compartments is considered unfavorable for transplantation and graft should be discarded [13].

Variables

We analyzed donor and KT recipient variables, and estimated glomerular filtration rate 3 months and 1 year after KT. The score obtained in each individual histological component and the cumulative score for pathological lesions of the preimplantation biopsy were collected. Kidney graft survival was defined as the time from transplant to graft failure, censoring for death with a functioning graft. Deceased patients with a functioning graft were considered as lost to follow-up. Glomerular filtration rate was estimated by the Modification of Diet in Renal Disease (MDRD-4) [16]. The KDPI score was calculated using the formula on the Organ Procurement and Transplantation Network website [17].

Statistical Analysis

Continuous variables are presented as mean and standard deviation or median and interquartile range as appropriate; categorical variables as frequencies and percentages. Categorical variables were compared using Fisher exact test or Chi-square test, and continuous variables using the Student's t-test, U Mann-Whitney, or ANOVA, according to normality and number of groups. For multiple comparisons in continuous variables, Bonferroni correction was conducted. Normality was analyzed by the Kolmogorov-Smirnov test.

The KDPI was analyzed as an absolute value and stratified according to quartiles. The biopsy score was stratified according to the mean value of the assessment scale (score = 3). Graft survival categorized by KDPI quartile and biopsy score were plotted using the Kaplan-Meier method and compared between groups by log-rank test. Pairwise testing over strata was performed if > 2 groups were compared in survival analysis.

To identify risk factors associated with graft failure univariable and multivariable analysis was performed using Cox regression. Pretransplant variables related to graft survival, KDPI, and biopsy score were included in the multivariable analysis as well as other covariates based on the criterion of p-value <0.1 in the univariable analysis. Several models were performed to analyze the global biopsy score, the different histological compartments, and the KDPI as a continuous and a categorical variable according to quartiles. In the models that included KDPI, donor variables already evaluated in the score (such as age and diabetes) were excluded.

Values significant $p < 0.05$ were considered. The statistical analysis was performed with SPSS v.25.

RESULTS

In the study period, 720 KT were performed in our center, of which 83 corresponded to living KT donors. In 267 no biopsy was performed and in 104 KT the biopsy was processed in another

TABLE 2 | Renal function at 3 months and 1 year after kidney transplantation. (A) Renal function according to KDPI quartile. (B) Renal function according to biopsy score.

MDRD at 3rd month[a], mean ± SD	Q1 (*n* = 47) 45.8 ± 16.5[ade]	Q2 (*n* = 54) 40.1 ± 20.3[bdf]	Q3 (*n* = 46) 37.6 ± 21.5[cef]	Q4 (*n* = 38) 27.3 ± 18.3[abc]	*p*-value a < 0.001 b = 0.005 c = 0.036 d = 0.087 e = 0.012 f = 0.297
MDRD at 1st year[a], mean ± SD	Q1 (*n* = 47) 46.8 ± 19.3[ade]	Q2 (*n* = 50) 39.6 ± 22[bdf]	Q3 (*n* = 43) 38.0 ± 24.3[cef]	Q4 (*n* = 36) 28.1 ± 19.3[abc]	*p*-value a<0.001 b = 0.039 c = 0.910 d = 1.000 e = 0.195 f = 0.213
MDRD at 3rd month, mean ± SD	Biopsy score ≤ 3 (*n* = 196) 43.6 ± 16.6		Biopsy score > 3 (*n* = 43) 32.3 ± 20.3		*p*-value 0.017
MDRD at 1st year, mean ± SD	Biopsy score ≤ 3 (*n* = 186) 40.1 ± 22.1		Biopsy score > 3 (*n* = 40) 31.7 ± 21.8		0.010

[a]*ANOVA test:* p < *0.001. Comparison between KDPI quartiles (Bonferroni correction).*
MDRD at 3rd month: [a]Q1 vs. Q4, [b]Q2 vs. Q4, [c]Q3 vs. Q4, [d]Q1 vs. Q2, [e]Q1 vs. Q3, [f]Q2 vs. Q3 and MDRD at 1st year: [a]Q1 vs. Q4, [b]Q2 vs. Q4, [c]Q3 vs. Q4, [d]Q1 vs. Q2, [e]Q1vs. Q3, [f]Q2 vs. Q3.
MDRD = 0 was considered in patients reinitiating hemodialysis.
Q, quartile; MDRD, modification of diet in renal disease; SD, standard deviation.

center. Finally, 266 KT met the criteria and were included. The median follow-up was 46 months.

