2014

Clinical and genetic determinants of tacrolimus pharmacokinetics and pharmacodynamics in the transplant population

Tatian Kirresh
University of Iowa

CLINICAL AND GENETIC DETERMINANTS OF TACROLIMUS PHARMACOKINETICS AND PHARMACODYNAMICS IN THE TRANSPLANT POPULATION

by

Tatian Kirresh

A thesis submitted in partial fulfillment
of the requirements for the Doctor of
Philosophy degree in Pharmacy
in the Graduate College of
The University of Iowa

May 2014

Thesis Supervisor: Associate Professor Daryl J. Murry

Graduate College
The University of Iowa
Iowa City, Iowa

CERTIFICATE OF APPROVAL

_____

PH.D. THESIS

_____

This is to certify that the Ph.D. thesis of

Tatian Kirresh

has been approved by the Examining Committee
for the thesis requirement for the Doctor of Philosophy
degree in Pharmacy at the May 2014 graduation.

Thesis Committee: _____
Daryl J. Murry, Thesis Supervisor

_____
Aliasger Salem

_____
Gary Milavetz

_____
Erika Ernst

_____
Brian Smith

To my family, for your unconditional love and support, every step of the way

ACKNOWLEDGMENTS

I would like to express my deepest gratitude to my advisor, Dr. Daryl Murry, for his unending support, guidance and patience, as life brought many changes and challenges, and without which the completion of this research project would not have been possible. You have my lifelong appreciation for your mentorship and compassion.

I am also grateful to my committee members, Dr. Aliasger Salem, Dr. Erika Ernst, Dr. Brian Smith and Dr. Gary Milavetz for their time and effort, and for their helpful ideas and intriguing discussions, and to Dr. Sony Tuteja for allowing me to be part of her valuable research projects and for her insightful comments.

I would also like to thank all my friends and colleagues for making this journey so memorable, with special thanks to Maha Alkeilani, Rowan Ejeilat and Janthima Methaneethorn for their tremendous support and encouragement throughout the years, and to Mark Schmidt, for always willing to help and answer my questions.

I will be forever grateful to my father for believing in me, my brother and sisters, for giving me strength to go on, and mostly to my mother, for being my best friend, I would not have made it through without your unconditional love and support. My deepest appreciation goes to my children, Naya and Adnan, for being my inspiration through this journey and being the reason for everything I do. Without the joy and smiles you bring to my life, none of this seemed possible. I would also like to thank my husband, Murad, for his support and company through all the challenges and hard times that we have been through.

ABSTRACT

Calcineurin inhibitors (CNIs) are the cornerstone of immunosuppressive therapy following transplantation; however, immunosuppressive drug regimens consist of multiple medications with narrow therapeutic indices and substantial inter-patient variability. Despite intensive therapeutic monitoring, considerable time can elapse before the desired therapeutic concentration is achieved, which increases the risk of graft rejection or drug-related toxicities. In addition, maintaining therapeutic concentrations of CNIs does not prevent the development of toxicities, such as nephrotoxicity.

Pharmacogenomics can greatly benefit solid organ transplant recipients through individualized drug therapy; tacrolimus is a widely used CNI and a substrate of cytochrome P450 3A (CYP3A) metabolizing enzymes and the efflux transporter p-glycoprotein (PGP) encoded by the ATP-binding cassette subfamily B member 1(ABCB1) gene. This dissertation describes work conducted in order to examine the effect of genetic variability in the above mentioned genes on the pharmacokinetics of tacrolimus and their contribution to a predisposition to adverse events or drug interactions in the transplant population.

Our retrospective study investigating the effect of genetic polymorphisms on the risk of CNI-induced renal dysfunction identified a time-sensitive effect for the CYP3A5 expressor genotype, which predicts increased renal tubular CYP3A5 expression, in modifying the risk for renal dysfunction in liver transplant patients.

This dissertation also examines the hypothesis that local tissue levels of tacrolimus and/or its major metabolite may be an improved indicator of nephrotoxicity, and through development of a robust and sensitive liquid chromatography/ mass spectrometry (LC/MS) analytical method to co-determine tacrolimus and its major metabolite, 13-O-demethyl tacrolimus (13-ODMT), in rat kidney tissues, we identified a possible relationship between tacrolimus dose and the extent of metabolite accumulation

in the kidneys of rats receiving tacrolimus intra-peritoneally, paving the way for examining this relationship in kidney transplant recipients with calcineurin inhibitor-induced nephrotoxicity (CNIT).

Overall, my research aims to identify biomarkers that might assist in early prediction of optimal tacrolimus starting and maintenance doses. Importantly, these studies provide the foundation for prospectively identifying patients at higher risk for adverse effects or drug interactions, with the ultimate goal of improving treatment outcome and quality of life for the transplant recipient receiving tacrolimus.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

13-ODMT: 13-O-demethyl tacrolimus

ABCB1: ATP-binding cassette subfamily B member 1

ACN: acetonitrile

ACR: acute cellular rejection

AUC: area under the curve

BCS: Biopharmaceutics Classification System

C/D: dose-normalized concentration

CNI: calcineurin inhibitor

CNIT: calcineurin inhibitor-induced nephrotoxicity

CNS: central nervous system

CV: coefficient of variation

CYP3A: cytochrome P450 3A

DeKAF: Long-Term Deterioration of Kidney Allograft Function

DM: diabetes mellitus

DNA: deoxyribonucleic acid

dNTP: deoxynucleoside triphosphate

eGFR: estimated glomerular filtration rate

ESI: electrospray ionization

FK506: tacrolimus

FKBP: FK-binding protein

HCC: hepatocellular carcinoma

HPLC: high performance liquid chromatography

HRS: hepatorenal syndrome

HTN: hypertension

HWE: Hardy-Weinberg equilibrium

IACUC: Institutional Animal Care and Use Committee

IS: internal standard

LC/MS: liquid chromatography/ mass spectrometry

LCMS-IT-TOF: liquid chromatography mass spectrometry ion-trap time-of-flight

LDLT: living donor liver transplant

LLOQ: lower limit of quantitation

MELD: Model for End-Stage Liver Disease

MOA: mechanism of action

MRM: multiple reaction monitoring

mRNA: messenger ribonucleic acid

mTOR: mammalian target of rapamycin

NASH: non-alcoholic steatohepatitis

NFAT: nuclear factor of activated T cells

NFSE: nifedipine-specific element

NODAT: new-onset diabetes after transplantation

OLT: orthotopic liver transplant

PBC: primary biliary cirrhosis

PCR: polymerase chain reaction

PGP: p-glycoprotein

POR: P450 oxidoreductase

PSC: primary schlerosing cholangitis

PXR: Pregnane X receptor

SNP: single nucleotide polymorphism

SrCr: serum creatinine

TDM: therapeutic drug monitoring

UGT1: UDP-glucuronosyltransferase 1

CHAPTER 1

INTRODUCTION

Immunosuppression in Solid organ Transplantation

The first successful kidney transplant was performed in 1954, and it was only possible because the donor and recipient were monozygotic twins[1]. However, absence of effective immunosuppressants meant early acute rejection and graft failure for transplants between non-identical patients. The introduction of 6-mercaptopurine and azathioprine, in addition to combination therapy with corticosteroids resulted in significant improvements in graft survival rates[2], but it was not until 1976 that the discovery of cyclosporine has revolutionized the field of solid-organ transplantation.

Currently, Immunosuppressive regimens include calcineurin inhibitors (CNIs), anti-metabolites, mammalian target of rapamycin (mTOR) inhibitors, corticosteroids and antibody-based therapies (Table 1). They all cause non-specific immunosuppression and target different steps in the immunological response. A schematic representation of the site of action of common immunosuppressants is shown in Figure 1.

Immunosuppressive drugs can be classified as induction, maintenance and antirejection therapies. CNIs represent the mainstay of maintenance immunosuppressive regimens, and they are used in more than 95% of centers upon discharge[3]with tacrolimus and cyclosporine being the two CNIs approved for use in organ transplantation.

Tacrolimus

Tacrolimus is a lactone antibiotic isolated from the fermentation of Streptomyces tsukubaesis[4] ($C_{44}H_{69}NO_{12}$, Molecular structure is shown in figure 2); it is widely used for its immunosuppressive properties to prevent organ rejection in transplantation. It is also used in a topical form in the treatment of moderate to severe atopic dermatitis[5]

Since its introduction in 1987[4] tacrolimus has become a valuable alternative to cyclosporine for immunosuppressive therapy in solid organ transplantation due to its higher potency (10 to 100 times more potent)[6] and better safety profile[7].

Mechanism of Action

Tacrolimus (FK506) causes immunosuppression through inhibition of calcineurin- a calcium/calmodulin-dependent protein phosphatase[8]. After entry into the cell, tacrolimus binds to its cytosolic partner: FK506-binding proteins FKBP-12 and FKBP-52. The complex binds to and inhibits the activity of the enzyme calcineurin, thereby inhibiting phosphatase-controlled translocation of nuclear factor of activated T cells (NFAT) into the nucleus. This prevents induction of cytokines that are required for activation and proliferation of lymphocytes and other immune cells. A schematic presentation of tacrolimus mode of action is depicted in Figure 3).

Physicochemical Properties

Tacrolimus is 23-membered macrolide lactone with a molecular weight of 804 Da. It has the chemical name [3S[3R*[E(1S*,3S*,4S*)], 4S*, 5R*, 8S*, 9E, 12R*, 14R*, 15S*, 16R*, 18S*, 19S*,26aR*]] 5,6,8,11,12,13,14,15,16,17,18,19,24,25,26,26a-hexadecahydro-5,19-dihydroxy-3-[2-(4-hydroxy-3methoxycyclohexyl)-1-methylethenyl]-14,16-dimethoxy-4,10,12,18-tetramethyl-8-(2-propenyl)-15,19epoxy-3H-pyrido[2,1-c][1,4] oxaazacyclotricosine-1,7,20,21(4H,23H)-tetrone, monohydrate[9].

It is a white crystalline powder soluble in methanol, ethanol, acetone, ethyl Acetate, diethyl ether, chloroform and dichloromethane, and virtually insoluble in water[10]. Tacrolimus is stable in the solid state, in methanol and in mildly acidic media, but tends to degrade under alkaline conditions[11]. It is available for oral, intravenous and topical administration.

As a highly lipophilic drug with high permeability and low aqueous solubility, tacrolimus is classified as a Class II drug according to the Biopharmaceutics

Classification System (BCS)[12]. Metabolism is the major route of elimination of class II drugs, but because of its limited intracellular solubility, tacrolimus is less likely to saturate the enzymes involved in its metabolism, giving genetic variability an important effect on drug disposition[13].

Pharmacokinetics

Upon oral administration, tacrolimus is rapidly absorbed, with peak concentrations achieved within 30- 60 minutes in most patients[11], but due to its poor aqueous solubility, extensive first pass metabolism and alterations in gastric motility in transplant[13], tacrolimus is characterized by a highly variable and erratic absorption, with bioavailability ranging between 5 and 93%. Lower half-life estimates are reported in studies carried out during one administration interval (12 hours) compared to single-dose studies in which drug concentrations are monitored until they are immeasurably low[14]. Its mean elimination half-life is 12.1 hours with a range of 3.5 to 40.5 hours for transplant patients and 34.2 ± 10 hours for healthy volunteers[15].

In the gut, tacrolimus is subjected to two barriers against its absorption: P-glycoprotein (PGP)[16] and cytochrome P450 (CYP) 3A enzymes[17] (mainly CYP3A4 and CYP3A5). PGP, the ATP-mediated efflux pump coded by the ATP-binding cassette subfamily B member 1(ABCB1) gene, pumps absorbed drug back out into the intestinal lumen and regulates tacrolimus access to CYP3A enzymes in order to maintain intracellular drug concentrations within their metabolizing capacity[14]. Repeated uptake and efflux of tacrolimus results in extensive first pass metabolism as the drug is continuously exposed to CYP3A enzymes in the intestinal wall[18].

Once in the systemic circulation, tacrolimus is extensively bound to erythrocytes (approximately 95%)[19], and whole blood concentrations are considerably higher than plasma concentrations of the drug, making its plasma volume of distribution much smaller than the whole blood volume of distribution[15].

In the liver, the most abundantly expressed CYP enzyme is CYP3A4, however, there is up to a 100-fold variability in its expression between individuals[20]. CYP3A5 also plays an important role in tacrolimus metabolism in individuals who express the enzyme[21]. Tacrolimus is extensively metabolized by CYP3A isoenzymes with less than 5% of the parent drug appearing in the urine or feces unchanged[22]. The drug undergoes hydroxylation, demethylation and oxidation reactions[23] producing first and second generation metabolites. Those modified in only one position are called first generation metabolites (figure 3). The major pathway of tacrolimus first step metabolism is 13-O-demethylation, followed by further demethylation to 13- and 15-didemethylated metabolite. The major metabolic pathway as proposed by Iwasaki et al[22] is shown in figure 4.

At least 8 different metabolites for tacrolimus have been identified. The major metabolite, 13-O-demethyl tacrolimus (13-ODMT), retains around 10% of tacrolimus immunosuppressant activity, and the mono-demethylated tacrolimus at position 31 is a minor metabolite with equipotent immunosuppressant activity[24]. Although the nephrotoxic potential of tacrolimus metabolites has not been studied yet[25], cyclosporine metabolites have been examined and some were shown to reduce glomerular filtration rate of isolated perfused rat kidneys[26] and to cause tubular vacuolization[27].

After hepatic metabolism more than 95% of the drug is eliminated, mainly as metabolites, by the biliary route, with less than 5% of the parent drug excreted in the urine unchanged[28].

Large interindividual variability in tacrolimus pharmacokinetics and exposure may be attributed to multiple sources, including a set of graft- and patient-related clinical and genetic factors[13] (Table 1). With such a high degree of pharmacokinetic variability and a narrow therapeutic index, whole blood level monitoring of tacrolimus trough levels (C◦) is standard clinical practice to maintain therapeutic levels of the drug while preventing toxicity. Recommended target trough concentrations range between 5-15

ng/mL, but vary depending on time after transplant, the type of transplant, and patients' immunosuppressive regimen[29].

Pharmacodynamics

Calcineurin is widely distributed in all tissues, and the inhibition of the calcineurin-NFAT pathway by tacrolimus is not specific to immune cells[30], which can lead to toxic changes in addition to immunosuppressive effects. The most common side effects include nephrotoxicity, neurotoxicity, diabetogenesis, gastrointestinal disturbances (most commonly diarrhea, nausea and constipation), hypertension, an increased risk of infections and malignant complications. Hirsutism, gingivitis and gum hyperplasia are cyclosporine-specific adverse effects that are rarely associated with tacrolimus[14]

New-onset diabetes after transplantation (NODAT) refers to abnormal glucose metabolism detected after transplantation[31]; the development of NODAT has an adverse effect on patient and graft survival in solid organ transplantation[32]. High tacrolimus trough concentrations represent a transplantation-related risk factor for NODAT, along with acute rejection during the first post-transplant year and high doses of corticosteroids[33]. High levels of FKBP-12 are present in pancreatic β-cells, and tacrolimus diabetogenic effect is thought to be the result of reduced insulin secretion.

Another important adverse effect of tacrolimus is nephrotoxicity, which can manifest as acute or chronic nephrotoxicity. The acute form results from arteriolar vasoconstriction and is almost always fully reversible[25]. The chronic form, conversely, is associated with irreversible changes in the form of interstitial fibrosis, tubular atrophy and hyaline arteriolopathy, which is considered the most specific pathological lesion of calcineurin-inhibitor induced nephrotoxicity (CNIT)[34] Hallmarks of CNIT are shown in Figure 5. Although pathologic diagnostic criteria have been described, it is difficult to

distinguish histologic changes associated with prolonged use of CNIs from those induced by conditions that may coexist with CNIT such as hypertension, diabetes or aging[35].

CNIT is one of the main factors contributing to long-term kidney allograft loss in renal transplant recipients, and is also associated with chronic kidney dysfunction in non-renal transplantation[13]. Several clinical risk factors for CNIT have been identified and are shown in table 2. Although renal metabolism of tacrolimus is unlikely to have a meaningful contribution in its systemic disposition[36], CYP3A5 and PGP are also expressed in renal proximal tubular cells and may affect intrarenal accumulation of the drug or its metabolites, thus modifying the risk of renal toxicity.

The most frequently encountered mild tacrolimus-induced neurotoxicity is tremor and it may occur in as many as 40 % of patients[37, 38]. Other mild symptoms include headache, insomnia, peripheral neuropathy and mood disturbances[39]. More severe neurotoxic effects such as epileptic seizures, visual hallucinations, cortical blindness, psychosis and coma are rare and are associated with higher tacrolimus concentrations[37, 38].

Pharmacogenetics

Pharmacogenetic studies investigate the effect of the individual's genetic inheritance on drug response[40] for the aim of optimizing drug therapy and minimizing adverse events. It is most relevant in cases where the drug has a narrow therapeutic index and incompletely explained large pharmacokinetic variability, which is the case of tacrolimus.

Influence of Genetic Polymorphisms on Tacrolimus Pharmacokinetics

Genetic associations with tacrolimus pharmacokinetics such as tacrolimus dose requirements and drug exposure have been extensively studied, and they mostly involve genetic polymorphisms in tacrolimus metabolizing enzymes (mainly CYP3A4 and

CYP3A5) and transporters (namely PGP) in the gut and the liver, which largely contribute to the large variability observed in tacrolimus pharmacokinetics.

CYP3A4

The most extensively studied CYP3A4 single nucleotide polymorphism (SNP) involves an A to G transition at position 392[41]. The wild-type allele is referred to as CYP3A4*1 and the variant allele as CYP3A4*1B, with in vitro studies suggesting that variant allele expression is associated with increased CYP3A4 transcriptional activity. CYP3A4*1B variant allele and the CYP3A5*1 wild-type allele are in linkage disequilibrium[42-44], which is when two alleles at closely linked loci occur together more frequently than expected by chance, and although current findings suggest that there may be an association between the CYP3A4 -392A>G SNP and tacrolimus pharmacokinetics, there is not enough data to determine whether this effect is rather related to the influence of genetic linkage with the CYP3A5 6986A>G SNP.

A new functional CYP3A4 polymorphism (CYP3A4*22) in intron 6 (rs35599367) associated with decreased CYP3A4 levels and activity has recently been found to significantly affect tacrolimus metabolism[45]. The reported allele frequency of CYP3A4*22 in Caucasians is 5% to 8%[46]. Several studies reported the absence of the mutant allele in Chinese[47, 48] and Japanese[49] populations. Carriers of the T allele require a 33% lower mean tacrolimus dose in the first year post transplant compared with wild-type patients, with higher dose-adjusted area under the curve $(AUC)_{0-12}$ in the mutant allele carriers compared to the wild-type[45].

CYP3A4*18B, a SNP only described in the Asian population[13], involves a G to A transition in intron 10 of CYP3A4. It is believed to be associated with increased CYP3A4 activity but its effect on tacrolimus pharmacokinetics still needs to be confirmed in the transplant population.

CYP3A5

To date, CYP3A5*1 genotype is the most important genetic determinant of tacrolimus exposure and dose requirements[50]. It is the most extensively studied CYP3A5 SNP, which involves an A to G transition at position 6986 within intron 3 of the CYP3A5 gene (rs776746)[41]. Heterozygous or homozygous carriers of the CYP3A5*1 wild-type allele express high levels of functional CYP3A5 protein (CYP3A5 expressers) whereas homozygous carriers of the CYP3A5*3 variant allele produce very low or undetectable levels (CYP3A5 non-expressers)[41]. The frequency of the CYP3A5*1 allele is largely dependent on ethnicity, with a reported frequency of 73% in African Americans[51], compared to only 5–15% of Caucasians[52].

CYP3A5 expressors experience lower dose-corrected tacrolimus exposure parameters and subsequently require higher doses of tacrolimus to maintain therapeutic levels[53]. Some studies recommend that patients with CYP3A5*3/3 genotype receive an initial tacrolimus dose that is roughly 50% lower than patients with one or two copies of the wild-type allele[54, 55], but because of considerable variation in tacrolimus clearance within CYP3A5 expressors, and the overlap that exists between the two groups [13], these recommendations have not yet been introduced into routine clinical application.

ABCB1

ABC transporter genes represent one of the largest transmembrane protein families; they encode membrane-bound proteins responsible for the efflux transport of a wide range of endogenous and exogenous substrates[56]. ABCB1 belongs to the B subfamily of the ABC transporters and encodes PGP, which has the ability to pump the immunosuppressive drug tacrolimus. The three most commonly studied SNPs in the ABCB1 gene in relation to tacrolimus pharmacokinetics include: a C to T transition in exon 26 at position 3435 and exon 12 at position 1236, and a G to T or A transition in exon 21 at position 2677[41]. These three variants are in linkage disequilibrium and

together they are referred to as ABCB1-13 haplotype. Variant alleles in ABCB1 3435C>T, 1236C>T and 2677G>T/A, are expected to minimize PGP activity[57]. However, available data on the effect of these SNPs on tacrolimus pharmacokinetics is inconsistent, with some studies reporting small but significant increase in dose-corrected tacrolimus exposure in carriers of the variant allele, while the majority of studies reported negative associations[53].

