Pharmacokinetics of Mycophenolic Acid in Patients post Hematopoietic Stem Cell Transplantation

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Daping Zhang

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Abstract

[*Purpose*] Mycophenolate mofetil (MMF), an ester prodrug of mycophenolic acid (MPA), is widely used as a maintenance immunosuppressive regimen in solid organ transplant patients. It is increasingly used for the prophylaxis and treatment of graft-versus-host disease (GVHD) in hematopoietic stem cell transplantation (HSCT) patients. MPA is metabolized primarily to phenolic MPA glucuronide (MPAG) and minorly to acyl MPA glucuronide (AcMPAG). In patients with normal renal and liver function, MPA and MPAG are highly bound to serum albumin, 97-99% for MPA and 82% for MPAG. The optimal MMF dosing and preferred targets are still under investigation in HSCT patients due to the substantial intra- and inter-individual pharmacokinetic variability of MPA and broad range of transplants. Therefore, the overall goal of this thesis is to elucidate the clinical pharmacokinetics of MPA in HSCT patients. To approach this goal, three specific aims are 1) To develop a reliable UPLC-MS/MS assay for the simultaneous monitoring of MPA, MPAG and AcMPAG in human plasma samples; 2) To evaluate the pharmacokinetic variations of MPA, from plasma protein binding and metabolism perspectives, in both pediatric and adult patients following HSCT; 3) To investigate population pharmacokinetics of unbound MPA in pediatric and adolescent HSCT patients.

[*Methods*] Plasma proteins were precipitated with acetonitrile and the chromatographic separation was achieved on a C_{18} column with a gradient elution. The detection was performed by a triple quadrupole mass spectrometer in the positive electrospray ionization (ESI) and multiple reaction monitoring (MRM) mode. Stability of MPA and its glucuronide

metabolites was also thoroughly evaluated in human blood and plasma samples under short- and long- term storage conditions. Twenty pediatric patients with a median age of 3 years (range, 0.2-12 years) and thirteen adult patients with a media age of 54 years (range, 18-63 years) were enrolled. Sparse sampling design was used in this study. Blood samples were collected on days 0, 7, 14, 21 and 30 after allogeneic HSCT. Total and free MPA, as well as MPAG were quantified using the validated UPLC-MS/MS assay. Nonlinear mixed-effects modelling (NONMEM) was employed to analyze MPA pharmacokinetic data. A total of 89 unbound MPA plasma concentration-time datum points from 23 patients with a median age of 3 years (range, 0.2-20 years) were available for model development.

[*Results*] Linearity of the assay was demonstrated over the range of 20-10,000 ng/ml for MPA and MPAG, and 2-1,000 ng/ml for AcMPAG in human plasma. The assay was precise and accurate with coefficient of variation and bias less than 15%. The plasma protein binding of MPA and MPAG did not change significantly in pediatric patients over the one month sampling period post HSCT. However, it increased in adult patients from day 7 to day 30 post HSCT, from 97.3±0.8% to 98.3±0.6% for MPA, and 74.6±9.4% to 82.9±8.1% for MPAG ($P < 0.05$). The plasma protein binding of MPA was significantly higher in males compared to females in both pediatric (98.3±1.1 vs 97.4±1.1%) and adult (98.1±0.7 vs 97.4±1.2%) patients ($P < 0.05$). The MPAG/MPA ratios on an mg/kg dose basis in adult patients were significantly higher than those in pediatric patients (4.3±3.4 vs 2.4±2.6; $P < 0.05$). Pharmacokinetics of unbound MPA was described by a two-compartment model with first-order elimination. Given the range of body sizes, clearance

and volume of distribution were scaled using standard weight-based allometric exponents. Final estimates in a standard 70 kg individual for clearance, inter-compartmental clearance, volumes of distribution in the central and peripheral compartments were 1720 L/h, 1180 L/h, 3260 L and 4120 L, respectively. No significant differences were observed in weight-adjusted clearance between males and females. Because of age-dependent differences in weight-adjusted clearance, the calculated unbound MPA AUC was higher in younger patients compared with those in older patients receiving 15 mg/kg MMF.

[*Conclusions*] The UPLC-MS/MS assay for simultaneous quantification of MPA and its glucuronide metabolites was developed and fully validated in human plasma samples per US FDA Guideline. Extended stabilization procedures were suggested to improve the accuracy of the analysis before routine application. Time-dependent plasma protein binding, sex and age-related differences in MPA metabolism, at least in part, impact the reported large inter- and intra-individual variability in MPA pharmacokinetics. The final population pharmacokinetic model successfully described unbound MPA population pharmacokinetics, which could be used to explore dosing guidelines for safe and effective immunotherapy in pediatric HSCT patients. Future research should be multi-institutional and focus on developing clinical decisions with adequate statistical power to improve clinical care of HSCT recipients.

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List of abbreviations

AcMPAG	Acyl MPA glucuronide
ACN	Acetonitrile
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BMT CTN	Blood and marrow transplant clinical trials network
BSA	Body surface area
BUN	Blood urea nitrogen
BW	Body weight
CE	Collision energy
CL	Clearance
CV	Coefficient of variation
CXP	Collision cell exit potential
CYP	Cytochrome P450
DBS	Dried blood spot
DP	Declustering potential
EHC	Enterohepatic circulation
EP	Entrance potential
ESI	Electrospray ionization
FOCE	First-order conditional estimation

GFR	Glomerular filtration rate
GI	Gastrointestinal
IV	Intravenous
IIV	Inter-individual variability
IMPDH	Inosine monophosphate dehydrogenase
IPRED	Individual predicted values
GVHD	Graft-versus-host disease
HSCT	Hematopoietic stem cell transplantation
LLOQ	Lower limit of quantification
MeOH	Methanol
MMF	Mycophenolate mofetil
MPA	Mycophenolic acid
MPAG	MPA glucuronide
MRM	Multiple reaction monitoring
MRP2	Multidrug resistance-associated protein-2
NONMEM	Nonlinear mixed-effects modelling
OFV	Objective function value
PBSC	Peripheral blood stem cell
PRED	Population predicted values
Q	Inter-compartmental clearance
QC	Quality control
RCCT	Randomized concentration-controlled trial

RSE	Relative standard error
SNPs	Single nucleotide polymorphisms
UGT	UDP-glucuronosyltransferase
UPLC-MS/MS	Ultra-performance liquid chromatography-tandem mass spectrometry
RUV	Residual unexplained variability
V_c	Central volume of distribution
V_p	Peripheral volume of distribution
WRES	Weighted residuals

Content

Chapter 1 Clinical pharmacokinetics of mycophenolic acid in hematopoietic stem cell transplantation recipients

1.1 Abstract

Mycophenolate mofetil (MMF), an ester prodrug of mycophenolic acid (MPA), is widely used as a maintenance immunosuppressive regimen in solid organ transplant patients. It is increasingly used for the prophylaxis and treatment of graft-versus-host disease (GVHD) in hematopoietic stem cell transplantation (HSCT) patients. MPA displays extensive binding to serum albumin and glucuronidation into the inactive MPA-7-O-glucuronide (MPAG). Here we review and discuss the pertinent information regarding the clinical pharmacokinetics/dynamics of MPA in HSCT patients. The pharmacokinetics of MPA is altered in HSCT patients with lower oral bioavailability, shorter half-life and higher clearance than those in healthy volunteers and renal transplant recipients. Moreover, clearance may be increased in young pediatric patients. The optimal MMF dosing and preferred targets are still under investigation in HSCT patients due to the substantial intra- and inter-individual pharmacokinetic variability of MPA and broad range of transplants (malignant vs. nonmalignant, related vs. unrelated donor, and human leukocyte antigen mismatch). The complex pharmacokinetics of MPA has partly hampered the efficient use of MMF and pharmacokinetic studies in HSCT patients have been limited in size and mostly inconclusive. Future research should be multi-institutional and focus on developing clinical decisions with adequate statistical power to improve clinical care of HSCT recipients.

1.2 Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) involves the intravenous (IV) infusion of stem cells obtained from a related or unrelated donor to reestablish hematopoietic function in patients whose bone marrow or immune system is damaged or defective (Figure 1). It is a potential curative therapy for many patients with defined congenital or acquired disorders of the hematopoietic system, or with cancers of the blood and bone marrow. Presently, cells for HSCT can be collected from bone marrow, peripheral blood, or umbilical cord blood. Approximately 30,000 allogeneic HSCTs will be performed worldwide in 2020; 20% of allogeneic HSCT are performed in the pediatric population. However, the efficacy of this procedure has been impeded by frequent and severe graft-versus-host disease (GVHD), a reaction of donor immune cells against host tissues. The exact risk is dependent on the stem cell source, age of the patient, conditioning, and GVHD prophylaxis used. Acute GVHD is measured by clinical symptoms in three organ systems: the skin, liver and gastrointestinal (GI) tract. About 35–50% of hematopoietic HSCT recipients will develop acute GVHD, which typically occurs before day 100 after the HSCT, but may occur later, and is often clinically indistinguishable from other causes of clinical symptoms such as conditioning regimen toxicity, infection, or medication. And 50% of patients with acute GVHD will eventually have manifestations of chronic GVHD, which typically happen 100 days after the HSCT [1].

Mycophenolate mofetil (MMF, CellCept®), an ester prodrug of mycophenolic acid (MPA), has been widely used since 1995 as an immunosuppressive drug to prevent acute rejection following solid organ transplantation. MPA is a purine analog, which can interfere

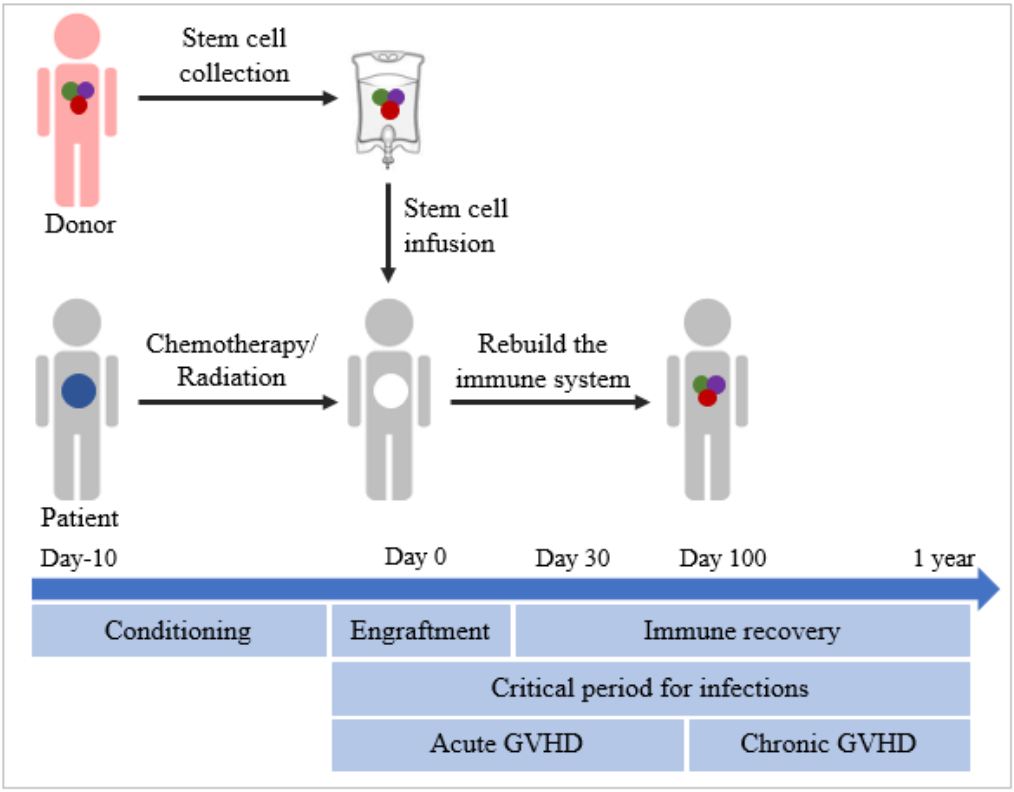


Figure 1. Procedure of allogeneic HSCT

with cell proliferation through the inhibition of inosine monophosphate dehydrogenase (IMPDH), thereby blocking the de novo purine synthesis in T- and B-cell lymphocytes [2]. The successful use of MMF in solid organ transplantation has triggered its increasing application in the prophylaxis and treatment of acute and chronic GVHD as well as to promote engraftment after HSCT, in which infections, development of GVHD and organ failure represent the major causes of morbidity and mortality [3-6]. MMF is frequently used in combination with a calcineurin inhibitor, such as cyclosporine or tacrolimus, as a maintenance immunosuppressive regimen. The pharmacokinetics of MPA in solid organ transplantation has been extensively reviewed elsewhere [7-12]. In HSCT patients, many factors may disturb the pharmacokinetics of MPA, leading to substantial variations in plasma MPA exposures. Potential factors include gastrointestinal mucosal injury induced by chemotherapy and/or radiation therapy, the presence/absence of GVHD, multiple drug interactions, severity of illness and loss of appetite. The current review intends to provide an overview of recent studies regarding pharmacokinetics/dynamics of MPA in HSCT patients.

1.3 Clinical pharmacokinetics

Orally administered MMF is rapidly absorbed and hydrolyzed by carboxylesterases to the active moiety MPA with maximum MPA peak concentrations generally occurring within 1 hour after administration. It is further metabolized by UDP glucuronosyl transferases in the liver, GI tract and kidneys, forming the major inactive metabolite MPA glucuronide (MPAG), being present in plasma at 20- to 100- fold higher concentrations than MPA, and to a smaller extent the active metabolite acyl-MPA glucuronide (AcMPAG). MPA is primarily

excreted in urine as MPAG (87%) metabolite, which could accumulate in patients with renal failure (Figure 2). Negligible amounts of unchanged MPA (<1% of dose) are excreted in urine [13, 14].

1.3.1 Enterohepatic circulation

MPAG is subject to enterohepatic circulation mediated by multidrug resistance-associated protein-2 (MRP2), resulting in a characteristic second peak at 6 to 12 hours after administration in MPA concentration-time profiles. Up to 40% (range: 10-60%) of the AUC may come from enterohepatic circulation in some populations [7, 15]. However, reduced enterohepatic circulation has been observed in HSCT patients, especially when co-administered with cyclosporine [16-18]. The shortened half-life and low trough levels of MPA further confirm a low prevalence of the second delayed peak from enterohepatic circulation in HSCT patients. The impaired enterohepatic circulation may be due to the mucosal damage associated with conditioning regimens (radiation and/or chemotherapy), and the destruction of intestinal flora caused by heavy use of broad-spectrum antibacterial agents. Moreover, cyclosporine interacts with MMF through inhibition of the biliary excretion of MPAG by the MRP2 transporter, leading to a further reduction of the enterohepatic circulation [19].

1.3.2 Oral bioavailability

The mean bioavailability of MPA from oral administration of MMF reported in 12 healthy volunteers is 94.1% relative to the intravenous route, leading to the manufacturer's recommendation that the conversion of i.v. to oral is 1:1 [13]. However, it is substantially

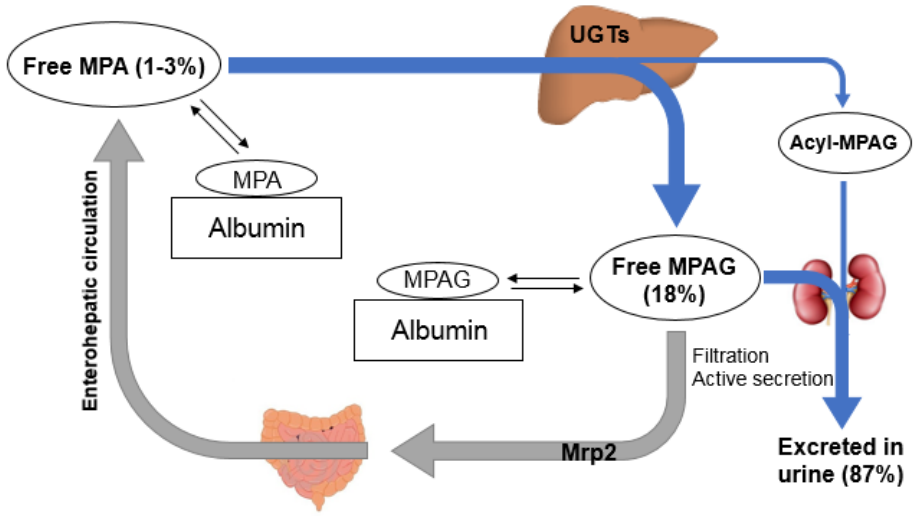


Figure 2. Metabolic pathway of MPA

lower with large inter-subject variation in adult HSCT patients co-treated with cyclosporine [18, 20, 21]. Possible reasons for low oral bioavailability include reduced enterohepatic circulation, concomitant drugs that are UGTs inducers or MRP2 inhibitors and presence of GVHD. Based on a population pharmacokinetic analysis, bioavailability decreases with increasing MMF doses (range: 250 to 2000 mg q12h) in renal transplant patients co-treated with cyclosporine or tacrolimus [22]. But it is not clear if this nonlinear pharmacokinetic property exists in HSCT patients. Because of these problems, the dose of MMF may need to be cautiously adjusted when converting from i.v. to oral administration in HSCT recipients.

1.3.3 Plasma protein binding

In patients with normal renal and liver function, MPA and MPAG are highly bound to serum albumin, 97-99% for MPA and 82% for MPAG [14]. Changes in protein binding may occur as a result of chemotherapy, severe illness, and renal and hepatic dysfunction. The unbound fraction of MPA in HSCT patients ranges from 0.3% to 7% based on results from several studies [18, 23, 24]. It is reported that liver is the major organ responsible for the systemic clearance of MPA from in vitro kinetic data [25]. Since MPA has a moderate extraction ratio (0.3-0.7), the metabolism following intravenous administration would be impacted by unbound fraction, intrinsic enzymatic activity of the liver and the blood flow to the liver. Assuming complete absorption and lack of extrahepatic clearance mechanisms, the apparent oral clearance (CL/F ratio, calculated as dose divided by AUC following oral administration) always depends on the former two factors (unbound fraction and intrinsic enzymatic activity) [10, 26, 27]. In reality, therapeutic monitoring of MPA mostly involves

measuring concentrations of total MPA (both protein-bound and free form). Given that only unbound MPA is responsible for the inhibition of IMPDH and total MPA concentrations do not always truly reflect unbound MPA concentrations, there is a need for monitoring unbound MPA levels in HSCT patients.

1.3.4 Pharmacokinetic variability

Total and unbound MPA exposures are lower in HSCT patients compared to those in renal transplant patients and healthy individuals receiving the same MMF dose [17, 23, 28]. The following factors may play a role. First, the lack of significant enterohepatic circulation may have contributed. A second factor may be the low oral bioavailability. Since the intravenous formulation of MMF still achieves significantly lower MPA exposures (AUC and predose levels) in HSCT patients than those in healthy volunteers and renal transplant patients, the low bioavailability might not be the main reason for lower MPA exposures in HSCT patients [29, 30].

As observed in solid organ transplant recipients, considerable intra- and inter-patient pharmacokinetic variability of total and unbound MPA has been reported in HSCT patients, leading to a significant therapeutic challenge [23, 24, 30]. Possible factors affecting MPA pharmacokinetics include one or more of the following situations: renal and hepatic function, albumin level, concomitant medications, pharmacogenetic factors, demographic information (weight, sex, age and race) and clinical status of HSCT patients (the intensity of conditioning, mucositis, infections and the occurrence of GVHD), although the majority remain unknown. Total and unbound MPA concentrations are lower in patients with GI or

liver GVHD relative to those with skin only involvement [3, 31]. Using a population pharmacokinetic modeling approach, plasma albumin and cyclosporine concentrations are able to explain the pronounced differences in total MPA clearance among adult renal transplant, HSCT and autoimmune disease patients [28]. These data are in accordance with the findings from other studies, that increased total MPA clearance could be partly explained by concomitant cyclosporine and decreased albumin concentrations in pediatric and adult HSCT patients [32, 33]. Weight, creatinine clearance and total bilirubin are identified as important clinical covariates affecting unbound MPA pharmacokinetics in pediatric and adolescent HSCT patients. The unbound MPA clearance is approximately 3-fold lower in patients with severe hepatic dysfunction than those with normal to mild hepatic impairment [34]. Another study conducted in adult HSCT patients further supports that creatinine clearance is a significant predictor of unbound MPA clearance [35].

MMF has been used in children, particularly off-label, for several years. Robust trials regarding the pharmacokinetics of MPA in pediatric HSCT patients are often lagging behind those in adult HSCT patients, partly due to concerns over blood volume for pharmacokinetic analysis [36]. The current method of dosing in pediatric patients is based on body weight or surface area. However, age-dependent pharmacokinetics of MPA has been observed in several studies. Children < 12 years old appear to have significantly higher weight-adjusted clearance and volume of distribution than those in the 12-16 years age group following intravenous MMF administration in combination with tacrolimus [37]. Jacobson et al also reported that pediatric HSCT recipients receiving 15 mg/kg every 8 hours (33% higher daily dose) have similar total and unbound MPA exposures to adult

HSCT recipients receiving 1 g every 12 hours intravenously, indicating higher MPA clearance in pediatric patients [30]. Based on a population pharmacokinetic analysis in HSCT patients, the predicted unbound MPA clearance adjusted by weight is higher in smaller children and declines with increasing body weight [34]. The mechanisms underlying these age-dependent differences are unclear but are probably related to developmental changes in all components of drug disposition.

