British Journal of Clinical Pharmacology

Br J Clin Pharmacol (2018) **84** 2433–2442 2433

ORIGINAL ARTICLE

# Mycophenolic acid concentrations in peripheral blood mononuclear cells are associated with the incidence of rejection in renal transplant recipients

**Correspondence** Assoc Prof B C Sallustio, Department of Clinical Pharmacology, Basil Hetzel Institute, The Queen Elizabeth Hospital, 28 Woodville Road, Woodville, SA 5011, Australia. E-mail: benedetta.sallustio@sa.gov.au

**Received** 9 April 2018; **Revised** 22 June 2018; **Accepted** 24 June 2018

Zaipul I. Md Dom[1,2], Janet K. Coller[1], Robert P. Carroll[3], Jonathan Tuke[4,5], Brett C. McWhinney[6], Andrew A. Somogyi[1,7] and Benedetta C. Sallustio[1,2]

*[1]Discipline of Pharmacology, Adelaide Medical School, The University of Adelaide, Adelaide, SA, 5005, Australia, [2]Department of Clinical Pharmacology, The Queen Elizabeth Hospital, Woodville, SA, 5011, Australia, [3]Centre for Clinical and Experimental Transplantation, Central Northern Adelaide Renal and Transplantation Service, Royal Adelaide Hospital, Adelaide, SA, 5000, Australia, [4]School of Mathematical Sciences, The University of Adelaide, Adelaide, SA, 5005, Australia, [5]ARC Centre of Excellence for Mathematical & Statistical Frontiers, School of Mathematical Sciences, The University of Adelaide, Adelaide, SA, 5005, Australia, [6]Department of Chemical Pathology, Pathology Queensland, Royal Brisbane and Women's Hospital, Brisbane, Queensland, Australia, and [7]Department of Clinical Pharmacology, Royal Adelaide Hospital, Adelaide, SA, 5000, Australia*

**Keywords** immunosuppression, pharmacokinetics, renal transplantation, therapeutic drug monitoring

## AIMS

Although therapeutic drug monitoring of plasma mycophenolic acid (MPA) concentrations has been recommended to individualize dosage in transplant recipients, little is known regarding lymphocyte concentrations of MPA, where MPA inhibits inosine monophosphate dehydrogenase (IMPDH). This study investigated the utility of measuring predose MPA concentrations in peripheral blood mononuclear cells ($C_{0C}$) and predose IMPDH activity, as predictors of graft rejection in renal transplant recipients.

## METHODS

Forty-eight patients commencing mycophenolate mofetil (1 g twice daily) in combination with tacrolimus and prednisolone were recruited. Blood was collected for determination of trough total ($C_{0P}$) and unbound ($C_{0u}$) plasma MPA concentrations. Peripheral blood mononuclear cells were isolated for determination of $C_{0C}$ and IMPDH activity. The incidence of rejection within 2 days of sample collection was determined histologically and classified according to the Banff 2007 criteria.

## RESULTS

There was no association between MPA $C_{0C}$ and $C_{0P}$ ($r_s = 0.28$, $P = 0.06$), however, MPA $C_{0C}$ were weakly correlated with MPA $C_{0u}$ ($r_s = 0.42$, $P = 0.013$). Multivariate analysis indicated that MPA $C_{0C}$ was the only covariate independently associated with rejection (FDR-adjusted $P = 0.033$). The receiver operating characteristic area under the curve (AUC) for the prediction of severe rejection using MPA $C_{0C}$ was 0.75 ($P = 0.013$), with 73% sensitivity and specificity at a $C_{0C}$ threshold of 0.5 ng $10^{-7}$ cells. However, predose IMPDH activity was not a predictor of rejection ($P > 0.15$).

## CONCLUSIONS

MPA $C_{0C}$ measurement within the early post-transplant period may be useful to facilitate early titration of MPA dosing to significantly reduce rejection.



DOI:10.1111/bcp.13704

### WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- Mycophenolic acid (MPA) is an immunosuppressant commonly used to prevent rejection following renal transplantation.
- MPA prevents lymphocyte proliferation by inhibiting inosine monophosphate dehydrogenase activity, the rate limiting enzyme for *de novo* purine synthesis.
- Several clinical trials have demonstrated that therapeutic drug monitoring of plasma MPA concentrations reduces the risk of rejection.

### WHAT THIS STUDY ADDS

- Peripheral blood mononuclear cell (PBMC) concentrations of MPA were better predictors of rejection risk, compared to measuring trough plasma MPA concentrations or PBMC inosine monophosphate dehydrogenase activity.
- Trough plasma MPA concentrations were relatively poor predictors of PBMC concentrations.
- Predose PBMC MPA concentrations $<0.5$ ng/$10^7$ cells predicted severe rejection with 73% sensitivity and specificity.

## Introduction

Mycophenolic acid (**MPA**), one of the primary immunosuppressants administered to prevent rejection following renal transplantation [1], exerts antiproliferative effects on lymphocytes as its major mode of action [2]. It is a potent, selective, reversible and noncompetitive inhibitor of inosine monophosphate dehydrogenase (**IMPDH**) type II, a rate-limiting enzyme involved in *de novo* purine synthesis that is selectively required for lymphocyte proliferation [2]. Inhibition of this pathway prevents the proliferation of lymphocytes and the activation of T-cells, which consequently contributes to the prevention of graft rejection.

The clinical use of MPA is compromised by MPA having a narrow therapeutic index and large inter-patient variability in pharmacokinetics [3]. Numerous studies have investigated the plasma MPA concentration-effect relationship, and recent consensus reports indicate significant benefits of monitoring area under the plasma concentration–time curve ($AUC_P$) with respect to rejection [3, 4]. In general, although there is also a relationship between MPA trough concentrations ($C_{0P}$) and the risk of graft rejection, the data are more equivocal due to the variable enterohepatic recirculation of MPA and its inhibition by cyclosporin (CsA; but not tacrolimus, TAC), potentially resulting in different relationships between $C_{0P}$ and $AUC_P$ when coadministered with CsA compared to TAC [3, 4].

