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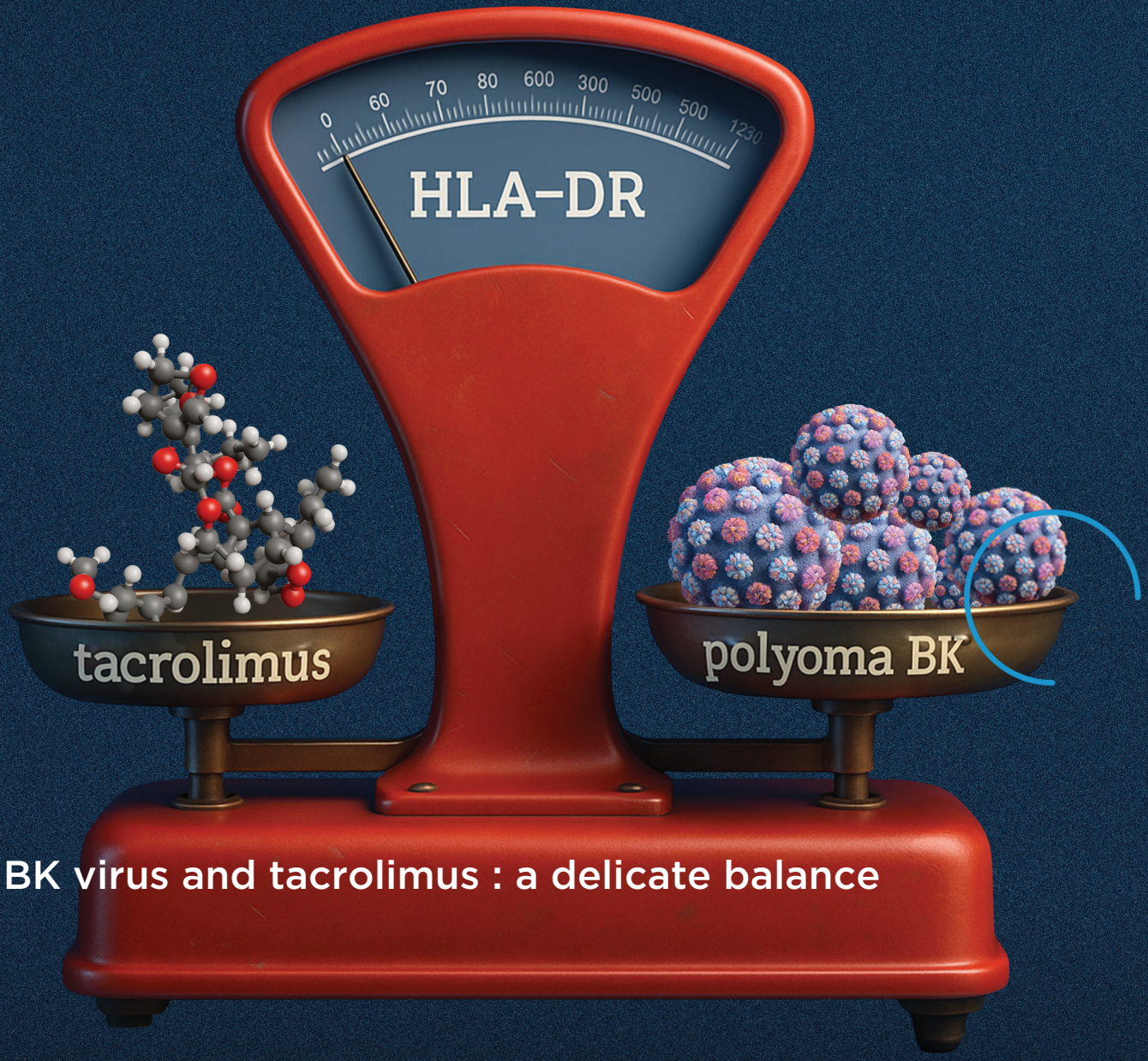

HLA, BK virus and tacrolimus : a delicate balance

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ORIGINAL RESEARCH
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T cell Activation Marker HLA-DR Reflects Tacrolimus-Associated Immunosuppressive Burden and BK Viremia Risk After Kidney Transplantation – An Observational Cohort Study

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Kidney transplantation (KT) is the current treatment of choice in patients with end-stage kidney disease. Immunosuppression is required to prevent acute rejection but is associated with a high incidence of adverse events. The immunosuppressive burden substantially differs between individuals, necessitating new immune monitoring strategies to achieve personalization of immunosuppression. To compare the evolution of T cell profiles in correlation with immunosuppression and clinical outcomes, 87 kidney transplant recipients were followed for 12 months after KT. Flow cytometry along with assessment of T cell activation markers and clinical data was performed before KT and during study visits 10 days, 2 months and 12 months after KT. Longitudinal T cell phenotyping revealed a significant decrease of T cell activation markers HLA-DR, FCRL3, and CD147 in $CD4^+$ effector T cells after KT. The most pronounced reduction (75%) was found for the activation-proliferation marker HLA-DR, which persisted throughout the observational period. The decrease in HLA-DR expression reflected immunosuppressive burden through strong associations with tacrolimus trough-level exposure (coeff = –0.39, $p < 0.01$) and BK viremia incidence (coeff = –0.40, $p < 0.01$) in multivariable regression analysis. T cell activation marker HLA-DR emerges as a potential biomarker for tacrolimus-related immunosuppressive burden in association with BK viremia risk following KT.

Keywords: immune monitoring, immunosuppression, kidney transplantation, translational nephrology, personalized medicine

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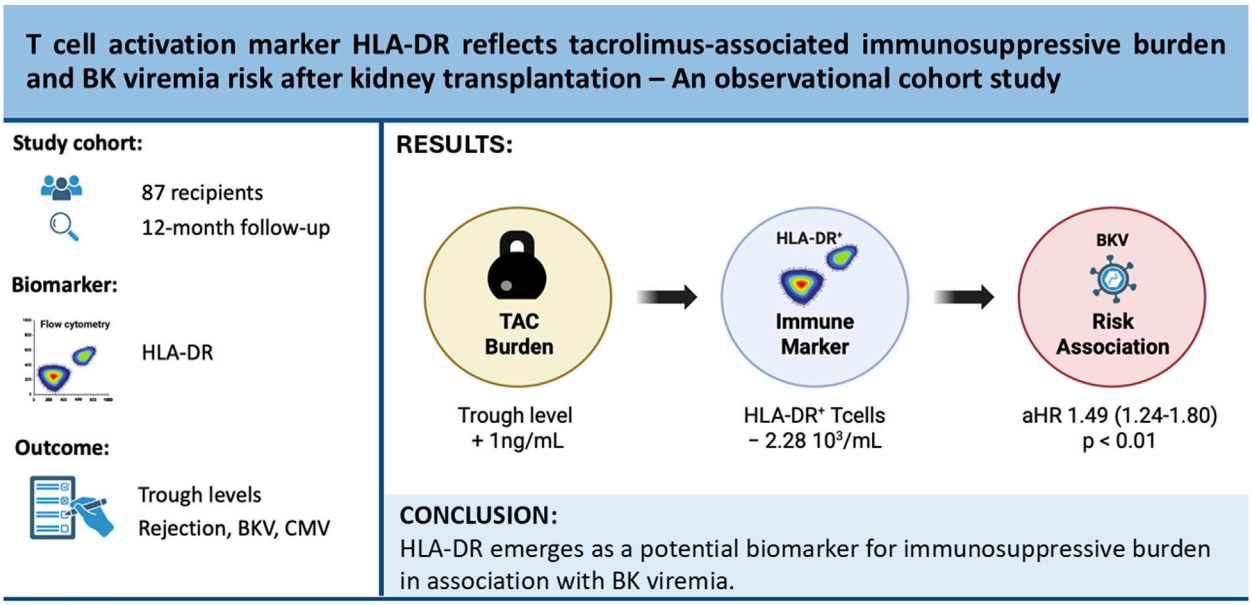

GRAPHICAL ABSTRACT |

INTRODUCTION

Kidney transplantation (KT) is the current treatment of choice in patients with kidney failure due to survival benefit and improved quality of life. Despite the administration of high-dose immunosuppressive therapy, acute rejection still affects over 10% of kidney transplant recipients (KTR) within the first 12 months [1]. Prolonged or repeated exposure to high-dose immunosuppression is associated with frequent adverse events including metabolic complications, susceptibility to infections and increased risk of malignancy [2]. The trough-level-guided use of calcineurin inhibitors is the cornerstone of T cell suppression in most immunosuppressive regimens. However, the biologically evident level of immunosuppression may vary substantially between individual patients. This variability demands biological effect measures to monitor the overall fitness of the immune system and guide treatment decisions in post-transplant care.

Assessment of the individual immune profile by immune cell phenotyping is currently emerging as a research field with prospects in autoimmunity, oncogenesis, and transplantation [3]. Single-cell sequencing and spatial transcriptomics of kidney allograft biopsies have been used to elucidate cellular interplay in acute rejection after KT, showing $CD4^+$ and $CD8^+$ T effector cells (T_{eff}) as well as innate immune cells (i.e., natural killer cells) expressing a variety of activation markers (i.e., FcγRIII, FCRL3, CD25, HLA-DR) [4, 5]. In peripheral blood $CD4^+$ and $CD8^+$ T_{eff} these activation markers have been shown to correlate with antigen-induced proliferation (i.e., HLA-DR) [6] and acute rejection (i.e., CD28, HLA-DR) [7, 8]. On the other hand, $CD4^+$ and $CD8^+$ T cells over-expressing markers of T cell senescence (i.e., TIGIT, LAP) [9, 10] correlate with exhaustion of donor-specific effector T cells positively impacting long-term graft tolerance [11], while activated regulatory $CD4^+$ T cells exert tolerogenic effects already early after KT [12]. The biological effect of tacrolimus has been demonstrated to significantly impact the differentiation and proliferative capacity of $CD4^+$ T cell populations [13], making $CD4^+$ T cells a potential surrogate marker for CNI-associated immunosuppressive burden in translational research. Other immune markers include Torque Tenov viral load starting 2–3 months after KT [14]. However, appropriate markers especially during the first 8 weeks after KT are still missing.

There is currently a lack of comprehensive data regarding differential biological effects of immunosuppressants on T cell profiles following transplantation. Exploring these changes may i) help to individualize CNI prescription in difficult-to-treat patient subgroups and ii) identify T cell markers correlating with immunosuppressive burden, which could be used as new immune monitoring tools after KT. We therefore chose to conduct a prospective, biologic effect study in a cohort of kidney transplant recipients (KTR) by correlating pharmacological data and clinical outcomes with longitudinal phenotyping of T cell activation markers before and after KT.

MATERIALS AND METHODS

Study Design and Population

A longitudinal, single-center cohort study evaluating immune cell subpopulations and short-term post-transplant outcomes in 87 KTR was conducted. The study was designed to prospectively enroll low-immunological risk KTR between September 2017 and August 2020 [15] (Study flowchart: **Supplementary Figure S1**). Patients receiving immunosuppression within the past 3 months, AB0-incompatible KT, repeated KT and high immunological risk patients were not included in the study (exclusion criteria are further detailed in the **Supplementary Material**). All patients received basiliximab or ATG, prednisone, mycophenolate, and tacrolimus per standardized protocols. Blood sampling and clinical data collection were performed pre-transplant (preKT), and at 10 days (D10), 2 months (M2), and 12 months (M12) post-transplant. Complete follow-up was obtained for 87 patients to perform a cohort analysis. The study protocol was approved by the Ethics Committee of the Medical University of Graz, Austria (ID 28-514 × 15/16).

T cell Phenotyping

Flow cytometry was conducted on peripheral blood mononuclear cells (PBMCs) isolated from whole blood samples, collected at study visits. Purified cells were stained with selected monoclonal antibodies (**Supplementary Table S1**) with BD LSR Fortessa Flow Cytometer (BD Biosciences, USA). T cell phenotyping included $CD4^+$ regulatory T cells (T_{reg}) defined as $CD3^+CD4^+CD127^-Foxp3^+$ according to OMIP-053 by Nowatzky et al [16], considering the interaction of T_{reg} marker CD25 with anti-CD25 antibody basiliximab [17]. $CD4^+$ effector T cells (T_{eff}) were conventionally defined as $CD3^+CD4^+CD25^-CD127^+CD45RA^-$ and confirmed as being $Foxp3^-$ (**Supplementary Figure S2**). Our selected antibody panels reflecting T cell activation status (including FCRL3, HLA-DR, CD147, CD15s, Ki67) were then separately studied on $CD4^+$ T_{reg} and T_{eff} populations (**Supplementary Table S2**). Gating and exploration of data using tSNE (t-distributed stochastic neighbor embedding) and FlowSOM/ClusterExplore algorithm were done by FlowJo analysis software (BD Biosciences, USA).

Tacrolimus Data

Tacrolimus dose and trough levels (TL) were recorded weekly to biweekly during the first 12 weeks after KT and at M12. Therapeutic drug monitoring of tacrolimus TL was performed by a validated LC-MS/MS assay. Tacrolimus TL targets were 8–10 ng/mL during the first 2 months and 6–9 ng/mL thereafter. The high granularity of tacrolimus TL data during the first 12 weeks after KT was transposed into a TL trendline. Tacrolimus-associated immunosuppressive burden was then estimated as the area under the curve (AUC) of the tacrolimus TL trendline by trapezoidal rule [18]. This estimate of cumulative tacrolimus TL exposure referred to as "TL AUC" throughout the manuscript.

Clinical Data

Occurrence and clinical data of biopsy-proven acute rejection (BPAR; using Banff 2019 classification [19]), CMV viremia (defined as ≥100 copies/mL), and BK-viremia (defined as ≥ 200 copies/mL) were documented at each study visit. Screening for viremia was done according to local practice guidelines every 7–14 days during the first two months, followed by readings every other month during the first year after KT. KTR with CMV D^+/R^- status received prophylaxis for 6 months, otherwise a preemptive strategy was followed. Kidney biopsies were performed by indication and at the local physician's discretion only.

Statistical Analysis

Baseline characteristics were summarized using descriptive analysis with mean ± standard deviation (SD) or median with interquartile range (IQR) for continuous variables and frequency tables for categorical variables. Continuous variables were tested for normality with Shapiro–Wilk tests and QQ plots. Parametric and non-parametric tests were used for group comparison where appropriate, with multiplicity adjustment by Holm-Sidak method. For the longitudinal assessment of T cell counts, a linear mixed-effects model was fitted using restricted maximum likelihood (REML) estimation, including time as a fixed effect and patients as random intercepts. Spearman correlation coefficient was used to assess the simple relationships between the independent variables TL AUC and T cell counts.

To further explore the underlying immunologic and pharmacologic relationships in a translational approach, we first assessed whether tacrolimus exposure (TL AUC) was associated with immune activation by modeling HLA-DR^+ T_{eff} counts as a dependent variable in a multivariable linear regression, with TL AUC as the main predictor. A cox regression was then used to assess whether HLA-DR^+ T_{eff} counts were associated with outcomes (BKV, CMV, BPAR) independent of TL AUC. The proportional hazards assumption using Schoenfeld residuals was confirmed. HLA-DR^+ T_{eff} counts measured on day 10 and month 2 post-transplant were modeled as time-dependent covariates, corresponding to event occurrence before and after month 2, respectively. Multivariable models were adjusted for immunosuppression-related confounders with known associations with both the exposures (tacrolimus exposure, T cell counts) and outcomes (BKV, CMV, BPAR), including induction agent, CNI formulation, mean mycophenolate mofetil dose, and cumulative steroid exposure. In addition, we assessed univariable associations of donor- and recipient-related characteristics. Among these, age, sex and KDRI met the inclusion threshold ($p < 0.20$) and were retained in multivariable models to balance clinical relevance with statistical parsimony to minimize overfitting. Time-dependent receiver operating characteristic (tdROC) curve was used to determine the predictive capability and cutoff of T cell counts for BK viremia risk. BK viremia incidence was then displayed by Kaplan-Meier curves above and below the predictive cutoff of day 10 (prior to any event) with log-rank test. All statistical analysis

TABLE 1 | Donor and recipient characteristics with immunosuppressive regimes are presented as mean (M) ± standard deviation (SD) when normally distributed and otherwise as median (MDN) and interquartile range (IQR) or absolute number (N) with relative percentage (%) for the whole cohort.

Recipient characteristics		**N = 87**
Female N (%)		32 (36.8%)
Male N (%)		55 (63.2%)
Age [years] MDN (IQR)		59 (53–66)
BMI [kg/m^2] MDN (IQR)		27.9 (23.6–29.1)
Hemodialysis		71 (82%)
Peritoneal dialysis		13 (14.7%)
Preemptive transplantation		3 (3.3%)
Dialysis vintage [mo] (MDN ± IQR)		29 (24–35)
Diabetes mellitus		16 (18%)
Arterial hypertension		84 (97%)
ADPKD		16 (18.4%)
Ethnicity N (%)		
Caucasian		82 (94%)
Turkish		2 (2.4%)
Asian		1 (1.2%)
Other		2 (2.4%)
Donor characteristics		**N = 87**
Age [years] MDN (IQR)		57.5 (49–67)
BMI [kg/m^2] MDN (IQR)		26.2 (24.1–28.5)
Expanded-criteria donor		51 (58.6%)
Donor after cardiac death		4 (4.6%)
KDRI MDN (IQR)		1.15 (1.02–1.23)
HLA mismatch N (%)		
0		2 (2.3%)
1		4 (3.4%)
2		6 (6.7%)
3		24 (28.6%)
4		35 (40.6%)
5		15 (17.2%)
6		1 (1.2%)
Immunosuppression		**N = 87**
Induction agent		
Basiliximab		82 (94.3%)
Anti-thymocyte globulin		5 (5.7%)
Maintenance regime		
Glucocorticoids		87 (100%)
Mycophenolic acid		87 (100%)
Tacrolimus	Twice daily	55 (63.2%)
	Once daily	32 (36.8%)

and data visualization was done with R Statistical language (version 4.3.2; R Foundation for Statistical Computing, Vienna, Austria). The following packages were utilized: "tidyverse", "lme4", "survminer", "survival", "Evalue" and "ggplot2". A p-value <0.05 was considered statistically significant.

RESULTS

Characteristics of the Study Cohort

Recipients were of Caucasian ethnicity (>90%), with a male preponderance (63%) and a median pretransplant dialysis vintage of 29 months (**Table 1**). The median recipient age was 59 years, the mean recipient BMI was 27 and the median KDRI was 1.15 (**Table 1**). Patients received basiliximab (94.3%) or low-dose ATG (5.7%) for induction, with an initial tacrolimus daily-dose of 0.1 mg/kg, alongside corticosteroids and mycophenolic acid for maintenance by standard protocol. Patients receiving ATG tended to be younger with a higher number of HLA-mismatches (**Supplementary Table S3**). Mean tacrolimus TL was 10.2 (±3.1) ng/mL at day 10, decreasing to 6.3 (±1.3) ng/mL by M12 (**Supplementary Table S4**). Recorded events included BPAR n = 16 (15 TCMR, 1 mixed TCMR-ABMR, median time-to-event 14 days), BKV n = 21 (median peak-level 1.1 log^4 and time-to-event 59 days) and CMV n = 48 (median peak-level 1.3 log^3 and time-to-event 67 days), (**Supplementary Table S5**; **Supplementary Figure S7**).

T cell Activation Marker HLA-DR Identifies Effector T cells Susceptible to Tacrolimus

To identify CD4$^+$ T cell subpopulations changing after induction therapy, we compared CD4$^+$ T_{eff} and CD4$^+$ T_{reg} immediately before transplantation (preKT) and 2 months after transplantation (M2) by unsupervised cluster-based analysis stratified by T cell activation status.

Among CD4$^+$ T_{eff}, activated clusters expressing activation markers CD147^{high}, FCRL3$^+$ and HLA-DR$^+$ were significantly reduced at M2, while non-proliferating and naive CD45RA$^+$ T cell clusters did not change (**Figure 1A**). Quantitative, longitudinal comparison of T cell subsets identified only HLA-DR$^+$ T_{eff} to significantly decrease already at D10 after KT and remain significantly reduced until M12 (**Figures 1B–D**), while FCRL3$^+$ and CD147^{high} T_{eff} returned to baseline by M12 (**Supplementary Figure S3**). Calculation of the relative change from baseline revealed that the nadir of HLA-DR$^+$ T_{eff} counts was reached at D10 (−75.6% from baseline), and cell counts showed an increasing trend at M2 (−64.7% from baseline), however, they remained significantly decreased at M12 (−22.3% from baseline), (**Supplementary Table S6**).

Among CD4$^+$ T_{reg}, a transient decrease of proliferative and activated Foxp3$^+$CD45RA$^-$CD15s$^+$ effector T_{reg} after KT with a general shift towards a CD45RA$^+$CD15s$^-$ resting phenotype (**Figure 2A**) was noted. However, proliferative and effector T_{reg} were fully replenished by M2 or between M2 and M12 (**Figures 2B-E**), and expression of Foxp3 followed the same trend (**Supplementary Figure S4**). The known interference of basiliximab with anti-CD25 monoclonal antibodies was evident at D10 and M2 in contrast to patients treated with ATG, however, no major differences were found in Foxp3$^+$ T_{reg} and HLADR$^+$ T_{eff} subsets (**Supplementary Figure S5**).

We next sought to explore the sustained decrease in HLA-DR$^+$ T_{eff} counts by testing the relation between cell quantity and immunosuppressive burden. Slope analysis of mean tacrolimus TL and HLA-DR$^+$ T_{eff} counts over 12 months revealed a decrease of 2.28 × 10^3/mL cells per 1 ng/mL increase in tacrolimus TL (**Supplementary Table S4**). A strong negative correlation between tacrolimus burden, estimated as TL AUC (**Figure 3A**), and the HLA-DR$^+$ T_{eff} counts during the first weeks until M2 after KT was observed ($r = -0.70$, $p = 0.008$; **Figure 3B**). To account for potential confounders related to recipient characteristics, donor

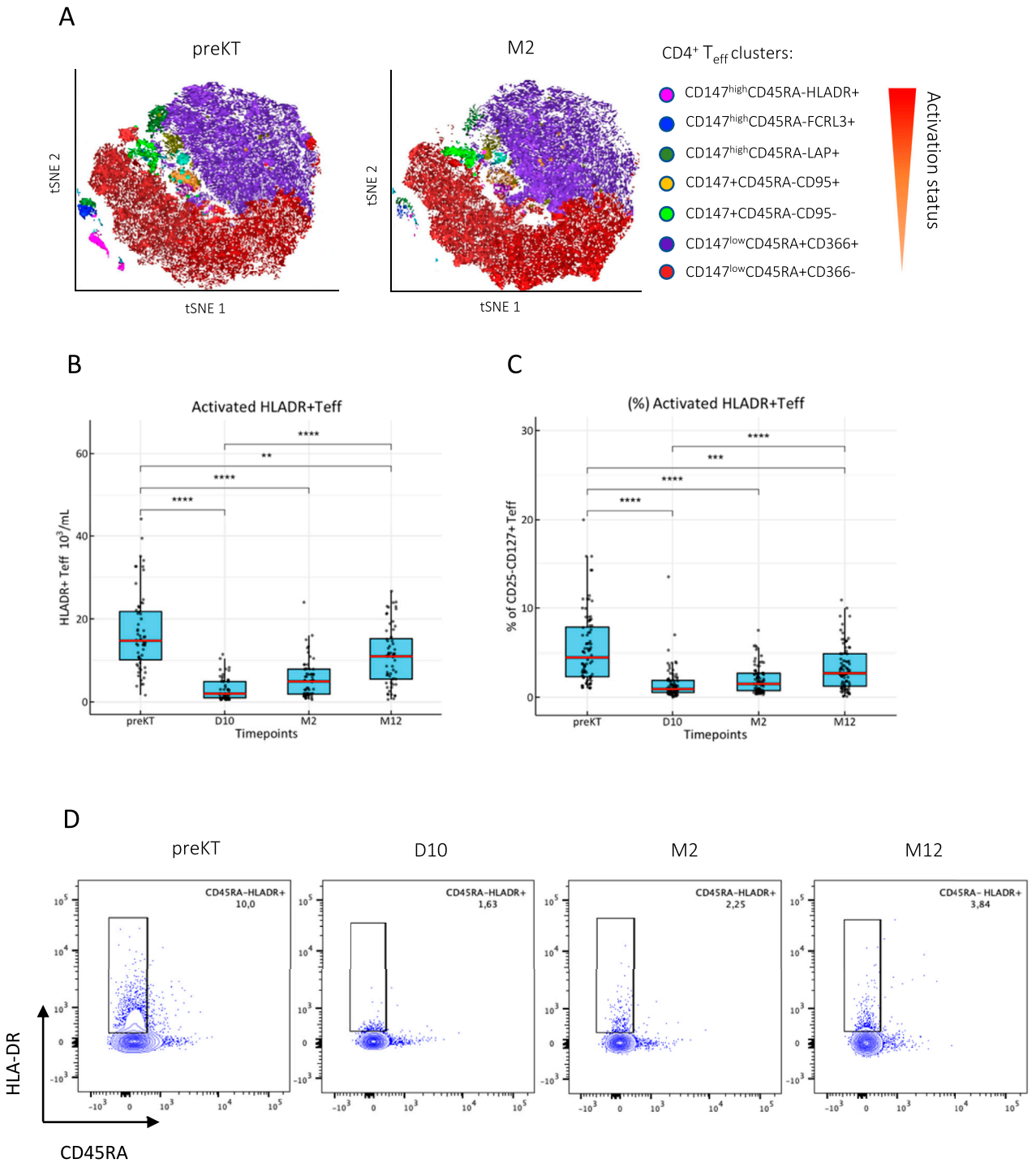

FIGURE 1 | T cell activation marker HLA-DR identifies a $CD4^+$ T cell subset susceptible to immunosuppression after KT. **(A)** $CD3^+CD4^+CD25^-CD127^+$ T_{eff} were clustered by activation status using FlowSOM algorithm and ClusterExplorer in FlowJo analysis software from peripheral PBMCs isolated immediately before and 2 months after KT. **(B, C)** The longitudinal evolution of absolute cell counts and frequencies are shown as box blots (MDN ± IQR) for all study visits with multiple group comparison by mixed-effects analysis; significant results are shown by asterisks (**) $p < 0.01$, (***) $p < 0.001$, (****) $p < 0.0001$. **(D)** Representative raw flow cytometry contour plots of one patient for each timepoint.

quality, and the immunosuppressive regimen, we performed multivariable linear regression. The significant association between HLA-DR^+ T_{eff} counts, and TL AUC remained robust across all models (β-coefficient = −0.39, p = 0.0002), (**Table 2**; **Supplementary Table S7**). No correlation was found for proliferative-effector T_{reg} counts (**Figure 3C**).

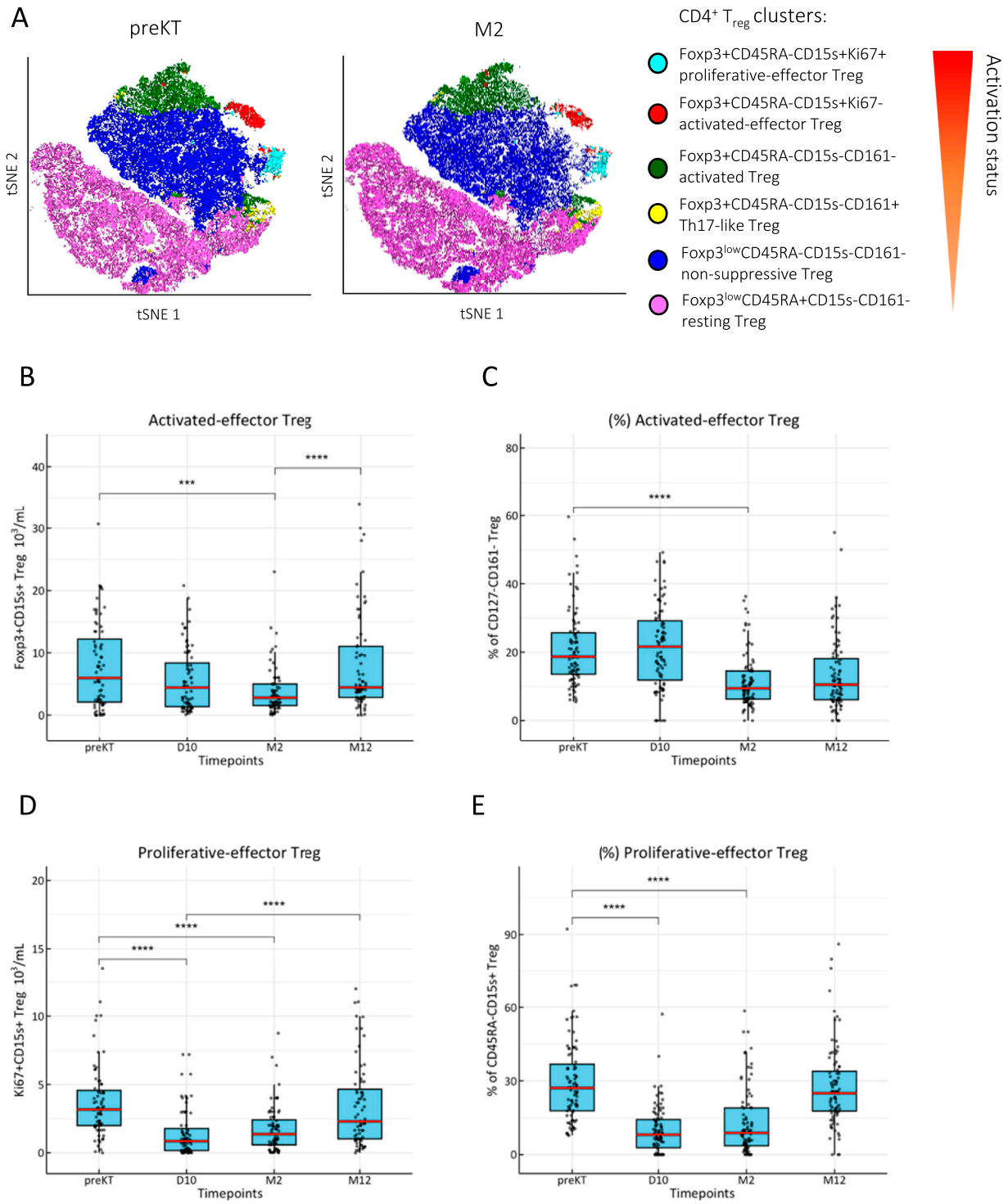

FIGURE 2 | Effector T_{reg} replenish after induction therapy. **(A)** $CD3^+CD4^+Foxp3^+CD127^-$ T_{reg} were clustered by activation status using FlowSOM algorithm and ClusterExplorer in FlowJo analysis software from peripheral PBMCs isolated immediately before (preKT) and 2 months after KT (M2). Temporary decrease of absolute counts and frequencies of **(B, C)**: activated $CD45RA^-CD15s^+$ T_{reg} and **(D, E)**: $Ki67^+$ proliferative-effector T_{reg} after KT; box blots (MDN ± IQR) for all study visits with multiple group comparison by mixed-effects analysis; significant results are shown by asterisks (***) $p < 0.0001$, (****) $p < 0.00001$.

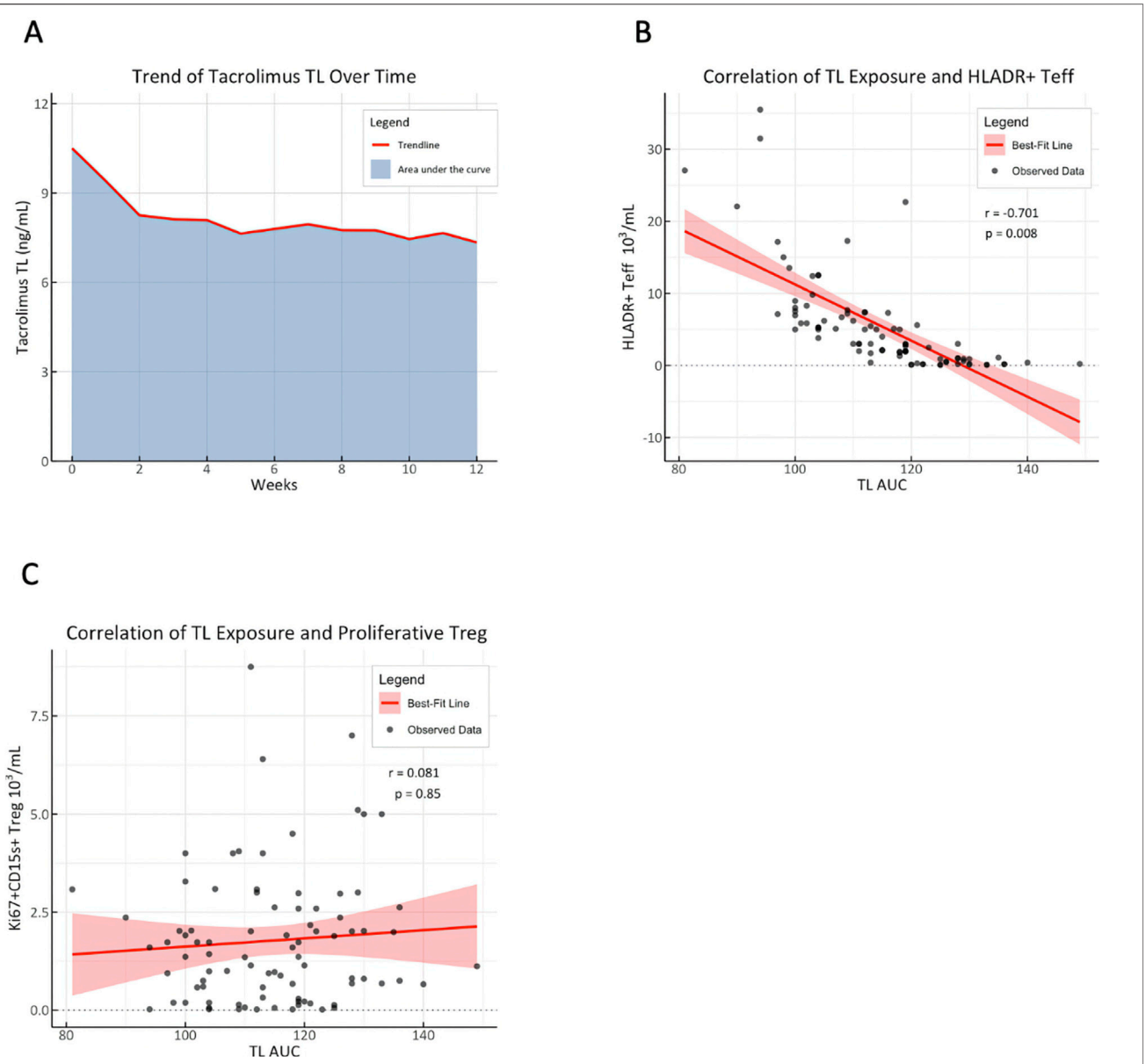

FIGURE 3 | HLA-DR$^+$ T_{eff} counts strongly correlate with tacrolimus trough level exposure. **(A)** Median tacrolimus trough level (TL) trend over time is shown as a red line. The area under the curve (AUC) was calculated by the trapezoidal rule (median AUC = 113.7 ng*t/mL) to represent tacrolimus TL exposure. TL exposure (TL AUC) was then plotted against the **(B)**: mean HLA-DR$^+$ T_{eff} count and **(C)**: proliferative-effector Treg counts of individual patients starting at D10 until M2; Spearman correlation coefficient (r) was calculated to determine the strength of the relation.

T cell Activation Marker HLADR Is Independently Associated With BK Viremia Risk

We further investigated outcome-oriented associations between the T-cell activation marker HLA-DR and immune-related events, including BK viremia, CMV infection, and BPAR. The HLA-DR$^+$ T_{eff} counts were significantly lower in patients who developed BK viremia compared to those who did not (**Figure 4A**). A similar trend was observed for CMV, although statistical significance was not reached (p = 0.09), while no difference was noted for BPAR (**Figure 4A**). Again, no difference was found for proliferative-effector T_{reg} counts (**Figure 4B**). To assess the association between HLA-DR$^+$ T_{eff} counts and BKV, CMV, and acute rejection (AR), a time-dependent multivariable cox regression was performed and adjusted for TL AUC and confounders. The significant association between HLA-DR$^+$ T_{eff} counts and BKV remained independent from TL AUC and confounders (fully adjusted HR = 1.49, p = 0.00002), (**Table 3**). No significant associations were identified between HLA-DR$^+$ T_{eff} counts and the occurrence of CMV or BPAR (**Table 3**).

TABLE 2 | HLA-DR$^+$ T_{eff} counts adjusted for recipient-, donor- and treatment-related covariates is associated with TL exposure.

Association of HLA-DR + Teff counts and TL exposure				
Model	**Coefficient**	**95% CI**	**p-value**	**R-squared**
Crude.	−0.419	−0.531 to −0.310	2.613 e-07	0.504
Model 1	−0.433	−0.523 to −0.303	5.021 e-06	0.552
Model 2	−0.403	−0.503 to −0.301	5.020 e-06	0.510
Model 3	−0.390	−0.528 to −0.310	2.612 e-04	0.484

Multivariable linear regression was used to adjust the crude association of HLA-DR$^+$ T_{eff} counts (dependent variable) and TL AUC for covariates; Model 1 = adjusted for sex + age; Model 2 = adjusted for Model 1+ KDRI; Model 3 = adjusted for Model 2 + ATG + TAC formulation +mean MMF dose + cumulative prednisolone dose.

Predicted probabilities of BKV by HLA-DR$^+$ T_{eff} counts were modeled from cox regression and depicted with a best-fit line to show the increase in BKV risk with decreasing HLA-DR$^+$ T_{eff} counts (**Figure 5A**). Time-dependent Receiver operating characteristic (ROC) analysis revealed an AUC of 0.75 (p = 0.001), with a specificity of 63% and sensitivity of 85% for an HLA-DR$^+$ T_{eff} count of 4.71 × 10^3 cells/mL at day 10 (**Supplementary Figure S6**). Stratification of the cohort based on HLA-DR$^+$ T_{eff} count above and below this cutoff demonstrated a significant difference in viremia-free survival, as shown by Kaplan-Meier curve analysis (**Figure 5B**).

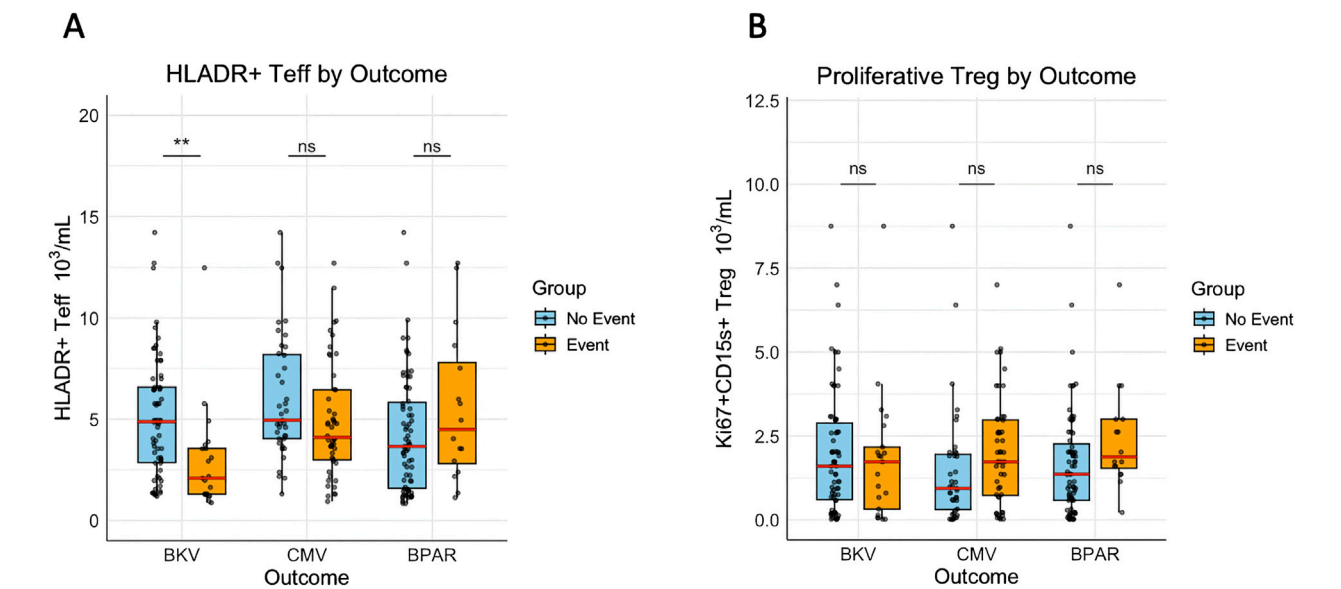

FIGURE 4 | HLA-DR$^+$ T_{eff} counts are significantly lower in patients developing BKV viremia. **(A)** The mean HLA-DR$^+$ T_{eff} counts and **(B)**: the mean proliferative-effector Treg counts between D10 and M2 of individual patients were pairwise compared between event and no event groups for BKV, CMV, and BPAR. (**) indicates $p < 0.01$.

TABLE 3 | BKV adjusted for TL exposure and covariates is independently associated with HLA-DR$^+$ T_{eff} counts.

Association of Outcome variables with HLA-DR + Teff counts					
Outcome	**Model**	**Events/Total (Censored)**	**Coefficient**	**HR (95% CI)**	**p-value**
BKV	Crude.	21/87 (66)	−0.717	0.488 (0.31–0.63)	0.00002
	Model 1		−0.425	0.654 (0.51–0.80)	0.0001
	Model 2		−0.377	0.686 (0.57–0.83)	0.0005
	Model 3		−0.402	0.669 (0.55–0.81)	0.0001
CMV	Crude.	48/87 (39)	−0.119	0.88 (0.67–1.17)	0.230
	Model 1		−0.080	0.91 (0.83–1.11)	0.594
	Model 2		0.016	1.03 (0.97–1.09)	0.774
	Model 3		−0.055	0.96 (0.90–1.03)	0.640
BPAR	Crude.	16/87 (71)	0.060	1.04 (0.83–1.20)	0.189
	Model 1		0.058	1.03 (0.86–1.20)	0.189
	Model 2		0.063	1.07 (0.88–1.19)	0.174
	Model 3		0.067	1.08 (0.89–1.20)	0.177

Time-dependent multivariable cox regression was used to test the association of HLA-DR$^+$ T_{eff} counts for the outcomes BKV, CMV, and BPAR. The crude model includes only HLADR$^+$ T_{eff}, Model 1 = adjusted for TL AUC, Model 2 = adjusted for Model 1 + age + sex + KDRI; Model 3 = adjusted for Model 2 + ATG + TAC formulation +mean MMF dose + cumulative prednisolone dose.

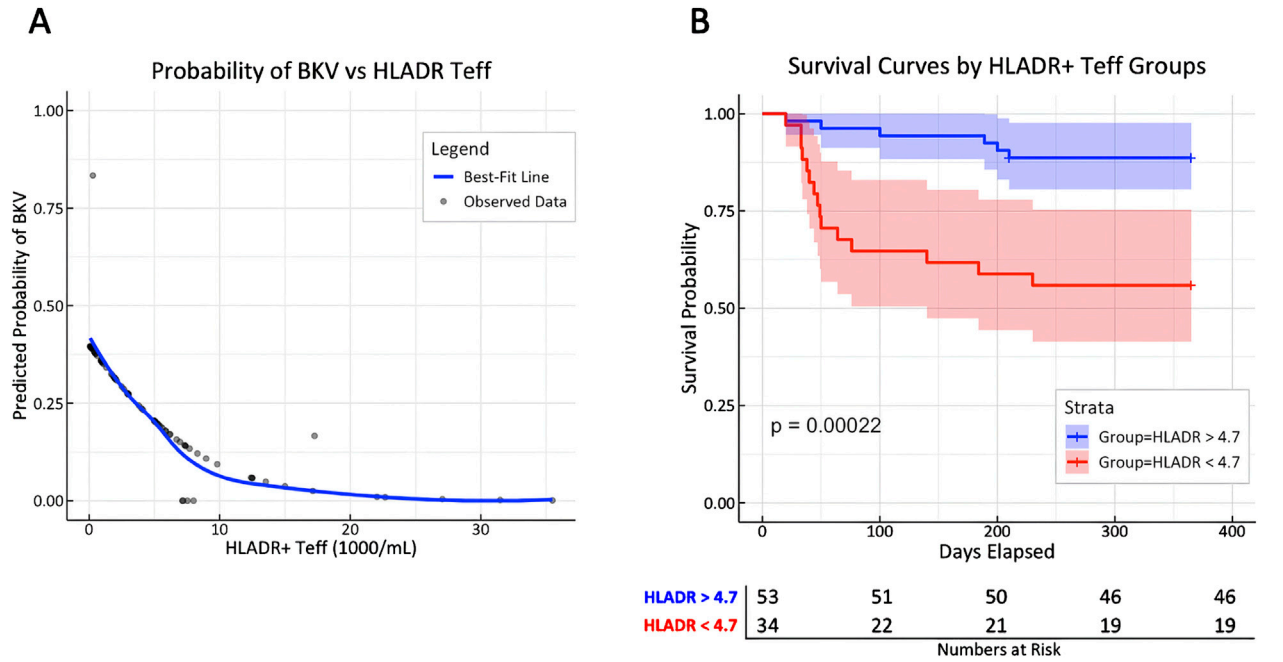

FIGURE 5 | T cell activation marker HLA-DR is associated with BK viremia, potentially allowing risk stratification early after KT. **(A)** Predicted probabilities for the first incident of BK viremia from cox regression stratified by HLA-DR$^+$ T_{eff} counts are depicted with a best-fit line (blue line); aHR = 1.49 [1.24–1.80] per unit decrease of HLA-DR, p = 0.00002. **(B)** Patients were stratified by HLA-DR$^+$ T_{eff} count at day 10 above or below 4.71 × 10^3/mL (cutoff determined by tdROC analysis of viremia incidence with AUC of 0.75; p = 0.002) to display the risk difference for experiencing BK viremia by Kaplan-Meier curves with log-rank analysis.

DISCUSSION

This observational cohort study employs longitudinal T cell phenotyping to identify immune markers correlating with immunosuppressive burden and clinical outcomes after KT. The study cohort included 87 prospectively enrolled, immunologically low-risk KTR receiving basiliximab- (94%) or ATG-based (6%) induction therapy with triple immunosuppressive maintenance therapy (steroids, tacrolimus, and mycophenolic acid). Suppression of T cell activation marker HLA-DR was associated with tacrolimus burden and was markedly aggravated in patients developing BK viremia, emerging as a potential immune monitoring tool.

Unsupervised cluster-based analysis of CD4$^+$ T_{eff} revealed significant changes among T cell activation markers following KT. The immediate decrease of HLA-DR$^+$ CD4$^+$ T_{eff} already at D10 indicated an early suppression of T cell proliferation, as HLA-DR expression has been shown to reflect T cell proliferative capacity with antigen stimulation after KT [6]. In contrast, other activation markers, such as FCRL3, and CD147 demonstrated a delayed timeline for observable change. In line with this observation, CNIs have been shown to decrease T cell proliferative capacity in stimulation assays [20]. However, stimulation assays are hampered by frequent preanalytical errors in clinical practice, underscoring the added practical value of using flow cytometry to provide a feasible indicator of the efficacy of immunosuppressive therapy in a real-world setting. In our study, the consistent, inverse relationship between TL exposure and HLA-DR$^+$ T_{eff} counts suggests a dose-dependent reduction of T cell quantity. The association between HLA-DR$^+$ T_{eff} counts and tacrolimus TL AUC remained robust even after adjusting for potential confounders. Together, these findings support that HLA-DR$^+$ T_{eff} count may serve as a surrogate biological measure of a CNI dose-immune effect. Notably, the observable changes in HLA-DR$^+$ T_{eff} cell counts within the first two months could complement other immune monitoring tools, such as TTV, which typically exhibit delayed responses to immunosuppression early after KT [21].

Building on this background, the use of tacrolimus TL AUC in our study may provide a more accurate estimation of immunosuppressive burden compared to single or averaged TL measurements. Recent evidence suggests that TL AUC reflects the immunosuppressive burden of CNI-based regimens, with demonstrated correlations to TTV levels and BK viremia risk in a retrospective cohort analysis of kidney transplant recipients [18]. The strong, inverse association between HLADR$^+$ T_{eff} cell counts and tacrolimus TL AUC in our study reflects these findings. However, the practical application of TL AUC is limited by its retrospective nature and the need for high data granularity, highlighting the importance of identifying a feasible and reliable surrogate marker for clinical monitoring and adverse event prediction. TTV viral load is currently evaluated as a promising immune monitoring tool after a calibration period of 8 weeks after KT [14].

In our study, the association between BK viremia and HLA-DR$^+$ T_{eff} counts remained robust after adjustment for TL AUC and confounding variables. This independent association as early as day 10 after KT is particularly intriguing, given that reduction of immunosuppression remains the mainstay of BKV management and could suggest that early reduction of immunosuppression could mitigate viremia in at-risk patients. Currently, our findings build a biologically plausible association between tacrolimus-based immunosuppression and activated T cell quantity reflected by immune marker HLA-DR, and BK viremia. Based on this association, the observed decrease of 2.28 × 10^3/mL in HLA-DR$^+$ T_{eff} cells per 1 ng/mL increase in tacrolimus TL over time provides valuable pilot data for estimating effect sizes in future studies. However, these findings are preliminary evidence and support the development of prospective investigations to validate and test HLA-DR$^+$ T_{eff} count as a biomarker for immunosuppressive burden to mitigate adverse events early after KT.

Previous studies have demonstrated that induction with the anti-CD25 monoclonal antibody basiliximab influences T_{reg} activation markers in CD4$^+$ T_{reg} [22], yet without impacting functionality [23]. This was confirmed by the absence of CD25$^+$ T_{reg} at day 10 in basiliximab-treated patients, whereas CD25$^+$ T_{reg} in ATG-treated patients and Foxp3$^+$ T_{reg} in the whole cohort were detectable. Concerning the evolution of Foxp3$^+$ T_{reg}, we observed a transient decrease of activated and proliferative T_{reg} markers following induction therapy, with reconstitution by month 2 or between month 2 and month 12. Previous studies suggested prognostic relevance of T_{eff}/T_{reg} ratio predicting acute rejection after KT [22], however, the reduction of T_{eff} cells was overall stronger than the reduction of T_{reg} in our study. In addition, there was no significant correlation between proliferative T_{reg} subsets and TL-AUC, and no differences were found for clinical outcomes.

From a pathomechanistic view, the stronger association between HLA-DR$^+$CD4$^+$ T_{eff} cells and BK viremia, compared to CMV infection, is noteworthy. It may reflect fundamental differences in host immune responses, suggesting a critical role of CD4$^+$ T cell immunity in the development of BK viremia. This is consistent with emerging strategies to restore BKV-specific immunity, including the use of allogeneic CD4$^+$ T-cell therapy [24]. Furthermore, the decrease in HLADR$^+$ T_{eff} counts with higher tacrolimus burden and BK viremia risk in our cohort aligns with findings from a previous observational study, suggesting a "CNI-first" approach to immunosuppression reduction as an effective treatment strategy for BK viremia and nephropathy [25]. Contrarily, a more pronounced involvement of CD8$^+$ T-cell-mediated immunity in CMV control has been suggested [26], as current investigations into interferon-gamma release assays as a monitoring tool for CD8$^+$ cellular immunity aim to guide decisions regarding pre-emptive or prophylactic therapy for CMV [27]. Similarly, TTV viral load is under evaluation as a potential immune monitoring tool for CNI-based immunosuppression, with predictive value for immune-related adverse events [14].

Finally, we identified a predictive threshold for HLA-DR$^+$ T_{eff} counts to stratify kidney transplant recipients (KTR) at risk of developing BK viremia. Specifically, an HLA-DR$^+$ T_{eff} count below 4.7 × 10^3/mL at day 10 post-transplantation was associated with meaningful risk prediction for BK viremia (median time to event: 59 days), potentially justifying early adjustment of immunosuppressive therapy. A comparable strategy has been reported in a prospective study, where the pretransplant abundance of CD28$^+$ T cells was shown to predict acute rejection risk in patients receiving belatacept (an anti-CD28 monoclonal antibody) compared to tacrolimus [8]. In this regard, our findings remain exploratory and provide preliminary data to support future studies investigating the utility of immune marker-guided CNI dosing and T-cell phenotyping as predictive tools for mitigating viral and immunological complications following kidney transplantation.

Limitations of our study include a small sample size, albeit comparable to other studies in the field. Nonetheless, a total of 348 blood samples for flow cytometry and more than 900 tacrolimus TL data were sufficient for comprehensive analysis. The prospective setting and the use of adjusted regression models to show a dose-immune effect strengthen the internal validity of our study. This analytical strategy was designed to reflect a biologically plausible and mechanistic pathway; however, causality can not be claimed, and residual confounding can not be entirely excluded. For sensitivity analysis, E-value analysis for the adjusted HR of 1.49 for BK viremia was 2.3 (1.8 lower bound), indicating that any unmeasured confounder would need to have a relative risk of at least 2.3 with both HLA-DR expression and BK viremia to fully account for the observed effect. Furthermore, the single-centre design with representation of a central European cohort may limit the overall comparability of our results. Therefore, we acknowledge that our results need further external validation, ideally with additional external cohorts and confirmation by a larger, multicentric trial. We also have to acknowledge that the implementation of flow cytometry may be hampered by technical reproducibility in clinical routine, and a higher frequency of flow cytometric measurements could have improved the granularity of the data. Our study does not include protocol biopsies, *de novo* DSA, tacrolimus single-dose AUC, T cell phenotyping of the CD8$^+$ lineage, or T cell stimulation assays, which could be the subject of a follow-up study.

In conclusion, T cell activation marker HLA-DR emerges as a potential biomarker for tacrolimus-associated immunosuppressive burden, yielding a strong association with BK viremia risk following kidney transplantation.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving humans were approved by Ethics committee Medical University of Graz, Austria (ID 28-514ex 15/16). 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

SA, KA, VP, BP, AR, and KE participated in research design, performance of the research, data analysis, statistical analysis and writing of the paper. SA, MS, AM, AK, and KK participated in patient recruitment, data analysis and writing of the paper. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

KE received an investigator-initiated research grant by Chiesi, congress-support and speaker fees by Chiesi and Astellas.

