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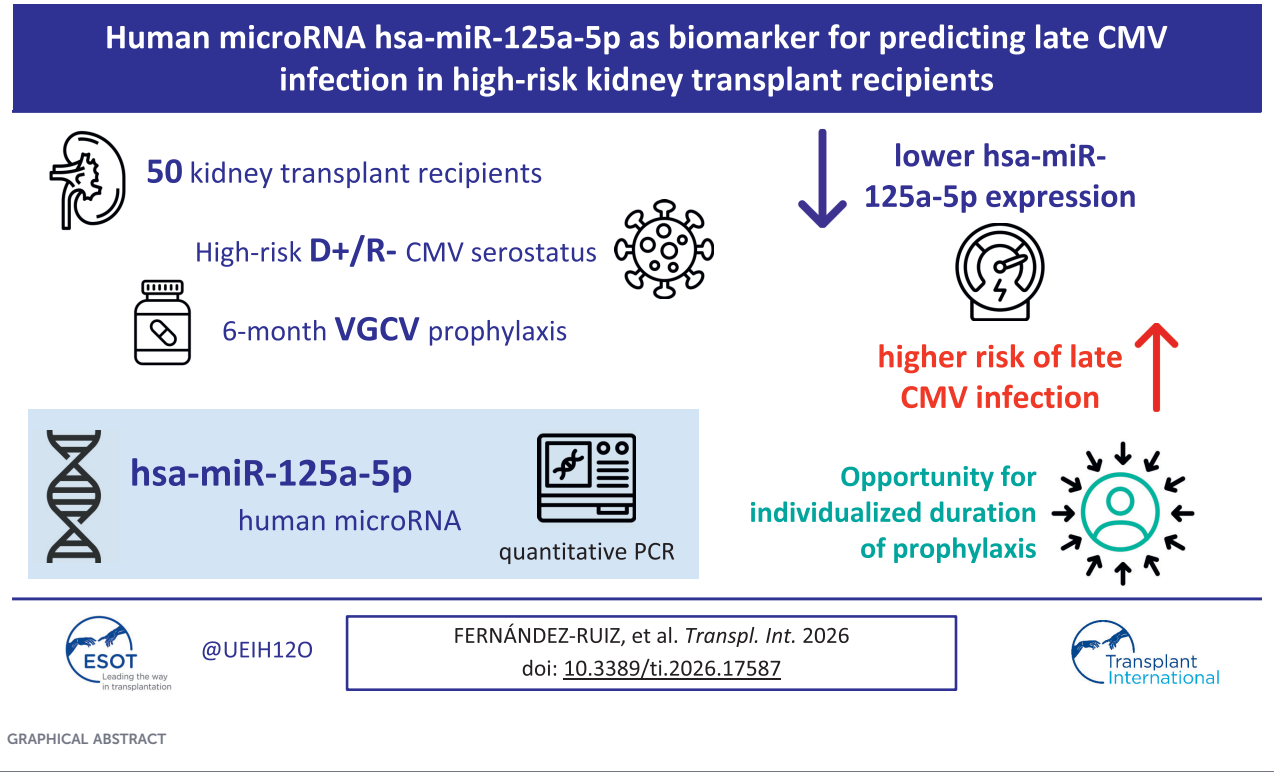
Human microRNA hsa-miR-125a-5p as biomarker for predicting late CMV infection in high-risk kidney transplant recipients

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Available strategies for predicting late-onset cytomegalovirus (CMV) infection in high-risk (seropositive donor/seronegative recipient [D+/R-]) kidney transplant recipients (KTRs) are suboptimal. We have previously shown that the expression level of human microRNA hsa-miR-125a-5p informs on the risk of CMV reactivation in seropositive (R+) KTRs. In the present work we assessed the predictive role of this microRNA in 50 consecutive D+/R- KTRs that received a 6-month course of valganciclovir prophylaxis. hsa-miR-125a-5p levels were measured by quantitative PCR in whole-blood samples obtained at the time of valganciclovir discontinuation. The cumulative incidence of late CMV infection through post-transplant month 12 was 46.0%. Patients who developed late CMV infection exhibited lower relative hsa-miR-125a-5p expression than those who remained free from this event (ΔCt values: 7.5 ± 1.3 vs. 6.7 ± 0.7, respectively; *P*-value = 0.005). The area under the receiver operating characteristic curve for predicting late CMV infection was 0.718 (95% confidence interval [CI]: 0.559–0.877). After multivariable adjustment, lower hsa-miR-125a-5p expression (ΔCt >7.623) was independently associated with the occurrence of late CMV infection (adjusted hazard ratio: 5.63; 95% CI: 2.28–13.91; *P*-value < 0.001). In conclusion, the relative expression of hsa-miR-125a-5p is useful to stratify the risk of late CMV infection in high-risk D+/R- KTRs.

KEYWORDS
biomarkers, cytomegalovirus, donor seropositive/recipient seronegative, kidney transplantation, microRNAs



Introduction

Cytomegalovirus (CMV) remains as an important cause of disease, graft loss or even mortality in solid organ transplant (SOT) recipients, particularly among high-risk groups [1]. In current clinical practice, risk stratification is primarily based on the combination of donor (D) and recipient (R) serostatus, with the highest incidence observed in CMV-seronegative recipients of organs from CMV-seropositive donors (D+/R-) [2, 3]. Although guidelines recommend either antiviral prophylaxis for 3–12 months (depending on the transplant type) or preemptive therapy as the prevention strategy in the D+/R- population [3, 4], prophylaxis is usually preferred due to logistical reasons [5]. However, the development of late CMV infection following the discontinuation of prophylaxis remains an unresolved concern [6, 7], with one-year cumulative incidence rates of CMV DNAemia and disease that reached 37.4% and 16.1%, respectively, among kidney transplant recipients (KTRs) recruited in the 200-day arm of the IMPACT trial [8]. In addition, late CMV disease has been associated with poorer graft outcomes [9, 10]. Accordingly, substantial efforts have been directed toward improving risk stratification for late CMV infection beyond serological status and therapeutic drug monitoring (TDM) of immunosuppressive agents, with the ultimate aim of tailoring the duration of prophylaxis rather than relying on a "one-size-fits-all" approach.

