ORIGINAL ARTICLE

Cytomegalovirus reactivation in liver transplant recipients due to hepatitis C cirrhosis is associated with higher cardiovascular risk – an observational, retrospective study

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SUMMARY

The association between cytomegalovirus (CMV) reactivation and cardiovascular risk has been reported in solid organ transplant populations; however, it has yet to be assessed in liver transplantation (LT). We aim to evaluate whether CMV reactivation is associated with cardiovascular events (CVE) in HCV-LT patients. LT patients (2010 and 2014) due to HCV cirrhosis were included. Clinically significant CMV (CS-CMV) was defined as viral load (VL) >5000 copies/ml, need of therapy or CMV disease. Baseline variables and endpoint measures (CVE, survival, severe recurrent hepatitis C, *de novo* tumors, and diabetes) were collected. One hundred and forty patients were included. At LT, a history of AHT was present in 23%, diabetes 22%, tobacco use 45%, obesity 20%, and renal impairment (eGFR < 60 ml/min) in 26.5%. CS-CMV reactivation occurred in 25% of patients. Twenty-six patients (18.5%) developed a CVE. Cox regression analysis revealed two factors significantly associated with CVE: Pre-LT DM [HR = 4.6 95% CI (1.6, 13), $P = 0.004$] and CS-CMV [HR = 4.7 95% CI (1.8, 12.5), $P = 0.002$]. CS-CMV was not independently associated with the remaining endpoints except for survival ($P = 0.03$). In our series, CS-CMV reactivation was associated with a greater risk of developing CVE, thus confirming data from other solid organ transplant populations and emphasizing the need for adequate CMV control.

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Key words
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Introduction

Cardiovascular events (CVE) represent a major source of morbidity and mortality following liver transplantation (LT), however, the optimal approach for assessing cardiovascular risk in this evolving patient population remains unclear [1,2]. So far, adequate control of known cardiovascular (CV) risk factors remains the only effective means to reduce their impact on patient survival.

Common CV risk factors, such as diabetes mellitus (DM), arterial hypertension (AHT), tobacco use, and dyslipidemia are well recognized in the literature; however, pathogenic mechanisms for CV disease are numerous, including immune-mediated vascular injury, oxidative stress, and inflammation of the vascular endothelium. In this regard, both chronic hepatitis C (HCV) and cytomegalovirus (CMV) infections have been related to the occurrence of CV manifestations through various different mechanisms. More specifically, in the nontransplant population, HCV infection has been associated with greater CV risk (especially in diabetic and hypertensive patients) [3,4], while CMV infection has been linked to an increased CV-related mortality due to endothelial dysfunction and ensuing accelerated atherosclerosis, which in turn result from a combination of the direct effects of the virus with the host's immunomodulatory and pro-inflammatory responses [5–7].

Previous murine transplant models have shown that CMV plays an important role in the development of atherosclerosis [8]. Furthermore, clinical studies involving kidney, heart, and lung transplant recipients have also found an association between CMV reactivation and CVE [9,10], although this association has not been described in the LT population [11].

In view of this, our hypothesis is that CMV reactivation or primo-infection produces a series of immunomodulatory changes that promote the development of CVE in HCV-LT recipients with *a priori* high baseline CV risk [12,13]. Hence, the primary objective of this study was to evaluate if CMV reactivation or primo-infection is associated with an increased medium-term risk of CVE in LT patients with active or past HCV infection who did not receive antiviral therapy for at least 1 year after transplantation. Our secondary objective was to evaluate if CMV reactivation/primo-infection was associated with other unfavorable events, such as aggressive recurrent hepatitis C, rejection, *de novo* DM, *de novo* tumors, and increased overall mortality.

Patients and methods

Study population

We conducted an observational, retrospective study at the Liver Transplant Unit of La Fe University Hospital which included all patients undergoing LT between January 2010 and June 2014 due to HCV cirrhosis. End of follow-up was set at December 2017 or patient death. The study was approved by our institutional ethics committee and the liver transplantation review board.

Inclusion criteria

Adult patients who underwent LT due to HCV cirrhosis between January 2010 and June 2014 (regardless of viremia at LT).

Exclusion criteria

(i) HBV or HIV co-infection, (ii) perioperative death related to surgery, (iii) re-LT, (iv) loss to follow-up, (v) fewer than five CMV viral load determinations during the first year, and (vi) CMV negative/negative Donor/Recipient serology.

Primary aim

All CVE occurring between LT and the end of follow-up were recorded, alongside baseline recipient and donor-related characteristics, type of immunosuppression, and data regarding CMV reactivation.