Despite application of therapeutic drug monitoring (TDM) strategies that target patients to narrow plasma MPA concentrations ($C_{0P}$: 1.9–3.5 mg $l^{-1}$) or $AUC_P$ ranges (0–12 h: 30–60 mg h $l^{-1}$) when coadministered with TAC [3, 5], rejection rates in Australian renal transplant recipients remain relatively high (13–24% in the first 6 months post-transplantation) [1]. This suggests that monitoring of plasma MPA concentrations alone may be inadequate to predict graft rejection and may not be the most appropriate predictor of target lymphocyte (site of action) concentrations, and hence, clinical outcomes. Direct measurement of lymphocyte MPA concentrations may provide a better understanding of its immunosuppressive efficacy and distribution during graft rejection. The importance of such an approach has already been demonstrated for CsA [6–9] and TAC [10, 11] by direct quantification of peripheral blood mononuclear cells (PBMCs), representing the target site of action. It has been suggested that lower PBMC CsA [9] and TAC [10] concentrations are associated with significantly higher incidences of graft rejection, which were not reflected by whole blood concentrations.

Little is known regarding the relationship between PBMC and plasma MPA concentrations. In 40 kidney transplant recipients, no association between MPA $C_{0P}$ and predose PBMC ($C_{0C}$) MPA concentrations was found [12]. However, a later study conducted by the same group reported significant correlations between plasma and PBMC MPA concentrations at 1.5 and 3.5 h postdose, but not between MPA $C_{0P}$ and $C_{0C}$ concentrations on days 2, 4 and 10 post-transplantation [13]. No other studies have investigated the factors that determine PBMC MPA concentrations. Two important modulators of these concentrations may include the binding of MPA to plasma albumin, and the possible role of PBMC uptake and efflux transporters in modulating intracellular concentrations. MPA is a substrate for the efflux transporter multidrug resistance-associated protein 2 (**MRP2**) [14], which is also expressed in lymphocytes [15]. Genetic variability in the *ABCC2* gene that encodes MRP2 may, therefore, influence PBMC MPA concentrations and modulate the efficacy and/or safety of MPA therapy.

Pharmacodynamic monitoring of MPA by the measurement of IMPDH activity has also been investigated in PBMCs [13, 16–21]. In general, there is an inverse relationship between plasma MPA concentrations and total PBMC IMPDH activity (**type I** and II). Within a dosing interval, maximum inhibition of activity coincides with the maximum plasma MPA concentration, and activity returns to predose levels by between 3.5 and 11 h postdose [13, 16, 19, 21]. Population pharmacokinetic–pharmacodynamic ($E_{max}$) modelling has been used to calculate $EC_{50}$ values for the inhibition of IMPDH activity by MPA, which range from 0.97 to 5.4 mg $l^{-1}$, in both adult and paediatric renal transplant recipients [16, 19, 21]. However, the relationship between trough plasma MPA concentrations and IMPDH activity is less clear [13, 18, 20]. Similarly, the relationship between IMPDH activity and clinical outcomes is not well defined. An increased risk of acute rejection in patients with high pretransplant IMPDH activities has been reported [17], but there appears to be no relationship post-

transplant between predose IMPDH activity and acute rejection [20].

This study aimed to investigate PBMC MPA concentrations and intracellular IMPDH activity as predictors of early graft rejection in kidney transplant recipients. We hypothesized that PBMC 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.

## Methods

### *Study population, pharmacokinetic and clinical data*

Forty-eight 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). The study was conducted in accordance with the Declaration of Helsinki and the Australian NHMRC Statement on Ethical Conduct in Human Research. Recipients were transplanted between June 2013 and November 2014 with kidneys from living and deceased donors. They received the MPA prodrug, mycophenolate mofetil (MMF, 1 g twice daily) for maintenance immunosuppression in combination with TAC and prednisolone. For each recipient, a fine-needle graft biopsy was taken between 5 and 22 days post-transplantation. On the same day, blood was drawn to assess trough plasma MPA concentrations. Total trough plasma MPA concentrations ($C_{0P}$) were determined using a validated high-performance liquid chromatography (HPLC) method [22], and matching unbound ($C_{0u}$) plasma MPA concentrations were determined following temperature controlled (37°C) ultrafiltration of plasma samples. MPA-d3 internal standard (Toronto Research Chemicals, Toronto, Canada) in methanol was added to ultrafiltrate, vortexed and centrifuged prior to analysis by liquid chromatography using an Acquity UPLC HSS T3 C18 analytical column (1.8 µm, 2.1 × 100 mm) and Acquity BEH C18 precolumn (1.7 µm, VanGuard 2.1 × 5 mm) maintained at 40°C, with gradient elution using 2 mmol $l^{-1}$ ammonium acetate and 0.1% formic acid in methanol (mobile phase B) or water (mobile phase A). Multiple reaction monitoring was carried out using positive electrospray ionization and detection of MPA 321.2 > 207.2 and MPA-d3 324.3 > 310.2 transitions. The assay was linear between 5–1500 µg $l^{-1}$ (10 µl injection) with intra-assay imprecision <4% and interassay imprecision <9% and inaccuracy <5%. Postpreparation sample stability was at least 24 h at 25 °C, carry-over was <0.1% at the highest calibrator, analyte recovery was 100–101%, and matrix effects were <10%. 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 leucocyte antigen (HLA) mismatches; cold ischaemia time (CIT); panel-reactive antibodies (PRA); plasma creatinine; albumin and bilirubin concentrations; pretransplant angiotensin II type-1 receptor (AT1R) antibody levels; donor-specific HLA antibodies; trough whole blood TAC concentrations; and the incidences of rejection and delayed graft function (DGF). Rejection status within the period of ±2 days from sample collection was determined based on histological evidence of rejection in protocol and for-cause biopsies (5–22 days post-transplant), and classified for severity according to Banff 2007 criteria [23] as: no rejection; subclinical or borderline; or clinically evident cellular/vascular rejection (Type 1A/2 or 2A/B). The incidence of DGF was identified by the lack of spontaneous decline in serum creatinine or requirement for haemodialysis within 7 days post-transplantation.

