ORIGINAL ARTICLE

The impact of everolimus in reducing cytomegalovirus events in kidney transplant recipients on steroid-avoidance strategy: 3-year follow-up of a randomized clinical trial

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SUMMARY

There is no evidence of whether everolimus (EVR) reduces cytomegalovirus (CMV) events in patients receiving steroid-free regimens. Besides, studies evaluating a tacrolimus (TAC) and EVR regimen are limited to 1-year follow-up. In this single-center prospective randomized trial, the incidence of CMV and 3-year efficacy and safety outcomes of EVR were compared to those of mycophenolate sodium (MPS) in a steroid-free regimen based on low-exposure TAC. Both groups received rabbit anti-thymocyte globulin (r-ATG) induction (6 mg/kg) and the steroids were withdrawn at day 7. Maintenance immunosuppression consisted of TAC (4–7 ng/ml until month 3 and 2–4 ng/ml thereafter) plus EVR (3–8 ng/ml) in the EVR group (n = 59); and TAC (4–7 ng/ml during all follow-up) plus MPS (1440 mg) in the MPS group (n = 56). The EVR group presented with a lower incidence of CMV events (18.6% vs. 50%, P = 0.001). No differences were observed in biopsy-proven acute rejection (6.8% vs. 3.6%, P = 0.680),graft loss (0.0% vs. 1.8%, P = 0.487),death (6.8% vs. 1.8%, P = 0.365), or estimated glomerular filtration rate at 36 months (61.1 ± 25.4 vs. 66.3 ± 24 ml/min/1.73 m^2, P = 0.369). A higher proportion of patients discontinued MPS treatment (8.5% vs. 26.8%, P = 0.013) for safety issues. In conclusion, EVR was associated with lower rates of CMV events in patients induced with standard dose r-ATG and a maintenance steroid-free regimen based on TAC. This regimen effectively prevented acute rejection and demonstrated a more favorable safety profile. (ClinicalTrials.gov:NCT02084446).

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Key words
cytomegalovirus, everolimus, kidney transplant, mTOR inhibitors, steroid

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Introduction

Cytomegalovirus (CMV) disease negatively impacts transplant outcomes, and the currently available strategies used to prevent it – prophylaxis and preemptive therapy – are associated with safety concerns, logistical difficulties, and high cost [1–4]. Therefore, using an immunosuppressive regimen associated with low incidence of CMV events seems an interesting strategy.

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Previous studies demonstrated that mammalian target of rapamycin inhibitors (mTORi) are associated with a low incidence of CMV events [5]. Some authors suggested that CMV-preventive strategies might be dispensable in patients receiving sirolimus or everolimus (EVR) [6,7]. However, only one prospective randomized study directly assessed the incidence of CMV events as the primary end-point in kidney transplant (KT) recipients. In this trial, two regimens based on tacrolimus (TAC), EVR, and steroids were compared with the standard-of-care immunosuppressive regimen based on TAC, steroids, and mycophenolate. EVR was shown to be independently associated with a lower risk of CMV. Interestingly, patients on EVR receiving induction therapy with low-dose rabbit anti-thymocyte globulin (r-ATG 3 mg/kg) presented better outcomes than those induced with basiliximab [7].

In our center, a steroid avoidance strategy was first adopted for low-risk patients in 2001. No prospective randomized study has evaluated the efficacy of a TAC-EVR regimen for preventing CMV events in a steroid-free strategy. In addition, studies evaluating this immunosuppressive regimen have a follow-up limited to 1 year [7–9].

This prospective randomized controlled study assessed the ability of EVR to prevent CMV infection and disease in patients receiving induction therapy with a standard dose of r-ATG as a steroid avoidance strategy. Patients were followed for 3 years to assess the medium-term efficacy and safety outcomes.

Materials and methods

Study design

This single-center open-label prospective randomized controlled clinical trial aimed to compare 36-month efficacy and safety outcomes of EVR or enteric-coated mycophenolate sodium (MPS) in *de novo* KT recipients receiving a steroid-free protocol based on low TAC exposure (ClinicalTrials.gov: NCT02084446). All subjects provided written informed consent before enrollment. The study was conducted according to Good Clinical Practice guidelines, met the ethical standards of the Declaration of Istanbul 2008 and 2000 Declaration of Helsinki, and was approved by the local ethics committee (CAAE: 07624412.0.0000.5040).

Population

Eligible patients were adults (aged 18–75 years) who received a primary allograft from nonhuman leukocyte antigen (HLA) identical living or deceased KT. The exclusion criteria were: focal segmental glomerulosclerosis as the chronic kidney disease etiology, ABO incompatibility, positive cytotoxic cross-match, multiorgan transplant, panel reactive antibodies >50%, and pre-transplant donor-specific anti-HLA antibodies (DSA) > 1500 mean fluorescence intensity (MFI) [10]. We also excluded patients who planned to be followed in another transplant center.

The sample size was calculated considering the CMV event incidences of 40% in the MPS group and 15% in the EVR group. With a two-sided type I error of 5% and 85% power to detect the difference, the estimated sample size was 56 patients per group. Assuming a 10% dropout rate, we randomized 124 patients.

