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Role of cytomegalovirus infection after kidney transplantation on the subsequent risk of atherosclerotic and thrombotic events☆

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ABSTRACT

Background and aims: Whether cytomegalovirus (CMV) infection increases the risk of cardiovascular complications after kidney transplantation (KT) through different indirect effects remains controversial.
Methods: We analyzed the incidence of post-transplant atherosclerotic (PAEs) and thrombotic events (PTEs) in 465 KT recipients according to the previous exposure to any level or high-level (≥1,000 IU/mL) CMV viremia (either asymptomatic or clinical disease) by means of landmark analysis beyond days 30, 180 and 360 after transplantation. Proportional hazards models were constructed with death and graft loss as competing risks.
Results: After a median of 722 days, the cumulative incidences of PAE and PTE were 6.0% each. Most PAEs (53.6%) occurred beyond post-transplant day 360, whereas most PTEs (60.7%) were diagnosed between days 30–180.The incidence of PAE beyond day 180 was higher among patients with previous CMV viremia compared to those without (two-year rates: 4.7% versus 0.4%; *P*-value = 0.035). This difference was more pronounced in recipients developing high-level viremia (6.3% versus 0.7%, respectively; *P*-value = 0.013). After multivariate adjustment for age, pre-transplant cardiovascular risk, antiplatelet and statin therapy and graft function, however, associations were not maintained either for any-level (hazard ratio [HR]: 1.84; 95% confidence interval [CI]: 0.48–7.05) or high-level CMV viremia (HR: 2.84; 95% CI: 0.78–10.36). No significant differences were found in the remaining landmark analyses (days 30 or 360) or for the outcome of PTE either.
Conclusions: Our study does not support that CMV infection independently contributes to the risk of PAE or PTE after KT.

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Abbreviations page

ATG	anti-thymocyte globulin
CI	confidence interval
CMV	cytomegalovirus
CMV-AUC	area under the curve of CMV viral load
D	donor
eGFR	estimated glomerular filtration rate
HR	hazard ratio
IQR	interquartile range
KT	kidney transplantation
NSTEMI	non-ST-segment elevation myocardial infarction
PAD	peripheral arterial disease
PAE	post-transplant atherosclerotic event
PCR	polymerase chain reaction
PTE	post-transplant thrombotic event
R	recipient
SD	standard deviation
SOT	solid organ transplantation
STEMI	ST-segment elevation myocardial infarction
VIF	variance inflation factor

Introduction

Cytomegalovirus (CMV) is the most relevant opportunistic agent complicating solid organ transplantation (SOT). Due to the long-term effect of immunosuppressive therapy, CMV can reactivate among seropositive recipients (R+) —the major source of post-transplant CMV exposure— or cause *de novo* infection in seronegative recipients that receive an organ from a seropositive donor (D+/R-) [1]. Several "indirect effects" not directly attributable to its cytopathic effect but rather to immune phenomena have been hypothesized to be driven by CMV, including accelerated atherosclerosis and cardiovascular mortality. This relationship is supported by the fact that CMV DNA may be detected in vascular smooth muscle cells, which suggests that the arterial wall itself may act as a site for viral latency [2]. Furthermore, CMV increases the local production of pro-inflammatory cytokines and cell-adhesion molecules and induces the accumulation of neutral lipids within the endothelial cells [3]. Endothelial inflammation promotes the expression of tissue factor and other coagulation factors [4,5] as well as platelet activation and aggregation and thrombin formation. Thus, it has been proposed that this inflammatory milieu would trigger atherosclerotic and thrombotic processes through sustained endothelial injury [6,7].

Cardiovascular disease is a leading cause of mortality after kidney transplantation (KT). The sole assessment of traditional cardiovascular risk factors, however, tends to underestimate the actual incidence of athero-thrombotic complications in this population [8–10]. The identification of novel risk factors would contribute to individualize prevention strategies among high-risk recipients [11]. In this regard, post-transplant CMV exposure is preventable by means of antiviral prophylaxis and the stringent application of preemptive therapy [1]. Some studies over the past decades (recently summarized [12]) provide biological and clinical support to the association between CMV infection and post-transplant atherosclerotic events (PAEs) and thrombotic events (PTEs) in the KT setting. It should be noted, however, that these previous studies used different methods to capture CMV exposure and outcome definitions and yielded rather conflicting results [13–17]. One of the most compelling pieces of evidence to date was provided by Courivaud et al., that reported that post-transplant CMV exposure —reflected by a positive serostatus in either the donor or the recipient or by documented CMV DNAemia— acted as an independent risk factor for PAE [18]. Nevertheless, most studies solely applied CMV serostatus as a surrogate for CMV exposure [19–23]. Although this approach can be useful in R-patients, it is not accurate enough to detect post-transplant CMV reactivation or superinfection among R+ patients. In contrast, other authors only considered episodes of active CMV replication or clinically evident disease [14,24,25]. On the other hand, various studies were performed more than a decade ago [15,19,21,25], compromising their generalizability to the current practices of cardiovascular prevention.

In view of the methodological heterogeneity of previous research and the lack of accuracy in the assessment of CMV exposure observed in most studies [12], we have analyzed the occurrence of PAE and PTE in a large contemporary cohort of KT recipients with close monitoring for CMV DNAemia through real-time polymerase chain reaction (PCR) during the first post-transplant year and prolonged follow-up beyond that point.

Materials and methods

Study population and setting

We performed a retrospective, observational cohort study at the "Hospital Universitario 12 de Octubre" (Madrid, Spain) based on a prospectively maintained database. All consecutive adult patients undergoing KT between November 2014 and January 2019 were eligible, including double organ (e.g. kidney-pancreas, liver-kidney, heart-kidney) recipients. Patients with primary graft non-function, death or graft loss within the first post-transplant week were excluded. The study was performed in accordance with the ethical standards laid down in the Declarations of Helsinki and Istanbul. Local Ethics Committee approved the study protocol (number 14/030) and informed consent was obtained from all participants.