Baseline characteristics of donors and recipients are presented in **Table 1**. Grafts were stratified by quartiles based on the KDPI score: Q1 = 86% (*n* = 66); Q2 = 95% (*n* = 72); Q3 = 99% (*n* = 83); Q4 = 100% (*n* = 45). The median KDPI was 95% and the median biopsy score was 2 points. Four donors had acute kidney injury at the time of donation. However, they did not present greater histological scores compared to donors with normal renal function (score 3 [2.25,3] vs. score 2 [2,3]; $p = 0.479$).

Kidney Graft Function

Renal function was worse at 3 months and 1-year post-KT, especially in grafts with a Q4 KDPI (**Table 2**). Similarly, kidneys with biopsy scores>3 presented worse eGFR in the 3rd month (−11.3 ml/min; $p = 0.017$) and after the first year post-KT (−8.4 ml/min; $p = 0.010$) (**Table 2**).

Kidney Graft Survival

Death-censored graft survival was 89.8% at 1 year and 85.4% at 5 years post-KT (**Figure 1A**). Regarding the survival of the allograft by quartile of KDPI, kidneys from donors in the lowest quartile presented better outcomes ($p = 0.001$). Pairwise testing did not show differences between other groups (**Figure 1B**).

We compared graft survival according to preimplantation biopsy score: score ≤3, $n = 214$ (80.5%) vs. score >3, $n = 52$ (19.5%). KT with a biopsy score >3 presented worse survival ($p = 0.018$) (**Figure 1C**). In addition, these biopsies corresponded to higher KDPI donors: the mean of the KDPI for score ≤3 was 86.4 ± 17.7%, vs. 93.8 ± 11.4%. for score >3 ($p < 0.001$).

Finally, KT survival was compared according to the quartile of KDPI and preimplantation biopsy scores simultaneously. Q1 donors with less histological injury (score ≤3) had the best survival rate, especially compared against Q3 with a biopsy score >3 and Q4 donors ($p = 0.014$) (**Figure 1D**).

We analyzed graft survival by histological compartments, comparing the absence (score 0) and the presence of histological injury (scores 1 and 2). The absence of glomerulosclerosis and hyaline arteriopathy were associated with a better graft survival ($p = 0.005$ and $p = 0.034$), but not the histological injury in the rest of the compartments (**Supplementary Table S1**).

Cox Regression Analysis

In the univariable analysis, donor age, diabetic donor, biopsy score >3, KDPI, glomerulosclerosis, and hyaline arteriopathy were related to a higher rate of graft loss (**Table 3**).

Models performed in the multivariable analysis are shown in **Table 3**. In the model that included KDPI and biopsy score, only KDPI was at the limit of statistical significance as a predictor of KT loss ($p = 0.081$) (Model 1). When KDPI was analyzed as quartiles, Q4 was an independent risk factor for graft survival ($p = 0.010$) (Model 2). Replacing score biopsy for glomerulosclerosis and hyaline arteriopathy, the presence of these lesions was related to a worse graft survival ($p = 0.007$; $p = 0.023$) (Model 3). Finally, when we included KDPI quartiles and the score of glomerulosclerosis and hyaline arteriopathy, glomerulosclerosis ($p = 0.016$), hyaline arteriopathy ($p = 0.047$), and Q4 KDPI ($p = 0.029$) remained as independent predictors for kidney graft survival (Model 4).

