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ORIGINAL ARTICLE

Influence of everolimus-based treatment on circulating regulatory T cells after liver transplantation: Comparative study with tacrolimus-based therapy

Clément Barjon[a,c,1], **Géraldine Dahlqvist**[a,b,*,1], **Khaldoun Ghazal**[a], **Faouzi Saliba**[d], **François Durand**[e], **Christophe Duvoux**[f], **Lynda Aoudjehane**[a], **Filomena Conti**[a,g]

[a] *UMR_S 938, CDR Saint-Antoine, F-75012 Paris, France*
[b] *Hepatogastroenterology Unit, Cliniques Universitaires Saint-Luc, Brussels, Belgium*
[c] *De Duve Institute, UCLouvain, 1200 Brussels, Belgium*
[d] *Centre Hépato-Biliaire, Hôpital Paul Brousse, AP-HP, Villejuif, France*
[e] *Liver Intensive Care Unit, Hôpital Beaujon, Assistance Publique-Hopitaux de Paris and University of Paris, Paris, France*
[f] *Department of Hepatology, Hôpital Henri Mondor, AP-HP, Créteil, France*
[g] *Liver Transplantation Unit, AP-HP, Hôpital de la Pitié-Salpêtrière, 75012 Paris, France*

KEYWORDS
Tolerance;
Regulatory T cells;
mTOR inhibitor;
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Abstract Liver transplantation remains the only treatment for terminal liver diseases. However, immunosuppressive drugs required for allograft acceptance are toxic and may be responsible for severe side effects. Modulating the immune system to induce tolerance is a promising approach to reduce immunosuppressive regimen. More particularly, promoting natural $CD4^+$ $CD25^+$ $FoxP3^+$ Tregs could be crucial in achieving tolerance. Contrary to calcineurin inhibitors, reports indicate that mTOR inhibitors may have a positive impact on Tregs.

Here we present the first randomized prospective clinical study where Tregs levels from liver transplanted patients receiving either tacrolimus or everolimus were monitored for 6 months, starting from the day of transplantation. A total of 30 patients from four centers were monitored. Blood samples were obtained at day 0, day 14, one month, three months and six

Abbreviations: CNI, calcineurin inhibitors; HD, healthy donors; IS, immunosuppression; Tregs, regulatory T cells; M, male; F, female; Tac, tacrolimus; Eve, everolimus; HCC, hepatocellular carcinoma; MELD, model for end stage liver disease.

* Corresponding author at: Cliniques Universitaires Saint-Luc, 10 Avenue Hippocrate, 1200 Brussels, Belgium.
E-mail address: geraldine.dahlqvist@uclouvain.be (G. Dahlqvist).

[1] Both authors equally contributed to this work.

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months post-transplantation. Flow-cytometry immunophenotyping of Tregs ($CD4^+$ $CD25^+$ $CD127^-$ $FoxP3^+$) and functional assays with Tregs were performed to assess their immunosuppressive capacity. Levels of Tregs were significantly reduced after one month of standard tacrolimus-based immunosuppressive regimen ($p < 0.05$). Four months after conversion, levels of Tregs from patients treated with everolimus was significantly higher than patients under tacrolimus ($p < 0.02$). Functional assays demonstrated that Tregs conserved their capacity to suppress the proliferation of activated PBMC.

Introduction

Immunosuppressive regimens mainly based on calcineurin inhibitors (CNI) have drastically reduced rejection rates after transplantantation [1,2]. However, transplanted patients must undergo a permanent drug-induced immunosuppression (IS) and suffers from deleterious side effects, such as nephrotoxicity and opportunistic infections. The immune status of the liver is unique, being considered a 'tolerogenic' organ. From 5% up to 63% of liver transplant patients are able to complete IS withdrawal without rejection of the graft [3,4]. The establishment of this 'operational tolerance' is one of the main focuses of research in transplantation. The operational tolerance may be defined as having a stable function of the graft one year after complete discontinuation of immunosuppression drug regimen, while maintaining a functional immune system [5]. Contrary to the process of 'true' tolerance which consists in the clonal deletion of autoreactive immune cells, it is believed that the operational tolerance is an active inhibitory process mediated by immunosuppressive cells, such as regulatory T cells.