1.3.5 Pharmacogenetic variability

Glucuronidation is considered a major metabolic pathway for MPA. It is mainly metabolized to MPAG by UGT1A family members, particularly UGT1A9 and UGT1A8, and minorly to AcMPAG by UGT2B7. MPAG could be excreted in bile via MRP2 and this transport is essential for the enterohepatic circulation [14]. The genetic factors control the level of UGT-mediated metabolism of MPA and the MRP2-mediated transport of MPAG, which may also explain, in part, the reported large inter-individual pharmacokinetic variability.

The impact of genetic variations on MPA pharmacokinetics has not been extensively evaluated in HSCT patients. To date, only one analysis has been published to evaluate pharmacogenetic factors as contributors to the variability of unbound MPA exposure in adult HSCT patients using a population pharmacokinetic modeling approach. They reported that genetic variations in UGT and MRP2 are not found to influence unbound MPA clearance in 132 adult HSCT patients [35]. The lack of pharmacogenetic effects may be attributed to drug-drug interactions that may mask or confound the effects of any

genetic variation on MPA pharmacokinetics. Another possible contributing factor is the relatively low frequency of several polymorphisms in the study population, leading to inadequate statistical power to detect more subtle genetic effects. Therefore, the information available to date is insufficient to draw any firm conclusions regarding the impact of genetic variability on MPA pharmacokinetics in HSCT patients.

Several polymorphisms in the genes encoding for UGT1A9, UGT1A8, UGT2B7 and MRP2 have been identified as contributing to the large inter-individual variability of MPA exposure in healthy volunteers and kidney transplant patients [38-41]. Some of the pharmacogenetic findings are not consistent. It should be noted that the study population is not homogenous in terms of age, gender, ethnicity and clinical status, resulting in the lack of coherent results across studies. A significant correlation between the presence of the UGT1A9 -275/-2152 allele and lower MPA exposure is demonstrated in healthy adults and renal transplant patients [39, 42]. It is also observed that renal transplant patients and healthy individuals carrying the -440/-331 single nucleotide polymorphisms (SNPs) of the UGT1A9 promoter region exhibit significantly higher MPA exposures than those not carrying these mutations [40, 43]. UGT1A8 is primarily expressed in GI tract. Several UGT1A8 variants are identified and could potentially account for the large inter-individual pharmacokinetic variability of MPA [44]. UGT2B7 is the enzyme primarily involved in formation of the pharmacologically active metabolite, AcMPAG. The healthy subjects with UGT2B7 211TT/TC exhibit higher AcMPAG exposures than those with UGT2B7 211CC, which could result in increased toxic effects of MMF [40]. In addition to genetic variation in metabolic phenotypes, polymorphisms in MRP2 may affect the pharmacokinetic of MPA

by interfering the active transport of MPAG in enterohepatic circulation. Reduced MPA exposures are observed in renal transplant recipients with C24T variant allele in MRP2, co-administered with tacrolimus or sirolimus. However, the effect of this genetic polymorphism is lost in patients co-administered with cyclosporine, suggesting a masking effect of cyclosporine against the C24T genetic polymorphism [45]. This is consistent with the findings of Baldelli et al, that C24T SNP has no impact on MPA exposure in renal transplant patients receiving cyclosporine as part of immunosuppression [43]. Therefore, study of different phenotypes resulting from the pharmacogenetic differences may provide additional information related to pharmacokinetic variations of MPA in HSCT patients. Further investigations in a larger population are required to characterize the impact of pharmacogenetic factors in HSCT patients with greater statistical confidence before any statement could be made.

1.3.6 Drug-drug interactions

HSCT patients are treated with complex medical regimens including chemotherapeutic, immunosuppressive and antimicrobial agents in various combinations. A high prevalence of clinically significant drug-drug interaction is identified as a consequence of the complexity of pharmacotherapy [46, 47]. Several drug interactions with MMF have been assessed, including calcineurin inhibitors, antibiotics, corticosteroids and antifungal agents. The most relevant drug interactions with MMF occur with calcineurin inhibitors. Cyclosporine inhibits MRP2-mediated enterohepatic circulation of MPAG and thus causes a decrease in plasma MPA exposures. In contrast, tacrolimus is a potent inhibitor of UGTs, leading to reduced MPA metabolism and increased MPA concentrations. From population

analyses of MPA in patients undergoing HSCT, concomitant cyclosporine is identified as a significant covariate to explain inter-individual variability in clearance. Clearance is higher in patients co-administered with cyclosporine compared to patients co-administered with tacrolimus [28, 32, 33, 48]. In addition, the broad-spectrum antibiotic therapy could destruct intestinal microflora, which plays an important role in enterohepatic circulation of MPAG. Concomitant antibiotic treatment may result in a reduction of MPA AUC due to the aggressiveness of bacterial obliteration [49]. Corticosteroids are potent inducers of UGTs enzymes, possibly resulting in enhanced MPAG formation and reduced MPA concentrations. Studies on pharmacokinetic interactions between corticosteroids and MPA have conflicting conclusions in solid organ transplant patients [50, 51]. In healthy volunteers, concomitant treatment with MMF and pantoprazole significantly lowers the peak concentrations and AUC of MPA due to the incomplete gastric dissolution of MMF [52]. A better understanding of enzymatic pathways responsible for these interactions will promote efficient use of MMF in patients undergoing HSCT.

1.4 Relationships between MPA exposures and clinical outcomes

Therapeutic targets for MPA have been firmly established in solid organ transplant recipients. When combined with cyclosporine, the recommended target ranges are 1 to 3.5 μg/ml and 30 to 60 μg·h/ml for total MPA trough concentrations and AUC, respectively. For the combination with tacrolimus, the target ranges of 1.9 to 4 μg/ml and 35 to 60 μg·h/ml for trough and AUC measurements, respectively, are optimal [8].

The association between MPA exposure and clinical outcome in HSCT patients has been evaluated by various investigators [23, 24, 53-55]. In 29 adult patients co-administered with tacrolimus, individual MMF targeting based on MPA AUC of 35-60 μg·h/ml is feasible and effective in the early phase after HSCT. The significantly higher doses required than those previously reported are not associated with dose-limiting toxicities [55]. In 34 childhood and adolescent HSCT patients, patients achieving steady state MPA trough concentrations of 1.0 to 3.5 μg/ml have a significantly reduced incidence of developing grade ≥ Ⅱ acute GVHD, when MMF is used in combination with tacrolimus [54]. Other studies confirm that total MPA trough levels correlate with the clinical response in the setting of both acute and chronic GVHD after HSCT [31, 56]. However, conflicting conclusions with the lack utility of trough concentrations predicting rejection/engraftment and/or risk of GVHD have also been reported, probably due to the poor correlation between MPA trough and AUC [18, 23, 57, 58]. Many of these studies are conducted under various conditions based on small sample sizes, making comparisons challenging. Therefore, studies with an adequately sized HSCT patient population are necessary to elucidate these associations.

Given that MPA is highly protein bound and only the unbound MPA is responsible for the inhibition of IMPDH and suppression of lymphocyte proliferation, Jacobson et al evaluated the associations between unbound MPA exposure and clinical outcomes in 87 adult HSCT recipients receiving 1g q12h MMF plus cyclosporine. Subjects with unbound MPA AUC_{0-6} less than 150 ng·h/ml have a higher cumulative incidence of Grade Ⅱ-IV GVHD than subjects with greater AUC (68% vs. 40%, p=0.02). An unbound AUC_{0-12} less than 300

ng·h/ml is also associated with more frequent GVHD (58% vs. 35%, p=0.05). Low total MPA trough concentrations are associated with poor engraftment, but not GVHD. Approximately 50% of the patients are below the unbound AUC targets with a nearly 5-fold variability at the MMF dose of 1g q12h [23]. In 308 HSCT patients receiving grafts from related (n=132) and unrelated donors (n=176), low total MPA C_{ss} (2.96 µg/ml) is associated with an increased risk of Grade III to IV acute GVHD and nonrelapse mortality in patients receiving unrelated donor grafts. In contrast, these findings are not observed in patients receiving related donor grafts [59]. An unbound MPA AUC of 200-250 ng·h/ml for q8h dosing, total MPA trough ≥1 µg/ml or total C_{ss} >3 µg/ml suggested by McCune et al, may be reasonable targets. They also suggested that centers adopt adult HSCT targets or targets used in pediatric solid organ transplant for pediatric HSCT patients until properly powered pediatric studies are available [60].

1.5 Dose optimization

The complexity of MPA disposition (absorption, distribution, metabolism and excretion) results in difficulties in defining the optimal MMF dosing. Current dosing of MMF (15 mg/kg or 600-900 mg/m^2 q8h for pediatric HSCT patients and 2-3 g q12h for adult HSCT patients) leads to substantial inter-patient variability and only some patients achieve therapeutic targets, suggesting alternative methods to estimate MMF dose are needed. Population pharmacokinetic modeling is able to estimate population values with intra- and inter-individual variability of pharmacokinetic parameters and identify patient-specific covariates. This powerful analysis provides a method to quantify how well a given dosing regimen will achieve a desirable target and how this dosing regimen can best be modified to meet an

individual patient's needs, which could facilitate personalized MMF dosing to a target plasma exposure [61]. Emerging pharmacometric methodologies could provide us with new venues for further research on the optimization of MMF therapy, reviewed by Dong et al [62].

In the majority of population pharmacokinetic models of MPA in HSCT patients, MPA clearance is associated with weight, albumin level, creatinine clearance and cyclosporine [32-35, 48]. Since the immunosuppressive activity of MMF is based on the reversible and selective inhibition of IMPDH, it is reported that renal transplantation patients with high pre-transplant IMPDH activity have a higher risk of rejection [63]. In renal transplant patients, IMPDH enzyme activity decreases with increasing MPA plasma concentration, with maximum inhibition coinciding with maximum MPA concentration [64]. The feasibility of evaluating IMPDH activity as a drug-specific biomarker of MPA-induced immunosuppression has also been established in HSCT patients. The relationship between MPA concentration and IMPDH activity is described by a direct inhibitory E_{max} model with an IC_{50} = 3.23 µg/ml total MPA and 57.3 ng/mL unbound MPA, based on a population pharmacokinetic/dynamic analysis [48]. Therefore, targeting IMPDH activity as a surrogate pharmacodynamic marker of MPA may provide an alternative strategy for dose individualization to improve clinical outcomes.

1.6 Conclusions

In summary, important aspects of the pharmacokinetics of MPA in HSCT patients include lower oral bioavailability and higher clearance than other populations with substantial intra-

and inter-individual variability. Also younger children require relatively higher MMF doses per body mass than older children and adults to achieve similar MPA exposures. The relationship between MPA exposures and its efficacy is still obscure and optimal targets have yet to be defined in HSCT patients, especially in pediatric patients. Current MMF dosing strategies warrant careful evaluation to ensure optimal plasma concentrations, dosing regimens, duration of therapy and upper limit of toxicity for individuals. To elucidate the advantage of MMF use in the HSCT population, multi-institutional trials including adequately powered pharmacokinetic and pharmacodynamics studies are needed to assess the effect plasma exposure of MPA on clinical outcomes in HSCT patients.

Chapter 2 Hypotheses and specific aims

2.1 Central hypothesis

Substantial intra- and inter-patient variability of MPA pharmacokinetics has been observed in both pediatric and adult patients post HSCT. We hypothesize that identification of the sources of variability in MPA pharmacokinetics and characterization of unbound MPA population pharmacokinetic parameters can provide important information for designing safe and effective MMF dosage regimens in both pediatric and adult HSCT patients.

2.2 Specific aims

2.2.1 Aim I

To develop and validate a highly sensitive and reliable UPLC-MS/MS assay for the simultaneous quantification of MPA, MPAG and AcMPAG in human plasma. The hypothesis is that a reliable assay can help to explain the reported large intra- and inter-patient pharmacokinetic variability of MPA and to better characterize the MPA therapeutic targets.

2.2.2 Aim II

To investigate the pharmacokinetic variability of MPA, from plasma protein binding and metabolism perspectives, in both pediatric and adult HSCT patients. The hypothesis is

that knowing the sources of pharmacokinetic variability of MPA can provide fundamental information to enhance our understanding of the complex pharmacokinetics of MPA.

2.2.3 Aim III

To develop a population pharmacokinetic model for unbound MPA in pediatric and adolescent HSCT patients. The hypothesis is that characterization of unbound MPA population pharmacokinetic parameters and evaluation of patient-specific covariates can help to make more informed decisions regarding appropriate dosing regimens of MMF in pediatric HSCT patients.

Chapter 3 Simultaneous quantification of mycophenolic acid and its glucuronide metabolites in human plasma by an UPLC-MS/MS assay

3.1 Abstract

Mycophenolic acid (MPA) is the active form of the immunosuppressant mycophenolate mofetil (MMF), which is used post solid organ and hematopoietic stem cell transplantations, as well as for the treatment of autoimmune diseases. MPA is metabolized primarily to phenolic MPA glucuronide (MPAG) and minorly to acyl MPA glucuronide (AcMPAG). Simultaneous monitoring of MPA and its metabolites is important to characterize the large intra- and inter-patient pharmacokinetic variability for rational individualization of the MMF dose. The aim of this study was to develop a reliable UPLC-MS/MS assay for the simultaneous monitoring of MPA, MPAG and AcMPAG in human plasma samples to support postmarketing clinical studies. Plasma proteins were precipitated with acetonitrile and the chromatographic separation was achieved on a C_{18} column with a gradient elution. The detection was performed by a triple quadrupole mass spectrometer in the positive electrospray ionization (ESI) and multiple reaction monitoring (MRM) mode. Stability of MPA and its glucuronide metabolites was also thoroughly evaluated in human blood and plasma samples under short- and long- term storage conditions. Linearity of the assay was demonstrated over the range of 20-10,000 ng/ml for MPA and MPAG, and 2-1,000 ng/ml for AcMPAG in human plasma. The assay was precise and accurate with coefficient

of variation and bias less than 15%. MPA and MPAG were stable at 25 °C up to 1 day in both heparin- and EDTA-treated blood. In heparin- and EDTA-plasma, MPA and MPAG were stable for at least 1 week at 25 °C and 4 °C, and 1 month at -20 °C. In contrast, 99% AcMPAG degraded in both heparin- and EDTA-blood as well as plasma when stored at room temperature for 1 day. All the analytes remained stable for at least 3 months in acidified EDTA-plasma at -80 °C. The assay was successfully applied to the quantification of MPA and its glucuronide metabolites in patients post hematopoietic stem cell transplantation.

3.2 Introduction

Mycophenolate mofetil (MMF, Figure 3) is used following solid organ transplantation and hematopoietic stem cell transplantation (HSCT) for prophylaxis of transplant rejection and acute graft-versus-host disease (GVHD). It is also used in the treatment of autoimmune diseases such as systemic lupus erythematosus, lupus nephritis and antineutrophil cytoplasmic antibody-associated systemic vasculitis. After oral administration, MMF is rapidly hydrolyzed to its active form mycophenolic acid (MPA, Figure 3), which reversibly inhibits inosine monophosphate dehydrogenase (IMPDH) activity by an uncompetitive mechanism and thus prevents the de novo purine biosynthesis of proliferating T and B lymphocytes [2, 65]. It is further metabolized by several uridine diphosphate glucuronosyltransferases (UGTs) in liver, gastrointestinal tract and kidneys to form primarily the pharmacologically inactive metabolite phenolic MPA glucuronide (MPAG, Figure 3), and to a smaller extent acyl MPA glucuronide (AcMPAG, Figure 3), that possesses pharmacological, toxicological and potentially pro-inflammatory activities. The

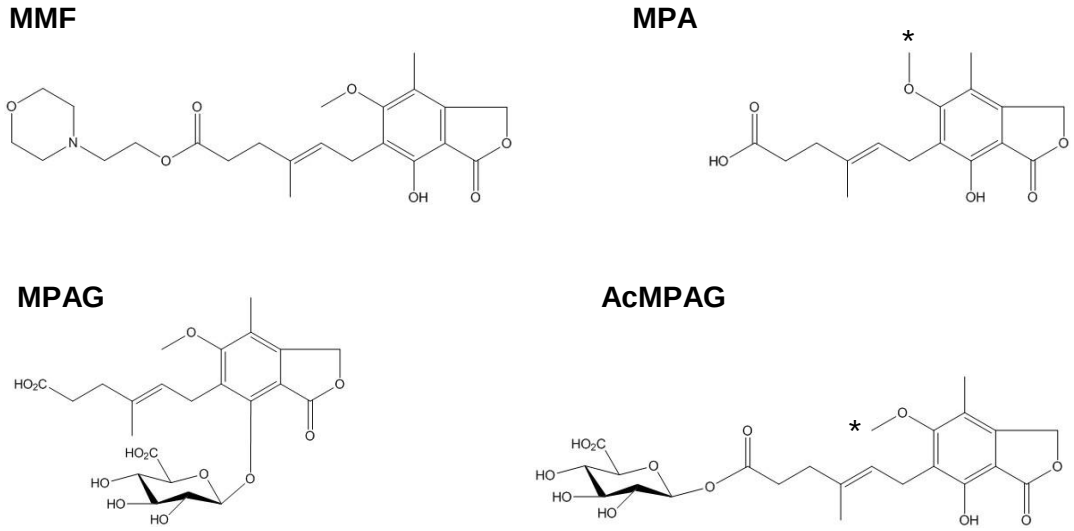


Figure 3. Chemical structures of MMF, MPA, MPAG and AcMPAG

* Deuterium labeled position for the respective internal standard

formed MPAG can be partly excreted into bile by MRP2 (multidrug resistance-associated protein), de-conjugated back to MPA by gut microflora β-glucuronidases or nonspecific esterases, and then reabsorbed back into systemic circulation during enterohepatic recycling [25, 66, 67].

Significant intra- and inter-patient variability in MPA pharmacokinetics has been observed, which could be related to different factors such as hepatic and renal function, protein binding, enterohepatic recirculation and concomitant medications. Therefore, determination of MPA and its conjugated metabolites is important to explain the reported large intra- and inter-individual pharmacokinetic variability and may offer a rational approach to optimize MMF therapy, based on this characterization.

Acyl glucuronide conjugates are chemically reactive electrophiles and have been shown, both in vivo and in vitro, to be susceptible to hydrolysis and intramolecular acyl migration in biological samples. The rates of hydrolysis and acyl migration can differ enormously among compounds and are dependent on pH, temperature and the nature of the solution (e.g., bile, blood, buffer, organic solvent, plasma, or urine) [68-70]. Such hydrolysis and intramolecular acyl migration may occur during sample handling, analytical workup and storage, which could result in an overestimation of the concentration of their parent compound. Therefore, the stability of acyl glucuronide conjugates should be thoroughly investigated at all stages of the analysis.

Several assays have been reported for the analysis of MPA and its metabolites by LC-MS/MS in different matrices [71-82]. But relatively few assays are available for

simultaneous quantification of MPA and its glucuronide metabolites with sufficient sensitivity for AcMPAG, which has a relatively low abundance in comparison to MPAG. Moreover, no detailed stabilization procedures are reported in these studies, which may yield inaccurate measurements of MPA as well as its glucuronides. To address this critical deficiency, we developed a high-throughput UPLC-MS/MS assay for simultaneous quantification of MPA, MPAG and AcMPAG in human plasma. In addition, detailed stability studies were conducted under various collection and storage conditions over short- and long-time intervals. The assay was successfully applied to quantify MPA and its glucuronide metabolites in patients post HSCT. Novel merits of this assay include the use of small sample volumes (50 µl human plasma) and dual stable isotopically labeled internal standards of the analytes (MPA-d_3 and MPAG-d_3) for reliable compensation of matrix effects of all analytes.

3.3 Materials and methods

3.3.1 Chemicals and reagents

MPA (98% purity), MPAG (96.4% purity), MPA-d_3 and MPAG-d_3 were purchased from Toronto Research Chemicals (North York, ON, Canada). AcMPAG (98.2% purity) was purchased from TLC PharmaChem (Vaughan, ON, Canada). Water, methanol (MeOH) and acetonitrile (ACN) of LC-MS grade were purchased from Merck EMD Millipore (Billerica, MA, USA). Heparin- and EDTA-treated human blood (blood collected in tubes treated with heparin and EDTA, respectively) as well as heparin- and EDTA-human

plasma (plasma collected from heparin- and EDTA-human blood, respectively) were purchased from Innovative Research (Novi, MI, USA).

3.3.2 UPLC-MS/MS conditions

The chromatographic analysis was performed on a Waters Acquity UPLC H-Class system with a flow-through-needle sample manager. The separation was achieved on a Waters Acquity UPLC BEH C_{18} column (2.1 X 50 mm, 300 Å, 1.7 µm, Waters, Milford, MA, USA). Mobile phase A (0.1% formic acid in water [v/v]) and mobile phase B (0.1% formic acid in MeOH [v/v]) were operated with a gradient elution at a flow rate of 0.5 ml/min as follows: 65% A→ 55% A (0–2.5 min), 55% A→20% A (2.5–4.0 min), 20% A→65% A (4.0–4.2 min), 65% A (4.2–5.0 min). The column temperature was set at 40 °C and the auto-sampler temperature was maintained at 10 °C. The total duration of each chromatographic run was 5.0 min. MPA-d_3 (1 µg/ml) was used as internal standard for the quantification of MPA and MPAG-d_3 (5 µg/ml) was used for the quantification of MPAG and AcMPAG.

