PHARMACOLOGICAL APPROACHES TO PRESERVE RENAL GRAFTS AND TO OPTIMIZE IMMUNOSUPPRESSION IN RENAL TRANSPLANTATION

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Renal transplantation has evolved as the best therapeutic option for patients with end stage renal diseases. Several risk factors such as ischemia reperfusion injury (IRI), delayed graft function, under or over-immunosuppression and drug toxicity can affect the renal graft function and survival. This work focused on pharmacological approaches to address two of the modifiable risk factors associated with early post-transplant graft survival, namely IRI and optimization of immunosuppression. The first part of work evaluated supplementation of the renal cold preservation solution with treprostinil to attenuate IRI injuries due to its vasodilatory, anti-platelet aggregatory and cytoprotective properties. Isolated rat kidneys were stored at 4°C for 24 h with or without treprostinil and reperfused *in vitro* to mimic post-transplant conditions. Cold stored kidneys showed a significant loss in renal function and treprostinil addition (20 ng/mL) to preservation significantly improved the filtration fraction, urine flow and showed a trend to increase the anionic and cationic tubular secretion. Treprostinil addition to storage and reperfusion attenuated the IR induced changes in certain gene expression, indicating protection against the IR induced effects and the need for a follow-up of its effects in an *in vivo* transplant setting. The second part of this work examined early (week 1) post-transplant pharmacologic measures in association with incidence of rejections (clinical and subclinical) at week-13 and infections at month-12 in renal transplant patients. The pharmacologic measures for exposure and efficacy to mycophenolic acid (MPA) and exposure to tacrolimus were obtained by sparse sampling. Patients with lower exposure to both drugs tended to have higher incidence and odds of rejection, when

compared to patients who had optimal exposure to both tacrolimus and MPA (46 vs 23 %, NS, odds ratio of 3.0; p =0.3414 for rejections). A composite scoring using pharmacologic measures such as the total 12 h exposure to MPA, tacrolimus, IMPDH activity and genotype of p-glycoprotein on peripheral blood mononuclear cells revealed an increasing incidence of rejections in patients with higher scores. This observation emphasizes potential role for early post-transplant monitoring using sparse sampling in individualized therapy of kidney transplant patients, in order to further improve overall outcomes.

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ABBREVIATIONS

°C	Degree Celsius
µ	Micrometer
µg	Microgram
µg*h/mL	Microgram per hour per millilitre
µl	Microliter
µls	Microlitres
µmol	Micro moles
AAG	alpha acid glycoprotein
ABCB1	ATP Binding cassette, sub-family B, member 1
ACN	Acetonitrile
ACR	Acute Clinical rejection
actinβ	Beta actin
ALT	alanine aminotransferase
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
API	Atmospheric pressure ionization
AST	Aspartate aminotransferase
ATN	Acute tubular necrosis
ATP	Adenosine tri phosphate
AUC	Area under the curve
bcrp	Breast Cancer Resistance Protein
BKV	BK virus
BPAR	Biopsy proven acute rejection
Br-AMP	8-bromoadenosine 5-monophosphate
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CI	Confidence Interval
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration
CL	Clearance
CL/F	Apparent total clearance of drug after oral administration
CMV	Cytomegalovirus
Co	Concentrations at time zero or Trough concentrations
CO_2	Carbon di oxide
Cox-2	cyclooxygenase 2
CPIC	Clinical Pharmacogenomic Implementation Consortium
cPRA	calculated panel reactive antibody values
CS	Cold Storage

Cxcl-10	C-X-C motif chemokine 10
cyp	Cytochrome P 450 enzyme
cyp24	Cytochrome P 450 family 24
cyp27b1	Cytochrome P 450 family 27 subfamily b member 1
CYP3A4	Cytochrome P450 Family 3 Subfamily A Member 4
CYP3A5	Cytochrome P450 Family 3 Subfamily A Member 5
d3-MPA	Deuterated Mycophenolic acid
DGF	Delayed graft function
DNA	Deoxy ribo nucleic acid
DPBS	Dulbecco's phosphate buffered saline
DPEC	Diethyl pyrocarbonate
DSA	Donor specific antibodies
DV	Dependant variable
eGFR	estimated glomerular filtration rate
ERCC	External RNA Controls Consortium
ES	Electron spray
FDA	Food and Drug Administration
FDR	False discovery rates
fM	Femtomoles
FR	Fractional reabsorption
f_u	Fraction unbound
g	Gram
G	Gauge
g/Dl	Grams per decilitre
gapd/gapdh	Glyceraldehyde 3-phosphate dehydrogenase
GFR	Glomerular filtration rate
glut2	Glucose transporter 2
h	Hours
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
ICAM-1	Intracellular adhesion molecule 1
IFN-gamma	Interferon gamma
IFTA	interstitial fibrosis and tubular atrophy
Il - 1beta	Interleukin 1 beta
IL-2	Interleukin 2
Il-6	interleukin 6
Il-8	interleukin 8
IMPDH	Inosine 5' monophosphate dehydrogenase
IPRED	Individual predictions
IPRK	Isolated Kidney Perfusion System
IR	Ischemia reperfusion
IRB	Institutional Review Board
irip	Ischemia reperfusion injury protein

IV	Intravenous
KH_2PO_4	Potassium dihydrogen phosphate
KHSB	Krebs Henseleit buffer with sodium bicarbonate
kim-1	Kidney injury molecule 1
L	Liter
L/h	Liters per hpur
LCMS/MS	Liquid chromatography mass spectrometry
log	Logarithm
LR	Linear Regression
LSS	Limited sampling strategy
M	Molar
MAP	maximum a posteriori
mapk14	Mitogen activated protein kinase 14
mate1	Multidrug and toxin extrusion 1
mate2k	Multidrug and toxin extrusion 2 splice variant
Mcp-1	Monocyte chemoattractant protein 1
MDAPE	median absolute prediction error
MDAPPE	Median absolute percentage prediction error
MDR1	Multidrug resistance protein-1 or P-glycoprotein
min	Minute
mL	Milliliter
mm	Millimeter
mM	Millimolar
MMF	Mycophenolate mofetil
MnSOD	Manganese superoxide dismutase
mol	Molecule
MPA	Mycophenolic acid
MPAG	Mycophenolic acid glucuronide
MPE	Mean prediction error
MRM	Multiple reaction monitoring
mRNA	Messenger ribonucleic acid
mrp4	Multi-drug resistant protein type 4
MS/MS	Tandem mass spectometry
n	Number of observations
N_2	nitrogen
Na	Sodium
Na/K-ATPase	Sodium Potassium Adenosine triphosphatase
NCA	Non-compartmental analysis
Nfk- beta	Nuclear factor kappa Beta
ng	Nanogram
ng.day/mL	Nano gram per day per millilitre
ng*h/mL	Nanogram per hour per millilitre
nhe3	sodium hydrogen exchanger

nm	Nanometer
NONMEM	Nonlinear mixed effect modeling
NPDE	normalized distribution of prediction errors
O_2	Oxygen
oat1	Organic anion transporter 1
oat3	Organic anion transporter 3
oct 2	Organic cation transporter 2
OD	Optical density
p65	Transcription factor p65
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase Chain Reaction
PD	Pharmacodynamics
PE	Polyethylene
pept1	Human Peptide Transporter 1
PGI2	Prostacyclin
pgk1	phosphoglyserate kinase 1
P-gp	P-glycoprotein
PK	Pharmacokinetics
PPAR	Peroxisome proliferator-activated receptors
pvcVPC	prediction and variability-corrected visual predictive check
qc	Quality control
RBC	Red blood corpuscles
RCC	Reporter code count (of Nanostring)
R_{CL}	Renal clearance
ring1	Ring finger protein 1
RNA	Ribo Nucleic Acid
RNase	Ribonuclease
ROS	Reactive Oxygen Species
rpm	Revolutions per minute
RT	Room temperature
RT-qPCR	Real time quantitative polymerase chain reaction
s	Seconds
SCR	Subclinical rejection
sd	Standard deviation
SD	Sprague Dawley
SNP	Single nucleotide polymorphism
Sod2	Superoxide dismutase
TCA	Trichloro acetic acid
TCMR	T Cell mediated rejection
TDM	Therapeutic Drug Monitoring
TNF-alpha	Tumor necrosis factor alpha
TR	Tubular reabsorption
UFR	Urine flow rate

ugt/UGT	Uridine 5'-Phospho glucuronosyltransferase
ugt1a1	Uridine 5'-Phospho glucuronosyltransferase family 1-member a1
urat1	Urate Transporter 1
UW	University of Wisconsin
V/F	Apparent volume of distribution of drug after oral administration
v/v	Volume by volume
Vdr	Vitamin D Receptor
w/v	Weight by volume
WRES	Weighted residuals
XMP	Xanthosine 5' monophosphate
$ZnSO_4 \cdot 7H_2O$	Zinc sulphate

1.0 INTRODUCTION

1.1 BURDEN OF RENAL DISEASE

Chronic kidney disease (CKD) is a major health problem with a global prevalence rate of around 11-13 % [1], when patients at all stages (1-5) of the disease are considered. The staging of kidney function is indicative of the progression in kidney disease and is based on an assessment of the renal function (estimated glomerular filtration rate- eGFR) and kidney damage (albumin creatinine ratio ACR)[1,2]. The normal healthy adult kidney has an eGFR around 120 ml/min/1.73m^2 with an ACR of < 3 mg albumin per mmol of creatinine. The respective cut-off values for the staging for eGFR expressed in "ml/min/1.73m^2" and ACR expressed in "mg/mmol" are: Stage-1 (eGFR>90 with ACR>30), Stage-2 (eGFR 60–89 with ACR>30), Stage-3 (eGFR 30–59), Stage-4 = (eGFR 29–15) and Stage-5 (eGFR<15). As of early 2016, the number of patients in United States reported with end stage renal disease (ESRD, Stage 5) was around 7 million[3]. ESRD primarily results from diabetes (37-46 %), hypertension (23 %), glomerulonephritis (14 %), cystic kidney (9 %) and other unknown reasons (18 %)[4,5]. Patients with ESRD often require "renal replacement therapy" that is offered by periodic dialysis or with renal transplantation. Renal transplantation is considered the best treatment choice for patients with ESRD.

1.2 RENAL TRANSPLANTATION

Renal transplantation offers survival advantage for patients with ESRD, with the risk of death being reduced by less than half in comparison to being maintained on dialysis[6]. In the long term, transplantation is also more cost effective with better quality of life than maintenance on dialysis, even after adjusting for differences in the quality of the transplanted kidney[7]. However,

due to the larger number of patients with ESRD relative to the number of available kidneys for transplantation, there is a long waiting list prior to someone receiving a kidney transplantation. Renal transplant data for 2018 shows that, out of an estimated active waiting list of around 65,500 patients with ESRD, only around 21,167 received a kidney transplant[8,9]. This included 6442 kidneys provided by living donors (30 %) with the remaining 70% coming from cadaveric donors[9]. Unlike in few other countries, the majority of transplanted kidneys in United States are obtained from deceased donors. With new patients constantly being added to the waiting list and with the growing demand for transplantable kidneys, the optimal use of cadaveric kidneys by increased utilization of marginal donors as well as decreasing the number of discarded kidneys, have all been pursued as viable alternatives[10]. As such kidneys are more susceptible to ischemia reperfusion injury (IRI), strategies aimed at preventing or minimizing IRI would be beneficial for post-transplant graft and patient survival. On the other hand, with higher number of transplants, the successful maintenance of good organ function over time is also of great importance[4,10] and requires careful optimization of immunosuppression in individual patients.

1.3 STATEMENT OF THE PROBLEM

Improving the long-term patient outcome is an ultimate goal in transplantation, as there are several immunological and non-immunological risk factors in the donor as well as the recipient that can affect the renal graft function and survival. Few of the risk factors are modifiable, such as ischemia reperfusion injury, delayed graft function, avoiding under or over-immunosuppression (rejection or infection), non-adherence and calcineurin inhibitor toxicity (Figure 1.1). The research work in this dissertation addresses two of the modifiable risk factors that can affect early graft

survival and benefit the recipients of kidney, namely ischemia reperfusion injury and optimal immunosuppression to avoid rejections and infections.

