PHARMACOKINETICS, LIMITED SAMPLING STRATEGIES, AND PHARMACOGENETICS OF MYCOPHENOLIC ACID IN THORACIC TRANSPLANT RECIPIENTS

by

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ABSTRACT

Mycophenolic acid (MPA), the active metabolite of mycophenolate mofetil, is an immunosuppressive agent known to exhibit wide inter-patient pharmacokinetic variability. The metabolism and transport of MPA and the phenolic (MPAG) and acyl (AcMPAG) glucuronides are mediated by UDP-glucuronosyltransferases (UGTs) and multidrug resistance-associated protein 2 (MRP2/ABCC2), respectively. Increasing evidence supports monitoring MPA area-under-the-concentration-time-curve; however, it is impractical and costly to implement. The objectives of this clinical study were to characterize MPA pharmacokinetics, develop MPA limited sampling strategies for estimating MPA exposure, and assess contribution of *UGT* and *ABCC2* genetics to MPA pharmacokinetics and clinical outcomes in thoracic transplant recipients.

Seventy thoracic (36 lung, 34 heart) transplant recipients were recruited. Eleven blood samples were obtained over a 12-hour dosing period at steady state. Plasma concentrations of MPA, MPAG, AcMPAG, and free MPA were measured by a high performance liquid chromatography-ultraviolet detection method, and conventional dose-normalized pharmacokinetic parameters were determined via non-compartmental methods. Limited sampling strategies were developed in 64 subjects by stepwise multiple regression analysis using the index group data, and tested in the validation group to determine bias and precision. Genetic polymorphisms in *UGT* and *ABCC2* were determined by sequencing and their contributions to pharmacokinetic variability were investigated in 68 thoracic transplant recipients using multivariate analysis.

Significantly lower MPA pharmacokinetic exposure was observed in lung transplant recipients (compared with heart), and in patients taking cyclosporine

(compared with tacrolimus). There was wide inter-patient variability of MPA, MPAG and AcMPAG pharmacokinetics. The best limited sampling strategies for both transplant populations were developed from the lung transplant group data; equations (Log C1.5, C2) and (Log C0, Log C1.5) were the best candidates for the heart and lung transplant population, respectively. For both transplant groups, *UGT2B7* variants 802T (*2a) and -138A (*2g) were associated with increased AcMPAG AUC (~3-fold) and AcMPAG/MPA (~10-fold) in multivariate analyses, respectively, and high AcMPAG exposure and metabolic ratio were associated with poor clinical outcomes.

UGT2B7 is a promising gene candidate that may influence MPA pharmacokinetics clinically; however, larger clinical pharmacogenetic studies in thoracic transplant subpopulations are warranted to corroborate the role of AcMPAG and *UGT2B7* variants in optimizing mycophenolate therapy.

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LIST OF ABBREVIATIONS

°C	degree Celsius
μg	microgram
μL	microliter
μmol	micromole
AcMPAG	acyl glucuronide of mycophenolic acid
AUC	area-under-the-concentration-time-curve
Cl/F	apparent clearance
Cmax	maximum concentration
Cmin	minimum concentration
CSA	cyclosporine
Cx	concentration taken at time x
DN	dose-normalized
DNA	deoxyribonucleic acid
EHC	enterohepatic recirculation
fMPA	free MPA
g	gram
GI	gastrointestinal
HPLC-UV	high performance liquid chromatography with ultraviolet detection
hr	hour
IMPDH	inosine monophosphate dehydrogenase
IS	internal standard
Kg	kilogram

L	liter
LSS	limited sampling strategy
ME	mean prediction error
mg	milligram
min	minute
mL	milliliter
MMF	mycophenolate mofetil
MPA	mycophenolic acid
MPAG	7-O-mycophenolic acid glucuronide
MRP2/ABCC2	multidrug resistance-associated protein 2
N or n	sample size
N/A	not available / not applicable
OATP/SLCO	organic anion transporting polypeptide
PD	pharmacodynamic
PG	pharmacogenetic
PK	pharmacokinetic
r	correlation coefficient
r^2	coefficient of determination
RMSE	root mean square error
SD	standard deviation
SRL	sirolimus
TAC	tacrolimus
TDM	therapeutic drug monitoring

Tmax	time to reach Cmax
UBC	University of British Columbia
UGT	UDP-glucuronosyltransferase
Vd/F	apparent volume of distribution
y	year(s)

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CO-AUTHORSHIP STATEMENT

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1. INTRODUCTION

1.1. Heart and Lung Transplantation

Heart transplantation is an aggressive treatment option for individuals with end-stage heart failure. The most common indications for heart transplantation include coronary heart failure, cardiomyopathy, refractory heart failure, myocardial infarction, valvular heart disease, and congenital heart disease.[1, 2] In Canada, 181 cardiac transplants were performed in 2007, and about 100 people remained on the waiting list.[3] Although patient and graft survival rates are still inferior to kidney transplantation, the patient 5-year survival rate for heart transplants has improved in recent years to about 80%.[2] Chronic cardiac rejection, characterized by allograft vasculopathy and associated graft failure, malignancies and infections are the major causes of mortality in heart transplant recipients.[1, 2, 4]

Lung transplantation has been an effective but aggressive treatment for end-stage lung diseases. Major indications for lung transplantation are emphysema, chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis, alpha-1-antitrypsin deficiency, primary pulmonary hypertension, and cystic fibrosis.[5-8] Indications for single-lung transplantation include chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis and emphysema. Bilateral-lung transplants are usually performed in patients with cystic fibrosis and primary pulmonary hypertension.[6, 7] In Canada, 172 single and bilateral lung transplants were performed in 2007, with over 200 people remaining on the waiting list.[3] Despite improvements in immunosuppression, surgical procedures and patient care, patient and graft survival rates are still relatively low compared to other

transplant populations. Chronic rejection, characterized by bronchiolitis obliterans syndrome, is a prominent problem that is responsible for patient morbidity and mortality.[9-11] The 5-year survival rate for lung transplant recipients is about 50%, with mortality attributed to bronchiolitis obliterans syndrome, acute rejection, and infections.[7, 12] Various factors may contribute to the challenges in managing lung transplant complications. The lungs are constantly in contact with the external environment, with increased exposure to allergens and antigens that leads to higher chances of infections. Large organ size and the presence of numerous antigen-presenting cells in donor organ also contribute to the poorer prognosis.[9, 10]

Heart and lung organ transplantation has become a manageable treatment option for end-state organ failure with greatly improved outcomes over the years.[12, 13] Since acute rejection is the main predictive factor, possibly the cause, of chronic rejection of heart and lung transplant,[12-14] insufficient immunosuppression always portends negative transplant outcomes. Effective maintenance immunosuppressive regimens are crucial in achieving positive long-term transplant outcomes. However, in addition to management of allograft chronic rejection, post-transplant morbidities pose extra challenges in heart and lung transplantation. Hypertension, hyperlipidemia, diabetes, renal insufficiency and malignancy are common co-morbidities in both heart and lung transplant groups, most of which are secondary to immunosuppressive drugs.[2, 7, 15] Other adverse effects and complications such as gastrointestinal toxicities and infections also impact negatively on patients' quality of life. Since immunosuppressive therapy is a life-long commitment for transplant recipients and treatment responses vary greatly between patients, finding the

delicate balance of an effective and safe maintenance immunosuppressive regimen suited for each individual remains the key challenge in transplantation.

1.2. Maintenance Immunosuppressive Agents

Maintenance immunosuppressive therapy combines agents with different mechanisms of action in inhibiting the activation and proliferation of T-lymphocytes, which is the key component in the cellular-mediated immune response against the allograft.[12, 14] Inhibition of cytokines such as interleukin-2 is a major pathway of deterring T-lymphocyte proliferation, and is the target of calcineurin and mammalian target of rapamycin (mTOR) inhibitors.[16, 17] Cyclosporine (CSA, Section 1.2.1) and tacrolimus (TAC, Section 1.2.2) are the two calcineurin inhibitors widely used in transplantation. Calcineurin is a calcium ion-dependent enzyme that is activated when T-lymphocytes are simulated. Calcineurin in turn de-phosphorylates nuclear factors such as NF-AT, which induces gene transcription of interleukin-2, completing the cycle in promoting differentiation and proliferation of T-lymphocytes.[16, 17] Sirolimus (SRL, Section 1.2.3) inhibits the kinase mTOR, which promotes cell propagation after receiving the cytokine (interleukin-2) stimulatory signal.[18] Mycophenolic acid (MPA, Section 1.2), the active moiety of mycophenolate mofetil (MMF), is an anti-metabolite that disrupts the cell-cycle by inhibiting *de novo* purine synthesis, thereby depleting guanine nucleotide supplies necessary for DNA replication and cell division.[17, 18] Corticosteroids (Section 1.2.4), the oldest immunosuppressive agents, bind to glucocorticoid receptors, and exhibit anti-inflammatory action by inhibiting the transcription of cytokines.[17]

A triple therapy consisting of a calcineurin inhibitor (CSA or TAC), an anti-metabolite (MPA), and corticosteroid, is the most common maintenance immunosuppressive regimen in heart or lung transplant recipients.[2, 7, 10, 15, 19] Tacrolimus is now the preferred calcineurin inhibitor to CSA, and MPA is now the main anti-proliferative agent used in heart and lung transplantation.[2, 7] The combination of TAC and MMF with or without steroids is used in about 50% of heart and 40% of lung transplant recipients, whereas a combination of CSA and MMF with or without steroids is used in about 30% of heart and 10% of lung transplant recipients.[2, 7] Sirolimus has also been incorporated in maintenance therapy in place of calcineurin inhibitors in recent years in about 10% of thoracic transplant recipients.[2, 7, 10]

1.2.1. Cyclosporine

Cyclosporine is a lipid-soluble cyclic polypeptide that originates from the fungus *Tolypocladium inflatum Gams*. The introduction of cyclosporine in the early 1980s has greatly improved transplant outcomes, allowing many patients to benefit from transplantation.[18, 20] Cyclosporine exerts its immunosuppressive effects by binding to the protein cyclophilin; the CSA-cyclophilin complex then binds to, and inactivates, the protein phosphatase calcineurin, thereby inhibiting the transcription of interleukin-2 and subsequently T-lymphocyte proliferation.[10, 17, 21, 22]

The original formulation Sandimmune® (Novartis Pharmaceuticals Corporation, East Hanover, NJ) has highly variable oral bioavailability and unpredictable pharmacokinetics; a newer microemulsion formulation, Neoral® (Novartis Pharmaceuticals Corporation, East Hanover, NJ), was developed and has improved oral bioavailability with more consistent pharmacokinetic properties.[16, 21, 22] Cyclosporine is

absorbed in the upper gastrointestinal (GI) tract, extensively distributed into tissues, and highly bound to lipoproteins (>90%) and erythrocytes; whole blood is therefore used in therapeutic drug monitoring of CSA.[21, 23] Cyclosporine is extensively metabolized by cytochrome P450 (CYP) 3A enzymes, primarily CYP3A4,[18] to over 30 metabolites, and is also subject to pre-systemic metabolism by intestinal CYP3A enzymes. Cyclosporine is also a known substrate and inhibitor of P-glycoprotein (encoded by the *ABCB1* gene) and an inhibitor of the multidrug resistance-associated protein 2, encoded by the *ABCC2* gene;[18, 21, 24, 25] there is therefore great potential for drug-drug interaction of CSA with other medications at both the metabolism and transport levels. Cyclosporine metabolites are eliminated mainly through the biliary route.[21, 23]

Major adverse effects associated with CSA are nephrotoxicity, hypertension, hyperlipidemia, diabetes mellitus, neurotoxicity and GI disturbances.[10, 17, 23] Nephrotoxicity is the main limitation of calcineurin inhibitor-based immunosuppressive regimens, and has prompted recent trends of rigorous therapeutic drug monitoring and calcineurin-sparing regimens.[6,10] Recently, it was suggested that CSA trough concentration does not correlate well with total drug exposure; on the contrary, monitoring of concentration at two hours post-dose (C2) has been advocated to be a superior predictor of CSA exposure, and is becoming the standard practice of CSA monitoring.[21, 26, 27]

1.2.2. Tacrolimus

Tacrolimus is another commonly used calcineurin inhibitor; it is a macrolide lactone isolated from the fungus *Streptomyces tsekubaensis*. Similar to CSA, TAC exerts its immunosuppressive effects via inhibition of calcineurin. Tacrolimus binds to the

intracellular FK-binding protein, and this TAC-protein complex binds to and inactivates calcineurin, thereby inhibiting subsequent transcription of pro-inflammatory interleukins.[28, 29] Tacrolimus was found to be 10 to 100 times more potent than CSA in lymphocyte inhibition *in vitro*, possibly due to the higher affinity of FK-binding protein complex to calcineurin.[10, 16]

Tacrolimus is absorbed in the small intestine; the extent of absorption is poor and TAC has a highly variable oral bioavailability averaging 17 – 22%. Food also impairs absorption of TAC.[28-30] Tacrolimus is distributed extensively into erythrocytes and lymphocytes, and TAC in plasma is highly bound (~99%) to alpha-1-acid-glycoproteins, lipoproteins and albumin.[28-30] Whole blood is therefore used in therapeutic drug monitoring of TAC. Tacrolimus is also a substrate of P-glycoprotein, and is extensively metabolized by CYP3A4 enzymes to multiple metabolites in the liver and GI tract. The metabolites are excreted mainly through the biliary tract.[28] Tacrolimus is susceptible to many pharmacokinetic drug-drug interactions through CYP, P-glycoprotein, and possibly the phase II metabolic enzymes UDP-glucuronosyltransferases (UGTs, Section 1.4).[29, 31]

Tacrolimus has a similar toxicity profile to CSA; common adverse effects include nephrotoxicity, neurotoxicity, hypertension, GI disturbances, and diabetes mellitus. Monitoring of TAC trough concentrations is a common practice in assuring optimal dosing in transplant recipients.[30]

1.2.3. Sirolimus

Sirolimus is a macrolide lactone produced by *Streptomyces hygroscopicus*. Its immunosuppressive activity is mediated via inhibition of the cytokine stimulation signaling cascade (especially interleukin-2), leading to a halt in the lymphocyte cell

cycle.[10, 18, 32] Sirolimus binds to FK-binding protein, and the SRL-protein complex inhibits the kinase mTOR, leading to a disruption of various signaling pathways that are involved in T-lymphocyte protein synthesis, DNA synthesis and cell propagation.[32, 33] Sirolimus complements the actions of calcineurin inhibitors, and was first approved by the US Food and Drug Administration to be used in conjunction with CSA; however, SRL has subsequently been approved to be used in lieu of calcineurin inhibitors to avoid renal toxicities.[33]

Sirolimus has an oral bioavailability of about 15%.[33, 34] Absorption is impacted by food intake, with a 35% increase in SRL exposure reported when taken with a high fat meal.[32] Over 90% of SRL is distributed among erythrocytes, and plasma SRL is highly bound to alpha-1-acid glycoprotein and lipoproteins.[32] Similar to calcineurin inhibitors, SRL is a substrate of both CYP3A4 and P-glycoprotein. It is extensively metabolized to more than 16 metabolites, which are subsequently eliminated via the biliary and fecal pathways.[32-34] Sirolimus has a relatively long elimination half-life (~60 hours), allowing a once-daily dosing regimen.[32, 34] Because of the shared pathway in metabolism and transport, pharmacokinetic interactions with CSA have been noted, and SRL is recommended to be taken 4 hours apart from CSA.[32]

Major adverse effects of SRL are hyperlipidemia, hypertriglyceridemia, bone marrow suppression, and impaired wound healing.[17, 18, 33] Since SRL blood concentrations are shown to correlate with clinical efficacy and adverse effects, therapeutic drug monitoring of SRL is recommended in optimizing SRL dosage.[33, 34]

1.2.4. Corticosteroids

Corticosteroids such as prednisone and methylprednisolone have played a major role in transplantation since the 1950s.[35] Their anti-inflammatory and immunosuppressive actions are non-specific, and the exact pathways of their impact on multiple systems are still not well understood. Corticosteroids have been suggested to alter leucocyte distribution and suppress production of various interleukins, growth factors and transcription factors involved in immune response.[17, 35] Corticosteroids exert their immune-modulation effects by binding to cytoplasmic glucocorticoid receptors; the complex then translocates to the nucleus and modulates transcription by binding directly to DNA regulatory elements.[17, 35]

The most commonly used corticosteroids in organ transplantation are prednisone, prednisolone and methylprednisolone. Absorption of the oral formulations prednisone and prednisolone is rapid, and prednisone is converted the active metabolite prednisolone in the liver.[35] Corticosteroids are rapidly metabolized by CYP3A4 enzymes, and may also be inhibitors and inducers of CYP3A4 and P-glycoprotein.[36] Although the elimination half-life of prednisone and methylprednisolone is short (a few hours), their long pharmacologic effect allows once-daily dosing.[35] Most of the metabolites are excreted in the urine.

Corticosteroids are notorious for their numerous adverse effects on multiple systems, especially with chronic use. These include weight gain, diabetes mellitus, hyperlipidemia, cosmetic effects, hypertension, osteoporosis, and mood swings.[6, 17, 35] While current trends are moving toward steroid avoidance,[37, 38] corticosteroids still play an important role in maintanence therapy and in treating acute rejection.[10, 17, 18]

1.3. Mycophenolate Mofetil

Mycophenolate mofetil (MMF) is a relatively new immunosuppressive agent that is now commonly used in combination with CSA and TAC in maintenance immunosuppression for solid organ transplant recipients.[39-42] MMF is a prodrug, a morpholinoethyl ester of the active metabolite mycophenolic acid (MPA) that is responsible for the immunosuppressive actions.[43, 44] MPA functions to inhibit proliferation of T-cells by selectively, reversibly and non-competitively inhibiting the enzyme inosine monophosphate dehydrogenase, and inhibits the type 2 enzyme more effectively than type 1.[45-47] Proliferation of lymphocytes relies heavily on the *de novo* pathway to synthesize purines for DNA and cell replication, and inosine monophosphate dehydrogenase is a key enzyme in the process to convert inosine to guanosine. While both types 1 and 2 enzymes have similar enzymatic activity, lymphocytes mainly express the type 2 enzyme (expression increased by ten-fold) when activated, rendering MPA a specific drug that targets activated lymphocytes.[45] In addition, MPA also inhibits B-lymphocytes from producing antibodies that would otherwise attack allograft antigens.[39, 44]

Although MPA is very effective in its immunosuppressive action, major adverse effects are GI toxicities, including GI bleeding, ulcers, diarrhea, and vomiting, as well as bone marrow suppression (leukopenia, anemia, and thrombocytopenia).[44, 48, 49] However, overall MPA has an excellent safety and efficacy profile, and is the predominant anti-metabolite of choice in maintenance therapy. It is used in about 75% of heart and 50% of lung transplant recipients.[2, 7, 39]

Unlike other immunosuppressive agents such as CSA and TAC that are dosed by body weight, MMF is typically given at fixed doses twice daily. When MMF was first approved in the United States and Canada in 1995, there were no guidelines regarding monitoring of MPA concentrations, and therapeutic drug monitoring was not deemed necessary.[44, 46] However, the wide inter-patient variability in MPA pharmacokinetics and its interactions with other immunosuppressive agents is now well-recognized (Sections 1.3.1 and 1.3.2).[50-56] While still controversial, there is evidence that MPA exposure correlates with treatment response and toxicities. This pharmacokinetic-pharmacodynamic relationship of MPA has been established in kidney, and to some extent heart, transplant recipients (Section 1.3.3).[49, 50, 57-63] The utility of MPA therapeutic drug monitoring and the use of limited sampling strategies (LSSs) have been advocated in recent years to guide MMF therapy (Sections 1.3.4 and 1.3.5).[57, 64-67]

1.3.1. Mycophenolic acid pharmacokinetics and variability

Mycophenolate mofetil is administered orally and is rapidly and completely absorbed in the stomach and upper GI tract within five minutes of ingestion.[44, 68, 69] It is then completely hydrolyzed by esterases in plasma and liver to MPA, the pharmacologically active moiety.[39, 69] Over 90% of MPA is metabolized by UDP-glucuronosyltransferase (UGT, Section 1.4) enzymes in the liver, GI tract and kidney via glucuronidation to the inactive metabolite mycophenolic acid glucuronide (MPAG). Both MPA and MPAG are highly bound to albumin, about 97% and 82%, respectively.[39, 44] In addition, <5% of MPA is metabolized to the acyl glucuronide of MPA (AcMPAG), which is pharmacologically active and has shown proinflammatory activities *in vitro*.[69-73] Although still controversial,[61, 74-76] it is suggested that AcMPAG may contribute to the

adverse reactions associated with MMF therapy. Recently, other minor glucoside metabolites of MPA have been identified, but the physiological effect of these minor metabolites is not well understood.[48, 70, 71, 77] It is also known that MPA goes through enterohepatic recirculation between 6 – 12 hours after drug administration, often resulting in a second absorption peak in the pharmacokinetic (PK) profile. The MPAG is released into the bile via the transporter multidrug resistance-associated protein 2 (encoded by *ABCC2*), and de-glucuronidated in the GI tract by β-glucuonidases to release MPA for re-absorption (Section 1.5).[39, 69] Virtually the entire MMF dose is excreted in the urine (93%) and feces (6%) in the form of MPAG (87% of the excreted dose); less than 1% of the dose is excreted as unchanged MPA.[44, 78] The chemical structures of MMF, MPA, MPAG and AcMPAG are presented in Figure 1.1, and the major metabolic pathways of MMF and MPA are depicted in Figure 1.2.

Wide inter-patient variability in MPA pharmacokinetics was first reported in the kidney transplant population and subsequently in other transplant populations. Most notable is the ten-fold MPA AUC difference and a trough concentration range of 0.24 – 7.04 μg/mL reported by Cattaneo et al.[79] In recent years, such PK variability has been extensively reviewed and discussed.[39, 65, 70, 80-82] As for the thoracic transplant population, the majority of studies were conducted in heart transplant recipients; a similar range of variability in MPA pharmacokinetics was observed in various studies, which are summarized in Table 1.1.[54, 55, 83-95] The range of MPA AUC observed in a study population varies from a 1.5-fold[83, 95] to 13-fold[88] difference in heart transplant recipients, and a 10 to 17-fold difference in lung transplant populations.[55, 88, 96]

Numerous factors have been postulated to contribute to the observed PK variability of MPA at the absorption, distribution, metabolism, enterohepatic recirculation, and elimination stages. These include interaction with concomitant immunosuppressive agents (such as CSA and TAC, Section 1.3.2), genetic variations in *UGT* and *ABCC2* (Sections 1.4 and 1.5), food intake, concomitant medication or supplements, co-morbidities (such as cystic fibrosis), albumin level/protein binding, liver function, and renal function.[39, 69, 70, 97-101] However, the exact mechanism and relative contribution of these factors to the MPA inter-patient variability remain to be elucidated.

1.3.2. Drug interactions with other immunosuppressive agents

In recent years, there has been growing concern that MPA pharmacokinetics can also be altered by other immunosuppressive agents, especially CSA and TAC, since co-administration of these drugs is commonly employed in maintenance immunosuppressive therapy.[39, 70, 97, 98, 101] An early study by Zucker et al.[58] reported that renal transplant recipients taking MMF with TAC have significantly higher MPA exposure [higher AUC and higher trough concentration (C0)] than those taking MMF with CSA, despite the same administered dosage of MMF. Subsequently, other studies conducted by different research groups,[31, 58, 102-106] including a pharmacokinetic study of lung transplant recipients at our centre,[89] found similar results. These pharmacokinetic studies suggest that CSA decreases MPA exposure, and TAC may increase MPA levels. Currently there is no known interaction between MPA and SRL.[107]

It is speculated that CSA reduces MPA exposure by inhibiting the ABCC2/MRP2 transporter, thereby reducing enterohepatic recirculation of MPA,[24, 105, 108-110] as

evidenced by various mechanistic studies in animals.[24, 40, 109, 111, 112] However, support for the interaction of MPA and TAC is less evident, with most of the observed differences attributed to decreased MPA exposures associated with CSA use.[29, 103, 113, 114] There are *in vitro* data and studies in renal transplant recipients showing that TAC may be an inhibitor of UGT enzymes, hence increasing MPA levels.[31, 58] Pharmacokinetic interactions of MPA and corticosteroids have also been suggested. Corticosteroids have been shown *in vitro* to induce UGTs in general;[115-118] however, the impact on specific UGTs that metabolize MPA remains unknown. A clinical study in renal transplant recipients reported increased MPA exposure as steroids were tapered and withdrawn;[115] however, the same trend was not observed in another steroid-tapering study.[119]

While drug-drug interactions play an important role in the wide inter-patient PK variability of MPA, the degree of effect remains unclear, and considerable variability remains to be elucidated.

1.3.3. Mycophenolic acid therapeutic drug monitoring

When MMF was first approved in the US and Canada, there were no guidelines regarding monitoring of MPA concentrations, and therapeutic drug monitoring was not deemed necessary.[44, 46] Unlike other immunosuppressive agents such as CSA and TAC, MMF typically is given at fixed doses twice daily. Although the utility and guidelines of PK monitoring of MPA are controversial,[57, 59, 65, 97, 100, 120] studies have shown a relation between graft rejection versus MPA AUC,[49, 121-129] trough concentrations,[60, 122, 124, 125, 129-139] or other limited sampling strategies.[50, 86, 127, 128, 137, 140-144] Associations between adverse effects and MPA AUC,[49, 130, 139, 145] trough concentrations,[63, 76, 124, 130, 133-135, 138, 145-153] or other limited sampling strategies[50, 61, 154, 155] have also been reported. Given the wide inter-

patient variability in MPA PKs observed in transplant recipients (Section 1.3.1) and the drug-drug interactions with concomitant immusuppressive agents (Section 1.3.2), therapeutic drug monitoring of MPA has been advocated to improve mycophenolate therapy management in various transplant populations.[63, 65, 66, 70, 134]

Since 1998, numerous reviews and consensus reports have been updated periodically on the status of MPA monitoring.[41, 46, 59, 64, 65, 67, 80, 82, 97, 100, 120, 156-159] For example, reports by Shaw et al. have suggested MPA monitoring schedules and guidelines.[41, 59, 67, 70] Currently, the MPA target trough levels proposed for kidney transplant recipients are 1 – 3.5 μg/mL for regimens with CSA, and 1.9 – 4.0 μg/mL for regimens with TAC; and 1.2 – 3.5 μg/mL for heart transplant recipients.[59, 64] The suggested total MPA exposure range is 30 – 60 μg*h/mL.[59, 64] However, results of MPA therapeutic drug monitoring studies were not unanimous, and the value of monitoring of MPA PK parameter(s) is debatable. Recently, an evidence report was commissioned by the Agency for Healthcare Research and Quality (AHRQ), United States Department of Health and Human Services, to systematically review whether therapeutic drug monitoring (AUC, trough concentrations, or limited sampling strategies) of MPA results in reduced incidence of rejection and adverse effects in solid organ transplant recipients.[57] The report summarizes that only three studies consisted of a concentration-monitoring group and a control (i.e., fixed dosages or no monitoring) group; two studies reported fewer rejection episodes in the concentration-controlled group, while one reported fewer rejection episodes in the control group.[57] Overall, randomized controlled trials[121-123, 160, 161] provide support for the association between MPA AUC(0-12) and rejection, but not

adverse effects. Trough levels of MPA did not correlate well with rejection, and results were equivocal for an association with adverse events.[57, 64, 120]

It is notable that virtually all published studies were not designed to compare the clinical outcomes of patients whose MPA was monitored and controlled versus fixed dosages. To date, there are three completed randomized controlled trials that aim to compare outcomes in renal transplant patients with fixed dose versus concentration-controlled MPA: the Fixed Dose versus Concentration-Controlled (FDCC) trial,[74, 162-164] the Adaptation de Posologie du MMF en Greffe Rénale (APOMYGRE) trial,[160] and the Opticept trial.[165-167]

The FDCC trial[164] was a multi-centre trial that randomized 901 *de novo* adult or pediatric kidney transplant recipients into the fixed-dose MMF treatment group (n=449) or concentration-controlled group (n=452) in which dosages were adjusted to target an MPA exposure of 45 μg*h/mL. The MPA AUC(0-12) was estimated from limited sampling strategies that utilized three timed concentrations. Participants were followed for 12 months post-transplant; primary outcome measures included biopsy-proven acute rejection, graft loss, death, and discontinuation of MMF, with secondary safety endpoints of incidences of infections, adverse events, and malignancies. A sub-study of the FDCC trial also investigated association of diarrhea with MPA and metabolite exposure.[74] The FDCC study reported that there was no difference in the proportion of patients reaching target MPA AUC(0-12) in both treatment groups, and one-third of patients had inadequate MPA exposure (below 30 μg*h/mL); consequently, no differences in the incidences of treatment failure (25.7% vs. 25.6%, p=0.81) or adverse events (92.3% vs. 94.7%, p=0.178) were observe at 12 months post-transplant between the fixed-dose and

concentration-controlled groups, respectively. The under-exposure to MPA was partly attributable to the fact that physicians did not increase MMF dosage enough to reach target range. However, the study did confirm that there was a significant relationship between incidences of acute rejection with lower MPA AUC and pre-dose concentrations within the first year, suggesting that therapeutic drug monitoring of MPA with adequate dosage changes could lead to positive treatment outcomes.[164]

The APOMYGRE trial[160] was a 12-month multi-centre study conducted in France, randomizing 137 *de novo* adult kidney transplant recipients into the fixed dose MMF treatment group (n=67) or concentration-controlled group (n=70). A Bayesian limited sampling strategy using three timed concentrations was used to estimate and target MPA AUC to 40 µg*h/mL. The primary treatment failure endpoints included death, graft loss, acute rejection and discontinuation of MMF, with secondary endpoints of adverse events and severity of acute rejection episodes. The APOMYGRE trial showed that at 12-month post-transplant, incidences of acute rejection and treatment failure were significantly lower in the concentration-controlled group (29.2% vs. 47.7% in fixed-dose group, p=0.03); however, there was no association between MPA exposures and most adverse events, except that a greater incidence of herpes infections was observed in the concentration-controlled group. Overall the concentration-controlled group was receiving higher dosages of MMF than the fixed-dose group. The authors concluded that MPA monitoring was beneficial in reducing treatment failures for renal transplant recipients.

The Opticept trial has been completed but results have not been published, with preliminary results available in abstracts.[165-167] It was a two-year multi-centre study randomizing 718 kidney transplant recipients into fixed-dose vs. concentration-controlled

treatment arms, using MPA trough concentration targets of ≥1.3 μg/mL and ≥1.9 μg/mL for CSA and TAC co-medication groups, respectively. Primary outcomes included biopsy-proven acute rejection, graft loss, death and renal function (indicated by calculated glomerular filtration rate), with secondary outcomes being incidences of adverse events. Preliminary results reported similar treatment and safety outcomes in both treatment groups.

The two published randomized controlled trials both corroborated that MPA total exposure, indicated by AUC(0-12), was correlated with incidences of acute rejection.[160, 164] Conversely, MPA trough levels were inadequate in monitoring for treatment outcomes, and currently no association is found between MPA PK parameters and adverse events. Monitoring of MPA AUC(0-12) is promising in better managing MMF therapy to avoid acute rejection; however, full AUC measurements are costly and impractical for clinical use. Since conventional trough level monitoring is not useful for MPA due to the lack of correlation with rejection, limited sampling strategies may be the optimal monitoring method to estimate total MPA exposure. In fact, both trials utilized validated limited sampling strategies to estimate MPA AUC(0-12). In order to implement routine monitoring of MPA clinically, development of limited sampling strategies specific to the patient population is an area worthy of exploration.

1.3.4. Limited sampling strategies of mycophenolic acid

Although total MPA exposure, estimated by AUC(0-12), is the best indicator of treatment efficacy (Section 1.3.3), it is impractical and costly to implement routine AUC(0-12) monitoring in the clinical setting. Alternatively, limited sampling strategies (LSSs) have the potential to improve MPA monitoring by using just a few timed samples

to estimate total MPA exposure. Generally there are two main approaches to developing LSSs: multiple regression analysis and Bayesian analysis.[168-171] Multiple regression analysis correlates the dependent variable (drug AUC) to the independent variables (concentrations at different time points) via stepwise regression, and describes the relationship as a linear function:

$$AUC = b + M_1C_{t1} + M_2C_{t2} + M_3C_{t3} + \dots M_iC_{ti}$$

where AUC is the estimated area under the concentration-time curve; b is a constant (y-intercept); C_{t1}, $C_{t2} \dots C_{ti}$ are concentrations obtained at times t_1, $t_2 \dots t_i$, and M_1, $M_2 \dots M_i$ are fitted constants associated with each timed concentration.[168-171] The major advantage of multiple regression analysis is the simple model development and straightforward calculations. It is limited by the inflexibility in sampling time deviations, and application is usually limited to the specific population and/or drug regimens in which LSSs are derived.[171] The Bayesian approach, on the other hand, utilizes population demographic and concentration data and their variability to establish the initial model; individual patient data are then incorporated to refine the model for prediction of drug exposure.[168, 169, 171] The advantages of a Bayesian-derived LSS include flexibility in sampling times, potential to be continuously refined, and incorporation of other factors into the model. However, Bayesian analysis requires population pharmacokinetic information for initial estimation, and this information may not always be available. In addition, specialized software and training are needed to develop and interpret results, which may be challenging for everyday clinical use.[168, 171, 172]

Full PK profile data obtained from multiple samples are used to develop LSS models, and these are usually validated with a separate data set to test the predictive

performance (bias and precision) of the LSS models. Alternatively, re-sampling procedures such as jack-knife or bootstrap methods are useful approaches in validation, especially for small sample sizes.[173] Bias and precision of LSSs are commonly evaluated according to guidelines suggested by Sheiner and Beal.[174] Absolute bias and precision are measured by the mean prediction error (ME), and absolute root mean squared prediction error (RMSE), respectively:

$$\mathrm{ME} = \frac{1}{\mathrm{N}}\sum(\mathrm{Pe_i})$$

$$\mathrm{RMSE} = \sqrt{\frac{1}{\mathrm{N}}\sum(\mathrm{Pe_i})^2}$$

where Pe = prediction error = predicted value – actual value; and N = number of data points. The relative bias and precision are calculated by converting ME and RMSE into percentages. A common acceptable range of relative ME and RMSE values in clinical studies is 15 – 20%.[172]

Ideally, an LSS should be properly validated in the target patient population, provide accurate and precise estimation of total drug exposure, and utilize convenient sampling times.[171, 175] To date, most LSSs established for MPA were developed in the kidney transplant population (Table 1.2);[56, 104, 175-190] a few studies reported LSS equations for the heart transplant population (Table 1.2),[84, 92, 95] but no LSS was established for the lung transplant population aside from the ones previously developed at our centre.[191] Of the reported LSSs, most would still be challenging to implement in a routine clinical setting due to inconvenient sampling times (i.e. beyond two hours post-

dose) (Table 1.2); only a relatively few number of studies in various transplant populations reported clinically convenient sampling times.[92, 95, 175, 177, 182, 183, 191]

The lack of MPA LSSs developed for the heart and lung transplant population warrants special attention, since an LSS established in a specific population (e.g. kidney transplant) is not always suitable for other populations, especially when the transplanted organs have different impact on drug metabolism. As the thoracic transplant population suffers from higher rejection and mortality rates,[2, 7, 9, 10] LSSs developed specifically in this group would provide a marked advance in facilitating therapeutic drug monitoring of MPA in order to improve treatment outcomes.

1.4. UDP-glucuronosyltransferases

The UDP-glucuronosyltransferase (UGT) enzymes are membrane-bound glycoproteins found on the luminal side of the endoplasmic reticulum.[192, 193] They are phase II metabolic enzymes responsible for biotransformation of numerous endogenous and exogenous compounds, including MPA, via glucuronidation. Common endogenous substrates of UGTs include bilirubin and steroid hormones. Approximately 35% of drugs that are metabolized by phase II reactions are substrates for UGTs.[192, 194] The UGTs catalyze the transfer of the ubiquitous co-substrate uridine diphospho-glucuronic acid to hydrophobic substrates, leading to the formation of more hydrophilic glucuronide derivatives for excretion in bile and/or urine.[194] The conjugation usually occurs at functional groups with oxygen, nitrogen, sulfur, and nucleophilic carbon atoms, such as phenols, alcohols, carboxylic acids, and amines.[194-196] Generally, glucuronidation leads to an inactivated compound; some exceptions include the glucuronidation of morphine to

morphine-6-glucuronide, which is more potent than morphine in its analgesic activity.[196, 197] For some carboxylic acids, such as acetylsalicylic acid and mycophenolic acid, glucuronidation by UGTs may also form acyl glucuronides. These metabolites may be reactive due to the acyl group, which is prone to chemical rearrangement and may form covalent adducts to proteins or other macromolecules, potentially leading to immunotoxicities.[195, 198-200]

The human UGT superfamily is encoded by four gene families *UGT1*, *UGT2, UGT3* and *UGT8*.[201] Of these, the UGT1 and UGT2 enzymes utilize glucuronic acid as a co-factor, and are the most significant enzymes in drug metabolism. There are at least 19 functional proteins, further classified into three subfamilies: UGT1A, UGT2A and UGT2B.[194, 201, 202] The entire UGT1 family is encoded by a single gene locus on chromosome 2 (2q37) and consists of nine functional isoenzymes: UGT1A1 and UGT1A3 – 1A10. All UGT1A isoenzymes are coded by a distinctive exon (exon 1) and promoter, and share four common downstream exons (exons 2 – 5). These isoenzymes are found in the liver and extrahepatic tissues (kidney, GI tract). There are three isoenzymes (UGT2A1, 2A2, and 2A3) characterized in the UGT2A subfamily and seven (UGT2B7, 2B11, 2B28, 2B10, 2B15, 2B17, 2B4) in the UGT2B subfamily, which are encoded by several genes on chromosome 4 (4q13) resulting in six exons.[192, 194, 201, 203, 204] UGT2A enzymes are found mainly in the nasal epithelium, and are proposed to be involved in terminating signals from odorant compounds;[194, 202] UGT2B enzymes are expressed in the liver and various extrahepatic tissues.[192, 194] UGTs share a high degree of sequence homology, sometimes reaching 95% between isoforms, and often showing overlapping substrate specificity but distinct expression patterns.[194]

The liver is the central organ of biotransformation, and the major hepatic UGTs involved in drug glucuronidation include UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B4 and 2B7.[205] However, extrahepatic glucuronidation may also play a significant role in drug disposition, as various UGTs are also expressed in the GI tract (UGT 1A1, 1A3, 1A4, 1A6, 1A8, 1A10, 2B4, 2B7, 2B10 and 2B15) and kidneys (UGT1A8, 1A9, 1A10 and 2B7).[192]

1.4.1. Role of UGTs in mycophenolic acid metabolism

Mycophenolic acid is primarily metabolized via glucuronidation to the inactive 7-O-mycophenolic acid glucuronide (MPAG) and the pharmacologically active acyl metabolite AcMPAG. While the liver is the main site of MPA metabolism, the kidneys and GI tract may also play a role in MPA elimination.[39, 48, 78, 206-208] Several studies have identified specific human UGT isoenzymes that have the highest metabolic activity toward MPA; however, results are inconsistent.[209-213] For example, Mackenzie[209] reported the extrahepatic UGT1A8 and 1A10 had the highest activity in MPA conjugation in cDNA-expressed enzymes in COS-7 cells, while the hepatic UGT1A9 also showed some capacity in MPA metabolism. Bernard et al.[211] systematically tested 16 cDNA-expressed UGT enzymes (UGT1A and 2B) in HEK-293 cell lines, and reported that UGT1A8 and 1A9 have the highest catalytic activities in the production of MPAG; while UGT1A1, 1A7 and 1A10 also contribute to the glucuronidation of MPA, they had much lower catalytic activities compared to UGT1A8 and 1A9. The study also showed that enzyme variants UGT1A9*3 and UGT1A8*3 had decreased metabolic activity compared to the reference enzymes (*1). A follow-up study[210] by the same research group demonstrated that UGT2B7 is the main enzyme that produces AcMPAG; in addition, UGT1A8 is also

capable of forming AcMPAG. Catalytic efficiencies of UGT2B7*1 and *2 are similar, while UGT1A8*3, *5, *7, *8 and *9 have impaired activities for MPAG and AcMPAG production. Similarly, Picard et al.[212] also reported that UGT1A9 had the highest efficiency in conjugating MPA to MPAG, and UGT2B7 is the only isoenzyme forming AcMPAG in UGT-transfected baculovirus-infected insect cells. Conversely, Basu et al.[213] reported that UGT1A7, 1A9, 1A8 and 1A10 are the major enzymes metabolizing MPA to AcMPAG in UGT-transfected COS-1 cells.

