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Kidney Transplantation in Western Balkans: A Regional Blueprint for Access, Capacity, and Equity

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There is no medical field where the impact of medical evolution is more palpable than in kidney transplantation. The pioneers of this procedure, 70 years ago, laid out the foundation for organ transplantation in general and kidney transplantation in particular. Despite the incredible advancements that have been made since, huge differences exist worldwide in terms of access, equity and quality of care. Nowhere are these disparities more prominent than in developing countries with limited resources, underfunded healthcare systems and transplantation infrastructures, particularly the Western Balkans. This position paper delineates the biggest barriers hindering the development of kidney transplantation in the Western Balkans, put forth and agreed upon by a group of regional experts on the field, based on the Modified Delphi Method. Limitations in training, infrastructure, restrictive and outdated legislative practices, lack of a centralized coordination network and fragmented regional collaboration, emerged as the principal challenges. Endorsed by European Society for Organ Transplantation (ESOT), this paper outlines a pragmatic and practical framework to overcome these obstacles, towards building robust and sustainable transplantation programs that ensure high-quality and equitable access to kidney transplantation, for all patients in this region.

Keywords: blueprint, equity, health systems, kidney transplantation, Western Balkans

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INTRODUCTION

Chronic kidney disease (CKD) represents a growing public health challenge, associated with significant morbidity and mortality rates and substantial implications across public healthcare systems. Affecting over 850 million people worldwide, recent data places CKD as the 7th leading cause of death globally and has shown a sharp rise in disability-adjusted life years (DALYs) and years of life lost (YLLs), particularly in low- and middle-income countries [1–4].

In light of this data, in 2024 the International Society of Nephrology (ISN), American Society of Nephrology (ASN) and European Renal Association (ERA) in a joint statement proposed to include CKD in the WHO's list of major non-communicable diseases, due to its significant contribution in premature cardiovascular-related deaths, expecting it to become the fifth leading cause of death worldwide by 2040 [5].

Kidney transplantation is widely recognized as the gold standard, but dialysis remains the cornerstone of CKD management. Nevertheless, evidence consistently shows transplantation to be associated with superior survival rates, improved quality of life, psychosocial wellbeing, social and workforce reintegration, as well as higher employment rates, compared to dialysis patients [6–9]. Moreover, from a health economics standpoint, transplantation proves to be more cost-effective in the long run. In Europe, the annual cost per patient for dialysis is estimated to vary between €50,000 to €90,000, while transplantation, despite higher upfront costs, yields lower long-term expenditures not only due to the ommited dialysis cost, but also to reduced hospitalization and complication rates [10, 11].

Between 10% and 30% of patients on waiting lists die before receiving a graft, reflecting persistent organ shortages and systemic inefficiencies (ERA Registry 2022; EDQM Newsletter Transplant 2024) [12, 13]. Despite these clear advantages, disparities in access, limited organ availability, inefficiencies and lack of resources across healthcare systems, legislative and cultural barrierscontinue to hinder its full potential [14]. A region facing these challenges is the Western Balkans (Albania, Bosnia and Herzegovina, Kosovo, Montenegro, North Macedonia, Serbia), where equity and access to kidney transplantation remains markedly lower than in the rest of the continent [9, 15, 16].

In this context, on May 24 -25, 2025 an important conference aiming to provide a better understanding of the current landscape and the challenges in kidney transplantation in the Western Balkans was held, in Tirana, Albania. Transplantation Without Borders: Balkan Initiative supported by the European Society for Organ Transplantation (ESOT), brought together regional experts and international leaders in the field, to address the structural, systemic and legislative barriers currently being faced in the Western Balkans. Additionally, it set to develop a blueprint on the steps that are required to move toward robust, active national programs and a unified regional collaboration to ensure faster and more equitable access, as well as better quality of care for patients in the region, in line with the ESOT Manifesto aiming to tackle inequality in organ transplantation [17].

This position paper summarizes the data on the current landscape of kidney transplantation in the Western Balkans, and outlines an actionable, collaborative regional strategy to improve outcomes through harmonized legislation, optimized living and deceased donor programs, and shared capacity- building frameworks.

MATERIALS AND METHODS

Study Design

This study employed a Modified Delphi Method, integrating quantitative and qualitative data collection to identify regional needs, barriers, and priorities for advancing kidney transplantation in the Western Balkans. The Delphi approach is designed to gather expert consensus through iterative rounds of structured feedback [18]. In this study, a modified format was adopted, consisting of a structured needs- assessment survey and expert roundtable discussions conducted during the regional conference "Transplantation Without Borders: Balkan Initiative" (Tirana, 2025) (**Figure 1**).

The objective of this methodological approach was to facilitate systematic consensus among nephrologists, transplant surgeons, immunologists, coordinators, and policymakers representing Western Balkan countries and regional model country (Croatia) on the current landscape and future strategic approach for kidney transplantation in the region.

Delphi Procedure

The first round was conducted through a structured online questionnaire, developed by EKITA-ESOT, distributed to transplant experts. The survey included five themes of questions:

1. Demographic and institutional characteristics;
2. Current national transplantation activity and infrastructure;
3. Barriers related to legislation, organization, training, and immunology;
4. Pharmacological and coordination system limitations;
5. Strategic priorities and feasible short-term actions.

The questionnaire combined closed questions, Likert-scale items assessing perceived importance or limitation, and open-ended questions that allowed for qualitative elaboration. Responses were analyzed descriptively to identify the most frequently cited barriers and priority areas.

The second round took place during the in-person conference sessions, where preliminary survey results were presented. Participants engaged in moderated roundtable discussions structured around five thematic domains: legislation, capacity building, infrastructure, pharmacological access, and coordination systems. Consensus was defined *a priori* as ≥75% agreement among participants on a given recommendation or priority area (**Supplementary Table 1**). Revised consensus statements were integrated into the final regional position framework.

Post-Conference Validation and Country-Specific Data Collection

Following the completion of the two Delphi rounds and the consensus meeting, an additional post- conference validation phase was conducted to complement and refine the findings. During this stage, national experts from each participating Western Balkan country were invited to provide updated and country-specific information through structured online communication.

Between May and October 2025, experts from Western Balkans submitted detailed written feedback via email

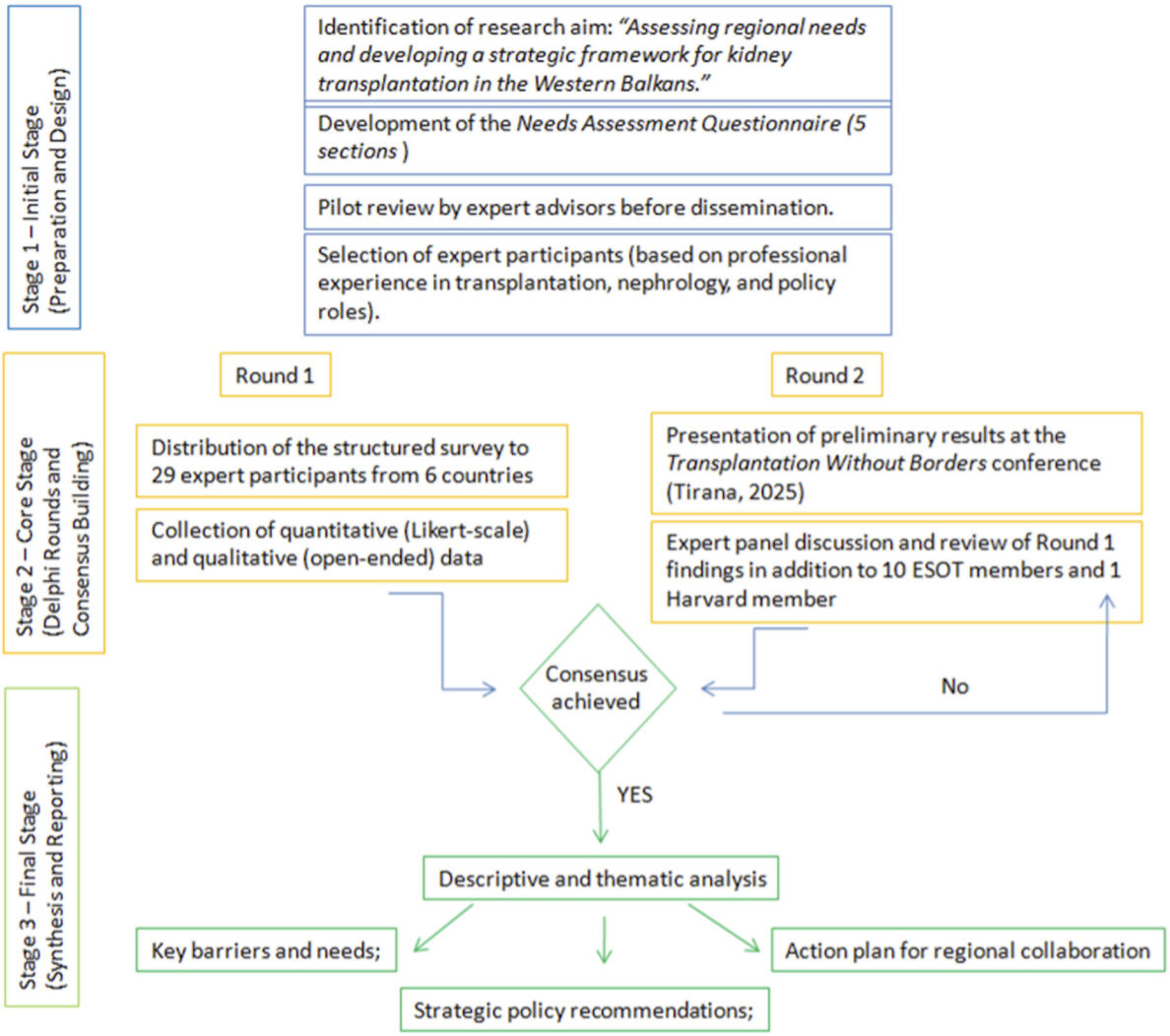

FIGURE 1 | Flow diagram illustrating the Modified Delphi Method applied to the Western Balkans kidney transplantation needs assessment.

correspondence coordinated by the study team. Each expert provided validated information on key indicators, including population data, dialysis prevalence, number of active transplant centers, annual kidney-transplantation activity, legal framework, coordination systems, and existing training infrastructure.

Data Analysis

Qualitative responses were subjected to thematic analysis using an inductive approach following Braun and Clarke's six-phase framework. Emerging categories were grouped into overarching themes corresponding to the main barriers and proposed solutions.

Country-specific expert reports in the post-conference validation were analyzed descriptively and merged into a unified regional dataset.

Raw country data were range normalized to a 0–1 scale. For each country, five indicators were compiled (CKD prevalence, dialysis burden, annual transplant rate, number of active centers, and deceased-donor program status) inverted so higher scores indicate greater capacity. Normalized values were plotted in Excel as a radar chart to visualize cross-country readiness.

Outcome

The Modified Delphi process resulted in a consensus-based regional framework outlining actionable recommendations for improving kidney transplantation across the Western Balkans. The findings serve as the foundation for the regional position statement "Transplantation without Borders: Balkan Initiative.

TABLE 1 | Demographic and professional characteristics of the 29 experts who participated in the Modified Delphi survey on kidney transplantation in the Western Balkans.

Characteristic	Category/description	N (%) (Total 29)
Gender	Male	10 (34%)
	Female	19 (66%)
Age (years)	Mean ± SD (range)	45.9 ± 10.2 (28–69)
Country of affiliation	Albania	8 (27.6%)
	Bosnia and Herzegovina	4 (13.8%)
	Kosovo	2 (6.9%)
	Montenegro	3 (10.3%)
	North Macedonia	5 (17.2%)
	Serbia	3 (10.3%)
	Croatia	4 (13.7%)
Professional role	Nephrologist	21 (72%)
	Transplant surgeon	3 (10%)
	Transplant coordinator	1 (3%)
	Laboratory/histocompatibility Specialist	1 (3%)
	Patient representative/ Organization	2 (7%)
	Other (resident/Fellow)	1 (3%)
Institution type	University hospital/Clinical center	23 (79%)
	National/ Academic/Research institute	6 (21%)

TABLE 2 | Summary of qualitative themes identified through the Modified Delphi process.

Theme	Description	Representative expert insights
Legislative and regulatory fragmentation	Variation in national frameworks limits living, deceased and kidney paired donation programs	"Our legal framework exists, but it's fragmented and not fully operational — we need harmonized regional standards."(Expert, Montenegro)
Immunological and pharmacological capacity gaps	Lack of advanced immunological testing and new therapeutic agents	"The need to outsource advanced immunological testing abroad, delays transplantation access to our patients."(Expert, Albania)
Workforce and training needs	Shortage of transplant coordinators, surgeons, and nephrologists; lack of structured professional training	"We need not only more specialists but also a defined career path in transplantation."(Expert, Bosnia and Herzegovina)
Limited infrastructure and coordination systems	Outdated or limited infrastructure and centralized coordination systems	"Even when we have trained specialists, the absence of a dedicated transplant center and staff significantly limits our transplantation services." (Expert, North Macedonia)
Regional cooperation	Absence of unified national registries, regional standardized protocols, and coordination systems limits the scope of transplantation activity	"In addition to empowering national programs, fostering regional collaboration is crucial in improving transplantation services in the region." (Expert, Serbia)

RESULTS

Participant Characteristics

A total of 29 experts participated in the Modified Delphi survey, representing six Western Balkan countries and Croatia. The panel comprised 66% female and 34% male participants, with a mean age of 45.9 ± 10.2 years (range 28–69 years). Most respondents were nephrologists involved in kidney transplantation (72%), while others included transplant surgeons, coordinators, immunologists, and patient organization representatives (**Table 1**).

Qualitative Analysis

Analysis of open-ended survey responses and expert discussions revealed five related themes reflecting shared regional challenges and strategic priorities for kidney transplantation in the Western Balkans. These themes, derived through the Modified Delphi process, highlight the experts' collective perspective on operational barriers, emphasized the need for harmonized legislative frameworks, improved infrastructure, and immunological capacity, strengthened workforce training and improved access to new pharmacological options across the region. A summary of these thematic domains and representative insights are presented in **Table 2**.

Country Specific Reports and Expert Feedback

Experts from each participating country provided quantitative and contextual updates on national kidney-transplantation indicators. The findings are summarized in **Figure 2** and **Table 3**, providing a comparative overview of population metrics, dialysis and transplantation activity, program structure, and the main challenges identified across the

FIGURE 2 | Kidney transplantation indicators in the Western Balkans (2025).

TABLE 3 | Country-specific summary of kidney transplantation indicators.

Country	Population (millions)	CKD Prevalence (%)	Dialysis patients (n)	Kidney transplants (total/annual)	Deceased donor program	Active centers (n)	PMP (per million population)
Albania	2.7	10.5	1800	400 total/ 25–30 per year	No (only living donor)	2 (both in Tirana)	9.3
Bosnia and Herzegovina	3.5	14	3,800	990 total/ 30–35 per year	Yes (limited)	2 (Sarajevo, Tuzla)	9.7
Kosovo	1.6	11	1,044	150 total/ ~8 per year (abroad)	No	0	5
Montenegro	0.63	10	495	200 total/ 10–12 per year	Inactive	1 (Podgorica)	15
North Macedonia	1.79	10%	1792	512 total/ 10 per year	Yes	1	5
Serbia	6.6	≈10–11	5,500	≈850 living patients/ ≈17 per year	Yes (limited activity)	8	3

Western Balkans. **Figure 2** shows that pmp rates remain very low across all active transplant centers in the Western Balkans. The country-level radar plots were generated to compare transplantation system readiness across five domains (CKD prevalence, dialysis burden, annual transplants, active centers, and deceased-donor status). Raw values were standardized to a 0–1 scale using range normalization, with limitation indicators (CKD, dialysis) inverted so that higher values uniformly indicate greater capacity. The charts visualize relative strengths and gaps across countries **Figure 3**.

Albania

> "*The need to outsource advanced immunological testing abroad, delays transplantation access to our patients.*" (Expert, Albania)

Albania has a population of approximately 2.7 million people, with a median age of 37.9 years. It is estimated that over 10.5% of the population is affected by CKD, with more than 200,000 people living with some stage of CKD. Among them,

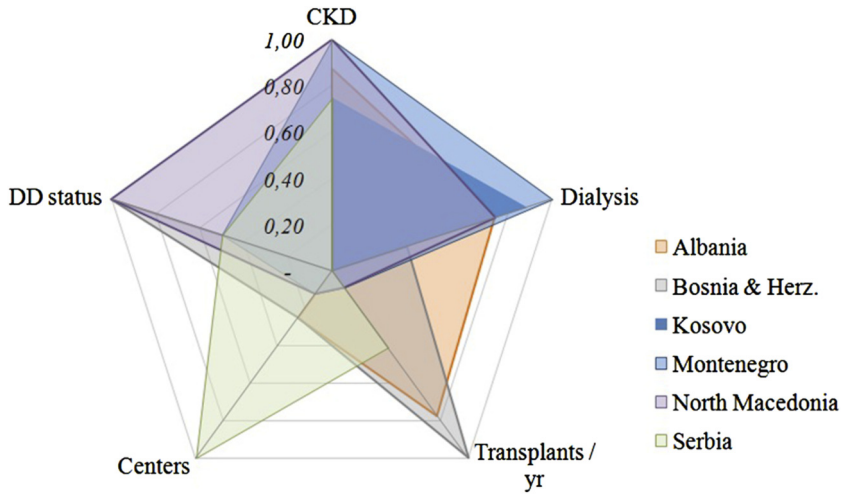

FIGURE 3 | Radar chart comparing kidney-transplantation readiness across five domains in six Western Balkan countries. Indicators were normalized to 0–1 (higher = greater capacity); CKD prevalence and dialysis burden were inverted. Data: Delphi survey and post-conference expert validation (2025).

1800 patients with end-stage kidney disease (ESKD) are currently receiving renal replacement therapy. Around 1,400 patients (75.9%) undergo hemodialysis with an estimated annual incidence increase between 5%-7%. Hemodialysis services are distributed across 14 active units nationwide. Thirty-five patients (1.9%) are on peritoneal dialysis (PD). A total of 400 patients (22.2%) have received a kidney transplant, with an average of 25–30 new cases conducted annually.

The basic legal framework on transplantation in Albania began to take shape in 1997, when the first legislation regulating organ transplantation was passed. A decade later, in 2007 the first kidney transplant in a public institution was performed as part of an international collaboration with the University Hospital of Bari supported by the INTERREG III Project. This initiative aimed to develop the necessary infrastructure and provide adequate training to healthcare providers involved in kidney transplantation. In 2011, the adoption of the National Law on Organs, Tissues and Cells by the Albanian Parliament paved the way for the formal introduction of kidney transplantation as a therapeutic modality for patients with ESKD.

However, despite the progress made, numerous challenges persist. At present, only living donor kidney transplantation (LDKT) is performed in Albania. Two active transplant centers, both located in Tirana, carry out all procedures. In 2024, 26 living donor kidney transplants were performed, corresponding to a rate of 9.3 per million population (pmp). There is currently no active deceased donor kidney transplantation (DDKT) program in the country.

Our survey identified insufficient training, infrastructure, and regulatory barriers as the main challenges limiting the development of kidney transplantation in the country. There are no formal training programs for transplant surgeons or nephrologists, and most healthcare professionals have obtained only short-term training abroad. Surgical procedures are performed by hybrid teams, combining local nephrologists and anesthesiologists with visiting surgeons from Turkey.

Significant infrastructural constraints, particularly in immunological evaluation, were also reported. While basic pre-transplant testing is performed locally, advanced assays are outsourced abroad due to limited equipment and capacity. Although standard immunosuppressive agents are available through public reimbursement, access to newer therapies for rejection, desensitization, and drug-resistant infections remains limited.

Participants emphasized the need to update national transplantation legislation, particularly to include paired donation and strengthen frameworks for both living and deceased donation. Finally, the absence of a centralized coordination system and dedicated transplant coordinators was identified as a critical barrier to improving inter-institutional collaboration and overall program efficiency.

Bosnia and Herzegovina

> "*We need not only more specialists but also a defined career path in transplantation.*" (Expert, Bosnia and Herzegovina)

Bosnia and Herzegovina, with a population of approximately 3.5 million and a median age of 49.7 years, faces a substantial burden of CKD, affecting an estimated 14% of the population, over 400,000 individuals. Among them, around 3,800 patients are living with ESKD and currently receive renal replacement therapy. Approximately 2,800 patients are treated with hemodialysis or peritoneal dialysis, with an annual incidence increase of 5.6%. Dialysis services are provided through 26 active centers nationwide. Sixty-eight patients are maintained on PD. To date, a total of 990 kidney transplants have been performed in the country, with an average of 30–35 new transplants per year.

The first kidney transplant in Bosnia and Herzegovina was performed in Sarajevo in 1974, marking the second in the former Yugoslavia. This early achievement established a solid foundation with trained professionals and a functioning transplant infrastructure. However, the Bosnian war interrupted program development, halting progress after nearly 300 kidney transplants had been performed. Following the war's end in 1995, transplantation activities resumed under the pre-existing National Law on Organ and Tissue Transplantation at three centers, Sarajevo, Tuzla, and Banja Luka, though national activity remained low, averaging 10–20 transplants per year. The first DDKT were carried out in the early 2000s. Current legislation permits living related donation (up to the fourth degree of consanguinity), spousal donation and deceased donation, while paired exchange transplantation is not yet legally allowed. In 2024, a total of 35 kidney transplants were performed (23 live related or unrelated donation transplants and 12 cadaveric kidney transplants), 2 liver transplants, 1 heart transplant, and 12 corneal transplants.

Our survey identified the lack of formal training, infrastructure, and regulatory support as the main barriers limiting the development of transplantation in Bosnia and Herzegovina. There is currently no structured clinical or surgical training program in kidney transplantation, and most healthcare professionals involved have received only short-term training abroad. Transplant teams typically include a urologist, vascular surgeon, nephrologist, anesthesiologist, immunologist, and specialized nurses. Due to staff shortages, the transplant program in Banja Luka has been suspended, leaving only Sarajevo and Tuzla as active centers.

Significant infrastructural and organizational limitations were also reported. Emerging transplant coordinators are not full-time professionals but perform coordination duties alongside routine clinical work, reducing program efficiency. While immunological testing capacity has improved, response times remain suboptimal. Standard immunosuppressive drugs are available through public funding, but access to newer agents for rejection, desensitization, and resistant infections is still limited. Legislative reform was highlighted as a top national priority, particularly the inclusion of kidney paired donation (KPD) to expand donor availability. Participants also emphasized the need for public awareness campaigns to promote organ donation, noting that current efforts led by the Donor Network of Bosnia and Herzegovina are constrained by insufficient funding.

Finally, the absence of a centralized national coordination system remains a major structural obstacle. Coordination exists only within the Federation of Bosnia and Herzegovina, which maintains a transplant waiting list, while Republika Srpska lacks a coordinator and registry. This administrative fragmentation continues to hinder national harmonization and the overall progress of transplantation in the country.

Kosovo

> "*By adopting an official law regulating organ transplantation and building capacity, kidney transplantation can become a realistic treatment option for every eligible patient in the country.*" (Expert, Kosovo)

With 1.59 million inhabitants and a median age of 34.8 years, Kosovo has Europe's youngest population. CKD affects approximately 11%, approximately 170,000 people. Among 1,044 patients receiving kidney replacement therapy, hemodialysis accounts for 85%–86%, around 900 patients), according to the European Renal Association registry 2022, with only 2 patients on PD. Approximately 150 patients have received transplants, with only around 8 new procedures annually, all conducted abroad.

Kosovo possesses a unique strength: a young population with strong cultural family ties. Large families are common, and relatives are willing to serve as living donors, creating a natural donor pool that could substantially increase transplantation if properly organized.

However, significant barriers prevent domestic transplantation. Kosovo lacks an officially adopted transplantation law; only a draft law exists in parliament. There is no formal surgical or clinical training in kidney transplantation and no domestic surgical team. While the nephrology team can assess transplant recipients and monitor post-transplant complications, immunological evaluation is limited with advanced testing outsourced abroad. Standard immunosuppressive drugs are publicly reimbursed, but newer agents for rejection, desensitization, and resistant infections remain article unavailable. Critically, there is no central coordination system or transplant coordinators for donor–recipient matching and follow-up.

To transform kidney transplantation into a sustainable, first-line treatment option, Kosovo must finalize and adopt comprehensive transplant legislation encompassing both paired and deceased donation. Equally important is the development of formal training programs for nephrologists, surgeons, and transplant coordinators, alongside investment in laboratory infrastructure to support advanced immunological testing. Expanding access to modern immunosuppressive therapies and establishing a centralized national transplant coordination system are also critical steps toward system-wide improvement.

Montenegro

> "*Our legal framework exists, but it's fragmented and not fully operational — we need harmonized regional standards.*" (Expert, Montenegro)

Montenegro, with a population of about 0.63 million and a median age of 39.7 years, has an estimated 10% CKD prevalence, roughly 6,000 individuals. Among them, 495 patients with ESKD receive renal replacement therapy, 295 patients (59.5%) undergo hemodialysis with an estimated annual incidence increase between 4%-8%, across 12 active units. On the other hand, there is currently no patient on PD. To date, around 200 kidney transplants have been performed (40.4%), with an average of 10–12 new cases conducted annually.

The legal framework for organ transplantation was introduced in 2010, and the first national legislation was enacted in 2012, marking the start of the country's modern transplant program. That same year, the first kidney transplant in a public institution was conducted through collaboration with the Clinical Hospital Center Zagreb and the Croatian Ministry of Health. Between 2012 and 2020, this partnership enabled 47 successful LDKT and two cadaveric donations, coordinated through Eurotransplant, allowing reciprocal access for Montenegrin patients to kidney, heart, liver, and lung transplants abroad.

Currently, only living donor transplantation is performed at the Clinical Center of Montenegro in Podgorica, which serves as the country's sole transplant facility. Their DDKT program remains inactive due to the low donor rate and limited public willingness for deceased donation. Major challenges include limited financial and infrastructural capacity, shortage of trained personnel, and regulatory gaps. There are no formal training programs in kidney transplantation, local teams collaborate with visiting surgeons from Croatia and Serbia. However, since 2019, a national HLA laboratory has been operational, ensuring basic and advanced immunological testing. Standard immunosuppressive therapies are widely available under public reimbursement schemes.

Recent legislative updates aim to strengthen the deceased donation framework, while a national public awareness campaign led by the Ministry of Health seeks to increase organ donation. The absence of a centralized transplant coordination system remains a key limitation, though efforts are ongoing to establish a national coordination network and improve donor detection and maintenance capacity in general hospitals.

North Macedonia

> "*Even when we have trained specialists, the absence of a dedicated transplant center and staff significantly limits our transplantation services.*" (Expert, North Macedonia)

North Macedonia, located in the center of the Balkan Peninsula, has a population of approximately 1.79 million and a median age of 36.5 years. In 2024, an estimated 1,792 patients were living with ESKD, with 1,516 receiving hemodialysis and 18 on PD. Hemodialysis services, first established in 1971, are currently provided through 23 active centers. To date, 512 kidney transplants have been performed, with an average of 7–41 transplants annually.

Kidney transplantation began in 1977 with a living donor, followed by the establishment of a deceased donor program in 1987. The legal foundation, initially based on Yugoslav legislation, was updated in 2011. Despite these developments, activity remained limited for decades, averaging 7.6 and 2.9 pmp for living and deceased donation, respectively. A major turning point was heralded by the country's integration into the Southeastern Europe Health Network (SEEHN) in 2009 and the Regional Health Development Center on Organ Donation and Transplant Medicine (RHDC) in 2011, which led to a steady rise in transplant rates, 12 pmp in 2012, 17.3 pmp in 2013, and 20 pmp in 2014 for living donors, alongside an annual 7.5 pmp for deceased donations. Activity continued to grow in subsequent years, and following the COVID-19 pandemic, a significant increase in transplantation activity was observed in both living (LD) and deceased donation (DD), with annual numbers of 8 living and 4 deceased transplants in 2020, 12 living and 16 deceased in 2021, 12 living and 10 deceased in 2022, and 14 living and 6 deceased in 2023, respectively.

In 2024, 10 kidney transplants were performed, 6 living donor and 4 deceased donor (annual 5 pmp), a temporary decline attributed to the renovation of the Urology and Transplant Departments. Additionally, 2 pediatric kidney transplants were conducted, and 282 kidney transplant recipients are currently under follow-up. The country has also expanded to other organ transplants, including liver, heart, and bone tissue procedures.

Survey responses identified several challenges: limited infrastructure, resources, and specialized training, absence of a dedicated national transplant center, and insufficient funding to sustain long- term program growth. While HLA testing and biopsy-based pathology are available, flow cytometry crossmatch remains limited to selected cases. Finally, religious and cultural beliefs continue to impede deceased donation, underscoring the need for public education and awareness campaigns to expand the donor pool and strengthen national self-sufficiency in transplantation.

Serbia

> "*In addition to empowering national programs, fostering regional collaboration is crucial in improving transplantation services in the region.*" (Expert, Serbia)

Serbia, a country located in the central Balkans with a population of approximately 6.6 million, continues to face significant challenges in organ transplantation. It is estimated that around 700,000 people in Serbia are living with CKD. Among the 5,500 patients undergoing hemodialysis nationwide, including 40 children, only a small fraction are listed for transplantation. Despite 36 officially designated donor hospitals, only a few are functionally active, and just five centers currently perform kidney, liver, heart, and corneal transplants. The total number of transplantations remains low, averaging 17 procedures per year over the past 5 years, corresponding to a national rate of 2.5–3 PMP, one of the lowest in Europe. There are currently 832 patients in Serbia, living with a functioning kidney.

Although the Law on Transplantation of Human Organs allows both living and deceased donation, the system has been constrained by a legal vacuum following the 2021 Constitutional Court ruling that suspended the presumed consent provision (Article 23). As a result, Serbia currently operates under an explicit consent, opt-in model, requiring family authorization in all cases. Before 2010, nearly two-thirds of kidney transplants were from living donors. Since then, initiatives such as continuous brain-death monitoring have increased the number of deceased donors, but donation levels remain insufficient and inconsistent from year to year.

Despite the inclusion of paired donation in the 2018 by-law, the program has not yet been implemented, representing a missed opportunity to expand the donor pool. Persistent deficiencies in donor identification, hospital coordination, and public awareness and trust continue to limit deceased donation. Moreover, lung transplantation is not yet available in Serbia, and pediatric procedures often require the assistance of foreign surgical teams.

Strengthening training programs for transplant professionals, investing in public education, enhancing transparency and coordination, and expanding living donor options are essential steps to ensure equitable access to transplantation and to rebuild public confidence in the national system.

DISCUSSION

Our study found that transplantation programs in the region are small, some still in their infancy, while others despite having been established for decades remain underdeveloped. They are often characterized by low numbers, both in living and deceased donation. While all countries in the region, except Kosovo, currently perform living donor kidney transplants, activity levels remain low compared to Western Europe.

Living donation represents a significant untapped potential in the Western Balkans. European data show that living kidney donation increased modestly from 8.1 to 9.6 transplants pmp, between 2010 and 2018, though regional variation remains large, with the highest rates observed in Northern and Western Europe [12, 18]. Our findings show that in the Balkans, less than 22% of patients requiring kidney replacement therapy have received a transplant, compared to approximately 40% across other European countries [12, 19]. Surveys indicate that attitudes toward living donation in the Balkans are generally favorable, supported by strong family-oriented cultural values [20]. Moreover, given the relatively young average age of the Balkan population and the presence of established surgical and nephrology infrastructures, investing in living donor programs could significantly expand access [20]. Promoting living donation would not only strengthen national transplantation capacity but also increase the rate of preemptive kidney transplantation, aligning regional practice with European standards [13, 18, 21, 22].

Furthermore, establishing national and ultimately regional KPD programs would represent a major step toward expanding access to kidney transplantation in the Western Balkans. As shown by our findings, there are currently no active paired donation programs in the region, largely due to limited legislative frameworks, inadequate infrastructure, and insufficient resources. Even in countries such as Serbia and North Macedonia, where enabling legislation exists, KPD has not yet been implemented.

Across Europe, KPD has become a central strategy to increase LDKT rates, particularly for highly sensitized and immunologically incompatible pairs [21, 23]. Mature regional schemes, such as the Scandiatransplant STEP program, demonstrate the effectiveness of cross-matching across national boundaries, achieving higher transplantation rates and improved donor–recipient compatibility [12, 18]. Importantly, paired donation is more cost-effective than extensive desensitization protocols and avoids the clinical risks associated with highly mismatched transplants [19, 24]. Countries such as Turkey have demonstrated the feasibility and scalability of KPD even in resource-constrained settings, underscoring its relevance for the Western Balkans [12, 24, 25].

Despite progress in living donor transplantation, the absence of robust deceased donor programs remains the Achilles heel of transplantation development in the Western Balkans. Our findings revealed that deceased donation is currently performed only in North Macedonia, Serbia, and Bosnia and Herzegovina, while no active programs exist in Albania and Kosovo. In Montenegro, a deceased donor framework exists but remains largely inactive due to the very low number of donors. This stands in sharp contrast to most European countries, where deceased donation dominates the transplantation landscape [12, 26, 27].

Across Europe, recent data demonstrate a steady increase in deceased donation, including a rise in donation after circulatory death (DCD) alongside donation after brain death (DBD), driven by broader donor acceptance criteria and the widespread use of hypothermic machine perfusion [22, 23]. These advances have significantly improved graft viability and recipient outcomes [13, 28, 29]. In the latest report, the average DDKT rate rose from 21.6 to 25 pmp, between 2010 and 2018 [26]. In 2023, Council of Europe member states collectively reported 26,243 kidney transplants from 12,592 deceased donors, corresponding to a regional average of roughly 30 DDKT pmp, with leading countries such as Spain (~60 pmp) and Croatia (~45 pmp) among the highest performers [13, 23, 30].

However, in the Western Balkans, deceased donation remains severely limited, with an average pmp of 1–5, mainly due to the lack of trained procurement teams, insufficient infrastructure, logistics, and organ preservation technologies. Even in countries where DDKT is performed, activity levels remain far below the European average, reflecting persistent barriers such as organ shortages in North Macedonia and Serbia, and restrictive legislation in Bosnia and Herzegovina [31]. Strengthening the training of procurement professionals, supported by regional collaboration and technical assistance, is therefore essential to overcome this bottleneck and build sustainable deceased donor programs in the region.

Building on these findings, our survey identified four principal barriers currently hindering the expansion and consolidation of kidney transplantation programs in the Western Balkans.

Formal Training and Capacity Building

Kidney transplantation training is theoretically included in both nephrology and surgical residency programs across the Western Balkans. However, the development of clinical competency is severely constrained by low procedural volume. With fewer than 25 kidney transplants performed annually in most countries, residents typically graduate having assisted with significantly fewer cases than international standards for competency, outlined by the European Union of Medical

Specialists (UEMS), Division of Transplant Surgery. European Training Requirements (ETR) for Transplant Surgery [32, 33]. This low-volume exposure particularly affects surgeons and anesthesiologists, whose procedural expertise relies heavily on repetition and hands-on experience [27, 34–38]. While short-term international fellowships and online educational resources exist for transplant nephrology, they remain insufficient to build the specialized, team-based skills required to manage a high-volume program. Currently, no formal or accredited pathways for transplantation training exist in the region. This lack of specialized training has even contributed to the closure of certain centers, such as Banja Luka (Bosnia and Herzegovina).

During the May 2025 Meeting, training and capacity building were identified as priority areas. In response, ESOT experts expressed commitment to supporting the development of structured regional training programs, adapted to each country's specific needs and current capacity level.

Strengthening Immunological Infrastructure and Access to Modern Therapies

Our survey identified significant limitations in immunological capacity across the Western Balkans. While most centers are equipped to perform basic immunological investigations, more advanced assays, are outsourced abroad due to the lack of specialized laboratories and trained staff.

Given the small population size and limited number of transplant centers in each country, the establishment of a centralized regional immunological reference laboratory serving all Western Balkan countries would be highly beneficial. Comparable models have been successfully implemented elsewhere in Europe [28]. The Eurotransplant Reference Laboratory (ETRL) functions as a quality assurance and coordination hub for 44 tissue-typing laboratories across eight member states [39]. A similar regional HLA immunology center could provide advanced immunological testing, quality assurance and training for local laboratories, centralized crossmatching and antibody analysis protocols, and support for a future regional PKD and acceptable mismatch programs [29, 40].

In addition, participants emphasized the importance of introducing new therapeutic agents for the management of rejection, infectious viral complications, sensitized recipients and new biomarkers [41, 42]. Wider access to novel immunosuppressive drugs and biologics, was identified as a regional priority to align treatment standards with those applied in leading European centers [13, 32, 33].

Developing Effective Coordination and Governance in Transplantation

Our survey revealed that Albania, Bosnia and Herzegovina, Montenegro and Kosovo, lack a centralized coordination mechanism and dedicated transplant coordinators, leading to fragmented communication between institutions, suboptimal donor identification, and prolonged waiting times for transplant candidates. Although North Macedonia and Serbia have established national coordination structures, both require more sustainable training and improved operational frameworks to function effectively.

Strengthening coordination at both national and inter-hospital levels is therefore a critical priority. The Croatian model provides a well-documented example of success. Following the introduction of a fully integrated national coordination system, including a central registry, standardized donor management protocols, and dedicated transplant coordinators, Croatia's deceased donor rate rose sharply to 33–36 per million population (pmp), surpassing most EU countries [43–45]. This transformation established Croatia among Europe's top-performing countries in organ donation and transplantation [46].

Adopting similar frameworks across the Western Balkans could optimize donor identification, ensure equitable organ allocation, and promote transparency and accountability at both the national and regional levels. Such systems would also provide a foundation for future cross-border collaboration and the gradual harmonization of donation and transplantation practices within the region.

Addressing Legislative and Cultural Barriers to Transplantation

Our findings reveal a fragmented legislative landscape that continues to constrain kidney transplantation across the Western Balkans. Kosovo currently lacks comprehensive transplantation legislation, with only a draft law under development, while Albania has no specific framework regulating deceased organ donation. The remaining four countries, Serbia, North Macedonia, Montenegro, and Bosnia and Herzegovina have enacted legislation permitting both living and deceased donation, yet implementation remains inconsistent and largely inadequate. Although paired donation is legally permitted in some countries, no national program has yet been established in the region. In contrast, EU member states with robust legislative frameworks, demonstrate how clear legal definitions of consent systems, centralized coordination, and continuous professional training can yield deceased donation rates exceeding 35–60 pmp, compared to approximately 1 – 5 pmp in most Western Balkan countries [13, 26, 43, 47]. Beyond legislative gaps, significant cultural and religious beliefs restrict deceased donation, particularly where public awareness remains low and mistrust toward healthcare institutions persists. Effective reform should therefore combine legislative modernization, with the establishment of active patient organizations emphasizing the advantages of kidney transplantation within the ESKD patient community, as well as participating in broad education and public awareness campaigns involving religious leaders, media, stakeholders,

policymakers and civil society organizations to build trust and promote organ donation [48, 49].

Strengthening Regional Integration in Transplantation

During the May 2025 Meeting, participants emphasized the need to strengthen regional collaboration in kidney transplantation, given the low procedural volumes and shared healthcare challenges across the Western Balkans. Establishing a regional network to facilitate experience exchange, collaboration on complex cases, and organize short-term, hands-on training programs was identified as a critical first step toward expanding national capacities [50, 51]. A successful precedent exists in North Macedonia, where transplantation activity increased following integration into the Southeastern Europe Health Network (SEEHN) and the Regional Health Development Center on Organ Donation and Transplant Medicine (RHDC). Building on this model, participants proposed annual regional meetings to review progress, harmonize practices, and address program-specific challenges [16, 52]. In the long term, implementing standardized clinical protocols and a regional KPD program, modeled after the Turkish and Scandiatransplant (STEP) systems, could significantly expand the donor pool and improve compatibility for highly sensitized patients [13, 31, 53]. In light of the current transplantation landscape across the Western Balkans, consistent with the findings of our survey, ESOT in collaboration with the WHO, launched in September 2025 an Operational and Technical Guidance for Developing a Transplant Programme in the Balkans. This comprehensive framework provides a roadmap for establishing larger, more efficient, and self- sustaining transplantation programs in the region [54].

CONCLUSION

This project represents the first comprehensive collection of real-world data and expert insights on the major challenges facing kidney transplantation in the Western Balkans. During this process, certain obstacles were identified toward a solid and sustainable kidney transplant program in the region. Fortunately, all of them can be overcome, especially in the context of EU membership to which all of Western Balkans avidly aspire.

We hope this initiative further solidified by the inspiring meeting in Tirana in May 2025, can serve as a steppingstone for regional progress guiding the development of realistic and practical solutions to address identified barriers and foster long-term growth and sustainability of transplantation services throughout the region. It is our mission to offer all our patients timely and equitable access to state- of-the-art transplant procedures in the Western Balkans. It will not be easy, but it will take more than that to stop us.

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: ER; data curation: ER, GS, DRe, ML, DRa, VG, AS, and AI; formal analysis: ER, KS, and JS; project administration: ER and DM; supervision: EA, DM, LP, and EC; writing – original draft: ER, KS, GS, DRe, ML, DRa,VG, AS, and AI; writing – review and editing: FO, CL, JS, JA, and EC. All authors contributed to the article and approved the submitted version.

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The author(s) declared that this work 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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The author(s) declared that generative AI was not used in the creation of this manuscript.

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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.2026.15952/full#supplementary-material

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SYSTEMATIC REVIEW AND META-ANALYSIS
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Impact of Vascular Anastomosis Time on Kidney Transplant Outcomes – A Systematic Review

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Anastomotic time (AT), also termed second warm ischaemic time (SWIT), is a potentially important intraoperative factor in kidney transplantation, yet its impact on outcomes has not been systematically synthesised. We conducted a systematic review to examine the association between AT and delayed graft function (DGF), graft survival, and patient survival. Cochrane, Embase, and Medline were searched to 21 July 2025. Nine retrospective cohort studies comprising 155,523 transplants were included. Across all donor types, longer AT was consistently associated with higher rates of DGF within individual studies. Several studies also reported poorer 1- and 5-year graft survival with prolonged AT, while findings for patient survival were equivocal. However, substantial heterogeneity across studies, including donor type, AT definitions, outcome reporting, and incomplete adjustment for key confounders, precluded formal meta-analysis. None of the included studies consistently adjusted for major determinants of graft outcomes. These findings suggest a potential link between prolonged AT and adverse graft outcomes, but high-quality prospective studies with standardised reporting and confounder adjustment are required before AT can be considered an independent determinant of transplant outcomes.

Systematic Review Registration: https://www.crd.york.ac.uk/prospero/, identifier PROSPERO CRD42024549222.

Keywords: anastomosis, second warm ischaemic time, surgical, surgical anastomosis, transplant

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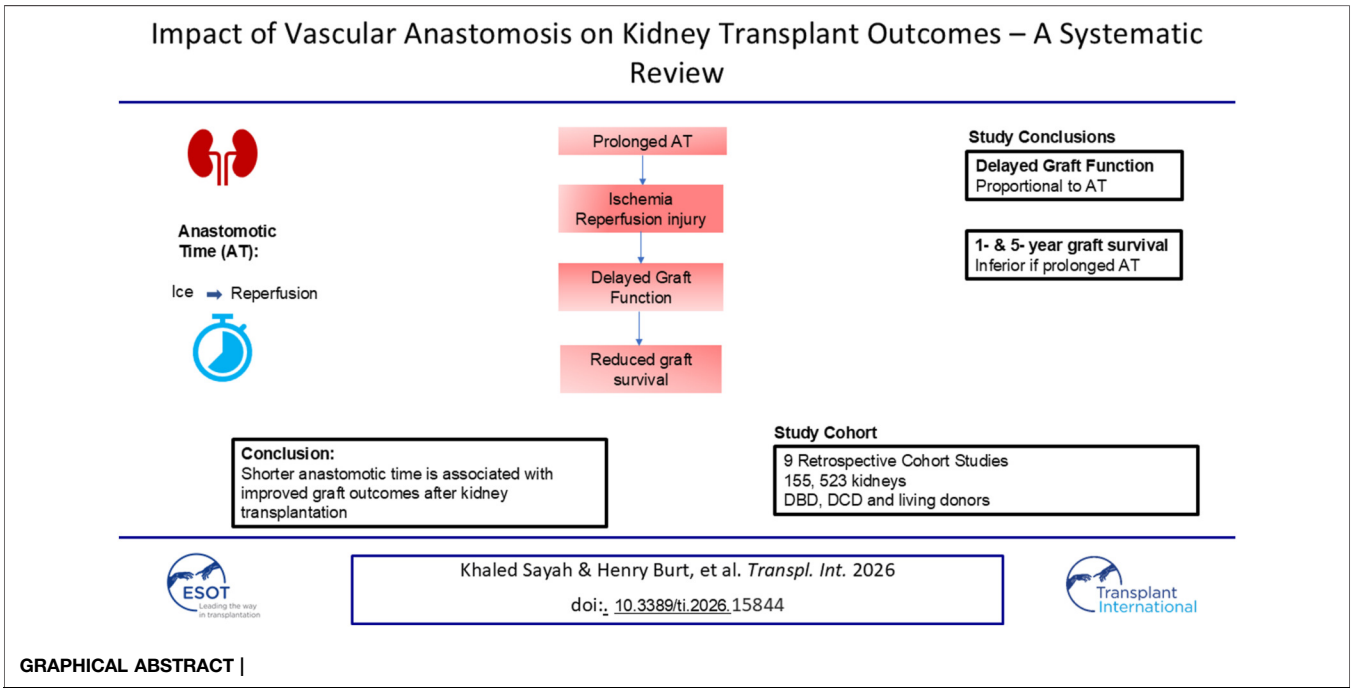
Abbreviations: AT or SWIT, Anastomosis time or second warm ischemic time; CIT, Cold ischemic time; CI, confidence interval; DBD, donation after brain death; DCD, donation after cardiac death; DGF, Delayed graft function; DWIT or FWIT, Donor warm ischemic time or first warm ischemic time; EGF, Early graft function; ESKD, End stage kidney disease; HR, Hazard ratio; IFTA, Interstitial fibrosis and tubular atrophy; IGF, Immediate graft function; NR, Not reported; OR, odds ratio; RR, Relative risk; SD, Standard deviation; SGF, Slow graft function; SWIM, systematic review without meta-analysis; TSANZ, Transplantation Society of Australia and New Zealand; Tx, Transplant outcomes.



GRAPHICAL ABSTRACT |

INTRODUCTION

Kidney transplantation is the gold standard treatment for end-stage kidney disease (ESKD), offering substantial improvements in both survival and quality of life compared with dialysis [1, 2]. However, the burden of ESKD continues to grow. In the United States alone, more than 808,000 people are living with ESKD [3]. The transplant waitlist continues to expand, with 90,323 patients listed in 2024, underscoring the critical importance of optimising graft outcomes and extending the longevity of transplanted kidneys [4–6].

Outcomes after transplantation are influenced by multiple donor and recipient factors, including age, donor pathway, HLA matching, sensitisation, and organ quality [7, 8]. Among perioperative factors, cold ischaemic time (CIT) is well established as a determinant of graft function and survival [9–12].

Vascular anastomosis time (AT), also referred to as the second warm ischaemic time (SWIT), has similarly been assumed to exert an influence, but has been comparatively under-investigated, and represents the interval from removal of the kidney from ice, until reperfusion in the recipient. It has been acknowledged as a modifiable risk factor, with prolongation of this interval contributing to ischemia-reperfusion injury that drives inflammatory cascades, interstitial fibrosis and tubular atrophy (IFTA), and ultimately reduces graft viability [8, 13–18]. The clinical manifestation of this injury is delayed graft function (DGF), often requiring dialysis within the first seven days after transplantation [13–15]. Beyond early graft dysfunction, longer SWIT are also associated with longer hospital stays, increased use of renal replacement therapy, and greater reliance on diagnostic resources such as imaging and biopsy, all of which compound the burden on patients and health systems [14].

The first study to explicitly link SWIT to kidney transplant outcomes was published as recently as 2015 [8]. Since then, only a limited number of retrospective cohorts have examined this association, and no systematic review has yet synthesised the available evidence despite the critical role SWIT has on outcomes. As SWIT is a modifiable surgical factor, clarifying its effect is of direct clinical relevance. To address this, we conducted a systematic review to evaluate its association with delayed graft function, one and five-year graft survival, and patient survival in adult kidney transplant recipients.

MATERIALS AND METHODS

The systematic review was conducted in adherence with the Preferred Items for Systematic Reviews (PRISMA) checklist and was registered with PROSPERO (CRD42024549222) in 2024 [16, 17].

Eligibility Criteria

Articles included in the systematic review were required to have been published between January 2000 – July 2025. AT (SWIT) was required to be reported with a clear description of the term, as well as type of donor graft (DCD, DBD, living donor) used in the study. Studies were required to have a sample size greater than 100, with adult recipients between 18 years or above. Transplant characteristics assessed included DGF, 1- and 5-year graft survival and patient survival. Review articles, articles with majority paediatric or elderly transplants, *en bloc* kidney

TABLE 1 | Baseline study characteristics.

Author and Year	Study type	Population and donor types	Recipient BMI (mean, SD) kg/m^2	Recipient Age (mean, SD) years	Donor BMI (Mean, SD) kg/m^2	Donor age (mean, SD) years	Cause of death	Donor Serum creatinine (mean, SD) μmoL/L	Serum Urea (μmoL/L)	eGFR (mL/min/ 1.73 m^2)	Cold ischemic time (mean, SD) hours
Heylen [19]	Retrospective cohort study	13,964 (DBD (12, 806), DCD (1,158))	25 ± 0.76	54.99 ± 2.6	25 ± 2	53 ± 2.6	NR	75.14 ± 2.3	NR	NR	13.7 ± 0.9
Weissenbacher [20]	Retrospective cohort study	1,245 DBD	23.67 ± 3.74	51.02	24.81 ± 3.52 (mean)	45 ± 3	DBD: Cerebrovascular accident (587), trauma (358), other (308)	83.98	2,939	NR	14.53 ± 5.69
Marzouk [14]	Retrospective cohort study	298 (DBD (n = 282), DCD (n = 16))	78 ± 17	51 ± 13	27 ± 6 (mean)	47 ± 17	NR	67 ± 31	NR	NR	12 ± 1.3
Kukla [21]	Retrospective cohort study	554 (DBD, DCD (breakdown not available))	24.4 ± 0.09	47.6 ± 4.2	25.5 ± 0.09	42.8 ± 0.37	CVD: 24.8 (23.5–26.2), trauma 24.6 (23.4–25.7), other causes 27.8 (24.8–30.8)	NR	NR	NR	18.9 ± 0.1
Tennankore [22]	Retrospective cohort study	131, 677 (living donor (50, 587), DBD (81,120), DCD (9199)	NR	50 ± 14	NR	39 ± 16	DBD: (Anoxia: 16, 447), (CVA: 29,375), (Head trauma: 32, 754), (other: 2,514). DCD: 9,199	NR	NR	NR	11 ± 2
Mahajan [23]	Retrospective cohort study	247 (DBD, DCD (breakdown not available))	NR	53 ± 3.5	NR	51.8 ± 4.4	NR	DGF: 78 ± 12; (–) DGF: 69.3 ± 6.1	NR	1-month eGFR 36.8 ± 3.6; 1-year eGFR 40 ± 3	DGF: 840 ± 63 min; No DGF: 824 ± 59 min
Heylen [8]	Retrospective cohort study	669 (DBD)	25 ± 5	55 ± 13	NR	48 ± 15	DBD	63 ± 23	NR	3-month eGFR 47 ± 17 (n = 646); 1-year eGFR 52 ± 18 (n = 598); 2-year eGFR 52 ± 18 (n = 512); 3-year eGFR 51 ± 20 (n = 373)	15 ± 4
Cron [24]	Retrospective cohort study	6, 397 (DCD)	28 ± 1.04	55.2 ± 2.4	26.8 ± 1.1	38.9 ± 3.2	Anoxia (n = 2,724. 42.58), CVA (n = 1,046. 16.35), other/unknown (n = 313. 4.89), trauma (n = 2,314. 36.17)	70.4 ± 5.5	NR	NR	17.6 ± 1.2; adjusted odds ratio (1.02) per 1 h
Hellegering [25]	Retrospective cohort study	472 (living donor)	24	44.8	25.5	50.1	N/A	NR	2 weeks: 157, 1 month: 137, 1 year:128	NR	NR

TABLE 2 | Comparing shorter to longer anastomosis times.

Study	Short anastomosis time (30–35 min)				Long anastomosis time (>35 min)			
	AT (minutes)	Number of participants	5-year survival [95% CI]	DGF	AT	Number of participants	5-year survival [95% CI]	DGF
Heylen [19]	<35	6,083	82%	NR	Q2 = 35–44 min Q3 = 45–54 min Q4 = ≥55 min	Q2 = 4,008 Q3 = 2050 Q4 = 1823	Q2 = 81% Q3 = 78% Q4 = 75%	NR
Weissenbacher [20]	<30	NR	80.60%	NR	>30 min	NR	NR	NR
Heylen [8]	34 IQR (30–40)	659	NR	17%	NR	NR	NR	NR
Cron [24]	≤30	1731	88.20%	36.70%	Q2 = 31–38 min Q3 = 39–47 min Q4 ≥ 48 min	Q2 = 1,506 Q3 = 1,584 Q4 = 1,576	Q4 = 84.8%	Q2 = 35.2% Q3 = 40.6% Q4 = 44.0%
Marzouk [14]	30 IQR (24–45)	311	NR	18%	NR	NR	NR	NR
Kukla [21]	25.2 IQR (24.3–26.1)	555	NR	29.2%	NR	NR	NR	NR
Mahajan [23]	NR	NR	NR	NR	43 IQR (35–48)	n = 247	NR	43.3%
Tennankore [22]	Q1 = <10 Q2 = 10 - <20 Q3 = 20 - <30	Q1 = 13,456 Q2 = 3,715 Q3 = 21,627	Q1 = 78% [77%–79%] Q2 = 80% [78%–81%] Q3 = 78% [78%–79%]	NR	Q4 = 30 - <40 Q5 = 40 - <50 Q6 = 50 - <60 Q7 ≥ 60	Q3 = 38,403 Q4 = 27,058 Q5 = 10,818 Q6 = 16,600	Q4 = 75% [75%–76%] Q5 = 74% [73%–75%] Q6 = 73% [72%–74%]	NR
Hellegering [25]	29.8 min	n = 477	NR	4.40%	NR	NR	NR	NR

TABLE 3 | Comparing short (<30 min) to longer AT (>30–35 min).

