APPLICATION OF PHARMACOMETRICS FOR COVARIATE SELECTION AND DOSE OPTIMIZATION OF TACROLIMUS IN ADULT KIDNEY TRANSPLANT RECIPIENTS

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Dedication

“To My Grandfather in Heaven”

Abstract

In spite of rigorous dose adjustments by way of therapeutic drug monitoring, a large proportion of kidney transplant recipients are unable to achieve the target tacrolimus trough concentrations. This is attributed to the narrow therapeutic window of the drug (10-15 ng/mL) and large inter-individual variability in pharmacokinetic parameter such as clearance. There is a need for development of clinical dosing models that can help prospectively predict the dose for an individual, especially in the critical period immediately post-transplant. Therefore, we established and quantified the effect of clinical and genetic factors on tacrolimus clearance (CL/F) using a large population of adult kidney transplant recipients. Tacrolimus troughs (n=11823) from 681 transplant recipients over the first 6-months post-transplant were analyzed using non-linear mixed effects modeling approach in NONMEM®. The troughs were characterized by a steady state infusion model. Covariates were analyzed using a forward selection ($p<0.0.1$) backward elimination ($p<0.001$) approach. We formulated an equation that predicts the CL/F of an individual based on the days post-transplant, presence of the highly influential CYP3A5*1 genotype, transplant at a steroid sparing center, age and concomitant use of a calcium channel blocker at the time of trough collection. The CL/F was seen to decrease with increasing days post transplant, transplant at a steroid sparing center and use of a calcium channel blocker. Transplant recipients with the CYP3A5*1/*3 and *1/*1 genotypes had a CL/F that was 70% and 100% higher, respectively, than those with the CYP3A5*3/*3 genotype. The dose required in order to achieve a particular target trough can be prospectively determined from this equation.

The above equation was validated in a separate cohort of adult kidney transplant recipients. The equation was assessed by predictive performance in 795 transplant recipients (n=13,968 troughs) receiving tacrolimus using bias and precision. Assessment was done for the initial troughs as well as for all troughs over the entire 6 months. The equation has low bias (0.2 ng/ml) and good precision (within ± 20% for a typical trough of 10 ng/mL) in predicting initial troughs and could be safely used to predict initial doses.

This is critical as an accurate initial dose will help the recipient to get to therapeutic range faster and reduce the number of out-of-range troughs. For all the troughs, over the 6 months post-transplant, the equation did better than a basic model with no covariates but had higher bias and imprecision than the prediction of initial troughs.

We were presented with 119 single nucleotide polymorphisms (SNP) in this study. Due to software limitations and impracticalities associated with such a large number of covariates, we developed and validated a novel "winnowing method" of covariate selection that is able to test and select SNPs in combination. This method uses random selection, repetitions of generalized additive modeling in the R statistical package and post-hoc estimates from NONMEM®. The salient feature of this method is the creation of an index, ranging from 0-1, that defines the relative importance of the SNP when tested in a combination. With this method, we were able to select 26 SNPs out of the 119 SNPs, which included the well-established CYP3A5*1 SNP. We validated this method using a simulated dataset. In the validation dataset, the winnowing method was able to select all the important SNPs. The type I and type II error rates were 9% and 0% respectively.

Although NONMEM® is the oldest and most widely used population pharmacokinetics software, several other software packages are now becoming available such as the Phoenix® NLME™. One desirable feature in this new software package is a graphical user interface and menu-driven covariate selection options. Therefore, we compared these two software packages in terms of covariates selected and predictive performance using both clinical and simulated data. For the tacrolimus data, NONMEM® predictions had lower bias and imprecision as compared to Phoenix® NLME™. For the clinical data, NONMEM® predictions had higher bias but were more precise than the Phoenix® NLME™ predictions.

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Chapter I

Introduction

1.1 Kidney transplantation

Kidney transplantation is recommended for patients with Stage 4 or 5 end stage chronic renal disease (ESRD) healthy enough to withstand transplant or without significant comorbidities. According to the Scientific Registry of Transplant Recipients (SRTR) Annual Data Report in 2010, 16,830 patients underwent kidney transplantation in 2009. Transplantation is associated with increased survival and improved quality of life for patients with ESRD [1, 2]. The five year survival rate for a recipient with a primary kidney transplant in U.S. is approximately 72% based on the reports from the Organ Procurement and Transplantation Network (OPTN).

A transplantation procedure involves the surgical placement of a kidney in the extra peritoneal space of a recipient [2]. The transplanted kidney can be donated by either a living or a deceased donor, the former being preferred. The five year allograft survival rate from a living donor is 80% whereas from a deceased donor the allograft survival is 67% [2]. Human leukocyte antigen (HLA) matching is done prior to selection of a specific donor kidney for a particular recipient since HLA markers play a vital role in humoral and cellular mediated immune responses [3].

1.2 Immunosuppression

An immunosuppressive regimen is initiated post-transplant to prevent the patient's own cells from rejecting the kidney graft. Induction immunosuppression is started immediately post-transplant using monoclonal or polyclonal antibodies or interleukin-2 (IL-2) receptor antagonists such as basiliximab. These are usually given for 2-3 doses within the first month post-transplant but are not continued long term [4]. Additionally, a maintenance immunosuppressive regimen is initiated with calcineurin inhibitors (CNIs, such as tacrolimus or cyclosporine), antiproliferative agents (such as azathioprine, mycophenolate) and steroids (such as prednisone) [5].

According to the 2010 OPTN and SRTR Annual Data Report, approximately 80% of the initial immunosuppressive regimens in adult kidney transplant recipients in 2009 consisted of a combination of tacrolimus and mycophenolate. The same source reported

that approximately 75% of the immunosuppressive regimens at one year post-transplant in 2008 were comprised of a combination of tacrolimus and mychophenolate. Several studies have also demonstrated the superiority of tacrolimus over cyclosporine in improving graft survival and decreasing the incidence of acute rejection [6, 7]. This highlights the importance of tacrolimus in immunosuppressive regimens after kidney transplantation. Because of its importance, it is the topic of this thesis.

The use of steroids for immunosuppression has been a subject of controversy. Long term steroid use is associated with numerous adverse effects such as hypertension, hyperlipidemia, osteoporosis, cataracts, osteonecrosis and infections. Several regimens that avoid or minimize the use of steroids have been proposed such as low-dose steroids, steroids on alternate days, tapering of steroids (between 3-6 months or later than 6 months) and steroid sparing regimens (no steroid use or steroids withdrawn within the first 7 days post-transplant) [8, 9] . Research in the past 10 years has focused on eliminating or using short courses of steroids in immunosuppressive regimens. However, this requires optimal use of other agents such as calcineurin inhibitors.

1.3 Risks associated with suboptimal immunosuppression

Optimal immunosuppression is critical for long-term allograft function and survival of the transplant recipient [10]. Immunosuppression above the target threshold increases the risk for infection and malignancies due to decreased immune responses. It also increases the risk for common toxicities associated with high tacrolimus troughs such as neurotoxicity, nephrotoxicity, diabetogenesis, gastrointestinal disturbances and hypertension [11]. Suboptimal immunosuppression increases the risk of acute rejection of the transplanted organ, leading to loss of allograft function [4, 10].

Rejection of the transplanted kidney is one the main complications arising post-transplant. Rejection can occur within minutes post-transplant (hyper acute), within days to weeks post-transplant (acute) or after months or years post-transplant (chronic) [12]. Clinical symptoms of rejection include fever, pain at the transplant site, decreased urine output and an increase in serum creatinine. Although these are some of the clinical symptoms, rejection is confirmed only with a kidney biopsy [12]. Common factors

increasing the risk of rejection include HLA mismatch between donor and recipient, non-compliance with immunosuppressive therapy, older age of the recipient and African-American (AA) race and low immunosuppressant levels [13].

1.4 Tacrolimus pharmacology

1.4.1 Mechanism of action

Tacrolimus (FK 506) is a calcineurin inhibitor and is a very common immunosuppressant used in kidney transplantation. It binds to the cytosolic immunophilin, FK506 binding protein (FKBP). The TAC-FKBP complex further binds to the calcium dependent phosphatase, calcineurin. Calcineurin is responsible for the activation and translocation of nuclear factor of activated T-cells (NF-AT) into the nucleus. This NF-AT is required for transcription of IL-2 and other cytokine genes responsible for stimulation of T-cells. The binding of FKBP to calcinuerin prevents the activation and translocation of NF-AT and thus inhibits the transcription of IL-2 and other cytokines leading to the immunosuppressive response [14].

1.4.2 Clinical pharmacology

Tacrolimus has poor mean oral bioavailability (25%) and absorption is decreased in the presence of food. It is rapidly absorbed with a peak blood concentration occurring in about 0.5-1 hours after administration [11]. After absorption, tacrolimus is extensively distributed to erthyrocytes (95%) and blood concentrations are on average 15 times higher than the corresponding plasma concentrations. It is also 99% plasma protein bound (both to albumin and α-1 acid glycoprotein). Tacrolimus also crosses the placenta and can be found in breast milk. It undergoes extensive metabolism by cytochrome P450 3A isoenzymes (primarily 3A5) and < 0.5% of the parent drug is eliminated unchanged [11, 15, 16].

Whole blood concentrations are measured due to extensive distribution into erythrocytes. Trough concentrations are used as a surrogate for systemic exposure for tacrolimus [10]. It is routine clinical practice to utilize trough concentrations to guide dose modifications for tacrolimus. Higher troughs (10-15 ng/ml) are targeted in the first three months post-

transplant due to a higher risk of acute rejection in the early period post-transplant. Relatively lower troughs (8-12 ng/mL in months 3-12 post-transplant and 5-10 ng/mL after a year post-transplant) are targeted in the later period post-transplant when the risk of acute rejection is lower. These targets are institution specific and may vary based on the recipient's immunologic risk, choice of induction therapy and other maintenance immunosuppressants (such as steroids) [11].

Tacrolimus has a narrow therapeutic window with a wide inter-individual variability in pharmacokinetics [17]. Initial doses are based on weight and subsequent doses are adjusted by the trial and error approach using therapeutic drug monitoring (TDM). Despite these rigorous adjustments, a large proportion of trough concentrations are above or below the therapeutic range, especially in the immediate period post-transplantation thus putting the patient at immunologic or toxicity risk. Hence, understanding the pharmacokinetics and variables the variables that affect pharmacokinetics are important.

1.5 Clinical factors affecting tacrolimus apparent clearance

Several clinical factors have been associated with tacrolimus apparent clearance (CL/F). These include hematocrit, days post-transplant, previous hepatitis C virus infection, diarrhea, race and drug interactions with certain classes of medications such as calcium channel blockers (e.g. diltiazem), antifungals (e.g. ketoconazole) and corticosteroids (e.g. prednisone) [4, 18, 19].

Low packed cell volume is known to be correlated with artifically high tacrolimus troughs when microparticle enzyme immunoassay (MEIA) is used for analysis. This might result in lowering of immunosuppression as a result of reduced doses due to falsely high concentrations [18]. Tacrolimus CL/F decreases with increasing days post-transplant. This may be related to increased bioavailability or increasing hematocrit due to resolution of recipient's anaemia in the weeks to months post-transplant [19]. Some studies have suggested that diarrhea results in increased tacrolimus troughs due to modeified gastrointestinal transit time[20]. AAs exhibit higher tacrolimus CL/F than non AAs. This is attributed to a difference in allelic distribution of CYP3A5 polymorphism between the AA and the non-AA recipients. Certain CYP3A inducers/inhibitors such as

antifungals and steroids that are commonly co-administered to transplant recipients are known to affect the trough concentrations.

1.6 Genetic factors affecting tacrolimus apparent clearance

Tacrolimus is extensively metabolized by the cytochrome P450 3A5 isoenzymes. The CYP3A5 6986A>G single nucleotide polymorphism (SNP) is associated with higher dose-adjusted tacrolimus exposure. Individuals with one or two CYP3A5*1 alleles are CYP3A5 enzyme expressors and have higher 3A5 metabolic capacity and lower tacrolimus exposure than individuals who are non-expressors (CYP3A5*3/*3) receiving the same dose [21]. In a study involving 178 kidney transplant recipients, 40% of CYP3A5 expressors were below their target tacrolimus trough concentrations as compared to < 10% of CYP3A5 non-expressors in the first week post-transplant [22]. In a study involving 136 kidney transplant recipients, tacrolimus daily dose after day 3 post-transplant was about 70% higher in CYP3A5 expressors as compared to non-expressors [23].

Tacrolimus is a P-glycoprotein (PGP) substrate. Polymorphisms in PGP, an efflux transporter present on the apical membrane of many cells, have been studied for their effect towards tacrolimus troughs. It is hypothesized that higher expression of PGP on kidney tubules could potentially lead to rejection due to efflux of tacrolimus out of its site of action. The investigated polymorphisms include ABCB1 3435C>T, 1236 C>T, and 2677 G>T/A. However, their effect on tacrolimus exposure is controversial and its role, if any, is likely minor [21, 24].

Therefore, numerous variables that affect tacrolimus troughs need to be assessed. This may be done using various tools like mixed effect models which can lead to clinical dosing models for tacrolimus in kidney transplant recipients.

1.7 Population pharmacokinetics

1.7.1 Introduction

Population pharmacokinetics (PopPK) is a quantitative assessment of pharmacokinetic parameters and variability in drug exposure and response. Traditional pharmacokinetic studies use the two-stage approach where pharmacokinetic parameters are obtained first from each individual. Mean and variability are calculated in the second stage. PopPK allows the estimation of pharmacokinetic information from sparse data arising out of the population at which the drug is targeted. It allows for estimation of a population mean pharmacokinetic parameters (typical value of clearance in the population) as well as interindividual variability (IIV) and residual unexplained variability (RUV) [25, 26]. A powerful aspect of PopPK is that various underlying demographic, environmental, pathophysiological and drug-related factors in the cohort can be explored in an attempt to identify the specific factors involved in variability of drug exposure and response [27].

PopPK analysis has several advantages over the traditional pharmacokinetic analysis. Most traditional pharmacokinetic analyses utilize data from a homogeneous cohort of subjects with intensive sampling at the same time points for all individuals. Unlike traditional studies, PopPK allows the quantification of pharmacokinetic parameters with sparse time point sampling and/or unbalanced designs collected as a part of a routine clinical study. It allows us to study special populations, such as elderly or neonates, in which the drug is to be administered but only a few blood samples can be obtained due to ethical or medical reasons. Due to its ability to analyze sparsely collected data, it is more cost effective than a traditional PK study that requires intensive sampling. Another advantage is that data from several studies (such as different centers, plasma and whole blood, bound and unbound concentrations, etc.) can be pooled together and analyzed using the PopPK approach [25].

1.7.2. Nonlinear mixed effects modeling

Non-linear mixed effects modeling is commonly utilized for PopPK analysis. It quantifies the drug concentration/exposure (PK) in the body which can be further used to explain its relationship to effects in the body (PD). The term “mixed effects” is used since both fixed and random effects are quantified simultaneously. There are 4 mains components to a mixed effects model [28]:

1. Structural model, which is the basic PK and/or PD model. This describes the overall trend in the data such as one-compartment PK model or an Emax PD model.

2. Covariate model, which attempts to explain the variability in the overall trend based on various demographic, environmental, pathophysiological and drug-related factors.

3. IIV model, which quantifies the between subject variability in the cohort

4. RUV model, which quantifies the remaining variability as a result of errors in the assay, inaccurate dose or sampling time, etc.

The typical value of a pharmacokinetic parameter for a drug, such as clearance (TVCL) or volume (TVV), is the estimate of the average (or typical) value of that particular parameter in the population. As an example, TVCL is estimated as:

$$TVCL = \theta_1 \quad (1)$$

If clearance is dependent on a covariate such as creatinine clearance (CrCL), we can describe that covariate relationship as shown below where θ_2 accounts for the effect of CrCl on drug clearance:

$$TVCL = \theta_1 + \theta_2 * CrCL \quad (2)$$

However, each individual has some IIV in clearance. Clearances for each individual in the study population, CL_i, can then be described as:

$$CL_i = TVCL + \eta_i \quad (3)$$

, where η_i is the IIV which is normally distributed with a mean of 0 and a variance of ω^2, i.e. $\eta_i \sim N(0, \omega^2)$.

Finally, the concentration of the ith individual at the jth time ($C_{obs,\,ij}$) point would differ from the prediction ($C_{pred,\,ij}$). This is referred to as residual error and quantified as:

$$C_{obs,ij} = C_{pred,ij} + \varepsilon_{ij} \quad (4)$$

The ε_{ij} is the RUV and is normally distributed with a mean of zero and a variability of σ^2, i.e. $\varepsilon_{ij} \sim N(0, \sigma^2)$.

1.8 Population pharmacokinetics software

1.8.1 NONMEM®

NONMEM® is a nonlinear mixed effects modeling software package that is distributed by ICON/ Globomax (Ellicott, MD). It is the oldest and most widely used PopPK analysis software program. It was developed by Lewis Sheiner and Stuart Beal in the 1980s and is written in ANSI Fortran 77. It has several components: 1) NONMEM, which the non-linear regression program; 2) NM-TRAN, a preprocessor that allows inputs in a user-friendly manner; and 3) PREDPP, which has pre-specified subroutines for handling PK data. NONMEM® has a versatile modeling platform. It allows users to implement pre-defined models or construct new models. Several interfaces are available that allow easy pre-processing and post-processing of NONMEM® runs. One example is PDx-POP that is an interface used to operate NONMEM®. It provides a user-friendly platform to create control streams and run models, to view results and to generate statistical and graphical diagnostics [29]. Xpose is an aid to model building in NONMEM® that is based on the R-language script. It acts as an add-on to NONMEM® by assisting in exploration, visualization, model diagnostics, covariate selection and model comparison [30]. It is impractical to test covariates within NONMEM® when the number of covariates is large because of the number of possible combinations. Xpose allows testing of covariates based on additive modeling for further testing in NONMEM®.

Parameters in NONMEM® are estimated by the minimization of the extended least squares (ELS) objective function value (OFV). The OFV is proportional to the -2 log likelihood of the data [31]. Likelihood estimates the probability of the data given the model parameters and a higher likelihood (or lower OFV) is indicative of a better fit of the model to the data. Since the observed measurement in most clinical study data is assumed to follow a normal distribution, the ELS OFV is given as:

$$OFV_{ELS} = \sum_{ij} \frac{(C_{obs,ij} - C_{pred,ij})^2}{\sigma^2} + \ln \sigma^2 \quad (5)$$

Random effects enter the model non-linearly and approximations to the non-linear model are conducted using Taylor series expansion methods. Most common methods are first-order (FO) estimation and first-order conditional estimation (FOCE) methods. FO involves first-order linearization around the expected mean of the η and ε, (i.e. 0) and although once widely used FOCE and FOCE-I are preferred. FOCE involves first-order linearization around the conditional values of the η [31]. FOCE and FOCE-I are more-time consuming than the FO method but they more accurate as observations per individual increases due to increased non-linearity of the system.

1.8.2 Phoenix® NLME™

Phoenix® (Pharsight Corporation, Cary, NC) is a recently developed software package. The package comprises of WinNonlin, NLME (non-linear mixed effects) and in-vitro in-vivo correlations (IVIVC) toolkit. Phoenix® NLME™ is the non-linear mixed effects software. It has a graphical as well as a textual interface to implement user defined models or use pre-defined models from the model library. The user can choose from an array of PopPK algorithms such as FO, FOCE-I, adaptive Gaussian quadrature, Lindstrom-Bates FOCE, etc. The software is easier to use since most features are menu-driven. Several covariate modeling algorithms such as running specific pre-defined scenarios, stepwise covariate search and shotgun covariate search come as built-in functions in Phoenix® NLME™ [32]. A maximum of 32 covariates can be tested in Phoenix® NLME™ in a given model.

1.9 Covariate modeling

Covariate modeling is an integral part of any PopPK study. A covariate is a characteristic of a subject in a study that could possibly influence a PK/PD outcome. Covariate modeling is critical as it can identify factors that can account for a portion of the variability. Identifying the source of variability can improve the predictive performance

of the model and can provide important contributions to the understanding of PK/PD in the population under study.

