

Declining Intracellular T-Lymphocyte Concentration of Cyclosporine A Precedes Acute Rejection in Kidney Transplant Recipients

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Background. We investigated cyclosporine A (CsA) concentrations at the site of action, inside T-lymphocytes, to evaluate its applicability as a new supplementary therapeutic drug monitoring method after renal transplantation.

Method. In this prospective single-center study, 20 kidney transplant recipients, mean age 54 (range 21–74) years, on CsA-based immunosuppression were included within 2 weeks posttransplant and followed for 3 months. Nine patients also had one full 12-hour pharmacokinetic profile performed. T-lymphocytes were isolated from 7 ml whole blood using Prepacyte and intracellular CsA concentrations were determined using a validated liquid chromatography double mass spectrometry method.

Results. Seven patients (35%) experienced acute rejections (all biopsy verified) during the first three months posttransplantation. Intracellular CsA concentrations tended to decline 1 week prior to acute rejection and the decrease was significant ($-27.1 \pm 14.6\%$, $P=0.014$) three days before the rejection episodes were recognized clinically. In addition, the intracellular CsA area under the curve 0–12 measured during stable phase was 182% higher in the rejection-free patients ($P=0.004$). There was no difference between patients experiencing rejection and the rejection-free patients with respect to CsA C2-levels, dose (mg/kg), human leukocyte antigen mismatch, donor age, recipient age, or *ABCB1* genotyping.

Conclusion. Intracellular CsA T-lymphocyte concentrations declined significantly 3 days prior to a rejection episode and there was a general lower intracellular exposure of CsA in recipients experiencing rejection. Intracellular measurement of CsA therefore seems to have a potential to further improve individualization of therapeutic drug monitoring. Larger studies are needed to elucidate the role for intracellular T-lymphocyte measurements in ordinary clinical care, for both CsA and other immunosuppressive drugs.

Keywords: Cyclosporine A, Renal transplantation, Acute rejection, Intracellular concentration, T-lymphocytes.

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Treatment with cyclosporine A (CsA) remains an important part of immunosuppressive therapy after organ transplantation. Routine therapeutic drug monitoring (TDM) of CsA is necessary because the drug exhibits great inter- and inpatient variations and has a narrow therapeutic index. Both reversible acute toxicity and irreversible tubulointerstitial fibrosis have been associated with short- and long-term overexposure of CsA (1). Underexposure of CsA, on the other hand, may lead to acute and/or chronic rejection (2). Different strategies of TDM have been in focus during the

last decade. Standard techniques have shifted from trough concentration (C0) measurements to C2-monitoring where concentrations are measured two hours after dosage. The C2-monitoring correlates more closely to the systemic exposure of CsA, which so far appears to be the most relevant pharmacokinetic parameter for the immunosuppressive effect (3–6), however the clinical benefit from C2- over C0-monitoring has still not been fully elucidated (7). Despite the improvement in the monitoring techniques, renal transplant patients still experience a rejection rate of 10–25% during the first postoperative year (8).

Routine CsA monitoring is performed by measuring whole blood concentrations. The immunosuppressive site of action for CsA, however, is inhibition of the phosphatase calcineurin intracellularly within T-lymphocytes (9). Many factors, other than CsA dose or total whole blood concentration, may have impact on the actual intracellular concentration of CsA. High hematocrit has been shown to lower the concentration of CsA in lymphocytes (10, 11). Changes in free-fraction of CsA in plasma may affect the distribution into the T-lymphocytes; because CsA is bound to plasma lipoproteins, changes in these proteins may also have an effect (12, 13). The efflux-transporter P-glycoprotein (P-gp), encoded by the *ABCB1* gene, for which CsA is both a substrate and an inhibitor, is expressed in T-lymphocytes (14–16). In lymphocytes isolated from renal transplant recipients with acute rejection an up-regulation of P-gp (leading to lower intracellular CsA) has been shown (17). Measurement of intracellular CsA concentration in T-lymphocytes could therefore in theory pro-

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Trial registry: www.clinicaltrials.gov (NCT00139009).