The mass spectrometric analysis was performed with a QTRAP® 5500 system (AB SCIEX, Framingham, MA, USA) in the positive electrospray ionization (ESI) and multiple reaction monitoring (MRM) mode. The MRM transitions monitored were m/z 321.1→207.0 for MPA and 519.1→343.2 for MPAG and AcMPAG. The compound-dependent parameters are shown in Table 1. The ion source-dependent parameters used in the QTRAP 5500 system were: IonSpray voltage of 5500 V, temperature of 350 °C, high collision gas, curtain gas of 30 Psig, ion source gas 1 of 50 Psig, ion source gas 2 of 60 Psig.

Table 1 Compound-dependent parameters in MRM

	Precursor ion (m/z)	**Product ion (m/z)**	**CE (volts)**	**DP (volts)**	**EP (volts)**	**CXP (volts)**
MPA	321.1	207.0	25.0	60	10	15
MPAG	519.1	343.2	27.0	20	10	15
AcMPAG	519.1	343.2	22.0	10	10	15
MPA-d_3	324.1	210.0	25.0	60	10	15
MPAG-d_3	522.1	346.2	25.0	20	10	15

CE: collision energy; DP: declustering potential; EP: entrance potential; CXP: collision cell exit potential

3.3.3 Preparation of standard solutions and quality controls

Stock solutions of all analytes and internal standards (1 mg/ml after adjustment for percent purity) were prepared in MeOH and stored at -80 °C. Working solutions were freshly prepared at 0.2, 0.4, 1, 5, 20, 50, 90, 100 µg/ml for MPA and MPAG and 0.02, 0.04, 0.1, 0.5, 2, 5, 9, 10 µg/ml for AcMPAG in ACN-water (30:70, v/v) with 0.1% formic acid. Calibration standards were freshly prepared by mixing 5 µl of the appropriate working solution with 50 µl of pooled EDTA-plasma pre-acidified with 1.0 N HCl (HCl:Plasma, 1:10 [v:v]) to give final concentrations of 20, 40, 100, 500, 2000, 5000, 9000, 10,000 ng/ml for MPA and MPAG, and 2, 4, 10, 50, 200, 500, 900, 1000 ng/ml for AcMPAG. LLOQ (20 ng/ml for MPA and MPAG, 2 ng/ml for AcMPAG) and quality control (QC) samples, denoted as LQC (60 ng/ml for MPA and MPAG, 6 ng/ml for AcMPAG), MQC (700 ng/ml for MPA and MPAG, 70 ng/ml for AcMPAG) and HQC (8000 ng/ml for MPA and MPAG, 800 ng/ml for AcMPAG), were prepared in acidified EDTA-plasma to evaluate assay accuracy and precision.

3.3.4 Sample preparation

To 50 µl of each calibration standard, QC sample or subject sample, 5 µl of IS working solution (mixture of 1 µg/ml MPA-d_3 and 5 µg/ml MPAG-d_3) was added. Plasma proteins were precipitated with 200 µl of ACN. After vigorous mixing and centrifugation (15 min at 18,000 × g), 15 µl of supernatant was transferred into pre-labeled tubes and diluted with 85 µl of ACN-water (30:70, v/v) with 0.1% formic acid. After centrifugation (15 min at 18,000 × g), 5 µl was injected into the UPLC-MS/MS system.

3.3.5 Method validation

The method was validated in terms of linearity, recovery, matrix effects, accuracy, precision, freeze and thaw stability, bench-top stability, processed sample stability and long-term stability according to FDA Guidance for Industry-Bioanalytical Method Validation [83]. Calibration curves ranging from 20-10,000 ng/ml for MPA and MPAG, and 2-1000 ng/ml for AcMPAG were constructed using peak area ratios of each analyte to its corresponding internal standard versus the nominal analyte concentrations. A weighted ($1/x^2$) least-squares linear regression analysis was used to obtain the slope, intercept and correlation coefficient. Accuracy and precision were evaluated by analyzing six QC samples at each concentration level on three separate days from freshly prepared calibration standards. Recovery was calculated by comparing peak areas from the extracted plasma samples of MPA, MPAG and AcMPAG with those containing the same amounts of analytes, which were spiked into the extracted blank matrix. Matrix effects were calculated by comparing peak areas of each analyte spiked into the extracted blank matrix with those in matrix-free neat solution at the same concentrations. Freeze and thaw (3-cycle) stability, bench-top stability (in an ice bath or at 25 °C for 6 h), processed sample stability (in the auto-sampler, at 10 °C for 72 h) and long-term stability (at -80 °C for 3 months) were also assessed in QC samples prepared in acidified EDTA-plasma.

3.3.6 Stability of MPA, MPAG and AcMPAG under various collection and storage conditions

Fresh human blood collected in heparin- or EDTA-treated tubes, was spiked with MPA, MPAG and AcMPAG, respectively, to give final concentrations of 5, 25 and 5 µg/ml, respectively. Aliquots (100 µl) of each analyte were stored at room temperature for the evaluation. At each time point (6 h and 1 day of storage), three aliquots of each analyte were taken and centrifuged at 1,860 g for 10 min to collect plasma before analysis. The separated plasma was processed by the same procedure described in section 3.3.4. Similarly, MPA, MPAG and AcMPAG were spiked into heparin or EDTA-plasma, to give final concentrations of 10, 50 and 5 µg/ml, respectively. Aliquots of 50 µl were stored at 25 °C, 4 °C and -20 °C, respectively. For each analyte, three aliquots from each condition were taken for analysis over short- and long-time intervals (up to 7 days at 25 °C and 4 °C, and 1 month at -20 °C).

3.3.7 Application

The method was applied to quantify MPA and its glucuronide metabolites in an adult patient post HSCT (20 years of age, 38.8 kg body weight) receiving an MMF steady-state maintenance dose of 1,000 mg twice daily. The plasma samples were supplied by Indiana University. Briefly, blood samples were centrifuged at 1,860 g for 15 min at 4 °C after collection and the separated plasma samples were stored at -80 °C prior to analysis. Plasma samples were processed by the same procedure described in section 3.3.4 for

analysis. The LQC, MQC and HQC samples were also analyzed in duplicate along with clinical samples.

3.4 Results

3.4.1 Method development

The precursor and product ion spectra of MPA and its glucuronide metabolites are shown in Figure 4. In positive ESI mode, the most abundant precursor and product ions for MPA were 321.1 and 207.0, respectively. Sodium adducts of MPAG and AcMPAG were selected as precursor ions due to their high abundancy. The most abundant fragment ions at m/z 343.2, $[M+Na-176]^+$, were detected and thus selected as product ions. The neutral loss of 176 Da corresponded to a monodehydrated glucuronic acid.

Methanol was used as the mobile phase constituent because it provided a better chromatographic resolution than ACN did. As shown in Figure 5A, simple protein precipitation provided relatively clean chromatograms with no interfering peaks at the retention time of each analyte. Representative chromatograms of a plasma sample from an adult patient post HSCT are shown in Figure 5B. The retention time was 3.8 min for MPA, 1.4 min for MPAG and 2.7 min for AcMPAG.

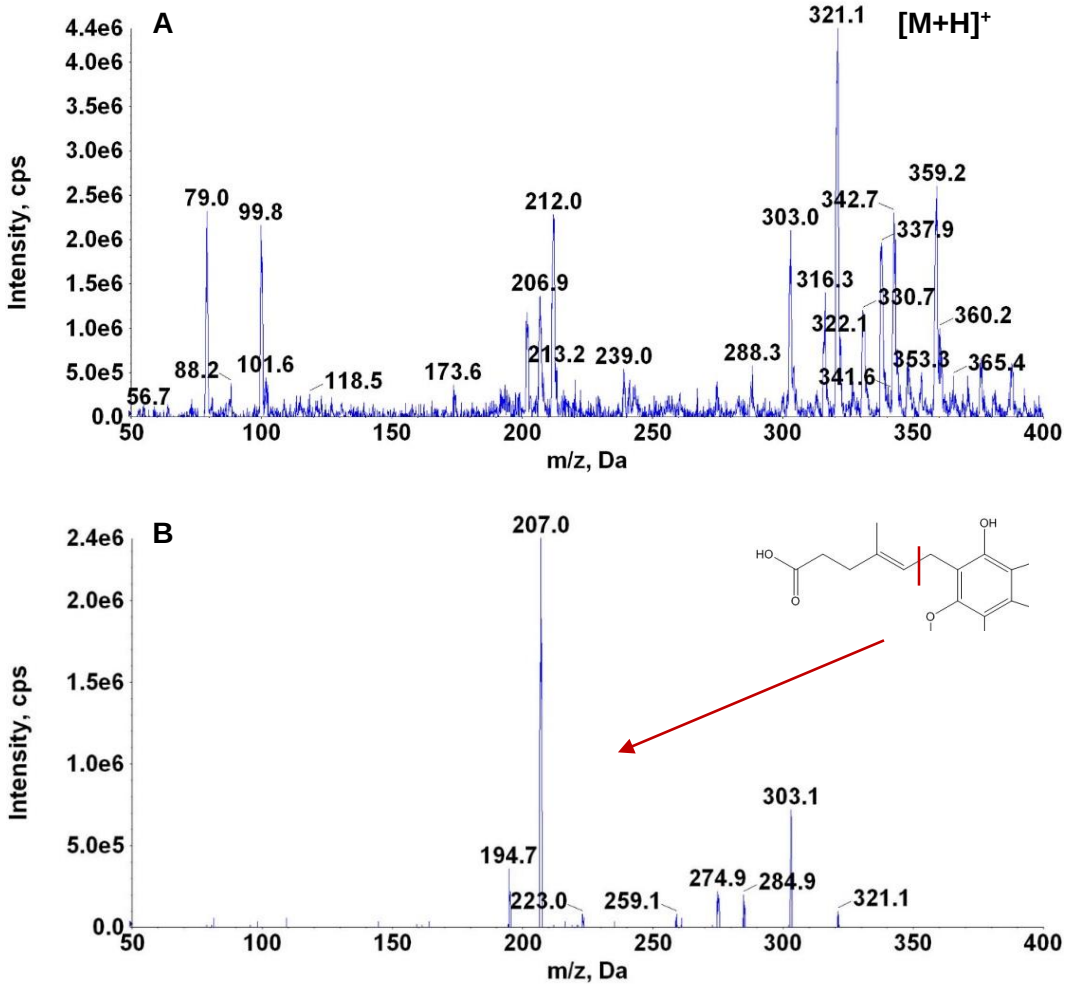
3.4.2 Method validation

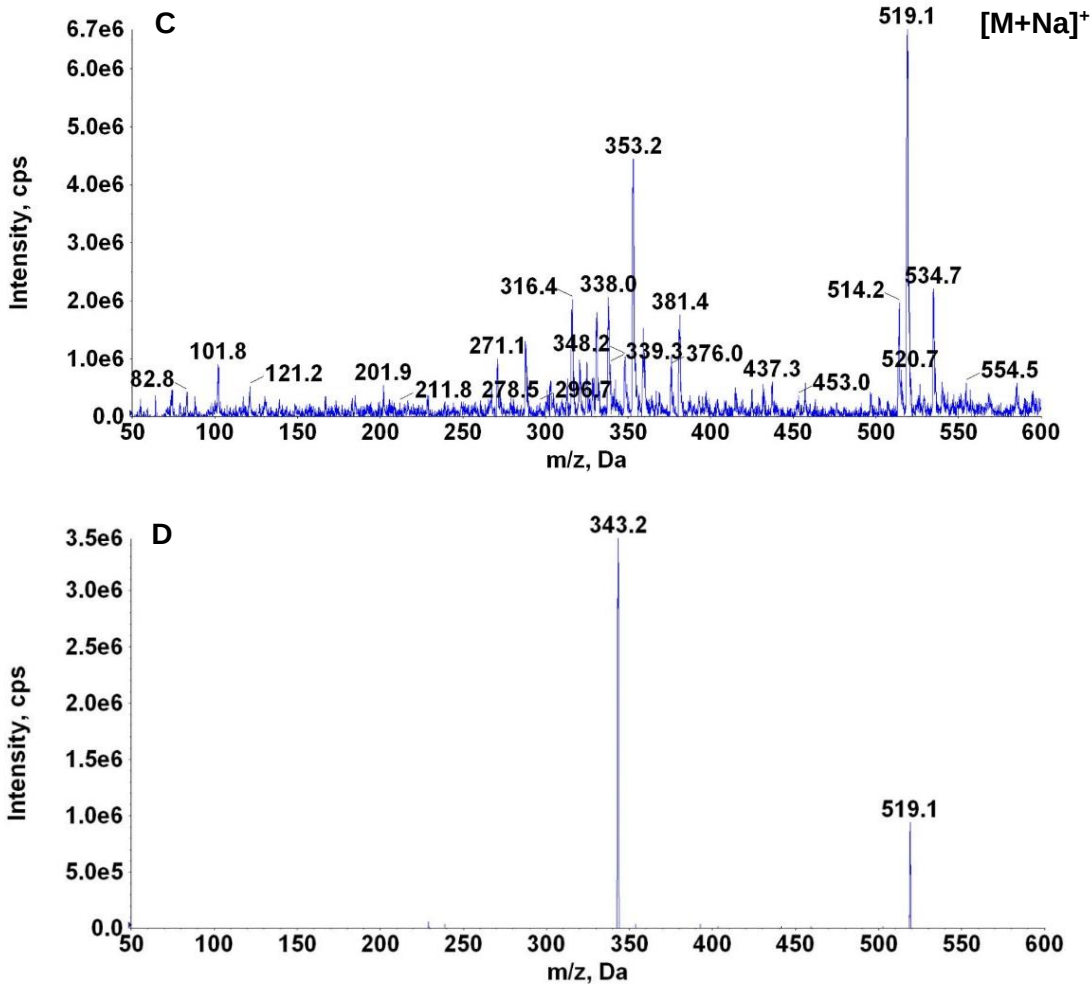
Calibration curves for all analytes (MPA, MPAG and AcMPAG) were generated from a weighted ($1/x^2$) least-squares linear regression analysis. Excellent linearity was observed

(r≥0.99) over the range of 20-10,000 ng/ml for MPA and MPAG, and 2-1000 ng/ml for AcMPAG. The percent recovery values of MPA and its glucuronide metabolites from plasma were above 85%. The matrix effects were within 15%.

The intra-day and inter-day accuracy and precision were established from analyzing six QC samples at different concentration levels on three separate days, and summarized in Table 2. The intra-day accuracy was 85.0-99.0% for MPA, 93.7-103.5% for MPAG and 93.6-98.6% for AcMPAG. The intra-day precision was 1.8-7.1% for MPA, 1.4-5.8% for MPAG and 2.4-8.0% for AcMPAG. The inter-day accuracy was 91.0-99.2% for MPA, 91.2-97.0% for MPAG and 95.7-103.6% for AcMPAG. The inter-day precision was 4.6-9.9% for MPA, 3.0-8.8% for MPAG and 4.7-11.3% for AcMPAG.

Stability of MPA and its glucuronide metabolites was also thoroughly assessed in QC samples prepared in acidified EDTA-plasma. The concentrations of all three analytes showed no significant changes after three cycles of repeated freeze and thaw. Short-term storage of acidified plasma samples at room temperature or in an ice bath did not compromise the integrity of the samples. The analytes in the injection solution were stable for at least 72 h in the auto-sampler at 10 °C. Long-term stability demonstrated that acidified QC samples were stable for at least 3 months at -80 °C. The detailed results for stability experiments are presented in Table 3.


A
[M+H]+
Intensity, cps
m/z, Da
321.1
359.2
79.0
99.8
212.0
342.7
303.0
337.9
316.3
206.9
330.7
322.1
360.2
213.2
239.0
288.3
88.2
101.6
118.5
173.6
353.3
365.4
341.6
56.7
B
207.0
HO
OH
O
O
303.1
194.7
274.9
284.9
223.0
259.1
321.1


C
[M+Na]+
519.1
353.2
316.4
338.0
381.4
514.2
534.7
271.1
348.2
339.3
376.0
437.3
520.7
554.5
101.8
121.2
201.9
211.8
278.5
296.7
453.0
82.8
Intensity, cps
m/z, Da
D
343.2
519.1
Intensity, cps
m/z, Da

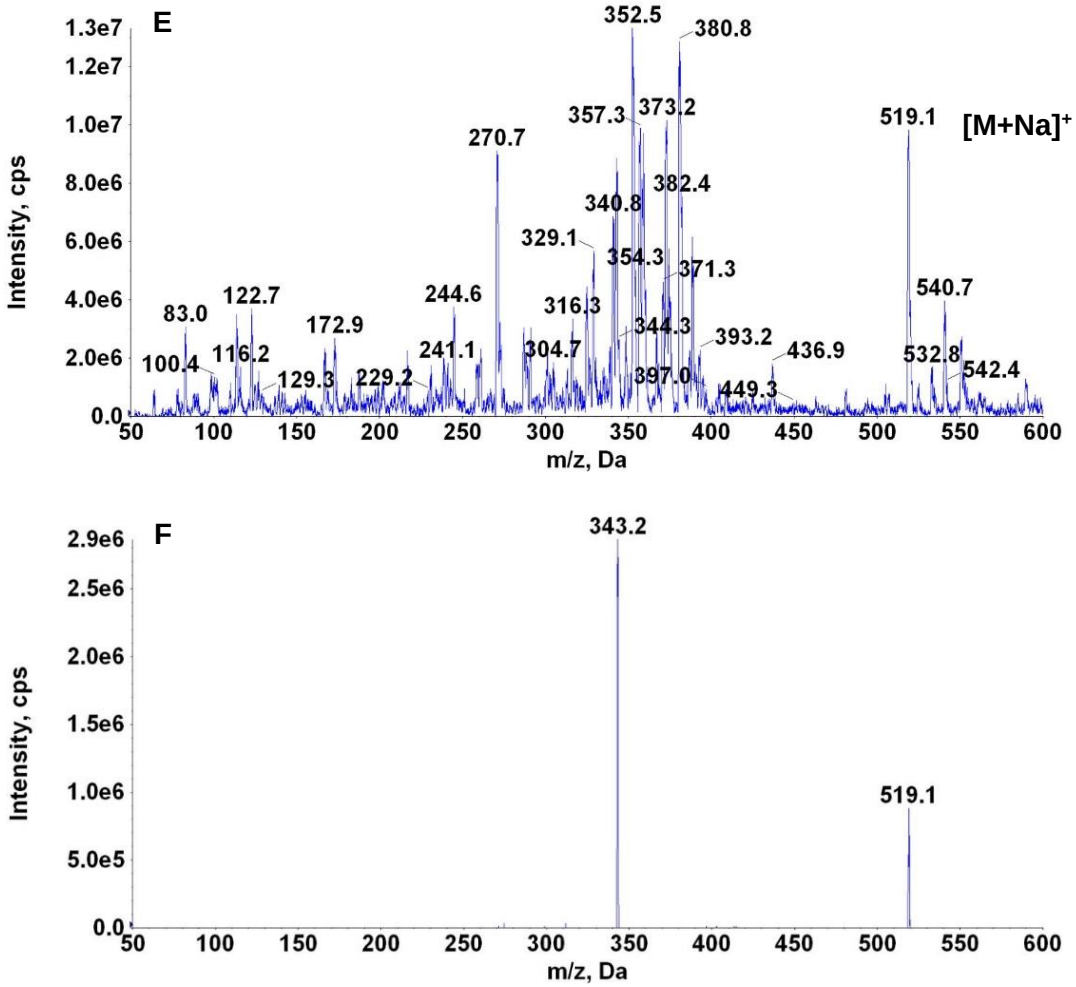


Figure 4. Precursor and product ion mass spectra: (A) precursor ion spectra of MPA, (B) product ion spectra of MPA, (C) precursor ion spectra of MPAG, (D) product ion spectra of MPAG, (E) precursor ion spectra of AcMPAG, (F) product ion spectra of AcMPAG