Immunosuppression

Eligible patients were randomized (1:1) as follows: (i) EVR group: TAC 0.05 mg/kg twice daily starting within 48–72 h post-transplant adjusted to maintain whole blood trough concentrations of 4–7 ng/ml for the first 3 months, then reduced to 2–4 ng/ml and then EVR 1.5 mg twice daily starting in the first 24 h after the surgery adjusted to maintain whole blood trough concentrations of 3–8 ng/ml; or the (ii) MPS group: TAC 0.05 mg/kg twice daily starting within 48–72 h post-transplant adjusted to maintain whole blood trough concentrations of 4–7 ng/ml and MPS 720 mg twice daily starting in the first 24 h after surgery.

Patients on both arms received r-ATG 1.5 mg/kg × 4 doses (6 mg/kg total) starting intraoperatively and continuing every other day. Methylprednisolone was given intravenously before the first three r-ATG doses (500, 250, and 125 mg). Prednisone 20 mg was given orally before the last r-ATG dose. No further corticosteroid use was planned after the first week post-KT unless patient was on long-term prednisone prior to the transplantation for another clinical reason.

CMV screening and clinical management

No patient received CMV prophylaxis. A preemptive strategy was used based on serial monitoring of CMV DNAnemia using a commercial quantitative CMV polymerase chain reaction assay (Q-CMV complete kit (Nanogen Advanced Diagnostics, Turin, Italy)) on whole blood samples taken every 2 weeks from week 2 up to 3 months and then at months 4, 6, 9, and 12. Intravenous gancyclovir or oral valgancyclovir was prescribed when the viral load was >5000 UI/ml in CMV

immunoglobulin G (IgG) D±/R+ patients or >2500 UI/ml in CMV IgG D+/R− patients. Patients with tissue invasive disease received intravenous gancyclovir regardless of DNAnemia values. All patients were treated until two negative assays performed with a 1-week interval were achieved, and invasive diseases were treated for at least 21 days. No patient received secondary prophylaxis. Conversion from MPS to EVR was performed in patients with antiviral therapy failure (defined as <3 log decline in DNAemia after 2 weeks of treatment) or recurrent episodes. No further immunosuppressive drug manipulation was routinely recommended. In critically ill patients, clinical management was delivered at the investigators' discretion.

Other infectious prophylaxis and screening

All patients received a daily dose of sulfamethoxazole 400 mg with trimethoprim 80 mg for at least 6 months for *Pneumocystis jirovecii* and *Toxoplasma gondii* prophylaxis. Patients with a latent tuberculosis infection or previous history of tuberculosis received isoniazid 300 mg for 9 months.

BK-polyoma (BK) virus viremia screening was performed using whole blood qualitative reverse transcription polymerase chain reaction at months 2, 4, 6, 9, 12, and 24 post-transplantation. An allograft biopsy and TAC minimization were indicated when the viral load was >10 000 copies/ml.

Definitions and procedures

Biopsy-proven acute rejection (BPAR) was defined as allograft dysfunction confirmed by a renal biopsy scored according to Banff 2009 criteria (grade IA or higher) and treated with methylprednisolone or r-ATG pulse therapy. Treated acute rejection included BPAR and treated allograft dysfunction without biopsy confirmation, including borderline infiltrates.

Delayed graft function (DGF) was defined as the need for dialysis during the first week post-transplantation [11].

Post-transplantation diabetes mellitus (PTDM) was defined as the need for glucose-lowering agents in patients without a previous diagnosis of diabetes.

Cytomegalovirus DNAemia or infection was defined as the detection of CMV DNA in whole blood samples according to the above-mentioned cut-offs used for preemptive treatment. CMV syndrome was defined as positive DNAemia and at least two of the following: (i) fever ≥38 °C; (ii) malaise or fatigue; (iii) leukopenia or neutropenia; (iv) atypical lymphocytosis; (v) thrombocytopenia; (vi) alanine transaminase or aspartate transaminase increase. Except for CMV retinitis, proven tissue CMV disease required histological confirmation. CMV events included DNAemia/infection, CMV syndrome, and/or tissue-invasive CMV disease. Recurrent CMV event was defined as a new CMV episode (infection, syndrome, or proven tissue disease) in a patient who had no evidence of viral replication after previous treatment [12].

For subclinical wound-healing complications, a routine abdominal ultrasound was performed in all patients between the second postoperative day and the hospital discharge.

The presence of circulating DSA was first analyzed using a screening test (LabScreen Mixed; One Lambda, Canoga Park, CA, USA). The cut-off for positive samples was the Normalized Background ratio advocated by the manufacturer (>3). Those serum samples that tested positive were subjected to single-antigen bead assays (LabScreen Single Antigen; One Lambda) on the Luminex platform. All reactions showing a MFI > 1500 were judged as positive provided that the antibodies were not directed against the self or denatured HLA antigens. For the *de novo* DSA (*dn*DSA) analysis, we selected serum samples collected 3 years post-transplantation or, in its absence, the last available sample collected after the first year.