DISCUSSION

This study presents one of the biggest cohorts and with the largest follow-up that analyzes the efficacy of the graft preimplantation

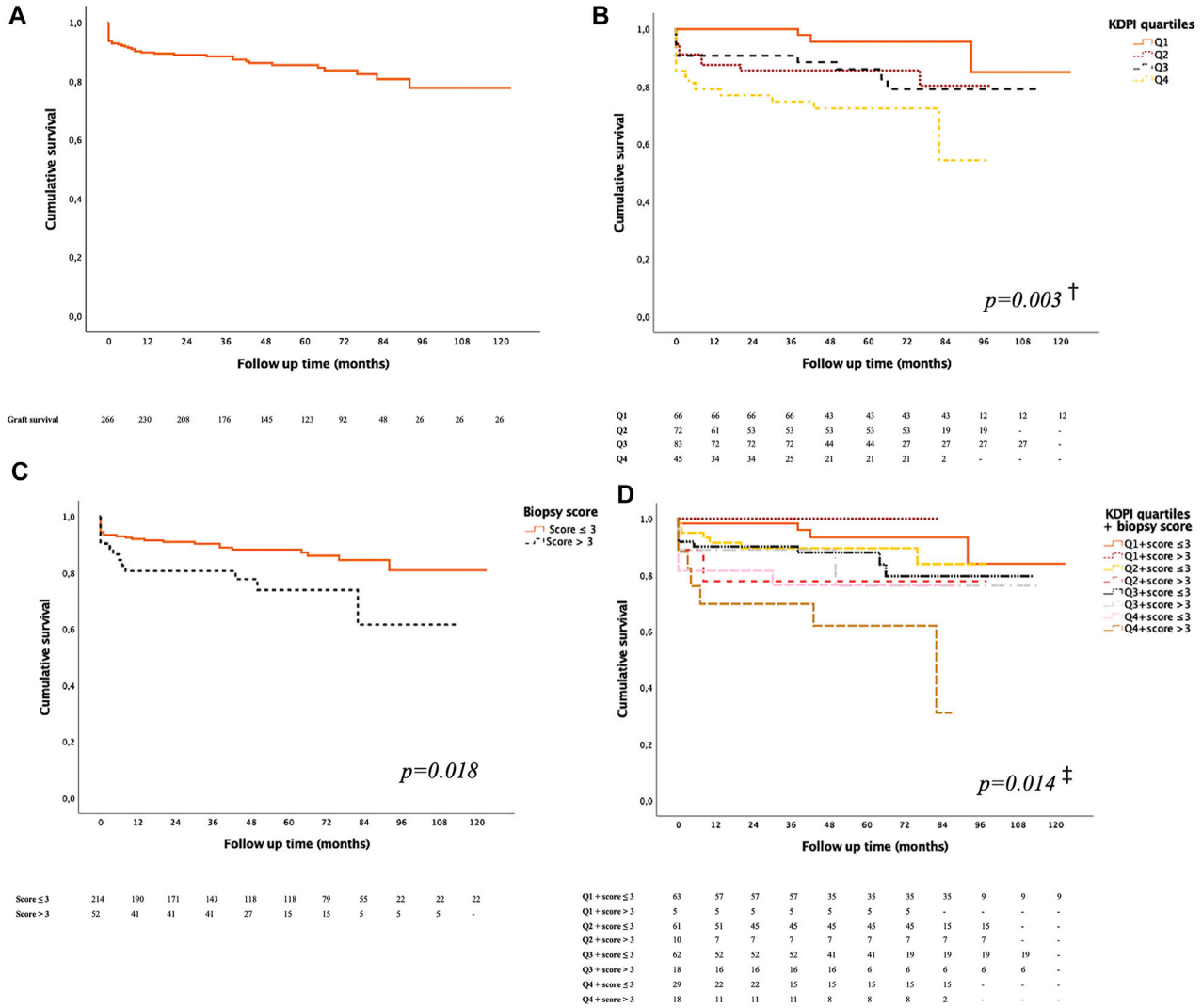


FIGURE 1 | Kidney graft survival function. **(A)** All kidney transplant patients. **(B)** According to the KDPI quartile. †Q1 vs. Q4; $p = 0.001$, Q1 vs. Q2; $p = 0.012$, Q1 vs. Q3; $p = 0.043$; the rest of pairwise comparisons were not significant: Q2 vs. Q3; $p = 0.876$, Q2 vs. Q4; $p = 0.110$, Q3 vs. Q4; $p = 0.192$. **(C)** According to the score of the preimplantation biopsy. **(D)** According to the combination of the KDPI quartile and the score of the preimplantation biopsy. ‡Q1 & score ≤3 vs. Q2 & score ≤3; $p = 0.019$, Q1 & score ≤3 vs. Q3 & score >3; $p = 0.023$, Q1 & score ≤3 vs. Q4 & score ≤3; $p = 0.009$, Q1 & score ≤3 vs. Q4 & score >3; $p < 0.001$, the rest of pairwise comparisons were not significant: Q1 & score ≤3 vs. Q1 & score >3; $p = 0.728$, Q1 & score ≤3 vs. Q2 & score >3; $p = 0.117$, Q1 & score ≤3 vs. Q3 & score ≤3; $p = 0.125$, Q1 & score >3 vs. Q2 & score ≤3; $p = 0.376$, Q1 & score >3 vs. Q2 & score >3; $p = 0.398$, Q1 & score >3 vs. Q3 & score ≤3; $p = 0.425$, Q1 & score >3 vs. Q3 & score >3; $p = 0.238$, Q1 & score >3 vs. Q4 & score ≤3; $p = 0.252$, Q1 & score >3 vs. Q4 & score >3; $p = 0.175$, Q2 & score ≤3 vs. Q2 & score >3; $p = 0.935$, Q2 & score ≤3 vs. Q3 & score ≤3; $p = 0.583$, Q2 & score ≤3 vs. Q3 & score >3; $p = 0.669$, Q2 & score ≤3 vs. Q4 & score ≤3; $p = 0.310$, Q2 & score ≤3 vs. Q4 & score >3; $p = 0.089$, Q2 & score >3 vs. Q3 & score ≤3; $p = 