Overall, it is recognized that UGT1A7, 1A8, 1A9, and 1A10 are involved in glucuronidation of MPA to the major metabolite MPAG, and UGT2B7 is the main isoenzyme that produces the minor metabolite AcMPAG.[39, 78]

1.4.2. UDP-glucuronosyltransferase genetic polymorphisms

Numerous polymorphisms in the UGT genes have been reported and reviewed, and some polymorphisms have been implicated in altering enzyme activities that are clinically significant in diseases or xenobiotic metabolism.[194, 204, 214-220] The most classic clinical example of *UGT* polymorphisms was observed in patients with Crigler-Najjar syndrome and Gibert's disease, which are characterized by unconjugated hyperbilirubemia.[194, 204, 219] One of the most extensively studied variants is the TATA box polymorphism in the promoter region of the *UGT1A1* gene (*1A1*28*), which was found to markedly reduce UGT1A1 transcription and enzyme expression, thereby impairing bilirubin metabolism and causing hyperbilirubinemia.[194, 204, 221] This polymorphism also has a direct effect on the rate of glucuronidation of SN-38, the active metabolite of anti-cancer agent irinotecan; the low promoter activity allele (*UGT1A1*28*) is associated with decreased SN-38 glucuronidation and severe toxicity.[194, 216, 222-224]

In addition to UGT1A1, different alleles in all other *UGT1A* genes (*UGT1A3* – *1A10*) and *UGT2B4, 2B7, 2B10, 2B15, 2B17* and *2B28* have been identified.[194, 204, 209, 214, 217] In fact, discovery and naming of UGT alleles are constantly being updated by the UGT Nomenclature Committee,[225] available at: http://www.pharmacogenomics.pha.ulaval.ca/sgc/ugt_alleles/. While the significance of most polymorphisms is unknown, functional studies have revealed certain variants that may have clinical significance. For example, *UGT1A6*2* has lower catalytic activity for methyl salicylate and β-blockers, and may modulate efficacy of non-steroidal anti-inflammatory drugs.[194] *UGT 1A7*3* and **4* variants are associated with decreased benzo(a)pyrene phenol metabolism, and may increase risk of oralaryngeal cancer by increasing the body's exposure to harmful phenol toxins.[204, 217, 221] The low-activity enzymes resulting from *UGT1A8* (**3*) and *1A9* (**2* and **3*) polymorphisms[210, 211, 226] are implicated in MPA pharmacokinetics and transplant outcomes (see also Section 1.6.1, Table 1.3). A higher incidence of SN-38 toxicities is also reported in patients with the *UGT1A9* -118 t_{10}-stretch variant.[224] The low-activity allele *UGT2B15*2* is associated with reduced oxazepam glucuronidation and increased risk of development of prostate cancer.[204, 214, 223]

A genotype-phenotype correlation is often difficult to establish; it is a complex pathway that starts with identification of a genetic variant, determination of its functional impact *in vitro*, and assessment of the clinical consequence *in vivo*, which is often the most challenging and controversial stage.[204] Currently, there is a lack of studies investigating associations between MPA pharmacokinetic parameters and genetic constitution; *UGT* polymorphisms are promising leads in elucidating variability in phase

II drug metabolism, and pharmacogenetic research is much needed to fully understand the clinical effects of these polymorphisms, as in the case of MPA metabolism (Section 1.6.1).

1.5. Multidrug Resistance-Associated Protein 2

The multidrug resistance-associated protein 2 (MRP2/ABCC2) is a member of the ATP-binding cassette (ABC) transporter family, which is comprised of least 49 transporters categorized into seven subfamilies, ABCA to ABCG.[227] The ABCC2 is one of nine transporters in the ABCC sub-family; it is a relatively large protein translated from 32 exons encoded by a gene on chromosome 10q24.[228-230] Also known as canalicular multispecific organic anion transporter, ABCC2 is an ATP-dependent organic anion transporter found predominantly in the canalicular membrane of hepatocytes. It is also expressed in apical membranes of enterocytes, gall bladder epithelial cells, renal proximal tubular cells, placenta, and possibly the blood-brain-barrier.[228-232] It has numerous endogenous and xenobiotic substrates, including glucuronide, sulfate and glutathion conjugates. Well known endogenous substrates include conjugated bilirubin, leukotriene C4, and steroids.[229, 232] The major function of ABCC2 is the biliary export of conjugated and non-conjugated compounds; ABCC2 plays a crucial role in the final elimination of drug and toxin metabolites, as well as enterohepatic recirculation of conjugated xenobiotics such as MPA.[227, 229, 231]

Since ABCC2 is also expressed in the intestine and renal tubule epithelia, it likely contributes to the absorption and urinary excretion of anionic compounds; however, given that there are various transporters involved, the relative role of ABCC2 in drug

absorption and renal elimination is unclear.[229] Nonetheless, intricate interactions of the ABCC2 transporter and phase II metabolic enzymes provide an efficient system in eliminating potentially toxic xenobiotics and toxins.[230]

1.5.1. Role of ABCC2 in mycophenolic acid disposition

Recent animal studies have elucidated that the ABCC2/MRP2 transporter is responsible for the biliary export of MPAG, thereby playing an essential role in MPA disposition. In addition, inhibition of ABCC2 by CSA was reported to be the mechanism of the CSA-MPA pharmacokinetic interaction.[24, 40, 109, 112] A study by van Gelder et al.[40] compared MPA PK profiles of normal Lewis rats in three co-medication groups: placebo, CSA and TAC. The authors noted that MPA PK profiles in rats also receiving CSA lacked the second re-absorption peak and had lower MPA exposure compared to the TAC co-medication and placebo groups; it was speculated that CSA inhibits biliary excretion of MPAG. A follow-up study[24] comparing MPA PK in transport-deficient (with non-functional Mrp2) rats taking CSA vs. TAC confirmed that the CSA-MPA interaction is at the Mrp2 level, since there was no difference in MPA PKs and MPAG metabolic ratio in Mrp2-deficient rats on CSA vs. TAC. Kobayashi et al.[109] compared biliary excretion of MPA and MPAG in normal and Mrp2-deficient rats and found that the biliary excretion of MPAG was markedly decreased in Mrp2-deficient rats, clearly demonstrating that MPAG is a substrate of the Mrp2 transporter. In addition, co-administration of CSA (compared with TAC) resulted in decreased biliary excretion of MPAG, suggesting Mrp2 as the site of interaction. Similarly, Westley et al.[112] used isolated perfused rat livers and showed that there was complete lack of MPAG in bile in Mrp2-deficient rats, compared to a 84% biliary recovery of MPA (in the form of MPAG) in normal rats, thus

corroborating that Mrp2 is indeed the major transporter for export of MPAG. The authors also showed that CSA inhibits Mrp2, and possibly hepatic glucuronidation of MPA, in rats.

In addition to biliary excretion, there is suggestion that ABCC2 may also be involved in the urinary excretion of MPAG and MPA via active tubular secretion.[39] Recent studies[112, 233, 234] suggest that other transporters, possibly P-glycoprotein, are also likely involved in the biliary and urinary excretion of MPA and MPAG; however, further studies are warranted.

The enterohepatic recirculation of MPA, mediated by biliary excretion of MPAG and its subsequent de-glucuronidation by β-glucurnoidase in the GI tract, contributes from 10 – 60% (average 40%) to total MPA AUC(0-12).[39, 69] This underscores the significance of ABCC2 in determining total MPA exposure, and emphasizes the need to consider the ABCC2 transporter when investigating MPA disposition (Section 1.6.2).

1.5.2. Multidrug resistance-associated protein 2 genetic polymorphisms

Genetic polymorphisms in ABCC2 were first noted in people with Dubin-Johnson Syndrome, a hereditary disease characterized by conjugated hyperbiliremia and hepatic accumulation of melanin-like pigment due to dysfunctional ABCC2.[235-237] Genetic variants in *ABCC2* and their role in drug disposition have been extensively reviewed.[229, 238-240] Of >40 polymorphisms reported for *ABCC2*, the most common include the variants C-24T (promoter region), G1249A ($Val^{417}Ile$), and the silent mutation C3972T ($Ile^{1324}Ile$), with allelic frequencies ranging from 15 – 30%.[237, 239-247]

The functional impact of *ABCC2* polymorphisms is poorly understood, with many studies showing inconsistent results.[228, 238] For example, the C-24T variant was associated

with diclofenac-induced hepatotoxicity,[245] however, no association with irinotecan-induced diarrhea or neutropenia was observed.[243] Niemi et al.[242] reported no impact of *ABCC2* C-24T on pravastatin pharmacokinetics but found the variant C1446G to be associated with lower pravastatin exposure. On the contrary, Fujita et al.[248] observed higher SN-38 (active metabolite of irinotecan) exposure in colorectal cancer patients carrying the C-24T variant; a study in renal transplant recipients[241] observed higher MPA trough levels in patients with C-24T, and the authors speculated that ABCC2 expression or activity may be up-regulated with the C-24T variant. However, a recent study in healthy volunteers found no link between C-24T and other *ABCC2* variants on the expression of mRNA or protein content in intestinal tissues.[249] As for the G1249A (Val[417]Ile) variant, no functional impact on pharmacokinetics of pravastatin[242] and MPA[241] or clinical outcomes of irinotecan[243] and diclofenac[245] was observed by several research groups. However, Zhang et al.,[250] Fujita et al.[248] and Haenisch et al.[249] reported increased MPA enterohepatic recirculation, decreased irinotecan exposure, and decreased bioavailability of talinolol, respectively, in patients with the variant G1249A. Naesens et al.[241] reported that the silent mutation C3972T (Ile[1324]Ile) had similar clinical impact as *ABCC2* C-24T, as the two polymorphisms are in moderate linkage disequilibrium. However, no significant clinical impact on MPA pharmacokinetics was observed in healthy volunteers.[251]

The inconsistent results may be partly attributed to the various pharmacokinetic or clinical outcome parameters being assessed, different *ABCC2* combinations of polymorphisms, and different substrates being investigated. *In vitro* experiments have suggested that some polymorphisms may impact on substrate specificity of ABCC2, and

retain the transport activity of the enzyme, thus only impacting on export of certain substrates.[228] While genetic polymorphism in ABCC2 may impact on the transporter function at the transcription level, translational regulation and transport of the protein to the apical membrane also contribute to the overall activity level of the transporter.[228] Wide inter-patient variability in the hepatic expression of ABCC2 is also observed;[252] it is therefore challenging to compare results between research groups.

1.6. Pharmacogenetics of Mycophenolic Acid

Since UGT and ABCC2 are the major proteins responsible for the metabolism and enterohepatic recirculation of MPA, genetic polymorphisms in the *UGT* (especially *UGT1A8*, *1A9* and *2B7*) and *ABCC2* genes may partially explain the pharmacokinetic variability of MPA. Identifying genetic polymorphisms that alter functional activities of the enzyme/transporter, and subsequently MPA pharmacokinetics, would provide valuable information to guide MMF regimen design. A number of *UGT* and *ABCC2* variants with potential functional impact have been identified in the regulatory and coding regions,[210, 211, 226, 231, 239, 241-245, 253-255] and several clinical studies have demonstrated their influence on MPA disposition *in vivo*. Due to the overlapping substrate specificity and lack of specific UGT isoenzyme antibodies, inter-individual variability in UGT expression has been difficult to determine. However, a recent study reported a 17-fold difference in UGT 1A9 expression in human liver microsomes (n=48), and the expression level was highly correlated to *UGT1A9* genetic polymorphisms in the promoter region,[253] underscoring the need for pharmacogenetic studies to investigate the role of *UGT* genetics in clinical drug metabolism. To date, all clinical pharmacogenetic

studies of MPA were conducted in renal transplant recipients. Results are inconsistent, however, underscoring the complexity of MPA pharmacogenetics (Tables 1.3 and 1.4).[118, 163, 181, 241, 250, 251, 256-263] The next two sections (Sections 1.6.1 and 1.6.2) provide a review of current literature on the clinical impact of various *UGT* and *ABCC2* polymorphisms on MPA pharmacokinetics.

1.6.1. Clinical impact of UDP-glucuronosyltransferase genetic polymorphisms on MPA metabolism

To date, a small number of clinical studies have evaluated the impact of *UGT* genetic polymorphisms on MPA pharmacokinetics and/or outcomes in healthy volunteers[251, 258] or renal transplant recipients;[118, 163, 181, 250, 257, 259-262] Table 1.3 summarizes the reported clinical impact of *UGT* genetic polymorphisms on MPA pharmacokinetics.

Most clinical studies have focused on polymorphisms in *UGT1A8*, *1A9* and *2B7*, the major enzymes involved in MPA glucurnoidation, with a few studies exploring the contribution of *UGT1A1*, *1A6* and *1A7* polymorphisms.[181, 251, 257] Miura et al.[181] investigated the association of *UGT, ABCC2, ABCB1* and *ABCG2,* and the organic anion transporting polypeptides (*OATP/SLCO*) polymorphisms with MPAG pharmacokinetics in 80 Japanese renal transplant recipients. For *UGT* variants, higher MPAG exposure and metabolic ratio (MPAG/MPA) were observed with *UGT1A1*6* and UGT*1A6*2* variants, however, no impact was noted with *UGT1A7*2*, *UGT1A7*3*, or *UGT1A9 I399T* variants. The contributions of UGT variants were not significant in multivariate analysis; on the contrary, the authors reported that variants in *SLCO1B1* and *SLCO1B3*, the genes that encode the organic anion transport proteins involved in hepatic uptake of organic anions,

play a significant role in MPA disposition. Similarly, Inoue et al.[257] did not observe any impact of *UGT1A7*2* or **3* on MPA pharmacokinetics in 80 Japanese renal transplant recipients, while Levesque et al.[251] reported a modest decrease (7%) of MPA exposure associated with *UGT1A7*2*, and no significant effect of *UGT1A7**3, in 47 healthy volunteers taking a single dose of mycophenolate mofetil. Overall, *UGT1A1*, *1A6* and *1A7* variants have negligible clinical impact on MPA pharmacokinetics.

The *UGT1A8*2* allele, with an allelic frequency of 15 – 25% in Caucasians, was reported to have little or no functional impact on MPA metabolism *in vitro*.[210, 226] In accordance with *in vitro* results, all clinical studies, except one, observed no significant impact of *UGT1A8*2* in MPA metabolism in healthy volunteers[258] or Japanese renal transplant recipients (Table 1.3).[181, 260] In fact, Miura et al.[181] observed no influence by UGT genetics in general. Kagaya et al.[260] studied MPA pharmacokinetics of two consecutive dosing periods in 72 Japanese renal transplant recipients and reported a much higher allelic frequency (59%) of *UGT1A8*2* in the Japanese population; no difference in MPA pharmacokinetics were found between the daytime and night-time dose. While Johnson et al.[259] reported 60% higher MPA trough concentrations associated with *UGT1A8*2* in kidney, pancreas and kidney-pancreas transplant recipients, the effect was observed only in patients also taking TAC (but not CSA), suggesting a contribution of co-medications. The uncommon *UGT1A8*3* variant (allelic frequency 1 – 2%) results in an enzyme with greatly impaired catalytic activity *in* vitro;[210, 226] however, clinical studies failed to demonstrate its impact on MPA metabolism. Levesque et al.[258] screened 305 healthy volunteers and selected people with specific *UGT* variants, including *UGT1A8*3*, for a one-dose MPA clinical pharmacokinetic study. The allelic frequency of *UGT1A8*3*

was 1.3% (observed in 4 subjects). Aside from lower free MPA (fMPA) AUC(6-12) in these subjects, there were no other significant differences in MPA or MPAG pharmacokinetics. Johnson et al.[259] observed no impact of *UGT1A8*3* on MPA trough concentrations in 117 kidney, pancreas and kidney-pancreas transplant subjects; the authors speculated that it was partly due to the low prevalence of the polymorphism (heterozygous genotype observed in 8 patients). In a retrospective study assessing association of *UGT1A8* genetics and clinical outcomes in 74 Brazilian renal transplant recipients, Betonico et al.[261] reported increased occurrences of infections associated with *UGT1A8*3* and in patients with the haplotype that contained the *1A8*3* variant (-999C / codon 55A / codon 277A), but only in patients taking 2 g/day MMF (and not those taking 1 g/day MMF); the authors suggested a dose-dependent effect of MPA pharmacokinetics. Generally, the most prominent *UGT1A8*2* and **3* variants showed very little impact on MPA pharmacokinetics *in vivo*. While the **2* variant was not expected to influence MPA metabolism, the lack of impact of the impairing *3 variant is likely due to its low prevalence, thus undermining the power to detect a difference.

Genetic variants in *UGT1A9* were the most studied polymorphisms in MPA pharmacokinetics, since UGT1A9 is the key hepatic UGT that glucuronidates MPA.[39, 211-213] The promoter variant C-2152T/T-275A, with an allelic frequency of 6 – 8% in Caucasians, was shown *in vitro* to increase hepatic expression of UGT1A9 and glucuronidation of MPA.[253] Clinical studies in healthy volunteers[258] and transplant recipients[259, 262] appeared to provide some support for the impact of this variant. In healthy volunteers, Levesque et al.[258] reported decreased MPA exposure during enterohepatic recycling [MPA AUC(6-12)] and decreased MPA, MPAG and AcMPAG

enterohepatic recirculation [estimated by AUC(6-12)/AUC(0-12)]; however, the decrease was relatively modest (~20%), and no changes in MPAG exposure was noted. Kuypers et al.[262] also observed decreased MPA exposure, enterohepatic recirculation and MPA trough concentrations in renal transplant recipients, but only in patients taking 2 g/day MMF and not those taking 1 g/day MMF. The authors noted that while the increase in UGT1A9 expression associated with this variant should result in higher enterohepatic recirculation (due to increased MPAG production), increased intestinal UGT1A9 expression could counter the effect, and may also contribute to the dose-dependent effect observed. Johnson et al.[259] also observed decreased MPA trough concentrations in kidney, pancreas and kidney-pancreas transplant recipients, but only in patients taking CSA and not TAC. Baldelli et al.,[263] however, reported no significant impact of C2152T/T-275A on MPA pharmacokinetics in renal transplant recipients. These studies suggest that while *UGT1A9* C-2152T/T-275A may be a clinically important variant, other factors such as MMF dosage and concomitant medications should also be considered while interpreting MPA pharmacokinetics.

Other promoter variants were found to increase expression or transcription of UGT1A9 *in vitro*, such as C-440T/T-331C, C-665T, and -118 t-stretch (9→10 repeats).[253, 264] Baldelli et al.[263] investigated the role of several UGT1A9 promoter variants (C-2152T/T-275A, T-1887G, C-665T, and C-440T/T-331C) in MPA pharmacokinetics, and found that renal transplant recipients who were homozygous for the common C-440T/T-331C variant (allelic frequency 41%) had significantly higher total MPA exposure (increased by 50%) and increased MPA absorption [indicated by AUC(0-2)] than patients with heterozygous or wild type genotypes. Other promoter

variants, however, did not influence MPA pharmacokinetics. Another clinical study[250] reported the clinical impact of the promoter *UGT1A9* -118 t-stretch (9→10 repeats) on MPA pharmacokinetics; this variant was also commonly observed (allelic frequency 40 – 50%), and was associated with increased UGT1A9 transcription, reduced tumour response, and higher incidence of irinotecan toxicity in colorectal cancer patients.[224, 253, 264] Zhang et al.[250] found that Chinese renal transplant recipients with this variant had increased MPA AUC(6-12) and increased MPAG exposure, suggesting more extensive enterohepatic recirculation. No effect from these additional UGT1A9 promoter variants was found in the study in healthy volunteers.[251]

The *UGT1A9*3* allele is a rare variant with an allelic frequency of 1 – 4%, and results in a dysfunctional enzyme with extremely low catalytic activity.[173, 211] Carriers of this polymorphism are expected to exhibit impaired MPA glucuronidation. Indeed, although the sample was small and exploratory, Levesque et al.[258] found significantly increased MPA and AcMPAG exposure in healthy volunteers (n=5) bearing this polymorphism, while MPAG exposure was not affected; the authors speculated that the impaired UGT1A9 activity was compensated by increased glucuronidation by other enzymes such as UGT1A8 and 2B7, thereby increasing AcMPAG production. Johnson et al.[259] did not observe significant changes in MPA trough concentrations, attributing this to the fact that allelic frequency was too low. Currently, little is known about the intronic variant IC399T, which was associated with higher SN-38 (active metabolite of irinotecan) glucuronidation *in vitro*. The variant is very common in the Asian population (allelic frequency 64%), however, no functional impact was observed on MPA or MPAG

pharmacokinetics in Japanese renal transplant recipients or in healthy volunteers.[181, 251, 257]

UGT2B7 is the major UGT that produces the active metabolite AcMPAG, and its genetic variants are of special interest since the acyl glucuronide may contribute to MPA toxicity.[48, 70, 71, 77] The most common polymorphism is the non-synonymous *UGT2B7**2a (C802T, His[268]Tyr), with an allelic frequency of 49 – 56% in Caucasians and 26 – 42% in Asians.[118, 250, 255, 258, 260] In addition, the *UGT2B7**2a variant is in complete reverse linkage with a group of promoter variants (-1306G, -1299C, -1112C, -900A, -327G, and -161C).[258] The clinical impact of *UGT2B7**2a on MPA pharmacokinetics is equivocal. While Levesque et al.[258] observed increased MPA, fMPA and MPAG exposure and increased AcMPAG urinary excretion in healthy volunteers, Djebli et al.[118] reported lowered AcMPAG exposure [AUC(0-9)] only in renal transplant recipients also taking SRL as co-medication; no impact on MPA pharmacokinetics was observed in Asian renal transplant recipients.[250, 260] van Agteren et al.[163] also found no significant impact of the G-900A variant (reversely linked to *UGT2B7**2a) on AcMPAG exposure, trough concentration or metabolic ratio, and no difference in incidence of MPA toxicities in renal transplant recipients. Since *UGT2B7**2a is also in complete reverse linkage with a set of promoter variations, the mechanism of gene modulation is unclear, and whether more promoter polymorphisms are involved is unknown. Currently there are no reports on the impact of *UGT2B7* G-138A on MPA metabolism; however, this uncommon promoter polymorphism has been associated with reduced UGT2B7 transcription or activity.[254, 265]

The current state of knowledge of UGT genetics and their impact on clinical pharmacokinetics of MPA is very limited, given only about a dozen pharmacogenetics studies have been published to date. While most studies were conducted in renal transplant recipients, the PK parameters, selection of candidate UGTs and associated genetic polymorphisms do differ between studies. Overall, the variants *UGT1A9* C-2152T/T-275A and *UGT2B7**2a appear to be promising candidates for further studies; in addition, much larger clinical studies are warranted to elucidate the clinical impact of rare polymorphisms such as *UGT1A8**3, *UGT1A9**3 and *UGT2B7* G-138A.

1.6.2. Clinical impact of multidrug resistance-associated protein 2 genetic polymorphisms in MPA disposition

The impact of the *ABCC2* transporter on MPA disposition has sparked research interest as it plays a significant role in the enterohepatic recirculation and excretion of MPAG and MPA.[24, 109, 112, 237, 241] To date, only a few clinical studies have assessed the role of ABCC2 genetics in MPA disposition; all but one[251] study were conducted in renal transplant recipients.[181, 241, 250] The reported clinical impact of *ABCC2* genetic polymorphisms on MPA pharmacokinetics is summarized in Table 1.4. The most common polymorphisms include C-24T, G1249A (Val[417]Ile), and the synonymous variant C3972T (Ile[1324]Ile), with allelic frequencies of about 20%, 20% and 30%, respectively.[241, 248, 251]

Naesens et al.[241] conducted an extensive study investigating the role of seven *ABCC2* single nucleotide polymorphisms (G-1549A, G-1023A, A-1019G, C-24T, G1249A, C3972T and G4544A) and three *UGT1A9* polymorphisms in MPA pharmacokinetics in 95 renal transplant recipients; a full 12-hour PK assessment was

performed on day 7, and abbreviated PK assessments (2 – 4 hours) on days 42, 90 and 360 days after transplantation. The authors noted no difference in MPA pharmacokinetics associated with any *ABCC2* polymorphisms on day 7. When stratifying patients by liver function, non-carriers of the *ABCC2* C-24T variant with mild liver dysfunction had lower MPA exposure, while no difference was detected in carriers of the variant regardless of liver function. At later periods post-transplant, patients with the *ABCC2* C-24T variant were reported to have higher MPA exposure and trough concentrations, and suffered from more episodes of diarrhea within the first year post-transplant. Since MPA exposure appeared elevated as patient stabilized over time, the authors speculated that the C-24T variant, with an observed allelic frequency of 23%, may be associated with increased *ABCC2* expression or activity and enhances the enterohepatic recirculation of MPA. Conversely, two studies in Asian populations[181, 250] and one in Caucasian renal recipients[263] did not observe an impact of *ABCC2* C-24T on MPA or metabolite pharmacokinetics. Although Miura et al.[256] also reported no difference in MPA pharmacokinetics or occurrences of diarrhea in Japanese renal transplant recipients bearing this variant, the apparent clearance of MPA was significantly lower in patients who were carriers of *ABCC2* C-24T and homozygous for the uptake transporter *SLOC1B3* 334G variant. Interestingly, Levesque et al.[251] observed increased AcMPAG exposure associated with *ABCC2* C-24T in healthy volunteers, suggesting involvement of *ABCC2* in the disposition of this minor but active metabolite. Currently, the impact and mechanism of this variant on transporter expression and/or activity remain to be elucidated.

Aside from *ABCC2* C-24T, a few polymorphisms in the coding region, including G1249A ($Val^{417}Ile$) and the synonymous variant C3972T ($Ile^{1324}Ile$), were candidates for MPA pharmacogenetic research due to their prevalence. A study in 98 Chinese renal transplant recipients reported increased AcMPAG exposure associated with the G1249A ($Val^{417}Ile$) variant (allelic frequency of 7% in this population); however no functional impact of this variant was observed in healthy volunteers taking a single dose of MMF[251] or in renal transplant recipients.[241, 263] Similarly, C3972T ($Ile^{1324}Ile$) was not found to influence MPA pharmacokinetics in healthy volunteers.[251] Naesens et al.[241] reported that C3972T ($Ile^{1324}Ile$) was in moderate linkage disequilibrium with C-24T (r^2=0.26) and observed the same clinical impact as C-24T, that carriers of the variant had higher MPA trough concentrations at later transplant time points, and non-carriers of this variant with liver dysfunction had lower MPA exposure (Table 1.4). The less common coding region polymorphism C4544A ($Cys^{1515}Tyr$) was found to have no significant clinical impact on MPA pharmacokinetics in healthy volunteers or renal transplant recipients.[241, 251]

Clinical studies assessing the role of *ABCC2* in MPA disposition are scarce, as transporter genetics is a novel research area compared to metabolic enzymes. From the few available studies, the *ABCC2* C-24T was the most promising candidate for further research. However, clinical results are often inconsistent, and there is still much to discover regarding the mechanism and interaction of genetic modulation.

1.6.3. Prospectus

Pharmacogenetics is a rapidly advancing area of research. However, the state of knowledge of MPA pharmacogenetics is in its infancy, and non-genetic and environmental factors also contribute to variability in its pharmacokinetics. To date, there

are no pharmacogenetic studies targeted at the thoracic transplant population, even though this transplant group suffers from worse transplant outcomes compared to the kidney transplant population. The ramifications of under-dosing or overdosing mycophenolate portend poor clinical outcomes, impaired quality of life and shortened survival for these patients. While it is a long journey with numerous challenges to overcome before genetic information can be utilized directly in individualizing mycophenolate therapy, pharmacogenetics provides powerful complementary information that can potentially improve immunosuppressive therapy and transplant outcomes. In the mean time, MPA PK variability is not well characterized in the thoracic transplant population, and monitoring of MPA exposure is challenging in the clinic. While LSSs provide a convenient alternative for monitoring MPA exposure, specific LSSs developed for the thoracic transplant group are lacking. This proposed study aims to fill the knowledge gap in MPA pharmacokinetic variability, limited sampling strategies and pharmacogenetics, thereby improving treatment response in thoracic transplant recipients.

1.7. Hypothesis

In thoracic transplant recipients on steady-state mycophenolate therapy, inter-patient variability in MPA pharmacokinetics can be partially explained by genetic variability of the UGTs and ABCC2 responsible for MPA's disposition.

1.8. Objectives

This study addressed the following objectives:

1. To characterize the pharmacokinetics of MPA and its two main metabolites (MPAG and AcMPAG) in stable thoracic transplant recipients. This was achieved by serial blood sampling over the MMF dosing period (12 hours). The AUC ratios of MPAG/MPA and AcMPAG/MPA were determined.
2. To genotype subjects for known and novel genetic polymorphisms in the *UGT* and *ABCC2* genes (promoter and coding regions) that are involved in MPA disposition.
3. To assess associations between *UGT* and *ABCC2* genotypes and the pharmacokinetics of MPA by stratifying subjects into different *UGT* genotypes and comparing the pharmacokinetics of MPA via multiple regression analysis.
4. To assess the relationship between PKs and clinical outcomes *(secondary)*.
5. Since full pharmacokinetic profiles are collected for this transplant population, an auxiliary objective is to utilize this valuable data source to update limited sampling strategies for MPA in heart and lung transplant populations for a convenient estimation of MPA exposure.

1.9. Tables

Table 1.1. Summary of mycophenolic acid pharmacokinetic studies in thoracic transplant recipients

Study	Subjects	Therapy	Compounds measured	MPA PK results summary
Armstrong et al.[83]	9 adult heart transplant recipients	MMF (3 g/day), CSA, prednisolone	MPA, MPAG, AcMPAG	Median (range) MPA AUC: 33.8 (26.6–40.3) µg*h/mL
Baraldo et al.[84]	9 adult heart transplant recipient, 44 PK profiles	MMF (2 – 3 g/day), CSA, prednisone	MPA	MPA Cmax 10.4 ± 6.6 mg/L MPA AUC: 45.9 ± 15.4 (range 13.4 – 91.7) mg*h/L
Cussonneau et al.[85]	7 heart transplant recipients	MMF (2 – 3 g/day), CSA, Corticosteroids	MPA, fMPA, MPAG, free MPAG	MPA free fraction 3.6±3.9%, free MPA concentration ranges 0.006 – 0.331 µg/mL
DeNofrio et al.[86]	38 heart transplant recipients	MMF (2 g/day), CSA, Prednisone (tapered over time)	MPA, fMPA	MPA free fraction 1.9±0.4% and fMPA AUC 0.83±0.30 µg*h/mL
Dosch et al.[87]	62 heart transplant recipients	MMF (3 g/day), CSA or SRL,	MPA	MPA AUC estimated by limited sampling strategy Dose-normalized MPA AUC: 31.92±16.12 (range 13.41 – 82.29) mg*h/L for CSA group; 60.95±27.42 (range 23.69 – 131.54) mg*h/L for SRL group Dose-normalized C0:

Study	Subjects	Therapy	Compounds measured	MPA PK results summary
				1.41±0.95 mg/L for CSA group; 5.1±3.4 mg/L for SRL group
Ensom et al.[54]	7 lung adult transplant recipients	MMF (1 – 3 g/day), CSA, Prednisone	MPA, fMPA	MPA free fraction 2.90±0.56% (range 2.00 to 3.40%) fMPA AUC of 1.29±0.50 (range 0.54 to 1.88 µg*h/mL) Dose-normalized AUC 23.57±15.76 (range 5.52 to 51.21 µg*h/mL)
Ensom et al.[55]	5 lung and 4 heart adult transplant recipients	MMF (2 – 3 g/day), CSA or TAC, Prednisone (tapering dose)	MPA, fMPA	Mean MPA free fraction 4.3 to 7.1% Mean MPA AUC 25.24 to 43.96 µg*h/mL
Gajarski et al.[90]	10 young adult and 16 pediatric heart transplant recipients	MMF (37.9±12.5 mg/Kg), CSA or TAC, Corticosteroid	MPA, MPAG	MPA levels higher in children taking TAC. MPAG/MPA ratios: 37.7±40.2 in young adult; 16.0±18.1 in pediatric heart transplant recipients; higher in patients taking CSA (vs. TAC)
Gerbase et al.[96]	30 adult lung transplant recipients	MMF (25 – 35 mg/Kg/day, maximum 2 g/day), CSA or TAC,	MPA trough levels	MPA trough levels ranged from ~1 – 11 µg/mL Patients with cystic fibrosis received

Study	Subjects	Therapy	Compounds measured	MPA PK results summary
		prednisone		significantly lower MMF dose, but no difference in MPA trough levels between patients with or without cystic fibrosis
Hummel et al.[91]	15 heart transplant recipients	MMF (0.25 – 3 g/day), CSA	MPA, MPAG	Mean MPA AUCs: 52.7, 71.3, and 80.0 µg*h/mL at weeks 2, 12, 52 post-transplant, respectively High inter-and intra-patient variability in MPA PKs
Kaczmarek et al.[92]	28 heart transplant recipients	MMF (0.25 – 2 g/day), TAC	MPA	Dose-normalized MPA AUC: 45.5±22.1 (range 8.1 – 87.9) mg*h/L Mean MPA C0: 2.0±1.2 mg/L
Lehmkuhl et al.[93]	154 heart transplant recipients	MMF (3 g/day) or enteric-coated MPA sodium (2.16 g/day), prednisone	MPA	Median MPA AUC: 71.3 µg*h/mL for MMF
Seebacher et al.[94]	7 heart transplant recipients	MMF (2 g/day)	MPA, MPAG	Development of high-performance liquid-chromatography assay for measurement of MPA and MPAG MPA AUC: 58.05 ± 11.05 and 46.75 ± 14.25 µg*h/mL after first and second MMF dose
Ting et al.[89]	21 adult lung transplant	MMF (1 – 3 g/day), CSA or TAC,	MPA, MPAG, AcMPAG	Dose-normalized MPA AUC: 23.4 ± 13.8 (range 3.3 – 57.1 µg*h/mL)

Study	Subjects	Therapy	Compounds measured	MPA PK results summary
	recipients	prednisone		
Ting et al.[88]	27 lung and 23 heart transplant recipients	MMF (0.5 – 3 g/day), CSA or TAC, and/or prednisone	MPA, MPAG, AcMPAG	Median (range) of dose-normalized MPA AUC: 27.85 (3.39 – 115.31) for lung transplant recipients; 79.00 (16.89 – 218.73) for heart transplant recipients
Wada et al.[95]	22 heart transplant recipients	MMF (1 – 3 g/day), CSA or TAC, corticosteroids	MPA	MPA AUC: 32.57±13.07 (range 13.11 – 50.98) µg*h/mL for CSA group; 58.55±17.51 (range 39.19 – 93.18) µg*h/mL for TAC group MPA Cmax: 8.82±4.10 µg/mL for CSA group; 14.23±7.23 µg/mL for TAC group

AcMPAG = acyl glucuronide of mycophenolic acid; AUC = area-under-the-concentration-time-curve; Cmax = maximum concentration; Cmin = minimum concentration; CSA = cyclosporine; fMPA = free MPA; MMF = mycophenolate mofetil; MPA = mycophenolic acid; MPAG = 7-O-mycophenolic acid glucuronide; PK = pharmacokinetic; SRL = sirolimus; TAC = tacrolimus

Table 1.2. Summary of limited sampling strategies for estimation of mycophenolic acid exposure in adult transplant recipients

Study	Patient population	LSS methods	Concentrations used	Comedications
LSSs developed in the kidney transplant population				
Chen et al.[176]	40 Chinese adults (72 PK profiles), liver transplant	Linear regression	(C1, C2, C6, C8)	MMF + TAC + steroids
Cho et al.[177]	10 Korean adults, kidney transplant	Linear regression	C0 or C1 or C8	MMF + CSA + steroids
Fleming et al.[178]	31 Indian adults (39 PK profiles), kidney transplant	Linear regression	AUC(0-6) calculated from (C0, C0.5, C1, C1.5, C2, C2.5, C3, C4, C5, C6)	MMF + prednisolone +/- CSA
Jiao et al.[179]	12 Chinese adults, kidney transplant	Linear regression	(C2, C4) for free AUC; (C1, C2, C3) for total AUC; (C2, C3, C4) and (C1, C2, C4) for estimating both free and total simultaneously	MMF + CSA + steroids
Johnson et al.[56]	10 adults, kidney transplant	Linear regression	(C0, C1, C3, C6)	MMF + CSA + prednisolone
Kuriata-Kordek et al.[104]	131 adults, kidney transplant	Not specified	(C2, C6) for CSA co-medication; (C4, C8, C12) for TAC co-medication	MMF + CSA or TAC + prednisone
Le Guellec et al.[180]	60 adults, kidney transplant	Bayesian	(C0.33, C1, C3) for both MPA and CSA	MMF + CSA +/- steroids

Miura et al.[266]	50 Japanese adults, kidney transplant	Linear regression	(C2, 4, 9) or (C0, 2, 4)	MMF + TAC + methylprednisolone
Muller et al.[182]	18 adults, kidney transplant	linear regression	(C0, C1, C2)	MMF + CSA + prednisone
Pawinski et al.[183]	21 adults (50 PK profiles), kidney transplant	Linear regression	(C0, C0.5, C2)	MMF + TAC
Premaud et al.[184]	44 adults, kidney transplant	Bayesian	(C0.33, C1, C3) for both MPA and CSA	MMF + CSA +/- steroids (depending on time post-tx)
Teshima et al.[185]	18 adults, kidney transplant	Linear regression	(C2, C7, C12)	MMF + TAC + steroids
Toda et al.[186]	6 Japanese adults (12 profiles), kidney transplant	Linear regression	(C0, C3, C6)	MMF + TAC + steroids (prednisolone)
van Hest et al.[175]	136 adults (257 PK profiles), kidney transplant	Linear regression	(C0, C0.67, C2)	MMF + CSA + steroids
Yeung et al.[187]	10 adults (29 PK profiles), kidney transplant	Linear regression	(C0, C1, C2, C4)	MMF + CSA + prednisolone
Zicheng et al.[189]	31 Chinese adults, kidney transplant	Linear regression	(C0.5, C1, C4, C10)	MMF + CSA + prednisone
Zicheng et al.[188]	53 adult (56 PK profiles), kidney transplant	Linear regression	(C0.5, C1, C4, C10)	MMF + CSA + steroids

Zhou et al.[190]	75 Chinese adults, kidney transplant	Linear regression	(C0.5, C2, C4) or (C0.5, C2, C8)	MMF + CSA + steroids
LSSs developed in the heart transplant population				
Baraldo et al.[84]	9 adults (44 PK profiles), heart transplant	Linear regression	(C1.25, C2, C6) or (C1.25, C2, C4, C6)	MMF + CSA + prednisone
Wada et al.[95]	22 adults, heart transplant	Linear regression	(C0, C1, C2) for CSA co-medication; (C1, C2, C4) for TAC co-medication	MMF + CSA or TAC + corticosteroids
Kaczmarek et al.[92]	28 adults, heart transplant	Linear regression	(C0.5, C1, C2) or (C0.5, C2)	MMF + TAC
LSSs developed in the lung transplant population				
Ting et al.[191]	21 adults, lung transplant	Linear regression	(C0, C2) or (C0, C1.5)	MMF + CSA or TAC + prednisone

AUC = area-under-the-concentration-time-curve; Cx = concentration at time x; CSA = cyclosporine; LSS = limited sampling strategy; MMF = mycophenolate mofetil; MPA = mycophenolic acid; PK = pharmacokinetic; SRL = sirolimus; TAC = tacrolimus

Table 1.3. Impact of *UGT* genetic variants on the pharmacokinetics of MPA – summary of clinical studies

Gene	Allele	Variant	Amino acid change	Reported functional impact	References
UGT1A1	*6	G211A	Gly^{71}Arg	↑ MPAG exposure and MPAG/MPA in Japanese renal transplant recipients[181]	181
UGT1A6	*2	T181A	Arg^{184}Ser	↑ MPAG exposure and MPAG/MPA in Japanese renal transplant recipients[181]	181
UGT1A7	*2	T387G/ C391A/ G392A	Asn^{129}Lys / Arg^{131}Lys	No significant impact on MPAG exposure or MPAG/MPA in Japanese renal transplant recipients[181] No significant impact on MPA pharmacokinetics in Japanese renal transplant recipients[257] Modest ↓ MPA exposure in healthy volunteers[251]	181, 251, 257
UGT1A7	*3	T387G/ C391A/ G392A/ T622C	Asn^{129}Lys / Arg^{131}Lys / Trp^{208}Arg	No significant impact on MPAG exposure or MPAG/MPA in Japanese renal transplant recipients[181] No significant impact on MPA pharmacokinetics in Japanese renal transplant recipients[257] No significant impact on MPA metabolism in healthy volunteers[251]	181, 251, 257
UGT1A8	*2	C518G	Ala^{173}Gly	No significant impact on MPA metabolism in healthy volunteers[258] ↑ MPA trough concentration in kidney, pancreas and kidney-	181, 258-260

