Population pharmacokinetic and pharmacogenetic analysis of tacrolimus in paediatric liver transplant patients

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- Although tacrolimus is the cornerstone immunosuppressant in children with liver transplantation, its use is complicated by its narrow therapeutic index and variable pharmacokinetics.
- Understanding the sources of pharmacokinetic variability is of prime importance in achieving therapeutic concentrations of tacrolimus and avoiding the risks of drug-related nephrotoxicity, treatment failure and organ rejection.

WHAT THIS STUDY ADDS

- Population pharmacokinetic modelling of tacrolimus in paediatric liver transplant patients has been conducted to evaluate the potential impact of demographic, clinical and genetically controlled factors on inter-patient pharmacokinetic variability within this population.
- The final model showed that in addition to weight and time post-transplantation, CYP3A5 genotype was a significant covariate that affected tacrolimus disposition in children following liver transplantation.

AIMS

To build a population pharmacokinetic model that describes the apparent clearance of tacrolimus and the potential demographic, clinical and genetically controlled factors that could lead to inter-patient pharmacokinetic variability within children following liver transplantation.

METHODS

The present study retrospectively examined tacrolimus whole blood pre-dose concentrations ($n = 628$) of 43 children during their first year post-liver transplantation. Population pharmacokinetic analysis was performed using the non-linear mixed effects modelling program (NONMEM) to determine the population mean parameter estimate of clearance and influential covariates.

RESULTS

The final model identified time post-transplantation and CYP3A5*1 allele as influential covariates on tacrolimus apparent clearance according to the following equation:

$$TVCL = 12.9 \times (\text{Weight}/13.2)^{0.75} \times \text{EXP}(-0.00158 \times \text{TPT}) \times \text{EXP}(0.428 \times \text{CYP3A5})$$

where TVCL is the typical value for apparent clearance, TPT is time post-transplantation in days and the CYP3A5 is 1 where *1 allele is present and 0 otherwise. The population estimate and inter-individual variability (%CV) of tacrolimus apparent clearance were found to be $0.977 \text{ l h}^{-1} \text{ kg}^{-1}$ (95% CI 0.958, 0.996) and 40.0%, respectively, while the residual variability between the observed and predicted concentrations was 35.4%.

CONCLUSION

Tacrolimus apparent clearance was influenced by time post-transplantation and CYP3A5 genotypes. The results of this study, once confirmed by a large scale prospective study, can be used in conjunction with therapeutic drug monitoring to recommend tacrolimus dose adjustments that take into account not only body weight but also genetic and time-related changes in tacrolimus clearance.

Introduction

With more than 45 years of accumulated experience since the first successful paediatric liver transplantation in 1967 [1], several advancements in surgical techniques, anaesthetic and immunosuppressive protocols have been accomplished that reflect positively on both patient and graft survival rates [2]. Tacrolimus is acknowledged to be a cornerstone immunosuppressant in most immunosuppressive protocols [3]. Since its introduction into clinical practice in the early 1990s, it has been shown to be efficacious and superior to ciclosporin in the prevention of rejection episodes in liver transplant patients in both adults and children [2, 3]. Despite these advantages, the use of this agent in the clinical setting is complicated by it having a narrow therapeutic window ($5\text{--}15\text{ ng ml}^{-1}$) and highly variable inter and intra-individual pharmacokinetics [4]. This high variability could lead to sub-therapeutic concentrations which may put the patient at a risk of rejection or high concentrations which may lead to severe side effects. These side effects include neurotoxicity, hyperglycaemia, infections and nephrotoxicity, which occurs in almost 50% of the patients taking this drug [5]. Therefore, a deep understanding of factors that influence tacrolimus pharmacokinetics is valuable in individualizing tacrolimus dosage regimens.

In the paediatric population, factors identified by previous population pharmacokinetic studies as having an important impact on tacrolimus apparent clearance include demographic and clinical parameters [6–11]. Genetic factors, although important, have not yet been fully explored in this vulnerable population. For example, tacrolimus is a substrate for the permeability-glycoprotein, P-gp and polymorphisms in this transporter may be implicated in the variable pharmacokinetics of the drug. P-gp, a product of the *ABCB1* gene (ATP binding cassette, subfamily B, member 1), acts as an efflux pump and, through the force of ATP, transports its substrates from the intracellular to the extracellular domain [12, 13]. Thus it is expected that genetic variations affecting P-gp expression or function will influence the pharmacokinetics of its substrates. Many single nucleotide polymorphisms (SNPs) have been identified in the *ABCB1* gene, of which three SNPs are of particular interest in relation to tacrolimus pharmacokinetics, i.e. two synonymous SNPs C3435T and C1236T, in addition to the non-synonymous SNP G2677T [12].

