The Impact of *CYP3A5* Variation and Pregnancy on the Metabolic Disposition of Calcineurin Inhibitors

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Abstract

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The calcineurin inhibitors (CNI) — cyclosporine A (CsA) and tacrolimus remain the backbone of immunosuppression therapy for most organ transplant patients despite their serious side effects, such as chronic calcineurin inhibitor nephrotoxicity (CNIT). CNIs are substrates for CYP3As and P-glycoprotein. Genetic and environmental factors affect the activity of these proteins and can contribute to inter-individual variability CNI clearance and pharmacological response. In addition, the active concentration of CNIs is affected by physiological changes that influence their binding to plasma and intracellular components.

In this dissertation project, the first objective was to investigate the impact of polymorphic *CYP3A5* expression on the metabolism of CNIs and to evaluate the hypothesis that *CYP3A5* genotype affects intrarenal CNI and metabolite accumulation.

The second objective was to characterize the impact of physiological changes induced by pregnancy on tacrolimus disposition and to evaluate *in utero* and neonatal tacrolimus exposure.

For objective one, CsA and tacrolimus were orally administered to 24 healthy participants selected based on their *CYP3A5* genotype. Compared to CYP3A5 nonexpressors, expressors had a comparable oral CsA clearance, but 30% higher $AUC_{metabolite}/AUC_{CsA}$ ratios for AM19 and AM1c9, and a 20.4% lower mean CsA apparent urinary clearance. For tacrolimus, CYP3A5 expressors had a 1.6-fold higher oral clearance, 2.0- to 2.7-fold higher metabolite/parent AUC ratios for 31-DMT, 12-HT and 13-DMT, and a 36% lower tacrolimus urinary clearance. A semi-physiological model of renal tacrolimus disposition was developed, which predicted that intrarenal tacrolimus exposure in CYP3A5 expressors is 53% of that in nonexpressors. Thus, with chronic therapy, intrarenal accumulation of CNIs and their metabolites will depend on the *CYP3A5* genotype of the liver and kidneys, which may contribute to inter-patient differences in the risk of CNIT.

The findings for objective two demonstrate that anemia and hypoalbuminemia during pregnancy increase the fraction of unbound tacrolimus. The clinical titration of dosage in pregnancy can lead to elevated unbound concentrations and possibly toxicity. In addition, tacrolimus crosses the placenta, with *in utero* exposure being approximately 71%, 20% and 20% of maternal exposure when comparing blood, plasma or unbound concentrations, respectively and only small amounts of tacrolimus are excreted into breast milk.

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LIST OF ABBREVIATIONS

12-HT: 12-hydroxy tacrolimus

13-DMT: 13-*O*-desmethyl tacrolimus

15-DMT: 15-*O*-desmethyl tacrolimus

31-DMT: 31-*O*-desmethyl tacrolimus

AAG*:* α_1-acid glycoprotein

AUC: area under the concentration–time curve

B cell: B lymphocyte

BCRP: breast cancer resistance protein

B/P ratio: blood to plasma concentration ratio

CD3: cluster of differentiation 3 cells

CD4: cluster of differentiation 4 cells

CD8: cluster of differentiation 8 cells

CL/F: oral clearance

C_{last}: blood concentration at the last sampling time after drug administration

CL_r: tacrolimus renal clearance

$CL_{urinary}$: urinary clearance

C_{max}: maximum blood concentration

CNIT: chronic calcineurin inhibitor nephrotoxicity

CNI: calcineurin inhibitor

C_P: plasma concentration

CrCL: renal creatinine clearance

C_{trough}: blood concentration at the end of each dosing interval

C_{WB}: whole blood concentration

CYP1A2: cytochrome P450 1A2

CYP2C19: cytochrome P450 2C19

CYP2C9: cytochrome P450 2C9

CYP2D6: cytochrome P450 2D6

CYP3A4: cytochrome P450 3A4

CYP3A5: cytochrome P450 3A5

CYP3A7: cytochrome P450 3A7

CYP3A+b5: CYP3A enzymes coexpressed with cytochrome b5

eGFR: estimated glomerular filtration rate

ELISA: enzyme-linked immunosorbent assay

ER: extraction ratio, the relative efficiency of eliminating the drug from the systemic circulation on a single pass through the organ

F: systemic bioavailability

FPIA: fluorescence polarization immunoassay

f_{ub}: unbound fraction in whole blood

f_{um}: unbound fraction of tacrolimus in breast milk

f_{up}: unbound fraction in plasma

GFR: glomerular filtration rate

Hct: hematocrit

HDL: high density lipoproteins

IL-10: interleukin 10

IL-13: interleukin 13

IL-2: interleukin 2

IL-4: interleukin 4

IL-6: interleukin 6

IL-8: interleukin 8

INF: interferon gamma

$K_{bc/p}$: the tacrolimus partitioning ratio between blood cells and plasma

LC-MS/MS: liquid chromatography-mass spectrometry/mass spectrometry

OAT: organic anion transporter

OCT: organic cation transporter

PBMCs: peripheral blood mononuclear cells

P-gp: P-glycoprotein

QTOF: Quadrupole-time-of-flight

RBC: red blood cell

SCr: serum creatinine

SD: standard deviation

T cell: T lymphocyte

TNF: tumor necrosis factor alpha

UGT: uridine 5'-diphospho-glucuronosyltransferase

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DEDICATION

To Mom and Dad

And Grandfather

Chapter 1

Introduction

1.1 Overview

Cyclosporine A (CsA) and tacrolimus are potent immunosuppressive agents that are widely used in reducing the incidence and severity of allograft rejection after solid-organ transplantation (Naesens et al., 2009; Staatz et al., 2010a). CsA is a lipophilic cyclic peptide of 11 amino acids, while tacrolimus is a macrolide antibiotic; both were originally isolated from fungi (Wenger, 1985; Thomson et al., 1995; Greenstein et al., 2008). The introduction of CsA in the 1980s and of tacrolimus in the 1990s has significantly improved the survival of transplanted organs (Hariharan et al., 2000; Scott et al., 2003). They exert their immunosuppressive effects by suppressing T-cell activation (Staatz et al., 2010a). CsA and tacrolimus are also used in the treatment of a variety of "autoimmune" and "inflammatory" diseases, including inflammatory bowl disease (IBD), skin diseases, asthma and rheumatoid arthritis (Greenstein et al., 2008).

Despite differences in their structure and *in vivo* potency, CsA and tacrolimus share a similar mechanism of action (**Figure 1.1**) (Sawada et al., 1987; Almawi and Melemedjian, 2000). Both drugs bind with high affinity to a family of cytoplasmic proteins present in most cells: cyclophilins for cyclosporine, and FK binding proteins (FKBP12) for tacrolimus (Sigal and Dumont, 1992). The drug-receptor complex specifically and competitively binds to and inhibits calcineurin, a calcium- and calmodulin-dependent phosphatase (Klee et al., 1998). This process inhibits the translocation of a family of transcription factors, nuclear factors of activated T cells (NFAT), leading to reduced transcriptional activation of early cytokine genes for interleukin (IL)-2, tumor necrosis factor alpha (TNF-alpha), IL-3, IL-4, CD40L, granulocyte-macrophage colony-stimulating factor, and interferon-gamma, which ultimately suppress the proliferation of lymphocytes (Clipstone and Crabtree, 1992; Cockerill et al., 1993; Splawski et al., 1996; Naesens et al., 2009; Staatz et al., 2010a).

Calcineurin and NFAT isoforms are not T-cell specific, and inhibition of this pathway by CsA and tacrolimus gives rise to biological effects (including toxicity) beyond immunosuppression (Liu et al., 2007). The principal adverse effects associated with calcineurin inhibitor therapy include acute and chronic calcineurin inhibitor nephrotoxicity (CNIT), neurotoxicity, diabetogenesis, hypertension, gastrointestinal disturbances, infection and malignancy in the long term (Staatz et al., 2010a). Acute CNI nephrotoxicity describes a reversible, hemodynamically mediated renal dysfunction, represented by a reversible decrease in GFR, that occurs in 17% to 50% of kidney transplant recipients who receive CNIs (Naesens et al., 2009; Jacobson et al., 2012). Histologically, it is characterized by necrosis and early hyalinosis of individual smooth muscle cells in the afferent arterioles, and/or isometric vacuolation of the proximal straight tubules (Liptak and Ivanyi, 2006).

Acute CNI nephrotoxicity is managed by lowering the CNI dose and discontinuing the CNI in severe cases (Mahalati et al., 1999). If untreated, acute toxicity can progress to chronic CNI nephrotoxicity, the pathological features of which include progressive and irreversible interstitial fibrosis typically associated with vacuolization of the cytoplasm in tubular epithelial cells, tubular atrophy and arteriolar hyaline changes (damaged media smooth muscle cells in afferent arterioles replaced by beaded medial hyaline deposits that bulge into the adventitia) (Burdmann et al., 2003; Liptak and Ivanyi, 2006; Naesens et al., 2009). These chronic effects of long-term CNI use have been confirmed both for cyclosporine (Farnsworth et al., 1984; Palestine et al., 1986) and tacrolimus (Starzl et al., 1990; Randhawa et al., 1993). By evaluating 62 renal biopsies at a median of 4 (range: 0.3–15.9) years after nonrenal solid organ transplantation, 35.5% of tissues showed the predominant features of chronic CNI nephrotoxicity and 38.7% patients had progression to end-stage renal disease (Kubal et al., 2012). This is similar to a finding that 5-

year risk of end stage renal disease ranges from 7% to 21% and is associated with a fourfold greater risk of death in nonrenal organ transplant recipients (Ojo et al., 2003). In kidney-pancreas transplant recipients, the 10-year incidence of chronic CNI nephrotoxicity was reported to be 100% (Nankivell et al., 2003).

At the cellular and molecular levels, CNI nephrotoxicity is associated with matrix accumulation and epithelial dedifferentiation via transforming growth factor-beta (TGF-β1) dependent and independent pathways (Khanna et al., 1999; McMorrow et al., 2005; Naesens et al., 2009; Djamali et al., 2012). CsA increases the expression of TGF-β1, which may be an important mechanism by which it causes renal fibrosis and long-term malignancy (Shehata et al., 1995; Hojo et al., 1999). It has been demonstrated that tacrolimus has nephrotoxic properties similar to those of CsA and induces similar histologic injury (Mihatsch et al., 1998; Naesens et al., 2009).

To optimize efficacy and minimize toxicity, therapeutic drug monitoring (TDM) of calcineurin inhibitors is routinely performed, with the drug dosage adjusted in order to achieve a target therapeutic whole-blood drug concentration and a consistent clinical response. Immunoassays are often used to determine the blood concentration of CsA and tacrolimus, with some cross-reactivity with their metabolites using the supposed 'specific' antibodies currently available, which tend to overestimate the actual concentrations of both drugs (Murthy et al., 1998; Steimer, 1999). High performance liquid chromatography-mass spectrometry remains the reference procedure and is employed by many medical centers, (Borrows et al., 2007), including UWMC in Seattle, WA. Despite advances in the analytical tools available to accurately measure blood CNI concentrations, routine TDM has not prevented some transplant recipients from developing CNIT (Ojo et al., 2003; Ekberg et al., 2007; Naesens et al., 2009). Therefore, a

mechanistic understanding of the underlying factors affecting the pharmacokinetics and pharmacodynamics of calcineurin inhibitors is crucial for not only achieving the recommended target range in regard to the outcome of acute rejection, but also for understanding an individual's susceptibility to drug toxicity such as CNIT.

CsA and tacrolimus display great interindividual variability in the blood drug concentration achieved with a given dose. In addition, because individual transplant recipients can respond differently to the same blood immunosuppressant concentration, the immunosuppressant toxicity or efficacy remains unpredictable (Naesens et al., 2009; Staatz et al., 2010a). Although a strong association has been reported between increasing CNI troughs and acute nephrotoxicity (Mahalati et al., 1999; Jacobson et al., 2012), there is no hard evidence to date that systemic exposure to CsA and tacrolimus represents the major determinant of the risk for chronic CNI nephrotoxicity (Naesens et al., 2009). One of the plausible explanations is that the effective drug concentration at the site of action (immunosuppression) or site of toxicity does not correlate well with whole blood concentrations. For example, CsA whole-blood concentrations have been shown to correlate only moderately ($r^2 = 0.30$) with CsA intracellular concentrations in the peripheral blood mononuclear cells, which might vary over a 10-fold factor for a given blood concentration (Crettol et al., 2008). Similarly, in 16 transplant patients, for whom blood CsA concentrations (C_2) were available within 1 day of the renal biopsy being performed, there was no significant correlation between CsA concentrations in blood and kidney tissue (Spearman $r = 0.168$, $P > 0.05$) (Noll et al., 2011).

There is emerging evidence that local tissue CNI concentrations matter most with regard to drug effect. Podder *et al*. demonstrated that higher local renal concentrations of CsA correlated significantly with decreased renal function and increased histologic damage (Podder et

al., 2001). For tacrolimus, in 146 adult liver transplant patients, tacrolimus tissue levels displayed excellent correlation ($r^2 = 0.98$) with the severity of the liver rejection, whereas blood levels did not and showed no correlation with liver tissue concentrations (Capron et al., 2007). The author hypothesized that there could be the presence of a redistribution of immunosuppressants from transplanted tissue to circulating and infiltrating T cells resulting in an equilibrium between both compartments (Capron et al., 2007). Considering that lymphocytes represent the major target for calcineurin inhibitor therapeutic effects, whereas kidney cells represent targets for the development of drug-related nephrotoxicity, factors that may influence the lymphocyte intracellular concentration and renal distribution/accumulation of calcineurin inhibitors are of clinical importance. Examples for consideration include P-gp mediated efflux activity in lymphocytes (Chaudhary et al., 1992) and the kidney, renal drug metabolism mediated by CYP3A, and protein/lipid binding that may modulate a slowly equilibrating intracellular "active" drug concentration.

Overall, clinical, genetic and environmental factors reported to influence the pharmacokinetics of calcineurin inhibitors include the transplant type (kidney, liver, heart, etc.), hepatic and renal function, use of concomitant medications such as corticosteroids, time after transplantation, patient age and race, levels of CYP3A and P-glycoprotein expression in the donor and the recipients, hematocrit and albumin concentrations, diurnal rhythm, food administration, and diarrhea (Staatz and Tett, 2004; Naesens et al., 2009).

1.2 The Metabolism of Cyclosporine A and Tacrolimus: Impact on Calcineurin Inhibitor Efficacy and Chronic Nephrotoxicity

1.2.1 Cytochrome P450 (CYP) 3A Isoenzymes

The hepatic microsomal cytochrome P450 superfamily (P450) of heme proteins includes components of the mixed-function oxidase system, which catalyzes the metabolism of numerous lipophilic endogenous and exogenous compounds (Kelly and Kahan, 2002). Cytochrome P450 (CYP) 3A isoenzymes CYP3A4 and CYP3A5 are largely responsible for the extensive metabolism of CsA and tacrolimus (Sattler et al., 1992; Dai et al., 2004; Dai et al., 2006). The CYP3A subfamily consists of at least four isoforms (Gellner et al., 2001): CYP3A4, CYP3A5, CYP3A7 and CYP3A43 with overlapping substrate specificity (Lamba et al., 2002). CYP3A4 was the first human CYP3A identified (Molowa et al., 1986) with another CYP3A member CYP3A5 subsequently identified, in part, because of its pronounced polymorphic tissue expression (Wrighton et al., 1989). CYP3A7, which is expressed at high levels in fetal liver, was also identified and cloned at about the same time (Komori et al., 1989). CYP3A43 was identified more recently (Domanski et al., 2001; Westlind et al., 2001), but its clinical relevance remains in doubt.

Functional CYP3A4 protein is found in the liver and small intestine of nearly all individuals, accounting for on average approximately 30% of the total cytochrome P450 activity in the liver and 70% of the cytochrome P450 activity in the small intestines (Zhang et al., 1999; Lamba et al., 2002). However, CYP3A4 expression is highly variable between individuals, with 10- to 100-fold differences in liver and up to 30-fold differences in small intestinal expression (Wacher et al., 1998). Differences in CYP3A4 content within the liver contribute to a 66% interpatient coefficient of variation in the clearance of CsA administered intravenously

(Thummel et al., 1994). There is also extensive and highly variable presystemic metabolism of CsA and tacrolimus by gastrointestinal cytochrome P450 (CYP) 3A isoenzymes (Bistrup et al., 2001; Tuteja et al., 2001; Zhang and Benet, 2001).

In contrast to CYP3A4, only some individuals express functional CYP3A5 protein in the liver, small intestine, and kidneys at levels significant enough to contribute to drug clearance; these individuals have been termed CYP3A5 expressors (Koch et al., 2002; Lamba et al., 2002). A single nucleotide polymorphism (SNP) in *CYP3A5,* resulting in low expression in homozygous *CYP3A5*3*/**3* individuals (CYP3A5 nonexpressors) compared with *CYP3A5*1* allele carriers (CYP3A5 expressors), contributes to the significant interindividual differences in the CYP3A5 enzyme expression (Kuehl et al., 2001; Wojnowski, 2004). A single nucleotide polymorphism (SNP) is a DNA sequence variation of a single nucleotide (adenine [A], thymine [T], cytosine [C] or guanine [G]), which occurs at a frequency of greater than 1% within the general population. SNPs may occur within the coding sequences of genes, within non-coding regions of genes, or in the inter-genic region between genes (Staatz et al., 2010a). In the case of CYP3A5, the non-coding A6986G polymorphism (rs776746) creates a cryptic consensus splice site in the pre-mRNA, resulting in the incorporation of 131 bp of intron 3 sequence (referred to as exon 3B and inserted between exon 3 and exon 4) in the mature mRNA, and the production of improperly spliced mRNA containing exon 3B (splice variant 1) and a small amount of properly spliced mRNA (wt-mRNA) (Kuehl et al., 2001; Lin et al., 2002). The insertion causes a frame shift and a predicted premature termination codon, so that the encoded protein is truncated at amino acid residue 102 with loss of enzyme activity (Kuehl et al., 2001; Lin et al., 2002). The frequency of the CYP3A5 6986A>G SNP is highly dependent on ethnicity, in that the

*CYP3A5*1* allele is present in approximately 5–15% of Caucasians, 45–73% of African Americans, 15–35% of Asians, and 25% of Mexicans (Lamba et al., 2002).

The expression of CYP3A7 accounts for between 30 and 50% of the total cytochrome P450 activity in human embryonic, fetal and newborn liver (Shimada et al., 1996; de Wildt et al., 1999). It is also expressed polymorphically in adult liver (apparent bimodal distribution) (Koch et al., 2002; Daly, 2006). In adult livers expressing CYP3A7, it has been suggested that this isoform may contribute up to 20% of the total CYP3A expression (Koch et al., 2002).

1.2.2 The Metabolic Pathways of CsA and Tacrolimus

In vitro, both CsA and tacrolimus are metabolized by CYP3A4 and CYP3A5 (Dai et al., 2004; Dai et al., 2006). *In vivo*, CsA is extensively (>99%) converted to more than 30 metabolites by CYP3A4 via hydroxylation, demethylation, sulfation, and position 1 cyclization, although the core cyclic structure of CsA is preserved (Christians and Sewing, 1995; Kelly and Kahan, 2002) (**Figure 1.2**). The primary metabolites are the monohydroxylated AM1 (M-17) and AM9 (M-1) and the N-demethylated AM4N (M-21). Further oxidation of AM1 and AM9 results in the dihydroxylated AM19 (M-8), AM49 (M-10), and AM69 (M-16) (Maurer et al., 1984; Maurer and Lemaire, 1986). AM1c and AM1c9 are cyclized metabolites from AM1 and AM19 (Christians and Sewing, 1995). AM1A has a carboxy group at amino acid 1, which is the major hepatic metabolite that is usually not detectable in blood of patients with normal liver function. Formation of AM1A from CsA requires several oxidation steps via AM1, including oxidation of AM1 to its aldehyde (Christians and Sewing, 1993). In whole blood, the concentration distribution of cyclosporine and its main metabolites are reported as follows: cyclosporine (27%), AM1 (24%), AM9 (14%)(Ryffel et al., 1988).

Tacrolimus is also extensively metabolized to at least 15 metabolites (**Figure 1.3**). (Iwasaki et al., 1995; Venkataramanan et al., 1995; Gonschior et al., 1996; Iwasaki, 2007). All have been isolated from human plasma, bile and urine and have been generated by human liver microsomes *in vitro* (Christians et al., 1991a; Christians et al., 1991c; Christians et al., 1991d; Christians et al., 1992). Tacrolimus undergoes O-demethylation, hydroxylation and/or oxidative metabolic reactions, predominantly by CYP3A4 and CYP3A5 in the liver and intestinal mucosa, with <0.5% of the parent drug appearing unchanged in the urine or feces (Sattler et al., 1992; Karanam et al., 1994; Iwasaki et al., 1995; Moller et al., 1999). More than 95% of tacrolimus metabolites are eliminated by the biliary route. Urinary excretion accounts for, on average, only 2.4% of tacrolimus elimination (Moller et al., 1999). Major pathways of metabolism are demethylation and oxidation along the two double bonds present in the molecule and hydroxylation (Tata et al., 2009). A two-step reaction is involved in the production of several metabolites: oxidation by cytochrome P450 3A enzymes destabilizing the macrolide ring, followed by its rearrangement (Iwasaki et al., 1995; Lhoest et al., 1998). 13-O-Demethyl-tacrolimus (13-DMT) appears to be the major breakdown product of tacrolimus in human liver microsomes and the blood (Christians et al., 1991a). Although CYP3A enzymes are highly efficient at metabolizing tacrolimus, it is considered a low-clearance drug, with clearance equivalent to 3% of liver blood flow on the basis of blood concentration data (Moller et al., 1999), because of its extensive plasma protein and blood cell binding.

The intrinsic clearance of CsA, calculated from total metabolite formation, is approximately 2.3-fold higher for CYP3A4 than for CYP3A5 (Dai et al., 2004), while the intrinsic clearance of tacrolimus is approximately 2-fold higher for CYP3A5 than for CYP3A4 (Dai et al., 2006). *In vivo*, CYP3A4 may play a more dominant role than CYP3A5 in the

metabolism of CsA, while CYP3A5 plays a more dominant role than CYP3A4 in the metabolism of tacrolimus (Staatz et al., 2010a). Although not typically considered, the *in vitro* metabolism of tacrolimus by CYP3A7 was reported to be 29% and 18% of that observed with CYP3A4 and CYP3A5, respectively (Kamdem et al., 2005).

The formation of CsA and tacrolimus metabolites by liver and kidney microsomes differs significantly as a function of the CYP3A5 genotype (Dai et al., 2004; Dai et al., 2006). For CsA, CYP3A4 catalyzes the formation of all three primary metabolites (AM1, AM9 and AM4N), whereas only AM9 is produced to any significant degree by CYP3A5. In addition, liver and kidney microsomes from individuals who express CYP3A5 generate a greater amount of the two major secondary metabolites of cyclosporine, AM19 (from incubation with AM1) and AMIc9 (from incubation with AM1c) (Dai et al., 2004). For tacrolimus, the formation rates of three of its four primary metabolites, 13-DMT, 31-DMT and 12-HT, were at least 1.7-fold higher in liver microsomes from individuals who are CYP3A5 expressors than in those from nonexpressors.

All CsA and tacrolimus metabolites identified to date are considerably less potent in immunosuppressive activity than the parent drug except for 31-DMT (Staatz et al., 2010a). How this translates into nephrotoxicity risk is unclear. The most active metabolites of CsA—AM1, followed by AM9—display only 10-20% of the immunosuppressive activity of the parent drug (Kelly and Kahan, 2002). At the trough time point, AM1 constitutes approximately 27% of the total concentration of CsA equivalents in blood (Kelly and Kahan, 2002). For CsA, elevated blood and urine levels of its secondary metabolites, AM19 and AM1c9 have been associated with renal dysfunction in CsA treated patients (Christians et al., 1991b; Kempkes-Koch et al., 2001; Vollenbroeker et al., 2005). In addition, for patients prescribed with CsA, the CYP3A5 6986A>G SNP may influence long-term survival, possibly because of toxic effects of CsA

metabolites over time (Staatz et al., 2010b). The systemic production of primary and related downstream metabolites of tacrolimus might also contribute to CNI nephrotoxicity (Kuypers et al., 2007; Kuypers et al., 2010; Min et al., 2010; Staatz et al., 2010b; Metalidis et al., 2011), although the *in vivo* blood metabolite profile for tacrolimus has not been well characterized based on CYP3A5 genotype. Nonetheless, a greater understanding of the systemic and local metabolite exposure in CYP3A5 expressors and nonexpressors may have significant value in defining individual risk of CNI-induced nephrotoxicity.

1.2.3 Genotype-dependent CYP3A5 mRNA and Protein Expression in the Kidney

A detailed analysis of the genotype-dependent CYP3A5 mRNA expression has been described for liver, intestine (Kuehl et al., 2001; Lin et al., 2002) and kidney samples (Koch et al., 2002; Bolbrinker et al., 2012). By investigating the expression of CYP3A mRNA species in the liver and in various other tissues using gene-specific TaqMan probes, Koch et al. found that only CYP3A5 was detected in the kidney (Koch et al., 2002). Moreover, in kidney samples from 93 patients obtained during surgical interventions, the highest levels of CYP3A5 mRNA were found in *CYP3A5*1/*1* genotyped individuals (n = 2), intermediate levels were found in heterozygotes (n = 10), and the lowest levels were found in *CYP3A5*3/*3* carriers (n = 81) (Bolbrinker et al., 2012).

Regarding enzyme levels, in microsomal preparations from 21 human kidneys, there is a significant difference in CYP3A5 protein content and activity between heterozygous *CYP3A5*1* allele carriers and *CYP3A5*3/*3* individuals (Givens et al., 2003). As mentioned above, immunohistochemistry analyses show little to no staining for the CYP3A4 enzyme in the human kidney (Schuetz et al., 1992; Haehner et al., 1996). In contrast, in normal kidneys,

immunohistochemistry revealed staining for CYP3A5 protein in all renal epithelia (Bolbrinker et al., 2012). In addition, compared with kidney samples from a donor with a *CYP3A5*3/*3* genotype, higher expression of CYP3A5 was detected in kidneys of *CYP3A5*1* allele carriers, which was observed exclusively in epithelial cells of the proximal tubule. There was no influence of CYP3A5 genotype on protein detection in the distal epithelium, collecting ducts, or glomeruli.

In a recent study (Metalidis et al., 2011), positive detection of CYP3A5 in the brush border of the proximal tubules, measured by immunohistochemistry, was found in 47% of chronic calcineurin inhibitor nephrotoxicity (CNIT) (N=32) and 14% of control biopsies (N=71). Brush border staining for CYP3A5 in distal tubules was present in 10% of CNIT and 39% of control biopsies. The authors suggested a protective role for CYP3A5 in distal tubules, but that local production of potentially toxic metabolites by proximal tubular CYP3A5 could contribute to CNIT (Kuypers et al., 2010; Metalidis et al., 2011). Since neither donor nor recipient genotype was associated with the immunohistochemical staining pattern of CYP3A5, with all patients showing some degree of CYP3A5 staining, the authors concluded that immunohistochemistry is an insufficiently sensitive (and specific) technique to detect the variability in the inherently limited renal *CYP3A5* gene expression (Metalidis et al., 2011).

Some groups have postulated that because of elevated *CYP3A5* mRNA, protein expression and activity in the kidneys of *CYP3A5*1* carriers (Koch et al., 2002; Givens et al., 2003; Bolbrinker et al., 2012), and no CYP3A4 kidney expression in either CYP3A5 expressors or nonexpressors (Schuetz et al., 1992; Haehner et al., 1996), CYP3A5 expressors may exhibit increased intrarenal metabolism of CsA and tacrolimus, decreased renal drug accumulation and thus decreased susceptibility to chronic drug-related nephrotoxicity compared to CYP3A5

nonexpressors (Joy et al., 2007; Naesens et al., 2009). *In vitro*, kidney microsomes from CYP3A5 expressors generate 13.5-fold higher concentrations of tacrolimus's major primary metabolite, 13-DMT (when incubated with tacrolimus) than those from nonexpressors (Dai et al., 2006). However, no study to date has provided clear evidence of intrarenal CNI metabolism, nor has evaluated the impact of intrarenal metabolism on the pharmacokinetics of CNI *in vivo*. It was noted that the interaction between the donor's and the recipient's genome may have a significant influence on the prediction of pre-transplant risk allocation to a transplant recipient (Naesens et al., 2012). In kidney transplant patients, it seems crucial to include the donor's genotype in the risk assessment of CNI nephrotoxicity as the transplanted kidneys is the site of toxicity (Naesens et al., 2012).

1.3 The Transport of Cyclosporine A and Tacrolimus: Other Factors Related to Calcineurin Inhibitor Efficacy and Chronic Nephrotoxicity

CsA and tacrolimus are also substrates for P-glycoprotein (ABCB1) efflux pump (Saeki et al., 1993). P-glycoprotein is encoded by the ATP-Binding Cassette gene, *ABCB1*. P-glycoprotein is an ATP-dependent efflux transporter located on the outer membrane of various cell types, including the canalicular surface of hepatocytes, the apical membrane of both the proximal and distal renal tubule cells, the luminal surface of columnar epithelial cells of the intestine (enterocytes), the luminal surface of capillary endothelial cells in the brain, and the cell surface of lymphocytes (Pauli-Magnus and Kroetz, 2004). P-glycoprotein appears to limit the access of calcineurin inhibitors to organs and body compartments such as the brain, testes, placenta, heart, liver and kidneys (Schinkel et al., 1995; Fromm, 2003; Christians et al., 2006). For example, in a knockout mouse model, absence of P-glycoprotein leads to a several-fold

increase in CsA and tacrolimus concentrations in the brain (Schinkel et al., 1995; Yokogawa et al., 1999).

P-glycoprotein shows significant interindividual variability, with 2- to 8-fold variation in protein content found in small intestinal biopsies from kidney transplant patients and healthy volunteers (Lown et al., 1997). In some studies, interindividual variability in local renal P-glycoprotein expression has been shown to contribute to susceptibility to CNI nephrotoxicity (Ernest and Bello-Reuss, 1998; Tsuruoka et al., 2001; Joy et al., 2005; Naesens et al., 2009; Staatz et al., 2010b). Because P-glycoprotein is found on the apical (luminal) membrane of renal tubular epithelial cells, it can regulate the export of drugs in the tubular filtrate and urine and influence intracellular drug concentrations.

ABCB1 is polymorphic, with at least 50 SNPs identified to date (Staatz et al., 2010a). No known *ABCB1* SNP results in total loss of P-glycoprotein expression or function, although certain SNPs appear to be associated with between-patient variability in P-glycoprotein transport capacity (Staatz et al., 2010a). The most common and extensively studied *ABCB1* SNPs include a C to T transition at position 3435 within exon 26 (rs1045642), a C to T transition at position 1236 within exon 12 (rs1128503) and a G to T or A transition at position 2677 within exon 21 (rs2032582) (Kroetz et al., 2003). In some studies, the homozygous *ABCB1* 3435 TT variant genotype has been associated with lowered intestinal P-glycoprotein expression or activity *in vivo* (Hoffmeyer et al., 2000; Hitzl et al., 2001; Tanabe et al., 2001; Fellay et al., 2002; Hitzl et al., 2004). However, other studies have demonstrated the opposite association or no association *in vitro* and *in vivo* (Staatz et al., 2010a).

Genomic SNPs are often not inherited individually, but rather as a haplotype – a conserved block of DNA sequence derived from the ancestral chromosome that has remained

together after meiotic recombination (Staatz et al., 2010a). Therefore, individual SNPs may interact with multiple other SNPs to elicit a biological effect or phenotype (Staatz et al., 2010a). Three of the variant alleles of *ABCB1*, 3435C>T, 1236C>T and 2677G>T, usually occur together, indicating that they are genetically linked (in linkage disequilibrium - LD) (Staatz et al., 2010a). The *ABCB1* 1236T-2677T-3435T (T-T-T) variant haplotype is present in approximately 32% of Caucasians, 5% of African Americans, 27% of Asian Americans and 35% of Mexican Americans (Kroetz et al., 2003). Variant alleles in *ABCB1* 3435C>T, 1236C>T and 2677G>T/A, whether present individually or in LD, were reported to significantly minimize P-glycoprotein activity (0–28% activity) when compared with *ABCB1* reference activity in transepithelial cells (Salama et al., 2006). In addition, studies have shown high frequencies of both the *ABCB1* 3435T and the CYP3A5*3 variant alleles in the same population, suggesting that these polymorphisms may be genetically linked (Yates et al., 2003; Anglicheau et al., 2004; Loh et al., 2008), although there is little biological plausibility for this hypothesis because of the significant physical distance between the two genes and the inherent limit of linkage disequilibrium.

Crettol et al. suggested that *ABCB1* allelic variants can affect CsA disposition within key cellular compartments, such as lymphocytes or kidney cells (Crettol et al., 2008). Because lymphocytes represent the major target for calcineurin inhibitor therapeutic effects, whereas kidney cells represent important targets for the development of drug-related nephrotoxicity, a number of clinical studies have been conducted to evaluate the impact of the *ABCB1* allelic variants on the pharmacokinetics and pharmacodynamics of calcineurin inhibitors.

The influence of *ABCB1* 3435C>T, 1236C>T and 2677G>T/A SNPs on the pharmacokinetics of cyclosporine and tacrolimus remains uncertain, with inconsistent results (Staatz et al., 2010a). It is possible that these polymorphisms may exert a small but combined

influence, which may be additive to the effects of the CYP3A5 6986A>G SNP (Staatz et al., 2010a). However, *ABCB1* SNPs exert no clear influence on either CsA or tacrolimus pharmacodynamics, with studies showing conflicting results in regard to the main parameters of acute rejection and nephrotoxicity (Staatz et al., 2010b). Similarly, an effect of *ABCB1* genotype/ haplotype on tacrolimus-related nephrotoxicity was shown in some studies (Hauser et al., 2005; Bandur et al., 2008; Hawwa et al., 2009; Gervasini et al., 2012), but not in others (Kuypers et al., 2007; Glowacki et al., 2011).

Because CYP3A5 and P-glycoprotein expression in the donor kidney may regulate exposure of calcineurin inhibitors to renal cells, consideration of the donor kidney genotype rather than the recipient genotype may be more important when assessing development of nephrotoxicity in kidney transplant patients. The majority of studies to date have evaluated the effects of individual SNPs only. With the possibility of multiple polymorphisms interacting to produce a combined effect, haplotype analyses that consider both donor and recipient genotype may be more informative (Staatz et al., 2010b).

Inconsistent pharmacogenetic results may also be due in part to low patient numbers recruited to the investigation. In addition, the use of serum creatinine levels as a biomarker of nephrotoxicity in several studies may not be accurate (Staatz et al., 2010b). A more standardized and sensitive approach to define and evaluate nephrotoxicity is needed. For example, changes in urine metabolite patterns as a molecular marker were shown to be sufficiently sensitive for the detection of the negative effects of CsA on the kidney after a single oral dose (Klawitter et al., 2010). In addition, proteomics and metabolomics have the potential to yield sensitive and specific diagnostic tools for monitoring early changes in cell signal transduction, regulation and biochemical pathways (Christians et al., 2011). Alternatively, in a study where biopsies were

graded for chronic allograft damage index parameters and for arteriolar hyalinosis, the risk of renal progression was associated with in situ damage measured by chronic damage index (Kubal et al., 2012). Such indices of renal injury may hold significant value for future CNI pharmacogenetic studies.

Taken together, variable expression of CYP3A and P-glycoprotein causes patient-to-patient variability in the absorption, metabolism and distribution of calcineurin inhibitors, which leads to a difference in the systemic and local drug exposure of both parent drugs and their metabolites. Given the estimation that genetics can account for 20–95% of variability in drug disposition and effects (Evans and McLeod, 2003), it seems particularly important to examine the impact of CYP3A and P-glycoprotein genetic polymorphisms on the pharmacokinetics and pharmacodynamics of calcineurin inhibitors. Local renal factors which appear important for susceptibility to CNI nephrotoxicity than systemic exposure to CsA and tacrolimus, include variability in P-glycoprotein and CYP3A4/5 expression or activity, older kidney age, salt depletion, the use of non-steroidal anti-inflammatory drugs (NSAIDs), and genetic polymorphisms in genes like *TGF*-β and angiotensin converting enzyme *(ACE)* (Naesens et al., 2009).

1.4 The Impact of Pregnancy on the Pharmacokinetics of Tacrolimus

While more than 14,000 pregnancies have been reported in organ transplant recipients, these remain high-risk pregnancies for both mother and fetus (McKay and Josephson, 2006; Coscia et al., 2010). Approximately 50,000 women of reproductive age in the United States are currently living after kidney transplantation (KT), and another 2800 undergo KT each year (Deshpande et al., 2011). No comprehensive study of tacrolimus pharmacokinetics in pregnancy

has been published to date. The available data are limited to case reports (Midtvedt et al., 1997; Fehrman-Ekholm and Nisell, 1998; French et al., 2003).

Beyond immediate clinical concerns for the mother and fetus, pregnancy provides a unique opportunity to further assess factors affecting the pharmacokinetics of tacrolimus based on previously reported physiological changes such as increase in intrinsic CYP3A activity (Hebert et al., 2008), decrease in plasma albumin, α_1-acid glycoprotein (*AAG)* concentrations, and hematocrit levels during gestation (Hytten, 1985; Feghali and Mattison, 2011). Similar to the change during pregnancy, hematocrit and α_1-acid glycoprotein concentrations are generally lower in kidney transplant patients immediately post-surgery and increase significantly as the patient recovers (Huang et al., 1988). In a study of 303 kidney transplant recipients, a correlation was found between relative clearance (i.e., the ratio of dose per kg/C_{trough}) and both hematocrit ($r = 0.81$) and albumin ($r = 0.74$) concentration over the first 12 weeks post-transplant (Undre and Schafer, 1998), consistent with the strong binding of tacrolimus to red blood cells and serum albumin (Staatz and Tett, 2004).

As tacrolimus concentrations are measured in whole blood, low hematocrit and albumin concentration will result in a reduction in total drug concentration in whole blood. In such a situation, whole blood drug clearance 'appears' to increase. In the absence of any changes in intrinsic metabolic processes, unbound drug clearance should remain the same (Staatz and Tett, 2004). Because of the extensive binding of tacrolimus to albumin and AAG (Weiss et al., 2008), changes in plasma protein concentrations in pregnancy may alter tacrolimus disposition kinetics. In particular, a change in the unbound drug concentration may affect its systemic clearance. Changes in hematocrit may also influence the metabolic clearance of tacrolimus (Chow et al., 1997). The investigation of tacrolimus pharmacokinetics in pregnancy should further enable us

to examine the contributors to interindividual variability in CNI pharmacokinetics and pharmacodynamics.

In summary, an understanding of the influence of genetic and physiological factors (including pregnancy) on the pharmacokinetics of calcineurin inhibitors could allow identification of the optimal immunosuppressant drug combination for a particular individual, assist in early prediction of the optimal starting dose and maintenance regimen, and help identify patients with an increased risk of chronic side effects, such as nephrotoxicity

1.5 Research Directions

In the chapters that follow, a series of investigations are presented where we explored the impact of polymorphic *CYP3A5* expression on the *in vivo* metabolism and disposition of cyclosporine A (CsA) and tacrolimus. The purpose of these studies was to better understand the inter-individual differences in CsA and tacrolimus dose requirements and to assess the pharmacokinetic basis for differential clinical observations in the occurrence of chronic calcineurin inhibitor nephrotoxicity between CYP3A5 expressors and nonexpressors. In addition, the study of tacrolimus pharmacokinetics in pregnancy, and the impact of physiological changes that occur during pregnancy, may provide valuable information on maternal and fetal drug exposure under the current dosing regimen and may have direct clinical applicability for tacrolimus dosing during gestation. Likewise, the assessment of *in utero* and neonatal tacrolimus exposure offer valuable information with respect to assessment of risk of neonatal exposure to tacrolimus in infants of mothers who are transplant recipients.

Research presented in **Chapter 2** examines how *CYP3A5* gene variation influences the systemic and intrarenal tacrolimus disposition in healthy subjects (N=24). An oral dose of

tacrolimus (5 mg) was administered to 24 healthy volunteers who were selected based on their *CYP3A5* genotype after extensive screening. A specific and sensitive analytical method utilizing LC-MS/MS was developed to quantify tacrolimus and its four primary metabolites in blood, plasma and urine. Ultracentrifugation was used to determine the unbound fraction of tacrolimus in plasma. Apparent urinary clearance, which is a function of both excretory and metabolic processes in the kidneys, served a focal parameter for comparing the impact of *CYP3A5* genotype on renal disposition of tacrolimus. A semi-physiological model was developed to evaluate the effect of the *CYP3A5* polymorphism on intrarenal metabolism and epithelial exposure to tacrolimus. *ABCB1* SNPs/haplotype was also identified to examine if they constitute a significant co-variate in determining the urinary clearance of tacrolimus.

Research presented in **Chapter 3** examines how *CYP3A5* gene variation affects the systemic and intrarenal CsA disposition in the same group of healthy subjects as described in **Chapter 2** (N=24). An oral dose of CsA (5 mg/kg) was administered to 24 healthy volunteers who were selected based on their *CYP3A5* genotype. A specific and sensitive analytical method utilizing LC-MS was developed to quantify CsA and its primary and secondary metabolites in blood, plasma and urine. The study results differ somewhat from those described in **Chapter 2**, both in terms of metabolite formation and parent drug disposition; thus, the impact and clinical relevance of the *CYP3A5* polymorphism on tacrolimus and CsA pharmacotherapy may differ.

In **Chapter 4**, a series of *in vitro* experiments were presented to aid in the interpretation of *in vivo* data described in **Chapter 2**, **Chapter 3** and **Chapter 7**. The disappearance clearance of tacrolimus and its primary metabolites was evaluated using CYP3A4, CYP3A5 and CYP3A7 Supersomes. The time course of tacrolimus product formation from these CYP3A enzymes was also compared. Importantly, for CsA, the formation of secondary metabolites was examined by

incubating the parent drug and its primary metabolites, individually, with CYP3A4 and CYP3A5 Supersomes in order to evaluate the kinetics of the sequential reactions and the impact of CYP3A5 genetic variation.

Research presented in **Chapter 5** explores how changes in albumin and hematocrit levels as well as the enzymatic activity change in pregnancy, affect tacrolimus disposition. This involved an opportunistic study of the pharmacokinetics of tacrolimus in pregnant women being prescribed tacrolimus for immunosuppression during early to late pregnancy (N=10) and postpartum (N=5). The study highlighted the critical changes in tacrolimus binding to erythrocytes and plasma proteins as driven by the variation in albumin concentration and hematocrit during the course of pregnancy and their impact on maternal tacrolimus pharmacokinetics.

Chapter 6 provides a discussion of the clinical relevance of the study findings described in **Chapter 5**. Particular emphasis is placed on the debatable issue of adjusting tacrolimus dosage during pregnancy in order to maintain whole blood tacrolimus concentrations in the usual therapeutic range, which results in an increase in circulating unbound drug concentration. The interpretation of tacrolimus concentration in the presence of anemia and hypoalbuminemia during pregnancy is also presented.

In **Chapter 7**, the *in utero* and neonatal drug exposure to tacrolimus was assessed by investigating the placental transfer of tacrolimus at term in pregnant women being prescribed tacrolimus for immunosuppression (N=8) and breast milk drug excretion postpartum (N=1). The umbilical venous cord blood-to-maternal blood concentration ratios reflect how much drug crosses the placenta *in vivo,* while the concentration differences between the umbilical vein and artery provide information on drug distribution or metabolism by the fetus. To elucidate the

difference in whole blood-to-plasma concentration ratios between maternal and cord blood, the partitioning of tacrolimus between blood cells and plasma was estimated using a classic model. In addition, the experimentally determined unbound drug concentration in milk and plasma may aid in understanding the mechanism of transfer of tacrolimus across the placenta and the mammary epithelia.

Finally, **Chapter 8** provides a summary of results in the above research chapters and potential directions for future studies that may further enhance our understanding of the contribution of both genetic and physiological factors to inter-individual differences in the metabolism and biological effects of calcineurin inhibitors.

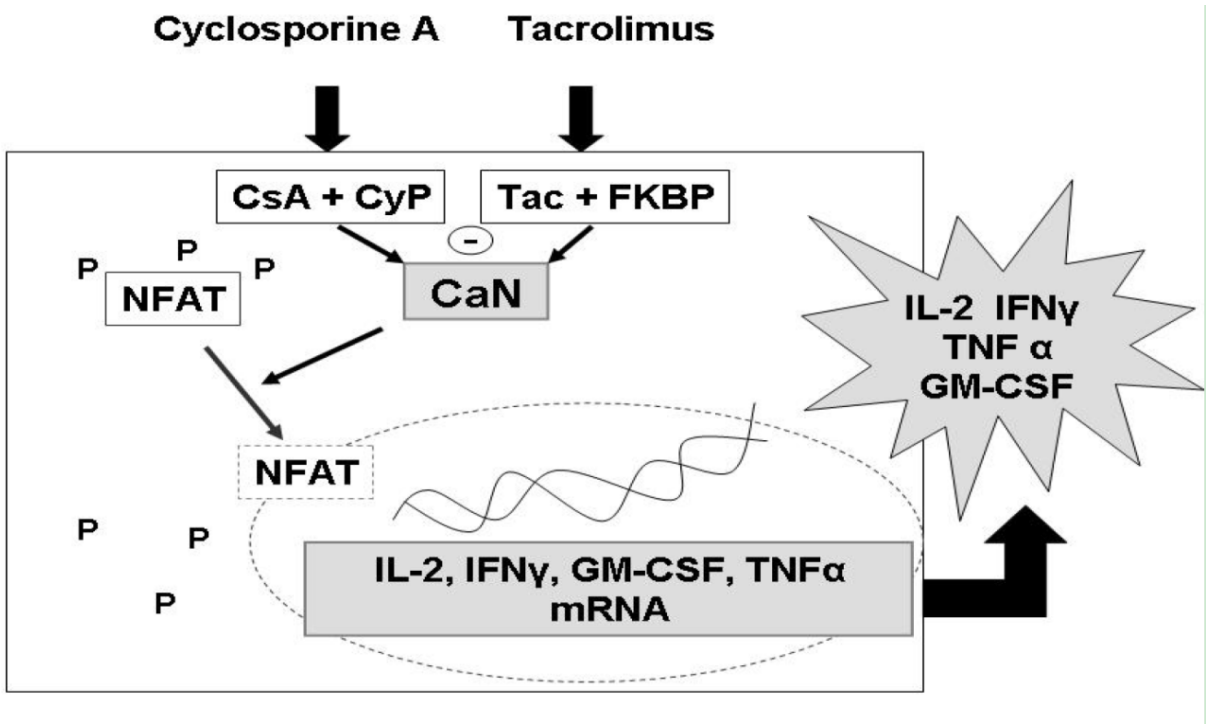


Figure 1.1 The principal action of calcineurin inhibitors, CsA and Tac, within the T-lymphocytes: inhibition of the phosphatase calcineurin.

This figure is adapted from a published illustration (Sommerer et al., 2010). CaN: the protein phosphatase calcineurin; CyP: CsA-cyclophilin protein complex; FKBP: FK506-binding protein complex; NFAT: nuclear factor of activated T-cells; IL-2: interleukin 2; IFNγ: interferon γ; GM-CSF: granulocyte-macrophage colony-stimulating factor.

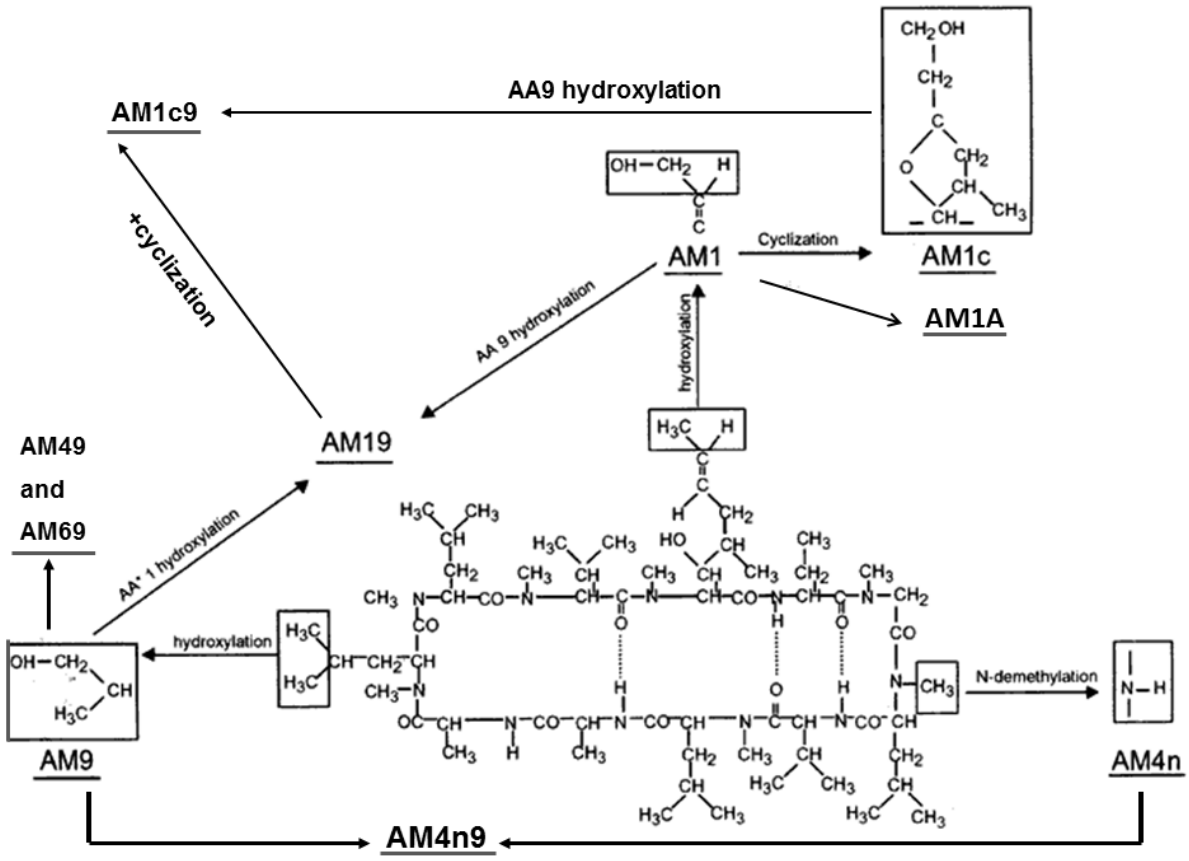
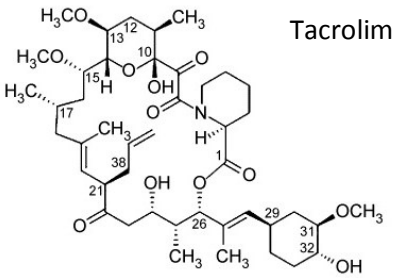


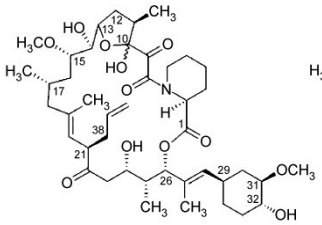
Figure 1.2 Metabolic pathway of Cyclosporine A (CsA).