### *ABCC2 genotyping and haplotype predictions*

Blood samples from recipients were also used for determination of the common *ABCC2* allele variants (–24 C > T, 1249 G > A and 3972 C > T) [24], and *ABCC2* haplotypes were inferred by the use of PHASE software version 2.1.1 [25]. The recipients were divided into high, wild-type (WT) or low MRP2 expressor groups according to *ABCC2* haplotypes reported previously by Laechelt *et al.* [26].

### *Isolation of PBMCs from whole blood*

Additional duplicate blood samples (2 × 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 individually within 4 h after collection to ensure maximal PBMC yield. PBMC 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 were diluted with an equal volume of 0.9% ice-cold NaCl, underlayered with 9 ml Lymphoprep and centrifuged without brakes at 1200 *g* at 4°C for 20 min. After centrifugation, PBMCs were harvested from the plasma/Lymphoprep interface and washed three times with 30 ml of 0.9% NaCl (centrifugation at 1200 *g* at 4°C for 10 min). The washed PBMC pellets were resuspended in 5 ml of 0.9% NaCl and 500 µl used for cell counting (in duplicate) on a haemocytometer. Each duplicate sample was centrifuged (1200 *g*, 4°C, 10 min), and each corresponding PBMC pellet was stored at –80°C for later determination of MPA $C_{0C}$ concentrations and IMPDH activity, respectively. Fresh blood (150 ml) obtained from healthy volunteers was used to isolate PBMCs (treated as 9 ml aliquots as described above) for the preparation of calibrator and quality control (QC) samples.

### *Measurement of predose PBMC MPA ($C_{0C}$) concentrations*

Measurement of $C_{0C}$ 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 with slight modifications [27]. On the day of the assay, frozen patient and blank PBMC 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 PBMC pellets (containing $10^7$ cells), to which 100 µl of PBS solution was added followed by 100 µl of MPA working solutions (prepared in 50% MeOH), to attain final concentrations of 0.1, 0.2, 0.5, 1.0, 3.0 and 5.0 ng $ml^{-1}$ for calibrators and 0.3, 1.5 and 2.0 ng $ml^{-1}$ for QC samples.

To each calibrator, QC and patient sample, 60 µl of 0.4 mol $l^{-1}$ HCl, 10 µl of 0.2 µg $ml^{-1}$ MPA-d3 internal standard and 1 ml of tertiary-butyl methyl ether were added. MPA extraction and cell lysis were performed by gently mixing on a roller mixer for 10 min followed by centrifugation at 1900 *g* at 4°C for 10 min. The organic layer was removed, transferred to a 5 ml disposable glass tube and evaporated to dryness using an evacuated centrifuge at 45°C for 20 min. The dried residues were reconstituted with 50 µl of 50% methanol, 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 [27].

The assay was fully validated according to the US Food and Drug Administration guidelines for bioanalytical methods [28] with assessment 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 intra- and interassay inaccuracy and imprecision were <15% ($n$ = 5). MPA extraction efficiency displayed good reproducibility, with coefficient of variations (CVs) ranging from 0.1 to 5.2% and matrix effects were minimal (< 10%); both were assessed at three MPA concentrations (1.0, 5.0 and 20.0 ng $ml^{-1}$) in duplicate. MPA was stable when spiked into blank PBMCs, with no significant degradation after 12 h at room temperature or 6 months at –80°C, nor in postprocessing samples left in the autosampler (4°C) for 24 h. 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 measured MPA $C_{0C}$ concentrations were adjusted according to the number of PBMC extracted and expressed as ng $10^{-7}$ cells.

## Measurement of PBMC IMPDH enzyme activity

Measurement of IMPDH activity from lysed PBMCs was based on previously published HPLC methods for the quantification of IMPDH activity in PBMCs [16, 29]. In brief, after thawing at 4°C, the PBMC pellets were resuspended in 900 µl ice-cold Millipore water and insoluble fragments of disrupted cells were removed by centrifugation at 15 800 *g* at room temperature (RT) for 2 min. The PBMC lysate was used for protein content (20 µl) and IMPDH enzymatic activity (50 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 PBMCs was determined from the conversion of inosine 5′-monophosphate (IMP) to xanthosine 5′-monophosphate (XMP) based on methods described previously [16, 29]. Briefly, the IMPDH incubation mixture (pH 7.4) consisted of 1 mmol $l^{-1}$ IMP, 0.5 mmol $l^{-1}$ $NAD^+$, 40 mmol $l^{-1}$ $NaH_2PO_4$ and 100 mmol $l^{-1}$ KCl. The enzymatic reaction was initiated by the addition of 50 µl of the PBMC lysate to 120 µl of reaction mixture and incubated at 37°C for 2.5 h. After incubation, the reaction was terminated by adding 20 µl of 4 mol $l^{-1}$ ice-cold $HClO_4$, vortexing for 10 s, and the deproteinised solution was centrifuged at 15 800 *g* at RT for 2 min. Subsequently, 170 µl of supernatant was neutralised by adding 17 µl of 5 mol $l^{-1}$ ice-cold $K_2CO_3$, vortexing for 10 s, and storing the samples for 30 min at –80°C. After thawing and centrifugation at 15 800 *g* at RT 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 µmol $l^{-1}$, 250 × 3 mm; Phenomenex, Lane Cove, NSW, Australia) maintained at 45°C on an Agilent HPLC system, with two mobile phases: A) 50 mmol $l^{-1}$ potassium phosphate ($KH_2PO_4$) and 7 mmol $l^{-1}$ TBA buffer (pH 5.5); and B) 100% MeOH. The mobile phases were pumped at a flow rate of 0.7 ml $min^{-1}$ using a semigradient programme of: 94% A and 6% B for 0–13.0 min; 80% A and 20% B for 13.1–23.0 min; and 95% A and 5% B for 23.1–40.0 min. Injection volume was 25 µl with ultraviolet detection at a wavelength of 254 nm.

