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Mycophenolic Acid Pharmacokinetics and Clinical Outcomes in Renal Transplantation: Effect of *ABCC2* Haplotype Analysis and Distribution into Lymphocytes and Kidney

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Discipline of Pharmacology

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Abstract

Mycophenolic acid (MPA) is an immunosuppressant commonly used to prevent rejection following renal transplantation. MPA prevents graft rejection through selective inhibition of inosine monophosphate dehydrogenase (IMPDH) enzyme in lymphocytes. It displays considerable inter-individual pharmacokinetic (PK) variability, and many factors, other than dose or total plasma concentrations, may impact on lymphocyte MPA concentrations. There is evidence that the multidrug resistance-associated protein 2 efflux transporter may influence MPA PK in plasma and affect its distribution into lymphocytes. Therefore, the major aim of this thesis was to investigate the influence of *ABCC2* genetic polymorphisms as a determinant of inter-individual variability in MPA treatment response, as well as clinical outcomes. This thesis also set out to investigate intra-lymphocyte pharmacokinetic-pharmacodynamic analyses of MPA as predictors of graft rejection.

The first major study of this thesis incorporated the development and validation of new LC-MS/MS methods for the quantification of MPA concentrations in human kidney biopsies (Chapter 2) taken as part of routine clinical procedures, and lymphocytes (Chapter 4), and applying these new methods in a pilot prospective clinical study of the utility of determining intra-renal and lymphocyte MPA concentrations as predictors of graft rejection.

For the second major study (Chapter 3) of this thesis, recipients (n = 60) prescribed MMF, tacrolimus and prednisolone-based immunosuppression, and their donors (n = 46) were retrospectively genotyped for three common single nucleotide polymorphisms (SNPs) of *ABCC2* (-24 C > T, 1249 G > A, 3972 C > T), haplotyped and patients were divided into wild-type, low or high *ABCC2* expressor group. Whilst no relationship was observed between donor expressor status and MPA PK parameters, recipients with the high *ABCC2* expressor haplotype (CAC, -24C/1249A/3972C, variant at 1249) had significantly higher

MPA trough (C_0) concentrations, presumably due to an increased enterohepatic recirculation of MPA, compared to recipients with low expressor haplotypes. The multivariate analyses indicated that MPA C_0 concentrations were associated with recipient *ABCC2* expressor phenotype, rejection incidence and peak panel reactive antibody.

The third major study (Chapter 4) of this thesis investigated the concentrations of MPA and IMPDH activity in lymphocytes, as predictors of rejection in 48 renal transplant recipients. Lymphocyte MPA concentration was the only covariate independently associated with rejection incidence in the multivariate analysis, suggesting that lymphocyte concentrations may be the best indicator of MPA therapeutic efficacy compared to monitoring plasma concentrations alone. ROC curve analyses revealed that lymphocyte MPA concentration was a better predictor of severe (cellular/vascular) rejection compared to IMPDH activity or plasma concentrations. IMPDH activity was not a significant predictor of graft rejection, however, a single measurement of IMPDH activity at pre-dose may have accounted for the lack of significant relationship between IMPDH activity and rejection.

In summary, *ABCC2* genetic variability may be important in determining exposure to MPA and may have clinically significant impacts on MPA treatment response. Despite monitoring plasma concentrations to maintain MPA efficacy and minimise the consequences of over-exposure, inter-individual differences in MPA plasma PK are still difficult to predict, but may be accounted for by directly measuring lymphocyte MPA concentrations.

Declaration

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Publications in support of this thesis

Refereed Journal Articles

Md Dom ZI, Noll BD, Coller JK, Somogyi AA, Russ GR, Hesselink DA, van Gelder T, Sallustio BC. Validation of an LC-MS/MS method for the quantification of mycophenolic acid in human kidney transplant biopsies. *J Chromatogr B Analyt Technol Biomed Life Sci* 2014; 945-946:171 – 177.

Refereed Conference Papers

Md Dom ZI, Coller JK, Somogyi AA, Sallustio BC. Impact of Recipient and Donor Multidrug Resistance Protein 2 Genetic Variability on Mycophenolic Acid Pharmacokinetics Following Kidney Transplantation. *Ther Drug Monit*. 2013; 35:657 – 735.

Md Dom ZI, Westley IS, Coller JK, Somogyi AA, Sallustio BC. Multidrug Resistance-Associated Protein 2 (MRP2/ABCC2) Haplotypes Significantly Affect the Pharmacokinetics of Mycophenolic Acid in Renal Transplant Recipients. *Public Health Genomics* 2015; 18:1 – 51.

Md Dom ZI, Coller JK, Carroll R, Somogyi AA, Sallustio BC. Intra-Lymphocyte Concentrations of Mycophenolic Acid Correlate With the Incidence of Early Graft Rejection in Renal Transplant Recipients, 2015 American Transplant Congress Abstracts. *Am J Transplant*. 2015; 15(S3):1 – 1.

Abbreviations

ABC	ATP-binding cassette
ABCC2	ATP-binding cassette, sub-family C, member 2
AcMPAG	Mycophenolic acid acyl glucuronide
Af	African
AMP	Adenosine 5'-monophosphate
AR	Acute rejection
As	Asian
AT1R	Angiotensin II type-1 receptor
ATP	Adenosine triphosphate
AUC	Area under the concentration-time curve
bp	Base pair
C_0	Trough
C_{0L}	Trough lymphocyte
C_{0P}	Trough plasma
Cau	Caucasian
C_{av}	Average plasma
CIT	Cold ischemia time
CL	Clearance
CL_R	Renal clearance
C_{max}	Maximum drug concentration
C_{Pmax}	Plasma MPA peak concentration
cMOAT	Canalicular multispecific organic anion transporter
CNI	Calcineurin inhibitor
CsA	Cyclosporine
C_u	Unbound
CV	Coefficient of variation
dGDP	Deoxyguanosine diphosphate
DGF	Delayed graft function
dGTP	Deoxyguanosine triphosphate
DJS	Dubin-Johnson syndrome
DNA	Deoxyribonucleic acid

dNTPs	Deoxynucleoside triphosphates
DSA	Donor-specific HLA antibodies
EC-MPS	Enteric-coated mycophenolate sodium
EDTA	Ethylenediaminetetraacetic acid
EHBR	Eisai hyperbilirubinemic
EMIT	Enzyme-multiplied immunoassay technique
F	Forward primer
FDA	United States Food and Drug Administration
FKBP	FK binding protein
f_u	Free fraction
g	Gram
G1	Gap 1 phase
G2	Gap 2 phase
GDP	Guanosine diphosphate
GFR	Glomerular filtration rate
GMP	Guanosine monophosphate
GMP	Guanosine monophosphate
GTP	Guanosine triphosphate
GY	Groningen yellow
hr	Hour
HEK	Human embryonic kidney
HGPRTase	Hypoxanthine guanine phosphoribosyltransferase
HLA	Human leukocyte antigen
HPLC	High-performance liquid chromatography
IL-2	Interleukin-2
ILAb	IL-2 antagonists
IMP	Inosine monophosphate
IMPDH	Inosine monophosphate dehydrogenase
IS	Internal standard
IV	Intravenous
kb	Kilobase
kDa	KiloDalton
k_m	Michaelis constant
LC-MS/MS	Liquid chromatography-tandem mass spectrometry

LLOQ	Limit of quantification
LSS	Limited sampling strategies
MAP	Maximum *a posteriori*
MeOH	Methanol
Min	Minute
MMF	Mycophenolate mofetil
MPA	Mycophenolic acid
MPA-d3	Deuterated mycophenolic acid
MPAC	Carboxy-butoxy ether mycophenolic acid
MPAG	Mycophenolic acid phenolic glucuronide
MRM	Multiple reaction monitoring
mRNA	Messenger RNA
MRP2	Multidrug resistance-associated protein 2
mTOR	Mammalian target of rapamycin
n	Number
N	Nucleotide
NFAT	Nuclear factor of activated T-cells
OATPs	Organic anion transporter polypeptides
OR	Odds ratio
PBMC	Peripheral blood mononuclear cell
PCR-RFLP	Polymerase chain reaction-restriction fragment length polymorphism
PD	Pharmacodynamics
*PE-d*HPLC	Primer extension denaturing HPLC
PGx	Pharmacogenetics
PK	Pharmacokinetics
PNP	Purine nucleoside phosphorylase
PPA	N-phthaloyl-L-phenylalanine
PRA	Panel reactive antibody
PRPP	5- Phosphoribosyl-1-pyrophosphate
QC	Quality control
R	Reverse primer
R^2	Coefficient of correlation
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid

ROC	Receiver operating characteristic
S	Synthesis phase
SD	Standard deviation
Sec	Second
SNP	Single-nucleotide polymorphism
SRL	Sirolimus
T	Time (hr)
TAC	Tacrolimus
TBA	Tetrabutylammonium hydrogen sulphate
t-BME	Tertiary-butyl methyl ether
TDM	Therapeutic drug monitoring
TGA	Therapeutic Goods Administration
t_{max}	Time to reach C_{max}
TR-	Transport-deficient
U	Unit
UGTs	Uridine diphosphate-glucuronosyltransferases
UV	Ultraviolet
V	Variant
V_{max}	Maximum velocity
WT	Wild-type
XMP	Xanthine monophosphate

Chapter 1: General Introduction

Chapter 1. General introduction

This thesis addresses renal disease, in particular transplantation, the only curative therapy for patients with end-stage renal disease. Immunosuppressive drug therapy (mainly cyclosporine (CsA), tacrolimus (TAC), and mycophenolic acid (MPA) – the subject of this thesis) is a major contributor to successful management of transplant recipients. Whilst the use of immunosuppressants has markedly improved graft and recipient survival following transplantation, there remains substantial inter-individual variability in outcomes partly due to lack of understanding of their tissue pharmacokinetics (PK) and pharmacogenetics (PGx) – mainly the kidney and lymphocytes. Therapeutic drug monitoring (TDM) has been implemented to individualise dosage selection based on plasma concentrations, although this often inadequately explains the variability in outcomes. Consequently, there is a need for clinical tools that are better predictors of kidney and lymphocyte MPA concentrations, since they are not necessarily mirrored by plasma concentrations, as currently monitored with TDM alone. In addition, a better understanding of the factors determining MPA distribution into lymphocytes and kidney, and hence, clinical outcomes, is essentially important.

The membrane efflux transporter, multidrug resistance-associated protein 2 (MRP2), is firmly established as a determinant of intestinal, biliary and renal transport, and hence, elimination of a broad range of substrates, including MPA, but also regulates drug distribution and affects drug response, an area still poorly researched. Therefore, the polymorphic *ABCC2* gene, encoding MRP2, provides a promising potential source of variability in MPA response.

Before outlining the hypotheses and aims of this thesis, this introduction will provide an overview on the clinical PK and pharmacodynamics (PD) of MPA, the potential role of PGx

for the individualisation of MPA therapy, and finally it will discuss areas where additional research is warranted.

1.1 Renal transplantation in Australia

According to the Global Burden of Disease Study in 2013 [1], renal disease is one of the 10 leading causes or risk factors of fatal burden in Australia, with two therapeutic treatments currently available, dialysis and renal transplantation. Relative to dialysis, renal transplantation is the preferred option for renal failure due to improved long-term outcomes and cost-effectiveness, and prolonged patient lifespan [2, 3]. In Australia, a record 914 renal transplantations (the highest number ever) were performed in 2014, as compared to 883 transplantations performed in 2013 [4]. Acute rejection episodes are still a major problem in renal transplant recipients especially in the early post-transplant period, with rejection rates of 13 – 24 % in the first six months of transplantation in 2013 [4].

1.1.1 Immunosuppressive therapy

To date, a range of immunosuppressant drugs has been developed and used in different combinations for induction and maintenance therapy, and for treatment of acute rejection episodes (Table 1.1). Antibody-based induction immunosuppression (such as anti-interleukin-2 receptor monoclonal antibody drugs) is initiated in the early post-transplant period, and is followed by maintenance immunosuppression, which typically involves triple therapy with mycophenolate mofetil (MMF), prednisolone and either a calcineurin inhibitor (CNI) (such as TAC or CsA) or less frequently a mammalian target of rapamycin (mTOR) inhibitor (such as sirolimus or everolimus). In Australia, MMF, TAC and prednisolone were the most commonly prescribed immunosuppressants at initial treatment for deceased donor grafts, accounting for 60 %, 88 % and 90 %, respectively, of maintenance immunosuppression in 2014 [4].

When the initial phase of graft stabilisation is achieved, the doses of maintenance immunosuppression are usually tapered down (as opposed to early post-transplantation) by: (1) dose reduction or discontinuation of MMF; (2) dose reduction or withdrawal of corticosteroid; (3) dose reduction or elimination of CNI; or (4) substitution of CNI with sirolimus in transplant recipients who are primarily sensitive to the nephrotoxicity of CNI [5].

Table 1.1 Immunosuppressants used for induction and maintenance therapy, and for treatment of acute rejection episodes following renal transplantation [6-8].

Drug class	Drug name	Mechanism of action
Anti-interleukin-2 (IL-2) receptor antibodies[1]	Basiliximab; Daclizumab	Bind to the α-subunit (CD25) on activated T cells, which then inhibit IL-2 binding, resulting in inhibition of IL-2 induced T-cell activation
Anti-lymphocyte antibodies[1]	Anti-thymocyte globulin[3]	Binds to and blocks T-cell membrane proteins, leading to lysis and T-lymphocyte cell death via apoptosis
	Muromonab-CD3[3,4]	Binds to T-cell-associated CD3 complex, resulting in CD-3 cell death by inducing antibody-mediated cytotoxicity and apoptosis
	Alemtuzumab	Binds to CD52 antigen on all B- and T-cells, causing cell lysis
Anti-proliferative agents[2]	Azathioprine	Blocks the *de novo* and salvage pathway of purine synthesis, resulting in inhibition of DNA/RNA synthesis
	Mycophenolate mofetil	Inhibitor of inosine monophosphate dehydrogenase which is the rate-limiting enzyme in the *de novo* pathway of purine synthesis
Calcineurin inhibitors[2]	Tacrolimus	Binds to immunophilin, FK506-binding protein 12 (FKBP12), resulting in inhibition of calcineurin phosphatase and T-lymphocyte activation

	Cyclosporine	Binds to cyclophilin, resulting in inhibition of calcineurin phosphatase and T-lymphocyte activation
Corticosteroids[2]	Prednisolone, Methyl-prednisolone[3]	Inhibit leukocyte infiltration and expression of cytokines, causing suppression of T-cell activation
Mammalian target of rapamycin (mTOR) inhibitors[2]	Sirolimus, Everolimus	Bind to immunophilin, FKBP12, resulting in formation of sirolimus- or everolimus- FKBP12 complex, which inhibit the activation of mTOR and suppression of T-cell proliferation

[1]Induction immunosuppression; [2]Maintenance immunosuppression; [3]Induction immunosuppression and treatment of acute rejection; [4]Muromonab-CD3 has been discontinued and was unavailable from 2011.

1.2 Mycophenolic acid

Mycophenolic acid (MPA), originally isolated from a fermentation product of *Penicillium* [9], was initially developed as an anti-viral [10], anti-cancer [11] and anti-psoriasis [12] agent before its development as an anti-proliferative agent. Currently, two mycophenolate oral formulations are commercially available: (1) the 2,4-morpholinoethyl ester pro-drug of MPA, mycophenolate mofetil (MMF, a prodrug to enhance the oral bioavailability of MPA [13]) (CellCept®, Hofmann-La Roche Pharmaceuticals, Basel, Switzerland); and (2) the sodium salt of MPA, formulated as enteric-coated tablets of mycophenolate sodium (EC-MPS, new formulation) (Myfortic®, Novartis, Basel, Switzerland).

MMF (the main focus as MMF was used for the studies performed in this thesis, hence details on EC-MPS will not be further discussed) has been approved by the United States Food and Drug Administration (FDA) and the Therapeutic Goods Administration (TGA) in Australia as well as in many other countries worldwide (e.g. Europe, Canada, Japan), and is commonly used for prophylaxis of organ rejection in patients receiving renal, heart or hepatic transplants. As discussed in Section 1.1.1, MMF is administered in combination with

a CNI (TAC or CsA) or less frequently mTOR inhibitors and corticosteroids [6-8]. Currently, the manufacturer's guidelines for MMF dosages are: (1) an oral dose of MMF 1 g twice daily in adult renal transplant recipients, and (2) in the paediatric transplant population, an oral dose of MMF suspension 600 mg/m^2 administered twice daily [14].

1.2.1 Chemistry

According to the manufacturer's information [14], the chemical name for MMF is 2-morpholinoethyl-(E)-6-(1,3-dihydro-4hydroxy-6-methoxy-7-methyl-3-oxo-5-iso-benzofuranyl)-4-methyl-4-hexenoate, with an empirical formula of $C_{23}H_{31}NO_7$ and a molecular weight of 433.50. The structural formula for MMF is shown in Fig. 1.1A. MMF is a white to off-white crystalline powder and is soluble in acetone, methanol and ethanol. The MMF solubility increases in acidic pH (4.27 mg/mL at pH 3.6), however its solubility is lower in water (0.043 mg/mL at pH 7.4). The pKa values for MMF are 5.6 and 8.5 for the morpholino and phenolic groups, respectively, and the apparent partition coefficient (log P) in 1-octanol/water (pH 7.4) is 238 [14].

The chemical name of MPA is 6-(4-hydroxy-6-methoxy-7-methyl-3-oxo-5-phthalanyl)-4-methyl-4-hexenoic acid (Fig. 1.1B). MPA has an empirical formula of $C_{17}H_{20}O_6$ and a molecular weight of 320.3 [15]. MPA is soluble in methanol (50 mg/mL), chloroform, dichloromethane, ethanol and sodium hydroxide (10 mg/mL), forming a colourless to a very faint yellow solution. The pKa values for MPA are 4.6 and 8.5 for the carboxylic acid [15] and phenolic [14] groups, respectively, and the apparent partition coefficient (log P) in 1-octanol/water (pH 7.4) is 1.6 [15].

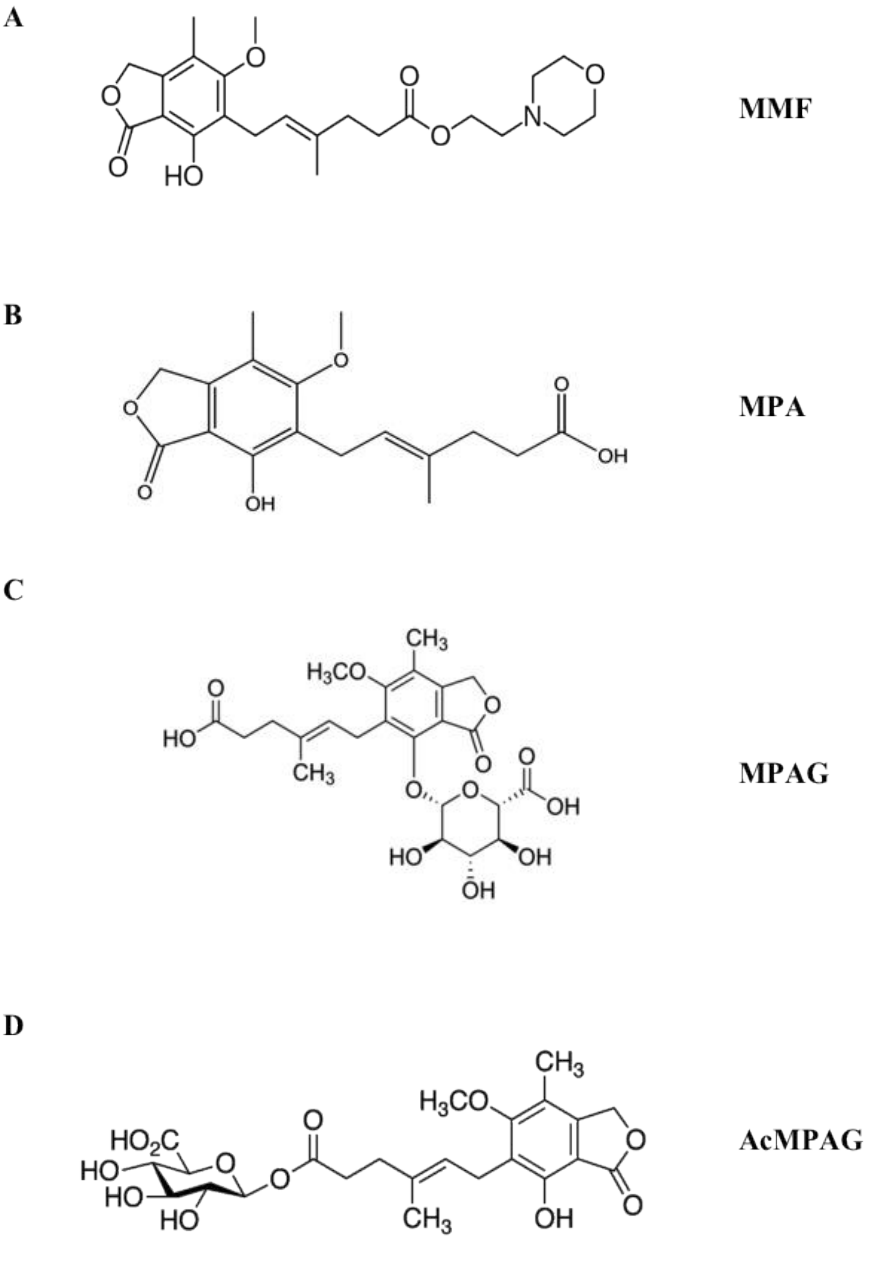

Figure 1.1 Chemical structures of (A) MMF, (B) MPA and its glucuronides: (C) the pharmacologically inactive phenolic glucuronide (MPAG) metabolite; and (D) the minor pharmacologically active acyl glucuronide (AcMPAG) metabolite.

1.2.2 Mechanism of action of MPA

1.2.2.1 Inhibition of inosine monophosphate dehydrogenase

Purine biosynthesis consists of two major pathways: the *de novo* and the salvage pathways (Fig. 1.2). The *de novo* purine pathway produces purine nucleotides that represent the building blocks of DNA synthesis and is fundamental to the production of DNA. 5-Phosphoribosyl-1-pyrophosphate (PRPP) synthetase, a crucial rate-limiting enzyme in the pathway of purine synthesis, is allosterically regulated by nucleotides, whilst inosine monophosphate (IMP) is a common intermediate in purine nucleotide biosynthesis and can be converted to guanosine monophosphate (GMP) as required [16].

MPA is a potent, selective, reversible, and non-competitive inhibitor of inosine monophosphate dehydrogenase (IMPDH), which is the rate-limiting enzyme in the *de novo* guanosine nucleotide pathway, converting IMP to GMP (Fig. 1.2) [17]. The *de novo* purine synthesis is required for lymphocyte progression through the cell cycle and proliferation, and GMP is an important building block for DNA, RNA and proteins. Unlike other cell types that can use the salvage pathway as an alternative supply of guanosine, T- and B-lymphocytes can only utilise the *de novo* pathway [16].

There are two types of IMPDH isoforms, IMPDH type I, which is expressed in all cell types and IMPDH type II, which is expressed only in activated lymphocytes [18-20]. MPA inhibits both IMPDH isoforms [17], however, the inhibition of IMDPH type II by MPA is approximately 5-fold greater than the type I isoform of IMPDH with the K_i values of 0.007 versus 0.033 μM, respectively [17]. Therefore, MPA has a more potent cytotoxic effect on lymphocytes than on other cell types, thereby preventing T- and B-lymphocyte proliferation, and inhibiting cell-mediated immune responses and antibody formation [21].

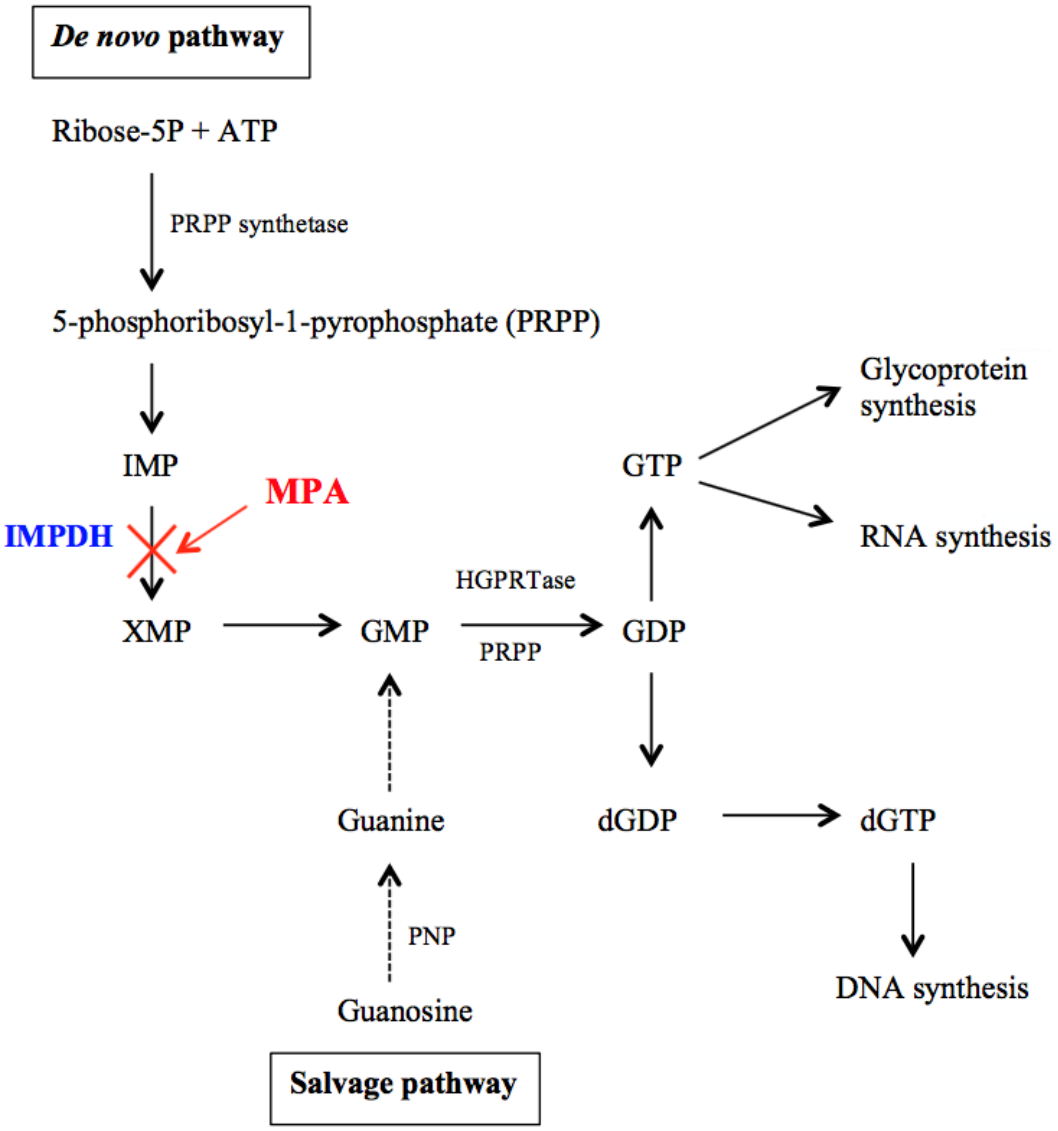

Figure 1.2 Schematic representation of purine synthesis consisting of two major cellular pathways: (1) the *de novo* and (2) salvage pathways of guanosine nucleotides biosynthesis. MPA inhibits IMPDH, a crucial enzyme involved in the *de novo* pathway. ATP, adenosine triphosphate; dGDP, deoxyguanosine diphosphate; dGTP, deoxyguanosine triphosphate; GDP, guanosine diphosphate; GMP, guanosine monophosphate; GTP, guanosine triphosphate; HGPRTase, hypoxanthine guanine phosphoribosyltransferase; IMP, inosine monophosphate; IMPDH, inosine monophosphate dehydrogenase; PNP, purine nucleoside phosphorylase; XMP, xanthine monophosphate.

1.2.2.2 Prevention of graft rejection

As discussed in Section 1.1.1, MPA is commonly administered in combination with CsA or TAC, and although TAC is structurally different to CsA, its mechanism of action is similar (Fig. 1.3). Similar to MPA, both CsA and TAC have potent immunosuppressive properties; CsA and TAC selectively bind to immunophilins; cyclophilin and FK506 binding protein, respectively, resulting in the formation of CsA-cyclophilin and TAC-FK506 complexes [22, 23]. Both drug-complexes inhibit the phosphatase activity of calcineurin within lymphocytes, thereby suppressing nuclear translocation and subsequent activation of nuclear factor of activated T-cells (NFAT) transcription factors (Fig. 1.3) [22, 23]. NFAT is the transcription factor required for the synthesis of interleukins, including IL-2 which is produced by activated T-cells and involved in the growth and proliferation of lymphocytes [24], and consequently, inhibition of IL-2 production produces immunosuppression. As discussed in Section 1.2.2.1, MPA is a potent inhibitor of IMPDH and selectively inhibits T- and B-lymphocyte proliferation. The inhibition of lymphocyte proliferation results in decreased expression of adhesion molecules and reduced recruitment of lymphocytes into the sites of inflammation [25], thus suppressing the production of nitric oxide and the formation of tissue-damaging peroxynitrite at rejection sites [26].

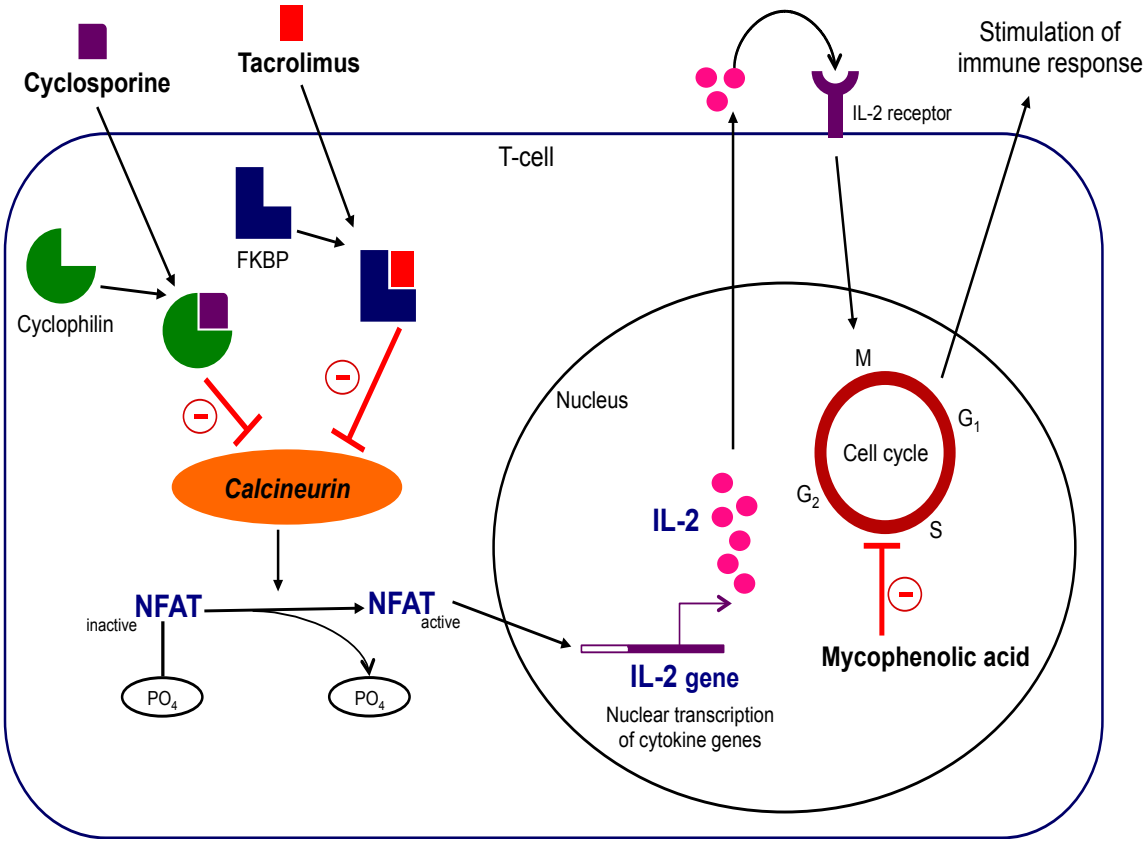

Figure 1.3 Mechanisms of T-cell-mediated immunosuppression showing the sites of action of CNIs and how MPA fits in with the mechanism of action of CNIs to inhibit lymphocyte proliferation. FKBP, FK binding protein; G1, gap 1 phase; G2, gap 2 phase; IL-2, interleukin-2; M, mitosis phase; NFAT, nuclear factor of activated T-cells; PO_4, phosphate; S, synthesis phase.

1.2.3 Clinical pharmacokinetics of MPA

1.2.3.1 Absorption and oral bioavailability

Following oral administration of MMF (Fig. 1.4), the drug is rapidly absorbed and completely metabolised by esterases in the liver, blood, stomach and small intestines to form the active entity, MPA [13]. Oral absorption of MMF is rapid and complete according to the manufacturer's information [14]. Following administration of a single oral dose of radiolabelled [mycophenolate$-^{14}$C] MMF to four healthy volunteers, a mean of 90.4 % of the total administered dose was recovered during the first 72 hours (hr) following administration. Following intravenous (IV) infusion over 48 hr, MMF rapidly disappears from the plasma and is undetectable after 10 – 30 minutes (min) [27]. In healthy volunteers receiving 1.5 g of MMF daily, the mean ± standard deviation (SD) absolute bioavailability of MPA following orally administered MMF relative to IV infusion of MMF based on MPA area under the plasma concentration-time curves over 24 hr (AUC_{0-24}) was 94.1 ± 16.2 % [27]. Another study in 9 cardiac transplant patients comparing the bioavailability of MPA (based on MPA AUC_{0-12}) between a 3 hr IV infusion (on day 3 and 5) with oral administration (on day 6 and 10) of MMF post-transplantation has reported the mean oral bioavailability of 95 % (range: 82.0 – 107.8 %) [28].

1.2.3.2 Distribution

The mean ± SD volume of distribution of MPA in healthy volunteers following oral and IV MMF administration was reported to be 3.6 ± 1.5 and 4.0 ± 1.2 L/kg, respectively [14]. In whole blood, 99.99 % of MPA is detected in plasma with only 0.01 % of MPA found in cellular elements [29, 30]. MPA is extensively bound to human serum albumin (97 – 99 %) in patients with normal renal and liver function [30, 31]. MPA does not bind significantly to

α_1-acid glycoprotein (750 mg/L) [30]. The primary metabolite of MPA, MPAG also binds approximately 82 % to human serum albumin in stable renal transplant recipients [32].

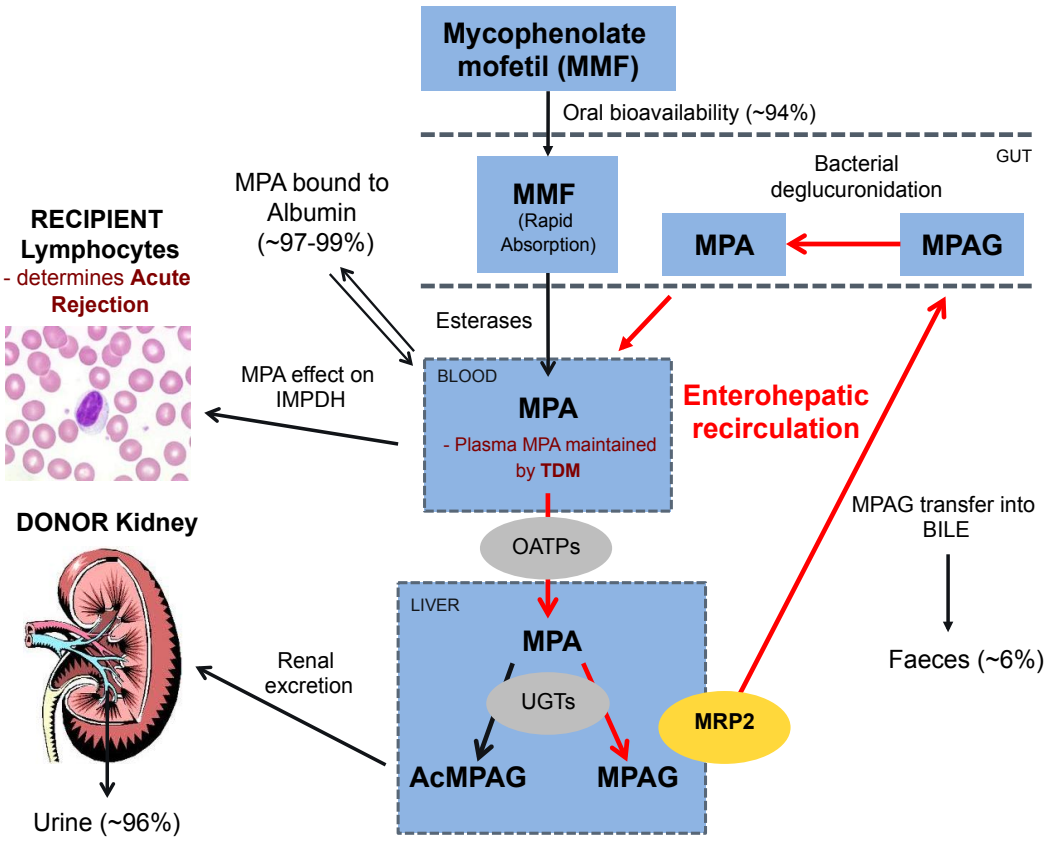

Figure 1.4 A graphical representation of pharmacokinetic behaviour of MMF. Pathways of enterohepatic circulation of MPA *via* the glucuronide conjugate metabolites are shown (red arrows). AcMPAG, mycophenolic acid acyl glucuronide; IMPDH, inosine monophosphate dehydrogenase; MMF, mycophenolate mofetil; MPA, mycophenolic acid; MPAG, mycophenolic acid phenolic glucuronide; MRP2, multidrug resistance-associated protein 2; OATPs, organic anion transporter polypeptides; TDM, therapeutic drug monitoring; UGTs, uridine diphosphate-glucuronosyltransferases.

Only free or unbound MPA is pharmacologically active and capable of inhibiting IMPDH [33]. Furthermore, higher exposure to free MPA results in an increased risk of MMF-related adverse events (e.g. leukopenia, infection) [34]. The percentage of free MPA is well correlated with plasma albumin concentrations [35]. The free fraction of MPA is decreased from 53.3 to 0.92 % with increasing concentrations of serum albumin (from 0.7 to 69 g/L). In contrast, the free fraction of MPA increased approximately 3- and 8-fold with increasing concentrations of MPAG (up to 475 mg/L) and sodium salicylate (from 10 to 500 mg/L), respectively [30]. The binding of MPA to plasma albumin is influenced by several factors including the availability of albumin binding sites and competition for these sites by urea and MPAG, which have been shown to compete with MPA for binding sites on serum albumin [36]. In addition, the binding of MPA and MPAG to plasma albumin can also be altered in patients with chronic renal dysfunction [37] and hypoalbuminemia [35], and because MPA is a restrictively cleared drug (refer to Section 1.2.3.5), these clinical conditions may cause an increase in the MPA free fraction (hence may affect total plasma concentrations but unbound MPA concentrations (C_u) are likely to be unaltered), and consequently a rise in the clearance (CL) of total MPA.