The functional assessment of CMV-specific T-cell-mediated immunity (CMV-CMI) has emerged as a promising tool for risk stratification [11]. However, there is still no consensus on the optimal method or monitoring strategy for clinical implementation [3, 4]. Evidence available for D+/R- patients remains limited, since most trials and observational studies have included other risk categories, such as seropositive (R+) recipients treated with T-cell-depleting agents [12–15]. In addition, only a minority of patients develop detectable CMV-CMI responses at the end of prophylaxis. As a consequence, the latest international guidelines formally recommend against routine CMV-CMI monitoring in the D+/R- subgroup [4]. On the other hand, currently available CMV-CMI assays are, to varying degrees, time-consuming and require specialized laboratory equipment [16].

Human *(Homo sapiens)* microRNAs (hsa-miRNAs) are small (approximately 17–25 nucleotides in length) non-coding RNA molecules that are highly conserved across species and exert regulatory functions at the post-transcriptional gene expression level [17]. Since their discovery in 1993, hsa-miRNAs have attracted increasing interest in a wide range of pathologies, including cancer, autoimmune disorders, and infection [18–20]. A growing body of evidence highlights the central role of hsa-miRNAs in patients with viral infections, such as hepatitis C, dengue, COVID-19, or Crimean-Congo hemorrhagic fever [21–25]. By modulating the expression of genes responsible for immune responses, hsa-miRNAs can promote or inhibit the

Abbreviations: auROC, area under receiver operating characteristic curve; CI, confidence interval; CMV, cytomegalovirus; CMV-CMI, cytomegalovirus-specific T-cell-mediated immunity; Ct: cycle threshold; D, donor; EPV, events-per-variable; HR, hazard ratio; hsa-miRNA, *Homo sapiens* microRNA; IFN, interferon; IGRA, interferon-γ release assay; IL, interleukin; IQR, interquartile range; KTR, kidney transplant recipient; mTOR, mammalian target of rapamycin; NPV, negative predictive value; PCR, polymerase chain reaction; PPV, positive predictive value; R, recipient; RT-qPCR, real-time quantitative polymerase chain reaction; SD, standard deviation; SOT, solid organ transplantation; TDM, therapeutic drug monitoring; TNF, tumor necrosis factor.

progression of infection. Moreover, some hsa-miRNAs can directly target viral genes [26]. Accordingly, hsa-miRNAs are investigated both as diagnostic and prognostic biomarkers and as novel therapeutic targets [27, 28].

In the SOT population, our group has recently described the role of a differentially expressed hsa-miRNA to stratify the risk of CMV infection in seropositive (R+) KTRs who developed viral reactivation despite exhibiting a theoretically protective CMV-CMI response measured by a commercial interferon-γ release assay (IGRA). We observed that hsa-miR-125a-5p was overexpressed in patients who remained free of CMV infection and defined an optimal cycle threshold (Ct) cut-off value of 7.375. The relative expression of hsa-miR-125a-5p yielded good predictive capacity, particularly in preemptively managed patients (area under the receiver operating characteristic curve [auROC] of 0.865) [29].

Given these results, the unmet need for clinically actionable biomarkers to individualize the management of high-risk patients and the limited value of CMV-CMI monitoring in this population, we investigated hsa-miR-125a-5p expression in a cohort of D+/R- KTRs at the time of discontinuation of antiviral prophylaxis. We further evaluated the performance of hsa-miR-125a-5p to predict the development of late CMV events during the 6 months following sample collection.

Materials and methods

Study population

The present study was based on a prospectively maintained database that recruited consecutive adult KTRs at our center since November 2014 [30–32]. The occurrence of primary graft non-function or early graft loss (within the first week) was an exclusion criterion. Patients were included at the time of transplantation and followed-up for a minimum period of 12 months (unless graft loss or death occurred earlier). Pre-transplant, peri-operative and post-transplant variables were collected by means of a standardized case report form and pseudo-anonymized before being entered into a secure REDCap database.

The study was performed in accordance to the Declarations of Helsinki and Istanbul. Participants provided informed consent and the local Clinical Research Ethics Committee approved the protocol (no. 14/030). Methods and results reporting adhered to the STREGA (STrengthening the REporting of Genetic Association studies) guidelines.

Study design and definitions

Consecutive KTRs with high-risk (D+/R-) serostatus at our institution between January 2015 and April 2021 were included. All the patients received prophylaxis with valganciclovir (900 mg once daily, with renal dose adjustment if needed) for 6 months [2, 3]. Whole blood samples were obtained at month 6 (±3 weeks) and stored at −80 °C until the present analysis.

The primary outcome of the present study was the occurrence of late CMV infection (defined as CMV DNAemia detectable at any level) during the first six months after the discontinuation of valganciclovir prophylaxis (i.e., by post-transplant month 12). Late high-level CMV infection (DNAemia ≥1,000 IU/mL) and late CMV disease during this period were analyzed as secondary outcomes.

The diagnosis of CMV DNAemia required polymerase chain reaction (PCR)-confirmed CMV replication in plasma or whole blood specimens regardless of the presence of attributable symptoms, as previously described [32]. CMV DNAemia was monitored monthly during the period of valganciclovir prophylaxis and after its discontinuation until the end of the first post-transplant year, with additional testing performed as clinically indicated. Breakthrough CMV infection was defined as the detection of CMV DNA by PCR while the patient was receiving valganciclovir prophylaxis. Description of immunosuppression and antimicrobial prophylaxis regimens, CMV PCR monitoring, and additional study definitions are available as Supplementary Material.

miRNA extraction

Total RNA was extracted from 50-µL whole-blood samples using the KingFisher Duo Prime platform (Thermo Fisher Scientific, Waltham, MA) and the MagMAX *mir*Vana™ Total RNA Isolation Kit (Applied Biosystems, Foster City, CA). RNA quality was measured in a UV-Vis NanoPhotometer™ N60 spectrophotometer (Implen, Munich, Germany).

