In Vivo CYP3A4 Activity, *CYP3A5* Genotype, and Hematocrit Predict Tacrolimus Dose Requirements and Clearance in Renal Transplant Patients

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Tacrolimus is metabolized by CYP3A4 and CYP3A5 and is characterized by a narrow therapeutic index and highly variable pharmacokinetics. This cross-sectional study in 59 renal transplant patients investigated the relationship among *in vivo* CYP3A4 activity (assessed using midazolam as a drug probe), *CYP3A5* genotype on the one hand, and tacrolimus pharmacokinetics on the other hand, taking into account other potential determinants of tacrolimus disposition. *In vivo* CYP3A4 activity and *CYP3A5* genotype explain 56–59% of variability in tacrolimus dose requirements and clearance, contributing ~25 and 30%, respectively. Hematocrit explains an additional 4–14%. These data indicate that CYP3A4- and CYP3A5-mediated tacrolimus metabolisms are major determinants of tacrolimus disposition *in vivo* and explain a substantial part of the clinically observed high interindividual variability in tacrolimus pharmacokinetics. Furthermore, these data provide a potential basis for a comprehensive approach to predicting tacrolimus dose requirement in individual patients and hence provide a strategy to tailor immunosuppressive therapy in transplant recipients.

Tacrolimus is characterized by highly variable pharmacokinetics and a narrow therapeutic index and displays a wide range of potentially severe drug-related toxicities.[1,2] Despite these unfavorable characteristics, it has become one of the cornerstone immunosuppressants in solid-organ transplantation.[3,4] Given the relationship between tacrolimus exposure on the one hand and toxicity and efficacy (i.e., prevention of rejection) on the other hand, therapeutic drug monitoring and concentration-controlled dosing have been routinely applied since its introduction in clinical practice in the early 1990s.[2,5]

In vitro studies have demonstrated that tacrolimus is metabolized by both CYP3A4 and CYP3A5[6,7] and that it is a substrate of the drug transporter p-glycoprotein (also known as ATP-binding cassette subfamily B member 1 (ABCB1)).[8] Because CYP3A4 and CYP3A5 as well as ABCB1 are expressed in the gastrointestinal tract and in the liver, and because of the important transporter–enzyme interplay occurring at both sites of drug metabolism, CYP3A iso-enzymes and ABCB1 are expected to be major determinants of tacrolimus disposition *in vivo*.[9–12]

Over the years, many factors affecting tacrolimus pharmacokinetics have been identified. These include both clinical (e.g., type of transplanted organ, time after transplantation, ethnicity, age, sex, concomitant medication and food, diarrhea, hepatic and renal dysfunction, hematocrit, and albumin) and genetic factors (e.g., *CYP3A4*, *CYP3A5*, and *ABCB1* single-nucleotide polymorphisms (SNPs).[13–15] Concerning the latter factors, it is well established that *CYP3A5* genotype has a marked impact on tacrolimus pharmacokinetics, whereas the impact of other genetic polymorphisms, including *CYP3A4* and *ABCB1* SNPs, is rather limited.[16,17] Carriers of a *CYP3A5*1*-allele (i.e., patients expressing CYP3A5) have a substantially higher tacrolimus clearance resulting in markedly higher tacrolimus dose requirements, as compared with *CYP3A5*3/*3* homozygous patients (i.e., patients not expressing CYP3A5).[18–22] On the basis of these observations, it has been hypothesized that individualizing the initial tacrolimus dosing based on the *CYP3A5* genotype (i.e., 0.30 mg/kg/day for *CYP3A5*1*-allele carriers and 0.15 mg/kg/day for *CYP3A5*3/*3* homozygous patients, instead of the standard 0.20 mg/kg/day for all patients), might help to avoid tacrolimus under- and overexposure early after transplantation. As

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the former is associated with an increased risk for acute rejection[23] and the latter with an increased risk for drug-related toxicities such as acute nephrotoxicity, neurotoxicity, and new-onset diabetes after transplantation,[24] this approach might improve clinical outcome. A recent prospective randomized study testing this hypothesis indeed demonstrated that pharmacogenetic adaptation of the initial tacrolimus loading dose according to the patient's *CYP3A5* genotype resulted in a faster achievement of target tacrolimus trough (C_0) levels and more patients within the desired tacrolimus target range early after transplantation.[25] However, despite pharmacogenetic dose adaptation, a substantial proportion of patients still did not have tacrolimus C_0 levels within the target range in the first days after tacrolimus introduction and this is probably one reason that the study failed to demonstrate improvement in drug-related clinical end points. It is indeed essential to realize that focusing solely on the *CYP3A5* genotype has some obvious limitations. First, the *CYP3A5* genotype cannot explain the important residual interindividual differences in tacrolimus pharmacokinetics observed within CYP3A5 expressers and CYP3A5 nonexpressers, the latter group accounting for 80–85% of patients in a Caucasian population. Second, it cannot explain intraindividual variability in tacrolimus pharmacokinetics, nor can it explain changes in tacrolimus pharmacokinetics over time in individual patients.[21,26]

Because we hypothesized that interindividual differences in tacrolimus pharmacokinetics might also be partially attributed to interindividual differences in *in vivo* CYP3A4 activity, we performed a cross-sectional study in renal transplant recipients in which we investigated the relationship between *in vivo* CYP3A4-activity (assessed using midazolam (MDZ) as a drug probe)[27–30] and the *CYP3A5* genotype on the one hand and tacrolimus pharmacokinetics (i.e., dose requirements, dose-corrected C_0 levels, area under the concentration-time curve $(AUC)_{0-12}$, and clearance) on the other hand, taking into account known clinical determinants of tacrolimus disposition.

RESULTS

Baseline characteristics

Table 1 shows the baseline characteristics for the entire study cohort ($n = 59$), as well as in the subgroups of CYP3A5 expressers (*CYP3A5*1*-allele carriers, $n = 10$) and CYP3A5 nonexpressers (*CYP3A5*3/*3* homozygous patients, $n = 49$). All patients included in this study were of Caucasian origin and were tested 3 months after transplantation. CYP3A5 expressers tended to be younger than CYP3A5 nonexpressers, but this trend did not reach statistical significance. Other clinical and biochemical baseline characteristics did not differ between subgroups.