1.2.3.3 Metabolism

MPA is mainly metabolised in the liver and to a lesser extent in the kidney and gastrointestinal tract [38] by uridine diphosphate-glucuronosyltransferase (UGT) enzymes primarily into its main inactive metabolite, the pharmacologically inactive phenolic-glucuronide of MPA (MPAG, Fig. 1.1C and 1.4) [39, 40], important for enterohepatic recycling of MPA (refer to Section 1.2.3.4), and into the pharmacologically active acyl-glucuronide of MPA (AcMPAG, Fig. 1.1D and 1.4) [41]. In humans, the UGT isoforms responsible for the formation of MPAG and AcMPAG are UGT1A9 and UGT2B7, respectively [40, 42]. UGT1A9 contributes 55 %, 75 % and 50 % to the formation of MPAG

by the liver, kidney and gastrointestinal tract, respectively [40] while UGT2B7 is the only isoform responsible for AcMPAG production [39, 40, 42]. UGT1A7, UGT1A8 and UGT1A10 are also involved in the production of MPAG [39, 42] but to a lesser extent than UGT1A9. However, UGT1A7, 1A8 and 1A10 are present only in the gastrointestinal tract and kidney [38] and therefore are not responsible for MPAG production in the liver.

The pharmacologically inactive MPAG metabolite has been shown to have no inhibitory effect on IMPDH [33] however, the active AcMPAG metabolite (concentrations in plasma: 5.6 – 32.5 μg/L) displayed somewhat variable inhibitory effect compared with that of MPA (median (range) inhibition/concentration ratio of AcMPAG to MPA = 0.91 (0.48 – 1.23), n = 21) [43]. The AcMPAG metabolite has also been proposed to cause hypersensitivity and drug-related toxicity as a result of covalent binding to nucleic acids, proteins, and lipids in patients receiving MPA therapy [44]. However, the mechanisms involved in the development of these side effects are still not yet fully understood and thus requires further investigation.

1.2.3.4 Enterohepatic recirculation

MPA is subject to enterohepatic recirculation via biliary excretion of MPAG [45] where following its hepatic formation, MPAG is excreted into bile, deconjugated back to MPA through the action of bacterial *β*-glucuronidases in the gastrointestinal tract, subsequently returned to the circulation following reabsorption from the colon. The enterohepatic recycling of MPA, on average, contributes approximately 37 % (range 10 – 61 % in humans) of the total dose-interval MPA AUC and is reflected as a secondary MPA peak observed 6 – 12 hr after oral administration [32]. The kidney, however, is the main organ responsible for the irreversible excretion of both MPAG and AcMPAG metabolites [32] and multidrug resistance-associated protein 2 (MRP2) has been shown to be involved in renal and biliary

excretion of MPAG [46] (refer to Section 1.3). However, it is yet to be clearly established whether AcMPAG metabolite is subjected to biliary excretion, resulting in enterohepatic recycling of MPA, therefore, the question of whether AcMPAG may be involved in the enterohepatic recirculation remains to be investigated.

1.2.3.5 Clearance and elimination

The liver is the main organ for MPA elimination via metabolism to MPAG and AcMPAG. The kidney plays a minor role in the elimination of MPA; however, it plays a major role in the irreversible elimination of MPAG and AcMPAG glucuronides. The clearance of MPA is correlated with the free fraction of MPA [34]. Following oral administration of radiolabelled [mycophenolate$-^{14}$C] MMF (as discussed in Section 1.2.3.1), of the total radioactivity recovered (> 90 %), an average of 96.3 % and 5.5 % of the dose were recovered in the urine (almost exclusively as MPAG (Fig. 1.1) and faeces, respectively [32]. MMF was not recovered in urine and approximately 0.6 % and 0.3 % of MPA and AcMPAG (Fig. 1.1), respectively, were detected as minor urinary components [32]. Several renal excretory pathways for MPA and MPAG have been proposed, including reabsorption [27] or glomerular filtration [32] for MPA. MPAG and AcMPAG are thought to be mainly excreted into the urine and cleared predominantly via active tubular secretion, although it is yet to be clearly established and an area requiring further investigation. The MRP2 efflux transporter has recently been shown to be involved in renal excretion of MPAG and MPA has been identified as a substrate of MRP2 [46].

Following IV administration, the mean ± SD elimination half-life and plasma CL of MPA were reported to be 16.6 ± 5.8 hr and 177 ± 31 mL/min, respectively. The mean ± SD apparent half-life and plasma CL of MPA were estimated to be 17.9 ± 6.5 hr and 193 ± 48 mL/min, respectively, following oral administration [27]. Following IV and oral

administration, the mean ± SD apparent plasma CL of MPAG were estimated to be 44.7 ± 14.3 and 40 ± 8.6 mL/min, respectively, which was 5- to 4-fold lower than that of MPA [27]. The apparent elimination half-life for MPAG was similar to MPA (mean ± SD: 16.1 ± 5.2 (oral) and 21.8 ± 19 (IV) hr) [27].

1.2.4 Factors influencing MPA pharmacokinetics

Demographic factors

Previous studies reported a significant association between MPA PK parameters and patient gender. The MPA trough (C_0) concentrations were significantly increased in female renal transplant recipients compared to males [47]. In a population PK model, males have been reported to have an 11 % increased MPA CL than females [48].

A number of studies have investigated the influence of ethnicity on the PK of MPA. The incidence of acute rejection was more frequent in African-American renal transplant recipients (31.8 %) receiving 1 g of MMF twice daily compared to Caucasian transplant patients (15.7 %) [49]. In addition, African-American patients administered with a higher dose of MMF (1.5 g twice daily) had increased efficacy, judged by a decreased rate of acute rejection (12.1 % vs. 31.8 %) compared to African-American patients receiving 1 g of MMF twice daily following renal transplantation [49]. In another study, there was no significant difference in dose-normalised MPA or MPAG AUC_{0-12} between African-American and Caucasian renal transplant recipients at 6 months following transplantation [50]. The influence of ethnicity on MPA PK parameters, however, is an area still poorly researched and thus requires further investigation.

Interactions with other immunosuppressive agents

The influence of concomitant immunosuppressant drugs on the PK of MPA is shown in Table 1.2. Higher MPA concentrations (1.2 – 4-fold higher) were observed in transplant recipients receiving MMF in combination with TAC or sirolimus than those receiving CsA with MMF [34, 51-55]. MPA C_0 was significantly higher in patients co-administered with TAC and interestingly, the second MPA peak as a result of the enterohepatic recirculation (as discussed in Section 1.2.3.4), is more pronounced in patients receiving TAC or sirolimus as compared to those receiving MMF in combination with CsA [53, 54]. One underlying mechanism was initially attributed to the inhibition of MRP2-mediated biliary excretion of MPAG by CsA, resulting in reduced enterohepatic recycling of MPAG back to MPA and subsequent accumulation of MPAG [56-58]. However, this is now thought to be due to the inhibition of MPAG uptake into hepatocytes by the organic anion transporting polypeptides (OATPs) [59].

Interactions with other co-medications

In addition to the interaction with other immunosuppressant drugs, drug-drug interactions may also explain part of the inter- and intra-individual variability in the PK of MPA. In one case report, a novel, clinically drug interaction was reported between rifampicin and MMF; there was a two-fold reduction in MPA exposure when co-administered with rifampicin as a result of inhibition of enterohepatic recirculation of MPA [60]. This observation has been explained by an inhibitory effect of rifampicin on MRP2 transport activity, most likely due to the fact that rifampicin is a substrate for MRP2 [61], and this competitive mechanism could have contributed to the observed decrease in enterohepatic recycling of MPA. Therefore, failure to recognise this drug interaction could potentially lead to MPA

underexposure and loss of clinical efficacy. Other non-immunosuppressant drugs that may affect MPA disposition include iron supplements [62], antacids [63] and antiviral drugs [64].

Other factors

Impaired renal function (GFR < 25 mL/min) significantly reduces renal excretion of MPAG, causing concentrations to become markedly increased by 3- to 6-fold [65]. Hypoalbuminaemia decreases the number of binding sites available to MPA and MPAG. A previous study has identified a significant relationship between low serum albumin (≤ 31 g/L) and increased free MPA percentage in renal transplant recipients [35].

Dialysis (e.g. removal of MPAG by haemodialysis [66]) and other factors such as diet (e.g. food resulted in a 25 % reduction in maximum plasma concentration (C_{max}) and a time to reach C_{max} (t_{max}) of MPA was slightly delayed [63]), time after transplant (e.g. 30 – 50 % lower MPA AUC_{0-12} reported in the first few weeks compared to the 1 – 6 months post-transplant [32]), and genetics (e.g. will be discussed in detail in Section 1.3.1.4), may also account for some of inter-individual differences in the PK of MPA.

Table 1.2 Pharmacokinetic measures of MPA in Caucasian renal transplant recipients receiving different concomitant medications.

Concomitant medication	No. of patients	Follow-up time	Assay method[1]	MPA C_0 (mg/L)	MPA AUC_{12} (mg.hr/L)	Reference
TAC	18	≥ 3 weeks	HPLC	2.8 ± 0.3	50 ± 3.9	[55]
CsA	7			1.2 ± 0.4*	32.1 ± 6.3*	
TAC	33	Month 3	EMIT	3.6 ± 2.6	-	[54]
CsA	107			2.1 ± 1.2*	-	
TAC	10	Day 5	HPLC	1.6 (1.4 – 3.0)[2]	25.6 ± 11.1[3]	[34]
CsA	32			0.8 (0.6 – 1.3)[2,*]	20.8 ± 8.4[3,*]	
TAC	51	-	HPLC	3.2 ± 2.2	23.2 ± 10.6[4]	[53]
CsA	97			1.7 ± 1.0*	21 ± 16.7[4,*]	
SRL	15	≥ 6 months	HPLC	4.7 ± 2.4	70.9 ± 19.3	[52]
CsA	12			2.8 ± 1.6*	51.7 ± 16.7*	
SRL	13	Week 2	EMIT	5.2 ± 3.0	81 ± 36	[51]
CsA	17			1.3 ± 0.5*	43 ± 11*	

All patients received MMF 1 g twice daily and *$p < 0.05$ versus TAC or SRL. Data are presented as mean (± SD) unless specified otherwise.
[1]The different analytical assays for MPA quantification are shown as EMIT is less specific than HPLC for parent MPA [67]; [2]Data are median (Inter-quartile range); [3]Value calculated from AUC_{0-6} only; [4]Value calculated from AUC_{0-4} only.
AUC, area under the concentration-time curve; C_0, trough; CsA, cyclosporine; EMIT, enzyme-multiplied immunoassay technique; HPLC, high-performance liquid chromatography; SRL, sirolimus; TAC, tacrolimus.

1.2.5 Pharmacokinetic parameter studies

Numerous studies have investigated the concentration-time profile of MPA in renal transplant recipients with the most common PK parameters reported are the C_0 concentration, AUC, C_{max} and t_{max}. Table 1.3 summarises the MPA PK parameters studies in renal transplant recipients. Following the administration of MMF, the MPA maximum concentration usually occurs around 1 – 2 hr post-dose. The concentration-time profile of MPA often shows two peaks [68], in which the secondary MPA peak, due to the enterohepatic recirculation of MPA, is observed 6 – 12 hr after oral administration and contributes approximately 10 – 61 % of the total dose-interval MPA AUC [32]. It has been shown that the enterohepatic recycling of MPA is reduced in patients co-administered with CsA due to the inhibition of biliary excretion of MPAG into the bile.

As shown in Table 1.3, MPA displays complex and large between- and within- subject PK variability despite all patients receiving the same MMF dosage of 1 g twice daily. The variability in exposure to MPA is influenced by many factors and is discussed in Sections 1.2.3.2 and 1.2.4. Despite considerable effort on the part of the manufacturer to introduce MMF as a "one-size-fits-all" drug, and although a fixed MMF dosage (1 g twice daily) may have contributed to the extensive use of MMF in organ transplantation, the data that continue to emerge in the literature suggest that this may not be the best optimal therapeutic option for MMF therapy. There has been heightened interest to consider individualisation of MMF therapy (refer to Section 1.2.8) as opposed to giving the standard MMF dosage as a means of improving clinical outcomes.

Table 1.3 Comparison of MPA pharmacokinetic parameters in Caucasian renal transplant recipients receiving 1 g MMF twice daily.

No. of patients	Follow-up time	R^2 between C_0 and AUC_{0-12}	C_{max} (mg/L)[1]	MPA C_0 (mg/L)[1]	MPA AUC_{0-12} (mg.hr/L)[1]	Reference
54[3,4]	1 week	0.65	9.9 (1.5 – 42.3)[2]	1.1 (0.11 – 4.1)[2]	36 (3.1 – 95)[2]	[69]
18[3,5]	3 weeks	0.64	9.6 ± 5.6	1.8 ± 1.4	41 ± 19.4	[70]
24[3,5]	≤ 30 days	0.72	13.8 ± 7.0	2.0 ± 1.2	47.6 ± 15.2	[71]
10[3,4]	2 days	0.36	11.1 ± 1.3	-	42.3 ± 2.3	[72]
	28 days		14.9 ± 1.9	-	37.5 ± 2.7	
10[3,4]	39 ± 45[1] months	0.39 – 0.66	12 ± 5.7	2.1 ± 1.2	49.8 ± 24.8	[73]
31[3,4]	6 days	-	12 ± 3.8	-	40.8 ± 11.4	[74]
31[3,5]	≤ 3 months	0.63	-	1.9 ± 1.3	44.8 ± 12.8	[75]
33[4,6]	1 week	0.57	10.3 (4.6 – 26.5)[2]	1.5 (0.1 – 5.0)[2]	43.8 (16.7 – 71.2)[2]	[76]

[1]Values are presented as mean (± SD) unless specified otherwise. [2]Values are median (range); [3]CsA co-therapy; [4]HPLC assay; [5]EMIT assay; [6]TAC co-therapy. AUC, area under the concentration-time curve; C_0, trough; C_{max}, maximum drug concentration; CsA, cyclosporine; EMIT, enzyme-multiplied immunoassay technique; HPLC, high-performance liquid chromatography; MPA, mycophenolic acid; R^2, coefficient of correlation; SD, standard deviation; TAC, tacrolimus.

1.2.6 Clinical pharmacodynamics of MPA

1.2.6.1 Efficacy

The pharmacodynamics (PD) of MPA have been extensively investigated in renal transplant recipients. Several studies have investigated the relationship between exposure to MPA and the incidence of acute rejection, with only a few studies failing to find any association between exposure to MPA and clinical efficacy (Table 1.4). The MPA concentration-effect relationship between AUC (and C_0) with the risk of acute rejection has been investigated in two randomised concentration-controlled studies in renal transplant recipients receiving concomitant CsA therapy [77, 78]. Patients (n = 156 and 150, respectively) were randomised into three AUC target groups; low, intermediate or high, with AUC targets of 16, 32 and 60 mg.h/L, respectively. Both studies reported similar observations; a higher proportion of patients experiencing acute rejection was observed in the low (31 % and 28 %) AUC group, as compared to intermediate (13 % and 15 %) and high (both at 12 %) AUC groups [77, 78]. These two pivotal concentration-controlled trials establishing a relationship between MPA exposure and the risk of rejection have provided the basis of the currently recommended therapeutic range of 30 – 60 mg.hr/L and 1 – 3.5 mg/L for MPA $AUC_{0\text{-}12}$ and C_0 concentrations, respectively.

In addition, a significant association between MPA $AUC_{0\text{-}12}$ and the likelihood of rejection was observed in a retrospective study of 27 renal transplant recipients receiving CsA-based therapy [79]. The MPA AUC < 30 mg.hr/L was associated with a 1.3-fold increased risk of rejection (P = 0.02), however no relationship between MPA C_0 concentration and rejection was observed (P = 0.21) [79].

Although the relationship between low MPA AUC values and the incidence of rejection has been well established especially in transplant recipients receiving concomitant CsA, it has

Table 1.4 Relationship between exposure of MPA and the risk of acute rejection in Caucasian renal transplant recipients.

No of patients	Co-medication	Follow-up time	Parameters investigated	Assay method	Clinical Outcomes	Reference
156	CsA	6 months	AUC_{0-12}	HPLC	Incidence of AR in low (16), intermediate (32), and high (60 mg.hr/L) AUC groups were 31, 13, and 12 %, respectively	[77]
150	CsA	6 months	AUC_{0-12}	HPLC	Incidence of AR in low (16), intermediate (32), and high (60 mg.hr/L) AUC groups were 28, 15, and 12 %, respectively	[78]
27	CsA	1 month	C_0, AUC_{0-12}	HPLC	Increased risk of AR with AUC < 30 mg.hr/L; No significant association between AR and C_0	[79]
51	TAC	3 months	C_0, AUC_{0-12}	HPLC	No association found between AR with either C_0 or AUC_{0-12}	[80]
33	TAC, daclizumab	1 year	AUC_{0-12}, Free AUC_{0-12}	HPLC	No significant concentration-effect relationship observed with either with AUC_{0-12} or free AUC_{0-12}	[76]
94	CsA, basiliximab	3 days	C_0, AUC_{0-12}	HPLC	AUC_{0-12} of 22 mg.hr/L was a threshold for discriminating patients with AR	[81]
100	TAC, daclizumab	1 year	C_0, AUC_{0-12}	EMIT	No significant concentration-effect relationship observed with either C_0 or AUC_{0-12}	[82]

Table 1.4 Cont'd

No of patients	Co-medication	Follow-up time	Parameters investigated	Assay method	Clinical Outcomes	Reference
42	CsA (n=32), TAC (n=10), basilixmab	5 days	AUC_{0-6}, Free AUC_{0-6}	HPLC	Neither free nor total AUC_{0-6} associated with AR	[34]
121	TAC	1 year	C_0	EMIT	Higher risk of AR associated with C_0 < 1.60 mg/L (sensitivity = 91 % and specificity = 83 %)	[83]
33	CsA (n=23), TAC (n=10)	3 months	C_0, AUC_{0-12}	HPLC	AUC_{0-12} of 27.5 mg.hr/L and C_0 of 1.1 mg/L were cut-off points for predicting of AR	[84]
137	CsA, basiliximab	12 months	AUC_{0-3}	HPLC	Increased risk (70 %) of AR with AUC < 30 mg.hr/L, as compared to intermediate (30 %) and high (0 %) AUC groups	[85]
901	CsA (n = 488), TAC (n = 413)	12 months	AUC_{0-12}	EMIT	Higher risk of AR in patients with AUC < 30 mg.hr/L (19 %) compared to patients with AUC > 30 mg.hr/L (13 %)	[86]

AR, acute rejection; AUC, area under the concentration-time curve; C_0, trough concentration; CsA, cyclosporine; EMIT, enzyme-multiplied immunoassay technique; HPLC, high-performance liquid chromatography; ILAb, IL-2 antagonists; TAC, tacrolimus.

been difficult to establish a similarly strong relationship between exposure to MPA and the likelihood of rejection in renal transplant recipients who received TAC as concomitant medication (Table 1.4). Neither AUC_{0-12} nor C_0 concentrations (determined within the first 2 weeks and 3 months after transplantation, and at the time of every rejection or side effect) was associated with rejection in a retrospective study of 51 renal transplant recipients receiving MMF in combination with TAC [80]. Consistent with this finding was the observation in a prospective study of 100 renal transplant recipients who received MMF, TAC and daclizumab (induction therapy). There was no significant difference in MPA AUC_{0-12} between patients who experienced rejection versus those who remained rejection-free (56.4 ± 45.4 vs. 46.0 ± 22.8 mg.hr/L, $P = 0.84$) [82].

Nevertheless, in TAC co-administered transplant patients, one study has reported positive findings [83]. Borrows *et al*. reported significantly lower MPA AUC_{0-12} and C_0 values were associated with the incidence of rejection. In a retrospective study of 121 renal transplant recipients, the median (range) MPA C_0 concentrations prior to rejection was significantly lower than patients who experienced rejection compared to those who did not experience rejection (1.1 (0.26 – 1.76) versus 1.85 (0.39 – 4.9) mg/L, $P < 0.001$) [83]. In addition, a threshold value of $C_0 < 1.60$ mg/L with a diagnostic sensitivity of 91 % and specificity of 83 % was chosen for discriminating patients with acute rejection [83].

Two prospective randomised trials have also investigated the MPA concentration-effect relationship between AUC and the incidence of acute rejection. The first, Adaption de Posologie du MMF en Greffe Renale (APOMYGRE) study, was a 12-month, multicentre, randomised trial involving 137 renal transplant recipients, performed in 11 centres in France [85]. Of the 10 incidences of acute rejection occurring in the first 3 months post-transplantation, a higher proportion of patients (70 %) experiencing acute rejection was associated with the MPA AUC < 30 mg.hr/L, as compared to intermediate (30 %) and high

(0 %) AUC groups. The second, Fixed-Dose versus Concentration-Controlled (FDCC) study, was a much larger trial involving 901 renal transplant recipients from 19 different countries [86]. In the FDCC study, there was a significant relationship between early MPA AUC values on day 3 and the incidence of acute rejection during the first month (P = 0.009) or the entire 12 months following transplantation (P = 0.006). Similar to APOMYGRE study, the risk of developing acute rejection was significantly higher (P = 0.018) in patients with a day 3 MPA AUC < 30 mg.hr/L (19 %), as compared to those with the MPA AUC > 30 mg.hr/L (13 %). The APOMYGRE and FDCC studies were landmark randomised concentration-controlled trials investigated the added benefit of TDM for MPA and these findings have resulted in proposals for drug monitoring of MPA and dosage adjustments in renal transplant recipients (discussed in Section 1.2.8).

1.2.6.2 Adverse events

The use of MPA has been associated with the frequent occurrence of both haematological and gastrointestinal adverse effects including leukopenia and diarrhoea. In daily clinical practice, MPA toxicity leads to dose reduction or discontinuation and therefore places patients at higher risk of experiencing kidney rejection. Several studies have investigated relationships between MPA PK and adverse events [75, 79, 82, 87]. In a prospective study involving 100 renal transplant recipients who received MMF in combination with TAC, a significant association between high MPA exposure and adverse events was reported at different time points after transplantation [82]. Patients with leukopenia had significantly higher MPA $AUC_{0\text{-}12}$ and C_0 at 3 and 12 months: at 3 months $AUC_{0\text{-}12}$: 61.4 ± 30.9 versus 42.3 ± 25.3 mg.hr/L, P = 0.01; and C_0: 4.1 ± 1.6 versus 2.9 ± 1.6 mg.hr/L, P = 0.01; and at 12 months $AUC_{0\text{-}12}$: 84.4 ± 45.6 versus 44.2 ± 21.9 mg.hr/L, P = 0.04; and C_0: 8.7 ± 7.9 versus 3.4 ± 1.9 mg.hr/L, P = 0.04. Similar observations were reported in anaemic patients (higher $AUC_{0\text{-}12}$ and C_0) at 3 and 12 months post-transplantation [82]. Another prospective

study in 31 CsA co-treated transplant recipients reported that MPA AUC was significantly higher in patients who presented with side effects (e.g. leukopenia, anaemia, diarrhoea) with mean ± SD AUC_{0-12} of 62.1 ± 21.1 versus 39.8 ± 15.2, P = 0.0005 [75].

In addition to investigating the relationship between MPA AUC and C_0 concentrations with the risk of rejection and adverse events, some studies have investigated relationships between the free or unbound (C_u) MPA concentration and clinical outcomes (both efficacy and adverse events). Although the monitoring of MPA C_u concentration is not widely used, it has been proposed to better predict immunosuppressive effect than total MPA AUC or C_0 as a risk factor for acute rejection or side effects, since only free or unbound MPA is pharmacologically active and capable of inhibiting IMPDH [33]. No previous studies have reported a relationship between MPA C_u concentration and the risk of rejection. In a study of 42 renal transplant recipients receiving concomitant CsA (n = 32) or TAC (n = 10) and induction therapy, there was no significant difference in the MPA C_u AUC_{0-6} between patients (median = 1.4 mg/hr/L) with and without rejection (0.9 mg/hr/L, P = 0.07) [34]. In addition, an open-label longitudinal study in 54 pediatric renal transplant recipients who received MMF in combination with CsA, there was no association between the risk of acute rejection and MPA C_u AUC_{0-12} values (P = 0.40) [88].

However, MPA C_u concentration was associated with adverse events, as reported in two clinical studies in both adult and pediatric renal transplant recipients [34, 88]. Significantly higher MPA C_u AUC_{0-6} values were observed in patients who experienced one or more adverse events (e.g. leukopenia, thrombocytopenia, or infection) in the first month post-transplantation (mean ± SD = 1.9 ± 0.3 mg/hr/L versus 1.1 ± 0.1 mg/hr/L, respectively, P = 0.004) [34]. Similar observations were reported in pediatric renal transplant recipients with higher MPA C_u AUC_{0-12} levels associated with an increased risk of developing adverse events (leukopenia or infection). A cut-off value of MPA C_u AUC_{0-12} > 0.4 mg.hr/L

(sensitivity of 92 % and specificity of 61 %) chosen for discriminating patients with leukopenia or infection [88], further supporting the utility of measuring the active, unbound concentration of MPA in comparison to total concentrations.

1.2.7 Summary

The PK of MPA have been extensively studied in renal transplant recipients, however, the findings from previous studies of concentration–effect and –toxicity relationships are inconclusive and demonstrated considerable inter-patient variability in the PK of MPA. The reported conflicting results are most likely due to: (1) the small population size and (2) low number of rejection episodes and adverse events, therefore some of these studies may not have been sufficiently powered to detect a difference; (3) the use of induction therapy (e.g. basiliximab and daclizumab); (4) the time after transplant (ranging from day 3 – 1 year post-transplant); (5) the majority of studies not assessing TAC or CsA concentrations; and finally (6) the different analytical assays (EMIT versus HPLC) used for determination of MPA plasma concentrations, as EMIT is less specific than HPLC and cross-reacts with AcMPAG [67], hence potentially overestimating MPA concentrations.

In addition, the therapeutic range of MPA co-administered with TAC had not been well defined and the effect of CsA on inhibiting enterohepatic recirculation reflects the two different C_0 concentration target ranges that have been proposed with 1.3 – 3.5 mg/L (associated with the AUC target of 30 – 60 mg.hr/L) when co-administered with CsA, but a higher C_0 concentration target range of 1.9 – 3.5 mg/L when co-administered with TAC to attain the same target AUC. The C_0 concentration range is higher (at least at the low end) when MMF is administered with TAC because a greater proportion of the exposure to MPA is in the latter part of the dosing interval (C_0 concentration may overestimate AUC), due to the greater enterohepatic recirculation compared to patients receiving MMF and CsA.

Exposure to MPA is essential to assess, as there is a large variability in C_0 concentrations or AUC measured in renal transplant recipients administered standard doses of MMF. The majority, but not all, of the clinical studies show a significant association between exposure to MPA with clinical efficacy and/or safety. MPA PK parameters such as C_0 concentration or AUC are generally the most commonly used tools for investigation of the MPA concentration-effect relationship in renal transplant recipients for the risk of rejection and adverse events. Based on the wide variability in the PK of MPA and the reported relationships between exposure to MPA and the incidence of rejection and/or adverse events, therapeutic drug monitoring (TDM) strategies have been implemented and this is discussed in the next section of this thesis.

1.2.8 Therapeutic drug monitoring of MPA

Maintenance of adequate exposure to MPA is very crucial especially in the early post-transplant period for prevention of acute rejection. Although strong relationships between MPA AUC_{0-12} and clinical outcomes had been reported previously, the application of TDM to MPA would provide clinical benefits in increasing the therapeutic potential of MPA (improved efficacy and reduced adverse events) in the early post-transplantation period, as compared to a fixed-dosage regimen. In current clinical practice, a fixed MMF dose of 1 g twice a day has been recommended for renal transplant recipients [14] in the early transplant period, and then monitored (tapered or maintained) accordingly at the time of major clinical events (rejection episode or adverse events). However, there are several reasons to consider individualisation of MMF doses (as opposed to giving a fixed dosage) as a tool in the management of transplant recipients: (1) MPA PK show a large inter-patient variability; (2) graft rejection relates to low MPA exposure; (3) adverse events relate to high concentrations; and (4) blood concentrations are poorly predicted by standard body measures (e.g. weight,

body mass index). Two approaches for MMF dose individualisation have been taken into consideration: (1) the PK approach (e.g. AUC_{0-12} or limited sampling strategy); and (2) PD monitoring (e.g. IMPDH activity).

Several analytical methods have been developed for the determination of MPA concentrations including the EMIT [89-91], HPLC [91-93] and HPLC with mass spectrometric detection [94]. In the clinical setting, determination of MPA concentrations by EMIT assay may be less laborious and time-consuming than HPLC, however, because of the cross-reactivity between the anti-MPA antibody used with the pharmacologically active AcMPAG metabolite, measurement of MPA concentrations are generally higher (5 – 40 % higher) compared to those measured from HPLC-based methods [67, 89, 91], suggesting that HPLC may be more suitable to provide the most accurate measurement of the parent compound MPA as compared to EMIT.

In 2001, a roundtable meeting to discuss the use of TDM to guide immunosuppressive therapy provided recommendations and strategies for applying TDM of MMF in solid organ transplantation [95]. Based on the roundtable discussion, the therapeutic window of 30 – 60 mg.hr/L and 1 – 3.5 mg/L for total MPA AUC_{0-12} and C_0 concentrations, respectively, have been proposed to minimise the risk of rejection, based mostly on findings in patients with MMF co-administered with CsA. Another roundtable discussion was held in 2004 to update and revise previous guidelines since TAC had since become the predominant CNI in renal transplantation [96]. The total MPA AUC_{0-12} of 30 – 60 mg.hr/L, MPA $C_0 \geq 1.3$ mg/L (CsA-based regimen) and $C_0 \geq 1.9$ mg/L (TAC-based regimen), measured using HPLC, was recommended in renal transplantation.

With the need for individualisation of MPA therapy, the question arises regarding which parameter is the most suitable as a marker for total MPA exposure. The most frequently used

means of MPA monitoring is C_0 concentration measurements in plasma. However, MPA C_0 concentrations correlate weakly with AUC_{0-12}, with R^2 values ranging from 0.23 to 0.63 in renal transplantation (no difference with regard to AUC and concomitant immunosuppression (e.g. CsA or TAC)) [75, 97-101], suggesting that the estimation of a full MPA AUC_{0-12} monitoring may be the most useful assessment of total MPA exposure than C_0 concentrations, to establish that adequate MPA exposure is present in the early post-transplantation period. Although low MPA exposure, as estimated by the AUC_{0-12}, has been shown to better correlate with the risk of rejection (refer to Section 1.2.6.1), MPA AUC_{0-12} monitoring requires multiple blood samples during a 12 hr dose interval, hence the use of full MPA AUC_{0-12} is restricted due to its impracticality in an out-patient clinical setting, time-consuming and costly. Therefore, there is a need for alternative limited sampling strategies (LSS) to allow full estimation of MPA AUC_{0-12} based on abbreviated AUC.

Several LSS have been published to estimate MPA AUC_{0-12} without taking multiple blood samples in the full 12 hr dose interval, based on multiple regression methods and mostly involving 3 or 4 blood samples drawn during the first 6 hr post-dose [97, 98, 100, 101]. However, some disadvantages of abbreviated LSS are that samples drawn during the first 6 hr of the dosing interval are likely to miss the enterohepatic recirculation as the concentration-time profile of MPA includes a secondary peak as a result of enterohepatic recycling, occurring 6 – 12 hr following administration. In addition, abbreviated LSS requires strict adherence regarding the timing of blood samples. Alternatively, a method of a maximum *a posteriori* (MAP) Bayesian estimation has been developed [102], which allows estimation of MPA AUC_{0-12} by combining a patient's own data with a population-PK model to estimate dosage prediction for each individual. One advantage of MAP Bayesian estimation is the flexibility with respect to the timing of sample collection [102], although the use of Bayesian estimation requires specific software programs to perform the analysis.

1.2.8.1 Pharmacodynamic monitoring of MPA

As previously discussed in Section 1.2.2, MPA produces potent, reversible and non-competitive inhibition of IMPDH, thereby suppressing the proliferative responses of T- and B-lymphocytes [16], antibody formation and the generation of cytotoxic T-cells are inhibited [21]. This is the principal mechanism by which MPA exerts immunosuppressive effects. Therefore, PD monitoring by measurement of IMPDH activity directly in lymphocytes may be a useful biomarker as it may correlate more closely to the biological response of MMF than MPA concentrations.

Determination of the activity of IMPDH in isolated peripheral blood mononuclear cells (PBMCs) has been investigated previously in renal transplant recipients, and is based on the chromatographic determination of the produced XMP [103-108]. However, there have been only two studies investigating relationships between MPA concentrations and IMPDH activity [103, 104]. A weak inverse correlation between post-transplant pre-dose IMPDH activity and MPA concentrations (R^2 = 0.24, P = 0.17) has been reported in 35 renal transplant recipients under MMF therapy [103]. In addition, this was the first study establishing a relationship between IMPDH activity and clinical outcome. Two main observations were made: (1) that a higher percentage of patients (63 %) with MMF dose reductions had lower pre-transplant IMPDH activity; and interestingly (2) that patients with high pre-transplant IMPDH activity (above a threshold value of 8.5 nmol/mg protein/hr) and MMF dose reduction had a higher risk of rejection (82 % versus 36 %, P < 0.01). In a prospective study involving 40 renal transplant recipients, there were significant inverse correlations between individual IMPDH activities and MPA plasma concentrations, taken at 1.5 and 3.5 hr on different days (2, 4 and 10) following transplantation [104]. However, there was no correlation found between MPA C_0 concentrations and pre-dose IMPDH activities. These observations suggest that determination of IMPDH activity may better

reflect clinically relevant information on the immunosuppressive efficacy of MPA and provides an additional tool in the future for MMF dose individualisation.

1.2.9 Summary

The inhibition of graft rejection by immunosuppressants is a key factor in successful transplant outcomes, in particular long-term graft survival is influenced by the incidence of acute rejection especially in the early post-transplantation period. Therefore, minimising, or preferably eliminating early acute rejection typically benefits graft survival. The PK of MPA are characterised by large inter-individual variability and due to its narrow therapeutic index, the application of TDM may help to overcome its PK variability, ensuring sufficient concentrations to prevent rejection but not too high to cause toxicity. To overcome this PK variability, relationships between plasma MPA concentrations and clinical outcomes have been demonstrated and target plasma concentration ranges have been proposed for dosage individualisation.

However, plasma MPA concentrations may be a relatively poor predictor of the target tissue concentration. There has been debate as to whether C_0 plasma concentrations are sufficient as an index of exposure to MPA (assumed to relate to $AUC_{0\text{-}12}$) for routine monitoring purposes and whether they relate to indices of clinical efficacy and safety. This discrepancy between plasma and tissue concentrations is most likely due to the presence of the efflux transporter MRP2, which at the two major effector sites of MPA, lymphocytes and kidney, may result in significant inter-individual variability in tissue concentrations and consequently overall PD of MPA, despite the use of TDM to overcome variability in bioavailability and clearance. Furthermore, time after transplant, co-medication and the binding of MPA to plasma albumin, may also add to the variability in total MPA response. Any PGx factor influencing the PK or PD pathways of MPA has the potential to affect a

patient's response to MPA treatment. This is discussed in the next section of this Introduction from the perspective of the genetic variability considered in this study, the MRP2 efflux transporter.

1.3 Drug transporters

Over the last decades, extensive work has been undertaken to identify single nucleotide polymorphisms (SNPs) in the genes encoding drug transporters to determine their allelic frequencies in different ethnic populations, and importantly to characterise their functional impact, if any, towards inter-individual variability in drug disposition and transport activity. Genetic polymorphisms in the genes encoding transporter proteins may potentially affect the function and/or expression of a given drug transporter, thereby contributing to the inter-individual variability in drug disposition [109, 110]. Members of the ATP-binding cassette (ABC) transporter family, especially MRP2, have an overlapping and remarkably broad substrate spectrum that are now increasingly recognised to play an important role in the absorption, distribution and elimination of several drug substrates. MRP2 is depicted in uppercase letters when the human protein is described, whereas Mrp2 (only the first letter is uppercase) is used in rodent species.

1.3.1 Multidrug resistance-associated protein 2

The multidrug resistance-associated protein 2 (MRP2) or canalicular multispecific organic anion transporter (cMOAT), encoded by *ABCC2* gene located on chromosome 10q24, is a member of the ABC transporter superfamily with a molecular weight of 190 – 200 kDa and comprised of 1545 amino acids spanning approximately 45 kb containing 32 exons [111, 112].

MRP2 has a very broad substrate specificity [113] and actively exports a wide variety of endogenous compounds as well as both conjugated and non-conjugated anionic drugs into bile including: gluthathione conjugates (e.g. reduced gluthathione and glutathione disulphide) [114, 115]; glucuronide conjugates [116]; bile salts conjugates [117]; non-conjugated anionic drugs (e.g. pravastatin) [118]; and anticancer drugs (e.g. cisplatin and methotrexate) [119]. Therefore, altered *ABCC2* functional activity would potentially affect the clearance of many clinically important drugs.

1.3.1.2 Localisation and function of MRP2

The membrane transporter MRP2 is predominantly expressed in the apical membrane of hepatocytes [120] and renal proximal tubule cells of the kidney [121]. MRP2 efflux transporter is also expressed in the apical membrane of enterocytes [122], lymphocytes [123], human placenta [124] and gall bladder epithelia [125]. Transport activities of *ABCC2* have been extensively studied using membrane vesicles and transfected mammalian cells [126] and also by comparing the Mrp2 transport activity between normal and Mrp2-deficient rats (e.g. such as Groningen yellow (GY) Eisai hyperbilirubinemic (EHBR) rats) [127] as a model to investigate the pathogenesis of Dubin-Johnson syndrome (DJS) in humans.

Alterations of MRP2 expression and/or function could potentially have clinically important effects. MRP2 is best known for its role in the hepatobiliary transport of conjugated bilirubin (a yellow-coloured bile pigment) and the absence of functional MRP2 from the hepatocytes results in DJS, an autosomal recessive disorder that is characterised by conjugated hyperbilirubinemia, which can result in clinically apparent jaundice (e.g. yellow colouring of skin, deposition of dark pigments in the liver) [128, 129]. The difference in the expression of MRP2 results in large inter-individual differences in MRP2 function. The expression of MRP2 was approximately 15-fold higher in patients undergoing surgical resection of liver

metastasis compared to controls (no histological evidence of liver disease) [130]. Inter-individual differences in the expression level of MRP2 have also been demonstrated in duodenal biopsies taken from 16 healthy volunteers who received 600 mg rifampicin daily [131]. There were 6- and 8-fold differences in the mRNA and protein levels, respectively. In addition, the administration of rifampicin induced duodenal mRNA and MRP2 protein levels in 14 (88 %) and 10 (63 %) individuals, respectively, out of 16 healthy volunteers.