Study design and outcomes

All patients were enrolled at transplantation and regularly seen at the outpatient transplant clinic at scheduled follow-up visits (baseline, every 2–3 weeks during the first 3 months, monthly during the first 6 months and at least every 2–3 months thereafter) or whenever clinically indicated. Previous comorbidities, clinical, laboratory and microbiological variables, post-transplant cardiovascular events (PAEs and PTEs) and other complications, and patient and graft outcomes were prospectively entered into our institutional database by using a standardized case report form. Details on traditional cardiovascular risk factors and the use of cardiovascular risk-modifying therapies (antiplatelet, lipid-lowering [statins] and anticoagulant agents) was retrospectively collected. To ensure the appropriate capture of cardiovascular events, electronic medical records of primary care physicians were also screened through the Madrid Electronic Health Record (HORUS) system, which integrates comprehensive patient information from the entire regional healthcare system. All the patients were followed-up for at least 18 months, unless death or graft loss occurred earlier.

CMV DNAemia was quantified by real-time PCR (as detailed below) monthly during the first 6 months and every 2–3 months thereafter until completing the first post-transplant year, as well as at any time when clinical or laboratory manifestations suggestive of CMV disease were present. Estimated glomerular filtration rate (eGFR) was performed using the Chronic Kidney Disease Epidemiology Collaboration equation [26].

The *study outcome* was the occurrence of PAE or PTE (as defined below) throughout the post-transplant period, with CMV exposure

as the explanatory variable of interest. Since the underlying pathogenic mechanisms for atherosclerotic and thrombotic complications are different, PAEs and PTEs were separately considered as study outcomes.

Study definitions

Post-transplant atherosclerotic events (PAEs) included acute coronary syndrome (ST-segment elevation [STEMI] and non-ST-segment elevation myocardial infarction [NSTEMI]), unstable angina, stable angina requiring revascularization, ischemic stroke, transient ischemic attack, symptomatic extracraneal artery stenosis requiring carotid endarterectomy, and/or lower limb peripheral arterial disease (PAD) with critical ischemia (i.e. ischemic rest pain, nocturnal recumbent pain, or ischemic skin lesions leading to ulceration or gangrene) [27]. Transplant renal artery stenosis was not included within the definition of PAE. *Post-transplant thrombotic events (PTEs)* included pulmonary embolism, deep venous thrombosis, retinal central vein thrombosis, and spontaneous jugular vein thrombosis. Post-transplant graft thrombosis, superficial venous thrombosis and vascular catheter-associated thrombosis were excluded. *Post-transplant anticoagulation therapy* was defined as the cumulative use of oral anticoagulants (vitamin-K antagonists or direct-acting agents) or low-molecular weight heparin for at least half of the period considered (censored at the time of patient death or graft loss). *Post-transplant antiplatelet therapy* also required the use of any antiplatelet agent (aspirin, clopidogrel and extended-release dipyridamole) for at least half of the period analyzed. *Post-transplant statin therapy* was analogously defined. *Cytomegalovirus (CMV) infection* required the demonstration of CMV DNAemia by PCR and comprised both asymptomatic viremia and clinical disease, as detailed in the Supplementary Material [28]. *Delayed graft function* was defined as the need for dialysis within the first two post-transplant weeks. *Acute graft rejection* was diagnosed by histological examination if possible or by response to empirical antirejection treatment. *Graft loss* was defined by the permanent return to dialysis and/or retransplantation.

Assessment of CMV exposure

Plasma CMV DNAemia was quantified by real-time PCR (RealStar® CMV PCR kit 1.0, Altona Diagnostics GmbH, Hamburg, Germany). DNA was extracted from 200 μL of sample with the NucliSENS® easyMag® instrument (bioMérieux Diagnostics, Marcy l'Etoile, France), according to the manufacturer's instructions. Viral loads were $\log_{10}$-transformed for statistical analyses. *High-level CMV viremia* was defined by a load ≥1,000 IU/mL. We calculated by means of the trapezoid rule the areas under the curve of CMV viral load (CMV-AUCs) —expressed as $\log_{10}$ IU x day/mL— from the time of transplantation to days 30 (CMV-$AUC_{0\text{-}30}$), 180 (CMV-$AUC_{0\text{-}180}$) and 360 (CMV-$AUC_{0\text{-}360}$) [29], as well as the peak CMV viral loads for each of these periods.

Immunosuppression and prophylaxis regimens

Details on the immunosuppression regimen are provided in Supplementary Methods. All patients received preoperatively a single dose of intravenous (IV) cefazolin (or ciprofloxacin in those with β-lactam hypersensitivity). Prophylaxis for *Pneumocystis jirovecii* pneumonia with trimethoprim-sulfamethoxazole (160/800 mg three times weekly) or monthly IV pentamidine was given during the first 9 months. Universal prophylaxis with oral valganciclovir (900 mg daily with dose adjustment for renal function) was administered for 3 (R+ patients that received induction therapy with anti-thymocyte globulin [ATG]) or 6 months (D+/R-patients). Intermediate-risk patients (R+ patients without ATG induction) underwent PCR-guided pre-emptive therapy, and IV ganciclovir (5 mg/kg/12 h) or oral valganciclovir (900 mg/12 h) for at least 2 weeks was initiated in the presence of high-level (≥1,000 IU/mL) or rapidly increasing CMV viral load.