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vide more relevant information on the immunosuppressive efficacy of CsA compared to whole blood concentrations.

A selective method has recently been developed to measure intracellular CsA concentration in T-lymphocytes (18). The assay has been suggested as a supplementary method for TDM of CsA after renal transplantation. To evaluate the clinical potential of this assay, the association between intracellular CsA concentrations and early acute rejections was investigated in a prospective pilot study of renal transplant patients.

MATERIALS AND METHODS

Study Design

A single-center prospective pilot study included 20 kidney transplant recipients, mean age 54 (range 21–74) years, on CsA-based immunosuppression, during the first or second posttransplant week (Table 1). The CsA treatment was initiated with 10 mg/kg orally on the day of transplantation followed by C2 monitoring with target concentrations as shown in Table 2. All enrolled patients had received a single

renal transplant at a mean of 5.2 (range 2–12) days prior to inclusion.

Sixteen patients received mycophenolate mofetil, 1.0 g BID from the day of transplantation. Mycophenolic acid concentration measurements were not scheduled but allowed. All patients received steroid in accordance to the following protocol; intravenous methylprednisolone at the day of transplantation and the first posttransplant day, followed by oral prednisolone from the second postoperative day, tapered from 80 to 20 mg/day during the first week, further tapered to 15 mg/day after 1 month, and 10 mg/day after 2 months, with an aim of further tapering down to 5 mg/day within the following months. Basiliximab was used as induction therapy in three patients (first dose of 20 mg intravenous within 2 hr pretransplant and 20 mg intravenous for 4 days posttransplantation).

Study specific blood samples (see below) were taken in association with standard routine C2 monitoring for clinical follow-up, typically three times a week the first 2 weeks, twice weekly for the next 4 weeks, and then once to twice a week until the 12th posttransplant week. Suspicion of an acute rejec-

TABLE 1. Demographic data at time of inclusion

	All	No-rejection group	Rejection group	P value
Gender (male/female)	14/6	9/4	5/2	
Weight (kg)	81.3±12.1	85.6±11.6	73.3±9.2	0.026
Age (years)	53.6±13.2	48.9±15.0	56.2±12.0	0.212
CsA dose (mg/day)	522±126	537±121	493±139	0.342
CsA dose (mg/kg/day)	6.4±1.1	6.3±1.0	6.7±1.3	0.452
HLA mismatch (A+B)	2.4±1.1	2.3±1.4	2.4±1.0	0.856
HLA mismatch (DR)	0.40±0.5	0.57±0.5	0.31±0.5	0.275
Plasma creatinine	248±187	215±150	308±243	0.303
Creatinine clearance	49.9±28.5	55.4±30.2	39.7±23.7	0.252
Hemoglobin (g/dL)	12.8±1.5	12.6±1.2	13.2±2.0	0.367
Leukocytes (10 ⁹ /L)	7.58±2.7	7.55±2.6	7.61±2.6	0.964
Serum urea	21.7±9.1	19.4±7.1	25.9±11.5	0.135
Albumin	38.0±8.4	37.8±6.7	38.4±11.5	0.872
Deceased donor (n)	19/20	13/13	6/7	
Treated with MMF (n)	16/20	10/13	6/7	
Treated with azathioprine (n)	1/20	1/13	0/7	
Treated with steroids (n)	20/20	13/13	7/7	
Induction therapy (basiliximab)	3/20	2/13	1/7	

Data are means±SD.

TABLE 2. Whole blood CsA C2 concentrations monthly after transplantation

	Rejection group	No-rejection group	Target level	P value
Whole blood (ng/mL)				
1 month	1606±359	1482±370	1500–2000	0.480
2 months	1414±197	1404±297	1400–1600	0.937
3 months	1201±246	1192±193	1000–1200	0.929
Intracellular (ng/10 ⁶ T-cells)				
1 month	102±88	149±83		0.102
2 months	107±60	121±158		0.634
3 months	148±200	125±37		0.380

Data are means±SD.

tion was set to a 20% increase in serum creatinine (excluding other explanatory causes: absence of urinary tract obstruction, nephrotoxic medication including inappropriate elevation of whole blood CsA levels, dehydration, or infection). All suspected acute rejections were verified with a biopsy and classified according to the Banff 97 criteria (19).