Previous animal studies have suggested that MPAG is a substrate for Mrp2 and CsA inhibited Mrp2-mediated transport through inhibition of MPAG biliary excretion via interaction with rat Mrp2 on the bile canalicular membrane [56, 58, 132]. The cumulative biliary excretion of MPAG-derived MPA was significantly decreased by approximately 20 % when MPA was co-administered with CsA compared to MPA alone [56, 132], suggesting that Mrp2 was important for MPAG biliary excretion and therefore the glucuronide is a likely substrate of Mrp2. Patel *et al*. has demonstrated that MPAG is a substrate of MRP2 (a K_m value of 224.2 ± 42.7 μM and V_{max} value of 2.7 ± 0.2 μmol/min) [133]. These findings indicate that MRP2-mediated transport for MPAG biliary excretion is an essential mechanism governing MPAG disposition, and therefore the MRP2 transporter may play a role in the enterohepatic recirculation of MPA (as discussed in Section 1.2.3.4) and renal excretion of MPAG.

A recent study using human embryonic kidney (HK293) cells has shown that MPA is a substrate of MRP2 (MRP2 transported MPA with a K_m value of 16 ± 4 μM and V_{max} value of 4.7 ± 0.7 μmol/min) [46]. In addition, the effect of different immunosuppressants has been tested on MRP2-mediated (^{3}H)-methotrexate transport using membrane vesicles isolated from HEK293 cells overexpressing MRP2. MPA, CsA and TAC inhibited MRP2-mediated transport with IC_{50} values (mean ± SD) of 0.065 ± 0.001, 41 ± 1 and 15.2 ± 0.1 μM, respectively [134]. Interestingly, the inhibition of MRP2-mediated transport by MPA, CsA

and TAC may suggest possible interactions at the renal and tubular efflux levels, however, since MPA is eliminated mainly in urine as MPAG (as discussed in Section 1.2.3.5) [134], the presence of a possible interaction between this glucuronide and MRP2 expression needs further investigation.

In summary, MRP2, located in the apical membrane of hepatocytes, kidney proximal tubules and intestinal epithelia, plays an important role in the detoxification and disposition by transporting a wide range of compounds and clinically relevant drugs including the immunosuppressant MPA, as a consequence, MRP2 is important in determining the pharmacological and side effects of drug substrates.

1.3.1.3 Genetic polymorphisms of *ABCC2*

Over the last few years, extensive work has been undertaken to identify SNPs in the *ABCC2* gene encoding MRP2 to determine its allelic frequencies in different ethnic populations, and importantly to characterise their functional impact, if any, towards inter-individual variability in drug disposition and transport activity. Genetic polymorphisms of the *ABCC2* gene may potentially affect the function and/or expression of the MRP2 efflux transporter, thereby contributing to inter-individual variability in drug disposition.

Several SNPs have been identified in both coding and non-coding regions of *ABCC2*, with significant linkage disequilbrium, some of which affect MRP2 transporter expression and/or function (Table 1.5) (based on information found in http://www.pharmgkb.org and http://www.ncbi.nlm.nih.gov/projects/SNP). These polymorphisms include deletions, nonsense mutations, premature stop codons and frame shift mutations. Among the *ABCC2* SNPs, the most commonly studied *ABCC2* SNPs are the promoter (-24 C > T, rs717620), exon 10 (1249 G > A, rs2273697) and exon 28 (3972 C > T, rs3740066) due to their high allelic frequency in Caucasian population [135, 136]. The 1249 G > A SNP is associated

with amino acid substitution from Valine to Isoleucine at 417 (Val417Ile), whilst 3972 C > T is a "silent" SNP (Ile1324Ile) in linkage disequilibrium with the -24 C > T SNP [137, 138]. In Caucasians, combinations of these SNPs make up 5 haplotypes (Table 1.6), the three most common being H1 (homozygous wild-type (WT)), H2 (variant at position 1249) and H9 (variant at position 3972) [139-141]. The functional effects of *ABCC2* polymorphisms on MRP2 expression and functional activity remain unclear, however, the -24 T allele has been associated with lower *ABCC2* mRNA expression in normal renal tissues ($P = 0.03$) and a 19 % reduction in transcriptional activity in HepG2 cells ($P = 0.003$) [135].

A recent study assessing *in vitro* expression and function of *ABCC2* haplotypes in transfected HEK293T/17 cells, has conclusively demonstrated that in comparison to *ABCC2* WT haplotype H1 (CGC; -24C/1249G/3972C), haplotype H2 (CAC; variant at 1249) resulted in increased protein expression and transport activity, haplotype H10 (TGC; variant at -24) resulted in lower protein expression, whilst haplotype H9 (CGT; variant at 3972) and H12 (TGT; variant at -24 and 3972) resulted in lower protein expression and transport activity (Table 1.6) [140]. Furthermore, to elucidate the impact of the most common *ABCC2* haplotypes, the same study has investigated the bioavailability and AUC of talinolol (an anti-hypertensive agent, β-blocker) *in vivo* in 24 healthy volunteers exhibiting at least one of the investigated haplotypes H1, H2, H9 and H12 [140]. Haplotype H2 decreased the bioavailability of talinolol, whilst haplotypes H9 and H12 increased the bioavailability of talinolol, however because of low number of individuals homozygous for defined haplotypes, this study may not have been sufficiently powered to detect a difference between these haplotype groups.

Recently, the effects of *ABCC2* polymorphisms on the PK of the TAC were investigated in 102 renal transplant recipients in a haplotype-specific manner [141]. The effect of individual *ABCC2* SNPs on TAC concentrations were initially investigated; the 1249 G > A and 3972

C > T SNPs was significantly associated with reduced dose-normalised TAC C_0 by 1.4- and 1.3-fold, respectively. Further investigation of the effects of *ABCC2* polymorphisms was then carried out in a haplotype-specific manner as reported previously [140]. It was shown that patients in the MRP2 high-activity group (H2/H2 and H1/H2 carriers) had significantly 1.5-fold lowered dose- corrected C_0 TAC concentrations ($P = 0.007$), in comparison to those patients in the low-activity and WT groups [141], further supporting the efficacy of employing an haplotype approach in place of individual SNPs.

In summary, the effects of *ABCC2* polymorphisms are variable and substrate specific. The MRP2 efflux transporter has an overlapping and remarkably broad substrate spectrum that is now increasingly recognised to play an important role in the absorption, distribution and elimination of several clinically important drug substrates. Genetic polymorphisms in the gene encoding MRP2 have been shown to cause significant inter-individual variations in drug disposition; however further investigations in a larger cohort are still required to elucidate and re-confirm the functional consequences of *ABCC2* haplotypes.

Table 1.5 Genetic polymorphisms in both coding and non-coding regions of *ABCC2*[1].

SNP	Nucleotide position	rs number	Amino acid change	Allelic frequency[2]	Effect on mRNA or protein level	Reference
-1549 G > A	5'-flanking	rs1885301		Cau: 0.43 As: 0.10 Af: 0.49	unknown	
-1019 A > G	5'-flanking	rs2804402		Cau: 0.43 As: 0.15 Af: 0.37	unknown	
-24 C > T	Promoter	rs717620		Cau: 0.19 As: 0.10 Af: 0.06	↓ mRNA expression	[135]
1249 G > A	Exon 10	rs2273697	Val417Ile	Cau: 0.47 As: 0.10 Af: 0.17	no change	[142]
1446 C > G	Exon 10	rs113646094	synonymous	Cau: 0.01[3] As: 0.00 Af: 0.00	↑ mRNA expression	[143]
2366 C > T	Exon 18	rs56220353	Ser789Phe	Cau: 0.00 As: 0.12 Af: 0.00	unknown	
3542 G > T	Exon 25	rs8187692	Arg1181Leu	Cau: 0.00 As: 0.00 Af: 0.09	unknown	
3563 T > A	Exon 25	rs17222723	Val1188Glu	Cau: 0.08 As: 0.00 Af: 0.07	↑ mRNA expression	[144]
3972 C > T	Exon 28	rs3740066	synonymous	Cau: 0.38 As: 0.20 Af: 0.27	unknown	
4348 G > A	Exon 31	rs56296335	Ala1450Thr	Cau: 0.00 As: 0.02 Af: 0.00	unknown	
4544 G > A	Exon 32	rs8187710	Cys1515Tyr	Cau: 0.08 As: 0.00 Af: 0.20	↑ mRNA expression	[144]

[1]Adapted and updated from Haufroid *et al*. [145]. [2]Based on http://www.ncbi.nlm.nih.gov/projects/SNP and http://www.pharmgkb.org.
[3]As reported by Lévesque *et al*. [139] - 1446 C > G SNP for Caucasian.
↑: increase; ↓, decreased; Af: African; As: Asian; Cau: Caucasian population.

Table 1.6 Frequencies (%) of *ABCC2* haplotypes [139-141] in Caucasians and their corresponding expressor phenotypes [140].

Haplotype	-24 C > T	1249 G > A	3972 C > T	Frequency	Influence on protein expression and transport activity[1]		Phenotype
$H1_{(WT)}$	C	G	C	31 – 54 %	Normal	Normal	-
H2	C	A	C	20 – 23 %	↑ Protein expression	↑ Transport activity	High expressor
H9	C	G	T	12 – 16 %	↓ Protein expression	↓ Transport activity	Low expressor
H10	T	G	C	7 – 19 %	↓ Protein expression	= Transport activity	Low expressor
H12	T	G	T	4 – 18 %	↓ Protein expression	↓ Transport activity	Low expressor

[1]In comparison to wild-type H1. The variant alleles are underlined. WT, wild-type; **=**, No significant difference; ↑, Increased *ABCC2* expression/activity; ↓, Decreased *ABCC2* expression/activity.

1.3.1.4 Influence of *ABCC2* on MPA exposure and effects

These sections will discuss the clinical studies conducted to date on the role of the MRP2 efflux transporter, aimed at elucidating the effect of *ABCC2* genetic variability on the PK of MPA and hence, clinical outcomes. As mentioned earlier in this thesis, the MRP2 efflux transporter is a major focus of this thesis and the functional significance of *ABCC2* genetic polymorphisms on MPA exposure are discussed in the context of Caucasians only, as this is the only ethnic population studied in this thesis.

1.3.1.4.1 Impact of *ABCC2* polymorphisms on MPA exposure

As discussed in Section 1.1.2.4, the MRP2 efflux transporter is involved in the enterohepatic recirculation of MPA and the biliary excretion of MPAG, therefore *ABCC2* genetic polymorphisms may affect the PK of MPA. There have been numerous studies investigating the association between the different *ABCC2* polymorphisms and the PK of MPA and/or clinical outcomes with some conflicting results. The majority of the studies have failed to find any significant association of the individual *ABCC2* SNPs with MPA PK parameters (or clinical outcomes) [146-150], however, there have been at least three positive studies [136, 139, 151] which will be discussed in detail below.

One study was conducted in 95 renal transplant recipients co-administered MMF and TAC using two doses of MMF, either 1 g (n = 63) or 2 g (n = 32) daily, with monitoring at days 7, 42, 90 and 360 after transplantation [136]. At day 7 post-transplant, under steady-state conditions, patients with mild liver disease and not carrying the -24 C > T SNP had significantly 2-, 3.2- and 2.4-fold lower dose-normalised MPA C_0 ($P = 0.045$), $AUC_{6\text{-}12}$ ($P = 0.002$) and $AUC_{0\text{-}12}$ ($P = 0.0007$) concentrations, respectively, and a 2.3-fold increased MPA CL ($P = 0.0007$), compared to those patients without liver dysfunction [136]. Their observations are consistent with a previous study in liver transplant recipients [152], in

which mild liver disease is associated with a decreased in exposure to MPA and a higher MPA CL. The authors reported that this effect is partly due to a lower enterohepatic recirculation, which is depicted by a reduction in the partial AUC_{6-12} [136]. An alternative explanation for the overall decrease in MPA AUC_{6-12} and AUC_{0-12} is that liver inflammation may affect the expression and transport activity of organic anion transporters via complex cytokine-mediated down-regulating signaling networks [153]. In addition, a similar observation was also reported in patients carrying the WT 3972 C > T SNP, where significantly decreased dose-normalised MPA C_0, AUC_{6-12} and AUC_{0-12} concentrations were observed in patients with mild liver dysfunction. As previously discussed in Section 1.3.1.3, the 3972 C > T is a "silent" SNP without any amino acid substitution, it is unlikely that this SNP affects the MRP2 expression or function, suggesting that the effect observed between the 3972 C > T SNP and MPA exposure may be mediated via linkage disequilibrium with the -24 C > T SNP [135, 137]. There were no significant effects of other *ABCC2* SNPs (-1549 G > A, -1023 G > A, -1019 A > A, 1249 G > A and 4544 G > A) on the PK of MPA in patients with or without liver dysfunction. However, the findings in this study may have been biased by the use of an EMIT assay for determination of MPA concentrations because of the cross-reactivity between MPA and AcMPAG measurement (as discussed in Section 1.2.8).

In a retrospective study involving 47 healthy volunteers who received a single 1.5 g dose of MMF, Lévesque *et al*. showed that, in comparison to the WT carriers (n = 27), individuals who were heterozygous carriers (n = 20) of the -24 C > T SNP had a 25 % increased AcMPAG AUC_{0-12} ($P \leq 0.05$ in multivariate analysis), suggesting the -24 T allele carriers may be exposed to higher concentrations of AcMPAG resulting in an increased risk of intestinal toxicity [139]. However, two of the patients also carried the *UGT1A9*3* variant, which was previously shown to be associated with an 85 % increased exposure to AcMPAG

[154], and therefore may suggest a potentially synergistic effect of the combined polymorphisms. This study provides evidence of the involvement of human MRP2 in the disposition of AcMPAG; MRP2/Mrp2 has previously been shown to be involved in the biliary excretion of MPAG (as discussed in Sections 1.2.3.4 and 1.3.1.2), and also in the canalicular excretion of AcMPAG in Wistar rats [132]. There were no significant differences between MPA and MPAG AUC_{0-12} values in patients carrying the -24T variant and the WT carriers [139].

Finally, in a group of 66 renal transplant recipients who received MMF in combination with CsA (n = 30), TAC (n = 13) or SRL (n = 23) with monitoring at day 7, and months 1 and 3 after transplantation, Lloberas *et al*. showed that under steady-state conditions, at month 3, heterozygous carriers (n = 16) of the -24 C > T SNP had significantly 1.4-fold lower (P = 0.023) MPA AUC_{0-12} than the WT carriers [151]. Their findings confirmed previous observations between the -24 C > T polymorphism and the oral CL of MPA in patients with mild liver dysfunction [136]. Patients with mild liver disease and carrying the -24 C > T SNP were protected from a reduction in exposure to MPA, suggesting a relationship between this SNP and lower oral CL of MPA under steady-state conditions [136]. At Day 7, free MPA AUC_{0-12} was statistically significantly 1.5-fold higher (P = 0.008) in the heterozygous carriers of the -24 C > T SNP [151]. In addition, a separate analysis of MPA PK parameters in the presence or absence of the -24 C > T SNP for each concomitant CNI (CsA versus TAC and SRL) was also investigated. The heterozygous carriers of the -24 C > T SNP co-administered with TAC and SRL had significantly lower free and total MPA AUC_{0-12} at month 3 (P = 0.001 and 0.003, respectively) compared to the WT carriers [151]. In contrast, at day 7, significantly higher free and total MPA AUC_{0-12} (P = 0.003 and 0.03, respectively) was observed in the heterozygous carriers under CsA co-treatment than the WT carriers [151]. However, this study has a relatively small number of patients, thus further

investigation in a larger prospective study is needed to reconfirm the presented differences observed in patients with the -24 C > T SNP co-treated with CsA or TAC.

1.3.1.4.2 Impact of *ABCC2* polymorphisms on clinical outcomes

In contrast to PK studies, data regarding the effects of *ABCC2* polymorphisms on MPA PD variability (adverse events) is relatively limited. A number of studies have investigated relationships between *ABCC2* polymorphisms and clinical outcomes [136, 148, 155, 156], however only one study has reported positive findings [136]. Naesens *et al*. demonstrated that patients carrying the variant -24 C > T SNP had a significantly higher incidence of diarrhoea compared to the non-carriers (29 % (12/41) versus 13 % (7/54), $P = 0.049$) in the first year post-transplantation [136]. The pathogenesis of MPA-associated diarrhoea is still unknown, however, it is believed that AcMPAG metabolite could have played a role in the incidence of diarrhoea through a secondary immunological mechanism [157]. There was no significant difference in the incidence of leukopenia or haematological disorders between the -24 C > T carriers and non-carriers [136].

Data on the effects of *ABCC2* SNPs on MPA PK parameters are limited with most studies considering only the impact of recipient genotype. Importantly, previous findings have been conflicting due to the lack of haplotype analysis and small patient groups. However, one study investigating the influence of both donor and recipient *ABCC2* polymorphisms on transplant outcome has been published [158], which reported that a donor haplotype CGACA (-24C/1249G/3563A/3972C/4544A, variant at 3563 and 4544) was more prevalent in patients with delayed graft function (DGF) ($P = 0.004$) compared to those patients without DGF. Multivariate regression analysis revealed that the donor 3563 T > A and 4544 G > A SNPs but none of the other polymorphisms (-24 C > T, 1249 G > A and 3972 C > T SNPs)

or clinical covariates tested were significantly associated with DGF (P = 0.006) [158], further confirming the superiority of haplotype analysis in predicting the incidence of DGF.

1.3.1.5 Summary

In summary, a full understanding of the findings in previous studies is hampered by the fact that the role of common *ABCC2* SNPs on exposure of MPA and MRP2 expression and function has not been extensively evaluated to date. In addition, although there are a large number of clinical studies on the effects of *ABCC2* polymorphisms on MPA PK, results are conflicting due to the lack of a haplotype approach and small patient numbers. Importantly, the majority of studies have investigated only the genotype of the recipient. The MRP2 efflux transporter is of particular clinical importance given its pivotal role in the biliary and renal excretion of MPAG, therefore transplant donor genotypes are likely to have the greatest impact on MPAG PK. While there are conflicting data regarding the impact of *ABCC2* polymorphisms on MPA PK, very limited studies have investigated the influence of *ABCC2* gene variants on clinical outcomes in particular the risk of rejection and adverse events.

1.4 Lymphocyte and kidney MPA concentrations

In addition to determining the effect *ABCC2* polymorphisms on the PK of MPA, membrane transporter expression in lymphocytes and the kidney may affect MPA PD despite targeting patients to narrow blood concentration ranges. Therefore, there is a need to move beyond individualising MPA dose to attain target plasma concentrations to the situation whereby distribution into target tissue is also factored into dosage adjustments. Due to a narrow therapeutic index (1.0 – 3.5 mg/L) and large inter-individual PK variability [96, 159], TDM has been recommended to individualise MPA dosing, ensuring sufficient concentrations to

prevent kidney rejection but not too high to cause toxicity. Routine MPA monitoring is performed by measuring C_0 concentrations in plasma, or even better by AUC determinations. The immunosuppressive site of action for MPA, however, is inhibition of IMPDH within lymphocytes. Therefore, obtaining intracellular MPA concentrations within lymphocytes may provide a better understanding of MPA distribution into target tissue and may have greater relevance for predicting MPA efficacy or toxicity than plasma concentrations.

The importance of this concept has been highlighted in a previous clinical study of 20 renal transplant recipients, which reported that intra-lymphocyte concentrations of CsA, another immunosuppressant that targets lymphocytes, decreased significantly 3 days before acute rejection was recognised clinically, and there was a significantly lower intracellular exposure to CsA in patients experiencing rejection (265 ± 18.2 versus 747 ± 221 ng/10^6 cells, P = 0.004) [160]. A similar observation between clinical rejection and decreased intra-lymphocyte concentrations of TAC (1.9 – 4.4-fold, $P < 0.05$) has also been reported in 90 liver transplant recipients [161], with this study further confirming the importance of obtaining intra-lymphocyte immunosuppressant concentrations in predicting acute rejection compared to measuring systemic concentrations alone.

In addition to intra-lymphocyte concentrations, tissue immunosuppressant concentrations within the transplanted organ may provide more clinically relevant information for predicting immunosuppressant efficacy than plasma or whole blood concentrations, as has been reported in previous studies with CsA [162, 163] and TAC [164, 165]. Although there was no significant association between blood concentrations and graft rejection, intra-renal immunosuppressant concentrations were found to be significantly lower (1.9 – 5.8-fold, $P \leq$ 0.02) in patients experiencing rejection versus those who did not experience rejection in both liver [163-165] and renal [162] transplantations.

At the time of commencing this PhD project in 2011, no previous studies had investigated the concentrations of MPA in human kidney biopsies, and there have been only two clinical studies investigating the utility of measuring lymphocyte MPA concentrations in renal transplant recipients. One study failed to find a relationship between trough lymphocyte (C_{0L}) and plasma (C_{0P}) MPA concentrations [166], however, another study reported some associations between lymphocyte and plasma MPA concentrations determined at 1.5 and 3.5 hr on days 2, 4 and 10 following transplantation, but not between MPA C_{0P} and C_{0L} concentrations [104]. Despite these two studies, the factors that determine lymphocyte MPA concentrations and variables predictive of rejection had not been previously investigated and thus remain unknown. Two important modulators of lymphocyte MPA concentration may include the binding of MPA to plasma albumin (hence unbound concentrations need to be used) and the possible role of uptake and efflux (e.g. MRP2) transporters in lymphocytes in modulating concentrations. There is yet to be a similar study investigating IMPDH activity and lymphocyte MPA concentrations, as predictors of graft rejection and importantly, including for the first time, establishing methods capable of measuring intracellular MPA concentrations in the human kidney biopsies and lymphocytes in renal transplant recipients – this will be a novel and innovative feature of this thesis.

1.5 Summary, Aims and Hypotheses

The application of TDM has been proposed to maximise the effectiveness of MPA to achieve target plasma concentrations that suppress rejection and minimise MPA-related toxicity. Whilst the use of TDM of plasma concentrations has been put forward to individualise MPA dosages, previous studies have shown that effective target plasma MPA concentrations are still highly variable between individuals. Consequently, there is a need for clinical tools that are better predictors of tissue, and lymphocyte, MPA concentrations that

are not necessarily mirrored by plasma concentrations as currently monitored with TDM alone. In addition, the application of PGx approaches to identify factors that affect the MPA concentration-effect relationship may explain some of the additional inter-individual variability in response to MPA. Therefore, knowledge of the multiple PGx factors that may influence an individual patient's response to immunosuppressants such as MPA and determine overall success of renal transplantation is needed.

The *ABCC2* gene provides a good candidate for this PGx approach, as its product, the MRP2 efflux transporter, plays an important role in determining MPA distribution into lymphocytes and kidney graft tissue, and hence, clinical outcomes. Furthermore, the gene is highly polymorphic with some genetic polymorphisms having a clinically relevant impact on MPA exposure.

Prior to commencing this PhD, several major gaps in the published knowledge of genetic polymorphisms in modulating MPA response were identified.

Firstly, the majority of research into the PGx of MPA had concentrated on genetic polymorphisms affecting MPA metabolism, not MPA distribution into lymphocytes and kidney graft tissue (Fig. 1.5) – an area still poorly researched.

Secondly, although there are numerous studies on the effects of MRP2 PGx on MPA PK and/or PD, reported findings are conflicting due to lack of haplotype analysis, with the majority of the studies considering only the impact of recipient genotype.

Thirdly, drug transporter genetic polymorphisms influencing MPA distribution into lymphocytes and kidney graft tissue had not been previously investigated, nor had the potential association (e.g. intra-lymphocyte and intra-renal concentrations) with clinical outcomes following renal transplantation.

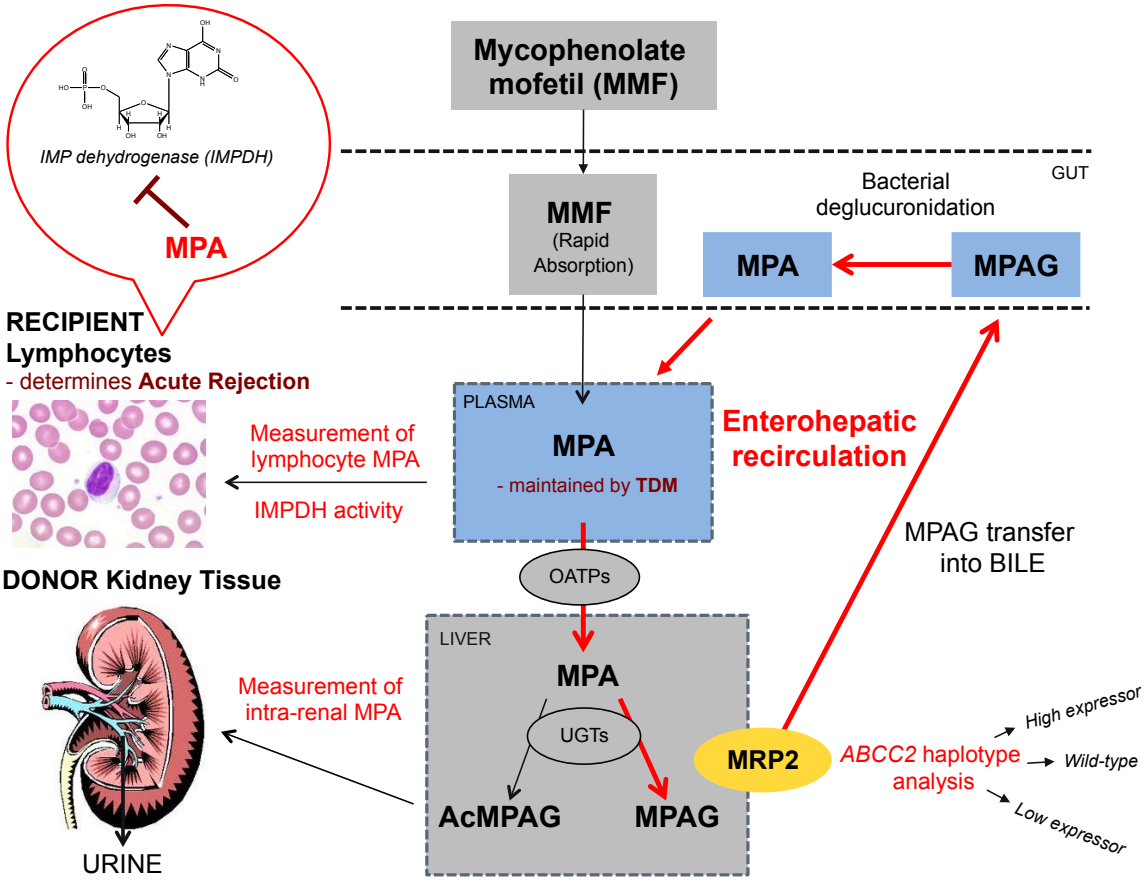

Figure 1.5 Summary of the proposed factors determining distribution of MPA in plasma, circulating lymphocytes and the kidney graft tissue. AcMPAG, mycophenolic acid acyl glucuronide; MPA, mycophenolic acid; MPAG, mycophenolic acid phenolic glucuronide; MRP2, multi-drug resistance protein 2; OATPs, organic anion transporter polypeptides; TDM, therapeutic drug monitoring; UGTs, uridine diphosphate-glucuronosyltransferases.

Finally, at the time of commencing this PhD, no studies had investigated the intracellular MPA concentrations in the human kidney and lymphocytes to determine whether these concentrations are better predictors of the incidence of rejection in renal transplant recipients.

Based on the existing literature, the major hypotheses to be tested for this PhD project were:

Hypothesis 1: Recipient *ABCC2* expression influences the plasma concentration of MPA (hence MMF dose): recipients with low expressor *ABCC2* haplotypes would have lower MPA concentrations as a result of decreased enterohepatic recirculation.

Hypothesis 2: Donor graft *ABCC2* haplotype expression or function influences the plasma concentration of MPAG: donors with high expressor *ABCC2* haplotypes would have decreased plasma concentrations of MPAG as a result of increased renal excretion of MPAG as renal excretion is the primary clearance mechanism for MPA metabolites.

Hypothesis 3: Lymphocyte MPA concentrations may have greater relevance for predicting IMPDH activity and the risk of graft rejection as compared to plasma MPA concentrations.

Hypothesis 4: Recipient *ABCC2* expressor status may influence the distribution of MPA into lymphocytes, therefore affecting lymphocyte MPA concentrations (hence the incidence of graft rejection).

Therefore, the main aims of this thesis were:

Aim 1: To develop and validate methods capable of accurately quantifying MPA concentrations in human kidney biopsies and human peripheral blood mononuclear cells (PBMCs), and intracellular IMPDH enzyme activity in PBMCs, from renal transplant recipients. Assay development and validation are presented in Chapters 2 and 4, for renal biopsies and PBMCs, respectively.

Aim 2: To investigate the influence of donor and recipient *ABCC2* haplotypes (formed by the common -24 C > T, 1249 G > A and 3972 C > T SNPs) on plasma MPA concentrations and clinical outcomes in renal transplant patients. This aim was addressed in a retrospective investigation of *ABCC2* genetic polymorphisms in patients receiving standard immunosuppression therapy consisting of MMF, TAC and prednisolone (Chapter 3).

Aim 3: To apply methods developed from Aim 1 to a pilot clinical investigation of the utility of determining lymphocyte MPA concentrations and intracellular IMPDH enzyme activity, as predictors of early graft rejection in renal transplant recipients (Chapter 4).

Chapter 2: Validation of an LC-MS/MS method for the quantification of mycophenolic acid in human kidney transplant biopsies

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Statement of Authorship is found in Appendix A.

2.1 Abstract

Mycophenolic acid (MPA) has a low therapeutic index and large inter-individual pharmacokinetic variability necessitating therapeutic drug monitoring to individualise dosing after transplantation. There is an ongoing discrepancy as to whether plasma MPA concentrations sufficiently predict kidney rejection or toxicity and whether immunosuppressant concentrations within the graft tissue may better predict transplant outcomes. The aim of the study was to develop an LC-MS/MS method for the quantification of MPA concentrations in human kidney biopsies taken as part of routine clinical procedures. A total of 4 surplus human kidney biopsies obtained from 4 different kidney transplant recipients were available to use for this study. MPA was also quantified in 2 kidney samples from rats administered MPA to assess tissue extraction reproducibility. Human kidney biopsies and rat kidneys were homogenized mechanically and underwent liquid-liquid extraction before analysis by LC-MS/MS. MPA-free human kidney tissue was used in calibrators and quality control samples. Analyte detection was achieved from multiple reaction monitoring of the ammonium adducts of both MPA (*m*/*z* 321.1 → 207.3) and N-phthaloyl-L-phenylalanine (PPA, internal standard, *m*/*z* 296.2 → 250.2) using positive electrospray ionisation. The method was linear (calibration curves $R^2 > 0.99$, n = 10), precise, and accurate with coefficients of variation and bias less than 15%. Extraction efficiencies for MPA and PPA were approximately 97 % and 86 %, respectively, and matrix effects were minimal. In 4 kidney transplant recipients, tissue MPA concentrations ranged from 1.3 – 7.7 ng/mg of tissue, however, the correlation between blood (C_0) and tissue MPA concentrations could not be established. The method was successfully applied to the quantification of MPA in human kidney biopsies without the need to alter current clinical protocols.

Keywords: Mycophenolic acid, LC-MS/MS, tissue MPA concentrations, transplantation.

2.2. Introduction

Mycophenolic acid (MPA) is a widely prescribed immunosuppressant for the prevention of rejection following kidney transplantation (Fig. 2.1) [167], usually in combination with other immunosuppressant drugs, most commonly a calcineurin inhibitor (cyclosporine or tacrolimus) and prednisolone. Due to a narrow therapeutic index and significant inter-subject pharmacokinetic variability [96, 159], MPA requires therapeutic drug monitoring (TDM) to individualize dosing, ensuring optimal plasma concentrations to prevent organ rejection and minimize the incidence of adverse events such as leukopenia and diarrhea [96, 168]. MPA pre-dose trough (C_0) concentrations are a relatively good indicator of total drug exposure [169] and the acceptable plasma therapeutic range for MPA C_0 is 1.0 to 3.5 mg/L [96]. Lower plasma C_0 MPA concentrations have been shown to correlate significantly with the risk of rejection whereas high MPA C_0 values were associated with an increased risk of toxicity [5, 47, 75]. Although TDM from plasma is necessary for individualization of MPA dosage, both acute rejection and adverse events still occur after kidney transplantation [170], further reinforcing that TDM alone may be inadequate in predicting target tissue concentrations and consequently therapeutic efficacy and/or safety. In contrast, MPA concentrations within lymphocytes or the graft tissue may provide more clinically relevant information on the immunosuppressive efficacy of MPA. Previous studies in liver and kidney transplant recipients suggest that rejection was associated with low concentrations of tacrolimus [161, 164, 165] and cyclosporine [162, 163, 171] within the graft tissue, but not with those in whole blood. To our knowledge, no previous studies have examined tissue MPA concentrations in human kidney biopsies and determined if the concentrations within the graft tissue correlate to the outcomes of rejection and toxicity compared to plasma concentrations.

The aim of this study was to develop and validate a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method capable of accurately quantifying MPA concentrations in human kidney biopsies from kidney transplant recipients taken as part of routine clinical procedures. The overall design of the method was primarily to assess whether MPA concentrations could be quantified in very small samples of kidney biopsy tissues, which are shared for histological assessments. In addition, we also assessed whether the potential in-source ion fragmentation of the metabolites of MPA (ether- and acyl-glucuronides) to MPA [172, 173] would result in an overestimation of MPA concentrations by LC-MS/MS.

2.3 Materials and Methods

2.3.1 Chemicals and reagents

Mycophenolic acid (MPA), tacrolimus, ammonium acetate, formic acid, tertiary-butyl methyl ether (t-BME) and N-phthaloyl-L-phenylalanine (PPA, internal standard (IS)) were sourced from Sigma-Aldrich (Castle Hill, NSW, Australia). MPA ether- (MPAG) and acyl- (AcMPAG) glucuronides were purchased from Roche Pharmaceuticals (Palo Alto, CA, USA). Cyclosporine and everolimus were purchased from Novartis Pharmaceuticals Australia Pty. Ltd. (North Ryde, Australia). Sirolimus was provided by Wyeth Research (NJ, USA). HPLC-grade methanol (MeOH) was purchased from Thermofisher Pty Ltd (Scoreby, VIC, Australia). Sodium chloride (NaCl), sodium dihydrogen orthophosphate ($NaH_2PO_4.H_2O$) and hydrochloric acid (HCl) were supplied by Ajax Finechem Pty Ltd (Taren Point, NSW, Australia). Sodium dihydrogen phosphate (NaH_2PO_4) and ethanol were obtained from Chem Supply (Gillman, SA, Australia). Potassium chloride (KCl) and potassium dihydrogen phosphate (KH_2PO_4) were purchased from Ajax Chemicals (Auburn, NSW, Australia). Ultrapure, de-ionized water (≥ 18.2 mOhms, Cascada AN-water Purification system, Pall, Cheltenham, VIC, Australia) was used throughout the assay.

2.3.2 Human and animals tissues

Human kidney tissue obtained from a non-transplant nephrectomy patient (The Queen Elizabeth Hospital Ethics of Human Research Committee, approval number 2008178) who was not administered MPA, was used to prepare the calibrators and quality control (QC) samples. Two rat kidney samples used as biological QCs in this study were obtained from a previous study [58]. Briefly, adult male transport-deficient (TR-) Wistar rats (HRD-AMC Abcc2) had been purchased from Harlan (Horst, the Netherlands) and ethics approval had

been obtained from the Animal Experiments Committee of the Eramus Medical Center. The rats received 20 mg/kg bodyweight mycophenolate mofetil (the prodrug of MPA; Cellcept®, Roche Bioscience, Palo Alto, CA, USA) in combination with 4 mg/kg bodyweight tacrolimus daily (oral microemulsion formulation, 10 mg/mL; Prograf®, Fujisawa Pharmaceutical Co. Ltd., Osaka, Japan), starting on day 7 and continuing through day 14 before being sacrificed. Kidney samples were then harvested and immediately stored at -80 °C until the day of assay. All harvested tissues were divided and assayed as biopsy-sized samples thereafter.

2.3.3 Measurement of tissue MPA concentrations

On the day of assay, frozen human and rat kidney specimens were thawed at room temperature and cut into biopsy-sized pieces as required (approximately 0.5-1.0 mm^3), using a clean scalpel blade and placed in a clean eppendorf tube (Sarstedt AG & Co, Nümbrecht, Germany). Patient biopsy specimens stored in TissueTek® solution (ProSciTech, Thuringowa, QLD, Australia) were removed from aluminium packaging and placed in an empty eppendorf tube to thaw. The biopsy specimens were displaced from the TissueTek® solution with a clean, unused pipette tip to the top of the eppendorf tube. The biopsy specimens were then rinsed with 2-3 drops of water, blotted dried on lint-free tissue and transferred to clean eppendorf tubes. Each tissue specimen was weighed before undergoing mechanical homogenisation. Briefly, a chilled mortar and pestle was used to grind the specimens to a fine powder. At this stage, 200 µL of phosphate buffered saline (pH 7.4) was added to each tissue specimen and was mixed thoroughly around the mortar with the pestle before being transferred into a 5 mL disposable glass tube, appropriately labeled. To each sample, 5 µL of PPA internal standard (0.1 mg/L), 60 µL of HCl (0.4 M) and 1 mL of t-BME were added. Samples were mixed on a roller mixer for 10 minutes at a gentle speed

ensuring that the samples were not mixed too vigorously as this may result in the formation of an emulsion. The tubes were then centrifuged at 4000 rpm (1900 x *g*) at 4 ºC for 10 minutes. The aqueous layer was then snap-frozen in an ethanol/dry ice bath, the organic layer removed and evaporated using an evacuated centrifuge at 45 ºC for approximately 20 minutes. The dried residues were reconstituted with 50 µL of 50/50 methanol/water, vortexed and transferred to vial-inserts. Ten microlitres of the reconstituted solutions were injected onto the LC-MS/MS for analysis.

2.3.4 Preparation of calibration curve standards and QC samples

All calibrators and quality control (QC) samples were prepared using a blank, MPA-free, human kidney tissue obtained from the non-transplant nephrectomy patient. MPA working solutions for calibration curve standards were prepared from MPA stock solution (100 mg/L in 100 % MeOH) and were then used to spike blank human kidney tissue in homogenisation buffer, to final concentrations of 0.6, 1.0, 2.0, 5.0, 10.0 and 20.0 ng/mL. Similarly, QC samples were prepared by spiking blank human kidney sample in homogenisation buffer with an independently prepared MPA stock solution (100 mg/L in 100 % MeOH) to final concentrations of 0.6, 2.0 and 10.0 ng/mL.

As well as spiked controls, two kidney samples obtained from rats administered MPA were initially assayed in replicates of 3 – 6 to determine the concentrations of MPA. Samples from these kidneys were then used consistently during method development as biological QCs to ensure tissue extraction reproducibility.

