Sustained inhibition of calcineurin activity with a Melt-Dose Once-daily Tacrolimus formulation in renal transplant recipients.

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Conflicts of interest

Authors declare that they do not have any relevant financial disclosure.

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Abbreviations: Tac, tacrolimus; TDM, therapeutic drug monitoring; PK, pharmacokinetic; C_0, pre-dose concentration; AUC, area under the curve; PD, pharmacodynamic; Tac-IR, immediate-release twice-daily tacrolimus formulation; Tac-ER, prolonged-release once-daily tacrolimus formulation; Tac-LCP, extended-release once-daily MeltDose tacrolimus formulation; FKBP, FK-binding protein; CN, calcineurin enzyme; CNA, calcineurin activity; HIV, human immunodeficiency virus; CYP, cytochrome P450 enzymes; UHPLC-MS/MS, ultra-high-performance liquid chromatography; PBMC, peripheral blood mononuclear cells; C24, concentration at time 24h, C_{max}, maximum concentration; T_{max}, time to reach maximum concentration; PTF, peak-trough fluctuation index; λ_z, apparent elimination rate constant; $t_{1/2z}$, apparent elimination half-life; I_0, pre-dose calcineurin activity at time 0 h; I_{24}, calcineurin activity at time 24 h; I_{min}, minimum calcineurin inhibition; I_{nadir}, maximum calcineurin inhibition; T_{nadir}, time to reach maximum calcineurin inhibition; AUE, area under the effect; Gof, goodness of fits; OFV, objective function value; df, degrees of freedom; E_0, the maximum change of % inhibition; IC_{50}, concentration to achieve a 50% of the maximum change; RSE, relative standard errors.

ABSTRACT

Background: Tacrolimus (Tac) is the cornerstone calcineurin inhibitor in transplantation. Extended-release Meltdose formulation (Tac-LCP) offers better bioavailability compared to immediate-release formulation (Tac-IR). We postulated that the less fluctuating pharmacokinetic profile of Tac-LCP might maintain a sustained inhibition of calcineurin activity (CNA) between dose intervals. Higher concentrations (C_{max}) after Tac-IR may not result in a more potent CNA inhibition due to a capacity-limited effect. This study was aimed at evaluating the pharmacodynamic/pharmacokinetic profiles of Tac-IR compared with Tac-LCP.

Methods: An open-label, prospective, non-randomized, investigator-driven study was conducted. Twenty-five kidney transplant recipients receiving Tac-IR were switched to Tac-LCP. Before and 28 days after conversion, intensive CNA-pharmacodynamic and pharmacokinetic sampling were conducted using UHPLC-MS/MS. Pharmacodynamic non-linear mixed effects model was performed in Phoenix-WinNonlin.

Results: Statistically significant higher C_{max} ($p<0.001$) after Tac-IR did not result in lower CNA as compared to after Tac-LCP ($p=0.860$). Tac-LCP showed a statistically more maintained CNA inhibition between dose intervals ($AUE_{0\text{-}24h}$) compared to Tac-IR, in which CNA returned to pre-dose levels after 4 hours of drug intake (373.8 vs 290.5 pmol RII· h/min·mg prot, Tac-LCP vs Tac-IR; $p=0.039$). No correlation was achieved between any pharmacodynamic and pharmacokinetic parameters in any formulations. Moreover, Tac concentration to elicit a 50 % of the maximum response (IC_{50}) was 9.24 ng/mL.

Conclusion: The higher C_{max} after Tac-IR does not result in an additional CNA inhibition compared to Tac-LCP attributable to a capacity-limited effect. Tac-LCP may represent an improvement of the pharmacodynamic of Tac due to the more sustained CNA inhibition during dose intervals.

Introduction

Tacrolimus (Tac) is the backbone of immunosuppressive therapy used after kidney transplantation. Therapeutic drug monitoring (TDM) is routinely performed for individualization of the Tac dose to maintain drug efficacy and minimize the consequences of overexposure or underexposure due to its narrow therapeutic index and its large interpatient and intrapatient pharmacokinetic (PK) variability [1,2]. In the clinical practice, TDM of Tac is based on measuring pre-dose blood concentration (C_0) in blood during the follow-up post-transplantation [3]. However, the correlation between C_0 and the area under the curve (AUC) of Tac exposure is not fully optimal and AUC correlates better with clinical outcomes [4,5]. Indeed, acute rejection and Tac-derived toxicity episodes occur in some patients although their C_0 levels are within the therapeutic range [4,6].

Tac is currently administered in different formulations which could also influence Tac PK and pharmacodynamic (PD) characteristics. Tac immediate-release administered twice-daily (Tac-IR, Prograf®, Astellas Pharma, Japan) has been the initial and the most commonly used formulation. To increase treatment adherence and ultimately leading to better prevention of graft rejection, a prolonged-release once-daily formulation was developed (Tac-ER, Advagraf®, Astellas Pharma, Japan). Recently, a new extended-release once-daily formulation (Tac-LCP, Envarsus®, Veloxis Pharmaceuticals, Denmark), using MeltDose® delivery technology has improved the solubility of Tac molecules, increased bioavailability and reduced fluctuation between maximum and pre-dose concentrations compared to Tac-IR and Tac-ER. Interestingly, Tac-ER has a PK profile similar to Tac-IR with lower AUC after 1:1 dose conversion [7]. In contrast Tac-

LCP has lower peak-concentration, fewer trough concentrations and improved bioavailability in comparison with Tac-IR and Tac-ER [7–13].

Tac is a calcineurin inhibitor that binds to FK-binding proteins (FKBP), mainly to FKBP-12, to inhibit calcineurin phosphatase enzyme (CN) [14,15]. This inhibition prevents the dephosphorylation and translocation of a nuclear factor of activated T-cells (NFAT) involved in the transcription of several cytokine genes that promote T-cell activation and expansion. Measuring the degree of CN inhibition may assess the PDs of Tac and could reflect the biological effect of Tac [14]. The majority of the studies in transplant recipients have been carried out with Tac-IR explaining that CN activity (CNA) and Tac concentrations in blood showed inverse profiles [16,17]. Although Tac concentrations and CNA profiles achieve similar inverse values, no correlation has been obtained so far between PK and PD parameters [18,19]. However, no study described the CN inhibition between dose intervals for 24 hours in Tac-IR formulation. On the other hand, little is known about PDs of the Tac once-daily formulations and it has been described only in Tac-ER formulation [20,21]. CNA cannot be completely inhibited, even in the presence of increasing Tac concentrations. This incomplete inhibition is due to a "capacity-limited effect" caused by restricted expression of FKBPs [22,23]. In this sense, previous studies observed no differences in CN inhibition in recipients with standard and low-dose of cyclosporine [24].