Quantification of hsa-miR-125a-5p expression in clinical samples

Complementary DNA (cDNA) was obtained by reverse transcription using the Taqman microRNA RT kit. The Fast Advanced Master Mix with specific TaqMan probes was used for RT-qPCR, according to the manufacturer´s instructions. All samples were run in triplicate and reactions were performed on a QuantStudio 3 real-time PCR system, with Ct values analyzed with the QuantStudio Design and Analysis Software v1.5.1 (all from Applied Biosystems, Foster City, CA). hsa-miRNA expression was normalized to the reference miRNA controls by means of the ΔCt method ($\Delta Ct = Ct_{miRNA} - Ct_{reference\ miRNA}$), with higher ΔCt values corresponding to lower miRNA expression [33].

Assessment of immunosuppression state and systemic inflammation

Peripheral blood lymphocyte subpopulations ($CD3^+$, $CD4^+$ and $CD8^+$ T-cell counts) were measured at month 6 by automated multicolor flow cytometry (BD MultitestTM 6-Color TBNK reagent kit) on the BD FACSCanto II instrument (all from BD Biosciences, San Jose, CA).

In a subset of patients randomly selected (n = 30), the levels of seven different inflammatory cytokines (IFN-γ, interleukin [IL]-1β, IL-2, IL-6, IL-18 and tumor necrosis factor [TNF]-α), along with the anti-inflammatory IL-10, were measured at months 1, 6 and 12. We performed a high-throughput Luminex bead-based multiplex assay (ProcartaPlex™ Human Th1/Th2 12plex kit) according to the manufacturer's instructions. Data acquisition was carried out using the Luminex MAGPIX System, and data analysis was performed with the ProcartaPlex Analysis App (all from Thermo Fisher Scientific, Waltham, MA). Plasma cytokine values below the lowest limit of quantification were assigned a value of 0.001 pg/mL.

Statistical analysis

Quantitative and qualitative variables were described using the mean ± standard deviation (SD), the median with interquartile range (IQR), or absolute and relative frequencies, as appropriate. The normality of the distributions was tested using the Kolgomorov-Smirnov test. Continuous variables were analyzed with either the Student's t-test or Mann-Whitney U test. Pearson's or Spearman's rank correlation coefficients were calculated to examine correlations between continuous variables. Differences in the relative expression of hsa-miR-125a-5p over time were analyzed using the Student's t-test for paired samples. The one-year cumulative incidence of study outcomes was estimated using the cumulative incidence function, accounting for death as a competing event.

The optimal cutoff value for hsa-miR-125a-5p expression (ΔCt) at month 6 for predicting the primary outcome was determined using the Youden's index (J = sensitivity + specificity - 1) [34], whereas discriminative capacity was assessed by the auROC (C-statistic). We calculated diagnostic accuracy in terms of sensitivity, specificity, and positive (PPV) and negative predictive value (NPV). We performed an internal validation analysis using 2,000 bootstrap resamples, in which the entire threshold-selection procedure was repeated. Specifically, the optimal ΔCt cut-off value was re-estimated within each bootstrap sample by maximizing Youden's index. The resulting threshold was subsequently applied to both the bootstrap sample and the original cohort to re-estimate sensitivity, specificity, PPV and NPV. Optimism was quantified as the average difference between the performance estimates obtained in the bootstrap and original samples and was subtracted from the apparent estimates.

Survival probabilities were estimated by the Kaplan-Meier method, and the log-rank test was used to assess differences between KTRs above or below the selected hsa-miR-125a-5p ΔCt cutoff value. The association with the primary outcome was confirmed using a Cox regression model adjusted for clinical variables with univariate *P*-values ≤ 0.05. The use of a mammalian target of rapamycin (mTOR) inhibitor-containing regimen during the first year after transplantation was modeled as a time-dependent covariate. The proportional hazards assumption was assessed using Schoenfeld residuals.

The selected hsa-miR-125a-5p ΔCt cut-off value was also evaluated for the secondary outcomes of late high-level CMV infection and late CMV disease. Given the limited number of events and the fact that this threshold was optimized for the primary outcome, these secondary analyses should be considered as exploratory.

Significance tests were considered significant at a *P*-value < 0.05, except for the multiple correlations between hsa-miR-125a-5p expression and cytokine levels at different time points, for which Bonferroni correction was applied to account for potential *P*-value inflation. Statistical analysis was performed with SPSS version 29.0.1.0 (Statistical Package for Social Sciences, Chicago, IL) and StataNow/MP version 19.5 (StataCorp, College Station, TX), and graphs were generated with Prism version 6.0 (GraphPad Software Inc., La Jolla, CA).

Results

Study cohort

We recruited 50 D+/R- KTRs, whose demographics and clinical characteristic are detailed in Table 1. Supplementary Figure S1 shows the results of TDM for tacrolimus during the first 6 months. All patients received prophylaxis with valganciclovir for a median of 181 days (IQR: 174.5–184.0). During the prophylaxis period, CMV PCR monitoring was performed at a median of 6 time points per patient (IQR: 3.4–7.3). Overall, 6 patients (12.0%) experienced breakthrough CMV infection.

The median interval from discontinuation of prophylaxis to hsa-miR-125a-5p quantification was 6 days (IQR: −2.5–25.5). During the one-year follow-up period (i.e., six months after the discontinuation of prophylaxis), one patient (2.0%) died at day 278 after transplantation.

Overall, 23 patients developed late CMV infection (primary outcome), with a mean DNAemia of 3.65 ± 1.03 $\log_{10}$ IU/mL. The one-year cumulative incidence, accounting for death as a competing event, was 46.0% (95% CI: 32.2–59.8). The median interval from prophylaxis discontinuation to infection was 54.0 days (IQR: 30.0–91.0).