Genotype

All SNPs were in Hardy–Weinberg equilibrium. There was significant linkage disequilibrium between *CYP3A5*1/*3* and *CYP3A4*1/*1b* ($r^2 = 0.47$, $D' = 1.00$) but not between *CYP3A4*1/*1b* or *CYP3A5*1/*3* and *ABCB1* SNPs ($r^2 < 0.10$, $D' < 0.30$). Consequently, there were significantly more *CYP3A4*1b* allele carriers in the CYP3A5 expressers as compared with the CYP3A5 nonexpressers (Table 1). In fact, 50.0% of *CYP3A5*1*-allele carriers also carried a *CYP3A4*1b* allele, whereas none of the *CYP3A5*3/*3* homozygous patients did ($P < 0.0001$). Concerning *ABCB1*, there was significant linkage disequilibrium

Table 1 Patient demographics

	All ($n = 59$)	*CYP3A5*1/*3* ($n = 10$)	*CYP3A5*3/*3* ($n = 49$)	P (**1/*3* vs. *3/*3*)
Age (years)	54.2 ± 10.9	49.9 ± 14.5	55.0 ± 10.0	0.18
Sex: female/male (%)	35.6/64.4	20.0/80.0	38.8/61.2	NS
Ethnicity: Caucasian/other (%)	100/0	100/0	100/0	NS
Weight (kg)	71.4 ± 13.7	71.7 ± 11.2	71.4 ± 14.3	NS
Length (m)	1.71 ± 0.14	1.73 ± 0.10	1.71 ± 0.09	NS
BMI (kg/m^2)	24.3 ± 4.0	24.1 ± 3.7	24.3 ± 4.1	NS
Time after transplantation (months)	3.1 ± 0.2	3.1 ± 0.2	3.1 ± 0.2	NS
Diabetes: yes/no (%)	11.9/88.1	0/100	14.3/85.7	NS
Hemoglobin (g/dl)	11.2 ± 1.7	11.1 ± 1.9	11.2 ± 1.6	NS
Hematocrit	0.35 ± 0.05	0.35 ± 0.06	0.35 ± 0.05	NS
Creatinine (mg/dl)	1.51 ± 0.43	1.57 ± 0.36	1.49 ± 0.45	NS
eGFR (ml/min/1.73 m^2)	48.4 ± 16.8	48.2 ± 15.4	48.5 ± 17.2	NS
Albumin (g/l)	44.0 ± 2.8	43.3 ± 3.4	44.2 ± 2.7	NS
*CYP3A4*1/*1B GG/GC/CC* (%)	91.5/8.5/0	50.0/50.0/0	100/0/0	<0.0001
ABCB1 -129TT/CT/CC (%)	84.5/13.8/1.7	70.0/20.0/10.0	87.5/12.5/0	0.06
ABCB1 1236 CC/TC/TT (%)	46.6/39.7/13.8	30.0/60.0/10.0	50.0/35.4/14.6	NS
*ABCB1 2677 GG/*G/*** (%)	44.6/39.3/16.1	33.3/55.6/11.1	46.8/36.2/17.0	NS
ABCB1 3435 CC/TC/TT (%)	28.8/54.2/16.9	30.0/60.0/10.0	28.6/53.1/18.4	NS

* = *T* or *A*; NS = $P > 0.2$.

BMI, body mass index; eGFR, estimated glomerular filtration rate.

between *ABCB1 1236* and *ABCB1 2677* ($r^2 = 0.81$, $D' = 0.92$), *ABCB1 1236* and *ABCB1 3435* ($r^2 = 0.33$, $D' = 0.73$), and *ABCB1 2677* and *ABCB1 3435* SNP ($r^2 = 0.52$, $D' = 0.85$).

Concomitant medication

Supplementary Table S1, online provides a detailed overview of concomitant immunosuppressive and nonimmunosuppressive medication in the different groups. All patients were treated with methylprednisolone and mycophenolic acid, and the methylprednisolone dose was similar in all groups (i.e., 4.1 ± 0.5 mg/day). The use of concomitant nonimmunosuppressive drugs was also equally distributed. As specified in the study protocol, no patient was treated with either an inducer and/or a strong or moderate inhibitor of CYP3A. Of note, several patients were treated with drugs for which there is some evidence that they might be weak CYP3A inhibitors. However, in this respect, there were no major differences between the groups.

MDZ and tacrolimus pharmacokinetics in CYP3A5 expressers and nonexpressers

Table 2 shows pharmacokinetic parameters for both MDZ and tacrolimus in the entire study cohort and in the subgroups of CYP3A5 expressers and nonexpressers. MDZ pharmacokinetic parameters, including systemic and apparent oral clearance and oral bioavailability, did not differ between groups (Table 2). For tacrolimus, however, there were marked differences between CYP3A5 expressers and nonexpressers. In CYP3A5 expressers, (weight-corrected) tacrolimus daily dose requirements and (weight-corrected) steady-state clearance were ~1.8-fold higher and dose-corrected tacrolimus C_0 level

