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Monitoring intracellular tacrolimus concentrations and its relationship with rejection in the early phase after renal transplantation

Marith I. Francke [a,b,*], Louise M. Andrews [c,d], Hoang Lan Le [c], Daan van de Velde [c], Marjolein Dieterich [a], Suwasin Udomkarnjananun [a,b,e], Marian C. Clahsen-van Groningen [b,f], Carla C. Baan [a,b], Teun van Gelder [g], Brenda C.M. de Winter [b,c], Dennis A. Hesselink [a,b]

[a] Department of Internal Medicine, Division of Nephrology and Transplantation, Erasmus MC, University Medical Center Rotterdam, the Netherlands
[b] Erasmus MC Transplant Institute, the Netherlands
[c] Department of Hospital Pharmacy, Erasmus MC, University Medical Center Rotterdam, Rotterdam, the Netherlands
[d] Department of Hospital Pharmacy, Meander Medical Center, Amersfoort, the Netherlands
[e] Division of Nephrology, Department of Medicine, Faculty of Medicine, Chulalongkorn University and King Chulalongkorn Memorial Hospital, Bangkok, Thailand
[f] Department of Pathology, Erasmus MC, University Medical Center Rotterdam, Rotterdam, the Netherlands
[g] Department of Clinical Pharmacy and Toxicology, Leiden University Medical Center, Leiden, the Netherlands

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ABSTRACT

Introduction: After kidney transplantation, rejection and drug-related toxicity occur despite tacrolimus whole-blood pre-dose concentrations ($[Tac]_{blood}$) being within the target range. The tacrolimus concentration within peripheral blood mononuclear cells ($[Tac]_{cells}$) might correlate better with clinical outcomes. The aim of this study was to investigate the correlation between $[Tac]_{blood}$ and $[Tac]_{cells}$, the evolution of $[Tac]_{cells}$ and the $[Tac]_{cells}/[Tac]_{blood}$ ratio, and to assess the relationship between tacrolimus concentrations and the occurrence of rejection.

Methods: In this prospective study, samples for the measurement of $[Tac]_{blood}$ and $[Tac]_{cells}$ were collected on days 3 and 10 after kidney transplantation, and on the morning of a for-cause kidney transplant biopsy. Biopsies were reviewed according to the Banff 2019 update.

Results: Eighty-three $[Tac]_{cells}$ samples were measured of 44 kidney transplant recipients. The correlation between $[Tac]_{cells}$ and $[Tac]_{blood}$ was poor (Pearson's $r = 0.56$ (day 3); $r = 0.20$ (day 10)). Both the dose-corrected $[Tac]_{cells}$ and the $[Tac]_{cells}/[Tac]_{blood}$ ratio were not significantly different between days 3 and 10, and the median inter-occasion variability of the dose-corrected $[Tac]_{cells}$ and the $[Tac]_{cells}/[Tac]_{blood}$ ratio were 19.4% and 23.4%, respectively ($n = 24$). Neither $[Tac]_{cells}$, $[Tac]_{blood}$, nor the $[Tac]_{cells}/[Tac]_{blood}$ ratio were significantly different between patients with biopsy-proven acute rejection ($n = 4$) and patients with acute tubular necrosis ($n = 4$) or a cancelled biopsy ($n = 9$; $p > 0.05$).

Conclusion: Tacrolimus exposure and distribution appeared stable in the early phase after transplantation. $[Tac]_{cells}$ was not significantly associated with the occurrence of rejection. A possible explanation for these results might be related to the low number of patients included in this study and also due to the fact that PBMCs are not a specific enough matrix to monitor tacrolimus concentrations.

1. Introduction

Despite therapeutic drug monitoring (TDM) and tacrolimus concentrations being within the therapeutic range, a considerable number of kidney allografts is rejected or injured by tacrolimus-related nephrotoxicity [1–5]. Therefore, it has been suggested that the tacrolimus concentration in a matrix other than whole-blood may correlate better with clinical outcomes [6–8]. Tacrolimus suppresses lymphocyte activation via intracellular inhibition of calcineurin. However, approximately 85% of tacrolimus measured in whole-blood is present within erythrocytes (which have a high content of the tacrolimus receptor FK-binding protein-12), but these are not involved in the alloimmune

* Corresponding author at: Dept. of Internal Medicine, Division of Nephrology & Renal Transplantation, Erasmus MC, University Medical Center Rotterdam, Room Rg-527, P.O. Box 2040, 3000 CA Rotterdam, the Netherlands..
E-mail address: m.francke@erasmusmc.nl (M.I. Francke).

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response [9–11]. The correlation between whole-blood tacrolimus concentrations ($[Tac]_{blood}$) and tacrolimus concentrations within peripheral blood mononuclear cells (PBMCs; $[Tac]_{cells}$) is poor to moderate [7,12–17]. Together, this suggests that the tacrolimus whole-blood concentration does not reflect the tacrolimus concentration within its target cell, which may explain why multiple studies could not find a correlation between whole-blood tacrolimus concentrations and clinical outcomes [16,18–20].

Tacrolimus concentrations within PBMCs may better correlate with the immunosuppressive effect of tacrolimus [6,8]. In a study in 213 kidney transplant recipients with a stable graft function, $[Tac]_{cells}$ was associated with T cell activation [7]. Moreover, in a study of 90 liver transplant recipients, lower $[Tac]_{cells}$ correlated with both the development and severity of rejection [16]. However, the latter finding has not been replicated in kidney transplant recipients [7,21]. In a previous study including 175 kidney transplant recipients, there was no correlation between $[Tac]_{cells,}$ measured at month 3 after transplantation, and the occurrence of biopsy-proven acute rejection (BPAR) [21]. However, an important limitation of that study was the fact that most rejections were diagnosed before $[Tac]_{cells}$ was measured. There was also no erythrocyte lysis step included in the preparation of the blood samples, and $[Tac]_{cells}$ was measured using immunoassays instead of the more sensitive LC-MS/MS method [21].