0.676$, Q2 & score >3 vs. Q3 & score >3; $p = 0.875$, Q2 & score >3 vs. Q4 & score ≤3; $p = 0.612$, Q2 & score >3 vs. Q4 & score >3; $p = 0.357$, Q3 & score ≤3 vs. Q3 & score >3; $p = 0.449$, Q3 & score ≤3 vs. Q4 & score ≤3; $p = 0.308$, Q3 & score ≤3 vs. Q4 & score >3; $p = 0.073$, Q3 & score >3 vs. Q4 & score ≤3; $p = 0.948$, Q3 & score >3 vs. Q4 & score >3; $p = 0.574$, Q4 & score ≤3 vs. Q4 vs. score >3; $p = 0.479$.

biopsy and the KDPI simultaneously in the evaluation of the ECD. Both variables are necessary for the assessment of non-optimal grafts. Additionally, we have identified a very high-risk group of donors, who are those with a KDPI greater than 99% and a biopsy score >3 points.

The usefulness of preimplantation biopsy as a predictive tool for graft survival has not been validated yet and some studies discuss its value for the assessment of the quality of organs from ECD [15,18-20]. In our case, all the grafts had a preimplantation biopsy and only those with a global score <7 were accepted. KT with a score <3 had better survival and allograft function, reflecting the potential value of the biopsy. However, in multivariable analysis, when donor clinical variables were included, the global score did not associate with graft survival. Previous studies did not find survival differences when comparing KT with mild and moderated lesions in preimplantation biopsy, so the discard of an allograft should not be done exclusively according to the histological analysis [21,22].

TABLE 3 | Univariable and multivariable Cox regression analysis for death-censored graft failure.