This figure is modified from a published illustration (Kelly and Kahan, 2002). The nomenclature for the conversion products is based upon CsA being cyclosporin A, followed by an M designating a metabolite, and a number indicating the amino acid position of the conversion. The use of two numbers indicates similar transformations at two positions. Unless designated by an "n" to signify demethylation or a "c" to signify cyclization, all other metabolites represent hydroxylations (Kahan et al., 1990). AM1, AM9, and AM4n are the predominant metabolites found in human blood and urine. Other metabolites of CsA result from further metabolism of the primary metabolites.

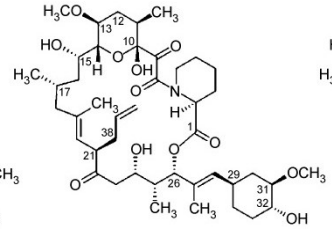


Tacrolimus

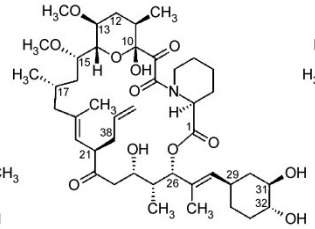
First Generation



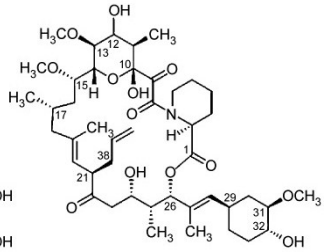
13-*O*-desmethyl tacrolimus



15-*O*-desmethyl tacrolimus

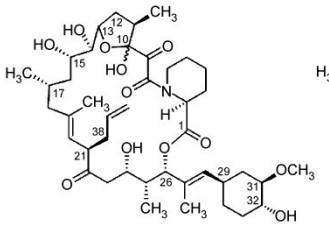


31-*O*-desmethyl tacrolimus

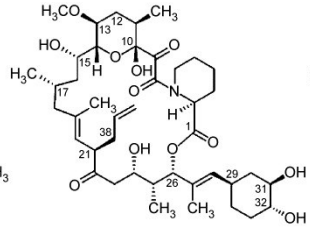


12-hydroxy tacrolimus

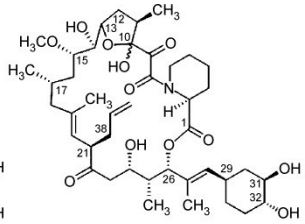
Second Generation



13, 15-*O*-Didesmethyl-tacrolimus



15, 31-*O*-Didesmethyl-tacrolimus



13, 31-*O*-Didesmethyl-tacrolimus

Figure 1.3 Cytochrome P450 3A-mediated hydroxylation, demethylation and oxidation of tacrolimus.

This figure is derived from a published illustration (Tata et al., 2009). The metabolites are classified as first- and second-generation metabolites. First generation metabolites are those directly derived from tacrolimus and are changed in one position (13-DMT, 13-*O*-desmethyl tacrolimus; 15-DMT, 15-*O*-desmethyl tacrolimus; 31-DMT, 31-*O*-desmethyl tacrolimus; 12-HT, 12-hydroxy tacrolimus). Metabolic changes in certain positions such as 13-*O*-demethyl tacrolimus, lead to secondary non-enzymatic rearrangement of the macrolide ring resulting in several isomers (Tata et al., 2009).

Chapter 2

CYP3A5 Gene Variation Influences both Systemic and Intrarenal Tacrolimus Disposition

Portions of Chapter 2 were submitted to *Clinical Pharmacology & Therapeutics.*

2.1 Abstract

We evaluated the hypothesis that CYP3A5 expression can affect intrarenal tacrolimus accumulation. An oral dose of tacrolimus was administered to 24 healthy volunteers who were selected based on their *CYP3A5* genotype. Compared to CYP3A5 nonexpressors, expressors had a 1.6-fold higher oral tacrolimus clearance and 2.0- to 2.7-fold higher metabolite/parent AUC ratios for 31-DMT, 12-HT and 13-DMT. In addition, the apparent urinary tacrolimus clearance was 36% lower in CYP3A5 expressors, compared to nonexpressors. To explore the mechanism behind this observation, we developed a semi-physiological model of renal tacrolimus disposition and predicted that tacrolimus exposure in the renal epithelium of CYP3A5 expressors is 53% of that for CYP3A5 nonexpressors, when normalized to blood AUC. These data suggest that at steady state, intrarenal accumulation of tacrolimus, and its primary metabolites, will depend on the *CYP3A5* genotype of the liver and kidneys. This may contribute to inter-patient differences in the risk of tacrolimus-induced nephrotoxicity.

2.2 Introduction to Chapter 2

The use of tacrolimus, a calcineurin inhibitor, to prevent solid organ transplant rejection is routinely guided by therapeutic blood level monitoring (TDM) (Scott et al., 2003). Despite close monitoring and individualization of dosing, TDM has not prevented some transplant recipients from developing chronic calcineurin inhibitor nephrotoxicity (CNIT) (Ojo et al., 2003; Ekberg et al., 2007).

The pathogenesis of CNIT is complex and largely unpredictable (Metalidis et al., 2011). However, there is evidence that the drug concentration in kidney tissue is more predictive of CNIT than systemic blood concentration (Fukudo et al., 2008; Naesens et al., 2009; Metalidis et

al., 2011). In addition, some studies suggest that a higher systemic exposure to tacrolimus metabolites or intrarenal production of potentially toxic metabolites may play a contributory role in the development of CNIT (Kuypers et al., 2007; Kuypers et al., 2010; Min et al., 2010; Metalidis et al., 2011). There is also evidence to suggest that inherited variation in an individual's genome contributes to the risk of nephrotoxicity (Kuypers et al., 2007; Naesens et al., 2009). This includes variation in CYP3A genes that influence the bioavailability and metabolic clearance of tacrolimus (Sattler et al., 1992). CYP3A4 is expressed in the liver and small intestine of nearly all individuals, but with substantial inter-individual variability that is largely unexplained to date by genetic factors (Lampen et al., 1995; Haehner et al., 1996; Kuehl et al., 2001; Lin et al., 2002; Givens et al., 2003). In contrast, polymorphic expression of CYP3A5 in the liver, small intestine, kidney and other organs is determined primarily by single-nucleotide variations that distinguish the "active" *CYP3A5*1* allele (inferred CYP3A5 expressor phenotype) from the "inactive" *CYP3A5*3*, **6* or **7* alleles (inferred CYP3A5 nonexpressor phenotype). Individuals carrying two loss-of-function *CYP3A5* alleles have a markedly reduced level of CYP3A5 tissue expression and catalytic activity (Lampen et al., 1995; Haehner et al., 1996; Kuehl et al., 2001; Lin et al., 2002; Givens et al., 2003). There are data suggesting a significant impact of the *CYP3A5* polymorphism on the metabolism of tacrolimus. Metabolism of tacrolimus is more efficient with recombinant CYP3A5 than recombinant CYP3A4. In addition, a higher metabolic clearance of tacrolimus has been observed in liver tissue obtained from CYP3A5-expressing organ donors than from nonexpressors (Kamdem et al., 2005; Dai et al., 2006). In contrast, in the kidneys, only CYP3A5 (and not CYP3A4) is found at levels thought sufficient to affect intra-tissue drug clearance (Haehner et al., 1996; Bolbrinker et al., 2012). Microsomes from *CYP3A5*1/*3* genotyped kidney tissues exhibited at least an 8-fold

higher CYP3A5 content and 18-fold higher midazolam or tacrolimus hydroxylation activity than microsomes from *CYP3A5*3/*3* genotyped tissues (Givens et al., 2003; Dai et al., 2006); an observation consistent with CYP3A5 being the predominant CYP3A isoform expressed in renal tissue (Haehner et al., 1996; Metalidis et al., 2011).

Pharmacokinetic studies in heart, lung, liver and kidney transplant patients have shown a significant difference in dose-normalized blood tacrolimus level between the CYP3A5 expressors versus nonexpressors; the CYP3A5 expressors typically required a larger tacrolimus dose to achieve the same, therapeutic trough blood concentration (Hesselink et al., 2003; Thervet et al., 2003; Haufroid et al., 2004; Staatz and Tett, 2004). Thus, inheritance of the *CYP3A5*1* allele could affect systemic and intrarenal concentrations of tacrolimus and its metabolites during drug therapy (Dai et al., 2006; Kuypers et al., 2010), and in turn the risk of CNIT.

To test this hypothesis, we examined the apparent urinary tacrolimus clearance in CYP3A5 expressors compared to CYP3A5 nonexpressors. The relationship between blood concentration and urinary excretion rate (i.e., apparent urinary clearance) should depend upon whether or not CYP3A5-dependent intrarenal tacrolimus metabolism is present; specifically, apparent urinary clearance of tacrolimus should be lower in CYP3A5 expressors compared to nonexpressors, reflecting intrarenal loss due to metabolism. In addition, we evaluated whether a CYP3A5-dependent difference in systemic tacrolimus clearance was associated with a difference in the accumulation of the known primary tacrolimus metabolites in blood that may also contribute to the risk of nephrotoxicity.

2.3 Materials and Methods

2.3.1 **Materials**

Tacrolimus for analytical use was kindly provided by Fujisawa USA Inc. (now Astellas Pharma US, Inc., Deerfield, IL). Tacrolimus internal standard (FK506-$^{13}C,D_2$) was purchased from Toronto Research Chemicals (catalog # F370002). The metabolites used for calibration curves were generated biologically and purified chromatographically (see "Isolation and Mass Spectrometric Analysis of Tacrolimus Metabolites"). Blank human (outdated) plasma and blood were purchased from Puget Sound Blood Center (Seattle, WA). Methanol (Optima grade), acetonitrile (Optima grade), ammonium acetate, sodium acetate and silanized inserts (catalog #03-375-3AS) were purchased from Fisher Scientific (Santa Clara, CA). Methyl tert-butyl ether (J.T. Baker) was purchased from VWR International.

2.3.2 Clinical Protocol

The human subjects protocol was approved by the University of Washington Institutional Review Board. Written informed consent was obtained from all study participants. A single oral dose of tacrolimus (5 mg) was administered to 24 healthy participants selected based on their *CYP3A5* genotype. None of the subjects had a significant medical history or abnormal clinical lab test results, and none had taken a known inhibitor, inducer, or activator of CYP3A4/5 (other than oral contraceptives) for at least 1 month preceding the start of and during the pharmacokinetic investigation, and all abstained from grapefruit and grapefruit juice and alcoholic beverages beginning at one week prior to the start until the end of the study. Sequential blood samples (5 ml) were collected in EDTA glass tubes just before and at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 14, 16, 22, 24, 48, 72 and 96 h after oral drug administration beginning at 8 am. Plasma was harvested from an aliquot of the blood samples after incubation at 37°C for 30 min

and centrifugation at 37°C. Urine was collected in silanized glass containers over the following post-dose intervals: 0-2 h, 2-4 h, 4-6 h, 6-12 h, 12-24 h, 24-48 h, 48-72h, and 72-96 h. All samples were stored at −80°C until analysis.

2.3.3 Genotyping

Buccal cell DNA was isolated using a DNeasy Blood & Tissue Kit or the Qiagen Gentra Puregene protocol (Qiagen, USA). Single-nucleotide polymorphisms (SNPs) in the *CYP3A5* gene (*3, *6 and *7 alleles) were determined from a buccal swab tissue sample, using either previously published methods by Lin *et al.* (Lin et al., 2002) or a validated Taqman® allelic discrimination assay from Applied Biosystems (Foster City, CA) (Hebert et al., 2008). The *ABCB1* C3435T and C1236T SNPs were genotyped using TaqMan® assays (Hebert et al., 2008). The *ABCB1* G2677T/A multivariate SNP was determined by PCR amplification and DNA sequencing using published oligonucleotides (Asano et al., 2003).

2.3.4 Tacrolimus and Metabolite Analysis

2.3.4.1 *Isolation of Metabolites*

Tacrolimus metabolites were generated by incubation of tacrolimus (0.215 mg/mL) with pooled human liver microsomes (3 mg/ml) and NADPH (1mM) for 1 hour. The reaction mixture was extracted twice with methyl-tert-butyl ether, and the organic layers were collected and evaporated to dryness under a stream of nitrogen at 30° C. The residue was reconstituted in methanol and injected onto an analytical column (Symmetry C18, 250 × 4.6 mm; Waters, Milford, MA) heated to 60° C. Metabolites were eluted with a gradient of two mobile phases: H_2O (A) and acetonitrile (B). The linear gradient extended from 37% B at 0 min to 62% B at 60

min; the column was washed between runs with 100% B for 30 min. The flow rate was 1 mL/min. Fractions containing UV visible metabolites were collected manually encompassing the following retention times: 27.2 min (13-DMT), 34.5-35.5 min (12-HT), 39.5 min (15-DMT), 46.5 min (31-DMT). Each was dried completely under a stream of nitrogen in heated evaporator, and re-suspended in methanol. The major metabolite in each fraction was purified further, using another analytical liquid chromatography column (Luna, C18(2), 5 μ, 100A, 250x4.6 mm; Phenomenex, Torrance, CA) heated to 60° C. The mobile phases were 20 mM NH_4Ac, pH 7.5 (A) and methanol (B). The metabolites were eluted with a linear gradient that extended from 40% B at 0 min to 90% B at 180 min; the flow rate was 1 mL/min. Metabolite peaks were collected encompassing 81 min (13-DMT), 92-93 min (12-HT), 94.5 min (15-DMT), 106.5 min (31-DMT), and the solvent was removed as described above. Isolated metabolites were re-suspended in methanol and stored at –80° C.

2.3.4.2 *Identification of Metabolites*

The isolated metabolites were identified using QTOF LC-MS analysis, comparing exact masses and retention times of the purified products with previously isolated and validated metabolites (Christians et al., 1991d; Lampen et al., 1995; Dai et al., 2006). Liquid chromatography was performed with an Agilent HP 1200 that was coupled to an Agilent QTOF 6520 mass spectrometer. The column (Luna, Phenyl-Hexyl 150 × 2 mm, 3 micron; Phenomenex, Torrance, CA) was heated to 60° C. A stepwise linear gradient was used to elute the metabolites: 60% B at 0 min, 90% B at 10 min, and held at 90% for 3 min, 100% B at 13.1 min and held for 5 min and then returned to initial conditions. Solvent A was 20 mM ammonium acetate (pH 7.5) in H_2O, and solvent B was 100% methanol. The flow rate was 0.25 mL/min and the total run time

was 26 minutes. The mass to charge ratios (m/z) of the MS spectra extracted from the total ion chromatograms were compared with compounds listed in METLIN: Metabolite and Tandem MS Database at the Scripps Center for Metabolomics website http://metlin.scripps.edu/. Using the "Metlin Metabolite Search: Simple," we searched for all positively charged ions with proton, sodium, potassium, and ammonium adducts and/or the neutral loss of one or two molecules of water. The MS spectra of the isolated metabolites gave m/z ion ratios that were within 4 ppm of the authentic standards. Concentrations of the isolated metabolite stock solutions were determined by UV absorbance, using a standard curve of tacrolimus peak areas and assuming a common extinction coefficient at 214 nm (Lampen et al., 1995; Dai et al., 2006).

2.3.4.3 *LC/MS-MS Quantification of Tacrolimus and Metabolites*

Tacrolimus and its metabolites in blood, plasma and urine samples were quantified by LC/MS/MS. A published extraction method (Chen et al., 2006) was adapted, with the following modifications: 0.5 mL of blood, plasma, or urine was mixed with 0.5 mL 0.01 M ammonium acetate, pH 7.5, 10 µL internal standard (250 ng/mL stock) and extracted with 4 mL of methyl-t-butyl ether, using glass screw-cap tubes. Samples were shaken for 20 minutes using a horizontal shaker and centrifuged (~2000 *g*) at room temperature. The top organic layer was dried, then re-suspended in 0.1 mL methanol and transferred to LC vials containing silanized glass inserts. A 15 µL aliquot of each extracted sample was injected into the LC–MS/MS system. Calibration standards were constructed from serial dilutions of methanolic stock solutions added to blank human blood, plasma or urine. These and quality control samples, were spiked with internal standard and extracted, as described above. The analytical instrument consisted of an Agilent series 1200 HPLC coupled to an Agilent series 6410 triple quadruple tandem mass spectrometer.

Solvent A was 20 mM ammonium acetate and 0.1 mM sodium acetate in H_2O (pH 7.5), and solvent B was 100% methanol. The chromatography column and gradient conditions were the same as that used for metabolite identification, described above. The elution times for 13-DMT, 12-HT, 15-DMT, 31-DMT, internal standard, and tacrolimus, were 8.5 min, 9.8 min, 9.8 min, 10.4 min, 11.4 min, and 11.4 min, respectively. Sodium adducts of tacrolimus and metabolites were detected with multiple reaction monitoring under the ESP^+ mode. The ion transition was *m/z* 812.2>602.4 for 13-DMT and 15-DMT, *m/z* 842.2>521.3 for 12-HT, *m/z* 812.2>616.3 for 31-DMT, *m/z* 830.2>620.4 for internal standard, and *m*/*z* 826.5 > 616.3 for tacrolimus (**Figure 2.7**).

Calibration curves for tacrolimus and its metabolites were generated by plotting the peak area ratios of tacrolimus or metabolite to internal standard against known standard tacrolimus or metabolite concentrations. Standard curve concentrations for tacrolimus ranged from: 0.005 ng/mL to 40 ng/mL. The limit of quantitation was 0.005 ng/mL for tacrolimus, and in the range of 0.003-0.04 ng/mL for 13-DMT, 15-DMT, 31-DMT and 12-HT in blood, urine and plasma samples. Intra-day and inter-day coefficients of variation for the assays were all less than 10%.

2.3.5 Pharmacokinetic Analysis

Noncompartmental pharmacokinetic analysis was performed using WinNonlin software version 5.2 (Pharsight, Mountain View, CA). Pharmacokinetic parameters determined for tacrolimus included the maximum concentration in blood (C_{max}), the time to reach maximum concentration (T_{max}), terminal half-life ($t_{1/2}$), AUC (0-96 h), AUC (0-infinity), and oral clearance (CL/F normalized to individual body weight in kg). In addition, $CL_{urinary}$ was calculated as the amount of drug or metabolite excreted in urine divided by AUC_{blood} over the collection interval.

2.3.6 Compartmental Model for Renal Metabolism

A semi-physiological model was developed to evaluate the effect of *CYP3A5* polymorphism on intrarenal metabolism and tubulo-epithelial exposure to tacrolimus. The model as shown in **Figure 2.4** consists of three compartments representing the systemic blood pool [*q1*], the tubular lumen [*q2*], and the tubular epithelium [*q3*]. Blood tacrolimus concentration-time data were entered as a forcing function (FF) for the blood compartment (Barrett et al., 1998), which obviated the need to model the systemic disposition kinetics of tacrolimus. CYP3A5-mediated metabolism is assumed to occur in the epithelial compartment as demonstrated by previous studies (Lohr et al., 1998; Bolbrinker et al., 2012). The transfer of drug between compartments and the metabolic process is assumed to follow first-order kinetics; the six rate constants are defined in **Figure 2.4**. The unidirectional transfer rate constant *k(2,1)* represents the glomerular filtration of tacrolimus. *k(3,1)* and *k(1,3)* are the bidirectional rate constants for the exchange of drug between the efferent arteriolar blood and the epithelium (i.e., basolateral transport). *k(3,2)* and *k(2,3)* are the bidirectional rate constants for the exchange of drug between the tubular epithelium and the lumen (i.e., apical transport). *k(0,3)* represents CYP3A5-mediated biotransformation of tacrolimus in the epithelium, which is effectively zero for CYP3A5 nonexpressors (Dai et al., 2006) and assumes a characteristic value for CYP3A5 expressors. Because blood concentration is used as the driver for the blood compartment, *k(2,1)* and *k(3,1)* have the unit of flow (ml/h). All the other first-order rate constants have the typical dimension of reciprocal time (h^{-1}).

In an effort to introduce a physiological framework for the model and at the same time provide realistic constraint on the parameter estimates, the rate constants for glomerular filtration *k(2,1)* and uptake from the efferent arteriole *k(3,1)* were re-parameterized as follows.

$$k(2,1) = f'_{ub} \cdot GFR$$

where f'_{ub} = exchangeable fraction of tacrolimus in blood, and *GFR* = glomerular filtration rate.

$$k(3,1) = ER_{Exp} \cdot Q_{effart} \quad \text{for the } \textit{CYP3A5} \text{ expressors}$$

$$k(3,1) = ER_{NEx} \cdot Q_{effart} \quad \text{for the } \textit{CYP3A5} \text{ nonexpressors}$$

where *ER* = extraction ratio of tacrolimus from the efferent arteriole for either a CYP3A5 expressor (*Exp*) or CYP3A5 nonexpressor (*NEx*); and Q_{effart} = efferent arteriolar blood flow. In turn, ER_{Exp} and ER_{NEx} can be expressed as a function of the apparent tissue-to-blood partitioning coefficient of tacrolimus ($K_{p,Exp}$ or $K_{p,NEx}$), epithelial volume (V_{epi}), f_{ub}, *GFR*, Q_{effart}, and the inter-compartmental transfer rate constants.

$$ER_{Exp} = \frac{K_{p,Exp} \cdot V_{epi} \cdot [k(1,3) + k(0,3)] \cdot k(3,2) - f_{ub} \cdot GFR \cdot [k(1,3) + k(0,3) + k(3,2) + k(2,3)]}{[k(2,3) + k(3,2)] \cdot Q_{effart}}$$

$$ER_{NEx} = \frac{K_{p,NEx} \cdot V_{epi} \cdot k(1,3) \cdot k(3,2) - f_{ub} \cdot GFR \cdot [k(1,3) + k(3,2) + k(2,3)]}{[k(2,3) + k(3,2)] \cdot Q_{effart}}$$

It should be noted that $K_{p,kid}$ is experimentally defined as

$$K_{p,kid}^{CYP3A5} = \frac{q_2^{SS} + q_3^{SS}}{V_2 + V_3}$$

where q_i^{SS} is the steady-state amount of tacrolimus in each of the renal tissue compartments, and V_i is the corresponding compartment volume. Given that CYP3A5 expressors have a metabolic sink in the epithelial compartment (*q3*), their apparent steady-state $K_{p,kid}$ is expected to be lower than that in CYP3A5 nonexpressors.

The above model was implemented using the general purpose compartmental modeling software SAAM II (The Epsilon Group, Charlottesville, VA). The model was fitted to the

amount of tacrolimus excreted in the successive urine collection periods over the 96 hours following a single dose of tacrolimus.

Individual estimated glomerular filtration rate (eGFR) was calculated using the Cockroft-Gault equation (Cockcroft and Gault, 1976), which incorporated the variables of sex, age, weight, serum creatinine concentration; it was also entered into model-fitting as fixed value.

Estimates for the physiological parameters: Q_{effart} and V_{epi} were taken from the literature, scaled to the individual body weight, and entered into model-fitting as fixed values for each individual. About 80% of total kidney volume is composed of tubular epithelial cells and cells within the interstitial space; most of the other cell types are associated with the rich vascular network of the kidney (Klahr and Morrissey, 2002). We assumed that CYP3A5 is expressed in all tubular epithelial cells, and assigned V_{epi} a value that equals 80% of the total kidney volume. The mean total kidney volume was obtained from Cheong *et al.* (Cheong et al., 2007), 202 ± 36 ml per kidney for men (weight = 90 ± 16 kg) and 154 ± 33 ml per kidney for women (weight = 73 ± 18 kg). The individual kidney volume entered into model-fitting as a fixed value was calculated as 202·(actual weight/90) for men and 154·(actual weight/73) for women.

Efferent arteriolar blood flow rate (Q_{effart}) was estimated as the afferent arteriolar blood flow rate (Q_{affart}) minus the glomerular filtration rate (Myers et al., 1975). Further recognition of the following relationship of Q_{affart} to GFR, filtration fraction (FF), and arterial hematocrit (44, 45) yields the estimating equation for Q_{effart}.

$$GFR = Q_{affart} \cdot (1 - Hct_{art}) \cdot FF$$

$$Q_{effart} = Q_{affart} - GFR = \frac{GFR}{(1 - Hct_{art}) \cdot FF} - GFR$$

The afferent arterial hematocrit is approximated by the hematocrit measured from venous blood for each individual. A mean value of 23.1 ± 0.7% was used as filtration fraction in healthy adults

(5 men and 7 women) aged 30.2 ± 4.6 years (Anastasio et al., 2001). Individual eGFR was used as the input as GFR in the above equations.

The exchangeable fraction of tacrolimus in blood available for glomerular filtration (f'_{ub}) was estimated from the nonlinear regression fit. We did consider setting f'_{ub} equal to the equilibrium free fraction determined *in vitro*. However, preliminary model fitting indicated that the effective f'_{ub} *in vivo* was lower than the *in vitro* equilibrium values.

While human renal tacrolimus tissue distribution data are not available, a $K_{p,kid}$ of 12.2 has been reported for male rats receiving oral administration of tacrolimus for four days (Qin et al., 2010). Tacrolimus is a substrate for rat CYP3A isoforms (Wu and Benet, 2003) and considering their expression and activity in the rat kidneys (Ghosh et al., 1995; Ronis et al., 1998), the rat $K_{p,kid}$ was entered as a mean for the prior distribution probability in CYP3A5 expressors, along with an assumed standard deviation of 15% that reflect the usual magnitude of analytical errors. The standard deviation for the Bayesian prior was not a sensitive parameter. All the inter-compartmental transfer rate constants, viz. *k(1,3)*, *k(2,3)*, *k(3,2)* and *k(0,3)*, were estimated from the nonlinear least-squares regression fit. A relative weighting scheme (FSD 0.1) was used, which assumes a constant coefficient of variation of 10%.

A preliminary model fit was conducted with mean urinary excretion data for the CYP3A5 expressor and nonexpressor groups. The use of mean excretion data offered the benefit of assessing the general plausibility of the three-compartment model in describing the urinary excretion data. It also had the advantage of allowing a simultaneous fit of data from the two CYP3A5 phenotype groups to evaluate the impact of a metabolic sink in the epithelial compartment on $K_{p,kid}$, about which we lack prior information.

Modeling of the individual data sets was then conducted as a two-stage process. In the first stage, data from the CYP3A5 nonexpressors were fitted to the three-compartment model with *k(0,3)* set to zero. The mean and standard deviation of each of the fitted parameters served as Bayesian priors for the second stage analysis with data from the CYP3A5 expressors; in so doing, k(0,3) became the only parameter that was left to float in the model fitting. The Bayesian prior estimate for $K_{p,kid}$ values in the CYP3A5 expressors was set to 12.2 ± 2.0, and the prior for $K_{p,kid}$ in CYP3A5 nonexpressors (23.4 ± 4.0) was set to be about twice of the expressor value based on the preceding simultaneous fit of mean data. The staged analysis avoided some of the modeling difficulties we encountered in the beginning and provided the most robust estimation of the inter-compartmental transfer rate constants.

In two of the 12 CYP3A5 nonexpressors, we encountered difficulty in estimating their *k(3,2)*. The problem was resolved by using the mean of the *k(3,2)* estimates from the other 10 subjects as Bayesian priors. In five of the 12 CYP3A5 expressors, mean estimates from the other seven subjects were used as priors in estimating *k (0,3)*. Convergence was reached all 24 subjects (**Figure 2.9**).

In an attempt to predict the impact of intrarenal metabolism on the exposure of the tubular epithelium to intact tacrolimus, the amount of tacrolimus in the epithelial compartment versus time was simulated for each subject using the final parameter estimates. The individually predicted time course of tacrolimus and the respective mean drug exposure in the renal epithelium were compared between the *CYP3A5* expressor and nonexpressor groups.

2.3.7 Statistical Analysis

Descriptive statistics are presented as mean ± standard deviation. Statistical comparisons were conducted using an unpaired two-sided Student's t-test (GraphPad Prism 5, La Jolla, CA). A *P* value less than 0.05 was considered significant.

2.4 Results

2.4.1 Demographic characteristics of healthy volunteers

Twenty-four healthy subjects, comprised of 12 CYP3A5 expressors (*CYP3A5*1*-allele carriers) and 12 nonexpressors (subjects who carry two copies of a loss-of-function *CYP3A5* allele: *CYP3A5*3*, *CYP3A5*6* or *CYP3A5*7*), were enrolled. There were no significant differences between the two CYP3A5 phenotype groups with respect to distribution of gender, body weight, serum creatinine, creatinine clearance, and estimated GFR (eGFR) (**Table 2.1**). However, the mean age of CYP3A5 expressors was older than that of nonexpressors (30.8 ± 9.9 vs. 23.5 ± 3.5 yrs, $P = 0.03$). The frequency of *ABCB1* 3435C>T, 1236C>T, 2677G>T/A SNPs and the 1236T-2677T-3435T (T-T-T) variant haplotype did not differ significantly between the CYP3A5 phenotype groups.

2.4.2 Systemic disposition of tacrolimus and its primary metabolites

The pharmacokinetics of tacrolimus and its metabolites differed between CYP3A5 expressors and nonexpressors. Blood or plasma tacrolimus concentrations were, on average, lower in CYP3A5 expressors compared to nonexpressors (**Figure 2.8**), as reflected in a 1.6-fold higher mean oral tacrolimus clearance (CL/F; which is dependent on both intestinal and hepatic

CYP3A activity) for CYP3A5 expressors (**Table 2.2**). The peak blood concentration (C_{max}) and time to peak concentration (t_{max}) of tacrolimus were not significantly different; however, the blood concentration at 96 hours after drug administration (C_{last}) was 1.9-fold higher in CYP3A5 nonexpressors compared to expressors (P = 0.0003). The mean blood concentration–time profiles of tacrolimus and its four primary metabolites after oral tacrolimus administration in CYP3A5 expressors and CYP3A5 nonexpressors are shown in **Figures 2.1A** and **2.1B**, respectively. The concentration of all metabolites was less than that of parent drug across all time points. As seen in **Figure 2.2,** the mean $AUC_{metabolite}/AUC_{parent}$ ratio for 31-DMT, 12-HT and 13-DMT, an indirect measure of the respective metabolite formation clearances, was 2.5-, 2.7- and 2.0-fold higher in CYP3A5 expressors than the ratios for CYP3A5 nonexpressors ($p < 0.001$ for all). In contrast, the mean ratio for 15-DMT did not differ between the two CYP3A5 phenotype groups.

2.4.3 Renal excretion of tacrolimus and its primary metabolites

The total amount of tacrolimus excreted in urine as unchanged drug over 96 hours after oral administration was 271.6 ± 147.4 (ng) and 642.6 ± 349.6 (ng) for CYP3A5 expressors and nonexpressors, respectively (P = 0.003); i.e., 58% lower in CYP3A5 expressors compared to nonexpressors. The mean apparent urinary tacrolimus clearance was 36%, based on blood AUC, or 49% based on plasma AUC, lower in CYP3A5 expressors compared to CYP3A5 nonexpressors (**Table 2.2**). Similarly, the apparent urinary tacrolimus clearance (based on blood AUC) normalized by eGFR was 42% lower in CYP3A5 expressors compared to CYP3A5 nonexpressors. The between-group difference in urinary tacrolimus clearance was evident over the successive urine collection time intervals (**Figure 2.3A and 2.3B**).

2.4.4 Semi-physiological model of renal tacrolimus disposition

A semi-physiological model of renal tacrolimus disposition was developed in an effort to evaluate how the presence or absence of intrarenal CYP3A5-mediated tacrolimus metabolism might impact epithelial exposure to tacrolimus. A three-compartment model (**Figure 2.4**) representing the blood pool, tubular lumen, and tubular epithelium and featuring glomerular filtration, epithelial transport, and metabolism within the epithelium is capable of explaining the relationship between the time course of blood concentration and urinary excretion, as illustrated by the goodness of fit between the observed amount of tacrolimus excreted in the successive urine collection periods and model prediction based on simultaneous fitting of the model to the mean data for the CYP3A5 expressor and nonexpressor groups (**Figure 2.5)**. The model was fit to individual tacrolimus urine excretion data according to a staged strategy as described in the Methods. Using the parameter estimates from individual model fitting (**Table 2.3**), a profile of the amount of tacrolimus in the epithelial compartment over time was simulated (**Figure 2.6A)**. The simulated tacrolimus exposure in the renal epithelial compartment of CYP3A5 expressors, when normalized to the blood AUC (0-96 h), was on average 53% of that for CYP3A5 nonexpressors (**Figure 2.6B)**. Large between-subjects variability in the maximum amount (29-fold) and normalized area under the amount-time curve (9-fold) in the epithelial compartment was observed, particularly when data from the two phenotype groups were combined.

There were no statistical differences in the model parameter estimates (**Table 2.3**) except for $K_{p,kidney}$, the apparent tissue-to-blood partitioning coefficient of tacrolimus. $K_{p,kidney}$ in CYP3A5 expressors was estimated to be about one-half of that in CYP3A5 nonexpressors.

The unbound fraction of tacrolimus in whole blood (f_{ub}), derived from *in vitro* measurements of unbound fraction in plasma (f_{up}, 2.1 ± 0.8%) and whole blood-to-plasma

distribution ratio (27.7 ± 5.0) (Minematsu et al., 2004), was 0.078 ± 0.026% (24), which is significantly higher than the *in vivo* f'_{ub} of 0.012 ± 0.011% estimated from modeling. Zahir *et al.* had previously reported an f_{up} of 1.20 ± 0.12% for tacrolimus (Zahir et al., 2001); the corresponding f_{ub} was calculated to be 0.044%. It should be noted that the exchangeable fraction *in vivo*, which reflects the interplay of drug binding and dynamic events at the glomerulus, does not necessarily equal the *in vitro* equilibrium unbound fraction (i.e., $f'_{ub} \neq f_{ub}$).

The other noteworthy observation with the model parameter estimates in **Table 2.3** is the comparison between the extraction ratio of tacrolimus from the efferent arteriolar flow (*ER*) and the estimated exchangeable fraction (f'_{ub}). The fact that *ER* was much greater than f'_{ub} (by ~400-fold) suggests an exceptionally efficient uptake of tacrolimus at the basolateral aspect of the renal tubular epithelium that goes well beyond the exchangeable fraction operating at the glomerulus.

2.5 Discussion

The risk of nephrotoxicity remains a major challenge to the long-term survival of organ transplant patients receiving chronic calcineurin inhibitor therapy despite the fact that tacrolimus dosage is titrated to achieve an accepted therapeutic range of trough blood concentrations. Currently, there is no effective way to identify those that will develop chronic nephrotoxicity from those that will not (Kuypers et al., 2010). Plausible susceptibility factors have been proposed, including individual differences in renal metabolism of the drug. In the present study, we evaluated how *CYP3A5* genetic variation and the corresponding enzyme phenotype affect systemic and intrarenal tacrolimus metabolism and exposure of kidney tubular epithelium to tacrolimus. A single dose study was conducted in healthy volunteers in order to avoid the

confounding effects of changing renal function or drug-drug interactions from concomitant therapies in organ transplant patients (Naesens et al., 2009).

Our results confirm that the mean oral tacrolimus clearance is higher in CYP3A5 expressors than in CYP3A5 nonexpressors, which explains the larger tacrolimus dose that CYP3A5 expressors require in order to maintain the same trough blood concentration as nonexpressors (Hesselink et al., 2003; Thervet et al., 2003; Haufroid et al., 2004; Staatz and Tett, 2004). The data also indicate that the *CYP3A5*1* genotype, and inferred high renal expression phenotype, is associated with a greater extent of renal tacrolimus metabolism and a lower apparent urinary tacrolimus clearance compared to those subjects lacking the active reference allele. Such a relationship between renal metabolism and apparent urinary clearance of unchanged drug was first reported by Sirianni *et al.,* who showed, in the isolated perfused rat kidney, that the urinary clearance of enalapril increased following inhibition of enalapril hydrolysis to enalaprilate by paraoxon (Sirianni and Pang, 1999). In our study, the mean apparent urinary tacrolimus clearance (based on blood AUC) was 36% lower in CYP3A5 expressors compared to CYP3A5 nonexpressors, which is highly indicative of intrarenal CYP3A5-dependent tacrolimus metabolism. Tacrolimus is a substrate of P-glycoprotein (P-gp), encoded by the polymorphic *ABCB1* gene and multiple studies, though with conflicting results, have related *ABCB1* SNPs to tacrolimus nephrotoxicity (Staatz et al., 2010b), suggesting P-gp may affect the renal secretion of tacrolimus. Our analysis of *ABCB1* genotype/ haplotype showed that they did not constitute a significant co-variate in determining the urinary clearance of tacrolimus, although the study was not powered to test for such a contribution. It follows then that there should be a corresponding difference in the exposure to tacrolimus in the metabolically competent cell types within the kidneys, viz., the tubular epithelia, resulting from the difference

in renal CYP3A5 expression. Results from our simulations using a semi-physiological model of renal tacrolimus disposition support this hypothesis. Importantly, the estimated value for the steady-state tissue-to-blood partitioning ratio, $K_{p,kidney}$ in CYP3A5 expressors was nearly 50% that calculated for CYP3A5 nonexpressors, which is a model prediction that can be evaluated by measuring tacrolimus concentrations in kidney biopsy and blood samples in genotyped patients receiving the immunosuppressant following kidney transplantation.

One predicted consequence of increased CYP3A5-dependent intrarenal tacrolimus metabolism is a reduced risk of tacrolimus-induced nephrotoxicity following solid organ transplantation, if renal tacrolimus concentration is a major driver of toxicity. To date, only a few studies in kidney recipients have considered donor *CYP3A5* genotype. Our prediction is supported by results from some of these studies (Fukudo et al., 2008; de Denus et al., 2011), but not by all (Klauke et al., 2008; Glowacki et al., 2011). Specifically, in sixty adult liver transplant patients receiving tacrolimus, the cumulative incidence of renal dysfunction within 1 year after transplantation was significantly lower in recipients predicted to be expressors of renal CYP3A5, but was not associated with liver donor's CYP3A5 genotype (Fukudo et al., 2008). Similarly, Simon *et al.* (de Denus et al., 2011) recently showed that in 160 heart transplant recipients treated with either cyclosporine or tacrolimus, recipient *CYP3A5* genotype (which predicts renal enzyme expression) was a significant determinant of eGFR after transplantation (p = 0.0002), with carriers of the *CYP3A5*1* allele exhibiting a higher eGFR. In contrast to these findings, Klauke *et al*. (Klauke et al., 2008) found no association between *CYP3A5* and post-transplant renal insufficiency in a case–control study after orthotopic heart transplantation (n = 106). In addition, in 209 kidney transplant patients, histological evaluation of biopsies revealed no significant association between tacrolimus toxicity features and donor or recipient *CYP3A5*

polymorphisms (Glowacki et al., 2011). Such discordant pharmacogenetic findings may reflect the additional complication that tacrolimus metabolites could exert independent effects on renal function. Notably, the significantly increased circulating concentrations of metabolites in CYP3A5 expressors (recipient's genotype in renal transplantation, for example) may counteract the protective role of CYP3A5 expression in the kidney (donor's genotype in renal transplantation). In addition, any effects of tacrolimus metabolites on renal function will depend on the efficiency by which they are cleared, as well as the efficiency of their formation systemically and intrarenally. It has been suggested previously that systemic production of primary and related downstream metabolites of tacrolimus might contribute to CNI nephrotoxicity (Kuypers et al., 2007; Kuypers et al., 2010; Min et al., 2010; Metalidis et al., 2011). A recent study showed that CYP3A5 expressing renal transplant patients with high early tacrolimus dose requirements, had a higher risk of developing CNIT compared with nonexpressors (Kuypers et al., 2007). CYP3A5 expressors are also, reportedly, at greater risk of developing *de novo* arteriolar hyalinization, a histologic sign of CNIT based on a study of 304 *de novo* renal graft recipients (Kuypers et al., 2010). In addition, Kuypers *et al.* observed an association between continuation of low-dose steroids and CNI nephrotoxicity, which could be related to induction of hepatic/intestinal CYP3A4 by steroids. Similar to the presence of the *CYP3A5*1* allele in liver/intestine, steroid use could result in higher systemic and tissue concentrations of tacrolimus metabolites (Kuypers et al., 2010).

The blood metabolite AUCs and $AUC_{metabolite}/AUC_{parent}$ ratios we observed after a single tacrolimus dose allow us to predict abundance of the CYP3A5-catalyzed metabolites in the blood circulation at steady-state. Based on that analysis, there should be greater absolute accumulations of three of the four primary tacrolimus metabolites (13-DMT, 31-DMT, and 12-HT) in CYP3A5

expressors at steady-state when the tacrolimus dose is titrated to achieve the same therapeutic tacrolimus concentration, a prediction consistent with *in vitro* results showing that the average formation rates of these metabolites were at least 1.7-fold higher in human liver microsomes with a *CYP3A5*1/*3* genotype compared to microsomes with a homozygous *CYP3A5*3/*3* genotype (Dai et al., 2006). Interestingly, we found that the 15-DMT/parent AUC ratio and renal excretion of 15-DMT in the two genotyped groups were similar, which is also consistent with *in vitro* metabolic data (Dai et al., 2006).

Although the nephrotoxic potential of tacrolimus metabolites has not been studied (Naesens et al., 2009), the metabolites of cyclosporine, another calcineurin inhibitor, has been examined. In a study by Sigal *et al.* (Sigal et al., 1991) with 61 cyclosporine analogs, it was shown that the ability to induce nephrotoxicity *in vivo* correlates with the immunosuppression activity of these agents. Another study by Kung *et al.* (Kung et al., 2001) showed an association between the degree of calcineurin inhibition in tissue homogenate (greatest in kidney) and susceptibility of that organ to cyclosporine toxicity. With regard to metabolites, in mouse mixed lymphocyte reaction studies, Iwasaki *et al.* (Iwasaki et al., 1995) found that 31-DMT had the same immunosuppressive activity as tacrolimus, whereas the IC_{50} values for other metabolites were at least 10-fold higher. Thus, a higher systemic steady-state level of 31-DMT in CYP3A5 expressors compared to nonexpressors is likely to influence renal exposure to this active metabolite with its entry to the renal tubular cells via either uptake from the efferent arteriole or reabsorption from the tubular lumen following glomerular filtration, and possibly contribute to the nephrotoxicity risk. However, one caveat to this interpretation is the observation that the metabolic clearance of 31-DMT by CYP3A5 was comparable to that of tacrolimus (**Figure 4.1**)

and thus effective elimination of the potential toxic metabolite in CYP3A5 expressors may have a protective effect on renal function.

In summary, our findings demonstrate that *CYP3A5* genotype has a significant impact on the metabolism and clearance of tacrolimus in the human kidney, as well as in previously studied and metabolically competent organs, the liver and small intestine. The extent of intrarenal metabolism in CYP3A5 expressing organ transplant patients is such that we predict a much lower accumulation of tacrolimus in the tubular epithelium compared to that in CYP3A5 nonexpressing patients. This difference may contribute to interindividual differences in tacrolimus-induced nephrotoxicity, although greater intrarenal exposure to primary and secondary tacrolimus metabolites in CYP3A5 expressors compared to nonexpressors may also have to be considered. Further prospective studies investigating the impact of *CYP3A5* genotype on tacrolimus nephrotoxicity studies would help to clarify this issue by identifying the *CYP3A5* genotype(s) of the recipients and their donor kidneys, as well as metabolite concentrations of tacrolimus in blood and renal tissue. A full elucidation of the pharmacogenomics of tacrolimus nephrotoxicity may lead to improved management of tacrolimus pharmacotherapy.

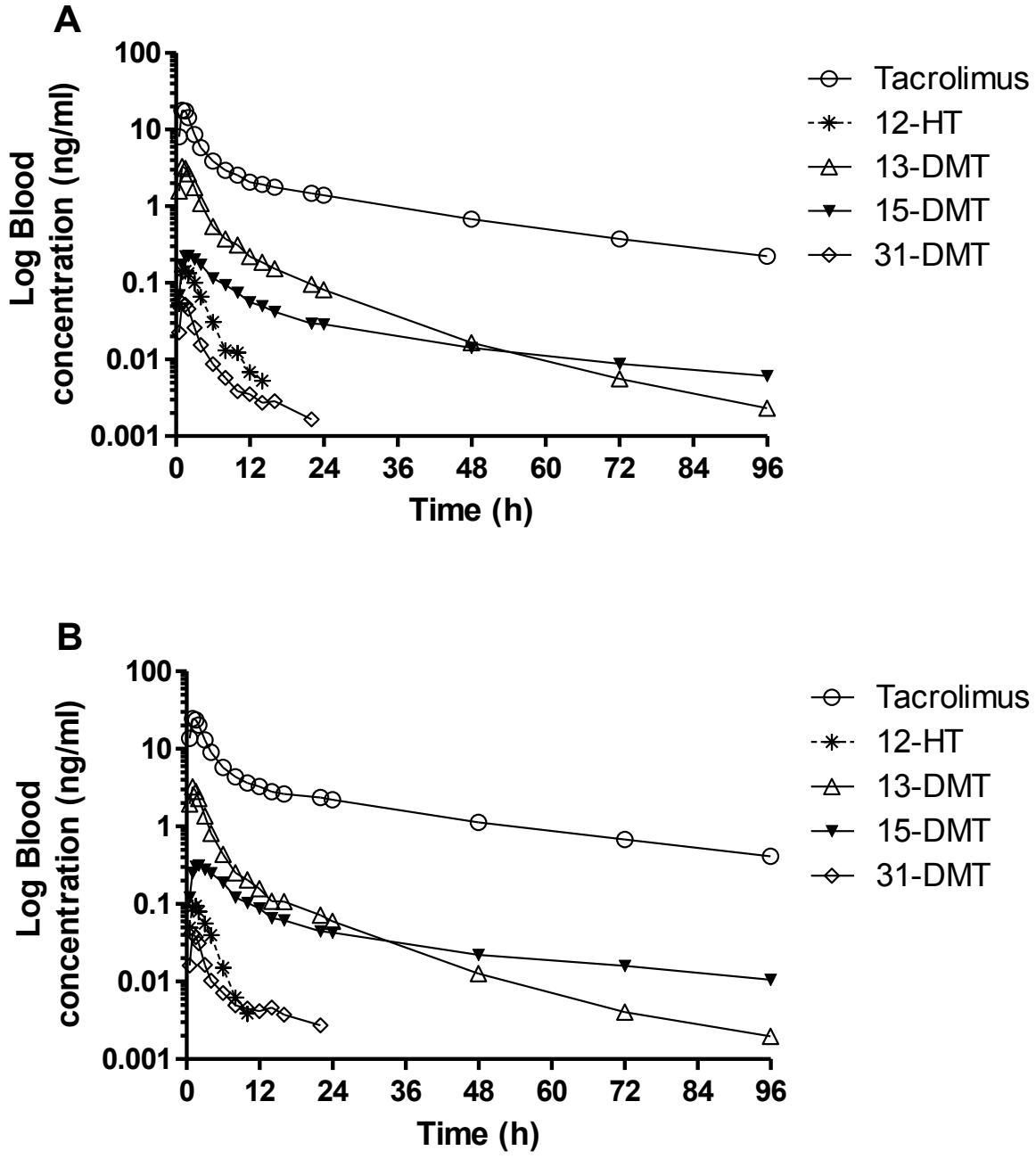


Figure 2.1 Mean log concentration–time profiles of tacrolimus and its four primary metabolites after oral tacrolimus administration in (A) CYP3A5 expressors (n = 12) and (B) CYP3A5 nonexpressors (n = 12).

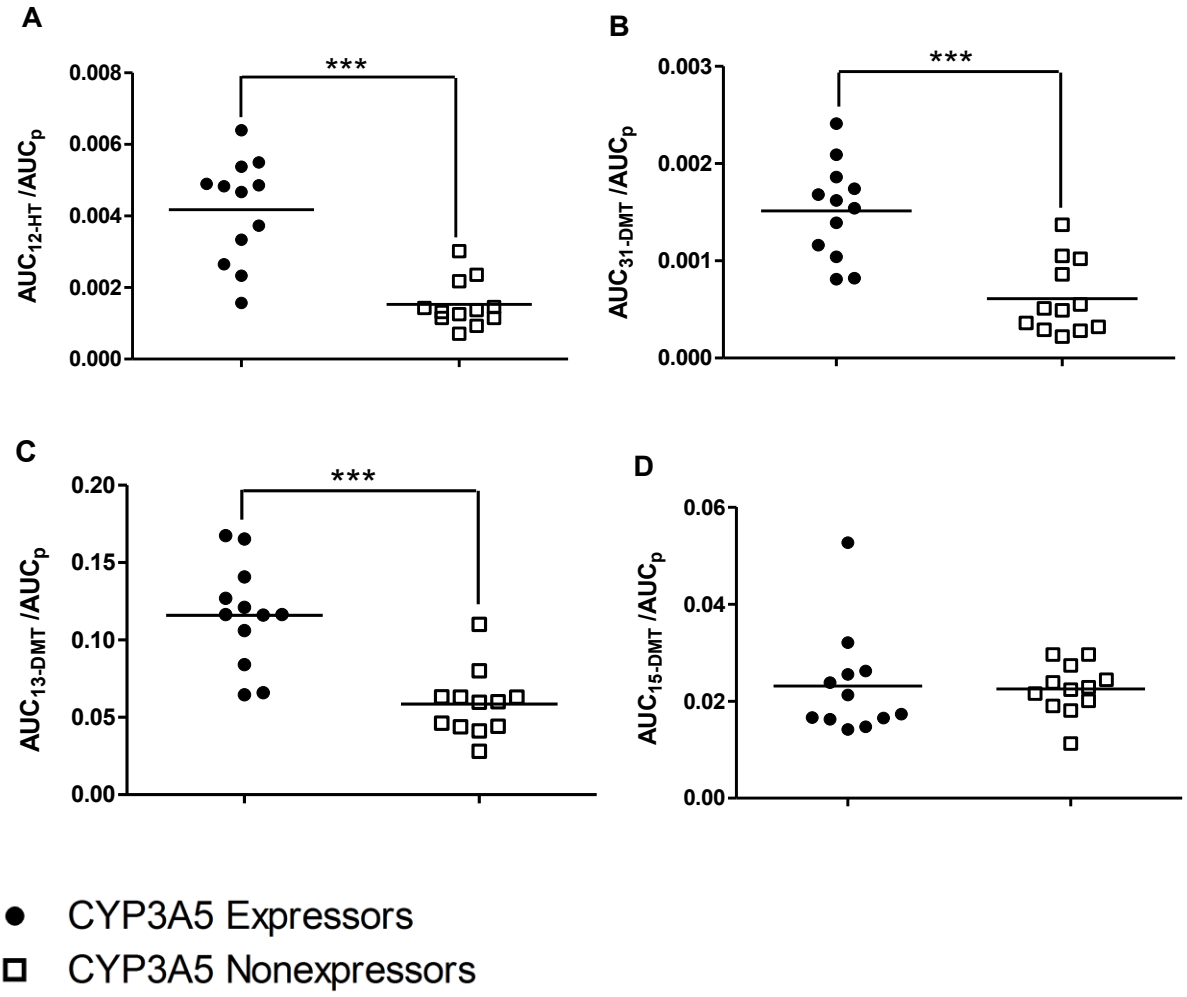


Figure 2.2 $AUC_{metabolite}$ to AUC_{parent} ratios for the four primary metabolites of tacrolimus : $AUC_{12\text{-}HT}$ /AUCp (A), $AUC_{31\text{-}DMT}$ /AUCp (B), $AUC_{13\text{-}DMT}$ /AUCp (C) and $AUC_{15\text{-}DMT}$ /AUCp (D). The solid line represents the mean ratios; *** $P < 0.0001$.