1.9.1 Types of covariates

Covariates may be continuous such as age, weight, creatinine clearance, etc. or categorical such as sex (male/female), race (African American or non-African American), and concurrent medication (yes/no). Covariates can also be either time-independent (e.g. sex) or they may change over time (e.g.: CrCL). It is important not only to identify significant covariates but also to determine the functional form of the covariate relationship. For example, age might be a significant covariate for clearance, but the relationship may be characterized by several types of relationships (i.e., linear or non-linear).

1.9.2 Approaches to covariate modeling

Several approaches can be used for covariate model building. These are discussed in detail below:

1. Graphical: This involves the use of scatter plots of the empirical Bayes estimates (EBEs)/ post-hoc estimates versus the covariate in order to visualize the strength and form of the relationship [33]. Scatter plots can be used to examine a possible relationship between covariates, if one exists. The relationship is quantified with the help of correlation coefficient (ρ). If a correlation is detected ($\rho > 0.7$), it may be essential to select one covariate from amongst the correlated factors. One major drawbacks of this method is that it is a subjective assessment and it is harder to detect a weak relationship that might exist. If the post-hoc estimates are poor then important covariates might not be picked up [34].

2. Generalized additive modeling (GAM): This technique is used to find the most appropriate model from a given set of covariates. This option can be implemented using Xpose. It also utilizes the EBEs to assess a relationship with a covariate. It involves regression of EBEs on the covariates.

$$EBE_i = \alpha + f_1(X_{1i}) + f_2(X_{2i}) + \ldots + f_n(X_{ni}), \quad (6)$$

where EBE_i is the post-hoc estimate for the i^{th} individual and $X_{1i}, X_{2i}, \ldots, X_{ni}$ are the 'n' covariates in the i^{th} individual. Model comparison is done using the Akaike Information Criteria (AIC) where 'p' is defined to be the number of parameters in the model:

$$AIC = -2\text{loglikelihood} + 2p \quad (7)$$

This method helps to identify possible significant covariates for analysis within NONMEM® when the initial number of covariates is large. This approach saves time by reducing the number of models that need to be tested in NONMEM®. Also, unlike multiple linear regression, it allows a non-linear/spline relationship between the covariates and EBEs [34]. However, the method relies on the post-hoc estimates for assessing relationships and does not account for time-varying covariates.

3. Stepwise selection method: This is a widely employed technique of covariate selection and involves two steps: forward selection and backward elimination. In forward selection, each covariate is entered into the model and the drop in OFV is noted. Based on an a priori set p-value, all significant covariates are retained in the model in forward selection. When a full model is obtained based on the forward selection criteria, backward elimination is carried out using a more stringent p-value to reduce the Type I error rate (false positives) in the model. This is done by removal of each covariate from the full model one by one. The increase in OFV is noted when each covariate is dropped from the model. The final model is obtained by retaining only the significant covariates during backward selection. Although this is a widely used method, it suffers from the drawback such as false findings, selection bias and problems in adjustment of p-values for multiple comparisons.

There are several methods to parameterize covariates within the context of population modeling. Continuous covariates such as weight can be centered (by subtracting the mean/median value from the covariate) or standardized (by dividing by the mean/median value of the covariate). This is done because PK parameters such as clearance and volume are functions of body size such as weight and can mask the effect of other

potentially significant covariates [35]. Furthermore, continuous covariates can be converted to categorical covariates to allow easy clinical interpretation or due to the inability to find a continuous functional form to explain the exact trend. Covariate modeling is a challenging and time-consuming aspect of a PopPK modeling project. It is necessary to maintain a balance between clinical relevance and statistical significance. There is no common consensus on the exact steps needed to conduct a covariate analysis. Determining a model that is most useful and clinically relevant model can be somewhat of an art.

1.10 Aims and scope of dissertation

Personalized medicine is an emerging field of healthcare that attempts to utilize an individual's characteristics to guide dosing [36]. Characteristics used can be demographic factors such as age, weight, race, co-medications, etc. and genotype. An important step towards implementing personalized medicine into clinical practice is to understand how these characteristics could explain the interindividual variability in drug response. This is especially true of drugs such as tacrolimus that have a narrow therapeutic index and show a large variability in their pharmacokinetics. An excellent example is the dosing of warfarin based on several clinical and genetic factors [37]. The warfarin algorithm uses each individual's unique set of clinical and genetic information to estimate a dose. We wanted to utilize a similar approach for estimating tacrolimus doses due its narrow therapeutic window, acute rejection/toxicity with sub-optimal trough concentrations and large interindividual variability in response.

The primary aim of this dissertation was to formulate a model for tacrolimus that predicts trough concentration and facilitates prediction of doses prospectively in adult kidney transplant patients. This is especially important in the immediate period post-transplant as the risk of acute rejection is high. This objective was achieved by analyzing numerous clinical factors and genetic variants in a large cohort of adult kidney transplant recipients that could influence tacrolimus troughs by affecting absorption, distribution, metabolism and excretion (ADME properties). This forms a basis of Chapter II of this thesis.

Once formulated, the model developed in Chapter II was validated. Chapter III focuses on this objective. The validation was done using an external dataset of adult transplant recipients. Predictive performance was used to assess the ability of the equation to predict troughs using prediction errors. The validation of the equation provides additional confidence of using the equation in clinical practice.

The PopPK analysis in Chapter II was carried out by non-linear mixed effects modeling using NONMEM®. Several limitations were identified when the number of covariates to be analyzed is large and is addressed in Chapter IV. These include both software limitations and practical constraints. As the number of covariates increases, the number of models that need to be tested increases non-linearly. With 'p' covariates, there are a total of 2^p possible models. If the number of covariates is large, it is impractical to test all subset of models. Built-in functionalities such as generalized additive modeling (GAM) in the NONMEM® add-in, Xpose, allow the selection of the "best" model with a given set of covariates. However, software limitations start to arise with too many covariates. In our dataset, we had 119 single nucleotide polymorphisms (SNPs). GAM procedure in Xpose was only able to test combinations of 25 SNPs at one time. Therefore, a method was developed that would allow screening of covariates for testing in NONMEM®. We referred to this method as a novel "winnowing method. The method uses an iterative process of random sampling and testing with GAM. This method is able to test for covariates in combination and screens covariates based on the number of times they appear in the "best model" when sampled. An index is created which ranks covariates according to their importance. The method can analyze a large number of covariates. Chapter IV deals with this novel winnowing method.

Finally, NONMEM® is the most commonly used software for PopPK analysis. However, new software with diverse features such as menu-driven options and newer functionalities are being continuously introduced. One such software is Phoenix® NLME™. We wanted to compare NONMEM® and Phoenix® NLME™ in terms of the clearance model obtained for tacrolimus and for clinically and/or statistical difference between the predictions of doses. This was done in order to ensure that similar results will be obtained when using one software package over another. A particular analysis

may demand extensive covariate selection which might be easily dealt with in Phoenix® NLME™. Chapter V of the thesis addresses the differences in models between NONMEM® and Phoenix® NLME™.

In summary, this thesis will present the development and validation of a model that attempts to prospectively predict tacrolimus trough concentrations, especially in the immediate period post-transplant, to improve dosing in patients. The long term goal of this work is to improve immunosuppressive regimen of tacrolimus for successful kidney transplantation. It presents the development and validation of a novel method that can be used to screen covariates for testing in NONMEM®. Finally, we compare NONMEM® and Phoenix® NLME™ for parameter estimates and final models obtained using both software packages.

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Chapter II

Dosing equation for tacrolimus using genetic variants and clinical factors

2.1 Introduction

Tacrolimus is the most widely used calcineurin inhibitor in kidney transplantation [1]. It has a narrow therapeutic window with wide inter-individual variability in pharmacokinetics and clearance (CL) [2, 3]. Higher troughs are associated with increased risk of toxicity whereas lower troughs are associated with increased risk of rejection [4]. Two recent studies showed that with contemporary immunosuppressive regimens (tacrolimus, mycophenolate and steroids ± antibody induction) low tacrolimus troughs in the first week post-transplant were associated with a greater risk of acute rejection [5, 6]. To better tailor therapy, multiple clinical factors have been explored to determine their effects on tacrolimus pharmacokinetics. It is generally acknowledged that drug interactions, hematocrit, corticosteroid therapy, day post-transplant, and race affect tacrolimus pharmacokinetics [3, 7-10]. It is also established that the cytochrome P4503A5 (CYP3A5)*1 allele is associated with significantly higher tacrolimus CL and lower systemic exposure [11-15]. However, because tacrolimus troughs are routinely monitored and dose adjusted based on trough measurements the effect of these factors are not regarded in a consistent manner by centers and a trial and error approach to dosing is still common practice. These factors have not been used to guide dosing primarily due to the lack of robust, clinically feasible dosing models that combine important factors. Because of the growing use of steroid sparing or avoidance protocols, the importance of early immunosuppression intensity provided by the calcineurin inhibitors and/or other immunosuppressive exposure is considered important in minimizing acute rejection [16].

A recent randomized trial studied CYP3A5 genotype guided tacrolimus dosing in kidney transplant recipients [17]. In the genotype guided group, patients with one or more CYP3A5*1 alleles received an initial tacrolimus dose of 0.3 mg/kg/day and those without a *1 allele received a dose of 0.15 mg/kg/day. The non-genotype guided group was administered 0.2 mg/kg/day. In the genotype guided group, 43.2% of subjects achieved the trough target compared to 29.1% in non-genotype guided group (p=0.03). Although using genotype guided dosing was significantly better, the overall proportion of patients achieving the therapeutic range may not be sufficient to justify the cost of incorporating genotyping into clinical practice. It is possible that addition of clinical factors and/or

other genotypes to dosing models may further improve the initial dose estimates and the number of patients achieving the therapeutic target. However, defining these factors and the development of a robust dosing model for clinical use requires a large study population. Therefore, we studied the effect of clinical and genetic factors on tacrolimus apparent clearance (CL/F) in a large kidney transplant population through a multicenter study. Our objective was to define the important clinical and genetic factors pertinent towards tacrolimus CL/F and develop a dosing model which would be suitable for the clinical setting.

2.2 Methods

2.2.1 Patient selection and recruitment

Subjects for this analysis were obtained from the first 1000 patients with end-stage renal dysfunction undergoing kidney or kidney-pancreas transplant enrolled in the Deterioration of Kidney Allograft Function (DeKAF) Genomics study. This is a multi-center observational trial to define genetic and clinical determinants associated with clinical outcomes after kidney transplant. Details of the trial have been published elsewhere and are registered at www.clinicaltrials.gov (NCT00270712). Patients were recruited from 2005-2007. A total of 681 patients, ≥18 years of age, who received tacrolimus at any time in the first 6 months post-transplant were selected for this analysis. The remaining subjects received cyclosporine and were not studied. Institutional Review Board Human Subjects Committee approval was obtained at each participating center. All recipients provided written informed consent.

Tacrolimus trough concentrations (n=11,823) in 681 patients were obtained during oral administration in the first 6 months post-transplant. All patients were administered Prograf either once or twice daily. Troughs were obtained as part of clinical care at the treating center. The initial tacrolimus dosing was based on an individual's body weight and subsequent doses were adjusted based on trough concentrations using institution specific targets. In general, troughs of 8-12 ng/mL were targeted in the first three months post-transplant and of 6-10 ng/mL were desired in the months 3-6 post-transplant. Trough concentrations, if available, were obtained twice in each of week's 1-8 post-

transplant and twice in each of months 3, 4, 5 and 6 post-transplant. There were a mean of 17 trough concentrations per patient (range 1-24). To ensure that tacrolimus was at or near steady state, only trough levels measured after day 2 post-transplant were used in this analysis. Trough concentrations were measured in the clinical laboratories of the participating centers. The majority (97.1%) of tacrolimus whole blood concentrations were obtained from centers using liquid chromatography-mass spectroscopy to measure trough concentrations. All troughs were measured in centers using CLIA certified assays or CLIA quality assays.

Recipient and donor demographics and clinical characteristics were obtained from medical records and are listed in Table 2.1. Baseline clinical factors collected were recipient age, weight, gender and race (AA or non-AA), donor type (living or deceased), donor gender, preemptive transplant, number of prior transplants, immunosuppressive regimen and enrolling center. The concomitant use of a CCB and day post-transplant at the time of each trough measurement was also obtained.

2.2.2 Genotyping and selection of variants for analysis

Recipient pre-transplant DNA was obtained from the lymphocytes isolated from peripheral blood. DNA was genotyped for 2,724 variants primarily using a customized Affymetrix GeneChip (Affymetrix, Santa Clara, CA) [18, 19]. Additional variants were genotyped using the SNPlex (Applied Biosystems, Foster City, CA) and Sequenom (Sequenom, San Diego, CA) systems. In a prior analysis, these variants were evaluated for their association with dose-normalized tacrolimus concentrations using standard regression analysis [20]. Since it was not feasible to evaluate 2,724 variants and their combinations in a population pharmacokinetic approach to estimate CL/F, the SNPs with a p-value <0.01 from the AA and non-AA populations from the regression analysis, were selected for this analysis. The variants evaluated towards tacrolimus were rs776746, rs12114000, rs3734354, rs4926, rs3135506 and rs2608555. The variants and their allele frequencies are given in Table 2.2.

2.2.3 Population modeling of troughs

The data were analyzed by a non-linear mixed-effects approach using the NONMEM® software (Version 7.1, NONMEM® Project Group, GloboMax LLC, MD) with a Visual Fortran compiler (Professional Edition for Windows 11.1, Intel®) and PdxPop (Version 4.0, ICON Development Solutions). R 2.4.1 was used for the generation of diagnostic plots. Given the long half-life of tacrolimus (12 hrs) [21, 22] relative to the dosing intervals, and the absence of pharmacokinetic sampling at times other than the troughs, the concentrations were analyzed according to a steady-state infusion model. It was assumed that the trough concentrations (C_{min}) were well approximated by the average steady-state plasma concentration ($C_{ss,\,av}$) of tacrolimus due to its long elimination half-life and were related to the tacrolimus dose through (CL/F)*:

$$C_{obs} = \text{Dosing Rate}/(CL/F)^*, \quad (1)$$

where C_{obs} is the observed tacrolimus trough concentration and dosing rate is the total daily dose of tacrolimus (in mg) divided by 24 hrs. The (CL/F)* is a regression parameter that predicts the trough concentrations. It approximates CL/F when $C_{min} \approx C_{ss,\,av}$, as in the case of drugs that have a long half-life such as tacrolimus. Using (CL/F)* as an approximation to CL/F has the advantage of giving the regression parameter clinically relevant meaning in terms of magnitude, units and interpretation. However, we caution the reader that the apparent clearance we describe in the manuscript is only an approximation of the actual apparent tacrolimus clearance.

The first order conditional approximation estimation with interaction (FOCE-I) was utilized. The pharmacokinetic parameter obtained was the CL/F for tacrolimus. The inter-individual variability (IIV) in CL/F was modeled using an exponential error model as shown in the following equation:

$$CL/F = TVCL^*e^{\eta(1)}, \quad (2)$$

where TVCL represents the typical value of CL/F in the population and $\eta(1)$ represents the IIV in tacrolimus CL/F, $\eta \sim N(0, \omega^2)$. An additive error model was utilized to characterize the residual unexplained variability (RUV) as shown in the following equation:

$$C_{ij} = C_{pred,ij} + \varepsilon_{ij}, \quad (3)$$

where C_{ij} is the j^{th} observed concentration in the i^{th} individual, $C_{pred,ij}$ is the j^{th} predicted concentration in the i^{th} individual and ε_{ij} is the RUV in tacrolimus CL/F, $\varepsilon \sim N(0, \sigma^2)$.

The clinical factors and the 6 variants described in Table 2.1 and 2.2 were tested for their influence on tacrolimus CL/F in the model building phase. Forward inclusion and backward elimination were used to analyze the covariates. A decrease in objective function value (OFV, a goodness-of-fit statistic) of 6.63 or more ($p<0.01$) was considered significant (χ^2: 1 degree of freedom, df) in the forward inclusion step. Backward elimination was performed using a more stringent increase in OFV of 10.83 or more ($p<0.001$, 1 df). Clinical factors that were tested included categorical covariates (day post-transplant of trough, recipient gender, race, donor type, donor gender, preemptive transplant, number of prior transplants and enrolling center) and continuous covariates (age and weight). Race (Caucasian, AA, Asian, Native American/Aleut/Inuit and Hawaiian/Pacific Islander) was categorized as AA or non-AA. Centers were designated as using steroid sparing immunosuppressive regimen if they administered steroids for ≤ 7 days post-transplant. Age was tested by standardizing it by the median age (50 years) in the population. Days post-transplant was converted to an ordered categorical covariate and classified as: immediate post-transplant (days 3-5), early post-transplant (days 6-10) and late post-transplant (days 11-180). Categorization was done since the model failed to converge when days post-transplant was modeled as a continuous function (such as a simple continuous function, Bateman function, Emax model, etc). The concomitant use of a CCB at the time of the trough measurement was also analyzed. Variants were initially classified as homozygous for the major allele, heterozygous for the minor allele or homozygous for the minor allele. However, the frequency of homozygous variant carriers in 5 of the 6 variants was $\leq 5\%$. Therefore, for these 5 variants, the genotypes were categorized as 2 variables (carriers or non-carriers of the minor allele). The minor allele frequency (MAF) of CYP3A5*1 was high, therefore, we were able to categorize the genotypes as CYP3A5*3/*3, CYP3A5*1/*3, or CYP3A5*1/*1.

2.2.4 Model building

The covariates were tested in a step-wise manner. In the first step, the OFV for the base model (pharmacokinetic model with no covariates) was observed. The covariates were analyzed next by forward inclusion by the following procedure: a) univariate analysis was performed, b) the insignificant covariates were removed from further consideration, c) the significant covariates (OFV >6.63 for 1 df) were ranked in terms of their significance towards tacrolimus CL/F, as assessed by the magnitude of drop in the OFV, d) the covariate with the highest rank (i.e. most significant) , was included into an updated model and e) the remaining significant covariates were tested for significance by adding them univariately to the updated model. Steps b) through e) were repeated until all the significant covariates were included into the forward inclusion model and all insignificant covariates were discarded. Backward elimination was then performed by removing each group within a covariate at a time (e.g. days 6-10 and 11-180 separately). An increase in OFV (>10.83 for 1 df) was used to yield the final model.

2.2.5 Model evaluation

For the purpose of model evaluation, a nonparametric bootstrap approach was employed. A single bootstrap run generated a new dataset, by random sampling with replacement from the original dataset. Bootstrap runs (n=1,000) were performed and nonparametric statistics, median and, 2.5^{th} and 97.5^{th} percentile were obtained from successful bootstrap runs (successful was defined as achieving both convergence and covariance step). A close agreement between the non-parametric statistics and the estimates from the original dataset (≤10%) were indicative of the stability and performance of the population model.

2.3 Results

Tacrolimus troughs (n=11,823) from 681 adult kidney transplant recipients obtained in the first 6 months post-transplant were studied. Patient characteristics, tacrolimus doses and troughs are given in Table 2.1. The mean ± S.D. daily doses over the 6 month period for the non African American (non-AA) and African American (AA) were 0.07 ± 0.05 and 0.09 ± 0.04 mg/kg, respectively. The mean ± SD troughs for the non-AA and AA patients were 8.66 ± 3.37 and 6.82 ± 3.51 ng/mL, respectively. A majority (59%) of the subjects received a living donor transplant and 21% of the population was AA. In our

study, in the first week post-transplant, 75% (n=413) of all transplant recipients, 69% (n=307) of non-AA and 96% (n=106) of AA, had a trough level <8 ng/mL. In the second week post-transplant, 55% (n=331) of all transplant recipients, 48% (n=228) of non-AA and 82% (n=103) of AA, had a trough <8 ng/mL. The 6 genetic factors and their allele frequencies are shown in Table 2.2. Factors affecting tacrolimus CL/F in order of their importance were day post-transplant, presence of CYP3A5*1 allele (rs776746), transplantation at a steroid sparing center, recipient age and calcium channel blocker (CCB) use (Table 2.3). The typical value of CL/F was 38.4 L/hr and decreased by 14% (0.86) in days 6-10 post-transplant and by 29% (0.71) in days 11-180 post-transplant, relative to the days 3-5 post-transplant. Patients who carried one or more CYP3A5*1 alleles had lower troughs than those with the CYP3A5*3 allele. Troughs and dose requirements by genotype are shown in Figure 2.1 (panels A and B). CL/F was increased by 69% (1.69) in subjects with the CYP3A5*1/*3 genotype and by 100% (2.00) in subjects with the CYP3A5*1/*1 genotype. CL/F was reduced by 30% (0.70) in patients undergoing transplantation at a center using a steroid sparing immunosuppressive regimen and by 6% (0.94) when a CCB was co-administered compared to no CCB. CL/F increased until the median age of 50 years and then decreased thereafter. Recipient weight, gender, race, donor type and gender, preemptive transplant, number of prior transplants, and the variants rs12114000, rs3734354, rs4926, rs3135506 and rs2608555 were not significant. The following equation describes the final model for estimation of tacrolimus CL/F in the first 6 months post-transplant.