Gene	Allele	Variant	Amino acid change	Reported functional impact	References
				pancreas transplant recipients also taking TAC (but not CSA)[259] No significant impact on MPAG exposure or MPAG/MPA in Japanese renal transplant recipients[181] No significant impact on MPA pharmacokinetics in Japanese renal transplant recipients[260]	
UGT1A8	*3	G830A	Cys^{277}Ile	No significant impact on MPA metabolism in healthy volunteers[258] No impact on MPA trough concentrations in in kidney, pancreas and kidney-pancreas transplant recipients[259] ↑ occurrences of infections with *3 allele and with haplotype (-999C/codon 55A/codon 277A) in renal transplant recipients taking 2g/day MMF[261]	[258, 259, 261]
UGT1A9		C-2152T/ T-275A	N/A	↓ exposure to MPA, ↓ EHC of MPA, MPAG and AcMPAG, but no changes in MPAG exposure in healthy volunteers[258] ↓ MPA trough concentrations in kidney, pancreas and kidney-pancreas transplant recipients also taking CSA (but not TAC)[259] ↓ MPA AUC, EHC [MPA(6-12)] and trough concentrations only in renal transplant subjects taking 2 g MMF/day (but not 1 g/day)[262] No significant impact on MPA pharmacokinetics in renal transplant	[258, 259, 262, 263]

Gene	Allele	Variant	Amino acid change	Reported functional impact	References
				recipients[263]	
UGT1A9		T-1887G	N/A	No significant on MPA pharmacokinetics in renal transplant recipients[263]	263
UGT1A9		C-665T	N/A	No significant on MPA pharmacokinetics in renal transplant recipients[263]	263
UGT1A9		C-440T/ T-331C	N/A	↑ MPA total exposure by 50% and ↑ MPA absorption [indicated by AUC(0-2)] in renal transplant recipients with homozygous variant genotype[263] No impact on occurrences of gastrointestinal toxicities[263] No significant impact on MPA metabolism in healthy volunteers[251]	251, 263
UGT1A9		-118 t-stretch (9>10)	N/A	↑ MPA AUC(6-12), MPAG(0-12) and MPAG AUC(0-6) in Chinese renal transplant recipients[250] No significant impact on MPA metabolism in healthy volunteers[251]	250, 251
UGT1A9	*3	T98C	Met[33]Tyr	↑ MPA and AcMPAG exposure in healthy volunteers[258] No impact on MPA trough concentrations in in kidney, pancreas and kidney-pancreas transplant recipients[259]	258, 259
UGT1A9		Intron C399T	N/A	No significant impact on MPAG exposure or MPAG/MPA in	181, 251, 257

Gene	Allele	Variant	Amino acid change	Reported functional impact	References
		(I399)		Japanese renal transplant recipients[181] No significant impact of MPA pharmacokinetics in Japanese renal transplant recipients[257] No significant impact on MPA metabolism in healthy volunteers[251]	
UGT2B7		G-900A (reversely linked to *2a)	N/A	↑ AcMPAG exposure [AUC(0-9)] in renal transplant recipients also taking SRL (but not TAC or CSA); polymorphism reversely linked to *UGT2B7**2a[118] No significant impact on AcMPAG exposure, AcMPAG trough concentration, AcMPAG/MPA; no difference in incidence of diarrhea or leucopenia with AcMPAG exposure or *UGT2B7* G-900A variant in renal transplant recipients[163]	118, 163
UGT2B7		G211T	Ala[71]Ser	No significant impact on MPA or MPAG pharmacokinetics in Chinese renal transplant recipients[250]	250
UGT2B7[a]	*2a	C802T	His[268]Tyr	↑ MPA, fMPA and MPAG exposure, and ↑ AcMPAG urinary excretion in healthy volunteers[258] No significant impact on MPA or MPAG pharmacokinetics in Chinese renal transplant recipients[250] No significant impact on MPA pharmacokinetics in Japanese renal transplant recipients[260]	118, 250, 258, 260

Gene	Allele	Variant	Amino acid change	Reported functional impact	References
				↓ AcMPAG exposure [AUC(0-9)] in renal transplant recipients taking also taking SRL (but not TAC or CSA); polymorphism reversely linked to *UGT2B7* G-900A[118]	

[a] *UGT2B7* C802T (His^{268}Tyr) is in complete linkage disequilibrium with variant promoter (-1306, -1299, -1112, -900, -327, -161) and in incomplete linkage disequilibrium with variant 372G (codon 124)

A = adenosine; AcMPAG = acyl glucuronide of mycophenolic acid; Ala = alanine; Arg = arginine; Asn = Asparagine; AUC = area-under-the-concentration-time-curve; C = cytosine; Cys = cysteine; EHC = enterohepatic recircultion; fMPA = free mycophenolic acid; G = guanosine; Gly = glycine; His = histidine; Ile = isoleucine; Lys = lysine; Met = methionine; MPA = Mycophenolic acid; MPAG = 7-O-mycophenolic acid glucuronide; N/A = not applicable; Ser = serine; T = thymine; Trp = tryptophan; Tyr = tyrosine; UGT = UDP-glucuronosyltransferase

Table 1.4. Impact of *ABCC2* genetic variants on the pharmacokinetics of MPA – summary of clinical studies

Gene	Variant	Amino acid change	Reported functional impact	References
ABCC2	A-1549G	N/A	No impact on MPA pharmacokinetics at 7 days post-transplant in renal transplant recipients with or without liver dysfunction[241]	241
ABCC2	G-1023A	N/A	No impact on MPA pharmacokinetics at 7 days post-transplant in renal transplant recipients with or without liver dysfunction[241]	241
ABCC2	A-1019G	N/A	No impact on MPA pharmacokinetics at 7 days post-transplant in renal transplant recipients with or without liver dysfunction[241]	241
ABCC2	C-24T	N/A	No impact on MPA pharmacokinetics or occurrences of diarrhea in Japanese renal transplant recipients; however, MPA apparent clearance was significantly lower in patients who were carriers of *ABCC2* -24T and homozygous for *SLCO1B3* T334G variants.[256] No impact on MPA, MPAG or AcMPAG exposures in Chinese renal transplant recipients[250] No significant impact on MPAG exposure or MPAG/MPA in Japanese renal transplant recipients[181] No significant impact on MPA pharmacokinetics in renal transplant recipients[263] No impact on MPA pharmacokinetics at 7 days post-transplant, and no difference in MPA pharmacokinetics between patients with and without liver dysfunction; In non-carriers of this variant, however, patients with	181, 241, 250, 251, 256, 263

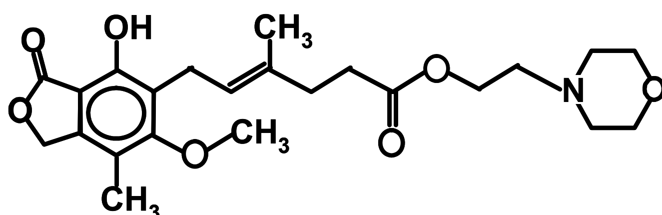
Gene	Variant	Amino acid change	Reported functional impact	References
			liver dysfunction had lower MPA exposure.[241] ↑ MPA trough levels and exposure in stable (>42 days post-transplant) renal transplant recipients[241] ↑ episodes of diarrhea within first year transplant in renal transplant recipients[241] ↑ AcMPAG exposure in healthy volunteers[251]	
ABCC2	G1249A	Val[417]Ile	↑ AcMPAG exposure in Chinese renal transplant recipients[250] No impact on MPA pharmacokinetics at 7 days post-transplant in renal transplant recipients with or without liver dysfunction[241] No significant impact on MPA pharmacokinetics in healthy volunteers[251] No significant impact on MPA pharmacokinetics in renal transplant recipients[263]	[241, 250, 251, 263]
ABCC2	C3972T	Ile[1324]Ile	Similar clinical impact as *ABCC2* C-24T as the two polymorphisms are in moderate linkage disequilibrium; no impact on MPA pharmacokinetics at 7 days post-transplant, and no difference in MPA pharmacokinetics between patients with and without liver dysfunction; In non-carriers of this variant, however, patients with liver dysfunction had lower MPA exposure. Also ↑ MPA trough levels and exposure in stable (>42 days post-transplant) renal transplant recipients[241]	[241, 251]

Gene	Variant	Amino acid change	Reported functional impact	References
			No significant impact on MPA pharmacokinetics in healthy volunteers[251]	
ABCC2	G4544A	$Cys^{1515}Tyr$	No impact on MPA pharmacokinetics at 7 days post-transplant in renal transplant recipients with or without liver dysfunction[241] No significant impact on MPA pharmacokinetics in healthy volunteers[251]	[241, 251]

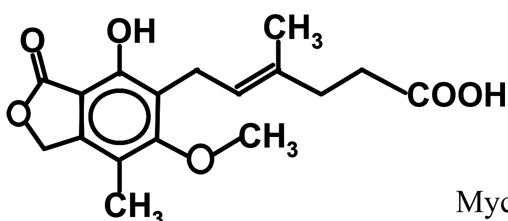
A = adenosine; ABCC2 = MRP2 = multidrug resistance-associated protein 2; AcMPAG = acyl glucuronide of mycophenolic acid; C = cytosine; Cys = cysteine; G = guanosine; Ile = isoleucine; MPA = mycophenolic acid; MPAG = 7-O-mycophenolic acid glucuronide; N/A = not applicable; SLCO = OATP = organic anion transport protein; T = thymine; Tyr = tyrosine; Val = valine

1.10. Figures

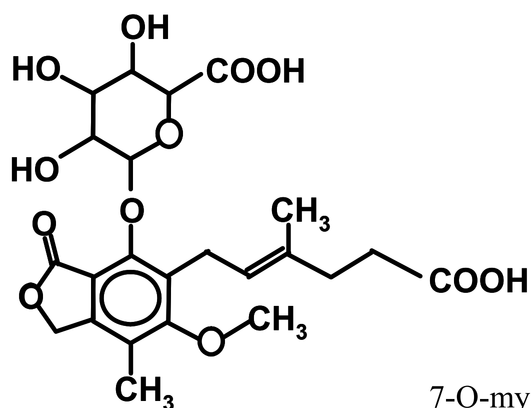
Figure 1.1. Chemical structures of MMF, MPA, MPAG and AcMPAG



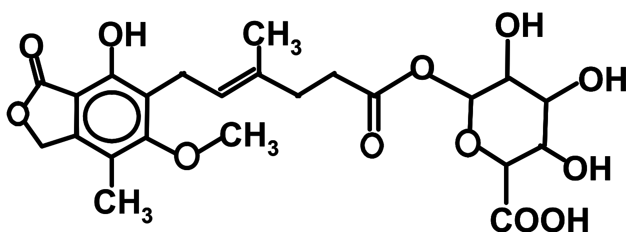
Mycophenolate mofetil (MMF)



Mycophenolic acid (MPA)

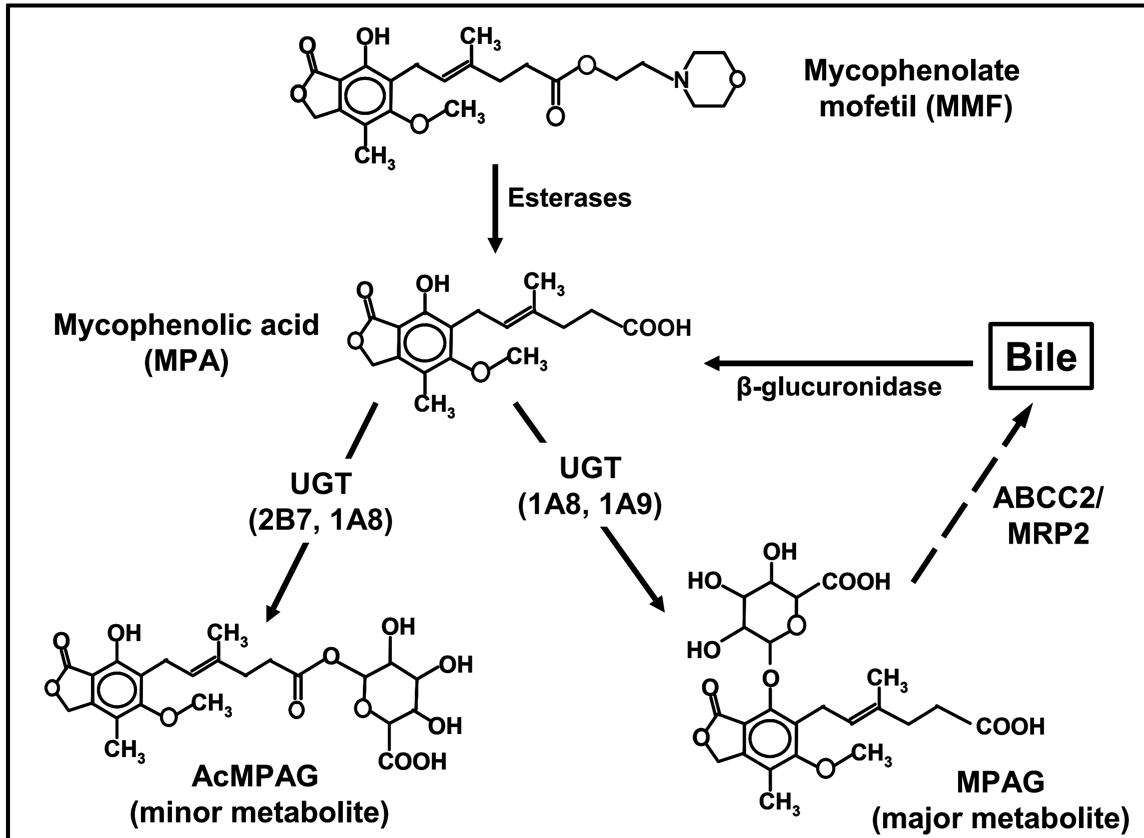


7-O-mycophenolic acid glucuronide (MPAG)



Acyl glucuronide of mycophenolic acid (AcMPAG)

Figure 1.2. Major metabolic pathways of mycophenolate mofetil



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2. PHARMACOKINETICS OF MYCOPHENOLIC ACID AND ITS PHENOLIC-GLUCURONIDE AND ACYL-GLUCURONIDE METABOLITES IN STABLE THORACIC TRANSPLANT RECIPIENTS[1]

[1] A version of this chapter has been published as a full manuscript. **Ting LS**, Partovi N, Levy RD, Riggs KW, Ensom MHH. Pharmacokinetics of mycophenolic acid and its phenolic-glucuronide and acyl-glucuronide metabolites in stable thoracic transplant recipients. *Ther Drug Monit* 2008; 30: 282 – 291.

2.1. Introduction

Mycophenolate mofetil (MMF; CellCept®) is an immunosuppressive agent commonly used in kidney, liver, heart and lung transplantation.[1-4] Approximately 50% of lung and liver as well as over 80% of kidney, heart or pancreas transplant recipients in the United States are prescribed MMF as part of a maintenance immunosuppressive regimen that may also include cyclosporine (CSA), tacrolimus (TAC) or sirolimus (SRL) plus or minus corticosteroids.[5-8]

MMF is the 2,4-morpholino-ester of mycophenolic acid (MPA), a potent and selective inhibitor of inosine monophosphate dehydrogenase. Inhibition of inosine monophosphate dehydrogenase prevents the *de novo* synthesis of guanine nucleotides, thereby inhibiting both T and B cell proliferation.[9, 10] After oral administration, MMF is rapidly and completely absorbed, and then hydrolyzed (by esterases in plasma, liver, and kidney) to MPA, the pharmacologically active moiety.[6, 11] The metabolism of MPA consists mainly of glucuronidation, catalyzed by UDP-glucuronosyltransferase (UGT) enzymes. The primary (inactive) metabolite is 7-O-mycophenolic acid glucuronide (MPAG), which is usually present in plasma in ~20- to 100-fold higher concentrations than MPA.[11, 12] At least two other minor metabolites exist, one of which, the acyl glucuronide (AcMPAG), exhibits immunosuppressive and proinflammatory activity.[11, 13-15] MPAG is released into the bile and de-glucuronidated in the gastrointestinal (GI) tract and reconverted into MPA in the gut lumen where it is then reabsorbed through enterohepatic recirculation. This enterohepatic circulation is reported to occur between 6 to 12 hours following drug administration and accounts for 10% to 60% of total MPA exposure.[12]

The importance of studying MPA's glucuronidated metabolites is underscored by MPAG's de-glucuronidation to MPA as well as AcMPAG's pharmacologic activity. Adverse effects of MMF such as GI disturbances and hematopoietic toxicity are commonly reported and may be associated with excessive MPA and AcMPAG concentrations.[4, 13, 16, 17] Many studies conducted primarily in kidney transplant recipients have evaluated the pharmacokinetics of MPA; however, relatively few have studied the pharmacokinetics of both MPAG and AcMPAG.[18-24] Furthermore, to our knowledge, virtually all published research reports in heart transplant recipients investigated only MPAG,[25-28] and only one also measured AcMPAG.[24] As well, we are aware of only two research studies,[29, 30] other than those from our investigative group,[31, 32] that have evaluated the pharmacokinetics of MPA specifically in lung transplantation; neither of these measured MPAG nor AcMPAG. Thus, to our knowledge, only two studies (a bioavailability study in 9 heart transplant recipients[24] and a preliminary pharmacokinetic study in 21 lung transplant recipients)[32] have characterized the pharmacokinetics of MPA, MPAG, and AcMPAG in thoracic transplant recipients.

Similarly, relatively little data are available on free MPA concentration (fMPA) in thoracic transplant recipients, despite fMPA being recognized as the pharmacologically active moiety.[33] We are aware of only 4 small studies (two in heart,[25, 34] one in heart and lung,[35] and one in lung[31] transplant recipients) that measured free MPA in addition to total MPA concentration.

In light of the relative paucity of pharmacokinetic data in thoracic transplant recipients, the primary objective of this study was to characterize and compare

pharmacokinetic parameters and metabolic ratios of MPA in stable lung or heart transplant recipients.

2.2. Materials and Methods

2.2.1. Subjects

This pharmacokinetics study was part of an open-label clinical study evaluating the pharmacogenetics of mycophenolate. The study was approved by the Clinical Research Ethics Board of the University of British Columbia (#C02-0568) and Vancouver Coastal Health Authority Clinical Trials Administration Office (#V03-0162) (Appendix A). Thoracic (heart or lung) transplant recipients were recruited via the Vancouver General Hospital lung transplant program, the St. Paul's Hospital heart transplant program, and the British Columbia Transplant Society. Subjects were included if they were on steady-state MMF (twice-daily for at least five days), at least 16 years old, provided informed consent, and not taking interacting medications (such as antacids or cholestyramine). Sample size was based on one of convenience.

2.2.2. Study protocol

Subjects reported to the British Columbia Transplant Society on the study day before their morning dose of MMF was administered. Subjects were asked to fast overnight before reporting to the British Columbia Transplant Society clinic on the study day, but there were no restrictions on activity or food intake during the study day. After obtaining written informed consent (Appendix B, UBC Clinical Research Ethics Board #C02-0568), an indwelling intravenous catheter was placed in a forearm vein for serial

blood collection. Blood samples were collected in 3-mL vacutainers containing ethylenediaminetetraacetic acid (BD Vacutainer® K_3EDTA, Franklin Lake, NJ) at pre-dose (time 0), and at 20, 40, 60 and 90 minutes, and 2, 4, 6, 8, 10 and 12 hours after taking their morning dose of MMF. The collection tubes were inverted several times and put on ice until centrifugation. The catheter line was then flushed with sodium chloride solution (U.S.P. 0.9%, Abbott Laboratory Bacteriostatic injection USP), followed by heparin lock flush solution (100 U.S.P. units/mL, heparin sodium injection BP, LEO Pharma Inc.). About 1 mL of blood was collected in the same manner and discarded immediately before the next sample collection to ensure no contamination with the heparin flush solution. Separation of plasma was achieved by centrifugation at 1380 x g for 5 minutes (Centrifuc Centrifuge 228, Fisher Healthcare, Houston, TX). In addition, urine was collected (and kept on ice) throughout the 12-hour study period for determination of MPA, MPAG and AcMPAG concentrations. The total volume of urine voided during the 12-hour study period was recorded.

Plasma and urine samples were collected in both non-acidified and acidified (pH 2-4 with 85% phosphoric acid solution, 20 µL acid per 1 mL plasma or urine) aliquots. At each collection time point, a 1 mL-aliquot of plasma was acidified, and the rest of the available plasma stored in non-acidified vials. For urine samples, two 25-mL acidified and one 25-mL non-acidified aliquots were collected. All plasma and urine samples were stored at -80 ^{o}C until analysis. Acidification was necessary to preserve the minor metabolite AcMPAG, and non-acidified samples were collected for free MPA measurements.

2.2.3. Chemicals

All solvents were HPLC grade. Orthophosphoric acid (85%), methanol (>99.9%), acetonitrile (99.9%) and water were purchased from Fisher Scientific Company (Ottawa, ON). Potassium dihydrogen phosphate (HPLC-grade, KH_2PO_4) crystalline and indomethacin (99%) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON). Analytical grade MPA, MPAG and AcMPAG were gifts from Roche Palo Alto LLC (Palo Alto, CA).

2.2.4. Plasma concentrations of MPA, MPAG and AcMPAG

The concentrations of MPA, MPAG, and AcMPAG were determined quantitatively in patient plasma samples by high performance liquid chromatography (HPLC) with ultraviolet detection, which was updated from a previously described method.[32] The HPLC instrumentation (Waters Alliance System, Waters Ltd., Mississauga, ON) consisted of a delivery pump, an automatic injector equipped with a 200 µL injector loop, an Atlantis dC18 (5µm, 4.6 x 150 mm) or a Symmetry C8 column, an Atlantis dC18 guard column (3.9 x 20 mm), and an ultraviolet detector set at 210 nm. An integrator was used to measure peak areas.

Solutions of 1 mg/mL of MPA, AcMPAG, MPAG and indomethacin (internal standard) were prepared in HPLC-grade methanol and kept at -20°C. Stock solutions of 100, 10, and 1 µg/mL of MPA and AcMPAG and 100 µg/mL of MPAG were prepared in HPLC-grade methanol. Calibration standards of the compounds were prepared by serial dilution in acidified plasma to obtain the following standard concentrations: 0.25, 0.50, 1.00, 2.00, 5.00, 10.00, 15.00, 20.00, and 30.00 µg/ml for MPA; 0.20, 0.25, 0.50, 1.00, 2.00, 5.00, 10.00, 15.00, and 20.00 µg/ml for AcMPAG and 5.00, 10.00, 20.00, 30.00,

40.00, 60.00, 80.00, 90.00 and 100.00 μg/ml for MPAG. Stock solutions also contained 5.00 μg/ml of the internal standard (IS) indomethacin. A set of standards was prepared for each analytical run.

Briefly, samples were kept on ice for the duration of the extraction for total MPA, MPAG and AcMPAG. Cold acetonitrile containing 5 μg/mL IS (1.2 mL, at -20°C) was added to 300 μL of plasma sample and vortex-mixed. The supernatant was separated by centrifugation at 1250 x g at 4ºC, and evaporated to dryness for 15 min at 37°C under 25 psi nitrogen flow. Samples were re-constituted in 300 μL of 20% acetonitrile. In addition, a 100 μL aliquot was further diluted 1:6 for detection of MPAG. Samples were filtered with Acrodisc® syringe filters (GHP membrane, 0.45μm) before injection (50 μL) onto the HPLC column. The mobile phase consisted of a gradient of 0%-60%: 100%-40% (v/v) acetonitrile : 0.01M phosphate buffer (KH_2PO_4, pH 3.0) at a flow rate of 2 mL/min:

0 minutes: 0% acetonitrile, 100% phosphate buffer

0 – 3.75 minutes: 62% acetonitrile, 38% phosphate buffer

3.75 – 8 minutes: 0% acetonitrile, 100% phosphate buffer

The retention times of MPA, MPAG, AcMPAG and indomethacin were 4.9, 3.4, 3.8, and 6.7 minutes, respectively.

Assay validation involved calibration curves for MPA, MPAG and AcMPAG with 9 standards and a blank at the beginning of each run. Calibration curves were generated by least-squares regression of the peak areas versus concentration of each standard. Precision and reproducibility of the assay were evaluated by running quality control samples at the lowest limit of quantification (LOQ), low, medium and high concentrations of 0.25, 4.00, 12.00, 25.00 μg/mL for MPA, 5.00, 20.00, 50.00, 75.00

µg/mL for MPAG, and 0.20, 3.00, 12.00, 18.00 µg/mL for AcMPAG, respectively, in quadruplicates for four days. Means, standard deviations, and coefficients of variation (CV) were calculated. Table 2.1 summarizes the intra-day and inter-day CV for each compound. Accuracies for MPA, MPAG and AcMPAG measurements were 87.90 – 99.40%, 89.60 – 97.03%, and 84.00 – 98.60%, respectively. The LOQs were 0.25, 5.00, and 0.20 µg/mL for MPA, MPAG and AcMPAG, respectively.

2.2.5. Free MPA analysis

Since the free fraction of MPA is known to be low (~3%) and concentration-independent, the plasma samples were spiked to ensure adequate free MPA concentrations within the analytical range of the HPLC assay.[36] Non-acidified plasma samples from subjects were pooled to obtain 1000 µL of plasma. The plasma was spiked with 25 µL of MPA stock solution (1 mg/mL) and vortex-mixed. The concentration of MPA in the spiked sample was at least 25 µg/mL, and would result in a free MPA concentration of at least 0.75 µg/mL, assuming a free fraction of 3%. A 400-µL aliquot of this spiked plasma was reserved for total MPA concentration quantification according to the procedure described above (Section 2.2.4). For free MPA concentration (fMPA), 500 µL of the spiked plasma was filtered with a Microcon YM-30 filter (30000 molecular weight cut-off, Millipore, Billerica, MA) under centrifugation for 75 minutes at 4°C and 1380 x g. An equal volume of 20% acetonitrile (in HPLC-grade water) containing 10 µg/mL indomethacin was added to the filtrate before injection (50 µL) onto the HPLC column.[36] Free fraction was calculated by dividing the fMPA by total MPA concentration in the spiked plasma.

2.2.6. Urine concentrations of MPA, MPAG and AcMPAG

The concentrations of MPA, MPAG, and AcMPAG were determined quantitatively in patient urine samples by high performance liquid chromatography (HPLC) with ultraviolet detection. The HPLC instrumentation (Waters Alliance System module 2695, Waters Ltd Mississauga, ON) consisted of a delivery pump, an automatic injector equipped with 200 µL injector loop, and an ultraviolet detector (Waters 2996 photodioade array detector, Waters Ltd. Mississauga, ON) set at 215 nm. An integrator was used to measure peak areas. An Atlantis dC18 (5µm, 4.6 x 150 mm) column was used for the MPAG assay, and an Aquity UPLC BEH C18 column (1.7 µm, 2.1 x 100 mm) for the MPA and AcMPAG assays.

Stock solutions of MPA, MPAG, AcMPAG and indomethacin (internal standard; IS) were prepared in HPLC-grade methanol. Calibration standards of the compounds were prepared by serial dilution in acidified urine. A separate method was developed to quantify MPAG, while MPA and AcMPAG were simultaneously assayed. Stock solutions of MPA (1000 µg/mL), MPAG (4000 µg/mL) and AcMPAG (1000 µg/mL) were prepared in HPLC-grade methanol. Indomethacin (50 µg/mL) in HPLC-grade methanol was used as internal standard.

For the MPAG assay, patient urine samples were first diluted 20 times before sample preparation. Two hundred and fifty microliters of methanol (HPLC-grade) was added to 250 µL of urine sample and vortex-mixed. The mixture was subject to centrifugation at 13000 rpm (16000 x g) for 5 min. A 200 µL aliquot of the mixture was further diluted with 200 µL of methanol, and 100 µL of indomethacin (50 µg/mL in methanol) was added. Samples were filtered with Acrodisc® syringe filters (GHP

membrane, 0.45 μm) before injection (30 μL) onto the HPLC column. The sample temperature and column temperature were kept at 10°C and 30°C, respectively. The mobile phase consisted of a gradient of 25%-70%: 75%-30% (v/v) acetonitrile: 0.01M phosphate buffer (pH 3.0) at a flow rate of 1.0 mL/min:

0 – 1.5 minutes: 25% acetonitrile, 75% phosphate buffer

1.5 – 7.5 minutes: 70% acetonitrile, 30% phosphate buffer

7.5 – 10.5 minutes: 70% acetonitrile, 30% phosphate buffer

10.5 – 10.6 minutes: 25% acetonitrile, 75% phosphate buffer

10.6 – 12.5 minutes: 25% acetonitrile, 75% phosphate buffer

The retention times of MPAG and indomethacin were 5.5 and 10.3 minutes, respectively. Each run consisted of a urine blank and a six-point MPAG calibration curve prepared in acidified urine at 6.25, 12.50, 25.00, 50.00, 100.00 and 200.00 μg/mL.

For the MPA and AcMPAG assay, 250 μL of methanol (HPLC-grade) was added to 250 μL of urine sample and vortex-mixed. The mixture was subject to centrifugation at 13000 rpm (16000 x g) for 5 min, and 100 μL of indomethacin (50 μg/mL in methanol) was added to a 400 μL aliquot of the mixture. Samples were filtered with Acrodisc® syringe filters (GHP membrane, 0.45 μm) before injection (20 μL) onto the HPLC column. Sample and column temperatures were kept at 10°C and 35°C, respectively. The mobile phase consisted of a gradient of 15%-100%: 85%-0% (v/v) acetonitrile: 0.01M phosphate buffer (pH 3.0) at a flow rate of 0.2 mL/min:

0 minutes: 15% acetonitrile, 85% phosphate buffer

0 – 8.5 minutes: 50% acetonitrile, 50% phosphate buffer

8.5 – 11.5 minutes: 85% acetonitrile, 15% phosphate buffer

11.5 – 12.0 minutes: 100% acetonitrile, 0% phosphate buffer

12.0 – 15.0 minutes: 100% acetonitrile, 0% phosphate buffer

15.0 – 15.1 minutes: 15% acetonitrile, 85% phosphate buffer

15.1 – 20.0 minutes: 15% acetonitrile, 85% phosphate buffer

The retention times of MPA, AcMPAG and indomethacin were 13.4, 10.8 and 15.7 minutes, respectively. Each run consisted of a urine blank and a six-point calibration curve prepared in acidified urine at 1.25, 2.50, 5.00, 10.00, 20.00 and 40.00 μg/mL of MPA and AcMPAG.

The HPLC validation involved preparing calibration curves for MPA, MPAG and AcMPAG with six standards and a blank at the beginning of each run. Calibration curves were generated by least-squares regression of the peak areas versus concentration of each stock solution, with a weighing factor of $1/x^2$. Accuracy of the assays was evaluated by running four sets of quality control samples at 6.25 (lower limit of quantitation), 9.00, 60.00 and 180.00 μg/mL for MPAG; 2.00 (lower limit of quantitation), 6.00, 16.00 and 30.00 μg/mL for MPA and AcMPAG. Precision of the assays was evaluated by four aliquots of pooled quality control samples at the above concentrations. Validation samples were repeated for three batches. Mean, standard deviation and coefficient of variation (CV) were calculated. The regression analysis of the HPLC assay validation showed linearity over the working range of concentrations, with coefficients of determination (r^2) greater than 0.988 for MPAG, and 0.989 for MPA and AcMPAG. The intra-day and inter-day CV for three different concentrations and the lower limit of quantitation were within acceptable limits. Table 2.2 summarizes the inter-day and intra-day CV for each compound. Accuracies for MPA, MPAG and AcMPAG measurements

were 93.55 – 101.97%, 95.63 – 102.12%, and 86.54 – 100.33%, respectively. Lower limits of quantitation were 2.00 μg/mL for MPA, 6.25 μg/mL for MPAG and 2.00 μg/mL for AcMPAG.

2.2.7. Pharmacokinetic parameters assessment

Pharmacokinetic parameters were determined by non-compartmental analysis using WinNonlin Professional Version 5.2 (Pharsight, Mountain View, CA), and normalized by MMF dose (per g of MMF) for presentation of results. These included: Dose-normalized (DN) AUCs and partial AUCs [AUC(0-6) and AUC(6-12)] for MPA, MPAG and AcMPAG, MPA AUC, maximum concentration (Cmax), minimum concentration (Cmin), time to maximum concentration (Tmax), apparent total body clearance (Cl/F), and apparent volume of distribution (Vd/F). Metabolic AUC ratios of MPAG/MPA and AcMPAG/MPA, MPA free fraction, and free MPA AUC (fMPA AUC) were also calculated. As well, the ratio of partial MPA AUC (6-12) to full AUC (0-12) was calculated to estimate the extent of enterohepatic recirculation. Patients were further stratified into lung or heart transplant groups and concomitant immunosuppressant (i.e. CSA or TAC) groups.

The total amount of analyte excreted in urine was calculated by multiplying the measured concentration by the total volume of urine collected. The renal clearances for MPA, MPAG and AcMPAG were calculated by dividing the amount of analyte excreted (Ae) by the respective plasma AUC (0-12); and the partial metabolic clearances for MPAG and AcMPAG were calculated by dividing the amount of metabolite excreted by the plasma MPA AUC (0-12).

2.2.8. Statistical analyses

Descriptive statistics were used for demographic and pharmacokinetic data. Mean comparisons were performed by Student's t-test or analysis of variance for parametric data; Wilcoxon rank sum test was used for nonparametric data. Normality was determined by Shapiro-Wilk W test. (JMP 6 software, SAS Institute Inc., Cary, NC). Statistical significance was deemed *a priori* at $p<0.05$.

2.3. Results

2.3.1. Patient characteristics

Seventy stable thoracic transplant recipients. Subjects consisted of 36 lung and 32 heart and two heart-kidney transplant recipients. All lung transplant recipients were also taking prednisone as their immunosuppressive regimen, while only four heart transplant recipients took prednisone. In addition to prednisone, 29 subjects were also taking CSA (11 lung, 18 heart), 39 taking TAC (25 lung, 14 heart) and two taking sirolimus (heart) as part of their immunosuppressive regimen (Table 2.3). Eighty-six percent of subjects were Caucasians, while others were of Asian, Indian, Native American, and Arab descent. The most common diagnoses for lung transplantation were cystic fibrosis, pulmonary fibrosis, chronic obstructive pulmonary disease/emphysema, and alpha 1-antitrypsin deficiency; common diagnoses for heart transplantation included ischemic cardiomyopathy, idiopathic dilated cardiomyopathy and unspecified cardiomyopathy.

Subject demographics are summarized in Table 2.3. The lung transplant group was younger in age, had a shorter time-since-transplant, had significantly more females

(Chi-square test, p=0.03), and were more likely to use prednisone (p<0.001) than the heart transplant group; lung transplant recipients were also given a significantly higher MMF twice-daily dose and dosage by weight, and had significantly lower albumin and serum creatinine levels than heart transplant recipients. The creatinine clearance (estimated by the Cockcroft-Gault equation[37]), however, was not different between the two groups.

2.3.2. MPA pharmacokinetics

Table 2.4 summarizes the dose-normalized (per g of MMF) pharmacokinetic parameters of MPA and the metabolites for the study patients. The two heart-kidney transplant recipients were included in the heart transplant group for subsequent pharmacokinetic analysis as they exhibited similar pharmacokinetics to the rest of heart transplant recipients. Free fraction was measured in a subset of 49 subjects (15 lung, 34 heart) as the collection of non-acidified plasma was not implemented for the first 21 lung transplant recipients.[36] The lung transplant group had significantly lower dose-normalized MPA AUC (full and partial), Cmax and Cmin, and higher apparent clearance, apparent volume of distribution, and MPAG/MPA metabolic ratio than the heart transplant group. There was no significant difference in the extent of enterohepatic recirculation between the lung and heart transplant groups. Figure 2.1 depicts the PK profiles of MPA, MPAG and AcMPAG in thoracic transplant recipients, stratified by transplant groups, lung and heart/heart-kidney transplant.

Within the lung transplant group, cystic fibrosis patients did not have statistically significant different MPA PKs when compared to the non-cystic fibrosis patients (data not shown). When patients with cystic fibrosis (n=10) were excluded from the lung

transplant group, most pharmacokinetic results remained similar and did not impact on the statistical results (data not shown). The only exceptions were apparent volume of distribution and MPAG/MPA metabolic ratio, which were no longer significantly different between the lung and heart transplant groups.

Pharmacokinetic data for lung and heart transplant groups were further stratified into co-medication groups, CSA and TAC (Table 2.4). The two heart transplant recipients taking SRL did not have significantly different PK parameters compared to their CSA or TAC counterparts, and were excluded from the table for clarity. Lung transplant recipients taking TAC had significantly higher dose-normalized MPA AUCs (full and partial) and Cmin, and lower apparent clearance and MPAG/MPA metabolic ratio than patients taking CSA. For heart transplant recipients, subjects taking TAC had a higher dose-normalized MPA AUC(0-12), AUC(6-12), Cmax and Cmin, while MPAG/MPA metabolic ratio MPAG/MPA was lower in the TAC co-medication group.

Figure 2.2 summarizes the PK profiles of MPA, MPAG and AcMPAG in lung transplant recipients, stratified by co-medications (CSA and TAC). Patients taking TAC had higher MPA concentrations than the CSA group; however, MPAG and AcMPAG concentrations were similar. The PK profiles of heart transplant patients, stratified by co-medications (CSA, TAC and SRL) are presented in Figure 2.3. In general, heart patients taking CSA had lower MPA concentrations than the TAC group. The MPAG concentrations were similar between groups, and AcMPAG concentrations were similar between CSA and TAC groups. One patient taking SRL had unusually high AcMPAG concentrations (Figure 2.3c).

2.3.3. Urine data

Urine data were available for 37 thoracic (13 lung, 24 heart) transplant recipients. Recovery of MPA (equivalent MMF dose) from urine was 124 ± 35%. Table 2.5 summarizes the dose-normalized total amount excreted of the compounds, renal clearances, and partial metabolic clearances, stratified by transplant type and by co-medication (CSA and TAC). All lung transplant recipients with available urine data were taking TAC. There was no significant differences in the amount of MPA, MPAG and AcMPAG excreted, renal clearances, or the partial metabolic clearances between the lung and heart transplant groups, although the heart transplant group tended to have higher excretion of MPA and MPAG, and lower AcMPAG renal clearance. The fractions of MMF dose excreted as MPA, MPAG and AcMPAG were 1.5%, 96.4%, and 2.1%, respectively.

2.4. Discussion

Despite the original guideline of fixed MMF dosing, numerous pharmacokinetic studies primarily in the renal transplant population have demonstrated large inter-patient variability in the pharmacokinetics of MPA.[31, 32, 38, 39] In contrast, detailed pharmacokinetic studies of MPA and its metabolites in thoracic transplant recipients are scarce. Since neither the lung nor heart is involved in MPA metabolism or excretion, the thoracic transplant subpopulation may exhibit unique pharmacokinetics.

In the current study of thoracic transplant recipients, there was wide inter-patient variability of MPA, MPAG and AcMPAG pharmacokinetics in total MPA exposure,

Cmin, Cmax, apparent clearance, MPA free fraction and metabolic ratios (Table 2.4). Other primary significant findings included: lower dose-normalized MPA AUC, Cmax and Cmin, higher apparent clearance, apparent volume of distrubution and MPAG/MPA metabolic ratio, in the lung versus heart transplant group; lower dose-normalized MPA AUC and Cmin, and higher apparent clearance and MPAG/MPA metabolic ratio in lung transplant recipients taking CSA versus TAC; lower dose-normalized MPA AUC, Cmax, Cmin, and higher MPAG/MPA metabolic ratio in heart transplant patients taking CSA versus TAC.

Despite wide large inter-patient variability, the mean dose-normalized MPA exposure (58.99 μg*h/mL/g) observed in our study subjects is in line with the range of values (7.5 to 94.7 μg*h/mL/g) reported in other kidney and heart transplant studies (Table 2.6).[24, 27, 34, 40-45] As well, the mean apparent total body clearance (Cl/F) value (35.5 L/h) is comparable to, albeit slightly higher than, the range of mean Cl/F values (11.9 to 34.9 L/h) found in population pharmacokinetics studies of kidney transplant recipients.[40] The extent of enterohepatic recirculation found in our study ranged from 10 to 61%, consistent with the 10 to 60% reported in the literature.[12]

To our knowledge, this is the first study to compare the pharmacokinetics of MPA and its glucuronidated metabolites in lung and heart transplant recipients. Interestingly, when the lung and heart groups were analyzed separately, significant pharmacokinetic differences were observed. The higher MPA exposure and lower metabolic ratios in heart transplant recipients may be attributed to various factors. While the use of other immunosuppressants (i.e. CSA, TAC and SRL) was similar in both groups, the prevalence of prednisone use was distinctly different (36 lung versus 4 heart,

respectively). Corticosteroids have been shown to induce the UGT enzymes that metabolize MPA;[6, 46-48] Cattaneo et al.[48] reported increasing MPA AUC with steroid tapering and withdrawal in kidney transplant recipients. While clinical data on such interactions are scarce, steroid use may contribute to the lower MPA and higher metabolite levels observed in the lung transplant group. In fact, in an exploratory stepwise multivariate analysis, prednisone use, co-medications (CSA, TAC or SRL) and age were the three significant factors that impact on dose-normalized MPA AUC; these factors combined contributed to about 30% of the variability in MPA exposure, with prednisone use alone contributing to about 15% of the variability ($r^2 = 0.150$), suggesting steroids may be an important determinant in MPA pharmacokinetics.