Other genetic determinants

P450 oxidoreductase (POR) is important for CYP-mediated oxidation. A SNP in the POR gene, POR*28 (rs 1057868; C>T), has been associated with tacrolimus pharmacokinetics in carriers of the CYP3A5 expressor genotype; carriers of the POR*28 variant allele had increased tacrolimus dose requirements compared to homozygous wild-type carriers in CYP3A5 expressors[58]. Most recently, POR*28 allele was found to be associated with increased in vivo CYP3A5 activity for tacrolimus in CYP3A5 expressers, with a significant gain of function of the enzyme, whereas POR*28 homozygosity (POR*28/28) in CYP3A5 nonexpressors was associated with a significantly higher CYP3A4 activity[59].

Polymorphisms in Pregnane X receptor (PXR)[60], P450 oxidoreductase (POR)[61] and UDP-glucuronosyltransferase 1 (UGT1) genes[62] have also been studied and their associations with tacrolimus exposure have been reported, but the clinical relevance of these findings and their impact on CNI metabolism and exposure remains to be validated.

Influence of Genetic Polymorphisms on Tacrolimus Pharmacodynamics

Genetic associations with tacrolimus pharmacodynamics and transplant outcome have also been studied but not as extensively as those related to tacrolimus pharmacokinetics.

Nephrotoxicity

A small number of studies have investigated the role of genetic polymorphisms in the risk to develop CNIT in renal transplant and chronic kidney disease in non-renal transplant.

Available data on the effect of CYP3A5*1 recipient and donor genotype is conflicting. In liver transplant, a higher incidence of nephrotoxicity in carriers of the CYP3A5*3/3 genotype has been reported, suggesting a protective role of CYP3A5 expression in the kidney[63]. On the other hand, CYP3A5*1 genotype of the recipient has been associated with the development of biopsy proven nephrotoxicity secondary to tacrolimus therapy in renal transplantation[43] suggesting that the presence of a CYP3A5*1 allele leads to higher drug clearance, higher dose requirements and possibly higher local tissue concentrations of tacrolimus metabolites. These discrepancies may be the result of population differences, small sample sizes and inconsistent definitions of nephrotoxicity[39].

Evidence supporting a protective effect of ABCB1 expression against increased tacrolimus concentration in renal cells is suggested, but available data on the effect of ABCB1 genotypes on the risk of tacrolimus-induced nephrotoxicity has been far from consistent. Kidneys with ABCB1 3435 TT genotype were reported to be at a significantly higher risk for nephrotoxicity[64] and chronic allograft damage[65] than those with CT or CC genotypes.

CYP2C8 enzyme is also expressed in the kidney, and involved in the metabolism of arachidonic acid to epoxyeicosatrienoic acids, the latter of which are believed to possess vasodilatory properties and may play a protective role against damaging processes in solid organ transplantation[66]. CYP2C8*3 is the most common variant in Caucasians, and was found to be positively associated with delayed graft function and worse creatinine clearance[67].

Diabetes Mellitus

The role of genetics in determining the risk of NODAT is complex and over-shadowed by demographic and clinical variables[39], especially in light of the fact that in addition to CNIs, other immunosuppressants such as glucocorticoids and mTOR inhibitors are also diabetogenic.

Neurotoxicity

ABCB1 in the blood brain barrier allows very little tacrolimus to enter the central nervous system (CNS) under normal circumstances[39]. ABCB1 genetic polymorphisms may affect tacrolimus entry into the CNS leading to neurotoxicity. ABCB1 1236 and 2677 homozygous wild-type allele carriers were reported to be at higher risk for neurotoxicity[68] but these results have not been consistently replicated.

Hypertension

CYP3A5*1 genotype[69] has been implicated in tacrolimus-induced hypertension in renal transplant patients, with CYP3A5 expressors experiencing higher systolic and diastolic blood pressures and requiring more antihypertensive medications than CYP3A5 non-expressors. But these associations between CYP3A5 genotype with post-transplant hypertension were not significant and remain to be validated.

Thesis Outline and Research Objectives

Knowledge on the effect of genetic polymorphisms in CNI metabolizing enzymes and transporters may help minimize the high inter-individual pharmacokinetic variation in tacrolimus blood and tissue concentrations in the transplant patient. The overall objective of this research is to better understand the interplay between clinical and genetic factors in defining tacrolimus pharmacokinetic properties and the risk of CNI induced renal dysfunction in the transplant population. The incorporation of genetic

information into individualized immunosuppressive drug dosing has the potential to improve treatment outcomes and minimize toxicities for the transplant patient.

As an intense amount of research has been directed towards this area, Chapter 2 presents a detailed review of the effect of CYP3A4, CYP3A5 and ABCB1 genetic polymorphisms on tacrolimus exposure in the transplant population, and addresses the potential to transform these genetic associations into clinically useful pharmacogenetic dosing strategies.

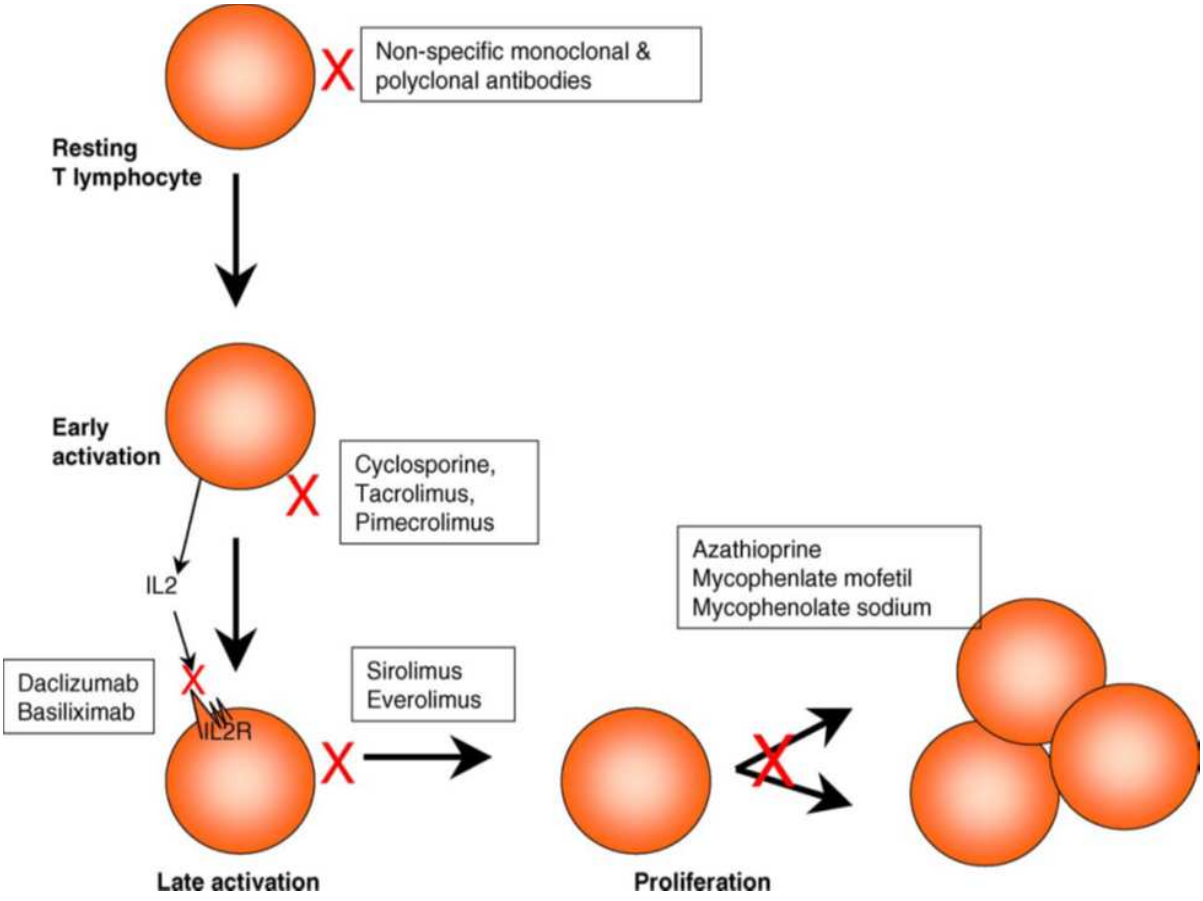
Genetic polymorphisms can also play a critical role in determining interindividual variation in predisposition to drug toxicities or drug interactions. In chapter 3, our aim was to investigate clinical and genetic factors, specifically polymorphisms in CYP3A and ABCB1 genes that may modify the risk for renal dysfunction in liver transplant patients receiving immunosuppressive treatment with calcineurin inhibitors.

Genetic polymorphisms may also correlate with local tissue concentrations of calcineurin inhibitors or their metabolites, which, despite being implicated in the risk of developing renal dysfunction, were not determined in any of the studies investigating genetic polymorphisms as risk factors for CNIT. In chapter 4, our aim is to develop and validate a sensitive and robust LC-MS/MS method to determine the concentrations of tacrolimus and its major metabolite 13-ODMT in rat kidney tissue. This is a proof-of-concept study to evaluate the relationship between increasing doses of tacrolimus with renal accumulation of the drug or its major metabolite.

Chapter 5 presents a summary of the obtained results in this thesis, and a proposed direction for application of the current findings in determining associations

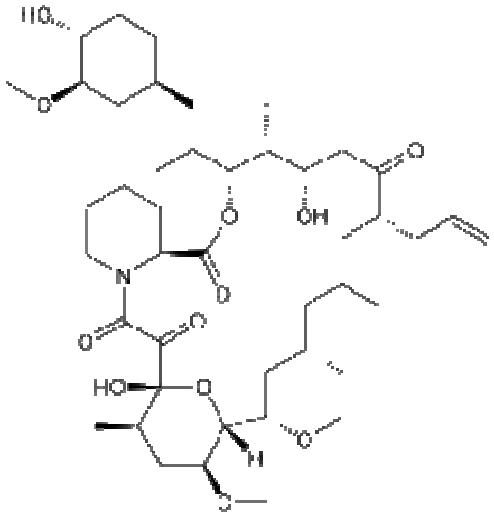
between genetic polymorphisms and renal tissue concentrations of tacrolimus and its major metabolite in defining the risk of renal toxicity in the transplant population.

Figure 1 Schematic sites of actions of common immunosuppressants



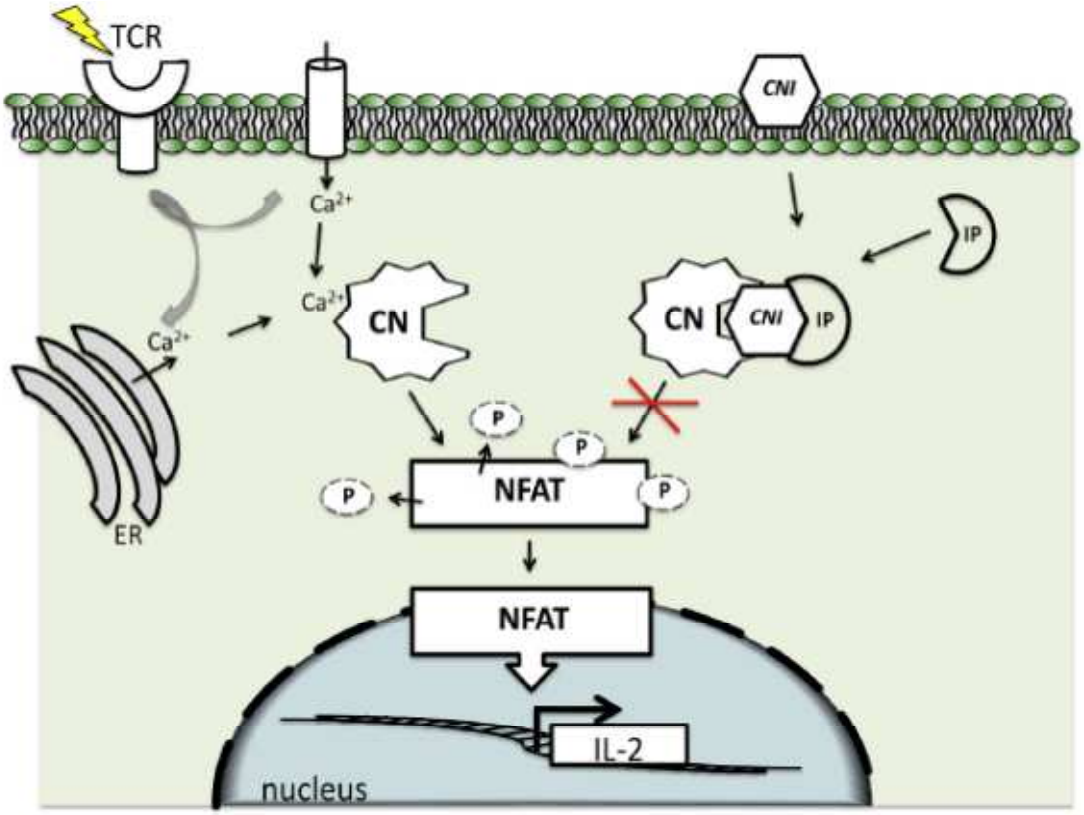
Source: Critical Reviews in Oncology/Hematology 56 (2005) 23–46

Figure 2 Tacrolimus chemical structure



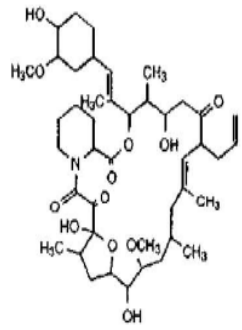
Source: N. Knops et al. / International Journal of Pharmaceutics 452 (2013) 14– 35

Figure 3 Schematic presentation of tacrolimus MOA

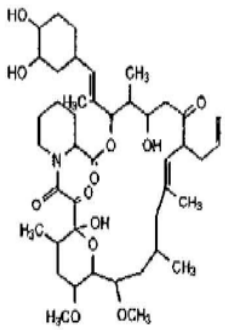


Source: N. Knops et al. / International Journal of Pharmaceutics 452 (2013) 14– 35
Abbreviations: TCR: T cell receptor; CN: calcineurin; NFAT: nuclear factor of activated T cells; CNI: calcineurin inhibitor; IP: immunophilin; P: phosphate group; IL-2: Interleukin 2 gene.

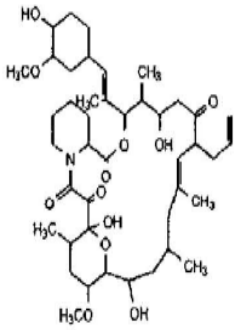
Figure 4 Structure of tacrolimus metabolites



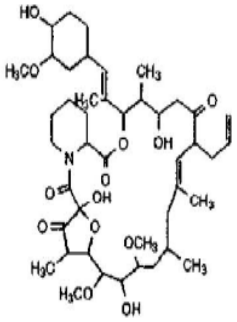
13-O-Demethyltacrolimus



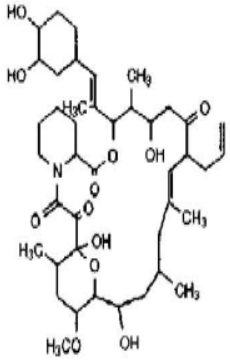
31-O-Demethyltacrolimus



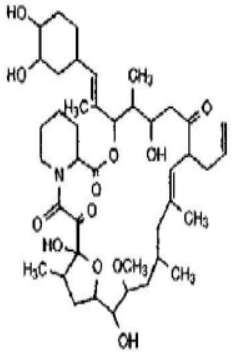
15-O-Demethyltacrolimus



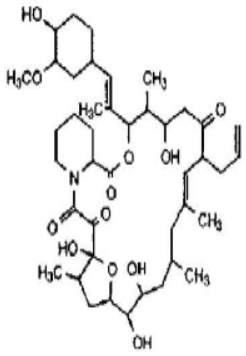
12-Hydroxytacrolimus



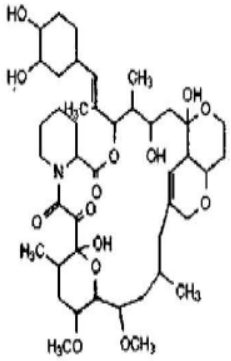
15, 13-O-Didemethyl tacrolimus



13, 31-O-Didemethyl tacrolimus



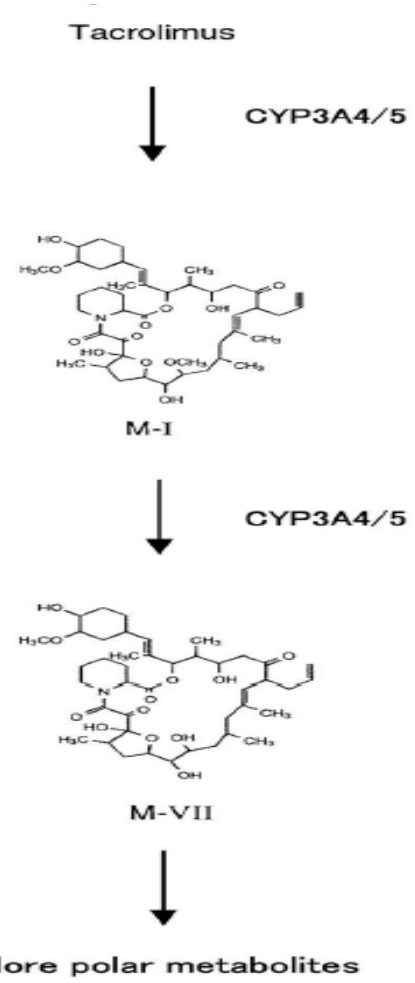
13, 15-O-Didemethyl tacrolimus



31-O-Demethyl,19-Hydroxy, 37, 39-Epoxytacrolimus

Source: Drug Metab. Pharmacokinet. 22(5): 328- 335 (2007)

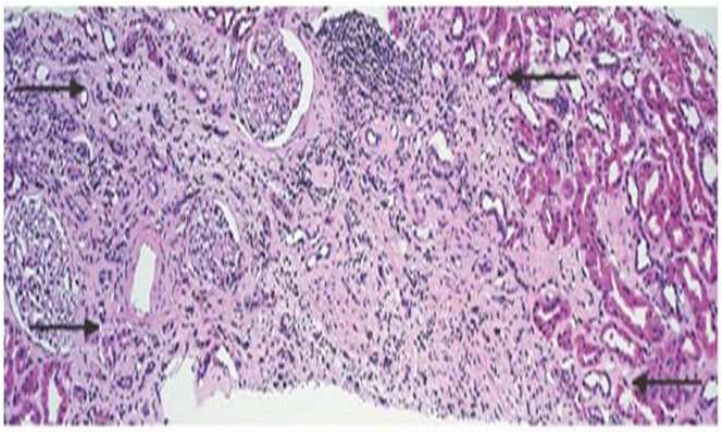
Figure 5 Proposed major metabolic pathway of tacrolimus by human liver microsomes



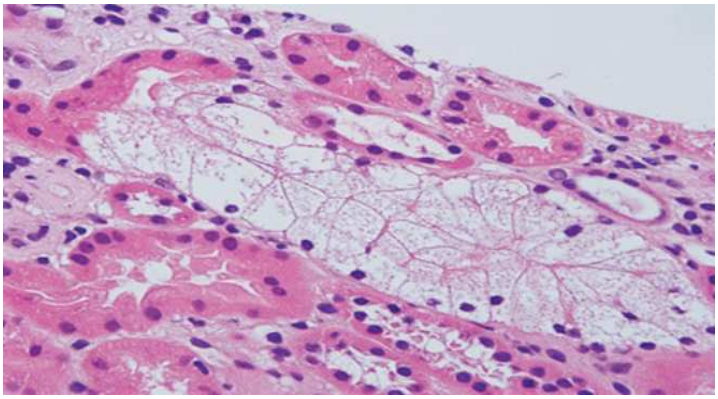
Source: Drug Metab. Pharmacokinet. 22(5): 328- 335 (2007)

Figure 6 Hallmarks of CNIT

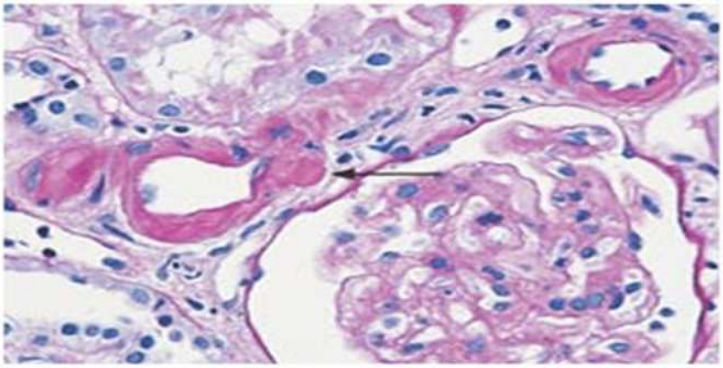
(a) Interstitial fibrosis and tubular atrophy in a band-like pattern