Additionally, in a subset of nine random patients, a full 12-hour pharmacokinetic profile was performed once during a phase of stable renal function. After the null sample, the patients received their individual morning dose of CsA and postdose samples were collected at: 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, and 12 hr after CsA administration. A standard hospital breakfast was given 2 hr after CsA administration. At this time, other prescribed drugs were also administered. At each sampling point, the CsA concentration was determined both in whole blood and intracellularly in T-lymphocytes.

The study was performed in accordance with the Declaration of Helsinki and all patients signed a written informed consent before study start. The study was evaluated by the Regional Committee for Medical Research Ethics, approved by the Norwegian Medicines Agency and registered on www.clinicaltrials.gov (NCT00139009).

T-Lymphocyte Isolation and CsA Determination

T-lymphocytes were isolated from 7 ml whole blood using Prepacite (BioE, St. Paul, MN) (20). To preserve the intracellular concentration during isolation, 100 $\mu\text{mol/L}$ verapamil was preadded to the heparin vacutainers to block P-gp activity (21) and the cells were isolated within 4 hr postsampling. CsA concentrations were measured in freshly isolated T-lymphocytes using a validated liquid chromatography (LC) double mass spectrometry (MS/MS) method (18). In brief, after protein precipitation with methanol and centrifugation, the supernatants were subjected to solid phase extraction using Oasis hydrophilic-lipophilic balance cartridges. CsA was all separated chromatographically before MS/MS detection. The intracellular levels of CsA were related to the number of T-lymphocytes ($\text{ng}/10^6$ cells). The lower limit of quantification for the intracellular CsA assay was 0.25 $\text{ng}/10^6$ cells. Whole blood CsA concentrations were analyzed using the CEDIA Cyclosporine PLUS assay (Cloned Enzyme Donor Immunoassay; Microgenics Corporation, Fremont, CA). The free fraction of CsA in plasma was estimated in every sample during the 12-hour pharmacokinetic investigation using equilibrium dialysis as previously described (12) and expressed as percentage of total plasma concentration. The clinical status of the patient (rejection/no rejection) was blinded until all analyses were completed.

Genotyping and ABCB1 Expression

Whole-blood (EDTA) was drawn once during the study for determination of the recipients *ABCB1* (G1199A, C1236T, G2677T, G2677A, G2677G, and C3435T) genotypes. Previously reported method and nucleotide sequences of primers were used to determine mutations by polymerase chain reaction (PCR)-restriction fragment length polymorphism assay (22). Restriction enzyme digestion generated DNA fragments that were separated by electrophoreses on 3% agarose gel. Positive controls were kindly supplied by Dr D. Katz, Abbott Laboratories, Abbott Park, IL (*MDR1*).

At inclusion, 2.5 ml whole blood was drawn in a PAXgene Blood RNA tube (Qiagen, Westburg, The Netherlands) for *ABCB1* mRNA quantification by reverse transcription PCR. Briefly, total RNA was isolated on the MagNA Pure instrument (Roche, Mannheim, Germany) and reverse transcribed using random primers (Roche). Target and housekeeping gene sequences were amplified in separate reactions on the LightCycler instrument (Roche), and specific hybridization probes were used for real-time product detection. The expression of *ABCB1* mRNA was calculated relative to a housekeeping gene index comprising the expression of 5-aminolevulinate synthase 1, β 2-microglobulin, and ribosomal protein L13.

Pharmacokinetic Calculations

The peak concentration (C_{max}), C_0 and C_2 , as well as the time to reach C_{max} (T_{max}), are given as observed values. The area under the whole blood concentration versus time curve from 0 to 12 hours postdose (AUC_{0-12}) was calculated in accordance with the trapezoidal rule on natural log transformed concentrations. Clearance was calculated using the noncompartment formula (clearance/bioavailability $[\text{CL}/F] = \text{Dose}/\text{AUC}_{0-12}$). The renal function was estimated using Nankivell-formula B (23).