CL/F (L/hr) = 38.4*[(0.86, if days 6-10) or (0.71, if days 11-180)]*[(1.69, if CYP3A5*1/*3 genotype) or (2.00, if CYP3A5*1/*1 genotype)]*(0.70, if receiving a transplant at a steroid sparing center)*[(Age in yrs/50)$^{-0.4}$]*(0.94, if CCB is present)

(4)

The total daily dose (TDD) requirement is then calculated from the estimated tacrolimus CL/F above and the desired goal trough concentration.

TDD (mg) = [CL/F (L/hr) * tacrolimus trough goal (ng/ml) * 24 hrs] / 1000

(5)

The base model had an IIV of 52.3%. in CL/F. The final model had an IIV of 40.1% in CL/F. Thus, the final model was able to explain 23.2% of the relative IIV in CL/F. Plots of the observed (OBS) concentrations versus the model predicted (PRED) concentrations and OBS versus the individual predicted (IPRED) concentrations are shown in Figure 2.2. A plot of the weighted residuals (WRES) versus time shows that most of the data lies within 3 units from the zero-ordinate (Figure 2.2). In addition, the mean parameter estimates from the study population and the 989 successful bootstrap runs are highly comparable thereby confirming the accuracy and precision of the estimates as well as stability of the final model (Table 2.3). The post-hoc estimates of CL/F from the final model by the 3 different genotypes are shown in Figure 2.3.

2.4 Discussion

We report here the first dosing model for tacrolimus using a combination of genetic and clinical factors in adult kidney transplant recipients that was developed from one of the largest tacrolimus pharmacogenetics studies (n=681) conducted to date. We found that CL/F was significantly influenced by day post-transplant, CYP3A5 genotype, transplantation at a steroid sparing center, recipient age, and the use of a CCB. The population CL/F was 38.4 L/hr and is close to estimates from other pharmacokinetic studies (ranging from 21-35 L/hr) [10, 23, 24]. Our estimate of CL/F is slightly larger than previously reported studies. This is expected because, as stated earlier, we model (CL/F)*, an approximation to the tacrolimus apparent clearance. In addition, differences in patient populations between the studies cannot be ignored, nor can the influence of covariates that have been included in our model.

In our study, 75% (n=413) and 55% (n=331) of the transplant recipients had tacrolimus trough concentrations <8 ng/mL in the first week and second week post-transplant, respectively. These troughs are below the usual trough goal in centers participating in this study (8-12 ng/mL in the first 3 months). These troughs were achieved through the use of the typical mg/kg dosing and it is possible that a higher number of patients would achieve troughs >8 ng/mL if dose individualization using clinical and genetic factors had been used.

Days post-transplant was the most significant clinical factor affecting tacrolimus CL/F and has been previously shown to be associated with CL/F [9, 10, 25, 26]. Tacrolimus CL/F decreased by 14% in days 6-10 post-transplant and by 29% in days 11-180 post transplant, relative to the immediate post-transplant period (days 3-5). This decrease in CL/F is consistent with previous findings [10, 26]. A decrease in CL/F may be attributable to an increase in tacrolimus bioavailability over time as the patients clinical status improves. CL/F may also decrease due to an increase in hematocrit and albumin concentrations with time as kidney function is restored.

The CYP3A5 genotype was also highly associated with CL/F. Individuals who carry one or more CYP3A5*1 alleles express CYP3A5 enzyme [4, 27]. Tacrolimus is a substrate for CYP3A5 and it is well established that individuals carrying one or more *1 alleles have a higher tacrolimus CL and lower trough concentrations [11-14, 17]. Several previous studies have highlighted the differences in CL/F and dose requirements for the CYP3A5*1 carriers compared to individuals with the CYP3A5*3/*3 genotype [24, 28-32]. In a study involving pediatric kidney transplant recipients, tacrolimus CL/F was about 50% higher in children with the CYP3A5*1/*1 or *1/*3 genotype as compared to the CYP3A5*3/*3 genotype [8]. A recent pharmacokinetic study found tacrolimus CL/F to be about 2-fold higher in CYP3A5 expressors (*1/*1 or *1/*3 genotype) as compared to the CYP3A5 non-expressors (*1/*1 genotype) [29]. In an earlier study on the pharmacokinetics of tacrolimus in healthy Japanese subjects, CL/F was about 1.5 times higher in the CYP3A5*1 carriers (*1/*1 or *3/*3 genotype) as compared to the CYP3A5*3/*3 genotype [27]. Most studies have combined genotype groups (*1/*1 with *1/*3) due to sample size limitations [13-15, 33]. Given our large sample size, we were able to define the differences in tacrolimus CL/F between three CYP3A5 genotype groups. In our study, subjects with the CYP3A5*1/*3 and *1/*1 genotypes had a CL/F that was 70% and 100% higher, respectively, than those with the CYP3A5*3/*3 genotype. These data demonstrate for the first time that the three CYP3A5 genotypes have distinctive CL/F estimates and dose requirements. The majority (85%) of our non-AA population had the CYP3A5*3/*3 genotype, whereas the majority (87%) of the AAs had the CYP3A5*1/*1 or the CYP3A5*1/*3 genotype. Despite this race was insignificant in our final dosing model most likely due to collinearity between CYP3A5

genotype and race. Although the CYP3A5 genotype is associated with tacrolimus CL/F, it is not associated with acute rejection [24, 29, 34]. The lack of association is not surprising given that multiple factors increase the risk of rejection (i.e. donor type, recipient age, HLA compatibility, low calcineurin inhibitor levels). Importantly though, data show that patients with the CYP3A5*1/*3 or *1/*1 genotype have earlier acute rejection and are slower to achieve target concentrations [14].

Our analysis found an important center effect. Centers were categorized by the use of a steroid sparing immunosuppressive regimen or not. A steroid sparing immunosuppressive protocol administers corticosteroids for a short period of time (typically 7-14 days) in the early post-transplant period whereas non-sparing centers maintain steroids throughout the first 6 months or longer [35, 36]. We found that CL/F was decreased by 30% in patients transplanted at a steroid sparing center. We hypothesize that the center effect is related to steroid use and that continuous therapy leads to an induction of CYP enzymes and higher tacrolimus CL/F. Various studies have demonstrated an induction of CYP3A enzymes by corticosteroid therapy [37-39]. Although controversial, studies have identified corticosteroid therapy as a significant factor towards tacrolimus CL/F [9, 40-42]. Clinical practice varies substantially between centers; therefore, the attribution of the effect to steroids cannot be confirmed in our study and must be directly tested in future analyses.

Age and CCB use were also identified as significant covariates towards CL/F. CL/F increased progressively up to age 50 years and then decreased thereafter. The concomitant use of a CCB was associated with a small (6%) decrease in tacrolimus CL/F. Multiple CCB were used in our study and we did not collect the specific CCB prescribed. The CCB, diltiazem, is a well known potent inhibitor of CYP3A enzyme and decreases tacrolimus CL/F [30, 31]. Other CCBs (such as amlodipine) are not potent inhibitors of CYP3A [43], therefore, the inhibitory effects of some CCBs may be overestimated whereas the effect of diltiazem is likely underestimated.

Since this study is based on a large population, we expect the estimates from this study to be fairly reliable. However, the data for this analysis came from an observational study

where supervision of trough measurements was conducted by the clinical teams. Hematocrit and aspartate aminotransferase (AST) have been previously shown to be important clinical factors [10] and neither was collected in our study. Future studies should also consider concomitant administration of anti-fungals given the potential for drug interactions [44, 45].

The dosing equation which we developed is simple and uses common clinical factors and one genotype. The availability of this equation now allows for the testing of clinical and genetic factor guided versus traditional weight-based initial dosing. Since the achievement of therapeutic levels early post-transplant is of importance, we would expect the use of individualized dosing to result in a higher proportion of patients within the target range and thereby lower the risk for acute rejection. This model was developed from data in adult individuals within the first 6 months post-transplant; therefore, whether it can be extrapolated beyond this time frame is not known. An advantage to our equation is that any tacrolimus trough may be targeted including patients where it is desirable to provide a higher level of immunosuppression as well as those who may be candidates for calcineurin sparing.

An example of how this model is applied clinically is as follows. Suppose we wish to determine prospectively the oral tacrolimus dose in a 50 year old, 85 kg kidney transplant recipient on day 3 post-transplant with a goal tacrolimus trough of 10 ng/mL and a genotype of CYP3A5*1/*1, in a steroid using center, receiving a CCB.

$$\text{CL/F (L/hr)} = 38.4*(2.00 \text{ for CYP3A5*1/*1 genotype})*[(50/50)^{-0.4}]*(0.94 \text{ for CCB use}) = 72.2 \text{ L/hr} \quad (6)$$

The total daily dose (TDD) requirement is:

$$\text{TDD (mg)} = [72.2 \text{ L/hr} * 10 \text{ ng/ml} * 24 \text{ hrs}] / 1000 = 17.5 \text{ mg} \quad (7)$$

The daily dose is 17.5 mg or 8.5-9 mg twice daily. Should this patient have received a typical weight based dosing (0.1 mg/kg/day) the estimated dose would be 8.5 mg/day or 4-4.5 mg twice daily which would have likely under-dosed this patient.

As another example, let us suppose that we wish to determine the oral tacrolimus dose in a 50 year old, 85 kg kidney transplant recipient on day 100 post-transplant with a goal tacrolimus trough of 8 ng/mL and a genotype of CYP3A5*3/*3, who underwent transplantation at a steroid sparing center, and is not receiving a CCB.

CL/F (L/hr) = 38.4*(0.71 for days 11-180**)*** (0.70 for transplant at a steroid sparing center) *$[(50/50)^{-0.4}]$ = 19.1 L/hr

(8)

The total daily dose (TDD) requirement is:

TDD (mg) = [19.1 L/hr * 8 ng/ml * 24 hrs] / 1000= 3.6 mg

(9)

The daily dose is 3.6 mg or 1.5-2 mg twice daily. Should this patient have received our typical weight based dosing (0.1 mg/kg/day) the estimated dose would be 8.5 mg/day or 4-4.5 mg twice daily which would have likely over-dosed this patient.

This model now requires confirmation in an independent population and prospective testing.

Table 2.1: Characteristics in all subjects, non-African Americans & African American

Clinical Characteristics		All subjects (Non-AA & AA)	Non-AA[1]	AA[2]
No. of subjects		681	540 (79.3%)	141(20.7%)
Age (yrs) of recipient[3]		50.2 ± 12.2	50.1 ± 12.2	46.9 ± 11.5
Baseline weight (kgs)[3]		81.3 ± 18.7	81.1 ± 18.8	81.9 ± 17.9
Gender of recipient		429 (63%) /	338 (63%) /	91 (65%) /
(Male/Female)		252 (37%)	202 (37%)	50 (35%)
No. of transplants	1	550 (81%)	417 (77%)	133 (94%)
	≥ 2	130 (19%)	122 (23%)	8 (6%)
No. transplanted at a steroid sparing center		205 (30%)	197 (36.5%)	8 (5.5%)
Living donor		398 (59%)	355 (66%)	43 (31%)
No. of troughs		11,823	9,523	2,300
Tacrolimus dose[4]		0.08 ± 0.05	0.07 ± 0.05	0.09 ± 0.04
Tacrolimus trough[4]		8.31 ± 3.48	8.66 ± 3.37	6.82 ± 3.51
No. of patients with	Week 1	413 (75%)	307 (69%)	106 (96%)
troughs <8 ng/mL	Week 2	331 (55%)	228 (48%)	103 (82%)
Calcium channel blocker use[5]		5082 (43%)	3915 (41%)	1167 (51%)
CYP3A5 genotype	*1/*1	72 (11%)	9 (2%)	63 (45%)
	*1/*3	129 (19%)	70 (13%)	59 (42%)
	*3/*3	476 (70%)	457 (85%)	19 (13%)

[1] Non African Americans, [2] African Americans, [3] reported age and baseline weight are those measured at the time of transplant and are mean ± S.D., [4] doses (mg/kg/day) and troughs (ng/mL) are over the 6 month study period and are mean ± S.D., [5] calcium channel blocker use at the time of trough collection

Table 2.2: Analyzed variants and their allele frequencies in all subjects, non-African Americans & African American

Reference Sequence (rs) No.	Gene Name	Allele	Overall Allele frequency[1] (%)	Non-AA Allele frequency[2] (%)	AA allele frequency[3] (%)
rs776746	CYP3A5	A	20.16	8.21	65.60
rs12114000	CYP3A4	A	3.82	0.09	18.09
rs3734354	SIM1	A	11.40	13.45	3.55
rs4926	SERPING1	A	23.45	26.63	11.35
rs3135506	APOA5	C	6.12	6.33	5.32
rs2608555	GAN	T	16.45	15.93	18.44

[1] allele frequency in the whole population, [2] allele frequency in the Non African American, [3] allele frequency in the African America

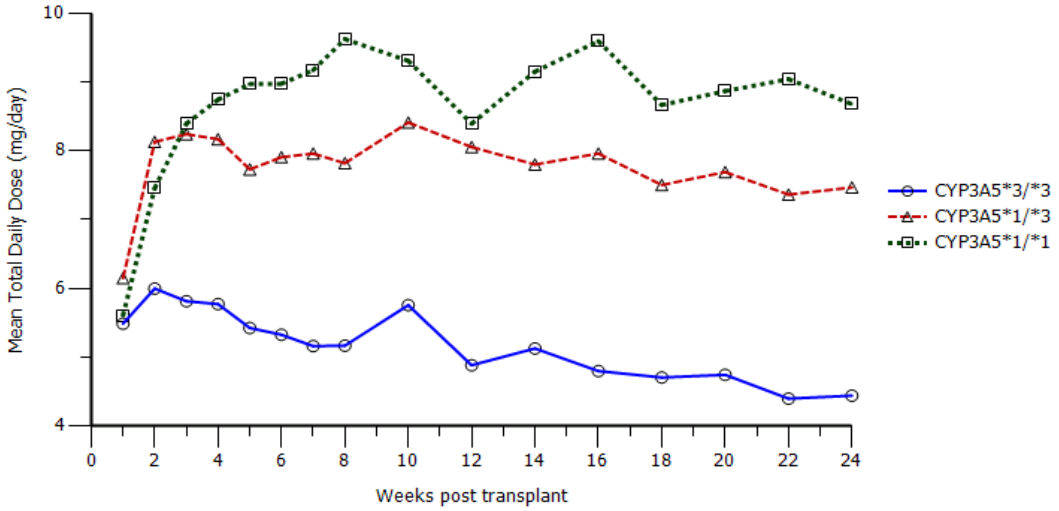
Table 2.3: Final parameter estimates and effect of genetic and clinical factors on tacrolimus CL/F

Parameter	Study population[1]		Bootstrap Analysis[2]	
	Estimate (%RSE)[3]	95% C.I.[4]	Median	2.5th- 97.5th percentiles
Tacrolimus CL/F[5]	38.4 (4.14)	35.3, 41.5	38.3	35.5, 41.7
Factors affecting CL/F				
6-10 DPT[6]	0.86 (4.13)	0.80, 0.93	0.86	0.80, 0.93
11-180 DPT[6]	0.71 (4.14)	0.66, 0.77	0.72	0.66, 0.77
CYP3A5*1/*3	1.70 (3.99)	1.56, 1.82	1.69	1.56, 1.82
CYP3A5*1/*1	2.00 (5.90)	1.77, 2.23	1.99	1.77, 2.23
Steroid sparing center	0.70 (3.50)	0.65, 0.75	0.70	0.65, 0.75
$(Age/50)^{\theta}$	-0.40 (13.5)	-0.50, -0.30	-0.39	-0.50, -0.29
CCB use[7]	0.94 (2.43)	0.89, 0.98	0.94	0.90, 0.99
IIV[8] for CL/F, %CV	40.1	37.4, 43.6	40.0	37.4, 43.6
RUV[9], additive, SD	3.19	3.07, 3.32	3.19	3.08, 3.32

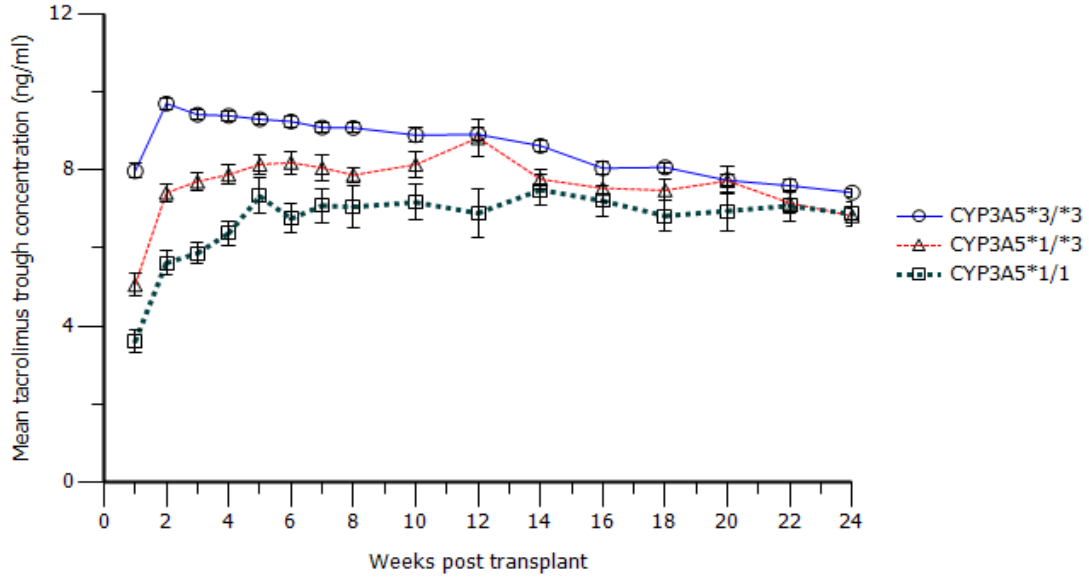
[1] 681 patients analyzed in this study, [2] results from 989 bootstrap runs with successful convergence and successful covariance step, [3] percent relative standard error, [4] confidence interval: estimate ± (1.96*standard error of estimate), [5] typical value of CL/F in L/hr, [6] days post- transplant, [7] calcium channel blocker use at the time of trough measurement, [8] inter-individual variability, [9] random unexplained variability

Figure 2.1: Tacrolimus doses and troughs by CYP3A5 genotype over the first 6 months post-transplant.