Considering the different PK profile between Tac-IR and Tac-LCP, the hypothesis of this study was that the higher peak Tac concentrations observed after Tac-IR would not result in higher CN inhibition, due to the capacity-limited effect. In contrast the more sustained and less fluctuating Tac concentrations observed after Tac-LCP would produce a more maintained inhibition of CNA during dose intervals. For this purpose, the primary aim of

143 this study was to evaluate the PD/PK profiles of Tac-LCP in stable renal transplant
144 patients compared with Tac-IR.

Methods

Study design

This is an open-label, single-centre, prospective, non-randomized, investigator-driven clinical trial (Figure 1; clinicalTrials.gov NCT02961608) comparing two Tac twice-daily formulations, Tac-IR (Prograf® or Adoport®, both Astellas Pharma, Japan) and once-daily Tac-LCP (Envarsus®, Chiesi Farmaceutici, Parma, Italy). The study was carried out in accordance with the Declaration of Helsinki and with approval from the local ethics committee of the Bellvitge University Hospital, Spain.

Eligible recipients were adults (≥ 18 years) who had received a renal transplant at least 6 months prior to inclusion, where Tac-IR formulation was administered and that showed C_0 between 5–10 ng/mL in steady-state conditions. Patients without signed informed written consent, with current infections, hepatitis B or C, severe gastrointestinal disorders, neoplasms, or HIV, patients receiving concomitant drugs that could interact with cytochrome P450 (CYP) CYP3A enzyme (antibiotics, antiepileptics, antihypertensive and anti-arrhythmic agents, antimycotic drugs, HIV protease inhibitors and theophylline) and pregnant or lactating women were excluded from this study.

Twenty-five stable kidney transplant recipients from Bellvitge University Hospital (Barcelona) receiving Tac-IR were subsequently switched to Tac-LCP. Before and four weeks after conversion, PD and PK intensive blood samplings were conducted for 24 hours (Pre-dose and at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 12.5, 13, 13.5, 14, 15, 20 and 24 h post-dosing) using two 3-mL EDTA-K_3-tubes for each sampling. Two weeks after conversion,

C_0 levels were measured to check if proper levels (5–10 ng/mL) were maintained with the current dosage (Figure S1).

Morning Tac doses were administered in fasting conditions the evening before, and again at least 1 hour before breakfast. All patients received the same Mediterranean diet (breakfast: 9:30 am, lunch: 2:00 pm, snack: 5:00 pm, dinner: 09:00 pm). Tac-IR intake was carried out every 12 hours (at 8:00 am and 08:00 pm) and Tac-LCP was administered once daily at 8:00 am.

Bioanalytical determination

Tac whole blood concentrations were measured using ultrahigh-performance-liquid chromatography mass spectrometry (UHPLC-MS/MS) (Acquity®-TQD® mass spectrometer) the method previously validated [25]. To measure CNA in peripheral blood mononuclear cells (PBMCs), a validated method was used by our group [26]. Briefly, PBMCs were isolated from blood using Ficoll density gradient and lysed with a hypotonic buffer. This lysate was incubated with a phosphorylated peptide (RIIp), as a substrate for the CN enzyme for 15 minutes. Finally, after phosphatase activity and following solid-phase extraction using Oasis HLB^{TM} µelution plates, dephosphorylated peptide (RII) and its corresponding internal-standard (RII-IS; an stable isotope-labelled form of RII) were detected by UHPLC-MS/MS. All the samples showed Tac and RII concentrations higher than the limit of quantification of both techniques (0.65 ng/mL and 0.04 µM, respectively) [25,26].

Pharmacokinetic data analysis

The following parameters were determined directly from Tac concentration-time profiles at steady-state: C_0, and C_{24} pre-dose concentration at 0 and 24 h; C_{max}, maximum concentration; T_{max}, time to reach C_{max}; $AUC_{0\text{-}24h}$, area under the time-curve from 0 to 24 h estimated by the trapezoidal rule; Peak-trough fluctuation index (PTF) was estimated as $PTF=100\times[(C_{max}-C_0)/C_{average}]$, where $C_{average}$ was obtained from $AUC_{0\text{-}24h}/\tau$ where τ=24 h; Swing fluctuation index was calculated as $\%\ Swing=100\times[(C_{max}-C_0)/C_0]$. The apparent elimination rate constant (λ_z) was estimated from the slope of the terminal phase of the linear logarithm concentrations-time plot (Tac-IR from 0-12 h; Tac-LCP from 0-24 h). Finally, the apparent elimination half-life ($t_{1/2z}$), estimated as $t_{1/2z}=\ln 2/\lambda_z$. Phoenix-WinNonlin 64 8.2. was used for these calculations.

Pharmacodynamic data analysis

The following parameters were determined directly from the observed CNA-time profiles at steady-state: I_0 and I_{24} pre-dose CNA at time 0 and 24 h; I_{min}, minimum CN inhibition; I_{nadir}, maximum CN inhibition; T_{nadir}, time to reach I_{nadir}. Percentages of CN inhibition at each time-point were calculated using two approaches (Equations 1-2).

$$\%\ Inhibition_{Imin} = \left[\frac{(I_{min}-I_x)}{I_{min}}\right] * 100 \qquad \text{Equation 1}$$

$$\%\ Inhibition_{Inadir} = \left[\frac{(Ix-I_{nadir})}{I_{nadir}}\right] * 100 \qquad \text{Equation 2}$$

where I_x was the CNA at each time point.

The overall PD response was evaluated by calculating the area under the percentage of CN inhibition vs time profiles during the 24 hours, to yield the area under the effect-time curve ($AUE_{0\text{-}24h}$). $AUE_{0\text{-}24h}$ was estimated using trapezoidal rule with Phoenix-WinNonlin.

Later, PD data was analysed by means of a modelling approach. The analyses were carried out with the non-linear mixed effects models implemented in Phoenix-WinNonlin. The first order conditional estimation method was used for population PD parameter estimation. The simple vs sigmoid inhibitory E_{max} models with baseline vs without baseline (Equation 3) were tested to characterize the relationship between Tac concentrations and responses given by the % inhibition $I_{nadir,}$ to remove the influence of different I_{nadir} values among patients. Interindividual variability associated with PD parameters was modelled by an exponential model. Multiplicative residual error models were employed. During the modelling process, the goodness of fits (Gof) of different models to the data were evaluated as follows: i) changes in the minimum objective function value (OFV) for hierarchical or nested models and the Akaike information criterion for non-hierarchical models ii) precision of parameter estimates iii) decreases in both inter-individual variability and residual variability iv) visual inspection of Gof plots, ie, observed vs population/individual predicted response values and conditional residuals against the population predicted effect. The difference in the OFV between two nested models has an approximate $\chi 2$ distribution with the number of degrees of freedom (df) equal to the difference in the number of parameters between the models. Based on $\chi 2$ distribution with df =1, a decrease in OFV of 7.8 units was considered as statistically significant with a significance level α of 0.005 [27,28].

