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Identification of factors affecting tacrolimus trough levels in Latin-American pediatric liver transplant patients

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Abstract

Tacrolimus is the milestone in pediatric liver transplant immunosuppression. Despite close monitoring, fluctuations in tacrolimus blood levels affect safety and efficacy of immunosuppressive treatments. Identifying the factors related to the variability in tacrolimus exposure may be helpful in tailoring the dose. The aim of the present study was to characterize the clinical, pharmacological, and genetic variables associated with tacrolimus systemic exposure in pediatric liver transplant patients.

De-novo transplant patients with a survival of more than one month were considered for inclusion and genotyped for CYP3A5. Peri-transplant clinical factors and laboratory covariates, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), hematocrit, and tacrolimus pre-dose steady-state blood concentrations collected 12 h after tacrolimus dose (C0), were recorded retrospectively between one month and two years post-transplant. A linear mixed effect (LME) model was used to assess the association of these

factors and the log-transformed tacrolimus dose-normalized C0 (logC0/D). Bootstrapping was used to internally validate the final model. External validation was performed in an independent group of patients that matched the original population. The developed LME model described that logC0/D increases as time post-transplant and ALT values increase (β=0.019, 95% CI, 0.010-0.028 and β=0.00030, 95% CI, 0.00002-0.00056, respectively), whereas it is significantly lower in graft CYP3A5-expressers compared to non-expressers [β= -0.349, 95% CI, -0.631-(-0.062)].

Conclusions: Donor CYP3A5 genotype, time post-transplant and, alanine aminotransferase values are associated with tacrolimus disposition between one month and two years post-transplant. A better understanding of tacrolimus exposure is essential to minimize the occurrence of an out-of-range therapeutic window that may lead to adverse drug reactions or acute rejection.

Introduction

Tacrolimus has become the cornerstone in immunosuppression in pediatric and adult liver transplant recipients to prevent allograft rejection. This calcineurin inhibitor has a narrow therapeutic index and presents large inter- and intra-individual pharmacokinetic variability (1, 2). In order to optimize its efficacy and minimize the occurrence of adverse events, therapeutic drug monitoring (TDM) is regularly performed in clinical practice based on trough concentrations (C0) determined before the next dose of tacrolimus (1). Trough concentrations have been selected as a measure of systemic exposure that correlates with clinical outcome (graft rejection and tacrolimus toxicity) (3). However, C0-based therapeutic ranges in children are defined based on adult clinical data (2), with subsequent empirical adaptation of the doses according to these trough concentrations.

Although the optimization of immunosuppressive therapies and the improvements in surgical procedures have contributed to longer overall and graft survival in pediatric liver transplantation, clinical issues, such as acute rejection and adverse drug reactions to tacrolimus, confer morbidity and mortality (1, 4). Moreover, previous reports have described a significant association between variability in tacrolimus C0 and the development of acute rejection and adverse drug reactions (1, 5-7). Furthermore, other factors including time post-transplant, body weight, hematocrit, age, liver function parameters, and type of graft contribute to its pharmacokinetic variability in pediatric liver transplant patients (1, 8-16).

Different polymorphisms of cytochrome P450 enzymes, especially in CYP3A5, affect tacrolimus clearance. This enzyme plays an important role in the metabolism of tacrolimus, and is mainly expressed in liver and intestine. A polymorphism in intron 3 of CYP3A5 (CYP3A5*3 allele) produces an abnormally spliced mRNA with a premature stop codon resulting in the absence of the CYP3A5 enzyme (17). Several studies have described higher tacrolimus trough concentrations in adult patients carrying the CYP3A5*3 allele (non-expressers) compared to the expressers (CYP3A5*1 carriers) (18-20). In addition, some studies have reported this association in Asian and European pediatric patients (9-12, 21-23). However, the behavior of tacrolimus variability in Latin-American pediatric liver transplant recipients is unknown and reports regarding safety and efficacy of immunosuppressive regimens are scarce (24).