End-points

The primary end-point was the incidence of CMV events during the follow-up period. Secondary end-points included: BPAR and treated acute rejection episodes, graft loss, death; loss to follow-up, treatment discontinuation for efficacy or safety reasons, DGF incidence and duration, surgical complications, renal function, BK virus viremia or nephropathy, spot urine protein/creatinine ratio, incidence of PTDM, incidence of dyslipidemia requiring statins, malignancy, *dn*DSA, and 3-year allograft and patient survival rates. End-points were conducted according to the intention-to-treat principle.

Statistical analysis

Continuous data are expressed as mean and standard deviation (SD), while median was used when the SD > mean. Comparisons were performed using Student's *t*-test or the Mann Whitney *U* test depending on the distribution. Categorical variables are expressed as frequencies and percentages and compared using χ^2 or Fisher's test. Renal function was assessed by glomerular filtration rate (Merchan, #1053) [13] estimated using the

4-variable Modification of Diet in Renal Disease formula. For patients who lost the graft, eGFR was scored as 0 ml/min; for those who died or were lost to follow-up, the last available eGFR was considered (last observation carried forward analysis). The intergroup comparison of eGFR over time was performed using repeated measures analysis of variance. A significantly statistical difference was assumed when the P value was <0.05.

Results

Population/Demographics

Between December 2012 and July 2014, 124 patients were recruited and 115 met the inclusion/exclusion criteria (EVR group = 59; MPS group = 56) (Fig. 1). Patients were predominantly young men with a low immunological risk who received kidneys from young deceased donors. Demographic characteristics were similar between groups (Table 1).

Immunosuppression

As shown in Fig. 2, mean TAC trough concentrations were similar between groups at months 1, 3, 6, 12, and 24 as follows: 7.3 ± 3.1 vs. 7.6 ± 2.6 (P = 0.657), 6.0 ± 2.1 vs. 5.9 ± 1.6 (P = 0.674), 5.3 ± 1.4 vs. 5.4 ± 2.0 (P = 0.685), 4.9 ± 1.2 vs. 5.3 ± 1.4 (P = 0.225), and 5.4 ± 1.9 vs. 5.4 ± 1.7 ng/ml (P = 0.934), respectively. Mean EVR concentrations in the EVR group in these months were 5.0 ± 1.6, 5.1 ± 1.3, 5.1 ± 1.9, 4.8 ± 1.4, and 4.8 ± 1.5 ng/ml, respectively, while mean MPS doses in the MPS group were 1102 ± 243, 991 ± 256, 1013 ± 251, 1038 ± 238, and 1071 ± 247 mg.

Six patients in the EVR group and five in the MPS group received prednisone as part of the initial immunosuppressive regimen, eight of these because of pre-transplantation indications (hypopituitarism, glomerulopathies, and autoimmune diseases). Three patients who underwent a second KT were maintained on steroids at the investigator's request.

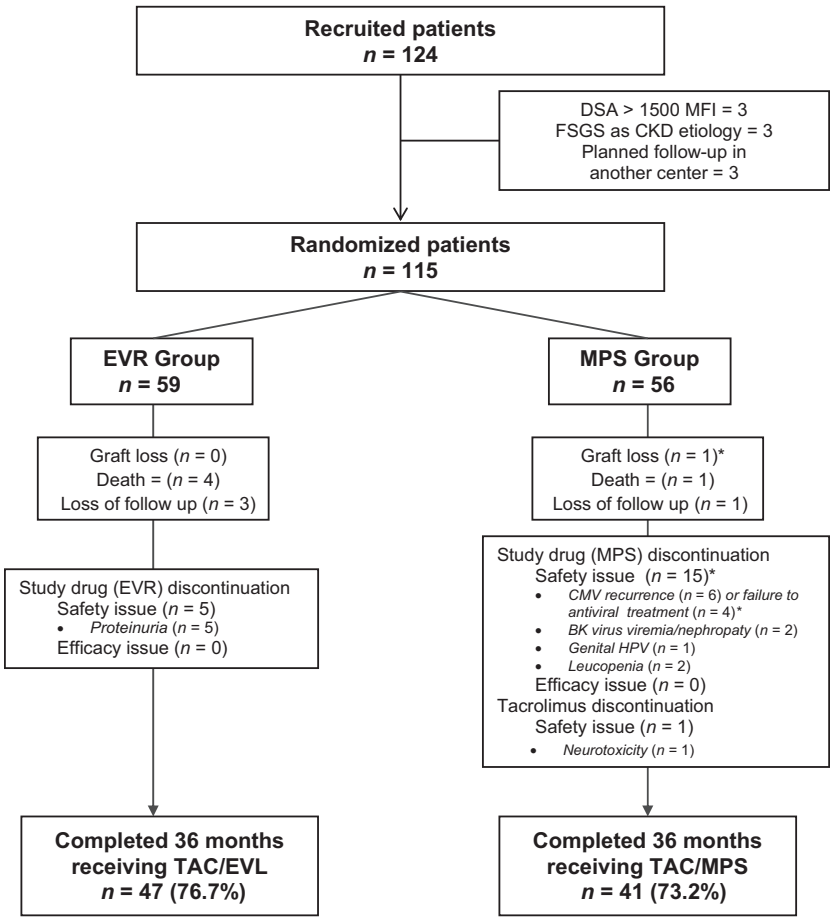

Figure 1 Patient disposition, losses and drug discontinuations. FSGS, focal segmental glomerulosclerosis; CKD, chronic kidney disease. *one patient with previous MPS discontinuation lost the graft.