(b) Isometric vacuolization of proximal tubular epithelial cells



(c) Beaded medial hyalinosis in afferent arteriolar profiles



Source: Clinical Practice Nephrology. Nature (2006) 2, 398-404

Table 1 Immunosuppressive agents used in solid organ transplantation

Class of agent	Agent
Corticosteroid	Prednisolone Prednisone Methyl prednisolone
Anti-proliferative	Azathioprine Mycophenolate mofetil Mycophenolate sodium
Calcineurin inhibitor	Cyclosporine Tacrolimus
TOR inhibitor	Sirolimus Everolimus
Polyclonal anti-lymphocyte antibodies	ALG ATG ALS
Monoclonal antibodies	Muromonab-CD3 Basiliximab Daclizumab

Source: Critical Reviews in Oncology/Hematology 56 (2005) 23–46

Table 2 Clinical covariates affecting tacrolimus disposition in solid organ transplantation

	Non-genetic clinical covariates affecting CNI clearance
Recipient	• Age • Bodyweight/surface area • Ethnicity • Diarrhea • Food ingestion • Hepatic dysfunction • Hematocrit • Albumin • Renal failure • Inflammation • Co-medication
Donor	• Delayed graft function/IRI (liver-kidney) • Age (liver) • Graft seize (liver) • Living versus deceased (liver-kidney)
Other	• Time after transplantation

Source: International Journal of Pharmaceutics 452 (2013) 14-35

Table 3 Clinical risk factors for calcineurin inhibitor nephrotoxicity

Risk Factors for Calcineurin Inhibitor Nephrotoxicity
Systemic overexposure to cyclosporine and tacrolimus
Local exposure to cyclosporine and tacrolimus
interactions with drugs interfering with ABCB1-mediated transport in the tubular epithelial cells (*e.g., m*TOR inhibitors)
ABCB1 genotype of the kidney
ABCB1 expression in renal tubular epithelial cells
Exposure to metabolites of cyclosporine and tacrolimus
CYP3A4/5 genotype of the patient
CYP3A5 expression in renal tubular epithelial cells
interactions with other drugs which lead to altered exposure to calcineurin inhibitor metabolites (e.g. ketoconazole)
Older kidney age
Use of nonsteroidal anti-inflammatory drugs
Salt depletion and diuretic use
Genetic polymorphisms of other genes (*e.g., TGF-β, ACE*)

Source: Clin J Am Soc Nephrol 4: 481–508, 2009

CHAPTER 2

THE PHARMACOGENETICS OF TACROLIMUS DOSING IN RENAL AND HEPATIC TRANSPLANTATION

Introduction

CNI therapy is the mainstay immunosuppressive treatment in solid organ transplantation. Tacrolimus, the most widely used CNI, is characterized by large interindividual variability in drug pharmacokinetics and exposure. It also has a narrow therapeutic index, making therapeutic drug monitoring (TDM) a vital part of CNI treatment in order to maintain therapeutic levels of these drugs while avoiding toxicities[70].

However, TDM is not without limitations; it will not help optimize drug exposure in the first 72 hours after transplantation[53] during that critical period before the therapeutic level is achieved. As a result, there is an increased risk of graft rejection in cases of underexposure or an increased incidence of adverse events in cases of overexposure[71].

Genetic variation in tacrolimus metabolizing enzymes (mainly CYP3A4 and CYP3A5 in the gut and liver)[17] and transporters (PGP)[16] is a major contributor to the marked interindividual variabilities in drug pharmacokinetics and pharmacodynamics[41]. The potential to use these genetic polymorphisms as markers to predict optimal doses of tacrolimus and individualize patient therapy is attractive. In a recent review by Ware and Macphee[53], the authors summarized study findings on the effects of CYP3A and ABCB1 genetic polymorphisms on dose-adjusted immunosuppressive drug exposure (tacrolimus, cyclosporine and sirolimus). In this review, we present an updated summary of the most recent findings(after 2009) on these effects on dose-corrected tacrolimus exposure, and we focus particularly on current advances in pharmacogenetic dosing strategies for

tacrolimus that, upon clinical application, may allow rapid achievement of target therapeutic drug concentrations.

The CYP3A4 Genotype and Pharmacokinetics

The CYP3A enzymes are the major enzymes involved in the metabolic transformation of tacrolimus. An A to G transition in CYP3A4 gene at position 392 alters the 10-basepair nifedipine-specific element (NFSE)[41] and is believed to be associated with increased CYP3A4 transcriptional activity. The wild-type allele is referred to as CYP3A4*1 and the variant allele as CYP3A4*1B. The CYP3A4*1B allele frequency varies according to ethnicity: 2–9.6% of Caucasians, 35–67% of Africans, 9.3–11% of Hispanics and 0% of Asians[72].

Multiple studies have shown linkage disequilibrium between the CYP3A4*1B variant allele and the CYP3A5*1 wild-type allele[42, 43, 73]. Current findings suggest that there may be an association between the CYP3A4 -392A>G SNP and tacrolimus pharmacokinetics (table 4), with some studies reporting decreased dose-adjusted tacrolimus exposure in patients with the mutant genotype. However, this effect may be related to the influence of genetic linkage with the CYP3A5 6986A>G SNP.

A CYP3A4 polymorphism in intron 6 (rs35599367) is associated with decreased CYP3A4 levels and activity and has recently been found to significantly affect tacrolimus metabolism[45]. The reported allele frequency of CYP3A4*22 in Caucasians was 5% to 8%[46]. Several studies reported the absence of the mutant allele in Chinese[47, 48] and Japanese [49] populations.

Elens et al[45] tested the effect of the CYP3A4*22 variant allele on tacrolimus dose requirements in 185 kidney transplant recipients. They reported that carriers of the T allele required a 33% lower mean tacrolimus dose in the first year post transplant compared with wild-type patients, independent of CYP3A5 allelic status. When CYP3A4 and CYP3A5 genotypes were combined, there was an overall increase of 179%, 101%,

and 64% in tacrolimus concentration to dose (C/D) ratio for poor metabolizers (CYP3A5 nonexpressors and carriers of the CYP3A4 T variant), intermediate metabolizers I (CYP3A5 nonexpressers and CYP3A4 CC carriers) and intermediate metabolizers II (CYP3A5 expressers carrying the CYP3A4 T allele), respectively, when compared to extensive metabolizers (CYP3A5 expressers with the CYP3A4 CC wild type).

Mutant allele carriers were found to have a higher dose-adjusted AUC_{0-12} compared to the wild-type allele carriers[74]. This study confirmed the impact of the CYP3A4*22 allele on tacrolimus exposure in the early period after renal transplantation, independent of the CYP3A5 genotype.

CYP3A4*18B**,** a SNP only described in the Asian population[13], involves a G to A transition in intron 10 of CYP3A4. It is believed to be associated with increased CYP3A4 activity. And despite the fact that strong linkage disequilibrium was detected between CYP3A4 mutant allele and the CYP3A5*1 allele, Shi et al[47] were able to report a higher tacrolimus clearance in healthy Chinese subjects independent of their CYP3A5 genotype. This effect, however, needs to be confirmed in the transplant population (table 4).

The CYP3A5 Genotype and Pharmacokinetics

An A to G transition at position 6986 within intron 3 of the CYP3A5 gene (rs776746)[41] creates an alternative splice site in the pre- messenger ribonucleic acid (mRNA) and leads to the production of aberrant mRNA with a premature stop codon, resulting in the absence of functional CYP3A5 from liver tissue. Carriers of one or two copies of the CYP3A5*1 wild-type allele produce high levels of full-length CYP3A5 mRNA and express high levels of functional CYP3A5 protein (CYP3A5 expressers), whereas homozygous carriers of the CYP3A5*3 variant allele produce very low or undetectable levels of functional CYP3A5 protein (CYP3A5 non-expressers)[41]. The CYP3A5*1 allele is found in approximately 45–73% of African Americans, 15–35% of

Asians and 5–15% of Caucasians[52]. In CYP3A5 expressors, CYP3A5 has a more dominant role than CYP3A4 in the metabolism of tacrolimus[41].

Polymorphisms in the gene encoding for CYP3A5 have been extensively studied and have been found to influence the dosing of tacrolimus[75]. An overwhelming majority of studies agree that CYP3A5 nonexpressors experience higher dose-corrected tacrolimus exposure parameters and subsequently require lower doses of tacrolimus to maintain therapeutic concentrations (table 4). Non-expressors also experience significant delays in achieving target drug concentrations[76] in the early post-operative period.

In a systematic review[29] of the effect of CYP3A5 genotype on the apparent oral clearance of tacrolimus in renal transplant recipients, a total of five studies were identified that reported a 48% lower mean apparent oral clearance of tacrolimus in CYP3A5 non-expressors compared to expressors. The authors recommended that patients with CYP3A5*3/3 genotype receive an initial tacrolimus dose that is roughly 50% lower than patients with one or two copies of the wild-type allele.

In liver transplant, both donor (hepatic) and recipient (intestinal) genotypes contribute to tacrolimus pharmacokinetic variability. In a recent meta-analysis[77] of the effect of CYP3A5 6986A>G genotype of both the donor and recipient on tacrolimus dose requirements in liver transplantation, the authors found that the donor rather than the recipient genotype influences dose-normalized tacrolimus concentration during the first month after transplantation, with a 1.3-2 times higher concentration to dose (C/D) ratio in CYP3A5 non expressors compared to expressors.

The ABCB1 Genotype and Pharmacokinetics

The three most common SNPs in the ABCB1 gene include: a C to T transition in exon 26 at position 3435 and exon 12 at position 1236, and a G to T or A transition in exon 21 at position 2677[41]. These three variants are in linkage disequilibrium and they comprise a common haplotype referred to as ABCB1*13[78]. The ABCB1 1236T-2677T-

3435T (T-T-T) variant haplotype is present in approximately 32% of Caucasians and 5% of African Americans. Variant alleles in ABCB1 3435C>T, 1236C>T and 2677G>T/A, are expected to minimize PGP activity[57]. A study by Masuda et al[79] identified an inverse correlation between ABCB1 intestinal mRNA levels and tacrolimus C/D ratio. They classified patients according to the median intestinal ABCB1 mRNA expression, and found that the oral dose of tacrolimus in the high-ABCB1 group was approximately twofold higher than that in the low-ABCB1 group, irrespective of the patients' ABCB1 genotype.

Research into the association between ABCB1 genotypes and tacrolimus exposure has yielded inconsistent results. Some studies reported increased tacrolimus exposure in carriers of ABCB1 variant alleles (table 5) but the majority of the studies reported an absence of a correlation between ABCB1 polymorphisms and tacrolimus C/D ratio (table 5), suggesting that local drug concentrations may reflect the effect of these SNPs better than their blood counterparts. This was shown by Elens et al[80] who investigated the effect of the donor genotype for 13 different polymorphisms on tacrolimus pharmacokinetics and hepatic concentrations in liver transplant patients in the first week after surgery. The authors found that ABCB1 1199G>A polymorphism was associated with a significantly higher hepatic concentration of tacrolimus and that transplanted livers carrying at least one mutated allele for 1236C>T and 2677G>T/A SNPs showed greater tacrolimus hepatic concentrations when compared with the homozygous wild-type. The impact of these ABCB1 genotypes on blood concentrations of tacrolimus was negligible.

Most recently, two meta-analyses[81, 82] reviewed the existing evidence of an association between ABCB1 C3435T genetic polymorphism and tacrolimus C/D ratio in renal transplant patients and came to different conclusions. Li et al[82] reported a definite correlation, with CT genotype carriers having a significantly lower tacrolimus C/D ratio compared to those carrying the TT genotype at 6 months post transplantation, which is

consistent with higher intestinal PGP expression and lower tacrolimus absorption expected in carriers of the wild-type allele. Terrazzino et al[81] found a modest effect of the C3435T polymorphism on tacrolimus solely during the first month post transplantation, however the effect was seen at later times.

In liver transplantation, a meta-analysis by Liu et al[83] reported a similar relationship between ABCB1 3435 polymorphism in the recipient and tacrolimus exposure, with CT genotype carriers having higher C/D ratio than those with CC and TT genotypes. However, the relation was not evident at different post-transplantation times. No significant associations were observed with donor ABCB1 C3435T genotype.

Pharmacogenetic dosing strategies

Genotype-based tacrolimus dosing has been proposed. Based on evidence from their retrospective trial in 2006, Haufroid et al[54] suggested that a first dose increase in CYP3A5 expressors (0.15 mg/kg b.i.d.), and reduction in nonexpressors (i.e., 0.075 mg/kg b.i.d.) would help optimize tacrolimus therapy. Based on these dosing recommendations, Thervet et al[55] carried out a randomized controlled trial comparing tacrolimus dosing based on CYP3A5 genotype with the standard practice of dosing tacrolimus based on the patient's body weight. The authors confirmed the benefit of pharmacogenetic adaptation of starting doses of tacrolimus in kidney transplant recipients, with a higher proportion of patients in the genotype-based dosing group achieving the targeted trough level at day 3 after initiation of tacrolimus (43.2% vs. 29.1%). They did not identify a potential benefit of prospective genotyping on clinical outcomes.

Although it is well established that CYP3A5 genotype is the major determinant of tacrolimus disposition, accounting for 29-35% of tacrolimus pharmacokinetic variability[50], it does not explain the remaining variability observed in tacrolimus disposition within CYP3A5 expressors and nonexpressors. Several other genetic and

clinical factors have been suggested to contribute to the variation in tacrolimus clearance rates such as CYP3A4 genotype, the patient's age, weight, ethnicity, co-medications, hemoglobin concentration, hematocrit, plasma albumin concentration and time post-transplant[84].

In a cohort of 96 renal transplant recipients, Wang et al[85] screened 768 SNPs in 15 candidate genes for association with tacrolimus dose. They generated a pharmacogenomic model that accounted for 50% of tacrolimus dosing variability. The final model used to predict a stable tacrolimus dose at the initiation of therapy included age, ethnicity, CYP3A5 genotype and co-medications. Upon validating their model in two cohorts of 77 and 64 patients, the authors concluded that it resulted in a better tacrolimus dose prediction compared to previous models[55].

Another algorithm described by Passey et al[86] predicts tacrolimus apparent clearance (CL/F) in order to inform the optimal starting dose of the drug. Their equation takes into account the age and CYP3A5 genotype of a patient, time after kidney transplantation, whether the transplantation was performed at a steroid-sparing center or not and whether the patient was treated with a calcium channel blocker. The authors evaluated the predictive performance of the clearance equation using an independent cohort of kidney transplant recipients[87]. While the bias and precision for the initial troughs (The initial trough was defined as the first trough measured at steady state in the first week post-transplant) was good, when all trough concentrations from the first 6 months post-transplant were used, the equation had a lower predictive performance.

Elens et al[88] tested the predictive power of the equation proposed by Passey et al[86] in 185 de novo kidney transplant recipients with and without accounting for CYP3A4*22 genotype and reported negative bias in CYP3A4*22 carriers. They suggested that CYP3A4*22 genotype should be included in the equation to improve its predictive ability. Based on the results of a study investigating the impact of CYP3A4*22 allele on tacrolimus PKs in 96 kidney transplant recipients in the first two weeks following

transplantation, Elens et al[74]suggested increasing the daily dose for CYP3A4*22 noncarriers and decreasing it for CYP3A4*22 carriers among CYP3A5 nonexpressers, with starting doses of 0.150, 0.080, and 0.070 mg/kg body weight twice daily for extensive, intermediate, and poor CYP3A-metabolizers.

Boughton et al[84] also tested the tacrolimus dosing algorithm proposed by Passey et al[86] in an independent cohort of 255 renal transplant recipients and found it to be poorly predictive of tacrolimus clearance. They compared predicted tacrolimus clearance based on the Long-Term Deterioration of Kidney Allograft Function Study (DeKAF) algorithm to dose-normalized trough whole blood concentrations on day 7 after transplantation and found weak correlation ($r=0.431$, $r^2=0.186$).

The important contribution of CYP3A4 metabolism to tacrolimus disposition and pharmacokinetic variability has also been shown by De Jong et al[50], who conducted a cross-sectional study in 59 renal transplant patients to investigate the relationship between in vivo CYP3A4 activity (assessed using midazolam) and CYP3A5 genotype with tacrolimus pharmacokinetics. They showed that CYP3A4- and CYP3A5-mediated tacrolimus metabolism are important determinants of tacrolimus disposition in vivo, accounting for 56–59% of the variability in tacrolimus dose requirements and clearance.

Based on these findings, it may be suggested that a polygenic algorithm that incorporates other genes known to affect tacrolimus clearance such as CYP3A4 genotype, may have a better predictive ability of the optimal initial dose of tacrolimus compared to one that is based only on CYP3A5 genotype[84]. In addition, all of the aforementioned validation studies of the algorithm proposed by Passey et al[86] were retrospective. Prospective randomized clinical trials are needed to validate the clinical applicability of any such algorithm and its potential to improve clinical outcome for the transplant recipient.

Conclusion

While much work has been done to improve tacrolimus dosing algorithms in the transplant patient, a better predictive model that includes both clinical and genetic factors is still needed to help optimize tacrolimus dosing and translate the role of genetic factors in tacrolimus pharmacokinetic variability into improved clinical outcomes.

In addition to optimized dosing, pharmacogenetic strategies can also be of valuable importance in the avoidance of CNI-induced toxicities. A number of studies evaluated the role of genetic factors in the differential susceptibility to CNI nephrotoxicity, a predominant side effect of CNIs, but with mixed results. Intracellular drug concentrations may be a better indicator of toxicity than systemic concentrations of CNIs and may help explain the role of genetic differences in drug transporters and metabolizing enzymes in determining the risk of CNI-induced nephrotoxicity

Table 4 Effect of CYP3A4/5 genetic polymorphisms on dose-corrected tacrolimus exposure

Population	n	Country	DNA source	SNP	Effect	Reference	Comments
Renal	103	Spain	R	CYP3A4*1/ 3A5*3	Increased	Gervasini et al[68]	
Liver	216	China	R	CYP3A5*3	Increased	Shi et al[47]	
				CYP3A4*22	neutral		No mutations found
Liver/	51	Italy	R/ D	CYP3A5*3	Increased	Provenzani et al[89]	Donor genotype
Renal	50		R	CYP3A5*3	Increased		
Renal	209	France	R/ D	CYP3A5*3	Increased	Glowacki et al[90]	Recipient Genotype
Renal	304	Belgium	R	CYP3A5*3	Increased	kuypers et al[43]	
Heart	15	Austria	R	CYP3A5*3	Increased	Kniepeiss et al[91]	Higher doses in *1/3
Renal	70	Korea	R	CYP3A5*3	Increased	Cho et al[48]	
				CYP3A4*18	neutral		
Renal	291	Mexico	R	CYP3A5*3	Increased	Garcia-Roca et al[92]	Adults and peds
Liver	58	South Korea	R/ D	CYP3A5*3	Increased	Ji et al[93]	Combined genotypes (C/D: $R_E D_E < R_N D_N$)

Table 4 - Continued

Renal	52	US	R	CYP3A5*3	Increased	Chitnis et al[94]	Combined effect
				CYP3A4*1B	Decrease		also examined
Liver	100	Iran	R	CYP3A5*3	neutral	Rahsaz et al[95]	
Renal	185	international	R	CYP3A4*22	increased	Elens et al[45]	
Renal	132	Korea	R	CYP3A5*3	increased	Kim et al[96]	No difference post 3m
Liver	98	Spain	R/D	CYP3A5*3	increased	Gomez-Bravo et al[97]	Both R and D
							genotypes
Renal	12	Korea	R	CYP3A5*3	increased	Yoon et al[98]	
				CYP3A4*18	neutral		
Renal	129	Korea	R	CYP3A5*3	increased		
Renal	96	Belgium	R	CYP3A5*3	increased	Elens et al[74]	
				CYP3A4*22	increased		
Renal	206	Spain	R	CYP3A5	increased	Tavira et al[99]	
				CYP3A4*1B	decreased		
				CYP3A4*22	neutral		

Table 4 - Continued

Renal	129	Korea	R	CYP3A5*3	increased	Kim et al[100]

*Combined effect of CYP3A4*22, CYP3A5*3 and ABCB1 C3435T showed a significant positive correlation with dose-corrected tacrolimus C_0 for all three mutations.