The significantly higher albumin levels observed in the heart transplant group may also contribute to the elevated MPA exposure, since an increase in protein binding has been shown to lead to decreased total clearance of MPA.[6, 46, 49, 50] Although no significant difference was observed in MPA free fraction between the two transplant groups, data were available for only 15 lung and 34 heart transplant patients. Serum creatinine was also significantly higher in the heart transplant group. Studies have shown a significant relation between decreased renal function and increased MPAG concentrations.[49, 51-54] As MPAG can compete with and displace MPA from albumin,[33, 52, 54] this may also explain the higher MPAG concentrations in the heart transplant recipients.[25] Similar to elevated MPA and MPAG concentrations in plasma, urinary excretion of MPA and MPAG were also higher, albeit not significantly, in the heart transplant group (Table 2.5). It was noted that the urinary recovery of MPA equivalent exceeded 100% of the dose (mean recovery 124%). Considering that patients were on a

multiple dosing regimen (at steady-state), enterohepatic recirculation of MPA was unpredictable, urine production in an arbitrary 12-hour period was variable, and there was variability associated with the analytical assay and sample dilution, the extra recovery was not unexpected.

To further explore the role of renal excretion in MPA pharmacokinetics, the Cockcroft-Gault equation was employed *post-hoc* to estimate creatinine clearance for each subject (Table 2.3). Although creatinine clearance was lower in the heart transplant group, the difference did not reach statistical significance. However, a correlation analysis revealed that creatinine clearance was negatively correlated ($p<0.05$) with DN MPAG AUC [correlation coefficient (r) = - 0.39] and DN fMPA AUC (r = - 0.36). Thus, creatinine clearance may still contribute to the pharmacokinetic differences observed. While patient age has not been shown to influence MPA pharmacokinetics,[55] it was positively correlated with DN MPA AUC (r = 0.34) and DN MPAG AUC (r = 0.38) in our patient population. This observation, however, is confounded by the fact that renal function may vary with age.

Although renal function, indicated by estimated creatinine clearance, was found to correlate with MPAG and MPA exposures, no differences were observed in the renal clearances of MPA, MPAG and AcMPAG between transplant groups or co-medication groups. Our renal clearance results were comparable to literature values from healthy volunteers.[11,12] Also in agreement with the literature,[12] >90% of the MMF dose was excreted as MPAG in our study population, suggesting that elimination of MPA and metabolites is relatively consistent. Therefore, the variability in MPA pharmacokinetics observed is likely due to alteration in MPA bioavailability and/or metabolism, and not at

the elimination stage. However, since urine data were available only for half (37/70) of our subjects, this observation warrants further confirmation.

Although other demographic differences were noted between lung and heart transplant patients, they were not expected to play a significant role in impacting the pharmacokinetics of MPA. For example, while cystic fibrosis patients are known to have impaired absorption and increased hepatic drug clearance,[29, 56] there was no significant difference in MPA pharmacokinetics between lung transplant recipients with and without cystic fibrosis. Gender may also play a role and it has been suggested that females have lower MPA clearance possibly due to competition of estrogen metabolism with UGTs; however, this literature is conflicting and clinical significance appears limited.[6, 46] We explored the impact of gender on various MPA PK parameters and observed no difference in dose-normalized MPA AUC, Cmax, Cmin, enterohepatic recirculation, metabolic ratios of MPAG/MPA, or AcMPAG/MPA between males and females (Wilcoxon rank sum test). Similarly, no difference was observed when the same comparison was repeated after stratification by co-medication (CSA or TAC), known to impact MPA pharmacokinetics. Although MPA AUC has been reported to increase with time in renal transplant recipients and in a heart transplant group (within 12 weeks post-transplant),[21, 27, 57, 58] our previous study in thoracic transplant recipients found no significant differences in any pharmacokinetic parameter between three sampling periods within the first 9 months post-transplant.[35] Similarly, we did not find a correlation of MPA exposure and time-post-transplant ($r = 0.16$) in this current study. Because the median time-since-transplant of our thoracic transplant recipients was 1.9 years, and only

one subject (lung) was studied within 3 months post-transplant, it was not a significant determinant of MPA pharmacokinetics in this study.

It is noteworthy that the heart transplant group was given a significantly lower MMF dose; in fact, 62% of heart transplant recipients were given a MMF twice-daily dose of 750 mg or less (250 – 750 mg), well below the recommended 1000 to 1500 mg.[59] The dose-normalized MPA PK parameters (AUC, Cmax and Cmin), are comparable to kidney transplant recipients taking low-dose MMF (500 mg twice-daily) with TAC.[60-62] It has been suggested that lung transplant recipients tend to require higher doses of immunosuppressive agents compared to other organ transplantation because of the poorer prognosis in mortality and graft loss.[63]

The pharmacokinetics of MPA are known to be influenced by concomitant immunosuppressive agents, namely CSA and TAC[30, 64-67]. Cyclosporine has been shown to decrease MPA exposure levels by inhibiting the MRP2 biliary transporter, thereby reducing the enterohepatic recirculation of MPA.[68-72] While less evident, a study (in renal transplant recipients and *in vitro*) suggests that TAC is an inhibitor of UGTs and therefore has the potential to augment MPA levels.[73, 74] As expected, when stratified into co-medication groups, lung transplant patients also taking CSA had significantly lower MPA exposure than those taking TAC; similar trends were observed in heart transplant recipients.

To date, few pharmacokinetic studies in the thoracic transplant population have investigated both MPA and its metabolites (Table 2.6). Although scarce, a few studies have measured MPAG.[24-27, 75] Gajarski et al.[26] reported MPAG/MPA ratios of 37.7±40.2 and 16.0±18.1 in 10 young adult and 16 pediatric heart transplant recipients, respectively.

In addition, patients on CSA had higher ratios than those on TAC (28.5 vs. 11.5; p=0.003).[26] The mean MPAG/MPA ratio, calculated from the data of Armstrong et al. for 9 adult heart transplant recipients on MMF and CSA was 30.5.[24] Likewise, mean MPAG/MPA ratios, calculated from the data of Hummel et al. for 15 heart transplants recipients on MMF and CSA, were 37.6, 26.2, and 23.9 at weeks 2, 12, and 52 post-transplant, respectively.[27] As well, Seebacher et al. found mean MPAG/MPA ratios of 12.2, 19.6, and 15.5 after the first, second, and combined doses of MMF in 7 heart transplant recipients (co-medication not reported).[75] Although the MPAG/MPA ratio observed in our study is slightly lower, it is still comparable to other reports due to the variability.

While several studies in kidney transplant recipients have measured AcMPAG concentrations,[18-22, 76-78] we are aware of only a small bioavailability study in 9 heart transplant recipients on MMF and CSA that measured AcMPAG.[24] The mean AcMPAG/MPA ratio, calculated from the data of Armstrong et al. for 9 adult heart transplant recipients on MMF and CSA, was 0.19. This is comparable to the median value of 0.18 in our 18 heart transplant recipients who were on CSA co-medication.

Several studies in kidney transplant recipients have measured free MPA concentration;[21, 38, 51, 52, 77-80] however, we are currently aware of only two studies in heart,[25, 34] one in heart and lung,[35] and another in lung[31] transplant recipients that measured fMPA in addition to total MPA concentration (Table 2.4). In the current study, free MPA fraction ranged from 0.1 to 14.6% (0.7 to 13.0% in lung and 0.1 to 14.6% in heart) and fMPA AUC from 0.05 to 18.89 µg*h/mL (0.24 to 2.87 in lung and 0.05 to 18.89 in heart). Cussonneau et al. reported free MPA fraction of 3.6±3.9% in 7 heart

transplant recipients.[25] DeNofrio et al. studied 38 heart transplant recipients who underwent surveillance endomyocardial biopsy and found mean (±SD) MPA free fraction of 1.9±0.4% and fMPA AUC of 0.83±0.30 μg*h/mL. Notably, patients with grade 2/3 rejection had a lower fMPA AUC compared with patients who had grade 0 rejection (0.49 ± 0.11 vs. 0.81 ± 0.25 μg*h/mL; $p < 0.05$) or grade 1 rejection (0.49 ± 0.25 vs. 0.95 ± 0.34 μg*h/mL; $p < 0.05$).[34] Ensom et al. found a mean (±SD) MPA free fraction of 2.90±0.56% (range, 2.00-3.40%) and fMPA AUC of 1.29±0.50 μg*h/mL (range, 0.54-1.88 μg*h/mL) in 7 stable lung transplant recipients on MMF and CSA co-medication.[31] In another study by our group, the mean MPA free fractions were 4.3 to 7.1% for 5 lung and 4 heart transplant recipients on CSA or TAC during the early post-transplant period.[35] Given this variability, the MPA free fraction and fMPA AUC in our thoracic transplant recipients are in reasonable agreement with the other studies, with the exception of DeNofrio et al., who reported a lower free MPA fraction and fMPA AUC. The difference in free fraction may be due to several factors, such as impaired renal function, albumin levels, and MPAG concentrations. While DeNofrio et al. reported a higher than normal creatinine levels for their patient group (185±70 μmol/L), the authors inferred that patient renal function was not impaired due to the low MPA free fraction measurements;[34] no information regarding albumin levels or MPAG concentrations was provided for their population, thus the discrepancy in the free fraction results could not be ascertained.

All of the participants of the current pharmacokinetic study were stable and already on a long-term steady-state maintenance MMF regimen. As such, we did not attempt to correlate MPA pharmacokinetic parameters with clinical outcomes. As

highlighted in several comprehensive reviews,[6, 46, 50, 81] the overwhelming majority of studies correlating MPA pharmacokinetics to outcomes have been performed in the kidney transplant subpopulation. The three multi-center randomized controlled trials comparing clinical outcomes of MMF fixed-dose versus MPA concentration-controlled (FDCC study, Apomygre study, and OptiCept study) were all being conducted in adult kidney transplant recipients and results may not be directly applicable to other solid organ transplant subpopulations.[19, 78, 82-86] This, coupled with the differences observed in MPA pharmacokinetics between lung and heart transplant recipients in the current study, underscore the need for studies linking pharmacokinetics to clinical outcomes in thoracic transplant recipients.

2.5. Conclusions

This current study demonstrated that while the inter-patient variability in MPA pharmacokinetics was large, there were significant differences between the lung and heart transplant groups. Specifically, the lung transplant patients had significantly lower MPA exposure than the heart transplant patients. In agreement with previous studies suggesting that CSA inhibits the enterohepatic recirculation of MPA, lower MPA levels were observed in patients also taking CSA as opposed to TAC or SRL. The higher albumin and serum creatinine levels, and less prednisone use in the heart transplant recipients may explain the differences in pharmacokinetics between the two transplant groups. In contract, gender and presence of cystic fibrosis did not have significant impact on MPA pharmacokinetics. However, in order to fully understand these underlying factors and to investigate whether different therapeutic drug monitoring guidelines should be

established for lung versus heart transplant groups, larger pharmacokinetic-pharmacodynamic and population pharmacokinetic studies are warranted.

2.6. Tables

Table 2.1. HPLC plasma assay validation: intra-day and inter-day coefficient of variation of MPA, MPAG and AcMPAG measurements

	Concentration of quality control samples(μg/mL)	Intra-day CV (%)	Inter-day CV (%)
MPA	0.25 (LOQ)	2.50	7.80
	4.00	2.60	6.00
	12.00	5.40	6.80
	25.00	2.10	2.70
MPAG	5.00 (LOQ)	6.40	10.40
	20.00	0.80	2.50
	50.00	3.30	3.10
	75.00	3.50	7.20
AcMPAG	0.20 (LOQ)	5.30	9.20
	3.00	2.20	8.30
	12.00	1.90	2.50
	18.00	2.10	4.70

AcMPAG = acyl glucuronide of mycophenolic acid; CV = coefficient of variation; LOQ = lower limit of quantitation; MPA = mycophenolic acid; MPAG = 7-O-mycophenolic acid glucuronide

Table 2.2. HPLC urine assay validation: intra-day and inter-day coefficient of variation of MPA, MPAG and AcMPAG measurements

	Concentration of quality control sample (μg/mL)	Intra-day CV (%)	Inter-day CV (%)
MPA	2.00 (LOQ)	3.11	5.09
	6.00	3.30	9.15
	16.00	3.04	9.47
	30.00	2.05	4.56
MPAG	6.25 (LOQ)	3.78	6.51
	9.00	3.65	10.45
	60.00	1.03	4.04
	180.00	4.27	13.36
AcMPAG	2.00 (LOQ)	3.85	3.85
	6.00	4.16	4.97
	16.00	3.94	2.33
	30.00	2.21	7.85

AcMPAG = acyl glucuronide of mycophenolic acid; CV = coefficient of variation; LOQ = lower limit of quantitation; MPA = mycophenolic acid; MPAG = 7-O-mycophenolic acid glucuronide

Table 2.3. Characteristics of study subjects

	All (n=70)	Lung (n=36)	Heart/Heart-kidney (n=34)
Number of subjects			
Gender (M/F)	49/21	21/15[a]	28/6[a]
CSA/TAC/SRL	29/39/2	11/25/0[a]	18/14/2[a]
Prednisone use	40	36[a]	4[a]
Median (range)			
Age (y)	56.5 (20.7 - 77.6)	51.7 (20.7 – 70.5)[b]	61.3 (23.2 - 77.6)[b]
Years since transplant (y)	1.9 (0.2 - 19.7)	1.4 (0.2 - 14.0)[b]	3.1 (0.3 - 19.7)[b]
Weight (Kg)	74.8 (46.0 - 109.2)	71.0 (46.0 - 109.2)	79.0 (50.0 - 109.1)
Height (m)	1.73 (1.49 - 1.85)	1.70 (1.51 - 1.85)	1.76 (1.49 - 1.85)
BMI	25.2 (18.0 – 34.0)	24.9 (18.0 – 34.0)	25.2 (19.8 – 33.6)
Twice-daily MMF dose (g)	1.00 (0.25 - 1.50)	1.50 (0.50 – 1.50)[b]	0.75 (0.25 - 1.5)[b]
MMF dosage (mg/Kg/day)	27.50 (5.50 - 53.96)	34.79 (12.50 - 53.96)[c]	19.32 (5.50 - 37.82)[c]
Serum creatinine (µmol/L)	115 (67 - 240)	100 (70 - 218)[b]	130 (67 - 240)[b]
Creatinine clearance (mL/min)[d]	62.0 (25.0 - 129.5)	69.0 (35.5 - 129.5)	57.2 (25.0 - 113.7)
Albumin (g/L)	41.0 (20.6 - 78.0)	38.0 (25.0 - 47.0)[b]	43.5 (20.6 - 78.0)[b]

[a] $p<0.05$, Pearson' Chi Square test
[b] $p<0.05$, lung vs. heart, Wilcoxon rank sum test
[c] $p<0.0001$, lung vs. heart, Student's t-test
[d] Estimated by the Cockcroft-Gault equation
BMI = body mass index; CSA = cyclosporine; F = female; M = male; MMF = mycophenolate mofetil; SRL = sirolimus; TAC = tacrolimus

Table 2.4. Pharmacokinetic parameters and metabolic ratios of MPA of thoracic transplant recipients, stratified by co-medication

	Thoracic transplant (n=70)			**Lung transplant (n=36)**			**Heart/heart-kidney transplant (n=32)[a]**		
Median (range)	**All lung (n=36)**	**All heart/heart-kidney (n=34)**	**p-value[b]**	**CSA (n=11)**	**TAC (n=25)**	**p-value[c]**	**CSA (n=18)**	**TAC (n=14)**	**p-value[c]**
Dose-normalized MPA PK parameters (per g of MMF)									
MPA AUC (μg*h/mL)	27.77 (3.39 - 212.14)	71.07 (16.89 - 218.73)	**<0.0001**	18.56 (3.39 - 35.11)	31.79 (8.30 - 212.14)	**0.033**	44.98 (16.89 - 218.73)	92.91 (29.92 - 180.50)	**0.046**
MPA AUC(0-6) (μg*h/mL)	18.46 (2.47 - 117.03)	44.87 (10.68 - 134.00)	**<0.0001**	14.29 (2.47 - 28.24)	22.65 (5.47 - 117.03)	**0.032**	32.06 (10.68 - 134.00)	55.87 (22.24 - 90.67)	0.055
MPA AUC(6-12) (μg*h/mL)	9.20 (0.92 - 95.11)	20.46 (5.32 - 102.16)	**0.001**	6.50 (0.92 - 16.79)	10.88 (1.94 - 95.11)	**0.015**	17.06 (5.32 - 102.16)	27.98 (7.68 - 89.83)	**0.042**
Ratio of partial MPA AUC (6-12)/full AUC	0.31 (0.11 - 0.56)	0.37 (0.10 - 0.61)	0.242	0.27 (0.12 - 0.55)	0.33 (0.11 - 0.56)	0.257	0.28 (0.10 - 0.49)	0.37 (0.21 - 0.61)	0.190

	Thoracic transplant (n=70)			Lung transplant (n=36)			Heart/heart-kidney transplant (n=32)[a]		
Median (range)	**All lung (n=36)**	**All heart/heart-kidney (n=34)**	**p-value[b]**	**CSA (n=11)**	**TAC (n=25)**	**p-value[c]**	**CSA (n=18)**	**TAC (n=14)**	**p-value[c]**
(0-12)									
Cmax (µg/mL)	7.62 (0.64 - 37.11)	18.00 (3.62 - 47.28)	**<0.0001**	5.66 (0.64 - 15.53)	7.81 (1.81 - 37.11)	0.272	14.78 (9.62 - 47.28)	22.14 (9.53 - 44.53)	**0.046**
Tmax (h)	1.1 (0.3 - 10.0)	1.0 (0.3 - 12.0)	0.773	1.0 (0.3 - 6.0)	1.1 (0.3 - 10.0)	0.918	1.5 (0.3 - 12.0)	1.0 (0.35 - 12.0)	0.531
Cmin (µg/mL)	0.73 (UD - 8.95)	1.52 (0.34 - 8.40)	**0.002**	0.44 (UD - 1.05)	1.04 (0.17 - 8.95)	**0.004**	0.93 (0.34 - 3.02)	2.85 (1.26 - 8.40)	**0.002**
Vd/F (L)[d]	196.8 (28.8 - 2686.8)[d]	100.6 (12.9 - 1141.1)[d]	**0.006**	248.1 (54.1 - 644.6)	168.1 (29.8 - 2686.8)	0.404	101.5 (29.3 - 1141.1)	83.64 (45.40 - 261.31)	0.630
Cl/F (L/h)[d]	36.12 (4.71 - 294.68)[d]	15.94 (4.57 - 59.19)[d]	**<0.0001**	53.88 (28.49 - 294.68)	30.17 (4.71 - 120.47)	**0.038**	21.17 (4.57 - 59.19)	14.26 (5.54 - 33.42)	0.544

	Thoracic transplant (n=70)			Lung transplant (n=36)			Heart/heart-kidney transplant (n=32)[a]		
Median (range)	**All lung (n=36)**	**All heart/heart -kidney (n=34)**	**p-value[b]**	**CSA (n=11)**	**TAC (n=25)**	**p-value[c]**	**CSA (n=18)**	**TAC (n=14)**	**p-value[c]**
MPA free fraction[e]	0.016 (0.007 - 0.130)[e]	0.030 (0.001 - 0.146)	0.368	N/A	0.016 (0.007 - 0.130)	N/A	0.025 (0.002 - 0.146)	0.034 (0.001 - 0.137)	0.704
fMPA AUC (ug*h/mL)[e]	1.26 (0.24 - 2.87)[e]	1.51 (0.05 - 18.89)	0.182	N/A	1.26 (0.24 - 2.87)	N/A	1.32 (0.05 - 12.26)	1.70 (0.05 - 18.89)	0.635
Dose-normalized MPAG PK parameters (per g of MMF)									
MPAG AUC(0-12) (µg*h/mL)	438.93 (71.94 - 928.11)	733.80 (49.90 - 1868.80)	**0.009**	387.44 (151.58 - 909.17)	440.76 (71.94 - 928.11)	1.000	736.30 (49.90 - 1868.80)	733.8 (113.3 - 1722.7)	0.662
MPAG AUC(0-6) (µg*h/mL)	252.03 (42.50 - 602.75)	407.40 (35.50 - 1142.50)	**0.010**	225.71 (69.00 - 494.87)	253.57 (42.50 - 602.75)	1.000	407.80 (35.50 - 1142.50)	406.78 (40.17 - 940.11)	0.894
MPAG AUC(6-12)	159.15 (29.44 -	302.67 (14.41 -	**0.019**	161.73 (61.13 -	154.01 (29.44 -	0.430	287.26 (14.41 -	331.13 (59.55 -	0.279

	Thoracic transplant (n=70)			**Lung transplant (n=36)**			**Heart/heart-kidney transplant (n=32)[a]**		
Median (range)	**All lung (n=36)**	**All heart/heart-kidney (n=34)**	**p-value[b]**	**CSA (n=11)**	**TAC (n=25)**	**p-value[c]**	**CSA (n=18)**	**TAC (n=14)**	**p-value[c]**
(µg*h/mL)	426.58)	828.93)		426.58)	389.42)		726.32)	828.93)	
Metabolic AUC ratio MPAG/MPA	13.79 (2.41 - 55.19)	10.29 (0.92 - 26.64)	**0.024**	28.49 (9.76 - 55.19)	12.69 (2.41 - 25.81)	**0.001**	13.66 (0.92 - 28.64)	8.93 (2.35 - 14.89)	**0.050**
Dose-normalized AcMPAG PK parameters (per g of MMF)									
AcMPAG AUC(0-12) (µg*h/mL)	8.26 (UD - 159.93)	18.16 (UD - 333.22)	**0.035**	8.71 (UD - 146.79)	7.81 (UD - 151.93)	0.864	17.94 (UD - 178.25)	16.14 (undectable - 113.64)	0.761
AcMPAG AUC(0-6) (µg*h/mL)	3.48 (UD - 101.08)	9.39 (UD - 101.35)	**0.020**	5.76 (UD - 76.65)	1.99 (UD - 101.08)	1.000	6.83 (UD - 101.35)	11.95 (UD - 80.29)	0.676
AcMPAG AUC(6-12)	3.21 (UD - 70.14)	7.95 (UD - 275.59)	0.094	2.95 (UD - 70.14)	3.47 (UD - 58.85)	1.000	6.56 (UD - 76.89)	6.88 (UD - 62.64)	0.894

	Thoracic transplant (n=70)			Lung transplant (n=36)			Heart/heart-kidney transplant (n=32)[a]		
Median (range)	**All lung (n=36)**	**All heart/heart-kidney (n=34)**	**p-value[b]**	**CSA (n=11)**	**TAC (n=25)**	**p-value[c]**	**CSA (n=18)**	**TAC (n=14)**	**p-value[c]**
(µg*h/mL)									
Metabolic AUC ratio AcMPAG/ MPA	0.21 (UD - 12.33)	0.24 (UD - 3.73)	0.810	0.39 (UD - 12.33)	0.21 (UD - 2.42)	0.559	0.18 (UD - 2.03)	0.24 (UD - 0.94)	0.676

[a] Two patients taking SRL did not have significantly different PK parameters compared to their CSA or TAC counterparts, and were excluded from the table for clarity
[b] Comparison between lung and heart transplant groups, Wilcoxon rank sum test
[c] Comparison between CSA and TAC groups, Wilcoxon rank sum test
[d] Data available for n=67 (35 lung, 32 heart transplant recipients)
[e] Data available for n=49 (15 lung, 34 heart transplant recipients)
[f] Urine data available n=37 (13 lung, 24 heart transplant recipients)
AcMPAG = acyl glucuronide of mycophenolic acid; AUC = area-under-the-concentration-time-curve; Cl/F = apparent clearance; Cmax = maximum concentration; Cmin = minimum concentration; CSA = cyclosporine; fMPA = free MPA; MPA = mycophenolic acid; MMF= mycophenolate mofetil; MPAG = 7-O-mycophenolic acid glucuronide; N/A = not applicable; TAC = tacrolimus; Tmax = time to reach Cmax; UD = undetectable; Vd/F = apparent volume of distribution

Table 2.5. Urine MPA, MPAG and AcMPAG data for 37 thoracic (13 lung, 24 heart) transplant recipients

	Thoracic transplant (n=37)			Heart/heart-kidney transplant (n=24)[b]		
Median (range)	**Lung [a] (n=13)**	**Heart/heart-kidney (n=24)**	**p-value[c]**	**CSA (n=15)**	**TAC (n=8)**	**p-value[d]**
Total urine volume collected (mL)	1300 (500 - 2325)	925 (425 - 2475)	0.085	950 (275 - 2475)	925 (425 - 1650)	0.332
DN amount MPA excreted in urine (mg/g MMF)	9.71 (3.91 - 25.59)	12.47 (2.63 - 45.30)	0.198	14.61 (2.63 - 45.30	10.48 (6.88 - 14.09)	0.087
DN amount MPAG excreted in urine (mg/g MMF)	1219.90 (779.10 - 1759.40)	1425.40 (442.90 - 2721.80)	0.089	1461.00 (442.90 - 2721.80)	1363.90 (890.70 - 1950.50)	0.540
DN amount AcMPAG excreted in urine (mg/g MMF)	29.33 (18.91 - 54.17)	27.97 (3.73 - 48.24)	0.365	30.20 (12.79 - 44.71)	27.97 (3.73 - 48.24)	0.628
MPA renal clearance (L/h)	0.23 (0.11 – 0.61)	0.18 (0.04 – 1.32)	0.787	0.29 (0.06 – 1.32)	0.16 (0.04 – 0.34)	0.114
MPAG renal clearance (L/h)	2.69 (1.07 – 4.64)	2.10 (0.61 – 16.33)	0.600	2.21 (0.61 – 9.03)	1.70 (0.67 – 16.33)	0.628

AcMPAG renal clearance (L/h)	4.55 (0.13 – 98.89)	1.40 (0.13 – 10.78)	0.126	1.40 (0.13 – 10.70)	1.71 (0.23 – 10.78)	1.000
MPAG partial metabolic clearance (L/h)	30.70 (4.65 – 83.24)	25.03 (6.12 – 80.79)	0.514	29.68 (8.25 – 80.79)	17.33 (6.12 – 53.99)	0.287
AcMPAG partial metabolic clearance (L/h)	0.92 (0.23 – 2.07)	0.47 (0.09 – 1.44)	0.063	0.66 (0.15 – 1.26)	0.31 (0.09 – 1.44)	0.114

[a] All lung transplant recipients who had urine data were taking TAC
[b] One patient taking SRL with urine data was excluded from this analysis
[c] Comparison between lung and heart transplant groups, Wilcoxon rank sum test
[d] Comparison between CSA and TAC groups, Wilcoxon rank sum test
AcMPAG = acyl glucuronide of mycophenolic acid; CSA = cyclosporine; DN = dose-normalized; MPA = mycophenolic acid; MPAG = 7-O-mycophenolic acid glucuronide; TAC = tacrolimus

Table 2.6. Summary of pharmacokinetic studies in thoracic transplant recipients

Study	Patient population	Immunosuppressive therapy	Compounds measured	Summary
Current study	36 lung, 34 heart adult transplant recipients	MMF (0.5 – 3 g/day), CSA, TAC or SRL, With/without prednisone	MPA, MPAG, AcMPAG, (fMPA for some subjects)	• Median (range) MPA AUC: 27.77 (3.39 – 212.14) for lung; 71.07 (16.89 – 218.73) for heart transplant recipients • MPA free fraction range: 0.7 to 13.0% in lung and 0.1 to 14.6% in heart • Median MPAG/MPA: 13.79 in lung, 10.29 in heart • Median AcMPAG/MPA: 0.21 in lung, 0.24 in heart • Lower MPA AUC, Cmax and Cmin, and higher apparent clearance, apparent volume of distribution and MPAG/MPA metabolic ratio in the lung versus heart transplant group • Lower MPA AUC and Cmin, and higher apparent clearance and MPAG/MPA metabolic ratio in lung transplant recipients taking CSA (vs. TAC) • Lower Cmin, Cmax, and higher MPAG/MPA metabolic ratio in heart transplant patients

Study	Patient population	Immunosuppressive therapy	Compounds measured	Summary
				taking CSA (vs. TAC)
Armstrong et al.[24]	9 adult heart transplant recipients	MMF (3 g/day), CSA, prednisolone	MPA, MPAG, AcMPAG	• Median (range) MPA AUC: 33.8 (26.6–40.3) µg*h/mL • Mean MPAG/MPA = 30.5 • Mean AcMPAG/MPA = 0.19
Baraldo et al.[41]	9 adult heart transplant recipients	MMF (2 – 3 g/day), CSA, prednisone	MPA	• 44 PK profiles • Cmax 10.4 ± 6.6 mg/L • MPA AUC: 45.9 ± 15.4 (range 13.4 – 91.7) mg*h/L
Cussonneau et al.[25]	7 heart transplant recipients	MMF (2 – 3 g/day), CSA, Corticosteroids	MPA, fMPA, MPAG, free MPAG	• MPA Free fraction 3.6±3.9% • MPAG free fraction 26.0±8.0%
DeNofrio et al.[34]	38 heart transplant recipients	MMF (2 g/day), CSA, Prednisone (tapered over time)	MPA, fMPA	• MPA free fraction 1.9±0.4% and fMPA AUC 0.83±0.30 µg*h/mL • Patients with grades 2/3 rejections had lower fMPA AUC and MPA AUC than grades 0/1

Study	Patient population	Immunosuppressive therapy	Compounds measured	Summary
Dosch et al.[42]	62 heart transplant recipients	MMF (3 g/day), CSA or SRL,	MPA	• MPA AUC estimated by limited sampling strategy • Dose-normalized MPA AUC: 31.92±16.12 (range 13.41 – 82.29) mg*h/L for CSA group; 60.95±27.42 (range 23.69 – 131.54) mg*h/L for SRL group • Dose-normalized C0: 1.41±0.95 mg/L for CSA group; 5.1±3.4 mg/L for SRL group
Ensom et al.[31]	7 lung adult transplant recipients	MMF (1 – 3 g/day), CSA, Prednisone	MPA, fMPA	• MPA free fraction 2.90±0.56% (range 2.00 to 3.40%) • fMPA AUC of 1.29±0.50 (range 0.54 to 1.88 µg*h/mL) • Dose-normalized AUC 23.57±15.76 (range 5.52 to 51.21 µg*h/mL)
Ensom et al.[35]	5 lung and 4 heart adult transplant recipients	MMF (2 – 3 g/day), CSA or TAC, Prednisone (tapering dose)	MPA, fMPA	• Mean MPA free fraction 4.3 to 7.1% • Mean MPA AUC 25.24 to 43.96 µg*h/mL
Gajarski et al.[26]	10 young adult and 16 pediatric heart	MMF (37.9±12.5	MPA, MPAG	• MPA levels higher in children taking TAC

Study	Patient population	Immunosuppressive therapy	Compounds measured	Summary

	transplant recipients	mg/Kg),
		CSA or TAC,
		Corticosteroid
				• MPAG/MPA ratios:
				  ○ 37.7±40.2 in young adult;
				  ○ 16.0±18.1 in pediatric heart transplant recipients
				  ○ Higher in patients taking CSA (vs. TAC)

Hummel et al.[27]	15 heart transplant recipients	MMF (0.25 – 3 g/day), CSA	MPA, MPAG	• Mean MPAG/MPA ratios: 37.6, 26.2, and 23.9 at weeks 2, 12, 52 post-transplant, respectively • Mean MPA AUCs: 52.7, 71.3, and 80.0 µg*h/mL at weeks 2, 12, 52 post-transplant, respectively • High inter-and intra-patient variability in MPA PKs
Kaczmarek et al.[43]	28 heart transplant recipients	MMF (0.25 – 2 g/day), TAC	MPA	• Dose-normalized MPA AUC: 45.5±22.1 (range 8.1 – 87.9) mg*h/L • Mean MPA C0: 2.0±1.2 mg/L
Lehmkuhl et al.[44]	154 heart transplant recipients	MMF (3 g/day) or enteric-coated MPA	MPA	• Compares efficacy and safety of enteric-coated

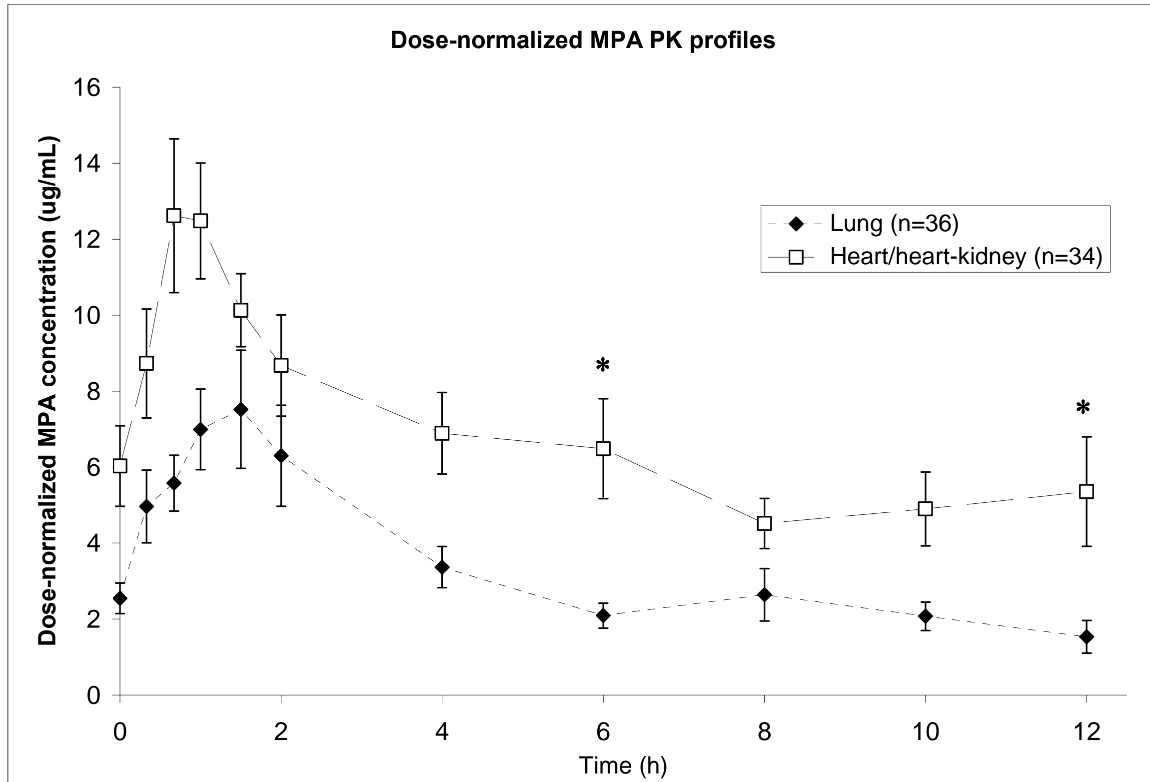
Study	Patient population	Immunosuppressive therapy	Compounds measured	Summary
		sodium (2.16 g/day), prednisone		MPA sodium vs. MMF • Median MPA AUC 71.3 µg*h/mL for MMF
Seebacher et al.[75]	7 heart transplant recipients	MMF (2 g/day)	MPA, MPAG	• Development of high-performance-liquid-chromatography assay for measurement of MPA and MPAG • Mean MPAG/MPA ratios: 12.2, 19.6, and 15.5 after the first, second, and combined doses of MMF • MPA AUC: 58.05 ± 11.05 and 46.75 ± 14.25 µg*h/mL after first and second MMF dose
Wada et al.[45]	22 heart transplant recipients	MMF (1 – 3 g/day), CSA or TAC, corticosteroids	MPA	• MPA AUC: 32.57±13.07 (range 13.11 – 50.98) µg*h/mL for CSA group; 58.55±17.51 (range 39.19 – 93.18) µg*h/mL for TAC group • MPA Cmax: 8.82±4.10 µg/mL for CSA group; 14.23±7.23 µg/mL for TAC group

AcMPAG = acyl glucuronide of mycophenolic acid; AUC = area-under-the-concentration-time-curve; Cmax = maximum concentration; Cmin = minimum concentration; CSA = cyclosporine; fMPA = free MPA; MMF = mycophenolate mofetil; MPA = mycophenolic acid; MPAG = 7-O-mycophenolic acid glucuronide; PKs = pharmacokinetics; TAC = tacrolimus

2.7. Figures

Figure 2.1 Dose-normalized pharmacokinetic profiles (mean± standard error of the mean) of 36 lung and 34 heart/heart-kidey transplant recipeints

Figure 2.1 a. Mycophenolic acid (MPA)



* p<0.05, at specified time point, lung vs. heart/heart-kidney, Wilcoxon rank sum test

Figure 2.1 b. 7-O-mycophenolic acid glucuronide (MPAG)

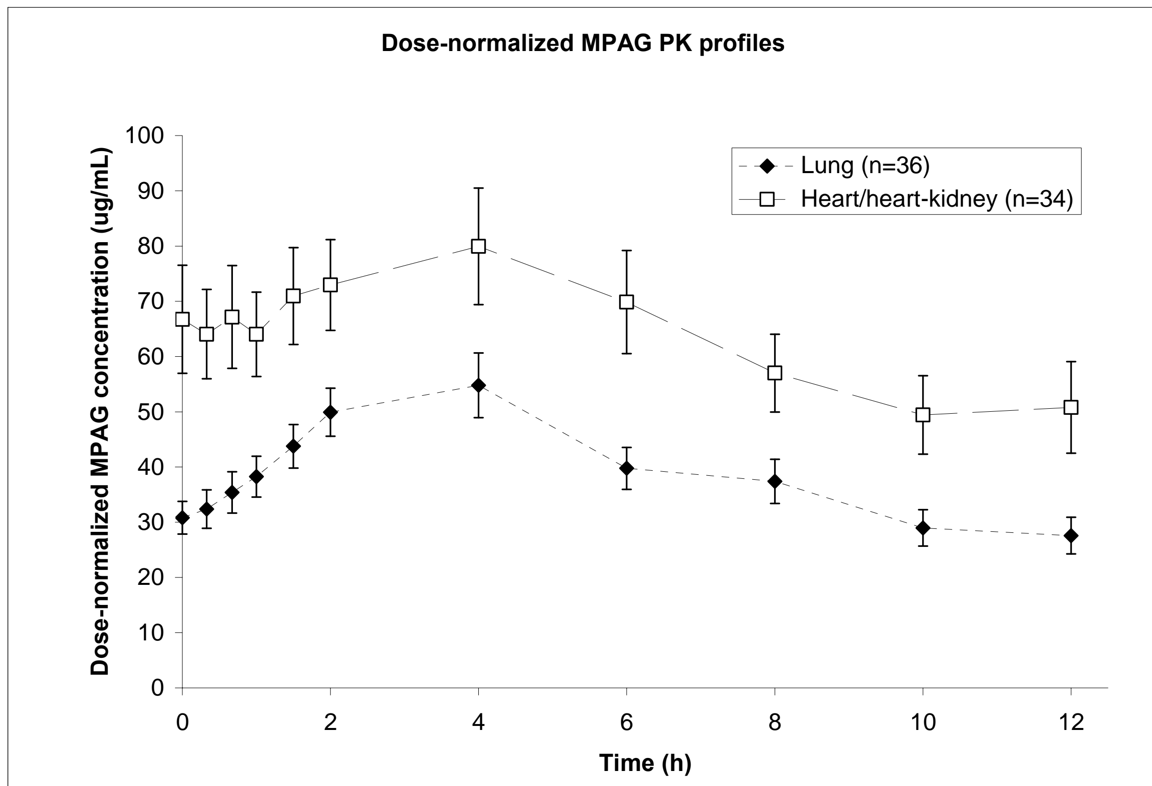


Figure 2.1 c. Acyl glucuronide of MPA (AcMPAG)