A. Total daily doses by CYP3A5 genotype

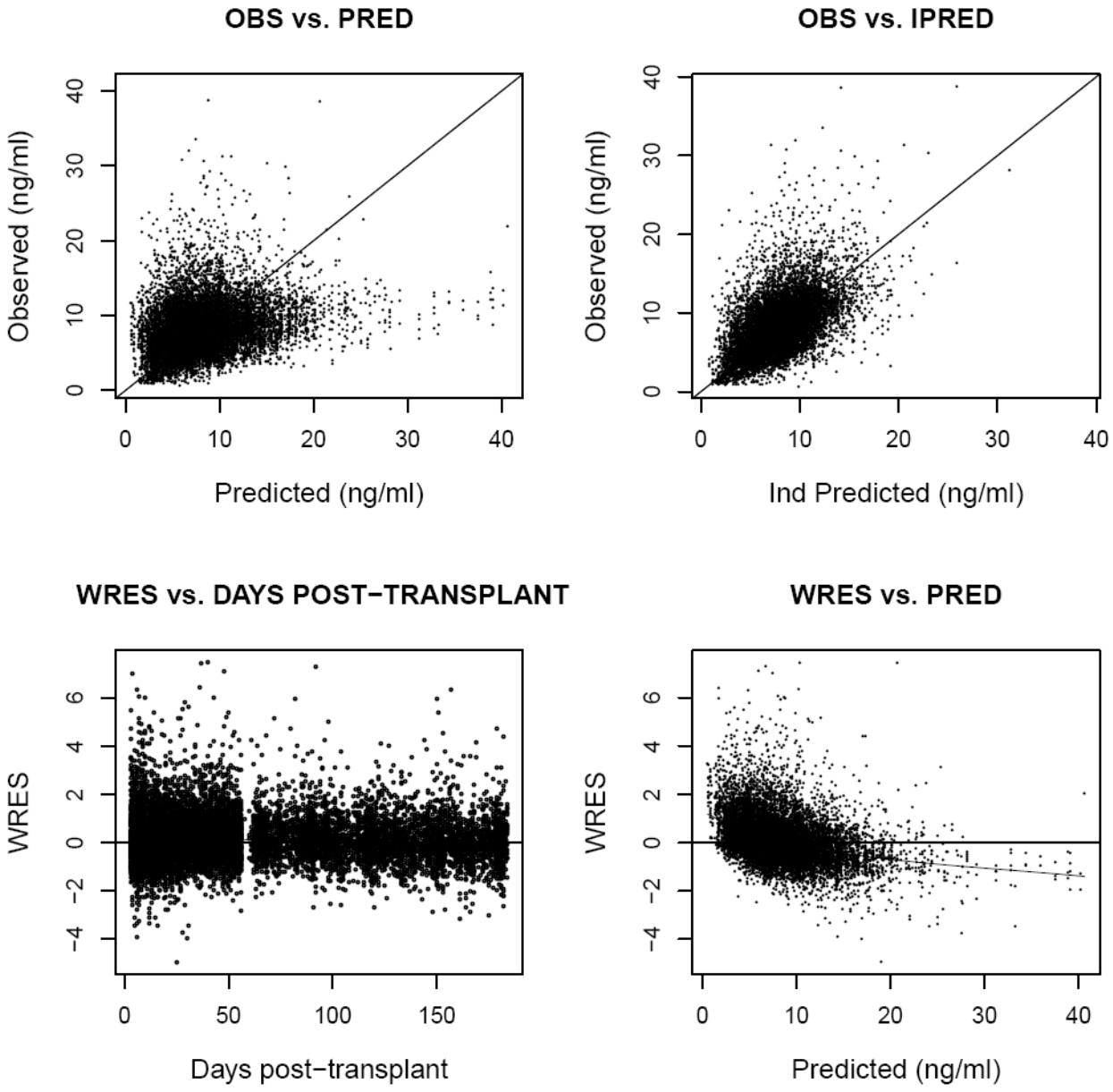


B. Trough concentrations by CYP3A5 genotype.



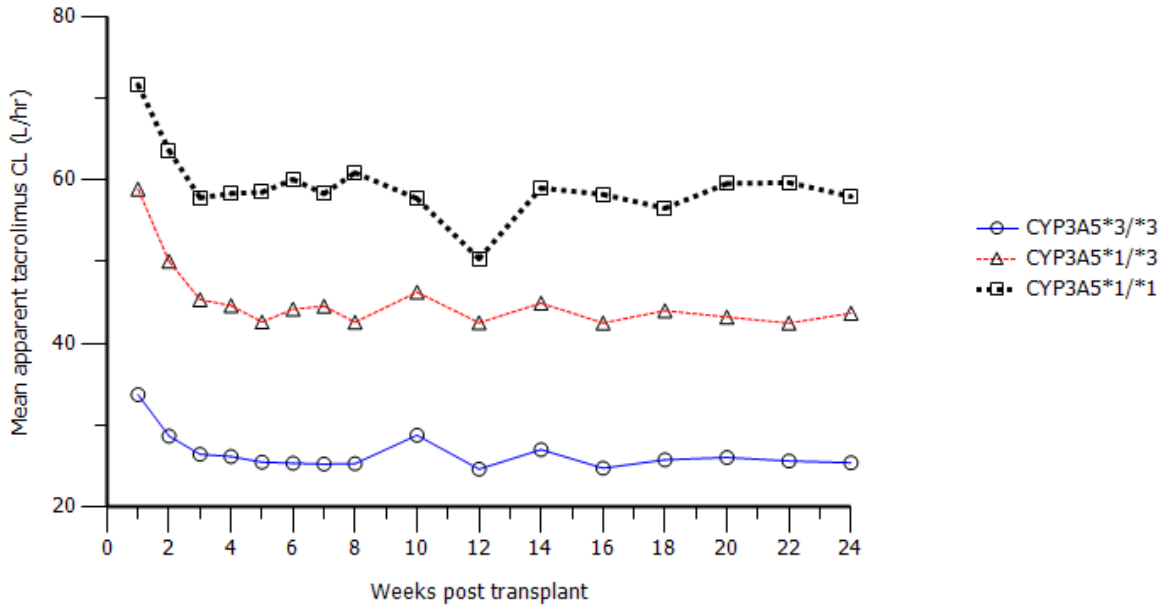
Data are mean and bars represent the standard error

Figure 2.2: Goodness-of-fit plots for the final tacrolimus model



IPRED: Individual predicted concentrations (ng/ml), WRES: Weighted residuals

Figure 2.3: Post-hoc estimates of tacrolimus CL/F from the final model over the first 6 months post-transplant by CYP3A5 genotype



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Chapter III

Validation of tacrolimus equation to predict troughs using genetic and clinical factors

3.1 Introduction

Inter-individual variability in drug exposure and response is a major limitation to drug therapy [1]. Although it is generally not clear why patients do not respond to therapy, a substantial portion of these individuals may have genetic variability that influences drug efficacy, pharmacology and/or systemic exposure [1, 2]. Genetic differences in metabolism enzymes and mechanistic pathways are well known to impact pharmacokinetics, efficacy and toxicity of warfarin. To reduce interpatient variability and improve efficacy, several dosing equations for warfarin which incorporate genetic polymorphisms in cytochrome (CYP) P450 2C9 and vitamin K epoxide reductase complex subunit 1 (VKORC1) along with clinical factors (e.g. age, body surface area, smoking status, race, age, concomitant medications) have been successfully developed [3-7]. Dosing of warfarin by genotype status has been given a level A rating by the pharmacogenetics implementation consortium which highlights the potential importance of genotype in dose determination [8]. Several studies have shown that genotype-guided warfarin dosing resulted in reduced time to stable anticoagulation and faster achievement of therapeutic international normalized ratio, and fewer and smaller dose changes [5, 9].

Tacrolimus is a widely used maintenance immunosuppressant in kidney transplantation [10]. It is rapidly absorbed from the gastrointestinal tract and has a poor oral bioavailability. It is highly bound to plasma proteins and mainly eliminated through metabolism by cytochrome P450 3A5 [11]. Several pharmacokinetic studies have estimated the tacrolimus apparent clearance (CL/F) to range from 21-35 L/hr [12-14]. Its use is complicated by its narrow therapeutic window and large inter-individual variability in pharmacokinetics [15, 16]. Elevated trough concentrations are associated with increased risk of toxicity while low troughs are associated with increased risk of rejection [17]. In the clinical setting, initial tacrolimus doses are based on body weight and subsequent doses are adjusted by therapeutic drug monitoring (TDM) of trough concentrations [15]. Despite these efforts, a large number of patients achieve troughs that are above or below the targeted therapeutic range, particularly in the early days post-transplant [18-20]. To address this problem, we developed an equation using 681 kidney

transplant recipients, enrolled through a multicenter consortium, using clinical factors and genetic variants that individualizes tacrolimus dosing [21]. Our equation estimates an apparent CL/F for an individual based on days post-transplant, CYP3A5*1 genotype status, transplantation at a steroid sparing transplantation center, age at the time of transplant and the use of calcium channel blocker (CCB) (Eq. 1). Then for any desired tacrolimus trough target, the total daily dose (TDD) requirement can be determined using the CL/F estimate (Eq. 2).

CL/F (L/hr) = 38.4*[(0.86, if days 6-10) or (0.71, if days 11-180)]*[(1.69, if CYP3A5*1/*3 genotype) or (2.00, if CYP3A5*1/*1 genotype)]*(0.70, if receiving a transplant at a steroid sparing center)*[(Age in yrs/50)$^{-0.4}$]*(0.94, if CCB is present)
(1)

TDD (mg) = [CL/F (L/hr) * tacrolimus trough goal (ng/mL) * 24 hrs] / 1000 (2)

A prerequisite for the clinical use of our clearance equation is to demonstrate its validity in predicting troughs. Therefore, the objective of this study was to evaluate the predictive performance of the clearance equation to predict troughs relative to actual observed troughs using an independent cohort of kidney transplant recipients. Validation of this equation will provide the additional confidence that our equation can be safely used, may reduce the number of out-of-range trough concentrations and ultimately reduce the number of troughs concentrations necessary to achieve optimal immune suppression. Randomized clinical trials would be a final step to demonstrate the clinical utility of the equation.

3.2 Methods

3.2.1 Study design

Patients in which the equation was developed and validated were recruited from the multi-center observational trial Deterioration of Kidney Allograft Function (DeKAF) Genomics study [21]. Details of the DeKAF Genomics study have been published

elsewhere [22-24] and are registered at www.clinicaltrials.gov (NCT00270712). The equation was developed in the 681 subjects who were selected from the first 1000 subjects enrolled in DeKAF Genomics. The 795 subjects reported here were selected from the next 1000 subjects and were used to validate the equation. Thus, the validation cohort was comprised of a completely separate set of transplant recipient than the development cohort. Approval was obtained from the Institutional Review Board Human Subjects Committees at each of the participating centers. All subjects provided written informed consent. Subject inclusion and exclusion criteria, data collection and analysis for the validation cohort were identical to that of the cohort of patients used for development of the equation [21]. Briefly, patients with end-stage renal dysfunction undergoing kidney or kidney-pancreas transplant who were ≥18 years of age and received tacrolimus (Prograf) at any time in the first 6 months post-transplant were selected for this validation analysis. Clinical data were prospectively collected from the medical records. Tacrolimus trough concentrations were obtained as part of clinical care at the treating center after either once or twice daily oral dosing of tacrolimus. Data were collected for the first 6 months post-transplant. Initial tacrolimus doses were based on body weight (mg/kg) as per standard clinical practice at each site. Subsequent doses were adjusted by TDM to achieve institution-specific targets (in general, troughs of 8–12 ng/mL in the first 3 months post transplant and 6–10 ng/mL in the months 3–6 post transplant). Two trough concentrations were extracted from the medical record in each of weeks 1–8 post-transplant and twice in each of months 3, 4, 5 and 6 post-transplant. Identical to the dosing equation development, only troughs after day 2 post transplant were included in this validation to ensure that tacrolimus was at or near steady state. Genotyping for CYP3A5*1 was conducted in all individuals and was previously described [21].

3.2.2 Statistical analysis

The data were analyzed using the R 12.2.0 statistical package. For each observed tacrolimus trough (C_{obs}) in the validation cohort, a corresponding CL/F was estimated using the previously developed tacrolimus equation (Eq.1). Our previously developed

equation estimated CL/F since tacrolimus was given by the oral route. From each CL/F estimate, a predicted trough (C_{pred}) was then determined for each C_{obs} in the validation cohort (Eq. 3).

C_{pred} (ng/mL) = Dose administered*1000/ (CL/F), (3)

where dose administered is the total daily dose of tacrolimus administered in mg divided by 24 hrs and CL/F is in L/hr.

Predictive performance of the equation was evaluated in two ways: 1) absolute performance for the initial trough concentrations prior to dose adjustments based on TDM information and, 2) relative performance for all trough concentrations over the entire 6 months post-transplant compared to a basic apparent clearance model. The initial trough was defined as the first trough measured at steady state in the 1st week post-transplant. To ensure that tacrolimus was at or near steady state, only troughs after day 2 post-transplant were used in this validation. The mean half-life of tacrolimus is approximately 12 hrs and therefore steady state would be achieved in about 60 hrs (2.5 days) [25]. We did not consider the concomitant use of cytochrome P450 inhibitors or inducers (e.g steroids) to make a determination of steady state. However, we indirectly accounted for the steroid effect in the development of the equation using the transplant center as a covariate, i.e. having a different CL/F for a recipient receiving transplant at a steroid sparing center versus not. The use of steroids (or not) was a center specific practice. The predictive performance of initial and all troughs was assessed by calculation of the bias and precision of the equation using prediction errors.

First, for the initial troughs (n=412 troughs), the absolute predictive performance was evaluated with prediction errors and given as bias and precision of the troughs predicted by the equation relative to the C_{obs}. Prediction error (PE_i) was defined to be the difference between C_{pred} obtained from the equation and the C_{obs} (which were derived from weight based dosing [mg/kg]) (Eq. 4). Bias and precision was calculated as the median prediction error (MPE, Eq. 5) and the median absolute prediction error (MAPE,

Eq. 6), respectively. A 95% confidence interval for these medians was calculated based on binomial probabilities [26].

$$PE_i = C_{pred,i} - C_{obs,i}\,, \quad (4)$$

where $C_{pred,i}$ is the equation-predicted i^{th} trough, and $C_{obs,i}$ is the observed i^{th} trough.

$$\text{MPE (Bias)} = \text{Median}\,(PE_i), \quad (5)$$

$$\text{MAPE (Precision)} = \text{Median}\,(|PE_i|) \quad (6)$$

Second, the relative predictive performance was evaluated over the entire 6 months (n=13,698 troughs). Relative performance of this model was assessed by comparing it to a basic apparent clearance model (derived from the original model before incorporation of clinical and genetic variables into the model, Eq. 7) [21].

$$\text{Basic tacrolimus apparent clearance, } CL/F = 29.7 \text{ L/hr} \quad (7)$$

The assessment was done using the Wilcoxon signed rank test applied to the paired differences (D_i) of the median errors (Eq. 8) for relative bias, and to the paired differences of the median absolute error (Eq. 9) for relative precision [27].

$$D_i = PE_{1i} - PE_{2i} \quad (8)$$

$$D_i = |PE_{1i}| - |PE_{2i}| \quad (9)$$

where PE_{1i} was the prediction error from the equation and PE_{2i} was the prediction error from the basic apparent clearance model in the i^{th} trough concentration.

For assessing relative bias, the null hypothesis (H_0) was that the median of the prediction errors of the two methods (equation and basic apparent clearance model) are the same. For the assessment of precision, the H_0 was that the median of the absolute prediction errors of the two methods (equation and basic apparent clearance model) are the same. A

$p<0.001$ was evidence in favor of rejection of H_0 and was indicative of a difference in bias and precision of the two methods.

3.3 Results

The demographics of subjects in the development and validation cohorts are shown in Table 3.1. The observed versus predicted troughs for the initial troughs are shown in Figure 3.1. The absolute predictive performance of the equation for the initial troughs is given in Table 3.2. For the initial troughs, the equation had a MPE (bias) of 0.2 ng/mL and a MAPE (precision) of 1.8 ng/mL. Thus, the initial troughs were slightly over-predicted by the equation on average by 0.2 ng/mL compared to the actual C_{obs}. The precision was within ± 18% for a trough of 10 ng/mL and within ± 15% for a trough of 12 ng/mL. The relative predictive performance of the equation for all the troughs in the first 6 months post-transplant is given in Table 3.3. The relative performance of the equation was assessed relative to a basic apparent clearance model (Eq. 7). The predictive performance of the equation to predict troughs had a MPE (bias) of 0.3 ng/mL and a MAPE (precision) of 2.9 ng/mL. Both MPE (0.5 ng/mL) and MAPE (3.6 ng/mL) were higher for the basic apparent clearance model in predicting trough as compared to the equation. From the Wilcoxon signed rank test for relative bias and precision, the p-value was <0.001 for bias as well as precision. This supported the rejection of H_0 and indicated that the median differences from the two methods were different. Since the bias and precision were better for the equation as compared to a basic apparent clearance model, the equation was superior to the basic apparent clearance model in predicting the trough concentration for all the observed trough concentrations in the first 6 months post-transplant.

3.4 Discussion

Low tacrolimus troughs in the early post-transplant period have been associated with a higher rate of acute rejection [20]. Mean trough concentrations in the first week post-transplant have been shown to be significantly different between rejectors and non-

rejectors [28, 29]. Assuring that all patients are within the therapeutic target in the early post-transplant period would improve therapy [29]. Therefore, we tested how closely our equation could predict the actual observed initial tacrolimus trough concentrations. The bias of the equation for the initial troughs was low (0.2 ng/mL). Moreover, the precision for the equation (1.8 ng/mL) was good (i.e. less than ± 20% for a trough of 10 ng/mL).

We also evaluated the performance of our equation using all troughs from the first 6 months post-transplant relative to troughs predicted using an basic apparent clearance model which was derived from our previous work in kidney transplant recipients (Eq. 7). This is a naïve predictor of CL/F since it does not take into account clinical or genetic factors that may affect apparent clearance. The bias and precision of the equation in predicting troughs was significantly better compared to the basic apparent clearance model. This is likely because the equation accounts for important clinical factors such as age, co-medications (such as calcium channel blocker) and genetic factors such as the CYP3A5*1 status [30]. A naïve CL/F is similar to current clinical practice where all subjects receive approximately the same tacrolimus dose (0.08-0.09 mg/kg/day) based on body weight. In our previous analyses, we were unable to show that weight was an important determinant of trough. Therefore the use of weight based dosing for tacrolimus is probably of limited benefit in tailoring therapy [18, 19, 21]. For all troughs in the first 6 months post-transplant, the equation had a bias of 0.3 ng/mL and a precision of 2.9 ng/mL (i.e. less than ± 30 % for a trough of 10 ng/mL). We believe that the relative performance may be more relevant than the absolute performance for all troughs since the bias and imprecision is expected to be inflated. This inflation is likely because the reference is a trough measurement that is derived from TDM which incorporates all available clinical information. It is not unexpected that it would have better performance than our equation which is based on a few covariates. The lower predictive performance of the equation using all troughs might be attributable to factors such as unknown drug interactions, non-compliance, and development of co-morbid conditions which are more likely to occur later post-transplant. In addition, dosing is less closely supervised in the

later months post-transplant which may affect the exactness of the trough measurements thereby reducing performance.

Predictive performance analyses suffer from the limitation that reasons for a low predictive performance are never clear. In our case, the lower predictive performance of the equation for all troughs may be attributed to the equation itself or inaccuracies in the reference (which in this case is the C_{obs}). For instance, the C_{obs} may not have been obtained at the ideal trough time point, the patient might be non-compliant, unknown drug interactions might be occurring and there might be substantial variability in the assay. When there is inaccuracy in C_{obs}, the bias and imprecision of the equation is overestimated [27].

Other possible reasons for poor predictive performance is that the equation is misspecified or lacks one or more relevant clinical or genetic factors. The equation does not contain adjustments for every possible medication that may interact with tacrolimus. However, many of the common potential interacting medications were tested during the development phase of the equation (e.g. ACE inhibitors and antiviral agents) and did not influence CL/F [18, 21]. There is a known drug interaction between tacrolimus and mycophenolic acid (MPA) which results in alterations in the MPA concentrations (not tacrolimus) and therefore concomitant MPA use is not relevant towards the equation [31]. Additionally, disease states that might affect absorption and/or gastrointestinal transit time (e.g: gastroparesis, malabsorption, diarrhea) was not recorded. We previously tested for race in the cohorts and it was not a significant covariate. However, African American race is highly correlated with CYP3A5*1 genotype. Therefore, it was difficult to distinguish between effects of race and genotype. Consequently, we are not able to quantify these types of effects with the available data.