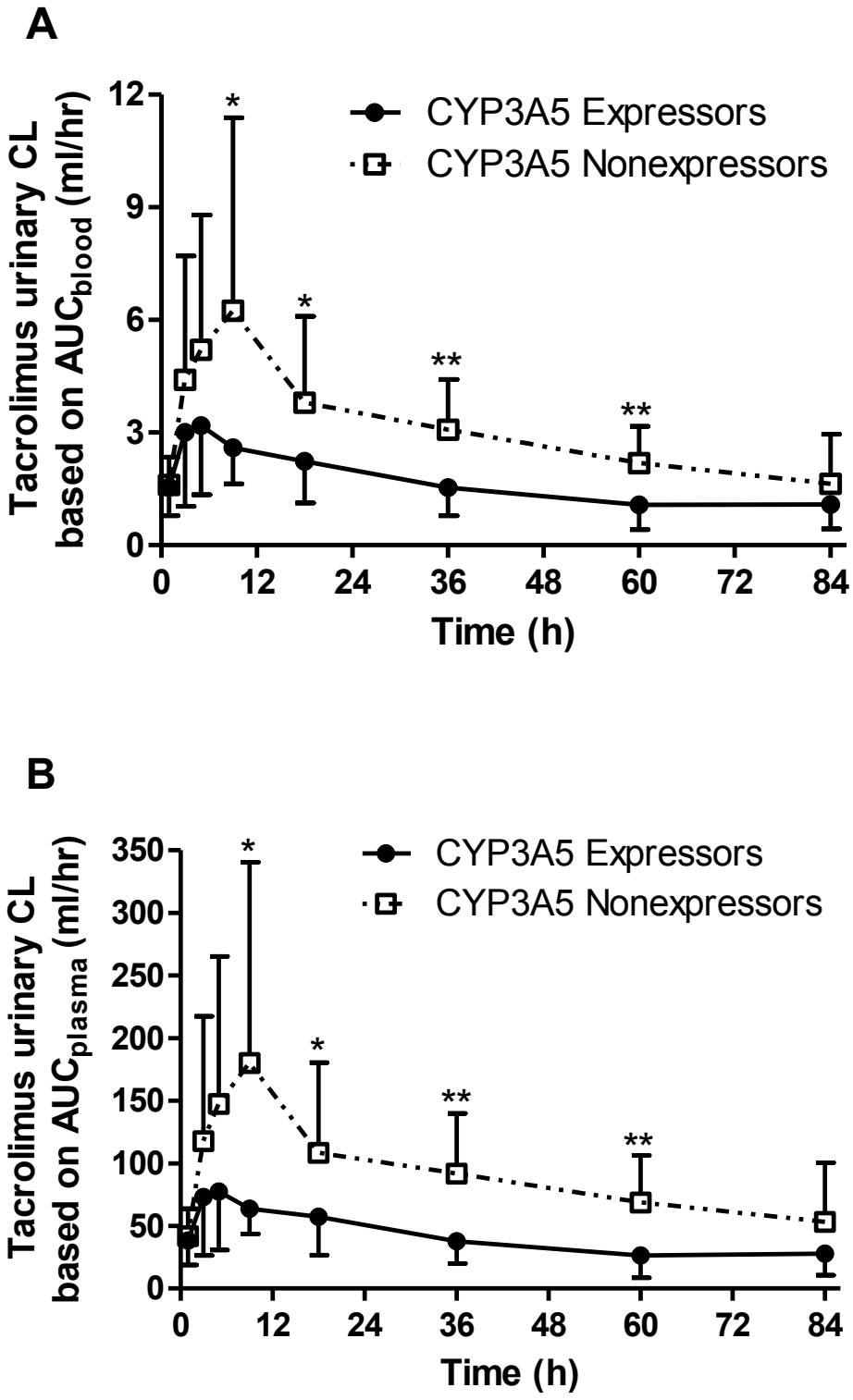
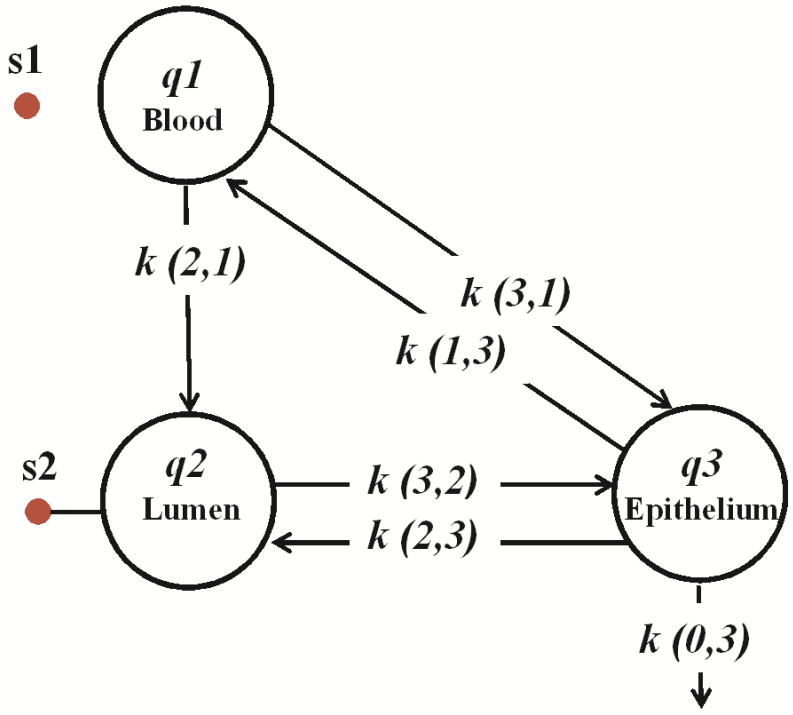


Figure 2.3 Serial calculations of the apparent urinary tacrolimus clearance based on (A) blood and (B) plasma concentrations; * $P < 0.05$; ** $P < 0.005$.



Compartments

q1 blood pool described by a forcing function (FF)

q2 tubular lumen

q3 renal epithelium

Rate Constants

k(2,1) glomerular filtration

k(3,1) secretion from efferent arteriole to the renal epithelium

k(1,3) back flux from the renal epithelium to efferent arteriole

k(2,3) secretion from the renal epithelium to tubular lumen

k(3,2) reabsorption of tacrolimus from tubular fluid back to the renal epithelium

k(0,3) intra-epithelial metabolism (=0 in CYP3A5 nonexpressors)

Sampling at s1 (blood), s2 (urine)

Figure 2.4 Compartmental model scheme for renal disposition. Rate constant, compartment labels and parameters are defined in the figure.

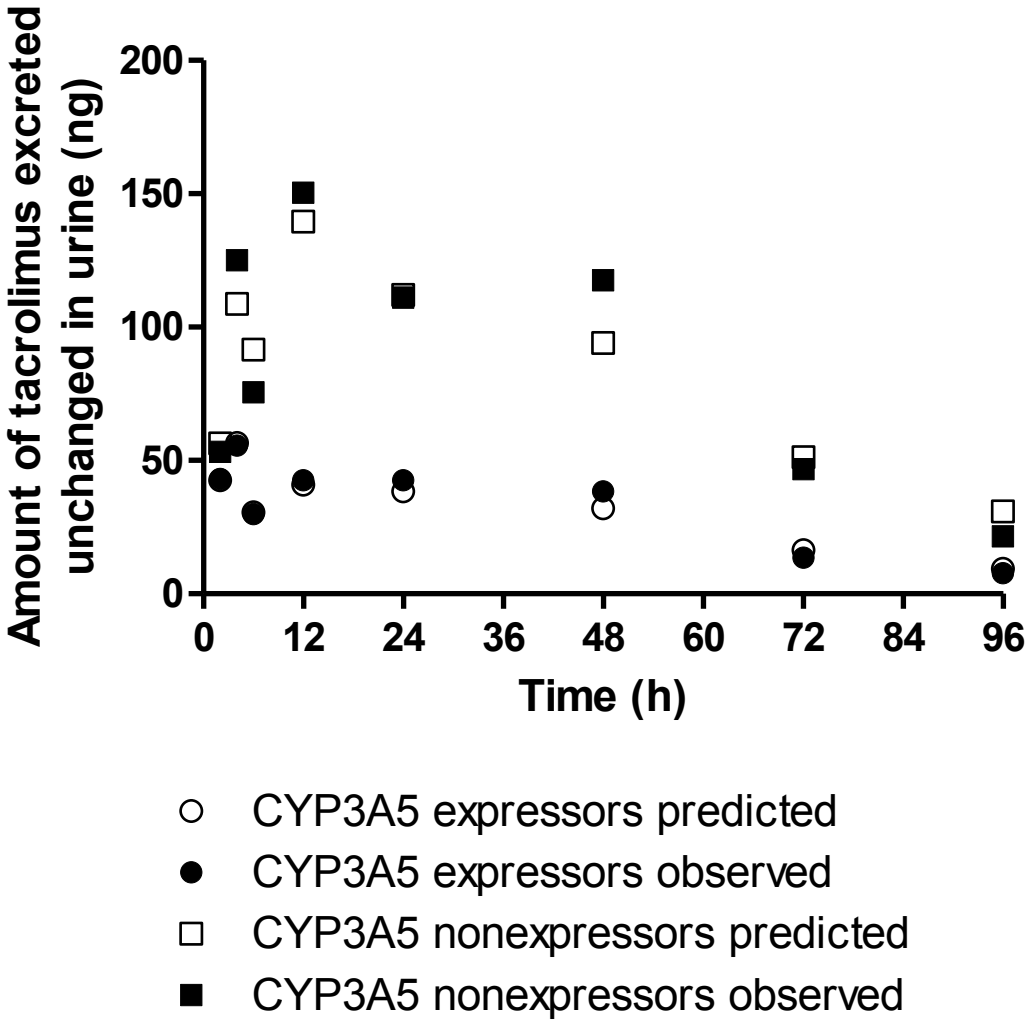


Figure 2.5 Model fit to the mean tacrolimus urine excretion data in CYP3A5 expressors and nonexpressors, simultaneously, using blood concentration as a forcing function.

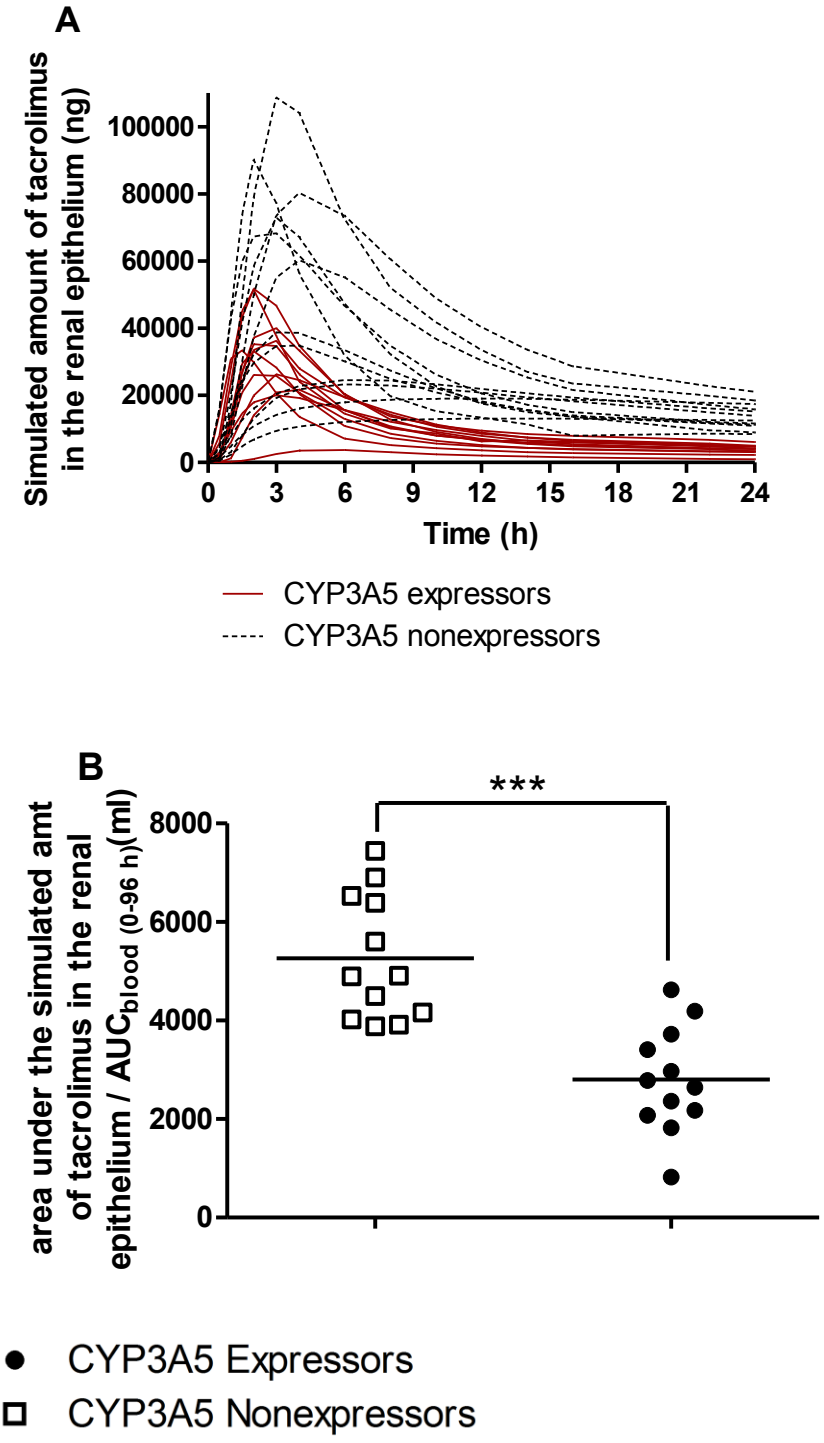


Figure 2.6 The simulated tacrolimus exposure in the renal epithelium. (A) Simulated tacrolimus amount in the renal epithelium (logarithmic amount shown in the upper insert) and (B) Area under the simulated amount $_{\text{renal epithelium}}$ - time curve normalized to blood AUC $_{(0\text{-}96h)}$; *** $P <$ 0.0001.

Table 2.1 Demographic characteristics of study participants.

	CYP3A5 expressors ***(n=12)***	**CYP3A5 nonexpressors** ***(n=12)***
Sex (male/female)	5/7	5/7
Age (years)	30.8 ± 9.9	23.5 ± 3.5
Race or ethnic group *(n, %)*		
Caucasian	6 (50%)	9 (75%)
Black	4 (33.3%)	1 (8.3%)
Asian	2 (16.7%)	2 (16.7%)
Body weight (kg)	71.6 ± 19.9	66.5 ± 13.5
Serum creatinine (mg/dl)	0.77 ± 0.15	0.86 ± 0.25
Creatinine CL (ml/min)	120.9 ± 27.9	132.9 ± 40.6
eGFR (ml/min)	129.4 ± 29.1	118.7 ± 26.4
Hematocrit (%)	40.0 ± 3.4	41.3 ± 3.4
***ABCB1* 1236C>T** *(n, %)*		
C/C	4 (33.3%)	1 (8.3%)
C/T	7 (58.3%)	7 (58.3%)
T/T	1 (8.3%)	4 (33.3%)
***ABCB1* 3435C>T** *(n, %)*		
C/C	4 (33.3%)	3 (25%)
C/T	6 (50%)	5 (41.7%)
T/T	2 (16.7%)	4 (33.3%)
***ABCB1* 2677G>T/A** *(n, %)*		
G/G	5 (45.5%)	3 (25%)
G/T	5 (45.5%)	7 (58.3%)
T/T or T/A	1 (9%)	2 (16.7%)
***ABCB1* 1236T-2677T-3435T** *(n, %)*	0 (0%)	2 (16.7%)

eGFR, estimated glomerular filtration rate calculated using the Cockroft-Gault equation; *ABCB1* 2677G>T/A was not determined for one subject because of a poor quality result.

Table 2.2 Tacrolimus pharmacokinetic parameters for study participants stratified by predicted CYP3A5 phenotype.

	Nonexpressors (N=12)	**Expressors (N=12)**	**Difference (%)**	***P* value**
$AUC_{0–96}$ (h•ng/ml)	209.2 ± 45.0	135.6 ± 60.4	-35.2%	0.003
$AUC_{0–inf}$ (h•ng/ml)	228.1 ± 50.0	144.8 ± 61.6	-36.5%	0.001
$t_{1/2}$ (h)	31.7 ± 4.3	29.9 ± 4.8		NS
t_{max} (h)	1.3 ± 0.4	1.4 ± 0.6		NS
C_{max} (ng/ml)	27.9 ± 7.7	20.8 ± 11.2		NS
C_{last} (ng/ml)	0.4 ± 0.1	0.2 ± 0.1	-46.2%	0.0003
CL/F (ml/h/kg)	355.4 ± 88.0	550.2 ± 127.7	54.8%	0.0003
$CL_{urinary}$(ml/h) (based on AUC_{blood})	3.15 ± 1.69	2.01 ± 0.57	-36.2%	0.04
$CL_{urinary}$/eGFR (%) (based on AUC_{blood})	0.048 ± 0.032	0.028 ± 0.012	-41.7%	0.05
$CL_{urinary}$ (ml/h) (based on AUC_{plasma})	97.71 ± 59.89	50.15 ± 13.67	-48.7%	0.01
$CL_{urinary}$/eGFR (%) (based on AUC_{plasma})	1.48 ± 1.10	0.58 ± 0.28	-53.9%	0.02

AUC, area under the concentration–time curve; t_{max}, time to reach the maximum blood concentration; C_{max}, maximum blood concentration; C_{last}, blood concentration at 96 hour after tacrolimus administration; CL/F, oral clearance; $CL_{urinary}$, tacrolimus urinary clearance for the 0 to 96-hour collection interval; eGFR, estimated glomerular filtration rate calculated using the Cockroft-Gault equation (Cockcroft and Gault, 1976); NS, not significant

Table 2.3 Parameter estimates (mean ± SD) from model fitting to the individual tacrolimus urine excretion data in CYP3A5 expressors and nonexpressors using blood concentration as a forcing function.

		CYP3A5 expressors	CYP3A5 nonexpressors
Parameters fixed to known physiological values	*GFR* (ml/h)	7761 ± 1748	7124 ± 1585
	Q_{effart} (ml/h)	48490 ± 11461	45731 ± 11158
	V_{epi} (ml)	250 ± 76	232 ± 52
Parameters from empirical Bayesian estimation	$K_{p,kidney}$	12.1 ± 0.3***	23.4 ± 0.04
Parameters from ordinary least-squares estimation	f_{ub} (%)	0.014 ± 0.015	0.011 ± 0.006
	k(0,3) (h^{-1})	0.55 ± 0.52	
	k(1,3) (h^{-1})	0.34 ± 0.05	0.36 ± 0.39
	k(2,3) (h^{-1})	0.0009 ± 0.0005	0.0012 ± 0.0008
	k(3,2) (h^{-1})	0.091 ± 0.063	0.106 ± 0.057
	ER (%)	5.1 ± 3.0	4.3 ± 4.7
Parameters derived from the above estimation	*k(2,1)* (ml/h)	0.94 ± 0.76	0.77 ± 0.51
	k(3,1) (ml/h)	2368 ± 1404	1800 ± 1734

GFR: values are from estimated glomerular filtration rate calculated using the Cockroft-Gault equation (Cockcroft and Gault, 1976)

Q_{effart} : efferent arteriolar blood flow rate

V_{epi} : the volume of renal epithelium

$K_{p,kidney}$: apparent tissue-to-blood partitioning coefficient of tacrolimus

f_{ub}: exchangeable fraction of tacrolimus in blood during glomerular filtration

k(0,3): rate constant representing CYP3A5-mediated intra-epithelial metabolism (=0 in CYP3A5 nonexpressors and assumes a characteristic value for CYP3A5 expressors.)

k(1,3): transfer rate constant representing back flux from the renal epithelium to efferent arteriole

k(2,3): transfer rate constant representing secretion from the renal epithelium to tubular lumen

k(3,2): transfer rate constant representing reabsorption of tacrolimus from tubular fluid back to the renal epithelium

ER: extraction ratio of tacrolimus from the efferent arteriole

k(2,1): transfer rate constant representing the glomerular filtration of tacrolimus

k(3,1): transfer rate constant representing secretion from efferent arteriole to the renal epithelium; *** $P < 0.0001$

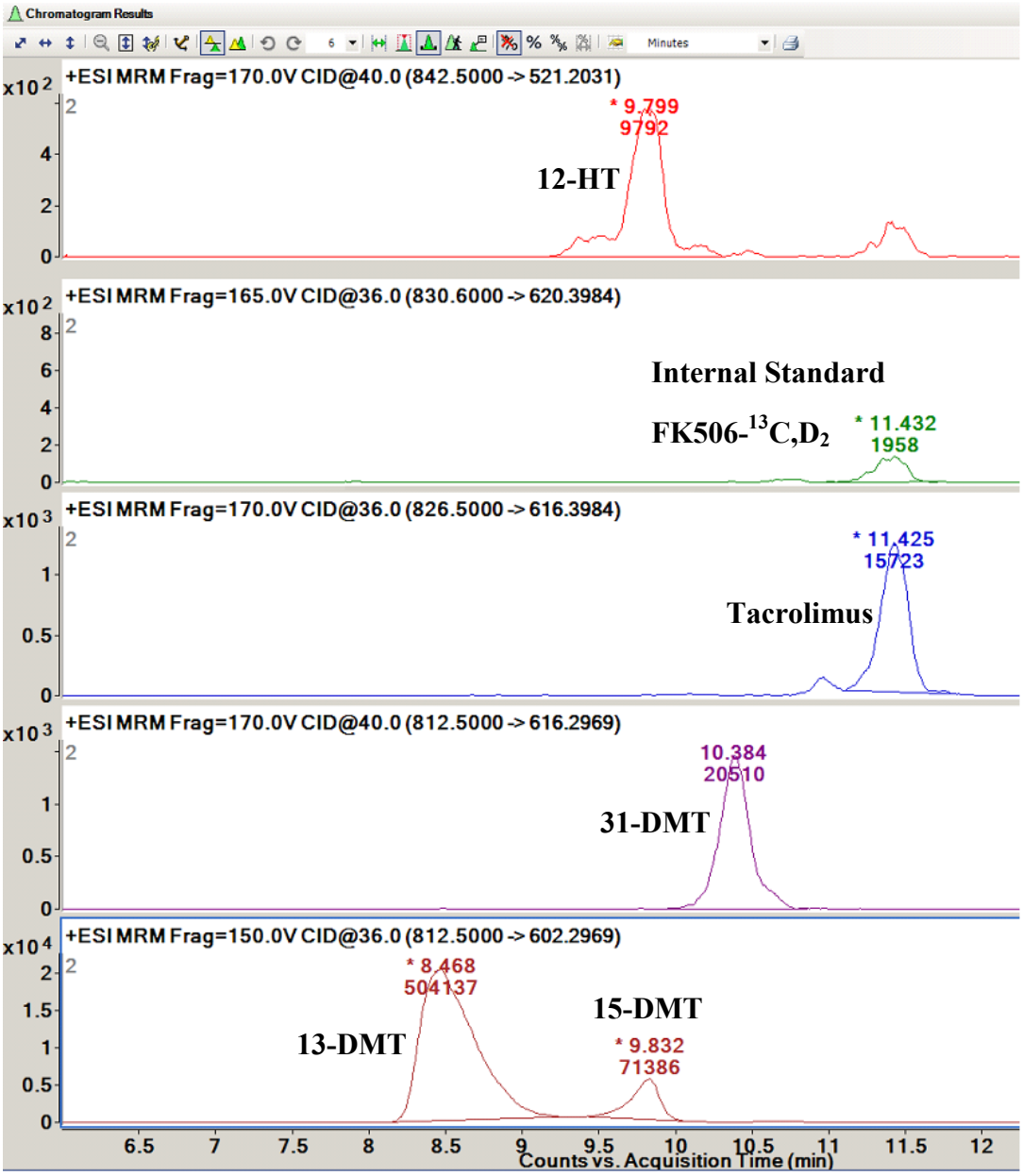


Figure 2.7 Representative chromatogram for tacrolimus and its primary metabolite quantification. The elution times for 13-DMT, 12-HT, 15-DMT, 31-DMT, internal standard, and tacrolimus, were 8.5 min, 9.8 min, 9.8 min, 10.4 min, 11.4 min, and 11.4 min, respectively. Sodium adducts of tacrolimus and metabolites were detected with multiple reaction monitoring under the ESP^+ mode. The ion transition was *m/z* 812.2>602.4 for 13-DMT and 15-DMT, *m/z* 842.2>521.3 for 12-HT, *m/z* 812.2>616.3 for 31-DMT, *m/z* 830.2>620.4 for internal standard, and *m/z* 826.5 > 616.3 for tacrolimus.

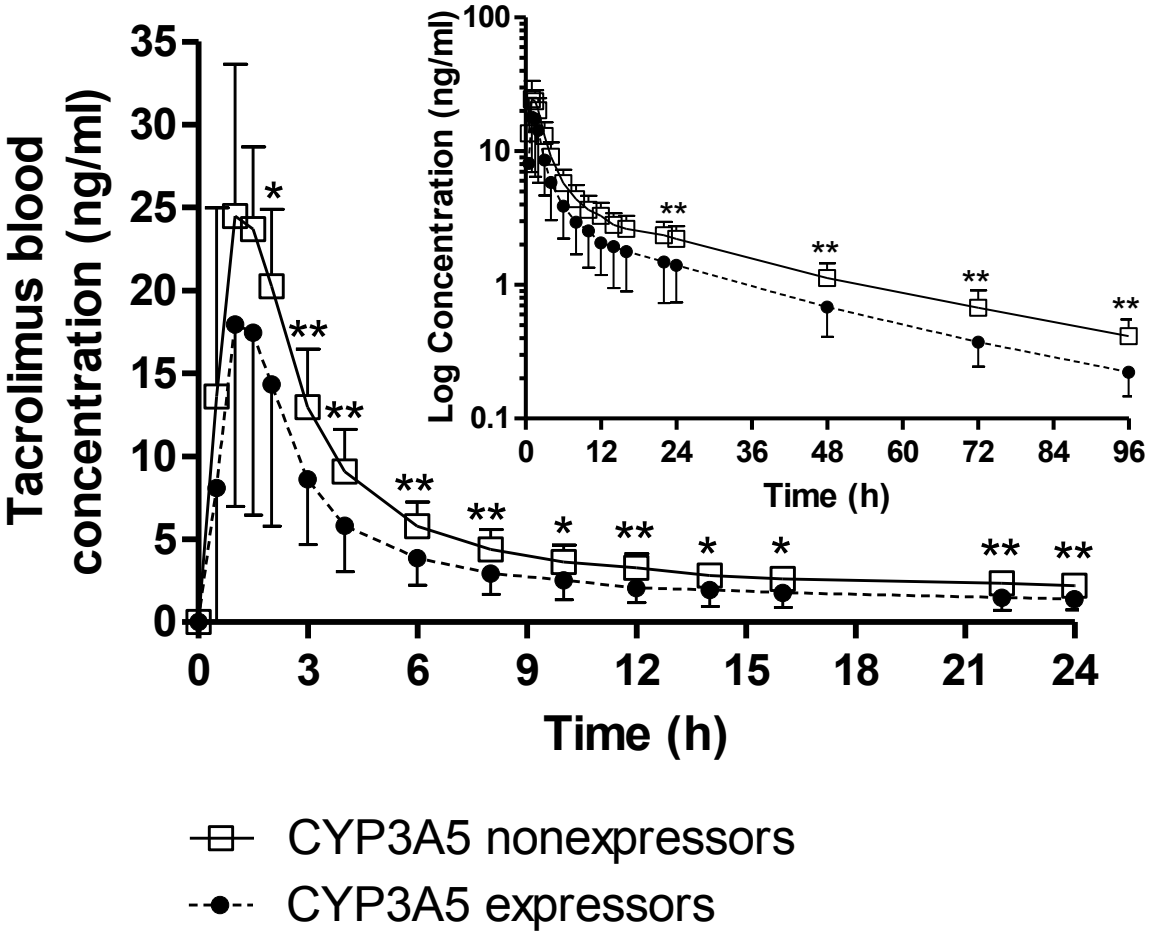
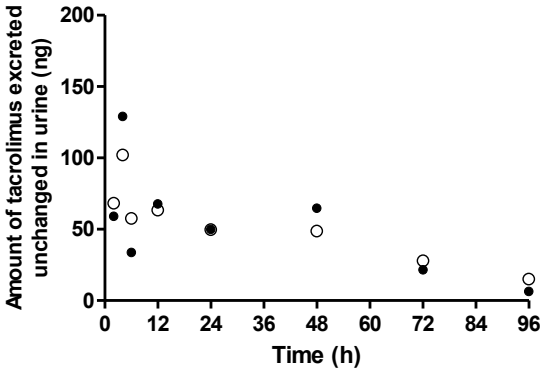


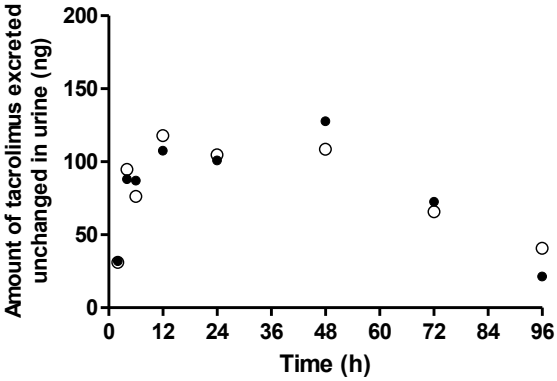
Figure 2.8 Tacrolimus concentration–time profiles (mean ± SD) after oral tacrolimus administration in CYP3A5 expressors (n=12) and CYP3A5 nonexpressors (n=12). Concentration–time profiles are displayed using a linear Y-axis and a logarithmic Y-axis (upper insert); * $P < 0.05$; ** $P < 0.005$

Figure 2.9 Model fit to the individual tacrolimus urine excretion data in CYP3A5 expressors and nonexpressors using individual blood concentration as a forcing function.

CYP3A5 Nonexpressors



○ Subject 002 predicted
● Subject 002 observed



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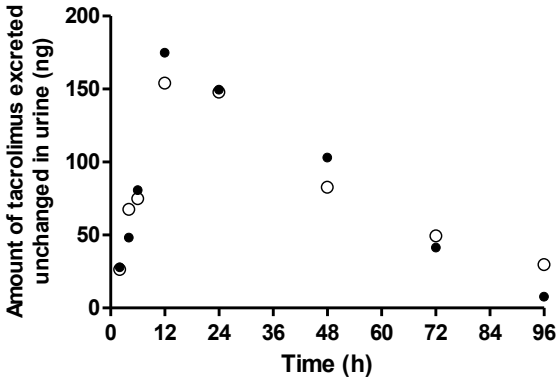

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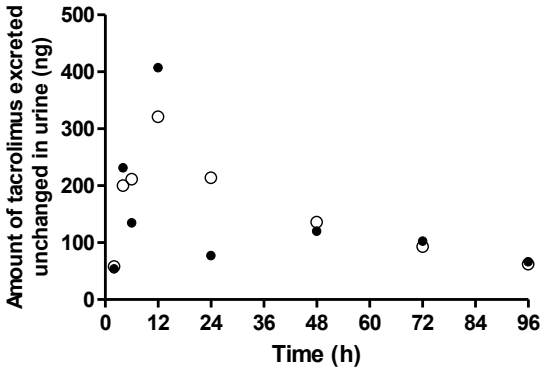

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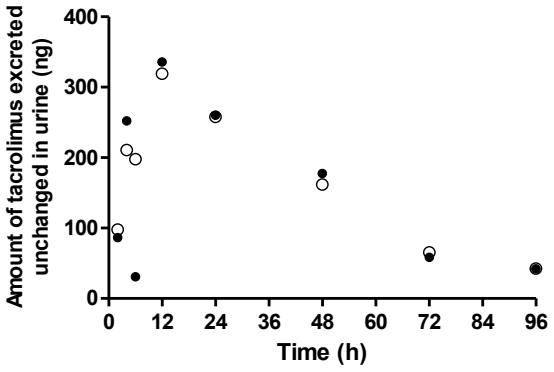

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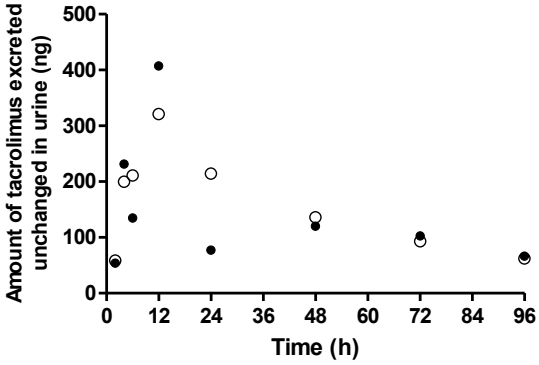

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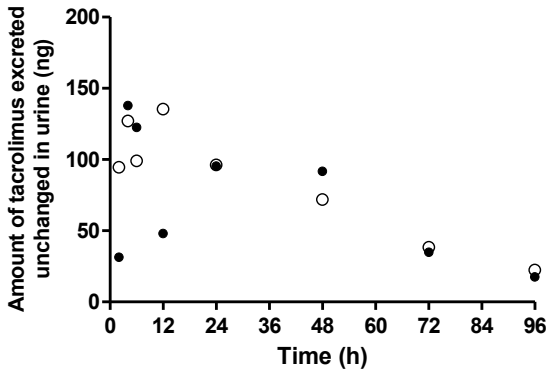

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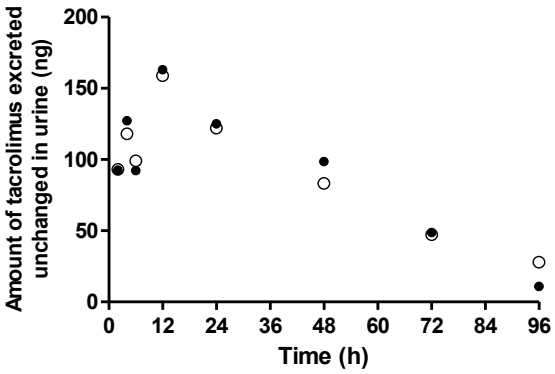

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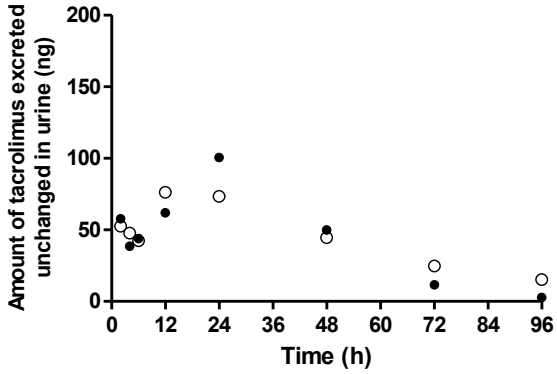

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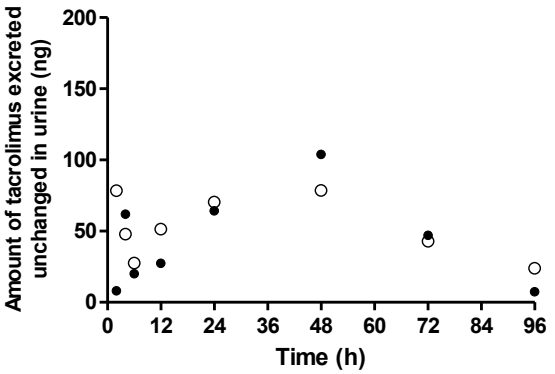

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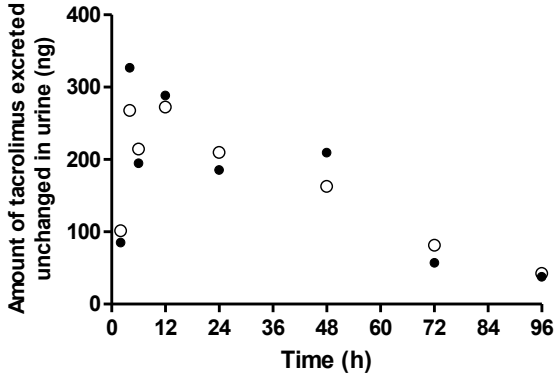

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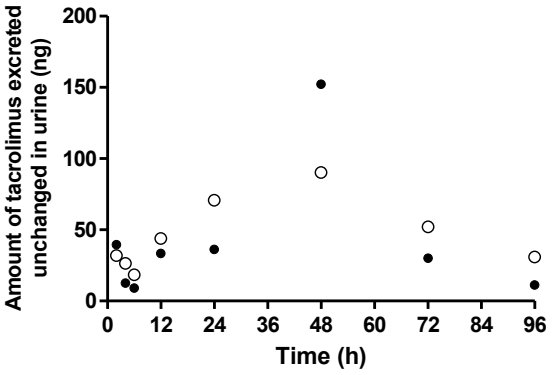

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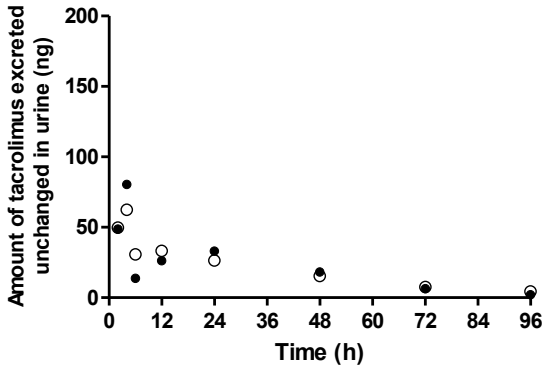
CYP3A5 Expressors



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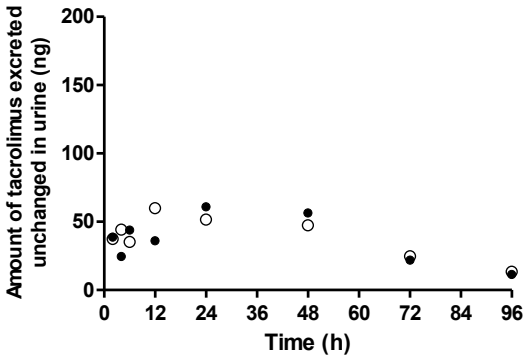

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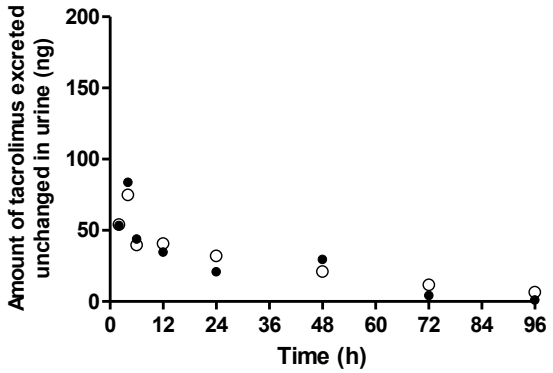

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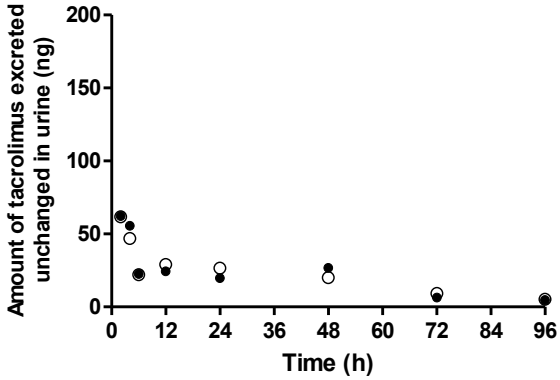

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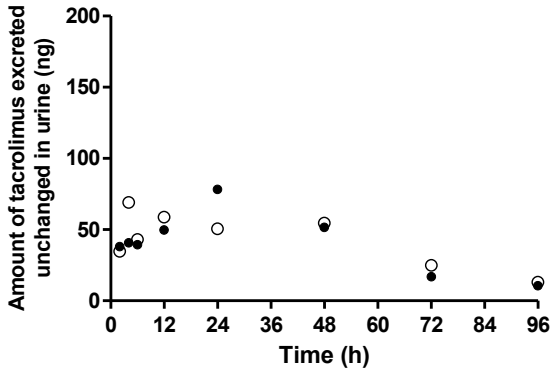

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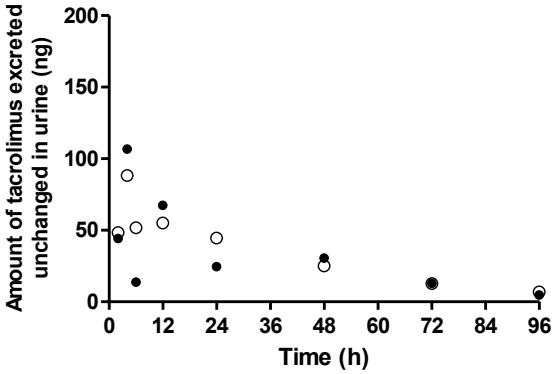

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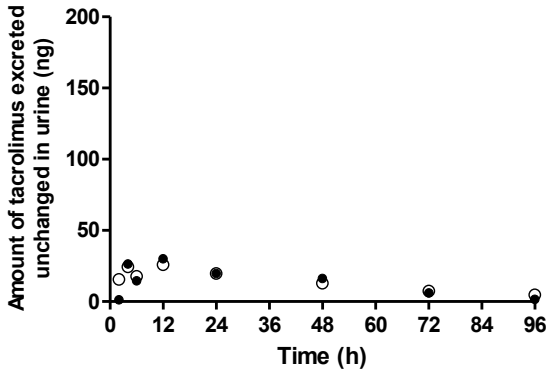

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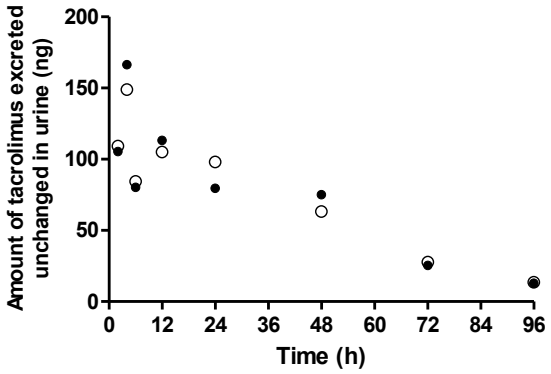

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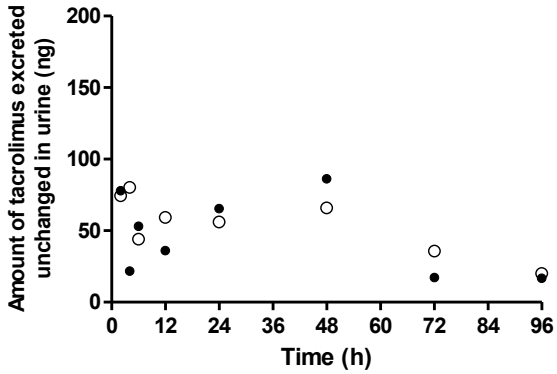

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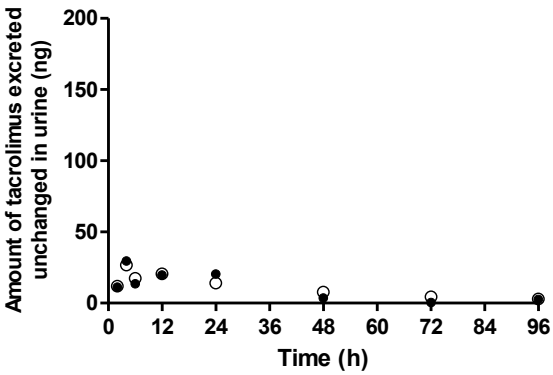

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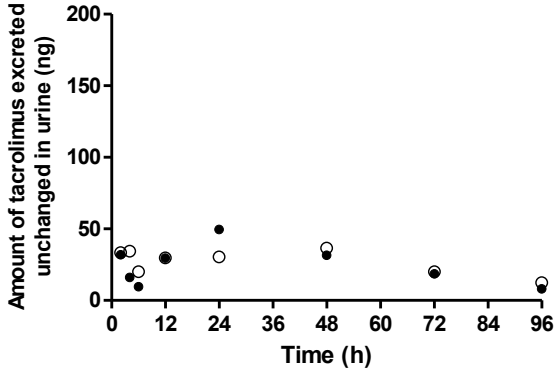

○ Subject 097 predicted
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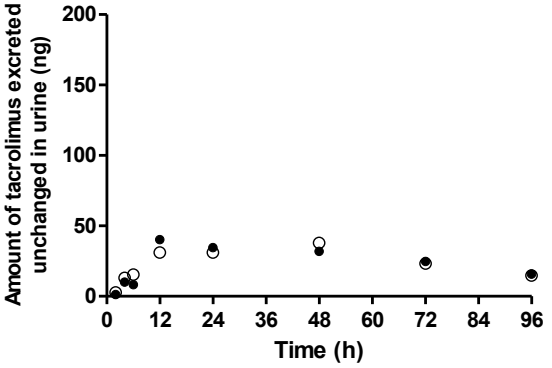


○ Subject 106 predicted
● Subject 106 observed

Chapter 3

CYP3A5 Gene Variation Influences Cyclosporine A Metabolite Formation and Renal Cyclosporine Disposition

Portions of Chapter 3 were submitted to *Transplantation.*

3.1 Abstract

Higher concentrations of AM19 and AM1c9, secondary metabolites of cyclosporine A (CsA), have been associated with nephrotoxicity in organ transplant patients. The risk of renal toxicity may depend upon the accumulation of CsA and its metabolites in the renal tissue. We evaluated the hypothesis that *CYP3A5* genotype, and inferred enzyme expression, affects systemic CsA metabolite exposure and intrarenal CsA accumulation.

An oral dose of CsA was administered to 24 healthy volunteers who were selected based on their *CYP3A5* genotype. CsA and its six main metabolites in whole blood and urine were measured by LC-MS. *In vitro* incubations of CsA, AM1, AM9 and AM1c with recombinant CYP3A4 and CYP3A5 were performed to evaluate the formation pathways of AM19 and AM1c9. The mean CsA oral clearance was similar between CYP3A5 expressors and nonexpressors. However, compared to CYP3A5 nonexpressors, the average blood AUC for AM19 and AM1c9 was 47.4% and 51.3% higher in CYP3A5 expressors (P = 0.040 and 0.011, respectively), corresponding to 30% higher $AUC_{metabolite}/AUC_{CsA}$ ratios for AM19 and AM1c9 in CYP3A5 expressors. The mean apparent urinary CsA clearance, based on a 48-hour collection, was 20.4% lower in CYP3A5 expressors compared to CYP3A5 nonexpressors (4.2 ± 1.0 and 5.3 ± 1.3 mL/min, respectively, P = 0.037), which is suggestive of CYP3A5-dependent intrarenal CsA metabolism.

In summary, at steady-state, intrarenal accumulation of CsA and its secondary metabolites should depend on the *CYP3A5* genotype of the liver and kidneys. This may contribute to inter-patient variability in the risk of CsA-induced nephrotoxicity.

3.2 Introduction to Chapter 3

Introduction of the calcineurin inhibitor cyclosporine A (CsA) in human kidney transplantation in the late 1970s revolutionized transplantation medicine and dramatically increased graft and patient survival (Calne et al., 1978). However, its use is associated with significant adverse side effects, in particular, acute and chronic calcineurin inhibitor nephrotoxicity (CNIT) (Klintmalm et al., 1981; Myers et al., 1984). Therapeutic immunosuppressant strategies that include CsA call for routine drug level monitoring. Nonetheless, many patients experience acute and chronic nephrotoxicity (Nankivell et al., 2003). Of note, susceptibility to CNIT is not ameliorated by keeping the CsA blood level within a narrow therapeutic range, implying that nephrotoxicity is not solely related to systemic exposure to CsA. It has been suggested that the local concentrations of CsA and its metabolites in kidney tissue may be more causally related to the risk of CNIT (Naesens et al., 2009).