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.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2025.14443/full#supplementary-material

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SYSTEMATIC REVIEW AND META-ANALYSIS
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Experimental Swine Models for Vascularized Composite Allotransplantation and Immunosuppression: A Systematic Review and Case Report of a Novel Heterotopic Hemifacial Swine Model

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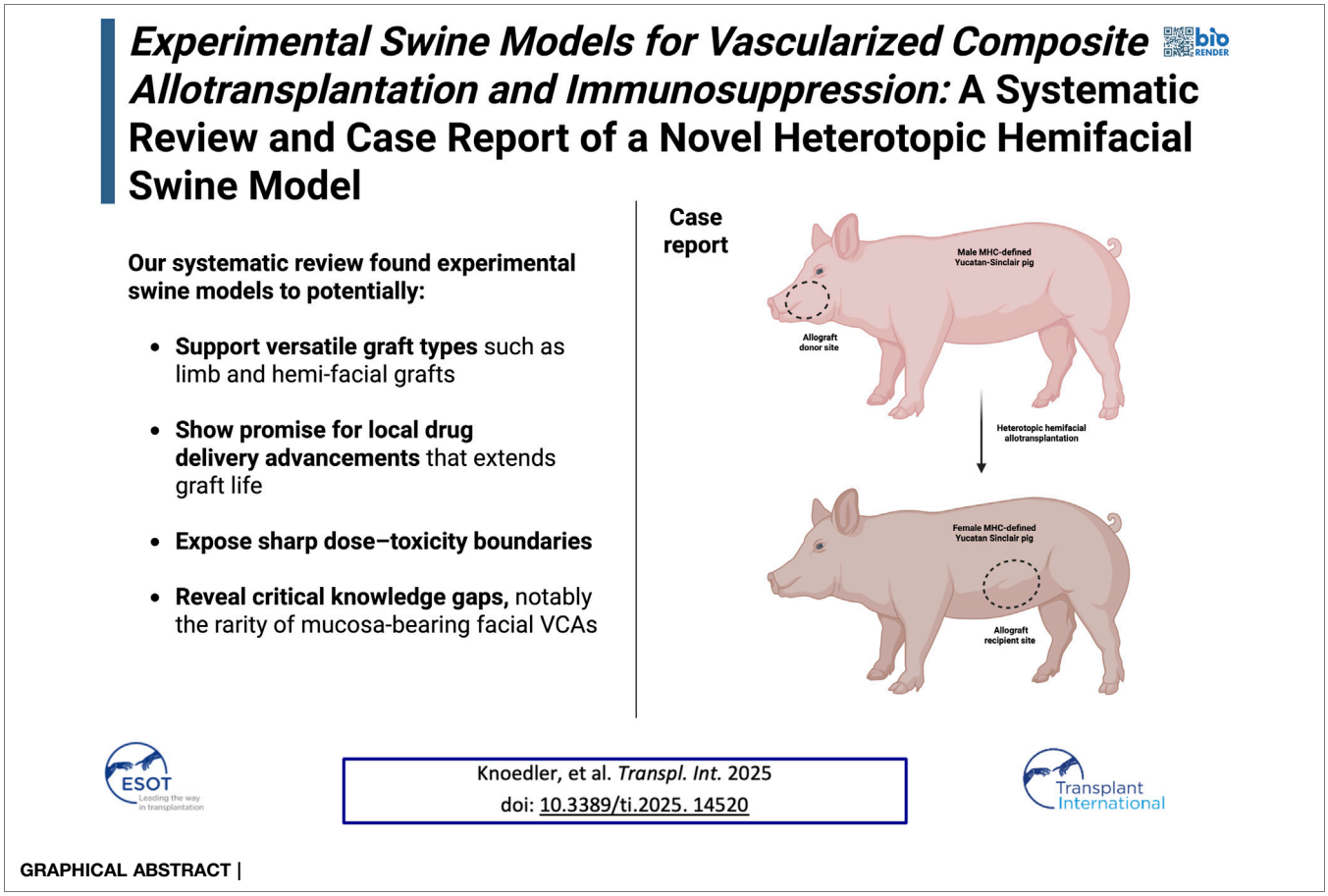
Lifelong immunosuppression is necessary to prevent rejection in vascularized composite allotransplantation (VCA). Animal models play a pivotal role in developing innovative immunosuppressive strategies. This systematic review and case report focuses on the most impactful swine VCA models while offering insights gained from the Yale Swine Allotransplantation Vascularized Experiment (Y-SAVE). 22 studies on swine VCA models were included. Key swine breeds included SLA-matched and mismatched MGH miniature swine, Yucatan miniature swine, and outbred domestic swine. Transplantation models varied, with 10 (45%) using osteomyocutaneous flaps and only 2 (9%) involving hemifacial flaps. While 16 (73%) studies utilized heterotopic models, 5 (23%) relied on orthotopic models. Novel strategies such as preconditioning and localized drug delivery emerged, alongside immunosuppression regimens combining tacrolimus with experimental therapies. We further introduced a modified heterotopic hemiface VCA model, demonstrating its feasibility for studying immune dynamics in facial transplants while preserving oral function and enabling serial skin and mucosal biopsies. Overall, our review highlights a notable gap in models that specifically investigate facial VCAs. Given the unique immunological environment of facial allografts, models such as the heterotopic hemiface transplant may offer critical insights into immune mechanisms and may provide a platform for refining targeted immunosuppressive strategies.

Keywords: swine models, vascularized composite allotransplantation, immunosuppression, graft rejection, preclinical research



GRAPHICAL ABSTRACT |

INTRODUCTION

Vascularized composite allotransplantation (VCA) represents an innovative surgical approach to restore form and function of patients with devastating deformities [1–5]. Moving beyond the boundaries of conventional reconstructive approaches (such as autologous free tissue transfer and local tissue re-arrangement), VCA surgery has emerged as a valuable therapeutic option for patients with severe injuries or irreversible tissue loss [6, 7]. Over the past decades, a growing number of VCAs have been performed, yielding positive short- and long-term outcomes [8, 9]. VCAs include different tissues such as skin, mucosa, muscle, bone, lymphatics, vasculature and nerves. The inclusion of different tissue types with varying antigenicity is associated with a strong immune response by the recipient [10, 11]. In particular, epithelial surface tissues such as the skin and mucosa seem to be the primary targets of alloreactivity, mainly via a lymphocyte mediated adaptive immune response [12, 13].

Graft rejection (both acute and chronic) persists as the main barrier in VCA surgery, limiting its more widespread application. To control allograft rejection, recipients are administered lifelong immunosuppressive (IS) regimens, typically consisting of tacrolimus, mycophenolate mofetil (MMF) and prednisolone [14, 15]. Such immunosuppressants have a variety of side effects, for instance nephrotoxicity and an increased risk of malignancy and opportunistic infections. Despite high intensity IS protocols, ~85% of VCA recipients still experience rejection episodes during the first year post-transplant and continue to reject almost annually, underlining the insufficiency of current immunomodulating strategies in VCA surgery [13, 16]. Besides acute graft rejection, patients face additional challenges such as chronic rejection which may lead to loss of functon and structure of the graft over time [17–19].

Large animal models, particularly swine, are invaluable for investigating novel immunomodulatory strategies with potential applications in human VCA recipients [20]. However, there is a notable lack of comprehensive research consolidating the current knowledge of swine models in this field. This gap represents an untapped opportunity to enhance *in vivo* experimentation and accelerate the translation of findings from the laboratory to clinical practice. To address this, we systematically reviewed the existing literature on experimental swine models in VCA, examining their indications, strengths, and limitations. Additionally, we detail the planning and outcomes of the Yale Swine Allotransplantation Vascularized Experiment (Y-SAVE). This research aims to advance the refinement of swine models and address persistent challenges in VCA surgery, ultimately improving their utility and translatability.

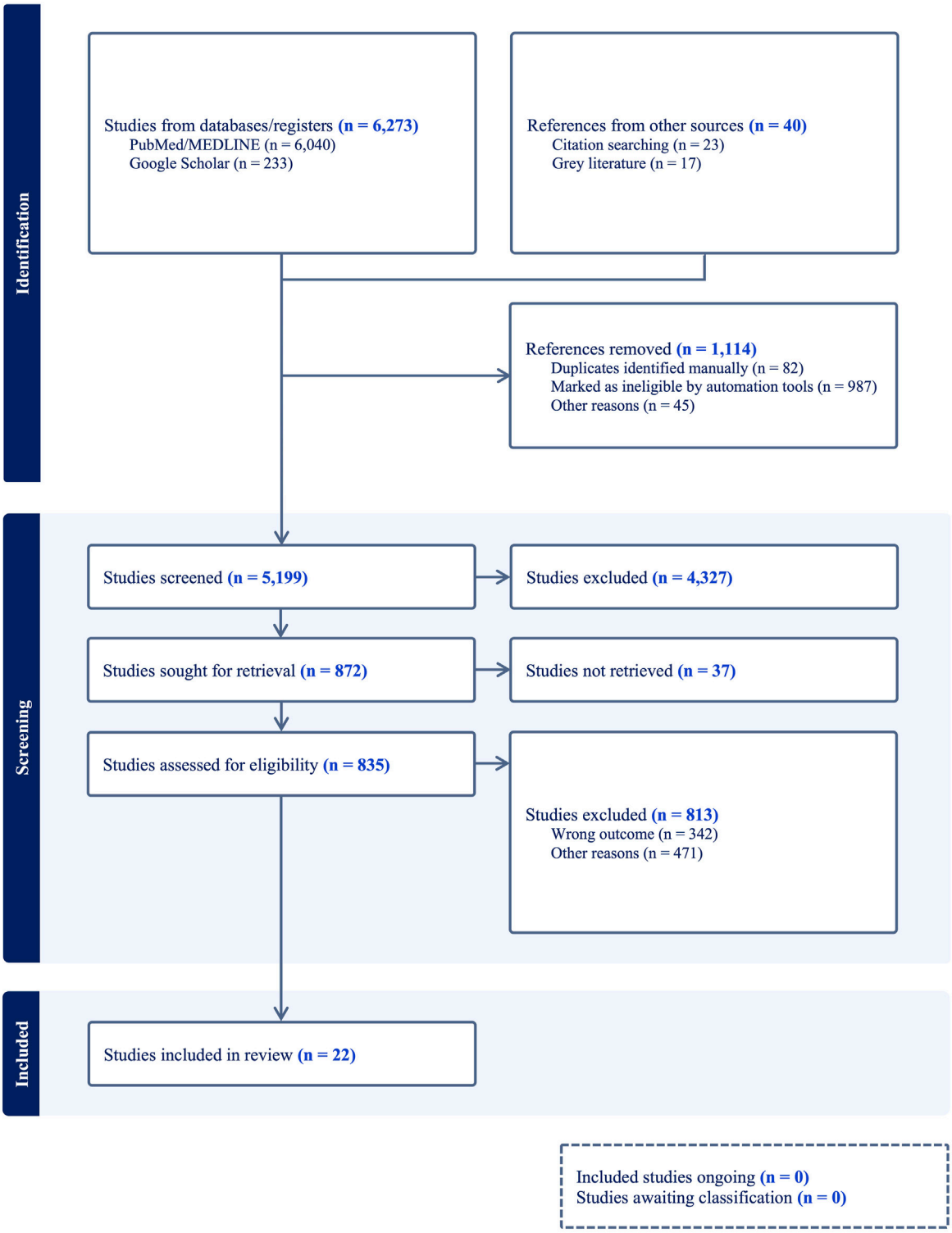


FIGURE 1 | PRISMA 2020 Flowchart of the conducted methodology.

METHODS

Search Strategy

This systematic review was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines [21]. The MEDLINE database (PubMed) and Google Scholar were queried for relevant articles published until November 13th, 2024. All studies had to be written in English. Only articles presenting original data were included. Only articles discussing experimental swine models for vascularized composite allotransplantation and immunosuppression were eligible.

Data Extraction and Quality and Bias Assessment

The search strategy for PubMed/MEDLINE and Google Scholar was developed (**Supplementary Table S1**). Two reviewers (LK, FK) independently screened all articles by title and abstract. Articles were subsequently analyzed in greater depth through full-text assessment to determine eligibility. Any disagreements regarding the inclusion of individual studies were resolved through consultation with a third author (MK). For included articles, citation searching was carried out on Google Scholar. Data extraction was performed independently by two authors (LK, FK) to ensure accuracy and consistency. During the blinded, dual-review process, we extracted the following variables for each study included: Digital Object Identifier (DOI), first author, study title, year of publication, region of publication, number of animals, mean age, gender, follow-up (mean and range), and the specifics of performed procedures. To evaluate the quality and risk of bias of the included studies, the SYRCLE risk of bias (RoB) tool for animal studies was employed [22]. The detailed risk of bias assessments for all studies are presented in **Supplementary Table S2**.

Case Report

To complement the findings of this systematic review, we included a representative case report describing a novel heterotopic hemifacial VCA model in swine. This model was developed in response to gaps identified in the literature, particularly the lack of large-animal models incorporating facial tissue and permitting mucosal assessment. The case report provides detailed procedural insights and demonstrates the feasibility of serial mucosal and skin biopsies in a controlled, minimally invasive manner. Its inclusion offers practical context and supports the translational relevance of emerging strategies for immune monitoring in facial allotransplantation.

RESULTS

After full-text analysis, a total of 22 eligible studies were included in the qualitative synthesis. A PRISMA flowchart of study identification, screening, and inclusion is presented in **Figure 1**.

Swine Models

We identified various swine breeds that were employed to examine VCA. The primary models included MGH miniature swine [23–26], Yucatan miniature swine [27, 28], and outbred domestic swine [29–31], which were selected for their genetic similarities to human immunologic responses. In particular, MGH miniature swine and Yucatan miniature swine were frequently chosen for their manageable size and robust immunological profiles. Other studies utilized outbred Yorkshire swine and Swiss Landrace pigs [32–34], adding diversity in immune response due to genetic variability, which allowed for a comprehensive analysis of transplantation outcomes across multiple immune phenotypes. Details are reported in **Table 1**.

Transplant Models and Interventions

Various transplantation models were utilized, including heterotopic hind-limb transplantation, gracilis myocutaneous flaps, vertical rectus abdominis myocutaneous (VRAM) flaps, osteomyocutaneous flaps, partial hindlimb models, forelimb models, and tibial VCA. Of the n = 22 studies included, n = 16 (73%) applied heterotopic VCA models, while n = 5 (23%) used orthotopic models [23, 24, 26–32, 34–44]. Notably, n = 1 study (5%) did not categorize the approach as either heterotopic or orthotopic and n = 10 (45%) studies included osteomyocutaneous VCAs [27, 28, 30–37]. Furthermore, n = 2 (9%) studies performed hemi-facial VCAs, with n = 1 involving transplantation of the maxillo-mandibular complex [29, 44]. Lastly, assessment of mucosal tissue was reported in n = 1 (5%) of studies included.

Orthotopic models were employed by fewer authors. Fries et al. utilized an orthotopic mismatched porcine forelimb VCA model in SH-mismatched Yucatan miniature pigs [35]. Kotsougiani et al. implemented an orthotopic tibial defect VCA model in SLA- and blood type-compatible Yucatan miniature pigs [28]. Tratnig-Frankl et al. used an orthotopic gracilis myocutaneous free flap model in MHC-defined miniature swine to assess the impact of antioxidant therapies on graft survival [39]. Interestingly, Kuo et al. employed an orthotopic hemi-facial chondromyocutaneous flap, including skin, muscle, ear cartilage, and parotid gland in Lan-Yu miniature swine to study rejection dynamics and Park et al. utilized an orthotopic hemi-facial osteochondrocutaneous flap, incorporating skin, mucosa, subcutaneous tissue, ear cartilage and the maxillo-mandibular complex in domestic swine to investigate vascular and skeletal fixation techniques [29, 44]. More information is provided in **Table 1**.

Immunosuppressive Strategies

Multiple immunosuppressive strategies were employed across different VCA models. These approaches included total body irradiation (TBI), thymic irradiation, T-cell depletion, bone marrow transplantation (BMT), and targeted drug therapies such as tacrolimus (TAC), cyclosporine A (CXA), mycophenolate mofetil (MMF), and mTOR inhibitors (e.g., rapamycin). Outcomes varied based on the immunosuppressive regimen and dosages used.

TABLE 1 | Overview of studies on Experimental Swine VCA models.

Author and year	Study design	Animals used	Transplant model	Heterotopic/ orthotopic	Facial VCA (yes/ no)	Tissue type	Donor	Recipient
Barone et al. [23]	*In vitro*	Complete MHC mismatched MGH miniature swine	Gracilis VCA transplanted to the cervical region	Heterotopic	No	Myocutaneous	MGH miniature swine	MGH miniature swine
Berkane et al. [33]	*Ex vivo*	Female Yorkshire pigs	Bilateral partial hindlimb VCA	N/A	No	Osteomyocutaneous	Female Yorkshire pigs	N/A
Blades et al. [43]	*In vivo*	Sinclair and Yucatan pigs	VRAM flap transplanted to the neck region	Heterotopic	No	Myocutaneous	Sinclair pigs	Yucatan pigs
Elgendy et al. [26]	*In vitro*	SLA- mismatched MGH miniature swines	VRAM flap transplanted to dorso-lateral neck region	Heterotopic	No	Myocutaneous	MGH miniature swine	MGH miniature swine
Fries et al. [35]	*In vivo*	SH- mismatched miniature swine	Radio-ulnar forelimb VCA	Orthotopic	No	Osteomyocutaneous	SH- mismatched Yucatan miniature pigs	SH- mismatched Yucatan miniature pigs with four SLA-HS
Ibrahim et al. [36]	*In vivo*	MHC-defined inbred MGH miniature swine	Hind limb VCA transplanted to subcutaneous abdominal wall pockets	Heterotopic	No	Osteomyocutaneous	Male MGH miniature swine	Female miniature swine
Kim et al. [25]	*In vivo*	Fully MHC mismatched MGH miniature swine	Hind-limb VCA model	Heterotopic	No	N/A	MGH mini-swine	MGH mini-swine
Kotsougiani et al. [28]	*In vivo*	Yucatan miniature pig	Tibial defect VCA model	Orthotopic	No	Osteomyocutaneous	Yucatan mini pig tibia (SLA- and blood type compatibility)	Yucatan mini pig tibia, age and size matched (SLA- and blood type compatibility)
Kuo et al. [29]	*In vitro*	Outbred miniature swine (genotypes: GPI-BB and PGD-AA)	Hind limb VCA transplanted to subcutaneous abdominal wall pockets	Heterotopic	No	Osteomyocutaneous	Outbred miniature swine (lan-yu strain; age 3 months; weight 12–20 kg)	Outbred miniature swine (lan-yu strain; age 3 months; weight 12–20 kg)
Kuo et al. [29]	*In vivo*	Outbred miniature swine (Lan-Yu and Hwa-Ban strains)	Hemi-facial flap (skin, muscle, ear cartilage, nerve, parotid gland, surrounding tissue)	Orthotopic	Yes	Chondromyocutaneous	Lan-Yu and Hwa-Ban strain	Lan-Yu strain
Kuo et al. [30]	*In vitro*	Outbred miniature swine	Hind limb VCA transplanted to subcutaneous abdominal wall pockets	Heterotopic	No	Osteomyocutaneous	Outbred miniature swine lan-yu strain; age 3 months; weight 12–20 kg	Outbred miniature swine lan-yu strain; age 3 months; weight 12–20 kg
Kuo et al. [31]	*Ex vivo*	Outbred miniature swine	Hind limb VCA transplanted to subcutaneous abdominal wall pockets	Heterotopic	No	Osteomyocutaneous	Female outbred miniature swine	Male outbred miniature swine
Leonard et al. [24]	*In vitro*	MGH miniature swine	Hind limb VCA transplanted to neck or abdominal wall region	Heterotopic	No	Fasciocutaneous	MGH miniature swine with PAA-positive SLA	MGH miniature Swine with PAA-negative SLA
Mathes et al. [32]	*In utero* and *in vitro*	MGH miniature swine and outbred Yorkshire sows and boars	Hind limb VCA transplanted to subcutaneous abdominal wall pockets	Heterotopic	No	Osteomyocutaneous	SLA homozygous MGH miniature swine	Outbred Yorkshire sow and boar fetuses (negative for SLA class I[c])

(Continued on following page)

TABLE 1 | (*Continued*) Overview of studies on Experimental Swine VCA models.

Author and year	Study design	Animals used	Transplant model	Heterotopic/ orthotopic	Facial VCA (yes/ no)	Tissue type	Donor	Recipient
Park et al. [44]	*In vivo*	Domestic swine	Hemi-facial flap (skin, mucosa, subcutaneous fat tissue, ear, maxilla and mandibular bone)	Orthotopic	Yes	Osteochondrocutaneous	Domestic swine	Domestic swine
Shanmugarajah et al. [38]	*In vitro*	Miniature MGH swine model	Hind limb VCA transplanted to the neck region	Heterotopic	No	Fasciocutaneous	HC miniature swine model (SLA^{gg} class I^{c}/II^{d})	HC miniature swine model (SLA^{cc} class I^{c}/II^{c})
Tratnig-Frankl et al. [39]	*In vivo/ ex vivo*	MHC-defined miniature swine	Gracilis VCA model	Orthotopic	No	Myocutaneous	MHC-defined miniature swine	MHC-defined miniature swine (group 1: class I and class II match; group 2: class I and class II missmatch)
Wachtman et al. [27]	*In vitro*	Yucatan miniature swine	Hind limb VCA transplanted to subcutaneous abdominal wall pockets	Heterotopic	No	Osteomyocutaneous	Yucatan miniature swine	Yucatan miniature swine
Waldner et al. [40]	*In vivo*	Partially inbred SLA–mismatched miniature swine (homozygous HC alleles)	VRAM flap transplanted to the neck region	Heterotopic	No	Myocutaneous	Miniature swine (hetero- and homozygous for HC; 2-3 months old; weight between 10 and 20 kg; full SLA mismatch	Miniature swine, (hetero- and homozygous for HC; 3-5 months old; weight between 20 and 30 kg; full SLA mismatch
Wang et al. [45]	*Ex vivo*	Yorkshire swines (SLA-mismatch in one)	Gracilis VCA transplanted to the neck region	Heterotopic	No	Myocutaneous	Yorkshire swine	Yorkshire swine
Wu et al. [42]	*In vivo*	SLA- mismatch swine	Gracilis VCA model	Heterotopic	No	Myocutaneous	Swine with single SLA mismatch	Swine with single SLA mismatch
Zhang et al. [34]	*In vivo*	MHC-mismatched Swiss landrace pigs	Knee VCA transplanted to subcutaneous abdominal wall pockets	Heterotopic	No	Osteomyocutaneous	Swiss landrace pigs (MHC-mismatched; aged 11–14 weeks)	Swiss landrace pigs (MHC-mismatched)

MGH, Massachusetts General Hospital; SLA, Swine leukocyte antigen; HC, histocompatibility complex; MHC, major histocompatibility complex; VCA, vascularized composite allotransplant; SH, single haplotype; PAA, pig allelic antigen; CS, Cold Storage; TAC, tacrolimus; MMF, Mycophenolate Mofetil; MPDN, Methylprednisolone; CXA, cyclosporine A; CD3-IT, CD3-Immunotoxin; (CTLA4-Ig), cytotoxic T-lymphocyte antigen 4 immunoglobulin; POD, postoperative day; AR, acute rejection; DSAs, donor specific antibodies; N/A, not applicable; TGMS, triglycerol monostearate; VRAM, vertical rectus abdominus myocutaneous flap, ASC, adipose-derived stem cell; AV, arteriovenous.

Starting with Barone et al., the authors combined low-dose total body irradiation (100cGy 2 days prior to surgery or 200cGy divided in 2 × 100 cGy doses on preoperative day 2 and 3), T-cell depletion with CD3 immunotoxin (0.05 mg/kg i.v., twice daily from preoperative day 4 to day 0), CXA (target level 400–800 ng/mL), and donor bone marrow cell infusion (7.8×10^8 to 4×10^9 cells/kg of recipient body weight) alongside VCA to achieve mixed chimerism, though this was insufficient for complete tolerance induction [23]. Ibrahim et al. employed short-term TAC monotherapy (target levels of 10–15 ng/mL) in a VCA model with intact vascularized bone marrow, demonstrating long-term graft survival with viable vascularized bone marrow and successful immune monitoring [36]. Kim et al. utilized a 30-day TAC course combined with adipose-derived stem cell (ASC) therapy (1.0×10^6 cells/kg administered intravenously on postoperative day (POD) 7), achieving rejection-free survival for over 200 days while significantly upregulating T-regulatory cells and donor-specific unresponsiveness. Elgendy et al. compared the efficacy of mTOR inhibitors, finding that TAC (0.1–0.125 mg/kg) significantly delayed acute rejection (grade I AR on POD 30 and grade IV AR on POD 74) compared to rapamycin (0.02–0.2 mg/kg), which led to rapid rejection (grade IV AR by POD 17-20) [26]. Conversely, Fries et al. employed low-dose TAC (49 mg) administered via an enzyme-responsive hydrogel platform, which prolonged graft survival, whereas high doses (91 mg) caused poor tolerance and complications

TABLE 2 | Overview of Interventions, Immunosuppressive Strategies and Outcomes of studies included.

Author and year	Interventions	Immunosuppresion	Outcomes	Complications
Barone et al. [23]	Bone marrow transplantation	Low-dose total body irradiation (100cGy 2 days prior to surgery or 200cGy divided in 2 × 100 cGy doses on preoperative day 2 and 3), T-cell depletion wirh CD3-IT (0.05 mg/kg), CXA (target level 400–800 ng/mL), donor bone marrow cells (7.8×10^8 to 4×10^9 cells/kg of recipient body weight)	Bone marrow infusion led to better clinical outcomes; chimerism detected but insufficient for tolerance	Mixed chimerism after bone marrow transplantat; VCA appeared insufficient for tolerance induction
Berkane et al. [33]	Two study groups: supercooling intervention group and cold storage control group undergoing subsequent normothermic machine perfusion	No immunosuppressive therapy used	Supercooled VCAs restored vascular flow and had lower resistance during machine perfusion	N/A
Blades et al., 2024 [43]	Investigation of possible surgical complications	No immunosuppressive therapy used	All flaps survived initially, with adequate perfusion for 4 days. Flap rejection occurred between POD 5 and POD 9 in all animals	Minimal erythema observed post-transplant, no surgery-related deaths or infections
Elgendy et al. [26]	Treatment with Co-stimulation blockade and mTOR inhibitor, with or without preceding short-term calcineurin inhibitor therapy	mTOR inhibitor (rapamycin [0.02–0.2 mg/kg] or tacrolimus [0.1–0.125 mg/kg])	TAC delayed AR (grade-I AR on POD 30, grade-IV on POD 74); rapid rejection with rapamycin (grade-I AR by POD 2 and 7, grade-IV AR by POD 17–20)	Rejection of allograft, erythema, severe necrotizing T cell mediated rejection with deep dermal arterial thrombosis
Fries et al. [35]	Tacrolimus eluting hydrogel implants with various concentrations (91 mg, high dose/49 mg, low dose)	Graft-implanted enzyme-responsive, TAC eluting hydrogel platform	Low-dose TAC prolonged survival; high-dose TAC caused poor tolerance (grade IV AR from POD 56–93)	High dose TAC group: one sample excluded due to flap failure on POD 1; four animals showed poor feeding and weight loss, requiring early euthanasia; four animals from high dose TAC group developed pancreatitis
Ibrahim et al. [36]	Development of novel tranlational VCA research model	Short-term tacrolimus monotherapy (target levels of 10–15 ng/mL) with or without bone marrow infusion	Long-term graft survival (>150 days) with viable vascularized bone marrow; successful immune monitoring	Venous thrombus in one case resolved by reanastomosis, no graft-versus-host disease
Kim et al. [25]	Treatment with tacrolimus for 30 days and ASC therapy (donor-derived ASCs [1.0 × 106 cells/kg])	TAC, ASC-therapy	Adipose-derived stem cells demonstrated grade IV AR on POD 119 and rejection-free survival over POD 200 as well as upregulated T-regulatory cells	The control group reached Banff grade 4 acute rejection by an average of 7.5 days after transplantation. Allografts treated with ASCs demonstrated grade 4 rejection on day 119
Kotsougiani et al. [28]	AV-bundle implantation in tibial allotransplant	TAC (target levels of 5–30 ng/mL), MMF (target levels of 1–3.5 ng/mL), MPDN (tapered to 0.1 mL)	Micro-CT showed bone formation and remodeling at the distal allograft junction; allograft survived without any healing problems or limited hindlimb perfusion during the 4-month follow-up	N/A
Kuo et al. [29]	Treatment with various dosages of mesenchymal stem cells, CXA, bone marrow transplantation and irradiation	Irradiation, bone marrow trnsplantation and CXA	Mesenchymal cells extended graft survival, combined CXA and stem cells showed significantly better survival, allografts with CXA exhibited delayed AR, examination of bromodeoxyuridine-labeled mesenchymal stem cells revealed donor mesenchymal stem cells engraftment into the recipient and donor skin	Graft-versus-host disease evident in CXA group
Kuo et al. [29]	Comparison of rejection in untreated, control and CXA-treatment groups	CXA in treatment group, untreated and control: N/A	100% survival rate, CXA treatment delayed flap rejection significantly (POD 38-49), no significant difference in rejection signs in allo-cartilage	Swelling for 2 weeks (postoperative saliva gland hypersecretion), control group: progressive rejection by POD 7-28, lymphoid gland tissue and skin were susceptible to early rejection

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TABLE 2 | (*Continued*) Overview of Interventions, Immunosuppressive Strategies and Outcomes of studies included.

Author and year	Interventions	Immunosuppresion	Outcomes	Complications
Kuo et al. [30]	Various combinations of mesenchymal stem cells cyclosporine or irradiation	Mesenchymal stem cells, CXA, irradiation	Mesenchymal stem cells with irradiation and CXA: significantly increased allograft survival compared with other groups (>120 days; $p < 0.01$); histology showed lowest degree of AR in grafted skin and interstitial muscle layers in mesenchymal stem cell/irradiation/ CXA group; significant increase in percentage of CD4+/CD25+ and CD4+/FoxP3+ T in the mesenchymal stem cell/irradiation/CXA group	Rejection episodes
Kuo et al. [31]	Various dosages of ASCs, tacrolimus or irradiation	TAC, irradiation	Multiple injections of adipose-derived stem cells, irradiation and TAC increased allograft survival significantly	Lymphocyte infiltration in the alloskin and interstitial muscle layers of treatment group
Leonard et al. [24]	Stem cell transfusion	100 cGy irradiation, T cell depletion with CD3-IT (50 μg/kg), hematopoietic cell transplantation (15×10^9 cells/kg)	Following withdrawal of immunosuppression both VCAs transplanted into stable chimeras Recipients of hematopoietic cell transplantation displayed no clinical signs of AR up to POD 504	Two animals developed skin graft versus host disease
Mathes et al. [32]	Treatment with CXA and bone marrow transplantation (2×10^9 cells/kg)	CXA (target levels of 400–800 ng/mL)	Donor cell engraftment and multilineage macro chimerism after *in utero* transplantation of adult bone marrow cells, and chimeric animals were unresponsive to donor antigens *in vitro*; both control VCAs rejected by POD 21; chimeric animals accepted VCAs (no DSAs or alloreactivity)	All grafts demonstrated some mild lymphocytic infiltration at the day 7 biopsy. All of the animals developed a severe dermal perivascular lymphocytic infiltration with scattered eosinophils and went on to reject their donor skin grafts
Park et al. [44]	Vascular anastomosis of the carotid artery and jugular vein, fixation of the maxillo-mandibular complex with titanium plates	No immunosuppressive therapy used	Successful transplant without early arterial or venous insufficiency, acute rejection from POD 7-8 onwards	Acute rejection POD 7-8, pink discoloration, edema, erythematous papule with flap necrosis on POD 14–18
Shanmugarajah et al. [38]	Hematopoietic stem cell transplantant, irradiation	T cell depletion with CD3-IT (50 μg/kg), 100 cGy TBI and 45 days of CXA (target levels of 400–800 ng/mL)	HC class II–mismatched chimeras were tolerant of VCAs; HC class I–mismatched animals rejected VCA skin, (infiltration of $CD8^+$ lymphocytes)	One HC class II mismatched model displayed clinical features of chronic graft versus host disease (euthanized on POD 190)
Tratnig-Frankl et al. [39]	Treatment with either saline (control), sodium iodide (NaI), or hydrogen sulfide (H_2S) injections	No postoperative immunosuppression	No effect of H_2S or NaI treatment in comparison to NaCl in delaying AR, flap survival and histology revealed no significant differences between the groups	One technical failure occurred in the saline MISMATCH subgroup
Wachtman et al. [27]	Bone marrow infusion and irradiation	Total body (100 cGy) and thymic (700 cGy) irradiation, bone marrow infusion, tacrolimus (0.1 mg/kg/day), CTLA4-Ig (20 mg/kg)	Experimental groups rejected allografts (skin and muscle) on POD 5 to 30; skin and muscle histology in all long-term survivors were normal	Rejection episodes
Waldner et al. [40]	Investigation of VRAM flap applicability in VCA research	TAC, rapamycin, CTLA4-Ig	POD 5: all grafts demonstrated pale-pink skin color without edema; follow-up showed improved correlation between clinical appearance and progression of graft rejection in histology	Intraoperative cardiac arrest in one sample (death due to anesthesia); one recipient experienced flap loss due to venous compromise; Banff grade I AR with erythemous and edematous grafts
Wang et al. [45]	Treatment with sub-normothermic *ex-vivo* perfusion using hyper-oxygenated University of Wisconsin (UW) solution	No immunosuppressive therapy used	Experimental group showed significantly later onset of grade 1 AR at 13.7 days ($SD = 0.52$, $p < 0.05$); by POD 15 75% of the flaps showed no evidence of grade 4 AR	Rejection episodes
Wu et al. [42]	Treatment with various dosages (28 mg/4cc and 49 mg/4cc) of tacrolimus-eluting hydrogel injected into the donor flap	TAC-eluting hydrogel (28 mg/4cc and 49 mg/4cc)	TAC-eluting hydrogel prolonged graft survival in both groups (grade 4 AR on average by POD 20 and 28)	Rejection episodes

(Continued on following page)

TABLE 2 | (*Continued*) Overview of Interventions, Immunosuppressive Strategies and Outcomes of studies included.

Author and year	Interventions	Immunosuppresion	Outcomes	Complications
Zhang et al. [34]	Treatment with various combinations of TGMS and TAC	Locally administered TAC-loaded on-demand drug delivery system	Repeated intra-graft TGMS-TAC administrations prolong graft survival	Grade III-IV rejection

MGH, Massachusetts General Hospital; SLA, Swine leukocyte antigen; HC, histocompatibility complex; MHC, major histocompatibility complex; VCA, vascularized composite allotransplant; SH, single haplotype; PAA, pig allelic antigen; CS, Cold Storage; TAC, tacrolimus; MMF, Mycophenolate Mofetil; MPDN, Methylprednisolone; CXA, cyclosporine A; CD3-IT, CD3-Immunotoxin; (CTLA4-Ig), cytotoxic T-lymphocyte antigen 4 immunoglobulin; POD, postoperative day; AR, acute rejection; DSAs, donor specific antibodies; N/A, not applicable; TGMS, triglycerol monostearate; VRAM, vertical rectus abdominus myocutaneous flap; ASC, adipose-derived stem cell; AV, arteriovenous.

such as weight loss and pancreatitis [35]. Kotsougiani et al. used a combination of TAC (target levels of 5–30 ng/mL), MMF (target levels of 1–3.5 ng/mL), and methylprednisolone (tapered to 0.1 mL for maintenance), achieving graft survival and enhancing vascular remodeling without rejection during the 4-month follow-up [28]. Meanwhile, Kuo et al. combined irradiation, BMT, and CXA with mesenchymal stem cells (MSCs) in variying dosages, resulting in significantly prolonged graft survival and reduced acute rejection. Here, increased regulatory T-cell populations ($CD4^+/CD25^+$ and $CD4^+/FoxP3^+$) were found [30, 31, 37]. Leonard et al. applied 100 cGy total body irradiation, T-cell depletion with CD3 immunotoxin (50 μg/kg), and hematopoietic cell transplantation (15×10^9 cells/kg), achieving stable mixed chimerism and long-term graft acceptance without signs of rejection up to POD 504 [24]. Mathes et al. pioneered an *in utero* bone marrow transplantation approach, achieving multilineage macrochimerism and donor-specific tolerance without prolonged post-transplant immunosuppression. The authors relied on CXA (target levels of 400–800 ng/mL) post-bone marrow infusion (2×10^9 cells/kg) to maintain donor-specific tolerance, demonstrating effective rejection prevention in chimeric animals [32]. Furthermore, Shanmugarajah et al. utilized T-cell depletion with CD3 immunotoxin (50 μg/kg), 100 cGy TBI, and a 45-day CXA regimen (target levels of 400–800 ng/mL) to achieve immune tolerance in MHC class II mismatched chimeras, although MHC class I mismatched animals experienced rejection [38]. Meanwhile, Kuo et al. demonstrated that CXA delayed rejection from POD 7 to 28 in untreated controls to POD 38 to 49 in their hemi-facial VCA model [29]. Additionally, strategies explored by Wu et al. focused on enzyme-responsive and TAC-eluting hydrogels. The authors demonstrated prolonged survival using hydrogel-administered TAC (28 mg/4 cc and 49 mg/4 cc), effectively delaying grade IV AR to POD 20 and 28 [42].

Overall, five studies did not administer immunosuppressive therapies. For instance, Blades et al. observed flap rejection between POD 5 and 9 without immunosuppressive treatment and Park et al. by POD 14 to 18 [43, 44]. Tratnig-Frankl et al. and Wang et al. did not administer immunosuppression to avoid skewing of rejection periods in novel treatment approaches. Tratnig-Frankl et al. investigated H_2S and NaI treatments but observed no significant differences in graft survival or immunological outcomes compared to saline controls [39]. In Wang et al. the experimental group received hyperoxygenated University of Wisconsin solution and showed significantly later onset of grade 1 AR, compared to the control group [45]. Lastly, in Berkane et al. the study protocol did not foresee immunosuppression [33]. Further information can be found in **Table 2**.

Major Findings

In several models, immunosuppressive therapies and interventions significantly improved graft survival, with some protocols achieving long-term graft acceptance and reduced acute rejection (AR). For instance, Ibrahim et al. reported long-term graft survival exceeding 150 days with short-term TAC therapy and bone marrow infusion, highlighting the effectiveness of combining localized and systemic immunosuppression [36]. Similarly, Kim et al. observed prolonged rejection-free graft survival beyond 200 days using adipose-derived stem cell therapy combined with TAC, correlating the upregulation of regulatory T-cells (Tregs) with sustained graft tolerance [25]. Leonard et al. demonstrated stable chimerism and long-term graft survival up to 504 days following hematopoietic cell transplantation with irradiation and T-cell depletion, suggesting the importance of chimerism in inducing robust immune tolerance [24]. Additionally, Mathes et al. demonstrated that cyclosporine treatment combined with *in utero* bone marrow transplantation resulted in long-term chimeric stability and VCA acceptance, highlighting the effectiveness of early hematopoietic intervention [32].

Most studies reported significant success in prolonging graft survival with tailored immunosuppressive regimens. For example, Wang et al. observed a delay in acute rejection in flaps treated with systemic immunosuppression, with 75% of experimental flaps showing no rejection by day 15 [45]. Treatments combining stem cells with immunosuppressantsoften resulted in prolonged graft survival with reduced rejection rates, as seen in the work of Kuo et al. [30, 31, 37] In contrast, Elgendy et al. found that TAC significantly delayed AR compared to rapamycin, where rapid AR was observed [26]. Additionally, Fries et al. revealed that while low-dose TAC via enzyme-responsive hydrogels prolonged graft survival, high doses led to poor outcomes, including weight loss, pancreatitis, and early euthanasia [35]. Localized immunosuppressive delivery methods demonstrated promising results. Wu et al. utilized tacrolimus-eluting hydrogels, which effectively prolonged graft survival and delayed grade IV AR [42]. Similarly, Zhang et al. employed a localized tacrolimus-loaded drug delivery system, resulting in repeated intra-graft administration that significantly extended graft survival [34]. Kuo et al. found CXA to significantly delay rejection of hemi-facial flaps. Lastly, some treatments failed to demonstrate

significant efficacy: Tratnig-Frankl et al. found no significant difference in graft survival or histological outcomes after antixodative therapy compared to controls [39]. Details are provided in **Table 2**.

Complications

Complications varied with the immunosuppressive approach and transplant model. While many studies reported successful outcomes in terms of prolonged graft survival and delayed rejection, complications often arose from either the intervention protocols themselves or the adverse effects of immunosuppressive regimens. For example, Fries et al. reported weight loss, poor feeding, and pancreatitis in animals subjected to high-dose TAC therapy, with some requiring early euthanasia [35]. Similarly, Elgendy et al. noted rapid rejection with rapamycin treatment compared to TAC [26]. Furthermore, vascular complications such as venous thrombosis were reported. These complications were resolved through re-anastomosis without long-term graft loss [36]. Blades et al. observed flap rejection between POD 5 and 9, despite initial adequate perfusion, and noted minimal erythema as an early rejection marker [43]. Additionally, Tratnig-Frankl et al. reported a technical failure in one saline subgroup, emphasizing the role of surgical precision in preventing graft loss [39]. Systemic complications related to immunosuppression were also observed. Shanmugarajah et al. documented chronic graft-versus-host disease (GvHD) in one animal, requiring euthanasia by POD 190 [38]. Similarly, Kuo et al. noted GvHD in animals treated with cyclophosphamide and irradiation, indicating the risks associated with preconditioning regimens [30, 31, 37]. Additionally, Wachtman et al. reported histological evidence of graft rejection in skin and muscle components, despite long-term survival in other grafted tissues [27]. Surgical mortality due to anesthesia-related complications was also recorded. Waldner et al. described an intraoperative cardiac arrest in one recipient, as well as venous compromise leading to flap loss in another case [40]. Furthermore, complications such as poor perfusion, erythema, and edema were commonly cited as markers of early graft rejection, requiring close monitoring for timely intervention [40, 43]. More information is presented in **Table 2**.

In summary, these studies underline the potential of swine models to explore VCA and immunosuppressive strategies, revealing that combinations of traditional drugs like TAC and cyclosporine with novel agents or delivery systems can extend graft survival and reduce immune responses.

Case Report

Based on these findings, we decided to perform a heterotopic hemiface transplantation procedure using an MHC-defined Yucatan Sinclair strain to establish a novel swine model consisting of heterotopic hemiface vascularized composite allotransplantation (VCA) to the groin area. This model specifically enables frequent biopsies due to the accessibility of the flap but more importantly including donor mucosa while allowing the receipient to ingeste, feeding, or persue related activities. In contrast, orthotopic transplantation risks confounding mucosal assessment, as the animal may chew on or manipulate the graft. This setup enables detailed analysis of immunological interactions at the skin and mucosa interfaces, thus providing valuable insights into tissue rejection dynamics and tolerance in a way that conventional graft sites may not accommodate as effectively. To conclude, the Yucatan Sinclair strain is furthermore well-suited for this purpose, given its immunologic compatibility in modeling human responses.

Animals

A heterotopic hemiface vascularized composite allotransplantation to the groin area was performed from a male donor pig to a female recipient of MHC-defined Yucatan Sinclair strain. We performed the study following the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health [46]. Experiments were conducted according to a protocol approved by Yale University's Institutional Animal Care and Use Committee (protocol number 2022-20476). More details are depicted in **Figure 2**.

Donor Preparation and Allograft Harvest

The donor pig was positioned supine on heat support, under isoflurane anesthesia (0.8%–2%). Following connection to monitoring equipment and IV fluid administration, the donor received prophylactic antibiotics (cefazolin) and analgesics (meloxicam, buprenorphine) alongside local anesthesia with bupivacaine at key surgical sites. Antiseptic preparation with povidone-iodine was applied to the head and neck.

A hemifacial flap was carefully marked on the donor pig's face. Skin incisions were made along the brachiocephalic muscle and the neck, sparing the ear and eye, while advancing dissection above the periosteal plane in the nasal and fronto-parietal areas. The dissection proceeded superiorly to the mandible, preserving the external jugular vein. In the facial region, meticulous incisions were made around the auricular, eyelid, and oral areas, incorporating buccal mucosa and securing the salivary glands. Further, the submandibular gland was removed after ligating its vascular branches, and the facial artery and nerve were identified. The facial nerve was transected near the stylomastoid foramen, and the external carotid artery and external jugular vein served as the flap's vascular pedicle. Following tissue elevations along the masseter muscle and excisions in the neck area, the sternomastoideus muscle was detached, exposing central vessels including the common carotid and its branches. Key arteries, such as the internal carotid, were ligated and transected.

The graft was perfused *in situ* until the recipient's vasculature was ready. The donor's central vessels were ligated, and the graft was flushed with heparin solution, followed by euthanasia with sodium pentobarbital as per established veterinary protocols.

Recipient Preparation and Hemiface Graft Inset

The recipient pig was anesthetized and positioned supine with a 30° rotation to expose the dorsolateral side, allowing simultaneous preparation with the donor. A groin incision

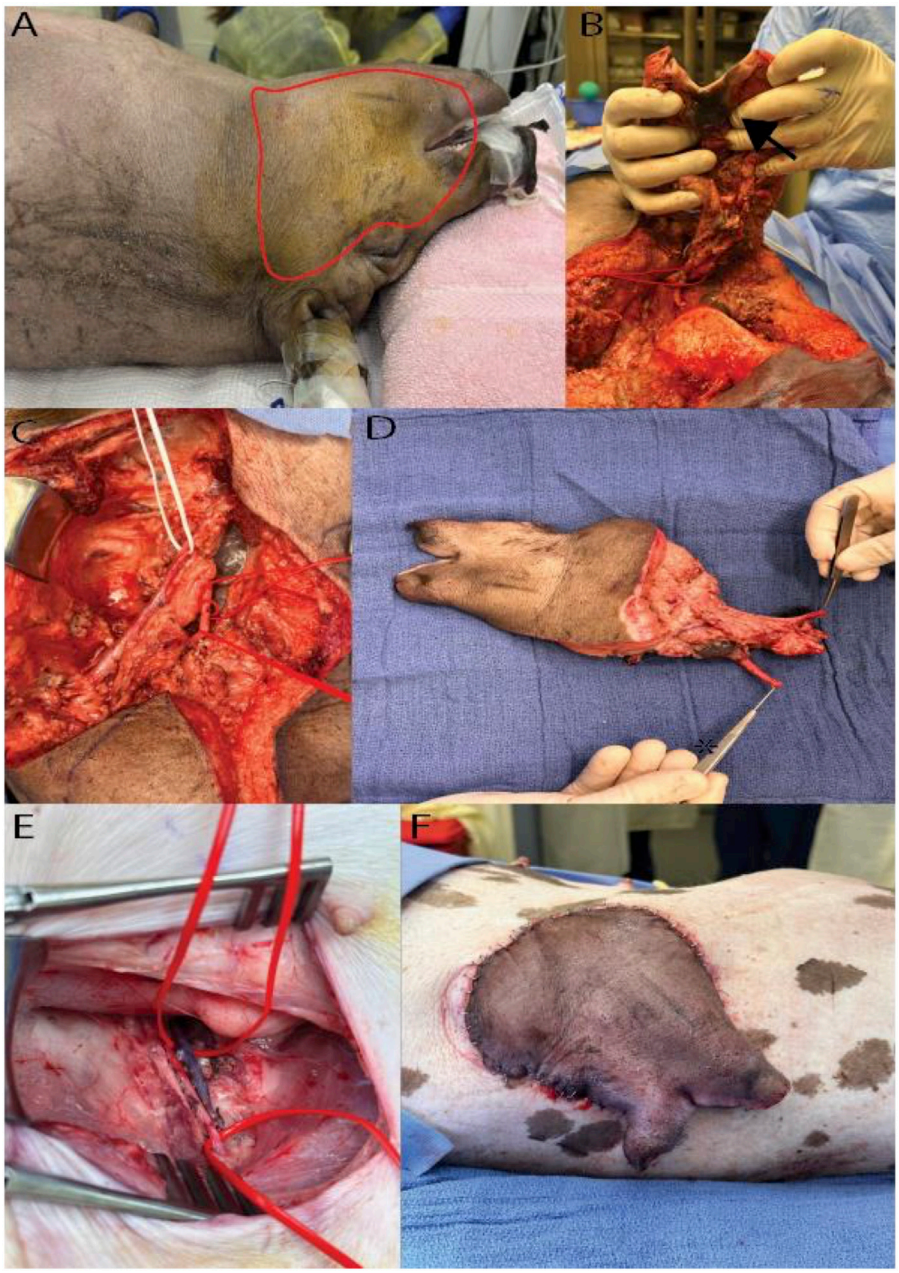

FIGURE 2 | Hemifacial Heterotopic Transplant Model. **(A)** Outline of the hemifacial transplant. **(B)** Underside of the hemifacial graft after dissection, with an arrowhead marking the intraoral mucosa. **(C)** Demonstration of the vascular pedicle of the graft, with white vessel loop identifying the external jugular vein and red loops marking the common carotid artery. **(D)** Explanted hemifacial graft showing the vascular pedicle. **(E)** Dissected femoral vessels used for vascular anastomosis. **(F)** Hemifacial graft inset in the lateral abdominal wall post-transplantation.

exposed the femoral vessels, isolated to allow anastomosis. A subcutaneous pocket was created from the groin to the dorsolateral abdominal wall, where the graft would be placed. The hemiface flap was inset dorsolaterally to facilitate immune monitoring.

Following ligation of the donor's femoral vessels, the graft was prepared for anastomosis. Venous anastomoses were conducted with a vascular coupling device (2.5 mm size), while arterial anastomoses were sutured with 9-0 nylon. Once vascular patency was confirmed, the graft was secured in place with sutures to the abdominal wall muscles while the skin and mucosa paddle were exteriorized for monitoring. The groin incision was closed in layers and covered with a Tegaderm® patch to prevent infection. Analgesia was administered via a fentanyl patch, and postoperative antibiotics were given. The recipient pig was monitored until full recovery.

The recipient pig recovered from the operation without any complications, exhibiting normal eating and drinking behavior and full mobility. Frequent monitoring revealed a viable flap, with the recipient site in the groin well-tolerated by the pig. After 24 h, the pig was euthanized according to protocol. These findings demonstrate the feasibility of our model for heterotopic hemiface vascularized composite allotransplantation in evaluating graft viability and immune response in a controlled and accessible site.

DISCUSSION

Our review highlights that specific experimental variables play a critical role in shaping long-term outcomes in swine VCA models. Graft composition emerged as a key determinant of immunogenicity and tolerance induction. Grafts that incorporate vascularized bone marrow (VBM) or osteomyocutaneous tissues consistently demonstrate enhanced tolerance induction, prolonged survival, and the establishment of mixed chimerism, compared to purely fasciocutaneous grafts. Multiple studies in large animal and rodent models show that inclusion of vascularized bone or bone marrow within the graft provides a continuous source of donor-derived hematopoietic stem cells, facilitating stable mixed chimerism and promoting donor-specific tolerance [47–49]. For example, in swine, protocols combining non-myeloablative conditioning, bone marrow infusion, and osteomyocutaneous VCA have achieved stable mixed chimerism and long-term graft survival across MHC barriers, with evidence of donor-specific hyporesponsiveness and regulatory T cell expansion. Similarly, in rodent models, VBM-containing grafts result in higher chimerism and longer allograft survival than non-osseous grafts, and removal of the VBM component abrogates tolerance [50, 51]. In contrast, purely fasciocutaneous or skin-only VCAs are more immunogenic and typically undergo earlier rejection, even under similar immunomodulatory protocols, and rarely achieve durable chimerism or tolerance [27, 49, 52]. The skin component remains the most challenging tissue for tolerance induction, and its rejection is accelerated in the absence of VBM [27, 38].

In addition, immunosuppressive regimens were found to vary widely, with tacrolimus serving as a cornerstone agent. Localized delivery of tacrolimus, such as via a hydrogel platform, has been shown to extend graft survival and reduce systemic toxicity compared to high-dose systemic regimens, as demonstrated by Fries et al. Low-dose tacrolimus hydrogel delayed acute rejection and was better tolerated, while high-dose regimens led to toxicity and poorer tolerability [35, 53–56]. Combination therapies, including tacrolimus with mycophenolate mofetil and methylprednisolone, are commonly used and have been associated with improved graft viability, bone remodeling, and minimal complications in large animal models, as described in systematic reviews and preclinical studies [57, 58]. Cellular therapies, such as mesenchymal stem cells (MSCs) and adipose-derived stem cells, have also been shown to modulate immune responses, promote regulatory T cell expansion, and extend rejection-free intervals, particularly when combined with short-course tacrolimus [59, 60].

Furthermore, monitoring strategies most commonly rely on clinical observation and histological grading of acute rejection, with relatively few studies employing serial biopsies or advanced immunophenotyping. The literature highlights that clinical assessment and histopathology—often using adaptations of the Banff criteria—are the mainstays for diagnosing and grading rejection, but there is a lack of standardized, reproducible protocols across studies, and serial or multimodal monitoring is not routine. Leonard et al. and Waldner et al. are exceptions to this general trend [58, 61, 62]. Leonard et al. correlated the presence of mixed chimerism with the histologic absence of acute rejection in swine VCA recipients, with tolerance and rejection-free survival documented up to postoperative day 504, integrating both chimerism analysis and histopathology for longitudinal monitoring. Waldner et al. specifically emphasized the correlation between clinical graft appearance and histological findings, using serial punch biopsies to confirm the progression of rejection in a swine myocutaneous VCA model [40, 63]. These variations in monitoring approaches highlight the need for standardized, reproducible protocols that integrate graft design, immunosuppressive regimens, and multimodal monitoring—including clinical, histological, and immunological parameters—to improve the translational value and comparability of swine VCA research.

Future research may also investigate swine VCA xenotransplants. Recent advancements in the field have introduced genetic engineering strategies to reduce the expression of swine xenogeneic antigens identifiable by human immunoglobulins, ultimately lessening the immunological rejection against xenotransplantation. For instance, Yoon et al. used CRISPR-CAS9 to target xeno-reactive genes GGTA1, CMAH, and B4GALNT2 from Jeju Native Pigs and develop triple-knockout pigs [64]. Genetically engineered pigs showed reduced expression of galactose-alpha-1,3-galactose and N-glycolylneuraminic acid, which have been previously identified as key drivers of xenorejection [65–67]. Overall, the removal of the three genes significantly reduced xenograft rejection and binding by human IgM and IgG antibodies [64]. Interestingly, another study used galactose-alpha-1,3-galactose/N-glycolylneuraminic acid double-knockout pig lungs that were perfused for up to 6 h with fresh heparinized human blood. The authors reported reduced antibody-mediated inflammation and activation of the coagulation cascade, as well as a delayed rise in pulmonary vascular resistance when compared to galactose-alpha-1,3-galactose single-knockout pig lungs [68]. Here, the authors highlighted that the additional N-glycolylneuraminic acid helps mediate the innate immune antigenicity in xenogenically perfused porcine lungs. Additional research has also underpinned the key role of the GGTA1, CMAH, β4GalNT2 and CIITA genes in activating human $CD4^+$ T cells in 4-gene knockout pigs [69]. With these recent advancements on the clinical horizon, wild-type pigs may become increasingly obsolete, both scientifically and due to evolving regulatory standards. Vice versa, knockout pigs may serve as a valuable donor pool to catalyze the widespread clinical adoption of VCA and pave the way toward the first vascularized composite xenotransplantation (VCX) case [2].

The insights gained from this systematic review and our heterotopic hemiface model underscore the importance of swine models in particular in translating immunosuppressive strategies for human VCA. Our model's ability to support serial biopsies of skin and mucosa provides a unique tool for examining the dynamics of immune tolerance and rejection, potentially improving clinical outcomes in patients undergoing complex tissue transplants. By optimizing immunosuppressive strategies to balance efficacy and safety, our model offers valuable guidance for refining VCA protocols, supporting the development of safer, more effective treatment paradigms in clinical transplantation.

Limitations

This study is limited by the inherent heterogeneity of the swine models and experimental protocols reviewed, which complicates direct comparisons and generalizations across studies. The small sample sizes across the included studies reduce the generalizability of our findings and limit our ability to perform a robust quantitative meta-analysis. The rarity of VCA studies in swine also introduces potential publication bias, as studies with negative or inconclusive outcomes may be underreported.

Additionally, our heterotopic hemiface model, while valuable for serial biopsies, may not fully represent the complexity of vascular integration seen in orthotopic models, potentially limiting its direct applicability to specific clinical scenarios in VCA. Moreover, while the described heterotopic hemifacial VCA model was primarily designed to ensure surgical feasibility and facilitate serial mucosal and skin biopsies, we acknowledge the limitation that the transplanted mucosa is no longer located within its native anatomical environment. As such, it is exposed to non-physiological conditions, including external microbial flora and mechanical influences at the abdominal implantation site. These factors may affect local immune responses and limit the interpretability of biopsy-derived data with respect to natural mucosal immunity. Nevertheless, the model remains valuable for studying epithelial immune activation and early alloimmune events in a controlled and accessible setting, and it offers an important proof-of-concept for future refinements toward orthotopic models.

CONCLUSION

Swine models have significantly advanced our understanding of VCA immunology through diverse composite grafts and immunomodulatory approaches. However, our review highlights a notable gap in models that specifically investigate facial VCAs, particularly those including the oral mucosa. Given the unique immunological environment of facial allografts, models such as the heterotopic hemiface transplant offer critical insights into immune mechanisms and provide a platform for refining targeted immunosuppressive strategies. By enabling serial biopsies and localized immune monitoring, this model addresses key challenges such as graft rejection and the systemic effects of immunosuppression. These advancements are essential for developing safer, more effective transplantation protocols, ultimately improving patient outcomes in facial VCA.

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 Yale University's Institutional Animal Care and Use Committee. The study was conducted in accordance with the local legislation and institutional requirements.