In the present study, the relationship between acute kidney transplant rejection and $[Tac]_{cells}$ was investigated as part of a prospective clinical trial [22]. In contrast to previous studies, samples for tacrolimus concentration measurement were drawn on the morning that the for-cause kidney transplant biopsy was performed. In addition, an improved method for $[Tac]_{cells}$ measurement was used, which incorporated an erythrocyte lysis step in the preparation of the sample, and LC-MS/MS was used to measure $[Tac]_{blood}$ and $[Tac]_{cells}$ rather than immunoassays [23].

2. Materials and Methods

2.1. Study design

This study was embedded in a prospective, single-arm, therapeutic intervention trial that investigated the efficacy of algorithm-based tacrolimus dosing in the early phase after kidney transplantation [22]. In this study, 60 kidney transplant recipients were prescribed a tacrolimus starting dose based on a dosing algorithm that included age, body surface area (BSA) and cytochrome P450 *(CYP)3A4* and *CYP3A5* genotype as covariates, rather than a standard, bodyweight-based starting dose [22,24]. As an integral and pre-planned part of this trial, samples for the measurement of $[Tac]_{cells}$ were collected during hospitalization from all participants on post-operative days 3 and 10, and on the morning of a for-cause kidney transplant biopsy (only on a weekday). No protocol biopsies were obtained.

2.2. Study endpoints

The aim of this study was to investigate 1) the correlation between $[Tac]_{blood}$ and $[Tac]_{cells}$ at day 3 and day 10 after transplantation, 2) the evolution of $[Tac]_{cells}$, and the $[Tac]_{cells}/[Tac]_{blood}$ ratio over time, and 3) the relationship between $[Tac]_{blood}$ and $[Tac]_{cells}$ and the occurrence of acute rejection.

2.3. Patient population

As described previously [22], patients were eligible for participation in the trial if they were at least 18 years old and were scheduled to undergo a single organ, HLA and blood group ABO compatible kidney transplantation with a living donor in the Erasmus MC, University Medical Center Rotterdam, the Netherlands. Patients had to receive tacrolimus as part of their initial immunosuppressive therapy and were excluded if they used tacrolimus or drugs interacting with tacrolimus in the 28 days prior to the kidney transplantation. Patients were only included in the present analysis if at least one $[Tac]_{cells}$ was measured during the study period.

2.4. Immunosuppressive treatment

As described previously [22], all patients received induction therapy with basiliximab (Simulect®; Novartis Pharma B.V., Arnhem, the Netherlands), followed by triple immunosuppressive therapy including tacrolimus (Prograft®; Astellas Pharma, Leiden, the Netherlands), mycophenolate mofetil (Cellcept®; Roche Pharmaceuticals, Woerden, the Netherlands), and glucocorticoids. The tacrolimus starting dose of all patients included in this trial was based on an internally and externally validated dosing algorithm that included age, BSA, and *CYP3A4* and *CYP3A5* genotype as covariates and was described by Andrews *et al.,* [24] rather than the standard, bodyweight-based starting dose (0.2 mg/kg/day). On day 3 after transplantation (considered the first steady state), the first tacrolimus whole-blood pre-dose concentration (C_0) was measured and thereafter, standard TDM of whole-blood pre-dose concentrations was performed, aiming for whole-blood tacrolimus C_0 of 7.5 to 12.5 ng/mL during post-operative weeks 1 and 2. No tacrolimus concentration measurements nor dose adjustments were allowed before post-operative day 3. Tacrolimus dose adjustments were made by the treating physician. In our center, tacrolimus C_0 ($[Tac]_{blood}$) is measured 3-times weekly during hospitalization and thereafter, at every visit to the outpatient clinic (which are scheduled weekly in the first 2 months after transplantation).

2.5. Blood sampling and tacrolimus measurement

During hospitalization, blood samples (6 mL, EDTA) were drawn on days 3 and 10, and on the morning of a for-cause renal transplant biopsy for the measurement of whole-blood tacrolimus concentrations. For the measurement of $[Tac]_{cells,}$ an additional blood sample (6 mL, heparin tube) was collected. All blood samples in this study were drawn prior to tacrolimus intake (*i.e.* only pre-dose concentrations were measured).

PBMCs were isolated from whole-blood samples using a mini Ficoll separation technique, which requires less blood compared to the standard Ficoll method which has been described previously [25]. In the mini Ficoll method, 2 times 1 mL blood was brought on top of 600 μL Ficoll-paque (GE Healthcare Bio-Sciences, Uppsala, Sweden), and centrifuged for 3 min at 16,000 g. Thereafter, the upper plasma layer was removed, and the middle layer (containing the PBMCs) was collected. The PBMCs were washed with 3 mL phosphate-buffered saline (PBS) and centrifuged for 7 min (1061*g*). The 2 samples were pooled and after adding 3 mL PBS, the pooled sample was centrifuged (5 minutes, 877 g). To eliminate a potential effect of the presence of erythrocytes on the tacrolimus concentration measurement, a red blood cell lysis step was included. In this step, 1 mL red blood cell (RBC) lysis buffer solution (eBioscience, Affymetrix, San Diego) was added to the cells. After 10 min, 3 mL PBS was added and the sample was centrifuged (5 min, 877 g) to wash out the lysed erythrocytes. Thereafter, PBMCs were counted (Sysmex XP-300 cell counter), and $1*10^6$ cells were resuspended in 1 mL PBS. The sample was centrifuged (1 min, 16,000 g) and the fluid was removed from the sample, leaving only the cell pellet. The whole procedure was performed at room temperature. Finally, the PBMCs were suspended in 50 μL PBS, snap-frozen in liquid nitrogen and stored until further analysis at −80 °C in aliquots of $1*10^6$ cells per vial. Previously, we determined the cell content and purity of Ficoll-separated PBMC isolates. Flowcytometric analysis of samples from healthy controls showed that the vast majority of cells of the interface consisted of mononuclear cells (88–100%) with lymphocytes accounting for > 85% [26]. The Sysmex XP-300 cell counter measures white blood cells in the analysis range 1.0–99.9 $x10^3$/μL with a precision (repeatability) of 3.5% (variation coefficient) or less [27–28].