Tacrolimus also undergoes extensive metabolism in hepatocytes and enterocytes by the cytochrome P450 3A5 (CYP3A5) enzyme [14]. The most common and functionally important genetic polymorphism in the CYP3A5 gene, is CYP3A5*3 (G6986A) which results in a cryptic splicing site that prematurely truncates the CYP3A5 protein and leads to loss of enzymatic activity [15]. Patients having at least one functioning allele (CYP3A5*1 allele) are termed CYP3A5 expressers, while those homozygous

for the variant allele (CYP3A5*3 allele) are termed CYP3A5 non-expressers.

Due to the importance of the aforementioned enzymes/transporters in the disposition and clinical outcomes of tacrolimus, the present study was conducted to investigate the influence of both CYP3A5 and *ABCB1* gene polymorphisms as well as patient specific factors (such as age, race, time post-transplantation, concomitant medications (steroid dose), liver enzyme levels, serum creatinine and type of grafted liver) on tacrolimus disposition. In addition, the influence of genetic variations on the frequency of adverse drug reactions (nephrotoxicity) and clinical outcomes (frequency of rejection episodes) have been investigated during the first year following liver transplantation. To our knowledge, this is the first population pharmacokinetic study to report the pharmacokinetics of tacrolimus in Caucasian children with liver transplants. The present study also expands the available knowledge by looking at the effect of CYP3A5 and *ABCB1* genotypes on tacrolimus disposition over the first year post-transplant. This was done by building a robust model to describe tacrolimus apparent clearance in a cohort of transplant children and then assessing whether the sources of variability in the pharmacokinetic parameter estimated were demographic, clinical or genetically controlled factors.

Methods

Patients and data collection

Ethics approval was granted by the National Health Services Office for Research Ethics Committees in England (NHSRECs). Written informed consent was obtained from the parents/guardians of all children included in the study. Furthermore, verbal assent was obtained from children older than 10 years. Data were collected from the records of 43 children who underwent liver transplantation at Birmingham Children's Hospital, Birmingham, UK. The immunosuppressive protocol consisted mainly of tacrolimus in combination with low dose steroids, with tacrolimus being administered orally in the form of capsules or as an oral suspension. The following information was collected for patients recruited in the study: tacrolimus dosing information, tacrolimus pre-dose concentrations, date of birth, body weight, gender, race, date of transplantation, serum creatinine, concomitant medications (steroid dose), history of rejection episodes and type of transplanted graft (whole/partial liver). In addition, biochemical parameters such as serum bilirubin, albumin, alanine transferase and aspartate transaminase levels were obtained from patient records (where available) and investigated in a subset analysis for their effect on tacrolimus pharmacokinetics.

Tacrolimus pre-dose steady-state blood concentrations (ng ml^{-1}), collected approximately 12 h after a tacrolimus dose were recorded retrospectively for all samples taken over 1 year post-transplantation. Tacrolimus blood concen-

trations were measured using enzyme immunoassay (Abbot IMx assay, Abbot Laboratories Ltd, Maidenhead, Berkshire, UK). The lower limit of quantification for the assay was 2 ng ml⁻¹ and the assay was linear up to 30 ng ml⁻¹. The inter-assay variability ($n = 4$) ranged between 4.7% and 11.8% for concentrations comprising the lower, middle and higher portions of the calibration range (4.1, 10.3 and 16.9 ng ml⁻¹, respectively). A total of 628 pre-dose concentrations were used in the analysis.

Genotyping ABCB1 and CYP3A5 SNPs

Genetic profiling of DNA samples took place within the Genomic Core Facility, Queen's University Belfast. All patients were screened for CYP3A5*3 in addition to the three ABCB1 SNPs (C3435T, C1236T and G2677T).

For each patient, a 1 ml blood sample was used to extract genomic DNA using QIAmp® DNA Blood Mini Kit (Qiagen, Hilden, Germany). The concentration of the extracted DNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Detection of the various single nucleotide polymorphisms (SNPs) was carried out using validated TaqMan® genotyping assays obtained from Applied Biosystems (ABI, Foster City, USA). The TaqMan® primer/probe set designed for each SNP allele was included in the kits. Their sequences are provided in Table 1.

Polymerase chain reactions (PCR) were performed in a 384-well plate format in 5 µl reaction mixture containing 2.5 µl TaqMan® master mix and using an amplification protocol of 95°C for 10 min, followed by 40 cycles of 92°C for 15 s and then 60°C for 1 min. An allelic discrimination analysis was carried out using the ABI7900HT sequence detection system (ABI, Foster City, USA).

Influence of ABCB1 and CYP3A5 SNPs on tacrolimus-related nephrotoxicity and the frequency of biopsy proven rejection episodes

Nephrotoxicity related to tacrolimus use was defined as a reduction in the estimated glomerular filtration rate (eGFR)

by 30% compared with the pre-transplant baseline. Serum creatinine concentrations (mmol l⁻¹) recorded for each patient were used to calculate eGFR values pre-transplant and at 3, 6 and 12 months post-transplant using the modified Counahan–Barrat formula [eGFR (expressed as ml min⁻¹ 1.73 m⁻²) = 40 × height (cm)/serum creatinine (mmol l⁻¹)] [16].