AUTHOR CONTRIBUTIONS

LK and FK conceived the study and developed the overall research strategy, including the design of the systematic review and case report. LH and MK-N were responsible for planning and executing the experimental procedures, from donor preparation to the performance of the heterotopic hemiface transplant model. SK, TS, and TN played key roles in the collection and management of experimental data. SB and SBr optimized the immunosuppressive regimens and assisted in monitoring graft function and animal recovery throughout the study. BP provided critical clinical insight, advising on surgical technique refinements and ensuring the translational relevance of our approach. MK-N supervised the entire project, coordinated ethical approvals, and led the manuscript drafting and revision process. 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.

GENERATIVE AI STATEMENT

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

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2025.14520/full#supplementary-material

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Risk Prediction and Management of BKPyV-DNAemia in Kidney Transplant Recipients: A Multicenter Analysis of Immunosuppressive Strategies

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BK polyomavirus (BKPyV) DNAemia remains a major complication in kidney transplantation (KT), requiring nuanced adjustments to immunosuppressive regimens to control viral replication while minimizing rejection risk. This retrospective multicenter cohort study included 8,027 KT recipients, of whom 1,102 developed BKPyV-DNAemia within the first year. Among them, 927 patients with complete therapeutic drug monitoring (TDM) data were categorized into three groups based on post- BKPyV-DNAemia immunosuppressive strategies: mycophenolic acid (MPA) control, sirolimus, and leflunomide. Multivariate logistic regression and Cox analyses identified risk factors for BKPyV-DNAemia treatment failure, acute rejection, and graft loss. Tacrolimus trough levels below 5 ng/mL and complete withdrawal of calcineurin inhibitors (CNIs) significantly increased rejection risk (OR = 2.65, P = 0.033). Maintaining tacrolimus levels between 5 and 7 ng/mL was associated with optimal viral control and lower rejection rates. Leflunomide substitution reduced BKPyV burden but increased rejection risk (OR = 2.14, P < 0.001). Sirolimus-based regimens with CNI withdrawal led to the highest rejection risk (OR = 6.00, P = 0.044) and a trend toward increased graft failure (HR = 4.37, P = 0.07). A tacrolimus target of ≥5 ng/mL emerged as optimal for balancing BKPyV-DNAemia suppression and long-term graft survival. While leflunomide is effective for viral control, its immunological risks warrant careful patient selection and monitoring.

Keywords: kidney transplantation, Bk virus, immunosuppressive therapy, calcineurin inhibitor, tacrolimus trough level

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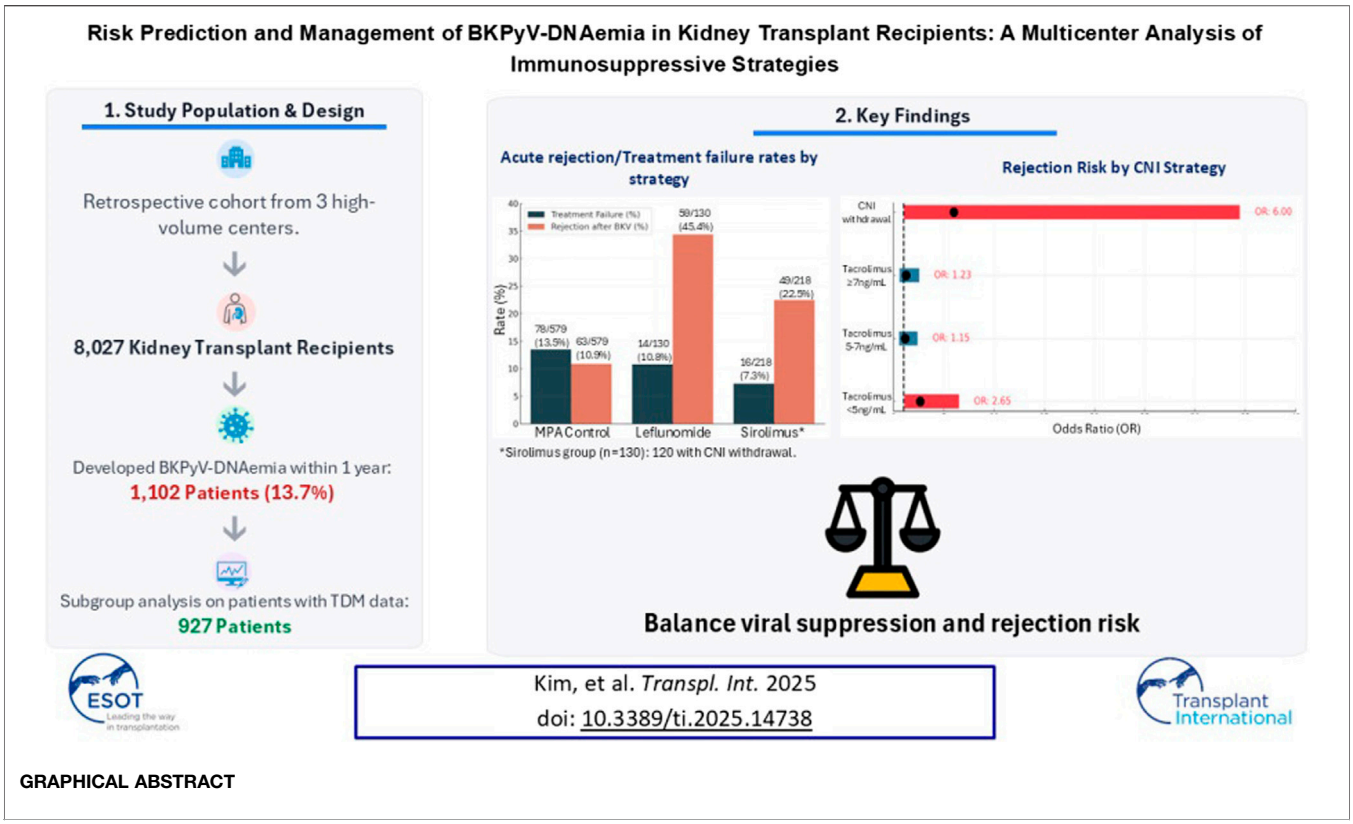
INTRODUCTION

Kidney transplantation (KT) is a vital treatment option for patients with end-stage renal disease, significantly improving both survival rates and quality of life [1, 2]. Despite its many advantages, post-transplant complications continue to pose challenges to graft longevity and patient outcomes [3, 4]. Among these complications, BK polyomavirus (BKPyV) DNAemia is recognized as a major concern affecting post-transplant outcomes [5–7]. The BKPyV, a member of the polyomavirus family, typically remains latent in renal tissue [8]. However, under conditions of immunosuppression, which are necessary to prevent graft rejection, the virus can reactivate [9].



GRAPHICAL ABSTRACT

This reactivation may lead to BK virus-associated nephropathy (BKVN), which is a leading cause of graft dysfunction.

The management of immunosuppression in KT recipients presents a critical clinical dilemma. Immunosuppressants, particularly calcineurin inhibitors (CNI) such as tacrolimus and mycophenolic acid (MPA), are essential for preventing organ rejection [10]. However, these same medications may inadvertently promote viral reactivation [11]. The challenge lies in reducing immunosuppression to mitigate the risk of BKPyV-DNAemia while simultaneously maintaining adequate immunosuppression to prevent rejection. Previous research has underscored the importance of maintaining optimal tacrolimus levels to maximize graft survival [12]. The present study builds upon this foundational work by offering a detailed analysis of risk factors, refining tacrolimus thresholds, and evaluating the efficacy of alternative immunosuppressive strategies that can minimize complications related to the BKPyV.

As the number of immunologically high-risk KT recipients continues to rise, BKPyV-DNAemia has become an increasingly critical concern for graft survival [13]. However, large-scale, multicenter studies addressing this issue are limited, and there is a notable lack of research on the relationship between CNI concentration and BKPyV-DNAemia outcomes. By leveraging clinical data from a large multicenter cohort, our study aims to establish the most effective immunosuppressive management following BKPyV-DNAemia onset by defining appropriate CNI trough levels and assessing the impact of different immunosuppressive regimens—such as leflunomide and sirolimus—on viral control, rejection risk, and long-term graft survival. Additionally, we seek to identify significant predictors and risk factors for BKPyV-DNAemia, enabling early detection and targeted intervention.

MATERIALS AND METHODS

Study Design and Population

This retrospective cohort study analyzed data collected over 15 years (2005–2020) from five transplant centers in South Korea that participated in a preceding study [12]. Of these five centers, only three had complete raw data on BKPyV; therefore, the final study population was limited to these three high-volume transplant centers. To ensure data integrity and relevance, strict inclusion and exclusion criteria were applied. Adult KT recipients (≥18 years of age) with at least 1 year of post-transplant follow-up were eligible for inclusion. A total of 8,027 recipients from the three institutions were included based on the inclusion criteria. For the subgroup analysis, 927 patients were selected after excluding those with missing therapeutic drug monitoring (TDM) data for CNI following the onset of BKPyV-DNAemia. These patients were then categorized into three groups based on their post-viremia immunosuppressive management strategies (**Figure 1**). The study was conducted in accordance with the principles outlined in the Declaration of Helsinki and was approved by the Institutional Review Board of Asan Medical Center (IRB number: 2022-0139).

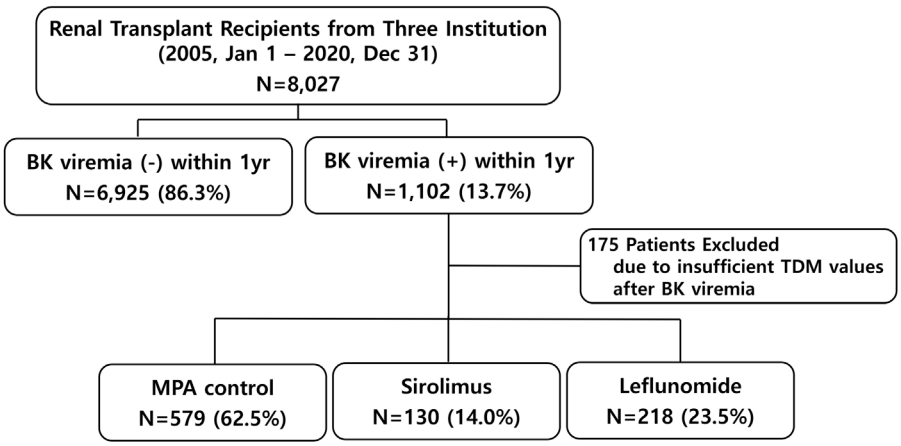


FIGURE 1 | Patient selection flowchart.

Data Collection and Processing

Data were extracted from centralized electronic medical records at the three participating centers using institutional clinical data warehouses. To ensure consistency, the investigators collaboratively defined key variables and operational definitions. Custom extraction algorithms facilitated the automated collection of recipient and donor demographics, transplant details, laboratory results, medication histories, and clinical outcomes. For this study, additional analyses were conducted using a refined dataset from a previous study [12], focusing specifically on raw data related to BKPyV-DNAemia, tacrolimus TDM results, and immunosuppressant prescription histories. All participating centers used quantitative polymerase chain reaction (qPCR) assays to monitor BKPyV-DNAemia in plasma specimens collected in EDTA tubes. While minor changes in assay platforms or reagents occurred over the 15-year study period due to technological updates, each center maintained internal quality control and calibration procedures to ensure consistency in viral load reporting. Inter-laboratory variability was minimized by interpreting BKPyV-DNAemia trends relative to each patient's baseline within the same institution, rather than applying absolute viral load cutoffs across centers.

Immunosuppressive Regimen and BKPyV-DNAemia Monitoring

The three participating institutions utilized similar immunosuppression protocols for KT, including maintenance immunosuppression and infection prophylaxis, with detailed methodologies referenced in prior studies [12, 14, 15]. For pretransplant desensitization in ABO- and HLA-incompatible recipients, rituximab (100–500 mg; Genentech, Inc., South San Francisco, CA, USA) was administered 1–2 weeks prior to plasmapheresis (PP; COBE® Spectra, Gambro BCT, Lakewood, CO, USA). PP continued until either IgM titers were ≤1:4 or IgG titers were ≤1:8 (ABOi), or until negative complement-dependent cytotoxicity crossmatch and T-cell flow-cytometric crossmatch (HLAi) were achieved. For induction therapy, basiliximab (20 mg on days 0 and 4) or anti-thymocyte globulin (ATG, 1.5 mg/kg/day) was used, with ATG reserved for high-risk patients. Maintenance immunosuppression consisted of a calcineurin inhibitor (tacrolimus or cyclosporin), mycophenolate mofetil (MMF), and corticosteroids. The detailed patterns and utilization of immunosuppressive agents among the study patients are described in a previous study [12]. At 2 months post-transplant, the most frequently observed tacrolimus trough level was ≥8.0 ng/mL in 40.0% of patients, followed by 7.0–7.9 ng/mL in 20.4% and 6.0–6.9 ng/mL in 16.6%. Notably, more than 60% of patients maintained an average tacrolimus trough level of at least 6.0 ng/mL for up to 10 months post-transplant.

At institutions participated in the present study, BKPyV monitoring was recommended at 1 and 2 weeks post-transplant, monthly until 6 months, and then every 2–3 months until 1 year post-transplant. However, testing intervals were adjusted in practice based on individual patient follow-up schedules and clinical judgment. Increased testing frequency was applied in cases of rising or high viral loads, while lower-risk patients were occasionally monitored less frequently. As such, the actual number of BKPyV-DNA tests per patient varied, and the total number of test results was substantially lower than the theoretical maximum. Across the three participating centers, a total of 34,355 BKPyV-DNAemia test results were obtained within the first post-transplant year for the 8,027 patients in this study (Center 1: 5,631 tests; Center 2: 4,562 tests; Center 3: 24,162 tests). This represents a substantial dataset for real-world BKPyV surveillance and supports the robustness of our virologic trend analysis.

Definitions

HLA-incompatible KT was defined as transplantation in recipients with a positive complement-dependent cytotoxicity crossmatch and/or flow cytometric crossmatch. BKPyV-

DNAemia positivity was identified as a log BKPyV PCR value greater than 3 within 1-year post-transplantation. Treatment failure was defined as a final follow-up log BKPyV PCR value greater than 3 persisting for at least 1 year after therapeutic intervention [16].

Subgroups were classified based on adjustments to primary immunosuppression following BKPyV-DNAemia detection. The Sirolimus group consisted of patients who transitioned from MPA to sirolimus within 6 months of BKPyV-DNAemia detection and remained on sirolimus-based therapy, including CNI withdrawal, for at least 6 months. Similarly, the Leflunomide group included patients who switched from MPA to leflunomide within 6 months of BKPyV-DNAemia detection and maintained leflunomide-based therapy for a minimum of 6 months. Lastly, the MPA control group included patients who underwent MPA tapering or discontinuation without transitioning to alternative therapies.

Statistical Analysis

Risk factor analyses for BKPyV-DNAemia and treatment failure were performed using univariate and multivariate logistic regression models. These models were employed to estimate odds ratios (OR) and 95% confidence intervals (CI) to identify independent predictors. Variables with a P-value <0.1 in the univariate analysis were included in the multivariate models to adjust for confounding factors. Long-term clinical outcomes, including biopsy-proven acute rejection (BPAR)-free survival and the efficacy of different CNI management strategies, were assessed using Kaplan-Meier survival analysis, with log-rank tests employed for group comparisons. Cox proportional hazards regression was used to quantify hazard ratios (HR) and 95% CIs for risk factors affecting BPAR-free survival. For subgroup analyses, the associations between immunosuppressive regimens and clinical outcomes were examined using chi-square tests or Fisher's exact tests for categorical variables, and Student's t-tests or one-way analysis of variance (ANOVA) for continuous variables, as appropriate. Multicollinearity was evaluated using variance inflation factors, and covariate interactions were analyzed to improve interpretability. Statistical significance was set at $P < 0.05$, with results reported as ORs, HRs, or mean differences. Analyses were performed using IBM SPSS (version 22.0, IBM Corp., Armonk, NY, United States).

RESULTS

Baseline Characteristics According to the Development of BKPyV-DNAemia

A total of 8,027 KT recipients from three centers met the inclusion criteria. Among these, 1,102 patients (13.7%) developed BKPyV-DNAemia within 1-year post-transplant. **Table 1** compares the baseline characteristics of patients who developed BKPyV-DNAemia with those who did not. The BKPyV-DNAemia group was older (49.1 ± 12.9 years vs. 45.6 ± 14.2 years, $P < 0.001$) and had a higher body weight (61.7 ± 12.6 kg vs. 60.6 ± 14.0 kg, $P = 0.016$). The proportion of females was lower in the BKPyV-DNAemia group (38.1% vs. 41.7%, $P = 0.024$). Hypertension was more common in this group (81.8% vs. 77.8%, $P = 0.003$). Other notable characteristics of the BKPyV-DNAemia group include a higher prevalence of pre-transplant dialysis ($P = 0.009$), longer pre-dialysis duration ($P < 0.001$), and higher proportions of patients with ABO incompatibility (17.6% vs. 14.4%, $P = 0.005$), HLA incompatibility (7.2% vs. 5.4%, $P = 0.018$), and the use of ATG for induction therapy (25.3% vs. 18.3%, $P < 0.001$). Significant differences in CNI utilization were also noted ($P = 0.005$), with tacrolimus use being more prevalent in the BKPyV-DNAemia group.

Univariate and Multivariate Analyses of Risk Factors for the Development of BKPyV-DNAemia Within One Year

Risk factors associated with BKPyV-DNAemia at 1 year were analyzed (**Table 2**). In the univariate analysis, older age, female sex, body weight, hypertension, pre-dialysis duration, ABO incompatibility, HLA incompatibility, basiliximab induction, ATG induction, tacrolimus TDM, desensitization, and rituximab use had P values smaller than 0.1. In the multivariate analysis, older age (OR = 1.02, $P < 0.001$) and longer pre-dialysis duration (OR = 1.02, $P = 0.023$) emerged as significant risk factors for BKPyV-DNAemia positivity at 1 year, while female sex was identified as a protective factor (OR = 0.82, $P < 0.001$). Induction therapy with ATG was significantly associated with an increased risk of BKPyV-DNAemia compared to basiliximab (OR = 3.57, $P < 0.001$). Among CNI regimens, tacrolimus TDM levels of 5–7 ng/mL (OR = 1.64, $P < 0.001$) and ≥7 ng/mL (OR = 1.20, $P = 0.023$) were significantly associated with BKPyV-DNAemia. Additionally, rituximab use showed a marginal association (OR = 1.02, $P < 0.001$).

Subgroup Analysis

After excluding 175 patients who lacked sufficient TDM data following BKPyV-DNAemia, a total of 927 patients were categorized into three groups according to the immunosuppressive management: MPA control (n = 579, 62.5%), sirolimus (n = 130, 14.0%), and leflunomide (n = 218, 23.5%). **Table 3** presents the baseline characteristics and clinical outcomes among the MPA, sirolimus, and leflunomide groups. The sirolimus group was older (51.4 ± 13.6 years, $P = 0.021$) and had a lower prevalence of ABO incompatibility (13.8%) compared to the other groups ($P = 0.05$). Induction therapy varied significantly across the subgroups ($P < 0.001$), with basiliximab being predominantly used in the MPA and leflunomide groups, while ATG was more common in the sirolimus group. BKPyV-DNA loads at first detection and at peak levels were higher in the leflunomide and sirolimus groups than in the MPA group ($P < 0.001$). CNI withdrawal was observed almost exclusively in the sirolimus group (92.3%, $P < 0.001$). Rejection rates following BKPyV-DNAemia were highest in the sirolimus group (34.4%), followed by leflunomide (22.5%) and MPA (10.9%) ($P < 0.001$). The

TABLE 1 | Baseline and clinical characteristics between kidney transplant recipients with and without BK viremia.

	BKPyV-DNAemia (–)	BKPyV-DNAemia (+)	P-value
Number of patients, n (%)	6,925 (86.3)	1,102 (13.7)	
Age, years (mean ± SD)	45.6 ± 14.2	49.1 ± 12.9	<0.001
Body weight, kg (mean ± SD)	60.6 ± 14.0	61.7 ± 12.6	0.016
Female, n (%)	2,888 (41.7)	420 (38.1)	0.024
Diabetes mellitus, n (%)	1777 (25.7)	307 (27.9)	0.12
Hypertension, n (%)	5,385 (77.8)	901 (81.8)	0.003
Pre-transplant dialysis, n (%)	5,738 (82.9)	948 (86.0)	0.009
Pre-dialysis duration, months (mean ± SD)	37.7 ± 49.7	44.3 ± 56.7	<0.001
ABO incompatibility, n (%)	997 (14.4)	194 (17.6)	0.005
HLA incompatibility, n (%)	374 (5.4)	79 (7.2)	0.018
Induction, n (%)			<0.01
none	375 (5.4)	13 (1.2)	
Basiliximab	5,251 (75.8)	806 (73.1)	
ATG	1,264 (18.3)	279 (25.3)	
Other[a]	35 (0.5)	4 (0.4)	
Calcineurin inhibitor, n (%)			0.005
Cyclosporin	2,511 (36.3)	366 (33.2)	
Tacrolimus	4,414 (63.7)	736 (66.8)	
Desensitization, n (%)	1,444 (20.9)	287 (26.0)	<0.001
Rituximab, n (%)	1,391 (20.1)	284 (25.8)	<0.001

Continuous data are presented as means ± standard deviations. Categorical data are presented as a number (%).
Abbreviations: ATG, anti-thymocyte globulin; TDM, therapeutic drug monitoring.
[a]*Other induction regimens include agents no longer in routine use, such as OKT3 (muromonab-CD3) and daclizumab (Zenapax), which were administered during the early years of the study period.*
Abbreviations: ATG, anti-thymocyte globulin; TDM, therapeutic drug monitoring.

TABLE 2 | Univariate and multivariate analyses identifying risk factors for 1-year BK viremia positivity.

	Univariate analysis		Multivariate analysis	
	OR (95% CI)	P-value	OR (95% CI)	P-value
Age, year	1.02 (1.01–1.02)	<0.001	1.02 (1.01–1.02)	<0.001
Female sex	0.86 (0.76–0.98)	0.025	0.82 (0.70–0.95)	0.010
Body weight, kg	1.01 (1.00–1.01)	0.016	1.00 (0.99–1.01)	0.75
Hypertension	1.28 (1.09–1.51)	0.003	1.14 (0.96–1.35)	0.15
Diabetes mellitus	1.12 (0.97–1.29)	0.12	–	–
Pre-transplant Dialysis	1.27 (1.06–1.53)	0.009	1.15 (0.95–1.40)	0.15
Pre-dialysis duration, year	1.03 (1.02–1.04)	<0.001	1.02 (1.00–1.03)	0.026
ABO incompatibility	1.27 (1.07–1.50)	0.005	0.75 (0.44–1.26)	0.27
HLA incompatibility	1.35 (1.05–1.74)	0.019	0.81 (0.48–1.38)	0.44
ATG vs. Basiliximab	4.80 (2.75–8.38)	<0.001	3.57 (2.03–6.27)	<0.001
Cyclosporin	Reference		Reference	–
Tacrolimus TDM <5	0.88 (0.44–1.78)	0.73	0.90 (0.44–1.82)	0.76
5 ≤ Tacrolimus TDM <7	1.46 (1.18–1.81)	<0.001	1.64 (1.31–2.06)	<0.001
Tacrolimus TDM ≥7	1.09 (0.95–1.26)	0.21	1.20 (1.03–1.39)	0.023
Desensitization	1.34 (1.16–1.55)	<0.001	1.68 (1.01–2.77)	0.044
Rituximab	1.38 (1.19–1.60)	<0.001	1.02 (1.01–1.02)	<0.001

Continuous data are presented as means ± standard deviations. Categorical data are presented as a number (%); All continuous variables were analyzed per unit increase: age (per 1 year), body weight (per 1 kg), and pre-dialysis duration (per 1 year).
Abbreviations: ATG, anti-thymocyte globulin; TDM, therapeutic drug monitoring.

higher proportion of ATG induction observed in the sirolimus group likely reflects both center-specific induction protocols and the clinical profile of patients selected for sirolimus conversion, who often presented with higher immunologic risk or CNI intolerance.

Among patients included in the subgroup analysis, the median first BKV PCR value was 3.27 log copies/mL (IQR 3.00–4.20), while the median maximum BKV PCR was 4.44 log copies/mL (IQR 3.52–5.40). The median duration of BKPyV DNAemia was 564 days (IQR 259–1,422). These metrics reflect the broad heterogeneity in viral kinetics observed in this population and underscore the need for individualized immunosuppressive strategies.

Notably, among patients with tacrolimus trough levels >5 ng/mL who underwent MPA reduction or discontinuation, no cases were identified in which leflunomide was concurrently initiated. This suggests that leflunomide use in our cohort was generally reserved for patients in whom both MPA and tacrolimus were reduced.

TABLE 3 | Characteristics and clinical outcomes of patients treated with MPA, sirolimus, or leflunomide in subgroup analysis.

	MPA	Sirolimus	Leflunomide	*P*-value
Number of patients	579 (62.5)	130 (14.0)	218 (23.5)	
Female sex	222 (38.3)	40 (30.8)	88 (40.4)	0.18
Diabetes mellitus	150 (25.9)	39 (30.0)	59 (27.1)	0.63
Hypertension	483 (83.4)	99 (76.2)	182 (83.5)	0.13
Age, years	49.4 ± 12.0	51.4 ± 13.6	50.1 ± 13.2	0.021
Body weight, kg	61.8 ± 12.7	62.3 ± 12.5	61.1 ± 11.5	0.61
ABO incompatibility	122 (21.1)	18 (13.8)	33 (15.1)	0.05
HLA incompatibility	31 (5.3)	12 (9.2)	14 (6.4)	0.25
Induction therapy				<0.001
None	12 (2.1)	0 (0)	1 (0.5)	
Basiliximab	497 (85.4)	40 (30.8)	183 (84.0)	
ATG	69 (11.9)	89 (68.5)	33 (15.1)	
Other	1 (0.7)	1 (0.8)	1 (0.5)	
First BKV PCR, log copies/mL	3.56 ± 0.9	4.01 ± 1.00	3.69 ± 1.2	<0.001
Maximum BKV PCR, log copies/mL	4.44 ± 1.4	4.98 ± 1.19	5.05 ± 1.34	<0.001
Desensitization	153 (26.4)	29 (22.3)	47 (21.6)	0.29
Rituximab	152 (26.3)	38 (29.2)	46 (21.1)	0.036
Calcineurin inhibitor				
Cyclosporin	61 (10.5)	1 (0.8)	27 (12.4)	<0.001
Tacrolimus TDM[a] <5	36 (6.2)	3 (2.3)	18 (8.3)	
5 ≤ Tacrolimus TDM[a] <7	207 (35.8)	4 (3.1)	98 (45.0)	
Tacrolimus TDM[a] ≥7	275 (47.5)	2 (1.5)	75 (34.4)	
CNI withdrawal	0 (0.0)	120 (92.3)	0 (0.0)	
Treatment failure	78 (13.5)	14 (10.8)	16 (7.3)	0.052
Rejection after BK viremia	63 (10.9)	59 (34.4)	49 (22.5)	<0.001

Continuous data are presented as mean ± standard deviation, while categorical data are presented as number (%).
Abbreviations: MPA; mycophenolic acid; ATG, anti-thymocyte globulin; TDM, therapeutic drug monitoring; CNI, calcineurin inhibitor.
[a]*TDM mean value: from first BKV, positive date to 1 year after.*

Risk Factors Associated With BKPyV-DNAemia Treatment Failure and Acute Rejection

Risk factors were analyzed by using univariate and multivariate logistic regression models, including demographic and clinical factors (age, sex, body weight, hypertension, diabetes mellitus, and pre-dialysis duration), immunologic factors (ABO and HLA incompatibility, induction therapy with basiliximab), immunosuppressive management (cyclosporin use, tacrolimus TDM levels <5 ng/mL, 5–7 ng/mL, ≥7 ng/mL, and desensitization with rituximab), BKPyV-DNAemia -related variables (first positive and highest BKPyV-DNA loads), and immunosuppressive regimen groups (MPA [reference], sirolimus, and leflunomide). Variables demonstrating a significance level of P <0.1 in univariate analysis were included in the multivariate model.

In the univariate analysis, both the initial and peak BKPyV-DNA loads were independently associated with treatment failure. An OR of 1.33 per $\log_{10}$ increase in first viral load indicates a 53% higher risk of persistent viremia for each 10-fold increase in initial BKPyV level. Similarly, an OR of 1.53 for maximum load implies a 33% increased risk per 10-fold rise in peak viral burden. These findings suggest that higher viral replication at presentation and over time both contribute to reduced viral clearance. In the multivariate analysis, BKPyV-DNAemia treatment failure was associated with maximum BKPyV-DNAemia PCR value (OR = 1.56, $P < 0.001$), while CNI withdrawal (OR = 0.05, $P < 0.001$) and the use of leflunomide were associated with a reduced risk (OR = 0.36, $P = 0.001$). Sirolimus use was also significantly associated with a higher risk of treatment failure (OR = 6.25, $P = 0.007$) in multivariate analysis (**Table 4**).

Table 5 presents the results of univariate and multivariate analyses evaluating the risk factors associated with acute rejection within 1 year following BKPyV-DNAemia. In the multivariate analysis, the maximum BKPyV PCR value (OR = 1.18, $P = 0.017$) was significantly associated with an increased risk of acute rejection, along with tacrolimus TDM <5 ng/mL (OR = 2.65, $P = 0.033$) and CNI withdrawal (OR = 6.00, $P = 0.044$). Leflunomide use was significantly associated with an increased rejection risk (OR = 2.14, $P < 0.001$), while sirolimus use did not show a significant association ($P = 0.68$). An exploratory analysis (**Supplementary Figure S1**) showed that patients who experienced acute rejection following BKPyV DNAemia had higher initial and peak viral loads compared to those without rejection, suggesting that early and substantial viral replication may contribute to subsequent immunologic injury.

Long-Term Graft Survival According to CNI Management

The CNI management groups were categorized as cyclosporin, tacrolimus TDM <5 ng/mL, tacrolimus TDM ≥5 ng/mL, and CNI withdrawal to evaluate long-term graft survival following

TABLE 4 | Univariate and Multivariate analysis of risk factors for BK viremia treatment failure.

	Univariate analysis		Multivariate analysis	
	OR (95% CI)	**P-value**	**OR (95% CI)**	**P-value**
Age, year	1.01 (0.99–1.03)	0.13	–	–
Female sex	0.97 (0.64–1.46)	0.87	–	–
Body weight, kg	1.00 (0.99–1.02)	0.65	–	–
Diabetes mellitus	0.90 (0.57–1.43)	0.66	–	–
Pre-transplant Dialysis	0.63 (0.38–1.06)	0.08	0.63 (0.36–1.11)	0.11
Pre-dialysis duration, year	0.98 (0.93–1.02)	0.30	–	–
ABO incompatibility	0.79 (0.46–1.37)	0.41	–	–
HLA incompatibility	0.89 (0.37–2.12)	0.79	–	–
ATG vs. Basiliximab	0.79 (0.17–3.60)	0.76		
First BKV PCR, log copies/mL	1.33 (1.11–1.59)	0.002	1.07 (0.88–1.31)	0.49
First BKV detection, months	0.95 (0.91–1.06)	0.675	1.01 (0.93–1.10)	0.79
Maximal BKV PCR, log copies/mL	1.53 (1.35–1.75)	<0.001	1.56 (1.36–1.80)	<0.001
Cyclosporin	Reference			
Tacrolimus TDM[a] <5	0.82 (0.31–2.19)	0.69	0.56 (0.19–1.61)	0.28
5 ≤ Tacrolimus TDM[a] <7	0.92 (0.47–1.80)	0.81	0.83 (0.41–1.69)	0.61
Tacrolimus TDM[a] ≥7	0.69 (0.35–1.36)	0.28	0.63 (0.31–1.30)	0.21
CNI withdrawal	0.47 (0.19–1.16)	0.10	0.05 (0.01–0.24)	<0.001
MPA group	Reference			
Sirolimus	0.78 (0.42–1.42)	0.41	6.25 (0.66–23.55)	0.007
Leflunomide	0.51 (0.29–0.89)	0.018	0.36 (0.20–0.66)	0.001
Desensitization	0.85 (0.53–1.37)	0.51	–	–
Rituximab	0.77 (0.47–1.25)	0.29	–	–

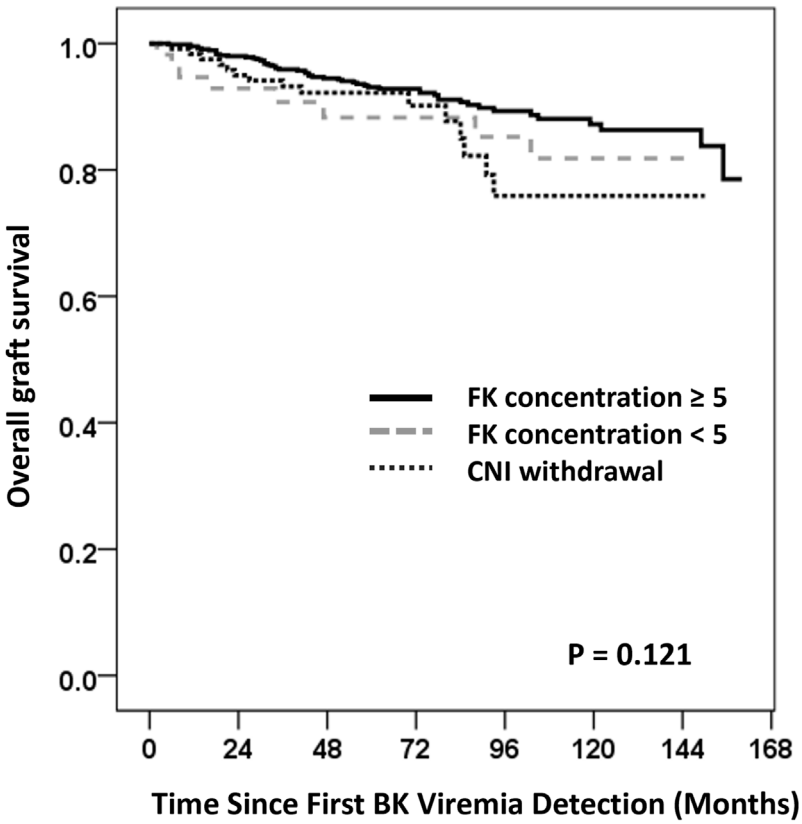
Abbreviations: ATG, anti-thymocyte globulin; BKV, BKPyV-DNAemia; CNI, calcineurin inhibitor; TDM, therapeutic drug monitoring; MPA; mycophenolic acid.
[a]TDM, mean value: from first BKV, positive date to 1 year after.

TABLE 5 | Univariate and multivariate analysis of risk factors for acute rejection within 1 year following BK viremia.

	Univariate analysis		Multivariate analysis	
	OR (95% CI)	**P-value**	**OR (95% CI)**	**P-value**
Age, year	1.02 (1.00–1.03)	0.024	1.01 (0.99–1.03)	0.07
Female sex	0.84 (0.60–1.19)	0.33	–	–
Body weight, kg	1.01 (0.99–1.02)	0.59	–	–
Diabetes mellitus	0.97 (0.67–1.42)	0.89	–	–
Pre-transplant Dialysis	0.88 (0.55–1.40)	0.59	–	–
Pre-dialysis duration, year	1.01 (0.99–1.01)	0.36	–	–
ABO incompatibility	1.00 (0.66–1.54)	0.99	–	–
HLA incompatibility	1.33 (0.70–2.53)	0.38	–	–
ATG vs. Basiliximab	1.01 (0.22–4.63)	0.99		
First BKV PCR, log copies/mL	1.32 (1.13–1.53)	<0.001	1.09 (0.91–1.31)	0.34
Maximal BKV PCR, log copies/mL	1.28 (1.15–1.43)	<0.001	1.18 (1.03–1.36)	0.017
Cyclosporin	Reference			
Tacrolimus TDM[a] <5	2.53 (1.07–6.01)	0.035	2.65 (1.08–6.51)	0.033
5 ≤ Tacrolimus TDM[a] <7	1.15 (0.56–2.33)	0.71	1.15 (0.55–2.38)	0.71
Tacrolimus TDM[a] ≥7	1.04 (0.51–2.10)	0.91	1.23 (0.59–2.55)	0.58
CNI withdrawal	6.42 (3.11–13.26)	<0.001	6.00 (1.05–34.45)	0.044
MPA group	Reference			
Sirolimus	6.81 (4.41–10.50)	<0.001	1.36 (0.27–6.85)	0.68
Leflunomide	2.38 (1.57–3.59)	<0.001	2.14 (1.40–3.29)	<0.001
Desensitization	1.33 (0.92–1.92)	0.13	–	–
Rituximab	1.36 (0.94–1.96)	0.10	1.41 (0.95–2.10)	0.09

Abbreviations: ATG, anti-thymocyte globulin; BKV, BKPyV-DNAemia; CNI, calcineurin inhibitor; TDM, therapeutic drug monitoring; MPA; mycophenolic acid.
[a]TDM, mean value: from first BKV, positive date to 1 year after.

BKPyV-DNAemia. In the Kaplan-Meier analysis, the overall log-rank test did not show a statistically significant difference in graft survival among the CNI management groups (P = 0.121) (**Figure 2**). Multivariate Cox regression analysis was conducted to identify predictors of graft failure following BKPyV-DNAemia (**Table 6**). CNI withdrawal was associated with borderline significance for worse survival compared to tacrolimus TDM ≥5 ng/mL (P = 0.067). In the multivariate



Number at risk	0 month	12 month	36 month	60 month	120 month
FK < 5	57 (100%)	53 (94.7%)	39 (90.7%)	33 (88.3%)	10 (81.8%)
FK ≥ 5	661 (100%)	646 (99.5%)	517 (9539%)	372 (93.1%)	100 (87.2%)
CNI withdrawal	120 (100%)	117 (98.3%)	100 (93.2%)	62 (92.2%)	6 (75.9%)

FIGURE 2 | Long-term Graft Survival Following BKPyV-DNAemia. Kaplan-Meier survival curves comparing long-term graft survival among patients stratified by CNI management strategies following BKPyV-DNAemia. The overall log-rank test was not statistically significant (P = 0.121). Pairwise comparisons showed that CNI withdrawal was associated with a trend toward worse survival compared to tacrolimus TDM levels of ≥5 ng/mL (P = 0.067). Note: No patients with tacrolimus trough levels >5 ng/mL underwent MPA reduction or discontinuation in combination with leflunomide initiation. Leflunomide use was limited to those with simultaneous reduction in both tacrolimus and MPA.

analysis, older age (HR = 1.02, P = 0.042), diabetes mellitus (HR = 2.11, P = 0.001), and the maximum BKPyV-DNAemia PCR value (HR = 1.24, P = 0.001) were identified as significant risk factors for long-term graft failure following BK viremia. Tacrolimus TDM ≥5 ng/mL was associated with a reduced risk of graft failure (HR = 0.54, P = 0.036), while CNI withdrawal showed a trend toward a higher risk of graft failure (HR = 4.37, P = 0.07). Additionally, sirolimus (HR = 2.12, P = 0.003) and leflunomide (HR = 1.94, P = 0.006) were associated with a higher risk of graft failure compared to MPA. Patients who experienced graft failure demonstrated higher median first and maximum BKPyV-DNA loads, suggesting that greater early or sustained viral replication may be associated with adverse long-term graft outcomes (**Supplementary Figure S2**).

DISCUSSION

Our multicenter retrospective study underscores the critical importance of personalized immunosuppressive strategies for managing BKPyV-DNAemia in KT recipients. Key risk factors for BKPyV-DNAemia included older age, induction therapy with ATG, and elevated tacrolimus levels, which should be considered for risk stratification and targeted surveillance. Notably, the balance between preventing rejection and minimizing BKPyV-DNAemia heavily depends on maintaining optimal concentrations of CNI. Maintaining tacrolimus TDM levels at or above 5 ng/mL was associated with a lower risk of graft failure. In contrast, CNI withdrawal, even with the use of sirolimus as an alternative, showed a trend toward increased

TABLE 6 | Univariate and Multivariate analysis of risk factors for overall graft failure following BK viremia.

	Univariate analysis		Multivariate analysis	
	HR (95% CI)	**P-value**	**HR (95% CI)**	**P-value**
Age, year	1.03 (1.01–1.05)	0.002	1.02 (1.00–1.04)	0.042
Female sex	0.74 (0.48–1.15)	0.18	–	–
Body weight, kg	1.01 (0.99–1.03)	0.32	–	–
Diabetes mellitus	1.83 (1.20–2.81)	0.005	2.11 (1.34–3.34)	0.001
Pre-dialysis duration, year	1.02 (0.98–1.06)	0.48	–	–
ABO incompatibility	0.81 (0.47–1.41)	0.46	–	–
HLA incompatibility	0.73 (0.27–2.00)	0.54	–	–
ATG vs. Basiliximab	0.89 (0.22–3.65)	0.87		
First BKV PCR, log copies/mL	1.15 (0.96–1.37)	0.12	–	–
Maximal BKV PCR, log copies/mL	1.37 (1.22–1.54)	<0.001	1.24 (1.09–1.42)	0.001
Cyclosporin	Reference			
Tacrolimus TDM[a] <5	0.88 (0.37–2.09)	0.78	0.79 (0.33–1.91)	0.60
5 ≤ Tacrolimus TDM[a]	0.55 (0.31–0.98)	0.044	0.54 (0.30–0.96)	0.036
CNI withdrawal	9.93 (4.75–20.75)	<0.001	4.37 (0.87–22.07)	0.07
MPA group	11.15 (6.31–19.72)	<0.001	1.58 (0.37–6.73)	1.58
Sirolimus	2.62 (1.62–4.24)	<0.001	2.12 (1.29–3.49)	0.003
Leflunomide	2.97 (1.93–4.57)	<0.001	1.94 (1.21–3.11)	0.006
Desensitization	0.96 (0.60–1.55)	0.88	–	–
Rituximab	0.98 (0.61–1.57)	0.93	–	–

Abbreviations: ATG, anti-thymocyte globulin; BKV, BKPyV-DNAemia; CNI, calcineurin inhibitor; TDM, therapeutic drug monitoring; MPA; mycophenolic acid.
[a]*TDM, mean value: from first BKV, positive date to 1 year after.*

graft failure. These findings suggest that adequate CNI exposure is crucial for balancing viral control and immune suppression. Substituting MPA with leflunomide effectively reduced BKPyV load but was associated with a higher risk of rejection and inferior long-term graft survival. These findings suggest that prolonged maintenance of leflunomide instead of MPA may increase the risk of acute rejection and compromise graft survival.

Building upon existing literature on tacrolimus TDM [12], we found that tacrolimus trough levels between 5 and <7 ng/mL may represent the "optimal range" to mitigate the risk of BKPyV-DNAemia while maintaining sufficient immunosuppression to prevent rejection. In line with our findings, Schaub et al. demonstrated the effectiveness of a CNI-focused strategy for managing BKV infection in KT recipients by prioritizing tacrolimus reduction. Tacrolimus levels were reduced in a stepwise manner, with adjustments to MMF considered only after achieving sufficient CNI reduction [17]. This approach, supported by evidence of tacrolimus's inhibitory effect on BKPyV-specific T cells, achieved a 92% clearance rate of BKPyV-DNAemia while maintaining stable allograft function over a median follow-up of 34 months [18, 19]. Moreover, the low clinical rejection rate of 8.6% and stable graft function despite subclinical inflammation further highlight the effectiveness of this strategy. The Kidney Disease Improving Global Outcomes (KDIGO) guidelines recommend reducing MPA first, followed by a reduction in CNI dosage, while the Second International Consensus Guideline presented both antimetabolite-first and CNI-first strategies as viable options. Additionally, KDIGO suggests a general 50% reduction in CNI dosage, whereas the International Consensus Guideline recommends target trough levels of tacrolimus (3–5 ng/mL) and cyclosporine (75–125 ng/mL) [20, 21]. Brennan et al. suggested that reducing antimetabolites before CNI reduction yields similar outcomes in BKPyV-DNAemia clearance compared to direct CNI reduction strategies [22]. These findings indicate that both approaches may be viable, emphasizing the need for individualized adjustments in immunosuppression. Furthermore, based on our study results, minimizing the duration of tacrolimus exposure below 5 ng/mL appears to be the most critical factor in optimizing post- BKPyV-DNAemia outcomes. Notably, large-scale studies stratifying outcomes by CNI levels are lacking, highlighting the significance of our findings in guiding immunosuppressive management.

A recent study from the Swiss Transplant Cohort proposed a five-group classification of BKPyV-DNAemia trajectories based on onset, duration, and clearance patterns [23]. This categorization demonstrated clinical relevance, showing that sustained or recurrent viremia, particularly among early-onset cases, was associated with higher rates of persistent replication and impaired graft function, whereas transient early-onset viremia correlated with more favorable outcomes. To explore this further, we performed a subgroup analysis among patients with early-onset BKPyV-DNAemia (≤90 days post-transplant), stratifying them by whether viremia resolved within 6 months or persisted thereafter. As shown in **Supplementary Figure S3**, the early-persistent subgroup exhibited a trend toward lower graft survival, although the difference did not reach statistical significance (log-rank $p = 0.26$). This suggests that duration of viremia may be a more critical determinant of outcome than timing of onset alone. Although we did not formally apply the full trajectory model used in the Swiss study, our findings support the clinical utility of integrating both onset and clearance patterns in future risk stratification frameworks.

Reduction or discontinuation of MPA in the treatment of BKPyV-DNAemia in KT recipients carries a risk of allograft

rejection, even after achieving BKPyV-DNAemia clearance. The incidence of acute rejection in patients treated with immunosuppression reduction for BKPyV-DNAemia has been reported to be approximately 10%–30%, with a higher risk observed in patients undergoing more aggressive reductions or conversions to alternative immunosuppressive regimens. [24–26]. In our study, the rejection rates for patients treated with MPA, sirolimus, or leflunomide were 10.9%, 34.4%, and 22.5%, respectively. Notably, the rejection rate in the MPA group (10.9%) was consistent with prior studies that reported rates of 9%–12% for tacrolimus-based regimens combined with MPA or azathioprine [24, 27]. This suggests that reducing immunosuppression in the context of MPA-based regimens can effectively mitigate the risk of rejection while maintaining control of BKPyV-DNAemia The rejection rate in the sirolimus group (34.4%) was significantly higher than that in the MPA group ($P < 0.001$). This discrepancy may be attributed to the elevated initial BKPyV PCR levels in the sirolimus group and the treatment strategy employed at one participating center, where sirolimus was initiated when BKPyV PCR levels reached ≥4, accompanied by the withdrawal of CNI. Consequently, many patients in the sirolimus group underwent CNI withdrawal, which likely contributed to the higher rejection rate. Notably, in the multivariate analysis for acute rejection, sirolimus itself was not identified as a significant risk factor. The leflunomide group exhibited the most effective response to BKPyV-DNAemia treatment, despite having the highest maximum BKPyV-DNAemia PCR levels. However, the risk of acute rejection within 1 year after the onset of BKPyV-DNAemia was 2.1 times higher compared to the MPA group, suggesting that substituting leflunomide may have a similar immunosuppressive effect as withdrawing MPA. These findings indicate that transitioning from MPA to leflunomide can be a highly effective treatment strategy for patients with elevated BKPyV PCR levels. Nevertheless, based on our results, reintroducing a low dose of MPA or maintaining appropriate CNI levels as BKPyV PCR stabilizes may be advisable to minimize the risk of rejection.

Leflunomide is an immunomodulatory and antiviral agent that inhibits dihydroorotate dehydrogenase, thereby suppressing BKPyV replication and lymphocyte proliferation. Its antiviral effects are particularly pronounced in renal tubular epithelial cells, where it reduces the replication of BKPyV by inhibiting DNA synthesis [28, 29]. This dual mechanism allows for a reduction in the use of immunosuppressive drugs without increasing the risk of rejection. While effective in high-risk cases, its use is still associated with rejection and graft dysfunction, and the absence of a clear correlation between serum levels and efficacy complicates its clinical application. Our subgroup analysis suggests that leflunomide is a promising option for managing BKPyV-DNAemia in KT recipients, especially in high-risk cases. Similarly, a study by Aldieri et al. reported a BKPyV-DNAemia clearance rate of 91.4%, including viral eradication in 8 of 11 patients with biopsy-proven BKVN, when leflunomide was used as an adjunct to reduced immunosuppression rather than complete discontinuation of antiproliferative agents [30]. Further evidence from a multicenter study [31] demonstrated a 76% BKPyV-DNAemia clearance rate in KT recipients treated with leflunomide after failing prior therapies. However, 11 patients experienced graft loss, with 9 of these cases attributed to BKVN; rejection episodes occurred in 33% of patients, emphasizing the challenges of balancing immunosuppression and antiviral efficacy. A systematic review [32] corroborated these findings, reporting BKPyV-DNAemia clearance rates ranging from 33% to 92%, although significant heterogeneity in dosing regimens and pharmacokinetics complicated the interpretation of results. Notably, adverse events such as hemolytic anemia and thrombotic microangiopathy were observed, highlighting the importance of monitoring during treatment. Smaller prospective studies further support the efficacy of leflunomide. Faguer et al. reported that 42% of KT recipients with BKVN achieved viral clearance, and 66.6% maintained stable or improved graft function after switching from MMF to leflunomide [33]. Our study suggests a potential role for leflunomide in BKPyV suppression while underscoring the need for careful monitoring to balance efficacy and safety. Study by Bischof et al. summarize contemporary treatment options and emphasize the importance of tailoring immunosuppressive reduction based on viral dynamics histologic severity, and graft function [34]. Their study highlights the limitations of a one-size-fits-all approach and outlines the variable efficacy of adjunctive therapies, such as leflunomide and immunoglobulin, especially in the absence of randomized controlled trials. Our findings align with this perspective, suggesting that while immunosuppressive modulation remains the cornerstone, its optimization requires greater clinical granularity and prospective validation.

The role of sirolimus in managing BKPyV-DNAemia has been highlighted in several studies, demonstrating both antiviral effects and potential benefits in specific patient populations. The TRANSFORM study, a randomized, multicenter trial, evaluated everolimus with reduced exposure to CNIs compared to MPA with standard CNI exposure in *de novo* KT recipients. While not primarily designed to assess BK virus infection, the study reported a significantly lower incidence of BKV replication, based on center-reported data, in the everolimus group compared to the MPA group (8.8% vs. 14.8%, $p < 0.001$). [35]. Similarly, a retrospective study by Tohme et al. demonstrated a lower incidence of BKPyV-DNAemia in patients converted to sirolimus-based regimens, with clinically significant BKPyV-DNAemia observed in only 4.3% of the sirolimus group compared to 17.9% in the tacrolimus group [36]. These findings suggest a potential role for sirolimus in reducing BKPyV replication, particularly in low-risk populations. The recent BKEver study further supports the effectiveness of early reduction of both MPA and tacrolimus as a first-line approach for managing new-onset BKPyV-DNAemia [37]. In this prospective multicenter cohort, 81.3% of kidney transplant recipients achieved viral clearance within 6 months without an increased incidence of acute rejection. Notably, patients converted to everolimus had a lower clearance rate of 55.7%, suggesting that mTOR inhibitor conversion may be less effective as an initial strategy. These findings are consistent with our results and reinforce the value of a measured, stepwise reduction in immunosuppression for achieving viral control while minimizing rejection risk.

Several *in vitro* studies further support the antiviral properties of sirolimus. One study reported that sirolimus inhibits v replication by impairing mTOR-SP6-kinase activation and

suppressing the expression of the BKPyV large T antigen in renal epithelial cells [38]. The inhibitory effects of sirolimus on BKPyV replication were most effective within 24 h of infection, particularly during early viral gene expression, but diminished during the late phase. These findings underscore a potential therapeutic window for sirolimus in the management of BKPyV-DNAemia In contrast, tacrolimus has been shown to activate BK viral replication via the same FKBP-12 pathway, highlighting a mechanistic divergence that could inform tailored immunosuppressive strategies. Moreover, sirolimus may modulate the immune response to the BKPyV through its effects on T-cell function. A study by Araki et al. demonstrated that rapamycin (sirolimus) enhances the formation of memory $CD8^+$ T cells, which exhibit superior antiviral functionality, higher expression of markers associated with long-lived immunity (e.g., CD127, CD62L, Bcl-2), and reduced expression of senescence markers such as KLRG-1 [39]. These findings suggest that sirolimus may augment the antiviral immune response while providing essential immunosuppression for transplant recipients. However, complete withdrawal of CNIs when using sirolimus, particularly in immunologically high-risk patients, may increase the risk of acute rejection and negatively impact long-term graft survival.

Our study also highlights the wide variability in BKPyV DNAemia dynamics. The median duration of DNAemia exceeded 1.5 years in our cohort, with some patients experiencing persistence for more than 4 years. The initial and peak viral loads were notably higher in patients requiring alternative immunosuppressive regimens such as sirolimus or leflunomide. These findings reinforce the notion that viral kinetics—not just presence or absence of viremia—may influence both treatment decisions and graft outcomes, and should therefore be considered in future prospective stratification models.

Interestingly, our study found that female sex was associated with a lower risk of developing BK viremia, which complements prior observations identifying male sex as a potential risk factor, such as those noted in the The Transplantation Society (TTS) guidelines [21]. Although the underlying mechanisms remain unclear, pharmacokinetic studies have reported that female recipients tend to exhibit higher tacrolimus exposure and slower clearance, which may affect immunosuppressive intensity and susceptibility to viral reactivation [40, 41]. Additionally, sex-based differences in antiviral immunity have been described. These biological factors may contribute to the observed association, though further investigation is warranted to clarify causality.

Prolonged pre-transplant dialysis duration may, in part, reflect underlying immunologic barriers—such as HLA or ABO incompatibility—that delay transplantation and potentially influence post-transplant infection risk. However, in our cohort, there was no statistically significant association between pre-transplant dialysis and the need for desensitization (Pearson $\chi^2 = 1.684$, $P = 0.194$), suggesting that pre-dialysis status was not primarily driven by immunologic risk factors.

The newly published consensus standard for BKPyV-associated nephropathy recommends not only timely reduction of immunosuppression upon BKPyV-DNAemia detection but also careful re-escalation of maintenance immunosuppression once viral clearance is achieved [21, 42]. In our retrospective cohort, data on post-clearance immunosuppressive intensification—including MPA reintroduction or increased tacrolimus dosing—were not consistently recorded. Moreover, substitution of MPA with leflunomide—a less potent immunosuppressant—may leave patients functionally under-immunosuppressed, potentially contributing to late acute rejection and graft loss. This observation highlights the need for closure of the immunosuppressive gap following viral clearance, ideally in line with expert guideline recommendations.

Despite its large sample size and extended follow-up, this study has several limitations. Its retrospective design and focus on Korean transplant centers may limit the generalizability of the findings to other populations. Normalization of BKV PCR values, such as using fold-change relative to the assay's lower limit of detection, can enhance cross-center comparability in multicenter studies. In our cohort, however, the distribution of viral load values was empirically consistent across institutions, supporting the validity of using absolute values for analysis without additional transformation. Additionally, transplant practices evolved over the 15-year study period, potentially introducing unmeasured confounders. Variability in BKPyV detection and management protocols across centers may have resulted in selection bias, particularly in the sirolimus group, where one center exclusively implemented MPA discontinuation, sirolimus initiation, and complete CNI withdrawal for patients with BKPyV PCR levels greater than 4. This approach likely resulted in more severe or refractory BKPyV infections at baseline, influencing treatment outcomes despite multivariate adjustments. We therefore attempted to address these biases through comprehensive multivariate analyses to ensure robust findings. Moreover, our cohort included a relatively high proportion of immunologically high-risk patients, which may have impacted both BKPyV-DNAemia incidence and rejection patterns compared to lower-risk populations. These factors highlight the complexity of immunosuppressive modifications in BKPyV-DNAemia management and underscore the need for individualized treatment strategies based on patient-specific risk profiles. We acknowledge that immune reconstitution after immunosuppression reduction may lead to antiviral inflammatory infiltrates that mimic T-cell mediated rejection (TCMR). In our study, rejection diagnoses were based on local Banff assessments without centralized or molecular review, limiting our ability to distinguish true TCMR from beneficial antiviral responses. This represents a limitation and highlights the need for more refined biopsy evaluation in future studies. Lastly, corticosteroid exposure, including pulse therapy for acute rejection, was not uniformly documented across centers and could not be systematically analyzed. While most centers followed standard protocols, the lack of detailed data on cumulative steroid burden is a limitation that future studies should address.