CsA undergoes extensive biotransformation to more than 30 products. The major metabolic pathways involve initial hydroxylation/N-demethylation and further oxidation, sulfation, and cyclization (Christians and Sewing, 1993). Formation of these metabolites is catalyzed principally by cytochromes P450 3A4 and 3A5 (CYP3A4 and CYP3A5), enzymes that are found mainly in the liver and the gastrointestinal tract. The expression of CYP3A5 is highly polymorphic and determined largely by single-nucleotide variations that distinguish the "active" *CYP3A5*1* allele (inferred CYP3A5 expressor phenotype in individuals heterozygous or homozygous for *CYP3A5*1*) from the "inactive" *CYP3A5*3*, **6* or **7* alleles (inferred CYP3A5 nonexpressor phenotype) (Lampen et al., 1995; Haehner et al., 1996; Kuehl et al., 2001; Lin et al., 2002; Givens et al., 2003). The

CYP3A5 polymorphism contributes to interindividual differences in the metabolic clearance of a number of drugs, including CsA. However, in the case of CsA, the *in vitro* intrinsic metabolic clearance calculated from total metabolite formation is approximately 2.3-fold higher for CYP3A4 than for CYP3A5 (Dai et al., 2004). Thus, CYP3A4 plays a more dominant role than CYP3A5 in the metabolism of CsA and the influence of the *CYP3A5* polymorphism on the bioavailability and total systemic clearance of CsA is limited (Staatz et al., 2010a).

Although the contribution of CYP3A5 to CsA oral clearance is modest, it might contribute more significantly to inter-individual variation in CsA metabolite tissue exposure because of marked differences between the product selectivity of CYP3A4 and CYP3A5. The primary CsA metabolites, AM1, AM9 and AM4N, and several secondary and tertiary metabolites, AM1c, AM19 and AM1c9, can be detected in the blood and urine (Lensmeyer et al., 1988). CYP3A4 catalyzes the formation of all three primary metabolites, whereas only AM9 is produced to a significant degree by CYP3A5 (Dai et al., 2004). Moreover, human liver microsomes from CYP3A5 expressors exhibit higher AM9 formation rates than liver microsomes from CYP3A5 nonexpressors (Dai et al., 2004). In the kidney, because CYP3A5, and not CYP3A4, is expressed in the tubular epithelium, the rate of AM9, AM19 and AM1c9 formation by human kidney microsomes is strongly associated with detection of CYP3A5 protein and presence of the *CYP3A5*1* allele (Dai et al., 2004). Thus, inter-individual variability in the systemic blood and renal concentration of CsA metabolites might be explained in part by differences in the expression and function of CYP3A5 in the major organs of drug elimination (Hebert, 1997).

High blood and urinary concentrations of AM19 and AM1c9 have been associated with renal dysfunction in CsA treated patients (Christians et al., 1991b; Kempkes-Koch et al., 2001; Vollenbroeker et al., 2005). However, it is unclear if greater than average metabolite exposure is the cause or the result of impaired kidney function. The primary and secondary metabolites of CsA are equivalent or less toxic than CsA in cultured renal epithelial cells (Bowers, 1990; Copeland et al., 1990). In contrast, AM19 and AM1c9 (but not CsA or its primary metabolites) have been shown to alter renal mesangial cell function by increasing endothelin release (Copeland and Yatscoff, 1992). Accordingly, the presence of CYP3A5 in the small intestine, liver and kidney may affect systemic and intrarenal concentrations of CsA and its putative nephrotoxic metabolites during drug therapy and, by inference, the risk of CNIT. To test this hypothesis, we measured and compared the concentrations of key CsA primary and secondary metabolites in blood and urine excretion among CYP3A5 expressors and nonexpressors. In addition, we evaluated the impact of *CYP3A5* genotype on intrarenal CsA metabolism *in vivo*, using the apparent urinary CsA clearance as a surrogate marker of intrarenal drug clearance.

3.3 Materials and Methods

3.3.1 Materials

CsA, bovine serum albumin, and β-nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate (NADPH), were purchased from Sigma-Aldrich (St. Louis, MO). Cyclosporine D (internal standard for clinical study) and cyclosporine B (internal standard for *in vitro* study) were purchased from LKT Laboratories (St. Paul, MN). The CsA metabolites used for calibration curves and quality

control were generated using a bioreactor approach (see below). Blank (outdated) human plasma and blood were purchased from Puget Sound Blood Center (Seattle, WA). Methanol (Optima grade), acetonitrile (Optima grade), ethyl acetate (Optima grade), ammonium acetate, hydrochloric acid, sodium hydroxide, potassium phosphate monobasic, potassium phosphate dibasic, and silanized glass LC inserts were purchased from Fisher Scientific (Santa Clara, CA). Methyl tert-butyl ether (J.T. Baker) was purchased from VWR International (USA). CYP3A4 + b_5 Supersomes™ (Catalog No. 456202) and human CYP3A5 + b_5 Supersomes ™ (Catalog No.456256) were purchased from BD Biosciences (Woburn, MA).

3.3.2 Clinical Protocol

This protocol was approved by the University of Washington Institutional Review Board. All study participants provided written informed consent and were selected based on their *CYP3A5* genotype. Subjects (n=24) received a single oral dose of CsA (5 mg/kg). None of the subjects had a significant medical history or abnormal clinical lab test results, and none had taken a known inhibitor, inducer, or activator of CYP3A4/5 (other than oral contraceptives) for at least 1 month preceding the start of and during the pharmacokinetic investigation, and all abstained from grapefruit products and alcohol one week prior to the start until the end of the study. Sequential blood samples (5 mL) were collected in EDTA tubes predose and at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 14, 16, 22, 24, and 48 hr after oral drug administration of the CsA dose. Urine was collected in silanized glass containers over the following post-dose intervals: 0-2 hr, 2-4 hr, 4-6 hr, 6-12 hr, 12-24 hr and 24-48 hr. All samples were stored at −80 °C until analysis.

3.3.3 Genotyping

Buccal cell DNA was isolated using a DNeasy Blood & Tissue Kit or the Qiagen Gentra Puregene protocol (Qiagen, USA). Single-nucleotide polymorphisms in the *CYP3A5* gene (*3, *6 and *7 alleles; rs776746, rs10264272 and rs41303343 respectively) and the *ABCB1* gene (C3435T, C1236T and G2677T/A) were determined from a buccal swab tissue sample, using previously published methods (Lin et al., 2002; Asano et al., 2003) or a validated Taqman® allelic discrimination assay from Applied Biosystems (Foster City, CA) (Hebert et al., 2008).

3.3.4 Isolation and Mass Spectrometric Analysis of Cyclosporine Metabolites

3.3.4.1 Isolation of Metabolites AM19 and AM1c9.

The CsA secondary metabolites AM19 and AM1c9 were generated by incubation of AM1 (6.7 µg/mL) and AM1c (9.5 µg/mL) respectively with human liver microsomes (1 mg/mL) in solutions containing 0.1 M potassium phosphate, and 1 mM EDTA (pH 7.4) supplemented with 1 mM NADPH for 2 hours at 37°C in a shaking water bath. The final volume was 1 mL/incubation tube (16 tubes for AM1, 4 tubes for AM1c, and 1 tube with no substrate). The reaction mixture was extracted twice with ethyl acetate (5 mL first, then 3 mL), and the organic layers were collected and evaporated to dryness under a stream of nitrogen at 40°C. The residue was reconstituted in methanol and injected (50 µL/injection) onto an analytical column (Symmetry C18, 250 × 4.6 mm; Waters, Milford, MA) heated to 60°C. Components of the mobile phases were: A, H_2O and B, 20%

methanol and 80% acetonitrile. The gradient was 60% B at 0 min and 85% B at 30 min at a flow rate of 1 mL/min. Elution fractions encompassing the NADPH-dependent UV visible peaks (λ= 214) with retention times of 9.6 min (AM19) and 10 min (AM1c9) were collected manually, dried completely under a stream of nitrogen, and resuspended in methanol. The column was washed between runs with 100% B for 10 min at 3 mL/min.

3.3.4.2 Isolation of Metabolite AM9.

Cyclosporine AM9 was generated by incubation of CsA (100 μM) with CYP3A5 Supersomes at 50 pmoles/mL in solutions containing 0.1 M potassium phosphate, and 1 mM EDTA (pH 7.4) supplemented with 1 mM NADPH in a final volume of 1 mL/incubation tube (11 tubes total, plus 1 tube which had no NADPH). After 1 hour, fresh NADPH was added (2 mM final concentration) and the incubation continued for another hour. The reaction mixture was stopped and extracted with 5 mL ethyl acetate, and the organic layers collected and evaporated to dryness under a stream of nitrogen at 40°C, then resuspended in methanol (200 μL/tube), pooled into one tube and dried again, then re-suspended in 130 μL of methanol. Extracted reaction products were injected onto the LC column and resolved using conditions described for AM19 and AM1c9 preparations, except that the column was heated to 70°C. Elution fractions encompassing the NADPH-dependent UV visible peak (λ= 214) with a retention time of 16.5 min were collected manually, dried completely under a stream of nitrogen, and resuspended in methanol.

3.3.4.3 *Verification of Metabolite Identities.*

The identity of the isolated metabolites was confirmed using time of flight (Q-TOF) mass spectrometric analysis and by comparing analyte masses, retention times and MS/MS fragmentation patterns to previously published data (Dai et al., 2004). LC-QTOF analysis was performed on an Agilent HP 1200 coupled to an Agilent QTOF 6520. The chromatography column (Luna, C18 (2) 250 × 2 mm, 5 micron; Phenomenex, Torrance, CA) was heated to 70°C. Solvent A was 25 mM ammonium acetate (pH 5) in H_2O, and solvent B was 60% methanol, 40% acetonitrile. A stepwise linear gradient was used: 60% B at 0 min, 80% B at 22 min, 90% B from 23 to 29 min, 100% B from 29.1 to 23.1 min, then returned to 60% B at 33.1 min. The flow rate was held constant at 0.3 mL/min for the first 33.1 min, increased to 0.5 mL/min for 2 min, and then returned to 0.3 mL/min at 35 min. Total run time was 36 min (analyte retention times described below). Using mass to charge ratios of the MS spectra of the chromatographic peaks of each metabolite, the neutral masses were determined to be 1217.8387, 1233.8317, 1233.8327, for AM9, AM19, AM1c9 respectively. From these spectra and extracted neutral masses, chemical formulas were generated using the "Identify Compounds, Generate Formulas" Method in Mass Hunter Qualitative Analysis software (Mass Hunter Workstation Software, Qualitative Analysis, Version B.04.00, Agilent Technologies, Inc.). The software generated the formulas $C_{62}H_{111}N_{11}O_{13}$, $C_{62}H_{111}N_{11}O_{14}$, $C_{62}H_{111}N_{11}O_{14}$ for AM9, AM19, and AM1c9 with a ppm of -2.01, -0.4, and -1.21, respectively.

For MS/MS fragmentation analysis, we used an Agilent 1200 HPLC coupled to an Agilent 6410 triple quadruple tandem mass spectrometer. Metabolite preparations were first scanned, and then injected on column to look at selected product ions that confirmed the location of site of metabolism (Dai et al., 2004). The same chromatography

column and gradient conditions described for Q-TOF analysis were employed, except that the aqueous mobile phase consisted of 10 mM ammonium acetate (pH 4.0). The isolated metabolites gave the expected fragmentation pattern for hydroxylation at the 9-position for AM9, and the 1- & 9-position for both AM19 and AM1c9.

Concentrations of the isolated metabolite stock solutions were assigned by UV absorbance using a standard curve of cyclosporine peak areas, assuming a common extinction coefficient at 214 nm for the parent drug and metabolites. This approach has been used for cyclosporine (Wallemacq et al., 1989; Brooks et al., 1993) when no authentic metabolite standards were available and gravimetric measurement was impossible because of the small amount of metabolites isolated. For this step, an analytical column (Symmetry C18, 3.5 μm, 150 × 4.6 mm; Waters, Milford, MA) was used for analyte separation and was heated to 70°C. The mobile phases were (A) water, and (B) 20% methanol and 80% acetonitrile. A stepwise linear gradient was used: 60% B at 0 min, 100% B at 15 min and maintained for 4 min, then returned to 60% B. The flow rate was 1 mL/min.

3.3.4.4 LC/MS Quantification of CsA and Metabolites in Blood, Plasma and Urine Samples.

Cyclosporine and metabolite concentrations were quantified by LC/MS with modifications from a previously published procedure (Chen et al., 2006). For blood, 10 μL internal standard solution (2 μg/mL Cyclosporine D) was added to 0.25 mL of standard, quality control, or unknown clinical blood samples, in 15 mL glass screw-capped tubes. The mixture was vortexed and then 0.5 mL of hydrochloric acid (0.18 M)

was added. The acidified mixture was vortexed and 5 mL of methyl-tert-butyl ether was added. Samples were shaken for 20 minutes using a horizontal shaker and centrifuged (~2000 *g*) at room temperature for 20 min. The solvent phase was transferred to clean glass tubes containing 1 mL NaOH (0.1 M) for further a clean-up step. The final solvent phase was transferred to clean glass tubes and evaporated to dryness under a stream of nitrogen in a heated evaporator. The residue was reconstituted in 0.1 mL methanol and transferred to LC vials containing silanized glass inserts. A 5 µL aliquot of each reconstituted sample was injected into the LC–MS system. For plasma, the same procedure was used, except that a single extraction with 0.25 mL plasma in 1 mL NaOH (0.1M) and 5 mL methyl-tert-butyl ether was conducted. For urine analysis, 0.1 mL was mixed with 0.9 mL bovine serum albumin (1 mg/mL) and extracted with 4 mL methyl-tert-butyl ether, as described in the plasma extraction. The concentrations of CsA and its metabolites in clinical samples were quantified using an Agilent 1100 LC/MS operated in the electrospray positive mode. The column and LC conditions were the same as that described for QTOF analysis (see "Verification of Metabolites"). The following proton adducts of CsA and metabolites were monitored: *m/z* 1235 for AM19 and AM1c9, *m/z* 1219 for AM1, AM9, and AM1c, *m/z* 1189 for AM4N, *m/z* 1127.3 for cyclosporine D (internal standard), and *m/z* 1203.2, for CsA. The retention times for AM19, AM1c9, AM1, AM9, AM1c, AM4N, CsA, and cyclosporine D, were 15.3 min, 16.7 min, 22.5 min, 23.0 min, 23.7 min, 27.1 min, 27.7 min, and 27.7 min. respectively (**Figure 3.4**).

Calibration curves for CsA and its metabolites were generated by plotting the peak area ratios (analyte/internal standard) against cyclosporine or metabolite concentrations. The limits of quantitation in blood, plasma and urine were below that of

the lowest standards used: 0.5 ng/mL for AM4N, AM1c, AM1c9, 5 ng/mL for AM1, AM9, AM19, and 3.6 ng/mL for CsA. Intra-day and inter-day variability for the quantification of cyclosporine and metabolites at concentrations within the range of the standard curve was less than 8%.

3.3.5 Pharmacokinetic Analysis

Noncompartmental pharmacokinetic analysis was performed using WinNonlin (version 5.2, Pharsight, Mountain View, CA). Pharmacokinetic parameters determined for CsA and metabolites included the maximum concentration in blood (C_{max}), the time to reach C_{max} (T_{max}), terminal half-life ($t_{1/2}$), the $AUC_{0\text{-}48\ hr}$, the $AUC_{0\text{-}inf}$, and oral clearance (CL/F normalized to individual body weight in kg). In addition, $CL_{urinary}$ was calculated as the amount of drug or metabolite excreted in urine divided by AUC_{blood} for the drug or metabolite over the collection interval.

3.3.6 Statistical Analysis

Descriptive statistics are presented as mean ± standard deviation. Normality of the data was confirmed before statistical analysis. Statistical comparisons were conducted using an unpaired two-sided Student's t-test by GraphPad Prism 5 (La Jolla, CA). A *P* value less than 0.05 was considered significant.

3.4 Results

3.4.1 Demographic Characteristics of Healthy Volunteers

The demographic characteristics of 24 healthy volunteers who participated in this study are presented in **Chapter 2** and are shown in **Table 2.1**.

3.4.2 Systemic Disposition of Cyclosporine A and Its Primary and Secondary Metabolites

Mean blood CsA concentration-time profiles for the CYP3A5 expressors and nonexpressors who received a single 5 mg/kg dose of CsA are shown in **Figure 3.1**. CsA concentrations were similar, as reflected by comparable oral clearance (CL/F) for CYP3A5 expressors and nonexpressors (**Table 3.1**). Other blood pharmacokinetic parameters for the two groups were also comparable.

The mean blood concentration–time profiles of CsA metabolites after oral CsA administration are shown in **Figure 3.1**. The circulating blood CsA metabolite concentrations were lower than those of the parent drug. AM1, AM9 and AM19 were the major circulating metabolites. Comparing CYP3A5 expressors and nonexpressors, the average blood AUC for the primary CsA metabolites (AM1, AM9, AM4N, and AM1c) were similar (**Table 3.2**), as was the $AUC_{metabolite}/AUC_{CsA}$ ratio for primary CsA metabolites, an indirect measure of the respective metabolite formation clearances (**Table 3.2**).

In contrast to results for CsA and its primary metabolites, the average blood AUC for the secondary metabolites AM19 and AM1c9 (**Table 3.2**) was 47.4% and 51.3% higher in CYP3A5 expressors compared to nonexpressors (P = 0.040 and 0.011,

respectively). In accordance, the $AUC_{metabolite}/AUC_{CsA}$ ratio for AM19 and AM1c9 was 33.1% and 30.7% higher in CYP3A5 expressors compared to nonexpressors (P = 0.016 and 0.025), respectively (**Table 3.2 and Figure 3.2C**). Similarly, the AUC_{AM19}/AUC_{AM1} (**Figure 3.2D**) and AUC_{AM1c9}/AUC_{AM1c} (not shown) ratio was 46.9% and 30.6% higher in CYP3A5 expressors compared to nonexpressors (P = 0.002 and 0.025), respectively.

Also of note, the mean oral clearance of CsA was 40.4% higher in female subjects than in male subjects (17.3 ± 4.6 vs. 12.4 ± 2.2 mL/min/kg, P = 0.004). Among CYP3A5 expressors, the mean CL/F of CsA was 42.9% higher in females than in males (16.9 ± 5.4 vs. 11.8 ± 1.2 mL/min/kg, P = 0.069). Among CYP3A5 nonexpressors, the mean CL/F of CsA was 38.2% higher in females than in males (17.8 ± 3.9 vs. 12.9 ± 2.9 mL/min/kg, P = 0.038).

3.4.3 Renal Excretion of CsA and Its Primary Metabolites

The total amount of intact CsA excreted in urine over 48 hours after oral administration was comparable between CYP3A5 expressors and nonexpressors (1445.9 ± 495.5 and 1677.0 ± 450.2 ng, respectively). However, the mean apparent urinary CsA clearance based on the 48-hour collection was 20.4% lower in CYP3A5 expressors compared to CYP3A5 nonexpressors (4.2 ± 1.0 and 5.3 ± 1.3 mL/min, respectively, P = 0.037) (**Figure 3.3A**). Similarly, the eGFR-normalized apparent urinary CsA clearance based on the 48-hour collection was 28.5% lower in CYP3A5 expressors compared to CYP3A5 nonexpressors (0.03 ± 0.01 and 0.05 ± 0.02, respectively, P = 0.035) (**Figure 3.3B**). Although the interindividual variability was large, CYP3A5 expressors exhibited increased intrarenal CsA metabolism compared to nonexpressors, as demonstrated by

increased urinary CsA clearances over discrete urine collection time intervals (**Figure 3.3C**).

The average cumulative amount of AM19 and AM1c9 excreted in urine was 48% and 50% higher in CYP3A5 expressors compared to nonexpressors (P = 0.077 and 0.069, respectively). This is in agreement with greater blood exposure for AM19 and AM1c9 in CYP3A5 expressors, compared to nonexpressors. For the other CsA metabolites, the average amount excreted in urine in the two predicted phenotype groups was comparable. Interestingly, there was no CYP3A5-dependent difference in the apparent urinary clearance (amount excreted/AUC_{blood}) for all of the primary and secondary CsA metabolites measured.

3.5 Discussion

Understanding the basis of interindividual differences in CsA clearance is an important step towards the goal of improving the safety and efficacy of immunotherapy. In the current study, we evaluated how *CYP3A5* genetic variation (and the predicted enzyme expression phenotype) affected systemic and intrarenal CsA metabolism and exposure to its metabolites in blood.

Results showed that the mean oral CsA clearance for CYP3A5 expressors and nonexpressors was similar. This is in general agreement with some previous findings (Hesselink et al., 2003; Anglicheau et al., 2004; Zhao et al., 2005; Loh et al., 2008), but not with others (Haufroid et al., 2004; Min et al., 2004). Because the AM9 pathway is only one of three primary CsA elimination routes and because CYP3A5 exhibits selective formation of only AM9 at an efficiency that is less than that of CYP3A4 (Dai et al.,

2004), one would expect the total metabolic clearance to the primary metabolites to be influenced only modestly by the *CYP3A5* genotype. In support of this prediction, both the AUCs and the $AUC_{metabolite}/AUC_{CsA}$ ratios for AM1, AM9, AM4N and AM1c were similar for the two CYP3A5 phenotype groups.

In contrast to what was seen for the primary CsA metabolites, the AUCs for both AM19 and AM1c9 were significantly higher in CYP3A5 expressors compared to nonexpressors. In addition, there were greater amounts of AM19 and AM1c9 excreted in the urine of CYP3A5 expressors compared to nonexpressors. Based on *in vitro* product formation rates and *in vivo* metabolite/parent AUC ratios, the predominant source of AM19 and AM1c9 appears to be through conversion of AM1 and AM1c to the secondary metabolites, reactions that can be catalyzed efficiently by both CYP3A4 and CYP3A5 (**Chapter 4, Figure 4.9**).

The above findings suggest that at steady-state, when CsA dose is adjusted to achieve a narrow therapeutic blood concentration range, there will be greater accumulation of AM19 and AM1c9 in the systemic blood of CYP3A5 expressors compared to nonexpressors. It has been previously suggested that the production and accumulation of the AM19 and AM1c9 secondary metabolites of CsA might contribute to drug-induced nephrotoxicity (Christians et al., 1991b; Copeland and Yatscoff, 1992; Kempkes-Koch et al., 2001; Vollenbroeker et al., 2005). For example, Vollenbroeker *et al.* reported that AM19 and AM1c9 were the only CsA metabolites to show a positive correlation with the concentration of C-reactive protein and interleukin 6 (biomarkers of organ inflammation) measured in 202 blood specimens from kidney transplant recipients (Vollenbroeker et al., 2005). Christians *et al.* found an inverse correlation between the

steady-state blood concentration of AM1c9 and renal function in liver transplant patients during the early post-operative period (Christians et al., 1991b). Likewise, Kempkes-Koch *et al.* found elevated urine AM19 levels in patients with histologically confirmed CsA nephrotoxicity late after renal transplantation (Kempkes-Koch et al., 2001). Elevated secondary metabolites of CsA in patients with impaired renal function could be the result, rather than the cause of CsA nephrotoxicity. Alternatively, individual variability in the formation and accumulation of secondary CsA metabolites in blood could contribute directly to differences in renal toxicity risk. With this in mind, formation of AM1c9 and AM19 may represent a toxification pathway.

Higher systemic levels of AM19 and AM1c9 in CYP3A5 expressors should enhance entry of these metabolites into the renal tubular cells either by secretion from the efferent arteriole or after reabsorption from the luminal side following glomerular filtration. This is turn, could influence nephrotoxicity risk. Results from combination therapy with ketoconazole and CsA support this hypothesis. In a prospective, randomized study, when systemic levels of CsA were maintained at a similar level compared with the control arms, renal function was significantly better in the ketoconazole co-treatment group compared to CsA treatment alone (el-Agroudy et al., 2004). Interestingly, in human liver microsomal incubations with CsA, ketoconazole inhibited the formation of secondary metabolites more than the formation of primary CsA metabolites (Omar et al., 1997), further suggesting that the secondary metabolites of CsA are contributory to CsA nephrotoxicity.

The relationship between *CYP3A5* genotype and CsA nephrotoxicity has been studied by several research groups. Some investigators report a significant inverse

association between *CYP3A5* expression and renal function, as measured by serum creatinine or eGFR or clinically-evident CsA-related nephrotoxicity (Hauser et al., 2005; Eng et al., 2006; Klauke et al., 2008), whereas others found a positive association (Kreutz et al., 2008; de Denus et al., 2011). The impact of CYP3A5 expression on CsA nephrotoxicity is likely complicated by CYP3A5's dual role in CsA clearance within the kidneys and in the systemic formation of active secondary metabolites. Moreover, in studies of kidney transplant recipients, the relationship between genotype and nephrotoxicity is complicated by the fact that the phenotype of the donor kidney may differ from the recipient's intestinal and hepatic phenotype (Staatz et al., 2010b). The kidney transplant recipient's *CYP3A5* genotype and hepatic and intestinal CYP3A5 activity should determine the concentrations of CsA and its metabolites to which the transplanted kidney is exposed. At the same time, the donor's renal CYP3A5 status would influence the amount of CsA and its metabolites formed locally in the renal tubular cells.

Results from the current study suggest that carriers of the *CYP3A5*1* allele, and an inferred high CYP3A5 renal expression phenotype, exhibit greater renal CsA metabolism and a lower apparent urinary CsA clearance compared to those subjects lacking the active *CYP3A5* allele. Such a relationship between renal metabolism and the apparent urinary clearance of unchanged drug was first reported by Sirianni *et al.,* who showed that the urinary clearance of enalapril was increased due to inhibition of its esterolysis by paraoxon in isolated perfused rat kidneys (Sirianni and Pang, 1999). In our study, the mean apparent urinary CsA clearance was 20.4% lower in CYP3A5 expressors, compared to CYP3A5 nonexpressors, consistent with significant intrarenal CYP3A5-

dependent CsA metabolism, presumably through AM9 formation (Dai et al., 2004). A semi-physiological model was developed to evaluate the effect of *CYP3A5* polymorphism on intrarenal metabolism and tubulo-epithelial exposure to tacrolimus, another calcineurin inhibitor (**Chapter 2**). In that case, the model fitting results supported the conclusion that reduced urinary tacrolimus clearance is due to increased intrarenal metabolism and decreased renal exposure to tacrolimus in metabolically competent cells, the tubular epithelia.

In individuals with significant renal CYP3A5 expression, one might expect higher intrarenal accumulation of AM19 and AM1c9, independent of an effect of intestinal and hepatic *CYP3A5* genotype on systemic accumulation of the secondary metabolites. Such a difference might affect the risk of renal toxicity. However, the effect from a higher level of putatively nephrotoxic secondary metabolites might be counteracted by lower intrarenal levels of CsA. In addition, it is also important to consider the role of renal P-glycoprotein, which can transport CsA and in the renal tubular epithelium would act to reduce intracellular concentrations by active efflux activity. Polymorphisms in the *ABCB1* gene, which putatively affect enzyme expression (Staatz et al., 2010a), have been associated with the risk of renal toxicity from CsA therapy (Hebert et al., 2003; Staatz et al., 2010b). High P-glycoprotein activity may independently influence intrarenal exposure to AM19 and AM1c9, if these metabolites are also substrates for active tubular efflux. This study was not designed to test the effect of *ABCB1* gene variation on renal CsA clearance (would require a much larger number of subjects), however we did conduct genotyping for the transporter and found, as expected, there were no significant difference in key genotype or haplotype frequencies between CYP3A5 expressor and

nonexpressor groups (**Table 2.1**). Thus, the *CYP3A5* expressor association that was observed should not have been influenced by the *ABCB1* genotype status.

In summary, we found that individuals expressing CYP3A5 exhibited enhanced formation of AM19 and AM1c9, secondary metabolites of CsA that have been associated with an increased risk of CsA-induced nephrotoxicity. Moreover, the same phenotype influenced the apparent urinary clearance of CsA, suggesting the presence of significant intrarenal CsA metabolism for individuals that carry the functional *CYP3A5*1* allele. These findings point towards the need for careful evaluation of the impact of both recipient and donor *CYP3A5* genotypes on renal function in organ transplant patients receiving chronic CsA immunotherapy.

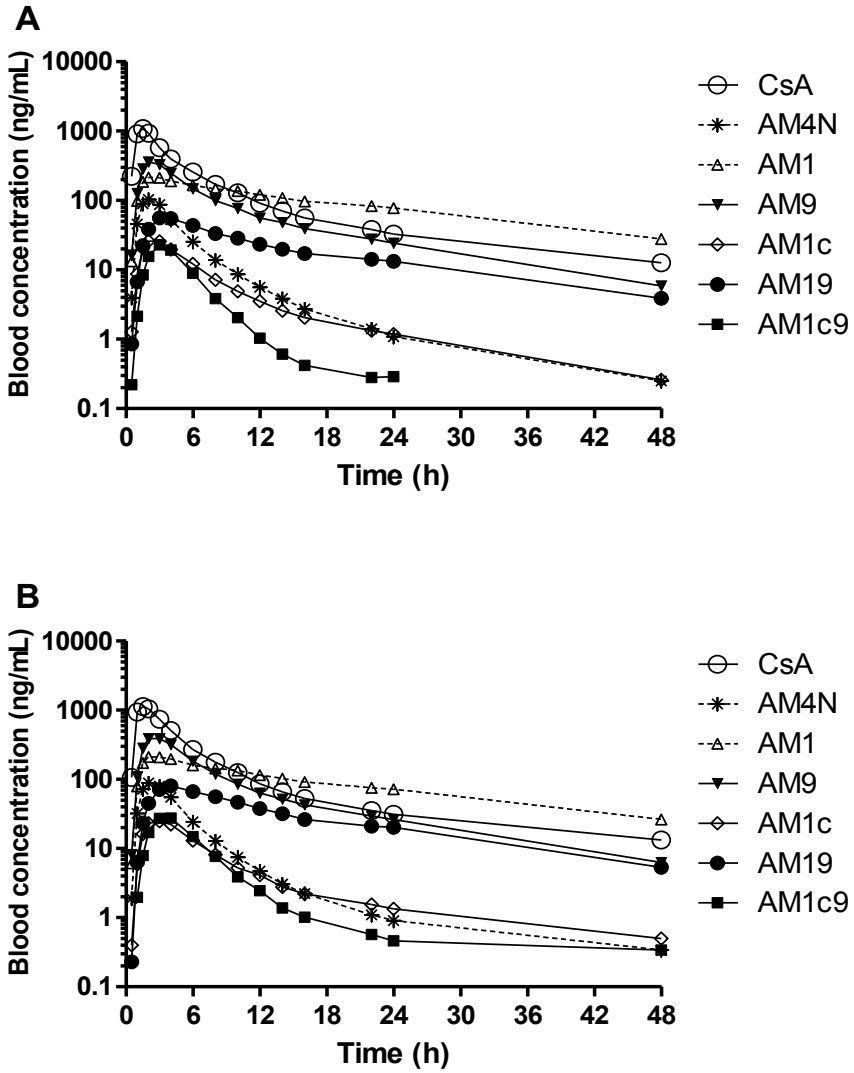


Figure 3.1 Mean log blood concentration–time profiles of cyclosporine A (CsA) and its metabolites after 5 mg/kg oral CsA administration in (A) CYP3A5 nonexpressors (n = 12) and (B) CYP3A5 expressors (n = 12).

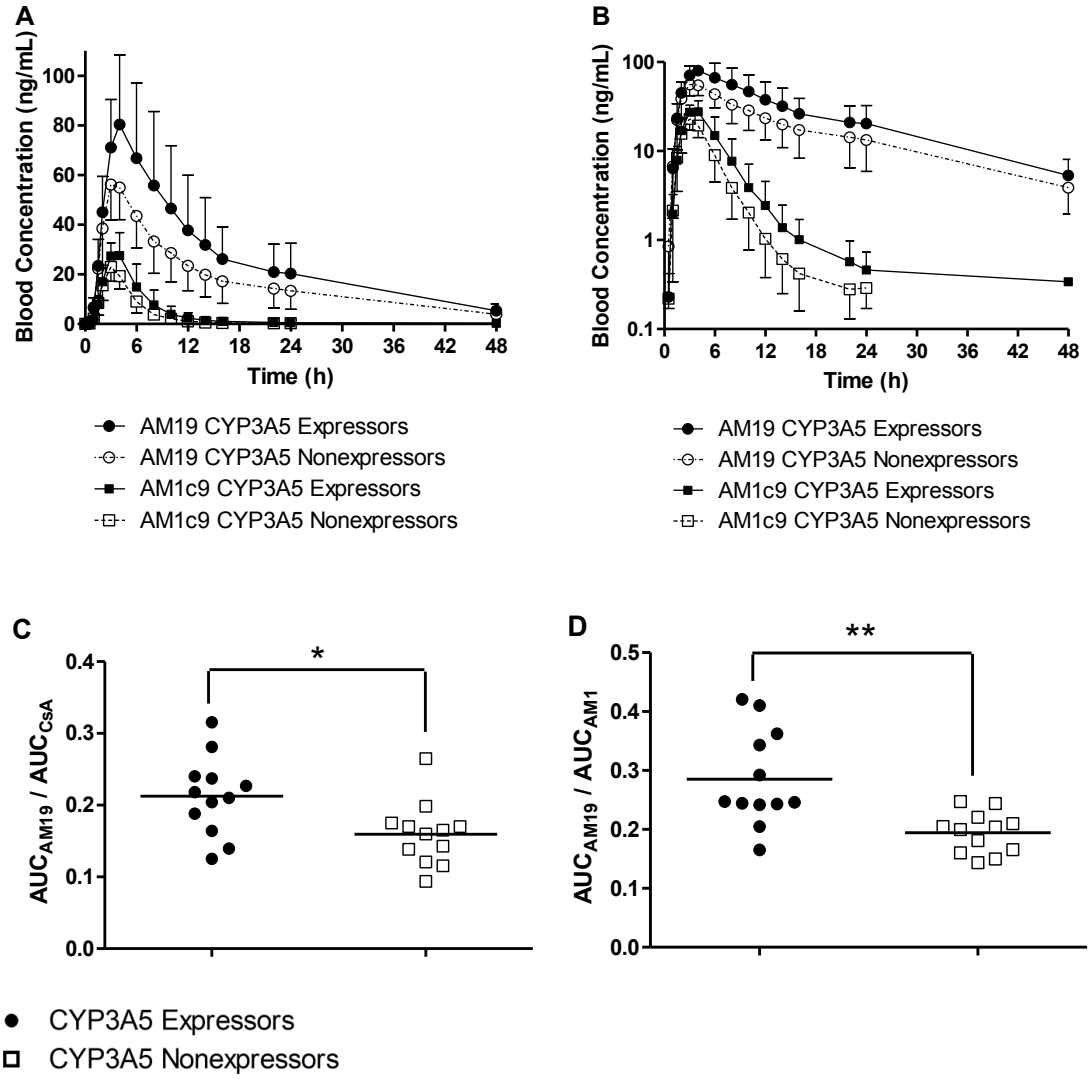


Figure 3.2 (A) Blood concentration–time profiles of AM19 and AM1c9 in CYP3A5 nonexpressors (n = 12) and CYP3A5 expressors (n = 12). (B) Blood concentration–time profiles of AM19 and AM1c9 displayed using a logarithmic Y-axis. Bars represent standard deviations. AUC ratios are shown for (C) AUC_{AM19}/AUC_{CsA} and (D) AUC_{AM19}/AUC_{AM1} by predicted CYP3A5 phenotype. The solid line represents the mean ratios; * $P < 0.05$; ** $P < 0.005$.

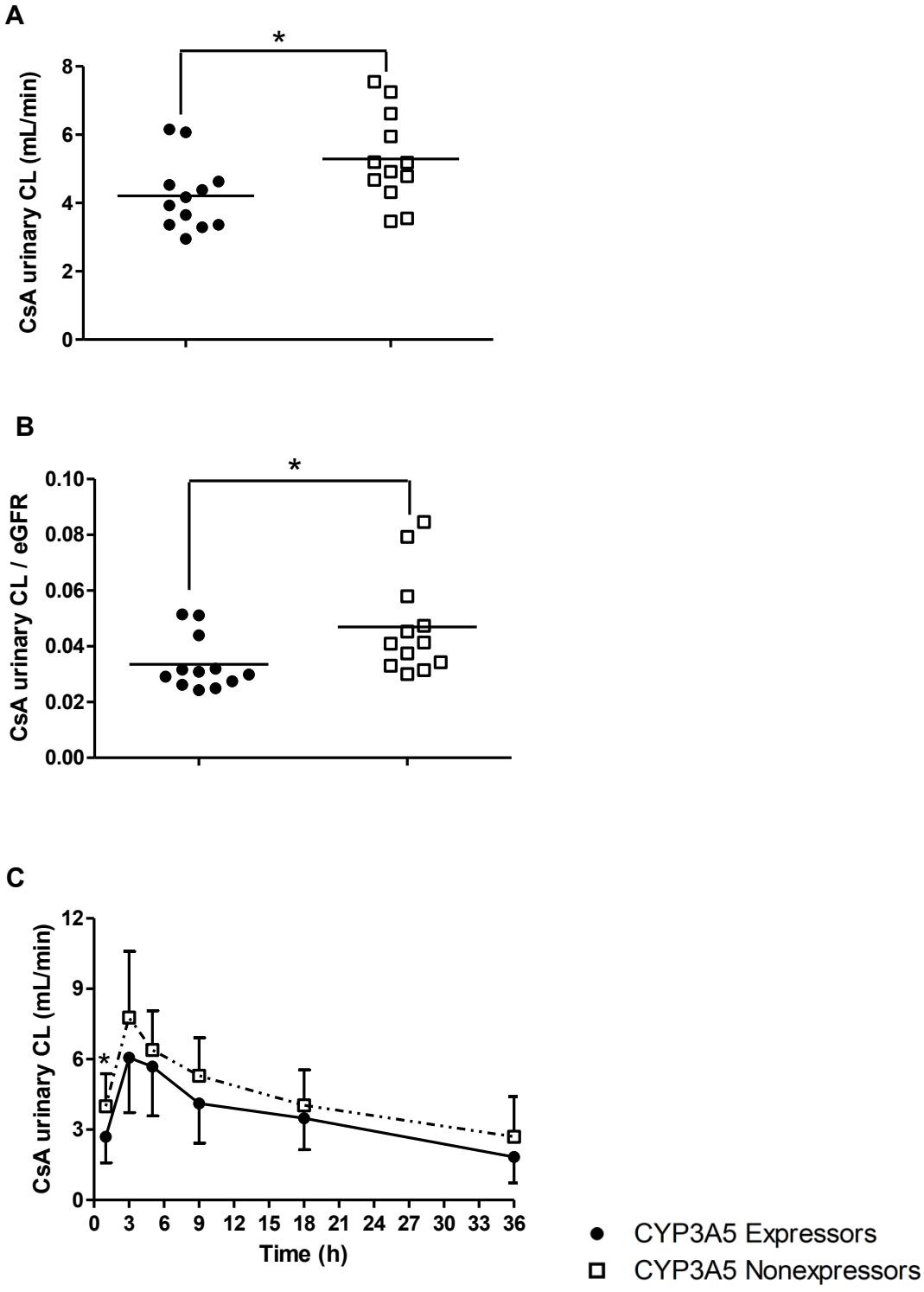


Figure 3.3 (A) Apparent urinary CsA clearance and (B) eGFR normalized CsA urinary clearance based on a 48-hour urine collection; (C) the time-course of urinary CsA clearance calculated based on discrete urine collection intervals. The solid line represents the mean ratios; * $P < 0.05$.

Table 3.1 Cyclosporine A blood pharmacokinetic parameters for study participants stratified by predicted CYP3A5 phenotype

	CYP3A5 Expressors (N=12)	CYP3A5 Nonexpressors (N=12)	*P* value
AUC_{0-48} (ng hr/mL)	5287 ± 1432	5780 ± 1444	0.41
AUC_{0-inf} (ng hr/mL)	5670 ± 1603	6098 ± 1509	0.51
$t_{1/2}$ (hr)	17.1 ± 4.1	17.8 ± 2.5	0.6
t_{max} (hr)	1.5 ± 0.3	1.6 ± 0.5	0.37
C_{max} (ng/mL)	1161 ± 221	1194 ± 319	0.77
C_{last} (ng/mL)	11.9 ± 4.5	12.6 ± 4.0	0.72
CL/F (mL/min/kg)	15.7 ± 4.2	14.8 ± 4.8	0.61

Data are presented as mean ± SD. AUC, area under the concentration–time curve; t_{max}, time to reach the maximum blood concentration; C_{max}, maximum blood concentration; C_{last}, blood concentration at 48 hour after Cyclosporine A administration; CL/F, oral clearance.

Table 3.2 $AUC_{0-infinity}$ and $AUC_{metabolite}/AUC_{CsA(0-infinity)}$ of Cyclosporine A and its metabolites for study participants stratified by predicted CYP3A5 phenotype.

	CYP3A5 Expressors (N=12)	CYP3A5 Nonexpressors (N=12)	*P* value
$AUC_{0-infinity}$			
CsA	6098 ± 1509	5670 ± 1603	0.51
AM1	4711 ± 1509	4900 ± 2188	0.81
AM9	3186 ± 766	2801 ± 712	0.22
AM4N	418 ± 118	456 ± 94	0.39
AM1c	197 ± 94	185 ± 79	0.74
AM19	1360 ± 602	923 ± 343	0.040
AM1c9	162 ± 62	107± 29	0.011
AUC_m/AUC_{CsA}			
AM1	0.76 ± 0.12	0.84 ± 0.23	0.29
AM9	0.52 ± 0.06	0.49 ± 0.08	0.35
AM4N	0.07 ± 0.02	0.08 ± 0.02	0.16
AM1c	0.03 ± 0.01	0.03 ± 0.01	0.82
AM19	0.21 ± 0.05	0.16 ± 0.04	0.016
AM1c9	0.03 ± 0.01	0.02 ± 0.01	0.025

Data are presented as mean ± SD. AUC, area under the concentration–time curve, expressed in units of ng hr/mL.

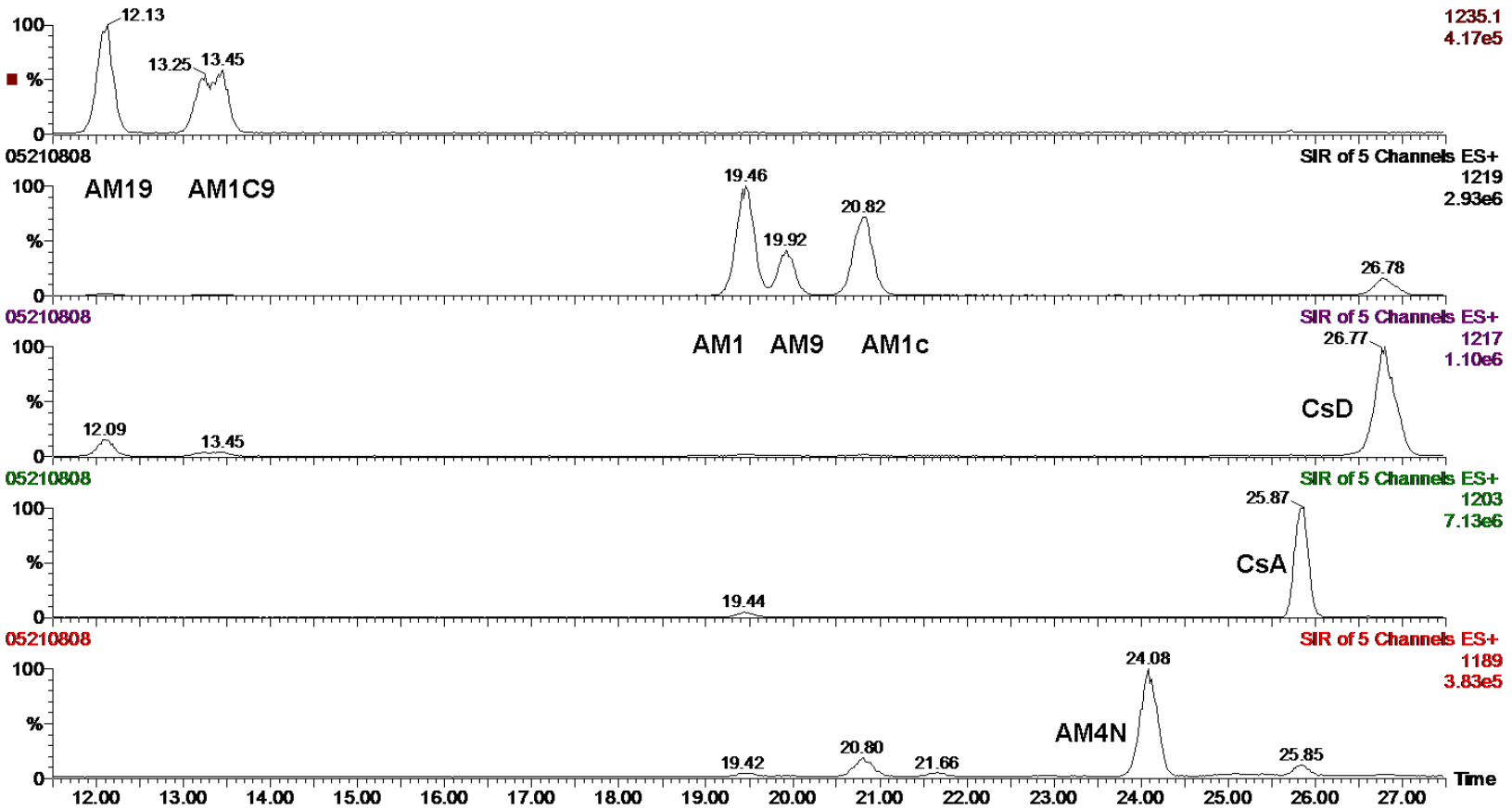


Figure 3.4 Representative chromatogram for CsA and its metabolite quantification.
The following proton adducts of CsA and metabolites were monitored: *m/z* 1235 for AM19 and AM1c9, *m/z* 1219 for AM1, AM9, and AM1c, *m/z* 1189 for AM4N, *m/z* 1127.3 for cyclosporine D (internal standard), and *m/z* 1203.2, for CsA.

Chapter 4

In Vitro Characterization of CsA and Tacrolimus Metabolism

Portions of Chapter 4 were submitted to *Clinical Pharmacology & Therapeutics* and *Transplantation.*

4.1 Abstract

To fully interpret the tacrolimus pharmacokinetic data, we conducted substrate depletion studies showing that the disappearance clearance of tacrolimus and 31-DMT catalyzed by CYP3A5 was similar and about 1.7- and 1.5-fold higher than that of CYP3A4. The disappearance clearance for the other primary metabolites was lower (ranging from 14% to 37%) than that of tacrolimus and 31-DMT, irrespective of the CYP3A isoform catalyzing the reaction. The results indicate that there may be sequential secondary intrarenal metabolism of the primary tacrolimus metabolites, particularly 31-DMT.

To fully interpret the CsA pharmacokinetic data, we evaluated the formation pathways of AM19 and AM1c9 by *in vitro* incubations of CsA, AM1, AM9 and AM1c with recombinantly expressed CYP3A4 and CYP3A5. The results suggested that the predominant source of AM19 and AM1c9 occurs through conversion of AM1 and AM1c to the secondary metabolites, respectively, reactions that can be catalyzed efficiently by both CYP3A4 and CYP3A5. AM9 does not contribute significantly to the formation of AM19 in CYP3A5 expressors, the pathway of which mediated by CYP3A5. In addition, CYP3A4 catalyzed the formation of AM1, AM9 and AM4N, whereas CYP3A5 exhibited marked product regioselectivity (AM9). At a substrate concentration of 8.33 μM, CYP3A4 generated AM1, AM9 and AM4N in a ~ 1:4:4 ratio, compared to a 2:34:1 ratio for CYP3A5. However, at lower CsA concentrations (200nM), the formation rate of AM9 from CsA mediated by CYP3A5 was, on average, 63.5% of that mediated by CYP3A4, consistent with the report that the intrinsic clearance of AM9 formation by CYP3A5 Supersomes is 57% of that by CYP3A4 Supersomes.

In addition, we showed that CYP3A7 is much less efficient at metabolizing tacrolimus than CYP3A4 and CYP3A5 *in vitro*. The disappearance clearance of tacrolimus at much lower than reported K_m catalyzed by CYP3A7 was 9.5 ± 2.8% and 6.0 ± 2.7 % of that of CYP3A4 and CYP3A5, respectively. At low tacrolimus concentrations, there was significant depletion of 31-DMT by CYP3A5, but not by CYP3A7, although the formation rates of 31-DMT by CYP3A5 and CYP3A7 were comparable. Furthermore, the metabolism of tacrolimus by CYP3A7+ b_5 showed substrate inhibition kinetics for the formation of 13-DMT, 15-DMT and 31-DMT. No 12-HT was formed. The formation of 31-DMT displayed very high affinity binding for CYP3A7 ($K_m \approx$ 10% of that with 13-DMT and 15-DMT formation) and a low V_{max} ($V_{max} \approx$ 3.6 % of that with 13-DMT formation), thus the CL_{int} of 31-DMT was a respectable 29% of that of 13-DMT.

4.2 Introduction to Chapter 4

The human cytochromes P450 (P450) CYP3A isoforms, CYP3A4, CYP3A5 and CYP3A7, exhibit varying degrees of catalytic capability and regioselectivity towards common substrates (Gorski et al., 1994; Kuehl et al., 2001; Williams et al., 2002). For example, CYP3A4 and CYP3A5 exhibit regioselective differences in tacrolimus metabolism (Dai et al., 2006). Both enzymes generate 13-DMT and 12-HT, but the formation rates are higher for CYP3A5 than CYP3A4. In addition, 31-DMT is formed predominantly from CYP3A5, whereas 15-DMT is generated almost exclusively by CYP3A4. With respect to CsA metabolism, it was shown that the two CYP3A isoforms (CYP3A4 and CYP3A5) also exhibit different product regioselectivity (Aoyama et al., 1989) and it was further demonstrated that three major metabolites (AM1, AM9 and AM4N) are formed by CYP3A4, while only AM9 is formed by CYP3A5 (Dai et al.,

2004). To date, only a few publications addressed the metabolism of tacrolimus by CYP3A7, showing that CYP3A7 is less efficient at metabolizing tacrolimus than CYP3A4 and CYP3A5 (catalytic efficiency 29% and 18%, respectively) (Kamdem et al., 2005). With respect to metabolite formation, it was reported that the formation of 13-DMT by CYP3A7+b_5 was 50-fold lower than that of CYP3A5 at a substrate concentration of 0.2 μM. The formation of other metabolites was negligible (Dai et al., 2006).