Regarding secondary study outcomes, 15 patients experienced late high-level CMV infection, corresponding to a one-year cumulative incidence of 30.0% (95% CI: 16.6–43.4) and a mean DNAemia level of 4.19 ± 0.87 $\log_{10}$ IU/mL. All but one of these patients (93.3% [14/15]) received antiviral therapy. The one-year cumulative incidence of late CMV disease was 24.0% (95% CI: 12.2–35.8). In detail, 12 patients experienced 9 episodes of viral syndrome, two episodes of probable CMV hepatitis, and one episode of probable CMV colitis.

hsa-miR-125a-5p is downregulated in D+/R- KTRs that developed late CMV infection

We first assessed the relative expression of hsa-miR-125a-5p at month 6 in relation to the reference control hsa-miR-425a-5p by RT-qPCR, as previously described [29]. As noted above, higher ΔCt values correspond to lower (i.e., downregulated) hsa-miR-125a-5p expression. The median interval from the sampling to the first episode of late CMV infection was 36.0 days (IQR: 0.0–52.0). hsa-miR-125a-5p expression was downregulated in patients experiencing late CMV infection within the 6 months after discontinuation of prophylaxis (i.e., post-transplant month 12) compared to those who did not develop this event (ΔCt: 7.5 ± 1.3 versus 6.7 ± 0.7, respectively; *P*-value = 0.005) (Figure 1A).

Regarding exploratory secondary outcomes, a similar—although not statistically significant—difference was observed when hsa-miR-125a-5p expression at month 6 was compared between patients who did and did not develop late high-level CMV infection (ΔCt: 7.5 ± 1.2 versus 6.9 ± 1.0, respectively; *P*-value = 0.060 (Figure 1B). The median interval from sampling to the diagnosis of high-level CMV infection was 36.0 days (IQR: 0.0–49.0). Finally, there were no differences between patients experiencing or not late CMV disease (ΔCt: 7.2 ± 1.4 versus 6.9 ± 0.9, respectively; *P*-value = 0.502) (Figure 1C). The median interval from sampling to the diagnosis of CMV disease was 42.0 days (IQR: 22.0–55.5).

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TABLE 1 Demographics and clinical characteristics of the study cohort (n = 50).

Variable	Values
Recipient age, years [mean ± SD]	49.9 ± 16.3
Recipient gender (male) [n (%)]	41 (82.0)
Prior or current smoking history [n (%)]	19 (38.0)
BMI at transplantation, Kg/m^2 [mean ± SD][a]	25.1 ± 3.7
Pre-transplant chronic comorbidities [n (%)]	
Hypertension	40 (80.0)
Dyslipidemia	20 (40.0)
Diabetes mellitus	18 (36.0)
Coronary artery disease	7 (11.7)
Malignancy	7 (14.0)
Cerebrovascular disease	3 (6.0)
Chronic respiratory disease	3 (6.0)
Previous kidney transplantation [n (%)]	7 (14.0)
Underlying end-stage renal disease [n (%)]	
Diabetic nephropathy	17 (34.0)
Glomerulonephritis	12 (24.0)
Polycystic kidney disease	5 (10.0)
Chronic tubulointerstitial nephropathy	3 (6.0)
Nephroangiosclerosis	2 (4.0)
Reflux nephropathy	1 (2.0)
Unknown	3 (6.0)
Other	7 (14.0)
Positive EBV serostatus (anti-EBNA IgG) [n (%)]	43 (86.0)
Positive HCV serostatus [n (%)]	2 (4.0)
Positive HBsAg status [n (%)]	2 (4.0)
Pre-transplant renal replacement therapy [n (%)]	42 (84.0)
Hemodialysis	28 (56.0)
Continuous ambulatory peritoneal dialysis	14 (28.0)
Time on dialysis, months [median (IQR)]	23.4 (12.3–43.5)
Donor age, years [mean ± SD]	49.3 ± 16.8
Type of transplantation [n (%)]	
Single kidney transplantation	39 (78.0)
Double kidney transplantation	1 (2.0)
Simultaneous kidney-pancreas transplantation	10 (20.0)
Type of donor [n (%)]	
DBD donor	35 (70.0)
Uncontrolled (Maastricht categories 1-2) DCD donor	3 (6.0)
Controlled (Maastricht categories 3) DCD donor	3 (6.0)
Living donor	9 (18.0)
Cold ischemia time, hours [mean ± SD]	13.1 ± 7.0
Number of HLA mismatches [median (IQR)]	4 (3–5)

(*Continued*)

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TABLE 1 Continued

Variable	Values
Induction therapy [n (%)]	
Antithymocyte globulin	25 (50.0)
Number of doses [median (IQR)]	5 (3–6)
Basiliximab	18 (36.0)
None	7 (14.0)
Primary immunosuppression regimen [n (%)]	
Prednisone, tacrolimus, E-MPS/MMF	40 (80.0)
Prednisone, tacrolimus, azathioprine	7 (14.0)
Prednisone, tacrolimus, everolimus	3 (6.0)
Mean tacrolimus trough levels over the first six post-transplant months, ng/mL [mean ± SD]	9.2 ± 1.8
Conversion to mTOR inhibitor [n (%)][b]	15 (30.0)
Median interval to conversion, days	201.0 (168.0–245.0)
Duration of valganciclovir prophylaxis, days [median (IQR)]	181 (174.5–184)
Post-transplant complications	
Delayed graft function [n (%)]	17 (34.0)
Early surgical reintervention [n (%)][c]	5 (10.0)
Renal artery stenosis requiring revascularization [n (%)][b]	5 (10.0)
Major atherothrombotic event [n (%)][b]	2 (4.0)
Acute graft rejection [n (%)][b]	3 (6.0)
Interval from transplantation, days [median (IQR)]	184 (84–299.5)
>1 episode [n (%)]	2 (4.0)
De novo cancer [n (%)][b]	3 (6.0)
All-cause mortality [n (%)][b]	1 (2.0)
Graft loss [n (%)][b]	1 (2.0)

BMI, body mass index; CMV, cytomegalovirus; DBD, donation after brain death; DCD, donation after circulatory death; EBV, Epstein-Barr virus; EBNA, EBV nuclear antigen; HLA, human leukocyte antigen; HBsAg, hepatitis B virus surface antigen; HCV, hepatitis C virus; IQR, interquartile range; E-MPS/MMF, enteric-coated mycophenolate sodium / mycophenolate mofetil; SD, standard deviation.

[a]Data on BMI was not available for 6 patients.

[b]At one-year follow-up.

[c]Within the first post-transplant month.