Another limitation of our study is the composition of the sirolimus group. The vast majority (92.3%) of patients receiving

sirolimus were managed in a CNI-withdrawal setting, with only 10 patients receiving sirolimus in combination with tacrolimus. No patients received cyclosporine plus sirolimus. Although the sirolimus + TAC subgroup showed a significantly higher rate of BKPyV treatment failure (40.0% vs. 8.3%, p = 0.002) but a lower rate of 1-year rejection (20.0% vs. 47.5%, p = 0.086) compared to the CNI-free sirolimus group, as shown in **Supplementary Table S1**, these results should be interpreted with caution due to the small sample size and the relatively high tacrolimus trough levels (mean TDM 7.6 ng/mL). Therefore, our study is not adequately powered to assess the effects of standard low-dose CNI + mTORi regimens and may not reflect their clinical efficacy.

This multicenter retrospective study highlights key risk factors for BKV and offers guidance on immunosuppressive strategies in kidney transplant recipients. Maintaining tacrolimus trough levels between 5 and 7 ng/mL balances BKPyV-DNAemia control and rejection risk. Adjusting or replacing MPA, including with leflunomide, may aid BKPyV-DNAemia management but carries long-term immunologic considerations. While CNI withdrawal may promote viral clearance, it raises rejection risk. We recommend individualized adjustment of immunosuppression based on BKPyV PCR trends. These findings support more personalized management approaches to improve long-term outcomes. Future prospective studies and incorporation of molecular diagnostics may enhance risk prediction and treatment optimization.

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 Institutional Review Board of Asan Medical Center (IRB number: 2022-0139). 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

J-MK, HeK, AH, HuK, and SM participated in the research design. J-MK, HeK, AH, YK, SS, YK, and KL participated in the performance of the research and data acquisition. J-MK, HeK, AH, HuK, and SM participated in data analysis and interpretation. JP, KL, HuK, and SM provided critical input on methodology. J-MK, HeK, HuK, and SM participated in the writing and critical revision of the manuscript for intellectual content. HuK is the guarantor of this work and takes full responsibility for the integrity of the data and the accuracy of the data 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.

GENERATIVE AI STATEMENT

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

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2025.14738/full#supplementary-material

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ORIGINAL RESEARCH
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Effector-Memory γδ T Lymphocytes Predict CMV Disease After the Withdrawal of Prophylaxis in Kidney Transplant Recipients

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Evaluation of CMV-specific cell-mediated immunity (CMI) has improved strategies to prevent post-transplant CMV disease. This study assessed the association between CMV disease and absolute count of TEMRA γδ T cells at the end of universal prophylaxis in kidney transplant recipients (KTR). We retrospectively analyzed 262 R^+ and 82 D^+/R^- KTRs who received antiviral prophylaxis and had TEMRA γδ T cells quantified at the end of prophylaxis. The primary endpoint was CMV disease within two years post-transplant. Post-prophylaxis CMV disease occurred in 43/344 (12.5%) patients. A threshold of 4.65/mm³ for TEMRA γδ T-cell count was identified by ROC analysis; higher counts were associated with reduced CMV disease incidence. While no significant association was found in the overall cohort, in R^+ patients, a count >4.65/mm³ was associated with a 97.7% positive predictive value for protection against CMV disease. Multivariate analysis confirmed its independent association with disease-free survival [HR: 0.27 (95% CI: 0.09–0.85), p = 0.0252]. Measuring TEMRA γδ T-cell counts at the end of prophylaxis may serve as a useful, accessible immune marker to guide CMV prevention strategies in R^+ kidney transplant recipients.

Keywords: CMV, infection, immunology, immunomonitoring, prophylaxis

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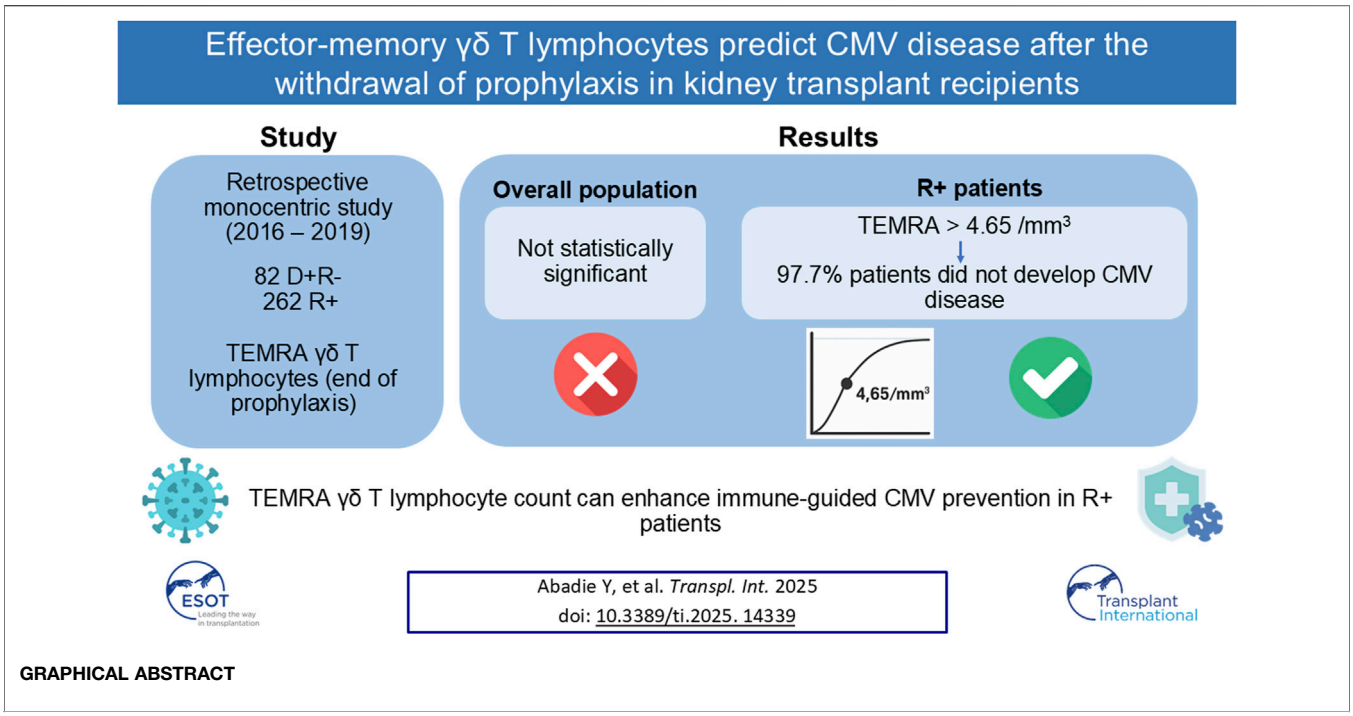
INTRODUCTION

Human cytomegalovirus (CMV) is a widespread virus within the general population [1]. Although the infection is mostly asymptomatic in immunocompetent hosts, it can have severe consequences for immunocompromised patients. In particular, kidney transplant recipients (KTR) are at risk, as CMV can cause direct, life-threatening organ damage (e.g., colitis, pneumonitis, encephalitis) [2], and contribute to indirect complications such as acute rejection [3] or post-transplant diabetes mellitus [4]. These complications significantly reduce both patient and graft survivals [5]. Fortunately, substantial progress has been made in preventing CMV disease with the advent of the universal prophylaxis [6, 7].

A better understanding of the anti-CMV immune response has enabled the development of biomarkers that can stratify the risk of developing CMV disease. The most commonly used



GRAPHICAL ABSTRACT

biomarker is based on donor and recipient serology, with donor-positive/recipient-negative (D+R-) patients being at the highest risk for CMV infection [3]. In recent years, additional biomarkers have been identified, focusing on the cellular component of the anti-CMV immune response, particularly the αβ T-cell response [8]. Two commercially available assays have been tested in various contexts. The QuantiFERON assay has demonstrated its value in 1) predicting protection against CMV disease in D+R- patients when performed at the end of prophylaxis [9, 10], 2) predicting spontaneous viral clearance in patients with low DNAemia [11], and 3) forecasting protection against clinical recurrence at the end of CMV treatment [12]. Similarly, ELISpot has proven effective in identifying KTR at very low risk of developing CMV disease [13–15].

Importantly, recent randomized trials have incorporated QuantiFERON or ELISpot to assess infection risk and personalize post-transplant CMV prevention strategies. Two trials confirmed the safety of discontinuing antiviral prophylaxis after 4–6 weeks in R+ patients who had received thymoglobulin, provided their QuantiFERON or ELISpot tests were positive, without increasing CMV infection rates [16, 17]. Moreover, Jarque et al. showed that R+ patients receiving basiliximab who had a positive ELISpot test 2 weeks post-transplant were protected from CMV infection [18].

Interestingly, some studies have shown that certain patients did not develop CMV disease despite the absence of any detectable CMV-specific αβ T-cell response, while others developed CMV disease despite having a CMV-specific αβ T-cell response [10]. These assays exclusively assess the αβ T-cell response, leading to the hypothesis that other components of the anti-CMV immune response may be essential to control the infection.

Our group has shown that the T cell immune response to CMV is also mediated by another subset of non-αβ T cells, namely the γδ T cells (and more specifically those negative for the Vδ2 TCR chain). The expansion of these cells during CMV infection correlates with the resolution of the viremia and the absence of recurrence [19]. *In vitro*, γδ T cells clones or cell lines have been shown to inhibit CMV replication and to kill CMV-infected cells [20]. This protective role has been confirmed by several mouse studies [21–23]. The expansion of the γδ T lymphocyte subset during CMV infection is accompanied by a very specific phenotypic change, including the acquisition of markers indicative of cytotoxic activity (perforin+, granzyme+) and of terminal effector differentiation characterized by the loss of CD27 and presence of CD45RA expression, ($CD27^{-}$, CD45RA+) [24, 25] so called T effector/memory expressing CD45RA (TEMRA) phenotype.

In this study, we aimed to analyze the occurrence of CMV disease in relation to the absolute count of TEMRA γδ T cells at the end of universal prophylaxis in KTR.

MATERIALS AND METHODS

Study Design and Population

We conducted this retrospective study at Bordeaux University Hospital (France). KTRs who received a deceased or living donor kidney between 1 September 2016 and 31 December 2019 were included if they were over 18 years old and if their CMV status was either D+R- or R+.

Induction therapy consisted of thymoglobulin for HLA-sensitized KTRs, and basiliximab for the others. Maintenance treatment included tacrolimus, targeting through level target of 8–10 ng/mL during the first year, followed by 6–10 ng/mL, along with mycophenolic acid (720 mg bid). Steroids were rapidly reduced to 5 mg/day and weaned in non-HLA-sensitized KTRs during the first month post-transplantation. Everolimus was used for a small number of KTRs with a through level target of 5–8 ng/mL.

All KTRs received universal prophylaxis with valganciclovir, aiming for 6 months in D+R- KTRs or 3 months in R+ KTRs. Valganciclovir dosage adjustments were made using the Cockcroft-Gault formula.

KTRs were excluded if they did not take antiviral prophylaxis for at least 6 weeks, if they experienced death, graft loss, or were lost-of follow-up before month 3, and if monitoring of the γδ T lymphocyte subset was not performed at the end of the antiviral prophylaxis. Notably, γδ T lymphocyte measurement at the end of the prophylaxis was part of the routine monitoring of KTRs during this period.

All clinical and biological variables were collected from the R@N database (with final approval from the French Data Protection Authority [CNIL], number 135715). All participants gave written informed consent. The study was performed in accordance with the ethical standards as laid down in the Declaration of Helsinki, and was approved by the Institutional Review Board of the Bordeaux University Hospital.

Endpoints

The endpoints were:

1) The incidence of CMV disease during the first 2 years post-transplantation, based on the absolute count of lymphocytes, Vδ2^{neg} γδ T lymphocytes, and TEMRA Vδ2^{neg} γδ T lymphocytes measured at the end of universal prophylaxis in the overall population.
2) The incidence of CMV disease in R+ KTRs, according to the same lymphocyte and T cell subsets counts.

Definitions

CMV disease was defined as "CMV syndrome" or "probable or proven end-organ CMV disease" using standardized criteria from international guidelines [26].

CMV syndrome was defined by the detection of a positive CMV PCR, with at least 2 additional criteria among the following: fever, malaise or fatigue, leukopenia or neutropenia, thrombopenia or elevation of hepatic aminotransferase.

Proven CMV end-organ disease was defined as the presence of appropriate clinical symptoms together with documentation of CMV in tissue from the relevant organ by immunohistochemistry.

Probable CMV end-organ disease was defined as the presence of appropriate clinical symptoms together with documentation of high viral DNA levels in tissue from the relevant organ by quantitative nucleic acid testing.

The onset of CMV disease was marked by the first detection of CMV DNAemia with CMV symptoms. The duration of CMV disease was the time from the first positive CMV DNAemia until symptom resolution and viral eradication following at least 2 weeks of treatment. The treatment duration was defined as the period during which KTRs received antiviral therapy for CMV disease. Recurrent disease referred to a new episode in KTRs who had previously achieved negative CMV DNAemia following treatment.

CMV Quantitative Nucleic Acid Testing

Various CMV quantitative nucleic acid testing (QNAT) methods were used throughout the study. Starting in September 2016, QNAT was performed with the LightMix® Human Cytomegalovirus Kit (TIB MOLBIOL GmbH, Berlin, Germany), with detection and quantification thresholds of 250 and 1000 IU/mL, respectively. From April 2019 onward, the CMV R-GENE® Kit (Biomerieux, France) was used, with thresholds of 150 and 200 IU/mL. All QNAT assays were conducted in the Department of Virology at Bordeaux University Hospital, adhering strictly to Quality Control for Molecular Diagnostics (QCMD, Glasgow, Scotland) standards since 2004. A CMV QNAT result below the quantification limit was considered negative.

Flow Cytometry Analysis of Vδ2^{neg} γδ T Cells at the End of the Prophylaxis

Lymphocyte and Vδ2^{neg} γδ T lymphocyte counts were analyzed at the end of universal valganciclovir prophylaxis (±1 month). Vδ2^{neg} γδ T lymphocyte counts were determined by flow cytometry in the Department of Immunology and Immunogenetics at Bordeaux University Hospital, as previously described [19]. To identify the Vδ2^{neg} γδ T lymphocyte subset and their TEMRA phenotype, we used a panel containing antibodies targeting CD3, γδ TCR, Vδ2 TCR, CD27, and CD45RA (Beckman Coulter, Marseille, France). The Vδ2^{neg} γδ T lymphocyte subset is rare in CMV-naïve subjects [24]. Results were reported as "not interpretable" (NI) when fewer than 300 events were detected in the Vδ2^{neg} γδ T lymphocyte gate (**Supplementary Figure S1**). For clarity, TEMRA Vδ2^{neg} γδ T cells are referred to as TEMRA γδ T cells throughout this report.

Other Variables Assessment

When comparing the incidence of CMV disease across different medication regimens or rejection episodes, only events occurring before the CMV disease onset were included in the "CMV disease" group. All rejection episodes were biopsy-proven. Preformed donor-specific antibodies (DSA) were defined as those present on the day of transplantation or earlier. Post-transplant estimated glomerular filtration rate (eGFR) was defined as the highest eGFR recorded during the prophylaxis period.

Statistical Analysis

KTRs characteristics are presented as medians and interquartile ranges (IQR) for quantitative variables and as percentages for qualitative variables. Fisher's exact test or McNemar's test was used to compare qualitative variables, while Student's t-test or the Mann–Whitney test was applied to quantitative variables. A

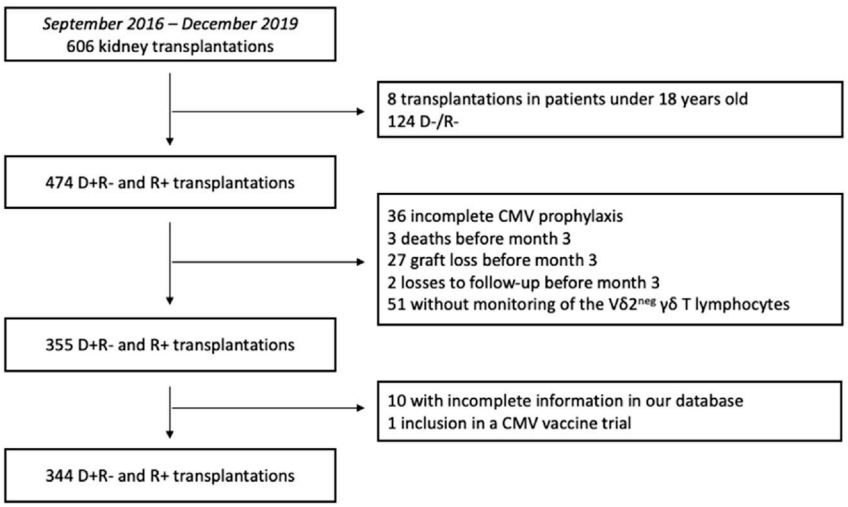


FIGURE 1 | Flowchart of the study design. D+R-: Donor positive and recipient negative for CMV serology. D-R-: Donor negative and recipient negative for CMV serology. R+: Recipient positive for CMV serology.

p-value <0.05 was considered statistically significant. The relationship between $V\delta2^{neg}$ γδ T lymphocyte counts was assessed using Spearman's correlation (rho). Receiver operating characteristic (ROC) curve analysis was conducted to evaluate the performance of lymphocyte counts and TEMRA $V\delta2^{neg}$ γδ T lymphocyte counts in predicting protection against CMV disease. The probability of CMV disease-free survival, based on lymphocyte levels, was estimated using the Kaplan-Meier method, and the log-rank test was used to compare hazards of CMV disease. Univariate Cox regression analysis was initially applied to identify variables associated with CMV disease. No continuous variable deviated from the assumption of linearity. Covariates with p-values <0.25 in univariate analysis were included in multivariate Cox regression analysis, and variables with p-values <0.05 were retained. Results are presented as hazard ratios (HR) with 95% confidence intervals (95% CI). All analyses were performed using RStudio (version 1.1.423; RStudio Inc., Boston, MA, United States) and Prism (version 10.0.2; GraphPad Software, Boston, MA, United States).

RESULTS

Study Population

Between September 2016 and December 2019, 606 kidney transplants were performed at Bordeaux University Hospital. Based on the inclusion and exclusion criteria, 344 KTRs were eligible for inclusion in the study (**Figure 1**).

Table 1 outlines the baseline characteristics of these patients. During the first 2 years post-transplantation, 43 out of 344 KTRs (12.5%) developed CMV disease, with a median onset of 79 days (IQR: 44.0–122.5 days) after discontinuing prophylaxis. Among these, 9 KTRs (20.9%) experienced CMV viral syndrome, and 34 KTRs (79.1%) developed CMV tissue-invasive disease. The median peak CMV DNAemia was 50,320 IU/mL (IQR: 12,432–281,010 IU/mL). The median disease duration was 29.5 days (IQR: 21.5–43 days), and the median treatment duration was 44 days (IQR: 24–55.5 days). CMV recurrence occurred in 6 of the 43 patients (13.6%).

CMV disease occurred in 31.7% (26/82) of D+R- KTRs and 6.4% (17/262) of R+ patients (p < 0.01). Conversely, CMV disease occurred in 4.6% (3/65) of patients treated with mTOR inhibitors and 14.3% (40/279) of patients not treated with mTOR inhibitors ($p = 0.03$). Interestingly, no significant differences were observed regarding the use of thymoglobulin or the number of treated acute rejection episodes between the groups.

CMV disease characteristics in D+R- and R+ subgroups are detailed in **Supplementary Table S1**.

TEMRA γδ T Lymphocyte Count at the End of the Prophylaxis Is Higher in KTRs Without CMV Disease

We did not observe any episode of CMV disease before the γδ T lymphocyte measurement at the end of the prophylaxis.

Table 2A describes immune profiles of the "CMV disease" and "No CMV disease" KTRs. $V\delta2^{neg}$ γδ T cells count was higher in the "No CMV disease" group than in the "CMV disease" group ($18.4 \pm 25.7/mm^3$ *versus* $6.0 \pm 7.9/mm^3$; $p < 0.01$). TEMRA γδ T lymphocytes count was also higher in the "No CMV disease" group ($23 \pm 26.8/mm^3$ *versus* $4.6 \pm 6.7/mm^3$; $p < 0.01$). Immune profiles in the D+R- and R+ subgroups are depicted in the **Tables 2B, C**.

It is worth noting that a significant number of immunophenotyping assays did not yield interpretable TEMRA γδ T lymphocyte counts (161/344, 46.8%). These results, labeled as NI (not interpretable), were evenly distributed between the "CMV disease" and "No CMV disease" groups. The proportion of NI patients was similar

TABLE 1 | Baseline characteristics in the study population.

Characteristics	Total (N = 344)	No CMV disease (N = 301)	CMV disease (N = 43)	*p* value
Age, y, mean (SD)	56.5 (14.5)	56.8 (14.6)	54.2 (13.9)	0.22
Sex, M/F, No.	217/127	189/112	15/28	0.86
Previous kidney transplantation	74 (21.5%)	67 (22.2%)	7 (16.2%)	0.43
Serostatus				<0.01
D + R-	82 (23.8%)	56 (18.6%)	26 (60.4%)	
R+	262 (76.2%)	245 (81.4%)	17 (39.6%)	
Prophylaxis duration, d, median (IQR)				
D +R-	181 (134.8–183.0)	181 (146.5–183.0)	181.5 (98.75–183.3)	0.74
R+	91.5 (89.0–92.0)	91 (89.00–92.00)	92 (89.50–94.00)	0.63
Donor sex, M/F, n	183/155	159/136	24/19	0.87
Donor age, y, mean (SD)	58.5 (16.2)	58.5 (16.3)	58.0 (15.3)	0.97
Donor status				
Living donor	63 (18.4%)	53 (17.6%)	10 (23.6%)	0.40
Standard criteria donor	108 (31.3%)	98 (32.6%)	10 (23.3%)	0.29
Extended criteria donor	173 (50.3%)	150 (49.8%)	23 (53.5%)	0.74
Immunological risk				
No donor-specific antibodies	267 (77.6%)	232 (77.0%)	35 (81.3%)	0.69
Donor-specific antibodies	77 (22.4%)	69 (23.0%)	8 (18.7%)	0.69
Induction therapy				
No induction therapy	9 (2.7%)	8 (2.6%)	1 (2.3%)	>0.99
Basiliximab	153 (44.4%)	135 (44.8%)	18 (41.9%)	0.75
Thymoglobulin	183 (53.1%)	159 (52.8%)	24 (55.8%)	0.74
Maintenance therapy				
Tacrolimus	293 (85.2%)	253 (84.0%)	40 (93.0%)	0.16
Ciclosporin	51 (14.8%)	48 (16.0%)	3 (7%)	0.16
Steroid	295 (85.7%)	260 (86.4%)	35 (81.4%)	0.35
Mycophenolate	317 (92.1%)	276 (91.7%)	41 (95.3%)	0.55
Azathioprine	22 (6.4%)	19 (6.3%)	3 (7.0%)	0.74
mTOR inhibitors	65 (18.9%)	62 (20.6%)	3 (7.0%)	0.03
Antibody-mediated rejection	12 (3.5%)	11 (3.6%)	1 (2.3%)	>0.99
T-cell mediated rejection	37 (10.8%)	31 (10.3%)	6 (14.0%)	0.44
Time to rejection, d, median (IQR)	117 (70–383.3)	242 (77.5–386.8)	76.5 (28.25–103)	0.08
Ischemia time, mn, median (IQR)	747 (470.5–1,015)	749.5 (472.0–1,022)	729 (268.5–1,439)	0.36
Post-transplantation eGFR, mL/min/1.73m^2, median (IQR)	35.0 (24.25–50.0)	35,0 (24.0–50.0)	39.0 (28.0–49.0)	0.48
2 years graft loss	13 (3.8%)	11 (3.7%)	2 (4.7%)	0.67
2 years death	17 (4.9%)	15 (5.0%)	2 (4.5%)	>0.99

SD: standard deviation.
M/F: Male/Female.
D+R-: Donor positive and recipient negative for CMV serology.
R+: Recipient positive for CMV serology.
IQR: interquartile range.
n: Number.
y: Year.
mn: Minutes.
mTOR: mammalian target of rapamycin.

between those who received thymoglobulin and those who did not (78/183 vs. 82/161, $p = 0.13$).

Patients with NI results had lower total lymphocytes counts and lower Vδ2^{neg} γδ T lymphocyte counts than patients with interpretable results, respectively 0.82 G/L vs. 1.01 G/L ($p < 0.01$) and 4.61/mm^3 vs. 27.4/mm^3 ($p < 0.01$) (**Supplementary Table S2**).

TEMRA γδ T Lymphocytes Count >4.65/mm^3 at the End of the Prophylaxis Is Associated With Protection Against CMV Disease

ROC curve analyses for total lymphocyte and Vδ2^{neg} γδ T lymphocyte counts regarding CMV disease occurrence showed low AUCs of 0.63 and 0.70, respectively (**Figures 2A,C**). Given the low AUC, we assessed CMV disease-free survival by comparing patients with values above or below the median (lymphocyte count: 761/mm^3; Vδ2^{neg} γδ T lymphocyte count: 7.95/mm^3). The probability of CMV disease-free survival was similar between the "high lymphocyte" and "low lymphocyte" groups ($p = 0.11$) (**Figure 2B**). However, KTRs with a Vδ2^{neg} γδ T lymphocyte count >7.95/mm^3 had a higher probability of CMV disease-free survival ($p < 0.01$) (**Figure 2D**).

The ROC curve for TEMRA γδ T lymphocyte count (excluding NI KTRs) yielded an AUC of 0.79 and defined an optimal threshold of 4.65/mm^3 (sensitivity 79.4%, specificity 78.9%) (**Figure 3A**). Of the 344 KTRs, 135 (39.2%) had a TEMRA γδ T lymphocyte count >4.65/mm^3. KTRs with a count >4.65/mm^3 had a higher probability of

TABLE 2 | Immune characteristics at the end of the prophylaxis, overall and according to serotype.

A) Overall	No CMV disease, n = 301	CMV disease, n = 43	*p* value
Lymphocytes count, G/L, mean, SD	0.92 (0.58)	0.76 (0.49)	0.06
Vδ2^{neg} γδ T lymphocyte count,/mm^3, mean, SD	18.4 (25.7)	6 (7.9)	<0.02
TEMRA γδ T lymphocyte count,/mm^3, mean, SD	23 (26.8)	4.6 (6.7)	<0.01
TEMRA γδ T lymphocyte not interpretable, n, %	137 (45.5%)	24 (55.8%)	0.3
B) D + R-	**No CMV disease, n = 56**	**CMV disease, n = 26**	***p* value**
Lymphocytes count, G/L, mean, SD	0.74 (0,42)	0.77 (0.54)	0.87
Vδ2^{neg} γδ T lymphocyte count,/mm^3, mean, SD	8.93 (17.17)	12.05 (13.74)	0.14
TEMRA γδ T lymphocyte count,/mm^3, mean, SD	13.52 (22.5)	14.31 (16.11)	0.57
TEMRA γδ T lymphocyte not interpretable, n, %	43 (76.8)	17 (65.4)	0.29
C) R+	**No CMV disease, n = 245**	**CMV disease, n = 17**	***p* value**
Lymphocytes count, G/L, mean, SD	0.97 (0.60)	0.79 (0.42)	0.18
Vδ2^{neg} γδ T lymphocyte count,/mm^3, mean, SD	22.51 (26.75)	10.56 (9.09)	0.04
TEMRA γδ T lymphocyte count,/mm^3, mean, SD	24.98 (27.01)	8.28 (8.04)	<0.01
TEMRA γδ T lymphocyte not interpretable, n, %	93 (37.9%)	7 (41.1%)	0.87
D) Comparison of D + R- and R+ patients	**D+ R- patients n = 82**	**R+ patients n = 262**	***p* value**
Lymphocytes count, G/L, mean, SD	0.74 (0.46)	0.95 (0.59)	<0.01
Vδ2^{neg} γδ T lymphocyte count,/mm^3, mean, SD	9.9 (6.15)	21.72 (26.1)	<0.01
TEMRA γδ T lymphocyte count,/mm^3, mean, SD	13.84 (19.72)	23.94 (26.54)	<0.01
TEMRA γδ T lymphocyte not interpretable, N, %	60 (73)	100 (38)	<0.01

SD: standard deviation.
n: Number.
D + R-: Donor positive and recipient negative for CMV, serology.
R+: Recipient positive for CMV, serology.
NI: not interpretable.

CMV disease-free survival compared to those with a count classified as NI or ≤4.65/mm^3 ($p < 0.01$) (**Figure 3B**).

We further analyzed the 161 NI KTRs and found that their Vδ2^{neg} γδ T lymphocyte counts were very low, similar to those of KTRs with TEMRA γδ T lymphocyte counts ≤4.65/mm^3, and much lower than those with counts >4.65/mm^3 [median: 3.2/mm^3 (IQR: 0.1–7.25), 2.9/mm^3 (IQR: 0.2–8.15), 24.4/mm^3 (IQR: 16.15–40.58), respectively] (**Figure 3C**). Thus, we grouped KTRs with TEMRA γδ T lymphocyte counts classified as NI and ≤4.65/mm^3. KTRs with TEMRA γδ T lymphocyte counts >4.65/mm^3 had significantly higher CMV disease-free survival rates than those in the combined NI and ≤4.65/mm^3 group ($p < 0.01$) (**Figure 3D**).

We conducted a univariate analysis to identify factors associated with CMV disease (**Table 3**). The following factors were included in the multivariate analysis: R+ serostatus [HR 0.16 (95% CI 0.09–0.29); $p < 0.01$], total lymphocyte count >761/mm^3 [HR 0.56 (95% CI 0.30–1.05); $p = 0.07$], Vδ2^{neg} γδ T lymphocyte count >7.95/mm^3 [HR 0.34 (95% CI 0.17–0.68); $p < 0.01$], TEMRA γδ T lymphocyte count >4.65/mm^3 [HR 0.18 (95% CI 0.07–0.46); $p < 0.01$], and the use of mTOR inhibitors [HR 0.27 (95% CI 0.06–1.11); $p = 0.07$]. In the multivariate analysis, only R+ serostatus [HR 0.23 (IQR 0.11–0.45); $p < 0.01$] remained independently associated with CMV disease. The TEMRA γδ T lymphocyte count was no longer significantly associated with CMV disease [HR 0.39 (95% CI 0.14–1.09); $p = 0.07$] (**Table 4**).

Differences of γδ T Lymphocyte Response Between D+/R- and R+ Patients at the End of the Prophylaxis

Total lymphocyte counts and Vδ2^{neg} γδ T lymphocyte counts were significantly lower in D+R- patients compared to R+ patients (0.74 ± 0.46 G/L vs. 0.95 ± 0.59 G/L, $p < 0.01$; Vδ2^{neg} : 9.9 ± 6.15 vs. 21.72 ± 26.1; $p < 0.01$ (**Table 2D**).

TEMRA : 13.84 ± 19.72 vs. 23.94 ± 26.54; $p < 0.01$ (**Figure 4A**). Among the 135 patients with TEMRA γδ T lymphocyte counts >4.65/mm^3, only 4 were D+R-, the majority being R+ (n = 131) ($p < 0.01$). Finally, the number of interpretable results was lower in D+R- patients compared to R+ patients (22 *versus* 161) (**Figure 4B**).

Based on these findings, we evaluated the predictive value of a TEMRA γδ T lymphocyte count >4.65/mm^3 for protection against CMV disease in the subgroup of R+ KTR, as detailed in **Table 5**.

TEMRA γδ T Lymphocytes Count >4.65/mm^3 at the End of the Prophylaxis Is Independently Associated With a Protection Against CMV Disease in R+ KTRs

Of the 262 R+ KTRs (including NI KTRs), 131 (50%) had a TEMRA γδ T lymphocyte count >4.65/mm^3. The sensitivity of

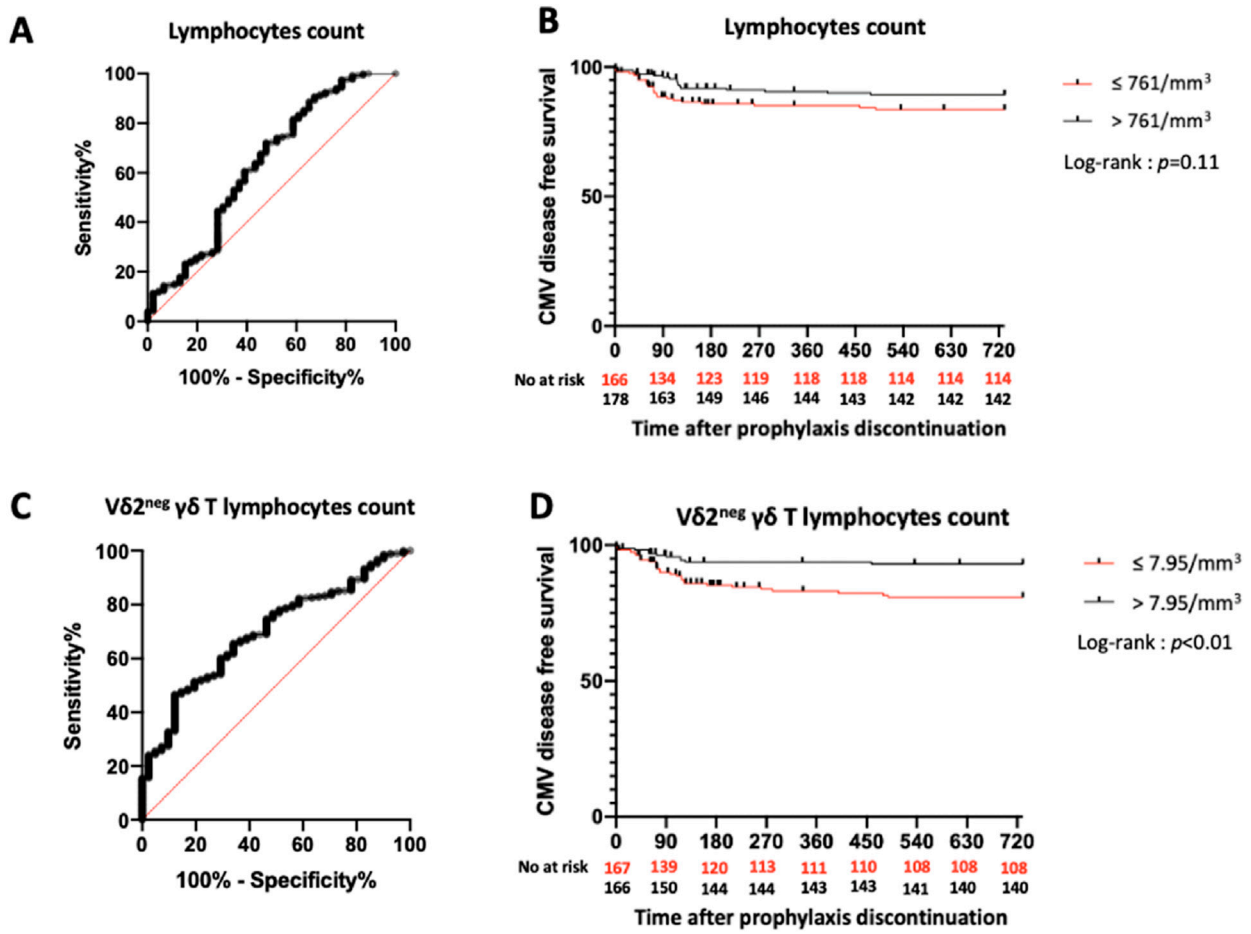


FIGURE 2 | Predictive value of total lymphocytes and Vδ2^{neg} γδ T lymphocytes count in the overall study population. **(A)**: ROC curve of total lymphocytes count. AUC = 0.63. **(B)**: Incidence of CMV disease according to lymphocytes count. **(C)**: ROC curve of Vδ2^{neg} γδ T lymphocytes count. AUC = 0.70. **(D)**: Incidence of CMV disease according to Vδ2^{neg} γδ T lymphocytes count. ROC: Receiver Operating Characteristic. AUC: Area Under Curve.

this test was low (52.2%), but specificity was high (82.3%). The positive predictive value (i.e., protection against CMV disease in KTRs with a TEMRA γδ T lymphocyte count >4.65/mm^3) was 97.7%, while the negative predictive value was only 10.7%. R+ KTRs with a TEMRA γδ T lymphocyte count >4.65/mm^3 had a significantly higher probability of CMV disease-free survival than those with counts classified as NI or ≤4.65/mm^3 ($p < 0.01$) (**Figure 4C**). The probability of CMV disease-free survival was also higher in R+ KTRs with TEMRA γδ T lymphocytes count > 4.65/mm^3 than in the group gathering R+ KTRs with TEMRA γδ T lymphocytes count "NI" and ≤4.65/mm^3 ($p = 0.02$) (**Figure 4D**).

Univariate analysis identified total lymphocyte counts >761/mm^3 [HR 0.35 (95% CI: 0.12–0.99); $p = 0.05$], Vδ2^{neg} γδ T lymphocyte counts >7.95/mm^3 [HR 0.31 (95% CI: 0.11–0.84); $p = 0.02$] and TEMRA γδ T lymphocyte counts >4.65/mm^3 [HR 0.28 (95% CI: 0.09–0.87); $p = 0.03$] as factors associated with CMV disease (**Table 6**). In the multivariate analysis of R+ KTRs, only a TEMRA γδ T lymphocyte count >4.65/mm^3 remained independently associated with protection against CMV disease [HR 0.27 (95% CI: 0.09–0.85); $p = 0.03$] (**Table 7**).

DISCUSSION

In this retrospective, single-center cohort study, KTRs with a TEMRA γδ T lymphocyte count greater than 4.65/mm^3 at the end of antiviral prophylaxis showed a significantly lower incidence of post-prophylaxis CMV disease during the first 2 years after transplantation. In the overall population, including both D+R- and R+ KTRs, this biomarker did not perform better than CMV serostatus in predicting the occurrence of CMV disease. However, it was independently associated with protection against CMV disease in the R+ population, demonstrating a predictive ability of 97.7% for CMV protection in this subgroup.

The usefulness of several immunomonitoring assays/biomarkers after prophylaxis withdrawal has been studied, but most of them were focused on the CD8$^+$ αß T lymphocytes. In 2009, Kumar et al. assessed both D+R- and R+ KTRs, showing that a positive QuantiFERON-CMV assay at the end of prophylaxis was associated with a decreased risk of CMV disease during the first 6 months post-transplantation [2/38 (5.3%) versus 16/70 (22.9%), $p = 0.038$] [9]. In this study, 32

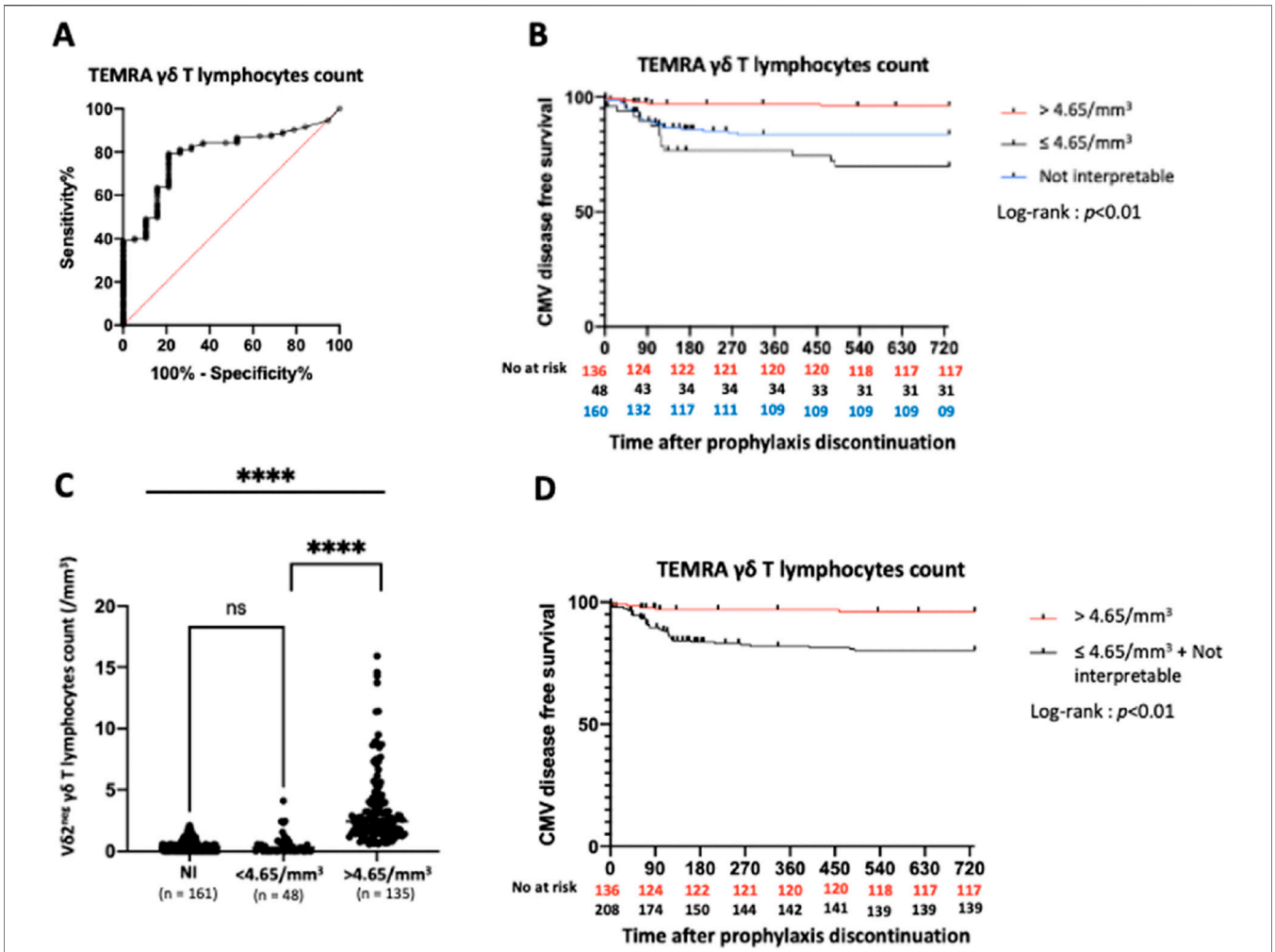


FIGURE 3 | Predictive value of TEMRA γδ T lymphocytes count in the overall study population. **(A)**: ROC curve of TEMRA γδ T lymphocytes count. AUC = 0.79. **(B)**: Incidence of CMV disease according to TEMRA γδ T lymphocytes count (3 groups). **(C)**: Vδ2^{neg} γδ T lymphocytes count according to Vδ2neg γδ T lymphocytes percentage ****: $p < 0.01$. **(D)**: Incidence of CMV disease according to TEMRA γδ T lymphocytes count (2 groups). ROC: Receiver Operating Characteristic. AUC: Area Under Curve. NI: Not interpretable.

TABLE 3 | Univariate analysis of CMV disease risk factors in the study population.

Variables	HR	95% CI	*p* value
Age	1	0.99–1.01	0.89
Sex (reference group: male)	1.05	0.56–1.97	0.87
Thymoglobulin	1.21	0.66–2.20	0.54
mTOR inhibitors before CMV disease	0.27	0.06–1.11	0.07
Steroids	0.96	0.49–1.91	0.91
Rejection before CMV disease	1.00	0.99–1.00	0.30
R+ patients	0.16	0.09–0.29	<0.01
Lymphocytes count >0.761 G/L	0.56	0.30–1.05	0.07
Vδ2^{neg} γδ T lymphocyte count >7.95/mm^3	0.34	0.17–0.68	<0.01
TEMRA γδ T lymphocyte count >4.65/mm^3	0.18	0.07–0.46	<0.01

HR: hazard ratio.
CI: confidence interval.
mTOR: mammalian target of rapamycin.
R+: Recipient positive for CMV, serology.

(29.6%) KTRs had indeterminate QuantiFERON-CMV results and were classified as negative. Following this initial study, Manuel et al. conducted a multicenter prospective study in 2013 focused on D+R- KTRs. In this study, QuantiFERON-CMV was performed at the end of prophylaxis, and KTRs were followed for 1 year. Among 127 KTRs, 31 (25%) had a positive QuantiFERON-CMV result, 81 (65.3%) were negative, and 12 (9.7%) had indeterminate results. During the first post-transplant year, KTRs with a positive result had a lower incidence of CMV disease than those with a negative or indeterminate result (6.4%, 22.2%, and 58%, respectively; $p < 0.001$). The assay had a high positive predictive value (93%) but a low negative predictive value (24%) [10]. More recently, Fernandez-Ruiz et al. assessed the post-prophylaxis QuantiFERON-CMV test in R+ KTRs receiving anti-thymocyte globulins. They found no significant difference in the incidence of CMV infection between QuantiFERON-CMV positive and negative groups during the

TABLE 4 | Multivariate analysis of CMV disease risk factors in the study population.

Variables	HR	95% CI	*p* value
mTOR inhibitors before CMV disease	0.28	0.07–1.15	0.08
R+ patients	0.22	0.11–0.45	<0.01
TEMRA γδ T lymphocyte count >4.65/mm^3 *versus* ≤4.65/mm^3 and NI	0.39	0.14–1.09	0.07

HR: hazard ratio.
CI: confidence interval.
mTOR: mammalian target of rapamycin.
R+: Recipient positive for CMV, serology.
NI: not interpretable.

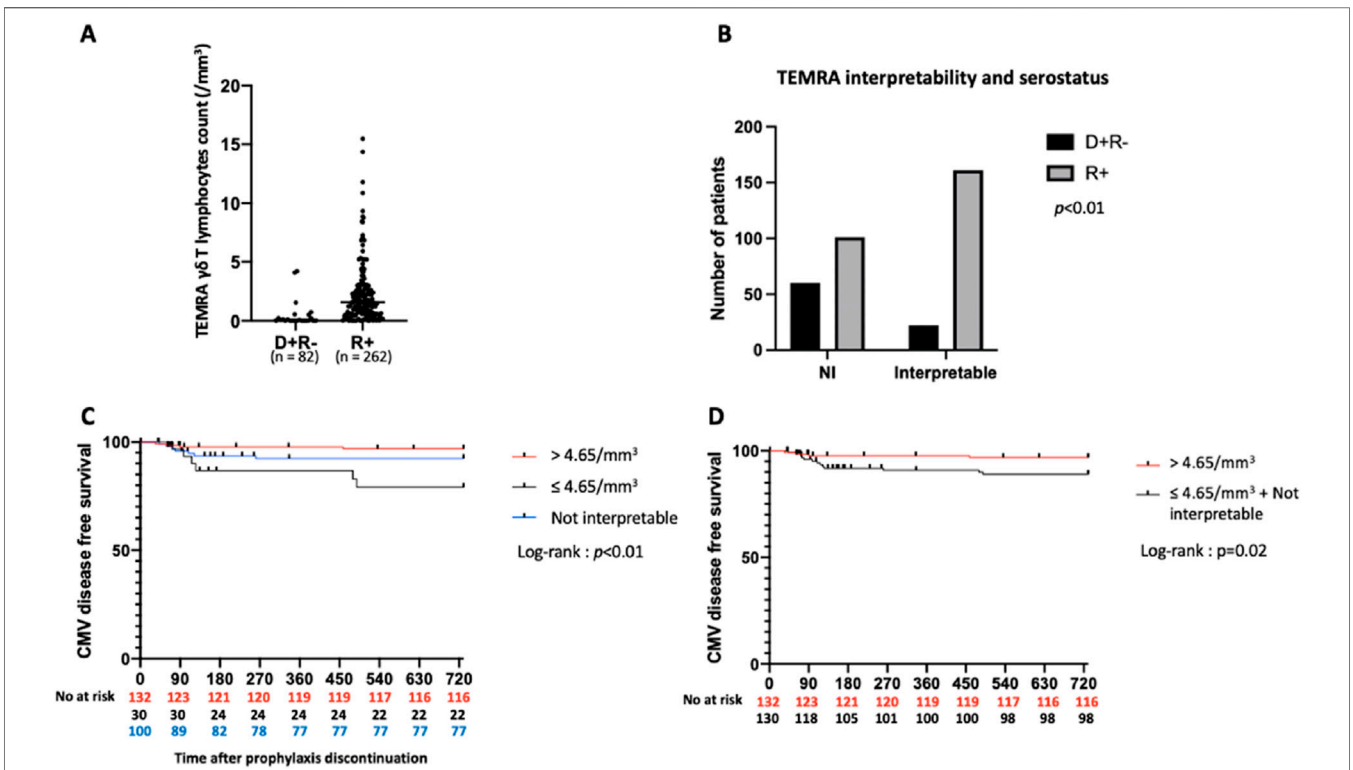


FIGURE 4 | Predictive value of TEMRA γδ T lymphocytes count in R+ patients. **(A)**: TEMRA γδ T lymphocytes count according to serostatus. $p < 0.01$. **(B)**: TEMRA γδ T lymphocytes count interpretability according to serostatus. **(C)**: Incidence of CMV disease according to TEMRA γδ T lymphocytes count in R+ patients (3 groups). **(D)**: Incidence of CMV disease according to TEMRA γδ T lymphocytes count in R+ patients (2 groups). NI: Not interpretable. D+R-: Donor positive and recipient negative for CMV serology. R+: Recipient positive for CMV serology.

first-year post-transplant (45.8% versus 36.1%; $p = 0.244$). The discrepancy with the study of Manuel et al. could be explained by differing endpoints: Fernandez-Ruiz et al. focused on CMV infection, while Manuel et al. focused on CMV disease [27].

Jarque et al. focused on the association of a positive ELISpot at the end of prophylaxis and the incidence of CMV disease during the first-year post-transplantation in R+ KTRs. They found significantly lower IFN-γ–producing T-cell frequencies against both IE-1 and pp65 CMV antigens in KTRs who later developed CMV infection. IE-1 cell-mediated immunity (CMI) was the strongest predictor of protection against late-onset CMV infection, with a positive predictive value of 90.8% [28]. Finally, Kumar et al. published a multicenter prospective study focusing on the predictive value of ELISpot at the end of prophylaxis in R+ and D+R- KTRs, finding a significantly lower incidence of CMV events in ELISpot positive R+ KTRs, with a positive predictive value above 97% [29].

In vitro, γδ T cells inhibit replication and kill infected cells [20], a protective role supported by animal studies [21]. Their expansion in peripheral blood parallels that of CD8$^+$ T cells following infection [25] and 8 weeks after treatment initiation, γδ T cell expansion is associated with the absence of CMV recurrence [19].

In this study, we tried to analyze the ability of γδ T cells to predict CMV disease at the end of prophylaxis. We found similar predictive performance for TEMRA γδ T lymphocyte counts above 4.65/mm^3 than ELISPOT at the end of prophylaxis in R+ KTRs, with a sensitivity of 52.2%, specificity of 82.3%, positive predictive value of 97.7%, and negative predictive value of 10.7%.

TABLE 5 | Baseline characteristics in the R+ population.

Characteristics	Total (N = 262)	No CMV disease (N = 245)	CMV disease (N = 17)	*p* value
Age, y, mean (SD)	57.1 (14.4)	57.1 (14.5)	57.6 (13.4)	0.94
Sex, M/F, No.	160/102 (61%/39%)	149/96 (61%/39%)	11/6 (64%/36%)	0.80
Previous kidney transplantation	62 (23.7%)	58 (23.7%)	4 (23.5%)	>0.99
Prophylaxis duration, d, median (IQR)	91.5 (89.0–92.0)	91 (89.00–92.00)	92 (89.50–94.00)	0.63
Donor sex, M/F, No	147/112	140/102	7/10	0.21
Donor age, y, mean (SD)	58.7 (12.6)	58.6 (15.7)	60.3 (14.5)	0.56
Donor status				
Living donor	44 (16.8%)	41 (16.7%)	3 (17.6%)	>0.99
Standard criteria donor	85 (32.4%)	82 (33.5%)	3 (17.6%)	0.28
Extended criteria donor	133 (50.8%)	122 (49.8%)	11 (64.8%)	0.31
Immunological risk				
No donor-specific antibodies	199 (76.0%)	191 (78.0%)	8 (47.0%)	<0.01
Donor-specific antibodies	63 (24.0%)	54 (22.0%)	9 (53.0%)	<0.01
Induction therapy				
No induction therapy	8 (3.0%)	7 (2.9%)	1 (5.9%)	0.41
Basiliximab	112 (42.7%)	107 (43.7%)	5 (29.4%)	0.31
Thymoglobulin	143 (54.6%)	132 (53.9%)	11 (64.7%)	0.45
Maintenance therapy				
Tacrolimus	228 (87.0%)	212 (86.5%)	16 (94.1%)	0.70
Ciclosporin	34 (13.0%)	33 (13.5%)	1 (5.9%)	0.70
Steroid	202 (77.0%)	187 (76.3%)	15 (88.2%)	0.37
Mycophenolate	241 (92.0%)	224 (91.4%)	17 (100%)	<0.01
Azathioprine	12 (4.5%)	12 (4.9%)	0 (0%)	>0.99
mTOR inhibitors	52 (19.8%)	51 (20.8%)	1 (5.9%)	0.20
Antibody-mediated rejection	10 (3.8%)	10 (4.1%)	0 (0.0%)	>0.99
T-cell mediated rejection	30 (11.5%)	25 (10.2%)	5 (29.4%)	0.03
Time to rejection, d, median (IQR)	116 (54–383)	117 (67–283)	62 (14–110)	0.26
Ischemia time, mn, median (IQR)	749.0 (495.5–1,013)	749.5 (491.3–1,015)	743 (395.0–1,004)	0.94
Post-transplantation eGFR, mL/min/1,73m^2, median (IQR)	36.5 (24.75–50.25)	38.0 (25.0–51.5)	28.0 (23.5–34.0)	0.02
2 years graft loss	10 (3.8%)	9 (3.7%)	1 (5.9%)	0.49
2 years death	10 (3.8%)	9 (3.7%)	1 (5.9%)	0.49

SD: standard deviation.
M/F: Male/Female.
D + R-: Donor positive and recipient negative for CMV, serology.
R+: Recipient positive for CMV, serology.
IQR: interquartile range.
n: Number.
y: Year.
mn: Minutes.
mTOR: mammalian target of rapamycin.

TABLE 6 | Univariate analysis of CMV disease risk factors in R+ patients.

Variables	HR	95% CI	*p* value
Age	1.01	0.97–1.04	0.76
Sex (reference group: male)	1.10	0.40–2.9	0.87
Thymoglobulin	1.63	0.60–4.40	0.34
mTOR inhibitors before CMV disease	0.16	0.11–3.66	>0.99
Steroids	1.08	0.35–3.33	0.89
Rejection before CMV disease	1.00	0.99–1.01	0.16
Lymphocytes count >0.761 G/L	0.35	0.12–0.99	0.05
Vδ2^{neg} γδ T lymphocyte count >7.95/mm^3	0.31	0.11–0.84	0.02
TEMRA γδ T lymphocyte count >4.65/mm^3 *versus* ≤4.65/mm^3 and NI	0.28	0.09–0.87	0.03

HR: hazard ratio.
CI: confidence interval.
mTOR: mammalian target of rapamycin.
R+: Recipient positive for CMV, serology.
NI: not interpretable.

The high positive predictive value reflects a low incidence of CMV disease in KTRs with TEMRA γδ T lymphocyte counts higher than 4.65/mm^3 within the first 2 years post-transplantation. This biomarker of the anti-CMV immune response could then complement the ELISPOT or QuantiFERON assays in order to better predict CMV disease

TABLE 7 | Multivariate analysis of CMV disease risk factors in R+ patients.

Variables	HR	95% CI	*p* value
Rejection before CMV disease	1.32	0.17–9.99	0.79
TEMRA γδ T lymphocyte count >4.65/mm^3 *versus* ≤4.65/mm^3 and NI	0.27	0.09–0.85	0.03

HR: hazard ratio.
CI: confidence interval.
NI: not interpretable.

and better guide the prevention strategy. It would be particularly interesting to analyze TEMRA lymphocytes levels in patients who do not develop disease despite lacking CD4+/CD8+ T-cell responses or those who develop disease despite having these responses.