Donor Type	Study	Number of participants	DGF (%)		1-year graft survival (%)		5-year graft survival (%)		1-year patient survival (%)		5-year patient survival (%)	
			Short AT	Long AT	Short AT	Long AT	Short AT	Long AT	Short AT	Long AT	Short AT	Long AT
DBD	Heylen [8]	669	17		95		85					
	Weissenbacher [20]	1,245	33.1		93	90	80.6	76.6			89.6	85.7
	Kukla [21]	554	29.2									
DCD	Cron [24]	6,397	36	42.3	96.7	94.7	89.1	85.9	96.7	96.2	87.8	87.6
DCD + DBD	Heylen [19]	13,964			88	86	82	79.5				
	Marzouk [14]	298	18.8									
	Mahajan [23]	279		43.3								
Living + deceased	Tennankore [22]	131,677			94	91.8	78.5	74.7				
Living only	Hellegering [25]	477	4.4%									

transplants, multi-organ transplants, or animal studies were excluded from the systematic review.

Literature Search and Study Selection

Supplementary Table 1 outlines the literature search strategy, developed using a combination of key words and MeSH terms. The following three databases were searched simultaneously via OVID: Cochrane databases for systematic reviews, Embase, and Medline. The final search result was performed on 21st July 2025. Results from the database search was uploaded to COVIDENCE for article screening, as outlined in **Supplementary Table 1**. Each included study was screened by at least two independent reviewers (KS, HB, YZ, LL), with any conflicts mediated by a third reviewer (HP).

Data Collection

Information from each study was extracted and collated in a standardized table. The following information was documented from each article: Author(s) and study year; type of study; donor type; number of patients in the study; anastomosis time; delayed graft function; 1-year and 5-year graft survival; 1-year and 5-year patient survival. Baseline study characteristics included were Recipient BMI, Recipient Age, Donor BMI, Donor Age, Cause of death (if applicable),

TABLE 4 | Newcastle-Ottawa assessment.

Newcastle-Ottowa clinical assessment									
References	**Representation of exposed cohort**	**Selection**		**Comparability**		**Outcome/Exposure**		**Adequecy of follow-up cohorts**	**Total score**
		Selection of non-exposed cohort	**Ascertainment of exposure**	**Demonstration that the outcome was not present at the start of the study**	**Comparability of cohort on the basis of the design or analysis controlled for confounders**	**Assessment of the outcome**	**Follow-up time was appropriate for outcomes to occur**		
Heylen [19]	1	1	1	1	2	1	1	0	8 good quality
Weissenbacher [20]	1	1	1	1	2	1	1	1	9 good quality
Marzouk [14]	1	1	1	1	2	0	0	0	6 fair quality
Kukla [21]	1	1	1	1	2	1	1	1	9 good quality
Tennankore [22]	1	1	1	1	2	1	0	1	7 good quality
Mahajan [23]	1	1	1	1	2	0	1	0	7 good quality
Heylen [8]	1	1	1	1	2	1	1	0	8 good quality
Hellegering [25]	1	1	1	1	2	1	1	0	8 good quality
Cron [24]	1	1	1	1	2	1	1	0	8 good quality

Donor serum creatinine, Serum urea, eGFR, and Cold Ischemic Time. This information is outlined on **Tables 1**, **2**, **3**. Data extraction was collected individually, then cross-checked by two independent reviewers before being documented on a joint spreadsheet. The terms SWIT and AT are used interchangeably throughout this manuscript.

Formulation of Results

Given substantial heterogenicity across included studies, including differences in donor type, AT definitions, comparator thresholds, missing covariate reporting, surgical complexity and inconsistent outcome stratification, formal meta-analysis was not undertaken following statistical consultation. Quantitative pooling of outcomes across studies was not performed, as doing so would risk biasing data or producing misleading estimates from clinically and methodologically incomparable data. Simple pooling approaches such as weighted arithmetic means were considered inappropriate for this dataset. Instead, a structured narrative synthesis was conducted. Outcomes were summarised within each study, and patterns of associated trends between AT and transplant outcomes were described across donor types. Studies were grouped by donor type (DBD, DCD, living donor and mixed cohorts) and by AT threshold where applicable. Direction and consistency of reported effects were assessed descriptively for delayed graft function, graft survival and patient survival.

Risk of Bias Assessment

Retrospective cohort studies were assessed for risk of bias using the Newcastle-Ottawa scale (**Table 4**) [18]. This tool evaluates bias across multiple domains, including the representation of exposed cohort, the selection of a non-exposed cohort, ascertainment of exposure, demonstration that the outcome was not present at the initial investigation of the study, comparability of included cohorts, and assessment of the outcome with adequate follow-up time [18].

Certainty of Evidence

For each comparison, certainty of evidence was assessed using the Grading of Recommendations Assessment, Development and Evaluation (GRADE) framework [26]. Outcome chosen for certainty assessments included Delayed Graft function, 1 and 5-year Graft Survival, and 1- and 5- year Patient Survival [26]. All included studies were retrospective observational cohorts and were therefore initially rated as low-certainty evidence. Longer anastomosis time was consistently associated with higher rates of delayed graft function and poorer graft survival within individual studies, but findings for patient survival were inconsistent. Certainty was downgraded due to residual confounding, heterogenicity in anastomosis time thresholds, and incomplete adjustment for key covariates, including cold ischaemic time, graft type and surgical complexity. Overall, the certainty of evidence across outcomes was rated as low to moderate, reflecting the observational design, heterogenicity and high likelihood of residual confounding. No pooled quantitative effect estimates were generated.

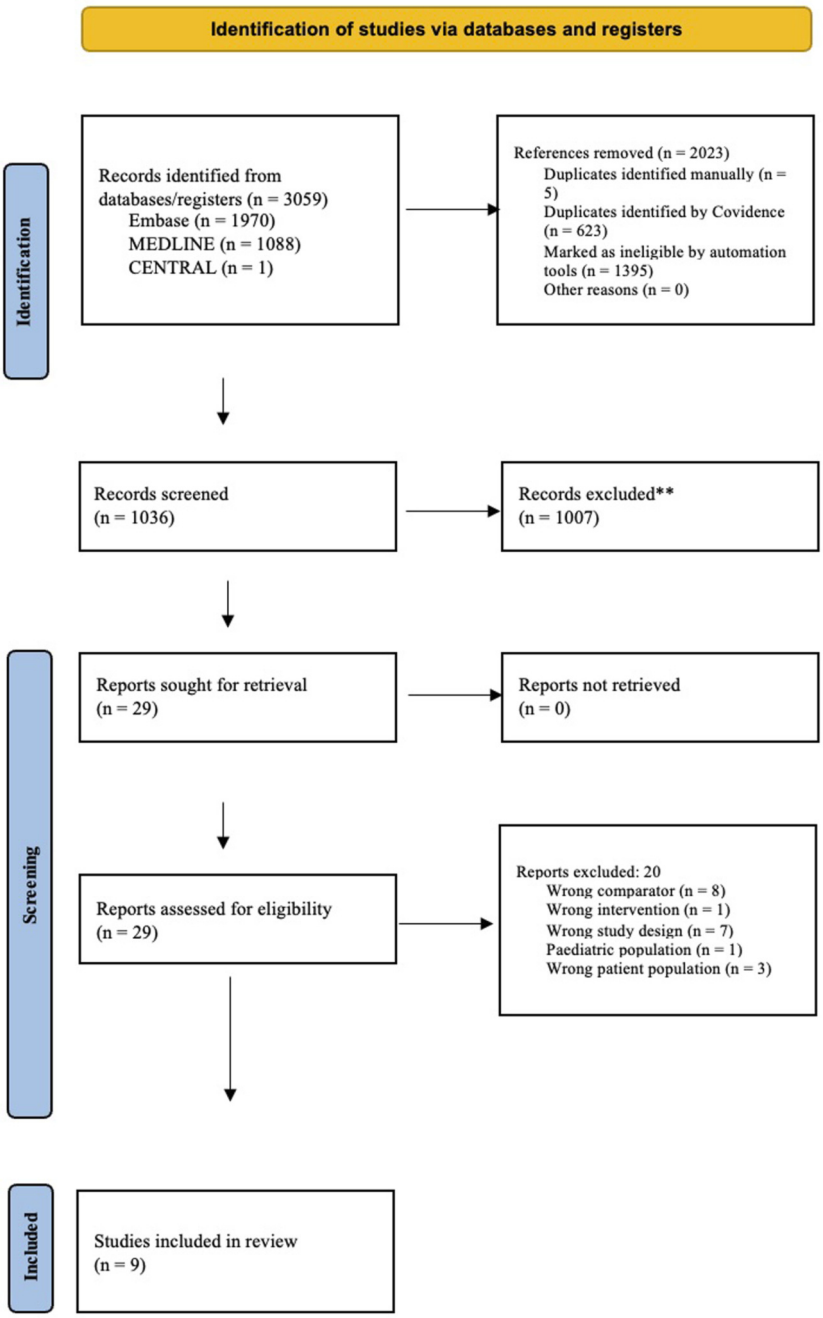


FIGURE 1 | PRISMA flowchart for literature assessment.

RESULTS

A total of 9 retrospective cohort studies were included in the systematic review, with a total combined data spread of 155, 523 patients across two continents of North America and Europe. The PRISMA flow chart is outlined in **Figure 1**.

Population Characteristics

Across the 9 retrospective cohort studies, population characteristics were sectioned into recipient and donor patient characteristics. Of the recipient population, reported BMI ranged from 23.7 kg/m^2 to 28.0 kg/m^2, and the median age was 51.2 IQR (48.8–55.0). With respect to the donor population, the median

TABLE 5 | Grading of recommendations, assessment, development and evaluations (GRADE).

Outcome type	No. of studies	Risk of bias/Quality of evidence	Consistency	Directness	Precision	Publication bias	Overall effect size estimate (95% CI)	Quality of evidence
Delayed graft function (DGF)	7 (7 cohort)	Low risk of bias; observational evidence	Minimal inconsistency	Direct (0)	Narrow CI; large sample size; high precision	No important publication bias	1.10 per 10 min (1.06–1.14)	Moderate
1-year Patient Survival	3 (3 cohort)	Low risk of bias; observational evidence, however	Minimal inconsistency	Direct (0)	Narrow CI; large sample size; high precision	No evident publication bias	1.021 per minute (1.006–1.037)	Moderate
5-year Patient Survival	4 (4 cohort)	Low risk of bias; observational study	Some inconsistency	Direct (0)	Narrow CI; large sample size	No evident publication bias	3.5 (1.6–7.3)	Moderate
5-year Graft Survival	4 (4 cohort)	Low risk of bias; observational study	Minimal inconsistency	Direct (0)	Narrow CI and large sample size	No evident publication bias	1.23 (1.15–1.33)	Moderate

age was 47 IQR (40.9–70.0). Donor types were recorded as living donors (n = 51, 059), donation after circulatory death (n = 16, 770), and donation after brain death (n = 96, 122). For the deceased donor population, the cause of death was included if available in the retrospective cohort study.

Study Characteristics

Three studies used a mixed kidney donor population of deceased donors consisting of both donation after circulatory death (DCD) and donation after brain death (DBD) [14, 19, 23]. Three studies used only DBD donors [8, 20, 21], one study limited the donor type to DCD [24], one utilized living donors [25], and the last study used a mix of both living and deceased donors [22].

Evidence and Reporting Bias

A summary of the Newcastle-Ottawa risk of bias assessment is provided in **Table 3**. All studies included provided a clear description of the donor type used in their respective studies and specified the AT intervention and comparator used in their analysis. Some studies failed to provide an adequate assessment of outcome and did not provide an adequate follow-up time for long term graft outcomes to be assessed. Eight studies included maintained a good quality score ranging from 7 to 9. One of the studies included was determined as Fair quality with a rating of 6 due to the above rationale.

Overall quality of evidence was summarized in accordance with the GRADE evidence profile on **Table 5** [26]. The quality of evidence was moderate for all measurable outcomes investigated. Study evidence was reduced due to all the studies included being retrospective cohort studies, and the different donor types used to assess each outcome.

Anastomosis Time Across Studies

Across the nine included studies, definitions of "short" and "long" AT varied, with thresholds ranging from <30 min to <35 min for shorter AT, and >30 min to >55 min for prolonged AT [8, 14, 21, 23–25]. Several studies reported AT quartiles rather than binary cut-offs [19, 22, 24]. In large registry-based cohorts, only a minority of recipients fell within the shortest AT category [22, 24]. Small, single centre studies reported median AT values ranging from 25 to 45 min, with wide interquartile ranges [8, 21, 23]. These findings suggest that achieving and AT <30 min is feasible in selected cases, particularly living donor transplantation, but may be uncommon in complex, deceased donor scenarios.

Anastomosis Time and Delayed Graft Function

Seven studies analysed the relationship between vascular AT and the incidence of DGF in kidney transplantation [8, 14, 21, 23–25]. Across all donor types, longer AT were associated with higher rates of DGF (**Table 2**). In one study examining DBD recipients who developed DGF had significantly longer median AT compared to those without [8]. In another study examining DCD recipients, a similar effect was noted, with higher rates of DGF across increasing AT quartiles [24]. These directional associations were observed in mixed donor cohorts [19, 23], and in living donor cohorts [25], although effect sizes varied across studies. Due to heterogenicity in AT thresholds, donor populations, and outcome definitions, no pooled estimates of DGF risk were calculated.

Anastomosis Time and Graft Survival

Five studies analysed the effect of AT on 1 and 5-year graft survival, with all studies reporting statistically significant findings, consistently demonstrating that graft survival at both time points was superior in cohorts with shorter AT [8, 19, 20, 22, 24] (**Table 2**). One study demonstrated a graded decline in 5-year graft survival across increasing AT quartiles [19]. One study reported inferior graft survival beyond an AT threshold of 30 min [20]. One study observed lower graft survival with prolonged AT in DCD transplantation [24]. Given differences in study design, donor populations, AT categorisations and outcome reporting, pooled graft survival estimates were not generated.

Anastomosis Time and Patient Survival

One study with the predetermined criteria reported data on 1-year patient survival [8, 20, 24], and four reported a 5-year patient survival [8, 20, 22, 24]. For patient survival, no study found an effect of AT at 1 year, and results at 5 years were mixed. Some cohorts reported poorer survival with prolonged AT, while others showed no independent association. Taken together, the evidence

suggests AT primarily influences graft-level outcomes, while its impact on patient-level survival remains uncertain [8, 20, 22, 24].

DISCUSSION

This review is, to our knowledge, the first systematic synthesis of vascular AT and kidney transplant outcomes across donor types. In the studies involved, prolonged AT was associated with higher rates of DGF across all donor types, and as expected the lowest incidence was seen in living donor grafts, intermediate rates in donation after brain death, and the highest rates in donation after circulatory death. In the studies involved, shorter AT, particularly under 30 min, were associated with superior graft survival, whereas both one-year and five-year graft survival declined once this interval was exceeded. Impacts on patient survival were less clear.

These findings support the interpretation that AT, DGF, and graft survival are best understood as a connected pathway rather than independent outcomes. Prolonged AT consistently increased the risk of DGF, and poorer long-term graft survival was observed in the same cohorts. Because DGF itself is a well-recognised predictor of graft loss, it is plausible that the adverse impact of prolonged AT on survival is mediated in part through its effect on early graft function. This biological plausibility is consistent with the known mechanisms of ischemia-reperfusion injury and lends coherence to the observed clinical outcomes [15].

The modifiability of vascular AT highlights its potential as a risk factor that could be improved through revised surgical techniques and guidelines. Current clinical guidelines from the Transplantation Society of Australia and New Zealand (TSANZ) do not specify an optimal vascular AT but emphasize the importance of minimising cold ischemic time [27]. Additionally, the guidelines highlight strong evidence that a short ischemic time may improve transplant outcomes, recommending that kidneys be transplanted as quickly as possible to mitigate prolonged cold ischemia [27]. However, various factors influencing vascular AT must be considered when promoting speed and precision. These include anatomical complexities (e.g., multiple renal arteries/veins, right-sided kidney grafts), recipient and donor characteristics (BMI, depth, and Age) [8, 28–30]. This emphasizes the need to recognize AT as a modifiable risk factor and incorporate its optimization into surgical planning within the transplant community and has impacts on transplant surgical training, as well as the introduction of robotic kidney transplantation more broadly.

Importantly, AT is not solely a function of surgical technique or efficiency, but also reflects surgical complexity. Prolonged AT commonly occurs in technically challenging scenarios, including ipsilateral re-transplantation, difficult iliac vessel exposure, high recipient BMI, deep iliac fossae, and the presence of calcified vessels, to name but a few [31]. In such cases, prolonged AT may be unavoidable and appropriate to ensure technical precision and haemostatic security. Accordingly, AT may act a s a surrogate marker of procedural complexity rather than an independent causal factor of poor transplant outcomes.

Cold ischaemic time is a well-established independent predictor of graft outcomes and is biologically distinct from AT [9, 11]. While AT and CIT are temporally separate, they are likely biologically synergistic. Prolonged AT may be particularly injurious in kidneys already exposed to extended cold storage, as warm re-ischemia following prolonged hypothermia may amplify ischemia reperfusion injury. This potential interaction, however, is beyond the scope of this review but remains a hypothetical, though clinically plausible mechanism, warranting prospective evaluation.

The feasibility of achieving AT below 30 min also warrants consideration. Registry based data suggests that a substantial proportion of deceased donor transplants exceed this threshold, particularly in DCD cohorts [8, 19, 20]. Short AT is most achievable in living donor cohorts, and straightforward deceased donor cases. Thresholds used across studies in this review, were arbitrary and heterogenous, and no evidence-based cut-off for “safe” AT currently exists.

These findings support viewing AT as a modifiable intra-operative factor with meaningful consequences for both early graft function and long-term graft durability. Minimisation can be encouraged through thorough preparation at the pre-operative briefing, with clear allocation of roles, readiness of instruments, and vascular exposure achieved before removal of the kidney from ice. Further, recording AT as a routine peri-operative quality measure would allow teams to monitor performance and provide constructive feedback. Workflow can also be streamlined, for example, by using a two-surgeon approach where possible and by preparing sutures or clamps in advance so that periods of non-productive time are reduced. An alternative approach would be to incorporate active methods of insulating and/or cooling the graft during anastomoses, to ameliorate the negative impacts of rapid graft rewarming and warm ischemia during this interval.

Several limitations must be recognised in this review, however. Important covariates such as cold ischaemic time, first warm ischemia, multiplicity of vessels, side of graft, recipient body mass index, machine perfusion, and centre or era effects were not consistently adjusted for. We also could not conduct formal meta-analyses and sensitivity analyses given significant differences in donor populations between different studies, variable use and reporting of technologies such as machine perfusion, and variable/inconsistent reporting of all relevant study outcomes stratified by AT thresholds and donor types.

Despite these limitations, the review has notable strengths. It is, to our knowledge, the first systematic review to focus specifically on vascular AT in kidney transplantation. Large and contemporary cohorts were included, stratified by donor type, and the overall risk of bias was low, with eight of the nine studies assessed as good quality. Taken together, this synthesis provides a structured overview of the available evidence and

highlights areas where prospective research with standardised definitions and reporting would add the most value.

CONCLUSION

This systematic review identified studies that found an inverse relationship between AT and graft survival. A shorter AT was associated with a superior immediate graft function, and 1-year and 5-year graft survival, across all types of donor recipients. Currently, there are no guidelines that define the significance of maintaining a short AT for optimal graft function and survival. This systematic review aims to inform the transplant community on the importance of maintaining a short AT, where possible, to provide the most optimal outcome post-operatively.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethical approval was not required for the study involving humans in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

KS, HB, and YZ were responsible for article screening and inclusion, with any conflicts resolved by a HP. AH provided statistical support. KS, HB, YZ, and LL were responsible for writing of the manuscript, with revision by TL, LY, CN, JY, WL, GW, AH, and HP. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

HP and AH would like to declare a conflict of interest in that they are shareholders in iiShield Ltd, a start-up company aiming to design an insulating jacket to be utilised during kidney transplantation.

The remaining author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

GENERATIVE AI STATEMENT

The author(s) declared that generative AI was not used in the creation of this manuscript.

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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.2026.15844/full#supplementary-material

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Longitudinal Monitoring of Donor-Derived Cell-Free DNA Supports Risk Stratification in Kidney Transplant Recipients With Allograft Dysfunction

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The prognostic value of donor-derived cell-free DNA (dd-cfDNA) for long-term kidney allograft outcomes after indication biopsy remains incompletely defined. In this prospective single-center cohort, 106 kidney transplant recipients with 108 indication biopsies were assessed for dd-cfDNA at biopsy and at 7, 30, and 90 days thereafter. dd-cfDNA was analyzed as a continuous, threshold-based, and longitudinal time-dependent variable. Clinical endpoints included ≥30% eGFR decline within 2 years, indication for re-biopsy, and graft failure. Persistent elevation of dd-cfDNA (≥0.5% at 90 days) occurred in 7.4% of patients, with 50% requiring re-biopsy and 37.5% developing graft failure. A single measurement ≥1.0% significantly predicted ≥30% eGFR decline (HR 2.28; 95% CI 1.03–5.05), whereas levels ≥0.5% were less discriminative. In multivariable time-dependent Cox models adjusted for age, sex, time from transplantation to biopsy, baseline eGFR, baseline proteinuria, and Banff domain scores, longitudinal dd-cfDNA remained independently associated with ≥30% eGFR decline (HR 1.68; 95% CI 1.12–2.51), re-biopsy (HR 1.88; 95% CI 1.38–2.55), and graft failure (HR 3.42; 95% CI 2.00–5.86). In conclusion, dd-cfDNA levels, particularly when assessed longitudinally, are associated with adverse allograft outcomes after indication biopsy and may provide relevant prognostic information beyond a single measurement.

Keywords: dd-cfDNA, donor-derived cell-free DNA, graft failure, kidney transplantation, rejection

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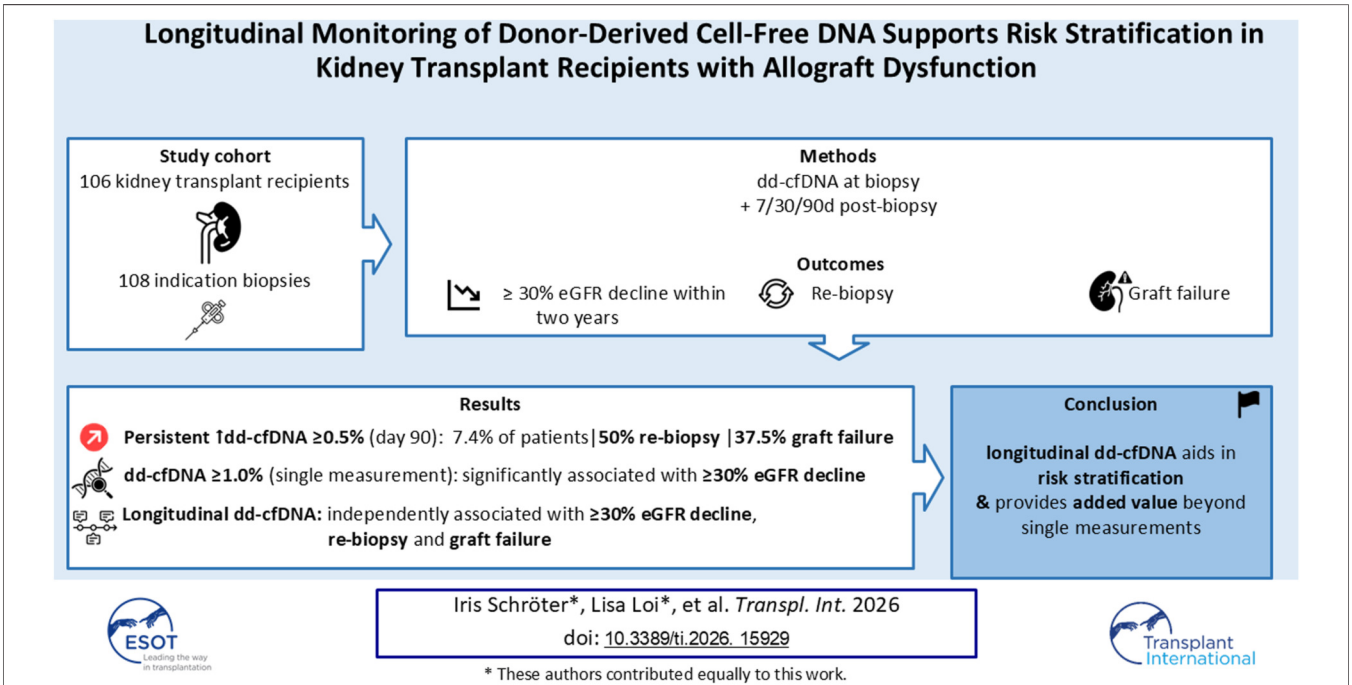
INTRODUCTION

Donor-derived cell-free DNA (dd-cfDNA) has emerged as a promising non-invasive biomarker for monitoring kidney allograft health, and has recently been shown to improve clinical decision-making beyond the standard of care in kidney transplantation [1–4]. Elevated levels of dd-cfDNA in the recipient's bloodstream, released during graft cell death, reflect ongoing graft injury, for example in cases of rejection, and often precede clinical or histological changes [5, 6]. Several studies have demonstrated the utility of dd-cfDNA in detecting acute rejection, particularly antibody-mediated



GRAPHICAL ABSTRACT |

rejection (AMR) [2–4, 7–11] with increasing dd-cfDNA levels reflecting the severity of microcirculation inflammation [1, 3, 5, 12]. Additionally, decreasing dd-cfDNA levels have been shown to indicate responses to rejection treatment and have been incorporated into recent clinical trials targeting AMR [13–17]. Further, there is emerging evidence that dd-cfDNA may also have prognostic value, for example in relation to the development of *de novo* donor-specific antibodies (DSA) and a subsequent decline in estimated glomerular filtration rate (eGFR) [18, 19].

However, data regarding the utility of particularly sequential dd-cfDNA measurements for longer-term outcomes such as graft failure, progressive allograft dysfunction indicating re-biopsy, or long-term eGFR decline remain limited. It is unclear whether fixed absolute thresholds are sufficient or whether the rate and magnitude of change over time (kinetics) provide greater prognostic value, and how dd-cfDNA testing should be optimally integrated into clinical decision-making.

To address these gaps, we analyzed both single time-point and longitudinal dd-cfDNA measurements in kidney transplant recipients with indication biopsies, evaluating dd-cfDNA as a continuous variable and through various threshold-based and longitudinal models to assess associations with key clinical outcomes.

MATERIALS AND METHODS

Study Design

This prospective single-center study included 106 kidney transplant recipients from the Department of Nephrology at Heidelberg University Hospital who underwent 108 clinically indicated allograft biopsies between November 2020 and December 2022. Biopsies were examined by two board-examined pathologists and reported using the BANFF 2018 reference guide [20]. Histopathology was not re-scored using later Banff updates, as clinical management during the study period was based on Banff 2018.

dd-cfDNA was measured at the time of biopsy (T_0) and at follow-up visits 7- (T_1), 30- (T_2), and 90-day (T_3) post-biopsy. Throughout the study period, treating physicians were blinded to dd-cfDNA testing results, as dd-cfDNA was measured for research purposes only and was not implemented in routine clinical care at our center. To quantify dd-cfDNA, plasma was isolated by sequential centrifugation and either stored at −80 °C or processed immediately for cfDNA extraction. dd-cfDNA was quantified using the AlloSeq cfDNA assay (CareDx), a multiplex PCR-based assay targeting 202 single nucleotide polymorphisms (SNPs). Sequence data was analyzed using the CareDx AlloSeq cfDNA software and all procedures were performed as described previously [4].

Clinical parameters, including serum creatinine, eGFR, and proteinuria, were assessed at the same initial time points and additionally at 180 days, 1 year, 2 years, and 3 years post-biopsy as part of an ongoing longitudinal follow-up. Detailed descriptions of the study setting as well as the results focusing on the initial follow-up period up to day 180, and correlations with histopathology have been published previously [4].

TABLE 1 | Patient characteristics stratified by dd-cfDNA thresholds at time of biopsy (T_0).

Variable	Total cohort N = 106	dd-cfDNA <0.5 N = 70	dd-cfDNA ≥0.5 N = 36	*P*-value	Available (N)
Demographics at time of biopsy (T_0)					
Age	49.2 ± 14.5	50.5 ± 14.8	46.5 ± 13.8	0.181	106
Male sex	71 (67.0)	50 (71.4)	21 (58.3)	0.254	106
Body mass index (kg/m^2)	26.0 ± 4.7	26.5 ± 5.0	25.0 ± 4.0	0.138	106
Prior Tx	16 (15.4)	7 (10.1)	9 (25.0)	0.061	104
Donor data					
Donor age	55.1 ± 13.4	55.3 ± 13.3	54.7 ± 13.8	0.842	96
Male donor	44 (44.0)	29 (43.9)	15 (44.1)	1.000	100
AB0i Tx	3 (2.9)	3 (4.3)	0 (0.0)	0.549	104
Deceased donation	68 (64.2)	44 (62.9)	24 (66.7)	0.089	106
Laboratory					
Baseline serum creatinine (mg/dL)	2.0 ± 0.8	1.9 ± 0.7 (62)	2.2 ± 1.0 (28)	0.091	90
T_0 serum creatinine (mg/dL)	3.0 ± 2.3	2.7 ± 1.7	3.7 ± 3.1	0.079	106
T_0 proteinuria (g/molCr)	141.8 ± 219.7	112.7 ± 158.1	209.3 ± 314.2	0.155	83
T_0 dd-cfDNA (%)	0.2 ± 0.1	0.8 ± 1.7	2.0 ± 2.6	**<0.001**	106
T_0 eGFR (mL/min/1.73m^2)	33.4 ± 17.5	30.5 ± 16.4	24.8 ± 12.6	**0.004**	106
Immunology					
Preformed antibodies	19 (22.9)	9 (15.5)	10 (40.0)	**0.031**	83
T_0 sCD30 (ng/mL)	33.9 ± 29.7	31.2 ± 26.7	39.0 ± 34.5	0.244	106
DSA >500 MFI	30 (29.4)	16 (23.5)	14 (41.2)	0.107	102
AB MM	2 ± 1	2 ± 1	2 ± 1	0.088	91
DR MM	1 ± 1	1 ± 1	1 ± 1	0.319	91
A/B/DR MM	2 ± 2	2 ± 2	3 ± 1	0.079	91
Immunosuppression					
Use of tacrolimus	82 (77.4)	52 (74.3)	30 (83.3)	0.418	106
Use of cyclosporine A	19 (17.9)	16 (22.9)	3 (8.3)	0.107	106
Use of mTOR inhibitors	7 (6.6)	6 (8.6)	1 (2.8)	0.418	106
Use of belatacept	3 (2.8)	1 (1.4)	2 (5.6)	0.266	106
Biopsy findings					
Acute rejection[a]	35 (33.0)	17 (24.3)	18 (50.0)	**0.0400**	106
Borderline changes	23 (21.7)	13 (18.6)	10 (27.8)	0.4600	106
AMR	7 (6.6)	2 (2.9)	5 (13.9)	**0.043**	106
TCMR	5 (4.7)	2 (2.9)	3 (8.3)	0.334	106
BKVAN	13 (12.3)	12 (17.1)	1 (2.8)	0.056	106
Follow-up data					
T_1 dd-cfDNA (%)	0.5 ± 0.7	0.2 ± 0.2 (59)	1.1 ± 0.9 (32)	**<0.001**	91
T_2 dd-cfDNA (%)	0.6 ± 0.8	0.3 ± 0.7 (50)	0.9 ± 0.9 (29)	**0.003**	79
T_3 dd-cfDNA (%)	0.4 ± 0.8	0.3 ± 0.5 (51)	0.7 ± 1.1 (27)	0.079	78
Patient death	6 (5.7)	4 (5.7)	2 (5.6)	1.0	106
Indication for Re-Biopsy	21 (19.8)	12 (17.1)	9 (25.0)	0.482	106
Re-biopsy with rejection	7 (6.6)	4 (5.7)	3 (8.3)	0.687	106

For each variable, perecentages were calculated based on the number of participants with data available for that variable; observations with missing values were excluded from the denominator. Results are given as N (%) or mean ± SD. In case of incomplete follow-up, the number of analyzed patients is indicated in parentheses. Abbreviations: A/B/DR MM, human leukocyte antigen mismatch score; AMR, antibody-mediated rejection; ATI, acute tubular injury; BKVAN, BK virus-associated nephropathy; dd-cfDNA, donor-derived cell-free DNA; DSA, donor-specific antibodies; g/molCr, g/molCreatinine; MFI, mean fluorescence intensity; mTORi, mTOR inhibitor; PRA, panel reactive antibody; sCD30, soluble CD30; TCMR, T cell-mediated rejection; Tx, transplantation. T_0 = at biopsy, T_1 = 7 days post-biopsy, T_2 = 30 days post-biopsy, T_3 = 90 days post-biopsy

*P-values less than 0.05 were considered statistically significant and are highlighted in **bold**; missing values were excluded; units/coding = measurement units or variable coding (continuous, categorical, %).*

[a]*Rejection cases include patients with borderline changes.*

Expanding on our initial short-term findings, this analysis explores longer-term dd-cfDNA patterns and their association with clinical outcomes beyond the early post-biopsy period. To explore these associations, patients were categorized into three groups based on dd-cfDNA levels: <0.5%, ≥0.5% to <1.0%, and ≥1.0%. These thresholds were applied to ensure comparability with previously published studies, in which these thresholds were used to evaluate dd-cfDNA as a prognostic marker and risk stratification tool in kidney transplant patients [18, 21, 22]. We then analyzed whether dd-cfDNA levels at biopsy, as well as their trajectories over time, could predict a ≥30% decline in eGFR slope 2 years post-biopsy, progressive allograft dysfunction requiring repeat biopsy, or the event of graft failure.

The study was approved by the ethics committee of the University of Heidelberg and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all study participants. The study is registered in the German Clinical Trials Register (DRKS00023604).

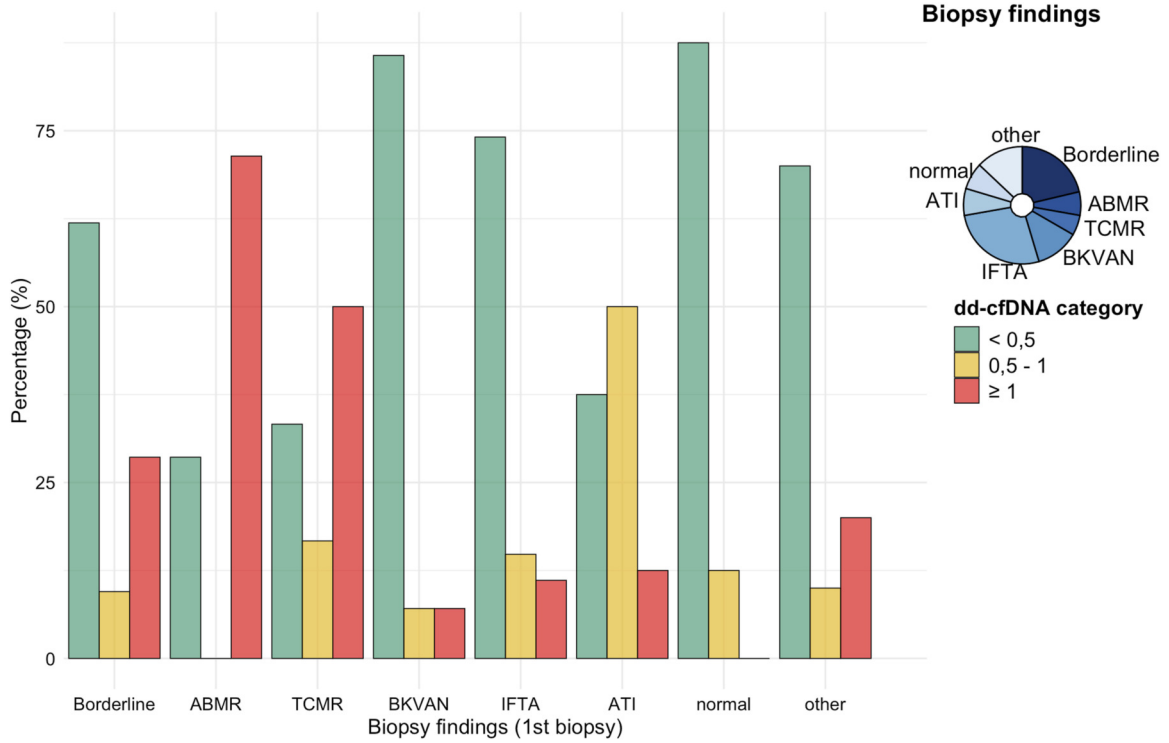


FIGURE 1 | % dd-cfDNA in relation to histological diagnoses at initial biopsy. Bars indicate the proportion of patients with dd-cfDNA <0.5%, 0.5%–1.0%, and ≥1.0% for each biopsy category. The inset pie chart shows the overall distribution of biopsy findings. Borderline, n = 23; AMR, n = 7; TCMR, n = 6; BKVN, n = 13; IFTA, n = 29; ATI, n = 8; normal, n = 8; other, n = 14. Other changes included recurrent disease (*N* = 4), infection (*N* = 1), CNI toxicity (*N* = 1), or IFTA with signs of CNI toxicity (*N* = 8). AMR = antibody-mediated rejection; TCMR = T-cell–mediated rejection; BKVAN = BK virus nephropathy; IFTA = interstitial fibrosis/tubular atrophy; ATI = acute tubular injury; CNI = Calcineurin inhibitor toxicity.

Statistical Analysis

Descriptive statistics were used to summarize baseline characteristics of the study population. Continuous variables were reported as mean ± standard deviation (SD) or median with interquartile range (IQR), depending on their distribution. Categorical variables were presented as counts and percentages. Group comparisons were performed using t-tests or Mann–Whitney *U* tests for continuous variables and chi-square or Fisher's exact tests for categorical variables, as appropriate.

Time-to-event outcomes were analyzed using Kaplan-Meier survival curves, with differences between groups assessed using the log-rank test. Time was defined as the interval from the initial biopsy to either the last clinical follow-up or the occurrence of the clinical endpoint. Missing dd-cfDNA values were not imputed, and patients contributed person-time only for intervals with an observed dd-cfDNA measurement. To assess potential bias from incomplete follow-up, baseline characteristics were compared between patients with complete versus incomplete dd-cfDNA sampling.

Three clinical endpoints were analyzed using Cox proportional hazards regression. Time was defined as the interval from the index biopsy to the occurrence of the respective endpoint or last available follow-up. Three clinical endpoints were examined: (A) a ≥30% decline in eGFR within 2 years after biopsy, (B) clinical indication for re-biopsy, and (C) graft failure.

To assess the association between dd-cfDNA and these outcomes, dd-cfDNA was evaluated using several complementary approaches:

i. dd-cfDNA was analyzed as a continuous variable measured at the initial timepoint (T_0).
ii. Threshold-based stratifications were applied using cutoffs of ≥0.5% and ≥1.0% at biopsy, reflecting commonly used thresholds where levels ≥0.5% suggest likely graft injury and levels ≥1.0% indicate a high risk of rejection [7, 18, 22, 23].
iii. To evaluate the impact of sustained elevation, "persistently high dd-cfDNA" was defined as values ≥0.5% at all measured timepoints (at biopsy as well as 7-, 30-, and 90-day post-biopsy).
iv. The increase in mean dd-cfDNA from the time of biopsy to 90 days post-biopsy was calculated and dichotomized at >0.3%. This cutoff was chosen to allow discrimination in a cohort with dd-cfDNA values predominantly <0.5% and is supported by prior evidence that rises >61% exceed the reference change value (RCV) for dd-cfDNA [24].

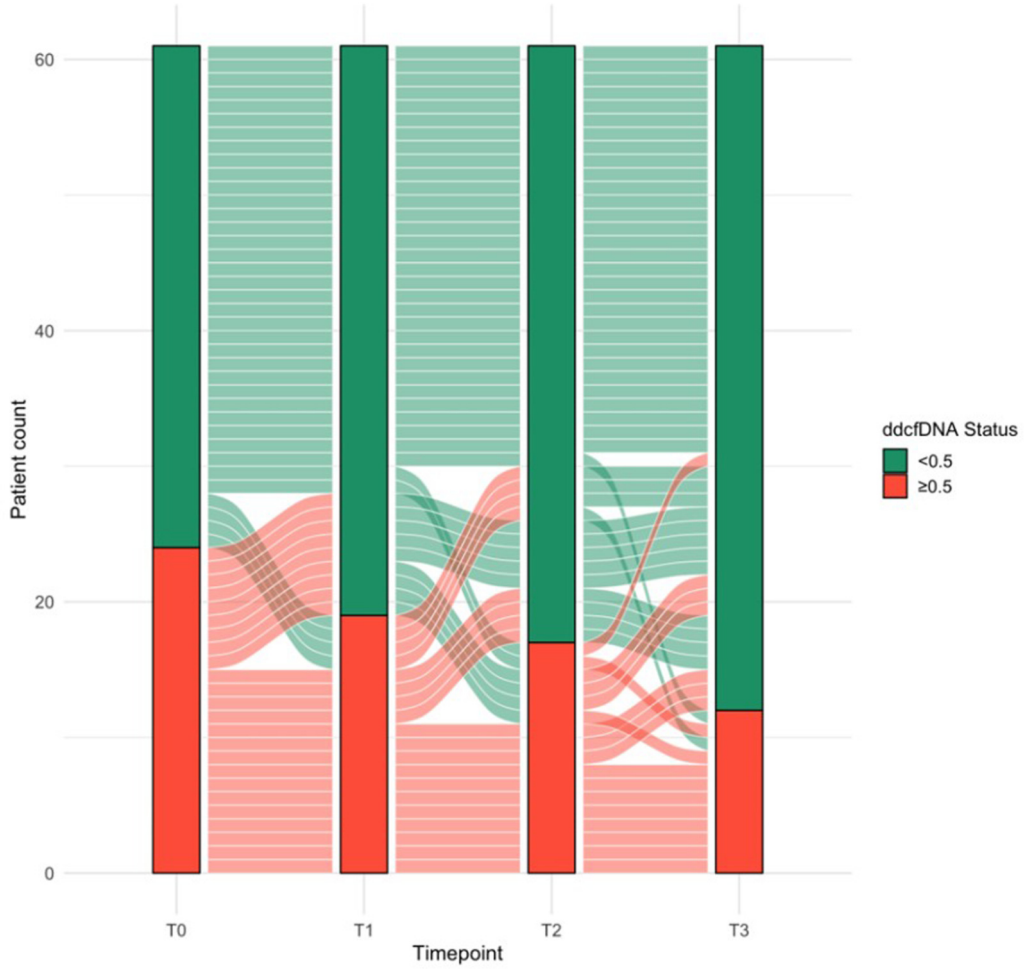


FIGURE 2 | Alluvial diagram illustrating longitudinal trajectories of donor-derived cell-free DNA (dd-cfDNA) status from biopsy (T_0) through follow-up (T_1 = day 7, T_2 = day 30, T_3 = day 90). Bars show the proportion of patients above (≥0.5%, red) or below (<0.5%, green) the threshold at each timepoint; flow bands illustrate patient transitions between categories. Persistent versus resolving elevation is thereby visualized.

v. Longitudinal dd-cfDNA trajectories were analyzed using time-dependent Cox regression models. For this purpose, dd-cfDNA measurements obtained at biopsy and during follow-up (7, 30, and 90 days) were reformatted into a start-stop data structure, with each interval assigned the most recent dd-cfDNA value. This approach allows for dynamic modeling of evolving dd-cfDNA levels in relation to subsequent clinical events. Univariable Cox models were initially fitted for each endpoint to explore associations between dd-cfDNA, clinical variables, laboratory measures, and histopathological findings. Multivariable models were then constructed using a prespecified, clinically motivated adjustment strategy. All primary multivariable models included recipient age and sex, time from transplantation to biopsy, baseline graft function, and baseline proteinuria, as these variables are plausibly associated with both dd-cfDNA levels and the studied endpoints. Histopathology was incorporated using aggregated Banff-based domain scores to reduce collinearity and avoid overfitting given the limited number of outcome events. TCMR-related activity/tubulointerstitial inflammation was summarized as the combined score of interstitial inflammation, tubulitis, and intimal arteritis (t + i + v); AMR-related activity/microvascular inflammation (MVI) was defined as the sum of glomerulitis, peritubular capillaritis und C4d positivity (g + ptc + C4d); and chronic injury burden was captured using a composite of Banff lesions indicating chronicity (ci + ct + cv + cg). To assess the robustness of the findings, prespecified sensitivity analyses were performed, including models excluding all histological variables and models adjusting only for chronic injury burden. Proportional hazards assumptions were assessed using Schoenfeld residuals for all multivariable models. Model discrimination was evaluated using

TABLE 2 | Univariate Cox Regression Analysis for ≥30% eGFR Decline within 2 years post-biopsy.

Variable	HR	95% CI	*P*-value
Demographics			
Recipient age (years)	1.00	0.97–1.02	0.7760
Male sex	1.18	0.56–2.5	0.6670
BMI >30 (kg/m^2)	0.89	0.41–1.94	0.7710
Prior Tx	1.13	0.51–2.52	0.7650
Donor data			
Donor age	1.00	0.98–1.03	0.7860
Male donor	0.79	0.36–1.72	0.5490
Deceased donation	1.21	0.54–2.7	0.6400
Biopsy findings			
Acute rejection[a]	3.18	1.52–6.64	**0.0021**
AMR	5.17	1.76–15.2	**0.0029**
TCMR	2.60	0.90–7.51	0.0781
Borderline changes	0.76	0.23–2.53	0.6590
BKVAN	0.86	0.30–2.47	0.7760
Immunology			
Preformed antibodies	0.59	0.17–2.05	0.4070
PRA ≥30%	0.55	0.16–1.92	0.3500
T_0 sCD30	1.01	0.99–1.03	0.2140
A/B/DR MM	1.07	0.84–1.36	0.6170
DSA >500 MFI	1.13	0.49–2.59	0.7680
de novo DSA	1.30	0.18–9.58	0.7980
Immunosuppression			
CNI	0.60	0.18–1.99	0.3990
mTORi	1.56	0.37–6.61	0.5430
Belatacept	2.37	0.72–7.87	0.1580
Laboratory			
T_0 eGFR (mL/min/1.73m^2)	0.98	0.95–1.01	0.1610
T_0 proteinuria (g/molCr)	1.00	1.00–1.01	**0.0013**
T_0 dd-cfDNA (%, continuous)	1.14	1.01–1.30	**0.0405**
T_0 dd-cfDNA ≥0.5%	1.67	0.79–3.54	0.1810
T_0 dd-cfDNA ≥1%	2.28	1.03–5.05	**0.0426**
Time-dependent dd-cfDNA % (T_0-T_3)	1.48	1.12–1.97	**0.006**
Persistently high dd-cfDNA ≥0.5% (T_0-T_3)	3.84	1.42–10.41	**0.0082**
dd-cfDNA T_0-T_3 >0.3%	2.27	0.81–6.30	0.1170
Baseline creatinine	1.15	0.73–1.81	0.5480
Histopathology/Banff composite scores			
TCMR/TI (t + i + v)	1.06	0.84–1.32	0.641
AMR/MVI (g + ptc + c4d)	1.30	0.96–1.77	0.094
Chronicity (ci + ct + cv + cg)	1.16	1.01–1.32	**0.030**

Abbreviations: A/B/DR MM, human leukocyte antigen mismatch score; AMR, antibody-mediated rejection; BKVAN, BK virus-associated nephropathy; BMI, body-mass index; CI, confidence interval; CNI, calcineurin inhibitor; dd-cfDNA, donor-derived cell-free DNA; DSA, donor-specific antibodies; g/molCr, g/mol Creatinine; HR, hazard ratio; MFI, mean fluorescence intensity; mTORi, mTOR, inhibitor; PRA, panel reactive antibody; sCD30, soluble CD30; TCMR, T cell-mediated rejection; Tx, transplantation. T_0 = at biopsy, T_1 = 7 days post-biopsy, T_2 = 30 days post-biopsy, T_3 = 90 days post-biopsy. Histopathology and Banff composite scores: Histological findings were assessed according to the Banff classification. To reduce collinearity and improve model stability, composite Banff domains were used in regression analyses. Banff composite domains were defined as: T-cell-mediated rejection (TCMR)/tubulointerstitial inflammation (TI) as t + i + v, where t = tubulitis, i = interstitial inflammation, v = intimal arteritis, antibody-mediated rejection (AMR)/microvascular inflammation (MVI) as g + ptc + c4d, where g = glomerulitis, ptc = peritubular capillaritis, c4d = C4d positivity, and chronicity as ci + ct + cv + cg, where ci = interstitial fibrosis, ct = tubular atrophy, cv = vascular fibrous intimal thickening, cg = transplant glomerulopathy. P-values less than 0.05 were considered statistically significant and are highlighted in ***bold***; *missing values were excluded; units/coding = measurement units or variable coding (continuous, categorical, %).*
[a]*Rejection cases include patients with Borderline changes.*

Harrell's concordance index (C-index). Results are reported as hazard ratios (HRs) with 95% confidence intervals (CIs; see **Supplementary Material**).

All statistical analyses were conducted using R Statistical Software (Version 2024.12.0 + 467).

RESULTS

Baseline Characteristics and dd-cfDNA Trajectories in Relation to Histopathology

A total of 106 kidney transplant recipients undergoing 108 indication biopsies with concurrent dd-cfDNA measurement were included. The mean age at biopsy was 49 ± 2 years, and 35/106 (33%) were female. Median time from transplantation to biopsy was 963 days (IQR 97–2853). The median clinical follow-up was 832 days (IQR 486–1,074).

Table 1 summarizes the clinical and demographic characteristics of the entire cohort as well as subgroups stratified by dd-cfDNA thresholds (<0.5% and ≥0.5%) at the time of indication biopsy. Compared to patients with dd-cfDNA <0.5%, those with levels ≥0.5% exhibited significantly lower eGFR (P = 0.004), a higher prevalence of preformed antibodies (P = 0.031), and an increased incidence of acute rejection (P = 0.040), particularly AMR (P = 0.043). No other baseline characteristics differed significantly between the two groups. Baseline characteristics between patients with complete versus incomplete dd-cfDNA follow-up were compared to assess potential bias but showed no significant differences (data not shown).

Of the 108 allograft biopsies evaluated, 36/108 (33.3%) showed histological evidence of rejection. Among these, borderline changes were diagnosed in 23/108 (21.3%) cases, while AMR and T-cell-mediated rejection (TCMR) were identified in 7/108 (6.5%) and 6/108 (5.6%) biopsies, respectively (**Figure 1**). Most biopsies (72/108; 66.7%) showed no histological signs of rejection.

The distribution of dd-cfDNA levels (<0.5%, ≥0.5%, and ≥1.0%) differed notably across histopathological diagnoses at the time of indication biopsy (T_0). At biopsy, dd-cfDNA was ≥0.5% in 38 cases (35.2%) and ≥1.0% or greater in 21 cases (19.4%). Among patients with AMR, the vast majority had dd-cfDNA levels ≥1.0%, while in patients with TCMR, most cases were associated with dd-cfDNA levels ≥0.5%, including a substantial fraction with levels ≥1.0%. Patients with borderline changes showed dd-cfDNA levels across all categories, but most were associated with values <0.5%. Histopathological diagnoses such as BK virus-associated nephropathy (BKVAN in 13/108; 12.0%), interstitial fibrosis and tubular atrophy (IFTA in 29/108; 26.9%), acute tubular injury (ATI in 8/108; 7.4%), and unspecific histology (in 22/108; 20.4%) occurred almost exclusively in patients with dd-cfDNA <0.5%, with only isolated cases in the ≥0.5% or ≥1.0% groups (**Figure 1**).

Figure 2 depicts the longitudinal dynamics of dd-cfDNA status (dichotomized at 0.5%) from the time of biopsy to 90 days post-biopsy. While most patients showed declining

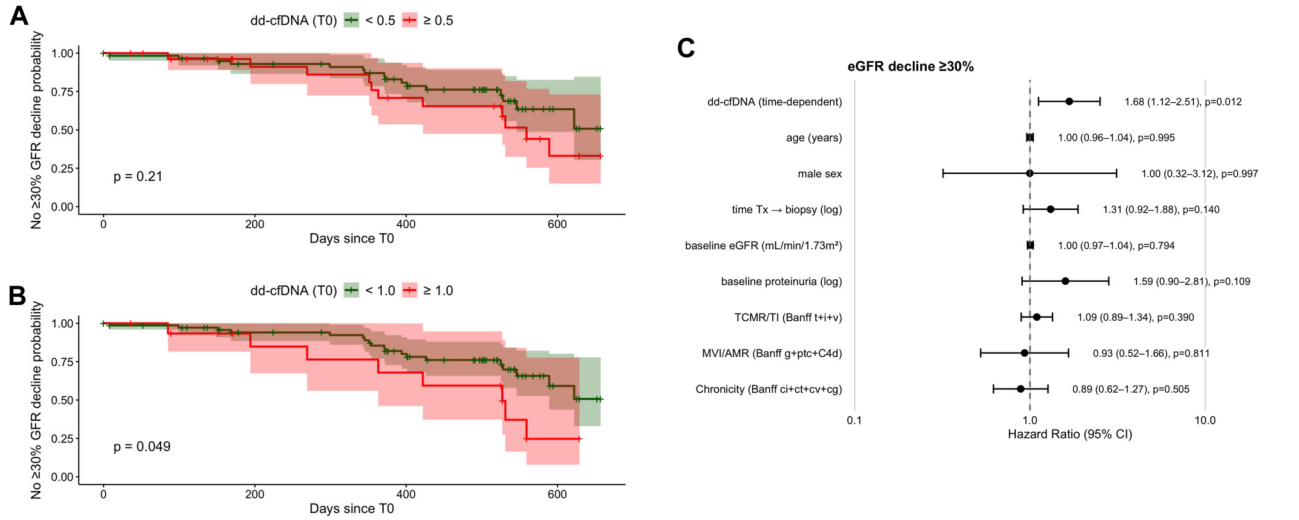


FIGURE 3 | Kaplan–Meier curves and multivariate time-dependent Cox regression for ≥30% eGFR decline within 2 years post-biopsy. **(A)** Kaplan–Meier estimates comparing patients with dd-cfDNA <0.5% vs. ≥ 0.5% at biopsy (log-rank $P = 0.18$). **(B)** Kaplan–Meier estimates for dd-cfDNA <1.0% vs. ≥ 1.0% at biopsy (log-rank $P = 0.04$). **(C)** Forest plot from time-dependent multivariate Cox regression including dd-cfDNA as a continuous longitudinal variable and clinical covariates. Hazard ratios (HR) with 95% confidence intervals (CI) are shown. dd-cfDNA, donor-derived cell-free DNA; eGFR, estimated glomerular filtration rate; HR, hazard ratio; CI, confidence interval; Tx, transplantation.

dd-cfDNA levels below 0.5% over time, a subset (n = 8) maintained persistently elevated levels across all measured timepoints. Among these, 4/8 (50%) showed progressive allograft dysfunction prompting a re-biopsy and 3/8 (37.5%) experienced graft failure ($P = 0.0863$ and $P = 0.678$, respectively). Conversely, patients whose dd-cfDNA normalized had more favorable clinical courses, with lower rates of re-biopsy (3/15; 20.0%) and graft failure (2/15; 13.3%).

dd-cfDNA and Risk of eGFR Decline

Twenty-eight patients experienced a ≥30% decline in eGFR within 2 years following biopsy (28/106; 26.4%). Among these, 11/28 (39.3%) showed dd-cfDNA levels ≥0.5% and 8/28 (28.6%) had dd-cfDNA levels ≥1.0% at biopsy. Higher baseline dd-cfDNA predicted a faster decline in eGFR over the subsequent year ($\beta = -2.91$; $P < 0.01$).