Regulatory T cells (Tregs) are $CD4^+$ T lymphocytes expressing a high level of the interleukin-2 (IL2) receptor (CD25) as well as the transcription factor FoxP3. They are also characterized by a low expression of the IL7 receptor (CD127). Tregs are able to efficiently suppress the immunological response through multiple mechanisms such as direct cytotoxicity and the secretion of the immunosuppressive cytokines IL-10 and active TGF-β. As such, Tregs have been described as promoters of graft tolerance. Preserving them as well as promoting their development might be crucial to achieve operational tolerance [6]. Multiple reports have shown that immunosuppressive drugs may influence Treg levels in transplanted patients [7—9]. Calcineurin inhibitors (CNI) such as tacrolimus (TAC) have mostly a deleterious effect on Tregs, while rapamycin analogues such as everolimus seem to preserve them. This disparate effect may be linked to the mechanism of action of the drugs on the IL-2 pathway, which is critical for the survival of Tregs. Indeed, mTOR inhibitors act downstream of the IL-2 pathway and do not prevent its production whereas CNI act upstream and prevent it.

The SIMCER study was a prospective, multicenter randomised clinical study aiming to compare a tacrolimus-based IS regimen to an everolimus-based regimen on multiple aspects, with an emphasis on nephrotoxicity [10,11]. The protocol included an ancillary study focusing on the evolution of Treg levels for 30 patients up to six months after liver transplantation. We present here the results of this ancillary study, which to our knowledge is the first to demonstrate the positive effect of everolimus on Tregs using a simultaneous randomised control group of patients who remained treated with tacrolimus in liver transplant patients.

Material and methods

Patients and clinical protocol

The SIMCER study was a 6-months, prospective, multicenter, randomized, open-label study in *de novo* liver transplant patients from 15 transplant centers in France between 2012 and 2015 (ClinicalTrials.gov identifier NCT01625377). The study protocol was approved by the French Health Products Safety Agency (Afssaps/ANSM) and the relevant institutional review board. Written informed consent was obtained from all participants. Blood samples from a total of 30 patients from four centers were analysed. Samples were obtained at day 0, day 14, 1 month, 3 months and 6 months post-transplantation. Immunosuppressive regimens were given as follows. Basiliximab induction (20 mg) at days 0 and day 4. Between transplantation and randomization at week 4, the patients received tacrolimus targeting a trough level of 6—10 ng/mL and mycophenolate mofetil at a minimal dose of 360 mg twice daily. At the moment of randomization, patients in the everolimus group received 1 mg of everolimus twice daily adjusted to target a trough level of 6—10 ng/mL and their tacrolimus dose was progressively reduced until discontinuation during the 12th week post-transplantation. Patients randomized in the tacrolimus group remained under the same IS regimen. The levels of circulating Tregs of patients were compared to the levels of circulating Tregs in healthy donors (HD) from buffy coats. These buffy coats were obtained according to the approval by the Ethics Committee (CPP) for Ile de France III.

Regulatory T cells immunophenotyping

Fresh PBMC from patients (EDTA tubes) were isolated from whole blood using Ficoll gradient centrifugation. The absolute number of cells (cells/L) were calculated as [number of cells/mL of sampled blood * 1000]. Cells were stained with

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anti-CD4, anti-CD25 and anti-CD127 (Miltenyi Biotech) for 30 min at 4 °C. After washing, cells were fixed and permeabilized using BD Cytofix/Cytoperm (BD Biosciences) then stained with anti-FoxP3 (eBiosciences) for 1 h at 4 °C. Flow cytometry data was acquired with Gallios Flow Cytometer (Beckman Coulter) and results were analysed using Kaluza software (Beckman Coulter). Tregs were defined as $CD4^+$ $CD25^+$ $CD127^{-/low}$ $FoxP3^+$ lymphocytes and expressed as percentages of $CD4^+$ lymphocytes (Supplementary Figure).

Regulatory T cells functional assay

Tregs from patients ($CD4^+$ $CD25^+$ T cells) were isolated from PBMC using magnetic microbeads (Miltenyi Biotech) when the volume of sampled blood was superior or equal to 1 mL. PBMC were co-cultured with Tregs in 96-wells microplates using 10^5 cells and $0{,}5 \times 10^5$ cells respectively (ratio 2:1). PBMC were activated using anti-CD3 (1 μg/mL) and anti-CD28 (0.1 μg/mL) antibodies (eBiosciences). Cells were cultured for 48 h in DMEM supplemented with 10% fetal bovine serum and H3 radioactive thymidine was added during the last 18 h to measure the proliferation. Wells containing PBMC only, activated PBMC only and Tregs only were used as controls. Results are expressed as inhibition percentages compared to wells containing activated PBMC only.