TEMRA γδ T lymphocytes appear to be a promising biomarker for the development of a γδ T lymphocyte-mediated adaptive response. However, it has been shown that TEMRA cells can display significant heterogeneity, with dysfunctional phenotypes (PD-1+, CD85j+) linked to an increased risk of CMV infections [30]. Further research is needed to refine the predictive value of TEMRA γδ T lymphocyte counts by incorporating the functional status of these cells. Notably, the functionality of these cells seems to improve in KTRs maintained on mTOR inhibitors compared to those on mycophenolate-based treatments, which may explain the lower CMV disease incidence associated with mTOR inhibitors in our study.

Our study has some limitations. Its retrospective, single-center design underscores the need for confirmation in prospective studies. The second limit is that this technique is not currently standardized. The third limitation is the large proportion of patients with non-informative (NI) results [160/344 (46.5%)]. Similar to the QuantiFERON-CMV assay, this result may be indicative of a weak CMV immune response, as these patients had lower total lymphocytes count, lower Vδ2^{neg} γδ T lymphocyte counts and exhibited more CMV disease than those with TEMRA γδ T lymphocyte counts above 4.65/mm^3. Since these non-significant findings are attributable to the insufficient number of circulating γδ T cells, resolving this issue may require increasing the number of cells analyzed through flow cytometry to improve sensitivity. Additionally, the main findings of our study apply to R+ KTRs, who are not the highest-risk group for CMV disease.

Future interventional studies are needed to determine whether TEMRA γδ T lymphocyte counts can improve CMV immune risk stratification and guide personalized CMV prevention strategies. Currently, the QuantiFERON-CMV and ELISpot-CMV assays can be used: at 4–6 weeks post-transplantation in R+ KTRs receiving thymoglobulin and universal prophylaxis to discontinue antivirals early in those with positive results [16], 2/at 2 weeks post-transplantation in R+ KTRs managed with a preemptive approach to stop PCR monitoring in those with a positive result [18]. Adding TEMRA γδ T lymphocyte counts to the arsenal of CMV cell-mediated immunity assays could enhance immune-guided CMV prevention, particularly in R+ KTRs with negative QuantiFERON-CMV or ELISpot-CMV results.

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 study was performed in accordance with the ethical standards as laid down in the Declaration of Helsinki, and was approved by the Institutional Review Board of the Bordeaux University Hospital.

AUTHOR CONTRIBUTIONS

YA, HK, LC, PM, and MC participated in the design of the study. YA, JV, and EW retrieved the data. YA and HK conducted the analysis. YA, HK, and LC wrote the manuscript, with some notes from JD-M and JV. 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.

GENERATIVE AI STATEMENT

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

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2025.14339/full#supplementary-material

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Preservation Fluid Bacteriology in Kidney Transplantation: Comparing Uncontrolled Donation After Circulatory Death With Donation After Brain Death

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Infectious complications remain a significant concern in organ transplantation, and preservation fluid (PF) has been identified as a potential source of microbial contamination. However, the clinical relevance of positive PF cultures, especially in kidney transplants from uncontrolled donation after circulatory death (uDCD), is not clearly established. This study aims to evaluate and compare the incidence and clinical implications of positive PF cultures in kidney transplants from uDCD and donation after brain death (DBD) donors. A prospective, single-center study was conducted, involving 497 kidney transplants—147 from uDCD and 350 from DBD donors. PF samples were systematically collected at the time of transplantation, cultured, and analyzed. The type of bacteria identified guided antibiotic treatment decisions. Recipients were monitored for the development of bacteremia within the first post-transplant week. Positive PF cultures were significantly more frequent in uDCD transplants (32.0%) compared to DBD (13.7%) ($p < 0.001$). Coagulase-negative staphylococci predominated in both groups. Despite this, bacteremia rates were comparable—8.5% in uDCD and 6.3% in DBD ($p = 0.673$)—with no culture-concordant cases. Antibiotics were administered to 10.6% of uDCD and 22.9% of DBD recipients ($p = 0.110$). Although uDCD kidneys had higher PF contamination, the clinical impact was minimal.

Keywords: kidney transplantation, organ procurement, preservation fluid, donation after circulatory death, infection

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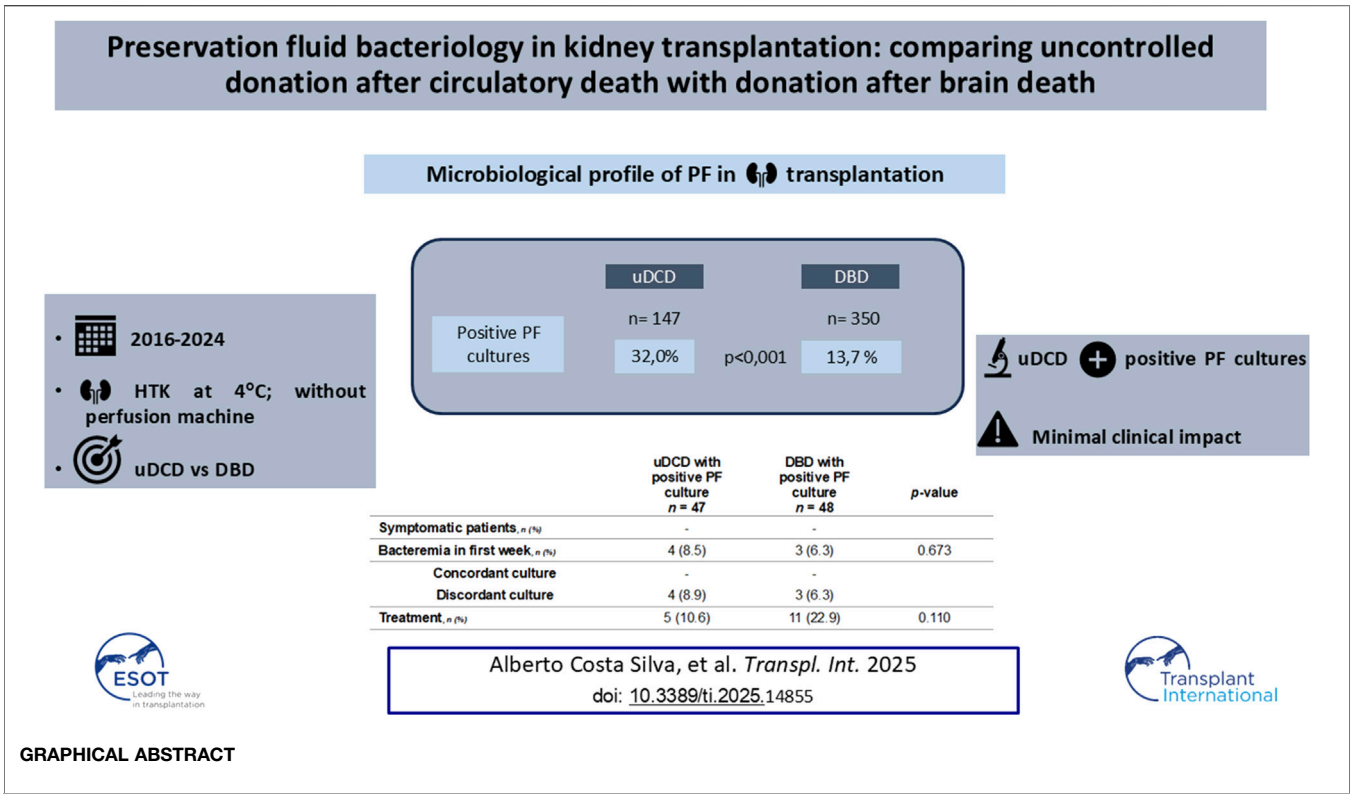
INTRODUCTION

Infectious complications are a significant cause of morbidity and mortality in organ transplant patients [1]. Preservation fluid (PF) is a critical component in maintaining organ viability after procurement but also poses a risk of infection, which can arise from microorganisms originating from the donor or introduced during recovery and handling [2, 3]. Routine screening of PF for microbial growth and the clinical implications of culture-positive results remains contentious issues.



GRAPHICAL ABSTRACT

The effects on recipients can range from asymptomatic colonization to mild or severe infections, potentially leading to graft failure and even death [4]. The incidence of culture-positive PF in solid organ transplantation has been reported to 7.2%–77.8%, with kidney transplantation alone showing positive cultures in up to 24% of cases, and pathogenic microorganisms identified in 10% [2, 5, 6].

Given the increasing demand for suitable grafts, kidneys from uncontrolled donation after circulatory death (uDCD) with *in situ* preservation using extracorporeal membrane oxygenation (ECMO) have emerged as a viable alternative, thereby expanding the organ donor pool [7, 8]. However, there is currently no data on the microbiological profile of PF in uDCD transplantation, particularly in the context of kidney transplantation.

This study aimed to assess the incidence of positive microbiological cultures in PF from uDCD kidney transplants compared to those from donation after brain death (DBD) and its clinical impact.

MATERIALS AND METHODS

A prospective study was conducted at a single center in accordance with the Declaration of Helsinki, following approval by the institutional review board and after obtaining informed consent from all participants. Transplants performed between January 2016 and December 2024 were included in the study. Exclusion criteria comprised transplants from living donors, cases from donors on antibiotics/with suspected infection and cases requiring surgical reintervention within the first 8 days post-transplant.

Transplants were categorized into two groups: uDCD and DBD, with the DBD group including both standard criteria donors (SCD) and expanded criteria donors (ECD).

All donors were from the same center. Donor surgical prophylaxis in the procurement was not made and recipients received single shot of cephazolin or ciprofloxacin in case of allergy. Preoperative skin cleansing was made with povidone-iodine antiseptic solution.

Following organ collection, they were placed in a sterile container, immersed in Custodiol® solution, and maintained at approximately 4°C without perfusion machine. At the time of transplantation, immediately before back-table surgery, a 20 mL sample of PF was collected and sent for culture within 4 h. This liquid was centrifuged and the pellet inoculated in solid media such as: non-selective, blood agar and selective, MacConkey agar and mannitol-salt agar and liquid, Brain-heart broth for 24-48 h at 37°C in aerobic conditions. The liquid media was then inoculated in blood agar and incubated for more 24 h in aerobic conditions. Identification of the colonies and antimicrobial susceptibility assay took place.

Patients were grouped according to whether their PF culture was positive or negative after 72 h of culture. At that time, for culture-positive patients, the type of bacteria was recorded, and an assessment of the recipient's blood culture was performed. Patients were classified as culture-concordant if the same bacteria was identified in PF and blood cultures, and culture-discordant if not. Patients were classified as symptomatic based on the

TABLE 1 | Bacteriological analysis of preservation fluid in uDCD and DBD transplants.

Microbiological status	uDCD n = 147	DBD *n* = 345	*p*-value
Culture-positive PF, *n (%)*	47 (32.0)	48 (13.7)	<0.001
Staphylococcus, *n (%)*			
S. epidermidis	21 (14.3)	18 (5.5)	
S. lugdunensis	10 (6.8)	6 (1.7)	
S. capitis	3 (2.0)	2 (0.6)	
S. warney	1 (0.7)	2 (0.6)	
S. caprae	1 (0.7)	2 (0.6)	
S. aureus	-	1 (0.3)	
S. hominis	-	1 (0.3)	
S. haemolyticus	-	1 (0.3)	
Streptococcus, *n (%)*			
S. constellatus	1 (0.7)	-	
S. viridans	1 (0.7)	-	
S. mitis	1 (0.7)	-	
Escherichia coli, n (%)	4 (2.7)	3 (0.9)	
Enterococcus, n (%)			
E. faecalis	1 (0.7)	3 (0.9)	
E. raffinosus	1 (0.7)	-	
Bacillus, n (%)			
B. licheniformis	-	1 (0.3)	
B. megaterium	-	1 (0.3)	
Biffidus, n (%)	1 (0.7)	-	
Serratia marcescens, n (%)	1 (0.7)	-	
Corynebacterium tuberculostearicum, n (%)	-	1 (0.3)	
Shewanella putrefaciens, n (%)	-	1 (0.3)	
Klebsiella aerogenes, n (%)	-	1 (0.3)	
Citrobacter braakii, n (%)	-	1 (0.3)	
Klebsiella pneumoniae + Escherichia coli + Enterococcus faecium, n (%)	-	1 (0.3)	
Klebsiella pneumoniae + Escherichia coli, n (%)	-	1 (0.3)	

DBD, donation after brain death; PF, preservation fluid; uDCD, uncontrolled donation after circulatory death.

presence of fever and elevated inflammatory markers, such as leukocyte count or C-reactive protein levels; wound site infection was also considered.

Treatment decisions were based on the bacteria identified in the PF culture; specifically, patients were treated for *Escherichia coli, Enterococcus faecium, Enterococcus faecalis, Staphylococcus aureus, Klebsiella pneumoniae, Klebsiella aerogenes, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* species. Antibiotic therapy was initiated upon availability of the PF culture results, typically 48–72 h after transplantation.

Demographic data were collected, as well as surgical extraction time (interval between the donor's surgical incision and kidney's transfer to cold storage), cold ischemia time (time between aortic clamping and transplantation), cause of chronic kidney disease (CKD) and type of dialysis. Induction immunosuppression for all uDCD and DBD recipients with high immunological risk was carried out using rabbit anti-thymocyte globulin. For DBD recipients without high immunological risk, basiliximab was administered. Maintenance therapy consisted of prednisolone, mycophenolate mofetil, and tacrolimus.

Data collection and analysis were performed using Statistical Package for the Social Sciences version 27 (IBM, Chicago, United States). The Kolmogorov–Smirnov test was used to evaluate the distribution of the parameters. Continuous variables with a normal distribution are presented as the mean ± standard deviation, while non-normally distributed variables are represented using the median and percentiles 25 and 75. The chi-square test was used to analyze categorical variables and to compare categorical and continuous variables, we employed the t-test or Kruskal–Wallis test, depending on the normality of the data. Statistical significance was set at $p < 0.05$.

RESULTS

PF cultures were analyzed from a total of 497 kidney transplants, including 147 from uDCD and 350 from DBD (171 from SCD and 179 from ECD). Positive PF cultures were significantly more frequent in the uDCD group, with 32.0% ($n = 47$) testing positive, compared to 13.7% ($n = 48$) in the DBD group ($p < 0.001$; **Table 1**).

The most commonly isolated bacteria in uDCD were *Staphylococcus* species, found in 24.5% (n = 36) of cases, followed by *E. coli* in 2.7% (n = 4). In DBD, *Staphylococcus species* was isolated in 9.6% (n = 33) of cases, with *E. coli* and *E. faecalis* both present in 0.9% (n = 3) each (**Table 1**).

Characteristics of patients with positive PF cultures are detailed in **Table 2**.

Despite the higher rate of positive PF cultures in uDCD, the incidence of bacteremia within the first week post-transplant was

TABLE 2 | Characteristics of patients with positive preservation fluid cultures.

Patients characteristics	uDCD with positive PF culture (*n* = 47)	DBD with positive PF culture (*n* = 48)
Donor age, years, median (P25-P75)	51.0 (41.0–57.0)	57.0 (43.3–67.0)
Donor sex, n (%)		
Male	36 (76.6)	42 (87.5)
Female	11 (23.4)	6 (12.5)
Cause of donor death		
Traumatic brain injury	-	15 (31.3)
Vascular cerebral event	-	11 (22.9)
Cerebral hypoxia	-	7 (14.6)
Cardiocirculatory	47 (100.0)	-
Others	-	15 (31.3)
Date of transplant		
2016-2020	32 (68.1)	33 (68.8)
2020-2024	15 (31.9)	15 (31.3)
Antibiotic prophylaxis		
Cephazolin	44 (93.6)	44 (91.6)
Ciprofloxacin	3 (6.4)	4 (8.4)
ICU length of stay, days, median (P25-P75)	-	2 (1–3)
Time on ECMO, minutes, median (P25-P75)	180.0 (15.0–180.0)	-
CIT, hours, median (P25-P75)	13.0 (11.0–16.0)	14.5 (12.0-17.8)
Surgical extraction time, minutes, median (P25-P75)	35.0 (20–47.5)	35.0 (30.0-60.0)
Multiorgan procurement, n (%)	-	24 (50.0)
Recipient age, years, median (P25-P75)	55.0 (48.0–61.0)	59.0 (50.0-64.8)
Recipient sex, n (%)		
Male	31 (66.0)	29 (60.4)
Female	16 (34.0)	19 (39.6)
Causes of recipient CKD, n (%)		
Diabetic nephropathy	4 (8.5)	8 (16.7)
Hypertensive glomerulosclerosis	2 (4.3)	1 (2.1)
Glomerulonephritis	10 (21.3)	13 (27.1)
Urological cause	2 (4.3)	-
Polycystic kidney disease	13 (27.7)	10 (20.8)
Others	4 (8.2)	2 (4.2)
Unknown	12 (25.5)	14 (29.2)
Type of dialysis, n (%)		
Hemodialysis	39 (83.0)	41 (85.4)
Peritoneal dialysis	8 (17.0)	6 (12.5)
Graft function, n (%)		
Delayed	34 (66.7)	17 (33.3)
Immediate	10 (25.6)	29 (74.4)
Non-function	3 (6.4)	2 (4.2)

CIT, cold ischemia time; CKD, chronic kidney disease; ECMO, extracorporeal membrane oxygenation; ICU, intensive care unit; PF, preservation fluid.

similar between groups, occurring in 8.5% (n = 4) of uDCD patients and 6.3% (n = 3) of DBD patients ($p = 0.673$). None of the bacteremia cases were culture-concordant with the bacteria found in the PF and none of the patients developed symptoms.

Based on PF cultures, antibiotics were administered to 10.6% (n = 5) in uDCD group compared to 22.9% (n = 11) in the DBD group ($p = 0.110$; **Table 3**).

In all treated uDCD cases, amoxicillin/clavulanic acid was used, and it was the most commonly prescribed agent in the DBD group (14.6% (n = 7); **Table 3**).

DISCUSSION

PF contamination can occur at various stages of the transplantation process, particularly during procurement and packaging. Potential sources include airborne transmission within the surgical environment, surgical instruments, or inadequate skin antisepsis. Donor gut ischemia can also result in the translocation of bowel flora into the bloodstream, posing a risk to other organs [2, 9]. Additionally, the biochemical characteristics of organ preservation PF can support the growth of microorganisms [10]. In the context of multiorgan procurement, kidneys are often the last to be retrieved, increasing their exposure to these contamination risks.

Several factors can distinguish uDCD from DCD, potentially affecting PF results. uDCD involves a femoral cannulation and ECMO, an invasive intervention, which can introduce microorganisms into the bloodstream. Additionally, uDCD can occur outside of hospital settings in non-sterile environments, exposing the body to external contaminants and increasing the risk of bacterial contamination. In contrast, DBD donors are typically managed in more controlled medical environments. Moreover, warm ischemia can lead to tissue damage,

TABLE 3 | Post-transplant bacteremia and antibiotic usage associated with positive preservation fluid cultures.

Post-operative status	uDCD with positive PF culture *n* = 47	DBD with positive PF culture *n* = 48	*p*-value
Symptomatic patients, *n (%)*	0	0	
Bacteremia in first week, *n (%)*	4 (8.5)	3 (6.3)	0.673
Concordant culture	0	0	
Discordant culture	4 (8.9)	3 (6.3)	
Treatment, *n (%)*	5 (10.6)	11 (22.9)	0.110
Amoxicillin/clavulanic acid	5 (10.6) 4 - *E. coli* 1 – *E. faecalis*	7 (14.6) *2 – E. faecalis* *2 - E.coli* 1 – E.coli + E.faecalis 1 – *K. pneumoniae + E. coli + E. faecium* *1 – S. aureus*	
Ciprofloxacin	0	1 (2.1) 1 - *K aerogenes*	
Piperacillin/tazobactam	0	2 (4.2) 1 – *K. pneumoniae + E. coli* 1 – *E. faecalis*	
Wound site infection	6 (12.8)	6 (12.5)	0.969

DBD, donation after brain death; PF, preservation fluid; uDCD, uncontrolled donation after circulatory death.

rendering kidneys more susceptible to bacterial contamination. This ischemic injury may increase the permeability of the intestinal mucosa, allowing bacteria to translocate from the gut into the bloodstream and potentially contaminate the kidneys and PF. Furthermore, reperfusion injury can exacerbate tissue damage and may release bacteria from previously ischemic areas into the PF. The sudden restoration of blood flow can also disseminate bacteria that translocated during the ischemic period, increasing the bacterial load in the PF. At our center, ECD grafts are biopsied, a process that may inadvertently serve as an entry point for bacterial contamination. Additionally, the waiting period for results prolongs the time the graft remains in preservation fluid, theoretically increasing the risk of contamination.

In our study, positive PF cultures were found in 32.0% of uDCD cases, which was significantly higher than the 13.7% observed in DBD cases. Most positive PF cultures contained staphylococci, suggesting possible contamination from the donor's skin flora.

No proven systemic infection related to PF was identified. Yu et al. reported that 2.9% of recipients developed infections that were defined as PF donor-derived infections [11]. Bacteremia was observed in 8.5% of uDCD patients and 6.3% of DBD patients with positive PF cultures, but none of the cases matched the bacteria found in the PF cultures. This finding is consistent with a previous study where no systemic infections were reported among 362 renal transplant patients [12]. According to a recent meta-analysis, despite the frequent contamination of PF in donated organs, the rate of PF-related infections remains low [10]. The lack of concordance between PF and blood cultures may indicate either that PF has a minimal role in developing systemic infections or that antibiotic treatment targeting the bacteria found in PF effectively prevents systemic dissemination. The incidence of wound site infections was comparable between the two groups.

Currently, routine culture of PF is not standard practice in most transplant centers [6, 13]. However, in France, since the 2008 guidelines from the Agency of Biomedicine, PF samples from kidney transplants are systematically collected for microbiological analysis, though no clear recommendations exist on how to manage positive bacteriological cultures from PF [14].

Despite a higher incidence of positive PF in uDCD (32.0% vs. 13.7% in DBD), antibiotics were administered to 10.6% of positive uDCD cases compared to 21.3% in the DBD group—a difference that was not statistically significant. This is likely because the predominant organisms in the uDCD group, such as *staphylococcus* and *streptococcus* species, were considered non-pathogenic and no antibiotic was given. Although some authors consider *Staphylococcus lugdunensis* to be pathogenic, no treatment was administered to the 10 patients in the uDCD group, and no related infections were observed.

One study reported that positive PF cultures resulted in antibiotic prescriptions in up to 35% of cases [15]. There is some evidence suggesting that prophylactic antibiotic use guided by PF cultures in asymptomatic patients does not reduce the rate of PF-related infections and can be part of an excessive antibiotics use [16, 17]. In other hand, some reports suggest that treating positive PF cultures can improve outcomes, including reducing infection, graft loss, and acute rejection, but only in cases involving high-risk bacteria such as Gram-negative bacilli and *Staphylococcus aureus* [10]. Despite conflicting findings, the literature identifies specific risk factors associated with positive PF cultures and the subsequent need for treatment, such as donor age, prolonged ICU stays, elevated preoperative creatinine levels, the presence of ESKAPE bacteria (*E. faecium*, *Staphylococcus aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter* species), elevated procalcitonin levels, hemofiltration, use of sirolimus, multiorgan procurement, and en-bloc transplant procedures [10, 18–20]. *Candida* species in PF are recognized

as potential causes of renal graft mycotic aneurysms [21]. While *Candida* species are known to grow on standard aerobic bacterial culture media, their identification may still be limited without the use of fungal-specific media and incubation conditions. In our study, no *Candida* species were identified, which may reflect these methodological constraints rather than their true absence. In fact, there were no reported cases of either mycotic aneurysms or post-operative candidemia.

Factors such as intestinal perforation during procurement, multiorgan procurement, and *en bloc* procurement are recognized as risk factors for PF-positive cultures [20]. In our sample, no instances of intestinal perforation were recorded during procurement, kidney *en bloc* retrieval is not performed, and only kidneys are procured in the uDCD setting. Consequently, it was impossible to assess these factors within our sample. While the use of a perfusion machine after organ procurement was suggested to reduce the risk of positive PF cultures [20], it is not used at our center, which precludes any analysis of its impact. Despite ongoing debate about the significance of bacteriological positivity in PF cultures, recent studies have explored decontamination methods using ultraviolet-C, ultrasound, and Ps80 detergent to reduce the microbial load [22]. The use of cephalosporins, such as cefazolin, for donor surgical prophylaxis can reduce the risk of organ contamination and subsequent infection in recipients. These antibiotics are renally excreted and may persist in renal tissue after procurement, providing continued antimicrobial protection [2]. The rate of positive PF cultures was higher during the first 4 years of the program, possibly due to the learning curve or procedural evolution.

Given the low treatment rates and minimal clinical impact, our findings support the literature questioning the necessity of routinely performing bacteriological analysis of PF. Minimizing antibiotic overuse is essential, particularly in kidney transplant recipients who are inherently vulnerable to multi-resistant bacterial infections. Currently, antibiotic prescription is based primarily on clinical judgment, leading to considerable variation between centers, and there are no universal guidelines for collecting PF cultures, determining the timing of collection before transplantation, selecting specific treatments, or monitoring recipients in the event of a positive PF culture.

The impact of the length of stay in intensive care as a risk factor for PF contamination could not be reliably assessed between groups, as uDCD donors do not stay in the ICU; instead, they are placed on ECMO and taken directly to the operating room.

Anaerobic cultures were not conducted in this study, consistent with many reports in the literature that do not routinely screen for anaerobes. The limited available data indicates a low incidence of infections caused by these agents [17, 20]. Transplants from controlled DCD were not included, as this type of transplantation is not regulated in the country. Patients who underwent early surgical reintervention were also excluded to avoid potential data misinterpretation due to additional contamination of the surgical site and blood cultures. The study's single-center design may limit generalizability. While the overall sample is robust, some subgroup analyses were conducted with relatively small sample sizes, which may reduce statistical power. The available literature primarily addresses other kidney transplantation donation settings, making direct data comparisons challenging, as this appears to be the first prospective publication focused on the incidence of positive microbiological cultures in PF from uDCD kidney transplants compared to those from DBD and its clinical impact. Future research should examine the differences in PF cultures between kidneys from the same donor and compare outcomes from multi-organ uDCD procurement with those from kidney-only procurement. Collecting culture samples immediately after harvesting could be important for comparison with samples taken at the time of transplant. Excluding patients who required surgical reintervention within the first 8 days post-transplant may have inadvertently omitted cases of donor-derived anastomotic infections, which are often associated with high-risk pathogens in PF cultures. This exclusion could introduce bias and limit the generalizability of the findings. The authors justified this decision by noting that reintervention may alter microbiological results, particularly blood cultures. Notably, among the cases requiring graft removal during early reintervention, no association was found between pathogenic organisms and histopathological analyses. The study's ability to assess the impact of high-risk pathogens is limited, as most peritoneal fluid cultures did not yield such organisms. However, this also suggests that the prevalence of these high-risk microorganisms is low.

In conclusion, PF contamination is common in kidney transplantation, particularly in the uDCD setting. Although positive PF cultures were more frequent in uDCD cases, most of the bacteria identified were non-pathogenic and antibiotic prescription rates were similar compared to the DBD group.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving humans were approved by Ethics commission Sao Joao Hospital. 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

Participated in research design: AC, TP-V, AP, AC, IF, MB, TA-L, SS, RR-A, CM-S, MP, and JA. Participated in the writing of the paper: AC, TP-V, AP, TA-L, and JA. Participated in the performance of the research: AC, TP-V, AP, TA-L, IF, AC, SS, MR, CM-S, MP, and JA. Participated in data analysis: AC, TP-V, AP, TA-L, IF, AC, IT, MB, SS, RR-A, CM-S, MP, and JA. 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.

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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Living Kidney Donation Practices in Europe: A Survey of DESCaRTES and EKITA Transplantation Working Groups

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Thorough evaluation of potential kidney donors ensures safety and graft quality, but European data on donor practices are lacking. An online survey was conducted to assess European practices regarding kidney function, risk assessment and follow-up. 56% of respondents (125 practitioners, 16 countries, ~3700 donations annually) use $eGFR_{CKD\text{-}EPI}$, 34% use creatinine clearance and 70% use measured GFR. Sixty-three percent have no upper age limits, 91% exclude candidates with hypertension with end-organ damage, and 78% candidates on ≥2 antihypertensives. BMI cut-offs of 30 (39%) and 35 kg/m^2 (42%) are common. Candidates are excluded for an HbA1c ≥ 53 mmol/mol (46%), glucose ≥7 (57%) or ≥11.1 mmol/L after glucose-tolerance test (59%). ApoL1-testing is not routine in 73%, and 38% perform a kidney biopsy if albuminuria/hematuria is present. Spot and 24-hour urine albumin is assessed in 38%. Hematuria is accepted when urological evaluation (15%), kidney biopsy (16%), or both (57%) are normal. Low-risk stones often do not preclude donation. Written informed consent is obtained by 95% of centers, with 65% asking consent for data. Lifetime follow-up is offered by 83%. This first study on evaluation and follow-up practices of donors in Europe shows variation between centers, suggesting a need for harmonization of donor practices.

Keywords: kidney function, living kidney donation, donor screening, risk assessment, donor follow-up

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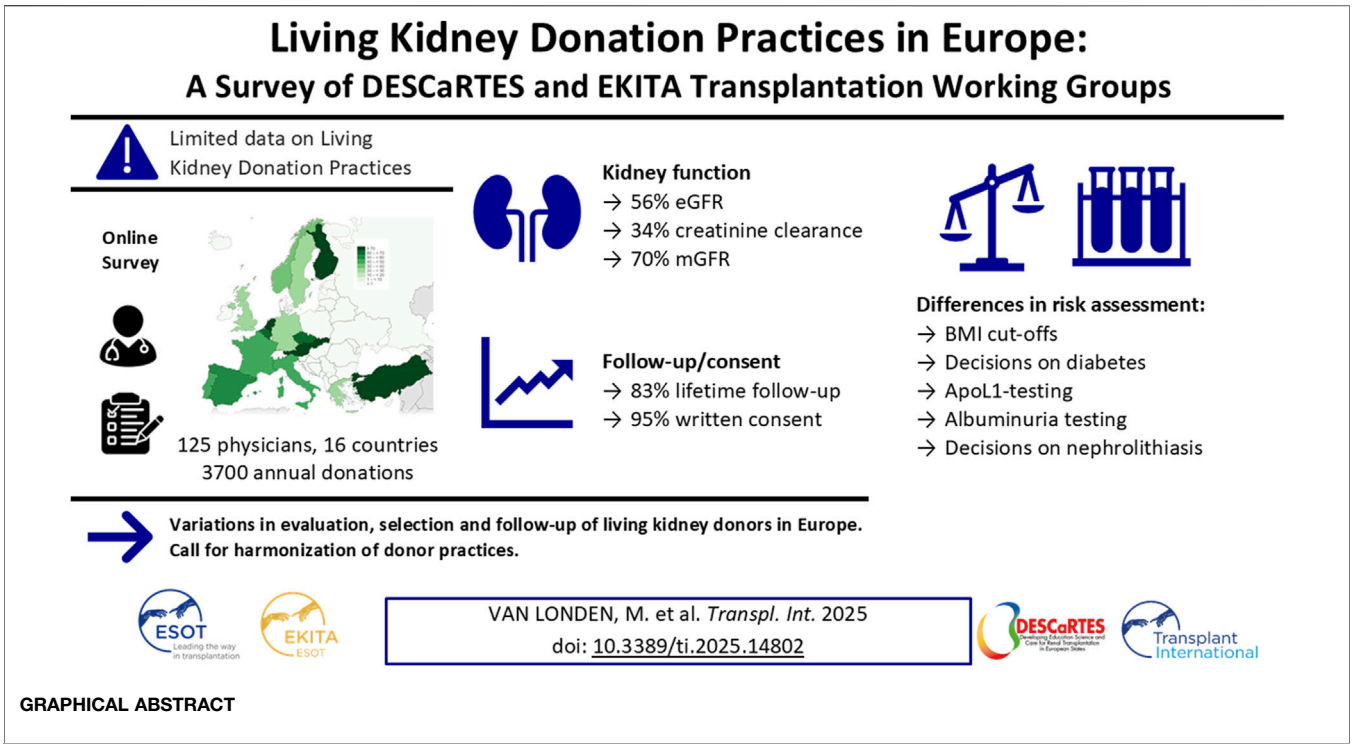
INTRODUCTION

Kidney transplantation with a graft from a living kidney donor (LKD) is the preferred treatment for most patients with end-stage kidney disease (ESKD) [1]. Due to superior outcomes for the transplant patient [2] and donor organ shortages, living kidney donation has become an important part of many transplant programs worldwide [1, 3]. The health outcomes of LKDs are favorable when compared



GRAPHICAL ABSTRACT

with the general population [4], but when compared with selected non-donors, donors may have increased risk of hypertension, cardiovascular disease, ESKD and mortality [5–9]. This underscores the importance of evaluating the potential LKDs to ensure the safety of the donor and the quality of the transplanted graft. For the evaluation of LKDs, national and international guidelines exist [10–13], but little is known about their use in clinical practice. In 2020, a survey on LKD practices in the United States was published [14], which revealed ample variation in LKD selection practices between centers. While this survey was, in fact, the third one to be conducted in the United States since 1995, no similar initiative has been conducted in Europe. Here, we report the results of the first survey on LKD kidney function measurement, donor risk assessment, and follow-up practices in Europe.

MATERIALS AND METHODS

Design of the Questionnaire

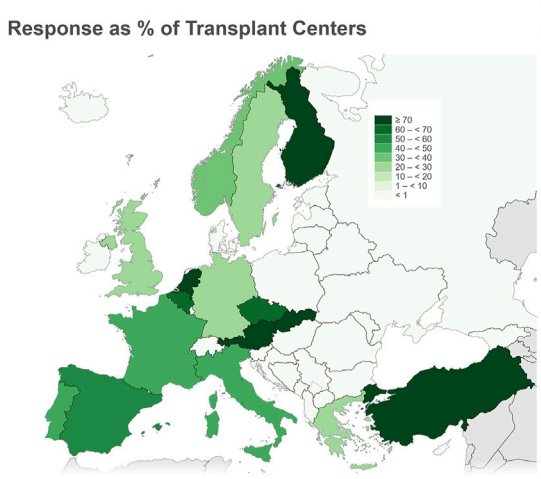
We used an online questionnaire to collect information on measurement of LKD kidney function, donor risk assessment, and post-donation follow-up practices in Europe. The questionnaire was administered to all relevant transplant professionals involved in the evaluation and/or follow-up of LKDs. Topics of the questionnaire were based on the 2017 evaluation of US donor practices [14] and were evaluated by the DESCaRTES working group of the European Renal Association (ERA) and EKITA working group of the European Society for Organ Transplantation (ESOT). Questions were entered, removed, or adapted in multiple rounds of discussion using the process of content validity through expert review. After agreement with the author group, the survey was tested by 10 transplant professionals (four nephrologists, four surgeons, and two clinical researchers in the field of kidney transplantation). The survey was designed, distributed and managed using REDCap electronic data capture tools hosted at the University Medical Center Groningen [15, 16].

The survey consists of 40–54 branched questions, with the number depending on previous answers. An overview of all the questions is provided in **Supplementary Table S1**. The questions were divided into five sections. The first section consists of questions on the center's LKD program in general, the second section concerns kidney function evaluation, the third section is about LKD risk assessment, the fourth section is on follow-up practices, and the final section concerns data collection practices.

Distribution of the Questionnaire

A link to the survey, accompanied by an introductory e-mail was dispatched to members of the DESCaRTES and EKITA working groups, who contacted members directly from their networks and asked them to forward the invitation for the questionnaire to relevant transplant professionals in their field. N = 125 complete responses were received, covering approximately 45% of European transplant centers (ESOT YPT Map of active European transplant centers, accessed at https://esot.org/map/). All respondents were invited to be recorded as collaborators in the final publication of the questionnaire.

Data are reported as percentages for all relevant questionnaire items. When a question has a numerical outcome, the median



Country	N	%*
Austria	4	3
Belgium	5	4
Czechia	4	3
Finland	1	1
France	17	14
Germany	11	9
Greece	1	1
Italy	20	16
Netherlands	6	5
Norway	1	1
Portugal	3	2
Slovakia	3	2
Spain	23	18
Sweden	2	2
Turkey	19	15
United Kingdom	5	4
Total	**125 respondents**	

FIGURE 1 | Overview of respondents. Overview of respondents as percentage of transplant centers per country (left) and absolute numbers with percentage of all reponses (right). Source: ESOT YPT Map of active European transplant centers (accessed at https://esot.org/map/). Created using IMAGE Interactive Map generator (accessed at: https://gisco-services.ec.europa.eu/image/screen/home).

[25th; 75th percentile] is given. The transplant region of all respondent centers (Eurotransplant, Scandiatransplant, Southern Alliance or "Other," including the United Kingdom and Turkey) was identified. Non-parametric tests were used to compare differences in responses between the centers (Kruskal-Wallis for continuous variables, Chi-squared test for categorical variables). A p-value of <0.05 was considered statistically significant. SPSS Statistics V23 (IBM, Armonk, United States), GraphPad Prism 8 (GraphPad Software, California, United States), and Microsoft Excel build 2406 (Microsoft, Redmont, United States) were used for data analyses and presentation.

RESULTS

General Characteristics

We collected data from 125 respondents of 124 transplant centers, representing 45% of European transplant centers (**Figure 1**). Of all respondents, n = 112 (90%) were nephrologists, n = 10 (8%) were surgeons, and n = 3 (2%) were other transplant practitioners (e.g., specialized nurses). Respondents represented n = 16 countries, screening an estimated combined number of 8141 potential LKDs per year and performing about 3700 LKD transplantations per year in the last 5 years.

The screening of potential LKDs takes a median of 10 [2; 48] hours, and the entire process takes 30 [8; 60] days, either as an inpatient evaluation (21%), outpatient evaluation (54%) or both, according to donor choice (25%). An overview of all professionals involved is shown in **Figure 2**. Most centers base their practice on guidelines; n = 62 (50%) used the KDIGO guidelines, n = 9 (7%) use the BTS guideline, n = 30 (24%) use both and n = 24 (19%) use local guidelines or a combination of guidelines. Most potential LKDs are asked for written informed consent for nephrectomy at the screening (30%), after being approved (36%), before surgery (19%) or repeatedly (10%). Five percent of centers do not routinely ask for written informed consent for donation. Most centers register LKD data locally (24%) or in national databases/registries (26%). 41% of centers register data in both, while 9% do not register data. In 65% of centers donors provide written informed consent for the registration of their data.

Evaluation of Kidney Function

For the evaluation of kidney function, most centers use the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI)-equation (n = 70, 56%), while a minority use the Modification of Diet in Renal Disease (MDRD)-equation (n = 5, 4%), the European Kidney Function Consortium (EKFC)-equation (n = 1, 1%) or the 24-hour creatinine clearance (CrCl, n = 43, 34%) and n = 6 (5%) did not specify the test. N = 51 (41%) centers use a combination of creatinine and cystatin C for GFR estimation, while n = 4 (3%) centers use cystatin C without creatinine. Centers not using cystatin C indicate a lack of availability (n = 20), no perceived added value (n = 21) or costs (n = 7) as arguments for not using cystatin C. N = 88 (70%) use measured GFR (mGFR, using an exogenous marker) in their practice, of which n = 60 (68%) routinely perform mGFR. An overview of practices regarding mGFR is shown in **Table 1**. Most centers use an age-dependent GFR threshold to select LKDs (n = 80, 64%). Centers using a fixed threshold most often use 80 mL/min/1.73 m^2 (n = 33, 26%). Centers in the United Kingdom, Norway, Spain, Germany, France, Finland, Czechia and Austria more frequently use mGFR-based screening. The use of CrCl is most common in Turkey, Portugal, the Netherlands and Italy (**Figure 3**). When differences between kidney sizes are found in imaging performed as part of the anatomical evaluation of the

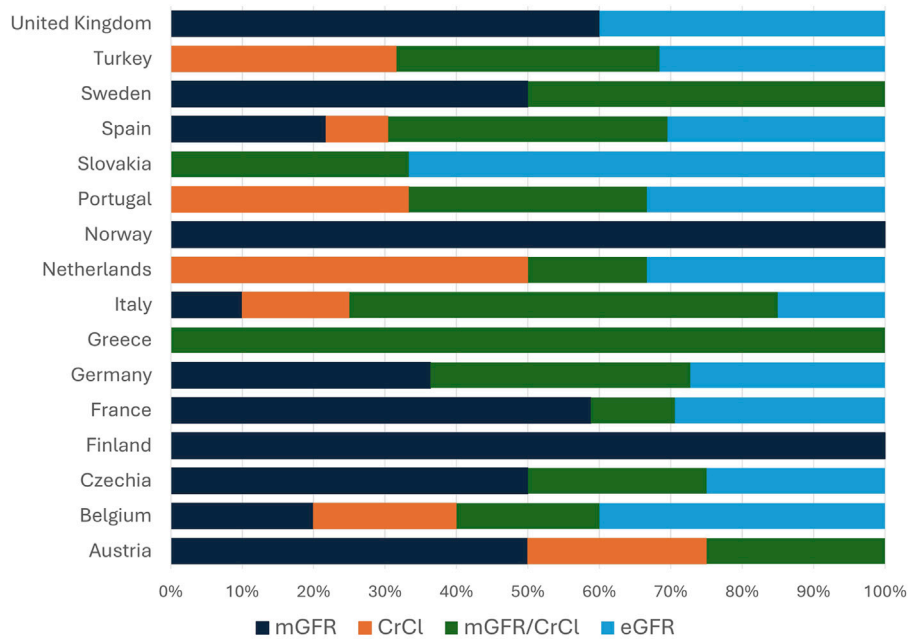


FIGURE 2 | Transplant professionals involved in living kidney donor decision making. Overview of transplant professionals routinely involved in the selection of living kidney donation, expressed as percentage of all respondents (n = 125). When "Other" was selected, respondents were asked to specify: 2 (2%) respondents answered, "social worker," 1 (1%) respondent answered "pharmacist," 1 (1%) respondent answered "vascular surgeon" and 1 (1%) respondent answered "healthcare ethics committee" to be routinely involved in the selection of living kidney donors.

TABLE 1 | Practices regarding measured GFR in living kidney donor candidates.

Variable	Centers
Use of mGFR, n (%)	
Incidentally	28 (22%)
Routinely	60 (48%)
Never	37 (30%)
Tracer used, n (% of 88 centers)	
Plasma 99mTC-DTPA clearance	52 (59%)
Urinary 99mTC-DTPA clearance	5 (6%)
Plasma iohexol clearance	19 (22%)
Urinary iohexol clearance	3 (3%)
Plasma 125I-iothalamate clearance	2 (2%)
Urinary 125I-iothalamate clearance	3 (3%)
Other	4 (5%)
Indexation of mGFR, n (% of 88 centers)	
Indexed for BSA	52 (59%)
Unindexed	16 (20%)
Use both	17 (19%)
No answer	3 (2%)
Use of confirmatory testing in decision-making	
mGFR and CrCl	42 (34%)
Mainly mGFR	32 (26%)
Only CrCl	17 (14%)
mGFR dependent on eGFR	9 (7%)
CrCl dependent on eGFR	14 (11%)
eGFR only	11 (9%)

mGFR, measured Glomerular Filtration Rate; BSA, Body Surface Area; CrCl, 24-hour creatinine clearance; eGFR, estimated Glomerular Filtration Rate.

donor candidate, most centers perform split kidney function testing (n = 88, 70%).

Assessment of LKD Risks

Most centers have a lower age limit of 18 years old (n = 60, 53%), with the range of lower age-limits between 18 and 40 years old. 15 centers (23%) do not have a lower age limit. Most centers (n = 79, 63%) do not use an upper age limit. Centers with upper limits use 70 (9%), 75 (13%) or 80 (10%) years of age. BMI cut-offs of ≥30 (39%) or ≥35 kg/m^2 (42%) are used to reject LKD candidates. Most centers offer weight loss interventions to overweight candidates (74%); responders provide dietary support (67%), exercise therapy/ training support (27%), endocrinological evaluation and/or medication (23%), or bariatric surgery (11%).

To assess the risk for diabetes, centers either use an oral glucose tolerance-test (OGTT) in all donor candidates (25%), in candidates with elevated fasting glucose (65%), elevated HbA1c (52%), a family history of diabetes (33%) or obesity (41%). A minority of centers perform an OGTT in potential LKDs with hypertension (6%), dyslipidemia (2%) or isolated microalbumuria without other abnormalities (16%). Centers usually reject donor candidates with a HbA1c ≥ 53 mmol/mol or 7% (46%), fasting glucose above 7 mmol/L or 126 mg/dL (57%), or glucose after an OGTT ≥11.1 mmol/L or 199 mg/dL (59%). 10% of centers reject candidates with gestational diabetes, and 11% of centers reject younger candidates if

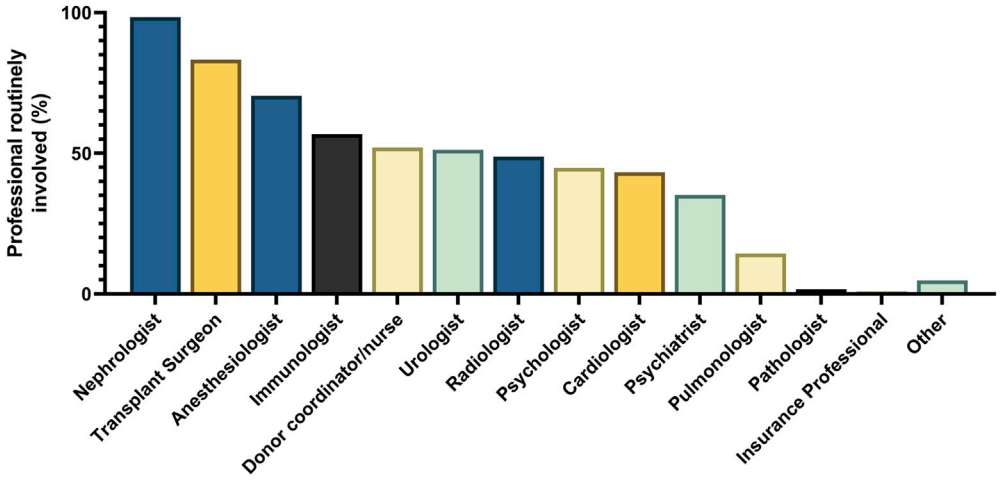


FIGURE 3 | Kidney function assessment per country. Overview of routinely used tests for decision-making regarding kidney function of a potential LKD. Centers were asked which test they mainly use for decision-making: measured GFR (dark blue), 24-hour creatinine clearance (orange), a combination of these (green) or estimated GFR (light blue). Answers are expressed as percentage of all respondents (n = 125).

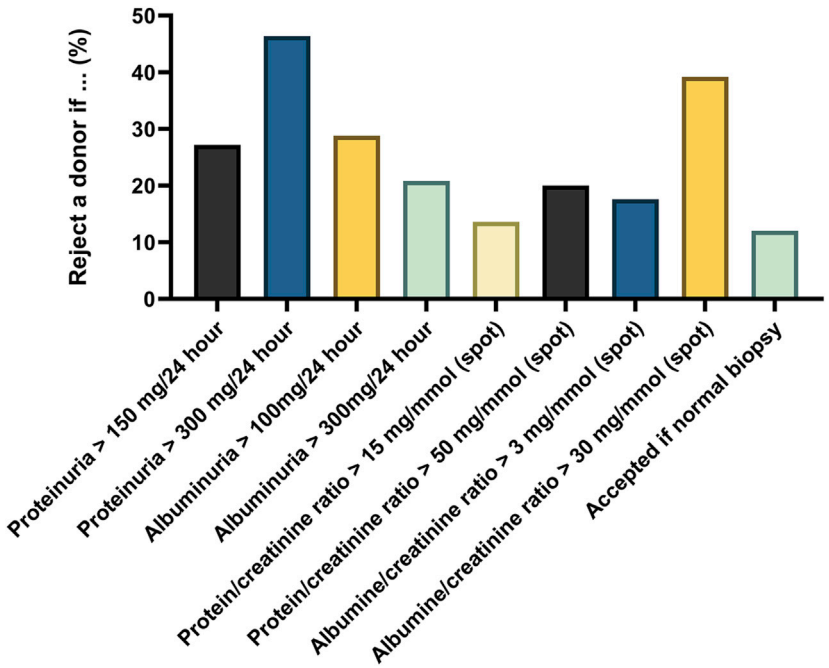


FIGURE 4 | Overview of decision-making regarding proteinuria and albuminuria. Overview of decision-making regarding proteinuria/albuminuria in 24-hour urine and/or spot urines. Centers were asked which of the answers best represents their practice regarding the exclusion of donors with proteinuria. Multiple answers could be given. Answers are expressed as percentage of all respondents (n = 125). 12% of centers would accept donors with any proteinuria/albuminuria if they have a normal biopsy result.

they have 'pre-diabetes', while some respondents (n = 9) indicated that this decision depends on the entire risk profile.

Blood pressure is usually assessed using automated or non-automated office blood pressure measurements (46% and 26%, respectively), while 24-hour ambulant blood pressure measurements are performed in 34% of centers. Almost all centers reject donor candidates with uncontrolled hypertension and/or signs of end-organ damage during screening (91%), 19% reject candidates using ≥2 antihypertensives and 78% candidates with ≥3 antihypertensives. Persistent borderline hypertension, without end-organ damage, was not indicated as reason to reject candidates.

38% of donor candidates undergo both spot urine and 24-hour urine test for proteinuria or albuminuria, while a minority undergoes 24-hour proteinuria/albuminuria testing (18% and 10%, respectively) or spot urine testing for proteinuria/albuminuria (9% and 24%, respectively). An overview of proteinuria-related decision-making is shown in **Figure 4**. 28 (22%) of centers base decision-making only on proteinuria (either 24-hour urine, spot urine or both).

Donor candidates with persistent isolated microscopic haematuria are mostly excluded in 5% of centers, while 42% of centers only exclude when urine sediment indicates a glomerular cause. Candidates with persistent isolated microscopic haematuria are usually accepted when they have no abnormalities in urological evaluation (15%), kidney biopsy (16%) or both (57%). 62% of centers do not perform kidney biopsies.

Donor candidates with a positive family history of Autosomal Dominant Polycystic Kidney Disease (ADPKD) are sometimes rejected outright (n = 4, 3%), depending on their age (n = 24, 19%), but most often receive additional testing with MRI (n = 28, 22%), ultrasound (n = 51, 41%) or MRI/ultrasound imaging depending on their age (n = 49, 39%) while some centers (n = 11, 9%) perform both imaging techniques. PKD-mutation analysis is performed in most of LKD candidates with a positive family history (n = 67, 54%).

27% of centers routinely perform ApoL1 testing for potential LKDs with African ancestry. For 11% of centers a high-risk ApoL1 genotype, when known, is a contra-indication for kidney donation.

2% of centers reject potential LKDs with any kidney stone, regardless of size or risk profile. Most centers accept donor candidates with a history of nephrolithiasis if no stones are present, the 24-hour urine profile is low-risk (36%) or when low-risk and stone-related symptoms were >5 years ago (29%). 29% of centers reject candidates with a history of bilateral stones.

NSAID use is accepted in 19% of centers when candidates are otherwise healthy. NSAID use is also accepted when a donor candidate has a rheumatological disease (2%), the use is infrequent (7%). 8% of centers accept some types of NSAIDs, while 61% of centers ask donors to stop NSAIDs completely. Smoking is a contra-indication for kidney donation in 3% of centers, whereas it is accepted (but strongly discouraged) in 78% of centers. Some centers ask LKD candidates to stop smoking 4 weeks before surgery, either with documentation of smoking-cessation (e.g., cotinine measurement, 3%) or without (16%).

A majority of centers do not routinely use online risk calculators to estimate lifetime risk of end-stage kidney disease (54%), 22% use the ESKD Risk Tool by Grams et al. [17] routinely and 22% for selected candidates. 2% use a different risk tool. Most centers do not use a fixed threshold for lifetime end-stage kidney disease in the donors, but rather an individualised risk leniency (57% use individualized thresholds, 1% report a threshold of 10%, 6% report a threshold of 5%, 5% report a threshold of 3% and 11% report a threshold of 1%). The remaining 21% do not use risk thresholds.

Follow-Up of LKDs

Most centers (n = 97, 83%) routinely offer lifetime follow-up of donors. In centers with living donor follow-up, most LKDs receive a follow-up visit every year (90%) or every 2–4 years (10%). Follow-up generally consists of blood pressure checks (98%), 24-hour urinalysis (34%), spot urine analysis (75%), eGFR (94%), CrCl (15%), mGFR (18%), blood tests (83%), body composition measurements (67%) and/or a medication review (70%). Psychosocial counselling is offered in 20%. Follow-up is mostly organized by nephrologists (82%), general practitioners (8%) or transplant surgeons (7%). Follow-up involves out-of-pocket payment for travel expenses in 6% of centers and all post operative care in 1% of centers. 3% of centers indicate that follow-up is not always performed.

Consensual and Controversial Practices

The highest consensus among centers was in the requirement of informed consent for kidney donation, decision making around hypertension, the exclusion of donor candidates <18 years of age and the use of routine (mostly annual) follow-up after kidney donation. Practices with low consensus include the use of kidney function testing, the routine use of CrCl and the acceptance policy of donor candidates with nephrolithiasis. Also, centers differ in assessment of albuminuria, use of cystatin C and BMI cut-off values. An overview of the consensus of all questionnaire items is provided in **Figure 5**.

Differences Between Transplant Regions

N = 29 (23%) of the respondents are part of Eurotransplant (ET), n = 4 (3%) of Scandiatransplant (ST), 67 (54%) of the Southern Alliance (SA), and 25 (20%) of other transplant regions. A detailed overview of questionnaire responses per transplant region can be found in **Supplementary Table S2** for general characteristics, **Supplementary Table S3** for kidney function assessment, **Supplementary Table S4** for risk assessment and **Supplementary Table S5** for donor follow-up. The number of LKD transplantations differed between respondents from the four identified transplant regions, with Scandiatransplant (median 36/year/center) performing the most LKD transplantations per center ($P < 0.001$, **Supplementary Table S2**). No differences were found when comparing the use of mGFR ($P = 0.06$), the use of mGFR tracer ($p = 0.40$) or GFR indexing ($p = 0.34$) or confirmatory testing ($P = 0.57$; **Supplementary Table S3**). Scandiatransplant more often performs OGTTs ($P = 0.045$, **Supplementary Table S4**), but no other differences in glucose testing were found. No differences were found between the regions regarding BMI cut-offs, but there were differences in weight loss interventions offered to LKD candidates (offered in 52% for ET, 25% for ST, 85% for SA, $P < 0.001$), with also more dietary interventions offered in the SA-region (45% vs. 0% vs. 78%, $P < 0.001$). No significant differences were found regarding ADPKD testing, nephrolithiasis, and haematuria testing ($P > 0.05$ for all analyses). Centers in the ET-region more often reject donors with a protein/creatinine ratio of >50 mg/mmol when no other abnormalities are present ($P = 0.03$), and centers in the ST- and SA-regions more often reject donors with an albumin/creatinine ratio >3 mg/mmol ($P < 0.001$). The ET-region

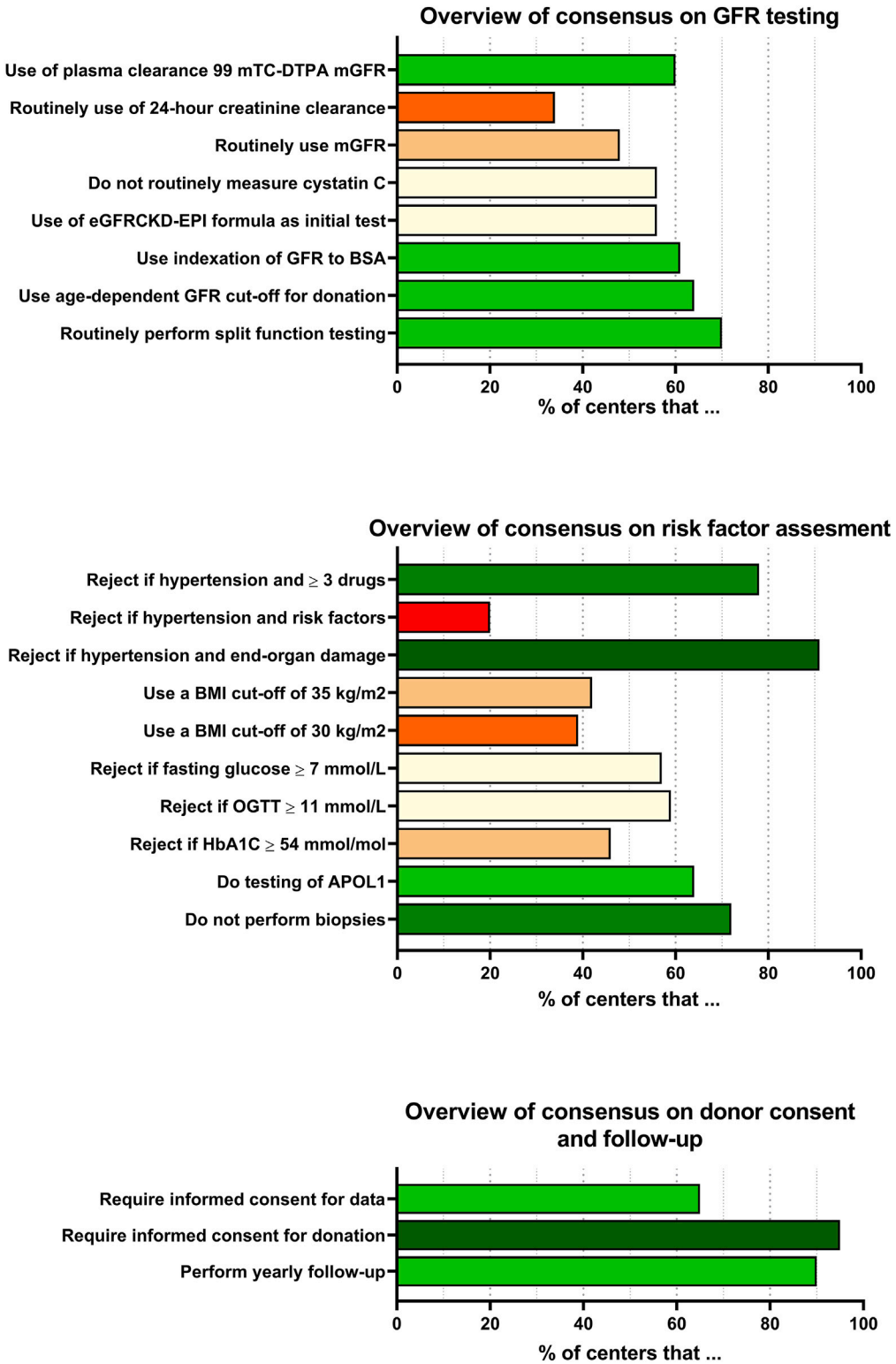


FIGURE 5 | Consensus overview of questionnaire items. Overview of rate of consensus on various questionnaire items. When multiple answers were possible, the most common answer is shown in the graph.