Statistical analysis

Quantitative data were depicted with the mean ± standard deviation (SD) or the median with interquartile range (IQR). Proportions were presented with 95% confidence intervals (CIs) computed by the modified Wald method. The normality of distribution was tested with the Kolmogorov-Smirnov test. Categorical variables were compared using the χ^2 test. Student's t-test or *U* Mann-Whitney test were applied for continuous variables. Time-to-event curves were plotted by the Kaplan-Meier method and inter-group differences were compared with the log-rank test.

Considering that both cardiovascular risk and CMV exposure dynamically evolve over time in KT recipients, we performed separate landmark survival analyses encompassing different post-transplant periods (beyond days 30, 180 and 360) and evaluated the association between cumulative CMV exposure (either any level or high-level viremia) and the subsequent occurrence of PAE or PTE. For each of these landmark analyses, competing-risks regression models were constructed according to the method of Fine and Gray [30]. Previous CMV exposure was considered the explanatory variable of interest and PAE and PTE the dependent variables. Death and graft loss were treated as competing risks. Models were adjusted in a two-step process. Firstly, a set of variables were assessed at the univariate level. These variables included demographics and clinical features (i.e. pre-transplant comorbidities, cause of end-stage renal disease, previous transplantation), donor age and type (i.e. donation after brain or circulatory death, living donor), surgical and peri-operative variables (i.e. cold ischemia time, delayed graft function), laboratory values (eGFR, lymphocyte count), cardiovascular risk-modifying therapies (statins, antiplatelet and anticoagulant agents), type of immunosuppression, and acute graft rejection. Secondly, those variables found to be significant at a *P*-value <0.05 in the univariate analysis were entered as covariates into multivariate models in a backward stepwise fashion. Multicollinearity among explanatory variables was assessed with the variance inflation factor (VIF), with VIF values < 3 being considered acceptable. Finally, the proportional-subhazards assumption was evaluated allowing time-varying coefficients and testing the time invariance for the coefficients. Associations were expressed by hazard ratios (HRs) with 95% CIs. Statistical analysis was performed with SPSS version 20.0 (IBM Corp., Armonk, NY) and stcrreg function of Stata version 16.1 (StataCorp, College Station, TX). Graphics were generated with Prism version 6.0 (GraphPad Software Inc., La Jolla, CA).

Results

Study population and atherothrombotic events

We included 465 KT recipients, whose clinical characteristics are summarized in Table 1. Median follow-up was 722 days (IQR: 576.5–1,644.5), totaling 482,436 transplant-days. Thirty-seven patients (7.9%) died at a median interval of 963 days (IQR: 208–1,673.5), resulting in two- and three-year survival rates of 94.5% and 90.8%, respectively. The most common causes of death were infection (14 patients), malignancy (7 patients) and cardiovascular events (4 patients). Graft loss occurred in 28 patients (6.0%), with two- and three-year death-censored graft survival rates of 93.8% and 93.0%, respectively. Overall, 225 (48.4%) and 129

Table 1
Demographics and clinical characteristics of the study cohort (n = 465).

Variable	
Age of recipient, years [mean ± SD]	55.0 ± 15.7
Gender of recipient (male) [n (%)]	316 (68.0)
Prior or current smoking history [n (%)]	169 (36.3)
BMI at transplantation, kg/m^2 [median (IQR)][a]	25.3 (22.3–28.4)
Pre-transplant chronic conditions [n (%)]	
Hypertension	386 (83.0)
Diabetes mellitus	136 (29.2)
Dyslipidemia	254 (54.6)
Coronary heart disease	49 (10.5)
Other chronic heart disease	70 (15.1)
Cerebrovascular disease	33 (7.1)
Lower limb PAD	32 (6.9)
Type of transplantation [n (%)]	
Single kidney	438 (94.2)
Double kidney	2 (0.4)
Simultaneous kidney-pancreas	20 (4.3)
Simultaneous liver-kidney	4 (0.9)
Simultaneous heart-kidney	1 (0.2)
Previous solid organ transplantation [n (%)]	72 (15.5)
Underlying end-stage kidney disease [n (%)]	
Glomerulonephritis	99 (21.3)
Diabetic nephropathy	94 (20.2)
Polycystic kidney disease	57 (12.3)
Nephroangiosclerosis	43 (9.2)
Congenital nephropathy	14 (3.0)
Reflux nephropathy	13 (2.8)
Lupus nephropathy	9 (1.9)
Vasculitis	7 (1.5)
Chronic interstitial nephropathy	4 (0.9)
Unknown	49 (10.5)
Other	76 (16.3)
CMV serostatus [n (%)]	
D+/R+	317 (68.2)
D-/R+	69 (14.8)
D+/R-	59 (12.7)
D-/R-	13 (2.8)
D unknown/R+	7 (1.5)
Positive EBV serostatus (anti-EBNA IgG) [n (%)]	411 (88.4)
Positive HCV serostatus [n (%)]	37 (8.0)
Positive HBsAg status [n (%)]	15 (3.2)
Positive HIV serostatus [n (%)]	5 (1.1)
Pre-transplant renal replacement therapy [n (%)]	413 (88.8)
Hemodialysis	333 (71.6)
Continuous ambulatory peritoneal dialysis	80 (17.2)
Time on dialysis, days [median (IQR)]	530.0 (239.0–1,057.5)
Age of donor, years [mean ± SD]	53.3 ± 17.1
Gender of donor (male) [n (%)]	262 (71.8)
Type of donor [n (%)]	
DBD donor	299 (64.3)
DCD donor	105 (22.6)
Living donor	61 (13.2)
Cold ischemia time, hours [median (IQR)]	16.9 (10.3–21.9)
Number of HLA mismatches [median (IQR)]	4.0 (3.0–5.0)
Intraoperative blood product transfusion [n (%)]	23 (4.9)
Induction therapy [n (%)]	
ATG	228 (49.0)
Basiliximab	181 (38.9)
Alemtuzumab	2 (0.4)
None	54 (11.6)
Primary immunosuppression [n (%)]	
Steroids	465 (100.0)
Tacrolimus	465 (100.0)
Mycophenolate mofetil/mycophenolic acid	439 (94.4)
Azathioprine	18 (3.9)
Everolimus	8 (1.7)
CMV antiviral prophylaxis [n (%)]	265 (56.9)
Post-transplant complications [n (%)]	
Delayed graft function	202 (43.4)
Number of dialysis sessions [median (IQR)]	2.0 (1.0–3.0)
Re-intervention within the first month	52 (11.2)
NODAT	75 (16.1)
Renal artery stenosis requiring revascularization	70 (15.1)
Acute graft rejection[b]	70 (15.1)
Time to the first episode, days [median (IQR)]	110.0 (17.8–172.8)
T-cell-mediated acute rejection	24 (5.2)
Antibody-mediated acute rejection	15 (3.2)