Statistics

Parameters in patients experiencing acute rejection and patients not experiencing acute rejection were evaluated using unpaired Student's *t* test on natural log transformed data, while effects within the rejection group were evaluated with paired Student's *t* test on natural transformed data. Wilcoxon signed rank test was used on untransformed data to evaluate the effect on T_{max} . The Mann-Whitney *U* test was used to investigate differences in *ABCB1* expression between the population with and without acute rejection episodes. Statistical significant difference was considered for *P* values <0.05 . All statistical analyses were performed using SPSS version 13.0.

RESULTS

Patient Demographics

Demographic data are shown in Table 1. Seven patients (35%) experienced nine biopsy-proven acute rejection episodes during the study at an average of 44 ± 32 days posttransplantation (range 8–90 days). No significant differences were observed between the rejection and no-rejection groups with respect to CsA dose (mg/kg), human leukocyte antigen mismatch, donor age, recipient age, or *ABCB1* expression or genotype. The average whole blood CsA C_2 -levels during the three study months are shown in Table 2. No differences between the rejection and no-rejection groups were detected. At the rejection episodes, the rejection group had CsA C_2 -levels of 1338 ± 184 ng/ml and the no-rejection patients had 1258 ± 190 ng/ml ($P=0.40$) at matched time points. During the study period (the first three posttransplant months), an average of 21 (range 6–32) samples per patient were analyzed for both intracellular and whole blood concentrations of CsA.

Intracellular T-Lymphocyte CsA Concentration

In the rejection group, the intracellular CsA concentrations tended to decline up to 1 week prior to an acute rejection episode, seen as increasing whole blood/intracellular

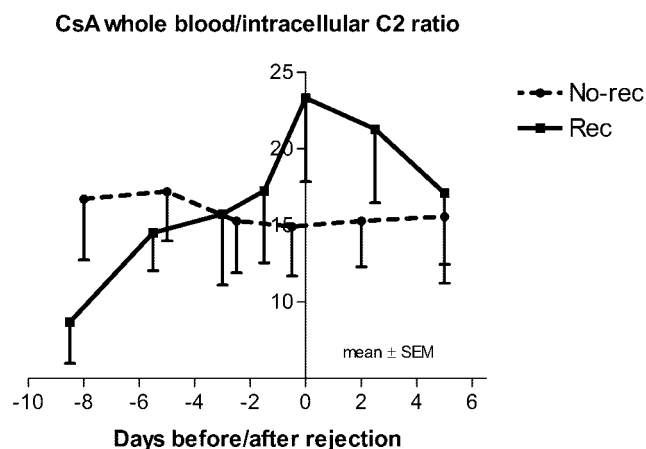


FIGURE 1. Average CsA whole blood/intracellular C2 ratio \pm SEM of the seven patients with rejection (—) and patients with no rejection (---). The x-axis shows time from rejection was detected (day 0), and for the control group day 0 is at the same posttransplant day as the mean rejection day for all rejections (posttransplant day 44). High levels of the ratio represent a drop in intracellular CsA concentration compared to whole blood concentration.

level ratio (Fig. 1). The increased ratio was mainly a result of decreased intracellular concentration, but also a slight increase in whole blood concentration. The intracellular levels of CsA were significantly lower ($-27.1 \pm 14.6\%$, $P=0.014$) 3 days before the rejection episodes could be detected clinically by an increase in p-creatinine.

Four of the nine patients, in whom a full 12-hour CsA pharmacokinetic profile was performed, experienced a rejection episode during the study period. Comparing patients in the rejection group ($n=4$) with the no-rejection group ($n=5$), there was a significant difference with regard to the intracellular CsA concentrations (Fig. 2, Table 3). The intracellular AUC_{0-12} was 182% higher in the no-rejection group (265 ± 18.2 vs. 747 ± 221 ng/ 10^6 lymphocytes \times h, $P=0.004$), and CL/F was 137% higher in the rejection group (716 ± 134 vs. $302 \pm 185 \times 10^6$ lymphocytes/h, $P=0.007$). Intracellular C_{max} , C_0 , and C_2 also tended to be lower in the rejection group. No differences were observed between the two groups with respect to CsA whole blood pharmacokinetic variables during this dosing interval (Table 3). However, the rejection group also tended to have lower plasma free fraction of CsA (3.1% vs. 3.8%, $P=0.21$) during the 12-hour pharmacokinetic investigation. No correlation was found between free fraction and intercellular concentrations (Fig. 3).