For all mentioned, we aimed to evaluate the impact of donor and recipient genetic polymorphisms in the CYP3A5 enzyme on tacrolimus C0 and to identify and characterize different clinical and biochemical variables associated with tacrolimus exposure after oral administration in pediatric liver transplant patients between one month and two years after liver transplantation.

1. Methods

This study is a retrospective, single-center cohort study conducted in accordance to the Helsinki Declaration at Hospital de Pediatría JP Garrahan (Buenos Aires, Argentina) after approval by the Institutional Review Board (Protocol #740). Written informed consent was obtained from parents or guardians.

Study Population

This study is part of a previous one that aimed to identify peri-transplant predictors of acute rejection and factors related to the risk of tacrolimus adverse drug reactions in pediatric liver transplant patients (6) in the context of the implementation of a new immunosuppressive protocol. Pediatric de-novo liver allograft recipients less than 18 years old at the time of transplantation were included during the period in which the CYP3A5 genotyping technique was available at the Hospital de Pediatría JP Garrahan. Patients included in the present analysis had at least four tacrolimus trough concentrations during the study period. Exclusion criteria included: less than 1 month of post-transplant survival, re-transplantation, combined or multivisceral transplants, interval of administration of tacrolimus other than every 12 h, and inappropriate follow-up or noncompliance, as previously defined (24). In addition, tacrolimus C0 levels obtained at times at which the patient was receiving simultaneous administration of azoles, macrolides, antiepileptic drugs, and/or calcium channel blockers, were excluded from the analysis. Follow-up data were collected between 1 month post-transplant and 2 years. All data were collected from the medical and nursing records, and a centralized database with restricted access was generated.

Immunosuppressive therapy

Tacrolimus (0.1 mg/kg/day) was initiated after reperfusion and kidney function normalization, administered in monotherapy with anti-CD25 induction (basiliximab) on day 0 and day 4, or in combination with corticosteroids and/or mycophenolate mofetil according to kidney and liver function (25), as depicted in **Table 1**. Concomitant drugs were sulfamethoxazole-trimethoprim, magnesium supplements, omeprazole (in all patients), acyclovir, and additional antibiotics, if needed.

Tacrolimus monitoring

For the analysis we used retrospective routine therapeutic drug monitoring (whole-blood 12-h tacrolimus C0). Patients were given oral tacrolimus (Prograft®, Astellas Laboratory, Killorglin, Co. Kerry, Ireland) twice daily. Data (tacrolimus doses, C0s, weight) were recorded after 30 days post-transplantation and every day during hospitalization and/or on out-patient visits for 2 years. At all times that a blood sample was obtained for assessment of tacrolimus C0, a complete blood sample test was performed including liver and renal function tests, hematocrit and hemoglobin levels. Characteristics of the patients enrolled in the study are presented in **Table 1**.

Tacrolimus trough concentrations were quantified using the chemiluminescent microparticle immunoassay (CMIA) (Architect; Abbott, Chicago, IL). Whole blood quality controls (Lyphochek Whole Blood Immunosuppressant; Bio-Rad, Irvine, CA) were daily assessed for assay acceptance. In addition, specimens were routinely assessed as part of an international proficiency testing program for the external quality control of tacrolimus (26). Total imprecision was less than 8%, and quality control values lied in the range of +/-2SD. Tacrolimus C0 target levels, defined based on adult clinical data (2), were 7-8ng/mL in the

first 6 months, 5-7ng/mL during the next 6 months, and 5ng/mL after the first year post-transplant(27, 28).

Biochemical, clinical, and genetic factors

Peri-transplant and post-transplant variables were studied including: *demographic features*: age, weight at transplant, gender, and primary diagnosis; *biochemical values*: hematocrit, albumin, serum creatinine, uremia, total bilirubin, liver function markers (aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), and gamma-glutamyl transpeptidase (GGT) activity); *transplant features*: type of graft (partial graft from a living or deceased donor vs. a whole graft from a deceased donor), type of donor (deceased vs. living donor), and days post-transplant; *clinical status*: Epstein bar virus and cytomegalovirus infections; and genotyping: CYP3A5*3 polymorphism in donors and recipients. CYP3A5 genotyping procedure was previously described (6).