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Table 1. Demographic characteristics.

	Total $n = 115$	Everolimus group $n = 59$	MPS group $n = 56$	P value
Recipient age (years old), mean ± SD	43.7 ± 14.2	45.8 ± 15.1	41.5 ± 12.9	0.107
Recipient gender – male, *n* (%)	92 (80)	45 (76.3)	47 (83.9)	0.356
Body mass index (kg/m^2), mean ± SD	23.9 ± 4.0	23.9 ± 3.9	23.9 ± 4.2	0.999
Recipient race, *n* (%)				
Caucasian	8 (7.0)	4 (6.8)	4 (7.1)	0.633
Afro-Brazilian	15 (13.0)	6 (10.2)	9 (16.1)	
Mixed race	92 (80.0)	49 (83.0)	43 (76.8)	
CKD etiology, *n* (%)				
Unknown	53 (46.1)	20 (33.9)	33 (58.9)	0.056
Hypertension	18 (15.7)	13 (22.0)	5 (8.9)	
Diabetes mellitus	15 (13.0)	9 (15.3)	6 (10.7)	
Chronic glomerulonephritis	16 (13.9)	8 (13.6)	8 (14.3)	
Other	13 (11.3)	9 (15.3)	4 (7.1)	
Time on dialysis (months), mean ± SD (median)	32.2 ± 31.2 (24)	29.9 ± 25.6 (24)	34.7 ± 36.2 (24)	0.614
Retransplantation, *n* (%)	5 (4.3)	3 (5.1)	2 (3.6)	1.000
CMV IgG serologic status, *n* (%)				
D+R+	85 (73.9)	41 (69.5)	44 (78.6)	0.066
D+/R−	13 (11.3)	10 (16.9)	3 (5.3)	
D−/R+	8 (7.0)	6 (10.2)	2 (3.6)	
D−/R−	6 (5.2)	1 (1.7)	5 (8.9)	
D_{unk}/R+	3 (2.6)	1 (1.7)	2 (3.6)	
PRA (%), mean ± SD (median)	5.4 ± 11.2 (0)	6.1 ± 12.0 (0)	4.6 ± 10.3 (0)	0.819
HLA MM, mean ± SD	3.9 ± 1.2	3.8 ± 1.2	3.9 ± 1.1	0.681
HLA-DR MM ≥ 1	82 (71.3)	40 (71.2)	42 (71.4)	1.000
Donor age (years old), mean ± SD	30.9 ± 11.9	30.5 ± 11.0	31.3 ± 12.9	0.820
Donor source – deceased, *n* (%)	111 (96.5)	55 (93.2)	56 (100)	0.119
Pulsatile machine perfusion, *n* (%)	61 (55.0)	30 (54.5)	31 (55.4)	1.000
Cold ischemia time (h), mean ± SD	23.7 ± 7.8	22.8 ± 7.6	24.6 ± 8.0	0.110

SD, standard deviation; CKD, chronic kidney disease; PRA, panel reactive antibodies; MM, mismatches; CMV, cytomegalovirus; R, recipient; D, donor; unk, unknown.

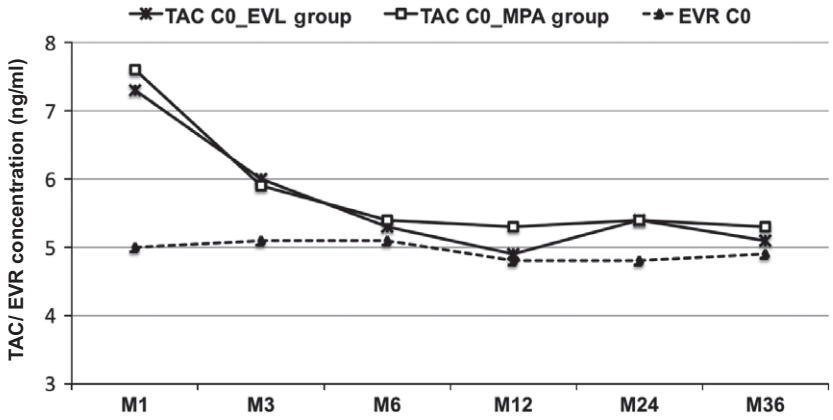
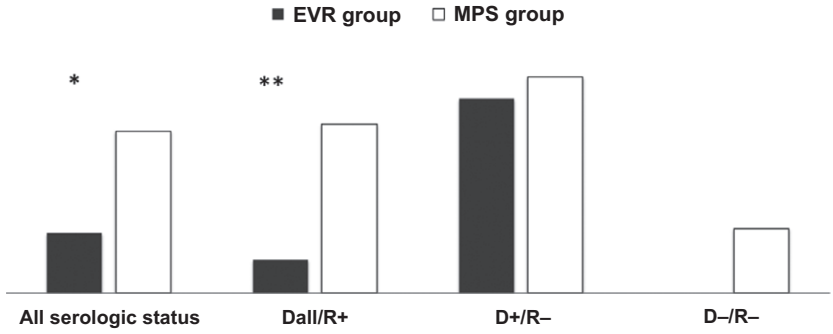


Figure 2 Mean tacrolimus (TAC) and everolimus (EVR) whole blood concentration. EVR C_0 was maintained within the target throughout the follow-up. Tacrolimus (TAC) C_0 was similar between groups at all periods. There was no adherence of investigators to the minimization scheduled for EVR group from month 3.