A validated liquid chromatography-tandem mass spectrometry method (LC-MS/MS) was used to determine both $[Tac]_{blood}$ and $[Tac]_{cells}$ in an ISO15189 certified laboratory [25]. The imprecision of this method is < 15% with a bias < 15% over the validated range 0.1–25.0 ng/mL (for $[Tac]_{blood}$), and 5–1250 pg/10^6 cells for the $[Tac]_{cells}$ measurement.

2.6. Rejection and clinical outcome

Biopsies were performed for-cause only and were assessed and graded according to the Banff 2019 update by a renal pathologist (M.C. C.-v.G.) [29]. The total follow-up period for rejection was 30 days after renal transplantation.

Patients were classified in 4 subgroups based on their biopsy results: 1) Patients with BPAR, 2) Patients diagnosed with borderline (suspicious) for acute T cell-mediated rejection (bTCMR) or with histomorphological signs suspicious for active antibody-mediated rejection (aABMR; *i.e.* microvascular inflammation or thrombotic microangiopathy), but not meeting the full Banff 2019 criteria for the diagnosis aABMR (*i.e.* no detectable donor-specific anti-HLA antibodies and a negative C4d staining), 3) Patients without signs of rejection in the biopsy and who received no anti-rejection treatment, 4) Patients in whom $[Tac]_{cells}$ was measured but in whom the biopsy was cancelled because of spontaneous clinical improvement.

2.7. Statistical analysis

All statistical analyses were performed in R (version 3.5.3) [30]. Categorical variables were described as number of cases with a percentage. Continuous variables were described as median with interquartile range (IQR). Normal distribution of the variables was determined with qq-plots and the Shapiro-Wilk test. Variables with a non-parametric distribution were log-transformed and again tested for normality. Pearson's correlation coefficient was used to determine correlations between variables with a log-normal distribution. To compare variables with a (log-)normal distribution, Student's *t*-test and ANOVA (for multiple groups) were used. To compare non-parametric variables, the Mann-Whitney U and the Kruskal-Wallis tests (for multiple groups) were used.

The inter-occasion variability was calculated for each patient with multiple $[Tac]_{blood}$ and $[Tac]_{cells}$ measurements to investigate the evolution of tacrolimus concentrations over time, by the following formula: coefficient of variation (CV) $\%=X_{sd}/X_{mean}*100$. Here, X_{sd} represents the standard deviation, and X_{mean} the mean of the $[Tac]_{cells}$, $[Tac]_{blood}$, or the $[Tac]_{cells}/[Tac]_{blood}$ ratios of an individual. The inter-patient variability was calculated at day 3 and day 10 after transplantation with the following formula: inter-patient variability $\% = Xt_{sd}/Xt_{mean}*100$, where Xt_{sd} and Xt_{mean} represent the standard deviation and the mean of the tacrolimus concentrations measured at a certain time point (day 3 or day 10).

2.8. Ethical considerations

This clinical trial was performed in accordance with the principles of the Declaration of Helsinki (seventh revision, October 2013, approved by the 64th WMA General Assembly, Fortaleza, Brazil) and the Medical Research Involving Human Subjects Act (WMO). Study procedures were performed in accordance with the ethical standards of the institutional research committee (Erasmus MC Medical Ethical Review Board, number 2018–157). The trial was registered (19–10-2018) in the Dutch national trial registry (https://www.trialregister.nl/trial/7360). Written informed consent was obtained from all patients prior to inclusion.

3. Results

Of the 60 patients who participated in the single-arm, prospective intervention trial, 44 patients were included in the present study. A total of 16 patients was excluded from this analysis (Supplementary Figure S1). In these 16 patients no $[Tac]_{cells}$ was measured because day 3 and day 10 after transplantation did not fall on a weekday. None of these excluded patients underwent a kidney transplant biopsy within the first 10 days after transplantation.

A total of 83 $[Tac]_{cells}$ was measured between day 2 and day 10 in the 44 kidney transplant recipients that were included in the analysis. The number of tacrolimus dose-adjustments per patient between day 3 and day 10 after transplantation ranged between 0 and 3 (0: n = 8; 1: n = 15; 2: n = 14; 3: n = 7).

3.1. Baseline characteristics

Table 1 presents the baseline characteristics of the 44 included patients. The majority of the included patients was male (n = 27; 61%) and received a kidney transplant for the first time (n = 43; 98%). The median age was 62.5 years (IQR 51.3–67.8).

3.2. The correlation between PBMC and whole-blood tacrolimus concentrations

On the third post-operative day, 35 patients had an available $[Tac]_{cells}$ and $[Tac]_{blood}$. The median $[Tac]_{cells}$ was 93.5 pg/10^6 cells (IQR 60.9–128.3) and the median $[Tac]_{blood}$ was 9.1 ng/mL (IQR 7.9–11.5). The correlation between $[Tac]_{cells}$ and $[Tac]_{blood}$ was poor (Pearson's r = 0.56; p < 0.05; n = 35; Supplementary Figure S2A). On day 10 after transplantation, both $[Tac]_{cells}$ and $[Tac]_{blood}$ were measured in 24 patients. The median $[Tac]_{cells}$ was 72.6 pg/10^6 cells (IQR 59.2–96.4) and the median $[Tac]_{blood}$ was 9.8 ng/mL (IQR 8.7–11.0). On the 10th post-operative day the correlation between $[Tac]_{cells}$ and $[Tac]_{blood}$ was not significant (Pearson's r = 0.20; p = 0.35; n = 24; Supplementary Figure S2B).