Specificity was tested in control incubations containing IMP in the absence of cosubstrate $NAD^+$, 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 for protein concentrations up to 2.7 mg $ml^{-1}$ and time of incubation up to 200 min. IMPDH activity was expressed as XMP produced (nmol) per incubation time (h) per mg protein (nmol $h^{-1}$ mg $protein^{-1}$).

## Data analyses

Normality of data distribution was assessed by the D'Agostino and Pearson omnibus normality test. Correlations between MPA $C_{0C}$, $C_{0P}$ and $C_{0u}$ concentrations and IMPDH activity were assessed using a Spearman's rank correlation ($r_s$). Differences in MPA $C_{0C}$, $C_{0P}$ 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 $C_{0C}$ concentrations or IMPDH activity to predict graft rejection. Associations between MPA $C_{0C}$, $C_{0P}$ and $C_{0u}$ concentrations, and IMPDH activity, with the severity of rejection (no evidence of rejection, subclinical or borderline rejection, and severe cellular or vascular rejection) were assessed using Kruskal–Wallis tests (with Dunn's *posthoc* for multiple comparisons). 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, donor-specific HLA antibodies post-transplant, type of 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, USA). Differences between MPA $C_{0C}$, $C_{0P}$ and $C_{0u}$ concentrations, and IMPDH activity, with the severity of rejection were also analysed using Jonckheere–Terpstra tests (SPSS, version 19, IBM, Armonk, NY, USA). All data are presented as either mean ± standard deviation for continuous parametric data, median (range) for continuous nonparametric data or frequencies (absolute numbers) for categorical data. Statistical significance was considered for *P*-values <0.05.

Multivariate analyses were performed using *R* [30], with false discovery rate (FDR) adjusted *P*-values <0.05 considered

significant. A step-up logistic regression model with step-wise addition of factors was used to compare current TDM practice using blood TAC and MPA $C_{0P}$ with MPA $C_{0C}$, as predictors of rejection. In addition, a step-up linear model of multivariate regression with step-wise addition of factors was used to investigate associations between recipient MRP2 phenotype, plasma albumin concentration, MPA $C_{0P}$ and MPA $C_{0C}$.

### Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY [31] and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 [32, 33].

## Results

### Patient characteristics

Patient demographic and biological characteristics are shown in Table 1. Thirty-nine patients (81%) were Caucasians and nine were Indigenous Australians. Sixteen patients (33%) experienced graft rejection during this study at an average ± standard deviation (range) of 12 ± 5 days (5–22 days) post-transplantation. There were no significant differences ($P \geq 0.15$) between the rejection and no rejection groups with respect to recipient and donor ages, sex, ethnicity, HLA mismatches, CIT, PRA, AT1R antibody levels, serum concentrations of creatinine, albumin or bilirubin, or trough whole blood ($C_{0B}$) TAC concentrations (Table 1). TAC doses had been adjusted according to TDM practice and, at the time of study, only four patients had concentrations below the recommended therapeutic range. No MMF dosage adjustment had been performed and all subjects received 2 g $day^{-1}$.

### ABCC2 polymorphisms and inferred MRP2 phenotype

Allele and genotype frequencies for the study cohort are shown in Supporting Table S1. Genotype frequencies did not deviate from Hardy–Weinberg equilibrium ($P \geq 0.1$). Eight *ABCC2* haplotypes were identified (Table S2), with the four most common being H1 CGC (44.3%, WT), H12 TGT (21.5%, low protein expression), H2 CAC (14.7% high protein expression) and H9 CGT (12.4%, low protein expression). The frequencies of patients with inferred high, moderate and low expressor phenotypes was 12.5, 48 and 39.5%, respectively.

## Table 1

Demographics and biological characteristics of 48 renal transplant recipients

| | All ($n$ = 48) | Rejection[a] group ($n$ = 15) | No rejection group ($n$ = 33) | $P$-value |
|---|---|---|---|---|
| **Pretransplant** | | | | |
| **Sex (male/female)** | 25/23 | 8/7 | 17/16 | 0.99 |
| **Ethnicity (Caucasian/Indigenous Australian)** | 39/9 | 12/3 | 27/6 | 0.99 |
| **Recipient age (years)** | 50.5 (20–69) | 42 (28–65) | 52 (20–69) | 0.15 |
| **Donor age (years)** | 48 (17–74) | 41 (18–71) | 50 (17–74) | 0.24 |
| **HLA mismatches** | 4 (0–6) | 4 (2–6) | 4 (0–6) | 0.45 |
| **PRA** | 0 (0–90) | 0 (0–3) | 0 (0–90) | 0.18 |
| **CIT (h)** | 11 (3–34) | 11 (4–26) | 11 (3–34) | 0.70 |
| **AT1R antibody level (U $l^{-1}$)** | 9 (0–40) | 11 (0–28) | 9 (0–40) | 0.61 |
| **Donor graft (living/deceased)** | 12/36 | 5/10 | 7/26 | 0.48 |
| **Post-transplant** | | | | |
| **DSA (present/absent)** | 13/35 | 4/11 | 9/24 | 0.99 |
| **DGF (yes/no)** | 19/29 | 5/10 | 14/19 | 0.75 |
| **Serum creatinine (μmol $l^{-1}$)**[b] | 150 (56–965) | 130 (58–913) | 159 (56–965) | 0.75 |
| **Albumin (g $l^{-1}$)**[b] | 32 (27–43) | 32 (27–43) | 32 (28–43) | 0.55 |
| **Bilirubin (μmol $l^{-1}$)**[b] | 8 (3–25) | 7 (3–25) | 8 (4–23) | 0.50 |
| **TAC $C_{0B}$ (μg $l^{-1}$)**[b] | 7.5 (2.5–23.6) | 6.7 (2.5–14.2) | 7.7 (4.3–23.6) | 0.23 |

