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Belatacept in kidney transplantation: balancing the scales

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TRANSPLANT TRIAL WATCH
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Transplant Trial Watch

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Keywords: randomised controlled trial, kidney transplantation, liver transplantation, hypothermic oxygenated machine perfusion, health related quality of life

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

RANDOMISED CONTROLLED TRIAL 1

Effect of an Exercise Intervention or Combined Exercise and Diet Intervention on Health-Related Quality of Life-Physical Functioning After Kidney Transplantation: The Active Care After Transplantation (ACT) Multicentre Randomised Controlled Trial.

by Knobbe, T. J., et al. The Lancet Healthy Longevity 2024 [record in progress].

Aims

The aim of this study was to determine the role of exercise intervention or exercise plus diet intervention on the physical functioning domain of health-related quality of life following kidney transplantation.

Interventions

Participants were randomised into three groups: usual care, exercise intervention, and exercise plus diet intervention.

Participants

221 kidney transplant recipients.

Outcomes

The primary endpoint was change in the physical functioning domain of health-related quality of life (HRQoL). The secondary endpoints were HRQoL composite scores, physical activity, physical fitness, cardiometabolic risk factors and body composition.

Follow-Up

15 months.

CET Conclusion

by Simon Knight

This multicentre RCT from the Netherlands randomised kidney transplant recipients to one of three groups: usual care, exercise or exercise plus diet. The exercise component comprised a 3-month supervised exercise program, with additional dietary counselling over 15 months in the exercise plus diet group. Interestingly for an RCT, the primary endpoint was a quality-of-life measure (the physical

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functioning component of the SF-36 questionnaire). At 3 months a small difference in physical functioning was seen in the exercise group, but this difference disappeared by 15 months. Study design and conduct are good, with variable block randomisation and allocation concealment via centralised randomisation. Interventions are well described, and primary analysis is by intent-to-treat. There are some limitations – 35% recruited patients were excluded from the intent-to-treat analysis due to missing primary outcome data at baseline or follow-up, a common issue in studies using QOL questionnaires. This may have led to a lack of statistical power. Also of note, the study only recruited patients in their first year post-transplant, so the results may not generalise to patients later post-transplant.

Jadad Score
3.

Data Analysis
Strict intention-to-treat analysis.

Allocation Concealment
Yes.

Trial Registration
ClinicalTrials.gov - NCT01047410.

RANDOMISED CONTROLLED TRIAL 2

Hypothermic Oxygenated Machine Perfusion Influences the Immunogenicity of Donor Livers In Humans.

by Elgosbi, M., et al. Liver Transplantation 2024 [record in progress].

Funding Source
Non-industry funded.

Aims
This observational study aimed to examine the influence of hypothermic oxygenated machine perfusion (HOPE) on the molecular profile of liver allografts as well as on the immune responses induced following liver transplantation.

Interventions
Participants from two randomised controlled trials comparing donor livers randomly assigned to either HOPE or to static cold storage (SCS), were included.

Participants
27 liver transplant recipients.

Outcomes
Molecular and immunogenic profiles of donor livers.

Follow-Up
3 months posttransplantation.

CET Conclusion
by Simon Knight

This interesting study investigated the immune responses in 27 liver transplant recipients participating in two randomised controlled trials of hypothermic oxygenated machine perfusion (HOPE) in a single centre. The investigators studied perfusate, liver biopsies and recipient T-cell profiles. They showed that, compared to static cold storage, HOPE livers demonstrated reduction in hepatic immune cells in the perfusate and a reduced activation of the reactive oxygen species pathway. In the recipient, there was upregulation in donor-specific T-reg cell expression following HOPE. These findings are interesting, but as this represents only a small single-centre subset of the overall RCT recruitment, can only be exploratory. The patients included only a very small number of DCD liver recipients. They are, however, in keeping with the reduction in acute rejection rates seen in other studies of HOPE in the liver and kidney.

Trial Registration
ClinicalTrials.gov - NCT01317342; ClinicalTrials.gov - NCT02584283.

Funding Source
Non-industry funded.

CLINICAL IMPACT SUMMARY

by Simon Knight

Ex-vivo machine perfusion of the liver has a number of potential benefits, including reconditioning, viability assessment and extended preservation durations. Whilst hypothermic oxygenated machine preservation (HOPE) may not afford the same extended preservation times or viability assessment as normothermic perfusion, it has shown the potential to reduce incidence of early allograft dysfunction and surgical complications, including the risk of non-anastomotic biliary strictures [1].

One area that is less studied is the immunological impact of machine perfusion. By increasing ATP storage and reducing ischaemia-reperfusion injury, it is possible that machine preservation has the potential to reduce the innate and adaptive immune response following reperfusion. This has been demonstrated in rodent liver transplant models, where lower doses of immunosuppression are required for successful transplantation following HOPE [2]. There is also some clinical evidence for this following hypothermic oxygenated machine preservation of the kidney, with the COMPARE study demonstrating a reduction in risk of acute rejection for HOPE compared to conventional hypothermic machine preservation [3].

In a recent, posthoc analysis of samples from 2 randomised clinical trials, Elgosbi et al. investigated the role of HOPE in the immunogenicity of liver transplantation [4]. HOPE resulted in lower presence of intrahepatic immune cells (liver mononuclear cells), compared to static cold

storage (SCS). Transcriptomic analysis demonstrated less activation of elements of the reactive oxygen species pathway, which translated to a later increase in expression of CD4+FOXP3+ regulatory T-cells and a reduction in alloreactive $CD8^+$ T cells.

The sample size in the study is too small to determine whether these immunological effects translate into a clinically meaningful difference in rejection rates or graft function, but nevertheless provide an interesting insight into the mechanisms behind reduced immune activation seen with oxygenated machine perfusion. The majority of patients in the cohort received DBD liver transplants, so it would be interesting to see if the benefit is greater in more injured DCD grafts.

Overall, this is an interesting study, and hopefully paves the way for more detailed analysis alongside future clinical trials of machine perfusion.

Clinical Impact

3/5.

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AUTHOR CONTRIBUTIONS

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

CONFLICT OF INTEREST

The author has undertaken previous paid consultancy work for OrganOx Ltd.

GENERATIVE AI STATEMENT

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

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ORIGINAL RESEARCH
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Biopsy-Proven T-Cell Mediated Rejection After Belatacept Rescue Conversion: A Multicenter Retrospective Study

Dominique Bertrand[1], Nathalie Chavarot[2], Jérôme Olagne[3], Clarisse Greze[4], Philippe Gatault[5], Clément Danthu[6], Charlotte Colosio[7], Maïté Jaureguy[8], Agnès Duveau[9], Nicolas Bouvier[10], Yannick Le Meur[11], Léonard Golbin[12], Eric Thervet[13], Antoine Thierry[14], Arnaud François[15], Charlotte Laurent[1], Mathilde Lemoine[1], Dany Anglicheau[2] and Dominique Guerrot[1]*

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After kidney transplantation, conversion to belatacept is a promising alternative in patients with poor graft function or intolerance to calcineurin inhibitors. The risk of acute rejection has not been well described under these conditions. Here we present a retrospective multicenter study investigating the occurrence of acute rejection after conversion in 901 patients (2011–2021). The incidence of cellular and humoral rejection was 5.2% and 0.9%, respectively. T-cell mediated rejection (TCMR) occurred after a median of 2.6 months after conversion. Out of 47 patients with TCMR, death-censored graft survival was 70.1%, 55.1% and 50.8% at 1 year, 3 years and 5 years post-rejection, respectively. Eight patients died after rejection, mainly from infectious diseases. We compared these 47 patients with a cohort of kidney transplant recipients who were converted to belatacept between 2011 and 2017 and did not develop rejection (n = 238). In multivariate analysis, shorter time between KT and conversion, and the absence of anti-thymocyte globulin induction after KT were associated with the occurrence of TCMR after belatacept

Abbreviations: CNIs, calcineurin inhibitors; DSA, donor specific antibody; ECDs, extended criteria donors; eGFR, estimated glomerular filtration rate; IQR, interquartile range; KTRs, kidney transplant recipients; MDRD, Modification of Diet in Renal Disease formula; OPI, Opportunistic infections; PML, progressive multifocal leukoencephalopathy.

conversion. The occurrence of rejection after conversion to belatacept appeared to be less frequent than with *de novo* use. Nevertheless, the risk of graft loss could be significant in patients with already low renal function.

Keywords: transplantation, kidney, belatacept, rejection, CNI toxicity

INTRODUCTION

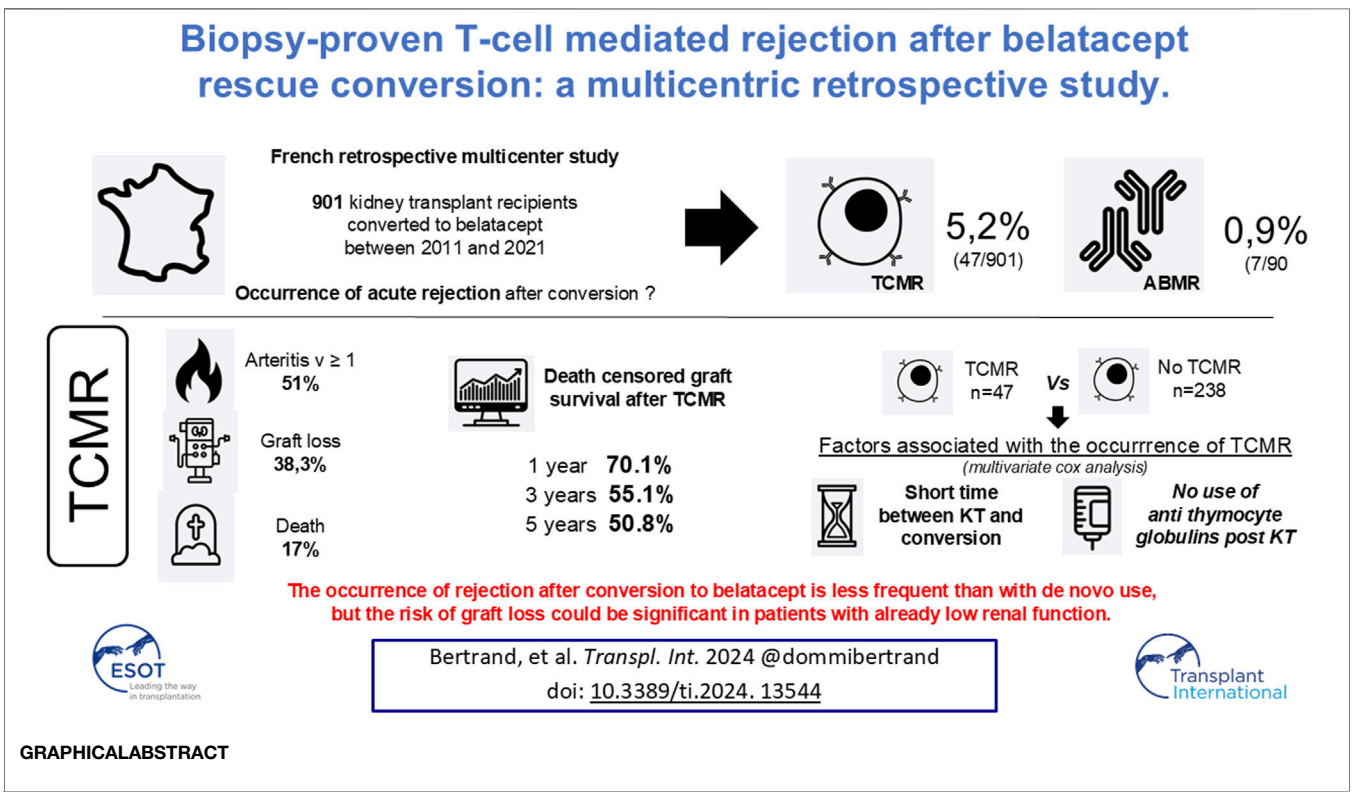
Belatacept is an immunosuppressive drug that blocks the costimulation pathway, preventing T cell activation. With this different mechanism of action, belatacept represents an alternative to calcineurin inhibitors (CNIs) after kidney transplantation and could have major advantages. When used as a *de novo* therapy post-transplantation, belatacept improved long-term graft function, graft survival and patient survival in the BENEFIT study [1]. Moreover its metabolic profile is better than CNIs [2] and the rate of *de novo* DSA is lower [3]. When used as a conversion strategy, the randomized study by Budde et al. [4] also reported benefits for graft function and for the rate of *de novo* DSA, in stable KTRs. Furthermore, there is growing evidence that CNIs to belatacept conversion is a valuable option as rescue therapy in patients with poor graft function [5]. A major pitfall and obstacle to more widespread use of belatacept in *de novo* KTRs is the particularly high rate of TCMR (T cell-mediated rejection) occurring in up to 24% of patients in the BENEFIT [1] PRINCEPS study. The rejection rate seems to be lower in conversion strategies ranging between 5.3% and 11.4 % according to various studies [4, 6–10] and was not significantly different between the belatacept and CNI arms in our retrospective study [5]. However some of these rejections are steroid-resistant TCMRs [11, 12] and could lead to accelerated graft loss. Unfortunately, there are no reports of risk factors or biomarkers associated with the occurrence of rejection in this context.

We designed a multicenter retrospective study in which we included all patients who were converted to belatacept over a 10-year period who presented with biopsy-proven rejection. The aims of the present study were to report the incidence of both TCMR and ABMR (antibody-mediated rejection) after conversion to belatacept in a rescue strategy, to depict the evolution of these patients and to identify factors associated with the occurrence of TCMR after conversion.

MATERIALS AND METHODS

Study Design: Flow Charts (Figure 1) and Patients

We conducted a retrospective study, between 2011 and 2021, in which all the kidney transplant recipients (KTRs) from the Spiesser group (13 French KT centers) who presented a



GRAPHICALABSTRACT

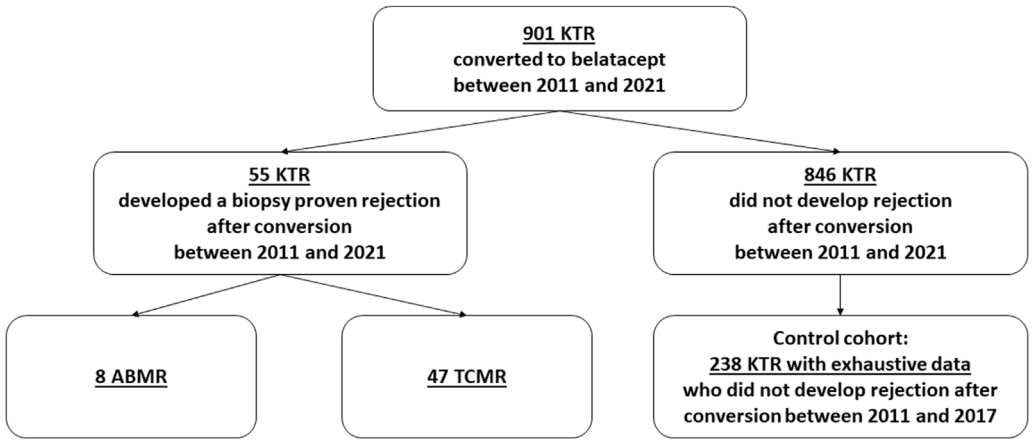


FIGURE 1 | Flow chart. KTRs, kidney transplant recipients; ABMR, antibody-mediated rejection; TCMT, T cell-mediated rejection.

biopsy-proven rejection after belatacept conversion were included (all were for cause biopsies). Conversion was performed for poor graft function and/or intolerance to calcineurin inhibitors. Histological features of the kidney allograft biopsies were scored according to the Banff classification [13]. During this period a total of 901 KTRs were converted to belatacept.

In accordance with French law (loi Jardé), because this was an anonymous retrospective study, institutional review board approval was not required. The clinical and research activities being reported are consistent with the Principles of the Declaration of Istanbul as outlined in the "Declaration of Istanbul on Organ Trafficking and Transplant Tourism."

Treatment

The CNI to belatacept conversion group consisted of 5 mg/kg of belatacept administered intravenously on days 1, 15, 29, 43, 57 and then 28 days thereafter [14]. CNIs were tapered as follows: 100% on day 1, 50% on day 2, 25% on day 15, and 0 from day 29 onwards. Other immunosuppressive medications, including corticosteroids, were maintained at existing doses unless modification was necessary. All patients were EBV seropositive before the conversion. Beginning in January 2019, all patients converted to belatacept received pneumocystis prophylaxis. Patients who received belatacept as *de novo* therapy were excluded.

Primary Outcome: Kidney Transplant Recipients With Rejection After Conversion

The primary endpoint was the rate of both TCMR and ABMR after conversion to belatacept. We excluded patients who experienced ABMR due to the small number of them and focused on TCMR in order to determine factors associated with graft loss. We defined graft failure as a return to chronic dialysis. We evaluated kidney graft function using eGFR (MDRD) [15].

Secondary Outcomes: Rate of Opportunistic Infection (OPI) and Factors Associated With the Occurrence of TCMR After Conversion to Belatacept

During the study period, 901 KTRs were converted to belatacept: 55 KTRs developed a biopsy-proven rejection while the 846 others did not. Among the 846 patients without rejection, 238 KTRs [6], well-phenotyped with exhaustive data, converted between 2011 and 2017, were analyzed and compared to the "rejection cohort" to identify the incidence of OPIs and factors associated with the occurrence of TCMR after conversion. Moreover, there was no difference between 2011–2017 and 2018–2021 regarding the protocol of conversion used (dose of belatacept, timing of discontinuation of CNIs).

All OPIs occurring under belatacept therapy were recorded in our medical charts. Infection was defined by a specific clinical/biological/radiological presentation and the finding of a causal infectious agent (bacterial, viral, fungal or parasitic). The nature of the infection (microbiological causative agents) and the localization of the infection were recorded. The lymphocyte count was reviewed at the time of the switch for all patients. We considered OPIs as described by Fishman in 2007 [16]: pneumocystis pneumonia; infection with herpes viruses (herpes simplex virus, varicella-zoster virus, Cytomegalovirus, Epstein-Barr virus and others); infection with *listeria*, *nocardia*, toxoplasma, strongyloides, leishmania, Trypanosoma cruzi; polyomavirus BK nephropathy; Cryptococcus neoformans infection; *Mycobacterium tuberculosis* or atypical mycobacteria infection; infection with aspergillus, atypical molds, mucor species; infection with JC polyomavirus [progressive multifocal leukoencephalopathy (PML)].

Statistical Methods

Quantitative data were presented as mean (SD), or median (interquartile range IQR) when data were not normally distributed. Qualitative data were presented as percentages.

Non-parametric Wilcoxon (quantitative data) and Mann-Whitney (qualitative data) tests were used to compare baseline characteristics. Univariate and multivariable Cox regression analyses were performed to determine independent covariates associated with the occurrence of TCMR: age, gender, time between KT and conversion, the use of anti-thymocyte globulin induction post KT, extended criteria donor, eGFR at conversion, lymphocyte counts at conversion, tacrolimus before conversion, the time between conversion and CNI discontinuation, MMF at time of TCMR or month 3 post-conversion in KTRs without TCMR, MMF dose, steroids at the time of TCMR or month 3 post-conversion in KTRs without TCMR, and steroid dose. All factors with $P < 0.1$ in the univariate analysis were included in the multivariate model. $P < 0.05$ was considered statistically significant in the multivariate model. Results were presented as a hazard ratio (HR) and a 95% confidence interval (CI). For Cox models, we tested the validity of the proportional hazards assumption using the Scaled Schoenfeld vs. time graph for each variable. There was no violation of the proportional hazards assumption. We tested the interaction between the variables in the final model using a parameter covariance matrix to show how much each parameter was correlated with each other. All analyses were performed using STATVIEW version 5.0 (SAS Institute, Cary, NC, United States) and GraphPad Prism version 8.0 software (GraphPad Software, San Diego, CA).

RESULTS

Incidence of Rejection After Belatacept Conversion During the Period 2011–2021

Between 2011 and 2021, 901 patients were converted from CNIs to belatacept after kidney transplantation. Of these 55 (6.1%) patients, who were converted after a median time of 3.6 months (IQR: 1.1–9.5) post-transplant developed a biopsy-proven acute rejection after a median time of 2.6 months post-conversion (IQR: 2.1–4.1 months). Of these, 47 (85.4%) developed TCMR and 8 (14.6%) ABMR. The incidence of TCMR and ABMR during this period was 5.2% and 0.9% respectively. None of the patients had a rejection prior to conversion.

We noted a substantial decrease in the rejection rate (TCMR) over time: 2011–2017: 18/256 = 7% and 2017–2021: 29/645 = 4.5%.

Kidney Transplant Recipients With TCMR: Clinical, Biological and Histological Characteristics at the Time of Diagnosis

Regarding TCMR, according to the Banff classification [13], we reported borderline lesions in 5 cases (10.7%), 9 grade IA (19.1%), 9 grade IB (19.1%), 7-grade IIA (14.9%), 11 grade IIB (23.4%) and 6 grade III (12.8%) TCMR. Biopsies of TCMR revealed v lesions in 24/47 cases (51%).

The general characteristics of KTRs with TCMR are reported in **Table 1**. Kidney transplant recipients with biopsy-proven rejection presented at the time of diagnosis with a decrease in eGFR from a median of 25.5 mL/min/1.73 m^2 (IQR: 14.5–32.1) at the time of conversion to 16.2 mL/min/1.73 m^2 (IQR: 9.9–24.6) at the time of rejection. Five KTRs (10.6%) required dialysis at the time of rejection.

Kidney Transplant Recipients With TCMR: Evolution After Treatment

All KTRs were treated with high doses of steroids after the diagnosis of TCMR: 43 (91.5%) with intravenous infusion and 4 (8.5%) with oral treatment. Moreover, of the 47 KTRs, 7 (14.9%) were treated with anti-thymocyte globulin. Twelve patients (25.5%) were resistant to treatment. After treatment, 33 patients (70.2%) recovered an eGFR at least equivalent to that at the time of the conversion, from 17.2 mL/min/1.73 m^2 (IQR: 12.6–28.9) at the time of rejection to 35.1 mL/min/1.73 m^2 (IQR: 24.3–43.2) after treatment. After treatment, belatacept was discontinued and CNIs were resumed in 18 KTRs (38.3%). Belatacept was continued in the remaining 29 KTRs (61.7%).

After TCMR, 8 deaths were reported within 13.3 months (IQR: 9.1–34.4) after rejection, 7 of which were of infectious origin: 3 deaths from invasive aspergillosis, 2 from bacterial pneumonia, one from uncontrolled bacterial osteitis and one from influenza virus. After TCMR, 18 graft losses were reported after a median time of 7.1 months (IQR: 1.3–15.9) after rejection. Death-censored graft survival was 70.1%, 55.1% and 50.8% at 1 year, 3 years and 5 years post rejection, respectively.

In KTRs without graft loss, median eGFR increased from 18.9 mL/min/1.73 m^2 (IQR: 14.1–29.7) at the time of rejection to 35.1 mL/min/1.73 m^2 (IQR: 28.9–45.7) after treatment and to 34.4 mL/min/1.73 m^2 (IQR: 24.3–41.4) 1-year post rejection.

Factors Associated With Graft Loss After TCMR

Characteristics of KTRs with TCMR and graft loss (n = 18) compared to those without graft loss (n = 29) are reported in **Table 2**. The discontinuation of belatacept after rejection and the eGFR at the time of rejection were significantly associated with graft loss after TCMR.

Factors Associated With the Occurrence of TCMR After Conversion to Belatacept

We compared the 47 KTRs with TCMR during the period 2011–2021 with a subset of the cohort converted to belatacept between 2011 and 2017 who did not develop rejection (n = 238) [6]. General patient characteristics are reported in **Table 1**.

Univariate and multivariate Cox analyses to determine factors associated with the occurrence of TCMR after belatacept conversion are reported in **Table 3**. In multivariate analysis, the time between KT and conversion, and the absence of anti-thymocyte globulin treatment as an induction after KT were associated with the occurrence of TCMR after belatacept conversion.

TABLE 1 | Clinical and biological characteristics of patients with and without T cell-mediated rejection (TCMR) (historical cohort).

	KTRs with TCMR n = 47	KTRs without TCMR (historical cohort) n = 238	*p*
Sex M/F n (%)	36 (76.6)/11 (23,4)	144 (60.5)/94 (39,5)	0.04
Age at conversion (years), mean ± SD	56.9 ± 13.9	56.2 ± 14.8	0.94
Mean time between KT and conversion (months) median time (IQR)	3.6 (1.0–9.1)	13.2 (4.1–51.3)	<0.0001
Conversion before 6 months post KT n(%)	28 (59.6)	82 (34.5)	0.001
Use of anti thymocyte globulins post KT	10 (21.3)	89 (37.4)	0.03
ECD n(%)	34 (72.3)	136 (57.1)	0.05
eGFR at conversion (MDRD. mL/min/1.73 m^2). mean ± SD	27.0 ± 17.4	27.3 ± 15.3	0.66
Lymphocytes count at conversion (/mm^3) mean ± SD	1,170 ± 613	1,070 ± 668	0.19
Treatment prior to conversion n (%)			0.04
Tacrolimus	40 (85.1)	167 (70.2)	0.66
MMF	44 (93.6)	208 (87.4)	0.22
Steroids	37 (78.7)	205 (86.1)	0.19
Mean time between conversion and CNI discontinuation (months)			0.71
Median time (IQR)	0.9 (0.5–1.1)	0.9 (0.88–1.0)	
Treatment at time of TCMR or at month 3 n(%)			0.48
MMF	42 (89.4)	220 (92.4)	0.47
Median dose (IQR)	1,250 (1,000–2,000)	1,000 (1,000–1,500)	0.57
Steroids	39 (82.9)	205 (86.1)	0.19
Median dose (IQR)	10 (7.5–10)	10 (5–10)	

M/F, male subjects/female subjects; KT, kidney transplantation; eGFR, estimated glomerular filtration rate; CNIs, calcineurin inhibitors; MMF, mycophenolate mofetil.

TABLE 2 | Clinical, biological and histological characteristics of patients with T cell-mediated rejection (TCMR) with or without graft loss.

	TCMR and graft loss n = 18	TCMR without graft loss n = 29	*p*
Sex M/F n (%)	12(66.7)/6 (33.3)	24 (82.8)/5 (17.2)	0.20
Age at conversion (years), mean ± SD	55.5 ± 14.0	59.1 ± 13.7	0.32
Mean time between KT and conversion (months) median time	3.3	4.3	0.70
Interval between rejection and conversion (months) median time	2.3	2.6	0.11
ECD n(%)	15 (83.3)	19 (65.5)	0.18
eGFR at the time of conversion (MDRD. mL/min/1.73 m^2) mean ± SD	23.9 ± 21.2	28.9 ± 14.7	0.07
eGFR at the time of rejection (MDRD. mL/min/1.73 m^2) mean ± SD	10.7 ± 6.5	23.7 ± 13.6	0.0001
Discontinuation of belatacept after TCMR	12 (66.6)	6 (20.7)	0.002
Banff lesion g+ptc median (IQR)	2.2 ± 1.5	1.4 ± 1.1	0.08
Banff lesion i+t median (IQR)	4.2 ± 1.7	3.8 ± 1.6	0.37
Banff lesion v median (IQR)	1.1 ± 1.1	1.2 ± 1.1	0.63
Banff lesion ci+ct median (IQR)	1.8 ± 1.9	2.6 ± 1.8	0.23
Banff lesion cv+ah median (IQR)	2.3 ± 1.5	2.8 ± 1.7	0.37

M/F, male subjects/female subjects; KT, kidney transplantation; eGFR, estimated glomerular filtration rate; CNIs, calcineurin inhibitors; MMF, mycophenolate mofetil; F, female; ECD, extended criteria donor; eGFR, estimated glomerular filtration rate; Banff scores: ah arteriolar hyalinosis, ci interstitial fibrosis, ct tubular atrophy, cv vascular fibrous intimal thickening; g, glomerulitis score; i, interstitial inflammation; ptc, peritubular capillaritis score; v, arteritis score.

Among KTRs with TCMR, 28/47 (59.6%) occurred in patients who were converted to belatacept during the first 6 months post transplantation (early conversion). We compared this population to the retrospective cohort in which 82 KTRs had early conversion to belatacept but no TCMR. In multivariate analysis (**Table 4**), lymphocyte count at the time of conversion and the dose of steroids used after the conversion were associated with the occurrence of TCMR after early belatacept conversion.

Among KTRs with TCMR 19/47 (40.4%) occurred in patients converted to belatacept after the first 6 months post-transplantation (late conversion). We compared this population to the retrospective cohort in which 156 KTRs were converted to belatacept late after transplantation but without TCMR. In multivariate analysis (**Table 5**), the absence of post-conversion use of steroids was associated with the occurrence of TCMR after belatacept late conversion.

Rate of Opportunistic Infections (OPIs)

The rate of OPIs was not different between the 2 groups (p = 0.25). In the TCMR group: 8 KTRs (8/47: 17%) developed 9 episodes of OPI, all occurring after the diagnosis of TCMR: 4 cases of CMV disease, 3 cases of invasive aspergillosis, 1 case of varicella-zoster infection and 1 case of HHV8 associated Kaposi sarcoma. In the control group, 26 KTR (27/238: 10.9%) developed 33 episodes of OPI: 14 cases of CMV disease, 10 cases of pneumocystis pneumonia, 2 cases of JC Virus associated PML, 2 cases of EBV-associated PTLD, 2 cases of varicella-zoster infection, 1 case of tuberculosis, 1 case of toxoplasmosis and 1 case of aspergillosis.

TABLE 3 | Univariate and multivariate Cox analyses for determining factors associated with the occurrence of T cell-mediated rejection (TCMR) after belatacept conversion.

	Univariate analysis			Multivariate analysis		
	HR	**IC 95%**	***p***	**HR**	**IC 95%**	***p***
Sex F	*0.48*	*0.25–0.95*	*0.03*	*0.68*	*0.32–1.41*	*0.29*
Age at conversion	1.01	0.98–1.02	0.69			
Time between KT and conversion	***0.97***	***0.95–0.99***	***0.002***	***0.97***	***0.94–0.99***	***0.01***
No use of anti thymocyte globulins post KT	***2.06***	***1.03–4.15***	***0.04***	***2.51***	***1.14–5.56***	***0.02***
Non ECD	*0.53*	*0.28–1.01*	*0.05*	*1.01*	*0.50–2.02*	*0.99*
eGFR at the time of conversion (MDRD. mL/min/1.73 m^2)	0.99	0.98–1.02	0.82			
Lymphocyte count at conversion	1.00	1–1.01	0.34			
No tacrolimus before conversion	*0.43*	*0.19–0.96*	*0.04*	*0.53*	*0.22–1.30*	*0.17*
Time between conversion and CNI discontinuation	1.05	0.89–1.24	0.56			
No MMF at the time of TCMR or at month 3	1.42	0.56–3.59	0.46	*1.1*	*0.96–1.26*	*0.17*
Dose of MMF	1.00	1–1.01	0.48			
No steroids at the time of TCMR or at month 3	1.21	0.56–2.58	0.62			
Dose of steroids	*1.14*	*1.02–1.28*	*0.02*			

F, female subjects; ECD, extended criteria donor; KT, kidney transplantation; MMF, mycophenolate mofetil; eGFR, estimated glomerular filtration rate. Italic values: significative in univariate analysis. Bold values: significative in multivariate analysis.

TABLE 4 | Univariate and multivariate Cox analyses for determining factors associated with the occurrence of T cell-mediated rejection (TCMR) after early belatacept conversion (<6 months post KT).

	Univariate analysis			Multivariate analysis		
	HR	**IC 95%**	***p***	**HR**	**IC 95%**	***p***
Lymphocytes count at conversion	***1.00***	***1.00–1.01***	***0.003***	**1.01**	**1.00–1.01**	**0.003**
No tacrolimus before conversion	0.58	0.20–1.68	0.35			
Time between conversion and CNI discontinuation	1.10	0.88–1.39	0.40			
No MMF at the time of TCMR or at month 3	1.63	0.49–5.39	0.42	***1.15***	***1.03–1.41***	***0.01***
Dose of MMF	1.00	0.99–1.01	0.96			
No steroids at the time of TCMR or at month 3	0.54	0.07–3.99	0.58			
Dose of steroids	***1.24***	***1.05–1.45***	***0.009***			

MMF, mycophenolate mofetil; eGFR, estimated glomerular filtration rate. Bold values: significative in multivariate analysis.

TABLE 5 | Univariate and multivariate Cox analyses for determining factors associated with the occurrence of T cell-mediated rejection (TCMR) after late belatacept conversion (>6 months post KT).

	Univariate analysis			Multivariate analysis		
	HR	**IC 95%**	***p***	**HR**	**IC 95%**	***p***
Lymphocytes count at conversion	1.00	0.99–1.01	0.77			
No tacrolimus before conversion	0.38	0.11–1.31	0.12			
Time between conversion and CNI discontinuation	1.01	0.75–1.37	0.94			
No MMF at the time of TCMR or at month 3	*3.51*	*0.81–15.20*	*0.09*	*1.59*	*0.36–6.97*	*0.54*
Dose of MMF	1.00	0.99–1.01	0.96	***2.58***	***1.01–6.62***	*0.04*
No steroids at the time of TCMR or at month 3	***3.43***	***1.39–8.44***	***0.007***			
Dose of steroids	1.14	0.69–1.11	0.28			

MMF, mycophenolate mofetil; eGFR, estimated glomerular filtration rate. Italic values: significative in univariate analysis. Bold values: significative in multivariate analysis.

DISCUSSION

This is the first report of the rate of kidney transplant rejection, both cellular and humoral, over a 10-year period in a large cohort of KTRs who were converted to belatacept as a rescue strategy. We confirm that the occurrence of acute rejection after conversion to belatacept appears to be less frequent than with *de novo* use. A major pitfall of the use of belatacept as a *de novo* strategy is the increased risk of TCMR compared to cyclosporine: in the BENEFIT study the rate of TCMR was 17%–24% at 1 year [1] and in the BENEFIT-EXT study, it was 18% at 1 year [17]. Nevertheless the occurrence of such rejection was not associated with worse graft survival or a poorer graft function at 8 years post KT. Regarding TCMR after conversion to belatacept in stable patients, the rate reported in the randomized study by Budde et al in a large cohort was 8% compared to 4% in the CNI arm [4]. When belatacept was used as a rescue strategy, the rate of TCMR was

between 5.3% and 11.4% according to different retrospective studies [7, 8, 18] and was not significantly different between the belatacept and CNI arms in our retrospective study (4.3% in both arms) [5] and in the recently published study by Divard et al (4% in both arms) [10].

In contrast to the data from the original princeps study, in our study the risk of graft loss or deterioration of renal function after rejection was significant. Almost 50% of the rejections had V lesions. We observed 8 graft losses after rejection and death-censored graft survival was nearly 50% at 5 years post rejection. Some refractory allograft rejections to steroids justified being very cautious. Rejection occurred very early after the conversion, as in the *de novo* use and therefore very close biological follow-up has to be implemented after conversion to belatacept. Nevertheless after treatment of TCMR (mainly with steroids) 70% of the patients recovered an eGFR at least equivalent to that at the time of the conversion. We identified 2 factors associated with graft loss after TCMR: eGFR on the day of rejection and the discontinuation of belatacept after treatment. In patients with a good response to treatment of the rejection, we believe that belatacept should be continued in this context in patients with features of CNI toxicity before conversion. Moreover, we also reported 8 deaths after rejection, 7 of which were due to infectious causes. Clinicians have to be extremely cautious about the overall infectious risk in the follow-up of these patients with poor graft function presenting TCMR. We and other authors already reported on the risk of OPIs after belatacept conversion as a rescue strategy, mainly due to CMV disease and pneumocystis pneumonia [6, 19]. Prophylaxis against these 2 pathogens must be implemented, if not, after the treatment of rejection in this context. Nevertheless the rate of OPI was not different between the TCMR group and the control group but one striking feature is the occurrence and death from invasive aspergillosis in three patients in the TCMR group. Rejection is already known to be a risk factor for invasive aspergillosis [20] but there are no data on the specific impact of costimulation blockade in this context, except in lung transplant recipients [21].