3.3. PBMC and whole-blood tacrolimus concentrations over time

A total of 83 $[Tac]_{cells}$ was measured (day 3: n = 35; day 10: n = 24; other day: n = 24), with a median $[Tac]_{cells}$ of 77.3 pg/10^6 cells (IQR 57.4–107.3). A total of 81 $[Tac]_{blood}$ was measured, with a median $[Tac]_{blood}$ of 9.4 ng/mL (IQR 8.1–11.7). Table 2 shows the $[Tac]_{cells}$ and $[Tac]_{blood}$ on the 3rd and 10th day after transplantation.

Table 1
Baseline characteristics.

Recipient characteristics	Study population (n = 44)
Gender	
Female/Male	17 (39%)/27 (61%)
Age (years)	63 (IQR 51–68)
Bodyweight (kg)	80.2 (IQR 70.3–89.3)
Length (cm)	176.0 (IQR 169.5–180.0)
BMI (kg/m^2)	26.3 (IQR 23.5–28.0)
BSA (m^2)	1.98 (IQR 1.83–2.07)
RRT prior to kidney transplantation	
Hemodialysis	12 (27%)
Peritoneal dialysis	5 (11%)
None (pre-emptive transplantation)	27 (61%)
Number of kidney transplantations	
1st/2nd/3rd	43 (98%)/0 (0%)/1 (2%)
Donor type	
Living related/Living unrelated	14 (32%)/30 (68%)
PRA current	
<15%/>15%	41 (93%)/3 (7%)
PRA peak	
<15%/>15%	42 (95%)/2 (5%)

BMI, body mass index; PRA, panel reactive antibodies; RRT, renal replacement therapy.
Continuous variables are described as median (IQR; range). Categorical variables as number of cases (%).

Table 2
PBMC and whole-blood tacrolimus concentrations at day 3 and day 10 after transplantation.

Parameter	Day 3 (n = 35)	Day 10 (n = 25)	p value
Last daily dose (mg/day)	10.0 (9.0–12.0)	11.0 (6.8–16.0)	0.877**
$[Tac]_{blood}$ (ng/mL)	9.1 (7.9–11.5)	9.8 (8.7–11.0)	0.431**
$[Tac]_{blood}$/dose (ng/mL per mg)	0.87 (0.58–1.23)	0.87 (0.59–1.57)	0.614*
$[Tac]_{cells}$ (pg/10^6 cells)	93.5 (60.9–128.3)	70.3 (58.3–96.0)	0.155*
$[Tac]_{cells}$ /dose (pg/10^6 cells per mg)	8.8 (5.6–11.8)	6.4 (4.6–11.7)	0.424*
$[Tac]_{cells}/[Tac]_{blood}$ (pg/10^6 cells per ng/mL)	10.3 (7.7–13.8)	7.6 (6.1–10.4)	0.115*

Data is shown as median (IQR).
PBMCs, peripheral blood mononuclear cells; $[Tac]_{blood}$, whole-blood tacrolimus pre-dose concentration; $[Tac]_{cells}$, tacrolimus pre-dose concentration within PBMCs.
* After logarithmic transformation to a normal distribution, p values were calculated using the *t* test.
** P values were calculated using the Mann Whitney *U* test.

The $[Tac]_{cells}$/dose was not significantly different between day 3 (median 8.8 pg/10^6 cells per mg; IQR 5.6–11.8; n = 35) and day 10 (median 6.4 pg/10^6 cells per ng/mL; IQR 4.6–11.7; n = 24) after transplantation (p = 0.42; Table 2). The $[Tac]_{cells}/[Tac]_{blood}$ ratio was not significantly different between day 3 (median 10.3 pg/10^6 cells per ng/mL; IQR 7.7–13.8; n = 35) and day 10 (median 7.6 pg/10^6 cells per ng/mL; IQR 6.1–10.4; n = 24) after transplantation (p = 0.12; Table 2).

3.3.1. Inter-patient and inter-occasion variability

Fig. 1 shows the evolution of the dose-corrected $[Tac]_{cells}$, $[Tac]_{blood}$, and the $[Tac]_{cells}/[Tac]_{blood}$ ratio over time. In 25 patients, multiple tacrolimus concentrations were measured during the first 10 post-operative days. The number of tacrolimus concentrations that was measured per patient ranged between 2 and 4 (2 occasions: n = 14; 3 occasions: n = 8; 4 occasions: n = 3). The median inter-occasion variability was 19.4% (IQR 11.7–27.9; n = 24), 23.6% (IQR 13.2–28.6; n = 23), and 23.4% (IQR 14.8–32.2; n = 24) for the dose-corrected $[Tac]_{cells}$, $[Tac]_{blood}$, and the $[Tac]_{cells}/[Tac]_{blood}$ ratio over time, respectively. The inter-patient variability on day 3 was 67.0%, 56.6%, and 47.1% for the dose-corrected $[Tac]_{cells}$, $[Tac]_{blood}$, and the $[Tac]_{cells}/[Tac]_{blood}$ ratio, respectively. The inter-patient variability on day 10 was 76.4%, 84.5%, and 51.7% for the dose-corrected $[Tac]_{cells}$, $[Tac]_{blood}$, and the $[Tac]_{cells}/[Tac]_{blood}$ ratio, respectively.