In general, the validity of equations can be tested by using either an internal or independent external population. We chose to validate our equation using an external cohort of subjects which is considered more rigorous than an internal validation. However, it has the caveat that if the predictive performances are not reasonable, it is

unfeasible to ascertain whether the unreasonable performance is due to a difference between the populations (development and validation cohort) or due to a model misspecification [32].

In conclusion, we conducted a validation of our equation in an independent cohort of kidney transplant recipients. The bias and precision was good for the initial troughs. The low bias (over-prediction of 0.2 ng/mL) and high precision (error of less than ± 20% for troughs of 10 ng/mL) demonstrated the ability of the equation to predict initial troughs. Consequently, we believe that the equation can be safely used for predicting initial troughs. Acute rejection is an early event with two-thirds of events occurring within the first 3 months post-transplant and fewer between months 4-6 post-transplant. It has been shown that low exposure to tacrolimus as early as the first week post-transplant is a predictor of early acute rejection [29]. Therefore any tools to predict troughs with an equation such as the one we developed may get patients to reach the therapeutic dose faster and reduce the number of out-of-range toughs and dose changes required. The ability to predict troughs based on an equation instead of the traditional trial and error dosing approach may lower overall costs of monitoring. The cost associated with genotyping may be recovered by the reduction in the number of troughs levels obtained and staff utilization. A study to prospectively apply the equation to dose selection is needed in the future. Comparison of the cost-benefit ratio of therapeutic drug monitoring with and without this equation was outside the scope of this study. The future of pharmacogenomics is dependent upon developing simple clinical tools that can translate genetic association findings into the clinical environment. Studies such as this demonstrate the potential value of genetic based dosing equations.

Table 3.1: Characteristics of subjects in the equation development and validation cohorts

Clinical characteristics		Development cohort	Validation cohort
No. of subjects		681	795
Age (yrs) of recipient[1]		50.2 ± 12.2	50.5 ± 13.2
Baseline weight (kgs) of recipient [1]		81.3 ± 18.7	83.0 ± 19.3
Gender of recipient (male/female)		429(63%)/ 252 (37%)	499(63%)/ 96 (37%)
Race (Non-African American /African American)		538(79%)/ 143(21%)	652(82%)/ 143(18%)
No. transplanted at steroid sparing center		205 (30%)	100 (12.6%)
Living donor		398 (59%)	465(58.5%)
No. of troughs		11,823	13,698
Mean tacrolimus daily dose in mg/kg[2]		0.08 ± 0.05	0.09 ± 0.05
Mean tacrolimus trough in ng/mL[2]		8.31 ± 3.48	8.53 ± 3.41
No. of patients with troughs <8 ng/mL	Week 1	413 (75%)	476 (60%)
	Week 2	331 (55%)	375 (47%)
Calcium channel blocker use[3]		5082 (43%)	5843 (43%)
CYP3A5 genotype (rs776746)	*1/*1	72 (11%)	75 (9.4%)
	*1/*3	129 (19%)	147 (18.5%)
	*3/*3	476 (70%)	573 (72.1%)

[1] reported age and baseline weight are those measured at the time of transplant and are mean ± S.D., [2] doses and troughs are over the 6 month study period and are mean ± S.D., [3]No. of troughs collected when calcium channel blocker was used

Table 3.2: Predictive performance of tacrolimus equation in predicting observed initial troughs (n=412 troughs in 412 subjects)

Absolute predictive performance	Equation (95% C.I.)
MPE (Bias)[1]	0.2 (0, 0.5)
MAPE (Precision) [2]	1.8 (1.6, 2.0)

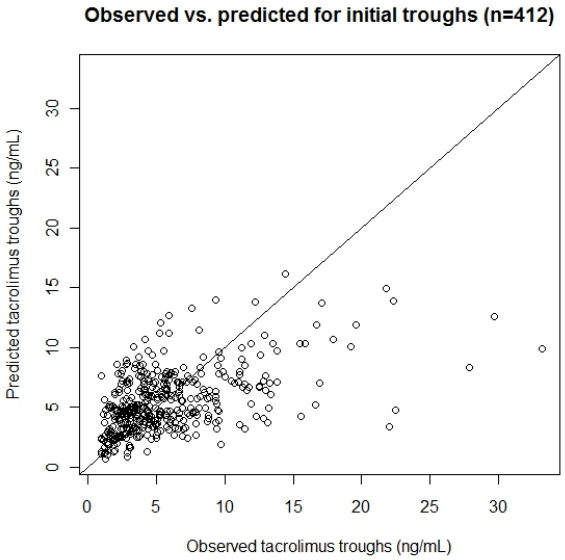
[1] Mean prediction error indicates the bias of the equation in predicting the initial troughs in ng/mL, [2] Median absolute prediction errors indicates the precision of the equation in predicting the initial troughs in ng/mL

Table 3.3: Predictive performance in predicting observed tacrolimus troughs (13,698 troughs in 795 subjects) using the tacrolimus equation relative to a basic apparent clearance model with no covariates in the first 6 months post-transplant

Absolute predictive performance	Equation (95% C.I.)	Basic clearance model (95% C.I.)
MPE (Bias)[1]	0.3 (0.2, 0.4)	0.5 (0.4, 0.6)
MAPE (Precision)[2]	2.9 (2.8, 2.10)	3.6 (3.5, 3.7)

[1] Mean prediction error indicates the bias of the equation in predicting all troughs in ng/mL, [2] Median absolute prediction errors indicates the precision of the equation in predicting all troughs in ng/mL.

Figure 3.1: Initial observed troughs versus the initial troughs predicted by the clearance equation (n=412)



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Chapter IV

A novel "winnowing method" of covariate selection using tacrolimus pharmacogenetics as a motivating example

4.1 Introduction

One of the main components of a population pharmacokinetics (PopPK) analysis is to quantify the relationship between covariates and pharmacokinetic parameters [1]. A covariate is a characteristic of the patient population such as age, co-medication, etc. that accounts for a portion of the variability seen in a drug's pharmacokinetic-pharmacodynamic response [1, 2]. Covariate selection is a rigorous process of any PopPK analysis. Several methods are available for covariate selection.

Preliminary analysis involves plotting of the post-hoc/empirical Bayes estimates (EBEs) of the individual clearances (CL_i) from the base model against covariates to determine the strength and shape of any relationship visually [3]. A commonly employed statistical method of investigation is the forward selection and backward elimination process using a critical cut-off p-value [1, 2, 4]. This method is performed either manually or in an automated fashion using Perl Speaks NONMEM (PsN) or other software packages such as Phoenix® NLME™. Xpose, an add-in for NONMEM®, selects the best model from a set of covariates based on the Akaike Information Criteria (AIC) using Generalized Additive Modeling (GAM) [5]. The covariates selected through GAM can be tested further in NONMEM® [6].

When the number of covariates is large, an exhaustive analysis of all possible combinations can be a time consuming or impractical approach. Software limitations start to arise when such a large number of covariates are to be tested. Processing time also increases exponentially as the number of covariates increases. As an example, we were presented with 119 single nucleotide polymorphisms (SNPs) that could have a potential role in tacrolimus apparent clearance (CL/F). This gives rise to $2^{119} \approx 10^{35}$ combinations. When tested using GAM (in Xpose), we were able to assess only 25 categorical SNPs (each with 3 levels) at a time. Thus, all SNPs could not be tested together in combination.

Therefore, the objective of this analysis was to devise and validate a method of covariate selection that can be used when there are a large number of covariates. This method should be able to select covariates in combination and reduce the processing time needed

to select the covariates. We used tacrolimus pharmacogenetics as a motivating example to develop this method and validated the method with the help of simulation studies.

4.2 Methods

4.2.1 Study Design

Patient enrollment was done from the multi-center genomics study Deterioration of Kidney Allograft Function (DeKAF) observational trial [7]. Details of the DeKAF study have been published elsewhere [8-10] and are registered at www.clinicaltrials.gov (NCT00270712). Recipient DNA was genotyped for 2,724 SNPs using a customized Affymetrix SNP chip. The top 119 SNPs based on a Bonferroni-corrected p-value were selected using repeated measures regression analysis in Statistical Analysis Software (SAS) and taken forward into the non-linear mixed effects modeling (NONMEM®) software package.

4.2.2 Software

NONMEM® (Version 7.1, NONMEM® Project Group, Globomax LLC) with a Visual Fortran Compiler (Windows Professional Edition 11.1) and PdxPop (Version 4.0, ICON Development Solutions) as an interface for NONMEM® was used. Additionally, the 'gam' package was installed in the R 12.2.1 statistical package and utilized.

4.2.3 Population pharmacokinetics modeling

The structural model was developed from the analysis of 11,823 troughs from 681 kidney transplant patients receiving tacrolimus and has been described elsewhere. Briefly, a steady state infusion model was utilized to describe the trough concentrations [7].

4.2.4 Screening of covariates

A novel "winnowing" scheme was followed to screen the genetic covariates for the significant SNPs from amongst the 119 potentially significant SNPs. Winnowing was devised to overcome the challenges associated with testing each of the 119 SNPs univariately and further in combination with other SNPs.

The first step was to obtain the base model (structural PK model with no covariates) model and EBEs from NONMEM®. Next, a series of GAM runs were devised using the 'gam' package in R.12.2.1. In the first GAM run, the EBEs of CL/F from the base model were regressed on a subset of 25 SNPs out of the 119 SNPs. This subset was a group of 25 randomly selected SNPs from the 119 SNPs (referred to as the *group size* of the random selection process). The most important combination of SNPs in a set of 25 randomly chosen SNPs was identified based on the AIC. The process was repeated 500 times (referred to as the *number of GAM repetitions*) with different subsets of 25 SNPs randomly selected each time in order to capture the various possible SNP combinations.

A SNP index (ranging from 0-1), computed as the number of times a given SNP was found in the best-case model, i.e. selected divided by the number of times the SNP was chosen at random from the 119 SNPs, i.e. sampled, was created to assign ranks for the individual SNPs (Eq. 1). Figure 4.1A shows the histogram of the SNP index after the first set of 500 GAM runs.

$$\text{SNP Index} = \frac{\text{Number of times SNP is selected}}{\text{Number of times SNP is sampled}} \quad (1)$$

After the first winnowing process, SNPs with an index of 0 (never important in a given set of SNPs) were eliminated from consideration. SNPs having an index of 1 (i.e. always important in a given set of SNPs) were incorporated into the revised model in NONMEM®. The covariate modeling within NONMEM® was done in a multiplicative fashion. The new model with the important SNPs incorporated was re-run in NONMEM® and the estimates of delta clearance (ΔCL, Eq. 2) were obtained.

$$\Delta CL_i = CL_i - TVCL \quad (2)$$

where TVCL is the typical value of the clearance and CL_i are the individual clearances.

The ambiguous SNPs (index 0.1- 0.9) were regressed on the estimates of ΔCL_i from the revised base model as the dependent variables. This was the second GAM run. Again, a group of 25 randomly selected SNPs were regressed on ΔCL_i. This process was repeated 500 times and a second SNP index was generated.

The process of running the revised model in NONMEM® based on the SNP index and testing the ambiguous SNPs in GAM was repeated until no ambiguous SNPs remained, i.e. all SNPs had a SNP index of either 1 or 0 (Figure 4.1B). This was the full covariate model from forward selection. Backward elimination was then performed for the full model. An increase in objective function ($p < 0.05$) over the base model was considered significant.

4.2.5 Validation of the method

PK data were simulated from a one-compartment intravenous (i.v.) bolus model. The dataset consisted of 500 individuals with 7 samples per individual drawn at 0.25, 0.5, 1, 2, 4, 6 and 8 hrs after administration of a 500 mg dose of the drug. The typical value of CL was set to 5 L/hr and the typical volume of distribution (V_d) was set to be 14.5 L/hr. A proportional error model was assumed for inter-individual variability (IIV) on CL and V_d and was set to 30% for both PK parameters. A proportional error model was assumed for random unexplained variability (RUV) and set to 10%. Using the R statistical package, 100 SNPs with specific allele frequencies were simulated. The effects of 10 important SNPs on CL were incorporated into the model and the corresponding observed concentrations were simulated using NONMEM®. The effect sizes of the 10 important SNPs and their corresponding allele frequencies were selected from published manuscripts and are listed in Table 4.1 [7, 11-16]. The remaining 90 SNPs were simulated either at a frequency of 70%, 20% and 10% or 60%, 30% and 10% for homozygous dominant, heterozygous recessive and homozygous recessive populations, respectively.

Once the simulated dataset with the specific SNP effects and IIV and RUV had been generated, the winnowing scheme was carried out as has been described above. A group size of 25 was chosen with 500 GAM repetitions similar to the analysis dataset. The objective was to determine if the 10 important SNPs were selected from the 100 SNPs available for analysis by this scheme. Type I (false positive) and type II (false negative) error rates (Eq. 3 and 4) were also calculated for the approach.

$$\text{Type I error } (\alpha) = 1 - \text{Specificit y} = \frac{\text{False positive}}{\text{False positive} + \text{True negative}} \quad (3)$$

$$\text{Type II error } (\beta) = 1 - \text{Sensitivit y} = \frac{\text{False negative}}{\text{False negative} + \text{True positive}} \quad (4)$$

4.2.6 Sensitivity analysis

4.2.6.1 Sensitivity to group size and number of GAM repetitions

The PK dataset used for this sensitivity analysis was the same as used for the validation. A sensitivity analysis was performed for the group size and the number of GAM repetitions. The group sizes that were tested were *5, 10 and 25 SNPs* randomly selected at one time. The number of GAM repetitions that were tested included *250, 500 and 1000 repetitions*. Thus a total of 9 combinations were tested. The type I and type II error rates were calculated as before (Eq. 3 and 4).

4.2.6.2 Sensitivity to minor allele frequency and covariate effect size

A sensitivity analysis was performed for the minor allele frequency and the SNP effect size that can be detected using this approach. To decrease the complexity arising out of the number of combinations possible, only two allele possibilities were assumed, i.e. either carrier of the recessive allele or not. Therefore, the minor allele frequencies that were tested included 5%, 10%, 15%, 20% and 30%. The covariate effect sizes that were assumed were an increase in CL of 20%, 30%, 40%, 50% and 75% for the carrier of the recessive allele. Thus, a total of 25 combinations were possible. The PK dataset was similar to that used in the validation analysis. A total of 1000 individuals with 6 samples per individual drawn at 0.25, 0.5, 1, 2, 4 and 6 hrs after administration of a 500 mg dose of the drug. Using the R statistical package, 100 SNPs with the allele frequencies specified above were simulated. The remaining SNPs were simulated with varying minor allele frequencies. The effect of the 25 important SNPs on CL was incorporated into the model and the corresponding observed concentrations were simulated using NONMEM®. The winnowing process was performed with a group size of 25 and 500 GAM repetitions similar to the tacrolimus analysis.

4.3 Results

4.3.1 Winnowing process for tacrolimus pharmacogenetics

The winnowing scheme selected 26 SNPs as the full model. The exact process followed to obtain these SNPs is depicted in Figure 4.2. It involved a total of 5 updated NONMEM® covariate models and 5 runs in GAM to attain the full model. Backward elimination was then performed to obtain the 19 SNPs affecting tacrolimus CL/F.

Amongst the 19 SNPs obtained from winnowing, 6 SNPs were associated with a 2-86% increase in CL/F from the base model, 6 SNPs were associated with a 6-38% decrease in CL/F from the base model and 7 SNPs were associated with ambiguous CL/F. The details of the clinical effects of individual SNPs and alleles are summarized in Table 4.2. Our approach selected the CYP3A5*1 SNP which is a well known polymorphism affecting tacrolimus CL/F. The role of other SNPs on tacrolimus CL/F is not well established. For the ambiguous SNPs, it is possible that the number of individuals in one of the three categories of the polymorphism was insufficient to establish a definite effect.

4.3.2 Winnowing process for validation dataset

A total of 18 SNPs were picked up the winnowing method for the full model in the validation dataset. All true positive SNPs (n=10) and 8 other false positive SNPs were selected by the winnowing approach. The type I and type II error rates were 9% and 0%, respectively.

The type I and type II error rates for the sensitivity analysis for the group size and number of GAM repetitions is shown in Figure 4.3. In general, for a given number of GAM repetitions, the type I error rate decreased with increasing the group size, i.e. fewer false positives were obtained with higher group size. For a group size of 25, type I error rate was 9% for all the GAM repetitions. For group sizes 5 and 10, the type II error rate increased with an increasing number of GAM repetitions, i.e. more false negatives were obtained with increasing GAM repetitions. The type II error rate was 0% for all GAM repetitions with a group size of 25.

For the sensitivity analysis for minor allele frequency and effect size, out of the 25 important SNPs, 23 SNPs were selected. The 2 SNPs that were not selected had a common effect size of 20% and a minor allele frequency of 5% and 10%. The type I and type II error rates for this winnowing scheme was 7% and 8% respectively.

4.4 Discussion

In this analysis, we developed and validated a novel "winnowing method" for selection of covariates when the initial number of covariates is large. We referred to this approach as a winnowing method since it is analogous to the winnowing of wheat where the whole grain is thrown in the air to separate it from the unwanted part, i.e. chaff. Here, we wanted to test the 119 SNPs, remove the unwanted SNPs from consideration and retain the important SNPs. These SNPs could be further tested in NONMEM®. We used tacrolimus pharmacogenetics as a motivating example to develop this method. Using this method, followed by backward selection, we were able to select 19 SNPs from the 119 SNPs. The SNPs selected included the widely established CYP3A5*1, which is well known to increase tacrolimus CL/F [17-19].

We validated this method using a simulation study. The assumptions used for the simulation study were reasonable and supported a well-designed pharmacokinetic study with precise analytical procedures. The method was able to select all of the 10 important SNPs from the 100 SNPs in the validation cohort thus yielding a 0% false negative rate. In addition to the true positive SNPs, 8 false SNPs were also selected putting the false positive rate of the method to be 10%.

We further carried out a sensitivity to explore the combination of iteration group size and number of GAM repetitions that would generate the least type I and type II error rates. The type I error rate was between 9-12% for all combinations. In general, we found that type I error rate was seen to decrease with higher group size for any given number of GAM repetitions. The type II error rate was between 0- 10% for all combinations. Type II error rate increased with the increase in the number of GAM repetitions for the 5 and 10 group size but was 0% for all GAM repetitions with the group sizes of 25. For the 25 group size, with all three GAM repetitions (250, 500 and 1000), the type I and type II

error rates were the same. Based on this a group size of 25 with any GAM repetitions seems to be the most accurate although 250 GAM repetitions will be the least time consuming.

We also carried out a sensitivity analysis to determine the lowest effect size and/or minor allele frequency that a SNP might have before it can be selected by the winnowing process. We tested a range of effect sizes (20-75% change in clearance of the recessive allele) and minor allele frequencies (5-30%). We saw that a SNP that has a minor allele frequency of <10% along with an effect size of ≤ 20% are not detected. Thus, out of the 25 SNPs that we had, 2 SNPs were not detected.

Covariates can be tested either within or outside of NONMEM®. The main disadvantage of testing within NONMEM® is that the process is intensive requiring long run times and is sometimes impractical with a large number of covariates. Additionally, when covariates are tested using stepwise method using a critical p-value in NONMEM®, testing is done on several model parameters (e.g. CL, V_d, etc.) and several functional forms are explored. This increases the overall probability of false positives due to multiple comparisons [20].

We created a SNP index that ranks SNPs based on the number of times the SNP is selected out of the number of times it is randomly sampled. This strategy reduces the type I error rate by decreasing the number of times a SNP is tested as compared to when it would have been tested if all combinations were accounted for. The remaining SNPs are regressed on the ΔCL until all SNPs are either included in the model or are removed from consideration since they have a SNP index of 0. Thus, this method utilizes continuous feedback from the NONMEM® generated EBEs and is different from simply testing all the covariates outside of NONMEM®.

This approach requires the use of the R statistical package. Therefore, the method is free to use and easy to implement. The R-script can be added to a NONMEM® add-in such as Xpose and can be easily implemented from there. However, the method has certain limitations. It utilizes EBEs and may not be suitable in cases where a substantial amount of shrinkage exists. Shrinkage may result in hidden, induced or distorted covariate

relationships. Also GAM is unable to handle time varying covariates or inter-occasion variability and may not be suitable when such covariates exist.