Regarding factors associated with the occurrence of TCMR, the time from transplantation to conversion appears to be essential. We already suspected that the proportion of acute cellular rejection is probably higher in early conversion (<6 months) [18]. In early conversion, the factor associated with the occurrence of TCMR was the lymphocyte count. This could be explained by the global level of immunosuppression before the conversion in KTRs: the higher the lymphocyte count, the lower the level of immunosuppression and the higher the risk of TCMR after the switch. Attention should be paid to the CNI tapering regimen, CNI exposure, and maintenance of mycophenolic acid dosing during conversion to prevent rejection [22]. In patients with a high lymphocyte count a more progressive discontinuation of CNIs could be proposed, for example, if antithymocyte globulins are not used as an induction. Such a protocol has already been used in the *de novo* use of belatacept with a reduction in the rejection rate [23]. The use of mTOR inhibitors instead of mycophenolate mofetil could be another possibility [24]. The use of a more intensive regimen of belatacept does not reduce the rejection rate in the PRINCEPS study [1]. In late conversion, the absence of steroids after conversion was associated with the occurrence of TCMR after conversion in multivariate analysis. Nevertheless the rejection rate after 6 months is low and we do not believe that reintroducing steroids in all KTRs converted is indicated but could be discussed in patients close to transplantation (conversion 6–12 months post KT?). We need biomarkers to assess the real risk of rejection in patients treated with belatacept (CD86 occupancy [25, 26]? Belatacept Drug Monitoring [27] ? Immunomonitoring of T cells resistant to costimulation blockade? [12]). Monitoring donor-derived cell-free DNA [28] or urinary chemokines [29] could be helpful in this situation, but has never been tested following belatacept conversion.

One of the benefits of belatacept use is the low incidence of *de novo* DSA, both in *de novo* use [3] and in the conversion protocol [5]. Budde et al. reported in their published conversion randomized control trial that the rate of *de novo* DSA in the belatacept arm was 1% compared to 7% in the CNI arm [4]. We confirmed this point in the case of rescue conversion strategy (7.4% in the belatacept group versus 15/64%–23.4% in the CNI group; $P = 0.01$) [5]. This is the first report of the incidence of ABMR in a large cohort of KTRs converted to belatacept as a rescue strategy and this rate was very low (<1%). This result is in line with the BELACOR study [30] in which sensitized patients with preformed DSA (Mean Fluorescence Intensity 500–3,000) received *de novo* belatacept infusion and none of them developed ABMR.

The retrospective nature of the study raises the concern of substantial bias. Nevertheless the high number of TCMR cases reported in this multicenter cohort allows us to find factors associated with graft loss in this context and also factors associated with the occurrence of TCMR in both early and late switching. Moreover, a strength of our study is the homogeneous conversion protocol used in all included centers regarding the dose of belatacept and the decrease protocol of CNIs. Future randomized studies including this particular population of KTRs, with poor graft function are highly needed to accurately report the rejection rate in this context and to avoid potential bias.

In conclusion, we have reported for the first time a low incidence of both TCMR (5%) and ABMR (<1%) in a very large and significant cohort of KTRs who were converted to belatacept as a rescue strategy. We have shown that for patients with TCMR after conversion, high doses of steroids are effective, but in some patients rejection impacted both graft and patient survival. eGFR at the time of rejection and continuation of belatacept after treatment are determining factors for graft survival. We also demonstrated that early switching (<6 months) is a more risky situation for TCMR occurrence compared to late switching (>6 months) and that the level of immunosuppression is probably essential. New markers are highly needed to better identify patients at risk of TCMR

post-conversion, in order to use this immunosuppressive drug with less fear.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

According to French law (loi Jardé), because this was an anonymous retrospective study, institutional review board approval was not required. 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

DB designed the study, collected and analyzed the data, and wrote the paper; all authors collected the data, provided feedback and critical review.

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CONFLICT OF INTEREST

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

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Evaluation of a Decentralized Donor-Derived Cell-Free DNA Assay for Kidney Allograft Rejection Monitoring

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Donor-derived cell-free DNA (dd-cfDNA) is an emerging non-invasive biomarker for allograft injury detection. This study aimed to evaluate a new, decentralized dd-cfDNA testing kit against a centralized dd-cfDNA testing service broadly utilized in the United States. Kidney transplant recipients with decentralized and centralized dd-cfDNA measurements and concomitant kidney allograft biopsies were included in the study. 580 kidney allograft recipients from 3 referral centers were included for 603 total evaluations. Correlation between assays was evaluated using r-squared (r^2) and Spearman's rank correlation test, and associations with rejection using logistic regression analyses and discrimination using area under the curve. Mean dd-cfDNA levels from decentralized and centralized tests were 0.51% ± 0.81% and 0.43% ± 0.78%, respectively. The assays were highly correlated, with r^2 = 0.95 and Spearman's rank correlation 0.88 (p < 0.0001). Both tests showed significant association with allograft rejection (p < 0.0001) and good and similar discriminations to predict rejection (AUC: 0.758 for the decentralized and AUC: 0.760 for the centralized dd-cfDNA; p = 0.8466). Consistency between the assays was also confirmed across clinical scenarios including post-transplant timepoint, allograft stability, and allograft rejection subcategories. This decentralized dd-cfDNA assessment demonstrates high accuracy and value to non-invasively monitor kidney recipients.

Keywords: AlloSeq, dd-cfDNA, liquid biopsy, allograft rejection, non-invasive diagnosis

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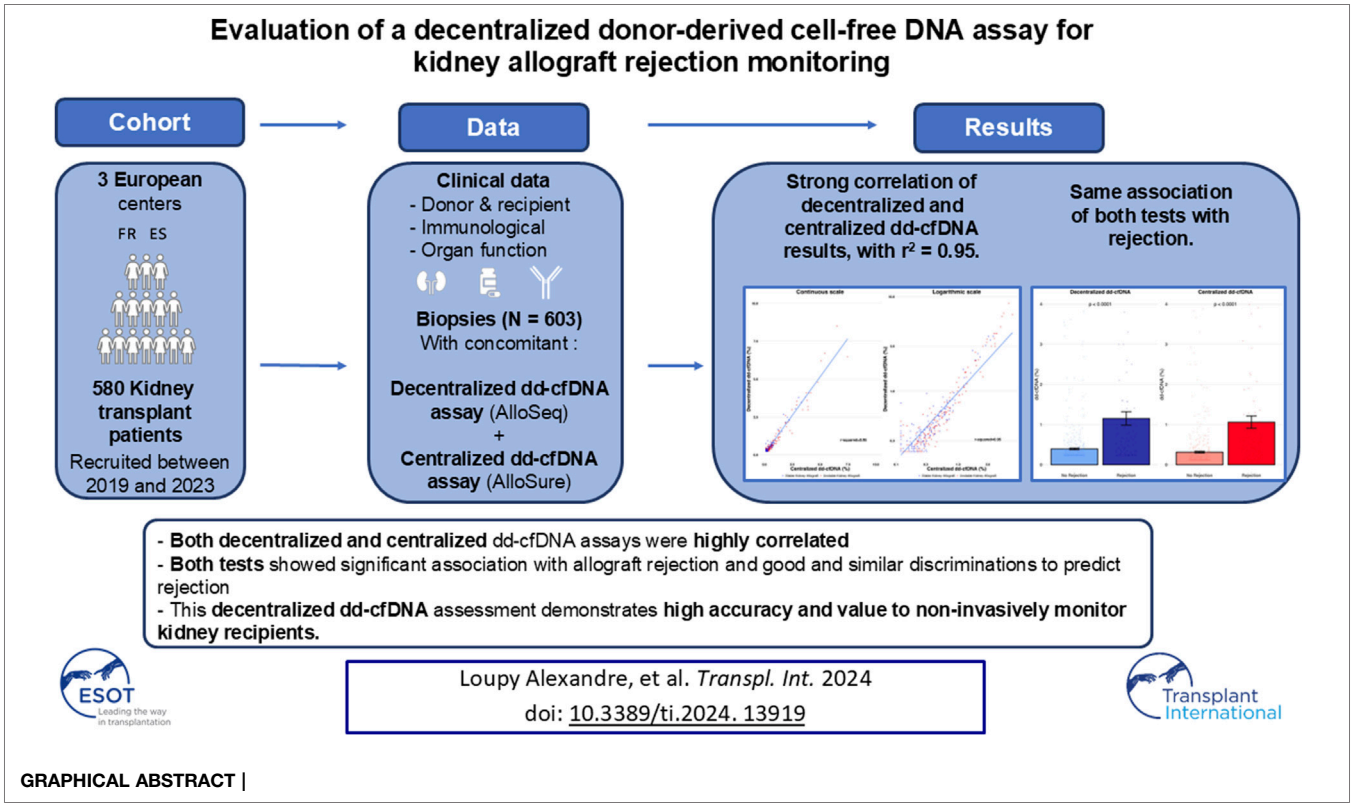

GRAPHICAL ABSTRACT |

INTRODUCTION

Allograft rejection remains the main cause of allograft loss after transplantation with detrimental consequences in terms of mortality, morbidity, and quality of life [1]. The gold standard to diagnose allograft rejection relies on tissue biopsy which is an invasive, costly procedure with potential pitfalls for interpretation [2, 3]. Measurement of various biomarkers offers non-invasive alternatives to the traditional biopsy with lower risk to the patient, less subjectivity, and greater convenience and flexibility. Serum creatinine (SC) and donor-specific antibodies (DSA) have been identified as informative biomarkers for kidney transplant monitoring, but SC has low sensitivity and specificity [4].

Donor-derived cell-free DNA (dd-cfDNA) has emerged as a clinically relevant biomarker in solid organ transplantation [5–8], and has been increasingly characterized with kidney transplant patients [9–13]. cfDNA naturally circulates in the bloodstream as a result of normal cell death mechanisms and can be influenced by factors like metabolic processes or overall health [14]. After transplantation, the allograft releases cfDNA, characterized as dd-cfDNA, into the recipient's blood stream at low levels, which increases in cases of injury, rejection, or other malfunction [9–12]. Genetically distinct from the recipient's own cfDNA (i.e., excluding cases where donor and recipient are monozygotic twins), dd-cfDNA can be detected and quantified at relatively low levels, making it an effective biomarker for routine post-transplant surveillance.

The American Society of Transplant Surgeons (ASTS) has recently recommended dd-cfDNA testing in adult kidney transplant recipients to monitor for rejection as a critical component of post-transplant surveillance [15], while the European Society for Organ Transplantation (ESOT) has advocated for the unmet need for non-invasive patient monitoring, the potential of dd-cfDNA in early detection of rejection, and its role in clinical decision-making [16]. Following those recommendations, centralized dd-cfDNA testing platforms are widely utilized by transplant physicians for routine post-transplant monitoring. Patient specimens are shipped from clinics and hospitals, dd-cfDNA testing is performed at the manufacturer's appropriately certified and accredited laboratory, and results are released to clinicians. Until recently, dd-cfDNA testing was primarily available through such centralized testing services [17]. However, the possibility of decentralized dd-cfDNA testing solutions hold key advantages including the convenience of onsite laboratory testing, enabling broader access and adoption, and enhanced flexibility and agility with clinical decisions.

The decentralized dd-cfDNA assay of focus in this study is a commercially available, CE-IVDD testing kit utilizing polymerase chain reaction (PCR) amplification of a proprietary single nucleotide polymorphism (SNP) panel and next-generation sequencing (NGS) technology to provide relative dd-cfDNA quantification for solid organ transplant recipients, without requiring genotyping. This assay offers up to 24-sample

throughput with turnaround time of 24 h from cfDNA sample to dd-cfDNA result (CareDx Instructions for Use: AlloSeq cfDNA Assay Instructions for Use IFU090 Version 6.0. Brisbane, CA) [18].

While decentralized dd-cfDNA testing technology opens avenues for onsite testing to facilitate therapeutic decisions for patient care [19], the direct comparison of its performance with respect to a well-characterized centralized dd-cfDNA testing platform [10, 20] and accuracy of clinical rejection detection have not previously been assessed on a single cohort. To address this timely issue, this large-scale, multicenter evaluation of a decentralized dd-cfDNA assay for kidney transplant patient monitoring was performed to assess both accuracy in comparison with a commonly used centralized dd-cfDNA testing platform and capacity to detect kidney allograft rejection.

MATERIALS AND METHODS

Study Design and Population

This large, multicenter trial included 580 kidney transplant patients from 3 centers. Patients were prospectively recruited in Necker Hospital, Paris, France; Saint-Louis Hospital, Paris, France; and Vall d'Hebrón University Hospital, Barcelona, Spain, between May 2019 and July 2023. Patients with combined organ transplantation or patient that had received a previous solid organ transplant (other than a kidney), pregnant women, recipients of a graft from a monozygotic twin, and patients who had received a bone marrow transplant were excluded. 792 samples with AlloSeq results were screened in the study. Evaluation without concomitant biopsies (n = 102, 12.9%) or AlloSure results (n = 87, 11.0%) were excluded. A total of 603 evaluation with AlloSeq and AlloSure results with a concomitant biopsy were included. All data were anonymised and prospectively entered at the time of transplantation, and were updated at several timepoints (3, 6, and 12 months post transplantation and annually thereafter), and at each clinical event using a standardised protocol to ensure harmonisation across study centers. Data were submitted for an annual audit to ensure data quality. Data were retrieved from the database in November 2023. The study was approved by the institutional review board of the Paris Transplant Institute for participating centers and was conducted in accordance with the Declaration of Helsinki. All patients provided written informed consent at the time of transplantation.

In the transplant referral center Vall d'Hebrón University Hospital, data were collected as part of routine clinical practice and entered in each center's database in compliance with local and national regulatory requirements and sent anonymised to the Paris Transplant Group.

Data Collection

Clinical data covered demographic parameters, including recipient age, sex, and transplant characteristics; biological parameters, including kidney allograft function, proteinuria, and anti-HLA DSA specificities and levels; and allograft pathology data, including Banff lesion scores and diagnoses. Kidney allograft function was assessed by the glomerular filtration rate estimated by the Modification of Diet in Renal Disease Study equation (eGFR) and proteinuria level using the protein/creatinine ratio. The presence of circulating DSA against HLA-A, HLA-B, HLA-Cw, HLA-DR, HLA-DQ and HLA-DP was assessed using single-antigen flow bead assays (One Lambda, Inc., Canoga Park, CA, United States) on a Luminex platform [21] at the time of dd-cfDNA evaluation. Beads with a normalized mean fluorescence intensity (MFI), a measure of donor-specific antibody strength, of greater than 500 units were judged as positive, as described previously [22]. HLA typing of the transplant recipients and donors was performed using an Innolipa HLA Typing Kit (Innogenetics, Ghent, Belgium). Allograft biopsies performed at the time of dd-cfDNA measurement were scored and graded from 0 to 3 according to the Banff 2019 classification [23] for allograft pathology for the following histological factors: glomerular inflammation (glomerulitis), tubular inflammation (tubulitis), interstitial inflammation, endarteritis, peritubular capillary inflammation (capillaritis), transplant glomerulopathy, interstitial fibrosis, tubular atrophy, arteriolar hyalinosis and arteriosclerosis. Additional diagnoses provided by the biopsy (e.g., the diagnoses of primary disease recurrence, BK virus nephropathy) were recorded. The biopsy sections (4 μm) were stained with periodic acid-Schiff, Masson's trichrome, and hematoxylin and eosin. C4d staining was performed via immunohistochemical analysis on paraffin sections using polyclonal human anti-C4d antibodies.

Circulating Nucleic Acid Extraction

Whole blood was drawn into Cell-Free DNA BCT (Streck, La Vista, NE, United States, 218997) following manufacturer instructions for use (IFU). Plasma was isolated within 7 days of blood draw according to Streck Double Spin Protocol 2. Filled blood tubes were centrifuged for 10 min at 1,600 × g at room temperature, the upper plasma layer was transferred to a new conical tube then centrifuged for 10 min at 16,000 × g at room temperature, and clarified plasma was transferred to a new, appropriately labeled screw-top tube for storage at −80°C. cfDNA extraction was performed with QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany, 55114) following the manufacturer protocol Purification of Circulating Nucleic Acids from 4 mL Plasma, using 25 μL Buffer AVE for elution. Purified cfDNA was stored at −80°C and shipped on dry ice with temperature monitors. Aliquots of the same cfDNA sample were used to perform centralized and decentralized dd-cfDNA tests.

Decentralized dd-cfDNA Measurement

AlloSeq cfDNA (CareDx, Brisbane, CA, United States) was performed following manufacturer IFU (CareDx Instructions for Use: AlloSeq cfDNA Assay Instructions for Use IFU090 Version 6.0. Brisbane, CA). Purified cfDNA samples were normalized to 0.625 ng/uL for 10 ng input in 16 μL. Multiplex PCR was performed to simultaneously amplify and index 202 SNP regions with the designated thermocycling protocol. The resulting PCR products were pooled with a fixed volume, then cleaned using the specified magnetic bead purification protocol. The final cleaned library pool was

quantified via Qubit dsDNA Quantification High Sensitivity (Thermo Fisher, Waltham, MA, United States, Q32851), then diluted and denatured for paired-end read sequencing on MiSeq (Illumina, San Diego, CA, United States) using MiSeq Reagent Kit v3, 150-cycle (Illumina, MS-102-3001). After each sequencing run, two post-run washes were performed to prevent index contamination between runs, the first using diluted bleach with the template line wash, and the second using detergent only without the template line wash.

Recipient and sample information, including genetic donor-recipient relationship, were entered into the AlloSeq cfDNA Software version 2.2.0 (CareDx), and FASTQ files generated from Illumina Real-Time Analysis software base calls were supplied following manufacturer IFU (CareDx Instructions for Use: AlloSeq cfDNA Software Instructions for Use IFU091 Version 6.0. Brisbane, CA). The proprietary data analysis algorithm, described below, automatically analyzed sequencing reads for each SNP region to determine the relative fraction of donor and recipient DNA in each sample. Target loci include 202 SNPs with genome-wide distribution (see **Supplementary Table S1**), multiethnic representation, high uniformity, and sufficient coverage to distinguish even genetically related donor-recipient pairs.

Calculation of dd-cfDNA was performed automatically within the AlloSeq cfDNA Software. Illumina short reads were trimmed for low quality ends and sequencing adaptors, then aligned to a custom reference assembly, containing the two expected alleles for each biallelic SNP, using a custom Needleman-Wunsch short read alignment algorithm. Mappings with both reads aligned were retained and the proportion of nucleotide signals at each targeted SNP locus were calculated. SNP results with low coverage or multiallelic (>2 allele) calls were excluded. Minor signals at homozygous SNPs were assumed as the dd-cfDNA fraction. Heterozygous SNP positions were excluded using a 30%–70% minor signal filter (only in cases where genotypes are not provided), and unexpected SNP results were assumed to be background noise and filtered out. Mean and standard deviation were calculated for the remaining minor signals, and any statistical outliers in the dataset were removed via z-score outlier detection (eliminating imbalanced heterozygous SNPs outside the 30–70 range, which could occur due to primer site differences), and mean and standard deviation recalculated, if necessary. The mean was adjusted for the expected 1:2:1 ratio (identical homozygous : heterozygous : opposite homozygous) of biallelic SNPs and for any genetic relatedness between recipient and donor, then reported as the final dd-cfDNA fraction in the software interface. Fully transparent visualization of results at every SNP locus and multiple QC metrics (too many outliers, markers passing filter, uniformity, average marker coverage, and total reads) were also reported.

Genotyping is not required for this assay, but recommended in cases of dd-cfDNA greater than approximately 30%, which are not clinically likely in cases of kidney transplant (CareDx Instructions for Use: AlloSeq Software Instructions for Use IFU091 Version 6.0. Brisbane, CA). Values in that high range would be reported as calculated by the AlloSeq cfDNA Software algorithm, but would only be considered accurate with one or more associated genotypes. In the absence of recipient and/or donor genotype(s), the minor fraction is automatically assigned to the donor (CareDx Instructions for Use: AlloSeq cfDNA Assay Instructions for Use IFU090 Version 6.0. Brisbane, CA; CareDx Instructions for Use: AlloSeq Software Instructions for Use IFU091 Version 6.0. Brisbane, CA).

Centralized dd-cfDNA Measurement

AlloSure Kidney (CareDx) was performed by trained CareDx R&D staff, following the same protocols as the CareDx CLIA-

TABLE 1 | Baseline patient characteristics of the cohort according to kidney allograft stability.

	Patients (N = 580)	Stable patients (N = 324)	Unstable patients (N = 256)	*P*-value
Recipient characteristics				
Age (y), mean (SD)	51.35 (16.63)	52.11 (16.22)	50.40 (17.12)	0.257
Male, number (%)	360 (62.07%)	192 (59.26%)	168 (65.62%)	0.122
Cause of end stage renal disease				
Glomerulopathy, number (%)	149 (25.69%)	91 (28.09%)	58 (22.66%)	
Polycystic kidney disease, number (%)	64 (11.03%)	37 (11.42%)	27 (10.55%)	
Interstitial nephritis, number (%)	31 (5.34%)	19 (5.86%)	12 (4.69%)	
Diabetes, number (%)	49 (9.45%)	26 (9.02%)	23 (8.98%)	
Vascular, number (%)	60 (10.34%)	37 (11.42%)	23 (8.98%)	
Other, number (%)	86 (14.83%)	46 (14.20%)	40 (15.62%)	
Unknown etiology, number (%)	141 (24.31%)	68 (20.99%)	73 (28.52%)	0.362
Donor characteristics				
Age (y), mean (SD)	53.61 (17.50)	54.14 (16.52)	52.95 (18.67)	0.661
Male, number (%)	339 (58.45%)	189 (58.33%)	150 (58.59%)	1
Deceased donor, number (%)	399 (68.79%)	210 (64.81%)	189 (73.83%)	0.024
Transplant baseline characteristics				
Prior kidney transplant, number (%)	102 (17.59%)	52 (16.05%)	50 (19.53%)	0.275
Cold ischemia time (h), mean (SD)	16.42 (13.36)	16.47 (14.12)	16.36 (12.54)	0.528
HLA-A/B/DR mismatch, mean (SD)	3.86 (1.44)	3.85 (1.49)	3.88 (1.39)	0.365
ABO incompatible transplant, number (%)	24 (4.67%)	15 (4.98)	9 (4.23)	0.833

Abbreviations: HLA, human leucocyte antigen.

TABLE 2 | Characteristics at the time of biopsy with concomitant decentralized and centralized dd-cfDNA measurements according to kidney allograft stability.

	Evaluations (N = 603)	Stable patients (N = 339)	Unstable patients (N = 264)	*P*-value
Time from transplant to evaluation (y), median (IQR)	0.39 (0.25–1.17)	0.27 (0.25–1.00)	1.18 (0.28–5.17)	<0.0001
Estimated GFR, mean (SD)	47.88 (20.69)	54.78 (20.05)	39.01 (17.98)	<0.0001
Proteinuria (g/g), median (IQR)	0.20 (0.10–0.57)	0.15 (0.08–0.30)	0.40 (0.14–1.08)	<0.0001
Positive anti-HLA DSAs, number (%)	304 (50.41%)	140 (41.30%)	164 (62.12%)	<0.0001
Biopsy findings				
Active AMR, number (%)	47 (7.79%)	19 (5.60%)	28 (10.61%)	
Chronic active AMR, number (%)	18 (2.99%)	2 (0.59%)	16 (6.06%)	
Inactive AMR, number (%)	3 (0.50%)	—	3 (1.14)	
Equivocal for diagnosis of AMR, number (%)	7 (1.16%)	1 (0.29%)	6 (2.27%)	
Acute TCMR, number (%)	9 (1.49%)	3 (0.88%)	6 (2.27%)	
Chronic active TCMR, number (%)	11 (1.82%)	2 (0.59%)	9 (3.41%)	
Mixed rejection, number (%)	7 (1.16%)	2 (0.59%)	5 (1.89%)	
Borderline lesions, number (%)	3 (0.50%)	2 (0.59%)	1 (0.38%)	
Viral nephritis, number (%)	12 (1.99%)	8 (2.36%)	4 (1.52%)	
Glomerulitis without rejection, number (%)	19 (3.15%)	13 (3.83%)	6 (2.27%)	
FSGS, number (%)	18 (2.99%)	2 (0.59%)	16 (6.06%)	
IF-TA, number (%)	222 (36.82%)	117 (34.51%)	105 (39.77%)	
No specific lesions, number (%)	227 (37.65%)	168 (49.56%)	59 (22.35%)	—

Abbreviations: GFR, glomerular filtration rate; HLA, human leucocyte antigen; DSA, Donor-Specific antibody; AMR, Antibody-mediated rejection; TCMR, T-Cell mediated rejection; FSGS, focal segmental glomerulosclerosis; IF-TA, interstitial fibrosis and tubular atrophy.

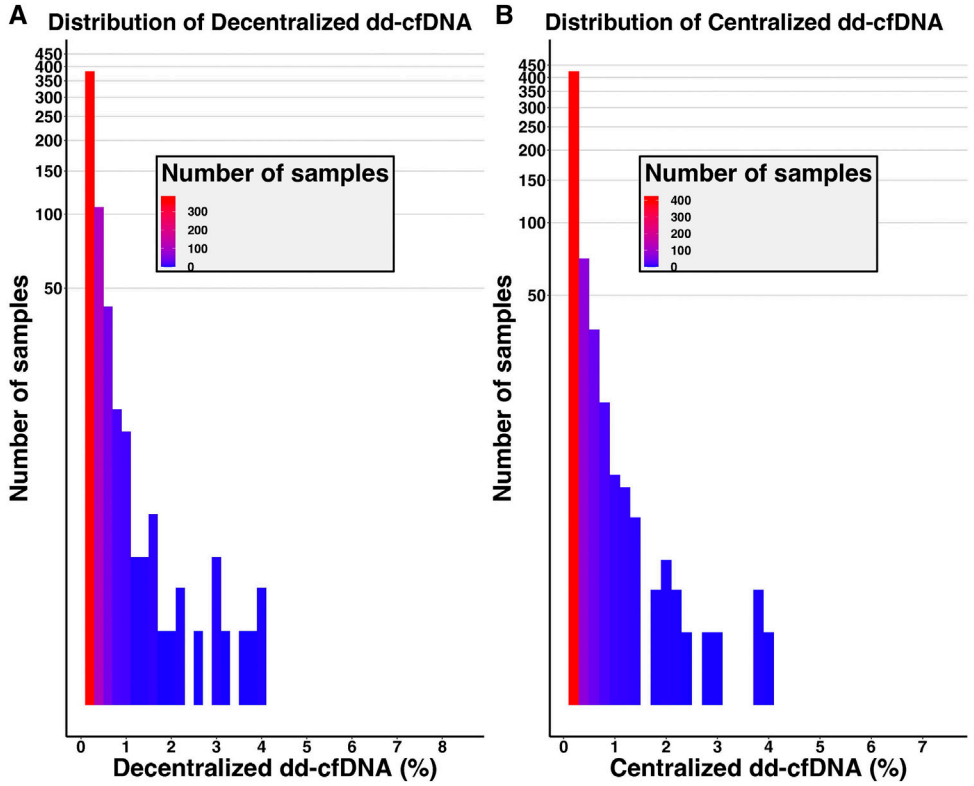

FIGURE 1 | Distribution of decentralized **(A)** and centralized **(B)** dd-cfDNA results among the cohort. The y-axis corresponds to the number of samples (logarithmic scale).

certified, CAP-accredited laboratory, as described previously [11, 24]. Purified cfDNA samples were used with fixed-volume input only and amplified via targeted PCR with primers for 405 SNP regions. Intermediate PCR products were cleaned with magnetic beads and indexing performed via another PCR with sequencing indexes and adapters. Resulting PCR products were pooled and cleaned with magnetic beads. The final cleaned library pool was quantified via Qubit dsDNA

Quantification High Sensitivity (Thermo Fisher, Q32851), then diluted for single read sequencing on NextSeq (Illumina) using NextSeq 500/550 Mid Output Kit v2.5, 150 cycles (Illumina, 20024904) or NextSeq 500/550 High Output Kit v2.5, 150 cycles (Illumina, 20024907), depending on sample throughput. Analysis pipeline and procedures were described previously [11, 24].

Statistical Analysis

Continuous variables were described using means and standard deviations or medians and interquartile ranges (IQR). Means and proportions were compared between groups using Student's t-test, analysis of variance (or Mann Whitney test if appropriate), or the Chi squared test (or Fisher's exact test if appropriate). The correlations between the decentralized and centralized dd-cfDNA results were assessed using the r-squared metric (r^2) and Spearman's rank correlation test. The associations of decentralized and centralized dd-cfDNA with rejection were assessed using logistic regression analyses. The discrimination ability was evaluated using area under the curve (AUC) [25]. All analyses were performed using R (version 4.1.2, R Foundation for Statistical Computing) and STATA (version 17). Values of $p < 0.05$ were considered significant, and all tests were two-tailed.

RESULTS

Characteristics of Patients at Baseline and at the Time of dd-cfDNA Measurement

The kidney transplant cohort was comprised of 580 patients and 603 evaluations with measurement of circulating dd-cfDNA post-transplant at the time of allograft biopsy. The mean recipient age was 51.35 ± 16.63 years. The mean donor age was 53.61 ± 17.50 years. A total of 399 (68.79%) patients received a kidney from a deceased donor, while 102 patients (17.59%) had a prior kidney transplant, and 24 (4.67%) were ABO incompatible. The mean cold ischemia time was 16.42 ± 13.36 h. The mean HLA-A/B/DR mismatch was 3.86 ± 1.44. The baseline characteristics of the recipients at the time of transplantation are summarized in **Table 1** with the comparison of patients with stable (Kidney graft instability was defined according to the acute kidney injury 2012 KDIGO guidelines [26] as an increase of serum creatinine of more than 0.3 mg per deciliter (>26.4 µmol/L) or of more than 50% from baseline and the presence of proteinuria) and unstable kidney function. The median time between kidney transplantation and dd-cfDNA assessment was 0.39 years (IQR 0.25–1.17). At the time of dd-cfDNA measurement, the mean estimated glomerular filtration rate was 47.88 ± 20.69 mL/min/

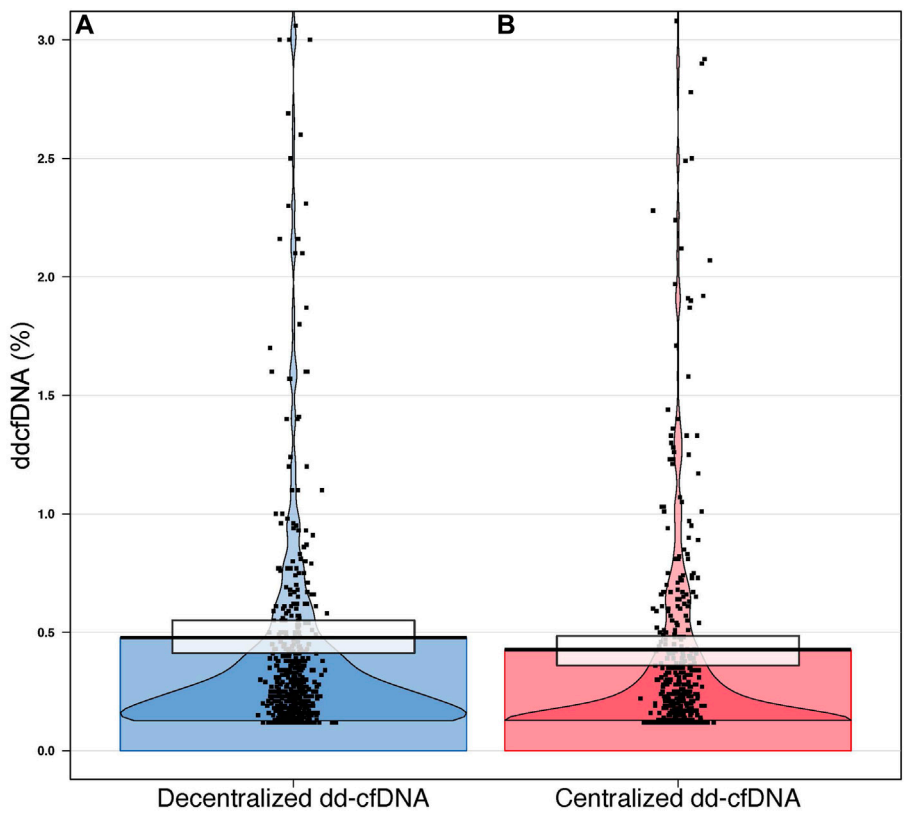

FIGURE 2 | Violin plot distribution of decentralized **(A)** and centralized **(B)** dd-cfDNA results. Each point represents a single sample. The black horizontal lines represent the central tendencies. The beans represent the smoothed densities, and the rectangles represent the inference intervals with confidence intervals (decentralized dd-cfDNA in blue, centralized dd-cfDNA in red).

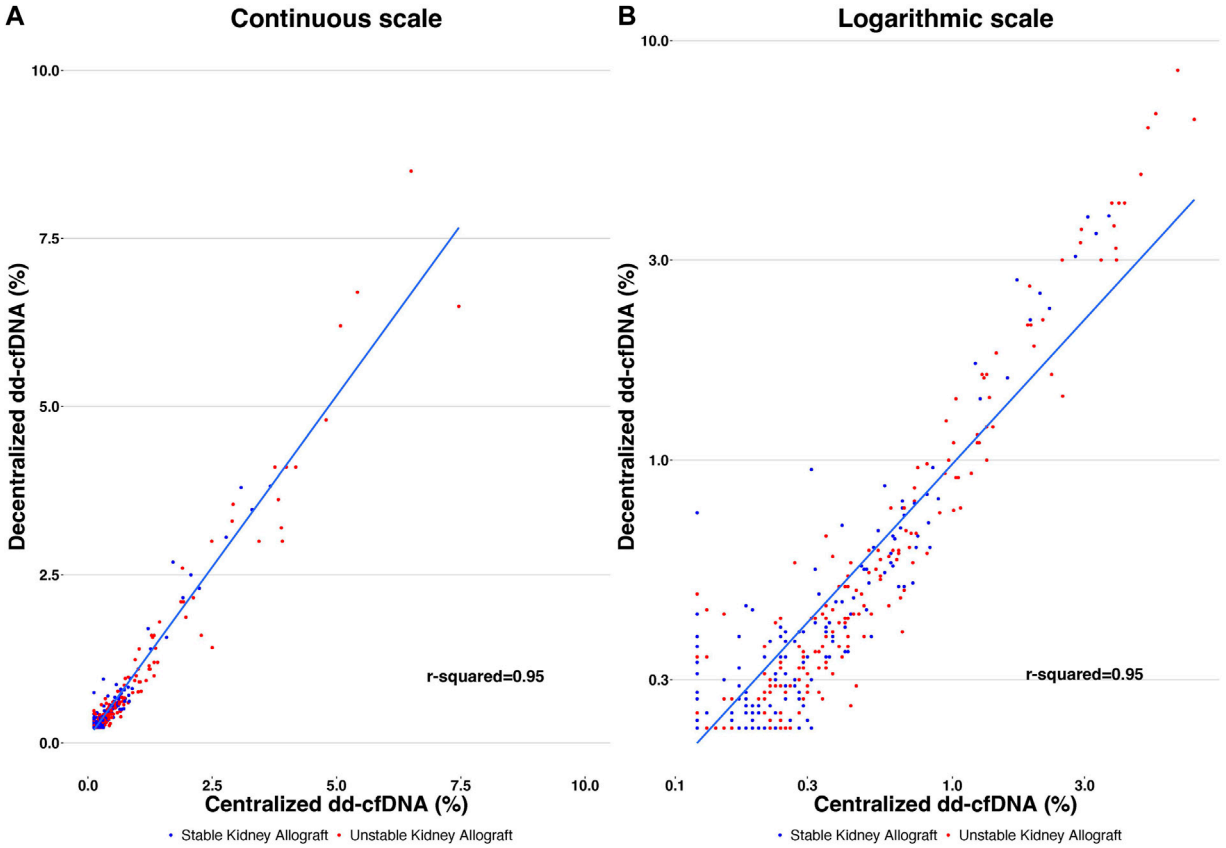

FIGURE 3 | Correlation of decentralized and centralized dd-cfDNA results, with r^2 = 0.95. Each point represents a single sample. The y-axis corresponds to the decentralized dd-cfDNA and the x-axis corresponds to the centralized dd-cfDNA using continuous scale **(A)** and logarithmic scale **(B)**.