Statistical analysis

Statistical analysis was performed using Prism 5 software for Mac OS X. Unpaired t-tests with Welch's correction were performed with p-value <0.05 considered as significant. For correlations, Spearman's rank correlation coefficient was calculated and tested with a t-test. P-value are noted (*) for $p < 0.05$ and (**) $p < 0.01$.

Results

Characteristics of the patients

Among the 30 patients included in this ancillary study, 21 patients were transplanted for alcoholic related liver disease, with 11 patients in the TAC group (73,3% of the patients in the group) and 10 patients in the EVR group (66,6% of the patients in the group). Six patients were transplanted for HCV-related cirrhosis with 2 HCV^+ patients in the TAC group and 4 HCV^+ patients in the EVR group. Two of the HCV-positive patients had a negative PCR result at the time of transplantation. The mean MELD score was 20.1 for the total cohort with a repartition not significantly different in both cohorts. There was no significant correlation in our cohort between MELD score and Treg levels (data not shown, $R^2 = 0.079$). Overall, the baseline characteristics of the patients were not significantly different (Table 1). Individual characteristics are presented in Supplementary Table 1.

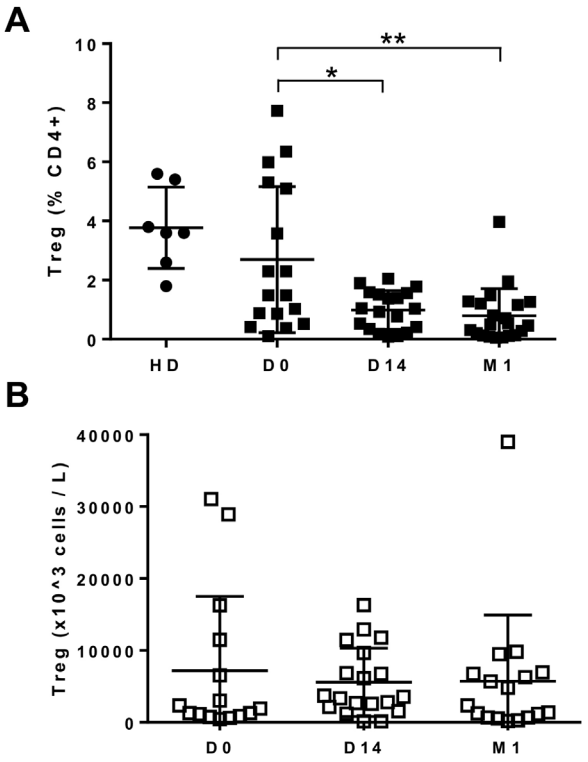

Fig. 1 (A) Treg levels of patients at day 0 (D0, baseline), day 14 (D14) and one month (M1) after transplantation. Treg levels of healthy donors (HD) are shown. Tregs are expressed as percentage of $CD4^+$ lymphocytes. (B) Tregs absolute counts of patients at baseline, D14 and M1, expressed as cells per liter of blood.

Regulatory T cells levels before conversion to everolimus

First, the circulating Treg levels of the patients were evaluated at baseline, expressed as percentage (%) of $CD4^+$ lymphocytes. All analyses were done on fresh samples. While 30 patients were included in the study, samples of all the patients were not available at each time points. Thus, the results presented here are pooled. The baseline Treg levels of the patients were measured the day of the liver transplantation in 17 out of 30 patients (D0). The mean baseline level at day 0 was 2.70% with a standard deviation of 2.4% (Fig. 1A). We observed a high variability in the patients, probably resulting from their diverse pathologies and their severity at the time of transplantation (Table 1 and Supplementary Table 1) [12]. Two patients were transplanted for ductal plate malformations and interestingly had higher Treg levels than cirrhotic patients (mean 6.52% ± 1.7). However, the difference between Tregs levels of HD and patients at D0 was not statistically significant. During the first month after transplantation, all patients received the same immunosuppressive (IS) regimen based on tacrolimus (see Section ''Material and methods''). The Treg levels of all patients were reduced significantly at D14 compared to D0 (mean level of 0.99%, $p < 0.01$ vs. HD; $p < 0.05$ vs. baseline) and further reduced one month (M1) after transplantation (mean level of 0.80%, $p < 0.001$; $p < 0.01$ vs. baseline). Tregs absolute numbers were also calculated (Fig. 1B). Although the

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Table 1 Patient's baseline data.