In addition, we registered concomitant immunosuppressive agents such as steroids (at least 30 consecutive days), azathioprine, mycophenolate mofetil, and sirolimus.

Relationship between tacrolimus C0 and predictor parameters

A linear mixed effect (LME) model was used to investigate the influence of CYP3A5 genotype, pharmacological factors, and clinical and laboratory parameters on log-transformed tacrolimus dose-normalized C0 concentrations (logC0/D).

Model development

The total dataset was randomly split into a model-building and a validation dataset. The model was initiated with the development of the base model in the model-building dataset to select the best structure for random effects. Different structural models were tested (random

intercept, slope with and without inter-model correlation) and the best model was selected based on the Akaike information criterion (AIC). Both continuous (time post-transplant, hematocrit, AST, ALT, ALP, and GGT activity) and categorical variables (administration of steroids and/or mycophenolate sodium or mofetil, Epstein-Barr virus infection, cytomegalovirus infection, type of donor, type of graft, and CYP3A5*3 polymorphism in donors and recipients) were considered in the analysis.

Covariates associated with a p value < 0.05 in the univariate analysis, and were therefore considered clinically relevant and biologically plausible, were included in the multivariate intermediate model. The final model was selected using a backward stepwise process based on the AIC. All statistical analyses and graphs were performed with RStudio Version 0.99.486, 2015, Inc (29, 30) using R (R Core Team, 2015), lme4 (31), and nlme (32).

Finally, all assumptions were checked in the final model, including linearity, absence of co-linearity, homoscedasticity, normality of residuals, absence of influential data points, and independence (30).

Model evaluation and external validation.

Once the final model was defined, a bootstrap was used to evaluate the stability and accuracy and to calculate the 2.5-97.5 percentiles of parameter estimates. The median values of the bootstrap parameters were compared to the values of the final model.

The performance of the model was visually assessed by comparing plots of the predicted concentration (Cpred) and the observed concentration (Cobs) to assess for bias (a systematic upward or downward deviation from the line of unity in these plots) and imprecision (a high degree of scatter of data points around the line of unity).

External validation was performed in the validation dataset that matched the data used for model development. The predictive performance of the model was assessed numerically

through calculation of the mean error (ME), the mean relative error (MRE), and the relative root mean squared error (RMSE) as previously reported (33).

Results

Overall, 89 patients were considered for inclusion based on the implementation of a new immunosuppressive protocol in 2010 and according to the availability of data as detailed below. Patients were excluded because of a survival shorter than 1 month (n=5), unavailable medical records (n=4), re-transplantation during the first month after surgery (n=2), absence of pharmacokinetic and clinical data (n=5), absence of genotyping data from donors and/or recipients due to limited amount of DNA or no availability of formalin-fixed paraffin embedded liver tissue (n=14), and non-adherence as previously defined (6) (n=6). Therefore, 53 patients were finally included in the analysis. Demographics, laboratory parameters, and clinical characteristics of the patients included in the building (n=40) and validation (n=13) of the dataset are shown in **Table 1**.

CYP3A5 polymorphism distribution in both donors and receptors included in this study is reported in **Table S1** (**Supporting Table 1**). The genotype frequencies of the CYP3A5 polymorphism did not deviate from the Hardy–Weinberg equilibrium ($p > 0.5$) as previously reported (6). According to the report of the Clinical Pharmacogenetics Implementation Consortium (34), the estimated allele frequency of CYP3A5*1 and *3 in our population was similar to that reported for the Latin American cohort of patients analyzed in the mentioned guideline. Specifically, our population showed an allele frequency of 0.254 and 0.746 for the CYP3A5*1 and *3 allele, respectively.

A base model was built using 824 tacrolimus trough concentrations obtained from the patients included in the model-building group. Random effects were included in the intercept for inter-individual variability and the slope for the effect of time post-transplant with a correlation between them.