When only the 11 patients in whom ≥ 3 tacrolimus concentrations were measured during the first 10 post-operative days were included, the results did not change significantly. The median inter-occasion variability was 18.1% (IQR 14.3–24.2; n = 10), 20.7% (IQR 13.7–24.0; n = 9), and 26.0% (IQR 16.3–33.9; n = 10) for the dose-corrected $[Tac]_{cells}$, $[Tac]_{blood}$, and the $[Tac]_{cells}/[Tac]_{blood}$ ratio over time, respectively.

3.4. Tacrolimus concentrations and the occurrence of rejection

All 44 patients that were included in the present analysis, survived the first post-transplant month with a functioning graft. In these patients, a total of 14 kidney transplant biopsies were obtained from 12 patients. Biopsies were obtained between day 2 and day 16 after transplantation (median 7 days; Supplementary Figure S3). Rejection was diagnosed in 4 kidney transplant biopsies of 3 patients. These 4 cases of BPAR were classified as aTCMR2A (in 2 patients) and aTCMR2B (in 2 consecutive biopsies of 1 patient). One patient was diagnosed with bTCMR and was subsequently treated with methylprednisolone. Two out of the 44 patients were classified as suspicious for active antibody-mediated rejection (aABMR). These two patients had histomorphological evidence of aABMR (*i.e.* microvascular inflammation or thrombotic microangiopathy) but did not have donor-specific anti-HLA antibodies or positive staining for C4d. These two patients were treated with methylprednisolone and immunoglobulins. Four patients had a kidney transplant biopsy showing acute tubular necrosis (ATN) and did not receive any anti-rejection treatment. In 8 patients who were scheduled to undergo a kidney transplant biopsy, a $[Tac]_{cells}$ was measured (n = 9, one patient had 2 scheduled biopsies), where after the biopsy was cancelled because of spontaneous improvement of their kidney function.

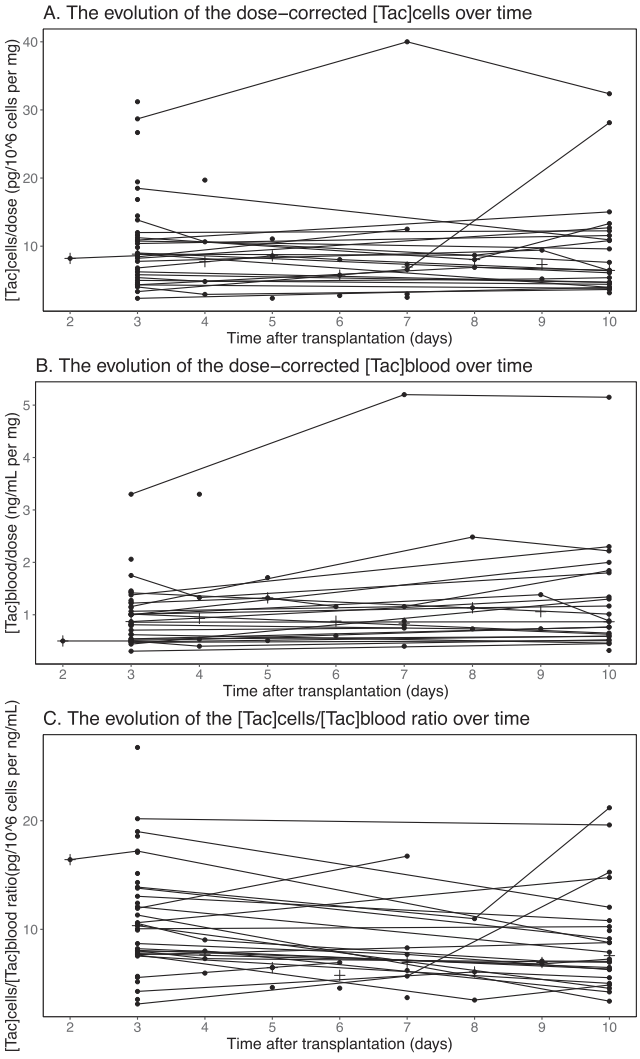

Fig. 1. Spaghetti plot of $[Tac]_{cells}$ (A), $[Tac]_{blood}$ (B), and the $[Tac]_{cells}/[Tac]_{blood}$ ratio (C) over time. The plus sign represents the median. $[Tac]_{blood}$, whole-blood tacrolimus pre-dose concentration; $[Tac]_{cells}$, tacrolimus pre-dose concentration within peripheral blood mononuclear cells.

Table 3 and Fig. 2 present the $[Tac]_{cells}$, $[Tac]_{blood}$, and the $[Tac]_{cells}/[Tac]_{blood}$ ratio of patients with BPAR (patients with BPAR, n = 3; biopsies with BPAR n = 4), ATN (n = 4), and those with a cancelled biopsy (n = 9). $[Tac]_{blood}$ was not significantly different between patients with BPAR (median 9.6 ng/mL; IQR 7.7–10.7; n = 4), ATN (median 13.0 ng/mL; IQR 11.1–14.6; n = 4), and a cancelled biopsy (median 9.9 ng/mL; IQR 8.0–11.4; n = 8), respectively (p = 0.36; Table 3). $[Tac]_{cells}$ was not significantly different between patients with BPAR (median 57.9 pg/10^6 cells; IQR 54.8–76.6; n = 4), ATN (median 96.0 pg/10^6 cells; IQR 82.0–104.3; n = 4), and a cancelled biopsy (median 72.3 pg/10^6 cells; IQR 56.5–74.8; n = 9), respectively (p = 0.70; Table 3). Also, the

Table 3
PBMC and whole-blood tacrolimus concentrations and the occurrence of rejection.