Table 5 Effect of ABCB1 genetic polymorphisms on dose-corrected tacrolimus exposure

Population	n	Country	DNA source	SNP	Effect	Reference	Comments
Renal	103	Spain	R	C3435T	neutral	Gervasini et al[68]	
				G2677T/A	neutral		
				C1236T	neutral		
				Haplotype (≥4variants)	reduced		In CYP3A5*3/3
Liver	216	China	R	C3435T	neutral	Shi et al[47]	
				C1236T	neutral		
Renal/	50	Italy*	R	G2677T/A	neutral	Provenzani et al[89]	Variant allele ↑ dose
				C3435T	neutral		Requirement
Liver	51		R/ D	G2677T/A	neutral		
				C3435T	neutral		
Renal	209	France	R/ D	C3435T	neutral	Glowacki et al[90]	
Renal	304	Belgium	R	C3435T	neutral	Kuypers et al[43]	
				G2677T/A	neutral		
				C1236T	neutral		

Table 5 - Continued

Renal	70	Korea	R	C3435T	neutral	Cho et al[48]	
				G2677T/A	neutral		
				C1236T	neutral		
Renal	52	US	R	C3435T	neutral	Chitnis et al[94]	
Liver	100	Iran	R	C3435T	neutral	Rahsaz et al[95]	
Renal	75	India	R	C3435T	increased	Singh et al[101]	At 1 week, 1, 3 and 6 months
				C1236T	increased		At 1 week and 3 months
				G2677T	Increased		At 1 month
Renal	185	International	R	C3435T	neutral	Elens et al[45]	no difference between CGC
				C1236T	neutral		and TTT haplotypes
				G2677T	neutral		
Renal	132	Korea	R	C3435T	neutral	Kim et al[96]	TTT carriers had lower
				C1236T	neutral		exposure on d3
				G2677T/A	increased		

Table 5 - Continued

Liver	98	Spain	R/D	C3435T	neutral	Gomez-Bravo et al[97]	
				C1236T	neutral		
				G2677T	neutral		
Renal	12	Korea	R	C3435T	neutral	Yoon et al[98]	
				C1236T	neutral		
				G2677T	neutral		
Liver	62	China (Han)	R	C3435T	increased	Yu et al[102]	*Haplotypes influenced PKs
				C1236T	neutral		
				G2677T	neutral		

*Patients carrying T-T haplotype and with an additional T/T homozygote at position C1236T or G2677A/T had lower C/D ratios and required higher tacrolimus doses.

CHAPTER 3

EFFECT OF CYP3A4/5 AND ABCB1 POLYMORPHISMS OF THE RECIPIENT ON THE RISK OF DEVELOPING CNI-INDUCED RENAL DYSFUNCTION FOLLOWING LIVER TRANSPLANTATION

Introduction

Liver transplantation has become a successful treatment option for patients with end-stage liver disease, with one-year graft survival rates exceeding 80%[103] largely due to advances in immunosuppressive therapy. CNI therapy in solid organ transplantation has dramatically improved short-term survival by decreasing rates of acute rejection[104]. They are currently prescribed as part of an immunosuppressive treatment regimen consisting of cyclosporin A or tacrolimus, an anti-proliferative agent (mycophenolate mofetil) and glucocorticoids[53].

Tacrolimus, an immunosuppressant widely used in liver transplantation, is a substrate of CYP3A (mainly CYP3A4 and CYP3A5) intestinal and hepatic metabolism[17]. In CYP3A5 expressors, CYP3A5 contributes more significantly to tacrolimus metabolism compared to CYP3A4[14]. A SNP in the CYP3A5 gene (6986G) displays a sequence variability in intron 3 that creates a cryptic splice site and results in the generation of CYP3A5 exon 3B; this CYP3A5*3 allele encodes an aberrantly spliced mRNA with a premature stop codon, leading to the absence of protein expression[105] . Polymorphisms in the gene encoding for CYP3A5 have been extensively studied and have been found to influence the pharmacokinetics[75] and pharmacodynamics[42, 63] of tacrolimus. A recently reported CYP3A4 polymorphism in intron 6 (rs35599367), associated with decreased CYP3A4 levels and activity, significantly affects tacrolimus metabolism[45]. The mutant allele carriers (also referred to as CYP3A4*22) required lower doses and experienced higher tacrolimus exposure compared to the wild-type.

Tacrolimus is also a substrate of PGP[16], encoded by the ABCB1 gene, where three partly linked polymorphisms located on exons 12, 21 and 26 account for the major haplotypes encountered in Caucasians[106]. The variant alleles are expected to result in reduced PGP activity[41]. Evidence concerning their functional significance is contradicting and the effect of ABCB1 polymorphisms on the pharmacokinetics of tacrolimus remains to be firmly established[13].

Genetic variation in drug transporters and metabolizing enzymes contributes to the large interindividual variability observed in tacrolimus pharmacokinetics and exposure[41]. In addition, CNIs have a narrow therapeutic index which makes whole blood level monitoring a necessity to maintain therapeutic levels of these drugs while preventing toxicity[70]. Despite TDM, nephrotoxicity is one of the most frequent and severe adverse events of CNIs and limits the use of CNIs in transplantation. Long-term studies of adult orthotopic liver transplant (OLT) recipients estimate the incidence of end-stage renal disease to be 10% at 10 years[107].

Risk factors for CNI-induced nephrotoxicity include systemic overexposure and/or local exposure to CNIs, older kidney age, salt depletion, and the use of nonsteroidal anti-inflammatory drugs[70]. Evidence suggests that local exposure to CNIs in the kidney could be more important than systemic exposure. Since CYP3A4/5 and PGP are found in renal tubules, inherited genetic variation in CYP3A[42, 43, 108] and ABCB1[65, 109] genes affects systemic and intrarenal exposure to tacrolimus and its metabolites. The local expression of CYP3A and PGP potentially modulates the risk of CNI-induced renal injury.

CYP3A5 is the predominant CYP3A isoform in renal tubular cells, with a primarily intracytoplasmic location of the enzyme[110]. Immunohistochemical analysis demonstrated its confinement to the proximal tubular cells[111], and a strong genotype-phenotype correlation between CYP3A5 genotype and its renal mRNA and protein expression levels, with more than 18-fold higher mean renal mRNA expression in

carriers of the CYP3A5*1 allele compared to CYP3A5*3/*3 carriers. Zheng et al[112] studied the effect of CYP3A5 genotype on intra-renal tacrolimus accumulation in healthy volunteers and reported that CYP3A5*1 genotype was associated with a greater extent of renal tacrolimus metabolism and a lower apparent urinary tacrolimus clearance. They also predicted, based on a semi-physiological model of renal tacrolimus disposition, that tacrolimus exposure in the renal epithelium of CYP3A5 nonexpressors is almost double that of CYP3A5 expressors, suggesting that both hepatic and renal CYP3A5 genotypes will determine tacrolimus intra-renal accumulation[13].

PGP is located on the brush border membrane of proximal tubular cells and facilitates active excretion of xenobiotics, including CNIs[113]. Theoretically, decreased expression or altered function of PGP could increase the nephrotoxic effects of tacrolimus by resulting in increased local accumulation of the drug in renal tubules.

Reduced intrarenal CYP3A5 expression has been suggested to increase the risk of nephrotoxicity in patients receiving CNIs[34, 114] and lower ABCB1 expression in renal tubular epithelial cells has been shown to be a risk factor for chronic histological damage in renal allografts[65]. Our hypothesis is that polymorphisms in the genes of the recipient allow for increased intra-renal exposure to tacrolimus or its metabolites leading to nephrotoxicity. Our specific aim in this study is to identify whether polymorphisms in CYP3A4, CYP3A5, and ABCB1 genes, that have previously been shown to impact tacrolimus dose requirements and modulate risk for nephrotoxicity, would be associated with the development of renal dysfunction following liver transplantation.

Methods

Patients

All subjects who received a liver transplant at UIHC liver transplant clinics between 1990 and 2009 eligible for study enrollment. Liver transplant recipients that were actively being followed by the transplant program were invited to participate in the

study. All study participants provided written informed consent. A retrospective review of the liver transplant database was conducted, and clinical data were evaluated. Patient information collected included: sex, age, gender, etiology of liver disease (hepatitis B, hepatitis C, autoimmune hepatitis, Laennec's disease, primary biliary cirrhosis (PBC), primary schlerosing cholangitis (PSC), fulminant hepatic failure, non-alcoholic steatohepatitis (NASH), hepatocellular carcinoma (HCC). The Model for End-Stage Liver Disease (MELD) score, pre-transplant co-morbidity (hypertension (HTN), diabetes mellitus (DM), dyslipidemia), baseline estimated glomerular filtration rate (eGFR), acute cellular rejection (ACR), hepatorenal syndrome (HRS) and immunosuppressive regimens (primary immunosuppression with tacrolimus or cyclosporine, and secondary immunosuppression with sirolimus, azathioprine or mycophenolate mofetil) were also collected. All data were collected pre-transplant, 1, 2, 3, 6 and 12 months post-transplant and every year thereafter for up to 19 years. Estimated GFR was calculated using Cockroft-Gault equation. Renal dysfunction was defined as eGFR < 60 ml/min.

Genotyping Analysis

Saliva was obtained from each subject and deoxyribonucleic acid (DNA) was extracted using the QIAamp DNA mini kit (Qiagen, Valencia, CA). Polymerase chain reaction (PCR) amplification and sequencing of the region of ABCB1, CYP3A4 and CYP3A5 genes containing the polymorphisms were carried out as described previously[74, 115] with minor modification.

Sequencing of ABCB1 and CYP3A5

Sequences of the primers and their annealing temperatures are given in table 6. PCR was carried out in a total volume of 25 µL using 50 ng of genomic DNA, 0.4 µM of each forward and reverse primer, 0.2 mM deoxynucleoside triphosphate (dNTP) (New England Biolabs, Ipswich, MA), 1X PCR buffer, and 1.0 unit of Taq DNA polymerase (New England Biolabs, Ipswich, MA). Amplification of gene fragments was carried out

on a Genius Techne (Techne Inc, UK). The unincorporated nucleotides and primers were removed by incubation with Antarctic phosphatase (New England Biolabs, Ispwich, MA) and exonuclease I (New England Biolabs, Ipswich, MA) for 30 minutes at 37°C followed by enzyme inactivation at 80°C for 15 minutes prior to sequencing. Sequencing was carried out on an Applied Biosystems Model 3730 through the University of Iowa Core DNA facility using the PCR primers for ABCB1 and nested primers for CYP3A5.

Sequencing of CYP3A4

Allelic discrimination analysis for the determination of CYP3A4*22 genotype was performed using TaqMan genotyping assay C_59013445_10 (Applied Biosystems, CA).

Statistical Analysis

Genotype groups were compared using Mann-Whitney U test. Continuous variables were analyzed by linear regression with corrections for multiple testing. Categorical, dichotomous and ordinal variables were analyzed by parametric or nonparametric tests as appropriate (Fisher's exact test, Chi-square test and Cochran Mantel Haenzel test).

CYP3A4/5 genotypes were evaluated by dividing patients into three groups: poor metabolizers (PM) who were CYP3A5 nonexpressors and carriers of the CYP3A4 intron 6 T variant, intermediate metabolizers (IM) who were CYP3A5 nonexpressors and carriers of the CYP3A4 intron 6 CC genotype or CYP3A5 expressors and carriers of CYP3A5 intron 6 variant allele, and extensive metabolizers (EM) who were CYP3A5 expressors and carriers of the CYP3A4 intron 6 CC genotype (Table 7).

Survival analysis for time to develop renal dysfunction (eGFR < 60 ml/min) during 60 months of follow-up following transplantation was performed using Kaplan-Meier and log-rank tests. Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium testing were performed using Statistical Genetics Utility programs.

Initially, univariate analyses were performed using Kaplan-Meier survival curves and log-rank tests for categorical predictors to screen for parameters that affect time to develop renal dysfucntion (eGFR< 60 ml/min) during 60 months of follow-up following transplantation, in order to include them in a multivariate Cox proportional hazards model. Three patients who were on dialysis prior to transplant were excluded from the analysis. The following variables were considered for evaluation in the univariate analysis: Gender, Age, CYP3A5 genotype, CYP3A4*22 genotype, primary diagnosis, ACR, HRS, primary immunosuppressant (tacrolimus versus cyclosporine), adjuvant immune-suppression, co-morbidities (HTN, DM, dyslipidemia) and whether the patient was on dialysis prior to OLT.

The continuous variables: age, MELD score and pre-OLT serum creatinine level were evaluated using univariate Cox regression analysis, and all variables that affected the incidence of eGFR<60 ml/min in the univariate analysis with a p-value of <0.1 were included in a multivariate Cox proportional hazards model using a backward elimination procedure and a significance level of <0.05. Data were censored at the end of follow-up. The proportional hazard assumption was assessed graphically for each potential predictor by examining the plot of the log (−log) survival function versus time and by including a time-dependent component for each covariate individually.

Results are expressed as mean ± SD unless noted otherwise and a two-sided p-value of <0.05 was considered statistically significant. Patients with missing values were excluded from the analysis. Data analysis was performed using SAS 9.3 software (SAS Institute Inc, Cary, NC).

Results

Patient Demographics

This study enrolled 146 patients with a mean age of 53.2 years. They were primarily Caucasian (95%) and 37% were females. Patient demographics are presented in

Table 8. A total of 87% were receiving tacrolimus, 43% on mycophenolate mofetil, 7% on daclizumab, 4% on sirolimus and 3% on azathioprine.

The incidence of DM, HTN and dyslipidemia was not significantly different between CYP3A5 expressers and non-expressers. Mean follow-up time was 98.2 months (with a follow-up range of 7- 240 months) and it did not differ significantly between CYP3A5 genotypes.

Genotype Frequencies of CYP3A5 G6986A, CYP3A4 intron 6, ABCB1 C1236T, ABCB1 G2677T/A and ABCB1 C3435T Single Nucleotide Polymorphisms

When considering the CYP3A4*22 allele, 122 patients of the 135 included participants were homozygote wild type (CYP3A4*1/*1). The remaining 13 patients were heterozygote CYP3A4*1/*22, yielding a variant allele frequency of 4.8%. No homozygous CYP3A4*22/*22 were detected and the genotype distribution did not deviate from HWE (χ^2 = 0.658, P = 0.417). For the CYP3A5*3 allele, 22 of 136 patients were heterozygote CYP3A5*1/*3, and 114 patients were CYP3A5*3/*3, giving a variant allele frequency of 91.9%. This genotype distribution did not deviate from the distribution predicted by the HWE (χ^2 = 1.939, P = 0.164).Our population genotype distribution was also in HWE for ABCB1 exon 12 (1236 C>T), exon 21 (2677 G>T, A) and exon 26 (3435 C>T). Variant allele frequencies and genotype distributions for all SNPs in the study population are shown in table 9.

Pairwise linkage disequilibrium was calculated for all SNPs. Strong linkage disequilibrium was observed between ABCB1 G2677T and C1236T SNPs (D'=0.84, r^2=0.68) and a moderately strong linkage disequilibrium was observed between ABCB1 C3435T and C1236T SNPs (D'=0.73, r^2=0.42), ABCB1 G2677T and C3435T SNPs (D'=0.79, r^2=0.5).

Effect of CYP3A5 Polymorphisms on Tacrolimus Dose Requirements

Cutoff values for tacrolimus dose ranges were defined as <0.1, 0.1≤ dose ≤0.2 and > 0.2 mg/kg/d. At two weeks post-transplant, the GG (non-expressor) genotype was 3.85 times more likely (OR 95% CL 0.988-14.97) to require a low dose (< 0.1 mg/kg/d) compared to GA (expressor) genotype (p=0.0418). The difference was not significant at 1 month post-transplant.

Effect of Combined CYP3A4/3A5 Genotype on Tacrolimus Dose Requirements

Upon combining CYP3A4 and CYP3A5 genotypes, EM tended to require higher doses of tacrolimus at three months post-transplant (36% of EM in the high dose group compared to 12.5% and 9.7% in the low and mid dose groups, respectively) but the effect was only marginally significant (Fisher's p-value=0.05).

Effect of ABCB1 Polymorphisms on Tacrolimus Dose Requirements

Tacrolimus dose at 1 and 3 months post-transplant did not differ significantly across ABCB1 2677, 3435 and 1236 genotypes in CYP3A5 non-expressors (results not shown). There was also no significant difference in tacrolimus dose at 1 and 3 months post-transplant between ABCB1 CGC-CGC and TTT-TTT haplotypes (p-value= 0.5895) in CYP3A5 non-expressors.

Effect of Higher Dose Requirement on the Risk of Developing Renal Dysfunction

Liver transplant recipients requiring higher tacrolimus doses (> 0.2 mg/kg) at three months post-transplant were 5.9 times more likely to have eGFR<60ml/min compared to those receiving lower doses (<0.2 mg/kg) (OR 95% CI 1.19-29.25, χ^2 p-

value = 0.0176). The effect of higher tacrolimus dose requirements on the incidence of eGFR < 60 ml/min was no longer significant at one year following transplantation.

Association of Renal Dysfunction with CYP3A5 Genotype

There was no difference in dose-normalized tacrolimus exposure at one year following OLT between CYP3A5 genotypes (Wilcoxon's p-value=0.073, table 10)

Liver transplant recipients with the CYP3A5*1/*3 genotype were 3.5 times more likely to have eGFR<60ml/min one year post transplantation compared to those with the CYP3A5*3/*3 genotype (OR 95% CI 1.2-10.22, χ^2 p-value = 0.0167, table 11). They were also 4.04 more likely to have serum creatinine (SrCr) ≥ 2mg/dl at one year post transplantation (OR 95% CI 1.2-12.74, Fisher's p-value=0.0221, table 12).

For each ABCB1 SNP, genotypes with at least one wild-type allele were combined and compared to the homozygous mutant genotype. None of the studied ABCB1 polymorphisms (1236 C>T, 2677 G>T, A and 3435 C>T) were individually associated with eGFR < 60 ml/min or SrCr ≥ 2.0 mg/dl at one year post transplant in CYP3A5 non-expressors.

In univariate analysis, CYP3A5 genotype was a significant predictor of the cumulative incidence of eGFR<60 ml/min during 60 months of follow-up following OLT (p-value= 0.04, figure 7), with the CYP3A5*1/*3 genotype significantly associated with increased incidence of eGFR<60 ml/min following OLT.

Based on the results from the univariate analyses, the following variables were considered for inclusion in multivariate Cox regression analysis: age, gender, pre-SrCr, ACR, dyslipidemia and CYP3A5 genotype (table 13). CYP3A5 genotype was found to violate the proportional hazard assumption and this was addressed by inclusion of a time-dependent component of that covariate in a multivariate Cox proportional hazards analysis. In a multivariate Cox proportional hazards model that included age, gender, pre-SrCr levels, CYP3A5 genotype and accounting for the fact that CYP3A5 genotype does

not satisfy the proportional hazards assumption, CYP3A5 expressors were at 4.1992 the risk of developing renal dysfunction compared to non-expressors (p-value= 0.0103, 95% CI =1.3975- 12.6177) starting at one month post transplantation (table 14).

Association of Renal Dysfunction and CYP3A4 genotype

There was no difference in dose-normalized tacrolimus exposure between CYP3A4 genotypes at one year following OLT (Wilcoxon's p-value= 0.3972). In univariate analysis, CYP3A4 genotype was not a significant predictor of the cumulative incidence of eGFR<60 ml/min during 60 months of follow-up following OLT (log-rank p-value 0.912, figure 8)

Effect of Combined CYP3A4/CYP3A5 Genotype on Renal Dysfunction

There was no difference in time to develop renal dysfunction (CrCl<60 ml/min) among extensive, intermediate and poor metabolizers (log-rank p-value= 0.1386). Once the PM and IM groups were combined, and compared against EM, there was a statistically significant difference in time to developing renal dysfunction (log-rank p-value=0.0466), with EM requiring significantly shorter times to develop renal dysfunction (figure 9). As with CYP3A5 genotype, CYP3A4/5 combined genotype was also found to violate the proportional hazard assumption in a multivariate Cox proportional hazards analysis and a time-dependent component of that covariate was included in the model. In a multivariate Cox proportional hazards model that included age, gender, pre-SrCr levels, combined CYP3A4/5 genotype (EM versus the remaining genotype groups), and accounting for the fact that CYP3A4/5 genotype does not satisfy the proportional hazards assumption, the EM genotype carriers were at 4.01 times the risk of developing renal dysfunction compared to non-expressors (95% CI =0.5737-2.9401) starting at one month following OLT (table 15).

Discussion

In liver transplant patients, both recipient (intestine) and donor (liver) CYP3A5 genotypes contribute to inter-individual variability in tacrolimus pharmacokinetics[116]. We examined the effect of recipient CYP3A5 (intestine) genotype on tacrolimus dose requirements post-transplantation. Liver transplant patients who are carriers of the CYP3A5 non-expressor genotype were significantly more likely to require lower doses of tacrolimus at two weeks post-operatively compared to CYP3A5 expressors. This may be due to a larger effect of recipient (intestine) rather than donor (liver) CYP3A5 genotype on tacrolimus dose requirements during the early post-operative phase until a recovery of the transplanted liver metabolic function occurs. This finding is consistent with a previous observation[63] where the intestinal CYP3A5*1 allele was reported to significantly correlate with an increase in dose requirements and reduced C/D ratio of tacrolimus at one and two weeks postoperatively. This finding supports a more important role for intestinal rather than hepatic CYP3A5 in tacrolimus first-pass metabolism immediately after living-donor liver transplant (LDLT). Moreover, studies evaluating the effect of intestinal CYP3A5 on postoperative tacrolimus trough levels in LDLT found that in patients with the same donor (hepatic) CYP3A5 genotype, those with an intestinal CYP3A5*1 allele tended to require a higher dose of tacrolimus compared to those who lack intestinal CYP3A5 expression[116].