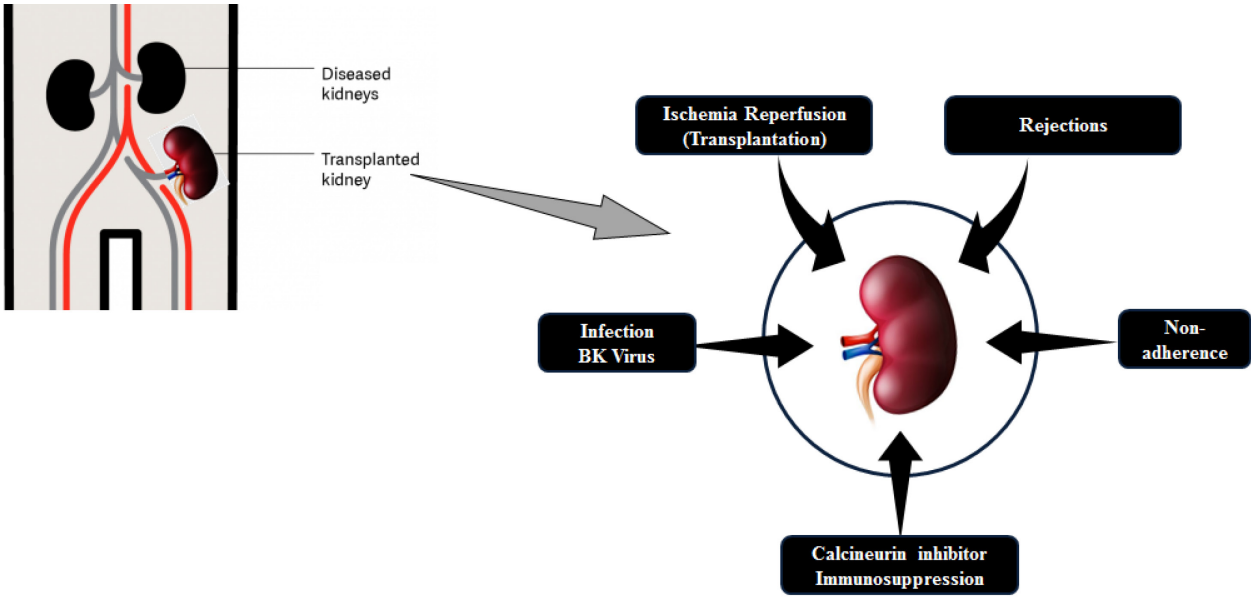


Figure 1.1 Factors that can alter the function and survival of renal allografts

1.3.1 Ischemia reperfusion injury

Ischemia reperfusion (IR) injury is an inevitable consequence of transplantation when a kidney is subject to sequential procedures like, isolation from donor, absence of blood flow, preservation in cold solution until transplantation and warm reperfusion with recipient blood during implantation. Reperfusion associated injury does not occur during ischemia, but during reperfusion at the time of implantation of kidney in the recipient. IR injury is also a known risk factor for acute kidney injury, delayed graft function (DGF) or acute and chronic rejection after transplant[11-13]. Hence, improving the cold preservation as well as attenuating injury during reperfusion offer potential opportunities to promote graft survival and decrease patient morbidity after renal transplant.

1.3.2 Acute cellular rejections

Renal transplant recipients receive immunosuppression with tacrolimus and mycophenolate mofetil (MMF) for prevention of graft rejection. Early after transplantation, the occurrence of rejection (acute) is confirmed on an indication biopsy, prompted by changes in graft function. However, silent (subclinical) rejections can occur without measurable changes in graft function and are only detected during a screening (protocol) biopsy. The occurrence of acute cellular rejections (clinical/subclinical) have been associated with chronic allograft injury[14,15] and reduced graft survival[14-16]. The presence of inflammation during early protocol biopsy, has also been associated with progression of fibrosis at 1 year[17] with an elevated risk for poor long-term outcomes[18]. The initial post-transplant graft conditions can thus impact the long-term graft survival but can be modified by providing optimal immunosuppression to the patients. Hence understanding the relationship of early acute cellular rejections (clinical + subclinical) to the combined immunosuppression exposure (tacrolimus + MPA) and to the efficacy of MPA (inhibition of IMPDH activity) at early post-transplant, will help in optimizing immunosuppression in individual patients.

1.3.3 Infections

Infectious complications remain a significant cause of morbidity and mortality in renal transplant patients. Immunosuppression predisposes renal transplant recipients to a variety of infections of bacterial, viral, fungal or parasitic origin from either the donor or nosocomial as well as community sources. Most infections are reported during the first year after transplantation with approximately 70% of the infections occurring within 3 months of transplantation[19]. In transplant

patients, apart from the exposure to infection, the main risk factor for infections is the dose, duration or the "intensity" of immunosuppression therapy[20]. Observational evidence has indicated that long-term graft survival have not improved in renal transplantation and this was attributable to the side effects related to the level of immunosuppression including opportunistic infections such as BK or CMV or JC virus[21].

It has been suggested that cadaveric kidney recipients are predisposed to infectious complications on account of increased immunosuppression needed due to higher HLA mismatching[22]. The risk of infections are also shown to vary with the immunosuppressive agent used. The initial induction therapy with lymphocyte depleting agents like thymoglobulin was shown to increase the risk of CMV viral infection in renal transplant patients[23]. Further, higher blood levels of tacrolimus or plasma levels of mycophenolic acid were also associated with risk of infections. For tacrolimus, a blood level above 10 ng/ml and a dose greater than 0.15 mg/kg/day prevented acute cellular rejections, but was shown to decrease renal function and increase infectious disease in first 30 days after transplantation[24]. Similarly, higher MPA exposure (AUC $\geq$ 60 µg*h/mL) was statistically associated with increased odds of viral infections, namely CMV and BKV viremia, and increased the risk of neutropenia[25]. The occurrence of CMV infection or BK nephropathy are associated with development of rejection and graft loss, independent of reduction of immunosuppression [26].

So, a delicate balance in immunosuppression is required as too little suppression can be associated with rejection and graft loss, while overimmunosuppression can lead to infections and malignancy in the long term. Hence, monitoring of immunosuppression after renal transplantation is necessary to prevent infectious complications.

1.4 BACKGROUND

1.4.1 ISCHEMIA REPERFUSION INJURY

1.4.1.1 Cold storage of donor kidneys

Kidneys isolated from donors are subject to warm ischemia owing to the lack of blood supply. The ischemia leads to a series of noxious events like ATP depletion, loss of the Na^+- K^+ pump, elevated hypoxanthine levels, increase in cytosolic calcium levels, cell swelling, and most organs lose complete function beyond 30-60 min[27]. To prevent progression of ischemic damage and cell death, immediately after isolation, kidneys are flushed in-situ at a rapid rate with a preservation solution, followed by rapid cooling to 4 °C. It has been shown that during hypothermia the demand for oxygen goes down by as much as 97% in comparison to renal perfusion at body temperature[28]. Energy dependent (ATP) metabolic reactions are almost halved for every 10º C decrease in temperature[29]. Thus, a prolonged ischemia is possible with cold storage and enables transport of the kidney to the recipient located elsewhere. The early organ preservation solution (Collins) had high concentrations of glucose and electrolytes resembling the normal physiological status[30]. The Collins solution was later improved to enable longer duration of cold storage as well as organs other than kidney with the formulation of University of Wisconsin solution (UW)[31]. Some of the improvements in UW solution included replacing glucose with an impermeant like lactobionate, along with raffinose and hydroxyethyl starch which prevented hypothermia induced cellular swelling, minimized acidosis and improved osmotic support[31]. Allopurinol and glutathione were included to suppress superoxide anions generated by xanthine oxidase during IR as well as from hypoxanthine, a breakdown product of adenosine present in UW solution.

1.4.1.2 Metabolic changes during cold storage

Static cold storage (SCS) of kidneys is still widely used due to simplicity and convenience when compared to machine perfusion that requires specialized perfusion devices. The hypothermia during static cold storage slows down the capacity of cells to supply energy, affects stability of proteins and inhibits enzymatic reactions that utilize cellular stores such as ATP, leading to metabolic uncoupling[32]. During hypothermia with hypoxia, oxidative phosphorylation becomes ineffective, since oxygen is not available for ATP generation and in addition, adenosine diphosphate cannot penetrate mitochondrial membrane due to the deactivation of the enzyme adenosine diphosphate translocase during hypothermic storage[8].

Hence, renal cells tend to produce ATP by anaerobic glycolysis, which in fact, is less efficient than aerobic glycolysis and leads to intracellular accumulation of lactic acid, further initiating noxious events like mitochondrial dysfunction and activation of lysosomal enzymes[33,34]. The presence of buffers in the preservation solution can help to counteract this acidosis. Consequent to the shortage of ATP, a functional loss of Na -K-ATPase activity occurs which can then lead to intracellular entry of sodium and chloride along with water leading to cellular edema[33]. Hence, presence of an impermeant like lactobionate or mannitol in preservation solution is essential to counteract this edema.

The rapid depletion of ATP is also associated with deactivation of calcium sodium exchange system, leading to massive influx of calcium into cytosol following the influx of sodium. Subsequent changes in the calcium-calmodulin complex can also generate mitochondrial and membrane dysfunction, damaging the phospholipid nature of these structures by activating the phospholipase pathway with production of prostaglandin derivatives[34], which can eventually affect the endothelial cells.

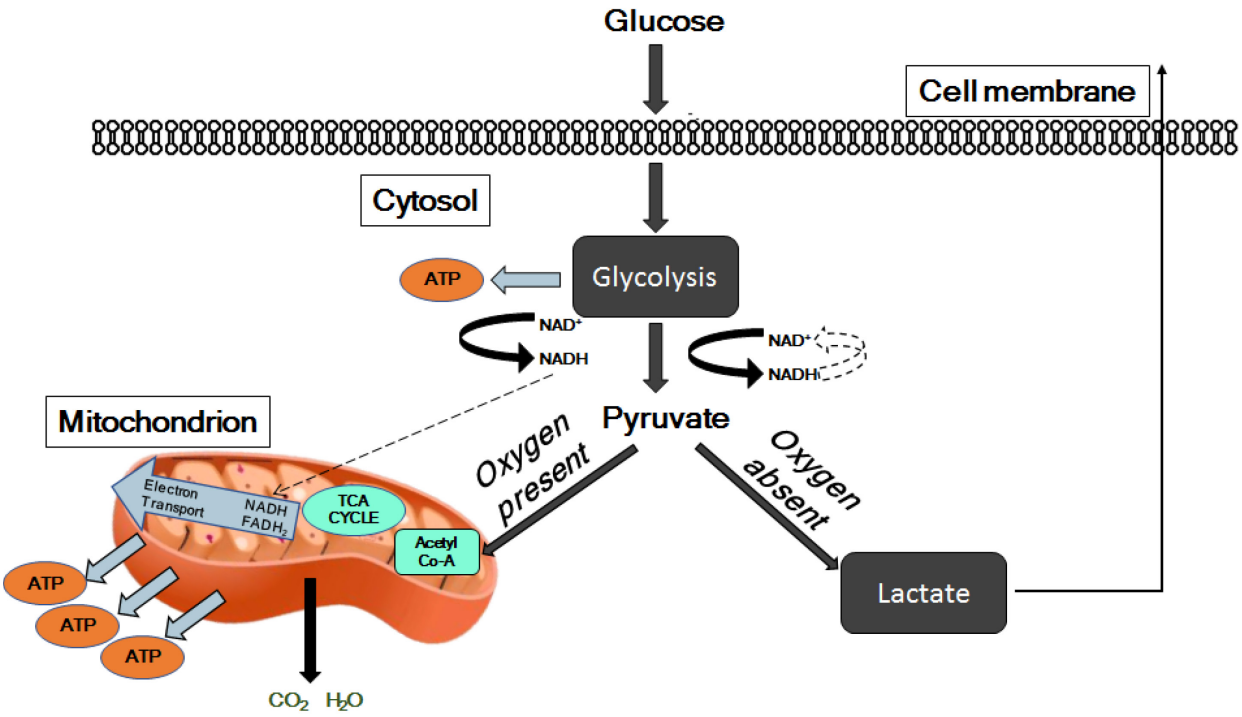


Figure 1.2. Anaerobic (oxygen absent) glycolysis during hypothermic preservation

1.4.1.3 Reperfusion injury

Reactive oxygen species (ROS)

Cold stored kidney is reperfused with warm recipient blood during implantation. Ironically, though reperfusion is critical for restoring the renal function, most of the damaging effects of IR injury occur and are manifested during and after reperfusion. IR injury is a complex and multi-factorial pathophysiological process that causes significant metabolic, structural and functional damage to the organ. The sudden increase in the availability of oxygen in an acidic environment with the accumulation of hypoxanthine during ischemia, triggers production of xanthine and superoxide anion by xanthine oxidase. The superoxide produced from oxygen introduced during reperfusion, can further react with itself to produce hydrogen peroxide that can yield hydroxyl radicals which can impair the cellular function by oxidizing cell membranes and proteins[34,35]. In addition, ROS can trigger the opening of mitochondrial membrane transition pores subsequent to

the increased mitochondrial calcium content due to hypoxia and can lead to cell death[36]. The ROS can trigger adhesion of leukocytes to renal vascular endothelium by production of mediators like platelet activation factor which can lead to migration of neutrophils and macrophages. Neutrophils can cause proteolytic damage to the kidney leading to microcirculatory disturbances and mediate local tissue injury.