Univariable Cox regression analyses (**Table 2**) showed that time-dependent dd-cfDNA was associated with ≥30% eGFR decline (HR 1.48; 95% CI 1.12–1.97; $P = 0.006$). Persistently elevated dd-cfDNA ≥0.5% (from biopsy to 90-day post-biopsy) showed a stronger association (HR 3.84; 95% CI 1.42–10.41; $P = 0.0082$), despite the small subgroup size (n = 8), whereas a >0.3% rise from biopsy to day 90 was not significant ($P = 0.1170$). Proteinuria at biopsy (HR 1.00 per g/mol creatinine; 95% CI 1.00–1.01; $P = 0.0013$) and acute rejection (HR 3.18; 95% CI 1.52–6.64; $P = 0.0021$), particularly AMR (HR 5.17; 95% CI 1.76–15.2; $P = 0.0029$), were also associated with eGFR decline.

When analyzing single measurements at biopsy, the 0.5% cutoff showed no significant association with eGFR decline (**Table 2**; **Figure 3A**). In contrast, single measurements at biopsy, both as a continuous variable (HR 1.14; 95% CI 1.01–1.30; $P = 0.0405$) and using the ≥1.0% cutoff (HR 2.28; 95% CI 1.03–5.05; $P = 0.0426$), were predictive of risk for eGFR decline (**Table 2**; **Figure 3B**).

In the multivariable time-dependent Cox model adjusting for age, sex, time from transplantation to biopsy, baseline eGFR, baseline proteinuria, and histological injury summarized by Banff domains, longitudinal dd-cfDNA remained independently associated with a ≥30% decline in eGFR within 2 years (HR 1.68; 95% CI 1.12–2.51; $P = 0.012$; **Figure 3C**). None of the prespecified Banff domain scores were independently associated with eGFR decline after adjustment.

dd-cfDNA and Risk for Progressive Allograft Dysfunction Prompting Re-Biopsy

Twenty-one patients (21/106; 19.8%) underwent re-biopsy at a median of 299 days (IQR 112-752) post-initial biopsy. In univariate analysis (**Table 3**), dd-cfDNA at biopsy was associated with re-biopsy (HR 1.24; 95% CI 1.06–1.46; $P = 0.0091$), and persistently elevated dd-cfDNA ≥0.5% (from biopsy to 90-day post-biopsy) showed a borderline association (HR 3.15; 95% CI 0.99–10.01; $P = 0.0521$). Other laboratory values at the time of biopsy, including eGFR and proteinuria, were not associated with a re-biopsy. TCMR at index biopsy (HR 6.13; 95% CI 1.70–24.7; $P < 0.001$), higher baseline creatinine (HR 1.80; 95% CI 1.04–1.96; $P = 0.0066$),

TABLE 3 | Univariate cox regression analysis for progressive allograft dysfunction requiring repeat biopsy.

Variable	HR	95% CI	*P*-value
Demographics			
Recipient age (years)	0.98	0.95–1.01	0.1520
Male sex	0.76	0.29–1.96	0.5670
BMI >30 (kg/m^2)	0.20	0.03–1.46	0.1110
Prior Tx	1.42	0.69–2.90	0.3420
Donor data			
Donor age	1.03	1.00–1.06	0.0890
Male donor	0.51	0.21–1.28	0.1520
Deceased donation	1.17	0.47–2.92	0.7380
Biopsy findings			
Acute rejection[a]	2.13	0.90–5.02	0.0850
AMR	0.00	0.00-Inf	0.9970
TCMR	6.13	1.70–24.70	**<0.001**
Borderline changes	1.27	0.42–3.82	0.6670
BKVAN	1.71	0.58–5.10	0.3340
Immunology			
Preformed antibodies	2.63	1.01–6.84	**0.0483**
PRA ≥ 30%	2.33	0.89–6.07	0.0839
T_0 sCD30	1.00	0.99–1.02	0.636
A/B/DR MM	0.89	0.68–1.16	0.3760
DSA >500 MFI	0.44	0.13–1.51	0.1940
de novo DSA	2.41	0.32–18.41	0.393
Immunosuppression			
CNI	1.44	0.19–10.73	0.7230
mTORi	1.14	0.15–8.58	0.8360
Belatacept	0.92	0.12–6.82	0.9310
Laboratory			
T_0 eGFR (mL/min/1.73m^2)	0.97	0.94–1.00	0.0828
T_0 proteinuria (g/molCr)	1.00	1.00–1.00	0.6200
T_0 dd-cfDNA (%, continuous)	1.24	1.06–1.46	**0.0091**
T_0 dd-cfDNA ≥0.5%	1.58	0.66–3.76	0.3000
T_0 dd-cfDNA ≥1%	1.21	0.44–3.30	0.7130
Time-dependent dd-cfDNA % (T_0-T_3)	1.63	1.33–2.00	**<0.001**
Persistently high dd-cfDNA ≥0.5% (T_0-T_3)	3.15	0.99–10.01	0.0521
dd-cfDNA T_0-T_3 >0.3%	0.95	0.35–2.58	0.9230
Baseline creatinine	1.80	1.04–1.96	**0.0066**
Histopathology/Banff composite scores			
TCMR/TI (t + i + v)	1.23	0.96–1.57	0.103
AMR/MVI (g + ptc + c4d)	1.04	0.67–1.63	0.853
Chronicity (ci + ct + cv + cg)	0.92	0.77–1.10	0.354

Abbreviations: A/B/DR MM, human leukocyte antigen mismatch score; AMR, antibody-mediated rejection; BKVAN, BK virus-associated nephropathy; BMI, body-mass index; CI, confidence interval; CNI, calcineurin inhibitor; dd-cfDNA, donor-derived cell-free DNA; DSA, donor-specific antibodies; g/molCr, g/mol Creaatinine; HR, hazard ratio; MFI, mean fluorescence intensity; mTORi, mTOR, inhibitor; PRA, panel reactive antibody; sCD30, soluble CD30; TCMR, T cell-mediated rejection; Tx, transplantation. T_0 = at biopsy, T_1 = 7 days post-biopsy, T_2 = 30 days post-biopsy, T_3 = 90 days post-biopsy. Histopathology and Banff composite scores: Histological findings were assessed according to the Banff classification. To reduce collinearity and improve model stability, composite Banff domains were used in regression analyses. Banff composite domains were defined as: T-cell-mediated rejection (TCMR)/tubulointerstitial inflammation (TI) as t + i + v, where t = tubulitis, i = interstitial inflammation, v = intimal arteritis, antibody-mediated rejection (AMR)/microvascular inflammation (MVI) as g + ptc + c4d, where g = glomerulitis, ptc = peritubular capillaritis, c4d = C4d positivity, and chronicity as ci + ct + cv + cg, where ci = interstitial fibrosis, ct = tubular atrophy, cv = vascular fibrous intimal thickening, cg = transplant glomerulopathy.
*P-values less than 0.05 were considered statistically significant and are highlighted in **bold**; missing values were excluded; units/coding = measurement units or variable coding (continuous, categorical, %).*
[a]Rejection cases include patients with Borderline changes.

and preformed antibodies (HR 2.63; 95% CI 1.01–6.84; P = 0.0483), however, were also associated with the indication for re-biopsy.

By contrast, single-timepoint cutoffs at biopsy using both the ≥0.5% and ≥1.0% cutoffs were not significantly associated with progressive allograft dysfunction prompting a re-biopsy (**Figures 4A, B**).

When longitudinal dd-cfDNA was entered into the prespecified multivariable time-dependent Cox model together with age, sex, time from transplantation to biopsy, baseline eGFR, baseline proteinuria, and aggregated Banff domain scores, higher dd-cfDNA levels were independently associated with an increased likelihood of re-biopsy due to progressive allograft dysfunction (HR 1.88; 95% CI 1.38–2.55; P < 0.001; **Figure 4C**). In contrast, histological injury domains did not retain independent prognostic significance after adjustment.

dd-cfDNA and Risk of Graft Failure

Graft failure occurred in 24/106 (22.6%) kidney transplant recipients with indication biopsy at a median of 624 days post-biopsy (IQR 336–829) and at a median of 1739 days post-transplant (IQR 1074–4,281).

Single-timepoint dd-cfDNA cutoffs at biopsy (≥0.5% and ≥1.0%) were not significantly associated with risk of graft failure (**Table 4**; **Figures 5A, B**). However, a non-significant trend toward increased risk of graft failure was observed in patients with dd-cfDNA levels ≥0.5% compared to those with levels <0.5% (28.9% vs. 18.6%, P = 0.098 **Figure 5A**). In univariate Cox regression analysis (**Table 4**), demographic variables were not associated with graft failure. Time-dependent dd-cfDNA (biopsy to day 90) was associated with higher graft failure risk (HR 1.51; 95% CI 1.11–2.04; P = 0.0080), while persistently elevated dd-cfDNA levels ≥0.5% showed a non-significant trend (HR 2.94; 95% CI 0.92–9.44; P = 0.0700). Baseline serum creatinine was also significantly associated with graft failure (HR 1.68; 95% CI 1.04–2.71; P = 0.0339). Among biopsy findings, only TCMR was significant (HR 3.93; 95% CI 1.15–13.38; P = 0.0287), whereas acute rejection overall and AMR were not. Other immunological markers such as preformed antibodies, panel reactive antibodies ≥30%, and sCD30 levels, as well as donor characteristics or immunosuppressive treatments showed no significant association with graft failure.

In adjusted time-dependent Cox analysis accounting for demographic factors, time from transplantation to biopsy, baseline graft function, baseline proteinuria, and Banff domain-based histological injury burden, longitudinal dd-cfDNA emerged as a strong independent predictor of graft failure (HR 3.42; 95% CI 2.00–5.86; P < 0.001; **Figure 5C**). None of the histological domain scores were independently associated with graft loss in the fully adjusted model.

DISCUSSION

In this longitudinal study of 106 kidney transplant recipients undergoing indication biopsies with concurrent dd-cfDNA measurement, we demonstrated that elevated donor-derived dd-cfDNA levels were strongly associated with adverse allograft outcomes, including subsequent eGFR decline, persistent allograft dysfunction requiring re-biopsy, and

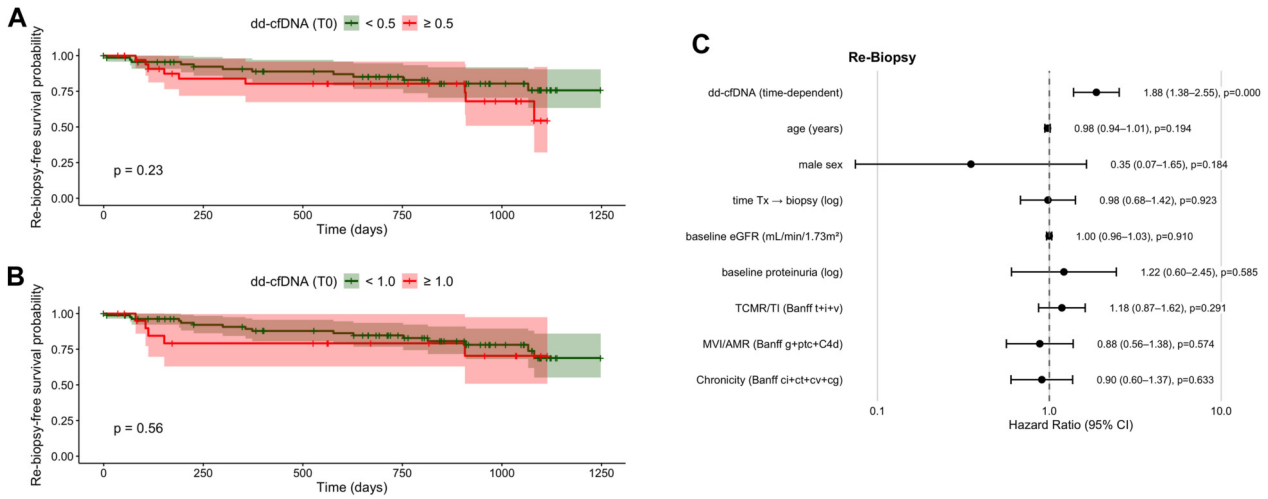


FIGURE 4 | Kaplan–Meier curves and multivariate, time-dependent Cox regression for re-biopsy–free survival after kidney transplantation. **(A)** Kaplan–Meier estimates stratified by dd-cfDNA <0.5% vs. ≥ 0.5% at biopsy (log-rank $P = 0.23$). **(B)** Kaplan–Meier estimates stratified by dd-cfDNA <1.0% vs. ≥ 1.0% at biopsy (log-rank $P = 0.56$). **(C)** Forest plot of time-dependent multivariate Cox regression including dd-cfDNA as a continuous longitudinal variable and clinical covariates. Hazard ratios (HR) with 95% confidence intervals (CI) are shown. dd-cfDNA, donor-derived cell-free DNA; eGFR, estimated glomerular filtration rate; HR, hazard ratio; CI, confidence interval; Tx, transplantation.

eventual allograft failure. These associations were most pronounced when dd-cfDNA was modeled as a time-dependent variable, with longitudinal trajectories capturing additional prognostically relevant information beyond single measurements.

Evidently, measured at a single time point, dd-cfDNA is very informative for identifying ongoing allograft injury, with high levels being primarily indicative for rejection [7, 18, 25, 26]. Consistent with these findings, dd-cfDNA levels in our cohort at time of biopsy were highest in patients with AMR [median (IQR) 2.00% (0.48–3.20)] and TCMR [median (IQR) 0.92% (0.19–11.25)] [4]. Notably, however, elevations in dd-cfDNA may also occur in other settings, such as ischemia-reperfusion injury (IRI), acute parenchymal injury seen with acute kidney injury, and sometimes severe cases of BKVAN [27, 28]. Interestingly though, even in these non-rejection settings, persistently elevated dd-cfDNA appears prognostically relevant: Cucchiari et al. showed for instance that early post-transplant dd-cfDNA kinetics reflected IRI severity as higher levels at 24 h and persistent elevation at day 7 were associated with delayed graft function, lower 6-month eGFR, and worse iBox-estimated 7-year graft survival, whereas normalization to <0.5% within the first week predicted better outcomes [29].

We also demonstrated that patients with dd-cfDNA ≥1.0% at biopsy more often presented with lower baseline eGFR, reflecting the clinical reality that conventional markers often prompt biopsy only once graft injury is already advanced. Declining eGFR or increasing proteinuria are late indicators of damage and frequently leave only limited therapeutic options once detected. In contrast, dd-cfDNA may provide earlier insight, as shown by Bromberg et al., who reported that dd-cfDNA elevations can precede biopsy-triggering changes in creatinine or proteinuria by several months, including up to 5 months before AMR diagnosis [5]. Interestingly, they also reported that elevated dd-cfDNA in non-rejecting patients was associated with decreased eGFR, suggesting that dd-cfDNA may capture broader forms of allograft injury beyond histological rejection and may serve as an early marker of functional decline. Interestingly, patients with "high immunological risk", such as those with *de novo* DSA, re-transplantation status, or C4d-positive biopsies, frequently had two or more consecutive dd-cfDNA measurements ≥1% [5], matching our data that patients with dd-cfDNA ≥1.0% at biopsy more often had preformed antibodies, and histopathological evidence of AMR, consistent with ongoing immune-mediated injury. It appears as if precisely these patients may be just subthreshold rejection. Even without meeting full diagnostic criteria, evidence suggests that they are already posed at risk and warrant close monitoring, a situation in which dd-cfDNA may help monitor graft injury and identify a critical window for therapeutic intervention before irreversible chronic damage occurs [5].

Building on the association between elevated dd-cfDNA at biopsy and adverse outcomes observed in our cohort and reported in prior studies [18, 21, 22], our longitudinal analysis further demonstrates that persistent dd-cfDNA elevation over time may also carry important prognostic significance, supporting the utility of serial monitoring. Specifically, longitudinal dd-cfDNA trajectories were independently associated with ≥30% eGFR decline, the need for re-biopsy, and graft failure, even after accounting for histological injury at the time of biopsy. This suggests dd-cfDNA reflects ongoing injury that may not be fully captured by a single biopsy, especially in patients with evolving damage. Among patients with persistently high levels, half required re-biopsies and more

TABLE 4 | Univariate Cox Regression analysis for Graft Failure.

Variable	HR	95% CI	*P*-value
Demographics			
Recipient age (years)	1.01	0.98–1.04	0.5170
Male sex	1.68	0.75–3.76	0.2070
Prior Tx	1.09	0.53–2.27	0.8100
BMI >30 (kg/m^2)	0.19	0.03–1.39	0.1020
Donor data			
Donor age	1.02	0.99–1.05	0.2440
Male donor	0.68	0.29–1.57	0.3680
Deceased donation	1.34	0.55–3.29	0.5230
Biopsy findings			
Acute rejection[a]	1.32	0.58–2.97	0.5060
AMR	2.97	0.68–12.98	0.1480
TCMR	3.93	1.15–13.38	**0.0287**
Borderline changes	0.38	0.09–1.64	0.1940
BKVAN	1.11	0.33–3.75	0.8640
Immunology			
Preformed antibodies	1.17	0.37–3.67	0.7920
PRA ≥30%	1.29	0.40–4.20	0.6670
T_0 sCD30	1.01	0.99–1.02	0.2080
A/B/DR MM	1.05	0.8–1.38	0.7400
DSA >500 MFI	0.82	0.32–2.07	0.6670
de novo DSA	1.71	0.23–12.77	0.6000
Immunosuppression			
CNI	0.70	0.16–2.99	0.6260
mTORi	1.90	0.44–8.14	0.4200
Belatacept	1.94	0.45–8.34	0.3740
Laboratory			
T_0 eGFR (mL/min/1.73m^2)	0.98	0.95–1.00	0.0965
T_0 proteinuria (g/molCr)	1.00	1.00–1.00	0.0852
T_0 dd-cfDNA (%, continuous)	1.66	1.20–2.29	**0.002**
T_0 dd-cfDNA ≥0.5%	1.83	0.83–4.40	0.1280
T_0 dd-cfDNA ≥1%	1.43	0.59–3.49	0.4270
Time-dependent dd-cfDNA % (T_0-T_3)	1.51	1.11–2.04	**0.0080**
Persistently high dd-cfDNA ≥0.5% (T_0-T_3)	2.94	0.92–9.44	0.0700
dd-cfDNA T_0-T_3 >0.3%	3.56	0.8–15.79	0.0965
Baseline creatinine	1.68	1.04–2.71	**0.0339**
Histopathology/Banff composite scores			
TCMR/TI (t + i + v)	1.01	0.77–1.34	**0.921**
AMR/MVI (g + ptc + c4d)	1.23	0.84–1.81	**0.295**
Chronicity (ci + ct + cv + cg)	1.21	1.04–1.42	**0.295**

Abbreviations: A/B/DR MM, human leukocyte antigen mismatch score; AMR, antibody-mediated rejection; BKVAN, BK, virus-associated nephropathy; BMI, body-mass index; CI, confidence interval; CNI, calcineurin inhibitor; dd-cfDNA, donor-derived cell-free DNA; DSA, donor-specific antibodies; g/molCr, g/molCreatinine; HR, hazard ratio; MFI, mean fluorescence intensity; mTORi, mTOR, inhibitor; PRA, panel reactive antibody; sCD30, soluble CD30; TCMR, T cell-mediated rejection; Tx, transplantation. T_0 = at biopsy, T_1 = 7 days post-biopsy, T_2 = 30 days post-biopsy, T_3 = 90 days post-biopsy. Histopathology and Banff composite scores: Histological findings were assessed according to the Banff classification. To reduce collinearity and improve model stability, composite Banff domains were used in regression analyses. Banff composite domains were defined as: T-cell-mediated rejection (TCMR)/tubulointerstitial inflammation (TI) as t + i + v, where t = tubulitis, i = interstitial inflammation, v = intimal arteritis, antibody-mediated rejection (AMR)/microvascular inflammation (MVI) as g + ptc + c4d, where g = glomerulitis, ptc = peritubular capillaritis, c4d = C4d positivity, and chronicity as ci + ct + cv + cg, where ci = interstitial fibrosis, ct = tubular atrophy, cv = vascular fibrous intimal thickening, cg = transplant glomerulopathy.

*P-values less than 0.05 were considered statistically significant and are highlighted in **bold**; missing values were excluded; units/coding = measurement units or variable coding (continuous, categorical, %).*

[a]*Rejection cases include patients with Borderline changes.*

than one-third lost their graft. These findings align with data from the ADMIRAL trial, where elevated dd-cfDNA (≥0.5%) was associated with a near threefold higher risk of *de novo* DSA development and persistent elevation with an almost twofold higher risk of >25% eGFR decline over 3 years [18]. Our results also parallel those of Bromberg et al., who linked sustained high dd-cfDNA-elevation to poor outcomes irrespective of histology [5], and of Bunnapradist et al., who demonstrated that dd-cfDNA trends following rejection were strongly associated with subsequent rejection or allograft dysfunction [21].

Notably, most biopsied patients (66.7%) in our cohort showed no histopathological signs of rejection, with correspondingly low dd-cfDNA levels in this subgroup. This naturally raises the question whether all such patients require a biopsy. Insights into this issue may be drawn from the multicenter Kidney Allograft Outcomes AlloSure Registry (KOAR) study, which reported a significantly higher rejection yield when dd-cfDNA was elevated (defined as a level ≥1% or dd-cfDNA ≥0.5% with a ≥61% increase from the prior test), with 39% vs. 7% rejection cases in the surveillance setting and 47% vs. 12% in the for-cause setting ($P < 0.001$) [30]. These results indicate that elevated dd-cfDNA may meaningfully improve the pre-test probability of histological rejection and the diagnostic yield of a biopsy, helping to identify patients most likely to benefit from histological assessment. This should not be interpreted as an argument against biopsy *per se*, as histology may still reveal other actionable findings such as calcineurin inhibitor toxicity, where therapeutic adjustments may still improve graft outcomes.

Taken together, our data indicate that repeated dd-cfDNA monitoring may provide complementary prognostic information beyond traditional markers such as allograft function, proteinuria, and histopathology. Consistent results from both single-center and multicenter studies position dd-cfDNA as a robust biomarker of graft injury [22, 31–33]. In clinical context, dd-cfDNA may help inform follow-up strategies and decisions about when to consider re-biopsy. However, realizing this potential clinical utility critically depends on appropriate patient selection. Patients with DSA or a history of rejection may particularly benefit from targeted dd-cfDNA surveillance, including monitoring AMR activity following anti-rejection therapy [2, 34, 35]. By contrast, in low-risk recipients with stable graft function, isolated dd-cfDNA elevations are often transient and not consistently associated with adverse outcomes, suggesting limited value for routine surveillance in this group [36]. These observations also align with the broader debate summarized by Naesens and Wong, emphasizing that the clinical utility of dd-cfDNA depends mainly on predictive values and likelihood ratios, which are strongly influenced by pre-test probability. The value of dd-cfDNA testing is therefore greatest in settings with a high prevalence of rejection, such as in patients with de novo DSA, where a positive result can raise the probability of rejection from ~50% to ~75%. By contrast, in low-prevalence, stable populations its impact is limited and false-positive results may potentially trigger unnecessary biopsies [37].

The strengths of our study include its longitudinal design with repeated measurements, integration of histological, immunological, and functional data, and use of time-dependent Cox regression to account for intra-individual variability. Our real-world, heterogeneous cohort reflects clinical practice. The main limitations of this study are the modest number of events and

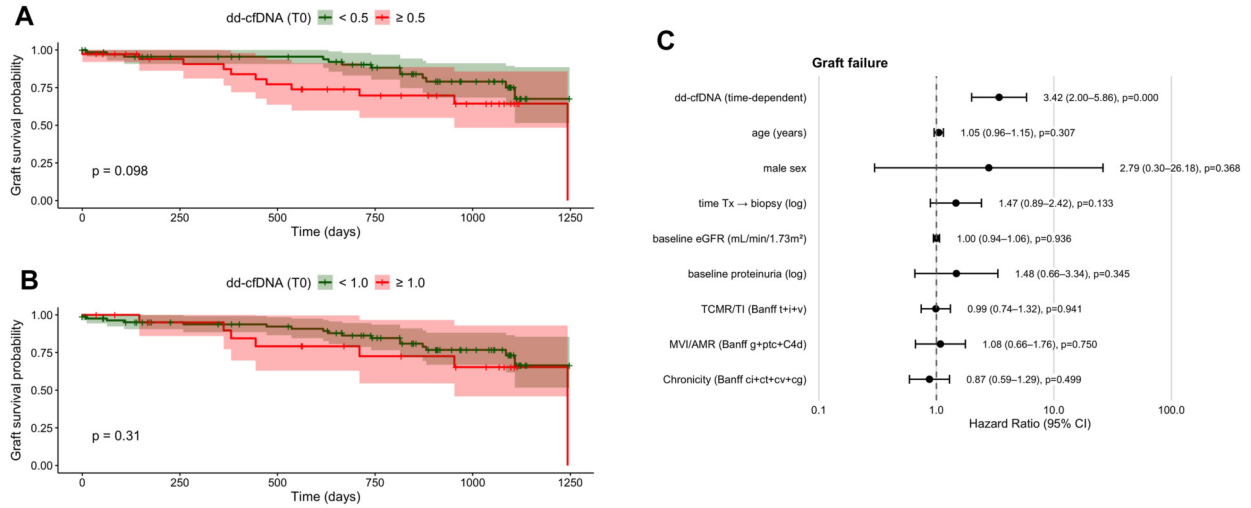


FIGURE 5 | Kaplan–Meier curves and multivariate time-dependent Cox regression for graft survival after kidney transplantation. **(A)** Kaplan–Meier estimates stratified by dd-cfDNA <0.5% vs. ≥ 0.5% at biopsy (log-rank $P = 0.098$). **(B)** Kaplan–Meier estimates stratified by dd-cfDNA <1.0% vs. ≥ 1.0% at biopsy (log-rank $P = 0.31$). **(C)** Forest plot from time-dependent multivariate Cox regression including dd-cfDNA as a continuous longitudinal variable and clinical covariates. Hazard ratios (HR) with 95% confidence intervals (CI) are shown. dd-cfDNA, donor-derived cell-free DNA; eGFR, estimated glomerular filtration rate; HR, hazard ratio; CI, confidence interval; Tx, transplantation.

limited numbers in certain subgroups, leading to uncertainty and wide confidence intervals for some estimates. In addition, dd-cfDNA was not available at every follow-up time point for all patients, which may limit some longitudinal analyses. Further, indication for re-biopsy is a clinician-driven endpoint, likely influenced by surveillance intensity and local practice, and should therefore be interpreted cautiously. Notably, strongest associations were observed for ≥30% eGFR decline and graft failure, which are less susceptible to indication bias and more directly reflect long term allograft prognosis. Finally, all analyses are exploratory and hypothesis-generating, intended to describe longitudinal patterns rather than providing definitive risk estimates. Larger multicenter cohorts with higher event rates are needed to obtain more precise estimates, to formally assess interactions, and to validate our observed associations.

Before our findings can be translated into practice, several important questions remain, including how dd-cfDNA can best be integrated into routine care, how often it should be measured, and whether decisions on clinical intervention should rely not only on absolute thresholds but also on patient-specific changes over time, as recently also outlined by the STAR working group [38]. The dd-cfDNA thresholds used in this study were chosen to align with commonly used clinical cutoffs and ensure comparability with existing literature, but although validated for detecting acute rejection, they were not specifically developed for prognostic assessment of long-term graft function. Future studies should therefore establish validated, outcome-specific (and potentially time-dependent) thresholds to support interpretation of dd-cfDNA in routine care. Further, regulatory hurdles persist, particularly the lack of cost-effectiveness analyses that clarify to what extent dd-cfDNA monitoring can reduce biopsies or improve their timing to secure the best therapeutic window [37, 39]. Comparative data between different assays, which could help drive competition and incentivize more cost-effective testing, are also scarce. Addressing these challenges will be essential to fully realize the promise of dd-cfDNA as a clinically meaningful tool that not only informs diagnosis and prognosis but also improves long-term care in kidney transplantation.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving humans were approved by the ethics committee of the University of Heidelberg. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

IS, LL, and LB designed the study. IS and LB analyzed and interpreted the data and drafted the manuscript. LL, MRe, MRu, and LB enrolled patients and collected the data. IS conducted the statistical analyses and modelling. Quantification of dd-cfDNA was performed at the Department of Transplantation Immunology under supervision of TT. CN, FK, CS, MZ, CM, and LB were responsible for the clinical

management of patients. MZ, CM, and LB supervised the project. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any other 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.2026.15929/full#supplementary-material

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ORIGINAL RESEARCH
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Diagnostic Potential of Urine CXCL10 and Donor-Derived cfDNA in Kidney Transplant Rejection

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Data suggests donor-derived cell-free DNA (dd-cfDNA) and urine CXCL10 outperform serum creatinine as a biomarker of antibody-mediated rejection (AMR) and T cell-mediated rejection (TCMR). We hypothesized that combining these biomarkers would improve the overall detection of rejection. We performed a retrospective two-center, case-controlled study of 103 adult renal transplant recipients who had for-cause or surveillance biopsies with corresponding urine and plasma samples. Rejection was classified by Banff 2022 criteria. While $\log_{10}$%dd-cfDNA correlated more strongly than $\log_{10}$CXCL10 with glomerulitis ($r = 0.55$, $p < 0.001$ vs. $r = 0.25$, $p = 0.01$) and peritubular capillaritis ($r = 0.47$, $p < 0.001$ vs. $r = 0.23$, $p = 0.02$), $\log_{10}$CXCL10 was a better correlate of tubulitis ($r = 0.28$, $p = 0.004$ vs. $r = 0.054$, $p = 0.59$). Both dd-cfDNA > 0.5% (OR 21.9, 95% CI 3.74–180, $p < 0.001$) and *de novo* DSA (OR 10.4, 95% CI 1.16–157, $p = 0.037$) were independently associated with AMR vs. no rejection (NR), while $\log_{10}$ serum creatinine and $\log_{10}$CXCL10 were not ($p > 0.05$). While dd-cfDNA >0.5% (OR 5.37, 95% CI 1.04–31.5, $p = 0.047$) was independently associated with Banff ≥1A TCMR vs. NR, $\log_{10}$CXCL10 was a significant predictor of TCMR in a model without %dd-cfDNA (OR 3.12, 95% CI 1.09–10.4, $p = 0.043$). Biomarker-guided screening strategies based on dd-cfDNA and urine chemokines such as CXCL10 for AMR (microvascular injury) and TCMR (tubulitis) warrant further study.

Keywords: CXCL10, dd-cfDNA, kidney transplantation, non-invasive immune monitoring, rejection

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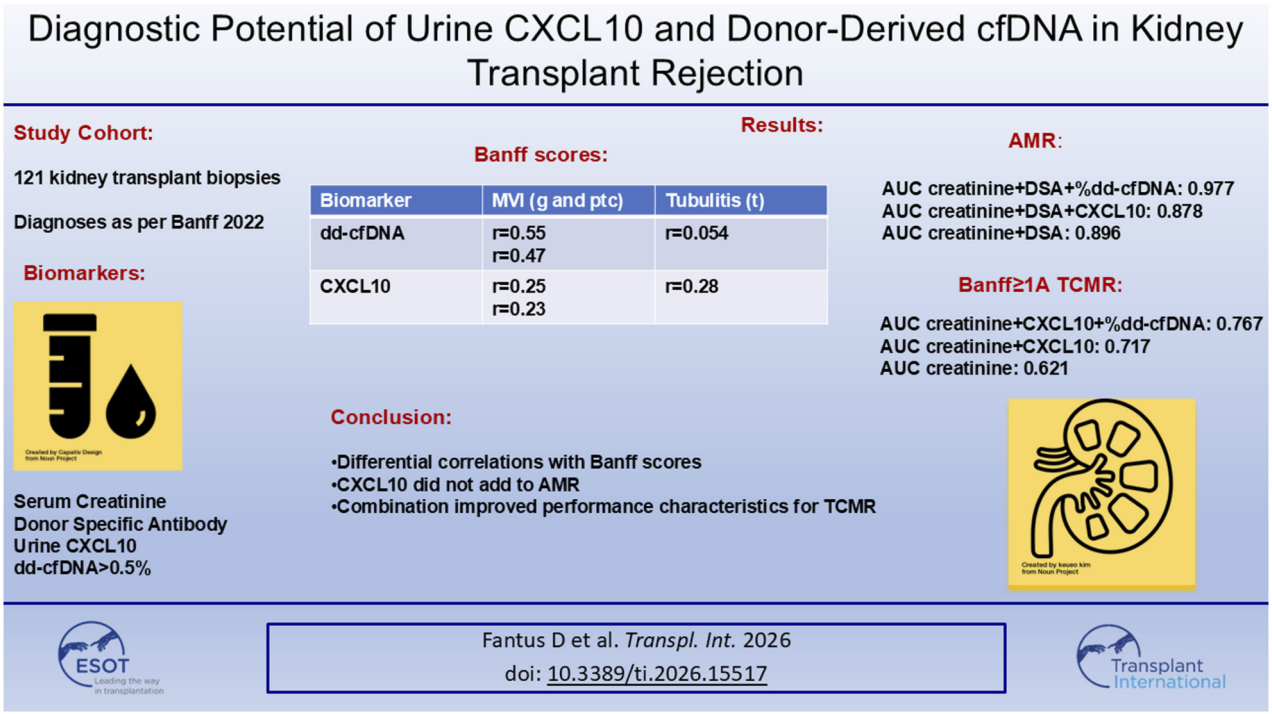
INTRODUCTION

Despite the widespread use of modern immunosuppressive drugs, alloimmune injury remains the most common cause of renal allograft failure today [1–6]. While a kidney biopsy remains the gold standard for rejection diagnosis, they are invasive, resource-intensive, subject to sampling error, and unsuited for longitudinal monitoring. Despite the need for non-invasive biomarkers, serum creatinine and donor specific antibody (DSA) remain the primary non-invasive tests of alloimmune renal injury used clinically [7]. Serum creatinine is a poor diagnostic biomarker of



GRAPHICAL ABSTRACT |

rejection [8, 9] and often only increases after significant allograft damage has occurred. DSA can be absent despite microvascular injury [10, 11], and detection of *de novo* DSA is only 50% predictive of subclinical AMR [12]. A novel non-invasive biomarker should be able to rule out rejection and accurately detect rejection early [13].

Urine C-X-C motif chemokine ligand 10 (CXCL10) is an interferon-gamma induced, CXC chemokine receptor 3 (CXCR3) chemokine that is highly expressed by infiltrating leukocytes and renal tubules and plays an important role in leukocyte trafficking and T helper cell type 1 (Th1) responses. Studies have shown that allograft inflammation from a variety of causes including BK viremia and urinary tract infection can upregulate urine CXCL10 [14–16]. During acute rejection, urine CXCL10 rises prior to serum creatinine [8, 17], can detect subclinical rejection [18–20], decreases after treatment of rejection [21], and remains elevated in patients who develop allograft dysfunction [17, 21–24]. Furthermore, in an unselected kidney transplant population, CXCL10 outperformed serum creatinine-based monitoring [20, 25]. However, urine CXCL10 largely reflects inflammation in the tubulointerstitial compartment [24]. As a result, arteritis and/or glomerulitis may be missed by this monitoring strategy.

Donor-derived cell-free DNA (dd-cfDNA) is a non-invasive biomarker of allograft injury that can be measured by assaying a preselected number of bi-allelic single nucleotide polymorphisms (SNPs) to distinguish donor vs. recipient cell-free DNA (cfDNA) in a sample extracted from plasma [26]. Cell death due to apoptosis and necrosis is thought to be the principal driver of cfDNA, including dd-cfDNA, release [27]. Multiple studies have shown that dd-cfDNA strongly correlates with rejection and even more strongly with AMR [28–32]. Further, dd-cfDNA has been show to increase between 1 and 5 months *before* biopsy-proven AMR [33] and decrease *after* treatment of rejection [34]. However, sensitivity is lower for TCMR, particularly low-grade and borderline TCMR [28, 35, 36].

Due to these different properties, we hypothesized that plasma dd-cfDNA and urine CXCL10 are complementary biomarkers. While elevated plasma dd-cfDNA reflects injury in the vascular compartment, urine CXCL10 reflects tubulointerstitial injury and inflammation. By directly comparing both the individual and combined diagnostic performance of plasma dd-cfDNA and urine CXCL10 when added to standard-of-care biomarkers (serum creatinine and DSA), we sought to assess how each biomarker complements rejection phenotypes.

MATERIALS AND METHODS

Study Population

We performed a two-site, case-controlled study selecting 121 biopsies with corresponding pre-biopsy urine and plasma samples in our transplant biobank. This study was approved by the research ethics board of the Centre hospitalier de l'Université de Montréal on 29 August 2022 (REB number MP-02-2023-10920).

Inclusion criteria were adults (18 or more years of age) who received a renal allograft between February 2011 and June

TABLE 1 | Demographic characteristics of the overall selected cohort and by diagnostic subgroup (no rejection, borderline TCMR, Banff ≥1A TCMR, and AMR).

Characteristic	Overall N = 103[a]	No Rejection N = 53[a]	Borderline N = 18[a]	TCMR N = 15[a]	AMR N = 17[a]	p-value[b]
Recipient age at biopsy	52 [40, 61]	54 [41, 60]	54 [42,63]	53 [40,69]	42 [34,53]	0.093
Recipient male sex	72 (70%)	39 (74%)	12 (67%)	9 (60%)	12 (71%)	0.8
Cause of ESRD						0.2
Polycystic kidney disease	26 (29%)	13 (31%)	5 (31%)	3 (20%)	5 (31%)	
Glomerulonephritis	25 (28%)	11 (26%)	2 (13%)	7 (47%)	5 (31%)	
Diabetes	18 (20%)	12 (29%)	2 (13%)	3 (20%)	1 (6.3%)	
Vascular	8 (9.0%)	2 (4.8%)	2 (13%)	1 (6.7%)	3 (19%)	
Genetic	5 (5.6%)	1 (2.4%)	2 (13%)	0 (0%)	2 (13%)	
Other	7 (7.9%)	3 (7.1%)	3 (19%)	1 (6.7%)	0 (0%)	
Unknown	14 (13.6%)	11 (20.8%)	2 (11.1%)	0 (0%)	1 (5.9%)	
Dialysis duration (months)	31 [13,59]	30 [13,54]	24 [15,65]	37 [15,72]	36 [12,59]	>0.9
Repeat transplant	5 (4.9%)	1 (1.9%)	0 (0%)	1 (6.7%)	3 (17.6%)	0.048
Donor age	51 [36, 57]	51 [40, 55]	54 [49, 64]	52 [39, 63]	35 [31, 52]	0.051
Donor male sex	53 (51%)	27 (51%)	11 (61%)	7 (47%)	8 (47%)	0.8
Living donor	25 (24%)	16 (30%)	2 (11%)	1 (6.7%)	6 (35%)	0.093
Delayed graft function	13 (13%)	4 (7.5%)	4 (22%)	5 (33%)	0 (0%)	0.009
Induction therapy						0.8
Basiliximab	79 (77%)	40 (75%)	13 (72%)	13 (87%)	13 (81%)	
Anti-thymocyte globulin (ATG)	13 (13%)	8 (15%)	2 (11%)	2 (13%)	1 (6.3%)	
Other	10 (9.8%)	5 (9.4%)	3 (17%)	0 (0%)	2 (13%)	
Maintenance immunosuppression						0.023
Triple therapy (tacrolimus, mycophenolate and prednisone)	84 (82%)	47 (89%)	14 (78%)	11 (73%)	12 (71%)	
Bitherapy (tacrolimus and either mycophenolate or prednisone)	9 (8.7%)	4 (7.5%)	2 (11%)	3 (20%)	1 (5.9%)	
Other	10 (10%)	2 (3.8%)	2 (11%)	1 (6.7%)	4 (24%)	
Surveillance biopsy	55 (53%)	44 (83%)	8 (44%)	2 (13%)	1 (5.9%)	<0.001
Months post-transplant	6 [4, 12]	6 [4, 12]	7 [4, 9]	3 [3, 9]	23 [8, 46]	0.001
Mean proteinuria (grams/L) (SD)	0.24 (0.61)	0.10 (0.25)	0.25 (0.44)	0.80 (1.65)	0.35 (0.44)	0.081
eGFR at biopsy (ml/min/1.73m^2)	49 [38, 60]	52 [44, 62]	43 [28, 53]	47 [33, 54]	42 [36, 53]	0.050

[a]*Median [Q1, Q3]; n (%), SD, standard deviation.*

[b]*Kruskal-Wallis rank sum test; Fisher's Exact Test for Count Data with simulated p-value (based on 2000 replicates); Pearson's Chi-squared test.*

ESRD, end-stage renal disease. eGFR, estimated glomerular filtration rate.

Exclusion criteria included simultaneous multi-organ transplantation, prior non-renal organ or bone marrow transplantation, pregnancy, and incomplete biomarker data (urine CXCL10, plasma dd-cfDNA, and DSA). For those patients who underwent multiple biopsies, only the last biopsy was included. ESRD = end-stage renal disease, eGFR = estimated glomerular filtration rate in mL/min/1.73m^2.

2021 and underwent a for-cause or protocol biopsy. We excluded recipients who were pregnant, simultaneous dual-organ transplants, or patients who were prior solid non-renal organ or bone marrow transplant recipients. We also excluded patients with high-grade BK viremia (>1000 copies/mL) and clinical cystitis/pyelonephritis at the time of biopsy, as both are associated with high urine CXCL10 levels. Our centers routinely perform one surveillance biopsy 3–6 months post-transplant.

The usual immunosuppressive protocol at our centers includes induction immunosuppression with either anti-thymocyte globulin or basiliximab and maintenance immunosuppression with mycophenolic acid, tacrolimus, and prednisone (see **Table 1**).

Dependent Variables

Cases were defined as patients with diagnoses of AMR, Banff ≥1A TCMR, or borderline TCMR as classified by the Banff 2022 criteria [11]. Ambiguous classifications were adjudicated by a second nephrologist (JH) and pathologist (IG). Inadequate biopsies not graded by adhering to the Banff criteria were excluded. Biopsy-confirmed BK nephropathy, pyelonephritis, and recurrent glomerular disease were also excluded.

Per Banff 2022 criteria, the AMR category included all diagnoses of active AMR, probable AMR, microvascular inflammation (MVI), mixed rejection, and probable mixed rejection [11]. Diagnoses of chronic AMR were not excluded provided there was a concomitant active component as characterized by glomerulitis, peritubular capillaritis or C4d positivity. Borderline TCMR was defined using the i1t1 threshold. The biopsy-proven acute rejection (BPAR) category was a combination of the AMR and Banff ≥1A TCMR categories with borderline TCMR excluded.

Controls (NR) were defined as patients with either normal histology or low-grade inflammation that did not meet the current Banff diagnostic criteria for BPAR or borderline TCMR.

Independent Variables

Urinary CXCL10

Urine samples were collected immediately prior to biopsy and supernatants processed and stored at −80 °C. Urine CXCL10 was measured at the Transplant Immunology Lab, Canadian Blood Services, University of Manitoba using the Meso Scale V-Plex assay, using previously reported methods [37]. The subclinical rejection threshold used to calculate sensitivity and specificity for

urine CXCL10 was > 13 pg/mL for male recipients over 2 weeks post-transplant, > 33 pg/mL for female recipients from 2 weeks to 5 months post-transplant, and > 13 pg/mL for female recipients > 6 months post-transplant [37, 38]. Units of measurement were picograms per ml.

Donor Derived Cell-free DNA

cfDNA was extracted from EDTA plasma samples collected immediately prior to the biopsy, following a protocol designed to avoid genomic DNA contamination. The percent dd-cfDNA was determined using the AlloSeq cfDNA Assay and Software (CareDx, Brisbane, CA) by the manufacturer. Due to the significant number of borderline TCMR cases (n = 18) in our study, coupled with the fact that our centers perform protocol biopsies as part of standard care, we selected an injury threshold of 0.5%. Others have previously compared sensitivity and specificity at various thresholds and have found sensitivity for rejection higher at this threshold [36, 39, 40]. Prior comparison of this assay versus the Allosure kidney assay has shown high correlation [41].

Donor-Specific Antibodies

DSA was assessed in samples collected either at the time of biopsy or in the preceding month. Analysis was conducted at the HLA laboratory of the McGill University Health Centre using a threshold of 1000 mean fluorescence intensity (MFI) units of the immunodominant DSA to dichotomize DSA as positive vs. negative per Canadian standards [42].

Statistical Analyses

Individual characteristics of the biopsies in each group were summarized within groups and overall using quartiles (Q1, median, and Q3) for numerical variables and counts and percentages for categorical variables. Kruskal-Wallis was used to compare the distribution of numeric variables between groups (or Wilcoxon tests for two-group comparisons). Chi-squared tests were used to compare categorical variables; Fisher's Exact test was used when cell sizes were smaller than 5.

Logistic regression models were used to examine relationships between plasma %dd-cfDNA, urine CXCL10, serum creatinine, and rejection events both with and without consideration of DSA depending on the rejection phenotype. Generalized estimating equations were used in sensitivity analyses to fit similar models to a dataset incorporating all biopsies with complete data, assuming an exchangeable working correlation matrix to account for within-participant correlations. Due to skewness, plasma % dd-cfDNA, urine CXCL10, and serum creatinine concentrations were analyzed on a $\log_{10}$ scale. Their association with Banff scores were described using Pearson correlations. Sensitivity and specificity for %dd-cfDNA and urine CXCL10 using thresholds as described above are also reported. As the small number of AMR events led to difficulties fitting conventional maximum likelihood logistic regression models, parameter estimates for the AMR vs. NR model were obtained via Firth's penalized likelihood method [43]. Differences in AIC (Akaike Information Criterion) values were used as a comparative measure of model fit, with lower AIC values indicating a better fitting model. Model discrimination was assessed by evaluating the AUC (area under the receiver-operator characteristic curve). Statistical analyses were performed using R (version 4.4.2) and the logistf package (version 1.26.0). In general, p-values less than 0.05 were described as statistically significant, without correction for multiple inference.

RESULTS

Between 26 February 2011 and 8 June 2021, 1,215 patients underwent renal transplantation at our institutions (**Figure 1**). Of these patients, 1,028 enrolled in our biobank and 1,259 biopsies were performed. Although our initial cohort of cases and controls encompassed 181 biopsies from 160 patients, 60 biopsies were ultimately excluded due to inadequate tissue and/or incomplete Banff scores (n = 23), insufficient cell free DNA for analysis (n = 32), or urine samples that were not collected (n = 5). As a result, 121 biopsies from 103 patients had measurements for all traditional and novel biomarkers as well as sufficient tissue to provide Banff scores. Demographic characteristics, other than shorter dialysis exposure and cause of end-stage renal disease, were similar between our cohort and excluded patients (see **Supplementary Table S1**). As there was no significant difference in our results when we used a generalized estimating equation (GEE) model to account for multiple biopsies per patient, our primary analytic cohort comprised 103 biopsies: one per patient, with the most recent biopsy selected (see **Supplementary Table S2**; **Supplementary Figure S1**). Within this cohort, 17.5% (n = 18) of the biopsies were categorized as Banff Borderline TCMR, 14.6% (n = 15) were Banff ≥1A TCMR, and 16.5% (n = 17) were AMR/mixed rejection (see **Table 1**).

Demographic characteristics of the overall cohort and by diagnostic category are presented in **Table 1**; **Supplementary Table S3**. All patients underwent ABO-compatible transplantation and had a negative flow cytometric or complement-dependent cytotoxicity crossmatch prior to transplantation as well as no pre-transplant DSA. AMR patients tended to be younger and were less likely to have diabetes and delayed graft function. They were also less likely to have been treated with maintenance immunosuppression consisting of tacrolimus, MMF, and prednisone at the time of rejection. Although most of the TCMR and AMR biopsies were performed due to graft dysfunction, the AMR biopsies were generally performed later post-transplant.

Biomarker levels are presented in **Table 2**; **Figure 2**, stratified by diagnostic category. The distribution of Banff scores by rejection category is also provided in **Table 2**. Log_{10}%dd-cfDNA is higher in patients with AMR while $\log_{10}$ serum creatinine and $\log_{10}$CXCL10 show smaller between-group differences (**Figure 2**).

Association With Acute and Chronic Banff Scores

Next, we sought to investigate the correlation between Banff scores and urine CXCL10 and plasma dd-cfDNA, respectively (**Figure 3**). Overall, $\log_{10}$%dd-cfDNA correlated more strongly than $\log_{10}$CXCL10 with glomerulitis ($r = 0.55$, $p < 0.001$ vs.

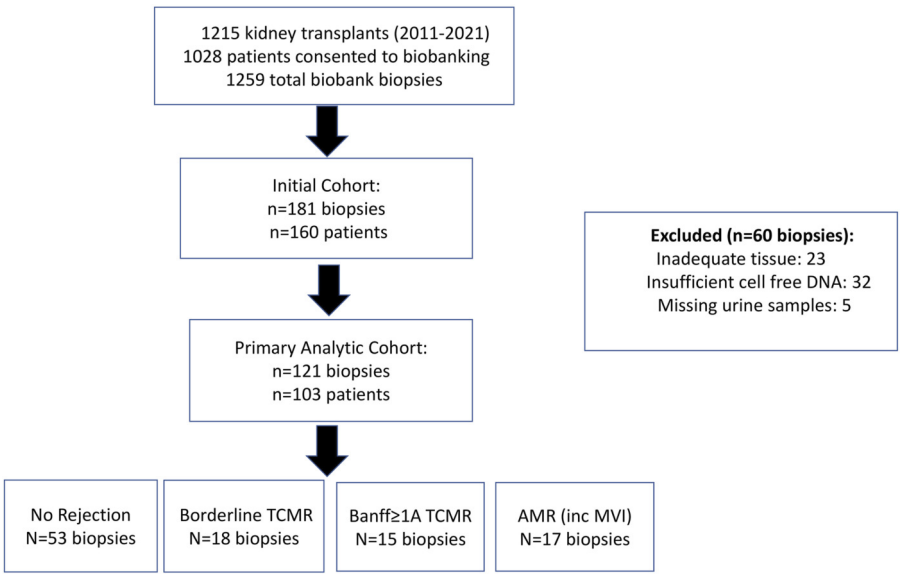


FIGURE 1 | Study Population. Out of a total of 1259 biopsies performed during the study period as part of our biobank, 181 biopsies were initially selected from 160 patients. However, 60 biopsies were subsequently excluded due to incomplete biomarker data or an inadequate biopsy not adhering to the Banff criteria. The remaining 121 biopsies were selected from 103 patients with one of the following diagnoses as per Banff 2022 criteria: no rejection, borderline TCMR, Banff ≥1A TCMR, and AMR (including probable AMR and MVI). The primary analytic cohort encompassed 103 biopsies from 103 patients. inc = included.

r = 0.25, p = 0.012), peritubular capillaritis (r = 0.47, p < 0.001 vs. r = 0.23, p = 0.019), peritubular capillary c4d staining (r = 0.47, p < 0.001 vs. r = 0.26, p = 0.008), and glomerular basement membrane double contours (r = 0.41, p < 0.001 vs. r = 0.12, p = 0.226). In contrast, although neither $\log_{10}$%dd-cfDNA nor $\log_{10}$CXCL10 correlated strongly with tubulitis, performance characteristics favored $\log_{10}$CXCL10 (r = 0.28, p = 0.004 vs. r = 0.054, p = 0.588). The correlation was similar when interstitial inflammation scores were compared (**Figure 3**). In contrast, there was poor correlation with both $\log_{10}$%dd-cfDNA and $\log_{10}$CXCL10 and chronic Banff scores (**Supplementary Figure S2**).

AMR vs. No Rejection

Because of the small number of AMR biopsies, the multi-predictor model for AMR vs. NR was fit using Firth's penalized likelihood method (**Figure 4**). The $\log_{10}$ serum creatinine OR was 1.33 (95% CI 0.000445 to 5480, p = 0.943), $\log_{10}$CXCL10 OR 0.794 (95% CI 0.0611 to 6.76, p = 0.842), DSA OR 13.0 (95% CI 1.34 to 220, p = 0.027), and $\log_{10}$%dd-cfDNA OR 40.8 (95% CI 4.13 to 925, p < 0.001). When %dd-cfDNA was used as a categorical variable, the $\log_{10}$ serum creatinine OR was 6.98 (95% CI 0.00417 to 34,500, p = 0.619), $\log_{10}$CXCL10 OR 1.01 (95% CI 0.109 to 8.82, p = 0.990), DSA OR 10.4 (95% CI 1.16 to 157, p = 0.037), and dd-cfDNA >0.5% OR 21.9 (95% CI 3.74 to 180, p < 0.001). These data demonstrated that measurement of % dd-cfDNA improved AMR diagnosis independent of DSA.

Furthermore, AUCs for distinguishing AMR vs. NR increased from 0.696 (95% CI 0.527–0.865) for $\log_{10}$ serum creatinine alone to 0.967 (95% CI 0.930–1.000) and 0.959 (0.915–1.000) when $\log_{10}$ serum creatinine and either $\log_{10}$%dd-cfDNA or dd-cfDNA >0.5% were combined, respectively (**Table 3**). In contrast, the AUC for detecting AMR was 0.706 (95% CI 0.544–0.867) when $\log_{10}$ serum creatinine and $\log_{10}$CXCL10 were combined.