We were able to select 26 SNPs by this process out of which 7 were removed by backward elimination. In the validation set, we were able to select all the 10 important SNPs using the winnowing method. Both the type I and type II error rates were within 12%. The selection process was sensitive to group size and both type I and type II error rates were seen to decrease with increase in the group size for a given number of GAM repetitions.

In conclusion, we developed and validated a novel winnowing method of covariate selection using GAM and EBEs from NONMEM. This approach can be utilized when there are a large number of covariates (> 25). The method can be made available as an add-in to NONMEM®. It can be used for initial screening of covariates and the selected covariates can be further tested in NONMEM®.

Table 4.1: Allele frequencies and effect sizes for the 10 significant SNPs used in the validation dataset

SNP	Allele frequency[1] (Homoz./Heteroz./Heteroz. Recessive)	% Change in CL
SNP 1	60/30/10	100%/ ↓ 40%/ ↓ 65%
SNP 2	95/5	100%/ ↓ 20 %
SNP 3	50/45/5	100%/ ↓ 15%/ ↓ 35%
SNP 4	95/5	100%/ ↓ 40%/
SNP5	70/20/10	100%/ ↑ 70%/ ↑ 100%
SNP 6	50/25/25	100%/ ↓ 30%/ ↓ 30%
SNP 7	85/10/5	100%/ ↓ 50% /↓ 75%
SNP 8	18/50/32	100%/ ↓ 15%/ ↓ 55%
SNP 9	25/50/25	100%/ 100%/ ↑ 20%
SNP 10	85/15	100%/ ↑ 30%

[1] Allele frequency represented as a % for the 10 significant SNPs

Table 4.2: SNPs associated with a change in tacrolimus CL/F

rs number	Gene	% change in CL/F if heteroz.	% change in CL/F if homoz.
rs776746_A	CYP3A5	↑ 80	↑ 86
rs7517376_G	FMO1	↑ 6	↑ 23
rs4646312_C	COMT	↑ 5	↑ 17
rs2295298_A	NFATC4	↑ 4	↑ 15
rs848291_T	FANCL	↑ 7	↑ 23
rs3136228_G	MSH6	↑ 2	↑ 18
rs3136516_A	F2	↓ 8	↓ 17
rs513349_G	BAK1	↓ 6	↓ 19
rs10211_G	CYP3A7	↓ 6	↓ 18
rs3734354_A	SIM1	↓ 11	↓ 38
rs6330_T	NGFB	↓ 8	↓ 15
rs12114000_A	CYP3A4	↓ 23	↓ 26
rs628816_A	DPYD	↓ 13	↑ 5
rs3135506_C	APOA5	↓ 16	↔
rs582537_A	IL12A	↑ 11	↑ 5
rs2332673_C	BCL2	↑ 17	↓ 13
rs491347_C	LRP5	↓ 6	↑ 2
rs6457815_C	PPARD	↓ 4	↑ 5

Figure 4.1.A. SNP index after first and last set of GAM runs

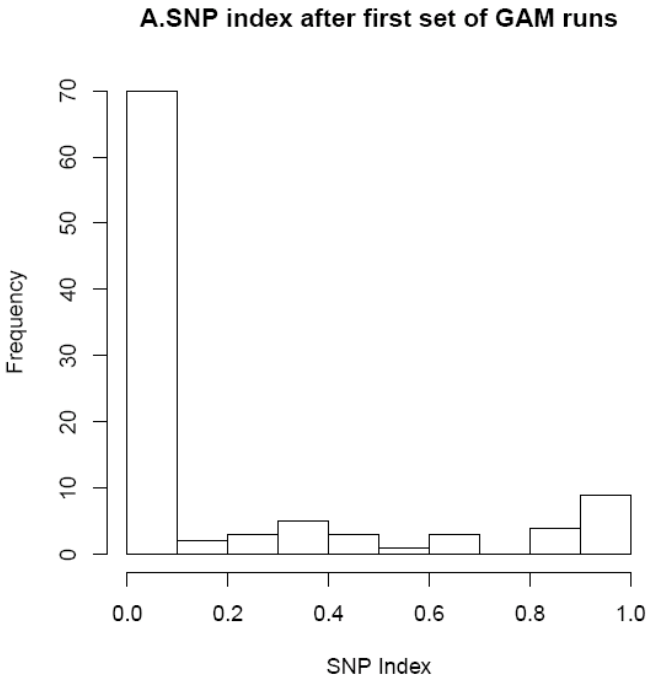


Figure 4.1.B. SNP index after first set of GAM runs

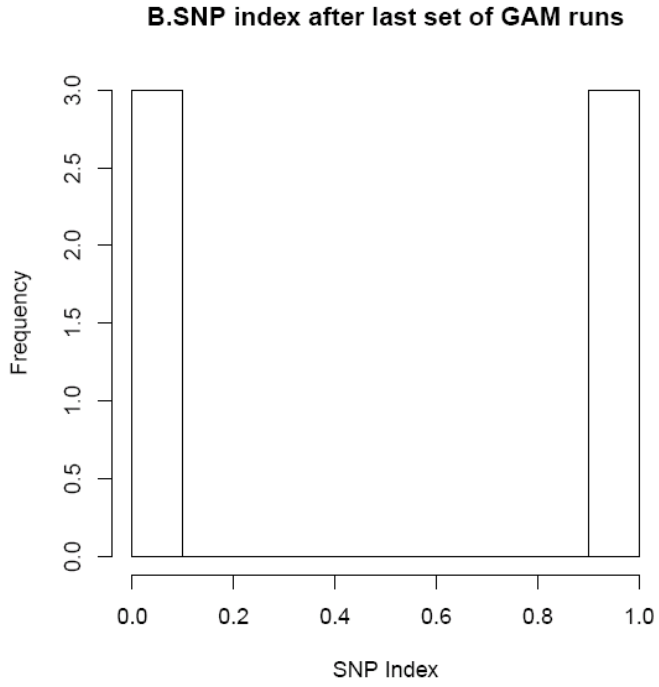


Figure 4.2: Schematic representing the winnowing process for the 119 SNPs for tacrolimus

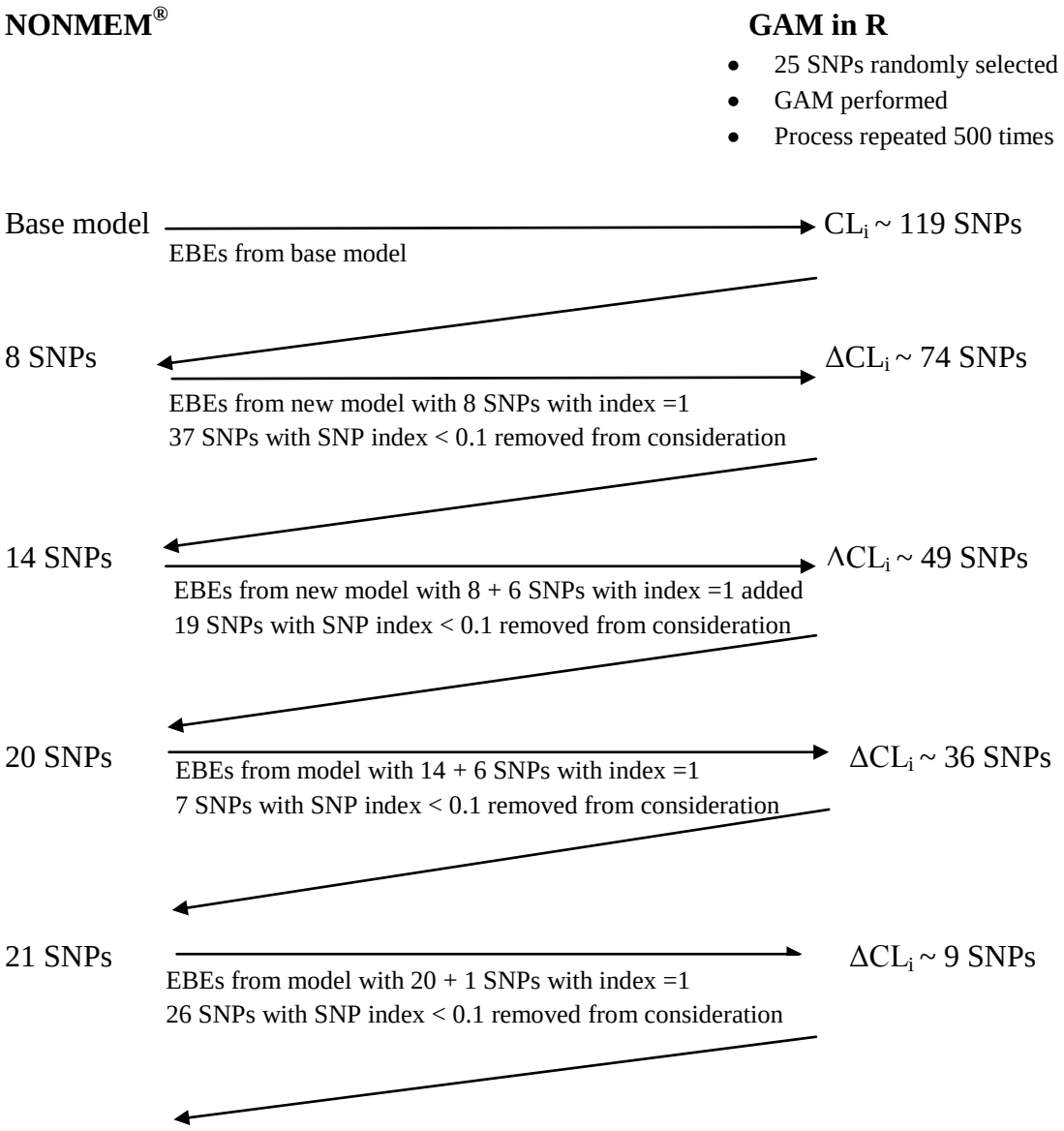
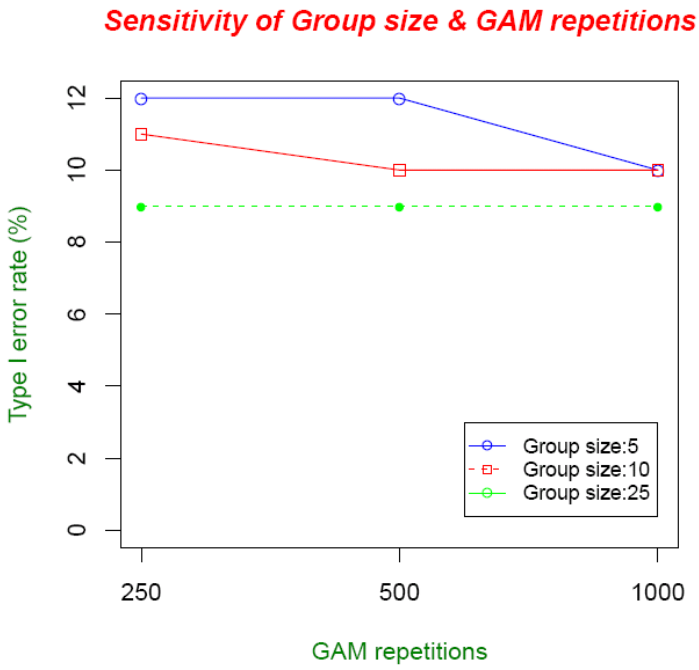
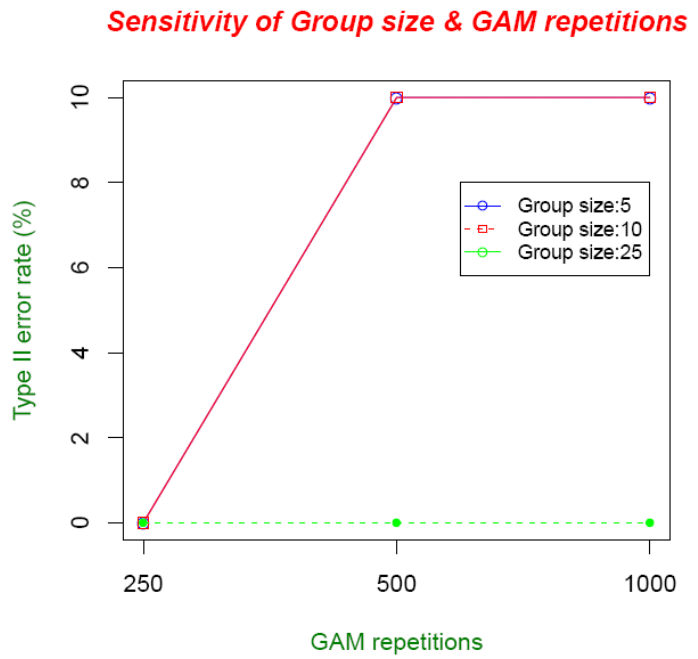


Figure 4.3: Sensitivity analysis of the winnowing process to group size and GAM repetitions as assessed by Type I and Type II error rates





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Chapter V

Comparison of covariate selection between two population pharmacokinetic programs, NONMEM® and Phoenix® NLME™, using clinical and simulated data

5.1 Introduction

Covariate modeling is an integral part of any population pharmacokinetics (PopPK) study. A covariate is any characteristic of a patient population that helps to explain a portion of the variability observed in a drug's pharmacokinetic/pharmacodynamic (PK/PD) response [1, 2]. Identifying the strength and mathematical form of a covariate relationship is a challenging and time consuming portion of any PopPK analysis [3-5]. Once significant covariates are identified as being important to the overall relationship, the equation from the final covariate model can be utilized to individualize dosing regimens in the clinic [1, 2, 4]. One of the most frequently employed methods for covariate modeling is the forward selection and backward elimination approach using a goodness-of-fit statistic (objective function value, OFV) [1, 2, 4]. Covariates are entered into the model in a stepwise manner based on a critical p-value. Once a full model has been obtained by forward selection, the covariates are eliminated sequentially using a more stringent critical p-value to avoid Type I error [4].

Several software packages are employed for PopPK modeling. The oldest and most widely used package is the non-linear mixed effects modeling software package (NONMEM®) [4, 6]. NONMEM® was developed by Lewis Sheiner and Stuart Beal at the University of California, San Francisco in the 1980s [6]. Owing to its existence for more than 30 years, NONMEM® has more support and discussion forums available than any other software package. However, its use requires considerable training and experience and is limited to trained users [7]. Other packages are available including Phoenix® NLME™ which was released in 2009 [8]. Phoenix® NLME™ has an interface with visual work flow and graphic models [7]. The modeling approach in Phoenix® NLME™ is similar to NONMEM® (non-linear mixed effects modeling). One desirable feature of Phoenix® NLME™ is the presence of multiple menu-driven covariate selection methods (scenario, automated stepwise and shotgun method). When there are a large number of covariate relationships to analyze, it is possible to exceed the limitations in both the software packages, such as number of fixed effects, or number of columns in data. A menu-driven graphical interface to manage and automate the covariate modeling process,

such as that available in Phoenix® NLME™, is especially useful since manual testing is very time consuming and computer-intensive. Therefore, it may be advantageous to use one software package over another for particular analyses.

Most of the analyses submitted for FDA approval utilize NONMEM®. In order to facilitate the use of Phoenix® NLME™ for a particular analysis (say involving a large number of covariates), comparison of results across software packages is essential in order to ensure consistency across platforms [9]. Furthermore, the first-order conditional estimation (FOCE) algorithms in NONMEM® and Phoenix® NLME™ are slightly different which may lead to different results [7]. During covariate selection, slight differences in algorithms may result in the selection of a different set of covariates or differences in the size of covariate effects. If these differences are substantial or the drug has a narrow therapeutic window, it could lead to variations in clinical interpretation such as dose recommendations. Comparison of Phoenix® NLME™ with the current gold standard (NONMEM®) is needed to ensure that results are comparable between software packages.

This analysis utilized data from a retrospective clinical study and a simulation study. The purpose was 1) to compare covariate selection by NONMEM® and Phoenix® NLME™ and 2) to identify differences in effect size of the covariates selected, when the same data was analyzed by both software packages. The differences were tested for their influence on clinical endpoint (such as dose adjustment) for the clinical study and for statistical significance (difference in predictive performance) for both the clinical and simulated study. Additionally, the automated stepwise selection method and shotgun covariate selection in Phoenix® NLME™ was explored.

5.2 Methods

5.2.1 Population pharmacokinetics

PopPK analyses consist of 4 main components: 1) determining the basic PK model which is also referred to as the structural model, 2) ascertaining the covariate model, which identifies the important covariate relationships in the data, 3) determining the inter-

individual variability (IIV) model and 4) determining the residual unexplained variability (RUV) model [8, 10].

In this analysis, we compared the aspect of covariate model determination using two different PopPK modeling software packages, NONMEM® and Phoenix® NLME™, using tacrolimus PK as an example. The objective for the clinical data was to determine covariates that would aid in prospectively predicting the dose for an individual by identifying those factors that affect tacrolimus apparent clearance (CL/F). The objective for simulated data was to identify factors affecting clearance (CL) for a simulated PK dataset. Structural model, error model and initial estimate were set identical in both programs.

5.2.2 Compound

Tacrolimus is a common maintenance immunosuppressant used in kidney transplantation and given for the life of the transplanted kidney. Initial doses are determined by body weight (mg/kg dosing) and subsequent doses are adjusted by therapeutic drug monitoring (TDM) [11, 12].

5.2.3 Study design

5.2.3.1 Clinical data

This was a seven-center study conducted in the U.S. and Canada. The details of this clinical study have been described in previous manuscripts [13, 14]. Briefly, tacrolimus troughs (n=11823) from 681 kidney recipients were analyzed. The covariates available for analysis were: recipient age, weight, sex and race (African American (AA) or non-AA), donor type (living or deceased), donor gender, pre-emptive transplant, number of prior transplants, enrolling center, concomitant use of a calcium channel blocker (CCB) at the time of each trough measurement, use of steroids at the time of each trough measurement, days post transplant (DPT) and 6 genetic variants: rs776746, rs12114000, rs3734354, rs4926, rs3135506 and rs2608555 [13].

5.2.3.2 Simulated data

PK data were simulated from a one-compartment intravenous (i.v.) bolus model. The dataset consisted of 100 individuals with 6 samples per individual drawn at 0.25, 0.5, 1, 2, 4 and 6 hrs after administration of a 500 mg dose of the drug. The typical value of clearance was set to 5 L/hr and the typical volume of distribution was set to be 14.5 L/hr. The covariates available for analysis were age, creatinine clearance (CrCL), smoking status (yes/no), sex, race (AA or non-AA) and co-medication (yes/no). Covariate effects were built into the simulated data. The covariates affecting CL were CrCL, smoking status and race. CrCL affected CL according to a power function (Eq. 1).

$$CL = \left(\frac{CrCL}{110} \right)^{0.7} \quad (1)$$

Smoking increased CL by 50% and AA race decreased CL by 30%. A proportional error model was assumed for IIV on clearance and volume and it was set to 30%. A proportional error model was assumed for RUV and set to 10%.

5.2.4 Software

NONMEM® (Version 7.1, NONMEM® Project Group, Globomax LLC) with a Visual Fortran Compiler (Windows Professional Edition 11.1) and PdxPop (Version 4.0, ICON Development Solutions) as an interface for NONMEM® were used. Phoenix® NLME™ (Version 1.2, Pharsight Corporation, Cary NC) was used for the comparator analyses.

5.2.5 Covariate model building

Covariates were modeled in a multiplicative fashion. Covariate selection in NONMEM® was performed using the traditional forward selection ($p<0.01$) and backward elimination ($p<0.001$) approach. The first order conditional estimation with interaction (FOCE-I) was utilized. Details of the model obtained by NONMEM® have been extensively reported in a prior publication [13]. Selection in Phoenix® NLME™ was done using the manual

stepwise selection: the forward selection ($p<0.01$) and backward elimination ($p<0.001$) approach similar to NONMEM®. The first order conditional estimation extended least squares (FOCE-ELS) algorithm, considered to be the counterpart of FOCE-I in NONMEM®, was used for Phoenix® NLME™. The same approach, i.e. forward selection ($p<0.05$) and backward elimination ($p<0.01$) with the FOCE-I and FOCE-ELS, was used for covariate model building for the simulated data.