Genotyping and ABCB1 mRNA Expression

Six of the seven patients in the rejection group were possible *ABCB1* TTT-haplotypes; however, no difference in pharmacokinetic parameters was observed compared to the other patients (data not shown). The expression of mRNA *ABCB1* in whole blood at the time of inclusion was not different between the rejection and no-rejection groups (0.75 ± 0.22 vs. 1.09 ± 0.26 , $P=0.144$).

DISCUSSION

The present study demonstrates a novel TDM-method of measuring CsA concentration within T-lymphocytes with

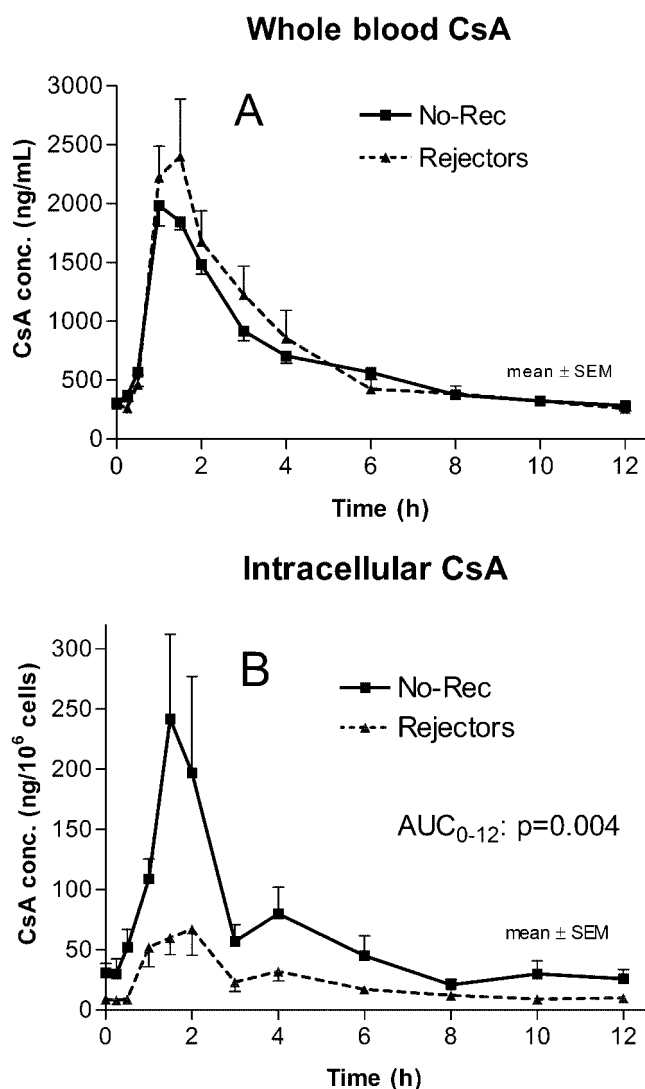


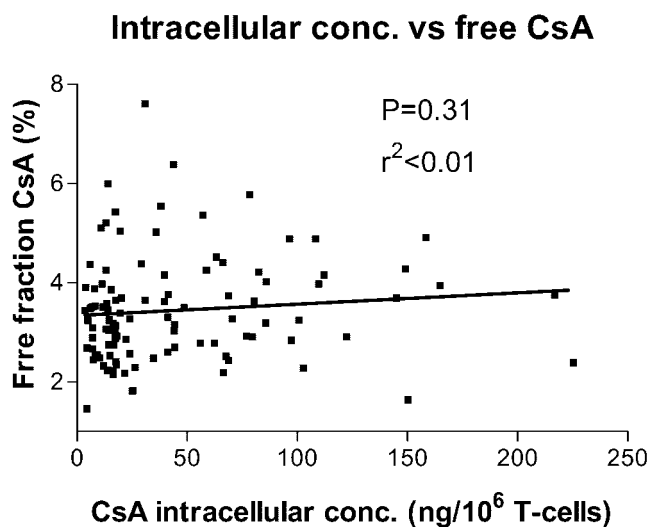
FIGURE 2. The 12-hour pharmacokinetic profile of the four patients who experienced rejection (Rec) and five no-rejection (No-Rec) patients. (A) CsA whole blood concentration. (B) Intracellular T-lymphocytes concentrations of CsA. Data are means \pm SEM.