Banff ≥1A TCMR Vs. No Rejection

Next, we compared the performance of serum creatinine, %dd-cfDNA, and urine CXCL10 in the detection of Banff ≥1A TCMR. AUCs and AICs were compared (**Figure 5**; **Table 3**). The $\log_{10}$ serum creatinine OR was 95.7 (95% CI 0.36 to 41,100, p = 0.120), $\log_{10}$CXCL10 OR 2.68 (95% CI 0.850 to 9.73, p = 0.110), and $\log_{10}$%dd-cfDNA OR 3.20 (95% CI 0.560 to 22.1, p = 0.200). When the 0.5% dd-cfDNA threshold was used, the $\log_{10}$ serum creatinine OR was 91.3 (95% CI 0.260 to 44,300, p = 0.130), $\log_{10}$CXCL10 OR 2.65 (95% CI 0.830 to 9.87, p = 0.120), and dd-cfDNA >0.5% OR 5.37 (95% CI 1.04 to 31.5, p = 0.047). Therefore, dd-cfDNA >0.5% was the only independent predictor of Banff ≥1A TCMR in this cohort. However, in a model without %dd-cfDNA, $\log_{10}$CXCL10 detected TCMR independently of serum creatinine (OR 3.12, 95% CI 1.09–10.4, p = 0.043) (data not shown).

AUCs for distinguishing TCMR vs. NR increased from 0.621 (95% CI 0.452–0.789) for $\log_{10}$ serum creatinine alone to 0.717 (95% CI 0.550–0.884) when $\log_{10}$ serum creatinine and $\log_{10}$CXCL10 were combined (**Table 3**). The AUC increased to 0.682 (95% CI 0.497–0.867) and 0.724 (95% CI 0.565–0.883) when $\log_{10}$ serum creatinine and either $\log_{10}$% dd-cfDNA or dd-cfDNA >0.5% were combined, respectively. When $\log_{10}$CXCL10 and either $\log_{10}$%dd-cfDNA or dd-cfDNA >0.5% were added to $\log_{10}$ serum creatinine, AUC increased to

TABLE 2 | A comparison of biomarker levels and Banff scores by diagnostic classification.

Characteristic	No Rejection N = 53[a]	Borderline N = 18[a]	TCMR N = 15[a]	AMR N = 17[a]	Overall N = 103[a]	p-value[b]
Urine CXCL10 (pg/mL)	7 [4, 14]	9 [1, 25]	17 [6, 42]	13 [9, 63]	9 [4, 22]	0.070
CXCL10 positive	13 (25%)	8 (44%)	8 (53%)	9 (53%)	38 (37%)	0.056
Serum creatinine (μmol/L)	119 [109,135]	138 [125, 203]	121 [116, 176]	152 [125, 172]	125 [114, 159]	0.008
%dd-cfDNA	0.20 [0.14, 0.30]	0.37 [0.17, 0.60]	0.34 [0.14, 0.58]	1.73 [0.76, 3.25]	0.26 [0.17, 0.56]	<0.001
%dd-cfDNA positive[c]	3 (5.7%)	7 (39%)	5 (33%)	15 (88%)	30 (29%)	<0.001
DSA positive (*de novo*)	1 (1.9%)	0 (0%)	0 (0%)	12 (71%)	13 (13%)	<0.001
Glomerulitis (g score)						<0.001
0	53 (100%)	18 (100%)	15 (100%)	6 (35%)	92 (89%)	
1	0 (0%)	0 (0%)	0 (0%)	6 (35%)	6 (5.8%)	
2	0 (0%)	0 (0%)	0 (0%)	4 (24%)	4 (3.9%)	
3	0 (0%)	0 (0%)	0 (0%)	1 (5.9%)	1 (1.0%)	
Interstitial inflammation (i score)						<0.001
0	52 (98%)	0 (0%)	1 (6.7%)	10 (59%)	63 (61%)	
1	1 (1.9%)	18 (100%)	3 (20%)	2 (12%)	24 (23%)	
2	0 (0%)	0 (0%)	9 (60%)	3 (18%)	12 (12%)	
3	0 (0%)	0 (0%)	2 (13%)	2 (12%)	4 (3.9%)	
Tubulitis (t score)						<0.001
0	32 (60%)	0 (0%)	1 (6.7%)	8 (47%)	41 (40%)	
1	14 (26%)	13 (72%)	2 (13%)	6 (35%)	35 (34%)	
2	6 (11%)	5 (28%)	6 (40%)	1 (5.9%)	18 (17%)	
3	1 (1.9%)	0 (0%)	6 (40%)	2 (12%)	9 (8.7%)	
Intimal arteritis (v score)						<0.001
0	52 (100%)	18 (100%)	9 (60%)	14 (82%)	93 (91%)	
1	0 (0%)	0 (0%)	2 (13%)	2 (12%)	4 (3.9%)	
2	0 (0%)	0 (0%)	4 (27%)	1 (5.9%)	5 (4.9%)	
Peritubular capillaritis (ptc score)						<0.001
0	51 (96%)	17 (94%)	9 (60%)	3 (18%)	80 (78%)	
1	2 (3.8%)	1 (5.6%)	3 (20%)	5 (29%)	11 (11%)	
2	0 (0%)	0 (0%)	2 (13%)	4 (24%)	6 (5.8%)	
3	0 (0%)	0 (0%)	1 (6.7%)	5 (29%)	6 (5.8%)	
c4d deposition in PTC						<0.001
0	53 (100%)	18 (100%)	14 (93%)	6 (38%)	91 (89%)	
2	0 (0%)	0 (0%)	0 (0%)	2 (13%)	2 (2.0%)	
3	0 (0%)	0 (0%)	1 (6.7%)	8 (50%)	9 (8.8%)	
GBM double contours (cg score)						<0.001
0	52 (98%)	17 (94%)	15 (100%)	9 (53%)	93 (90%)	
1	1 (1.9%)	1 (5.6%)	0 (0%)	3 (18%)	5 (4.9%)	
2	0 (0%)	0 (0%)	0 (0%)	2 (12%)	2 (1.9%)	
3	0 (0%)	0 (0%)	0 (0%)	3 (18%)	3 (2.9%)	
Interstitial fibrosis (ci score)						<0.001
0	35 (66%)	6 (33%)	3 (20%)	4 (24%)	48 (47%)	
1	17 (32%)	9 (50%)	12 (80%)	12 (71%)	50 (49%)	
2	0 (0%)	3 (17%)	0 (0%)	1 (5.9%)	4 (3.9%)	
3	1 (1.9%)	0 (0%)	0 (0%)	0 (0%)	1 (1.0%)	
Tubular atrophy (ct score)						0.002
0	25 (47%)	1 (5.6%)	3 (20%)	3 (18%)	32 (31%)	
1	27 (51%)	16 (89%)	12 (80%)	12 (71%)	67 (65%)	
2	0 (0%)	1 (5.6%)	0 (0%)	2 (12%)	3 (2.9%)	
3	1 (1.9%)	0 (0%)	0 (0%)	0 (0%)	1 (1.0%)	
Arterial intimal thickening (cv score)						0.4
0	20 (40%)	4 (24%)	3 (20%)	5 (31%)	32 (33%)	
1	19 (38%)	5 (29%)	8 (53%)	8 (50%)	40 (41%)	
2	10 (20%)	7 (41%)	3 (20%)	2 (13%)	22 (22%)	
3	1 (2.0%)	1 (5.9%)	1 (6.7%)	1 (6.3%)	4 (4.1%)	
Arteriolar hyalinosis (ah score)						0.069
0	29 (55%)	8 (44%)	6 (40%)	4 (24%)	47 (46%)	
1	20 (38%)	8 (44%)	6 (40%)	6 (35%)	40 (39%)	
2	4 (7.5%)	1 (5.6%)	2 (13%)	5 (29%)	12 (12%)	
3	0 (0%)	1 (5.6%)	1 (6.7%)	2 (12%)	4 (3.9%)	

[a]*Median [Q1, Q3]; n (%).*

[b]*Kruskal-Wallis rank sum test; Pearson's Chi-squared test; Fisher's exact test; NA.*

[c]*dd-cf DNA >0.5%.*

Absolute biomarker levels for urine CXCL10 (pg/mL), serum creatinine (μmol/L), and plasma dd-cfDNA (%) are compared among the four major diagnostic classifications (no rejection, borderline TCMR, Banff ≥1A TCMR, and AMR). The percentage of patients positive for urine CXCL10 and %dd-cfDNA are based on pre-defined thresholds. The subclinical rejection threshold used for urine CXCL10 was >13 pg/mL for male recipients over 2 weeks post-transplant, >33 pg/mL for female recipients from 2 weeks to 5 months post-transplant, and >13 pg/mL for female recipients >6 months post-transplant. An injury threshold of 0.5% dd-cfDNA was used. DSA is presented as a categorical variable with positivity defined as a minimum of 1000 MFI units of the immunodominant DSA.

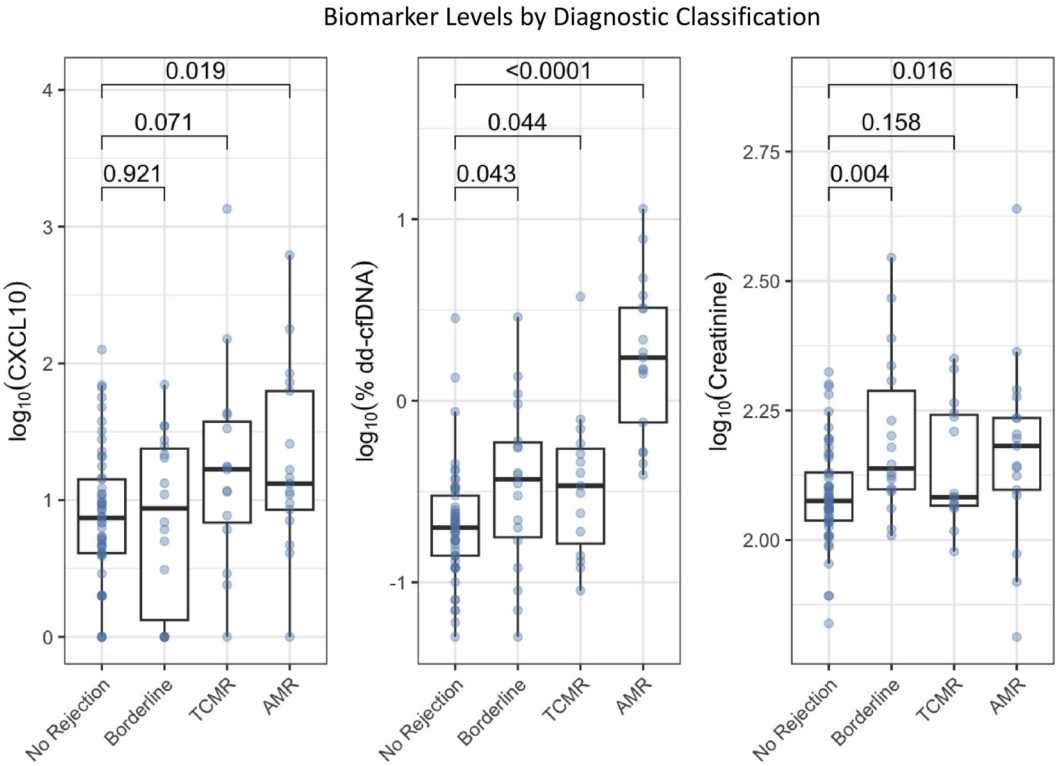


FIGURE 2 | A comparison of log-transformed biomarker levels (urine CXCL10, plasma %dd-cfDNA, and serum creatinine) by diagnostic classification (no rejection, borderline, TCMR, and AMR).

0.725 (95% CI 0.554–0.896) and 0.767 (95% CI 0.604–0.931), respectively. That is, there was an improvement in the diagnosis of Banff ≥1A TCMR with the addition of both urine CXCL10 and %dd-cfDNA to serum creatinine.

BPAR vs. No Rejection

We also assessed performance in the diagnosis of BPAR (AMR and Banff ≥1A TCMR) vs. NR. Models were fit using various combinations (**Figure 6**; **Table 3**). In a multi-predictor logistic regression model, the $\log_{10}$ serum creatinine OR was 17.4 (95% CI 0.160–2640, p = 0.200), $\log_{10}$CXCL10 OR 2.00 (95% CI 0.680–6.32, p = 0.200), DSA OR 8.58 (95% CI 0.870–200, p = 0.092), and $\log_{10}$% dd-cfDNA OR 11.1 (95% CI 2.24–75.1, p = 0.006). Similarly, when dichotomized, dd-cfDNA >0.5% OR 13.4 (95% CI 3.14–75.8, p = 0.001) remained the only significant predictor of BPAR.

AUCs for distinguishing BPAR vs. NR increased from 0.661 (95% CI 0.534–0.787) for $\log_{10}$ serum creatinine alone to 0.833 (95% CI 0.729–0.937) and 0.849 (95% CI 0.756–0.941) when $\log_{10}$ serum creatinine and either $\log_{10}$%dd-cfDNA or dd-cfDNA >0.5% were combined, respectively (**Table 3**). AUC increased to 0.713 (95% CI 0.592–0.834) when $\log_{10}$CXCL10 was added to $\log_{10}$ serum creatinine.

Borderline TCMR vs. No Rejection

Similar analyses were performed comparing borderline TCMR vs. NR. In a multi-predictor logistic regression model, the $\log_{10}$ serum creatinine OR was 939 (95% CI 5.43–309732, p = 0.014), $\log_{10}$CXCL10 OR 1.19 (95% CI 0.360–3.96, p = 0.800), and $\log_{10}$% dd-cfDNA OR 2.59 (95% CI 0.520–14.1, p = 0.200) (**Figure 7**). When the 0.5% dd-cfDNA threshold was used, the $\log_{10}$ serum creatinine OR was 386 (95% CI 1.57–180,000, p = 0.043), $\log_{10}$CXCL10 OR 1.27 (95% CI 0.380–4.33, p = 0.700), and dd-cfDNA >0.5% OR 5.85 (95% CI 1.19–33.5, p = 0.032). In this model, dd-cfDNA >0.5% was a significant independent predictor of borderline TCMR.

AUCs for distinguishing borderline TCMR vs. NR increased from 0.729 (95% CI 0.590–0.868) for $\log_{10}$ serum creatinine alone to 0.768 (95% CI 0.631–0.906) when $\log_{10}$ serum creatinine and dd-cfDNA >0.5% were combined (**Table 4**).

Sensitivity, Specificity and Bivariate Scatter Plot for CXCL10 and dd-cfDNA

Sensitivities and specificities, using the aforementioned thresholds, were calculated for each biomarker alone as well as together, if either were positive, for AMR, Banff ≥1A TCMR, and borderline TCMR (see **Table 5**). While performance characteristics for AMR favored %dd-cfDNA alone, sensitivity for both TCMR and borderline TCMR increased when urine CXCL10 was added to %dd-cfDNA. Sensitivity increased from 0.33 to 0.67 for TCMR and from 0.39 to 0.72 for borderline TCMR. Given each diagnostic category was compared to NR,

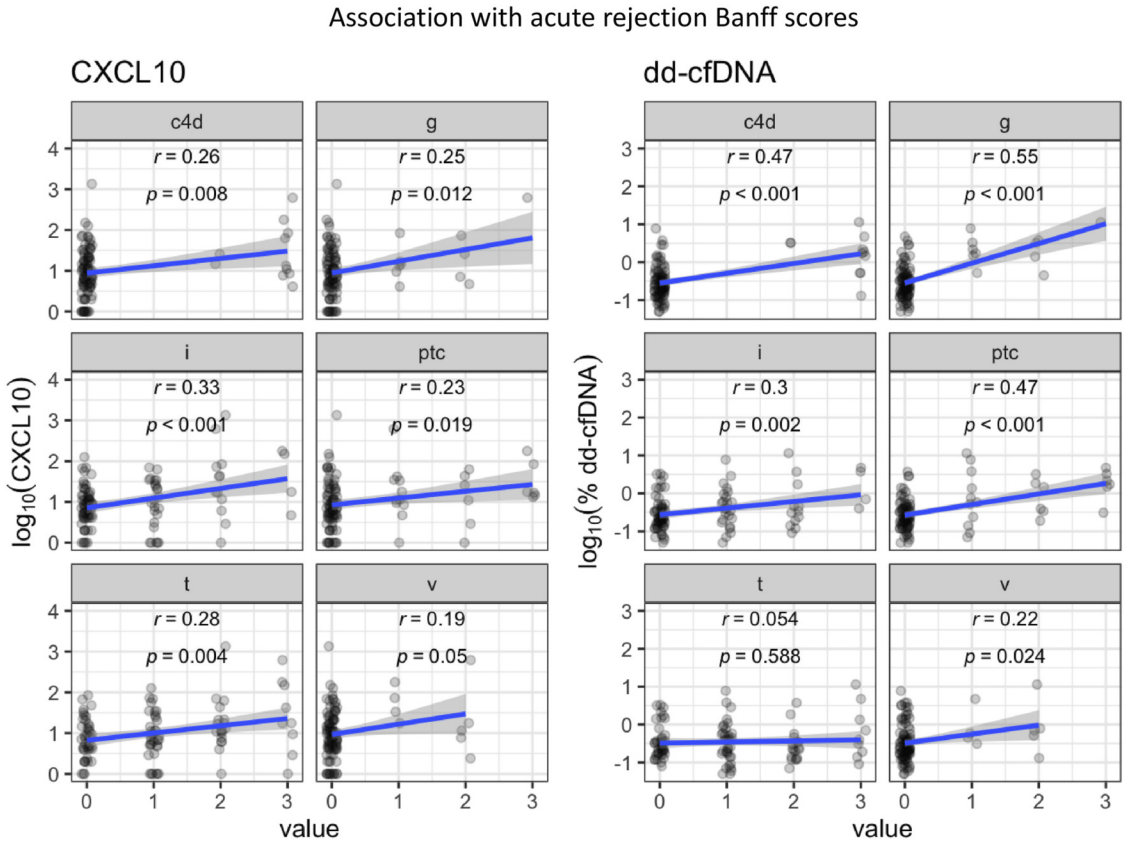


FIGURE 3 | A comparison of the association of log-transformed biomarker levels (urine CXCL10 and plasma %dd-cfDNA) with the Banff scores required to diagnose acute rejection (g = glomerulitis, i = interstitial inflammation, ptc = peritubular capillaritis, t = tubulitis, and v = intimal arteritis).

specificities did not change across groups. A bivariate scatter plot of $\log_{10}$CXCL10 vs. $\log_{10}$%dd-cfDNA, with ellipses centered at the mean values for each group, for AMR, Banff ≥1A TCMR, borderline TCMR, and no rejection (see **Figure 8**) corroborates these observations, with differences associated with AMR favoring dd-cfDNA. For differences associated with Banff ≥1A TCMR, both urine CXCL10 and dd-cfDNA performed modestly. Differences associated with borderline TCMR slightly favored dd-cfDNA.

DISCUSSION

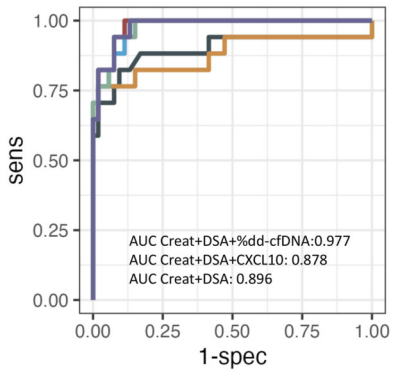
In this case-controlled study, we have shown that %dd-cfDNA is a better non-invasive biomarker of AMR than serum creatinine and urine CXCL10. However, when Banff ≥1A TCMR was assessed, %dd-cfDNA and urine CXCL10 were synergistic. Although performance characteristics for TCMR slightly favored %dd-cfDNA in our logistic regression model, their combination enhanced sensitivity and AUC. Over the last 15 years, various non-invasive diagnostic biomarkers of kidney transplant rejection have been evaluated, with the most data on urine chemokines, plasma dd-cfDNA, and blood gene expression profiling [13, 44]. Positive predictive values have remained low due to the low incidence of rejection under modern immunosuppression, the inherent limitations of histological sampling which may underestimate rejection, and confounders such as infection and non-alloimmune acute kidney injury. As a result, they appear to be more promising as non-invasive "rule out" tests for rejection in stable patients rather than as stand-alone diagnostic tests. At the same time, further study on how these tests may be used as adjuncts to histology are necessary.

Despite advances in our understanding of the diagnostic accuracy of urine CXCL10 and dd-cfDNA in kidney transplant rejection, few studies [45] have simultaneously measured these two biomarkers in the same cohort. In this study we showed urine CXCL10 was a better correlate of tubulitis than %dd-cfDNA and, when compared to serum creatinine alone, independently improved the non-invasive diagnosis of Banff ≥1A TCMR but did not add to AMR detection. Conversely, plasma %dd-cfDNA correlated more strongly than urine CXCL10 with microvascular inflammation and independently improved the non-invasive diagnosis of both AMR and Banff ≥1A TCMR. Overall, our histology correlations are consistent with what others have reported for both

A AMR Logistic Regression Model

Biomarker	OR (95% CI), P value[1]	OR (95% CI), P value[2]
Log_{10} Serum Creatinine	1.33 (0.000445-5480), 0.943	6.98 (0.00417-34,500), 0.619
Log_{10} Urine CXCL10	0.794 (0.0611-6.76), 0.842	1.01 (0.109-8.82), 0.990
DSA	13.0 (1.34-220), 0.027	10.4 (1.16-157), 0.037
Log_{10} %dd-cfDNA[1] or %dd-cfDNA>0.5[2]	40.8 (4.13-925), <0.001	21.9 (3.74-180), <0.001

B AMR Receiver Operating Characteristic Curves



C AMR Akaike Information Criterion Plot

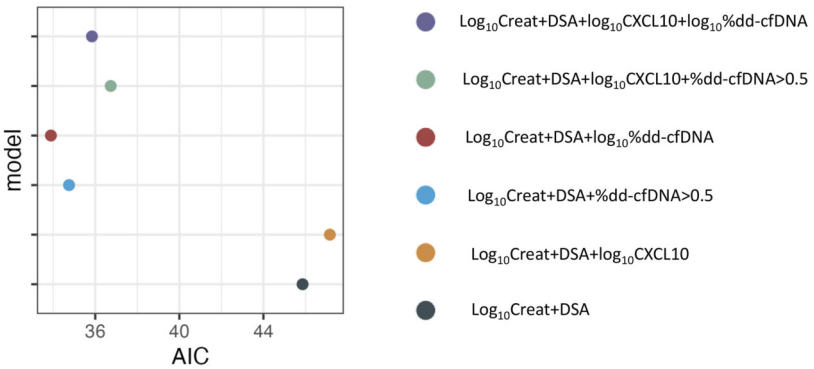


FIGURE 4 | Logistic regression analysis for AMR. **(A)** Odds ratio for log_{10}serum creatinine, log_{10}urine CXCL10, DSA, and either log_{10}%dd-cfDNA or %dd-cfDNA >0.5 using Firths penalized likelihood method. **(B)** Concomitant Receiver Operating Characteristic (ROC) analysis using various combinations of biomarkers. **(C)** Concomitant Akaike Information Criterion (AIC) analysis. (Creat = creatinine).

TABLE 3 | Comparison of AUCs with 95% confidence intervals for all models of AMR, Banff ≥1A TCMR, and BPAR (Banff ≥1A TCMR and AMR), with percent dd-cfDNA included as either a continuous variable or applying a threshold of 0.5%.

Model	AMR (95%CI)	TCMR (95%CI)	BPAR (95%CI)
Log_{10}serum creatinine	0.696 (0.527–0.865)	0.621 (0.452–0.789)	0.661 (0.534–0.787)
DSA	0.844 (0.730–0.957)		0.678 (0.591–0.765)
%dd-cfDNA >0.5	0.913 (0.828–0.998)	0.638 (0.510–0.766)	0.784 (0.693–0.875)
Log_{10} (%dd-cfDNA)	0.973 (0.941–1.000)	0.672 (0.493–0.851)	0.832 (0.729–0.934)
Log_{10} (cxcl10)	0.691 (0.540–0.841)	0.654 (0.482–0.826)	0.674 (0.552–0.796)
Log_{10} serum creatinine + DSA	0.896 (0.774–1.000)		0.763 (0.649–0.878)
Log_{10}serum creatinine + %dd-cfDNA >0.5	0.959 (0.915–1.000)	0.724 (0.565–0.883)	0.849 (0.756–0.941)
Log_{10}serum creatinine + log_{10} (%dd-cfDNA)	0.967 (0.930–1.000)	0.682 (0.497–0.867)	0.833 (0.729–0.937)
Log_{10}serum creatinine + log_{10} (cxcl10)	0.706 (0.544–0.867)	0.717 (0.550–0.884)	0.713 (0.592–0.834)
DSA + %dd-cfDNA >0.5	0.932 (0.847–1.000)		0.791 (0.699–0.883)
DSA + log_{10} (%dd-cfDNA)	0.979 (0.954–1.000)		0.830 (0.728–0.932)
DSA + log_{10} (cxcl10)	0.851 (0.705–0.996)		0.750 (0.631–0.869)
Log_{10} (cxcl10) + %dd-cfDNA >0.5	0.920 (0.808–1.000)	0.725 (0.560–0.889)	0.828 (0.724–0.933)
Log_{10} (cxcl10) + log_{10} (%dd-cfDNA)	0.972 (0.940–1.000)	0.688 (0.516–0.860)	0.831 (0.729–0.934)
Log_{10} (cxcl10) + DSA + %dd-cfDNA >0.5	0.931 (0.820–1.000)		0.829 (0.724–0.934)
Log_{10} (cxcl10) + DSA + log_{10} (%dd-cfDNA)	0.979 (0.953–1.000)		0.829 (0.725–0.933)
Log_{10}serum creatinine + DSA + %dd-cfDNA >0.5	0.977 (0.949–1.000)		0.855 (0.765–0.945)
Log_{10}serum creatinine + DSA + log_{10} (%dd-cfDNA)	0.981 (0.957–1.000)		0.832 (0.729–0.935
Log_{10}serum creatinine + log_{10} (cxcl10) + DSA	0.878 (0.748–1.000)		0.793 (0.682–0.904)
Log_{10}serum creatinine + log_{10} (cxcl10) + %dd-cfDNA >0.5	0.959 (0.915–1.000)	0.767 (0.604–0.931)	0.862 (0.771–0.953
Log_{10}serum creatinine + log_{10} (cxcl10) + log_{10} (%dd-cfDNA)	0.967 (0.930–1.000)	0.725 (0.554–0.896)	0.831 (0.727–0.936)
Log_{10}serum creatinine + log_{10} (cxcl10) + DSA + %dd-cfDNA >0.5	0.978 (0.951–1.000)		0.866 (0.775–0.956
Log_{10}serum creatinine + log_{10} (cxcl10) + DSA + log_{10} (%dd-cfDNA)	0.980 (0.955–1.000)		0.826 (0.720–0.933

%dd-cfDNA and urine CXCL10 [20, 29, 46]. Although Rabant et al. demonstrated that urine CXCL10 independently improves AMR diagnosis, %dd-cfDNA was not included in that analysis [47]. Furthermore, the AUC for AMR in this study, when compared to %dd-cfDNA performance, was relatively modest at 0.702.

A TCMR Logistic Regression Model

Biomarker	OR (95% CI), P-value[1]	OR (95% CI), P-value[2]
Log_{10} Serum Creatinine	95.7 (0.360-41,100), 0.120	91.3 (0.260-44,300), 0.130
Log_{10} Urine CXCL10	2.68 (0.850-9.73), 0.110	2.65 (0.830-9.87), 0.120
Log_{10} %dd-cfDNA[1] or %dd-cfDNA>0.5[2]	3.20 (0.560-22.1), 0.200	5.37 (1.04-31.5), 0.047

B TCMR Receiver Operating Characteristic Curves **C** TCMR Akaike Information Criterion Plot

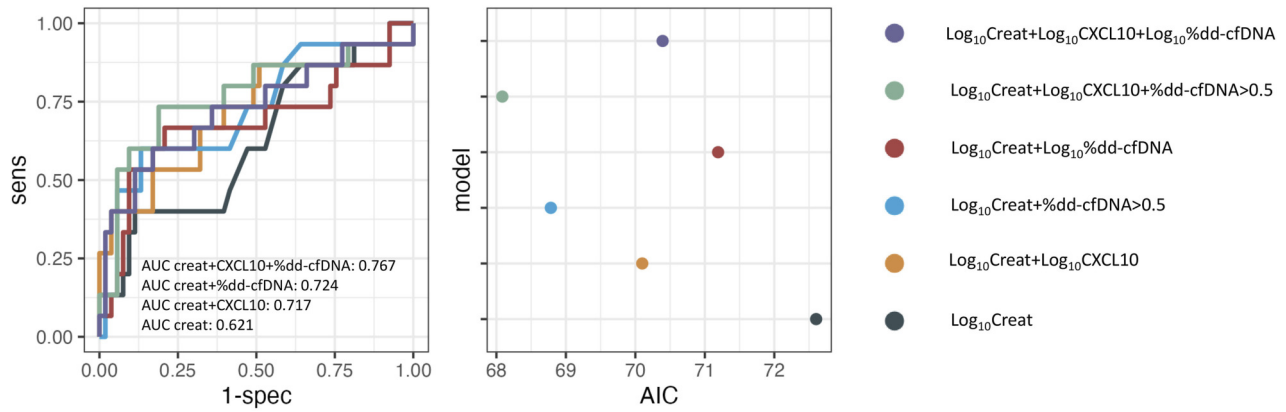


FIGURE 5 | Logistic regression analysis for Banff ≥1A TCMR. **(A)** Odds ratio for log_{10}serum creatinine, log_{10}urine CXCL10, and either log_{10}%dd-cfDNA or %dd-cfDNA >0.5 using logistic regression. **(B)** Concomitant Receiver Operating Characteristic (ROC) analysis using various combinations of biomarkers. **(C)** Concomitant Akaike Information Criterion (AIC) analysis. (Creat = creatinine).

A BPAR (≥1A TCMR and AMR) Logistic Regression Model

Biomarker	OR (95% CI), P value[1]	OR (95% CI), P value[2]
Log_{10} Serum Creatinine	17.4 (0.160-2640), 0.200	68.3 (0.500-13,000), 0.100
Log_{10} Urine CXCL10	2.00 (0.680-6.32), 0.200	2.10 (0.72-6.74), 0.200
DSA	8.58 (0.870-200), 0.092	6.39 (0.58-157), 0.200
Log_{10} %dd-cfDNA[1] or %dd-cfDNA>0.5[2]	11.1 (2.24-75.1), 0.006	13.4 (3.14-75.8), 0.001

B BPAR Receiver Operating Characteristic Curves **C** BPAR Akaike Information Criterion Plot

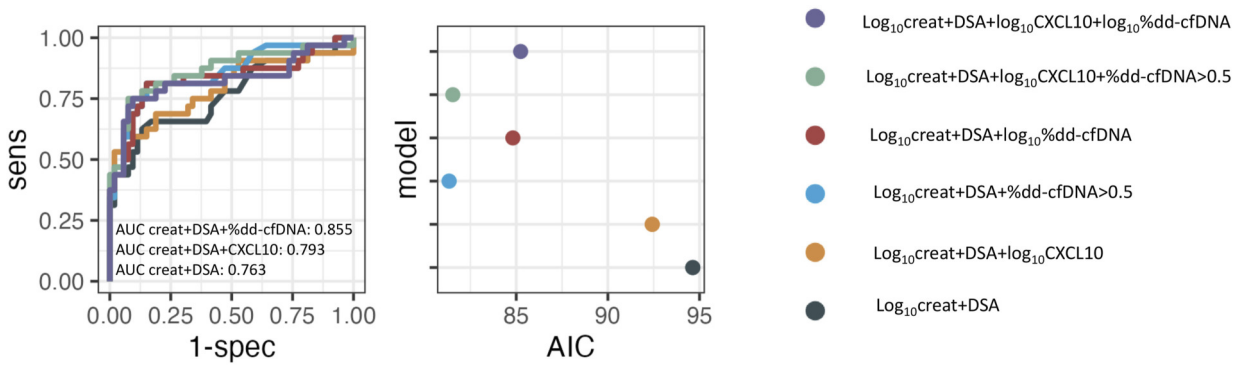


FIGURE 6 | Logistic regression analysis for BPAR (Banff ≥1A TCMR and AMR). **(A)** Odds ratio for log_{10}serum creatinine, log_{10}urine CXCL10, DSA, and either log_{10}% dd-cfDNA or %dd-cfDNA >0.5 using logistic regression. **(B)** Concomitant Receiver Operating Characteristic (ROC) analysis using various combinations of biomarkers. **(C)** Concomitant Akaike Information Criterion (AIC) analysis. (Creat = creatinine).

A Borderline TCMR Logistic Regression Model:

Biomarker	OR (95% CI), P-value[1]	OR (95% CI), P-value[2]
Log_{10} Serum Creatinine	939 (5.73-341,000), 0.014	386 (1.57-180,000), 0.043
Log_{10} Urine CXCL10	1.19 (0.360-3.96), 0.800	1.27 (0.380-4.33), 0.700
Log_{10} %dd-cfDNA[1] or %dd-cfDNA>0.5[2]	2.59 (0.520-14.1), 0.200	5.85 (1.19-33.5), 0.032

B Borderline TCMR Receiver Operating Characteristic Curves **C** Borderline TCMR Akaike Information Criterion Plot

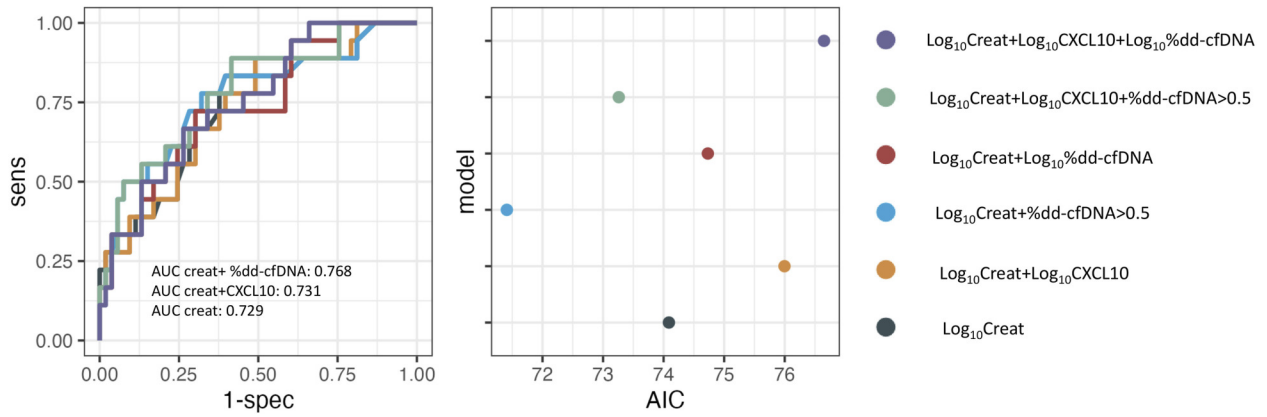


FIGURE 7 | Logistic regression analysis for Borderline TCMR. **(A)** Odds ratio for log_{10}serum creatinine, log_{10}urine CXCL10, and either log_{10}%dd-cfDNA or %dd-cfDNA >0.5 using logistic regression. **(B)** Concomitant Receiver Operating Characteristic (ROC) analysis using various combinations of biomarkers. **(C)** Concomitant Akaike Information Criterion (AIC) analysis. (Creat = creatinine).

TABLE 4 | Comparison of AUCs with 95% confidence intervals for all models of borderline TCMR with percent dd-cfDNA included as either a continuous variable or applying a threshold of 0.5%.

Model	AUC	95% CI
Log_{10}serum creatinine	0.729	0.590–0.868
%dd-cfDNA >0.5	0.666	0.546–0.786
Log_{10} (% dd-cfDNA)	0.661	0.486–0.836
Log_{10}(CXCL10)	0.508	0.329–0.687
Log_{10}serum creatinine + %dd- cfDNA >0.5	0.768	0.631–0.906
Log_{10}serum creatinine + log_{10} (%dd-cfDNA)	0.737	0.603–0.871
Log_{10}serum creatinine + log_{10}(CXCL10)	0.731	0.595–0.866
Log_{10}(CXCL10) + % dd-cfDNA >0.5	0.724	0.573–0.876
Log_{10}(CXCL10) + log_{10} (% dd-cfDNA)	0.647	0.471–0.822
Log_{10}serum creatinine + log_{10}(CXCL10) + % dd-cfDNA >0.5	0.781	0.654–0.908
Log_{10}serum creatinine + log_{10}(CXCL10) + log_{10} (%dd- cfDNA)	0.753	0.627–0.879

Overall, this study—the first to simultaneously measure these two novel biomarkers in a meticulously characterized clinical-pathological cohort including protocol biopsies—demonstrates that urine CXCL10 may not be helpful as a diagnostic biomarker of AMR when plasma %dd-cfDNA and DSA are available. Our study also adds to prior work demonstrating that %dd-cfDNA improves the non-invasive detection of AMR independently of serum creatinine and DSA [10, 39].

In contrast, both urine CXCL10 and %dd-cfDNA were modest predictors of Banff ≥1A TCMR. These data are consistent with recent studies showing modest performance for both urine CXCL10 and %dd-cfDNA for detecting borderline TCMR and/or Banff ≥1A TCMR [28, 35, 36, 48]. The large proportion of TCMR without vascular lesions in our study may explain the lower %dd-cfDNA diagnostic performance compared to other studies [29]. The decrease in performance of urine CXCL10 when %dd-cfDNA was included in our multivariable analysis suggests some diagnostic overlap. This may be explained by the fact that both correlate similarly with interstitial inflammation (**Figure 3**). In addition, the inclusion of biopsies with low-grade inflammation in the NR group, 40% of which had tubulitis scores ≥ t1 and would have met the 2015 definition of borderline, although appropriate, may have decreased the performance characteristics of urine CXCL10.

TABLE 5 | Sensitivity and specificity for %dd-cfDNA and urine CXCL10 in AMR, Banff ≥1A TCMR, and borderline TCMR.

AMR vs. NR	Sensitivity	Specificity
%dd-cfDNA positive	0.88 (0.64–0.99)	0.94 (0.84–0.99)
CXCL10 positive	0.53 (0.28–0.77)	0.75 (0.62–0.86)
%dd-cfDNA or CXCL10 positive	0.94 (0.71–1.00)	0.72 (0.58–0.83)
TCMR vs. NR		**Sensitivity**
%dd-cfDNA positive		0.33 (0.12–0.62)
CXCL10 positive		0.53 (0.27–0.79)
%dd-cfDNA or CXCL10 positive		0.67 (0.38–0.88)
Borderline vs. NR		**Sensitivity**
%dd-cfDNA positive		0.39 (0.17–0.64)
CXCL10 positive		0.44 (0.22–0.69)
%dd-cfDNA or CXCL10 positive		0.72 (0.47–0.90)

The percentage of patients positive for urine CXCL10 and dd-cfDNA are based on pre-defined thresholds. The subclinical rejection threshold used for urine CXCL10 was >13 pg/mL for male recipients over 2 weeks post-transplant, >33 pg/mL for female recipients from 2 weeks to 5 months post-transplant, and >13 pg/mL for female recipients >6 months post-transplant. A threshold of 0.5% dd-cfDNA was used.

Nonetheless, its greater sensitivity compared to %dd-cfDNA for both Banff ≥1A and borderline TCMR supports its potential as a screening tool. Although other diagnostic advantages of urine CXCL10 may lie in its higher correlation with tubulitis, its early identification of post-transplant infections such as BK and ease of sample collection, in our study, %dd-cfDNA, due to its higher specificity, more effectively categorized low-grade inconsequential inflammation such as isolated tubulitis as no rejection [15, 16]. As a result, one approach might be to use urine CXCL10 and %dd-cfDNA sequentially to more accurately non-invasively detect Banff ≥1A and borderline TCMR. Given that both of these rejection phenotypes are clinically important due to the risk of developing persistent chronic active TCMR, *de novo* DSA, or AMR [49–51], defining optimally targeted screening strategies remains a priority.

Limitations of our study include potential over-estimation of diagnostic performance based on a curated case-control cohort with exclusion of non-rejection diagnoses. The cohort consisted of 47% clinical indication biopsies wherein the decision to biopsy was already made using serum creatinine and clinical judgement. Furthermore, as centers that perform protocol biopsies, there is a relative over-representation of more severe rejection phenotypes (AMR and Banff ≥1A TCMR) compared to borderline TCMR. While borderline TCMR is the most common rejection phenotype we see within the first year (unpublished data), AMR is less frequent yet increases over time. In addition, while urine CXCL10 was selected due to having an assay with well-developed performance characteristics and pre-analytical sample handling data and the potential to be used in clinical labs [37], exclusion of urine CXCL9, a urine chemokine with more favorable diagnostic characteristics [48] is a limitation. Further, due to the small size of our cohort, we were unable to attempt to distinguish among AMR subsets, e.g., our AMR group includes a heterogeneous mix of mixed rejection as well as DSA negative, c4d negative microvascular inflammation (MVI). We do know that these phenotypes have different long-term prognoses and that performance characteristics of the biomarkers may differ [52]. Finally, this exploratory study was not designed or powered to assess whether absolute biomarker levels reflect degree of injury and whether changes post-therapy reflect resolution vs. persistent inflammation. Nevertheless, this analysis offers new and important insights into the relative associations of urine CXCL10 and plasma dd-cfDNA with different types of rejection.

Taken together, the potential for individualized biomarker-guided screening strategies for AMR and TCMR deserves further investigation in larger unselected prospective observational cohort studies to provide real-world diagnostic performance characteristics.

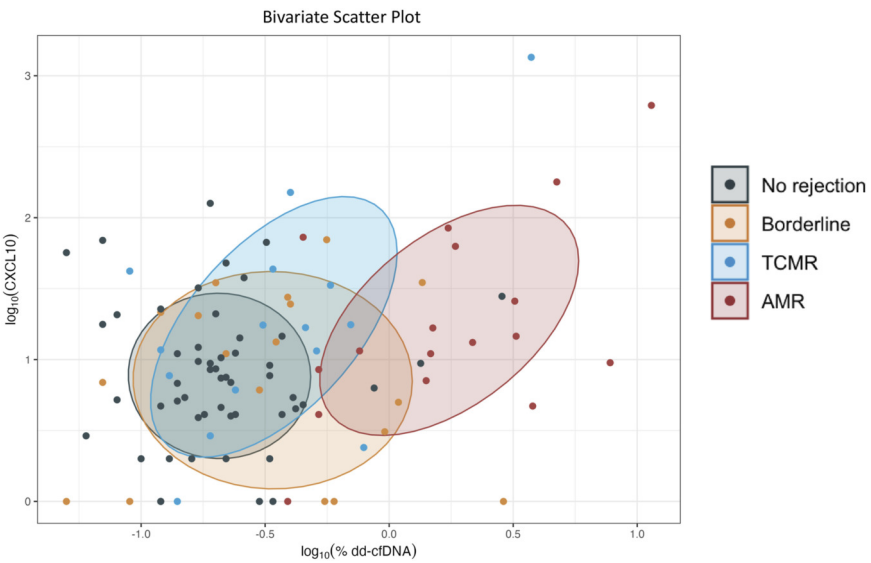


FIGURE 8 | Bivariate scatter plot of $\log_{10}$ urine CXCL10 versus $\log_{10}$ %dd-cfDNA for no rejection, borderline TCMR, Banff ≥1A TCMR, and AMR. Ellipses are centered at the mean values in each target group.

Such studies will enhance practical clinical implementation of these biomarkers into post-transplant care [44, 53].

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving humans were approved by Research ethics board of the Centre hospitalier de l'Université de Montréal. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DF and RB: Participated in research design, writing of the paper, the performance of the research, and data analysis. CS, NT, TV, and CD: Participated in performance of the research, contributed reagents and analytic tools, and participated in data analysis. MB: Participated in performance of the research. FG: Participated in performance of the research and contributed reagents and analytic tools. JB: Participated in performance of the research and participated in data analysis. SC, CL, and HC: Participated in writing of the paper and data analysis. JH: Participated in research design and writing of the paper, contributed reagents and analytic tools, and participated in data analysis. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

NT, TV, and SC were employed by the company CareDx.

The remaining author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

GENERATIVE AI STATEMENT

The author(s) declared that generative AI was not used in the creation of this manuscript.

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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.2026.15517/full#supplementary-material

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ORIGINAL RESEARCH
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Discovery of Donor-Derived Exosomal DNA as an Exploratory Biomarker of Kidney Graft Rejection: A Cross-Sectional Study

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Circulating donor DNA has emerged as a valuable tool for clinical decision-making in kidney transplantation. While most studies focus on cell-free DNA, the role of donor DNA associated with extracellular vesicles (EVs) remains unexplored. To address this, we analyzed donor-derived exosomal DNA (dd-exoDNA) in 100 kidney transplant recipients (KTR) undergoing surveillance or indicated biopsies. Serum exosomes were isolated using precipitation-based technology, and dd-exoDNA was analyzed via digital PCR targeting donor/recipient HLA-DRB1 mismatches. Dd-exoDNA levels were higher in rejection versus non-rejection (2.66 [0.56–7.10] $\times 10^{-3}$ vs. 0.69 [0.28–1.71] $\times 10^{-3}$, p = 0.004) and were associated with Banff score items: glomerulitis ≥1 (p = 0.037), peritubular capillaritis ≥1 (p = 0.040), and tubulitis ≥2 (p = 0.043). In multivariate analysis, dd-exoDNA remained independently associated with rejection, although with wide confidence intervals (OR [95%CI] 3.68 [1.32–10.26], P = 0.013). Exploratory threshold analyses suggested moderate discriminative performance. These findings indicate that donor DNA associated with circulating EVs may offer complementary information to existing biomarkers, warranting validation in external cohorts and comparison with established assays.

Keywords: biomarker, exosomal DNA, exosomes, extracellular vesicles, kidney transplant

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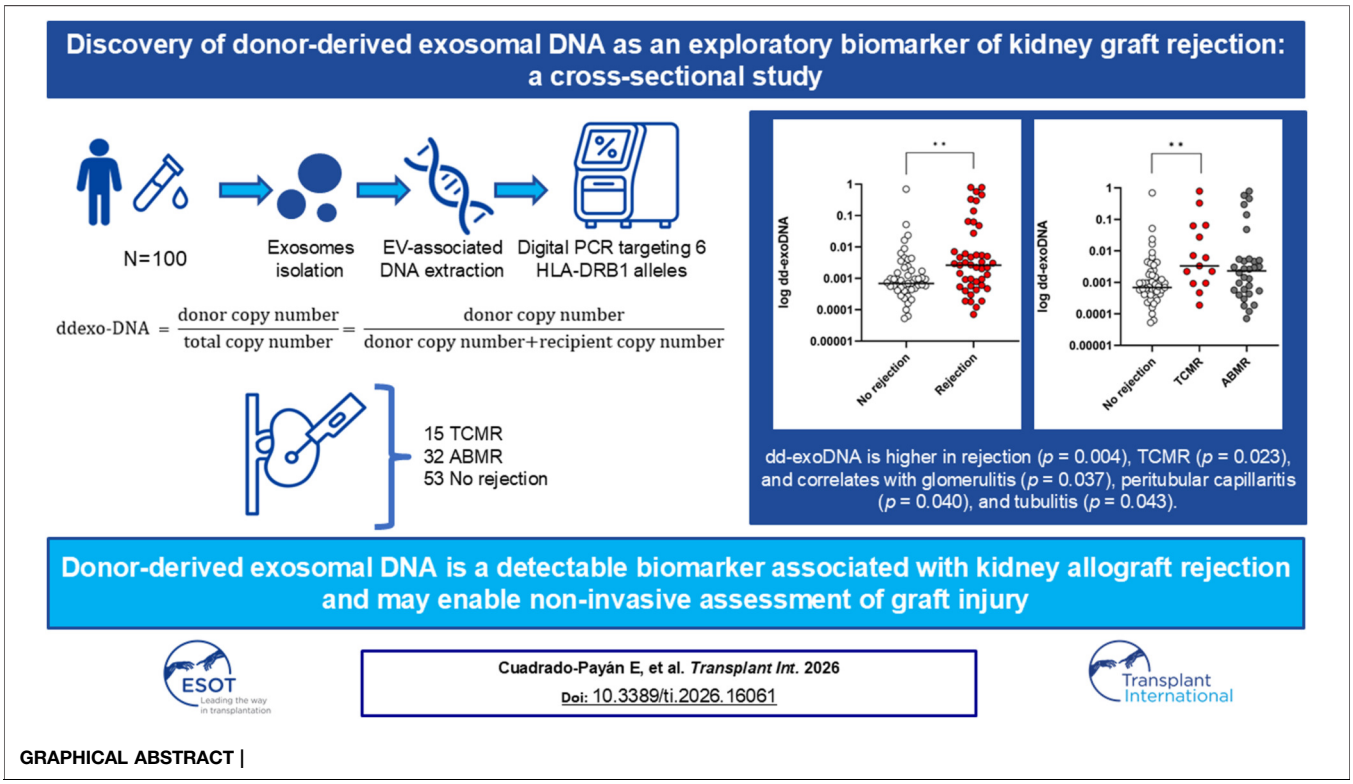
INTRODUCTION

Early identification of allograft rejection is essential for timely treatment and improved graft outcomes. Although biopsy remains the diagnostic gold standard, its invasive nature limits both routine and repeated use [1]. Consequently, recent efforts have focused on non-invasive biomarkers to enable earlier detection of rejection [1–3].



GRAPHICAL ABSTRACT |

While most studies have focused on circulating cell-free DNA (cfDNA) as a non-invasive biomarker, its performance is limited by variable specificity. Although emerging approaches allow inference of tissue of origin, sensitivity remains suboptimal, particularly for T cell–mediated rejection (TCMR) [4].

In this context, extracellular vesicles (EVs), particularly exosomes, have emerged as valuable sources of biologically informative circulating signals [5, 6]. Produced in the endosomal compartment of both resting and activated cells, exosomes play a crucial role in intercellular communication [6, 7]. They carry a diverse molecular cargo, including nucleic acids, proteins, and lipids, that reflects the molecular landscape of the parental cells [8]. In kidney transplantation, exosomes mediate innate and adaptive immune responses [9, 10], facilitating antigen presentation via the semi-direct pathway and transmitting allogeneic or tolerogenic signals, highlighting their involvement in immune processes relevant to graft rejection [9–14]. Building on this, exosomes have been shown to participate in tissue repair [15], regulate fibrosis [16], modulate renal injury [17], and reflect residual humoral immunity after desensitization [18]. However, exosomes should be interpreted as dynamic mediators of intercellular signaling rather than direct indicators of cell death or tissue injury.

Notably, exosomes have been shown to carry circulating DNA protected from degradation by their phospholipid bilayer, although EV-associated DNA may also localize on the vesicle surface [19–23]. While exosome DNA has shown promise as a biomarker in certain types of tumors [24–26], infections [19, 27], pregnancy [28], and prenatal diagnosis [29], its biological function and diagnostic value in solid organ transplantation remain largely unexplored, particularly regarding its potential to monitor kidney graft health.

Given these gaps, our study was designed as an exploratory, proof-of-concept evaluation to investigate the existence, detectability, and preliminary diagnostic relevance of donor-derived exosomal DNA (dd-exoDNA) by identifying HLA-DRB1 mismatches within circulating exosomes as an unequivocal signature of the donor's origin [30] and evaluating whether this signal correlates with biopsy-proven rejection, the clinical gold standard.

MATERIALS AND METHODS

Patient Recruitment and Sample Collection

This single-center, cross-sectional study conducted at Hospital Clínic Barcelona analyzed serum and histological samples from KTRs who underwent graft biopsies between 2018 and 2023. Inclusion criteria required age >18 years, a pathological diagnosis other than borderline rejection, and HLA-DRB1 alleles with available commercial probes. Exclusion criteria included pregnancy, multi-organ or dual kidney transplants, donor–recipient pairs identical for all four HLA-DRB1 alleles that could not be distinguished using this panel, and borderline rejection due to heterogeneity and limited diagnostic reproducibility. In patients with previous grafts, prior HLA-DR was not shared with the current donor.

Biopsies were performed for routine surveillance at 3 and 12 months post-transplantation or for clinical indications suchs as declining renal function, increasing proteinuria, or newly detected donor-specific antibodies (DSAs). Both surveillance and for-cause biopsies were included to evaluate whether dd-exoDNA correlates with histologic rejection, regardless of clinical presentation. Analyses were stratified by biopsy indication to account for potential differences in biomarker performance.

Serum samples were collected immediately before biopsy in serum separator tubes with gel separator (BD Vacutainer SST), aliquoted, and stored at −80 ˚C until processing.

Comprehensive demographic, clinical, laboratory, and immunological data were collected from electronic health records. The Pathology Department at Hospital Clínic de Barcelona carried out histological assessments of all biopsy samples, strictly adhering to the 2019 Banff classification criteria [31].

The study adhered to the Declaration of Helsinki. Pre-analytical procedures for sample collection, handling, and storage adhered to the guidelines established by the International Society for Extracellular Vesicles [32]. The local Ethical Committee approved the study (registry code HCB/2022/0044). All transplant procedures were performed in accordance with the Declaration of Istanbul.

Exosome Isolation, Quantification, and Characterization

Exosomes were isolated using the precipitation method with the ExoGAG kit (Nasasbiotech, Spain), which interacts with surface glycosaminoglycans [33]. Serum samples were centrifuged at 2000g for 10 min. 2 mL of supernatant were mixed with ExoGAG reagent at a ratio of 1:2, incubated at 4 ˚C for 5 min, and centrifuged at 23,500 g for 30 min. EV pellet was resuspended in 200 µL of 0.1 µm-filtered phosphate-buffered saline (PBS).

Particle concentration and size distribution were determined by nanoparticle tracking analysis (NTA) using a NanoSight LM10 instrument (Malvern, UK) equipped with a 488 nm Blue laser and a SCMOS camera. For each sample, five 50-second videos were captured. Data were analyzed with NTA software v3.4. Exosomes' morphology was assessed by transmission electron microscopy (TEM), as previously described by our group [34, 35].

Phenotypic characterization was performed using the MACSPlex Human Exosome Kit (Miltenyi Biotec), following the manufacturer's recommendations. A total of 1×10^9 EVs were incubated with 15 µL of antibody-coated beads overnight, followed by APC-labeled antibodies against CD9, CD63, and CD81 for 1 h. Data were acquired with a BD LSRFortessa SORP cytometer and analyzed using FACS DIVA software (BD Biosciences). Median fluorescence intensity (MFI) for each marker was normalized to average tetraspanin levels per sample.