Software packages were assessed in terms of the covariates influencing tacrolimus CL/F and their effect size. For the clinical data, comparison of the two programs was done by assessing both clinical and statistical differences. Clinical difference was judged by determining the difference in tacrolimus daily doses needed to achieve a typical tacrolimus trough of 10 ng/mL in the first two weeks post-transplant for 681 recipients, using covariate effects from the final models. The equations from the final CL/F model were used to determine a total daily dose (TDD) according to a steady state infusion model (Eq.2). The details of this method have been described elsewhere [14].

TDD (mg) = [CL/F (L/hr) * tacrolimus trough goal (ng/mL) * 24 hrs] / 1000 (2)

where TDD is the total daily dose of tacrolimus administered in mg divided by 24 hrs, target tacrolimus trough goal is 10 ng/mL and CL/F is in L/hr from the final covariate models. The proportion of levels where doses predicted by NONMEM® and Phoenix® NLME™ differed by ≥ 0.5 mg in the first two weeks post-transplant for a typical trough of 10 ng/mL was calculated.

For both clinical and simulated data, statistical difference was assessed using relative predictive performance of the covariate model selected by manual stepwise selection in Phoenix® NLME™ compared to NONMEM® with the use of prediction errors. Prediction error (PE_i) was defined to be the residual ($C_{obs,\,i} - C_{pred,\,i}$) at each of the 'n' trough concentrations. Bias and precision of the two methods were calculated as the mean prediction error and the root mean squared prediction error, respectively (Eq. 3 & 4).

$$\text{Bias} = \frac{1}{n}\sum_{i}^{n} PE_i \tag{3}$$

$$\text{Precision} = \sqrt{\frac{1}{n}\sum_{i}^{n} PE_i^2} \tag{4}$$

A 95% confidence interval based on standard errors (Eq. 5 & 6) was calculated for difference between the bias (ΔME) of Phoenix® NLME™ and NONMEM®. ME_1 was defined to be the bias from NONMEM® and ME_2 was defined to be the bias from selection in Phoenix® NLME™.

$$\Delta\text{ME} = \text{ME}_1 - \text{ME}_2 \tag{5}$$

$$\text{se}_{\Delta\text{ME}} = \sqrt{\frac{1}{\text{n(n - 1)}}\sum_{i=1}^{n}[(\text{PE}_{1i} - \text{PE}_{2i}) - \Delta\text{ME}]^2} \tag{6}$$

Similarly, a 95% confidence interval based on standard errors (Eq. 7 & 8) for the difference between the precision (ΔMSE) of Phoenix® NLME™ and NONMEM® was calculated. MSE_1 was defined to be the squared precision from NONMEM® and ME_2 was defined to be the squared precision from selection in Phoenix® NLME™.

$$\Delta\text{MSE} = \text{MSE}_1 - \text{MSE}_2 \tag{7}$$

$$\text{se}_{\Delta\text{MSE}} = \sqrt{\frac{1}{\text{n(n - 1)}}\sum_{i=1}^{n}[(\text{PE}_{1i}^2 - \text{PE}_{2i}^2) - \Delta\text{MSE}]^2} \tag{8}$$

If the confidence intervals did not include zero, the bias and/or precision was significantly different and the method with the lower bias or imprecision was significantly better [15].

5.3 Results

5.3.1 Clinical data

The final CL/F model from the manual forward selection and backward elimination approach in both software packages selected the same covariates, i.e. days post-transplant, CYP3A5 genotype, transplant at a steroid sparing center, age at the time of transplant and the concomitant use of a calcium channel blocker (CCB) at the time of trough (Eq. 8) [13].

CL/F (L/hr) = TVCL*[(θ_1, if days 6-10) or (θ_2, if days 11-180)]*[(θ_3, if CYP3A5*1/*3 genotype) or (θ_4, if CYP3A5*1/*1 genotype)]*(θ_5, if receiving a transplant at a steroid sparing center)*[(Age in yrs/50)$^{-\theta_6}$]*(θ_7, if CCB is present)

(8)

where TVCL is the typical value of clearance in the population and θ represents the magnitude of the covariate effect sizes. The covariate effect sizes, percent relative standard errors (%RSE) and results from 1000 bootstrap runs (median, 2.5th and 97.5th percentile) are listed in Table 5.1. Parameter estimates were similar for both software packages. Comparison of population and individual predictions from the two software packages is shown in Figure 5.1.

In terms of the clinical difference, about 20% of the levels (n=405) differed in predicted doses by ≥ 0.5 mg with NONMEM® as compared to Phoenix® NLME™ for a target trough of 10 ng/mL in the 1st two weeks post transplant (n=2040). The absolute performance from the two software packages is listed in Table 5.2. In general, the troughs by NONMEM® were over-predicted by 0.08 ng/mL while those by Phoenix® NLME™ were over-predicted by 0.09 ng/mL. For a trough of 10 ng/mL, the precision was within ± 47.7% and ± 47.8% for NONMEM® and Phoenix® NLME™, respectively. In terms of the statistical measures of relative performance for comparison between NONMEM® vs. Phoenix® NLME™, the 95% C.I. for the difference in bias (ΔME) and precision (ΔMSE) was (0.007, 0.012) and (-0.34, -0.23), respectively. Since the confidence interval did not include zero, it indicated that there was a significant difference in bias and precision between predictions from NONMEM® and Phoenix® NLME™ (p-value<0.05). The method with the lower value of bias and precision was concluded to be significantly

better. Therefore, the NONMEM® predictions had significantly lower bias and better precision than Phoenix® NLME™ predictions.

When the same clinical data was analyzed using the automated stepwise selection in Phoenix® NLME™, two additional covariates, i.e. steroid use at the time of trough collection and recipient's weight at the time of transplant were found to be important. The covariate effects and median, 2.5th and 97.5th percentiles from 1000 bootstrap runs are listed in Table 5.3 for the automated stepwise selection method in Phoenix® NLME™. In terms of absolute performance, the troughs by Phoenix® NLME™ automated selection method was over-predicted by 0.04 ng/mL. For a trough of 10 ng/mL, the precision was within ± 47.2% for Phoenix® NLME™ automated selection.

5.3.2 Simulated data

The final CL/F model from the manual forward selection and backward elimination approach in both software packages selected the same covariates, i.e. CrCl, smoking status and race. The covariate effect sizes, %RSE and results from 1000 bootstrap runs (median, 2.5th and 97.5th percentile) are listed in Table 5.4. Parameter estimates were similar for both software packages except CrCL. Comparison of population and individual predictions from the two software packages for the simulated data is shown in Figure 5.2. The estimate for CrCL was predicted more accurately by NONMEM® and had a smaller %RSE than Phoenix® NLME™.

The absolute performance is listed in Table 5.5. In terms of absolute performance, the concentrations were under-predicted by NONMEM® by 0.24 mg/L and were over-predicted by Phoenix® NLME™ by 0.12 mg/L. The precision was 6.45 and 6.50 mg/L for NONMEM® and Phoenix® NLME™, respectively. In terms of the statistical measures of relative performance for comparison between NONMEM® vs. Phoenix® NLME™, the 95% C.I. for the difference in bias (ΔME) and precision (ΔMSE) was (0.30, 0.41) and (-1.10, -0.32), respectively. Since the confidence interval did not include zero, it indicated that there was a significant difference in bias and precision between predictions from NONMEM® and Phoenix® NLME™ (p-value<0.05). The method with the lower value of

bias and precision was concluded to be significantly better.. Therefore, the NONMEM® predictions had higher bias but they were more precise than Phoenix® NLME™ predictions.

The automated stepwise approach in Phoenix® NLME™ yielded the same model as the manual method. The shotgun method in Phoenix® NLME™ tested 64 models (2^6 models) and the best model that could be selected based on ΔOFV was the same as the manual or automated stepwise selection scenario.

5.4 Discussion

This study utilized clinical and simulated data to compare the covariate model building aspect of NONMEM® and Phoenix® NLME™. We wanted to identify differences in results, if any, when using the same covariate modeling approach and algorithms from both software packages. When the manual forward selection backward elimination approach with FOCE-I/FOCE-ELS was utilized in both software packages, the predictions from NONMEM® had lower bias and were more precise than Phoenix® NLME™ for the clinical data. When the same approach was followed in both software packages for the simulated data, the predictions from NONMEM® had higher bias but were more precise than Phoenix® NLME™.

In order to utilize alternate software packages and features such as the covariate selection in Phoenix® NLME™ for PopPK analysis, comparisons against the gold standard, (i.e., NONMEM®) need to be performed. This is important in order to confidently utilize functionalities that different packages might offer. Since the FOCE algorithms in NONMEM® and Phoenix® NLME™ are slight variants of the same algorithm, this study compared the covariate model building aspects of the two software packages in selecting covariates as well as the covariate effect sizes so as to allow prospective dose determination for tacrolimus. We compared the forward selection-backward elimination method of covariate selection. This is one of the most commonly used methods for covariate selection. Since this had already been implemented in NONMEM® previously

[13], we followed the same approach in Phoenix® NLME™ to determine if similar results are obtained.

Additionally, Phoenix® NLME™ offers other methods of covariate selection. These include 1) scenarios, 2) stepwise selection, and 3) shotgun covariate selection. The scenario option allows us to run certain pre-specified scenarios. The list of all possible covariate relationships is generated when covariate information is entered into Phoenix® NLME™, and the user can choose a subset of these to consider. The stepwise selection mode automates the forward selection and backward elimination procedure to find a statistically significantly better model from among the subset of covariate relationships that have been chosen for consideration. It generates a text output which describes each step of the selection process and lists the best model. The shotgun method tests all combinations of scenarios that are possible with a given set of covariates. Since it tests all possible combinations, it should only be used when testing a small number of covariates. If the number of covariates is large, there could be too many combinations to test in a reasonable amount of time. Due to the large number of covariates in this analysis, we also performed the automated stepwise selection method in Phoenix® NLME™ to obtain the covariate model. Additionally, with the simulated data, we performed the shotgun covariate search which tests all possible combinations of covariates.

For the clinical data, Phoenix® NLME™ yielded similar covariates as NONMEM® with the manual forward selection-backward elimination process. However, when the automated covariate selection method in Phoenix® NLME™ was employed, two additional covariates, steroid use at the time of transplant and weight at the time of transplant, were significant. This might be due to differences in the way the forward selection-backward elimination was performed manually. The manual method has been described previously and was not identical to the automated procedure in Phoenix® NLME™ [13]. Additionally, the final model obtained by the manual selection method was based on both clinical judgment and statistical significance. The automated stepwise method in Phoenix® NLME™ was based solely on statistical significance. For the

simulated data, identical results were obtained from both the software packages. The stepwise automated selection procedure also gave the same covariate model.

Clinical difference was ascertained between the two methods by determining the proportion of troughs in the 1st two weeks post-transplant in which the dose predictions differed by ≥ 0.5 mg between NONMEM® and Phoenix® NLME™ manual stepwise selection. Since 0.5 mg is the smallest capsule size for tacrolimus dosing, predictions need to differ by 0.5 mg before dose change is feasible [16, 17]. In approximately 20% of the troughs, a different dose was predicted with NONMEM® versus Phoenix® NLME™ manual selection. For a patient who is at either end of a therapeutic range, especially for a narrow therapeutic range drug such as tacrolimus, a small change of 0.5 mg could result in a more favorable or less favorable clinical outcome (no rejection/toxicity). However, we want to caution the readers that the clinical impact of this difference in dose from the two software packages is drug specific and may not be important in case of a drug that has a wide therapeutic window.

With the simulated data, the estimates and %RSE were similar for the simple selection in NONMEM® and Phoenix® NLME™. The coefficient of CrCL was slightly different between NONMEM® (0.68, 95% C.I.: 0.46, 0.90) and Phoenix® NLME™ (0.52, 95% C.I.: 0.28, 0.76). The estimation for coefficient of CrCL from NONMEM® was more accurate. Other estimates were very similar between both software packages.

Predictive performance was assessed by bias and precision. In terms of relative performance, NONMEM® had better precision than Phoenix® NLME™ manual selection for both datasets but the bias was higher with NONMEM® for the simulated dataset. The reasons for a low predictive performance are never evident. Inaccurate predictions may be attributed to model misspecification or to inaccuracies in the reference concentration, i.e. trough concentrations. When trough concentrations are inaccurate, the bias and imprecision of the model are inflated [15]. Therefore, we used simulated data for the comparator analysis.

The use of two different software packages to analyze the same dataset allows direct comparison across software packages. Additional factors such as cost of the software package and the feasibility of performing a particular analysis in one software package versus the other will also contribute in software choice. For example, the menu driven automated covariate selection in Phoenix® NLME™ can be used when the number of covariates is large (say > 15). This would aid in finding the most important covariates which can be further tested based on clinical relevance. This process of covariate selection requires less time. In the present analysis, the overall time for the automated stepwise selection was about 4.5 hours for the clinical data.

In conclusion, we compared covariate model building with NONMEM® and Phoenix® NLME™. We observed similar parameter estimates and %RSE for both software packages. Automated stepwise selection, available in Phoenix® NLME™, yielded two additional covariates for the clinical data but could be used as a full model for further testing based on clinical relevance. For the clinical data, NONMEM® had a lower bias and was more precise than Phoenix® NLME™. For the simulated data, Phoenix® NLME™ had a lower bias but NONMEM® was more precise.

Table 5.1: Effect sizes of covariates obtained from NONMEM® and Phoenix® NLME™ for clinical data

	NONMEM®		***Phoenix® NLME™***	
Covariate	**Est. (%RSE)**	**Median (C.I.)***	**Est.(%RSE)**	**Median (C.I.)***
TVCL	38.4 (4.1)	38.8 (35.5, 41.7)	37.4 (3.7)	37.7 (34.4, 41.8)
Factors affecting CL				
DPT (6-10)	0.86 (4.1)	0.86 (0.80, 0.93)	0.90 (3.1)	0.90 (0.81, 0.99)
DPT (11-180)	0.71 (4.1)	0.72 (0.66, 0.77)	0.73 (2.9)	0.73 (0.66, 0.80)
*CYP3A5*1/*3*	1.7 (4.0)	1.69 (1.56, 1.82)	1.69 (4.0)	1.69 (1.56, 1.82)
*CYP3A5*1/*1*	2.00 (5.9)	1.99 (1.77, 2.23)	2.03 (5.3)	2.02 (1.80, 2.24)
Center	0.70 (3.5)	0.70 (0.65, 0.75)	0.71 (3.5)	0.70 (0.65, 0.75)
$(Age/50)^{-\theta}$	0.40 (13.5)	0.39 (0.50, 0.29)	0.39 (12.5)	0.38 (0.50, 0.28)
CCB	0.94 (2.4)	0.94 (0.90, 0.99)	0.94 (1.3)	0.94 (0.90, 0.98)

TVCL: Typical value of clearance, * Bootstrap results with 1000 simulations with the 2.5^{th} and 97.5^{th} percentile, DPT: Days post-transplant, CYP3A5: CYP3A5 genotype, Center: Transplant at a steroid sparing center, Age: Age at the time of transplant (in years), CCB: Calcium channel blocker use at the time of trough collection, Weight: Weight at the time of transplant, Steroid: Steroid use at the time of trough collection

Table 5.2: Absolute performance for NONMEM® and Phoenix® NLME™ manual selection for clinical data

Absolute performance	Bias[1] (95% C.I.)	Precision[2] (95% C.I.)
NONMEM®	-0.08 (-0.16, 0.01)	4.77 (4.68, 4.87)
Phoenix® NLME™	-0.09 (-0.17, -0.001)	4.78 (4.68, 4.88)

[1]Bias (in ng/mL) of the CL/F equation in predicting the troughs , calculated as the mean prediction error, [2] Precision (in ng/mL) of the equation in predicting the troughs, calculated as the root mean squared error

Table 5.3: Effect sizes of covariates obtained from Phoenix® NLME™ automated stepwise search for clinical data

Phoenix® NLME™ automated stepwise search		
Covariate	**Est.(%RSE)**	**Median (C.I.)***
TVCL	26.9 (2.82)	26. 9 (25.4, 28.6)
Factors affecting CL		
DPT (6-10)	1.02 (28)	1.02 (0.99, 1.04)
DPT (11-180)	1.22 (3.8)	1.22 (1.08, 1.38)
*CYP3A5*1/*3*	1.70 (4.0)	1.67 (1.54, 1.80)
*CYP3A5*1/*1*	2.00 (5.9)	2.00 (1.79, 2.23)
Center	0.70 (3.58)	0.70 (0.65, 0.75)
$(Age/50)^{-\theta}$	0.42 (17.6)	0.42 (0.31, 0.52)
CCB	0.95 (1.27)	0.94 (0.90, 0.99)
$(Weight/70)^{\theta}$	0.31(32.7)	0.30 (0.16, 0.46)
Steroid	1.14 (2.37)	1.14 (1.05, 1.25)

TVCL: Typical value of clearance, * Bootstrap results with 1000 simulations with the 2.5^{th} and 97.5^{th} percentile, DPT: Days post-transplant, CYP3A5: CYP3A5 genotype, Center: Transplant at a steroid sparing center, Age: Age at the time of transplant (in years), CCB: Calcium channel blocker use at the time of trough collection, Weight: Weight at the time of transplant, Steroid: Steroid use at the time of trough collection

Table 5.4: Effect sizes of covariates obtained from NONMEM® and Phoenix® NLME™ for simulated data using manual forward selection backward elimination approach

	NONMEM®		Phoenix® NLME™	
Covariate	**Est. (%RSE)**	**Median (C.I.)***	**Est.(%RSE)**	**Median (C.I.)***
TVCL	5.1 (4.4)	5.06 (4.56, 5.55)	4.8 (4.5)	5.00 (4.5, 5.5)
Factors affecting CL				
(CrCL/110)$^{\theta}$	0.68 (16.5)	0.69 (0.44, 0.92)	0.52 (23.5)	0.66 (0.45, 0.92)
SMK	1.6 (7.3)	1.60 (1.39, 1.85)	1.45 (7.3)	1.57 (1.34, 1.84)
RACE	0.7 (5.8)	0.7 (0.63, 0.79)	0.78 (7.5)	0.71 (0.63, 0.81)
TVV	14.6 (2.9)	14.6 (13.8, 15.4)	14.5 (3.0)	14.6 (13.7, 15.5)

TVCL: Typical value of clearance, TVV: Typical value of volume, CrCL: Creatinine clearance (mL/min) standardized by the median value, SMK: Fractional change in CL for a smoker, RACE: Fractional change in CL for AA, * results from 1000 bootstrap runs reported as the median and the 2.5th and 97.5th percentile

Table 5.5: Absolute performance for NONMEM® and Phoenix® NLME™ manual selection for simulated data

Absolute performance	Bias[1] (95% C.I.)	Precision[2] (95% C.I.)
NONMEM®	0.24 (-0.28, 0.75)	6.45 (5.73, 7.08)
Phoenix® NLME™	-0.12 (-0.64, 0.40)	6.50 (5.81, 7.12)

[1]Bias (in mg/L) of the CL/F equation in predicting concentrations, calculated as the mean prediction error, [2] Precision (in mg/L) of the equation in predicting concentrations, calculated as the root mean squared error

Figure 5.1: Comparison of population and individual predictions from NONMEM® and Phoenix® NLME™ for the clinical data