Innate and adaptive immune response

Consequent to free radical and neutrophil mediated tissue injury there is a release of cellular injury factors and damage associated molecular patterns (DAMPs). DAMPs can activate toll like receptors (TLRs) that can further recruit molecules within cytoplasm to activate transcription factors like NFKB, MAP kinase and Interferon I pathways [37]. The TLRs are usually expressed on immune cells such as macrophages, neutrophils, dendritic cells but are also expressed by the injured renal tubular cells and mesangial cells during IRI [38].

The transcription factor NFKB plays a central role in both the tubular epithelial cells and inflammatory cells in generation of an inflammatory response by activating pro-inflammatory factors such as IL-1β, tumor necrosis factor (TNF)-α, or interferon (IFN)-γ, chemokines such as IL-8, MCP-1 [39] and genes related to cell adhesion and growth control [38].

Vascular components of IRI

Endothelial cells play an important role in vascular tone and leukocyte function and with an injury there is an enhanced vasoconstriction leading to small vessel occlusion. IR leads to endothelial cell swelling, loss of glycocalyx, breakdown of the actin cytoskeleton and fluid leak into the interstitium [40]. Further, activation of endothelial-leukocyte interactions and coagulation results in local compromise of the microcirculation and regional ischemia, especially in the outer renal medulla [12]. Hence during reperfusion, though cortical circulation is established,

hypoperfusion still persists in the outer part of medulla, characterized by congestion of peritubular capillaries with swelling of the endothelial cells, increased paracellular permeability and increased expression of adhesive molecules, such as intercellular adhesion molecule 1 (ICAM-1), E- and P-selectins and leukocyte accumulation with platelet aggregation[39]. Excessive platelet aggregation and release of platelet-derived mediators can exacerbate tissue injury following ischemia and reperfusion. The proximal tubular injury also results in an inadequate sodium reabsorption with more solute delivery to the distal nephron leading to tubulo-glomerular feedback in reducing the forces for filtration [12].

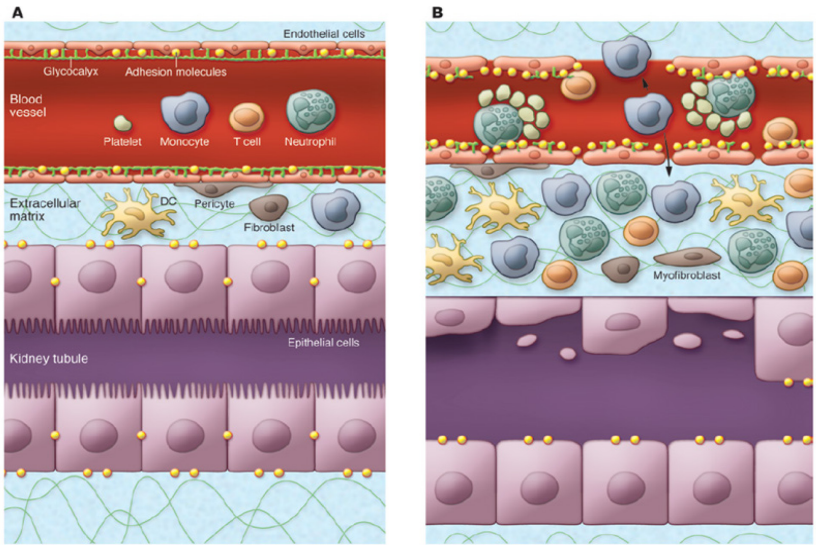


Figure 1.3. Renal endothelium, interstitum and tubule in normal kidney (A) and after ischemia reperfusion (B)
[With copyright permission from Bonventre and Yang[12]]

1.4.1.4 Post-transplant impact of Ischemia-Reperfusion Injury

Delayed graft function (DGF)

Delayed graft function in the immediate post-transplant period is a consequence of IR injury, primarily due to tubular dysfunction but may also be due to enhanced graft immunogenicity, dysfunction and rejection [40]. DGF is defined as the failure of the transplanted

kidney to function immediately, hence necessitating renal replacement therapy within the first week after transplant surgery. Previous studies have identified extended cold ischemic time (time between cessation of donor circulation and vascular reperfusion in the recipient) as an independent risk factor for DGF [5]. ECD kidneys from old donors are also at a higher risk of DGF after IR [41]. Hence, there is a great interest in strategies for improved preservation of allograft during cold storage in order to prevent DGF. The relationship of DGF with acute rejections of kidney is controversial, however, the combined occurrence of DGF with T-cell mediated rejection or graft inflammation is reported to worsen graft survival with poor outcomes[42,43].

Immunogenicity of the allograft

The IR mediated injury can augment graft immunogenicity subsequent to the activation of adaptive immunity and thereby increases the possibility of immunologic recognition and graft rejection [11]. Following IRI, apoptotic cells are not rapidly cleared after the initial injury and secondary necrosis occurs due to loss of membrane integrity leading to the release of endogenous adjuvants (damage-associated molecular patterns-DAMPs). Inflammatory DAMPs, including Heat-Shock proteins (HSP), HMGB-1 and DNA fragments, are recognized by specific receptors such as TLR-2 and TLR-4 which, in turn, trigger inflammatory and cytotoxic responses. The massive release of pro-inflammatory cytokines, such as IL-1α and tumor necrosis factor (TNF), from damaged cells during ischemia-reperfusion injury causes donor dendritic cells to become activated and migrate from the donor graft to the recipient lymph nodes and spleen, thus sensitizing recipient T-cells which can recognize donor MHC molecules on the surface of donor antigen-presenting cells (APCs), leading to an immune response.

Renal transporters

Renal clearance is a predominant pathway of drug elimination which includes three concurrent processes occurring in the nephron, viz. glomerular filtration, tubular secretion, and tubular reabsorption. Glomerular filtration is a passive process while tubular secretion, and sometimes reabsorption, involves a variety of transporters located on the basolateral and luminal membranes of the tubular epithelium. These transporters are predominantly expressed in the proximal tubule and work in tandem to eliminate drugs from the blood circulation to the urine and to reabsorb essential substrates. Both basolateral and apical transporters tend to be charge selective for anionic and cationic drugs, although studies indicate some degree of overlap. The major renal drug transporters engaged in the secretion of anionic drugs include organic anion transporters 1 and 3 (hOAT1 and hOAT3), multidrug resistance-associated proteins 2 and 4 (hMRP2 and hMRP4) and breast cancer resistance protein (BCRP). The transporters for cationic drugs include organic cation transporter 2 (hOCT2), multidrug and toxin extrusion proteins 1 and 2-K (hMATE1 and hMATE2-K) and P-glycoprotein (P-gp) (facilitate excretion of larger and more hydrophobic cations)[44]. In addition, several closely related transporters/co-transporters like urate anion transporter (URAT), sodium dependent glucose transporter (SGLT), monocarboxylate transporter (MCT), renal sodium dependent dicarboxylate transporter (NADC) and sodium-hydrogen antiporter (NHE) are present in the proximal tubules that contribute to renal handling of endogenous substrates and metabolic wastes.

After IR, morphological changes can occur in the proximal tubules, including loss of polarity, loss of brush border as well as redistribution of integrins and Na/K-ATPase to the apical membrane. Impaired Na^+ reabsorption during IR is due to dysregulation of the basolateral Na^+/K^+ ATPase, leading to disruption of the concentration gradient for apical Na^+ uptake. This could have

been due to the impaired mitochondrial function with a decrease in ATP synthesis after IR, as the activity of Na^+/K^+-ATPase per se was shown not to be affected[45]. Further, IR induced inhibition of several Na^+ dependent cotransporters can impair reabsorption of glucose and influx of metabolic substrates like alpha keto glutarate and succinate[46].

Ischemia mediated renal injury has been shown to decrease clearance of the organic anion transporter substrate para-aminohippurate (PAH) for at least 7 days after transplantation. The delayed elimination of PAH was shown to result from downregulation of *oat1* and *oat3* after IR in preclinical studies. The mRNA and protein expression levels of organic anion transporters, oat1 and oat3 were suppressed in a rat model of IR and was accompanied by significant elevation in serum levels of indoxyl sulfate, a uremic toxin that is a substrate of the above anion transporters[47]. Transporter expression, tubular function and usually GFR can recover within a few days after IR. In addition to IR, inflammation and immune activation due to complications associated with renal transplantation may down regulate renal anionic transporters and this effect may be more pronounced in renal allografts with BK virus nephropathy or TCMR, as shown by decreased renal secretion of a OAT1/3 and MRP2/4 substrate, cidofovir in allografts with BK virus nephropathy[48].

1.4.1.5 Studies with ex-vivo supplementation to preservation solution

The improvement in organ preservation after retrieval and attenuation of reperfusion injury are two primary objectives for the supplementation of preservation solution with pharmacological agents. Due to the multifactorial nature of IR associated injury, several approaches targeting different pharmacological pathways have been attempted to improve the organ preservation. Examples include targets involved in inflammation, coagulation, oxidative stress, mitochondrial protection, apoptosis and the hypoxia response following an ischemia–reperfusion sequence[49].

Polyethylene glycol (PEG) is a polymer of ethylene oxide with a hydroxyl terminal having oncotic properties and can bind to phospholipids on cell membranes. Addition of PEG 35 was shown decrease ROS, improve renal cell viability and preserve the ATP content of tissue in porcine kidneys. Supplementation of Trimetazidine, an anti-ischemic agent into UW or EC solutions was shown to improve function of the reperfused rat and pig kidneys. Further in a pig transplant model, trimetazidine was shown to decrease DGF, due to its effect of preserving Ca^{2+} homeostasis and mitochondrial function during cold storage[50]. Supplementation of the UW solution with a unique combination of trophic factors like Substance-P, IGF-I, nerve growth factor and bactenecin resulted in marked suppression of cold ischemic injury in an experimental dog kidney transplant model, possibly due to the preservation of graft ATP content during cold storage. Melatonin is an endogenous antioxidant, also shown to mitigate the pathological effects of IRI in experimental models of supplementation to UW solution. Melatonin was shown to effectively preserve rat kidney for up to 48 h with minimal production of malondialdehyde, suggesting its antioxidant effect.

Preservation trials have also been carried out in presence of alternate gases like carbon monoxide (CO), xenon, hydrogen sulphide (H_2S). Xenon was shown to upregulate HIF-1 alpha and decrease histologic changes with improvement of graft function in lewis rat model[51]. Though CO is not an antioxidant by itself, it can induce the expression of antioxidant genes like superoxide dismutase or catalase, but due to its unfavorable effect on hemoglobin, other synthetic CO releasing molecules (CORM) have been evaluated. Supplementation of CORM to UW solution was shown to improve renal perfusion and glomerular filtration rate. Hydrogen sulfide (H_2S) showed protective effects as supplementation to UW solution in both syngeneic and allogeneic

models of rat kidney transplantation, due to its modulatory effects on inflammation and oxidative stress[52].

1.4.1.6 Prostacyclins

Prostacyclin (PGI_2), is endogenously produced by vascular endothelial and smooth muscle cells and has a potent vasodilator and anticoagulant effect. PGI2 mediates its biological effects through binding to cell surface prostacyclin receptors (IP), coupled to stimulatory G protein and increases the intracellular cyclic adenosine monophosphate (cAMP) signaling, which acts as a second messenger to inhibit platelet aggregation, cell proliferation and inflammatory mediator release. The IP receptor is located on a variety of cells, including platelets, vascular smooth muscle, and endothelial cells, where PGI2 has local effects. At the endothelial level, prostacyclin exerts anti-inflammatory and anti-platelet activity and promotes an antithrombotic surface, which is required for proper function and maintenance of vascular integrity.

Treprostinil (Remodulin®) is a chemically stable analog of prostacyclin (PGI2), with potent pulmonary and systemic vasodilation and anti-platelet aggregation effects along with cytoprotective properties. Treprostinil is approved by the U.S. Food and Drug Administration for the treatment of pulmonary artery hypertension (PAH). Treprostinil has improved stability and has a longer elimination half-life of around 4 h. Treprostinil has a favorable pharmacological profile for activity against several pathways that are known to be activated during IR. Treprostinil has antioxidant and anti-inflammatory effects, decreases intracellular calcium levels leading to vascular smooth muscle relaxation, inhibits platelet aggregation and downregulates pro-inflammatory cytokines. Hence, it may favorably decrease ischemic effects by restoring blood flow and minimize damaging effects of reactive oxygen species and inflammatory mediators, during reperfusion. Previous studies have shown treprostinil to attenuate the significant decrease

in ATP content of 24 h cold preserved livers and decreased hepatic IR injury in an orthotopic rat liver transplant model[53]. With favorable pharmacological profile and encouraging previous evidence, treprostinil was chosen for ex vivo addition to cold preservation solution as well as for treatment during reperfusion of rat kidney in an isolated rat kidney perfusion model.