2.3.5 LC-MS/MS conditions

Detection of MPA and PPA IS in rat kidneys and human kidney biopsies was achieved using an API 3200 tandem mass spectrometer (AB Sciex, Mount Waverley, VIC, Australia) operating in positive electrospray ionization mode. The spectrometer was coupled to a Shimadzu UPLC system (Shimadzu USA Manufacturing Inc, Canby, OR, USA) consisting of a dual Shimadzu LC pump, a degasser (DGU-20A3), an autosampler (SIL-20AC) and a column oven (CTO-20AC). The analytical column used was a 2 x 30 mm Luna phenyl-hexyl, 5 µm particle size column (Phenomenex, Lane Cove, NSW, Australia) maintained at 60 ºC. Mobile phases A and B consisted of 100 % water or 100 % methanol, respectively, with both solutions also containing 2 mM ammonium acetate and 0.1 % formic acid, which assisted the formation of positive ammonium adducts used for MPA and PPA detection by the mass spectrometer. The mobile phases were filtered, degassed and pumped at a flow rate of 0.5 mL/min with the ratio of mobile phase A:B at 50:50 for 0.0 – 1.2 minutes and then changed to the ratio of 5:95 (A:B) for the remaining 1.2 – 2.2 minutes of the sample run time. The analytical column was washed using 50 % mobile phase A for a further 30 seconds prior to the next sample injection. Ten microlitres of reconstituted solutions were injected onto the analytical column and Analyst v1.5.1 software (AB Sciex, Mount Waverley, VIC, Australia) was used for data acquisition and quantitation.

The collision energy was set at 25 V, the ionspray voltage was set at 5500 V and the declustering potential was set at 25 V and 15 V for MPA and PPA IS, respectively, allowing optimal detection of MPA and PPA by the mass spectrometer. The desolvation temperature was set at 375 ºC with nitrogen as both the curtain and collision gas. Analyte detection was obtained from multiple reaction monitoring (MRM) of the ammonium adducts of both MPA (*m*/*z* 321.1→207.3) and PPA IS (*m*/*z* 296.2→250.2), and calibration curves were constructed

using weighted 1/X least-squares linear regression analysis of peak area ratios (MPA/PPA internal standard) versus MPA concentrations. MPA tissue concentrations were converted from ng/mL of MPA in digestion buffer to ng/mg of MPA in tissue for all unknown human kidney biopsies and rat kidney samples.

2.3.6 Method validation

The assay development and validation were performed following the principle of the Food and Drug Administration (FDA) guidelines for bioanalytical methods [174]. The method was assessed for accuracy, precision, linearity, extraction efficiency, matrix effects and stability.

2.3.6.1 Accuracy, precision and linearity

The highest and lower limit of quantification (LLOQ) calibrators and QC samples were assayed in replicates of 6 in a single analytical run to obtain intra-day inaccuracy and reproducibility as well as over 6 consecutive days to obtain inter-day inaccuracy and reproducibility. The assay was deemed reproducible if intra- and inter-day inaccuracy and imprecision for calibrators and QC samples were both within 15 % (or 20 % for the LLOQ). Linearity was assessed and acceptable if the coefficient of determination (R^2) for each of the calibration curves was greater than 0.99 and calibrator concentrations did not differ by more than 15 % (or 20 % for the LLOQ) from the nominal concentrations. Tissue weighing accuracy and reproducibility had been assessed previously [171]. Two rat kidney tissues were also assayed in 6 replicates within a single analytical run as well as over 6 separate days to ensure intra- and inter-day tissue extractions were reproducible. Results were accepted if these biological QCs had a coefficient of variation below 15 %.

2.3.6.2 Extraction efficiency and matrix effects

Extraction efficiency and matrix effects were estimated using a post-extraction analyte addition approach, as previously described [175]. Three sets of samples were prepared at low (2 ng/mL) and high (20 ng/mL) concentrations. Samples in set 1 consisted of standards containing the analytes at concentrations of 2 and 20 ng/mL prepared in 50/50 methanol/water; samples in set 2 consisted of MPA-free tissue acquired from rat kidney and human kidney tissues, which were spiked with 100 µL of spiking solution to give final analyte concentrations of 2 and 20 ng/mL, thereafter, the samples were extracted, as described previously, and the dried residues were reconstituted in 100 µL of 50/50 methanol/water; samples in set 3 consisted of MPA-free tissue from the same source as used in set 2 that were first extracted followed by reconstitution of the dried residues with 100 µL of 50/50 methanol/water containing the analytes of interest at amounts of equivalent to the 2 and 20 ng/mL calibrators. Extraction efficiency was estimated by comparing MPA and internal standard peak areas from the samples of set 3 to those from the corresponding samples of set 2. Matrix effects were determined by comparing the peak areas of the samples of set 1 to those from the corresponding samples of set 2. Process efficiency was determined by comparing sets 3 and 1.

Potential interference from other immunosuppressants that may be co-administered with MPA was also investigated using post-extraction addition. Based on previous measurement of cyclosporine [171] and tacrolimus [176] concentrations in human kidney biopsies, duplicate samples (0.7 mg) of drug-free human kidney tissue were homogenised and spiked with 100 µL of 50/50 methanol/water alone or methanol/water containing cyclosporine (50 ng/mg tissue), tacrolimus, sirolimus and everolimus (all at 1 ng/mg tissue). The samples were then extracted as described above, and spiked with MPA and PPA during reconstitution

using 50 µL of a 50/50 methanol/water solution containing the equivalent concentrations of a 2 ng/mL calibrator. MPA and PPA peak areas in the reconstituted extracts were also compared to direct injection of the methanol/water spiking solution.

Assessment of matrix effects was also estimated using a post-column analyte infusion technique [175, 177] that involves post-column infusion of MPA and internal standard against injections of reconstituted blank tissue extract. The sample was mechanically extracted and reconstituted in 50/50 methanol/water. Pure solutions of MPA and internal standard were introduced separately onto the LC-MS/MS system by an infusion syringe placed after the chromatographic column and before mass spectrometer ionization source in a constant flow [175]. The reconstituted tissue sample without added analyte was injected into the LC-MS/MS system, and the response from the analyte was recorded and compared to direct injection of pure 50/50 methanol/water.

2.3.6.3 Stability

The stability of MPA in tissues was investigated over a 2 month period in biological QCs that were stored at -80 °C, with replicate samples (n = 3 – 6) thawed and analysed at 0, 1 and 2 months together with freshly prepared calibration curves. The stability of MPA during chromatographic analysis was investigated by repeated injection of an extracted calibrator sample over 24 hr.

Stability of MPA glucuronide metabolites was checked using authentic MPA ether- (MPAG) and acyl- (AcMPAG) glucuronides. Three separate sets of samples were prepared for each MPA glucuronide. Samples in set 1 consisted of pure solutions containing each of the glucuronides at a concentration of 100 ng/mL prepared in 50/50 methanol/water and kept at

-20 ºC until ready for injection onto the LC-MS/MS; samples in set 2 consisted of MPA-free tissue spiked with AcMPAG or MPAG before being homogenized mechanically; samples in set 3 consisted of MPA-free tissue spiked with AcMPAG or MPAG after undergoing mechanical homogenisation; thereafter, samples in sets 2 and 3 were extracted as described previously and the dried residues were reconstituted in 50 µL of 50/50 methanol/water.

2.3.7 Clinical application of the method to biopsy specimens from kidney transplant recipients

A total of 4 excess biopsy specimens obtained from 4 different kidney transplant recipients were available for use in this study. Three of the 4 kidney transplant recipients were taking MPA, everolimus and prednisolone-, and the other, sirolimus and prednisolone-based immunosuppression. The kidneys were transplanted from living (n = 1) and deceased (n = 3) donors between February 2006 and April 2010. Informed written consent was obtained from the recipients and living donor, and where the kidney was obtained from a deceased donor, the recipient was asked to provide consent for the use of the biopsy specimen. The study was approved by the Ethics of Human Research Committee of the Queen Elizabeth Hospital (approval number 2008178). Biopsies were taken in cases of suspected rejection, with 1 of 4 biopsies taken at 8 days post-transplant, 2 taken within the first year post-transplant, and 1 taken after 4 years post-transplant. Rejection was defined according to Banff 2007 criteria [178]. Fine core needle kidney biopsies were routinely divided by pathology staff and part embedded in paraffin for histological assessment with the remaining biopsy tissue snap frozen at -80 ºC in TissueTEK® solution until the day of the assay.

2.4. Results

2.4.1 Assay performance

PPA and MPA eluted at 1.80 and 1.83 minutes, respectively, and total chromatographic analysis time was 2.2 minutes. Figure 2.2 (A) shows a representative chromatogram of an extracted patient kidney biopsy tissue, with added internal standard (light grey). The calibration curves were linear over the concentration range of 0.6 to 20.0 ng/mL, with coefficients of determination, R^2, greater than 0.997 (mean ± SD; 0.999 ± 0.00096, n = 10) routinely obtained. Intra- and inter-day inaccuracy and reproducibility for calibration curves and QC samples was less than 15 % (Table 2.1). Tissue extraction reproducibility was determined using two rat kidneys from abcc2-deficient rats administered MPA. The mean (± SD) kidney MPA concentrations (n = 3 – 6 replicates) were 0.35 (± 0.04) and 3.5 (± 0.26) ng/mg of tissue. Tissue extraction from the rat kidney samples was considered reproducible and within acceptable limits (CV < 13 %, n = 6) (Table 2.1). These rat kidneys were used consistently during method development and validation as biological QCs.

Extraction efficiency, matrix effects and process efficiency were all acceptable, as shown in Table 2.2. There was no evidence for a matrix effect in human kidney tissue. However, for rat kidney, a small matrix effect in the form of ion enhancement was observed at a higher concentration of MPA (Table 2.2), although it was well within the acceptable limit of ± 15 % as discussed previously [175, 179], and was similar for both MPA and PPA. Other immunosuppressants likely to be co-administered with MPA had no significant effects on either MPA or PPA peak areas, except sirolimus, which appeared to interfere with the detection of MPA, decreasing peak area by 39 %. In the post-column infusion technique, Fig. 2.3 shows a comparison of an injection of analyte-free human kidney tissue extract

(light grey) and mobile phase (black) for MPA (A) and PPA IS (B). MPA and PPA IS co-eluted outside of the area of ion suppression.

The stability of MPA glucuronide metabolites was checked by comparing the peak areas of both metabolites in pure solution, before and after undergoing mechanical homogenisation. There was no difference in the peak areas of MPA metabolites before and after mechanical homogenisation and no MPA peak was observed at its retention time on the chromatograms (Fig. 2.2). In contrast, a peak was observed in the MPA MRM corresponding to the retention times of MPAG or AcMPAG, 1.51 and 1.63 minutes, respectively, as shown in Fig. 2.2 (B) and (C), respectively. This could be due to in-source ion fragmentation of the glucuronides to MPA during the ionization procedure inside the mass spectrometer. However, since we were able to separate both glucuronide metabolites from MPA chromatographically; this in-source fragmentation did not interfere with quantification of MPA.

2.4.2 Method application to biopsy specimens from kidney transplant patients

Mean (± SD, range) patient biopsy weights were 0.18 (± 0.1, 0.11 to 0.33) mg, and tissue MPA concentrations measured in each patient biopsy from 4 kidney transplant recipients were 1.25 (biopsy taken at 8 days post-transplant; co-administered with everolimus), 1.77 (524 days post-transplant; co-administered with sirolimus), 6.67 (693 days post-transplant; co-administered with everolimus) and 7.67 (1651 days post-transplant; co-administered with everolimus) ng/mg of tissue, as shown in Fig. 2.4. Tissue MPA concentrations were all within the calibration curve range. Two of the 4 MPA biopsy concentrations were of a similar magnitude to the plasma therapeutic range for C_0 (1 – 3.5 mg/L) and the other two biopsies concentrations were above this range. Plasma MPA C_0 concentrations were not

available for all patients and therefore the correlation between plasma (C_0) and tissue MPA concentrations could not be established in this study.

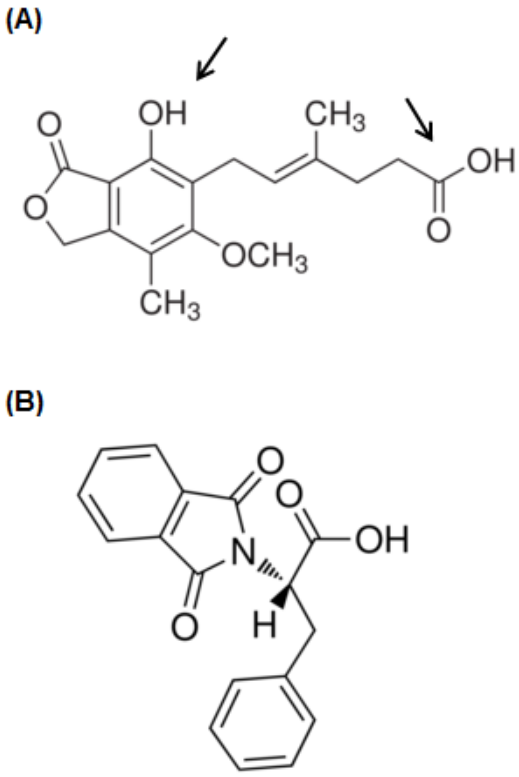

Figure 2.1 The chemical structures of (A) mycophenolic acid (MPA) and (B) N-phthaloyl-L-phenylalanine (PPA, internal standard). Sites for MPA glucuronidation are shown by the arrow.

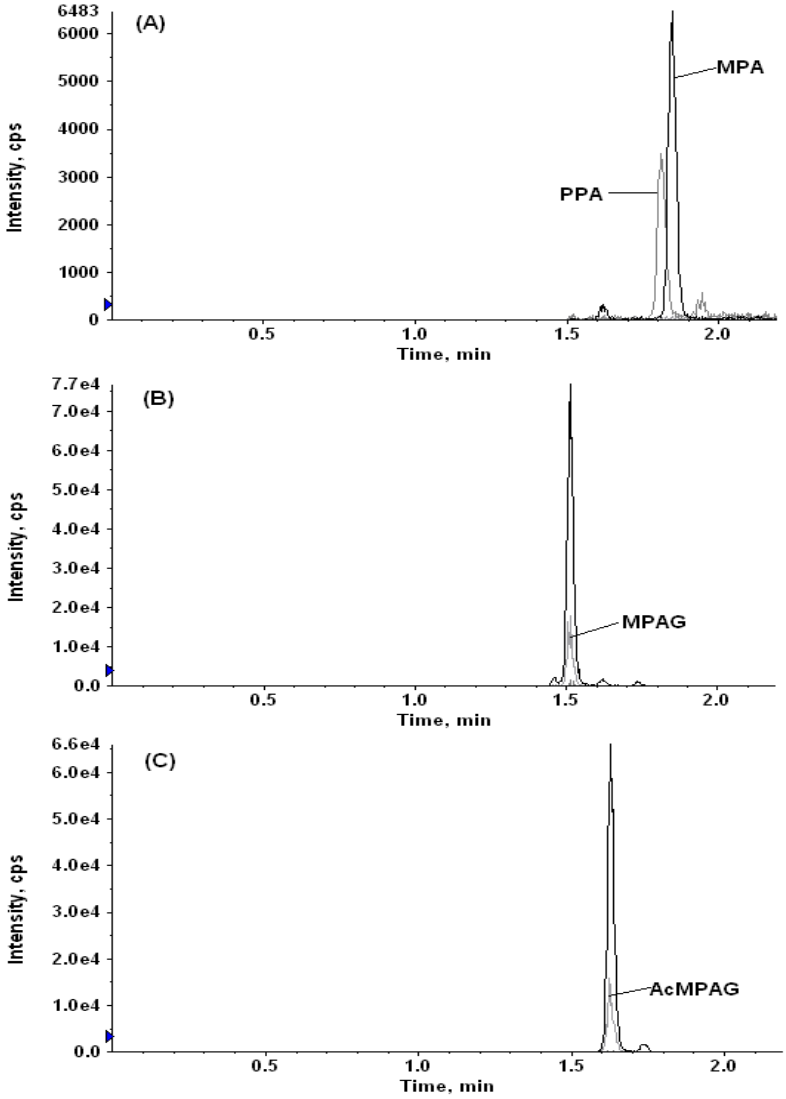

Figure 2.2 Representative LC-MS/MS chromatograms from (A) an extracted patient kidney biopsy tissue (weight 0.33 mg) with a measured MPA concentration of 11.0 ng/mL in extraction buffer, equating to 6.7 ng/mg in the biopsy; and MPA-free human kidney tissues spiked with (B) MPA ether-glucuronide (MPAG) at the retention time of 1.51 min, or (C) MPA acyl-glucuronide (AcMPAG) at the retention time of 1.63 min. In panels (B) and (C) the MPAG or AcMPAG MRMs are shown in light grey.

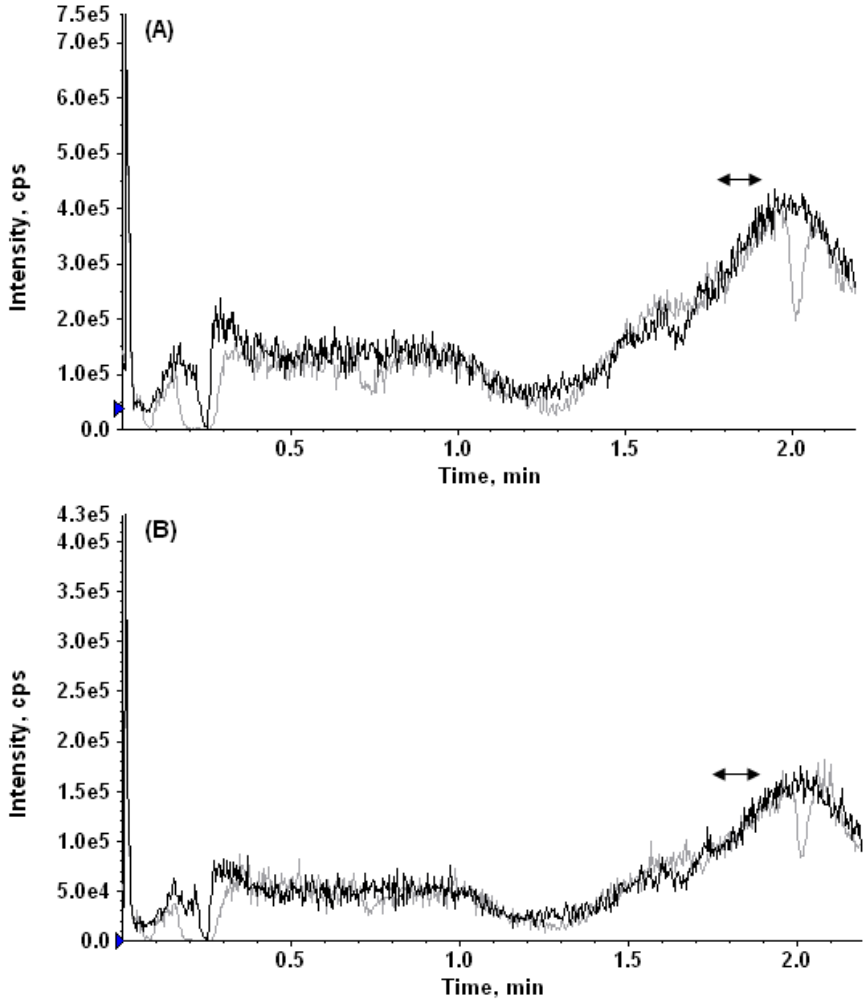

Figure 2.3 Post-column infusion traces of (A) MPA and (B) PPA IS following injections of analyte-free human kidney tissue extract (light grey) and mobile phase (black). Expected analyte peak widths are indicated by double arrow on the chromatograms.

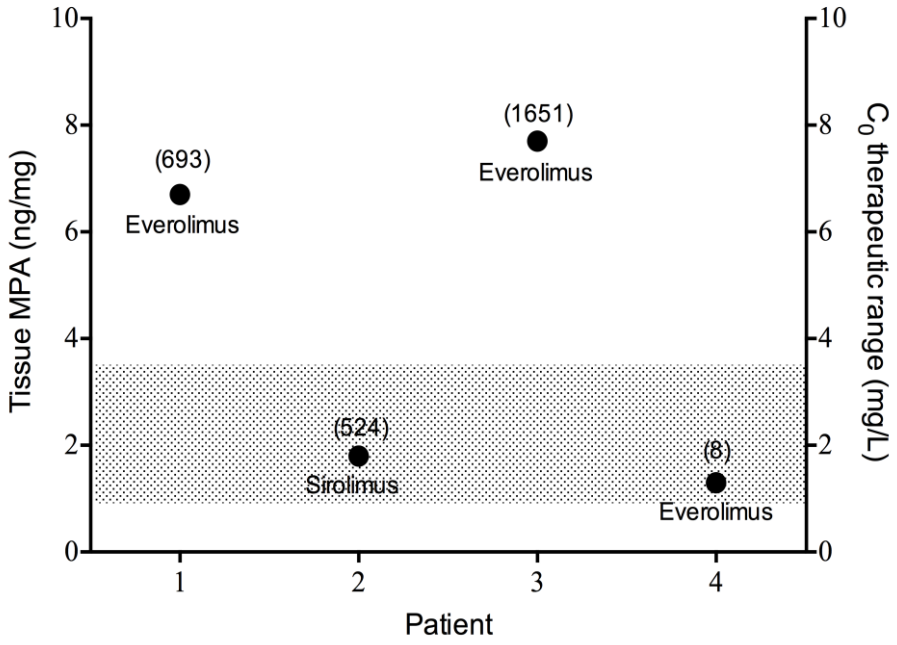

Figure 2.4 Kidney (•) MPA concentrations measured in 4 kidney transplant recipients over 8 – 1651 days post-transplantation. Numbers in parenthesis indicate the time biopsies were taken post-transplantation (days) and the shading (1.0 to 3.5 mg/L) indicates the plasma therapeutic range for C_0.

Table 2.1 Intra- and inter-day inaccuracy (bias) and imprecision of the lowest and highest calibration and low, middle and high QC samples for MPA, and two kidney samples obtained from rats administered MPA (Biological QCs).

	Intra-day (n = 6)			**Inter-day (n = 6)**		
	Mean ± SD	**CV (%)**	**Bias (%)**	**Mean ± SD**	**CV (%)**	**Bias (%)**
Calibrators (ng/mL)						
0.6	0.61 ± 0.07	10.7	2.2	0.59 ± 0.05	4.6	-0.4
20	20.1 ± 0.66	3.3	0.6	19.9 ± 0.87	4.4	-0.5
QCs (ng/mL)						
0.6	0.60 ± 0.05	8.6	0.1	0.57 ± 0.05	9.5	-5.4
2	1.86 ± 0.25	13.3	-7.3	2.0 ± 0.11	5.5	1.9
10	10.5 ± 0.82	7.8	5.1	10.3 ± 0.49	4.8	2.8
Biological QCs (ng/mg)						
A	0.35 ± 0.05	12.8	-	0.35 ± 0.04	12.9	-
B	3.5 ± 0.26	7.3	-	3.6 ± 0.22	6.1	-

Table 2.2 Extraction efficiency, matrix effects, and process efficiency of the LC-MS/MS assay in human and rat kidney samples using the post-extraction analyte addition approach [175] at MPA concentrations of 2 (low) and 20 (high) ng/mL.

		Human Kidney		**Rat kidney**	
		Low	**High**	**Low**	**High**
Extraction efficiency	MPA	97 %	98 %	94 %	89 %
	PPA	88 %	86 %	83 %	86 %
	MPA/PPA	103 %	104 %	100 %	103 %
Matrix effect	MPA	- 4 %	2 %	0 %	10 %
	PPA	- 2 %	9 %	- 5 %	10 %
	MPA/PPA	- 2 %	- 6 %	5 %	0 %
Process efficiency	MPA	94 %	99 %	95 %	98 %
	PPA	94 %	95 %	92 %	95 %
	MPA/PPA	101 %	105 %	103 %	105 %

2.5 Discussion and Conclusions

The quantification of MPA concentrations within the graft tissue may provide a better understanding of MPA distribution during kidney rejection. Previous studies in liver and kidney transplant recipients showed that intra-graft tacrolimus [161, 164, 165] and cyclosporine [162, 163, 171] concentrations are better predictors of transplant outcomes compared to whole blood concentrations. No study to date has examined tissue MPA concentrations in kidney transplant recipients to determine whether MPA concentrations within the graft tissue may better reflect or predict transplant outcomes. This study aimed to develop a method suitable for the quantification of MPA in human kidney biopsies from kidney transplant recipients taken as part of routine clinical care. Several analytical methods have been reported for the quantification of MPA concentrations in plasma [92, 93, 180-182]; however, to our knowledge, this is the first study to measure tissue MPA concentrations in human kidney biopsies taken as part of routine clinical procedures.

The method has been comprehensively assessed following the principle of the FDA criteria for industry on bioanalytical methods [174] and several aspects of this method are based on our previous method for the quantification of the calcineurin inhibitor tacrolimus in human kidney biopsy specimens [176]. Like tacrolimus, detection of MPA was also based on MRM monitoring of the ammonium adducts, similar to a several other published MPA methods measuring MPA in plasma [94, 183, 184] to enhance greater overall immunosuppressant assay flow-through on the LC-MS/MS system. However, several changes to the tacrolimus method were also necessary to maximise analyte peak intensity and improve the sensitivity. This method employed mechanical homogenisation using a mortar and pestle followed by liquid-liquid extraction of the drug in biopsy samples. Enzymatic tissue digestion had been used previously for the tacrolimus method [176] and has some advantages over mechanical

homogenisation particularly minimizing sample handling and analyte loss that may occur in the process. However, the solubilisation is carried out at alkaline pH and may hydrolyse the relatively unstable AcMPAG during sample preparation. This study demonstrated that the mechanical homogenisation technique prevented degradation of the glucuronides and produced excellent extraction efficiency (approximately 97 %) and high process efficiency, and therefore can be considered as an alternative sample preparation technique for the analysis of small biopsy-sized samples. Furthermore, the use of biological QCs obtained from rats administered MPA also provided additional assessment and validation of the reproducibility of the tissue extraction procedures. Liquid-liquid extraction has been used in our sample preparation as it has been shown to minimize potential matrix interference in comparison to the solid-phase extraction technique [185] and this method demonstrated no significant matrix effects in human samples and practically negligible ion enhancement (≤ 10 %) in rat kidney samples. Initially, carboxy-butoxy ether mycophenolic acid (MPAC) had been selected as the internal standard, however MPAC produced an interfering peak in the MPA trace (most likely due to in-source fragmentation) and was replaced by PPA, which provides overall accuracy, precision and specificity well within acceptable limits in both human and rat tissues. The use of stable labeled internal standards (MPA-d3) is highly recommended to adequately compensate for matrix effects, and may contribute to the improved performances, however deuterated internal standard was not available at the time of developing the method.

We have demonstrated no interference in the quantification of tissue MPA concentrations by cyclosporine, tacrolimus and everolimus, the other commonly co-administered immunosuppressants. However, sirolimus appeared to cause underestimation of MPA and the method may require further validation before MPA concentrations can be measured in

tissues also exposed to sirolimus. Regarding potential analytical interference from the metabolites of MPA, the in-source ion fragmentation of drug metabolites to their respective parent analyte has been discussed previously [172] and a previous study by Vogeser and colleagues [173] has demonstrated that the in-source ion fragmentation of MPAG can interfere with the quantification of MPA. We have checked with pure MPAG and AcMPAG solutions that both metabolites did not interfere with the detection and quantification of MPA. Although in-source ion fragmentation of the glucuronides into MPA was observed at the retention times of MPAG and AcMPAG, the chromatographic separation of the two MPA glucuronides from MPA resulted in no interference with the quantification of MPA. This factor must be assessed critically during method development to ensure a correct detection and quantification of the actual MPA peak, since falsely high MPA concentrations could lead to lower dose administration and potentially an increased risk of rejection in kidney transplant recipients.

The LC-MS/MS method has allowed for the accurate and precise quantification of MPA in biopsy-sized tissue samples taken as part of routine clinical care. As same-day plasma MPA C_0 concentrations were not available for the biopsy specimens in this small study, we were unable to investigate any relationships between kidney MPA concentrations and those in plasma. However, it appears that graft MPA concentrations were of a similar magnitude to the C_0 therapeutic range for MPA. In comparison, we have previously reported tissue to blood concentration ratios of 4 and 20 for cyclosporine [171] and tacrolimus [176], respectively, with no correlation between their concentrations in whole blood and those within the kidney, consistent with other published studies [163-165]. However, small sample size was a limitation for this and our two previous studies [171, 176]. In addition, this study

may also have been limited by the age of the biopsy samples, as the stability of MPAG and AcMPAG during long-term storage could not be assessed.

In summary, this method has been successfully used for quantification of MPA concentrations in core needle biopsies from kidney transplant recipients taken as part of routine clinical care. In contrast to previously published studies [162, 165], clinical practice in this study did not allow prospective acquisition of excess biopsy specimen. In addition, due to shared histological assessments and processing of biopsy specimens, this method was primarily designed to quantify MPA in routine core needle biopsies of kidney transplant recipients weighing as little as 0.1 mg. Nonetheless, these limitations have ensured that our assay benefits unaltered biopsy sampling and handling to fit into current clinical practice, and therefore will allow further investigations on the immunosuppressant MPA concentrations in the transplanted organ and their relationship with clinical outcomes following kidney transplantation.

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Chapter 3: Multidrug Resistance-Associated Protein 2 (MRP2/*ABCC2*) Haplotypes Significantly Influence the Pharmacokinetics of Mycophenolic Acid in Renal Transplant Recipients

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Statement of Authorship is located in Appendix B.

Manuscript in Preparation

3.1 Abstract

This retrospective study aimed to investigate whether donor and recipient *ABCC2* haplotypes (formed by the common -24 C > T, 1249 G > A and 3972 C > T SNPs) influence MPA and MPAG concentrations, and the potential associations between *ABCC2* genetic polymorphisms and kidney transplant outcomes. Sixty patients gave informed consent having been prescribed mycophenolate mofetil, tacrolimus and prednisolone-based immunosuppression. Blood or graft tissue was collected for *ABCC2* genotyping, and pharmacokinetic analysis was based on therapeutic drug monitoring data from recipients in whom abbreviated AUC (0 – 6 hr) monitoring had been carried out within 14 days of transplantation. *ABCC2* genotyping (-24 C > T, 1249 G > A, 3972 C > T) was performed with PCR-RFLP. *ABCC2* haplotypes were inferred using PHASE v2.1.1 and patients were classified as low, wild-type or high expressors. MPA trough (C_0) concentrations were 2.3-fold higher in recipients with high- (n = 5) compared to low-expressor (n = 30) *ABCC2* haplotypes (P = 0.04). In addition, a significant gene-dose effect was observed with an inverse step-wise relationship between *ABCC2* expression and MPA C_0 concentrations (Jonckheere-Terpstra test, P = 0.01). There was also a significant gene-dose effect between *ABCC2* expression and MPAG/MPA C_0 ratio (P = 0.04). In contrast, there was no effect of donor expressor status on either MPA or MPAG plasma concentrations. Multivariate analyses indicated that MPA C_0 was associated with recipient *ABCC2* expressor haplotype-inferred phenotype, rejection and peak panel reactive antibody (P = 0.008), whilst MPAG exposure was associated with delayed graft function and cold ischemia time (P = 0.0002). Recipient *ABCC2* haplotypes are associated with variability in MPA concentrations and rejection and may be an important factor to consider in dosage adjustment.

Keywords: MPA, MRP2, *ABCC2*, mycophenolic acid, haplotype, pharmacogenetics, pharmacokinetics, rejection, renal transplantation.

3.2 Introduction

Mycophenolic acid (MPA), administered either as the morpholinoethyl ester prodrug or as the sodium salt, is now the principal immunosuppressive agent used concomitantly with calcineurin inhibitors, either tacrolimus (TAC) or cyclosporine (CsA), to prevent rejection following renal transplantation [4]. MPA produces potent, selective and noncompetitive inhibition of inosine monophosphate dehydrogenase (IMPDH), which catalyses the *de novo* synthesis of guanosine nucleotides required for T and B lymphocyte proliferation [16]. MPA is mainly metabolised in the liver and to a lesser extent in the kidney and gastrointestinal tract [38] by UDP-glucuronosyltransferase enzymes primarily into its main inactive metabolite, the phenolic-glucuronide of MPA (MPAG) and the pharmacologically active acyl-glucuronide of MPA (AcMPAG) [41]. MPA is also subject to enterohepatic recirculation via biliary excretion of MPAG [45] and its deconjugation back to MPA through the action of bacterial β-glucuronidase in the gastrointestinal tract, and subsequent reabsorption into the circulation. The enterohepatic recycling of MPA, on average, contributes approximately 37 % (range 10 – 61 % in humans) of the total dose-interval MPA area under the concentration-time curve (AUC) and is reflected as a secondary MPA peak observed 6 – 12 hours (hr) after oral administration [32]. The kidney, however, is the main organ responsible for the irreversible excretion of both MPAG and AcMPAG metabolites [32] and multidrug resistance-associated protein 2 (MRP2) has been shown to be involved in renal and biliary excretion of MPAG [46].

Relationships between plasma MPA concentrations (trough (C_0) or AUC) with both rejection [78, 83] and adverse events [82, 83] have been demonstrated in various studies. Increased rejection rates and higher incidence of adverse events are associated with low and high MPA concentrations, respectively, with proposed target ranges to avoid these effects of

30 – 60 mg.hr/L for AUC_{0-12} [78] corresponding to C_0 concentration targets of 1.3 – 3.5 mg/L (when co-administered with CsA) or 1.9 – 3.5 mg/L (when co-administered with TAC), determined by high-performance liquid chromatography assay [96]. Because of the narrow therapeutic index and large inter-individual variability in pharmacokinetics (PK) [32], therapeutic drug monitoring (TDM) [96] has been proposed to individualise doses of MPA based on the above target ranges. The large variability in MPA PK has been at least partially explained by differences in the type of concomitantly used calcineurin inhibitor (TAC or CsA) [186], kidney graft [187] or hepatic [188] function, plasma protein binding [35] and drug-drug interactions [60]. In addition, there is a growing awareness that multiple genomic factors may also influence the PK and pharmacodynamics (PD) of immunosuppressants such as MPA [189], and therefore determine the overall success of renal transplantation.

The membrane transporter MRP2, encoded by the *ABCC2* gene, is a member of the ABC membrane transporter superfamily and the protein is predominantly expressed in the apical membrane of hepatocytes and renal proximal tubular epithelium [111]. This efflux transporter is of particular clinical importance given its pivotal role in the biliary and renal excretion of MPAG [46]. Several single nucleotide polymorphisms (SNPs) in the *ABCC2* gene have been identified and among them, the SNPs most frequently observed in Caucasians are the promoter (-24 C > T, rs717620, 20 % frequency), exon 10 (1249 G > A, rs2273697, 16 %) and exon 28 (3972 C > T, rs3740066, 30 %) [135, 136]. The functional effects of *ABCC2* polymorphisms on MRP2 expression and functional activity remain unclear. For instance, the promoter polymorphism has been shown to increase [190], decrease [135, 136] or have no influence [148] on MRP2 expression and/or functional activity. However, a recent report assessing *in vitro* expression and function of haplotypes

demonstrated that in comparison to *ABCC2* wild-type (WT) haplotype H1 (CGC; -24C/1249G/3972C), haplotype H2 (CAC; variant at 1249) resulted in increased protein expression and transport activity, haplotype H10 (TGC; variant at -24) resulted in lower protein expression, while haplotypes H9 (CGT; variant at 3972) and H12 (TGT; variant at -24 and 3972) resulted in lower protein expression and transport activity [140]. These outcomes suggest that consideration of *ABCC2* haplotypes in place of individual SNPs is more likely to accurately reflect MRP2 protein expression and/or transport activity.

Although there are a large number of clinical studies evaluating the influence of individual *ABCC2* SNPs on MPA and/or MPAG PK [136, 146, 148-151], results have been conflicting due to lack of haplotype analysis and small patient cohorts. Importantly, previous studies of the effects of *ABCC2* SNPs on clinical outcomes have focused mostly on the impact of recipient genotype. Only one study investigated the influence of both donor and recipient *ABCC2* genotype variants on kidney transplant outcomes [158]. Two main observations of this study were: i) a donor haplotype CGACA (-24C/1249G/3563A/3972C/4544A, variant at 3563 and 4544) was more frequent in patients with delayed graft function (DGF) compared to those without DGF; and ii) variant alleles (A/A) at 3563/4544 of the donor genotype were strongly related to the incidence of DGF but not with any of the other genetic or clinical parameters tested [158]. Since enterohepatic recirculation, involving biliary MPAG excretion, is an important component of MPA PK, recipient *ABCC2* expression and/or function may influence the PK of MPA. In contrast, donor *ABCC2* expression and/or function may have the greatest impact on MPAG PK, as renal excretion is the primary clearance mechanism for MPA metabolites [32].

Therefore, the aims of this retrospective study were to investigate the influence of donor and recipient *ABCC2* haplotypes (formed by the common (-24 C > T, 1249 G > A and 3972 C > T SNPs) on MPA and MPAG PK, and the potential associations between *ABCC2* genetic polymorphisms and kidney transplant outcomes. It was hypothesised that recipients with low expressor *ABCC2* haplotypes (H9, H10 and H12) would have lower MPA concentrations as a result of decreased enterohepatic recirculation, whilst high expressor donor *ABCC2* haplotypes would decrease the plasma concentrations of MPAG as a result of increased renal excretion of MPAG.

3.3 Methods

3.3.1 Study population, pharmacokinetic data and blood/graft collection

In total, 60 kidney transplant recipients, and 46 donors, who had undergone kidney transplantation between May 2004 and March 2011, were included in this retrospective study. Each recipient and living donor gave written informed consent to participate in this study. Where the kidney graft was from a deceased donor, consent for the use of tissue sample for genotyping was obtained from transplant recipients at the time of surgery. The study protocol was approved by the Royal Adelaide Hospital Research Ethics Committee (approval number 100117) and the Queen Elizabeth Hospital Human Research Ethics Committee (approval number 2008178). All transplant recipients received a fixed dose of MMF (1 g twice daily) maintenance immunosuppression in combination with TAC and prednisolone. Patient clinical notes were used to obtain demographic data (sex, age and ethnic background), MMF dose, TAC pre-dose (C_0) concentrations and transplant-related data including albumin and bilirubin concentrations, human leukocyte antigen (HLA) mismatch, cold ischemia time, panel reactive antibody (PRA) and donor type (living or deceased). The incidence of rejection within 0 – 26 days post-transplantation was defined based on clinical assessment and/or histology (e.g. protocol or for-cause biopsies) and classified for severity according to Banff 2007 criteria [178], as no rejection, subclinical borderline, clinically evident, or severe vascular rejection. The incidence of DGF was identified by the lack of spontaneous fall in serum creatinine or haemodialysis within 7 days post-transplantation.