A substrate like CsA or tacrolimus may undergo sequential cytochrome P450-dependent oxidative reactions to generate secondary or tertiary metabolites. Such reactions may occur by two different kinetic mechanisms (Sugiyama et al., 1994). In mechanism 1, the primary metabolite-enzyme complex is activated and then converted to the secondary metabolite before the primary metabolite is released from the enzyme. In mechanism 2, very little of the secondary metabolite may be formed initially; instead, most of the secondary metabolite is formed from the released primary metabolite that subsequently reassociates with the enzyme to form complexes that lead to the formation of the downstream metabolite (Sugiyama et al., 1994). Although secondary metabolites may be formed by different cytochrome P450 enzymes, the oxidation reactions frequently are catalyzed by the same enzyme (Sugiyama et al., 1994). Significant non-dissociative sequential metabolism (mechanism 2) generally results in low or no detectable levels of the primary metabolites and emergence of secondary and tertiary metabolites both *in vitro* and in the blood circulation *in vivo* (Pang and Gillette, 1979; Pang, 1995). Both CsA and tacrolimus are known to undergo sequential oxidative metabolism, although the degree to which it is non-dissociative or even catalyzed by the same enzyme is largely

unknown. The issue is potentially important, as some of the secondary metabolites (e.g., AM19 and AM1c9 for CsA and 31-DMT for tacrolimus) have been associated with adverse effects from chronic CNI pharmacotherapy. In this regard, polymorphic CYP3A5 and CYP3A7 expression could influence systemic and renal exposure to primary and secondary CNI metabolites, both in adults and in the developing fetus (Leeder et al., 2005). However, teasing out the contributions of a single enzyme to product formation from *in vivo* pharmacokinetic data can be challenging when sequential metabolism occurs.

In chapter 2, although we observed a lower urinary tacrolimus clearance in CYP3A5 expressors compared to CYP3A5 nonexpressors, we did not see statistically significant differences in the urinary clearance of tacrolimus's primary metabolites. One possible explanation is that sequential intrarenal metabolism of the primary tacrolimus metabolites to secondary tacrolimus metabolites masked their local formation from tacrolimus. In this chapter, we characterized the sequential metabolism of primary tacrolimus metabolites by substrate depletion studies using CYP3A4 and CYP3A5 Supersomes™. In addition, we presented the time course of primary metabolite disappearance by incubating tacrolimus with recombinant CYP3A enzymes, which was illustrative of the sequential metabolism of tacrolimus.

In **Chapter 3**, we observed that the average blood AUC for the secondary CsA metabolites, AM19 and AM1c9, was 47.4% and 51.3% higher in CYP3A5 expressors compared to nonexpressors. In accordance, the $AUC_{metabolite}/AUC_{CsA}$ ratio for AM19 and AM1c9 was 33.1% and 30.7% higher in CYP3A5 expressors compared to nonexpressors. Similarly, the AUC_{AM19}/AUC_{AM1} and AUC_{AM1c9}/AUC_{AM1c} ratio was 46.9% and 30.6%

higher in CYP3A5 expressors compared to nonexpressors. In order to evaluate the kinetics of the sequential reactions and the impact of CYP3A5 genetic variation, the formation of secondary metabolites was examined by incubating the parent drug (CsA) and its primary metabolites (AM1, AM9 and AM1c) with CYP3A4 and CYP3A5 Supersomes. We also evaluated the primary and secondary CsA metabolite formation at both high and low substrate (CsA) concentrations.

Finally, in **Chapter 7**, we report that the arterial-to-venous drug concentration ratios did not vary with the time interval between dosing and sample collection and arterial umbilical cord blood concentrations of tacrolimus were 100 ± 12% of venous concentrations (ranged 81–113%). Although there is no clear evidence of significant fetal tacrolimus metabolism, we characterized the kinetic parameters estimated from tacrolimus primary metabolite formation by CYP3A7 and further compared the disappearance clearance of tacrolimus by three CYP3A isoforms at clinically relevant concentration (1 ng/mL). Because CYP3A7 is produced polymorphically in livers and small intestines of ~15% of adult Caucasians (Kuehl et al., 2001; Burk et al., 2002), our data should also provide insight into the metabolic potential of CYP3A7 in adult.

4.3 Materials and Methods

4.3.1 Materials

The source of cyclosporine A, tacrolimus and their metabolites was described in **Chapter 2** and **Chapter 3**. CYP3A4 + b_5 Supersomes™ (Catalog No. 456202, b_5 content is 700 pmol/mg protein), human CYP3A5 + b_5 Supersomes ™ (Catalog No.456256, b_5 content is 1400 pmol/mg protein), and human CYP3A7 + b_5

Supersomes™ (Catalog No. 456237, b_5 content is 260 pmol/mg protein) were purchased from BD Biosciences (Woburn, MA).

4.3.2 CYP3A-Dependent Tacrolimus and Primary Metabolite Disappearance Kinetics

Experimental Conditions

All experiments were performed using glass tubes to reduce non-specific binding. Tacrolimus and its primary metabolites were dissolved in methanol; the final methanol concentration was less than 1%.

In experiment 1, tacrolimus and its primary metabolites (13-DMT, 15-DMT, 31-DMT, 12-HT) (1ng/mL) were incubated with 10 pmol/mL of human CYP3A4 + b5 Supersomes™ and human CYP3A5 + b5 Supersomes ™ for 0, 15 and 30 min, in duplicate, with a solution containing 0.1 M potassium phosphate, pH 7.4, and 1 mM EDTA in a shaking water bath maintained at 37°C. In experiment 2, tacrolimus and 31-DMT were incubated with 10 pmol/mL of human CYP3A4 + b5 Supersomes™, CYP3A5 + b5 Supersomes™ and CYP3A7 + b5 Supersomes™ for 2.33 min, 5.33 min, 10.33 min and 20.33 min. Again, all incubations were performed in duplicate using the same buffer described for experiment 1.

The final reaction volume was 1 mL. All reactions were initiated by adding NADPH (final concentration 1 mM) or buffer (as negative control) after 5 min of preincubation and were terminated by the addition of 1 mL of ice-cold acetonitrile. Tacrolimus and its metabolites were extracted and quantified by LC-MS/MS using the

method described in chapter 2, with minor modification (excluded the addition of ammonium acetate buffer).

Kinetic Analysis of Substrate Disappearance in CYP3A Incubations

For substrate disappearance experiments, the apparent elimination rate constant (K_e, time^{-1}) was calculated from the slope of the logarithm of remaining substrate concentration versus incubation time plot after addition of 1 ng/mL tacrolimus and NADPH (Obach and Reed-Hagen, 2002). K_e was estimated using the time range that conferred log-linear substrate disappearance with respect to time. The apparent disappearance clearance (CL_{Disapp}^{CYP3A}) was calculated from: $CL_{Disapp}^{CYP3A} = \frac{K_e}{[\text{supersomal protein}]}$.

4.3.3 Tacrolimus Metabolite Formation Kinetics for CYP3A7

Experimental Conditions

All experiments were performed using glass tubes. Incubations of recombinant enzyme preparation (human CYP3A7 + b_5 Supersomes™) with each specified concentration of tacrolimus were performed in duplicate in solutions containing 0.1 M potassium phosphate, pH 7.4, and 1 mM EDTA in a shaking water bath maintained at 37°C. Tacrolimus was first purified by HPLC before its use in the kinetic studies. All metabolites contained in the purified stock tacrolimus were under 0.01%, as confirmed by LC-MS/MS analysis. Tacrolimus was dissolved in methanol; the final methanol concentration was less than 0.5%. Tacrolimus (0.01, 0.05, 0.1, 0.2, 0.5, 1, 5, 10, 50, 100 μM) was incubated 5 pmol/mL CYP3A7+b_5 for 2 min. The final reaction volume was 1 mL. All reactions were initiated by adding NADPH (final concentration 1 mM) or buffer (as negative control) after 5 min of preincubation and were terminated by the addition of

4 mL of ice-cold methyl tert-butyl ether after 2 min. The average reaction velocity from replicate incubations was computed for each substrate concentration. Tacrolimus metabolites were extracted and quantified by LC-MS/MS, using the method described in chapter 2 with minor modification (without adding the ammonium acetate buffer). No internal standard (IS) was employed in the extraction process because of significant area overlap in selective metabolite mass (*m/z)* channels at high IS concentrations. Instead, the absolute areas of metabolites were used for plotting calibration curves. Full tacrolimus concentration-metabolite formation rate profile experiments were conducted in two independent sets of experiments.

Kinetic Analysis of Metabolite Formation in CYP3A7 Incubations

Enzyme kinetic parameters for tacrolimus primary metabolite formation were computed using GraphPad Prism 5 (GraphPad, La Jolla, CA) nonlinear regression analysis. For each of the replicate CYP3A7 experiments, a simple hyperbolic model (eq. 4.1) and a Michaelis-Menten model with noncompetitive substrate inhibition (eq. 4.2) (von Moltke et al., 1996) were fit to the metabolite formation data:

$$V = \frac{V_{max} \times [S]}{(K_m + [S])} \qquad \text{(eq. 4.1)}$$

$$V = \frac{V_{max} \times [S]}{(K_m + [S] \times (1 + [S]/K_s))} \qquad \text{(eq. 4.2)}$$

[*S*] is the substrate concentration (0.01–100 μM). Vmax is the maximum enzyme velocity. K_m is the Michaelis constant, expressed in the same units as substrate concentration. K_s is the dissociation constant for substrate binding, where two substrate molecules can bind to an enzyme with the same affinity and expressed in the same units

as K_m. Average parameter values and computer-generated standard error estimates were calculated from the replicate experiments. For tacrolimus metabolite formation in incubations using heterologously expressed CYP3A7, intrinsic clearances CL_{int}^{CYP3A} were calculated as $CL_{int}^{CYP3A} = \frac{V_{max}}{K_m}$ and the unbound intrinsic clearances $CL_{int,u}^{CYP3A}$ were calculated as $CL_{int,u}^{CYP3A} = \frac{V_{max}}{K_{m,u}}$.

The unbound K_m ($K_{m,u}$) was estimated from fitting the model described by $V = \frac{V_{max} \times [S]_u}{(K_{m,u} + [S]_u \times (1 + [S]_u / K_s))}$ to the metabolite formation data. In this case, $[S]_u$ is the unbound substrate concentration ($= f_{u,app} \times [S]$), as described in (Dai et al., 2006).

By defining f_s as the fraction of total amount of drug added to the tube that remained in the aqueous environment, f_u as the fraction of unbound drug in the aqueous mixture (relative to $[S]_s$), and $f_{u.app}$ as the apparent unbound drug fraction in the mixture that was used to correct the concentration-dependent metabolite formation data, the relationship between $f_{u.app}$, f_u and f_s can be expressed as $f_{u.app} = f_s \times f_u$ (Dai et al., 2006).

The mean $f_{u,app}$ was used to adjust tacrolimus concentration from 0.01 to 10 μM (mean $f_{u,app}$ ranged from 4% to 14%, **Figure 4.6**). It was noted that the major factor contributing to the nonlinearity of tacrolimus unbound drug concentration was nonspecific binding of tacrolimus to the experimental apparatus ($1 - f_s$), rather than binding to the microsomal/supersomal protein ($1 - f_u$) (Dai et al., 2006). f_s varied 5-fold (ranging from 10 to 50%) and f_u was relatively constant over the tacrolimus concentration range (mean $f_u \approx 25\%$). In a previous study (Dai et al., 2006), 15 mL polypropylene centrifuge tubes were used for incubation instead of the glass tubes employed in the

current experiment and binding of tacrolimus to the experimental apparatus ($1 - f_s$) was different (less binding to the glass compared to polypropylene surface). Therefore, $[S]_u$ was also calculated by using the mean f_u value by assuming that binding to the Supersomal protein accounted for all the non-specific substrate binding.

4.3.4 **Formation of CsA metabolites by CYP3A4 and CYP3A5**

All incubations were performed in duplicate with solutions containing 0.1 M potassium phosphate, pH 7.4, and 1 mM EDTA in glass tubes. The incubation temperature was 37°C. CsA was dissolved in methanol and added into the incubation solutions to the desired concentrations (8.33 μM or 200 nM). The final reaction volume was 0.25 mL and the final methanol concentration was 2%. CYP3A4 and CYP3A5 Supersomes concentrations were 5 pmol/mL. The mixture was preincubated at 37°C for 5 min before addition of NADPH (final concentration 1 mM) or buffer (as negative control) to initiate the reaction. Reactions with an 8.33 μM CsA concentration were terminated after 10 min or 60 min by the addition of cold acetonitrile. Reactions with a 200 nM CsA concentration were terminated after 5, 10 or 15 min by the addition of cold acetonitrile.

4.3.5 **Formation of Secondary Metabolites of CsA by CYP3A4 and CYP3A5**

To quantify rates of formation of secondary metabolites of CsA (parent drug), the primary metabolites, AM1, AM9 and AM1c (1 μM) were incubated in duplicate with CYP3A4 (1000 pmol/mL co-expressed with cytochrome b_5), and CYP3A5 (1000 pmol/mL co-expressed with cytochrome b_5). The final reaction volume was 0.25 mL and the final methanol concentration was 2%. CYP3A4 and CYP3A5 Supersomes

concentrations were 5 pmol/mL. The reactions were initiated by addition of NADPH or buffer after a 5-min preincubation period and were terminated after 1 hr. A lower concentration of AM1, AM9 or AM1c (200 nM) was also incubated with CYP3A4 and CYP3A5 Supersomes co-expressed with cytochrome b_5 for a shorter incubation of 30 min. The final reaction volume and CYP3A4 and CYP3A5 Supersomes concentrations were the same as above (0.25 mL and 5pmol/mL, respectively). Reaction products were extracted with ethyl acetate and measured by LC-MS, as described in **Chapter 3**.

4.4 Results

4.4.1 Disappearance Clearance of Tacrolimus and Its Primary Metabolites by CYP3A4 and CYP3A5

Results from substrate depletion studies showed that the disappearance clearance of tacrolimus and 31-DMT catalyzed by CYP3A5 was similar and about 1.7- and 1.5-fold higher than that of CYP3A4. The disappearance clearance for the other primary metabolites was lower (ranging from 14% to 37%) than that of tacrolimus and 31-DMT, irrespective of the CYP3A isoform catalyzing the reaction (**Figure 4.1A**). The results indicate that there may be sequential secondary intrarenal metabolism of the primary tacrolimus metabolites, particularly 31-DMT, thus rendering the urinary recovery of only primary metabolites an unreliable indicator of total renal tacrolimus metabolism and intrarenal metabolite exposure. This may partially explain why the mean urinary clearance of the tacrolimus metabolites was comparable between CYP3A5 expressors and nonexpressors.

Considering that the total dose of tacrolimus that the kidney is exposed to may be much smaller than what the liver and the small intestine see during first pass, 13-DMT and other metabolites formed in the kidney might be relatively insignificant compared to these metabolites (pre-)formed by liver and/or the small intestine. Furthermore, the metabolites formed in the kidney may passively diffuse into blood, becoming available for systemic circulation, which may also mask their detection by examining urinary clearance.

4.4.2 Disappearance Clearance of Tacrolimus and 31-DMT by CYP3A7

The disappearance clearance of tacrolimus catalyzed by CYP3A7 was 9.5 ± 2.8% and 6.0 ± 2.7 % of that of CYP3A4 and CYP3A5, respectively (**Figure 4.2**). Because the incubation concentration (1 ng/mL) is much lower than the reported K_m (Dai et al., 2006), the disappearance clearance should reflect the catalytic efficiency of the metabolizing enzymes (Obach and Reed-Hagen, 2002). This finding, that CYP3A7 is much less efficient at metabolizing tacrolimus than CYP3A4 and CYP3A5, is in qualitative agreement with the results of Kamdem et al., although their reported activity of CYP3A7 relative to either CYP3A4 or CYP3A5 is higher (Kamdem et al., 2005). Those investigators reported a lower affinity and capacity of CYP3A7 toward tacrolimus, suggesting that the enzyme will play no role in tacrolimus metabolism *in vivo* (Kamdem et al., 2005). We also observed a significantly lower enzyme efficiency for CYP3A7 than that of CYP3A4 and CYP3A5, and both findings are consistent with the observation that arterial umbilical cord blood concentrations of tacrolimus were similar to the venous concentrations shown in **Chapter 7**.

We also quantified metabolite formation from a 1 ng/mL tacrolimus concentration (**Figure 4.3**). By converting all metabolite concentrations to nM and using the sum of formation rate of primary metabolites at 2.33 min to calculate the relative ratios (**Table 4.1**), the sum of formation rate of primary metabolites by CYP3A7 was 17.9 % and 4.7 % of that by CYP3A4 and CYP3A5, respectively. This is in general agreement with the ratios calculated based on substrate depletion data. Small differences could be the result of sequential secondary metabolism.

4.4.3 Formation Clearance of Tacrolimus Primary Metabolites by CYP3A7

Although CYP3A7 was shown as not efficient at metabolizing tacrolimus (Kamdem et al., 2005), the metabolite formation of tacrolimus, particularly formation of the active metabolite, 31-DMT, has not been evaluated. Accordingly, we determined the enzyme kinetic parameters for 13-*O*-demethylation, 15-*O*-demethylation and 31-*O*-demethylation of tacrolimus catalyzed by baculovirus-expressed CYP3A7+b5. In this experiment, the tacrolimus concentration range varied from 0.01 to 100 μM.

The formation of 13-DMT, 15-DMT and 31-DMT was NADPH-dependent. However, at higher tacrolimus concentrations, there was significant non-enzyme generated appearance of signals in the 15-DMT and 31-DMT channels, whereas non-enzyme generated appearance of signals in the 13-DMT was less. 12-HT was not detected under these incubation conditions. At low tacrolimus concentrations (0.01 and 0.05 μM), the formation rates of 13-DMT and 31-DMT were comparable (1.7 ± 0.4 and 5.8 ± 0.8 pmol/min/nmol for 13-DMT 1.4 ± 0.7 and 3.6 ± 0.7 pmol/min/nmol for 31-DMT, respectively), and about 6-10 times higher than that of 15-DMT (0.6 ± 0.1

pmol/min/nmol at 0.05 μM). As shown in **Figure 4.2**, at low tacrolimus concentrations, there was significant depletion of 31-DMT by CYP3A5, but not by CYP3A7. Also, the formation rates of 31-DMT by CYP3A5 and CYP3A7 were comparable (**Table 4.1**). Because of the abundant expression of CYP3A7 in the fetal liver, one could speculate that 31-DMT may accumulate to a greater extent in the fetal liver containing only CYP3A7 compared to those with both CYP3A7 and CYP3A5 expression (genetically determined).

Metabolite formation by CYP3A forms in some cases demonstrated atypical kinetic behavior where a plot of velocity versus substrate concentration does not follow the typical Michaelis-Menten hyperbola. The types of nonhyperbolic kinetics observed for some CYP3A substrates include autoactivation and substrate inhibition. It has been hypothesized that this nonhyperbolic behavior reflects the binding of two substrate molecules simultaneously in the CYP3A4 active site (Korzekwa et al., 1998). The metabolism of tacrolimus by CYP3A7+ b_5 showed substrate inhibition kinetics for the formation of 13-DMT, 15-DMT and 31-DMT (**Figure 4.5**). Kinetic parameter estimates (**Table 4.2**) indicated a relatively high affinity (K_m ranged from 0.19 to 2.03 μM) for binding that led to product formation, and low affinity binding (K_s ranged from 18.5 to 229.4 μM) that reduced the reaction rate.

Kamdem et al. previously reported that the K_m and V_{max} values for 13-DMT formation calculated from Michaelis–Menten plots were 1.5 μM and 0.72 nmol/min/nmol for CYP3A4, and 1.4 μM and 1.1 nmol/min/nmol for CYP3A5. CYP3A7 exhibited some catalytic activity, but its velocity was 10- to 20-fold lower [0.084 nmol/min/nmol], whereas the K_m value was 4.3- to 8.3-fold higher (6 μM) than those of CYP3A4 and

CYP3A5 (Kamdem et al., 2005). In our system, V_{max} and K_m for 13-DMT formation was 0.23 nmol/min/nmol and 1.55 μM, respectively.

There are several possible explanations for the discrepancies we observed. The reaction reported previously was allowed to proceed for 10 min (Kamdem et al., 2005) instead of 2 min used in our study. Also, the b_5 content used in the two studies was different. Other factors that may contribute to the discordant findings include the material of the incubation apparatus (affecting non-specific substrate binding), and the quantitative methods employed.

Quantitation of the unbound concentration of tacrolimus ($[S]_u$) in the various incubation systems is critical for accurate prediction of the intrinsic tacrolimus clearance (Dai et al., 2006). In our study and the study by Kamdem et al., the unbound concentration of tacrolimus was not characterized experimentally. In order to compare the current kinetic parameter estimates to those reported by Dai et al., $K_{m,u}$ and $CL_{int,u}$ were estimated using f_u described earlier (Dai et al., 2006) (mean $f_u \approx 25\%$), assuming that binding to the Supersomal protein accounts for all the binding; $K_{m,u'}$ and $CL_{int,u'}$ were estimated using $f_{u,app}$ described in (Dai et al., 2006) (**Figure 4.6**, ranged from 4% to 14%).

Under this condition, V_{max} for 13-DMT formation by CYP3A7+b5 was 0.23 nmol/min/nmol, 2.8% of that by CYP3A4+b5 and 1.3% of that by CYP3A5+b5. The estimated unbound intrinsic clearance for 13-DMT formation by CYP3A7+b5 was 0.60 or 1.95 mL/min/nmol, about 0.7% or 2.3% of that by CYP3A5+b5 (82 mL/min/nmol) (Dai et al., 2006). It was also reported that the formation of 13-DMT by rCYP3A7+b_5 was 50-fold lower than that of rCYP3A5 at a substrate concentration of 0.2 μM. The formation of other metabolites was negligible (Dai et al., 2006). The current experiment

was overall in agreement with the previous finding, in that estimated unbound intrinsic clearance for 13-DMT formation by CYP3A7+b_5 was 136- to 42-fold lower than that of CYP3A5+b5. When 1 ng/mL of tacrolimus was incubated with Supersomes, the formation rate of 13-DMT at 2.33 min by CYP3A7+b_5 was 39-fold lower than that of CYP3A5+b5 (**Table 4.1**), suggesting that the unbound intrinsic clearance for 13-DMT formation by CYP3A7+b_5 is about 2.6% of that by CYP3A5+b5.

The formation of 31-DMT displayed very high affinity binding for CYP3A7 (K_m ≈ 10% of that with 13-DMT and 15-DMT formation, **Table 4.2**) and a low V_{max} (V_{max} ≈ 3.6 % of that with 13-DMT formation, **Table 4.2**), thus the CL_{int} of 31-DMT was a respectable 29% of that of 13-DMT. Notably, the formation of 31-DMT was inhibited to a much greater extent at higher substrate concentrations (K_s ≈ 8% of that with 13-DMT formation and ≈ 22% of that 15-DMT formation, **Table 4.2**).

4.4.4 Formation of CsA Metabolites by CYP3A4 and CYP3A5

CYP3A4 catalyzed the formation of AM1, AM9 and AM4N, whereas CYP3A5 exhibited marked product regioselectivity (AM9). At a substrate concentration of 8.33 μM, CYP3A4 generated AM1, AM9 and AM4N in a ~ 1:4:4 ratio, compared to a 2:34:1 ratio for CYP3A5 (**Figure 4.7**). The formation rate of AM9 from CsA (8.33 μM) mediated by CYP3A5 at 10 min was 280% of that mediated by CYP3A4. The major limitation of this study is that substrate CsA concentration (8.33 μM) was much higher than the reported apparent K_m value of CYP3A4 for AM1 and AM9 formation (around 1 μM), but slightly less than the 11.3 μM K_m value of CYP3A5 for AM9 formation (Dai et al., 2004). Because of the issue of enzyme saturation and substrate concentration that may

not represent *in vivo* CsA concentration during first pass and in the systemic circulation, the contribution of CYP3A5 to AM9 formation was further examined at a lower CsA concentration of 200 nM (**Figure 4.8**), when the formation rates of all metabolites should be linearly related to CL_{int}. Under this condition, CYP3A5 does not form measurable levels of AM1 and AM1c within the first 15 minutes of incubation (**Figure 4.8B**). However, the formation rate of AM9 from CsA mediated by CYP3A5 was, on average, 63.5% of that mediated by CYP3A4, consistent with the report that the intrinsic clearance of AM9 formation by CYP3A5 Supersomes is 57% of that by CYP3A4 Supersomes (122 ± 46 and 214 ± 110 μl/min/nmol, respectively) (Dai et al., 2004).

Finally, it is noteworthy that CYP3A5 catalyzed AM1 formation at high concentrations (8.33 μM), which was not reported previously when CsA was incubated at 20 μM and higher concentrations. Thus, it will be important to characterize the kinetics of AM1 formation by CYP3A5 to understand its relative contribution to CsA clearance *in vivo*.

4.4.5 Formation of AM19 and AM1c9 by CYP3A4 and CYP3A5

At a substrate concentration of 1 μM, CYP3A5 Supersomes converted AM1 to AM19 at a rate similar to that of CYP3A4 Supersomes (23.9 ± 5.3 vs. 28.5 ± 4.7 pmol/min/nmol, respectively). No AM1c9 was detected when AM1 was incubated with either CYP3A4 or CYP3A5 Supersomes with or without NADPH.

AM9 was converted to AM19 much more efficiently by CYP3A4 (9.4 ± 1.0 pmol/min/nmol), compared to CYP3A5 (1.0 ± 0.2 pmol/min/nmol). AM1c9 was detected when AM9 was incubated with both CYP3A4 and CYP3A5 Supersomes even without

the addition of NADPH. Only CYP3A4 formed AM1c9 (0.6 ± 0.3 pmol/min/nmol) when the non-NADPH dependent formation of AM1c9 was accounted for.

The formation of AM1c9 from AM1c by CYP3A4 and CYP3A5 was also comparable (17.1 ± 4.6 vs. 10.8 ± 0.1 pmol/min/nmol, respectively) (**Figure 4.9A**). Similar results were found when 200 nM of AM1, AM9 and AM1c were incubated with CYP3A4 and CYP3A5 Supersomes for a shorter incubation of 30 min (**Figure 4.9B**).

The above results suggested that the predominant source of AM19 and AM1c9 seems to be through conversion of AM1 and AM1c to the secondary metabolites, respectively, reactions that can be catalyzed efficiently by both CYP3A4 and CYP3A5. The observation that the average blood AUC for the secondary CsA metabolites, AM19 and AM1c9, was 47.4% and 51.3% higher in CYP3A5 expressors compared to nonexpressors in **Chapter 3** is consistent with the *in vitro* data, in that CYP3A5 is equally capable of converting AM1 to AM19, and AM1c to AM1c9 as CYP3A4. AM9 should not contribute to the formation of AM19 in CYP3A5 expressors to a significant extent. Because AM19 and AM1c9 are the final forms of metabolites that are excreted through bile or urine, we evaluated the ratios of AUC_{AM19}/AUC_{AM1} and AUC_{AM1c9}/AUC_{AM1c} in **Chapter 3**. Not surprisingly, the AUC_{AM19}/AUC_{AM1} and AUC_{AM1c9}/AUC_{AM1c} ratio was 46.9% and 30.6% higher in CYP3A5 expressors compared to nonexpressors, further supporting the formation pathways of AM19 and AM1c9 as described above.

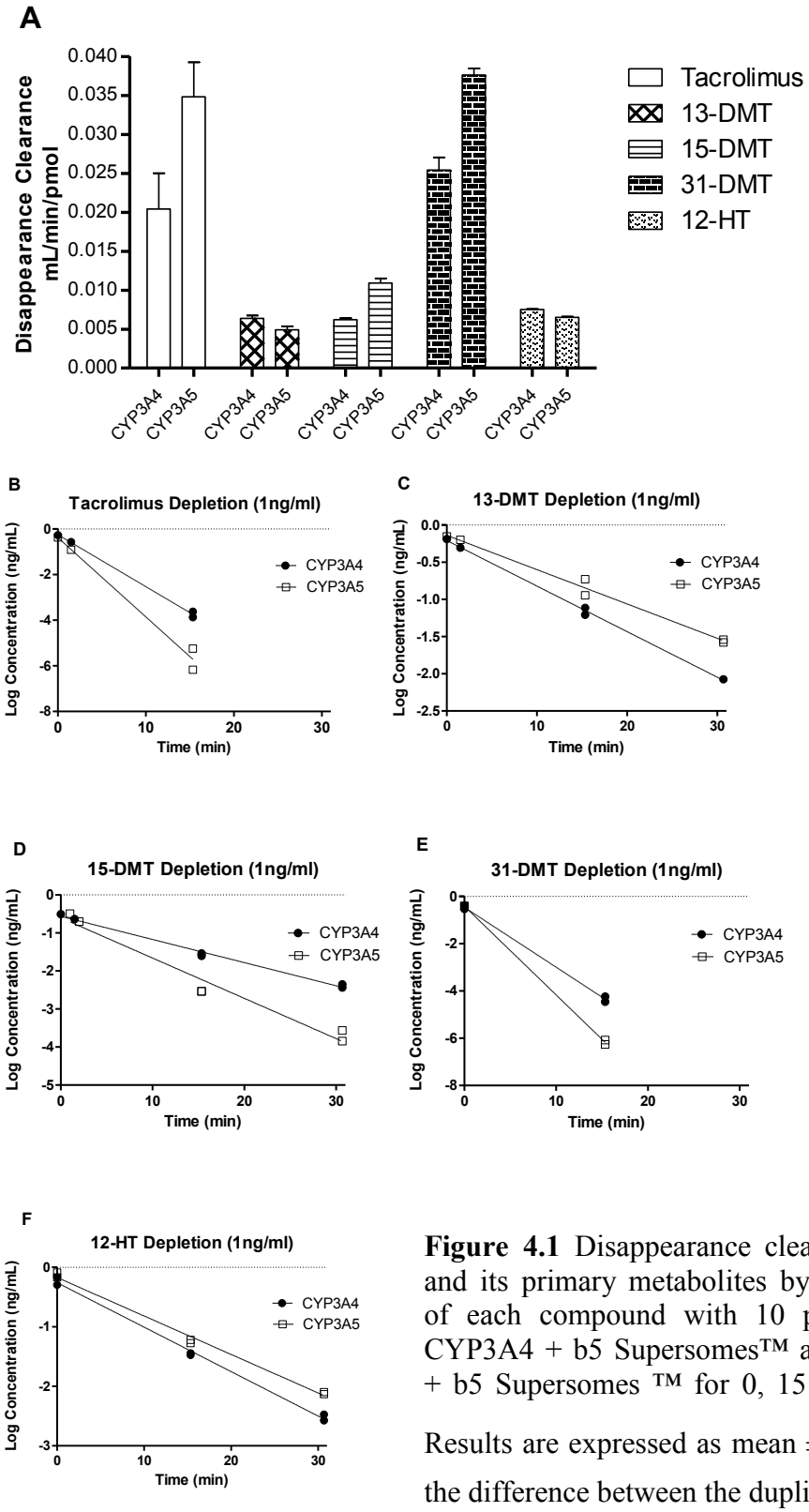


Figure 4.1 Disappearance clearance of tacrolimus and its primary metabolites by incubating 1ng/mL of each compound with 10 pmol/mL of human CYP3A4 + b5 Supersomes™ and human CYP3A5 + b5 Supersomes ™ for 0, 15 and 30 min (N=2). Results are expressed as mean ± $\sqrt{\frac{d^2}{2}}$, in which d is the difference between the duplicates (A).

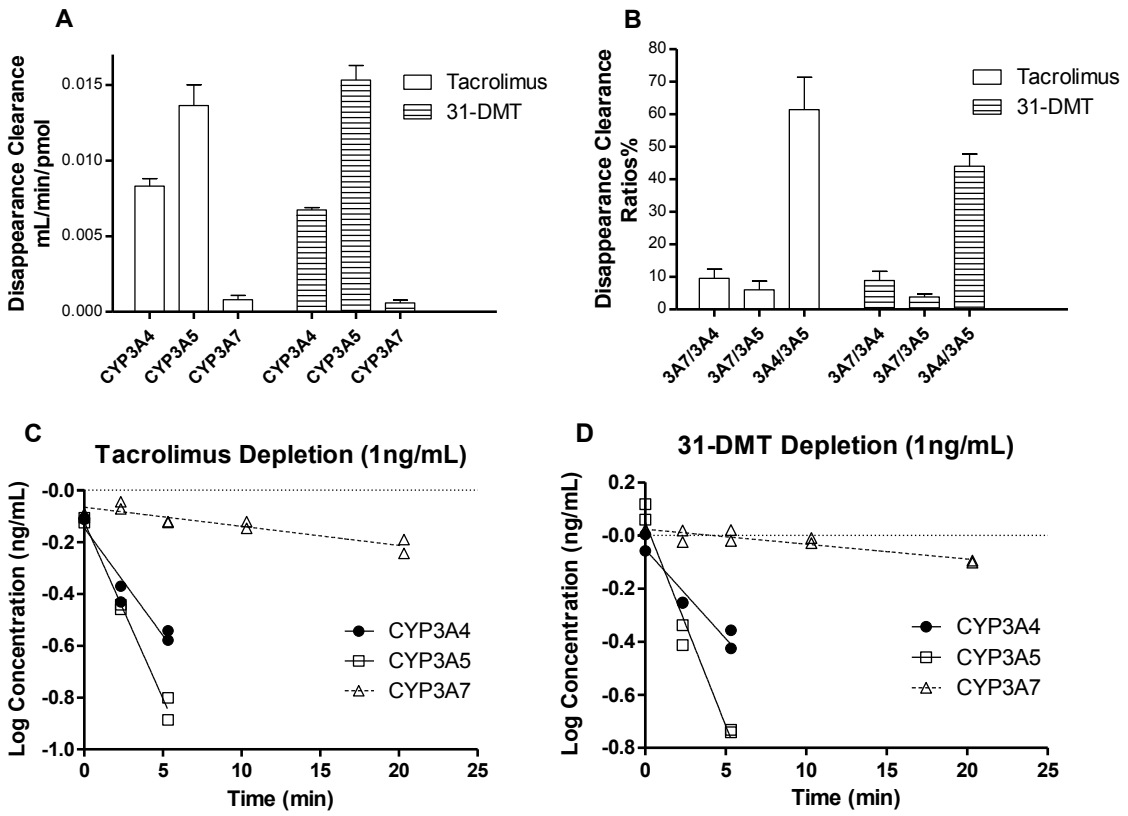


Figure 4.2 Disappearance clearance of tacrolimus and 31-DMT measured by incubating 1ng/mL of each compound with 10 pmol/mL of human CYP3A4 + b5 Supersomes™, human CYP3A5 + b5 Supersomes ™ and human CYP3A7 + b5 Supersomes ™ for 2.33 min, 5.33 min, 10.33 min and 20.33 min (N=2 at each timepoint and the incubation volume was 1 mL). Results are expressed as mean ± $\sqrt{\frac{d^2}{2}}$, in which *d* is the difference between the duplicates.

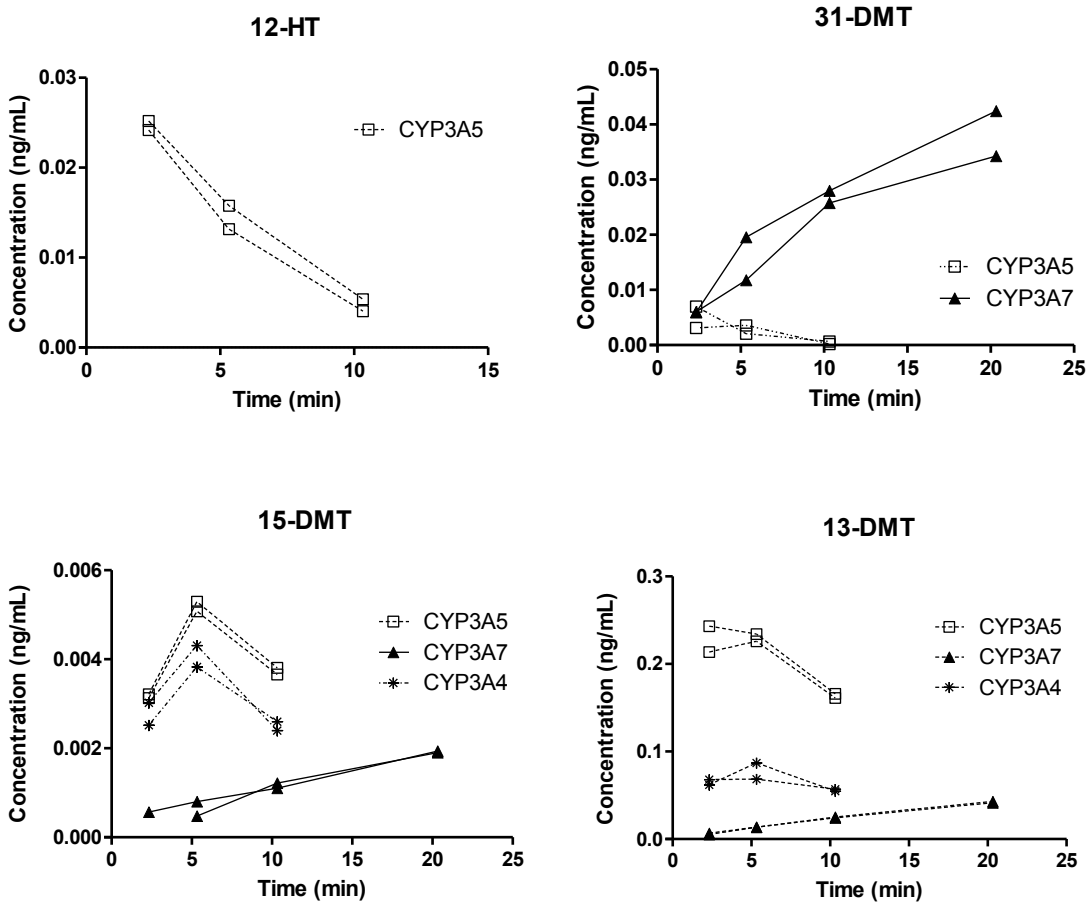


Figure 4.3 Tacrolimus primary metabolite formation (time-concentration profile) generated by incubating 1 ng/mL of tacrolimus with 10 pmol/mL of human CYP3A4 + b5 Supersomes™, human CYP3A5 + b5 Supersomes ™ and human CYP3A7 + b5 Supersomes ™ for 2.33 min, 5.33 min, 10.33 min and 20.33 min (N=2 for each time point and the incubation volume was 1 mL).

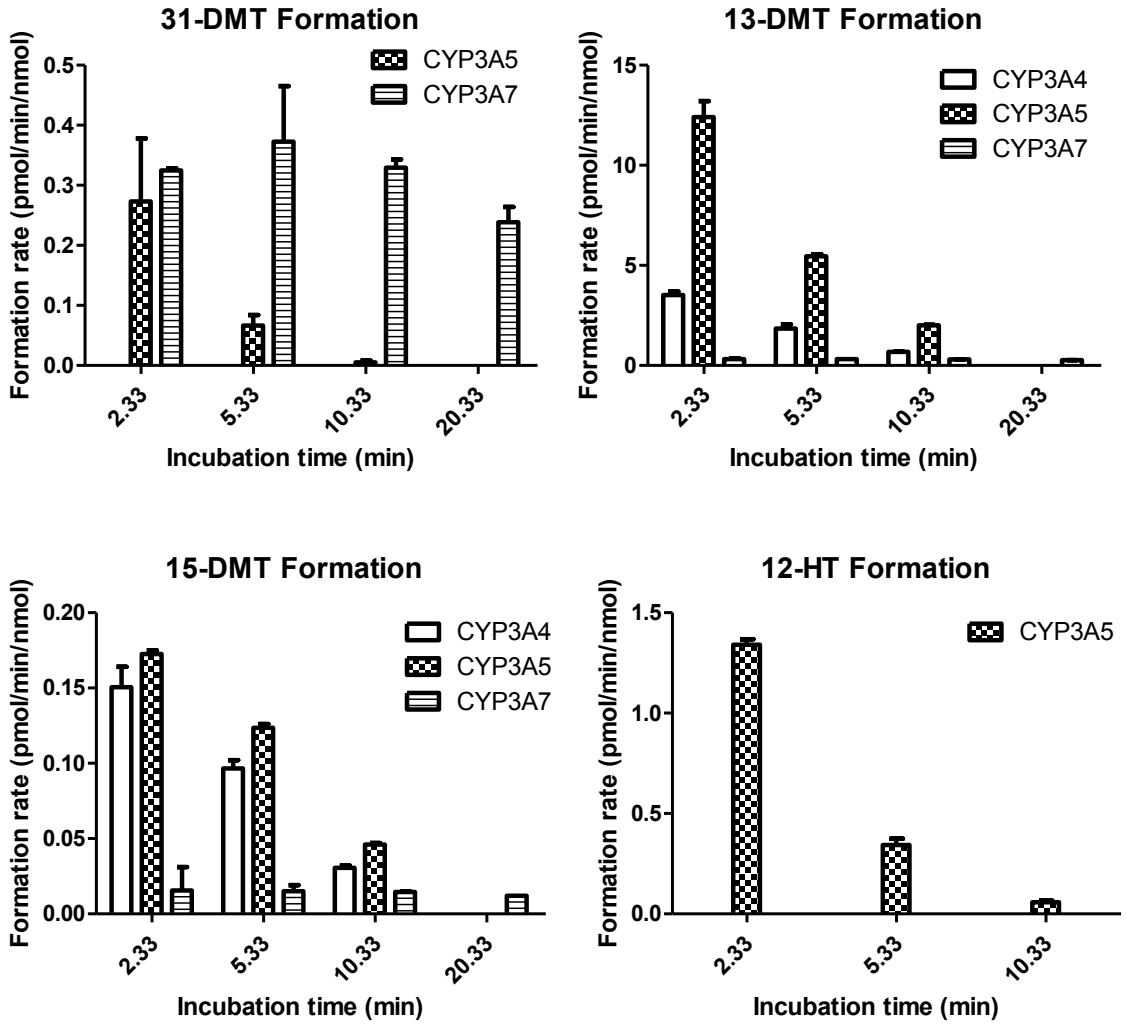


Figure 4.4 Tacrolimus primary metabolite formation rate (pmol/min/nmol) generated by incubating 1 ng/mL of tacrolimus with 10 pmol/mL of human CYP3A4 + b5 Supersomes™, human CYP3A5 + b5 Supersomes ™ and human CYP3A7 + b5 Supersomes ™ for 2.33 min, 5.33 min, 10.33 min and 20.33 min (N=2 for each time point and the incubation volume was 1 mL).

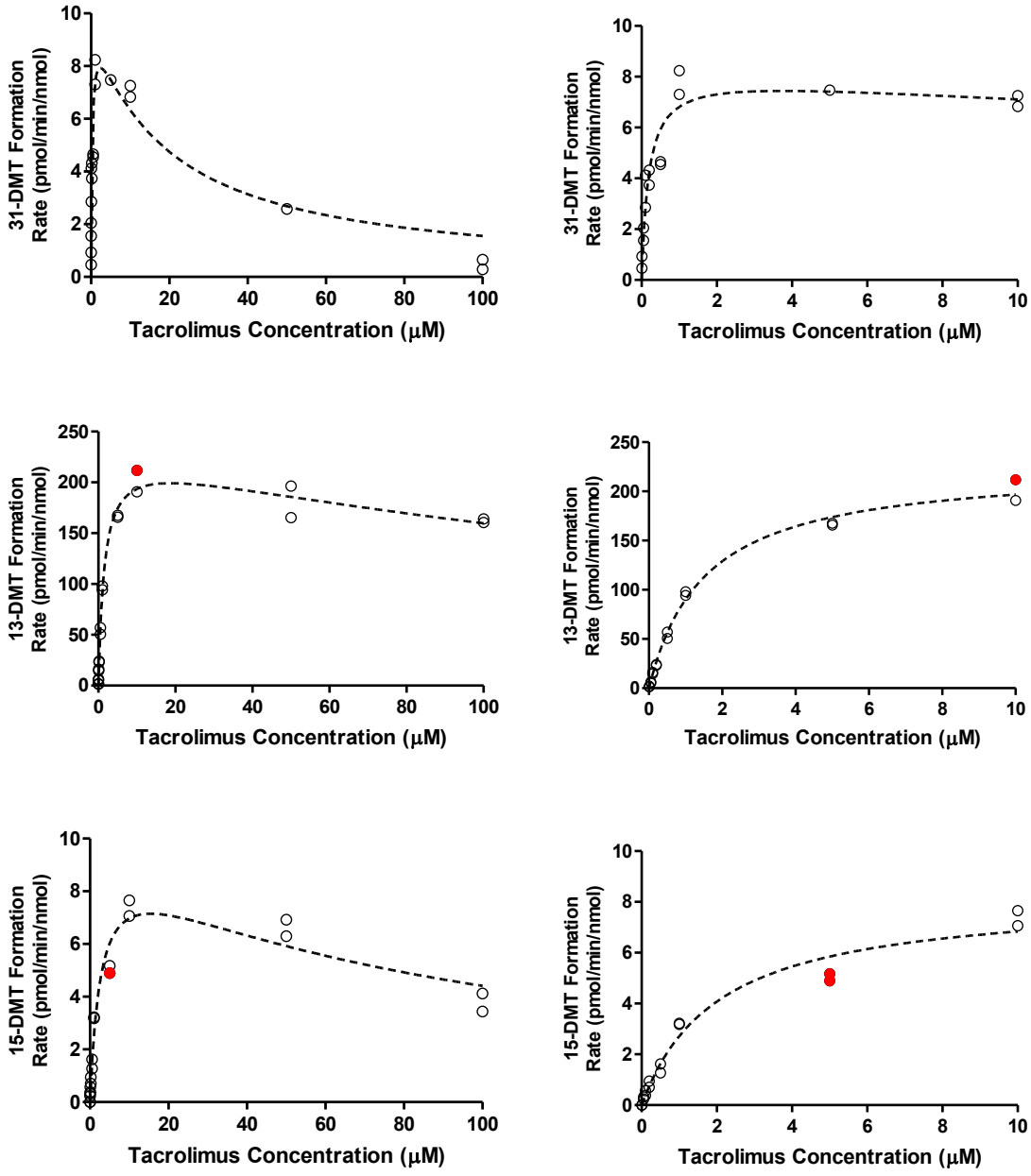


Figure 4.5 Tacrolimus primary metabolite formation catalyzed by incubating tacrolimus (0.01, 0.05, 0.1, 0.2, 0.5, 1, 5, 10, 50, 100 μM) with 5 pmol/mL CYP3A7+b_5 for 2 min (N=2 for each substrate concentration and the incubation volume was 1 mL). A Michaelis-Menten kinetic model with noncompetitive substrate inhibition was fit to the metabolite formation data using GraphPad Prism 5 (GraphPad, La Jolla, CA) nonlinear regression analysis.

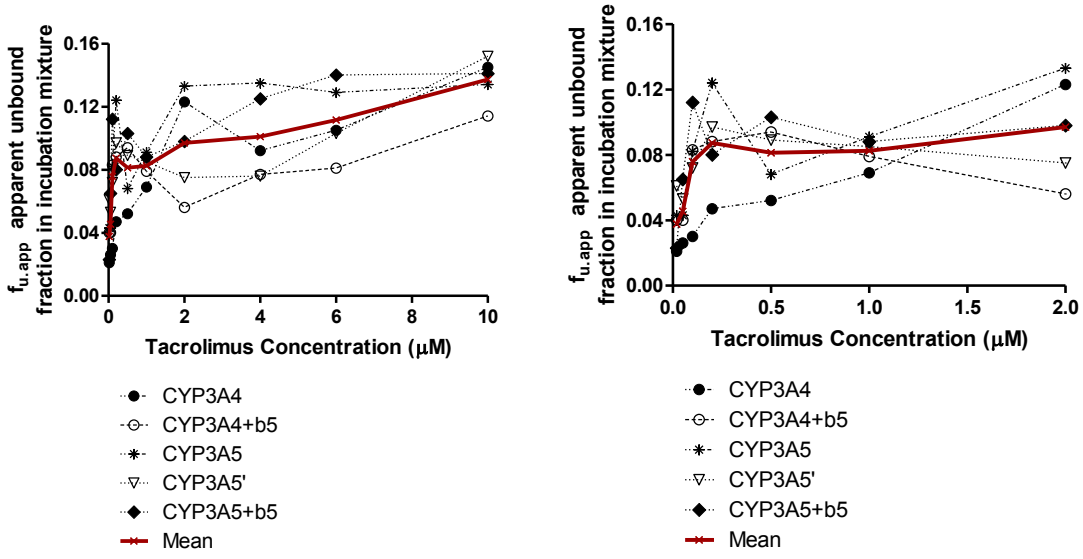


Figure 4.6 $f_{u.app}$, the apparent unbound drug fraction in the incubation mixture that was used to correct the concentration-dependent metabolite formation data, as described in (Dai et al., 2006).