	HR (95% CI)	*p*-value
Univariable analysis		
Female donor	1.643 (0.882–3.059)	0.118
Donor age	1.055 (1.014–1.098)	0.008
HBP donor	0.795 (0.388–1.630)	0.531
DM donor	2.654 (1.277–5.516)	0.009
Smoking donor	0.761 (0.355–1.633)	0.484
Non-heart beating donor	1.095 (0.458–2.616)	0.839
KDPI	1.034 (1.003–1.066)	0.029
Female recipient	1.074 (0.566–2.037)	0.828
Recipient age	1.031 (0.997–1.065)	0.071
Time of RRT	1.000 (1.000–1.001)	0.194
Cold ischemia time	1.000 (0.999–1.001)	0.986
Biopsy score > 3	2.173 (1.120–4.218)	0.022
Glomerulosclerosis ≥1	2.305 (1.033–5.143)	0.041
Hyaline arteriopathy ≥1	1.349 (1.090–5.059)	0.029
Myointimal elastosis ≥1	1.524 (0.463–5.021)	0.489
Tubular atrophy ≥1	0.661 (0.307–1.420)	0.289
Interstitial fibrosis ≥1	1.304 (0.589–2.886)	0.512
Multivariable analysis		
Model 1		
Cold ischemia time	1.000 (1.000–1.001)	0.576
KDPI	1.032 (0.996–1.069)	0.081
Recipient age	0.998 (0.957–1.039)	0.910
Time of RRT	1.000 (1.000–1.001)	0.181
Biopsy score > 3	1.719 (0.855–3.456)	0.128
Model 2		
Cold ischemia time	1.000 (1.000–1.001)	0.710
Recipient age	0.987 (0.945–1.031)	0.548
Time of RRT	1.000 (1.000–1.001)	0.210
Biopsy score > 3	1.414 (0.680–2.940)	0.354
KDPI Q2[a]	2.503 (0.738–8.492)	0.141
KDPI Q3[a]	3.136 (0.842–11.680)	0.088
KDPI Q4[a]	6.684 (1.583–28.229)	0.010
Model 3		
Cold ischemia time	1.000 (1.000–1.001)	0.593
KDPI	1.030 (0.994–1.068)	0.101
Recipient age	0.996 (0.956–1.038)	0.858
Time of RRT	1.000 (1.000–1.001)	0.236
Hyaline arteriopathy ≥1	2.322 (1.124–4.794)	0.023
Glomerulosclerosis ≥1	2.861 (1.330–6.154)	0.007
Model 4		
Cold ischemic time	1.000 (0.999–1.001)	0.722
Recipient age	0.988 (0.947–1.032)	0.595
Time of RRT	1.000 (1.000–1.001)	0.233
Hyaline arteriopathy ≥1	2.136 (1.010–4.516)	0.047
Glomerulosclerosis ≥1	2.614 (1.193–5.729)	0.016
KDPI Q2[a]	2.217 (0.650–7.559)	0.203
KDPI Q3[a]	3.111 (0.850–11.388)	0.086
KDPI Q4[a]	4.767 (1.177–19.315)	0.029

[a]*Reference KDPI Q1.*

KDPI, kidney donor profile index; RRT, renal replacement therapy; HR, hazard ratio; CI, confidence interval.

We observed that glomerulosclerosis and hyaline arteriopathy was related to shorter graft survival. Some groups have defended the value of glomerulosclerosis as the main parameter in the evaluation of the preimplantation biopsy, showing that a percentage of glomerular sclerosis >20% is associated with a worse graft evolution [23-25]. Bröcker et al. stated that patients with hyaline arteriopathy usually had worse renal function [26]. Our results remained even when KDPI was included in the model, so not only determining the global biopsy score is important but which histological compartments are most affected.

Regarding a typical frozen sections' biopsy analysis, subtle findings such as interstitial fibrosis and tubular atrophy, thickening of capillary walls, mesangial cellularity, and histological lesions related to diabetes and other comorbidities are often more difficult to identify compared to correctly fixed and stained sections such as microwave paraffin embedding [15,24,27]. In our case, the use of the freezing technique may have underestimated the degree of some histological injuries. However, in all grafts, a wedge biopsy was performed to ensure the proper quality of the sample. New ultra-fast freezing techniques seem to offer better results with a higher quality of the histological sample, although their use has not yet become widespread in kidney donor biopsies [28]. Our results show that glomerulosclerosis and hyaline arteriopathy should be considered the main histological compartments in biopsies processed with the most commonly used freezing methods.