Similar tacrolimus exposure was achieved in CYP3A5 expressor and non-expressor genotype groups, nonetheless, patients with CYP3A5 expressor genotype were more likely to have eGFR< 60 ml/min and SrCr ≥ 2.0 mg/dl at one year post-transplant compared to non-expressors. This suggests that the presence of a CYP3A5*1 allele in the kidney results in higher renal tissue concentrations of CNI metabolites which could be responsible for the increased susceptibility to nephrotoxicity in CYP3A5 expressors. It has been shown that CYP3A5*1 genotype, and high-renal-expression phenotype, are associated with a greater extent of renal tacrolimus metabolism and a lower apparent

urinary tacrolimus clearance as compared with subjects lacking enzyme expression. Although the nephrotoxic potential of tacrolimus metabolites has not been examined, it has been suggested by several studies[42, 43] which report an increased risk of developing CNIT in renal transplant recipients carrying the CYP3A5 expressor genotype. Recipient CYP3A5 genotype in renal transplant patients reflects intestinal and hepatic levels of the enzyme, which will be elevated in CYP3A5 expressors resulting in higher systemic concentrations and renal accumulation of tacrolimus metabolites. The donor kidney CYP3A5 genotype in these studies was not determined.

In liver transplant patients, Fuduko et al[63] investigated the effect of recipient CYP3A5 genotype on the incidence of renal dysfunction and found opposite results, with a significantly lower incidence of renal dysfunction in CYP3A5 expressors, suggesting that CYP3A5 expression in the kidney plays a protective role in the development of renal dysfunction, probably by reducing exposure of renal cells to tacrolimus. This suggests that exposure to the parent drug, rather than the metabolites, modulates the risk of nephrotoxicity. More recently, Shi et al[47] also reported that the non-expressor CYP3A5 genotype was associated with higher urine transferrin levels and a higher risk for early renal injury in Chinese liver transplant recipients. This discrepancy with the current findings may be partially explained by varying definitions of renal dysfunction used in these studies. The lack of an association between ABCB1 SNPs and the risk of renal dysfunction following liver transplantation, however, was a unanimous finding.

Our data suggest that the prognostic ability of CYP3A5 genotype varies with time for our cohort, violating the proportional hazards assumption for Cox regression. CYP3A5 expressor genotype has a non-significant lower hazard for renal dysfunction at time of transplant, which increases linearly each month resulting in a significantly higher risk to develop renal dysfunction in CYP3A5 expressors starting from one month post-transplant compared to non-expressors. In the early period following transplantation, patients undergo extensive immunosuppression with high dose corticosteroids, resulting

in CYP3A4 induction and increased tacrolimus clearance, possibly masking the prognostic value of CYP3A5 genotype in predicting the risk of renal dysfunction due to renal accumulation of tacrolimus metabolites until a more stable phase is achieved. A possible effect of donor liver genotype and whether the CYP3A5 genotypes of the donor and the recipient were concordant or discordant may also contribute to this observation.

Based on the current findings, age at time of transplant is an important predictor of renal function in liver transplant patients. Normal aging processes and co-existing medical conditions might accelerate progression to renal dysfunction in the transplant patient. Gender was also an important predictor of renal dysfunction in this patient cohort, with females having double the risk for renal dysfunction compared to males. Female gender has been previously reported[117] to be a significant predictor of severe chronic kidney disease in an Australian OLT population (OR=29.6, 95% CI=2.1– 407, P=0.01). It was also found that every unit increase in pre-transplant serum creatinine levels in this patient cohort is associated with a 40% increase in the hazard of developing renal dysfunction. However, when eGFR is used an indirect measure of kidney function, the effect of risk factors that are also incorporated in the estimates of the outcome variable should be interpreted with caution.

Similar results were obtained when the CYP3A5 genotype was replaced with the CYP3A4/5 combined genotype (EM versus other), where the combined genotype was also found to violate the proportional hazards assumption and had a prognostic ability that also varies with time for this patient cohort resulting in a significantly higher risk to develop renal dysfunction in EM (CYP3A5 expressors and carriers of the CYP3A4 intron 6 CC wild-type genotype) starting at one month post-transplant compared to the other groups.

Our current study has several limitations that preclude drawing firm conclusions from our data. Donor CYP3A5 genotyping, which reflects hepatic levels of the enzyme that would largely affect tacrolimus blood concentrations and dose requirements, was not

performed in this study. However, all patients had their tacrolimus dose adjusted to achieve a target trough concentration. In addition, since no routine kidney biopsies are performed in the non-renal transplant setting, our definition of renal dysfunction is not based on histologic findings and does not prove a causal role of the studied polymorphisms on CNI-induced renal injury. Thirdly, tissue levels of tacrolimus and its metabolites were not determined, and further studies investigating local accumulation of the drug and its metabolites are needed to clarify and properly identify the role of genetic variation in modulating the risk of nephrotoxicity in organ transplantation.

Conclusion

In conclusion, this study has demonstrated that CYP3A5 genotype *1/*3, which predicts increased renal tubular CYP3A5 expression, and a combined CYP3A4/5 high expressor genotype, predispose patients to CNI-induced renal dysfunction following liver transplantation in a time-sensitive manner. Prospective genotyping in the transplant population may help improve transplant outcomes and safety profiles of immunosuppressive regimens.

Figure 7 Cumulative incidence of renal dysfunction (eGFR <60 ml/min) according to recipient CYP3A5 genotype of liver transplant patients (P-value= 0.04)

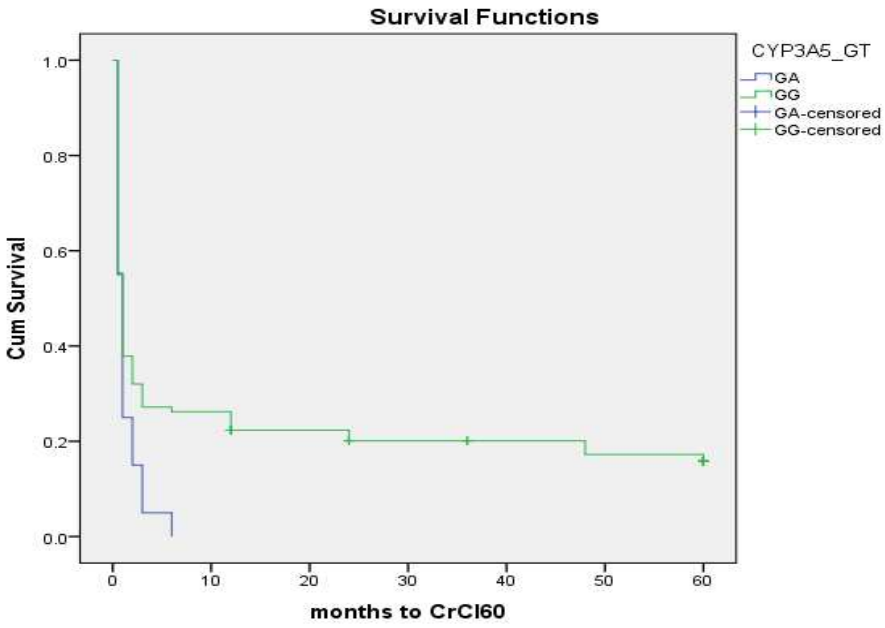


Figure 8 Cumulative incidence of renal dysfunction (eGFR < 60 ml/min) according to recipient CYP3A4 genotype of liver transplant patients (P-value = 0.912)

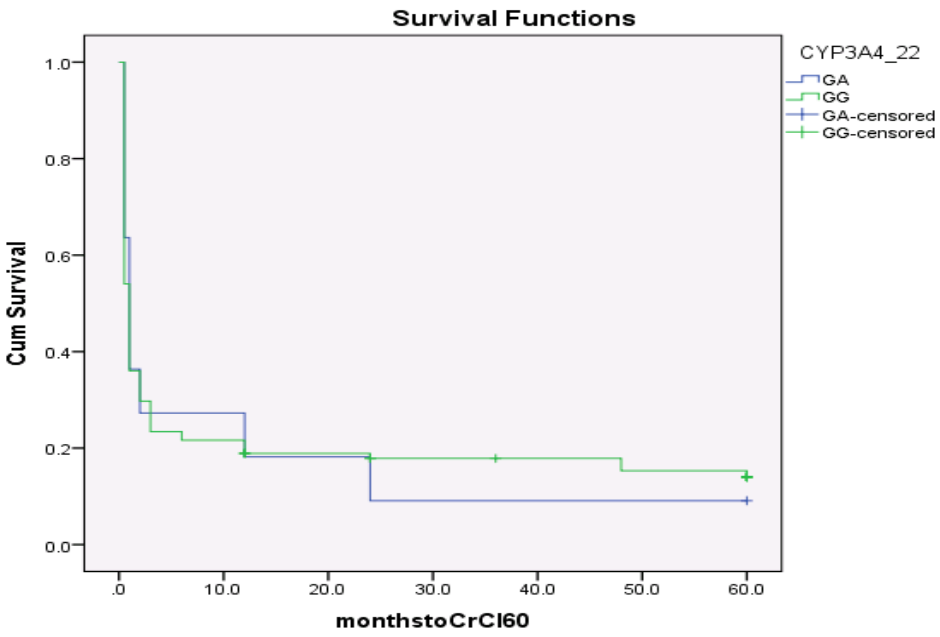


Figure 9 Cumulative incidence of renal dysfunction according to combined CYP3A4/5 genotype of liver transplant patients (group 1 = EM, group 2 = PM + IM, P-value = 0.047)

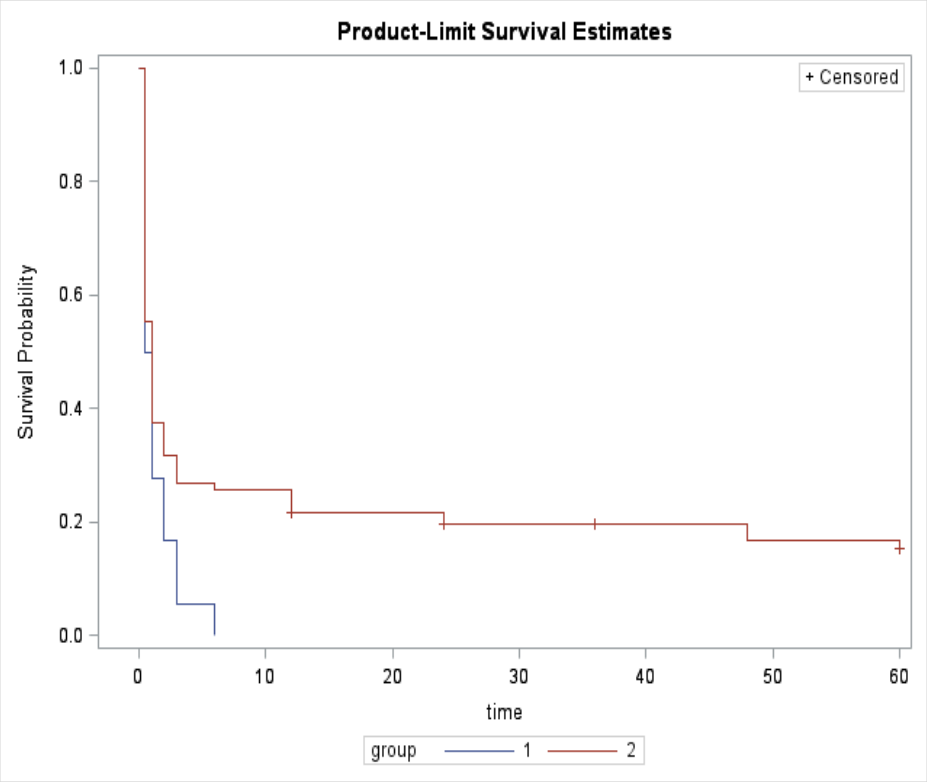


Table 6 Primer sequences used for PCR amplification of *ABCB1* and *CYP3A5* genes

SNP	Primer	Sequence	Annealing Temp (° C)
ABCB1 C1236T rs1128503	Forward	5’- TCCTGTGTCTGTGAATTGCCTTG-3’	55
	Reverse	5’-GCTGATCACCGCAGTCTAGCTGCG-3’	
ABCB1 G2677T/A rs2032582	Forward	5′-GCAGGCTATAGGTTCCAGGCT-3′	59
	Reverse	5′-TGAGGAATGGTTATAAACACAT-3′	
ABCB1 C3435T rs1045642	Forward	5′-TCACAGTAACTTGGCAGTTTCAG-3′	58
	Reverse	5′-ACTATAGGCCAGAGAGGCTG-3′	
CYP3A5 G6986A rs776746	Forward	5′-CCTGCCTTCAATTTTTCACTG-3′	61
	Reverse	5′-GCAATGTAGGAAGGAGGGCT-3′	
	Nested (F)	5′-CCTGCCTTCAATTTTTCACTG-3′	
	Nested (R)	5′-CATTCTTTCACTAGCACTGTTC-3	

Table 7 CYP3A4/CYP3A5 combined genotype groups

CYP3A5	CYP3A4	
	CT	CC
GG	PM (n=10)	IM (n=91)
GA	IM (n=1)	EM (n=18)

Abbreviations: PM = poor metabolizers, IM: intermediate metabolizers, and EM: extensive metabolizers.

Table 8 Patient demographics

Demographics	All Patients	CYP3A5*1/*3 Genotype	CYP3A5*3/*3 Genotype	P-value
	(n=136)	(n= 22)	(n= 114)	
Age (years)	53.21±11.01	55.56±10.89	52.76±11.02	0.1708
Sex (M/F)	85/51	14/8	71/43	0.9043
Weight (kg)	88.11±21.79	83.96±16.07	88.91±22.7	0.3643
DM (%)	34 (25)	9 (41)	25 (22)	0.0598
HTN (%)	68 (50)	15 (68)	53 (46)	0.0625
LIPID (%)	22(16)	5 (23)	17 (15)	0.3539
FU time (m)	98.23±58.91	113.7±52.35	93.36±59.91	0.14
Pre-SrCr (mg/dl)	1.38±1.04	1.29±0.43	1.4± 1.12	0.5311
Pre-eGFR (ml/min)	74.68±30.44	67.27±22.7	75.8±32.16	0.2375

Table 9 Frequency and distribution of the studied polymorphisms in the study population

Gene	Polymorphism	Variant allele freq	Genotype distributions		
			wt/wt	wt/mt	mt/mt
ABCB1	1236C>T	0.447	39/142	79/142	24/142
	2677 G>T	0.461	40/140	71/140	29/140
	3435 C>T	0.515	32/135	67/135	36/135
CYP3A5	6986A>G	0.919	0/136	22/136	114/136
CYP3A4	Intron 6 C>T	0.048	122/135	13/135	0/135

Table 10 Dose-normalized tacrolimus exposure according to CYP3A5 genotype

CYP3A5 genotype	n	mean Level (ng/ml)	Mean Dose (mg/kg/d)	C/D ratio
GG	69	10.87	0.1032	153.5 ± 123.8
GA	14	9.32	0.1388	119.5 ± 123.9

Table 11 Incidence of eGFR<60 ml/min at one year post transplant according to CYP3A5 genotype

CYP3A5 genotype	eGFR<60 ml/min at one year	
	Yes (%)	No (%)
GA	18 (85.71)	3 (14.29)
GG	69 (62.16)	42 (37.84)

Table 12 Incidence of SrCr≥ 2.0 mg/dl at one year post transplant according to CYP3A5 genotype

CYP3A5 genotype	SrCr≥ 2.0 mg/dl at one year	
	Yes (%)	No (%)
GA	6 (28.6)	15 (71.4)
GG	10 (9.01)	101 (90.99)

Table 13 Results of univariate analysis on time to develop renal dysfunction during 60 months following OLT

Risk Factor	P-value
Age	0.0022
CYP3A5 genotype	0.0400
Gender	0.0143
Pre-SrCr	0.0522
ACR	0.0803
Dyslipidemia	0.0932

Table 14 Multivariate Cox Proportional Hazards Model of risk factors for renal dysfunction (Total number of events= 102)

Variable	Hazard Ratio (95% CI)	P-value
Per unit increase in pre-SrCr (mg/dL)	1.377 (1.011-1.874)	0.0423
Per additional year of age	1.038 (1.016-1.060)	0.0005
CYP3A5 GA genotype	0.757 (0.338-1.695)	0.1111
CYP3A5 GA genotype < 1 m	1.230 (0.682-2.218)	0.4920
CYP3A5 GA genotype > 1 m	4.199 (1.398-12.618)	0.0106
Female gender	1.977 (1.275-3.066)	0.0022

Table 15 Multivariate Cox Proportional Hazards Model of risk factors for renal dysfunction

Variable	Hazard Ratio (95% CI)	P-value
Per unit increase in pre-SrCr (mg/dL)	1.384 (1.013-1.890)	0.0414
Per additional year of age	1.039 (1.017-1.061)	0.0004
CYP3A4/5 EM genotype	0.800 (0.348-1.840)	0.5997
CYP3A4/5 EM genotype < 1 m	1.224 (0.655-2.287)	0.5257
CYP3A4/5 EM genotype > 1 m	4.0052 (1.334-12.025)	0.0134
Female gender	1.890 (1.215-2.938)	0.0047

CHAPTER 4

DEVELOPMENT AND VALIDATION OF AF LIQUID CHROMATOGRAPHY-MASS SPECTROMETRIC ASSAY FOR SIMULTANEOUS DETERMINATION OF TACROLIMUS AND 13-O-DESMETHYL TACROLIMUS IN RAT AND HUMAN KIDNEY TISSUE

Introduction

Therapeutic drug monitoring (TDM) of immunosuppressants is critical to achieving optimal patient care following transplantation because of their narrow therapeutic index and significant variability in blood concentrations between individuals. The analytical methods available for monitoring tacrolimus levels are divided into two categories: immunoassays and liquid chromatography-based methods. High performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) has become an increasingly important tool for TDM of immunosuppressants in the past years due to its high specificity and sensitivity compared to other available analytical methodologies, including immunoassays, which tend to overestimate drug concentrations due to nonspecific cross-reaction from their metabolites[118].

Despite TDM, CNIT is one of the main factors contributing to long-term kidney allograft loss and limits the use of CNIs in transplantation. A number of studies[42, 43] have investigated the effect of genetic polymorphisms in the CYP3A4 and CYP3A5 genes on the development of CNIT and have implicated local tissue levels of tacrolimus metabolites in increasing the risk for CNIT development. Our results in chapter 3 demonstrate that the CYP3A5 expressor genotype of the recipient, both alone and in combination with CYP3A4 expressor genotype, are associated with an increased risk of CNI-induced renal dysfunction following liver transplantation. In a study by Kuypers et al, CYP3A5 expressor genotype of the recipient was also associated with the development of biopsy proven nephrotoxicity secondary to tacrolimus therapy in renal

transplantation[43] suggesting that the presence of a CYP3A5*1 allele leads to higher drug clearance, higher dose requirements and possibly higher local tissue concentrations of tacrolimus metabolites. Intra-renal drug concentrations were not determined in these studies.

At least 8 different metabolites for tacrolimus have been identified[24], four of which are primary metabolites; 13-, 31- and 15-monodesmethylated and 12-hydroxylated tacrolimus. These in turn, undergo further metabolism to form secondary metabolites[22]. The major metabolite in human liver microsomes was found to be 13-ODMT[119] which retains around 10% of tacrolimus immunosuppressant activity[22]. A number of LC-MS/MS methods have been described to determine tacrolimus concentration in human whole blood and liver tissue[120]. Capron et al[120] reported an HPLC-MS/MS method to quantify tacrolimus in liver biopsies after hepatic transplantation to evaluate the predictive value of tissue or blood tacrolimus for rejection. Accurate measurement of tacrolimus and its metabolites in renal tissue is essential to support the hypothesis of their contribution to the risk of CNIT development.

Our hypothesis is that the extent of tacrolimus and 13-ODMT accumulation in rat kidney tissue will vary according to local metabolism and transport mechanisms and will not depend on the administered tacrolimus dose. Our specific aim in this study is to establish a sensitive and robust LC-MS/MS method for the determination of tacrolimus and 13-ODMT in rat and human kidney tissues and to apply this method to study the accumulation of these analytes in kidney tissues of rats receiving increasing doses of tacrolimus intra-peritoneally and for the evaluation of biopsy proven CNIT in renal transplant recipients.