Statistical analysis

The geometric mean of all the recipient data [95% geometric mean interval confidence] was used to summarize PD/PK parameters except in the case of the categorical variables (T_{max} and T_{nadir}). Statistical comparisons of log-transformed values of PD/PK parameters such as CNA and Tac concentrations between both formulations were performed by means of a paired t-test with IBM SPSS v23 and Graphpad Prism 6.0. Comparisons of biochemical variables between occasions were compared using a paired-t-student when the assumptions of normality and homogeneity of variances were fulfilled or Wilcoxon signed-rank test if not. Furthermore, a parametric Pearson's or non-parametric Spearman's correlations were applied to analyze the potential correlation between PD/PK parameters. Statistical significance was set at $\alpha=0.05$.

Results

Population characteristics

Twenty-five recipients were recruited between October 2016 and September 2018. After the first PD/PK profile with Tac-IR, one recipient was excluded due to the lack of compliance and no data from Tac-ER was obtained. A second patient failed to complete Tac sampling for PK analysis. Demographic characteristics are shown in Table 1. All patients were treated with an immunosuppressive drug regimen consisting of oral twice-daily Tac (Prograf®/Adoport®) combined with mycophenolate mofetil (Cellcept®) or mycophenolate sodium (Myfortic®) and glucocorticoids according to the local protocol (Table 1). One patient received Tac monotherapy. Biochemical parameters were not statistically different after switching between both occasions (Table 1). Moreover, no relevant clinical events were observed after conversion. Overall, patients were converted at dose ratio 1:0.7 [Tac-IR:Tac-LCP] following the labelling requirements. However, five recipients received doses above 1:0.7 conversion ratio, whereas six patients received doses below this ratio.

Pharmacokinetic analysis

A total number of 789 Tac concentrations (422 for Tac-IR and 376 for Tac-LCP) were analyzed for the PK study. As previously described, the main differences between both formulations were observed in the first 12 hours after morning dose intake (Figure 1A and 1B). After Tac-IR, a rapid increase to the peak in Tac concentrations was observed followed by a fast decay, providing a peak-like profile. In contrast, concentrations

283 increased slowly after Tac-LCP with a gradual decay of Tac levels after C_{max} (Figure 1A).
284 When apparent terminal phase slopes (Figure 1B) and half-life values were compared, a
285 longer half-life was found for Tac-LCP than for Tac-IR (Table 2). This confirmed the
286 occurrence of a flip-flop phenomenon due to a slower rate of release/absorption for Tac-
287 LCP than its rate of elimination from the body [29]. Consequently, the persistence of Tac in
288 the body become dependent on absorption rather than on elimination. In accordance, a
289 statistically significant higher fluctuation and swing parameters were observed after Tac-
290 IR compared to Tac-LCP (Table 2). Tac-LCP showed statistically significant lower and
291 delayed C_{max} compared to Tac-IR. Moreover, the ratio of C_{max}/C_0 was significantly higher
292 after Tac-IR (C_{max} was almost three times higher than C_0) in comparison with Tac-LCP
293 (approximately two times higher). By contrast, there were similarities between both
294 formulations with regards to pre-dose concentrations (both at 0 and 24 h) and $AUC_{0\text{-}24h}$.
295 The total exposure for 24 hours ($AUC_{0\text{-}24h}$) adjusted by total daily dose was 27% higher
296 after Tac-LCP than after Tac-IR (Table 2).
297
298 In both formulations, pre-dose concentrations were correlated with $AUC_{0\text{-}24h}$ (Table 2).
299 Furthermore, a significant correlation was also observed between C_{max} and $AUC_{0\text{-}24h}$ in
300 both formulations. However, C_{max} was only correlated with C_0 in Tac-LCP (Table 2). No
301 correlation was found between C_{max} of Tac-IR and C_{max} of Tac-LCP (data not shown).
302
303 *Pharmacodynamic analysis*
304
305 A total of 759 CNA measurements (397 for Tac-IR and 362 for Tac-LCP) have been
306 determined. After morning drug intake, CNA diminished fast in both formulations with
307 similar pre-dose inhibition (I_0) (Figure 2A). The main PD differences between both

formulations occurred after 3 h post-dose, when CNA recovered to I_0 levels after Tac-IR, whereas CN inhibition was maintained until 12 h post-dose after Tac-LCP. In the period between 4 and 12 h, Tac-LCP showed statistically significant lower levels of CNA. In contrast, no significant differences were obtained in CN inhibition between both formulations after 12 h (Figure 2A). No significant differences in pre-dose CN inhibitions (I_0 and I_{24}) and I_{min} were observed between formulations (Table 3). In addition, similar I_{nadir} was shown between both formulations, although T_{nadir} occurred later after Tac-LCP compared to Tac-IR. The inhibition intensity of CN for 24 hours considering I_{min} as baseline ($AUE_{0\text{-}24h}$ I_{min}) was greater after Tac-LCP compared to Tac-IR (Figure 2B, Table 3). In addition, CN inhibition during drug doses interval was more sustained along the I_{nadir} value after Tac-LCP ($AUE_{0\text{-}24h}$ I_{nadir}) although without reaching statistical significance ($p = 0.06$) (Figure 2C, Table 3).

Positive correlations were observed between I_0 and I_{nadir} in both formulations (Table 3). In contrast to PK, CNA showed weaker correlations between pre-dose CN inhibitions and PD AUEs. The $AUE_{0\text{-}24h}$ I_{min} was only negatively correlated with I_0 and I_{24} after Tac-LCP. In contrast, the I_{24}/I_{nadir} ratio did not correlate with $AUE_{0\text{-}24h}$ I_{min} in any formulation. The $AUE_{0\text{-}24h}$ I_{nadir} correlated with I_{24} but not the I_0 in both formulations. Similarly, good correlation between I_{24}/I_{nadir} ratio and $AUE_{0\text{-}24h}$ I_{nadir} was observed in both formulations (Table 3).

Pharmacodynamic/Pharmacokinetic analysis

For the first hours post-dose, a rapid decrease in CNA was observed in both formulations, however while after Tac-IR, Tac concentrations increased rapidly, after Tac-LCP, Tac

levels increased slightly (Figure 3A). Moreover, at 1.5 h despite Tac levels rising to 16.9 ± 5.5 ng/mL after Tac-IR and after Tac-LCP reaching 7.6 ± 3.3 ng/mL, both formulations showed similar CNA levels (240.8 ± 47.1 vs 245.7 ± 40.3 pmol RII/min·mg prot). Similarly, after the evening dose of Tac-IR, CNA diminished rapidly, however Tac concentrations slightly increased (Figure 3A).