1.73 m^2, median urinary protein-to-creatinine ratio was 0.20 g/g (IQR 0.10–0.57). At the time of dd-cfDNA measurement, 339 (56.22%) patients were clinically stable while 264 (43.78%) were unstable, with 65 (10.78%) presenting with antibody mediated rejection (AMR) and, 27 (4.48%) showing T-cell mediated rejection (TCMR) or mixed rejection. Functional, immunological, and histological characteristics at the time of dd-cfDNA evaluation are summarized in **Table 2**. Patients with unstable kidney allograft function showed significantly lower eGFR, higher proteinuria, and more positive anti-HLA DSA ($p < 0.0001$ for all comparisons).

Comparison of Decentralized and Centralized dd-cfDNA Results

The mean dd-cfDNA levels from decentralized and centralized assays were 0.51% ± 0.81% [median: 0.23, interquartile range (IQR): 0.23–0.42] and 0.43% ± 0.78% (median: 0.17, IQR: 0.12–0.37), respectively. **Figure 1** shows the distribution of decentralized and centralized dd-cfDNA results and **Figure 2** shows the violin plots of the two tests. The decentralized assay showed high correlation with centralized dd-cfDNA results with r^2 = 0.95 and a Spearman's rank correlation of 0.88 ($p < 0.0001$). Correlation between the two tests is represented in **Figure 3**.

Association and Discrimination of Decentralized and Centralized dd-cfDNA Results With Rejection

Mean dd-cfDNA levels of the decentralized assay were 1.15% ± 1.60% (median: 0.54, IQR: 0.26–1.10) for biopsies showing rejection (AMR, TCMR and mixed rejection) and 0.39% ± 0.48% (median: 0.23, IQR: 0.23–0.36) for biopsies without rejection ($p < 0.0001$). Mean dd-cfDNA levels of the centralized assay were 1.06% ± 1.47% (median: 0.48, IQR: 0.22–1.04) for biopsies showing rejection and 0.31% ± 0.49% (median: 0.14, IQR: 0.12–0.29) for biopsies without rejection ($p < 0.0001$) (**Figure 4**). The decentralized and centralized assays showed strong correlation among biopsies both without concurrent allograft rejection (r^2 = 0.94) and with ongoing rejection (r^2 = 0.95).

Both assays showed significant and strong association with allograft rejection using a logistic regression: decentralized dd-cfDNA (log transformation) (OR = 3.293, 95% CI: 2.453–4.421; $p < 0.0001$) and centralized dd-cfDNA (OR = 2.722, 95% CI:

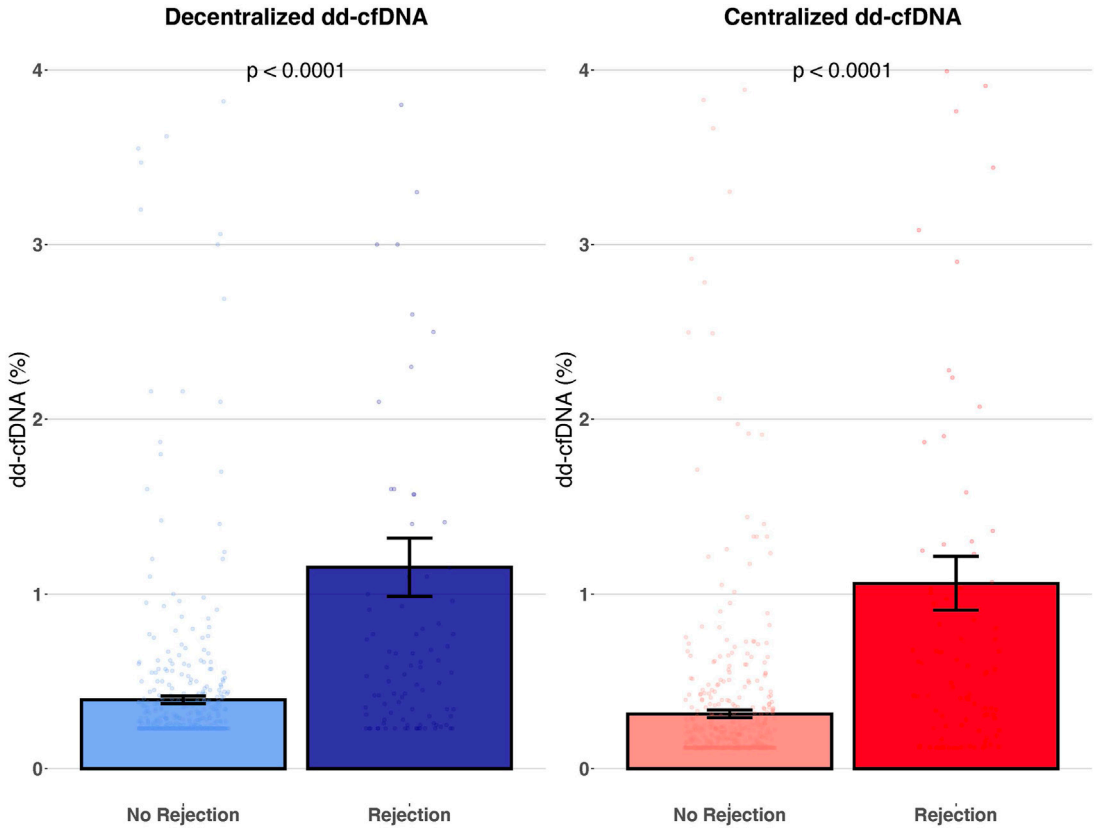

FIGURE 4 | Association of decentralized and centralized dd-cfDNA with rejection. This figure represents the mean level of decentralized and centralized dd-cfDNA according to the presence or absence of rejection. Each dot corresponds to an individual dd-cfDNA value. Data are presented as mean values ± SEM.

2.146–3.454; $p < 0.0001$). The discriminations of decentralized dd-cfDNA (log transformation) and centralized dd-cfDNA to detect rejection were similar without significant difference (AUC: 0.758 and 0.760, respectively; $p = 0.8466$) (**Figure 5**).

Sensitivity Analyses

Various analyses were performed to further confirm the robust correlation between dd-cfDNA results from decentralized and centralized assays in different clinical scenarios. With respect to post-transplant timepoint of evaluation, the high correlation between decentralized and centralized dd-cfDNA results remained when assessed within ($r^2 = 0.97$) or beyond ($r^2 = 0.94$) the first year post-transplantation. Regarding allograft stability, good correlation was observed for patients with stable ($r^2 = 0.95$) compared to unstable ($r^2 = 0.95$) renal function. Among different rejection phenotypes, the good correlation was maintained for AMR ($r^2 = 0.96$) versus TCMR and/or mixed rejection ($r^2 = 0.97$).

Bland-Altman plot was assessed to visualize the differences between the decentralized and centralized dd-cfDNA measurements. The average difference was 0.08 (95% CI: 0.06–0.09), the upper limit of agreement was 0.43 (95% CI: 0.41–0.45), and the lower limit of agreement was −0.26 (95% CI: −0.24 to −0.28) (**Supplementary Figure S1**). A Passing-Bablok regression was performed for the comparison of the decentralized and centralized dd-cfDNA test (**Supplementary Figure S2**). The slope of the fitted regression was 0.76 (95% CI: 0.70–0.82) and the intercept was 0.139 (95% CI: 0.132–0.145).

DISCUSSION

This large, multi-national study has demonstrated the accuracy of decentralized dd-cfDNA solution AlloSeq cfDNA compared to heavily utilized centralized dd-cfDNA platform cfDNA AlloSure Kidney. The generalisability of the decentralized dd-cfDNA assay has been validated in distinct cohorts and various clinical scenarios [27–40]. This is the first large, multi-center validation study on a European cohort directly comparing the analytical correlation and clinical assay performance of these two assays.

The decentralized and centralized dd-cfDNA assays yielded good correlation overall and both tests were strongly associated with allograft rejection, including AMR and TCMR and/or mixed rejection. Results were also consistent across several clinical scenarios including the interval between transplantation and the timing of dd-cfDNA evaluation, in stable and unstable patients, further highlighting the robustness and value of this decentralized dd-cfDNA assay. However, the correlation of these two assays and their associations with other situations, such as BK virus associated nephropathy, urinary tract infection, or sepsis,

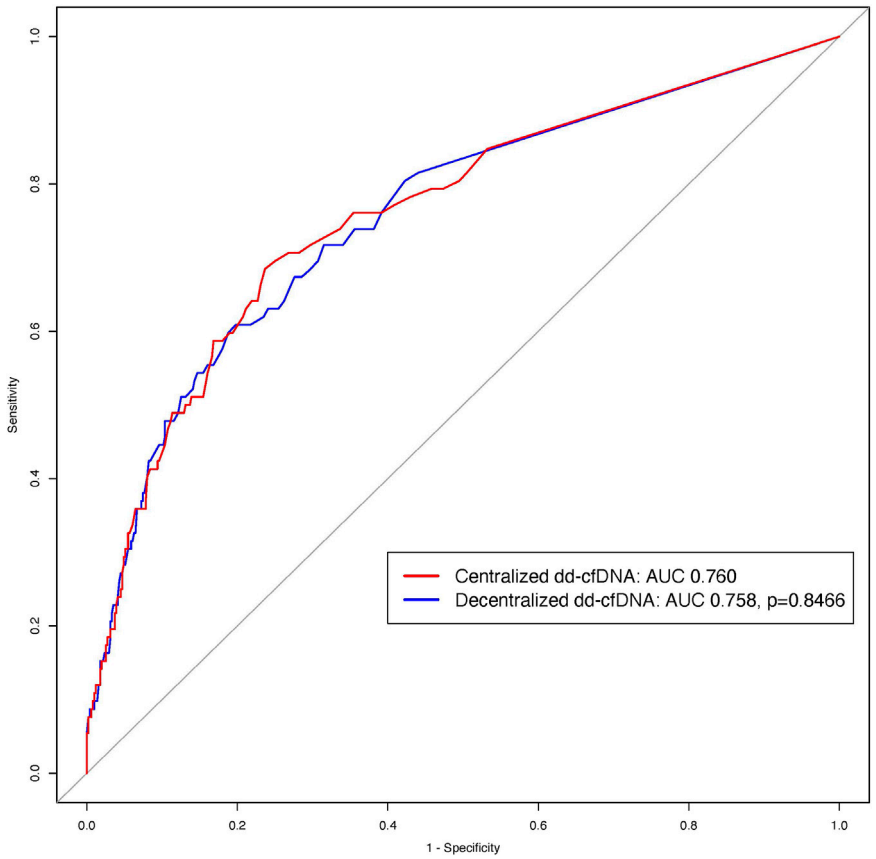

FIGURE 5 | ROC curves of the centralized and decentralized dd-cfDNA to detect rejection. The red ROC curve corresponds to centralized dd-cfDNA alone and the blue curve to decentralized dd-cfDNA alone for the detection of rejection. There was no difference between the two tests (p = 0.8466).

were not assessed in this study because no association was shown in a previous study [13].

The observed slope of 0.76 for the correlation between decentralized and centralized dd-cfDNA results indicates that the centralized dd-cfDNA method tends to provide lower values compared to the decentralized method, particularly at higher levels. However, values exceeding 1% represent a threshold beyond which the probability of rejection is high [9]. Therefore, the lack of agreement at these higher values is of limited clinical concern, as any results in that range from either method would lead to further diagnostic actions, such as a biopsy, regardless of the discrepancy.

dd-cfDNA testing is an efficient, informative, and minimally invasive solution for post-transplant monitoring in kidney transplant patients [13]. Implementation of this biomarker for routine surveillance to inform on potential injury or rejection enables clinicians to more promptly modify immunosuppressive treatment [41–43]. The predicate centralized dd-cfDNA testing platform is well-characterized and broadly used for solid organ transplant patients in the United States [9–12]. The decentralized dd-cfDNA assay evaluated here is a commercial kit with international availability and support, offering an efficient solution for decentralized dd-cfDNA quantitation. Both assays share the same fundamental biochemistry. Curated panels of SNP loci are amplified via PCR, then resulting amplicons are indexed using NGS barcodes, pooled, and purified in preparation for NGS. The decentralized dd-cfDNA assay targets 202 SNPs, performs amplification and indexing in a single PCR, and has been validated with Illumina MiSeq and MiniSeq, while the centralized dd-cfDNA assay targets 405 SNPs, requires two independent PCR steps, and uses Illumina NextSeq [24].

These decentralized and centralized dd-cfDNA assays have been compared in internal manufacturer studies, yielding strong concordance with r^2 = 0.9136 for clinical samples, r^2 = 0.9458 for analytical samples near the limit of detection, and r^2 = 0.9991 in the range 1%–70% for linearity (unpublished manufacturer data). With respect to other dd-cfDNA testing methods, various studies have revealed that this decentralized dd-cfDNA assay was well-correlated with digital droplet PCR (ddPCR) [39], quantitative fluorescent PCR (QF-PCR) amplification of short tandem repeats (STR) [31], and high-throughput sequencing [33], with higher sensitivity than both ddPCR [39] and QF-PCR [31]. Several other recent studies have also demonstrated the use of this decentralized dd-cfDNA assay for kidney transplant monitoring [27–30, 32, 34–38, 40].

The results from this study demonstrate the accuracy of this decentralized dd-cfDNA assay with respect to the predicate centralized dd-cfDNA assay. This decentralized assay leverages low input, NGS sensitivity, and associated analysis software to yield accurate dd-cfDNA results, offering increased convenience in clinical practice compared to centralized assays. Onsite testing

would allow transplant centers flexibility in both throughput and testing schedule, enabling implementation for not only standard patient monitoring but also clinical trials. However, further studies are needed with the comparisons between the two assays regarding the efficiency in terms of turnaround time and cost. The utilization of decentralized dd-cfDNA assays, such as the one evaluated here, addresses the current need for a powerful and efficient tool to expand access and broaden adoption of dd-cfDNA testing for kidney transplant surveillance.

CONCLUSION

The decentralized dd-cfDNA assay evaluated in this study shows strong correlation with the well-characterized and broadly used centralized dd-cfDNA assay. Though this behaviour cannot be generalized to other assays or methods without further study, the good concordance demonstrated here illustrates the potential of decentralized dd-cfDNA testing. Moreover, owing to its high accuracy to detect rejection, this decentralized dd-cfDNA assay proves to be a significant asset in clinical practice to enhance monitoring and care of kidney transplant patients.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving humans were approved by the institutional review board of the Paris Transplant Institute for participating centers. 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

AL and OA designed the study. AL, AC, and OA performed the data analysis and wrote the manuscript. AL, AC, CU-D, OB, DK, CL, and OA, contributed to data acquisition and interpretation. All authors contributed to the article and approved the submitted version.

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The author(s) declare financial support was received for the research, authorship, and/or publication of this article. OrganX Foundation.

CONFLICT OF INTEREST

CareDx participated in dd-cfDNA testing by providing reagents for decentralized testing, blinded to clinical information, and reviewed the manuscript and data analysis. Authors NT, EV, CM, SC, TH, and TV were employed by the company CareDx.

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

GENERATIVE AI STATEMENT

The author(s) declare that no Generative AI was 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.2024.13919/full#supplementary-material

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Assessing the Predictive Power of PIRCHE-II Scores for the Development of *De Novo* Donor-Specific Antibodies After Simultaneous Pancreas-Kidney Transplantation

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The molecular HLA epitope mismatch is an advanced measure for developing *de novo* donor-specific antibodies (dnDSA) after kidney transplantation. Its relevance in simultaneous pancreas/kidney transplant recipients (SPKTRs) remains unclear. We investigated dnDSA development in 72 SPKTRs and 383 kidney transplant recipients (KTRs) and used the Predicted Indirectly Recognizable HLA-Epitopes (PIRCHE-II) algorithm to calculate the mismatch load of HLA-derived epitopes in total, per HLA-class, and per HLA-locus. At 1 year post-transplant, SPKTRs exhibited an increased dnDSA incidence (11.2% vs. 3.1%, $p = 0.011$); but not at 10 years post-transplant. In SPKTRs, preformed DSA (HR 2.872, $p = 0.039$) and younger donor age (HR 0.943, $p = 0.017$) were independent risk factors for developing dnDSA. PIRCHE-II scores for HLA-DQ correlated with dnDSA development upon univariate analysis ($p = 0.044$). Among 455 KTRs/SPKTRs, multivariate analysis identified PIRCHE-II scores for HLA-DQ (HR 1.023, $p = 0.025$) and ciclosporine use (HR 2.440, $p = 0.001$) as independent predictors of dnDSA development. Simultaneous pancreas/kidney transplantation (SPK) was an independent risk factor in case of preformed DSA only (HR 2.782, $p = 0.037$). High PIRCHE-II scores for HLA-DQ are crucial for dnDSA development in both SPKTRs and KTRs. The lack of an independent association of total PIRCHE-II scores urges caution in implementing it in post-transplantation risk assessment.

Keywords: simultaneous pancreas and kidney transplant, preformed DSA, *de novo* DSA, epitope matching, PIRCHE-II score

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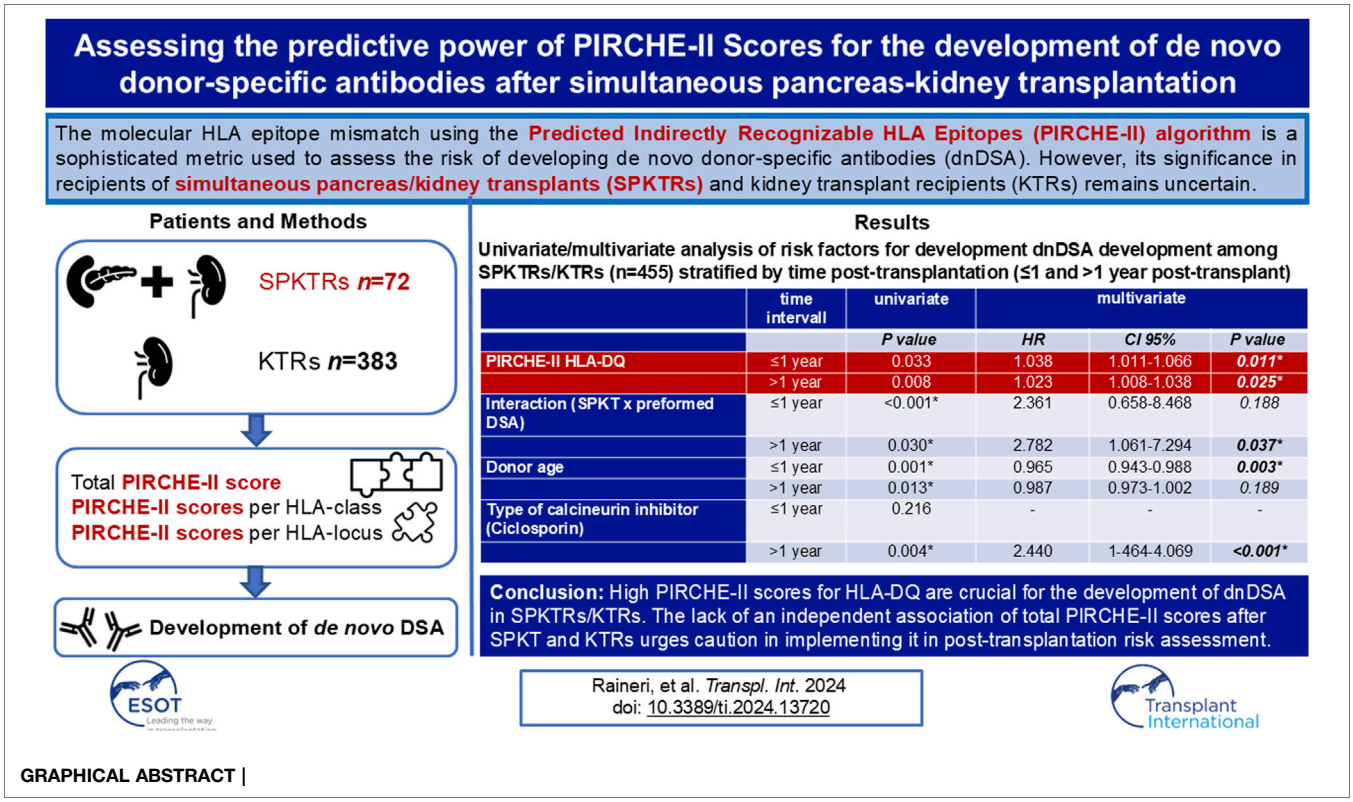
Abbreviations: ABMR, antibody-mediated rejection; BMI, body mass index; CNI, calcineurin inhibitor; dnDSA, *de novo* donor-specific anti-HLA-antibodies; eGFR, estimated glomerular filtration rate; HbA1c, hemoglobin A1c; KT, kidney transplantation; KTRs, kidney transplant recipients; MFI, mean fluorescence intensity; MMF, mycophenolate mofetil; MPA, mycophenolic acid; NGS, next-generation sequencing; PIRCHE, Predicted Indirectly ReCognizable HLA Epitopes; PT, pancreas transplantation; PTA, pancreas transplantation alone; SPK, simultaneous pancreas/kidney transplant; SPKTRs, simultaneous pancreas/kidney transplant recipients; SSO, sequence-specific oligonucleotide; SSP, sequence-specific primer; TCMR, T-cell mediated rejection.



GRAPHICAL ABSTRACT |

INTRODUCTION

In June 2021, the First World Consensus Conference on Pancreas Transplantation provided evidence-based guidelines, offering directions for clinical practice after pancreas transplantation (PT) [1, 2]. The primary message emphasized that both simultaneous pancreas/kidney transplantation (SPK) and pancreas transplantation alone (PTA) lead to improved quality of life [3, 4] and long-term patient survival [5, 6] compared to other medical interventions. Experts conclude that according to empirical evidence, preformed donor-specific anti-HLA antibodies (DSA) with MFI level <5000 in recipients with negative T and B cell flow cytometric crossmatches should not be a prohibitive factor for pancreas transplantation, based on the fact that evidence regarding negative impact of pretransplant DSA on transplant outcomes is lacking [2]. Contrarily, detection of dnDSA has been associated with worse outcomes, including graft rejection and failure [7, 8], Hence, rigorous post-transplant surveillance is recommended. In the setting of SPK, the relevance of HLA mismatching is a matter of debate as it did not translate into improved overall graft outcome, even though associated with reduced development of dnDSA and reduced graft rejection [9, 10]. Concerning immunosuppression, tacrolimus and mycophenolate, compared to ciclosporin and azathioprine, showed superior immunological results, i.e., reduced risk of developing dnDSA [11, 12]. Early tapering of corticosteroids was found suitable for a specific subset of pancreas transplant recipients, demonstrating viability without concomitant compromise in outcomes but improved metabolic parameters in the long term [13, 14].

Our study aims to assess the impact of the Predicted Indirectly ReCognizable HLA Epitopes (PIRCHE-II) scores for the first time in predicting the development of dnDSA and graft outcomes in simultaneous pancreas/kidney transplantation recipients. The PIRCHE-II score is an established algorithm to calculate HLA epitope mismatches for certain HLA antigen mismatches. It estimates the number of indirectly recognizable, donor HLA-derived T cell epitopes and predicts T cell-related immune responses against the donor HLA-derived peptides. Moreover, the PIRCHE-II score has demonstrated the ability to predict the incidence of dnDSA in kidney transplantation (KT) independently and was associated with kidney allograft survival in a cohort of kidney transplantation [15, 16].

In our study, we attempted to address the following questions: 1) What is the incidence of dnDSA among SPKTRs vs. KTRs? 2) What risk factors are associated with the development of dnDSA among SPKTRs at 1-year post-transplantation and in the long-term? 3) What risk factors are associated with the development of dnDSA among the whole cohort of SPKTRs/KTRs at 1-year post-transplantation and in the long term?

MATERIALS AND METHODS

Patients

Our study was approved by the Cantonal Ethic Commission Review Board of Zurich, Switzerland (KEK-ZH Number 2020-02817) and has complied with the Declaration of Helsinki.

We conducted a retrospective study of 72 SPKTRs who underwent a first deceased donor SPK and 383 KTRs who underwent a first deceased-donor single kidney transplantation at the University Hospital of Zurich between May 2009 and December 2019. Allograft outcome was evaluated in terms of 1) kidney allograft function, survival, and graft rejection, 2) pancreas allograft function, survival, and graft rejection, and 3) the development of dnDSA.

Post-transplant care was carried out according to a standardized scheme with appointments in our outpatient clinic twice a week at weeks 2 and 3, once a week at weeks 4, 5, 6, 8, 10, 12, once a month at months 4, 5, 6, 8, 10 and 12, with at least 16 visits within the first year after transplant. Subsequently, quarterly check-ups were performed in cooperation with the nephrologists close to the patient's home, with at least annual follow-up visits in our outpatient clinic. At any appointment, kidney function was evaluated by measuring serum creatinine, serum urea, and proteinuria. Pancreas graft function was defined as insulin-free survival and was assessed by the measurement of serum lipase and fasting plasma glucose levels. In addition, HbA1c values were routinely checked at the first visit, at week 12, at months 6, 9, and 12, annually, and at any time pancreas dysfunction was suspected.

Induction and Maintenance Immunosuppression

Among both SPKTRs and KTRs, a peak MFI cut-off of 1,000 of any historic preformed DSA was applied for acceptance of an organ offer. All 72 SPKTRs received lymphocyte-depleting induction immunosuppression. The maintenance immunosuppression consisted of a dual-drug combination of a calcineurin inhibitor (CNI, tacrolimus) and antimetabolite (MMF, mycophenolate mofetil) or (MPA, mycophenolic acid) or azathioprine. Early steroid withdrawal within the first post-transplant week was performed in all SPKTRs.

Regarding the individually defined immunologic risk, 383 KTRs received lymphocyte-depleting induction or induction with interleukin-2 receptor blockade. The primary immunosuppression consisted of a dual-drug combination of a calcineurin inhibitor (CNI, tacrolimus, or ciclosporin) and antimetabolite (MMF, mycophenolate mofetil) or (MPA, mycophenolic acid) or azathioprine, and steroids. Steroids were reduced over 12 weeks to 5 mg prednisone/day. KTRs underwent steroid withdrawal at +6 months post-transplantation unless 1) preformed DSA persisted with an MFI >500, 2) dnDSA developed with an MFI >500, or 3) KTRs had glomerulonephritis as the underlying disease.

Assessment of Kidney and Pancreas Allografts Function and Survival

Kidney allograft function, survival, and rejection were evaluated based on the best serum creatinine (μmol/L), best proteinuria (mg/day), and eGFR (mL/min) at 1 year post-transplant. The best serum creatinine and best proteinuria were calculated as the median of the 3 lowest serum creatinine and proteinuria values in the first post-transplant year. Additionally, kidney graft outcomes were evaluated based on the need for re-transplant, dialysis treatment, or patient death.

Pancreas allograft function, survival, and rejection were evaluated based on the need for insulin therapy, best HbA1c value in the first 2 years post-transplant, and the need for pancreas re-transplant or patient death. The best HbA1c value was calculated as the median of the 3 lowest HbA1c values in the 1- and 2-years post-transplant.

HLA Typing, Anti-HLA Antibody Analysis and Calculation of Predicted Indirectly ReCognizable HLA Epitopes (PIRCHE-II)

The HLA-derived mismatched peptide epitopes presented by SPKTRs HLA-molecules were calculated using the PIRCHE-II algorithm. In addition to the standard donor HLA typing, further typing was performed to assess additional loci if the recipient developed anti-HLA antibodies after transplantation against an HLA locus that had not been previously typed. For each HLA locus, the presentation of both HLA class I (HLA-A, B, C) and HLA class II-derived peptides (HLA-DR, DQ) were calculated and designated PIRCHE-II-A, B, C, DR, and DQ, respectively. HLA typing of donors and recipients was determined using either sequence-specific oligonucleotide (SSO), sequence-specific primer (SSP), or Next-generation sequencing (NGS) technologies depending on when they were transplanted. For all PIRCHE-II calculations only low-resolution HLA typing was entered and the high-resolution typing was imputed according to the standard PRICHE-II algorithm. The PIRCHE-II algorithm is available online.[1] For class I scores, the PIRCHE-II score is the sum of HLA-A, HLA-B, and HLA-C scores, while for class II scores, it is the sum of HLA-DRB1 and HLA-DQB1 scores. The total PIRCHE-II score is the sum of all loci scores for each donor-recipient pair.

The anti-HLA antibodies testing was routinely performed with the use of a Luminex-based single bead assay (One Lambda, Canoga Park, CA, United States) on the day of the transplant, at months 3, 6, 12, annually after that, and at any other time in case of unexplained deterioration of allografts function. Positivity of dnDSAs were defined by the presence of dnDSA targeting the HLA loci A, B, C, DRB (including DRB345), DQB and DPB with a normalized mean fluorescence intensity (MFI) exceeding 500. The dnDSA detected post-transplant were analyzed individually by a specialist in transplantation immunology in a blinded fashion. Here, it was determined if the antibody showed true donor specificity by analyzing the pattern of single-bead reactivity and comparing it to the HLA typing of the donor.

SPKTRs/KTRs with 0 HLA-antigen mismatches, 0 HLA-antigen mismatches for HLA-class I, and 0 HLA-antigen mismatches for HLA-classes II were excluded for the distinct analyses.

[1]https://www.pirche.org

TABLE 1 | Clinical characteristics of SPKTRs and KTRs at transplantation.

	Total (n = 455)	SPKTRs (n = 72)	KTRs (n = 383)	*P*-value
Recipient characteristics				
Time post-transplant, months*	70 (6–158)	74 (6–158)	68 (11–157)	0.349
Recipient age, years*	53 (17–75)	43 (23–58)	55 (17–75)	<**0.001***
Recipient, male sex, n (%)	278 (62)	36 (50)	242 (63)	**0.048***
Underlying kidney disease, n (%)				
Type 1 diabetes	70 (15)	68 (94)	2 (1)	<**0.001***
Type 2 diabetes	29 (6)	3 (4)	26 (7)	0.598
Other	356 (78)	1 (1)	355 (93)	<**0.001***
BMI pre-transplant, kg/m^2	25 (16.44–41.21)	24 (16.95–34.14)	25 (16.44–41.21)	<**0.001***
Deceased donation, n (%)	455 (100)	72 (100)	383 (100)	1
Cold ischemia time h:min*	9 h 27 min (567 min)	9 h 54 min (594 min)	9 h 12 min (554 min)	0.239
Induction IS, n (%)				
Lymphocyte depletion	189 (42)	72 (100)	117 (31)	<**0.001***
IL-2 receptor blockade	266 (58)	0 (0)	266 (69)	<**0.001***
Maintenance IS, n (%)				
Tacrolimus	396 (87)	72 (100)	324 (85)	<**0.001***
Everolimus	1 (0)	0 (0)	1 (0)	1
Ciclosporin	58 (13)	0 (0)	58 (15)	<**0.001***
MMF	372 (82)	22 (31)	350 (91)	<**0.001***
EC-MPA	81 (18)	49 (67)	32 (8)	<**0.001***
Azathioprine	2 (0)	1 (1)	1 (0)	0.291
Donor Characteristics				
Donor age, years*	52 (10–88)	34 (11–57)	55 (10–88)	<**0.001***
Donor male sex, n (%)	268 (59)	53 (74)	215 (53)	**0.006***
Immunocompatibility				
Total HLA Mismatches *	6 (0–10)	6 (2–10)	5 (0–10)	<**0.001***
Total PIRCHE-II Score*	71.32 (0–233.55)	60.495 (20.63–165.83)	73.47 (0–233.55)	**0.009***
PIRCHE-II A*	14.95 (0–62.52)	10.56 (0–48.59)	15.63 (0–62.52)	**0.008***
PIRCHE-II B*	14.72 (0–54.19)	11.16 (0.05–35.93)	15.09 (0–54.19)	0.088
PIRCHE-II C*	12.63 (0–75.06)	11.65 (0–50.00)	13.00 (0–75.06)	0.059
PIRCHE-II HLA I	13.85 (0–75.06)	11.32 (0–50.00)	14.62 (0–75.06)	0.059
PIRCHE-II DR*	12.00 (0–56.13)	11.26 (0–46.51)	12.00 (0–56.13)	0.999
PIRCHE-II DQ*	19.00 (0–80.60)	17.05 (0–52.84)	19.30 (0–80.60)	0.268
PIRCHE-II HLA II	14.16 (0–80.60)	14.07 (0–52.84)	14.20 (0–80.60)	0.237
Preformed DSA, n (%)[a]	139 (31)	16 (22)	123 (32)	0.124
HLA-A	42 (9)	3 (4)	39 (10)	0.122
HLA-B	31 (7)	3 (4)	28 (7)	0.448
HLA-Cw	25 (5)	1 (1)	24 (6)	0.153
HLA-DRB	39 (9)	2 (3)	37 (10)	0.064
HLA-DR51-53	30 (7)	5 (7)	25 (7)	0.800
HLA-DQB	56 (12)	9 (13)	47 (12)	1
HLA-DP	9 (2)	0 (0)	9 (2)	0.366
Persistence of preformed DSA after transplantation	74 (53)	13 (81)	61 (50)	0.606
HLA-A	6 (1)	1 (1)	5 (1)	1
HLA-B	2 (0)	0 (0)	2 (1)	1
HLA-Cw	11 (2)	1 (1)	10 (3)	1
HLA-DRB	9 (2)	1 (1)	8 (2.)	0.566
HLA-DR51-53	14 (3)	3 (4)	11 (3)	0.472
HLA-DQB	30 (7)	7 (10)	23 (6)	0.296
HLA-DP	2 (0)	0 (0)	2 (1)	1
Maximum peak of preformed DSA after transplantation*	—	1,610 (865–25,767)	1,353 (551–8,173)	—
HLA-A*	—	2,648 (600–2,855)	1,353 (509–10,299)	—
HLA-B*	—	1,092 (640–1,545)	1904 (551–7,386)	—
HLA-Cw*	—	5007 (5,007–5,007)	1,060 (518–5,348)	—
HLA-DRB*	—	1,352 (1,352–1,352)	1,009 (563–4,179)	—
HLA-DR51-53*	—	3,070 (1,448–4,089)	1,154 (682–16,815)	—
HLA-DQB*	—	1,610 (865–25,767)	1820 (516–21,358)	—
HLA-DP*	—	0	2,285 (852–8,173)	—

**median (range).*

[a]Preformed DSA: DSA against the current kidney/pancreas allograft with MFI>500 at any time before transplantation. Each percentage refers to the total number of patients in the SPKTRs/KTRs cohort. No cases of either preformed DSA, directed against HLA-DQA or HLA-DPA were identified among SPKTRs and KTRs.

TABLE 2 | Outcomes of SPKTRs and KTRs.