1.4.2 IMMUNOSUPPRESION IN RENAL TRANSPLANTATION

1.4.2.1 Induction and maintenance

Renal transplant recipients require chronic immunosuppression to prevent rejection of the transplanted kidney, excepting in some rare instances of donor being the identical twin. The initial therapy (induction) is usually started before or immediately after transplantation, with a biological agent like a lymphocyte depleting agent (anti-thymocyte globulin, Alemtuzumab) or an interleukin 2 receptor antagonist (basiliximab, daclizumab) to prevent T-cell immune response against the graft at the time of implantation. Induction therapy is completed within the first few days after transplantation.

Maintenance immunosuppression is a chronic therapy usually with one or a combination of several drugs with different mechanisms of action to avoid the rejection of kidney graft and to ensure optimal graft function. Currently, four classes of drugs are available for maintenance immunosuppression, viz. corticosteroids (prednisone), calcineurin inhibitors (cyclosporine, tacrolimus), antimetabolites (azathioprine, mycophenolic acid) and target of rapamycin inhibitors (sirolimus, everolimus). A carefully monitored dosing of immunosuppressive drug is essential to balance the risk of rejection with that of the side effects. The most notorious adverse post-transplant events (e.g., infection, malignancy) are associated with the net state of immunosuppression; hence it is essential that maintenance immunosuppression be gradually

decreased after the initial post-transplant period and then maintained in an optimal range to avoid rejections as well as adverse events. In renal transplant patients, the current standard maintenance immunosuppressive therapy includes tacrolimus with mycophenolic acid with steroids being optionally used based on risk factors in the patient.

1.4.2.2 Tacrolimus

Tacrolimus (FK506) is a 23-membered macrocyclic lactone that binds to a cytosolic immunophilin (FK binding proteins 12 and 52). The complex of TAC–FK binding protein inhibits the phosphatase activity of calcineurin and interrupts the calcium-dependent signal transduction pathway in T-cells to produce immunosuppressive effect. The pharmacokinetic parameters of tacrolimus are highly variable among patients. Much of the variability is due to the pre systemic metabolism by cytochrome P450 (CYP) 3A4/3A5 isoenzymes and efflux transport by P-glycoprotein (P-gp) in the intestinal mucosa. Tacrolimus has rapid absorption (peak concentration occurring at 0.5 – 1 h) from the gastrointestinal tract, but has a poor oral bioavailability ranging between 5 - 93% [54] and the bioavailability is decreased by food [55].

Tacrolimus is primarily distributed within the erythrocytes and is approximately 83 % (range 75-95 %) bound to the erythrocytes. Due to this extensive binding to red blood cells, transplant patients have a significantly higher blood tacrolimus concentrations (mean 15-fold; range 4 to 114-fold) than the corresponding plasma concentrations. Binding of tacrolimus to erythrocytes may be due to an affinity for a protein similar to FKBP present within the erythrocytes and this binding could protect tacrolimus from hepatic metabolism. Around 16 % of tacrolimus is reported to be bound to plasma components such as lipoproteins, α-acid glycoprotein, albumin and globulin [56]. On an average, around 0.6 % of tacrolimus in blood is associated with the lymphocyte fraction with the remaining 0.4 % being the unbound or free fraction.

The lymphocytes are the proposed site of action of tacrolimus, and also are reported to show a high variability in the percent tacrolimus content (0.1 – 1.5%). It was also reported that tacrolimus lymphocyte content is significantly less in liver transplant patients experiencing rejection in comparison with stable recipients (0.3-0.5 vs 0.8 %, p=0.012)[56]. It was further proposed that pulse steroid doses in patients with rejection, could impact on the expression of MDR1 and CYP 3A enzyme in the lymphocytes, contributing to the variability in lymphocyte distribution of tacrolimus[56]. In transplant patients, the inherent variability in the activity of MDR1 expressed on the lymphocytes, based on genotype of patients, can further limit tacrolimus entry into the lymphocyte and cause differences in immunosuppression despite identical whole blood concentrations.

The whole blood clearance (CL) of tacrolimus also shows high interindividual variability and ranges from 3–35 L/h. Tacrolimus is primarily metabolized by CYP3A4/3A5 isozymes in the liver with at least 15 metabolites, with the major metabolite being 13-O-demethyl-tacrolimus. Many factors have been identified to be associated with the variability in tacrolimus pharmacokinetics, with the CYP3A5 being the most prominent with carriers of *1 allele requiring almost a two-fold higher dose[57]. In addition, CYP3A4*22 has also been shown to influence tacrolimus dose requirements and pre-dose concentrations in white renal transplant recipients, though to a lesser extent than CYP3A5[58]. Other factors include age, gender, body mass index, adherence, drug-interactions, to name a few. In renal transplant recipients, a good correlation was reported between CL and hematocrit (r=0.8) as well as albumin (r=0.7) and oral steroid doses (r=0.9) over the first 12 weeks post-transplant[59]. Biliary excretion and fecal elimination are responsible for the major clearance of the metabolites of tacrolimus, whereas renal excretion plays

only a small role [55]. The terminal half-life of tacrolimus in kidney transplant recipients is approximately 15.6 h but can range between 8 - 19 h[54,60].

Exposure to tacrolimus and outcomes in renal transplant patients

The Area under curve ($AUC_{0\text{-}12\ h}$) is generally considered as the ideal marker of drug exposure but it is difficult to obtain in patients due to practical and cost considerations. Since tacrolimus has a narrow therapeutic index and has a high inter-individual variability, monitoring trough whole blood concentrations is recommended in all transplant patients, as trough levels show a good correlation with total exposure. Ideally, target concentrations of 8-12 ng/mL are desired in the first 3 months post-renal transplant but the target concentrations are lowered beyond that time point[61,62], based on empirical observations, to maintain efficacy with a good allograft function and at the same time decreasing the adverse effects.

The correlation between trough and AUC for tacrolimus is not always consistent and is reported to be better in the earlier post-transplant phase than at later time period[62,63]. C_0 of tacrolimus, is still considered as a surrogate for the AUC. The use of trough (C_0) blood concentrations to adjust tacrolimus dose is based on historical observations on the association between a target C_0 and clinical outcome of acute rejections. In an early investigative trial for C_0 of tacrolimus, decreasing rejection was observed with increasing C_0 while increasing toxicity occurred beyond a limit, thereby a range of 5-15 ng/mL was proposed during the first 7 weeks post-renal transplant[64]. Multiple studies have shown lower tacrolimus trough levels at first week, or at discharge or at first month and during first 6 weeks post-transplant to be significantly associated with higher incidence of biopsy proven acute rejections (BPAR) [54,65]. A significant relationship between tacrolimus trough concentrations and the development of rejection in the first month after transplant was also shown with median concentration of 9.2 ± 3.5 ng/ml in non-

rejectors versus 5.1 ± 1.2 ng/mL in patients with BPAR [65,66]. Another study[67] also derived an effective target C_0 of 9.3 ng/mL on day 5 post-renal transplantation and C_0 values below the cut-off were associated with a shorter graft survival. Beyond the initial 3 months, tacrolimus target goal is decreased in stable renal transplant patients, and a study[68] showed association of time-varying tacrolimus levels with acute rejections, as there was a 7% increase in the risk of rejection for every unit decrease in average tacrolimus trough levels during the first 6 months post-transplant.

In contrast to the above studies, few other studies did not show a significant association between C_0 and rejection [69,70]. One of the reason could be the evolution of tacrolimus target trough concentrations, that has slowly seen a shift from higher concentrations of around 20 ng/mL in earlier years of use to lesser concentrations during recent times mainly with co-administration with another immunosuppressant drug, mycophenolic acid[71]. Further, the target C_0 associated with avoiding rejections were comparatively lower in some studies performed at later post-transplant periods, owing to the lack of steroid co-therapy. Overall, during the first year post-transplant, on co-therapy with mycophenolate, steroids and anti-IL-2 induction therapy, a target C_0 between 5-10 ng/mL during the first year could be considered as the therapeutic range for tacrolimus[62]. In contrast to rejections, tacrolimus trough concentration have been shown to be significantly, but not consistently, higher in recipients with an incidence of infection in week 6 post-transplant, though the mean daily drug dose did not differ between the patient sub-groups[72].

Though trough blood concentrations (C_0) of tacrolimus are routinely used in TDM for clinical practicality, the association of C_0 with the true exposure ($AUC_{0\text{-}12\ h}$) as well as the clinical outcomes has been a matter of debate[62,63,73,74]. The relationship of tacrolimus exposure to clinical outcome has also been investigated in several studies. It has been shown that patients experiencing

rejections have lower tacrolimus AUC during the early post-transplant period [75]. An AUC exceeding 200 ng*h/mL was suggested for tacrolimus during the early post-transplant period for avoiding the risk of acute rejections[75,76]. Another study had shown exposures of 210 ng*h/mL and 125 ng*h/mL as optimal limit for tacrolimus, until the first 6- and 52-weeks post-transplant, respectively [73]. In another study of 100 de novo renal transplant recipients, patients with tacrolimus AUC below 150 ng*h/mL and MPA AUC below 45 µg*h/mL on day 7 post-transplant, tended to have higher incidence of rejections (26 %) in comparison to patients that reached both target values (8 %) [72]. The authors also reported significantly higher AUC in patients with incidence of CMV or herpes infections during the first 6 weeks post-transplant (average 197.4 vs 160.5 ng*h/mL).

Abbreviated exposure methods for tacrolimus

Even after vast clinical experience with tacrolimus, there is a lack of clear target AUC that can be used during both early as well as late phase of renal transplantation and due to a lack of prospective studies focusing on targeting a specific AUC in relation to outcomes. In addition, performing a total exposure study is too tedious, hence calculation of AUC based on a limited number of blood samples strategy (LSS), is a possible alternative. Bayesian estimation is an analytical technique that allows the use of previous information in a population in conjunction with observed data in individual patients to estimate the most likely value of pharmacokinetic parameters of interest in those patients. On the other hand, linear regression (LR) based methods can be used to derive equation from many drug concentration measurements within a steady-state interval in several patients. Initially a reference AUC is determined by the trapezoidal method, followed by multiple regression analysis of all combinations of time points to identify the combination with the best correlation between the estimated AUC and the reference AUC.

The Bayesian estimation using LSS sample concentrations provides more clinical flexibility in time points than the regression method which is simpler yet practically difficult to implement due to strict restrictions on the sampling point deviations. In general, with a compromise between accuracy of LSS and clinical feasibility it is preferable to limit the clinical sampling times to less than 4 hours post dosing.

Several studies have reported on the estimation of AUC using Bayesian approach with an appropriate pharmacokinetic model for tacrolimus derived in kidney transplant patients and a review [77] of these methods revealed satisfactory performance in the estimation of tacrolimus AUC. The nature of studies used to develop population models for tacrolimus differed over a wide range, including post-transplant days ranging from immediate to several years after transplantation and sample size ranging from 12- 262 patients, with almost 80% of studies having less than 100 patients. Further, retrospective data collection and the use of trough concentrations were widely utilized in tacrolimus model developments. The structural model mostly used one and two compartment models with a first order elimination. The most common covariates identified for tacrolimus whole blood CL/F were the post-transplant time, CYP3A5 genotype, hematocrit, body weight and Aspartate aminotransferase.

The application of Bayesian estimation in clinical study also ranged from early to several years post-transplant, however a majority were within the 1 year of transplant. The D-optimality based selection of time points that could in theory give more valuable information on exposure were used for the LSS and studies identified 0, 1 and 3 h or 1,3 and 6 h or 1,3 and 8 h or 0, 1.5 or 4 h as the optimal time points, where 0 is the steady state trough (12 h) concentration. The combination of time points at pre-dose, 1 and 3 h post-dose were found to consistently show lower bias and imprecision values (< 15 %)[77], when considered across the different transplant

populations. Despite developments in LSS application for AUC estimation, there are no reports on their regular application for TDM of tacrolimus, excepting a few reports of unpublished inhouse applications. These reports do show the feasibility of LSS in accurate estimation of total AUC for tacrolimus, which is required for assessing various clinical outcomes.

1.4.2.3 Mycophenolic acid

After oral administration, MMF is rapidly absorbed and undergoes hydrolysis by esterases into the active mycophenolic acid (MPA). MPA is 98-99% bound to plasma proteins, mainly to albumin[78]. The major route of metabolism of MPA involves glucuronidation to the pharmacologically inactive MPA 7-O glucuronide (MPAG), by the hepatic UGT1A9 in addition to UGT1A8, UGT1A1, UGT1A7 and UGT1A10 in intestine and kidneys[79]. MPAG is approximately 80% bound to serum albumin and is excreted by the kidney via glomerular filtration and tubular secretion. MPAG is subjected to enterohepatic recirculation mediated by multidrug-resistant protein-2 (MRP-2) and can be converted back to MPA in the gastrointestinal tract. MPA is also metabolized to a minor active acyl-glucuronide (AcMPAG) metabolite, primarily by UGT2B7.