No correlation was found between C_{max} and I_{nadir} in any formulation (Table 4). Therefore, recipients who showed higher C_{max} did not exhibit higher CN inhibition (lower I_{nadir}). In fact, patients with higher C_{max}/C_0 ratio did not exhibit a higher I_0/I_{nadir} ratio. Furthermore, no correlation was seen between different PD AUEs and the PK AUC or C_0 in any formulation (Table 4).

Pharmacodynamic model

The model that best described the relationship between Tac concentrations and the response given by % inhibition I_{nadir} was a simple inhibitory E_{max} model without baseline (Equation 3).

$$E = E_0 \cdot \left[1 - \frac{\cdot C}{IC50 + C}\right] \qquad \text{Equation 3}$$

where E_0 is the maximum change of % inhibition I_{nadir}, C is the Tac concentration at each time, IC_{50} is the Tac concentration to achieve a 50% of the maximum change of % inhibition I_{nadir}.

Final parameter estimates were estimated with accurate precision (Figure 3B, Figure S2). Relative standard errors (RSE) of fixed parameters (IC_{50} and E_0) were lower than 25%

and values lower than 31% were found for RSE of random parameters (ω^2,σ^2).The maximum change of the measured response from baseline was 44.32 % and the concentration to elicit a 50 % of the maximum response (IC_{50}) was 9.24 ng/mL. Figure 3B shows the goodness-of-fit plot of the response values vs Tac concentrations which suggest a good fit of the final PD model. Moreover, no bias was found when observed vs population predictions, observed vs individual predictions and conditional weighted residuals vs population predictions of the % inhibition I_{nadir} were plotted (Figure S2).

According to these results, when C_0 values for each formulation were considered (6.61 for Tac-IR and 6.21 ng/mL for Tac-LCP), the % of inhibition with respect to the I_{nadir} were of 25.84 and 26.51 %, respectively. Considering the mean C_{max} values (18.18 for Tac-IR and 12.31 ng/mL for Tac-LCP), the % of inhibition with respect to the I_{nadir} were of 14.93 and 19%, respectively. The % of inhibitory effect with respect to I_{nadir} of 25% and 10% can be reached when Tac concentrations of 7.14 and 31.72 ng/mL were achieved, respectively.

Discussion

To our knowledge, this is the first study that has analysed the PD profile of CNA inhibition during doses interval in kidney transplant recipients converted from the classical formulation Tac-IR to new extended-release Tac-LCP. Moreover, to our knowledge, no report has evaluated the CNA for 24 hours in both formulations.

As expected, PK profiles obtained in our study were in accordance with previous studies [7,30,31]. A higher fluctuating profile during dose intervals was observed after Tac-IR due to the large differences in the absorption/elimination rate [32]. The prompt release of Tac molecules in the proximal gut after Tac-IR provided an early high absorption rate with a high and early C_{max}. In contrast, the sustained release achieved after Tac-LCP produced a continuous absorption along more distal parts of the gut with a lower and delayed C_{max} with a gradual and slow decrease of Tac concentrations between the 24 h dose intervals. Moreover, Tac-LCP showed a greater bioavailability (30%) compared to Tac-IR supporting 1:0.7 [Tac-IR:Tac-LCP] conversion dose ratio as has been previously described in order to obtain the same exposure [2,7,13,33].

As previously described by other authors, our results showed that Tac-IR-treated patients reached significant reduction of CNA in the first hours after drug intake (1–3 h) and a recovery to baseline levels after 4 h [17,34–36]. This rapid recovery could be clinically relevant, especially in non-adherent recipients in which the evening drug intake was delayed. In this context, a prolonged under-immunosuppression during that interval could promote the activation of alloreactive T-cells and, ultimately, contribute partly to graft rejection [37]. In contrast, Tac-LCP showed a longer significant CNA inhibition (2–8 h).

The non-fluctuating PK profile of Tac-LCP was translated into more sustained and less fluctuating CN inhibition within the 24 hours of dose intervals. Our results showed that with similar PK $AUC_{0\text{-}24h}$, Tac-LCP achieved better overall inhibition during doses interval, characterised by a higher $AUE_{0\text{-}24h}$ I_{min} and lower $AUE_{0\text{-}24h}$ I_{nadir}. Therefore, lower doses of Tac-LCP achieved higher CN inhibition.

In addition, the higher C_{max} achieved after Tac-IR did not correspond to a more relevant potent effect on the CN inhibition, reaching similar I_{nadir} in both formulations. This result is in accordance with the capacity-limited effect, indicating that higher Tac concentrations do not imply higher CNA inhibition as the binding substrate of Tac, FKBP12, is limited [22,23]. Although most patients only showed maximum CN inhibition between 20-35 % with respect to I_0 value [38], previous studies reported that even though CNA is only inhibited at around 20 %, it can produce a strong effect on cytokines secretion related to CN pathways like IL-2 and IFN-γ [14,22,24,39,40]. Once these differences in CNA inhibition have been observed between both formulations, further studies analysing NFAT translocation and cytokines synthesis would additionally illustrate Tac PD mechanisms.

The capacity-limited effect observed in CN inhibition is in consonance with the PD E_{max} model previously reported describing the relationship between Tac concentrations and CN activity. These studies showed an IC_{50} between 18-27 ng/mL of Tac; however, these concentrations were clinically toxic for patients. Using our data, similar PD relationship between Tac concentrations and CNA using an inhibitory E_{max} model led to similar results than previously reported (data not shown) [36,38,40–42]. Nevertheless, when this model was applied to % of CN inhibition with respect to I_{nadir}, to remove the influence of different I_{nadir} values between patients, an IC_{50} value of 9.24 ng/mL was found. Thus, higher Tac

concentrations do not result in a more potent effect. This low IC_{50} supported the PD benefits of Tac-LCP, with more sustained Tac concentrations around 8–10 ng/mL during a prolonged drug dose interval in contrast to more fluctuating Tac concentrations observed after Tac-IR. Moreover, the E_{max} model corroborates that although C_{max} was higher after Tac-IR compared to Tac-LCP (18.18 vs 12.31 ng/mL) similar % inhibition with respect to I_{nadir} was obtained, illustrating the capacity-limited effect. Furthermore, no evidence of hysteresis occurrence was observed in our model as should be expected at steady-state conditions, where the distribution equilibrium to the biophase has been achieved.

As other authors have mentioned, no correlations in any studied parameter were found between PD/PK in any formulation [19,35,36,38,43,44]. Explanation for this are the following: the capacity-limited effect, the delay observed in some patients between Tac concentrations and their corresponding CNA effect and the high interpatient variability observed either in PD or PK.