	All serologic groups	Dall/R+	D+/R–	D–/R–
EVR group	11/59 (18.6%)	5/48 (10.4%)	6/10 (60%)	0/1 (0%)
MPS group	28/56 (50%)	25/48 (52.2%)	2/3 (66.7%)	1/5 (20%)

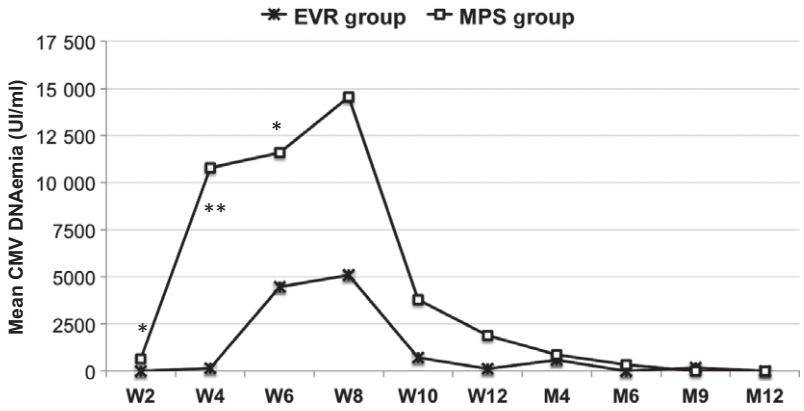
Figure 3 Cytomegalovirus incidence according to pretransplant serologic status. The incidence of CMV events was inferior in everolimus group when considered all serologic status and in recipients IgG+, irrespective of donor serostatus (Dall/R+). *P = 0.001; **P < 0.001. CMV, cytomegalovirus; D, donor; R, recipient; all, negative, positive or unknown.

Primary end-point

Patients on EVR had a significantly lower incidence of CMV events (18.6% vs. 50.0%, P = 0.001). This difference was most pronounced in those with pre-transplantation positive IgG CMV serology regardless of donor serology (D+/R+, D−/R+, and D_{unk}/R+) (10.4% vs. 52.2%, P < 0.001). The incidence of CMV events in CMV IgG D+/R− patients were similar between groups (60.0% vs. 66.7%) (Fig. 3). Interestingly, a greater difference was also observed in patients with zero mismatches in the HLA-DR locus (5.9% vs. 56.3%, P = 0.002) compared to those with one or more DR mismatches (23.8% vs. 47.5%, P = 0.037). The single patient with zero HLA-DR mismatches who developed a CMV event (viremia) was CMV IgG negative before KT and received a kidney from a positive CMV IgG donor (D+/R−).

Of the 11 first CMV events observed in the EVR group, 10 were infections and one was a syndrome. In this group, five of the 11 patients (45.5%) presented with recurrent CMV episodes. In the MPS group, 25 of the 28 episodes were infections, three were syndromes, and one was gastrointestinal invasive disease. Four patients were converted to EVR due to ganciclovir/valganciclovir treatment failure during the first CMV event. Seven of the 28 patients (25%) presented with recurrent CMV episodes; six were converted to EVR for this reason and one was previously converted due to treatment failure. All CMV events were treated with antiviral drugs.

The significant difference between groups in the incidence of CMV events was maintained after exclusion of the 11 patients who received steroids (20.8% vs. 52.9%, P = 0.001).



	W2	W4	W6	W8	W10	W12	M4	M6	M9	M12
EVR (M±SD)	0 ± 0	141 ± 851	4470 ±15 988	5103 ± 19 165	714 ± 3271	119 ± 541	584 ± 3236	0 ± 0	173 ± 787	0 ± 0
MPS (M±SD)	639 ± 1976	10802 ± 38 430	11 597 ± 27 481	14 556 ± 55 730	3790 ± 14 286	1893 ± 8605	849 ± 2511	325 ± 2327	0 ± 0	0 ± 0

Figure 4 Kinetics of CMV viral load. MPS group presented earlier and higher CMV DNAemia peaks. CMV, cytomegalovirus; M, mean; SD, standard deviation. *$P < 0.05$ **$P < 0.001$.

The success rate of CMV monitoring including all defined periods was 89.8% (91.4% in the EVR group and 88% in the MPS group). The kinetics of the viral load showed that patients on MPS presented earlier and had higher CMV DNAemia peaks than patients on EVR (Fig. 4).