Mycophenolic acid blocks DNA synthesis or replication by non-competitive and reversible inhibition of ionosine monophosphate-5′-dehydrogenase (IMPDH) types 1 and 2 and thereby inhibiting proliferation of T and B-lymphocytes. MPA is five times more potent as an inhibitor of the type II isoform of IMPDH, which is expressed in activated T and B lymphocytes, than of the type I isoform, that is expressed in most cell types[80]. Hence, MPA suppresses both cell and antibody-mediated responses, which are major factors in acute and chronic allograft rejections.

Variability in clinical PK of Mycophenolic Acid

A range of clinical factors are known to contribute to the large interpatient and within patient variability in MPA PK in renal transplant patients. Mycophenolic acid released from MMF has a different pharmacokinetic profile from EC-MPA (delayed release) and as such, they are not considered bioequivalent, even though the amount of active MPA (739 mg) from 1 g of MMF is approximately equal to the 720 mg released from EC-MPA. Unbound MPA is hypothesized to be responsible for MPA efficacy, however due to the cost and difficulty, determination of free MPA is not a clinically routine procedure. Further as only free MPA is subject to metabolism, and total MPA concentration may fluctuate in the absence of a change in free concentration (due to protein-binding displacement by concurrent albumin-binding drugs like MPAG or uremic toxins and hypoalbuminemia), for a low extraction drug like MPA, there is an alteration in total but not free MPA clearance and this makes it difficult to establish a therapeutic range based on total MPA.

A slowly recovering kidney graft function is observed after renal transplant, which also could differ between patients. Many studies have reported accumulation and impaired renal excretion of the metabolite MPAG or uremic toxins in patients with delayed graft function that can displace MPA from the binding with albumin, thus increasing the free MPA available for hepatic glucuronidation, during early post-transplant period. With improvement in renal function and subsequent recovery in albumin levels, MPAG excretion as well as MPA binding are increased leading to a comparative decrease in MPA clearance or an increase in total MPA exposure compared to the immediate post-transplantation, after the same dose of MMF. Hence differences in renal function recovery, albumin as well as steroid doses are factors suggested to contribute to the interpatient variability, during early post-transplant[81]. The 8 h exposure of total MPA immediately after renal transplant was around 30-50% of those in the 3-month post-transplant

period, due to impact of the above clinical variables. However, beyond 3 months, the PK of MPA after oral administration in renal transplant patients was reported to be comparable with healthy individuals[82].

In addition to the above factors, variation in the formation and disposition of the glucuronide metabolites can increase interpatient variability in MPA concentrations. MPA glucuronides are excreted unchanged in the urine, but can also undergo enterohepatic recirculation (EHR), mediated via MRP-2 mediated biliary secretion and hydrolysis by β-glucuronidase in the gut, resulting in the regeneration of a significant amount of MPA. The exposure of MPA can decrease by 30-40% when combined with cyclosporine due to its effect on the inhibition of EHR mediated MPA formation from MPAG. In addition, the variability in the metabolism and subsequent EHR process due to various polymorphisms in UGTs and MRP-2 can also contribute to variability in the plasma drug concentration-time curves of MPA[83]. Schaik et al. had shown low exposure of MPA due to high clearance in patients with genotypic variants of UGT1A9 (−275 T>A and -2152C>T), which was further associated with significantly higher odds of acute rejection in renal transplant patients (OR 13.3, 1.1–162.3; $P<0.05$)[84].

In summary, due to the variability in clinical PK of MPA in renal transplant patients, monitoring of MPA was suggested at end of first post-transplant week, 2nd week and at months 1 and 2[85].

Population pharmacokinetics of Mycophenolic Acid

The initial population pharmacokinetic (POPPK) models for MPA were published in 1999 and since then, most of the studies used parametric estimation approach, using NONMEM as the software for the non-linear mixed effects modeling. The objectives of POPPK models for MPA were to estimate typical values for PK parameters like clearance (CL), volume of distribution (V),

absorption (Ka, Tlag), in addition to determination of the between subject or within subject variability in parameters and characterizing the influence of various covariates on the PK of MPA. The models for MPA in the literature for renal as well as other transplant populations were developed after consideration of the nature of study from which MPA concentrations were obtained including the time post-transplant, range of available time points across the patients, co-administered drugs (cyclosporine versus tacrolimus), the formulation of MPA used (MMF or EC-MPS), the covariates that were available to explain variability in MPA PK, to name a few. The availability of sensitive and accurate LCMS methods to analyze concentrations of MPA and its metabolites also has an impact on the evolution of POPPK models. As a result, even within renal transplant population, the structural part of the models for MPA were evolving continually from simple 1 or 2-compartment models with first order absorption and elimination to more complex models for absorption or considering multiple compartments with inclusion of free MPA, metabolites and enterohepatic recirculation (EHR) to describe the PK of total MPA, free MPA as well as the phenolic or acyl glucuronide metabolites. Most of POPPK models for MPA have found the 2-compartment model to adequately describe the PK of MPA.

An overview of POPPK models in literature, revealed numerous attempts for characterizing the complex PK characteristics of MPA, including EHR, absorption after MMF or EC-MPS, protein binding and metabolite profile, in order to improve the estimation of parameters like CL/F and V/F. A majority of the early as well as recent population models for MPA are developed in the renal transplant patient population after dosing of MMF with the co-administered drugs, either tacrolimus or cyclosporine and steroids. Further most of models used the first order conditional estimation with or without interaction. One of the first population model for MPA by Funaki [86] was an attempt to model enterohepatic recirculation of MPA after dosing of MMF. Other

published models in early 2000s included attempts to model MPA using bi-exponential elimination with absorption lag time [87,88], zero order absorption model[89], two compartment model with first order absorption [90,91] or four compartment model including MPAG with EHR[92]. A double gamma absorption model was used by Premaud et al[93] using an alternative in-house software for describing MPA profiles in recent as well as stable renal transplant patients using a single compartment. Later POPPK studies on MPA in renal transplant patients focused more on including metabolite levels of both glucuronides [94,95], better description of EHR[96], inclusion of genotypes[97] and modeling of free MPA[98].

Examination of the population estimates for CL/F in adult Caucasian renal transplant recipients in various studies ranged between 13 to 33 L/h, with studies done at early post-transplant or with cyclosporine co-administration contributing to higher values of the CL/F [99-101]. In the same sub-set of population, estimates for the central V/F ranged between 12-98 L whenever a 2-compartment model was used to describe the profile of MPA. Considering the 20 years of POPPK characterization of MPA with models including early post-renal transplant data, factors that have repeatedly and significantly correlated with and decreased the inter-patient variability in MPA clearance or volume of distribution, included albumin concentration, creatinine clearance (renal function), cyclosporine dose and in some cases, the body weight. The most promising application of the developed POPPK models is the Bayesian prediction for dose optimization and monitoring of MPA exposure to improve the clinical outcomes.

Exposure to MPA and outcomes in renal transplantation

The objective of PK monitoring in clinical care is to ascertain appropriate drug exposure in relation to clinical endpoints or outcomes. For MPA, several studies have shown the total drug exposure (12 h AUC) to have a good correlation with the risk of acute rejection.

One of the earliest reports on association for MPA steady-state exposure to early acute rejection was reported in Japanese renal transplant patients [102], with lower rejection rates when AUC was greater than 40 µg*h/mL and higher CMV infection when AUC exceeded 90 µg*h/mL. Other reports after analysis of data from early pilot phase I trials [103,104] revealed a sigmoidal relationship of $AUC_{0\text{-}12\ h}$ in the logistic regression analysis for the probability of decreasing the acute rejections. From the fitted curve, it was further determined that, with exposure of 30 µg*h/mL, a 50% reduction in rejection could be achieved, corresponding to mean exposures after 1 g twice daily and with an exposure of 55 µg*h/mL, a 90% reduction in rejection could be achieved, corresponding to mean exposures after 1.5 g twice daily. However, after consideration of dose related adverse events like leucopenia, diarrhea, tissue invasive CMV infections, 1 g twice daily dose was considered optimal, though the concentrations of MPA and MPAG were not different tin patients with or without adverse events. When exposures were examined, the clinical outcome of acute rejections in 1 g twice daily group occurred in patients with the lowest quartile of $AUC_{0\text{-}12\ h}$, that was considered an impact of interindividual variation or non-compliance[82].

Large scale retrospective analysis of studies in renal transplant patients, further confirmed the predictive value of MPA AUC in the risk for developing acute rejections [105]. It was concluded that maintaining renal transplant patients on a target MPA AUC of 30-60 µg*h/mL can assure a less than 10% risk of acute rejections, in combination with cyclosporine and steroids[106]. Further, no gain from increasing exposure beyond 60 µg*h/mL was obtained in terms of decreasing risk for acute rejections, though an increase in frequency of side effects did occur. In another investigation of MPA exposure in 33 renal transplant patients, majority of patients with rejections had an exposure less than 30 µg*h/mL[107].

In the first combination trial with tacrolimus at University of Pittsburgh, renal transplant patients treated with MPA/tacrolimus/steroids showed a significant reduction in the incidence of rejection compared with regimen without MPA (44 vs 27 %, p=0.014), when MMF was given at 1 g twice daily[108]. In a multicentered, prospective, randomized fixed-dose concentration-controlled (FDCC) trial [109] with MMF/cyclosporine/ steroids with 150 renal transplant patients, the risk of acute rejection was increased in patients if MPA AUC_{0-12} on day 3 post-transplantation was less than 30 µg*h/mL. Again, exceeding exposure beyond the upper limit of ≥ 60 µg*h/mL did not provide a gain in efficacy but was significantly associated with increased risk of leukopenia, diarrhea and viral infections, CMV and BKV viremia. In another trial with 46 renal transplant patients, the 12 h exposure on day 5, though was not associated with rejections [110] while in 27 patients on similar therapy and time post-transplant, the 6 h MPA exposure showed significant relation with acute rejections [111]. Another prospective study, APOMYGRE, targeted AUC of 40 µg*h/mL after 1 gm twice daily, using Bayesian estimation and physician dose adjustment in comparison to fixed dosing of 1 gm twice daily to a total of 137 renal transplant recipients[112]. The target-based dose adjustment group had a higher exposure for MPA within 3 months post-transplant with a significantly lower incidence of BPAR (7.7 vs 24.6 % in the fixed dosing group). Another prospective study the fixed dose–concentration controlled (FDCC) showed significant associations of MPA AUC on day 3 with BPAR on first month as well as first year[113]. In a study of MMF with tacrolimus in 100 de novo renal transplant patients, Kuypers et al.[72] did not find any significant differences in the day 7 exposure to tacrolimus (mean of 168 vs 168 ng*h/mL) and MPA (mean of 56 vs 46 µg*h/mL) in patients with rejection versus rejection-free patients. However, a trend to increased incidence of rejections was observed in patients having a low exposure to both MPA and tacrolimus (26 % vs 8 % in high exposure for both, p>0.05).

Most of above studies confirmed relationship of MPA exposure on the risk of reducing acute rejections during the first 3 months post-transplant, while a study on cyclosporine withdrawal beyond 3 month post-transplant also showed a significantly higher risk of acute rejection in patients having subclinical rejections together with a lower MPA exposure (mean 43 vs 58 µg*h/mL)[114].

In a Japanese study with 36 patients on cyclosporine or tacrolimus co-therapy, the exposure to MPA was significantly less in patients with rejection (mean of 34.2 vs 28.2 µg*h/mL) at 2 weeks post-renal transplantation[115]. Similar significant differences in exposure of MPA (mean 41 µg*h/mL in rejectors vs 54 µg*h/mL in non rejectors) was observed in Chinese renal transplant recipients on tacrolimus or cyclosporine co-therapy and after < 1-month post-transplant [116]. Several other studies with cyclosporine co-therapy also showed significant relationship with total MPA AUC and acute rejections but the free MPA AUC was related to incidence of infections/leucopenia [117,118].

Most of the studies showed good correlation of total MPA exposure ($AUC_{0\text{-}12\ h}$) with the risk of acute rejections, but the relationship between total exposure and adverse events, including leucopoenia and gastrointestinal symptoms, is less clear. In all three prospective trials, APOMYGRE, OPTICET and FDCC trials, no correlation was observed between MPA exposure and adverse events in spite of differences in exposure, probably because of multi-causality and imprecise definitions for the adverse events[119]. However, in the absence of an upper exposure limit, a risk for resurgence of viral infections including CMV and BK virus can occur at higher exposures and lowering the dose of MMF has shown to be a viable strategy during BK infections[120]. Other reasons are also associated with MMF dose reductions though may or may not be related to exposure like, GI complications and hematologic abnormalities.