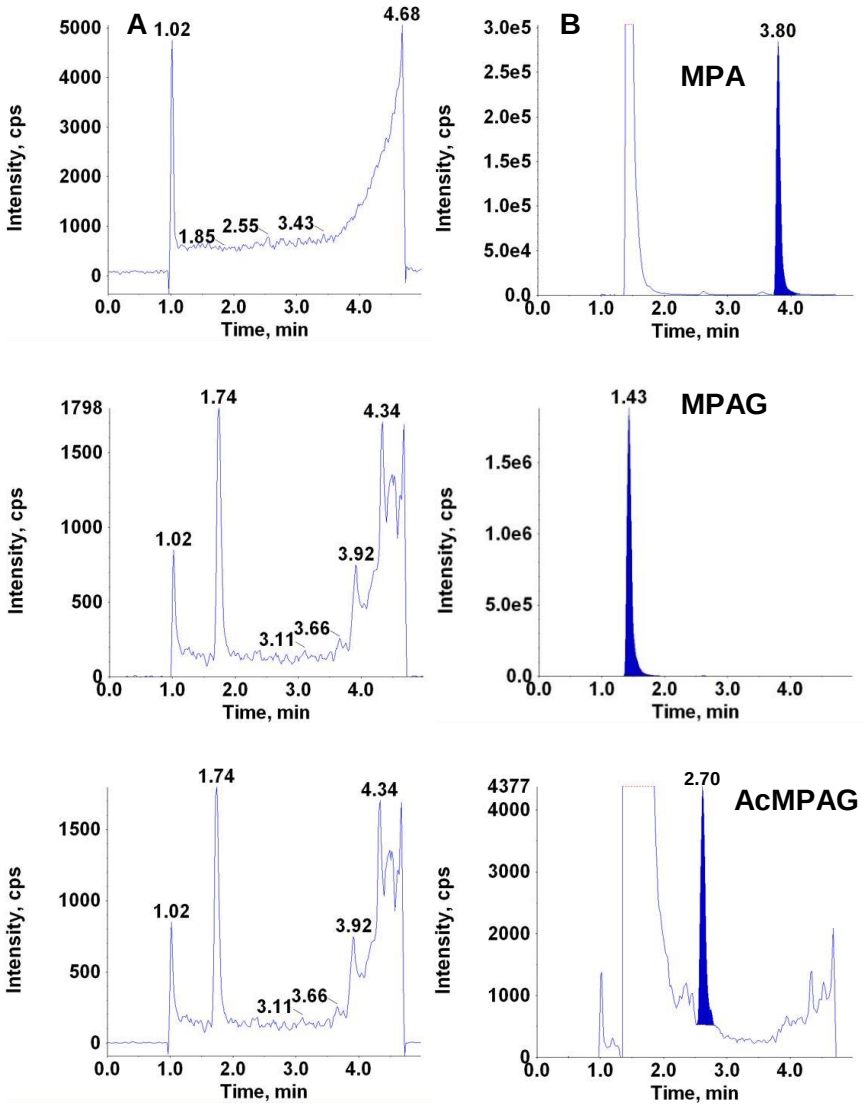


Figure 5. Representative chromatograms: (A) double blank, (B) a patient sample with measured concentration of MPA, MPAG and AcMPAG 2.5 µg/ml, 89.8 µg/ml and 13.0 ng/ml, respectively

Table 2 Intra-day (n=6) and inter-day (n=18) accuracy and precision for MPA, MPAG and AcMPAG in acidified EDTA-treated human plasma

		Intra-day run (n=6)			Inter-day run (n=18)		
	Normal concentration (ng/ml)	Measured concentration (mean ± SD)	Accuracy (%)	Precision (CV %)	Measured concentration (mean ± SD)	Accuracy (%)	Precision (CV %)
MPA	20	17.0 ± 1.2	85.0	7.1	18.2 ± 1.8	91.0	9.9
	60	56.7 ± 1.0	94.5	1.8	59.5 ± 5.1	99.2	8.6
	700	693.3 ± 13.2	99.0	1.9	693.6 ± 56.7	99.1	8.2
	8000	7188.3 ± 155.0	89.8	2.2	7348.4 ± 336.8	91.8	4.6
MPAG	20	20.7 ± 1.2	103.5	5.8	19.4 ± 1.7	97.0	8.8
	60	56.4 ± 2.2	94.1	3.9	55.6 ± 1.7	92.7	3.1
	700	674.0 ± 20.2	96.3	3.0	647.6 ± 29.4	92.5	4.5
	8000	7498.3 ± 104.8	93.7	1.4	7297.8 ± 219.8	91.2	3.0
AcMPAG	2	1.9 ± 0.2	96.8	8.0	2.1 ± 0.2	103.6	11.3
	6	5.6 ± 0.2	93.6	4.4	5.8 ± 0.3	97.5	5.2
	70	67.6 ± 4.1	96.5	6.1	67.0 ± 4.1	95.7	6.0
	800	788.7 ± 19.1	98.6	2.4	781.4 ± 36.4	97.7	4.7

Table 3 Freeze and thaw stability, bench-top stability, processed sample stability and long-term stability for MPA, MPAG and AcMPAG in acidified EDTA-treated human plasma

			MPA			MPAG			AcMPAG		
	Temp.	Time	Nominal concentration (ng/ml)			Nominal concentration (ng/ml)			Nominal concentration (ng/ml)		
			60	700	8000	60	700	8000	6	70	800
Freeze and thaw stability		0	100	100	100	100	100	100	100	100	100
		Cycle 3	108.7	111.5	102.2	99.3	107.7	104.0	104.0	107.0	106.4
Bench-top stability	25°C	0	100	100	100	100	100	100	100	100	100
		6 h	100.7	105.3	99.2	94.0	105.7	104.4	96.7	104.0	105.9
	Ice bath	6 h	102.3	105.3	101.0	100.2	104.4	101.0	94.5	99.0	101.4
Processed sample stability	10°C	0	100	100	100	100	100	100	100	100	100
		72 h	89.3	90.7	87.5	86.3	96.1	89.5	89.3	90.7	87.5
Long-term stability	-80°C	0	100	100	100	100	100	100	100	100	100
		3 months	107.7	112.0	93.7	104.3	107.3	107.0	94.7	111.3	107.3

3.4.3 Stability of MPA, MPAG and AcMPAG under various collection and storage conditions

At room temperature, MPA and MPAG were stable up to 1 day in human blood collected in heparin or EDTA-treated tubes (Figures 6A and B). AcMPAG was unstable with degradation of 50% by 6 h and 99% on day 1 at room temperature (Figure 6C). In heparin- or EDTA-plasma, MPA and MPAG were stable for at least 1 week at 25 °C and 4 °C, and 1 month at -20 °C (Figures 7A, B, C and D). However, near to 50% of AcMPAG degraded at 6 h and 99% degraded on day 1 when stored at 25 °C (Figures 7E and F). This trend for AcMPAG stability in plasma was similar to that in heparin- and EDTA-blood stored at room temperature (Figure 6C). When plasma samples pre-spiked with AcMPAG were stored at 4 °C, the rate of degradation significantly decreased. Near to 80% of AcMPAG degraded in heparin-plasma on day 7, compared to EDTA-plasma (20%), which suggested that EDTA is a preferred anticoagulant for sample collection. In contrast, AcMPAG was stable up to 1 month in heparin- or EDTA plasma when stored at -20 °C.

3.4.4 Application

The developed method was used to monitor MPA and its glucuronide metabolites in an adult patient post HSCT. The chromatograms of MPA, MPAG and AcMPAG at 3 h post oral administration of MMF are shown in Figure 5B. The measured concentrations of MPA, MPAG and AcMPAG were 2.5 µg/ml, 89.8 µg/ml and 13.0 ng/ml, respectively. The method was specific and sensitive enough to simultaneously monitor all the three analytes.

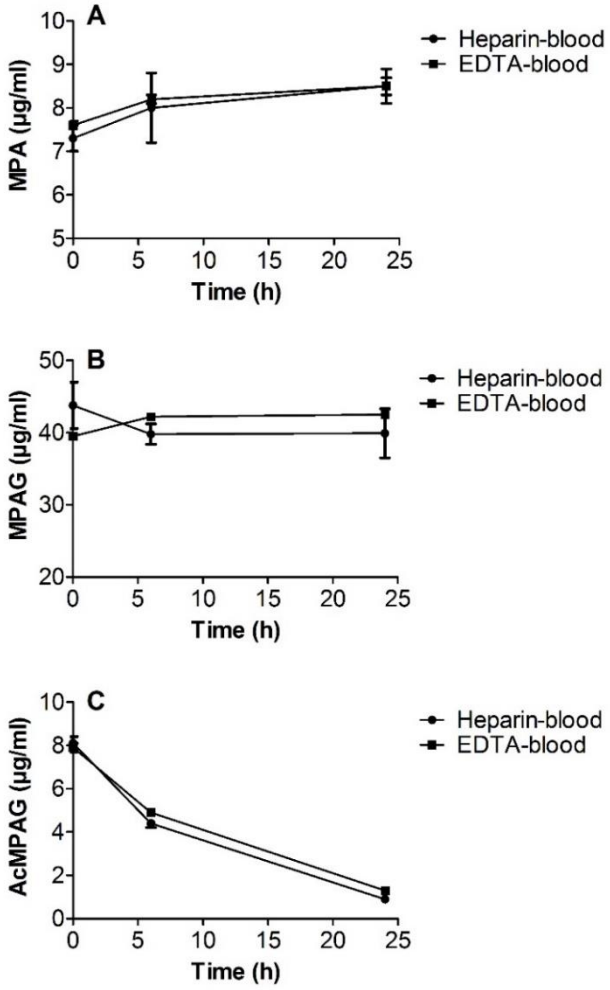


Figure 6. Stability of (A) MPA, (B) MPAG and (C) AcMPAG in heparin- and EDTA-blood stored for up to 24 h at 25 °C

Data are displayed as mean and SD (n=3)

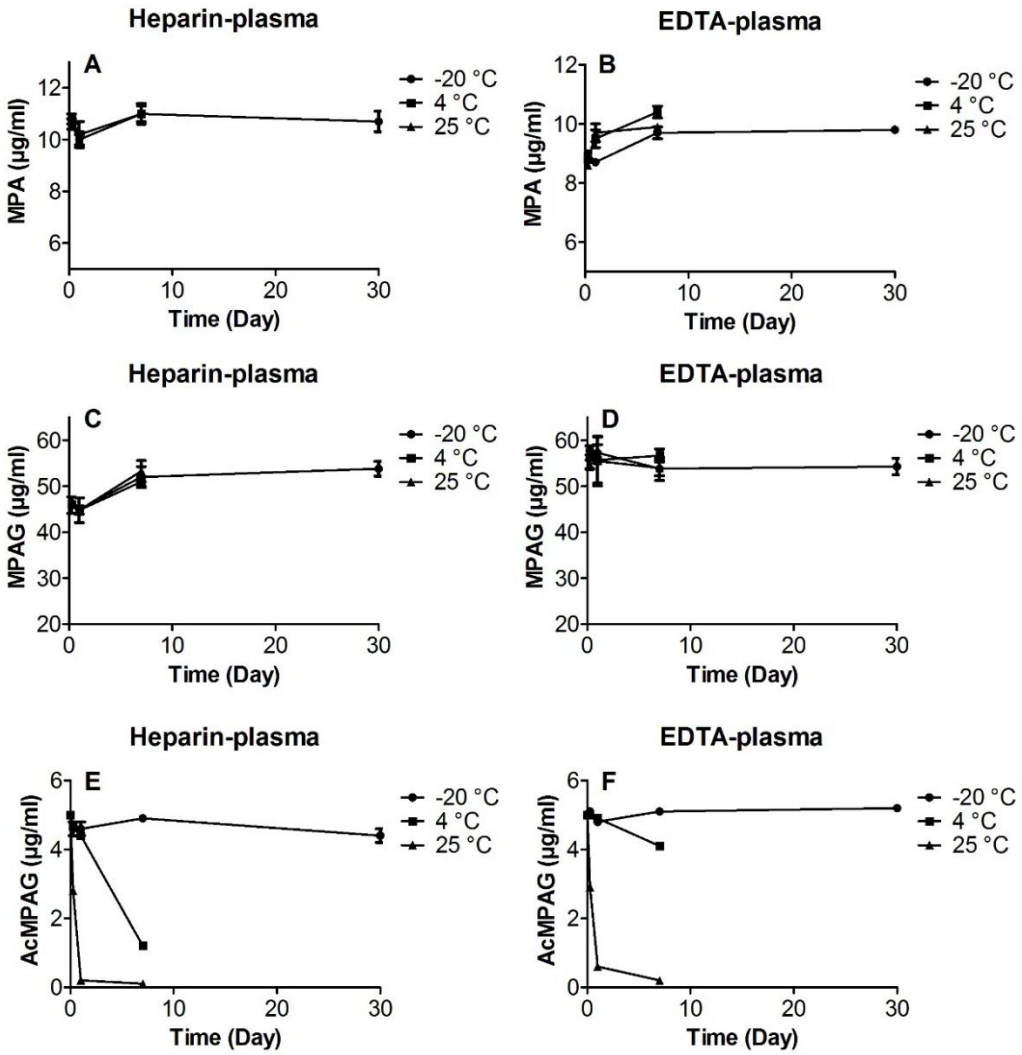


Figure 7. Stability of MPA in (A) heparin- and (B) EDTA-plasma, MPAG in (C) heparin- and (D) EDTA-plasma, AcMPAG in (E) heparin- and (F) EDTA-plasma, stored at 4 and 25 °C for up to 1 week, and at -20 °C for up to 1 month. Samples at 4 °C and 25 °C were discontinued at 1 week

Data are displayed as mean and SD (n=3)

3.5 Discussion

Efficient ionization is needed before analytes enter into the mass spectrometer. ESI is the preferred choice for the analysis of glucuronide conjugates due to its soft ionization, which can minimize in-source fragmentation of the relatively weak glucuronide ether or ester bonds. Generally, negative ESI mode can also be used due to the presence of the acidic carboxylic functional group. However, the negative ionization is often less sensitive than the positive one. Moreover, fragmentation mechanisms in negative mode are hard to predict and understand. Therefore, positive ionization mode was used to develop methods for quantification of glucuronides in this study.

The glucuronides are susceptible to interferences from the co-eluting matrix components due to their polar and hydrophilic nature. Therefore, the isotope labeled analog MPAG-d_3 was used as internal standard to thoroughly compensate the matrix effects for MPAG and AcMPAG.

Glucuronides are often unstable in the ion source since high temperatures could cause the cleavage of the glucuronides and formation of its parent drug. Because of this potential deconjugation in the ion source, chromatographic separation is necessary to ensure the accurate quantification of both parent drug and its glucuronide conjugates. The unavoidable in-source fragmentations of MPAG and AcMPAG were shown by signal of m/z 321.1→207.0 trace within their respective peak, attributing to the cleavage of MPA glucuronides and the formation of MPA. With a gradient elution, a good chromatographic separation of conjugates from their parent molecule was achieved and thus no

chromatographic interferences between analytes and no false over-estimation of MPA from the deconjugation in MS ion source (no contribution of MPAG or AcMPAG to the MPA peak area). Therefore, quantification of the analytes was not affected by the in-source fragmentations.

Acyl glucuronides are known to have a limited stability under neutral or slightly alkaline conditions. Either a hydrolysis or an acyl migration can occur [68, 84-86]. Hydrolysis leads to the regeneration of the pharmacologically active parent drug. Therefore, in vitro degradation of AcMPAG during sample handling and storage could compromise the accurate quantification of both AcMPAG and MPA. Our method was validated in acidified EDTA-plasma. However, acidification may not be feasible immediately after the sample collection in clinical settings. To thoroughly investigate the stability of MPA and its glucuronide metabolites, we spiked each analyte into heparin- or EDTA-blood as well as plasma. Sample stability was then assessed under different storage conditions over short- and long-time intervals. MPA and MPAG were stable under all conditions studied. In both heparin- and EDTA-blood as well as plasma, AcMPAG was unstable with degradation of 50% by 6 h and 99% on day 1 at room temperature, consistent with that reported in literature with 12% decrease within 15 min in human whole blood [76]. Therefore, storage of whole blood samples or non-acidified plasma samples at room temperature would compromise the accurate quantification with an over-estimation of MPA concentration and under-estimation of AcMPAG concentration. Our results are in agreement with previous findings by de Loor et al [86]. When the plasma samples were acidified to contain 10% of 1.0N HCl, no significant AcMPAG degradation was observed (versus 50% degradation in

non-acidified plasma samples) after 6 h at room temperature, which is of importance with respect to the handling and assay procedures. The acidified samples remained stable up to 3 months at -80 °C (Table 3). Our results reconfirm the finding that sample acidification is effective in stabilizing the sample for AcMPAG quantification reported by Brandhorst et al [75]. Therefore, blood samples should be centrifuged immediately to collect plasma post sampling, acidified and stored at -20 °C or lower, in order to minimize AcMPAG hydrolysis and acyl migration to ensure the accurate collection of pharmacokinetic and toxicological data.

3.6 Conclusions

The UPLC-MS/MS assay for simultaneous quantification of MPA and its glucuronide metabolites (MPAG and AcMPAG) was developed and fully validated in human plasma samples per US FDA Guideline. Furthermore, detailed stability studies were conducted under various collection and storage conditions over short- and long-time intervals. Extended stabilization procedures are suggested to improve the accuracy of the analysis before routine application. The merits of this assay include the efficient sample clean up by protein precipitation, high sensitivity with LLOQ of 2 ng/ml for AcMPAG, small sample volume of 50 µl for processing, and short chromatographic run time of 5 min. The method can be easily extended with slight modifications to simultaneously monitor MPA and its glucuronide metabolites in extracts of blood, ultrafiltrate and dried blood spot (DBS) samples for clinical and pharmacokinetic evaluations.

Chapter 4 Pharmacokinetic variability of mycophenolic acid in pediatric and adult patients with hematopoietic stem cell transplantation

4.1 Abstract

The aim of this study was to evaluate the pharmacokinetic variations of mycophenolic acid (MPA), the active metabolite of mycophenolate mofetil (MMF), in both pediatric and adult patients following hematopoietic stem cell transplantation (HSCT). Twenty pediatric patients with a median age of 3 years (range, 0.2-12 years) and thirteen adult patients with a median age of 54 years (range, 18-63 years) were enrolled. Blood samples were collected on days 0, 7, 14, 21 and 30 after allogeneic HSCT. Total and free (unbound) MPA, as well as MPAG were quantified using a validated LC-MS/MS assay. The plasma protein binding of MPA and MPAG did not change significantly in pediatric patients over the one month sampling period post HSCT. However, it increased in adult patients from day 7 to day 30 post HSCT, from 97.3±0.8% to 98.3±0.6% for MPA ($P < 0.05$), and 74.6±9.4% to 82.9±8.1% for MPAG ($P < 0.05$). The plasma protein binding of MPA was significantly higher in males compared to females in both pediatric (98.3±1.1 vs 97.4±1.1%) and adult (98.1±0.7 vs 97.4±1.2%) patients ($P < 0.05$). The MPAG/MPA ratios on an mg/kg dose basis in adult patients were significantly higher than those in pediatric patients (4.3±3.4 vs 2.4±2.6; $P < 0.05$). Time-dependent plasma protein binding and age-related differences in MPA metabolism, at least in part, impact the reported large intra- and inter-

individual variability in MPA pharmacokinetics. These patient and pharmacologic factors, if incorporating into MMF regimen design and modification, may contribute to the rational dose selection of MMF in HSCT patients.

4.2 Introduction

Mycophenolate mofetil (MMF) is an immunosuppressive drug approved by the FDA in 1995 to prevent acute rejection in renal allograft recipients. Besides in solid organ transplantation, MMF is increasingly used in the prevention and treatment of acute and chronic graft-versus-host disease (GVHD) post allogeneic hematopoietic stem cell transplantation (HSCT) [5]. MMF itself is biologically inactive and must be metabolized by carboxylesterases to mycophenolic acid (MPA), which is a potent, reversible, uncompetitive inhibitor of the rate-limiting enzyme inosine monophosphate dehydrogenase (IMPDH) in the de novo purine biosynthesis. Inhibition of IMPDH blocks the T- and B-lymphocyte proliferations, and reduces the antibody production and the generation of cytotoxic T lymphocytes, consequently contributing to the prevention of allograft rejection and treatment of ongoing rejection [87, 88].

MPA metabolism occurs primarily in the liver but also to some extent in the intestine and kidney [25]. A major fraction is converted to the inactive 7-O-glucuronide (MPAG) and a minor fraction is converted to the active acyl glucuronide (AcMPAG). UGT1A9, 1A8, 1A1, 1A7 and 1A10 produce MPAG in significant amounts, with UGT 1A9 being the most active isoform. UGT 2B7 is the only isoform producing AcMPAG in a significant amount [14, 89]. UGT1A8 expressed in the kidney and throughout the GI tract, and UGT1A9 expressed in

the liver, intestine and kidney, are believed to be the major isoforms involved in MPA glucuronidation [43, 90]. MPAG is mainly excreted in urine via active tubular secretion and glomerular filtration. It could be partly excreted into the bile by Mrp2 (multidrug resistance-associated protein), de-conjugated back to MPA by the gut microflora β-glucuronidases, and then reabsorbed into the portal circulation, characterized as enterohepatic circulation (EHC). In humans, the mean contribution of EHC to the overall AUC of MPA is 37% (ranging from 10 to 61%) [10].

MPA extensively binds to human serum albumin and has a free fraction of <3% in patients with normal renal and liver function. Only unbound MPA is capable of inhibiting IMPDH in vitro and in vivo [66, 91]. Changes in albumin levels may potentially change activity or toxicity. MPAG also displays high serum albumin binding (82%) in stable patients. Therefore, competition for albumin binding between MPA and MPAG may exist. AcMPAG forms an irreversible covalent bound with albumin, which makes the measurement of the free fraction technically challenging [92].

Many centers use standard MMF dose (1,000 mg, q12h) for adult HSCT patients and 15 mg/kg q8h for pediatric HSCT patients. However, the pharmacokinetics of MPA and the relationships between dose, plasma concentration and exposure are poorly understood in HSCT patients, especially in pediatric HSCT patients [4]. The standard dose (2000 mg/day) in adult HSCT patients achieves significantly lower MPA exposure compared with renal transplant patients [28]. Increased dose to 3000 mg/day with cyclosporine still fails to achieve therapeutic plasma exposure in many adult HSCT patients [16]. The physiologic differences between kidney and HSCT recipients including renal function, pre-HSCT

chemotherapy effects, prophylactic antibiotic use and higher severity of illness, may affect MPA disposition. In pediatric HSCT patients, a 15 mg/kg MMF dose every 12 hours intravenously with cyclosporine achieves significantly lower total and unbound MPA exposure than pediatric renal transplant recipients receiving 600 mg/m^2 every 12 hours. Although q8h dosing improves exposure, it does not consistently obtain MPA plasma exposure similar to that in adults [30]. Another study demonstrates that MMF administration of 900 mg/m^2 q6h in combination with tacrolimus achieves similar MPA plasma exposure to that obtained in adults [37]. Therefore, despite the increased use of MMF, the optimal dose has not been clearly established in both pediatric and adult HSCT patients.