Because of the controversy about the efficacy of histologic evaluation for predicting graft survival, the KDPI has been implemented in the United States as an effective system for evaluating the quality of deceased donors [5]. Along with our experience, kidney allografts from donors with a lower KDPI had better survival. Additionally, we observed a negative relationship between KDPI and graft function. In the United States, a kidney with a KDPI >85% is considered suboptimal and it is likely rejected. However, there are many transplant centers that currently utilize high KDPI kidneys [29,30]. In our study, the median KDPI score was 95%, but one-year graft survival was 89.8% and 85.4% at 5 years, which is higher than reported in other series with a lower KDPI [31]. Notwithstanding, the KDPI is a tool developed by the American Transplantation Registry, so its usefulness in Spain is limited by the difficulty to extrapolate this score to other countries with different healthcare systems and transplant programs [6,9].

Due to the technical limitations of the preimplantation biopsy and the lack of accuracy of KDPI, it seems reasonable the combination both variables for the assessment of the ECD. We analyzed simultaneously KDPI and biopsy scores in an ECD cohort and we identified a group at high risk of graft failure: KDPI greater than 99% and biopsy score >3. However, a KT with a very high KDPI score that does not present these lesions in the biopsy can offer an acceptable medium-term survival (5-year graft survival: 78.7%), especially through an old-for-old allocation program or for high estimated post-transplant survival (EPTS) recipients [32]. On the other hand, dual KT could be the best choice to improve the results of transplantation with grafts with both high KDPI and high biopsy scores [33].

Our study presents several limitations. It is a retrospective, single-center study, with the limitations that inherently may exist in data collection. Second, biopsies were not re-evaluated retrospectively by a single pathologist in order to reduce the

interobserver variability. However, only a limited group of expert pathologists analyzed the biopsies. Therefore, to avoid further biases, we excluded kidneys with biopsies analyzed in other centers where there could be differences in the preparation and interpretation of the histological samples. In third place, according to our protocol, the grafts with a very high biopsy score were not implanted, so we cannot be sure what function they might have had. Fourth, the use of the frozen-section analysis has drawbacks that have already been discussed. Last, when we analyzed the KDPI and the biopsy score as continuous in the multivariate models, we found no significant differences. However, when both variables were stratified, survival inequalities were observed. These divergences in the results may be due to the multicollinearity between both variables.

In conclusion, the KDPI and a greater histological injury in the preimplantation biopsy, especially glomerular and vascular lesions, were related to a higher rate of KT graft loss coming from ECD. Both parameters were related to graft function and survival. As long as a kidney donor evaluation index more adapted to our country is not available as well as more rapid and precise histological techniques, we suggest that both the clinical and histological variables should be considered together in the pretransplant assessment of ECD with a high KDPI.

DATA AVAILABILITY STATEMENT

The data underlying this article will be shared on reasonable request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Puerta del Mar Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AM designed the study. FV, LV, and JC collected the data. FV, LV, and AM analyzed the data and wrote the paper. FV, LV, JC, JN, LA, AG, MEM, MCM, TG, and AM revised the paper, contributed important reagents, and approved the final version of the manuscript.

CONFLICT OF INTEREST

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

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2022.10056/full#supplementary-material

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Transferring an ICU Patient at the End of His Life for the Purpose of Organ Donation: Could It Be Considered?

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Dear Editors,

Controlled donation after circulatory death (cDCD) refers to organ donation from patients whose death is defined by circulatory criteria after the planned withdrawal of life-sustaining treatments (WLST) in intensive care units (ICUs) [1]. The development of this type of donation has varied from country to country due to their different legal, ethical, and organizational frameworks, which explain diverse activity levels and transplant outcomes [2]. France began its cDCD program in 2015 with ethical and technical aspects leading to a nationwide protocol. The underlying principle is that the decision to withdraw LST must be made in the patient's best interest, independently from any consideration regarding organ donation, and that cDCD must not alter end-of-life care [3]. The challenge is not only to identify potential cDCD donors, but also to provide support to grieving families and to give caregivers a reassuring ethical framework [4]. Yet, caregivers can feel particularly uncomfortable when, in practice, end-of-life care and organ donation overlap.