Overall, though the AUC is widely regarded as the best measure of MPA exposure, it is highly impractical to obtain blood samples until 12 h during routine clinical applications. Hence, surrogates for AUC are applied in the clinic similar to trough concentrations for tacrolimus. Similar strategies like single time point trough monitoring or alternatively collected 2-3 samples in a limited sampling design have all been attempted for MPA monitoring.

Utility of Trough concentrations of MPA

In an analysis of serial trough samples from 121 renal transplant patients, Borrows et al[25] reported significant association of lower median trough levels (C_0) to acute rejection within 1 month post-transplant. In the same study, median trough levels were also found to be higher in patients with anemia and viral infections. In the multi-centered prospective trial, the trough levels were significantly associated with acute rejections but less significantly in comparison to AUC, because of the higher variability observed in trough levels[109]. Pawinski et al[121] had investigated the effect of MPA AUC versus trough on the risk of graft rejection using receiver operating characteristic, which was 0.85 and 0.63 for the AUC and trough, respectively. A couple of studies with cyclosporine coadministration had shown significant association of trough levels with acute rejection in 22 and 48 renal transplant patients, respectively[122,123]. Yet another study[124] showed that mean MPA trough levels during the first week was significantly lower in patients who developed rejection (1.5 ± 0.1 vs 2.1 ± 0.1 µg/mL) and higher in those with gastrointestinal effects (4.1 ± 0.5 µg/mL). The MPA trough level was also shown to be associated with formation of donor specific antibodies[125]. Further, a trough level of 1.4 µg/mL was suggested to be representative of an AUC of 30 µg*h/mL that is required to prevent rejection and as well capable of 50 % inhibition of IMPDH[125]. Relationships between high MPA trough and adverse events have also been reported by some investigators. In a retrospective study of 30 renal transplant patients,

the mean MPA trough was significantly higher in individuals who experienced adverse effects like CMV, and leukopenia compared with those who did not[126].

However, a relationship between MPA trough and acute rejection had been reported by many but not all studies. However, in the prospective trials including FDCC, OPTICET and APOMYGRE, no correlation of trough concentrations to adverse events were observed, again probably because of multi-causality and imprecise definitions for the adverse events[119].

The correlation between trough concentrations and total AUC show a wide range of values (r = 0.07-0.66[81]) when compared amongst several studies in renal transplant patients, probably because of the different post-transplant periods of sample collection. Another hypothesis was that the poor correlation with AUC as well as rejection might be due to higher variability consequent to differences in enterohepatic recirculation[82]. Unlike early transplant, trough MPA concentrations may be of more utility in stable renal transplant patients, though currently, there is no recommended target range. Kuypers et al.[72] has reported trough MPA concentrations to be poorly predictive of the 12 h AUC, as despite the MPA day 7 exposure being significantly different from exposure at 12 months, the corresponding trough concentrations did not reflect the difference.

There has been no consensus in literature about the exposure measure that best correlated with adverse events or infections. In early post-transplant period, the total AUC of MPA is the most useful exposure measure as it shows a stronger relationship with risk of acute rejection than the trough concentrations. Overall in the case of rejections, single time point trough concentration has shown poor correlation and hence, other monitoring strategies that use limited sampling from first few hours and allow prediction of AUC could be more useful to improve clinical outcomes.

Limited sampling strategies to estimate MPA 12 h exposure

The lack of single time points like trough or early time points like 1, 2 h samples to effectively predict the AUC had motivated a number of studies to examine the application of limited sampling strategies (LSS) (or alternatively called sparse sampling strategies), in the estimation of the 12 h exposure to MPA and predict clinical outcomes. Two different options have been presented for consideration in studies involving LSS for MPA exposure after MMF dosing. One is the selection of time points for blood sampling and the other option is the selection of methodology to estimate exposure using MPA plasma concentrations obtained at those time points. The more convenient option for sampling time points in the clinic would be to limit 2-3 samples within the first 2-4 hours post-dosing of MMF. Similarly, the easier methodology would be to use a simple regression equation to calculate exposure by incorporating MPA concentrations determined at specific time points (linear regression method – LR). The best time points for LR are determined from full PK profile of MPA and statistical regression-based selection of the time points that best describes the full exposure.

Despite its convenience, LR is seriously limited by its practicality and thereby affecting its accuracy. The timing of sampling should be precise in order to reflect the original equation and hence it is difficult to implement in a clinical setting. A change in the covariates like cyclosporine or tacrolimus co-administration or the age of patients in comparison to the study from which the LR was derived, was shown to impair accuracy and result in poor AUC predictions [117,127]. Other issues also include, inability to calculate AUC if a single time point is missed leading to loss or exclusion of the patient.

On the other hand, the Maximum a posteriori (MAPB) bayesian estimation can estimate AUC from a limited number of time points but it requires complex calculations using a

pharmacostatistic model. The main advantage though, would be the flexibility in timing of blood sample as well as avoidance of patient exclusion due to loss of random samples, makes it a more suited methodology for clinical implementation. The MAPB method calculates the individual PK parameters for MPA based on previous MPA population parameters in combination with concentrations at the sparse time points, with other demographic or covariate information obtained from the individual patient and can be used for estimation of exposure or recommendation of dose for that patient. Hence, provision of individual information from the patients will improve the model from reliance on just the population parameters and move the estimations towards individual parameters. However, a drawback with using MAPB method for a drug with complicated profile like MPA, is the availability of an accurate model that can be more specific, like for example, to renal transplant patients at certain time periods post-transplant or with externally validated and clinically feasible time points for MPA as well as co-administered drugs like tacrolimus.

The literature provides many examples for estimation of AUC for MPA with LSS, especially in adult renal transplant patients. Initial studies developed LR time points for abbreviated AUCs using predose, 30 or 40 min and 2 h [128,129] for cyclosporine co-therapy with r^2 versus total AUC ranging from 0.74-0.86. Few studies later included 6-8 h sampling point for MPA during tacrolimus co-therapy to account for the EHR[130] however seriously limiting their clinical application. The LSS by LR method was used retrospectively to evaluate the association of estimated MPA exposure with clinical outcomes in several studies with cyclosporine co-therapy. Weber et al[117] used 0, 0.5 and 2 h to estimate MPA AUC which was shown to be associated with the acute rejections but not the adverse events. Another retrospective study by Kiberd et al [131] estimated MPA AUC using 0, 1, 2 and 4 h and showed association of exposure

with acute rejections but not the side effects. The utility of LR was further extended for therapeutic monitoring in a prospective study, by estimating samples collected at predose, 40 m and 2 h for the estimation of MPA AUCs with cyclosporine or tacrolimus[132]. The same LR estimation was also used for MPA with tacrolimus co-therapy in another prospective study with 100 de novo renal transplant patients by Kuypers et al[133] and an r^2 of 0.78 was reported with MPA AUC.

Initial Bayesian model proposed by Le gullec et al [89] used 3 samples at 20 m, 1 and 3 h along with patient body weight to estimate MPA AUC in stable renal transplant patients beyond 6 months of transplantation. The estimates were compared in a different set of patients having a full AUC and the authors reported acceptable precision (bias 7.7 %) and precision (rmse 12.4 %). In another earlier study utilizing Bayesian estimation in de novo renal transplant recipients[93], one compartment model with double gamma absorption was used with 3 samples like the earlier study. The authors reported higher bias (>20 %) at day 7 post-transplant which subsequently decreased to 10 % at month 3. Several other population models were used for Bayesian estimation in the clinic and accuracy was validated by comparison with full MPA exposure estimated by trapezoid rule. Most but not all models used 2-compartment disposition for MPA in stable and de novo renal transplant patients with various absorption models and using different types of mixed modeling software[99], with some models utilizing GFR, body weight or co-therapy as covariates [89].

A large-scale retrospective analysis had reported evidence that clinical application of bayesian estimation-based dosing by physicians, resulted in MPA AUCs within target range (30-60 μg*h/mL) in comparison to routine procedures[134]. The analysis by authors also led to the suggestion that monitoring of adult renal transplant recipients at least every 2 weeks during the first month post-transplant followed by every 1–3 months during the first year, for a 75% chance that patients are within target window for up to 1 year after the previous dose adjustment.

Hence, the LSS were proposed as an alternative for full AUC determination during post-transplant periods at recommended intervals such as day-7 post-transplant, months 1 and 2 at time of change in MMF dose or during rejections, infections, adverse events or suspicion of non-compliance [85].

Pharmacodynamic monitoring – IMPDH activity in renal transplant patients

MPA is a selective, reversible, noncompetitive inhibitor of inosine monophosphate dehydrogenase (IMPDH), the rate-limiting enzyme needed to produce guanosine, depleting the intracellular guanosine triphosphate stores. Although guanosine may be synthesized in other cells through a salvage pathway, T-lymphocytes don't have that capacity and interruption of IMPDH enzyme activity preferentially limits lymphocyte proliferation [135] and migration to areas of inflammation or foreign tissue. Human IMPDH is encoded as two isoforms, type I and II, located on two different chromosomes with 85% of their amino acid sequence conserved, but the recombinant IMPDH2 was 4.8-fold more sensitive to MPA than IMPDH1. Earlier studies had expressed the IMPDH activity within PBMC cells as mol/gm of protein but with the availability of sensitive LCMS methods to determine AMP content in PBMC cells, the IMPDH activity in recent studies are expressed after normalization to AMP instead of the protein.

Studies determining IMPDH enzyme activity in transplant patients were mainly performed to determine its utility for predicting rejections or adverse events as well as to determine utility of pretransplant IMPDH in accounting for differences in outcomes in patients on MMF therapy. Some of the earlier studies reported that, despite considerable inter-patient variability in the pre-transplant IMPDH activity, the intra-patient variability in IMPDH activity is low. A retrospective study in 48 renal transplant recipients with a 3-year follow up revealed, an association between higher pre-transplant IMPDH activity and increased incidence of rejections, whereas a lower activity was associated with MMF dose reduction[136]. Hence determining IMPDH prior to

transplant could be beneficial as would be the pharmacodynamic monitoring of IMPDH activity along with the PK monitoring for MPA [137]. The practicality of monitoring IMPDH activity is currently hampered by technical requirements, cost, time and tediousness involved in the procedure.

The median IMPDH activity at baseline was higher in healthy individuals (74.8 μmol/sec/M AMP) in comparison to stem cell transplant recipients on a steady state dose of MMF (45.2 μmol/sec/M AMP)[138]. The variability in transplant patients (5.3 fold) was also higher than healthy individuals (4 fold). The inhibition of IMPDH activity was well correlated to MPA exposure as reported in a serial pharmacokinetic-pharmacodynamic study in pediatric renal recipients done for until 6 months after transplant [139]. In adult renal transplant patients, an inverse correlation between MPA concentration and IMPDH activity was observed, and at around 1.5 h post-dose of 1000 mg MMF, an average 50% peak inhibition in IMPDH activity has been reported[140].

In the literature, there has been limited information on the utility of quantifying inhibition of IMPDH activity as a biomarker to guide therapy with MPA. However various retrospective and prospective studies in renal transplant patients have shown the relation of MPA-IMPDH during MMF therapy in consideration to clinical outcomes.

A study on the long term (15 month) follow-up of the PK and PD in 54 renal transplant patients showed an increasing trend in the predose IMPDH activity in rejecting as compared to the non-rejecting patients on chronic MMF therapy ($p=0.019$) [141]. The authors suggested that due to high interpatient variability in IMPDH activity of around 42%, monitoring the trend in repeat predose IMPDH activity could help in prognosis of rejection.

In a study for intensified versus standard dose of EC-MPS, the intensified dosing regimen (days 0 through 14: 2880 mg/day; days 15 through 42: 2160 mg/day; followed by 1440 mg/day) resulted in higher MPA exposure with lower IMPDH activity on day 3 of transplantation, in comparison to the standard dosing (1440 mg/day). The incidence of BPAR was lower (2.6%) in the intense regimen group as compared to standard dosing regimen (13.5%)[142].

In a 3-month follow-up study in 52 de novo renal transplant patients on EC-MPS, the IMPDH activity was determined by abbreviated samples at 0, 0.5, 1, 2 and 4 h, on day 7 followed by 1 and 3 months. A significantly lower inhibition of IMPDH was observed on day 7, in the group with BPAR (26.5 ± 11% vs. 56.7 ± 18%, $p<0.001$), however, neither the calculation method or time points used for determination of these values was stated. No correlation of MPA abbreviated AUCs or trough concentrations or the $IMPDH_{(0\text{-}4\ h)}$ area under efficacy curve was noted with BPAR [143].