[a]Rejection status within ±2 days from sample collection
[b]At same time as MPA sample collection
Data are given as median (range) or frequencies (absolute numbers) depending on data type
AT1R, angiotensin II type-1 receptor; CIT, cold ischaemia time; DGF, delayed graft function; DSA, donor-specific HLA antibodies; HLA, human leucocyte antigen; PRA, panel reactive antibodies; TAC $C_{0B}$: trough whole blood tacrolimus concentrations

### *Correlations between MPA $C_{0C}$, $C_{0P}$ and $C_{0u}$ concentrations, and IMPDH activity*

MPA $C_{0C}$ concentrations ranged from 0.1 to 3.9 ng $10^{-7}$ cells (median = 0.68 ng $10^{-7}$ cells). The corresponding MPA $C_{0P}$ concentrations ranged from 0.45 to 6.5 mg $l^{-1}$ (median = 2.1 mg $l^{-1}$), with 19 (40%) below, 17 (35%) within and 12 (25%) above the notional therapeutic range. Pre-dose IMPDH activity ranged from 0.9 to 33.9 nmol $h^{-1}$ $mg^{-1}$ protein (median = 11.9 $nmol^{-1}$ $h^{-1}$ mg protein), similar to previous studies [16, 17]. There was no correlation between MPA $C_{0C}$ and $C_{0P}$ concentrations ($P = 0.055$, Table 2), and no correlations between predose IMPDH activity and either $C_{0C}$ ($P = 0.066$) or $C_{0P}$ concentrations ($P = 0.64$, Table 2). There was no effect of inferred MRP2 phenotype on either $C_{0C}$ or the ratio of $C_{0C}/C_{0P}$ ($P > 0.43$, data not shown).

MPA $C_{0u}$ plasma concentrations ($n = 34$) ranged from 1.0 to 166.4 μg $l^{-1}$ (median = 19.2 μg $l^{-1}$) and were significantly correlated with MPA $C_{0C}$ and MPA $C_{0P}$ ($P = 0.013$ and $< 0.0001$, Table 2, Figure 1) concentrations, but not with predose IMPDH activity ($P = 0.878$, Table 2).

### *Relationships between rejection and MPA $C_{0C}$, $C_{0P}$ and $C_{0u}$ concentrations, and IMPDH activity*

In the rejection group ($n = 15$), 11 (73%) patients developed severe cellular or vascular rejection, and four (27%) patients were classified as subclinical or borderline at the time of protocol or for-cause biopsies. There was no difference in MPA $C_{0P}$, $C_{0u}$ or predose IMPDH activity between recipients with or without rejection ($P > 0.197$, Table 3). However, median MPA $C_{0C}$ were 59% lower in recipients with rejection compared to those without rejection ($P = 0.029$), and there was a statistically significant concentration-effect relationship between MPA $C_{0C}$ and the severity of rejection (Jonckheere–Terpstra trend test, $P = 0.015$, Table 3, Figure 2).