Exo-DNA Isolation and Pre-Amplification

ExoDNA was extracted using QIAamp DNA Mini Kits (Qiagen), following the manufacturer's instructions, including protease treatment to reduce non-vesicular protein–DNA complexes potentially co-isolated during ExoGAG precipitation. No DNase I treatment of intact EVs was performed prior to lysis; therefore, the assay captures total EV-associated DNA, including both intravesicular DNA and DNA associated with the vesicle surface. Concentration and DNA integrity were assessed using Qubit single-strand molecular probes (Invitrogen) and an Agilent 4200 TapeStation System (Agilent), respectively, at the Functional Genomics Platform of the August Pi Sunyer Biomedical Research Institute (IDIBAPS). Quality thresholds were verified, with a main peak at ~160–180 bp. DNA quantity was normalized to EV yield and stored at −80 ˚C.

HLA-Specific Probes

HLA alleles in exoDNA were detected and quantified via dPCR using a panel of six TaqMan probes targeting specific HLA-DRB1 alleles (Bio-Rad): HLA-DRB1*01, HLA-DRB1*07, HLA-DRB1*08, HLA-DRB1*11, HLA-DRB1*13, and HLA-DRB1*15, based on Zout et al [36]. Each probe had HEX and FAM versions, enabling simultaneous detection of donor and recipient alleles. Of note, the HLA-DRB1*15 assay was also specific for the HLA-DRB1*16 allele. As previously described, 2 ng of exoDNA were pre-amplified using SSoAdvancedPreAmp Supermix (Bio-Rad) [36, 37].

QuantStudio Absolute Q Digital PCR Assay

Digital PCR assays were conducted using the QuantStudio Absolute Q platform (Thermo Fisher Scientific), with microfluidic array plates for sample partitioning, following the protocol reported by Zou et al [36] and the HLA Expert Design datasheet. Each 9 µL reaction included 1X dPCR Master Mix, allele-specific probes, and exoDNA. PCR cycling conditions: 95 ˚C for 3 min, 40 cycles of 94 ˚C for 10s and 55 ˚C for 30s, followed by 72 ˚C for 5 min. Data were analyzed using the Absolute Q Digital PCR Software.

Fraction Calculation

The percentage of dd-exoDNA was calculated according to the following formula, as previously described for dd-cfDNA [36]:

$$\text{dd-exoDNA (fraction)} = \frac{\text{donor copy number}}{\text{total copy number}} = \frac{\text{donor copy number}}{\text{donor copy number} + \text{recipient copy number}}$$

The number of copies for alleles present with two copies per genome was adjusted to a single copy per diploid genome. For alleles shared by the donor and recipient, calculations were adjusted assuming equal allele contributions per genome.

Statistical Analysis

Due to the exploratory nature of the study and the absence of prior dd-exoDNA data, no formal sample size calculation was performed. The cohort included 100 patients with well-defined histological diagnoses, comparable to those in early-phase biomarker studies [38]. The Kolmogorov-Smirnov test was used to assess normality. Normally distributed variables are presented as mean ± standard deviation (SD), and non-

TABLE 1 | Main baseline characteristics of the included patients.

Variable	N = 100
Recipient age at transplantation (years)	58.7 ± 14.6
Sex (male)	64 (64)
Recipient race	
Caucasian	85 (85)
Hispanic	10 (10)
Black	3 (3)
Asian	2 (2)
Etiology of CKD	
Unknown or uncertain	26 (26)
Glomerular disease	21 (21)
Urological	14 (14)
ADPKD	12 (12)
Diabetic nephropathy	10 (10)
Congenital nephropathy	8 (8)
Nephroangiosclerosis	6 (6)
Other	4 (4)
Interstitial nephropathy	3 (3)
Previous transplant	
0	80 (80)
1	16 (16)
2	3 (3)
3	1 (1)
cPRA	
0%	44 (44)
1%–49%	18 (18)
50%–89%	15 (15)
90%–98%	10 (10)
99%–100%	13 (13)
Donor type	
Living	24 (24)
Brain death	33 (33)
Type II circulatory death	9 (9)
Type III circulatory death	34 (34)
Induction immunosuppression	
Thymoglobulin	44 (44)
Basiliximab	56 (56)
Maintenance immunosuppression	
Prednisone + Tacrolimus + MMF	39 (39)
Prednisone + Tacrolimus + mTORi	61 (61)
Histological diagnosis	
Nonspecific changes	28 (28)
IFTA	16 (16)
Chronic vascular changes	7 (7)
Diabetes-related complications	2 (2)
ABMR	32 (32)
TCMR	15 (15)
DSAs at biopsy	16 (16)

Data are expressed as mean ± standard deviation or absolute number and percentage (ADPKD, autosomal dominant polycystic kidney disease; CKD, chronic kidney disease; cPRA, panel reactive antibodies; DSA, donor-specific antibodies; MMF, Mycophenolate mofetil; mTORi, mTOR inhibitors; IFTA, interstitial fibrosis with tubular atrophy; ABMR, antibody-mediated rejection; TCMR, T cell-mediated rejection).

normally distributed variables as median and interquartile range (IQR). Qualitative variables are expressed as absolute and relative frequencies.

Between-group comparisons used Student's t-test, Mann-Whitney U, or Kruskal-Wallis tests, and χ^2or Fisher's exact test as appropriate. When the Kruskal–Wallis test was applied, post-hoc pairwise comparisons were conducted using Dunn's test with Bonferroni correction for multiple comparisons. ROC curves were used to evaluate diagnostic performance (AUC, 95% CI), with optimal thresholds determined by Youden's Index. Associations were assessed with Spearman correlation.

For the MACSPlex analysis of 37 surface markers, unadjusted p values were first calculated using the Mann–Whitney U test. To control the false discovery rate, p values were adjusted using the Benjamini–Hochberg procedure; both unadjusted and adjusted p values are reported.

The clinical utility of dd-exoDNA was evaluated using Decision Curve Analysis (DCA) with logistic regression models including DSA and eGFR, with or without dd-exoDNA. Covariates for multivariable logistic regression were selected *a priori* based on clinical relevance. Sensitivity analyses confirmed robustness. No correction for multiple comparisons was applied for Banff lesion analyses due to the exploratory design; therefore, these findings should be interpreted as hypothesis-generating.

Statistical analyses were performed using IBM SPSS Statistics v31.0 (IBM Corp., Armonk, NY), except for DCA, which was conducted using the rmda package in R (v4.4.1). Graphs were generated with GraphPad v9.5.1 (GraphPad Software, La Jolla, CA). Two-tailed $p < 0.05$ was considered statistically significant.

RESULTS

Baseline Characteristics of the Population

This study included serum and allograft biopsies from 100 KTR, comprising 34 surveillance and 66 clinically indicated biopsies performed at a median of 4 [1–13.5] months post-transplant. The baseline characteristics of the included patients are summarized in **Table 1**.

The mean age of this cohort at the time of transplant was 58.71 ± 14.6 years, with predominantly male sex (64.0%) and recipients of a first transplant in 80.0% of cases. Thirty-eight patients had positive panel reactive antibodies (cPRA) ≥50% at the time of biopsy.

Histopathological analysis revealed rejection in 47 biopsies. Among these, 15 were TCMR, and 32 were classified as antibody-mediated rejection (ABMR). No mixed rejection cases were identified. Of the ABMR cases, 11 met Banff criteria and had detectable DSAs. The remaining 21 cases were classified as ABMR based on the presence of microvascular inflammation and/or positive C4d staining. TCMR cases were further classified into 8 acute and 7 chronic-active. Regarding ABMR, 22 cases were active, and 10 were chronic-active.

Among the 53 biopsies without evidence of rejection, the most frequent findings were non-specific changes (28 cases) and interstitial fibrosis with tubular atrophy (IFTA, 16 cases). Other diagnoses included chronic vascular changes (7 cases) and diabetes-related complications (2 cases).

Quantitative and Qualitative Evaluation of Circulating Serum Exosomes

Serum exosomes were quantified by NTA, and TEM revealed their typical round shaped membrane particles (**Figures 1A,B**).

Exosomes concentration was 1.26×10^{11} [0.883×10^{11}-1.67×10^{11}] particles/mL. When stratified by biopsy results, exosome

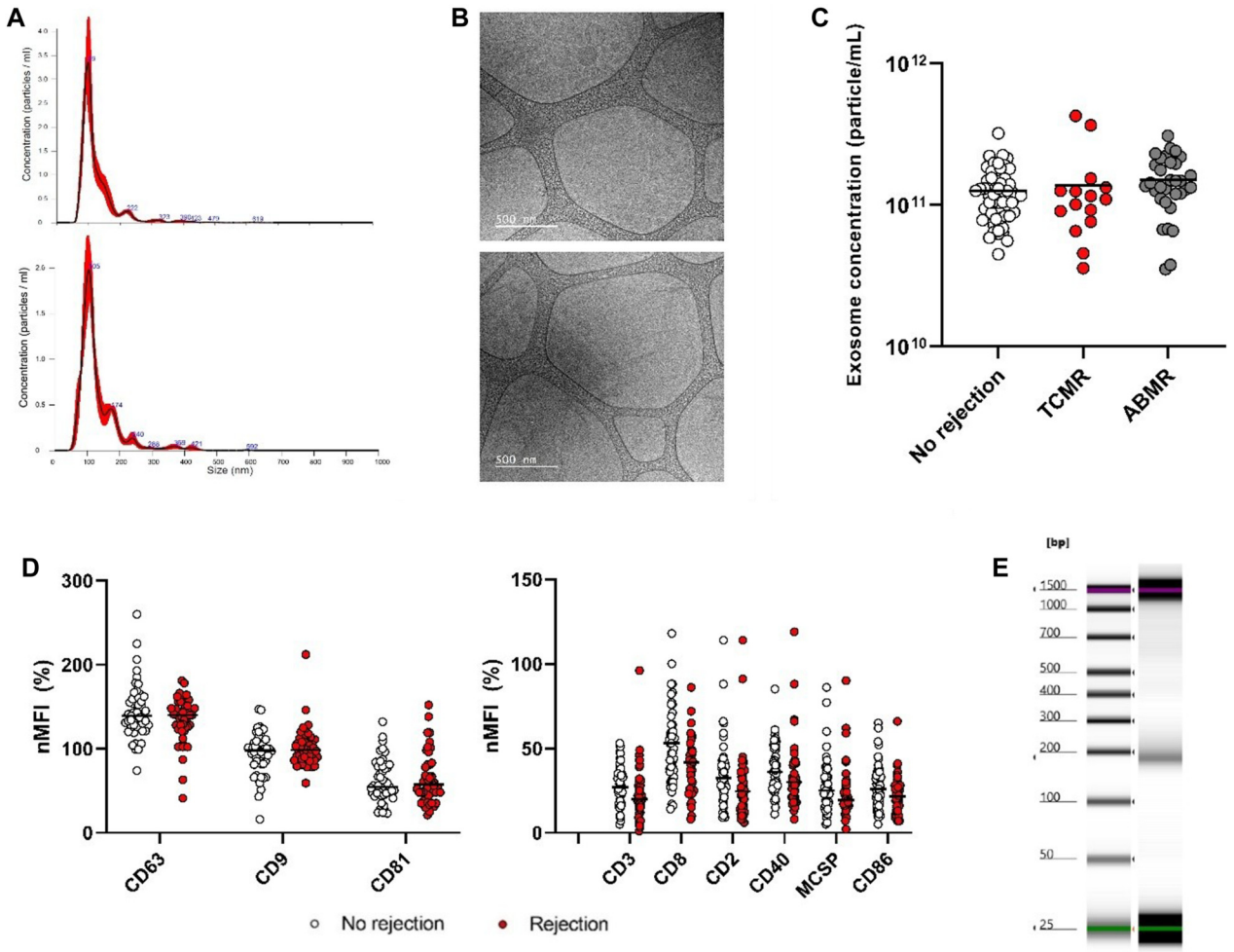


FIGURE 1 | Quantification and characterization of serum exosomes (TCMR; T cell-mediated rejection, ABMR; antibody-mediated rejection, nMFI; normalized median fluorescence intensity). One-way Kruskal-Wallis test followed by multiple pairwise comparisons was used. **(A)** Representative size distributions of exosomes in one sample from the rejection (upper panels) and the non-rejection group (lower panels) with Nanoparticle Tracking Analysis. **(B)** Transmission electron microscopy visualization of the grid with an irregular distribution of hole sizes and shapes containing exosomes of various sizes (scale bars 500 nm) from one sample from the rejection (up) and the non-rejection group (down). **(C)** Quantification of serum exosomes depending on biopsy results, showing a non-significant higher concentration in ABMR cases (p = 0.065). **(D)** MFI signal of tetraspanins (left) and common immune cell-derived surface exosomes' antigens (right) detected using the MACSPlex kit according to rejection or not (only 6 out of 37 surface antigens that displayed a nominal (unadjusted)difference between the two groups are shown). **(E)** Electrophoresis of exo-DNA sample, with electronic ladder showing specific sizing standards (left) and exo-DNA showing an average DNA fragment length ranging between 160 and 200 bp (right).

concentrations were numerically higher in cases with rejection compared to those without (1.30×10^{11} particles/mL (9.8×10^{10}–1.84×10^{11} particles/mL) vs. 1.16×10^{11} particles/mL (8.3×10^{10}–1.51×10^{11} particles/mL), although this difference did not reach statistical significance ($p = 0.19$). Further differentiation between cellular and humoral rejection revealed a similar trend toward higher exosome concentrations in ABMR compared to TCMR (1.4×10^{11} [1.16×10^{11}–1.98×10^{11}] vs. 1.1×10^{11} [8.3×10^{10}–1.3×10^{11}] particles/mL; $p = 0.065$) (**Figure 1C**).

Further exosome characterization was performed using the MACSPlex kit, which analyzes 37 common surface and immunological exosome biomarkers. Several markers showed unadjusted differences between rejection and non-rejection groups, with lower median expression levels observed in the rejection group for CD3, CD8, CD2, CD40, MCSP, and CD86 (**Figure 1D**). When stratified by rejection type, CD40 serum exosome antigen was also significantly lower in patients with ABMR than in those without rejection. However, after adjustment for multiple comparisons, no marker remained statistically significant (**Supplementary Table S1**).

Quantification of exoDNA in Stable and Rejection Patients

The average DNA fragment length of exo-DNA ranged between 160 and 200 base pairs (bp) (**Figure 1E**), while the median exoDNA concentration was 0.917 [0.48–1.50] ng/μL. No differences were observed in terms of exoDNA concentrations between patients with and without rejection (1.01 [0.49–1.56] ng/μL for control versus 0.83 [0.37–1.48] ng/μL for rejection ($p = 0.62$). Additionally,

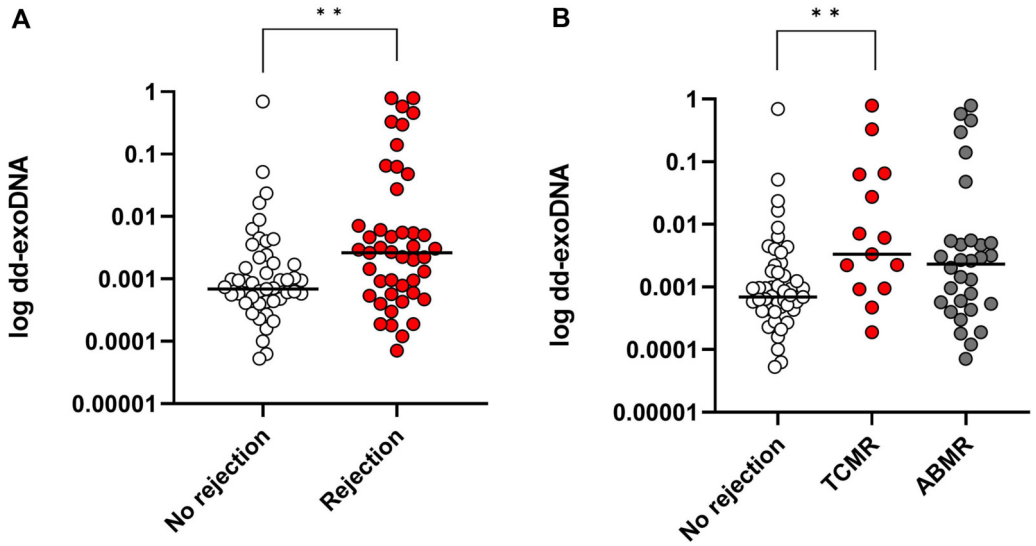


FIGURE 2 | Dd-exoDNA fraction levels according to biopsy results (TCMR; T cell-mediated rejection, ABMR; antibody-mediated rejection). **(A)** Higher dd-exoDNA fraction levels were observed in rejection cases (p = 0.004) compared to no-rejection. **(B)** A significant difference between no-rejection and TCMR group is seen (p = 0.022). One-way Kruskal-Wallis test followed by multiple pairwise comparisons was used.

exoDNA concentrations were higher in TCMR than in ABMR (1.32 [0.58–2.33] vs. 0.75 [0.36–1.40] ng/μL), although this difference was not statistically significant (p = 0.32).

Detection of Donor-Derived ExoDNA (Dd-ExoDNA) and Association With Kidney Graft Rejection

Dd-exoDNA was consistently detected in all patients 0.92 [0.47–1.51] × 10^{-3}). No differences were observed between living donors and deceased donors (1.02 [0.58–3.6] ×10^{-3} vs. 0.92 [0.40–4.56] ×10^{-3}, p = 0.35), nor among the different types of deceased donors. In patients with any-type rejection, dd-exoDNA was significantly higher (2.66 [0.56–7.10] × 10^{-3}) compared to patients without rejection (0.69 [0.28–1.71] × 10^{-3}), with a p = 0.004 (**Figure 2**).

Dd-exoDNA levels showed substantial dispersion within the TCMR group, particularly in chronic-active TCMR, with a right-skewed distribution characterized by a subset of cases exhibiting markedly elevated values. In line with this heterogeneity, the global Kruskal–Wallis test did not reach statistical significance (p = 0.09); however, post-hoc pairwise comparisons identified a significant difference between the no-rejection and TCMR groups (p = 0.022), while no significant differences were observed for the remaining comparisons (**Table 2**).

To evaluate whether extreme observations drove these findings, a sensitivity analysis excluding outliers identified using an IQR–based rule applied to log-transformed dd-exoDNA values was performed. After exclusion, the difference between the TCMR and non-rejection groups was attenuated, indicating that a limited subset of TCMR cases accounted for the elevated dd-exoDNA levels.

TABLE 2 | Dd-exoDNA levels across different rejection categories.

Rejection category	Dd-exoDNA fraction	p value
No rejection (n = 53)	0.69 [0.28–1.71] × 10^{-3}	Ref
Rejection (n = 47)	2.66 [0.56–7.10] × 10^{-3}	**0.004**
TCMR (n = 15)	3.32 [0.93–45.03] × 10^{-3}	**0.022**
Active (n = 8)	2.78 [0.71–34.38] × 10^{-3}	0.95
Chronic-active (n = 7)	7.09 [1.57–178.74] × 10^{-3}	0.17
ABMR (n = 32)	2.01 [0.49–4.89] × 10^{-3}	0.13
Active (n = 22)	1.20 [0.40–4.74] × 10^{-3}	1
Chronic-active (n = 10)	3.17 [1.32–5.55] × 10^{-3}	0.25

(TCMR, T cell-mediated rejection; ABMR, antibody-mediated rejection; Ref: reference). Data are expressed as median [interquartile range]. One-way Kruskal-Wallis test followed by multiple pairwise comparisons was used. Significantly higher levels of dd-exoDNA were observed among patients with rejection, particularly in those with TCMR. Median dd-exoDNA levels tended to be higher in chronic-active forms, although pairwise comparisons did not reach statistical significance. Bold values indicate statistically significant results (p < 0.05).

Among patients with ABMR, no significant differences in dd-exoDNA levels were observed between DSA-negative and DSA-positive cases (2.63 [0.40–5.43] × 10^{-3} vs. 2.02 [0.77–4.06] × 10^{-3}, respectively; p = 0.755).

Donor-Derived exoDNA (Dd-ExoDNA) Fraction and Banff Classification Scores

The relationship between dd-exoDNA fraction and individual Banff classification scores was also assessed. The median dd-exoDNA was 0.81 [0.40–2.42] × 10^{-3} in the absence of glomerulitis (g), while it was significantly higher (2.43 [0.50–16.87] × 10^{3}) in those with g ≥ 1 (p = 0.037). Similarly, for peritubular capillaritis (ptc), the median dd-exoDNA was 0.92 [0.29–2.63] × 10^{-3} in samples with no inflammatory activity, increasing to 2.82% [0.58–16.47] × 10^{-3}

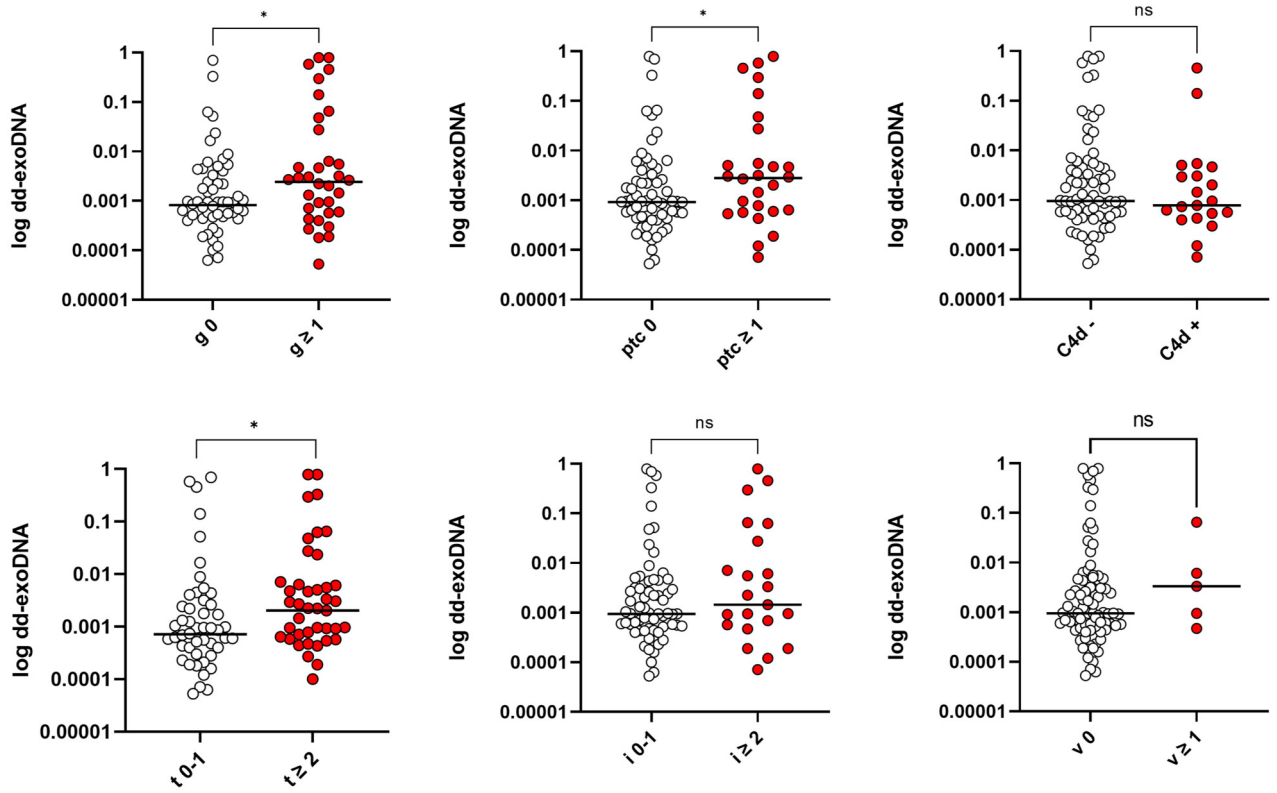


FIGURE 3 | Relationship between dd-exoDNA fraction and individual Banff classification scores (g; glomerulitis, ptc; peritubular capillaritis, t; tubulitis, i; interstitial inflammation, v; vasculitis). Significant differences in dd-exoDNA were observed for glomerulitis (p = 0.037), peritubular capillaritis (p = 0.040), and tubulitis (p = 0.043), but no significant differences were found for complement fraction C4d deposition (p = 0.807), interstitial inflammation (p = 0.45), and vasculitis (p = 0.253). Mann-Whitney U test was used.

in patients with ptc ≥1 (p = 0.040). For tubulitis (t), a significant difference was also observed, with values reaching 2.93 [1.19–17.23] × 10^{-3} in cases with moderate to severe (t ≥ 2), compared to 0.89 [0.40–4.36] × 10^{-3} in patients with mild or absent involvement (p = 0.043). In contrast, no significant differences were found regarding complement fraction C4d deposition (p = 0.807), interstitial inflammation (i) (p = 0.45), and vasculitis (v) (p = 0.253) (**Figure 3**).

Diagnostic Performance of Donor-Derived ExoDNA (Dd-exoDNA)

To assess the diagnostic performance of dd-exoDNA in distinguishing rejection from non-rejection, the area under the ROC curve (AUC) was calculated. The AUC for discriminating rejection from non-rejection was 0.67 (95% CI: 0.56–0.79). For differentiating TCMR or ABMR specifically from non-rejection, dd-exoDNA demonstrated an AUC of 0.67 (95% CI: 0.51–0.83) and 0.60 (95% CI: 0.48–0.73), respectively (**Figure 4**).

Compared with DSAs' diagnostic ability at the time of biopsy, dd-exoDNA showed numerically higher AUC, although the confidence intervals overlapped, as DSAs yielded an AUC of 0.59 (95% CI: 0.47–0.70). Similarly, the estimated glomerular filtration rate (eGFR) showed poor diagnostic accuracy, with an AUC of only 0.37 (95% CI: 0.23–0.51) (**Supplementary Figure S1**).

Notably, dd-exoDNA performance varied depending on biopsy indication. In clinically indicated biopsies, its discriminative ability improved significantly (AUC 0.70 [95% CI: 0.57–0.83]), whereas for surveillance biopsies, the AUC was lower (0.52 [95% CI: 0.30–0.74]) (**Supplementary Figure S2**). It should be noted that among the 34 surveillance biopsies, only 5 were rejection cases, compared with 42 among the 66 clinically indicated biopsies.

We aimed to determine a clinically significant threshold for identifying samples potentially indicative of kidney graft rejection. Using a threshold of 1.9 × 10^{-3}, dd-exoDNA showed a specificity of 77.4% and a sensitivity of 57.4% for distinguishing rejection from non-rejection. Among the 100 dd-exoDNA determinations, 39 were above the 1.9 × 10^{-3} threshold. Of these, 27 corresponded to patients with biopsy-proven rejection, while the remaining 12 were from patients without rejection. The positive predictive value (PPV) for identifying rejection was 69.2%, while the negative predictive value (NPV) was 67.2%. Among patients without histological rejection, those with exoDNA values above the 90th percentile showed heterogeneous biopsy findings. Two patients presented chronic graft lesions, including moderate chronic vascular changes and moderate interstitial fibrosis with

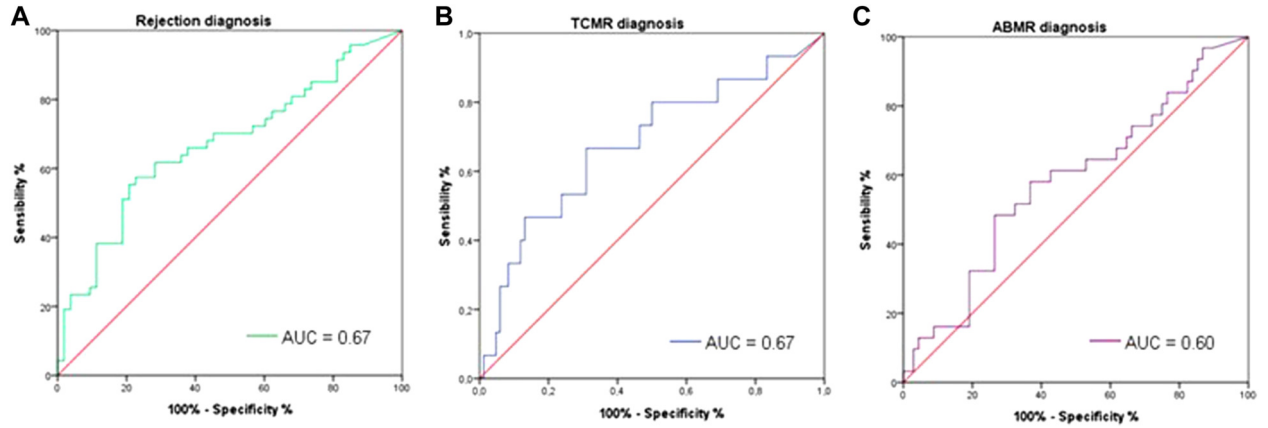


FIGURE 4 | ROC curves for rejection diagnosis of dd-exoDNA fraction. (TCMR; T cell-mediated rejection, ABMR; antibody-mediated rejection). **(A)** An area under the curve of 0.67 (95% CI: 0.56–0.79) for distinguishing rejection from non-rejection is seen (green line). **(B)** For diagnosis of TCMR, dd-exoDNA shows an area under the curve of 0.67 (95% CI: 0.51–0.83) (blue line). **(C)** For ABMR, the area under the curve is 0.60 (95% CI: 0.48–0.73) (purple line).

TABLE 3 | Univariable and multivariable logistic regression analysis of factors associated with rejection (cPRA, panel reactive antibodies; DSA, donor-specific antibodies; mTORi, mTOR inhibitors; MMF, Mycophenolate mofetil; eGFR, estimated glomerular filtration rate; OR, odds ratio).

Variables	Univariable		Multivariable	
	OR [95% CI]	p value	OR [95% CI]	p value
Sex (male)	0.98 [0.43–2.23]	0.97		
Age	1.00 [0.98–1.03]	0.73		
Previous transplants	0.70 [0.26–1.89]	0.48		
cPRA	1.00 [0.99–1.01]	0.67		
Donor type (deceased vs. living)	0.68 [0.27–1.72]	0.42		
Induction immunosuppression (thymoglobulin vs. basiliximab)	1.55 [0.69–3.44]	0.28		
Maintenance immunosuppression (mTORi vs. MMF)	0.32 [0.14–0.73]	**0.007**	0.4 [0.15–1.18]	0.101
Biopsy indication (clinical vs. surveillance)	10.15 [3.47–29.69]	**<0.001**	7.43 [2.21–25.02]	**0.001**
DSAs at biopsy	4.20 [1.25–14.11]	**0.02**	3.96 [0.84–18.52]	0.080
eGFR at biopsy	0.96 [0.94–0.99]	**0.004**	0.97 [0.94–1.005]	0.094
dd-exoDNA ≥1.9 x 10^{-3}	4.61 [1.94–10.95]	**0.001**	3.68 [1.32–10.26]	**0.013**

Bold values indicate statistically significant results ($p < 0.05$).

tubular atrophy. In contrast, the remaining three showed no histological evidence of rejection or other relevant abnormalities.

To further assess whether dd-exoDNA fraction was independently associated with rejection, we conducted a multivariable logistic regression analysis adjusting for relevant clinical and immunological variables. In the adjusted model, dd-exoDNA remained independently associated with rejection, with an odds ratio (OR) of 3.68 (95%CI 1.32–10.26, p = 0.013). Biopsies performed for clinical indication were also significantly associated with rejection (OR 7.43, 95% CI 2.21–25.02, p = 0.001). Maintenance immunosuppression with mTOR inhibitors, although associated with a lower risk of rejection in the univariable analysis (OR 0.32, p = 0.007), did not retain statistical significance after adjustment (OR 0.4, p = 0.101). Likewise, eGFR at biopsy and DSAs at biopsy did not remain statistically significant after adjustment (**Table 3**). Decision curve analysis showed that the model including dd-exoDNA provided a higher net benefit than the baseline model (DSA + eGFR) across a range of probability thresholds (**Figure 5**).

DISCUSSION

Unlike other extensively studied kidney biomarkers [1, 5, 39–41], the role of dd-exoDNA remains largely unexplored, offering an opportunity to deepen our understanding of exosome biology and its clinical implications in kidney transplantation. Studies in heart and lung transplantation suggest that donor-derived exosomes may detect rejection before histological changes, although standardized assays and large-scale validation are still lacking [5, 42, 43]. To our knowledge, this single-center, cross-sectional study is the first to report the detection of donor-specific HLA, particularly donor-derived HLA-DR alleles, in exosomes isolated from KTR serum using dPCR. Although exploratory and proof-of-concept, our findings support the biological plausibility

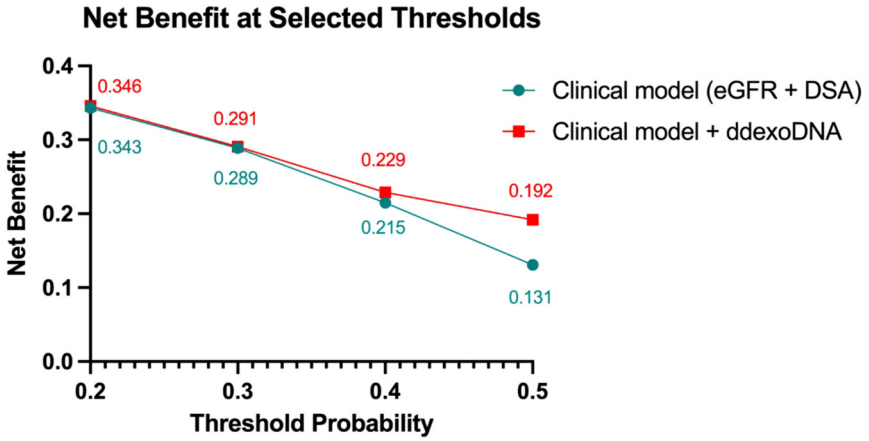


FIGURE 5 | Decision Curve Analysis of Predictive Models. (eGFR: estimated glomerular filtration rate, DSA: donor-specific antibodies). Decision Curve Analysis comparing the net benefit of the clinical model (eGFR + DSA) versus the combined model including dd-exoDNA. The model with dd-exoDNA showed higher net benefit across clinically plausible thresholds (e.g., 0.20–0.30). Higher thresholds are shown for completeness.

of dd-exoDNA as a promising complementary biomarker of allograft injury.

HLA-DR was selected due to its clinical relevance, as mismatches in this allele play a critical role in kidney allograft rejection and survival [44], and its limited polymorphism allows broad population coverage through validated probes [36, 37].

Moreover, the association between dd-exoDNA levels and biopsy-proven rejection supports its potential complementary role. Patients with TCMR tended to show higher dd-exoDNA levels, a finding that should be interpreted cautiously given the limited subgroup size and wide confidence intervals, particularly in chronic-active forms, which encompass variable degrees of ongoing inflammation and tissue injury. This variability likely reflects biological heterogeneity within TCMR. Increased dd-exoDNA levels may relate to the immunogenic role of renal tubular epithelial cells (RTECs), which can express HLA-DR under pathological conditions, including allograft rejection [45–49]. Previous studies have demonstrated HLA-DR expression in a substantial proportion of tubular cells in TCMR, supporting their role in antigen presentation and immune modulation [47, 49]. As RTECs are a major source of exosomes in the kidney microenvironment, their heightened immunogenic activity may contribute to increased exosomal DNA release in TCMR.

In contrast, the absence of differences between DSA-positive and DSA-negative ABMR cases likely reflects the biological heterogeneity of ABMR, where microvascular injury may occur independently of circulating antibodies. The substantial overlap observed between rejection and non-rejection groups is consistent with the biological heterogeneity of graft injury and supports the interpretation of dd-exoDNA as a complementary biomarker rather than a standalone diagnostic test.

The correlations observed between dd-exoDNA and selected Banff lesions (g, ptc, t) provide additional mechanistic support. These lesions represent endothelial and tubular inflammatory injury, both of which could contribute donor-derived material to the EV compartment. Significantly higher values in tubulitis, glomerulitis, and peritubular capillaritis suggest dd-exoDNA may also derive from endothelial injury, as endothelial cells can upregulate class II MHC expression under inflammatory conditions [50]. These observations align with prior studies of cell-free DNA dynamics [41, 51] and reflect the complexity of exosomal DNA release in both tubular and microvascular compartments. This discrepancy likely reflects differences between continuous lesion scores and categorical diagnoses, as well as the greater biological heterogeneity of ABMR compared with high-grade TCMR. Some biopsies classified as non-rejection despite elevated dd-exoDNA levels showed chronic or injury-related features that did not meet formal Banff rejection criteria, suggesting that dd-exoDNA may capture graft injury-related processes not fully reflected in categorical diagnoses. These observations should therefore be viewed as hypothesis-generating.

In terms of diagnostic performance, dd-exoDNA showed modest discrimination between rejection and non-rejection, consistent with early-phase non-invasive biomarkers [1, 39], particularly in TCMR, where current biomarkers have well-recognized limitations [39, 40, 51]. Predictive values were influenced by the relatively high rejection prevalence in our cohort (47%), whereas sensitivity and specificity indicated moderate diagnostic accuracy. Together, these findings support dd-exoDNA as a complementary biomarker rather than a stand-alone diagnostic tool with performance likely dependent on clinical context and pretest probability. Performance improved in clinically indicated biopsies, where pretest probability of rejection and the severity of histological lesions are typically higher. In contrast, performance in surveillance biopsies was limited, consistent with the small number of rejection cases and the expected challenge of detecting subtle subclinical injury in a cross-sectional design.

Dd-exoDNA positivity ($\geq 1.9 \times 10^{-3}$) remained independently associated with rejection, even after adjustment for potential confounders including biopsy indication, immunosuppressive regimen, DSA status, eGFR at biopsy, and baseline immunological risk (OR 3.68, 95% CI 1.32–10.26, $p = 0.013$).

Although statistically significant, the wide confidence intervals indicate limited precision of effect estimates, likely reflecting the modest sample size and the number of rejection events. Accordingly, effect magnitude should be interpreted cautiously and considered exploratory pending validation in larger cohorts. In decision curve analysis, including dd-exoDNA improved net benefit relative to the baseline model across probability thresholds. Clinically relevant benefit was mainly observed at lower thresholds (≈0.20–0.30), which better reflect real biopsy decision ranges, whereas higher thresholds primarily illustrate model behavior.

Beyond DNA quantification, we explored whether EV surface antigen profiles differed between rejection and non-rejection samples. Several immune-related markers (CD3, CD8, CD2, CD40, MCSP, and CD86) showed nominally lower expression in rejection; however, none remained significant after correction for multiple comparisons, underscoring the exploratory nature of the MACSPlex analysis. Despite this, the consistent directionality across markers suggests that EV surface phenotypes may reflect underlying immunological processes during rejection. The trend toward lower CD40 expression in ABMR is biologically plausible, given its role in antigen presentation and B-cell activation, but requires confirmation in larger cohorts. Prior studies have demonstrated that EV surface profiles may carry prognostic information in solid organ transplantation, including in cardiac [7] and kidney transplant [52] settings. Importantly, by leveraging donor–recipient HLA-DR mismatches, our study uniquely assigns graft origin to circulating EV-associated DNA, providing a framework for future integrated immune monitoring.

This work must be interpreted in the context of several limitations. First, the relatively small sample size and cross-sectional design preclude assessment of temporal dynamics and limit subgroup analyses and causal inferences. Second, DNase treatment was not performed prior to vesicle lysis; therefore, the assay quantifies total EV-associated donor-derived DNA rather than intravesicular DNA alone. Although current evidence suggests that a substantial fraction of EV-associated DNA may be localized on the vesicle surface, this approach does not allow definitive discrimination between surface-bound and intraluminal DNA fractions. In addition, the low DNA input limits analytical resolution at low donor fractions, potentially affecting patient-level interpretation and threshold robustness. Third, the reliance on HLA-DRB1 mismatches limits applicability in donor–recipient pairs identical for this locus (10.5% in our cohort), underscoring the need for expanded probe panels targeting additional HLA loci to enable broader clinical implementation. Fourth, the mixed inclusion of surveillance and clinically indicated biopsies generated a non-representative rejection prevalence, which directly influences predictive values. Therefore, PPV and NPV estimates should be considered context-specific and not extrapolated to broader transplant populations without external validation. Moreover, while ROC-based discrimination (AUC) is not mathematically dependent on disease prevalence, the mixed case-mix may introduce spectrum effects that influence AUC estimates; thus, the observed discrimination should also be interpreted as context-specific. Finally, the cost-effectiveness of dd-exoDNA testing remains to be established, given the specialized equipment required. From a practical perspective, assay costs are mainly driven by HLA-specific probes and access to digital PCR technology. Using precipitation-based exosome isolation and dPCR, the workflow can be completed within a single working day, allowing short turnaround times and potential integration into centralized clinical laboratories.

In conclusion, despite these limitations, our findings provide proof of principle that donor-derived DNA can be reliably detected in the EV-enriched fraction of serum and is associated with histological graft injury. As interest in the diagnostic and therapeutic potential of extracellular vesicles continues to grow, dd-exoDNA represents a biologically grounded and technically feasible avenue for non-invasive immune surveillance. Future studies should incorporate longitudinal sampling, mechanistic characterization of exosomal DNA release, integration with established biomarkers like dd-cfDNA, and validation in external cohorts. Such work will determine whether dd-exoDNA can ultimately complement existing tools to refine rejection monitoring and enhance precision in post-transplant care.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The local Ethical Committee approved the study (registry code HCB/2022/0044). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

EC-P, DC, and IR designed the hypothesis and objectives, drafted the original protocol, analyzed the data and wrote the manuscript; EC-P, MR-B, EB-M, and JR organized sample collection, analysis, data generation and interpretation; EC-P, NH, DS-J, and MA collaborated on sample collection and processing; DC, IR, MR-B, EB-M, JB and FD collaborated on protocol draft and data analysis; EM-H, DR-E, CA, AM-A, AG-R, NE, VT, and PV-A recruited patients and contributed to data analysis; EG-R and EP revised the protocol draft and contributed to data analysis. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

The author(s) declared that this work 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.2026.16061/full#supplementary-material

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Risk Assessment of Delayed Graft Function in Pediatric Kidney Transplantation – a CERTAIN Research Network Analysis

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Delayed graft function (DGF) in pediatric kidney transplantation is a serious complication with negative impact on graft survival. Currently, there are no reliable methods available to assess the risk of DGF in children. We performed a retrospective analysis of data from the Cooperative European Paediatric Renal Transplant Initiative (CERTAIN) registry to develop a DGF risk assessment model for pediatric kidney transplantation, based on parameters available within the first 24 h post-transplant. The model was developed by forward selection and logistic regression. This study included n = 694 patients. The overall rate of DGF was 8.5%. The following key parameters were selected for the DGF risk assessment model: (i) occurrence of post-transplant surgical complications, (ii) immediate graft urine production, (iii) rate of change in recipient's serum creatinine, (iv) initial calcineurin inhibitor therapy. The significance of these parameters was confirmed by calculating adjusted odds ratios. In the training cohort and the internal validation cohort the ROC-AUCs were 0.9043 and 0.878. This multivariable model based on early post-transplant parameters can predict the occurrence of DGF in pediatric kidney transplant recipients with high accuracy and may facilitate future interventional trials of targeted pharmacological strategies against ischemia-reperfusion injury in this population.

Keywords: delayed graft function, risk assessment model, pediatric kidney transplantation, post-transplant complications, ischemia-reperfusion injury

Abbreviations: CERTAIN, Cooperative European Paediatric Renal Transplant Initiative; DGF, Delayed graft function; HLA, Human leukocyte antigen; ROC-AUC, Area under the receiver operating characteristic curve.

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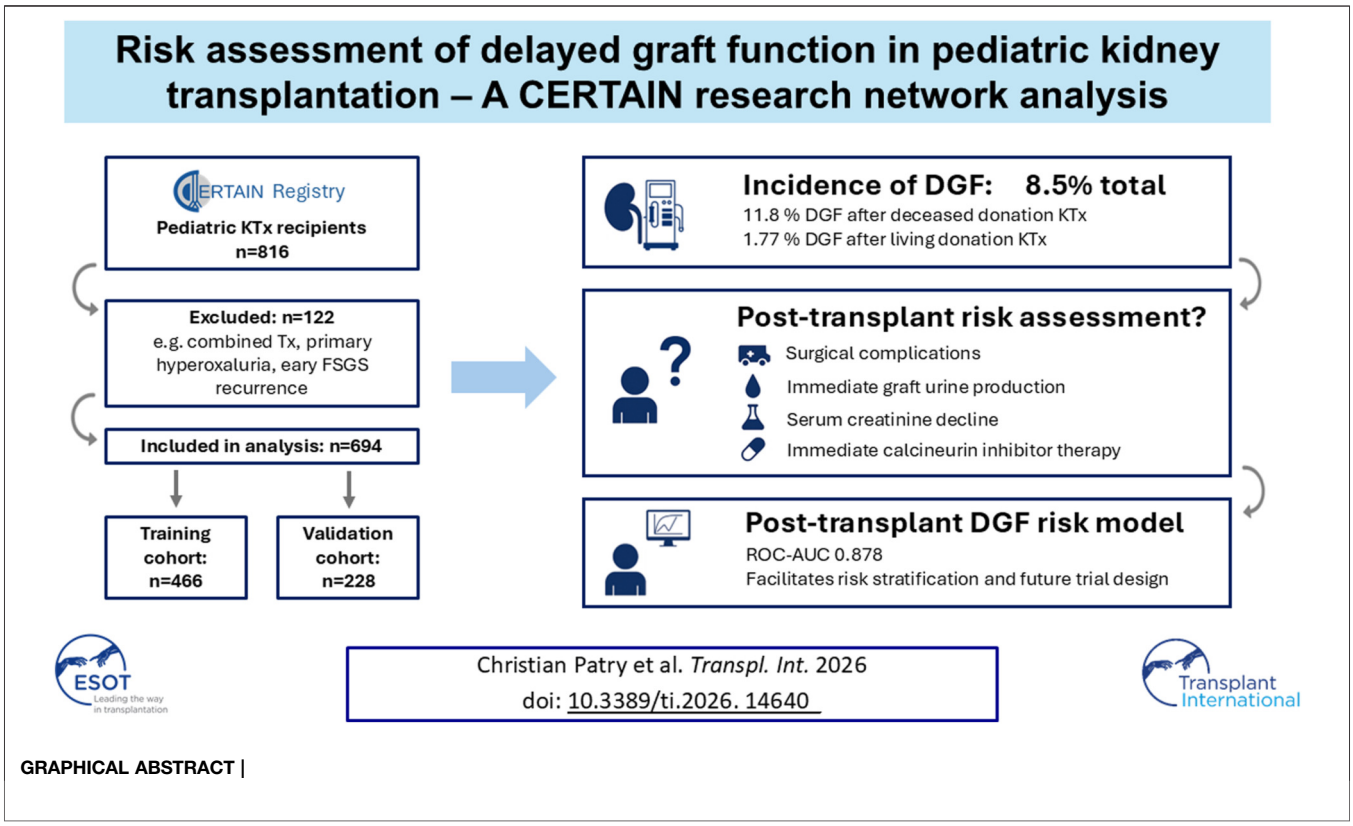

GRAPHICAL ABSTRACT |

INTRODUCTION

Delayed graft function (DGF) in newly transplanted kidneys is currently defined as the need for dialysis within the first 7 days after transplantation [1]. In children, the reported incidence of DGF after deceased donor transplantation ranges from 7.5% to 19.7%, with a decreasing incidence in recent years [2]. According to data from UK and US databases, it is lower in living-related transplants (4%) [2, 3]. DGF in children is associated with adverse long-term outcomes, such as higher rates of rejection and decreased graft survival [4, 5].

DGF is primarily caused by ischemia-reperfusion injury, which involves microvascular inflammation, the production of reactive oxygen species and cell death [6]. Currently, both preclinical and clinical research is exploring potential therapeutic targets to intervene early in these pathways [7, 8]. Since DGF is a risk factor for graft dysfunction in both short- and long-term follow-up [4], children with a high likelihood of experiencing ischemia-reperfusion injury and subsequent occurrence of DGF may benefit from such therapeutic strategies for ischemia-reperfusion injury in the first days after transplantation surgery. Methods to identify this respective high-risk pediatric subpopulation early in the initial clinical course after transplantation would facilitate future interventional clinical trials with targeted treatment options and could also provide additional guidance for immediate patient-specific management post-transplant. A limited number of studies in adults have examined factors associated with ischemia-reperfusion injury for their potential to aid in the assessment of the likelihood of the occurrence of DGF, such as delayed graft urine production [9–11]. However, factors associated with DGF in adult kidney transplant recipients cannot be uncritically extrapolated to children, as important variables unique to the pediatric population may be overlooked. In pediatric kidney transplantation, there is a complete lack of statistical models based on post-transplant parameters that could rapidly identify those children who are likely to have experienced ischemia-reperfusion injury and therefore are at increased risk of developing DGF.

Therefore, we designed this study based on data from the Cooperative European Paediatric Renal Transplant Initiative (CERTAIN) registry [12–14]. We had two aims: First, we wanted to investigate peri- and post-transplant parameters that might be associated with graft ischemia-reperfusion injury and thus might help predict the occurrence of DGF. Second, we wanted to develop a predictive model for DGF in children based on these peri- and/or post-transplant parameters.

MATERIALS AND METHODS

Patients and Data Collection

This retrospective, multicenter, longitudinal cohort study included pediatric kidney transplant recipients enrolled in the

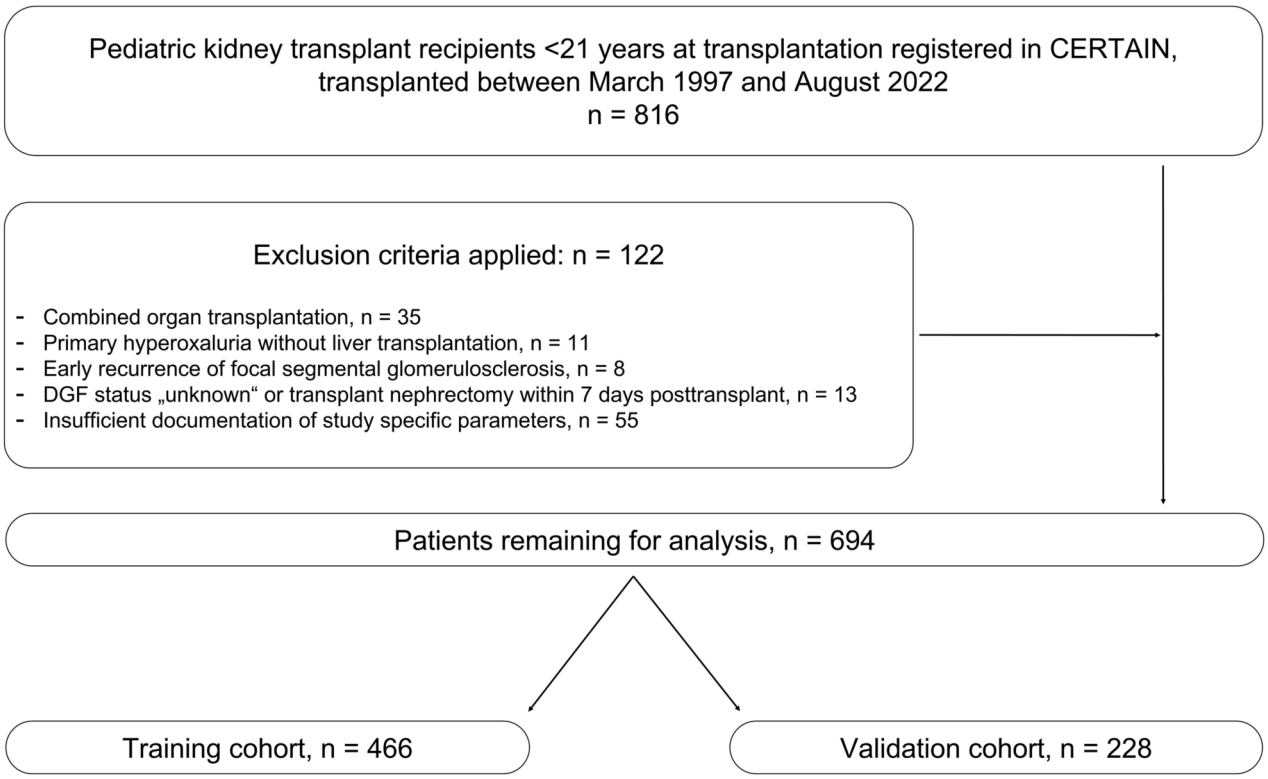

FIGURE 1 | Study flowchart for the development of the post-transplant pediatric DGF risk assessment model. Post-transplant data were evaluated specifically for this model in n = 816 patients. After application of exclusion criteria, n = 694 patients remained, which were further divided 2:1 into a training cohort and a validation cohort. DGF, delayed graft function.

CERTAIN registry. The CERTAIN registry collects detailed longitudinal clinical and laboratory data and applies rigorous validity checking procedures[1]. Participation in the CERTAIN registry was approved by the ethics committee at each center. Informed consent was obtained from the parents or legal guardians prior to enrollment, with assent from patients when appropriate for their age. This general ethics approval for participation in the registry fully covers the use of the collected data for this registry-based study. This general ethics approval for the registry fully covers the use of the collected data for the present study. The time points of data collection, and the corresponding time intervals were as follows: baseline (pre-transplant), at months 1, 3, 6, 9, 12 and every 6 months thereafter up to 5 years post-transplant. One graft per patient was analyzed. A more detailed description of the CERTAIN registry with respect to data quality and completeness can be found in the **Supplementary Appendix**.

Patients were eligible for the study if they received a kidney transplant before the age of 21 years. Exclusion criteria were early graft nephrectomy within the first week after transplantation, primary hyperoxaluria as primary kidney disease, combined transplants, or early recurrence of focal segmental glomerulosclerosis post-transplant (**Figure 1**).

Study Design

Data from both deceased and living donor transplants in children transplanted between March 1997 and August 2022 were included. The study was designed, analyzed and reported according to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines[2]. Patients who experienced DGF are referred to as the "DGF group," and the others as the "no-DGF group". We focused on risk factors occurring within the first 24 h post-transplant to allow early identification of at-risk patients who might benefit from pharmacologic intervention. The following post-transplant parameters were selected based on evidence from the available literature [9–11] and/or clinical plausibility to be evaluated in recipients during the first 24 h after kidney transplantation surgery: post-transplant complications requiring re-operation, rate of change in recipient's serum creatinine, systolic and diastolic blood pressure, central venous blood pressure, need for vasopressor therapy, urine production, furosemide therapy, and start of calcineurin inhibitor therapy within the first 24 h after

[1]http://www.certain-registry.eu/

[2]https://www.strobe-statement.org

TABLE 1 | Parameters examined in the analysis of peri- and post-transplant risk factors for DGF.