Secondary end-points

There was no intergroup difference on BPAR or treated acute rejection incidence, graft loss, death, or loss to follow-up (Table 2). However, a higher proportion of patients on MPS discontinued the initial immunosuppressive regimen (8.5% vs. 26.8%, $P = 0.013$) (Fig. 1). In the MPS group, 15 patients were converted from MPS to EVR due to safety issues: CMV treatment failure/recurrence in 10, severe genital HPV infection in one, BKV viremia in one, BKV nephropathy in one, and severe leukopenia in two. Conversions from MPS to EVR occurred a mean 178.1 ± 269.8 (median = 87) days after KT. One patient in the MPS group was converted from TAC to EVR due to neurotoxicity. In thte EVR group, all conversions were from EVR to MPS due to proteinuria (mean 375 ± 155 days post-KT).

The groups were also similar regarding DGF incidence and duration, length of hospital stay after KT, wound-healing complications requiring or not requiring surgical intervention, PTDM, dyslipidemia requiring statin therapy, proteinuria, BK virus events, and malignancy (Table 2). The eGFR values and their variation over the months were similar in the EVR and MPS groups (Fig. 5). At the end of 36 months, eGFR was also similar between groups (61.1 ± 25.4 vs. 66.3 ± 24 ml/min/1.73 m^3, $P = 0.369$).

Twenty-seven patients in the EVR group (45.7%) and 30 patients in the MPS group (53.6%) had serum available for *dn*DSA analysis collected ≥1 year post-transplantation (mean 2.2 years in both groups). Two patients in the EVR group (one class I, one class II; 7.4%) and four patients in the MPS group (one class I, three class II; 13.3%) presented with *dn*DSA.

Over the 3 years of follow-up, prednisone was added to the immunosuppressive regimen in five patients: four in the EVR group (three after acute rejection episodes, one due to suspected recurrent glomerular disease) and one in the MPS group (after a acute rejection episode).

After the exclusion of the 11 patients who received steroids since the transplant, the results of the secondary outcomes remained similar except for DGF, in which a trend toward a higher incidence in the EVR group emerged (22.0% vs. 7.8%, $P = 0.054$).

Discussion

Our results showed that, compared with MPS, EVR was associated with a lower incidence of CMV in patients receiving induction therapy with r-ATG at a standard dose and a steroid-free maintenance regimen based on a reduced TAC dose. This effect appeared more evident in patients with pre-transplantation positive IgG CMV serology and in those with zero HLA-DR mismatches. In addition, the tested regimen demonstrated similar efficacy at preventing acute rejection and a more

Table 2. Secondary end-points.

	Total *n* = 115	Everolimus group *n* = 59	MPS group *n* = 56	*P* value
BPAR, *n* (%)	6 (5.2)	4 (6.8)	2 (3.6)	0.680
Banff IA	2 (1.7)	2 (3.4)	0 (0)	
Banff IIA	1 (0.9)	1 (1.7)	0 (0)	
AMR	2 (1.7)	1 (1.7)	1 (1.8)	
Mixed AR: IIA/AMR	1 (0.9)	0 (0)	1 (1.8)	
Treated AR, *n* (%)	12 (10.4)	7 (11.9)	5 (8.9)	0.763
DGF, *n*/*N**(%)	28/111 (25.2)	17/55 (30.9)	11/56 (19.6)	0.275
Dialysis sessions after KT	2.4 ± 1.9	2.6 ± 1.9	2.1 ± 2.1	0.472
Length of hospital stay, mean ± SD	14.3 ± 9.2	14.7 ± 8.3	13.9 ± 10.1	0.666
Wound-healing complication, *n* (%)	27 (23.5)	17 (28.8)	10 (17.9)	0.192
Wound-healing complication requiring surgery *n* (%)	6 (5.2)	5 (8.5)	1 (1.8)	0.207
PTDM, *n*/*N*†(%)	19/100 (19)	12/50 (24)	7/50 (14)	0.308
Statin therapy at 36 months, *n* (%)	62 (53.9)	36 (61)	26 (46.4)	0.137
Urine protein/creatinine ratio at 36 months (mg/g), mean ± SD (median)	319.4 ± 850.1 (113)	410.1 ± 1058.5 (124)	228.5 ± 571.1 (91)	0.157
BK virus event, *n* (%)	6 (5.2)	3 (5.1)	3 (5.4)	1.000
Viremia	4 (3.5)	2 (3.4)	2 (3.6)	
Nephropathy	2 (1.7)	1 (1.7)	1 (1.8)	
Malignancy, *n* (%)	2 (1.7)	1 (1.7)	1 (1.8)	1.000
Basal cell carcinoma (skin)	2 (1.7)	1 (1.7)	1 (1.8)	
Death-censored graft survival (%)	99.1	100	98.2	0.326
Patient survival (%)	95.7	93.1	98.1	0.183
Graft loss, *n* (%)	1 (0.9)	0 (0)	1 (1.8)	0.487
Post-transplant GN	1 (0.9)	0 (0)	1 (1.8)	
Death, *n* (%)	5 (4.3)	4 (6.8)	1 (1.8)	0.365
Infection	3 (2.6)	3 (5.1)	0 (0)	
Unknown	2 (1.7)	1 (1.7)	1 (1.8)	
Loss to follow-up, *n* (%)	4 (3.5)	3 (5.1)	1 (1.8)	0.619
eGFR at 36 months (ml/min/1.73 m^2)‡	64.1 ± 24.7	62.1 ± 25.4	66.3 ± 24	0.369

SD, standard deviation; BPAR, biopsy-proven acute rejection; AR, acute rejection; AMR, antibody-mediated rejection; DGF, delayed graft function; KT, kidney transplant; PTDM, post-transplant diabetes mellitus; GN, glomerulonephritis; eGFR, estimated glomerular filtration rate.