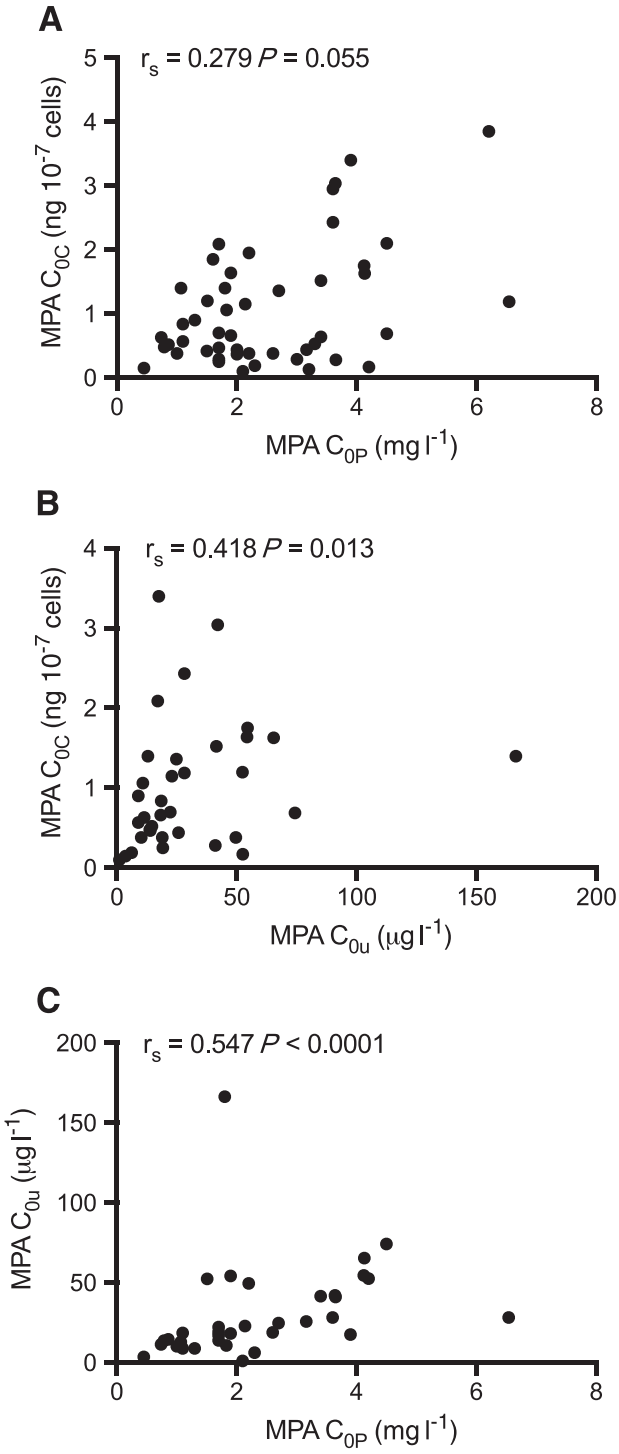
ROC curve analyses were performed to provide threshold data for predicting the risk of rejection using MPA $C_{0C}$. The ROC area under the curve (AUC) for the prediction of all rejection using MPA $C_{0C}$ concentrations was 0.70 ($P = 0.03$), with a threshold of 0.55 ng $10^{-7}$ cells providing 70% sensitivity, 67% specificity and a likelihood ratio of 2.09 (Figure 3A). The ROC AUC for the prediction of severe (cellular/vascular) rejection using MPA $C_{0C}$ concentrations was 0.75 ($P = 0.013$; Figure 3B), with a $C_{0C}$ threshold of 0.5 ng $10^{-7}$ cells providing 73% sensitivity and specificity, and a likelihood ratio of 2.68.

## Table 2

Spearman rank correlation coefficients ($r_s$) and *P*-values for mycophenolic acid (MPA) pharmacokinetic variables and predose inosine monophosphate dehydrogenase (IMPDH) activity

| | $C_{0P}$ | $C_{0u}$ | IMPDH |
|---|---|---|---|
| $C_{0C}$ | $r_s = 0.279$<br>$P = 0.055$ | $r_s = 0.418$<br>$P = 0.013$ | $r_s = -0.267$<br>$P = 0.066$ |
| $C_{0P}$ | | $r_s = 0.638$<br>$P < 0.0001$ | $r_s = -0.070$<br>$P = 0.636$ |
| $C_{0u}$ | | | $r_s = -0.027$<br>$P = 0.878$ |

$C_{0C}$, trough PBMC MPA concentration; $C_{0P}$, trough plasma MPA concentration; $C_{0u}$, trough unbound plasma MPA concentration; IMPDH, predose PBMC IMPDH activity




## Figure 1

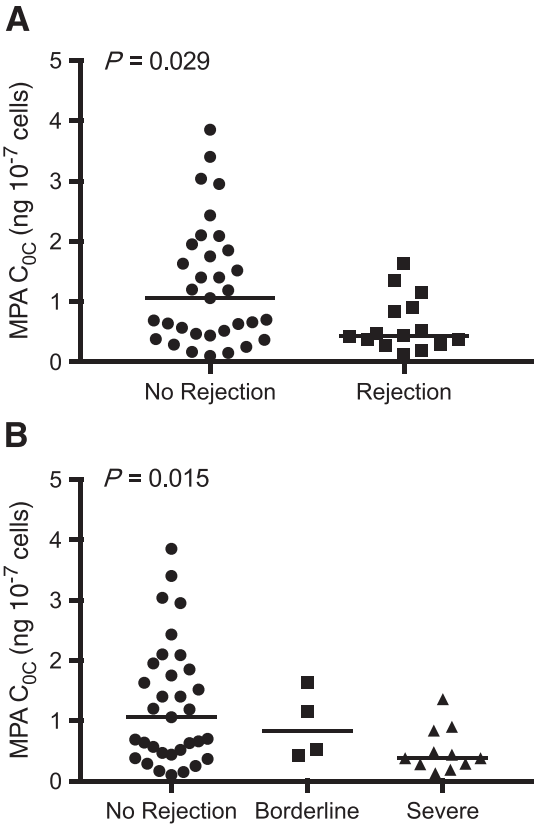
Spearman rank correlations between: (A) trough plasma mycophenolic acid (MPA) concentrations ($C_{0P}$) and predose MPA PBMC concentrations ($C_{0C}$); (B) trough plasma unbound MPA concentrations ($C_{0u}$) and $C_{0C}$; and (C) $C_{0P}$ and $C_{0u}$ in 48 renal transplant recipients

## Table 3

Median (range) mycophenolic acid (MPA) pharmacokinetic parameters and predose inosine monophosphate dehydrogenase (IMPDH) activity in renal transplant recipients with no, all, borderline and severe rejection