	Tacrolimus (n = 15)	Everolimus (n = 15)	p Value
Age (Years, Mean ± SD)	52.07 ± 9.63	55 ± 6.72	0.34
Gender (Male (%)	14 (93)	11 (73,3)	0.3295
HCV + (%)	2 (13.33)	4 (26.67)	0.6513
Mean MELD score	19.93 ± 9.7	20.27 ± 10.18	0.77
Alcoholic cirrhosis (%)	11 (73.3)	10 (66.67)	1
HCC (%)	5 (33,33)	2 (13.33)	0,3898

Patient's baseline data.

mean level of Tregs per litre of blood tended to decrease during the first month of tacrolimus based IS regimen, no significant differences could be observed at this step.

Regulatory T cells levels after conversion to everolimus

One month after the transplantation, all patients were randomized into two groups: the first group remained treated with a tacrolimus-based IS regimen (TAC) while the second group switched progressively to an everolimus-based IS regimen [13]. Treg levels of patients from the TAC group remained statistically lower than both the baseline Treg level and HD Treg level three months (M3) after the transplantation ($p < 0.05$ and $p < 0.01$ respectively) (Fig. 2A). This was confirmed six months (M6) after the transplantation. Treg levels of patients from the EVR group were significantly higher than Treg levels of the TAC group at M3, with a mean of 3.98% vs. 1.19% respectively ($p < 0.05$). This significant difference was confirmed at M6 with a mean Tregs level of 3.62% for the EVR group and a mean Treg level of 1.02% for the TAC group ($p < 0.05$). Treg levels of patients from the EVR group were similar to HD Tregs levels (mean of 3.77%). Finally, the absolute Treg counts of patients in the EVR group was statistically higher to the Tregs absolute count of patients in the TAC group at M6 (mean of $14{,}118 \times 10^3$ cells/L vs. mean of 5170×10^3 cells/L respectively; $p < 0.05$) (Fig. 2B).

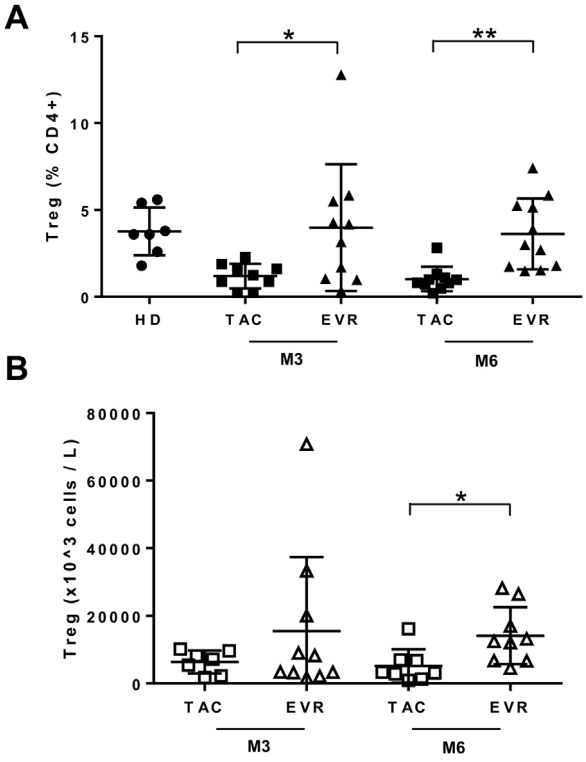

Fig. 2 (A) Tregs levels of patients three months (M3) and six months (M6) after transplantation. TAC group includes patients who remained treated with tacrolimus, EVR group includes patients who were switched from tacrolimus to everolimus at one month post-transplantation. Tregs are expressed as percentage of $CD4^+$ lymphocytes. (B) Tregs absolute counts of patients at M3 and M6 from TAC and EVR groups, expressed as cells per liter of blood.

Regulatory T cells levels in HCV^+ patients

Six patients were transplanted for HCV-related cirrhosis. Two of them had a negative HCV-PCR at the time of transplantation.

When considering Treg levels of the HCV^+ patients at M3 compared to the non-HCV patients, we observed a significant difference in the percentage in favour of the HCV^+ patients (5.698% ± 2.52 vs. 1.59% ± 0.36; $p = 0.01$) but this difference was not maintained at M6.