Table 2 Midazolam and tacrolimus pharmacokinetic parameters

	All($n = 59$)	*CYP3A5*1/*3*($n = 10$)	*CYP3A5*3/*3*($n = 49$)	*P*(**1/*3* vs. **3/*3*)
MDZ PK after i.v. administration				
MDZ i.v. AUC_{0-480} (ng × min/ml)	2,365 ± 833	2,420 ± 857	2,354 ± 837	0.82
MDZ i.v. AUC_{0-480}/dose (ng × min/ml/mg)	2,365 ± 833	2,420 ± 857	2,354 ± 837	0.82
MDZ i.v. $AUC_{0-\infty}$ (ng × min/ml)	3,019 ± 1,434	3,273 ± 1,957	2,965 ± 1,318	0.42
MDZ i.v. $AUC_{0-\infty}$/dose (ng × min/ml/mg)	3,019 ± 1,434	3,273 ± 1,957	2,965 ± 1,318	0.42
MDZ i.v. CL (ml/min)	388 ± 142	389 ± 190	388 ± 132	0.97
MDZ i.v. CL/weight (ml/min/kg)	5.51 ± 2.05	5.56 ± 2.79	5.50 ± 1.90	0.93
MDZ PK after p.o. administration				
MDZ p.o. T_{max} (min)	33.5 ± 17.0	30.0 ± 17.3	34.3 ± 17.0	0.47
MDZ p.o. C_{max} (ng/ml)	21.3 ± 7.8	23.6 ± 8.3	20.8 ± 7.7	0.31
MDZ p.o. AUC_{0-480} (ng × min/mL)	2,725 ± 1,183	2,900 ± 1,552	2,689 ± 1,117	0.61
MDZ p.o. AUC_{0-480} /dose (ng × min/ml/mg)	1,363 ± 591	1,450 ± 761	1,334 ± 559	0.61
MDZ p.o. $AUC_{0-\infty}$ (ng × min/ml)	3,353 ± 1,754	3,762 ± 2,657	3,270 ± 1,533	0.48
MDZ p.o. $AUC_{0-\infty}$/dose (ng × min/ml/mg)	1,677 ± 877	1,881 ± 1,328	1,635 ± 767	0.48
F (%)	56.4 ± 12.2	56.9 ± 12.8	56.3 ± 12.2	0.45
MDZ p.o. CL/F (ml/min)	718 ± 282	707 ± 332	721 ± 274	0.88
MDZ p.o. CL/F / weight (ml/min/kg)	10.3 ± 4.4	10.1 ± 5.1	10.4 ± 4.3	0.89
Tacrolimus PK after p.o. administration				
Tacrolimus dose (mg/day)	9.85 ± 4.83	15.75 ± 3.81	8.64 ± 4.09	<0.0001
Tacrolimus dose/weight (mg/day/kg)	0.141 ± 0.075	0.227 ± 0.075	0.124 ± 0.063	<0.0001
Tacrolimus C_0 (ng/ml)	12.5 ± 2.9	11.6 ± 2.31	12.7 ± 3.0	0.29
Tacrolimus T_{max} (h)	1.60 ± 0.61	1.35 ± 0.53	1.65 ± 0.61	0.15
Tacrolimus C_{max} (ng/ml)	43.4 ± 12.5	50.3 ± 9.7	42.0 ± 12.6	0.05
Tacrolimus C_{avg} (ng/ml)	20.9 ± 4.4	21.5 ± 3.9	20.8 ± 4.5	0.62
Tacrolimus AUC_{0-12} (ng × h/ml)	251 ± 53	259 ± 47	249 ± 54	0.62
Tacrolimus C_0/dose (ng/ml/mg)	1.65 ± 1.06	0.77 ± 0.24	1.83 ± 1.07	<0.0001
Tacrolimus C_{max}/dose (ng/ml/mg)	10.1 ± 4.8	6.7 ± 2.0	10.8 ± 4.9	<0.0001
Tacrolimus C_{avg}/dose (ng/ml/mg)	5.11 ± 2.77	2.83 ±0.78	5.58 ± 2.81	<0.0001
Tacrolimus AUC_{0-12}/dose (ng × h/ml/mg)	61.3 ± 33.2	34.0 ± 9.3	67.0 ± 33.7	<0.0001
Tacrolimus CLss (l/h)	20.1 ± 9.0	31.4 ± 8.5	17.8 ± 7.2	<0.0001
Tacrolimus CLss/weight (l/h/kg)	0.291 ± 0.140	0.454 ± 0.154	0.258 ± 0.111	<0.0001

AUC, area under the concentration-time curve; C_0, trough level; C_{avg}, average-time concentration; CL, clearance; CL/F, apparent oral clearance; C_{max}, maximum concentration; CLss, steady-state clearance; F, oral bioavailability; MDZ, midazolam; PK, pharmacokinetics; T_{max}, time to reach maximum concentration.

and AUC_{0-12} were ~2.0-fold lower, as compared with those of CYP3A5 nonexpressers (Table 2).

Identification of covariates associated with tacrolimus pharmacokinetics: univariate correlation analysis

In univariate correlation analysis (weight-corrected) systemic and apparent oral MDZ clearance, reflecting *in vivo* CYP3A4 activity, correlated with (weight-corrected) tacrolimus daily dose requirements, dose-corrected tacrolimus C_0 level and AUC_{0-12}, and (weight-corrected) tacrolimus steady-state clearance ($P < 0.001$ for all). In addition, the following clinical, biochemical, and genetic covariables were correlated with all studied tacrolimus pharmacokinetic parameters at the $P < 0.20$ level: age, hemoglobin/hematocrit, creatinine/estimated glomerular filtration rate, *CYP3A4*1/*1b*, *CYP3A5*1/*3*, and *ABCB1 -129T>C*. Finally, length was correlated with dose-corrected tacrolimus AUC_{0-12} and tacrolimus steady-state clearance ($P < 0.20$).

MDZ clearance and *CYP3A5* genotype predict tacrolimus pharmacokinetics: uni- and bivariate linear regression analysis

Figure 1 shows the scatterplots of (weight-corrected) tacrolimus daily dose requirements (Figure 1a,b), dose-corrected tacrolimus C_0 level and AUC_{0-12} (Figure 1c,d), and (weight-corrected) tacrolimus steady-state clearance (Figure 1e,f) vs. (weight-corrected) apparent oral MDZ clearance in CYP3A5 expressers (red dots) and nonexpressers (blue dots). In CYP3A5 nonexpressers (weight-corrected) apparent oral MDZ clearance explains 31–43% of the variability in tacrolimus pharmacokinetic parameters (blue regression lines). In CYP3A5 expressers, similar trends were observed but did not reach statistical significance for most tacrolimus pharmacokinetic parameters (red regression lines). Bivariate linear regression models incorporating (weight-corrected) apparent oral MDZ clearance and *CYP3A5* genotype as explanatory variables explained 56–59% of the variability in tacrolimus pharmacokinetic parameters (Table 3; Figure 1a–f). (Weight-corrected) apparent oral MDZ clearance and *CYP3A5* genotype contributed equally to these models (i.e., partial R^2 = 0.23–0.29 for apparent oral MDZ clearance and partial R^2 = 0.29–0.35 for *CYP3A5* genotype).

MDZ clearance, *CYP3A5* genotype, and hematocrit predict tacrolimus pharmacokinetics: multivariate linear regression analysis

Table 4 shows the multivariate linear regression models predicting (weight-corrected) tacrolimus daily dose requirements, dose-corrected tacrolimus C_0 level and AUC_{0-12}, and (weight-corrected) tacrolimus steady-state clearance. Hematocrit was the third explanatory variable to stay in the model, after *CYP3A5* genotype and (weight-corrected) apparent oral MDZ clearance, for all tacrolimus pharmacokinetic parameters. For dose-corrected tacrolimus C_0 level age stayed in the model as well. The different multivariate linear regression models explain 60–72% of the variability in tacrolimus pharmacokinetic parameters.