ATG: antithymocyte globulin; BMI: body mass index; CMV: cytomegalovirus; D: donor; DBD: donation after brain death; DCD: donation after circulatory death; EBV: Epstein-Barr virus; HCV: hepatitis C virus; HBsAg: hepatitis B virus surface antigen; HIV: human immunodeficiency virus; HLA: human leukocyte antigen; IQR: interquartile range; NODAT: new-onset diabetes after transplantation; PAD: peripheral arterial disease; SD: standard deviation; R: recipient.

[a] Data on BMI not available for 53 patients.

[b] Includes 16 patients with borderline acute rejection and 17 with empirically-treated episodes not confirmed by biopsy.

(27.7%) recipients were followed-up with a functioning graft for at least two and three years, respectively.

Details on the use of antiplatelet and anticoagulant agents and statins are provided as Supplementary Material (Table S1). Almost half of the patients (208 [44.7%]) met the criteria for post-transplant statin therapy during the first year (i.e. cumulative use for ≥50% of the period), with lower figures for antiplatelets (162 [34.8%]) and anticoagulants (44 [9.5%]).

Fifty-three patients developed 28 PAEs and 28 PTEs during the follow-up period, accounting for an overall cumulative incidence of 11.4% (95% CI: 8.8–15.6). Two patients consecutively experienced PTE and PAE more than a year apart, whereas one further patient was diagnosed with a deep venous thrombosis and two days later developed critical lower limb PAD. Clinical characteristics, timing and incidence rates for both types of events are separately detailed in Table 2. Most PAEs (53.6% [15/28]) occurred beyond post-transplant day 360, whereas most PTEs (60.7% [17/28]) were diagnosed between days 30–180.

CMV events

Two hundred and sixty-five patients received valganciclovir prophylaxis for a median of 96 days (IQR: 90–161.5). The total number of monitoring points for CMV DNAemia were 5,458, with a median of 9 (IQR: 8–12) and 11 (IQR: 9–14) points per patient throughout the first post-transplant year and the entire follow-up period, respectively. Overall, 222 patients (47.7%) had at least one episode of CMV infection, either as asymptomatic viremia (72.5% [161/222]) or clinical disease (27.4% [61/222]). Table S2 details the incidence, clinical characteristics and viral kinetics of these events. About one third of patients with asymptomatic CMV infection (36.0% [58/161]) required pre-emptive therapy at any time.

CMV exposure and subsequent development of PAE

First, we explored the potential association between CMV donor/recipient serostatus and the occurrence of PAE throughout the follow-up period. There were no significant differences across increasing risk categories, with two-year incidence rates of 7.7% for D-/R-, 4.5% for R+ and 3.5% for D+/R-patients (log-rank *P*-value = 0.823) (Fig. S1a).

Next, the association between the actual occurrence of CMV infection and subsequent PAE was assessed through fixed intervals after transplantation. While no differences were observed for the analysis focused on the first 30 days (log-rank *P*-value = 0.577), the incidence of PAE beyond day 180 was significantly higher among patients with previous CMV viremia at any level compared to those without (two-year incidence rates: 4.7% versus 0.4%, respectively; log-rank *P*-value = 0.035). This difference was no longer significant beyond day 360 (two-year incidence rates: 3.5% versus 0.4%; log-

Table 2
Description of PAEs and PTEs occurring during follow-up in the study population.

Post-transplant atherosclerotic events (PAEs)	
Cumulative incidence, % (95% CI)	6.0 (4.2–8.6)
Total number of events	28
Interval from transplantation to the first episode, days [median (IQR)]	423.0 (11.0–856.5)
Distribution of events over time [n (%)]	
Number of events within the first 30 post-transplant days	8/28 (28.6)
Number of events between post-transplant days 30–180	4/28 (14.3)
Number of events between post-transplant days 180–360	1/28 (3.6)
Number of events beyond post-transplant days 360	15/28 (53.6)
Type of event [n (%)]	
NSTEMI	8/28 (28.6)
STEMI	1/28 (3.6)
Unstable angina	5/28 (17.8)
Stable angina requiring revascularization	3/28 (10.7)
Stroke/transient ischemic attack	4/28 (14.3)
Lower limb PAD with critical ischemia	6/28 (21.4)
Non-arteritic anterior ischemic optic neuropathy	1/28 (3.5)
Post-transplant thrombotic events (PTEs)	
Cumulative incidence, % (95% CI)	6.0 (4.2–8.6)
Total number of events	28
Interval from transplantation to the first episode, days [median (IQR)]	84.0 (44.0–177.8)
Distribution of events over time [n (%)]	
Number of events within the first 30 post-transplant days	4/28 (14.3)
Number of events between post-transplant days 30–180	17/28 (60.7)
Number of events between post-transplant days 180–360	3/28 (10.7)
Number of events beyond post-transplant days 360	4/28 (14.3)
Type of event [n (%)]	
Pulmonary embolism	11/28 (39.3)
Deep venous thrombosis	15/28 (53.6)
Retinal central vein thrombosis	1/28 (3.6)
Jugular vein thrombosis	1/28 (3.6)

CI: confidence interval; IQR: interquartile range; NSTEMI: non-ST-segment elevation myocardial infarction; STEMI: ST-segment elevation myocardial infarction; PAD: peripheral arterial disease.

rank *P*-value = 0.222) (Fig. 1).