Interestingly, there was no difference in HCV^+ patients at M3 and M6 compared to HD while there was a significant reduction of Tregs levels in non-HCV patients compared to HD at both time points ($p = 0.0025$ and 0.0118 respectively) (Fig. 3).

Influence of the serum concentration of the IS drugs on Tregs levels

The multicenter characteristic of this study resulted in conversion dynamics of the patients from tacrolimus to everolimus that were not exactly the same in all medical centers involved. As a result, not all patients in the EVR group at the time point M3 were cleared of tacrolimus and had received the same dose of everolimus. A subgroup of patients from one centre had higher blood level of tacrolimus and/or a lower blood level of everolimus than other EVR patients (Fig. 4). Those patients had the lowest level of Tregs of the EVR group at M3. However, the coef-

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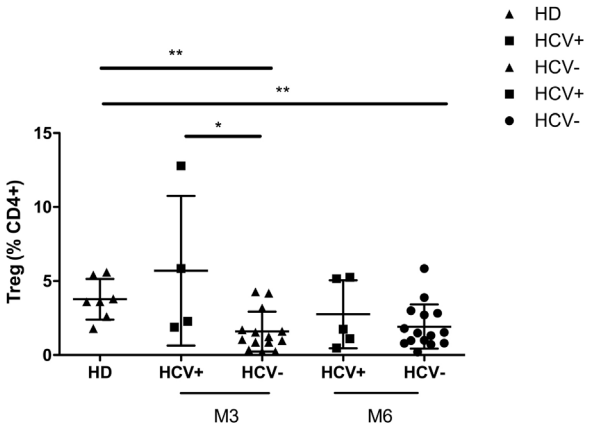

Fig. 3 Tregs levels of patients at three months (M3) and six months (M6) after transplantation depending on their HCV antibody status (HCV^+ vs. HCV^-). Treg levels of healthy donors (HD) are shown. Tregs are expressed as percentage of $CD4^+$ lymphocytes.

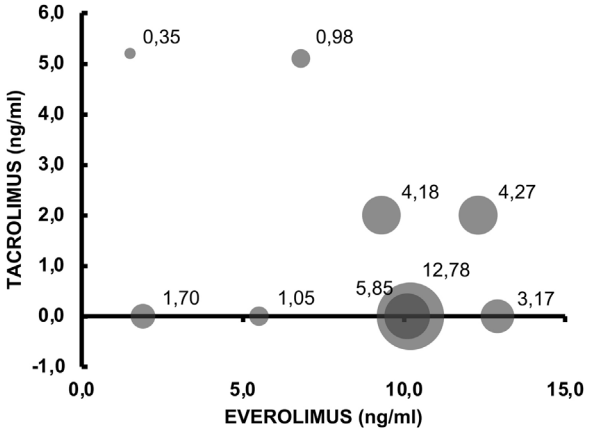

Fig. 4 Treg levels (% of $CD4^+$ T cells) of patients from the EVR group at M3. Levels are plotted according to the blood dosage of everolimus and tacrolimus at M3. The size of the plot indicates the level of Treg, larger size meaning higher level.

ficient of correlation between tacrolimus concentration in the EVR group at M3 and the percentage of Tregs was not statistically significant ($r = -0.5230$, $p = 0.1475$).

Functional characteristics of Tregs isolated from patients

In parallel of monitoring the quantity of circulating Tregs in the patients, we monitored their ''quality'' using a functional assay. Tregs were isolated from patient blood when enough material was available (n = 14 at D0, n = 7 at D14, n = 8 at M3 and n = 9 at M6) and the results were pooled per group. An autologous proliferation assay was performed using TCR-activated total PBMCs from patients at a PBMC:Tregs ratio of 2:1 (Fig. 5). Overall, no significant differences could be established indicating that the immunosuppressive treatments did not affect the functions of the circulating Tregs.