DISCUSSION

This cross-sectional study in renal transplant recipients demonstrated that *in vivo* CYP3A4 activity, as reflected by apparent oral MDZ clearance, and genetically determined CYP3A5 expression explain 56–59% of variability in (weight-corrected) tacrolimus daily dose requirements, dose-corrected tacrolimus C_0 levels and AUC_{0-12}, and (weight-corrected) tacrolimus steady-state clearance. In addition, within the subgroups of CYP3A5 expressers (*CYP3A5*1* allele carriers) and CYP3A5 nonexpressers (*CYP3A5*3/*3* homozygous patients), *in vivo* CYP3A4 activity explains 35–52% of variability in tacrolimus daily dose requirements. Taking into account other potential clinical and genetic determinants of tacrolimus disposition, *in vivo* CYP3A4 activity and *CYP3A5* genotype remain the most important independent predictors of tacrolimus disposition. In multivariate analysis, hematocrit is the only other variable that independently predicts tacrolimus disposition, explaining an additional 4–14% of variability in tacrolimus pharmacokinetic parameters. Combined *in vivo* CYP3A4 activity, *CYP3A5* genotype, and hematocrit explain 60–72% of variability in (weight-corrected) tacrolimus total daily dose requirements, dose-corrected tacrolimus C_0 levels and AUC_{0-12}, and (weight-corrected) tacrolimus steady-state clearance.

First, our data indicate that CYP3A4- and CYP3A5-mediated tacrolimus metabolism are both important determinants of tacrolimus disposition *in vivo*. This is in line with the result of a study in healthy volunteers investigating tacrolimus disposition after oral and intravenous administration of ^{14}C-labeled tacrolimus, which showed that <1% of the administered dose leaves the body unchanged and that eventually >95% of the administered dose appears in the feces as tacrolimus metabolites.[31]

Second, our study confirms that the *CYP3A5* genotype is a major determinant of tacrolimus disposition,[17–22] as it explains 29–35% of the variability in the studied tacrolimus pharmacokinetic parameters. Conversely and importantly, this also implies that 65–71% of the variability in tacrolimus disposition cannot be attributed to the *CYP3A5* genotype. Indeed, the *CYP3A5* genotype cannot explain the significant residual interindividual differences in tacrolimus pharmacokinetics observed within CYP3A5 expressers and CYP3A5 nonexpressers, respectively. Of note, this consideration probably explains why in a recent prospective randomized trial investigating the potential benefit of optimizing the initial tacrolimus dose based on the *CYP3A5* genotype alone (i.e., 0.30 mg/kg/day for *CYP3A5*1*-allele carriers and 0.15 mg/kg/day for *CYP3A5*3/*3* homozygous patients, instead of the standard 0.20 mg/kg/day for all patients), a substantial proportion of patients (i.e., 56.8%) still did not have tacrolimus C_0 levels within the desired target range in the first days after tacrolimus loading.[25] This, at least in part, explains the lack of improvement in clinical outcome in this study. However, the relatively low allelic frequency of the *CYP3A5*1*-allele in the predominantly Caucasian study cohort, the delayed introduction of tacrolimus, and, importantly, the low risk of rejection due to the use of induction therapy and the high doses of mycophenolate mofetil administered in all patients were not favorable to