Univariate analysis showed a significant linear association between logC0/D and ALT values, time post-transplant, total bilirubin values, donor CYP3A5 polymorphism, and Epstein Barr Virus (EBV) infection status ($p<0.05$). All covariates significantly related to logC0/D are listed in **Table 2**. The positive associations between logC0/D and time post-transplant and ALT are shown in Figure 1 A and B, respectively. The figures show an increase in logC0/D with time post-transplant or with liver dysfunction assessed by ALT. As depicted in **Figure 1 C,** logC0/D was lower in patients with a CYP3A5-expresser graft compared to non-expressers ($p<0.05$). In more detail, **Figure 1 D** shows the bivariate model of logC0/D according to time post-transplant and donor CYP3A5 genotype**.** Patients with CYP3A5 non-expresser grafts presented significantly higher logC0/D compared to CYP3A5 expressers between one month and two years post transplantation.

After backwards elimination, the best multivariate model describing tacrolimus exposure retained the following covariates that independently correlate with logC0/D: time post-transplantation, alanine aminotransferase values and donor CYP3A5 expression. **Table 3** summarizes the final model estimates. Visual inspection of residual plots did not reveal any obvious deviations from homoscedasticity or normality (**Supporting Figure S1**). The predicted concentrations as a function of the observed concentrations of tacrolimus showed that the model performed well in terms of fitness of the data (**Figure 2**).

The internal validation of the final model by bootstrapping (1000 successful runs) gave satisfactory results as shown in **Table 3**. Moreover, in the external validation, the predictive performance of the final model was successfully assessed: the mean error (ME) was 0.213, the mean relative error (MRE) was 2.05%, and precision, expressed as the relative root mean squared error (RMSE) was 15.4% for the predictive model.

Discussion

In this study, for the first time in a Latin-American pediatric liver transplant population, we identified different factors that significantly influence tacrolimus exposure. We developed and validated a model that showed a positive association between log-transformed tacrolimus dose-normalized trough concentrations and ALT values as well as time post-transplant, while a negative association with donor CYP3A5 expression (expressers vs. non-expressers) was found between 1 month and 2 years post-transplantation.

Liver function tests, ALT and AST, are traditional markers of acute liver damage secondary to different events including acute rejection episodes, viral infections, and/or liver fibrosis (35). As 98-99% of tacrolimus is metabolized in the liver (36), it is expected that tacrolimus C0 increases with liver dysfunction. Previously, apparent clearance was found to decrease exponentially with the increase of AST in adult transplant patients (35). In our study, elevated ALT levels, compatible with impaired liver function, positively correlated with logC0/D due to a deficit in tacrolimus metabolism.

Few studies have detected a relationship between tacrolimus exposure and time post-transplant (11, 16). In our case, we observed that time post-transplant was retained in the final model and the ratio logC0/D increased with time, in line with a reduction in tacrolimus doses (data not shown). In adult transplant patients, reduced tacrolimus dose requirements have been routinely found in the first year after transplantation (37-39). This observation may be

explained by a decrease in tacrolimus clearance due to drug-drug interactions, increased bioavailability over time, or both (12, 40). Regarding drug-drug interactions, introduction or discontinuation of steroids may play an important role. The concurrent use of tacrolimus with mild CYP3A inducers, such as prednisone, may result in decreased tacrolimus trough concentrations whereas the discontinuation of steroids may result in an increased tacrolimus exposure (41). In our study, we registered the administration of steroids in the immunosuppressive maintenance treatment. We tested for the significance of concomitant steroids in tacrolimus logC0/D but it was not retained in the final model ($p>0.05$, Table 3). Thus, we were not able to confirm a drug-drug interaction effect of steroids in tacrolimus pharmacokinetics over time. On the other hand, tacrolimus largely binds to red blood cells and plasma proteins. Thus, the increase over time in oral bioavailability may potentially be due to an increased hematocrit and albumin concentration (10, 12, 42). In our study, we tested for the significance of hematocrit in tacrolimus C0 and did not find a significant relation ($p>0.05$, Table 2) to confirm the change of tacrolimus clearance over time. Nevertheless, the mechanisms responsible for the change in apparent clearance over time are only partly known (38-40) and further studies are required (12).