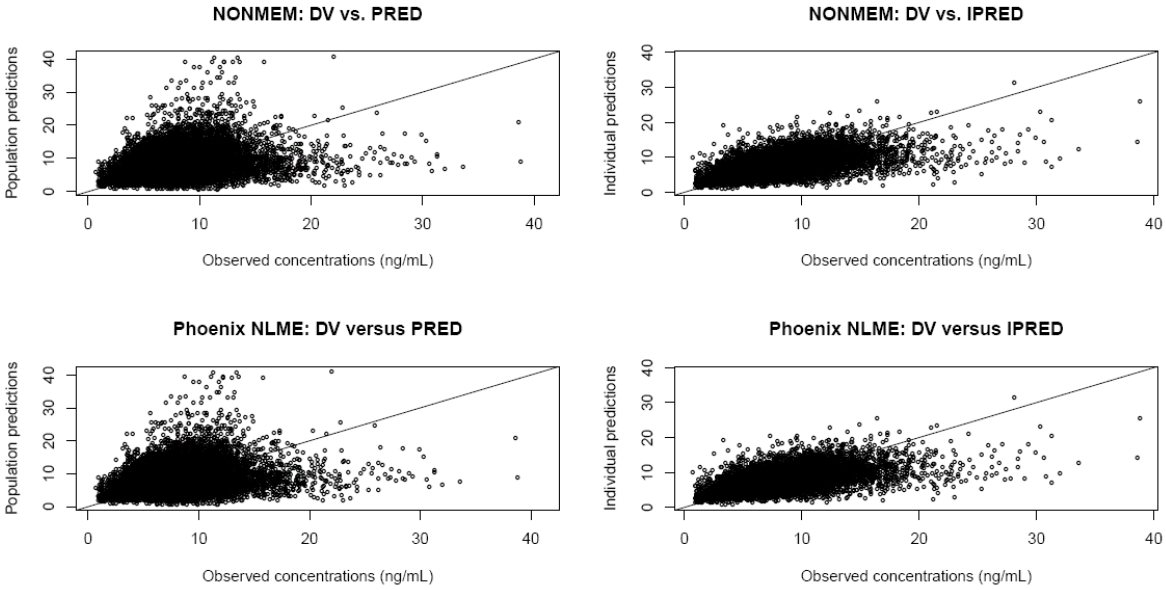
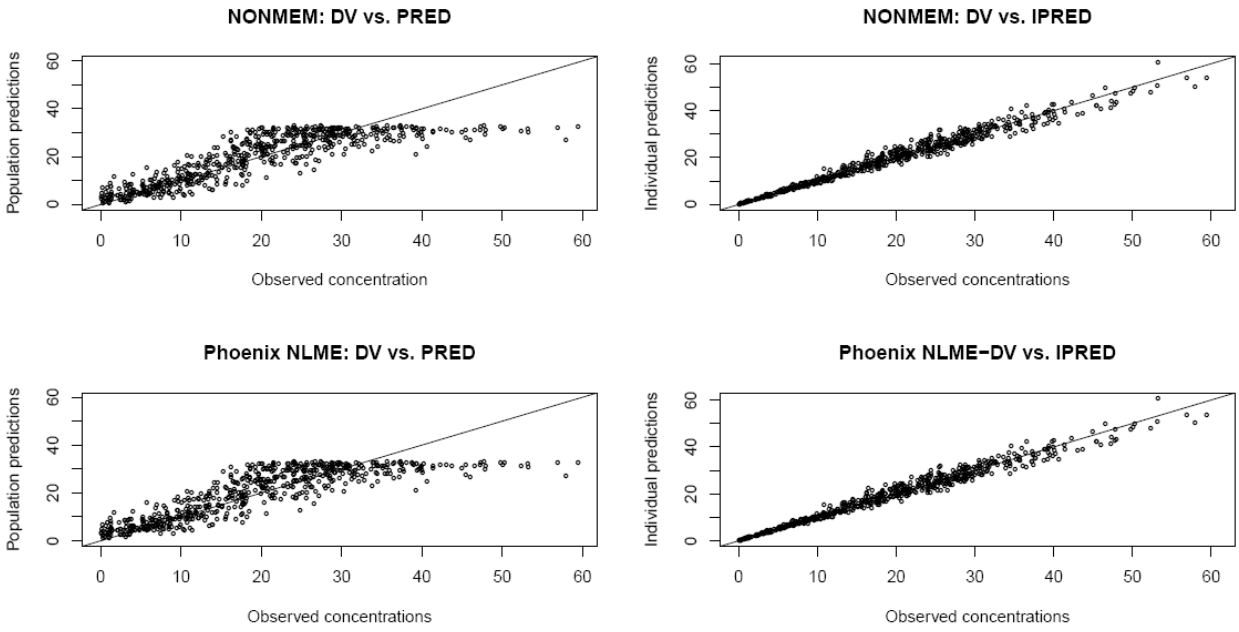


Figure 5.2: Comparison of population and individual predictions from NONMEM® and Phoenix® NLME™ for the simulated data



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Chapter VI

Conclusions

The overall objective of this work was to formulate and validate a clinical dosing model for tacrolimus in kidney transplant recipients by identifying the clinical and genetic covariates that might influence tacrolimus CL/F. Tacrolimus is a common maintenance immunosuppressant, used widely in transplant recipients. Patients with suboptimal tacrolimus troughs are at a higher risk of rejection of the allograft or at a higher risk of toxicity [1]. Therefore, achieving the therapeutic range is important, especially in the immediate post-transplant period when the risk of rejection is higher [2].

The use of tacrolimus is complicated by its narrow therapeutic window (10-15 ng/ml) and wide inter-individual variability in pharmacokinetics. Traditionally, the initial dose of tacrolimus is based on mg/kg dosing and subsequent doses are adjusted by therapeutic drug monitoring. In spite of such rigorous monitoring, a large proportion of transplant recipients are below/above the therapeutic drug levels. Multiple clinical factors (such as days post-transplant, co-medications) and genetic variants (CYP3A5*1) have been previously explored to explain inter-individual variability in trough concentrations [3, 4]. However, the contribution of these established factors and other possible influential factors have not been studied in a combined and consistent manner to generate evidence-based guidelines that can prospectively predict the dose for a transplant recipient.

The data for this study came from one one of the largest tacrolimus pharmacogenetics studies conducted to date (681 subjects and 11,823 trough concentrations). Recipients were genotyped for 2,724 SNPs using a SNP chip. Two types of covariates were available for analysis: routine clinical variables (age, weight, co-medication, etc.) and 119 SNPs. These SNPs were selected from 2,784 SNPs based on a Bonferroni corrected p-value by multiple linear regressions of SNPs on dose normalized trough concentrations. The top 6 SNPs were selected for the building of the dosing model due to software limitations within NONMEM® to analyze the large number of SNPs. The clinical dosing model was obtained by non-linear mixed effects modeling of the tacrolimus troughs using NONMEM®. The troughs were characterized by a steady state infusion model. Clinical factors and 6 genetic variants were screened for importance towards tacrolimus clearance (CL/F) using the forward selection backward elimination approach.

Our study presents the first dosing model for tacrolimus using a combination of genetic variants and clinical factors in adult kidney transplant recipients.

CL/F (L/hr) = 38.4*[(0.86, if days 6-10) or (0.71, if days 11-180)]*[(1.69, if CYP3A5*1/*3 genotype) or (2.00, if CYP3A5*1/*1 genotype)]*(0.70, if receiving a transplant at a steroid sparing center)*[(Age in yrs/50)$^{-0.4}$]*(0.94, if calcium channel blocker is present) (1)

The total daily dose (TDD) requirement is then calculated from the estimated tacrolimus CL/F above and the desired goal trough concentration.

TDD (mg) = [CL/F (L/hr) * tacrolimus trough goal (ng/ml) * 24 hrs] / 1000 (2)

We found that tacrolimus CL/F in kidney transplant recipients was significantly influenced by days post-transplant, CYP3A5 genotype, transplantation at a steroid sparing center, recipient age, and the use of a calcium channel blocker. CL/F decreased with increasing days post-transplant, likely due to an increase in bioavailability with improved gastrointestinal motility after transplant**.** Our large sample size enabled us to define the distinct differences in tacrolimus CL/F between three CYP3A5 genotype groups (*1/*1, *1/*3 and *3/*3). The presence of *1/*3 genotype increased CL/F by 70% and the presence of *1/*1 genotype increased CL/F by 100% as compared to the *3/*3. A majority of our non-AA population possessed the CYP3A5*3/*3 genotype and a majority of the AA population had the CYP3A5*1/*1 or the CYP3A5*1/*3 genotype. However, race was not found to be a significant covariate, possibly due to a correlation between race and CYP3A5 genotype. CL/F was seen to decrease with calcium channel blocker use and transplant at steroid sparing center. This is most likely attributed to drug interaction as these classes of medications are CYP3A substrates. Our CL/F model is simple and utilizes 4 clinical factors and 1 genetic variant. The predicted CL/F can be used to prospectively predict dose for a transplant recipient in order to achieve a target trough concentration.

The next step was to validate the developed equation in an external dataset of transplant recipients. We validated the genetic-based tacrolimus equation that predicts troughs in an independent cohort of 795 kidney transplant recipients receiving tacrolimus (n= 13698 troughs). The patient characteristics were similar for the development and validation cohort. The performance of the equation to predict initial troughs was assessed by calculating bias and precision of the equation. For all troughs in the first 6 months post-transplant, a comparison was made between the troughs predicted using the equation versus those predicted using a basic apparent clearance model with no covariates. The initial troughs were over-predicted by the equation on an average by 0.2 ng/mL. For a typical trough of 10 ng/mL the precision was within ± 20 %. For all troughs over the 6 months post-transplant, the equation predicted troughs significantly better than the basic CL/F model. Therefore, the tacrolimus equation had good bias and precision in predicting initial troughs and performed better than a basic apparent clearance model for all troughs. The equation can be safely used for predicting initial clearance from which initial dose can be determined. The validation of the equation provided additional confidence to use the equation for predicting troughs. Randomized clinical trials where traditional approach (mg/kg dosing followed by TDM) will be compared with our equation would be a final step to confirm clinical utility.

The standard tool for population pharmacokinetic analysis, i.e. NONMEM®, is utilized to determine the association of the clinical and genetic covariates with tacrolimus troughs. It has limitations on the number of covariates that can be modeled at one time. Moreover, with even 20 covariates for consideration, the number of possible combinations rise exponentially and the modeling becomes very time intensive. Due to software and time limitations associated with the large number of SNPs (n=119) and SNP combinations ($\approx 2^{119}$) in this study, we devised a "winnowing method" that would enable screening of the covariates in a much more time-efficient and practical manner. The method uses GAM to compare models arising out of randomly selected sub-group of covariates. Next, another sub-group of covariates (SNPs) is selected randomly again and GAM is repeated. This process of random selection and GAM is repeated several times (say 500) to capture various combinations of covariates. The results from these repetitions are summarized by creating a SNP index. An index, ranging from 0-1, is the proportion of the number of

times a SNP is selected by GAM (i.e. appears in the final model) to the number of times it is randomly selected (i.e. appears in the subgroup). This index defines the relative importance of the SNPs. The important SNPs (index =1) are included in an updated NONMEM model, unimportant SNPs (index=0) are removed from consideration and remaining SNPs (index= 0.1-0.9) are re-tested by regression on the EBE's from the updated NONMEM model. This process is repeated until all SNPs are categorized as 0 or 1 index SNPs. The full model is obtained by this process and greatly reduces the number of SNPs that need to be tested in NONMEM. We further validated this approach using a simulated dataset with 10 important SNPs in a group of 100 SNPs. The approach was able to identify all the important SNPs. The type I and type II error rates were 9 % and 0 % respectively.

The method is run using NONMEM and the R statistical package. It is easy to use and reduces the rate of type I error by decreasing the amount of multiple testing [5]. It decreases the number of covariates that need to be tested within NONMEM and uses feedback from NONMEM (in the form of updated EBEs) to select or reject future covariates. Since, the method requires the use of EBEs, it may not however be appropriate to use in the presence of high shrinkage. Time varying covariates or inter-occasion variability can also not be handled with this approach.

Finally, we compared covariate model building within NONMEM® and Phoenix® NLME™ using clinical and simulated data. NONMEM®, is the oldest and most widely used PopPK software [6]. Newer software packages, such as Phoenix® NLME™, are being developed that provide a graphical user interface and menu-driven options for covariate selection [7]. Additionally, several methods of manual and automated covariate selection are available within Phoenix® NLME™, which makes it much more favorable to use in an analysis with a large number of covariates.

We used the tacrolimus troughs data as well as a simulated dataset to compare both these software packages when the same algorithm (FOCE-I in NONMEM®/FOCE-ELS in Phoenix® NLME™) and same covariate selection method (manual forward selection backward elimination) was used in both the software packages. Both software packages

selected the same covariates with the manual selection method. With the clinical data, about 20% of the troughs differed in a dose prediction of ≥ 0.5 mg with NONMEM® compared to Phoenix® NLME™. With the clinical data, we saw that NONMEM® predictions had better bias and precision than Phoenix® NLME™. When we used the automated stepwise selection method in Phoenix® NLME™, two additional covariates were selected as being significant and may be attributed to the difference in which the covariate selection was carried out manually versus the automated manner. This method was faster and could be used as a full model for further testing in NONMEM®. NONMEM® predictions had higher bias and lower precision than predictions from Phoenix® NLME™ automated stepwise method. With the simulated data, the same covariates were selected. Phoenix® NLME™ predictions had better bias but NONMEM® predictions had better precision.

In conclusion, we developed and validated a genetics-based model that predicts troughs and can be used for prospectively determining tacrolimus doses in kidney transplant recipients. We developed and validated a novel "winnowing method" of covariate screening for testing within NONMEM®. We compared covariate selection using NONMEM® and Phoenix® NLME™ based on clinical and statistical differences.

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Appendix

Chapter II: NONMEM Code for final model for tacrolimus troughs

```
;Model Desc: SS ASSUMPTION NO PREDPP
;Project Name: comb_de_add_final
;Project ID: Tacrolimus troughs_final

$PROB RUN#064B
$INPUT C ID PID DAY DV RATE DOSE=DROP MDV TDD=DROP SERN=DROP
SEX WT WMZ=DROP WMO=DROP WMT=DROP WMS=DROP HT=DROP
HGT=DROP HTDF=DROP DONR ETHN=DROP RACE CNTR AGE CACB ROID
PREM DGEN NTRP R354=DROP R926=DROP R746 R000=DROP R506=DROP
R555=DROP
$DATA CNITAC2_DE_edited.csv IGNORE=C
$SUBROUTINES
$PRED

;Convert days post transplant to a categorical variable

APOD=0
BPOD=0
CPOD=0

IF(DAY.LE.5)APOD= DAY
IF(DAY.GT.5.AND.DAY.LE.10)BPOD=DAY
IF(DAY.GT.10)CPOD=DAY

TVCL=THETA(1)
IF(DAY.EQ.BPOD)TVCL=TVCL*THETA(2)
IF(DAY.EQ.CPOD)TVCL= TVCL*THETA(3)
IF(R746.EQ.1)TVCL=TVCL*THETA(4)
IF(R746.EQ.2)TVCL=TVCL*THETA(5)
IF(CNTR.EQ.3)TVCL=TVCL*THETA(6)
TVCL=TVCL*(AGE/50)**THETA(7)
IF(CACB.EQ.1)TVCL=TVCL*THETA(8)

 CL=TVCL*EXP(ETA(1))
CLML=CL*1000

CSS=RATE/CL
F=CSS
Y = F + ERR(1)
 IPRED=F

$EST METHOD=1 INTERACTION PRINT=5 MAX=9999 SIG=2 MSFO=064B.MSF
$THETA
```

```
  (0,30) ;[CL]
  (0,1) ;[CL]
  (0,1) ;[CL]
  (0,1) ;[CL]
  (0,1) ;[CL]
  (0,1) ;[CL]
  (-1,0.1) ;[CL]
  (0,1) ;[CL]

$OMEGA
  0.5 ;[P] omega(1,1)

$SIGMA
  0.5 ;[A] sigma(1,1)

$COV
$TABLE ID CL CLML DAY WT AGE SEX IPRED CWRES APOD BPOD CPOD
ONEHEADER NOPRINT FILE=064B.tab
$TABLE ID CLML FIRSTONLY NOAPPEND NOPRINT FILE=064B.par
$TABLE ID ETA1 FIRSTONLY NOAPPEND NOPRINT FILE=064B.eta
```

Chapter IV: R code for carrying out the GAM repetitions

```
setwd("C:/Documents and Settings/Chaitali/Desktop/Sim_IV")
library(nlme)
 rm(list=ls())

####read in file that contains covariates and post-hoc estimates from NONMEM model
df.test1 <- read.csv(file="SIM1.csv")

######Gead in the covariates that you want to run the gam on (100 SNPs in our
example)
gam.cov <- c("G1","G2","G3","G4","G5","G6","G7","G8","G9","G10","G11","G12",
"G13","G14","G15","G16","G17","G18","G19","G20","G21","G22","G23","G24","G25
","G26","G27","G28","G29","G30", "G31", "G32", "G33", "G34", "G35", "G36",
"G37","G38","G39","G40","G41","G42","G43","G44","G45","G46","G47","G48",
"G49","G50","G51","G52","G53","G54","G55","G56","G57","G58","G59",
"G60","G61","G62","G63","G64","G65","G66","G67","G68","G69","G70",
"G71","G72","G73","G74","G75","G76","G77","G78","G79","G80","G81",
"G82","G83","G84","G85","G86","G87","G88","G89","G90","G91","G92",
"G93","G94","G95","G96","G97","G98","G99","G100")

n.gam.cov <- length(gam.cov)
run <- "iter_1"

###Open the gam library
library(gam)

###iter size: Group size
###n.iter: Number of GAM repetitions
list.iter.size <- c(25)
list.n.iter <- c(500)

runs <- list()

for(i in list.iter.size) {
  for(j in list.n.iter) {
    runs[[length(runs)+1]] <- list(
      iter.size=i,
      n.iter=j,
      results.file=paste(run,"gam",i,j,"r", sep='.'),
      summary.file=paste(run,"gam",i,j,"r","csv", sep='.')
      )
     }
  }

#### Run the GAM on CL or delta CL
```

```
df.fact <- as.data.frame(mapply(df.test1[,gam.cov], FUN=factor))
df.fact$DELC <- df.test1$CL
gam.obj <- gam(DELC~1,data=df.fact)
gam.iter <- function(iter.size=NULL) {

 this.gam.cov <- sample(gam.cov, iter.size, replace=F)
 scope <- lapply(this.gam.cov, FUN=function(x) {
     eval(parse(text = paste("~", paste(c(1,x), collapse="+"))))
 }
 )
 names(scope) <- this.gam.cov
 step.gam.obj <- step.gam(gam.obj, scope=scope, trace=F)
 selected.snips <- names(step.gam.obj$xlevels)
 list(selected = selected.snips, sampled = this.gam.cov)

}
summarize.gam.run <- function(results.file=NULL) {
 res <- dget(file=results.file)

 sampled <- lapply(res, FUN=function(x) {
          x$sampled
  }
 )
 list.sampled <- unlist(sampled)

 selected <- lapply(res, FUN=function(x) {
          x$selected
   }
 )
 list.selected <- unlist(selected)

 u.snip <- unique(list.sampled)

 summ <- lapply(u.snip, FUN=function(x) {
      n.select <- length(list.selected[list.selected==x])
      n.sample <- length(list.sampled[list.sampled==x])
      data.frame(snip=x,n.select=n.select, n.sample=n.sample,
index=signif(n.select/n.sample, digits=3))
   }
 )
 df.summ <- data.frame(do.call('rbind', summ))
 df.summ <- df.summ[order(df.summ$index,decreasing=T),]
 df.summ
}
```

```
### Do all of the GAMS of more than one group size and GAM repetition is selected
big.run <- lapply(runs, FUN=function(this.run) {
   start.date <- date()
   res <- lapply(1:this.run$n.iter, FUN=function(x) {
         gam.iter(iter.size=this.run$iter.size)
     }
   )

   dput(file=this.run$results.file, res)
   end.date <- date()
   res$start <- start.date
   res$end <- end.date
   res
  }
)
dput(file=paste(run,"big.run","r",sep='.'), big.run)


# Give us summarized results
results.files <- unlist(lapply(runs, FUN=function(this.run) {this.run$results.files}))


results <- lapply(runs, FUN=function(this.run) {
       this.result <- summarize.gam.run(results.file=this.run$results.file)
       write.csv(file=this.run$summary.file, this.result, row.names=F, quote=F)
       this.result
  }
)
names(results) <- results.files
```