Parameters of interest	DGF, n = 59	no DGF, n = 635	*P* value
Recipient			
Age at kidney transplantation [years], mean ± SD	10.5 ± 5.9	9.8 ± 5.3	0.365
Type of donor kidney, n (%)			0.665
Left donor kidney	27 (47)	311 (50)	
Right donor kidney	31 (51)	317 (50)	
Missing values	1	7	
Arterial vessel anastomosis, n (%)			0.447
Aorta	21 (37)	261 (42)	
Iliac vessels	36 (63)	360 (58)	
Missing values	2	14	
Venous vessel anastomosis, n (%)			0.460
Vena cava	27 (47)	354 (57)	
Vena iliac externa	11 (19)	110 (18)	
Vena iliac communis	13 (23)	98 (16)	
Other venous vessel	6 (11)	58 (9)	
Missing values	2	15	
Surgical complications post-transplant			<0.001
No	51 (88)	616 (99)	
Yes	7 (12)	7 (1)	
Missing value	1	12	
Rate of change in recipient's serum creatinine, mean ± SD (DGF, n = 59; no-DGF, n = 632)	−0.008 ± 0.025	−0.064 ± 0.092	<0.001
Systolic blood pressure, mean ± SD [mmHg]			
Mean pressure between 0 and 12 h (DGF, n = 54; no-DGF, n = 518)	123 ± 18	121 ± 18	0.342
Mean pressure between 12 and 24 h (DGF, n = 52; no-DGF, n = 498)	126 ± 17	123 ± 17	0.336
Minimum pressure between 0 and 12 h (DGF, n = 54; no-DGF, n = 518)	113 ± 21	112 ± 19	0.602
Minimum pressure between 12 and 24 h (DGF, n = 51; no-DGF, n = 487)	121 ± 18	118 ± 18	0.310
Maximum pressure between 0 and 12 h (DGF, n = 54; no-DGF, n = 518)	113 ± 18	130 ± 20	0.290
Maximum pressure between 12 and 24 h (DGF, n = 51; no-DGF, n = 487)	132 ± 19	128 ± 18	0.137
Diastolic blood pressure, mean ± SD [mmHg]			
Mean pressure between 0 and 12 h (DGF, n = 54; no-DGF, n = 518)	68 ± 18	66 ± 15	0.563
Mean pressure between 12 and 24 h (DGF, n = 52; no-DGF, n = 498)	72 ± 16	70 ± 14	0.397
Minimum pressure between 0 and 12 h (DGF, n = 54; no-DGF, n = 518)	61 ± 20	60 ± 16	0.599
Minimum pressure between 12 and 24 h (DGF, n = 51; no-DGF, n = 487)	68 ± 17	65 ± 15	0.252
Maximum pressure between 0 and 12 h (DGF, n = 54; no-DGF, n = 518)	74 ± 18	72 ± 16	0.554
Maximum pressure between 12 and 24 h (DGF, n = 51; no-DGF, n = 487)	77 ± 16	75 ± 15	0.299
MAP, mean ± SD [mmHg]			
Mean pressure between 0 and 12 h (DGF, n = 50; no-DGF, n = 457)	85 ± 17	85 ± 15	0.821
Mean pressure between 12 and 24 h (DGF, n = 50; no-DGF, n = 465)	89 ± 15	88 ± 14	0.752
Minimum pressure between 0 and 12 h (DGF, n = 50; no-DGF, n = 457)	78 ± 18	77 ± 15	0.848
Minimum pressure between 12 and 24 h (DGF, n = 49; no-DGF, n = 451)	85 ± 16	84 ± 15	0.470
Maximum pressure between 0 and 12 h (DGF, n = 50; no-DGF, n = 457)	93 ± 17	93 ± 16	0.836
Maximum pressure between 12 and 24 h (DGF, n = 49; no-DGF, n = 451)	94 ± 15	92 ± 16	0.347
Central venous blood pressure, mean ± SD [mmHg]			
Mean pressure between 0 and 12 h (DGF, n = 40; no-DGF, n = 358)	6.7 ± 3.7	5.1 ± 3.6	0.010
Mean pressure between 12 and 24 h (DGF, n = 39; no-DGF, n = 326)	6.9 ± 3.4	5.7 ± 4.3	0.045
Minimum pressure between 0 and 12 h (DGF, n = 40; no-DGF, n = 358)	5.4 ± 3.9	3.9 ± 3.8	0.031
Minimum pressure between 12 and 24 h (DGF, n = 36; no-DGF, n = 300)	6.6 ± 3.8	4.9 ± 3.7	0.015
Maximum pressure between 0 and 12 h (DGF, n = 40; no-DGF, n = 358)	8.1 ± 3.9	6.3 ± 4.1	0.007
Maximum pressure between 12 and 24 h (DGF, n = 36; no-DGF, n = 300)	7.7 ± 3.4	7.1 ± 7.3	0.411
Vasopressor therapy, n (%)			<0.001
None	20 (35)	302 (56)	
(Nor-)epinephrine	27 (47)	115 (21)	
Dopamine	9 (16)	115 (21)	
Other vasopressors	1 (2)	11 (2)	
Missing values	2	92	
Urine production, n (%)			<0.001
No	27 (69)	64 (17)	
Yes	12 (31)	308 (83)	
Missing values	20	263	
Furosemide therapy, n (%)			0.182
No	8 (15)	122 (23)	
Yes	45 (85)	406 (77)	
Missing values	6	107	
Initial calcineurin inhibitor therapy, n (%)[1]			0.293

(*Continued*)

TABLE 1 | Continued

Parameters of interest	DGF, n = 59	no DGF, n = 635	*P* value
No	3 (6)	16 (3)	
Yes	50 (94)	520 (97)	
Missing values	5	99	

For binary and categorical variables, numbers and percentages are given. For continuous variables, mean and standard deviation are presented. A two-sided t-test was calculated for metric variables, chi-squared test was applied to compare non-metric variables. For all tests P < 0.05 was considered significant. [1]Initial calcineurin inhibitor therapy: 85% tacrolimus, 15% cyclosporin.
MAP, mean arterial pressure.

kidney transplantation (**Table 1**). Parameters not documented in the CERTAIN registry minimum data set were documented in a study-specific electronic case report form designed specifically for this project. In addition, we assessed perioperative parameters specific to this study, namely, "type of donor kidney (left/right)", "type of arterial vessel anastomosis (aorta/iliac)", and "type of venous vessel anastomosis (cava/iliac externa/iliac communis/other)" (**Table 1**). Finally, we developed a DGF risk assessment model based on the analyzed parameters. We report this model according to the guidelines of the Transparent Reporting of a Multivariable Prediction Model for Individual Prognosis or Diagnosis (TRIPOD) statement (see the **Supplementary Appendix**) [15]. In a subcohort of 680 patients (n = 56 in the DGF group, n = 624 in the no DGF group), we investigated whether pre-transplant factors that might influence the occurrence of DGF post-transplant could further enhance the predictive accuracy of the developed prediction model (**Supplementary Figure 1**). The following pre-transplant parameters were included in this extended analysis: pre-transplant dialysis mode, donor type, donor sex, recipient age, duration of cold ischemia time, donor hemodynamic instability (see definition below) and number of HLA-DR mismatches (**Supplementary Table 1**). No patient was treated following a steroid avoidance protocol.

Definitions of Study-Specific Variables

The variable "complications after kidney transplantation requiring reoperation" included at least one of the following events: thrombosis (DGF group, n = 5; no DGF group, n = 2), bleeding (DGF group, n = 3; no DGF group, n = 4), or vascular stenosis (DGF group, n = 0; no DGF group, n = 2) within 24 h post-transplant. If any of these three complications occurred, the patient was classified as having a post-transplant complication. There were no other reasons for reoperation within the first 24 h post-transplant. "Immediate urine production" was defined as urine production by the graft, either during transplant surgery or in the first 24 h thereafter. Urine production was assessed in n = 552 patients (79.5%) by direct drainage through a catheter stenting the graft ureter (n = 507 patients, 79.8% in the DGF group and n = 45 patients, 76.3% in the no DGF group). **Supplementary Table 2** shows the type of graft ureteral stent used. The variable "vasopressor therapy" had four categories, which were "none," "(nor)epinephrine," "dopamine," and "other vasopressors." If the patient received more than one vasopressor including (nor)epinephrine, they were classified as treated with (nor)epinephrine. If the patient received combination therapy that included dopamine but not (nor)epinephrine, the patient was classified as receiving dopamine. If a vasopressor other than (nor)epinephrine or dopamine was used, the patient was classified in the "other vasopressor" category. In n = 17 patients, the parameter: "other vasopressor" included dobutamine. The variable "rate of change in recipient's serum creatinine" was mathematically modeled using **Formula 1**, assuming an exponential decay process:

$$\sqrt[(T)]{1-\frac{\text{recipient's serum crea pretransplant}\left[\frac{\text{mg}}{\text{dl}}\right]-\text{recipient's serum crea at timepoint T posttransplant}\left[\frac{\text{mg}}{\text{dl}}\right]}{\text{recipient's serum crea pretransplant}\left[\frac{\text{mg}}{\text{dl}}\right]}}-1 \quad (1)$$

Definitions: T = time point of recipient's serum creatinine measurement within the first 24 h after transplant surgery [hours]. The time point of graft reperfusion after transplantation surgery was defined as T = 0; the time point 24 h after reperfusion was defined as T = 24. Crea = creatinine.

In the subgroup analysis of pre- and post-transplant predictors of DGF the variable "donor hemodynamic instability" was defined as the presence of at least one of the following parameters: absence of spontaneous cardiac activity, occurrence of hypotensive episodes as documented in CERTAIN, treatment of the donor with norepinephrine and/or dopamine.

Statistical Analyses

The risk assessment model was developed using multiple logistic regression based on forward selection of individual variables. Patient and graft characteristics were described by mean and standard deviation for continuous variables and relative and absolute frequencies for categorical variables. First, the peri- and post-transplant parameters assessed in this study were compared between DGF and no-DGF patients using univariable t-tests or chi-squared tests, as appropriate. Second, these parameters were analyzed using the variable selection process. To develop the risk assessment model, patients were randomly assigned to a training and a test cohort in a 2:1 ratio to ensure equal prevalence of the outcome "occurrence of DGF". Multiple imputation was then performed on the training data. Imputation was based on the fully conditional specification method of Van Buuren et al. [16] and performed with the

TABLE 2 | Comparison of baseline recipient and donor characteristics in patients with and without DGF.

Characteristics	DGF n = 59	no DGF n = 635	*P* value
Recipient			
Age [years], mean ± SD	10.5 ± 5.9	9.8 ± 5.3	0.316
Male sex, n (%)	43 (72.0)	378 (59.5)	0.045
Height [cm], mean ± SD	130.1 ± 33.5	126.2 ± 31.4	0.363
Weight [kg], mean ± SD	33.8 ± 20.2	30.8 ± 17.7	0.228
BMI [kg/m^2], mean ± SD	17.9 ± 3.4	17.6 ± 3.2	0.478
Number of previous kidney transplantations, n > 0 (%)	6 (10.2)	55 (8.6)	0.302
Patients receiving anti-thymocyte therapy, n (%)	1 (1.69)	10 (1.57)	0.943
At least one HLA mismatch			
HLA-A	34 (57.7)	424 (66.8)	0.258
HLA-B	45 (76.2)	501 (78.9)	0.567
HLA-DR	38 (64.4)	462 (72.7)	0.035
Primary kidney disease, n (%)			0.2717
Renal hypoplasia/dysplasia	15 (25.4)	174 (27.4)	
Glomerular disease	18 (30.5)	148 (23.3)	
Obstructive uropathy/vesicoureteral reflux	6 (10.2)	93 (14.6)	
Polycystic kidney disease	9 (15.3)	89 (14.0)	
Not specified/unknown	1 (1.7)	71 (11.2)	
Tubular disorder	4 (6.8)	21 (3.3)	
Systemic disorders including vasculitis	2 (3.4)	13 (2.0)	
Kidney tumor	2 (3.4)	13 (2.0)	
Other	2 (3.4)	13 (2.0)	
Dialysis prior to KTx, n (%)			<0.001
Preemptive KTx	2 (3.4)	181 (28.5)	
Hemodialysis	26 (44.1)	183 (28.8)	
Peritoneal dialysis	31 (52.2)	267 (42.1)	
Unknown	0 (0.0)	4 (0.6)	
Donor			
Age [years], mean ± SD	31.0 ± 16.6	30.0 ± 17.6	0.683
Type of donor			
Deceased donor after brain death, n (%)	43 (72.9)	305 (48.0)	<0.001
Deceased donor after cardiac death, n (%)	2 (3.39)	10 (1.57)	<0.001
Deceased donor, unknown reason, n (%)	10 (16.9)	98 (15.4)	<0.001
Living donor, n (%)	4 (6.8)	222 (35.0)	<0.001
Cold ischemia time [min], mean ± SD	821 ± 359.8	610 ± 424	<0.001

For binary and categorical parameters, numbers and percentages are presented. For continuous variables, mean and standard deviation are presented.
A two-tailed t-test was calculated for metric variables. Chi-squared test was used to compare non-metric variables. For all tests, p < 0.05 was considered significant.
BMI, body mass index; KTx, kidney transplantation; HLA, human leukocyte antigen.

"mice" package (using 20 imputations). Forward selection was applied to the imputed data sets for logistic regression modeling. Forward selection based on 1000-fold subsampling was performed on 63.2% (1-1/e) of the cohort for each imputed data set. Variables selected for the final model had to be both selected in all imputed data sets for the full training cohort and also to be confirmed in the subsampling process. The final model was built by pooling the respective sub-models derived from the multiple imputation process. To control for interdependence of model parameters and to provide transparency following the guidelines of the TRIPOD statement [15], we calculated adjusted odds ratios for each parameter selected for the model. Internal validation of the final model was performed (i) only on complete cases with no missing data in the test cohort, (ii) on each imputed dataset (same procedure as for the training cohort), and (iii) on a combined multiple imputed data set, which was derived by averaging. Validation was assessed by the area under the receiver operating characteristic curve (ROC-AUC). Model calibration was assessed using calibration plots. All analyses were performed with R version >4.0.0.

RESULTS

Patient and Transplant Characteristics

We screened n = 816 patients. After applying the exclusion criteria, n = 694 children remained for further analysis (**Figure 1**). The overall rate of DGF in the entire patient cohort was 59 out of 694 patients (8.5%); the rate of DGF after deceased donation was 11.8% (55 out of 468 patients), after living donation 1.77% (4 out of 226 patients) ($P < 0.001$). **Table 2** shows the baseline characteristics of patients with and without DGF and the corresponding donor characteristics. Patients with DGF were more likely to be male, less likely to have at least one HLA-DR mismatch, and less likely to have undergone preemptive transplantation. 93.2% of patients with DGF had received a transplant from a deceased donor and 6.8%

TABLE 3 | Parameters selected for the DGF risk assessment model.

Parameters	Regression coefficient	Odds ratio [95% CI]	*P* value
Intercept	*2.316*	10.132 [1.899–50.364]	0.008
Surgical complications post-transplant			
No	*Reference*	-	-
Yes	1.694	5.443 [1.236–26.208]	0.026
Immediate urine production			
No	*Reference*	-	-
Yes	−2.408	0.090 [0.033–0.211]	<0.001
Initial calcineurin inhibitor therapy			
No	Reference	-	-
Yes	−2.978	0.051 [0.011–0.256]	<0.001
Rate of change in recipient's serum creatinine	0.233	1.262 [1.107–1.467]	<0.001

The reference category describes the variable characteristic to which the risk calculation of the other parameters refers. The reference category was randomly selected.

from a living donor. Cold ischemia time in patients with DGF (821 ± 360 min) was 34.6% longer ($P < 0.001$) than in patients without DGF (610 ± 424 min). **Supplementary Table 3** shows the timepoint of initiation of post-transplant dialysis within the first 7 days after transplant surgery in the DGF cohort.

Peri- and Post-Transplant Risk Factors for DGF

Table 1 shows the analyzed peri- and post-transplant risk factors for DGF. Post-transplant complications requiring reoperation were significantly more frequent in the DGF group (12%) than in the no-DGF group (1%, $P < 0.001$). The rate of decline in recipient's serum creatinine (calculated by **Formula 1**, see above) during the first 24 h post-transplant was significantly slower in the DGF group (−0.008 ± 0.025 = 0.8% ± 2.5% decline per hour) than in the no-DGF group (−0.064 ± 0.092 = 6.4% ± 9.2% decline per hour; $P < 0.001$). The frequency of vasopressor use was significantly different between the DGF and the no-DGF groups. Norepinephrine and epinephrine were used more frequently in the DGF group (47%) than in the no-DGF group (21%, $P < 0.001$), whereas dopamine was used more frequently in the no-DGF group (21%) than in the DGF group (16%, $P < 0.001$). Immediate urine production was significantly more frequent in the no-DGF group (83%) than in the DGF group (31%, $P < 0.001$). Several measures of central venous pressure were significantly higher in the DGF group than in the no-DGF group, whereas the measures systolic blood pressure, diastolic blood pressure, and mean arterial pressure (MAP) were comparable between groups (**Table 1**).

Parameter Selection and Performance of the Model

The total patient population was divided 2:1 into a training cohort (n = 466 patients) and a validation cohort (n = 228) (**Figure 1**). The DGF risk assessment model was trained in the training cohort using logistic regression analysis on the parameters listed in **Table 1**. The following combination of four key post-transplant parameters was selected for the DGF risk assessment model by the forward selection process: (i) occurrence of post-transplant surgical complications, (ii) immediate urine production, (iii) rate of change in recipient's serum creatinine, (iv) initial calcineurin inhibitor therapy (**Table 3**). In addition, the significance of the association of each of these four post-transplant parameters with the occurrence of DGF was confirmed by calculating adjusted odds ratios (**Table 3**). Post-transplant surgical complications and slower rate of decline in recipient's serum creatinine were associated with increased odds of DGF, whereas immediate urine production and initial calcineurin inhibitor were associated with decreased odds of DGF.

TABLE 4 | ROC-AUCs of the DGF risk assessment model in different patient cohorts.

Type of cohort	ROC-AUC
ROC-AUC on training cohort	0.9043
ROC-AUC on validation cohort with complete cases	0.878
Mean ROC-AUC of imputation validation datasets	0.8765
ROC-AUC on merged imputed validation cohort	0.9104

ROC-AUC, area under the receiver operating characteristic curve.
There were no missing data in the selected predictors.

In the training cohort, the corresponding ROC-AUC of the final model was 0.9043. In a second step, the imputation procedures generated several validation cohorts with imputed data for parameters with missing data. The mean ROC-AUC of the model used on these imputed datasets was 0.8765. After merging the imputed datasets, an imputed validation cohort was generated with a ROC-AUC of 0.9104 in the model. In comparison, the ROC-AUC of the validation cohort without imputed data was 0.878 (**Table 4**; **Figure 2**). The corresponding risk of developing DGF based on post-transplant parameters can be calculated with the model using the following formula (**Formula 2**) together with the coefficients written below and shown in **Table 3**.

$$\text{Linear Score} = 2.316 + \text{Variable A} + \text{Variable B} + \text{Variable C} + (0.233 \times Variable\ D)$$

$$\text{P (DGF)} = \frac{1}{1 + e^{-\text{Linear Score}}} \quad (2)$$

Definitions:

- P (DGF) = probability of developing DGF predicted by the DGF risk assessment model (range: 0–1)

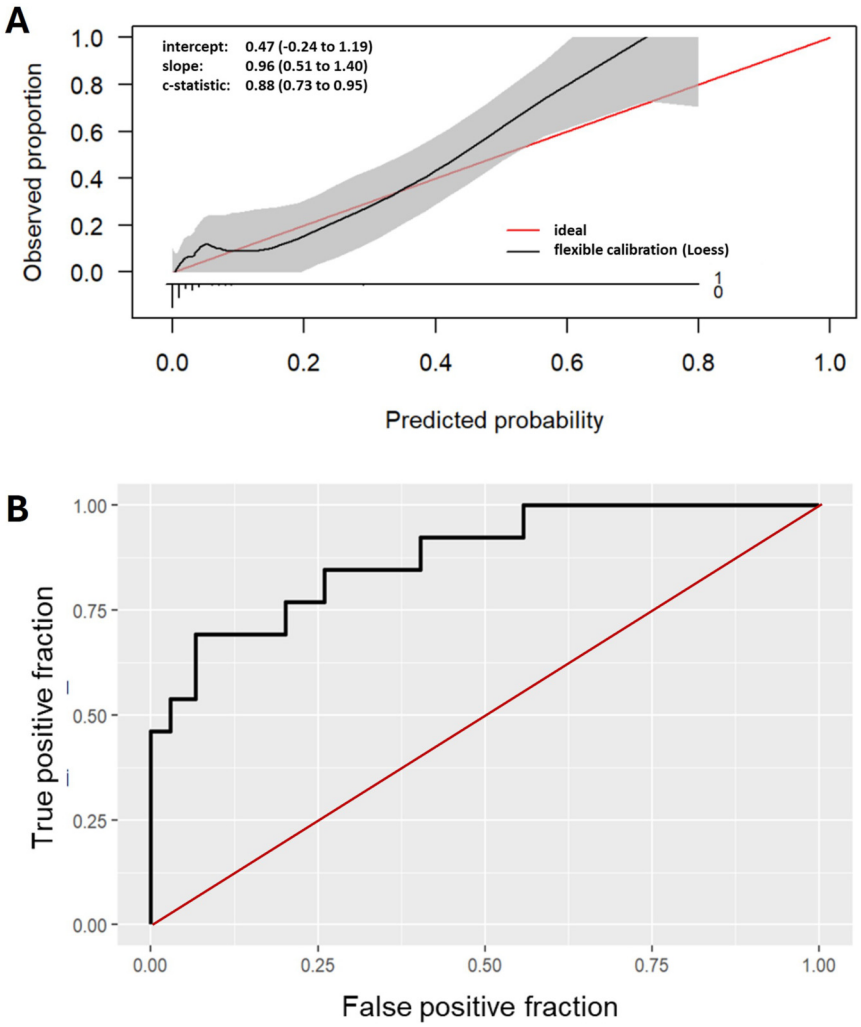

FIGURE 2 | (A) shows the calibration plot of the pediatric DGF risk assessment model. **(B)** shows the ROC-AUC curve for the model, illustrating the discriminative performance. ROC-AUC, area under the receiver operating characteristic curve.

- Variable A: post-transplant surgical complications occurred = 1.694; post-transplant surgical complications did not occur = 0
- Variable B: immediate urine production = −2.408; no immediate urine production = 0
- Variable C: initial calcineurin inhibitor therapy = −2.978; no initial calcineurin inhibitor therapy = 0
- Variable D: rate of change in recipient's serum creatinine (see **Formula 1**)

Abbreviations: DGF, delayed graft function. An online calculator based on **Formula 2** will be available on the CERTAIN website at the time of publication of this article.

Calibration of Final Risk Assessment Model

The calibration of this final pediatric DGF risk assessment model was evaluated using calibration plots. The corresponding calibration plot is shown in **Figure 2**. The model showed the following performance in terms of calibration: Intercept: 0.47; slope: 0.96. Due to the low prevalence of DGF in the study cohort, there are some deviations at very low and high predicted probabilities. Additionally, the confidence intervals for the intercept (−0.24 – 1.19) and slope (0.51–1.40) are relatively broad. Although the C-statistic of 0.88 indicates that the model has high discriminative power, at very high predicted probabilities (>0.8), the model appears to slightly overestimate the probability of the event (**Figure 2**).

Extended Analysis of Combined Predictors for DGF

In the extended analysis of combined pre- and post-transplant risk factors for DGF, we used the same statistical methods and procedures as used in the main analysis (described above). We were able to demonstrate that the inclusion of additional pre-transplant parameters did not improve the predictive performance of the primarily developed prediction model based on post-transplant parameters alone, as identified in the main analysis (see above). **Supplementary Table 1** shows all pre-

and post-transplant parameters examined in this extended analysis. For the combined model, only the number of HLA-DR mismatches was additionally selected by the forward selection procedure (**Supplementary Table 4**). The adjusted odds ratio for the selected pre-transplant parameter "HLA-DR mismatches" was not statistically significant (**Supplementary Table 4**). The AUC (0.8782 in the validation cohort without imputed data) of this combined pre and post-transplant model was comparable to the AUC (0.878 in the validation cohort without imputed data) of the developed post-transplant prediction model. **Supplementary Figure 2** shows the calibration plot of the combined pre -and post-transplant pediatric DGF risk prediction model.

Sensitivity Analysis After Exclusion of the Variable "Initial Calcineurin Inhibitor Therapy"

Sensitivity analysis after exclusion of the variable 'initial calcineurin inhibitor therapy: To assess the impact of the predictor 'start of calcineurin inhibitor therapy within 24 h post-transplant' on model performance, we repeated the forward selection procedure as in the main analysis. Then, we excluded the CNI variable post-selection and refitted the model. The three remaining predictors were highly significant ($p < 0.001$ for each parameter) and had regression coefficients comparable to those in the main model (surgical complications: 1.574 vs. 1.694; immediate urine production: −2.049 vs. −2.408; rate of change in serum creatinine: 0.233 vs. 0.233). The corresponding adjusted odds ratios were also comparable (surgical complications: 4.827 [95% CI: 1.147–21.35] vs. 5.443 [1.236–26.21]; immediate urine production: 0.128 [0.055–0.277] vs. 0.09 [0.033–0.211]; rate of change in serum creatinine: 1.263 [1.112–1.462] vs. 1.262 [1.107–1.467]; **Supplementary Table 5**). The discriminative performance of this three-variable model remained good, closely resembling that of the four-variable model (ROC-AUCs: 0.882 vs. 0.904 in the training cohort; 0.891 vs. 0.878 in the validation cohort with complete cases; a mean of 0.885 vs. 0.877 across imputed validation datasets; 0.917 vs. 0.910 in the merged imputed validation cohort; **Supplementary Table 6**.

DISCUSSION

This study is the first to develop a multivariable prediction model based on early (within the first 24 h after kidney transplant surgery) post-transplant parameters to assess the likelihood of DGF in pediatric recipients. This model can predict the occurrence of DGF in children with high accuracy, independent of pre-transplant risk factors. After external validation, this model can be used in two different clinical settings: First, the model can identify children at high risk for DGF; thus, it can serve as a valuable tool to guide patient-specific clinical decision-making after transplantation and to tailor management strategies accordingly. Second, the reliable identification of pediatric patients at high risk for DGF is important for immediate post-transplant clinical decision making and future interventional trials of targeted pharmacological strategies against ischemia-reperfusion injury. Ischemia-reperfusion injury resulting in DGF after kidney transplantation is a currently unmet clinical need. Several approaches to targeting ischemia-reperfusion injury pathways in the context of kidney transplantation are currently being evaluated in preclinical or clinical trials, including inhibition of complement pathways, modulation of complement regulatory proteins, or interference with the hepatocyte growth factor pathway [6, 17, 18]. Thus, this model may facilitate the conduct of such trials in children in the future.

We found that several early post-transplant parameters were significantly associated with the development of DGF. One of these factors was hemodynamic instability as indicated by the need for circulatory support with inotropes such as epinephrine or norepinephrine in the recipient. It is likely that hemodynamic instability in the recipient leads to hypoperfusion of the renal allograft. In addition, the use of inotropic agents such as norepinephrine may itself impair graft perfusion mediated by pharmacologically induced arteriolar constriction [19].

The DGF risk assessment model was trained in the training cohort using logistic regression analysis, selecting the following four key post-transplant parameters: (i) occurrence of post-transplant surgical complications, (ii) immediate urine production, (iii) initial calcineurin inhibitor therapy, (iv) rate of change in recipient's serum creatinine. The significance of the association of each of these four parameters with the occurrence of DGF was confirmed by calculating adjusted odds ratios. The occurrence of postoperative complications requiring reoperation on the first day after transplantation had the highest odds ratio, indicating a 5.4-fold increase in the odds of DGF. A slower rate of decline in the recipient's serum creatinine level on the first day post-transplant was associated with a 26% increase in the odds of DGF, while urine production immediately post-transplant was associated with a 90% decrease in the odds of DGF. Both parameters can be interpreted as markers of ischemia-reperfusion injury-mediated graft dysfunction. Initial immunosuppressive therapy with calcineurin inhibitors had an adjusted odds ratio of 0.051, indicating a 95% reduction in the odds of DGF. However, only 3 patients (6%) in the DGF cohort did not receive a calcineurin inhibitor compared to 16 patients (3%) in the no-DGF cohort (**Table 2**). Because of these small numbers, this finding should be interpreted with caution. In addition, children identified by treating physicians as being at high risk for DGF may have initially received a CNI-free immunosuppressive regimen. This may have confounded the observed association between CNI use and reduced risk of developing DGF. To further assess the impact of the immediate initiation of CNI treatment after KTx on overall model performance, we performed a sensitivity analysis that excluded the CNI variable after forward selection. This analysis showed that initial CNI use did not substantially impact the strength or

direction of the associations between the other predictors and DGF. This finding supports the internal validity of the final pediatric post-transplant DGF risk assessment model. Accordingly, the model should not be interpreted as evidence of a protective effect of CNI, but rather as a tool for DGF risk stratification in children in the early post-transplant setting.

In a dedicated extended analysis, we evaluated established or putative pre-transplant risk factors for DGF development such as cold ischemia time and donor type [20] for their potential to improve the predictive accuracy of the post-transplant model developed in this study. None of the additional pre-transplant parameters improved the performance of the post-transplant prediction model. Among the pre-transplant factors analyzed, only the number of HLA-DR mismatches was included in the combined model using the forward selection process. However, this combined prediction model did not outperform the post-transplant model, as evidenced by a nearly identical AUC in the internal validation. Furthermore, the inclusion of HLA-DR mismatches in the combined prediction model, as suggested by forward selection, is unlikely to provide meaningful clinical benefit given the lack of statistical significance in an adjusted odds ratio analysis performed in parallel. Therefore, the results of this extended analysis of combined risk factors for DGF suggest that the investigated post-transplant parameters alone are sufficient for accurate prediction of DGF in pediatric kidney transplantation within 24 h after transplantation surgery.

The post-transplant DGF risk assessment model developed in this study demonstrated robust discrimination performance, but limitations regarding calibration, accuracy, generalizability, and clinical applicability must be considered. First, our study is retrospective and therefore subject to the biases inherent to patient registries. Variability in donor and recipient characteristics, transplant practices, or local protocols may limit the external validity of prediction models. Therefore, even if this DGF risk assessment model performs well in the internal validation process, a generalizability of its use will require an additional external validation step based on diverse pediatric transplant populations. Second, it is important to note that the predictive accuracy of this DGF risk assessment model is limited to the variables included in the analysis itself. Factors not analyzed in this study may also contribute to the development of DGF. However, we were unable to examine the method of organ procurement in living donors because the number of DGF events in the subset of living donors in this patient cohort was too small for a meaningful statistical analysis. In addition, the landscape of pediatric kidney transplantation continues to evolve, which may introduce other new potential variables for the development of DGF in the future. Potential changes in post-transplant treatment regimens in the future must be considered in a DGF risk prediction model to keep it current and ensure its continued applicability. Third, the absolute number of patients with DGF in this study cohort was relatively small. To avoid an overly optimistic estimation of the predictive accuracy of the DGF risk assessment model developed in this study, we performed a calibration analysis. Despite the low prevalence of DGF in our cohort, the analysis revealed a general alignment between the predicted and observed frequencies of DGF.

Fourth, the forward selection process applied in our study optimizes the model's predictive performance but does not evaluate individual parameters independently for causality. The observed associations between the selected variables do not reflect isolated causal effects of single parameters, but their combined predictive contribution to the DGF risk in our study population. This is especially relevant regarding the observed negative association between early CNI use and DGF risk. The model should not be used to determine whether CNI therapy can be initiated or should be withheld. The model captures patient-specific factors present on day 1 post-transplant, including already taken clinical decisions such as withholding CNI probably due to an individually perceived risk of DGF by the transplant team. As such, the model does not necessarily give evidence of a protective effect of CNI against DGF and furthermore it is not intended for guiding immunosuppressive management. Importantly, a sensitivity analysis showed that excluding the CNI variable from the final model did not substantially impact its performance.

Fifth, it should be noted that the adjusted odds ratios of the predictors should not be interpreted as risk ratios or direct measures of risk reduction in the final prediction model. Rather they should be viewed as indicators that confirm the direction and relative strength of the associations between the predictors and DGF.

Sixth, model parameters such as serum creatinine and immediate urine output may have been biased by an early post-transplant dialysis procedure in children with DGF. In a dedicated sensitivity analysis, we excluded the 8 patients for whom the date of kidney transplantation and the date of the first post-transplant dialysis coincided. We then re-ran the model and excluded the CNI variable after forward selection. Both the discriminative performance (ROC-AUCs) and the direction of the associations of the selected predictors remained unchanged compared to the main model (**Supplementary Tables 7,8**), indicating that the inclusion of patients undergoing post-transplant dialysis on day 1 did not introduce relevant bias into the final model.

In conclusion, we developed a multivariable prediction model based on early (within the first 24 h after kidney transplantation) post-transplant parameters to assess the likelihood of DGF in pediatric kidney transplant recipients. This model can predict the occurrence of DGF in children with high accuracy, independent of pre-transplant risk factors. Thus, this model is a valuable tool for guiding patient-specific clinical decision-making post-transplant and it may facilitate future interventional trials of targeted pharmacological strategies against ischemia-reperfusion injury.

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

Participation in the CERTAIN registry was approved by the ethics committee at each center. Written informed consent was obtained from the parents or legal guardians prior to enrollment, with assent from patients when appropriate for their age. This general ethics approval for participation in the registry fully covers the use of the collected data for this registry-based study (reference number: S-388/2010).

AUTHOR CONTRIBUTIONS

All authors participated in the design of the study. MB collected patient data. CP, BT, AF, and CS analysed and interpreted the data. All authors contributed to the article and approved the submitted version.

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The remaining author(s) declared that this work 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.2026.14640/full#supplementary-material

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ORIGINAL RESEARCH
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Effect of Normothermic Machine Perfusion on Glycocalyx Shedding During Liver Transplantation – A Prospective Pilot Study

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Ischemia–reperfusion injury (IRI) plays a pivotal role in liver transplantation by inducing oxidative stress and inflammation, thereby contributing to impaired graft function and postoperative complications. A key element of IRI is degradation of the endothelial glycocalyx, resulting in microcirculatory dysfunction. This study investigated the impact of normothermic machine perfusion (NMP) on glycocalyx integrity and its association with early postoperative outcomes. Thirty grafts undergoing NMP prior to transplantation were analyzed. Syndecan-1 and heparan sulfate were quantified in perfusate and recipient serum. Donor-related factors influencing glycocalyx injury during NMP were assessed, and correlations with outcomes established. Syndecan-1 levels increased during NMP and remained significantly elevated in grafts from circulatory-death (DCD) donors compared with brain-death (DBD) donors. Receiver operating characteristics revealed predictive potential for early allograft dysfunction (EAD) with a syndecan-1 cut-off of 4,796.13 ng/mL after 6 h of NMP. In contrast, heparan sulfate concentrations showed no relevant changes. Postoperatively, syndecan-1 levels in recipient serum were elevated immediately after transplantation but declined over subsequent days, while heparan sulfate remained stable. These findings indicate that glycocalyx injury develops during NMP, particularly in DCD livers, with elevated syndecan-1 reflecting endothelial vulnerability and a potentially modifiable aspect of graft physiology relevant to future protective strategies.

Clinical Trial Registration: www.Clinicaltrials.gov, identifier NCT: 04764266.

Keywords: glycocalyx, heparan sulfate, liver transplantation, normothermic machine perfusion, syndecan-1

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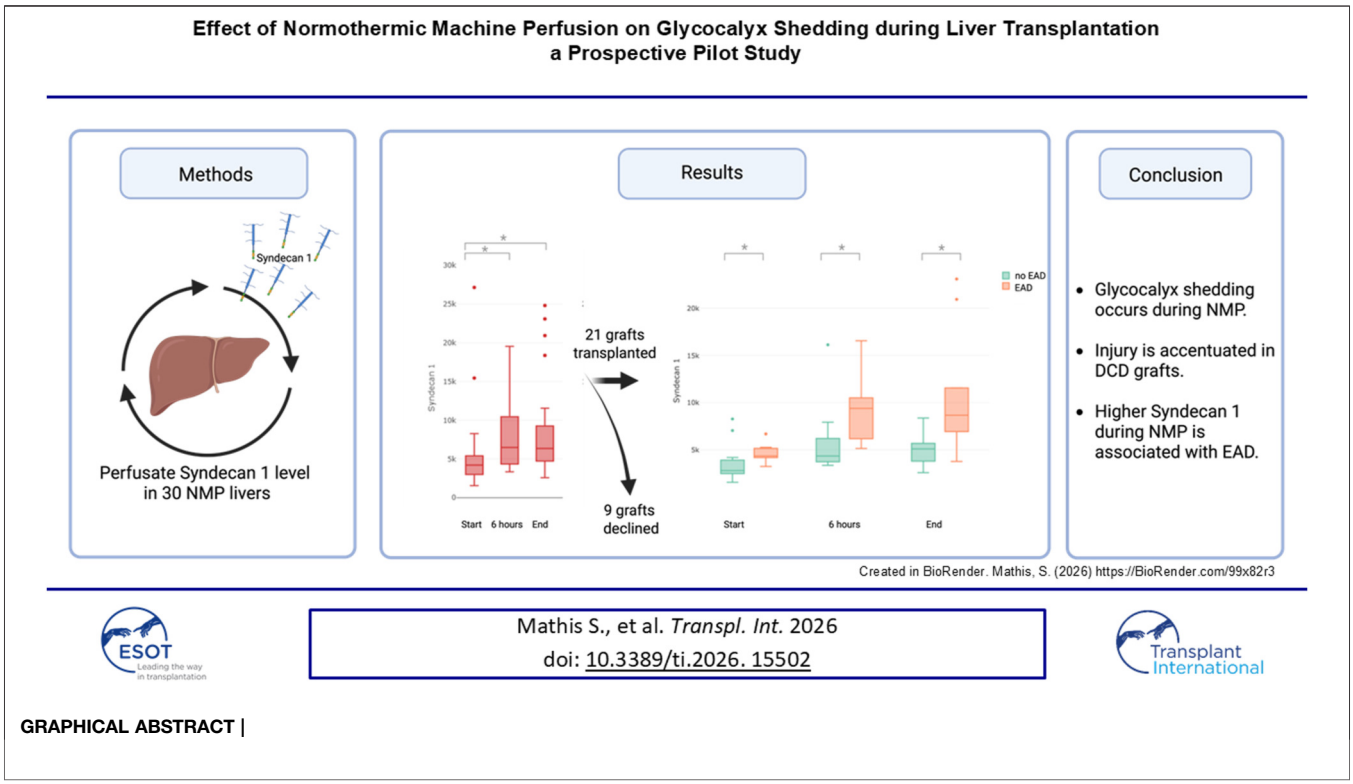
Abbreviations: AKI, acute kidney injury; ALT, alanine aminotransferase; aPTT, activated partial thromboplastin time; AST, aspartate aminotransferase; CRP, C-reactive protein; DBD, Donation after brain death; DCD, Donation after cardiocirculatory death; EAD, early allograft dysfunction; ECD, extended criteria donor; GGT, glutamyl transferase; ICU, intensive care unit; IRI, ischemia reperfusion injury; LDH, lactate dehydrogenase; NMP, normothermic machine perfusion; NO, nitric oxide; PT, prothrombin time; ROS, reactive oxygen species; SCS, static cold storage.



GRAPHICAL ABSTRACT |

INTRODUCTION

Ischemia-reperfusion injury (IRI) represents an unavoidable pathological process in liver transplantation, known to negatively affect graft function and patient outcome [1–3]. The abrupt restoration of blood flow after ischemic storage triggers a cascade of oxidative stress and inflammatory responses, characterized by the excessive production of reactive oxygen species (ROS) and the activation of immune pathways [4].

Probably one of the first structural damages in the onset of IRI, and certainly one of the key components of IRI is the disruption of the endothelial glycocalyx, a thin layer of proteoglycans, glycoproteins and glycosaminoglycans that lines the luminal surface of vascular endothelial cells [5–7]. During IRI, glycocalyx components, e.g., syndecan-1 and heparan sulfate are sheared from the endothelium and released into the blood. Glycocalyx shedding leads to microcirculatory disturbances, resulting in altered capillary perfusion and leukocyte-mediated tissue inflammation [8–10]. These microcirculatory disturbances have been shown to correlate with organ dysfunction after liver transplantation [11]. Microcirculatory dysfunction is aggravated by a reduction of shear stress mediated nitric oxide (NO) release, which further deteriorates capillary perfusion [12]. Although the exact pathomechanism is unclear, glycocalyx disruption also appears to be involved in the development of interstitial graft edema due to loss of barrier function and development of capillary leakage [13–15].

In recent years, normothermic machine perfusion (NMP) of the liver has emerged as a well-established procedure in many transplant centers. Beyond its recognized benefits for assessing organ function, NMP is gaining increasing attention for its potential role in mitigating IRI, as the process of organ reperfusion is shifted from the recipient to the perfusion device. This not only allows for better characterization of IRI but also has the potential to identify possible therapeutic targets. Studies showed that in contrast to static cold storage (SCS), NMP seems to mitigate IRI through multiple pathways [16–18]. Specifically, a shift in gene expression from a pro-inflammatory to a regenerative profile and a reduction in necrosis was observed in NMP liver grafts [18].

Although glycocalyx damage after SCS has been the focus of numerous studies, the extend of glycocalyx damage during NMP remains to be elucidated. To investigate this, we analyzed glycocalyx alterations during NMP, with a specific focus on identifying donor characteristics that may contribute to glycocalyx degradation [19, 20]. Additionally, we aimed to evaluate whether changes in glycocalyx integrity influence postoperative outcomes in liver transplant recipients.

MATERIALS AND METHODS

In this study, 30 livers from deceased donors subjected to NMP were included after the allocated recipients of the organs provided written informed consent to participate in the study. The study

was approved by the local ethics committee (approval number 1382/2020) and registered with ClinicalTrials.gov (www.Clinicaltrials.gov; NCT: 04764266, Simon Mathis MD; February 17th, 2021). It was conducted in accordance with the Declarations of Helsinki and Istanbul. Grafts allocated to patients younger than 18 years of age and organs allocated to patients undergoing re-transplantation were excluded.

The primary endpoint of the study was the temporal profile of the glycocalyx components syndecan-1 and heparan sulfate in the perfusate during normothermic machine perfusion. Secondary endpoints included donor factors influencing the extent of glycocalyx injury during NMP; in transplanted grafts, the temporal profile of glycocalyx components in the recipient and associations between the extent of glycocalyx injury and recipient outcome were analyzed as secondary endpoints.

NMP Data

NMP for the livers was initiated using the OrganOx metra® liver perfusion device (OrganOx, Oxford, UK) in a back-to-base approach as previously described [21–23]. The perfusion fluid consisted of 840 mL packed red cells and 500 mL gelatin solution (Gelofusin®, B. Braun, Melsungen, Germany). In addition, 10,000 IU heparin, calcium gluconate and sodium bicarbonate were added to the priming. Measurements of the glycocalyx shedding parameters syndecan-1 and heparan sulfate were performed at three time points during NMP: Immediately after initiation of NMP (NMP 1), after 6 h of NMP (NMP 2) and right before the end of NMP (NMP 3). For each time point, 4.5 mL of perfusate was collected, centrifuged and stored at −80 °C until analysis. Perfusate electrolyte and metabolic composition were assessed at the same time points. The decision to accept or reject a graft for transplantation during NMP was at the discretion of the transplant surgeon on duty based on local SOP [24]. Even if the graft was declined for transplantation, the data collected during NMP were included in the analysis.

Donor and Recipient Data

Donor characteristics (age, sex, weight, donor type), graft characteristics (SCS-time, NMP-time) and recipient demographics (age, sex, weight, height, Meld score, etiology of liver disease) were collected.

Liver transplantation was performed according to local standard operating procedure using the total cava replacement technique or the piggyback technique without the use of a cavo-caval shunt.

Postoperative data were collected daily during the first seven postoperative days and included bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), glutamyl transferase (GGT), alkaline phosphatase, acetyl cholinesterase, c-reactive protein (CRP), procalcitonin and coagulation parameters (PT, aPTT, fibrinogen). In addition, the need for renal replacement therapy, occurrence of early allograft dysfunction (EAD), length of ICU stay, 30-day and 90-day mortality were recorded [25].

To assess glycocalyx degradation in the recipient, 4.5 mL of EDTA blood was collected from the recipient before induction of anesthesia [Baseline (BL)], on admission to the intensive care unit (ICU) and on postoperative days 1 (D1), 2 (D2) and 3 (D3). All samples were centrifuged and frozen at −80 °C until subsequent analysis. Syndecan-1 concentrations were measured in perfusate and patient plasma by Human CD138 ELISA KIT, Diaclone SAS, Besancon Cedex, France; heparan sulfate levels were determined using Human Heparan Sulfate Proteoglycan (HSPG) ELISA KIT, Reddot Biotech inc., Kelowna, Canada.

Statistics

Sample size estimation for assessing glycocalyx changes during NMP was based on data from two previously published studies [19, 22]. Assumptions were made regarding a statistical power of 80% and a significance level of 5%.

Statistical analysis was performed using "R" (version 4.1.2). Binary data are presented as numbers/total numbers and percent; continuous data are presented as medians (25th-75th percentile). For binary variables, their influence on glycocalyx shedding parameters has been examined by performing group comparisons employing Wilcoxon Rank Sum Tests. Adjustment for multiple testing was performed according to the Benjamini-Hochberg procedure. The relationship between continuous variables and glycocalyx shedding parameters was assessed by evaluating Pearson´s product-moment correlation coefficient. Stepwise changes over time in syndecan-1 and heparan sulfate were analyzed using a Friedman Rank Sum Test, followed by pairwise assessments employing a Wilcoxon Singed Rank Test with paired samples.

RESULTS

Thirty organs were included in the study. NMP was initiated for donor-related reasons in 15 cases, for logistical reasons in 13 cases, and for recipient-related reasons in 4 cases, with multiple indications applicable in several instances. No additional perfusion modalities (Hypothermic oxygenated perfusion or Normothermic regional perfusion) were used during procurement. In summary 88 timepoints were analyzed. Due to protocol violations regarding the sampling time points, one value at the end of NMP and one value on admission to ICU had to be excluded from analysis.

Donor and Recipient Data

Donor data are presented in **Table 1**. Twenty-two grafts were from DBD donors and 8 from DCD donors. Nineteen donors were classified as extended criteria donors (ECD) [26]. Median cold ischemic time was 330 (262.26–438.25) minutes prior to perfusion, and NMP time was 1,058.5 (710.25–1,200) minutes. One graft was not transplanted due to extremely high transaminase levels. Two grafts were declined due to absent lactate clearance combined with an inability to maintain stable pH levels during perfusion. Two grafts were not used due absent lactate clearance and impaired parenchymal appearance. Two grafts were declined based solely on histopathological findings regarded as unsuitable for transplantation. One graft was not transplanted because of a malignancy diagnosed in the donor, and another transplantation was cancelled due to lymph node

TABLE 1 | Donor and Perfusion characteristics.

Age (years)	61 (5.8–70.3)
Sex	
Female	21 (70%)/30
Male	9 (30%)/30
BMI	26.5 (23–30)
Donor type	
DBD	22 (73.3%)/30
DCD	8 (26.7%)/30
Extended criteria donor	19 (63.3%)/30
DCD	8 (42.1%)/ 19
Donor ICU stay >7 days	3 (15.8%)/19
Donor age >65 years	9 (47.4%)/19
Elevated transaminases	4 (21.1%)/19
CS time (min)	330 (262.25–439.25)
NMP time (min)	1038.5 (710.25–1200)

TABLE 2 | Recipient characteristics.

Age (years)	63.5 (48.75–67.25)
Sex (female/male)	6 (28.57%)/15 (71.43%)
Body weight (kg)	76.5 (62–83)
Body height (cm)	174.5 (166.5–178.5)
Meld score	15.5 (8.75–17.25)
Etiology of liver disease	
Alcohol	7 (33.3%)/21
Hepatocellular carcinoma	7 (33.3%)/21
Primary sclerotic cholangitis	3 (14.3%)/21
Polycystic liver disease	2 (9.5%)/21
Other	2 (9.5%)/21

metastases of the allocated recipient. Characteristics of the 21 patients who were finally transplanted are presented in **Table 2**.

Recipient´s median ICU and hospital length of stay were 3 (3–9) and 21 (17–37.25) days; 7 recipients required postoperative renal replacement therapy. Biliary complications, including anastomotic strictures, non-anastomotic strictures, bile leaks, episodes of cholangitis, and cholangiopathy, were observed in 7 recipients (33.3%), whereas 14 had none. Thirty-day and 90-day survival rates were 100% and 90.5%, respectively. Two early deaths occurred: One patient died 51 days after transplantation due to progressive graft dysfunction in acute rejection and 1 patient died on day 82 due to a cardiac event.

In total, 5 recipients died during the follow up period (median follow up: 23.7 months (19.7–27.8)).

Glycocalyx Damage Parameters During NMP

Syndecan-1

Syndecan-1 significantly increased during NMP, rising from 4,219.8 (2,999.3–5,414.8) ng/mL to 6,491.2 (4,338.9–10,468.2) ng/mL after 6 h ($p = 0.001$). There was no further increase in syndecan-1 until the end of NMP, with levels reaching 6,355.6 (4,720.2–9,260) ng/mL ($p = 0.25$; **Figure 1**).

In grafts from DCD donors, syndecan-1 levels were constantly higher throughout the entire NMP period compared to grafts from DBD donors (NMP 1: 5,337.96 ng/mL vs. 4,032.17 ng/mL, $p = 0.031$; NMP 2: 10,695.6 ng/mL vs. 5,801.39 ng/mL, $p < 0.001$; NMP 3: 9,727.55 ng/mL vs. 5,174.07 ng/mL, $p = 0.025$). Neither sex, laboratory parameters (AST, ALT, GGT, creatinine, leukocytes, CRP), nor pre-existing conditions (arterial hypertension, diabetes mellitus) of the donor correlated with the dynamics of syndecan-1 during NMP. No association between cold storage-time and syndecan-1 in perfusate could be shown. The classification as ECD appears to have no influence on syndecan-1 levels during NMP.

Heparan Sulfate

Heparan sulfate levels were 4.2 (3.7–5.4) ng/mL after start of NMP and 4.8 (3.8–7.4) ng/mL after 6 h of NMP; at the end of NMP heparan sulfate concentrations were 5.95 (3.96–8.79) ng/mL. No significant dynamics of heparan sulfate were observed during NMP ($p = 0.100$) (**Figure 4**) No difference in heparan sulfate levels was detected between DBD and DCD donors during the first six hours of NMP. However, higher levels were measured in DCD grafts at the end of NMP (DBD: 5.23 (3.55–7.23) ng/mL vs. DCD: 8.99 (7.01–10.16) ng/mL; $p = 0.047$). No other recorded donor characteristics or laboratory parameter appeared to influence the level of heparan sulfate during NMP. Furthermore, no association was found between heparan sulfate levels in perfusate and the recipient´s postoperative course.

Correlation Analysis of Syndecan-1 and Routine Perfusate Markers

Median perfusate glucose concentrations declined from 23.3 (17.65–28.5) mmol/L at the beginning of perfusion to 8.2 (5.07–11.85) mmol/L at its end. Perfusate sodium concentrations were 142 (134.75–143) mmol/L, 150 (142.75–156.5) mmol/L, and 150.5 (145.25–160) mmol/L at the beginning of perfusion, after 6 h, and at the end, respectively. No correlation between perfusate glucose or sodium and syndecan-1 measured at the same time points was observed ($p = 0.138$ for glucose; $p = 0.95$ for sodium).

Syndecan-1 showed a consistent association with established markers of hepatocellular injury. At the beginning of perfusion, syndecan-1 correlated with aspartate aminotransferase (AST) ($r = 0.406$, $p = 0.04$), with this relationship strengthening after 6 h ($r = 0.51$, $p = 0.009$) and persisting at the end of perfusion ($r = 0.448$, $p = 0.026$). Correlations with alanine aminotransferase (ALT) emerged at later time points, namely, after 6 h ($r = 0.481$, $p = 0.014$) and at the end of perfusion ($r = 0.4$, $p = 0.049$). A significant association with bilirubin was observed only at the end of perfusion ($r = 0.488$, $p = 0.013$). Syndecan-1 also correlated with lactate dehydrogenase throughout the perfusion course - at the start ($r = 0.428$, $p = 0.03$), after 6 h ($r = 0.510$, $p = 0.009$), and at the end ($r = 0.468$, $p = 0.019$). In contrast, neither pH nor lactate showed any correlation with syndecan-1.

Outcome Associations of Glycocalyx Injury During NMP

There was a significant correlation between syndecan-1 measured at the end of NMP and recipients AST levels on ICU admission ($r =$

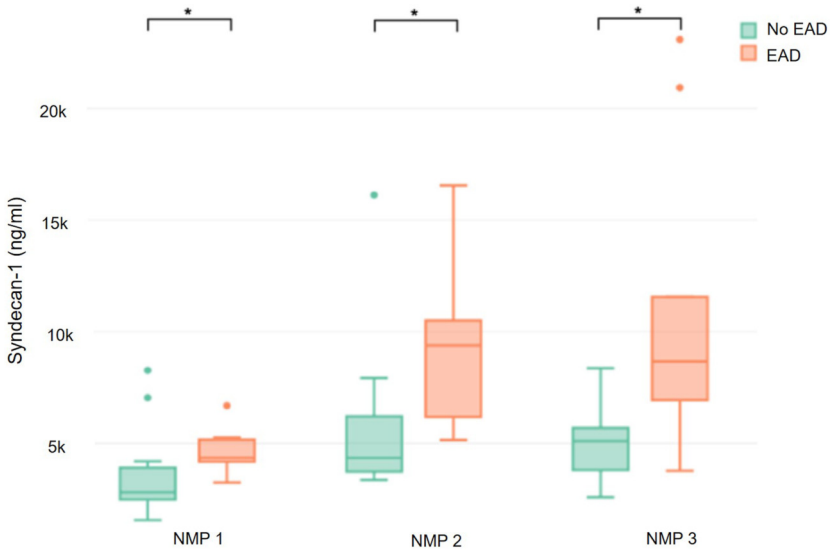


FIGURE 1 | Progression of syndecan-1.