The histopathology reports of 31 patients were reviewed and the occurrence of biopsy proven rejection episodes was recorded and correlated with the genotypes of interest.

Pharmacokinetic analysis

Population pharmacokinetic analysis was performed by means of non-linear mixed effect modelling using first order conditional estimation with interaction (NONMEM; version 7.2, ICON Development Solutions, Ellicott City, MD, USA). Other modelling tools included Perl Speaks NONMEM (PsN) toolkit [17] and Pirana [18] which was used to manage different runs, generate summary tables and reports. Xpose® (version 4.3.2) [19] was also used as a graphical tool to generate various plots throughout the modelling process.

Selecting the base model

A one compartment model with first order elimination and absorption was chosen to fit the data. Since the observations collected were pre-dose concentrations from therapeutic drug monitoring records, no information could be derived relating to the apparent volume of distribution or absorption rate constant (k_a) and hence their values were fixed according to literature values. k_a was fixed to 4.5 h⁻¹ [9], while the apparent volume of distribution was fixed to 30 l kg⁻¹ [7, 8].

Inclusion of weight in the base model was attempted using linear, power in addition to allometric equations. When the allometric approach was used, apparent clearance was standardized by the median weight of the

Table 1

ABCB1 and CYP3A5 SNPs characteristics and sequences of TaqMan® probes designed for allele detection as supplied by the manufacturer

SNP	Location	NCBI reference	Probe sequence	Reporter 1 dye	Reporter 2 dye
ABCB1 C (1236)->T	Chr7	rs1128503	GCCCACTCTGCACCTTCAGGTTTCAG[A/G]CCCTTCAAGATCTACCAGGACGAGT	VIC	FAM
ABCB1 C (3435)->T	Chr7	rs1045642	TGTTGGCCTCCTTTGCTGCCCTCAC[A/G]ATCTCTTCTGTGACACCAACCCGGC	VIC	FAM
CYP3A5 A (6986)->G	Chr7	rs776746	ATGTGGTCCAACAGGGAAGAGATA[T/C]TGAAAGACAAAAGAGCTCTTTAAAG	VIC	FAM
			Primer sequence	Probe sequence	
ABCB1 G (2677)->T	Chr7	rs2032582	F: 5'-GTCTGGACAAGCACTGAAAGATAAGA-3' R: 5'-CTTAGAGCATAGTAAGCAGTAGGGAGT-3'	TTCCAG[C/A]ACCTTC	VIC FAM

ABCB1, ATP-binding cassette sub-family B member 1, Chr, chromosome. NCBI, National Centre for Biotechnology Information. VIC and FAM are two different fluorescent reporter dyes available commercially from Applied Biosystems® (P/N 4316033).

studied population (13.2 kg) and adjusted for size using the following equation:

$$CL_i = CL_{std} \times (Wt_i / Wt_{std})^{pwr}$$

where CL_i is the apparent clearance in the i th individual with weight Wt_i , CL_{std} is the apparent clearance in an individual with a standard weight of 13.2 kg, pwr is the allometric power coefficient fixed to 0.75 or estimated as an additional theta (θ) in the model. The allometric approach is well established and has been used successfully to describe the pharmacokinetics of several drugs given to the paediatric population [20, 21]. Using allometric equations in which weight is used as a covariate for size as suggested by Fukudo *et al.* was also attempted [11].

An exponential error model was chosen for inter-individual variability as the distribution of pharmacokinetic parameters usually follows a log normal distribution. The residual variability, which represents the difference between the observed and model predicted concentrations, was modelled using the additive, proportional and combined error structures:

$$C_{it} = C_{pred\ it} + \varepsilon_{it}$$

$$C_{it} = C_{pred\ it} \times (1 + \varepsilon_{it})$$

$$C_{it} = C_{pred\ it} \times (1 + \varepsilon_{it}) + \varepsilon_{it}$$

where C_{it} , $C_{pred\ it}$ are the measured and model predicted tacrolimus concentrations, respectively, of the i th individual at the t th sampling time. The term ε_{it} is the residual error term where ε is a normally distributed random variable with a mean of zero and a variance of σ^2 . Residual variability can arise from assay errors, random fluctuation in an individual's parameter values, inaccuracy in recording the timing of sample collection and dose administration, non-compliance to therapy or model misspecification. Inter-occasion variability on clearance was also examined in the model.