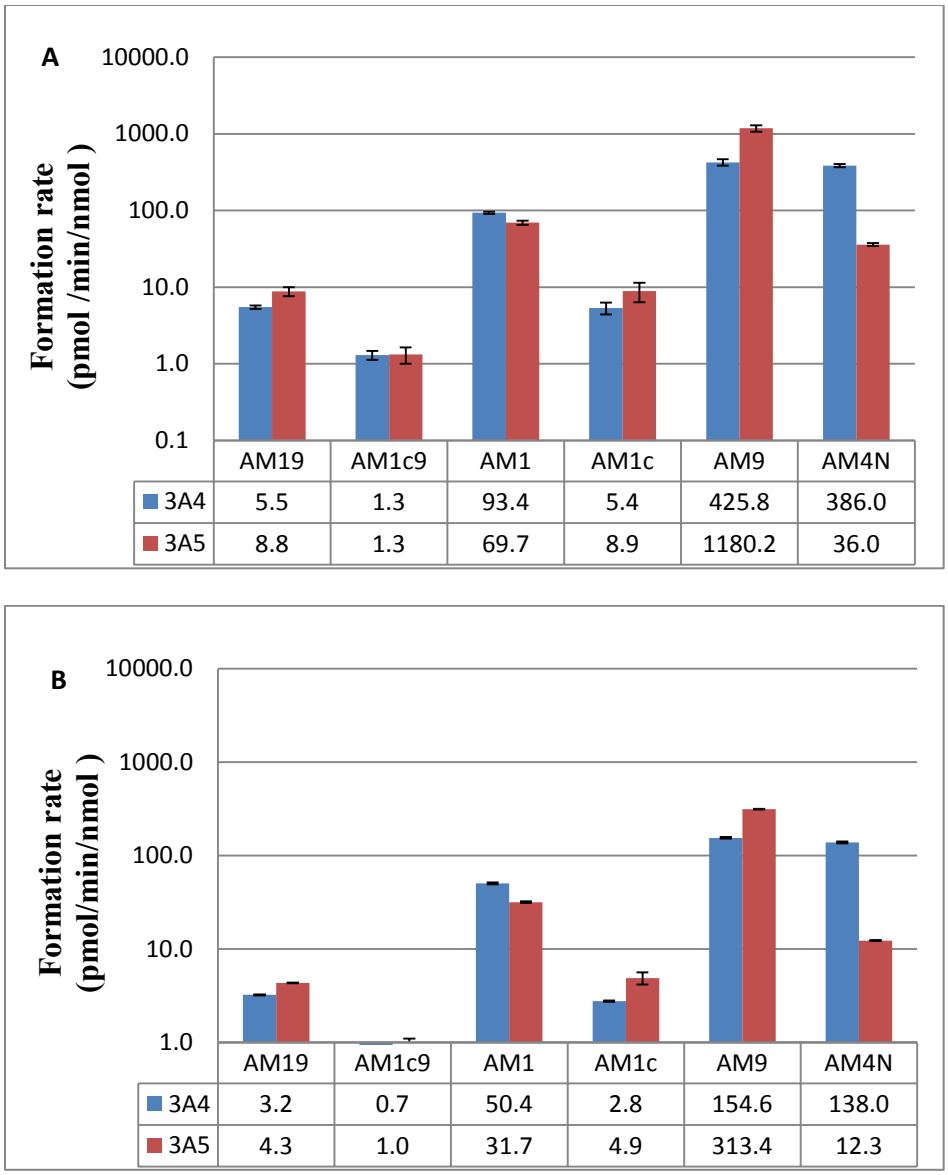
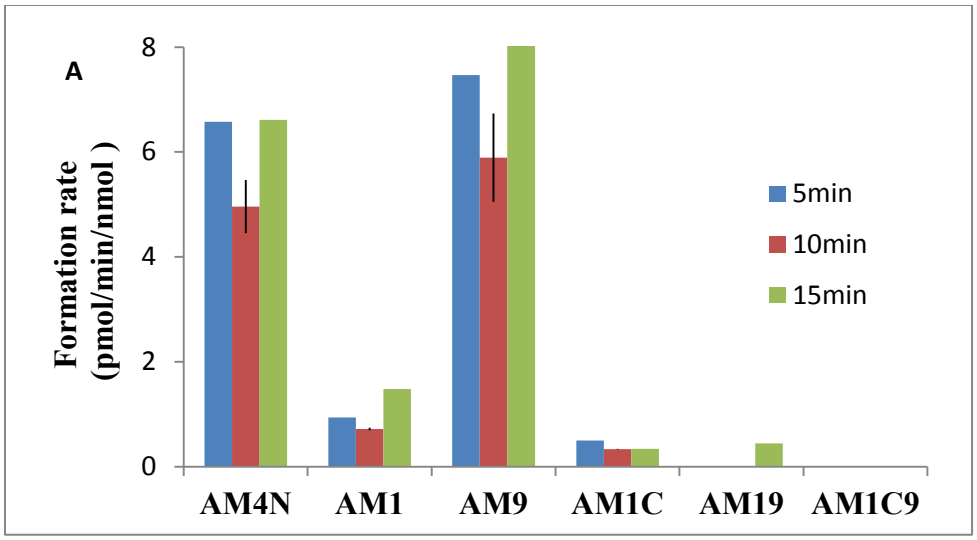


Figure 4.7 CsA primary and secondary metabolite formation rates generated by incubating 8.33 µM CsA with 5 pmol/mL of CYP3A4+b_5 and CYP3A5+b_5 for 10 min (A) and 60 min (B) (N=2 for each time point and the incubation volume was 0.25 mL).



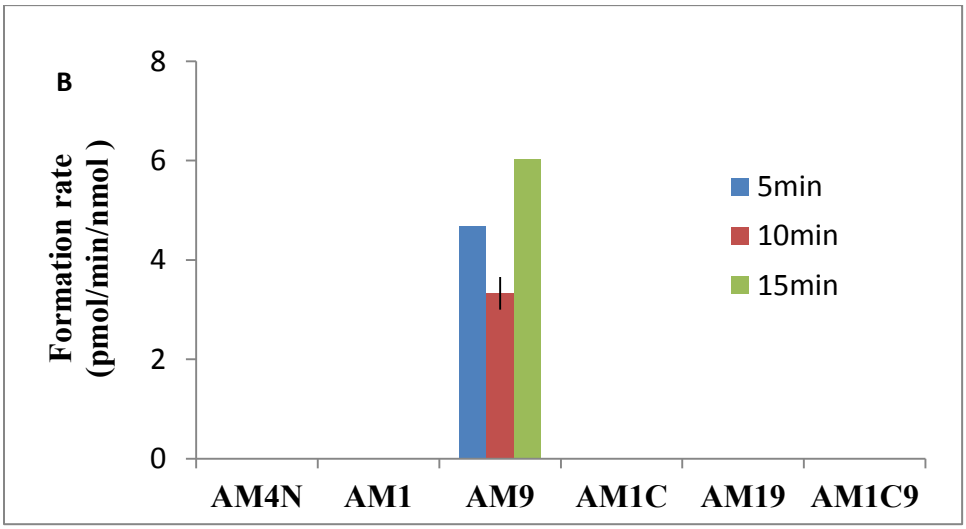
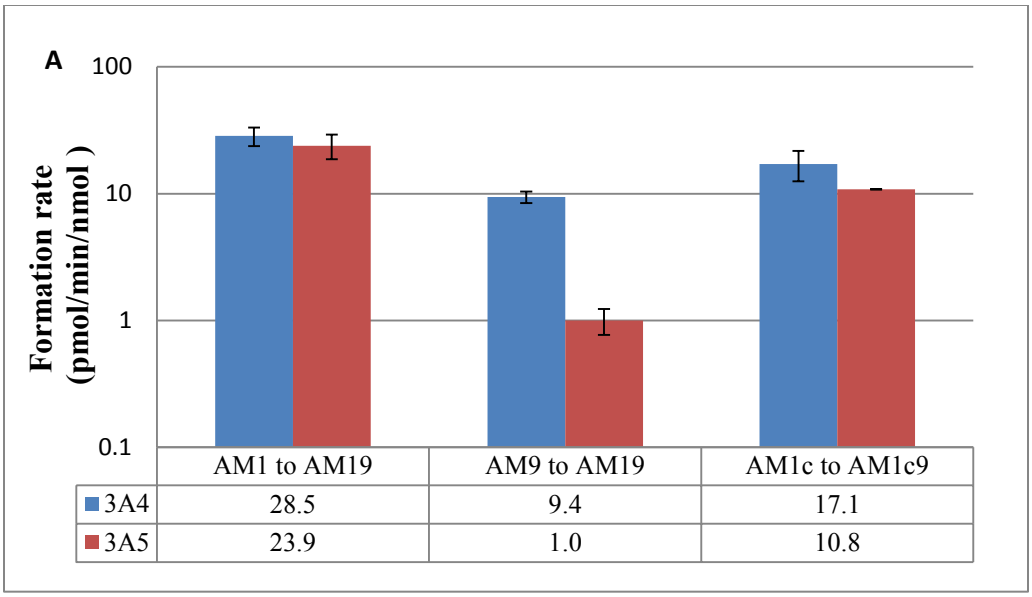


Figure 4.8 CsA primary and secondary metabolite formation rates generated by incubating 200 nM CsA with 5 pmol/mL of CYP3A4+b_5 (A) and CYP3A5+b_5 (B) for 5, 10 and 15min and 60 min (the incubation volume was 0.25 mL).



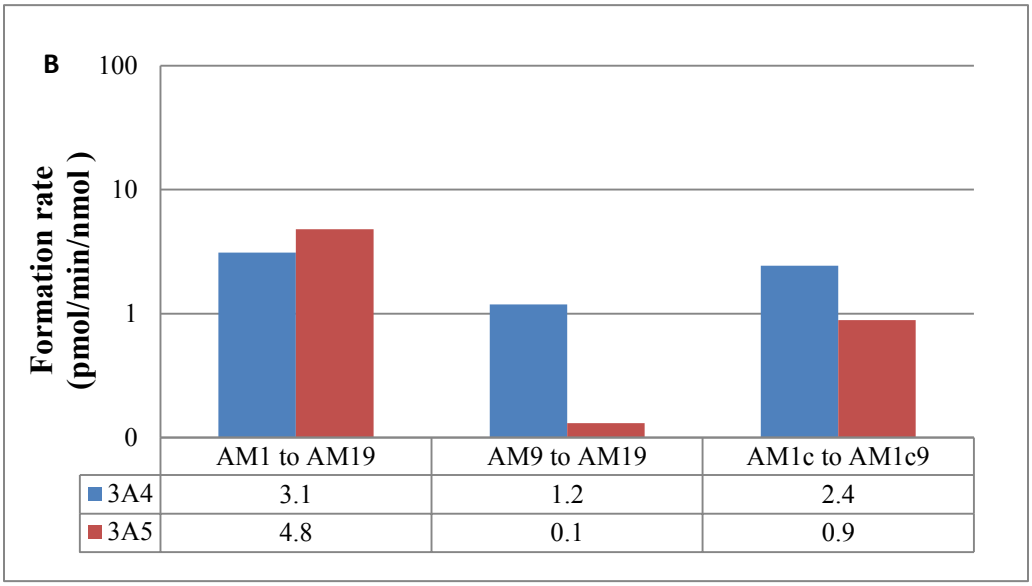


Figure 4.9 CsA secondary metabolite formation rate generated by incubating 1 μM AM1, AM9 and AM1c with 5 pmol/mL of CYP3A4+b_5 and CYP3A5+b_5 for 60 min (A), or by incubating 200 nM AM1, AM9 and AM1c with 5 pmol/mL of CYP3A4+b_5 and CYP3A5+b_5 for 30 min (B) (the incubation volume was 0.25 mL).

TABLE 4.1 Formation rate (pmol/min/nmol) of 13-DMT, 15-DMT, 31-DMT and 12-HT by incubating tacrolimus (1ng/mL) for 2.33 min with CYP3A4+b5, CYP3A5+b5 and CYP3A7+b5

	CYP3A4	**CYP3A5**	**CYP3A7**
13-DMT	3.52 (7%)	12.41(9%)	0.32 (17%)
15-DMT	0.15 (13%)	0.17 (2%)	0.015
31-DMT		0.27 (55%)	0.33 (2%)
12-HT		1.34 (3%)	

Each reported value represents the average of values obtained from duplicated experiments ($\sqrt{\frac{d^2}{2}}$, in which *d* is the difference between the duplicates are shown in parentheses, expressed as %CV).

TABLE 4.2 Kinetic parameters estimated from 13-DMT, 15-DMT and 31-DMT formation by CYP3A7+b5

	V_{max} nmol/min/nmol	K_m μM	K_s μM	CL_{int} mL/min/nmol	$K_{m,u}$ μM	$CL_{int,u}$ mL/min/nmol	$K_{m,u'}$ μM	$CL_{int,u'}$ mL/min/nmol
13-DMT	0.23 (3.7%)	1.55 (11.1%)	229.4 (18.5%)	0.15	0.39	0.60	0.11	1.95
15-DMT	0.0082 (6.5%)	2.03 (19.9%)	84.26 (28.7%)	0.0040	0.51	0.016	0.15	0.051
31-DMT	0.0082 (11.5%)	0.19 (31.9%)	18.5 (38.3%)	0.043	0.047	0.17	0.013	0.62

A Michaelis-Menten kinetic model with noncompetitive substrate inhibition was fit to the metabolite formation data using GraphPad Prism 5 (GraphPad, La Jolla, CA) nonlinear regression analysis. Each reported parameter and computer-generated standard error estimate (numbers in parentheses, expressed as %CV) represents the average of values obtained from duplicated experiments. $K_{m,u}$ and $CL_{int,u}$ were estimated using f_u described in (Dai et al., 2006) (mean $f_u \approx$ 25%), assuming that binding to the Supersomal protein accounts for all of the non-specific binding; $K_{m,u'}$ and $CL_{int,u'}$ were estimated using $f_{u,app}$ described in (Dai et al., 2006) (**Figure 4.6**, ranged from 4% to 14%), assuming that tacrolimus binds non-specifically to the glass incubation tubes and the Supersomal protein in the same way that it binds to polypropylene centrifuge tubes.

Chapter 5

Pharmacokinetics of Tacrolimus during Pregnancy

Portions of Chapter 5 were submitted to *Therapeutic Drug Monitoring.*

5.1 Abstract

Information on the pharmacokinetics of tacrolimus during pregnancy is limited to case reports despite the increasing number of pregnant women being prescribed tacrolimus for immunosuppression. We conducted a pharmacokinetics study of tacrolimus in women treated with oral tacrolimus during early to late pregnancy. Blood, plasma and urine samples were collected over one steady-state dosing interval from women treated with oral tacrolimus during early to late pregnancy (n = 10) and postpartum (n = 5). Total and unbound tacrolimus as well as metabolite concentrations in blood and plasma were assayed by a validated LC/MS/MS method. A mixed effect linear model was used for comparison across gestational age and using postpartum as the reference group.

The mean oral clearance (CL/F) based on whole blood tacrolimus concentration was 39% higher during mid- and late-pregnancy compared to postpartum (47.4 ± 12.6 vs. 34.2 ± 14.8 L/h, $P < 0.03$). Tacrolimus free fraction increased by 91% in plasma (f_P) and by 100% in blood (f_B) during pregnancy (P = 0.0007 and 0.002, respectively). Increased f_P was inversely associated with serum albumin concentration (r = – 0.7, P = 0.003), which decreased by 27% during pregnancy. Pregnancy related changes in f_P and f_B contributed significantly to the observed gestational increase in tacrolimus whole blood CL/F (r^2 = 0.36 and 0.47 respectively, $P < 0.01$). In addition, tacrolimus whole blood CL/F was inversely correlated with both hematocrit and red blood cell counts, suggesting that binding of tacrolimus to erythrocytes restricts its availability for metabolism. Treating physicians increased tacrolimus dosages in study participants during pregnancy by an average of 45% in order to maintain tacrolimus whole blood trough concentrations

in the therapeutic range. This led to striking increases in unbound tacrolimus trough concentrations and unbound AUC, by 112% and 173%, respectively during pregnancy (*P* = 0.02 and 0.03, respectively).

In Summary, Tacrolimus pharmacokinetics are altered during pregnancy. Dose adjustment to maintain whole blood tacrolimus concentration in the usual therapeutic range during pregnancy increases circulating free drug concentrations, which may impact clinical outcomes.

5.2 Introduction to Chapter 5

Tacrolimus, a macrolide immunosuppressant, is used widely to prevent rejection following solid organ transplantation (Scott et al., 2003). While more than 14,000 pregnancies have been reported in organ transplant recipients, these remain high risk pregnancies for both mother and fetus (McKay and Josephson, 2006; Coscia et al., 2010).

Tacrolimus dosing is routinely titrated to maintain whole blood trough concentrations within an individualized therapeutic range, due to its narrow therapeutic index and high intra- and inter-patient variability in its pharmacokinetics (Machida et al., 1991; Jusko et al., 1995b). Whole blood samples are used in therapeutic monitoring due to difficulty in accurately measuring plasma tacrolimus concentrations, primarily because of temperature dependent distribution into the red blood cell and challenges with assay sensitivity (Machida et al., 1991; Akbas et al., 2005). Whole blood concentrations are used as surrogate markers for the concentration of active drug at the site(s) of action, despite the knowledge that <0.1% of tacrolimus is unbound in blood and therefore available for receptor binding (Undre et al., 1999a; Undre et al., 1999b; Undre, 2003).

Cytochrome P450 3A (CYP3A) isoforms are responsible for the extensive metabolism of tacrolimus *in vivo* (Sattler et al., 1992). The two dominant CYP3A isoforms in adult human tissues are CYP3A4 and CYP3A5. Expression of CYP3A4 in the liver and small intestine is unimodally distributed across populations with substantial inter-individual variability (Lampen et al., 1995; Haehner et al., 1996; Kuehl et al., 2001; Lin et al., 2002; Givens et al., 2003). In contrast, expression of CYP3A5 in the liver, small intestine, kidneys and other organs is polymorphic and determined largely by a single-nucleotide polymorphism (A6986G) that distinguishes the "active" *CYP3A5*1* allele (CYP3A5 expressing) from the "inactive" *CYP3A5*3* allele. Along with other non-expressing *CYP3A5*6* and **7* alleles, this accounts for a markedly reduced cellular CYP3A5 protein expression and function in some individuals (Lampen et al., 1995; Haehner et al., 1996; Kuehl et al., 2001; Lin et al., 2002; Givens et al., 2003).

Tacrolimus is an excellent substrate for both CYP3A4 and CYP3A5,(Dai et al., 2006) with CYP3A5 expressors exhibiting a 1.5- to 2-fold higher tacrolimus apparent oral clearance (CL/F), lower trough concentrations, and higher dosage requirement than nonexpressors with two *CYP3A5*3*, **6* or **7* alleles (Staatz et al., 2010b). Because both enzymes can be found in the gastrointestinal tract, pre-systemic intestinal metabolism of tacrolimus can be considerable (Floren et al., 1997; Tuteja et al., 2001). The oral absorption of tacrolimus is also influenced by the activity of P-glycoprotein (P-gp), an efflux transporter that transfers tacrolimus from the enterocyte back into the gut lumen (Undre et al., 1999a). Thus, extensive pre-systemic metabolism and P-gp efflux limits the oral bioavailability of tacrolimus in non-pregnant women and men to approximately 14 ± 6% (Floren et al., 1997; Hebert et al., 1999).

Pregnancy is accompanied by an increase in maternal blood volume as well as significant changes in maternal renal and hepatic function, which in some cases influence the dosage of the medication (Fuchs and Coustan, 2007). Previous work suggests that intrinsic CYP3A activity increases by 25-100% during pregnancy using CYP3A probe substrates such as midazolam (Hebert et al., 2008), dextromethorphan (Tracy et al., 2005), and nelfinavir (Hirt et al., 2006; Villani et al., 2006). As stated above, tacrolimus is also a substrate of the efflux transporter, P-glycoprotein (P-pg) (Saeki et al., 1993; Yokogawa et al., 1999). Although intestinal P-gp activity during pregnancy has not been evaluated, our group has shown that renal P-gp activity, assessed by net renal tubular secretion of digoxin, approximately doubles during pregnancy(Hebert et al., 2008). Based on these findings, the metabolism and transport of tacrolimus might be expected to change substantially during pregnancy. Because of the expression of P-gp on peripheral blood lymphocytes (Chaudhary et al., 1992), potential lymphocytic P-gp activity change in pregnancy, although not currently known, may also affect the distribution of tacrolimus into lymphocytes, the site of calcineurin inhibition.

In pregnancy, both albumin and α_1-acid glycoprotein (AAG) concentrations in plasma decrease significantly. This is likely related in part to increased plasma volume and increased urinary albumin excretion (Feghali and Mattison, 2011). In plasma, tacrolimus has been shown to bind to AAG and albumin (Piekoszewski and Jusko, 1993; Weiss et al., 2008). Accordingly, changes in plasma protein concentrations in pregnancy may alter tacrolimus plasma protein binding and also affect its systemic clearance. In addition, red blood cell count and hematocrit decrease in pregnancy (Hytten, 1985). This could significantly influence tacrolimus distribution within blood (Zahir et al., 2004) such

that patients with lower hematocrit will have a lower tacrolimus whole blood-to-plasma ratio (Jusko et al., 1995a) (Piekoszewski et al., 1993). This change may also affect the metabolism and clearance of tacrolimus in pregnancy (Chow et al., 1997). Not surprisingly, therapeutic monitoring of the immunosuppressive drugs becomes more complicated in pregnancy (McKay and Josephson, 2006).

No comprehensive study of tacrolimus pharmacokinetics in pregnancy has been published to date. The available data are limited to case reports (Midtvedt et al., 1997; Fehrman-Ekholm and Nisell, 1998; French et al., 2003). Because of the significant physiological and enzymatic changes that occur during pregnancy, and the unique binding characteristics of tacrolimus to blood cells and plasma proteins and its metabolism and transport by CYP3A enzymes and P-gp, we conducted a study to characterize the pharmacokinetics of tacrolimus during pregnancy and postpartum. Our findings may help in the clinical interpretation of tacrolimus concentrations.

5.3 Materials and Methods

5.3.1 Subjects

This study was approved by the Institutional Review Boards at the University of Washington and Georgetown University and was conducted in accordance with their guidelines. All subjects gave written informed consent. We examined the steady-state pharmacokinetics of oral tacrolimus in the blood, plasma and urine of pregnant (n = 10) and postpartum (n = 5) women who were receiving the drug for therapeutic reasons. Women were excluded from the study if their screening hematocrit was less than 28%. Blood, plasma and urine samples were collected during early- (10–14 weeks gestation),

mid- (22–26 weeks gestation), and/or late-pregnancy (34–38 weeks gestation), as well as >3 months postpartum.

Sequential blood samples (5 mL) were collected in EDTA glass tubes just before (0 h) and at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12 h after a regular oral dose. The duration of blood sampling was truncated based on the subject's dosage interval, which was either 8 or 12 h. After mixing, 2 mL of blood was aliquoted and frozen in glass cryovials. The remaining blood was incubated at 37° C for 30 min. After centrifugation at 37° C, plasma samples were aliquoted and frozen in glass cryovials. Urine was collected in silanized glass jars over the dosing interval and then frozen in silanized glass vials. Glass pipettes were used for all liquid transfers. All blood, plasma and urine samples were stored at −80° C until analysis.

The number of study participants at each gestational age varied due to differences in the timing of each subject's enrollment during gestation and subject availability. Five of the subjects participated in a single pharmacokinetic study day, three participated in 2 study days, and two participated in all 4 study days. Of the five women who were studied postpartum, two participated in the early pregnancy study, three participated in the mid pregnancy study, and four participated in the late pregnancy study.

5.3.2 Dosing Regimen

Tacrolimus dosages ranged from 3 to 12 mg/day. Oral tacrolimus capsule (Prograf®, Astellas) were provided by the investigators for the 3 days before each study. The subjects were instructed to avoid alcohol, grapefruit, and grapefruit juice for 3 days before each study day and to fast starting 5 h before study drug administration until 1 h

after the tacrolimus dose on each study day. Clear liquids were allowed and encouraged during the fasting portion of the study.

5.3.3 Genotyping Methods

Buffy coats or buccal cell DNA was isolated using a DNeasy Blood & Tissue Kit (Qiagen, USA). *CYP3A5*3* (rs776746, 6986A>*G*) polymorphism was determined using a validated Taqman® allelic discrimination assay from Applied Biosystems (Foster City, CA) as described previously.(Hebert et al., 2008) With individuals that were identified as having the *CYP3A5*1* allele, additional genotyping for the *CYP3A5*6* (14690G>A) allele was conducted. The *CYP3A5*6* genotype was performed using the same methodology as described for *CYP3A5*3* (Taqman® allelic discrimination assay).(Hebert et al., 2008)

5.3.4 Determination of Tacrolimus Free Fraction in Plasma

Tacrolimus unbound fraction in plasma (f_P) was determined using an ultracentrifugation procedure adapted from Nakai *et al.*(Nakai et al., 2004) Duplicate plasma samples at the peak and the second highest concentrations for all subjects were measured in two independent experiments. Duplicates of selected plasma samples at the trough were evaluated when quantifiable and were compared with f_P determined at the peak concentrations. Plasma samples (230 µL) were centrifuged at 100,000 rpm (435,630 *g*) for 140 min at 37° C (Thermo Scientific S100-AT3 rotor, Sorvall micro-ultracentrifuge). Tacrolimus concentration in the supernatant after ultracentrifugation (60

μL), and in the corresponding plasma incubated for 140 min at 37° C, were analyzed by the LC-MS/MS method described above.

5.3.5 Pharmacokinetic Analysis

The unbound fraction in whole blood (f_B) was determined from the relationship: $f_B = (C_P/C_{WB})\, f_P$, where f_P is the unbound fraction in plasma, C_P is the plasma concentration, and C_{WB} is the whole blood concentration (Minematsu et al., 2004). For each subject, a single measured value for f_P was assigned at each stage of pregnancy. Steady-state pharmacokinetic parameters were estimated using standard non-compartmental techniques as described previously (Hebert et al., 2008). Creatinine clearance (CrCL) was estimated as follows: CrCL = [(urine volume)(urine creatinine concentration)]/[(serum creatinine concentration)(duration of the collection interval)]. Because tacrolimus was administered orally, its clearance and volume of distribution could not be estimated independent of its oral bioavailability (F), so we report CL/F (calculated as $Dose_{oral}$/AUC).

5.3.6 Statistical Analysis: a Mixed Effect Linear Model Using R

Because this was an opportunistic study, the number of subjects participating in each stage of pregnancy varied according to their availability. Because repeated measurements on the same subject are correlated, we analyzed the data according to a mixed effect linear model using R (R Development Core Team, 2009), specifically the

packages nlme and lme4 (Pinheiro & Bates, http://cran.r-project.org/web/packages/nlme/index.html; Bates & Maechler, http://CRAN.R-project.org/package=lme4). Time (early-pregnancy, mid-/late-pregnancy and postpartum) was treated as a fixed effect in the model. Paired-comparisons were performed using postpartum study visit as the reference group. When the states of pregnancy and postpartum were compared, mid- and late-pregnancy data were pooled. If a subject completed both mid- and late-pregnancy study days, values were averaged to determine pharmacokinetic parameter estimates for the individual during pregnancy. For subjects who only completed either mid- or the late-pregnancy study, the single value was used as their pregnancy datum for statistical analysis. Log transformations of data were applied when proved to be a better fit to model assumptions of normality. Linear regression analysis was conducted using GraphPad Prism version 5 (GraphPad, La Jolla, CA). Results are reported as a mean ± standard deviation (SD), unless otherwise indicated. A value of $P < 0.05$ was considered significant for all tests.

5.4 Results

A total of 10 pregnant subjects (5 non-Hispanic White, 3 Hispanic, 1 non-Hispanic Black, 1 Asian), age 26.9 ± 6.2 years and height 161.4 ± 7.9 cm, participated in this study. All subjects were solid organ transplantation recipients (five kidney, one kidney/pancreas, three liver, and one heart). All subjects, except for one, had *CYP3A5*3/*3* genotype. One subject carried SNPs associated with the *CYP3A5*3* and *CYP3A5*6* alleles. Based on known haplotype patterns, it is likely that the *CYP3A5*3* SNP and the *CYP3A5*6* SNP are on different chromosomes, making this subject a

complex heterozygous nonexpressor (*CYP3A5*3/*6*) (Thompson et al., 2006). Other subject characteristics are described in **Table 5.1**. Of the subjects who were studied postpartum, five were lactating and one of them was studied again 42 weeks after cessation of lactation. Because the data are limited and the pharmacokinetic parameters did not seem to differ between the lactation and post-cessation of lactation study days (data not shown), we report the means of the parameters obtained during the postpartum days for these subjects.

Tacrolimus whole blood concentration-time profiles during early- and mid-/late-pregnancy and postpartum in women treated with the immunosuppressant every 12 hours are shown in **Figure 5.1**. The estimated tacrolimus pharmacokinetic parameters during pregnancy and postpartum are reported in **Table 5.2** (whole blood) and **Table 5.3** (plasma). With clinical dosage adjustments, the mean whole blood tacrolimus C_{max} and C_{trough} were comparable throughout gestation and postpartum (**Figure 5.1A**). The mean tacrolimus CL/F based on whole blood concentration was higher by 39% during mid/late pregnancy compared to postpartum (47.4 ± 12.6 L/h vs. 34.2 ± 14.8 L/h, $P < 0.03$) (**Table 5.2 and Figure 5.2A**). Moreover, the AUC of unbound tacrolimus in blood was 2.7 times higher during mid/late pregnancy than postpartum (0.44 ± 0.19 ng·h/mL vs. 0.16 ± 0.06 ng·h/mL, $P = 0.03$, **Figure 5.1C**). In addition, the unbound tacrolimus trough concentration in blood was on average 112% higher during mid-/late-pregnancy than postpartum (0.018 ± 0.010 ng/mL vs. 0.009 ± 0.003 ng/mL, $P = 0.02$). No statistically significant difference was evident in unbound CL/F when comparing pregnancy to postpartum (**Table 5.2 and Figure 5.2B**). In addition, there was no apparent change in the formation clearance of tacrolimus' four primary metabolites as inferred from the lack

of any trend for changes in metabolite/parent AUC ratios across pregnancy periods and postpartum (**Figure 5.3**).

Mean tacrolimus renal clearance estimated based on either AUC_{blood} or AUC_{plasma} was not significantly different between pregnancy and postpartum (**Table 5.2 and 5.3**). The corresponding unbound renal clearance calculated based on free fraction in plasma (f_P) was lower during mid-pregnancy compared to postpartum (14.9 ± 6.9 mL/min vs. 38.5 ± 16.3 mL/min, P = 0.02) (**Table 5.3**). Tacrolimus renal clearance based on AUC_{plasma} did not correlate with creatinine clearance. The percent dose recovered unchanged in urine, although very low, trended toward a decrease in pregnancy compared to postpartum (0.008 ± 0.004% vs. 0.013 ± 0.004%, P = 0.07).

On average, there was a 91% increase in the tacrolimus percent unbound in plasma (f_P) during mid- and late-pregnancy compared to postpartum (5.4 ± 0.7% vs. 2.8 ± 0.4%, P = 0.0007) (**Figure 5.4A**). The average percent unbound in plasma did not differ significantly between the peak and trough samples, consistent with previous report that the fraction unbound of tacrolimus in plasma was independent of the concentration in the range of 0.065–10.5 ng/mL(Zahir et al., 2001). The calculated tacrolimus percent unbound in blood (f_B) during mid- and late-pregnancy was, on average, 100% higher than that for the postpartum period (0.4 ± 0.1% vs. 0.2 ± 0.1%, P = 0.002) (**Figure 5.4B**). A good correlation was found between the CL/F and the percent unbound in plasma (f_P, r = 0.6, P = 0.007) and in blood (f_B, r = 0.7, P = 0.001) (**Figure 5.4C and 5.4D**).

We observed the expected significant decrement in serum albumin, red blood cell count, and hematocrit during pregnancy (**Table 5.1, Figures 5.5A and 5.6A**). An inverse correlation was observed between f_P and the serum albumin concentrations (r = – 0.7, P =

0.003, **Figure 5.5B**). The mean tacrolimus blood/plasma concentration ratio (15.9 ± 4.2) increased with increasing red blood cell counts (r = 0.6, P = 0.02, **Figure 5.6B**). Tacrolimus CL/F was inversely correlated with red blood cell counts (r = – 0.7, P = 0.002, **Figure 5.6C**). Tacrolimus CL/F tended to be inversely correlated with hematocrit (%) (r = – 0.4, P = 0.09), but this did not reach significance. When individual patient data were examined, the blood-to-plasma tacrolimus concentration ratio did not differ significantly between peak and trough times (data not shown), nor did it vary from pregnancy to postpartum. However, when data from all the subjects were pooled, the blood-to-plasma tacrolimus concentration ratio appeared to be somewhat higher at plasma concentrations below 0.5 ng/mL (**Figure 5.7**).

5.5 Discussion

Despite the increasing number of women being prescribed tacrolimus for immunosuppression during pregnancy, data describing the effects of pregnancy on tacrolimus disposition are scarce. Our study characterized tacrolimus pharmacokinetics during pregnancy and demonstrated that physiological changes occurring during pregnancy indeed alter tacrolimus PK. Our major findings suggest that pregnancy can result in lower whole blood tacrolimus concentrations but no significant change in unbound concentrations if no adjustment in dosage is made. In the clinical setting, providers routinely increase the tacrolimus dosage during pregnancy to maintain target whole blood trough concentrations, ignoring possible changes in unbound tacrolimus, since these are not measured in clinical practice. As observed in our study, this approach results in approximate doubling of the unbound tacrolimus trough concentrations. The

mechanisms by which tacrolimus pharmacokinetic are changed during pregnancy are rather complex.

Judging by whole blood clearance estimates, tacrolimus is considered a low-clearance drug (clearance equivalent to ~3% of liver blood flow) (Moller et al., 1999). Classic pharmacokinetic models predict that for drugs eliminated exclusively by metabolism in the liver their apparent clearance following oral administration (i.e., CL/F) should be governed by the intrinsic hepatic clearance of unbound drug and unbound fraction of drug in blood, and independent of hepatic blood flow (Wilkinson, 1987; Benet and Zia-Amirhosseini, 1995). However, these predictions do not account for intestinal metabolism, and/or the possibility of slow equilibration of drug between plasma and erythrocytes.

We found that the mean tacrolimus CL/F increased by 39% during mid-/late-pregnancy compared to postpartum, which could not be explained by known factors such as drug interactions. Postpartum values were comparable to those previously reported in non-pregnant subjects (21-38 L/h) (Staatz et al., 2002; Staatz et al., 2003; Tada et al., 2005; Passey et al., 2011). Previous investigations with midazolam, dextromethorphan and nelfinavir have suggested that intrinsic hepatic/intestinal CYP3A activity may be increased by 25% to 100% during pregnancy (Tracy et al., 2005; Hirt et al., 2006; Villani et al., 2006; Hebert et al., 2008). Contrary to expectations, the estimated unbound oral clearance of tacrolimus ($CL/F/f_B$) did not change in pregnancy. There are several possible explanations for this discrepancy. First, the inference of intrinsic hepatic clearance from oral clearance of unbound drug relies on the assumption that equilibrium in plasma protein binding and erythrocyte-to-plasma partitioning is maintained at all times. Plasma

protein binding and erythrocyte distribution can restrict drug extraction from the blood by an organ (Shen et al., 1997). For tacrolimus, blood-to-plasma equilibration is reported to be slow (Beysens et al., 1991; Chow et al., 1997); thus, equilibrium may not be fully reached in the time it takes for blood to pass through the liver. Therefore, it is possible that the effective exchangeable fraction in blood may be different than what is indicated by our *in vitro* equilibrium experiments. Furthermore, the discrepancy between *in vivo* exchangeable fraction and *in vitro* equilibrium fraction may differ between pregnancy and postpartum. Given the complex binding behavior of tacrolimus *in vivo*, the apparent lack of change in the unbound oral clearance based on *in vitro* equilibrium measurements may belie a fundamental difference in the intrinsic hepatic clearance between pregnancy and postpartum.

Second, it is possible that an increase in the oral bioavailability of tacrolimus may have occurred during pregnancy. An increase in oral bioavailability could result from an increase in the absorption of tacrolimus from the gastrointestinal tract, and/or a decrease in the metabolism of tacrolimus by CYP3A in the intestinal mucosa. Intestinal metabolism is a significant part of tacrolimus first pass in rats, with over 30% of the absorbed tacrolimus dose metabolized in the small intestine (Hashimoto et al., 1998). In humans, intestinal metabolism has been shown to play a significant role in the first pass of cyclosporine, a calcineurin inhibitor with a similar metabolic fate as tacrolimus (Kolars et al., 1991). Drug interaction studies have demonstrated that tacrolimus first pass metabolism can be either inhibited or induced (Lampen et al., 1995; Floren et al., 1997; Tuteja et al., 2001). Co-administration of ketoconazole, a potent CYP3A inhibitor, increased the oral bioavailability of tacrolimus by 47% in kidney transplant recipients

(Tuteja et al., 2001) or 114% in healthy volunteers (14% vs. 30%) (Floren et al., 1997). In addition, co-administration of rifampin significantly decreased tacrolimus bioavailability (14.4% vs. 7.0%), most likely through induction of CYP3A and/or P-glycoprotein in the small intestine (Hebert et al., 1999). In both of the above described tacrolimus interaction studies in healthy volunteers, the available fraction across the liver ($F_{hepatic}$) was estimated apart from the fraction of the oral dose absorbed and available fraction across the gut (F_{abs} x F_{gut}). The changes seen in both of these drug interaction studies suggest that the largest effects occurred in the intestine. In the present context, a reduction in gut extraction and increased systemic availability would have to occur in pregnancy to offset the presumed CYP3A-related increase in intrinsic hepatic clearance, with the end result of no net change in the CL/F of unbound tacrolimus. There is currently no evidence supporting the hypotheses of an increase in oral absorption or reduced intestinal CYP3A-medaited first-pass metabolism of tacrolimus during pregnancy.

Intestinal P-gp activity appears to affect the oral bioavailability of tacrolimus in mice (Yokogawa et al., 1999), rats (Arima et al., 2001) and perhaps in human (Floren et al., 1997; Tuteja et al., 2001). Research conducted in pregnant mice (Zhang et al., 2008) and non-human primates (Zhang et al., 2009) showed that there was no change in P-gp protein expression or activity in the liver or kidneys during gestation. In contrast, renal P-gp activity is elevated during pregnancy in humans (Hebert et al., 2008). The regulation of human intestinal P-gp during pregnancy has not been investigated. If the activity of intestinal P-pg in pregnancy were to follow that in the kidneys, a decrease rather than an increase in absorption is expected.

Changes in unbound drug in blood appear to be the main driver in the pharmacokinetic changes seen with tacrolimus during pregnancy. Tacrolimus percent unbound in plasma was nearly two-fold higher during mid- and late-pregnancy compared to postpartum (5.4 ± 0.7% vs. 2.8 ± 0.4%, P =0.0007). For comparison, in healthy male and female subjects, f_P was found to be 2.1 ± 0.8% using the same ultracentrifugation method (Zheng *et al.*, Clinical Pharmacology and Therapeutics). Also, mean f_P values of 3.7 ± 0.8% have been reported previously using equilibrium gel filtration (Weiss et al., 2008) and 1.20 ± 0.12% using equilibrium dialysis (Zahir et al., 2001). We found a significant correlation between CL/F of tacrolimus based on whole blood concentration and the percent unbound in plasma (r = 0.6, P = 0.007) and in blood (r = 0.7, P = 0.001). This strongly suggests an increased delivery of tacrolimus to intra-hepatic CYP3A during pregnancy that resulted in enhanced hepatic extraction and systemic clearance and reduced mean oral AUC.

Although it was previously suggested that plasma albumin concentration was not an important factor in tacrolimus distribution (Piekoszewski and Jusko, 1993), unbound fraction in plasma was inversely correlated with the decrease in serum albumin levels during pregnancy (r = – 0.7, P = 0.003). This is consistent with results from a study of non-pregnant liver transplantation recipients receiving tacrolimus who were randomized post-transplantation to 2 weeks of human serum albumin infusion or an artificial plasma expander. The authors suggested that the unbound fraction of tacrolimus in the artificial plasma expander group may be greater.(Trull et al., 2002) Similarly, in a study with kidney transplant patients, an inverse correlation was found between the Dose (mg/kg)/C_{min} ratio and albumin concentration (r = - 0.74, P = 0.047) (Undre and Schafer,

1998). indicating that changing albumin concentrations could affect the clearance of tacrolimus. It is likely that both the reduction in albumin and α_1-acid glycoprotein (AAG) concentrations caused the increase in tacrolimus plasma unbound fraction during pregnancy. Weiss *et al.* reported that AAG contributed substantially to the binding of tacrolimus in plasma (Weiss et al., 2008). There is also the finding that in liver transplantation recipients the unbound fraction of tacrolimus in plasma is inversely correlated with α_1-acid glycoprotein ($r^2 = 0.50$) as well as HDL cholesterol levels (HDL = high density lipoproteins) ($r^2 = 0.55$) (Zahir et al., 2004). In pregnancy, both albumin and *AAG* concentrations decreased significantly (Feghali and Mattison, 2011).

The calculated tacrolimus percent unbound in blood during mid-/late-pregnancy was much higher than postpartum (0.4 ± 0.1% vs. 0.2 ± 0.1%, $P = 0.002$) reflecting the fact that the change in f_B was mainly driven by the change in f_p. However, in pregnancy, red blood cell count and hematocrit also decrease as a result of volume expansion, which results in the relatively greater increase in plasma volume than red cell mass (Hytten, 1985). Strong binding of tacrolimus to erythrocytes prevents it from concentrating in plasma despite its affinity for plasma proteins (Piekoszewski et al., 1993). Although the blood-to-plasma tacrolimus concentration ratio in our study did not vary significantly from peak to trough times, nor from pregnancy to postpartum for an individual patient, an analysis of pooled data in this study and the literature (Jusko et al., 1995a; Chow et al., 1997; Zahir et al., 2004) suggests concentration-dependent sequestration by erythrocytes. In our analysis, the blood-to-plasma tacrolimus concentration ratio was found to vary between 35.2 and 7.6 over a plasma concentration range of 0.14 to 2.52 ng/mL. Moreover, the lower red blood cell counts during pregnancy were associated with lower tacrolimus

blood-to-plasma concentration ratio (r = 0.6, P = 0.015, **Figure 5.6B**). This is consistent with previous work demonstrating a positive correlation between erythrocyte-associated fraction of tacrolimus and hematocrit (r^2 = 0.47) or red blood cell count (r^2 = 0.49) (Zahir et al., 2004), an inverse correlation between the Dose (mg/kg per day)/Cmin ratio and hematocrit (r = - 0.81) over the first 12 weeks post-kidney transplantation (Undre and Schafer, 1998), and likewise between Dose/C_{WB} and hematocrit in living donor liver transplantation (r = −0.53; P < 0.001) (Minematsu et al., 2004). In our study, we found that tacrolimus CL/F was inversely correlated to red blood cell count (r = – 0.7, P = 0.002). Moreover, the calculated unbound tacrolimus trough concentrations in blood were on average 112% higher during mid-/late-pregnancy than postpartum. Similarly, the AUC of free drug in blood during mid-/late-pregnancy was 2.7-fold higher than that seen postpartum. This could potentially impact both immunosuppressant efficacy and adverse response to tacrolimus. Accordingly, the relationship between drug response and tacrolimus concentrations (total and free) need to be investigated during pregnancy before an informed decision on dose adjustment can be made. Future studies that characterize lymphocytic P-gp activity and measure intra-lymphocytic tacrolimus concentrations during pregnancy should provide insight towards the impact of increased tacrolimus unbound fraction on the distribution of tacrolimus into lymphocytes. Quantification of changes in IL-2 production in stimulated blood should provide mechanistic understandings of the impact of pregnancy on the pharmacodynamic effects of tacrolimus.

5.6 Conclusions

Gestational increases in whole blood apparent oral clearance, most likely caused by an increase in the unbound fraction of tacrolimus, usually lead to increased tacrolimus dosages in response to observed decrements in its trough concentration. Lower albumin concentrations in pregnancy contribute to an increased plasma tacrolimus free fraction. The normal reduction in red blood cell count also has an effect on the unbound fraction in blood, which, together with lower plasma protein binding, explains much of the variance in oral tacrolimus pharmacokinetics during pregnancy and postpartum. As a result of dosage titration to maintain a target whole blood trough concentrations during gestation and postpartum, unbound tacrolimus concentrations are increased by nearly two-fold, which may have important clinical implications.

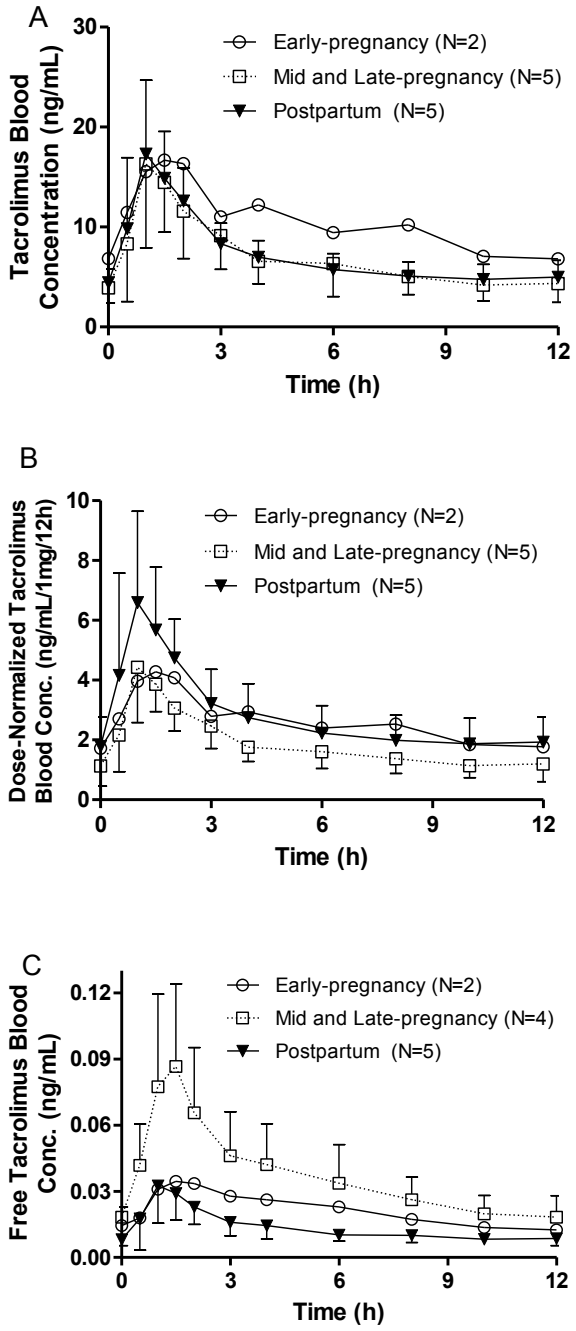


FIGURE 5.1 Tacrolimus blood concentration-time profiles during early- and mid-/late-pregnancy as well as > 3 months postpartum in women treated with tacrolimus every 12 hours (A); and dose-normalized (to 1 mg over 12 hours) tacrolimus blood concentration-time curves (B), and unbound tacrolimus blood concentration-time profile (C). Individual data points on all graphs represent the mean ± SD from study subjects when n > 2. The mean data were presented when n = 2. Only data from subjects with a 12-hour dosing interval were included. The unbound concentration is not available for one subject in late pregnancy due to hemolyzed plasma collection

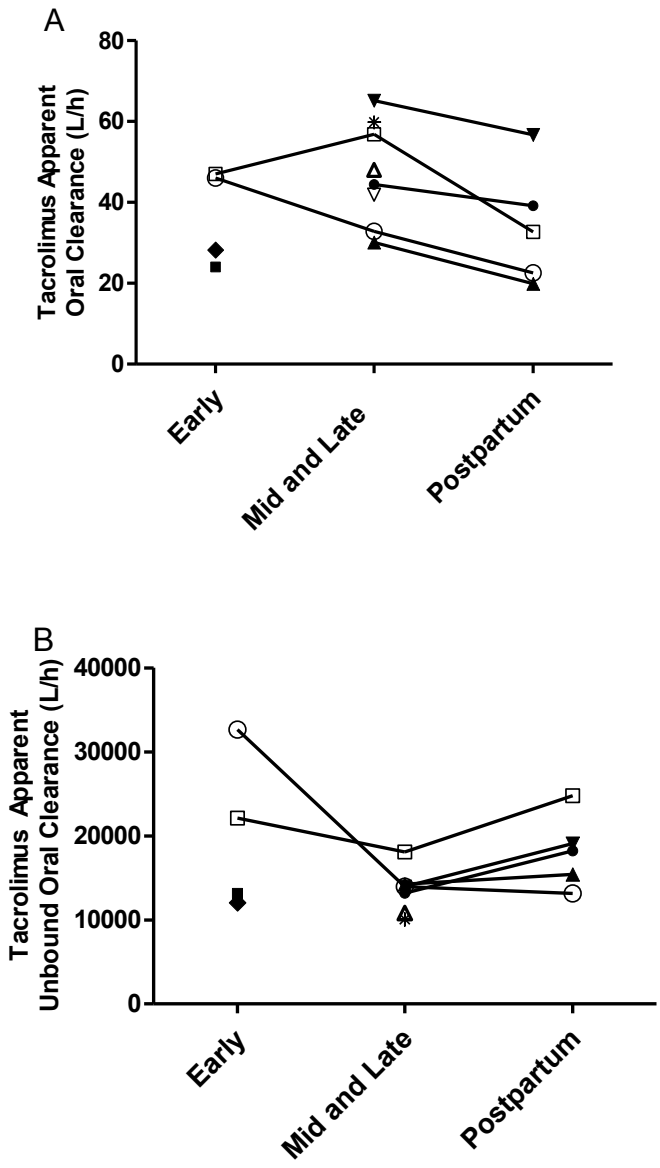


Figure 5.2 Tacrolimus oral clearance (A) and unbound oral clearance (B) based on AUC_{blood} for subjects during pregnancy (n = 10) and postpartum (n = 5).

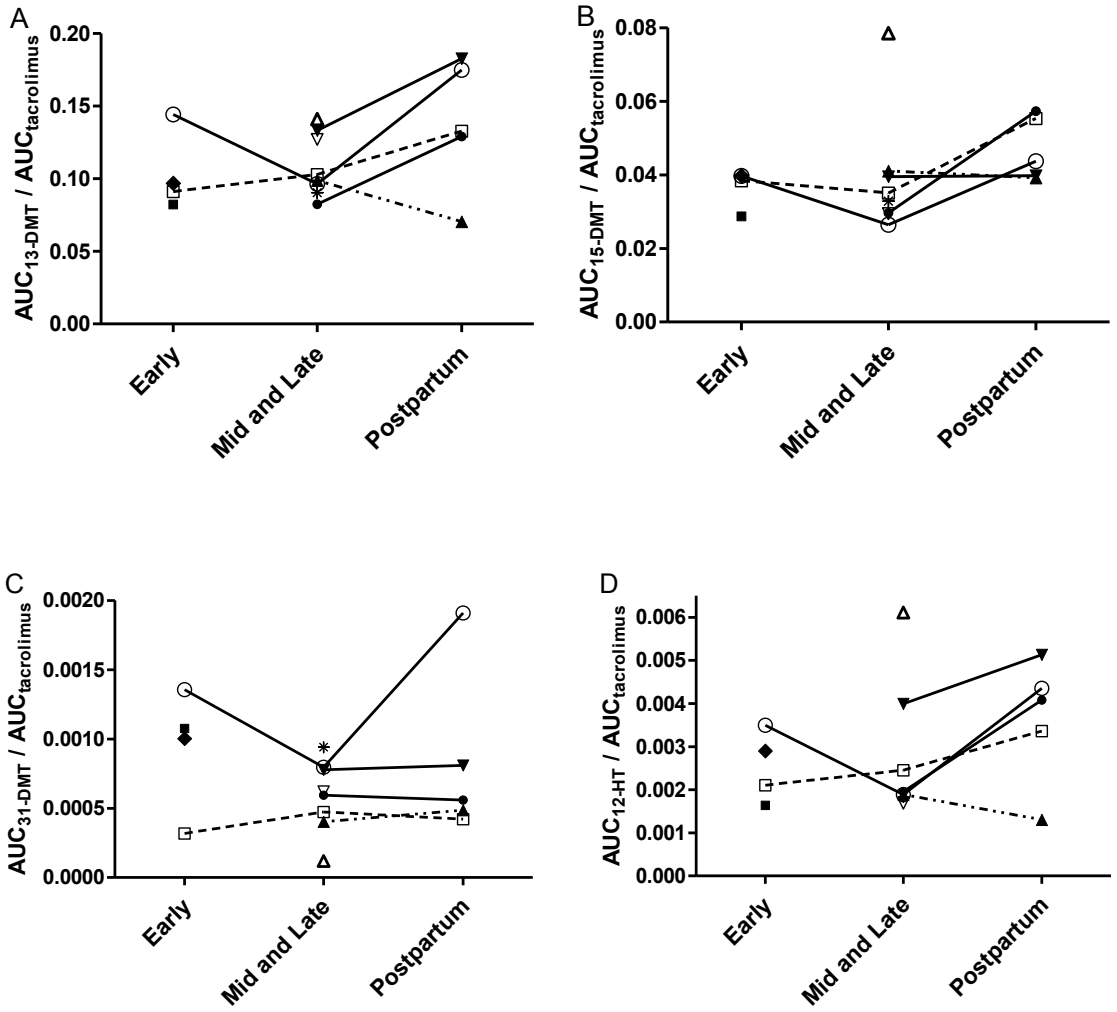


Figure 5.3 The blood metabolite/parent AUC ratio for tacrolimus's primary metabolites: (A) 13-DMT, (B) 15-DMT, (C) 31-DMT, and (D) 12-HT. AUC values were calculated for the 12-hour, steady-state dose interval during pregnancy (early-pregnancy n = 4, mid-pregnancy n=8, and late-pregnancy n=5) and postpartum (n = 5).