MPA and MPAG plasma concentrations for TDM were determined by high-performance liquid chromatography (HPLC) [93]. Pharmacokinetic analyses were based on TDM data

from transplant recipients in whom abbreviated AUC monitoring had been carried out within 14 days of transplantation. In brief, blood samples (patients were on a fixed dose at the time of sampling) were collected at pre-dose, 1, 2, 3, and either 4 or 6 hr after daily MMF administration. Because of the differences in the last sampling time between individuals, the MPA and MPAG average plasma concentrations (C_{av}) were used in the data analysis and determined as follows: $C_{av} = AUC_0^n$ (mg/L) ÷ sampling time (n), where n is the time of the last sample (4 or 6 hr). Same-day TAC C_0 concentrations were also obtained from TDM data.

3.3.2 *ABCC2* genotyping

Blood samples from all 60 kidney transplant recipients and 15 living donors were available for *ABCC2* genotyping. For transplants from deceased donors (n = 31), a small sample of excess donor kidney blood vessel was collected at the time of surgery for genotyping. Of the 46 donor blood/graft and 60 recipient blood samples, 24 and 26, respectively, had already been genotyped as part of a previous study aimed to develop a primer extension denaturing HPLC (*PE-d*HPLC) assay to determine the common variants (-24 C > T, 1249 G > A and 3972 C > T SNPs) of the *ABCC2* gene [191]. The remaining samples were genotyped by the use of polymerase chain reaction (PCR) – restriction fragment length polymorphism (RFLP) assays as previously described [136]. In brief, genomic deoxyribonucleic acid (DNA) was isolated from 400 μL of whole blood collected in EDTA tubes, or ≤ 25 mg of donor tissue samples by use of a QIAamp® DNA mini kit according to manufacturer's protocol (QIAGEN, Clifton Hill, Australia). The primer sequences and the size of the amplified fragments for PCR-RFLP assay are shown in Table S3.1 (see Supplementary Tables). All PCR reactions were prepared on ice to prevent premature polymerisation and primer binding and were carried out in a 30 μL volume containing: 50 ng purified genomic DNA; 50 μM

dNTPs (Finnzymes, distributed by Genesearch, Arundel, QLD, Australia); 0.1 μM of -24 C > T and 1249 G > A or 0.5 μM of 3972 C > T forward and reverse primers; 2.5 U of Taq DNA Polymerase (New England Biolabs, distributed by Genesearch) for the -24 C > T SNP or 1 U of Taq DNA Polymerase for the 1249 G > A and 3972 C > T SNPs; and 1 X ThermoPol Reaction Buffer, (New England Biolabs). All PCR reactions were run in a PTC-100 or PTC-200 Peltier Thermal Cycler (MJ Research Inc., distributed by GeneWorks, Thebarton, SA, Australia) at optimal temperature programs for the specific PCR assay. PCR cycling conditions were as follows: 12 min at 95 °C; 35 cycles of a) 30 sec at 93 °C, b) 35 sec at 55 °C (-24 C > T), 63 °C (1249 G > A) or 58 °C (3972 C > T), and c) 30 sec at 72 °C; and 5 min at 72 °C. All PCR assays incorporated a negative (no DNA template) control to ensure no contamination of reagents and random samples were sequenced (BigDye v3.0 sequencing kit with analysis on an ABI Prism 7700 DNA sequencer (Applied Biosystems, Scoresby, VIC, Australia) at SA Pathology (Adelaide, SA, Australia) to confirm the presence of SNPs and subsequently used as positive controls (WT or variant) for each PCR assay.

After amplification, the PCR products were separated and visualised on a 4 % 2:1 (w:w) Omnigel-Sieve (Adelab Scientific Pty Ltd., Thebarton, SA, Australia):Agarose I (AMERSCO Inc., distributed by Adelab Scientific Pty Ltd.) gel containing ethidium bromide. A pUC19/HpaII DNA molecular weight marker (GeneWorks) was used as reference. PCR products were then digested with the restriction enzymes and their optimal digestion conditions for each PCR-RFLP assay are shown in Table S3.2 (see Supplementary Tables). Digested fragments were visualised by gel electrophoresis on a 4 % 3:1 Omnigel-Sieve:Agarose I gel containing ethidium bromide, with a pUC19/*Hpa*II DNA molecular

weight marker as reference. A comparison of the *ABCC2* genotypes was performed between PCR-RFLP and *PE-d*HPLC assays, and the results were 100 % concordant between assays.

3.3.3 Data analysis

Chi-squared analyses were performed to test for genotype deviations from Hardy-Weinberg equilibrium. *ABCC2* haplotype predictions were inferred by the use of PHASE software version 2.1.1 [192], which allows the estimation of haplotypes from population genotype data. The validity of PHASE haplotype estimations was checked as per PHASE recommendations, and a PHASE case-control permutation test was run to detect any significant differences in inferred *ABCC2* haplotypes between the recipient and donor groups. If the permutation test gave a significant result, post-hoc analysis of individual *ABCC2* haplotype frequencies was performed using Odds Ratios and Fisher's Exact Tests. Pairwise linkage disequilibrium was assessed using the Arlequin program (Version 3.5) [193] which was able to calculate the D' and R^2 coefficients of linkage disequilibrium as well as perform a test of linkage disequilibrium for statistical significance for each pairwise comparison of loci. Odds Ratios and Fisher's Exact Test were used to examine any differences in *ABCC2* SNP allele, genotype and haplotype frequencies between recipients and donors.

The associations between donor or recipient *ABCC2* haplotypes with MPA and MPAG pharmacokinetics (C_0, C_{av} and the corresponding MPAG/MPA ratios) were investigated in the first two weeks following transplantation using the Kruskal-Wallis test (with Dunn's multiple comparisons post hoc). The recipients and donors were divided into three groups according to the *ABCC2* haplotypes analysis as reported previously by Laechelt et al. [140]: *ABCC2* high expressor group (H2/H2 and H1/H2), low expressor group (H9/H9, H10/H10,

H12/H12, H1/H9, H1/H10 and H1/H12) and WT group (H1/H1). Gene-dose effects were analysed using Jonckheere-Terpstra Test (SPSS, version 19, IBM).

The associations between MPA and MPAG PK with genetic and clinical parameters were investigated using a general linear model of multivariate regression with step-wise addition of factors. The variables included in the step-wise addition procedure were: age, sex, genetic (donor and recipient phenotypes), rejection, DGF, MMF dose, human leukocyte antigen (HLA) mismatches, cold ischemia time (CIT), panel reactive antibody (PRA) and TAC C_0 concentration. Data are presented showing the overall *P* value and also individual *P* values for each covariate. A $P > 0.05$ for an individual covariate included in the model indicates that, whilst not independently associated with the outcome being investigated, the variable significantly improved the overall final linear model. Analysis was performed using *R* [194].

Data are presented as median (range) (unless indicated otherwise) and $P < 0.05$ (two-tailed) was considered statistically significant for all analyses.

3.4 Results

3.4.1 Frequency distribution of the *ABCC2* polymorphisms

Patient characteristics and transplant-related data are shown in Tab1e 3.1. The allele and genotype frequencies of *ABCC2* genetic polymorphisms in recipients and donors are summarised in Table 3.2. Genotype frequencies in both recipient and donor groups did not deviate from Hardy-Weinberg equilibrium ($P \geq 0.13$). There were no significant differences between the recipient and donor groups in either allele ($P \geq 0.2$) or genotype ($P \geq 0.1$) frequencies at each locus. Five and six *ABCC2* haplotypes were identified in recipients and donors, respectively, with no significant difference in population *ABCC2* haplotype frequency distributions between the recipient and donor groups ($P = 0.34$, Table 3.3). However, only 37 % of donor-recipient pairs (n = 17) had identical *ABCC2* haplotypes. The four most common *ABCC2* haplotypes in recipients and donors, respectively, were H1 CGC (-24C/1249G/3972C, both at 48 %, WT), H12 TGT (18 and 21 %, low expressor), H2 CAC (both at 14 %, high expressor) and H9 CGT (13 and 12 %, low expressor) (Table 3.3). In addition, haplotype TAT has also been observed in our study cohort and was present in 4 % of donors.

3.4.2 Linkage disequilbrium

Significant linkage disequilibrium was observed between the -24 C > T and 3972 C > T SNPs which had moderate-to-high values for both D' and R^2 (Recipient: D' = 0.64, R^2 = 0.29; Donor: D' = 0.88, R^2 = 0.33, $P < 0.0001$). No two loci were in complete linkage disequilibrium.

3.4.3 Effects of *ABCC2* haplotypes on MPA and MPAG pharmacokinetics

Recipient ABCC2 haplotypes. MPA C_0 concentrations in the recipient *ABCC2* high expressor group (n = 5) were 2.3-fold higher than those in the *ABCC2* low expressor group (n = 30, $P = 0.04$); but not different to those in the WT group (n = 8, $P > 0.99$), respectively (Fig. 3.1A). There was no significant difference between the *ABCC2* low expressor groups and WT group ($P = 0.36$). However a significant gene-dose effect relationship was observed among the three *ABCC2* haplotype groups (Fig. 3.1A, Jonckheere-Terpstra test, $P = 0.01$), with an inverse step-wise relationship between *ABCC2* expression and MPA C_0 concentrations. *ABCC2* haplotypes did not significantly affect MPAG C_0 concentrations ($P = 0.74$) (Fig. 3.1B). There was also a significant gene-dose effect between *ABCC2* expression and MPAG/MPA C_0 ratio (Fig. 3.1C, Jonckheere-Terpstra test, $P = 0.04$). There were no significant differences in either MPA ($P = 0.74$) or MPAG ($P = 0.62$) C_{av} concentrations, or the MPAG/MPA C_{av} ratio ($P = 0.99$) between the three *ABCC2* haplotype groups (Fig. 3.1D-F), respectively.

Donor ABCC2 haplotypes. Donor *ABCC2* haplotypes had no effect on MPA and MPAG C_0 ($P = 0.28$ and 0.90, respectively) or C_{av} ($P = 0.12$ and 0.22, respectively) concentrations (Fig. 3.2A, B, D and E). In addition the ratios of C_0 or C_{av} MPAG/MPA were not different between donor *ABCC2* haplotypes ($P = 0.31$ and 0.51) (Fig. 3.2C and 3.2F).

3.4.4 Effects of demographics, genetic and clinical covariates on MPA and MPAG pharmacokinetics

Rejection occurred in 22 kidney transplant recipients, giving an overall incidence of 37 %. Of these, 18 were biopsy-proven defined by Banff 2007 guidelines (Banff grade IA or

higher) and four were borderline or suspicious rejection and were treated with methyl-prednisolone. The overall incidence of DGF was 7 %.

A step-up linear regression model selection procedure was used to identify variables associated with MPA and MPAG PK. Of the variables examined (refer to Section 3.3.3), MPA C_0 was significantly associated with rejection, PRA peak and recipient *ABCC2* expression phenotypes (explaining 23 % of variance, $P = 0.008$). Patients who experienced rejection had a 1.1 unit increase in MPA C_0 concentrations ($P = 0.017$) compared to those who had no rejection (given all other predictors were held constant). MPA C_0 values increased as PRA peak increased ($P = 0.096$). Recipients with low and WT *ABCC2* expressor haplotypes had 1.1 and 0.2 units, respectively, lower MPA C_0 concentrations ($P = 0.10$ and 0.79, respectively), compared to high expressor recipients.

MPAG C_0 was significantly associated with the incidence of DGF (explaining 46 % of variance, $P = 7.9 \times 10^{-7}$); patients who had DGF had a 181 unit increase in MPAG C_0 values compared to those who had no DGF. There were no significant associations between the MPAG/MPA C_0 ratio and clinical parameters, hence no modelling of the data was performed.

Of the demographic variables tested, age was the only significant variable associated with MPA C_{av} (explaining 44 % of variance, $P = 1.0 \times 10^{-5}$). As the age of the patient increased by 1 year, MPA C_{av} decreased by 1.4 units.

MPAG C_{av} was significantly related with DGF and CIT; these variables explained 36 % of variance in MPAG values ($P = 0.0002$). Patients who experienced DGF had a 168 unit increase in MPAG C_{av} concentrations ($P = 0.00014$) compared to those who had no DGF. In

addition, patients with optimal CIT (≤ 18 hr) had a 118 unit decrease in MPAG C_{av} concentrations ($P = 0.039$) compared to those with non-optimal CIT.

Finally, the ratio of MPAG/MPA C_{av} was also significantly associated with DGF and CIT (explaining 22 % of variance in the ratio of MPAG/MPA C_{av} concentrations, $P = 0.006$). Patients who experienced DGF had a 1.2 unit increase in log (MPAG/MPA C_{av}) values ($P = 0.01$) compared to those who did not experience DGF, and as CIT increased by 1 unit, patients had a 0.04 unit increase in log (MPAG/MPA C_{av}) values ($P = 0.087$).

Table 3.1 Demographic, pharmacokinetic and transplant related data for the study population.

	Patients n = 60
Sex	
Male	35 (58.3)
Female	25 (41.7)
Age (years)	56 (19 – 72)
MMF dose (gram/day)	2 (1.5 – 3)
TAC C_0 (μg/L)	9.4 (2.5 – 21.2)
HLA mismatches	4 (0 – 6)
PRA	
At transplantation	0 (0 – 98)
Peak	0 (0 – 98)
Cold ischemia time (hr)	9.8 (3 – 23.3)
Donor graft	
Living	19 (31.7)
Deceased	41 (68.3)
Rejection	
Yes	22 (37)
No	38 (63)
DGF	
Yes	4 (6.7)
No	56 (93.3)

DGF, delayed graft function; HLA, human leukocyte antigen; MMF, mycophenolate mofetil; PRA, panel reactive antibody; TAC C_0, tacrolimus trough whole blood concentration.

Data are given as absolute values with percent data in parentheses or median (range).

Table 3.2 Recipient and donor allele and genotype frequencies (%) for the *-24 C > T*, *1249 G > A* and *3972 C > T* SNPs of *ABCC2*.

		Allele Frequency (%)				Genotype Frequency (%)				
SNP	Group	WT	V	OR (95 % CI)	*P*	WT/WT	WT/V	V/V	χ^2	*P*-value
-24C>T	Recipient	75.0	25.0	1.1 (0.56 – 2.0)	1.0	51.7	46.7	1.7	2.4	0.3
	Donor	73.9	26.1			47.8	52.2	0.0		
1249G>A	Recipient	85.0	15.0	0.90 (0.42 – 2.0)	1.0	75.0	20.0	5.0	1.1	0.6
	Donor	85.9	14.1			78.3	15.2	6.5		
3972C>T	Recipient	69.2	30.8	1.5 (0.83 – 2.7)	0.2	43.3	51.7	5.0	4.9	0.1
	Donor	59.8	40.2			32.6	54.3	13.0		

OR (95 % CI), Odds ratio (95 % confidence interval); V, Variant; WT, Wild-type; χ^2, Chi-square. *P*-value from Fisher's exact test.

Table 3.3 *ABCC2* haplotype frequencies (%) in 60 kidney transplant recipients and 46 donors.

ABCC2 haplotype	*-24 C > T*	*1249 G > A*	*3972 C > T*	Frequency (%)	
				Recipient	Donor
H1	C	G	C	47.5	47.8
H2	C	A	C	14.2	14.1
H9	C	G	T	13.3	12.0
H10	T	G	C	7.5	1.1
H12	T	G	T	17.5	20.7
	T	A	T	0.0	4.3

H1, wild-type; H2, high expressor; H9, H10 and H12, low expressor [140].

The variant nucleotides in each haplotype are underlined.

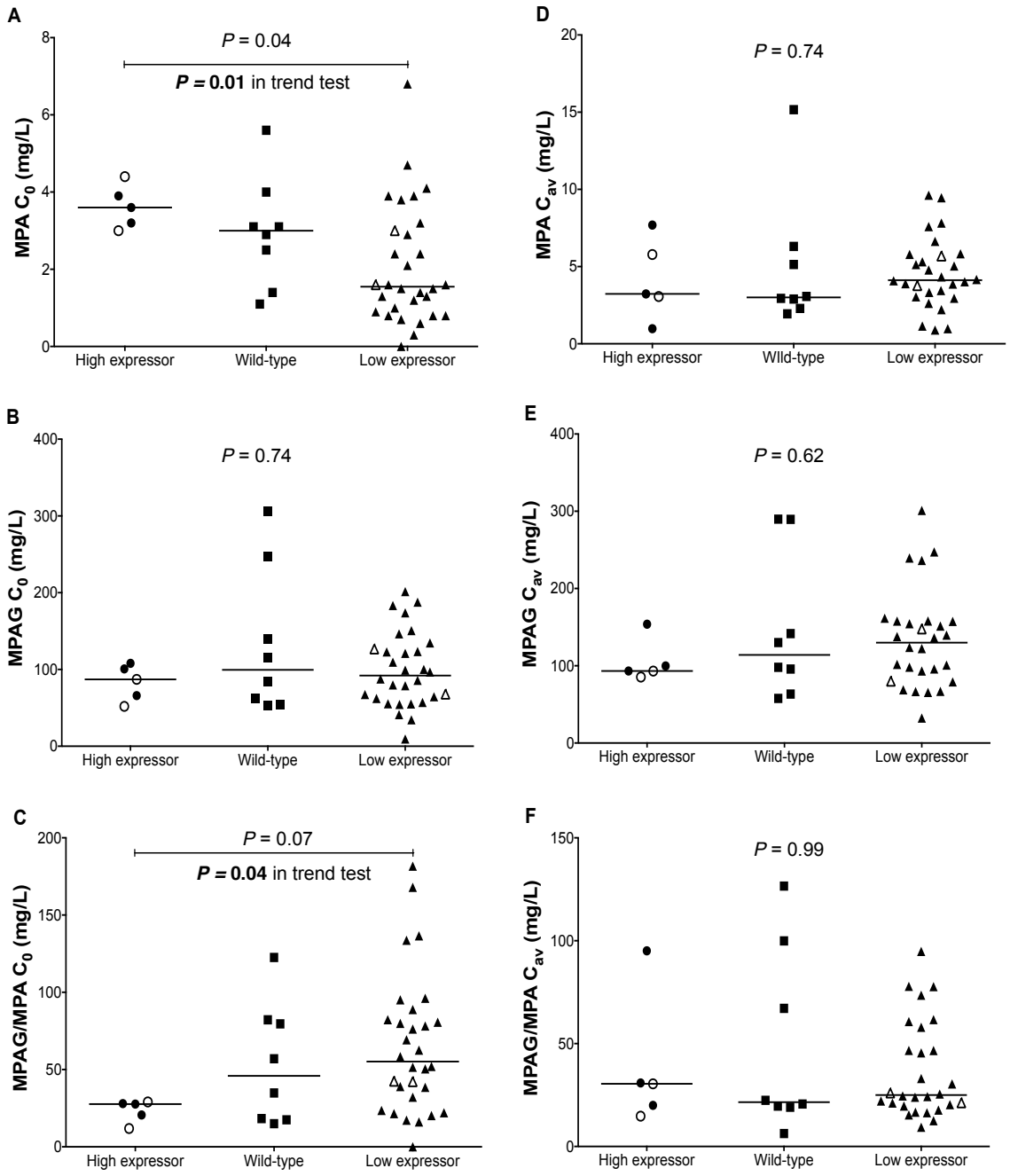

Figure 3.1 Influence of the **recipient** *ABCC2* haplotypes on (A) MPA, (B) MPAG and (C) ratio of MPAG/MPA trough (C_0) concentrations (mg/L), and (D) MPA, (E) MPAG and (F) ratio MPAG/MPA average (C_{av}) plasma concentrations (mg/L). Notes: Lines indicate median values. Open squares and triangles are homozygous high- and low-expressor *ABCC2* haplotypes, respectively. *P*-values are shown for both Kruskal-Wallis analysis (plain text) and Jonckheere-Terpstra (trend) test (bold text).

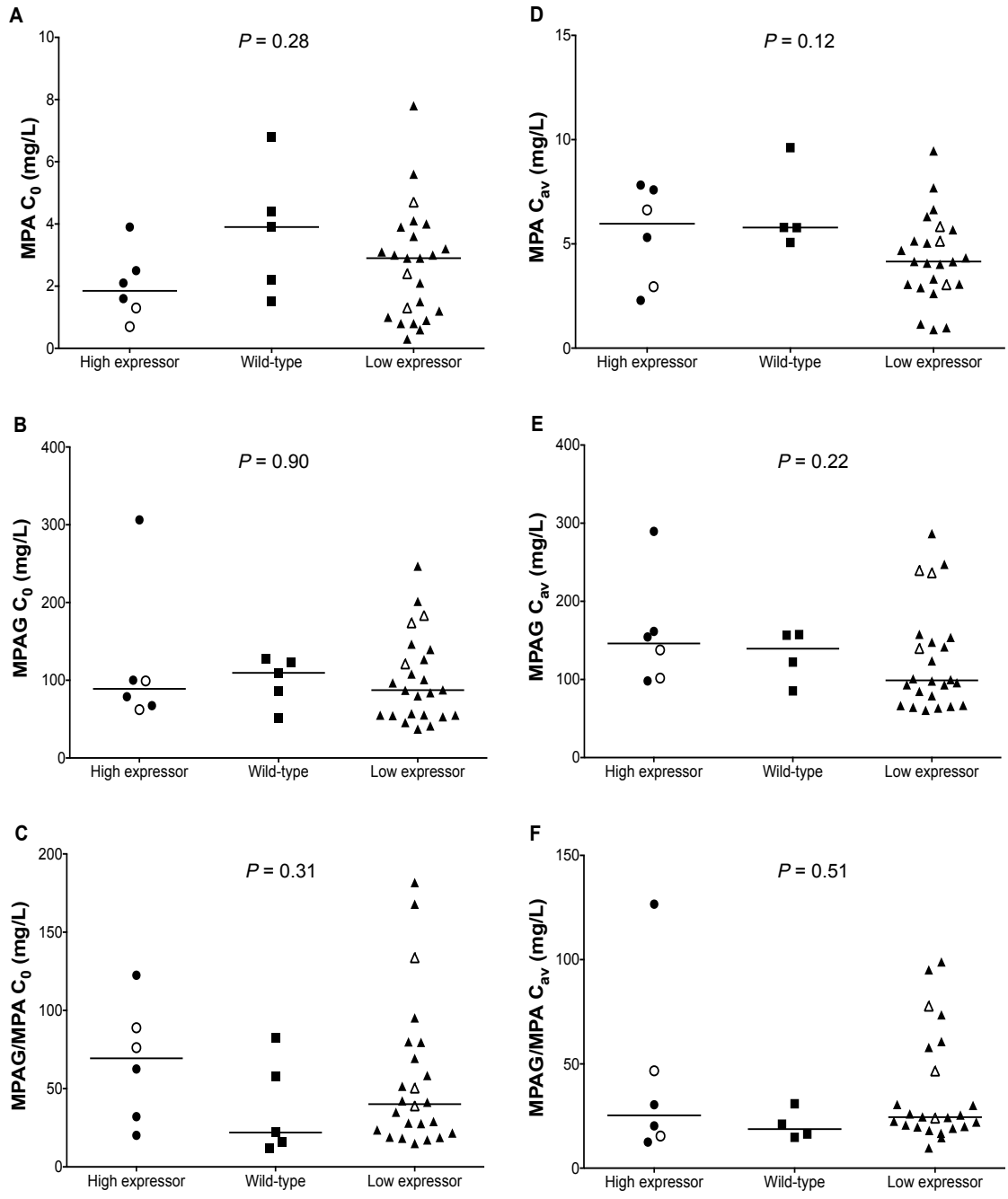

Figure 3.2 Influence of the **donor** *ABCC2* haplotypes on (A) MPA, (B) MPAG and (C) ratio of MPAG/MPA trough (C_0) concentrations (mg/L), and (D) MPA, (E) MPAG and (F) ratio of MPAG/MPA average (C_{av}) plasma concentrations (mg/L). Notes: Lines indicate median values. Open squares and triangles are homozygous high- and low-expressor *ABCC2* haplotypes, respectively. *P*-values are shown for Kruskal-Wallis analysis.

Supplementary Tables

Table S3.1 Primer sequences and the amplified fragment size for PCR-RFLP assays.

SNP	Region	Primer Sequences	Fragment size
-24 C>T	Promoter	F 5'-CTGTTCCACTTTCTTTGATGA-3'	211 bp
		R 5'-TCTTGTTGGTGACCACCCTAA-3'	
1249 G>A	Exon 10	F 5'-GGGCAAAGAAGTGTGTGGAT-3'	303 bp
		R 5'-ACATCAGGTTCACTGTTTCTCCCA-3'	
3972 C>T	Exon 28	F 5'-AACTTACTTCTCATCTTGTCTCCTTGC-3'	184 bp
		R 5'-CTCCACCTACCTTCTCCATGCTATC-3'	

bp, base pair; F, forward primer; R, reverse primer.

Table S3.2 Restriction enzymes and digest conditions used for restriction fragment length polymorphism (RFLP) analysis.

SNP	Restriction enzymes	Recognition sequence	Digest conditions	Fragment length (*bp*)
-24 C>T	*BbsI*	5'-GAAGACNN^NNN-3'	2.5 U: 16 hr at 37 °C, 20 min at 65 °C	WT: 151 + 60 V: 211
1249 G>A	*NcoI*	5'-AT^CG_AT-3'	5 U: 16 hr at 37 °C, 20 min at 65 °C	WT: 208 + 95 V: 208 + 69 + 26
3972 C>T	*ClaI*	5'-C^CATG_G-3'	1 U: 16 hr at 37 °C, 20 min at 65 °C	WT: 158 + 26 V: 184

bp, base pair; N, can be any nucleotide; U, unit; V, variant fragment; WT, wild-type fragment; ^ , cleavage site.

3.5 Discussion

Previous pharmacogenetic studies of *ABCC2* genetic variability on MPA and MPAG PK have reported conflicting findings with the majority of studies having investigated only the genotype of the recipient and the influence of *ABCC2* genetic polymorphisms in isolation. To our knowledge, this is the first study to investigate the role of *ABCC2* haplotypes of both the donor graft and the transplant recipient on MPA and MPAG pharmacokinetics and kidney transplant outcomes.

In the present study, the three SNPs investigated (-24 C > T, 1249 G > A and 3972 C > T) are the most commonly observed in the Caucasian population. The allele and genotype frequencies observed were not significantly different between the recipients and donors, and variant allele frequencies were comparable to those previously published in healthy Caucasians [135] and in Caucasian kidney transplant recipients [136]. As reported in previous studies [135, 140], the four most common *ABCC2* haplotypes identified were the CGC (WT), CAC (high expressor), CGT (low expressor) and TGT (low expressor). In addition, although there was no significant difference in overall haplotype frequencies between donor and recipient groups, only 37 % of recipients had identical *ABCC2* haplotype to their donor graft. This mismatching on the basis of *ABCC2* haplotype indicates that *ABCC2* expression and/or functional activity may differ significantly between the transplant recipient and the donor graft. Although previous studies have investigated the frequencies of *ABCC2* haplotypes [135, 140, 158, 195], comparison across studies is limited due to the different combinations of *ABCC2* SNPs included in the analyses. Despite this, the frequencies of the WT (CGC) (48 % versus 39 % [140] and 47 % [135]), CAC (14 % versus 20 % [140] and 21 % [135]) and TGT (18 – 21 % versus 18 % [140] and 17 % [135]) haplotypes (combination of -24 C > T, 1249 G > A and 3972 C > T SNPs) were comparable

to those reported previously. In agreement with previous findings [135, 195], significant linkage disequilbrium was observed between the -24 C > T and 3972 C > T SNPs and although the 3972 C > T SNP results in a silent mutation, this linkage may be inferred in altered MRP2 expression and/or functional activity.

The investigation of the possible associations between *ABCC2* haplotypes and MPA PK revealed new and significant findings. Of the prior investigations that have focused on individual *ABCC2* SNPs, conflicting observations on the influence on MPA PK have been reported [136, 146, 149, 151]. To our knowledge, the present study provides the first evidence that the recipient's MRP2/*ABCC2* affects the PK of MPA in a haplotype-specific manner. This study has demonstrated for the first time that recipient haplotype CAC (-24C/1249A/3972C), exhibiting significantly higher protein expression [140], is associated with 2.3-fold higher MPA C_0 concentrations, compared to recipients with low expressor haplotypes. This association was also confirmed in the multivariate analysis, and is consistent with increased enterohepatic recirculation due to increased biliary excretion of MPAG and reabsorption of MPA after deglucuronidation of MPAG in the gastrointestinal tract. The lack of significant differences in MPA C_0 concentrations between recipients with the WT (CGC) and low expressor haplotypes (CGT, TGC and TGT) may suggest that the variant A at position 1249 could have accounted for the high levels of MPA C_0 concentrations. In addition, significant gene-dose effects were observed between recipient *ABCC2* expression with MPA C_0 concentrations and MPAG/MPA C_0 ratio.

In contrast, *ABCC2* recipient haplotypes were not associated with MPA C_{av} concentrations. The concentration-time profile of MPA includes a secondary peak as a result of enterohepatic recirculation, occurring 6 – 12 hr following administration [32]. Thus,

recipient *ABCC2* gene variants are most likely to affect this later part of the dosing interval, which in this study is seen as differences in C_0 concentrations, rather than the early-hour samples (C_{1-3}), which are more closely related to the rate of absorption. These findings are of practical clinical interest and may be informative for groups assessing MPA exposure through abbreviated AUC monitoring rather than MPA C_0 monitoring. In addition, the calcineurin inhibitor co-administered with MMF may also influence the enterohepatic recirculation of MPA and should be taken into consideration. CsA, but not TAC, decreases MPA and increases MPAG plasma concentrations [54, 186]. Although this interaction was initially attributed to the inhibition of MRP2-mediated biliary excretion of MPAG by CsA [58], this is now thought to be due to the inhibition of MPAG uptake into hepatocytes by the organic anion transporting polypeptides (OATPs) [59]. However, this was not expected in the present study as all patients were receiving TAC as part of their immunosuppressant therapy.

There was no association between recipient *ABCC2* haplotypes with either MPAG C_0 or C_{av} concentrations. This seems contradictory to expectations as recipients with low expressor *ABCC2* haplotypes, associated with lower protein expression and transport activity [140], may be expected to have higher plasma MPAG concentrations than high expressor recipients. These findings, however, are not unexpected since hepatic clearance only plays a minor role in the clearance of MPAG.

This is the first study investigating the potential influence of donor *ABCC2* genetic polymorphisms on the PK of MPA and MPAG. The efflux MRP2 transporter is expressed in the apical membrane of kidney proximal tubules [111], hence the donor *ABCC2* genotypes determine kidney graft MRP2 expression and/or function, and consequently influence the

renal clearance (CL_R) of MPAG. However, in the present study, no association was observed between donor *ABCC2* haplotypes and either MPA or MPAG PK. The renal clearance of MPAG may involve passive filtration from plasma and active tubular secretion (potentially MRP2-mediated) and/or reabsorption. In healthy subjects, the mean unbound fraction of MPAG in plasma (f_u) is approximately 0.18 [30], and the CL_R of MPAG was reported between 25 – 34 mL/min [27]. Assuming an average glomerular filtration rate (GFR) of 120 mL/min in healthy subjects, active tubular secretion may be inferred if CL_R is greater than GFR x f_u (approximately 22 mL/min). Thus, for MPAG, the contribution of active tubular secretion to overall CL_R may be relatively small, and a large change in *ABCC2* expression and/or MRP2 functional activity may be necessary to significantly affect MPAG plasma concentrations.

In addition to identifying associations between *ABCC2* haplotypes and MPA PK, the present study also investigated the effects of demographics and other clinical covariates on MPA and MPAG PK using a step-up linear regression model selection procedure. Recipient MRP2/*ABCC2* expression phenotypes, PRA peak and rejection contributed individually towards the association with MPA C_0 values, and collectively explained 23 % of MPA C_0 variability. Supporting our initial findings, the multivariate analysis revealed that higher MPA C_0 concentrations were associated with high recipient MRP2 expression phenotype and, somewhat surprisingly, also related to the risk of rejection and high PRA peak. It is unclear whether rejection may have played a role to cause higher MPA C_0 concentrations, or whether having a high expressor MRP2 phenotype *per se* may predispose patients to rejection, particularly in patients with high PRA peak, who are already at greater risk of rejection [196]. Alternatively, the effect of MPR2 phenotype on the enterohepatic recirculation of MPA may alter the relationship between C_0 and AUC, as previously

suggested for the interaction between MPA and CsA or TAC [96], such that a slightly higher C_0 concentration target range may be necessary for patients who are high MRP2 expressors compared to those who are low MRP2 expressors.

Interestingly, the multivariate analysis did not show an association between MPA C_{av} concentrations and the incidence of rejection, potentially reflecting the importance of incorporating MPA concentrations between 6 – 12 hr post-dose to accurately determine total MPA exposure in patients co-administered with TAC. Age of the patients was the only significant variable associated with MPA C_{av} concentrations, in agreement with another recent study, which revealed that higher recipient age was associated with increased clearance of MPA [197]. As expected, DGF and CIT were associated with MPAG concentrations as renal clearance is the final irreversible clearance of the glucuronide. DGF was associated with increased MPAG C_0 and C_{av} concentrations and conversely, CIT (optimal CIT $\leq$ 18 hr) was linked to decreased MPAG C_{av} concentrations. CIT plays an essential role in the success of renal transplantation and prolonged CIT is a risk factor for the development of DGF [198]. Recent studies have shown a correlation between longer CIT (20 – 30 hr and > 30 hr) and increased incidence of DGF [199] and each additional hour of CIT can increase the risk of graft failure [200].

Despite the novel observations from this study, several limitations need to be discussed. Firstly, blood samples were only collected up to 6 hr post-dose and therefore may have underestimated the effects of *ABCC2* haplotypes on the enterohepatic recycling of MPA. Furthermore, the use of C_{av} concentrations based on the last sample taken at either 4 or 6 hr post-dose may potentially confounding the results, such that the 6- but not the 4-hr sample, may be picking up the secondary peak on the enterohepatic recirculation of MPA. The use of

an AUC_{0-12} would be a more reliable tool to adequately reflect changes in total MPA exposure but remains an impractical barrier. For example, a decrease in the partial AUC_{6-12} may have better indicated changes in the enterohepatic recycling of MPA, which could result in MPA under-exposure and altered dosage requirements. Secondly, it is possible that detecting associations between *ABCC2* haplotypes (e.g. MRP2 expressor groups) and PK or clinical outcomes may have been limited by the relatively small sample size and low frequency of the high expressor phenotype (approximately 15 %). In addition, both recipient and donor *ABCC2* haplotypes could have a combined effect on the PK of MPAG and MPAG/MPA ratio, however, combining recipient/donor haplotype expressor groups would result in extremely small numbers in some groups. Finally, this study was limited to examination of only three SNPs, and it is possible that other functionally relevant SNPs (e.g. 3563 T > A and 4544 G > A) [201] are associated with clinical outcome [158] and also contribute to interindividual variability in MPA PK.

In conclusion, recipient *ABCC2* haplotypes impacted on MPA C_0 concentrations and may be useful to optimise the starting dose of MMF, resulting in faster achievement of target concentrations. In our study, there was no significant influence of donor *ABCC2* haplotypes on kidney transplant outcomes and matching of the *ABCC2* haplotypes between recipients and donors was uncommon, suggesting that haplotype analysis of donors may be of limited additional benefit in patients already undergoing TDM. In contrast, the previously reported association between donor haplotype and DGF, and the combined contribution of rejection and recipient high expressor *ABCC2* haplotype on MPA C_0 concentrations warrants further investigation in a larger and internationally collaborative validation cohort.

3.6 Acknowledgements

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Chapter 4: Intra-Lymphocyte Concentrations of Mycophenolic Acid Correlate with the Incidence of Early Graft Rejection in Renal Transplant Recipients

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Statement of Authorship is located in Appendix C.

Manuscript in Preparation

4.1 Abstract

Although therapeutic drug monitoring of mycophenolic acid (MPA) concentrations in plasma has been recommended to individualise MPA doses in transplant recipients, little is known regarding MPA concentrations at the site of action within lymphocytes, where MPA inhibits inosine monophosphate dehydrogenase (IMPDH). This study investigated the utility of measuring trough lymphocyte MPA (C_{0L}) concentrations and IMPDH activity, as predictors of graft rejection in renal transplant recipients. Forty-eight patients commencing mycophenolate mofetil (1 g twice daily) for maintenance immunosuppression in combination with tacrolimus and prednisolone were recruited. Blood was collected for determination of total (C_{0P}) and unbound (C_{0u}) plasma MPA concentrations. Lymphocytes were isolated for determination of C_{0L} concentrations and IMPDH activity. The incidence of rejection within 2 days of sample collection was determined histologically and classified according to the Banff 2007 criteria. There was no significant correlation between MPA C_{0L} and C_{0P} concentrations (Spearman r = 0.18, P = 0.22), however, MPA C_{0L} concentrations were significantly correlated with MPA C_{0u} concentrations (Spearman r = 0.36, P = 0.034). Multivariate analysis indicated that MPA C_{0L} concentration was the only covariate independently associated with rejection incidence (33 % of patients had rejection, P = 0.03), whilst MPA C_{0P} concentration was the only significant variable associated with MPA C_{0L} concentration (P = 0.009). The ROC area under the curve (AUC) for the prediction of severe rejection using MPA C_{0L} concentrations was 0.71 (P = 0.045), with a C_{0L} threshold of 0.5 ng/10^7 cells providing 71 % sensitivity and 70 % specificity. However, the ROC analysis for IMPDH activity was not significant (AUC = 0.66, P = 0.12). MPA C_{0L} measurement within the early post-transplant period may be one factor to facilitate early titration of MPA dosing to significantly reduce rejection.

Keywords: Mycophenolic acid (MPA), inosine monophosphate dehydrogenase (IMPDH), lymphocyte MPA, intracellular MPA, rejection, renal transplantation.

4.2 Introduction

Mycophenolic acid (MPA), one of the primary immunosuppressants administered to prevent rejection following renal transplantation [4], exerts anti-proliferative effects on lymphocytes [17]. MPA is a potent, selective, reversible, and non-competitive inhibitor of inosine monophosphate dehydrogenase (IMPDH), a rate-limiting enzyme involved in *de novo* purine synthesis that is selectively required for lymphocyte proliferation [17]. Inhibition of this pathway prevents the proliferation of lymphocytes and the activation of T-cells, which consequently contributes to the prevention of graft rejection. Similar to MPA, calcineurin inhibitors such as cyclosporine (CsA) and tacrolimus (TAC), also produce immunosuppression by targeting lymphocytes and causing selective inhibition of the nuclear transcription factor calcineurin, thus preventing the formation and secretion of interleukin-2 [22, 23].

Immunosuppression following renal transplantation typically involves triple therapy with mycophenolate mofetil (MMF), prednisolone and a calcineurin inhibitor (TAC or CsA) [4]. The clinical use of immunosuppressants, such as MPA, however, is complicated by a narrow therapeutic index and large inter-patient variability in pharmacokinetics (PK) [5, 96]. Numerous studies have investigated the plasma MPA concentration-effect relationships, and in general have reported relationships between MPA exposure and the risk of graft rejection [77, 78, 80, 82, 83]. Consequently, MPA may benefit from therapeutic drug monitoring (TDM) to individualise MPA dosing in order to achieve target plasma concentration ranges that suppress graft rejection and minimise MPA-related toxicity [96]. Routine MPA monitoring is performed using either trough plasma (C_{0P}) concentrations, or even better, using the area under the plasma concentration-time curve (AUC_P) determinations [96].