Inclusion of covariates

Covariates were initially tested for the presence of correlations using both visual inspection and statistical analysis. A stepwise forward inclusion and backward elimination procedure was then employed to test the influence of certain covariates on tacrolimus apparent clearance. In the forward inclusion, each candidate covariate was introduced individually into the base model to form an intermediate model. The difference in the value of the

minimum objective function (OBJF) between the intermediate and base model was used to compare statistically these hierarchical models. This difference is proportional to $-2 \log$ likelihood of the data which approximates a chi square distribution with degrees of freedom equal to n , where n equals the number of fixed effect parameters (θ) introduced into the model. A decrease in the OBJF by 3.841 points was considered to be statistically significant ($P < 0.05$, 1 degree of freedom) at this stage. The covariate that caused the largest decrease in objective function was included in the model and the process repeated until no covariate was able to reduce the objective function by more than 3.841 points. The model obtained at the end of this step was used for the backward elimination step where each covariate was removed from the model individually by fixing its value to zero. Covariates that failed to increase the objective function by more than 6.635 were removed from the model one at a time. This process was repeated until all the remaining covariates increased the objective function by more than 6.635 points when removed ($P < 0.01$, 1 degree of freedom).

Each continuous covariate was tested in a proportional, additive, exponential or power form with or without centring to the median covariate value. Categorical covariates were tested using the additive or proportional forms only.

Model evaluation

Once the model was finalized, it was inspected for the presence of influential observations or individuals that could affect parameter estimates or the parameter covariate relationships.

To investigate the impact of each patient in the data set, case deletion diagnostics was conducted where each patient was removed individually from the model and the model was re-run. For each patient removed, the percentage change in parameter estimates relative to the final model (with all the individuals included) was calculated. A cut-off point of 20% was used to define influential individuals [22].

A bootstrap technique was also used to evaluate the stability and accuracy of the final model and to calculate the 95% confidence interval (CI) of parameter estimates. The mean values of the parameters obtained from the bootstrap were then compared with the values obtained from the final model. The final model was then used in a simulation study to obtain the changes in apparent clearance over time in both CYP3A5 expressers and non-expressers. The model was simulated 1000 times for two typical individuals (expresser vs. non-expresser) assuming that a dose of 2.3 mg day⁻¹ was administered.

Statistical analysis

Statistical analysis was performed using SPSS® software (version 19, SPSS Inc, USA). For each SNP, deviation from Hardy–Weinberg Equation (HWE) was tested using either the chi square or Fisher's exact test as appropriate. The

Table 2

Demographic and pharmacokinetic characteristics of the study population data

Demographic data*	
Number of patients	43
Age (years)	5 (0.65–17.56)
Weight (kg)	21.6 (6.06–69.95)
Gender	19 females 24 males
Time post-transplantation (TPT) (days)	153.48 [15–364]
Type of transplant	Full 9 Partial 34
Race	31 Caucasian 9 Asian 3 African Caribbean
Pharmacokinetic data	
Total tacrolimus samples	628
Tacrolimus blood concentrations (ng ml ⁻¹)	8.93 (1.2–26.4)
Number of samples per patient	15 [3–28]
Tacrolimus dose (mg day ⁻¹)	4.6 (0.4–30)
Tacrolimus dose (mg kg ⁻¹ day ⁻¹)	0.25 (0.03–1.23)
Corticosteroid dose	5.71 [0–30]

*Expressed as mean (range) as appropriate.

Mann-Whitney U-test was used to assess the statistical significance of the difference in dosage requirements between CYP3A5 expressers and non-expressers. Haploview 4.2 program [23] was used to calculate the frequency of the ABCB1 haplotypes (C1236T-G2677T-C3435T) and the absolute standardized linkage disequilibrium coefficient [D'] between the different pairs of ABCB1 SNPs. Correlations among covariates were tested using a Pearson correlation test for continuous covariates and a chi-square test for categorical covariates. A chi-square test was also used to assess the differences in the frequency of variant alleles amongst patients who developed nephrotoxicity and biopsy proven rejection episodes as opposed to those who did not.

Results

Patient characteristics

A total of 43 paediatric patients were recruited in this study. The demographic and pharmacokinetic characteristics of the recruited patients are summarized in Table 2. The mean $\pm$ SD tacrolimus concentration measured was 8.93 ± 3.75 ng ml⁻¹ while mean value for dosages administered was 0.25 ± 0.19 mg kg⁻¹ day⁻¹. There was a significant difference ($P < 0.05$) in dosage requirements between CYP3A5*3 expressers and non-expressers. Figure 1 shows the median monthly changes in dosage requirements in patients having either genotype over time.

ABCB1 and CYP3A5 genotype frequencies

All patients were screened for the CYP3A5*3 and the three ABCB1 SNPs of interest. In all cases, the observed genotype

Figure 1

Median value for $C:D$ over time post-transplantation. $C:D$ ratio is defined as pre-dose concentration over dose per kg and represents a measure of dose requirement. Open circles (red) represent mean $C:D$ for patients carrying *3/*3 genotype, while closed circles (blue) represent individuals carrying the *1/*3 and *1/*1 genotypes

Table 3

Genotype frequencies of ABCB1 and CYP3A5 SNPs in the study patients

RS number	Genetic variation	Genotype	Frequency (%)
1128503	C3435T	C/C	34.9
		C/T	41.9
		T/T	23.2
1128503	C1236T	C/C	34.9
		C/T	37.2
		T/T	27.9
2032582	G2677T	G/G	30.2
		G/T	41.9
		T/T	27.9
776746	CYP3A5	*1/*1	4.7
		*1/*3	20.9
		*3/*3	74.4

frequencies were not significantly different from those predicted by the Hardy–Weinberg equilibrium. The frequency of each genotype is shown in Table 3. Linkage analysis of the ABCB1 (C1236T, G2677T, C3435T) showed that the D' between C1236T and G2677T was 1 and between G2677T and C3435T was 0.745 while D' was 0.737 between C1236T and C3435T. This indicates that the SNPs are in linkage disequilibrium. Out of the eight possible haplotypes the C–G–C haplotype was the most frequent (47%) followed by the (T–T–T) haplotype (36.9%).