Currently, Tac monitoring is based on the measurement of Tac concentrations at time pre-dose due to their fine correlation with the achieved exposure during drug doses interval ($AUC_{0\text{-}24h}$) [2,3]. Our results confirmed a good correlation between C_0 or C_{24} and $AUC_{0\text{-}24h}$, and slight differences were observed between formulations [45,46]. Similarly, the pre-dose of PD (I_{24}) also correlated with their previous $AUE_{0\text{-}24h}$ I_{nadir}, as it has been previously described [19,20,36,47]. In contrast, a good correlation between I_0 or I_{24} and $AUE_{0\text{-}24h}$ I_{min} was only observed after Tac-LCP. Although some relations were seen between pre-dose time-points and AUEs, these relations were weaker than those obtained in PK ($r<0.800$). These results could be relevant as some studies reported that recipients with higher CNA

exhibited more incidence of acute rejection and patients with lower CNA developed Tac-associated toxicity despite C_0 concentrations being within the therapeutic range [20,41,43,48,49]. Interestingly, recipients that developed acute rejection suffered an increment of CNA a few days before [42]. These findings reinforced the importance of PD to refine Tac TDM [2].

To summarize, the higher C_{max} achieved after Tac-IR was not translated into higher CN inhibition (lower I_{nadir}) because the capacity-limited effect restricted additional CN inhibition. Tac-LCP showed PD benefits during the first 12 hours after drug intake due to a more prolonged and sustained inhibition of CNA compared to Tac-IR, which showed a rapid recovery of CN inhibition after 4 hours post-dose. Finally, the lack of correlation between PD and PK AUCs proved that the patient TDM based on Tac C_0 did not reflect the biological effect of Tac on its molecular target. Further studies including population PKPD model are needed to give a clear guidance of therapeutic trough concentration to achieve the optimal CN inhibition and the variability of both Tac formulations.

Highlights

- *What is the current knowledge on the topic?* Extended-release Meltdose Tac formulation (Tac-LCP) offers better bioavailability and less fluctuating pharmacokinetic profile compared to immediate-release formulation (Tac-IR). The restricted expression of Tac binding protein may limit the capacity of calcineurin activity (CNA) inhibition.

- *What question did this study address?* Does the different pharmacokinetic profile of immediate- and extended-release lead to different pharmacodynamic profile of CNA inhibition?

- *What does this study add to our knowledge?* High peak concentrations achieved after Tac-IR are not translated into higher CNA inhibition. Tac-LCP shows a more sustained CNA inhibition between dose intervals compared to Tac-IR. Tac concentration needed to elicit a 50 % of the maximum response (IC_{50}) was 9.24 ng/mL.

- *How might this change clinical pharmacology or translational science?* Tac-LCP formulation showed a more sustained CNA inhibition during dose intervals which represents an improvement of Tac pharmacodynamics.

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Wrote Manuscript: PF, HC, RR, OB, JG, NL

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Performed Research: PF, HC, RR, OB, AV, LV, GC, CP, NM, EM, AM, MM, AC, JC, JT, JG, NL

Analysed Data: PF, HC, RR, OB, AV, LV, GC, JT, JG, NL

Contributed New reagents/Analytical Tools: PF, HC, RR, GC, NL

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Figure legends

Figure 1: A. Pharmacokinetic profiles of twice-daily tacrolimus (Tac-IR) and once-daily tacrolimus (Tac-LCP). Time-course assay of tacrolimus (Tac) concentration in whole blood (ng/mL) on all patients receiving Tac-IR and their conversion to Tac-LCP and B, using logarithm scale for Tac concentrations. Each point is the geometric mean of all the patients ± 95 % confidence interval. Paired t-test between both formulations was applied. ** $p < 0.05$*.

Figure 2: A. Pharmacodynamic profiles of twice-daily tacrolimus (Tac-IR) and once-daily tacrolimus (Tac-LCP). Time-course assay of calcineurin (CN) activity (pmol RII/min·mg prot) on all patients receiving Tac-IR and their conversion to Tac-LCP. Each point is the geometric mean of all patients ± 95 % confidence interval. Paired T-test comparing both formulations in each time point was assessed. **$p < 0.05$*; B. Time-course assay of CN inhibition in which each time-point values of a Tac formulation (I_x) were subtracted from their corresponding minimum inhibition point (I_{min}) and corrected by the patient's I_{min}, 100 x $[(I_{min}-I_x)/I_{min}]$; C. Time-course assay of CN inhibition in which the maximum inhibition value (I_{nadir}) was subtracted from I_x and corrected by the patient's I_{nadir}, 100 x $[(I_x-I_{nadir}/I_{nadir})]$.

Figure 3: A. Pharmacodynamic (PD) and pharmacokinetic (PK) profiles of twice-daily tacrolimus (Tac-IR) and once-daily tacrolimus (Tac-LCP). Blue and green continuous lines represent calcineurin (CN) activity (pmol RII/min·mg prot) on the left axis of Tac-IR and Tac-LCP, respectively. Blue and green discontinuous lines show tacrolimus (Tac) concentrations in whole blood (ng/mL) on the right axis of Tac-IR and Tac-LCP,

684 respectively. Each point is the geometric mean of all patients. B. Individual predicted
685 (IPRED:lines) and observed (DV:open circles) % inhibition I_{nadir} versus Tac blood
686 concentrations (IVAR). Tac population pharmacodynamic parameter estimates for the
687 base and final models. E_0 = maximum change of % inhibition I_{nadir}; IC_{50} = Tac
688 concentration to achieve a 50% of the maximum change of % inhibition I_{nadir}; ω^2 =
689 standard deviation of variance of between-patient variability; σ^2 = variance of
690 proportional component of residual. All final parameter estimates are shown with the
691 coefficient of variation (CV) indicated in parentheses.
692
693 Figure S1: Schematic representation of the study follow-up. On day 1, pharmacodynamic
694 (PD) and pharmacokinetic (PK) profile for 24 hours (0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 12.5,
695 13, 13.5, 14, 15, 20 and 24 h) from twice-daily tacrolimus (Tac-IR) was assessed. Later,
696 patients were converted to once-daily tacrolimus (Tac-LCP). On day 14, trough levels
697 (C_0) were measured to check the proper dosage. On day 28, PD/PK profile from Tac-LCP
698 was assessed.
699
700 Figure S2: Goodness of fit plots of the final pharmacodynamic model. A, B. Solid line of
701 DV vs PRED and DV vs IPRED plots represents the identity line. Goodness-of-fit plots
702 of the final model proved that the model adequately described the data. Data were
703 randomly distributed around the identity line both in DV vs PRED and DV vs IPRED
704 plots. C. CWRES were randomly distributed around y=0 and most of them were within
705 the ± 2 interval. DV, observed values of % inhibition I_{nadir}; PRED, population predicted
706 values of % inhibition I_{nadir}; CWRES, population conditional weighted residuals of %
707 inhibition I_{nadir}; IPRED, individual predicted values of % Inhibition I_{nadir}.
708