Without TDM, patients are at greater risk of either inadequate immunosuppression resulting in graft rejection, or over-exposure and subsequent immunosuppression toxicity [96].

Despite application of TDM strategies that target patients to narrow plasma concentrations ranges (1.9 – 3.5 mg/L for C_{0P} or 30 – 60 mg.hr/L for $AUC_{0\text{-}12P}$, when co-administered with TAC) [95], rejection rates in Australian renal transplant recipients remain high (13 – 24 % in the six months post transplantation) [4]. This suggests that only monitoring of plasma concentrations may be inadequate to predict graft rejection and may not be the most appropriate predictor for target tissue concentrations, and hence, clinical outcomes. Consequently, there is a need for clinical tools that are better predictors of target tissue concentrations and these could include direct drug measurement at the target site of action (e.g. lymphocytes) and identification of pharmacodynamic (PD) biomarkers (e.g. IMPDH activity).

Direct measurement of lymphocyte MPA concentrations may provide a better understanding of MPA immunosuppressive efficacy and its distribution during graft rejection, compared to plasma concentrations. The importance of such an approach has already been demonstrated for the calcineurin inhibitors CsA [160, 202-204] and TAC [161, 165] by direct quantification in lymphocytes, representing the target site of action. It has been suggested that lower lymphocyte CsA [160] and TAC [161] concentrations are associated with significantly higher incidences of graft rejection, which were not reflected by whole blood concentrations. Indeed, a previous clinical study of 20 kidney transplant recipients demonstrated that lymphocyte CsA concentrations ($AUC_{0\text{-}12L}$) were approximately 3-fold lower in patients experiencing rejection (35 %) compared to those who did not experience rejection ($P = 0.004$), during the first three months following transplantation [160]. A similar

observation has also been reported in 90 liver transplant recipients, where low lymphocyte TAC concentrations were associated with a 2-fold higher incidence of clinical rejection (P = 0.01) in the early post-transplant period, despite adequate concentrations in whole blood [161].

Prior to commencing this study in 2013, little was known regarding the relationship between lymphocyte and plasma MPA concentrations. A study in 40 kidney transplant recipients failed to find an association between MPA C_{0P} and trough lymphocyte (C_{0L}) MPA concentrations (P = 0.17) [166]. However, a later study conducted by the same group reported some correlations between plasma and lymphocyte MPA concentrations determined at 0, 1.5 and 3.5 hour (hr) on days 2, 4 and 10 post-transplantation [104]. There were significant correlations between plasma and lymphocyte MPA concentrations at 1.5 and 3.5 hr, but not between MPA C_{0P} and C_{0L} concentrations [104]. Despite these two findings, no previous studies had investigated the factors that determine lymphocyte MPA concentrations. Two important modulators of these concentrations may include the binding of MPA to plasma albumin (hence unbound concentrations need to be used) and the possible role of uptake and efflux transporters in lymphocytes in modulating concentrations. The efflux transporter multidrug resistance-associated protein 2 (MRP2), encoded by the *ABCC2* gene, for which MPA is a substrate [46], is expressed in lymphocytes [205], hence genetic variability in *ABCC2* gene may influence lymphocyte MPA concentrations and could therefore modulate the efficacy and/or safety of MPA therapy.

The PD monitoring of MPA by the measurement of IMPDH activity has also been investigated in whole blood [206] and lymphocytes [103, 104, 106, 107], however only two studies have investigated relationships between MPA plasma concentrations and IMPDH

activities [103, 104]. A study in 35 renal transplant recipients reported a weak inverse correlation between post-transplant pre-dose IMPDH activity and MPA C_{0P} concentrations (R^2 = 0.24, P = 0.17) [103]. Relationships between MPA plasma concentrations and IMPDH activities (samples taken at multiple time points (0, 1.5, and 3.5 hr) on different days (2, 4 and 10 post-transplant) have also been investigated in a prospective study involving 40 renal transplant recipients receiving MMF therapy (1 g daily) [104]. There were significant inverse correlations between IMPDH $T_{1.5}$ activity and MPA $C_{1.5}$ or $AUC_{0\text{-}3.5}$ ($P < 0.01$ or P = 0.027, respectively), however, no correlation was found between MPA C_{0P} concentrations and pre-dose IMPDH activities. To the best of our knowledge, no previous studies have investigated the utility of determining both lymphocyte MPA concentrations and IMPDH activity, and examined their relationship to clinical outcomes after renal transplantation.

Therefore, this study aimed to investigate the utility of measuring lymphocyte MPA concentrations and intracellular IMPDH activity, as predictors of early graft rejection in kidney transplant recipients. We hypothesised that determination of lymphocyte MPA concentrations within the early post-transplant period may have greater relevance for predicting IMPDH activity and the incidence of graft rejection, compared to plasma MPA concentrations. Using a general linear model of multivariate regression with step-wise addition of factors, the present study also examined variables predictive of rejection and lymphocyte MPA concentrations, as well as investigated associations between MPA C_{0L}, C_{0P}, C_{0L}/C_{0P} ratio, average (C_{Pav}) and unbound (C_{0u}) plasma concentrations with data on patient demographics, biological characteristics, and *ABCC2* genetics.

4.3 Methods

4.3.1 Chemicals and reagents

Mycophenolic acid (MPA), tertiary-butyl methyl ether (t-BME), adenosine 5'-monophosphate (AMP), inosine 5'-monophosphate (IMP), β-nicotinamide adenine dinucleotide hydrate (NAD^+), perchloric acid ($HClO_4$) and tetrabutylammonium hydrogen sulphate (TBA) were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). MPA-d3 internal standard was obtained from Toronto Research Chemicals (Toronto, Canada). Sodium chloride (NaCl) and hydrochloric acid (HCl) were supplied by Ajax Finechem Pty Ltd (Taren Point, NSW, Australia). Sodium dihydrogen phosphate (NaH_2PO_4) was sourced from Chem Supply (Gillman, SA, Australia). HPLC-grade methanol and xanthosine 5'-monophosphate (XMP) were purchased from Thermo Fisher Scientific Australia Pty Ltd (Scoreby, VIC, Australia). Potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4) and potassium carbonate (K_2CO_3) were obtained from Ajax Chemicals (Auburn, NSW, Australia). Ultrapure, de-ionised water (Millipore, ≥ 18.2 mΩ, Cascada AN-water purification system, Cheltenham, VIC, Australia) was used throughout the assay.

4.3.2 Study population, pharmacokinetic and clinical data

In total, 48 kidney transplant recipients gave written informed consent to participate in this prospective clinical study, which was approved by the Royal Adelaide Hospital Research Ethics Committee (approval number 130109). Recipients were transplanted between June 2013 and November 2014 with kidneys from living and deceased donors. They received a fixed dose of MMF (1 g twice daily) maintenance immunosuppression in combination with TAC and prednisolone. Total (C_{0P}) plasma MPA concentrations were determined (refer to Section 4.3.3) using a validated high-performance liquid chromatography (HPLC) method,

[93] and unbound (C_{0u}) plasma MPA concentrations were determined by the Analytical Chemistry Unit, Pathology Queensland, Royal Brisbane and Women's Hospital, Brisbane QLD, using a validated liquid chromatography-tandem mass spectrometry method (unpublished assay). Demographic, pharmacokinetic and clinical data were obtained from original patient case notes and the data collected were: recipient and donor ages; sex; ethnicity (self-report); donor type (living or deceased); human leukocyte antigen (HLA) mismatches; cold ischemia time (CIT); panel reactive antibody (PRA); plasma creatinine; albumin and bilirubin concentrations; angiotensin II type-1 receptor (AT1R) antibody levels; donor-specific HLA antibodies (DSA); trough whole blood TAC concentrations; and the incidences of rejection and delayed graft function (DGF). The incidence of rejection within ±2 days from sample collection (median (range): 13 (5 – 22) days post-transplant) was defined based on clinical assessment and/or histology (e.g. protocol or for-cause biopsies), and classified for severity according to Banff 2007 criteria [178] as no rejection, subclinical borderline, clinically evident, or severe vascular rejection. The incidence of DGF was identified by the lack of spontaneous fall in serum creatinine or haemodialysis within 7 days post-transplantation.

4.3.3 MPA pharmacokinetic calculations

Briefly, abbreviated AUC_P monitoring had been carried out within 5 - 22 days of transplantation as part of the recipients' routine clinical care. Blood samples (patients were on a fixed dose at the time of sampling) were collected at pre-dose (C_{0P}), 1, 2, 3, and either 4 or 6 hr after MMF administration. As there were differences in the last sampling time between patients, the average MPA plasma concentration (C_{Pav}) was determined as follows: $C_{Pav} = AUC_0^n$ (mg/L) ÷ sampling time (n), where n is the time of the last sample (4 or 6 hr).

4.3.4 *ABCC2* genotyping and haplotype predictions

Blood samples collected from transplant recipients were also used for determination of the common *ABCC2* allele variants (-24 C > T, 1249 G > A and 3972 C > T). A previously reported method was used to determine mutations by polymerase chain reaction (PCR) – restriction fragment length polymorphism (RFLP) assay [136] and *ABCC2* haplotypes were inferred by the use of PHASE software version 2.1.1 [192]. The recipients were divided into high, wild-type (WT) or low MRP2 expressor groups according to *ABCC2* haplotypes as reported previously by Laechelt et al. [140].

4.3.5 Isolation of lymphocytes from whole blood

Additional blood samples (2 x 9 mL) were collected in EDTA tubes at the same time as the C_{0P} sample (median (range): 13 (5 – 22) days post-transplant) and were processed within 4 hr after collection to ensure maximal lymphocyte yield. Lymphocytes were isolated with Lymphoprep™ (Axis-Shield, Oslo, Norway) according to the manufacturer's protocol with several modifications. In brief, 9 mL of patient's blood was diluted with an equal volume of 0.9 % ice-cold sodium chloride (NaCl) and 18 mL of this suspension was underlayered with 9 mL Lymphoprep™ and centrifuged without brakes at 1200 x *g* at 4 °C for 20 minutes (min). After centrifugation, lymphocytes were harvested from the plasma/Lymphoprep™ interface and washed three times with 30 mL of 0.9 % NaCl (centrifugation at 1200 x *g* at 4 °C for 10 min). The cell pellets were resuspended with 5 mL of 0.9 % NaCl and 500 µL of this suspension was used for cell counting (in duplicate) on a haemocytometer. After final centrifugation (1200 x *g*, 4 °C, 10 min), the two cell pellets for determination of MPA C_{0L} concentrations and IMPDH activity, respectively, were kept at −80 °C until analysis.

Calibrators and quality control (QC) samples were prepared from blank lymphocytes isolated from 150 mL of fresh blood, and treated as 9 mL aliquots as described above.

4.3.6 Measurement of trough intra-lymphocyte MPA concentrations

Measurement of C_{0L} concentrations from patient samples was based on our previously published liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the quantification of MPA in human kidney transplant biopsies (refer to Chapter 2) with slight modifications [207]. On the day of the assay, frozen patient and blank lymphocyte pellets were thawed at 4 °C and placed on ice. Subsequently, 200 µL of ice-cold phosphate buffered saline (PBS) pH 7.4 was added to each thawed patient cell pellet and was mixed thoroughly. The calibrators and QC samples were prepared from blank lymphocyte pellets (containing 10^7 cells), which had been spiked with MPA working solutions (100 µL of MPA in 50 % methanol/50 % water plus 100 µL of PBS solution) prepared from MPA stock solution (100 mg/L in 100 % methanol) to concentrations of 0.1, 0.2, 0.5, 1.0, 3.0 and 5.0 ng/mL for calibrators and 0.3, 1.5 and 2.0 ng/mL for QC samples. To each calibrator, QC and patient sample, 60 µL of 0.4 M HCl, 10 µL of 0.2 µg/mL MPA-d3 internal standard and 1 mL of tertiary-butyl methyl ether (t-BME) were added. MPA extraction and cell lysis were performed by gently mixing on a roller mixer for 10 min. The samples were then centrifuged at 1900 x *g* at 4 ºC for 10 min. After centrifugation, the organic layer was removed, transferred to a 5 mL disposable glass tube and evaporated to dryness using an evacuated centrifuge at 45 ºC for approximately 20 min. The dried residues were reconstituted with 50 µL of 50 % methanol/50 % water, vortexed for 2 min and 10 µL of the reconstituted solutions injected onto the LC-MS/MS for analysis. Analytical and LC-MS/MS conditions are described elsewhere (refer to Chapter 2) [207].

The assay was fully validated according to the U.S. Food and Drug Administration (FDA) guidelines for bioanalytical methods [174]. The assay was assessed for linearity, accuracy, precision, extraction efficiency, matrix effects and stability. The calibration curves were linear, with coefficients of determination (R^2) greater than 0.99 (n = 5), and within- and between-assay inaccuracy and imprecision were less than 15 % (n = 5). MPA extraction efficiency displayed a good reproducibility, with coefficient of variations (CVs) ranging from 0.1 to 5.2 % and matrix effects were minimal (< 10 %), both assessed at 3 concentrations (1.0, 5.0 and 20.0 ng/mL) in replicates of 2, using two different blank lymphocytes. MPA was stable when spiked into blank lymphocytes, with no significant degradation after 12 hr at room temperature or 6 months at –80 °C, nor in post-processing samples left in the autosampler (4 °C) for 24 hr. Other immunosuppressants likely to be administered with MPA (i.e. TAC, CsA, prednisolone, sirolimus and everolimus) had no significant effects on either MPA or MPA-d3 peak areas. The MPA C_{0L} concentrations measured were adjusted according to the number of lymphocytes extracted and expressed as ng/10^7 cells.

4.3.7 Measurement of intracellular IMPDH enzyme activity

Measurement of intracellular IMPDH activity from lysed lymphocytes was based on previously published high performance liquid chromatography (HPLC) methods for the quantification of IMPDH activity in peripheral mononuclear cells [106, 208]. In brief, after thawing at 4 °C, the lymphocyte pellets were resuspended in 900 µL ice-cold Millipore water and insoluble fragments of disrupted cells were removed by centrifugation at 15,800 x *g* for 2 min. The lymphocyte lysate was used for enzymatic assay (50 µL) and protein content (20 µL) determinations. The measurement of lysate protein concentration was performed with Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories, California, USA)

using bovine serum albumin as standard according to the manufacturer's protocol. IMPDH activity in lymphocytes was determined from the conversion of inosine 5'-monophosphate (IMP) to xanthosine 5'-monophosphate (XMP) according to methods described previously [106, 208]. Briefly, the IMPDH incubation mixture (pH 7.4) consisted of 1 mM IMP, 0.5 mM NAD^+, 40 mM NaH_2PO_4 and 100 mM KCl. The enzymatic reaction was initiated by the addition of 50 µL of the lymphocyte lysate to 120 µL of reaction mixture and incubated at 37 °C for 2.5 hr. After incubation, the reaction was terminated by adding 20 µL of 4 M ice-cold $HClO_4$, vortexing for 10 seconds (sec), and the deproteinised solution was centrifuged at 15,800 x g for 2 min. Subsequently, 170 µL of supernatant was neutralised by adding 17 µL of 5 M ice-cold K_2CO_3, vortexing for 10 sec, and storing the samples for at least 30 min at −80 °C. After thawing and centrifugation at 15,800 x g for 2 min, 25 µL of the supernatant was immediately injected onto the HPLC column for analysis.

Chromatographic detection of XMP production was achieved using a Synergi HydroRP 80A column (4 µM, 250 × 3 mm) (Phenomenex, Lane Cove, NSW, Australia) maintained at 45 °C on an Agilent HPLC system, with mobile phases consisting of buffer (pH 5.5) containing 50 mM potassium phosphate (KH_2PO_4) and 7 mM TBA (Mobile phase A) and 100 % methanol (Mobile phase B) [208]. The mobile phases were pumped at a flow rate of 0.7 mL/min using a semi-gradient program of 94 % mobile phase A and 6 % mobile phase B (0 – 13.0 min), changed to 80 % mobile phase A and 20 % mobile phase B (13.1 – 23.0 min) and then finally back to 95 % mobile phase A and 5 % mobile phase B (23.1 – 40.0 min). Injection volume was 25 µL with ultraviolet (UV) detection at a wavelength of 254 nm.

Specificity was tested in control incubations containing substrate IMP in the absence of co-substrate NAD^+ or containing NAD^+ in the absence of IMP. No endogenous XMP was

detected in samples incubated without IMP or NAD^+ and no interfering peaks were observed at the retention time of XMP. Linearity of XMP formation with protein content was confirmed by using four serial dilutions of the lymphocyte lysate (protein concentration ranged from 0.18 – 2.7 mg/mL). XMP formation (protein concentration 2.7 mg/mL) also increased linearly with the time of incubation (0 – 200 min). IMPDH activity was expressed as XMP produced (nmol) per incubation time (hr) per mg protein (nmol/hr/mg protein).

4.3.8 Statistical analyses

Normality of data distribution was assessed by the D'Agostiono and Pearson omnibus normality test. Correlations between MPA C_{0L}, C_{0P} and C_{0u} concentrations and IMPDH activity were assessed using a Spearman's rank correlation. Differences in MPA C_{0L}, C_{0P} and C_{0u} concentrations, and IMPDH activity, between patients with and without graft rejection were evaluated using Mann-Whitney rank sum test. Receiver operating characteristic (ROC) curve analysis was performed to assess the ability of either MPA C_{0L} concentrations or IMPDH activity to predict graft rejection. Associations between MPA C_{0L}, C_{0P} and C_{0u} concentrations, and IMPDH activity, with the severity of rejection (no evidence of rejection, subclinical borderline rejection, and severe cellular or vascular rejection) were assessed using Kruskal-Wallis tests (with Dunn's multiple comparisons post hoc). The Mann-Whitney rank sum test (CIT, PRA, creatinine, bilirubin, AT1R and TAC C_0 concentration), unpaired *t*-test (recipient and donor ages, HLA and albumin) and Fisher's Exact test (sex, ethnicity, DSA post-transplant, donor graft and DGF) were used to investigate the differences in demographic and clinical covariates between patients with or without rejection episodes. All analyses were performed using Prism version 6.0 (GraphPad Software, Inc, La Jolla, CA). Differences between MPA C_{0L}, C_{0P} and C_{0u} concentrations, and IMPDH activity,

with the severity of rejection were also analysed using Jonckheere-Terpstra Test (SPSS, version 19, IBM, Armonk, NY).

A step-up linear model of multivariate regression with step-wise addition of factors was used to identify predictors associated with rejection incidence as well as variables that determine lymphocyte MPA concentrations. Similar models were also used to investigate associations between MPA PK (C_{0P}, C_{Pav}, C_{0L} and C_{0u}) and IMPDH enzyme activity with genetic and clinical variables. The variables included in the step-wise addition procedures were: recipient and donor ages; sex; ethnicity; donor graft (living or deceased); genetics (donor and recipient *ABCC2* phenotypes); HLA mismatches; CIT; PRA; creatinine, albumin and bilirubin concentrations; AT1R antibody levels; DSA; trough TAC concentrations, the incidences of rejection and DGF, and the severity of rejection. The Chi-square test was used to test for each additional factor. Data are presented showing the overall *P* value and also individual *P* values for each covariate. A $P > 0.05$ for an individual covariate included in the model indicates that, whilst not independently associated with the outcome being investigated, the variable significantly improved the overall final linear model. Analysis was performed using *R* [194].

All data are presented as either mean ± SD for continuous parametric data, median (range) for continuous non-parametric data or frequencies (absolute numbers) for categorical data. Statistical significance was considered for *P*-values < 0.05 (two-tailed).

4.4 Results

4.4.1 Patient characteristics

Patient demographic and biological characteristics are shown in Table 4.1. Thirty-nine patients (81 %) were Caucasians and nine patients were Indigenous Australians. Sixteen patients (33 %) experienced graft rejection during this study at an average ± SD (range) of 12 ± 5 days (5 – 22 days) post-transplantation. There were no significant differences ($P \geq 0.066$) between the rejection and no rejection groups with respect to recipient and donor ages, sex, ethnicity, HLA mismatches, CIT, PRA, AT1R antibody levels, and serum creatinine, albumin or bilirubin concentrations; however, TAC C_0 whole blood concentrations were significantly lower in patients experiencing rejection ($P = 0.028$) compared to those patients in the no rejection group (Table 4.1).

4.4.2 Correlations between MPA C_{0L}, C_{0P} and C_{0u} concentrations, and IMPDH activity

MPA C_{0L} concentrations ranged from 0.1 to 3.9 ng/10^7 cells (median = 0.68 ng/10^7 cells) and the corresponding MPA C_{0P} concentrations ranged from 0.45 to 6.5 mg/L (median = 2.1 mg/L). Trough IMPDH activity ranged from 0.9 to 33.9 nmol/h/mg protein (median = 11.9 nmol/hr/mg protein). There was no significant correlation between MPA C_{0L} and C_{0P} concentrations (Spearman $r = 0.18$, $P = 0.22$, Fig. 4.1A), and trough IMPDH activity was not significantly correlated with either C_{0L} (Spearman $r = 0.23$, $P = 0.11$, Fig. 4.1B), or C_{0P} (Spearman $r = 0.22$, $P = 0.13$, Fig. 4.1C), concentrations.

MPA C_{0u} plasma concentrations (n = 34) ranged from 3.6 to 166.4 μg/L (median = 20.8 μg/L) and were significantly correlated with MPA C_{0L} (Spearman $r = 0.36$, $P = 0.034$, Fig.

4.2A) and MPA C_{0P} (Spearman r = 0.68, P < 0.0001, Fig. 4.2B) concentrations, but not with the trough IMPDH activity (Spearman r = 0.14, P = 0.42, Fig. 4.2C).

4.4.3 Relationships between rejection and MPA C_{0L}, C_{0P} and C_{0u} concentrations, and IMPDH activity

In the rejection group (n = 16), ten (63 %) patients developed severe cellular or vascular rejection, and six (37 %) patients were classified as subclinical borderline at the time of protocol or for-cause biopsies. In the total 48 patients recruited, there was no difference between either MPA C_{0L} concentrations (P = 0.052, Fig. 4.3A), MPA C_{0P} concentrations (P = 0.26, Fig. 4.3B), trough IMPDH activity (P = 0.057, Fig. 4.3C) or MPA C_{0u} concentrations (P = 0.57 (n = 34), Fig. 4.3D) with the incidence of graft rejection. However, there was a significant concentration-effect relationship between MPA C_{0L} concentrations and the severity of rejection (Jonckheere-Terpstra (trend) test, P = 0.030, Fig. 4.4A). There was no significant difference between MPA C_{0P} concentrations (trend test, P = 0.18, Fig. 4.4B), trough IMPDH activity (trend test, P = 0.075, Fig. 4.4C) or MPA C_{0u} concentrations (trend test, P = 0.43, Fig. 4.4D), between patients with no evidence of rejection (n = 32, 67 %), subclinical borderline rejection (n = 6, 13 %) and severe cellular or vascular rejection (n = 10, 20 %).

Receiver operating characteristic (ROC) curve analyses were performed to provide threshold data for both MPA C_{0L} concentrations and IMPDH activity that might predict the risk of rejection with optimal sensitivity and specificity. The ROC area under the curve (AUC) for the prediction of severe (cellular/vascular) rejection using MPA C_{0L} concentrations was 0.71 (P = 0.045, Fig. 4.5B), with a C_{0L} threshold of 0.5 ng/10^7 cells providing 71 % sensitivity and 70 % specificity, and a likelihood ratio of 2.37. However, the ROC AUC for the

prediction of all rejection using MPA C_{0L} concentrations and IMPDH activity were not significant (AUC = 0.67, *P* = 0.052), Fig. 4.5A, and AUC = 0.67 (*P* = 0.057), Fig. 4.5C, respectively). In addition, the ROC analysis of IMPDH activity for the prediction of severe rejection showed a non-significant AUC of 0.66 (*P* = 0.12, Fig. 4.5D).

4.4.4 Variables associated with rejection, lymphocyte and plasma MPA concentrations

Rejection

Variables: recipient and donor ages; sex; ethnicity; donor graft type; ABCC2 genetics; HLA mismatches; CIT; PRA; creatinine, albumin, bilirubin and trough TAC concentrations; AT1R; DSA; the incidences of rejection and DGF, the severity of rejection, MPA C_{0P}, C_{0L}, C_{0L}/C_{0P} *ratio,* C_{0u}, C_{Pav} *and IMPDH activity.*

Of the above variables, MPA C_{0L} concentration was the only independent predictor of rejection, such that the occurrence of rejection was associated with a decrease in MPA C_{0L} concentrations (*P* = 0.03). This agrees with the data shown on Fig 4.4A.

MPA C_{0L} ***concentrations***

Variables: recipient and donor ages; sex; ethnicity; donor graft type; ABCC2 genetics; HLA mismatches; CIT; PRA; creatinine, albumin, bilirubin and trough TAC concentrations; AT1R; DSA; the incidences of rejection and DGF, the severity of rejection, MPA C_{0P}, C_{0u}, *and IMPDH activity.*

Of the above variables, MPA C_{0P} concentration was the only significant variable associated with MPA C_{0L} concentration (explaining 12 % of variance, P = 0.009), such that as MPA C_{0P} concentration increased by 1 unit, MPA C_{0L} concentration increased by 0.25 units.

MPA C_{0P} concentrations

Variables: recipient and donor ages; sex; ethnicity; donor graft type; ABCC2 genetics; HLA mismatches; CIT; PRA; creatinine, albumin, bilirubin and trough TAC concentrations; AT1R; DSA; the incidences of rejection and DGF, the severity of rejection, and IMPDH activity.

Of the above variables, donor type (living) and recipient age were the only significant covariates associated with MPA C_{0P} concentrations, explaining 21 % of variance (P = 0.002). With regard to individual predictors, patients who had received a graft from a living donor had a 1.1 unit decrease in MPA C_{0P} concentrations compared to those who had received a graft from a deceased donor (given all other predictors were held constant, P = 0.015). In addition, as the age of patients increased by 1 year, MPA C_{0P} concentrations increased by 0.03 units (P = 0.04).

MPA C_{0L}/C_{0P} ratio

Variables: recipient and donor ages; sex; ethnicity; donor graft type; ABCC2 genetics; HLA mismatches; CIT; PRA; creatinine, albumin, bilirubin and trough TAC concentrations; AT1R; DSA; the incidences of rejection and DGF, the severity of rejection, and IMPDH activity.

The ratio of MPA C_{0L}/C_{0P} concentration was significantly associated with ethnicity, and this variable explained 23 % of variance in the ratio of C_{0L}/C_{0P} concentration (P = 0.0004).

Patients who were Caucasian had a 0.43 unit decrease in the ratio values compared to those who were Indigenous Australians.

MPA C_{0u} concentration

Variables: recipient and donor ages; sex; ethnicity; donor graft type; ABCC2 genetics; HLA mismatches; CIT; PRA; creatinine, albumin, bilirubin and trough TAC concentrations; AT1R; DSA; the incidences of rejection and DGF, the severity of rejection, MPA C_{0P} concentrations and IMPDH activity.

Of the above variables, AT1R score was the only significant factor associated with MPA C_{0u} concentrations (explained 13 % of variance, P = 0.02), such that as the AT1R score increased by 1 unit, MPA C_{0u} concentrations decreased by 1.1 units.

Table 4.1 Patient demographics and biological characteristics of 48 renal transplant recipients.

	All (n = 48)	Rejection[1] group (n = 16)	No rejection group (n = 32)	*P*-value
Sex (Male/Female)	25/23	7/9	18/14	0.54
Ethnicity (Caucasian/Indigenous Australian)	39/9	13/3	26/6	1.0
Recipient age (years)	49 ± 12	45 ± 10	51 ± 13	0.066
Donor age (years)	48 ± 15	43 ± 15	41 ± 14	0.12
HLA mismatches	3.8 ± 1.5	4 ± 1.4	3.7 ± 1.6	0.53
PRA	0 (0 – 90)	0 (0 – 3)	0 (0 – 90)	0.080
CIT (hr)	11 (3 – 34)	11 (4 – 26)	10 (3 – 34)	0.95
Serum creatinine (mL/min)	244 (34 – 913)	244 (55 – 913)	286 (34 – 864)	0.76
Albumin (g/dL)	31 ± 3.5	31 ± 3.5	32 ± 3.5	0.24
Bilirubin (mg/dL)	8 (3 – 23)	8 (3 – 23)	8 (4 – 18)	0.62
AT1R antibody level (U/L)	9 (0 – 40)	10 (0 – 28)	9 (0 – 40)	0.31
TAC C_0 (µg/L)	7.5 (4 – 24)	6.6 (3 – 14)	7.9 (4 – 24)	0.028
DSA post-transplant (Present/Absent)	13/35	5/11	8/24	0.74
Donor graft (Living/Deceased)	12/36	5/11	7/25	0.50
DGF (Yes/No)	19/29	6/10	13/19	1.0

[1]Rejection occurred within the period of ±2 days from sample collection.
Data are given as mean ± SD, median (range) or frequencies (absolute numbers) depending on data type.
AT1R, angiotensin II type-1 receptor; CIT, cold ischemia time; DGF, delayed graft function; DSA, donor-specific HLA antibodies; HLA, human leukocyte antigen; PRA, panel reactive antibody; TAC C_0: tacrolimus trough whole blood concentrations.

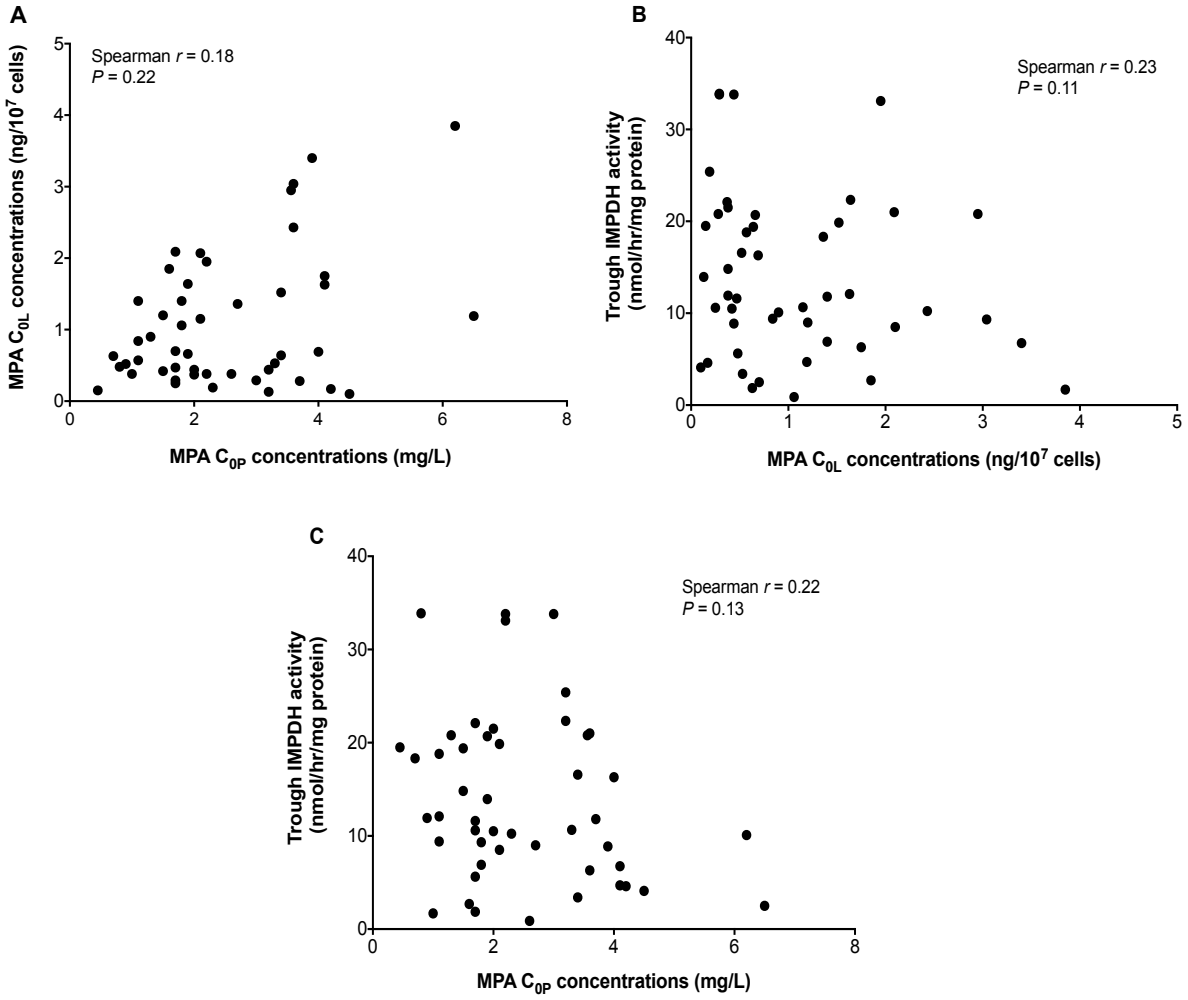

Figure 4.1 Correlations between (A) trough intra-lymphocyte (C_{0L}, ng/10^7 cells) and corresponding trough plasma (C_{0P}, mg/L) MPA concentrations, and trough IMPDH activity (nmol/hr/mg protein) with either (B) MPA C_{0L} concentrations or (C) MPA C_{0P} concentrations, in 48 renal transplant recipients.

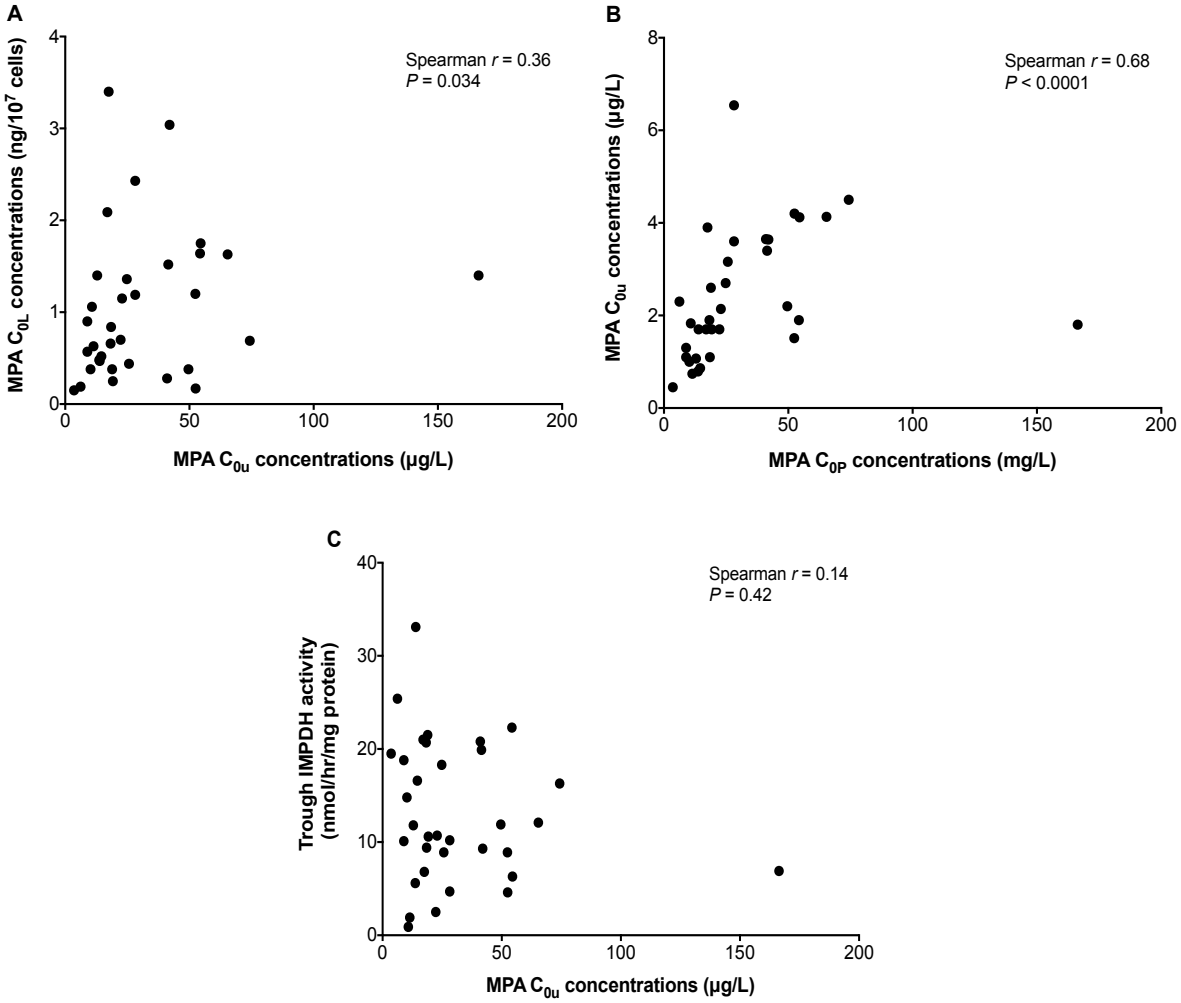

Figure 4.2 Correlations (n = 34) between unbound (C_{0u}) MPA plasma concentrations (µg/L) with (A) MPA C_{0L} (ng/10^7 cells), (B) MPA C_{0P} (mg/L) plasma concentrations, and (C) trough IMPDH activity (nmol/hr/mg protein).