Influence of ABCB1 and CYP3A5 SNPs on tacrolimus-related nephrotoxicity and the frequency of biopsy proven rejection episodes

Single nucleotide analysis over the first post-transplant year showed that the incidence of nephrotoxicity at

6 months was higher among patients carrying the G2677->T (64.7% in nephrotoxic patients vs. 35.3% in controls, $P = 0.009$), C3435->T (64.7% in nephrotoxic patients vs. 35% in controls, $P = 0.009$) and the C1236->T (70.6% in nephrotoxic patients vs. 29.4% in controls, $P = 0.024$). Haplotype analysis has also shown a significant association between T-T-T haplotype and the incidence of nephrotoxicity, as higher incidence of nephrotoxicity was noted in carriers of the T-T-T haplotype at 6 months post-transplantation (56.8% in nephrotoxic patients vs. 29.9% in controls, $P = 0.026$). On the other hand, a lower incidence of nephrotoxicity was associated with carriers of the C-G-C haplotype at 6 months post-transplant (28.3% in nephrotoxic patients vs. 57.4% in controls, $P = 0.018$). No such association was detected at 3 or 12 months or with CYP3A5 genotypes. In addition, there was no significant difference in the frequency of variant alleles or haplotypes in the genotypes of interest amongst patients who experienced rejection episodes and those who did not.

Population pharmacokinetic analysis

NONMEM analysis was performed using 628 blood concentrations relating to the 43 children enrolled. A one compartment model with first order absorption and elimination adequately described the disposition of tacrolimus in whole blood. As mentioned earlier, the lack of samples collected shortly after tacrolimus administration did not enable evaluation of the distribution phase and hence a two compartment model could not be used to fit the data.

Since the additive term of residual error variance approached 0, it was removed resulting in a proportional error model that was considered adequate to describe

residual variability. In the early stages of model development inclusion of weight in the model was attempted in various forms. Inclusion of weight in the base model as an allometric function with fixed exponent resulted in a significant improvement in the fit and reduced the inter-individual variability by 22.9%. Investigation of other models including where the power values were not fixed but included as additional θ s did not result in any significant improvement in model fit. No correlation was detected between race and the various genotypes tested. However, a moderate inverse correlation was observed between time post-transplantation and corticosteroid dose ($r = -0.405$, $P < 0.05$).

Covariate selection and final pharmacokinetic model

Univariate analysis of the covariates tested in the present study showed that inclusion of time post-transplantation (TPT), race, age, ABCB1 and CYP3A5 polymorphisms each caused a significant decrease in the objective function ($P < 0.05$), Table 4.

TPT was first included in the base model as it gave the largest decrease in OBJF ($P < 0.001$) followed by CYP3A5 polymorphism ($P < 0.01$). Subsequent additions of other covariates did not result in a significant decrease in the objective function. In the backward elimination step, the OBJF increased significantly ($P < 0.01$) when either of TPT or CYP3A5 was removed from the model. Hence, both covariates were retained in the final model. The results of the subset analysis showed that none of the tested biochemical parameters influenced tacrolimus apparent clearance and hence they were not included in the subsequent analysis. In addition, the effect of concomitantly

Table 4

Summary of main models evaluated during covariate selection

Model structure	-2LLD ^{1st} selection*	-2LLD ^{2nd} selection*	-2LLD ^{3rd} selection
$CL/F = \theta_1 \times (\text{weight}/13.2)^{0.75}$	–	–	
$CL/F = \theta_1 \times (\text{weight}/13.2)^{0.75} \times e^{\theta_2 \times \text{TPT}}$	–150.707*		
$CL/F = \theta_1 \times (\text{weight}/13.2)^{0.75} \times \text{Age}^{\theta_2}$	–52.225	–0.092	
$CL/F = \theta_1 \times (\text{weight}/13.2)^{0.75} \times e^{\theta_2 \times \text{CYP3A5}}$	–9.331	–8.22*	
$CL/F = \theta_1 \times (\text{weight}/13.2)^{0.75} + \text{C1236T} \times \theta_2$	–21.486	–0.05	
$CL/F = \theta_1 \times (\text{weight}/13.2)^{0.75} + \text{G2677T} \times \theta_2$	–10.156	–1.335	
$CL/F = \theta_1 \times (\text{weight}/13.2)^{0.75} + \text{C3435T} \times \theta_2$	–21.192	–0.796	
$CL/F = \theta_1 \times (\text{weight}/13.2)^{0.75} + \text{Race} \times \theta_2$	–5.017	–5.08	–1.935
$CL/F = \theta_1 \times (\text{weight}/13.2)^{0.75} \times (1 + \theta_2 \times \text{Type})$	–1.71		
$CL/F = \theta_1 \times (\text{weight}/13.2)^{0.75} + \text{Gender} \times \theta_2$	–0.493		
$CL/F = \theta_1 \times (\text{weight}/13.2)^{0.75} + \text{HAP} \times \theta_2$	–1.012		
$CL/F = \theta_1 \times (\text{weight}/13.2)^{0.75} \times e^{\theta_2 \times \text{CSD}}$	–37.99	–0.006	