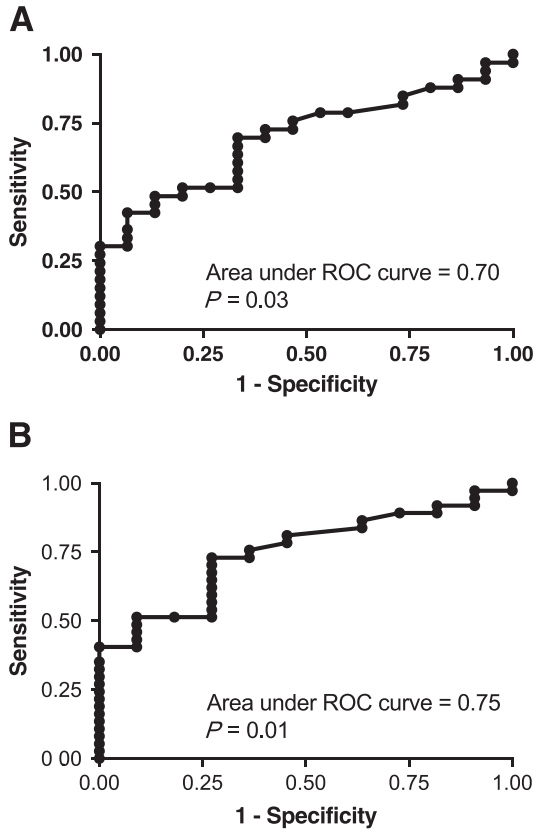
| | No rejection | All rejection | Borderline rejection | Severe rejection | *P*-value No *vs.* all rejection (Mann–Whitney) | *P*-value No, borderline, severe (Jonckheere–Terpstra) |
|---|---|---|---|---|---|---|
| **$C_{0C}$ (ng $10^{-7}$ cells)** | 1.06 (0.10–3.85) ($n$ = 33) | 0.44 (0.13–1.64) ($n$ = 15) | 0.84 (0.42–1.64) ($n$ = 4) | 0.38 (0.13–1.36) ($n$ = 11) | 0.029 | 0.015 |
| **$C_{0P}$ (mg $l^{-1}$)** | 2.20 (0.45–6.54) ($n$ = 33) | 2.00 (0.79–3.65) ($n$ = 15) | 2.02 (1.5–3.3) ($n$ = 4) | 2.00 (0.79–3.65) ($n$ = 11) | 0.197 | 0.177 |
| **$C_{0u}$ (μg $l^{-1}$)** | 19.2 (1.0–166.4) ($n$ = 25) | 20.7 (6.2–54.3) ($n$ = 10) | 38.6 (22.9–54.3) ($n$ = 2) | 16.1 (6.2–49.6) ($n$ = 8) | 0.583 | 0.324 |
| **IMPDH (nmol $h^{-1}$ $mg^{-1}$)** | 10.6 (0.9–33.8) ($n$ = 33) | 13.7 (3.4–33.9) ($n$ = 15) | 10.6 (3.4–22.3) ($n$ = 4) | 13.9 (5.6–33.9) ($n$ = 11) | 0.197 | 0.151 |

$C_{0C}$, trough PBMC MPA concentration; $C_{0P}$, trough plasma MPA concentration; $C_{0u}$, trough unbound plasma MPA concentration; IMPDH, predose PBMC IMPDH activity




## Figure 2

Comparison of mycophenolic acid $C_{0C}$ (ng $10^{-7}$ cells) with (A) all rejection and (B) severity of graft rejection. Lines indicate median values. *P*-values are shown for Mann–Whitney (A) and Jonckheere–Terpstra test for trend (B)




## Figure 3

Receiver operating characteristic (ROC) curves using MPA $C_{0C}$ concentrations for the prediction of (A) all rejection and (B) severe (cellular/vascular) rejection

### *Multivariate analyses of pharmacokinetic variables associated with rejection, and PBMC MPA concentrations*

Multivariate logistic regression analysis demonstrated that MPA $C_{0C}$ ($P$ = 0.011, FDR-adjusted $P$ = 0.033) was the only significant independent predictor of rejection with lower MPA $C_{0C}$ predicting rejection with a ROC AUC of 0.72. In contrast, there was no association between rejection and TAC $C_{0B}$ (FDR-adjusted $P$ = 0.395) or MPA $C_{0P}$ (FDR-adjusted $P$ = 0.129), used for current TDM. With regard to prediction of MPA $C_{0C}$, the final multivariate regression model incorporating only MPA $C_{0P}$, predicted 19% of variability in MPA $C_{0C}$ (FDR-adjusted $P$ = 0.003). There was no significant effect of recipient MRP2 phenotype (FDR-adjusted $P$ = 0.691) or plasma albumin concentrations (FDR-adjusted $P$ = 0.482) on MPA $C_{0C}$. Since there was a strong association between $C_{0P}$ and $C_{0u}$, and there were several missing $C_{0u}$ values, $C_{0u}$ was not tested as a predictor of $C_{0C}$ in the multivariate model.

## Discussion

There has been considerable effort to understand the relationship between MPA pharmacokinetics and pharmacodynamics, in an attempt to reduce the risk of rejection after renal transplantation. As for other immunosuppressants (TAC or CsA), MPA concentrations in PBMCs may better predict efficacy compared to blood/plasma concentrations [9–11], as they may better reflect intralymphocyte immunosuppressant concentrations. This study investigated differences in the associations between MPA $C_{0C}$ or IMPDH activity and graft rejection, versus MPA $C_{0P}$ and graft rejection.

The primary outcome of this study supports our hypothesis that obtaining PBMC MPA concentrations may provide greater prediction of graft rejection compared to measuring trough MPA concentrations alone, as there was a concentration–effect relationship between MPA $C_{0C}$ and severity of rejection, but no relationship between MPA $C_{0P}$ and rejection. In addition, multivariate analysis confirmed that MPA $C_{0C}$ was the dominant pharmacokinetic factor predicting rejection in our study population, who were already receiving TDM for trough blood TAC concentrations.