*Including only deceased donor transplants.

†Excluding patients with pretransplant diabetes.

‡LOCF analysis.

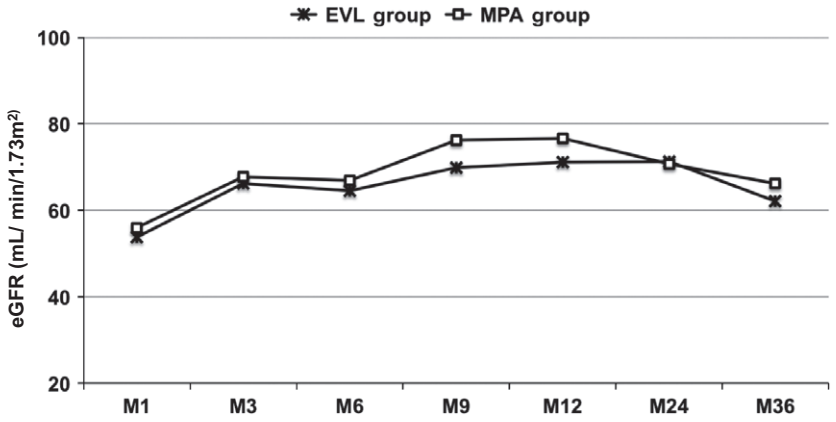


Figure 5 Estimated glomerular filtration rate over the time. LOCF analysis. Repeated measurements ANOVA: time effect <0.05; group effect >0.05; interaction >0.05.

favorable safety profile, resulting in an inferior discontinuation rate.

There are some potential mechanisms to explain the anti-CMV effect of mTORi: (i) mTORCi inhibition leads to inhibition of viral protein synthesis and viral DNA beyond the induction of apoptosis [14]; (ii) inhibition of the mTOR pathway increases the level and quality of virus-specific $CD8^+$ memory T-cells [15]; (iii) mTORi increases the yield and effector function of human gamma delta T cells, which are capable of killing CMV-infected cells [16]; and (iv) the inhibition of mTOR may also interfere with innate immunity by increasing pro-inflammatory cytokines (nuclear factor kappa β, interleukin [IL]-12, IL-23, tumor necrosis factor α, and IL-6) and suppressing anti-inflammatory cytokines (IL-10) [17,18].

Despite the significant reduction in CMV events in patients on EVR, the incidence in our patients was almost fourfold higher than that found by Tedesco-Silva *et al.* [7] (18.6% vs. 4.7%) in patients receiving similar immunosuppressive regimen based on low-dose TAC plus EVR but remained on steroids. This should be a consequence of the higher r-ATG dose (6 vs. 3 mg/kg), higher percentage of CMV IgG D+/R− transplants (16.9% vs. 5.0%), known risk factors for CMV disease [19], and the use of distinct assays for CMV screening (antigenemia vs. DNAemia) [20]. In addition, DNAemia cut-off directly affects the CMV infection incidence and there is no consensus on CMV DNA threshold loading to be used to trigger the inception and cessation of preemptive antiviral treatment and no standard cut-off was established [21,22]. Importantly, the cut-off used in our study may have impacted the absolute incidence of events, but this was not evident in the intergroup comparison.

The sub-analysis of CMV events according to CMV IgG pre-transplantation serologic status showed that the protective effect was clearer in CMV IgG D±/R+ transplants. There was no impact of EVR use among D+/R− transplants. It is possible that in high-risk situations (D+/R−), the protective effect of mTORi is insufficient to control the primary infection. However, in an intermediate risk situation, these drugs might shift the balance toward the control. The magnitude of the mTORi effect may be associated with the presence of CMV-specific memory cells [23]. To note, the small sample size does not allow us to drawn definitive conclusions about it. Clinical trials with larger samples would be interesting to confirm these findings. In addition, experimental studies would be useful to explain the molecular mechanisms of this effect.

Previous studies have shown the association between HLA-DR matching and CMV events and that it is consistent with the important role of CD4+ T cells in controlling CMV infections [24]. In fact, patients with zero HLA-DR mismatches in our cohort presented a reduced incidence of CMV events, and this reduction was more apparent in patients receiving EVR.

Kinetic analyses of whole blood CMV DNA load showed that patients on EVR presented evidence of lower exposure to CMV replication with later onset of CMV DNAemia, lower viral load peaks, and sooner remission. Remarkably, no patient on EVR presented with CMV DNAemia before week 4.