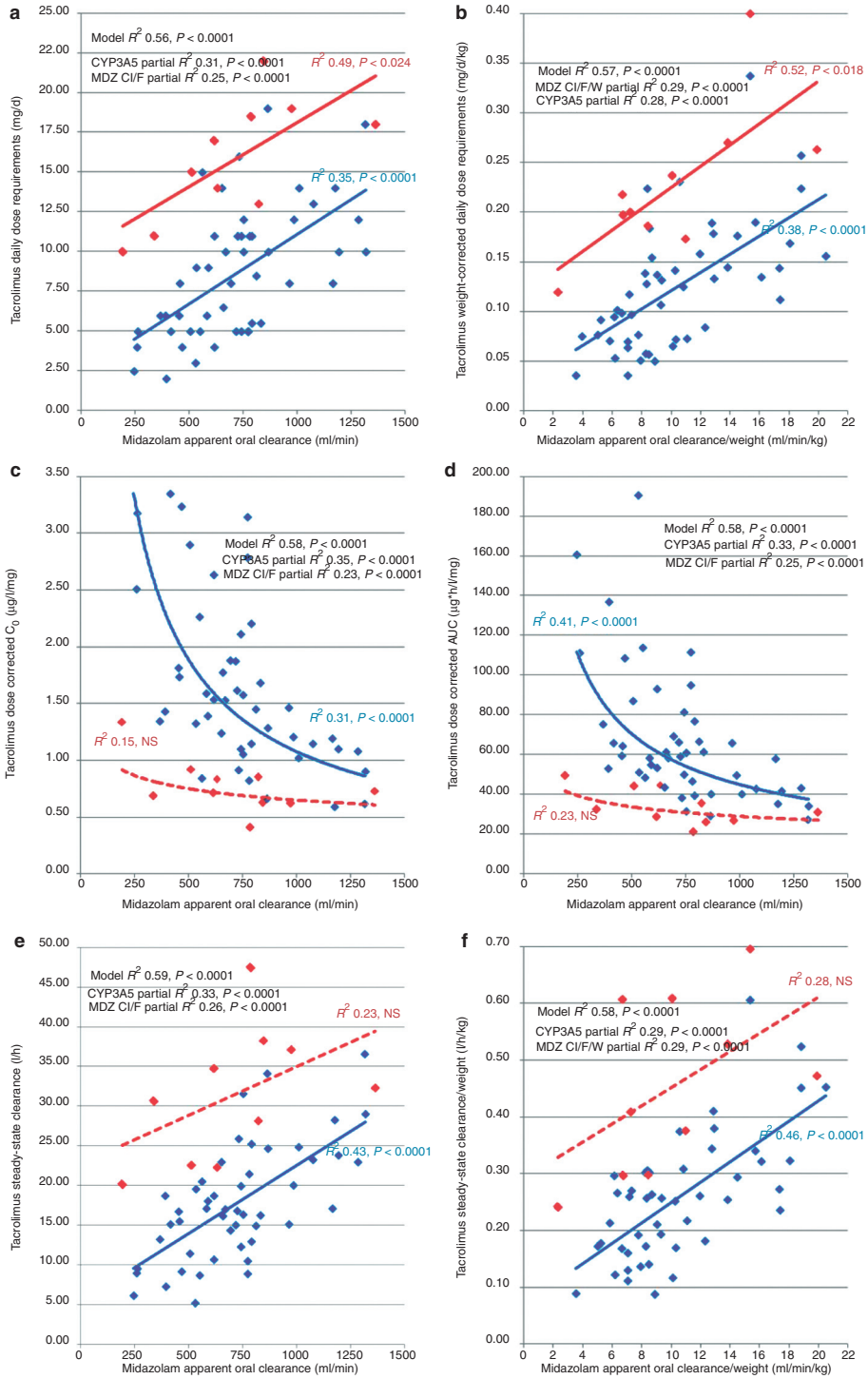

Figure 1 (**a** and **b**) Scatterplots of (weight-corrected) tacrolimus total daily dose requirements, (**c** and **d**) dose-corrected tacrolimus trough (C_0) levels and AUC_{0-12}, and (**e** and **f**) (weight-corrected) tacrolimus steady-state clearance vs. (weight-corrected) apparent oral midazolam clearance in CYP3A5 expressers (red dots) and nonexpressers (blue dots). The red and blue regression lines with corresponding R^2 and P values depict the relationship between (weight-corrected) apparent oral midazolam clearance and the various tacrolimus pharmacokinetic parameters in CYP3A5 expressers and nonexpressers, respectively. Finally, the bivariate models (i.e., model R^2, partial R^2, and P values) based on *CYP3A5* genotype and (weight-corrected) apparent oral midazolam clearance, are also reported in each panel. AUC, area under the concentration-time curve.

detect the potential benefit of prospective genotyping on clinical outcomes either.[25]

Third, our study confirms that *CYP3A5* genotype does not affect MDZ clearances (H. de Jonge, H. de Loor, K. Verbeke, Y. Vanrenterghem, and D.R. Kuypers, unpublished data; refs. 32–40) but does have a major impact on tacrolimus pharmacokinetics in renal transplant patients (H. de Jonge, H. de Loor, K. Verbeke, Y. Vanrenterghem, and D.R. Kuypers, unpublished

Table 3 Bivariate regression analysis

Dependent/explanatory variable	Model r^2/ partial r^2	*P/P*	Parameter estimate
Tacrolimus dose (mg/day)	0.562	<0.0001	
*CYP3A5*1* carrier (no/yes)	0.310	<0.0001	7.23
MDZ CL/F (ml/min)	0.252	<0.0001	0.00862
Tacrolimus dose/weight (mg/day/kg)	0.567	<0.0001	
MDZ CL/F/W (ml/min/kg)	0.291	<0.0001	0.00949
*CYP3A5*1* carrier (no/yes)	0.276	<0.0001	0.104
1/[Tacrolimus C_0/dose (ng/ml/mg)]	0.580	<0.0001	
*CYP3A5*1* carrier (no/yes)	0.352	<0.0001	0.663
MDZ CL/F (ml/min)	0.228	<0.0001	0.000712
1/[Tacrolimus AUC_{0-12}/dose (ng × h/ml/mg)]	0.577	<0.0001	
*CYP3A5*1* carrier (no/yes)	0.330	<0.0001	0.0136
MDZ CL/F (ml/min)	0.247	<0.0001	0.0000164
Tacrolimus Clss (l/h)	0.587	<0.0001	
*CYP3A5*1* carrier (no/yes)	0.331	<0.0001	13.9
MDZ CL/F (ml/min)	0.256	<0.0001	0.0161
Tacrolimus Clss/weight (l/h/kg)	0.578	<0.0001	
*CYP3A5*1* carrier (no/yes)	0.286	<0.0001	0.202
MDZ CL/F/W (ml/min/kg)	0.292	<0.0001	0.0175

AUC, area under the concentration–time curve; C_0, trough level; CL/F, apparent oral clearance; MDZ, midazolam; W, weight.

Table 4 Multivariate regression analysis

Dependent/explanatory variable	Model r^2/ partial r^2	*P/P*	Parameter estimate
Tacrolimus dose (mg/day)	0.604	<0.0001	
*CYP3A5*1* carrier (no/yes)	0.310	<0.0001	0.00875
MDZ CL/F (ml/min)	0.252	<0.0001	7.16
Hematocrit	0.042	0.0187	−19.9
Tacrolimus dose/weight (mg/day/kg)	0.621	<0.0001	
MDZ CL/F/W (ml/min/kg)	0.291	<0.0001	0.00953
*CYP3A5*1* carrier (no/yes)	0.276	<0.0001	0.103
Hematocrit	0.053	0.0074	−0.347
1/[Tacrolimus C_0/dose (ng/ml/mg)]	0.722	<0.0001	
*CYP3A5*1* carrier (no/yes)	0.352	<0.0001	0.663
MDZ CL/F (ml/min)	0.228	<0.0001	0.000712
Hematocrit	0.117	<0.0001	−3.36
Age (years)	0.025	0.0328	−0.00695
1/[Tacrolimus AUC_{0-12}/dose (ng*h/ml/mg)]	0.689	<0.0001	
*CYP3A5*1* carrier (no/yes)	0.330	<0.0001	0.0136
MDZ CL/F (ml/min)	0.247	<0.0001	0.0000164
Hematocrit	0.113	<0.0001	−0.0594
Tacrolimus Clss (l/h)	0.696	<0.0001	
*CYP3A5*1* carrier (no/yes)	0.331	<0.0001	13.7
MDZ CL/F (ml/min)	0.256	<0.0001	0.0165
Hematocrit	0.110	<0.0001	−59.4
Tacrolimus Clss / weight (l/h/kg)	0.715	<0.0001	
*CYP3A5*1* carrier (no/yes)	0.286	<0.0001	0.198
MDZ CL/F/W (ml/min/kg)	0.292	<0.0001	0.0176
Hematocrit	0.137	<0.0001	−1.03