This association was more pronounced when only CMV viremia ≥1,000 IU/mL within the first 180 days was considered (two-year incidence rates: 6.3% versus 0.7% for those with and without prior high-level CMV infection, respectively; log-rank *P*-value = 0.013). We found a similar, although non-significant, trend in the landmark survival analyses beyond days 30 (two-year rates: 7.1% versus 2.8%; log-rank *P*-value = 0.175) and 360 (two-year rates: 4.6% versus 0.7%; log-rank *P*-value = 0.107) (Fig. S2).

A number of clinical factors were associated at the univariate level with the development of PAE in the different landmark survival analyses (Table S3). When proportional hazard regression models (with death and graft loss as competing risks) were adjusted by these factors, the development of any level (HR: 1.84; 95% CI: 0.48–7.05; *P*-value = 0.371) or high-level CMV viremia during the first 180 days (HR: 2.84; 95% CI: 0.78–10.36; *P*-value = 0.115) were no longer associated with the subsequent occurrence of PAE (Table 3).

To further explore these associations, we assessed whether cardiovascular risk factors and use of risk-modifying therapies were over-represented in the group of patients that experienced any level (Table S4) or high-level CMV viremia (Table S5) during the first 180 days. Patients with CMV infection were older, showed higher body mass index, and had higher prevalence of some risk factors (male gender, smoking habit or dyslipidemia) and previous atherosclerotic disease.

Within the group of recipients with CMV infection, no significant differences were observed in CMV-AUCs measured throughout the first 30, 180 and 360 days after transplantation (Fig. S3 a-c) according to the subsequent development of PAE. Peak viral loads in each of these periods were also similar between patients with or without this outcome (Fig. S4 a-c).

CMV exposure and subsequent development of PTE

There were no significant differences in the two-year incidence rate of PTE according to the category of CMV donor/recipient serostatus (7.7%, 6.5% and 1.7% for the D-/R-, R+ and D+/R-patients, respectively; log-rank *P*-value = 0.339) (Fig. S1b).

No significant differences were observed between patients experiencing or not experiencing any-level CMV viremia in the incidence of PTE beyond days 30 (log-rank *P*-value = 0.699), 180 (log-rank *P*-value = 0.888) and 360 (log-rank *P*-value = 0.313) (Fig. 2). The lack of association persisted for high-level CMV viremia (Fig. S5). After adjustment for those factors identified at the univariate level (Table S6), no association was observed between CMV exposure and PTE in any of the landmark analyses (Table 3).

Regarding viral kinetics, no differences were found in the CMV-AUCs (Fig. S3d) or peak CMV viral load (Fig. S4d) through the first 180 days after transplantation according to the subsequent occurrence of PTE.

Discussion

The development of CMV infection after SOT has been linked to atherosclerotic and thrombotic events as part of the so-called “indirect effects” attributed to this pathogen [19,31,32]. Such deleterious effects are presumably derived from immune and non-immune actions that induce endothelial cell injury, chronic inflammation and a hypercoagulable state [33,34]. The supporting evidence, however, is scarce and conflicting [13,35,36] and contains several methodological flaws, such as inadequate follow-up and heterogeneous —and often imprecise— designs. These studies applied a variety of approaches to capture CMV exposure (from donor-recipient serostatus to pp65 antigenemia assay or molecular