There were no significant intergroup differences in recurrence rates. However, four patients were converted from MPS to EVR during the first CMV episode due to antiviral treatment failure, which probably affected our results. Nearly half of the patients in the EVR group experienced recurrent CMV, which can be explained by

the high percentage of D+/R− transplants and lack of secondary prophylaxis.

Robust evidence has shown that, in patients on steroids, EVR and MPS present the same anti-rejection efficacy when used with cyclosporine (CsA) [25] or TAC [26]. The results of patients on steroid-free regimens are conflicting [27,28]. Our data show that EVR was as effective as MPS in the absence of steroids in patients induced with r-ATG. However, this study was insufficiently powered to allow definitive conclusions about this outcome.

We also found no differences in 3-year patient and graft survival rates. It is noteworthy that our follow-up duration is one of the main strengths of this study since the follow-up durations of the other available studies testing this combination (TAC-EVR) are limited to 1 year [8, 9].

Different from studies demonstrating better renal function in patients on EVR versus MPS in combination with CsA, we observed no benefits on renal function. In agreement with our findings, previous studies using TAC instead of CsA also did not report renal function improvements in EVR regimens [29]. A possible explanation for this finding would be the potential lower nephrotoxic effect of therapeutic doses of TAC compared to CsA [30]. However, we highlight inadequate adherence to the TAC protocol-defined target range after month 3 (Fig. 2). Similar noncompliance to predetermined TAC levels were observed in previous studies [8,29]. In fact, one of the challenges of this association in current clinical practice is to determine the optimal mTORi and calcineurin inhibitor concentrations to maintain a good efficacy–toxicity balance.

There were also no statistically significant differences in the incidence of wound-healing complications and PTDM, known mTORi-associated adverse events [31–33]. The urine protein/creatinine ratio at 3 years was similar between groups, but five patients in the EVR group were discontinued due to proteinuria.

High discontinuation rates were previously reported in trials testing mTORi, mainly when conversion strategies were adopted [34]. Regimens using *de novo* mTORi combined with calcineurin inhibitor at low concentrations are better tolerated [25]. In our study, the EVR group presented significantly lower treatment discontinuation rates than the MPS group. Our excellent patient and donor profile can explain these results. In fact, the safety of mTORi regimens in expanded criteria donors remains under debate. In addition, most adverse events associated with this drugs are high exposure-related, and we kept EVR levels closely within the target range [35]. On the other hand, safety issues were the main concern in the MPS group. In fact, the poor tolerability to MPS regimens is a real challenge in our setting. Although the results were not statistically significant, Tedesco-Silva *et al.* [7] showed that 12.9% of patients on TAC-MPS presented an adverse event leading to drug discontinuation at 1-year follow-up. It is noteworthy that 10 of the 15 MPS discontinuations in our study were motivated by CMV infection treatment failure or recurrence according to our clinical protocol. We also emphasize that our study was not powered to support robust conclusions about this secondary end-point.

Some limitations of our study were described above. Other aspects should be considered when interpreting the results: (i) its single-center nature; (ii) our peculiar donor and recipient demographics; (iii) exclusion of higher immunological risk patients; (iv) randomization not stratified based on CMV serostatus and the EVR group presenting a higher percentage of D+/R− transplants, although CMV events were less prevalent in this group; (v) 9.5% of patients were on steroids, but we performed a sub-analysis excluding these patients and the results remained similar; and (vi) our results cannot be extrapolated to patients receiving universal prophylaxis. Importantly, this strategy is not adopted worldwide because it involves high cost with nonreimbursable drugs, adverse events, and late disease [36]. Recent data from a large global transplant registry showed that only 37% of patients received prophylaxis as a prevention strategy for CMV disease. Even in the highest risk group (D+/R−), 25.3% did not receive prophylaxis [37]. A similar reality was reported in a recent European survey [22]. And finally, logistical difficulties in the collection and shipment of serum for the *dn*DSA analysis resulted in a high percentage of missing data, precluding any conclusions about it.

As strengths, this is the first study to prospectively evaluate TAC-EVR in a steroid-free regimen in patients receiving induction therapy with a standard dose of r-ATG; and to the best of our knowledge, this trial tested this combination with the longest follow-up to date.

In conclusion, compared with MPS, EVR was associated with a reduced incidence of CMV events in patients receiving a steroid-free regimen and induction therapy with r-ATG 6 mg/kg. Although current guidelines recommend universal prophylaxis or preemptive treatment for all KT recipients, logistical difficulties and high cost with nonreimbursable drugs make these

strategies infeasible in several centers worldwide. Our findings ratify previous suggestions on mTORi as an alternative to costly currently available CMV prevention strategies.

Authorship

RME and PMAP: designed the study. TVSF, PMAP, MLMBOS, CMG, EFC, and RME: performed the study. TVSF, PMAP, CMG, EFC, and RME: collected data. TVSF and RME: analyzed data. TVSF and RME: wrote the paper.

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Conflict of interest

TVSF and RME received speaker's fees and/or support for travel expenses for educational purposes from Novartis and Pfizer. PMAP, MLMBOS, EFC, and CMG declare no conflicts of interest.

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