1. The following were considered CVE: development of ischemic heart disease, stroke, heart failure, *de novo* cardiac arrhythmias, and peripheral arterial disease. All CVE occurring in the setting of sepsis or hemorrhage were not included. Likewise, any perioperative cardiac arrhythmias that resolved shortly after surgery with conservative management were not considered as a CVE [14]. CVE occurring after CMV reactivation or primo-infection were the only ones that were considered as outcome endpoints in the analysis.
2. The following Pre-LT variables were recorded: donor and recipient demographics, presence of DM pre-LT according to WHO criteria [15], renal insufficiency according to Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation [16], tobacco use according to chart review, AHT according to WHO criteria [17], body mass index (BMI), dyslipidemia, presence of

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hepatocellular carcinoma (HCC), Child-Pugh score, MELD score, and pre-LT antiviral treatment.

3. Post-LT variables included those related to immunosuppression and CMV such as:

(i) *CMV reactivation* or primo-infection was defined as a minimum of one CMV viral load >400 copies/ml during the first year post-LT and was considered either an early or late reactivation depending on whether it happened before or after the first 3 months post-LT; (ii) *Clinically significant (CS) CMV reactivation* or primo-infection was established when CMV viral load exceeded 5000 copies/ml, when treatment was necessary, or when the patient developed CMV disease requiring hospital admission for IV treatment; and (iii) *CMV disease* was confirmed by immunohistochemistry or PCR of the tissue sample.

To monitor CMV replication, all patients underwent weekly CMV viral load determinations during the first month, every 2 weeks during the second and third months and monthly thereafter for a total of 6 months. Monitoring of viral load was carried out in the microbiology laboratory using quantitative plasma PCR with the Argent and Geneproof kits. The cutoff value in plasma samples was 400 copies/ml. The CMV prevention strategies were those established by international consensus [18–20]: (i) in high-risk patients [CMV Donor/Recipient (D/R): +/−], treatment with valganciclovir 900 mg/day adjusted to renal function for 3 months; (ii) in patients who received steroid boluses or IL-2 receptor antibodies (basiliximab), regardless of serological status, treatment with valganciclovir 900 mg/day for 14 days; (iii) in low-risk patients (CMV D/R: +/+ or CMV D/R: −/+) a preemptive approach was preferred: when CMV viral load showed signs of progression or reached 5000 copies/ml, therapy was instituted with valganciclovir 900 mg every 12 h until two negative viremias were obtained in different weeks. CMV disease was initially treated with IV gancyclovir eventually switching to oral valganciclovir in case of good response for a total of 14–21 days.

Primary prophylaxis and preemptive treatment were considered as potential variables associated with CVE.

(iv) *Immunosuppression-related variables*: Over-immunosuppression (IS) was considered when the patient received a triple IS regimen aiming for standard trough levels of Tacrolimus (Tac)/Cyclosporine (Csa), when quadruple therapy was used, or when IS levels were elevated during the first 15 postoperative days (median Csa > 250 ng/ml, median Tac > 10 ng/ml or Tac > 20 ng/m at any single time point) [21].

Secondary aims

Severe recurrent hepatitis C occurring within the first year post-LT, biopsy-proven rejection, *de novo* DM, *de novo* tumors, and overall mortality were recorded. Associations between these events and baseline features and/or CMV reactivation or primo-infection were analyzed. Outcome measures were defined as follows:

1. *Severe recurrent hepatitis C*: defined as fibrosis ≥1 in a METAVIR scale, or fibrosis = 0 with moderate to severe necroinflammatory or fibrosing cholestatic hepatitis (FCH) during the first year. Elastography values during the first LT year >14 kPa were also considered as severe disease [22].

2. *Cellular rejection*: defined according to histologic Banff criteria. Protocol liver biopsies at day 7 post-LT were not performed [23].

3. *De novo DM after-LT*: need for insulin or oral antidiabetic agents or presence of altered glucose metabolism according to WHO criteria (fasting plasma glucose >126 mg/dl or three consecutive values >200 mg/dl at any time during the day) in patients who were nondiabetic prior to LT [15].

4. *De novo tumors*: any solid or hematologic tumor diagnosed during follow-up.

Statistical analysis

A descriptive analysis of quantitative variables was carried out using means or medians (1st, 3rd quartile) with their corresponding standard deviations, respectively. Categorical variables are reported as absolute and relative frequencies. Survival as a function of CS-CMV reactivation or primo-infection was studied using the Kaplan–Meier curves. The curves were compared using the log-rank test.