A number of variables could affect MPA pharmacokinetics, including renal and hepatic function, albumin concentration, magnitude of EHC, concomitant immunosuppressive therapy, and genetic polymorphisms in drug metabolizing enzymes and transporters. Because of the complex pharmacokinetics of MPA, high intra- and inter-patient pharmacokinetic variability of MPA has been observed in organ transplant patients, childhood-onset systemic lupus erythematosus patients and HSCT patients. MPA exposure could vary more than 10-fold between patients, leading to a significant therapeutic challenge [17, 28, 64, 93-96]. This study was conducted to gain insights into the pharmacokinetic variability of MPA, from plasma protein binding and metabolic perspectives, in both pediatric and adult HSCT patients. Identifying the patient and pharmacologic characteristics that significantly affect MPA pharmacokinetics would allow for more rational decisions on MMF dosing in both pediatric and adult HSCT patients.

4.3 Methods

4.3.1 Study subjects

This study was conducted as an open-label and inpatient/outpatient clinical study in HSCT patients. The main objective was to evaluate the intra- and inter-patient variability of MPA in pediatric and adult patients post HSCT. Twenty pediatric patients with a median age of 3 years (range: 0.2 to 12 years) and thirteen adult patients with a median age of 54 years (range: 18-63 years), undergoing HSCT from both related and unrelated donors, were enrolled at Indiana University Hospital and Riley Hospital for Children (Table 4). All adult patients and nearly half of pediatric patients (9/20) were diagnosed with malignancies. The study was approved by the Institutional Review Boards of participating centers (IRB # 1111007321). Informed consent was obtained from each patient (or parent/guardian for pediatric patients) and assent was obtained from children who are at least 7 years of age before enrollment.

4.3.2 Study protocol

MMF (CellCept®, Roche) was initiated by a 2-hour intravenous infusion at 15 mg/kg every 8 hours for pediatric patients, and at an oral fixed dose of 1,000 mg twice daily for adult patients prior to transplantation. In this study, 17 pediatric patients were co-administered with cyclosporine, and other pediatric patients with tacrolimus as a concomitant immunosuppressive therapy. Nine adult patients were co-administered with cyclosporine, and other adult patients with tacrolimus. The sparse sampling design was employed. One

Table 4 Patient demographic and clinical characteristics

Characteristics	**Pediatric patients (n=20)**	**Adult patients (n=13)**
Age at transplant (years)	3 (0.2-12)	54 (18-63)
Body weight (kg)	13.7 (5.4-33.3)	91.9(38.4-113.3)
Sex (Male/Female)	12/8	7/6
Race		
White	16	13
Asian	2	0
Black	2	0
Ethnicity		
Hispanic	4	0
Non-Hispanic	12	13
Others	4	0
MMF Dose		
in mg/kg (2-hr infusion, TID)	15	
in mg(BID)		1000
Transplant source		
Bone marrow	1	0
Cord or double cord	19	2
Peripheral blood stem cells	0	11
Donor type		
Related sibling	1	7
Unrelated donor	19	6
Malignant/Non-malignant	9/11	13/0
Alive/Deceased	18/2	8/5
Blood chemistry		
Albumin (g/dL)	3.6 (2.4-4.4)	3.5 (2.3-4.5)
Creatinine (mg/dL)	0.3 (0.2-1.7)	0.9 (0.4-2.7)
Total bilirubin (mg/dL)	0.6 (0.2-7.7)	0.6 (0.2-18.5)
BUN (mg/dL)	17.5 (3-42)	17(3-78)

AST (Units/L)	24 (9-119)	21 (8-173)
ALT (Units/L)	20.3 (6-220)	18 (9-343)
ALP (Units/L)	130.3 (63-324)	84 (34-153)

Data are given as median (range)

blood sample was collected from each patient on day 0 of transplant, and days 7, 14, 21 and 30 post transplant. The sampling time fell into one of the following three time intervals: 2-4 h, 4-6 h and 6-8 h. After centrifugation, plasma samples were collected and kept at –80 °C until analysis. Pre- and post-operative biochemical parameters indicative of liver and renal function (albumin, serum creatinine, total bilirubin, blood urea nitrogen [BUN], aspartate aminotransferase [AST], alanine aminotransferase [ALT], alkaline phosphatase [ALP]) were measured in all patients.

4.3.3 Assessment of GVHD

In this study, clinical staging for each organ/system involved in acute GVHD and overall grading were based on a modified Keystone Grading Criteria (Table 5). Chronic GVHD was defined by the technical manual of procedures edited by the Blood and Marrow Transplant Clinical Trials Network (BMT CTN, Table 6) [97]. Symptoms of chronic GVHD if present were reported using the GVHD symptom record.

4.3.4 Total and free MPA and MPAG analysis

MPA and MPAG concentrations were measured by a validated liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method (Chapter 3). Briefly, the chromatographic separation was achieved on a C_{18} column with a gradient elution, and the detection was performed by a triple quadrupole mass spectrometer in the positive electrospray ionization (ESI) and multiple reaction monitoring (MRM) mode. Linearity of the assay was demonstrated over the range of 0.02-10 µg/ml for MPA and MPAG in human plasma. The lower limit of quantification (LLOQ) for this method was 0.02 µg/ml

Table 5 Staging and grading of acute GVHD

Stage	Skin	Liver (bilirubin)	Lower GI (stool output/day)*	Upper GI
0	No rash	≤ 2 mg/dl	Adult: < 500 ml/day Child: < 10 ml/kg/day	No protracted nausea and vomiting
1	Maculopapular rash < 25% BSA	2.1–3 mg/dl	Adult: 500–1000 ml/day Child: 10–19.9 ml/kg/day	Persistent severe nausea, vomiting with a positive upper GI biopsy
2	Maculopapular rash 25–50% BSA	3.1–6 mg/dl	Adult: 1001–1500 ml/day Child: 20–30 ml/kg/day	
3	Generalized erythroderma (> 50% BSA)	6.1–15 mg/dl	Adult: > 1500 ml/day Child: > 30 ml/kg/day	
4	Generalized erythroderma (> 50% BSA) plus bullous formation and desquamation > 5% BSA	> 15 mg/dl	Severe abdominal pain with or without ileus, or grossly bloody stool or melena (regardless of stool volume)	
Grade				
0	None	None	None	
I	Stage 1–2	None	None	
II	Stage 3 or	Stage 1 or	Stage 1	
III	Stage 0–3 skin with	Stage 2–3 or	Stage 2–3	
IV	Stage 4 or	Stage 4 or	Stage 4	

*for GI staging:

- The “adult” stool output values should be used for patients > 50 kg in weight.

- Use 3 day averages for GI staging based on stool output. If stool and urine are mixed, stool output is estimated to be 1/3 of total stool/urine mix.

Table 6 Definition and possible manifestation of chronic GVHD

Organ system	Definite manifestations of chronic GVHD	Possible manifestations of chronic GVHD
Skin	Scleroderma (superficial and fasciitis), lichen planus, vitiligo, scarring alopecia, hyperkeratosis pilaris, contractures from skin immobility, nail bed dysplasia	Eczematoid rash, dry skin, maculopapular rash, hyperpigmentation, hair loss
Mucous Membranes	Lichen planus, non-infectious ulcers, corneal erosions, non-infectious conjunctivitis	Xerostomia, keratoconjunctivitis sicca
GI tract	Esophageal strictures, steatorrhea	Anorexia, malabsorption, weight loss, diarrhea, abdominal pain
Liver	None	Elevation of alkaline phosphatase, transaminitis, cholangitis, hyperbilirubinemia
GU	Vaginal stricture, lichen planus	Non-infectious vaginitis, vaginal atrophy
Musculoskeletal/serosa	Non-septic arthritis, myositis, myasthenia, polyserositis, contractures from joint immobilization	Arthralgia
Hematologic	None	Thrombocytopenia, eosinophilia, autoimmune cytopenias
Lung	Bronchiolitis obliterans	Bronchiolitis obliterans with organizing

for both MPA and MPAG. The assay was accurate and precise with bias and %CV less than 15%.

For total MPA and MPAG analysis, 5 µl of internal standard working solution (mixture of 1 µg/ml MPA-d_3 and 5 µg/ml MPAG-d_3) was added to 50 µl of each calibration standard, quality control (QC) sample or subject sample. The plasma proteins were precipitated with acetonitrile (ACN) and the supernatant was transferred into pre-labeled tubes and evaporated to dryness after vigorous mixing and centrifugation. Samples were reconstituted with 100 µl of 30% ACN with 0.1% formic acid, centrifuged at 18,000 × g for 15 min, and the supernatant was injected into the UPLC-MS/MS system. The proportions of MPA and MPAG bound to plasma proteins in clinical samples were evaluated after 30 min of incubation at 37 °C. One hundred and fifty (150) µl of plasma sample was filtered with a Centrifree® ultrafiltration device (Millipore, Bedford, MA) assembled with a regenerated cellulose membrane (molecular weight cut-off, 30 kDa) under centrifugation (2,000 × g, Eppendorf centrifuge 5810 R equipped with a swing-bucket rotor A-4-62) for 15 min. The plasma ultrafiltrates were diluted with 30% ACN with 0.1% formic acid and then directly injected into the UPLC-MS/MS system. Samples with concentrations above the upper limit of linearity were diluted and reanalyzed.

4.3.5 Statistical methods

Non-normally distributed variables were expressed as median and range, and normally distributed variables as mean and SD. All statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). For 2-group comparisons,

continuous variables were analyzed by Student's t-test or Mann-Whitney U test, if applicable. For multi-group comparisons, continuous variables were analyzed by ANOVA or Kruskal-Wallis test and post hoc comparisons, if applicable. A p-value of 0.05 was considered statistically significant. Probability of acute and chronic GVHD, relapse and survival was estimated with the Kaplan-Meier method, performed with GraphPad Prism 5.0.

4.4 Results

4.4.1 Patients

A total of 20 pediatric patients and 13 adult patients received allogeneic HSCT were included in the current analysis. The age range was 0.2-12 years (median, 3 years) and 18-63 years (median, 54 years) for pediatric and adult patients, respectively. The sex distribution was 12/8 and 7/6 males/females for pediatric and adult patients, respectively. A total of 84 blood samples were collected from pediatric patients, and 45 were from adult patients. At the time of pharmacokinetic sampling, 17 pediatric patients were co-administered with cyclosporine and 3 with tacrolimus as a concomitant immunosuppressive therapy. For adult patients, 9 were co-administered with cyclosporine, and 4 with tacrolimus. Demographics and transplant characteristics of the study population are summarized in Table 4.

4.4.2 Plasma protein binding

During the 1-month sampling period post HSCT, a large variation in serum albumin levels was observed (Figure 8A) and the free fraction (% unbound) of MPA and MPAG did not change significantly (Figures 9A and 9C) in pediatric patients. For adult patients, a temporary drop in serum albumin levels was observed in the early period post HSCT and the serum albumin levels reached to the normal range (3.5–5.0 g/dL) after Day 21 post HSCT (Figure 8B). A significant increase of protein binding was observed in adult patients from day 7 to day 30 post HSCT, from 97.3±0.8% to 98.3±0.6% for MPA ($P < 0.05$), and 74.6±9.4% to 82.9±8.1% for MPAG ($P < 0.05$), resulting in a significantly decreased percentage of unbound MPA and MPAG (Figures 9B and 9D).

Sex-related differences in serum albumin levels and plasma protein binding of MPA and MPAG were observed. Significantly higher serum albumin levels were observed in pediatric males than those in pediatric females (3.6±0.5 vs 3.1±0.5; Figure 10A). However, similar serum albumin levels were observed between adult males and adult females (3.5±0.4 vs 3.5±0.4; Figure 10B). In pediatric patients, males displayed significantly higher plasma protein binding of MPA and MPAG compared to females (98.3±1.1 vs 97.4±1.1% for MPA and 78.7±8.7 vs 73.3±9.4% for MPAG), resulting in lower percentage unbound in males than females (1.7±1.1 vs 2.6±1.1% for MPA and 21.3±8.7 vs 26.7±9.4% for MPAG; Figures 11A and 11C). In adult patients, the plasma protein binding of MPA and MPAG was also significantly higher in males compared to females (98.1±0.7 vs 97.4±1.2% for MPA and 81.5±8.3 vs 73.8±10.1% for MPAG), resulting in lower percentage unbound in

males than females (1.9±0.7 vs 2.6±1.2% for MPA and 18.5±8.3 vs 26.2±10.1% for MPAG; Figures 11B and 11D).

4.4.3 MPAG/MPA ratios

Very high plasma concentrations of MPAG (1.3-168 µg/ml) in comparison to MPA (0.04-23.5 µg/ml) were observed in all the patients studied. The MPAG/MPA ratios were similar between males and females in pediatric (30.1±30.9 vs 39.9±38.0; Figure 12A) and adult (58.6±35.0 vs 40.4±29.3%; Figure 12B) patients. The MPAG/MPA ratios on an mg/kg dose basis were significantly higher in adult patients than those in pediatric patients (4.3±3.4 vs 2.4±2.6; $P < 0.05$; Figure 13).

4.4.4 Acute and chronic GVHD

One pediatric patient and one adult patient had graft failure. Acute GVHD was observed in 4 pediatric patients (Grade I, n=3; Grade II, n=1) and 8 adult patients (Grade I, n=2; Grade II, n=5; Grade III, n=1). During the study period, the incidence of grade I to IV acute GVHD was 21% (4/19) and 67% (8/12), in pediatric and adult patients, respectively (Figure 14). One pediatric patient (use of cord as the graft source) and six adult patients (use of PBSC as the graft source) developed chronic GVHD.

4.4.5 Relapse and survival

In the present study, 5 adult and pediatric patients experienced relapse of disease. Three of 20 pediatric patients and 6 of 13 adult patients died due to GVHD, infection, relapse disease progression, graft failure and organ failure (Figure 15).

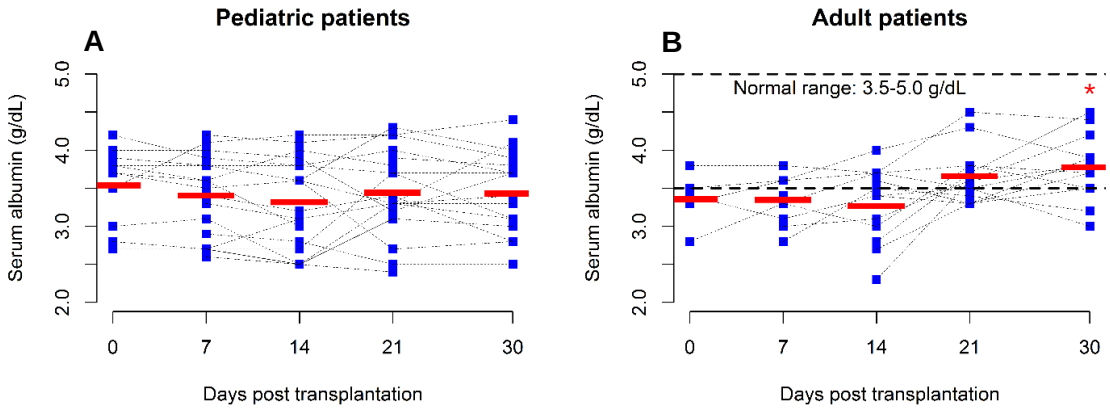


Figure 8. Changes in serum albumin levels over 1-month sampling period in (A) pediatric and (B) adult patients after HSCT

Horizontal solid lines indicate mean values.

Reference range of serum albumin concentrations for adult patients: 3.5–5.0 g/dL

* Kruskal-Wallis test at $p<0.05$

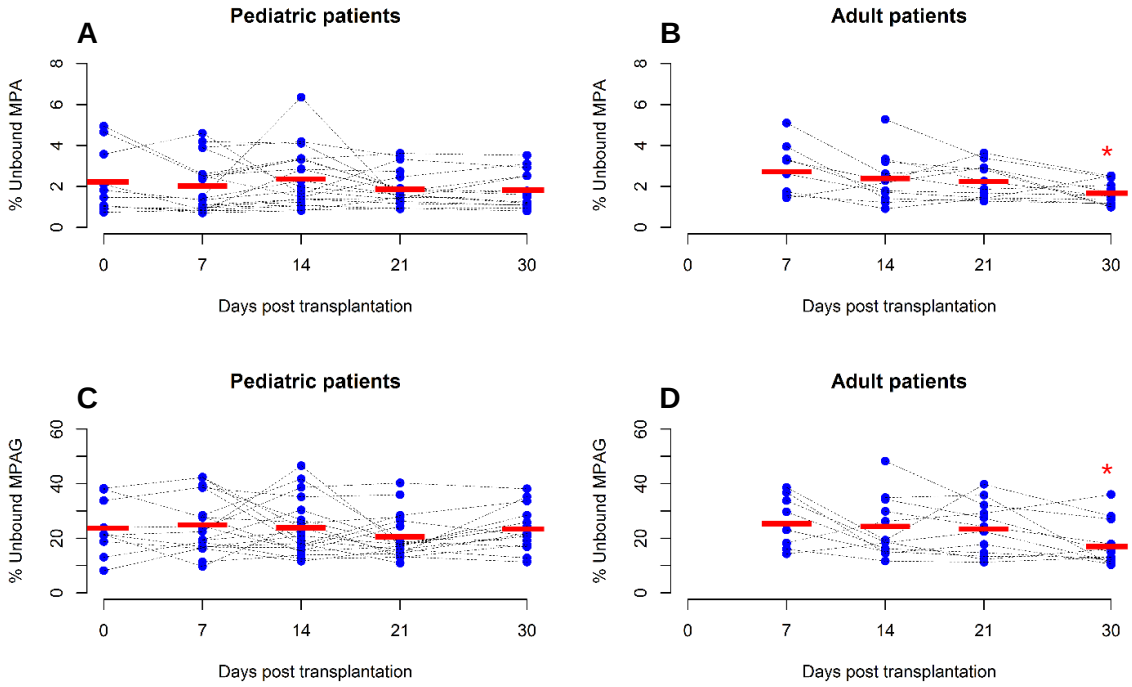


Figure 9. Changes in percent unbound MPA and MPAG in pediatric (A and C, respectively) and adult (B and D, respectively) patients over 1-month sampling period after HSCT

Horizontal solid lines indicate mean values of pharmacokinetic parameters

* Kruskal-Wallis test at $p<0.05$

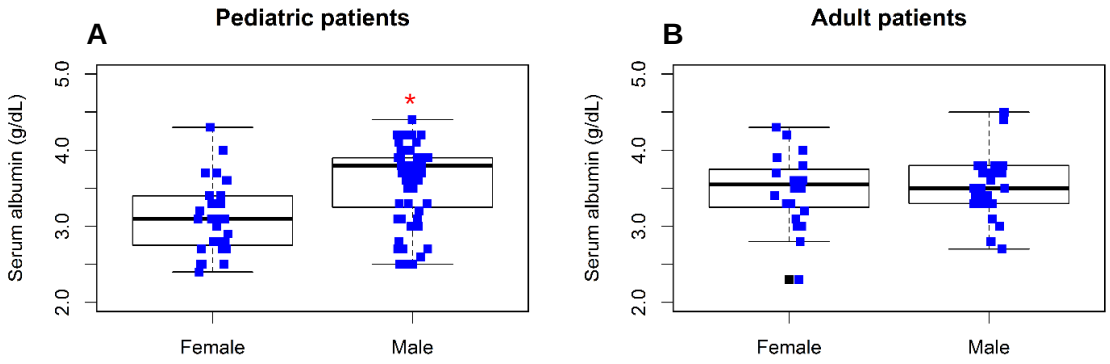


Figure 10. Sex differences in serum albumin levels in (A) pediatric and (B) adult patients after HSCT

* Unpaired t-test at $p<0.05$

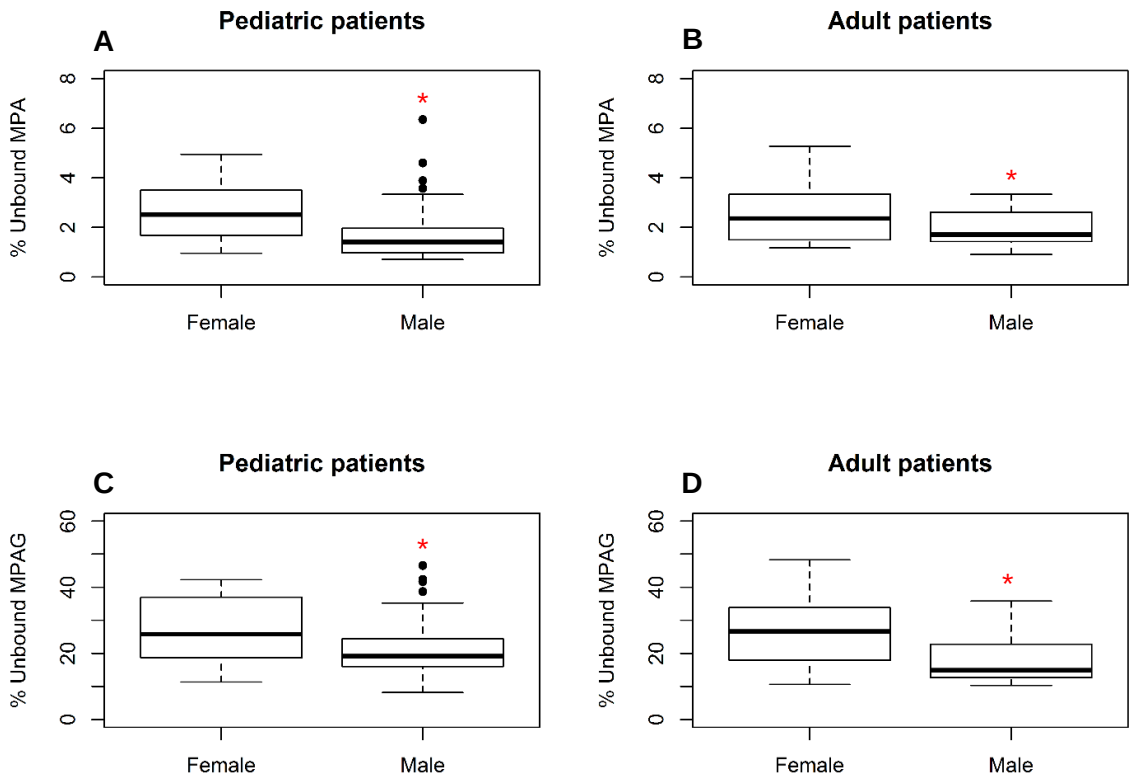


Figure 11. Sex differences in percent unbound MPA and MPAG in pediatric (A and C, respectively) and adult (B and D, respectively) patients after HSCT