Figure 1

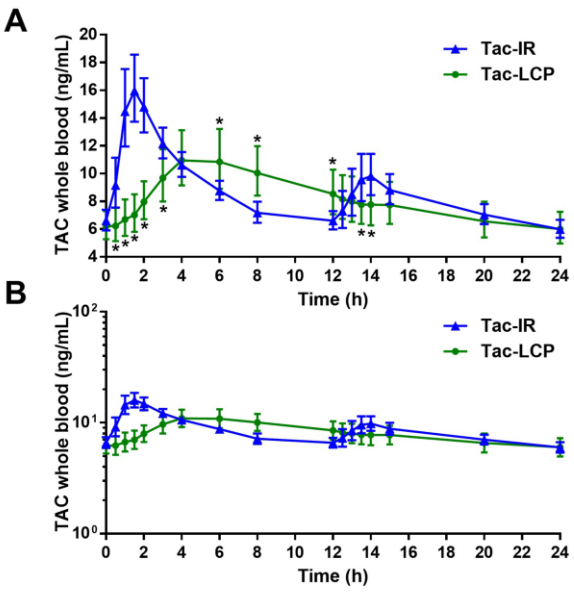

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Figure 2

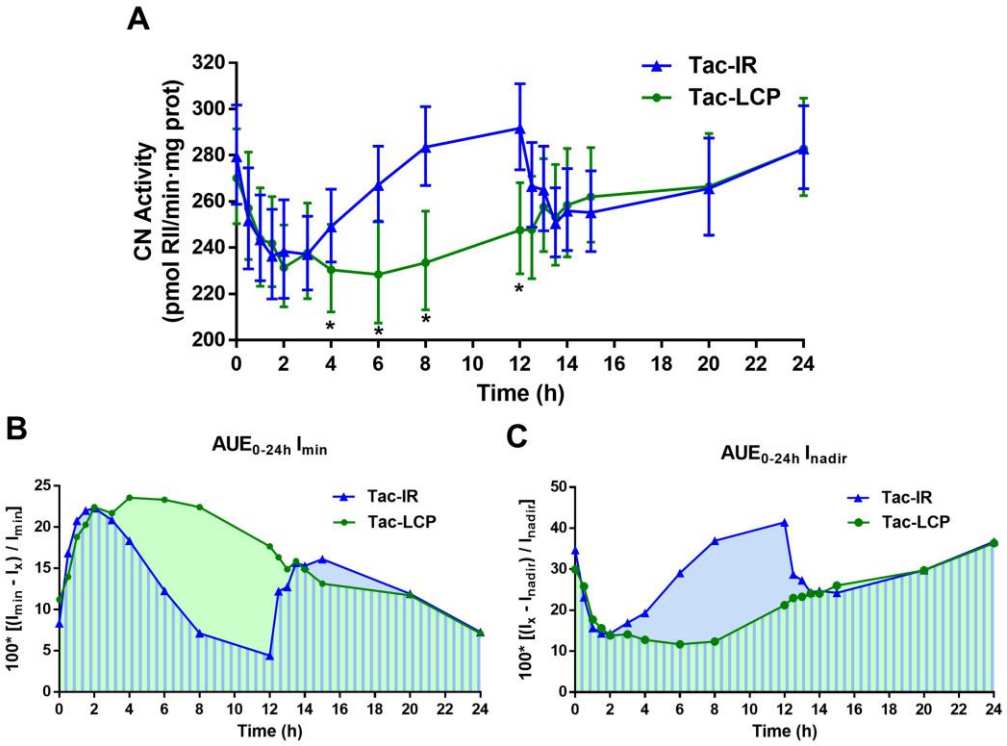

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Figure 3

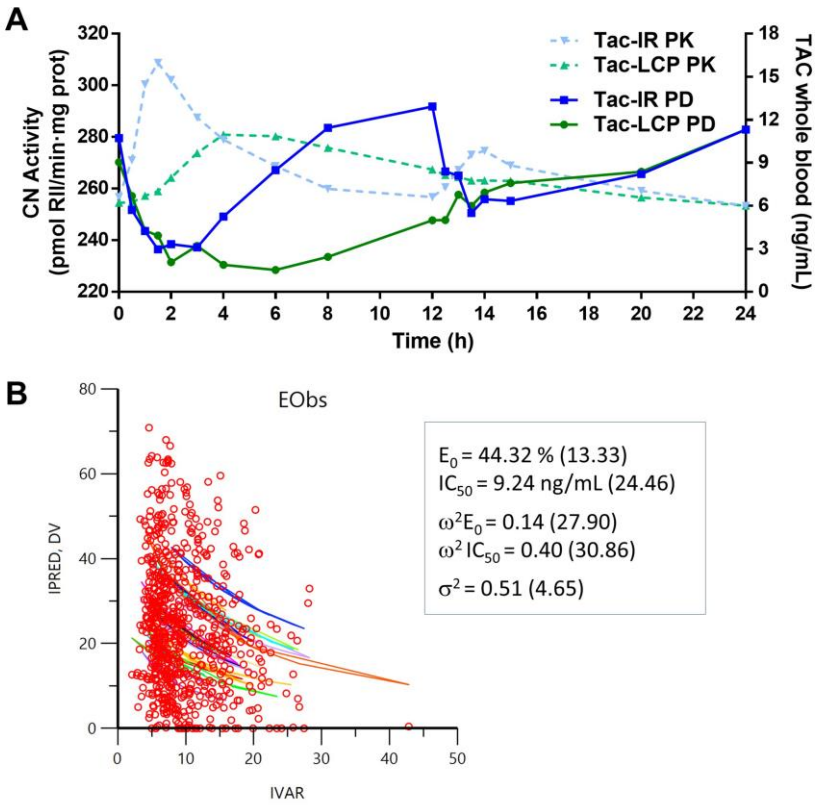

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Figure S1

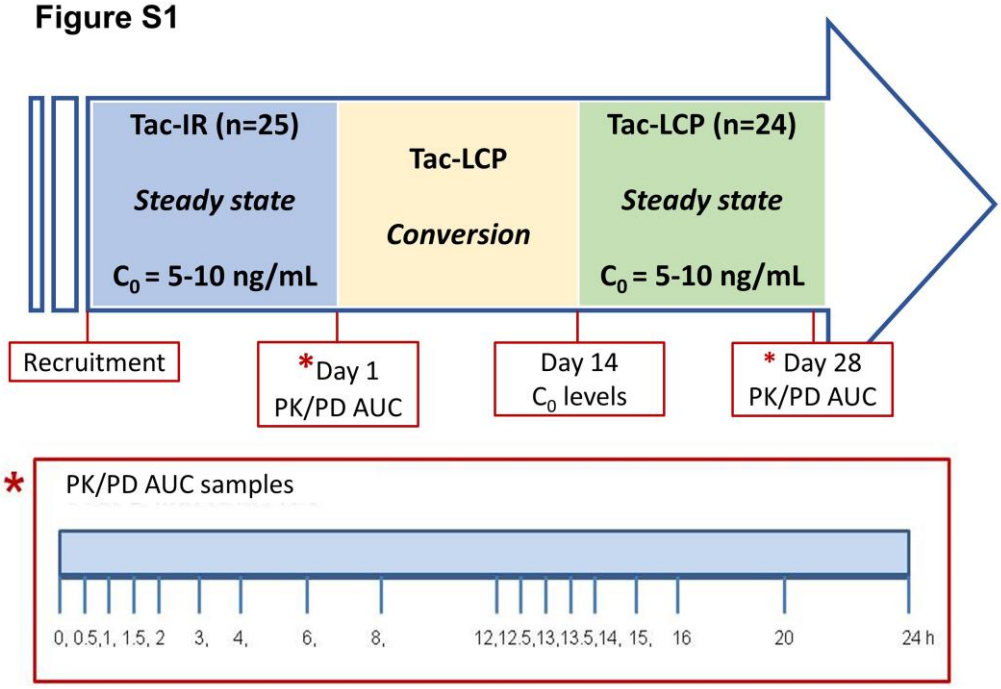

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Figure S2

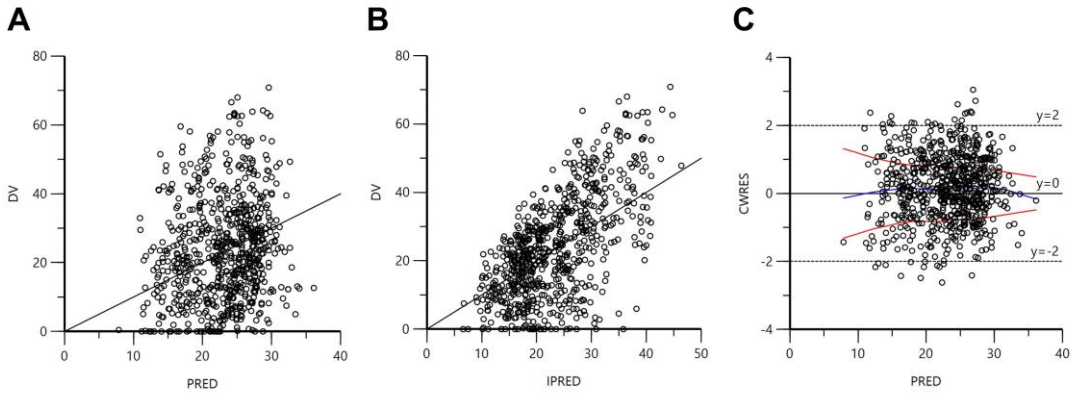

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Table 1:

Variables	**N=25**		
Sex – Male/Female (%)	18/7 (72/28)		
Age (years)	58.47 ± 13.14		
Time after transplantation (years)	1.84 [0.96 – 3.88]		
Type of donor - Deceased/Living (%)	22/3 (88/12)		
Tacrolimus formulation – Prograf®/Adoport® (%)	9/16 (36/64)		
Concomitant mycophenolate (%)			
• Mycophenolate mofetil	22 (88)		
• Mycophenolate sodium	2 (8)		
• Without mycophenolate	1 (4)		
Other concomitant drugs (%)			
• Prednisone	19 (76)		
• Omeprazole	20 (80)		
	Tac-IR	**Tac-LCP**	**p**
Haematocrit (%)	39.40 [36.90-44.90]	39.60 [37.10-44.55]	0.347[a]
Glomerular filtrate (mL/min)	47.00 [33.50-56.00]	48.50 [41.25-60.25]	0.965[a]
Creatinine (µmol/L)	137 [106-172]	133 [106-159]	0.564[b]
Albumin (g/L)	45.00 [42.50-46.50]	44.00 [42.00-46.75]	0.662[a]
ALT (µkat/L)	0.27 [0.19-0.47]	0.26 [0.19-0.72]	0.692[b]
GGT (µkat/L)	0.49 [0.33-1.04]	0.56 [0.36-1.18]	0.793[b]