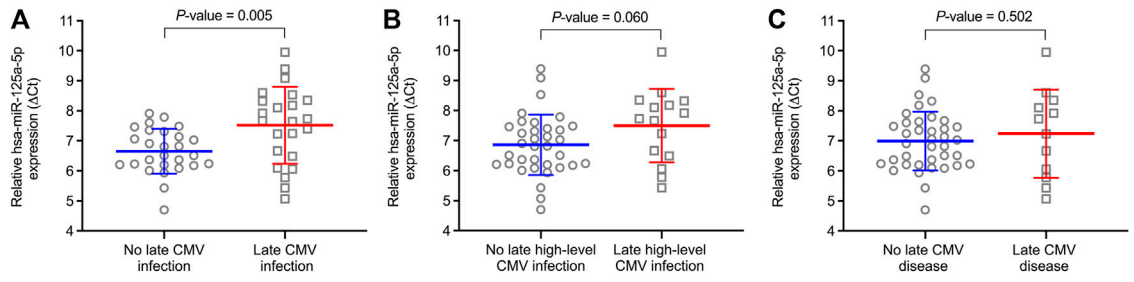


FIGURE 1
Comparison of relative expression (ΔCt values) of hsa-miR-125a-5p by RT-qPCR at post-transplant month 6 according to the occurrence of study outcomes within 6 months following discontinuation of antiviral prophylaxis (i.e., by post-transplant month 12): **(A)** late CMV infection (primary outcome); **(B)** late high-level (DNAemia ≥1,000 IU/mL) CMV infection and **(C)** late CMV disease (secondary outcomes). Horizontal bars and whiskers represent the mean value and SD, respectively. CMV: cytomegalovirus; Ct: cycle threshold.

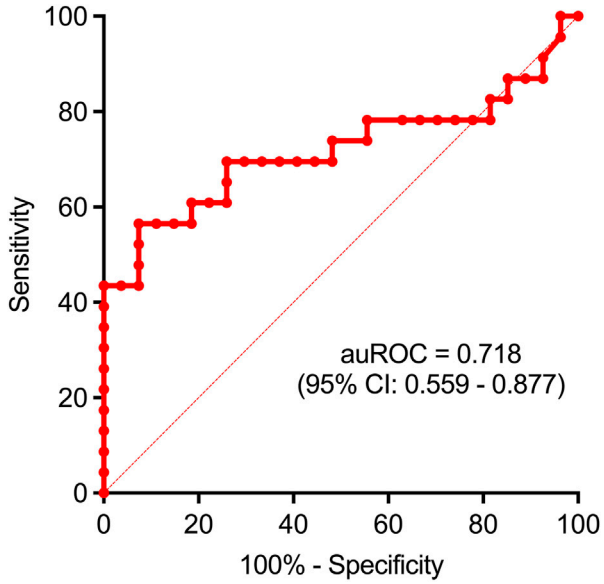


FIGURE 2
Discriminative capacity of the relative expression (ΔCt values) of hsa-miR-125a-5p at post-transplant month 6 to predict the occurrence of late CMV infection. auROC: area under receiver operating characteristic curve; CI: confidence interval.

Lower hsa-miR-125a-5p expression acts as an independent predictor of late CMV infection

We analyzed the discriminative capacity of the relative expression of hsa-miR-125a-5p at month 6 to predict the occurrence of late CMV infection within 6 months after discontinuation of prophylaxis. The auROC was 0.718 (95% confidence interval [CI]: 0.559–0.877) (Figure 2). Internal bootstrap validation yielded an optimism-corrected auROC of 0.718 (95% bootstrap percentile CI: 0.557–0.879), which suggests very limited optimism in the estimation of the discriminatory capacity of the biomarker. When considering the secondary outcome of high-level CMV infection, the auROC decreased to 0.667 (95% CI: 0.477–0.856). The optimism-corrected auROC estimated by bootstrapping was 0.667 (95% bootstrap percentile CI: 0.484–0.849).

On the basis of the Youden's index, we established at ΔCt >7.623 the optimal cut-off value for hsa-miR-125a-5p expression. We evaluated whether stratifying KTRs according to this threshold could predict the occurrence of late CMV infection. Indeed, patients with lower hsa-miR-125a-5p expression (ΔCt >7.623) at month 6 were less likely to remain free of late CMV infection through month 12 (log-rank test P-value < 0.001) (Figure 3A). Similar differences were also found for the secondary outcomes of late high-level CMV infection (log-rank test P-value = 0.001) (Figure 3B) and late CMV disease (log-rank test P-value = 0.048) (Figure 3C).

For the primary outcome of late CMV infection, a ΔCt >7.623 for hsa-miR-125a-5p expression yielded a sensitivity and specificity of 56.5 (95% CI: 34.5–76.8) and 92.6 (95% CI: 75. 7–99.1), respectively, and a PPV of 86.7 (95% CI: 62.0–96.3) (Table 2).