Parameter	BPAR (n = 4[***])	ATN (n = 4)	Cancelled (n = 9[***])	P value
Dose (mg/day)	9.5 (IQR 8.3–11.5)	14.0 (IQR 11.8–16.5)	9.0 (IQR 7.0–13.5)	0.21[**]
$[Tac]_{blood}$ (ng/mL)	9.6 (IQR 7.7–10.7)	13.0 (IQR 11.1–14.6)	9.9 (IQR 8.0–11.4)	0.36[**]
$[Tac]_{blood}$/dose (ng/mL per mg/kg)	0.9 (IQR 0.7–1.2)	0.8 (IQR 0.7–1.0)	1.2 (IQR 0.7–1.3)	0.75[*]
$[Tac]_{cells}$ (pg/10^6 cells)	57.9 (IQR 54.8–76.6)	96.0 (IQR 82.0–104.3)	72.3 (IQR 56.5–74.8)	0.70[*]
$[Tac]_{cells}$ /dose (pg/10^6 cells per mg/kg)	7.3 (IQR 6.2–8.4)	6.7 (IQR 4.6–8.9)	8.6 (IQR 5.8–9.4)	0.86[*]
$[Tac]_{cells}/[Tac]_{blood}$ (pg/10^6 cells per ng/mL)	8.7 (IQR 6.2–11.1)	6.8 (IQR 5.8–9.4)	6.9 (IQR 6.0–8.3)	0.87[*]

Data is shown as median (IQR).
ATN, acute tubular necrosis; BPAR, biopsy-proven acute rejection; $[Tac]_{blood}$, whole-blood tacrolimus pre-dose concentration; $[Tac]_{cells}$, tacrolimus pre-dose concentration within peripheral blood mononuclear cells.
[*] After logarithmic transformation to a normal distribution, p values were calculated using the Anova test.
[**] P values were calculated using the Kruskal-Wallis test.
[***] two samples are from the same patient.

$[Tac]_{cells}/[Tac]_{blood}$ ratio was not significantly different between patients with BPAR (median 8.7 pg/10^6 cells per ng/mL; IQR 6.2–11.1; n = 4), ATN (median 6.8 pg/10^6 cells per ng/mL; IQR 5.8–9.4; n = 4), and a cancelled biopsy (median 6.9 pg/10^6 cells per ng/mL; IQR 6.0–8.3; n = 8), respectively (p = 0.87; Table 3).

In a second analysis, the $[Tac]_{cells}$, $[Tac]_{blood}$, and the $[Tac]_{cells}/[Tac]_{blood}$ ratio of patients with either BPAR, bTCMR, or with histological signs suspicious for aABMR (together classified as rejection; n = 7) were compared to those of patients with ATN (n = 4), and those of patients with a cancelled biopsy (n = 9; Supplementary Table S1 and Supplementary Figure S4). $[Tac]_{blood}$ was not significantly different between patients with rejection (median 8.8 ng/mL; IQR 7.8–11.1; n = 7), ATN (median 13.0 ng/mL; IQR 11.1–14.6; n = 4), and a cancelled biopsy (median 9.9 ng/mL; IQR 8.0–11.4; n = 8), respectively (p = 0.47.; Supplementary Table S1). $[Tac]_{cells}$ was not significantly different between patients with rejection (median 56.5 pg/10^6 cells; IQR 47.3–78.9; n = 7), ATN (median 96.0 pg/10^6 cells; IQR 82.0–104.3; n = 4), and a cancelled biopsy (median 72.3 pg/10^6 cells; IQR 56.5–74.8; n = 9), respectively (p = 0.54; Supplementary Table S1). The $[Tac]_{cells}/[Tac]_{blood}$ ratio was not significantly different between patients with rejection (median 6.2 pg/10^6 cells per ng/mL; IQR 5.8–8.7; n = 7), ATN (median 6.8 pg/10^6 cells per ng/mL; IQR 5.8–9.4; n = 4), and a cancelled biopsy (median 6.9 pg/10^6 cells per ng/mL; IQR 6.0–8.3; n = 8), respectively (p = 0.99; Supplementary Table S1).

Finally, the $[Tac]_{cells}$, $[Tac]_{blood}$, and the $[Tac]_{cells}/[Tac]_{blood}$ ratio of patients with BPAR were compared to those of patients who had not been scheduled for a biopsy during the whole follow-up period (*i.e.* in whom only protocol samples were collected on days 3 and 10; Supplementary Table S2 and Supplementary Figure S5). $[Tac]_{blood}$ was not significantly different between samples of patients with BPAR drawn on the day of the biopsy (median 9.6 ng/mL; IQR 7.7–10.7; n = 4), samples of patients without a biopsy drawn on day 3 (median 9.1 ng/mL; IQR 7.8–12.4; n = 22), and samples of patients without a biopsy drawn on day 10 (median 9.5 ng/mL; IQR 8.5–10.3; n = 14), respectively (p = 0.99; Supplementary Table S2). $[Tac]_{cells}$ was not significantly different between patients with BPAR (median 57.9 pg/10^6 cells; IQR 54.8–76.6; n = 4), samples of patients without a biopsy drawn on day 3 (median 105.5 pg/10^6 cells; IQR 77.0–167.2; n = 22), and samples of patients without a biopsy drawn on day 10 (median 79.5 pg/10^6 cells; IQR 63.8–93.9; n = 14), respectively (p = 0.10; Supplementary Table S2). Also, the $[Tac]_{cells}/[Tac]_{blood}$ ratio was not significantly different between patients with BPAR (median 8.7 pg/10^6 cells per ng/mL; IQR 6.2–11.1; n = 4), samples of patients without a biopsy drawn on day 3 (median 10.5 pg/10^6 cells per ng/mL; IQR 8.1–14.9; n = 22), and a cancelled biopsy (median 8.3 pg/10^6 cells per ng/mL; IQR 6.4–10.7; n = 14), respectively (p = 0.26; Supplementary Table S2).