To assess the effect of CMV on post-LT outcome measures, survival analysis was carried out by means of Cox regression models for CVE as well as for recurrent hepatitis C, rejection, DM, and HCC recurrence. These models were constructed taking into consideration relevant clinical variables and potential confounders. Moreover, several Cox regression models were performed at landmark analysis at 3, 6, and 12 months. Hazard ratio together with 95% confidence intervals was reported. We only considered outcome measures that appeared after CS-CMV reactivation or primo-infection. R software (version 3.4.3) was used to perform the statistical analysis. All *P*-values below 0.05 were considered statistically significant.

Results

Baseline features and LT outcome

The study population included 140 patients of a total of 201 who underwent LT due to HCV cirrhosis (117 viremic and 23 nonviremic) between January 2010 and June 2014. Reasons for exclusion were: (i) HBV or HIV co-infection, $n = 20$; (ii) perioperative death, $n = 4$; (iii) CMV serology mismatch D/R−/−, $n = 3$; (iv) re-LT, $n = 13$; (v) loss to follow-up, $n = 1$; (vi) fewer than five CMV determinations during the first year after LT, $n = 20$.

Median age at LT was 57 years, 72% were men, and the median MELD score was 16. Hepatocellular carcinoma was present in 58% of patients, 22% were diabetics, 23% had AHT, 45% were either actual smokers or had a history of tobacco use, and the median BMI was 27 kg/m^2. In most patients, baseline IS consisted of a calcineurin inhibitor (mainly tacrolimus) in combination with mycophenolate mofetil, however, 13% of patients received therapy with mTOR inhibitors. Nearly 34% of patients were considered over-immunosuppressed (Table 1). The median follow-up period was 4.1 years (2.6, 5.59 1st, 3rd Q).

Thirty-eight of the 140 patients died during the follow-up period. The main causes of death were: primary recurrent disease including HCV ($n = 12$, 31.5%) or HCC ($n = 5$, 13%), infections ($n = 7$, 18%), *de novo* tumors ($n = 5$, 13%), CVE ($n = 2$, 5%), and others ($n = 7$, 18%), (Table 2). Median time to death was 1.18 years (0.49, 2.06 1st, 3rd Q).

A total of 26 patients (18.5%) suffered a CVE during follow-up, of which nine were arrhythmias, four myocardial infarctions, seven strokes, four episodes of angina, one case of peripheral artery disease, and one case of heart failure.

In addition, 81 of the 117 viremic patients, (69%) developed severe recurrent hepatitis C. Of the overall population, 10 (7%) developed a *de novo* tumor, eight (6%) had HCC recurrence, and 37 (27%) *de novo* diabetes (Table 2).

Variables associated with cardiovascular events

We assessed whether CVE were associated with baseline factors, type of IS or CMV-related factors (Table 3). Of the 26 patients with CVE, 12 had experienced a prior CS-CMV reactivation (n=11) or primo-infection (n=1). The median time to CVE was 6.36 months (1st Q 1.8, 3rd Q 29.76), and the median time to CMV reactivation was 1.8 months (1st Q 1.1, 3rd Q 3.1).

The Kaplan–Meier curve showed that the rate of CVE since liver transplantation was higher in those with CS-CMV reactivation ($P = 0.001$) (Fig. 1). By Cox regression analysis, CS-CMV reactivation or primo-infection was significantly associated with the development of CVE [HR = 4.7, 95% CI (1.8, 12.5), $P = 0.002$]. Pre-LT diabetes mellitus was also associated with the development of CVE [HR = 4.6, 95% CI (1.6, 13), $P = 0.004$] (Table 3). The other variables analyzed (donor and recipient age, history of tobacco use, HCV-RNA positive at LT, AHT, dyslipidemia, and over-immunosuppression) were not associated with CVE post-LT. Furthermore, several models were carried out to analyze the effect of CS-CMV reactivation or primo-infection on the development of CVE at landmarks of 3, 6, and 12 months and the association persisted at the three time points [3 months: HR = 4.7, 95% CI (0.7, 29.4), $P = 0.09$, 6 months: HR = 6.08, 95% CI (1.08, 34), $P = 0.04$, 12 months: HR = 5.4, 95% CI (1.02, 28.9), $P = 0.04$]. (Tables S1-S3).