In the multivariate analysis, MPA $C_{0P}$ was the only significant pharmacokinetic variable associated with MPA $C_{0C}$. However, it only explained 19% of the variability in MPA $C_{0C}$; consistent with no correlation observed between MPA $C_{0C}$ and $C_{0P}$. The lack of prediction of graft rejection by MPA $C_{0P}$ concentrations is consistent with previous findings in renal transplant recipients also receiving TAC [34, 35], and suggests that MPA $C_{0P}$ concentrations may not be the best predictor of rejection. The current MPA therapeutic range is derived primarily from plasma total AUC data [3, 4], which is a better predictor of rejection than trough plasma concentrations. However, full AUC monitoring in clinical practice is impractical (requiring intense sampling during a 12 h dosing interval), labour-intensive and costly. Alternatively, a Bayesian population forecasting model [36] may have been more suitable to predict individual MPA exposures and should be compared to $C_{0C}$ in future studies.

Although univariate regression analysis showed a weak correlation between MPA $C_{0C}$ and $C_{0u}$ concentrations, $C_{0u}$ was not a significant predictor of rejection in univariate analyses. This was surprising as it is unbound MPA which exerts pharmacological effects [37] to inhibit IMPDH activity. Nonetheless, this observation is in agreement with previous findings [38, 39]. It is possible that the smaller number of recipients for whom $C_{0u}$ (73% of cohort) were available may have resulted in a type II statistical error. Alternatively, it is possible that carrier-mediated MPA uptake into or efflux out of PBMCs complicates the relationship between $C_{0u}$ and unbound $C_{0C}$ (not measured in this study). MPA is a substrate of the efflux pump, MRP2 [14], so that *ABCC2* polymorphisms affecting the expression and/or function of MRP2, may be a source of the variability observed in MPA $C_{0C}$. For example, the *ABCC2* CAC (-24C/1249A/3972C) haplotype is associated with significantly higher MRP2 expression and activity [26], and may therefore modulate MPA immunosuppressive efficacy. However, in this study, recipient *ABCC2* haplotypes were not significantly associated with MPA $C_{0C}$, which may therefore reflect a weak expression of MRP2 in PBMCs [15], or a subtle effect of *ABCC2* genetics that is not detected by our small sample size. MPA is also a substrate for P-glycoprotein [40] that is similarly expressed in PBMCs [41]. Determination of differences in the expression and/or function of this transporter may also help explain some of this MPA pharmacokinetic variability.

This study also investigated the potential for IMPDH activity in PBMCs as a useful biomarker, as it may correlate more closely to the biological response of MPA than plasma concentrations. Although predose IMPDH activity was not a significant predictor of rejection, our relatively small sample size could have resulted in a type II statistical error. However, in a study of 101 renal transplant recipients, Sombogaard *et al*. similarly found no association between post-transplant predose IMPDH activity and acute rejection [20]. It may be that the time at which IMPDH activity is assessed is important. Glander *et al*. reported that patients with high pretransplant IMPDH activity (hence requiring greater inhibition post-transplant) had a 3.6–fold higher incidence of graft rejection compared to patients with low pretransplant IMPDH activity [17]. In addition, many studies have demonstrated that minimum IMPDH activity occurs shortly after MMF administration, coinciding with peak plasma MPA concentrations, followed by recovery of IMPDH activity by 3.5–11 h, to predose levels [13, 16, 19, 21]. Thus, our study may have been limited using only a single predose measurement taken 5–22 days post-transplantation. Consequently, the measurement of IMPDH activities at multiple time points, which we were not able to perform in this study, may be needed to further elucidate the predictive nature of this factor.

Measurement of IMPDH messenger RNA (mRNA) has also been investigated as a possible predictor of rejection, particularly to differentiate between the two types of IMPDH isoforms: type I, which is expressed in all cell types; and type II, which is expressed only in activated lymphocytes [2]. In isolated human T lymphocytes, both IMPDH type I and type II mRNA is increased following activation, correlating with increased total IMPDH activity [42]. However, like the measurement of IMPDH activity, the relationship between

IMPDH mRNA expression and rejection is not clear. One study in renal transplant recipients found that low post-transplant expression of both type I and II mRNA was associated with acute rejection, although there was no correlation between total IMPDH activity and type I or II mRNA expression [20], whilst a later study reported an association between rejection and high pretransplant expression of both type I and II mRNA [18].

In conclusion, the results from our study suggest that MPA $C_{0C}$ may represent an additional tool for individualization of MMF dose following renal transplantation. Its clinical application may be most practical as a single test during the early post-transplantation period, when the risk of developing graft rejection is highest, and when protocol or for-cause biopsies are also usually performed.

## Competing Interests

There are no competing interests to declare.

*The authors would like to acknowledge the work of the following people: Prof G. Russ, Ms T. East, Ms D. Spellacy, Drs C. Hope and A. Fuss, transplant surgeons and clinical staff at the Central and Northern Adelaide Renal and Transplantation Service, Adelaide SA, for their assistance with blood and tissue sample collection; and Prof A. Vinks and Ms S. Cox at the Cincinnati Children's Hospital Medical Center, Cincinnati OH, for their help in setting up the IMPDH assay. Z.I.M.D. was the recipient of an Endeavour Postgraduate Scholarship funded by the Australian Government.*

## Contributors

Z.I.M.D., patient recruitment, acquisition of data, analyses and interpretation; contribution to study design, drafting of manuscript. J.K.C., patient recruitment, data analysis and interpretation, drafting and critical review of manuscript. R.P.C., data analysis and interpretation, critical review of manuscript. J.T., data analysis and interpretation, critical review of manuscript. B.C.M., acquisition of data, analysis and critical review of manuscript. A.A.S., data interpretation, drafting and critical review of manuscript. B.C.S., study conception, design and coordination, data interpretation, drafting and critical review of manuscript.

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## Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

http://onlinelibrary.wiley.com/doi/10.1111/bcp.13704/suppinfo.

**Table S1** Recipient allele and genotype frequencies (%) for the *C-24 T*, *G1249A* and *C3972T* SNPs of *ABCC2*
**Table S2** *ABCC2* haplotype frequencies (%) in 48 kidney transplant recipients