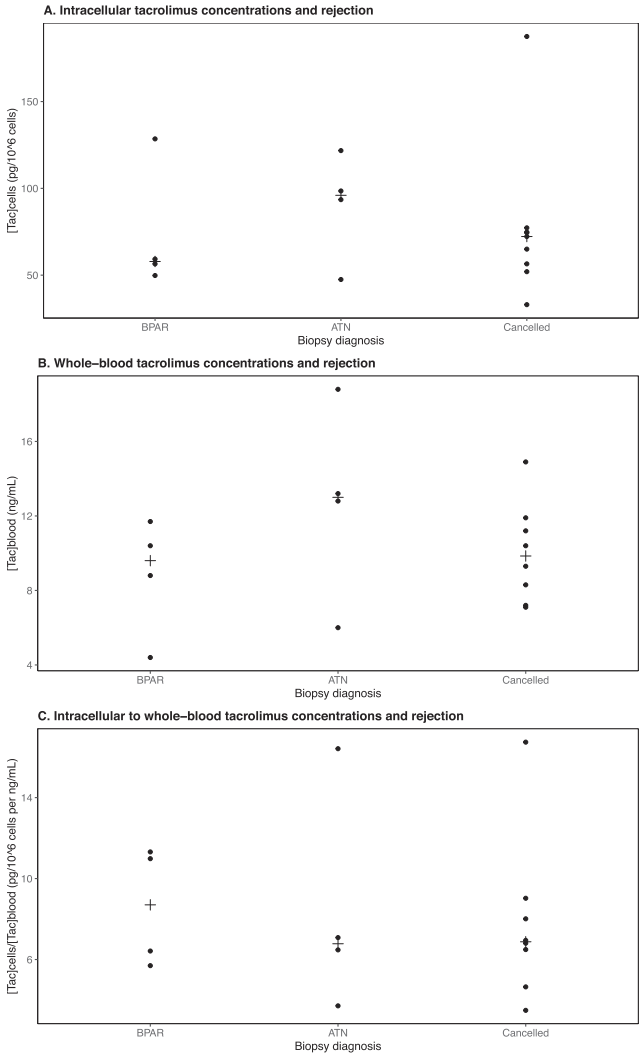

Fig. 2. $[Tac]_{cells}$ (A), $[Tac]_{blood}$ (B), and the $[Tac]_{cells}/[Tac]_{blood}$ ratio (C) for different (biopsy) diagnoses. The plus sign represents the median tacrolimus concentration. BPAR, biopsy-proven acute rejection; ATN, acute tubular necrosis; Cancelled, the biopsy was cancelled after a blood sample was drawn; $[Tac]_{blood}$, whole-blood tacrolimus pre-dose concentration; $[Tac]_{cells}$, tacrolimus pre-dose concentration within peripheral blood mononuclear cells.

4. Discussion

In this study, we demonstrate that $[Tac]_{cells}$ was not significantly different between patients with BPAR and patients with ATN or a cancelled biopsy nor between patients with BPAR and patients who were never scheduled to undergo a kidney transplant biopsy. The correlation between $[Tac]_{cells}$ and $[Tac]_{blood}$ was poor, and the dose-corrected tacrolimus exposure and distribution appeared to be stable during the first 10 days after transplantation.

The poor correlation at day 3 and the absence of a significant correlation at day 10 between $[Tac]_{cells}$ and $[Tac]_{blood}$ in the early phase after transplantation is in line with previous studies, which reported

poor to moderate correlations between $[Tac]_{cells}$ and $[Tac]_{blood}$ (r ranging between −0.32 and 0.59; r^2 ranging between 0.014 and 0.51) measured within the first 3 months after solid organ transplantation [12,21,15–17]. Moderate correlations between $[Tac]_{cells}$ and $[Tac]_{blood}$ were observed in a previous study in our center (r = 0.31 at month 3, r = 0.41 at month 6, and r = 0.61 at month 12), in patients 1 year after kidney transplantation (r = 0.53), and in kidney transplant recipients with a stable kidney function (r = 0.67) [7,12,21]. Although the correlation between $[Tac]_{cells}$ and $[Tac]_{blood}$ might improve with time after transplantation, the correlation is not strong enough to state that $[Tac]_{blood}$ reflects the tacrolimus concentration within PBMCs. This means that $[Tac]_{cells}$ and $[Tac]_{blood}$ cannot be used interchangeably.

The high inter-patient variability in the dose-corrected $[Tac]_{cells}$ and the $[Tac]_{cells}/[Tac]_{blood}$ ratio, can be explained by factors affecting the tacrolimus distribution. ABCB1 is an efflux transporter protein, which is present in mononuclear cell membranes. Inter-individual differences in ABCB1 activity may result in differences in the distribution of tacrolimus [31–33]. In a study of Capron *et al.* in 96 kidney transplant recipients, single-nucleotide polymorphisms (SNPs) in the *ABCB1* gene were associated with reduced ABCB1 activity and higher $[Tac]_{cells}$, while having no effect on $[Tac]_{blood}$ [31]. Other factors that have been associated with the $[Tac]_{cells}/[Tac]_{blood}$ ratio are age, albumin, hematocrit, sex, and total plasma protein concentration [7,21,31].