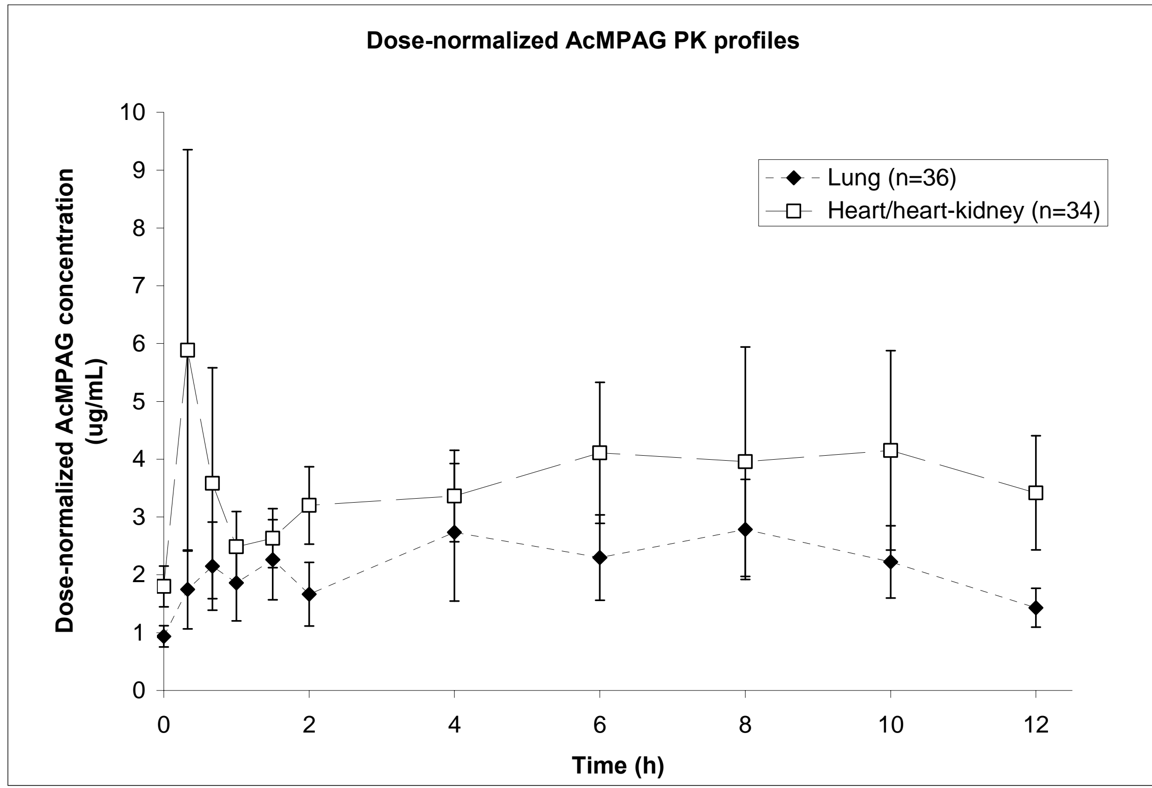
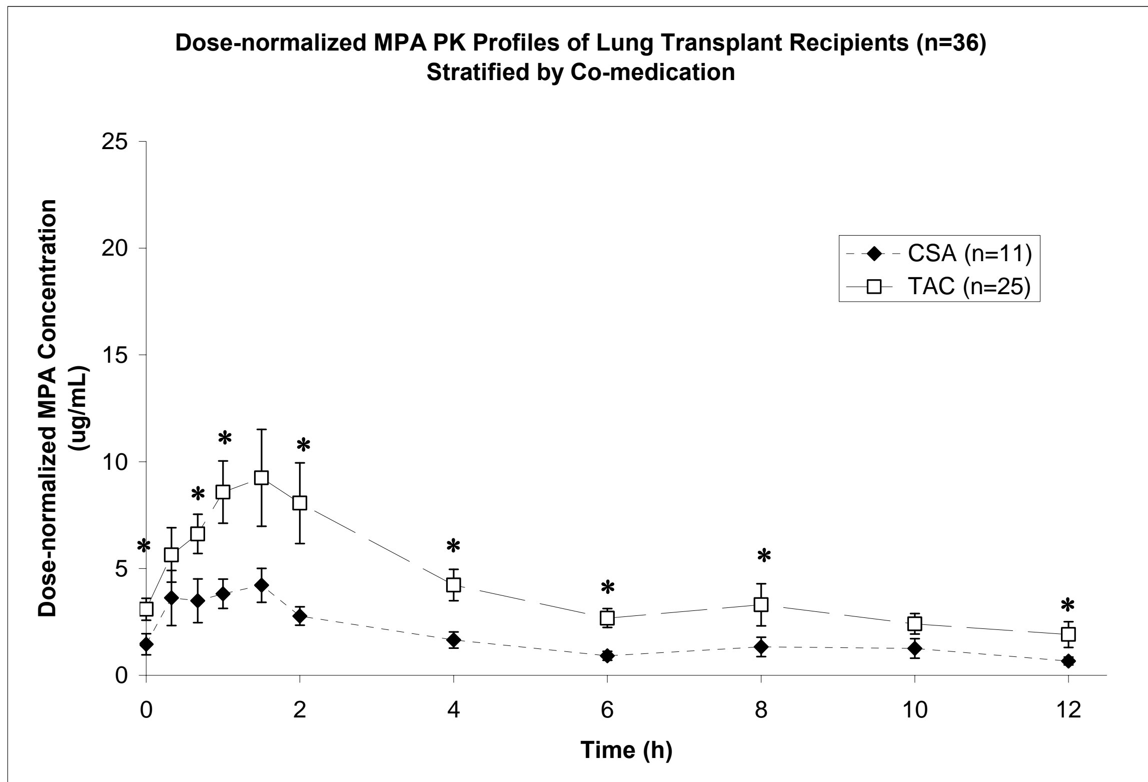


Figure 2.2. Dose-normalized pharmacokinetic profiles (mean± standard error of the mean) of 36 lung transplant recipients, stratified by co-medication cyclosporine (CSA) or tacrolimus (TAC)

Figure 2.2 a. Mycophenolic acid (MPA)



* p<0.05, at specified time point, CSA vs. TAC, Wilcoxon rank sum test

Figure 2.2 b. 7-O-mycophenolic acid glucuronide (MPAG)

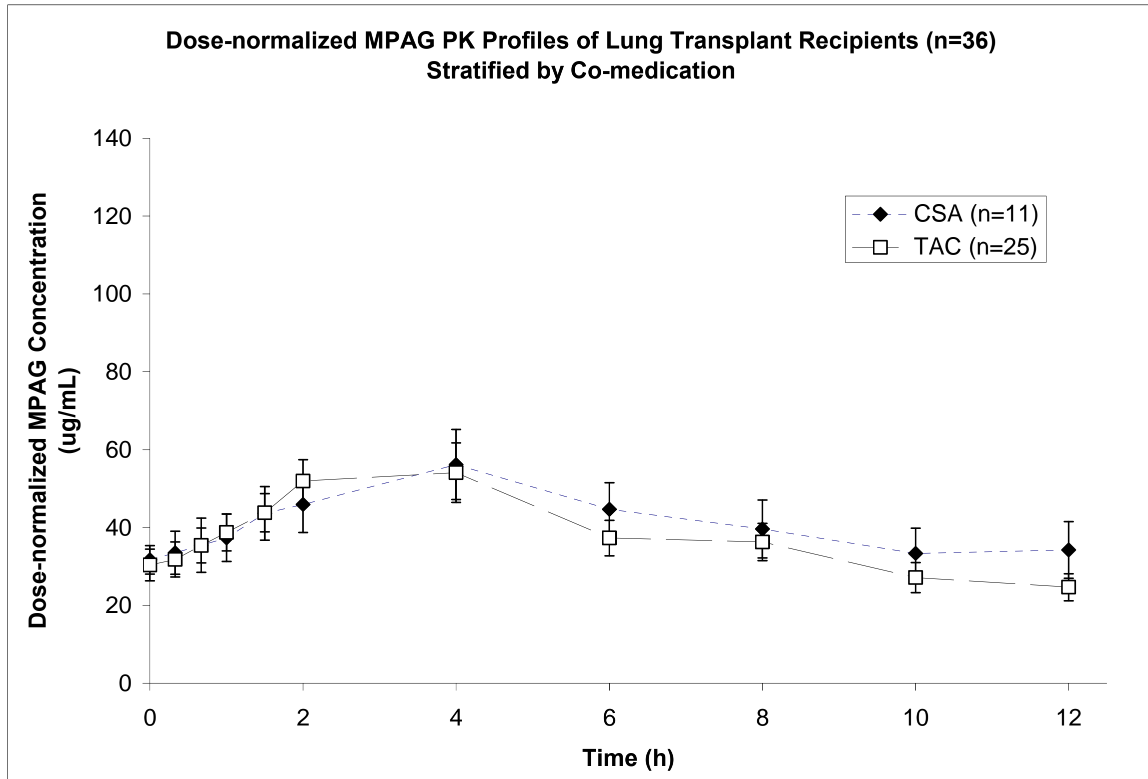


Figure 2.2 c. Acyl glucuronide of MPA (AcMPAG)

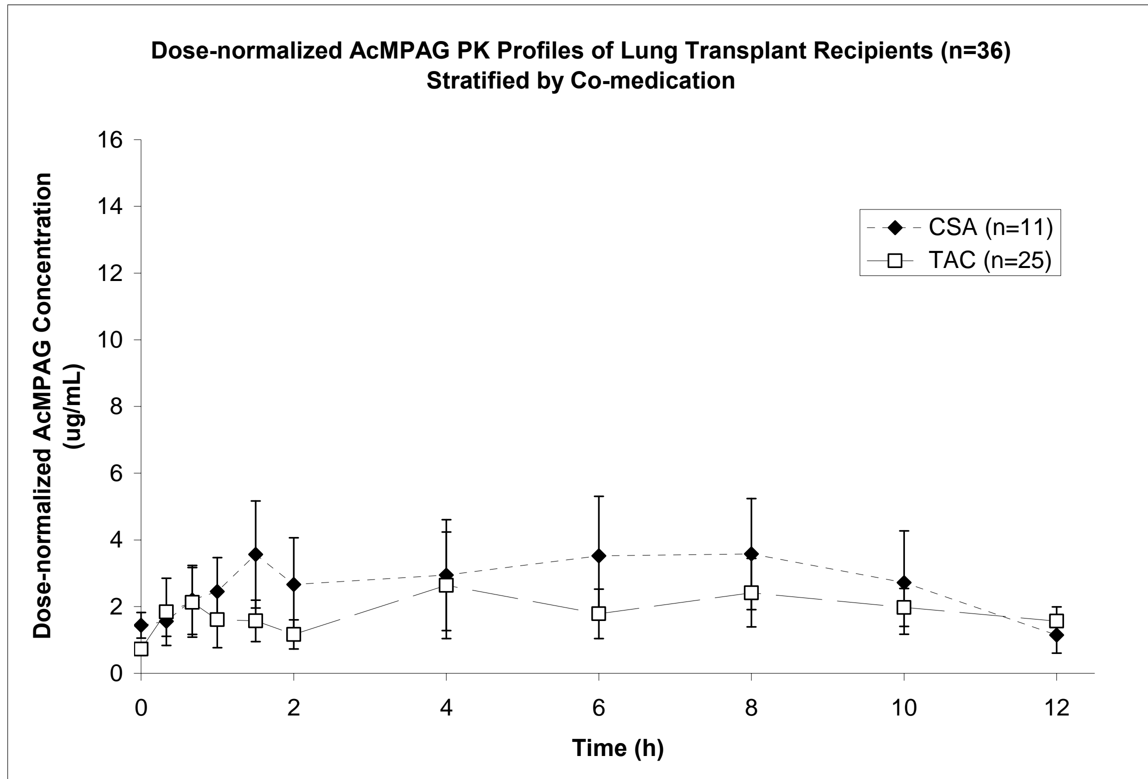
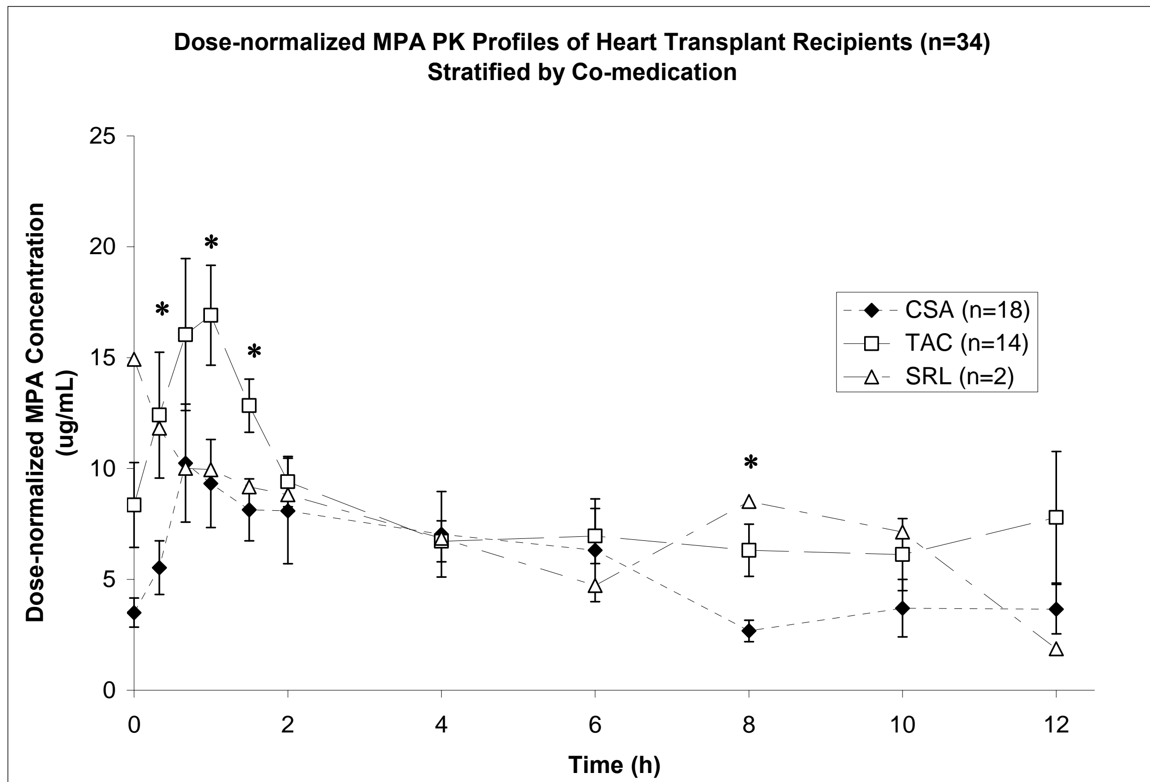


Figure 2.3. Dose-normalized pharmacokinetic profiles (mean± standard error of the mean) of 32 heart transplant recipients, stratified by co-medication cyclosporine (CSA), tacrolimus (TAC) or sirolimus (SRL)

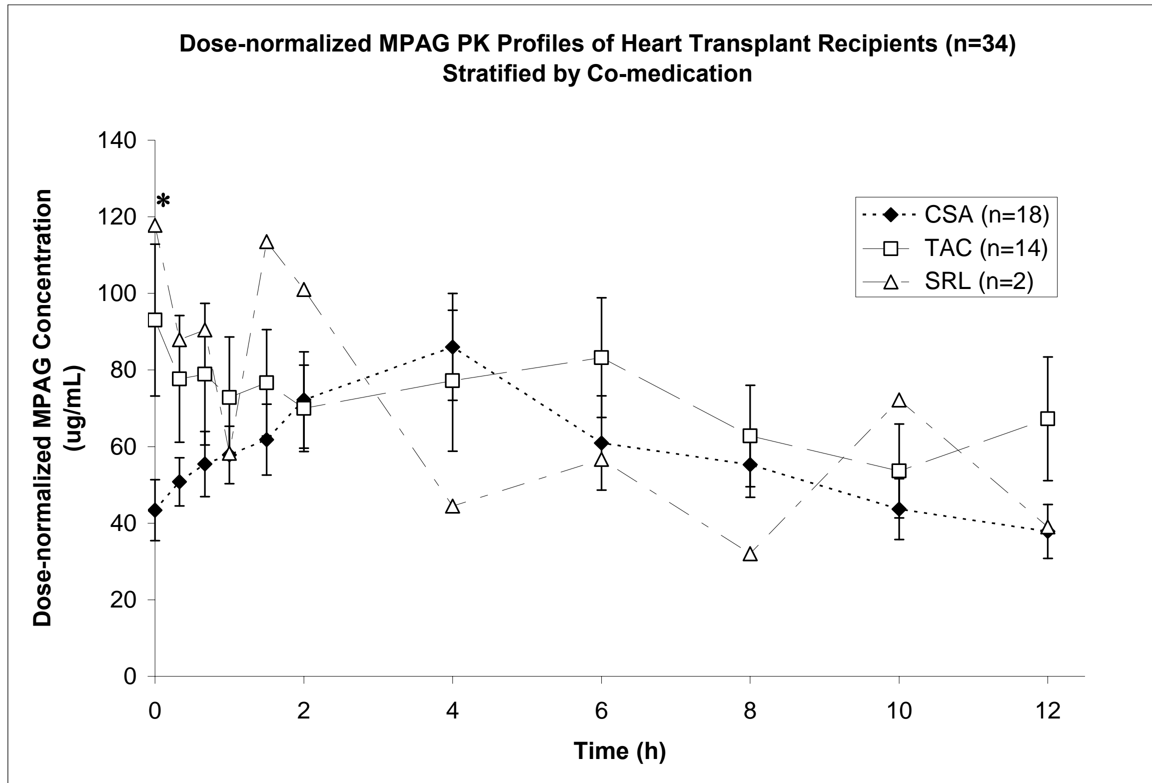
Figure 2.3 a. Mycophenolic acid (MPA)



* $p<0.05$, at specified time point, CSA vs. TAC, Wilcoxon rank sum test

Standard error bars for SRL group were omitted due to the small n, and for clarity

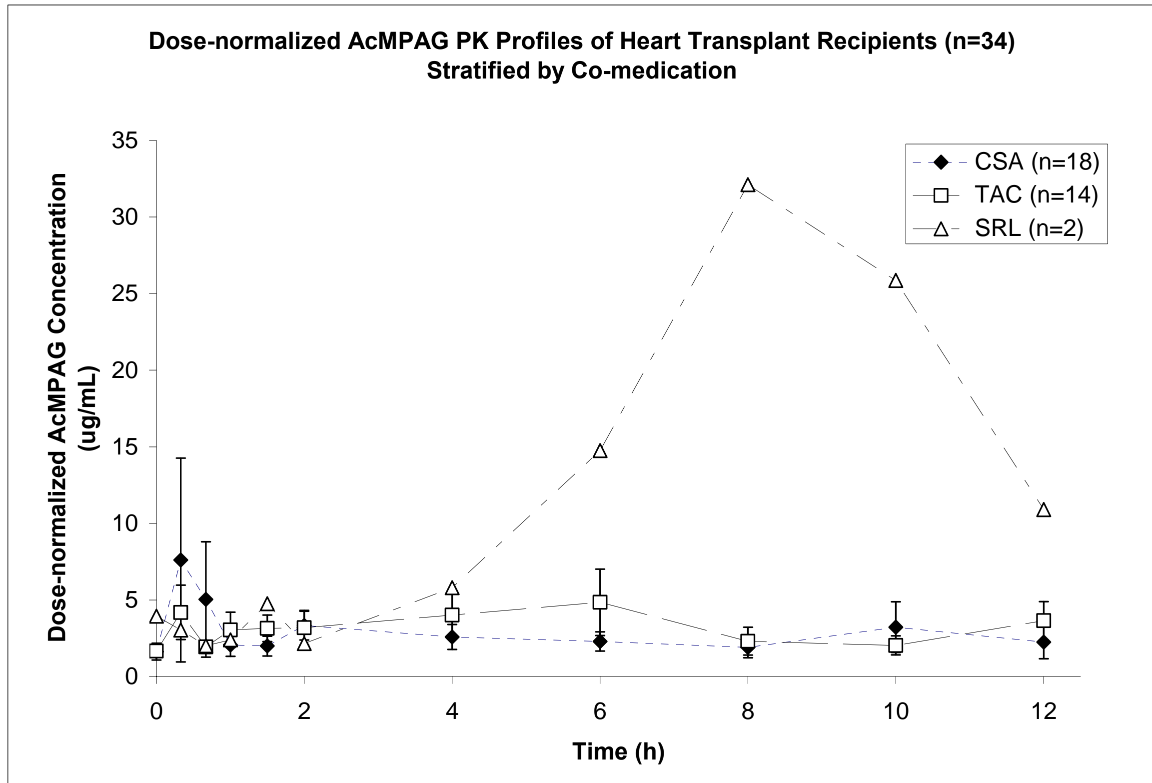
Figure 2.3 b. 7-O-mycophenolic acid glucuronide (MPAG)



* p<0.05, at specified time point, CSA vs. TAC, Wilcoxon rank sum test

Standard error bars for SRL group were omitted due to the small n, and for clarity

Figure 2.3 c. Acyl glucuronide of MPA (AcMPAG)



Standard error bars for SRL group were omitted due to the small n, and for clarity

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3. LIMITED SAMPLING STRATEGY FOR PREDICTING MYCOPHENOLIC ACID AREA-UNDER-THE-CURVE IN ADULT THORACIC TRANSPLANT RECIPIENT[1]

[1] Versions of this chapter have been published as a full manuscript and a short communication:

Ting LSL, Partovi N, Levy R, Riggs KW and Ensom MHH. Limited sampling strategy for predicting mycophenolic acid area-under-the-curve in adult lung transplant recipients. *Pharmacotherapy* 2006; 26(9): 1232 – 1240.

Ting LSL, Partovi N, Levy RD, Ignaszewski AP, Ensom MHH. Performance of limited sampling strategies for predicting mycophenolate area under the curve in thoracic transplant recipients. *J Heart Lung Transplant* 2008; 27(3): 325 – 328.

3.1. Introduction

Mycophenolate mofetil (MMF), the prodrug of mycophenolate acid (MPA), is now commonly used in solid organ transplantation. It exerts its immunosuppressive effects by inhibiting the enzyme inosine monophosphate dehydrogenase, which is essential in the *de novo* synthesis of purines for DNA replication when cells (especially lymphocytes) proliferate.[1-3] MPA is metabolized by UDP-glucuronosyltransferases (UGTs) mainly to the inactive metabolite mycophenolate glucuronide (MPAG). A minor but pharmacologically active metabolite, the acyl glucuronide of MPA (AcMPAG), has been recently identified.[4-6] A second concentration peak of MPA is often observed due to its enterohepatic recirculation as MPAG is de-glucuronidated in the intestine. Adverse effects of MPA are mainly gastrointestinal (GI), including diarrhea, vomiting, ulcers and GI bleeding.[2, 7] The specific actions on lymphocytes and lack of nephro- and hepato-toxicity of MPA have led to its success in immunosuppressive therapy in kidney, heart, lung, and other transplantation populations.[2, 8-10]

MMF is typically administered orally twice a day, at a dosage of 2 to 3 g daily. It is the only immunosuppressive agent that is not dosed by body weight.[7, 11, 12] Although no recommendations on therapeutic drug monitoring (TDM) were made initially, recent study findings indicate the benefits of MPA TDM. Positive clinical outcomes (lower rates of rejection, infection and adverse effects) have been established with adequate MPA exposure guided by monitoring plasma MPA levels.[10, 13, 14] It is generally agreed that the area under the MPA concentration versus time curve (AUC) is a good predictor of treatment response. Other more convenient predictors, such as the trough concentration (C0), have also been investigated; however, its correlation with AUC and

treatment outcomes is poor.[12, 15, 16] Since obtaining a full 12-hour pharmacokinetic (PK) profile for estimation of AUC is inconvenient and costly, a limited sampling strategy (LSS) that provides unbiased and precise estimation of AUC would greatly simplify TDM and benefit patients.

Limited sampling strategies for MPA have been suggested by several groups.[17-23] However, all these studies were mainly conducted in the kidney transplant population, and some suggest sampling times that are not clinically convenient (e.g. beyond two hours post-dose). Until our study, no LSSs had been developed for the lung transplant population, even though about 50% of the lung transplant population has MMF as part of their immunosuppressive therapy.[24] In addition, LSSs for the heart transplant population is scarce; to date, there are only three studies that report LSSs developed specifically in the heart transplant population.[25-27] Typically an LSS established in a specific population is not always suitable for other populations, especially when function of the transplanted organs may have a different impact on drug metabolism. We therefore strived to establish clinically convenient LSSs that utilized blood samples within two hours post-dose for the heart and lung transplant population at our centre. In addition, the predictive performance of LSSs published in the literature was evaluated in our heart transplant population.

3.2. Materials and Methods

3.2.1. Patient population

This was an open-label clinical study approved by the Clinical Research Ethics Board of University of British Columbia (#C02-0568) and Vancouver Coastal Health

Authority Clinical Trials Administration Office (#V03-0162) (Appendices A and B). Seventy stable adult lung or heart transplant recipients receiving MMF therapy twice-daily were recruited. A subject was deemed "stable" if he/she was an outpatient, free from acute rejection, and on a steady-state immunosuppressive regimen at the time of study. They were recruited via the Vancouver General Hospital Lung Transplant Clinic, St. Paul's Hospital Heart Transplant Program, and the British Columbia Transplant Society. Patients were included if they were at least 16 years of age, provided informed consent, and were not taking interacting medications (e.g. antacid, cholestyramine etc.). In addition to MMF, patients were also taking prednisone and tacrolimus (TAC), cyclosporine (CSA) or sirolimus (SRL) for their immunosuppressive therapy, and were at steady state (taking the current drug regimen for at least 5 days) on the day of the study. The detailed study protocol is described in Sections 2.2.1 and 2.2.2. Briefly, patients were asked to fast overnight before reporting to the BC Transplant Society Clinic on the study day. After obtaining written informed consent, an indwelling intravenous catheter was placed in a forearm vein for serial blood collection. Blood samples were taken immediately before (time 0) the morning dose of MMF, and at 20, 40, 60 and 90 min, and 2, 4, 6, 8, 10 and 12 hours post-dose. Plasma was separated and analyzed for MPA concentrations. Of the 70 participants, six were excluded due to incomplete blood sampling (less than 12 hours or missing timed samples); therefore, 64 full 12-hour pharmacokinetic profiles were obtained for final analysis.

3.2.2. Plasma concentration of MPA

The concentration of MPA was determined quantitatively in patient plasma samples by high performance liquid chromatography with ultraviolet detection (HPLC-UV).[28] The detailed methodology is described in Sections 2.2.3 and 2.2.4.

3.2.3. Pharmacokinetic parameters assessment

The AUC of MPA was determined for each patient by the trapezoidal method via non-compartmental analysis using WinNonlin Professional version 5.2 (Pharsight, Mountain View, CA).

3.2.4. Limited sampling strategy determination

Multiple regression analysis was used to determine LSSs for MPA. The Bayesian method was not chosen because no population PK data (the *priors*) for MPA were available in thoracic transplant recipients. Since there were considerable differences in MPA pharmacokinetics between the heart and lung transplant groups (see Chapter 2, section 2.3.2), and preliminary development of limited sampling strategies using combined thoracic data was unsuccessful, limited sampling strategies were developed separately for the heart and lung transplant groups. Seventeen and sixteen patient profiles from the heart and lung transplant group, respectively, were randomly assigned as the index groups to establish the corresponding limited sampling strategies. Multiple regression analysis was performed using JMP 6.0.0 (SAS Institute Inc., Cary, NC). The AUC was the dependent variable while the timed concentrations were the independent variables. When the data did not yield acceptable LSSs from the analysis, the AUC and timed concentrations were log-transformed. Only concentrations taken on or before 2

hours post-dose were considered for the clinically convenient LSS, and a maximum of 3 concentrations were used. The backward elimination method (probability to leave=0.05) was used to determine the best initial regression equation. All timed concentrations were used initially for the regression analysis; concentrations were then removed one by one from the equation and the coefficient of determination (r^2) was re-calculated. Timed concentrations that did not affect the r^2 were omitted from the regression equation. In addition, different combinations of timed concentrations on or before 2 hours post-dose (3 maximum) were manually entered for regression analysis to determine the correlation with AUC. Only equations utilizing a maximum of three timed concentrations taken within two hours post-dose, and with $r^2 \geq 0.75$ were considered further for validation.

3.2.5. Validation of LSS

The remaining MPA PK profiles (16 patients in heart transplant group and 15 patients in lung transplant group) were used to validate the developed LSSs for the corresponding transplant groups. The predicted AUC obtained from the LSS was compared to the observed AUC. The bias and precision of the LSS was determined according to guidelines proposed by Sheiner and Beal.[29] Absolute bias was measured by the mean prediction error (ME), and absolute precision was measured by the root mean square prediction error (RMSE), according to equations 1 and 2 presented below. Acceptable bias and precision were deemed to be ±15%.[30] In addition, the percentage of validation profiles with acceptable bias was determined for each LSS, and the limit was deemed to be ≥ 85% (i.e. at least 85% of the tested profiles had a prediction bias of ±15%).

Equation 1: $ME = \frac{1}{N}\sum(Pe_i)$

Equation 2: $RMSE = \sqrt{\frac{1}{N}\sum(Pe_i)^2}$

where Pe_i = predicted AUC – actual AUC, and N = number of data points.

In addition, LSS equations developed from heart transplant patients reported by Baraldo et al. (equations 3 and 4),[26] Wada et al. (equations 5 and 6),[25] and Kaczmerak et al. (equations 7 and 8)[27] were tested using our heart transplant recipients data:

Equation 3: AUC = 5.568 + 0.902 C1.25 + 2.022 C2 + 4.594 C6

r^2=0.926

Equation 4: AUC = 3.80 + 1.015 C1.25 + 1.819 C2 + 1.566 C4 + 3.479 C6

r^2=0.948

Equation 5 [for cyclosporine co-medication]:

AUC = 0.10 + 11.15 C0 + 0.42 C1 + 2.80 C2

r^2=0.96

Equation 6 [for tacrolimus co-medication]:

AUC = 23.56 + 1.05 C1 + 1.25 C2 + 2.53 C4

r^2=0.73

Equation 7: AUC = 1.09 C0.5 + 1.19 C1 + 3.60 C2

r^2=0.84

Equation 8: AUC = 1.65 C0.5 + 4.74 C2

r^2=0.75

Since blood samples at 1.25 and 0.5-hour post-dose were not collected at our centre, C1.25 was estimated by averaging the MPA concentrations at 1 and 1.5 hours post-dose,

and C0.5 was estimated by averaging MPA concentrations at 0.3 and 0.6 hours post-dose, in order to test equations 5 – 8. Patients also taking sirolimus were included to test the above equations.

3.3. Results

3.3.1. Study subjects characteristics

Characteristics of the index and validation groups for heart and lung transplant recipients are summarized in Tables 3.1 and 3.2, respectively. There were no differences between the index and validation groups for either heart or lung transplant recipients except for albumin levels; the heart validation group had a higher albumin level, whereas the lung validation group had a lower albumin level, compared to the respective index groups. For the lung transplant index and validation groups, there were similar numbers of patients with and without cystic fibrosis.

3.3.2. Limited sampling strategy candidates determined by stepwise regression

Heart transplant group

When original (untransformed) data were used, none of the LSSs resulted in an adjusted r^2 of ≥0.75. Data were therefore log-transformed; however, none of the LSSs met the criteria of $r^2 \geq 0.75$.

To further explore possibilities of LSSs for the heart transplant population, C4 was added to the analysis; the best LSS candidate determined, that had an adjusted $r^2=0.875$, is presented in equation 9:

Equation 9: Log AUC = 0.2982 Log C1 + 0.2477 Log C2 + 0.3192 Log C4 + 1.0504

In addition, eight other three-concentration LSSs and one two-concentration LSS met the criteria. These were subsequently tested with the validation group.

Lung transplant group

When original (untransformed) data were used, 17 three-concentration, nine two-concentration, and two one-concentration LSSs met the criteria of adjusted $r^2 \geq 0.75$, and were subsequently tested with the validation group (Table 3.3). The best LSS candidate determined by stepwise regression (adjusted r^2=0.957) was:

Equation 10: AUC = 2.2940 C0 + 0.9308 C0.3 + 3.2379 C2 + 10.0927

In addition, log-transformed data yielded 15 three-concentration, 8 two-concentration and two one-concentration LSS candidates (Table 3.4). The best LSS candidate determined by stepwise regression (adjusted r^2=0.809) was:

Equation 11: Log AUC = 0.6769 Log C2 + 1.1381

All LSS candidates were tested for their predictive performances, bias and precision.

3.3.3. Limited sampling strategies using a single concentration

Heart transplant group

The correlations between AUC and single concentrations were generally poor. Log-transformed data yielded adjusted r^2 values of 0.196 to 0.679, with the best being C8 (r^2=0.679). The adjusted r^2 values for C0 and C12 were 0.280 and 0.583, respectively.

Lung transplant group

The correlations between AUC and single concentrations were poor for untransformed data (adjusted r^2 ranging from -0.005 to 0.886) except for C1.5 (r^2=0.904), C2 (r^2=0.886) and C4 (r^2=0.871). However, these three single-concentration LSSs

yielded poor bias and precision results when validated (Table 3.3). The adjusted r^2 for C0 and C12 were 0.064 and 0.166, respectively.

As for log-transformed data, C1.5, C2 and C4 yielded acceptable r^2 values of 0.759, 0.809 and 0.813, respectively (Table 3.4). While these LSSs yielded acceptable bias when tested with the validation group, their precision did not meet the criteria of ≤15%. The rest of the single time points had poor correlation with log AUC (adjusted r^2 ranged from 0.169 to 0.623); the adjusted r^2 values for C0 and C12 were 0.231 and 0.445, respectively.

Even though the single-concentration LSSs utilizing C4 and Log C4 were beyond 2 hours post-dose, they were included in the validation since they provided acceptable r^2 values. Although the convenient sampling times at 1.5 or 2 hours post-dose correlated well with AUC, validation results did not meet the acceptable criteria of bias, precision or percentage of profiles within acceptable limits.

3.3.4. Limited sampling strategies using two concentrations

Heart transplant group

No two-concentration LSS yielded satisfactory correlation with AUC when utilizing concentrations within the first two hours post-dose for log-transformed data. When C4 was included in the analysis, the only two-concentration LSS combination was (Log C1, Log C4), which yielded acceptable bias (-3.51%), precision (8.54%) and percentage of profiles within acceptable limits (93.8%).

Lung transplant group

For untransformed data, nine two-concentration LSSs had acceptable r^2 (≥0.75) and were considered further for validation. A summary of validation results for the LSSs

are presented in Table 3.3. Of the nine combinations of two-concentration LSSs, none passed the validation with acceptable bias and precision.

For log-transformed data, eight combinations of two-concentration LSSs were validated (Table 3.4). While all had minimal bias, four LSSs had RMSE exceeding 15%, indicating poor precision; in addition, four LSSs had less than 85% validated profiles within acceptable limits. The only combination that yielded acceptable results was (Log C0, Log C1.5), which provided clinically convenient sampling times, and minimally biased and highly precise estimation of AUC (Table 3.4).

3.3.5. Limited sampling strategies using three concentrations

Heart transplant group

No three-concentration LSS yielded satisfactory correlation with AUC when utilizing concentrations within the first two hours post-dose for log-transformed data. When C4 was included in the analysis, however, there were nine three-concentration LSS combinations with acceptable predictive performances. All three-concentration LSSs yielded similar validation results, with a bias and precision within ±11%.

Lung transplant recipients

Seventeen three-concentration LSSs derived from untransformed data were considered for validation. However, the predictive performances were poor, with bias ranging from -14.9% to 41.9%, and precision ranging from 41% to 68% (Table 3.3).

For log-transformed data, 15 combinations of three-concentration LSSs were validated (Table 3.4). Of these, five LSS combinations passed the validation requirements: (Log C0, Log C1.5, Log C2), (Log C0.3, Log C1.5, Log C2), (Log C0, Log C0.6, Log C1.5), (Log C0, Log C0.3, Log C1.5), and (Log C0, Log C1, Log C1.5). They all had

similar precision of ~12%, and equal number of validation profiles was within the acceptable ±15% range. The combination of (Log C0, Log C0.3, Log C1.5) had the least bias (1.66%); however, the LSSs with the most convenient and conventional sampling times were (C0, C1.5, C2) and (C0, C1, C1.5).

3.3.6. Predictive performance of other LSSs in heart transplant recipients

Since the heart transplant data did not yield convenient LSSs that used concentrations within two hours post-dose, we evaluated the predictive performance of those LSSs derived from the lung transplant when applied to the heart transplant population. All the LSSs developed from lung transplant data yielded a bias within 5% when applied to the heart transplant group, while precision was within 15%; in addition, seven three-concentration LSSs and three two-concentration LSSs had at least 85% of the pharmacokinetic profiles tested within acceptable limits, meeting all the criteria of an acceptable LSS (Table 3.5).

Predictive performances of LSSs for heart transplant published in the literature were also evaluated (Table 3.6). When applying equations reported by Baraldo et al.[26] to our heart transplant data, prediction had a negative bias within 15% and precision within 24%. Wada et al.[25] developed LSSs in Japanese subjects specific to concomitant immunosuppressants of cyclosporine or tacrolimus. Both LSSs from Wada et al.[25] yielded good accuracy (bias <15%); however, precision of the prediction was poor. When the two patients taking sirolimus were excluded from the testing of equation 6, bias worsened to 21.9% while precision improved to 28.7%. Neither equation reported by Kaczmarek et al.[27], developed in patients taking tacrolimus, performed well in our heart transplant population. Bias and precision both exceeded the acceptable limits of 15%;

similar results were obtained when the two patients taking sirolimus were excluded from the validation.

3.4. Discussion

Although standardized guidelines regarding the therapeutic range of MPA AUC are lacking, a range of 30 – 60 µg*h/mL has been suggested.[10, 13, 15, 31] Since it is challenging to determine AUC on a routine basis, limited sampling strategies are a useful tool to abbreviate pharmacokinetic profiling.

In this study, we have developed limited sampling strategies for estimation of MPA AUC in stable heart and lung transplant recipients separately. Successful LSSs should be practical and clinically convenient, and still provide relatively unbiased and precise estimation of AUC. In brief, LSSs should be developed in the patient population of interest, and validated using a separate dataset. The convenience of sampling times and minimal number of samples required are crucial for a successful LSS. We have provided a detailed discussion of the different approaches to establishing LSSs and guidelines in developing LSSs in a separate systemic review of LSSs for immunosuppressive agents.[30]

Due to the various challenges in acquiring LSSs, convenient and valid LSSs for immunosuppressive agents are wanting. Although LSSs for estimation of MPA AUC have been established recently by various groups (Table 3.7),[17, 19, 20, 25-27, 32-41] most of the LSSs were centre-specific, as suggested sampling times differed between research groups. This is probably due to the different sampling times used in the PK profiling, and the varying number of samples, ranging from 7 – 13 (Table 3.7). As MPA is absorbed mainly in the first 2 hours, and reabsorbed at 6 – 12 hours post-dose, an accurate characterization

of AUC depends on when and how frequently samples were taken during these phases. In addition, the suggested sampling times were not always practical, and some utilized more than 3 blood samples. While it is recognized that MPA undergoes enterohepatic recirculation, and concentrations obtained during the first absorption phase may not be fully indicative of the extent of MPA re-absorption, successful LSSs that utilized only concentrations within the first four hours post-dose have been reported.[19,20,25,27,33,37,38,40] Since a clinically convenient sampling strategy is easier to implement and is likely to encourage patient adherence, we limited ours to a maximum of three samples drawn within two hours post-dose. Furthermore, most published LSSs were developed in kidney transplant recipients. Given that the liver and kidney, but not lungs or heart, are involved in elimination of MPA, the pharmacokinetics of MPA are likely to be different in the thoracic transplant population.

In this study, subjects were randomized to index and validation groups in order to balance parameters such as co-medication and disease state. The demographics of the index and validation groups were similar for both heart and lung transplant groups (Tables 3.1 and 3.2). While the albumin levels were different, the median values for both index and validation groups were within the normal range of 35 – 50 g/L[42], and impact on LSS development and validation was unlikely.

Limited sampling strategies were developed in the two transplant groups separately because combining all the data yielded no LSSs that matched our criteria. We established and validated our LSSs in all heart or lung transplant recipients who were on steady-state MMF therapy, regardless of concomitant immunosuppressive agents. Data were not stratified according to co-medications due to the small sample size. It is also

desirable to develop robust LSSs that are applicable to all patients for clinical convenience. In our analysis, both index and validation groups had a balanced number of patients taking CSA or TAC in heart and lung transplant groups. To explore the impact of co-medication on the LSSs, the data were re-analyzed by separating the PK profiles into CSA and TAC groups to see if there was less variability in the prediction. Original, non-transformed data were used to determine whether stratifying the data precluded the need to log-transform data. Of the 14 heart transplant recipients taking TAC, seven profiles were randomly assigned to each of the index and validation group. However, none of the LSSs had an acceptable of $r^2 \geq 0.75$. Similarly, 17 heart transplant recipients also taking CSA were randomized into index (n=9) and validation (n=8) groups, and no LSS were considered further for validation. For the lung transplant group, 23 patients also taking TAC were randomized into index (n=12) and validation (n=11) groups, resulting in nine potential LSSs using original data. However, none of the LSS fulfilled the criteria of an acceptable LSS; the % bias ranged from -6.2 to -16.8%, and % precision from 39.0 to 52.9%.