	Total (455)	SPKTRs (n = 72)	KTRs (383)	*P*-value
Pancreas allograft function/survival				
Pancreas allograft loss/use of insulin, n (%)	12 (3)	12 (17)	—	—
Time to pancreas loss, months*		6		
Cause of pancreas allograft loss	6 (1)	6 (8)	—	—
Thrombosis, n (%)	11 1 (0)	1 (1)	—	—
Leakage, n (%)	1 (0)	1 (1)	—	—
Steroid-induced diabetes mellitus, n (%)	4 (1)	4 (6)		—
Others/unknown, n (%)				
Pancreas retransplantation, n (%)	4 (1)	4 (6)	—	—
History of HbA1c, %				
1 year post-transplant *	—	5.3	—	—
2 years post-transplant *	—	5.3	—	—
Best value since transplantation *	—	4.9	—	—
BMI post-transplant, kg/m^2	25 (14.97–43.31)	23 (14.97–34.66)	—	—
Kidney allograft function/survival				
Kidney allograft loss, n (%)	22 (5)	1 (1)	21 (5)	0.226
dialysis treatment	68 (15)	4 (6)	64 (17)	**0.011***
patient's death	73 (16)	2 (3)	71 (19)	**<0.001***
Kidney retransplantation graft survival after the first transplantation	380 (84)	70 (97)	310 (81)	**<0.001***
Baseline creatinine 1-year post-transplant, μmol/L *	117 (48–626)	98.5 (57–380)	120 (48–626)	**<0.001***
Baseline proteinuria 1-year post-transplant, mg/day *	83 (0–1,693)	83 (0–780)	83 (0–1,693)	0.394
eGFR (CKD-Epi) at 1-year post-transplant, mL/min *	55 (6–120)	67 (15–116)	52 (6–120)	**<0.001***
Rejection in KTRs biopsy, n (%)	91 (20)	11 (15)	80 (21)	0.336
TCMR, n (%)	57 (13)	7 (10)	50 (13)	0.561
ABMR, n (%)	34 (7)	4 (6)	30 (8)	0.630
Steroid free at 1 year, n (%)	242 (53)	51 (78)	191 (50)	**0.0012***
De novo DSA, n (%)[a]	75 (16)	16 (22)	59 (15)	
HLA-A	14 (3)	2 (3)	12 (3)	—
HLA-B	17 (3)	5 (7)	12 (3)	—
HLA-Cw	3 (1)	0 (0)	3 (1)	—
HLA-DRB	27 (4)	6 (8)	21 (5)	—
HLA-DR51-53	15 (3)	4 (6)	11 (3)	—
HLA-DQB	48 (11)	12 (17)	36 (9)	—
HLA-DP	6 (1)	2 (3)	4 (1)	—
Peak of *de novo* DSA after transplantation*	1,638 (514–17,553)	1,550 (548–14,458)	2,188 (514–17,553)	—
HLA-A*	—	573 (511–788)	1,030 (551–9,549)	—
HLA-B*	—	1,443 (505–3,345)	809 (507–8,760)	—
HLA-Cw*	—	—	2,359 (724–4,527)	—
HLA-DRB*	—	796 (527–1,708)	762 (501–2,520)	—
HLA-DR51-53*	—	682 (549–1,408)	746 (515–16,082)	—
HLA-DQB*	—	1,383 (510–14,458)	1,867 (549–17,553)	—
HLA-DP*	—	1,076 (528–1,551)	955 (501–3,902)	—

**median (range).*

[a]De novo *DSA: DSA against the current kidney/pancreas allograft with MFI>500 at any time before transplantation. Each percentage refers to the total number of patients in the SPKTRs/KTRs cohort.* De novo *DSA against HLA-DQA were detected in 1 and 3 SPKTRs and KTRs, respectively, while* de novo *DSA against HLA-DPA were found in 0 and 1 SPKTRs and KTRs, respectively.*

Statistical Methods

Clinical characteristics are expressed as numbers (%) and were compared across groups using Fisher's exact test for categorical variables. Continuous variables are expressed as median (range: minimum-maximum) and were compared using Mann Whitney-U Test. Statistical analysis was performed using IBM SPSS Version 28.0.1.1. Survival was analyzed using the Kaplan-Meier method and compared with the LogRank test. Univariable and multivariable Cox proportional hazards models with the enter method were used to investigate factors associated with survival. Bonferroni adjustment was applied to account for multiple comparisons, restricting the correction to the analyses involving the different PIRCHE-II scores. Variables with a p-value ≤0.05 in the univariable analysis were included in the multivariable model. Statistical significance was assumed for a two-tailed p-value <0.05 for all tests.

RESULTS

Overall Patient Characteristics

Tables 1, **2** shows the clinical characteristics and outcomes of SPKTRs and KTRs. In the SPKTR cohort, all patients underwent

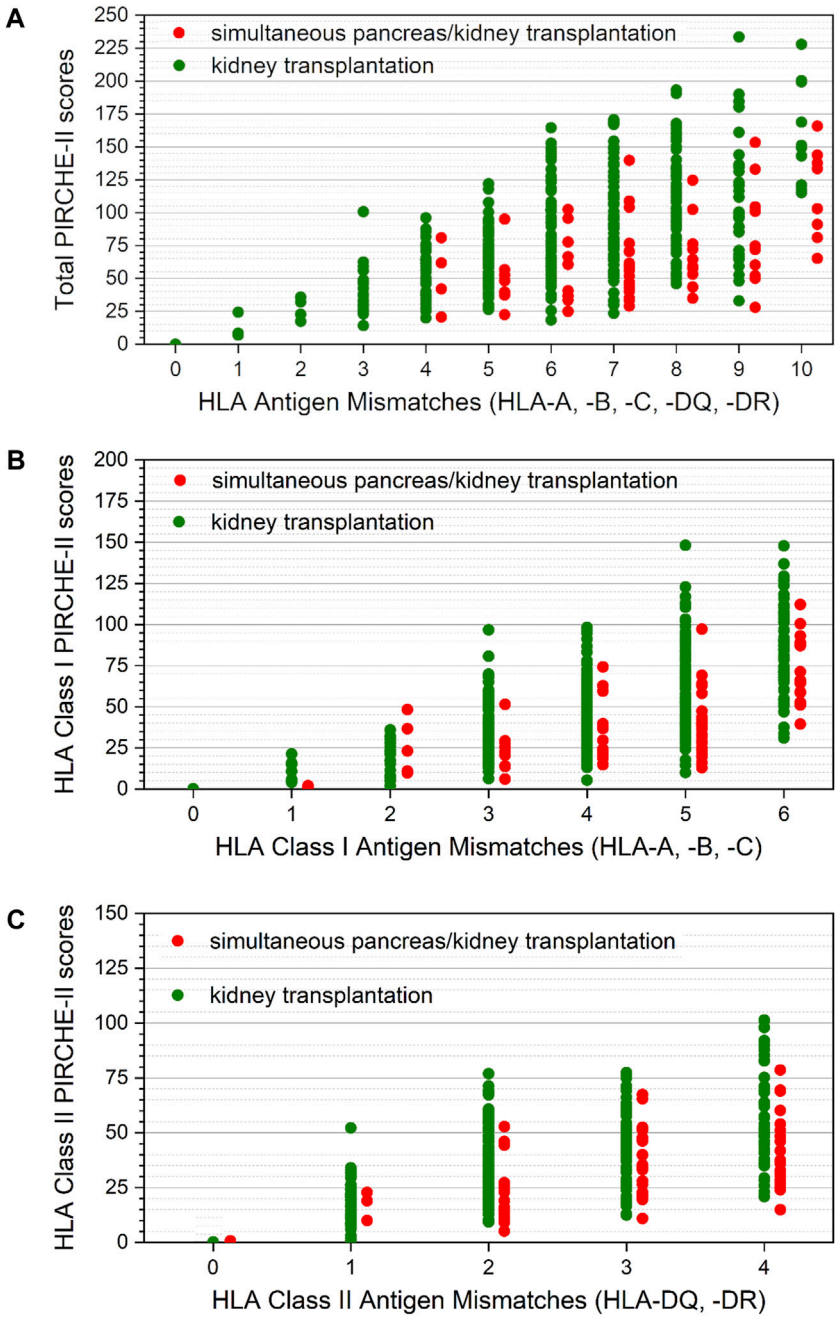


FIGURE 1 | (A) Distribution of total PIRCHE-II scores compared to total HLA-mismatches. Total PIRCHE-II scores and the number of HLA mismatches were calculated from HLA class I (HLA-A, B, C) and HLA class II (HLA-DQ, DR) mismatches. Median PIRCHE-II scores for SPKTRs (red) and KTRs (green) were 60.25 (IQR 43.29–92.08) and 73.19 (IQR 53.47–107.25), respectively. **(B)** Distribution of total PIRCHE-II scores for HLA-class I antigens compared to HLA-class I mismatches. PIRCHE-II scores for HLA-class I and the number of HLA-class I mismatches were calculated from HLA-class I (HLA-A, B, C) mismatches. Median PIRCHE-II scores for HLA-class I for SPKTRs (red) and KTRs (green) were 36.59 (IQR 21.64–58.75) and 43.76 (IQR 28.73–68.65), respectively. **(C)** Distribution of total PIRCHE-II scores for HLA-class II antigens compared to HLA-class II mismatches. PIRCHE-II scores for HLA-class II and the number of HLA-class II mismatches were calculated from HLA-class II (HLA-DQ, DR) mismatches. Median PIRCHE-II scores for HLA-class I for SPKTRs (red) and KTRs (green) were 26.92 (IQR 18.22–40.45) and 31.26 (IQR 18.16–47.38), respectively.

thymoglobulin induction immunosuppressive therapy and received tacrolimus as maintenance calcineurin inhibitor therapy. Moreover, none of the SPKTR patients received prednisone for maintenance therapy. Additionally, the donors' age in the SPKTR cohort was significantly younger than in the KTRS cohort.

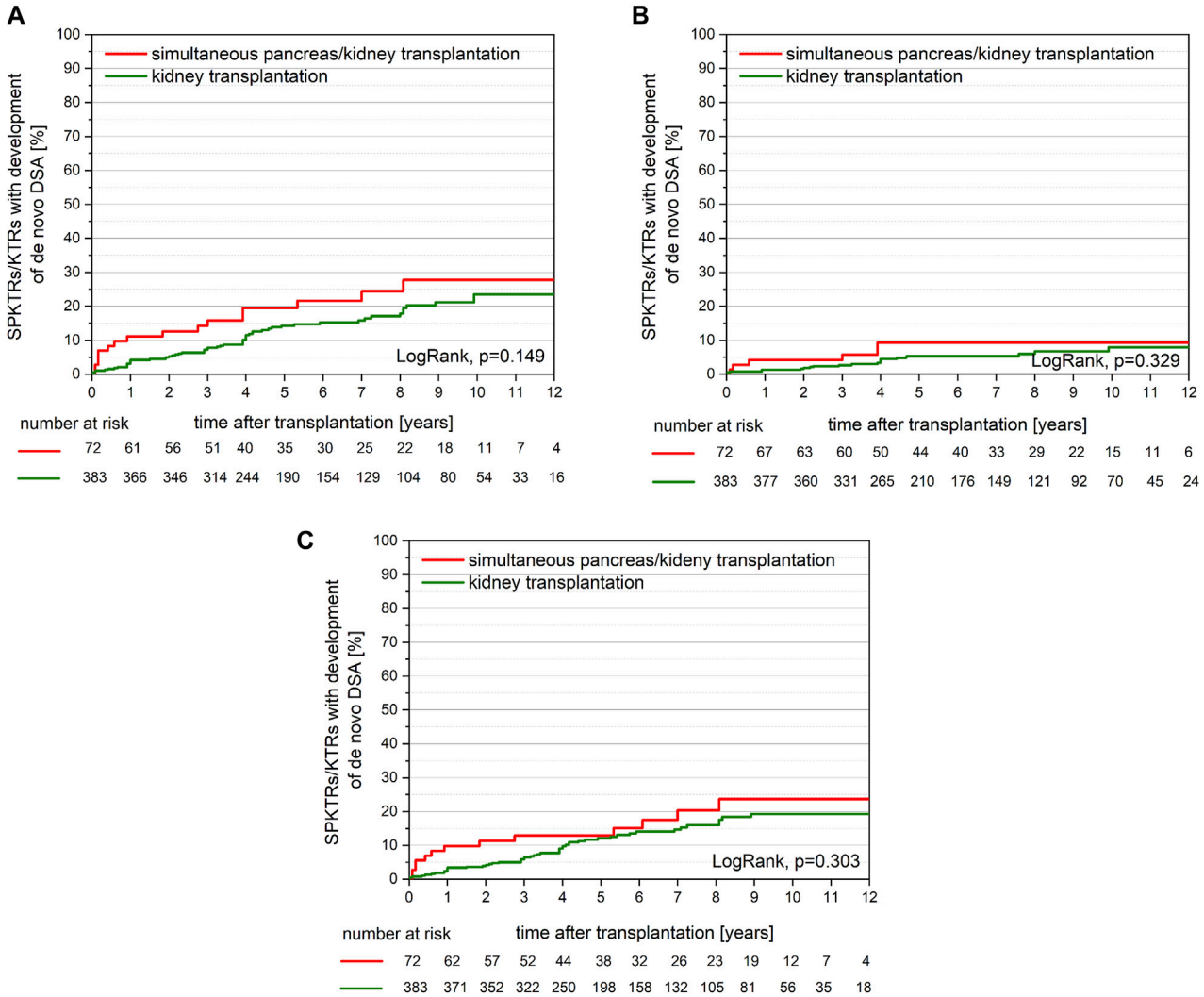


FIGURE 2 | (A) Development of *de novo* DSA was comparable between SPKTRs (red) and KTRs (green; $p = 0.149$) with 27.7% vs. 23.5% at 10 years post-transplant, respectively. Interestingly, *de novo* DSA at 1-year post-transplantation were detectable in 11.2% of SPKTRs vs. 3.1% of KTRs ($p = 0.011$). **(B)** Development of *de novo* DSA against HLA-class I was comparable between SPKTRs (red) and KTRs (green; $p = 0.329$) with 9.3% vs. 8.0% at 10 years post-transplant, respectively. *De novo* DSA at 1 year post-transplantation were detectable in 4.2% of SPKTRs vs. 1.3% of KTRs ($p = 0.086$). **(C)** Development of *de novo* DSA against HLA-class II was comparable between SPKTRs (red) and KTRs (green; $p = 0.303$) with 23.7% vs. 19.3% at 10 years post-transplant, respectively. Interestingly, *de novo* DSA at 1-year post-transplantation were detectable in 9.8% of SPKTRs vs. 3.4% of KTRs ($p = 0.012$).

The median total PIRCHE-II score of SPKTRs was 60.495 (range: 20.63–165.83), with PIRCHE-II for HLA-class I antigens of 36.56 (1.02–112.17) and PIRCHE-II for HLA-class II antigens of 26.92 (0–78.56; **Figures 1A–C**). Preformed DSA were detected in 16/72 (22%) SPKTRs, of which 9/72 (12.5%) SPKTRs showed preformed DSA against HLA-DQ.

The median total PIRCHE-II scores of KTRs was 73.47 (range: 0–233.55), with PIRCHE-II for HLA-class I antigens of 44.00 (0–148.23) and PIRCHE-II for HLA-class II antigens of 31.53 (0–101.82; **Figures 1A–C**). Preformed DSA were defined as DSA against graft(s) with MFI >500 at any time before transplantation. Preformed DSA were detected in 123 of 383 (32%) KTRs, of which 47 of 383 (12%) KTRs showed preformed DSA against HLA-DQ.

The median total PIRCHE-II score and the median PIRCHE-II score for HLA-class I antigens significantly differed between SPKTRs and KTRs (respectively, $p = 0.009$ and $p < 0.001$), while no significant difference was detected for PIRCHE-II Score for HLA class II antigens ($p = 0.526$).

Graft Outcome

During the observation period of 10 years, 4 of 72 (6%) SPKTRs died, 12 of 72 (17%) SPKTRs lost their pancreas allograft function, and 1 of 72 (1%) SPKTRs returned to dialysis. During the observation period of 10 years, 64 of 383 (17%)

TABLE 3 | Univariate and multivariate analysis of risk factors for the development of dnDSA at 1-year post-transplantation among SPKTRs (n = 72).

	Univariate	Multivariate		
	***P*-value**	**HR**	**CI 95%**	***P*-value**
PIRCHE-II HLA-A[§]	0.712	—	—	—
PIRCHE-II HLA-B[§]	0.158	—	—	—
PIRCHE-II HLA-C[§]	0.913	—	—	—
PIRCHE-II HLA-class I[§]	0.506	—	—	—
PIRCHE-II HLA-DR[§]	0.572	—	—	—
PIRCHE-II HLA-DQ[§]	0.655	—	—	—
PIRCHE-II HLA-class II[§]	0.559	—	—	—
Total PIRCHE-II[§]	0.403	—	—	—
Preformed DSA	0.017*	4.432	0.975–20.137	0.054
Recipient age	0.439	—	—	—
Donor age	0.100	0.969	0.907–1.034	0.339

[§]P-values were adjusted for multiple comparisons using the Bonferroni correction, with a corrected significance level of 0.0056 (0.05/8) applied to the analyses involving the different PIRCHE-II scores. P-values ≤ 0.00625 are considered statistically significant.

TABLE 4 | Univariate and multivariate analysis of risk factors for the development of dnDSA among SPKTRs overall (n = 72).

	Univariate	Multivariate		
	***P*-value**	**HR**	**CI 95%**	***P*-value**
PIRCHE-II HLA-A	0.241	—	—	—
PIRCHE-II HLA-B	0.249	—	—	—
PIRCHE-II HLA-C	0.399	—	—	—
PIRCHE-II HLA-class I	0.236	—	—	—
PIRCHE-II HLA-DR	0.246	—	—	—
PIRCHE-II HLA-DQ	0.284	—	—	—
PIRCHE-II HLA-class II	0.211	—	—	—
Total PIRCHE-II	0.179	—	—	—
Preformed DSA	**0.001***	**2.872**	**1.053–7.831**	**0.039***
Recipient age	0.208	—	—	—
Donor age	**0.004***	**0.943**	**0.899–0.990**	**0.017***

*[§]P-values were adjusted for multiple comparisons using the Bonferroni correction, with a corrected significance level of 0.0056 (0.05/8) applied to the analyses involving the different PIRCHE-II scores. P-values ≤ 0.00625 are considered statistically significant. * statistically significant.*

KTRs died, and 21 of 383 (5%) KTRs returned to dialysis, being not significantly different as compared to SPKTRs (p = 0.226). Data about the development of TCMR and ABMR between SPKTRs and KTRs are shown in **Supplementary Figures 1A, B**.

Development of dnDSA in SPKTRs and KTRs

Overall, SPKTRs showed a trend towards a higher incidence of dnDSA compared to KTRs over the whole observation period. 16/72 (22%) SPKTRs developed dnDSA (**Table 2**) as compared to 59 of 383 (15%) KTRs. Yet, within the first year post-transplantation 8/72 (11%) SPKTRs developed dnDSA as compared to 16/383 (4%) SPKTRs/KTRs (p = 0.011; **Figure 2A**). Both dnDSA directed against HLA-class I (4% vs. 1%, p = 0.086) and HLA-class II dnDSA (10% vs. 3%, p = 0.012) were more frequently observed in SPKTRs as compared to KTRs in the first post-transplant year (**Figures 2B, C**). However, this difference did only reach statistical significance for dnDSA directed against HLA-class II.

Risk Factors for the Development of dnDSA in SPKTRs

In our univariate analysis, PIRCHE-II scores per HLA locus, per HLA class, and total PIRCHE-II scores did not show an association with the development of dnDSA at 1 year post-transplantation or throughout the entire study period (**Tables 3**, **4**). Conversely, the presence of preformed DSA significantly increased the risk of developing dnDSA both at 1 year post-transplantation (HR 4.432, CI 0.975–20.137, p = 0.054) and over the entire study period (HR 2.872, CI 1.053–7.831, p = 0.039). Additionally, a younger donor age was associated with a higher incidence of dnDSA over the study period (HR 0.943, CI 0.899–0.990, p = 0.017).

Similarly, PIRCHE-II scores per HLA locus, per HLA class, and total PIRCHE-II scores were not linked to the development of dnDSA against HLA class I in the univariate analysis (**Table 5**).

TABLE 5 | Univariate and multivariate analysis of risk factors for the development of dnDSA against HLA-class I among SPKTRs (n = 72).

	Univariate	Multivariate		
	***P*-value**	**HR**	**CI 95%**	***P*-value**
PIRCHE-II HLA-A	0.318	—	—	—
PIRCHE-II HLA-B	0.276	—	—	—
PIRCHE-II HLA-C	0.900	—	—	—
PIRCHE-II HLA-class I	0.241	—	—	—
PIRCHE-II HLA-DR	0.609	—	—	—
PIRCHE-II HLA-DQ	0.830	—	—	—
PIRCHE-II HLA-class II	0.448	—	—	—
Total PIRCHE-II	0.699	—	—	—
Preformed DSA	0.688	—	—	—
Recipient age	0.577	—	—	—
Donor age	0.655	—	—	—

[§]P-values were adjusted for multiple comparisons using the Bonferroni correction, with a corrected significance level of 0.0056 (0.05/8) applied to the analyses involving the different PIRCHE-II scores. P-values ≤ 0.00625 are considered statistically significant.

TABLE 6 | Univariate and multivariate analysis of risk factors for the development of dnDSA against HLA-class II among SPKTRs (n = 72).

	Univariate	Multivariate		
	***P*-value**	**HR**	**CI 95%**	***P*-value**
PIRCHE-II HLA-A	0.825	—	—	—
PIRCHE-II HLA-B	0.603	—	—	—
PIRCHE-II HLA-C	0.244	—	—	—
PIRCHE-II HLA-class I	0.907	—	—	—
PIRCHE-II HLA-DR	0.096	0.998	0.936–1.064	0.947
PIRCHE-II HLA-DQ	0.044*	1.040	0.989–1.094	0.124
PIRCHE-II HLA-class II	0.034*	—	—	—
Total PIRCHE-II	0.301	—	—	—
Preformed DSA	**0.001***	**4.700**	**1.397–15.811**	**0.012***
Recipient age	0.252	—	—	—
Donor age	0.015*	0.963	0.914–1.014	0.152

*[§]P-values were adjusted for multiple comparisons using the Bonferroni correction, with a corrected significance level of 0.0056 (0.05/8) applied to the analyses involving the different PIRCHE-II scores. P-values ≤ 0.00625 are considered statistically significant. * statistically significant.*

TABLE 7 | Univariate and multivariate analysis of risk factors for the development of dnDSA among SPKTRs/KTRs (n = 455) stratified by time post-transplantation (≤1 year post-transplant and >1 year post-transplant).

	Time interval	Univariate	Multivariate		
		P-value	HR	CI 95%	*P*-value
PIRCHE-II HLA-A[§]	≤1 year	0.350	—	—	—
	>1 year	0.125	—	—	—
PIRCHE-II HLA-B[§]	≤1 year	0.169	—	—	—
	>1 year	0.506	—	—	—
PIRCHE-II HLA-C[§]	≤1 year	0.192	—	—	—
	>1 year	0.503	—	—	—
PIRCHE-II HLA-class I[§]	≤1 year	0.689	—	—	—
	>1 year	0.516	—	—	—
PIRCHE-II HLA-DR[§]	≤1 year	0.130	—	—	—
	>1 year	0.115	—	—	—
PIRCHE-II HLA-DQ[§]	≤1 year	**0.033**	**1.038**	**1.011–1.066**	**0.011***
	>1 year	**0.008**	**1.023**	**1.008–1.038**	**0.025***
PIRCHE-II HLA-class II[§]	≤1 year	0.027	—	—	—
	>1 year	0.018			
Total PIRCHE-II[§]	≤1 year	0.130	—	—	—
	>1 year	0.080			
Simultaneous pancreas/kidney transplantation (SPKT)	≤1 year	0.015*	1.020	0.996–1.043	0.765
	>1 year	0.978	—	—	—
Preformed DSA	≤1 year	0.297	—	—	—
	>1 year	0.894	—	—	—
Interaction (SPKT x preformed DSA)	≤1 year	<0.001*	2.361	0.658–8.468	0.188
	>1 year	**0.030***	**2.782**	**1.061–7.294**	**0.037***
Recipient age	≤1 year	0.235	—	—	—
	>1 year	0.078	1.003	0.996–1.011	0.403
Donor age	≤1 year	**0.001***	**0.965**	**0.943–0.988**	**0.003***
	>1 year	0.013*	0.987	0.973–1.002	0.189
T-cell depleting induction	≤1 year	0.014*	0.516	0.234–1.137	0.101
	>1 year	0.821	—	—	—
Type of calcineurin inhibitor (Ciclosporin)	≤1 year	0.216	—	—	—
	>1 year	**0.004***	**2.440**	**1–464–4.069**	**<0.001***

[§]*P-values were adjusted for multiple comparisons using the Bonferroni correction, with a corrected significance level of 0.0056 (0.05/8) applied to the analyses involving the different PIRCHE-II scores.* P-*values ≤ 0.00625 are considered statistically significant.*

** statistically significant.*

However, in the univariate analysis, PIRCHE-II scores per HLA locus DQ were associated with an increased risk of developing dnDSA against HLA class II (p = 0.044). Multivariate analysis revealed that only preformed DSA remained independently associated with an increased risk of developing dnDSA against HLA class II (HR 4.700, CI 1.397–15.811, p = 0.012, **Table 6**).

Risk Factors for the Development of dnDSA in SPKTRs/KTRs

Among the whole cohort SPKTRs/KTRs multivariate analysis revealed that PIRCHE-II scores for HLA locus DQ and younger donor age were significantly associated with the development of dnDSA at 1 year post-transplantation (HR 1.038, CI 1.0011–1.066, p = 0.011; HR 0.965, CI 0.943–0.988, p = 0.003) and HLA locus DQ was significantly associated with the development of dnDSA after 1 year post-transplantation (HR 1.023, CI 1.008–1.038, p = 0.025) (**Table 7**). Additionally, using ciclosporin for maintenance immunosuppression was associated with an increased risk of developing dnDSA after 1 year post-transplantation (HR 2.440, CI 1.464–4.069, p < 0.001). Simultaneous pancreas-kidney transplantation (SPK) was not associated with dnDSA development in the multivariate analysis. However, SPK and the presence of preformed DSA independently increased the risk for the development of dnDSA after 1-year post-transplantation (HR 2.782, CI 1.061–7.294, 0.037).

DISCUSSION

A well-established correlation has been suggested in kidney transplantation between a higher number of HLA epitope mismatches [17] and an increased risk of developing dnDSA associated with AMR and allograft loss [18–21]. Lachmann et al. revealed in their paper a strong correlation between the total PIRCHE-II score (considering HLA-locus A, HLA-locus B, HLA-locus C, HLA-locus DR, HLA-locus DQ) and an increased risk of development dnDSA, primarily directed against HLA-DQ, followed by HLA-DR, HLA-A, and HLA-B mismatches. This was confirmed in subsequent studies [22, 23].

In contrast to the investigations carried out in a kidney transplantation cohort, the PIRCHE-II scores' prognostic value in predicting dnDSA development and graft outcomes following

other solid organ transplantations is not well studied. Particularly in pancreas transplantation, data regarding the relevance of PIRCHE-II scores is scarce. Based on suggested risk assessment according to the recently published First World Consensus Conference on pancreas transplantation [1, 2], less importance has been attributed to HLA mismatching and preformed DSA. In this context, our study aims to evaluate risk factors for developing dnDSA among SPKTRs. To the best of our knowledge, this study represents the first effort to investigate the influence of the PIRCHE-II scores, adjusted for both HLA class I and II, and HLA locus-specific, on the development of dnDSA in a cohort of SPKTRs.

Firstly, our results indicate that, despite SPKTRs having fewer preformed DSA and lower median total PIRCHE-II scores, there was a higher incidence of dnDSA against HLA class I and II in the first post-transplant year compared to KTRs. Notably, dnDSA were predominantly directed against the HLA-locus DQ, consistent with previous studies [24]. Several factors contribute to this finding. The pancreas allograft is a highly immunogenic organ, and its beta cells can prompt a strong alloimmune response, contributing to a higher incidence of dnDSA development. Pancreatic inflammation and injury, common in the early post-transplant period, can further activate alloreactivity, leading to the development of dnDSA as the immune system reacts to the inflamed or injured pancreatic tissue. Although SPKTRs receive more intense induction immunosuppressive therapy, rapid steroid withdrawal might not be suitable for all SPKTRs and allow DSA development. Given the observed differences in alloreactivity during the early post-transplant period, it is particularly crucial to study the impact of PIRCHE-II scores on dnDSA formation in SPKTRs. To reduce the potential risk of overestimation of dnDSA with an MFI cut-off of 500, all dnDSA were analyzed individually by a specialist in transplantation immunology in a blinded fashion. Here, 1) analyzing the pattern of single-bead reactivity and comparing it to the HLA typing of the donor, 2) investigating for epitope specificity to determine alpha chain binding antibodies in the setting of HLA-DQ and DP, 3) determining unspecific reactivity by comparing the pattern of reactivity to lot-specific reactivity patterns in non-immunized males that are continuously tracked in our transplant laboratory, and 4) incorporating the reactivity to the recipient's own HLA antigens was applied to reduce overestimation.

Secondly, regarding the risk factors associated with the development of dnDSA in SPKTRs, the presence of preformed DSA and younger donor age were independently associated with an increased risk. Interestingly, total PIRCHE-II scores, PIRCHE-II scores per HLA class, and PIRCHE-II scores per HLA locus were not independently associated with an increased risk for the development of dnDSA. However, PIRCHE-II scores for HLA class II, particularly HLA-locus DQ, may predict the development of dnDSA against HLA class II, although the sample size in our analysis was not sufficient to show this association independently upon multivariate analysis. This finding aligns with the observation that dnDSA against HLA-locus DQ exhibited the highest incidence among all HLA loci. Conversely, the lack of association for HLA class I is likely attributable to the low incidence of dnDSA against HLA class I and the small sample size of our cohort. Nonetheless, our results highlight other factors associated with the development of dnDSA that should be considered in future studies when evaluating the predictive and additive value of PIRCHE-II scores.

Notably, preformed DSA increased the risk of dnDSA development among SPKTRs but not KTRs. Factors associated with SPKT, preformed DSA, and the combination of both may likely explain this elevated risk of dnDSA development. Factors associated with SPKT include 1) the transplantation of two organs, which presents more allo-antigens compared to kidney transplantation alone, and 2) the pancreas as a highly vascularized organ, that may provoke a stronger alloimmune response compared to the kidney alone. Factors associated with preformed DSA include 1) the presence of an already activated immune system with presence of memory B cells, that may get stimulated by the increased antigen load in SPKT, and 2) a high number of shared HLA-epitopes, that contribute to the potential of HLA antibodies cross-reacting with other HLA antigens [25]. Our results suggest, that the combination of SPKT and preformed DSA is decisive for the increased risk of dnDSA development. 1) Preformed DSA may precipitate subclinical or clinical TCMR and AMR, particularly in the pancreas allograft, which can further induce an inflammatory microenvironment that may stimulate antigen presentation and immune activation, leading to dnDSA development. 2) The rapid steroid withdrawal in SPKTRs may also be critical in cases with preformed DSA, facilitating this inflammatory microenvironment that may allow the formation of dnDSA. Organs from younger donors tend to have higher immunogenicity due to increased expression of HLA antigens and costimulatory molecules. Additionally, the presence of more active dendritic cells and other antigen-presenting cells, coupled with increased cellular proliferation, can enhance antigen exposure and the recipient's immune activation, thereby increasing the risk of DSA formation.

Thirdly, when considering the entire cohort of SPKTRs/KTRs, simultaneous pancreas/kidney transplantation did not independently increase the risk of developing dnDSA. However, our data demonstrated an association between HLA epitope mismatching and dnDSA development, consistent with the literature [15]. Unlike previous studies, we observed the most pronounced association between PIRCHE-II scores for HLA-locus DQ and the development of dnDSA against HLA-class II. Ladowski et al. reported similar results but primarily focused on PIRCHE-II for HLA class II, especially HLA-DQB mismatches [24]. The concept of HLA epitope mismatch load and the impact of dnDSA is most clearly shown for HLA-locus DQ. It remains unknown whether the number of HLA epitope mismatches or the increased likelihood of more immunogenic HLA epitopes contributes to this increased risk [26]. Current evidence suggest HLA-DQ combinations that are more immunogenic than others [27].

To our knowledge, we are among the first to include PIRCHE-II for both HLA-DQB and HLA-DR in the SPKTRs population and demonstrate their role in predicting dnDSA formation against both HLA class I and II. Additionally, Chaigne et al. demonstrated in a cohort of pancreas recipients that the

formation of anti-HLA class I antibodies was unrelated to PIRCHE-II scores. In contrast, the development of anti-HLA class II antibodies was influenced by PIRCHE-II scores [28]. We did not observe an association between PIRCHE-II scores for HLA-locus C, which may explain the lack of significance for total PIRCHE-II scores in our cohort. This finding aligns with previous studies, such as those by Lachmann et al., who also considered HLA-A, B, C, DR, and DQ when calculating the PIRCHE-II score [15]. Thus, our observation highlights the lack of association with the PIRCHE-II score for HLA-C, which can potentially contribute to misleading interpretations of total PIRCHE-II scores. Moreover, we observed an increased risk of dnDSA with the use of ciclosporin and lower donor age [29]. These findings from our multivariate analysis are significant because the two most cited studies, by Lachmann et al. [15] and Unterrainer et al [30], are based on data from patients primarily under ciclosporin-based immunosuppression and with incomplete recipient and donor typing. These earlier studies may not fully reflect the current state of transplantation practices.

Our study possesses several strengths. First, it stands as one of the first analyses focusing on the development of dnDSA in SPKTRs compared to KTRs. Second, we included a well-characterized cohort of SPKTRs spanning over a decade, adhering to a standardized immunosuppressive protocol without the use of ciclosporin, and maintained close clinical and immunological post-transplant monitoring, thus providing high data density. Third, our study explored, for the first time in SPKTRs, the total PIRCHE-II scores, PIRCHE-II scores per HLA-class, and PIRCHE-II scores per HLA-locus. Yet, there are also limitations warranting consideration. Most importantly, the retrospective study design, small sample size and the single-center bias concerning allograft allocation, immunosuppressive strategy based on a steroid-free immunosuppression regimen in SPKTRs. Our study also relied on imputed high-resolution HLA alleles for the PIRCHE-II calculation, which could potentially influence our results due to errors in the imputation. The population in our study was predominantly Caucasian and a recent study has suggested that the potential difference in PIRCHE-II score association with dnDSA development in this setting would be minimal [31].

In summary, SPKTRs exhibit a higher incidence of *de novo* dnDSA in the first year post-transplantation, which is not linked to an increased HLA-epitope mismatch load. The correlation with preformed DSA indicates a higher immunologic risk, particularly under a steroid-free regimen, favoring dnDSA development. Over the long term, a high HLA-epitope mismatch load for HLA locus DQ is similarly crucial for dnDSA development in both SPKTRs and KTRs. The lack of association between the total PIRCHE-II score, PIRCHE-II scores for HLA classes, and other HLA loci suggests that these biomarkers should at the moment not be used for risk stratification post-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 Cantonal Ethic Commission Review Board of Zurich, Switzerland (KEK-ZH Number 2020-02817) and has complied with the Declaration of Helsinki. 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

FR: participated in data collection, participated in data analysis, participated in writing the paper. LF and JN: participated in data collection, participated in writing the paper. FR, CC-W, and SvM: participated in review and editing. TS: participated in research design, participated in data collection, participated in data analysis, participated in writing of the paper. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

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

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2024.13720/full#supplementary-material

Supplementary Figure S1 | (A) Development of TCMR between SPKTRs (red) and KTRs (green; p = 0.415) with 14.2% vs. 10.6% at 10 years post-transplant, respectively. **(B)** Development of ABMR was comparable between SPKTRs (red) and KTRs (green; p = 0.447) with 8.1% vs. 11.1% at 10 years post-transplant, respectively.