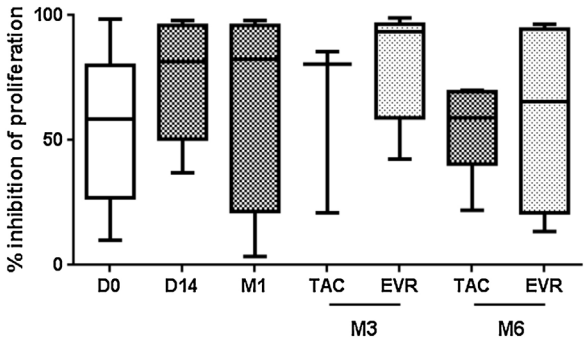

Fig. 5 Tregs functional assay. $CD4^+$ $CD25^+$ cells were isolated from the blood of patients using magnetic beads. Tregs were co-cultured with autologous total PBMC activated with anti-CD3 and anti-CD28 antibodies at a ratio 1:2 respectively. Proliferation index was determined by measuring the incorporation of radioactive thymidine. Results are expressed as inhibition percentages of the proliferation of PBMC cultured without Tregs.

Discussion

Here we describe an ancillary study of a prospective, randomized and multicenter study comparing Treg levels of two liver transplant patient groups receiving concurrently either a TAC-based IS regimen or an EVR-based IS regimen. While several clinical studies have shown that mTOR inhibitors favour the increase of Tregs in patients converted from calcineurin inhibitors, a 'natural' recovery of Tregs could not be excluded [14—16]. This study demonstrates that patients under TAC treatment display a low level of Tregs even six months after the transplantation. Conversely, patients who were converted to an EVR treatment one month after transplantation recover a level of Tregs similar to the Tregs level of healthy donors. While most of the studies on EVR after liver transplantation have focused on HCC recurrence, other have shown a positive impact of mTOR inhibitors on Tregs [2,17—19].

Interestingly, our study suggests that two other factors might directly influence Tregs levels in liver transplanted patients. Firstly, the hepatis C virus (HCV) infection status is a cause of higher Tregs levels. In the EVR subgroup, patients infected with HCV had a higher level of Tregs than uninfected patients, one of them displaying the highest level of Tregs of the whole study, with 12.78% of $CD4^+$ T cells at M3. Indeed, HCV infection is known to rapidly reinfect the graft after transplantation and to increase Tregs levels, potentially favouring graft acceptance [19—21]. An HCV eradication effect was possibly observed as the difference in Tregs levels was not significant at M6 between non-HCV and HCV^+ patients. However, when comparing Tregs levels in the EVR group and the TAC group without the HCV^+ patients, the difference at M3 and M6 remained significantly different. Nevertheless, these conclusions should be taken with caution due to the low number of HCV^+ patients in this cohort.

Secondly, the drug dose might directly influence circulating Tregs levels [22]. Drug doses are constantly being adjusted in recently transplanted patients and thus one sample at one given time point might not reflect the overall quantity of the drug received. Additionally, the fact that this

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study was multicenter resulted in slightly different dosage adjustments during the TAC to EVR conversion. However, we could not demonstrate a global quantitative correlation between drug doses and Tregs levels in this study.

One limit of our study is that we only had access to circulating Tregs and not intrahepatic Tregs. While comparing both levels of Tregs would be interesting, it is known that in steady state circulating Treg levels are representatives of intrahepatic levels after liver transplantation outside of a situation of acute rejection, in which circulating Treg levels are lower as Tregs are homed to the liver graft [23—25]. Only one patient in the EVR group experienced one episode of acute rejection at M6 and had a lower percentage of circulating Tregs compared to M3 (1.8% vs. 3.7%).

Other limitations of our results were the limited sample size as well as the unavailability of samples at certain time points for certain patients. However, this study clearly suggests that EVR-based IS helps to increase Tregs levels compared to TAC based IS. Together with the recent encouraging clinical results obtained, these results makes EVR an interesting alternative to the calcineurin-inhibitor based IS to potentially improve the long-term quality of life of transplanted patients.

In our study population, during the six months of follow-up, we did not observe any dropout and the side effects were not significantly different between the two groups.

While the positive effect of mTOR inhibitors on circulating Tregs levels is largely accepted, future studies should involve longer term analysis to determine if expanding circulating and intrahepatic Tregs can lead to graft tolerance and become a tool to predict better immunological outcome.

Conflict of interest

Faouzi Saliba has received speaker's honoraria and/or research grants from Roche, Novartis, Astellas, Gilead, Merck Sharp & Dohme, Gambro, Baxter and Vital Therapies. Christophe Duvoux has received speaker's honoraria and/or research grants from Novartis, Astellas, and Chiesi. François Durand received research grants, speaker's fees or consultant fees from Novartis, Astellas, Gilead and BMS.

The other authors have no conflicts of interest to disclose.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.clinre.2020.10.004.

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