CL/F: apparent clearance, θ : Fixed effect parameter estimated by NONMEM, TPT: time post-transplantation (measured in days), CYP3A5: coded as 1 in case of presence of at least one *1 allele 0 otherwise, C1236T, G2677T, C3435T: coded as 1 in case at least 1 variant allele (T) is present 0 otherwise, Race: is coded as 1 for Asian and African Caribbean patients and 0 for Caucasian patients, Type: is the type of grafted liver (whole/partial) coded as 1 in cases where the patient received a whole liver or 0 in cases of a partial liver. HAP: ABCB1 haplotype (C1236T-G2677T-C3435T), patients carrying at least one copy of the variant haplotype were coded 1 otherwise 0. CSD: corticosteroid dose (mg). -2LLD: -2 log likelihood difference (a reduction in the value of the objective function).

*The covariate with the highest decrease in objective function was included in the model for subsequent analysis.

administered corticosteroid therapy was not significant enough to be retained in the final model. The final model was:

$$\text{Apparent clearance (CL/F)} (\text{lh}^{-1}) = 12.9 \times (\text{weight}/13.2)^{0.75} \times e^{-0.00158 \times \text{TPT}} \times e^{0.428 \times \text{CYP3A5}}$$

Our results showed that the only influential genetic covariate is the CYP3A5 genotype since neither the ABCB1 SNPs nor their haplotypes had a significant influence on the apparent clearance. This model can be used in clinical practice (if validated externally) to make dosage recommendations that account for the genetic and time dependent change in clearance. For example assume that tacrolimus trough concentrations have to be maintained at 5 ng ml⁻¹ in two children who weigh 13.2 kg and who are non-expressers for the CYP3A5. The first child had his transplant 14 weeks ago while the second child had his transplant 40 weeks ago. The calculated apparent clearance for the first child according to the developed model is:

$$\begin{aligned} \text{CL/F} (\text{lh}^{-1}) &= 12.9 \times (13.2/13.2)^{0.75} \times e^{-0.00158 \times 14 \times 7} \times e^{0.428 \times 0} \\ &= 11.05 \text{lh}^{-1}. \end{aligned}$$

The total dose requirement per day, therefore, would be = 11.05 l h⁻¹ × 5 ng ml⁻¹ × 24 h = 1.3 mg day⁻¹. Using similar calculations, the apparent clearance for the second child is 8.29 l h⁻¹ and the total daily dose requirement is 1 mg day⁻¹. Should the same two children have been CYP3A5 expressers, the total dose requirement would need to be increased to 2 mg day⁻¹ for the first child and to 1.5 mg day⁻¹ for the second child.

The inter-individual variability of clearance in the final model was 40%, while the residual (unexplained) variability between the observed and model predicted concentration was found to be 35.4%. When included in the model, the inter-occasion variability on clearance was below 15% and did not result in any significant change in the parameter estimated.

Model evaluation

The plots of observed against both population and individual predicted tacrolimus concentrations in the final model (Figure 2), demonstrated a reasonable agreement around the line of identity. The scatter plots of residuals vs. model-predicted tacrolimus concentrations showed that 99% of the observations were within the acceptable limit of ± 3 units of the null ordinate of perfect agreement.

The results of the case deletion diagnostics (influence analysis) indicated that none of the patients showed profound influence on any of the parameters estimated by NONMEM as the percentage change in parameter estimates, when each individual was removed, relative to the final

model (with all individuals included) was less than 20% in all parameter estimates across all individuals.

All of the 2000 bootstrap runs minimized successfully and the parameter estimates of the final model fell within the 95% CI obtained from the bootstrap analysis. Furthermore the median value for the structural and statistical parameters obtained from the bootstrap analysis was almost identical to those obtained from the final model (% bias was less than 6% for all model parameters), suggesting that the model is accurate and robust. Table 5 summarizes the final model estimates in addition to the results obtained from the bootstrap analysis.

The results of the simulation study are shown in Figure 3. Apparent clearance decreased with time and was always higher in CYP3A5 expressers.