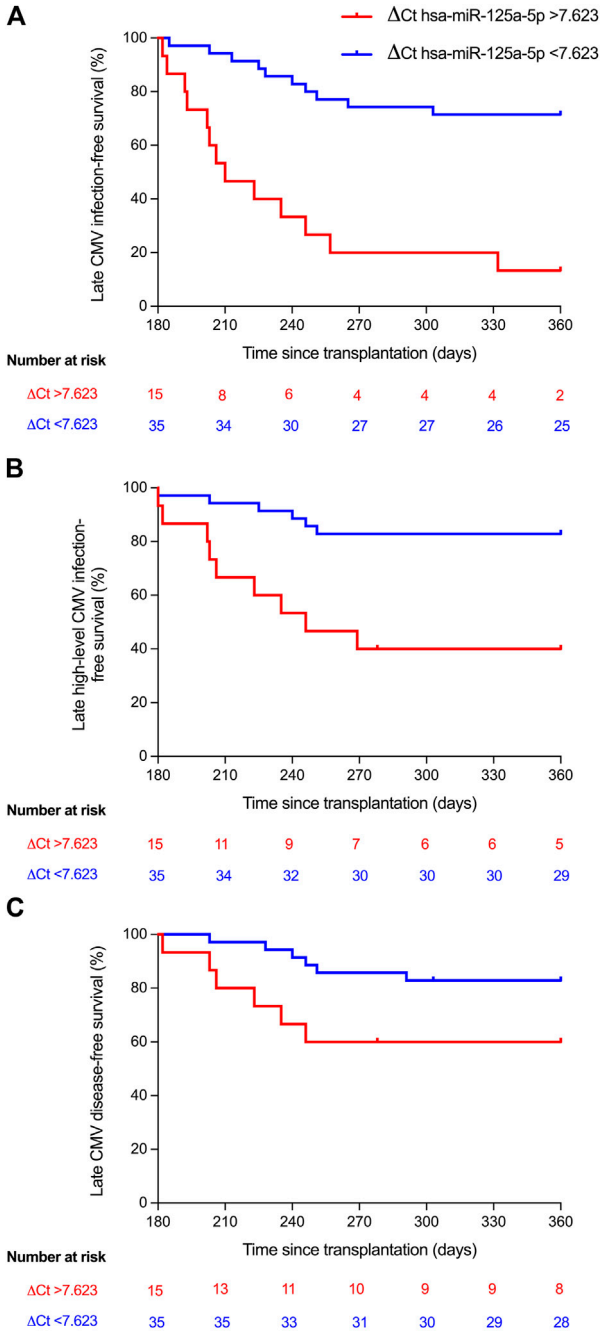


FIGURE 3
Kaplan-Meier event-free survival curves according to the optimal cut-off value for hsa-miR-125a-5p expression at month 6 (ΔCt 7.623): **(A)** late CMV infection (log-rank test P-value < 0.001); **(B)** late high-level CMV infection (log-rank test P-value = 0.001) and **(C)** late CMV disease (log-rank test P-value = 0.048). CMV: cytomegalovirus.

The median bootstrap-derived optimal cut-off was ΔCt >7.623 (IQR: 7.376–7.634), closely matching the original threshold. The estimated optimism was 3.4, 3.7, 4.8, and 2.4 percentage points for sensitivity, specificity, PPV, and NPV, respectively. Accordingly, the optimism-corrected specificity and PPV were 88.9% and 81.9% (compared with the original estimates of 92.6% and 86.7%).

To determine whether the association between the relative expression of hsa-miR-125a-5p at month 6 and late CMV

TABLE 2 Diagnostic accuracy of the optimal cut-off value for hsa-miR-125a-5p expression (ΔCt 7.623) at month 6 to predict the primary and secondary study outcomes.

Predicted outcome	Sensitivity (% [95% CI])	Specificity (% [95% CI])	PPV (% [95% CI])	NPV (% [95% CI])	PLR (95% CI)	NLR (95% CI)
Late CMV infection	56.5 (34.5–76.8)	92.6 (75. 7–99.1)	86.7 (62.0–96.3)	71.4 (60–8 – 80.1)	7.6 (1.9–30.4)	0.5 (0.3–0.8)
Late high-level CMV infection	60.0 (32.3–83.7)	82.9 (66.4–93.4)	60.0 (39.4–77.6)	82.9 (71.9–90.1)	3.5 (1.5–8.1)	0.5 (0.3–0.9)
Late CMV disease	50.0 (21.1–78.9)	76.3 (59.8–88.6)	40.0 (22.9–59.8)	82.9 (72.8–89.7)	2.1 (0.9–4.7)	0.7 (0.4–1.2)

CI, confidence interval; CMV, cytomegalovirus; NLR, negative likelihood ratio; NPV, negative predictive value; PLR, positive likelihood ratio; PPV, positive predictive value.

infection remained after multivariable adjustment, we first identified the clinical risk factors through univariate analysis (Supplementary Table S1). In the Cox regression model adjusted for pre-transplant diagnosis of coronary heart disease and chronic lung disease and use of an mTOR inhibitor during the first post-transplant year (time-dependent covariate), lower hsa-miR-125a-5p expression (ΔCt >7.623) at month 6 acted as an independent predictor of late CMV infection (adjusted hazard ratio [HR]: 5.63; 95% CI: 2.28–13.91; *P*-value < 0.001). The final model included four parameters, yielding an events-per-variable (EPV) ratio of 5.8. No substantial deviations from the proportional hazards assumption were detected (*P*-value for global test = 0.315).

This association remained essentially unchanged after forcing tacrolimus levels at month 6 into the model (adjusted HR: 5.27; 95% CI: 1.88–14.72; *P*-value = 0.002), or the occurrence of breakthrough CMV infection during the preceding prophylaxis period (adjusted HR: 6.23; 95% CI: 2.40–16.18; *P*-value < 0.001). Of note, these additional inclusions further reduced the EPV ratio, and potentially increased the risk of model overfitting.

We performed a sensitivity analysis using a restricted adjustment set to assess the robustness of the association between hsa-miR-125a-5p and the primary outcome. To this end, we specified a parsimonious model comprising a minimal set of clinically relevant covariates with established associations with late CMV infection—recipient age and induction therapy with antithymocyte globulin—in addition to hsa-miR-125a-5p ΔCt >7.623. The association between the biomarker and the primary outcome remained statistically significant (adjusted HR: 5.93; 95% CI: 2.54–13.84; *P*-value <0.001), consistent with the findings of the primary multivariable analysis.

Within the three-week window allowed between the discontinuation of prophylaxis and blood sampling for hsa-miR-125a-5p quantification, three patients (6.0%) experienced CMV infection. To preserve the independency of observations with study outcomes, we performed a second sensitivity analysis excluding these three patients, which did not substantially alter the association found between ΔCt >7.623 for hsa-miR-125a-5p expression at month 6 and late CMV infection (adjusted HR: 4.26; 95% CI: 1.79–11.96; *P*-value = 0.002).

When hsa-miR-125a-5p expression at month 6 was analyzed as a continuous variable rather than dichotomized according to the optimal cut-off value, the association with late CMV infection remained significant in both univariable (HR [per one-unit increase in ΔCt]: 2.42; 95% CI: 1.49–3.93; *P*-value < 0.001) and multivariable models (adjusted HR [per one-unit increase in ΔCt]: 2.26; 95% CI: 1.32–3.87; *P*-value = 0.03).

hsa-miR-125a-5p expression is relatively stable over time and independent of the degree of immunosuppression, systemic inflammation, and previous breakthrough CMV infection

To assess the kinetics of hsa-miR-125a-5p expression over time, we also measured ΔCt values by RT-qPCR in samples obtained at baseline (within the 3–4 h before to transplantation) and at post-transplant months 1 and 12 (available for 35, 46 and 45 patients, respectively). There was a significant increase between months 1 and 6 (ΔCt: 6.5 ± 1.2 versus 7.1 ± 1.1, respectively; *P*-value for repeated measures = 0.012), whereas no significant differences were found between any other time points (Figure 4).