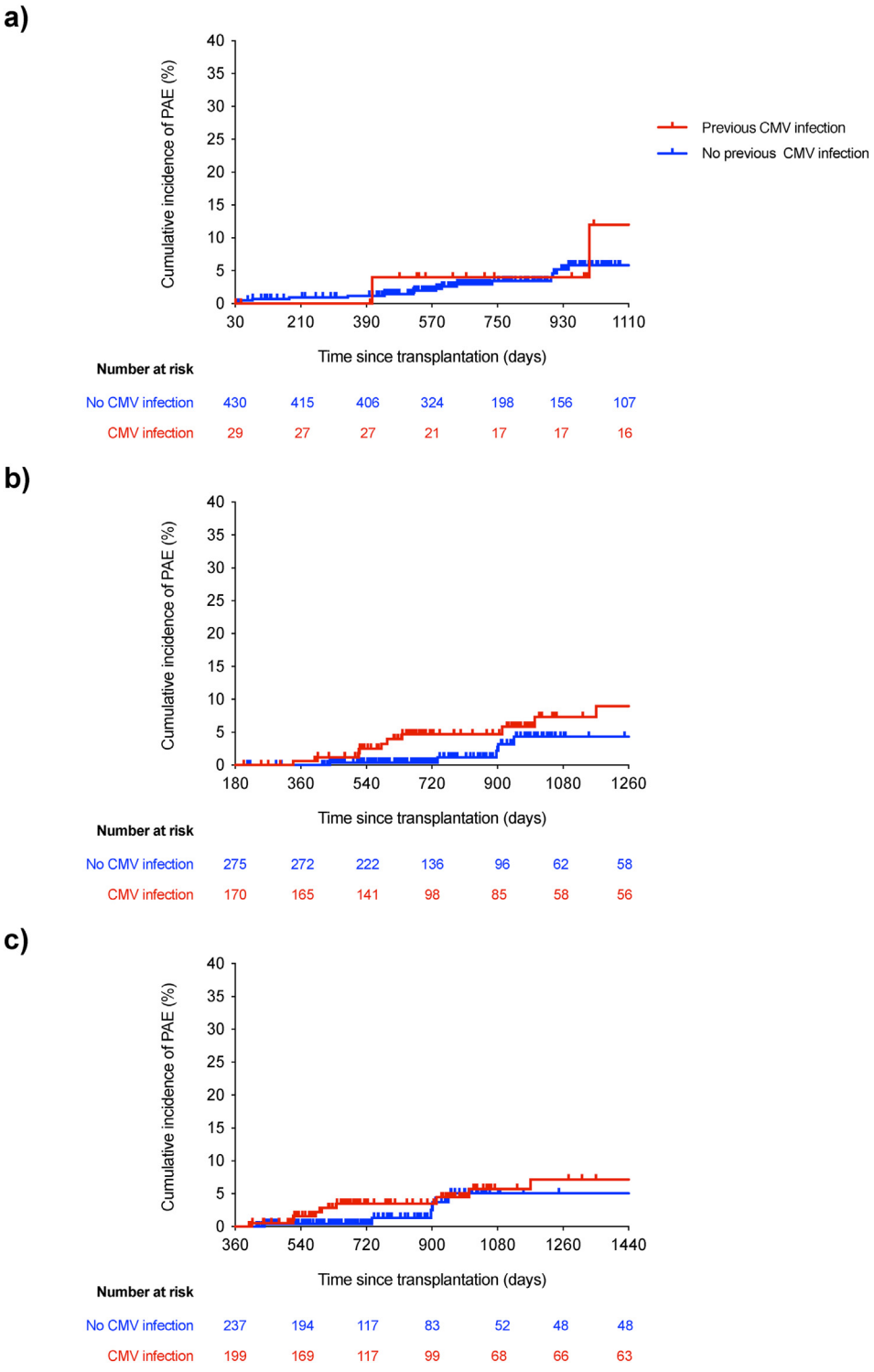

Fig. 1. Cumulative incidence of PAE beyond **(a)** day 30 (log-rank *P*-value = 0.577), **(b)** day 180 (log-rank *P*-value = 0.035) and **(c)** day 360 after transplantation (log-rank *P*-value = 0.222) according to the previous development of CMV viremia at any level. CMV: cytomegalovirus; PAE: post-transplant atherosclerotic event.

methods) and did not attempt to explore the temporal association between the presence, level and duration of CMV viremia and the subsequent incidence of cardiovascular complications. Moreover, a variety of events with different pathophysiologic mechanisms were often combined as a sole outcome, such as major cardiovascular events or atherosclerotic, thrombotic and/or embolic episodes [15,19,22,25,37]. In the present study we included a large cohort of KT recipients and used a thorough methodological approach to take into account the impact of cumulative CMV exposure at different time frames on the incidence of two separated outcomes, PAE and PTE, in the long-term follow-up.

In our study, patients experiencing any level of CMV viremia (either symptomatic or not) during the first 180 days exhibited an increased incidence of PAE during the subsequent period, and this

Table 3
Unadjusted and adjusted models for the association between prior CMV exposure and the development of PAE and PTE, with death and graft loss as competing risks.

	Unadjusted models			Adjusted models[a]		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Post-transplant atherosclerotic events (PAEs)						
CMV viremia at any level						
First 30 days	1.52	0.36–6.48	0.571	0.45[b]	0.09–2.31	0.340
First 180 days	2.97	1.00–8.77	0.049	1.84[c]	0.48–7.05	0.371
First 360 days	1.93	0.64–5.81	0.240	1.21[d]	0.40–3.68	0.730
High-level CMV viremia						
First 30 days	2.65	0.62–11.38	0.190	0.88[b]	0.15–5.38	0.894
First 180 days	3.28	1.17–9.20	0.024	2.84[c]	0.78–10.36	0.115
First 360 days	2.27	0.79–6.51	0.128	2.06[d]	0.66–6.45	0.214
Post-transplant thrombotic events (PTEs)						
CMV viremia at any level						
First 30 days	0.67	0.09–4.83	0.696	0.33[e]	0.05–2.32	0.265
First 180 days	1.11	0.23–5.29	0.892	11.29[f]	0.31–417.97	0.188
First 360 days	0.33	0.03–3.48	0.355	–	–	–
High-level CMV viremia						
First 30 days	1.26	0.18–8.79	0.815	0.60[e]	0.08–4.24	0.609
First 180 days	0.47	0.05–4.03	0.488	0.79[f]	0.07–8.71	0.846
First 360 days	0.67	0.06–7.57	0.750	–	–	–

CI: confidence interval; CMV: cytomegalovirus; HR: hazard ratio.
[a] Models were adjusted for variables with univariate *P*-values <0.05 (Tables S3 and S4).
[b] Model adjusted for recipient age, pre-transplant diabetes, pre-transplant atherothrombotic disease, previous SOT, cold ischemia time, delayed graft function, antiplatelet therapy, and eGFR at day 30 (donor age was not included due to significant collinearity with recipient age).
[c] Model adjusted for recipient age, pre-transplant diabetes, pre-transplant atherothrombotic disease, previous SOT, cold ischemia time, delayed graft function, antiplatelet therapy, statin therapy, and eGFR at day 180 (donor age was not included due to significant collinearity with recipient age).
[d] Model adjusted for recipient age, pre-transplant diabetes, pre-transplant atherothrombotic disease, cold ischemia time, delayed graft function, antiplatelet therapy, and eGFR at day 360 (donor age was not included due to significant collinearity with recipient age).
[e] Model adjusted for recipient age, pre-transplant dyslipidemia, pre-transplant atherothrombotic disease, underlying nephroangiosclerosis, anticoagulant therapy, and eGFR at day 30 (donor age was not included due to significant collinearity with recipient age).
[f] Model adjusted for smoking habit.