The variability in MPA PK parameters, which potentially influences the predictive performance of the LSSs tested, did not improve when the data were stratified into CSA and TAC groups. None of the LSSs from the stratified analysis provided acceptable LSSs. Nevertheless, as shown by the validation results from all data, the LSSs developed were robust enough for prediction of MPA AUC in all transplant recipients, regardless of co-medication.

To our knowledge, there are only three published LSSs for heart transplant recipients developed by Baraldo et al.[26], Wada et al.[25] and Kaczmarek et al.[27], and the

only published LSSs for lung transplant recipients was developed by our group.[43] Since there were no suitable LSSs established from our heart transplant population, LSSs developed from our lung transplant group and from the available literature were re-validated in our heart transplant population to assess their applicability.

From the re-validation results, LSSs appeared to be centre-specific. Application of our lung transplant LSSs to the heart transplant population yielded satisfactory prediction results (bias and precision within ±15%, Table 3.5), which showed the LSSs were robust for both the heart and lung transplant populations. Inclusion of two heart-kidney transplant recipients did not affect the predictive performance of our LSSs. The LSSs developed by other research groups, however, did not perform as well when applied to our population. Acceptable prediction was observed in only half or fewer of our patients (Table 3.6). This could be attributed to several factors. Firstly, while the LSSs were developed in heart transplant recipients, immunosuppressive therapies differ between studies. Interactions of immunosuppressants are well-recognized, and concomitant medications could impact on MPA pharmacokinetics.[44-47] All patients in Baraldo's study were taking cyclosporine and prednisone, patients in Wada's study were all taking prednisolone and either cyclosporine or tacrolimus, patients in Kaczmarek's study were taking tacrolimus only, whereas our patients were taking cyclosporine, tacrolimus or sirolimus, and use of prednisone was uncommon. Secondly, a drawback of LSSs developed by multiple regression analysis is the requirement of exact sampling times.[30] Since the LSSs developed by Baraldo et al.[26] and Kaczmarek et al.[27] utilized timed blood samples that were not collected at our centre (1.25 hour and 0.5 hour post-dose, respectively), the accuracy and precision of the prediction was likely compromised as we

could only estimate C1.25 and C0.5. Thirdly, while the recommended twice-daily dose of mycophenolate mofetil for heart transplant patients is 1 or 1.5 g,[7] as was the dose used in Baraldo's and Wada's studies, our patients were generally given a lower mycophenolate mofetil dose (range 0.25 – 1.5 g). Since there is poor relationship between MMF dosages and MPA pharmacokinetics[31], LSSs established for certain dosages of MMF may not be suitable for others. Finally, the PK variability is likely different between the transplant populations from different centres; we found that log-transformation of concentration and AUC data was necessary for our population to develop acceptable equations, while all the other equations reported in the literature[25-27] do not involve data transformation. Application of those equations may therefore yield sub-optimal predictive performance in our patient population.

The concentrations and AUCs were log-transformed in this study in order to normalize the data for more reliable prediction. The Food and Drug Administration guidelines on statistical approaches to establishing bioequivalence recommend transformation of the AUC and concentration data if sample size is small.[48] A small sample size precludes normal distribution of PK parameters, and variance in the timed concentrations may not be uniform. In fact, when LSSs were developed using the untransformed data in this study, none of the LSS provided acceptable predictive performance; application of equations (using untransformed data) from reported literature to our data also yielded poor results.

For the heart transplant group, the best overall LSS (considering predictive performance, number of samples and sampling times) was derived from the lung transplant data utilizing two concentrations: (Log C1.5, Log C2):

Equation 13: Log AUC = 0.1817 Log C1.5 + 0.4994 Log C2 + 1.1132

This LSS used only two samples, had convenient sampling times, and exhibited good accuracy (% bias = -3.30%) and precision (% RMSE = 11.12%) when validated with 33 heart transplant PK profiles. In addition, the LSS predicted AUCs within ±15% accuracy in 29 out of 33 profiles (Table 3.5).

For the lung transplant group, five three-concentration and one two-concentration LSSs met the criteria of a clinically acceptable LSS. Of these, the LSSs using two concentrations (Log C0, Log C1.5) provided the best combination of cost, convenience, and predictive performance (% bias = 4.71%; % RMSE = 12.79%):

Equation 14: Log AUC = 0.1677 Log C0 + 0.5657 Log C1.5 + 1.0830

Adding a sample did not improve predictive performance significantly (Table 3.4) but increased the burden of sample collection, handling, and processing.

3.5. Conclusions

This study developed minimally biased, highly precise and convenient LSSs for predicting MPA AUC in lung transplant recipients. In addition, these LSSs performed well when applied to the heart transplant population, whereas other published LSSs developed in heart transplant recipients yielded less optimal results when applied to our population. While numerous LSSs provided acceptable predictive performance, the LSSs utilizing two concentrations were superior when convenience and cost were taken into consideration. Specifically, the LSSs (Log C1.5, C2) and (Log C0, Log C1.5) were the best candidates for the heart and lung transplant population, respectively.

3.6. Tables

Table 3.1. Characteristics of heart transplant recipients in the index and validation groups

	All heart subjects (n=33)	**Index group (n=17)**	**Validation group (n=16)**
Male (%)	81.8	76.5	87.5
CSA/TAC/SRL (n)	17/14/2	9/7/1	8/7/1
Age (y)	60.9 (23.2 – 77.6)	62.4 (23.2 – 77.6)	56.9 (26.5 – 70.6)
Years since transplant (y)	3.3 (0.3 – 19.7)	4.5 (0.5 – 12.4)	2.2 (0.3 – 19.7)
Weight (Kg)	78.0 (50.0 – 109.1)	78.0 (53.6 – 109.1)	76.4 (50.0 – 95.5)
MMF BID dose (mg)	750 (250 – 1500)	750 (500 – 1500)	750 (250 – 1250)
Serum creatinine (μmol/L)	130 (67 – 240)	130 (67 – 240)	131 (83 – 177)
Albumin (g/L)	43 (21 – 78)	42 (21 – 69)[a]	45 (41 – 78)[a]
MPA AUC (ug*h/mL)	45.27 (16.89 – 190.01)	39.50 (16.89 – 85.79)	49.34 (22.31 – 190.01)
Dose-normalized MPA AUC (ug*h/mL)	71.99 (16.89 – 218.73)	70.15 (16.89 – 112.90)	87.14 (22.56 – 218.73)

AUC = area-under-the-curve; BID = twice a day; CSA = cyclosporine; TAC = tacrolimus; MMF = mycophenolate mofetil; MPA = mycophenolic acid; SRL = sirolimus
Data expressed as median (range)
[a] Chi Square test for categorical data, Wilcoxon's rank sum test for continuous data, $p<0.05$

Table 3.2. Characteristics of lung transplant recipients in the index and validation groups

	All lung subjects (n=31)	**Index group (n=16)**	**Validation group (n=15)**
Male (%)	64.5	62.5	66.7
CSA/TAC (n)	8/23	4/12	4/11
Age (y)	53.3 (24.0 – 70.5)	49.5 (24.0 – 70.5)	54.7 (27.4 – 67.4)
Years since transplant (y)	1.4 (0.2 – 12.3)	1.9 (0.2 – 12.3)	0.7 (0.3 – 7.6)
Weight (Kg)	72.7 (46.0 – 109.2)	75.2 (55.6 – 109.1)	68.5 (46.0 – 109.2)
MMF BID dose (mg)	1250 (500 – 1500)	1500 (500 – 1500)	1000 (750 – 1500)
Serum creatinine (μmol/L)	102 (78 – 218)	101 (78 – 218)	102 (79 – 193)
Albumin (g/L)	37 (25 – 47)	39 (33 – 47)[a]	36 (25 – 44)[a]
MPA AUC (ug*h/mL)	31.26 (9.45 – 172.96)	32.80 (12.45 – 172.96)	26.66 (9.45 – 159.11)
Dose-normalized MPA AUC (ug*h/mL)	27.85 (8.30 – 212.14)	31.14 (8.30 – 115.31)	26.33 (6.30 – 212.14)

AUC = area-under-the-curve; BID = twice a day; CSA = cyclosporine; TAC = tacrolimus; MMF = mycophenolate mofetil; MPA = mycophenolic acid; SRL = sirolimus

Data expressed as median (range)

[a] Chi Square test for categorical data, Wilcoxon's rank sum test for continuous data, $p<0.05$.

Table 3.3. Predictive performance of limited sampling strategies using untransformed data for lung transplant recipients

Timed concentrations	LSS equation (ug*h/mL for AUC, ug/mL for Cx)	Adjusted r^2	% Bias (% ME)[a]	% Precision (% RMSE)[a]	Number (%) of profiles (out of 15) within ±15% bias[a]
C0, C0.3, C2	AUC = 2.2940 C0 + 0.9308 C0.3 + 3.2379 C2 + 10.0927	0.957	27.70	48.96	7 (46.7%)
C0, C0.6, C2	AUC = 2.896 C0 + 0.9680 C0.6 + 2.9355 C2 + 88464	0.953	23.55	44.04	6 (40.0%)
C0.3, C0.6, C2	AUC = 2.9739 C0.3 - 2.1163 C0.6 + 3.9285 C2 + 15.6056	0.949	41.91	69.86	4 (26.7%)
C0, C0.6, C1.5	AUC = 2.3529 C0 + 0.8833 C0.6 + 2.5351 C1.5 8.5594	0.948	**11.59**	41.00	5 (33.3%)
C0, C0.3, C1.5	AUC = 1.9428 C0 + 0.7191 C0.3 + 2.7619 C1.5 + 9.974	0.946	15.27	44.55	2 (13.3%)
C0, C1, C1.5	AUC = 3.0305 C0 + 1.2614 C1 + 2.2817 C1.5 + 6.2652	0.956	17.57	43.09	3 (20.0%)
C0, C1, C2	AUC = 3.5900 C0 + 1.1613 C1 + 2.6971 C2 + 7.2655	0.944	29.73	44.85	4 (26.7%)
C0.3, C1.5, C2	AUC = 1.2531 C0.3 + 1.0890 C1.5 + 2.0474 C2 +	0.942	27.87	53.92	6 (40.0%)

Timed concentrations	LSS equation (ug*h/mL for AUC, ug/mL for Cx)	Adjusted r^2	% Bias (% ME)[a]	% Precision (% RMSE)[a]	Number (%) of profiles (out of 15) within ±15% bias[a]
	13.5227				
C0.3, C1, C2	AUC = 1.4073 C0.3 – 0.2030 C1 + 3.3931 C2 + 14.5464	0.939	33.56	61.69	6 (40.0%)
C0, C1.5, C2	AUC = 3.3261 C0 + 1.3010 C1.5 + 1.7055 C2 + 11.8190	0.938	28.16	50.05	5 (33.3%)
C0.3, C1, C1.5	AUC = 0.0170 C0.3 + 0.3394 C1 + 2.6934 C1.5 + 12.1599	0.934	**8.66**	49.67	6 (40.0%)
C0.3, C0.6, C1.5	AUC = 1.4053 C0.3 – 0.4440 C0.6 + 2.9249 C1.5 + 13.5091	0.933	20.98	54.36	3 (20.0%)
C0.6, C1.5, C2	AUC = 1.2747 C0.6 + 1.7135 C1.5 + 0.9465 C2 + 13.7106	0.926	22.97	54.14	6 (40.0%)
C0.6, C1, C1.5	AUC = 1.1734 C0.6 + 0.1950 C1 + 2.4666 C1.5 + 12.7877	0.924	18.93	53.97	4 (26.7%)
C0.6, C1, C2	AUC = 1.4589 C0.6 – 0.1110 C1 + 2.9244 C2 + 14.9252	0.917	31.80	62.04	7 (46.7%)
C1, C1.5, C2	AUC = 1.2027 C1 + 2.4328 C1.5 –	0.900	32.87	68.04	2 (13.3%)

Timed concentrations	LSS equation (ug*h/mL for AUC, ug/mL for Cx)	Adjusted r^2	% Bias (% ME)[a]	% Precision (% RMSE)[a]	Number (%) of profiles (out of 15) within ±15% bias[a]
	0.0301 C2 + 14.9812				
C0, C0.3, C0.6	AUC = 6.744 C0 – 6.6927 C0.3 + 8.9226 C0.6 – 1.1271	0.790	**-14.94**	50.30	1 (6.7%)
C0.3, C2	AUC = 1.3626 C0.3 + 3.3046 C2 + 13.9379	0.943	33.28	60.20	6 (40.0%)
C0, C2	AUC = 3.6519 C0 + 3.2024 C2 + 12.2269	0.938	34.94	57.64	7 (46.7%)
C0.3, C1.5	AUC = 1.0792 C0.3 + 2.8168 C1.5 + 13.1612	0.938	19.66	53.30	5 (33.3%)
C0, C1.5	AUC = 2.9869 C0 + 2.7496 C1.5 + 11.56	0.937	20.85	49.68	3 (20.0%)
C0.6, C1.5	AUC = 1.2319 C0.6 + 2.5225 C1.5 + 13.2709	0.930	18.57	54.21	3 (20.0%)
C0.6, C2	AUC = 1.4244 C0.6 + 2.8863 C2 + 14.6326	0.923	31.83	61.46	6 (40.0%)
C1, C1.5	AUC = 1.1996 C1 + 2.4085 C1.5 + 15.0154	0.908	33.03	68.07	2 (13.3%)
C1.5, C2	AUC = 2.5229 C1.5 + 0.3907 C2 +	0.897	38.01	76.42	2 (13.3%)

Timed concentrations	LSS equation (ug*h/mL for AUC, ug/mL for Cx)	Adjusted r^2	% Bias (% ME)[a]	% Precision (% RMSE)[a]	Number (%) of profiles (out of 15) within ±15% bias[a]
	20.2118				
C1, C2	AUC = 1.2796 C1 + 2.7538 C2 + 17.1738	0.889	48.46	78.19	3 (20.0%)
C1.5	AUC = 2.8521 C1.5 + 19.9346	0.904	35.95	75.72	3 (20.0%)
C2	AUC = 3.3126 C2 + 22.8386	0.886	54.57	92.43	3 (20.0%)
C4	AUC = 8.2987 C4 + 11.3261	0.871	**10.04**	39.01	1 (6.7%)

AUC = area-under-the-curve; Cx = concentration at hour x post-dose; ME = mean prediction error; RMSE = root mean square prediction error

[a] Values within acceptable limits are in bold.

Table 3.4. Predictive performance of limited sampling strategies using log-transformed data for lung transplant recipients

Timed concentrations	LSS equation (ug*h/mL for AUC, ug/mL for Cx)	Adjusted r^2	% Bias (% ME) [a]	% Precision (% RMSE) [a]	Number (%) of profiles (out of 15) within ±15% bias [a]
Log C0, Log C0.3, Log C2	Log AUC = 0.1057 Log C0 + 0.1098 Log C0.3 + 0.5910 C2 + 1.0855	0.846	**6.33**	**11.53**	12 (80.0%)
Log C0.3, Log C0.6, Log C2	Log AUC = 0.2331 Log C0.3 – 0.1398 Log C0.6 + 0.6805 C2 + 1.0874	0.838	**7.06**	**13.73**	12 (80.0%)
Log C0, Log C1.5, Log C2	Log AUC = 0.1386 Log C0 + 0.2106 Log C1.5 + 0.4084 Log C2 + 1.1027	0.834	**7.13**	**12.59**	**13 (86.7%)**
Log C0, Log C1, Log C2	Log AUC = 0.1585 Log C0 + 0.0964 Log C1 + 0.5562 Log C2 + 1.0887	0.833	**8.07**	**12.73**	12 (80.0%)
Log C0.3, Log C1.5, Log C2	Log AUC = 0.1423 Log C0.3 + 0.1977 Log C1.5 + 0.4344 Log C2 + 1.0494	0.832	**5.40**	**12.20**	**13 (86.7%)**
Log C0, Log C0.6, Log C2	Log AUC = 0.173 Log C0 + 0.0751 Log C0.6 + 0.5649 Log C2 + 1.1041	0.831	**7.20**	**12.40**	12 (80.0%)
Log C0, Log C0.6, Log C1.5	Log AUC = 0.1839 Log C0 + 0.1359 Log C0.6 + 0.4927 Log 1.5 + 1.0320	0.829	**2.10**	**11.46**	**13 (86.7%)**

Timed concentrations	LSS equation (ug*h/mL for AUC, ug/mL for Cx)	Adjusted r^2	% Bias (% ME) [a]	% Precision (% RMSE) [a]	Number (%) of profiles (out of 15) within ±15% bias [a]
Log C0.3, Log C1, Log C2	Log AUC = 0.1788 Log C0.3 – 0.0931 Log C1 + 0.6622 C2 + 1.1011	0.829	**6.23**	**13.43**	12 (80.0%)
Log C0, Log C0.3, Log C1.5	Log AUC = 0.1338 Log C0 + 0.1258 Log C0.3 + 0.5413 Log C1.5 + 1.0307	0.823	**1.66**	**12.06**	**13 (86.7%)**
Log C0, Log C1, Log C1.5	Log AUC = 0.1995 Log C0 + 0.1493 Log C1 + 0.4868 Log C1.5 + 1.0192	0.821	**3.86**	**12.21**	**13 (86.7%)**
Log C0.6, Log C1.5, Log C2	Log AUC = 0.0524 Log C0.6 + 0.2024 Log C1.5 + 0.4474 Log C2 + 1.0912	0.793	**7.92**	15.43	**13 (86.7%)**
Log C1, Log C1.5, Log C2	Log AUC = 0.0269 Log C1 + 0.1833 Log C1.5 + 0.4841 Log C2 + 1.1013	0.789	**8.92**	16.47	11 (73.3%)
Log C0.3, Log C1, Log C1.5	Log AUC = 0.1881 Log C0.3 – 0.0480 Log C1 + 0.5951 Log C1.5 + 1.0303	0.784	**1.52**	**13.81**	12 (80.0%)
Log C0.3, Log C0.6, Log C1.5	Log AUC = 0.1873 Log C0.3 – 0.0292 Log C0.6 + 0.5882 Log C1.5 + 1.0214	0.782	**5.99**	**13.99**	12 (80.0%)
Log C0.6, Log C1, Log	Log AUC = 0.0590 Log C0.6 – 0.0287 Log C1 + 0.6559 C2	0.781	**9.37**	17.38	11 (73.3%)

Timed concentrations	LSS equation (ug*h/mL for AUC, ug/mL for Cx)	Adjusted r^2	% Bias (% ME) [a]	% Precision (% RMSE) [a]	Number (%) of profiles (out of 15) within ±15% bias [a]
C2	+ 1.1293				
Log C0, Log C2	Log AUC = 0.1332 Log C0 + 0.6166 Log C2 + 1.318	0.835	**8.70**	**14.60**	12 (80.0%)
Log C0.3, Log C2	Log AUC = 0.1392 Log C0.3 + 0.6286 Log C2 + 1.0778	0.834	**5.92**	**13.55**	12 (80.0%)
Log C0, Log C1.5	Log AUC = 0.1677 Log C0 + 0.5657 Log C1.5 + 1.0830	0.806	**4.17**	**12.79**	**13 (86.7%)**
Log C1.5, Log C2	Log AUC = 0.1817 Log C1.5 + 0.4994 Log C2 + 1.1132	0.804	**9.02**	16.76	11 (73.3%)
Log C0.3, Log C1.5	Log AUC = 0.1667 Log C0.3 + 0.5800 Log C1.5 + 1.0194	0.798	**1.99**	**13.96**	12 (80.0%)
Log C0.6, Log C2	Log AUC = 0.0398 Log C0.6 + 0.6529 Log C2 + 1.1238	0.798	**9.62**	17.49	11 (73.3%)
Log C1, Log C2	Log AUC = 0.0246 Log C1 + 0.6644 Log C2 + 1.1274	0.796	**10.24**	18.17	11 (73.3%)
Log C0.6, Log C1.5	Log AUC = 0.1098 Log C0.6 + 0.5754 Log C1.5 + 1.0502	0.764	**4.19**	15.14	12 (80.0%)
Log C2	Log AUC = 0.6769 Log C2 + 1.1381	0.809	**10.33**	18.49	11 (73.3%)

Timed concentrations	LSS equation (ug*h/mL for AUC, ug/mL for Cx)	Adjusted r^2	% Bias (% ME) [a]	% Precision (% RMSE) [a]	Number (%) of profiles (out of 15) within ±15% bias [a]
Log C1.5	Log AUC = 0.6294 Log C1.5 + 1.0907	0.759	**5.73**	16.52	12 (80.0%)
Log C4	Log AUC = 0.7069 Log C4 + 1.2344	0.813	**-0.22**	**10.34**	12 (80.0%)

AUC = area-under-the-curve; Cx = concentration at hour x post-dose; ME = mean prediction error; RMSE = root mean square prediction error

[a] Values within acceptable limits are in bold.

Table 3.5. Predictive performance of limited sampling strategies in 33 heart transplant recipients using log-transformed data derived from lung transplant recipients

Timed concentrations	LSS equation (ug*h/mL for AUC, ug/mL for Cx)	Adjusted r^2	% Bias (% ME) [a]	% Precision (% RMSE) [a]	Number (%) of profiles (out of 33) within ±15% bias[a]
Log C0, Log C0.3, Log C2	Log AUC = 0.1057 Log C0 + 0.1098 Log C0.3 + 0.5910 C2 + 1.0855	0.846	**-3.13**	**10.50**	**30 (90.9%)**
Log C0.3, Log C0.6, Log C2	Log AUC = 0.2331 Log C0.3 – 0.1398 Log C0.6 + 0.6805 C2 + 1.0874	0.838	**-3.66**	**11.21**	26 (78.8%)
Log C0, Log C1.5, Log C2	Log AUC = 0.1386 Log C0 + 0.2106 Log C1.5 + 0.4084 Log C2 + 1.1027	0.834	**-2.90**	**10.65**	**31 (93.9%)**
Log C0, Log C1, Log C2	Log AUC = 0.1585 Log C0 + 0.0964 Log C1 + 0.5562 Log C2 + 1.0887	0.833	**-2.66**	**10.07**	**30 (90.9%)**
Log C0.3, Log C1.5, Log C2	Log AUC = 0.1423 Log C0.3 + 0.1977 Log C1.5 + 0.4344 Log C2 + 1.0494	0.832	**-3.93**	**10.95**	**30 (90.9%)**
Log C0, Log C0.6, Log C2	Log AUC = 0.173 Log C0 + 0.0751 Log C0.6 + 0.5649 Log C2 + 1.1041	0.831	**-2.71**	**10.33**	**30 (90.9%)**
Log C0, Log C0.6, Log C1.5	Log AUC = 0.1839 Log C0 + 0.1359 Log C0.6 + 0.4927	0.829	**-3.56**	**12.55**	27 (81.8%)

Timed concentrations	LSS equation (ug*h/mL for AUC, ug/mL for Cx)	Adjusted r^2	% Bias (% ME) [a]	% Precision (% RMSE) [a]	Number (%) of profiles (out of 33) within ±15% bias[a]
	Log 1.5 + 1.0320				
Log C0.3, Log C1, Log C2	Log AUC = 0.1788 Log C0.3 – 0.0931 Log C1 + 0.6622 C2 + 1.1011	0.829	**-3.60**	**11.35**	26 (78.8%)
Log C0, Log C0.3, Log C1.5	Log AUC = 0.1338 Log C0 + 0.1258 Log C0.3 + 0.5413 Log C1.5 + 1.0307	0.823	**-4.03**	**13.19**	28 (84.8%)
Log C0, Log C1, Log C1.5	Log AUC = 0.1995 Log C0 + 0.1493 Log C1 + 0.4868 Log C1.5 + 1.0192	0.821	**-3.44**	**12.69**	27 (81.8%)
Log C0.6, Log C1.5, Log C2	Log AUC = 0.0524 Log C0.6 + 0.2024 Log C1.5 + 0.4474 Log C2 + 1.0912	0.793	**-3.48**	**10.89**	**31 (93.9%)**
Log C1, Log C1.5, Log C2	Log AUC = 0.0269 Log C1 + 0.1833 Log C1.5 + 0.4841 Log C2 + 1.1013	0.789	**-3.35**	**10.93**	**29 (87.9%)**
Log C0.3, Log C1, Log C1.5	Log AUC = 0.1881 Log C0.3 – 0.0480 Log C1 + 0.5951 Log C1.5 + 1.0303	0.784	**-4.74**	**14.15**	25 (75.8%)
Log C0.3, Log C0.6, Log C1.5	Log AUC = 0.1873 Log C0.3 – 0.0292 Log C0.6 + 0.5882 Log C1.5 + 1.0214	0.782	**-4.74**	**14.17**	25 (75.8%)
Log C0.6,	Log AUC = 0.0590	0.781	**-3.10**	**11.18**	28

Timed concentrations	LSS equation (ug*h/mL for AUC, ug/mL for Cx)	Adjusted r^2	% Bias (% ME) [a]	% Precision (% RMSE) [a]	Number (%) of profiles (out of 33) within ±15% bias[a]
Log C1, Log C2	Log C0.6 – 0.0287 Log C1 + 0.6559 C2 + 1.1293				(84.8%)
Log C0, Log C2	Log AUC = 0.1332 Log C0 + 0.6166 Log C2 + 1.318	0.835	**-2.57**	**10.91**	28 (84.8%)
Log C0.3, Log C2	Log AUC = 0.1392 Log C0.3 + 0.6286 Log C2 + 1.0778	0.834	**-3.60**	**10.71**	**29 (87.9%)**
Log C0, Log C1.5	Log AUC = 0.1677 Log C0 + 0.5657 Log C1.5 + 1.0830	0.806	**-3.43**	**12.66**	27 (81.8%)
Log C1.5, Log C2	Log AUC = 0.1817 Log C1.5 + 0.4994 Log C2 + 1.1132	0.804	**-3.30**	**11.12**	**29 (87.9%)**
Log C0.3, Log C1.5	Log AUC = 0.1667 Log C0.3 + 0.5800 Log C1.5 + 1.0194	0.798	**-4.70**	**14.11**	25 (75.8%)
Log C0.6, Log C2	Log AUC = 0.0398 Log C0.6 + 0.6529 Log C2 + 1.1238	0.798	**-3.10**	**11.04**	**29 (87.9%)**
Log C1, Log C2	Log AUC = 0.0246 Log C1 + 0.6644 Log C2 + 1.1274	0.796	**-3.05**	**11.12**	28 (84.8%)
Log C0.6, Log C1.5	Log AUC = 0.1098 Log C0.6 + 0.5754 Log C1.5 + 1.0502	0.764	**-4.24**	**13.55**	24 (72.7%)
Log C2	Log AUC = 0.6769	0.809	**-3.00**	**11.39**	26 (78.8%)

Timed concentrations	LSS equation (ug*h/mL for AUC, ug/mL for Cx)	Adjusted r^2	% Bias (% ME) [a]	% Precision (% RMSE) [a]	Number (%) of profiles (out of 33) within ±15% bias[a]
	Log C2 + 1.1381				
Log C1.5	Log AUC = 0.6294 Log C1.5 + 1.0907	0.759	**-4.09**	**13.69**	25 (75.8%)
Log C4	Log AUC = 0.7069 Log C4 + 1.2344	0.813	**-0.42**	**9.83**	**29 (87.9%)**

AUC = area-under-the-curve; Cx = concentration at hour x post-dose; ME = mean prediction error; RMSE = root mean square prediction error

[a] Values within acceptable limits are in bold.

Table 3.6. Predictive performance of LSS equations from the literature when applied to 33 heart/heart-kidney transplant recipients

Literature LSS equation	Timed concentrations used	% Bias (%ME)	% Precision (%RMSE)	Number (%) of profiles (out of 33) within ±15% bias
Equation 3	C1.25[a], C2, C6	-14.31	23.92	13 (39.4%)
Equation 4	C1.25[a], C2, C4, C6	-13.32	20.44	17 (51.5%)
Equation 5	C0, C1, C2	12.90	68.00	6 (35.3%)[b]
Equation 6	C1, C2, C4	6.50	38.17	9 (56.3%)[c]
Equation 7	C0.5[d], C1, C2	-30.62	38.11	9 (27.3%)
Equation 8	C0.5[d], C2	-20.80	35.56	11 (33.3%)

[a] C1.25 was estimated by averaging C1 and C1.5
[b] Equation 5 developed for patients also taking cyclosporine; number of profiles tested = 17
[c] Equation 6 developed for patients also taking tacrolimus; number of profiles tested = 16, which included two patients taking sirolimus
[d] C0.5 was estimated by averaging C0.3 and C0.6.

Table 3.7. Selected MPA LSSs developed by multiple regression analysis in adult transplant recipients from other research groups

Research group	Patient population	Sampling times (hr)	Suggested concentrations	LSS equation (for estimation of AUC_{0-12})
Baraldo et al.[26]	Heart transplant	0, 0.5, 1.25, 2, 4, 6, 8, 12	(C1.25, C2, C6) and (C1.25, C2, C4, C6)	AUC = 5.568 + 0.902 C1.25 + 2.022 C2 + 4.594 C6 AUC = 3.80 + 1.1015 C1.25 + 1.819 C2 + 1.566 C4 + 3.479 C6
Kaczmarek et al.[27]	Heart transplant	0, 0.5, 1, 2, 3, 4, 6, 8, 10, 12	(C0.5, C1, C2) and (C0.5, C2)	AUC = 1.09 C0.5 + 1.19 C1 + 3.60 C2 AUC = 1.65 C0.5 + 4.74 C2
Kuriata-Kordek et al.[34]	Kidney transplant	0, 0.6, 1, 2, 4, 6, 8, 10, 12	(C2, C6) and (C4, C8, C12)	CSA co-medication: AUC = 11.73 C6 + 2.92 C2 – 0.274 TAC co-medicaiton: AUC = 7.06 C4 + 6.77 C8 + 3.76 C12 + 15.3
Le Guellec et al.[19]	Kidney transplant	0, 0.3, 0.6, 1, 1.5, 2, 3, 4, 6, 9	(C0.3, C1, C3) for both MPA and CSA	AUC = 0.58 C0.3 + 0.97 C1 + 6.64 C3 + 3.48
Pawinski et al.[20]	Kidney transplant	0, 0.5, 1, 2, 3, 4, 6, 8, 9, 10, 11, 12	(C0, C0.5, C2)	AUC = 7.75 + 6.49 C0 + 0.76 C0.5 + 2.43 C2
Van Hest et al.[33]	Kidney transplant, diabetic	0, 0.3, 0.6, 1.25, 2, 6, 8, 12	(C0, C0.6, C2)	AUC = 7.182 + 4.607 C0 + 0.998 C0.6 + 2.149 C2
Wada et al.[25]	Japanese heart transplant	0, 1, 2, 4, 6, 8, 12	(C0, C1, C2) and (C1, C2, C4)	CSA co-medication: AUC = 0.10 + 11.15 C0 + 0.42 C1 + 0.28 C2 TAC co-medication:

Research group	Patient population	Sampling times (hr)	Suggested concentrations	LSS equation (for estimation of AUC_{0-12})
				AUC = 23.56 + 1.05 C1 + 1.25 C2 + 2.53 C4
Willis et al.[32]	Kidney, 1st month post-transplant	0, 0.25, 0.75, 1, 1.25, 1.5, 2, 3, 4, 6, 8, 10, 12	(C0, C1, C3, C6)	AUC = 9.02 + 3.77 C0 + 1.33 C1 + 1.68 C3 + 2.96 C6
Teshima et al.[35]	Kidney transplant	0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12	(C2, C7, C12)	AUC = 2.05 C2 + 8.51 C7 + 2.29 C12 + 4.24
Zicheng et al.[36]	Liver transplant	0, 0.5, 1, 1.5, 2, 4, 6, 8, 10, 12	(C1, C2, C6, C8)	AUC = 6.03 + 0.89 C1 + 1.94 C2 + 2.24 C6 + 4.64 C8
Jiao et al.[37]	Chinese kidney transplant	0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12	(C2, C3, C4) and (C1, C2, C4)	AUC = 10.403 + 0.841 C2 + 1.105 C3 + 0.447 C4 AUC = 3.504 + 1.098 C1 + 0.670 C2 + 5.659 C4
Chen et al.[38]	Chinese liver transplant	0, 0.5, 1, 1.5, 2, 4, 6, 8, 10, 12	(C1, C2, C4)	AUC = 10.776 + 0.749 C1 + 1.604 C2 + 4.116 C4
Ng et al.[39]	Hematopoietic cell Transplant	0, 1, 2, 4, 6, 8, 12	(C0, C1, C2, C6)	AUC = 4.43 + 2.76 C0 + 0.51 C1 + 1.97 C2 + 4.27 C6
Miura et al.[40]	Japanese kidney transplant	0, 1, 2, 3, 4, 6, 9, 12	(C2, C4, C9) and (C0, C2, C4)	AUC = 1.77 C2 + 2.34 C4 + 4.76 C9 + 15.94 AUC = 2.25 C2 + 1.92 C4 + 7.27 C9 + 6.61
Zhou et al.[41]	Chinese kidney	0, 0.5, 1, 1.5, 2, 4, 6, 8, 10,	(C0.5, C2, C5) and (C0.5, C2,	AUC = 14.81 + 0.80 C0.5 + 1.56 C2 +

Research group	**Patient population**	**Sampling times (hr)**	**Suggested concentrations**	**LSS equation (for estimation of AUC_{0-12})**
	transplant	12	C8)	4.80 C4 AUC = 11.29 + 0.51 C0.5 + 2.13 C2 + 8.15 C8

AUC = area under the concentration-time curve
Cx = concentration at hour x post-dose

3.7. References

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4. PHARMACOGENETICS OF MYCOPHENOLATE AND *UGT* AND *ABCC2* POLYMORPHISMS IN THORACIC TRANSPLANT RECIPIENTS[1]

[1] A version of this chapter will be submitted for publication. **Ting LSL**, Benoit-Biancamano MO, Bernard O, Riggs KW, Guillemette C and Ensom MHH. Pharmacogenetics of Mycophenolate and *UGT* and *ABCC2* Polymorphisms in Thoracic Transplant Recipients.

4.1. Introduction

Mycophenolate mofetil (MMF) is a commonly used immunosuppressive agent, in combination with cyclosporine, tacrolimus, or sirolimus in maintenance immunosuppression for solid organ transplant recipients.[1] MMF is a an ester prodrug of the active metabolite mycophenolic acid (MPA), which is responsible for the immunosuppressive actions.[2, 3] Mycophenolic acid inhibits the proliferation of T-lymphocytes by inhibiting the enzyme inosine monophosphate dehydrogenase type-2, a key enzyme in the *de novo* pathway of purine synthesis.[1, 2] Although MPA is very effective in its immunosuppressive action, management of MMF therapy is challenged by the wide inter-patient variability in the pharmacokinetic (PK) parameters of MPA.[1, 4-7] Since recent studies support an association between MPA exposure and clinical outcomes,[8-10] PK monitoring of MPA is becoming an important tool in optimizing MMF therapy. However, the source of the wide PK variability is not fully elucidated, and prediction of MPA exposure and drug response remains difficult.[11-13]

Over 90% of MPA is metabolized by UDP-glucuronosyltransferase (UGT) enzymes in the liver, gastrointestinal tract and kidneys via glucuronidation to the inactive metabolite 7-O-mycophenolic acid glucuronide (MPAG). Mycophenolic acid also goes through enterohepatic recirculation as MPAG is released into the bile and de-glucuronidated in the GI tract. In addition, about 5% of MPA is metabolized to the acyl glucuronide of MPA (AcMPAG), which is pharmacologically active and has shown proinflammatory activities. It has been postulated that AcMPAG may be responsible for some adverse reactions associated with MMF therapy.[14-17] Recently, other minor

glucoside metabolites of MPA have been identified, but the physiological effect of these minor metabolites is not well understood.[18, 19]

The UGT isoforms 1A8, 1A9, and 2B7 are the main enzymes that metabolize MPA. UGT1A9 is considered to play an essential role in the hepatic clearance of MPA via glucuronidation to MPAG; the extrahepatic UGT1A8 is involved in formation of MPAG, and to a lesser extent, AcMPAG, in the kidneys and gastrointestinal tract; the minor metabolite AcMPAG is predominantly formed by UGT2B7, which is expressed in the liver, GI tract, and kidneys.[20-24] The multidrug resistance-associated protein 2 (MRP2), which exports glucuronidated compounds into the bile, also contributes to the disposition of MPA, MPAG and AcMPAG.[25-27] This could impact MPA pharmacokinetics significantly, as the enterohepatic recirculation of MPA, mediated by biliary excretion and deglucuronidation of MPAG, contributes from 10 – 60% of total MPA exposure.[3] The significance of the MRP2 transporter is further emphasized as it is also involved in the drug-drug interaction between cyclosporine and MPA. .[25-28]

Since these proteins are responsible for the systemic clearance and enterohepatic recirculation of MPA, genetic polymorphisms in the *UGT* and *ABCC2* (MRP2) genes may partially explain the pharmacokinetic variability of MPA. A number of *UGT* and *ABCC2* variants with potential functional impacts have been identified in the regulatory and coding regions;[20, 21, 24, 28-37] several clinical studies have demonstrated their influence on MPA disposition *in vivo*. Results are not consistent, however, underscoring the complexity of MPA pharmacogenetics.

To our knowledge, all of the *in vivo* MPA studies on genetic polymorphisms in the *UGT* and *ABCC2* genes have been performed in renal transplant recipients or healthy

subjects,[28, 38-45] and no data are available in the thoracic transplant subpopulation. Unlike the kidney, the heart or lung is not involved in the metabolism or excretion of MPA and its glucuronidated metabolites; thus, the thoracic transplant population may exhibit unique pharmacokinetics. Given the multiple gene targets and polymorphisms, clinical pharmacogenetic studies that consider both *UGT* and *ABCC2* gene candidates are warranted. The objective of this study was to investigate the contributions of *UGT* and *ABCC2* genetics to the inter-patient variability of MPA pharmacokinetics in thoracic transplant recipients on steady-state mycophenolate therapy. As a secondary objective, we also explored whether *UGT* and *ABCC2* genetics impact on clinical outcomes directly.

4.2. Materials and Methods

This was an open-label clinical study. This study was approved by the UBC Clinical Research Ethics Board (#C02-0568) (Appendices A and B), Vancouver Coastal Health Authority Clinical Trials Administration Office (#V03-0162), Providence Health Care Research Ethics Board (#P04-0190), and CHUL Research Center and Laval University (#104.05.06).

4.2.1. Subjects

Sixty-eight thoracic transplant recipients from British Columbia were recruited from the Vancouver General Hospital lung transplant program, St. Paul's Hospital heart transplant program, and British Columbia Transplant Society. Details are described in Sections 2.2.1 and 2.2.2. Inclusion criteria stipulated that subjects be: at least 16 years of

age, on steady-state MMF (taking the same dosage of MMF for at least five days prior to the study), able to provide informed consent, and not taking antacids or cholestyramine.

4.2.2. Study protocol

The detailed study protocol is described in Sections 2.2.1 and 2.2.2. Briefly, subjects reported to the British Columbia Transplant Society for their study visit after an overnight fast and before taking their morning dose of MMF. After obtaining written informed consent, an indwelling intravenous catheter was placed in a forearm vein for serial blood collection. Eleven blood samples were collected in 3-mL vacutainers containing ethylenediaminetetraacetic acid at time 0 (before medication intake), 20, 40, 60, and 90 minutes, and 2, 4, 6, 8, 10 and 12 hours after taking MMF. Plasma was separated by centrifugation and collected immediately. Two aliquots were prepared, one of which (1 mL) was acidified (pH 2-4 with 85% phosphoric acid solution, 20µL per 1 mL of plasma). Samples were stored at - 80°C until analysis for total MPA, fMPA, MPAG and AcMPAG concentrations. In addition, two 10-mL heparinized (BD Vacutainer® Heparin, Franklin Lake, NJ) whole blood samples were collected pre-dose (time 0) for genetic analysis; these samples were stored at -80°C until analysis.

4.2.3. Clinical data collection

Occurrences of clinical events were collected qualitatively (event vs. no event) from subjects' medical charts. These included occurrences of: gastrointestinal toxicities (nausea, vomiting, diarrhea or abdominal cramps), leucopenia, thrombocytopenia, anemia, infections (e.g. cytomegalovirus and herpes infections), and biopsy-proven acute rejection episodes. Only clinical events occurring between date of last

immunosuppressant medication (MMF and cyclosporine, tacrolimus or sirolimus) change and pharmacokinetic sampling day were considered for analysis.