Effect of prophylaxis on the development of CVE

A total of 16 patients received primary prophylaxis against CMV reactivation, while the remainder of patients were followed up with a preemptive strategy. Five (31%) of those on primary prophylaxis as well as 21(17%) on preemptive strategy developed a CVE. Due to the small number of patients receiving primary prophylaxis ($n = 16$), it was not possible to draw a firm conclusion with regard to the beneficial effect of primary versus preemptive prophylaxis on CVE development.

Other outcome measures and CS-CMV reactivation

Several models were constructed to analyze whether CS-CMV reactivation or primo-infection was associated with the remaining outcome measures, including severe recurrent hepatitis C, *de novo* diabetes mellitus, HCC recurrence, and *de novo* tumors. No association was found between CS-CMV reactivation/primo-infection and these outcomes. Post-LT survival although was significantly lower in patients with CS-CMV reactivation or primo-infection compared to those without ($P = 0.03$).

Discussion

This study intends to examine the impact of CMV reactivation on HCV cirrhosis LT recipients. The following are, in our view, the most relevant findings:

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Table 1. Characteristics of the study population.

Variables	*N* = 140
Recipient age, years (median, 1st, 3rd Q)	57 (50.2, 63)
Gender, male, *n* (%)	101 (72%)
Child-Pugh classification (%)	A = 46 (33%) B = 29 (21%) C = 64 (46%)
MELD score (median, 1st, 3rd Q)	16 (10, 21)
HCC (%)	80 (58%)*
Creatinine clearance (ml/min) (median, 1st, 3rd Q)	95 (70, 108.5)
eGFR < 60% (%)	26.5%
Pre-LT DM, *n* (%)	31 (22%)
Pre-LT AHT, *n* (%)	32 (23%)
Pre-LT Tobacco use, *n* (%)	63 (45%)
BMI at LT (median, 1st, 3rd Q) (kg/m^2)	27 (24, 30)
BMI > 30%	27 (20%)
History of dyslipidemia, *n* (%)	11 (8%)
Pre-LT antiviral treatment, *n* (%)	82 (63.5%)
Donor age, years (median, 1st, 3rd Q)	58.5 (50, 67)
Donor–Recipient Serological status	
CMV D/R +/+	109 (78%)
CMV D/R −/+	19 (13.5%)
CMV D/R +/−	12 (8.5%)
CMV-prophylaxis	16 (11%)
D/R +/− Mismatch	12
IL-2 receptor antibody therapy	3
Rejection therapy	1
CNI at discharge, *n* (%)	125 (90%)
Tac	65 (52%)
Csa	60 (48%)
MMF at discharge, *n* (%)	105 (75%)
Steroids at discharge, *n* (%)	86 (62%)
M-TOR inhibitors at discharge, *n* (%)	16 (11.5%)
Basiliximab use, *n* (%)	12 (9%)
Immunosuppression (%)	
High	46 (34%)
Low	51 (37.5%)
Optimal	39 (29%)

eGFR, estimated glomerular filtrate rate; HCC, hepatocellular carcinoma; D, donor; R, recipient; LT, liver transplantation; CMV, cytomegalovirus; DM, diabetes mellitus; CNI, calcineurin inhibitors; Tac, tacrolimus; Csa, cyclosporin; MMF, mycophenolate mofetil; BMI, body mass index; AHT, arterial hypertension.

*Intra MILAN, *n* = 66 (48%), Extra MILAN, *n* = 14 (10%).

1. CVE occurred more frequently in patients with CS-CMV reactivation post-LT. 2. In contrast, other outcome measures, such as severe recurrent hepatitis C, *de novo* tumors, and HCC recurrence were not associated with CMV reactivation after LT.
2. Moreover, survival was associated with CS-CMV reactivation or primo-infection.