We explored whether the relative expression of hsa-miR-125a-5p may be influenced by the net state of immunosuppression—as assessed by TDM and immunological parameters—or the plasma inflammatory *milieu*. There was no significant correlation between hsa-miR-125a-5p levels (as measured by the ΔCt values) and tacrolimus trough levels at month 6 (Pearson's coefficient: −0.228; *P*-value = 0.136), nor with mean tacrolimus levels during the first six post-transplant months (Pearson's coefficient: 0.015; *P*-value = 0.918). Likewise, no correlations were observed with $CD3^+$ (Spearman's Rho: −0.153; *P*-value = 0.290), $CD4^+$ (Spearman's Rho: −0.168; *P*-value = 0.234), or $CD8^+$ T-cell counts (Spearman's Rho: −0.062; *P*-value = 0.667) measured at month 6.

Regarding the potential association between the relative expression of hsa-miR-125a-5p and plasma cytokine levels measured at different time points, only moderate correlations with IL-1β and IL-6 levels at month 1 remained significant after Bonferroni correction (Spearman's rho = 0.549; *P* = 0.0016 for both cytokines). Of note, no correlations were observed between hsa-miR-125a-5p expression at month 6 and the plasma cytokine *milieu* at that point (Supplementary Figure S2).

Finally, we confirmed that the relative expression of hsa-miR-125a-5p at month 6 did not differ according to the occurrence of breakthrough CMV infection during the preceding prophylaxis period (ΔCt: 7.1 ± 1.4 versus 7.1 ± 1.1 for patients with and without breakthrough infection, respectively; *P*-value = 0.989).

Discussion

In the present study we show that hsa-miR-125a-5p downregulation (i.e., higher ΔCt value) at the time of discontinuing antiviral prophylaxis was associated with an

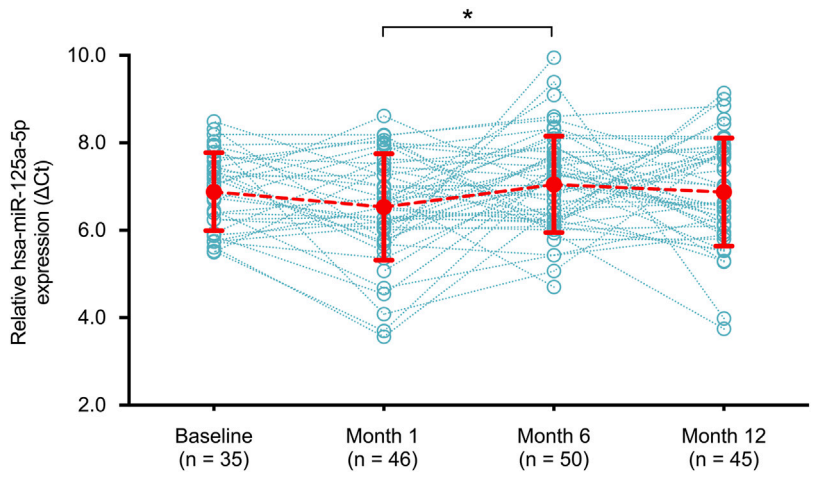


FIGURE 4
Individual trajectories of hsa-miR-125a-5p relative expression (ΔCt values) measured at baseline (before transplantation) and at different post-transplant time points. * *P*-value < 0.05. Ct: cycle threshold.

increased incidence of late CMV infection during the subsequent 6 months among high-risk (D+/R-) KTRs. These findings are in line with those previously reported by our group in seropositive (R+) KTRs with detectable CMV-CMI [29] and further support the predictive role of this hsa-miRNA for stratifying the risk of CMV events across different D/R constellations.

As discussed above, the current approach to selecting the optimal CMV prevention strategy relies primarily on the donor and recipient serostatus [3, 4]. Patients at risk of donor-derived infection in the absence of preexisting CMV-specific immunity (D+/R-) typically benefit from the use of antiviral prophylaxis with valganciclovir (or letermovir) for various months [8, 35, 36]. Although preemptive therapy has the theoretical advantage of allowing the progressive mounting of CMV-CMI under controlled viral replication [37], the rapid kinetics of CMV DNAemia following primary infection—with a median doubling time of 1.54 days compared with 2.67 days in R+ recipients [38]—require very close monitoring and a low threshold for treatment initiation, limiting the feasibility of this approach in clinical practice. Unfortunately, late CMV infection frequently occurs after discontinuation of prophylaxis, with a substantial risk of progression to clinically overt disease. The role of the so-called "hybrid approach", which sequentially combines prophylaxis and surveillance, has not been established in this population [39, 40]. Similarly, the implementation of immunoguided strategies for individualizing prophylaxis duration according to the CMV-CMI status is hampered by limited supporting evidence, the lack of standardized protective thresholds across different techniques, and economic barriers [12–15].

Because of their stability and ease of detection in different blood components and biological fluids (plasma, serum or urine), the application of hsa-miRNAs as diagnostic, prognostic or treatment-response biomarkers is attracting growing interest in multiple areas [18, 41–43]. Among the various methods available for miRNAs detection (including Northern blot analysis, oligonucleotide microarrays, or *in situ* hybridization), RT-qPCR may be considered the gold standard due to its cost-effectiveness, accuracy, and large dynamic input range [44]. In the SOT setting, hsa-miRNAs are increasingly being investigated as non-invasive biomarkers for predicting graft outcomes and guiding individualized immunosuppressive strategies, as recently reviewed [45]. Whereas CMV microRNA expression profiles have been characterized in SOT and hematopoietic stem-cell transplant recipients [46–48], the relationship between hsa-miRNAs and the susceptibility to CMV has received comparatively little attention. By using a microarray chip, Egli et al. identified 142 differentially expressed hsa-miRNAs—including hsa-miR-125a-5p—in peripheral blood at the onset of CMV replication. The levels of several hsa-miRNAs changed dynamically in parallel with decreases in CMV DNAemia, consistent with the proviral and antiviral effects observed *in vitro*. However, no individual hsa-miRNA was found to be strongly associated with CMV viremia clearance or progression [49]. More recently, Barison et al. performed a transcriptomic analysis in endomyocardial specimens from heart transplant recipients and found that two miRNAs (hsa-mir-93-5p and hsa-mir-345-5p) were significantly downregulated in patients with CMV DNAemia and upregulated in those experiencing acute graft rejection [50]. None of these studies addressed the potential utility of these miRNAs as predictive biomarkers, focusing instead on expression profiles once CMV infection had been established.