4.2.4. Plasma concentrations of MPA, MPAG and AcMPAG

The concentrations of total MPA, MPAG and AcMPAG were determined quantitatively in patient plasma samples by a Waters Alliance System (Waters Ltd., Mississauga, ON) high performance liquid chromatography with ultraviolet detection (HPLC-UV) as described in Sections 2.2.3 and 2.2.4.[46, 47] Free fraction of MPA was determined by ultracentrifugation [Microcon YM-30 filter (30000 molecular weight cut-off, Millipore, Billerica, MA)] of spiked non-acidified plasma (to 25 µg/mL of MPA) from each subject. Concentration of MPA was determined in an aliquot of the spiked plasma and the filtrate.[46, 48] Free fraction was calculated by dividing free MPA (fMPA) concentration by total MPA concentration in the spiked plasma. For detailed methodology, please see Section 2.2.5.

4.2.5. Pharmacokinetic analysis

Conventional pharmacokinetic parameters were calculated for each subject by non-compartmental analysis using WinNonlin version 5.2 (Pharsight, Mountain View, CA), and normalized by MMF dose (per g of MMF) for presentation of results. Total and partial AUCs for MPA, MPAG and AcMPAG were determined via the linear trapezoidal method. Enterohepatic recirculation was estimated by AUC(6-12)/AUC(0-12).

4.2.6. Pharmacogenetic analysis of **UGT1, UGT2B7***, and* **ABCC2** *genes*

Genomic DNA was extracted from whole blood using the QIAmp blood Maxi Kit (Qiagen Inc., Chatsworth, CA). The concentration of DNA was determined by an

Eppendorf BioPhotometer (Eppendorf, Hamburg, Germany). Coding and/or regulatory regions of *UGT1A1, 1A7, 1A8*, *1A9*, *2B7* and *ABCC2* genes as well as the 3' untranslated region of the *UGT1* gene were amplified by polymerase chain reaction using specific primers.[39, 49](Table 4.1) In brief, polymerase chain reactions were carried out with 1 µL of DNA (25 µg/µL). The reaction volume for the PCR was 50 µL containing 2 mmol/L $MgCl_2$, 0.2 mmol/l of each deoxynucleotide triphosphate, 0.4 µmol/L of each primer, and 2 units of Taq polymerase. The reaction was incubated at 94ºC for 3 minutes, followed by 35-40 cycles at 94ºC for 30 seconds, 52 - 60ºC for 30-40 seconds, and 72ºC for 30-60 seconds, with a final elongation step at 72ºC for 7 minutes. Genotyping was achieved by direct sequencing, performed using the automated sequencer ABI 3700 (Applied Biosystems, Foster City, CA); sequences were analyzed by visual inspection and using the Staden preGap4 and Gap4 software programs (Open Source Technology Group, http://staden.courceforge.net/).[39, 49] Reference sequences obtained from GenBank (AF297093 for *UGT1*, AC11000 for *UGT2B7*, and NT030059 for *ABCC2*) were used to compare and detect genetic variations. Ambiguous sequences were subsequently re-amplified and re-sequenced.

4.2.7. Statistical analysis

While the study sample size was one based on convenience, a sample size of at least 32 was estimated to be statistically sufficient to discriminate differences of 75% between UGT2B7*1 homozygous subjects (control group) and subjects carrying the UGT2B7*2 allele with a statistical power of 80% and an alpha of 5%. Statistical analyses were carried out with JMP 6 software (SAS Institute Inc., Cary, NC). Descriptive statistics were used for demographic and pharmacokinetic data. Mean comparisons were

performed by Wilcoxon rank sum test for nonparametric data. For parametric data, mean comparisons were performed by Student's t-test (for two groups) or analysis of variance (for more than two groups). Stepwise multiple regression analysis was performed to assess contribution of genetic variants and other demographic covariates (with $p \leq 0.1$ in univariate analysis) to the inter-subject variability of MPA pharmacokinetic parameters. Normality of distribution was assessed using the Shapiro-Wilk W test. Distribution was normalized by log or Box Cox Y transformation of the parameter values for which a normal distribution was not observed. When a variant was present in less than 10% of subjects, homozygous and heterozygous carriers were grouped together. Data were analyzed separately for lung and heart transplant subjects. Results were considered statistically significant if $p \leq 0.05$.

For pharmacokinetic-clinical outcomes analysis, subjects were categorized as having clinical event vs. no event, and high vs. low AcMPAG exposure. Since no current guidelines exist for AcMPAG levels, the cutoff values (50 µg*h/mL for AcMPAG AUC, and 2 for AcMPAG/MPA) were arbitrarily determined by observation of data distribution. Association of clinical events with PK parameters was performed by Fisher's exact test.

For genetic-clinical data comparison, Pearson's Chi Square test or Fisher's exact test was used in univariate analysis. Nominal logistics regression was subsequently used to assess impact of multiple genetic variants and other demographic covariates (with $p \leq 0.1$ in univariate analysis) on clinical events. Odds ratios were determined from the β-coefficients of nominal logistics regression models.

4.3. Results

4.3.1. Subject characteristics

Sixty-eight stable adult thoracic transplant recipients receiving twice-daily MMF were studied. Subjects consisted of 36 lung and 32 heart transplant recipients. Eighty-eight percent of the subjects were Caucasians; the rest included four Asians, two East Indians, one Arabian and one Native American. Subjects were also taking cyclosporine (n=28), tacrolimus (n=38), and sirolimus (n=2) as their co-medication. All lung transplant recipients were also taking prednisone in their immunosuppressive regimen, while only two heart transplant recipients took prednisone.

Subject demographics and diagnoses for transplantation are summarized in Table 4.2. There were more females, more tacrolimus and prednisone use in the lung transplant group; lung transplant recipients were given a significantly higher MMF twice-daily dose and dosage than heart transplant recipients, and had significantly lower serum creatinine and albumin levels (Table 4.2).

4.3.2. MPA pharmacokinetics

Pharmacokinetic results from 27 lung and 23 heart transplant recipients have been reported elsewhere.[4, 46] Dose-normalized pharmacokinetic parameters of MPA and the metabolites MPAG and AcMPAG of lung and heart transplant recipients are summarized in Table 4.3. Free MPA fraction was available for 47 subjects (32 heart and 15 lung transplant recipients). Large inter-patient variability was observed in all pharmacokinetic parameters of MPA, MPAG, and AcMPAG.

The lung transplant recipients had lower dose-normalized MPA pharmacokinetics than the heart transplant group, as characterized by significantly lower Cmax, Cmin, and total MPA AUC(0-12), and higher apparent clearance (Table 4.3). Although the MPAG metabolite exposure and MPAG/MPA ratio were significantly higher in the lung transplant group, no difference was observed for the metabolite AcMPAG. When subjects were further stratified into co-medication groups (cyclosporine, tacrolimus, or sirolimus), we observed lower dose-normalized MPA AUC and Cmin, and higher MPA apparent clearance and MPAG/MPA metabolic ratio in lung transplant recipients taking cyclosporine versus tacrolimus. A lower dose-normalized Cmin in heart transplant patients taking cyclosporine versus tacrolimus was also observed (See also chapter 2, Section 2.3.2).

4.3.3. Influence of genetic factors on MPA PK parameters in lung and heart recipients.

Allelic frequencies and functional impact of genetic variants in the studied population are listed in Table 4.4. The allelic frequencies observed were in Hardy-Weinberg equilibrium. Variants that were genotyped but not observed in this study population included: *UGT1A8* $Ala^{144}Val$, $Ala^{169}Thr$ and $Thr^{240}Ala$; *UGT1A9* -2208, $Lys^{75}Lys$; *UGT2B7* $Lys^{25}Asn$, $Asp^{121}Asn$, $Thr^{123}Asn$; *ABCC2* $Leu^{407}Leu$, $Arg^{412}Gly$ and $Lys^{430}Arg$.

The most significant factors that impact on MPA and metabolites pharmacokinetics in both lung and heart transplant groups are summarized in Table 4.5. For both transplant groups, co-medication (cyclosporine) was associated with lower MPA Cmin. The variant *UGT2B7* G-138A (observed in three lung and one heart transplant

recipients) was associated with higher AcMPAG/MPA metabolic ratio (reference vs. variant): 0.6±1.4 vs 5.6±5.8 and 0.3±0.4 vs 3.7 µg·h/mL in lung and heart transplant group, respectively; and *UGT2B7*2* (codon 268) was associated with higher AcMPAG AUC (reference vs. variant): 12.5±16.9 vs 30.6±50.9 µg*h/mL and 20.2±29.1 vs 73.8±100.3 µg*h/mL in the lung and heart transplant group, respectively.

In addition, various demographic factors (gender, age, serum creatinine level) and genetic polymorphisms had significant impact on MPA and metabolite pharmacokinetics specific to the lung or heart transplant group (Table 4.4); the percentage of contribution to the variability of the pharmacokinetic parameter ranged from 10 – 32% in the lung transplant, and 7 – 20% in the heart transplant group (Appendix C).

Of the studied polymorphisms, *UGT1A7*2* (129K/131K), *UGT1A8*2* (173G) and **3* (277I), *UGT1A9 -275A/-2152T*, *-440T/-331C*, *-118T*$_{9\text{-}10}$ and **3 (33T)*, and *ABCC2 -24T* were not found to markedly modify MMF pharmacokinetics in our multivariate model.

4.3.4. Influence of genetic factors on clinical events lung and heart recipients

Occurrences of GI toxicities, infection, rejection, anemia and leucopenia were observed in 24%, 22%, 15%, 38% and 12% of the 68 thoracic transplant recipients, respectively. Significant genetic and demographic factors that were associated with clinical events for all thoracic (n=68) transplant recipients are summarized in Table 4.6. No significant factors were associated with GI toxicities, and no individual significant factors associated with infection in multivariate analysis. Important associations included cyclosporine use, *UGT2B7* T-125C variant and *UGT2B7* G-138A variant with rejection; prednisone use, *ABCC2* Ile^{1324}Ile variant and *UGT1A7* Trp^{208}Arg variant with anemia;

and *ABCC2* Val[1188]Glu variant and *UGT* 3'UTR T1813C variant with leucopenia. Thrombocytopenia was observed only in one subject, and therefore was not tested.

4.3.5. Influence of AcMPAG exposure on clinical outcome in thoracic transplant recipients

As *UGT2B7* variants had a significant impact on AcMPAG exposure and metabolic ratio, we further explored the association of AcMPAG exposure and clinical outcomes in all thoracic transplant recipients (n=68). Significant association was observed with AcMPAG AUC (>50 μg*h/mL) and occurrences of infections (p=0.0105) and rejection (p=0.0132); AcMPAG/MPA metabolic ratio (>2) was also significantly associated with occurrences of infection (p=0.0375), anemia (p=0.0107) and leucopenia (p=0.0307).

4.4. Discussion

Positive pharmacogenetic results from healthy volunteers[39, 49] have corroborated the investigation of *UGT* and *ABCC2* genetics in the transplant population. Highly-controlled subject selection and genetic screening ensure minimal confounding factors in determining the impact of UGT genetics; however, in the clinical setting, other factors (e.g. multiple dosing, different dosages, co-medications, morbidities, organ function, and demographic factors) contribute to the pharmacokinetic variation of MPA. To date, there are only a few other clinical MPA pharmacogenetic studies, all in renal transplant subjects (see also Table 1.3).[28, 38, 40-45, 50, 51] To our knowledge, this is the first clinical study investigating the contribution of several *UGTs* and *ABCC2* (MRP2) to the

pharmacokinetics of MPA (and its glucuronidated metabolites) in thoracic transplant recipients. The thoracic transplant group was of special interest because neither the lung nor heart is involved in MPA disposition, whereas UGTs and ABCC2 are found in the kidney, liver and GI tract; interpretation of pharmacogenetic studies in kidney and liver transplant recipients can be challenging with the introduction of donor genes.

It was noted initially that the lung and heart transplant recipients in this study had different MPA pharmacokinetics; lung transplant recipients had significantly lower dose-normalized MPA PK parameters, including MPA AUC, Cmax and Cmin, MPAG AUC, and higher MPA apparent clearance and MPAG/MPA ratio than did the heart transplant group (Table 4.3). In addition, use of concomitant immunosuppressive agents (cyclosproine, tacrolimus, and sirolimus) was different in the two groups, and prednisone use was much more common in the lung transplant subjects. Dosages of MMF were also significantly lower in the heart transplant group. Lung transplant patients also taking cyclosporine had significantly lower MPA exposure than those taking TAC. While the results were less prominent in heart transplant recipients, similar trends were observed. In light of this observation, separate analyses were performed for the two transplant groups. Indeed, impact of most of the *UGT* and *ABCC2* genetic variants investigated did differ in the two groups (Table 4.4, Appendix C).

With our patient population, we were able to detect the common alleles, as well as less common alleles such as *UGT1A8*3*, *UGT1A9*3*, and *UGT2B7* G-138A. The allelic frequencies of the polymorphisms observed were comparable to other studies.[20, 24, 29-31, 38, 39, 44, 52-54]

In the multivariate analysis considering both genetic and demographic factors, the most significant genetic factors observed in both transplant groups were the *UGT2B7* G-138A and *UGT2B7*2* (codon 268) variants, which were associated with higher AcMPAG/MPA metabolite ratio and AcMPAG AUC, respectively. In addition, cyclosporine use was associated with lower MPA Cmin (Table 4.5). The *UGT2B7*2* (His[268]Tyr), presents at an allelic frequency of 64% in our population, was associated with a 2.4 and 3.6-fold increase in AcMPAG exposure in the lung and heart transplant population, respectively (Table 4.5). This observation may be of clinical importance as *UGT2B7* is the major isoenzyme in the production of the active metabolite AcMPAG,[20-22] and its impact on AcMPAG exposure was observed in both transplant groups. A previous clinical study by Levesque et al. also observed significantly higher total and free MPA AUC, MPAG AUC, and AcMPAG urinary excretion in healthy volunteers with the *UGT2B7*2* allele.[39] Djebli et al. observed an effect of *UGT2B7* genotypes (*G-900A* and **2*, reversely linked) on AcMPAG exposure only in renal transplant patients taking sirolimus, but not in those on cyclosporine or tacrolimus; this suggests that the influence of genetics could be masked by drug-drug interactions.[41] Conversely, Kagaya et al. and Zhang et al. did not observe a contribution of *UGT2B7* genotypes to MPA exposure in Japanese and Chinese renal transplant subjects, respectively.[40, 43] Although *in vitro* and other clinical studies reported limited functional impact of the coding region variant (*UGT2B7*2*) allele,[20, 31, 40, 41] its potential impact on UGT2B7 modulation and AcMPAG formation *in vivo* merits further investigation in a larger population. Involvement of other *UGT2B7* variants are also likely, as *UGT2B7*2* (His[268]Tyr) is in tight reverse linkage with several promoter variations (-1306G, -1299C, -1112C, -900A, -327G, and -161C)

that influence transcriptional activity of the *UGT2B7* promoter in liver and colon cells.[39, 41]

The uncommon *UGT2B7* G-138A variant, with an allelic frequency of ~4% in Caucasians, was reported to have reduced transcription of the enzyme *in vitro*,[30, 52] but limited impact *in vivo*.[30] On the contrary, we observed a significantly higher AcMPAG/MPA AUC ratio (~10-fold increase) associated with the *UGT2B7* G-138A variant in both transplant groups (n=4, three lung and one heart transplant recipients), suggesting an increased AcMPAG production. Of the four patients bearing this variant, three patients also had the *UGT2B7*2/*2* and one *UGT2B7*1/*2* genotypes. Interestingly, *UGT2B7* G-138A was also associated with occurrences of rejection, anemia and leucopenia in univariate analysis (data not shown), and remained a significant factor for occurrences of rejection in multivariate analysis (Table 4.6). The mechanism of G-138A in *UGT2B7* modulation is unclear; although this variant was observed in only a few patients and clinical data are limited, the magnitude of its impact on AcMPAG PKs warrants attention and corroboration in future studies.

The functional impact of other *UGT* variants was less pronounced. The *UGT1A8*2* allele, with an allelic frequency of 15 – 25% in Caucasians, was reported to have little or no functional impact on MPA metabolism.[20, 29, 39, 40, 44, 45] In agreement with these reports, direct influence on MPAG formation was not observed in our study subjects. The rare *UGT1A8*3* allele produces a low activity enzyme;[20, 21, 29] likely due to its low allelic frequency (2.9% in our population), studies in healthy volunteers,[39] kidney/pancreas/kidney-pancreas transplant recipients[44] and the present study in thoracic

transplant patients did not show a significant impact of this polymorphism on MPA metabolism.

Interestingly, none of the candidate *UGT1A9* variants (-275/-2152 and **3* allele) contribute significantly to MPA's pharmacokinetic variability in either transplant group. However, it is worth noting that the *UGT1A9*3* allele was found in only three heart and one lung transplant subjects. Therefore, the impairing effect of this allele may not be detectable due to the small sample size. Although *in vitro* studies showed a higher glucuronidation activity associated with the promoter variant at -275/-2152,[24, 53] the clinical impact still warrants further investigation. While the current study observed no significant influence of this variant on MPA pharmacokinetics in thoracic transplant recipients, the study in healthy volunteers by Levesque et al.[39] showed a significant decrease in MPA AUC(6-12) in carriers of this variant, and enterohepatic recirculation of MPA was decreased. Kuypers et al.[38] also observed a lower MPA AUC and estimated enterohepatic recirculation, and higher MPA clearance in renal transplant recipients with the *UGT1A9* -275/-2152 variant; however, this effect was dose-dependent, and was significant only in subjects taking 2 g MMF but not in those taking 1 g MMF daily. Johnson et al.[44] reported a decreased MPA trough concentration in kidney, pancreas and kidney-pancreas transplant recipients with the *UGT1A9* -275/-2152 variant who were taking cyclosporine as co-medication, but observed no impact overall when all patients were included. The impact of the *UGT1A9* -275/-2152 variant on MPA metabolism appears to be equivocal, and likely depends on other factors such as MMF dose and concomitant immunosuppressants.

Genetic polymorphisms in *ABCC2* and their role in drug disposition have been reviewed recently.[35, 37, 55, 56] Of >50 polymorphisms reported for *ABCC2*, the most commonly studied include the variants C-24T (promoter region), G1249A (Val[417]Ile), and the silent mutation C3972T (Ile[1324]Ile), with allelic frequencies ranging from 15 – 30%.[28, 32-37] To date, we are aware of a few clinical studies in renal transplant subjects[28, 43, 45, 57] and a study in healthy volunteers[49] that investigated the potential role of *ABCC2* polymorphisms on MPA disposition. In the current study, the candidate *ABCC2* variants [*ABCC2 -24T, G1249A* (Val[417]Ile), and *C3972T* (Ile[1324]Ile)] did not influence MPA pharmacokinetics in this study population, and their reported impact varies in other clinical studies in renal transplant subjects[28, 43, 45, 57] and healthy volunteers.[49] Generally, the *ABCC2 -24* variant was not found to be a significant factor in MPA pharmacokinetics;[43, 45, 57] however, Levesque et al.[49] reported elevated AcMPAG exposure (by 25%) in healthy volunteers, and Nasesen et al.[28] observed higher MPA exposure and MPA trough levels associated with this variant (in linkage with *ABCC2* C-3972T), but only in renal transplant patients with liver dysfunction. The authors speculated that the -24 variant up-regulates the expression or activity of ABCC2. Zhang et al.[43] noted increased MPA AUC(6-12) in Chinese renal transplant subjects with the *ABCC2 G1249A* (Val[417]Ile) variant, while Miura et al.[45, 57] observed no impact of *ABCC2* or *UGT* variants in Japanese renal transplant recipients; however, the uptake transporters genetic variants in *OATP/SLCO1B1* and *OATP/SLCO1B3* appeared to play a significant role in MPA and MPAG AUCs, revealing another layer of complexity of MPA pharmacogenetics.

The disparity of results is likely complicated by the fact that ABCC2 is also a site of drug-drug interaction between cyclosporine and MPA.[25, 26, 58, 59] Indeed, when co-medication was considered in the multivariate analysis, cyclosporine use was a significant covariate of MPA Cmin in both heart and lung transplant population (Table 4.5).

Additional variants in the *UGT1A 3'UTR*, *UGT1A1*, and *UGT1A7* were also investigated in this study to provide a complete view of UGT genetics and MPA pharmacokinetics. However, results differ between the lung and heart transplant groups, and their contribution to MPA pharmacokinetics was unclear.[42, 45, 49] While *UGT1A1* and *UGT1A7* showed limited conjugation activity for MPA *in vitro*,[21, 22] and have no effect on MPA pharmacokinetics in Japanese renal transplant recipients,[42, 45] in our study *UGT1A1* -3279 and *UGT1A1*28* contribute to the variability of MPAG AUC in lung and heart transplant recipients, respectively (Table 4.4). In a recent study of healthy volunteers, *UGT1A7* Asn^{129}Lys/Arg^{131}Lys was shown to have a modest effect on MPA exposure, and in our study *UGT1A7* Asn^{129}Lys/Arg^{131}Lys and Trp^{208}Arg also had an impact on MPA pharmacokinetic parameters in the two transplant groups (Table 4.4). The inconsistent results from different study populations emphasize the need to genotype several *UGT* genes that may contribute *in vivo* to MPA disposition in order to account for the vast inter-patient variability observed clinically.

Since the ultimate goal of understanding MPA variability is to improve clinical outcomes of transplant recipients, the role of *UGT* and *ABCC2* genetics and demographic factors in MMF efficacy and safety outcomes was explored with a retrospective chart review. To date, only one study has linked *UGT* genetics to clinical outcomes (adverse

effects) of MPA: Betonico et al.[50] observed greater occurrence of infections in renal transplant recipients with UGT1A8*3 and the UGT1A8 haplotype (–999C/codon 55A/codon 277A), but not other polymorphisms.

In the current study, the lack of association of specific genetic or demographic factors with GI toxicities and infection is likely due to numerous aspects that were not accounted for, such as diet, other medications and supplements, and environmental exposures to pathogens. Interestingly, *UGT 3'UTR* and *ABCC2* variants that had no impact on MPA or metabolite pharmacokinetics were associated with occurrence of leucopenia, and their roles remain to be elucidated. On the contrary, occurrences of anemia, the most commonly observed adverse effect in our population, was associated with lack of prednisone use, *ABCC2* (Ile^{1324}Ile) variant, and *UGT1A7* Trp^{208}Arg. However, the impact of these genetic variants is currently unclear; although occurrences of anemia appeared to be lower in homozygous carriers, the effect in heterozygous carriers is uncertain. Corticosteroids have been used to treat some forms of anemia;[60, 61] since the exact cause and type of anemia in these patients were unknown, the mechanism of prednisone's protective effect cannot be confirmed. Occurrence of rejection was associated with cyclosporine use and *UGT2B7* T-125C and G-138A variants. Indeed, incidence of acute rejection was more common in cyclosporine-based regimens in both lung and heart transplant populations, and tacrolimus has surpassed cyclosporine as the calcineurin inhibitor of choice.[62-66] The role of *UGT2B7* variants in increased rejection may be due to their impact on AcMPAG exposure (Table 4.5), which has pro-inflammatory properties. Despite the pronounced impact of these variants on clinical

outcomes, as indicated by the significantly elevated odds ratios (Table 4.6), their low frequencies warrant larger studies to corroborate these results.

4.5. Conclusions

The divergence in effects of *UGT* and *ABCC2* genes on MPA pharmacokinetics and clinical outcomes in patient subpopulations is not unexpected, since demographic, physiological and pharmacotherapeutic factors also contribute to drug disposition and clinical outcomes. Currently, clinical pharmacogenetic studies of MPA are particularly scarce in thoracic transplant recipients. It is apparent that multiple polymorphisms in *UGT* and *ABCC2,* and possibly interactions of polymorphisms, are involved in determining MPA disposition. Management of MMF therapy is a complex issue; in addition to the pharmacogenetic information acquired, non-genetic components such as demographic, physiological and pharmacotherapeutic factors should be considered collectively to help explain and predict the wide pharmacokinetic variability of MPA. Studies investigating multiple aspects, including pharmacogenetics, pharmacokinetics and clinical outcomes of MPA, are much needed for a more complete understanding of MMF therapy.

4.6. Tables

Table 4.1. Primers used for polymerase chain reaction and sequencing[39, 49]

Location	Sense	Sequence (5' to 3')	Position in reference sequence[a]	Annealing temperature
UGT1A 3'UTR	F[b]	GTTCATACCACAGGTGTTCCAG	186967 to 187853	66 °C
	R[b]	GCTGTTCTCAGTGCACTCCAAG		
UGT1A1 promoter	F	CTGGGGATAAACATGGGATG	171386 to 171991	59 °C
	R[b]	CACCACCACTTCTGGAACCT		
UGT1A1 TATA box	F[b]	GAGGTTCTGGAAGTACTTTGC	174774 to 175128	53 °C
	R[b]	TCCACTGGGATCAACAGTATCT		
UGT1A7 exon 1	F	CGCTGGACGGCACCATTG	98840 to 99351	59 °C
	R[b]	GGCATCACGGGTTTGGGATAC		
UGT1A8 exon 1	F	GTCAGGTTTTGTGCCTGTAG	34179 to 34615	54°C
	R	TAAAAAAACCATTGGATGAACTCAG		
UGT1A8 exon 1	F	CTGGACCGGGAATTCATGGA	34519 to 35004	54°C
	R[b]	GTGGCTGTAGAGATCATATGCT		

Location	Sense	Sequence (5' to 3')	Position in reference sequence[a]	Annealing temperature
UGT1A8 exon 1	F[b] R	TTCGCCAGGGGAATAG ATTTGCTCTAGGGGGTC	34765 to 35446	54°C
UGT1A9 -2152	F R	GTAGGTCTTTTACATTTCCC CCTGAAACAGCAAAACCAA	85902 to 86892	53°C
UGT1A9 -275	F[b] R	GAGCCCCAATTTAGGAGGTTA CAGTAGGTGGGAGAAATACCA	88043 to 88491	58°C
UGT1A9 exon 1	F R[b]	GTGCTGGTATTTCTCCC GTCAAAAATGTCATTGTATGAACC	88467 to 88896	54°C
UGT2B7 promoter and exon 1	F R	CAAAAATATGTGGACCATGTTTAGTC CAATCAGTAACATGAAGCTCTAACTTATTT	48270 to 49427	56°C
UGT2B7 promoter and exon 1	F[c] R[c]	GACTGTACTGGCATCTTC CATCATGCAACAGATTAA	48821 and 48946	-
UGT2B7 codon 268	F R	GTAATTATCTTGTGTCATC GACTATAGAATCATTTCTACTG	50636 to 50896	52°C

Location	Sense	Sequence (5' to 3')	Position in reference sequence[a]	Annealing temperature
ABCC2 -24	F R[b]	TAATTGGTTGGGATGAAAGGTC AGCTTTAGACCAATTGCACATC	20290979 to 20291282	51°C
ABCC2 exon 10	F[b] R	GTCCATATGGAGCACATCCTTCC TGAGATGGTAGAAAGTCTTCCACC	20312191 to 20312683	55°C
ABCC2 exon 25	F[b] R	GGAGCCTCTCATCATTCTGC TTTCACACCACTAGCCATGC	20344205 to 20344670	55°C
ABCC2 exon 28	F[b] R	TGGGACACTGCTACCCTTCTC GGCTGCTATCCTTCCCTCTGA	20352499 to 20352924	55°C
ABCC2 exon 32	F[b] R	CTGCTTTGTAGCCTTGTCTGA TGAAATTCAGGACAGTGGTTG	20359672 to 20360171	55°C

F = forward; R= reverse
[a] Reference sequence for *UGT1A*: AF297093; reference sequence for *UGT2B7*: AC111000; reference sequence for *ABCC2*: NT030059; position relative to the first nucleotide of each primer.
[b] Primers used for both PCR and sequencing
[c] Primers used for sequencing only

Table 4.2. Demographic data and diagnoses of pharmacokinetic study subjects

	All (n=68)	**Lung (n=36)**	**Heart (n=32)**
Number of subjects			
Gender (M/F)	47/21	21/15[a]	26/6[a]
CSA/TAC/SRL	28/38/2	11/25/0[a]	17/13/2[a]
Prednisone	38	36[a]	2[a]
Median (range)			
Age (y)	56.5 (20.7 - 77.6)	51.7 (20.7 – 70.5)[b]	61.3 (23.2 - 77.6)[b]
Year since transplant (y)	1.8 (0.2 - 19.7)	1.4 (0.2 - 14.0)[b]	2.7 (0.3 - 19.7)[b]
Weight (Kg)	73.6 (46.0 - 109.2)	71.0 (46.0 - 109.2)	78.6 (50.0 - 109.1)
Height (m)	1.73 (1.49 - 1.85)	1.70 (1.51 - 1.85)	1.76 (1.49 - 1.85)
BMI (Kg/m^2)	25.15 (17.97 - 34.00)	24.93 (17.97 - 34.00)	25.22 (19.8 - 33.56)
Twice-daily MMF dose (mg)	1.00 (0.25 - 1.50)	1.50 (0.50 – 1.50)[b]	0.75 (0.25 - 1.5)[b]
MMF dosage (mg/Kg/day)	27.75 (5.50 - 53.96)	34.79 (12.50 - 53.96)[b]	20.44 (5.50 - 37.82)[b]
Serum creatinine (mg/dL)	112 (67 - 240)	100 (70 - 218)[b]	130 (67 - 240)[b]
Albumin (g/dL)	41.0 (20.6 - 78.0)	38.0 (25.0 - 47.0)[b]	43.5 (20.6 - 78.0)[b]
Common diagnoses for lung or heart transplantation			
Lung transplant subjects (number of subjects)			
	Cystic fibrosis	10	N/A
	Pulmonary fibrosis	10	N/A

	Lung (n=36)	Heart (n=32)
COPD/emphysema	6	N/A
Alpha 1-antitrypsin deficiency	5	N/A
Others[c]	5	N/A
Heart transplant subjects (number of subjects)		
Ischemic cardiomyopathy	N/A	12
Idiopathic dilated cardiomyopathy	N/A	11
Unspecified cardiomyopathy	N/A	2
Myocardial infarction	N/A	2
Others[d]	N/A	5

[a] $p<0.05$, lung vs. heart, Chi Square test

[b] $p<0.05$, lung vs. heart, Wilcoxon rank sum test

[c] Other diagnoses for lung transplantation included: bronchiolitis obliterans (n=1), fibrosing alveolitis (n=1), lymphangioleiomyomatosis (n=1), pulmonary hypertension (n=1), and sarcoidosis (n=1).

[d] Other diagnoses for heart transplantation included: congenital heart disease (n=2), hypertropic cardiomyopathy (n=1), ischemic coronary artery disease (n=1), and valvular heart disease (n=1).

BMI = body mass index; COPD = Chronic obstructive pulmonary disease; CSA = cyclosporine; F = female; M = male; MMF = mycophenolate mofetil; N/A = not applicable; SRL = sirolimus; TAC = tacrolimus

Table 4.3. Dose-normalized pharmacokinetic parameters of 68 thoracic transplant recipients taking twice-daily MMF

Median (range)	**All (n=68)**	**Lung (n=36)[a]**	**Heart (n=32)[a]**	**p-value[b]**
Total MPA AUC(0-12)	42.12 (3.39 – 218.73)	27.77 (3.39 - 212.14)	71.07 (16.89 - 218.73)	**<0.001**
fMPA AUC(0-12)[c]	1.51 (0.05 – 18.89)	1.26 (0.24 - 2.87)	1.52 (0.05 - 18.89)	0.141
MPAG AUC(0-12)	523.80 (49.90 – 1868.80)	438.93 (71.94 - 928.11)	733.80 (49.90 - 1868.80)	**0.014**
AcMPAG AUC(0-12)	11.47 (UD – 333.22)	8.26 (UD - 159.93)	16.14 (UD - 333.22)	0.068
MPA Cmax (µg/mL)	11.83 (0.64 – 47.28)	7.62 (0.64 - 37.11)	18.01 (3.62 - 47.28)	**<0.001**
MPA Cmin (µg/mL)	1.06 (UD – 8.95)	0.73 (UD - 8.95)	1.52 (0.34 - 8.40)	**0.001**
MPA EHC [AUC(6-12)/AUC(0-12)]	0.32 (0.10 – 0.61)	0.31 (0.11 - 0.56)	0.37 (0.10 - 0.61)	0.266
MPA apparent clearance (L/h)	24.10 (4.57 – 294.68)	36.12 (4.71 - 294.68)	14.26 (4.57 - 59.19)	**<0.001**
MPAG/MPA metabolic ratio	12.87 (0.92 – 55.19)	13.79 (2.41 - 55.19)	10.29 (0.92 - 26.64)	**0.024**
AcMPAG/MPA metabolic ratio	0.21 (UD – 12.33)	0.21 (UD - 12.33)	0.22 (UD - 3.73)	0.966

[a] Results from 27 lung and 23 heart transplant recipients have been reported elsewhere previously.[46]

[b] Comparison between lung and heart groups, Wilcoxon rank sum test
[c] n=47; free MPA concentration measurements available for 15 lung and 32 heart transplant recipients

AcMPAG = acyl glucuronide of MPA; AUC = area under the concentration-time curve; Cmax = maximum concentration; Cmin = minimum concentration; EHC = enterohepatic recirculation; fMPA = free MPA; MMF = mycophenolate mofetil; MPA = mycophenolic acid; MPAG = mycophenolic acid glucuronide; N/A = results not applicable; SD = standard deviation; UD = undetectable

Table 4.4. Pharmacokinetic impact and allelic frequencies of *UGT* and *ABCC2* genes in 68 thoracic transplant recipients

Gene	Region	Nucleotide change	Amino acid change	Number of variant carriers (n=68)[a]	Allelic frequency (n=68)	Impact on MPA PKs in lung transplant recipients[b] (n=36)	Impact on MPA PKs in heart transplant recipients[b] (n=32)
UGT1A	3'UTR	T1813C/ G1941C/ G2042C		28/27/29	0.235/ 0.228/ 0.243	NS	NS
UGT1A1	Promoter	C-3440A		12	0.088	NS	NS
	Promoter	G-3279T		35 heterozygous, 18 homozygous	0.522	↑ MPAG AUC 1.5x (reference vs. homozygous variant)	↑ free fraction 11.2% (reference vs. homozygous variant)
	Promoter	G-3156A		35	0.287	NS	NS
	Promoter (TATA box)	-53 6→7 (*28)		39	0.316	NS	↑ MPAG AUC 1.66x
UGT1A7	Exon 1	T387G/ CG391AA	Asn^{129}Lys/	31 heterozygous, 8 homozygous /	0.346	NS	↑ MPA AUC 1.8x (reference vs. homozygous variant) ↑ MPA Cmax 1.9x

Gene	Region	Nucleotide change	Amino acid change	Number of variant carriers (n=68)[a]	Allelic frequency (n=68)	Impact on MPA PKs in lung transplant recipients[b] (n=36)	Impact on MPA PKs in heart transplant recipients[b] (n=32)
			$Arg^{131}Lys$	31 heterozygous, 8 homozygous			(reference vs. homozygous variant) ↓ MPA clearance 22% (reference vs. homozygous variant)
	Exon 1	T622C	$Trp^{208}Arg$	33 heterozygous, 23 homozygous	0.581	↓ MPA AUC 15% (reference vs. homozygous variant) ↓ MPA clearance 17% (reference vs. homozygous variant)	NS
UGT1A8	Exon 1	C518G (*2)	$Ala^{173}Gly$	30	0.250	NS	NS
	Exon 1	A709C	$Thr^{237}Thr$	1	0.007	N/A	↓ MPA Cmax 77.7%, n=1 ↓ Free MPA AUC 98.9%, n=1
	Exon 1	G830A	$Cys^{277}Ile$	4	0.029	NS	↑ Cmin 1.5x, n=2

Gene	Region	Nucleotide change	Amino acid change	Number of variant carriers (n=68)[a]	Allelic frequency (n=68)	Impact on MPA PKs in lung transplant recipients[b] (n=36)	Impact on MPA PKs in heart transplant recipients[b] (n=32)
		(*3)					
UGT1A9	Promoter	T-2188C		3	0.022	NS	↑ MPAG AUC 2.6x, n=1
	Promoter	C-2152T/ T-275A		12/11	0.088/ 0.081	NS	NS
	Promoter	T-1887G		23	0.169	NS	NS
	Promoter	T-1818C		28	0.243	NS	NS
	Promoter	C-440T/ T-331C		34/35	0.299/ 0.301	NS	NS
	Promoter	-118 9→10 (T stretch)		31 heterozygous, 8 homozygous	0.346	NS	↓ AcMPAG/MPA 22.9%
	Exon 1	T98C (*3)	$Met^{33}Tyr$	4	0.029	↑ Vd/F 10.4x, n=1	NS
UGT2B7	Promoter	G-138A		4	0.029	↓ MPA AUC 76%, n=3	↓ MPA Cmin 84%,

Gene	Region	Nucleotide change	Amino acid change	Number of variant carriers (n=68)[a]	Allelic frequency (n=68)	Impact on MPA PKs in lung transplant recipients[b] (n=36)	Impact on MPA PKs in heart transplant recipients[b] (n=32)
		(*2g)				↓ MPA Cmax 73%, n=3 ↓ MPAG AUC 32%, n=3 ↑ MPA clearance 3.9x, n=3 ↑ AcMPAG/MPA 9.8x, n=3	n=1 ↓ MPA Vd/F 92%, n=1 ↑ AcMPAG/MPA 10.8x, n=1
	Promoter	T-125C		3	0.022	NS	NS
	Intron 1	IVS1+ T829C		37 heterozygous, 23 homozygous	0.610	NS	NS
	Intron 1	IVS1+ A985G		7	0.051	NS	NS
	Intron 1	IVS1+ C999A		14	0.125	↑ MPA AUC 2x ↑ MPA Cmin 2.4x	NS

Gene	Region	Nucleotide change	Amino acid change	Number of variant carriers (n=68)[a]	Allelic frequency (n=68)	Impact on MPA PKs in lung transplant recipients[b] (n=36)	Impact on MPA PKs in heart transplant recipients[b] (n=32)
						↓ MPA clearance 54% ↑ MPA EHC 1.3x	
	Exon 2	C802T[c] (*2a)	His^{268}Tyr	35 heterzygous, 26 homozygous	0.640	↑ AcMPAG AUC 2.4x	↑ AcMPAG AUC 3.6x ↑ AcMPAG/MPA 3.7x
ABCC2	Promoter	C-24T		21	0.169	NS	NS
	Exon 10	G1249A	Val^{417}Ile	25	0.213	↑ Vd/F 3.1x	↑ MPAG/MPA 1.6x
	Exons 25/32	T3563A/ G4544A	Val^{1188}Cys/ Glu^{1515}Tyr	6/6	0.044	NS	NS

[a] Unless specified otherwise, variant carriers consist of heterozygous and homozygour carriers.
[b] Multiple regression analysis, $p \leq 0.05$
[c] *UGT2B7* C802 is in complete linkage disequilibrium with variant promoter (-1306, -1299, -1112, -900, -327, -161) and in incomplete linkage disequilibrium with variant 372G (codon 124).

Variants that were genotyped but not observed included: *UGT1A8* Ala^{144}Val, Ala^{169}Thr and Thr^{240}Ala; *UGT1A9* -2208, Lys^{75}Lys; *UGT2B7* Lys^{25}Asn, Asp^{121}Asn, Thr^{123}Asn; *ABCC2* Leu^{407}Leu, Arg^{412}Gly and Lys^{430}Arg.