* Unpaired t-test at $p<0.05$

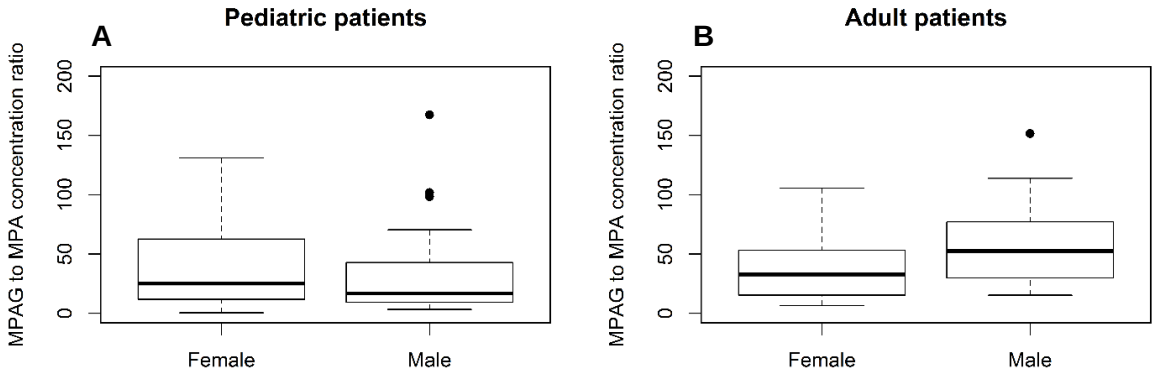


Figure 12. Sex differences in MPAG to MPA concentration ratios in (A) pediatric and (B) adult patients after HSCT

Unpaired t-test at $p<0.05$

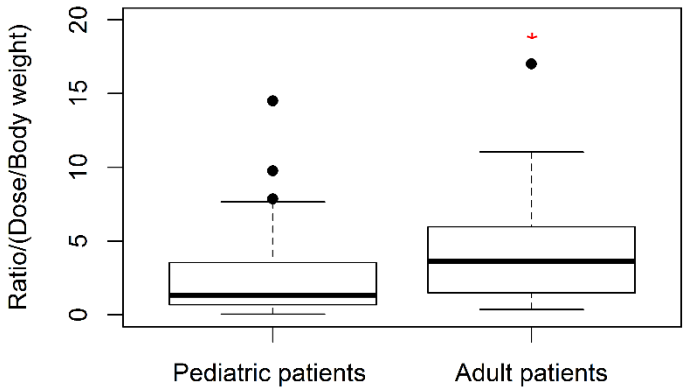


Figure 13. Differences in MPAG to MPA concentration ratios in pediatric and adult patients after HSCT. MPAG to MPA concentration ratios normalized by dose/body weight

* Unpaired t-test at $p < 0.05$

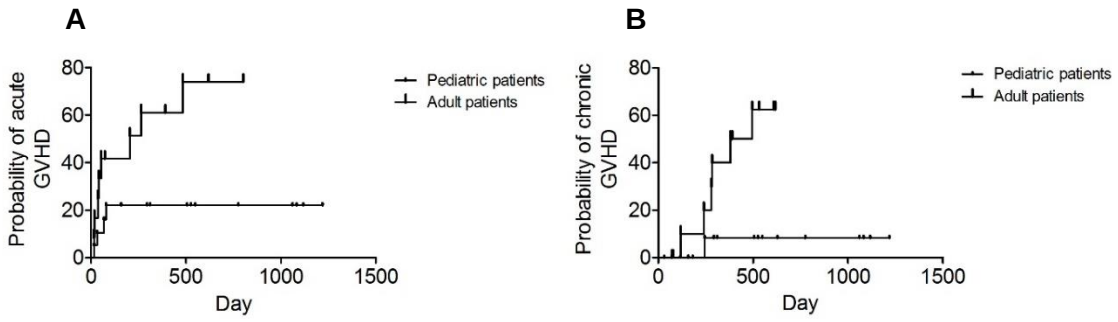


Figure 14. The probability developing (A) grade I to IV acute GVHD or (B) chronic GVHD in pediatric and adult patients

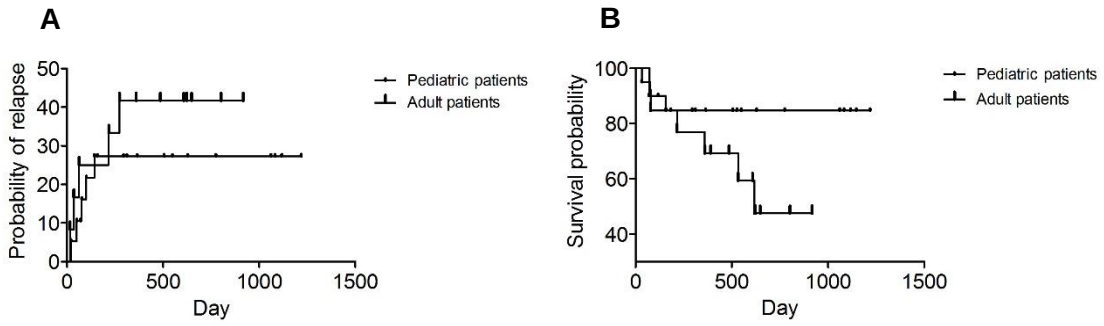


Figure 15. The probability of (A) relapse and (B) survival in pediatric and adult patients

4.5 Discussion

Extensive plasma protein binding is an important pharmacokinetic property of MPA. The inhibition of IMPDH depends on the free MPA. Renal function, albumin level and MPAG concentration may all affect the protein binding of MPA, leading to considerable alterations of free MPA concentration in vivo [98, 99]. In renal transplant recipients, MPA protein binding negatively correlates with urea and creatinine concentration and positively correlates with albumin concentration. MPA free fraction is highly affected by the free and total MPAG $AUC_{0\text{-}6}$ [100]. Li et al. found total MPA clearance increased with decreased serum albumin concentration in HSCT patients, most likely due to the increased fraction of unbound MPA [32]. Impaired renal function can lead to an accumulation of MPAG, which may displace MPA from its protein binding sites or increase EHC of MPAG, resulting in an increase in total MPA concentration, observed in liver transplant recipients with mild to moderate renal dysfunction [101]. In vitro data have shown that MPA plasma protein binding is not affected by other common immunosuppressant medications (cyclosporine, tacrolimus and prednisone) [98].

In the early period post HSCT in adult patients, conditioning therapy including chemotherapy with or without radiation might lead to a temporary drop in serum albumin level, resulting in a temporary decrease in protein binding (increase in % unbound). After 1 month post HSCT, the observed decrease in MPA free fraction in adult patients might be due to the increased albumin concentration offering high binding capacity for MPA and decreased competition of MPAG from albumin binding sites. Kuypers et al. also reported serum albumin levels initially decreased and recovered by week 6 in renal transplant

recipients [102]. Assuming that the liver is the major organ involved in MPA elimination, the hepatic extraction ratio of MPA (the fraction of MPA that is metabolized during a single pass through the liver) is approximately 0.3 to 0.7, indicating that MPA can be either restrictive or nonrestrictive [10]. Therefore, its hepatic clearance is affected by free fraction, intrinsic enzymatic activity of the liver, and the blood flow to the liver. The decrease in % unbound MPA one month post HSCT in adult patients observed in this study, may lead to a decrease in glucuronidation rate, resulting in an decrease in MPA clearance and an increase in total MPA exposure in the patients.

In pediatric males, the higher plasma protein binding of MPA might result from the higher serum albumin levels than in pediatric females. In adult patients, the plasma protein binding of MPA was also significantly higher in males compared to females, even though similar serum albumin levels were observed between males and females. Therefore, the plasma protein binding of MPA is not only affected by the serum albumin level, but also by some other factors. It has been observed that the percentage of free MPA correlates with red blood cell and leukocyte counts in renal transplant recipients [103]. Increasing hemoglobin causes a decrease in MPA clearance in renal transplant patients, indicating that MPA binds not only to albumin but also to hemoglobin or red blood cells [99].

Glucuronidation is the major elimination pathway for MPA. Studies evaluating the effect of sex on MPA pharmacokinetics give conflicting results. Morissette et al. reported sex related differences in MPAG/MPA ratio. It was significantly higher in males than in females of kidney transplant patients co-administered with tacrolimus [104]. The effect of sex on MPA clearance has been described by developing a population pharmacokinetic model in

renal transplant patients following oral administration of MMF. Based on the final population pharmacokinetic model, it appears that males have an 11% higher MPA clearance than females [105]. Tornatore et al. reported rapid apparent MPA clearance in males than in females in Caucasians (13.8±6.27 vs 8.70±3.33 L/h) and African Americans (10.2±3.73 vs 9.71±3.94 L/h) post renal transplantation [106]. A possible effect is that the lower metabolism of MPA in females may be due to the competition of estrogen metabolism with UGTs. The sex related difference in clearance, with males exhibiting a more rapid clearance, could contribute to the large inter-individual pharmacokinetic variability. No effect of sex on MPA clearance has been found by other studies. The dose-adjusted AUC in females is slightly higher than in males, but this difference fails to reach statistical difference in renal transplant patients [107]. In a population pharmacokinetic meta-analysis containing 13,346 MPA concentration-time datum points from 468 renal transplant patients, no significant relationship is established between sex and MPA exposure [99]. In this study, the MPAG/MPA ratios were similar between males and females in both pediatric and adult patients.

Pediatric patients display different pharmacokinetics from that of adult patients. Different MMF disposition rates are expected in pediatric patients compared to adult patients, based on the ontogeny of human hepatic UGTs. Higher MPAG/MPA ratios on an mg/kg dose basis were observed in adult patients than those in pediatric patients in this study. Gajarski et al. also found that MPAG/MPA ratios were higher for adults compared with children in heart transplant recipients [108]. This could be due to higher amounts of glucuronide-conjugating enzymes in the liver of adult patients than those of pediatric patients. Further

studies are still needed to better understand the underlying developmental changes of hepatic UGTs activity.

Metabolic drug-drug interaction may exist when co-administered with other immunosuppressants including cyclosporine and tacrolimus. Cyclosporine, an Mrp2 inhibitor, can cause a decrease in the biliary secretion of MPAG, resulting in an increase in MPAG exposure and a decrease in MPA exposure [109]. Tacrolimus, though mainly metabolized by the cytochrome P450 (CYP) 3A subfamily, is reportedly a good inhibitor of MPA conjugation both in vitro and in vivo. Co-administration with tacrolimus can decrease the intrinsic UGT enzymatic clearance of MPA and consequently augment the bioavailability of MMF [110, 111]. On the same MMF dose basis, total and free MPA concentrations are lower when co-administered with cyclosporine, but higher with tacrolimus in organ transplant patients [93, 112].

Following an oral administration of MMF, the average plasma half-life in liver and renal transplant patients is about 6 and 11 h, respectively, and the concentration-time profile of MPA often shows two peaks, the first peak occurring within 2 h post-dose and the second one at 6-12 h due to EHC. In clinical HSCT studies, a plasma MPA half-life ranging from 1 to 4 h is observed. Compared to solid organ recipients, MPA exposure is lower and the EHC is markedly reduced or absent in HSCT patients receiving an equivalent dose of MMF [16, 18, 29]. In our study, no secondary peak was observed on MPA concentration-time profile when co-administered with cyclosporine or tacrolimus. The reasons, however, were still unclear. Physiological changes including gut GVHD and damaged epithelium of the intestine due to high-dose chemotherapy and/or the reduction in bacterial flora in the

gastrointestinal tract from broad-spectrum antibiotic use could reduce the contribution of EHC, resulting in lower MPA exposure. Additional studies are needed to determine the pathophysiological mechanisms responsible for the altered MPA pharmacokinetics in HSCT patients.

The current study certainly has several potential limitations. The relatively small number of clinical evaluable HSCT patients may affect our statistical power. Since measurement of MPA exposure using a full set of samples requires considerable volume of blood, which is not feasible for pediatric patients, the relatively sparse sampling approach used in this study limits our ability to characterize the reabsorption kinetics of MPA due to the EHC. Another potential limitation is that no pharmacokinetic parameters were derived for cyclosporine and tacrolimus. The inhibition of Mrp2 and UGT by cyclosporine and tacrolimus, respectively, may vary among patients. Despite these limitations, our study provides useful findings for pharmacokinetic variability, from plasma protein binding and metabolism perspectives, in both pediatric and adult HSCT patients.

4.6 Conclusions

Mycophenolic acid is a commonly used immunosuppressant with complex pharmacokinetics and substantial intra- and inter-patient variability. This study provides preliminary data to explain intra- and inter-patient pharmacokinetic variability of MPA in both adult and pediatric HSCT patients. We have observed time-dependent changes of protein binding and age-related differences on metabolism of MPA post HSCT. Time-dependent changes in plasma protein binding could contribute to the intra-individual

variation in adult patients post HSCT. Age-dependent metabolic ability, as well as sex-related plasma protein binding could contribute to the inter-individual variation. In order to achieve a reliable immunosuppression and less toxic side effects in HSCT patients, we believe that effective drug monitoring for MPA needs to be established for the optimal use of MPA. Incorporating these patient and pharmacologic factors into MMF dosage regimen design may contribute to the more effective individualization of MMF dosing in pediatric and adult HSCT patients.

Chapter 5 Population pharmacokinetics of unbound mycophenolic acid in pediatric and adolescent patients post hematopoietic stem cell transplantation

5.1 Abstract

Mycophenolate mofetil, a prodrug of mycophenolic acid (MPA), is increasingly used in patients post hematopoietic stem cell transplantation (HSCT) for the prophylaxis and treatment of acute and chronic graft-versus-host disease (GVHD). However, limited information is available for unbound MPA pharmacokinetics and guidance for optimal dosing of MMF is still lacking in pediatric HSCT patients. The aim of this study was to investigate population pharmacokinetics of unbound MPA in pediatric and adolescent HSCT patients. Sparse sampling design was used in this study. Nonlinear mixed-effects modelling (NONMEM) was employed to analyze MPA pharmacokinetic data. A total of 89 unbound MPA plasma concentration-time datum points from 23 patients with a median age of 3 years (range, 0.2-20 years) were available for model development. Pharmacokinetics of unbound MPA was described by a two-compartment model with first-order elimination. Given the range of body sizes, clearance and volume of distribution were scaled using standard weight-based allometric exponents. Final estimates in a standard 70 kg individual for clearance, inter-compartmental clearance, volumes of distribution in the central and peripheral compartments were 1720 L/h, 1180 L/h, 3260 L and 4120 L, respectively. No significant differences were observed in weight-adjusted

clearance between males and females. Because of age-dependent differences in weight-adjusted clearance, the calculated unbound MPA AUC was higher in younger patients compared with those in older patients receiving 15 mg/kg MMF. The final population pharmacokinetic model successfully described unbound MPA population pharmacokinetics, which could be used to explore dosing guidelines for safe and effective immunotherapy in pediatric HSCT patients.

5.2 Introduction

For more than a decade, mycophenolic mofetil (MMF, CellCept®) has been successfully used in solid organ transplant patients to prevent acute allograft rejection. After oral administration, MMF is rapidly and extensively (94%) converted by esterases, found in blood, gut wall, liver and other tissues, to the active moiety MPA, which is a selective, reversible, and noncompetitive inhibitor of inosine monophosphate dehydrogenase (IMPDH). Inhibition of IMPDH blocks the de novo purine biosynthesis in T and B-cell proliferation and causes immunosuppression, eventually contributing to the prevention of graft rejection [2, 87, 113].

The disposition of MPA is complex, leading to difficulties in defining the optimal MMF doses. In patients with normal renal and liver function, 97-99% MPA is bound to plasma proteins [98]. It is mainly metabolized into the phenolic glucuronide conjugate (MPAG) by uridine glucuronosyl-transferase (UGT) enzymes, with substantial inter-individual variability. MPAG also displays significant binding to serum albumin (82% in stable patients). MPAG is either excreted into the urine or carried back into intestinal lumen via

bile through multidrug resistant protein transporters (MRPs), specifically MRP2. In the intestine, MPAG may be deconjugated back to MPA and reabsorbed into the systemic circulation through enterohepatic circulation [14]. MPAG is pharmacologically inactive, and only unbound MPA can distribute into the extravascular space and is responsible for pharmacological activity. Patients developing protein binding changes, resulting from chemotherapy, severe illness, and renal and hepatic dysfunction, may have normal total MPA concentrations but high unbound MPA concentrations. Weak correlation (r^2=0.2) between total and unbound MPA concentrations has been observed [23]. Therefore, unbound MPA concentrations are a better reflection of immunosuppressive activity.

The success of MMF use in solid organ transplantation has triggered an increasing use of MMF in allogeneic HSCT, in which acute or chronic GVHD is the main cause of morbidity and mortality after transplantation. Storb et al. conducted preclinical studies in a canine HSCT model to show that MMF in combination with cyclosporine has activity in preventing acute GVHD after non-myeloablative conditioning [114, 115]. Subsequently, MMF was introduced for prophylaxis of acute GVHD and/or the treatment of acute or chronic GVHD after allogeneic HSCT, administrated in combination with other immunosuppressive agents [3, 54, 116-122]. In adult HSCT recipients, standard fixed doses of MMF 1 g twice daily achieve MPA exposures that are significantly lower compared with other populations [28]. Children undergoing HSCT are suggested to receive an MMF dose of at least 15 mg/kg every 8 hours intravenously [30]. In solid organ transplant recipients, the AUC_{0-12} of total MPA between 30 and 60 mg·h/L is recommended by the Randomized Concentration-Controlled Trial (RCCT) [123]. Target values in HSCT are still under

investigation primarily because of the reported large intra- and inter-patient pharmacokinetic variability and broad ranges of transplant characteristics (malignant vs nonmalignant, related vs unrelated donor) [30, 53, 60].

In renal transplant recipients, total MPA clearance is significantly impacted by renal function and plasma albumin concentration. Total MPA exposure, determined by total MPA clearance, significantly decreases with decreasing renal function, decreasing albumin level and increasing cyclosporine predose level [99, 105, 124]. Low albumin concentration and accumulation MPAG, resulting from impaired renal function, decrease plasma protein binding of MPA. The subsequent increase in the fraction unbound of MPA produces an increase in total MPA clearance and, consequently, results in decreased total MPA exposure. Moreover, cyclosporine, an MRP2 inhibitor, can cause an increase in total MPA clearance. When cyclosporine is used in combination with MMF, the MPA levels decrease due to the interference of cyclosporine with MRP2-mediated hepatic recycling of MPAG [28]. In a population pharmacokinetic analysis containing 4,496 MPA concentration-time datum points from 408 HSCT patients, serum albumin level and concomitant cyclosporine are also identified as important covariates influencing total MPA clearance [32].

Infants and very young children are very difficult to treat due to the age-related differences in drug disposition and engraftment kinetics. To date, most published population pharmacokinetic studies of MPA have been conducted in adult HSCT patients. In general, large intra- and inter-individual variability have been observed in HSCT patients, as seen in organ transplant patients. Compared with adults, infants and young children often have

larger pharmacokinetic variability and possibly altered pharmacodynamics. Pharmacokinetic disposition of MPA in pediatric populations remains limited and robust trials in pediatric HSCT patients are often lagging behind those involving adults. Even less is known about the pharmacodynamics in this group, especially in those patients with nonmalignant disease in whom the rarity of the diseases makes properly powered trials unfeasible in a single center. Moreover, measuring 12 h MPA AUC is impractical due to the ethical and logistical constraints in obtaining adequate blood volume needed for pharmacokinetic analysis, which can further limit the enrollments of studies in pediatric HSCT patients.

Characterization of the pharmacokinetics of unbound MPA is of clinical relevance to further optimize MMF dosing regimens, firstly because the unbound MPA concentrations are ultimately responsible for the inhibition of IMPDH and prevention of graft rejection, and secondly because significant intra-patient and inter-patient variability in the bound fractions of MPA have been observed in our previous study. Despite the increased use of MMF, only very limited information is available regarding the pharmacokinetics of unbound MPA in pediatric HSCT patients, especially the very young. The objective of this study was to investigate population pharmacokinetics of unbound MPA following intravenous infusion of MMF in pediatric and adolescent patients undergoing HSCT. Of particular interest was the comparison of the unbound MPA AUC between different age groups, with the current body weight-based dosing regimen.