We have found that the downregulation of hsa-miR-125a-5p (ΔCt value > 7.623) at month 6 was associated with the subsequent development of CMV infection of any level or high-grade CMV DNAemia through month 12, as well as CMV disease. The association with the primary outcome of late CMV infection persisted after adjustment for clinical covariates with a well-established impact on the risk of CMV events, such as the use of mTOR inhibitor or tacrolimus levels. Although the auROC estimate indicated only moderate overall discriminatory ability, the selected cut-off value yielded excellent specificity and PPV (92.6% and 86.7%, respectively), suggesting a strong ability to identify D+/R− KTRs at particularly high risk of CMV infection following discontinuation of antiviral prophylaxis. Therefore, this subset of patients with low

relative hsa-miR-125a-5p expression in whom the potential benefits of extended prophylaxis or intensified surveillance strategies warrant evaluation in future studies. On the other hand, the relatively low sensitivity and NPV (56.5% and 71.4%, respectively) implies that a substantial proportion of late CMV events might not be identified by the proposed cut-off. Interestingly, the optimally selected threshold for hsa-miR-125a-5p expression in our previous R+ cohort (>7.375) [29] was very similar to that found in the present study. Taken together, these results suggest that hsa-miR-125a-5p expression in whole blood is stable over time and remains instrumental in modulating host susceptibility to CMV infection—either viral reactivation or superinfection in R+ patients, or primary infection in the D+/R- group—regardless of pre-transplant serological status. Indeed, the serial assessment of hsa-miR-125a-5p ΔCt values (including samples obtained immediately before transplantation) revealed little or no longitudinal changes. On the other hand, we found no apparent association between hsa-miR-125a-5p expression and the net state of immunosuppression, as reflected by lymphocyte subset counts or tacrolimus exposure, or the inflammatory cytokine *milieu* in plasma. The only exception was a moderate correlation with IL-1β and IL-6 levels at month 1, although this association was not reproduced at other time points, particularly at month 6, coinciding with discontinuation of prophylaxis and the onset of the period at risk for CMV infection.

Breakthrough CMV infection was documented in a substantial proportion of our patients (12.0%), likely reflecting the high frequency of CMV PCR monitoring during prophylaxis. However, hsa-miR-125a-5p expression at month 6 did not differ according to the previous occurrence of breakthrough CMV infection, and additional adjustment for this event in the multivariable model did not materially alter the magnitude or statistical significance of the observed association. Therefore, we are reasonably confident that the predictive value of hsa-miR-125a-5p ΔCt was not substantially confounded by the development of CMV-CMI in response to precedent subclinical viral replication. This possibility, however, cannot be entirely excluded, since we lacked data on CMV seroconversion or CMV-CMI assessment at the time of hsa-miR-125a-5p testing or during the subsequent follow-up.

This study has some noteworthy limitations. The sample size was small, although the high CMV seroprevalence rates in our setting [51, 52] and the low proportion of D+/R- recipients in contemporary series—approximately 10%–15% [53, 54]— limit the feasibility of recruiting larger cohorts within this specific group. Furthermore, internal validation indicated low optimism in observed C-statistics. A further limitation is the suboptimal frequency of DNAemia monitoring after discontinuation of prophylaxis for capturing self-resolving episodes of CMV infection. We did not observe significant differences in the relative expression of hsa-miR-125a-5p according to the occurrence of CMV disease. In addition to the limited statistical power, it is plausible that clinical and genetic factors may further contribute to the progression from asymptomatic CMV primary infection to clinically evident disease [55]. Although the ΔCt threshold retained a significant ability to discriminate CMV disease-free survival, this was a secondary analysis exploratory in nature. Finally, as previously acknowledged, we lacked a direct measurement of CMV-CMI. In this regard, it is worth noting that our previous study found no correlation between the magnitude of IFN-γ responses measured by the CMV-specific IGRA and hsa-miR-125a-5p ΔCt values [29], supporting the notion that this hsa-miRNA provides independent predictive value.

The present proof-of-concept work provides preliminary evidence supporting the potential role of hsa-miR-125a-5p as a biomarker for predicting late CMV infection in high-risk D+/R- KTRs following discontinuation of antiviral prophylaxis. Validation of in independent cohorts is still required before the proposed cut-off for ΔCt value > 7.623 can be considered for clinical decision-making. Meanwhile, ongoing work is aimed at elucidating the biological mechanisms through which this hsa-miRNA modulates susceptibility to CMV infection in the setting of post-transplant immunosuppression, as a prerequisite for the eventual incorporation of hsa-miR-125a-5p quantification by RT-qPCR into risk-stratification strategies.

Data availability statement

The datasets presented in this article are not publicly available due to privacy restrictions. Requests to access the datasets should be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by Clinical Research Ethics Committee, Instituto de Investigación Sanitaria Hospital "12 de Octubre" (imas12), Madrid, Spain. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

MF-R, PM-S, and NR designed the study; PM-S, LM-R, PP-J, TR-M, and NR performed the laboratory analyses; MF-R, IR-G, MP-J, FL-M, EG, JS, RS, AA, and JA participated in patient selection and clinical management; MF-R, PM-S, IR-G, TR-M, MP-J, FL-M, EG, JS, RS, and AA contributed to clinical data collection; MF-R, PM-S, and NR performed data analysis; MF-R and PM-S drafted the first version of the manuscript; and IR-G, TR-M, MP-J, FL-M, EG, JS, RS, AA, and JA critically reviewed the manuscript and provided substantial intellectual input. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2026.17587/full#supplementary-material

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