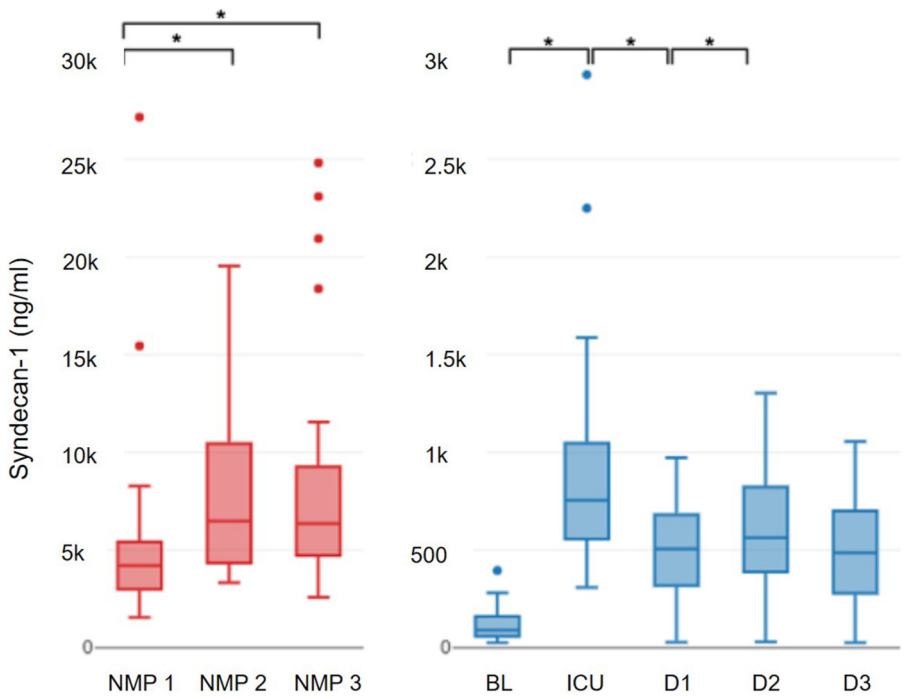


FIGURE 2 | Syndecan-1 during NMP depending on the occurrence of EAD.

0.55; p = 0.026) and the first three postoperative days (day 1: r = 0.58 p = 0.026; day 2: r = 0.48, p = 0.033; day 3: r = 0.51, p = 0.032). Furthermore, perfusate syndecan-1 values where significantly higher in grafts of recipients who developed EAD (n = 10) compared to those without EAD (n = 11) (4,331.5 (4,180.3–5,033.7) ng/mL vs. 2,800.7 (2,480.5–3,618.7) ng/mL; p = 0.024 and 9,379.7 (6,181–104,923) ng/mL vs. 4,338.9 (3,730–6,201.3) ng/mL; p = 0.013 and 8,663.6 (6,926.2–11,551.9) ng/mL vs. 5,096.5 (3,795–5,679.5) ng/mL; p = 0.013) (**Figure 2**). In contrast, syndecan-1 concentrations during NMP did not differ between recipients who subsequently developed acute kidney injury (AKI) grade II or III and those with no AKI or grade I.

Grafts that were classified as transplantable (n = 23) exhibited significantly lower syndecan-1 levels at the onset of perfusion

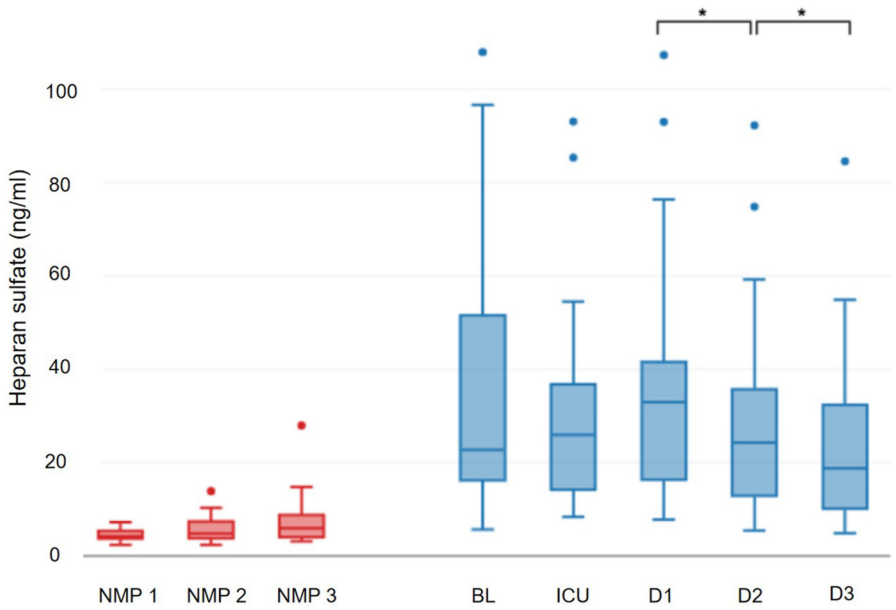


FIGURE 3 | Comparison of syndecan-1 during NMP between organs classified as transplantable and non-transplantable.

(3,892.6 (2,883.7–4,561.2) ng/mL) and after six hours of perfusion (5,915.7 (4,082.2–9,379.7) ng/mL) when compared to grafts that were classified as unsuitable for transplantation due to their metabolic profile (5,653.7 (5,074–11,706.6) ng/mL; p = 0.001 and 7,764.8 (7,133.6–12,740.7) ng/mL; p = 0.037) (**Figure 3**) [24]. Of note, two grafts, which had been approved for transplantation based on metabolic performance, were not transplanted due to the presence of malignancies in the donor or recipient.

Glycocalyx Damage Parameters in the Recipient

Syndecan-1 levels in recipients blood significantly increased from baseline (90.69 (58–161) ng/mL) to ICU admission (755.87 (556.3–1,046.8); p < 0.001), followed by a notable decline on the first (563.3 (389–822) ng/mL; p = 0.008) and second (506.3 (319–680) ng/mL; p = 0.002) postoperative day. Between the second and the third postoperative day, syndecan-1 levels remained constant (485.8 (278.75–699.3) ng/mL; p = 0.366) (**Figure 1**). From postoperative day 1 onwards, syndecan-1 concentrations differed significantly between recipients who developed no or grade I AKI compared to those who progressed to AKI grade II or III (404.8 (227.7–529.4) ng/mL vs. 790.9 (562–1,108.9) ng/mL; p = 0.01); this divergence persisted through postoperative day 3 (322 (85.4–408.7) ng/mL vs. 691.7 (586.6–832.5) ng/mL; p = 0.003).

In contrast, recipients plasma levels of heparan sulfate did not increase during transplantation (25.9 (14.6–36.7) ng/mL; p = 0.508) or on postoperative day 1 (32.9 (17.6–41.3) ng/mL; p = 0.926). However, from postoperative day 1 to postoperative day 2 (24.3 (13.2–35.1) ng/mL; p = 0.006) and postoperative day 3 (18.7 (10.1–30.8) ng/mL; p = 0.008), heparan sulfate levels experienced a decrease, reaching values below the preoperative baseline (p = 0.006) (**Figure 4**).

DISCUSSION

The main finding of this prospective observational study was that the degree of glycocalyx alteration during NMP is predictive for EAD after orthotopic liver transplantation. Furthermore, grafts that were ultimately classified as non-transplantable exhibited significantly higher syndecan-1 levels at the start of perfusion in comparison to those that were released for transplantation. Syndecan-1 and heparan sulfate both increased during NMP, indicating ongoing glycocalyx damage. Not surprising, the glycocalyx of DCD organs appeared to be more severely damaged compared to DBD grafts, exhibiting significantly higher syndecan-1 and heparan sulfate levels compared to DBD grafts. Elevated syndecan-1 levels decreased postoperatively; heparan sulfate levels were comparable to the patients' preoperative baseline and decreased even further during the first two postoperative days.

Despite the fact that NMP is already in widespread clinical use, a significant number of pathophysiological processes during NMP have not yet been investigated. The present study provides preliminary evidence of progressive damage to the endothelial glycocalyx, as indicated by increased glycocalyx components in perfusate.

Two hypotheses may be introduced to explain these findings: First, the introduction of NMP results in the establishment of a non-physiological laminar flow; whereas pulsatile flow seems to protect glycocalyx integrity, laminar flow seems to be damaging [27, 28].

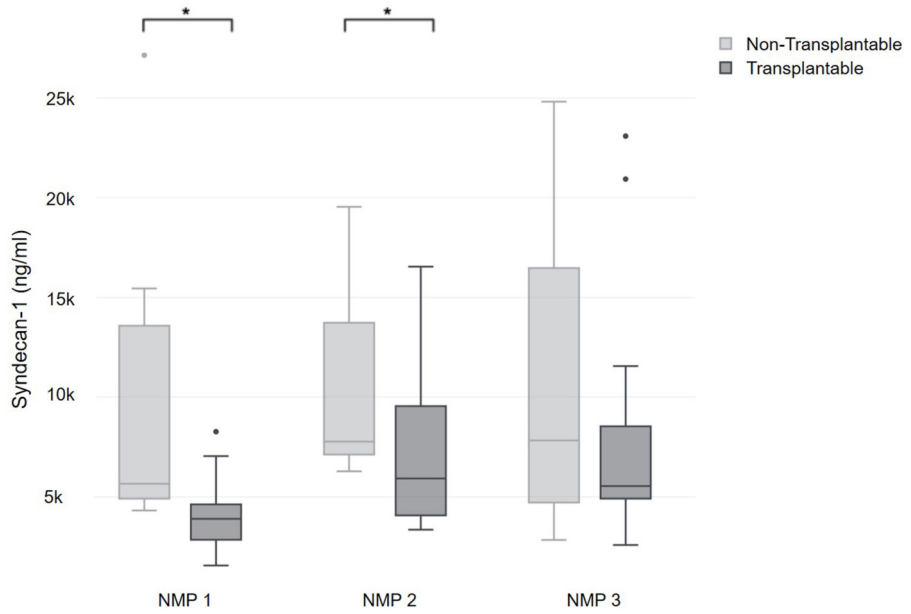


FIGURE 4 | Progression of heparan sulfate.

Second, the composition of the perfusate may play a role in maintaining glycocalyx stability [29, 30]. It was shown that fresh frozen plasma and various plasma components positively influence glycocalyx integrity by promoting endothelial stabilization [31, 32]. In addition, animal studies indicate a positive effect of hydroxyethyl starch (HES 130/0.4) a synthetic colloid [33, 34]. Whether this effect extends to gelatin, the colloid used in the perfusion fluid of our study, remains uncertain.

A further major component of the perfusate are red blood cells. In a study on hemorrhaged rats investigating the effects of resuscitation with blood products on the microcirculation, unwashed red blood cells have been shown to restore the glycocalyx [35]. However, this effect was no longer observed when the red blood cells were washed and all residual plasma components removed. The authors concluded that the effects of unwashed erythrocytes on the glycocalyx are primarily attributable to residual plasma components, rather than red blood cells themselves [35].

The specific biochemical environment within the perfusate may also affect glycocalyx stability, particularly regarding to glucose, sodium, and oxygen levels: Marked hyperglycemia, hypernatremia, and hyperoxia were observed during *ex situ* perfusion, each representing a distinctly unphysiological condition. Experimental and clinical studies have demonstrated that excessive glucose exposure, elevated sodium concentrations, and supraphysiological oxygen tensions can induce structural disruption, shedding, or functional impairment of the endothelial glycocalyx [36–38]. It is therefore plausible that the constellation of these factors during perfusion may have amplified the extent of glycocalyx damage detected in the present cohort.

Further studies are needed to investigate the effects of different perfusion fluid components on the integrity or restoration of the endothelial glycocalyx.

The observed correlations between syndecan-1 and established markers of hepatocellular injury suggest that glycocalyx disruption represents a distinct yet integrative component of graft injury during NMP. Syndecan-1 release reflects damage to the endothelial barrier, a microstructural interface that differs fundamentally from hepatocellular injury captured by AST, ALT, bilirubin, or LDH [39]. The consistent association between syndecan-1 and these conventional markers indicates that endothelial glycocalyx loss accompanies and may contribute to broader cellular injury processes within the graft. By identifying this vulnerable structural layer as a quantifiable element of perfusion-related damage, the present findings highlight a potentially actionable target for future interventions. Strategies aimed at preserving glycocalyx integrity during NMP could therefore serve as a complementary approach to reducing overall graft injury.

A central finding of this study was that the levels of syndecan-1 at the end of NMP correlated with the development of EAD. This finding is in line with previous studies in which levels of syndecan-1 measured in graft efflux during the flushing phase after SCS were associated with the occurrence of EAD [19]. Our study demonstrates that during the course of NMP, a noticeable difference in glycocalyx damage can be observed between grafts that develop EAD and those that do not. However, it must be acknowledged that the sample size is limited, and the discriminatory ability may be overstated. While these associations are noteworthy, the study was not designed to evaluate long-term graft function or to propose glycocalyx markers as decision-making tools for graft acceptance. Rather,

the primary intention was to characterize a vulnerable endothelial structure that remains poorly explored in the context of NMP and to assess whether its injury profile is associated with early postoperative trajectories. The observed correlations therefore underscore that glycocalyx disruption during NMP may reflect a biologically meaningful process, supporting further mechanistic research and the development of perfusion strategies aimed at protecting this important microvascular interface.

In the present cohort, syndecan-1 concentrations did not differ during NMP between recipients who later developed AKI grade II–III and those with no or only grade I AKI; however, from postoperative day 1 to day 3, markedly higher syndecan-1 levels were observed in those who progressed to AKI grade II–III. This postoperative pattern is consistent with observations previously reported by Schiefer et al [20]. The temporal separation emerging only after transplantation suggests that postoperative systemic factors, including bleeding, hemodynamic instability, and inflammatory activation, are linked to both glycocalyx degradation and renal dysfunction, indicating a shared pathophysiological background rather than a direct causal relationship [40, 41]. These observations reinforce the notion that postoperative endothelial vulnerability represents a relevant pathophysiological mechanism and may constitute a target for perioperative strategies aimed at mitigating AKI following liver transplantation [42].

The observation that grafts ultimately not used for transplantation exhibited elevated syndecan-1 levels in the perfusate at the commencement of NMP could suggest that this value may serve as a predictor of the future metabolic performance of the graft at an early stage of perfusion. The provision of earlier information on the condition of the organ could prove advantageous in two respects. Firstly, it could facilitate the resolution of logistical challenges. Secondly, it could initiate any therapies related to the graft during NMP, a subject that is currently the focus of intensive research, at an earlier stage [43].

It is noteworthy that the course of heparan sulfate levels markedly diverged from that of syndecan-1: During NMP heparan sulfate levels showed no dynamics. Following admission to ICU heparan sulfate levels were comparable to the recipients' baseline and even decreased over the next 3 days, reaching levels that were even lower than baseline. As syndecan-1 carries heparan sulfate as side chains, it could be assumed that cleavage of syndecan-1 would simultaneously lead to cleavage of heparan sulfate. Interestingly, in our data, heparan sulfate levels only increased at the end of NMP but not at any other time point. Similar discrepancies have been reported in other studies where both glycocalyx markers were measured [44, 45]. Passov et al. extensively investigated the dynamics of heparan sulfate levels in the context of liver transplantation [46]. They observed lower heparan sulfate levels in the efflux of the graft, compared to levels in the portal vein; Passov proposed that heparan sulfate may be reabsorbed into the graft. It is currently unclear whether this finding represents a glycocalyx restoration mechanism. However, cell culture experiments support this hypothesis [47]. Nevertheless, postoperative heparan sulfate values below baseline may also indicate enhanced liver function after transplantation compared to the recipient's preoperative condition. In fact, Schiefer et al. demonstrated that glycocalyx components in patients scheduled for liver transplantation were markedly elevated compared to a healthy control population [20].

The results of this study point towards new possibilities for maintaining physiological functioning of the graft during NMP. In recent years several substances with possible glycocalyx restoring characteristics have been identified [48]. NMP offers the advantage of safely adding these substances to the perfusate. Data regarding these new possibilities, however, are still lacking.

Glycocalyx shedding has already been described in liver grafts after SCS. Schiefer et al. demonstrated significantly increased levels of syndecan-1 in the perfusion fluid of SCS grafts right before transplantation [26]. However, our study differs from this previous study, in that SCS was followed by NMP. It is evident that a direct comparison between the results of this work an those of previously published studies is not feasible. However, it is pertinent to acknowledge that despite similar baseline values, syndecan-1 levels at ICU admission were significantly lower in our study compared to a previous study of SCS graft recipients [19]. This finding may be attributed to the fact that in NMP grafts the very "first" reperfusion occurs with start of NMP; in SCS grafts however, reperfusion entirely happens in the recipient. It could be hypothesized that reperfusion occurring in the machine is more controlled compared to the intraoperative setting where bleeding and hemodynamic perturbations may pose a further challenge.

Our study has several limitations. First, the number of grafts analyzed was small, with 30 grafts included in the study. The small number of grafts and the heterogeneity of the cohort may introduce substantial bias and limit the generalizability of the findings; therefore, this limitation must be explicitly acknowledged, and the results should be interpreted with caution. This study was conceived with the objective of investigating the phenomenon of glycocalyx destruction during normothermic machine perfusion. However, the extent of glycocalyx destruction during machine perfusion appears to have a greater influence on the postoperative outcome of recipients than was previously assumed. Even though the study was not designed to establish a correlation between glycocalyx damage and the outcomes of the recipients, and that the exploratory data analysis was primarily intended to generate further hypotheses, the results were so interesting, that they have been included in the present publication. A further limitation is the detection of the endothelial glycocalyx itself. Direct imaging of the glycocalyx would be advantageous; however, this is a challenging procedure due to its vulnerable nature and was not feasible in the present study; the measurement of shedded glycocalyx components is a commonly employed approach and accepted as a surrogate marker instead. A further constraint arises from the limited size of the transplanted patient cohort, which renders long-term outcome measures, such as overall survival, difficult to interpret and highly susceptible to the influence of single individual outcomes, however, analyses from the same center have demonstrated that overall mortality is generally within the expected range [21]. In future studies, the intervals utilized for the analysis of parameters during NMP may benefit from more precise alignment. This would facilitate the

creation of a more precise representation of the course and would ensure that already falling values are not misinterpreted as peaks.

In summary, our data show that the endothelial glycocalyx is damaged during NMP liver transplantation. Shedding of glycocalyx proteins appears to be particularly pronounced in grafts derived from DCD donors. Furthermore, in the patient cohort under investigation, higher levels of glycocalyx shedding parameters during NMP significantly correlated with the development of EAD. This correlation was detected as early as 6 h after start of NMP which could potentially impact future donor recipient decisions. However, given the limited sample size and the heterogeneity of the studied cohort, the findings must be interpreted with caution. Further studies with larger and more homogeneous populations are required to validate these observations and to determine their clinical relevance.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving humans were approved by Ethics committee of the Medical University Innsbruck. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SM participated in research design, performance of research, writing of the paper and data analysis. GP participated in research design, performance of research and writing of the paper. LG participated in research design, performance of research and writing of the paper. NS participated in writing of the paper. LS participated in data analysis. PT participated in data analysis. RB participated in performance of research and writing of the paper. BC participated in performance of research and writing of the paper. AK participated in performance of research. RO participated in performance of research. TR participated in performance of research. SS participated in performance of research. JM participated in research design, performance of research, writing of the paper and data analysis. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

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Evaluation of the Prevalence of Occult Fibrin in Donor Organs, Its Origins, and Consequences: Insights From the COPE Studies

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Microthrombi are often observed in the glomerular tufts of peri-transplant renal biopsies, and occult fibrin has been described in livers undergoing normothermic perfusion, with its presence associated with cholangiopathy and poorer transplant survival. To further examine the phenomenon, we measured D-dimers in the perfusates of kidneys and livers that were part of organ perfusion studies conducted by the Consortium for Organ Preservation in Europe. Both kidneys and livers were found to contain variable amounts of D-dimers. The need for dialysis in kidneys donated after circulatory death (DCD) was associated with higher levels of D-dimers in the hypothermic kidney perfusate. Higher amounts of D-dimers in the liver perfusate were associated with poorer liver transplant survival. There was no significant difference in D-dimer release from livers and kidneys between donors who died from head trauma, stroke, or hypoxia. Organs from donors who died by euthanasia had significantly fewer D-dimers. This study shows that occult fibrin is

Abbreviations: COMPARE, Cold Oxygenated Machine Perfusion of Aged REnal transplants; COPE, Consortium for Organ Preservation in Europe; CVA, Cerebrovascular accident; DBD, Donation after brain death; DCD, Donation after circulatory death; FP7, Seventh Framework Programme; HMP, Hypothermic machine perfusion; HMPO2, Oxygenated hypothermic machine perfusion; NMP, Normothermic machine perfusion; NRP, Normothermic regional perfusion; POMP, Pre-implantation Oxygenated Hypothermic Machine Perfusion; QUOD, Quality in Organ Donation; TPA, Tissue plasminogen activator.

common in both livers and kidneys from deceased donors and has adverse consequences. The different D-dimer loads by donor cause of death suggest a donor origin for at least some of the occult fibrin.

Keywords: D-dimers, machine perfusion, microthrombi, organ donation, transplantation

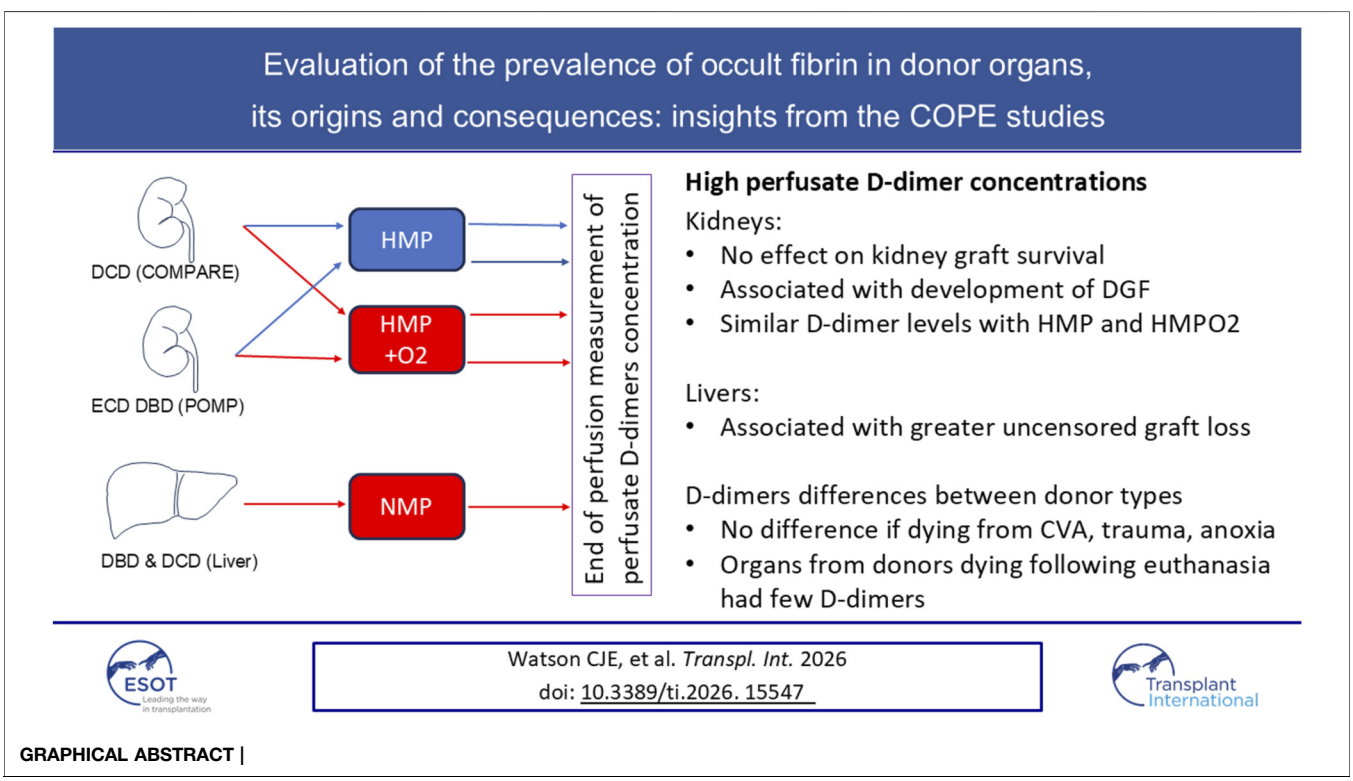
INTRODUCTION

It has long been recognized that donor kidneys may harbor fibrin microthrombi, most commonly observed within the glomerular tufts of early renal biopsies. However, their origin, significance, and prevalence are not clear. Early reports have suggested a recipient origin related to ciclosporin [1], while more recently they have been suggested to be of donor origin or to develop during cold storage; their significance has also been questioned [2–6]. Studies of livers undergoing end-ischemic *ex situ* normothermic machine perfusion (NMP) have also revealed the presence of microthrombi, which manifest as D-dimers in the perfusate [7].

D-dimers are breakdown products of polymerized fibrin. The D-dimer assay has a high sensitivity but low specificity for thromboembolic disease, as it is elevated in other conditions, such as cancer and infection. It is likely that, even in these conditions, an elevated D-dimer level signals the presence of fibrin polymer breakdown products. The test is simple to perform and is in routine use for the diagnosis of thromboembolic disease, where a negative test result excludes venous thrombosis or embolism. In the context of *ex situ* organ perfusion, the presence of D-dimers is highly likely to represent occult fibrin being flushed out of the organ.

The release of large amounts of D-dimers into the liver perfusate has been associated with a higher incidence of post-transplant cholangiopathy and poorer transplant survival compared to livers with less D-dimer release, and by extrapolation, less occult fibrin [7]. Fibrin plugs in the peribiliary vascular plexus, coinciding with areas of stromal necrosis, have been noted in livers donated after circulatory death (DCD) that underwent brief periods of NMP [8], supporting the causative role of fibrin in transplant cholangiopathy. D-dimers have also been observed to be released during hypothermic liver perfusion [7, 9]. These observations raise several questions:

1. Are D-dimers in the NMP perfusate associated with adverse outcomes in other series of perfused livers? If so, are they present in livers undergoing NMP from the point of recovery at the donor hospital, even with minimal exposure to cold ischemia?
2. What is the prevalence of D-dimers/fibrin in organs other than the liver, and if so, are they a marker of poor outcomes?
3. If fibrin originates in the donor, is its prevalence affected by donor cause of death?



GRAPHICAL ABSTRACT |

In an attempt to address these questions, we sought samples from the biorepository created as part of the Consortium for Organ Preservation in Europe (COPE), a European Union Seventh Framework Programme (FP7)-funded research program that includes three preservation studies where perfusate samples of both livers and kidneys were taken during machine perfusion and stored for future analysis [10–12]. In addition, we looked at our own data on D-dimers released from livers undergoing NMP, particularly referencing donor cause of death to look for possible associations. Finally, D-dimers were also measured in pre-retrieval blood samples from donors after brain death (DBD) and circulatory death obtained from the Quality in Organ Donation (QUOD) biorepository, whose livers underwent NMP at our institution, to see whether they were present in the donors and, if so, whether their prevalence corresponded to D-dimer load in the liver.

MATERIALS AND METHODS

The Consortium for Organ Preservation in Europe (COPE) was an FP7-funded program of research that included two randomized multicenter trials of hypothermic kidney perfusion and one randomized multicenter study of normothermic liver perfusion. The details of these studies have been published elsewhere [10–12] but are summarized below. All three trials had received the appropriate ethics approvals (POMP: 14/SC/1072; COMPARE: 14/SC/1056; and Liver: 14/LO/0182). In the COPE studies, perfusate samples were taken at pre-defined time points, centrifuged, and the supernatant was frozen and transferred to the biorepository at Oxford, where they were thawed once for aliquoting and stored at −80 °C.

The COPE Cold Oxygenated Machine Perfusion of Aged REnal Transplants (COMPARE) Study

The COMPARE study was a randomized controlled trial in which pairs of kidneys from the same DCD donor were randomized such that one underwent oxygenated hypothermic machine perfusion (HMPO2) and the other underwent non-oxygenated hypothermic machine perfusion (HMP) from the donor hospital until removed for transplantation at the recipient centre using Machine Perfusion University of Wisconsin solution and the XVIVO Kidney Assist Transport [11]. Perfusate samples were taken 15 min after the start of perfusion and at the end of perfusion.

The COPE Pre-Implantation Oxygenated Hypothermic Machine Perfusion (POMP) Study

The POMP study involved expanded criteria DBD donor kidneys, which were randomized on arrival at the recipient center to either remain stored on ice in their original cold storage solution, or to undergo end-ischemic oxygenated cold perfusion (HMPO2) until removed for implantation [12]. The XVIVO Kidney Assist Transport was also used for this study. Perfusate was collected after 15 min of perfusion and at the end of perfusion and processed as in the COMPARE study.

The COPE Liver Study

The COPE liver study was a device-to-donor study of the *metra*® (OrganOx, Oxford, UK) that randomized livers from both DBD and DCD donors to either standard cold storage in a University of Wisconsin solution or normothermic machine perfusion on the *metra* from the point of retrieval at the donor hospital until implantation at the recipient center [10]. Samples of perfusate were collected in ethylene diamine tetra-acetic acid (EDTA) after 1 h of perfusion.

Liver NMP Cases

For additional comparison of D-dimer concentrations released from livers of different deceased donors, we also compared a cohort of NMP liver perfusions undertaken in a back-to-base setting at our own institution. Perfusate samples had been taken after 2 h of NMP, centrifuged, and the supernatant stored at −80 °C. The 2-h time point was chosen for sampling because previous work had suggested it to be a time point after which D-dimer concentrations tended to plateau in some livers; it also corresponded to the previously used time point [7]. Cases receiving tissue plasminogen activator and those that had previously undergone normothermic regional perfusion were excluded.

QUOD Samples

The Quality in Organ Donation (QUOD) initiative is a biorepository in the United Kingdom that collects blood, urine, and tissue samples from consenting deceased organ donors to facilitate research. Samples include blood taken prior to organ recovery [13]. The pre-retrieval donor D-dimer content was assayed in the last EDTA sample from 78 donors who were selected based on high or low D-dimer levels detected after 2 h of normothermic liver perfusion. These livers included those that had and had not been exposed to alteplase plus fresh frozen plasma thrombolysis. This allowed us to assess the prevalence of fibrin degradation products in the donors and their relation to those in the liver perfusate.

D-dimer measurement

D-dimers were measured using latex immunoturbidimetry with the D-dimer HS assay on the ACLTOP 750CTS analyzer (Werfen, Barcelona, Spain) according to the manufacturer's instructions.

Statistical Analysis

Statistical analysis and graphing were performed using GraphPad Prism version 10.5 for macOS (GraphPad software, Boston, USA). Comparisons between multiple groups were undertaken with the Kruskal-Wallis test, while the Kolmogorov-Smirnov test was used for comparisons between two groups, since not all groups were normally distributed. Survival was compared with the Log-rank test. The Wilcoxon matched pairs test was used to

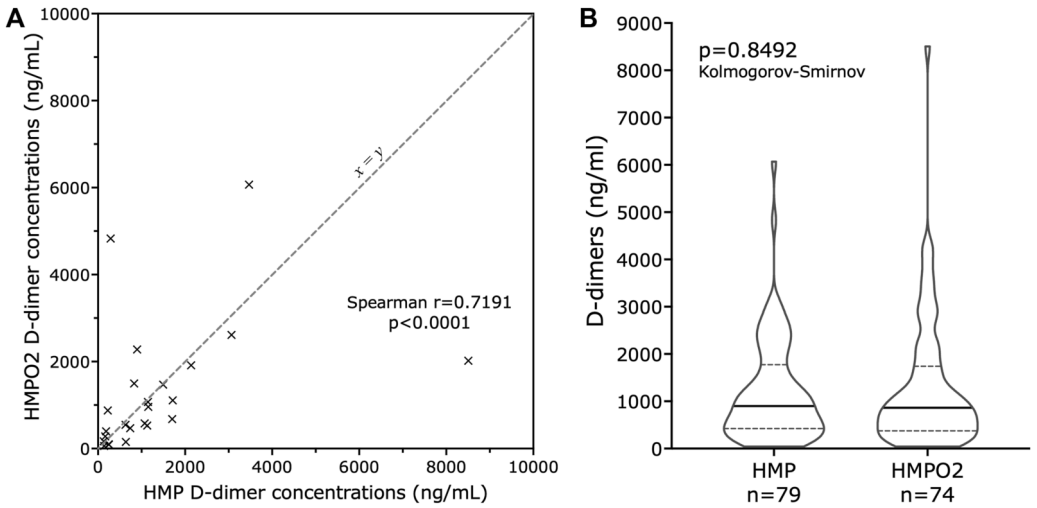


FIGURE 1 | D-dimer concentrations in kidneys undergoing HMP and HMPO2 in the COMPARE study. Panel **(A)** shows the D-dimer concentrations in the perfusate at the end of perfusion in 27 pairs of DCD donor kidneys from the same donor, where one kidney underwent HMP with oxygen (HMPO2) and one kidney underwent HMP without. There is a strong correlation (r = 0.731) between the two D-dimer concentrations. The dashed line is the line of equivalence (x = y). Panel **(B)** is a violin plot showing the D-dimer concentrations at the end of perfusion for all kidneys undergoing HMPO2 and HMP, with no significant difference between the groups (p = 0.885). The horizontal dashed lines (- -) represent the interquartile range, and the solid line (—) represents the median values.

compare pairs of kidneys from the same donor with different treatments.

RESULTS

Not all organs in each of the three COPE trials had perfusate samples taken, nor were all perfused organs transplanted.

D-dimers in COMPARE Study Kidneys

The COMPARE study included 112 samples taken 15 min after perfusion and 157 samples taken at the end of perfusion (median duration of perfusion: 547 min; IQR: 392–691), including 68 kidneys with samples taken at both time points. Of those 68 kidneys, there were more D-dimers present in the perfusate at the end of perfusion (median: 878 ng/mL; IQR: 335–2069) than at 15 min (median: 91 ng/mL; IQR: 52–217), with a close correlation between the samples taken at 15 min and at the end of perfusion (Spearman r = 0.7826; p < 0.0001; **Supplementary Figure S1**).

There were also samples from 24 pairs of kidneys from the same donor, one of which had undergone HMPO2 and the other HMP alone from the time of retrieval. End-of-perfusion D-dimer concentrations were closely correlated (Spearman r = 0.7191, p < 0.0001; **Figure 1A**), with no significant difference between groups (p = 0.4223, Wilcoxon matched pairs). Overall, of the 157 end-of-perfusion samples, 74 were from kidneys that underwent HMPO2, and 79 were from kidneys that underwent HMP alone. Two other kidneys received unspecified interventions, and two were described as having received cold storage alone but with perfusate samples; the true nature of these last two is unclear, and they were excluded from our analysis. There was no difference in D-dimer concentrations between the end of perfusion samples for HMPO2 and HMP kidneys (p = 0.8847, **Figure 1B**), which also suggests that oxygenating the perfusate did not affect D-dimer concentrations.

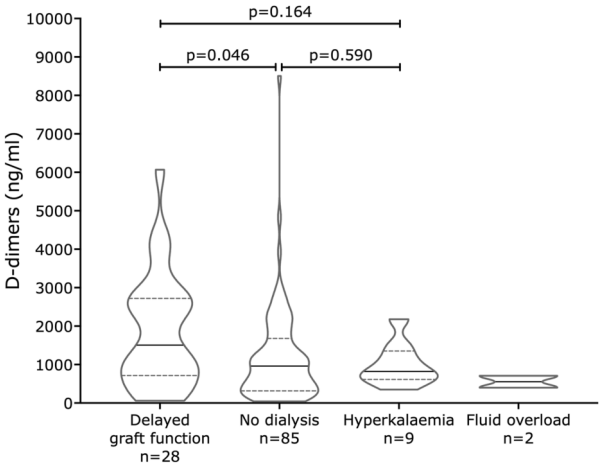


FIGURE 2 | Violin plots of end-of-perfusion D-dimer levels in COMPARE kidneys by early graft function classification. There were significantly greater D-dimer concentrations at the end of perfusion in kidneys that were defined as having delayed graft function due to the need for multiple dialysis episodes compared to those not requiring dialysis. In this truncated violin plot, the horizontal dashed lines (- -) represent the interquartile range, and the solid line (—) represents the median values.

Investigators had classified the need for dialysis in the first week post-transplant into four groups: no dialysis (n = 85); delayed graft function (DGF, n = 28); hyperkalemia (a single post-transplant dialysis due to high serum potassium, n = 9); and

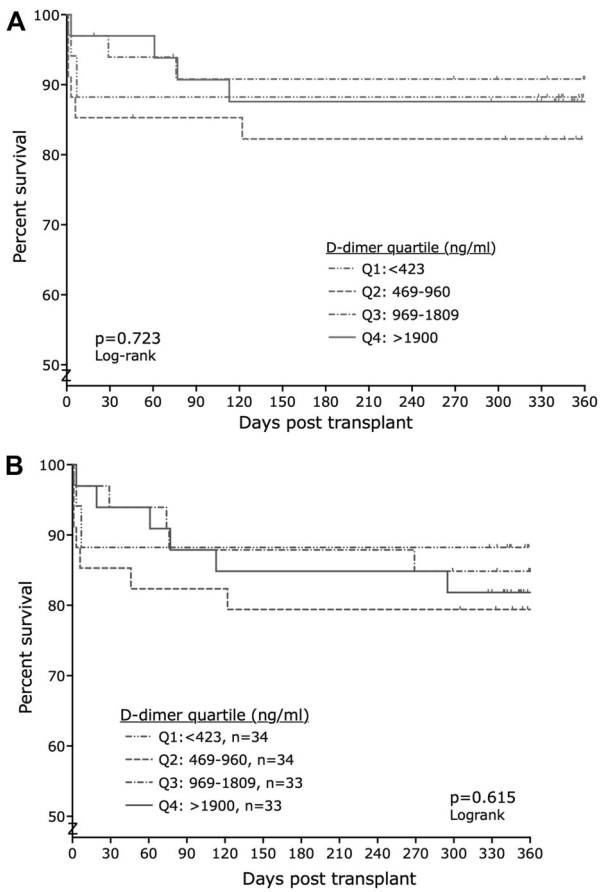


FIGURE 3 | There was no significant difference between groups in either **(A)** graft survival (censored for death) or **(B)** transplant survival (graft survival not censored for death).

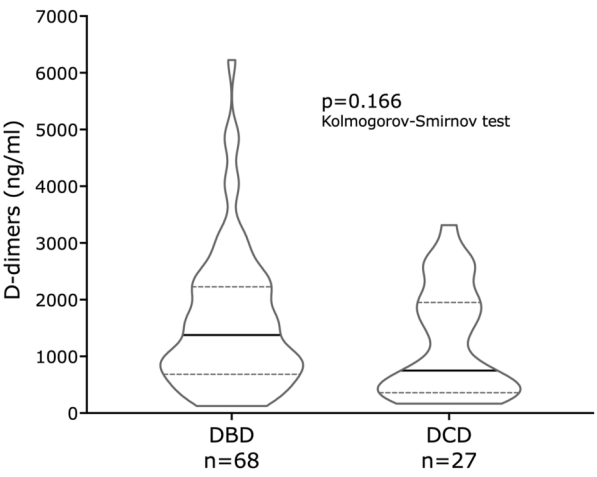


FIGURE 4 | D-dimer concentrations in the COPE liver machine perfusion study. Panel 4 is a violin plot of D-dimer concentrations after 1 h of perfusion in DBD and DCD donor livers, where the horizontal line represents the median value and the dotted lines represent the interquartile ranges. There is no significant difference in D-dimer concentrations between DBD and DCD donor livers.

fluid overload (the need for a single dialysis episode to remove excess fluid, n = 2). Eleven kidneys that failed within the first seven days were excluded, as were 21 kidneys for which follow-up consent was not given. Of the 11 kidneys lost in the first week, 4 were due to technical reasons related to vascular anastomosis, 4 were described as having an immunological cause, 2 had primary nonfunction, and one had recurrent focal segmental glomerulosclerosis. There was a significant difference in D-dimer concentrations between kidneys with and without delayed graft function (p = 0.0461, Kolmogorov-Smirnov, **Figure 2**).

There was no significant difference by D-dimer quartile in glomerular filtration rate at 12 months, as calculated from the Chronic Kidney Disease Epidemiology Collaboration (CKD-Epi) formula (data not shown). Graft survival was characterized by more early graft loss within 30 days in the two quartiles with the lowest D-dimer concentrations and more late graft loss in the quartiles with the higher D-dimers, but overall there was no significant difference in graft or transplant survival (for graft survival p = 0.7234, for transplant survival p = 0.6147, logrank test; **Figures 3A,B**).

D-dimers in POMP Study Kidneys

The POMP study included 74 perfusate samples taken 15 min after the start of perfusion and 74 samples taken at the end of perfusion (median: 289 min; IQR: 188–439), including 71 kidneys for which both beginning and end of perfusion samples were available. There was a statistically significant, moderate correlation between the 15-minute and end-of-perfusion samples (Spearman r = 0.5857, p < 0.0001, **Supplementary Figure S2**), with the end-of-perfusion samples containing more D-dimers. There was also a significant, moderate correlation between the duration of end-ischemic HMPO2 and D-dimer concentrations (Spearman r = 0.4443, p = 0.0001, **Supplementary Figure S3**). In the POMP study, the need for dialysis was classified as due to delayed graft function (n = 16), fluid overload (n = 1), other (n = 1), or no dialysis required (n = 56) (**Supplementary Figure S4**). There was no correlation between delayed graft function and end-of-perfusion D-dimer concentrations.

Only three of the kidneys for which perfusate was available were lost during the study period. These included the kidney with the highest recorded D-dimer concentration (3,875 ng/mL), which was lost to venous thrombosis on day 8.

The median 15-minute perfusate D-dimer concentration in the COMPARE study was slightly higher than that in the POMP study (COMPARE: 90 ng/mL, IQR 52 to 217; POMP: 65 ng/mL, IQR 50 to 109, p = 0.0168). The end-of-perfusion perfusate D-dimer concentrations were much higher in the COMPARE study than in the POMP study (COMPARE median 878 ng/mL, IQR 334–2069; POMP 300 ng/mL, IQR 174–556, p < 0.0001). When interpreting these data, it is important to remember that the COMPARE kidneys were from DCD donors, while the POMP kidneys were from extended criteria DBD donors, and that the duration of perfusion was longer in the COMPARE study

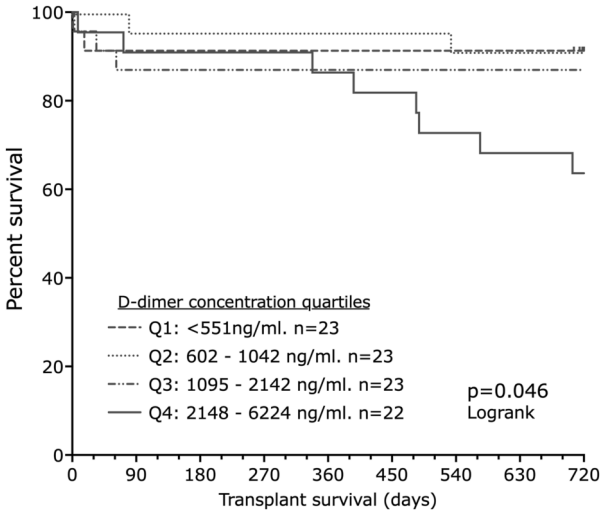


FIGURE 5 | Liver transplant survival by D-dimer quartile. There is a significant increase in transplant loss (death or graft failure) at 2 years with increasing D-dimer quartiles (Log-rank p = 0.046).

than in the POMP study (median duration in the COMPARE study was 547 min vs. 289 min in the POMP study).

D-dimers in the COPE Liver Study

Perfusion samples were collected 1 h after the start of perfusion from 95 livers (68 from DBD donors and 27 from DCD donors). The median D-dimer concentration was 1,376 ng/mL (IQR: 684–2228) for DBD livers and 750 ng/mL (IQR: 360–1950) for DCD livers, a difference that was not significant (**Figure 4**; p = 0.1657, Kolmogorov-Smirnov).

Of the 95 livers for which samples were received, 91 were transplanted. Two-year follow-up data were available for all transplanted livers. The transplanted livers were divided into quartiles based on D-dimer concentrations. Transplant failure (graft loss (n = 2) or death (n = 13)) was more likely in the quartile with the highest D-dimer concentration (p = 0.0458, Logrank, **Figure 5**).

Of the livers for which a perfusate sample was obtained, 59 had undergone magnetic resonance cholangiopancreatography (MRCP) at 6 months. None of these livers had definite cholangiopathy affecting the peripheral ducts; however, three had hilar strictures, which may have been secondary to cholangiopathy or related to the placement of the bile cannula before perfusion; the D-dimer concentrations of these three livers were 246, 669, and 1,950 ng/mL, respectively.

D-dimers by Cause of Death

D-dimer concentrations were examined by donor cause of death in organs from the COPE liver study, the COMPARE and POMP kidney studies, and a cohort of perfused livers from our own center (**Figure 6**). There was no difference observed in D-dimer concentrations between donors who died from cerebrovascular accidents (CVAs), hypoxia, or trauma in any of the study populations. Both the COMPARE kidney study and the COPE liver study had separate donor cohorts who died from euthanasia, and organs from those donors were associated with significantly lower D-dimer concentrations than organs from donors who died from other causes. For example, in the COMPARE study, donors who died following euthanasia had significantly lower D-dimers than donors who died from hypoxia ($p < 0.0001$), trauma ($p < 0.0003$), or cerebrovascular accidents ($p = 0.002$, all tested using the Kolmogorov-Smirnov test). In contrast, donors who died of meningitis tended to have higher D-dimer concentrations. The COPE liver study also included three donors described as having died of chest infections who also had high D-dimer concentrations.

D-dimers in QUOD Donors

Pre-donation samples were obtained from 77 liver donors whose livers subsequently underwent NMP at our institution; all had pre-donation D-dimer levels above the normal range (<250 ng/mL), suggesting the presence of intravascular thrombus breakdown products in their circulation, with donors who died from trauma having the highest D-dimer concentrations (**Supplementary Figure S5**). DBD donors had higher D-dimers than DCD donors (**Supplementary Figures S5, S6**, p = 0.046, Kruskal-Wallis), but this was a selected group of donors based on liver D-dimer release; therefore, no inference about differences between DBD and DCD donors should be made. There was no significant correlation between D-dimer concentrations in donors and D-dimer concentrations in the liver perfusate of TPA-treated (n = 37) or untreated (n = 40) livers.

DISCUSSION

In this study, we demonstrated that the release of D-dimers from organs of deceased donors during *ex situ* perfusion is common and occurs in both kidneys and livers. In addition, we confirmed the previous observation from the Cambridge study that the presence of high concentrations of D-dimers in livers is associated with increased transplant failure (graft loss not censored for death). Unlike the Cambridge study, the COPE study livers were placed on NMP at the donor hospital, with very short periods of cold storage, suggesting that the fibrin formation is probably not secondary to cold storage alone, although a contribution from cold storage cannot be ruled out. The D-dimer concentrations were also much lower in the COPE livers than in Cambridge livers. While this may reflect a contribution of cold storage-induced fibrin in the Cambridge livers, it is also possible that the shorter period of NMP before samples were taken (1 h vs. 2 h) may have meant there was less time for flushing out the vasculature.

We also demonstrated that D-dimer concentrations increased with time in both kidney cohorts, being lower after 15 min of perfusion and higher at the end of perfusion, and that there was a correlation between the early and late perfusion D-dimers, supporting the notion that long hypothermic perfusions are associated with greater washout of pre-existing intravascular fibrin. This is further supported by the observation that the extended criteria DBD kidneys undergoing oxygenated

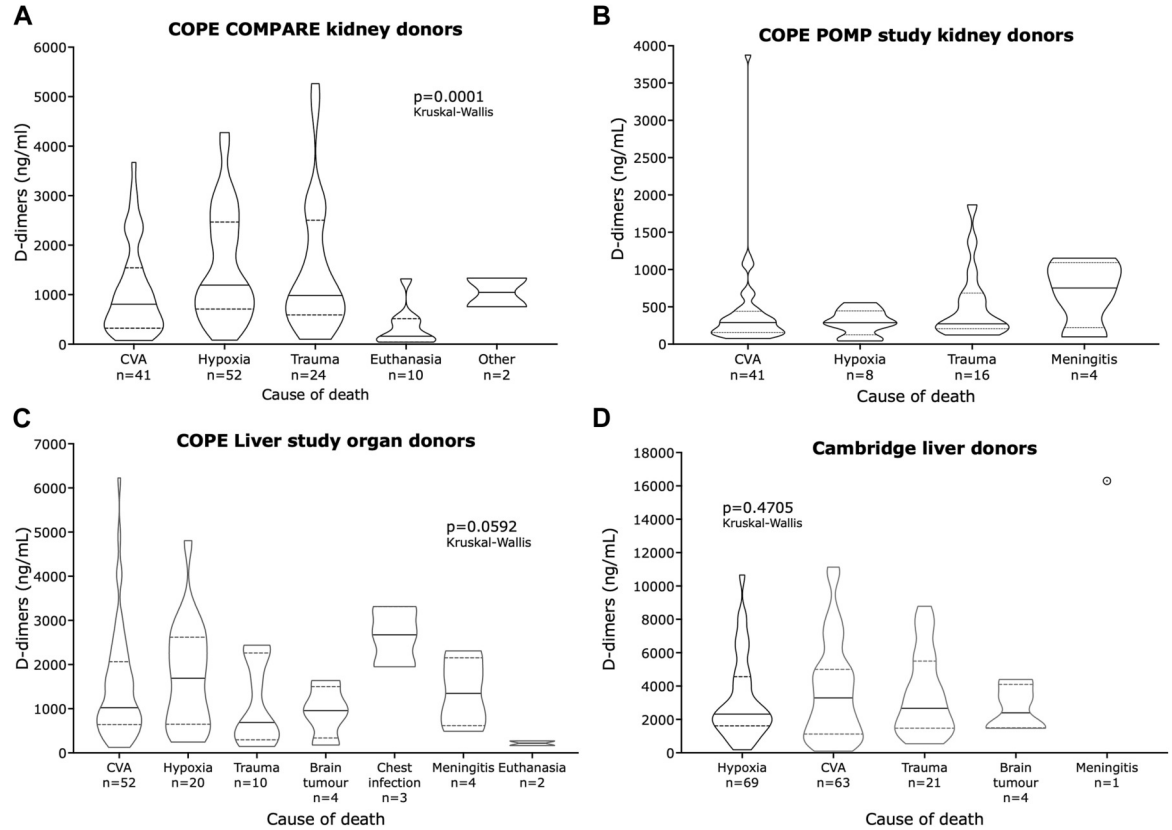


FIGURE 6 | Violin plots of four groups of organs and their D-dimer concentrations by cause of donor death. **(A)** End of perfusion samples in the COMPARE study; **(B)** End of perfusion samples in the POMP study; **(C)** One-hour perfusate samples in the COPE liver study, and **(D)** 2-h perfusate samples in the investigators' liver NMP cohort.

perfusion at the recipient center in the POMP study tended to release fewer D-dimers than DCD kidneys undergoing HMP or HMPO2 from the donor hospital in the COMPARE study, during which the duration of perfusion was much longer (the median duration in the COMPARE study was 547 min vs. 289 min in the POMP study). It is also possible that this difference relates to a greater burden of fibrin in the DCD donor kidneys in the COMPARE study, or both factors may be at play.

There was no difference in D-dimer concentrations in the COMPARE study between kidneys undergoing HMP and those undergoing HMPO2, suggesting that oxygenation during hypothermic perfusion does not affect the prevalence of fibrin. Kidneys defined as having delayed graft function appeared to have higher D-dimer loads than kidneys categorized as needing dialysis for hyperkalemia or fluid overload, supporting the researchers' differing indications for dialysis. The D-dimer load did not translate into poorer graft survival.

The difference in D-dimer amounts in organs according to the cause of death of the organ donor was also notable. There was no significant difference in D-dimer release in all the studies between donors who died from stroke (cerebrovascular accident), trauma, or hypoxia; however, there was a significant lack of D-dimers in donors who died by euthanasia. It is unclear whether this relates to treatment at the time of donation [14–16] or to the absence of a circulatory insult in the time leading up to death, following which a clot forms while hospitalized or as a result of stress. Further investigation is required. Donors undergoing normothermic regional perfusion were excluded from the study due to prior evidence showing an association between this procedure and low levels of D-dimer release [7]. In the absence of NRP, we demonstrated here that DCD donor organs shed equivalent amounts of D-dimers as DBD livers.

The subgroup of kidneys in the COMPARE study from euthanized donors was reported to have lower vascular resistance during perfusion compared to other kidneys in that same study [17] and it is possible that the absence of microthrombi may contribute to this observation and explain the variable resistance seen during HMP of donor kidneys in general. This is the subject of investigation by other researchers.

Another observation, based on a small number of cases, is that organs from five donors who died from meningitis and three donors with active chest infections due to chronic pulmonary disease appeared to release higher concentrations of D-dimers, suggesting a contribution of donor sepsis to their etiology. This observation requires substantiation in a larger cohort.

All organ donors in the QUOD study had abnormally high concentrations of D-dimers, possibly reflecting the cause of death, such as trauma; it may also reflect thromboembolic complications in the intensive care unit. The lack of correlation between D-dimers released from the liver during NMP and those in the donor's blood suggests that screening donors to identify at-risk organs would not be worthwhile. This and other observations pose a challenge for neuro-intensivists, since disseminated thrombosis appears to be common, and its contribution to neurological outcomes is uncertain.

Thrombi are often observed in the glomeruli of deceased donor kidneys [4, 5, 18], but their prevalence has hitherto been unknown. A recent study of over a thousand pre-implantation and implantation punch biopsies revealed a <2% incidence of microvascular thrombi, the majority of which had no thrombotic clinical sequelae [19]; this is consistent with other studies. Nevertheless, sampling glomeruli may miss thrombi in peritubular capillaries. Occlusion of these capillaries and of the arteries within the glomeruli may contribute to acute tubular necrosis. Our data suggest that fibrin microthrombi are common and that they may have an etiological role in DGF. They may contribute to the different degrees of resistance during hypothermic perfusion. These microthrombi, together with vascular spasm, probably contribute to the blotchy appearance of the kidneys during reperfusion, during which the slow flow of arterial blood results in desaturation, with a resultant blue hue to the kidney until the prevailing arterial pressure manages to clear some of the occluding microthrombi.

Our data, particularly the observations relating to donors who died as a consequence of euthanasia, provide strong evidence to support the fact that at least some of the fibrin originates in the donors before donation. These data align with observations that thromboelastography of both DCD and DBD donor blood before retrieval is hypercoagulable [20]. We have not been able to determine whether organs also acquire fibrin microthrombi during cold storage, something that would contribute to the adverse effects of extended periods of cold ischemia. Nevertheless, the prevalence of microthrombi in donor organs suggests attention should be paid to the management of patients with severe neurological injury to minimise the risk of thrombi formation in all organs.