Supplement Figure S2 | Development of *de novo* DSA against HLA-class II DP was comparable between SPKTRs (red) and KTRs (green; p = 0.184) with 8.7% vs. 5.8% at 10 years post-transplant, respectively. *De novo* DSA at 1 year post-transplantation were detectable in 1.4% of SPKTRs vs. 0.8% of KTRs (p = 0.606).

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ORIGINAL RESEARCH
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A Multidrug Donor Preconditioning Improves Steatotic Rat Liver Allograft Function and Recipient Survival After Transplantation

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The scarcity of donors has prompted the growing utilization of steatotic livers, which are susceptible to injuries following orthotopic liver transplantation (OLT). This study aims to assess the efficacy of multidrug donor preconditioning (MDDP) in alleviating injuries of steatotic grafts following rat OLT. Lean rats were subjected to a Western-style diet with high-fat (HF) and high-fructose (HFr) for 30 days to induce steatosis. Both lean and steatotic livers were implanted into lean recipients fed with a chow diet after OLT. The HF + HFr diet effectively elevated blood triglyceride and cholesterol levels and induced fat accumulation in rat livers. Our results demonstrated a significant decrease in alanine aminotransferase levels ($p = 0.003$), aspartate aminotransferase levels ($p = 0.021$), and hepatic Suzuki scores ($p = 0.045$) in the steatotic rat liver allograft group following MDDP treatment on post-operation day (POD) 7. Furthermore, the survival rates of steatotic rat liver allografts with MDDP (19/21, 90.5%) were significantly higher than those in the steatotic control (12/21, 57.1%, $^*p = 0.019$). These findings indicate that MDDP treatment improves steatotic rat liver allograft function and recipient survival following OLT.

Keywords: allograft function, donor shortage, rat steatotic liver donor, ischemia reperfusion injury, multidrug donor preconditioning, orthotopic liver transplantation

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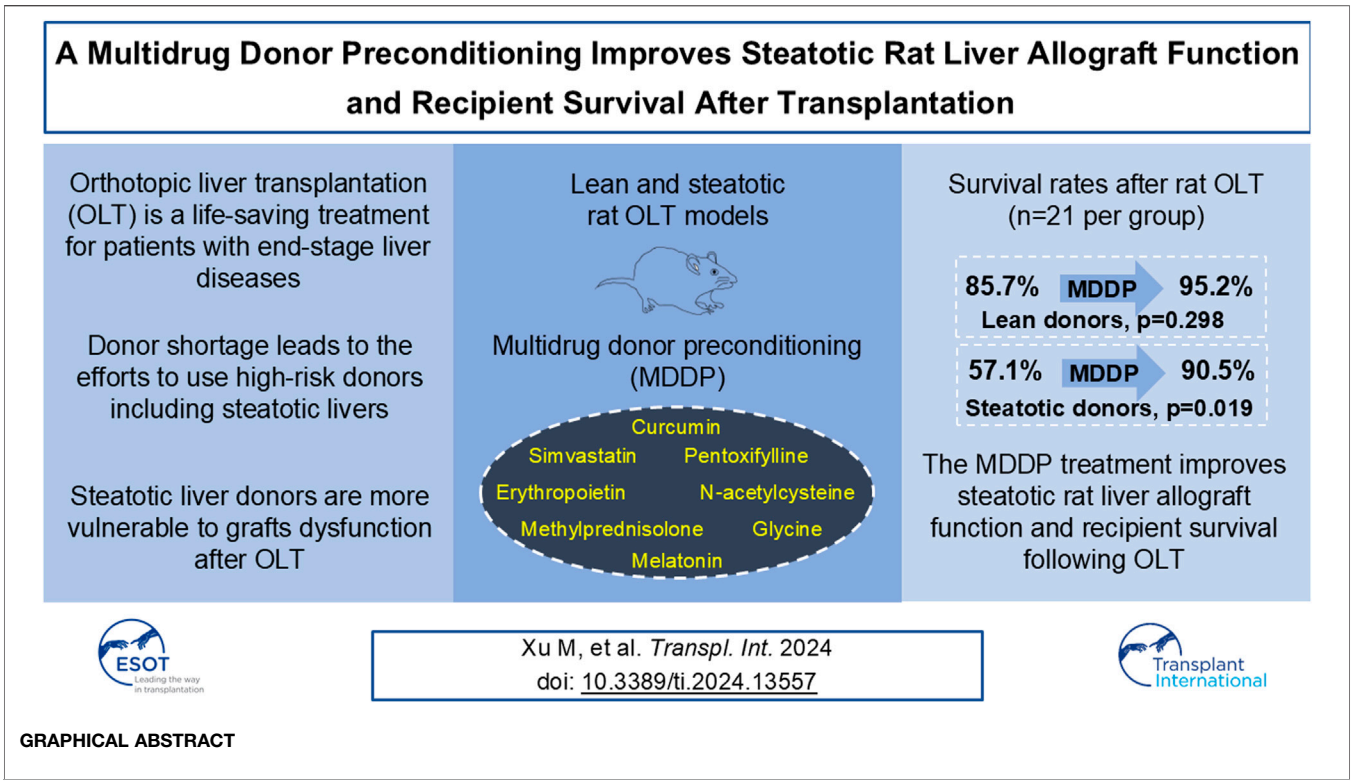
INTRODUCTION

Orthotopic liver transplantation (OLT) stands as a crucial life-saving intervention for individuals suffering from end-stage liver disease. However, its widespread application is constrained by the scarcity of donors, a challenge exacerbated by the growing number of patients awaiting transplantation [1]. To alleviate this issue, marginal donors, including those with steatotic livers, advanced age, or prolonged ICU stays, have been increasingly considered for transplantation [2]. Nonetheless, these marginal donors are particularly susceptible to ischemia-reperfusion injury (IRI),

Abbreviations: OLT, Orthotopic liver transplantation; MDDP, Multidrug donor preconditioning; IRI, Ischemia-reperfusion injury.



GRAPHICAL ABSTRACT

which triggers the generation of free radicals upon liver reoxygenation, leading to lipid peroxidation and hepatocellular damage, with cold ischemia contributing to endothelial cell injury [3].

Metabolic dysfunction-associated steatotic liver disease (MASLD) has become a prevalent global health concern, propelled by factors such as sedentary lifestyles and consumption of high-fat and fructose-rich diets [4]. Western societies exhibit a heightened risk of MASLD due to dietary habits characterized by high fat and glucose intake, with fructose recently implicated in the development of necrotic inflammation and fibrosis in nonalcoholic steatohepatitis [5]. MASLD prevalence ranges from 10% to 30%, with rates soaring to 75%–92% among obese populations [6]. The escalating incidence of hepatic steatosis in the general populace is mirrored in the pool of potential liver donors. However, due to heightened vulnerability to hypoxia-reperfusion injury, hepatocytes laden with fat present a considerable challenge when utilized as donor allografts [7, 8]. Various animal models, such as the Lieber-DeCarli diet model [9] and fructose model [10–12], have been employed to study MASLD.

Multidrug treatment strategies have demonstrated efficacy in managing complex conditions such as HIV, cancer, and ischemic injury. These approaches have been extended to recondition liver donors to mitigate hepatic fat content and IRI ahead of transplantation. Over the past decades, several agents have been incorporated into perfusion and preservation solutions to reduce IRI risk in fatty liver donor rat OLT models, including melatonin [13], Treprostinil [14], carvedilol [15], cyclic RGD peptide [16], IL-6 [17], among others. Additionally, a multidrug cocktail comprising curcumin, simvastatin, N-acetylcysteine, erythropoietin, pentoxifylline, melatonin, glycine, and methylprednisolone has exhibited promise in diminishing IRI in fatty liver donors *in vitro* liver machine perfusion studies [18, 19]. Furthermore, the multidrug treatment approach has been implemented to decrease rat hepatic fat content during *ex vivo* normothermic machine perfusion for potential implantation [20, 21]. This indicates a promising method to broaden the liver donor pool by facilitating the utilization of steatotic livers while mitigating associated risks.

In this study, rats were fed either a standard chow diet or a Western-style diet rich in high-fat and fructose content to induce hepatic steatosis. Lean and steatotic liver allografts, with or without multidrug donor preconditioning (MDDP), were subsequently transplanted into lean recipients to investigate their effects on IRI. Our findings indicate that MDDP treatment effectively improved the transaminase levels of steatotic rat allograft on POD7 and recipient survival following rat OLT.

MATERIALS AND METHODS

The Ethics and Source of Animal Used in the Present Study

Male Sprague-Dawley rats weighing between 270 and 350 grams were procured from Charles River, Sulzfeld, Germany. These rats were accommodated in conventional cages under standard

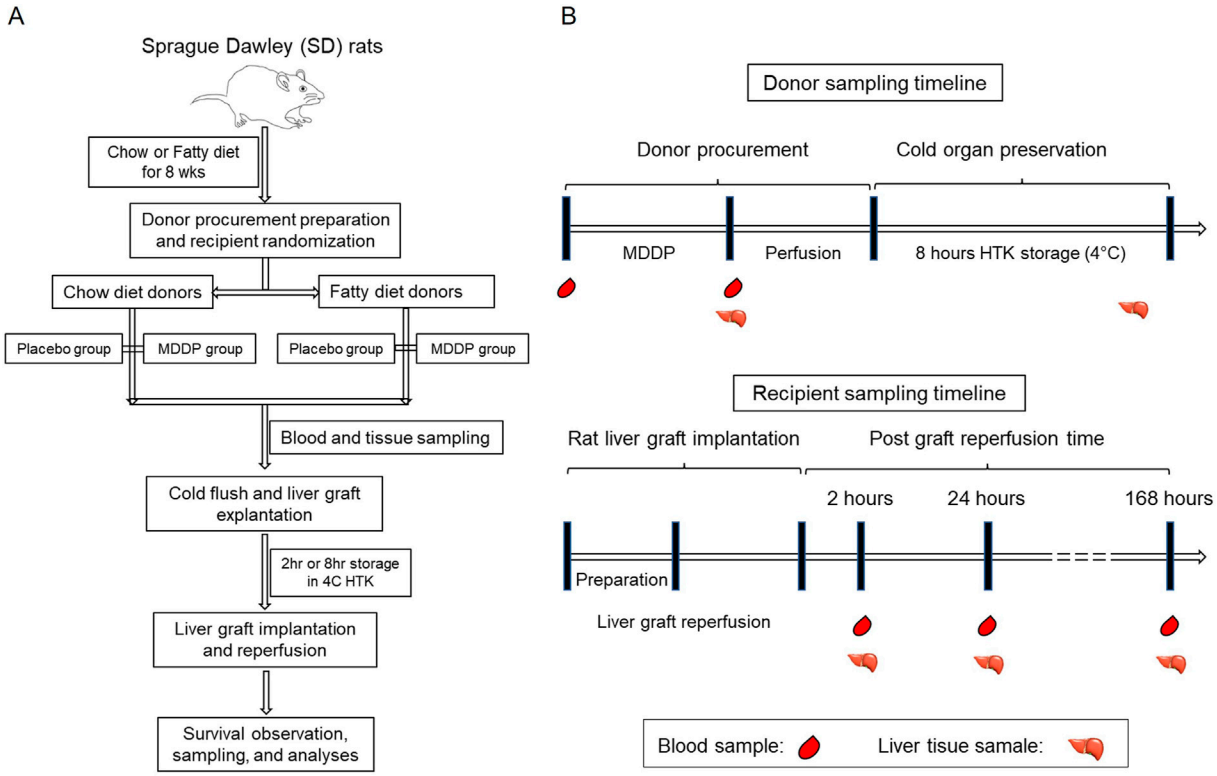


FIGURE 1 | Overall experimental design of the present study. **(A)** The flow chart of rat fatty liver model fed with chow or high fructose + high fat (HF + HFr) diets, multidrug donor preconditioning (MDDP) treatment, and rat orthotopic liver transplantation (OLT). **(B)** The rat blood and liver samples collection plan.

laboratory conditions at a temperature of 23°C ± 2°C, with a 12-hour light-dark cycle, ensuring their welfare and care aligned with the principles outlined in the "Guide for the Care and Use of Laboratory Animals" by the National Academy of Sciences, as published by the National Institutes of Health. Ethical approval for all experiments was obtained following the regulations and guidelines of the Georg-August-University of Göttingen (UMG).

Western-Style Food and the Development of Rat Fatty Liver Model

Rats were randomly divided into two dietary groups: a normal chow food group and a high-fat and fructose (Lieber-DeCarli, LDC) diet group. Approximately 90% of the total energy intake (J) was derived from the LDC diet, with the remaining 10% J coming from fructose, replacing a portion of the maltodextrin included in the LDC diet. The LDC diet, provided in powder form, was obtained from Ssniff Spezialdiaeten GmbH, Soest 59494, Germany. The proportions of energy from protein, fat, and carbohydrates were consistent with our previous reports [22, 23]. Animals were provided with pre-weighed food in bottles *ad libitum* for a period of 30 days.

Various combinations of cold ischemia time (CIT) and graft perfusion site were chosen to induce specific degrees of graft injury while maintaining an acceptable survival rate. This was done with the aim of developing an optimized model for steatotic rat liver transplantation for further research. The rats were divided into four groups: lean liver donors, lean donors with multidrug donor preconditioning (MDDP) treatment, fatty liver donors, and fatty liver donors with MDDP treatment (**Figure 1A**).

The Perioperative Donor Multidrug Donor Preconditioning (MDDP), Procurement, and Cold Storage

The animal underwent anesthesia with a flow rate of 1.5 L/min oxygen and 4% Sevoflurane for induction, followed by maintenance with 2% Sevoflurane. The conception of MDDP was derived from our former colleagues (Prof. Dr. Kollmar, et al) in two *ex vivo* studies. Specifically, simvastatin was utilized to lower hepatic cholesterol levels by inhibiting HMG-CoA reductase and increasing eNOS and heme oxygenase 1 expression [24]; curcumin [25], N-acetylcysteine [26], erythropoietin [27], and melatonin [28] acted against oxidation; erythropoietin [27], pentoxifylline [29], and glycine inhibited cytokine [30] release; curcumin [31], erythropoietin [27], and pentoxifylline [32] inhibited apoptosis; and methylprednisolone inhibited inflammation at various stages [33, 34]. Details regarding the routes and timing of MDDP treatment are outlined in **Table 1**.

TABLE 1 | The detail of multidrug donor preconditioning (MDDP) in the present study. The MDDP dosage, administration routes, and timing, together with the potential mechanisms of action.

Medication	Dosage	Administration route	Administration time	Mechanism of action
Curcumin	50 mg/kg	Intragastric (i.g.)	30 min prior liver HTK cold flush (4°C)	Anti-oxidation and apoptosis; activates HSP
Simvastatin	5 mg/kg			HMG-CoA reductase inhibitor, lowering hepatic cholesterol
N-acetylcysteine	150 mg/kg	Intraperitoneal (i.p.)		Anti-oxidation
Erythropoietin	3000 IU/kg			Inhibit oxidation, apoptosis, and TNFα production, stimulating eNOS expression
Pentoxifylline	50 mg/kg			Inhibits TNFα, Leukocytes recruitment, and apoptosis
Melatonin	10 mg/kg			Anti-oxidation
Glycine	100 mg/kg	Intravenous (i.v.)	10 min prior to liver HTK cold flush (4°C)	Attenuates Kupffer cell activation
Methylprednisolone	5 mg/kg			Anti-inflammation

The animal's abdomen was shaved and secured to the operating table with tape. A transverse and midline incision was made in the abdomen to fully expose the liver. A bile duct stent was inserted, and branches of the portal vein (PV) and right adrenal vein/renal artery/renal vein were ligated. The inferior caudate lobe was ligated using 6-0 silk thread, resected, and then fixed in 10% neutral formalin for histological analysis. Subsequently, the abdominal artery (AA) was dissected, and 300 units of heparin were injected through the dorsal vein of the penis. Following this, 20 mL of HTK solution (Custodiol) was infused through the AA under a pressure of 10 cm H_2O until the entire liver turned uniformly yellow. The excised livers were stored in 4°C HTK solution for 2 or 8 h prior to implantation. During cold storage, cuffs were made for the PV and infrahepatic inferior vena cava (IHIVC).

Rat Orthotopic Liver Implantation, Samples Collection, *In Vivo* Microscopic Study, and Recipient Survival Follow-Up

The procedure for preparing and exposing the recipient's liver followed similar steps as described for the donor operation. Once the liver was completely freed, 3 mL of normal saline and 10 units of heparin were injected intravenously. Concurrently, livers from the donors were flushed with 10 mL of normal saline. The IHIVC, PV, and suprahepatic inferior vena cava (SHIVC) were occluded. The SHIVC was anastomosed by running a 7-0 suture. Cuffs for the PV and IHIVC were inserted into the recipient's vessels and secured with circumferential 6-0 silk sutures. The bile duct cannula of the graft was also connected. The abdominal cavity was flushed with 42°C normal saline, and a running suture was used to close it. Post-surgery, hydration of recipients, volume supplementation, and warm-up procedures were considered critical, as described in previous studies [35]. Additionally, metabolic acidosis was observed after the operation due to the clamping of the portal vein and inferior hepatic vena cava. To address this, recipient rats in this study were administered a dose of intravenous 0.5 mL bicarbonate along with 1.5 mL normal saline to improve their behavior. Subcutaneous injection of analgesia (Buprenorphine, 0.05 mg/kg) continued until the 3rd day post-operation, and a solution of 1 mL metamizole was added to the drinking water (100 mL) until the 7th day post-operation. Samples were collected as depicted in **Figure 1B**. The *in vivo* microscopic study was conducted as previously described [36] on post-operation day (POD) 1, and recipient survival status was checked daily until POD 7 after OLT. All recipient rats were fed a chow diet following transplantation.

Blood Chemistry Assays for the Liver Function Panel

The measurement of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) activity, serving as biomarkers for liver injury, was conducted in the core laboratory of our institute. Furthermore, markers for biliary injury and obstruction, such as γ-glutamyl transpeptidase (GGT) and alkaline phosphatase (ALP) activity (non-specific), along with liver function tests (albumin and bilirubin levels, and prothrombin time), were analyzed. Additionally, serum samples were assessed for total lipid profile (triglycerides, HDL-cholesterol, LDL-cholesterol) and ferritin levels during the first 7 days post-transplantation using automated systems in the Department of Clinical Chemistry at the University Medical Center Göttingen, Germany.

Histopathological Studies of Hepatic Steatosis and Reperfusion Injuries and Image Interpretation

The collected liver tissue was Formalin-fixed and paraffin-embedded (FFPE). Subsequently, the FFPE tissue specimens were sectioned into serial slices measuring 5 μm in thickness using a microtome. These sections were then deparaffinized in xylene, followed by rehydration through a graded series of ethanol, and subsequently stained with H&E. After mounting using xylene-based media, the slides were examined under a light microscope (Olympus BX43) equipped with an internal digital camera (Olympus DP21). Hematoxylin stained the nuclei blue-purple, while the cytoplasms were nonspecifically counterstained pink-red with eosin. The H&E-stained liver sections were evaluated for steatosis, hepatic vacuolization, apoptosis, and necrosis in a blinded manner.

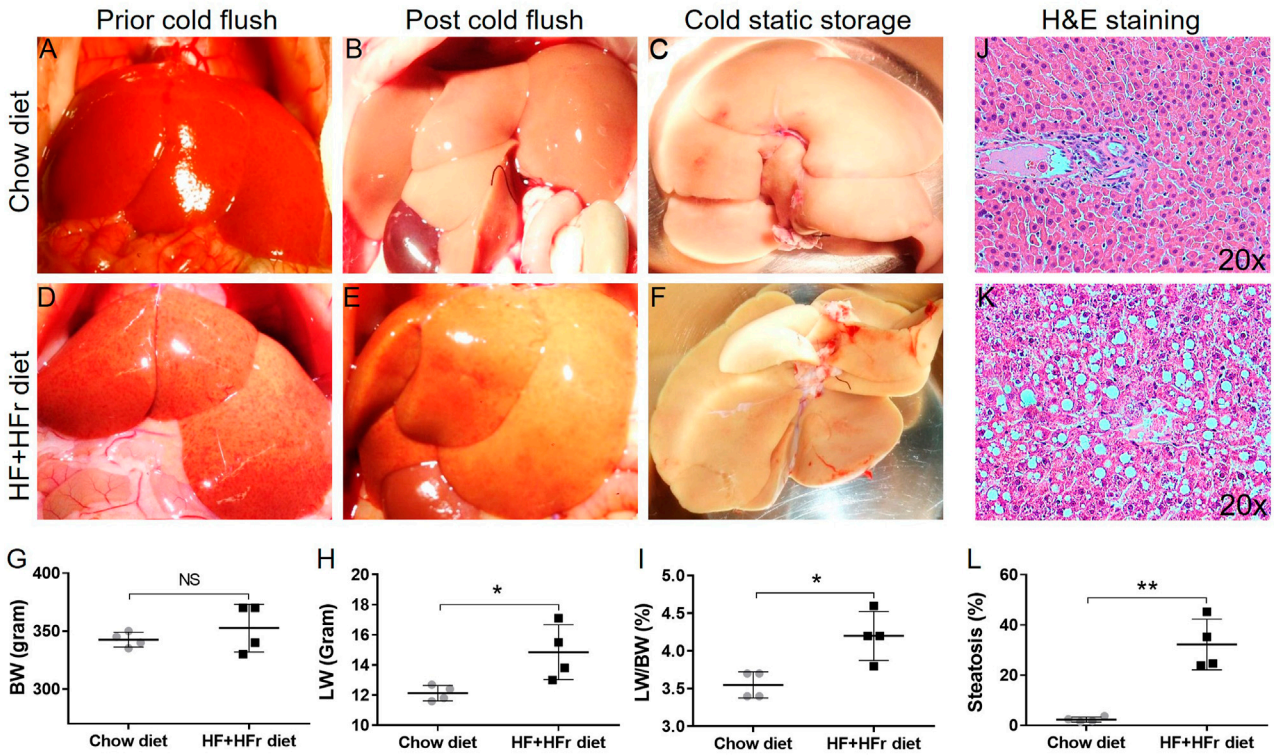


FIGURE 2 | Macroscopic and microscopic features of rat liver allografts fed with chow and HF + HFr diets. The gross appearance of lean **(A–C)** and steatotic **(D–F)** rat liver allografts prior, post-HTK perfusion, and in cold storage. The body weight [BW, **(G)**], liver weight [LW, **(H)**], and LW/BW ratio **(I)** of lean and steatotic groups. The H&E staining images (×20) of lean **(J)** and fatty **(K)** rat liver allografts and quantitative analyses of steatosis [**(L)**, n = 4 per group; $^{*}p < 0.05$ or $^{**}p < 0.01$, t-Test].

Data Presentation and Statistical Analysis

All the numeric data were presented in the format of mean ± standard deviation (SD). The statistical analysis was performed using the Student's t-test with the setting of 2-tailed distribution and 2-sample equal variance. The difference was considered significant when the *p*-values were less than 0.05.

RESULTS

Macroscopic and Microscopic Features of Diet-Induced Rat Fatty Liver Donors

During the explantation procedure, rat livers from the chow diet group exhibited a uniform red coloration with sharp hepatic edges (**Figures 2A–C**), while those from the HF + HFr diet group for 4 weeks appeared pale with interspersed red spots (**Figures 2D–F**). Despite no significant difference in body weight (BW, **Figure 2G**), both liver weight (LW, **Figure 2H**, $^{*}p = 0.029$) and LW/BW ratio (**Figure 2I**, $p = 0.012$) were markedly increased in the HF + HFr diet group compared to the chow diet group. Moreover, histological examination using HE staining (**Figures 2J, K**) revealed a significantly higher percentage of steatosis in the HF + HFr diet group compared to the chow diet group (**Figure 2L**, $^{**}p = 0.001$). Notably, no evidence of inflammatory infiltration or fibrosis was observed in either the livers from the HF + HFr or chow diet groups. These results suggest that the HF + HFr diet successfully induced steatotic liver in rat model.

The Blood Chemistry of Lean and Steatotic Rat Liver Donors

To assess the impact of MDDP treatment on liver function, we analyzed blood samples taken just before the cold HTH flush for various chemistry parameters, including ALT, AST, LDH, ALP, bilirubin, triglycerides, LDL, and HDL (**Figure 3**). While the ALT levels remained unchanged (**Figures 3A, B**), AST levels showed a marginally significant increase in the fatty + MDDP group compared to the fatty control group, indicating a potential hemolytic process resulting from MDDP treatment ($p = 0.045$). Additionally, triglyceride (TG) and LDL levels were notably elevated in the fatty control group compared to the lean control group (**Figures 3F, G**), a trend significantly mitigated by MDDP treatment ($^{***}p < 0.001$, respectively). The rapid decrease in donor blood TG and LDL levels might stem from synergistic drug interactions. For instance, curcumin could enhance the effectiveness of statins in lowering cholesterol [37]. Additionally, pentoxifylline has demonstrated synergistic effects with simvastatin in cancer therapy [38]. Our findings also indicate that there were no significant differences in ALT, LDH, ALP, bilirubin, or HDL levels between the MDDP treatment group and the control group, regardless of whether the livers were lean or fatty.

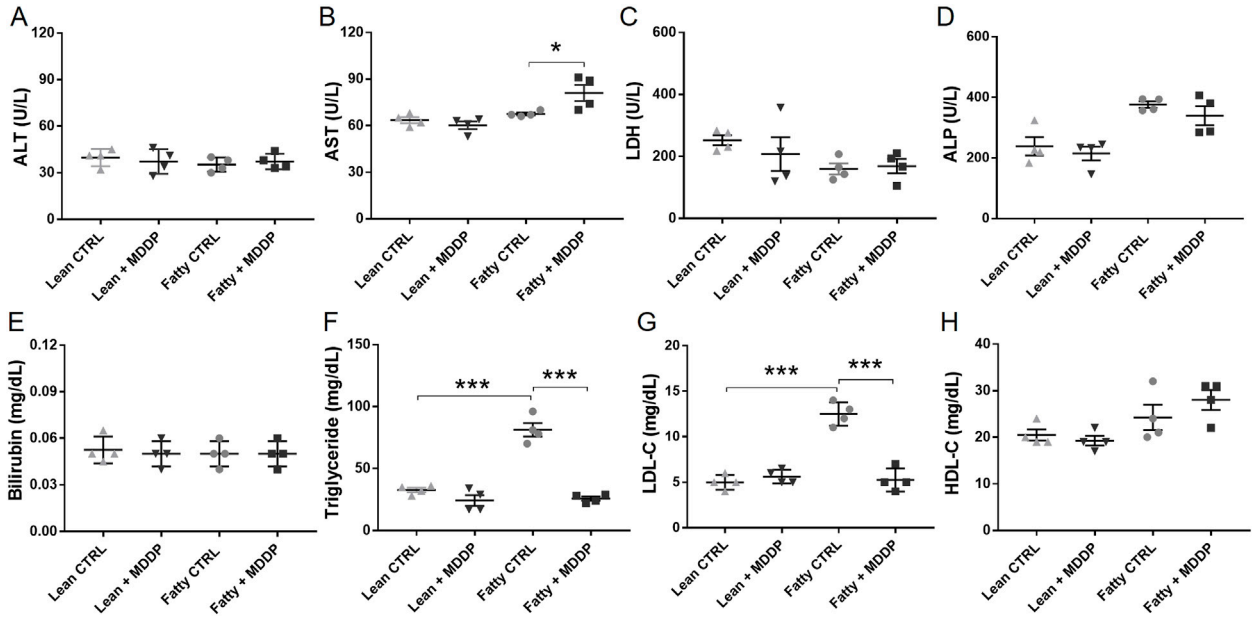


FIGURE 3 | The clinical chemistry parameters of rat liver donor blood samples. The liver function panel includes ALT **(A)**, AST **(B)**, LDH **(C)**, ALP **(D)**, Bilirubin **(E)**, Triglyceride **(F)**, LDL **(G)**, and HDL **(H)** of lean and steatotic rats fed with chow or HF + HFr diets shortly before liver explantation (n = 4 per group, $^{*}p < 0.05$ or $^{***}p < 0.001$, t-Test).

The Effects of MDDP on Rat Liver Allograft Reperfusion and Histological Findings

We conducted a comparison of reperfusion dynamics between lean and steatotic rat liver allografts without MDDP treatment, revealing a noticeable delay in reperfusion for the steatotic livers compared to the lean ones (**Figures 4A–D**). Specifically, patchy areas were observed on all lean and steatotic rat livers shortly after portal reperfusion, potentially caused by small air embolisms, vasospasms, or mechanical injuries. This could be a systematic error that would not affect the statistical comparisons between the different groups. However, at the 3rd-minute post-reperfusion initiation, the steatotic liver allograft (**Figure 4C**) exhibited more areas of non-reperfusion compared to the lean graft (**Figure 4A**). By the 15th minute post-reperfusion, the steatotic rat liver (**Figure 4D**) displayed more dark areas compared to the lean liver (**Figure 4B**), indicating poorer reperfusion and potentially more severe reperfusion injury in the steatotic liver allografts than in the lean liver allografts.

Additionally, *in vivo* microscopy was employed to investigate hepatic micro-reperfusion (**Figure 4E**) and leukocyte status (**Figure 4F**) at 24 h post-rat liver OLT. Remarkably, sinusoidal leukostasis was significantly higher in the steatotic liver allografts compared to the lean liver allografts ($p = 0.016$, **Figure 4H**). However, MDDP treatment did not induce significant changes in sinusoidal reperfusion (**Figure 4G**), sinusoidal leukostasis (**Figure 4H**), or venule leukocyte adhesion (**Figure 4I**), irrespective of whether lean or steatotic rat liver allografts were transplanted, at 24 h post-transplantation. Further examination of H&E images on POD 1 and 7 revealed that MDDP treatment did not alter hepatic vacuolization, architecture, apoptosis, or necrosis in either lean or steatotic rat liver transplantation (**Figures 5A–D**). We further found that the hepatic fat contents in both the control and MDDP groups were significantly decreased on POD 7 than on POD 1 (**Figure 5E**, both $^{***}p < 0.001$, respectively). Moreover, the hepatic fat contents in the MDDP-treated rat livers appeared to be lower than that in the control group at both POD 1 and POD 7 (**Figure 5E**, $^{**}p = 0.002$ and $^{***}p < 0.001$, respectively). In addition, the Suzuki scores were significantly lower in the MDDP group than in the control group on POD 7 (**Figure 5F**, $^{*}p = 0.046$).

The Recipient Rats' Blood Chemistry and Recipient Survival

To evaluate the impact of MDDP treatment on liver allograft function, we analyzed blood samples collected on POD 1 and 7 for various chemistry parameters. Our findings revealed a significant reduction in ALT (**Figure 6A**, $^{**}p = 0.003$) and AST (**Figure 6B**, $^{*}p = 0.021$) levels in the steatotic rat liver allograft group following MDDP treatment on POD 7. However, these reductions were not observed in the lean rat liver allograft group. Furthermore, MDDP treatment led to a significant decrease in ALP levels in both the steatotic rat liver allograft ($^{*}p = 0.011$) and lean rat liver allograft ($^{**}p = 0.001$) groups on POD 7 (**Figure 6D**). MDDP treatment significantly increased HDL levels in the steatotic rat liver allograft group on POD 7 (**Figure 6H**, $^{***}p < 0.001$). In contrast, no such effect was observed in the lean rat liver allograft group. Furthermore, the MDDP did not significantly affect the recipient's blood levels of

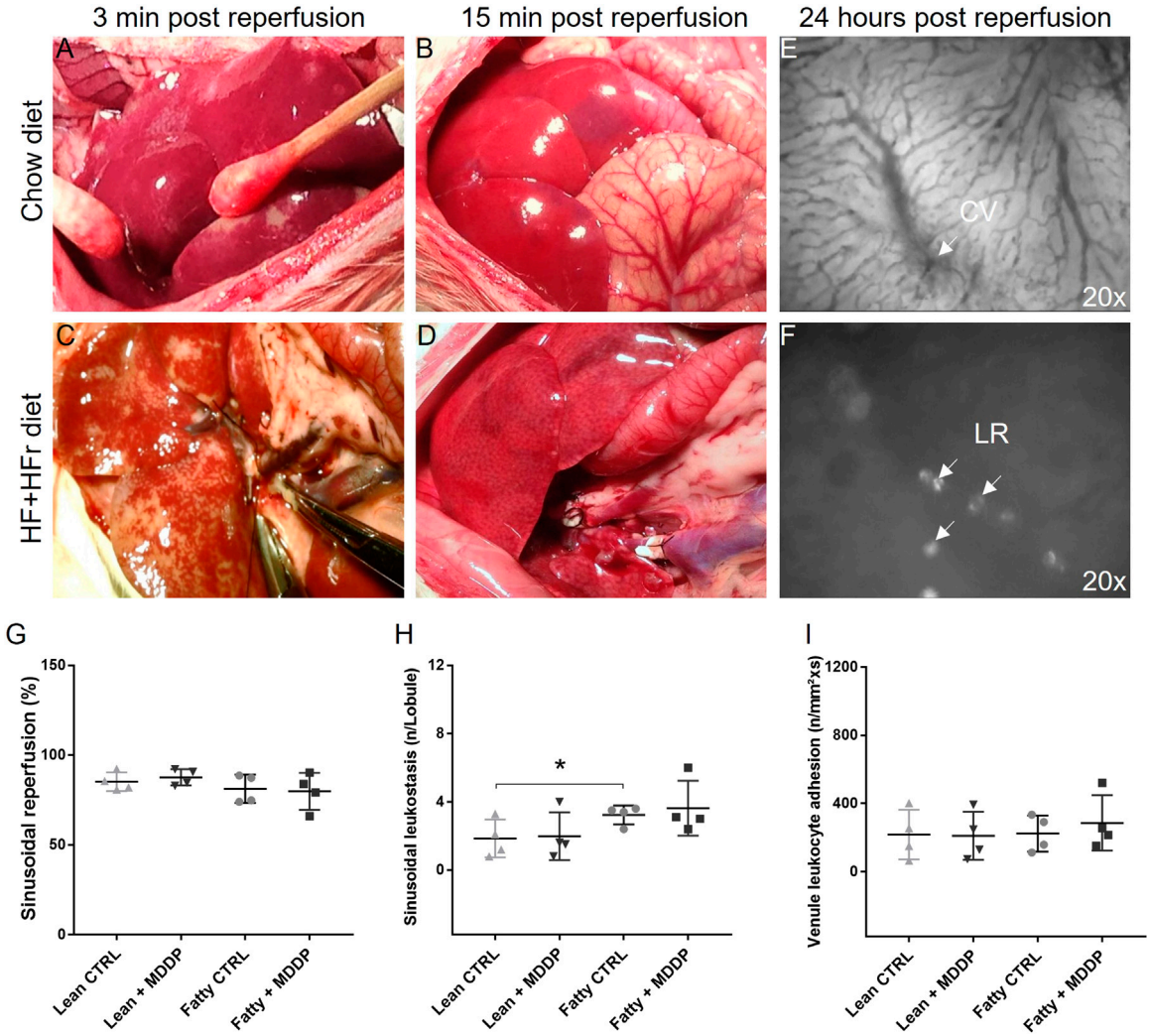


FIGURE 4 | The reperfusion of rat liver allografts and *in vivo* microcirculation study. The lean **(A, B)** and steatotic **(C, D)** allografts at 3 min and 15 min after reperfusion. **(E, F)** *In vivo* microcirculation studies of the rat liver allograft 24 h after transplantation showed the hepatic sinus, central vein (CV, 20x), and Hepatic leukocyte rolling (LR, 20x). **(G–I)** The quantitative analyses of sinusoidal reperfusion **(G)**, sinusoidal leukostasis **(H)**, and venule leukocyte adhesion [**(I)**, n = 4 per group, $^*p < 0.05$, t-Test].