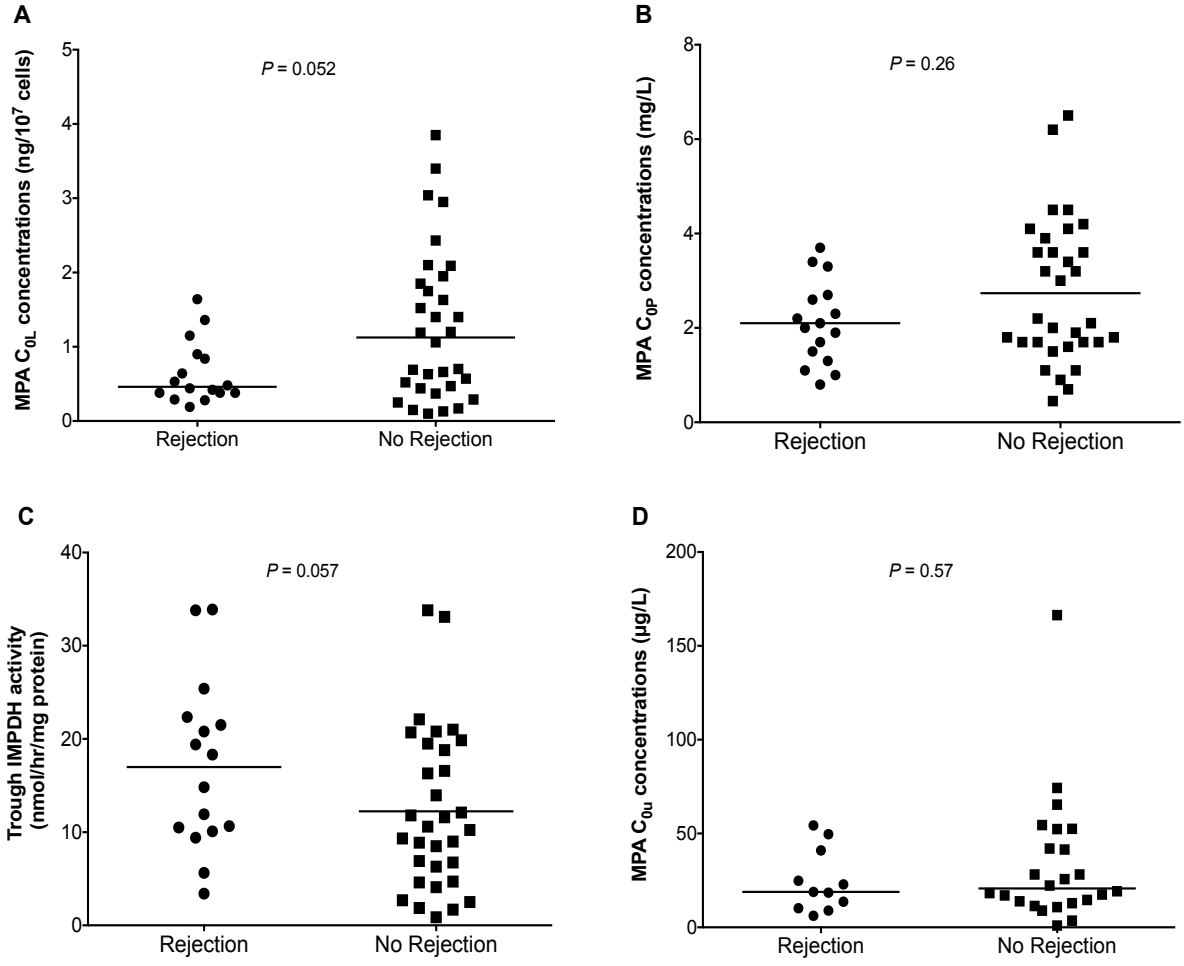

Figure 4.3 Comparisons between (A) MPA C_{0L} (ng/10^7 cells), (B) MPA C_{0P} (mg/L) concentrations, (C) trough IMPDH activity (nmol/hr/mg protein) and (D) MPA C_{0u} concentrations (µg/L) (n = 34), in recipients who did or did not experience graft rejection after transplantation. Lines indicate median values.

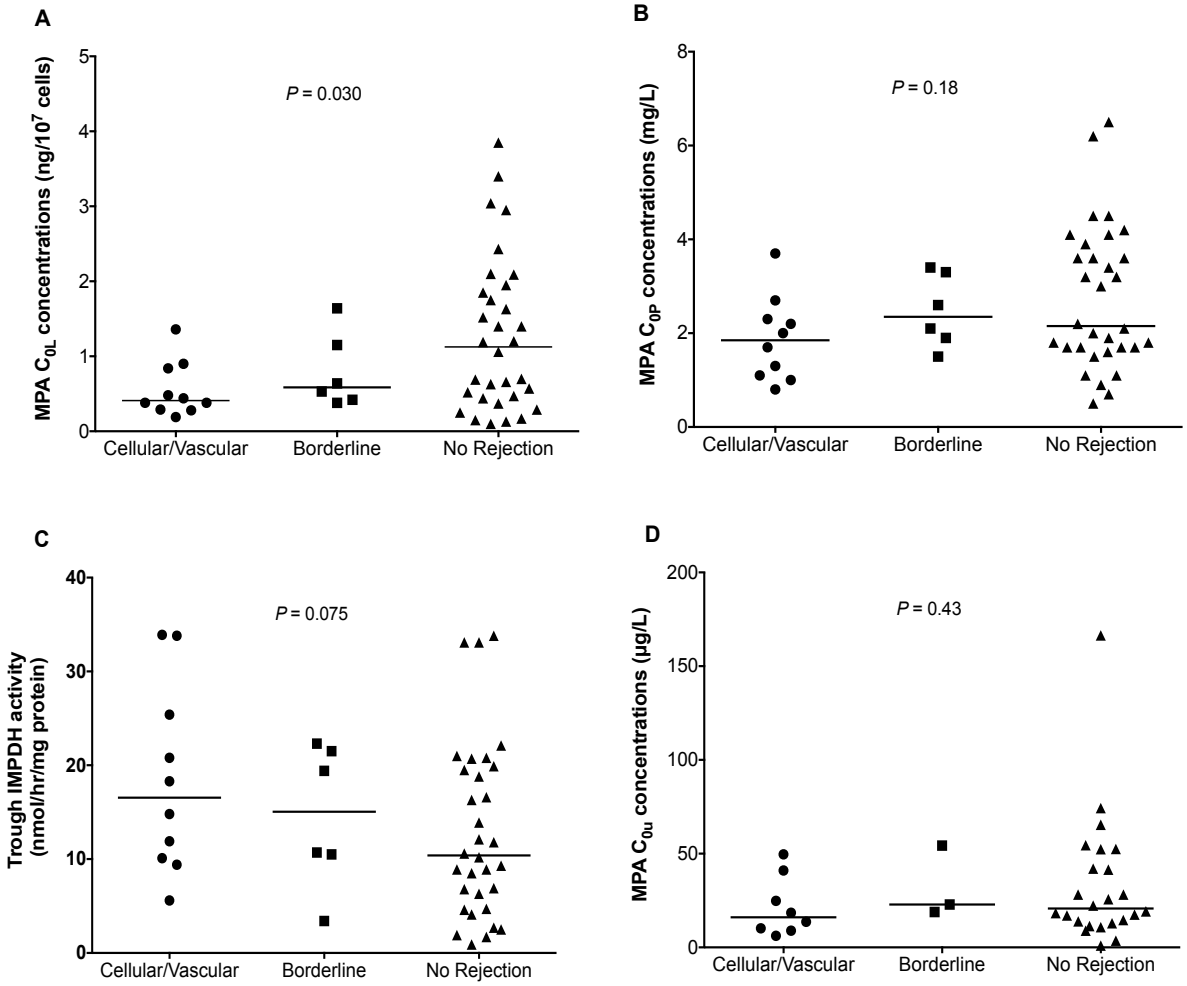

Figure 4.4 Comparisons between (A) MPA C_{0L} (ng/10^7 cells), (B) MPA C_{0P} (mg/L), (C) trough IMPDH activity (nmol/hr/mg protein) and (D) MPA C_{0u} plasma concentrations (µg/L) (n = 34), in recipients who had no evidence of rejection, subclinical borderline rejection, or severe cellular/vascular rejection. Lines indicate median values. *P*-values are shown for Jonckheere-Terpstra test for trend.

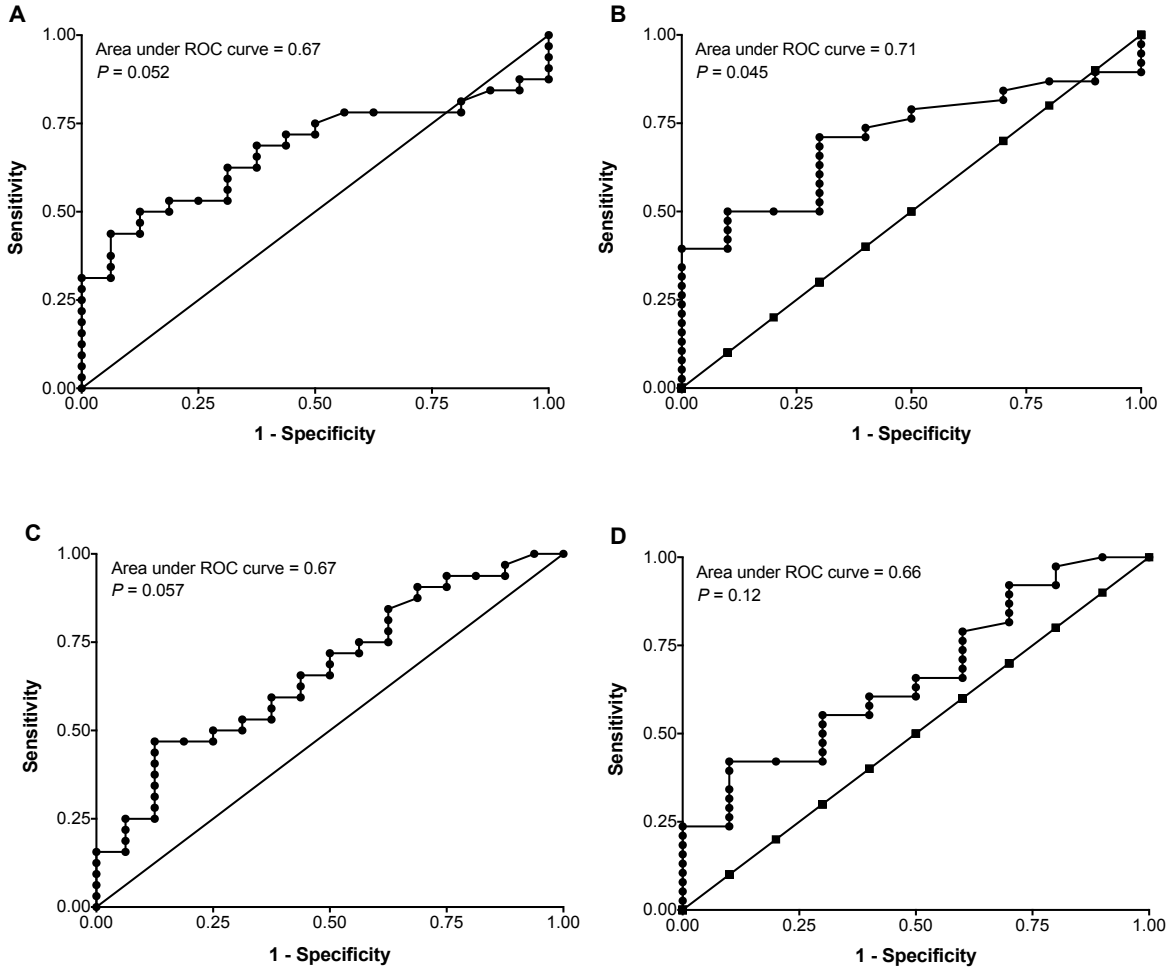

Figure 4.5 Receiver operating characteristic (ROC) curves for the use of (A and B) MPA C_{0L} concentrations and (C and D) trough IMPDH activity for the prediction of all rejection (A and C) and severe (cellular/vascular) rejection (B and D).

4.5 Discussion

Considerable effort has been made into the understanding of MPA PK-PD in an attempt to reduce the risk of rejection and MPA-induced toxicity after transplantation. Relationships between MPA concentrations in plasma and clinical outcomes have been demonstrated supporting dosage individualisation based on target plasma concentration ranges. However, plasma concentrations may not be the best indicator of therapeutic efficacy and/or toxicity. It has been suggested previously for other immunosuppressants, TAC or CsA, that concentrations in lymphocytes may provide greater relevance in predicting efficacy and/or toxicity than whole blood concentrations [160, 161, 165]. The present study set out to investigate the potential of lymphocyte MPA concentrations and IMPDH activity, versus plasma concentrations, to predict the incidence of graft rejection following renal transplantation.

This study provides the first evidence that MPA C_{0L} concentration may be a better predictor of graft rejection, compared to either MPA C_{0P} or C_{0u} concentrations. The multivariate analysis showed that MPA C_{0L} concentration was the only covariate independently associated with rejection, supporting our initial hypothesis that obtaining lymphocyte MPA concentrations may have greater relevance for predicting graft rejection compared to measuring systemic concentrations alone. This finding was supported in the univariate analyses, such that there was a significant-effect relationship between MPA C_{0L} concentrations and the severity of rejection, and ROC analysis indicated 70 % specificity and 71 % sensitivity for the prediction of severe rejection using a cut-off C_{0L} concentration of 0.5 ng/10^7 cells. Although univariate analysis showed that TAC C_0 concentrations were significantly lower in patients experiencing rejection, they were not a significant predictor of rejection in the multivariate analysis, hence confirming that MPA C_{0L} concentration was the

only dominant factor for rejection in our study population. MPA C_{0P} concentrations not predicting the incidence of graft rejection supports previous findings in renal transplant recipients [80, 82], and suggests that MPA C_{0P} concentrations may not be the best predictor of clinical outcomes. The use of AUC_{0-12} values would be preferred and might be a more reliable assessment of total MPA exposure, however the full AUC_{0-12} monitoring in clinical practice is restricted due to being impractical (e.g. requires intense sampling during a 12 hr dose interval), labour-intensive and costly. Although univariate regression analysis showed a significant correlation between MPA C_{0L} and C_{0u} concentrations, C_{0u} was not a significant predictor of rejection, even though it is the unbound MPA which exhibits pharmacological effects [30] and is capable of inhibiting IMPDH [17].

In addition, the present study has also demonstrated that MPA C_{0u} concentrations were strongly correlated with total MPA C_{0P} concentrations, although the multivariate analysis showed that total MPA C_{0P} concentrations were not a significant predictor of MPA C_{0u} concentrations. As such, TDM based on total plasma concentrations may be inappropriate to account for changes in percentage of unbound MPA in plasma, and hence, MPA distribution into lymphocytes. In the present study, MPA C_{0u} concentrations were not correlated with graft rejection (in univariate and multivariate analyses); in agreement with the previous findings of Atcheson *et al* [34]. The AT1R score was the only significant variable associated with MPA C_{0u} concentrations in the multivariate analysis, suggesting lower MPA C_{0u} concentrations with increasing AT1R antibody levels. A previous study has shown that renal transplant patients who had the AT1R antibody level > 10 U pre-transplant had a 1.9-fold higher risk of developing acute rejection, independently of other standard immunological risk parameters such as HLA mismatch or PRA [209]. It is difficult to provide mechanistic answers to the question of how high AT1R antibody levels may have played a role to cause

lower MPA C_{0u} concentrations, and how high pre-transplant AT1R antibody levels may predispose patients to rejection. However, AT1R antibodies may be directly involved in the pathogenesis of rejection by binding to the allograft immediately after transplantation and initiating biological processes (e.g. pro-inflammatory actions on vascular cells) leading to graft rejection [210].

The measurement of IMPDH activity directly within lymphocytes may also be a useful biomarker as it may correlate more closely to the biological response of MPA than plasma concentrations. In the present study, there was a trend for a higher post-transplant IMPDH activity (P = 0.057) in patients who experienced rejection compared to those who did not experience rejection. Although IMPDH activity was not a significant predictor of rejection in the multivariate analysis, our finding may represent a type II statistical error and further investigation in larger subject numbers is required to evaluate the added value of monitoring IMPDH activity. Determination of pre-transplant IMPDH activity (versus post-transplant) may be a promising new approach and help to improve MMF therapy after transplantation. Glander *et al*. reported that patients with high pre-transplant IMPDH activity (hence higher IMPDH activity has to be inhibited post-transplant) had a 3.6-fold higher incidence of graft rejection compared to patients with low pre-transplant IMPDH activity [103]. The investigation of IMPDH messenger RNA (mRNA) could also form an attractive alternative method and may provide reliable information to predict rejection. Previous studies have shown that increased expression of IMPDH genes resulted in increased IMPDH activity [211], and the IMPDH type I and type II mRNA levels were significantly 2.4- and 3.4-fold lower in patients with graft rejection, respectively (P = 0.026 and P = 0.007, respectively), compared to patients without rejection [212].

An alternative explanation of our findings is that there are two types of IMPDH isoforms, IMPDH type I, which is expressed in all cell types and IMPDH type II, which is expressed only in activated lymphocytes [17]. MPA inhibits both IMPDH isoforms, however, the inhibition of IMDPH type II by MPA is approximately 5-fold greater than the type I isoform with the K_i values of 0.007 versus 0.033 μM, respectively [17]. However, the use of total IMPDH activity in our study may itself be less than ideal such that the overall enzyme activity measured may be due to the residual IMPDH type I activity, and therefore could have accounted for the lack of significant association between IMPDH activity and rejection. In addition, the IMPDH activity in this study may have been limited as only a single measurement at pre-dose (C_0) taken 5 – 22 days post-transplant was used. The measurement of IMPDH activities at multiple time points, which we were not able to assess in this study, may possibly be a significant predictive factor of rejection. A previous study in 40 renal transplant recipients has shown that the minimum IMPDH activity occurred shortly after MMF administration and coincided with the plasma MPA peak concentration (C_{Pmax}) at 1.5 hr, suggesting maximal inhibition of IMPDH activity coinciding with MPA peak levels, and there was a striking recovery of IMPDH activity to pre-dose levels over the 3.5 hr dosing interval [104]. In addition, it would be interesting to investigate the IMPDH activity in renal transplant recipients not receiving MMF as part of their immunosuppression regimen to determine if patient characteristics and other clinical covariates have any influence on the IMPDH activity in the same way as patients treated with MPA.

Although MPA C_{0L} was the only independent predictor of rejection, little is known regarding the factors that determine lymphocyte MPA concentrations. In the present study, there was considerable inter-patient PK variability in lymphocyte (CV = 86 %) and plasma (CV = 54 %) MPA concentrations. In the multivariate analysis, MPA C_{0P} concentration was the only

significant variable associated with MPA C_{0L} concentration, explaining only 12 % of variability in lymphocyte MPA concentration; hence this may explain the poor correlation observed between MPA C_{0L} and C_{0P} concentrations. The poor correlation found between plasma and lymphocyte MPA concentrations may also reflect inter-individual variability in plasma protein binding of MPA or transporter-mediated uptake into and efflux from lymphocytes. MPA is extensively bound to albumin (97 – 99 %) in patients with healthy renal function [30]. The binding of MPA to plasma albumin is influenced by several factors including hypoalbuminemia, elevated MPAG concentrations and renal function [30, 37, 66], and consequently, this could account for the variability in intracellular MPA concentrations. This study confirmed the importance of plasma protein binding in determining lymphocyte MPA concentrations, demonstrating a significant but moderate correlation between total MPA C_{0L} and MPA C_{0u} concentrations, although MPA C_{0u} concentrations were not a significant predictor of MPA C_{0L} concentrations in the multivariate analysis. The discrepancy between results from the different analyses (univariate versus multivariate) may be due to having to drop out data in the multivariate analysis because of lower number of recipients with MPA C_{0u} concentrations, and hence the model is not sufficiently powered to pick up the difference, or by introducing more patient characteristics and other clinical covariates to the model, MPA C_{0u} concentration may not be the most important predictor in determining lymphocyte MPA concentrations.

Importantly, inter-individual differences in the expression and/or function of drug transporters could be a possible additional cause of MPA PK variability. MPA is a substrate of the efflux pump, MRP2 [46], so that *ABCC2* polymorphisms, coding for MRP2, may be an additional cause of variability observed in lymphocyte MPA concentrations by affecting the expression or function of MRP2. The CAC (-24C/1249A/3972C) haplotype has

previously been shown to be associated with significantly higher protein expression and transport activity [140], and may therefore modulate the immunosuppressive efficacy of MPA therapy. However, *ABCC2* haplotypes were not significantly associated with any of the MPA PK parameters and IMPDH activity in the multivariate analysis, and may therefore reflect a weak expression of MRP2 in lymphocytes [205]. Determination of differences in the expression and/or function of uptake (e.g. organic anion-transporting polypeptide) and other efflux pumps (e.g. P-glycoprotein) may help explain some of this PK variability such that MPA is also a substrate of P-glycoprotein efflux transporter [213], which is highly expressed in lymphocytes [214].

The present study also investigated the effects of demographics and other clinical covariates on MPA PK parameters (C_{0P}, C_{Pav} and C_{0u}) and IMPDH activity. Multivariate analyses indicated that MPA C_{0P} was associated with donor type (living) and age of the patients. We currently have no good explanation for these results; however, kidneys from live donors may have a greater chance of maintaining their function than deceased donor kidneys (may have poorer function), thus affecting the protein binding of MPA to plasma albumin. Several age-related factors such as a reduction in liver function with aging may have significant effects on the PK of MPA. A previous study employing a population PK approach has shown that the clearance of MPA was significantly influenced by the recipient age [197]. Ethnicity of the patients was the only significant variable associated with the ratio of MPA C_{0L}/C_{0P} concentrations, such that Caucasian patients had a 0.43 unit decrease in the ratio values compared to those who were Indigenous Australians. Higher ratio values were observed in Indigenous Australians, representing lower and higher lymphocyte and plasma MPA concentrations, respectively, compared to Caucasians. These observations may help explain the proclivity of Indigenous Australians to experience rejection and infection at the same

time following transplantation (RP Carroll, personal communication). The effect of ethnicity on MPA PK parameters, however, is an area still poorly researched and thus requires further investigation.

In conclusion, the results from our study suggest that, despite TDM to minimise inter-individual pharmacokinetic variability in systemic MPA concentrations, lymphocyte MPA concentrations may represent an additional tool to further improve individualisation of MMF dose following renal transplantation. However, even if lymphocyte MPA concentration was a better biomarker to predict graft rejection, facilitating early titration of MMF dosing, its clinical application may only practically be feasible as a single test during the early post-transplant period, where the risk of developing graft rejection is higher, or when the protocol or for-cause biopsies are performed at the time of rejection.

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Chapter 5: General Discussion

Chapter 5. General Discussion

Mycophenolic acid (MPA) has a narrow therapeutic index and large inter-patient pharmacokinetic (PK) variability necessitating therapeutic drug monitoring (TDM) to individualise dosing following renal transplantation. Although TDM is a necessary measure for allowing better individualisation of MPA therapy, early graft rejection and immunosuppressant toxicity still occur after transplantation. Consequently, there is a need for clinical tools that are better predictors of target tissue MPA concentrations that are not necessarily mirrored by plasma concentrations, as currently monitored with TDM. The multidrug resistance-associated protein 2 (MRP2) efflux transporter has been established as potentially influencing intestinal and biliary excretion of MPA, and hence MPA PK, but also regulates MPA distribution into lymphocytes and graft tissue, an area still poorly researched. As such, genetic variability in the *ABCC2* gene (encoding MRP2) may explain some of the additional inter-individual variability in response to MPA.

Therefore, the primary aims of this thesis were: (1) to establish two new methods for determining intra-renal and intracellular MPA concentrations in human kidney biopsies and lymphocytes, respectively; and to investigate (2) the impact of recipient and donor *ABCC2* genetic variability (formed by the common -24 C > T, 1249 G > A and 3972 C > T SNPs) on MPA PK and distribution, and potential associations with kidney transplant outcomes; as well as (3) the utility of measuring lymphocyte MPA concentrations and IMPDH activity, as compared to plasma concentrations, as predictors of graft rejection.

5.1 LC–MS/MS methods for the quantification of MPA in human kidney transplant biopsies and lymphocytes

In order to fulfill the aims of this thesis, several new and adapted methods had to be developed and validated. Firstly, a new robust and reproducible LC-MS/MS method was successfully established and validated for the quantification of MPA concentrations in very small samples of human kidney biopsies taken as part of routine clinical protocols from renal transplant recipients. The biopsies were taken from the kidney cortex tissue at the time of validating the method; therefore the assay performance (e.g. sensitivity, specificity) may have been different if the biopsy samples were taken from the different region of the kidney. The kidney biopsies used in this study were taken in cases of suspected rejection, however, the biopsies (protocol biopsies) are now routinely taken immediately pre-transplant, and at 1 week, 1 and 6 months post-transplant, as well as at times of rejection. All biopsy samples were collected for histological assessments. Kidney tissue MPA concentrations were of a similar magnitude to the plasma therapeutic range (MPA C_0: 1 – 3.5 mg/L). However, we were unable to investigate if changes in kidney MPA concentrations reflected those concentrations in plasma, as corresponding plasma concentrations were not available at the time of validating the method. In addition, the uptake of MPA in kidney biopsy tissues during rejection versus non-rejection kidneys could not be assessed, as all kidney biopsies used in the study were primarily collected in cases of suspected rejection. Nevertheless, the established method was linear, accurate, and precise, with minimal matrix effects. In addition, the retrospective analysis of MPA in Mrp2-deficient rats has allowed additional validation of the reproducibility of the tissue extraction procedures.

The method presented in Chapter 2 was the first study to measure tissue MPA concentrations in human kidney biopsies and was primarily designed to accurately quantify MPA in routine

core needle biopsies of kidney transplant patients weighing as little as 0.1 mg. Unfortunately, for the prospective clinical study presented in Chapter 4, we were unable to examine the routine core needle biopsies of kidney transplant patients taken as part of the clinical procedures, as the protocol allograft biopsies (stored in the last 12 months) may get requests for further testing and this extended past the end date of experiments. Nonetheless, the establishment of this protocol for accurately quantifying MPA concentrations in excess biopsy tissues taken as part of routine clinical care has ensured that our method benefits unaltered biopsy sampling and handling, to fit into current clinical practice. This will therefore allow further studies directed toward understanding the mechanisms determining the tissue PK of MPA and their relationship with clinical outcomes.

Secondly, prior to commencing this thesis, no protocols for determining intracellular MPA concentrations in lymphocytes, representing the target site of action, had previously been established. Therefore, for the prospective clinical study of Chapter 4, a new selective and sensitive analytical method was successfully developed and validated for the quantification of lymphocyte MPA concentrations in renal transplant recipients. This method, like our previous method for the detection of MPA in kidney transplant biopsies, demonstrated sensitivity, specificity, and reproducibility to allow the accurate and precise measurement of MPA concentrations within lymphocytes.

5.2 *ABCC2* genetic polymorphisms as a determinant of variability in MPA concentrations

One of the major aims of this thesis was to investigate the associations between *ABCC2* genetic polymorphisms with MPA and MPAG PK parameters. More specifically, the primary goal was to examine the influence of donor and recipient *ABCC2* haplotypes (as opposed to individual SNP analysis) on the PK of MPA and its metabolite MPAG, and their relationships with kidney transplant outcomes.

The retrospective clinical study presented in Chapter 3 revealed several new and promising findings, with the most significant results related to the CAC (-24C/1249A/3972C, variant at 1249) haplotype of *ABCC2*. Recipient carriers of this haplotype, which has been associated with increased MRP2 protein expression and transport activity, had 2.3-fold increased MPA C_0 concentrations, as opposed to recipients with low expressor haplotypes. As such, CAC carriers appear to have increased enterohepatic recirculation of MPA, as a result of increased biliary excretion of MPAG. Support for these findings was obtained in the multivariate analysis, which revealed that MPA C_0 concentrations were significantly associated with recipient *ABCC2* expression phenotypes, and also with rejection and PRA peak. It was also found that there was a significant gene-dose effect among the three *ABCC2* haplotype groups (Jonckheere-Terpstra test for trend), with an inverse step-wise relationship between recipient *ABCC2* expression and MPA C_0 concentrations. In the clinical context, the observed haplotype effects were relatively modest, equating to only 17 – 57 % differences in MPA C_0 concentrations; as such, these justify further investigations to elucidate the influence of *ABCC2* haplotypes on MPA PK. Nevertheless, the findings of Chapter 3 provided the first evidence that the recipient's *ABCC2* affects MPA PK in a haplotype-specific manner, enabled a more informative haplotype approach to investigating *ABCC2* pharmacogenetics

(PGx) compared to individual SNP analysis. The consideration of *ABCC2* haplotypes in place of individual SNPs are more likely to accurately reflect MRP2 expression and/or function and should now be part of all studies on *ABCC2* PGx.

In addition to identifying associations between recipient *ABCC2* haplotypes and MPA PK, the role of donor *ABCC2* genetic variability in influencing the PK parameters of MPAG metabolite was also investigated (as renal excretion is the primary clearance mechanism for MPA metabolites), but revealed no significant relationship between the two. The findings suggest that a large change in *ABCC2* expression in the donor graft may be required to significantly affect the concentrations of MPAG and that the contribution of active tubular secretion may be relatively small to overall renal clearance of MPAG.

Due to the relatively small sample size (n = 5) and low frequency (12 %) of the high expressor phenotype, it is difficult to evaluate the usefulness of pre-transplant *ABCC2* haplotype analysis to inform MPA initial dosage prior to transplantation. In addition to providing some mechanistic support for the association between the CAC haplotype and MPA C_0 concentrations, the interpretation is made difficult due to the lack of clear understanding of the functional consequences of *ABCC2* haplotypes. In the present study, it was not possible to actually confirm that MRP2 expression was accurately predicted by the assigned phenotypes based on haplotype analysis, and other factors may also have affected MRP2 expression. The combined investigation of mRNA expression, MRP2 protein expression and transport activity in the recipient and donor graft kidney tissue may provide a more useful insight into the exact mechanisms behind the functional consequences of *ABCC2* haplotypes. Therefore, further investigations are required to determine whether *ABCC2* haplotypes, along with other genetic and environmental factors, are likely to provide

clinical benefit in guiding dosing of MPA prior to transplantation in addition to TDM to achieve improved clinical outcomes, including MPA toxicity.

5.3 Intra-lymphocyte pharmacokinetic-pharmacodynamic (PK-PD) analyses of MPA as predictors of graft rejection

The final aim of this thesis, as described in the prospective clinical study presented in Chapter 4, was to investigate the utility of measuring lymphocyte MPA concentrations and IMPDH activity (as opposed to plasma concentrations), as predictors of graft rejection following renal transplantation.

The prospective pilot study provided the first evidence of a positive association between lymphocyte MPA concentrations and the incidence of graft rejection. The findings of this thesis revealed that MPA C_{0L} concentration was the only significant covariate independently associated with rejection in our study population, further confirming that the monitoring of lymphocyte MPA concentrations may more accurately predict therapeutic response than plasma concentrations alone, as currently monitored with TDM. ROC curve analyses revealed that MPA C_{0L} concentration was a better predictor of severe (cellular/vascular) rejection compared to IMPDH activity or plasma concentrations. In addition, there was a significant concentration-effect relationship between MPA C_{0L} concentrations and the severity of rejection. Based on these findings (in univariate and multivariate analyses), it can be concluded that MPA C_{0L} concentrations may be a more superior parameter to predict clinical outcomes than plasma concentrations. As such, based on the findings of Chapter 4 and other international studies investigating intra-lymphocyte immunosuppressant concentrations, it appears that intra-lymphocyte concentrations may provide information regarding mechanisms important to rejection that plasma concentration data do not provide, which then may be used as a potential clinical optimisation tool in future individualisation procedures that could be incorporated as part of TDM.

Although the multivariate analysis indicated that MPA C_{0L} concentration was the only independent predictor of graft rejection, little is known regarding the factors that determine lymphocyte MPA concentrations. A large inter-patient variability in MPA C_{0L} and C_{0P} concentrations has been observed in this study. The multivariate analysis indicated that MPA C_{0P} concentrations was the only independent predictor associated with C_{0L} concentrations (explaining 12 % of variance), thus this may explain the poor correlation coefficient found between MPA C_{0L} and C_{0P} concentrations. The pre-dose IMPDH activity was not significantly associated with MPA C_{0L} concentrations, suggesting that lymphocyte MPA concentrations may not provide any better correlation with IMPDH activity, than plasma concentrations. Whilst several studies have previously employed total lymphocytes as a form of analysis, the measurement of MPA in specific subsets of lymphocytes (e.g. CD4+ cells) would be more beneficial rather in lymphocytes as a whole; however, its application may not likely be performed for analytical (e.g. analytical challenge of sensitivity) and practical (e.g. the need to draw large blood volumes) reasons.

MPA exerts its immunosuppressive effect by inhibition of IMPDH within lymphocytes, which then inhibits lymphocyte proliferation. It is generally thought that the unbound or free MPA is presumed to be the pharmacologically active species and capable of inhibiting IMPDH; therefore, the unbound MPA concentrations should have a better correlation with IMPDH activity than total MPA plasma concentrations. The present study could not confirm the inverse relationship between IMPDH activity and MPA plasma levels as there was no significant correlation between IMPDH activity and either total, unbound or lymphocyte MPA concentrations. Based on these findings, it remains unclear whether unbound MPA concentration or IMPDH activity should be measured for appropriate MPA monitoring, as well as whether or not IMPDH activity measured on a single time point is better correlated

with total or unbound MPA concentrations. It would be interesting to study the AUC of IMPDH activity, as it may likely be better correlated with unbound or total MPA AUC. Nevertheless, data collected in this thesis may be used in further PK-PD population analyses using total and unbound MPA, and also MPAG and AcMPAG concentrations, in combination with the existing data on patient characteristics and other clinical covariates. These analyses could therefore provide a better comparison and understanding on which concentration of MPA, total or unbound, is related to the inhibition of IMPDH activity.

The PD monitoring of MPA by the measurement of IMPDH activity would be strengthened if IMPDH activity was also a better predictor of graft rejection. The pre-dose IMPDH activity was slightly but not significantly higher in patients experiencing graft rejection compared to those patients without rejection incidence. In addition, the multivariate analysis demonstrated that IMPDH activity was not a significant covariate independently associated with rejection in our study population. On the basis of the observed findings, it can be speculated that IMPDH activity may not be a more sensitive parameter to predict graft rejection than lymphocyte MPA concentration. This needs however to be confirmed in a larger prospective study. Previous investigation has shown a correlation between pre-transplant IMPDH activity and the incidence of rejection although this observation has not been confirmed and replicated in larger multi-centre prospective studies. Consequently, it is important to further investigate if IMPDH activity (pre- and post-transplant) could potentially provide a decision support tool for guiding MPA therapy prior to transplantation, in order to achieve an individualised approach to maximising clinical outcomes following renal transplantation.

5.4 Summary

In the light of current findings in this thesis, a revised summary of the hypothetical mechanisms originally outlined in Chapter 1, Figure 1.5, of factors affecting MPA PK and distribution into lymphocytes and kidney graft, and their association with kidney transplantation outcome, is shown in Figure 5.1 below.

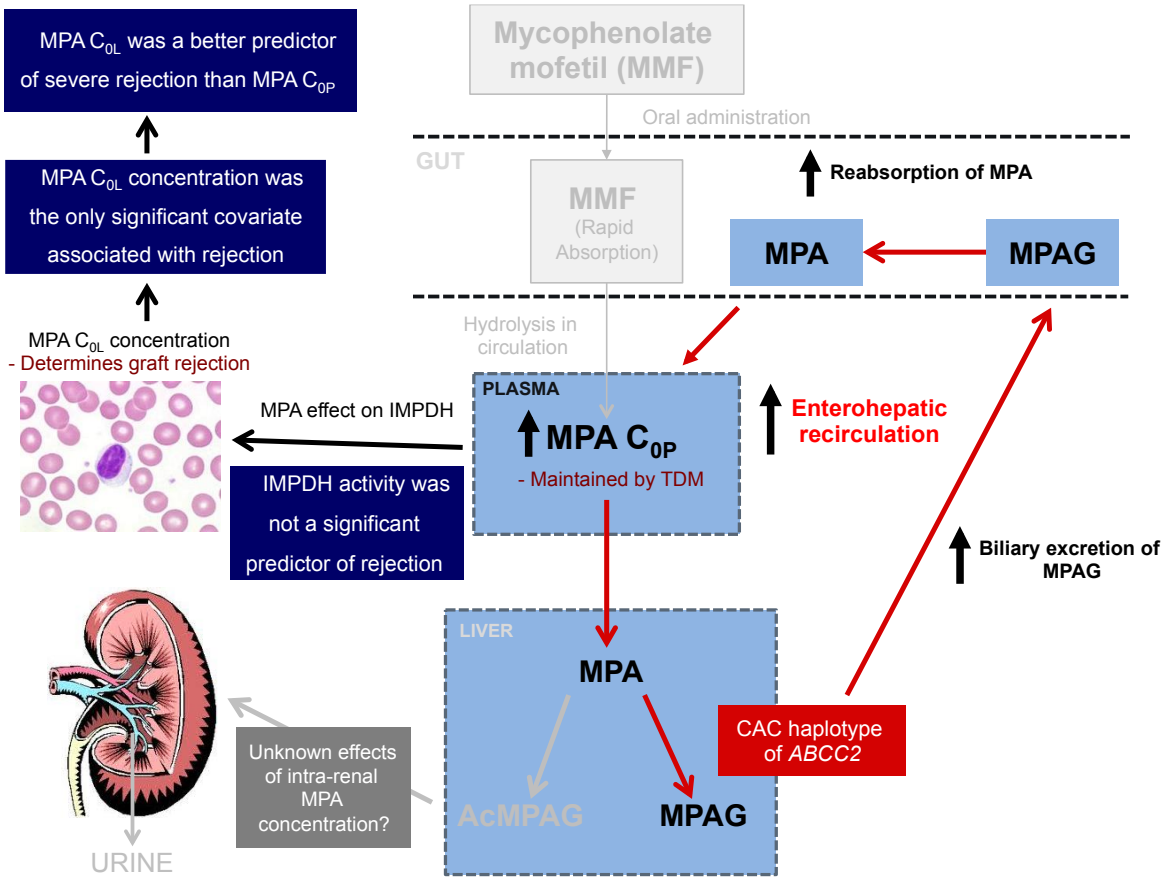

Figure 5.1 Revised summary of the hypothetical mechanisms of factors of both the transplant recipient and donor graft that determine concentrations of MPA in plasma, circulating lymphocytes and the kidney graft tissue based on thesis findings.

5.5 Conclusion

In conclusion, this thesis has provided sufficient evidence to justify further prospective investigation of the role of *ABCC2* haplotypes as an important clinical tool for further individualising immunosuppressant regimens. There is a need to move beyond individualising dose to attain target plasma concentrations to the situation whereby distribution into lymphocytes (and graft kidney tissue) is also factored into dosage adjustments. The association between IMPDH activity and clinical outcomes requires further investigation before prospective validation of individualised MPA therapy based on PD monitoring may be initialised. It is hoped that the research outcomes of this thesis might one day allow better tailoring of target concentration ranges based on recipient genotype and measurements of intracellular and target tissue MPA concentrations (by more efficient drug and dosage selection), and consequently improving clinical outcomes following renal transplantation.

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Appendix A

Statement of Authorship – Chapter 2

Statement of Authorship

Title of Paper	Validation of an LC-MS/MS method for the quantification of mycophenolic acid in human kidney transplant biopsies
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Principal Author

Name of Principal Author (Candidate)	Zaiput Md Dom		
Contribution to the Paper	Performed the experimental works* and statistical analyses, interpreted data, prepared and wrote the draft manuscript *Contributed to the development and validation of LC-MS/MS method		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	7.4.16

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that the candidate's stated contribution to the publication is accurate (as detailed above) and permission is granted for the candidate to include the publication in the thesis.