Dose (mg/day)	3.00 [2.25-5.00]	2.00 [1.62-3.50]	<0.001[b]
Conversion rate		0.70 [0.67-0.80]	

Table 1. Demographic characteristics of 25 recipients enrolled in the study. Biochemical characteristics of recipients enrolled in the study and their daily dose of tacrolimus within the two formulations are compared (immediate-release, Tac-IR; and extended-release, Tac-LCP). In brackets is represented the percentage. The recipients' age is expressed as mean ± standard deviation whereas the other numerical parameters are expressed as median [interquartile range]. CKD-EPI calculation was used for glomerular filtrate estimation. ALT, alanine aminotransferase; GGT, γ-glutamil-transferase**.**

[a] Paired t-test

[b] Wilcoxon test

Table 2:

Variables	Tac-IR	Tac-LCP	p
C_0 (ng/mL)	6.61 [5.91-7.40]	6.21 [5.28-7.30]	0.351[a]
C_{24} (ng/mL)	5.99 [5.39-6.67]	6.00 [4.97-7.25]	0.987[a]
C_{max} (ng/mL)	18.18 [15.89-20.81]	12.31 [10.40-14.56]	<0.001[a]
T_{max} (h)	1.50 [1.01-1.96]	4.25 [3.95-6.00]	<0.001[b]
AUC_{0-24h} (ng·h/mL)	209.3 [191.2-229.1]	201.1 [170.0-237.9]	0.473[a]
C_{max}/C_0	2.75 [2.39-3.16]	1.98 [1.74-2.26]	<0.001[a]
PTF (%)	115.5 [85.0-140.9]	75.7 [48.7-97.5]	<0.001[b]
Swing (%)	164.2 [100.1-254.3]	97.5 [56.9-132.5]	<0.001[b]
λ_z	0.058 [0.047-0.071]*	0.026 [0.021-0.032]	<0.001[a]
$t_{1/2\lambda z}$ (h)	11.89 [9.70-14.58]*	26.57 [21.66-32.57]	<0.001[a]
AUC_{0-24h}/TDD (ng·h/mL/mg)	62.77 [50.44-78.10]	82.85 [68.92-99.59]	<0.001[a]