a potential to detect acute rejection episodes several days earlier than possible with the standard clinical methods used today. In addition the results indicate that patients experiencing an acute rejection during the first three months posttransplantation have a significantly lower intracellular CsA AUC_{0-12} than patients not experiencing rejections at time-points distinctly separate from the time of rejection. This variable may therefore be a new diagnostic marker for patients at high risk of rejection.

The intracellular concentrations of CsA in T-lymphocytes appear to provide information about processes important to rejection that whole blood concentrations data do not provide. CsA whole blood concentrations actually tended to be slightly higher in the rejection patients during the study and did not correlate with intracellular concentrations. Higher whole blood free fraction of CsA would in theory give higher cell penetration and more drug delivery inside T-lymphocytes. The

TABLE 3. Pharmacokinetic parameters from the 12-h pharmacokinetic investigation

	Rejection group	No-rejection group	P value
Whole blood			
Plasma creatinine ($\mu\text{mol/L}$)	146.5 \pm 30.3	120.2 \pm 33.3	0.286
Days after transplant	42.5 \pm 19.2	36.2 \pm 18.2	0.556
CsA dose (mg)	188 \pm 25.0	205 \pm 99.1	0.744
Days from rejection treatment	-27, -6, +9, +24		
AUC ₀₋₁₂ (ng/mL \times h)	8388 \pm 2668	8031 \pm 1058	0.790
AUC ₀₋₁₂ /dose (ng/mL/mg \times h)	46.8 \pm 21.9	45.6 \pm 17.7	0.930
C _{max} (ng/mL)	2577 \pm 880	2123 \pm 265	0.304
C0 (ng/mL)	294 \pm 62	349 \pm 105	0.391
C2 (ng/mL)	1678 \pm 520	1483 \pm 182	0.454
T _{max} (h)	1.26 \pm 0.33	1.37 \pm 0.32	0.625
CL/F (mL/min)	369 \pm 172	411 \pm 151	0.703
Free fraction CsA (%)	3.09 \pm 0.33	3.77 \pm 0.94	0.211
Intralymphocyte			
AUC ₀₋₁₂ (ng/mL \times h)	265 \pm 18.2	747 \pm 221	0.004
AUC ₀₋₁₂ /dose (ng/mL/mg \times h)	1.44 \pm 0.33	4.51 \pm 2.59	0.053
C _{max} (ng/mL)	86.1 \pm 27.5	256 \pm 149	0.061
C0 (ng/mL)	8.6 \pm 4.0	26.5 \pm 17.3	0.087
C2 (ng/mL)	67.2 \pm 42.6	197 \pm 179	0.204
T _{max} (h)	2.1 \pm 1.3	2.15 \pm 1.07	0.989
CL/F (10 ⁶ cells/h)	716 \pm 134	302 \pm 185	0.007

Data are means \pm SD.**FIGURE 3.** Correlation between intracellular CsA concentration and free fraction of CsA in plasma. The figure shows all samples (n=110) obtained during the full 12-hour pharmacokinetic investigation. The result of a linear regression analysis is displayed.

free fraction of CsA during the 12-hour pharmacokinetic interval tended to be higher (not significant) in the no-rejection group compared to the rejection group, and may only partly explain the lower intracellular CsA concentrations found in the rejection group. P-gp (which is coded by the *ABCB1*-gene) is a transmembrane pump that transports CsA out of the cell. Genotype and the expression analyses of *ABCB1* were performed in recipient whole blood. The TTT haplotype has previously been suggested to be a less functional genotype and would therefore be associated with a higher immunosuppressive efficacy. However, no differences in the possibly less functional genotype of P-gp between the groups were observed, nor were any significant differences in whole blood *ABCB1* mRNA expression at baseline. Other potentially relevant biochemical parameters (Hb, triglyceride, or cholesterol) were not significantly different between the groups (data not shown).