In this article, we have shown that hypothermic machine perfusion does flush out fibrin fragments, and previous reports have confirmed the same observations with hypothermic oxygenated liver perfusion (HOPE) [7, 9]. The relatively high incidence of cholangiopathy following HOPE in the European randomized trial, 14% at 5 years, may reflect the relatively short perfusion period [21]. Flushing out fibrin fragments probably does contribute to superior kidney outcomes with prolonged preservation using HMP compared to static cold storage alone [22].

An alternative treatment is *ex situ* normothermic perfusion with recombinant tissue plasminogen activator (TPA) and a plasminogen source. This has been shown to release more D-dimers from livers than normothermic perfusion without thrombolytics [7, 23], but further work is required to define optimal protocols for clinical use, with their efficacy proven in a randomized setting. TPA treatment may also play a role in kidney transplantation, given the association of D-dimers with delayed graft function, and warrants further investigation. When thrombolysis is not undertaken, measuring D-dimers during perfusion may prove a valuable diagnostic tool for the assessment of the viability of deceased donor organs.

A retrospective analysis of prospectively collected samples such as this has limitations. One major limitation is the lack of standardization in the timing of perfusate sampling. Future studies should include serial sampling at standard time points.

In summary, this study confirms the previous observation that occult fibrin in donor livers is associated with adverse graft outcomes and also demonstrates that it is associated with delayed graft function in kidneys. We demonstrated that all of the organ donors studied had high levels of D-dimers, suggesting intravascular fibrin. These levels were not correlated with the levels found in the livers of the same donors. Perfusion, whether hypothermic or normothermic, appears to reduce the fibrin burden and may thus contribute to improved outcomes. Finally, we showed that donors who died by euthanasia have no fibrin burden, in contrast to those donors who died from other causes. The next challenge is to rid the organs of their occult fibrin before it can affect post-transplant outcomes.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, subject to reasonable request.

ETHICS STATEMENT

Five ethics committees were involved in approval for the underlying studies and the use of perfusates for future research POMP: REC 14/SC/1072 - South Central - Hampshire B Research Ethics Committee; COMPARE: EC 14/SC/1056 - South Central - Hampshire B Research Ethics Committee; COPE Liver: REC 14/LO/0182 - NRES Committee London - Dulwich; QUOD: 23/ NW/0097 (North West - Greater Manchester Central Research Ethics Committee) and 20/SS/0105 (Scotland A Research Ethics Committee). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

AUTHOR CONTRIBUTIONS

CW, SM, AB, RG, AP, and VK were involved in the research design, CW, IJ, AP, and VK were involved in writing the paper which all authors reviewed before submission. All authors except AP contributed to performing the research while CW performed

the data analysis. All authors contributed to the article and approved the submitted version.

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AUTHOR DISCLAIMER

The views expressed are those of the author(s) and do not necessarily reflect those of the NIHR, NHS Blood and Transplant, or the Department of Health and Social Care.

CONFLICT OF INTEREST

CW is a paid consultant to Organox Ltd.; PF is the founder of Organox Ltd.

The remaining author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

GENERATIVE AI STATEMENT

The author(s) declared that generative AI was not used in the creation of this manuscript.

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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.2026.15547/full#supplementary-material

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CNI Trough Variability Does Not Reliably Reflect Medication Adherence: Insights From a 3-Year Follow-Up Study

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Calcineurin inhibitor (CNI) trough concentrations and their variability are frequently used as adherence proxies, despite limited validation. We evaluated the association between self-reported adherence and CNI exposure during the first year after kidney transplantation. We included 619 patients from two prospective French cohorts (14,829 C_0 values). Adherence was assessed using the MMAS-4 questionnaire. CNI exposure was evaluated via C_0 levels, intra-patient variability (IPV; CV threshold = 30%), and underexposure rates. Cross-sectional and longitudinal analyses were performed. No significant differences in C_0, IPV, or underexposure were observed between adherent and non-adherent patients, regardless of the CNI used or analytical approach. In longitudinal analysis, IPV was similar (31.3% [25.5–38.2] vs. 31.6% [23.6–38.9], p = 0.68), as was the proportion of patients with high IPV (55.5% vs. 51.5%, p = 0.5). At 3 years, high IPV was not significantly associated with rejection (HR 1.02 [0.67–1.55], p = 0.93). Self-reported adherence was not associated with CNI C_0 levels, IPV, or underexposure. CNI C_0 variability alone cannot reliably detect non-adherence and should not be interpreted as a standalone adherence marker. Multimodal strategies combining pharmacokinetics with validated self-report tools are needed to evaluate adherence.

Keywords: adherence, calcineurin inhibitor (CNI), kidney transplantation, trough concentrations (C_0), variability

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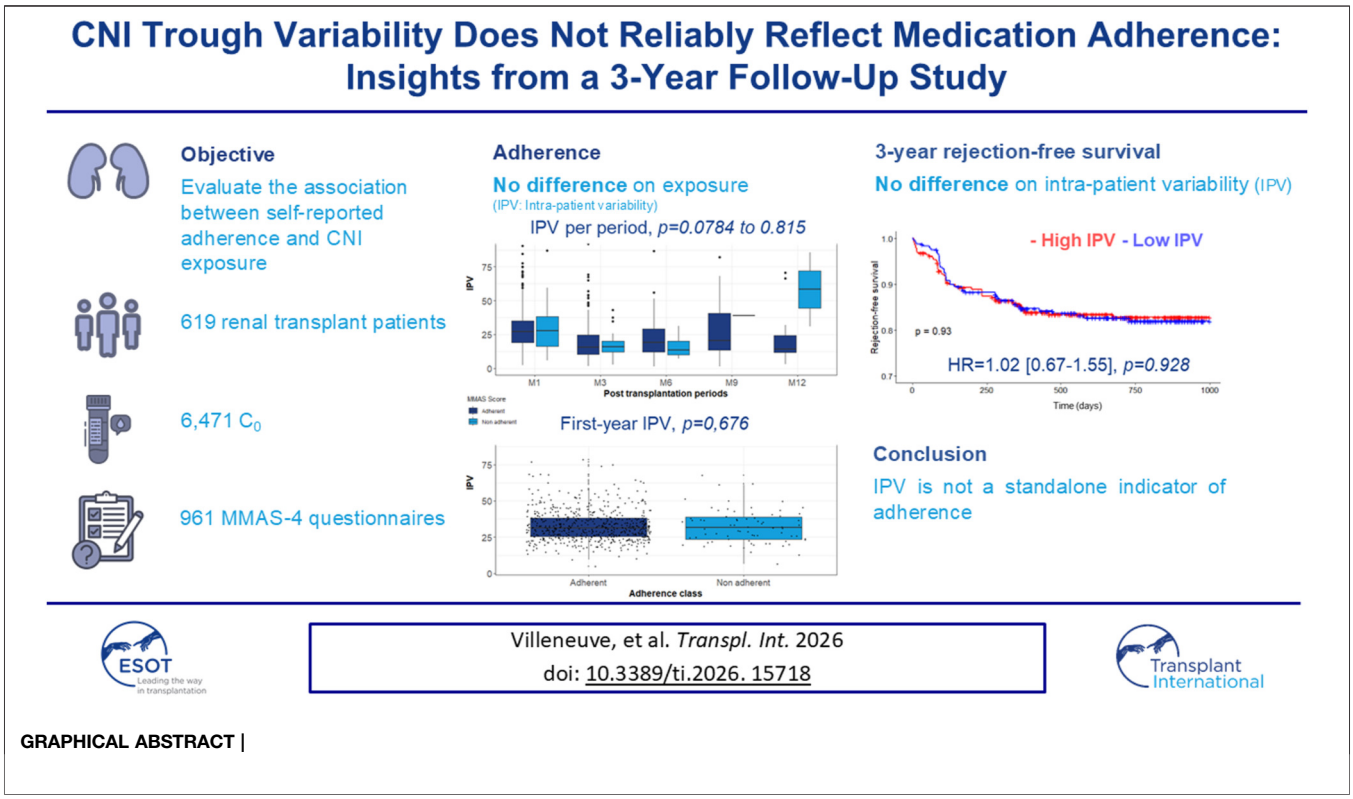
Abbreviations: BAASIS, Basel Assessment of Adherence to Immunosuppressive Medication Scale; BIC, Bayesian Information Criterion; C_0, trough concentrations; CNI, Calcineurin inhibitor; CV, Coefficient of variation; HR, Hazard ratio; IPV, Intra-patient variability; IQR, Interquartile range; ITAS, mmunosuppressive Therapy Adherence Scale; MMAS, Morisky-Green-Levine Medication Adherence Scale; MEMS, Medication Electronic Monitoring Systems; MMF, mycophenolate mofetil.



GRAPHICAL ABSTRACT |

INTRODUCTION

Over the past years, medication adherence has emerged as a major concern for clinicians, policymakers and health managers. In kidney transplantation, the prevalence of poor adherence ranges between 15% and 43% in adults and between 40% and 60% in adolescents [1–7]. It is a critical determinant of post-transplant outcomes, contributing to late rejection, chronic allograft dysfunction, nephropathy and ultimately, graft loss [1, 8, 9]. It is also associated with psychosocial complications, reduced quality of life, and substantial healthcare costs. Consequently, improving adherence may yield benefits comparable to those of optimized or new pharmacological interventions [10].

Conducting studies on adherence requires clear definitions and robust and validated measurement tools. The World Health Organization originally defined adherence as "the extent to which a person's behavior–taking medication, following a diet, and/or executing lifestyle changes, corresponds with agreed recommendations from a healthcare provider" [10]. The concept of medication adherence has since evolved to encompass a dynamic process, comprising initiation, implementation and discontinuation [11]. Nevertheless, no gold standard for adherence measurement has been established to date [12]. A wide variety of tools have been employed in transplant populations, including pill counts, pharmacy refill databases, Medication Electronic Monitoring Systems (MEMS), immunosuppressant blood concentrations and validated questionnaires such as the BAASIS, ITAS or MMAS [9, 13–15]. Composite scores combining questionnaires and pill count or drug concentrations have also been used [14, 16].

Among these tools, trough concentrations (C_0) of calcineurin inhibitors (CNIs), particularly tacrolimus, have been proposed as a proxy of adherence. Several approaches have been proposed: (i) evaluating intra-patient variability (IPV) via coefficient of variation over a period [9, 13, 14, 17, 18]; (ii) assessing mean C_0 levels [19]; (iii) estimating the proportion of C_0 values outside of the therapeutic range [13, 20]; (iv) calculating the time spent outside of the therapeutic range [14, 21]. While exposure to immunosuppressants and its variability are known to influence transplant patients outcomes [18, 22–24], the association between drug exposure biomarkers and non-adherence remains inconsistent. Most studies rely on cross-sectional designs whereas non-adherence is a dynamic phenomenon that may be isolated or repeated over time [1]. In this regard, longitudinal analysis of drug exposure variability may better reflect therapeutic implementation patterns. However, correlating long-term variability with a single-point questionnaire is methodologically questionable. Moreover, the pharmacokinetics of tacrolimus is influenced by numerous factors, and the proportion of variability attributable solely to non-adherence is likely to be small.

In clinical practice, poor adherence can be suspected when tacrolimus concentrations are unexpectedly low. However, in research settings, the validity of using drug levels as surrogate

adherence markers remains questionable. Despite this, both clinicians and researchers continue to infer adherence behaviors from drug exposure data [12, 18], and C_0 monitoring remains a widely used -albeit imperfect- proxy [25].

In this context, the objective of our study was to explore the relationship between adherence and exposure to calcineurin inhibitors (CNIs) during the first year after kidney transplantation. Because the clinical consequences of high CNI intra-patient variability–whether driven by non-adherence or other factors–may only emerge later, we also examined the association between first-year CNI exposure and graft rejection occurring up to 3 years post-transplantation.

MATERIALS AND METHODS

Design and Study Population

This study included patients from two successive multicenter prospective cohorts: EPIGREN, conducted between 2007 and 2011 in three French kidney transplantation centers, and EPHEGREN, conducted between 2012 and 2017 in the same centers plus four additional ones. Both studies were sponsored by Limoges University Hospital (CHU Limoges) and complied with the legal requirements of the Declaration of Helsinki. Regulatory approvals were received from the French Medicine Agency (authorization no. 060566, dated 08/08/2006), the French National Data Protection Authority (CNIL; no. 907275 ACT in 2006 for EPIGREN, no. 912242 ACT in 2012 for EPHEGREN) and the relevant regional Ethics Committee (no.06-040 on 19/05/2006 for EPIGREN and no. 130-2013-30 on 20/11/2013 for EPHEGREN).

All *de novo* kidney transplant patients followed-up in the participating centers were eligible for inclusion, except if they met the following non-inclusion criteria: (i) inability to understand written French; (ii)inability to understand the protocol; (iii) impossibility to be followed in one of the seven investigating centers (refusal of regular long-term follow-up, moving, etc.). Eligible patients were enrolled within the first month post-transplantation during a routine consultation, after receiving information about the study and giving their written consent.

A total of 820 patients were recruited in these two cohorts (**Figure 1**). Their immunosuppressive regimens were left to the discretion of the investigators and complied with the standard of care for transplanted patients (mainly CNIs -either tacrolimus or cyclosporine- in combination with mycophenolate). Dose adjustments of CNIs were based on trough concentrations, using the recommended target ranges [22, 26, 27].

Data Collection

Following inclusion, patients were scheduled for up to eight visits over a three-year period after the transplantation: at 1 month (M1), 3 months (M3), 6 months (M6), 12 months (M12), 24 months (M24), and 36 months (M36). An additional visit at 9 months (M9) was included for patients in the EPIGREN cohort.

At each visit, clinical outcomes -including graft rejection- and biological data, were collected from the medical records and patient-reported outcomes assessed using self-administered questionnaires.

Self-Reported Adherence

Self-reported adherence was assessed using the 4-Item Morisky-Green-Levine Medication Adherence Scale (MMAS-4) (**Supplementary Material**) [28]. The MMAS-4 is straightforward to administer and interpret, has been formally validated in French and among transplant populations, and is well suited for repeated longitudinal assessments [1]. The questionnaire was self-administered during scheduled follow-up visits in accordance with the study protocol and completed by patients without assistance from clinicians or family members. Participants filled out the questionnaire in the waiting room prior to their visit. These research visits were synchronized with routine clinical follow-up. A score of 0 was considered indicative of adherence, whereas any score greater than 0 indicated non-adherence.

Both cross-sectional and longitudinal approaches were employed in this study. Therefore, only patients who had completed the MMAS-4 at least twice during the three-year post-transplantation follow-up period were considered (**Figure 1**). In the cross-sectional approach, adherence was evaluated independently at each follow-up visit. In the longitudinal approach, patients were categorized into two groups based on their previously established adherence trajectories over time, identified using a mixed-effects modeling framework with latent processes and latent classes, as described by Villeneuve et al. [1] (**Supplementary Material**). The two resulting categories were: (i) patients with a consistently good and stable adherence over time; (ii) patients with a poor and worsening adherence.

Exposure to Calcineurin Inhibitors

Both IPV and underexposure to CNIs were analyzed. To be eligible for this analysis, patients had to have at least three C_0 measurements of either cyclosporine or tacrolimus over the first year post-transplantation. Patients not meeting this criterion were excluded (**Figure 1**). In addition, extreme C_0 values (≥500 μg/L for cyclosporine and ≥20 μg/L for tacrolimus) were considered outliers and excluded from the dataset.

IPV was quantified using the coefficient of variation (CV), calculated as the ratio of the standard deviation (SD) to the mean (μ) of C_0 values. Variability was classified as high (CV ≥30%) or low (CV <30%), in line with previously published thresholds [17]. Underexposure was assessed based on the proportion of C_0 values below the lower bound of the therapeutic window. For cyclosporine, these thresholds were 200 μg/L at M1, 150 μg/L between M2 and M6 and 100 μg/L beyond M6. For tacrolimus, a fixed threshold of 4 μg/L was applied across all time points [22, 26, 29].

Missing Data Management

CNI trough levels were obtained as part of routine clinical care and were considered exhaustive for the study period, as all trough concentrations available from routine therapeutic drug monitoring during follow-up were included in the analyses. For the MMAS, patients were invited to complete the questionnaire at each visit. For the longitudinal analysis, we used mixed models with

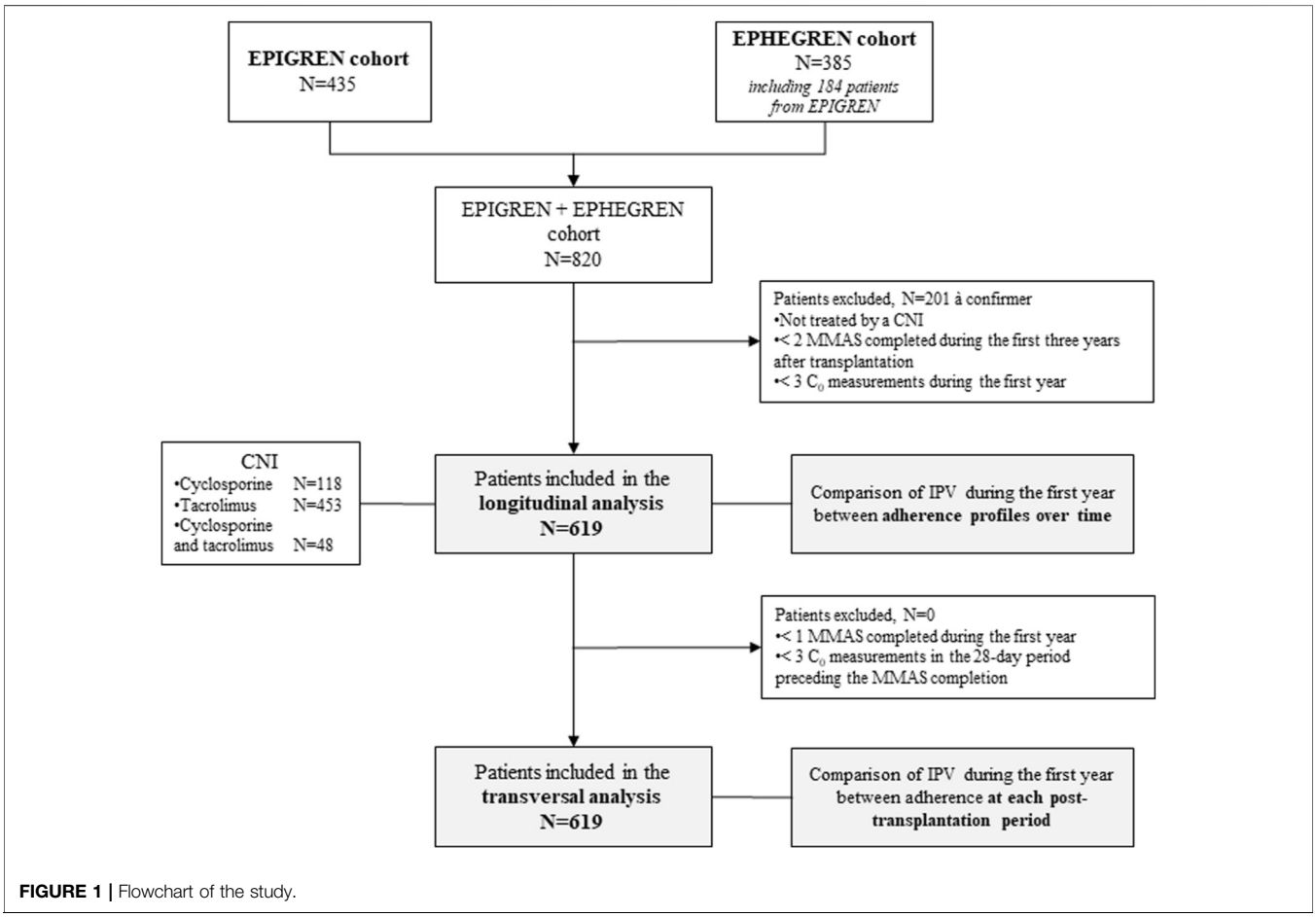


FIGURE 1 | Flowchart of the study.

latent processes and latent classes to characterize adherence profiles over time. This modeling framework accommodates incomplete longitudinal observations. Parameters were estimated by maximum likelihood, under the assumption that missing data were missing at random.

Statistical Analysis

All analyses were performed using R version 4.2(R Foundation for Statistical Computing: http://www.r-project.org). Categorical variables are reported as frequencies and percentages, and continuous data as their median and interquartile range [IQR] otherwise. Group comparisons were performed using Pearson's chi-square or Fisher's exact tests for categorical data, and Student's t-test or Kruskal-Wallis test for continuous variables, as appropriate. Bonferroni risk correction was applied in the case of multiple comparisons.

The relationship between adherence and CNI exposure was explored using both cross-sectional and the longitudinal approaches. In the cross-sectional analysis, adherence at each visit (based on MMAS-4) was compared to C_0 values collected during the preceding 28-days. In the longitudinal analysis, adherence profiles were compared to C_0 values collected during the first year after transplantation. Both analyses were performed for the entire cohort and for each CNI. To ensure the robustness of intra-patient variability (IPV) estimation, a minimum of three trough concentrations per analysis window was required. A single IPV estimate was calculated for each patient and each analysis window, ensuring that each individual contributed only one IPV value, irrespective of the number of available measurements.

The relationship between IPV and graft rejection at 3 years post-transplantation was explored using a Cox proportional hazards model. An initial univariate analysis was conducted, followed by inclusion of variables with $p < 0.20$ in a multivariable model. Final model selection was performed using backward stepwise elimination based on the Bayesian Information Criterion (BIC). Model robustness was assessed through 1,000 bootstrap resamples, each followed by backward stepwise selection using the same procedure. The hazard ratios (HR) and 95% confidence intervals (95% CI) derived from the final model and the percentage of selection of each covariate in the bootstrap procedure were calculated [30] Candidate covariates included recipient-related, treatment-related, and immunological variables routinely collected in the cohort. Time-to-rejection was estimated using Kaplan-Meier survival analysis and high IPV vs. low IPV patients were compared using the log-rank test. The proportionality of risks in the final models was verified using the Schoenfeld residuals.

Two-sided p-values <0.05 were considered to be statistically significant.

RESULTS

Description of the Population

A total of 619 patients were included in this study, contributing to 14,829 C_0 measurements during the first year post-transplantation (**Figure 1**). The main sociodemographic and clinical characteristics of the cohort are summarized in **Table 1**.

Among the participants, 118 patients received cyclosporine only, 453 tacrolimus only, and 48 received both CNIs at different times during the first year (**Supplementary Table S1**; **Supplementary Figure S1**). Almost all patients were also treated with mycophenolate as part of their immunosuppressive regimen (**Supplementary Table S2**).

The median [IQR] C_0 was 8.6 [6.8–10.6] μg/L for tacrolimus (11,618 C_0 values) and 161 [124–217.0] μg/L for cyclosporine (3,211 C_0 values). Outlier values, as defined in the Methods, represented less than 1% of cyclosporine concentrations and less than 2% of tacrolimus concentrations.

TABLE 1 | Characteristics of the cohort (N = 619). Continuous variables are reported in median [IQR], categorial variables are reported in numbers (%).

N	619	Missing data (%)
Age (years)	54.0 [44.5–64.0]	0.0
Gender, male	398 (64.3)	0.0
Height (cm)	170 [163–176]	29.4
Weight (kg)	72 [62–82]	1.3
Body Mass index	25.1 [22.0–28.3]	29.4
Occupational status		20.7
Active	86 (17.5)	
Retired or without any professional activity	387 (78.8)	
Other	18 (3.7)	
Rank of kidney transplantation		0.0
0	535 (86.4)	
1	78 (12.6)	
2	6 (1.0)	
Primary kidney disease		25.2
Glomerulonephritits	138 (29.8)	
Genetic disease	129 (27.9)	
Vascular nephropathy	43 (9.3)	
Interstitial nephritis	38 (8.2)	
Diabetic nephropathy	22 (4.8)	
Others	93 (20.1)	
History of hypertension before transplantation	455 (92.3)	20.4
History of diabetes before transplantation	64 (13.0)	20.4
Immunosuppressive strategy		0.0
Cyclosporine	118 (19.1)	
Tacrolimus	453 (73.2)	
Switch cyclosporine/tacrolimus	48 (7.8)	
Patients on MMF (%)	603 (97.4)	0.0
MMF formulation (%)		2.6
Cellcept	411 (68.2)	
Myfortic	144 (23.9)	
Cellcept/Myfortic	48 (8.0)	
Patient therapeutic education, before transplantation	175 (35.5)	20.4
Patient therapeutic education, after transplantation	120 (24.3)	20.4
Functional graft at the end of study	476 (96.6)	20.4

Cross-Sectional Approach

Description of Adherence and Exposure Data

At least three C_0 measurements were available during at least one 28-day period preceding MMAS-4 administration in 619 patients, who were included in this analysis (**Figure 1**). This dataset comprised 961 adherence questionnaires and a total of 6,471 C_0 values – 4,252 for tacrolimus and 1,225 for cyclosporine (**Table 2**). The median [IQR] number of C_0 values per 28-day period decreased over time, from 7 [5–8] in the first month to 3.5 [3, 4] between M6 and M12.

The proportion of patients classified as non-adherent tended to increase over time, rising from 4.1% during the first month to 11.1% between M9 and M12 (**Table 2**). However, this trend did not reach statistical significance across the 5 follow-up periods (p = 0.83).

No significant difference in median C_0 values was observed between adherent and non-adherent patients, regardless of the period or the CNI administered (**Table 2**).

Relationship Between Adherence and Intra-patient Variability

The median IPV, as well as the proportion of patients with high IPV (CV ≥30%) were not significantly different between post-transplant periods (**Table 2**). Furthermore, no significant differences in IPV (median CV or proportion with high IPV) were found between adherent and non-adherent patients during any of the evaluated time periods (**Table 2**).

Relationship Between Adherence and Underexposure

The proportion of C_0 values below the therapeutic target in the 28 days preceding each MMAS-4 assessment did not differ significantly between adherent and non-adherent patients at any post-transplantation time point. At M1, the proportion of subtherapeutic C_0 values for each patient was higher in adherent patients compared to non-adherent patients (33.3% [IQR: 19.2–57.1] vs. 17.1% [11.5-36.5] (p = 0.04). However, this difference was not significant when considering patients on cyclosporine and tacrolimus separately. Moreover, no significant differences were found either between adherent and non-adherent patients from M3 to M12 (**Table 2**). Overall, these findings suggest that underexposure to CNIs was comparable in both groups and was not significantly associated with self-reported adherence at any time point.

Longitudinal Approach

Description of Adherence and Exposure Data

Based on longitudinal adherence profiling, the majority of patients (551/619) were classified as adherent over time.

There were no significant differences in baseline characteristics between adherent and non-adherent patients, with the exception of age (p < 0.001) and pre-transplant diabetes (p = 0.024): non-adherent patients were significantly younger than adherent patients and had more frequently pre- transplant diabetes.

TABLE 2 | Comparison of IPV (whatever the CNI) in adherent vs. non-adherent patients according to follow-up periods after the transplantation, using the cross-sectional approach. Continuous variables are presented in median [IQR] and categorial variables are presented in numbers. Continuous variables are compared using Wilcoxon test and categorial variables are compared using Chi^2 test.

Period	Status	Patients (n)	IPV median [IQR]	p	High IPV(n)	p	% C_0	p
a. Overall								
M1	A	490	27.2 [19.2–35.0]	0.82	190	0.86	33.3 [19.2–57.1]	**0.04**
	NA	21	27.6 [16.2–38.4]		12		17.1 [11.5–36.5]	
M3	A	306	15.7 [10.3–24.6]	0.61	43	0.67	50.0 [25.0–100.0]	0.65
	NA	27	15.8 [12.1–20.1]		3		75.0 [40.0–80.0]	
M6	A	80	19.2 [12.2–29.1]	0.17	19	0.63	87.5 [50.0–100.0]	0.40
	NA	7	13.6 [9.8–20.2]		1		83.3 [75.0–91.7]	
M9	A	11	20.6 [13.5–40.7]	0.67	4	0.86	50.0 [43.8–62.5]	0.81
	NA	1	39.1 [39.1–39.1]		1		45.0 [42.5–47.5]	
M12	A	16	14.1 [11.6–24.3]	0.08	3	0.11	100 [68.8–100]	0.34
	NA	2	58.3 [44.5–72.0]		2		50.0 [50.0–50.0]	

Period	Group	Patients (n)	Number of C_0	C_0 median [IQR] (µg/L)	p	IPV median [IQR] (%)	p	High IPV (n)	p	% C_0 < target median [IQR]	p
b. For cyclosporin											
M1	A	113	820	214 [157–273]	0.71	27.6 [21.1–40.4]	0.46	49	0.83	55.6 [33.3–77.8]	0.36
	NA	4	32	221 [178–268]		22.1 [13.9–34.9]		1		37.5 [20.0–60.0]	
M3	A	63	263	166 [128–211]	0.16	15.9 [10.9–27.5]	0.53	11	1.00	75.0 [33.3–100.0]	0.44
	NA	6	24	144 [121–180]		14.0 [12.8–16.0]		1		80.0 [75.0–100.0]	
M6	A	15	56	145 [122–198]	0.84	26.3 [10.8–35.0]	0.63	6	1.00	100.0 [50.0–100.0]	0.82
	NA	1	3	148 [138–154]		11.1 [11.1–11.1]		0		66.7 [66.7–66.7]	
M9	A	2	6	145.0 [138.0–187.2]	0.17	52.1 [37.2–67.0]	1.00	1	1.00	50.0 [50.0–50.0]	NA
	NA	1	4	101.5 [83.8–122.5]		39.1 [39.1–39.1]		1		50.0 [50.0–50.0]	
M12	A	4	17	122.0 [97.0–147.0]	Not estimable	23.0 [15.5–37.5]	Not estimable	1	Not estimable	100.0 [93.8–100.0]	Not estimable
	NA	0	0	–		–		0		–	
c. For tacrolimus											
M1	A	370	2576	8.9 [6.9–11.1]	0.04	26.9 [18.3–34.4]	0.87	141	0.59	20.0 [12.5–33.3]	0.74
	NA	24	171	9.6 [7.1–11.8]		27.8 [18.1–38.4]		11		14.3 [12.5–33.3]	
M3	A	239	1031	8.8 [7.4–10.5]	0.93	15.7 [10.2–24.1]	0.87	32	0.65	29.2 [20.0–52.5]	Not estimable
	NA	25	111	8.8 [7.6–10.3]		16.4 [11.7–21.0]		2		0	
M6	A	63	243	8.2 [6.1–10.1]	0.99	17.1 [12.3–28.4]	0.31	13	0.94	66.7 [50.0–100.0]	0.36
	NA	8	31	8.1 [6.7–9.2]		14.3 [9.3–22.5]		1		100.0 [100.0–100.0]	
M9	A	9	35	8.0 [6.1–10.4]	Not estimable	19.1 [12.4–37.3]	Not estimable	3	1.00	62.5 [43.8–81.3]	Not estimable
	NA	0	0	–		–		0		–	
M12	A	12	47	6.9 [5.5–9.2]	0.58	13.8 [11.6–18.7]	0.09	2	0.12	100.0 [38.3–100.0]	0.832
	NA	2	7	7.2 [4.2–8.4]		58.3 [44.5–72.0]		2		50.0 [50.0–50.0]	

Bold values indicate statistical significance ($p < 0.05$).

When stratified by CNI, the patients classified in the non-adherence class where respectively 42 on tacrolimus, 10 on cyclosporine and 6 who switched from one CNI to the other.

No significant differences in median C_0 values over the first year were observed between adherent and non-adherent patients for either tacrolimus (8.6 [6.8–10.6] vs. 8.7 [6.9–10.6] µg/L, p = 0.434) or cyclosporine (163 [124–218] vs. 155 [122–202] µg/L, p = 0.117). This absence of difference was consistent across most follow-up periods, except at M6 and M12 for patients on tacrolimus.

Relationship Between Adherence and Intra-patient Variability

The overall IPV did not differ significantly between adherent and non-adherent patients: 31.3% [25.5–38.2] vs. 31.6% [23.6–38.9], p = 0.676. Similar findings were observed when stratified by CNI: for cyclosporine, the median IPV was 32.8% [26.8–42.9] in adherent vs. 36.9% [26.1–45.5] in non-adherent patients (p = 0.531), and for tacrolimus, the median IPV was 31.0% [25.0–37.4] in adherent vs. 30.7% [22.5–37.4] in non-adherent patients (p = 0.459).

The proportion of patients with high IPV was also comparable between groups: 55.5% in adherent vs. 51.5% in non-adherent patients (p = 0.5). Similar results were found when examined by CNI: 59.3% in adherent vs. 68.8% in non-adherent patients on cyclosporine (p = 0.5) and 54.0% in adherent vs. 50.0% in non-adherent patients on tacrolimus (p = 0.6).

Relationship Between Adherence and Underexposure

Overall, the proportion of C_0 values below the lower limit of the therapeutic window was comparable between adherent and non-adherent patients (11.7% vs. 10.3%, p = 0.12). When stratified by treatment, a significant difference was observed among patients on tacrolimus (2.6% in adherent vs. 3.8% in non-adherent

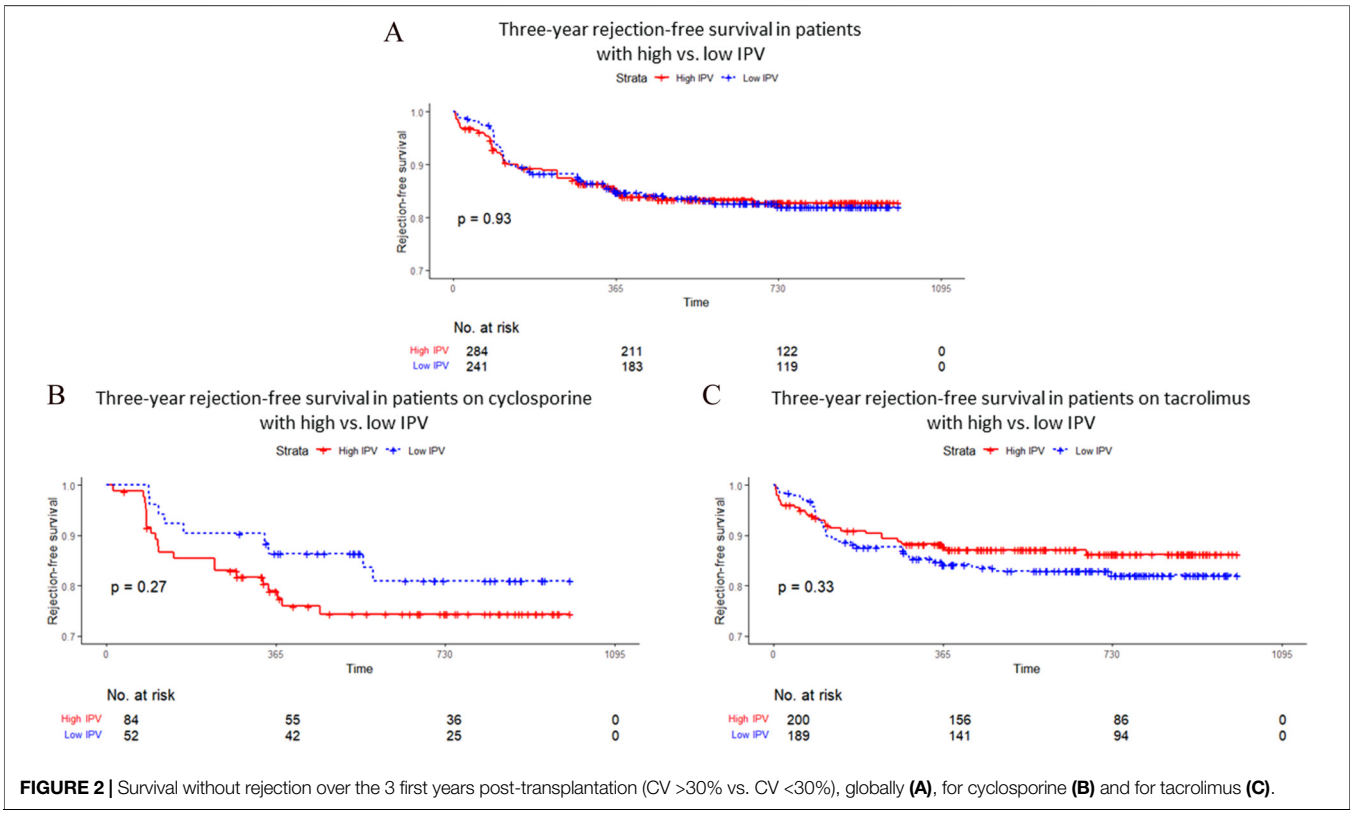


FIGURE 2 | Survival without rejection over the 3 first years post-transplantation (CV >30% vs. CV <30%), globally **(A)**, for cyclosporine **(B)** and for tacrolimus **(C)**.

patients, p = 0.026), whereas no significant difference was found for cyclosporine (39.3% vs. 39.7%, p = 0.935).

CV and Rejection

Rejection-free survival at 3 years post-transplantation did not differ significantly between patients with low versus high IPV during the first year, both overall and when stratified by type of CNI (HR $_{low}$ = 1.02 [0.67–1.55], p = 0.928, **Figure 2**). In multivariate Cox analysis, rejection-free survival was significantly associated with HLA mismatch and *de novo* DSA (both associated with poorer outcomes) whereas therapeutic patient education was independently associated with improved outcomes (**Table 3**).

DISCUSSION

Several previous studies have suggested an association between immunosuppressant C_0 and medication adherence. In the present study, we tested this hypothesis using data from 619 kidney transplant recipients enrolled in two prospective cohorts, and who benefited from regular follow-up visits from M1 to M36 post-transplantation. Our analysis focused primarily on CNI exposure data collected during the first year, a period during which adherence was also repeatedly assessed. Both cross-sectional and longitudinal approaches were applied, allowing for a detailed evaluation of these two dynamic and time-dependent phenomena. Adherence was measured using the MMAS-4 questionnaire, while exposure to CNIs was assessed through C_0, intra-patient variability (IPV), and the proportion of subtherapeutic C_0 values.

In the cross-sectional approach, C_0 were compared between adherent (MMAS = 0) and non-adherent (MMAS >0) patients at each follow-up visit during the first year. In the longitudinal analysis, adherence profiles were derived from MMAS-4 data collected over the full three-year follow-up period, while CNI exposure was restricted to the first year post-transplantation. Across both analytical approaches, no significant differences were observed in C_0 values, IPV, or the proportion of subtherapeutic C_0 measurements between adherent and non-adherent patients.

Our findings are consistent with several previous studies that questioned the reliability of CNI trough concentrations as a proxy for adherence. For example, Reese et al. used electronic monitoring of pill bottle openings to assess adherence in a study comparing a control group to two intervention groups receiving reminder notifications. While non-adherence rates differed significantly between groups, no differences were observed in mean tacrolimus concentrations [31]. Similarly, Schäfer-Keller et al. used electronic monitoring as the reference standard and evaluated various methods for detecting non-adherence, including immunosuppressant blood level monitoring. None of the tested methods, however, demonstrated acceptable sensitivity or specificity [32].

Leino et al. applied a previously proposed cutoff for tacrolimus IPV to identify patients at high risk of non-adherence but found no significant differences in adherence between patients with

TABLE 3 | Univariate and multivariate Cox analyses of the association of rejection-free survival and potential risk factors over the first 3 years post-transplantation.

Characteristic	N	HR[a]	CI 95%	p value	HR	CI 95%	p value	% Bootstrap selection
Age (years)	525	0.99	0.98, 1.01	0.361				
Gender (male)	525	0.96	0.62, 1.48	0.840				
Rank of kidney transplantation (vs. 0)	525							
1		1.28	0.69, 2.35	0.433				
2		1.57	0.22, 11.3	0.656				
Functional graft at the end of study (YES)	395	0.08	0.04, 0.15	<0.001				
MMF speciality (vs. Cellcept)	511							
Switch Cellcept to myfortic		1.90	0.97, 3.74	0.062				
Myfortic		0.98	0.60, 1.59	0.933				
First-line CNI (vs. cyclosporine)	525							
Switch (cyclosporine to tacrolimus)		2.39	1.28, 4.48	0.007				
Tacrolimus		0.81	0.45, 1.46	0.492				
IPV (low)	525	1.02	0.67, 1.55	0.928				
Pre-transplant hypertension (yes)	395	0.57	0.21, 1.59	0.285				
Pre-transplant diabetes (yes)	395	0.36	0.11, 1.15	0.083				
Pre-transplant therapeutic education (yes)	**395**	**0.55**	**0.30, 0.98**	**0.043**	**0.47**	**0.24, 0.91**	**0.026**	**85.5**
Post-transplant therapeutic education (yes)	395	1.18	0.66, 2.11	0.586				
Adherence class (non-adherent)	525	0.96	0.46, 1.98	0.902				
HLA mismatch	**433**	**1.31**	**1.11, 1.53**	**<0.001**	**2.35**	**1.18, 4.65**	**0.014**	**76.0**
Cold ischemia time >1000 (yes)	437	1.00	1.00, 1.00	0.201				
Delayed graft function (yes)	525	0.74	0.36, 1.53	0.420				
Pre-transplant DSA (yes)	525	1.37	0.63, 2.96	0.428				
De novo DSA (yes)	**456**	**2.40**	**1.42, 4.06**	**0.001**	**1.23**	**1.01,1.50**	**0.044**	**79.0**

[a]*HR, Hazard Ratio; CI, Confidence Interval. Bold values indicate statistical significance ($p < 0.05$).*

high versus low IPV [33]. This was likely due to a highly adherent study population with limited variability in tacrolimus exposure. In our study, we similarly observed no significant difference in the proportion of patients with high CNI IPV between adherent and non-adherent groups.

Scheel et al., using the BAASIS questionnaire -which is conceptually comparable to the MMAS-4 and ITAS- found that rejection was associated with patient-reported non-adherence and the percentage of subtherapeutic C_0. However, they did not observe any association between non-adherence and either IS concentration variability or the percentage of subtherapeutic levels [13]. Similarly, several other studies using questionnaires (BAASIS or ITAS), often combined with interviews or electronic monitoring (MEMS), have reported no significant differences in CNI IPV between adherent and non-adherent patients [9, 13, 14, 16]. Our results support these findings, as neither IPV nor the proportion of subtherapeutic C_0 values differed significantly between patients classified as adherent and non-adherent. This conclusion holds true for patients treated with tacrolimus. However, in the subgroup receiving cyclosporine, adherent patients exhibited higher IPVs, possibly reflecting the well-documented higher pharmacokinetic variability associated with cyclosporine.

McGillicuddy et al. explored whether a mobile health intervention could improve adherence and observed a reduction in tacrolimus IPV along with improved clinical outcomes [25]. However, it remains unclear whether these benefits were due to enhanced adherence or simply reduced pharmacokinetic variability.

In a comprehensive review, Gonzales et al. proposed high tacrolimus IPV as a potential proxy for non-adherence [17] However, they also acknowledged that most supporting studies focused on the early post-transplant period, with limited evidence regarding the long-term predictive value of tacrolimus IPV.

While non-adherence is frequently cited as a major contributor to poor outcomes in kidney transplantation [34], several studies attributing poor outcomes to non-adherence did not directly measure adherence, limiting the strength of their conclusions. In addition, variability in CNI exposure also arises from multiple sources beyond patient behavior, including food-related changes in bioavailability, drug–drug interactions, variability in absorption and metabolism, epigenetic factors [35, 36], and clinical decision making. In routine practice, clinician-driven dose adjustments in response to intercurrent infections, rejection episodes, adverse effects, or evolving therapeutic targets are an integral component of therapeutic drug monitoring and are expected to generate variability in trough concentrations independently of adherence. This multidimensional nature of CNI variability likely explains, at least in part, why C_0 and IPV performed poorly as proxies for adherence in our study. Rather than reflecting adherence alone, CNI exposure metrics integrate biological, pharmacokinetic, and clinical factors, thereby limiting their specificity as adherence markers when used in isolation.

Despite a consensual definition of adherence, the absence of a universally accepted gold standard for its measurement contributes to the heterogeneity of findings in the literature. As emphasized by several authors, identifying non-adherence reliably requires a multimethod approach, as each individual method -including pharmacokinetic monitoring- has well-documented limitations [13, 32, 37].

The association between high IPV of CNI C_0 and poor clinical outcomes has been widely reported in kidney transplantation. Several studies have shown that patients with high IPV are at increased risk of rejection and graft loss, supporting the value of CNI therapeutic drug monitoring as a practical and accessible tool for identifying patients at risk [13, 38–40]. However, not all studies have confirmed this association. For instance, some authors found no significant correlation between high IPV and graft rejection [9] highlighting the complexity of interpreting IPV in isolation.

In our study, patients with lower IPV (CV <30%) tended to have better rejection-free survival at 3 years post-transplantation, although the difference did not reach statistical significance. This trend is consistent with the prevailing literature in kidney transplantation, where high IPV is generally associated with adverse outcomes. By contrast, evidence for this association is less consistent in liver transplantation, where pharmacokinetic variability appears to have a more limited prognostic value [33, 41].

One of the main strengths of our study is the longitudinal evaluation of both adherence and CNI C_0 from the first month up to 1 year post-transplantation. While previous studies have often excluded the early post-transplant period due to greater pharmacokinetic fluctuations, particularly during the first 3 months [42], our approach allowed for a more comprehensive characterization of adherence profiles and variability in drug exposure over time. By including this early period, we captured clinically relevant fluctuations in C_0 that may influence long-term outcomes. Notably, CNI trough levels were collected as part of routine follow-up, regardless of the underlying clinical context, which may partly explain the observed variability and the difference in subtherapeutic C_0 at 1 month post-transplant between adherent and non-adherent patients. In addition, the early post-transplant period is characterized by greater clinical instability and frequent dose adjustments, which could further contribute to these findings. Furthermore, information on medication prescriptions or dispensing history was not available, limiting our ability to distinguish pharmacokinetic variability related to clinical management from variability related to medication-taking behavior. Another strength lies in the granularity of our dataset: each patient contributed a large number of C_0 measurements, which improves the accuracy of IPV estimates, in line with published recommendations [17]. Moreover, our analysis encompassed both tacrolimus and cyclosporine, offering a broader view of variability patterns across the two most commonly used CNIs in kidney transplantation. This dual-CNI perspective enhances the generalizability of our findings, whereas many prior studies focused on only one agent.

Our study also has some limitations. A key limitation is the use of a single method to assess adherence -namely, the MMAS-4 self-reported questionnaire. While widely used, self-reported tools are inherently subject to recall bias and social desirability effects. Although the literature emphasizes the value of combining multiple adherence assessment methods, such cross-validation is rarely implemented. To address this limitation, a subgroup of patients from the EPIGREN cohort also underwent face-to-face interviews conducted by a trained pharmacologist. In this subset, adherence estimates from MMAS-4 and interview-based assessments were highly concordant, with strong sensitivity and specificity, supporting the reliability of our primary adherence measure. Repeated administration of self-reported questionnaires may reduce response rates and data quality over time; however, the very short format of the MMAS (four items) likely limited, though did not fully eliminate, attrition-related bias.

Another limitation concerns the time frame of exposure assessment. While adherence trajectories were modeled over a three-year period, CNI exposure and variability were analyzed only during the first year. This may have limited our ability to capture late changes in pharmacokinetics or adherence behavior. As this was an observational study, causal relationships cannot be established, and residual confounding remains possible despite robust cohort design and analytic approaches.

Adherence was analyzed as a longitudinal and dynamic behavior, allowing changes over time to be captured regardless of their underlying causes, including intercurrent clinical events. However, the complex bidirectional relationship between post-transplant complications and adherence was not formally evaluated in this study.

Moreover, this study is based on real-world cohort data, and the exact timing of drug intake and blood sampling was not systematically recorded, which may have introduced variability in trough concentration measurements. Although samples were routinely collected immediately before the morning dose under standardized clinical practice, residual variability related to dosing habits cannot be fully excluded and could not be adjusted for in the analyses.

Finally, analyses conducted beyond 6 months should be interpreted descriptively rather than as supporting formal statistical comparisons, due to the reduced sample size and the progressive stabilization of CNI exposure and therapeutic drug monitoring practices over time [42, 43]. The absence of a formal sample size calculation, combined with progressive attrition over time, limits statistical power in later follow-up periods. Despite these limitations, the study provides valuable real-world insights into post-transplant outcomes in kidney transplant recipients.

In conclusion, no significant association was observed in this study between adherence -assessed both cross-sectionally and longitudinally using the MMAS-4- and CNI exposure parameters, including C_0 and IPV, during the first year following kidney transplantation. Although therapeutic drug monitoring (TDM) may help identify isolated episodes of non-adherence -particularly in cases of markedly low C_0- it remains insufficient for capturing adherence patterns over time. Our findings support the consensus that characterizing adherence requires a multimodal approach, integrating pharmacokinetic data with validated patient-reported measures and clinical interviews.

We also confirmed that high CNI variability is associated with a reduction in rejection-free survival, reinforcing the prognostic value of TDM for risk stratification beyond its role in dose individualization. These results underscore the importance of interpreting TDM in context, and not as a standalone indicator of adherence. Our findings also highlight the need for the development of integrated, scalable adherence monitoring

strategies that can be feasibly implemented in routine care. Future work should explore combining digital adherence tools, behavioral interventions, and pharmacometric modeling to better identify at-risk patients and guide personalized adherence support.

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 humans were approved by Comité de Protection de Personnes (CPP) du Sud-Ouest et Outre Mer IV cppsoom4@ch-esquirol-limoges.fr http://www.cpp-soom3.u-bordeaux2.fr/. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CV participated in the conceptualization of the study, data analysis and manuscript writing. CM participated in the coordination of the EPHEGREN cohort, conceptualization of the study and manuscript writing. PM coordinated the EPHEGREN cohort. J-pR, LC, NK, IE, P-FW, MB, LE and AT participated in the performance of the research.

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CONFLICT OF INTEREST

The author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

GENERATIVE AI STATEMENT

The author(s) declared that generative AI was not used in the creation of this manuscript.

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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.2026.15718/full#supplementary-material

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LETTER TO THE EDITOR
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Sexual Dimorphism in Renal Progenitors: Do Immunosuppressants Erase the Female Advantage?

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Keywords: estrogen, immunosuppression, kidney transplantation, renal progenitors, sex differences

Dear Editors,

Sex-based differences in chronic kidney disease (CKD) progression are well recognized: women generally experience slower renal function decline and greater resistance to podocyte injury than men. Paradoxically, this biological advantage largely disappears after kidney transplantation, where graft outcomes become broadly comparable between sexes [1]. The mechanisms underlying this loss of sexual dimorphism remain poorly understood.

Recent mechanistic studies on estrogen-regulated renal progenitors offer a unifying explanation [2]. Estrogen exerts renal protection through two complementary molecular axes. First, estrogen activates the ERα–PI3K/AKT–mTOR pathway, promoting renal progenitor proliferation and podocyte renewal, counterbalancing podocyte loss and preserving glomerular integrity. Elegant lineage-tracing and functional studies demonstrate that estrogen-dependent progenitor activation contributes directly to female renal resilience and adaptive capacity [3–6]. Second, estrogen physiologically suppresses calcineurin–NFAT signaling, limiting pro-inflammatory transcription, cytoskeletal destabilization, and maladaptive hypertrophy in podocytes [7, 8]. Together, these pathways establish a coordinated balance between regeneration and immune restraint, forming the biological basis of female renal advantage (**Figure 1**).

Testosterone, by contrast, drives these same pathways toward maladaptive outcomes. It stimulates mTORC1/S6K1 activity, but rather than sustaining progenitor renewal, the result is glomerular hypertrophy and fibrotic signaling. Simultaneously, testosterone enhances calcineurin/NFAT activation, amplifying pro-inflammatory transcription and accelerating glomerulosclerosis [9, 10]. This dual effect helps explain the well-documented male disadvantage in CKD progression.

Kidney transplantation introduces a pharmacologic environment that inadvertently neutralizes both estrogen-mediated protective axes. Calcineurin inhibitors (CNIs), the backbone of transplant immunosuppression, uniformly suppress NFAT signaling. In men, this mimics estrogen's inhibitory effect; in women, it represents a redundant blockade that abolishes a uniquely protective pathway. Simultaneously, mTOR inhibitors directly counteract estrogen-driven progenitor proliferation, eliminating the regenerative advantage observed in female kidneys. Thus, transplantation creates an artificial biological equivalence not by enhancing male resilience, but by pharmacologically suppressing female resilience.

This framework suggests that sexual dimorphism in transplant outcomes is not inherently absent but rather masked by immunosuppressive strategies that converge on hormone-sensitive pathways. Importantly, registry studies increasingly suggest sex-specific differences in transplant benefit and long-term outcomes, supporting the biological plausibility of this interaction.

We propose that future transplant studies should systematically stratify outcomes by sex, hormonal status, and immunosuppressive regimen. Tailoring mTOR inhibition or adjusting its timing may preserve regenerative capacity in women without compromising graft protection. By recognizing that women may lose their intrinsic regenerative advantage under current regimens, the field can move toward truly individualized and sex-specific immunosuppression strategies that protect graft survival without disrupting inherent biological strengths.

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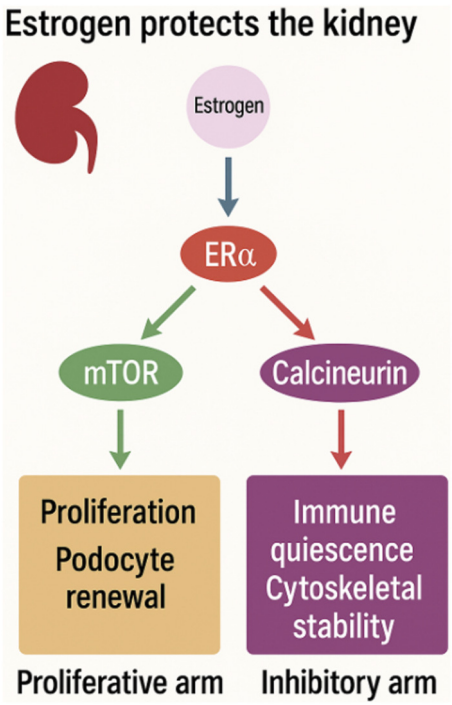

FIGURE 1 | Estrogen protects renal integrity by coordinating dual molecular pathways.

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

ZU conceived the idea for this manuscript, performed the literature review, developed the conceptual and mechanistic framework, and wrote the manuscript. The author critically revised the content and approved the final version of the manuscript.

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