Materials and methods

Chemicals and Reagents

Tacrolimus and the internal standard (IS) ascomycin were purchased from Cerilliant (Cerilliant Corp., Round Rock, TX).13-ODMT was provided by Astellas (Osaka, Japan). Chemical structures are provided in figure 10. Zinc sulfate heptahydrate, acetonitrile (ACN), methanol and ethyl ether were obtained from Fischer Scientific (Fair Lawn, NJ, USA). Formic acid (≥88%) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Analytical-grade water was produced by Milli-Q Plus water system (Millipore Corporation, Bedford, MA, USA).

Preparation of Standard and Quality Control Samples

Stock solutions of tacrolimus and 13-ODMT were prepared by dissolving an accurately weighed amount of drug in ACN. Appropriate amounts of tacrolimus and 13-ODMT were weighed on a Mettler Toledo AG 104 analytical balance (Mettler Toledo Inc., Hightstown, NJ, USA) and dissolved in ACN, to make a 1 mg/mL stock solution of each drug. Fresh stock samples were prepared by further diluting both drugs with ACN to obtain working solutions at 10 ng/ml, 100 ng/ml and 1µg/ml to spike tissue homogenates. A stock solution of 1 mg/mL IS in ACN was prepared and further diluted to reach a 1 µg/mL working solution. All stock solutions were stored at -70°C. Standard and quality control samples were prepared by spiking tissue homogenates with appropriate amounts of working solutions (table 16, 17).

Rat Study

Young (4 to 8-week old) male Sprague Dawley rats (200-250 g body weight) were given tacrolimus intraperitoneally in doses of 0.5 mg/kg (n=3) and 2 mg/kg (n=3). Four hours following dosing, rats were anesthetized by pentobarbital (150 mg/kg), and kidneys were removed and snap frozen in liquid nitrogen. Animal procedures were

performed according to the Institutional Animal Care and Use Committee (IACUC) guidelines at the University of Iowa (protocol number 0911253).

Tacrolimus Extraction from Rat Kidney Tissue

Samples were prepared using a procedure previously described by Qin et al[121] with several modifications. Briefly, frozen rat kidneys were thawed at room temperature and accurately weighed. They were homogenized with analytical grade water at a ratio of 1:2 to obtain a concentration of 0.35 mg of tissue per µL. Then, 20 µL of 1µg/ml IS was added to 142.5 µl tissue homogenate (containing 50 mg of tissue) and vortexed for 30 seconds followed by the addition of 400 µl of 0.1 M $ZnSO_4$ solution and 400 µl of ACN and vortex mixing for 1 minute. The samples were allowed to stand at room temperature for 10 minutes then centrifuged at 2500 rpm for 5 minutes. Clear supernatant was extracted with 1.5 ml diethyl ether (100%) and vortexed for 1 minute. After standing at room temperature for 10 minutes and centrifuging at 2500 rpm for 10 min, the organic phase was transferred to a new glass tube (10X13mm) and evaporated under stream of nitrogen at 20º C. Dry residues were reconstituted in 1000 µl mobile phase and 100 µl of the reconstituted solution were transferred into an LC-MS vial. A 10 µl aliquot of the solution was injected into the LCMS-Ion Trap (IT)-Time of Flight (TOF) (Shimadzu, Columbia, MD, USA) system.

Instrumentation and Assay

LC-MS-IT-TOF System and Conditions

An LCMS-IT-TOF system operated in ESI mode was used for analysis of tacrolimus and 13-ODMT in rat kidney tissue. Separation was done through a Phenomenex Kinetex column (2.6 µm C18 100 Å, 100 X 2.1 mm, Phenomenex, Torrance CA) and a guard column, maintained at 55ºC. 10 µL of the reconstituted solution was injected into the system and eluted at a flow rate of 0.3 mL/min. Mobile phase was a 1:1

mixture of organic and aqueous phases, both containing 0.1% formic acid. The organic phase was an equal mixture of acetonitrile and methanol and the aqueous phase was HPLC grade water. Organic phase percentage is increased from 50% at start to 90% at four minutes then gradually decreases to 50% from 6 to 6.5 minutes. The method had a total run time of 9 minutes, with retention times of 3.7 minutes for 13-ODMT and 5.1 minutes for both tacrolimus and the IS. Specific product ions resulting from the fragmentation of precursor ions of tacrolimus, 13-ODMT and IS were detected using Multiple Reaction Monitoring (MRM) acquisition mode (tacrolimus m/z 616.31, 13-ODMT m/z 602.29, IS m/z 604.3).

Nebulizing gas flow was 1.5 L/min and detector voltage was 1.7 kV. CDL, heat block and interface temperatures were 230°C, 230°C and 400°C, respectively.

Renal Tacrolimus LC-MS/MS Assay Validation

Selectivity

Six blank kidney tissue samples and six zero samples (blank samples fortified with the IS) obtained from six different rats were extracted and analyzed to assess for interferences with endogenous matrix components.

Calibration Curves

The Calibration curve covered the range from 0.25 to 25 ng/ml. Each calibration curve included a blank sample (drug-free homogenate sample without IS), a zero sample (drug-free homogenate sample with IS), and eight spiked samples at a concentration of: 0.25, 0.375, 0.5, 1, 2.5, 5, 20 and 25 ng/ml for tacrolimus and 13-ODMT. The peak area ratios of tacrolimus to IS and of 13-ODMT to IS were plotted against nominal drug concentrations to construct the calibration curves, which were obtained by weighted $1/X^2$ least-squares linear regression analysis.

Limits of Quantification and Detection

The lower limit of quantitation (LLOQ) is the minimum concentration at which the analyte can be reliably determined with precision and accuracy that do not exceed 20% variation from the nominal concentration and at which the response is at least 5 times that for the blank sample.

Accuracy and Precision

To assess the accuracy and precision of the method, five samples of controls at each concentration level of 0.75, 10, and 17.5 ng/ml were prepared and analyzed. Within-batch accuracy was calculated from the difference between the mean observed and nominal concentrations at a given concentration, and the mean had to be within 15% of the nominal concentration. Within-batch precision was determined by calculating the coefficient of variation which was accepted if it did not exceed 15%.

Extraction Efficiency

Extraction efficiency of tacrolimus and 13-ODMT from rat kidney tissue was determined by comparing area ratios of tacrolimus/IS and 13-ODMT/IS for extracted samples with unextracted standards in replicates of three at the three concentrations (0.75, 10, and 17.5 ng/ml). At each concentration level, six blank samples were prepared; three were fortified only with the IS and three others were fortified with tacrolimus, 13-ODMT and IS. Upon extraction, samples fortified with tacrolimus, 13-ODMT and IS were reconstituted with1000 µl mobile phase. Those fortified only with IS were reconstituted with 1000 µl mobile phase containing the respective nominal amount of tacrolimus and its metabolite. The latter were used as 100% controls.

Stability

Freeze-Thaw Stability

Five aliquots at each of the three quality control concentrations (0.75, 10, and 17.5 ng/ml) underwent three freeze-thaw cycles to calculate accuracy and precision in order to determine freeze-thaw stability for tacrolimus and 13-ODMT in rat tissue homogenate. Samples were stored at -70°C for 24 hours and thawed unassisted at room temperature. They were refrozen at the same temperature for 24 hours and this cycle was repeated two times after which analysis was performed.

Short-Term Temperature Stability

Spiked tissue homogenate samples in replicates of three at each quality control concentration were stored at room temperature for 6, 24 and 72 hours before being analyzed and their stability was tested by comparing their response with that of freshly prepared solutions.

Auto-Sampler Stability

Extracted samples in replicates of five at low, mid and high control concentrations were stored in the auto-sampler for 24 hours and they were analyzed against a calibration curve obtained from freshly prepared calibration standards.

Data Analysis

Data acquisition and analysis was performed using LCMS solution software version 3.5 SP2 (Shimadzu, Columbia, MD, USA).

Results

Analytical Performances

Selectivity

Analysis of a blank and zero samples from each of six different sources of the biological matrix showed no significant interference at the expected retention times of the analytes.

Linearity and Lower Limit of Quantitation

Three calibration curves were prepared and analyzed on three separate days. The curves were linear with a correlation coefficient greater than 0.99 (as calculated by weighted linear regression). Standards were between -14.44% and 5.77% deviation from nominal concentration for tacrolimus and between -13.96% and 8.9% for the metabolite. The LLOQ for tacrolimus and 13-ODMT was 0.25 ng/ml at which the response was > 5 times the blank response. Calibration curves for tacrolimus and 13-ODMT on day three are shown in figure 11.

Accuracy and Precision

Accuracy and precision data shows that intra- and interassay bias and coefficient of variation (CV) were <20% for LLOQ and <15% for quality control samples (table 18).

Extraction Efficiency

Mean extraction efficiency ranged from 67% to 74.9 % and from 66.7% to 78.4% for tacrolimus and 13-ODMT, respectively (table 19).

Stability

Freeze-Thaw Stability

Spiked tissue homogenates undergoing three freeze-thaw cycles had a CV of ≤7.98% and an accuracy range of 6.74% to 9.04% for tacrolimus and ≤14.45% CV and from1.85% to 9.24% accuracy for the metabolite (table 20). Both analytes are stable for three freeze-thaw cycles in rat kidney tissue homogenates.

Short-Term Temperature Stability

Tacrolimus and 13-ODMT were found to be stable in tissue homogenates only at ambient temperature for 6 hours as shown in table 21. Spiked tissue homogenate samples stored at room temperature for 6 hours had an accuracy of -5.03% and 7.13% for tacrolimus and of -3.23% and 5.95% for 13-ODMT with a CV of ≤ 10.7% and ≤ 9.54% for tacrolimus and 13-ODMT, respectively.

Spiked tissue homogenate samples stored at room temperature for 24 and 72 hours failed to meet acceptance criteria for precision and accuracy with a lower response at 24 and 72 hours compared to that at 6 hours, indicating possible degradation of the analytes at these storage conditions. The concentration of tacrolimus and 13-ODMT could not be reliably determined after storage at room temperature for 24 or 72 hours.

Auto-Sampler Stability

Quality control samples stored for 24 hours in the auto-sampler were within ± 15% deviation from nominal concentrations for precision and accuracy (table 22) when compared against freshly prepared standards. This indicates that extracted samples are stable over the longest expected run times for validation samples.

Rat Samples

Intra-renal concentrations of tacrolimus and 13-ODMT in rats receiving a single tacrolimus dose of 0.5 or 2 mg/kg intra-peritoneally were successfully determined (table

23). Median concentrations of 11.54 ng/ml for tacrolimus and 0.72 ng/ml for 13-ODMT were obtained at the 0.5 dose level, and median concentrations of 8.89 ng/ml and 1.5 ng/ml for tacrolimus and 13-ODMT, respectively, were observed at a dose level of 2 mg/kg (Representative chromatograms of a blank sample, a standard containing 0.25 ng/ml tacrolimus and 13-ODMT and a rat sample containing 11.79 ng/ml tacrolimus and 1.86 ng/ml are shown in figure 12)

Discussion

This is the first report of a validated LC-MS/MS method for the simultaneous determination of tacrolimus and its major metabolite, 13-ODMT, in rat kidney tissues.

One of the challenges encountered during the course of method development was the separation of phospholipids from the analytes of interest, which resulted in phospholipid build-up and loss of chromatographic reproducibility[122] . The issue was resolved by the addition of zinc sulfate salt[123], which allowed for successful precipitation and removal of phospholipids. In addition, the choice of ethyl ether as an extraction solvent helped minimize retention of phospholipids and their ion-suppression effects.

Another issue was the initial failure of 13-ODMT LLOQ samples to meet precision and accuracy requirements, although tacrolimus LLOQ and quality control samples were all within acceptable limits. A possible explanation is that tacrolimus and the internal standard ascomycin are structurally similar with compatible retention times of 5.1 minutes each, while 13-ODMT on the other hand had a different retention time of 3.7 minutes which may have maximized the effect of possible suppressors of ionization[123]. The addition of a washout step with isopropyl alcohol: tetrahydrofuran (90:10) in between runs has helped minimize build-up of ion-suppressing matrix components which seemed to have a more pronounced effect at lower analyte concentrations.

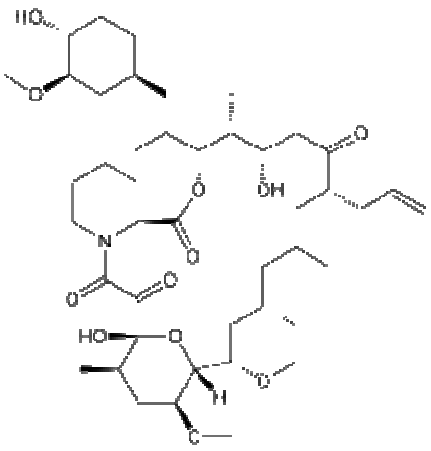
The rat study was performed on young (4 to 8-week old) male Sprague Dawley rats (200-250 g body weight) which were given tacrolimus intra-peritoneally in doses of 0.5 mg/kg (n=3) and 2 mg/kg (n=3). Our results show that a higher tacrolimus dose did not result in higher intra-renal tacrolimus concentration. Tacrolimus is a substrate of CYP3A2 enzyme and PGP in rats[124]. The lack of correlation between tacrolimus dose level and its renal tissue concentration requires further investigation in a larger group of animals to elucidate whether it is an artifact of a small sample size, or related to an underlying physiological mechanism such as up-regulation of transporter proteins. A study[125] in rats showed that daily subcutaneous injections of cyclosporine, a CNI, resulted in a dramatic increase in PGP expression levels in renal brush border membranes. A better correlation was observed between tacrolimus dose and intra-renal metabolite concentration compared to that with the parent drug. The metabolite concentration was at least twice as high for the higher tacrolimus dose level indicating a possible association between local metabolite levels and the toxic effects of the drug and although these results may not be extrapolated to local tacrolimus or metabolite accumulation in human kidneys, this is a proof of concept study that will allow us to use readily available rat kidney tissue in the validation and application of this method in human kidney biopsies, using human tissue controls against rat tissue standards.

Conclusion

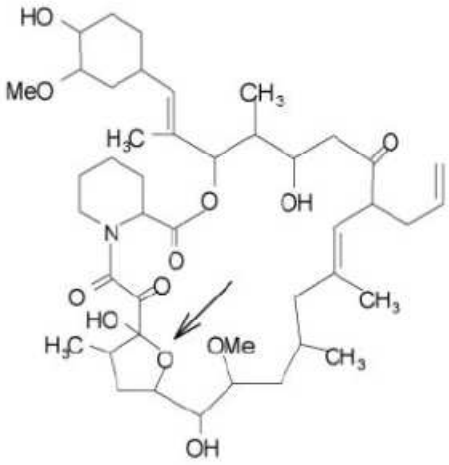
A sensitive and robust LC-MS/MS method was developed to simultaneously determine the concentrations of tacrolimus and its major metabolite in rat kidney tissue. Ion-suppression effects of phospholipids were successfully eliminated and the method was successfully applied to study the renal accumulation of tacrolimus and 13-ODMT in rats.

Figure 10 Chemical Structures of (a) Tacrolimus (b) IS and (c) 13-ODMT.

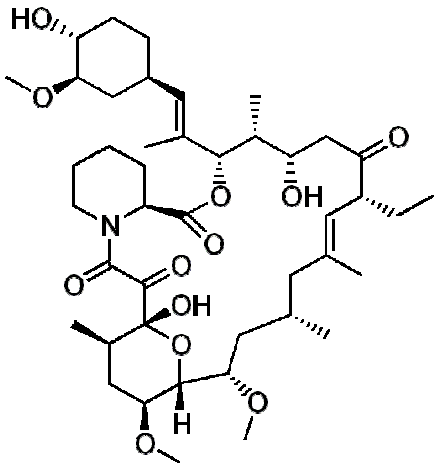
(a)



(c)



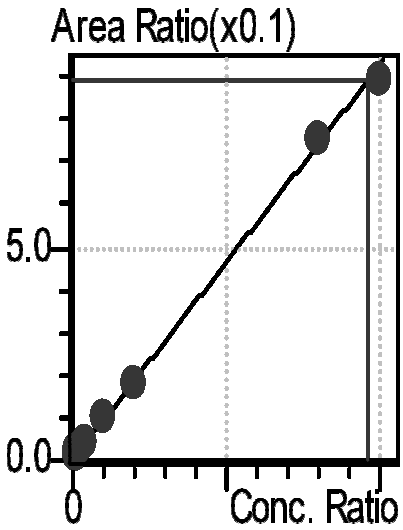
(b)



Source: G. L. Lensmyer and M. A. Poquette/ Ther Drug Monit, Vol. 23, No. 3, 2001

Figure 11 Day 3 calibration curves for a) Tacrolimus (r^2 =0.9958) and b) 13-ODMT (r^2=0.9939)

a) F(x)=0.0018494X+0.0015755



b) F(x)= 0.0011404X+0.0044477

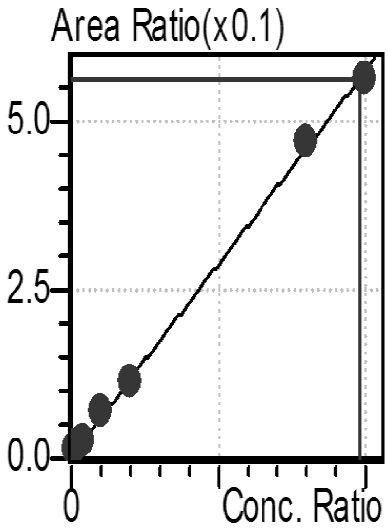


Figure 12 Chromatograms representing (a) a blank sample, (b) a standard containing 0.25ng/ml tacrolimus and 13-ODMT and (c) a rat sample containing 11.79ng/ml tacrolimus and 1.86 ng/ml 13-ODMT

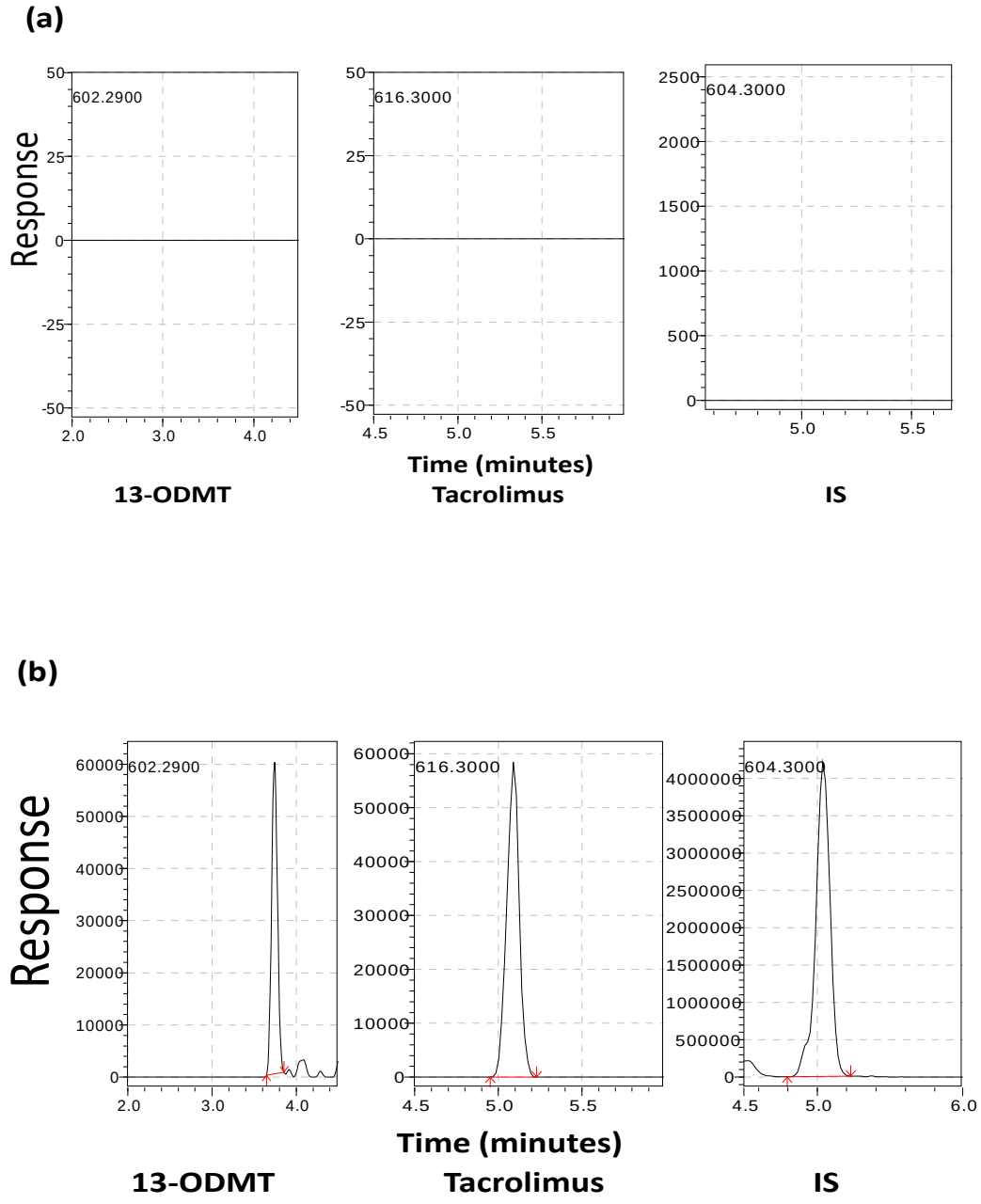


Figure 12 - Continued

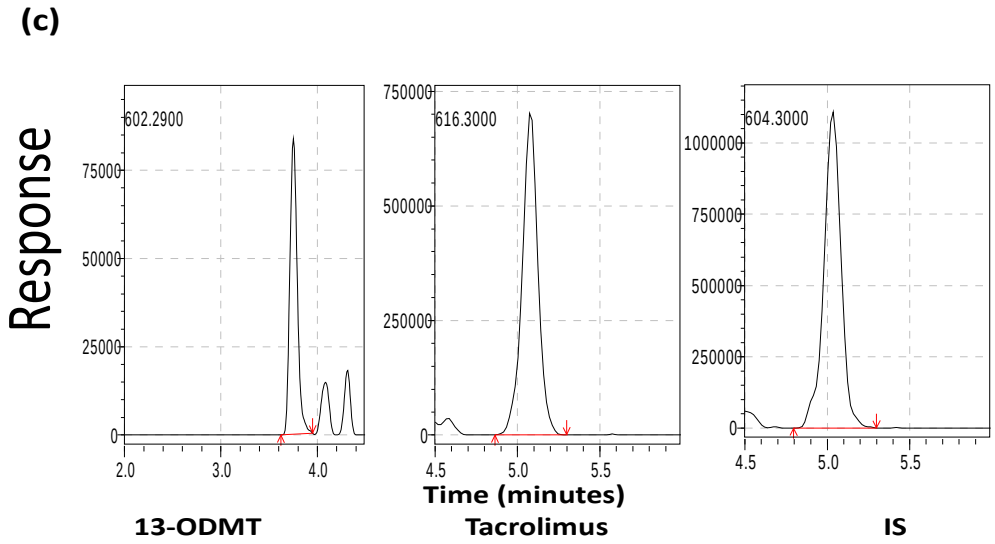


Table 16 Preparation of calibration standards for tacrolimus and 13-ODMT

Concentration (ng/ml)	Tacrolimus and metabolite
Blank	None
0	None
0.25	25 µLof 10 ng/mL
0.375	37.5 µLof 10 ng/mL
0.5	5 µLof 100 ng/mL
1	10 µLof 100 ng/mL
2.5	25 µLof 100 ng/mL
5	5 µLof 1µg/mL
20	20 µLof 1µg/mL
25	25 µLof 1µg/mL