Within individual patients, the dose-corrected tacrolimus exposure and distribution appeared to be stable in the early phase after transplantation. Both dose-corrected $[Tac]_{cells}$ and the $[Tac]_{cells}/[Tac]_{blood}$ ratio were not significantly different between day 3 and day 10, and the inter-occasion variability was relatively low (19.8% and 23.4%) compared to previous studies (39–45% for the $[Tac]_{cells}/[Tac]_{blood}$ ratio [12,21]). This may be in part explained by the short time period during which the tacrolimus concentrations were measured in this study compared to the previous studies (1 week *versus* 1 year). An important observation is that the inter-patient variability of the dose-corrected $[Tac]_{cells}$ is higher than the inter-occasion variability. The inter-occasion variability consists of both the intra-patient variability and the variability in the analytical method of the tacrolimus concentration measurement (the PBMC isolation as well as the LC-MS/MS analysis). As the intra-patient variability is part of the inter-occasion variability, this means that the intra-patient variability is also lower than the inter-patient variability. This in turn, makes it possible to predict an individual's tacrolimus dose requirement to reach a certain (intra-PBMC) target exposure, based on previously measured tacrolimus concentrations and factors affecting the tacrolimus distribution. Moreover, as the inter-occasion variability in the $[Tac]_{cells}/[Tac]_{blood}$ ratio was low, we might be able to identify patients with a low $[Tac]_{cells}/[Tac]_{blood}$ ratio based on factors affecting the distribution of tacrolimus.

In the present study, and in contrast to previous investigations, we measured $[Tac]_{cells}$ at the time of a for-cause kidney transplant biopsy, rather than at a fixed point in time. With a fixed sampling time point, the tacrolimus dose, and thus the tacrolimus exposure, may have changed between the moment of blood sampling and the moment of diagnosis, whereas sampling at the time of a for-cause biopsy makes it possible to investigate the relationship between rejection and tacrolimus exposure at the time of diagnosis. Moreover, we included a RBC lysis step in the work-up of the blood sample. Despite these methodological and analytical improvements (compared to our previous study [21]), we did not find a significant difference in either $[Tac]_{blood}$, $[Tac]_{cells}$ or the $[Tac]_{cells}/[Tac]_{blood}$ ratio between patients with and without BPAR. There are multiple explanations for these findings, including low statistical power due to the low number of BPAR cases, and experimental variability in the measurement of intracellular tacrolimus concentrations. Another explanation for these results might be that PBMCs are not the ideal matrix to monitor tacrolimus exposure. Tacrolimus exerts its immunosuppressive effect via the inhibition of calcineurin within lymphocytes, and more specifically within T lymphocytes. Recent studies showed that tacrolimus concentrations vary between different subsets of cells [34–35]. As PBMCs consist of multiple cell subsets (T lymphocytes, B lymphocytes, NK cells, monocytes), the intra-PMBC tacrolimus concentration is determined by tacrolimus present in cells other than T lymphocytes. In fact, in a study in 20 kidney transplant recipients, no significant correlation (r = 0.218, p = 0.40) was observed between the tacrolimus concentration within PBMCs and $CD4^+$ T lymphocytes [34]. Moreover, the effect of tacrolimus on cell activation and cell function differs per cell-type [36–37]. Consequently, the total PBMC tacrolimus concentration may not be specific enough to monitor tacrolimus' immunosuppressive effect. Theoretically, the tacrolimus concentration within the T lymphocyte subset best reflects the concentration within the target cell and may therefore be the optimal matrix to monitor tacrolimus exposure. However, so far, this has not been demonstrated. Assuming that $[Tac]_{cells}$ correlates with clinical outcomes (which remains to be demonstrated), one might expect a higher $[Tac]_{cells}$ in patients with ATN, as this observation can indicate tacrolimus-related nephrotoxicity.

The main limitation of the present study is the small number of patients with BPAR and ATN. Therefore, this study might be underpowered to show a difference in tacrolimus concentrations between these groups. This means that if there would be a real difference in $[Tac]_{cells}$ between patients with and without BPAR, we might have been unable to detect this difference. Ideally, we would have included a control group, consisting of patients without clinical problems with blood samples drawn on the same day as those of the patients undergoing a kidney transplant biopsy. However, this requires very frequent blood sampling. To get the best estimation we did two analyses, one in which patients with a cancelled biopsy were chosen as controls and one in which patients who were not scheduled for a biopsy were used as controls. Ideally, we would have included a "negative" control group consisting of patients with a $[Tac]_{cells}$ measurement but without clinical problems and no abnormalities on a protocol biopsy. In our center, however, protocol biopsies early after transplantation are not standard of care. Finally, we were not able to collect blood samples from all patients at all time points, as a large number of patients was no longer hospitalized at the 10th day after transplantation, or postoperative days 3 and 10 were not on a weekday.

5. Conclusion

In conclusion, in this prospective clinical study, the correlation between $[Tac]_{cells}$ and $[Tac]_{blood}$ was poor, indicating that $[Tac]_{blood}$ does not reflect the concentration at the target site. The dose-corrected tacrolimus exposure and the $[Tac]_{cells}/[Tac]_{blood}$ ratio appeared to be stable in the early phase after kidney transplantation. This is the first study in which samples for tacrolimus concentration measurement were prospectively collected on the morning of a for-cause kidney transplant biopsy. This made it possible to investigate the relationship between tacrolimus exposure and rejection at the time of the diagnosis and before changes in the tacrolimus dose were made. Despite the methodological and analytical improvements of the present study, $[Tac]_{cells}$ was not significantly associated with the occurrence of rejection. Possible explanations for our findings might be that PBMCs are not a specific-enough matrix to monitor tacrolimus concentrations or the lack of statistical power. Future studies should investigate the relationship between intracellular tacrolimus concentrations in lymphocyte subsets (*i.e.* T lymphocytes) rather than PBMCs.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clinbiochem.2021.12.002.

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