The 12 h MPA exposure and IMPDH activity were determined in 66 renal transplant recipients on EC-MPS and cyclosporine therapy[144] during a 12 week follow up. Abbreviated sampling was determined for both MPA and IMPDH activity and time points at predose, 1, 2, 3 and 4 h post dose were found optimal (r^2=0.8 for both MPA and IMPDH). Patients with BPAR showed significantly lower exposure in comparison to uneventful versus patients with infections, the median AUCs being 28 vs 40 vs 65 μg*h/mL, respectively. A low 12 h IMPDH AUEC was associated with an increased frequency of gastrointestinal side-effects (median 43 vs 75 nmol.h/mg protein/h). IMPDH AUEC was shown to be associated with adverse events rather than the BPAR, in this study.

Due to the high variability in IMPDH activity, studies have hypothesized that inter-individual variability may be attributed to the SNPs in genes coding for IMPDH I and II. However,

results on the relationship of IMPDH genotype to pharmacokinetic and clinical outcomes are contradictory. A study evaluating 8 SNPs of IMPDH in de novo kidney transplant patients identified the SNP, 3757T>C for IMPDH II with a variant allele (C) to be associated with increased IMPDH activity in de-novo kidney transplant recipients[145]. For IMPDH I, 2 SNPs, rs2278293 and rs2278294, were shown to be significantly associated the incidence of BPAR within a year after kidney transplantation and the SNP rs2278294 SNP was shown to be associated with lower risk of rejection and a higher risk of leucopenia during the first post-transplant year as well[146]. The correlations of IMPDH1 polymorphisms and MPA exposure with subclinical acute rejection (SCR) were investigated in 82 Japanese recipients, on day 28 post-transplantation[147]. It was reported that patients with the IMPDH1 rs2278293 G⁄G genotype tend to develop SCR, on the contrary the risk of SCR was found to be lower in patients with the IMPDH rs2278293 A⁄A genotype for whom MMF was considered more effective.

Few studies have come around the tedious and time-consuming methods for determination of IMPDH activity by alternative measurement of IMPDH mRNA for type 1 and 2, using quantitative real-time polymerase chain reaction (RT-PCR) methods.

In a study of 12 renal transplant recipients over 2 years, the IMPDH-I mRNA expression was increased during the first 3 months following transplantation and reached maximal levels during acute rejection episodes, whereas type II IMPDH mRNA was stable [148]. The authors suggested monitoring of IMPDH RNA over time may provide reliable information to predict acute rejection. In another cohort of 101 renal transplant patients, the IMPDH mRNA was found to be marginally associated with acute rejections, with higher pre-transplant expression values of type 1 and 2 IMPDH mRNA in patients who had acute rejections [145]. On the contrary, IMPDH activity was not correlated with acute rejections.

In 35 de novo renal transplant recipients followed up for 6 months, the IMPDH activity and mRNA expression was determined at weeks 2, 4, 12 and 24, using samples at predose and 2 h after MMF administration. Patients with BPAR in the first 4 weeks exhibited significantly higher pre-transplant expression of genes for IMPDH I and II[149]. The authors proposed pre-transplant IMPDH mRNA expression for prediction of response to MPA and cautioned to the impact of post-transplant steroid therapy on limiting utility of post-transplant mRNA expression.

The availability of more sensitive non-radioactive methods has facilitated MPA pharmacodynamic monitoring by IMPDH activity. The determination of IMPDH inhibition may provide a better indicator of degree of immunosuppression in comparison to the MPA plasma concentrations. Apart from rejections, IMPDH activity may be related to MPA dose-related toxicity and could allow individualizing exposure while limiting side effects. The evidence so far has not provided a conclusive determination of IMPDH target inhibition due to the high interpatient variability and suggests longitudinal patient wise monitoring.

1.4.2.4 Indication versus protocol biopsy

Biopsy of kidney tissue is an invasive procedure to find histological changes in the transplanted graft and to guide further changes in immunosuppression therapy. An indication biopsy is performed in response to a change in patients lab parameters like increase in serum creatinine or proteinuria or any change in patient's clinical condition that may be indicative of allograft dysfunction including recurrent renal disease, rejections or BK nephropathy. However, protocol (scheduled) biopsies are obtained at predefined intervals, regardless of renal function, except if it is contraindicated (like in patients on anticoagulants or antiplatelet agents). Hence, protocol biopsies have revealed clinically inapparent rejections (subclinical) with Banff grade 1A or higher lesions or inflammation in the graft. Treatment of subclinical rejections identified before

3 months had decreased chronic tubulointerstitial score at 6 months and decreased the 2-yr serum creatinine in comparison to no treatment[150]. The exposure to immunosuppression at early post-transplant is a likely determinant of the subclinical rejections at the 3-month protocol biopsy. It was also observed that patients who had inflammation in the protocol biopsy had significantly lower tacrolimus trough levels and higher blood creatinine levels versus those without inflammation in their protocol biopsy[10]. The biopsy is also the gold standard for diagnosing BK virus nephropathy. In response to this diagnosis, studies have indicated a stepwise reduction in MMF and tacrolimus or conversion to cyclosporine (which can also reduce MPA levels) to prevent risk of acute rejections precipitated by this infection [151]. Due to the insensitivity of creatinine levels to detect subclinical rejections and in the absence of non-invasive markers to diagnose rejections, protocol biopsies provide information on adequacy of the immunosuppression therapy.

1.5 HYPOTHESIS AND AIMS

The overall goal of this research is to improve the early post-transplant graft function in kidney transplant recipients. Several immunological and non-immunological risk factors in renal transplant patients can impact early graft survival and subsequently affect the long-term outcomes. In this dissertation, the focus is on two of the modifiable risk factors in renal transplantation, (i) ischemia reperfusion injury and (ii) acute cellular rejections associated with current immunosuppressive therapy.

Ischemia reperfusion injury (IR) is an inevitable process during transplantation and has consequences on graft survival[68,152,153]. The IR of kidneys is a known risk factor for delayed graft function (DGF) after transplant as well as acute and chronic rejection of the kidney[12,13]. With the

increased demand for transplantable kidneys, the use of extended criterion donors (age >60 yrs with hypertension or creatinine >1.5 mg/dL or death due to cerebrovascular causes) and donation after circulatory death has increased. Kidneys from such donor types may be subject to prolonged warm and cold ischemic times with an increase in IR injury effects, thereby leading to higher incidence of DGF and suboptimal kidney function[154,155]. Hence, strategies for reduction in IR injuries will improve the graft utility, survival and decrease patient morbidity.

Strategies like pre-treatment of donors done prior to organ retrieval are shown to reduce ischemia effects. However, ethical and practical issues of dealing with the donor post-mortem, is a major limitation. Alternatively, pharmacological treatments focused on targeting IR pathways is possible during preservation (static cold storage or machine perfusion) and transport of the kidney. If appropriate, the pharmacological treatment may also be continued during reperfusion (implantation as well as after transplantation), with informed consent of the patient. Further, if beneficial effects are demonstrable in kidneys of pre-clinical models, the target concentration of ex-vivo pharmacological treatment are easily translatable for testing with human kidneys.

Prostacyclin (PGI_2), is endogenously produced by vascular endothelial and smooth muscle cells and has a potent vasodilator and anticoagulant effect. Treprostinil is a synthetic PGI_2 analogue with better stability, improved potency and a longer half-life and with a pharmacological activity against several pathways that are known to be activated during IR. The anticipated effects of treprostinil as a pharmacological additive during IR include, activation of PPAR-gamma induced anti-inflammatory and antioxidant effects, cAMP generation to prevent depletion of ATP levels apart from the vasodilatory and anti-coagulation effects. Treprostinil has been shown to attenuate the significant decrease in ATP content of 24 h cold preserved livers and decrease hepatic IR injury in an orthotopic rat liver transplant model[53]. Hence, overall hypothesis is that the ex-vivo addition

of treprostinil to cold storage solution can attenuate the IR injury and preserve the function of the kidney graft by its vasodilatory action on renal endothelial cells and by decreasing the reperfusion induced activation of inflammatory and coagulation pathways.

The hypothesis was tested after establishing an isolated perfusion of rat kidney (IPRK) as a model for IR. Further, the beneficial effect of ex-vivo addition of treprostinil to storage solution and reperfusion, was tested by functional evaluation of cold stored renal graft using the IPRK model, in chapter 2. The effects of prolonged cold storage (24 vs 72 h), IR injury and treprostinil on the gene expression of important renal targets including renal drug transporters, metabolism enzymes, receptors, antioxidant enzymes and inflammatory mediators was quantitatively compared using a Nanostring® based analysis in chapter 3.

The second part of dissertation evaluated baseline maintenance immunosuppression that is currently being followed as a standard after renal transplantation to minimize acute rejections. Tacrolimus, mycophenolate mofetil and steroids are usually used in renal transplant patients to avoid rejection of the graft. As MPA and tacrolimus have differing mechanisms of action, they achieve overall better efficacy when given in combination, leading to a decrease in the frequency of acute cellular rejection episodes[156,157] and increase in graft survival at 1 year. Despite standardized immunosuppression with MMF and tacrolimus, the early 3-month protocol biopsies have revealed histological lesions associated with inflammatory changes and presence of immune cells in the kidney graft even before an impact on graft function is observed. Grading of these lesions in protocol biopsies reveal histological changes meeting the Banff criterion for rejection but without any observable change in graft function (subclinical rejections). Previous reports have shown that the inflammation detected in 1- or 4-month biopsies to be associated with progression of fibrosis[17] and early subclinical inflammation is a risk factor for progression of chronic interstitial

damage[158]. It has previously been shown that baseline immunosuppression (cyclosporine versus tacrolimus) influences the degree of inflammation in early protocol biopsies and further determines incidence of acute clinical rejections[159]. A re-assessment of standardized early immunosuppression (MMF + tacrolimus) taking histological findings (clinical + subclinical rejections) in early post-transplant biopsies into consideration is necessary to improve long term outcomes.

In conjunction with this standardized immunosuppression regimen, the pathological analysis of transplanted graft is performed in a few transplant centers only, at early post-transplant time periods such as 3 months as well as 6- or 12-months post-transplant. These protocol (screening) biopsies provide an accurate diagnosis for the graft conditions in response to the standard immunosuppression therapy and are also done to confirm the presence or absence of acute cellular rejections.

The current immunosuppression protocol includes regular therapeutic drug monitoring (TDM) of tacrolimus and adjustment of dosing regimen to maintain a therapeutic range in order to minimize rejection and toxicity. MPA however is administered at a fixed dose and no TDM is performed. Further, there has been limited information on the utility of quantifying IMPDH activity inhibition as a biomarker to guide therapy with MPA, though several studies in renal transplant patients have shown the relation of MPA-IMPDH in consideration to clinical outcomes. Our hypotheses are: (a) relationship of tacrolimus exposure and outcome (clinical/subclinical rejections) will be influenced by genotype of the MDR1 gene expressed on the lymphocytes (PBMC) (b) it is important to personalize MMF dosing based on exposure (abbreviated area under the curve (AUC) and/or inhibition of IMPDH activity.

First, exposure to tacrolimus was determined by sparse sampling during early post-transplant week-1-2 and followed up during the 3-month biopsy on week-13. Patients with same

blood levels might differ in target site concentrations within the peripheral blood mononuclear cells (PBMCs), due to efflux by P-glycoprotein expressed on PBMCs. Hence, the genotyping of patients for P-glycoprotein along with estimation of total exposure to tacrolimus by maximum a posteriori (MAP) bayesian method was performed (in chapter 4). Next, concentrations of MPA along with inhibition of IMPDH activity was determined at the same sparse sampling points as for tacrolimus and patients were also genotyped for UDP, IMPDH I and II enzymes. The individual exposures to total MPA was determined by MAP estimation and individual inhibition of IMPDH activity was determined, in chapter 5. The associations of individual and combined exposures to MPA, tacrolimus and inhibition of IMPDH activity with histological lesions were analyzed by univariate and multivariate logistic regression in chapter 6. The summary of work, limitations and recommended future directions are discussed in chapter 7.

Overall, we anticipate that the study findings can (a) extend utility of treprostinil to improve the preservation of renal grafts and decrease reperfusion associated post-transplant morbidities and (b) can suggest the utility of pharmacological measures obtained during early post-transplant in optimizing the maintenance immunosuppression with an impact on long term outcomes in renal transplant patients.