1. Cardiovascular events and *de novo* tumors are emerging as the leading causes of long-term mortality post-LT. More specifically, cardiovascular complications are the main cause of non-graft-related deaths, accounting for approximately 21% of deaths among patients who survive at least 3 years [1,2]. The association between CMV reactivation and CVE has been described in other solid organ transplants, such as heart, lung, or kidney transplantation [24–26], but had not been previously assessed in LT patients. In our study, 16 of 26 patients who developed a CVE during the follow-up period had a prior history of CMV reactivation after LT, 12 of whom considered clinically significant. Moreover, in the Cox regression analysis, CMV reactivation was independently associated with the development of CVE (HR = 4.7, $P = 0.002$). The reason why CMV contributes to increased CV risk in the LT setting is not entirely clear. Several well-established CV risk factors have been reported in the general population with cirrhosis, namely: metabolic syndrome, NASH, cirrhotic cardiomyopathy, diabetes mellitus, dyslipidemia, and renal insufficiency. Recently, epidemiological studies have suggested that HCV infection should also be considered a CV risk factor in the nontransplant population without comorbidities [12,13]. Evidence of a link between CMV and CV risk has been described in experimental studies performed in immunocompetent and transplant populations [5–7]. As endothelial cells are a known target of CMV, it seems plausible that CMV may cause an inflammation of the intima layer with subsequent endothelial dysfunction, which may ultimately contribute to the progression of atherosclerosis in the nontransplant population [5]. In the post-transplant setting, experimental studies with murine models have provided compelling evidence that CMV plays an important role in the vascular disease process due to the alloreactive immune response to CMV inducing an acceleration of transplant vascular sclerosis [8]. In clinical studies, CMV reactivation has been associated with higher incidence of CVE, especially in heart transplant patients [24,26] due to cardiac allograft vasculopathy and/or direct endothelial injury; some studies have also reported a significant increase in medium-term mortality in different organ transplant patients with CMV reactivation [27]. In addition, recently, Desai and cols [11] have reported that positive CMV serology has an

impact on long-term mortality, specifically due to CVE deaths in renal, cardiac, and pulmonary transplant patients, but not in LT patients. Although an increase in CVE related to CMV reactivation in the post-LT has not been described, some studies have described an association between CMV reactivation and endothelial complications not in the heart but in other endothelial sites. More specifically, CMV reactivation has been associated with increased risk of late arterial thrombosis [28]. A direct thrombogenic effect together with a hypercoagulable state associated with impaired fibrinolysis may be the underlying reasons. Pretransplantation stratification of CV risk is a relevant goal in liver transplantation as many factors already present in the pre-LT period typically worsen after surgery, due to chronic exposure to immunosuppression treatment or changes in lifestyle, and that CV events represent a major source of morbidity and mortality following LT.

In fact, in our HCV-LT population, both factors, pre-LT hepatitis C cirrhosis or recurrent hepatitis C and CS-CMV reactivation or primo-infection, added to the described CV risk factors which generally increase in the post-LT, may explain the results of our study. In our study population, the prevalence of traditional CV risk factors was similar to those described in other LT populations due to causes other than HCV [14]. Our findings, which should be confirmed in larger series including other indications for LT, emphasize the need for an adequate CMV prophylactic and/or preemptive strategy not only to prevent the typical consequences of CMV reactivation but also to reduce the medium-to-long-term increased risk of CVE. Meaningful conclusions related to the potential beneficial effect of a universal prophylaxis versus preemptive therapy, at least in patients with high CV risk, cannot be drawn from this study because of the small number of patients on primary prophylaxis.

2. While initially, one of our goals was to evaluate the potential association between CMV reactivation and HCV-related disease severity [29–34], we understand that this is no longer relevant in an era of universal use of extremely efficacious therapies against HCV [29–34]. Interestingly, we did not find an association between

Table 2. Post-transplant endpoint events.

	N = 140 (%)
CVE (%)	26 (18.5)
Severe recurrent Hepatitis C* (%)	81/117 (69)
Post-LT Antiviral treatment (% yes)	100 (88)
Types	
IFN – free	65 (65)
IFN-based therapies	35 (35)
SVR (%)	87 (87)
De novo DM (%)	37 (27)
DM at end of follow-up (%)	57 (41)
Cellular rejection (%)	20 (15)
Treated cellular rejection (%)	14 (70)
De novo neoplasia (%)	10 (7)
HCC recurrence (%)	8 (6)
Re-LT (%)	9 (6)
CMV reactivation or primo-infection	65 (46)
CS-CMV reactivation or primo-infection	35 (25)
Deaths (%)	38 (27)

CS, clinically significant; CVE, cardiovascular events; HCC, hepatocellular carcinoma; DM, diabetes mellitus; LT, Liver Transplantation; IFN, Interferon; SVR, sustained virological response.

*In HCV-RNA positive.

Table 3. Cardiovascular events post-LT: Cox regression analysis.