TABLE 2 | Overview of National and International guidelines for the selection of Living Kidney Donors [10].

Title	Year	Organization	Reach/Origin	Source
KDIGO Clinical Practice Guideline on the Evaluation and Care of Living Kidney Donors	2017	KDIGO	Global	[11]
BTS/RA Living Donor Kidney Transplantation Guidelines 2018	2018	BTS/RA	United Kingdom	[12]
Recommandations d'aide à la pratique clinique pour le don de rein du vivant	2023	Agence de la Biomédecine	France	[13]
European Renal Best Practice Guideline on kidney donor and recipient evaluation and perioperative care	2015	ERBP	Europe	[18]
Samenvatting van aanbevelingen in de Britse richtlijn "Living Donor Kidney Transplantation"	2020	Nederlandse Transplantatie Vereniging	Netherlands	[19]

more often accepts candidates with proteinuria if they have no abnormalities on biopsy (P = 0.03). Only in the ET-region do centers exclude smokers from donation (14% vs. 0% in other regions). The intensity, specialty in charge of follow-up and medical items part of follow-up differ per transplant region (**Supplementary Table S5**).

DISCUSSION

In this study, we show differences in the evaluation, selection and follow-up practices for LKDs across Europe. We report marked differences in the use of confirmatory kidney function testing (eGFR, creatinine/cystatin C, mGFR, creatinine clearance), albuminuria assessment, and the acceptance policy of donors with nephrolithiasis. These results point to opportunities for harmonization and future studies.

High standards for the acceptance of LKD candidates is paramount for ensuring the safety of LKDs and improving the quality of the transplanted graft. Although national and international guidelines exist for LKD evaluation (**Table 2**), our study highlights the differences in guideline application across Europe. Consistent with guideline recommendations, all centers have a dedicated team for the evaluation of LKD candidates, although the professionals involved in this team differ. In line with recommendations, centers obtain informed consent for donation, although consent for data use is inconsistent.

The guidelines stated in **Table 2** show differences in kidney function testing recommendations, which are also clear from the responses to our survey. Overall, an individualized assessment of kidney function is performed, but centers and particularly countries differ in their technique, for example, eGFR (creatinine, cystatin C or both), 24-hour urinary creatinine clearance or measured GFR for decision-making. In 2022, the DESCARTES working group of the ERA released a position paper, where they advocated an individualized (and age-dependent) GFR threshold and recommend mGFR for LKD assessment [20]. Most centers (68%) routinely perform mGFR in donor candidates, using multiple possible tracers. In light of challenges in the production of radioactive tracers, and a publication calling for standardization of mGFR from the European Kidney Function consortium [21], iohexol plasma clearance (currently used in 22% of respondents) may become more common. Guidelines advocate normalizing kidney function for body surface area [11], and we report similar normalization rates compared to the United States (71% BSA-normalization in our survey vs. 75% in the US) [14].

Most centers (63%) do not set an upper age limit for kidney donation, reflecting a change in guidelines over time. While perioperative risks are higher for older donors, lifetime risks for end-stage kidney disease will always be higher for younger donors, due to remaining life-span being longer [7, 8, 17]. Accordingly, several centers reported stricter selection in younger donors. While obesity is a well-known risk factor for adverse outcomes in donation [22, 23], the presence of obesity is handled differently across transplant-centers and regions: cut-offs for BMI of 30 kg/m^2 or 35 kg/m^2 are both used. The importance of a healthy weight for LKDs is recognized; 74% of centers offer weight-loss interventions for LKDs, most often in the Southern Alliance. However, weight-loss interventions vary greatly between respondents: 67% offer dietary support, 27% offer exercise therapy/training, 23% offer endocrinological evaluation/medication and 11% offer bariatric surgery. While research on bariatric surgery in future LKDs is expanding [24, 25], secondary hyperoxaluria from bariatric surgery and corresponding nephrolithiasis/nephrocalcinosis are risk factors for CKD [26, 27]. If bariatric surgery is necessary for LKD candidates, sleeve gastrectomy reduces hyperoxaluria risk compared to Roux-en-Y bypass [28].

While risks of diabetes and hypertension are differently assessed between respondents, there is a consensus on the acceptance of LKD candidates with these comorbidities. In line with guidelines, candidates with uncontrolled hypertension and/or with signs of end-organ damage, are not accepted for donation. Candidates with diabetes are also excluded from donation. In line with the KDIGO guidelines, most centers reject donors with an abnormal OGTT, HbA1c, or fasting glucose. Guidelines differ in their recommendations on proteinuria testing: the KDIGO guideline advise using albuminuria and not proteinuria, because of standardization issues and evidence about albuminuria as an independent risk factor. The French guideline underscores this (grade B level of evidence), while the British Transplant Society guideline considers the measurement of total protein in the urine to be an acceptable alternative (grade A1 level of evidence). This is reflected in the answers to our survey, where decision-making is based on both, with most centers performing 24-hour assessment of protein/albumin excretion. Hematuria can be acceptable for LKD candidates, if no other abnormalities are found on urinalysis, urological evaluation and/or kidney biopsy. Candidates with persistent asymptomatic hematuria are rejected in a minority of centers, in line with most guidelines. Not all centers perform kidney

biopsies in donors with hematuria, as recommended in the BTS guideline and suggested in the French/KDIGO guidelines [12, 13, 29]. Acceptance of LKD candidates with nephrolithiasis varies although most centers accept candidates with historical stone disease provided the recurrence risk is deemed low, in line with the guidelines and supported by the literature [30]. ApoL1 testing for donors with African ancestry is routinely performed in a minority (27%), while it is considered in the risk profile when known. In comparison, in the 2017 survey in the United States, 13% of respondents routinely performs ApoL1 genotyping and 32% perform this for selected candidates [14]. Interestingly, NSAID use is acceptable in 19% of centers and conditionally accepted in another 17%, in line with data from the US [14]. While smoking is an important modifiable risk factor for cardiovascular and kidney disease [17], it is not generally a contra-indication for kidney donation. Some centers ask donors to stop 4 weeks before the surgery, possibly because of the increased risk of complications found in non-donation surgery [31].

A minority use the end-stage kidney disease (ESKD) Risk Tool by Grams et al. either routinely or for selected candidates (45%) [17], which is slightly less than in the US survey [14]. Most centers using thresholds for ESKD risk leniency reported individualized thresholds, or no numerical threshold at all. Limitations of the ESKD Risk tool and other calculators include lack of validation outside the cohorts they were developed in (a non-donor US population), and a lack of consensus on relevant thresholds for individual candidates [20]. Also, long-term risk for ESKD is impossible to capture from baseline data in younger donors [32, 33]. Use of an ESKD Risk tool may therefore falsely re-assure donors and clinicians of limited risks. In younger donors, lifelong follow-up is of special importance, even if they have an apparent low risk of ESKD [20].

Long-term follow-up of kidney donors is considered necessary and often mandatory, although specifics vary between centers. While 17% of centers do not promote lifetime follow-up, 10% organize follow-up every 2–4 years. Follow-up is mainly managed by the nephrologist but may be organized by general practitioners, most often in the Eurotransplant region, likely due to the local practice and reimbursement policies. Follow-up generally includes a medication review, cardiovascular risk assessment, spot urine analysis and blood tests. A minority of centers also incorporate psychosocial counselling.

This study has several limitations. While it offers broad representation across Europe (**Figure 2**), the Eastern part of Europe is underrepresented. This limitation may be cause by not having sufficient contact details in this area and could indicate more necessity for outreach by European transplant professionals and organisations. Survey fatigue could also have been a reason for a limited response rate in some areas. We aimed to limit this, by choosing one respondent for a transplant center to answer, which could induce bias itself: The questionnaire was designed to identify practice variation between centers, rather than between individuals within centers. The questionnaire format is subject to social desirability bias and recall bias. Also, statistical analyses comparing transplant regions were limited by power and multiple testing (increasing the chance of type I error). Donor evaluation and follow-up decisions are often individualized and may not apply uniformly across cases, a complexity not fully captured by questionnaires. When developing the questions, we specifically attempted to recognize this caveat. Our survey was inspired by the initiative from the United States to evaluate the LKD practices [14], but results between the US and Europe cannot be compared directly because ours was more recent (2023 vs. 2017) and the healthcare systems in Europe and the US differ [34]. Our survey benefits from a high response rate (**Figure 1**), a wide range of assessed topics and the addition of data on follow-up practices.

The current study provides a snapshot of current living kidney donor practices across Europe and can help to inform healthcare professionals on prevalent practices. Our findings may support the development of healthcare policies aimed at improving the quality of LKD information, selection and follow-up. Future studies should focus on the role of cultural, social or logistical factors in living kidney donor practices, for example, on how the availability of resources influencing kidney function testing. Our results underscore the importance of harmonization of living donor care using evidence-based practice. We also advocate for the establishment of a European registry of LKD outcomes to further study LKD practices and outcomes [35, 36].

In conclusion, this is the first study on practices in the evaluation, selection and follow-up of LDKs in Europe. The selection of LKDs is a balancing act between the benefits for the donor- and the recipient on one hand, and short-term and long-term risks of donor nephrectomy on the other hand [33]. Our study identified several areas with considerable heterogeneity between centers and regions, especially in confirmatory kidney function testing, use of 24- hour creatinine clearance and the acceptance policy of donors with nephrolithiasis. Heterogeneity was also apparent in the assessment of albuminuria, use of cystatin C and BMI cut-offs. This heterogeneity can be used as a basis for future studies and should serve to dynamically inform professionals, help design healthcare policies and improve the overall quality of information, selection and follow-up of living donors. We, therefore, support harmonization of living donor management using evidence-based practice and call for a European registry of LKD outcomes [35, 36].

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

Conceptualization: ML, FL, GM, and CM; Methodology: ML, FL, GM, and CM; Investigation (data collection): ML, FL, GZ, GO, IG, LF, JS, DC, LH, GM, and CM; Data curation: ML; Writing (original draft): ML, CM; Writing (review and editing): FL, GZ, GO, IG, LF, JS, DC, LH, GM, and CM; Supervision: GM and CM, members of the DESCaRTES and EKITA working groups; Project administration: ML and CM. All authors contributed to the article and approved the submitted version.

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GENERATIVE AI STATEMENT

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

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The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2025.14802/full#supplementary-material

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ORIGINAL RESEARCH
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Comparative Analysis of Islet Auto-Transplantation Outcome Classification Systems: Evaluating Concordance, Feasibility, and a Data-Driven Approach

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A standardized approach to assessing islet autotransplantation outcomes is crucial for evaluating graft function and guiding clinical decisions. This study compares the performance of existing classification systems—Milan, Minneapolis, Chicago, Leicester, Igls, and a novel Data-Driven approach—by evaluating their ability to differentiate transplant outcomes using metabolic and insulin secretion parameters. Our analysis shows strong concordance among Milan, Minneapolis, Chicago, and Igls, primarily due to minor variations in C-peptide thresholds. The Leicester and Data-Driven systems, however, exhibit greater divergence, with the Leicester system simplifying assessment by excluding severe hypoglycemic events and HbA1c, and the Data-Driven approach offering a more dynamic framework without predefined thresholds. Fasting C-peptide levels emerged as a highly reliable predictor of graft function, with the arginine test proving more effective than Mixed Meal Tolerance Test for additional evaluation. The Data-Driven approach provided superior stratification of outcomes, highlighting the importance of residual insulin secretion in metabolic control. These findings suggest that refining classification systems, particularly by considering insulin sensitivity and residual secretion, could enhance long-term patient monitoring and improve our understanding of beta-cell replacement therapies. Further validation across diverse cohorts is essential for broader clinical adoption.

Keywords: islet autotransplantation, graft function, classification systems, C-peptide, insulin secretion

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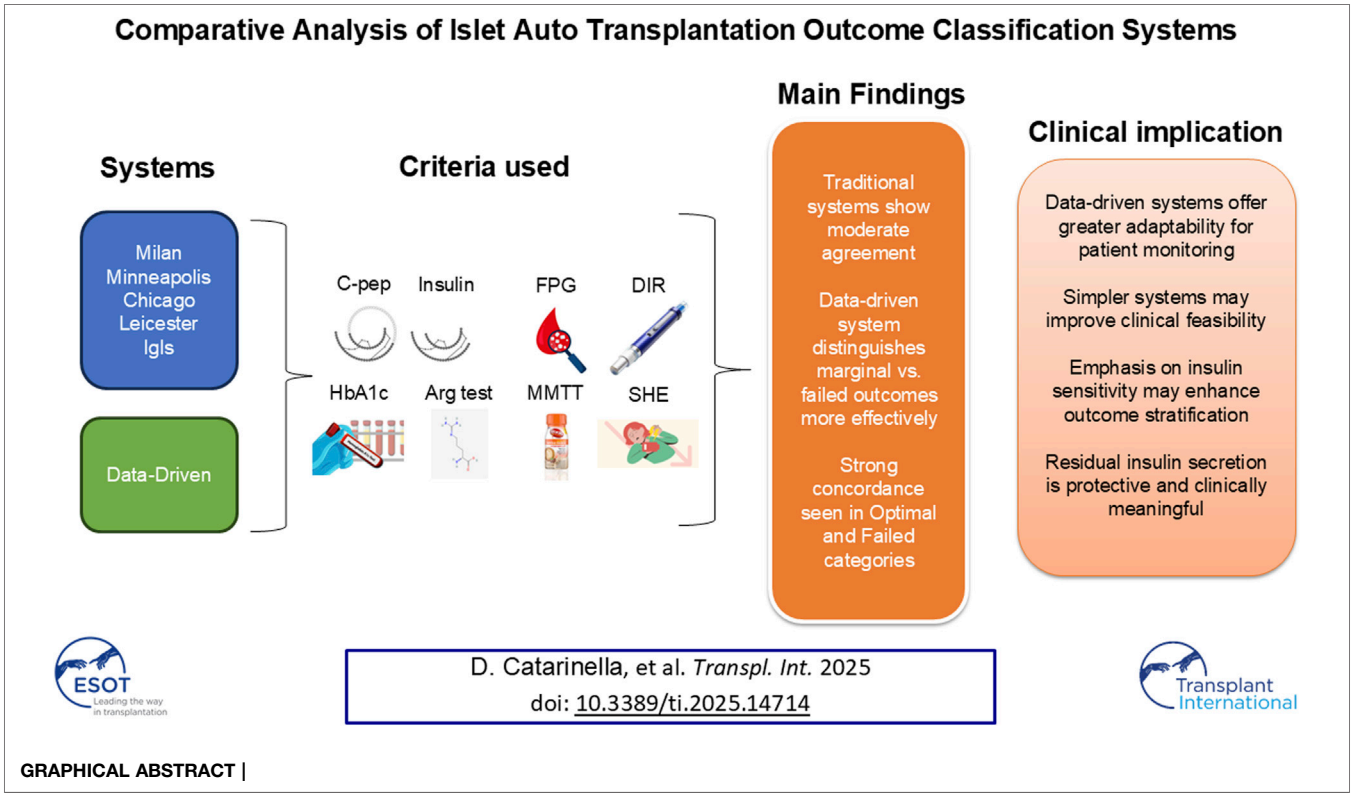


GRAPHICAL ABSTRACT |

INTRODUCTION

A standardized approach for evaluating the outcomes of beta-cell replacement therapies is essential for enabling comparisons across centers and treatment modalities, including pancreas transplantation, islet transplantation, and stem cell-based interventions [1]. In the context of allotransplantation, a collaborative effort led to the establishment of the Igls criteria, a classification system incorporating key metabolic parameters such as HbA1c levels, frequency of severe hypoglycemic events, insulin requirements, and C-peptide levels [2, 3]. However, the direct application of the Igls criteria presents challenges in the setting of islet autotransplantation (IAT) [4–8]. In IAT, insulin-producing cells from the patient's pancreas are transplanted, usually after the pancreas is surgically removed [9]. This helps restore insulin production and improve blood sugar control [10]. Unlike allotransplant recipients, patients undergoing pancreatectomy typically do not have pre-existing diabetes and often retain measurable C-peptide secretion prior to the procedure. As a result, the original Igls framework, which evaluates improvements relative to a pre-transplant baseline, may not be suitable for assessing graft function in these patients, since measuring a reduction in insulin requirements or an increase in C-peptide levels relative to pre-pancreatectomy values is not feasible. To address these limitations, several centers have proposed modifications to the Igls criteria to better suit the context of IAT. Notably, institutions in Milan [11], Minneapolis [12], Chicago [13], and Leicester [14] have developed adapted frameworks aimed at more accurately assessing graft function in these patients. Moreover, the original Igls criteria were recently revised to broaden their scope and applicability [4]. These revised approaches consider the unique characteristics of individuals undergoing IAT, ensuring a more appropriate evaluation of post-transplant outcomes. Despite these efforts, a comparative evaluation of the performance of these modified scoring systems remains absent. To bridge this gap, we conducted our study to systematically assess and compare the effectiveness of these adapted criteria in evaluating graft function following IAT. The study sought to determine how well each classification method reflects the functional outcomes of islet transplantation and its capacity to differentiate graft performance using metabolic markers and graft function scores. Additionally, we aimed to develop a Data-Driven classification system to overcome the limitations of arbitrarily defined thresholds traditionally used in graft assessment. By identifying natural clusters within the data, we sought to create a scoring system that more accurately captures the spectrum of graft function and provides an objective, adaptive framework for evaluating post-transplant outcomes.

In some IAT settings, particularly in patients with chronic pancreatitis, outcomes such as pain relief, quality of life, and reduction in narcotic use are central to post-transplant evaluation. However, these aspects are not relevant to our cohort, which—according to the Milan protocol—includes predominantly patients undergoing pancreatectomy for pancreatic neoplasms, high-risk surgical procedures, or

postoperative complications, rather than chronic pain. Accordingly, this study focuses exclusively on graft function evaluation through metabolic and insulin secretion parameters.

MATERIALS AND METHODS

Study Objective

The primary aim of this study was to conduct a comparative evaluation of the proposed classification systems for autologous islet transplantation, with the goal of assessing their concordance and their ability to distinguish transplant performance based on the available parameters.

Study Design

This retrospective observational study included adult patients who underwent total or partial pancreatectomy with IAT at IRCCS Ospedale San Raffaele, Milan, between November 2008 and June 2023 (Clinical Trial. gov: NCT01702051). Data was sourced from a previously published cohort [11]. The study population consisted of patients who underwent pancreatectomy with IAT for indications such as painful chronic pancreatitis, post-surgical pancreatic complications, high-risk pancreaticoduodenectomy, or benign/borderline neoplasms. Eligibility criteria required at least one post-operative follow-up assessment starting from month 1, with sufficient data for the calculation of graft function scores and the availability of at least one standardized stimulation test, either the mixed-meal tolerance test (MMTT) or the arginine stimulation test. Both the MMTT and the arginine stimulation test were used to assess different facets of beta-cell function. The MMTT reflects physiological postprandial insulin secretion in response to mixed nutrients and is therefore more representative of daily metabolic challenges. In contrast, the arginine stimulation test evaluates the maximal insulin secretory response under standardized conditions, making it less susceptible to variations in glucose absorption or gastrointestinal function. This dual approach was employed to capture complementary information on residual islet function across a heterogeneous post-pancreatectomy population. At each available follow-up time point, data were extracted based on the criteria above, enabling the assessment of metabolic and functional parameters. These included fasting plasma glucose, glycated hemoglobin (HbA1c), fasting and stimulated C-peptide levels (measured during both the Arginine and MMTT tests), as well as fasting insulin and proinsulin levels. Beta-cell function was evaluated through the calculation of the area under the curve (AUC) of C-peptide over the first 120 min following the MMTT or arginine test, the insulin peak time during the MMTT, and the acute insulin response to arginine (AIR-arg) during the arginine test. Insulin resistance and beta-cell function indices were derived using the Homeostatic Model Assessment (HOMA), including HOMA-IR for insulin resistance and HOMA-β for beta-cell function, calculated using both C-peptide and insulin levels [15]. All biochemical analyses were performed according to standardized laboratory protocols. The extracted data were used to compare various classification systems for graft function. Continuous glucose monitoring (CGM) data were not included in the present analysis due to the retrospective nature of the study and the lack of standardized CGM use throughout the cohort. During the study period, CGM was not routinely implemented in post-IAT follow-up, particularly in patients without overt diabetes, resulting in incomplete and non-comparable data.

Mixed Meal Tolerance Test (MMTT)

The MMTT was performed following an overnight fast (≥8 h), using a 250-kcal test meal, consisting of approximately 52% carbohydrates, 11% fats, and 37% proteins. Specifically, the "Boost High Protein Rich Chocolate Balanced Nutritional Drink" (Nestlé Health Science) was used. The drink was consumed within 10 min, and blood samples were collected at baseline (−10 and 0 min), followed by 10, 20, 30, 60, 90, 120, and 180 min after ingestion. The overall beta-cell response to the mixed meal was assessed by calculating the AUC of C-peptide levels over the 120-min test period. The highest C-peptide measurement during the test, referred to as the C-peptide peak, was also recorded.

Arginine Test

The arginine test was performed following an overnight fast, with insulin therapy suspended prior to the test. A 30-g intravenous bolus of arginine hydrochloride was administered over 30 min. Blood samples for insulin, glucose, and C-peptide concentrations were collected at baseline and at the following time points: 5, 10, 20, 30, 40, 50-, 60-, 90-, and 120-min post-infusion. The acute insulin response to arginine (AIR-arg) was calculated as the incremental AUC of insulin between 0 and 10 min. The overall beta-cell response to the arginine stimulus was assessed by calculating the AUC of C-peptide during the 120-min test period [16, 17].

Classification Methods

Graft function was assessed using five classification systems, including frameworks developed by institutions in Milan [11], Minneapolis [12], Chicago [13], and Leicester [14], as well as the revised Igls criteria [4]. These classification methods were applied to the study cohort to assess their concordance and ability to distinguish transplant performance. A summary of the classification criteria is provided in **Table 1**. All systems categorized graft function into four levels. In four of them, the categories were defined as Optimal, Good, Marginal, and Failed. The Leicester classification used a different nomenclature (Good, Partial, Poor, and Failed), which was standardized to align with the four-tier grading of the other systems. Graft function was primarily evaluated based on fasting C-peptide levels, although some classifications allowed for the inclusion of stimulated values. However, given that two different stimulation tests were utilized in this study, and their stimulated C-peptide responses differ, fasting C-peptide was selected as the standard parameter for comparison.

Development of a Data-Driven Classification System

To identify natural clusters within the data, an agglomerative hierarchical cluster analysis was performed using three key

TABLE 1 | Modified Igls classification after islet Auto-transplantation.

Classification	HbA1c	Severe Hypo episodes (SHE)	Insulin dose	Fasting C-peptide (stimulated)
Igls updates				
Optimal	≤6.5%	None	0 U/kg/d	Any
Good	<7%	None	Any	≥0.2 ng/mL (>0.5 ng/mL)
Marginal	≥7%	≥1	Any	≥0.1 ng/mL (>0.3 ng/mL)
Failed	-	-	Any	<0.1 (≤0.3 ng/m)
Chicago Auto-Igls				
Optimal	≤6.5%	None	0 U/kg/d	>0.5 ng/mL[a]
Good	<7%	None	<0.5 U/kg/day	>0.5 ng/mL[a]
Marginal	≥7%	≥1	≥0.5 U/kg/day	>0.5 ng/mL[a]
Failed		-	-	≤0.5 ng/mL[a]
Minnesota Auto-Igls				
Optimal	≤6.5%	None	None	≥0.2 ng/mL (>0.5 ng/mL)
Good	<7%	None	<0.5 U/kg/d	≥0.2 ng/mL (>0.5 ng/mL)
Marginal	≥7%	≥1	≥0.5 U/kg/d	≥0.2 ng/mL (>0.5 ng/mL)
Failed	-	-	-	<0.2 ng/mL (≤0.5 ng/mL)
Milan Auto-Igls				
Optimal	≤6.5%	None	None	>0.5 ng/mL
Good	<7%	None	<0.5 U/kg/d	>0.5 ng/mL
Marginal	≥7%	≥1	≥0.5 U/kg/d	>0.3 ng/mL
Failed	-	-	-	≤0.3 ng/mL
Leicester Auto-Igls				
Good	–	–	None (up to 5 years)[b]	≥0.2 ng/mL (>0.5 ng/mL)
Partial	–	–	<20 U/d	≥0.2 ng/mL (>0.5 ng/mL)
Poor	–	–	20–40 U/d (within 5 years)[b]	≥0.2 ng/mL (>0.5 ng/mL)
Failed	–	–	–	≤0.5 ng/mL

[a]*The fasting C-peptide value was used.*
[b]*The time range was not considered for the calculation.*

TABLE 2 | Data-Driven classification after islet auto-transplantation.

HbA1c (%)		DIR (U/kg/d)		Fasting C-peptide (ng/mL)	
Value	**Score**	**Value**	**Score**	**Value**	**Score**
>7.15	1	>0.57	1	<0.25	1
6.44–7.15	2	0.1–0.57	2	1.52–0.25	2
5.85–6.45	3	0.03–0.1	3	1.89–1.53	3
<5.85	4	<0.03	4	>1.89	4
Glucometabolic outcome classification based on composite score					
Category			Composite score		
Optimal			12		
Good			9 - <12		
Marginal			6 - <9		
Failed			3 - <6		

metabolic variables: HbA1c, fasting C-peptide, and Daily Insulin Requirement (DIR). The optimal number of clusters was determined through dendrogram analysis, which identified four categories (Clusters A, B, C, and D), each exhibiting significant differences in metabolic parameters, as shown in **Supplementary Figure S1**; **Supplementary Table S1**. These clusters were ranked in descending order of metabolic outcomes, with Cluster C demonstrating the most favorable profile, followed by A, B, and D. To establish threshold values for each metabolic parameter, Receiver Operating Characteristic (ROC) curve analysis was employed (**Supplementary Figure S2**). The optimal cut-off points were selected to achieve a specificity of 80%, ensuring reliable differentiation between the clusters. These threshold values for HbA1c, fasting C-peptide, and DIR were subsequently used to assign a score to each variable, with scores ranging from 1 to 4, where higher scores indicated better glucometabolic control. The sum of the scores across the three parameters resulted in a composite glucometabolic score ranging from 3 to 12. The composite glucometabolic score was used to categorize patients into four distinct outcome groups: failure (scores 3–6), marginal control (scores 6–9), good control (scores 9–12), and optimal control (score of 12). This methodology offers a refined and data-driven framework for evaluating glucometabolic regulation, as detailed in **Table 2**.

Statistical Methods

Data are presented as mean ± standard deviation or median (25th–75th percentile). The clustering process was performed using Euclidean distance to compute pairwise dissimilarities, while Ward's method was applied for cluster merging, minimizing intra-cluster variance to ensure the formation of homogeneous groups. Threshold values for HbA1c, fasting C-peptide, and DIR for Data-Driven classification were determined through Receiver Operating Characteristic (ROC) curve analysis. The optimal cut-off points were selected to achieve a specificity of 80%. A specificity of 80% was chosen based on common practice in clinical classification studies, where it is widely used as a balanced threshold to ensure clinical reliability while preserving model generalizability. In the context of graft function monitoring, this level of specificity allows for confident identification of impaired metabolic profiles without excessively compromising sensitivity. Agreement between different

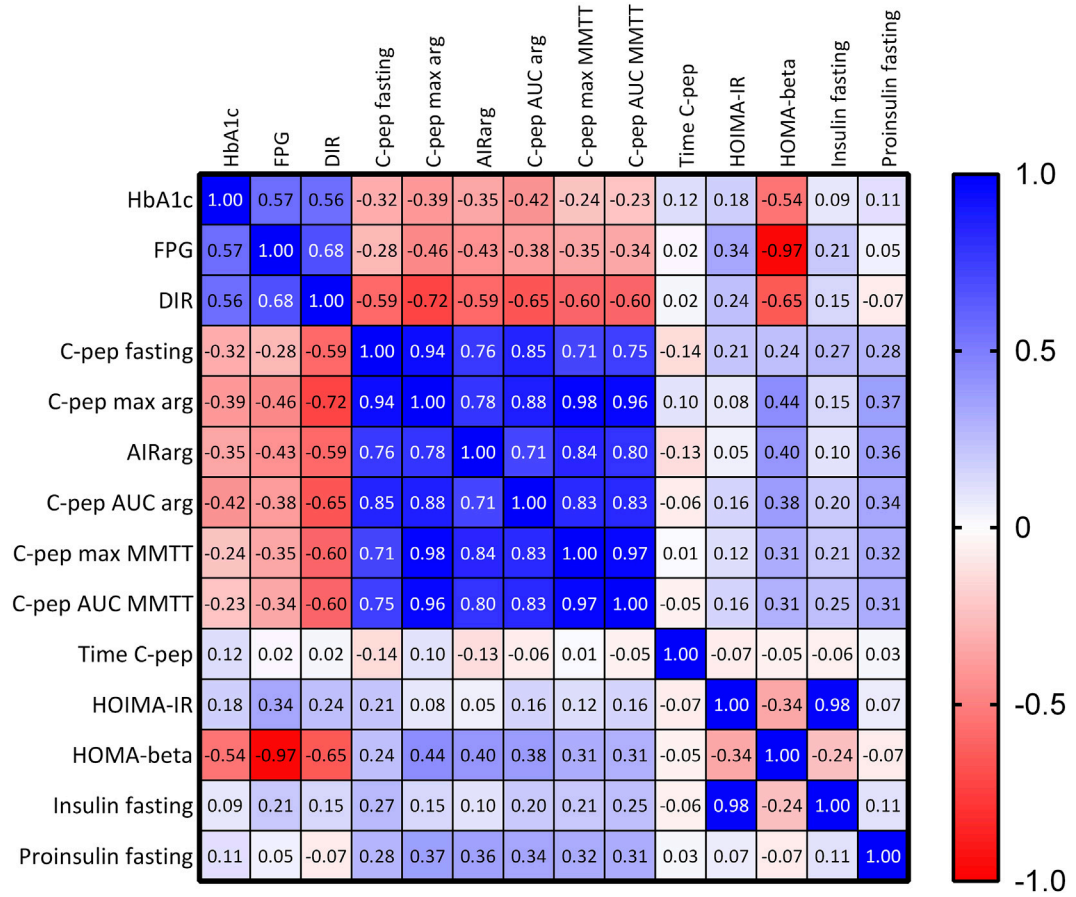


FIGURE 1 | Correlation of Clinical Outcomes in Islet Autotransplantation Patients. The correlation matrix displaying the Spearman R values represented as a heatmap. The color gradient reflects the strength and direction of correlations, with dark blue indicating a strong positive correlation, dark red indicating a strong negative correlation, and lighter shades representing weaker correlations. The numerical values in each cell represent the Spearman correlation coefficient (R), with values close to +1 indicating a strong positive correlation, −1 indicating a strong negative correlation, and 0 indicating no correlation.

classification systems was assessed using Fleiss' Kappa for multiple raters or Cohen's Kappa for pairwise comparisons. Variability was evaluated using the median coefficient of variation (CVM), calculated as the ratio of the median absolute deviation (MAD) to the median, expressed as a percentage. Differences in dispersion were analyzed using the Brown-Forsythe test. To compare metabolic and secretion parameters across classification groups, the Kruskal-Wallis test was used, followed by Dunn's multiple comparison test for *post hoc* analysis.

RESULT

Study Population

The analysis was conducted on data from 88 patients, with a mean age of 59.4 ± 13.8 years, including 40 females. The cohort had a mean BMI of 25.2 ± 4.1 and a mean eGFR of 95.8 ± 30.2. Patients received a median of 1,561 (1,076–2,162) IEQ/kg, with a pre-pancreatectomy fasting C-peptide level of 2.8 ± 1.99 ng/mL and an HbA1c of 5.4% ± 0.58%. Among the patients, 59 had malignant condition, and 72 underwent total or subtotal pancreatectomy. A total of 356 observation points were collected during the follow-up period. Of these, 189 (53%) were gathered within the first-year post-transplant, 117 (33%) between the first and fifth years, and 50 (14%) after 5 years. Among the observation points, 169 (47%) included a MMTT, and 204 (58%) involved an arginine test. Fasting plasma glucose data were available for 342 (96%) of the time points, fasting insulin for 355 (100%), fasting proinsulin for 234 (66%). HbA1c, insulin requirements, and fasting C-peptide levels were available for all patients (100%) as per protocol. A correlation matrix for metabolic outcomes is shown in **Figure 1**, demonstrating the expected correlation between secretion parameters (resting and stimulated C-peptide) and metabolic outcomes (DIR, FPB, and HbA1c).

Concordance Between Classification Systems

A comparative analysis was conducted to evaluate the concordance among six classification systems (Igls, Chicago,

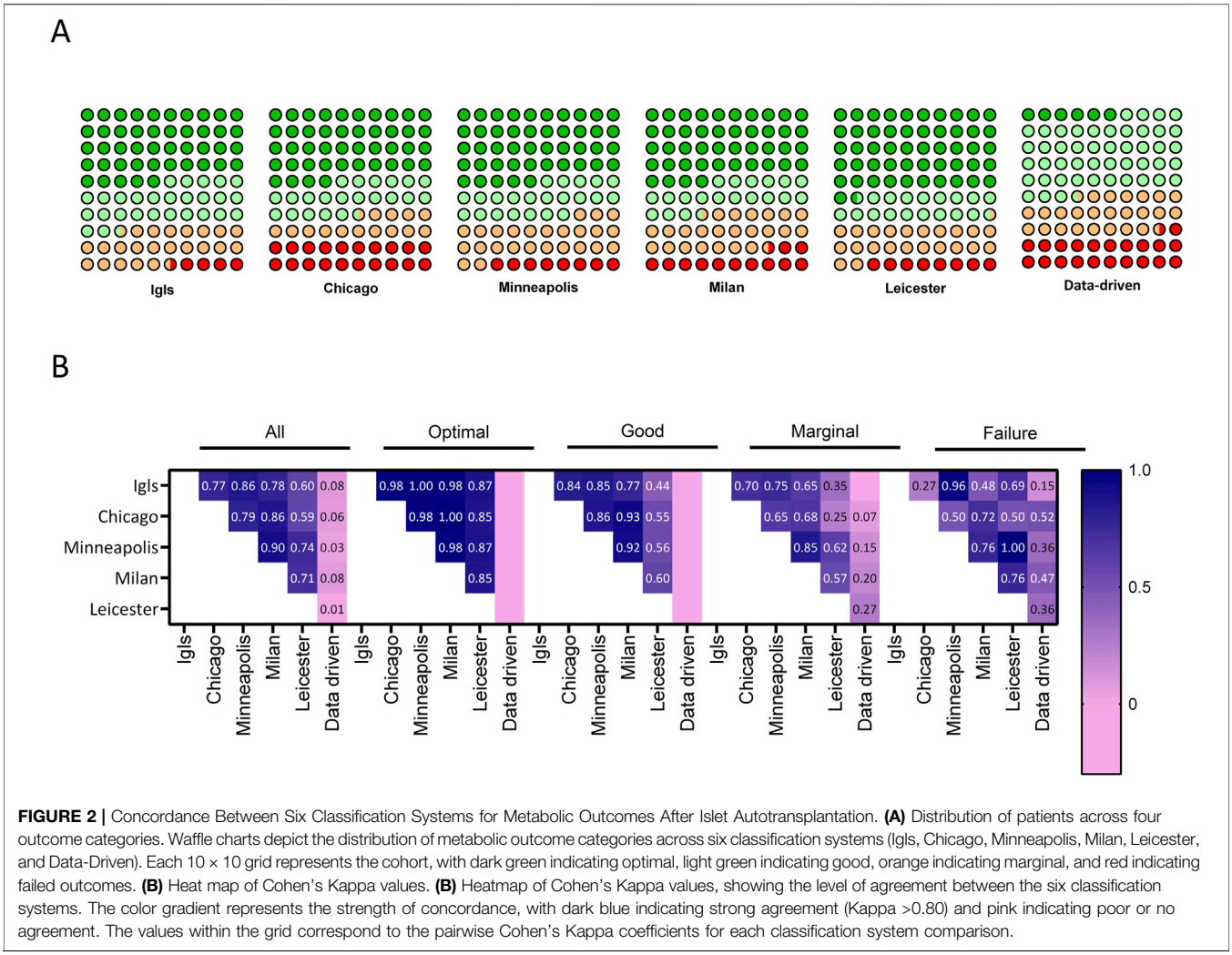
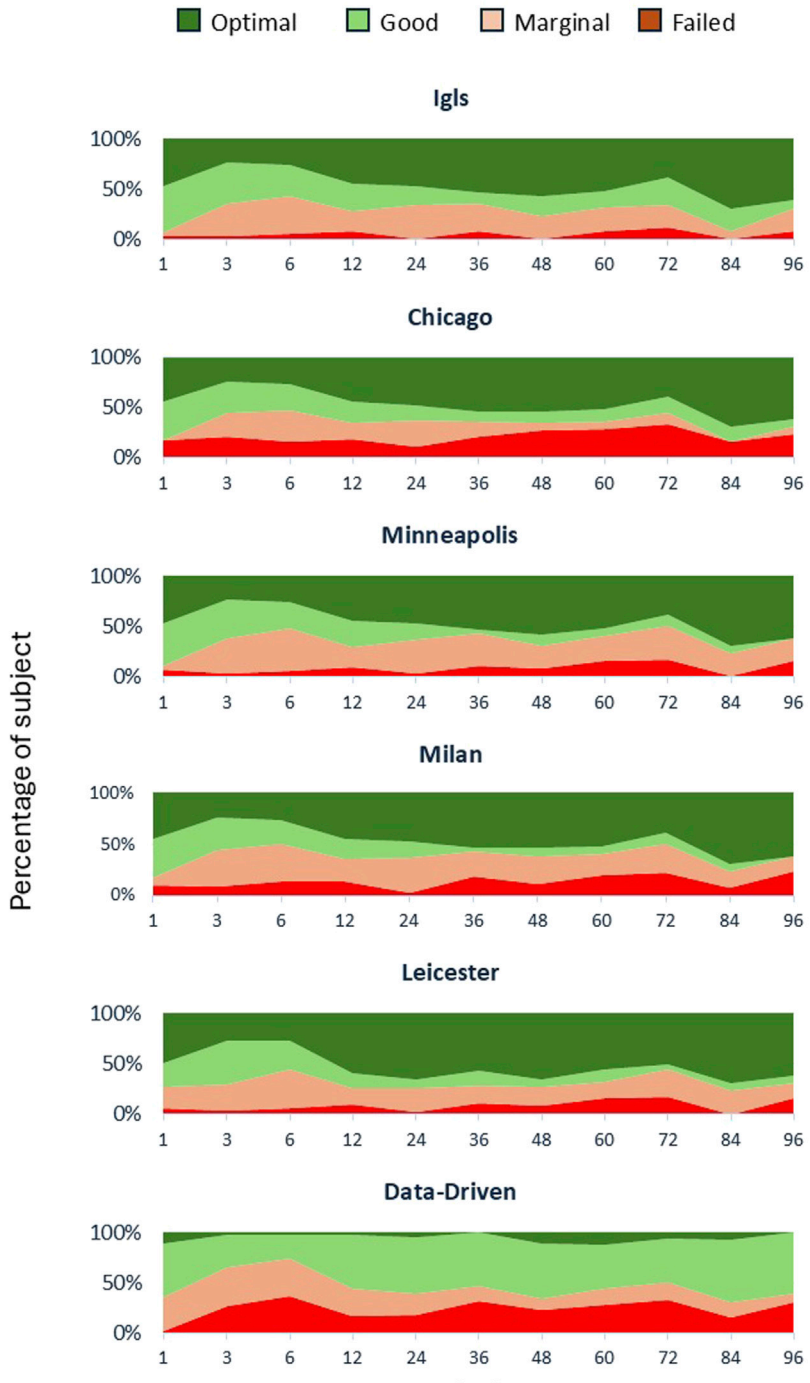


FIGURE 2 | Concordance Between Six Classification Systems for Metabolic Outcomes After Islet Autotransplantation. **(A)** Distribution of patients across four outcome categories. Waffle charts depict the distribution of metabolic outcome categories across six classification systems (Igls, Chicago, Minneapolis, Milan, Leicester, and Data-Driven). Each 10 × 10 grid represents the cohort, with dark green indicating optimal, light green indicating good, orange indicating marginal, and red indicating failed outcomes. **(B)** Heat map of Cohen's Kappa values. **(B)** Heatmap of Cohen's Kappa values, showing the level of agreement between the six classification systems. The color gradient represents the strength of concordance, with dark blue indicating strong agreement (Kappa >0.80) and pink indicating poor or no agreement. The values within the grid correspond to the pairwise Cohen's Kappa coefficients for each classification system comparison.

Minneapolis, Milan, Leicester, and a Data-Driven approach) in patients undergoing autologous islet transplantation. A visual summary in the form of a comparative schematic that illustrates the key components and thresholds used in each classification system is reported in the **Supplementary Table S2**. The distribution of patients across the four outcome categories (optimal, good, marginal, and failed) for each classification system is illustrated in **Figure 2A**. Overall, Fleiss' Kappa revealed moderate overall agreement among the systems ($K = 0.51$, $p < 0.001$). When categorized by outcome, the highest concordance was observed in the optimal ($K = 0.68$, $p < 0.001$) and failed groups ($K = 0.53$, $p < 0.001$), while lower agreement was found in the marginal ($K = 0.45$, $p < 0.001$) and good categories ($K = 0.36$, $p < 0.001$), indicating greater variability in classifying intermediate outcomes. This finding was further validated by the analysis of beta cell function over the 8-year follow-up period (**Figure 3**), which considered each time point and demonstrated that performance remained consistent over time, eliminating the possibility of time-related bias. To evaluate and compare the overall performance of different classification methods, a heat map of Cohen's Kappa values was generated (**Figure 2B**), providing a clear visualization of agreement patterns and key trends. Cohen's Kappa values demonstrated strong agreement among Igls, Chicago, Minneapolis, and Milan classification, with Leicester showing slightly lower concordance. In contrast, the Data-Driven approach exhibited poor agreement with all conventional classifications, underscoring fundamental differences in classification criteria.

Evaluation of the Consistency and Differentiation Capacity of the Classification Systems Based on Glucose Control Parameters

To evaluate the consistency of classification systems in identifying actual outcomes, we performed two types of analyses. The first analysis aimed to assess the ability of each classification system to differentiate categories based on glycemic control parameters, including HbA1c, DIR, and fasting glucose. The second analysis focused on determining whether the absolute values of these



Fleiss' Kappa

N	63	34	38	54	38	28	26	25	18	13	13
All	0.52	0.51	0.48	0.52	0.52	0.46	0.42	0.53	0.51	0.36	0.45
Optimal	0.72	0.76	0.77	0.60	0.59	0.61	0.62	0.71	0.68	0.58	0.59
Good	0.47	0.47	0.39	0.37	0.36	0.02	0.14	0.20	0.26	0.08	-0.12
Marginal	0.16	0.44	0.42	0.52	0.62	0.48	0.35	0.40	0.41	0.34	0.41
Failed	0.51	0.33	0.35	0.62	0.33	0.60	0.43	0.67	0.61	0.27	0.69

FIGURE 3 | Consistence of Outcome Classification Over Time. The β-cell graft function of 88 IAT cases was assessed and classified as "optimal," "good," "marginal," or "failure" based on the revised criteria (**Tables 1**, **2**). The analysis examined classification consistency at each time point over a 96-month follow-up period. The panels depict β-cell graft function outcomes over time, with Fleiss' Kappa values reported at the bottom for each time point, evaluated across all categories as well as separately for each category.

TABLE 3 | Evaluation of the consistency and differentiation capacity of the classification systems based on glucose control parameters.

	HbA1c		FPG		DIR	
	%	CVM (%)	mg/dL	CVM (%)	U/kg/day	CVM (%)
Igls						
Optimal	5.8 (5.3–6)	6.9	100 (91–112)	10.3	0 (0–0)	0
Good	6.4 (5.9–6.7)	4.7	136 (115–169)	17.8	0.18 (0.11–0.32)	83.3
Marginal	7.5 (7.2–8.5)	7.3	156 (126–220)	22.6	0.43 (0.23–0.67)	40.7
Failed	6.6 (6.2–7.7)	9.0	175 (117–274)	31.5	0.44 (0.35–0.65)	23.9
p	<0.0001[a]	<0.0001[b]	<0.0001[a]	<0.0001[b]	<0.0001[a]	<0.0001[b]
Chicago						
Optimal	5.8 (5.3–6.1)	6.9	101 (91–112)	10	0 (0–0)	0
Good	6.4 (5.9–6.6)	4.7	131 (114–160)	16	0.17 (0.10–0.26)	64.7
Marginal	7.7 (7.3–8.8)	7.8	153 (133–222)	20.1	0.38 (0.20–0.59)	46.1
Failed	6.8 (6.3–7.8)	8.8	165 (122–218)	27.7	0.44 (0.35–0.65)	29.5
	<0.0001[a]	<0.0001[b]	<0.0001[a]	<0.0001[b]	<0.0001[a]	<0.0001[b]
Minneapolis						
Optimal	5.8 (5.3–6)	6.9	100 (91–112)	10.3	0 (0–0)	0
Good	6.4 (5.9–6.6)	4.7	131 (112–157)	16	0.18 (0.12–0.22)	60
Marginal	7.3 (7–8.1)	8.2	160 (131–221)	22.5	0.47 (0.38–0.72)	50.5
Failed	6.8 (6.4–7.9)	8.8	174 (120–226)	28.6	0.44 (0.35–0.65)	27.3
	<0.0001[a]	<0.0001[b]	<0.0001[a]	<0.0001[b]	<0.0001[a]	<0.0001[b]
Milan						
Optimal	5.8 (5.3–6.1)	6.9	101 (91–112)	10	0 (0–0)	0
Good	6.4 (5.8–6.6)	4.7	128 (112–152)	13.1	0.17 (0.1–0.26)	41.2
Marginal	7.3 (6.7–7.9)	8.2	159 (129–221)	23	0.38 (0.20–0.59)	47.4
Failed	6.9 (6.4–7.9)	8.7	165 (120–218)	30.3	0.44 (0.35–0.65)	29.5
	<0.0001[a]	<0.0001[b]	<0.0001[a]	<0.0001[b]	<0.0001[a]	<0.0001[b]
Leicester						
Optimal	5.8 (5.3–6.2)	6.9	102 (92–113)	10.5	0 (0–0)	0
Good	6.5 (5.9–7)	8.5	128 (112–154)	14.4	0.18 (0.12–0.22)	25.7
Marginal	6.9 (6.3–7.8)	10.8	169 (137–224)	23.4	0.47 (0.38–0.72)	24.7
Failed	6.8 (6.4–7.9)	8.8	174 (120–226)	28.6	0.44 (0.35–0.65)	27.3
	<0.0001[a]	<0.0001[b]	<0.0001[a]	<0.0001[b]	<0.0001[a]	<0.0001[b]
Data-Driven						
Optimal	5.7 (5.4–5.8)	1.8	102 (92–110)	8.1	0 (0–0)	0
Good	5.9 (5.3–6.2)	6.8	104 (93–117)	11.3	0 (0–0)	0
Marginal	6.5 (6.1–6.9)	6.2	141 (117–180)	20.1	0.28 (0.15–0.44)	50.9
Failed	7.5 (7–8.35)	9.3	165 (126–225)	27	0.43 (0.31–0.65)	37.9
	<0.0001[a]	<0.0001[b]	<0.0001[a]	<0.0001[b]	<0.0001[a]	<0.0001[b]
Among different classification						
	p[a]		p[a]		p[a]	
Optimal	0.0832		0.91		-	
Good	<0.0001[h]		<0.0001[h]		<0.0001[h]	
Marginal	<0.0001[c,e,h]		0.023[i]		<0.0001[c,e,f,l,m]	
Failed	0.0008[m]		0.97		0.85	

[a]*Kruskal-Wallis test.*

[b]*Brown-Forsythe test.*

Significant at Dunn's multiple comparisons test: [c]Igls vs. Leichester; [d]Chicago vs. Milan;[e] Chicago vs. Leichester;[f] Milan vs. Leicester; glgls vs. Milan;[h] Data-Driven vs. all others; [i] Leicester vs. Data drive; [l]Minneapolis vs. Data-Driven; [m]Leicester vs. Data-Driven; [n]Chicago vs. Data-Driven.

parameters varied significantly within the same category across different classification systems. The detailed findings are presented in **Table 3**; **Figure 4**. When considered collectively, all classification systems significantly differentiate metabolic parameters of glycemic control across the various outcome categories, although with substantial dispersion in values, which increases progressively from the "optimal" to the "failed" category across all classifications. However, *post hoc* analysis provided valuable insights. Most classification systems successfully differentiated between the "optimal" and "good" categories, except for the Data-Driven approach, and between these two and the "failed" category. In contrast, differentiating between the "marginal" and "failed" categories, and to some extent between the "good" and "marginal" categories based on glycemic control, proved challenging. The Data-Driven classification system, however, showed the highest accuracy in making these distinctions. The analysis of absolute values within the same functional category across different classification systems revealed that the values were not always directly comparable. In the "optimal" category, glycemic control parameters were consistently similar across all systems, while in the "Good" and "Marginal" categories, there was greater variability. Overall, the data-driven classification system exhibited the most deviation compared to the others.

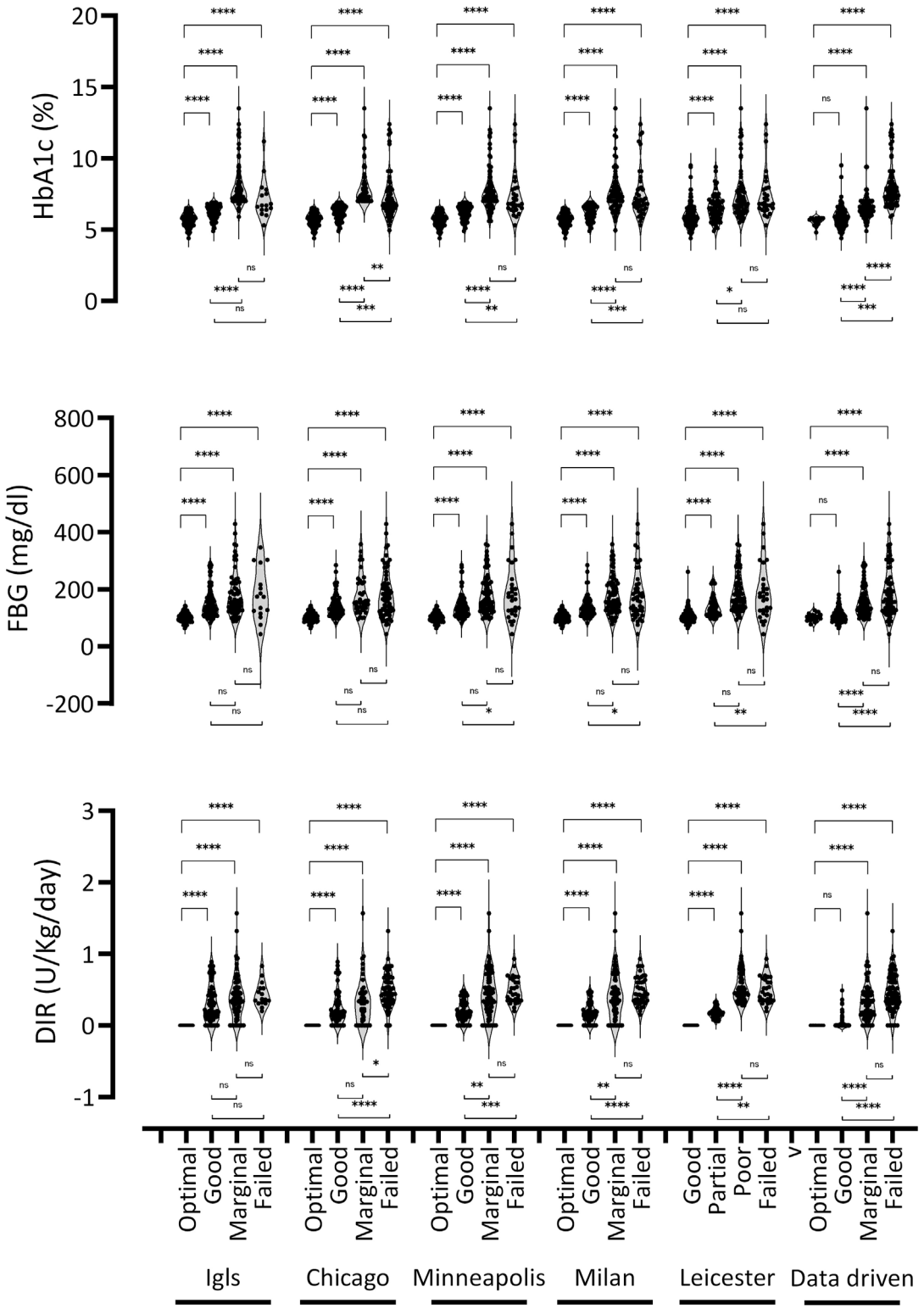


FIGURE 4 | Evaluation of the consistency and differentiation capacity of classification systems based on glucose control parameters. Violin plots depicting the distribution of HbA1c, fasting blood glucose (FBG), and Daily Insulin Requirement (DIR) across the four categories identified by each classification system. Each violin represents a distinct category, with individual data points shown as dots. The width of each violin corresponds to the data density at different values. Statistical differences between categories were assessed using the Kruskal-Wallis test, followed by *post hoc* Dunn's test. Asterisks indicate statistical significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

TABLE 4 | Evaluation of the consistency and differentiation capacity of the classification systems based on insulin secretion parameters.