The metabolism of tacrolimus largely occurs in the liver. CYP3A5 plays a more dominant role in the metabolism of tacrolimus than CYP3A4 (43) and has a significant effect on tacrolimus pharmacokinetics in adult and pediatric transplant patients (10-12, 18, 21, 22, 44, 45). Specifically in liver transplantation, it has been reported that donor CYP3A5 genotype has a more dominant effect than the recipient genotype on tacrolimus pharmacokinetics (10, 11, 18, 21, 22, 45). This result implies that after day 30 post-transplant, recipients of a graft expressing CYP3A5 have a lower logC0/D compared to recipients of a non-expresser graft. Therefore, higher tacrolimus doses are required in patients with grafts carrying CYP3A5*1 allele compared to non-expressers (CYP3A5*3) to achieve the target C0 according to time

post transplantation. In these cases, therapeutic drug monitoring is performed as a tool to aid tacrolimus titration until reaching the target range. In agreement with our results, others reported that donor CYP3A5 expression significantly decreased tacrolimus C0/D due to a 30% increase in tacrolimus clearance in a Japanese pediatric liver transplant population (11) and that tacrolimus daily dose requirement was higher among French children who received a liver expressing CYP3A5 compared with those with a CYP3A5*3 liver (21). The association between donor CYP3A5 genotype and tacrolimus disposition was also reported specifically on the first day after transplantation pediatric liver (46). On the other hand, considering the effect of the recipient CYP3A5-expression stratification we observed no association with tacrolimus exposure in our cohort of patients. However, previous studies in different populations did describe this association. In this sense Caucasian pediatric liver transplant recipients with CYP3A5 expression presented with an increased apparent clearance of tacrolimus compared with non-expressers (12). Furthermore, studies in Chinese pediatric liver transplant patients reported that CYP3A5 genotyping both in recipients and donors was necessary to establish a personalized tacrolimus dosage regimen (22). Therefore, donor genotype in addition to the patient genotype may play an important role in determining the tacrolimus pharmacokinetic response but results varied among studied populations. This highlights the necessity of further studies on the relationship between tacrolimus exposure and pharmacogenetics in both donors and liver pediatric transplant recipients.

Some covariates identified as influential on tacrolimus pharmacokinetics were not retained in our final model. One of the most important pharmacokinetic properties of tacrolimus is its high binding capacity to red blood cells. Several pharmacokinetic studies have reported a significant effect of hematocrit on tacrolimus dose requirements (10, 14). This effect was not observed in the present population, which may be explained in part by the partial recovery of hematocrit levels after the first month post-transplant during which considerable variation in

hematocrit is observed and multiple transfusions are required. The type of donor (living donor/deceased donor) was tested for potential significant association to tacrolimus C0 based on a potential impact of regeneration of the graft (liver) leading to improvement of hepatic function(47). Nonetheless, this variable was not significantly associated with tacrolimus exposure measured as C0 as shown in Table 2.

There are some limitations to be acknowledged in this study. First, because of its retrospective nature, it has all the limitations inherent to this type of descriptive study. Second, the area under the blood concentration-time curve (AUC) is expected to be a better marker of systemic exposure to tacrolimus than C0 (2); however, the AUC is difficult to obtain due to practical and ethical reasons in the pediatric population. Nevertheless, in children tacrolimus TDM is based on monitoring C0. Therefore, our results should be interpreted with caution. Third, we focused on the period after 30 days post-transplant in order to avoid the variability produced by hemodynamic alterations, interruption of doses, and different frequency intervals of tacrolimus administration. Finally, we also acknowledge that one of the reasons of the limited number of studied patients was the discontinuation of the genotyping technique due to reagents unavailability.

The starting dose of tacrolimus is usually based on bodyweight and then adjusted by means of therapeutic drug monitoring. Recently, a model was developed to predict the individual starting dose of tacrolimus in pediatric renal transplantation and the final model included bodyweight (48). However, limited information is available in pediatric liver transplant patients. Therefore, our study provides novel information about tacrolimus dosing based on body weight in children with liver transplant.