AUC, area under the concentration-time curve; C_0, trough level; CL/F, apparent oral clearance; CLss, steady state clearance; MDZ, midazolam; W, weight.

data; refs. 17–22). This suggests that *in vivo* MDZ should be considered a probe drug for CYP3A4 activity rather than total CYP3A activity (i.e., combined CYP3A4 and CYP3A5 activity). *In vivo* CYP3A4 activity, was assessed using MDZ as a drug probe, and *CYP3A5* genotype might therefore independently predict the pharmacokinetics of the dual CYP3A4 and CYP3A5 substrate tacrolimus.[6,7] Our study indeed demonstrated that in multivariate analysis apparent oral MDZ clearance, reflecting *in vivo* CYP3A4 activity, explains an additional 23–29% of the variability in the studied tacrolimus pharmacokinetic parameters. These results are similar to those of a previous study in renal transplant recipients investigating the relationship between MDZ clearance and the clearance of cyclosporin A,[41] another calcineurin inhibitor that has the same mechanism of action as tacrolimus and shares the same pathways for metabolism, distribution, and excretion, which are, however, not notably affected by *CYP3A5* genotype.[5] In the latter study, apparent oral MDZ clearance explained 25% of the variability in cyclosporine clearance, albeit in univariate analysis.[41] By comparison, in our study apparent oral MDZ clearance explained 43–46% of the variability in tacrolimus clearance within the subgroup of CYP3A5 nonexpressers.

Fourth, the fact that hematocrit is the third and only other variable that independently predicts tacrolimus disposition is consistent with the strong binding of tacrolimus to red blood cells (blood/plasma ratio ~20:1) and the fact that tacrolimus concentrations are measured in whole blood instead of plasma.[13] Of note, the selection of the study population might be responsible for the fact that no other clinical or biochemical variable predicted tacrolimus disposition in our study. By selecting a population of adult renal transplant recipients of Caucasian ancestry who were all tested 3 months after transplantation, some variables known to be associated with tacrolimus pharmacokinetics (i.e., pediatric vs. adult patients, type of solid-organ transplantation, ethnicity, and time after transplantation) could not affect tacrolimus disposition in our study. In addition, patients with medical or surgical gastrointestinal or hepatic disorders were excluded, which implies that there were no patients with hepatic dysfunction and/or diarrhea, two other variables that are associated with tacrolimus pharmacokinetics.[13] Furthermore, major drug–drug interactions were avoided because the use of drugs and substances that are known to either induce or inhibit CYP3A iso-enzymes or to interfere with the absorption, distribution, metabolism, or excretion of tacrolimus, other than corticosteroids, was prohibited and because all participants were treated with a similar dose of methylprednisolone. Finally, food–drug interactions were controlled by testing under standardized conditions. Importantly, this does not imply that these results would

differ if the study were repeated in a more diverse population, as some of the clinical factors mentioned above are likely to exert their effect through alterations in *in vivo* CYP3A4 activity, which was measured in this study. Of course, this hypothesis needs to be tested in appropriate and independent study cohorts.

Besides providing mechanistic insights into the important role that CYP3A-mediated tacrolimus metabolism plays in tacrolimus disposition in renal transplant patients, our findings may potentially have clinical implications. To improve clinical outcome, the transplant community is constantly looking for tools that allow the individualization of immunosuppressive therapy to the specific needs of the individual patient. One of these tools, which is routinely applied in clinical practice, is tacrolimus therapeutic drug monitoring and concentration-controlled dosing.[2,5] This approach helps to avoid both under- and overexposure and reduces the pharmacokinetic component of inter- and intraindividual variability in drug response. Therefore, therapeutic drug monitoring is thought to optimize efficacy while reducing drug-related toxicity. However, therapeutic drug monitoring has its limitations. First, it cannot predict the optimal initial tacrolimus dosing and as a consequence many patients have C_0 levels below or above the desired target range in the first few critical days after transplantation. The former might imply an increased risk of acute rejection, as suggested by the shorter time to acute rejection observed in *CYP3A5*1*-allele carriers,[23] and the latter implies an increased risk of tacrolimus-related toxicities, such as acute nephrotoxicity, neurotoxicity, and the development of new-onset diabetes after transplantation.[24] Our data suggest that predicting initial tacrolimus dosing based on the patient's *CYP3A5* genotype, *in vivo* CYP3A4 activity, and hematocrit is likely to be far more accurate and hence might allow more patients to be brought into the desired target range early after transplantation. Of course, this needs to be confirmed in studies in which *in vivo* CYP3A4 activity and tacrolimus pharmacokinetics are assessed before and early after transplantation, especially because *in vivo* CYP3A4 activity might change substantially after transplantation. Second, some drug-related toxicities are not (only) associated with tacrolimus exposure but rather seem to be related to tacrolimus dose requirements. For example, the development of chronic calcineurin inhibitor–associated nephrotoxicity is associated with early tacrolimus dose requirements but not with tacrolimus exposure.[42] Hence, preemptive identification of patients who are likely to have high tacrolimus dose requirements based on *in vivo* CYP3A4 activity and *CYP3A5* genotype might allow identification of patients who will benefit the most from a calcineurin-free immunosuppressive regimen,[43,44] in terms of prevention of chronic calcineurin inhibitor–associated nephrotoxicity and improved renal graft function.

In summary, this study demonstrated that *in vivo* CYP3A4 activity, assessed using MDZ as a drug probe, and *CYP3A5* genotype explain 56–59% of the variability in tacrolimus dose requirements and clearance, both contributing ~25–30%. Hematocrit explains another 4–14%. Combined *in vivo* CYP3A4 activity, *CYP3A5* genotype, and hematocrit explain 60–72% of variability in tacrolimus dose requirements and tacrolimus clearance. These data indicate that CYP3A4- and CYP3A5-mediated tacrolimus metabolism are both important determinants of tacrolimus disposition *in vivo* and explain a substantial part of the clinically observed high interindividual variability in tacrolimus pharmacokinetics. Furthermore, these data provide a potential basis for a comprehensive approach in predicting tacrolimus dose requirements in individual patients, which might facilitate tailoring immunosuppressive therapy to the specific needs of an individual patient.