LDH (**Figure 6C**), bilirubin (**Figure 6E**), triglycerides (**Figure 6F**), or LDL (**Figure 6G**) on POD 1 and POD 7.

In our studies, recipients of lean rat liver donors with a 2-hour CIT had an AST level of 318.4 ± 81.4 U/L on POD1 and achieved nearly 100% recipient survival over 3 months (n = 10). As depicted in **Figure 7**, no significant difference in recipient survival was observed between the control group (18/21, 85.7%) and the MDDP group (20/21, 95.2%) using lean donors with 8 h CIT (**Figure 7A**, $p = 0.298$, Log-rank test). However, the survival rates of steatotic rat liver allografts with 8 h of CIT and MDDP treatment (19/21, 90.5%) were significantly higher than those in the steatotic control group (12/21, 57.1%, **Figure 7B**, $^*p = 0.019$, Log-rank test). These findings suggest that MDDP treatment improves steatotic rat liver allograft function and recipient survival following OLT.

DISCUSSION

The acceptance of steatotic liver donors for transplantation in patients with end-stage liver disease has risen, yet these liver allografts exhibit heightened vulnerability to ischemia-reperfusion injury (IRI) post-OLT. In addressing this challenge, we employed a multidrug donor preconditioning approach to mitigate IRI in steatotic rat liver allografts following OLT in a rat model. Our study revealed that MDDP treatment significantly improved the function of steatotic rat liver allograft and recipient survival post-transplantation.

We first successfully induced a rat steatotic liver model by administering a Western-style diet abundant in fat and fructose as previously described [22, 23]. This induction was characterized by elevated blood triglyceride and cholesterol levels, as well as the

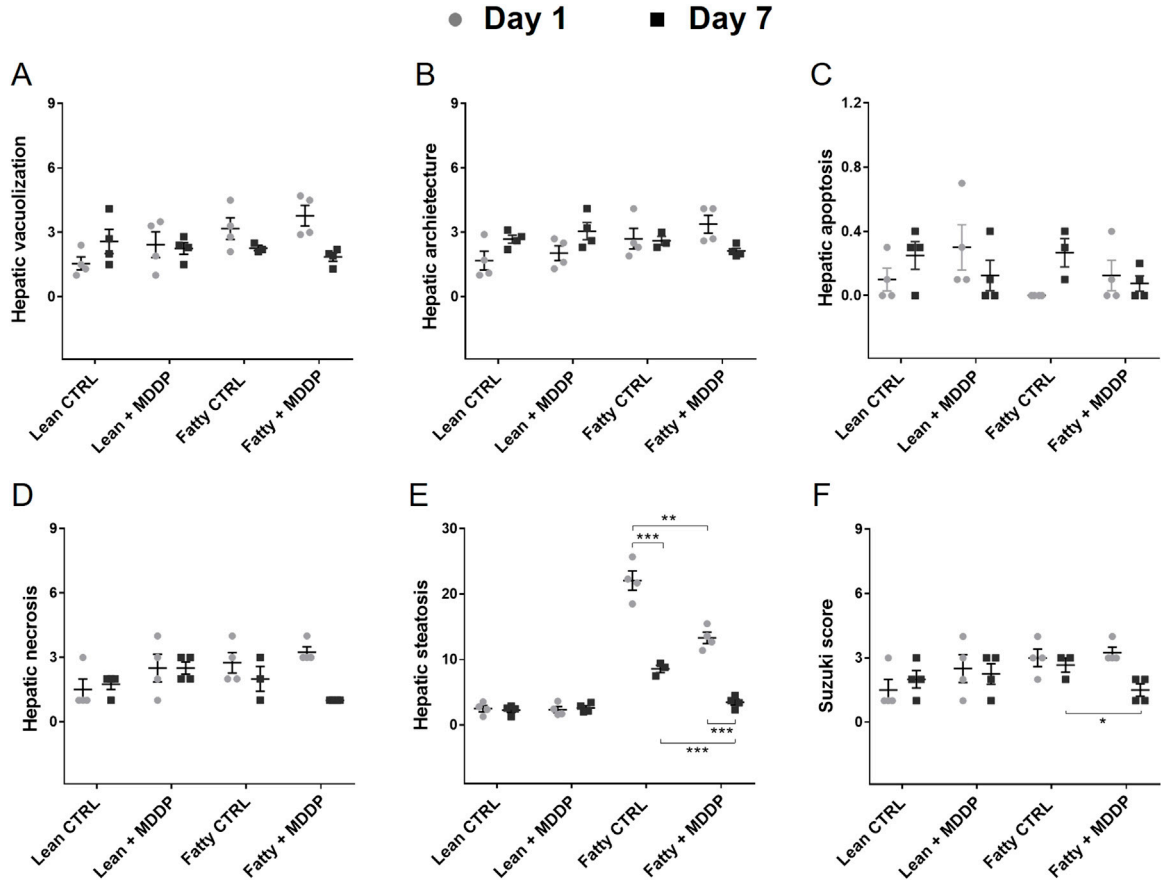


FIGURE 5 | The histological studies of rat liver allografts after transplantation. Histology analyses on POD 1 and 7 after transplantation, including Hepatocyte vacuolization **(A)**, Hepatic architectures **(B)**, Hepatic apoptosis **(C)**, Hepatic necrosis **(D)**, Hepatic fat contents **(E)**, and Suzuki scores [**(F)**, n = 3 or 4 per group, $*p < 0.05$, $**p < 0.01$, or $***p < 0.001$, respectively, t-Test].

accumulation of both microvesicular and macrovesicular fatty droplets within the hepatocytes. Subsequently, the implanted steatotic rat liver allografts exhibited delayed and uneven reperfusion, which was associated with compromised graft function and diminished survival post-OLT, which are consistent with previous studies [39, 40]. This model provided an excellent platform for investigating strategies aimed at mitigating the risks associated with steatotic liver donor transplantation. Notably, the MDDP treatment significantly reduced donor blood triglyceride and cholesterol levels without impacting liver-specific enzymes, indicating a favorable therapeutic outcome without notable hepatotoxic effects. Interestingly, we also observed a mild increase in blood AST levels in the steatotic donors with MDDP treatment compared to the steatotic control group, which may be due to an increased hemolytic process. It has been revealed that a strong positive correlation between blood cholesterol levels and RBC rigidity could affect the cell membrane fluidity, thus affecting the deformability of erythrocytes [41]. The RBC deformability can be further increased by an increase of RBC membrane cholesterol content in response to the lipid-lowering drug simvastatin, which can result in an increased risk of hemolysis [42]. However, the evaluation of donor blood AST levels associated with MDDP treatment was relatively mild.

The IRI of liver allografts involves a multifaceted process characterized by various pathways, including the exacerbation by steatosis of reactive oxygen species (ROS), the release of proinflammatory cytokines by activated Kupffer cells, and occurrences of leukocyte adhesion, vasoconstriction, apoptosis, and necrosis [43–46]. Given the complexity of IRI and the involvement of numerous molecular pathways, we employed a pharmacological combination of multidrug donor conditioning aimed at multiple pathways in allograft IRI following OLT. Furthermore, we employed a prolonged cold ischemia time (CIT) to induce significant liver damage, allowing us to assess the therapeutic effects of MDDP treatment. As expected, on POD1, there was a marked elevation of transaminase levels in the lean and steatotic controls with 8 h CIT compared to the lean control with 2 h CIT. Elevated AST levels exceeding 7500 U/L on POD1 have been associated with reduced recipient survival following OLT [47]. Although not statistically significant, the overall transaminase levels appeared paradoxically lower in the steatotic group (Control + MDDP) with 8 h CIT compared to the lean group (Control + MDDP) under the same conditions. This suggests that lean and

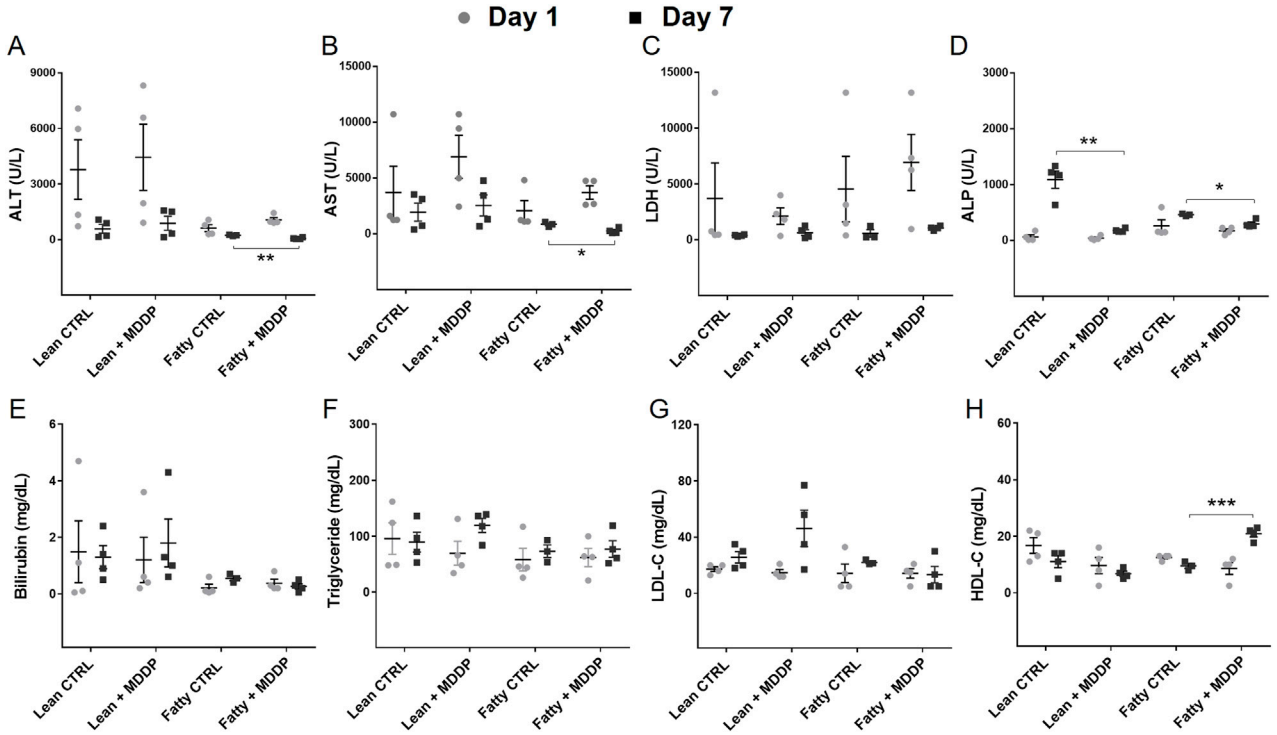


FIGURE 6 | The clinical chemistry parameters of rat recipient blood samples. The liver function panel includes ALT **(A)**, AST **(B)**, LDH **(C)**, ALP **(D)**, Bilirubin **(E)**, Triglyceride **(F)**, LDL **(G)**, and HDL **(H)** of lean and steatotic rats fed with chow or HF + HFr diets on POD 1 and 7 after OLT without or with MDDP (n = 3 or 4 per group, $^{*}p < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$, t-Test).

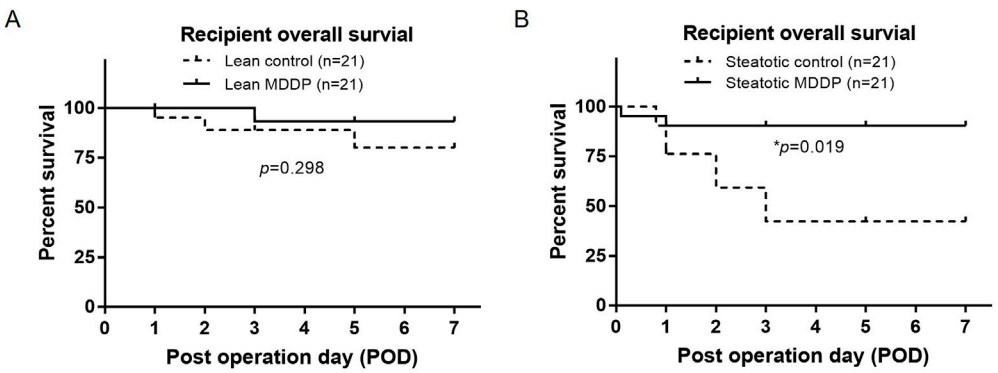


FIGURE 7 | The rat recipient's survival time after orthotopic liver transplantation (OLT). Rat recipient survival after OLT of lean **(A)** and steatotic **(B)** liver allografts with 8 h of cold ischemia and without or with MDDP treatment (n = 21 per group, $^{*}p < 0.05$, Log-rank test).

steatotic livers may respond differently to prolonged CIT. The exact mechanism behind the relatively lower transaminase levels in the steatotic control group on POD 1 remains unclear. It is speculated, however, that steatotic hepatocytes may have reduced transaminase reserves due to impaired synthetic function [48], similar to the drop in transaminase levels observed in patients with liver failure [49]. Interestingly, we observed a significant reduction in blood transaminase levels and hepatic Suzuki scores on POD 7, along with improved survival in recipients of steatotic rat liver grafts treated with MDDP compared to those who did not receive MDDP treatment. However, these effects were not significant in lean rat liver allograft transplantation, suggesting the benefits of this MDDP treatment may be limited in steatotic liver transplantation.

Another noteworthy finding was the significant decrease in steatosis of rat liver grafts following OLT on POD 1, which was further reduced with MDDP treatment. This finding aligns with previous studies, which show that 4–8 h of normothermic machine perfusion (NMP) with a defatting solution can reduce hepatic steatosis by up to 40% in discarded human livers [50, 51] and rat fatty liver models [20, 21]. In addition, no significant histological

changes, including hepatic inflammation, apoptosis, and necrosis, were observed at 24 h post-transplantation, potentially attributable to the inappropriate timing of tissue examination as the hepatic necrosis may occur up to 48 h after reperfusion [52–54].

A notable limitation of this study is the difficulty in identifying the precise mechanism by which MDDP treatment improves rat liver allograft function and survival post-transplantation, due to the complex nature of ischemia-reperfusion injury and the multifaceted mechanisms of action of the drug combination. Moreover, our study is also limited by a lack of translational capacity, as it would be unrealistic to deliver intra-gastric and intra-peritoneal medications during human organ procurement. The potential effects of the MDDP on other organs remain unknown. Further studies with *ex vivo* normothermic liver machine perfusion and transplantation are required to validate the efficacy and possible toxicity of MDDP treatments.

In summary, we established a diet-induced steatotic rat liver transplantation model with satisfactory liver damage and survival rate after OLT, enabling further exploration in pharmacological studies. Our findings demonstrate that MDDP treatment effectively improved the steatotic rat liver allograft function and recipient survival following 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 animal study was approved by Georg-August-University of Göttingen (UMG). The study was conducted in accordance with the local legislation and institutional requirements.

AUTHOR CONTRIBUTIONS

MX: designing and development of the rat fatty liver model and transplantation, acquisition/analysis of the data, and writing the manuscript; SA: experimental design for rat fatty liver model, data analysis, and writing the manuscript; M-HK: data analysis and critical review; OK: designed the animal models and helped in data interpretation and final approval. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

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

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Antiplatelet Prophylaxis Reduces the Risk of Early Hepatic Artery Thrombosis Following Liver Transplantation in High-Risk Patients

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The prevention of hepatic artery thrombosis (HAT) is pivotal for graft survival immediately after liver transplantation (LT). This study aimed to identify risk factors (RF) for early HAT (eHAT) and assess the benefit of antiplatelet prophylaxis (AP). This retrospective single-center study included 836 adult patients who underwent LT between 2007 and 2022. AP was administered for 3 months in N = 127 patients for surgical reasons. In total, 836 patients underwent LT, of whom 5.5% developed eHAT. In multivariable analysis, arterial anastomotic redo (aHR = 4.33), arterial reconstruction (aHR = 3.72) and cryptogenic liver cirrhosis (aHR = 4.25) were independent RFs for eHAT and AP appeared to be protective (aHR = 0.18). Indeed, in patients with at least one RF who received AP (RF+AP+, n = 94), the eHAT rate was significantly lower (3.2% vs. 21.3%, $p < 0.001$) than in those with RF who did not receive AP (RF+AP−, n = 89). The effect was even more pronounced when focusing on surgical RF alone (i.e., redo and/or reconstruction) with an additional improvement in 1 year graft survival of 85.3% vs. 70.4%, $p = 0.02$. AP did not pose an increased risk of bleeding. In conclusion, the main RFs for eHAT include arterial anastomotic redo, arterial reconstruction and cryptogenic liver cirrhosis as LT indications. Our results suggest that AP may protect against eHAT development in these high-risk patients.

Keywords: liver transplantation, hepatic artery thrombosis, risk factors, antiplatelet prophylaxis, 1-year graft survival, hepatic artery thrombosis

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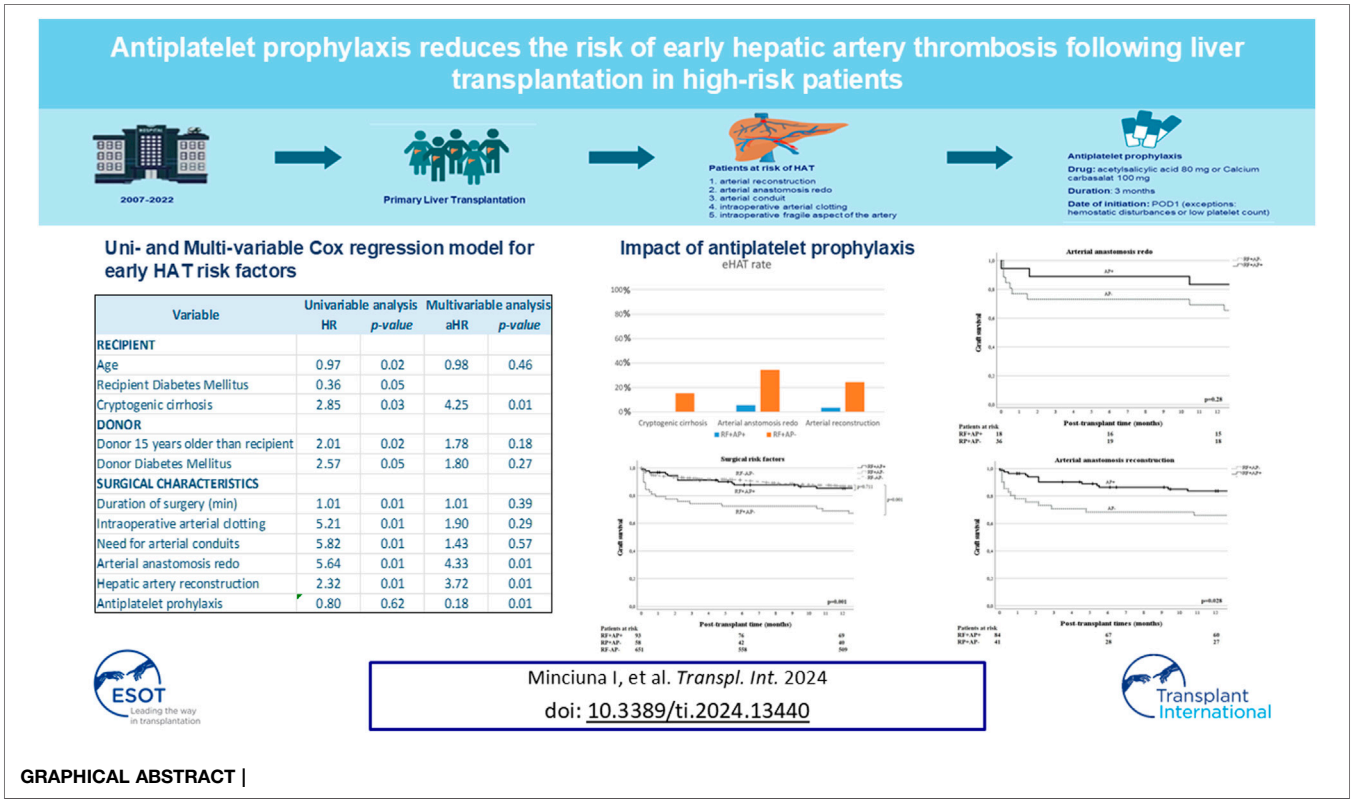

Variable	Univariable analysis		Multivariable analysis	
	HR	*p-value*	aHR	*p-value*
RECIPIENT				
Age	0.97	0.02	0.98	0.46
Recipient Diabetes Mellitus	0.36	0.05		
Cryptogenic cirrhosis	2.85	0.03	4.25	0.01
DONOR				
Donor 15 years older than recipient	2.01	0.02	1.78	0.18
Donor Diabetes Mellitus	2.57	0.05	1.80	0.27
SURGICAL CHARACTERISTICS				
Duration of surgery (min)	1.01	0.01	1.01	0.39
Intraoperative arterial clotting	5.21	0.01	1.90	0.29
Need for arterial conduits	5.82	0.01	1.43	0.57
Arterial anastomosis redo	5.64	0.01	4.33	0.01
Hepatic artery reconstruction	2.32	0.01	3.72	0.01
Antiplatelet prohylaxis	0.80	0.62	0.18	0.01

GRAPHICAL ABSTRACT |

INTRODUCTION

Despite advancements in surgical technique, postoperative care, and immunosuppression, liver transplantation (LT) continues to be associated with morbidity and mortality, particularly in the early postoperative period [1].

The most feared complications are vascular in nature and can lead to graft dysfunction, graft loss or even recipient death [2]. With a reported incidence of 4.4%–9%, hepatic artery thrombosis (HAT) is a severe complication that can result in liver necrosis, abscess formation, ischemic biliopathy and graft failure requiring re-transplantation in up to 50% of cases [3, 4]. Depending on the time of occurrence, HAT can be subdivided as early (i.e., within 2 months) and late (i.e., beyond 2 months post-LT) [3]. At the core of this division are the differences in terms of risk factors (RFs), clinical presentation, treatment options and potential outcomes.

Therefore, prevention, early detection and timely management of early HAT (eHAT) are of paramount importance for graft and patient survival, especially in patients at high risk of developing HAT. Many transplant teams, including ours [5] have implemented close surveillance of all vascular anastomoses with color Doppler to provide early detection in the immediate post-operative period, and facilitate timely intervention. Moreover, it is known that during transplant, constant platelet activation and aggregation result in thromboxane development and fibrinogen activation, which subsequently predispose to arterial thrombosis and ischemia/reperfusion injury [6]. Prevention of eHAT by antiplatelets (i.e., antiplatelet prophylaxis, AP) has therefore received considerable attention. However, there is unfortunately considerable heterogeneity in the reported studies, the majority of which are retrospective and all of which are observational in nature, in terms of study populations (adults, children, living or deceased donors), in- and exclusion criteria, reported outcomes (early HAT, late HAT, any HAT), type of antiplatelet therapy and duration of therapy. In fact, only 4 studies [7–10] have evaluated the effect of AP on the development of HAT in adult deceased donor liver transplant populations, of which only 1 [9] assessed the effect of early HAT and the remainder on HAT at any time point. Based on these, and other studies (in pediatric or living donor populations), the most cited review from the ILTS group ERAS4OLT recently recommended antiplatelet prophylaxis in all liver transplant patients [11]. However, largely due to the same heterogeneity, the group judged their recommendation as low-quality evidence and the effect size as small. In addition, a recent multicenter study by Oberkofler et al. showed that the benefits of antiplatelets may extend beyond thromboprophylaxis, as the authors observed a reduction in acute cellular rejection rates [12]. However, in this multicenter study, only 4 centers used aspirin routinely in all patients, while the other 13 administered AP only at the surgeon's discretion. It is therefore still unclear whether AP is beneficial for all patients or only for those with a high risk of HAT. Moreover, there are some concerns regarding the risk of bleeding with the use of antiplatelets, in particular in the early postoperative period [13].

Therefore, the **aim** of this study was to identify risk factors for the development of eHAT and assess whether and in whom antiplatelet prophylaxis (AP) reduces the risk of eHAT.

MATERIALS AND METHODS

Study Population

All patients aged 18 years or older who underwent LT at our center between January 2007 and September 2022 were included in the study. Exclusion criteria included re-transplantation, combined organ transplantation, and chronic use of antiplatelets for non-liver-related (cardiovascular) reasons. As we were interested in early HAT only, patients who developed late HAT (i.e., after 2 months) were also excluded.

Antiplatelet prophylaxis (AP, i.e., acetylsalicylic acid 80 mg or carbasalate calcium 100 mg) was administered for 3 months to patients for any of the following surgical reasons: 1) need for arterial reconstruction (defined as any additional arterial anastomosis between the donor and recipient hepatic arteries in the case of an anatomical variant), 2) arterial anastomosis redo (defined as immediate remaking of the arterial anastomosis in the case of suboptimal arterial inflow during the transplantation), 3) arterial conduit, 4) intraoperative arterial thrombus formation developed during implantation prompting immediate thrombectomy, or 5) a fragile aspect of the artery [e.g., due to previous transarterial radio- (TARE) or chemo-embolization (TACE) or atherosclerosis]. The arterial anastomosis was kept as short as possible and performed in an end-to-end manner to prevent kinking while considering the diameters of both the donor and recipient arteries. The most frequent site of anastomosis was at the level of the recipient's proper hepatic artery, just above the gastroduodenal artery.

Arterial flow was assessed intra-operatively by *in situ* Doppler Ultrasound, placing the probe directly on the hepatic artery. Immediately after abdominal closure [referred to as postoperative day (POD) 0], as well as on POD1 and POD7, arterial flow was assessed routinely by Doppler ultrasound performed by transplant hepatologists with extensive ultrasonography experience. This was followed by a contrast-enhanced computed tomography (CT) scan if the results suggested the presence of a vascular complication within the graft. After discharge, all patients remained life-long in follow-up at our center.

Data Collection

Data were collected retrospectively from electronic patient records. The primary endpoint was early HAT (eHAT), defined as a thrombotic occlusion of the hepatic artery, resulting in the absence of a hepatic arterial signal at the hilum or in the intrahepatic arterial branches on Doppler Ultrasound and/or a non-enhancing filling defect on contrast-enhanced CT scan, occurring within 2 months after LT. Secondary outcomes included graft and recipient survival. Patients were followed from the time of transplant until re-transplantation (i.e., graft failure), death (i.e., recipient mortality) or last follow-up (September 2022). Graft survival was calculated from the time of transplantation until re-transplantation or death, with censoring at the time of the last follow-up. Patient survival was calculated from the time of transplantation until death or last follow-up, irrespective of re-transplantation.

The following recipient variables were collected at the time of LT: age, gender, BMI, ethnicity, blood group, transplant indication, MELD score, type of graft [i.e., donation after brain death (DBD), donation after circulatory death (DCD) or living donor liver transplantation (LDLT)], metabolic co-morbidities (i.e., hypertension, Type II diabetes mellitus, obesity, dyslipidemia), prothrombotic condition (protein C or S deficiency, JAK2 mutation, Factor V Leiden mutation, antiphospholipid syndrome, antithrombin III deficiency), history of pre-LT vascular interventions (TACE, TARE), CMV and EBV mismatch status. The following donor characteristics were collected: age, gender, BMI, Donor Risk Index (DRI) [9], diabetes mellitus and smoking status. The collected data at the time of surgery included cold ischemia time, warm ischemia time, duration of surgery, arterial reconstruction, need for arterial anastomosis redo, use of arterial conduit, intraoperative arterial thrombus formation, use of *ex-situ* machine perfusion or normothermic regional perfusion, blood-loss volume, use of perioperative blood products and the percent of graft steatosis. Other variables collected post-LT included total duration of hospitalization, hemorrhagic events during the first 3 months following LT, need for re-transplantation, and 1-year graft and patient survival.

Statistical Analysis

The primary outcome was the development of eHAT. Secondary outcomes were graft and patient survival, calculated by the Kaplan-Meier method. Quantitative variables were expressed as medians with extreme values (range) and compared using Student's t-test or Wilcoxon test as appropriate. Qualitative variables were expressed as numbers and percentages and compared using Chi-square or Fisher's exact tests, as appropriate. Patients who developed eHAT and those who did not were compared with regard to recipient, donor and surgical factors.

Risk factors (RFs) for the development of eHAT were detected by first performing univariable Cox regression analyses on all variables of interest, taking into account the time to eHAT. Subsequently, factors that were statistically significant ($p < 0.05$) in the univariable analysis were considered for inclusion in a multivariate COX regression analysis to identify independent predictors of eHAT. As we were interested in the effect of antiplatelet prophylaxis (the variable of primary interest), we decided to add this variable to the multivariable model, regardless of the univariate results. As we predicted that we would run into the risk of overfitting in the multivariable model due to the small number of events and many potential risk factors, we decided to go for a multivariable model with the best fit, as defined as the smallest AIC (Akaike Information Criterion), and the highest Area Under Receiver Operating Characteristic (AUROC). Variable selection was done by back-step, forward-step and manual methods, to keep all options open and find the one model with the best fit.

TABLE 1 | Baseline characteristics of 836 patients undergoing primary liver transplantation at our institution between 2007 and 2022.

Variables	Overall n = 836	Early HAT n = 46	No HAT n = 790	*p*-value
Recipient characteristics				
Age (years)	54 (18–72)	50 (19–70)	55 (18–72)	0.23
Recipient sex (male)	523 (62.6%)	32 (69.6%)	491 (62.2%)	0.31
Ethnicity				
Caucasian	575 (68.8%)	36 (78.3%)	539 (68.2%)	0.15
Asian	27 (3.2%)	0	27 (3.4%)	0.20
Black	40 (4.8%)	3 (6.5%)	37 (4.7%)	0.92
Other	85 (10.2%)	4 (8.7%)	81 (10.3%)	0.73
BMI (kg/m^2)	25.5 (15.4–46.8)	25.9 (19.4–39.8)	25.4 (15.4–46.8)	0.30
Blood type				
O	346 (41.4%)	19 (41.3%)	327 (41.4%)	0.99
A	326 (39.0%)	18 (39.1%)	308 (39.0%)	0.98
B	109 (13.0%)	6 (13.0%)	103 (13.0%)	0.99
AB	55 (6.6%)	3 (6.5%)	52 (6.6%)	0.98
Liver disease etiology				
Viral	143 (17.1%)	6 (13.0%)	137 (17.3%)	0.45
ALD	137 (16.4%)	5 (10.9%)	132 (16.7%)	0.29
MASH	69 (8.3%)	3 (6.5%)	66 (8.4%)	0.66
PBC/PSC	203 (24.3%)	9 (19.6%)	194 (24.6%)	0.44
AIH	24 (2.9%)	1 (2.2%)	23 (2.9%)	0.77
Acute liver failure	76 (9.1%)	4 (8.7%)	72 (9.1%)	0.92
Metabolic	39 (4.7%)	2 (4.3%)	37 (4.7%)	0.91
Vascular	6 (0.7%)	0	76 (0.8%)	0.55
Cryptogenic	35 (4.2%)	5 (10%)	30 (3.8%)	**0.02**
HCC	268 (32.1%)	14 (30.4%)	254 (32.2%)	0.80
Pre-LT TACE/TARE	112 (13.4%)	4 (8.7%)	108 (13.7%)	0.33
MELD Score	22 (6–40)	24 (8–40)	22 (6–40)	0.43
Prothrombotic RF	10 (1.2%)	0	10 (1.3%)	0.44
Hypertension	131 (15.7%)	6 (13.0%)	125 (15.8%)	0.61
Diabetes Mellitus	174 (20.8%)	4 (8.7%)	170 (21.5%)	**0.04**
Obesity	39 (4.7%)	2 (4.3%)	37 (4.7%)	0.91
Dyslipidemia	17 (2%)	1 (2.2%)	16 (2%)	0.94
CMV mismatch	144 (17.2%)	5 (10.9%)	139 (17.6%)	0.24
EBV mismatch	32 (3.8%)	1 (2.2%)	31 (3.9%)	0.54
Donor characteristics				
Age (years)	53 (7–88)	51 (8–78)	53 (7–88)	0.52
Sex (male)	425 (50.8%)	19 (41.3%)	406 (51.4%)	0.18
BMI (kg/m^2)	25 (10–42)	25 (19–35)	25 (10–42)	0.68
Donor Risk Index (DRI)	1.8 (0.9–3.3)	1.85 (0.9–2.5)	1.84 (0.9–3.3)	0.79
Diabetes mellitus	39 (5.3%)	5 (12.2%)	34 (4.9%)	**0.04**
Smoking	398 (47.7%)	22 (47.8%)	376 (47.7%)	0.70
Graft steatosis	309 (39.2%)	17 (40.5%)	292 (39.1%)	0.86
Donor 10 years older	231 (28.4%)	17 (37%)	214 (27.9%)	0.18
Donor 15 years older	178 (21.3%)	16 (34.8%)	162 (20.5%)	0.02
Type of graft				
DBD	527 (63.0%)	29 (63.0%)	498 (63.0%)	0.99
DCD	274 (32.8%)	14 (30.4%)	260 (32.9%)	0.72
Living Donor	34 (4.1%)	3 (6.5%)	31 (3.9%)	0.38
Domino	1 (0.1%)	0	1 (0.1%)	0.80
Surgical characteristics				
Surgery duration (min)	358 (154–760)	389 (234–570)	357 (154–760)	**0.03**
Machine perfusion	105 (12.6%)	3 (6.5%)	102 (12.9%)	0.20
DHOPE	77 (9.2%)	5 (10.8%)	74 (9.3%)	0.88
NRP	24 (2.8%)	0	24 (3.0%)	0.21
NMP	4 (0.5%)	0	4 (0.5%)	0.61
Blood loss (L)	3.5 (0.3–58)	3.9 (0.3–20)	3.5 (0.4–58)	**0.04**
Cold ischemia (min)	362 (109–1,031)	373 (124–759)	362 (109–1,031)	0.32
Warm ischemia (min)	28 (14–80)	28 (14–57)	28 (14–80)	0.95
RBC transfusion	620 (74.2%)	34 (73.9%)	586 (74.2%)	0.96
RBC units	4 (1–48)	4.5 (1–20)	4 (1–48)	0.94
FFP use	587 (70.2%)	33 (71.7%)	554 (70.1%)	0.81
FFP units	6 (1–56)	6 (1–25)	6 (1–56)	0.68
Plt transfusion	404 (48.3%)	17 (37.0%)	387 (49.0%)	0.11
Plt units	2 (1–11)	2 (1–4)	2 (1–11)	0.64

(Continued on following page)

TABLE 1 | (*Continued*) Baseline characteristics of 836 patients undergoing primary liver transplantation at our institution between 2007 and 2022.

Variables	Overall n = 836	Early HAT n = 46	No HAT n = 790	*p*-value
Fibrinogen use	403 (48.2%)	16 (34.8%)	387 (49.0%)	0.06
Tranexamic acid	610 (73.0%)	37 (80.4%)	573 (72.5%)	0.24
Prothrombin complex	120 (14.4%)	6 (13.0%)	114 (14.4%)	0.79
Intraoperative arterial Thrombus formation	27 (3.2%)	6 (13.0%)	21 (2.7%)	**0.01**
Arterial conduit	15 (1.8%)	4 (8.7%)	11 (1.4%)	**0.01**
Supraceliac conduit	11 (1.3%)	2 (4.3%)	9 (1.3%)	0.06
Infrarenal conduit	4 (0.5%)	2 (4.3%)	2 (0.3%)	**0.01**
Arterial redo	44 (5.3%)	10 (21.7%)	34 (4.3%)	**0.01**
HA reconstruction	125 (15.0%)	13 (28.3%)	112 (14.2%)	**0.01**
Peri-anastomotic bile leak[a]	23 (2.8%)	3 (6.5%)	20 (2.5%)	0.11

Results are expressed as N (%) or median (range). Variables were compared between patients who developed eHAT (n = 46) and those who did not (n = 790).
(e)HAT, (early) hepatic artery thrombosis; LT, liver transplantation; BMI, body mass index; ALD, alcohol-related liver disease; MASH, metabolic dysfunction associated steatohepatitis; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis; AIH, auto-immune hepatitis; HCC, hepatocellular carcinoma; TACE, trans arterial chemoembolization; TARE, trans arterial radioembolization; MELD, Model for end-stage liver disease; RF, risk factor; CMV, cytomegalovirus; EBV, Epstein-Barr virus; DBD, donation after brain death; DCD, donation after cardiac death; DHOPE, dual hypothermic oxygenated machine perfusion; NRP, normothermic regional perfusion; NMP, normothermic machine perfusion; RBC, red blood cells; FFP, fresh frozen.
[a]*Bile leak preceding HAT.*
The bold values indicate statistical significance.