After external validation, this model could be used in clinical practice to make dosage recommendations accounting for the liver enzyme levels (ALT), genetics, and time post-transplant-dependent change in the log-C0/D of tacrolimus. For instance, we may assume two random patients in our study population with a body weight of 16.3 kg. The ALT level (UI/L) of Patient 1 is 1200 who underwent liver transplant 3.3 months before, whereas the ALT level in Patient 2 is 40 UI/L after receiving a liver transplant 9.3 months earlier. None of them is a graft expresser of CYP3A5. If tacrolimus trough concentrations have to be maintained at 5ng/ml, we could use the present model to calculate the tacrolimus doses. According to our model, the calculated dose for the first child would be 0.35 mg bid, while for the second child it would be 1.11 mg bid. This is in line with a setting in which lower tacrolimus doses are recommended in liver dysfunction to avoid systemic accumulation. If both patients have ALT levels of 40 UI/L, underwent surgery 4 months previously, and Patient 1 has a non-expresser graft while Patient 2 has an expresser graft, tacrolimus doses would be 0.6 mg bid and 1.7 mg bid, respectively.

In conclusion, in the present report we have developed a model to describe tacrolimus pharmacokinetics in children who underwent liver transplantation. This final LME model presented a suitable performance and predictive ability to adequate tacrolimus doses in future patients in order to minimize the occurrence of an out-of-range therapeutic window that may lead to adverse drug reactions or acute rejection. The results of this study may be used in the clinical setting in conjunction with therapeutic drug monitoring and may contribute to the development of programs to optimize tacrolimus dosing, taking into account not only patient body weight but also time post-transplant, genotype, and liver function.

Competing Interests

There are no competing interests to declare.

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Figure legends

Figure 1. Relation between Log-transformed dose normalized tacrolimus trough concentrations according to time post-transplantation (A), alanine aminotransferase values (B), and donor CYP3A5 genotype (C). Bivariate linear mixed effect model according to time post-transplant and donor CYP3A5 genotype.

* $p < 0.05$

Abbreviations: ALT: alanine aminotransferase; C0: tacrolimus trough concentrations; CYP3A5: donor CYP3A5 genotype (0: non-expressers, 1:expressers).

Figure 2. Goodness of fit plot of the final model. Observed vs. individual predicted tacrolimus concentrations.

Abbreviations: C0: tacrolimus trough concentrations (ng/ml); D: single tacrolimus dose (mg/kg).

Supporting information.

Supporting Figure S1. Plots of the standardized residuals *versus* fitted values (A), histogram of residuals (B) and quartile-quartile plots (QQ-plots) (C).

Table 1. Demographics and relevant medical history

Characteristics/Parameters	Model-building Data Set	Validation Data Set
Number of subjects	40	13
Age (years)† ¶	2.2 (0.5-17.6)	3.7 (0.8-12.2)
Sex (female/male)	24/16	10/3
Weight (kg)† ¶	16.3 (6.0-75.0)	19 (6.8-74)
Type of donor (deceased/living)	31/9	10/3
Follow-up time (months)† ¶	18.6 (1.3-25.9)	18.6 (1.4-30.9)
Graft type (complete/technical variant)	17/23	2/11
Primary Diagnosis	Number (%)	
Biliary atresia	16 (40)	6 (46)
Acute liver failure	9 (23)	2 (15)
Cholestatic cirrhosis‡	4 (10)	3 (23)
Hepatic cirrhosis: autoimmune and cryptogenic	6 (15)	1 (8)
Malignancies§	3 (7)	1 (8)
Metabolic disease: metabolic liver failure	2 (5)	0 (0)
Immunosuppressive therapy		
Basiliximab (10 to 20 mg/doses at days 0 and 4 after transplantation)	28 (70)	10 (77)
Tacrolimus (0.1 mg/kg/day)	40 (100)	13 (100)
Prednisone (1.25–3.75 mg/kg/day)	35 (88)	11 (86)
Mycophenolate mofetil (20–40 mg/kg/day)	20 (50)	7 (54)
Azathioprine (1-2 mg/kg/day)	3 (8)	2 (15)
Sirolimus (0.1 mg/kg/day)	4 (10)	1 (8)
Liver function and blood parameters¶	Mean (SD)	
AST (UI/L)	88.8 (120.5)	82.3 (100.9)
ALT (UI/L)	135.1 (153.3)	120.6 (139.2)
GGT (UI/L)	234.0 (305.4)	295.4 (346.4)
Total bilirubin (mg/dL)	2.3 (1.8)	2.3 (4.5)
Direct bilirubin (mg/dL)	0.9 (2.2)	2.3 (4.8)
Albumin (g/dL)	3.6 (0.6)	3.4 (0.6)
Hematocrit (%)	32.7 (4.6)	32.8 (5.1)
Serum creatinine (mg/dL)	0.4 (0.2)	0.5 (0.2)
Pharmacokinetic data ¶	Mean (SD)	
Total number of tacrolimus samples	824	352
Number of samples per patient†	16 (4-71)	29 (4-48)
Tacrolimus blood concentrations (ng/ml)	6.3 (2.6)	6.8 (2.7)
Tacrolimus daily dose (mg)	2.6 (2.1)	2.9 (2.3)
Tacrolimus daily dose normalized (mg/kg)	0.15 (0.10)	0.14 (0.10)
Dose normalized tacrolimus trough concentration [(ng/ml)/(mg/kg)]	114.39 (96.99)	149.27 (122.75)
Log-transformed dose normalized tacrolimus trough concentration	4.50 (0.68)	4.70 (0.80)