Correlations	Tac-IR		Tac-LCP	
	r	p	r	p
C_0 vs AUC_{0-24h}	0.806[c]	<0.001	0.781[c]	<0.001
C_{12} vs AUC_{0-24h}	0.879[c]	<0.001	-	-
C_{24} vs AUC_{0-24h}	0.706[c]	<0.001	0.860[c]	<0.001
C_{max} vs AUC_{0-24h}	0.626[c]	<0.001	0.800[d]	<0.001
C_0 vs C_{max}	0.372[c]	0.067	0.624[d]	0.001

Table 2. Values of pharmacokinetic parameters of tacrolimus (Tac) in whole blood and their correlations after immediate-release, Tac-IR; and extended-release, Tac-LCP. Data is represented as geometric mean [95% CI] unless T_{max}, PTF and swing that are expressed as median [interquartile range]. C_0, Tac concentration at time pre-dose (0h); C_{12}, Tac concentration at time 12h; C_{24},Tac concentration at time 24 h; C_{max}, maximum concentration of Tac; T_{max}, time to reach C_{max}; AUC_{0-24h}, area under the curve (AUC) from 0 to 24 hours of Tac concentration during drug doses intervals; PTF, peak-trough fluctuation index defined as $[(C_{max} – C_0)/C_{average}]$; Swing index defined as [(C_{max} –

C_0)/C_0]; λ_z, elimination rate constant; $t_{1/2\lambda z}$, elimination half-life; TDD, total daily dose.

*λ_z and $t_{1/2\lambda z}$ were estimated from the pharmacokinetic profile of 0-12 h.

[a] Paired t-test

[b] Wilcoxon-test

[c] Pearson's correlation test

[d] Spearman's correlation test

Table 3:

Variables	Tac-IR	Tac-LCP	p
I_0 (pmol RII/min·mg prot)	279.5 [258.8-301.8]	270.2 [250.5-291.4]	0.134[a]
I_{24} (pmol RII/min·mg prot)	282.9 [265.5-301.4]	282.9 [262.6-304.8]	0.932[a]
I_{min} (pmol RII/min·mg prot)	299.2 [281.9-317.7]	288.8 [268.3-310.8]	0.111[a]
I_{nadir} (pmol RII/min·mg prot)	216.3 [202.6-231.0]	218.0 [201.2-236.1]	0.860[a]
T_{nadir} (h)	2.21 [1.69-2.90]	4.19 [3.05-5.77]	0.002[b]
$AUE_{0\text{-}24h}$ I_{min} (pmol RII·h/min·mg prot)	290.5 [256.9-328.5]	373.8 [321.1-435.2]	0.039[a]
$AUE_{0\text{-}24h}$ I_{nadir} (pmol RII·h/min·mg prot)	617.6 [510.7-746.9]	471.8 [382.3-582.1]	0.063[a]

Correlations	Tac-IR		Tac-LCP	
	r	p	r	p
I_0 vs I_{nadir}	0.880[c]	<0.001	0.867[c]	<0.001
I_0 vs $AUE_{0\text{-}24h}$ I_{min}	0.044[c]	0.835	-0.497[c]	0.013
I_{24} vs $AUE_{0\text{-}24h}$ I_{min}	-0.133[c]	0.526	-0.486[c]	0.016
I_{24}/I_{nadir} vs $AUE_{0\text{-}24h}$ I_{min}	-0.028[c]	0.894	0.185[c]	0.386
I_0 vs $AUE_{0\text{-}24h}$ I_{nadir}	-0.197[c]	0.345	0.305[c]	0.147
I_{24} vs $AUE_{0\text{-}24h}$ I_{nadir}	0.816[d]	<0.001	0.569[c]	0.004
I_{24}/I_{nadir} vs $AUE_{0\text{-}24h}$ I_{nadir}	0.724[c]	<0.001	0.700[c]	<0.001

Table 3. Values of pharmacodynamic parameters of calcineurin (CN) activity and their corresponding correlations in immediate-release, Tac-IR; and extended-release, Tac-LCP; treated patients. Data is represented as geometric mean [95% CI] unless T_{nadir} that is expressed as median [interquartile range]. I_0, CN activity at time before drug intake (0 h); I_{24}, CN activity at time 24 h; I_{nadir}, maximum inhibition of CN activity; T_{nadir}, time to reach I_{nadir}; $AUE_{0\text{-}24h}$ I_{min}, area under the activity curve (AUE) from 0 to 24 hours of CN inhibition using I_{min} as baseline; $AUE_{0\text{-}24h}$ I_{nadir}, AUE of CN inhibition using I_{nadir} as baseline.

[a] Paired t-test

[b] Wilcoxon test

[c] Pearson's correlation test

[d] Spearman's correlation test

Table 4:

	Tac-IR		Tac-LCP	
	r	**p**	**r**	**p**
C_0 vs I_0	-0.140[a]	0.504	-0.033[a]	0.881
C_{max} vs I_{nadir}	-0.014[a]	0.946	-0.126[b]	0.566
C_{max}/C_0 vs I_0/I_{nadir}	0.026[a]	0.903	-0.015[b]	0.945
C_0 vs $AUE_{0\text{-}24h}$ I_{min}	0.193[a]	0.355	0.127[a]	0.562
$AUC_{0\text{-}24h}$ vs $AUE_{0\text{-}24h}$ I_{min}	0.095[a]	0.652	0.333[a]	0.121
C_0 vs $AUE_{0\text{-}24h}$ I_{nadir}	0.286[a]	0.166	0.146[a]	0.506
$AUC_{0\text{-}24h}$ vs $AUE_{0\text{-}24h}$ I_{nadir}	0.247[a]	0.233	0.142[a]	0.519

Table 4. Correlation between pharmacokinetic and pharmacodynamic parameters in immediate-release, Tac-IR; and extended-release, Tac-LCP; treated patients. C_0, tacrolimus (Tac) concentration at time pre-dose (0h); I_0, calcineurin (CN) activity at time pre-dose (0h); C_{max}, maximum concentration of Tac; I_{nadir}, maximum inhibition of CN activity; $AUC_{0\text{-}24h}$, area under the curve (AUC) from 0 to 24 hours of Tac concentration during drug doses intervals; $AUE_{0\text{-}24h}$ I_{min}, area under the activity curve (AUE) from 0 to 24 hours of CN inhibition using I_{min} as baseline; $AUE_{0\text{-}24h}$ $I_{nadir,}$ AUE of CN inhibition using I_{nadir} as baseline.

[a] Pearson's correlation test

[b] Spearman's correlation test