5.3 Methods

5.3.1 Patients

The study was conducted as an open-label clinical study in HSCT patients. Twenty-three pediatric and adolescent patients with a median age of 3 years (range, 2 months to 20 years) with malignant/nonmalignant conditions, undergoing HSCT from both related and unrelated donors, were enrolled at Indiana University Hospital and Riley Hospital for Children (Table 7). The study was approved by the institutional review board at each center. Informed consent was obtained from each patient (or parent/guardian for pediatric patients) and assent was obtained from children who are at least 7 years of age before enrollment.

5.3.2 Study protocol

MMF (CellCept®, Roche) was initiated at 15 mg/kg by a 2-hour intravenous infusion every 8 hours. Twenty patients were coprescribed with cyclosporine, and three were coprescribed with tacrolimus as a concomitant immunosuppressive therapy. Random sparse PK sampling design strategy was used in this study. One blood sample was collected from each patient on day 0 of transplant, and days 7, 14, 21 and 30 post transplant. The sampling time fell into one of the following three time intervals: 2-4 h, 4-6 h or 6-8 h. During the study visit, the total amount of blood drawn for research purpose could not exceed 19 ml as based on a minimal body weight of 40 kg, 13 ml based on body weight range of 25-40 kg, 10 ml based on body weight range of 10-25 kg, 8 ml if body

Table 7 Demographic and clinical characteristics of the study population

Characteristics	**number/median (range)**
Age at transplant (years)	3 (0.2-20)
≤ 2	7
2.5-4	8
7-12	6
18-20	2
Body weight (kg)	17.5 (5.4-113.3)
Sex (Male/Female)	14/9
Race	
White	18
Asian	2
Black	3
Ethnicity	
Hispanic	4
Non-Hispanic	14
Transplant source	
Bone marrow	1
Cord or double cord	22
Donor type	
Related sibling	1
Unrelated donor	22
Malignant/Non-malignant	12/11
Alive/Deceased	19/4
Blood chemistries	
Albumin (g/dL)	3.4 (2.4-4.4)
Creatinine (mg/dL)	0.3 (0.2-1.7)
Total bilirubin (mg/dL)	0.6 (0.2-18.5)
Blood urea nitrogen (mg/dL)	19 (3-78)
Aspartate aminotransferase (AST, Units/L)	23 (8-79)
Alanine aminotransferase (ALT, Units/L)	23 (6-147)
Alkaline phosphatase (ALP, Units/L)	124 (28-324)

weight less than 10 kg. Blood samples were centrifuged at 2,000 g for 15 min at 4 °C. After centrifugation, plasma samples were harvested and kept at -80 °C until analysis.

5.3.3 Bioanalytical assay

After 30 min of incubation at 37 °C, 150 µl of plasma sample was filtered with a Centrifree® ultrafiltration device (Millipore, Bedford, MA) assembled with a regenerated cellulose membrane (molecular weight cut-off, 30 kDa) under centrifugation (2,000 × g, Eppendorf centrifuge 5810 R equipped with a swing-bucket rotor A-4-62) for 15 min. The plasma ultrafiltrates were diluted with 30% acetonitrile (ACN) with 0.1% formic acid and then unbound MPA concentrations were measured by a previously established and validated ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method. Briefly, the chromatographic separation was achieved on a C_{18} column with a gradient elution, and the detection was performed by a triple quadrupole mass spectrometer in the positive electrospray ionization (ESI) and multiple reaction monitoring (MRM) mode. The lower limit of quantification (LLOQ) was 0.5 ng/ml. Linearity of the assay was demonstrated over the range of 0.5-500 ng/ml. Intra- and inter-day precision and accuracy for quality control (QC) samples were always < 15%.

5.3.4 Population pharmacokinetic analysis

The population pharmacokinetic analysis was conducted using nonlinear mixed-effects modelling software program NONMEM® version 7.3.0 with PDx-Pop® version 5.0 (ICON Development Solutions, LLC, Ellicott City, MD, USA) interfaced with Xpose® version 4.5.3 (Uppsala University, Uppsala, Sweden) and R version 3.2.3 (Free Software Foundation,

Inc., Boston, MA, USA). Because NONMEM estimated pharmacokinetic parameters for the active moiety MPA, the dose of intravenous MMF formulation was converted to the equivalent MPA content by multiplying the MMF dose by 0.682 considering the fractional difference in molecular mass between MMF and MPA. The first-order conditional estimation (FOCE) method was implemented throughout the model building and evaluation procedure. Four significant digits were specified for the estimation procedure.

5.3.5 Structural model development

The plot of observed concentration-time data of unbound MPA was initially examined. One-compartment (NONMEM subroutine ADVAN1 TRANS2) and two-compartment (NONMEM subroutine ADVAN3 TRANS4) pharmacokinetic models, with first order elimination, were compared to investigate the best fit of the concentration-time data. A two-compartment model was finally selected, as also described in the literature [34, 35]. Typical values for the pharmacokinetic parameters including clearance (CL), central volume of distribution (V_c), inter-compartmental clearance (Q) and peripheral volume of distribution (V_p) were estimated. To improve the model stability, body weight (BW) was included as a primary covariate in the structural model. Studies have shown that body weight plays an important role in determining pharmacokinetic parameter estimates and consequently drug doses for the pediatric population. Body weight was standardized to 70 kg and its effects on CL, V_c, Q and V_p were described by the allometric model using a coefficient of 0.75 and 1.0 for clearance and volume of distribution terms, respectively [125-127].

5.3.6 Inter-individual and residual unexplained variability

Inter-individual variability (IIV), expressed as a coefficient of variation (CV %), was assessed using an exponential error model (Equations 1-5) for all pharmacokinetic parameters, which assumes a log-normal distribution on inter-individual variability.

$$P_i = \theta_P \times \left(\frac{BW_i}{70}\right)^{0.75 \text{ for CL and } 1.0 \text{ for V}} \times \exp^{\eta_{P_i}} \quad (1)$$

$$CL_i = \theta_{CL} \times \left(\frac{BW_i}{70}\right)^{0.75} \times \exp^{\eta_{CL_i}} \quad (2)$$

$$V_{c_i} = \theta_{V_c} \times \left(\frac{BW_i}{70}\right)^{1.0} \times \exp^{\eta_{V_{c_i}}} \quad (3)$$

$$Q_i = \theta_Q \times \left(\frac{BW_i}{70}\right)^{0.75} \times \exp^{\eta_{Q_i}} \quad (4)$$

$$V_{p_i} = \theta_{V_p} \times \left(\frac{BW_i}{70}\right)^{1.0} \times \exp^{\eta_{V_{p_i}}} \quad (5)$$

where P_i represents the pharmacokinetic parameter of the i^{th} individual, θ_P represents the typical value of the pharmacokinetic parameter for a 70 kg individual, BW_i is the body weight of individual i in kg, and η_{Pi} is the inter-individual random effect with mean 0 and variance ω^2.

Residual unexplained variability (RUV) between observed and predicted unbound MPA concentrations, was described by a proportional error model (Equation 6) and expressed as a CV%.

$$Y_{ij} = C_{pred,ij} \times (1 + \varepsilon_{ij}) \quad (6)$$

where Y_{ij} is the observed and $C_{pred,\ ij}$ is the individual predicted unbound MPA concentrations and ε_{ij} is the residual random error with mean 0 and variance σ^2.

In this study, body weight for each patient was often obtained on the same day that the blood sample was collected for quantification. However, when it was not, the weight recorded before or after the blood sample collection was used. Although blood samples were collected on more than 1 occasion, no attempt was made to model between-visit variability due to the limited data available for analysis.

5.3.7 Covariate analysis

Once the effects of body weight on pharmacokinetic parameters were considered in the structural model, a stepwise forward inclusion and backward elimination approach was used to examine the effects of other covariates on unbound MPA pharmacokinetic parameters, based on the likelihood ratio test and a pre-specified alpha level. Patient-specific factors considered for covariate testing included age, sex, and measurements of albumin, creatinine, total bilirubin, blood urea nitrogen, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP). If the difference in objective function value (OFV) between nested models was larger than the critical value from a chi-squared distribution with degrees of freedom equal to the difference in the number of estimated parameters, the models are significantly different from each other. During forward inclusion, each covariate was tested one at a time. The most significant covariate was kept in the model and the process was repeated to test the remaining covariates until no significant covariates remained. All of the significant covariates from

stepwise forward inclusion were included in the full model. Next, a stepwise backward elimination step was performed, in which the covariate with the smallest contribution to the change in the OFV was removed from the model and the remaining parameters in the model were retested until removal of any other covariate produced a statistically inferior model. A statistical significance criterion of $P < 0.01$ ($\Delta OFV = 6.63$) was used during forward inclusion and backward elimination. All significant covariates following stepwise forward inclusion and backward elimination were included in the final full model.

Model adequacy was further internally evaluated by plausible parameter estimates, adequate parameter precision and inspection of goodness-of-fit plots. The following diagnostic plots were used to visually assess model fit, observed (DV) vs. population predicted (PRED) and individual predicted (IPRED) values, weighted residuals (WRES) vs. time after dose and IPRED. Changes in estimates of inter-individual variability and residual unexplained variability were also examined.

5.4 Results

5.4.1 Patient characteristics

A total of 23 pediatric and adolescent patients with malignant and nonmalignant disease received allogeneic HSCT. Twenty subjects were coprescribed with cyclosporine and 3 with tacrolimus at the time of pharmacokinetic sampling. In total, 89 unbound MPA concentration-time samples were obtained from 23 HSCT recipients. The age range of the subjects was 0.2-20 years with a median age of 3 years. For the calculated unbound MPA exposure, we defined the patient subpopulations as follows: infants (≤ 2 years, n=7),

children (2.5-11 years, n=12), adolescents (12-20 years, n=4). The sex distribution was 14 males and 9 females. Demographic and clinical characteristics of the study population are summarized in Table 7.

5.4.2 Model development

A two-compartment model with first-order elimination was determined to be the most appropriate structural pharmacokinetic model for unbound MPA concentration-time data. Using standard principles of allometric scaling, body weight was built into the structural model a priori and scaled to a reference patient having a weight of 70 kg, as described previously. Standard fixed allometric exponents of 0.75 for clearance and 1.0 for volume of distribution were applied. During stepwise forward inclusion and backward elimination, no covariate was found to be significant. Given this finding, the final pharmacokinetic model was the structural pharmacokinetic model (defined by Equations 2-6). An exponential error model was used to estimate IIV on the pharmacokinetic parameters (including CL, V_c, V_p and Q) and a proportional error model was used to estimate RUV.

The values for the pharmacokinetic parameters including CL, V_c, Q and V_p, as well as their inter-individual variability are presented in Table 8. Parameter uncertainty expressed as the relative standard error (RSE) of estimates was small (less than 28%) for all the population pharmacokinetic parameters except for Q (48%), indicating a much higher degree of uncertainty in inter-compartmental clearance estimate. Goodness-of-fit plots did not show systematic bias for the final pharmacokinetic model predictions (Figure 16). The population-predicted concentrations were symmetrically distributed around the line of

Table 8 Unbound MPA population pharmacokinetic parameter estimates

Parameter (unit)	Estimate	RSE (%)*
CL (L/h)	1720	8.0
V_c (L)	3260	21.5
Q (L/h)	1180	47.7
V_p (L)	4120	27.7
Inter-individual variability (IIV)		
CL, CV%	16.2	71.2
V_c, CV%	54.1	67.1
Q, CV%	46.3	35.1
V_p, CV%	13.4	57.0
Residual unexplained variability (RUV), %CV	65.6	23.5

*RSE, relative standard error, the ratio of the standard error to the mean

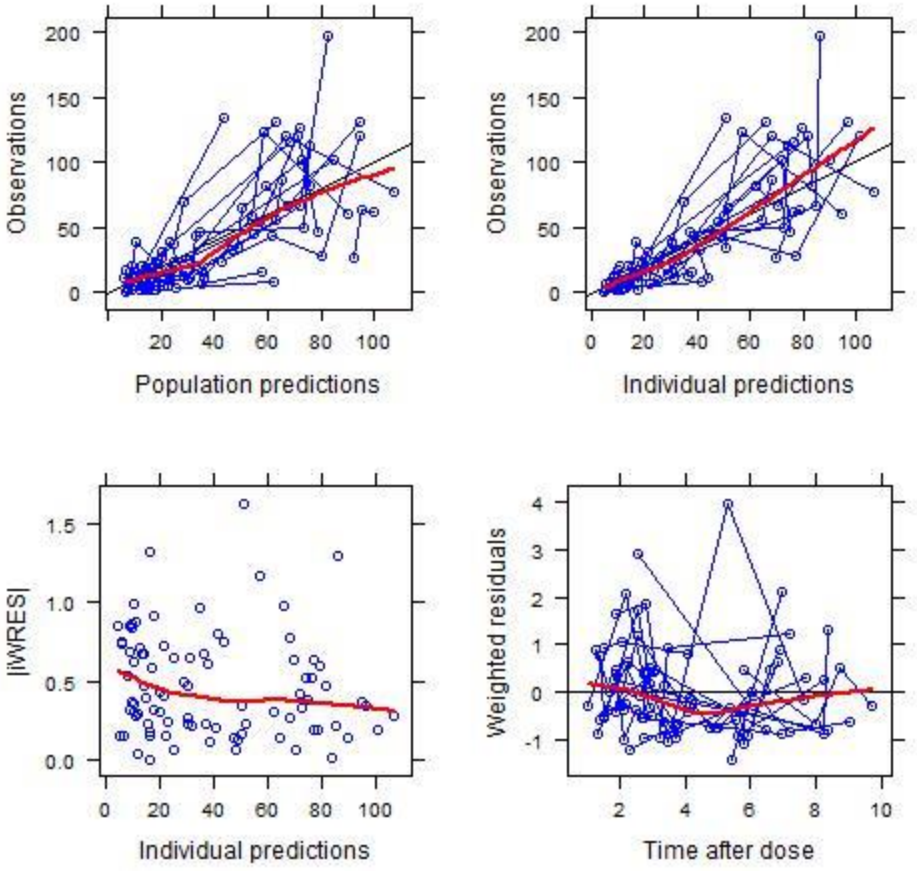


Figure 16. Goodness-of-fit plots of the final population pharmacokinetic model

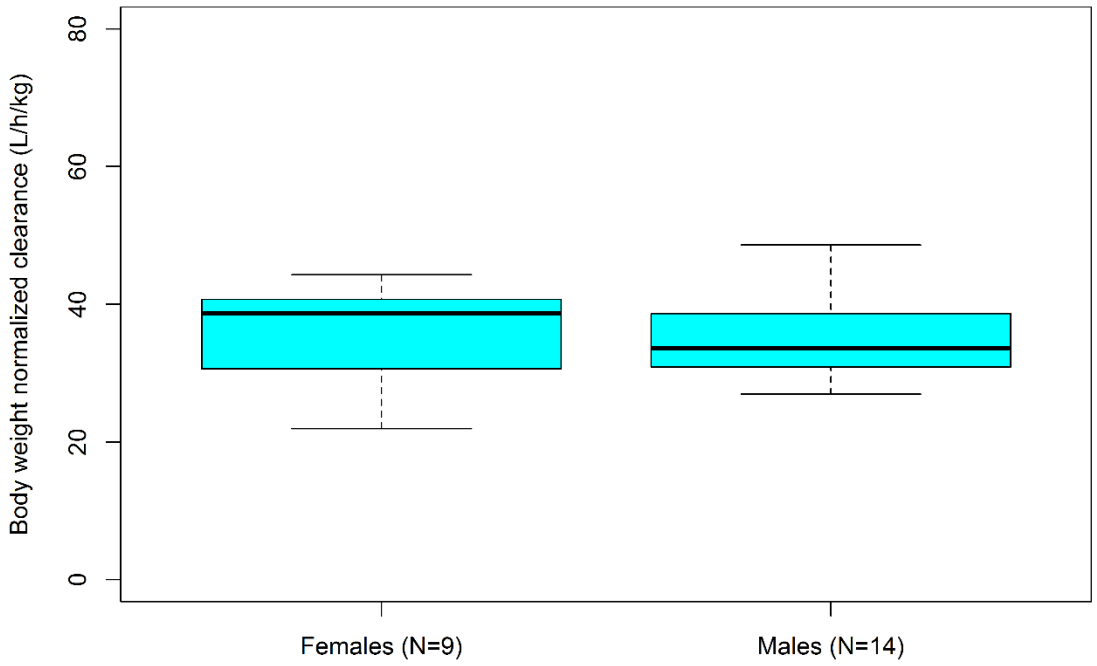


Figure 17. Boxplot of individual predicted clearance adjusted by body weight between females and males

Student t-test at $p<0.05$

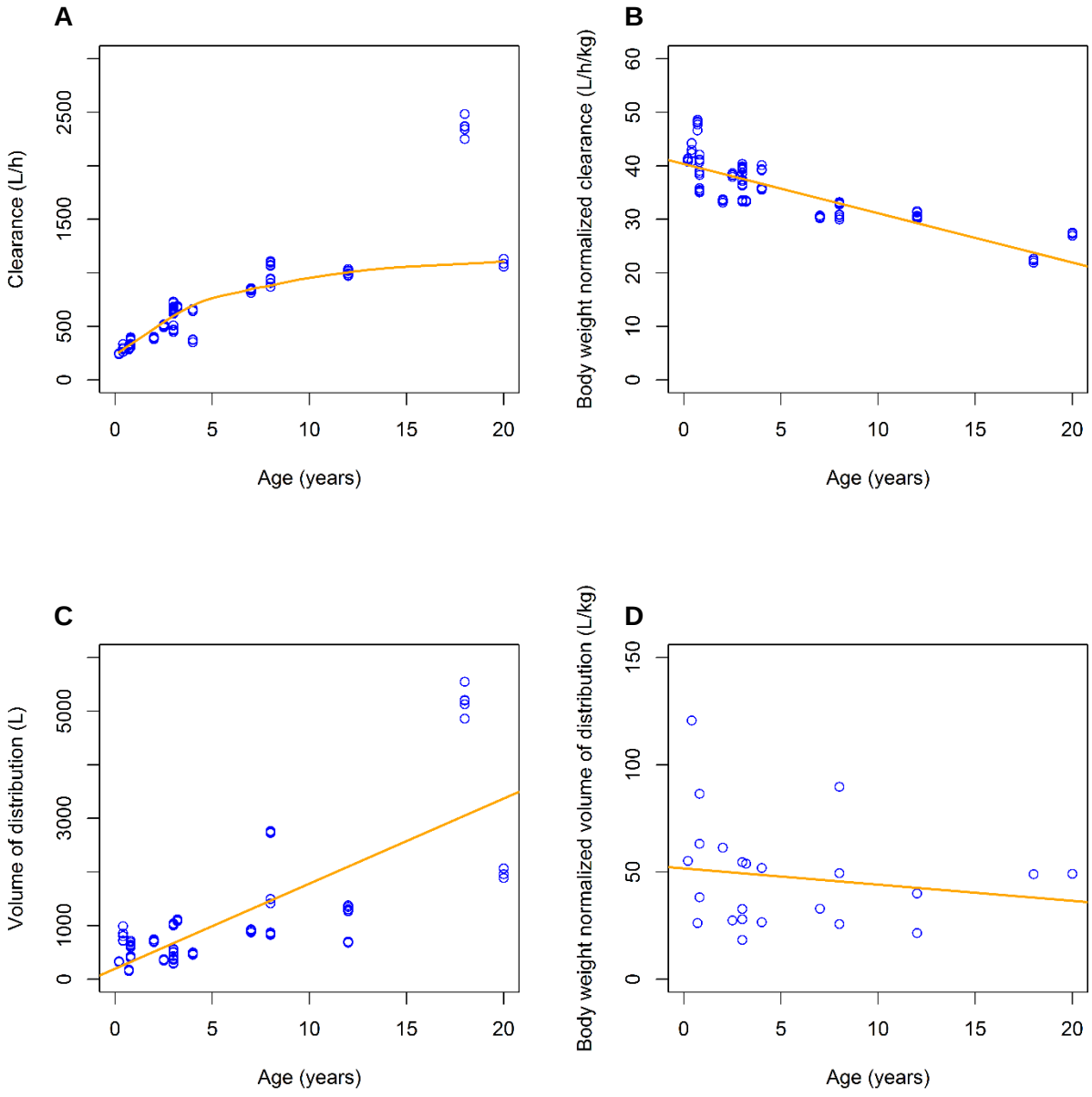

A
Clearance (L/h)
0
500
1500
2500
0
5
10
15
20
Age (years)
B
Body weight normalized clearance (L/h/kg)
0
10
20
30
40
50
60
0
5
10
15
20
Age (years)
C
Volume of distribution (L)
0
1000
3000
5000
0
5
10
15
20
Age (years)
D
Body weight normalized volume of distribution (L/kg)
0
50
100
150
0
5
10
15
20
Age (years)

Figure 18. Scatter plots of (A) the individual predicted clearance vs. age (B) the individual predicted clearance normalized by body weight vs. age (C) the individual predicted central volume of distribution vs. age (D) the individual predicted central volume of distribution normalized by body weight vs. age

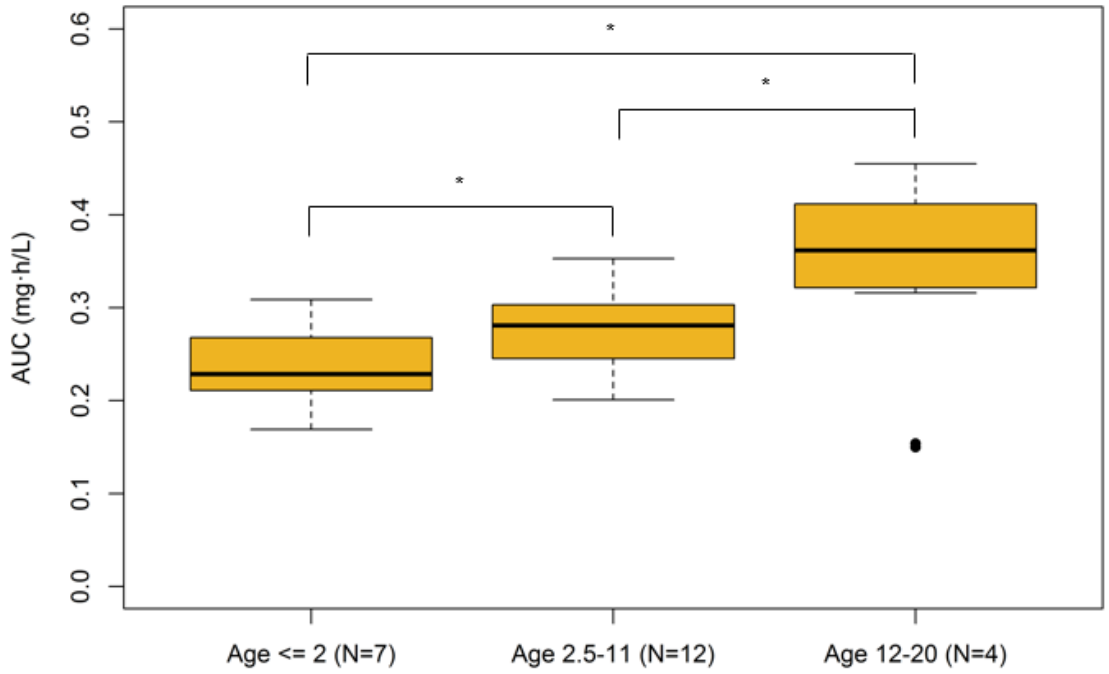


Figure 19. Boxplot of individual predicted AUC among different age groups

*One-way ANOVA at P < 0.001

identity, indicating that the model adequately described the concentration-time profile of unbound MPA. All WRES fell within 3 standard deviations, demonstrating good predictability of the model. No particular trend was observed in checking residuals.