The intracellular CsA concentration showed a general high inter- and inpatient variability. This is at least partly explained by the complex isolation procedure and the low level of automatization of the method. However, it can not be ruled out that intracellular concentrations also have a true high biological variation. Despite the high variability, the results showed significantly decreased intracellular concentrations in rejection patients and large differences in intracellular CsA AUC₀₋₁₂ between the rejection and the no-rejection groups.

Barbari et al. have earlier shown that acute rejection after renal transplantation is associated with a relative low CsA lymphocyte maximum level and high total lymphocyte count (11, 24, 25). Their findings are based on analysis of monthly CsA sampling with a less specific T-lymphocyte isolation and CsA analysis method than used in the present study (18, 26, 27). The present study is the first to show a decline in intracellular T-lymphocyte CsA days before a rejection could be clinically verified and a difference in intracellular CsA AUC levels between rejection and no-rejection groups, which may be used as a risk assessment tool in future individualization protocols. A limitation of the presented study is that whole blood analysis of routine C2-monitoring and whole blood measurements during the pharmacokinetic profile used different assays. However, the results from routine and pharmacokinetic whole blood concentrations were evaluated separately and never compared with each other, so this should not affect the conclusion drawn.

Studies have indicated that total P-gp expression in T-lymphocytes increases during an acute rejection (28). However, the role of P-gp with respect to the immunosuppressive effect of calcineurin inhibitors is not clear (17, 28-30). In the present study, the expression of *ABCB1* in whole blood at the initiation of CsA therapy was not significantly different between the group with and without rejection episodes. Determination of *ABCB1* expression in T-lymphocytes during CsA treatment may provide better understanding of the mechanisms for the reduced intracellular concentration in association to rejections.

Free fraction of CsA should theoretically be an important parameter for CsA efficacy. Our data, however, do not support this hypothesis. The rejection group tended to have lower plasma free fraction of CsA (3.1% vs. 3.8%, $P=0.21$) during the 12-hour pharmacokinetic investigation. This 22%

difference in free fraction between the groups could only partly explain the 182% difference in intracellular CsA since no correlation was found ($r^2 < 0.01$) between free fraction and intracellular concentration CsA concentration when performing a linear regression analysis (Fig. 3). We currently have no good explanation for this finding; however, our present hypothesis is that up-regulation of the gene expression of the P-gp pump (*ABCB1*) might affect the intracellular concentration.

If this novel TDM method proves clinically applicable for early detection of rejections, it may have major implication on clinical care of transplanted patients. The possibility of actually being able to predict renal allograft rejection will most likely make it possible to better treat and even avoid these episodes. However, before the results of this study can be confirmed as a functional TDM tool in a larger study, the time from sampling to result need to be reduced by introduction of a more automatic cell separation technique.

Another promising application might be to evaluate the individual risk of rejection based on AUC_{0-12} , or maybe even CsA whole blood/intracellular ratio early after transplantation. A low intracellular AUC_{0-12} CsA concentration or high whole blood/intracellular ratio seem to indicate a high-risk patient, and more intense immunotherapy should be applied for these patients. Contrary, patients with a high intracellular AUC_{0-12} or high whole blood/intracellular ratio may benefit from a lowering CsA dose. This would give the clinicians a new tool to individualize and improve immunosuppressive therapy. Similar methods may perhaps be applied for other immunosuppressive drugs that target intracellular receptors.

This pilot study shows that intracellular T-lymphocyte monitoring has a potential of predicting rejections several days before they are recognized clinically. Further optimization and evaluation of the method, however, is needed to elucidate the future role for intracellular T-lymphocyte measurements for both CsA and other immunosuppressive drugs in ordinary clinical care.

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