A = adenosine; AcMPAG = acyl glucuronide of MPA; ABCC2 = multiple resistance-associated protein 2; Ala = alanine; Arg = arginine; Asn = Asparagine; AUC = area under the concentration-time curve; Cmax = maximum concentration; EHC = enterohepatic

recirculation; G = guanosine; Glu = glutamic acid; Gly = glycine; His = histidine; Ile = isoleucine; Lys = lysine; Met = methionine; MPA = mycophenolic acid; MPAG = 7-O-mycophenolic acid glucuronide; N/A = not applicable (allele not observed or allelic frequency too low); NS = not significant; Ser = serine; T = thymine; Thr = threonine; Trp = tryptophan; Tyr = tyrosine; UGT = UDP-glucuronosyltransferase; UTR = un-translated region; Val = valine; Vd/F = apparent volume of distribution

Table 4.5. The most significant pharmacogenetic-pharmacokinetic results from multivariate analysis for both lung and heart transplant recipients

Multivariate analyses	**Lung (n=36)**			**Heart (n=32)**		
Covariates	*p*	r^2	**Impact**	*p*	r^2	**Impact**
MPA exposure						
Cmin						
Co-medication	0.007	0.2002	MPA Cmin ↓ 70% for CSA	0.002	0.2851	MPA Cmin ↓ 62% for CSA
MPA metabolites						
AcMPAG AUC_{0-12}						
UGT2B7 Codon 268	0.0073	0.1759	AcMPAG AUC ↑ 2.4x	0.0065	0.2358	AcMPAG AUC ↑ 3.6x
AcMPAG/MPA ratio						
UGT2B7 G-138A	0.001	0.3002	AcMPAG/MPA ↑ 9.8x, n=3	0.0221	0.1275	AcMPAG/MPA ↑ 10.8x, n=1

A = adenosine; AcMPAG = acyl glucuronide of MPA; AUC = area under the concentration-time curve; Cmin = minimum concentration; G = guanosine; MPA = mycophenolic acid; r^2 = coefficient of determination

Table 4.6. Significant pharmacogenetic and demographic factors that impact on clinical outcomes in 68 thoracic transplant recipients

	Covariates used in multivariate analysis	Variant carriers (68 subjects total)	Significant covariates in multiple regression analysis	p-value (Odds ratio)[a]	Effect of variant	Nominal logistic (multivariate) whole model test R^2 (p-value)[b]
GI	N/A	N/A	N/A	N/A	N/A	N/A
Infection	Gender	47 M	N/A	N/A	N/A	**0.1505 (0.0129)**
	UGT 2B7*2 ($His^{268}Tyr$)	26				
	UGT 2B7 IV1S+999	14				
Rejection	Cyclosporine use	28	Cyclosporine use	<0.0001 (5.6×10^4)	↑	**0.4381 (<0.0001)**
	UGT 2B7 T-125C	3	*UGT* 2B7 T-125C	0.0001 (1.4×10^5)	↑	
	UGT 2B7 G-138A	4	*UGT* 2B7 G-138A	0.020 (4.3×10^2)	↑	
Anemia	Cyclosporine use	28				**0.4388 (<0.0001)**
	Cr Clearance >60 mL/min[c]	36				
	Prednisone use	38	Prednisone use	0.0029 (0.26)	↓	

	Covariates used in multivariate analysis	Variant carriers (68 subjects total)	Significant covariates in multiple regression analysis	p-value (Odds ratio)[a]	Effect of variant	Nominal logistic (multivariate) whole model test R^2 (p-value)[b]
	UGT 1A8*2 (Ala^{173}Gly)	30				
	UGT 2B7 G-138A	4				
	UGT 2B7*2 (His^{268}Tyr)	26				
	ABCC2 Val^{417}Ile	25				
	ABCC2 Ile^{1324}Ile	33 heterozygous /7 homozygous	*ABCC2* Ile^{1324}Ile	0.0108 (?)	?	
	UGT 3' UTR T1813C	28				
	UGT 1A7 Trp^{208}Arg	23	*UGT* 1A7 Trp^{208}Arg	0.0256 (?)	?	
Leucopenia	*UGT* 2B7 G-138A	4				**0.3933 (0.0007)**
	UGT 2B7*2 (His^{268}Tyr)	26				
	ABCC2 Val^{1188}Glu	6	*ABCC2* Val^{1188}Glu	0.0135 (4.5)	↑	
	UGT 3' UTR T1813C	28	*UGT* 3' UTR T1813C	0.0237 (0.0036)	↓	

[a] Multivariate analysis

[b] In nominal logistic modeling, R^2 ranges from 0 to 1, and indicates the uncertainty that is attributed to the whole multivariate model. R^2 of 1 indicates that factors/covariates completely predict categorical response, and 0 indicates no prediction.

[c] Estimated by Cockcroft-Gault fomula

↑ = increased occurrence; ↓ = decreased occurrence; ? = impact unclear; ABCC2 = multiple resistance-associated protein 2; N/A = not available; Cr = creatinine; GI = gastrointestinal; UGT = UDP-glucuronosyltransferase

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5. CONCLUDING CHAPTER

5.1. General Discussion

Mycophenolic acid is now a mainstay anti-proliferative agent used in combination with calcineurin inhibitors and/or corticosteroids in maintenance immunosuppression.[1-3] Despite its superior efficacy and safety profile, the wide-inter-patient variability in MPA pharmacokinetics and pharmacokinetic interactions with concomitant medications (e.g. CSA, TAC) makes prediction of MPA exposure and drug response very challenging. As inadequate MPA exposure is associated with acute rejection, which results in greater risk of chronic rejection and ultimately leads to graft failure, the current practice of fixed mycophenolate mofetil dosing needs to be re-evaluated. Although still controversial, therapeutic drug monitoring via measurement of MPA levels is advocated by some groups[4-9] to help manage MMF therapy. The focus of considerable research is to explain such pharmacokinetic variability by determining and characterizing factors, including genetics, that influence pharmacokinetics and pharmacodynamics of MPA. Naturally, the goal is to personalize MMF therapy for transplant recipients. Pharmacogenetic testing is a promising tool as its advantages include potential prediction of drug response before treatment begins, convenient and non-invasive sample collection, and requirement of only one assessment that gives unvarying information for each patient.

Among all transplant populations, pharmacotherapeutic studies in the thoracic transplant population are wanting. Therefore, this open-label clinical study strived to characterize MPA pharmacokinetics (Chapter 2), develop and update MPA limited sampling strategies (Chapter 3), and assess contribution of *UGT* and *ABCC2* genetics to

MPA pharmacokinetics (Chapter 4) in 70 thoracic transplant recipients. In addition, associations between MPA pharmacokinetics and clinical outcomes were assessed. As expected, there was wide inter-patient variability of MPA, MPAG and AcMPAG pharmacokinetics, with coefficients of variation exceeding 50% for most PK parameters measured. Interestingly, we observed significantly lower MPA exposure in lung compared to heart transplant recipients, suggesting the two transplant groups exhibit different MPA pharmacokinetics. Pharmacokinetic data also agree with previous studies that co-medication with CSA (compared with TAC or SRL) decreases MPA exposure.

Since the large PK variability was confirmed at our centre, monitoring MPA exposure is a potential tool for MMF therapy management. However, trough levels are known to be a poor predictor of clinical outcomes, and a 12-hour AUC is impossible to obtain for routine MPA monitoring in the clinical setting; we therefore proceeded to develop limited sampling strategies for our patient population as an alternative. Given the significant pharmacokinetic differences between lung and heart transplant groups, separate limited sampling strategies were developed, with the criteria that only a maximum of three concentrations within the first two hours post-dose were used, and that bias and precision be within ±15%. While there were numerous candidate LSSs for the lung transplant population, none developed from the heart transplant group were acceptable. However, application of LSSs developed from the lung transplant group yielded excellent results when applied to the heart transplant group. Application of limited sampling strategies from other research groups yielded less optimal results, reinforcing the need to re-establish or re-validate limited sampling strategies for each

specific centre. The best limited strategy equations for lung and heart transplant groups were (Log C0, Log C1.5) and (Log C1.5, Log C2), respectively.

Although limited sampling strategies provide a convenient alternative for MPA drug monitoring, they are merely instruments used in managing MMF therapy after dose initiation. As such, the main focus of the current study was to identify factors that contribute to MPA's pharmacokinetic variability. Since MPA is metabolized and transported by the UGTs and ABCC2, respectively, the contribution of genetic polymorphisms in *UGT* and *ABCC2* to the pharmacokinetic variability and clinical outcomes was investigated in 68 thoracic transplant recipients. In both lung and heart transplant groups, *UGT2B7* variants 802T (*2a) and -138A (*2g) were associated with increased AcMPAG AUC and AcMPAG/MPA, respectively, in multivariate analyses. In turn, high AcMPAG exposure and metabolic ratio were associated with clinical outcomes, namely occurrences of rejection, infection, anemia and leucopenia. Various other *UGT* and *ABCC2* genetic variants also influenced MPA and metabolite pharmacokinetics; however, their contribution ranged from 7 – 32% in lung and heart transplant groups, and their functional impact was unclear. Cyclosporine use was also found to be a significant factor in determining MPA pharmacokinetics. Due to the relatively small sample size and low prevalence of some polymorphisms, larger clinical pharmacogenetic studies in thoracic transplant subpopulations are warranted to corroborate the role of AcMPAG and *UGT2B7* variants in optimizing MMF therapy.

Aside from a few exceptional examples of pharmacogenetic predictors, such as *UGT1A1**28 and irinotecan toxicities,[10, 11] it is apparent that pharmacogenetic markers play only a modest part in clinical pharmacokinetics. This is one of the reasons why

pharmacogenetics-driven dosing has experienced low clinical utility thus far. Even in the case of *cytochrome P450* (*CYP) 2C9* polymorphisms and warfarin dosing, which is relatively well-established,[12, 13] *CYP2C9* genetics alone may explain as little as 10% of warfarin dose variability.[14] In fact, given the many levels of genetic control and complex interactions between genes and environment in a biological system, it is unrealistic to expect genetics of *UGT* and *ABCC2* alone to predict clinical outcomes of mycophenolate treatment. Genetic effects may also be drug-specific. For example, *CYP3A5* is a promising pharmacogenetic predictor in individualizing TAC therapy; *CYP3A5* functional non-expressers (*CYP3A5**3) have significantly higher TAC exposure compared to functional expressers (*CYP3A5**1).[15, 16] The impact of *CYP3A5* variants was subsequently confirmed in various clinical studies, including a population pharmacokinetic study.[15] However, even though CSA and SRL share similar metabolic pathways as TAC, studies have failed to observe an association between *CYP3A5* variants and CSA or SRL exposures.[15, 16]

In this study, inter-patient variability in MPA pharmacokinetics could be partially explained (~30%) by genetic variability of the *UGTs* and *ABCC2* responsible for MPA's disposition. Thus, the pharmacokinetic and pharmacogenetic data from our study supplement each other in our understanding of mycophenolate therapy. The next logical step is to build on the pharmacogenetic information acquired, and incorporate non-genetic components.

Non-genetic factors such as disease state, protein-binding, concomitant medication (e.g. CSA or TAC), renal and hepatic function, are highly influential in MPA disposition and should be considered when determining an optimal dose.[17] Indeed, in an

exploratory multiple regression analysis of demographic factors, prednisone use, concomitant immunosuppressive agent (CSA or TAC), and age were significant parameters that influence MPA exposure in our thoracic transplant population. Population pharmacokinetics, which characterizes pharmacokinetic response and its variability in the whole population, offers a more comprehensive approach to characterize the host of factors contributing to MPA pharmacokinetic variability. These factors may include disease states, renal and liver function, demographics, and lifestyle information.[18] Population pharmacokinetics not only identify specific factors (or covariates) and the extent of their contribution to the pharmacokinetic variability, but also allow use of sparse, complete, combination, or difference sources of pharmacokinetic data. Results from population pharmacokinetic modeling complement the pharmacogenetic component and provide measurable parameters that can be used to predict pharmacokinetic and treatment response when applied to individual patients.[19] To date, population pharmacokinetic modeling of MPA has focused on the kidney transplant group, and no studies have integrated both genetic and non-genetic data. The most common covariates significantly affecting MPA parameters were serum albumin, bodyweight, and CSA co-medication.[20-28]

Clearly, there exists a data gap regarding pharmacogenetics of mycophenolate in transplantation, and pharmacogenetics-guided dosing for MPA is immature and controversial; conversely, MPA exposure monitoring via limited sampling strategies is immediately applicable to thoracic transplant patient care. While MPA AUC is considered the best indicator of mycophenolate efficacy (association with rejection), the current study found an association of the minor metabolite AcMPAG with efficacy and

safety outcomes. Since the clinical data were retrospectively collected, the role of AcMPAG and other pharmacokinetic parameters, such as metabolic ratios and free concentration of MPA, in predicting safety outcomes is still unclear and warrants corroboration.

5.2. Strengths and Weaknesses of Thesis Research

To our knowledge, the current study was the first prospective clinical research study to investigate the role of both *UGT* and *ABCC2* genetics in the disposition of MPA in thoracic transplant recipients. In addition to the high priority genes *UGT1A8*, *1A9*, *2B7* and *ABCC2*, other *UGT*s reported to have modest or little contribution to MPA metabolism (including *UGT1A1*, *1A7* and *3'UTR*) were also tested for a comprehensive investigation. To date, studies in this specific patient group are scarce, despite the fact that the lung and the heart are not involved in MPA disposition, and patient outcomes are inferior to kidney transplant recipients.[2, 3] Since transplant populations differ in pathology, treatment responses, and possibly pharmacokinetics, studying this neglected transplant population sheds new light on management of mycophenolate therapy. This study also measured total MPA, free MPA and the two major metabolites, MPAG and AcMPAG, in a full 12-hour pharmacokinetic study in order to fully characterize the drug's fate during an entire steady-state dosing period. In addition, limited sampling strategies were developed from the valuable 12-hour pharmacokinetic data; since full AUC is useful in predicting acute rejection episodes yet unfeasible for routine measurement, the limited sampling equations provide a practical solution that can be directly implemented into routine patient care to improve patient management.

This study was limited by the relatively small sample size, which is one based on convenience. The number of lung and heart transplant recipients on mycophenolate therapy followed in British Columbia was about 70 and 150, respectively. While a ~30% (n=70) recruitment rate was considered quite successful, the two groups were studied separately since they exhibited different MPA pharmacokinetics, thereby further reducing the sample size. As a result, only common genetic variants such as *UGT2B7**2 carried sufficient power to be conclusive, while pharmacogenetic-pharmacokinetic results from uncommon variants remained exploratory. In addition, study patients were on steady-state mycophenolate therapy and relatively healthy; patients intolerant to mycophenolate mofetil, who may carry genetic variants of interest and exhibit special pharmacokinetics, may have been overlooked. Since this was a one-time study only, and patients were on various other medications and different immunosuppressive regimens that were subject to adjustments, the retrospective clinical outcomes data collection was limited to the time period in which no dosage changes were made. Hence, the data collection periods differed for each patient, and clinical events were not quantified. Although the clinical outcomes aspect was retrospective and exploratory in nature, this study encompassed pharmacokinetics, pharmacogenetics, and clinical outcomes of MPA, and established important associations between these areas, thereby bridging multiple aspects involved and improving understanding of MPA therapy.

5.3. Status of Working Hypotheses

The current understanding of MPA pharmacogenetics is still in its infancy. Genotype status of metabolizing enzymes and transporters may be useful in

characterizing drug disposition in an individual, thereby reflecting drug efficacy and/or risks of adverse effects. Based on available data, there are several promising gene candidates that may influence MPA pharmacokinetics clinically; however, whether their impact is sufficient to merit development and implementation of pharmacogenetic testing is uncertain.[14, 15, 29-33] While we reported association of *UGT2B7* variants (*2a and -138A) with increased AcMPAG exposure in both lung and heart transplant recipients, and association of AcMPAG exposure with clinical outcomes, these results need to be confirmed in larger clinical studies. While pharmacogenetics has potential to predict drug response in patients with functional variants, the predictive values of *UGT* and *ABCC2* genetics specifically in MPA pharmacokinetics need to be clearly established in order to support application of genetic testing.

There are still controversies and challenges to overcome before genetic information can be translated into routine clinical practice. In general, utilization of pharmacogenetics in clinical medicine is limited, due to various factors.[14] Firstly, the contribution of genetic variations to heterogeneity in drug response may be modest or insignificant, or the frequency of impairing genetic variants is too low to justify routine screening. Secondly, genetic results are rarely straightforward; association of genotypes with pharmacokinetics or clinical outcomes (efficacy or toxicity) is often ambiguous due to various clinical factors that may mask the effect of genotypes, limiting the observation of direct association between genotype and phenotype. Currently, a lack of guidelines for clinicians in the interpretation of genetic results poses another challenge in implementing pharmacogenetics into clinical practice. Involvement of multiple genes (e.g. metabolizing enzymes and transporters) and/or gene-gene interactions further complicates issues; in

transplantation, donor genotypes may also play a significant role in calcineurin inhibitor toxicities, as in the case of kidney and liver transplant recipients.[16] Thirdly, while genotyping techniques have advanced rapidly over the years, they are still too costly and laborious for clinic use. The US Food and Drug Administration-approved Amplichip® (Roche Molecular Systems, Pleasanton CA), a diagnostic microarray chip for detection of *CYP2D6* and *CYP2C19* genotypes, is estimated to cost US$500 per test, with instrumentation setup cost of ~US$219000.[34] The Invader® *UGT1A1* Molecular Assay (Third Wave Technologies Inc., Madison, WI), also approved by the US Food and Drug Administration to test for the *UGT1A1**28 variant, costs ~US$255 per test.[35] Other diagnostic tests including CodeLink™ Human P450 SNP Bioarray (GE Healthcare, US), DrugMet™ Genotyping Test (Jurilab Ltd., Finland), Tag-It™ Mutation Detection Kits (Tm Bioscience, Canada), and the Invader® CYP450 2D6 Analysis Panel (Third Wave Technologies Inc., US) are in development.[34] While these pharmacogenetic assays will become more convenient and economical in the future, their implementation for clinical use and cost-effectiveness remains to be validated. Although it is currently premature to implement routine clinical pharmacogenetic testing, contribution of pharmacogenetics to drug response should not be undermined. Pharmacogenetics can provide crucial and valuable information in completing our understanding of complex pharmacotherapy, as in the case of mycophenolic acid in transplantation.

5.4. Potential Applications of Research Findings

Since transplant patients are taking immunosuppressive agents for life, and suboptimal dosing may have dire results such as graft loss and death, it is highly desirable

to achieve the most suitable dosage regimen in a timely fashion. The ultimate goal of this study was to contribute to the individualization of mycophenolate dosage regimens for thoracic transplant recipients to achieve optimal treatment response and minimal toxicities. It has been established that inadequate MPA total exposure (estimated by AUC) is predictive of acute rejection;[4, 36-38] however, the inconvenience and cost of obtaining AUC preclude its use in routine care. The limited sampling strategies developed in this study utilized only two concentrations at convenient times, (Log C0, Log C1.5) and (Log C1.5, Log C2) for lung and heart transplant population, respectively, thus providing convenient alternatives for estimation of MPA AUC. These equations can be applied directly in the clinic for monitoring of MPA to prevent occurrences of acute rejection, thereby improving immunosuppressive therapy and improving patient care. Monitoring of MPA is probably most advantageous during initial dosing, when dosages are altered, or when interacting medications are changed.

The current research also added valuable information regarding the roles of *UGT* and *ABCC2* genetics in determining MPA pharmacokinetics and clinical outcomes. Although associations of genetic constitution and MPA treatment outcomes are still in the research and exploratory phase, the combination of MPA pharmacokinetics, pharmacogenetics and clinical outcomes data obtained from this study will contribute to the development of an MMF dosing algorithm that integrates multiple aspects of MMF therapy. For example, an algorithm developed by Barnes-Jewish Hospital at Washington University Medical Center (http://www.warfarindosing.org/Source/Home.aspx) provides estimation of optimal warfarin dosages by incorporating various clinical factors (e.g. presence of liver disease) and genotype status of the *CYP2C9* and *vitamin K epoxide*

reductase genes.[39] The estimation could be further refined by entering subsequent measurements of blood coagulation tendency (international normalized ratio). In the future, a similar algorithm for MMF may be attainable by incorporating important clinical factors such as transplant type, co-medication, liver and renal function biomarkers, pharmacodynamic biomarkers (see Section 5.6), selected *UGT2B7* and *ABCC2* genotypes, other genetic targets (see Section 5.6), and subsequent MPA concentrations (obtained via therapeutic drug monitoring) to estimate the optimal starting dosage for *de novo* transplant recipients. In addition, development of a diagnostic chip to detect genotypes of candidate *UGT* and/or *ABCC2* genes based on our findings is an exciting future possibility.

5.5. Overall Significance of Thesis Research

Although success rates of solid organ transplantation have improved over the years, graft rejection remains the foremost challenge in transplant patient management. Rejection (acute and chronic) and infection are the two main causes of post-transplant morbidity and mortality; the delicate balance of modulating the immune system to minimize rejection and infection is the axis of clinical transplantation and research. The use of immunosuppressive agents such as MPA is crucial, yet the unpredictable pharmacokinetics and drug response present great challenges in maximizing efficacy and minimizing toxicities. Although MMF has been used for 14 years and wide inter-patient variability in its pharmacokinetics is well-recognized, clinicians still rely on empirical dosing with minimum guidance in tailoring MMF therapy. Our knowledge of genetic and non-genetic factors and their relative contribution to the variable MPA drug response

remains poor. There exists an apparent knowledge gap in the pharmacogenetics of MPA in all transplant groups, and pharmacokinetic data are particularly limited in the thoracic transplant population compared with the renal transplant population. In this study, we found that genetics in *UGT* and *ABCC2* contribute to ~30% of the observed MPA PK variability. Hence, the current study added valuable knowledge to MMF management, and laid the groundwork for developing future studies to further characterize MPA pharmacokinetics, pharmacogenetics, and their contribution to clinical outcomes, bringing us one step closer to the ultimate goal of individualizing immunosuppressive regimens.

5.6. Future Directions

There is still much to learn about MPA pharmacotherapy before personalized therapy is realized. While the major pharmacogenetic research focuses on the metabolizing enzymes (UGTs) and transporters (ABCC2), other potentially significant areas that warrant research attention include: genetics and/or activities of hepatic uptake transporters (e.g. organic anion transporting polypeptides, OATP/SLCO); other efflux transporters; and drug target enzyme (inosine monophosphate dehydrogenase, IMPDH). Hepatic OATP1B1 and 1B3 are located in the basolateral membrane, and mediate the uptake of a broad spectrum of organic anions into hepatocytes for biotransformation.[40, 41] Polymorphisms in the *SLCO1B1* and *1B3* genes, which encode for OATP1B1 and 1B3, have been reported, and may have functional impact on drug disposition.[40] Although *in vitro* studies have not confirmed the involvement of OATP1B1 and 1B3 in MPA pharmacokinetics, Miura et al.[41] reported an association of *SLCO1B3* and *ABCC2*

polymorphisms with MPA oral clearance. Uwai et al.,[42] on the other hand, reported that MPAG is a substrate of human OAT3, which is expressed in the renal proximal tubules, suggesting its role in MPA elimination. Other hepatic efflux transporters such as ABCC3, ABCC4 and ABCC6 in the basolateral membrane of hepatocytes are involved in the export of organic anions and their conjugates into the bloodstream for renal elimination, and may also play a role in MPA pharmacokinetics.[40]

On the pharmacodynamic level, genetic polymorphisms and variable enzymatic activities of inosine monophosphate dehydrogenase, the drug target of MPA, have been described. While the clinical impact of these polymorphisms is poorly characterized to date, the activity of this enzyme has been associated with MPA treatment outcomes.[15] Since acute rejection is mainly mediated by CD4 T-lymphocytes with involvement of interleukin-2 and various signaling molecules,[43] monitoring these immunologic markers may help improve overall immunosuppressive therapy in transplant recipients.

Drug effects are multifactorial and complex; a pharmacogenetics-based dosing strategy is useful only if multiple genes, haplotype analysis, or maybe even a whole genome approach, are considered.[14, 15] In addition to exploring population pharmacokinetics of MPA and incorporating pharmacogenetic information (Section 5.1), important areas of future research would be to further characterize uptake and efflux transporters, determine their role in MPA elimination, characterize relevant drug-drug interactions, identify pertinent pharmacodynamic biomarkers, and develop convenient assay platforms for pharmacodynamic monitoring. By incorporating new knowledge from these areas, it will be possible to develop a dosing algorithm for transplant recipients according to relevant *UGT*, *ABCC2*, *SLCO* and/or *IMPDH* polymorphisms,

along with non-genetic factors identified via population PK modeling. In addition, the study of *de novo* transplant recipients instead of patients at steady state would fully characterize clinical efficacy and toxicities of pharmacotherapy, and may capture important genetic determinants that are otherwise overlooked. These multivariate models would need to be further validated in prospective, randomized controlled trials in *de novo* transplant recipients in order to evaluate whether algorithm-guided dosing of MMF is superior to empirical dosing in improving patient outcomes.

5.7. References

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APPENDICES

Appendix A. University of British Columbia Research Ethics Board

Certificates of Approval

PROVIDENCE HEALTH CARE
Research Institute

UBC-Providence Health Care Research Institute
Office of Research Services
11th Floor Hornby Site - SPH
c/o 1081 Burrard St.
Vancouver, BC V6Z 1Y6
Tel: (604) 806-8567
Fax: (604) 806-8568

ETHICS CERTIFICATE OF EXPEDITED APPROVAL: ANNUAL RENEWAL

PRINCIPAL INVESTIGATOR:	DEPARTMENT:	UBC-PHC REB NUMBER:
Mary H. Ensom	UBC/Pharmaceutical Sciences Clinical Pharmacy	H04-50190

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT:
N/A
Other locations where the research will be conducted:
N/A

CO-INVESTIGATOR(S):
N/A

SPONSORING AGENCIES:
Michael Smith Foundation for Health Research - "Pharmacogenetics of Mycophenolate In Thoracic Transplant Recipients: Role of UDP-Glucuronosyltransferase Genetic Polymorphisms"

PROJECT TITLE:
Pharmacogenetics of Mycophenolate In Thoracic Transplant Recipients: Role of UDP-Glucuronosyltransferase Genetic Polymorphisms

EXPIRY DATE OF THIS APPROVAL: July 2, 2009

APPROVAL DATE: July 2, 2008

CERTIFICATION:

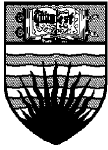
1. The membership of the UBC-PHC REB complies with the membership requirements for research ethics boards defined in Part C Division 5 of the Food and Drug Regulations of Canada.
2. The UBC-PHC REB carries out its functions in a manner fully consistent with Good Clinical Practices.
3. The UBC-PHC REB has reviewed and approved the research project named on this Certificate of Approval including any associated consent form and taken the action noted above. This research project is to be conducted by the principal investigator named above at the specified research site(s). This review of the UBC-PHC REB have been documented in writing.

The UBC-PHC Research Ethics Board Chair or Associate Chair, has reviewed the documentation for

the above named project. The research study, as presented in the documentation, was found to be acceptable on ethical grounds for research involving human subjects and was approved for renewal.

Approval of the UBC-PHC Research Ethics Board or Associate Chair, verified by the signature of one of the following:

Dr. I. Fedoroff, Chair	Dr. J. Kernahan, Associate Chair	Dr. Kuo-Hsing Kuo, Associate Chair



The University of British Columbia
Office of Research Services and Administration
Clinical Research Ethics Board

Certificate of Expedited Approval: Amendment

PRINCIPAL INVESTIGATOR	DEPARTMENT	NUMBER
Ensom, M.H.H.	Pharmaceutical Sci	**C02-0568**

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT

BC Transplant Society, Vancouver Acute (VCHA)

CO-INVESTIGATORS:

Guillemette, Chantal, ; Levy, Robert, Medicine; Partovi, Nilufar, Pharmaceutical Sci; Riggs, Wayne, Pharmaceutical Sci; Ting, Lillian

SPONSORING AGENCIES

Dean of Pharmaceutical Sciences

TITLE :

Pharmacogenetics of Mycophenolate in Lung Transplant Recipients: Role of UDP-Glucuronosyltransferase Genetic Polymorphisms

APPROVAL DATE (yy/mm/dd)	TERM (YEARS)	AMENDMENT:	AMENDMENT APPROVED:
03-01-08	1	Consent form version 3 dd 23 June 2003, co-investigator, sponsor	July 25 2003

CERTIFICATION:
In respect of clinical trials:

1. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations.

2. The Research Ethics Board carries out its functions in a manner consistent with Good Clinical Practices.
3. This Research Ethics Board has reviewed and approved the clinical trial protocol and informed consent form for the trial which is to be conducted by the qualified investigator named above at the specified clinical trial site. This approval and the views of the this Research Ethics Board have been documented in writing.

The amendment(s) for the above-named project has been reviewed by the Chair of the University of British Columbia Clinical Research Ethics Board and the accompanying documentation was found to be acceptable on ethical grounds for research involving human subjects.

The CREB approval period for this amendment expires on the one year anniversary date of the CREB approval for the entire study.

Approval of the Clinical Research Ethics Board by one of:
Dr. P. Loewen, Chair
Dr. A. Gagnon, Associate Chair

Appendix B. Informed Consent and Assent Forms

THE UNIVERSITY OF BRITISH COLUMBIA

Faculty of Pharmaceutical Sciences
2146 East Mall
Vancouver, B.C. Canada V6T 1Z3
Tel: (604) 822-3183
Fax: (604) 822-3035

INFORMED CONSENT FORM

Pharmacogenetics of Mycophenolate in Thoracic Transplant Recipients: Role of UDP-Glucuronosyltransferase Genetic Polymorphisms

Principal Investigator
Mary H.H. Ensom, Pharm.D., FASHP, FCCP, FCSHP, Faculty of Pharmaceutical Sciences, University of British Columbia and Department of Pharmacy, Children's and Women's Health Centre of British Columbia, (604) 875-2886
Co-Investigators
Chantal Guillemette, Ph.D., Faculty of Pharmacy, Laval University, (418) 656-4141 ext. 6348
Nilufar Partovi, Pharm.D., FCSHP, Faculty of Pharmaceutical Sciences, University of British Columbia and Department of Pharmacy, Vancouver General Hospital, (604) 875-4293
Lillian Ting, M.Sc., Faculty of Pharmaceutical Sciences, University of British Columbia, (604) 875-3198
K. Wayne Riggs, Ph.D., Faculty of Pharmaceutical Sciences, University of British Columbia, (604) 822-2061
Robert D. Levy, MD, FRCP(C), Faculty of Medicine, University of British Columbia and Department of Medicine, Respiratory Division, St. Paul's Hospital, (604) 806-9151
Andrew P. Ignaszewski, MD, FRCP(C), FACC, Faculty of Medicine, University of British Columbia and Department of Medicine, Cardiology Division, St. Paul's Hospital, Cardiology Division, (604) 806-8605
Sponsor:
Canadian Institutes of Health Research
Name and 24 Hour Telephone Number of Contact Person:
Dr. Nilu Partovi (604) 875-4293

Background
You are being invited to participate in this study because you are a thoracic (lung or heart) transplant recipient and take the medication mycophenolate (Cellcept®). Mycophenolate is one of several "immunosuppressants" used together that help prevent rejection of your

transplanted organ(s). You would be prescribed mycophenolate as standard treatment even if you did not participate in this study.

Previous research studies have shown that different people handle mycophenolate in their bodies differently. Monitoring mycophenolate blood levels may help your doctor and pharmacist know what dose of mycophenolate works best for you. This means that knowing your mycophenolate blood levels may help determine whether there is enough drug in your body to prevent rejection but not too much to cause unwanted drug effects.

Each person has a different genetic make-up. Therefore, one of the possible explanations for why different people handle mycophenolate differently may be genetic variation in the enzymes used to break down this drug in the body.

Purpose
The purpose of this study is to improve mycophenolate therapy in thoracic transplant subjects by finding out how your body handles this drug. This study will determine if a person's genetic make-up can explain why s/he may handle the drug differently than another individual. To achieve this, urine samples and 11 mycophenolate blood levels and will be collected over a 12-hour period from 150 subjects.

Study Procedures
You will have the option of participating in this study if you are a thoracic transplant recipient, older than 16 years of age, who is being treated with mycophenolate (Cellcept®). (See also Exclusions below).

If you choose to participate in the study, then the only procedure that will be different than usual transplant care is obtaining blood samples and urine samples once over a 12-hour period. You will be scheduled to visit the BC Transplant Society Office research clinic as an outpatient for your blood sampling. The study visit day will take approximately 13 hours. During the study visit day, you will be eating a standard breakfast, lunch, and dinner.

A study visit appointment will be made with you by a study nurse or one of the investigators. You are asked not to eat anything after midnight on the evening before your morning appointment and report to the research clinic about an hour before your usual morning dose of Cellcept®.

When you arrive, the nurse will place a tiny catheter (identical to those used in the hospital after your transplant) into a vein in your forearm. This will allow easier blood collection and avoid having many "needle pokes" during the visit or you can choose to have "needle pokes" instead. If you have a "central line", then all blood samples can be drawn from the "central line" instead of needing a catheter. You will have your first blood sample collected right before you take your usual morning dose of Cellcept®. After this dose of Cellcept®, you will have 10 more blood samples taken later at 20, 40, 60, 90 minutes, and at 2, 4, 6, 8, 10, and 12 hours. All blood samples, except for one, require only about 3 ml (or one-half teaspoonful) each. An extra 20 ml (or 4 teaspoonsful)

for the genetic analysis will be drawn during one of the blood sampling times. Thus, a total of about 53 ml (less than 2 ounces) of blood will be collected during the study visit day. In addition, you will be asked to collect all your urine during the 12-hour study period in provided containers. Physical activity will be limited to walking within the building.

On the clinic visit day, a nurse will also complete a questionnaire with you to assess if you have had any unwanted drug effects with Cellcept®.

Exclusions
You must be excluded from study participation if:
You refuse to or are unable to give written informed consent.
You are younger than 16 years of age.
Your mycophenolate (Cellcept®) therapy is not at steady state. "Steady state" means that you must have taken Cellcept® for at least 5 days without a dosage adjustment.
You are taking other medications (e.g., antacids, cholestyramine, etc.) that can interact with mycophenolate.

Risks
The only risks associated with my participation in this study that are beyond your risks if you were not to participate would be the risks related to blood collection and catheter placement. These risks are considered rare and mild but may include the following: slight bruising, temporary feeling of faintness, slight pain, and/or infection.

There may also be other adverse reactions or risks that could arise which are not predictable. If new information arises during my involvement in this study which could affect your desire to continue, you will be given such new information. See New Findings.

Benefits
The direct benefits to you as a participant of this study cannot be guaranteed, but may include improving your doctor's understanding of how your body handles Cellcept® when we provide your doctor with your study results. This information may be used to provide dosage recommendations specific for you. Your participation is also expected to help find whether genetic variation in the enzymes used to break down mycophenolate in the body can explain differences in handling of this drug by different people.
.
Alternative Treatments
If you decide not to participate or to withdraw at some later date, you will continue to receive Cellcept® as standard treatment to prevent rejection.

Confidentiality
Your confidentiality will be respected. No information that discloses your identity will be released or published without your specific consent to the disclosure. However, research records and medical records identifying you may be inspected in the presence of the investigator or his or her designate by representatives of the Canadian Institutes of Health

Research, and the UBC Research Ethics Board for the purpose of monitoring the research. However, no records which identify you by name or initials will be allowed to leave the investigators' offices.

Remuneration/Compensation
You will receive $100 total for the successful completion of this study to help offset costs for travel, meals, and parking required on the study visit day. There will be no other costs to you for participating in this study and you will not be charged for any research procedures.

Compensation for Injury
If you become ill or injured during the study, needed medical treatment will be available at no extra cost to you through my medical plan.
Signing this consent form in no way limits your legal rights against the sponsor, investigators, or anyone else.

Contact
If you have any questions, need more information about the study, or if you experience any adverse effects, you should contact Dr. Ensom at (604) 875-2886, Dr. Partovi at (604) 875-4293, Dr. Levy at (604) 806-9151, or Dr. Ignaszewski at (604) 806-8605.
If you have any concerns about your treatment or rights as a research subject, you may contact the Research Subject Information Line at the University of British Columbia at (604) 822-8598.

New Findings
If you choose to enter this study and, at a later date, a more effective treatment becomes available, it will be offered to you. You will also be advised of any new information that becomes available that may affect your desire to remain in this study.

Subject Consent
Participation in this study is entirely voluntary and that you may refuse to participate or you may withdraw from the study at any time without any consequences to your continuing medical care.
You have received a copy of this consent form for your own records. You consent to participate in this study.

____
Subject Signature Name (Print) Date

____
Guardian Signature (for subjects under the age of 19) Name (Print) Date

____
Witness Signature Name (Print) Date

____
Investigator Signature Name (Print) Date

THE UNIVERSITY OF BRITISH COLUMBIA

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Fax: (604) 822-3035

ASSENT FORM FOR SUBJECTS AGED 16-19

Pharmacogenetics of Mycophenolate in Thoracic Transplant Recipients: Role of UDP-Glucuronosyltransferase Genetic Polymorphisms

Everybody is different in how his/her body reacts to drugs, and this is often due to our different genetic makeup. The purpose of this study is to find out how different people handle mycophenolate (Cellcept®) in their body differently. We hope to improve treatment with mycophenolate by finding out the right amount of drug for each individual. This may help reduce unwanted drug effects. To study this, urine samples and 11 blood samples will be drawn from you during the day of visit. The amount of mycophenolate in your urine and blood and your genetic makeup will be determined from the blood samples. This will require one or more "needle pokes" as the nurse sets up a line in your arm for blood draws. The whole study will take about 13 hours, and you will be staying at the BC Transplant Society Office research clinic for the day. Due to the "needle poke(s)" and blood draw, you may get slight bruising, pain, infection and/or feeling of faintness.

Your participation is totally voluntary, and if at any time you don't feel like continuing the study, you can stop the procedure without giving any reasons. No one will be angry if you decide to leave, there is no penalty and there will be no change in your health care.

If you have any questions before or during the study, please feel free to ask or discuss with your parents/guardians, friends, doctors, nurses or the researchers. We will try our best to answer all your questions and concerns.

Your participation will be kept private, and nobody in the study is allowed to give away your name, your information or your involvement in this study.

Your participation will be greatly appreciated. If you understand this study and would like to participate, please read and sign the statement below.

SUBJECT'S ASSENT TO PARTICIPATE IN RESEARCH

I have had the opportunity to read this consent form, to ask questions about my participation in this research, and to discuss my participation with my parents/guardians. All my questions have been answered. I understand that I may withdraw from this research at any time, and that this will not interfere with the availability to me of other health care. I have received a copy of this consent form. I assent to participate in this study

---

Subject Signature | Name (Print) | Date

Appendix C. Significant Pharmacogenetic and Demographic Factors that Impact on MPA Pharmacokinetics in Multivariate Analysis

Multivariate analyses	Lung (n=36)		Heart (n=32)	
Covariates	***p***	**r^2**	***p***	**r^2**
MPA exposure				
Total MPA AUC(0-12)				
Gender			0.027	0.083
Co-medication			0.017	0.146
Age			0.011	0.197
UGT2B7 G-138A	0.004	0.218		
UGT2B7 IVS1 +C999A	0.021	0.105		
UGT1A7 $Asn^{129}Lys$			0.013	0.182
UGT1A7 $Trp^{208}Arg$	0.028	0.108		
Cmax				
Co-medication			0.008	0.172
UGT1A8 $Thr^{237}Thr$			0.044	0.084
UGT 2B7 G-138A	0.005	0.213		
UGT1A7 $Asn^{129}Lys$			0.008	0.211
Cmin				
Gender			0.049	0.068
Co-medication	0.007	0.200	0.002	0.285
Age			0.018	0.131
UGT1A8 $Cys^{277}Ile$			0.026	0.100
UGT1A9 C-2152T/T-275A			0.044	0.056
UGT2B7 G-138A			0.044	0.063
UGT2B7 IVS1+C999A	0.040	0.100		
MPA Clearance				
Age			0.013	0.194
UGT 2B7 G-138A	0.005	0.218		
UGT2B7 IVS1+999	0.025	0.102		
UGT1A7 $Asn^{129}Lys$			0.047	0.107
UGT1A7 $Trp^{208}Arg$	0.026	0.113		
fMPA AUC(0-12)	N/A	N/A		
UGT1A8 $Thr^{237}Thr$			0.015	0.181
MPA Free fraction	N/A	N/A		

Multivariate analyses	Lung (n=36)		Heart (n=32)	
Covariates	***p***	**r^2**	***p***	**r^2**
UGT1A1 G-3279T			0.018	0.174
MPA EHC				
Age			0.028	0.151
UGT2B7 IVS1+C999A	0.030	0.131		
MPA metabolites				
MPAG AUC_{0-12}				
UGT1A9 T-2188C			0.016	0.177
UGT1A1 G-3279T	0.006	0.205		
*UGT1A1**28 (TATA)			0.047	0.107
UGT2B7 G-138A	0.002	0.206		
MPAG/MPA ratio				
ABCC2 $Val^{417}Ile$			0.029	0.149
Co-medication	<0.001	0.315		
AcMPAG AUC_{0-12}				
Serum Creatinine	0.012	0.188		
UGT2B7 $His^{268}Tyr$	0.007	0.176	0.007	0.236
AcMPAG/MPA ratio				
UGT1A9 -118 t-stretch			0.014	0.124
UGT2B7 G-138A	0.001	0.300	0.022	0.128
UGT2B7 $His^{268}Tyr$			0.007	0.236

A = adenosine; ABCC2 = multiple resistance-associated protein 2; AcMPAG = acyl glucuronide of mycophenolic acid; Arg = arginine; Asn = Asparagine; AUC = area-under-the-concentration-time-curve; C = cytosine; Cmax = maximum concentration; Cmin = minimum concentration; Cys = cysteine; EHC = enterohepatic recircultion; fMPA = free mycophenolic acid; G = guanosine; His = histidine; Ile = isoleucine; Lys = lysine; MPA = Mycophenolic acid; MPAG = 7-O-mycophenolic acid glucuronide; N/A = not applicable; T = thymine; Trp = tryptophan; Tyr = tyrosine; UGT = UDP-glucuronosyltransferase; Val = valine