METHODS

Study population. Stable tacrolimus-treated renal allograft recipients were considered for participation in this study. All patients were tested 3 months after transplantation and were of Caucasian ancestry. The minimum age for inclusion was 18 years. Combined-organ transplants were excluded, as were females of childbearing potential not using an acceptable method of birth control and pregnant women. Patients with medical or surgical gastrointestinal or hepatic disorders were excluded from the study, as were patients with significant comorbidity (severe chronic lung disease or heart failure with or without respiratory insufficiency). Anemia and hypoalbuminemia were also exclusion criteria, as were documented noncompliance; addiction to any known drug, nicotine, or alcohol (>7 units/week); the use of opioid or antipsychotic drugs; and known allergy to or intolerance of MDZ. The use of drugs and substances known to either induce or inhibit CYP3A isoenzymes or to interfere with the absorption, distribution, metabolism, or excretion of tacrolimus other than corticosteroids was prohibited. All patients were treated with tacrolimus (Prograf or Prograft, Astellas Pharma Europe, Staines, UK), combined with mycophenolic acid administered as its prodrug mycophenolate mofetil (Cellcept, Roche, Basel, Switzerland) and a low dose of methylprednisolone (Medrol, Pfizer, New York, NY).

This study was performed according to the Declaration of Helsinki and was approved by the ethics committee of the University Hospitals Leuven, Faculty of Medicine, Catholic University Leuven, Belgium, and the Belgian Federal Agency for Medicines and Health Products (EudraCT 2007-004069-16, https://eudract.ema.europa.eu). Informed consent was obtained from all participants.

Study design. This was a cross-sectional study in tacrolimus-treated renal transplant recipients investigating the relation between *in vivo* CYP3A4 activity (assessed using MDZ as a drug probe) and *CYP3A5* genotype on the one hand, and tacrolimus pharmacokinetics (i.e., dose requirements, dose-corrected C_0 levels and AUC_{0-12}, and clearance) on the other hand. Other potential clinical (i.e., sex, age, weight, length, body mass index, diabetes, creatinine/estimated glomerular filtration rate, albumin, and hemoglobin/hematocrit) and genetic (i.e., *CYP3A4* and *MDR-1* SNPs) determinants of tacrolimus pharmacokinetics were also considered.

Of note, based on *in vitro* data that showed that MDZ is metabolized by both CYP3A4 and CYP3A5 but is not a substrate of other drug-metabolizing enzymes or drug transporters, it is traditionally considered a phenotypic drug probe for total *in vivo* CYP3A activity (i.e., combined CYP3A4 and CYP3A5 activity).[27–30] However, more recent *in vivo* studies demonstrated that *CYP3A5* genotype, and therefore CYP3A5 expression, does not affect MDZ pharmacokinetics (H. de Jonge, H. de Loor, K. Verbeke, Y. Vanrenterghem, and D.R. Kuypers, unpublished data; refs. 36–44), which suggests that *in vivo* MDZ should be considered a drug probe reflecting CYP3A4 activity, rather than total CYP3A activity. Hence, systemic MDZ clearance after intravenous administration is a measure for the role of CYP3A4 in systemic elimination of a drug (i.e., *in vivo* hepatic CYP3A4 activity) (H. de Jonge, H. de Loor, K. Verbeke, Y. Vanrenterghem, and D.R. Kuypers, unpublished data; ref. 27) whereas apparent oral MDZ clearance after oral administration is a measure for the role of CYP3A4 in both presystemic and systemic elimination of an orally administered drug (i.e., combined hepatic and intestinal CYP3A4 activity) (H. de Jonge, H. de Loor, K. Verbeke, Y. Vanrenterghem, and D.R. Kuypers, unpublished data; refs. 28–30). Finally,

MDZ oral bio-availability (F) can be considered a measure for the role of CYP3A4 in presystemic elimination of an orally administered drug. Because the aim of the current study was to investigate determinants of tacrolimus disposition *in vivo*, and because tacrolimus is almost exclusively administered orally in routine clinical practice, we considered apparent oral MDZ clearance, which reflects both presystemic and systemic elimination, the most appropriate measure of *in vivo* CYP3A4 activity for these analyses.

Pharmacokinetic study. Following an overnight fast, patients presented at our outpatient clinic. A full physical examination was performed, and an intravenous citrate locked nonpolyurethane catheter was placed in an antecubital vein for blood sampling. Blood samples were drawn for a full biochemical analysis, including hematology, serum creatinine, serum albumin, liver tests, electrolytes, and lipids. Estimated glomerular filtration rate was calculated using the modification of diet in renal disease formula.[45] All concomitant medication was registered. Before testing, participants had to abstain from consuming alcohol and grapefruit-containing products for at least 7 days. In addition, they were not allowed to take any herbal products or over-the-counter medication. On day 1 of the study, 2 mg of MDZ (2 ml of a 1 mg/ml MDZ solution, Dormicum, Roche) mixed in 30 ml of a 5% glucose solution was administered orally from a glass container followed by 100 ml of water to rinse the glass. Immediately thereafter, patients took their usual morning dose of tacrolimus and their other immunosuppressive medication. Two 4-ml blood samples were collected in ethylenediaminetetraacetic acid–containing tubes before and at 0.25, 0.5, 1, 1.5, 2, 3, 4, 5, 6, and 8 h after MDZ administration. For each time point, one sample was centrifuged for 10 minutes at 1,860*g*, at 4°C, and plasma was stored at −80°C pending analysis of MDZ plasma concentrations. The other sample was stored as whole blood at −80°C pending analysis of tacrolimus whole-blood concentrations. On the second day, 1 mg of MDZ (1 ml of a 1 mg/ml MDZ solution, Dormicum, Roche) diluted in 4 ml of a 0.9% sodium chloride solution was injected slowly over 15–30 s through a second intravenous access point. Plasma was obtained from blood samples drawn at the same 11 time points as on day 1. On both days, the subject's blood pressure, pulse, and oxygen saturation were monitored throughout the first hour after MDZ administration. Patients were kept fasting until 2 h after the tests were initiated, but they were allowed to drink water. At that time, a standard breakfast was provided, and patients were allowed to take their concomitant nonimmunosuppressive medication. Patients were not allowed to perform any exercise during the entire duration of the test.