Discussion

To our knowledge, this is the first population pharmacokinetic study which investigates the influence of genetic variations in both the ABCB1 and CYP3A5 genes on tacrolimus disposition and clinical outcomes in Caucasian children with liver transplants. The observed frequencies of the variant alleles of ABCB1 and CYP3A5 SNPs did not deviate significantly from those expected by the Hardy–Weinberg equation and were similar to those reported in the literature [11, 24]. A one compartment model with linear absorption and elimination was used to describe the data. Body weight, time post-transplantation and CYP3A5 genotype were able to explain part of the inter-individual variability in the apparent clearance. The value of apparent clearance in a typical individual in this population was 0.977 l h⁻¹ kg⁻¹ which is within the reported range of apparent clearance (0.075–1.85 l h⁻¹ kg⁻¹) [6–11] in children with liver transplants. As expected, this value is almost three times that reported in the adult population (0.36 l h⁻¹ kg⁻¹) [25, 26] which explains why children need as much as a 2.7 to 4.4 times higher dose per kg than adults to reach the same pre-dose concentration [27].

This study found that the apparent clearance of tacrolimus increased 1.53 times in an individual carrying the wild-type allele compared with an individual homozygous for the variant allele. This strong influence of the recipient's genotype was also reported by Li *et al.* [28] in adult patients where tacrolimus apparent clearance increased by 56% in adult recipients expressing the CYP3A5 enzyme. In the clinical setting, those results suggest that carriers of the CYP3A5*1 allele require a higher dose than patients carrying the *3/*3 genotype (Figure 1) which in theory means that those patients are at a higher risk of having rejection episodes if the dose was not increased. However, such correlation between CYP3A5 genotypes and rejection episodes was not observed in a subset of 31 patients investigated in the present study. This

Figure 2

Goodness of fit plots. Plot of conditional weighted residuals vs. population prediction (A) and individual prediction (B) of the final model. Plot of observed vs. population predicted concentration (C) and individual predicted concentration (D) of the final model. The black solid line represents the line of identity

Table 5

Parameter estimates of the final population pharmacokinetic model and bootstrap results

Parameter	Estimate (%RSE)	Median bootstrap estimate	95% CI	% Bias
θ_1 (l h ⁻¹)	12.92 (4.8%)	12.928	10.8, 15.2	0.03
θ_2 (day ⁻¹)	-0.00158 (13.8)	-0.00159	-0.002, -0.001	0.42
θ_3	0.4282 (25.5)	0.4281	0.14, 0.73	-0.02
ω^2	0.16 (19.8)	0.151	0.1, 0.21	-5.55
σ^2	0.125 (11.9)	0.123	0.09, 0.153	-0.96

θ_1 : Mean value of apparent clearance; θ_2 : factor of TPT (day⁻¹) on apparent clearance; θ_3 : factor of CYP3A5*1 on apparent clearance; ω^2 : inter-patient variance of the individual apparent clearance around the population apparent clearance; σ^2 : intra-patient variance of the observed pre-dose concentration around the predicted concentration; 95% CI: 95% confidence interval for each parameter calculated as 2.5, 97.5 percentiles of the bootstrapped dataset; % Bias: relative bias of the estimates of the final population pharmacokinetic model to the median estimates of the 2000 bootstraps.

Figure 3

Time related changes of tacrolimus apparent clearance in a typical individual taking a dose of 2.3 mg day^{-1} ($0.17 \text{ mg kg}^{-1} 12 \text{ h}^{-1}$). At each time point, 1000 simulations were conducted and the mean of each simulation and 95% CI are shown. The upper line (blue) represents the pattern of change in tacrolimus apparent clearance in a typical patient carrying the CYP3A5 $*3/*1$ or $*1/*1$ genotypes while the lower line (red) represents a patient carrying the $*3/*3$ genotype

could be explained by the fact that in the case of liver transplant patients, the donor genotype in addition to the recipient genotype can play an important part in determining the pharmacokinetic and pharmacodynamic response of tacrolimus. This highlights the necessity for extensive future research on the complex interplay between tacrolimus pharmacogenetics in both the donor and recipient of the liver transplant along with the pharmacokinetic/pharmacodynamic aspects of drug metabolism and response.

A recent literature review on ABCB1 pharmacogenetics by Shuker *et al.* [13] reported that studies so far have failed to establish a clear relationship between tacrolimus pharmacokinetics and ABCB1 pharmacogenetics in kidney and heart transplant patients. As for liver transplants, the majority of studies conducted to date have investigated the initial postoperative period (1–5 weeks post-transplant) and found a lack of association between ABCB1 pharmacogenetics and tacrolimus pharmacokinetics [29]. The current study has extended the period of investigation to include the whole first postoperative year, and it has shown that the ABCB1 SNPs do not significantly influence tacrolimus pharmacokinetics over the first year post-transplantation. The effect of these SNPs, however, might be stronger over the longer term (3 and 4 years post-transplantation) [29]. As for the relationship between the pharmacogenetics and clinical outcomes, single marker analysis showed that all the three ABCB1 SNPs were associated with nephrotoxicity at 6 months. This was also confirmed by the results of haplotype analysis which is