Table 17 Preparation of controls for tacrolimus and 13-ODMT

Concentration (ng/ml)	Tacrolimus and metabolite
0.75	7.5 µL of 100 ng/mL
10	10 µL of 1µg/mL
17.5	17.5 µL of 1µg/mL

Table 18 Intra- and interassay precision and bias of LC-MS determination of tacrolimus and 13-ODMT in rat kidney tissue

Conc.	Tacrolimus				13-ODMT			
(ng/ml)	Intra-assay (n=5)		Inter-assay (n=15)		Intra-assay (n=5)		Inter-assay (n=15)	
	Precision CV (%)	Bias (%)	Precision CV (%)	Bias (%)	Precision CV (%)	Bias (%)	Precision CV (%)	Bias (%)
0.25 (LLOQ)	≤ 13.86	0.77 to 14.64	10.94	7.35	≤ 14.77	5.01 to 13.79	11.35	8.01
0.75	≤ 12.65	5.52 to 7.66	9.93	6.64	≤ 13.49	-0.98 to 5.39	8.37	3.13
10	≤ 8.13	-11.32 to -8.21	6.27	-10.00	≤ 8.56	-12.08 to -3.19	7.54	-8.25
17.5	≤ 2.59	-11.53 to 3.32	6.96	-4.93	≤ 4.52	-13.68 to 3.01	8.37	-4.15

Table 19 Extraction Efficiency

Concentration (ng/ml)	Tacrolimus Extraction Efficiency (%)	13-ODMT Extraction Efficiency (%)
0.75	70.9	66.7
10	74.9	78.4
17.5	67	67.6

Table 20 Freeze-thaw stability

Concentration	Tacrolimus		13-ODMT	
(ng/ml)	Bias (%)	CV (%)	Bias (%)	CV (%)
0.75	6.74	3.56	9.24	14.48
10	7.86	5.48	2.99	3.29
17.5	9.04	7.98	1.85	7.53

Table 21 Short-term temperature stability at 6 hours

Concentration	Tacrolimus		13-ODMT	
(ng/ml)	Bias (%)	CV (%)	Bias (%)	CV (%)
0.75	-5.03	10.70	-3.23	9.54
10	7.13	3.82	5.95	5.88
17.5	6.52	4.12	2.25	5.31

Table 22 Auto-sampler stability

Concentration (ng/ml)	Tacrolimus		13-ODMT	
	Bias (%)	CV (%)	Bias (%)	CV (%)
0.75	12.04	4.17	1.82	2.4
10	-8.32	5.15	-9.45	5.29
17.5	-5.95	3.59	-9.34	3.09

Table 23 Rat samples

Dose (mg/kg)	Tacrolimus concentration[a] (ng/ml)	13-ODMT concentration[a] (ng/ml)
0.5	11.54 (8.37- 13.4)	0.72 (0.35- 0.86)
2	8.89 (5.68- 11.79)	1.5 (1.49- 1.86)

[a]Results are expressed as median (range)

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

All patients undergoing organ transplantation have to undergo lifelong treatment by immunosuppressants, which are characterized by a highly variable pharmacokinetic profile and a narrow therapeutic index, making it necessary to closely monitor their blood concentrations to avoid the clinical consequences of a sub-therapeutic or supra-therapeutic tacrolimus blood level.

An overview of tacrolimus pharmacokinetics and pharmacodynamics is presented in this dissertation, followed by a description of the genetic determinants of drug exposure and transplant outcome. A significant impact of the CYP3A5 A6986G polymorphism on tacrolimus blood levels has been described, in contrast to the conflicting results about the contribution of individual ABCB1 polymorphisms or haplotypes. It is possible that a minor influence of the ABCB1 polymorphisms exists but is masked by the large variation in the transplant populations examined, the type of transplantation performed, sample size, time after transplantation and co-medication. CYP3A4 plays an important role in tacrolimus metabolism. A new CYP3A4*22 polymorphism recently found to significantly affect tacrolimus blood levels may help explain part of the residual variability in tacrolimus pharmacokinetics.

Tacrolimus blood concentrations are most variable in the early period following transplantation. A decrease in this variation may be achieved by a better selection of the initial starting dose for each individual patient. Based on a comprehensive review of the recent literature on the effect of CYP3A5, CYP3A4 and ABCB1 polymorphisms on dose-corrected tacrolimus exposure and their potential for use in genotype-based tacrolimus dosing, we conclude that a better predictive model that includes both clinical and genetic factors is still needed to optimize tacrolimus dosing, and translate the role of genetic factors in tacrolimus pharmacokinetic variability into improved clinical outcomes for the transplant population.

Secondly, our results confirm that genetic inheritance will affect CNI pharmacodynamics and may predispose patients to drug toxicities or drug interactions. CYP3A5 expressor genotype of the recipient, which predicts increased renal tubular expression, predisposes patients to CNI-induced renal dysfunction following liver transplantation, but due to the importance of local kidney tissue levels of tacrolimus or its metabolites in supporting these findings, a need to develop a sensitive and robust LC-MS/MS method to simultaneously determine the concentrations of tacrolimus and its major metabolite could not be overlooked.

Lastly, a robust and sensitive LC-MS/MS method that allowed for co-determination of tacrolimus and 13-ODMT was successfully developed and validated and the method was successfully applied to study the relationship between tacrolimus dose level and the extent of accumulation of tacrolimus and its major metabolite (13-ODMT) in rat kidney tissue. Despite a lack of correlation between tacrolimus dose level and its renal tissue concentration, a better correlation was observed between tacrolimus dose level and 13-ODMT accumulation.

Future Directions

As previously discussed, not even precise monitoring of CNI levels prevents the development of CNIT. Local tissue levels do not correlate with systemic levels and are believed to be significantly higher. The modification and successful application of the above mentioned LC-MS/MS method to quantify tacrolimus and tacrolimus metabolite levels in kidney biopsies of renal transplant recipients with CNIT will help determine the predictive value of local tacrolimus concentrations in CNIT development. It will be interesting to explore whether significant differences exist in intra-renal concentrations of tacrolimus and 13-ODMT between patients with and without biopsy-proven CNIT, and to determine if there's a correlation between those local tissue levels and the donor genotype of the transplanted kidney. This will provide the needed evidence to support the hypothesis that tacrolimus and/or its metabolite contribute to development of nephrotoxicity.

REFERENCES

1. Taylor AL, Watson CJ, Bradley JA. Immunosuppressive agents in solid organ transplantation: Mechanisms of action and therapeutic efficacy. Crit Rev Oncol 2005;56(1):23-46.

2. Bloom RD, Goldberg LR, Wang AY, Faust TW, Kotloff RM. An overview of solid organ transplantation. Clin Chest Med 2005;26(4):529-43.

3. Pillai AA, Levitsky J. Overview of immunosuppression in liver transplantation. World Journal of Gastroenterology: WJG 2009;15(34):4225.

4. Kino T, Hatanaka H, Hashimoto M, Nishiyama M, Goto T, Okuhara M, Kohsaka M, Aoki H, Imanaka H. FK-506, a novel immunosuppressant isolated from a streptomyces. I. fermentation, isolation, and physico-chemical and biological characteristics. J Antibiot (Tokyo) 1987 Sep;40(9):1249-55.

5. Hultsch T, Kapp A, Spergel J. Immunomodulation and safety of topical calcineurin inhibitors for the treatment of atopic dermatitis. Dermatology 2005;211(2):174-87.

6. Almawi WY, Melemedjian OK. Clinical and mechanistic differences between FK506 (tacrolimus) and cyclosporin A. Nephrology Dialysis Transplantation 2000;15(12):1916-8.

7. Webster AC, Woodroffe RC, Taylor RS, Chapman JR, Craig JC. Tacrolimus versus ciclosporin as primary immunosuppression for kidney transplant recipients: Meta-analysis and meta-regression of randomised trial data. Bmj 2005;331(7520):810.

8. Siekierka JJ, Hung SH, Poe M, Lin CS, Sigal NH. A cytosolic binding protein for the immunosuppressant FK506 has peptidyl-prolyl isomerase activity but is distinct from cyclophilin. 1989.

9. Möller A, Iwasaki K, Kawamura A, Teramura Y, Shiraga T, Hata T, Schäfer A, Undre N. The disposition of 14C-labeled tacrolimus after intravenous and oral administration in healthy human subjects. Drug Metab Disposition 1999;27(6):633-6.

10. Tanaka H, Kuroda A, Marusawa H, Hashimoto M, Hatanaka H, Kino T, Goto T, Okuhara M. Physicochemical properties of FK-506, a novel immunosuppressant isolated from streptomyces tsukubaensis. Transplant Proc 1987 Oct;19(5 Suppl 6):11-6.

11. Venkataramanan R, Swaminathan A, Prasad T, Jain A, Zuckerman S, Warty V, McMichael J, Lever J, Burckart G, Starzl T. Clinical pharmacokinetics of tacrolimus. Clin Pharmacokinet 1995;29(6):404-30.

12. Wu C, Benet LZ. Predicting drug disposition via application of BCS: Transport/absorption/elimination interplay and development of a biopharmaceutics drug disposition classification system. Pharm Res 2005;22(1):11-23.

13. Knops, Noël Levtchenko, Elena van den Heuvel,Bert Kuypers, Dirk. From gut to kidney: Transporting and metabolizing calcineurin-inhibitors in solid organ transplantation. Int J Pharm 2013;452(1-2):14-35.

14. Staatz CE, Tett SE. Clinical pharmacokinetics and pharmacodynamics of tacrolimus in solid organ transplantation. Clin Pharmacokinet 2004;43(10):623-53.

15. Wallemacq PE, Verbeeck RK. Comparative clinical pharmacokinetics of tacrolimus in paediatric and adult patients. Clin Pharmacokinet 2001;40(4):283-95.

16. Rao US, Scarborough GA. Direct demonstration of high affinity interactions of immunosuppressant drugs with the drug binding site of the human P-glycoprotein. Mol Pharmacol 1994;45(4):773-6.

17. Sattler M, Guengerich FP, Yun C, Christians U, Sewing KF. Cytochrome P-450 3A enzymes are responsible for biotransformation of FK506 and rapamycin in man and rat. Drug Metab Disposition 1992;20(5):753-61.

18. Benet LZ, Izumi T, Zhang Y, Silverman JA, Wacher VJ. Intestinal MDR transport proteins and P-450 enzymes as barriers to oral drug delivery. J Controlled Release 1999;62(1):25-31.

19. Nagase K, Iwasaki K, Nozaki K, Noda K. Distribution and protein binding of FK506, a potent immunosuppressive macrolide lactone, in human blood and its uptake by erythrocytes. J Pharm Pharmacol 1994;46(2):113-7.

20. Wacher VJ, Silverman JA, Zhang Y, Benet LZ. Role of P-glycoprotein and cytochrome P450 3A in limiting oral absorption of peptides and peptidomimetics. J Pharm Sci 1998;87(11):1322-30.

21. Lin YS, Dowling AL, Quigley SD, Farin FM, Zhang J, Lamba J, Schuetz EG, Thummel KE. Co-regulation of CYP3A4 and CYP3A5 and contribution to hepatic and intestinal midazolam metabolism. Mol Pharmacol 2002;62(1):162-72.

22. Iwasaki K. Metabolism of tacrolimus (FK506) and recent topics in clinical pharmacokinetics. Drug Metabolism and Pharmacokinetics 2007;22(5):328-35.

23. Shiraga T, Matsuda H, Nagase K, Iwasaki K, Noda K, Yamazaki H, Shimada T, Funae Y. Metabolism of FK506, a potent immunosuppressive agent, by cytochrome P450 3A enzymes in rat, dog and human liver microsomes. Biochem Pharmacol 1994;47(4):727-35.

24. Iwasaki K, Shiraga T, Nagase K, Tozuka Z, Noda K, Sakuma S, Fujitsu T, Shimatani K, Sato A, Fujioka M. Isolation, identification, and biological activities of oxidative metabolites of FK506, a potent immunosuppressive macrolide lactone. Drug Metab Disposition 1993;21(6):971-7.

25. Krejci K, Tichy T, Bachleda P, Zadrazil J. Calcineurin inhibitor–induced renal allograft nephrotoxicity. Biomedical Papers 2010;154(4):297-306.

26. Roby KA, Shaw LM. Effects of cyclosporine and its metabolites in the isolated perfused rat kidney. J Am Soc Nephrol 1993 Aug;4(2):168-77.

27. Copeland KR, Thliveris JA, Yatscoff RW. Toxicity of cyclosporine metabolites. Ther Drug Monit 1990;12(6):525-32.

28. Wallemacq PE, Reding R. FK506 (tacrolimus), a novel immunosuppressant in organ transplantation: Clinical, biomedical, and analytical aspects. Clin Chem 1993 Nov;39(11 Pt 1):2219-28.

29. Barry A, Levine M. A systematic review of the effect of CYP3A5 genotype on the apparent oral clearance of tacrolimus in renal transplant recipients. Ther Drug Monit 2010;32(6):708-14.

30. Liu EH, Siegel RM, Harlan DM, O'Shea JJ. T cell–directed therapies: Lessons learned and future prospects. Nat Immunol 2007;8(1):25-30.

31. Räkel A, Karelis A. New-onset diabetes after transplantation: Risk factors and clinical impact. Diabetes Metab 2011;37(1):1-14.

32. Kasiske BL, Snyder JJ, Gilbertson D, Matas AJ. Diabetes mellitus after kidney transplantation in the united states. American Journal of Transplantation 2003;3(2):178-85.

33. van Hooff JP, Christiaans MH, van Duijnhoven EM. Tacrolimus and posttransplant diabetes mellitus in renal transplantation. Transplantation 2005;79(11):1465-9.

34. Issa N, Kukla A, Ibrahim H. Calcineurin inhibitor nephrotoxicity: A review and perspective of the evidence. Am J Nephrol 2013;37(6):602-12.

35. Gaston RS. Chronic calcineurin inhibitor nephrotoxicity: Reflections on an evolving paradigm. Clin J Am Soc Nephrol 2009 Dec;4(12):2029-34.

36. Dai Y, Hebert MF, Isoherranen N, Davis CL, Marsh C, Shen DD, Thummel KE. Effect of CYP3A5 polymorphism on tacrolimus metabolic clearance in vitro. Drug Metab Disposition 2006;34(5):836-47.

37. Bechstein WO. Neurotoxicity of calcineurin inhibitors: Impact and clinical management. Transplant Int 2000;13(5):313-26.

38. Wijdicks EF. Neurotoxicity of immunosuppressive drugs. Liver Transplantation 2001;7(11):937-42.

39. Hesselink DA, Bouamar R, Elens L, van Schaik RH, van Gelder T. The role of pharmacogenetics in the disposition of and response to tacrolimus in solid organ transplantation. Clin Pharmacokinet 2013:1-17.

40. Wang J, Wei D, Chou K. Pharmacogenomics and personalized use of drugs. Current Topics in Medicinal Chemistry 2008;8(18):1573-9.

41. Staatz CE, Goodman LK, Tett SE. Effect of CYP3A and ABCB1 single nucleotide polymorphisms on the pharmacokinetics and pharmacodynamics of calcineurin inhibitors: Part I. Clin Pharmacokinet 2010 Mar;49(3):141-75.

42. Kuypers D, de Jonge H, Naesens M, Lerut E, Verbeke K, Vanrenterghem Y. CYP3A5 and CYP3A4 but not MDR1 single-nucleotide polymorphisms determine long-term tacrolimus disposition and drug-related nephrotoxicity in renal recipients. Clinical Pharmacology & Therapeutics 2007;82(6):711-25.

43. Kuypers DR, Naesens M, de Jonge H, Lerut E, Verbeke K, Vanrenterghem Y. Tacrolimus dose requirements and CYP3A5 genotype and the development of calcineurin inhibitor-associated nephrotoxicity in renal allograft recipients. Ther Drug Monit 2010;32(4):394-404.

44. Hesselink DA, van Schaik RH, van der Heiden, Ilse P, van der Werf M, Gregoor PJS, Lindemans J, Weimar W, van Gelder T. Genetic polymorphisms of the CYP3A4, CYP3A5, and MDR-1 genes and pharmacokinetics of the calcineurin inhibitors cyclosporine and tacrolimus. Clinical Pharmacology & Therapeutics 2003;74(3):245-54.

45. Elens L, Bouamar R, Hesselink DA, Haufroid V, van der Heiden, Ilse P, van Gelder T, van Schaik RH. A new functional CYP3A4 intron 6 polymorphism significantly affects tacrolimus pharmacokinetics in kidney transplant recipients. Clin Chem 2011;57(11):1574-83.

46. Elens L, van Gelder T, Hesselink DA, Haufroid V, van Schaik RH. CYP3A4*22: Promising newly identified CYP3A4 variant allele for personalizing pharmacotherapy. Pharmacogenomics 2013 Jan;14(1):47-62.

47. Shi Y, Li Y, Tang J, Zhang J, Zou Y, Cai B, Wang L. Influence of CYP3A4, CYP3A5 and MDR-1 polymorphisms on tacrolimus pharmacokinetics and early renal dysfunction in liver transplant recipients. Gene 2012.

48. Impact of cytochrome P450 3A and ATP-binding cassette subfamily B member 1 polymorphisms on tacrolimus dose-adjusted trough concentrations among korean renal transplant recipients. Transplantation proceedingsElsevier; 2012. .

49. Okubo M, Murayama N, Shimizu M, Shimada T, Guengerich F, Yamazaki H. The CYP3A4 intron 6 C> T polymorphism (CYP3A4* 22) is associated with reduced CYP3A4 protein level and function in human liver microsomes. J Toxicol Sci 2012;38(3):349-54.

50. de Jonge H, de Loor H, Verbeke K, Vanrenterghem Y, Kuypers D. In vivo CYP3A4 activity, CYP3A5 genotype, and hematocrit predict tacrolimus dose requirements and clearance in renal transplant patients. Clinical Pharmacology & Therapeutics 2012.

51. Roy JN, Lajoie J, Zijenah LS, Barama A, Poirier C, Ward BJ, Roger M. CYP3A5 genetic polymorphisms in different ethnic populations. Drug Metab Dispos 2005 Jul;33(7):884-7.