association was more clear for a DNAemia level over 1,000 IU/mL. However, after adjusting for clinically relevant covariates —such as recipient age, cardiovascular risk factors, pre-transplant atherosclerotic disease, or antiplatelet and statin therapy— the effect of previous CMV exposure was no longer significant. Since only 6.2% of patients experienced CMV infection during the first 30 days, it is to be expected that the potential effect of CMV infection on the cardiovascular risk would be only evident after longer exposure periods. However, no association was found in the landmark analysis beyond day 360 either, which may suggest that the impact of late CMV replication (if any) may be diluted by the cluster of cardiovascular risk factors among CMV-exposed patients. In fact, recipients experiencing CMV viremia during the first months exhibited a worse cardiovascular risk profile than those that remained free from this event. Although a previous study reported a relationship between CMV exposure and the occurrence of PTE [38], our research does not support this association, either with CMV donor-recipient serostatus or viral replication. Unlike PAE, most cases of PTE in our cohort occurred during the first 180 days and therefore the number of late events was too low to draw firm conclusions on this relationship in the long-term. When we evaluated other parameters eventually reflecting a dose-response gradient between the amount of CMV replication (peak CMV viral load or CMV-AUC) and study outcomes, no association was observed in any of the landmark analyses.

It could be argued that the use of valganciclovir prophylaxis in 56.9% of the patients would have contributed to obscure any causal association that could have been actually present. However, one of the strengths of our study was that patients receiving prophylaxis were also monitored for CMV DNA, allowing us to identify and include in the analysis episodes of breakthrough viremia. In fact, the median number of PCR assays performed during the first year was similar between patients that received primary prophylaxis and those pre-emptively managed (10 and 9 monitoring points, respectively).

Donor-recipient CMV serostatus at transplantation did not exert a meaningful effect on the incidence of PAE or PTE across increasing risk categories (D-/R-, R+ and D+/R-groups). This lack of association contrasts with some previous experiences. In a recent study on 392 CMV-seronegative recipients, Belga et al. found that patients receiving an organ from a seropositive donor (D+/R-) had a three-fold higher risk of developing PAE and PTE jointly considered compared to those that received an organ from a seronegative donor (D-/R-). On note, a similar association was not observed for the presence of PCR-proven CMV viremia during follow-up, and CMV infection actually preceded the development of the event in only 11 out of 35 patients within the D+/R-group [38]. Courivaud et al. used a composite definition for “CMV exposure” based on serology (R+ and D+/R-) and/or the documentation of CMV DNAemia by PCR, and concluded that exposed patients were at an increased risk of PAE, being this association more evident for those experiencing CMV replication (HR: 2.18; *P*-value = 0.042). Of note, the temporal relationship between CMV viremia and PAE was not detailed by the authors, the study period extended for 15 years, and 44% of the patients were seronegative at transplantation (with almost half of them receiving an organ from a seropositive donor) [18]. In comparison, as many as 84.5% of KT recipients in our cohort were seropositive. Given this high prevalence and the currently widespread use of antiviral prophylaxis, we consider that the assessment of CMV viremia after transplantation constitutes a more appropriate marker for CMV exposure than the donor-recipient serostatus, which may reflect the impact of the D+/R- group but ignores the vast majority of R+ patients experiencing CMV reactivation [16].

A number of clinical factors —including older age, previous history of atherosclerotic events and lower eGFR— acted as independent risk factors for PAE. All of them are well-established cardiovascular risk factors with high prevalence in the KT population