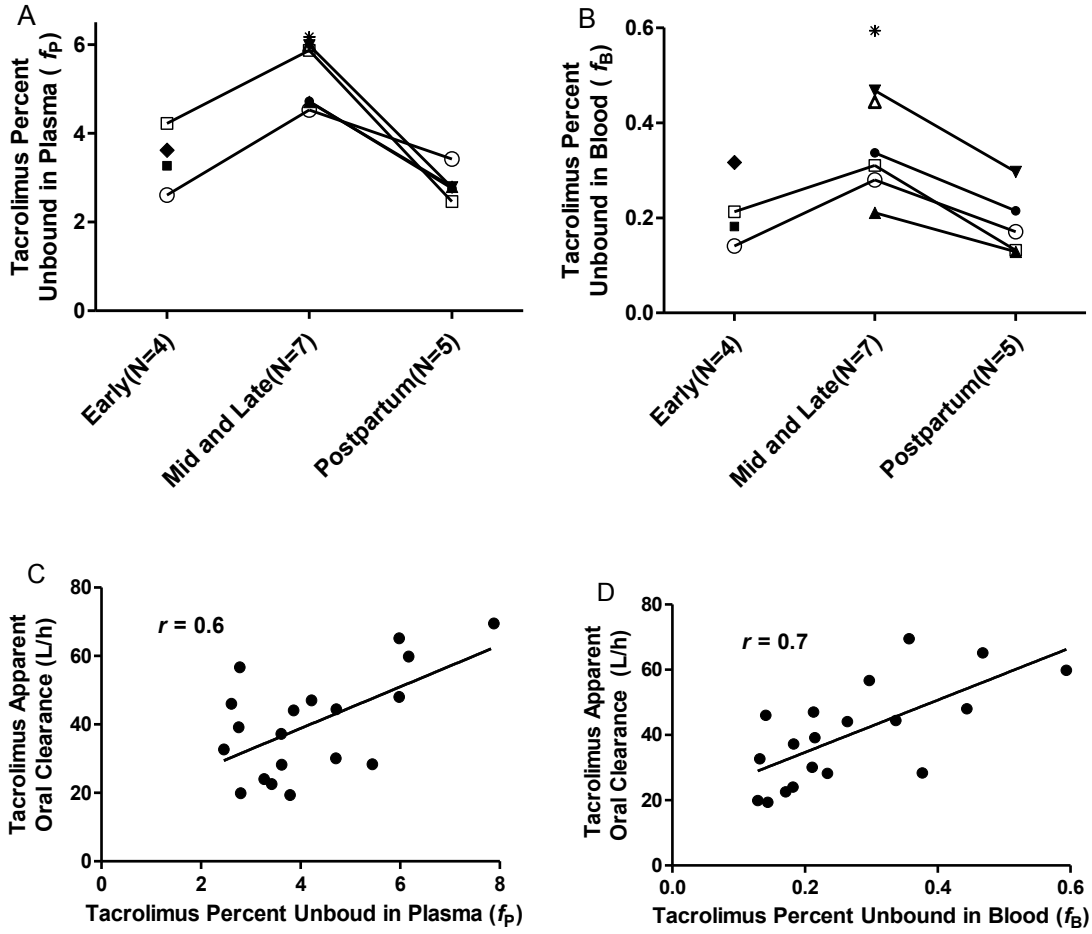


Figure 5.4 Tacrolimus percent unbound in plasma (A) and blood (B). The correlation between tacrolimus free fraction in plasma and oral clearance (C) and the correlation between free fraction in blood and tacrolimus oral clearance. The free fraction of tacrolimus could not be determined for one subject because of hemolyzed plasma samples

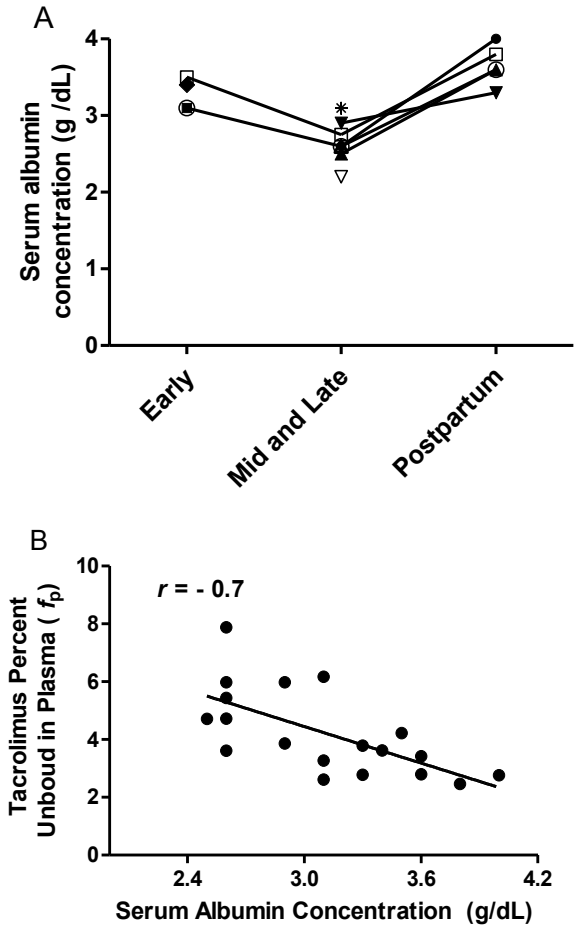


Figure 5.5 Serum albumin concentrations during early- (n = 4) and mid-/late-pregnancy (n = 8) as well as postpartum (n = 5) (A) and correlation between serum albumin concentrations and tacrolimus percent unbound in plasma during pregnancy and postpartum (B) in 10 solid organ transplantation recipients.

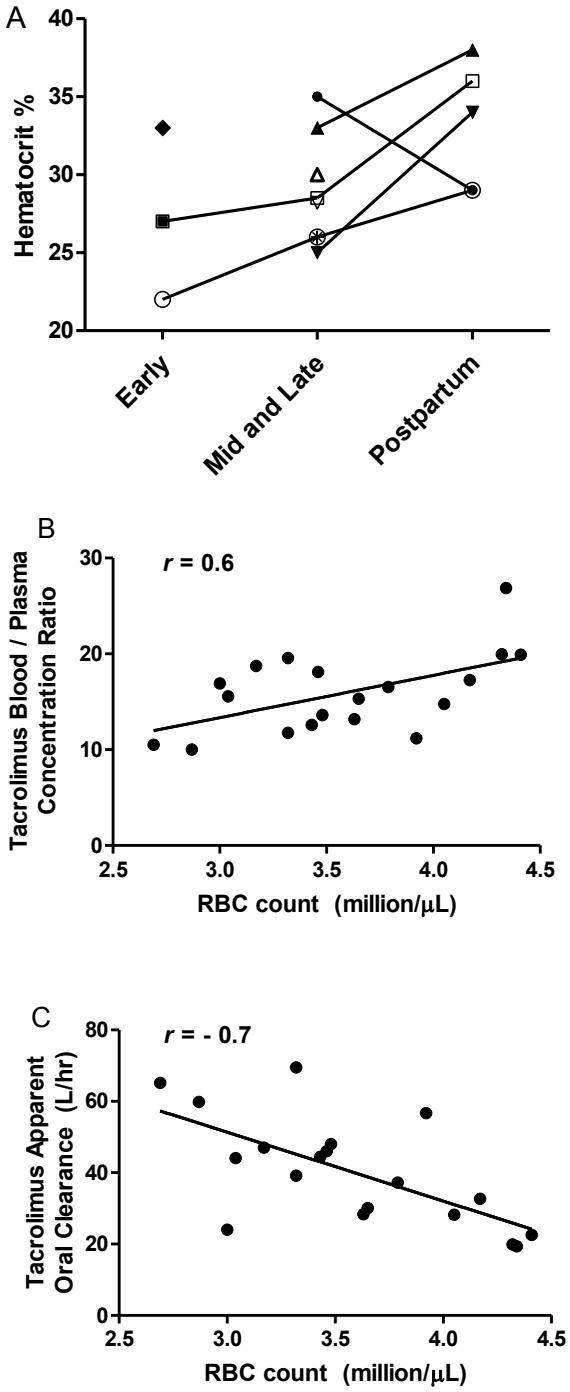


Figure 5.6 Hematocrit during early- (n = 4) and mid-/late-pregnancy (n = 8) as well as postpartum (n = 5) (A) and correlation between red blood cell counts and mean tacrolimus blood / plasma concentration ratio (B). Correlation between red blood cell counts and tacrolimus oral clearance (C) during pregnancy and postpartum in 10 solid organ transplantation recipients.

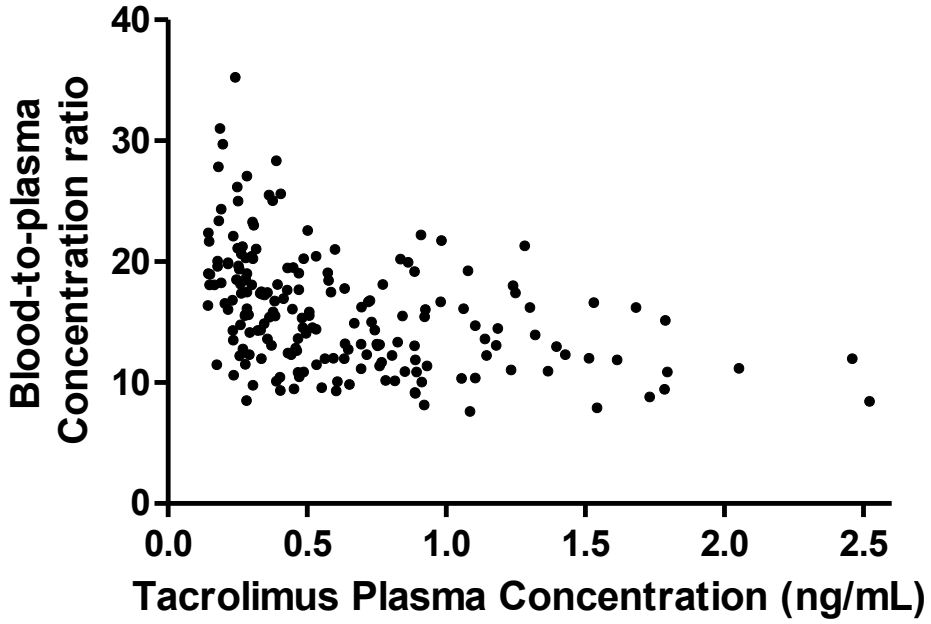


Figure 5.7 The blood to plasma tacrolimus concentration ratio versus plasma concentration in solid organ transplantation recipients during pregnancy. All measured plasma concentrations were pooled and plotted against the respective blood to plasma concentration ratio.

TABLE 5.1 Characteristics of study subjects (n = 10)**.**

Parameter	Early Pregnancy (10–14 Weeks, n = 4)	Mid and Late Pregnancy (22–38 Weeks, n =8)	Postpartum (n = 5)
Actual body weight (kg)	63.1 ± 15.2 (N.S.)	73.9 ± 14.3 (P =0.0004)	66.7 ± 16.3
Creatinine clearance (mL/min)	129.1 ± 67.5 (N.S.)	115.2 ± 54.3 (N.S.)	129.8 ± 52.2
Serum creatinine (mg/dL)	0.8 ± 0.6 (N.S.)	0.9 ± 0.4 (N.S.)	0.9 ± 0.4
Serum albumin concentration (g/dL)	3.3 ± 0.2 (N.S.)	2.7 ± 0.3 (P = 0.001)	3.7 ± 0.3
Hematocrit (%)	27.3 ± 4.5 (P =0.04)	29.0 ± 3.5 (N.S.)	33.2 ± 4.1
Red blood cell count (million/μL)	3.4 ± 0.5 (N.S.)	3.3 ± 0.4 (P = 0.02)	4.0 ± 0.4
Median tacrolimus dose (mg/day)	6.8	8.6	6
Mean tacrolimus dose (mg/day)	6.9 ± 2.5 (N.S.)	8.1 ± 3.1 (P < 0.02)	5.6 ± 1.8
Median dosing interval (h)	8	8	12
Mean dosing interval (h)	10.0 ± 2.3 (N.S.)	10.5 ± 2.1 (N.S.)	12.0 ± 0.0

P values: comparisons between early and the postpartum study days or mid/late pregnancy and the postpartum study days. N.S., not significant. Results are expressed as means ± standard deviation. The values from one subject in early pregnancy were not included in the calculation of creatinine clearance and serum creatinine because of her rejection after kidney transplant.

TABLE 5.2 Estimated steady-state, whole blood tacrolimus pharmacokinetic parameters during pregnancy and postpartum

Parameter	Early Pregnancy (10–14 Weeks, $n = 4$)	Mid and Late Pregnancy (22–38 Weeks, $n = 8$)	Postpartum ($n = 5$)
Unbound fraction in whole blood (f_B)	0.19 ± 0.04% (N.S.)	0.38 ± 0.13% ($P = 0.002$)	0.19 ± 0.07%
Oral clearance (L/h)	36.3 ± 11.9 (N.S.)	47.4 ± 12.6 ($P = 0.03$)	34.2 ± 14.8
Unbound oral clearance (L/h/f_B)	19990 ± 9563 (N.S.)	13470 ± 2617 (N.S.)	18133 ± 4392
Renal clearance based on AUC_b (mL/h)	3.6 ± 1.4 (N.S.)	2.8 ± 1.0 (N.S.)	3.5 ± 1.4
Unbound renal clearance (mL/min/f_B)	40.1 ± 21.5 (N.S.)	14.4 ± 8.6 ($P < 0.05$)	28.0 ± 9.8
Percent dose recovered in urine unchanged (%)	0.013 ± 0.006 (N.S.)	0.008 ± 0.004 (N.S.)	0.013 ± 0.004

P values: comparisons between early and the postpartum study days or mid/late pregnancy and the postpartum study days. N.S., not significant. Results are expressed as means ± standard deviation.

TABLE 5.3 Estimated steady-state plasma tacrolimus pharmacokinetic parameters during pregnancy and postpartum.

Parameter	Early Pregnancy (10–14 Weeks, $n = 4$)	Mid and Late Pregnancy (22–38 Weeks, $n = 7$)	Postpartum ($n = 5$)
Unbound fraction in plasma (f_P)	3.4 ± 0.7% (N.S.)	5.4 ± 0.7% ($P = 0.0007$)	2.8 ± 0.4%
Oral clearance based on AUC_p (L/h)	632.8 ± 249.5 (N.S.)	630.5 ± 194.8 (N.S.)	494.3 ± 103.3
Unbound oral clearance (L/h/f_P)	19160 ± 9414 (N.S.)	11700 ± 2760 ($P = 0.03$)	17718 ± 4914
Renal clearance based on AUC_p (mL/h)	77.6 ± 34.5 (N.S.)	45.9 ± 20.8 (N.S.)	64.4 ± 22.4
Unbound renal clearance (mL/min/f_P)	41.1 ± 26.9 (N.S.)	14.9 ± 6.9 ($P = 0.02$)	38.5 ± 16.3

P values: comparisons between early and the postpartum study days or mid/late pregnancy and the postpartum study days. N.S., not significant. Results are expressed as means ± standard deviation.

Chapter 6

Interpreting Tacrolimus Concentrations During Pregnancy and Postpartum

Portions of Chapter 6 were submitted to *Transplantation.*

6.1 Abstract

Pregnancy following solid organ transplantation, although considered high risk for maternal and fetal complications, has been quite successful. Tacrolimus pharmacokinetic changes during pregnancy make interpretation of whole blood trough concentrations particularly challenging. Both anemia and hypoalbuminemia during pregnancy increase the fraction of unbound tacrolimus. The clinical titration of dosage to maintain whole blood tacrolimus trough concentrations in the usual therapeutic range can lead to elevated unbound concentrations and possibly toxicity in pregnant women with anemia and hypoalbuminemia. Measurement of plasma or unbound tacrolimus concentrations for pregnant women might better reflect the active form of the drug, though these are technically-challenging and often unavailable in usual clinical practice. Tacrolimus crosses the placenta with *in utero* exposure being approximately 71% of maternal blood concentrations. The lower fetal blood concentrations are likely due to active efflux transport of tacrolimus from the fetus toward the mother by placental P-glycoprotein. To date, tacrolimus has not been linked to congenital malformations, but can cause nephrotoxicity and hyperkalemia in the newborn. In contrast, very small amounts of tacrolimus are excreted in the breast milk and are unlikely to elicit adverse effects in the nursing infant.

6.2 Introduction to Chapter 6

There is a growing body of evidence describing successful pregnancies in solid organ transplant recipients with tacrolimus based immunosuppression(Kainz et al., 2000b; Jain et al., 2003; Jain et al., 2004; Gutierrez et al., 2005; Coscia et al., 2010).

Nevertheless, these pregnancies are at increased risk for maternal, fetal and neonatal complications such as preeclampsia, hypertension, renal impairment, rejection, infection, post-pregnancy graft loss, miscarriage, preterm birth, stillbirth, intrauterine growth restriction, low birth weight, reversible renal dysfunction as well as hyperkalemia in the newborn and rarely neonatal death(Kainz et al., 2000b; Jain et al., 2003; Jain et al., 2004; Gutierrez et al., 2005; Coscia et al., 2010). Given the severity of the complications, a critical evaluation and optimization of tacrolimus therapy during pregnancy is warranted.

6.3 Physiologic Changes in Pregnancy that Alter Drug Disposition

There are a number of physiologic changes that occur during pregnancy that alter biotransformation, renal clearance, volume of distribution and protein binding. Pregnancy appears to increase the activities of some drug-metabolizing enzymes (e.g. CYP3A, CYP2D6, CYP2C9 and UGT), whereas others (e.g. CYP1A2 and CYP2C19) are decreased(Tracy et al., 2005; Hebert et al., 2008; Buchanan et al., 2009; Hebert et al., 2009; Claessens et al., 2010; S Zheng et al., 2012a). Previous work utilizing probe substrates suggests that intrinsic CYP3A activity increases by 25-100% during pregnancy (Tracy et al., 2005; Hirt et al., 2006; Villani et al., 2006; Hebert et al., 2008).

Not only are there changes in drug metabolism during pregnancy, but also in renal clearance(Hebert et al., 2005; Andrew et al., 2007; Hebert et al., 2008; Eyal et al., 2010). Normal pregnancy is characterized by renal vasodilation (Conrad, 2004) and a 35-40% increase in cardiac output(Easterling et al., 1990). Effective renal plasma flow increases significantly by 6 weeks gestation, reaching a peak ~50-85% above non-pregnant values, with a corresponding increase in glomerular filtration rate (GFR)(Davison and Dunlop,

1980; Sturgiss et al., 1994; Chapman et al., 1998). Normal serum creatinine during pregnancy is ≤ 0.7 mg/dL. In addition, there appears to be an increase in the activity of several renal transporters (e.g. P-glycoprotein)(Andrew et al., 2007; Hebert et al., 2008; Eyal et al., 2010). Changes in enzyme and transporter activities, as well as renal filtration can increase or decrease concentrations of many drugs (Hebert et al., 2005; Hebert et al., 2008; Hebert et al., 2009).

Weight gain during pregnancy often exceeds the recommended 11-40 lbs. The majority of the weight gain during pregnancy is water (~62%), followed by fat (~30%) and protein (~8%). Along with the increase in total body water (~7-9 L) are parallel increases in extracellular fluid and blood volumes (Hytten et al., 1966; Davison and Dunlop, 1980). Changes in body composition along with those in protein binding can result in changes in the volume of distribution for some drugs. Although an increase in volume of distribution alone will not alter average steady state drug concentrations, it will lead to lower peaks and higher troughs.

Changes in drug binding in blood are most clinically relevant when the percentage bound is high (>80%). Drugs can bind to plasma proteins such as albumin and a_1-acid glycoprotein (AAG) or to cellular components such as erythrocytes or lymphocytes. Albumin concentrations in normal pregnancy decrease by ~1-13% across gestation and by much more in some allograft recipients(1990:468; S Zheng et al., 2012a). Pregnancy also leads to lower plasma AAG concentrations (~52% lower at 30-36 weeks gestation) (Aweeka et al., 2010). Hemoglobin is known to fall by ~11% during normal pregnancy (nadirs mid-pregnancy); however, much greater declines and anemia have been reported following solid organ transplantation(1990:468; S Zheng et al., 2012a). Depending on the

extraction ratio (ER) of the drug across the eliminating organ (liver, kidney), changes in drug binding may or may not affect drug clearance, volume of distribution, first-pass metabolism and the clinical interpretation of drug concentrations.

For most drugs, particularly those that exhibit low ER, a decrease in drug binding to plasma proteins can lead to lower total drug concentrations, but no change in unbound concentrations (active form of the drug)(S Zheng et al., 2012a). A well-documented example of this is phenytoin. For phenytoin, low albumin concentrations result in lower total drug concentrations but no change in unbound concentrations (ME, 2004:511). For this reason, clinicians either correct the total phenytoin concentrations for changes in binding or measure unbound concentrations and titrate to the unbound therapeutic range. Like phenytoin, there are a number of other highly bound drugs (e.g. tacrolimus) for which similar pharmacokinetic changes can occur.

6.4 Tacrolimus Pharmacokinetics in Pregnancy

The complexity of tacrolimus pharmacokinetics makes it particularly interesting from the research perspective, but challenging from the clinical perspective. Tacrolimus is a substrate for CYP3A4, CYP3A5 and P-glycoprotein. (Sattler et al., 1992; Saeki et al., 1993) It is also highly bound to plasma proteins and erythrocytes. In addition, it has temperature and concentration dependent partitioning between plasma and red blood cells (RBCs). Using blood for analysis, tacrolimus is considered to be a low ER drug. However with plasma, it could be considered a high ER drug. These characteristics, along with the slow tacrolimus uptake and release by erythrocytes, make predicting the pharmacokinetic changes during pregnancy difficult.

Many case reports discuss the effects of pregnancy on tacrolimus concentrations. Some describe no change in tacrolimus concentrations or dosage while others report lower concentrations and / or the need for dosage escalation during pregnancy (Yoshimura et al., 1996; Jain et al., 1997; Midtvedt et al., 1997; Casele and Laifer, 1998; Kainz et al., 2000b; Jain et al., 2003; Jain et al., 2004; Garcia-Donaire et al., 2005; Jabiry-Zieniewicz et al., 2006; Grimer, 2007). Interpretation of these reports has been limited by differences in the biologic fluid (blood, plasma, or serum), non-specific methodologies (ELISA, FPIA), lack of attention to temperature dependent distribution into RBCs and failure to account for changes in plasma proteins or RBC concentrations on drug binding. These issues have been addressed by Zheng et al. (S Zheng et al., 2012a). This work highlights several important findings thereby explaining the discrepancies in previous reports. First, tacrolimus fraction unbound and whole blood oral clearance increase during pregnancy. Second, the changes in whole blood drug concentrations reflect changes in drug binding due to low RBC count, albumin and perhaps AAG concentrations during pregnancy. Third, there is no change in whole blood, unbound oral clearance. The following discussion will address the clinical implications of these findings in pregnant allograft recipients. We will also comment on tacrolimus *in utero* exposure and neonatal exposure through breast milk.

6.5 Tacrolimus Distribution in Blood

Tacrolimus protein binding issues are even more complicated than for phenytoin. Tacrolimus concentrates in erythrocytes, with blood-to-plasma ratio being 4-42(Sam et al., 2006). Tacrolimus binding to erythrocytes accounts for 85-95% of the drug in blood,

which slowly equilibrates with plasma (Beysens et al., 1991; Piekoszewski et al., 1993; Chow et al., 1997). Patients with higher RBC counts or lower tacrolimus concentrations exhibit greater tacrolimus blood:plasma ratios (Jusko et al., 1995a; S Zheng et al., 2012a). In pregnancy, RBC counts decrease as a result of rapid volume expansion and relatively greater increase in plasma volume than RBC mass (Hytten, 1985). Tacrolimus is highly protein bound (< 3% unbound) to both albumin and AAG in plasma, accounting for 5-15% of the drug in blood (Chow et al., 1997; Undre, 2003; S Zheng et al., 2012a). Changes in albumin, AAG and RBCs will alter tacrolimus binding (Undre and Schafer, 1998; S Zheng et al., 2012a). Therefore, if there is no change in the unbound intrinsic clearance of tacrolimus, a decrease in albumin, AAG and / or RBC count should lead to a fall in whole blood concentration, but no change in unbound concentration. **Figure 6.1A-D** depict this concept by comparing the distribution profile within the blood in normal, anemic and / or hypoalbuminemic patients without any adjustment in dosage or change in intrinsic clearance of unbound drug. **Figure 6.1A** is a state of normal RBC count and albumin concentration. Tacrolimus is concentrated in the RBCs and most of the tacrolimus in plasma is bound to plasma proteins. In this example, the whole blood concentration is 10, plasma concentration is 3, and unbound concentration is 1. **Figure 6.1B** and **6.1C** are examples of the same situation except the patient has anemia and hypoalbuminemia, respectively. The active form of the drug (unbound concentration) in both cases is still 1, but the whole blood concentrations are now 7 and 9, respectively. In **Figure 6.1D** the patient has anemia and hypoalbuminemia as is often seen during pregnancy in allograft recipients. In this example, the unbound concentration stayed the same at 1, but the whole blood concentration fell to 6. Conceptually, this is the same situation for

tacrolimus during pregnancy in allograft recipients as described by Zheng et al. (S Zheng et al., 2012a).

Clinically, trough tacrolimus concentrations are measured in whole blood, thus dosage titration to maintain trough concentrations in the therapeutic range leads to an increase in unbound tacrolimus concentrations. With this increase comes the potential for tacrolimus toxicity. To illustrate the clinical implications of this, the subjects participating in the Zheng et al. study are presented in **Figure 6.2A-D**. The whole blood oral clearance for tacrolimus is 39% higher during pregnancy compared to postpartum. The consequence of the increase in oral clearance in pregnancy can be graphically seen in **Figure 6.2A**, in which the dose-normalized, whole blood, trough concentrations are lower during pregnancy than postpartum. If no dosage adjustments had been made, the whole blood trough concentrations would have fallen. However, as this started to occur, the clinicians increased the tacrolimus dosage (**Figure 6.2B**). In doing so, they were quite successful at maintaining whole blood trough concentrations within the target range as seen in **Figure 6.2C**. However, because the tacrolimus whole blood unbound fraction increased by ~100% during pregnancy and there was no change in tacrolimus unbound oral clearance in blood, without realizing it, these dosage adjustments resulted in a markedly higher unbound tacrolimus trough concentrations (active form), as shown in **Figure 6.2D**. Although maintaining whole blood tacrolimus trough concentrations in the usual therapeutic range during pregnancy has been routine in most transplant centers, it is unclear if this is the optimum approach.

6.6 Immune Response

Immune changes in pregnancy are complex and are not detailed here in their entirety. A recent review of this topic has been published by Pazos et al. and can be refered to for a more detailed discussion (Pazos et al., 2012). In brief, pregnancy creates a unique immune environment in which the fetus can be tolerated by down regulation of some T-cell mediated immune responses, while other components of the immune system such as monocytes and neutrophils are activated (Luppi, 2003; Kraus et al., 2012). T cells, B cells, neutrophils, dendritic cells, monocytes and natural killer cells are transcriptionally regulated by estrogen (Cvoro et al., 2008; Pierdominici et al., 2010). CD3, CD4 and CD8 T cell concentrations in blood are decreased during pregnancy, but the CD4:CD8 ratio is maintained (Kraus et al., 2012). The effects of pregnancy on cytokines are mixed. Some report increased concentrations of IL-2, IL-6 and IL-8 while others describe down regulation of INFg, TNFa, IL-6, IL-10 and IL-13 but no change in IL-4(Wegmann, 1990; Opsjon et al., 1993; Austgulen et al., 1994; Luppi et al., 2002a; Luppi et al., 2002b; Kraus et al., 2012). Pregnancy appears to promote a generalized activation of circulating leukocytes with up-regulation of adhesion molecules. B cell lymphopoiesis and total B cell counts are reduced (Medina et al., 1993; Kraus et al., 2012); however, estrogen has been shown to increase antibody production *in vitro* and in animal models (Kanda and Tamaki, 1999; Grimaldi et al., 2001). Thus, it is possible that pregnancy decreases production of new B cells while at the same time enhancing antibody production from mature B cells (Kraus et al., 2012). Although there is good evidence to suggest that some aspects of the immune response are dampened during pregnancy leading to decreased viral clearance and alleviation of symptoms for some

autoimmune diseases, other parts of the innate immunity are enhanced(Kraus et al., 2012; Pazos et al., 2012).

There is a general aversion to using medications during pregnancy; however, discontinuation of immunosuppression during pregnancy has led to rejection and at least 2 maternal deaths, suggesting that some level of immunosuppression is required (Medina et al., 1993; Kanda and Tamaki, 1999). Published data with a variety of immunosuppressives, given alone or in combination, with or without dosage escalation during pregnancy suggest similar rejection rates as in the non-pregnant transplant population(Sims, 1991; McKay and Josephson, 2006). Rejection rates during pregnancy range from 2-8% (Armenti et al., 2006; Deshpande et al., 2011). Post-pregnancy graft loss at 2 years is approximately 8% (Deshpande et al., 2011). The effect of pregnancy on allograft function remains controversial (Galdo et al., 2005; Deshpande et al., 2011). Establishing the optimum therapeutic range for tacrolimus during pregnancy is not possible based on the paucity of available data.

The impacts of tacrolimus unbound and plasma concentrations have been evaluated for efficacy in the non-pregnant population. The concentration of tacrolimus in the blood primarily reflects RBC concentration, which does not necessarily reflect lymphocyte concentration or its availability to interact with intracellular targets (Ichimaru et al., 2001; Christians et al., 2002). Several experts suggest that unbound concentration of tacrolimus better correlates with incidence of rejection (Morozumi, 1993; Erden et al., 1994; Kershner and Fitzsimmons, 1996). Unbound drug, which is available for cellular diffusion and cellular distribution, may be more reflective of target site concentration. Indeed, tacrolimus plasma concentrations in patients are in the range of *in vitro*

lymphocyte proliferation inhibitory concentrations (Sawada et al., 1987; Eiras et al., 1990; Piekoszewski and Jusko, 1993). Zahir et al. reported that the percentage of tacrolimus associated with the lymphocytes and unbound concentration in blood were significantly higher in stable allograft recipients than in those experiencing rejection (Zahir et al., 2004). Tsunoda *et al.* suggested that concentrations in the transplanted organ itself might be more predictive of the pharmacological effect of tacrolimus (Tsunoda and Aweeka, 1996). Hepatic tissue concentrations of tacrolimus were found to be significantly higher in patients without rejection than in patients experiencing rejection episodes after liver transplantation(Sandborn et al., 1995). Based on the data from (S Zheng et al., 2012a), it is expected that if the tacrolimus dose were not increased during pregnancy to maintain a supposed therapeutic whole blood trough concentrations, the unbound tacrolimus concentration in blood would be comparable during pregnancy and postpartum. This assumes that no other factors alter the unbound tacrolimus concentrations. Given the high variability in tacrolimus pharmacokinetics, this assumption will not always hold. Even so, in many patients, the therapeutic benefit of tacrolimus may not be compromised by withholding dosage adjustment unless warranted by other confounding variables. Consistent with this, Jain et al. (Jain et al., 2004) reported no rejection episodes in 21 pregnancies in which tacrolimus dosage was not adjusted during gestation despite lower trough concentrations.

6.7 Toxicity

Not only is it important to consider the effects of tacrolimus concentrations on efficacy, but toxicity as well. Trull et al. (Trull et al., 2002) reported that in non-pregnant liver allograft recipients receiving tacrolimus who were randomized post-transplantation to 2 weeks of albumin infusion or artificial plasma expander, those with the albumin infusions and corresponding higher serum albumin concentrations had lower serum creatinine concentrations and higher rejection rates. No significant differences were observed in whole blood tacrolimus concentrations between groups. It was suggested that the greater unbound fraction of tacrolimus in the artificial plasma expander group might have led to an increase in nephrotoxicity and lower rejection rate by increasing the active form of the drug. Excluding the patient experiencing rejection during pregnancy from the subjects studied by Zheng et al. (S Zheng et al., 2012a), **Figure 6.3A-C** demonstrates an apparent correlation between serum creatinine concentration and tacrolimus trough whole blood, plasma and unbound blood concentrations, respectively. Although the data are limited, tacrolimus nephrotoxicity during pregnancy appears to correlate best with trough plasma concentrations. In normal pregnancies, a substantial increase is expected in creatinine clearance. Not surprisingly, given the doubling of unbound tacrolimus concentrations observed by Zheng et al. (S Zheng et al., 2012a), the expected increase in creatinine clearance during mid-/late-pregnancy (115.2 ± 54.3 mL/min) as compared to postpartum (129.8 ± 52.2 mL/min) was not observed. Accordingly, the attenuated creatinine clearance during pregnancy may reflect tacrolimus nephrotoxicity. Backman et al. (Backman et al., 1994) suggested that monitoring tacrolimus plasma concentrations would be superior to whole blood, based the reduction

in GFR correlating with yearly mean tacrolimus plasma but not with whole blood concentrations or dosage. Notably, Zheng et al. (S Zheng et al., 2012a) observed a strong correlation between tacrolimus plasma and unbound concentrations in blood ($r = 0.9$, $P < 0.0001$), whereas a weaker correlation existed between whole blood and unbound concentrations ($r = 0.6$, $P = 0.004$).

6.8 Tacrolimus Therapeutic Range in Pregnancy

It is unbound drug that is available to cross cellular membranes and bind receptors. As described above, it appears that for both efficacy and toxicity, whole blood tacrolimus concentrations may be less predictive of optimal drug dosing than plasma or unbound blood concentrations. Unfortunately only whole blood concentrations are currently available in the clinical setting, thus careful consideration should be given to those patients that have significant anemia and hypoalbuminemia. Zheng et al. (S Zheng et al., 2012a) found an inverse correlation between the fraction unbound in plasma and serum albumin concentrations. In addition, multiple studies have reported correlations between RBC count and tacrolimus concentrations (Undre and Schafer, 1998; Minematsu et al., 2004; S Zheng et al., 2012a). This was confirmed by Zheng's finding of an inverse correlation between tacrolimus whole blood oral clearance and RBC counts (S Zheng et al., 2012a). Because there is no change in the whole blood unbound oral clearance, the unbound concentration should not change if no dosage adjustments are made and no other factors occur that would alter tacrolimus concentrations. Due to the variability in tacrolimus concentrations during pregnancy and the potential for tacrolimus whole blood concentrations to fall below the lower limits of assay capability, clinicians are likely to be

concerned about not adjusting the tacrolimus dosage. If the decision is made to maintain whole blood trough concentrations in the usual therapeutic range during pregnancy, it must be recognized that unbound concentrations are likely to be higher in hypoalbuminemic and/or anemic patients than prior to pregnancy. For these patients, tacrolimus toxicity should be monitored closely. The choice of target whole blood concentrations should take into account the individual patient's history, current medical conditions, concomitant medications and the potential impact of high or low unbound concentrations. As an alternative, monitoring of tacrolimus plasma concentrations during pregnancy would remove one of the key variables (RBC count) that can confound the interpretation of tacrolimus concentrations. Utilizing an assay for unbound tacrolimus concentrations would remove both factors (RBC count and low plasma proteins), but is more costly and time-consuming.

6.9 Fetal Exposure

Tacrolimus, like most drugs, crosses the placenta, with umbilical cord concentrations being ~71%, 23% and 19% of maternal concentrations for whole blood, plasma and unbound, respectively (S Zheng et al., 2012b). The downward concentration gradient from maternal circulation to umbilical cord probably reflects the active efflux of tacrolimus from the fetus toward the mother by placental P-glycoprotein as well as the difference between maternal and fetal hematocrits. Even with some transfer across the placenta, tacrolimus does not appear to cause congenital malformations (Kainz et al., 2000b). However, tacrolimus has been reported to cause reversible neonatal renal

impairment and hyperkalemia (Jain et al., 1993; Jain et al., 1997; Resch et al., 1998; Kainz et al., 2000b) .

6.10 Breast Milk

Tacrolimus is excreted in the breast milk with infant ingestion reported to be < 1% of the maternal weight-adjusted dosage (French et al., 2003; Gardiner and Begg, 2006; S Zheng et al., 2012b). Breastfeeding does not appear to contribute to tacrolimus concentrations in the infant postpartum (Chusney et al., 2011). A limited number of cases report no adverse effects in the nursing infants while the mother was receiving tacrolimus therapy(Gardiner and Begg, 2006; Gouraud et al., 2011). The American Academy of Pediatrics has listed cyclosporine in their table entitled "Cytotoxic drugs may interfere with cellular metabolism of the nursing infant" (Ward et al., 2001; Gouraud et al., 2011). Theoretically, calcineurin inhibitors might alter the immune benefits transferred to the nursing infant, although no reports were found. For tacrolimus, the amount that is excreted through the breast milk is extremely low and unlikely to have any effect on the nursing infant. Infant exposure during lactation is expected to be far lower than *in utero* exposure. Blood level monitoring of the infant while being mindful of the effects of plasma proteins and RBC count on drug binding could be considered if concerns arose.

6.11 Conclusions

If properly handled and measured, plasma or unbound tacrolimus trough concentrations, although more costly and less readily accessible, might better predict

drug efficacy and toxicity during pregnancy than whole blood concentrations(Sawada et al., 1987; Backman et al., 1994; Hebert et al., 2005; S Zheng et al., 2012a). pregnancy, if tacrolimus whole blood concentrations are the only available assay, clinical interpretation of trough tacrolimus concentrations should take into account serum albumin concentration and RBC count. For allograft recipients who develop anemia and hypoalbuminemia during pregnancy, unbound tacrolimus trough concentrations are variable and can increase by as much as 2-fold if dosage titration follows the conventional practice of maintaining stable whole blood concentrations within the generally accepted therapeutic range. Tacrolimus crosses the placenta, but umbilical cord blood concentrations at the time of delivery are lower than maternal concentrations, most likely due to P-glycoprotein efflux across the placenta. Very low amounts of tacrolimus are excreted in the breast milk.

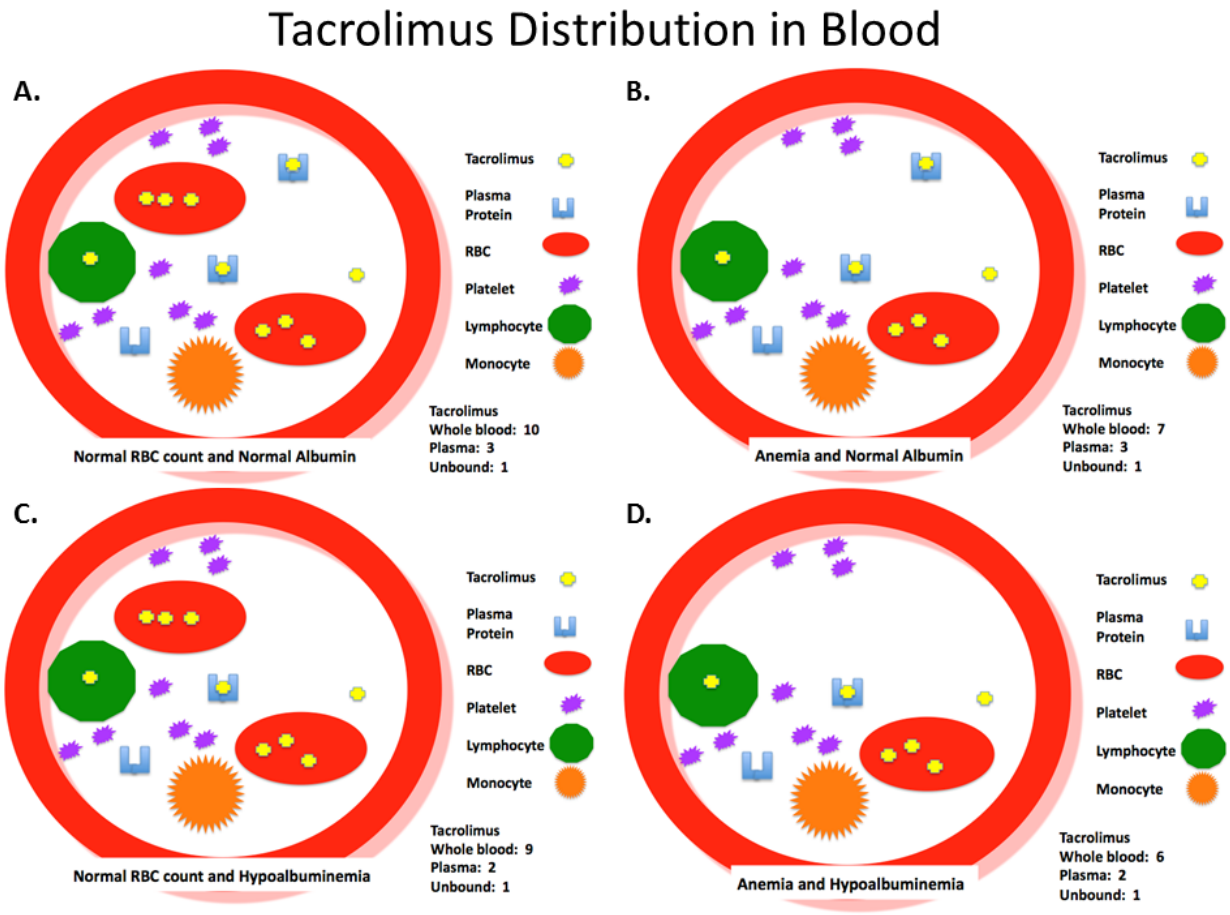


Figure 6.1 Depicts a simulated scenario of tacrolimus distribution in blood of patients with A. normal red blood cell count and normal albumin, B. anemia and normal albumin, C. normal red blood cell count and hypoalbuminemia and D. anemia and hypoalbuminemia.

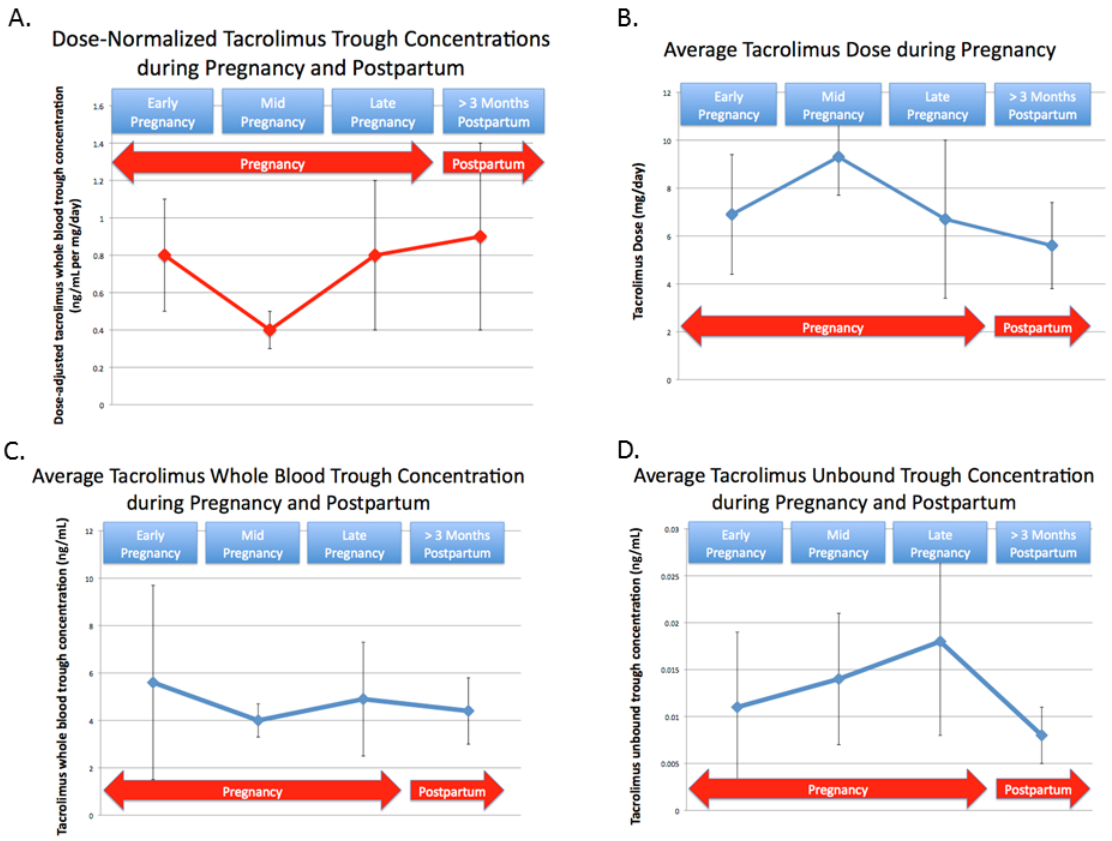


Figure 6.2 A. Average (± SD), dose-normalized (1 mg/day), whole blood tacrolimus trough concentrations during early-, mid-, and late-pregnancy as well as > 3 months postpartum. B. Average (± SD) tacrolimus dose (mg/day) during early-, mid-, and late-pregnancy as well as > 3 months postpartum. C. Average (± SD) whole blood tacrolimus trough concentrations during early-, mid-, and late-pregnancy as well as > 3 months postpartum. D. Average (± SD) unbound tacrolimus trough concentrations during early-, mid-, and late-pregnancy as well as > 3 months postpartum.

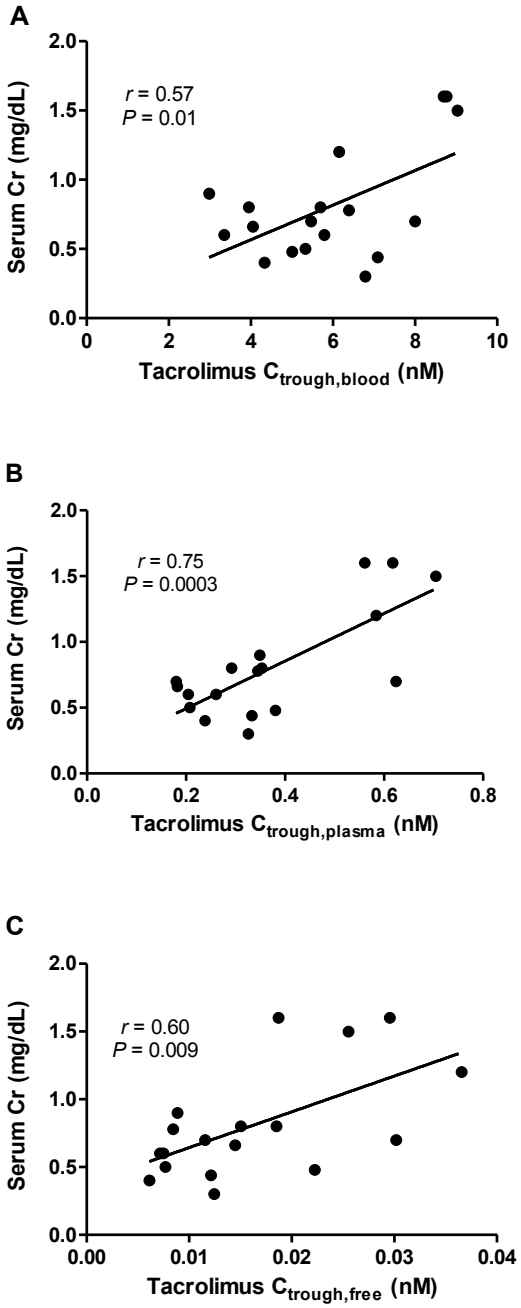


Figure 6.3 Correlations between serum creatinine concentrations during pregnancy and postpartum and A. blood, B. plasma and C. unbound tacrolimus trough concentrations in non-rejecting transplantation recipients.

Chapter 7

Tacrolimus Placental Transfer at Delivery and Neonatal Exposure through Breast Milk

Portions of Chapter 7 are being submitted to *Pediatrics*.

7.1 Abstract

The current investigation aims to provide new insights into *in utero* exposure to tacrolimus by evaluating maternal and umbilical cord blood, plasma and unbound concentrations at delivery. This study also presents a case report of tacrolimus excretion via breast milk. Maternal and umbilical cord samples were obtained at delivery from eight allograft recipients to measure tacrolimus and metabolite bound and unbound concentrations in blood and plasma. Tacrolimus pharmacokinetics in breast milk were conducted in one subject.