	Fasting C peptide		Arginine peak C-peptide		MMTT peak C-peptide	
	ng/mL	CVM (%)	ng/mL	CVM (%)	ng/mL	CVM (%)
Igls						
Optimal	1.6 (1.2–2.1)	29	4.25 (2.9–5.2)	26	5.4 (4.1–7.7)	25
Good	1.3 (0.6–2)	54	1.8 (0.8–3)	57	4.2 (2.7–6.4)	41
Marginal	0.6 (0.3–0.9)	55	1.02 (0.6–1.6)	38	1.1 (0.3–3.1)	77
Failed	0 (0–0)	0	0 (0–0.1)	0	0.2 (0–0.3)	55
p	<0.0001[a]	<0.0001[b]	<0.0001[a]	<0.0001[b]	<0.0001[a]	0.0778[b]
Chicago						
Optimal	1.6 (1.2–2.1)	27	4.2 (2.9–5.2)	26	5.5 (4.2–7.8)	26
Good	1.6 (0.95–2.1)	40	2.6 (1.4–3.4)	39	4.3 (2.9–6.5)	40
Marginal	0.85 (0.6–1.3)	34	1.4 (1–2.4)	33	2.9 (1.4–5)	52
Failed	0.25 (0.1–0.35)	44	0.5 (0.3–0.7)	42	0.3 (0.2–0.7)	55
	<0.0001[a]	<0.0001[b]	<0.0001[a]	<0.0001[b]	<0.0001[a]	0.0038
Minneapolis						
Optimal	1.6 (0.2–2.1)	29	4.3 (2.9–5.2)	26	5.4 (4.1–7.7)	25
Good	1.3 (0.7–2.1)	48	2.3 (1.12–3.3)	47	4.5 (2.6–6.7)	41
Marginal	0.6 (0.4–1.1)	44	0.98 (0.7–1.4)	39	2.9 (0.8–4.3)	67
Failed	0.07 (0–0.2)	86	0.15 (0–0.5)	93	0.2 (0–0.3)	43
	<0.0001[a]	<0.0001[b]	<0.0001[a]	<0.0001[b]	<0.0001[a]	0.0019
Milan						
Optimal	1.6 (1.2–2.1)	27	4.2 (2.9–5.2)	26	5.4 (4.1–7.7)	25
Good	1.6 (0.95–2.2)	41	2.6 (1.5–3.4)	35	4.5 (2.6 6.7)	41
Marginal	0.6 (0.4–1.2)	39	1.1 (0.8–1.6)	32	2.9 (0.8–4.3)	67
Failed	0.17 (0.03–0.2)	53	0.34 (0.1–0.5)	49	0.2 (0–0.3)	43
	<0.0001[a]	<0.0001[b]	<0.0001[a]	<0.0001[b]	<0.0001[a]	0.0019
Leicester						
Optimal	1.6 (1.2–2.2)	29	4.21 (3.-5.1)	24	5.4 (4.2–7.6)	26
Good	1.3 (0.7–1.8)	44	2.44 (1.1–3.4)	47	2.7 (1.2–3.7)	48
Marginal	0.5 (0.3–0.9)	42	0.9 (0.6–1.4)	41	2 (0.7–4)	72
Failed	0.1 (0–0.2)	86	0.1 (0–0.5)	93	0.2 (0–0.3)	43
	<0.0001[a]	<0.0001[b]	<0.0001[a]	<0.0001[b]	<0.0001[a]	0.033
Data-Driven						
Optimal	2.2 (1.9–2.4)	11	4.9 (4.3–5.6)	13	6.3 (5.9–7.7)	14
Good	1.6 (1.2–2.2)	31	3.8 (2.7–5.1)	28	5.4 (4.1–7.5)	27
Marginal	0.9 (0.5–1.4)	48	1.2 (0.8–2.5)	58	3.3 (1.8–4.8)	44
Failed	0.3 (0.2–0.6)	60	0.8 (1.3–0.4)	49	0.3 (0.2–0.8)	69
	<0.0001[a]	<0.0001[b]	<0.0001[a]	0.0069[b]	<0.0001[a]	0.0008[b]
Among different classification						
	p[a]		p[a]		p[a]	
Optimal	0.0030[h]		0.7346		0.6832	
Good	0.007[d,i]		<0.0001[h]		0.0007[i]	
Marginal	<0.0001[e,i,m,n,o]		0.0023 [f,o]		0.0993	
Failed	<0.0001[e,g,i,l,m,n,,o,p]		<0.0001[n,h]		0.0687	

[a]*Kruskal-Wallis test.*

[b]*Brown-Forsythe test.*

Significant at Dunn's multiple comparisons test:[c] *Igls vs. Leichester;*[d] *Chicago vs. Milan;*[e] *Chicago vs. Leichester;*[f] *Milan vs. Leicester;* [g]*Igls vs. Milan;*[h] *Data-Driven vs. all others;*[i] *Leicester vs. Data drive;* [l]*Minneapolis vs. Data-Driven;*[m]*Igls vs. Data-Driven;* [n] *Igls vs. Chicago;* [o] *Chicago vs. Minneapoli;* [p] *Milan vs. Data-Driven.*

Evaluation of the Consistency and Differentiation Capacity of the Classification Systems Based on Insulin Secretion Parameters

Fasting and peak C-peptide levels after stimulation were assessed, with detailed findings presented in **Table 4**; **Figure 5**. Like glucose control parameters, all classification systems, when considered collectively, significantly differentiated parameters of insulin secretion across the various outcome categories, albeit with substantial variability. However, *post hoc* analysis revealed key differences: unlike glucose control, insulin secretion parameters struggled to distinguish between the "optimal" and "good" categories but effectively differentiated between "good," "marginal," and "failed" outcomes. This pattern was partially confirmed by the evaluation of more complex insulin secretion parameters, such as Acute Insulin Response to arginine (AIRarg) and the 2-h C-peptide AUC, although these were less effective than peak C-peptide in differentiating between categories (**Supplementary Figure S3**; **Supplementary Table S3**). Additionally, the response to arginine stimulation generally correlated better with classification categories than the response to MMTT. As observed with glucose control parameters, absolute insulin secretion values within the same

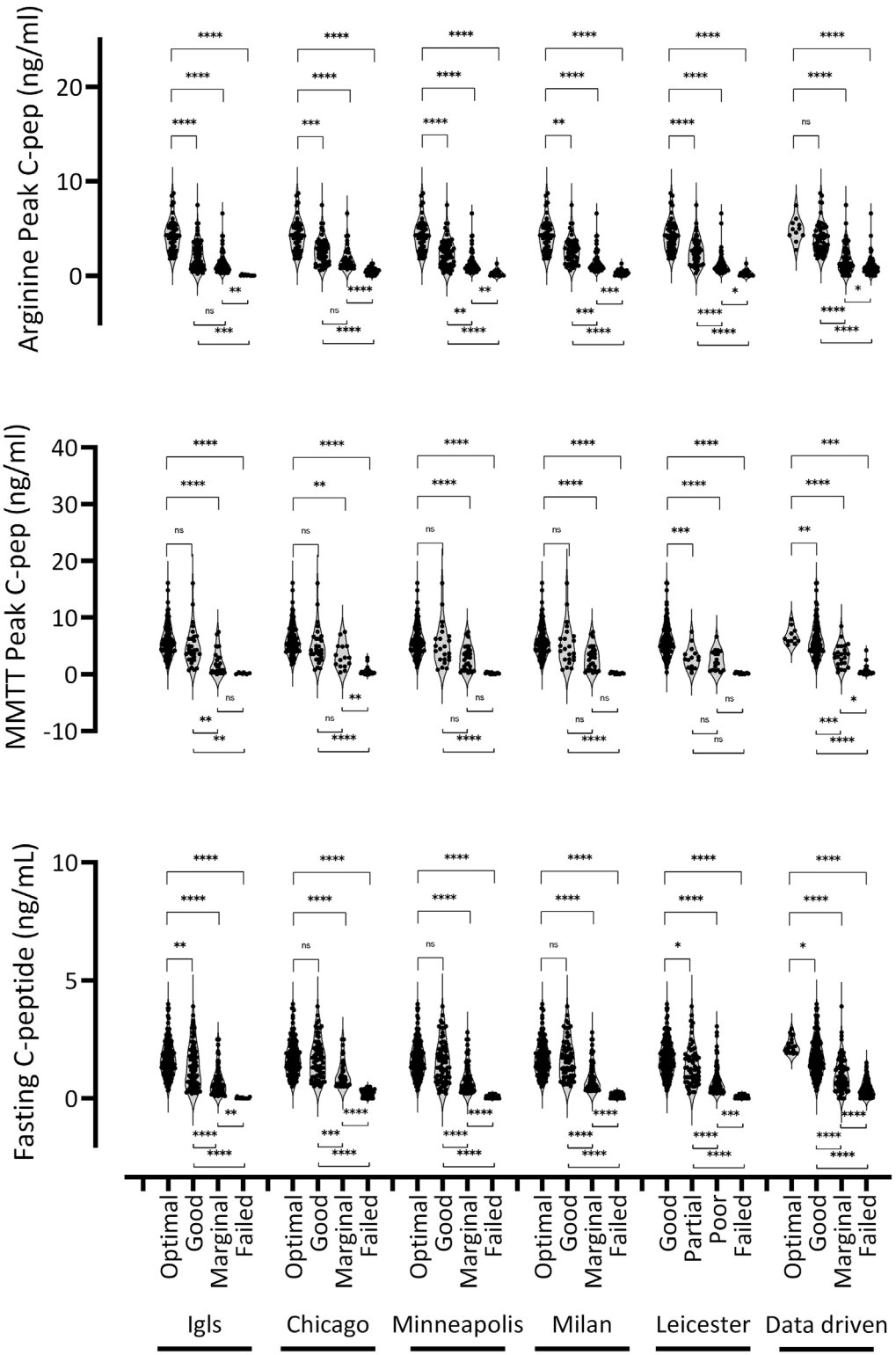


FIGURE 5 | Evaluation of the consistency and differentiation capacity of classification systems based on C peptide secretion parameters. Violin plots illustrate the distribution of fasting C peptide, peak C peptide during an arginine test, and peak C peptide during an MMTT test across the four categories identified by each classification system. Each violin represents a distinct category, with individual data points shown as dots. The width of each violin reflects the data density at various values. Statistical differences between categories were determined using the Kruskal-Wallis test, followed by *post hoc* Dunn's test. Asterisks denote statistical significance (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

functional category varied across classification systems, making direct comparisons difficult. In the "optimal" category, insulin secretion parameters were relatively consistent across all classification methods, whereas greater variability was observed in the "good" and "marginal" categories. Notably, the data-driven classification system exhibited the greatest deviation from the others. Further analyses were conducted to assess insulin resistance and β-cell function, including Insulin HOMA-IR, C-peptide HOMA2-%B, fasting insulin, and proinsulin levels (**Supplementary Figure S4**; **Supplementary Table S4**). Among these, only C-peptide HOMA2-%B followed the trend of direct insulin secretion parameters, while the others showed less distinct stratification across the "optimal" to "failed" categories. Notably, insulin resistance, as measured by HOMA-IR, was consistently higher in the "good" category compared to the "optimal" category, while no significant differences were observed among the other groups.

DISCUSSION

The comparative evaluation of classification systems for IAT presented in this study underscores the value of multiple existing frameworks while also highlighting key differences that may influence their practical utility. The choice of one classification system over another appears to be dictated less by its intrinsic ability to differentiate metabolic outcomes and more by considerations of feasibility, simplicity, and the number of parameters required for implementation [18]. This aspect is particularly relevant in the clinical setting, where the complexity of obtaining certain metabolic parameters can impact the widespread applicability of a given classification method [10].

One of the most notable findings of this study is the advantage conferred by classification systems that exclude severe hypoglycemic events (SHE) as a criterion, such as the Data-Driven approach and the Leicester system. SHE remains one of the most challenging variables to standardize, as its assessment relies heavily on patient-reported data, which can be prone to subjectivity and recall bias. Nonetheless, the clinical relevance of preventing SHEs should not be overlooked, particularly in insulin-treated patients following total pancreatectomy. Importantly, the impact of SHEs on long-term outcomes has been significantly reduced in recent years with the introduction of advanced diabetes technologies, including CGM, insulin pumps, and hybrid closed-loop systems. These tools have markedly improved hypoglycemia detection and prevention, which may partly justify the omission of SHEs from simplified classification systems in selected clinical contexts. The Leicester system further reduces complexity by not requiring glycated hemoglobin as a mandatory parameter, thereby increasing its practicality in real-world applications. These considerations suggest that classification systems prioritizing feasibility and ease of calculation may be more suitable for routine clinical use, particularly in settings with limited resources or less frequent metabolic monitoring.

From a conceptual standpoint, the strong correlation observed among the Milan, Minnesota, Chicago, and Igls classifications is not surprising, given that they primarily differ in their thresholds for fasting and stimulated C-peptide levels. This convergence reinforces the robustness of C-peptide as a central biomarker in graft function assessment [19]. However, an interesting observation emerged when comparing the "good" and "optimal" outcome categories across all classifications: while these groups exhibited no significant differences in insulin secretion, they did show distinct variations in glucose control. This suggests that factors beyond insulin production—such as insulin sensitivity—may play a critical role in differentiating these groups. The finding that HOMA-IR was significantly higher in the "good" group than in the "optimal" group supports the hypothesis that differences in insulin resistance, rather than secretion capacity, may contribute to variations in glycemic control. This insight is particularly relevant in the broader context of beta-cell replacement and diabetes management, where therapeutic strategies often focus on preserving or enhancing residual insulin secretion without always accounting for the impact of insulin sensitivity on metabolic outcomes.

Equally significant is the differentiation between the "marginal" and "failed" categories. Unlike the distinction between "good" and "optimal," which appears to be driven by insulin resistance, the primary factor separating "marginal" from "failed" function is the presence of residual insulin secretion. This observation aligns with existing literature on beta-cell replacement therapies, where even minimal levels of residual C-peptide secretion have been associated with protection against severe hypoglycemia and reduced progression of microvascular complications. Fasting C-peptide values ranging from 0.09 to 0.2 ng/mL have been reported as sufficient for these protective effects, reinforcing the clinical significance of residual beta-cell function. This finding has broader implications beyond IAT, extending to the field of type 1 diabetes treatment, where preservation of C-peptide at disease onset is increasingly recognized as a therapeutic goal [20–24]. In the context of IAT, where classification serves primarily as a descriptive tool rather than a determinant of therapeutic interventions, understanding the long-term impact of residual insulin secretion on patient health may provide valuable insights into post-transplant metabolic outcomes.

Another critical consideration is the role of fasting *versus* stimulated C-peptide in classification. Our findings suggest that fasting C-peptide alone is highly informative and may be sufficient for functional assessment in many cases, reducing the necessity for more complex stimulation tests. However, when stimulation is required, the Arginine test appears to provide better differentiation than the MMTT. This is a key observation, as the arginine test is generally easier to standardize and less time-consuming than a full MMTT, making it a more practical choice for post-transplant metabolic evaluations.

A particularly intriguing outcome of this study is the performance of the Data-Driven classification system, which avoids predefined threshold values by leveraging natural clustering of metabolic parameters. This methodology offers a flexible and adaptive framework that may better capture the heterogeneity of post-transplant metabolic function. While the Data-Driven system was more restrictive in defining "optimal"

outcomes compared to conventional classifications, it demonstrated superior granularity in distinguishing between different levels of graft function. This suggests that data-driven approaches could serve as powerful tools for refining outcome assessments in IAT. However, further validation in larger and more diverse cohorts is necessary before widespread adoption can be considered.

Despite its strengths, this study has several limitations that should be acknowledged. First, the analysis was conducted in a single-center cohort, which may limit generalizability to other institutions with different patient populations, surgical techniques, or follow-up protocols. Additionally, while the study incorporated many metabolic parameters, it did not evaluate long-term clinical outcomes such as quality of life, diabetes-related complications, or the durability of graft function beyond 8 years. Future studies should aim to address these gaps by integrating patient-reported outcomes and long-term metabolic trajectories. Finally, while the Data-Driven classification demonstrated promising results, its reliance on retrospective data raises questions about its applicability in prospective clinical settings. Further research is needed to determine whether this approach can be successfully implemented in real-time decision-making.

This study provides a comprehensive evaluation of existing classification systems for IAT and introduces a novel Data-Driven approach that may offer advantages in terms of adaptability and differentiation. The findings highlight the strengths and limitations of different frameworks, emphasizing that the choice of a classification system should consider both scientific validity and practical feasibility. The insights gained from this analysis contribute to a broader understanding of beta-cell function assessment and may inform future refinements in transplantation and diabetes care. Ultimately, continued research and collaborative efforts will be essential to optimize graft function evaluation and improve long-term outcomes for patients undergoing IAT.

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 Comitato Etico Ospedale san Raffaele. 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

DC, RC, and LP contributed to conceptualization and study design. PM, RM, AM, and LV contributed to data collection. ST, CG, VP, and FD contributed to the methods. LP and FA had access to raw data. SS contributed to data curation. LP analyzed the data. LP, DC, and RC contributed to data interpretation. Funding was acquired by LP. LP wrote the original draft of the report. DC, FA, and RC reviewed and edited the report. DC and LP are responsible for final submission of the manuscript for publication and all authors approved the final version before submission. LP, DC, and RC accessed and verified the underlying study data. 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.

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.2025.14714/full#supplementary-material

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ORIGINAL RESEARCH
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The Impact of Post-Operative Phrenic Nerve Dysfunction on Lung Function Parameters and Long-Term Outcomes After Lung Transplantation

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A rare but important complication after lung transplantation (LTx) is postoperative phrenic nerve dysfunction (PND). Diaphragmatic plication (DP) is a well-established treatment option for PND, however, the long-term effect of PND and DP on lung function parameters and survival after LTx are currently unknown. We retrospectively reviewed 1400 LTx recipients transplanted at Medical University of Vienna between 01/2003 and 12/2022. Fluoroscopy and/or phrenic nerve conduction studies confirmed PND when chest radiographs after extubation showed a unilateral heightened diaphragm. We identified 25 patients with post-operative PND, of whom 12 underwent DP. The remaining 1,375 patients served as a control group. Median ICU-stay and hospital-stay were significantly longer in the PND groups (DP: 20 and 57 days; non-DP: 27 and 43 days; control group: 7 and 25 days; $P = 0.001$/$P < 0.001$). PND led to consistently lower %TLC in lung function tests performed within the first three years after LTx. DP was associated with lower %FEV1.0 early after LTx but it aligned to %FEV1.0 of the other groups during follow-up. Although PND significantly affected postoperative recovery after LTx, it did not impair long-term survival outcomes.

Keywords: lung transplantation, phrenic nerve dysfunction, lung function parameters, diaphragmatic plication, surgery

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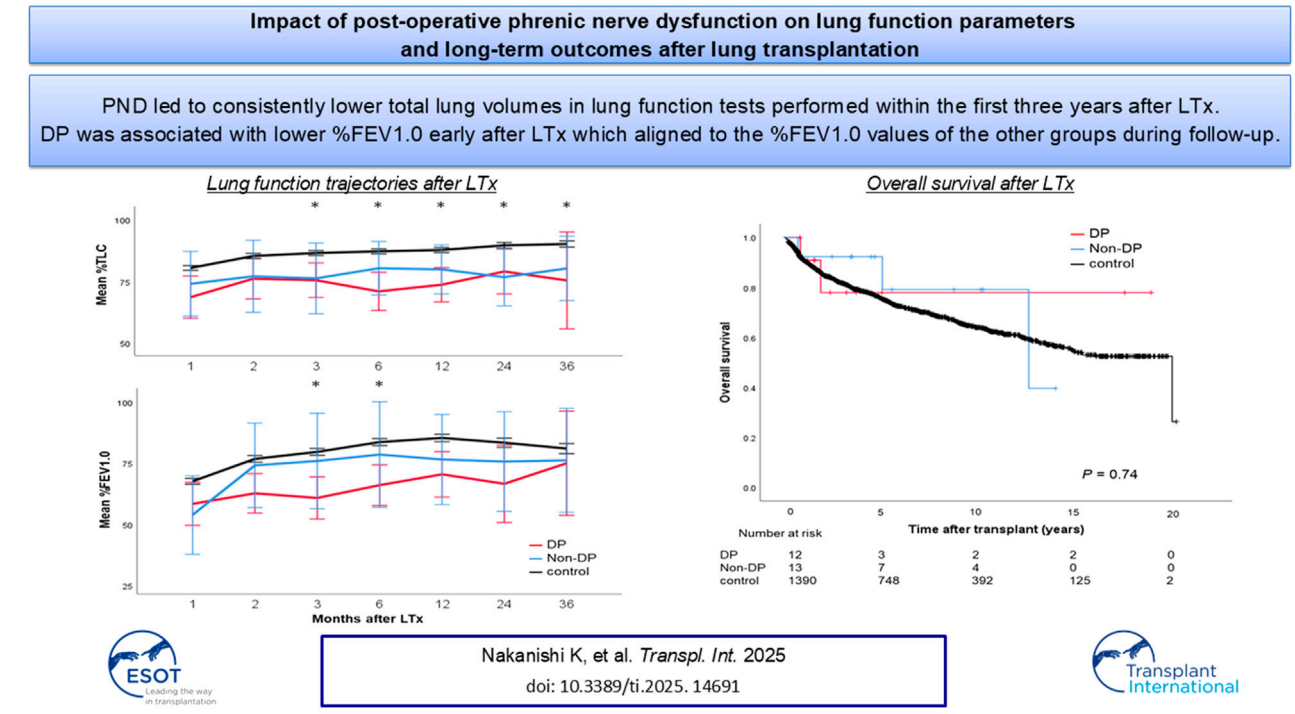
INTRODUCTION

Lung transplantation (LTx) has evolved to a well-established treatment option for patients with end-stage pulmonary disease [1, 2]. A rare but important complication of LTx is postoperative phrenic nerve dysfunction (PND). The incidence of PND ranges from 3% to 9% after LTx, although it is more common in combined heart-lung transplant [3–5]. Mechanical injury of the phrenic nerve can be caused during pericardial manipulation, sternum retraction, and/or mediastinal dissection. Severe PND after LTx is associated with persistent lobar atelectasis, failure to clear airway secretions, and recurrent infections, potentially causing allograft dysfunction. PND has previously been shown to prolong post-operative intensive care unit- (ICU) and hospital-stay [6].

Diaphragmatic plication (DP) was first described in the 1980s as a surgical option for PND [7, 8]. The procedure is considered safe and effective in preventing atelectasis and improving symptoms in



GRAPHICAL ABSTRACT

patients with PND. Recently, Lawrence et al. suggested two indications for DP in LTx recipients: DP for functional indications (symptomatic diaphragmatic dysfunction) and to overcome severe graft oversizing [9]. Other experiences of postoperative DP in patients receiving LTx are limited to case reports, mostly with favorable short-term results [10, 11]. However, the long-term effect of DP on lung function parameters and survival is still unknown.

Therefore, this study aimed to evaluate perioperative and long-term outcomes of LTx recipients with PND who received DP, LTx recipients with PND who did not receive DP, and a control group of LTx recipients with normal postoperative diaphragmatic function.

MATERIALS AND METHODS

Study Population

Overall, 1,841 patients underwent LTx at the Medical University of Vienna between January 2003 and December 2022. As illustrated in **Figure 1**, the following patients were excluded: (i) patients who died within 30 days (n = 58), (ii) patients whose data according to lung function follow-up was incomplete due to transition to and follow-up in a local hospital close to the patients' home (n = 318), (iii) patients who underwent combined transplantation (heart-lung, n = 11 or liver-lung, n = 3), and (iv) patients aged <15-year (n = 51). Size-reduction, single-lung transplantation, and lobar transplantation were not considered exclusion criteria. Eventually, 1,400 patients were included in the present study. Patients were divided into three groups: (i) patients who were diagnosed with PND after LTx and underwent DP (DP group), (ii) patients who were diagnosed with PND after LTx but did not undergo DP (non-DP group), and (iii) patients who did not develop PND after LTx (control group). PND was tested by fluoroscopy and/or phrenic nerve conduction studies (PNCS) whenever chest radiographs after extubation showed a unilateral heightened diaphragm. Phrenic nerve conduction studies were performed in accordance with the manufacturer's instructions (Dantec Keypoint, Natus, Middleton, USA). Surface electrical stimulation was applied in the supraclavicular region, targeting the cervical portion of the phrenic nerve. Compound muscle action potentials were recorded at the costal margin along the anterior axillary line, typically between the 7th and 8th intercostal spaces, with the patient in a supine position and breathing spontaneously. As the procedure is non-invasive, we are not aware of any associated risks. Nerve conduction studies were conducted at the discretion of the treating physician. Regarding timing, nerve conduction testing is performed after weaning from the respirator, irrespective of the supplementary oxygen requirement. The indications for DP were (i) inability to wean from respirator or (ii) significant lower lobe atelectasis in computed tomography (CT) scans. The present study was approved by the ethics board on human research from the Medical University of Vienna (approval No. EK 1639/2023). Patient written consent for the publication of the study data was waived by the institutional ethics board due to the retrospective nature of the study.

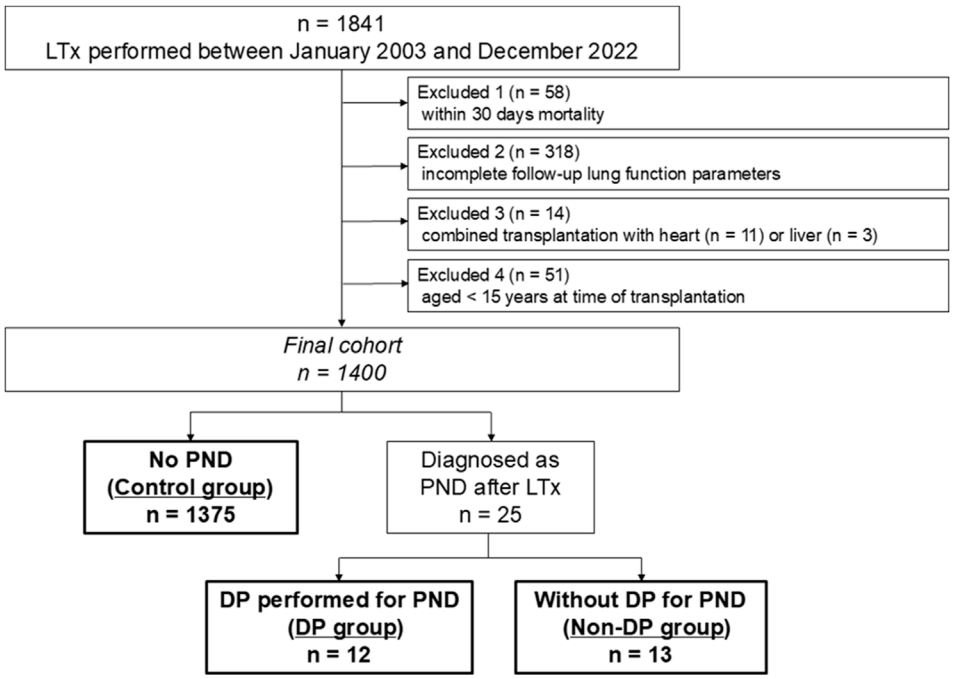


FIGURE 1 | Patient selection. LTx, lung transplantation; PND, phrenic nerve dysfunction; DP, diaphragmatic plication.

TABLE 1 | Donor characteristic.

		DP group (n = 12)	Non-DP group (n = 13)	Control group (n = 1,375)	*P* value
Age, years	Median (IQR)	49 (33–60)	41 (31–51)	44 (31–53)	0.433
Sex female: male ratio	n (%)	7:5 (58:42)	7:6 (54:46)	681:694 (50:50)	0.781
Height, cm	Median (IQR)	170 (168–180)	170 (165–178)	170 (165–180)	0.554
Blood group	n (%)				0.480
O		2 (17)	5 (38)	552 (40)	
A		8 (66)	5 (38)	578 (42)	
B		2 (17)	3 (24)	179 (13)	
AB		0	0	66 (5)	
Donation type	n (%)				0.466
DBD		11 (92)	13 (100)	1,317 (96)	
DCD		1 (8)	0	58 (4)	
Cause of death	n (%)				0.367
Cerebrovascular/Stroke		8 (66)	8 (61)	851 (62)	
Anoxia/Cardiac arrest		2 (17)	0	42 (3)	
Trauma		2 (17)	3 (23)	345 (25)	
Suicide		0	1 (8)	41 (3)	
Others		0	1 (8)	96 (7)	
Smoking history	n (%)				0.156
Yes		5 (42)	6 (46)	649 (47)	
No		6 (50)	7 (54)	429 (31)	
Unknown		1 (8)	0	297 (22)	
Total intubation days	Median (IQR)	3 (3–11)	3 (2–5)	3 (1–5)	0.101
Last PaO2 at 1.0 FiO2, mmHg	Median (IQR)	400.6 (351.2–480.5)	438.0 (362.0–520.1)	439.0 (373.5–506.0)	0.539
Last PaCO2 at 1.0 FiO2, mmHg	Median (IQR)	39.4 (35.9–43.0)	39.8 (34.9–41.4)	39.0 (35.0–42.9)	0.933

DP, diaphragmatic plication; IQR, interquartile range; DBD, donor after brain death; DCD, donor after circulatory death.

Data Collection

A review of medical charts, including preoperative examinations as well as intraoperative and postoperative data, from the hospital documentation system and the institutional transplant database was conducted. The outcome parameters analyzed were re-intubation and tracheostomy rates, ICU- and hospital-stay, % forced expiratory volume in one second (%FEV1.0) and % total lung capacity (%TLC) during follow-up lung function tests

TABLE 2 | Recipient characteristics.

		DP group (n = 12)	Non-DP group (n = 13)	Control group (n = 1,375)	*P* value
Age, years	Median (IQR)	59 (56–66)	48 (31–60)	54 (39–60)	0.039
Female: male ratio	n (%)	3:9 (25:75)	6:7 (44:56)	602:773 (44:56)	0.488
Height, cm	Median (IQR)	175 (165–178)	168 (159–179)	170 (163–176)	0.409
Diagnosis	n (%)				0.674
COPD		7 (59)	5 (39)	548 (40)	
Fibrosis		3 (25)	2 (15)	325 (24)	
Cystic fibrosis		0	3 (23)	228 (16)	
PAH		1 (8)	1 (8)	70 (5)	
Others		1 (8)	2 (15)	204 (15)	
ECLS bridge-to-LTx	n (%)	2 (17)	1 (8)	98 (7)	0.261
LAS	Median (IQR)	37.1 (32.7–61.3)	38.6 (32.7–42.4)	35.4 (32.3–43.4)	0.728
Transplant era	n (%)				0.040
		2 (17)	2 (15)	399 (29)	
2003–2009					
		1 (8)	5 (39)	514 (37)	
2010–2016					
		9 (75)	6 (46)	462 (34)	
2017–2022					
Type of LTx	n (%)				0.702
Double-lung		12 (100)	13 (100)	1,270 (92)	
Single-lung		0	0	105 (8)	

DP, diaphragmatic plication; IQR, interquartile range; COPD, chronic obstructive pulmonary disease; PAH, pulmonary arterial hypertension; ECLS, extracorporeal life support; LTx, lung transplantation; LAS, lung allocation score.

TABLE 3 | Transplant procedure.

		DP group (n = 12)	Non-DP group (n = 13)	Control group (n = 1,375)	*P* value
EVLP	n (%)	0	2 (15)	58 (4)	0.186
Approach	n (%)				0.109
Clamshell		7 (58)	12 (92)	935 (68)	
Thoracotomy		5 (42)	1 (8)	440 (32)	
Size reduction	n (%)				0.014
Whole lungs		4 (34)	7 (54)	765 (55)	
Extra-anatomical size reduction		7 (58)	5 (38)	477 (35)	
Lobar		0	1 (8)	133 (10)	
Others		1 (8)	0	0	
Type of intraoperative support	n (%)				0.552
No support		1 (8)	1 (8)	298 (22)	
Intraoperative ECMO		11 (92)	12 (92)	1,056 (77)	
CPB		0	0	21 (1)	
Total preservation time 2nd lung, min	Median (IQR)	410 (364–508)	455 (381–513)	386 (338–450)	0.071
Duration of surgery, min	Median (IQR)	338 (292–405)	300 (248–436)	295 (250–349)	0.100
Intraoperative transfusions					
RBC units	Median (IQR)	3 (2–5)	6 (3–8)	4 (2–6)	0.283
FFP concentrates	Median (IQR)	8 (5–15)	10 (6–12)	9 (5–12)	0.625
Induction	n (%)				0.250
Yes		6 (50)	8 (62)	960 (70)	
No		6 (50)	5 (38)	415 (30)	

DP, diaphragmatic plication; EVLP, ex vivo *lung perfusion; ECMO, extracorporeal membrane oxygenation; CPB, cardiac pulmonary bypass; IQR, interquartile range; RBC, red blood cell; FFP, frozen fresh plasma.*

performed 1, 2, 3, 6, 12, 24 and 36 months after LTx as well as overall and Chronic lung allograft dysfunction (CLAD)-free survival. Pulmonary function tests were performed by certified pulmonary function technicians. %FEV1.0 and %TLC were calculated as the ratio of actual to predicted values using European Respiratory Society formulas [12], which are as follows:

FEV1.0:

Male: 4.3*height (m) - 0.029*age - 2.49.

Female: 3.95*height (m) - 0.025*age - 2.6.

TLC:

Male: 7.99*height (m) - 7.08.

Female: 6.60*height (m) - 5.79.

Surgical Procedure

Organ procurements and transplant procedures were performed according to the standardized institutional protocol published elsewhere [13, 14]. Donor lungs were perfused with low-potassium dextran solution and stored on ice, as moderate hypothermic storage at 10° was only established after 2022 in our institution. *Ex vivo* lung perfusion (EVLP) was performed in selected cases. For bilateral lung transplantation, a clamshell incision or bilateral anterior thoracotomies in the fourth intercostal space were made. Single-lung transplantation was performed through an anterolateral thoracotomy. Slightly oversized grafts were tailored by extra anatomical downsizing of the middle lobar and/or lingular resection. The need for downsizing was ultimately decided by the implantation team before closing the chest. Basic immunosuppression consisted of a triple-drug regimen with cyclosporine (or tacrolimus), mycophenolate mofetil, and corticosteroids. Alemtuzumab or anti-thymocyte globulin was used for induction therapy in most recipients. DP was performed as previously published based on the common principle of lowering the entire diaphragmatic dome by suturing the redundant part from the posterior costophrenic angle to the cardio-phrenic angle [15] at a median of 27 days (IQR, 15–125) after LTx. The procedure was carried out via a separate lateral or posterolateral thoracotomy through the 6th intercostal space, distinct from the original transplant incision.

TABLE 4 | Outcome parameters.

		DP group (n = 12)	Non-DP group (n = 13)	Control group (n = 1,375)	*P* value
Localization of PND	n (%)				0.751
Right		6 (50)	6 (46)	-	
Left		4 (33)	6 (46)	-	
Bilateral		2 (17)	1 (8)	-	
Time from LTx to plication, days	Median (IQR)	27 (15–125)	-	-	
Tracheostomy	n (%)	4 (33)	7 (54)	268 (20)	0.005
Re-intubation	n (%)	4 (33)	8 (62)	228 (17)	<0.001
ICU-stay, days	Median (IQR)	20 (9–57)	27 (6–38)	7 (4–15)	0.001
Hospital-stay, days	Median (IQR)	57 (23–93)	43 (29–60)	25 (19–36)	<0.001
5-year overall survival	%	77.9	92.3	75.7	0.742
5-year CLAD-free survival	%	64.9	92.3	70.3	0.633

DP, diaphragmatic plication; ICU, intensive care unit; PND, phrenic nerve dysfunction; LTx, lung transplantation; IQR, interquartile range; CLAD, chronic lung allograft dysfunction.

Statistical Analysis

All statistical analyses of data were performed using the SPSS Statistics 25 software (IBM Corporation, Armonk, NY). Categorical variables were compared using the Fisher's exact test. For continuous variables, the Student's t-test was used. One-way analysis of variance (ANOVA) and the Kruskal-Wallis test were applied to compare the means or medians of more than two samples, respectively. For the comparison of pulmonary function parameters among the groups, one-way ANOVA was performed. Overall survival (OS) and CLAD-free survival were analyzed by the Kaplan-Meier method, and log-rank tests were used to compare survival. OS was defined as the time from surgery to death due to any cause. CLAD-free survival was defined as the time from surgery to either the first development of CLAD or death due to any cause. For all analyses, a *P*-value of <0.05 was considered statistically significant.

RESULTS

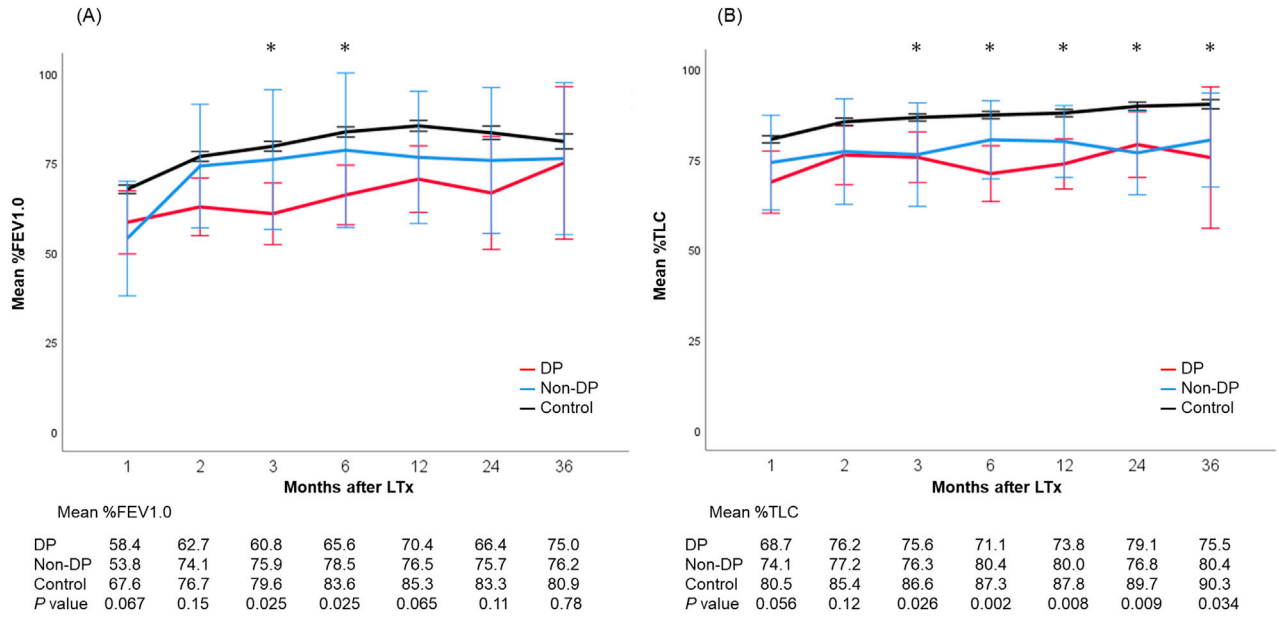
We identified 25 patients with PND (1.8%), of whom 12 underwent DP. The remaining 1,375 patients served as a control group. Donor characteristics are summarized in **Table 1**. There were no significant differences in terms of age, sex, height, blood type, cause of death, smoking history, and total intubation days observed between the three groups. Last PaO2 at 1.0 FiO2 was slightly worse in the DP group compared to the non-DP and control group (median, 400.6 mmHg [IQR, 351.2–480.5] vs. 438.0 mmHg [IQR, 360.0–520.1] vs. 439.0 mmHg [IQR, 373.5–506.0]), but the difference did not reach significant ($P = 0.54$).

Basic recipient demographic data and surgical characteristics of the three study groups are provided in **Tables 2**, **3**. There were no significant differences in sex, height, diagnosis, extracorporeal life support bridge-to-LTx, lung allocation score, and type of LTx. Patients of the non-DP group were significantly younger compared to patients of the DP and control group (median, 48 years [IQR, 31–60] vs. 59 years [IQR, 56–66] vs. 54 years [IQR, 39–60]; $P = 0.039$). Chronic obstructive pulmonary disease was the most common indication for LTx in all three groups (DP: 59%, non-DP: 39%, control: 40%). DP was performed more often in later years ($P = 0.04$). Single-lung transplantations were in general rarely performed and only found in the control group (8%). Two cases of lung retransplantation were included in the control group. Size reduction of the donor lungs was performed most frequently in the DP group compared to others (58% vs. 38% vs. 35%) ($P = 0.014$). Most patients underwent transplantation with the use of central venoarterial extracorporeal membrane oxygenation (VA-ECMO), with 92% in both the DP and non-DP groups, and 77% in the control group (**Table 3**). There were no significant differences in terms of total preservation time, duration of surgery, intraoperative transfusions, and induction therapy between the three groups.

Postoperative outcomes are presented in **Table 4**. There was no difference in laterality of PND between DP and non-DP patients ($P = 0.75$). Both, median ICU-stay as well as hospital-stay, were significantly longer in the PND groups (DP: 20 [IQR, 9–57] and 57 [IQR, 23–93] days; non-DP: 27 [IQR, 6–38] and 43 [IQR, 29–60] days; control group: 7 [IQR, 4–15] and 25 [IQR, 19–36] days; $P = 0.001/P < 0.001$). Re-intubation rate was the highest in the non-DP group compared to the DP and control group (62% vs. 33% vs. 17%, $P < 0.001$) as well as the tracheostomy rate (54% vs. 33% vs. 20%, $P = 0.005$). Trajectories of lung function parameters are shown in **Figure 2**. % FEV1.0 at three and six months after LTx was significantly worse in the DP group compared to the other groups. However, it gradually improved over time, and there was no significant difference any longer between the three groups 36 months after LTx. In contrast to this, the measured %TLC remained consistently lower in the PND groups. Of note, the % TLC was not different between patients of the DP and non-DP groups. The 5-year OS/CLAD-free survival rates were 77.9%/64.9% in the DP group, 92.3%/92.3% in the non-DP group, and 75.7%/70.3% in the control group ($P = 0.74/P = 0.63$) (**Figures 3A,B**).

DISCUSSION

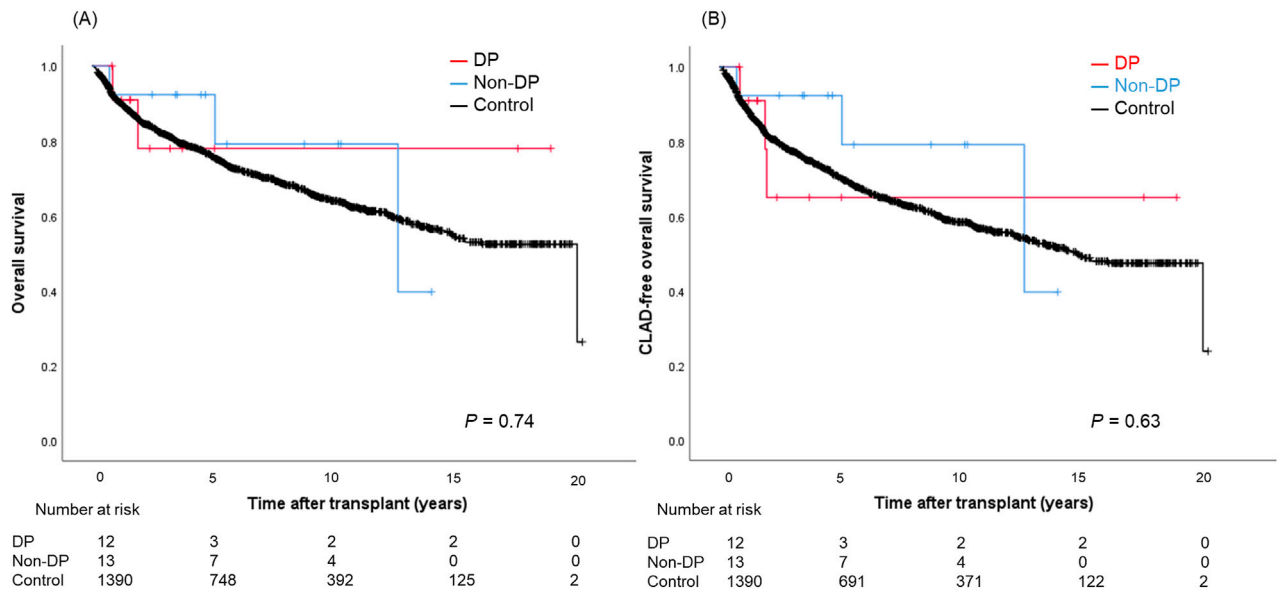
This study demonstrated that PND–despite having a significant impact on perioperative recovery–did not impair long-term overall and CLAD-free survival after LTx. This is well in line with trajectories of lung function tests, showing lower % FEV1.0 and %TLC early after LTx. Interestingly, % FEV1.0 seems to recover over time, whereas %TLC remained lower in non-DP and DP patients compared to the control group. To the best of our knowledge, this is the first study to



Mean %FEV1.0							
DP	58.4	62.7	60.8	65.6	70.4	66.4	75.0
Non-DP	53.8	74.1	75.9	78.5	76.5	75.7	76.2
Control	67.6	76.7	79.6	83.6	85.3	83.3	80.9
P value	0.067	0.15	0.025	0.025	0.065	0.11	0.78

Mean %TLC							
DP	68.7	76.2	75.6	71.1	73.8	79.1	75.5
Non-DP	74.1	77.2	76.3	80.4	80.0	76.8	80.4
Control	80.5	85.4	86.6	87.3	87.8	89.7	90.3
P value	0.056	0.12	0.026	0.002	0.008	0.009	0.034

FIGURE 2 | Trajectories of lung function parameters including mean %FEV1.0 **(A)** and %TLC **(B)** after lung transplantation. Each bar shows the standard error of the mean. *, <0.05. DP, diaphragmatic plication.



Number at risk					
DP	12	3	2	2	0
Non-DP	13	7	4	0	0
Control	1390	748	392	125	2

Number at risk					
DP	12	3	2	2	0
Non-DP	13	7	4	0	0
Control	1390	691	371	122	2

FIGURE 3 | Overall survival **(A)** and CLAD-free survival **(B)** curves in patients with the DP group, the non-DP group, and the control group. DP, diaphragmatic plication; CLAD, chronic lung allograft dysfunction.

investigate lung function parameters and long-term outcomes of LTx recipients with PND and DP.

The true incidence of PND after LTx is not clear. This complication was first systematically examined in LTx recipients in 1995, with a reported incidence as high as 29% [16]. Subsequent series have reported much lower rates ranging from 3% to 9% [3, 5]. PND was observed in only 1.8% of our patients. This variability in post-transplant PND might be explained by the heterogeneity of underlying diseases, improvements in surgical technique, as well as differences in the definition and diagnostic methods used. The most recent study of PND after LTx was published by the Santander Lung Transplant Program. In a well-conducted prospective observational study covering over 4 years, the group could

show that some degree of diaphragmatic impairment determined by systematic phrenic nerve conduction studies was evident in 43.3% of subjects and 29.0% of operated hemithoraces [17]. The main risk factors identified by this study were female gender, double-LTx, right grafts, clamshell incisions, and mediastinal adhesions. Morbidity was increased in PND without any difference in mortality. The significant lower numbers of PND in our cohort might be attributed to the fact that only patients with an elevated diaphragm were tested, thus, mild or temporary dysfunctions were not captured.

Several surgical principles have to be respected to avoid injury to the phrenic nerve. When the chest is opened by a clamshell incision, the anterior mediastinum should be mobilized to reduce the tension on the nerves. Pushing the heart should be reduced to a minimum, as the nerve can also be damaged by extensive pressure. Any dissection close to the nerve should be done with scissors or in a blunt way. The use of cautery should be limited to a minimum. Intraoperative electrophysiological phrenic nerve monitoring, which has previously been tested during cardiac surgery, could also be a tool to prevent damage to the phrenic nerve but its applicability in LTx needs to be determined [18].

The limited number of published series on PND after LTx found an increased length of ICU-stay, increased readmission rates to the ICU, and increased duration of hospitalization [3, 4, 6, 17]. Studies on the long-term effects of PND on lung function parameters are scarce. Lawrence et al. reported that patients with DP had consistently lower FEV1.0 than those without DP at 1-, 2-, and 3-year post-LTx although the gap between the two groups appeared to remain stable [9]. Furthermore, 1-, 2-, and 3-year survival as well as 3-year CLAD-free survival were similar. In our cohort, %FEV1.0 was significant lower early after LTx in DP patients compared to the other groups. Interestingly, % FEV1.0 seemed to improve over time. %TLC was significantly worse in both PND groups compared to the values of the control group. Furthermore, there was no difference in terms of %TLC between DP and non-DP patients. An important caveat of this interpretation is that patients in the non-DP group were significantly younger and patients in the DP group were transplanted in the most recent era (2017–2022). These bias may account for differences in the long-term lung function trajectories.

DP is a well-established treatment option for PND and is frequently performed in non-LTx patients. Freeman et al. conducted a single-center retrospective study to assess the impact of DP on the functional and physiologic outcomes in symptomatic patients [19]. In this study, mean FEV1.0 and TLC improved by 23% and 19%, respectively, when measured 6 months after surgery. The authors concluded that DP significantly improved pulmonary function, symptoms of dyspnea, and patient functional status. We performed DP liberally in patients with a confirmed PND and difficulties weaning from respirator or significant lower lobe atelectasis in CT scans. The optimal timing for DP after LTx remains unclear due to limited evidence in the literature. In our study, DP was performed at a median of 27 days post-transplant (IQR: 15–125 days). To our knowledge, only one previous study has reported the timing of DP, with a median of 16 days (range: 1–34 days), but it did not evaluate long-term outcomes [9]. Our study is the first to investigate the long-term impact of DP on pulmonary function after LTx. Based on our limited experience, we believe that DP should be considered when patients demonstrate persistent diaphragmatic dysfunction that interferes with respirator weaning or leads to significant atelectasis. If the patient's general condition, including wound healing and immunosuppression status, is acceptable, proceeding with DP within the first month appears to be reasonable. Furthermore, early DP may contribute to improved respiratory outcomes, as it facilitates weaning in patients previously unweanable from mechanical ventilation.

An interesting finding from long-term lung function trajectories is that FEV1 slightly improved in our DP patients. It is known that the function of accessory muscles of respiration improved by the rehabilitation after LTx. These muscles can compensate for the loss of diaphragmatic function and prevent detrimental long-term sequelae. Therefore, post-LTx rehabilitation is an essential part of successful lung transplant programs. Pulmonary rehabilitative exercises focus on restoring the strength and function of the diaphragm [20]. Furthermore, inspiratory muscle training can be performed to improve diaphragmatic weakness due to PND and is an important treatment option, particularly in cases of prolonged mechanical ventilation [21].

This study has several limitations. First, this retrospective study introduces several potential biases. There might have been a selection bias, particularly in pulmonary function tests, in which patients who have a longer survival have better function results. Second, the numbers of patients in the DP and non-DP groups are low, which may have limited the power to detect statistically significant differences. Due to the small sample size, we were also unable to perform multivariate analyses to adjust for potential confounding factors, including recipient age, transplant year, and diagnosis. Moreover, we might have missed some cases due to the exclusion criteria of 30-day mortality. However, this study aimed to examine long-term outcomes and lung function trajectories. Third, this study cannot account for surgical improvements, advances in perioperative care, and innovations in immunosuppressive therapy during the long study period of almost 20 years. Furthermore, we did not collect detailed information on patients' history of prior thoracic surgery or the presence of preoperative PND. Previous thoracic surgeries, such as lung resections or cardiac operations, could potentially cause phrenic nerve injury, and it is possible that some patients had impaired phrenic nerve function before LTx. Although we excluded cases of combined heart-lung transplantation based on the prior report [4] indicating a higher incidence of phrenic nerve injury in such procedures, we did not systematically screen for other types of prior thoracic surgical interventions. Therefore, we cannot entirely exclude the possibility that some of the postoperative phrenic nerve dysfunction observed in this study may have originated from preexisting conditions. Subsequent studies from other high-volume LTx centers or even a multicenter approach are warranted to confirm and validate our findings in independent patient cohorts.

In conclusion, we demonstrated that PND was associated with complicated recovery after LTx. PND led to slightly but consistently lower total lung volumes in lung function tests performed within the first three years after LTx. Despite this, PND was not associated with impaired long-term and CLAD-free survival.

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 Medical University of Vienna (approval No. EK 1639/2023). The studies were conducted in accordance with the local legislation and institutional requirements. Patient written consent for the publication of the study data was waived by the institutional ethics board due to the retrospective nature of the study.

AUTHOR CONTRIBUTIONS

KN: Project administration, Data curation, Investigation, Methodology, Writing—original draft. CH: Data curation, Investigation. TS: Data curation, Investigation. SS: Formal analysis, Supervision, Validation, Data curation, Investigation. ST: Investigation, Methodology, Writing—review and editing. PJ: Formal analysis, Supervision, Validation, Investigation, Methodology. AB: Data curation, Investigation. TC-Y: Investigation, Methodology, Writing—review and editing. KH: Conceptualization, Project administration, Supervision, Data curation, Investigation, Methodology, Writing—review and editing.

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CONFLICT OF INTEREST

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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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ORIGINAL RESEARCH
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Developing an Adult Living Donor Liver Transplant Program in Western Europe: The Rotterdam Experience

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Liver transplantation (LT) is curative for end stage liver disease. Expanding LT indications with limited deceased donor grafts has created organ shortages. Living donor liver transplant (LDLT) increases available organs. In 2019, we restarted our adult LDLT program. We describe our steps to create a successful LDLT program, and our outcomes. Critical steps of program development included market analysis, creation of protocols based on best care practices and a rigorous education program. Patients and donors were then actively recruited for LDLT. Outcomes were measured as morbidity (≥3 on the Clavien-Dindo grading system) and mortality. Between January 2019 and August 2024, 54 LDLT were performed. 2 (3%) donors experienced grade 3A and 7 (12%) donors experience grade 3B complications. There was no donor mortality. 22 (41%) patients were transplanted for PSC, the average MELD score was 13 (6–32). 35 (65%) patients had Roux-en-Y reconstructions. 25 (46%) complications were experienced in 22 (40%) patients, there were 2 recipient deaths. Patient and graft survival after LDLT was 97% and 97%, respectively. This paper reported the successful establishment of a LDLT program in the Netherlands. Establishing a LDLT program brings its own unique challenges, with careful planning and persistence, these challenges can be overcome.

Keywords: living liver donation, living donor liver transplantation (LDLT), liver transplant, liver donation, live donor liver transplant

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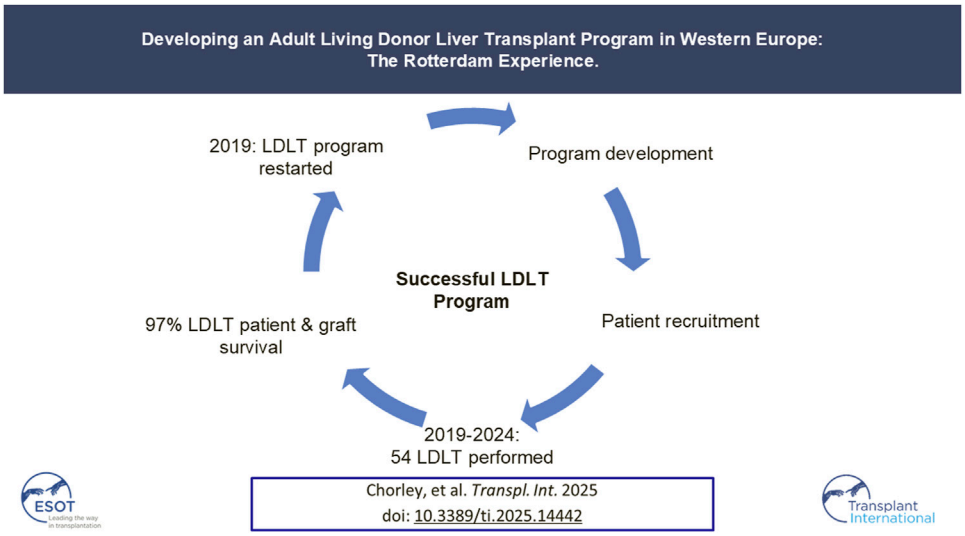
Abbreviations: BMI, body mass index; CT, computer tomography scan; ECG, electrocardiogram; GRWR, graft-to-recipient weight ratio; HCC, hepatocellular carcinoma; ICU, Intensive care unit; LDLT, Living donor liver transplant; LLD, living liver donor; LT, Liver transplant; MELD, model for end stage liver disease; MRCP, magnetic resonance cholangiopancreatography; MRI, magnetic resonance imaging; OR, operating room; PSC, Primary sclerosing cholangitis; WL, Waiting list.