Name of Co-Author	Benjamin Noll		
Contribution to the Paper	Contributed to the development of work and data interpretation		
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Contribution to the Paper	Conceived the study, supervised the development of work, and participated in drafting the manuscript and critical review. Acted as a corresponding author.		
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Validation of an LC–MS/MS method for the quantification of mycophenolic acid in human kidney transplant biopsies

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ABSTRACT

Mycophenolic acid (MPA) has a low therapeutic index and large inter-individual pharmacokinetic variability necessitating therapeutic drug monitoring to individualise dosing after transplantation. There is an ongoing discrepancy as to whether plasma MPA concentrations sufficiently predict kidney rejection or toxicity and whether immunosuppressant concentrations within the graft tissue may better predict transplant outcomes. The aim of the study was to develop an LC–MS/MS method for the quantification of MPA concentrations in human kidney biopsies taken as part of routine clinical procedures. A total of 4 surplus human kidney biopsies obtained from 4 different kidney transplant recipients were available to use for this study. MPA was also quantified in 2 kidney samples from rats administered MPA to assess tissue extraction reproducibility. Human kidney biopsies and rat kidneys were homogenised mechanically and underwent liquid–liquid extraction before analysis by LC–MS/MS. MPA-free human kidney tissue was used in calibrators and quality control samples. Analyte detection was achieved from multiple reaction monitoring of the ammonium adducts of both MPA (m/z 321.1 → 207.3) and N-phthaloyl-L-phenylalanine (PPA, internal standard, m/z 296.2 → 250.2) using positive electrospray ionisation. The method was linear (calibration curves $R^2 > 0.99$, $n = 10$), precise, and accurate with coefficients of variation and bias less than 15%. Extraction efficiencies for MPA and PPA were approximately 97% and 86%, respectively, and matrix effects were minimal. In 4 kidney transplant recipients, tissue MPA concentrations ranged from 1.3 to 7.7 ng/mg of tissue, however, the correlation between blood (C_0) and tissue MPA concentrations could not be established. The method was successfully applied to the quantification of MPA in human kidney biopsies without the need to alter current clinical protocols.

1. Introduction

Mycophenolic acid (MPA) is a widely prescribed immunosuppressant for the prevention of rejection following kidney transplantation (Fig. 1) [1], usually in combination with other immunosuppressant drugs, most commonly a calcineurin inhibitor (cyclosporine or tacrolimus) and prednisolone. Due to a narrow therapeutic index and significant inter-subject pharmacokinetic variability [2,3], MPA requires therapeutic drug monitoring (TDM) to individualise dosing, ensuring optimal plasma concentrations to prevent organ rejection and minimise the incidence of adverse events such as leukopenia and diarrhoea [3,4]. MPA pre-dose trough (C_0) concentrations are a relatively good indicator of total drug exposure [5] and the acceptable plasma therapeutic range for MPA C_0 is 1.0–3.5 mg/L [3]. Lower plasma C_0 MPA concentrations have been shown to correlate significantly with the risk of rejection whereas high MPA C_0 values were associated with an increased risk of toxicity [6–8]. Although TDM from plasma is necessary for individualisation of MPA dosage, both acute rejection and adverse events still occur after kidney transplantation [9], further reinforcing that TDM alone may be inadequate in predicting target tissue concentrations and consequently therapeutic efficacy and/or safety. In contrast, MPA concentrations within lymphocytes or the graft tissue may provide more clinically relevant information on the immunosuppressive efficacy of MPA. Previous studies in liver and kidney transplant recipients suggest that rejection was associated with low concentrations of tacrolimus [10–12] and cyclosporine

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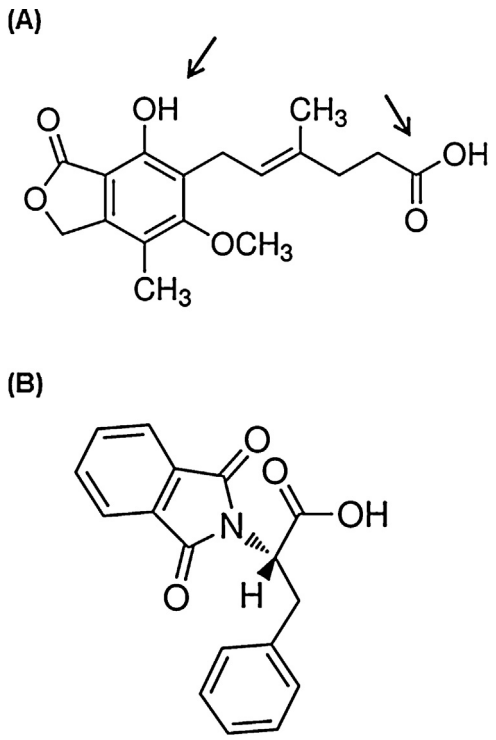

Fig. 1. Chemical structures of (A) mycophenolic acid (MPA) and (B) N-phthaloyl-L-phenylalanine (PPA, internal standard). Sites for MPA glucuronidation are shown by the arrows.

[13–15] within the graft tissue, but not with those in whole blood. To our knowledge, no previous studies have examined tissue MPA concentrations in human kidney biopsies and determined if the concentrations within the graft tissue correlate to the outcomes of rejection and toxicity compared to plasma concentrations.

The aim of this study was to develop and validate a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method capable of accurately quantifying MPA concentrations in human kidney biopsies from kidney transplant recipients taken as part of routine clinical procedures. The overall design of the method was primarily to assess whether MPA concentrations could be quantified in very small samples of kidney biopsy tissues, which are shared for histological assessments. In addition, we also assessed whether the potential in-source ion fragmentation of the metabolites of MPA (ether- and acyl-glucuronides) to MPA [16,17] would result in an overestimation of MPA concentrations by LC–MS/MS.

2. Materials and methods

2.1. Chemicals and reagents

Mycophenolic acid (MPA), tacrolimus, ammonium acetate, formic acid, tertiary-butyl methyl ether (t-BME) and N-phthaloyl-L-phenylalanine (PPA, internal standard (IS)) were sourced from Sigma–Aldrich (Castle Hill, NSW, Australia). MPA ether- (MPAG) and acyl- (AcMPAG) glucuronides were purchased from Roche Pharmaceuticals (Palo Alto, CA, USA). Cyclosporine and everolimus were purchased from Novartis Pharmaceuticals Australia Pty. Ltd. (North Ryde, NSW, Australia), and sirolimus from Wyeth Research (NJ, USA). HPLC-grade methanol (MeOH) was purchased from Thermofisher Pty Ltd (Scoreby, VIC, Australia). Sodium chloride (NaCl), sodium dihydrogen orthophosphate ($NaH_2PO_4{\cdot}H_2O$) and hydrochloric acid (HCl) were supplied by Ajax Finechem Pty Ltd (Taren Point, NSW, Australia). Sodium dihydrogen phosphate (NaH_2PO_4) and ethanol were obtained from Chem Supply (Gillman, SA, Australia). Potassium chloride (KCl) and potassium dihydrogen phosphate (KH_2PO_4) were purchased from Ajax Chemicals (Auburn, NSW, Australia). Ultrapure, de-ionised water (≥18.2 mΩ, Cascada AN-water Purification system, Pall, Cheltenham, VIC, Australia) was used throughout the assay.

2.2. Human and animals tissues

Human kidney tissue obtained from a non-transplant nephrectomy patient (The Queen Elizabeth Hospital Ethics of Human Research Committee, approval number 2008178) who was not administered MPA, was used to prepare the calibrators and quality control (QC) samples. Two rat kidney samples used as biological QCs in this study were obtained from a previous study [18]. Briefly, adult male transport-deficient (TR-) Wistar rats (HRD-AMC Abcc2) had been purchased from Harlan (Horst, the Netherlands) and ethics approval had been obtained from the Animal Experiments Committee of the Eramus Medical Center. The rats received 20 mg/kg bodyweight mycophenolate mofetil (the prodrug of MPA; Cellcept®, Roche Bioscience, Palo Alto, CA, USA) in combination with 4 mg/kg bodyweight tacrolimus daily (oral microemulsion formulation, 10 mg/mL; Prograf®, Fujisawa Pharmaceutical Co. Ltd., Osaka, Japan), starting on day 7 and continuing through day 14 before being sacrificed. Kidney samples were then harvested and immediately stored at −80 °C until the day of assay. All harvested tissues were divided and assayed as biopsy-sized samples thereafter.

2.3. Measurement of tissue MPA concentrations

On the day of assay, frozen human and rat kidney specimens were thawed at room temperature and cut into biopsy-sized pieces as required (approximately 0.5–1.0 mm^3), using a clean scalpel blade and placed in a clean eppendorf tube (Sarstedt AG & Co, Nümbrecht, Germany). Patient biopsy specimens stored in TissueTek® solution (ProSciTech, Thuringowa, QLD, Australia) were removed from aluminium packaging and placed in an empty eppendorf tube to thaw. The biopsy specimens were displaced from the TissueTek® solution with a clean, unused pipette tip to the top of the eppendorf tube. The biopsy specimens were then rinsed with 2–3 drops of water, blotted dried on lint-free tissue and transferred to clean eppendorf tubes. Each tissue specimen was weighed before undergoing mechanical homogenisation. Briefly, a chilled mortar and pestle was used to grind the specimens to a fine powder. At this stage, 200 μL of phosphate buffered saline (pH 7.4) was added to each tissue specimen and was mixed thoroughly around the mortar with the pestle before being transferred into a 5 mL disposable glass tube, appropriately labelled. To each sample, 5 μL of PPA internal standard (0.1 mg/L), 60 μL of HCl (0.4 M) and 1 mL of t-BME were added. Samples were mixed on a roller mixer for 10 min at a gentle speed ensuring that the samples were not mixed too vigorously as this may result in the formation of an emulsion. The tubes were then centrifuged at 4000 rpm (1900 × *g*) at 4 °C for 10 min. The aqueous layer was then snap-frozen in an ethanol/dry ice bath, the organic layer removed and evaporated using an evacuated centrifuge at 45 °C for approximately 20 min. The dried residues were reconstituted with 50 μL of 50/50 methanol/water, vortexed and transferred to vial-inserts. Ten microlitres of the reconstituted solutions were injected onto the LC–MS/MS for analysis.

2.4. Preparation of calibration curve standards and quality control (QC) samples

All calibrators and QC samples were prepared using a blank, MPA-free, human kidney tissue obtained from the non-transplant nephrectomy patient. MPA working solutions for calibration curve

standards were prepared from MPA stock solution (100 mg/L in 100% MeOH) and were then used to spike blank human kidney tissue in homogenisation buffer, to final concentrations of 0.6, 1.0, 2.0, 5.0, 10.0 and 20.0 ng/mL. Similarly, QC samples were prepared by spiking blank human kidney sample in homogenisation buffer with an independently prepared MPA stock solution (100 mg/L in 100% MeOH) to final concentrations of 0.6, 2.0 and 10.0 ng/mL.

As well as spiked controls, two kidney samples obtained from rats administered MPA were initially assayed in replicates of 3–6 to determine the concentrations of MPA. Samples from these kidneys were then used consistently during method development as biological QCs to ensure tissue extraction reproducibility.

2.5. LC–MS/MS conditions

Detection of MPA and PPA IS in rat kidneys and human kidney biopsies was achieved using an API 3200 tandem mass spectrometer (AB Sciex, Mount Waverley, VIC, Australia) operating in positive electrospray ionisation mode. The spectrometer was coupled to a Shimadzu UPLC system (Shimadzu USA Manufacturing Inc, Canby, OR, USA) consisting of a dual Shimadzu LC pump, a degasser (DGU-20A3), an autosampler (SIL-20AC) and a column oven (CTO-20AC). The analytical column used was a 2 × 30 mm Luna phenyl-hexyl, 5 μm particle size column (Phenomenex, Lane Cove, NSW, Australia) maintained at 60 °C. Mobile phases A and B consisted of 100% water or 100% methanol, respectively, with both solutions also containing 2 mM ammonium acetate and 0.1% formic acid, which assisted the formation of positive ammonium adducts used for MPA and PPA detection by the mass spectrometer. The mobile phases were filtered, degassed and pumped at a flow rate of 0.5 mL/min with the ratio of mobile phase A:B at 50:50 for 0.0–1.2 min and then changed to the ratio of 5:95 (A:B) for the remaining 1.2–2.2 min of the sample run time. The analytical column was washed using 50% mobile phase A for a further 30 s prior to the next sample injection. Ten microlitres of reconstituted solutions were injected onto the analytical column and Analyst v1.5.1 software (AB Sciex, Mount Waverley, VIC, Australia) was used for data acquisition and quantitation.

The collision energy was set at 25 V, the ionspray voltage was set at 5500 V and the declustering potential was set at 25 V and 15 V for MPA and PPA IS, respectively, allowing optimal detection of MPA and PPA by the mass spectrometer. The desolvation temperature was set at 375 °C with nitrogen as both the curtain and collision gas. Analyte detection was obtained from multiple reaction monitoring (MRM) of the ammonium adducts of both MPA (m/z 321.1 → 207.3) and PPA IS (m/z 296.2 → 250.2), and calibration curves were constructed using weighted 1/X least-squares linear regression analysis of peak area ratios (MPA/PPA internal standard) versus MPA concentrations. MPA tissue concentrations were converted from ng/mL of MPA in digestion buffer to ng/mg of MPA in tissue for all unknown human kidney biopsies and rat kidney samples.

2.6. Method validation

The assay development and validation were performed following the principle of the Food and Drug Administration (FDA) guidelines for bioanalytical methods [19]. The method was assessed for accuracy, precision, linearity, extraction efficiency, matrix effects and stability.

2.6.1. Accuracy, precision and linearity

The highest and lower limit of quantification (LLOQ) calibrators and QC samples were assayed in replicates of 6 in a single analytical run to obtain intra-day inaccuracy and reproducibility as well as over 6 consecutive days to obtain inter-day inaccuracy and reproducibility. The assay was deemed reproducible if intra- and inter-day inaccuracy and imprecision for calibrators and QC samples were both within 15% (or 20% for the LLOQ). Linearity was assessed and acceptable if the coefficient of determination (R^2) for each of the calibration curves was greater than 0.99 and calibrator concentrations did not differ by more than 15% (or 20% for the LLOQ) from the nominal concentrations. Tissue weighing accuracy and reproducibility had been assessed previously [15]. Two rat kidney tissues were also assayed in 6 replicates within a single analytical run as well as over 6 separate days to ensure intra- and inter-day tissue extractions were reproducible. Results were accepted if these biological QCs had a coefficient of variation below 15%.

2.6.2. Extraction efficiency and matrix effects

Extraction efficiency and matrix effects were estimated using a post-extraction analyte addition approach, as previously described [20]. Three sets of samples were prepared at low (2 ng/mL) and high (20 ng/mL) concentrations. Samples in set 1 consisted of standards containing the analytes at concentrations of 2 and 20 ng/mL prepared in 50/50 methanol/water; samples in set 2 consisted of MPA-free tissue acquired from rat kidney and human kidney tissues, which were spiked with 100 μL of spiking solution to give final analyte concentrations of 2 and 20 ng/mL, thereafter, the samples were extracted, as described previously, and the dried residues were reconstituted in 100 μL of 50/50 methanol/water; samples in set 3 consisted of MPA-free tissue from the same source as used in set 2 that were first extracted followed by reconstitution of the dried residues with 100 μL of 50/50 methanol/water containing the analytes of interest at amounts of equivalent to the 2 and 20 ng/mL calibrators. Extraction efficiency was estimated by comparing MPA and internal standard peak areas from the samples of set 3 to those from the corresponding samples of set 2. Matrix effects were determined by comparing the peak areas of the samples of set 1 to those from the corresponding samples of set 3. Process efficiency was determined by comparing sets 3 and 2.

Potential interference from other immunosuppressants that may be coadministered with MPA was also investigated using post-extraction addition. Based on previous measurement of cyclosporine [15] and tacrolimus [21] concentrations in human kidney biopsies, duplicate samples (0.7 mg) of drug-free human kidney tissue were homogenised and spiked with 100 μL of 50/50 methanol/water alone or methanol/water containing cyclosporine (50 ng/mg tissue), tacrolimus, sirolimus and everolimus (all at 1 ng/mg tissue). The samples were then extracted as described above, and spiked with MPA and PPA during reconstitution using 50 μL of a 50/50 methanol/water solution containing the equivalent concentrations of a 2 ng/mL calibrator. MPA and PPA peak areas in the reconstituted extracts were also compared to direct injection of the methanol/water spiking solution.

Assessment of matrix effects was also estimated using a post-column analyte infusion technique [20,22] that involves post-column infusion of MPA and internal standard against injections of reconstituted blank tissue extract. The sample was mechanically extracted and reconstituted in 50/50 methanol/water. Pure solutions of MPA and internal standard were introduced separately onto the LC–MS/MS system by an infusion syringe placed after the chromatographic column and before mass spectrometer ionisation source in a constant flow [20]. The reconstituted tissue sample without added analyte was injected into the LC–MS/MS system, and the response from the analyte was recorded and compared to direct injection of pure 50/50 methanol/water.

2.6.3. Stability

The stability of MPA in tissues was investigated over a 2 month period in biological QCs that were stored at -80 °C, with replicate samples (n = 3–6) thawed and analysed at 0, 1 and 2

months together with freshly prepared calibration curves. The stability of MPA during chromatographic analysis was investigated by repeated injection of an extracted calibrator sample over 24 h.

Stability of MPA glucuronide metabolites was checked using authentic MPA ether- (MPAG) and acyl- (AcMPAG) glucuronides. Three separate sets of samples were prepared for each MPA glucuronide. Samples in set 1 consisted of pure solutions containing each of the glucuronides at a concentration of 100 ng/mL prepared in 50/50 methanol/water and kept at -20 °C until ready for injection onto the LC–MS/MS; samples in set 2 consisted of MPA-free tissue spiked with AcMPAG or MPAG before being homogenised mechanically; samples in set 3 consisted of MPA-free tissue spiked with AcMPAG or MPAG after undergoing mechanical homogenisation; thereafter, samples in sets 2 and 3 were extracted as described previously and the dried residues were reconstituted in 50 μL of 50/50 methanol/water.

2.7. Clinical application of the method to biopsy specimens from kidney transplant recipients

A total of 4 excess biopsy specimens obtained from 4 different kidney transplant recipients were available for use in this study. Three of the 4 kidney transplant recipients were taking MPA, everolimus and prednisolone-, and the other, sirolimus and prednisolone-based immunosuppression. The kidneys were transplanted from living ($n = 1$) and deceased ($n = 3$) donors between February 2006 and April 2010. Informed written consent was obtained from the recipients and living donor, and where the kidney was obtained from a deceased donor, the recipient was asked to provide consent for the use of the biopsy specimen. The study was approved by the Ethics of Human Research Committee of the Queen Elizabeth Hospital (approval number 2008178). Biopsies were taken in cases of suspected rejection, with 1 of 4 biopsies taken at 8 days post-transplant, 2 taken within the first year post-transplant, and 1 taken after 4 years post-transplant. Rejection was defined according to Banff 2007 criteria [23]. Fine core needle kidney biopsies were routinely divided by pathology staff and part embedded in paraffin for histological assessment with the remaining biopsy tissue snap frozen at −80 °C in TissueTEK® solution until the day of the assay.

3. Results

3.1. Assay performance

PPA and MPA eluted at 1.80 and 1.83 min, respectively, and total chromatographic analysis time was 2.2 min. Fig. 2(A) shows a representative chromatogram of an extracted patient kidney biopsy tissue, with added internal standard (light grey). The

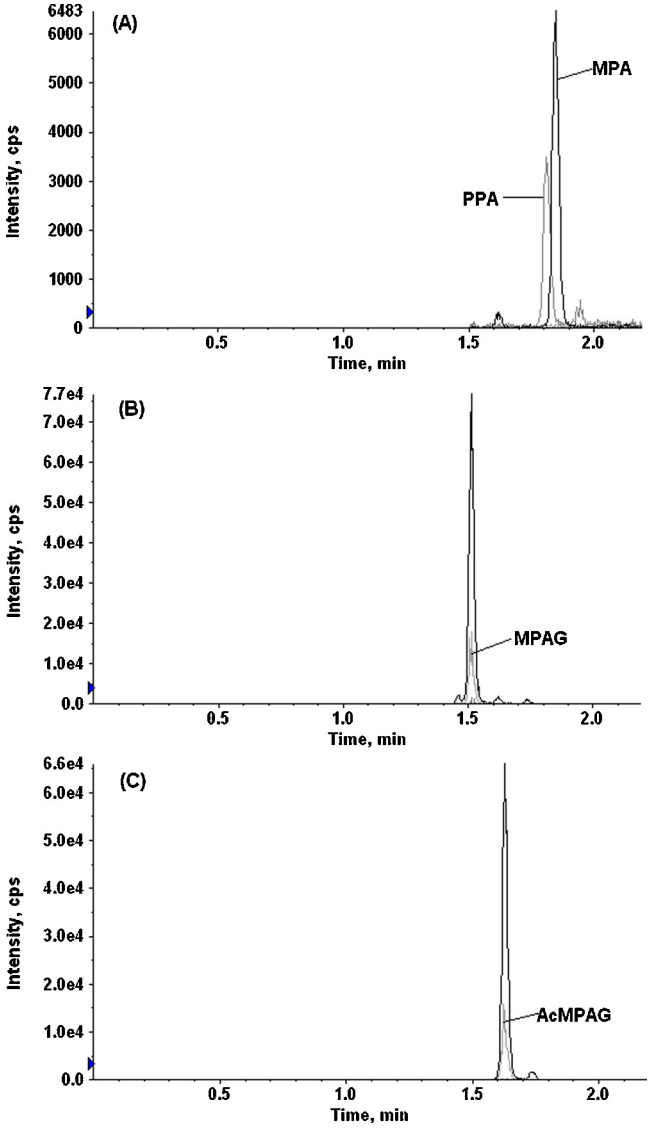

Fig. 2. Representative LC–MS/MS chromatograms from (A) an extracted patient kidney biopsy tissue (weight 0.33 mg) with a measured MPA concentration of 11.0 ng/mL in extraction buffer, equating to 6.7 ng/mg in the biopsy; and MPA-free human kidney tissues spiked with (B) MPA ether-glucuronide (MPAG) at the retention time of 1.51 min, or (C) MPA acyl-glucuronide (AcMPAG) at the retention time of 1.63 min. In panels (B) and (C) the MPAG or AcMPAG MRMs are shown in light grey.

Table 1
Intra- and inter-day inaccuracy (bias) and imprecision of the lowest and highest calibration and low, middle and high QC samples for MPA, and two kidney samples obtained from rats administered MPA (biological QCs).

	Intra-day ($n = 6$)			Inter-day ($n = 6$)		
	Mean ± SD	CV (%)	Bias (%)	Mean ± SD	CV (%)	Bias (%)
Calibrators (ng/mL)						
0.6	0.61 ± 0.07	10.7	2.2	0.59 ± 0.05	4.6	−0.4
20	20.1 ± 0.66	3.3	0.6	19.9 ± 0.87	4.4	−0.5
QCs (ng/mL)						
0.6	0.60 ± 0.05	8.6	0.1	0.57 ± 0.05	9.5	−5.4
2	1.86 ± 0.25	13.3	−7.3	2.0 ± 0.11	5.5	1.9
10	10.5 ± 0.82	7.8	5.1	10.3 ± 0.49	4.8	2.8
Biological QCs (ng/mg)						
A	0.35 ± 0.05	12.8	–	0.35 ± 0.04	12.9	–
B	3.5 ± 0.26	7.3	–	3.6 ± 0.22	6.1	–

Table 2
Extraction efficiency, matrix effects, and process efficiency of the LC–MS/MS assay in human and rat kidney samples using the post-extraction analyte addition approach [20] at MPA concentrations of 2 (low) and 20 (high) ng/mL.

		Human kidney		Rat kidney	
		Low	High	Low	High
Extraction efficiency	MPA	97%	98%	94%	89%
	PPA	88%	86%	83%	86%
	MPA/PPA	103%	104%	100%	103%
Matrix effect	MPA	−4%	2%	0%	10%
	PPA	−2%	9%	−5%	10%
	MPA/PPA	−2%	−6%	5%	0%
Process efficiency	MPA	94%	99%	95%	98%
	PPA	94%	95%	92%	95%
	MPA/PPA	101%	105%	103%	105%

calibration curves were linear over the concentration range of 0.6–20.0 ng/mL, with coefficients of determination, R^2, greater than 0.997 (mean ± SD; 0.999 ± 0.00096, $n = 10$) routinely obtained. Intra- and inter-day inaccuracy and reproducibility for calibration curves and QC samples was less than 15% (Table 1). Tissue extraction reproducibility was determined using two rat kidneys from abcc2-deficient rats administered MPA. The mean (± SD) kidney MPA concentrations ($n = 3$–6 replicates) were 0.35 (±0.04) and 3.5 (±0.26) ng/mg of tissue. Tissue extraction from the rat kidney samples was considered reproducible and within acceptable limits (CV < 13%, $n = 6$) (Table 1). These rat kidneys were used consistently during method development and validation as biological QCs.

Extraction efficiency, matrix effects and process efficiency were all acceptable, as shown in Table 2. There was no evidence for a matrix effect in human kidney tissue. However, for rat kidney, a small matrix effect in the form of ion enhancement was observed at a higher concentration of MPA (Table 2), although it was well

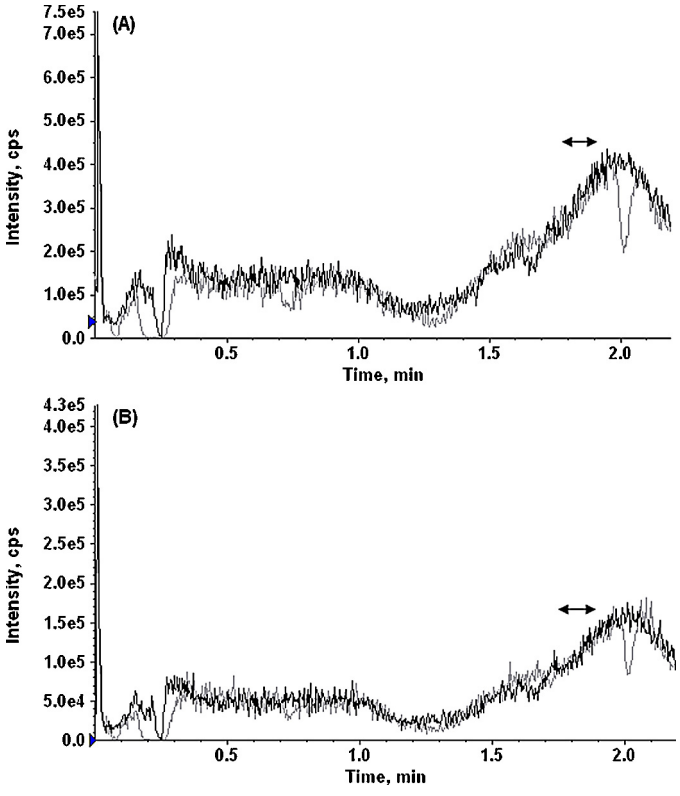

Fig. 3. Post-column infusion traces of (A) MPA and (B) PPA IS following injections of analyte-free human kidney tissue extract (light grey) and mobile phase (black). Expected analyte peak widths are indicated by double arrow on the chromatograms.

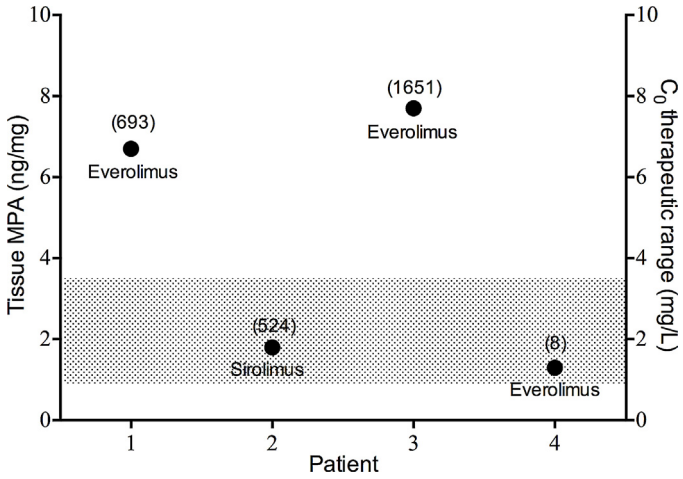

Fig. 4. Kidney (●) MPA concentrations measured in 4 kidney transplant recipients over 8–1651 days post-transplantation. All recipients also received prednisolone and one other immunosuppressant (as indicated). Numbers in parenthesis indicate the time biopsies were taken post-transplantation (days) and the shading (1.0–3.5 mg/L) indicates the plasma therapeutic range for C_0.

within the acceptable limit of ±15% as discussed previously [20,24], and was similar for both MPA and PPA. Other immunosuppressants likely to be co-administered with MPA had no significant effects on either MPA or PPA peak areas, except sirolimus, which appeared to interfere with the detection of MPA, decreasing peak area by 39%. In the post-column infusion technique, Fig. 3 shows a comparison of an injection of analyte-free human kidney tissue extract (light grey) and mobile phase (black) for MPA (A) and PPA IS (B). MPA and PPA IS co-eluted outside of the area of ion suppression.

MPA in tissues stored at −80 °C was stable over a 2 month period, with mean measured MPA concentrations varying by less than 15%. MPA was similarly stable at room temperature during chromatographic analysis with peak areas varying 4.2–16.5% for repeated injections over 24 h.

The stability of MPA glucuronide metabolites was checked by comparing the peak areas of both metabolites in pure solution, before and after undergoing mechanical homogenisation. There was no difference in the peak areas of MPA metabolites before and after mechanical homogenisation and no MPA peak was observed at its retention time on the chromatograms (Fig. 2). In contrast, a peak was observed in the MPA MRM corresponding to the retention times of MPAG or AcMPAG, 1.51 and 1.63 min, respectively, as shown in Fig. 2(B) and (C), respectively. This could be due to in-source ion fragmentation of the glucuronides to MPA during the ionisation procedure inside the mass spectrometer. However, since we were able to separate both glucuronide metabolites from MPA chromatographically; this in-source fragmentation did not interfere with quantification of MPA.

3.2. Method application to biopsy specimens from kidney transplant patients

Mean (±SD, range) patient biopsy weights were 0.18 (±0.1, 0.11–0.33) mg, and tissue MPA concentrations measured in each patient biopsy from 4 kidney transplant recipients were 1.25 (biopsy taken at 8 days post-transplant; co-administered with everolimus), 1.77 (524 days post-transplant; co-administered with sirolimus), 6.67 (693 days post-transplant; co-administered with everolimus) and 7.67 (1651 days post-transplant; co-administered with everolimus) ng/mg of tissue, as shown in Fig. 4. Tissue MPA concentrations were all within the calibration curve range. Two

of the 4 MPA biopsy concentrations were of a similar magnitude to the plasma therapeutic range for C_0 (1–3.5 mg/L) and the other two biopsies concentrations were above this range. Plasma MPA C_0 concentrations were not available for all patients and therefore the correlation between plasma (C_0) and tissue MPA concentrations could not be established in this study.

4. Discussion and conclusions

The quantification of MPA concentrations within the graft tissue may provide a better understanding of MPA distribution during kidney rejection. Previous studies in liver and kidney transplant recipients showed that intra-graft tacrolimus [10–12] and cyclosporine [13–15] concentrations are better predictors of transplant outcomes compared to whole blood concentrations. No study to date has examined tissue MPA concentrations in kidney transplant recipients to determine whether MPA concentrations within the graft tissue may better reflect or predict transplant outcomes. This study aimed to develop a method suitable for the quantification of MPA in human kidney biopsies from kidney transplant recipients taken as part of routine clinical care. Several analytical methods have been reported for the quantification of MPA concentrations in plasma [25–29]; however, to our knowledge, this is the first study to measure tissue MPA concentrations in human kidney biopsies taken as part of routine clinical procedures.

The method has been comprehensively assessed following the principle of the FDA criteria for industry on bioanalytical methods [19] and several aspects of this method are based on our previous method for the quantification of the calcineurin inhibitor tacrolimus in human kidney biopsy specimens [21]. Like tacrolimus, detection of MPA was also based on MRM monitoring of the ammonium adducts, similar to several other published MPA methods measuring MPA in plasma [30–32] to enhance greater overall immunosuppressant assay flow-through on the LC–MS/MS system. However, several changes to the tacrolimus method were also necessary to maximise analyte peak intensity and improve the sensitivity. This method employed mechanical homogenisation using a mortar and pestle followed by liquid–liquid extraction of the drug in biopsy samples. Enzymatic tissue digestion had been used previously for the tacrolimus method [21] and has some advantages over mechanical homogenisation particularly minimising sample handling and analyte loss that may occur in the process. However, the solubilisation is carried out at alkaline pH and may hydrolyse the relatively unstable AcMPAG during sample preparation. This study demonstrated that the mechanical homogenisation technique prevented degradation of the glucuronides and produced excellent extraction efficiency (approximately 97%) and high process efficiency, and therefore can be considered as an alternative sample preparation technique for the analysis of small biopsy-sized samples. Furthermore, the use of biological QCs obtained from rats administered MPA also provided additional assessment and validation of the reproducibility of the tissue extraction procedures. Liquid–liquid extraction has been used in our sample preparation as it has been shown to minimise potential matrix interference in comparison to the solid-phase extraction technique [33] and this method demonstrated no significant matrix effects in human samples and practically negligible ion enhancement ($\leq$10%) in rat kidney samples. Initially, carboxy-butoxy ether mycophenolic acid (MPAC) had been selected as the internal standard, however MPAC produced an interfering peak in the MPA trace (most likely due to in-source fragmentation) and was replaced by PPA, which provides overall accuracy, precision and specificity well within acceptable limits in both human and rat tissues. The use of stable labelled internal standards (MPA-d3) is highly recommended to adequately compensate for matrix effects, and may contribute to improved performances, however deuterated internal standard was not available at the time of developing the method.

We have demonstrated no interference in the quantification of tissue MPA concentrations by cyclosporine, tacrolimus and everolimus, the other commonly co-administered immunosuppressants. However, sirolimus appeared to cause underestimation of MPA and the method may require further validation before MPA concentrations can be measured in tissues also exposed to sirolimus. Regarding potential analytical interference from the metabolites of MPA, the in-source ion fragmentation of drug metabolites to their respective parent analyte has been discussed previously [16] and a previous study by Vogeser and colleagues [17] has demonstrated that the in-source ion fragmentation of MPAG can interfere with the quantification of MPA. We have checked with pure MPAG and AcMPAG solutions that both metabolites did not interfere with the detection and quantification of MPA. Although in-source ion fragmentation of the glucuronides into MPA was observed at the retention times of MPAG and AcMPAG, the chromatographic separation of the two MPA glucuronides from MPA resulted in no interference with the quantification of MPA. This factor must be assessed critically during method development to ensure a correct detection and quantification of the actual MPA peak, since falsely high MPA concentrations could lead to lower dose administration and potentially an increased risk of rejection in kidney transplant recipients.

The LC–MS/MS method has allowed for the accurate and precise quantification of MPA in biopsy-sized tissue samples taken as part of routine clinical care. As same-day plasma MPA C_0 concentrations were not available for the biopsy specimens in this small study, we were unable to investigate any relationships between kidney MPA concentrations and those in plasma. However, it appears that graft MPA concentrations were of a similar magnitude to the C_0 therapeutic range for MPA. In comparison, we have previously reported tissue to blood concentration ratios of 4 and 20 for cyclosporine [15] and tacrolimus [21], respectively, with no correlation between their concentrations in whole blood and those within the kidney, consistent with other published studies [10,11,13]. However, small sample size was a limitation for this and our two previous studies [15,21]. In addition, this study may also have been limited by the age of the biopsy samples, as the stability of MPAG and AcMPAG during long-term storage could not be assessed.

In summary, this method has been successfully used for quantification of MPA concentrations in core needle biopsies from kidney transplant recipients taken as part of routine clinical care. In contrast to previously published studies [11,14], clinical practice in this study did not allow prospective acquisition of excess biopsy specimen. In addition, due to shared histological assessments and processing of biopsy specimens, this method was primarily designed to quantify MPA in routine core needle biopsies of kidney transplant recipients weighing as little as 0.1 mg. Nonetheless, these limitations have ensured that our assay benefits unaltered biopsy sampling and handling to fit into current clinical practice, and therefore will allow further investigations on the immunosuppressant MPA concentrations in the transplanted organ and their relationship with clinical outcomes following kidney transplantation.

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Appendix B

Statement of Authorship – Chapter 3

Statement of Authorship

Title of Paper	Multidrug Resistance-Associated Protein 2 (MRP2/*ABCC2*) Haplotypes Significantly Influence the Pharmacokinetics of Mycophenolic Acid in Renal Transplant Recipients
Publication Status	Unpublished and Unsubmitted work written in manuscript style
Publication Details	Md Dom Z, Coller JK, Westley IS, Tuke J, Somogyi AA, Sallustio BC (2016). Multidrug Resistance-Associated Protein 2 (MRP2/*ABCC2*) Haplotypes Significantly Influence the Pharmacokinetics of Mycophenolic Acid in Renal Transplant Recipients

Principal Author

Name of Principal Author (Candidate)	Zaipul Md Dom		
Contribution to the Paper	Carried out the experimental works*, statistical analyses, interpreted data, prepared and wrote the draft manuscript *Performed DNA extraction, genotyping assay and haplotype analysis. Collected data from clinical notes and prepared data for modeling analyses		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	19.7.16

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that the candidate's stated contribution to the publication is accurate (as detailed above) and permission is granted for the candidate to include the publication in the thesis.

Name of Co-Author	Janet K Coller		
Contribution to the Paper	Advised on the methodology, helped in modeling analyses and data interpretation, participated in drafting the manuscript and critical review		
Signature		Date	22.7.16

Name of Co-Author	Ian S Westley		
Contribution to the Paper	Performed genotyping assay and reviewed the manuscript		
Signature		Date	19/7/2016

Name of Co-Author	Jonathan Tuke		
Contribution to the Paper	Helped in modeling analyses and data interpretation, reviewed the manuscript		
Signature		Date	25/7/16

Name of Co-Author	Andrew A Somogyi		
Contribution to the Paper	Contributed to drafting the manuscript and critical review		
Signature		Date	25/07/2016

Name of Co-Author	Benedetta C Sallustio		
Contribution to the Paper	Conceived the study, contributed to its design and coordination, supervised development of work, and participated in drafting the manuscript and critical review. Acted as a corresponding author.		
Signature		Date	20.7.2016

Appendix C

Statement of authorship – Chapter 4

Statement of Authorship

Title of Paper	Intra-lymphocyte Concentrations of Mycophenolic Acid Correlate with the Incidence of Early Graft Rejection in Renal Transplant Recipients
Publication Status	Unpublished and Unsubmitted work written in manuscript style
Publication Details	Md Dom Z, Coller JK, Carroll RP, Tuke J, Somogyi AA, Sallustio BC (2016). Intra-lymphocyte Concentrations of Mycophenolic Acid Correlate with the Incidence of Early Graft Rejection in Renal Transplant Recipients

Principal Author

Name of Principal Author (Candidate)	Zaipul Md Dom		
Contribution to the Paper	Carried out the experimental works*, patient recruitment, statistical analyses, interpreted data, prepared and wrote the draft manuscript		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that the candidate's stated contribution to the publication is accurate (as detailed above) and permission is granted for the candidate to include the publication in the thesis.

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Contribution to the Paper	Helped in modeling analyses and data interpretation, reviewed the manuscript		
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Contribution to the Paper	Conceived the study, contributed to its design and coordination, supervised development of work, participated in drafting the manuscript and critical review. Acted as a corresponding author.		
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