Mean (± SD) tacrolimus blood concentrations at the time of delivery in the umbilical cord vein (6.6 ± 1.8 ng/mL) were 71 ± 18% (range 45–99%) of maternal blood concentrations (9.0 ± 3.4 ng/mL). The mean fetal plasma (0.09 ± 0.04 ng/mL) and unbound drug concentrations (0.003 ± 0.001 ng/mL) were approximately one fifth of the respective maternal concentrations. Arterial umbilical cord blood concentrations of tacrolimus were 100 ± 12% of venous concentrations. In addition, infant exposure to tacrolimus through the breast milk was less than 0.3% of the mother's weight-adjusted dose.

In Summary, lower umbilical cord than maternal concentrations may be explained in part by placental P-gp function, greater red blood cell partitioning and higher hematocrit levels in the venous cord blood. The neonatal drug exposure of tacrolimus in breast milk is very low and likely does not represent a health risk to the breastfeeding infant.

7.2 Introduction to Chapter 7

Tacrolimus is an immunosuppressive agent widely used for the prevention of rejection in kidney, liver, heart, and other solid organ transplant recipients.(Scott et al., 2003) Although usually managed successfully, pregnancy following transplantation is associated with many complications.(Jain et al., 1997; Kainz et al., 2000a; McKay and Josephson, 2006) Limited data have been published characterizing tacrolimus and metabolite transfer across the placenta, metabolism by the fetus, and excretion into breast milk. Utilizing a non-specific immunoassay, mean umbilical cord plasma concentrations have been reported to be 0.71 ± 0.77 ng/mL (49% of maternal plasma concentrations).(Jain et al., 1997) Routine clinical immunoassays are not specific for the parent compound nor are they accurate at low concentrations.(French et al., 2003; Borrows et al., 2007) Since tacrolimus crosses the placenta, one potential concern is the effect of tacrolimus *in utero* exposure on the developing fetus.(McKay and Josephson, 2006)

There is an array of drug transporters on placental syncytiotrophoblasts. Some of these transporters, such as P-gycoprotein (P-gp), minimize fetal exposure by effluxing drug back into the maternal circulation.(Wang et al., 2007) Tacrolimus is a substrate for P-gp.(Saeki et al., 1993) We utilized venous umbilical cord-to-maternal blood, plasma and unbound tacrolimus and metabolite concentration ratios at the time of delivery to characterize placental transfer *in vivo*.(Hutson et al., 2011) To our knowledge, no other study has evaluated *in vivo* placental transfer of tacrolimus by measuring both blood and plasma concentrations, as well as the unbound concentration, utilizing a highly sensitive, specific assay. We report tacrolimus pharmacokinetics in allograft recipients at the time

of delivery and postpartum utilizing a high-performance liquid chromatography-mass spectrometry assay.

Maternal CYP3A4/5 metabolic activities in the liver and small intestine are the major determinants of steady-state circulating maternal drug concentrations following oral drug administration.(Haufroid et al., 2004; Kamdem et al., 2005; Rodriguez-Antona et al., 2005; Dai et al., 2006) In the fetus, CYP3A7 is the major cytochrome P450 enzyme expressed in the liver, accounting for ~50% of the total cytochrome P450 content.(Rodriguez-Antona et al., 2005) CYP3A7 is less efficient at metabolizing tacrolimus than CYP3A4 and CYP3A5 (catalytic efficiency 29% and 18%, respectively), which are the major maternal isoforms of the enzyme.(Kamdem et al., 2005) Measurement of tacrolimus and metabolite concentration gradients between the umbilical vein and artery can provide valuable information on fetal metabolism of tacrolimus and its impact on fetal exposure.(Hutson et al., 2011)

Tacrolimus crosses into breast milk (French et al., 2003; McKay and Josephson, 2006) and some women have been advised against breastfeeding during therapy.(Jain et al., 1997; Gardiner and Begg, 2006) Given the many benefits of breastfeeding, it is of value to expand our understanding of the extent of infant exposure to tacrolimus via breast milk.(Gardiner and Begg, 2006) In the current study, we characterized both the tacrolimus milk-to-blood and milk-to-plasma area under the concentration-time curve ratios. In addition, we determined unbound drug concentration in milk and plasma to gain an understanding of the mechanism by which tacrolimus is transferred into human milk across the mammary epithelia.(Koshimichi et al., 2011)

7.3 Materials and Methods

7.3.1 Subjects of the Study

The study was approved by the Institutional Review Boards at the University of Washington, Georgetown University, and the University of Texas Medical Branch in Galveston and was conducted in accordance with their guidelines. We examined the concentrations of tacrolimus in the maternal and umbilical cord blood and plasma samples at the time of delivery. All subjects gave written informed consent. One woman participated in the breast milk sample collection for measurement of tacrolimus concentrations.

7.3.2 Sample Collection

Maternal and umbilical cord (arterial and venous) blood samples were collected immediately after delivery in all cases but one. For this one subject whose delivery occurred 4.5 hours after tacrolimus dosing, maternal blood was collected 30 min before delivery and venous cord blood was collected 30 min after delivery. For one subject, 45 weeks and 1 day after delivery, a breast milk pharmacokinetic study was completed. Sequential blood samples (5 mL) were collected over one dosing interval in EDTA glass tubes just before (0 h) and at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10 and 12 h after oral drug administration. Blood and plasma samples were collected, processed and frozen as previously reported (**Chapter 5**). Breast milk collections were performed using the Medela Classic double electric breast pump, into glass bottles every 3 hours, over one dosing interval. Both breasts were completely emptied of milk during each collection.

The subject did not breastfeed her infant during the study day. Glass pipets were used for all transfers to avoid adsorptive loss of tacrolimus. All samples were stored at −80° C until analysis.

7.3.3 Tacrolimus and Metabolite Analysis

Tacrolimus and metabolite concentrations in blood, plasma and milk samples were quantified utilizing a previously reported LC-MS/MS procedure that was modified to improve sensitivity.(Chen et al., 2006) An ultracentrifugation procedure adapted from Nakai *et al.* (Nakai et al., 2004) was used to determine unbound fraction of tacrolimus in plasma (f_{up}) and in milk (f_{um}). The unbound fraction in whole blood (f_{ub}) was determined from the relationship: $f_{ub} = (C_P/C_{WB})\, f_{up}$, where f_{up} is the unbound fraction in plasma, C_P is the plasma concentration, and C_{WB} is the whole blood concentration. This calculation assumes that unbound drug concentration in plasma is always at partitioning equilibrium with drug sequestered in blood cells. A full description of the modified LC-MS/MS assay, ultracentrifugation procedure, along with the procedure for isolation and purification of the tacrolimus metabolites for use as analytical standards, is provided in **Chapter 2**.

7.3.4 Pharmacokinetic Analysis

Noncompartmental pharmacokinetic analysis was performed using WinNonlin software version 5.2 (Pharsight, Mountain View, CA). Measured milk concentrations for each 3-hour interval were used to determine the amount excreted (milk concentration × expected milk volume). The estimated amount excreted daily was the sum of the 3 hour intervals over the 12 hour sampling period multiplied by two. The tacrolimus dose

ingested by a typical, exclusively breastfed 3-month old infant was also estimated, by assuming a breast milk ingestion of 150 mL/kg/day.

To elucidate the difference in whole blood-to-plasma concentration ratios between maternal and cord blood, the partitioning of tacrolimus between blood cells and plasma ($K_{bc/p}$) was estimated by $K_{bc/p} = K_{b/p}/Hct - 1/Hct + 1$.[(Hinderling, 1997)] $K_{b/p}$ is the blood-to-plasma concentration ratio and *Hct* represents the hematocrit. For maternal blood, *Hct* was not available for samples at term. Instead, pre-term hematocrit values (ranging from 25% to 35%) for the same individual collected during pregnancy (56 ± 53 days before delivery) were used when available. For venous umbilical cord blood, an average reported hematocrit value of 50% was used.(Jahazi et al., 2008)

7.3.5 Statistical Analysis

Descriptive statistics are presented as mean ± standard deviation (SD), unless otherwise indicated. Statistical comparisons were conducted using an unpaired two-sided Student's T-test (GraphPad Prism 5, La Jolla, CA). A *P* value less than 0.05 was considered significant.

7.4 Results

7.4.1 Patient Demographics

A total of 8 pregnant subjects (3 non-Hispanic White, 3 Hispanic, 1 non-Hispanic Black, 1 Asian), age 25.4 ± 6.2 years and height 158.4 ± 8.8 cm, participated in this study.

All subjects were solid organ transplantation recipients (four kidney, one kidney/pancreas, one kidney/heart, two liver) and were receiving the tacrolimus orally for immunosuppression. The mean gestational age at delivery was 36.8 ± 2.9 weeks.

7.4.2 Placental Transfer of Tacrolimus

Maternal (N=8) and umbilical cord blood (N=7 for venous and N=5 for arterial) were collected at the time of delivery. The average tacrolimus dosage was 7.5 ± 2.6 mg/day. For one patient who gave birth to twins, two umbilical cord venous blood samples were collected, analyzed separately and counted as one. The difference between the twin venous cord blood concentrations was 4.9% and the mean concentration was used for calculating all ratios. All samples were assayed for blood concentrations. In addition, plasma was harvested from maternal (N=7) and umbilical venous cord (N=6) blood. Plasma isolated from arterial blood was hemolyzed and was not included in analysis. Unbound tacrolimus concentrations were determined in maternal (N=7) and umbilical cord venous (N=5) plasma samples. Total and unbound tacrolimus concentrations in maternal, umbilical cord blood and plasma are presented in **Table 7.1**.

The mean tacrolimus concentration in maternal blood and venous umbilical cord blood was 9.0 ± 3.4 ng/mL and 6.6 ± 1.8 ng/mL, respectively; i.e., venous umbilical cord blood concentration of tacrolimus was, on average, 71 ± 18% of maternal blood concentrations (ranged 45–99%). In comparison, venous cord plasma concentrations were only 23 ± 11% of maternal plasma concentrations (ranged 12–44%). The percent unbound of tacrolimus in maternal plasma was 4.4 ± 2.8%, comparable to that in umbilical cord venous plasma (3.6 ± 0.8%). Accordingly, the ratio of unbound tacrolimus

concentration in venous cord plasma and maternal plasma was 0.19 ± 0.10 (ranged 0.09–0.36), which is about the same as with the ratio of total plasma concentrations.

The tacrolimus venous umbilical cord blood-to-plasma concentration ratios were on average 274% higher than the maternal blood-to-plasma concentration ratios (**Figure 7.2A**). $K_{bc/p}$, the tacrolimus partition ratio between blood cells and plasma, was calculated to be 75.4 ± 19.0 in maternal blood and 152.3 ± 46.2 in venous umbilical cord blood (**Figure 7.2B**).

The umbilical cord venous-to-maternal (UV/M, **Figure 7.1**) or arterial-to-venous (UA/UV, data not shown) drug concentration ratios did not vary with the time interval between dosing and sample collection. Arterial umbilical cord blood concentrations of tacrolimus were 100 ± 12% of venous concentrations (ranged 81–113%).

Of the four primary metabolites of tacrolimus, 13-DMT and 15-DMT could be measured in all fetal samples, while 31-DMT was quantifiable in limited samples. The UV/M blood ratios of 31-DMT, 13-DMT and 15-DMT were 0.47 ± 0.08, 0.67 ± 0.15 and 1.09 ± 0.45, respectively. Similar to the parent drug, venous umbilical cord plasma concentrations of 13-DMT and 15-DMT were only 13 ± 8% and 9 ± 7% of maternal plasma concentrations, respectively. The umbilical cord arterial/venous blood ratios of 13-DMT and 15-DMT were both 1.13 ± 0.10.

7.4.3 Excretion of Tacrolimus into Milk

The amount of tacrolimus excreted in the breast milk over a steady-state dosing interval was determined in one patient, treated with 1.5 mg of oral tacrolimus twice daily. Tacrolimus concentration in breast milk peaked later than blood and plasma (6 hours, 1

hour and 1 hour respectively). Peak and trough concentrations in milk were 1.11 and 0.78 ng/mL, respectively (**Figure 7.3A**). The average concentration of tacrolimus in milk was 0.93 ng/mL, comparable to previously reported values.(French et al., 2003; Gardiner and Begg, 2006) The breast milk-to-blood and milk-to-plasma AUC ratios in this subject were 0.13 and 2.89, respectively. Tacrolimus proportions unbound in milk and plasma were 3.7 ± 0.6% (N=3) and 2.7 ± 0.4% (N=3), respectively. The calculated unbound tacrolimus milk-to-plasma AUC ratio was 3.96 (**Figure 7.3B**).

The amount of tacrolimus excreted in milk over 24 hours was 32.0 ng, corresponding to a relative infant dose of 0.059% of the mother's weight-adjusted dose (54.4 μg/kg/d). The estimated tacrolimus dose that an average 3-month old exclusively breast-fed infant would ingest was 0.14 μg/kg/d, which is 0.3% of the mother's weight-adjusted dose. The mean concentration of 13-DMT in milk was 0.03 ng/mL (trough to peak concentration ranged from 0.01 to 0.04 ng/mL), which is much lower than that of parent drug. The other metabolites were under the limit of quantitation in breast milk.

7.5 Discussion

Fetal exposure to tacrolimus, a widely used immunosuppressant for pregnant transplantation recipients, has been evaluated in only a limited number of studies.(Jain et al., 1997) The current investigation characterizes whole blood, plasma and unbound tacrolimus concentrations in maternal and umbilical cord blood samples at delivery, providing new insights into the *in utero* exposure to tacrolimus. This study also presents a case report of an infant being breast-fed by a mother receiving tacrolimus-based immunosuppressive therapy.

Many factors are known to influence drug transfer across the placenta, including placental barrier permeability in relation to the physicochemical properties of the drug, facilitated or active drug transport, and drug biotransformation.(Hutson et al., 2011) Tacrolimus is lipophilic with a molecular weight of 804 Da. Although its molecular weight exceeds the cut off (500 Da) for facile diffusion across the placenta, some degree of passive diffusion is still expected.(Danesi and Del Tacca, 2004) For fetal development, the distribution, function and toxicity of tacrolimus in different organs, such as the interruption of T cell development at critical phases within the fetal thymus, potential functional changes in the fetal brain, renal insufficiency and neonatal hyperkalemia need to be considered.(Jain et al., 1997; Mckay and Josephson, 2008) Although National Transplantation Pregnancy Registry (NTPR) database analysis reported that prematurity occurred in 53% of 170 infants born to kidney transplant recipients taking tacrolimus, and 46% had low birth weight,(Coscia et al., 2010) the incidence of major malformations is not much higher than that in the general population.(Mckay and Josephson, 2008) Jain *et al.* concluded that complications such as renal insufficiency and hyperkalemia in the newborn were minor and congenital anomalies were rare.(Jain et al., 2003) Robert *et al.* reported no birth defects or noteworthy health problems in the offspring among solid organ transplant recipients in British Columbia.(Humphreys et al., 2012) Although it is clear that tacrolimus in maternal blood can pass through the placenta, the unbound tacrolimus concentration in cord blood was only ~20% of that found in maternal blood, which may explain the relatively low incidence of major fetal health problems with tacrolimus use in pregnancy.(Coscia et al., 2010)

Tacrolimus is a P-gp substrate(Saeki et al., 1993) and thus should be subject to the actions of the placental transporter, which serves to protect the fetus from exposure to xenobiotics in the maternal circulation by its active efflux action at the microvillous, maternal facing plasma membrane of the syncytiotrophoblast.(Audus, 1999) Indeed, the observed mean unbound drug concentration ratio between umbilical cord venous blood and maternal blood was 0.19 ± 0.10, indicative of efficient tacrolimus efflux activity. The placental transfer of cyclosporine A, another calcineurin inhibitor and P-gp substrate,(Saeki et al., 1993) has also been studied. Umbilical cord-to-maternal blood concentration ratio at delivery has been reported to be 0.2 at 6 hours after the last dose,(Bourget et al., 1990) 0.56 at 8 and 10 hours after the last dose,(Venkataramanan et al., 1988) and approximately 1 at 20 hour after the last dose.(Flechner et al., 1985) Although an unbound concentration ratio was not published, the data for cyclosporine, like tacrolimus, are consistent with fetal to maternal P-gp mediated drug efflux. Because the placenta is at its thinnest and the expression of P-gp is substantially decreased towards term, tacrolimus concentration in the fetal circulation in the third trimester may represent greater exposure compared to earlier gestational ages.(Sun et al., 2006; Hutson et al., 2011)

The differences observed in the venous umbilical cord to maternal concentration ratio based upon blood (0.71 ± 0.18), plasma (0.23 ± 0.11) and unbound drug (0.19 ± 0.10) measurements may be explained by greater partitioning of tacrolimus into blood cells and a higher hematocrit in cord blood, compared to the maternal blood. $K_{bc/p}$ expresses a drug's binding to constituents in RBCs and other blood cell types versus binding to plasma proteins (viz. albumin and α_1-acid glycoprotein).(Hinderling, 1997)

Previous studies have shown that RBC uptake accounts for >90% of the cellular fraction of tacrolimus in blood from healthy non-pregnant subjects. [Zheng *et al.*, Therapeutic Drug Monitoring] Hence, the 2-fold higher $K_{bc/p}$ value in venous umbilical cord blood compared to that in maternal blood most likely reflects a greater uptake of tacrolimus into fetal RBCs, compared to maternal RBCs. The biochemical mechanism of this difference in RBC to plasma partitioning is unclear. The absolute lymphocyte count is on average 2.7 times greater in human umbilical cord blood than in adult peripheral blood.(D'Arena et al., 1998) However, because tacrolimus associated with the lymphocyte fraction is very low (0.61%) compared to the erythrocyte fraction (83.2%),(Zahir et al., 2004) it cannot account for the 2-fold higher $K_{bc/p}$.

The concentration of tacrolimus in whole blood does not necessarily reflect its concentration at the intracellular targets within lymphocytes, the site of action for calcineurin inhibition.(Kelly et al., 1995; Ichimaru et al., 2001; Christians et al., 2002) *In utero* immunosuppression from maternal tacrolimus administration may be better correlated with the unbound tacrolimus concentration. In this and other studies, tacrolimus concentration in plasma has been shown to reflect unbound concentration better than whole blood concentration.(Minematsu et al., 2004) It has been demonstrated that the dose of tacrolimus required for a 50% inhibition of IL-2 release by T cells was 10-fold higher in cultures with RBC than without,(Ahmed et al., 2001) suggesting that fetal immunosuppression may be limited by greater drug RBCs partitioning and a higher hematocrit in cord blood.

Differential protein binding between the fetal and maternal circulations can be a critical factor in the placental transfer of drugs when it is the unbound drug that

equilibrates across the placenta.(Hill and Abramson, 1988; Hutson et al., 2011) The concentration of both albumin and α_1-acid glycoprotein, the plasma binding proteins of tacrolimus,(Piekoszewski and Jusko, 1993; Weiss et al., 2008) gradually increase throughout gestation in fetal plasma and vary across trimester in maternal circulation. (Krauer et al., 1984) However, we did not see a significant difference in the unbound fraction of tacrolimus between maternal and umbilical cord plasma. Similarly, Hutson *et al.* suggested that protein binding may not be a significant factor in establishing the umbilical cord-to-maternal concentration ratio at steady state when efflux transporters limit drug transfer.(Hutson et al., 2011)

Arterial and venous umbilical cord blood concentrations for tacrolimus were comparable, suggesting that fetal liver CYP3A7 does not play a quantitatively important role in fetal tacrolimus disposition. Studies of greater sample sizes are needed to further examine whether significant metabolism of tacrolimus, and local metabolite formation, occurs in the fetus.

The safety of tacrolimus for the nursing infant warrants special consideration.(Mckay and Josephson, 2008) The amount of tacrolimus excreted in breast milk in our case report was only 0.059% of the mother's weight-adjusted dose and for an exclusively breast-fed infant at 3-months of age, the estimated tacrolimus dose ingested via breast milk was estimated be 0.3% of the mother's weight-adjusted dose. This is consistent with a previous case report of a breast-fed baby receiving approximately 0.5% of the weight-adjusted maternal dose.(Gardiner and Begg, 2006) French *et al*.(French et al., 2003) reported that at 2.5 months of age, the infant was developing well both physically and neurologically. Armenti *et al*.(Armenti et al., 2003) reported five cases of

kidney recipients nursing their infants while receiving tacrolimus, with no reports of problems in the children. Our data indicate that the infant exposure to tacrolimus via breast milk is extremely low and should not lead to adverse effects in the nursing infant, although long-term follow up studies in these children have yet to be conducted. In the first neonatal week, however, clinicians can expect to see some residual tacrolimus in newborn blood samples from *in utero* exposure, which is much higher than the exposure via breast milk, particularly in the first week when ingested breastmilk volumes are relatively low.

The observation that the total milk-to-plasma and unbound milk-to-plasma AUC ratios were 2.89 and 3.96, consistent with the calculated milk-to-plasma ratio (2.2).(Koshimichi et al., 2011) These ratios suggest active transport of tacrolimus in the mammary gland and / or movement into milk through fat from the mammary gland. P-glycoprotein is located in the apical membrane of mammary epithelial cells, where it can actively extrude a variety of compounds.(Breedveld et al., 2006) P-gp mRNA (Alcorn et al., 2002b; Alcorn et al., 2002a) and protein expression are detected in human mammary gland epithelial cells, but the protein expression levels are thought to be too low to support quantifiable drug transporting activity.(Ito and Alcorn, 2003) Jeffrey *et al.* demonstrated protein expression of P-gp in lactating rat mammary tissue, but concluded that it does not have a significant role in the secretion of nelfinavir into rat milk. Nonetheless, the unbound milk to plasma concentration ratio data are most consistent with some form of mammary efflux activity, mediated by P-gp or other unidentified transporters.

7.6 Conclusions

The *in utero* fetal blood exposure of tacrolimus is on average 71% of the maternal exposure at term, while the mean fetal plasma and unbound drug concentrations are approximately one-fifth of the respective maternal concentrations. These observations may be explained in part by P-gp function at the placenta, by greater red blood cell partitioning and higher hematocrit levels in the venous cord blood. There is no clear evidence of significant fetal tacrolimus metabolism. In addition, the neonatal drug exposure to tacrolimus in breast milk is very low and likely does not represent a health risk to the breastfeeding infant.

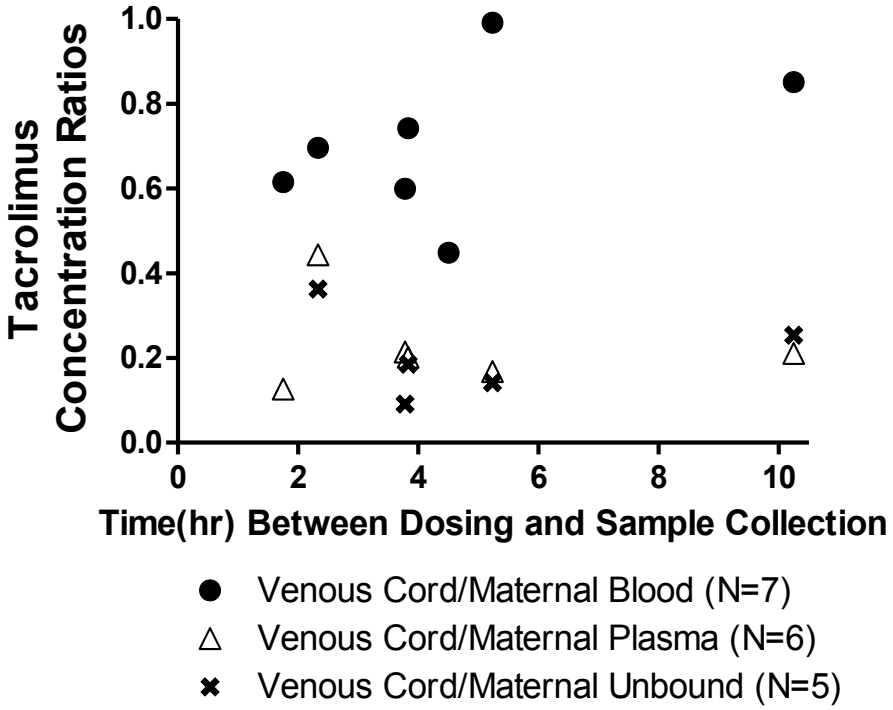


Figure 7.1 Tacrolimus venous umbilical cord-to-maternal blood, venous umbilical cord-to-maternal plasma and venous umbilical cord-to-maternal unbound drug concentration ratios in 8 patients, taking tacrolimus at the time of delivery.

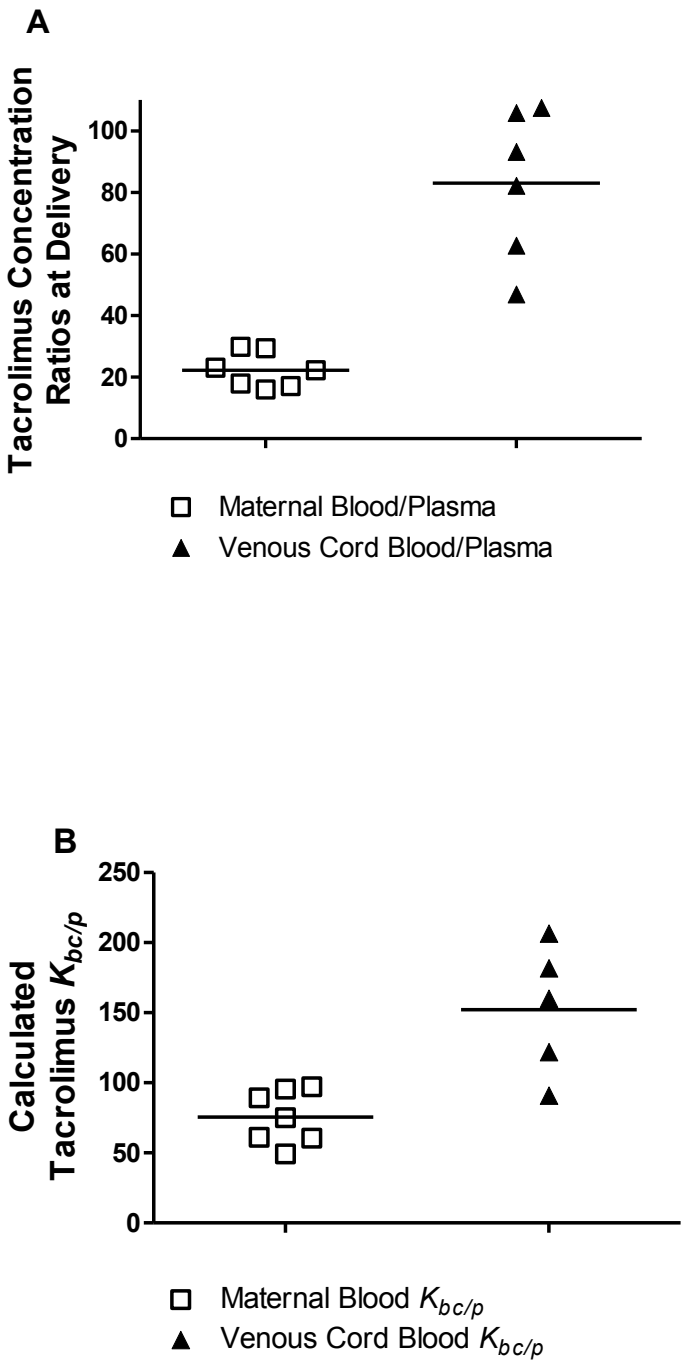


Figure 7.2 Tacrolimus maternal blood-to-plasma, venous umbilical cord blood-to-plasma concentration ratios in 8 patients, taking tacrolimus at the time of delivery (A) and the calculated $K_{bc/p}$, the tacrolimus partitioning ratio between blood cells and plasma, in maternal blood and venous umbilical cord blood (B).

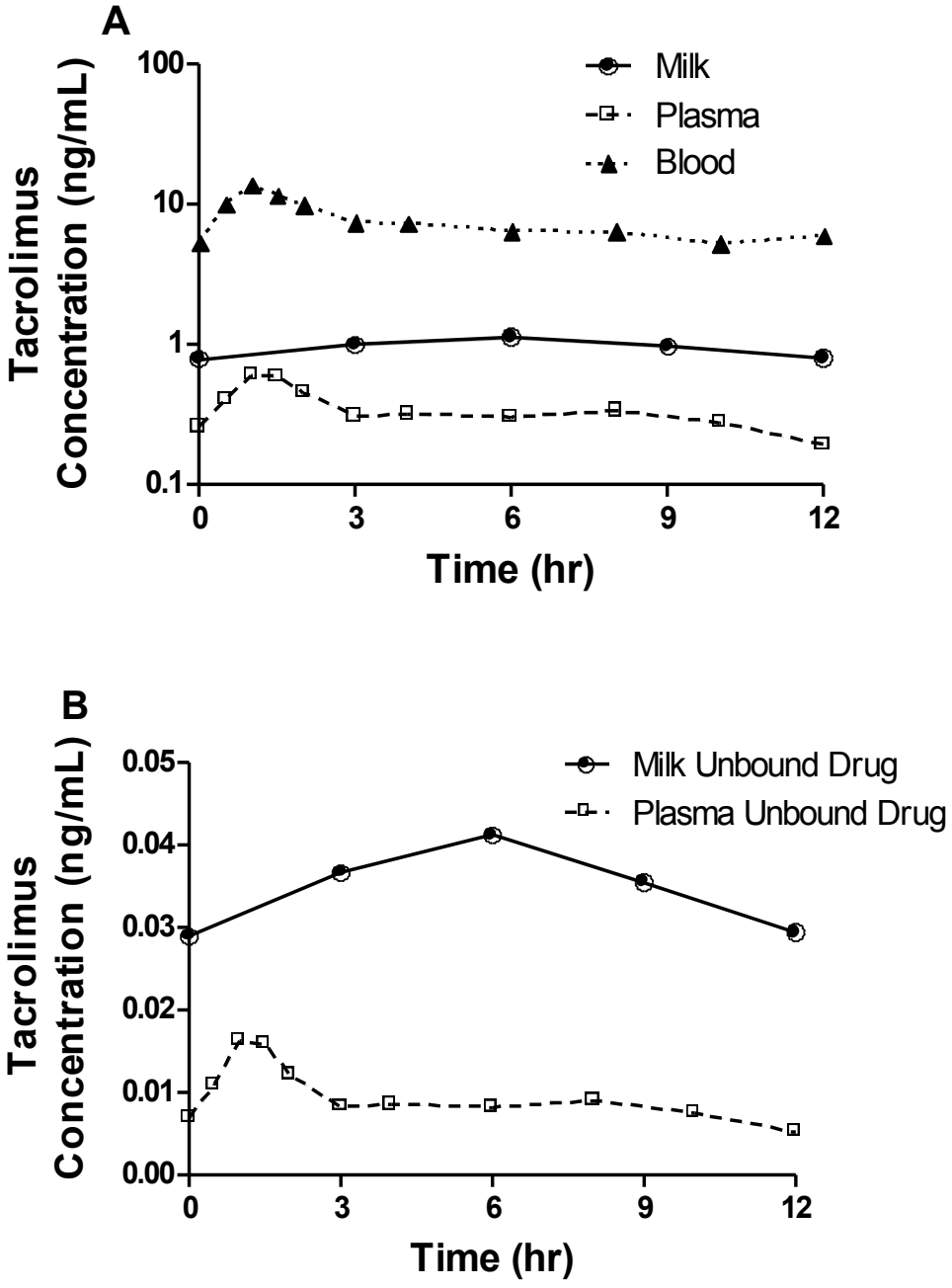


Figure 7.3 Tacrolimus concentration in maternal blood, plasma and breast milk (A) and steady state unbound tacrolimus concentrations in maternal plasma and breast milk (B) of one subject over a single dosing interval. The subject was treated with 1.5 mg of tacrolimus twice daily for immunosuppression.

Table 7.1 Tacrolimus concentrations (ng/mL) and concentration ratios in maternal, umbilical cord blood and plasma samples.

Concentration	Maternal Venous	Umbilical Cord Venous	Umbilical Cord Arterial
Blood	9.0 ± 3.4	6.6 ± 1.8	5.7 ± 1.0
Plasma	0.40 ± 0.20	0.09 ± 0.04	
Unbound drug	0.017 ± 0.010	0.003 ± 0.001	
Ratio	Venous Umbilical Cord/Maternal	Umbilical Cord Arterial/Venous	
Blood	0.71 ± 0.18	1.00 ± 0.12	
Plasma	0.23 ± 0.11		
Unbound drug	0.19 ± 0.10		

Chapter 8

Summary

The calcineurin inhibitors (CNI) — cyclosporine A (CsA) and tacrolimus remain the backbone of immunosuppression therapy for most organ transplant patients despite their many serious side effects, such as chronic calcineurin inhibitor nephrotoxicity (CNIT) (Staatz et al., 2010a). The accumulation of CNIs and their metabolites in the renal tissue is affected by both systemic and local CNIs metabolism and disposition, which may play important roles in defining an individual's susceptibility to CNIT (Staatz et al., 2010b). CNIs are substrates for CYP3As and P-glycoprotein (Saeki et al., 1993; Dai et al., 2004; Dai et al., 2006). Genetic and environmental factors affect the activity of these proteins and contribute to the high degree of inter-individual variability in CNI clearance.

CYP3A5 is the predominant CYP3A isoform in renal tubular cells, with primarily an intracytoplasmic localization (Murray et al., 1999; Metalidis et al., 2011). It was hypothesized that increased renal CYP3A5 expression protects the kidney against CNIT by enhanced metabolism of the drug and reducing local CNI accumulation (Staatz et al., 2010b; Metalidis et al., 2011). However, it was also hypothesized that proximal tubular CYP3A5 activity towards CNIT could result in the local production of toxic metabolites and increase nephrotoxicity risk (Kuypers et al., 2010; Metalidis et al., 2011). Precedence exists for the local production of a toxic metabolite that leads to nephrotoxicity; namely, conversion of the anti-cancer drug ifosfamide to chloroacetaldehyde, which is the primary contributor to ifosfamide-induced nephrotoxicity (McCune et al., 2005). In addition, the active unbound form of CNIs may be affected by physiological changes, such as those induced by pregnancy, that influence their binding properties to plasma and cellular components such as albumin and erythrocytes.

The first objective of my dissertation project was to investigate the impact of polymorphic *CYP3A5* expression on the *in vivo* metabolism of CNIs and to evaluate the hypothesis that *CYP3A5* genotype (vis-à-vis the inferred enzyme expression) affects intrarenal CNI and metabolite accumulation (**Chapter 2 and 3**). The second objective was to characterize the impact of physiological changes that occur during pregnancy on tacrolimus pharmacokinetics and to evaluate *in utero* and postnatal tacrolimus exposure in offspring of mothers who were transplant recipients receiving tacrolimus therapy (**Chapter 5, 6 and 7**). A series of *in vitro* metabolic experiments were also conducted (**Chapter 4**) to aid in the interpretation of *in vivo* data described in the other chapters.

To assess the pharmacokinetic basis for a difference in the occurrence of CNIT between homozygous *CYP3A5*3/*3* individuals (CYP3A5 nonexpressors) and *CYP3A5*1* allele carriers (CYP3A5 expressors), an oral dose of CsA and tacrolimus was administered sequentially to 24 healthy participants who were selected based on their *CYP3A5* genotype, after a washout period of at least 2 weeks. *ABCB1* SNPs/haplotype was also identified to determine if they constitute a significant co-variate of the urinary clearance of CsA and tacrolimus.

For tacrolimus (**Chapter 2)**, compared to CYP3A5 nonexpressors, expressors had a 1.6-fold higher oral tacrolimus clearance and 2.0- to 2.7-fold higher metabolite/parent AUC ratios for 31-DMT, 12-HT and 13-DMT. In addition, the apparent urinary tacrolimus clearance was 36% lower in CYP3A5 expressors, compared to nonexpressors. To explore the mechanism behind this observation, we developed a semi-physiological model of renal tacrolimus disposition, which demonstrated that the apparent urinary tacrolimus clearance can be used as a surrogate marker of intrarenal drug metabolism.

The model predicted that, with chronic drug therapy, tacrolimus exposure in the renal epithelium of CYP3A5 expressors should be 53% of that for CYP3A5 nonexpressors at comparable blood tacrolimus AUCs. One predicted consequence of CYP3A5-dependent intrarenal tacrolimus metabolism is a reduced risk of tacrolimus-induced nephrotoxicity following solid organ transplantation, provided renal tacrolimus concentration is the major driver of toxicity. However, the significantly increased circulating concentrations of tacrolimus metabolites in renal CYP3A5 expressors, particularly the most pharmacologically active metabolite, 31-DMT, may counteract any protective role that a reduction in renal tacrolimus concentration might confer. However, one caveat to this interpretation is the observation that the metabolic clearance of 31-DMT by CYP3A5 is comparable to that of tacrolimus (**Chapter 4**, **Figure 4.1**) and thus effective elimination of the potentially toxic metabolite in CYP3A5 expressors may have a protective effect on renal function. Further prospective studies investigating the impact of *CYP3A5* genotype on tacrolimus nephrotoxicity would help to clarify this issue. In such a study it would be important to identify the *CYP3A5* genotype(s) of the recipients and their donor kidneys, as well as tacrolimus metabolite concentrations in blood and renal tissue.

For CsA (**Chapter 3)**, compared to CYP3A5 nonexpressors, expressors had a comparable oral CsA clearance while the average blood AUC for AM19 and AM1c9 was 47.4% and 51.3% higher in CYP3A5 expressors, corresponding to 30% higher $AUC_{metabolite}/AUC_{CsA}$ ratios for AM19 and AM1c9 in CYP3A5 expressors. Higher systemic levels of AM19 and AM1c9 in CYP3A5 expressors should enhance entry of these metabolites into the renal tubular cells either by secretion from the efferent arteriole or after reabsorption from the luminal side following glomerular filtration. In individuals

with significant renal CYP3A5 expression, one might expect higher intrarenal accumulation of AM19 and AM1c9, independent of an effect of intestinal and hepatic *CYP3A5* genotype on systemic accumulation of the secondary metabolites. Because higher concentrations of AM19 and AM1c9 have been previously associated with nephrotoxicity in organ transplant patients in a series of clinical studies, such a difference in AM19 and AM1c9 formation between CYP3A5 expressors and nonexpressors might affect the risk of nephrotoxicity. However, the impact of a higher level of nephrotoxic secondary metabolites might be counteracted by lower intrarenal levels of CsA. As presented, the mean apparent urinary CsA clearance was 20.4% lower in CYP3A5 expressors, which is suggestive of CYP3A5-dependent intrarenal CsA metabolism based on the semi-physiological model developed for tacrolimus renal disposition in **Chapter 2.** Future studies that test the robustness of the renal drug metabolism and disposition model can be carried out by fitting the model to CsA urinary excretion data. In that case, it will be interesting to compare the parameter estimates from the CsA model fitting to those reported in **Chapter 2**.

Although the development and application of the semi-physiological based model of renal CNI metabolism improved our understanding of intra-renal CNI disposition, it also generated some anomalous results. For example, the unbound fraction of tacrolimus in whole blood (f_{ub} , 0.078 ± 0.026%), derived from our *in vitro* measurements of unbound fraction in plasma (f_{up}, 2.1 ± 0.8%) and whole blood-to-plasma distribution ratio (27.7 ± 5.0), was about 6.5 times higher than the *in vivo* derived f'_{ub} of 0.012 ± 0.011% estimated from Least-Square Minimization in modeling without assigning any prior f_{ub} values. Thus, our *in vitro* measurements of unbound fraction in plasma (f_{up}, 2.1 ± 0.8%)

determined by ultracentrifugation may not define the true plasma water tacrolimus concentration. Ultracentrifugation does not spin down all of the plasma components that tacrolimus can bind to; thus, the concentration measured in the apparent plasma "water" may include bound tacrolimus. Of note, an alternative f_{ub} (=0.044%) calculated based on f_{up} (1.20 ± 0.12%) for tacrolimus (Zahir et al., 2001), was only 3.7 times that of our model predicted f_{ub}. Going forward, it would be interesting to determine the f_{ub} for CsA experimentally and compare it to the model predicted value. Further evaluation of the semi-physiological model for renal disposition and metabolism of tacrolimus would involve the simultaneous fitting of tacrolimus metabolite urine excretion data. Because the unbound fraction of tacrolimus metabolites can be determined, this model fitting exercise could provide greater mechanistic understandings of how f_{ub} affects the urinary clearance of CNIs. Theoretically, the parameter estimates generated for the metabolites can be compared to those from tacrolimus model fitting results, thus providing some evaluation and comparison of their potential for renal secretion and reabsorption.

To fully interpret the CsA pharmacokinetic data, we evaluated the formation pathways of AM19 and AM1c9 by *in vitro* incubations of CsA, AM1, AM9 and AM1c with recombinantly expressed CYP3A4 and CYP3A5 (**Chapter 4**, **Figure 4.9**). The results suggested that the predominant source of AM19 and AM1c9 occurs through respective conversion of AM1 and AM1c to the secondary metabolites, which are reactions that can be catalyzed efficiently by both CYP3A4 and CYP3A5. AM9 does not contribute significantly to the formation of AM19 in CYP3A5 expressors, which is a pathway mediated by CYP3A5.

Finally, our analysis of *ABCB1* genotype/haplotype showed that they did not constitute a significant co-variate in determining the urinary clearance of tacrolimus and CsA. However, the study was not powered to test for such a contribution. The lack of a significant difference in *ABCB1* genotype/haplotype frequencies between the two CYP3A5 phenotype groups suggests that there was no uncontrolled bias in our interpretation of the CYP3A5 genotype association, but it does not address the question of whether or not P-gp function (and *ABCB1* genotype/haplotype) influences the intra-renal disposition of CNIs and their metabolites. A far larger number of subjects would need to be studied to fully address the question of a multigene pharmacokinetic trait.

In summary, findings presented in **Chapters 2, 3 and 4** demonstrate that, with chronic, stable CNI dosing, intrarenal accumulation of CNIs and their metabolites (AM19 and AM1c9 for CsA; 31-DMT, 12-HT and 13-DMT for tacrolimus), will depend on the *CYP3A5* genotype of the liver and kidneys. This may contribute to inter-patient differences in the risk of CNIT.

For the second dissertation objective, blood, plasma and urine samples were collected over one steady-state dosing interval from women treated with oral tacrolimus during early to late pregnancy (n = 10) and postpartum (n = 5). Maternal and umbilical cord samples were obtained at delivery from some of the study participants (n = 8). Also, tacrolimus distribution into breast milk was evaluated in one subject. Total and unbound tacrolimus as well as metabolite concentrations in blood, plasma and milk were assayed. A mixed effect linear model was used for comparison across gestational age, using postpartum as the reference group.

As shown in **Chapter 5**, the mean oral tacrolimus blood clearance (CL/F) was 39% higher during mid- and late-pregnancy compared to postpartum. Tacrolimus free fraction increased by 91% in plasma (f_P) and by 100% in blood (f_B) during pregnancy. The increased f_P was inversely associated with serum albumin concentration ($r = -0.7$), which decreased by 27% during pregnancy. Pregnancy related changes in f_P and f_B contributed significantly to the observed gestational-age dependent increase in tacrolimus whole blood CL/F ($r^2 = 0.36$ and 0.47 respectively). In addition, tacrolimus whole blood CL/F was inversely correlated with both hematocrit and red blood cell counts, suggesting that binding of tacrolimus to erythrocytes restricts its availability for metabolism. For these allograft recipients, the treating physician increased the tacrolimus dosage during pregnancy by an average of 45% in order to maintain tacrolimus whole blood trough concentrations in the therapeutic range. This led to a marked increase in unbound tacrolimus trough concentrations and unbound AUC, by 112% and 173%. In normal pregnancies, a substantial increase in creatinine clearance is expected. Given the doubling of unbound tacrolimus concentrations observed, the expected increase in creatinine clearance during mid-/late-pregnancy (115.2 ± 54.3 mL/min), as compared to postpartum (129.8 ± 52.2 mL/min), was not observed. This anomaly may reflect tacrolimus nephrotoxicity.

As discussed in **Chapter 6,** if the decision is made to maintain whole blood trough concentrations in the usual therapeutic range during pregnancy, it must be recognized that unbound concentrations are likely to be higher in hypoalbuminemic and/or anemic patients than what existed prior to pregnancy. For these patients, tacrolimus toxicity should be monitored closely. The choice of target whole blood

concentrations should take into account the individual patient's history, current medical conditions, concomitant medications and the potential impact of high or low unbound concentrations. As an alternative, monitoring of tacrolimus plasma concentrations during pregnancy would remove one of the key variables (RBC count) that can confound the interpretation of tacrolimus concentrations. Utilizing an assay for unbound tacrolimus concentrations would remove both factors (RBC count and low plasma proteins), but is more costly and time-consuming.

With respect to future studies, multiple reports have shown that the concentration of tacrolimus in whole blood does not necessarily reflect its concentration at the intracellular targets within lymphocytes, the postulated site of action for calcineurin inhibition (Kelly et al., 1995; Ichimaru et al., 2001; Christians et al., 2002). Capron et al. observed that the tacrolimus level in human liver biopsies showed no correlation with blood concentrations (Capron et al., 2007). *Plasma* may better reflect the drug concentration available for cellular diffusion and distribution than blood (Minematsu et al., 2004). The quantification of changes in IL-2 production in stimulated blood may provide mechanistic understandings of the impact of increased unbound fraction on the pharmacodynamic effects of tacrolimus. Measurement of the intra-lymphocytic tacrolimus concentrations should also provide more insight towards the disposition and distribution of tacrolimus within different blood cell components. Our characterization of the pharmacokinetics of tacrolimus during pregnancy have provided important rationale for conducting new studies that are aimed to characterize both the intra-lymphocytic tacrolimus concentrations and IL-2 production in stimulated blood. It will also be important to quantify the changes in P-gp expression on the cell membrane of

lymphocytes during pregnancy. One plausible hypothesis is that the intra-lymphocytic tacrolimus concentrations may remain unchanged, despite higher unbound extracellular concentrations, if P-gp expression on the cell membrane of lymphocytes (and CNI efflux) increases during pregnancy.

Regarding *in utero* and neonatal tacrolimus exposure, mean (±SD) tacrolimus blood concentrations at the time of delivery in the umbilical cord vein (6.6 ± 1.8 ng/mL) were 71 ± 18% (range 45–99%) of maternal blood concentrations (9.0 ± 3.4 ng/mL). The mean fetal plasma (0.09 ± 0.04 ng/mL) and unbound drug concentrations (0.003 ± 0.001 ng/mL) were both approximately one fifth of the respective maternal concentrations. Lower umbilical cord than maternal concentrations may be explained in part by placental P-gp function or higher hematocrit levels and greater red blood cell partitioning in the venous cord blood than in maternal blood. Although it is clear that tacrolimus in maternal blood can pass through the placenta, unbound tacrolimus concentration in cord blood is only ~20% of that found in maternal blood. This may explain the relatively low incidence of major fetal health problems with tacrolimus use in pregnancy (Coscia et al., 2010). Future studies that evaluate our hypothesis of greater tacrolimus red blood cell partitioning in cord blood based on classical model calculations (**Chapter 7**, **Figure 7.2)** are warranted.

It was also noted that arterial umbilical cord blood concentrations of tacrolimus were 100 ± 12% of venous concentrations, which is consistent with *in vitro* findings that CYP3A7 is much less efficient at metabolizing tacrolimus than CYP3A4 and CYP3A5 (**Chapter 4**, **Figure 4.2**). Also, as shown in Figure 4.2, at low tacrolimus concentrations, there was significant depletion of 31-DMT by CYP3A5, but not by CYP3A7, although

the formation rates of 31-DMT by CYP3A5 and CYP3A7 were comparable (**Table 4.1**). Because of the abundant expression of CYP3A7 in the fetal liver, one could speculate that 31-DMT may accumulate to a greater extent in the fetal liver containing only CYP3A7 compared to those with both CYP3A7 and CYP3A5 expression. Fetal liver microsomes prepared from liver tissues with only CYP3A7 and with both CYP3A7 and CYP3A5 expression could be used to further characterize 31-DMT formation and elimination in these two phenotype groups. Additionally, the hypothesis may be evaluated by measuring 31-DMT concentrations in fetal liver tissues.

Finally, infant exposure to tacrolimus through the breast milk was less than 0.3% of the mother's weight-adjusted dose and is not likely to pose a health risk to the breastfeeding infant. Notably, the unbound milk to plasma concentration ratio data (3.96) are most consistent with some form of mammary efflux activity, mediated by P-gp or other unidentified transporters.

In conclusion, this dissertation research further enhanced our understanding of the contribution of both genetic and physiological factors to inter-individual differences in the metabolism and pharmacological effects of calcineurin inhibitor therapy. A full elucidation of the pharmacogenomics of CNIT may lead to improved management of tacrolimus pharmacotherapy. Based on results presented in this dissertation, we hypothesize that the accumulation of tacrolimus in the kidney of organ transplant patients will depend on the genotype of the kidney. This will be tested in an ongoing study (not part of this dissertation) by quantifying the tissue and blood levels of tacrolimus in kidney transplant patients and correlating those findings with the *CYP3A5* genotype of the kidney. We predict that that the kidney/blood tacrolimus concentration ratio will be

higher in CYP3A5 nonexpressors, compared to that for CYP3A5 expressors. Results from that study should further improve our understanding of the contribution of inter-individual differences in CYP3A5 function to the intrarenal metabolism and toxicity of tacrolimus following long-term drug use. If there is apparent benefit from knowing the donor *CYP3A5* genotype for managing a kidney transplant recipient, then future studies applying this technique may result in improved kidney transplant outcomes. Ultimately, measuring the expression of CYP3A5 enzyme in the donor organ, at baseline, or in protocol biopsies, may better predict the individual risk of developing CNIT and guide immunosuppressive therapy (Metalidis et al., 2011).

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VITA

Songmao Zheng was born in P. R. China. He graduated from Sichuan University with a Bachelor of Science degree in Biological Sciences in 2007, during which he had a one year exchange, under the guidance of Dr. C. Anthony Blau at the University of Washington (UW).

He joined the Department of Pharmaceutics at the UW in the fall of 2007. During his graduate training, he received an Amgen travel award to the AAPS National Biotechnology Conference for his internship project on PK/PD Modeling of Antibody Drug Conjugates with Dr. Baiteng Zhao at Seattle Genetics. He also received an AAPS PPDM travel award to the AAPS Annual Meeting for his thesis work on CYP3A5 and *in vivo* tacrolimus metabolism. For his work on pregnancy and tacrolimus disposition led by Dr. Mary F. Hebert, he attended the 8th Annual IHDCYH Summer Institute in Maternal-Fetal and Pediatric Pharmacology.

Under the advisorship of Dr. Kenneth E. Thummel, he earned a Doctor of Philosophy degree in 2012.