Finally, within the group of patients with at least one of the identified independent RFs (i.e., RF+), we compared eHAT development in those who received AP (RF+AP+) to those who did not (RF+AP−), based on the Chi-square test. Similarly, 1-year graft and patient survival were compared using the log-rank test.

All statistical analyses were performed using commercially available statistical software (SPSS Inc., Chicago, IL). A p-value of <0.05 was considered statistically significant.

RESULTS

In total, 839 patients who underwent primary liver-only LT were initially included in the study. For the purposes of our study, we decided to exclude 3 patients who developed HAT within hours after liver transplantation, given the fact that they did not have a chance of being exposed to AP, even if indicated, thus leading to a final number of 836 patients included in the analysis. These were followed for a median time of 48.6 months (range 0.02–189.84). Overall, 1 year graft survival was 85.7% (95% CI: 84.5–86.9) and patient survival was 90% (95% CI: 88.9–91.1).

Recipient characteristics, donor characteristics and surgical details of the total population are presented in detail in **Table 1**. Briefly, patients were an average of 54 (18–72) years old, and were mainly men (62.6%) with HCC (32.1%) and cholestatic liver disease (24.3%) being the main indications. The median MELD was 22 (6–40) and 63% received a graft from a DBD and 32.8% from a DCD donor.

In total, 127 (15.2%) patients received AP for 3 months, for the following reasons: arterial reconstruction (n = 84, 66.1%), and/or arterial anastomosis redo (n = 18, 14.2%), and/or arterial conduit (n = 5, 3.9%), and/or thrombectomy of intraoperatively formed arterial thrombus (n = 13; 10.2%) or fragility of the artery (n = 7, 5.5%). The majority of patients (55.9%) had a combination of the above. In total, 90.6% of these patients started AP on POD 0–5 (range: POD 0–18), with the exception of n = 12 subjects who were delayed to POD 7–18 due to fear of bleeding. In addition, all patients received high-dose prophylactic LWMH (i.e., nadroparin 5700 IU) during the ICU stay and normal dose (i.e., nadroparin 2850 IU) on admission. Patients receiving AP had significantly lower intraoperative blood loss (median 2,800 vs. 3,500 mL, p = 0.03), higher DRI (median 1.96 vs. 1.81, $p < 0.05$) but similar postoperative coagulation parameters such as median INR (2.0 vs. 1.9, p = 0.49), factor V (0.27 vs. 0.27, p = 0.68), antithrombin (0.38 vs. 0.39, p = 0.89) and platelet count (101.7 × 109 vs. 97 × 109, p = 0.52) than those who did not receive AP. Moreover, the use of AP was not associated with increased hemorrhagic events in the first 3 months post-LT (10.2% vs. 9.6%, p = 0.82).

Characteristics of the Population That Developed Early HAT

In the total population, 46 (5.5%) patients developed eHAT. The median time to diagnosis was 4 days (range 0–50) and 71.7% of HAT occurred within the first week.

Patients who developed eHAT were more likely to have cryptogenic cirrhosis (10.0% vs. 3.8%, p = 0.02) but less likely to have pre-LT diabetes mellitus (8.7% vs. 21.5%, p = 0.04), than those without eHAT (**Table 1**). Moreover, patients who developed eHAT were significantly more likely to undergo hepatic artery reconstruction (28.3% vs. 14.2%, $p < 0.01$), arterial anastomosis redo (21.7% vs. 4.3%, $p < 0.01$), arterial conduit placement (8.7% vs. 1.4%, $p < 0.01$), or thrombectomy of an intra-operatively formed arterial clot (13% vs. 2.7%, $p < 0.01$). Similarly, the overall duration of surgery was significantly longer (389 vs. 357 min, p = 0.03) and patients had more intraoperative blood loss (3,912 vs. 2,500 mL, p = 0.04). As for donor factors, patients with eHAT were significantly more likely to receive a graft from a diabetic donor (12.8% vs. 5.2%, p = 0.04). Although both recipient and donor ages were not significantly different between the groups, we also evaluated the impact of an age

difference between donor and recipient in 5-year increments. While a difference of 5 or 10 years was not significant for either older donors (p = 0.76 and p = 0.14, respectively) or recipients (p = 0.64 and p = 0.33, respectively), a difference in age with a donor 15 years older than the recipient was significantly more common in patients with eHAT compared to those without (34.8% vs. 20.5%, p = 0.02). The reverse situation (i.e., recipient 15 years older) was not found to be associated with eHAT development (p = 0.51).

Interestingly, there was no statistically significant difference in AP administration between patients with eHAT (13%) vs. those without eHAT (15.3%; p = 0.67).

As expected, patients who developed eHAT were more likely to require re-transplantation (52.2% vs. 6.2%; p < 0.01) than those without eHAT. Similarly, eHAT was associated with a lower graft survival at 1 year of 47.3% (95% CI: 39.9–54.7) compared to 87.9% (95% CI: 86.7–89.1) in those without eHAT, (p < 0.01). One-year patient survival was, however, not affected (82.4% vs. 90.5%, p = 0.07). The remainder of the patients with eHAT were treated with surgical revascularization (50%), endovascular therapy (5%) and prolonged anti-platelet therapy/anticoagulation (45%).

Identifying Risk Factors for eHAT Including AP

In the total population (N = 836), we performed univariable Cox regression analysis to identify risk factors for eHAT. We found that recipient age (HR 0.97, 95% CI: 0.95–0.99) cryptogenic cirrhosis as the underlying liver disease (HR 2.85, 95% CI: 1.12–7.21), duration of surgery (HR 1.004; 95% CI: 1.001–1.007), intraoperative arterial thrombus formation (HR 5.21; 95% CI: 2.21–12.29), arterial conduit (HR 5.82; 95% CI: 2.09–16.23), hepatic artery reconstruction (HR 2.32; 95% CI: 1.22–4.41), arterial anastomosis redo (HR 5.64; 95% CI: 2.80–11.38), donor-recipient age difference greater than 15 years (HR 2.01; 95% CI: 1.09–3.68) and donor diabetes mellitus (HR 2.57; 95% CI: 1.01–6.52) were significantly associated with an increased risk of eHAT (**Table 2**). In contrast, AP was not associated with eHAT in the univariable analysis in the whole population (HR 0.80; 95% CI: 0.34–1.89), nor was the use of DCD grafts (HR 0.89, 95% CI: 0.47–1.70), nor DRI (HR 1.1, 95% CI: 0.55–2.19) nor graft steatosis (HR 1.06, 95% CI: 0.57–1.96). Next, we fitted multiple multivariable models (see methods), with the final model being selected by the lowest AIC (508.20) and highest AUROC (0.681). We found that the use of AP (aHR = 0.18; 95% CI: 0.05–0.59) was protective against eHAT while arterial redo (aHR = 4.33; 95% CI: 1.69–11.07), hepatic artery reconstruction (aHR = 3.72; 95% CI: 1.50–9.22), together with cryptogenic cirrhosis as the underlying liver disease (aHR = 4.25; 95% CI: 1.60–11.25) were consistently and independently associated with increased eHAT development (**Table 3**).

The Effect of Antiplatelet Prophylaxis in Patients With Risk Factors for eHAT

Given that AP was not a significant predictor of eHAT in the univariable analysis of all (i.e., unselected) patients, but appeared to be a significant predictor in the multivariate model, we were interested in identifying in which population AP may be most beneficial. Therefore, we compared eHAT rates and survival outcomes between those who had identified risk factors and were given AP (RF+AP+) and those with RF not receiving AP (RF+AP–). First, in patients with cryptogenic cirrhosis (n = 35), only n =

TABLE 2 | Univariable Cox proportional hazards survival analysis of potential risk factors for eHAT in the overall population.

Variable	Univariable analysis		
	HR	95% CI	*p*-value
Age (years)	0.97	0.95–0.99	**0.02**
Cryptogenic cirrhosis	2.85	1.12–7.21	**0.03**
BMI (kg/m^2)	0.99	0.94–1.06	0.92
MELD Score	1.005	0.97–1.04	0.77
Pre-LT TACE/TARE	0.63	0.22–1.76	0.38
Recipient Diabetes Mellitus	0.36	0.13–1.01	0.05
Type of graft			
DBD	1.003	0.55–1.82	0.99
DCD	0.89	0.47–1.70	0.72
Donor age (years)	0.99	0.97–1.01	0.27
Donor sex (male)	0.68	0.37–1.23	0.20
Donor BMI (kg/m^2)	1.01	0.94–1.09	0.73
Donor 15 years older than recipient	2.01	1.09–3.68	**0.02**
Donor Diabetes Mellitus	2.57	1.01–6.52	**0.05**
Donor smoking	1.08	0.58–2.00	0.79
Donor Risk Index (DRI)	1.10	0.55–2.19	0.78
Donor steatosis (any degree)	1.06	0.57–1.96	0.85
Surgery duration (min)	1.004	1.001–1.007	**0.01**
Blood loss (L)	1.00	1.00–1.00	0.82
Fibrinogen use	0.55	0.31–1.03	0.06
Intraoperative arterial thrombus formation	5.21	2.21–12.29	**0.01**
Arterial conduit	5.82	2.09–16.23	**0.01**
Arterial redo	5.64	2.80–11.38	**0.01**
HA reconstruction	2.32	1.22–4.41	**0.01**
Peri-anastomotic bile leak[a]	2.48	0.77–8.01	0.12
Antiplatelet prophylaxis	0.80	0.34–1.89	0.62

Results are expressed as hazard ratio (HR) and 95% confidence interval (CI).
eHAT, early hepatic artery thrombosis; HR, hazard ratio; BMI, body mass index; MELD, Model for end-stage liver disease; DBD, donation after brain death; DCD, donation after cardiac death; HA, hepatic artery.
[a]Bile leak before hepatic artery thrombosis.
The bold values indicate statistical significance.

TABLE 3 | Final multivariable Cox proportional hazards survival model for risk factors for eHAT in the overall population.

Variable	aHR	95% CI	*p*-value
Age	0.98	0.95–1.02	0.46
Donor 15 years older than recipient	1.78	0.75–4.20	0.18
Cryptogenic liver cirrhosis	**4.25**	**1.61–11.25**	**0.01**
Surgery duration	1.002	0.99–1.005	0.39
Intraoperative arterial thrombus formation	1.90	0.57–6.25	0.29
Donor diabetes mellitus	1.80	0.63–5.15	0.27
Arterial conduit	1.43	0.40–5.15	0.57
Arterial anastomosis redo	**4.33**	**1.69–11.07**	**0.01**
Hepatic artery reconstruction	**3.72**	**1.50–9.22**	**0.01**
Antiplatelet prophylaxis	**0.18**	**0.05–0.59**	**0.01**

Results are expressed as adjusted hazard ratio (aHR) and 95% confidence interval (95% CI). This model had an AIC of 508.20 and an AUROC of 0681.
AP, antiplatelet prophylaxis; eHAT, early hepatic artery thrombosis.
The bold values indicate the variables with statistical significance.

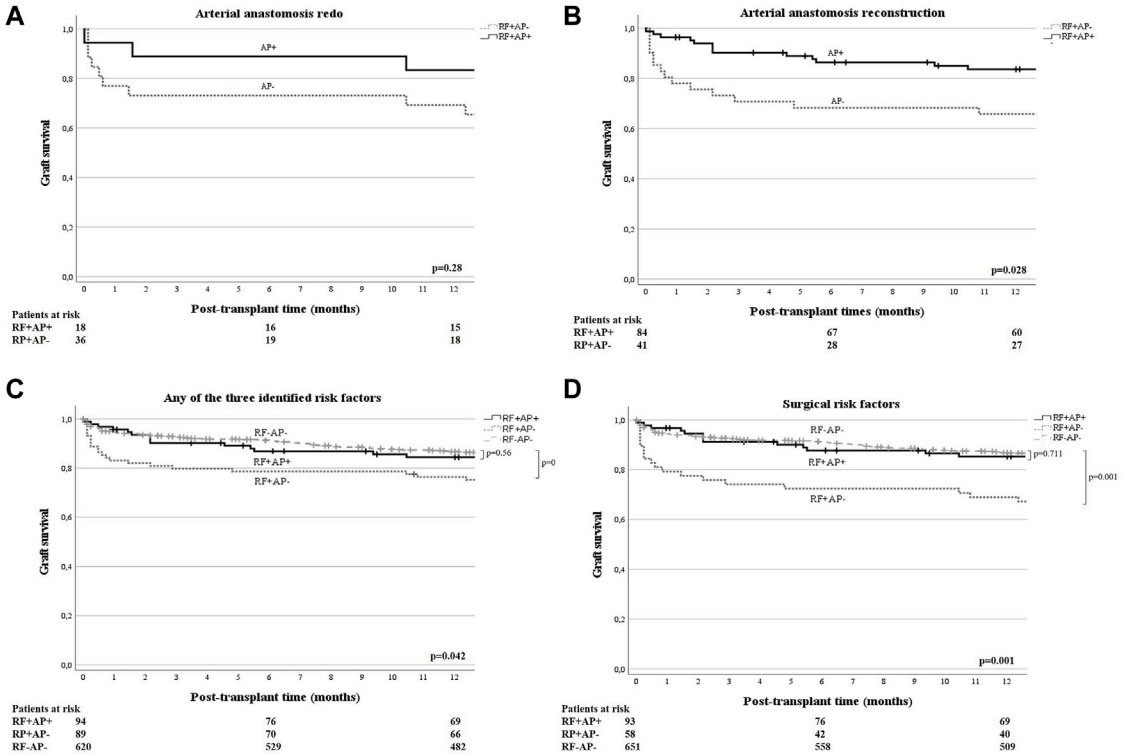

FIGURE 1 | (A, B) One-year graft survival of patients with arterial redo [**(A)**, n = 44], or arterial reconstruction [**(B)**, n = 125], (i.e., independent eHAT RF derived from the multivariate model), stratified by use of antiplatelet prophylaxis (AP+/–) and compared by log-rank test. One year graft survival for RF+AP+ vs. RF+AP– was 83.3% (95% CI: 74.5–92.1) versus 69.2% (95% CI: 60.1–78.3) in patients with redo anastomosis [$p = 0.28$; **(A)**]; and 83.7% (95% CI: 79.5–87.9) versus 67.8% (95% CI: 60.4–75.2) in patients with arterial reconstruction [$p = 0.03$, **(B)**], respectively. **(C)** One year graft survival of patients with either cryptogenic liver cirrhosis and/or arterial reconstruction and/or anastomotic redo (i.e., anyRF+, n = 183), stratified according to the use of antiplatelet prophylaxis (AP+/–) and compared by log-rank test. An additional comparison was made with all other patients who did not have the identified risk factors and did not receive AP (surgRF–AP–, n = 620). One-year graft survival was 84.4% (95% CI: 80.6–88.2) for anyRF+AP+ versus 77.4% (95% CI: 70–81.8) for anyRF+AP– vs. 86.6% (95% CI: 85.2–88) $p = 0.042$. **(D)** One year graft survival of patients with either arterial reconstruction and/or anastomotic redo (i.e., surgRF+, n = 151), stratified by use of antiplatelet prophylaxis (AP+/–) and compared by log-rank test. An additional comparison was made with all other patients who did not have these surgical risk factors and did not receive AP (surgRF–AP–, n = 651). One year graft survival was as follows: in surgRF+AP+ 85.3% (95% CI: 81.5–89.1), in surgRF+AP– 70.4% (95% CI: 64.4–76.4), and in surgRF-AP 86.8% (95% CI: 85.5–88.1). Graft survival in patients with surgRF+AP+ was significantly better than in patients with RF+AP– ($p = 0.018$) and equal to all surgRF–AP– ($p = 0.71$).

2 patients received AP (i.e., ccRF+AP+) and n = 33 did not (ccRF+AP–). Although limited by low numbers, we found that the eHAT rates were not significantly different (0% vs. 15.2%, $p = 0.55$) and there was no significant difference in 1-year patient ($p = 0.10$) or graft survival ($p = 0.19$; **Figure 1A**) between those with and without AP. Second, in those with arterial anastomosis redo (n = 44), 18 received AP (redoRF+AP+) and 26 did not (redoRF+AP–). Here, the eHAT rate was significantly lower in redoRF+AP+ (5.6%) vs. redoRF+AP– (34.5%, $p = 0.02$). However, 1-year patient survival ($p = 0.90$) and graft survival ($p = 0.28$) were similar between the two groups (**Figure 1B**). Third, in patients who underwent arterial reconstruction (N = 125), 84 received AP (reconRF+AP+) and 41 (reconRF+AP–) did not. Again, the eHAT rate was significantly lower in the reconRF+AP+ group (3.5%) than in the reconRF+AP– group (24.4%; $p < 0.01$). Moreover, those with reconRF+AP+ had an improved 1-year graft survival of 83.7% (95% CI: 79.5–87.9) vs. 67.8%, (95% CI: 60.4–75.2; $p = 0.03$) in reconRF+AP (**Figure 1C**). Patient survival remained unchanged ($p = 0.29$).

Next, we evaluated the effect of AP in patients with at least one of the three risk factors (anyRF; n = 183) and found a significantly lower eHAT rate of 3.2% in anyRF+AP+ (n = 94) compared to the rate of 21.3% in anyRF+AP– (n = 89; $p < 0.01$) but no difference in 1-year patient ($p = 0.96$) or graft survival ($p = 0.17$) (**Figure 1D**). Following this observation, we then compared these two groups to the remaining patients in our cohort who did not have any of these three risk factors and who did not receive antiplatelet therapy (i.e., anyRF–AP–,n = 620), and found that those who had anyRF+AP– had a significantly worse graft survival (77.4% vs. 86.6%, $p = 0.01$), while graft survival in patients with anyRF+AP+ was similar to that in those without any RF (84.4% vs. 86.6%, $p = 0.56$). Finally, when evaluating the effect of AP in those with surgical RF only (i.e., either arterial redo or reconstruction, n = 151), the difference in eHAT rate became even greater with 3.2% in surgRF+AP+ (n = 93) versus 25.8% in surgRF+AP– (n = 58; $p < 0.01$). Moreover, surgRF+AP+ showed a 1-year graft survival of 85.3% (95% CI: 81.5–89.1) which was equivalent to the graft survival of 86.8% (95% CI: 85.5–88.1; $p =$

0.71) in patients who did not have any of the two surgical RF and no AP (surgRF−AP−, n = 651), whereas graft survival was significantly compromised in surgRF+AP-(70.4%; 95% CI: 64.4–76.4; $p = 0.02$) (**Figure 1E**). There was again no effect on 1-year patient survival (88.7% vs. 84.5% vs. 90.5%, respectively, $p = 0.33$).

DISCUSSION

In our study, which included 836 patients after liver transplantation, the eHAT rate was 5.5% and 15.2% received AP for surgical reasons. Although we did not find a significant association between the overall eHAT rate and the use of AP in the uncontrolled (univariable) analysis, AP was found to be independently associated with reduced eHAT rate (aHR = 0.18) in the multivariable model. In contrast, arterial anastomosis redo (aHR = 4.33), hepatic artery reconstruction (aHR = 3.72), and cryptogenic cirrhosis as the underlying liver disease (aHR = 4.25) were associated with an increased risk of eHAT. Interestingly, we showed that administration of AP in patients with any one of these risk factors significantly mitigated the risk of eHAT, especially in those who underwent either arterial redo and/or reconstruction. In this high-risk group, an 8-fold decrease in the rate of eHAT (3.2% vs. 21.3%) and an absolute difference in 1-year graft survival of 14.9% (85.3% vs. 70.4%) were seen in favor of AP. Indeed, after AP, graft survival in these high-risk patients became equivalent to that of patients without any of these eHAT risk factors. Therefore, our results suggest that AP may be recommended in all patients who underwent an arterial redo or reconstruction during transplant surgery.

While the real pathogenesis of eHAT remains unclear, it is typically attributed to a combination of donor, surgical, and recipient factors. Among the identified non-surgical RFs, we only found cryptogenic liver cirrhosis as an independent risk factor for eHAT. Although patients with so-called cryptogenic liver cirrhosis were labeled as such because no specific etiology could be identified at the time, we now know that in retrospect, a large proportion of this group of cryptogenic cirrhosis may have been suffering from metabolic dysfunction associated steatohepatitis MASH, since the typical clinicopathological features of MASH are known to fade once decompensated cirrhosis is established [14]. Indeed, among the patients in our cohort, 23% had DM and 20% had obesity. MASH, together with the other associated co-morbidities and systemic changes (systemic inflammatory milieu, intestinal dysbiosis, insulin resistance), may all contribute to a chronic inflammatory status that favors endothelial cell activation, lipid-derived oxidative injury, necroapoptosis, and ultimately, prothrombotic changes [15, 16]. So, while it is tempting to speculate that preceding MASH may have, at least in part, contributed to the increased risk of eHAT, we did not find a higher rate of eHAT in patients with confirmed MASH. Additionally, we could not identify a protective effect of AP due to the very small number of patients who received it (n = 2). Larger studies are needed to confirm these findings before firm conclusions can be drawn.

The most important RFs were, however, surgical in nature. The need to perform an arterial anastomosis redo during the transplantation surgery was found to be significantly associated with the development of eHAT, both in univariable and multivariable analysis. A redo is usually needed for technical issues such as anastomotic angulation or traction, or suboptimal arterial inflow resulting from spasm, intimal dissection or instant thrombus formation. Our results suggest that in this situation, the increased risk of HAT and graft failure can be mitigated by the administration of AP in the post-transplant setting. To the best of our knowledge, this factor has not been previously examined as a separate potential risk factor in other studies. Finally, in agreement with previous studies [3, 17], bench reconstruction of an anatomical variant or damaged hepatic artery also increased the risk of eHAT development, probably due to the increased number of arterial anastomoses combined with an abnormal morphology compared to the standard end-to-end/single arterial anastomosis technique [18, 19].

The most important finding in this study was the protective effect of AP on the rate of eHAT in high-risk patients, while this did not appear to be the case in the overall population. Although AP was mainly used for a variety of surgical difficulties during arterial anastomosis, not all patients with these difficulties actually received AP. This may have increased the number of eHAT in the group without AP, rendering it not beneficial in the overall population. Our findings are consistent with recent publications. Wolf et al. assessed the use of AP in 354 consecutive, and thus unselected, LT recipients and, like us, did not identify any benefit [13]. However, a more recent study found that prophylaxis with 325 mg/day of aspirin initiated immediately after surgery and continued for 3 months in 439 unselected patients led to a decreased eHAT incidence from 3.6% to 0%, without increasing the risk of bleeding [20]. However, such high dosing may come at the expense of other adverse events such as peptic ulcerations and liver/kidney toxicity and is probably not to be recommended in all post-LT patients.

On the other hand, in selected high-risk patients, AP was shown to be very beneficial. Indeed, when we selected patients who had at least one of the independent risk factors for eHAT, AP was associated with an 8-fold decreased rate of eHAT (3.2% vs. 25.8%) compared to those with the same RFs who did not receive AP. Also, 1-year graft survival was significantly improved in the AP group while risks of bleeding were similar. Our study is in line with another retrospective single-center study that found an 82% relative risk reduction in high-risk patients (defined as those who received grafts from donors after a cerebrovascular accident and/or use of an iliac conduit at transplantation), without any recorded bleeding episodes during follow-up [17]. Our results therefore confirm that AP should be reserved for these selected high-risk patients.

Two other previously described surgical risk factors for eHAT (i.e., the use of arterial conduit and intraoperative arterial thrombosis) [21, 22], were identified as potential risk factors for eHAT in our univariable analysis, but failed to remain independent risk factors in the multivariable analyses. This may be due to the small number of patients in each group (n = 15 and n = 27, respectively). However, in retrospect, we

observed that 33% and 48% of these patients, respectively, received AP, and none (0%) of the patients who received AP developed eHAT compared to 40% in those with an arterial conduit and 42.9% in those with intraoperative arterial thrombosis who did not receive AP. Although not a direct result of our study, it is reasonable to assume that AP may be protective in these situations as well, something that could be further explored in larger datasets with more events.

Moreover, the use of antiplatelets may also have long-term additional protective effects on these patients in terms of preventing cardiovascular events [6] and even reducing the incidence of acute rejection episodes as suggested by the study of Oberkofler et al [12].

Our study has several strengths and limitations that need to be addressed. Strengths of this study include a relatively large and uniform dataset with complete follow-up and comprehensive data collection on a large subset of potentially important recipient, donor and surgical RFs. Despite this, our study was limited by the fact that the event rate was still low, resulting in limited power and potential overfitting in the case of multivariable analysis as many potential risk factors were identified from the univariable analyses. We tried to overcome this by fitting multiple models and using the AIC and AUROC to select the best model fit. Second, due to the retrospective nature of this study, there are missing data that could have underestimated the role of some potential RFs. Third, we could not completely retrieve the individual reasons why some patients with potential surgical RFs were not prescribed AP, which could have introduced potential confounding by indication. Although intraoperative blood loss was higher in those not receiving AP (indicating fear of postoperative bleeding as a possible reason) none of the post-LT coagulation parameters indicated worse coagulation or potentially higher risk of bleeding in these patients. Finally, the observational and retrospective, rather than interventional, nature of our study does not allow us to draw definite conclusions about the beneficial effects of AP and larger, prospective studies may be needed to confirm our findings.

CONCLUSION

Patients who underwent arterial redo or hepatic artery reconstruction, or who had cryptogenic liver cirrhosis as an indication for LT have an increased risk of eHAT. In selected high-risk patients, AP was associated with an 8-fold reduced risk of eHAT and significantly improved graft survival. Our results warrant increased vigilance for eHAT in the presence of these RFs and suggest a possible protective role of antiplatelet prophylaxis in these selected cases.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving humans were approved by the Medical Ethics Review Committee. 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

Conceptualization: SD and IM; Provision of study materials or patients: IM, SD, JD, CD, RM, WP, RP, and HJ; Collection of data: IM and SD; Data analysis: IM and SD; Manuscript writing: All authors; Supervision: SD; All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

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

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Fumagillin Shortage: How to Treat *Enterocytozoon bieneusi* Microsporidiosis in Solid Organ Transplant Recipients in 2024?

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Intestinal microsporidiosis caused by *Enterocytozoon bieneusi* is an opportunistic infection that especially affects solid organ transplant (SOT) recipients. Management revolves around tapering the immunosuppressive regimen and/or using a specific anti-microsporidia treatment, but only fumagillin has demonstrated efficacy for treatment of this infection. Since fumagillin has been commercially discontinued, nitazoxanide is increasingly being used in this indication. We aimed to describe therapeutic management of *E. bieneusi* infections in this context. We conducted a French nationwide observational retrospective study on reported cases of *E. bieneusi* infections in SOT recipients. We identified 154 cases: 64 (41.6%) were managed by simply modifying the immunosuppressive regimen, 54 (35.1%) were given fumagillin, and 36 (23.4%) were given nitazoxanide. Clinical remission rate ranged from 77.8% to 90.7% and was not significantly different between therapeutic strategies but tended to be lower with nitazoxanide. Stool negativization rate was highest with fumagillin (91.7%) and lowest with nitazoxanide (28.6%). Relapses occurred in 6.9% of cases and were more frequent with nitazoxanide (14.3%). This study shows that tapering immunosuppression can result in a satisfactory remission rate but is sometimes accompanied by relapses. Nitazoxanide had limited effectiveness, whereas fumagillin had good results that provide a solid rationale for bringing fumagillin back to market.

Trial Registration Number: ClinicalTrials.gov ID: NCT05417815.

Keywords: solid organ transplant, nitazoxanide, microsporidiosis, Enterocytozoon bieneusi, infectious diarrhea

INTRODUCTION

Enterocytozoon bieneusi is by far the most common microsporidia species causing intestinal microsporidiosis, which manifests as profuse watery diarrhea and abdominal pain [1]. *Enterocytozoon bieneusi* microsporidiosis can occur in both immunocompetent and immunocompromised individuals, but significantly affects immunocompromised solid organ transplant (SOT) recipients [2, 3]. While in the immunocompetent, the infection will generally result in acute diarrhea, in the immunocompromised, it will become chronic, which can lead to significant weight loss and dehydration.

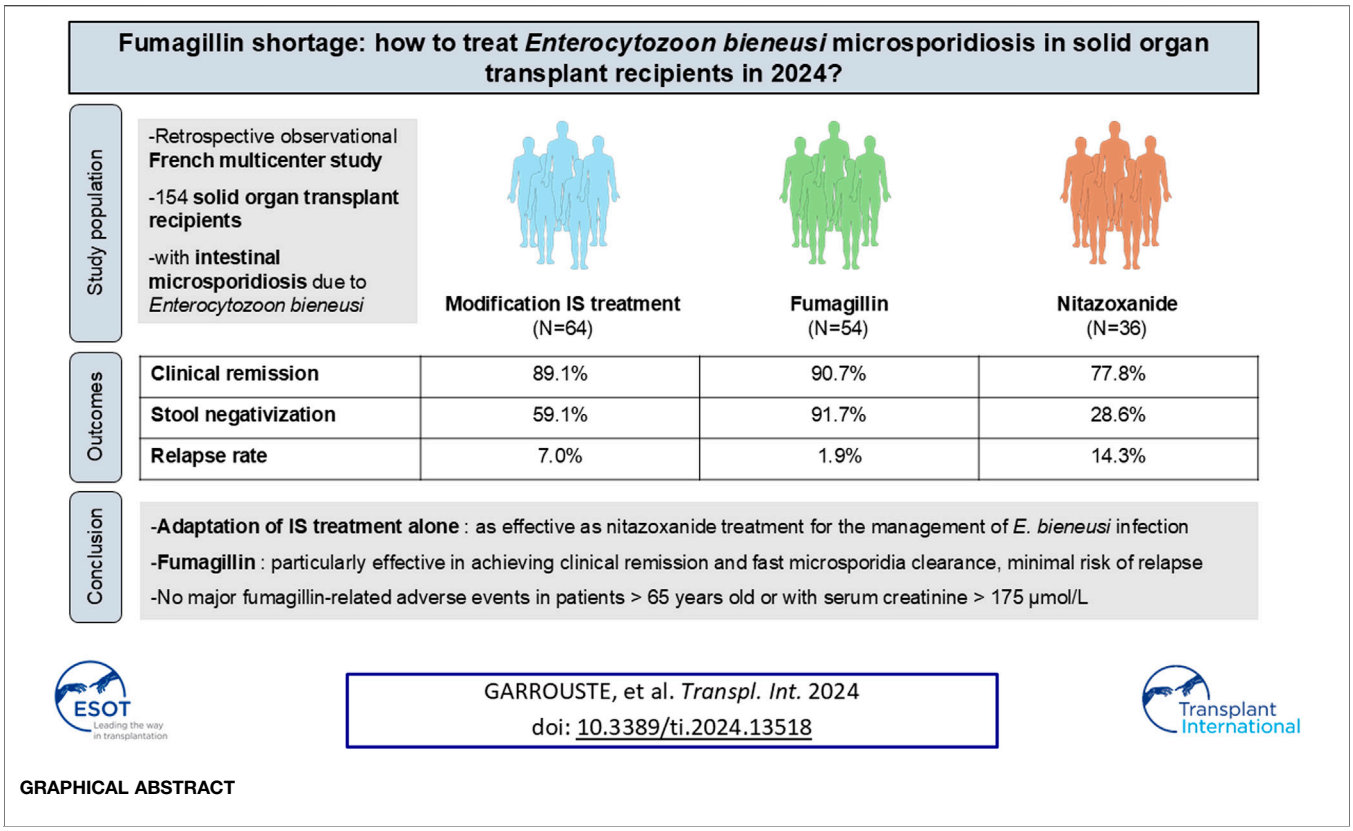
Management of these infections in SOT patients is not fully standardized but involves tapering immunosuppression, possibly also associated with specific treatment. Only fumagillin, a mycotoxin produced by the fungus *Aspergillus fumigatus*, has demonstrated effectiveness against *E. bieneusi* infections in clinical trials [4, 5]. Serious adverse events were observed in 25% of patients and especially included dose-related hematologic toxicity that manifested as thrombocytopenia and/or neutropenia, requiring hospitalization for the duration of treatment [6]. Fumagillin has been out of stock on several occasions in the past and has not been commercialized since 2019, in some cases leading to therapeutic impasse. In the absence of fumagillin, the proposed alternative is nitazoxanide, a broad-spectrum antiparasitic drug effective against a broad range of protozoan and helminthic infections. However, the effectiveness of nitazoxanide against microsporidia has never been formally evaluated, but some successes have been described in case reports [7–11]. Nitazoxanide has few reported serious side effects, but there have been occasional reports of nitazoxanide-induced liver injury [12].

Given this context of difficulty treating intestinal microsporidioses, the French National Reference Center for microsporidioses is regularly asked to advise on therapeutic options for SOT patients. Here, to address this need, we worked with the French network of transplant recipients to conduct a retrospective observational study of *E. bieneusi* infections and therapeutic management in a French cohort of SOT patients.

PATIENTS AND METHODS

Ethical Statement

This retrospective study was approved by our local research ethics committee ("Comité de Protection des Personnes Sud-Est VI," France) and performed in compliance with French data privacy policy (approval #MPP220505).



GRAPHICAL ABSTRACT

Study Population

We used the French National Reference Center (NRC) for Microsporidiosis, the Spiesser and Divat groups, and the "Groupe français de recherche en greffe de foie" to retrospectively collect microsporidiosis cases in adult SOT recipients in France between 2018 and 2021 where *E. bieneusi* was identified as the causal agent. Briefly, since 2018 and on a voluntary basis, French laboratories diagnosing a case of microsporidiosis report their case to the NRC (by sending samples and providing clinical data). The network of participating laboratories is sufficiently extensive to allow representation of the entire French territory (mainland and overseas). The NRC case register was used to identify centers to contact for the present study. At the same time, two French transplant medicine networks, Spiesser and Divat, and the "Groupe français de recherche en greffe de foie", were also contacted to identify cases corresponding to the study inclusion criteria. So these two approaches allowed to identify as many cases of microsporidiosis as possible. As fumagillin production was discontinued in 2019, the number of patients treated with fumagillin over the 2018–2021 period was low compared to other management strategies. We therefore also included fumagillin-treated patients from the TRANS-SPORE registry, a previous retrospective observational study on microsporidiosis in French kidney transplant recipients for the period 2005–2017 carried out in six university hospital centers [2]. Cases were defined by the presence of persistent diarrhea (*i.e.*, ≥3 liquid stools per day for more than 2 weeks) and detection of microsporidia spores by microscopic examination of fecal smears (after Van Gool chemiluminescent staining, Weber's modified trichrome staining, or immunofluorescent staining) and/or molecular methods. Identification to species level was achieved using species-specific antibodies or PCR. Exclusion criteria were age <18 years, extraintestinal microsporidiosis, and microsporidiosis caused by a species other than *E. bieneusi*.

Patient demographics and medical records were retrieved from the hospital registries, and the following data were recorded: age, gender, type of organ transplant, retransplantations, date of current transplant, clinical presentation associated with microsporidiosis, values of biological parameters at diagnosis (hemoglobin, lymphocyte and CD4 counts, neutrophil count, platelet count, C-reactive protein (CRP), serum creatinine, residual concentrations of immunosuppressive drugs before and at diagnosis) and after treatment (platelet count, serum creatinine), microsporidiosis treatment, associated infections, relapses, and graft and patient outcomes at 1 year.