GRAPHICAL ABSTRACT |

INTRODUCTION

Liver transplantation (LT) is the only curative treatment option for end stage liver disease and selected malignancies and is a proven treatment alternative in certain metabolic diseases [1]. More than 7000 LT procedures are performed annually in Europe [1]. Since its inception, both patient and graft survival following LT have improved significantly, owing to advancements in surgical techniques, anaesthesia, immunosuppressive regimens, and the timely detection and management of complications, particularly through minimally invasive methods [2].

Initially, living donor liver transplantation (LDLT) was performed to reduce waitlist (WL) mortality in paediatric patients who faced restricted access to deceased donor organs due to size mismatches [3]. Over time, LDLT has evolved into an increasingly attractive option for adult patients and their healthcare providers, especially in locations where the demand for liver transplant exceeds the availability of deceased donor organs.

Deceased organ donation remains the predominant source for transplantation worldwide. Yet, in certain regions of the world, deceased organ donation rates remain suboptimal, often due to social, religious, logistic and cultural factors [4, 5]. This disparity has led to the growing use of LDLT, particularly in Asia and the Middle East [6]. The first successful adult LDLT's were performed in Asia and the United States of America [7]. The favourable outcomes for both donors and recipients prompted many European LT centres to initiate their own LDLT programs during the 1990s [8]. Notably, programs in Germany and Belgium became prominent reference points for patients and their healthcare providers in Europe, contributing significantly to the field of LDLT [8]. However, due to the complexities of donor surgery, the risk of donor related complications and reports of live donor fatalities in the United States, most European transplant programs discontinued their LDLT programs [9]. LDLT is only performed in a limited number of European centres, accounting for less than 5% of total LT procedures across the continent [8].

Erasmus University Medical Centre (Erasmus MC), which performed its first LT in 1986, has since carried out more than 1,700 liver transplants. Erasmus MC first introduced LDLT in 2004, successfully performing 10 procedures between 2004 and 2011. In response to an increasing wait list mortality and the growing demand for liver transplants, Erasmus MC renewed its commitment to LDLT in 2018. This decision marked the beginning of efforts to re-establish a sustainable, successful and safe LDLT program. The aim of this article is to outline the steps undertaken to develop this LDLT program and to present the outcomes of our initial 54 LDLT procedures.

MATERIALS AND METHODS

Program Development

Market Analysis and Rationale for Initiating a LDLT Program

Before launching a living donor program, it is essential to create a development plan. This plan should include market analysis to determine the feasibility and necessity of a LDLT program. According to Eurotransplant, approximately 20% of patients on the Dutch liver transplant waiting list either die or are delisted before they can receive a LT. This unmet need persisted despite advances such as machine perfusion, use of extended criteria donors, and the recent transition from an opt-in to an opt-out organ donation system in the Netherlands. These developments, while beneficial, have not sufficiently expanded the deceased organ donor pool to meet the growing demand for LT. Prior to the initiation of the LDLT program at Erasmus MC, patients were typically only eligible for LT screening and WL

placement if their Model for End Stage Liver Disease (MELD) score was above 15 – except in situations where hepatocellular carcinoma (HCC) or cholangiocarcinoma was the indication for LT. This policy was based on the recognition that patients with lower MELD scores had minimal or no access to deceased donor liver transplants. As a result, cirrhotic patients with low MELD scores–such as primary sclerosing cholangitis (PSC) patients with recurrent cholangitis, or patients with metabolic liver diseases–were largely underserved. This indicated that the patient population lacking access to LT was significantly larger than the 20% who were delisted or died while on the WL. The introduction of a LDLT program would help to address this gap by offering LT options to patients with a lower MELD score already on the WL, provide access to LT for those previously deemed ineligible due to low MELD scores, and ensure timely transplantation for patients with progressive diseases such as HCC, potentially avoiding death or delisting due to disease progression.

The Netherlands presents a favourable environment for launching a LDLT program. As a multicultural society, it supports a diverse patient and donor base. Importantly, the Dutch legal framework permits all forms of living donation–related directed, unrelated directed, and unrelated undirected donation–unlike some other European countries where regulations are more restrictive. Furthermore, the success of large living kidney donor programs in The Netherlands indicated both public awareness and acceptance of the concept of living donation. These factors combined suggest a receptive donor population and a clear, unmet medical need among LT recipients. This market analysis strongly supported the initiation of a LDLT program as both a necessary and viable addition to liver transplant services in the Netherlands.

Program Development and Resource Allocation for LDLT Initiative

Following the completion of the market analysis, the next critical phase in launching a LDLT program involved identifying structural, human and procedural requirements. These would be necessary for successful implementation and long-term sustainability of the LDLT program. To ensure that the program would be well supported, all relevant multidisciplinary stakeholders were invited to share their concerns and perspectives, this collaborative approach allowed the LDLT program to be integrated into the broader LT program. Key staff members were recruited to establish the programs' foundation, including the recruitment of an experienced LDLT surgeon and LDLT nurse coordinator. These individuals were tasked not only with the establishment and day-to-day running of the program, but also with ensuring its continuity through training of existing medical personnel involved in the deceased liver transplant program.

Institutional support for the LDLT program was both early and robust. The LDLT initiative received endorsement from the board of directors, chief executive officer and leadership within both the liver transplant surgery and hepatology departments. In accordance with international ethical guidelines and standards, an independent live donor advocate was also appointed to protect the interests and autonomy of all potential donors throughout the evaluation and donor screening process [10].

TABLE 1 | Manpower involved in donor screening and follow up.

Screening phase	Nurse practitioner
	Live donor surgeon
	Social worker
	Psychologist
	Anaesthesiologist
	Radiologist
	Cardiologist
Peri-operative phase	Live donor surgeon
	Liver transplant surgeon
	Nurse practitioner
	Nurses
	Anaesthesiologist
After care phase	Nurse practitioner
	Live donor surgeon
	Social worker and psychologist (as needed)

A comprehensive workflow analysis of the existing deceased donor LT program was undertaken to identify similarities and gaps. Based on this assessment, strategic recruitment efforts were undertaken to expand the multidisciplinary team. This included two specialized radiologists proficient in high resolution MRCP and CT scans as well as radiology technicians trained in liver volumetry techniques. To optimize patient outcomes and perioperative care, an intensivist was brought onboard to provide specialist oversight during the ICU stay for the donor and recipient, serving as the primary liaison during their respective ICU stays. A social worker and psychologist were integrated into LDLT program to support potential donors during the screening process, ensuring holistic psychosocial evaluation and preparation. These healthcare professionals would also be available to support donors if needed after donation as well. **Table 1** details the individuals involved in each stage of donor screening and follow-up.

In anticipation of clinical activities, all necessary surgical and supportive equipment–including specialised instruments, foot pump devices, incentive spirometers–was procured prior to the enrolment of patients and donors in the program. This preparatory phase ensured operational readiness and underscored the institutions commitment to delivering safe, ethical and sustainable LDLT program.

Education and Capacity Building

A comprehensive education and training program was implemented to ensure all health professionals involved in the care of potential living liver donors and recipients possessed the required knowledge, clinical competencies and ethical awareness to manage this complex patient population effectively. The education program was designed to foster deep understanding of the principles, procedures, ethics and psychological dimensions unique to a LDLT program.

All staff members who would have contact with donors and recipients were targeted for training and education. Specialised education and training sessions were delivered to nursing staff in

the operation room (OR), intensive care unit (ICU) outpatient clinic and inpatient care settings. This approach aimed to standardise clinical care pathways, enhance communication between teams and ensures both donors and recipients received consistent, high-quality care throughout the entire donation and transplant process.

Workflows and Protocol Development

A critical component in the establishment of a LDLT program was the development and implementation of standardised workflows and protocols to guide the evaluation and clinical management of both potential living liver donors and potential LDLT recipients. These protocols clearly defined the eligibility parameters, indications and contraindications for donation and LDLT, as well as specific clinical, ethical and psychosocial considerations to be evaluated throughout the assessment process. Clinical pathways were established to outline the specific day-to-day care of post donation donors and LDLT recipients. These pathways were designed to standardize care delivery, facilitate multidisciplinary coordination, and ensure that each patient received high quality, patient centred treatment in all clinical settings. Given Erasmus MC status as an international training centre, all procedural documents and clinical materials were available in both Dutch and English. Additionally, patient resources–including detailed, user-friendly information booklets for both donors and recipients–were produced to as a tool to enhance patient understanding and program transparency. These booklets outlined the structure of the LDLT program, provided educational information about LDLT and donation, and included information on national resources available within the Netherlands such as the Dutch Transplant Foundation. The financial impact of living donation is not to be underestimated; therefore, information on the financial impacts and subsidies available for donors was also included in education materials. The risks, potential complications, and long-term implications of both donation and transplantation were addressed in detail as part of the pre-screening and consent process. Although written consent prior to medical procedures is not a legal requirement in the Netherlands, the unique complexity of LDLT and the interdependence of the donor and recipient procedures prompted the adoption of a formal written informed consent process for both donors and recipients. This decision reflects the program's commitment to ethical standards, respect for patient autonomy, and the safeguarding of all individuals involved in the donation and transplant process.

Risk Assessment and Mitigation Planning

Prior to the initiation of clinical activity, a comprehensive risk assessment was performed to systematically identify, evaluate and address potential pitfalls and complications that could occur once the living donor program was functioning at Erasmus MC. This evaluation involved extensive consultation with all relevant stakeholders, including surgery, hepatology, anaesthesiology, intensive care, radiology, psychosocial, and administrative teams. Each discipline was invited to provide their input on potential risks within their domain of expertise using the risk assessment tool RISKID. Participants could anonymously enter potential risks and hazards from their perspective into this system. Once potential hazards had been identified, these risks could be evaluated based on the likelihood of occurrence, who might be harmed and how severe the consequences of the event would be. All findings were recorded, and actions were implemented based on the risk levels. Existing protocols and procedures were rigorously reviewed to confirm that anticipated complications—both routine and exceptional—had been adequately addressed prior to clinical activity. Finally, the risk assessment and updated protocols were updated and reviewed by all stakeholders participating in LDLT. This risk assessment is updated periodically in line with institutional requirements, after near miss incidents, or when new processes are implemented.

To further enhance preparedness, a Crisis Management Plan was developed and documented. This plan outlined clear, stepwise procedures for responding to major complications in living donors, including intraoperative adverse events and severe postoperative morbidity. A formal crisis response statement was also prepared, highlighting Erasmus MC's institutional commitment to transparency, ethical accountability, and donor protection.

This risk assessment process reinforced a culture of safety, readiness, and continuous quality improvement as foundational principles of the LDLT program.

Implementation

Patient Recruitment and Candidate Selection

Following the establishment of clear eligibility criteria for both living liver donors and LDLT recipients, the program progressed to the active recruitment of potential donors and recipients. This phase was designed to identify suitable donor and recipient pairs, while maintaining safety, transparency, and ethical integrity. In October 2018, an initial cohort of 20 patients was selected from the deceased donor WL based on a comprehensive review of their diagnosis, previous medical and surgical history, MELD score and Child-Pugh score. These 20 patients were identified as potentially appropriate candidates for LDLT, given their clinical profiles and likelihood of limited access to deceased donor grafts. Each of the selected patients was invited to the outpatient clinic for a detailed consultation, where their own hepatologist and the LDLT surgeon explained the concept of LDLT including the risks, benefits, possible complications, and donor criteria. This personalized approach ensured patients were given the opportunity to make informed decisions, based on accurate information. Subsequently, a structured and ongoing LDLT screening process was also initiated, where all patients currently on the deceased donor waiting list as well as all new referrals to the transplant centre were reviewed on a weekly by the LDLT nurse coordinator. This continuous review process enabled the early identification of potential new candidates for LDLT. This strategic and patient-centred approach to recruitment allowed for early identification of donor-recipient pairs and contributed to the broader goals of expanding the LDLT program and access to LT.

TABLE 2 | Donor and recipient selection criteria.

Donor suitability criteria	Recipient suitability criteria
18–55 years	Indications following international criteria
BMI <30 kg/m^2	Formally listed with Eurotransplant
Remnant liver volume ≥30%	No re transplantation, no expected arterial/venous jump grafts
Blood group compatible with recipient	
Psychologically and physically healthy, no previous major surgery	

BMI: Body Mass Index.

Donor Selection and Evaluation

Donor selection and evaluation are the ethical and clinical cornerstones of any LDLT program. The screening process must be methodical, evidence based and sufficiently stringent to exclude any individual for whom the donation procedure poses an elevated or unacceptable risk. Furthermore, donor evaluation must prioritise the long-term health, safety and quality of life, of the donor, ensuring that no compromises are made in pursuit of recipient benefit. To uphold these principles, the donor evaluation process at Erasmus MC was designed to proceed in a stepwise manner, with the explicit goal of identifying and excluding unsuitable donors as early as possible in the screening process. This approach minimizes unnecessary testing and reduces the physical and psychological burden on potential donors.

The donor selection criteria included individuals who were related, unrelated directed and unrelated undirected to their recipient, aged between 18 and 55 years, with a body mass index (BMI) of less than 30, blood group compatible with the recipient, and to ensure the absence of any major medical history or surgical procedures (**Table 2**). Donors would be accepted if they voluntarily came forward to donate and were physically and psychologically fit to provide informed consent. Potential donors must have a clear ability to understand the risks, benefits and long-term complications associated with donation. These criteria were applied uniformly across all donor types (related directed, unrelated directed and unrelated undirected donors) to maintain consistency and safeguard donor welfare.

The structured and ethical approach to donor evaluation reflects the program's commitment to the principle of *primum non nocere*—first, do no harm—while enabling access LDLT through safe and responsible living donation.

Beyond ensuring medical and surgical suitability, the overarching goal of any living donor program is the steadfast commitment to donor safety, autonomy and wellbeing. At Erasmus MC, donor voluntariness is regarded as a fundamental prerequisite for participation in the screening process and subsequent donation. It is imperative that all potential donors engage in the donation process free from coercion, external pressure, or undue influence of any kind. In alignment with international ethical standards, Erasmus MC does not actively solicit or recruit living donors. The presence of any form of coercion—be it emotional, social, or financial—automatically makes a potential donor unsuitable for living liver donation. Financial incentives or indirect compensation are explicitly prohibited in the LDLT program at Erasmus MC, any indication of incentives or compensation for organ donation results in an immediate discontinuation of the evaluation process. To further safeguard donor autonomy, a donor advocate is integrated into the live donor team to provide additional oversight as needed. Additionally, all potential living donors are explicitly informed–during a private consultation with the LDLT surgeon—that they may withdraw from the process at any point without the need to justify their decision to the LDLT team or the recipient. Any withdrawal from the donation process can be framed as a medical contraindication, thereby protecting the donor from social or familial repercussions.

All potential liver donors were self-referred, no referral is needed from another health professional to begin the donor screening process. The majority of living liver donors are family members or close family friends of the intended recipient. Potential donors initially contact the LDLT nurse coordinator directly, where donors were pre-screened for suitability in terms of age, BMI, blood group compatibility, previous medical/surgical history (**Table 2**). Potential donors who met the initial selection criteria were invited for a structured intake and information session with a qualified LDLT surgeon and LDLT nurse coordinator in the outpatient clinic. This session provided potential donors with a detailed overview of the donation process, surgery, associated risks, and the expected recovery period. Potential donors then underwent extensive blood testing, which included but was not limited to blood group typing, renal and liver function, haematological investigations, extensive coagulopathy screening, virology, and infectious screening. Donors with satisfactory blood test results who expressed a willingness to proceed with donor screening, were then planned for the next screening phase. An interview with social worker determined if the potential donor had adequate support systems in place to manage the pre, peri, and post-operative periods. A comprehensive psychological evaluation with a psychologist assessed the potential donors' motivation for donation, expectations, and current relationship with recipient, coping mechanisms, and any previous life events or psychiatric history which may affect decision making, delay or inhibit recovery after donation. To ensure the donor could safely undergo anaesthesia, lung function tests, a chest X-ray and electrocardiogram were performed. Advanced radiological imaging played a pivotal role in determining the anatomical and technical feasibility of living donation and transplant. A four phase CT scan was performed to confirm the absence of focal liver lesions, abnormal pathology in the abdomen, and to assess the liver quality, venous, and arterial anatomy. Liver volumetry was performed on the CT images to calculate segmental liver volumes using specialised volumetric software. All donors underwent an MRCP to determine biliary anatomy and rule out structural anomalies. Donors with an estimated remnant liver volume of less than 30% were excluded from donation due to unacceptable risk. Additionally, the estimated graft recipient weight ratio was required to exceed >0.7 to ensure adequate liver function post-transplant. Potential donors who satisfied the

criteria in the first two phases of the screening process were then referred to anaesthesia for clearance, and an echocardiogram was performed. The final stage of evaluation included a liver biopsy, which allowed for assessment of steatosis, fibrosis, inflammation, iron overload or alpha-1 antitrypsin in the liver–any of which could be a contraindication for donation. Only after successful completion of all screening phases and multidisciplinary team review were potential donors formally approved to undergo living liver donation surgery.

In line with ethical and clinical best practices, any incidental findings during screening—such as previously undiagnosed medical conditions—triggered referral to the appropriate specialists within Erasmus MC for further evaluation and management.

LDLT Recipient Evaluation

Prior to being considered for LDLT, all potential recipients must undergo evaluation for LT in accordance with the national liver transplant screening protocol and be placed on the Eurotransplant waiting list [11, 12]. This ensures that LDLT candidates are first deemed appropriate for LT based on national and international standards. To further optimize outcomes in the early phase of the LDLT program, specific inclusion, and exclusion criteria for LDLT were established. In the initial phase of the program, patients anticipated to present significant surgical complexity–re transplantations, polycystic liver disease patients, and patients with complete portal vein thrombosis–were excluded as candidates for LDLT (**Table 2**).

Potential LT candidates are referred by hepatologists in peripheral hospitals to Erasmus MC when their MELD score exceeds 15. Direct referrals are also accepted for patients with hepatocellular carcinoma (HCC) or cholangiocarcinoma, given the time sensitive nature of these indications. Upon referral, new potential recipients who meet the criteria for LDLT are introduced to the LDLT program during their first visit by the LDLT nurse coordinator, who provides detailed information about the program including LDLT risks and benefits, donor criteria and donor screening processes (**Table 3**). Potential recipients who meet criteria for LT then proceed to a 2–3 days inpatient evaluation at Erasmus MC. Recipient evaluation includes, but is not limited to CT and Magnetic resonance imaging (MRI) scans, echocardiogram and electrocardiogram (ECG), appointments with social worker, anesthesia, infectious diseases specialist, liver transplant surgeon, dentist, and ear nose throat specialist, bone density scan, gastroscopy and colonoscopy, lung function tests, chest X-ray, and blood tests. At the completion of screening, each potential recipient is presented in a multidisciplinary team meeting consisting of anesthesiologists, hepatologists, social work, and transplant surgeons. This team collaboratively determines the patients' suitability for LT based on medical, surgical, psychosocial, and logistical factors. Recipients deemed eligible for LT are then placed on the Eurotransplant liver transplant waiting list, with any potential living liver donors evaluated in parallel where appropriate.

Donor Surgery

Surgery was performed under general anesthesia. A central venous catheter, arterial line urinary catheter and peripheral intravenous cannula were placed for safety and hemodynamic control at the beginning of and during the donor surgery. An upper midline incision was used with the Thompson Retractor®. After inspection and palpation of the liver, the right or left lobe of the liver is fully mobilized. The gallbladder is mobilized off the liver bed and an intraoperative cholangiogram is performed to verify biliary anatomy. Depending on a right or left liver lobe donation, the right or left hepatic artery and portal vein are dissected and encircled. The right hepatic vein is encircled with an umbilical tape running between with liver and IVC, which is used during the parenchymal transection. The transection line in our center is on the right side of the middle hepatic vein keeping the latter always to the left liver lobe. Extending to the mid-point of the gallbladder fossa, is marked and liver dissection is performed with Cavitron Ultrasonic Surgical Aspirator (CUSA). The liver graft was procured and flushed with University Wisconsin solution via the right/left portal vein and right/left hepatic artery. A Jackson Pratt (JP) drain was placed at the end of surgery, with the tip of the drain next to the resected liver. After surgery was completed, trans-abdominal plain blocks or rectal sheath catheters were placed by anesthesia for pain relief.

Recipient Surgery

Surgery was performed under general anesthesia. Peripheral intravenous catheter, arterial line, central venous catheter, pulmonary artery catheter was placed for monitoring and hemodynamic control; transesophageal echocardiogram monitoring was performed during surgery where indicated. A reversed L-shape incision was used. After mobilization of the left and right lobes, hepatica artery, portal vein, and bile duct were dissected, and divided as high as possible. After the hepatectomy, the hepatic vein reconstruction was performed with polene 5.0. The portal vein was anastomosed using prolene 6.0 or 7.0. After portal-venous reperfusion, the hepatic artery was reconstructed with interrupted prolene 8.0 sutures. An intraoperative Doppler ultrasound of the liver was performed to confirm patency of all blood vessels. Duct-to-duct anastomosis or Roux-en-Y anastomosis was employed for the biliary reconstruction with interrupted PDS 7.0 sutures. Two abdominal drains were placed intra operatively in the recipient: one in the liver hilum and the second behind the liver lobe.

Post-Operative Management

Initially, both living liver donors and recipients were admitted to the Intensive care unit (ICU) for overnight monitoring following surgery. However, in response to the increased demand for ICU resources during the COVID-19 pandemic, a revised protocol was implemented. Under this new protocol, donors are now admitted to the post anesthesia care unit (PACU) for the first postoperative night before returning to the surgical ward on day 1 to continue recovery.

The focus of donor post-operative care is ensuring donor safety and comfort. Postoperative management is initially focused

TABLE 3 | Donor screening phases.

Donor screening
Phase 1: Intake interview, information conversation, blood testing
Phase 2: Social work and psychological screening, Chest X-ray, ECG, lung function tests, CT scan, MRCP
Phase 3: Surgical clearance, anaesthesia clearance, echocardiogram
Phase 4: Liver biopsy

ECG, electrocardiogram; CT scan, computerized tomography scan; MRCP, Magnetic resonance cholangiopancreatography.

on adequate pain control, prompt mobilization, correction of electrolyte imbalances due to the rapid regeneration of liver tissue and prevention of complications. Donors are extubated in the operating room prior to transfer to the PACU. A mild elevation in lactate levels is common immediately postoperatively which is routinely managed through aggressive fluid resuscitation. Pain management is multifaceted. A patient-controlled analgesia pump provides continuous and bolus breakthrough pain management which is typically kept for 2–3 days–dosages are reduced daily before switching to oral pain relief when patient-controlled analgesia is ceased. Post-operative pain is also managed with transverse abdominis plane (TAP) blocks or rectal sheath catheters–these are refilled with a local anesthesia agent such as ropivacaine every 8 h and provides targeted pain relief for 3–4 days postoperatively.

After donation surgery, all living donors undergo daily monitoring of key clinical, biochemical parameters to ensure the prevention, (early) detection and management of complications. Laboratory tests are performed to assess liver function, renal function, electrolytes, coagulation, and infection parameters. In addition to laboratory monitoring, an abdominal ultrasound is performed on day 0 and day 5 to ensure vascular patency (hepatic artery, portal vein and hepatic veins) and to identify the presence of any peri-hepatic fluid collections. Prophylactic antibiotic therapy is administered until the abdominal drain is removed, in line with the infection prevention protocol in our LDLT program. Early mobilization is a key component of postoperative care and begins on post-operative day 1 facilitated by a physiotherapy team. Physical activity is progressively increased each day to support circulation, pulmonary function, and overall recovery. Donors are typically discharged between postoperative day 5 and 7, depending on their clinical recovery. Following discharge, donors are then followed intensively in the outpatient setting for the first year after donation. After completing the first postoperative year without complications, donors have the option to return to Erasmus MC yearly for an appointment or complete blood testing via their general practitioner followed by a remote consultation with the LDLT nurse coordinator. This follow-up protocol ensures comprehensive short- and long-term monitoring of donor health and underscores Erasmus MC's commitment to donor safety and wellbeing.

Following LDLT, recipients are typically admitted to the ICU for 2–3 days for close observation. In cases where surgery proceeds uneventfully, recipients may be extubated on the OR table. Otherwise, recipients are extubated within 8–12 h postoperatively, once clinically stable. Postoperative care is delivered by a multidisciplinary team including the attending and consultant hepatologist and nurse practitioner, as well as live donor nurse practitioner/LDLT surgeons. This collaborative approach ensures continuity of care and supports the early identification and management of potential complications. To monitor for vascular complications, daily liver ultrasounds are performed from postoperative day 0 to day 7. Recipients receive a standardized immunosuppression regimen consisting of induction with methylprednisolone and basiliximab that is given day 0 and day 4 and maintenance with mycophenolic acid and prednisone from D0 followed by tacrolimus beginning on day 5 post-operatively. Once two adequate trough levels of tacrolimus have been achieved, mycophenolic acid is discontinued. Prednisone is tapered over a 3–6-month period, depending on the clinical course. Recipients also receive prophylactic antibiotics until the drains are removed.

Most recipients are discharged from the hospital within 14 days following LDLT, assuming a stable recovery without significant complications. After discharge, patients are closely monitored in the outpatient with regular laboratory investigations, imaging, and appointments with hepatologists and nurse practitioners. Immunosuppressant levels and compliance are monitored to ensure optimum graft function and long-term success.

The study was reviewed and approved by the Medical Ethics Review Committee of the Erasmus MC (MEC-2023-0774). The donors and patients provided written informed consent to participate in this study.

RESULTS

Outcomes from LDLT procedures performed between January 2019 and August 2024 were included in this analysis. A total of three donor procedures were aborted intra-operatively due to the identification of abnormal biliary anatomy, which was previously undetected on pre-operative imaging. To ensure accuracy and consistency of results, these donors and their corresponding recipients have been excluded from the reported data. In the final quarter of 2020 and the first half of 2021, our ability to perform LDLT was significantly impacted by the COVID-19 pandemic, which placed considerable strain on hospital resources. Specifically, operating room availability, ICU bed capacity, and admission scheduling were constrained due to prioritization of critical care for COVID-19 patients (**Figure 1**). Despite these challenges, the program demonstrated resilience and adaptability, with a subsequent rebound in case numbers as hospital operations normalized.

Donor Outcomes

Donor characteristics are reported in **Table 4**. Most donors were related to their recipients, with 36 donors (66%) being female. The median donor age was 33 years (range 18–58 years), and the mean BMI was 27 kg/m^2 (range 17–31 kg/m^2). Fifty-two right lobe donations took place, as well as two left lobe donations and two domino LDLT. The median length of hospital stay was 6 days

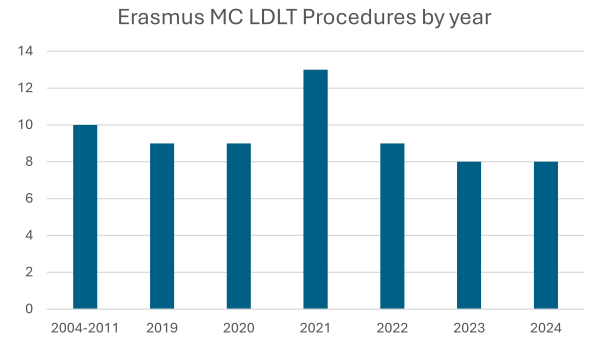


FIGURE 1 | LDLT procedures performed at Erasmus MC, by year.

(range 5–11 days). The mean blood loss was 350 mL (range 50–2,500 mL). Complications were graded using the Clavien-Dindo scale [13–15]. Donor complications have been divided into postoperative complications (within 90 days) and complications that occurred >90 postoperatively. Donor complications are detailed in **Tables 5**, **6**. 9 (16%) donors experienced complications within 90 days of surgery, and 3 (5%) donors experienced complications 90 days or more post donation surgery. Immediate postoperative complications included three grade one complications–one donor had a symptomatic urinary tract infection and received oral antibiotics, and two donors received antibiotics for wound infections. Two donors experienced grade 3A and 4 donors experienced grade 3B complications within 90 days of surgery (**Table 5**). Two (3%) donors required drainage of fluid collections via interventional radiology 2 weeks after donation surgery, and one donor presented with a diaphragmatic hernia 7 weeks post donation surgery. One donor required re-laparotomy for a persistent bile leak 6 weeks postoperatively, and one donor required a re-laparotomy day 1 post living liver donation for refixation of the left liver lobe post right lobe donation. One donor developed an incisional hernia 1 month post donation surgery and underwent surgical repair. Three donors experienced complications 90 days or more post their donation surgery (**Table 6**). One donor developed a diaphragmatic hernia 8 months post liver donation, and two donors required incisional hernia repairs 17 months and 3 years after donation. There were no grade 4 or 5 complications in living liver donors. There was no donor mortality (average follow up 35 months, range 12 weeks–5 years 7 months).

TABLE 4 | Donor characteristics.

Sex	
Female	36 (66%)
Donation type	
Right lobe	52 (96%)
Left lobe	2 (4%)
Relationship to recipient	
Related directed	35 (65%)
1st degree relative	30 (56%)
2nd degree relative	5 (9%)
Unrelated directed	17 (31%)
Partner	6 (11%)
Friend	6 (11%)
Sister/brother-in-law	4 (7%)
Stepfather	1 (2%)
Unrelated undirected	2 (4%)
Age (years)	33 (18–58)
BMI (kg/m2)	27 (17–31)

TABLE 5 | Post-operative complications in liver donors (within 90 days).

Clavien-Dindo classification (grade)	No. of complications
1	3
2	0
3A	
Drainage of biloma	2
3B	
Diaphragmatic hernia	1
Incisional hernia	1
Re-laparotomy	2
4A	0
4B	0
5	0

TABLE 6 | Complications >90 days postoperatively in living liver donors.

Clavien-Dindo classification (grade)	No. of complications
1	0
2	0
3A	
Drainage of biloma	0
3B	
Diaphragmatic hernia	1
Incisional hernia	2
4A	0
4B	0
5	0

Recipient Outcomes

In total 54 LDLT were performed between January 2019 and August 2024. Recipient characteristics are reported in **Table 7**. End-stage liver disease secondary to Primary Sclerosing Cholangitis (PSC) and HCC were the most common indications for LDLT in 22 (41%) and 11 (20%) of patients respectively. The median MELD score was 13 (range 6–32). Mean time on the LT WL was 1 year (range of 4 days–15 years, 1 month) and the mean length of hospital stay after transplantation was 21 days (range of 10–52 days).

The mean cold ischemia time was 163 min (range 115–290 min), the mean warm ischemia time was 35 min (range 21–55 min), mean OR time 508 min (355–760 min). The mean blood loss was 3.3L (range 0.2–31.5 L), mean actual graft-to-recipient weight ratio (GRWR) 1.08 (range 0.55–1.75). Thirty-five LDLT (65%) recipients had Roux-en-Y reconstructions, 17 (31%) patients had a duct-to-duct biliary anastomosis. One recipient had a combination of duct to duct, and Roux-en-Y biliary anastomosis and one recipient had a duct

TABLE 7 | Recipient characteristics.

Sex	
Female	27 (50%)
Aetiology	
PSC	22
ASH	4
NET	4
MMA	4
NASH	3
AIH/PSC	3
HBV	3
GSD (type 1a and 1b)	2
SBC	2
PBC	2
PFIC type 3	1
HCV	1
Caroli Disease	1
Polycystic liver disease	1
Hemochromatosis	1
HCC (included in above)	11
Age (years)	42 (16–71)
MELD Score	13 (6–32)

PSC, primary sclerosing cholangitis; HCC, hepatocellular carcinoma; MMA, methylmalonic aciduria; GSD, glycogen storage disease; PFIC, primary familial intrahepatic cholestasis; HCV, hepatitis C virus; HBV, hepatitis B virus; ASH, alcoholic liver cirrhosis; NET, neuroendocrine tumour; NASH, non-alcoholic steatohepatitis; SBC, secondary biliary cholangitis; MELD, model for end stage liver disease.

TABLE 8 | Post-operative complications in transplant recipients.

Clavien-Dindo classification (grade)	No. of complications
3A	
Bile duct stenosis	4
Bile leaks	11
Fluid collection	3
3B	
Hepatic artery thrombosis	3
Post-operative bleeding	2
Incisional hernia repair	2
Re-laparotomy for intra-abdominal abscess	1
4A	
CVVH	1
4B	0
5	2

CVVH, Continuous Veno-Venous Hemofiltration.

to duodenum biliary anastomosis. The mean ICU stay was 3.7 days (range 1–40 days).

25 (46%) complications were observed in 22 patients (40%). There were 14 grade 3A, 8 grade 3B, 1 grade 4A, and 2 grade 5 complications as shown in **Table 8**. Three (5%) recipients developed hepatic artery thrombosis 2-, 5- and 7-day post LDLT. All thromboses were urgently managed in the OR with thrombectomy saving the living liver grafts. Following the thrombectomy in the OR, all hepatic arteries were patent. Biliary complications occurred in 11 patients (bile leaks in 6 (11%) patients and biliary stricture in 5 (9%) patients). All bile leaks occurred within 3 months of the LDLT, 2 out of 4 biliary strictures occurred within 3 months of surgery and the remaining 2 were late onset strictures. Bile duct stenosis was diagnosed based on MRCP findings or recurrent cholangitis, while bile leaks were diagnosed if the bilirubin level in the drain was >3 times the serum bilirubin level. 4 patients with bile leaks were treated conservatively, with the surgical drain remaining in place until the bile leak has resolved. Two patients required percutaneous transhepatic cholangiography drainage for their bile leak. Recipients with biliary stenosis were managed with progressive stenting protocols via endoscopic retrograde cholangiopancreatography, or with percutaneous transhepatic cholangiography drainage. None of the patients with biliary complications required surgical revision of the anastomosis. One recipient who had a hepatic artery thrombosis 7 days post LDLT developed biliary complications and underwent a re-transplantation 16 months later with a deceased donor liver transplant. A second LDLT recipient developed chronic rejection and ischemic type biliary lesions (ITBL). The patient was listed for re transplantation 15 months after LDLT and underwent re-transplantation 2 years after her LDLT. There were two LDLT recipient deaths, neither of whom developed biliary of vascular complications. One recipient had an acute cellular rejection 1 month after LDLT, she was treated for her rejection with rabbit anti-thymocyte globulin (r-ATG). However, she had a severe adverse reaction to r-ATG with a therapy resistant systemic inflammatory reaction, which resulted in resuscitation and transfer to the ICU for extra corporeal membrane oxygenation (ECMO). Unfortunately, she passed away 6 weeks after LDLT. A second recipient was found to have metastatic gallbladder cancer during the LDLT, after the living donor hepatectomy had already been performed. Even retrospectively, this metastatic gallbladder disease could not be visualized on the preoperative scans. He initially recovered well after surgery, but experienced respiratory complications 1 week after LDLT. Due to the poor prognosis, active treatment was withdrawn, and he passed away soon after. There was no further recipient mortality (average follow up 35 months, range 12 weeks–5 years 7 months).

DISCUSSION

This study reports the outcomes of 54 living liver donation and LDLT surgeries performed at Erasmus University Medical Centre between January 2019 and August 2024. It also outlines several key steps essential for the safe and effective implementation–and subsequent expansion–of a LDLT program. The introduction of a LDLT program represents a valuable addition to any existing LT program. It has the potential to enhance access to LT and improve outcomes–particularly relevant given the persistently high wait list delisting and mortality seen in the Netherlands [16].

Ensuring donor safety and minimizing the risk of complications remain the most critical priorities of any live donor program [17]. Institutional experience–including rigorous donor selection processes and comprehensive post donation care–is fundamental to the safety and overall success of LDLT programs [18]. At Erasmus MC, the majority of donations and transplants have involved right lobe grafts, primarily due to the liver volume required by recipients.

Establishing a successful and sustainable LDLT program requires deliberate strategies to address and overcome professional resistance [19]. A persistent concern in many Western countries has been the ethical dilemma of subjecting healthy individuals to the inherent risk of major surgery. However, this resistance tends to diminish when transplant teams are confident that donor safety is prioritized above all else, and when living liver donation is clearly based on informed consent and a deep respect for individual autonomy [19]. At our centre, all donors have expressed satisfaction with their decision to donate and none have reported regret. Donor follows up at Erasmus MC focuses not only on physical recovery, but also emotional and psychological wellbeing. Donors' quality of life is actively assessed through self-reported questionnaires which donors complete pre donation, 6 weeks, 3 months, 6 months, 12 months and yearly post donation. Notably, we have had not observed major late-term complications aside from incisional hernias. From the inception of the program, a steadfast commitment to the principle "donor safety first" has fostered widespread acceptance and support of living liver donation and LDLT within our institution. With increasing experience, we have gradually expanded the program to include more complex recipient cases, for example, recipients after liver resections, after Whipple procedures, patients with polycystic liver disease and patients with partial portal vein thrombosis. Our growing expertise has also enabled us to perform more technically advanced procedures including domino LDLTs, manage small-for-size syndrome after LDLTs, perform left lobe donations, and reconstruction of segment 5/8 veins in right lobes using PTFE grafts. Looking ahead, we anticipate that we will be able to offer LDLT to increasingly complex recipients, such as patients requiring re-transplantation [20].

LDLT recipient outcomes at our centre have been highly encouraging, with excellent patient and graft survival and an acceptable rate of postoperative complications. LDLT offers a substantial survival benefit to patients with end stage liver disease. Even recipients with MELD scores as low as 11 have an additional 13–17 years of life expectancy compared to similar patients at our centre who did not receive a LDLT [21]. Within the LDLT program at Erasmus MC, the 1-year graft and patient survival after LDLT was 97%. LDLT outcomes typically improve with increasing experience; centres performing less than 20 LDLT annually usually report poorer outcomes [22]. Our high success rate is likely attributed to the significant planning and development that preceded our first LDLT; as well as the strict selection criteria applied to our LDLT recipients.

Despite the high one-year graft and patient survival rates, postoperative complications were observed in 40% of patients within the first 90 days after LDLT (**Table 8**). This is consistent with existing literature, where complication rates of up to 47% within the first 90 days postoperatively have been reported–most commonly biliary, vascular and haemorrhagic complications [18]. A significant number of recipients underwent hepaticojejunostomies, likely due to the high prevalence of patients with PSC as an indication for LT for whom this technique is routinely employed. While this procedure is also often performed in the DDLT setting at our institution, it is well documented that this approach carries an increased risk of biliary complications [23]. Although limiting donor selection to those with favourable anatomy could potentially reduce the incidence of biliary complications–by increasing the feasibility of duct-to-duct anastomosis–it is challenging to justify excluding otherwise ideal donors based solely on biliary anatomy, especially given the already stringent donor criteria in place.

CONCLUSION

We successfully established a LDLT program in the Netherlands, achieving excellent early outcomes for both donors and recipients. One year graft and patient survival was 97% and 97% respectively, and no donor mortality was observed. The number of LDLTs increased annually, reflecting growing confidence and experience among both patients and healthcare providers. The importance of allowing time for all stakeholders to adapt to and gain trust in the LDLT process cannot be overstated. While the establishment of an LDLT program presents many unique challenges, these can be successfully overcome through careful planning, dedication and commitment.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

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

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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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LETTER TO THE EDITOR
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Should Systematic HSV Serological Screening of Donors Be Implemented to Manage Mismatched HSV D+/R– Liver Transplants?

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Dear Editors,

Solid organ transplant (SOT) recipients are at high risk of infection due to immunosuppressive therapy, particularly in the early post-transplant period. Although guidelines for cytomegalovirus (CMV) are well-established, herpes simplex virus (HSV) recommendations are less clear and vary among countries. CMV risk management strategy for SOT recipients depends on the serological status of the donor (D) and the recipient (R). In liver transplantation, mismatched CMV-D+/R- or R+ patients may receive valganciclovir prophylaxis which is typically shorter for R+. Alternatively, they may undergo a preemptive approach based on regular CMV PCR monitoring [1–4]. Current guidelines HSV-specific prophylaxis (acyclovir or valacyclovir) primarily target HSV-R+ patients without prophylaxis for CMV and some of them propose to also consider HSV-D+/R- patients (**Supplementary Material S1**) [1–3, 5–8]. Transmission may occur through close contact or be donor-derived and can lead to moderate or severe infections, particularly in immunocompromised patients. However, while screening for several viral infections is routinely performed in donors, HSV serology is not consistently included—often due to the presumed high seroprevalence. This letter aims to highlight this paradox, which hinders the optimal management of patients following the transplantation. To illustrate, we describe our 2023 liver transplant (LT) cohort—from the second largest center nationally—based on CMV and HSV serological status and discuss the management of two mismatched HSV-D+/R- cases.

In 2023, our center performed 122 LT, with recipients averaging 55.3 ± 12.1 years of age, the majority of whom were men (83/122; 68.0%). HSV1/2 IgG testing (Liaison® XL HSV-1/2 IgG, DiaSorin), is included in recipient screening prior to transplantation, without distinction between anti-HSV-1 and HSV-2 IgG. Among the 122 LT recipients tested, 77.0% (94/122) were positive, 19.7% (24/122) negative, and 3.3% were uncertain (4/122) as signals were near the assay's detection threshold (**Table 1A**). Regarding CMV IgG status, 39.3% (48/122) of patients were positive and 60.7% (74/122) negative (**Table 1A**). HSV-R+ patients benefit from a clinical monitoring regardless of CMV prophylaxis. Among HSV-seronegative or uncertain status recipients, 21.4% (6/28) were CMV-D±/R+, 39.3% (11/28) CMV-D-/R- and 39.3% (11/28) CMV-D+/R- (**Table 1B**). Mismatched CMV-D+/R-patients received valganciclovir prophylaxis (450-mg twice daily if normal renal function) for 3 months, which may be effective in preventing HSV infection. No specific HSV virological follow-up was planned. Patients with CMV-R+ status benefit from a preemptive approach and CMV-D-/R-patients only benefit from a clinical follow-up. In all cases, there is no HSV systematic monitoring. Notably, 60.7% (17/28) of HSV-R- did not receive any antiviral prophylaxis or

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TABLE1 | Serological status for Herpes Simplex Virus (HSV) and Cytomegalovirus (CMV) in patients undergoing liver transplantation in the year 2023 in a French center. Data extracted from laboratory information system. Serologies performed on the Liaison XL, DiaSorin®.

A	anti-HSV 1/2 IgG		anti-CMV IgG	
	n	%	n	%
Total	**122**	-	**122**	-
Positive	94	77.0	48	39.3
Negative	24	19.7	74	60.7
Uncertain	4	3.3	0	0

B	HSV negative or uncertain serostatus (n = 28)			
	n	%	Recipient management	Protection against HSV
CMV R+	6	**21.4**	CMV virological monitoring with pre-emptive treatment	No
CMV D-/R-	11	**39.3**	No special follow-up (CMV PCR if clinical signs)	No
CMV D+/R-	11	**39.3**	**Prophylaxis by valganciclovir during 3 months**	**Yes**

A. Anti-HSV IgG (with no distinction between HSV-1 and 2) and anti-CMV IgG status of the 122 liver transplants patients. B. Anti-CMV IgG donor-recipient status in HSV-seronegative patients and management recipient in the centre. R, recipient; D, donor; uncertain, ratio around the technique's detection threshold.

specific follow-up to prevent a possible primary HSV infection that could eventually be transmitted by the donor or close contact (**Table 1B**). In our cohort, knowledge of the HSV donor status could have been relevant for at least 14% (17/122) of the recipients but clinicians requested HSV serology for only two cases. In the first case, a 67-year-old male recipient was HSV seronegative, prompting the clinician to request HSV serology. The donor was HSV seropositive. The patient ultimately received valganciclovir prophylaxis due to a CMV D+/R–status. In contrast, the second case, a 43-year-old female did not receive any prophylaxis because she was CMV-D-/R- (**Supplementary Material S2**). She developed primary HSV-1 infection 27 days post-transplantation (DPT) and presented with acute hepatitis and gingivostomatitis. Retrospective HSV nucleic acid test (NAT) revealed that active HSV infection began very early (6 DPT). After two negative plasma HSV NAT results during acyclovir treatment and clinical improvement, a secondary prophylaxis with valacyclovir was continued for 1 month. After the diagnosis of HSV infection in the recipient, retrospective testing of the donor confirmed HSV-seropositivity. HSV DNA was also detected in the donor's respiratory and blood (<107 copies/mL; ct = 35; HSV-1 HSV-2 R-GENE®) samples, potentially contributing to early transmission and infection in the recipient. This case highlights the potential benefit of systematic HSV donor screening to guide early post-transplant management.

Guidelines for HSV prophylaxis in SOT recipients generally recommend prophylaxis for HSV-R+ based on CMV status. Although prophylaxis reduces the risk of HSV reactivation, the lack of data on HSV-related morbidity in HSV-R+ limits our ability to fully assess the disease burden underlying this recommendation [9]. American Society of Transplantation recommends HSV-specific prophylaxis for at least 1 month in HSV-R+ without CMV prophylaxis and suggest it only at the clinician's discretion for R-patients, without specifying a duration [1]. Recent European guidelines recommend HSV prophylaxis (e.g., valacyclovir) for D+/R– recipients without CMV prophylaxis. If the donor's status is unknown and the recipient is seronegative, management should follow the D+/R– approach [3]. This is consistent with the proposals by Arana et al. in 2022 [5]. Although HSV donor serology may be useful for diagnostic purposes, there are currently no guidelines recommending universal HSV screening in donors. Only the Swiss Transplant Infectious Diseases working group modified its national recommendations and proposed pretransplant HSV serostatus determination in liver recipients and donors to guide HSV prophylaxis in HSV-D+/R- mismatches [6]. Indeed, HSV serologic testing in donors is generally not recommended based on the known globally high HSV seroprevalence. Nonetheless in Northern countries, fewer than 60% and 20% of people under the age of 50 are infected with HSV-1 and HSV-2, respectively [10–12]. Besides, HSV-1/2 seroprevalence is decreasing by 1% per year, particularly among young people in Europe and the United States [1, 10, 11]. In 2023, about 1 in 5 LT recipients were HSV-seronegative in our center, a proportion expected to rise. As donor age increases, young organ recipients may face a higher risk of contracting HSV from HSV-D+. Despite the constantly evolving landscape of infectious disease screening, the lack of recommendations for HSV screening in donors highlights inconsistencies in HSV prevention.

HSV infection in SOT recipients should not be overlooked, especially given the decreasing HSV seroprevalence. Based on our experience and in line with the recent recommendations from the European council and Switzerland, we advocate routine HSV serological testing of donors to guide early post-transplant management, especially in HSV-D+/R-mismatched patients. In absence of CMV prophylaxis (Valganciclovir), HSV-specific prophylaxis (e.g., Valacyclovir) should be initiated for at least 1 month (**Supplementary Material S3**). Upon detection of CMV DNAemia during follow-up, valacyclovir can be stopped when valganciclovir is initiated. While universal prophylaxis for seronegative recipients without known donor status is recommended in some centers, including in European and Australian guidelines, we prefer a more individualized approach to limit patient exposure to multiple pharmacological agents [2]. Further studies are needed to determine whether a systematic approach to HSV donor screening, universal prophylaxis or a preemptive approach (weekly HSV PCR monitoring, similar to CMV protocols), would be the most cost-effective and clinically appropriate strategy for early post-transplant care. Given HSV's tropism for certain grafts—particularly the liver—and the potential severity of primary infection, prevention should remain a priority. This is especially important as CMV prophylaxis duration may be shortened in the future based on CMV-specific cell-mediated immunity (CMV-CMI) results, which must not distract from the risk of HSV infection. Since novel CMV antivirals like letermovir—currently recommended for

kidney transplant prophylaxis and expected to be approved for other organs—do not cover HSV, ensuring adequate HSV-specific prophylaxis remains essential to prevent HSV infection [4]. Lastly, behavioral counseling should be provided to reduce the risk of transmission in all HSV-seronegative recipients.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving humans were approved by Ethics Committee of the Rennes University Hospital. 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 interpretation of the studies and review of the manuscript; CP conceptualized the design, JB collected datas and wrote the manuscript, PH-D, CC, and BG were in charge of the patients. All authors contributed to the article and approved the submitted version.

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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.2025.14835/full#supplementary-material

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BK Virus: Beyond Nephropathy Metastatic BK Virus-Induced, Donor-Derived Bellini's Carcinoma in a Kidney Allograft Recipient: Boosting Rejection to Treat the Cancer

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Dear Editors,

BK virus (BKV), present in 80%–90% of the population, establishes a lifelong persistent infection in the kidney and urinary tract after a subclinical primary infection. It can reactivate and cause *de novo* infection in immunocompromised kidney transplant recipients (KTRs) lacking neutralizing antibodies against the donor strain [1], causing nephropathy (BKVAN) in 4%–8% of cases. Persistent BKV infection increases the risk of urothelial carcinoma and collecting duct carcinoma (CDC) [2].

A 73-year-old KTR was admitted for asthenia, acute kidney injury (creatinine 320 μmol/L), inflammatory syndrome (CRP 130 mg/L), and anaemia (Hb 75 g/L). He was followed for a KT performed 9 years earlier, complicated by biopsy-proven BKVAN at month 10. Mycophenolate mofetil was switched to everolimus (3–8 ng/mL), then to leflunomide, and tacrolimus to ciclosporin (80–120 ng/mL). The viral load decreased over 5 months and BKV was never detected again in the blood. At admission, MRI revealed a hypovascular mass in the graft with central necrosis and retroperitoneal inflammation. Biopsy confirmed a tumour composed of irregular tubular structures, trabeculae and single cells (**Figure 1**). The nuclei had a high mitotic index. Necrotic changes were observed. This tumour proliferation infiltrated between non-tumour and dysplastic premalignant tubules (**Figure 1A**). Immunohistochemistry showed diffuse positivity of tumour cells for PAX8, CK7, INI1, fumarate hydratase, and SDHB, and focal positivity for GATA3 (**Figure 1B**), but negativity for CK20, p504S, p63, or ALK. Only tumour cells showed strong nuclear staining with anti-SV40 large T-antigen (**Figure 1C**), leading to the diagnosis of BKV-associated CDC. No metastases were initially found, and transplantectomy was performed. On pathological examination, the tumour invaded the surgical margins of the transplantectomy. Immunosuppressive therapy was tapered by withdrawing leflunomide and reducing tacrolimus trough levels, but not entirely discontinued in order to minimize the risk of donor-specific alloimmunization. Two months later, PET/CT showed iliac, retroperitoneal, pelvic lymph node metastases, and a right ischiopubic bone metastasis. Bulk HLA genotyping of the biopsy revealed that the tumour was

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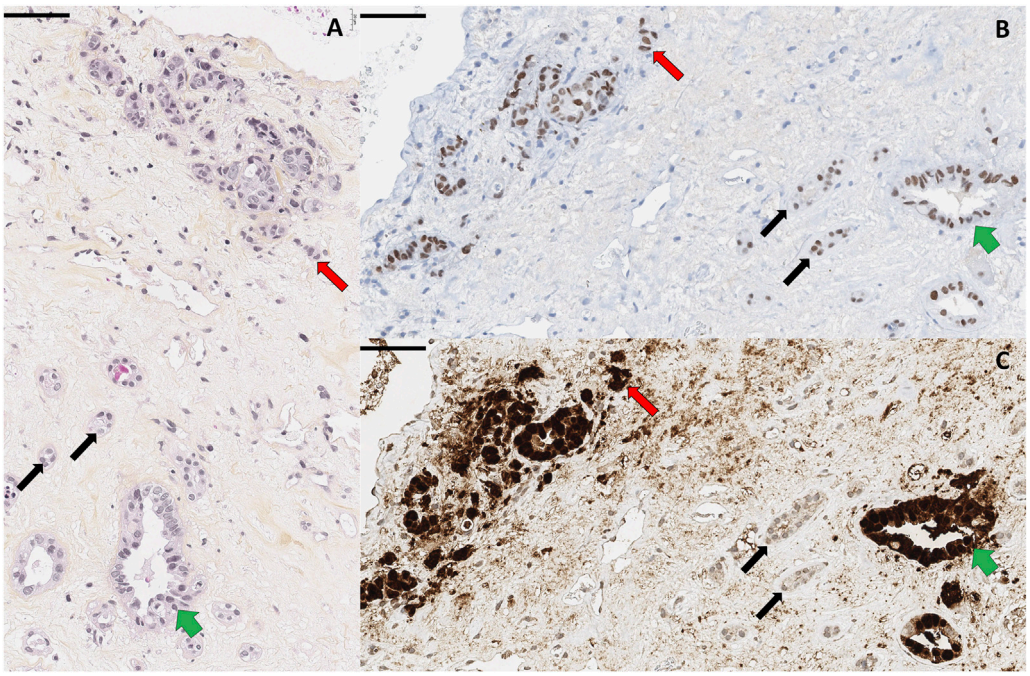

FIGURE 1 | Pathological findings. **(A)** Haematoxylin-Eosin-Safran staining showing infiltrative carcinomatous cells (red arrows), dysplastic premalignant (green arrows) and normal (black arrows) renal tubules. **(B,C)** Immunohistochemical examinations showing PAX8 [renal origin, **(B)**] and Sv40 [viral antigen, **(C)**] labelling of the tumour. Scale bars 60 µm.

not of recipient origin. Immunosuppression was completely withdrawn to stimulate the allo-immune anti-tumoral response, and the patient achieved complete metastatic regression within 3 months. At 2 years, he remained recurrence-free.

This is a very rare case of metastatic donor-derived BKV-induced CDC in a KTR, successfully managed without chemotherapy nor immunotherapy. Bellini's CDC is a rare (<1%) and aggressive variant of renal cell carcinoma [2]. It has been hypothesized that CDC could be linked to BKV in transplanted patients [3]. No other specific risk factor have been identified. The tumorigenesis induced by BKV is known. Polyomaviruses encode 2 viral oncogenes, the small and the large T-antigen [4, 5]. They can inactivate tumour suppressor genes p53 and pRb. Deletion of p53 and pRB leads to gene instability and replication errors that contribute to oncogenesis. Dysregulation of large T-antigen, with persistent over-expression in non-lytic cells, promotes cell growth, genetic instability and neoplasic transformation [6, 7]. The high levels of large T-antigen expression in tumour nuclei is visualized by SV40 staining in immunohistochemistry. Microdissected samples of neoplasic cells usually contain DNA sequences specific for segments of BK-polyomavirus large T-antigen and VP1 genes. On the contrary, no BKV DNA sequences are detected in microdissected normal renal parenchyma [8]. Donor-derived tumours in KTRs are rare (<0.1%) and may arise from donor cells predisposed to oncogenesis. Key oncogenic drivers occur as early as late childhood and early adolescence. Then, late events during transplantation and under immunosuppression, such as BKV infection and genomic integration, may promote further oncogenesis in donor renal cells [9]. These donor-derived tumours offer a unique treatment opportunity: withdrawal of immunosuppression led to spontaneous alloimmune tumour rejection by enabling the immune system to target the graft through alloimmune and antitumour responses. Ortega *et al* reported remission of a metastatic donor-derived urothelial tumour after transplantectomy and immunosuppression withdrawal [10]. Meier *et al* achieved similar success in a metastatic Bellini carcinoma by boosting the anti-tumour immune response with IL-2 immunotherapy [3] (**Supplementary Table S1**).

This case highlights the specificity of urological tumours in KTRs. Identifying donor-derived malignancies may refine treatment strategies, reducing reliance on aggressive therapies. The clinical history reported in this case suggests pragmatic management, although this is by no means a recommendation. Firstly, given the very unfavourable prognosis of these tumours, it seems legitimate to perform surgery and completely stop immunosuppression. The two expected benefits of surgery are the removal of the largest possible tumour mass, and the avoidance of symptomatic toxic graft rejection. The addition of immunotherapy or chemotherapy should be discussed on a case-by-case basis, after evaluating the efficacy of the initial treatment. Given BKV's oncogenic potential, long-term monitoring should

extend beyond the risk of nephropathy to include surveillance for malignancy. Options could include annual urinary cytology screening, early invasive urological evaluation in the event of haematuria and potentially biannual imaging of the graft.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

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

FL and XC were responsible for data collection and interpretation and drafting the article. FL and XC generated the figure. 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.

GENERATIVE AI STATEMENT

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

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2025.14664/full#supplementary-material

SUPPLEMENTARY TABLE S1 | table summarizing post-transplant CDC cases linked to BK virus.

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