No significant difference was observed in body weight-adjusted clearance between females (median 38.6 L/h/kg [21.9-44.3]) and males (median 33.6 L/h/kg [26.9-48.6]), as shown in Figure 17. As expected, absolute clearance (L/h) and central volume of distribution (L) for unbound MPA increased with age due to the increasing body size (Figures. 18A & C). Absolute clearance reached a plateau in children older than 5 years of age (Figure 18A), which was comparable to that in adults. In contrast, body weight-normalized clearance decreased with increasing age (Figure 18B), leading to increases in unbound MPA exposure (Figure 19). At 15 mg/kg of MMF, unbound MPA exposure was significantly lower in infants (median 0.23 mg·h/L [0.17-0.31]) compared to children (median 0.28 mg·h/L [0.20-0.35]) and adolescents (median 0.36 mg·h/L [0.15-0.45]) at $p < 0.001$ (Figure 19). A weak trend towards an increased patient age with lower body weight-adjusted central volume of distribution was identified (Figure 18D).

5.5 Discussion

In this study, a population pharmacokinetic model was developed for unbound MPA in pediatric and adolescent HSCT patients. Patient recruitment for pediatric trials is a challenge at multiple levels, including the ethical and logistical difficulties in obtaining adequate samples. The present study was performed in 23 subjects undergoing intravenous infusion of MMF. Sparse sampling design strategy was employed, in which

randomly timed but accurately recorded blood samples were collected. Population pharmacokinetic analysis is well-suited to analyze both sparsely and intensively sampled data to provide estimates of pharmacokinetic parameters, intra- and inter-individual variability in these parameters, and patient-specific covariates explaining inter-individual variability. It is well-known that only the unbound drug is pharmacologically active and the total drug concentrations only partially reflect unbound concentrations. This study is of special clinical relevance as the pharmacokinetics of unbound MPA were well characterized over a wide age range of patients (2 month-20 years).

The data were well described by a two-compartment structural pharmacokinetic model with first-order elimination, which is consistent with the previous population analyses of unbound MPA in either pediatric or adult HSCT patients. Weight, creatinine clearance and total bilirubin have been reported as important covariates affecting unbound MPA pharmacokinetics in two previous population pharmacokinetic studies in pediatric and adult HSCT patients [34, 35]. But, even after accounting for these clinical covariates in the final model, the variability in unbound MPA pharmacokinetics remains large in those studies. No covariate except weight was found to be significant in the present analysis, which may be due to several factors, including the small sample size, hepatic and renal function, clinical status, and disease severity of the study population. The estimation of CL for a typical adult subject weighing 70 kg was comparable with one of the previously published models for unbound MPA (1720 L/h vs. 1610 L/h) [35]. However, our population estimates of inter-compartmental clearance and volumes of distribution in the central and peripheral compartments were inconsistent with values previously published in the

literature. This could be due in part to the range of body weights represented in the study as a result of including infants, children and adults. In addition, the sparsely sampled data provided limited information about distribution parameters under a two-compartment model. The estimates of inter-individual variability for all pharmacokinetic parameters were high (RSE ranging from 35 to 71%), indicating a higher degree of uncertainty. The estimate of residual unexplained variability, representing a combination of intra-individual variability, measurement error and model misspecification, was high (approximately 66%), which could be partly attributed to the nature of the clinical study design. Other clinical or patient-specific factors that were not tested in this analysis might also play a role. Potential factors include changes in the patients' clinical status and differences in coadministered medications. Evaluation the effects of coadministered medications is of clinically importance given that HSCT patients may receive many other medications concurrently with MMF. Corticosteroids may induce the expression of several UGT enzymes, resulting in enhanced unbound MPA clearance [12]. Cyclosporine, an MRP2 inhibitor, reduces the biliary excretion of MPAG and enterohepatic circulation of MPA. Zeng et al combined three groups of patients (blood or marrow/kidney/liver transplant recipients) to detect significant covariate effects in a population pharmacokinetic approach, and found that co-administered cyclosporine as opposed to tacrolimus resulted in a mean increase in total MPA clearance of 63% [33]. Moreover, chemotherapy, infection and disease states such as acute GVHD may also alter unbound MPA disposition. However, these factors were not evaluated in this study due to the small sample size and a lack of detailed information on clinical status.

Growth and development can be investigated using easily observable demographic factors such as weight and age. In children, the allometric model is commonly used to predict pharmacokinetic parameters from body weight and adult values, and thus to predict doses. Weight can be centered at 70 kg and standard fixed allometric exponents of 0.75 for clearance terms and 1.0 for volume of distribution terms are applied [125]. A broad range of weight (5.4-113.3 kg) was observed in our study population. In order to obtain a stable base model, an allometric size adjustment with fixed coefficients of 0.75 for clearance and 1.0 for volume of distribution, was performed a priori within the development of the structural model before other covariates were evaluated. This is crucial in screening other potential covariate effects due to the frequently observed high collinearity between size and other developmental- or maturation-related covariates in pediatric pharmacokinetic data [127]. This approach has often been used in population-based analyses of data obtained from pediatric patients [128, 129]. There is controversy over whether or not allometric coefficients should be fixed or estimated independently for the drug of interest. Estimates of the allometric coefficient may be quite imprecise depending very much on the number of subjects and the distribution of weight in the sample. The exponents of 0.75, 0.80 and 0.85 have been reported to provide the same degree of accuracy of error in the prediction of clearance in children [125]. Given the small sample size (n=23) and weight ranges (5.4-113.3 kg) of our study population, the allometric coefficient was fixed to 0.75 for clearance terms and 1.0 for volume distribution terms.

The allometric model plays a significant role in determining the pharmacokinetics of many drugs and consequently drug doses for children, based on weight and adult estimates. However, it is important to appreciate its limitations on neonates and infants because progressive changes in organ maturation, body composition, and ontogeny of drug elimination pathways have marked effects on pharmacokinetic parameters in the first few years of life. Expression of UGT enzymes undergoes an independent rate and pattern of maturation. Most enzymes have matured to adult activity levels by the first 2 years of life [130-132]. Dramatic increases in renal function occur in the ensuing postpartum period, and glomerular filtration rate (GFR) normalized by body weight has approached the adult value by 6 months of age [133]. Total body water constitutes 75% of the body weight in the full-term neonates, decreases to approximately 60% at 5 months, and remains relatively constant after 5 months. Albumin, lipoprotein and glycoprotein concentrations change over the first year, which could affect drug binding. The fixed 3/4 allometric function is inappropriate to predict drug clearance in young pediatric patients because it may overestimate the clearance for neonates and underestimate the clearance for infants, as noted in other studies [134-137]. Neonates tend to have approximately 1.3- to 2.8- fold larger distribution volumes (per unit body weight) as compared to adults [138]. Consequently, allometric scaling alone is insufficient to predict pharmacokinetic parameters in neonates and infants. Ideally, pharmacokinetic parameters should be described in an individual reflecting the effects of size, maturation and organ function [126].

In this study, the weight-adjusted central volume of distribution decreased with age, which might be due to differences in body composition or protein binding. About 30% of subjects

were younger than 2 years of age in the study population. We attempted to evaluate the patient age as a covariate to characterize the potential maturation effect. However, the model was over-parameterized and successful estimation and covariance steps could not be achieved, probably due to the small sample size. Future studies should be pursued to include a large number of patients less than 2 years of age, as these analyses may yield important information about the ontogeny of hepatic and renal systemic clearance pathways related to the unbound MPA pharmacokinetics.

There have been contradictory reports regarding the effect of sex on MPA clearance. Tornatore et al reported that total MPA clearance in adults was influenced by sex with males having more rapid apparent clearance than females in renal transplant recipients [106, 139]. In another report by van Hest et al, an 11% total higher MPA apparent clearance was observed in males than females, who underwent kidney transplant [105]. In our study, sex was not a significant covariate during likelihood ratio test and was not included in the final pharmacokinetic model. This is consistent with one of the previously published studies on unbound MPA pharmacokinetics in HSCT patients [35]. However, this finding must be verified in larger studies, because the small sample size may contribute to our inability to identify other significant covariates beyond body weight.

Therapeutic targets for mycophenolate therapy in pediatric and adult HSCT remain poorly defined. Using similar total AUC targeting of 30-60 mg·h/L as in solid organ transplantation, Haentzschel et al individualized MMF dose regimens for GVHD prophylaxis in 29 adult HSCT patients. The results suggest that targeting of MPA exposure is feasible and effective in the early phase after HSCT [55]. Given their small sample size (29 patients)

and significantly higher doses than previously reported to achieve the target AUC, it may be premature to suggest MMF dosing guidelines. A relationship between unbound MPA AUC and the clinical outcome was defined by Jacobson et al in 87 adult HSCT patients who were receiving 1 g of MMF twice daily with concomitant cyclosporine. It is reported that an unbound AUC_{0-6} less than 0.15 mg·h/L (150 ng·h/mL) of unbound AUC_{0-12} less than 0.3 mg·h/L (300 ng·h/mL) in the first week post HSCT are both associated with a greater cumulative incidence of grade II-IV acute GVHD than the patients with a greater AUC ($P \leq 0.05$), indicating the current practice of dosing regimen in adult patients (1g twice daily) provides inadequate plasma concentrations [23].

In the present study, all patients were given a 15 mg/kg intravenous infusion MMF dose. Less than 50% of patients achieved an unbound AUC_{0-8} above 0.3 mg·h/L, calculated based on individual dose and clearance, suggesting that the dose increases might be beneficial for pediatric patients. Based on a population pharmacokinetic/dynamic modelling approach, Li et al described the overall relationship between MPA concentration and IMPDH activity by a direct inhibitory E_{max} model with an IC_{50} = 3.23 μg/ml of total MPA and 57.3 ng/ml of unbound MPA in adult HSCT recipients [48]. The IMPDH activity displays a high inter-individual variability (coefficient of variation 40.2%) in healthy pediatric (2.0–17.9 years, n=106) and adult (18.7–67.3 years, n=106) subjects, but there is no age-related differences in IMPDH activity in these healthy individuals. In addition, a comparable inhibition of IMPDH activity (expressed by IC_{50}) by total MPA has been observed between children (2.0–11.9 years, n=8) and adolescents (12.0–18.9 years, n=9) early post renal transplantation [140]. If we assume the same relationship between

unbound MPA concentration and IMPDH activity exists for all HSCT patients, less than 50% of the unbound MPA concentrations observed in our study were above the reported IC_{50}. Given the small number of subjects and significant heterogeneity among subjects, the relationships between unbound MPA pharmacokinetic parameters and clinical outcomes after HSCT were not evaluated in this analysis. Therefore, large-scale prospective pharmacokinetic-pharmacodynamic studies in pediatric HSCT patients are urgently needed to better understand the relationships between unbound MPA exposure and clinical outcomes, and to define therapeutic targets in HSCT patients.

In the present study, the weight-adjusted unbound MPA clearance continuously decreased with increasing age, and was the highest for the youngest patients, resulting in substantial differences in the unbound MPA AUC among different age groups, with lower values for younger patients. Bhatia et al demonstrated age-dependent total MPA pharmacokinetics in HSCT patients. Following intravenous or oral administration of MMF, patients under 6 years of age exhibited a significantly higher weight-adjusted clearance compared to older children and adolescents [37]. Zeng et al reported that children with a bodyweight of 10 kg receiving standard MMF dose regimens after HSCT, achieved an total MPA AUC below the target range (30-60 mg·h/L), suggesting children who weigh below 10 kg might have a higher total MPA clearance and need a higher MMF dose compared to heavier children [33]. Filler et al also demonstrated a negative correlation between the dose required for total MPA AUC of 60 mg·h/L and age, when studying 27 renal transplant recipients (1.8-20.7 years) receiving a combination therapy of MMF and tacrolimus. The data suggest that young children under 2 years of age require almost

double the dose of MMF in combination with tacrolimus when compared with adolescents [141]. Enhanced excretory capacity of the kidney has been reported in toddlers (1- 3 years) when normalized to body weight, in which there is an “overshoot” of the glomerular filtration rate well above the levels observed in older children and adults, and there is an early reaching of adult levels in active drug secretion. Due to the high GFR in toddlers, it is not surprising that they often require higher weight-normalized doses for renally excreted drugs compared with adults [142]. Therefore, body weight-adjusted MMF dosing is not reliable for the post-transplant immunosuppression in pediatric HSCT patients. To achieve similar MPA exposure as in adult HSCT recipients, either the MMF dose would need to be increased or the dosing interval can be shortened in pediatric HSCT patients.

5.6 Limitations

The main limitation of this study was the sparse sampling study design, which may have contributed to our inability to develop more complex models and identify other significant covariates for population pharmacokinetics of the unbound MPA. A more robust design will improve population and individual estimates under a two-compartment model, and allow us to detect other important covariate-parameter relationships explaining inter-individual variability in unbound MPA pharmacokinetics. In addition, the validity of the final model was evaluated using goodness-of-fit plots. We failed to use bootstrapping to validate the final model due to the diversity of subjects in the dataset (e.g., heterogeneous and sparse data), and the time-varying nature of the clinical status, which would result in bootstrap datasets not representative for the original dataset.

5.7 Conclusions

In conclusion, unbound MPA population pharmacokinetics in pediatric and adolescent HSCT patients were well described by a two-compartment structural model. Allometric scaling of pharmacokinetic parameters allowed for simultaneous analysis of concentration-time data from pediatric and adolescent patients. Prescribed MMF doses are still empirical, because therapeutic targets for mycophenolate therapy are yet to be defined in HSCT. The current dose regimens may not be optimal for pediatric patients to achieve the exposures similar to those achieved in adults. This model may have important clinical implication for further development of individualized MMF dosing strategies in pediatric and adolescent HSCT patients.

Chapter 6 Summary

MMF (CellCept®) was approved by the FDA in 1995 for acute rejection in renal transplant patients. Recently, it is increasingly used in the prophylaxis and treatment of acute and chronic GVHD after HSCT. Following oral administration, the prodrug MMF is rapidly absorbed and hydrolyzed to the active moiety MPA. MPA is further metabolized to the major inactive metabolite MPAG by UGTs and excreted in the urine as MPAG (87%). Extensive plasma protein binding has been observed in patients with normal hepatic and renal function, 97-99% for MPA and 82% for MPAG. In healthy individuals and organ transplant patients, MPAG undergoes significant enterohepatic circulation. However, the pharmacokinetics of MPA is altered in HSCT patients. Due to the significant pharmacokinetic variability and broad ranges of transplants, the optimal MMF dosing and preferred targets are still under investigation in HSCT patients. In this thesis work, we aim to identify the sources of pharmacokinetic variability and characterize the population pharmacokinetics of unbound MPA in HSCT patients. These information, if incorporating into MMF regimen design and modification, may contribute to the rational dose selection of MMF in HSCT recipients.

Chapter 1 extensively reviews the pertinent information regarding the pharmacokinetics/dynamics of MPA in HSCT patients. Important aspects of pharmacokinetics in HSCT recipients include decreased enterohepatic circulation, lower oral bioavailability, shorter half-life and higher clearance compared to healthy individuals and renal transplant patients. In addition, clearance appears to be higher in younger

patients than in adult patients. So they may require relatively higher MMF doses per body mass to achieve similar MPA exposures as adults. The relationship between MPA exposures and clinical efficacy is still obscure and optimal targets have yet to be defined in HSCT patients, especially in pediatric HSCT patients. Emerging pharmacometric methodologies, especially population pharmacokinetic models, could provide us with new venues for further research on the optimization of MMF therapy. Hence, multi-institutional trials including adequately powered pharmacokinetic and pharmacodynamic studies are required to assess the impact of MPA exposures on clinical outcomes of HSCT and further help justify appropriate clinical dosing regimens.

Acyl glucuronide conjugates are known to have a limited stability under neutral or slightly alkaline conditions. In chapter 3, we developed and validated a reliable UPLC-MS/MS assay for simultaneous quantification of MPA and its glucuronide metabolites (MPAG and AcMPAG) in human plasma samples per US FDA Guideline. Furthermore, we proposed extended stabilization procedures to improve the accuracy of the analysis before routine application. The merits of this assay include the efficient sample clean up by protein precipitation, high sensitivity with LLOQ of 2 ng/ml for AcMPAG, small sample volume of 50 µl for processing, and short chromatographic run time of 5 min. Based on this assay, the reported large intra- and inter-individual pharmacokinetic variability could be characterized.

MPA displays extensive plasma protein binding and glucuronidation into MPAG. In chapter 4, we investigated the pharmacokinetic variability of MPA, from plasma protein binding and metabolism perspectives, in both pediatric and adult HSCT patients. Since lower free

and total AcMPAG concentrations were observed in the study population, only the phenolic glucuronide metabolite were quantified in this study. Plasma protein binding was found to increase 1 month post engraftment in adult HSCT patients. Males had significantly higher plasma protein binding than females for both pediatric and adult HSCT patients. Moreover, the MPAG/MPA ratios on an mg/kg dose basis in adult patients were significantly higher than those in pediatric patients. Therefore, time-dependent changes in plasma protein binding could contribute to the intra-individual variation in adult patients post HSCT. Age-dependent metabolic ability, as well as sex-related plasma protein binding could contribute to the inter-individual variation. Knowing these sources of pharmacokinetic variability of MPA can provide fundamental information to enhance our understanding of the complex pharmacokinetics of MPA, and further contribute to the more effective individualization of MMF dosing in pediatric and adult HSCT patients.

Characterization of the pharmacokinetics of unbound MPA is of clinical importance to further optimize MMF dosing regimens, firstly because the unbound MPA concentrations are ultimately responsible for the inhibition of IMPDH and prevention of graft rejection, and secondly because substantial intra-patient and inter-patient variability in the bound fractions of MPA have been observed in our study. Despite the increased use of MMF, only very limited information is available regarding the pharmacokinetics of unbound MPA in pediatric HSCT patients, especially the very young. In chapter 5, we developed a population pharmacokinetic model for unbound MPA in pediatric and adolescent HSCT patients. Allometric scaling of pharmacokinetic parameters allowed for simultaneous analysis of concentration-time data from pediatric and adolescent patients. Similar weight-

adjusted clearance was observed between males and females. Because of age-dependent differences in weight-adjusted clearance, the calculated unbound MPA AUC was higher in younger patients compared with those in older patients receiving 15 mg/kg MMF. Therefore, the current dose regimens may not be optimal for pediatric patients to achieve the exposures similar to those achieved in adults. This model may have important clinical implication to make more informed decisions regarding appropriate dosing regimens of MMF in pediatric HSCT patients.

Taken together, this thesis represents great efforts towards a better understanding of pharmacokinetic variability of MPA, and provides unique knowledge about the population pharmacokinetics of unbound MPA in pediatric and adolescent HSCT patients. We anticipate that our research may have important clinical implication for further development of individualized MMF dosing strategies in HSCT patients.

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