Today, the scarcity of donor organs and the good transplantation outcomes [5, 6] legitimately support the development of cDCD in a context where WLST decisions occurs more and more frequently in ICUs worldwide [7]. This development is limited by technical and organizational aspects, in particular related to the systematic use of normothermic regional perfusion (NRP), which requires technology (NRP device) and expertise (NRP settings and vessels cannulation) not available in all hospitals. It is also likely limited by many ethical issues as cDCD reshapes end-of-life care by introducing the issue of organ donation before the time of death. Thus, cDCD may potentially affect not only the WLST decision-making process but also other end-of-life care practices, such as sedative practices, and the acceptance by relatives and caregivers [8, 9].

For the further development of cDCD, more hospitals should have the technical and organizational capacity to achieve regulatory approval for cDCD. However, beyond ethical

Abbreviations: cDCD, controlled donation after circulatory death; ICU, intensive care units; NRP, normothermic regional perfusion; WLST, withdrawal of life-sustaining treatments.

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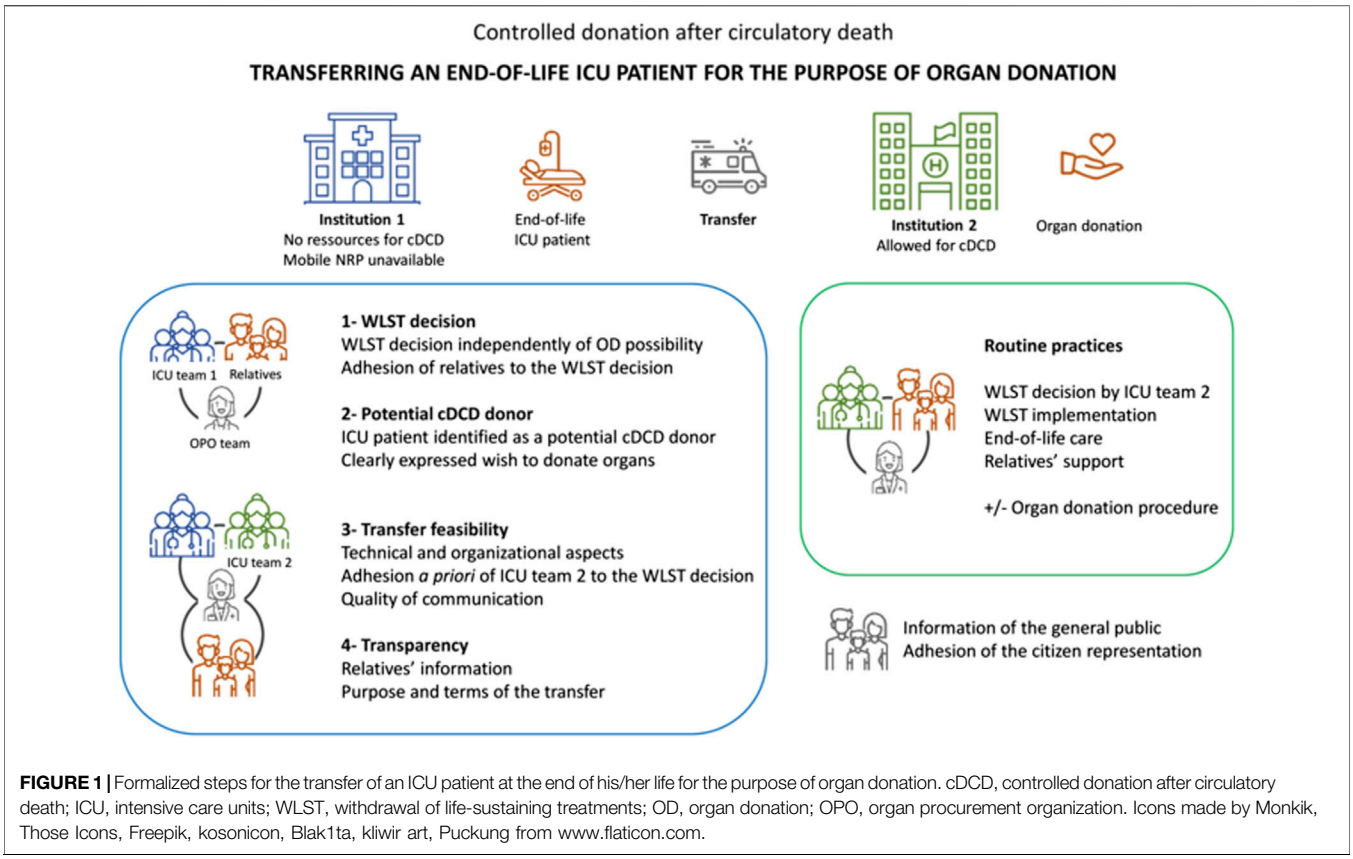