Definitions of Groups

The included patients were divided into three groups according to therapeutic management. (i) Patients who did not receive any specific drug against *E. bieneusi* and who were managed with a modification of immunosuppressive (IS) treatment that could be carried out in a context of a too high IS trough levels at diagnosis of microsporidiosis or in a context of 'normal' trough levels per standard-of-care [13, 14], belonged to the "MIT" group. (ii) Patients who received fumagillin, with or without modification of the IS treatment, were included in the "FUM" group. (iii) Patients

TABLE 1 | Clinical characteristics of the patients.

	All patients (n = 154)	MIT (n = 64, 41.6%)	Fumagillin (n = 54, 35.1%)	Nitazoxanide (n = 36, 23.4%)	p
Age at diarrhea onset (years) (n = 145)	56.2 ± 14.5	53.6 ± 15.0	56.6 ± 15.3	60.5 ± 11.4	0.09
Male sex	86 (55.8)	31 (48.4)	35 (64.8)	20 (55.6)	0.20
Previous transplant	26 (16.9)	8 (12.5)	11 (20.4)	7 (19.4)	0.47
Transplant(s)					
Kidney	144 (93.5)	59 (92.2)	51 (94.4)	34 (94.4)	0.92
Heart	8 (5.2)	3 (4.7)	1 (1.9)	4 (11.1)	0.17
Liver	6 (3.9)	3 (4.7)	3 (5.6)	0 (0.0)	0.50
Lung	1 (0.6)	1 (1.6)	0 (0.0)	0 (0.0)	1.00
Pancreas	3 (1.9)	2 (3.1)	0 (0.0)	1 (2.8)	0.46
Multi-organs transplant	7 (4.5)	3 (4.7)	1 (1.9)	3 (8.3)	0.43
Baseline serum creatinine (μmol/L) (n = 101)	142 [112; 180]	135 [110; 175]	146 [112; 200]	146 [127; 175]	0.68
Time between last transplantation and first positive PCR (years) (n = 151)	6 [2; 10]	6 [2; 10]	6 [3; 11]	5 [3; 10]	0.98

Data are presented as number of patients (percentages), mean ± standard deviation, or median [25th; 75th percentiles]. Comparisons between groups were made with Chi-squared test or Fisher's exact test for categorical data, and with ANOVA, or Kruskal-Wallis test for quantitative data. In the first column, "n" is the number of available data when there is missing data. MIT: modification of immunosuppressive treatment; PCR: polymerase chain reaction.

who received nitazoxanide, with or without modification of the IS treatment, belonged to the "NTZ" group. Patients who received one treatment then the other were classified under their second treatment group (considering that the first had not been effective).

Three criteria were studied to evaluate the effectiveness of therapeutic management: (i) the resolution of clinical manifestations (*i.e.*, resolution of diarrhea) at the end of the treatment/modification of the IS treatment, (ii) the stool negativization (*i.e.*, stool clearance), determined by PCR during and/or after treatment, and (iii) the clinical relapse (reappearance of diarrhea) rate.

Statistical Analysis

No sample size estimation was performed. As this is a rare disease, we aimed for the exhaustiveness. Statistical analysis was performed using Stata software (version 15; StataCorp, College Station, TX). All tests were two-sided, with an alpha level set at 5%. Categorical data are reported as number of patients and percentages, and quantitative data are reported as mean ± standard deviation or median [25th; 75th percentiles]. Baseline between-groups comparisons were performed using a Chi-squared test or Fisher's exact test for categorical data, and ANOVA or a Kruskal-Wallis test for quantitative data. Between-groups comparisons on outcomes were performed using linear or generalized linear mixed models, with hospital center as random effect. In particular, two multivariable analyses were performed on clinical remission using a mixed effects logistic regression. In these analyses, the fixed effects were: group, age, sex, and serum creatinine at diagnosis, in the first model; and group, age, sex, and renal failure, in the second model. Finally, time to stool negativization and time to clinical remission (censored data) were estimated by the Kaplan-Meier method and compared between groups using the log-rank test.

RESULTS

Study Population

The characteristics of the patient cohort are given in **Table 1**. A total of 154 patients from 26 French hospital centers were included. They were mainly males (55.8%), aged 56.2 ± 14.5 years, and the vast majority kidney transplant recipients (93.5%).

The MIT group included 64 patients (41.6%), the FUM group included 54 patients (35.1%) and the NTZ group included 36 patients (23.4%) (**Table 1**).

There were 27 patients treated with fumagillin from the TRANS-SPORE registry [2] and 27 from the French National Reference Center for microsporidiosis for the 2018–2021 period. There were no differences between these two subcohorts on any of the criteria tested, *i.e.*, clinical characteristics (age, gender, transplant, time to onset of microsporidiosis), clinical remission rate, creatinine at 3 months, relapse, organ failure, and death (data not shown).

Clinical and Biological Presentation at Microsporidiosis Diagnosis

Microsporidiosis onsetted at a median time of 5.6 [2.3; 9.7] years following transplantation (**Table 1**). In addition to diarrhea, more than half (53.9%) of the patients presented weight loss (approximately 8% [5; 12] of ideal weight) (**Table 2**). The other reported symptoms were nausea/vomiting (37.7%), asthenia (20.8%), abdominal pain (20.1%), fever (5.2%), anorexia (3.9%), dehydration (3.2%), bloating (1.9%), hypotension (1.3%), and dysphagia (1.3%). There were no between-group differences in these symptoms except for abdominal pain ($p = 0.045$, **Table 2**). Microsporidia diagnosis was performed with a median delay of 19 [12; 39] days after the onset of diarrhea. At diagnosis, median values of hematological and inflammatory parameters were normal, except for lymphocyte count which was decreased (**Table 2**). More than half of the patients (n = 55/100, 55.0%) had acute renal failure according to the acute kidney injury network (AKIN) classification [15], of which 34 (34.0%) had AKIN 2 or 3 renal failure (**Table 2**). Overall, median serum creatinine value was 187 [124; 293] μmol/L at diagnosis. Note that even though fumagillin is contraindicated for creatinine values above 175 μmol/L, median serum creatinine value in the

TABLE 2 | Clinical presentation of microsporidiosis and laboratory characteristics at diagnosis.

	All patients (n = 154)	MIT (n = 64)	Fumagillin (n = 54)	Nitazoxanide (n = 36)	p
Symptoms					
Diarrhea	154 (100)	64 (100)	54 (100)	36 (100)	NA
Fever (>38.5°C)	8 (5.2)	5 (7.8)	1 (1.9)	2 (5.6)	0.41
Nausea/vomiting	58 (37.7)	24 (37.5)	18 (33.3)	16 (44.4)	0.57
Asthenia	32 (20.8)	15 (23.4)	10 (18.5)	7 (19.4)	0.79
Abdominal pain	31 (20.1)	16 (25.0)	5 (9.3)	10 (27.8)	0.045
Weight loss	83 (53.9)	29 (45.3)	33 (61.1)	21 (58.3)	0.19
Weight loss (%) (n = 75)	8 [5; 12]	7 [5; 13]	9 [5; 11]	6 [5; 11]	0.71
Hospitalization rate	127 (82.5)	47 (73.4)	50 (92.6)	30 (83.3)	0.02
Serum creatinine (µmol/L) (n = 150)	187 [124; 293] (43; 838)	182 [136; 285] (85; 653)	191 [129; 278] (43; 838)	216 [118; 311] (85; 676)	0.88
Renal failure	55/100 (55.0)	27/49 (55.1)	14/23 (60.9)	14/28 (50.0)	0.74
AKIN stage					0.22
1	21/55 (38.2)	14/27 (51.9)	3/14 (21.4)	4/14 (28.6)	
2	14/55 (25.4)	7/27 (25.9)	4/14 (28.6)	3/14 (21.4)	
3	20/55 (36.4)	6/27 (22.2)	7/14 (50.0)	7/14 (50.0)	
CRP (mg/L) (n = 115)	4 [1; 11]	3 [1; 11]	5 [2; 15]	3 [1; 8]	0.31
Hemoglobin (g/dL) (n = 150)	11.6 [10.1; 12.9]	11.4 [10.0; 12.4]	11.7 [10.3; 13.4]	11.9 [10.1; 12.9]	0.87
Platelets (G/L) (n = 133)	226 [169; 268]	236 [168; 279]	219 [165; 264]	217 [173; 263]	0.96
PNN (G/L) (n = 133)	4.7 [3.3; 6.6]	4.7 [3.1; 7.1]	5.3 [3.3; 8.1]	4.5 [3.3; 5.8]	0.33
Lymphocyte (G/L) (n = 130)	1.08 [0.64; 1.60]	1.19 [0.77; 1.82]	1.10 [0.64; 1.81]	1.01 [0.51; 1.30]	0.08

Data are presented as number of patients (percentages), or median [25th; 75th percentiles] (range). Comparisons between groups were made with Chi-squared test or Fisher's exact test for categorical data, and with Kruskal-Wallis test for quantitative data. In the first column, "n" is the number of available data when there is missing data. AKIN: acute kidney injury network (score 3 is more serious than score 1); CRP: C-reactive protein; MIT: modification of immunosuppressive treatment; NA: not applicable; PNN: polynuclear neutrophil.

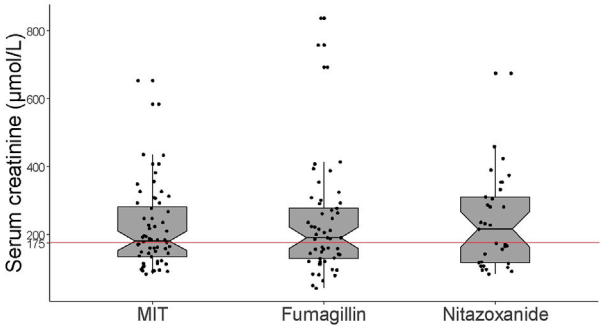


FIGURE 1 | Serum creatinine values at microsporidiosis diagnosis according to therapeutic management group. The red line at 175 µmol/L represents the maximum creatinine value beyond which fumagillin is contraindicated according to the safety notice. MIT: modification of immunosuppressive treatment.

FUM group was 191 [129; 278] µmol/L (**Figure 1**). In the MIT group, 51.9% of patients (n = 14/27) had AKIN 1 renal failure, whereas 50.0% of patients (n = 7/14) in both the FUM and NTZ groups had AKIN 3 renal failure. Nearly a third of patients (30.5%) had another concurrent infection (**Supplementary Table S1**).

Therapeutic Management

A high proportion of patients were hospitalized (82.5%), particularly patients receiving fumagillin (92.6% vs. 73.4% in the MIT group vs. 83.3% in the NTZ group, $p = 0.02$) (**Table 2**), which must be interpreted in light of the recommendations concerning its use. The vast majority of these hospitalizations (98.4%) were in conventional wards (only two patients were in intensive care units) for a median hospital stay of 7 [5; 14] days.

There were significantly fewer patients who received tacrolimus in the NTZ group (**Table 3**). Steroids dose was significantly higher in the FUM group compared to the others ($p = 0.003$). Nearly half of patients (n = 60/121, 49.6%) had significant tacrolimus trough levels (>10 ng/L) at the time of microsporidiosis diagnosis (**Table 3**).

Median duration of treatment with nitazoxanide and fumagillin was 14 [13; 21] and 14 [12; 14] days, respectively. Regarding nitazoxanide, three (8.3%) patients received less than 7 days of treatment, 17 (47.2%) patients received more than 14 days, and three (8.3%) patients received treatment several times for at least 14 days. Regarding fumagillin, seven (13.0%) patients received 7 days of treatment, and five (9.3%) patients received more than 14 days. The reasons for treatment durations of 7 days, whether for nitazoxanide or fumagillin, are not known.

Safety and Treatment Interactions

One patient (kidney transplant recipient) (2.8%) treated with nitazoxanide experienced hepatic adverse effects attributed to the treatment, but this did not lead to treatment discontinuation. Regarding patients treated with fumagillin, as expected, thrombocytopenia was reported in 86.8% (n = 33/38) of cases (**Supplementary Figure S1**). Median nadir platelet count was 70 [40; 124] G/L, and severe (*i.e.*, < 50 G/L) thrombocytopenia was observed in 34.2% (n = 13/38) of patients. Severe thrombocytopenia led to premature stoppage of treatment in four patients, after 10 days of treatment for two of them and 13 days for the other two. Only one patient developed a hemorrhagic event (an intra-alveolar hemorrhage which occurred a few days after stopping fumagillin when the

TABLE 3 | Management and follow-up.

	All patients (n = 154)	MIT (n = 64)	Fumagillin (n = 54)	Nitazoxanide (n = 36)	p
Immunosuppressive treatment at day 0					
Tacrolimus	134 (87.0)	58 (90.6)	50 (92.6)	26 (72.2)	0.02
Trough levels (ng/L) (n = 121)	10.0 [6.6; 14.7]	9.9 [7.1; 13.9]	9.8 [5.5; 14.7]	11.2 [6.5; 14.8]	0.85
Trough levels >10 ng/L	60/121 (49.6)	25/52 (48.1)	24/48 (50.0)	11/21 (52.4)	0.94
Mycophenolate mofetil	128 (83.1)	51 (79.7)	49 (90.7)	28 (77.8)	0.17
Dose (g/day) (n = 124)	1.0 [1.0; 1.5]	1.0 [1.0; 1.0]	1.0 [1.0; 1.5]	1.0 [0.8; 1.5]	0.63
Steroids	105 (68.2)	45 (70.3)	38 (70.4)	22 (61.1)	0.58
Dose (mg/day) (n = 103)	5.0 [5.0; 10.0]	5.0 [5.0; 5.0]	7.5 [5.0; 10.0]	5.0 [5.0; 10.0]	0.003
Everolimus	15 (9.7)	9 (14.1)	2 (3.7)	4 (11.1)	0.14
Dose (mg/day) (n = 15)	2.0 [1.5; 3.0]	2.0 [2.0; 4.0]	2.5 [2.0; 3.0]	1.5 [1.3; 1.8]	0.15
Cyclosporine	14 (9.1)	3 (4.7)	2 (3.7)	9 (25.0)	0.002
Dose (mg/day) (n = 13)	120 [100; 200]	160 [60; 400]	85 [50; 120]	120 [110; 200]	0.41
Azathioprine	5 (3.3)	2 (3.1)	2 (3.7)	1 (2.8)	1.00
Dose (mg/day) (n = 4)	88 [63; 100]	63 [50; 75]	100	100	0.26
Belatacept	4 (2.6)	1 (1.6)	3 (5.6)	0	0.36
Dose (mg/day) (n = 4)	340 [265; 395]	380	300 [230; 410]	—	0.65
Follow-up and outcome					
Clinical remission	134 (87.0)	57 (89.1)	49 (90.7)	28 (77.8)	0.23
Time from first symptoms to clinical remission (days) (n = 76)	10 [5; 21]	8 [5; 15]	14 [7; 24]	9 [5; 25]	0.28
Serum creatinine at month 3 (µmol/L) (n = 126)	144 [115; 203]	137 [110; 195]	159 [116; 203]	139 [117; 209]	0.69
Microsporidia stool monitoring	72 (46.8)	22 (34.4)	36 (66.7)	14 (38.9)	0.08
Stool negativization rate	50/72 (69.4)	13/22 (59.1)	33/36 (91.7)	4/14 (28.6)	0.002
Relapse	10/145 (6.9)	4/57 (7.0)	1/53 (1.9)	5/35 (14.3)	0.13
Organ failure at month 12	1/101 (1.0)	0/50 (0.0)	0/23 (0.0)	1/28 (3.6)	0.51
Death at month 12	1/124 (0.8)	0/50 (0.0)	1/47 (2.1)	0/27 (0.0)	0.60

Data are presented as number of patients (percentages), or median [25th; 75th percentiles]. Comparisons between groups for immunosuppressive treatment at day 0 were made with Chi-squared test or Fisher's exact test for categorical data, and with Kruskal-Wallis test for quantitative data. Comparisons between groups for outcomes were made with linear or generalized linear mixed models, with the center as random effect. In the first column, "n" is the number of available data when there is missing data. MIT: modification of immunosuppressive treatment.

thrombocytopenia worsened). For all patients, thrombocytopenia was reversible several days after stopping treatment. There was no effect of serum creatinine value at initiation of fumagillin treatment on fumagillin tolerance.

No drug–drug interactions were reported. In particular, there were no reported cases of difficulty obtaining the therapeutic target concentrations of IS drugs in the presence of fumagillin or nitazoxanide.

Follow-Up and Outcome

Three months after diagnosis, median serum creatinine value was 144 [115; 203] µmol/L. Symptoms associated with microsporidiosis disappeared for 134 (87.0%) patients, within a median of 10 [5; 20] days following the start of specific treatment and/or modification of IS treatment. Clinical remission rate was 77.8% for the NTZ group, 89.1% for the MIT group, and 90.7% for the FUM group, with no significant between-group differences (**Table 3**; **Figure 2**). By adjusting the effect of age, sex and serum creatinine, there is no difference in terms of clinical remission (n = 154; p = 0.49). Likewise, no difference was observed by adjusting the effect of age, sex and renal failure (n = 98; p = 0.78).

Microsporidia monitoring on patient stools was only carried out in less than half of the patients (46.8%). Stool negativization rate was significantly higher in the FUM group compared to MIT and NTZ groups (91.7% vs. 59.1% vs. 28.6% respectively, p = 0.002) (**Table 3**; **Figure 3**).

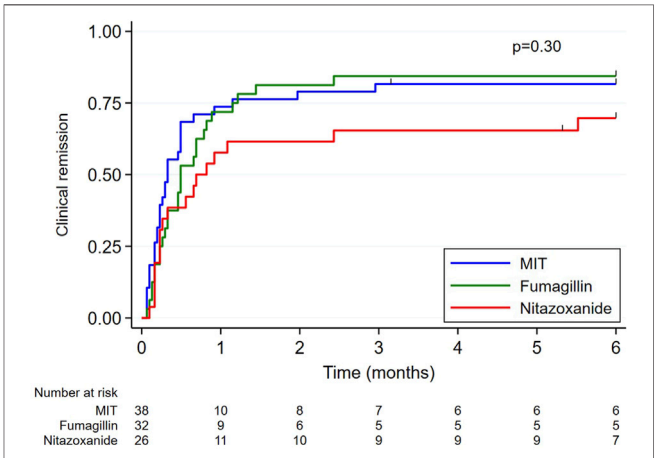


FIGURE 2 | Graphical representation of clinical remission times according to treatment. Clinical remission times (censored data) were estimated by the Kaplan-Meier method and compared between groups using the log-rank test. The analysis was carried out on 96 data, 58 clinical remission times not being specified. MIT: modification of immunosuppressive treatment.

Ten out of 145 patients (6.9%) relapsed. Median time to relapse was 150 [102; 526] days after the first positive-test sample of the first episode. Relapses tended to be more frequent in the NTZ group than in the MIT and FUM groups (14.3% vs. 7.0% vs. 1.9% respectively, p = 0.13). All relapsed

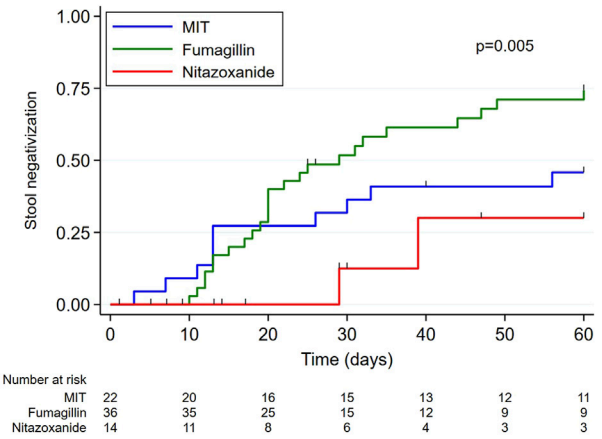


FIGURE 3 | Graphical representation of stool negativization times according to treatment. Times to stool negativization (censored data) were estimated by the Kaplan-Meier method and compared between groups using the log-rank test. MIT: modification of immunosuppressive treatment.

patients in the NTZ group received at least 14 days of nitazoxanide (two had received nitazoxanide for 30 days). Conversely, the lowest rate of relapses was observed in the FUM group, with only one case (1.9%). Note that this patient received fumagillin only during 7 days and relapsed after 294 days.

Graft and Patient Survival

Six kidney transplant recipients experienced acute graft rejection during the 3 months following microsporidiosis diagnosis (five MIT and one NTZ). All the rejection cases were attributed to the reduction in IS dose.

One patient, a kidney-pancreas transplant recipient belonging to NTZ group and diagnosed with chronic rejection before the onset of microsporidiosis, presented kidney transplant failure after 5 months.

One kidney transplant recipient in the FUM group died of hemorrhagic stroke at 5 months, with no direct link to microsporidiosis or treatment.

DISCUSSION

In the context of a fumagillin shortage since several years, we conducted this study to describe management practices and challenges with *E. bieneusi* microsporidiosis in SOT recipients.

More than 40% of the *E. bieneusi* microsporidiosis patients in this study were managed by adapting their IS treatment only, and the outcome was favorable in 89.1% of cases. Interestingly, patients treated with a specific anti-microsporidia drug (fumagillin or nitazoxanide) presented more severe clinical (*i.e.*, hospitalization rate, tri-therapy immunosuppressant regimen) and/or biological (*i.e.*, severe acute renal failure AKIN 3) status at the time of microsporidiosis diagnosis. Note that all six graft rejections identified in the 3 months following microsporidiosis were attributed to IS reduction as infection management strategy.

Clinical remission rates were not significantly different between groups but nitazoxanide tended to be the least effective strategy, with a clinical remission rate of 77.8%. Interestingly, nearly half of patients treated with nitazoxanide received more than 14 days of treatment, suggesting that 14 days may be too short. Dosage and duration of nitazoxanide treatment have not been defined for microsporidiosis, but previously published cases reported success following treatment with 1,000 mg nitazoxanide twice daily for 60 days, or 500 mg twice daily usually for 14 days but also up to more than a year [7–11]. Fumagillin was not more effective than IS management alone in obtaining clinical remission (90.7% and 89.1%, respectively). However, fumagillin was the most effective treatment for achieving stool negativization (91.7% vs. 28.6% for nitazoxanide alone and 59.1% for IS management alone). Nitazoxanide treatment also had the highest relapse rate, at 14.3%, compared to 1.9% for fumagillin and 7.0% for IS management alone, but the difference was not statistically significant. The only relapse after fumagillin treatment was associated with premature discontinuation of treatment, whereas fumagillin was effective for the other six patients who were also prematurely discontinued. This raises the question of the necessary duration of treatment in SOT recipients, as the recommended duration of 14 days had initially been defined from a cohort consisting mainly of HIV patients [4]. Finally, note that there were no graft rejections reported in fumagillin-group patients. Importantly, these comparisons between therapeutic strategies must also take into account that patients of each treatment group have different baseline characteristics, which introduces potential confounding factors and may influence the success or failure of treatment. However, by adjusting the effect of age, sex and renal failure, there is no difference in terms of clinical remission.

Interestingly, at the time of microsporidiosis diagnosis, almost 50% of patients had elevated tacrolimus levels (above 10 ng/mL). Over-dosing of tacrolimus was previously described in patients with infectious diarrhea [16, 17], including microsporidiosis [2]. This phenomenon is caused by the impaired function of P-glycoprotein efflux proteins, leading to an increase in intestinal absorption of tacrolimus between 90 and 360 min after intake [18].

Even though fumagillin proved very effective against *E. bieneusi*, its side effects and contraindications warrant caution. The tolerance and effectiveness of fumagillin have not been established for patients over 65 years old [19]. Likewise, serum creatinine higher than 175 µmol/L contraindicates the use of fumagillin, although there is apparently nothing in the summary of characteristics of the medicinal product to justify this point [19]. In our retrospective study, 18 fumagillin-group patients were between 65 and 83 years old and did not develop more adverse events than their younger counterparts [3/33 (9.1%) for patients under 65 and 1/18 (5.6%) for patients 65 and over]. Interestingly, median serum creatinine at initiation of fumagillin treatment was 191 [129; 278] µmol/L (maximum serum creatinine was as high as 838 µmol/L). Thrombocytopenia was observed in 86.8% of cases after 14 days of treatment, and as previously described by Maillard et *al.*, severe thrombocytopenia occurred

more frequently in patients with an initial platelet count below 200 G/L (**Supplementary Figure S1**) [6].

This study has certain limitations, mainly due to its retrospective design. Some results may be due to center-dependent effects, for example, fumagillin co-prescribed with high-dose steroids. The absence of systematic microsporidia follow-up (particularly at the end of the treatment) was also a limitation to confirming stool negativization after clinical remission, and it underscores the lack of clear recommendations on microsporidia follow-up of patients during the course of treatment. Such follow-up would also be informative to evaluate whether a fumagillin treatment duration of 14 days is necessary to achieve stool negativization in SOT patients. This lack of microsporidia follow-up further complexified the diagnosis of relapse: as no samples from the initial infection and relapse were available to compare the strains, we were unable to formally distinguish between a relapse and a new infection event on the one hand, but also between a relapse and a treatment failure on the other hand.

In conclusion, *E. bieneusi* infections in SOT recipients remain life-threatening diseases, as all cases of acute graft rejection in our cohort were attributed to the reduction in IS treatment required to manage microsporidiosis. However, adaptation of IS treatment alone was as effective as nitazoxanide treatment for the management of *E. bieneusi* infection. Fumagillin was particularly effective for achieve clinical remission and fast microsporidia clearance with minimal risk of relapse. Moreover, no major fumagillin-related adverse events were observed in patients over 65 years old or with serum creatinine above 175 μmol/L. The unavailability of fumagillin remains a problem for treatment, particularly for patients for whom it is not possible to modify IS treatment or with the most severe clinical presentation.

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 Comité de Protection des Personnes Sud-Est VI, France. 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

Conceptualization: CG, PP, and CN; methodology: CG, PP, CL, and CN; formal analysis: CG, CL, and CN; investigation: CG, PP, CU-C, XI, NK, JuB, EvC, SL, LL, BA, LR, M-FD, CDa, FM, CDe, AM-S, NO, DC, DB, AC, PG, MeR, EM, JD, ES, AS, SH, LC, CéD, CP, CL'O, VM, LD, HK, EsC, LE, JBr, SC, SV, CT, NA, QR, MaR, JN, AB, FB, MMa, A-PB, TC, JL, AL, AD, MN, JC, MMo, and CN; resources: CG, PP, CU-C, XI, NK, JuB, EvC, SL, LL, BA, LR, M-FD, CDa, FM, CDe, AM-S, NO, DC, DB, AC, PG, MeR, EM, JD, ES, AS, SH, LC, CéD, CP, CL'O, VM, LD, HK, EsC, LE, JBr, SC, SV, CT, NA, QR, MaR, JN, AB, FB, MMa, AP-B, TC, JL, AL, AD, MN, JC, MMo, and CN; data curation: CG, CL, and CN; writing–original draft: CG and CN; writing–review and editing: CG, PP, CU-C, XI, NK, JuB, EvC, SL, LL, BA, LR, M-FD, CDa, FM, CDe, AM-S, NO, DC, DB, AC, PG, MeR, EM, JD, ES, AS, SH, LC, CéD, CP, CL'O, VM, LD, HK, EsC, LE, JBr, SC, SV, CT, NA, QR, MaR, JN, AB, FB, MMa, AP-B, TC, JL, AL, AD, MN, JC, MMo, CL, and CN; visualization: CG and CN; supervision: CG and CN; project administration: CG and CN. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

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

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2024.13518/full#supplementary-material

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LETTER TO THE EDITOR
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Can We Noninvasively Rule Out Acute Rejection? External Validation of a Urinary Chemokine-Based Model

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Keywords: biomarkers, chemokine CXCL9, chemokine CXCL10, graft rejection, kidney transplantation (KT)

Dear Editors,

One of the major unmet needs of kidney transplantation is the availability of validated biomarkers for the noninvasive diagnosis of rejection [1]. This is especially true in clinically stable patients at low immunological risk [2], who are less likely to benefit from invasive surveillance biopsies. Emerging evidence support the combined use of noninvasive biomarkers and clinical parameters for risk-stratification [3–5].

A large multicentric cohort study showed that adding plasma donor-derived cell-free DNA (dd-cfDNA) to a standard of care prediction model improves discrimination for acute rejection in kidney transplant recipients (KTRs) [4]. However, dd-cfDNA is less sensitive in detecting T-cell-mediated rejection (TCMR) compared to antibody-mediated rejection (ABMR), especially when early and borderline lesions are present [6, 7].

Therefore, interest in alternative biomarkers of TCMR, including urinary chemokines CXCL9 and CXCL10, is growing [5, 8, 9]. Thanks to the availability of the Ella Automated Immunoassay System, multiple urinary chemokines can be inexpensively quantified in urine supernatant [3]. Recently, a large single-center prospective cohort study developed a predictive model for acute rejection (AR) based on integrating urinary chemokines with routine clinical markers, such as BK Polyoma virus (BKPyV) DNAemia, presence of circulating donor-specific anti-HLA antibodies (DSAs), and eGFR (MDRD formula). The model has a high diagnostic discriminatory value for detecting AR (ROC AUC 81.3%) [3]. The authors argued that implementing this model would allow avoiding 59% of the biopsies, as patients classified at low AR risk could safely skip the biopsy [3]. One potential limitation of this model is the fact that BKPyV DNAemia and urinary chemokine measurements may suffer from large inter-laboratory variability. Therefore, the predictive performance of the model might deteriorate upon validation in external and completely independent cohorts that use different labs.

Herein, we aimed to externally validate the model in a consecutive series of KTRs who underwent a for-cause or surveillance kidney biopsy at the University Hospital of Parma, Parma, Italy. The study was approved by the local Institutional Review Board (IRB) (Protocol #46898, 24/11/2020), and all the patients signed informed consent to the study.

Mid-stream urinary samples were collected on the day of the biopsy (before the procedure) for urinary chemokine analyses. The samples were centrifuged, and the urine supernatants were frozen at −80°C within 4 h from the collection, as previously described [8]. Thawed samples were run in batches on Simple Plex assay for dual detection kit for CXCL9 and CXCL10 (Biotechne, Minnesota, USA. cat# SPCKC-PS-001623). For the analyses, we considered the average of the triplicate values.

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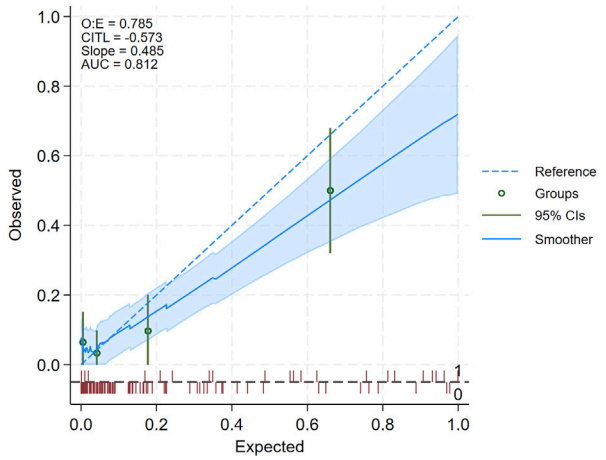

FIGURE 1 | Calibration plot summarizing the results of model external validation. The diagonal dotted blue line represents the line of identity between observed and expected acute rejection (AR) positive biopsies, while the solid blue line represents the smoothed regression line: a perfect model prediction would cause the solid blue and dotted blue line to overlay exactly. When the solid blue line is above the dotted blue lines, the model underestimates the AR risk, if it is below, it overestimates the risk. The shaded area represents the 95% confidence interval of the regression line: if the dotted line falls within the margin of the shaded area, then the difference between the observed and predicted can be regarded as statistically non-significant. Another hint to infer whether the difference is statistically significant is based on the green dots and the green vertical lines representing, for each quartile of AR risk, the estimated observed risk and 95% confidence interval: if the vertical green line does not cross the dotted blue line, then the difference between observed and expected can be regarded as statistically non-significant. The red rug (spike) plot at the bottom represents the number of patients, with positive (=1, above the dotted gray horizontal line) and negative (=0, below the gray horizontal line) biopsies. In the upper left corner are reported the ratio of observed to expected positive biopsies (O:E), Calibration-In-The-Large (CITL) namely, the average predicted AR risk is compared with the overall event rate, the slope of the regression line of observed vs. expected, and the Area Under (AUC) of the ROC curve.

BKPyV DNAemia copies were detected using real-time PCR and DSAs were detected by Luminex xMAP (LIFEcodes Class I and II kit, Immunocor).

We included 124 kidney transplant recipients (N = 21 with AR), aged 48.5 ± 12.7 years. As shown in **Supplementary Table S1**, 62.1% were males, 10.5% received a living donation, 12.9% were re-transplantation, and 3 patients (2.4%) received ABO/HLA incompatible kidneys. The patients with a diagnosis of AR received more often Thymoglobulin induction (35.0% vs. 13.6%, P = 0.045). Acute rejection episodes were T-cell mediated in 10 (47.6%) of the cases and antibody mediated or mixed in the remaining ones. At the time of biopsy, DSAs were detected more often in the rejecting patients (28.6% vs. 6.8%, P = 0.009), while there was no difference in MDRD eGFR at the biopsy and in BKPyV DNAemia positivity or copies/mL (**Supplementary Table S1**). The diagnostic performance of urinary chemokines in this cohort is reported in the **Supplementary Material**. **Figure 1** shows the calibration plot of observed against expected probabilities of AR [10]: calibration is plotted in groups across the AR risk spectrum, and via a smoothed regression line, both with the associated 95% confidence intervals (see **Supplementary Material**, for further details).

The plot shows that the model's expected and observed AR risks align in patients at the lower risk end of the spectrum. Consistently, the shaded blue area, which represents the 95% confidence interval of the regression line, and the 95% confidence interval of the quartile of AR risk (vertical green line), included the line of identity for the lower bounds of AR risk (left-hand side of the plot). In contrast, for expected AR risk above approximately 0.4 (i.e., 40%, right-hand side of the plot), the model tended to overestimate the risk of AR. The upper left corner of the plot reports the performance statistics which confirmed that predicted AR risk slightly overestimated observed AR risk, as the value of the observed to expected ratio (O:E) and of the slope were both below 1, and the value of the CITL (Calibration-In-The-Large, i.e., average predicted AR risk is compared with the overall event rate) was below zero. On the other hand, the AUC of the ROC curve (81.2%) showed good model discrimination.

We acknowledge that model validation was carried out on a limited number of subjects compared to the original cohort. However, this is, to the best of our knowledge, the first attempt to validate an integrated model based on urinary chemokines CXCL9 and CXCL10 in an independent cohort of subjects. Moreover, our findings are remarkably similar to those of the original cohort. In fact, discriminatory capacity was identical to that estimated in the original cohorts (AUC of the ROC curve 81.2% [95 percent confidence interval: 69.1 to 93.2] vs. 81.3% of the original study). The model on average, overestimates the risk of AR, a trend which was also partially observed in the original study [3]. However, overestimation occurred only for patients at the higher AR risk of the spectrum. We also drew a Decision Curve Analysis (**Supplementary Figure S1**), which confirmed that the model is useful for decision-making purposes for threshold probabilities up to 50% (the threshold probability is the minimum probability of AR at which a decision-maker would take the decision to perform a biopsy).

In conclusion, our findings on an independent cohort of patients support the utility of this model for identifying patients at low risk of AR in whom biopsy can be safely avoided.

DATA AVAILABILITY STATEMENT

Datset will be made available to other researchers following publication upon request. Further inquiries can be directed to the corresponsing author.

ETHICS STATEMENT

The studies involving humans were approved by the Comitato Etico Area Vasta Emilia Nord. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; IG, BM, and OB conducted the experiments. UM performed the statical analysis. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

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

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2024.13810/full#supplementary-material

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