Abbreviations: AST, aspartate aminotransferase; ALT, alanine transaminase; GGT, gammaglutamyl transpeptidase.

†Data are expressed as median (range). ‡Including Alagille syndrome, congenital hepatic fibrosis, and sclerosing cholangitis. §Including hepatoblastoma and hepatocellular carcinoma. ¶ Continuous demographic data and clinical laboratory data recorded during the complete study period did not significantly differ between model-building and validation data sets (Mann-Whitney U test, $p>0.05$).

Table 2. Univariate linear mixed models for the log-transformed dose-normalized tacrolimus trough concentrations in pediatric liver transplant patients

Variables	Estimate (β)	Standard error	p-value
Laboratory parameters			
ALT (UI/L)	0.0005	0.0001	<0.001
Hematocrit (%)	-0.0005	0.0043	0.90
Total bilirubin (mg/dL)	0.032	0.012	0.01
Immunosuppressive scheme			
Co-administration of steroids and/or Mycophenolate mofetil/sodium (yes vs no)	0.115	0.043	0.01
Clinical parameters			
CMV infection (yes vs no)	-0.094	0.095	0.33
EBV infection (yes vs no)	-0.152	0.061	0.01
Time post-transplant (months)	0.023	0.004	<0.001
Transplant variables			
Type of donor (living vs deceased)	-0.163	0.207	0.44
Graft type (complete=1 vs technical variant=0)	0.143	0.171	0.41
Genetic variables			
Donor CYP3A5 polymorphism (expressers vs non-expressers)	-0.413	0.168	0.02

Abbreviations: ALT: alanine aminotransferase; CMV: cytomegalovirus; EBV: Epstein Barr Virus.

Table 3. Parameter estimates of the final linear mixed effects model and bootstrap results.

Parameters (units) ***Fixed effects***	**Estimates (%SE)**	**P value**	**2.5, 97.5 percentiles of the bootstrap**
(Intercept)	4.424 (10.644)	<0.001	4.186, 4.647
Time post-transplant per month	0.019 (0.437)	<0.001	0.010, 0.028
ALT (UI/L)	0.00030 (0.014)	0.03	0.00002, 0.00056
Donor CYP3A5 polymorphism (expressers vs non-expressers)	-0.349 (13.840)	0.02	-0.631,-0.062
Interaction term (Time*ALT)	0.00005 (0.002)	0.004	0.00001, 0.00008
Random effects			
Random effect on subject	0.580		0.439, 0.708
Random effect on slope	0.018		0.010, 0.025
Correlation between random effects	-0.852		-1.000, -0.645
Residual variability	0.350		0.332, 0.369

Abbreviations: ALT: alanine aminotransferase; SE: Standard error.