Identification of selected *CYP3A5*, *CYP3A4*, and MDR-1 SNPs. Genomic DNA was isolated from whole-blood samples using a salting-out procedure.[46] Participants were genotyped for the *CYP3A5*1/*3* SNP (*rs776746*, *6986A>G*) using a previously published PCR restriction fragment-length polymorphism method.[21] In addition, all participants were genotyped for *CYP3A4*1/*1b* (*rs2740574*, *-290A*>T), *ABCB1 -129T>C* (*rs3213619*), *ABCB1 1236C>T* (*rs1128503*), *ABCB1 2677G>T/A* (*rs2032582*), and *ABCB1 3435C>T* (*rs1045642*) (**Supplementary Table S2,** online).

Quantification of MDZ plasma concentrations. MDZ plasma concentrations were measured using a recently published high-performance liquid chromatography–tandem mass spectrometry method.[47,48] A brief description of this method is available in the **Supplementary Materials and Methods** online.

Quantification of tacrolimus whole-blood concentrations. Tacrolimus whole-blood concentrations were measured using a commercially available validated liquid chromatography–tandem mass-spectrometry kit that complies with National Committee for Clinical Laboratory Standards and FDA guidelines. This kit is designed specifically for tacrolimus therapeutic drug monitoring in transplant recipients (MassTrak Immunosuppressants Kit, Waters, Zellik, Belgium).[49] A brief description of this method is available in the **Supplementary Materials and Methods** online. The analytical performance of the kit was validated by successful participation of our laboratory in the International Tacrolimus Proficiency Testing Scheme provided by Analytical Services International (London, UK).

Determination of pharmacokinetic parameters. The concentration–time data were evaluated by standard noncompartmental methods (WinNonlin 5.2.1, Pharsight, Mountain View, CA). The maximum concentration (C_{max}) and time to reach maximum concentration (T_{max}) after oral MDZ and tacrolimus administration were determined by visual inspection of the data. The terminal elimination-rate constant (λ_Z) was determined by linear regression of the log concentration vs. time data. The AUC was calculated using a combination of linear and logarithmic trapezoidal methods ("linear up/log down"). The MDZ AUC_{0-480} was calculated from time of drug administration to the last sampling time (8 h or 480 min) and was then extrapolated to infinity ($AUC_{0\to\infty}$). Systemic clearance of i.v. MDZ was MDZ i.v. CL = $Dose_{i.v.}/AUC_{0\to\infty i.v.}$, and apparent oral clearance of orally administered MDZ was MDZ p.o. CL/F = $Dose_{p.o.}/AUC_{0\to\infty p.o.}$. MDZ oral bioavailability was F = $((AUC_{0\to\infty p.o.}/Dose_{p.o.})/(AUC_{0\to\infty i.v.}/Dose_{i.v.})) \times 100\%$. The tacrolimus AUC_{0-8} and tacrolimus AUC_{0-12} were calculated from time of drug administration to the last sampling time (8 h) and assuming $C_{12} = C_0$, respectively. Estimates of tacrolimus steady-state clearance were obtained assuming $C_{12} = C_0$ as well.

Statistical analysis. Data are expressed as mean ± SD except when stated otherwise. Distribution of continuous variables was evaluated according to Shapiro–Wilks and parametric and nonparametric tests were applied as appropriate. Hardy–Weinberg equilibrium test was performed using appropriate χ^2 test. Pairwise r^2 and D' values for linkage disequilibrium were calculated using JMP genomics (version 5.1, SAS institute, Cary, NC). *CYP3A5* genotype groups were compared using a two-sample *t*-test or the Wilcoxon–Mann–Whitney test for continuous variables and using nonparametric tests (Fisher's exact test and χ^2) for categorical variables. A two-sided $P < 0.05$ was considered statistically significant. Exploratory univariate correlation analysis (Spearman's correlation coefficient) was performed to explore whether a specific covariate potentially affected tacrolimus pharmacokinetics. Dose-corrected tacrolimus C_0 and AUC_{0-12}, (weight-corrected) tacrolimus daily dose requirements, and (weight-corrected) tacrolimus steady-state clearance were used as dependent variables. All covariates correlated with tacrolimus pharmacokinetic parameters at a P value <0.2 in univariate correlation analysis were retained and entered in uni-, bi-, and multivariate linear regression models. To make linear regression possible, 1/x transformation was performed on dose-corrected tacrolimus C_0 and AUC_{0-12}. In addition, categorical covariates were coded with a dummy variable set arbitrarily at 0 or 1 depending on the absence or presence of a specific feature. In multivariate regression analysis, significant covariates of tacrolimus pharmacokinetics were selected by backward elimination procedure. SAS software (version 9.2, SAS institute) was used for all statistical analyses.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/cpt

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AUTHOR CONTRIBUTIONS

H.d.J., H.d.L., K.V., Y.V., and D.R.K. wrote the manuscript; H.d.J. and D.R.K. designed the research; H.d.J. and H.d.L. performed research; H.d.J. and D.R.K. analyzed data; H.d.L. contributed new reagents/analytical tools.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

✓ Tacrolimus is metabolized by CYP3A4 and CYP3A5 and is characterized by a narrow therapeutic index and highly variable pharmacokinetics. The *CYP3A5* genotype is a major determinant of tacrolimus disposition but cannot explain the important residual interindividual differences within CYP3A5 expressers (*CYP3A5*1*-allele carriers) and CYP3A5 nonexpresser (*CYP3A5*3/*3* homozygous patients).

WHAT QUESTION DID THIS STUDY ADDRESS?

✓ To what extent do *in vivo* CYP3A4 activity, assessed using MDZ as a drug probe, and *CYP3A5* genotype predict tacrolimus pharmacokinetics in renal allograft recipients?

WHAT THIS STUDY ADDS TO OUR KNOWLEDGE?

✓ *In vivo* CYP3A4 activity and *CYP3A5* genotype explain 56–59% of variability in tacrolimus dose requirements, dose-corrected exposure, and clearance, contributing ~25 and 30%, respectively. Hematocrit explains an additional 4–14%. Combined *in vivo* CYP3A4 activity, *CYP3A5* genotype, and hematocrit explain 60–72% of variability in tacrolimus pharmacokinetics.

HOW THIS MIGHT CHANGE CLINICAL PHARMACOLOGY AND THERAPEUTICS?

✓ These data provide a potential basis for a comprehensive approach in predicting the tacrolimus dose requirement in individual patients and hence provide a strategy to tailor immunosuppressive therapy in transplant recipients.

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