52. Lamba JK, Lin YS, Schuetz EG, Thummel KE. Genetic contribution to variable human CYP3A-mediated metabolism. Adv Drug Deliv Rev 2012.

53. Ware N, MacPhee I. Current progress in pharmacogenetics and individualized immunosuppressive drug dosing in organ transplantation. Curr Opin Mol Ther 2010;12(3):270-83.

54. Haufroid V, Wallemacq P, VanKerckhove V, Elens L, De Meyer M, Eddour D, Malaise J, Lison D, Mourad M. CYP3A5 and ABCB1 polymorphisms and tacrolimus pharmacokinetics in renal transplant candidates: Guidelines from an experimental study. American Journal of Transplantation 2006;6(11):2706-13.

55. Thervet E, Loriot M, Barbier S, Buchler M, Ficheux M, Choukroun G, Toupance O, Touchard G, Alberti C, Le Pogamp P. Optimization of initial tacrolimus dose using pharmacogenetic testing. Clinical Pharmacology & Therapeutics 2010;87(6):721-6.

56. Shuker N, Bouamar R, Weimar W, van Schaik RH, van Gelder T, Hesselink DA. ATP-binding cassette transporters as pharmacogenetic biomarkers for kidney transplantation. Clinica Chimica Acta 2012;413(17):1326-37.

57. Salama NN, Yang Z, Bui T, Ho RJ. MDR1 haplotypes significantly minimize intracellular uptake and transcellular P-gp substrate transport in recombinant LLC-PK1 cells. J Pharm Sci 2006;95(10):2293-308.

58. de Jonge H, Metalidis C, Naesens M, Lambrechts D, Kuypers DR. The P450 oxidoreductase* 28 SNP is associated with low initial tacrolimus exposure and increased dose requirements in CYP3A5-expressing renal recipients. Pharmacogenomics 2011;12(9):1281-91.

59. Elens L, Hesselink DA, Bouamar R, Budde K, de Fijter JW, De Meyer M, Mourad M, Kuypers DR, Haufroid V, van Gelder T, et al. Impact of POR*28 on the pharmacokinetics of tacrolimus and cyclosporine A in renal transplant patients. Ther Drug Monit 2014 Feb;36(1):71-9.

60. Barraclough KA, Isbel NM, Johnson DW, Campbell SB, Staatz CE. Once-versus twice-daily tacrolimus. Drugs 2011;71(12):1561-77.

61. Miller WL, Agrawal V, Sandee D, Tee MK, Huang N, Choi JH, Morrissey K, Giacomini KM. Consequences of POR mutations and polymorphisms. Mol Cell Endocrinol 2011;336(1):174-9.

62. Laverdière I, Caron P, Harvey M, Lévesque É, Guillemette C. In vitro investigation of human UDP-glucuronosyltransferase isoforms responsible for tacrolimus glucuronidation: Predominant contribution of UGT1A4. Drug Metab Disposition 2011;39(7):1127-30.

63. Fukudo M, Yano I, Yoshimura A, Masuda S, Uesugi M, Hosohata K, Katsura T, Ogura Y, Oike F, Takada Y. Impact of MDR1 and CYP3A5 on the oral clearance of tacrolimus and tacrolimus-related renal dysfunction in adult living-donor liver transplant patients. Pharmacogenetics and Genomics 2008;18(5):413.

64. Hauser IA, Schaeffeler E, Gauer S, Scheuermann EH, Wegner B, Gossmann J, Ackermann H, Seidl C, Hocher B, Zanger UM. ABCB1 genotype of the donor but not of the recipient is a major risk factor for cyclosporine-related nephrotoxicity after renal transplantation. Journal of the American Society of Nephrology 2005;16(5):1501-11.

65. Naesens M, Lerut E, de Jonge H, Van Damme B, Vanrenterghem Y, Kuypers DR. Donor age and renal P-glycoprotein expression associate with chronic histological damage in renal allografts. Journal of the American Society of Nephrology 2009;20(11):2468-80.

66. Rahman M, Wright JT, Douglas JG. The role of the cytochrome P450-dependent metabolites of arachidonic acid in blood pressure regulation and renal function. American Journal of Hypertension 1997;10(3):356-65.

67. Gervasini G, Garcia M, Macias RM, Benitez J, Caravaca F, Cubero JJ. CYP2C8* 3 polymorphism and donor age are associated with allograft dysfunction in kidney transplant recipients treated with calcineurin inhibitors. The Journal of Clinical Pharmacology 2013.

68. Gervasini G, Garcia M, Macias RM, Cubero JJ, Caravaca F, Benitez J. Impact of genetic polymorphisms on tacrolimus pharmacokinetics and the clinical outcome of renal transplantation. Transplant Int 2012.

69. Effect of CYP3A5* 1/* 3 polymorphism on blood pressure in renal transplant recipients. Transplantation proceedingsElsevier; 2012. .

70. Naesens M, Kuypers DR, Sarwal M. Calcineurin inhibitor nephrotoxicity. Clinical Journal of the American Society of Nephrology 2009;4(2):481-508.

71. Schwartz M, Holst B, Facklam D, Buell D. FK 506 in liver transplantation: Correlation of whole blood levels with efficacy and toxicity. the US multicenter FK 506 dose optimization. Transplant Proc 1995 Feb;27(1):1107.

72. Ball SE, Scatina J, Kao J, Ferron GM, Fruncillo R, Mayer P, Weinryb I, Guida M, Hopkins PJ, Warner N. Population distribution and effects on drug metabolism of a genetic variant in the 5′ promotor region of CYP3A4. Clinical Pharmacology & Therapeutics 1999;66(3):288-94.

73. Hesselink DA, Van Schaik RH, Van Der Heiden, Ilse P, Van Der Werf M, Gregoor PJS, Lindemans J, Weimar W, Van Gelder T. Genetic polymorphisms of the CYP3A4, CYP3A5, and MDR-1 genes and pharmacokinetics of the calcineurin inhibitors cyclosporine and tacrolimus. Clinical Pharmacology & Therapeutics 2003;74(3):245-54.

74. Elens L, Capron A, van Schaik RH, De Meyer M, De Pauw L, Eddour DC, Latinne D, Wallemacq P, Mourad M, Haufroid V. Impact of CYP3A4*22 allele on tacrolimus pharmacokinetics in early period after renal transplantation: Toward updated genotype-based dosage guidelines. Ther Drug Monit 2013 Oct;35(5):608-16.

75. Anglicheau D, Legendre C, Beaune P, Thervet E. Cytochrome P450 3A polymorphisms and immunosuppressive drugs: An update. 2007.

76. MacPhee IA, Fredericks S, Tai T, Syrris P, Carter ND, Johnston A, Goldberg L, Holt DW. The influence of pharmacogenetics on the time to achieve target tacrolimus concentrations after kidney transplantation. American Journal of Transplantation 2004;4(6):914-9.

77. Rojas LE, Herrero MJ, Boso V, Garcia-Eliz M, Poveda JL, Librero J, Alino SF. Meta-analysis and systematic review of the effect of the donor and recipient CYP3A5 6986A>G genotype on tacrolimus dose requirements in liver transplantation. Pharmacogenet Genomics 2013 Oct;23(10):509-17.

78. Kroetz DL, Pauli-Magnus C, Hodges LM, Huang CC, Kawamoto M, Johns SJ, Stryke D, Ferrin TE, DeYoung J, Taylor T. Sequence diversity and haplotype structure in the human ABCB1 (MDR1, multidrug resistance transporter) gene. Pharmacogenetics and Genomics 2003;13(8):481-94.

79. Initial dosage adjustment for oral administration of tacrolimus using the intestinal MDR1 level in living-donor liver transplant recipients. Transplantation proceedingsElsevier; 2005. .

80. Elens L, Capron A, Van Kerckhove V, Lerut J, Mourad M, Lison D, Wallemacq P, Haufroid V. 1199G> A and 2677G> T/A polymorphisms of ABCB1 independently affect tacrolimus concentration in hepatic tissue after liver transplantation. Pharmacogenetics and Genomics 2007;17(10):873-83.

81. Terrazzino S, Quaglia M, Stratta P, Canonico PL, Genazzani AA. The effect of CYP3A5 6986A> G and ABCB1 3435C> T on tacrolimus dose-adjusted trough levels and acute rejection rates in renal transplant patients: A systematic review and meta-analysis. Pharmacogenetics and Genomics 2012;22(8):642-5.

82. Li Y, Hu X, Cai B, Chen J, Bai Y, Tang J, Liao Y, Wang L. Meta-analysis of the effect of MDR1 C3435 polymorphism on tacrolimus pharmacokinetics in renal transplant recipients. Transpl Immunol 2012;27(1):12-8.

83. Liu Y, Li C, Cui Z, Fu X, Zhang S, Fan L, Ma J, Li G. The effect of ABCB1 C3435T polymorphism on pharmacokinetics of tacrolimus in liver transplantation: A meta-analysis. Gene 2013;531(2):476-88.

84. Boughton O, Borgulya G, Cecconi M, Fredericks S, Moreton-Clack M, MacPhee I. A published pharmacogenetic algorithm was poorly predictive of tacrolimus clearance in an independent cohort of renal transplant recipients. Br J Clin Pharmacol 2013.

85. Wang P, Mao Y, Razo J, Zhou X, Wong ST, Patel S, Elliott E, Shea E, Wu AH, Gaber AO. Using genetic and clinical factors to predict tacrolimus dose in renal transplant recipients. Pharmacogenomics 2010;11(10):1389-402.

86. Passey C, Birnbaum AK, Brundage RC, Oetting WS, Israni AK, Jacobson PA. Dosing equation for tacrolimus using genetic variants and clinical factors. Br J Clin Pharmacol 2011;72(6):948-57.

87. Passey C, Birnbaum AK, Brundage RC, Schladt DP, Oetting WS, Leduc RE, Israni AK, Guan W, Matas AJ, Jacobson PA. Validation of tacrolimus equation to predict troughs using genetic and clinical factors. Pharmacogenomics 2012;13(10):1141-7.

88. Elens L, Hesselink DA, van Schaik RH, van Gelder T. The CYP3A4*22 allele affects the predictive value of a pharmacogenetic algorithm predicting tacrolimus predose concentrations. Br J Clin Pharmacol 2012 Nov 16.

89. Provenzani A, Notarbartolo M, Labbozzetta M, Poma P, Biondi F, Sanguedolce R, Vizzini G, Palazzo U, Polidori P, Triolo F. The effect of CYP3A5 and ABCB1 single nucleotide polymorphisms on tacrolimus dose requirements in caucasian liver transplant patients. Ann Transplant 2009;14(1):23-31.

90. Glowacki F, Lionet A, Buob D, Labalette M, Allorge D, Provôt F, Hazzan M, Noël C, Broly F, Cauffiez C. CYP3A5 and ABCB1 polymorphisms in donor and recipient: Impact on tacrolimus dose requirements and clinical outcome after renal transplantation. Nephrology Dialysis Transplantation 2011;26(9):3046-50.

91. Kniepeiss D, Renner W, Trummer O, Wagner D, Wasler A, Khoschsorur GA, Truschnig-Wilders M, Tscheliessnigg K. The role of CYP3A5 genotypes in dose requirements of tacrolimus and everolimus after heart transplantation. Clin Transplant 2011;25(1):146-50.

92. García-Roca P, Medeiros M, Reyes H, Rodríguez-Espino BA, Alberú J, Ortiz L, Vásquez-Perdomo M, Elizondo G, Morales-Buenrostro LE, Mancilla Urrea E. < i> CYP3A5</i> polymorphism in mexican renal transplant recipients and its association with tacrolimus dosing. Arch Med Res 2012;43(4):283-7.

93. Ji E, Choi L, Suh K, Cho J, Han N, Oh JM. Combinational effect of intestinal and hepatic CYP3A5 genotypes on tacrolimus pharmacokinetics in recipients of living donor liver transplantation. Transplantation 2012;94(8):866-72.

94. Chitnis SD, Ogasawara K, Schniedewind B, Gohh RY, Christians U, Akhlaghi F. Concentration of tacrolimus and major metabolites in kidney transplant recipients as a function of diabetes mellitus and cytochrome P450 3A gene polymorphism. Xenobiotica 2012;43(7):641-9.

95. Rahsaz M, Azarpira N, Nikeghbalian S, Aghdaie MH, Geramizadeh B, Moini M, Banihashemi M, Darai M, Malekpour Z, Malekhoseini SA. Association between tacrolimus concentration and genetic polymorphisms of CYP3A5 and ABCB1 during the early stage after liver transplant in an iranian population. Experimental and Clinical Transplantation: Official Journal of the Middle East Society for Organ Transplantation 2012;10(1):24-9.

96. Kim I, Moon YJ, Ji E, Im Kim K, Han N, Kim SJ, Shin WG, Ha J, Yoon J, Lee HS. Clinical and genetic factors affecting tacrolimus trough levels and drug-related outcomes in korean kidney transplant recipients. Eur J Clin Pharmacol 2012;68(5):657-69.

97. Gómez-Bravo MA, Salcedo M, Fondevila C, Suarez F, Castellote J, Rufian S, Pons JA, Alamo JM, Millán O, Brunet M. Impact of donor and recipient CYP3A5 and ABCB1 genetic polymorphisms on tacrolimus dosage requirements and rejection in caucasian spanish liver transplant patients. The Journal of Clinical Pharmacology 2013;53(11):1146-54.

98. Yoon S, Cho J, Kwon O, Choi J, Park S, Kim Y, Yoon Y, Won D, Kim C. CYP3A and ABCB1 genetic polymorphisms on the pharmacokinetics and pharmacodynamics of tacrolimus and its metabolites (MI and M-III). Transplantation 2013.

99. Tavira B, Coto E, Diaz-Corte C, Alvarez V, López-Larrea C, Ortega F. A search for new CYP3A4 variants as determinants of tacrolimus dose requirements in renal-transplanted patients. Pharmacogenetics and Genomics 2013.

100. Kim I, Noh H, Ji E, Han N, Hong SH, Ha J, Burckart GJ, Oh JM. Identification of factors affecting tacrolimus level and 5-Year clinical outcome in kidney transplant patients. Basic & Clinical Pharmacology & Toxicology 2012;111(4):217-23.

101. Singh R, Srivastava A, Kapoor R, Mittal RD. Do drug transporter (ABCB1) SNPs influence cyclosporine and tacrolimus dose requirements and renal allograft outcome in the posttransplantation period? The Journal of Clinical Pharmacology 2011;51(4):603-15.

102. Yu X, Xie H, Wei B, Zhang M, Wang W, Wu J, Yan S, Zheng S, Zhou L. Association of MDR1 gene SNPs and haplotypes with the tacrolimus dose requirements in han chinese liver transplant recipients. PloS One 2011;6(11):e25933.

103. Waki K. UNOS liver registry: Ten year survivals. Clin Transpl 2006:29-39.

104. Hariharan S, Johnson CP, Bresnahan BA, Taranto SE, McIntosh MJ, Stablein D. Improved graft survival after renal transplantation in the united states, 1988 to 1996. N Engl J Med 2000;342(9):605-12.

105. Kuehl P, Zhang J, Lin Y, Lamba J, Assem M, Schuetz J, Watkins PB, Daly A, Wrighton SA, Hall SD. Sequence diversity in CYP3A promoters and characterization of the genetic basis of polymorphic CYP3A5 expression. Nat Genet 2001;27(4):383-91.

106. Op den Buijsch, Robert AM, Christiaans MH, Stolk LM, De Vries JE, Cheung CY, Undre NA, Van Hooff JP, Dieijen-Visser V, Marja P, Bekers O. Tacrolimus pharmacokinetics and pharmacogenetics: Influence of adenosine triphosphate-binding cassette B1 (ABCB1) and cytochrome (CYP) 3A polymorphisms. Fundam Clin Pharmacol 2007;21(4):427-35.

107. Gonwa TA, Mai ML, Melton LB, Hays SR, Goldstein RM, Levy MF, Klintmalm GB. END-STAGE RENAL DISEASE (ESRD) AFTER ORTHOTOPIC LIVER TRANSPLANTATION (OLTX) USING CALCINEURIN-BASED IMMUNOTHERAPY1: Risk of development and treatment. Transplantation 2001;72(12):1934-9.

108. Hebert MF, Dowling AL, Gierwatowski C, Lin YS, Edwards KL, Davis CL, Marsh CL, Schuetz EG, Thummel KE. Association between ABCB1 (multidrug resistance transporter) genotype and post-liver transplantation renal dysfunction in patients receiving calcineurin inhibitors. Pharmacogenetics and Genomics 2003;13(11):661-74.

109. Min S, Kim SY, Ahn SH, Min S, Kim SH, Kim YS, Moon KC, Oh JM, Kim SJ, Ha J. CYP3A5* 1 allele: Impacts on early acute rejection and graft function in tacrolimus-based renal transplant recipients. Transplantation 2010;90(12):1394-400.

110. Murray GI, McFadyen MC, Mitchell RT, Cheung YL, Kerr AC, Melvin WT. Cytochrome P450 CYP3A in human renal cell cancer. Br J Cancer 1999 Apr;79(11-12):1836-42.

111. Bolbrinker J, Seeberg S, Schostak M, Kempkensteffen C, Baelde H, de Heer E, Kreutz R. CYP3A5 genotype-phenotype analysis in the human kidney reveals a strong site-specific expression of CYP3A5 in the proximal tubule in carriers of the CYP3A5* 1 allele. Drug Metab Disposition 2012;40(4):639-41.

112. Zheng S, Tasnif Y, Hebert M, Davis C, Shitara Y, Calamia J, Lin Y, Shen D, Thummel K. Measurement and compartmental modeling of the effect of CYP3A5 gene variation on systemic and intrarenal tacrolimus disposition. Clinical Pharmacology & Therapeutics 2012;92(6):737-45.

113. Saeki T, Ueda K, Tanigawara Y, Hori R, Komano T. Human P-glycoprotein transports cyclosporin A and FK506. J Biol Chem 1993 Mar 25;268(9):6077-80.

114. Joy MS, Hogan SL, Thompson BD, Finn WF, Nickeleit V. Cytochrome P450 3A5 expression in the kidneys of patients with calcineurin inhibitor nephrotoxicity. Nephrology Dialysis Transplantation 2007;22(7):1963-8.

115. Zheng H, Zeevi A, Schuetz E, Lamba J, McCurry K, Griffith BP, Webber S, Ristich J, Dauber J, Iacono A. Tacrolimus dosing in adult lung transplant patients is related to cytochrome P4503A5 gene polymorphism. The Journal of Clinical Pharmacology 2004;44(2):135-40.

116. Uesugi M, Masuda S, Katsura T, Oike F, Takada Y, Inui K. Effect of intestinal CYP3A5 on postoperative tacrolimus trough levels in living-donor liver transplant recipients. Pharmacogenetics and Genomics 2006;16(2):119-27.

117. Chronic kidney disease following liver transplantation: A south australian experience. Transplantation proceedingsElsevier; 2010. .

118. Yang Z, Wang S. Recent development in application of high performance liquid chromatography-tandem mass spectrometry in therapeutic drug monitoring of immunosuppressants. J Immunol Methods 2008;336(2):98-103.

119. Vincent SH, Karanam BV, Painter SK, Chiu SL. < i> in vitro</i> metabolism of FK-506 in rat, rabbit, and human liver microsomes: Identification of a major metabolite and of cytochrome P450 3A as the major enzymes responsible for its metabolism. Arch Biochem Biophys 1992;294(2):454-60.

120. Capron A, Lerut J, Verbaandert C, Mathys J, Ciccarelli O, Vanbinst R, Roggen F, De Reyck C, Lemaire J, Wallemacq PE. Validation of a liquid chromatography-mass spectrometric assay for tacrolimus in liver biopsies after hepatic transplantation: Correlation with histopathologic staging of rejection. Ther Drug Monit 2007;29(3):340-8.

121. Qin X, Bi H, Wang C, Li J, Wang X, Liu L, Chen X, Huang M. Study of the effect of wuzhi tablet (schisandra sphenanthera extract) on tacrolimus tissue distribution in rat by liquid chromatography tandem mass spectrometry method. Biomedical Chromatography 2010;24(4):399-405.

122. Increased bioanalytical throughput using selective phospholipid depletion. 57th ASMS conference on mass spectrometry; 2009. .

123. Taillon M, Furtado M, Garofolo F. Challenges of developing a bioanalytical method for a macrolide immunosuppressant compound by LC-MS/MS. Bioanalysis 2011;3(11):1201-15.

124. Zhou Y, Zhang B, Li J, Zuo X, Yuan H, Yang G, Cheng Z, Liu Z, Li P, Tan H. Effect of amlodipine on the pharmacokinetics of tacrolimus in rats. Xenobiotica 2013(0):1-6.

125. Jette L, Beaulieu E, Leclerc J, Beliveau R. Cyclosporin A treatment induces overexpression of P-glycoprotein in the kidney and other tissues. American Journal of Physiology-Renal Physiology 1996;270(5):F756-65.