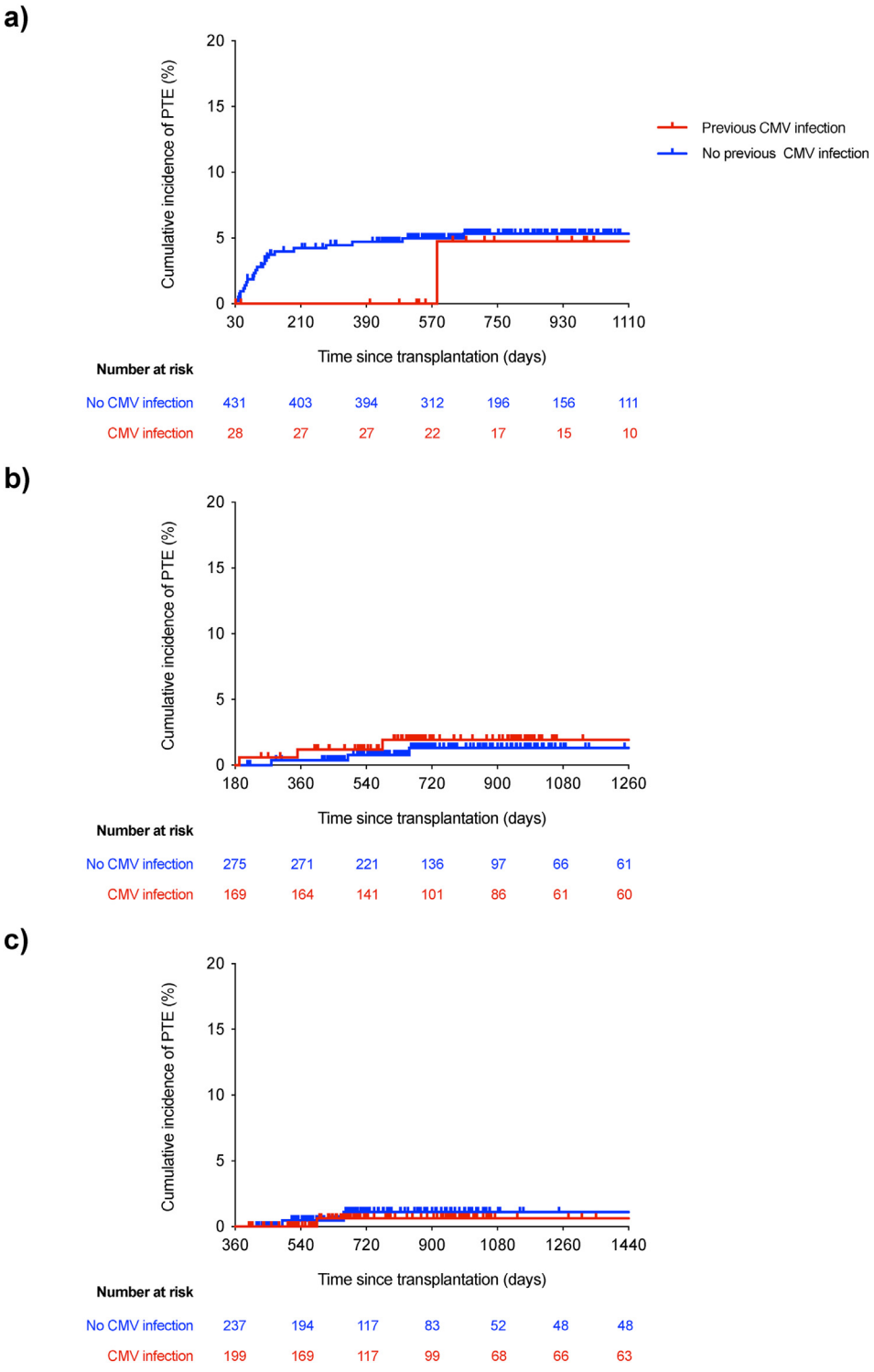

Fig. 2. Cumulative incidence of PTE beyond **(a)** day 30 (log-rank *P*-value = 0.699), **(b)** day 180 (log-rank *P*-value = 0.888) and **(c)** day 360 after transplantation (log-rank *P*-value = 0.313) according to the previous development of CMV viremia at any level. CMV: cytomegalovirus; PTE: post-transplant thrombotic event.

[9]. It is plausible that the exposure to CMV replication during the first months could have accelerated the pro-atherogenic inflammatory milieu already settled before transplantation. In fact, the use of antiplatelet agents and statins were also found to predict the occurrence of PAE beyond day 180. Our findings would support the notion that CMV infection, particularly at high level, early after KT may act as a contributing rather than a causative factor accelerating the atherosclerotic process in combination with traditional cardiovascular risk factors.

The present study has some limitations. Firstly, this was a single-center study and the differential impact of local CMV monitoring and cardiovascular prevention practices cannot be excluded. Since a high proportion of the R+ patients received ATG induction, immunosuppression and prophylaxis regimens may not

be comparable to other institutions. Secondly, due to its observational design, CMV monitoring by PCR was performed as usual clinical practice and we cannot rule out that some episodes of low-level asymptomatic viremia that resolved spontaneously in the time interval between two consecutive monitoring points may have been missed. In addition, the CMV IgG serostatus was determined at pre-transplantation only for risk stratification purposes, and seronegative patients at risk of primary infection were not retested during follow-up. Although PCR-based quantitative nucleic acid amplification testing is considered as the preferred method for the post-transplant monitoring of CMV viremia [39], the rate of seroconversion was not available as an additional measurement of CMV exposure. Thirdly, we were not able to adjust the proportional hazards models for the levels of immunosuppressive drugs due to the lack of collected data. Fourthly, the absolute number of cardiovascular events during follow-up were low, limiting the statistical power. Some studies have reported a positive correlation between CMV-specific IgG titters —which might indicate frequent or recent virus reactivation— and the risk of coronary artery events in the general population [40], although we are not aware of similar studies performed in the transplant population. Of note, we did not quantify CMV IgG titters with or without neutralizing activity nor enumerate CMV-specific T-cell counts. Therefore, the potential protective effect of adaptive immune response on the risk of PAE or PTE was not tested. Finally, the median follow-up period of our cohort was around two years. Thus, it cannot be excluded long-term effects derived from CMV infection on the atherothrombotic risk over the next years or decades.

Nonetheless, our conclusions are strengthened by the prospective design of a large and well-characterized cohort of consecutive KT recipients, the comprehensive assessment of CMV exposure by means of different variables to capture changing CMV kinetics, the considerable follow-up, the differentiation between atherosclerotic and thrombotic events as separate outcomes, and the application of landmarking and competing risk survival analysis.

In conclusion, our results suggest that CMV exposure does not independently increase the risk of PAE or PTE after KT. CMV viremia (especially in the presence of viral load over 1,000 IU/mL), might contribute to the subsequent development of PAE when added to traditional cardiovascular risk factors. Further studies are needed to evaluate whether extending universal prophylaxis against CMV infection to KT recipients with poorer cardiovascular health reduces the incidence of this complication.

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Author contributions

IRG participated in the study concept and design, data collection and analysis, and manuscript writing, drafting and reviewing; FLM, EG, AS, AH, RSJ, TRM, PP, and EG participated in the clinical management of patients, and manuscript editing and reviewing; LC participated in data collection and manuscript editing and reviewing; MDF participated in the laboratory procedures, and manuscript editing and reviewing; NR, AA, JMA participated in the study concept and design, manuscript editing and reviewing; DL participated in data analysis and interpretation, manuscript editing and reviewing; and JMA and MFR participated in the study concept and design, data analysis and interpretation, manuscript writing, editing and reviewing. All authors have read and approved the submitted manuscript.

Declaration of competing interest

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.athplu.2022.03.003.

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