FIGURE 1 | Formalized steps for the transfer of an ICU patient at the end of his/her life for the purpose of organ donation. cDCD, controlled donation after circulatory death; ICU, intensive care units; WLST, withdrawal of life-sustaining treatments; OD, organ donation; OPO, organ procurement organization. Icons made by Monkik, Those Icons, Freepik, kosonicon, Blak1ta, kliwir art, Puckung from www.flaticon.com.

issues, this may be limited by technical or organizational aspects, and/or by the relatively small number of potential cDCD donors, and subsequent procedure failure risks. The question is: which ways could be considered to allow cDCD for an ICU end-of-life patient hospitalized in an institution that does not have the resources for cDCD? The first strategy is the use of mobile normothermic regional perfusion and should be preferred as ethical issues are limited in this scenario [10]. When this is not possible, another strategy could be to allow the transfer of an ICU end-of-life patient for the purpose of organ donation to another hospital allowed for cDCD.

The transfer of an ICU end-of-life patient for the purpose of organ donation raises many ethical issues as its potential impact on the patient himself, on his/her relatives and on ICU caregivers. There are many risks, including: not complying with the wishes of a patient unable to express himself; considering the patient from a purely utilitarian perspective; changing end-of-life practices so that the death occurs with a timeframe that allows organ donation; transferring the patient under the presumption of consent, even though the patient is on the registry of refusals (which is only consultable after death in France); affecting experiences and perceptions of relatives through geographic and/or relational discontinuity; having an impact on the experience of caregivers and their motivation to be involved in organ donation.

Overall, in contexts of potential organ donation, end-of life support must always be preserved. The transfer of an ICU end-of-life patient for the purpose of organ donation should remain an exception. Moreover, this exception may only be justified by the aim of complying with the clearly expressed wish of the patient to donate his/her organs after-death. A sole presumption of consent (as stated by French law for conventional organ donation procedures) may not be sufficient. The individual's values and preferences regarding end-of-life and organ donation must be respected over any utilitarian considerations. This is a key issue while France has adopted the opt-out system. Concerning relatives, they must be clearly informed at each stage of this complex process. Support for them must always be provided. Facilities for transport and accommodation must be offered to them, as well as the return of the body must be mandatory after death and organ procurement. Concerning caregivers, the principle of separation between WLST decisions and organ donation possibility must strictly be respected, end-of-life practices must be applied as they are formalized independently from the possibility of organ donation. The training and support of caregivers involved is a central goal.

Finally, it could be possible if the following four conditions are met:

(1) Arrangements that favor the proximity of the patient to his/her relatives and ICU caregivers, such as the use of a mobile NRP, cannot be implemented locally for technical or organizational reasons.

(2) The clearly expressed wish of the patient to donate organs after death (first-person or relatives testimony, living will, advance directives).
(3) The adherence to a formalized procedure described in **Figure 1** that involves the end-of-life patients, the relatives, the ICU team 1 working in the hospital 1, the ICU team 2 working in the hospital 2 allowed for cDCD, and the organ procurement organization. Sharing the details of the case should ensure that ICU team 2 adheres a priori to the decision of ICI team 1 to withdraw LST. Particular attention must be paid to the quality of communication between the two ICU teams, the organ procurement organization (OPO) team and the relatives.
(4) The transparency of the procedure is ensured.

The future development of cDCD needs to address, beyond the technical and organizational aspects, the ethical tension between end-of-life care and organ donation. The future developments of cDCD are ethically reasonable as long as end-of life support is preserved. The information of the general public and the adhesion of the citizen representation to the procedure are crucial and must be pursued.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

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

CONFLICT OF INTEREST

MD, GA, and LM are member of the Steering Committee of the French cDCD Program.

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

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