	Estimate	HR	CI 95%	*P*-value
CS-CMV reactivation or primo-infection	1.561	4.76	(1.80, 12.56)	0.002
Recipient age	0.045	1.046	(0.98, 1.11)	0.17
Donor age	−0.004	0.99	(0.964, 1.028)	0.78
Pre-LT DM	1.526	4.6	(1.62, 13.06)	0.004
HCV-RNA negative at LT	0.649	1.914	(0.506, 7.248)	0.339
Pre-LT creatinine clearence	−0.003	0.997	(0.98, 1.015)	0.775
Pre-LT tobacco use	−0.444	0.642	(0.25, 1.647)	0.356
Pre-LT dyslipidemia	−0.793	0.543	(0.121, 1.699)	0.24
Pre-LT AHT	−0.33	0.717	(0.25, 2.052)	0.535
High immunosuppression	−0.238	0.788	(0.214, 2.901)	0.72
Optimal immunosuppression	−1.002	0.367	(0.088, 1.537)	0.17

CS, clinically significant; DM, diabetes mellitus; AHT, arterial hypertension; LT, Liver Transplantation.

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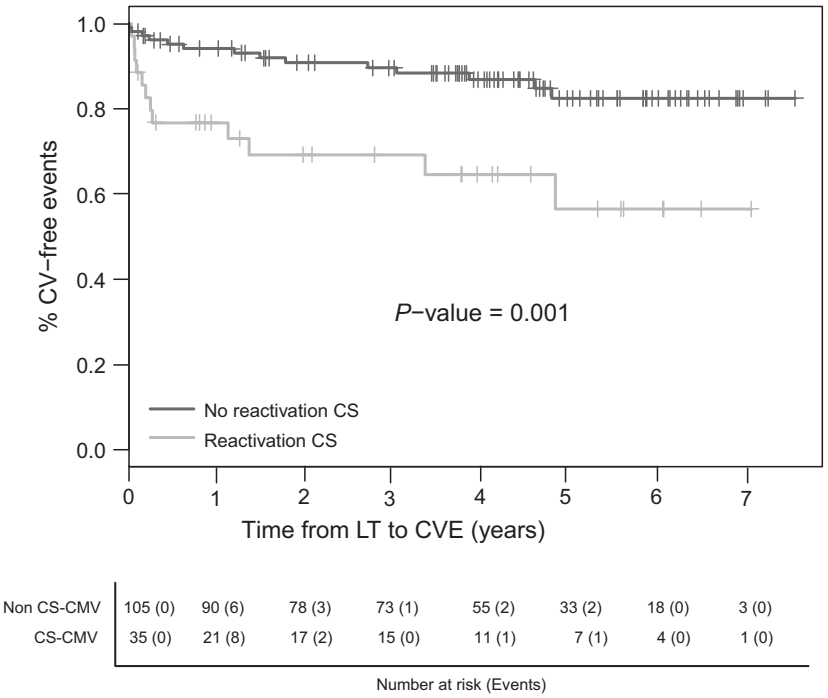

Figure 1 Kaplan–Meier curve showing the probability of being free of cardiovascular event (CVE) since liver transplantation. The rate of CVE was higher in those with clinically significant-cytomegalovirus reactivation ($P = 0.001$).

other endpoints, such as *de novo* tumors, rejection, or post-LT DM. However, an association with survival was observed as previously reported by other authors [27].

Our study has limitations such as the sample size or the selection of an HCV-infected target population. There are several reasons we chose to specifically analyze the association between CMV reactivation and post-LT outcome in HCV-infected patients. First, at the time we designed the study, new oral antivirals were only emerging in the market; second, it is by far the largest LT indication in our center; third, patients chronically infected with HCV are *a priori* individuals with an increased risk of CVE.

In conclusion, in HCV-LT patients, CMV reactivation was associated with a greater risk of developing CV events. Our results should be confirmed in larger series including non-HCV patients given the significant impact that CV events have on long-term mortality. Furthermore, extreme care is encouraged with regard to CMV monitoring to prevent clinically significant CMV reactivation, at least in patients considered at increased risk of developing either CV events or CMV reactivation.

Authorship

VA: research design and performance, literature search, data collection and paper writing. TM: research design, data collection and paper writing. IC: research design, data collection and paper writing. CP: manuscript review. AC-G: manuscript review. AC: manuscript review. CV: manuscript review. MG: manuscript review. SB: manuscript review. AR: manuscript review. LG-D: manuscript review. JMM: microbiological procedures. LP: manuscript review. VF-F: statistical analyses. JL-L: manuscript review. MP: manuscript review. MB: research design, literature search and wrote paper.

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Conflict of interest

The authors have declared no conflicts of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Cardiovascular events post-LT: Cox regression analysis at 3 months.

Table S2. Cardiovascular events post-LT: Cox regression analysis at 6 months.

Table S3. Cardiovascular events post-LT: Cox regression analysis at 12 months.

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