considered to be superior to single SNP analysis. It has been reported that following liver transplantation in children, the reduction in the eGFR reaches its maximum at 6 months post-transplant but then gradually improves as the tacrolimus dose is decreased over time [30]. This time-related change in eGFR could explain why the influence of the examined genotypes was easier to detect at 6 months following transplantation. The current results are in concordance with our previous findings [29]. To date, the influence of CYP3A5 SNP on nephrotoxicity following liver transplant has only been investigated in adults by Fukudo *et al.* [31]. This is the first study to investigate its effect in paediatric liver transplant patients. The results of this study have shown that CYP3A5 in the enterocytes was not related to the incidence of nephrotoxicity. Of note, however, such a result could be specific to the way nephrotoxicity was defined in the present study (i.e. 30% reduction in eGFR from the pretransplant baseline) as opposed to other ways that can be used for defining nephrotoxicity.

Similar values of apparent clearance per kg were previously obtained in Asian and Caucasian adult liver transplant patients [25, 26] indicating lack of racial influence on this parameter. In this study we are able to demonstrate a similar relationship in children with liver transplant. In a previous study by Staatz *et al.* [8] the apparent clearance of tacrolimus was higher in patients receiving a full paediatric liver graft compared with those receiving a split liver from an adult source. The authors attributed this finding to the higher metabolic capacity of the CYP enzymes present in the liver from younger donors as compared with those present in the liver from adult donors. Our results and the results of Wallin *et al.* [7] have shown that graft type is not an influential covariate, as its inclusion into the model did not result in a significant improvement in the model fit. This discrepancy, however, could be explained by the fact that the majority of children receiving a full paediatric liver in the current study received it from older children.

Few studies have detected a relationship between tacrolimus apparent clearance and time post-transplantation. The exact nature of this relationship, however, is still controversial as tacrolimus clearance was found to decrease with time in one study [10] while other studies showed an initial increase followed by a plateau, an increase in linear or even a sigmoidal manner [6, 7, 11]. While higher corticosteroid doses given in the first few weeks following transplant may result in metabolic induction which could explain the initial increase in apparent clearance, it is evident that more studies are required to establish the exact pattern of time related changes in tacrolimus clearance, which is of critical importance in determining an individualized dosage regimen for patients. Most of the studies to date have investigated the effect of time in the immediate post-operative period, e.g. Fukudo *et al.* collected data for their study during the

first 50 postoperative days and demonstrated a time-dependent increase of apparent clearance of tacrolimus within the first 3 weeks post-transplant. In the present study, scarcity of collected data within initial period (as demonstrated by increased variability in the weighted residual plot during the third week) did not allow us to note the initial increase in clearance over the first 3 weeks. However, the different time frame investigated during this study (2 weeks–1 year) with the majority of observations being collected after the first month of transplant allowed us to investigate the effect of time over longer periods beyond the initial 50 days post-transplant. We found that tacrolimus apparent clearance decreases in an exponential manner with time. This is confirmed by the significant decrease in dosage requirements over the first year in the present study and previous adult studies. For instance, as much as a 63% reduction in dose requirements within the first year post-transplantation has been observed in adult liver transplant patients [32]. Such an observation could be explained by a decrease in tacrolimus clearance or increased bioavailability over time or both.

The present study is limited by the fact that the data were collected retrospectively from patient records generated during routine therapeutic monitoring. As a consequence, only trough concentrations were available for analysis. This allowed us to estimate only the apparent clearance of tacrolimus while other pharmacokinetics parameters such as the volume of distribution and absorption rate constant were obtained from the literature. These data, however, are extremely valuable especially in the paediatric population where prospective pharmacokinetic studies are difficult to conduct due to ethical and financial limitations. Tacrolimus blood concentrations in the present study were measured using enzyme immunoassay. Immunoassays are known to have some degree of non-specificity as they tend to react with the parent compound and its metabolites. Assay errors could contribute to increased residual (unexplained) variability and might have impacted on the ability of our model to detect additional covariates. Although the cross reactivity has not been determined experimentally for the current study, previous population pharmacokinetic studies in kidney transplant patients have reported that the cross reactivity of tacrolimus immunoassay for other metabolites is less than the minimum detectable sensitivity [33].

In conclusion, we have developed a stable and robust model to describe tacrolimus pharmacokinetics in children who have undergone liver transplantation. This is the first population pharmacokinetic study to evaluate the influence of the ABCB1 polymorphisms on tacrolimus apparent clearance in paediatric liver transplant patients and to demonstrate the influence of the CYP3A5 recipient genotype. The results of this study, if verified by a prospective study, can be used in the clinical setting in conjunction with therapeutic drug monitoring for making dosage

adjustments that take into account not only body weight but also time related changes in tacrolimus clearance and patient genotype.

Competing Interests

All authors have completed the Unified Competing Interest form at http://www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare no support from any organization for the submitted work.

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