2.0 EVALUATION OF TREPROSTINIL IN AMELIORATING RENAL GRAFT INJURY FOLLOWING REPERFUSION OF COLD STORED RAT KIDNEYS

2.1 ABSTRACT

Ischemia reperfusion injury after renal transplantation impairs graft function and the post-transplant recovery of the recipient. Treprostinil, a prostacyclin (PGI2) analogue has shown promising effects by attenuating ischemia reperfusion after orthotopic liver transplantation in rats. The aim of this study was to examine the ischemia reperfusion injury in the rat kidneys and to evaluate the effects of treprostinil addition either to preservation (storage) solution or preservation as well as during reperfusion in an *ex vivo* kidney perfusion model. Kidneys cold preserved (CS) for 24 h (4°C) were functionally evaluated using an isolated kidney system under perfusion with oxygenated buffer. Perfusate flow and renal functions including glomerular filtration rate (GFR by inulin clearance), tubular secretion (clearance of PAH, metformin) were measured during 2 h perfusion. Tissues at the end of preservation or perfusion were subjected to histological and mRNA expression analysis for oat1/mrp4 and oct2/mate1 transporters. Following reperfusion CS kidneys showed a significant loss in recovery of renal function as assessed by the fall in GFR, reabsorption of sodium and glucose and tubular secretion, to less than 10% of control values. Although a trend in improvement of renal function was observed with treprostinil addition to storage (CS-20 and CS-50), the effects were not statistically different from CS. Significant upregulation of oct2/mate1 during storage, and oat1 during reperfusion of CS kidneys, were attenuated by CS-50 and CS-50-R (treprostinil to both storage and reperfusion). Treprostinil addition to CS improved some parameters like urine flow and altered mRNA expression, but failed to improve renal function and histological damage by CS during this *ex vivo* evaluation. A longer follow up of renal function recovery using *in vivo* transplant model might provide a more conclusive evidence on the beneficial effects of treprostinil.

2.2 INTRODUCTION

Deceased donor transplant in US contributes to more than 60% of the kidneys that are transplanted every year[160]. The deceased donor kidneys are flushed out of blood and stored in cold storage solution, until transplantation. The logistic demands of transplant necessitate varied duration of cold storage, but all kidneys are invariably exposed to reperfusion with warm recipient blood, thus cold storage and warm reperfusion are inevitable sequential events in every cadaveric kidney transplant. During cold storage, the metabolic demands of the kidneys are lowered with hypothermic state (4°C) but there is also oxygen deprivation and a decrease in ATP production, setting the stage for ensuing damages initiated during warm reperfusion[161]. During reperfusion in the recipient, sub-optimal renal ATP levels, a sudden exposure to reactive oxygen radicals and activation of inflammatory mediators can lead to cellular dysfunction, acute tubular necrosis (ATN) and delayed graft function (DGF)[162,163]. Clinically, the cold ischemia reperfusion (IR) mediated injury can be a significant contributor for ATN and DGF after renal transplant, which in turn can prolong the post-transplant patient recovery, delay adjustment to optimal immunosuppression as well as impact graft outcomes[42].

The pathophysiology of IR injury has offered a plethora of pharmacological interventional studies using vasodilators, anti-inflammatory agents, antioxidants, mitochondrial protectors and colloids, to name a few. The pharmacological intervention by simple addition to cold storage solution offers an easy prophylactic approach which if required, can also be extended to treat patients post-transplantation.

Prostacyclin analogues are known to act on endothelial cells and platelets with potent vasodilatory, anti-thrombotic and anti-inflammatory effects which may be of benefit in targeting ischemic reperfusion mediated injury. Treprostinil is a potent PGI2 analogue with improved

chemical stability and a longer half-life. Previous study in rat liver transplant model showed that pretreatment of donor and recipient rats with treprostinil protected the grafts from early reperfusion mediated injuries. Treprostinil treated rats showed decreased mRNA expression of inflammatory mediators like TNF-alpha, IFN-gamma, ICAM-1 and in addition improved the hepatic tissue levels of cAMP, ATP, ADP, AMP and total adenine nucleotides in comparison to the placebo treated rats[53]. The potential for treprostinil to minimize cold ischemia reperfusion of renal grafts has not been demonstrated yet. Treprostinil is FDA approved for treatment of pulmonary arterial hypertension and can be considered as safe for addition to the cold storage solution during kidney preservation. It is hypothesized that addition of treprostinil either to preservation solution only or additionally during reperfusion, can improve the viability of the kidney graft by promoting cAMP mediated intracellular ion homeostasis, prevention of ATP loss and thereby improve the functional recovery of graft during reperfusion.

Our objective in this study is to establish a model for renal ischemia reperfusion injury and examine the effect of treprostinil addition during cold storage and warm reperfusion, on the functional recovery of rat kidneys. The kidneys preserved in University of Wisconsin solution at 4° C were reperfused in an isolated rat kidney perfusion system for simultaneous evaluation of glomerular and tubular functions.

2.3 MATERIALS

2.3.1 Chemicals

Remodulin® vials containing 20 mL of 1mg/mL was kindly provided by the manufacturer, United Therapeutics Corporation (Silver Spring, MD). The University of Wisconsin (UW) solution (CoStorSol™) was obtained from Preservation solutions Inc. (Elkhorn, WI). Lactated Ringer's solution was obtained from Baxter healthcare (IL, USA). The components of Krebs-Henseleit buffer and sodium bicarbonate were of analytical grade and obtained from Sigma Aldrich, US. A kit containing 21 L-amino acids was obtained from Supelco, Millipore Sigma, US. Inulin (from Dahlia tubers), P-Aminohippuric acid, metformin and dextran (from Leuconostoc spp). was obtained from Millipore Sigma, US. Bovine serum albumin (protease-free) was obtained from Fisher BioReagents, US.

2.3.2 Perfusate solution

The perfusate (300 mL) was prepared fresh, a couple of hours before the perfusion of the kidney. The perfusate consisted of Krebs-Henseleit buffer with sodium bicarbonate (KHSB), containing BSA (40 g/L) with dextran (16.5 g/L) as oncotic agents and a mixture of 20 Amino acids[164] to improve the stability and function of the perfused kidney[165,166]. The oncotic agents were dissolved in 150 mL of Milli-Q water under constant stirring for at least 30 minutes until complete solubility without any cloudiness was observed. KHSB was prepared separately and mixed with the oncotic fluid, followed by the amino acid mixture. The pH was adjusted to 7 with HCl and passed through 0.11 μ filter. The perfusate was equilibrated through the recirculating perfusion

system for at least 30 minutes for adequate oxygenation and the final pH was 7.4 prior to connecting the kidney.

2.3.3 Perfusion system

Kidneys were reperfused with the acellular perfusate prepared earlier, in a recirculation mode using the perfusion system (Radnoti LLC, CA). The system comprised of a peristaltic pump, a thermostatic controlled water jacket and an oxygenator to maintain perfusate flow, temperature (37°C) as well as to enrich perfusate with carbogen (95%O2/ 5%CO2), respectively. The total pressure within the perfusion system was monitored with an inline sphygmomanometer and an inline syringe filter (37 mm Acrodisc, Pall life sciences) was used to avoid any particulates and tissue debris from entering the renal vasculature during perfusion. The perfusion system was equilibrated with perfusate for 30 min to ensure adequate oxygenation, the baseline pressure was set to zero and the system was verified for absence of bubbles or pressure fluctuations, prior to connecting the kidney.

2.4 METHODS

2.4.1 Animals and grouping

Adult male Sprague Dawley rats were used in this study, performed with the approval of the Institutional animal usage and care committee at the University of Pittsburgh. All rats were housed under standard conditions (light, temperature, humidity, diet and free access to food and

water) for a period of at least 3 days prior to the experiment. A total of 20 rats (weight range 250–280 g) were divided into 5 groups, each consisting of four animals. Two groups were used for determination of baseline values, a control group with no preservation and a CS group with 24 hours of cold preservation. Three additional groups were evaluated after 24 hours cold preservation with treprostinil added at final concentrations of 20, 50 ng/mL and 50 ng/mL plus an additional 10 ng/mL during reperfusion, denoted as CS-20, CS-50 and CS-50-R, respectively.

2.4.2 Isolation and cold storage of kidneys

Briefly, rats were anesthetized with a mixture of isoflurane/oxygen and ventilated on a table top research anesthesia machine (Parkland Scientific, FL) with 1 liter/min O2 in air, until isolation of both kidneys. The procedures for surgery and perfusion was performed according to methods described in Taft et al.[164]. A midline abdominal incision was made to expose the right kidney. The intestine was gently moved aside and wrapped in a gauze, soaked with saline. The right ureter was identified for cannulation with a polyethylene cannula (PE-10), prefilled with saline and the urine flow was verified, before proceeding further. In few instances, urine flow was initiated by gentle suction within PE-10 via a tuberculin syringe. Next, the superior mesenteric artery at the branching of aorta was isolated and used for accessing the right renal artery with a 20G cannula. The branching to the adrenal artery was ligated and cannula was secured at both renal artery and the superior mesenteric artery. The right kidney was flushed with 15 mL of cold saline to remove blood via the incised renal vein, followed by perfusion with 15 mL of cold UW solution. The renal vein was clamped, the cannula locked, and kidney was gently isolated along with the ureter to be placed into 30 mL of cold UW solution at 4 °C for 24 h. A whole-body perfusion was then performed for 3 minutes with cold UW solution via cannulation of the left

ventricle and flushing out of blood via an incision at the inferior vena cava. The left kidney was then isolated, cleared of adhering tissues and stored together with the right kidney. At the end of 24 hours, the right kidneys were perfused for functional evaluation and left kidneys were stored for mRNA expression and histology, as described below. Freshly isolated kidneys of the control group were not subjected to cold storage prior to perfusion or storage for mRNA and histology.

2.4.3 Reperfusion of the isolated rat kidney (IPRK)

Only control rat kidneys were perfused immediately after isolation, whereas kidneys from all other groups were reperfused after 24 h of CS. In the case of control kidneys, cannulation of renal artery was performed under 20 mL/min of perfusate flow to avoid uninterrupted renal perfusion. After the cannula was secured in the renal artery and superior mesenteric artery, the kidney was quickly excised and mounted onto the perfusion chamber. On the other hand, CS kidneys were slowly perfused for 10 minutes with 10 mL of cold (4°C) lactated ringer solution to wash out all the UW solution, connected to a 10 mL/min perfusate flow and immediately mounted onto the perfusion chamber. The venous outflow was directed back into the perfusion reservoir to complete the recirculation. During the initial 15 minutes of equilibration, perfusate flow was adjusted to maintain a stable renal perfusion pressure at 100 mm Hg.

2.4.4 Functional evaluation during IPRK

Following equilibration, marker compounds namely inulin, PAH and metformin, were added to the perfusate at final circulating concentrations of 750, 100 and 5 µg/mL, respectively. The perfusate of CS50-R group, evaluating the effect of treprostinil during reperfusion, contained

10 ng/mL of the drug in perfusate, in addition. Following an additional equilibration of 5 minutes, the kidneys were perfused for 120 minutes, for functional evaluation by calculated renal clearance of marker compounds, described later. Urine flow was collected into pre-weighed 1.5 mL Eppendorf tubes for immediate determination of pH and gravimetry based volume. The urine was collected at 20 min time intervals and perfusate was sampled with replacement, at the midpoint of every urine collection time interval. At the end of perfusion, kidney was removed and weighed. A small section of kidney was stored in formalin and the rest was flash frozen in liquid nitrogen and stored in -80 °C, for mRNA analysis. Aliquots of the perfusate and urine samples were frozen in -20°C until further analysis.

2.4.5 Bioanalysis of marker compounds

a) Colorimetric assay for Inulin: Inulin in perfusate and urine samples was quantified with a colorimetric assay[167] published earlier, with few modifications for accommodating analysis of urine samples. Urine samples (50 µls) were diluted to 250 µls with Milli-Q water prior to analysis. Briefly, perfusate (100 µls) or diluted urine (250 µls) were mixed with 50 mM of indole acetic acid and incubated at 37 °C for 1 h in presence of 2 mL of 12 N HCl. After cooling for 10 min at RT, the incubates were transferred to 96-well plate and OD readings were obtained at 530 nm. Blank perfusate and blank urine from IPRK were used for preparation of standard and quality control (qc) concentrations. The standards in perfusate ranged from 0.2 – 2 mg/mL with 0.3 and 0.9 mg/mL as qcs. The standards in urine ranged from 0.2-8 mg/mL with 0.3, 0.9 and 5 mg/mL as qcs.

b) Analysis of glucose and sodium: Perfusate and urine samples were analyzed for glucose and sodium ion, by using hexokinase method and ion selective electrode, respectively.

c) HPLC assay for PAH: The perfusate and urine levels of PAH were measured using previously published HPLC methods, with minor modifications[168,169]. Briefly, 20 µls of perfusate or urine sample was diluted 5 times with blank matrix and precipitated with 10 µls of 10% (w/v) trichloroacetic acid (TCA). This mixture was shaken in a vortex for 2 min, centrifuged at 13,000 rpm for 6 min at RT and 40 µls of supernatant was injected on to a Symmetry C18 column (5 µm, 250 mm × 4.6 mm i.d.) (Waters Corp., Milford, MA, USA) attached with a guard column Zorbax SBC18 (5 µm, 12.5 mm × 4.6 mm i.d.). The mobile phase consisted of 1 M acetic acid (pH 3.2) and ACN in a gradient mode at a constant flow rate of 0.7 mL/min and effluent was monitored at 270 nm. Standards for PAH ranged between 10-250 µg/mL in both matrices, as shown in Figure 2.1.

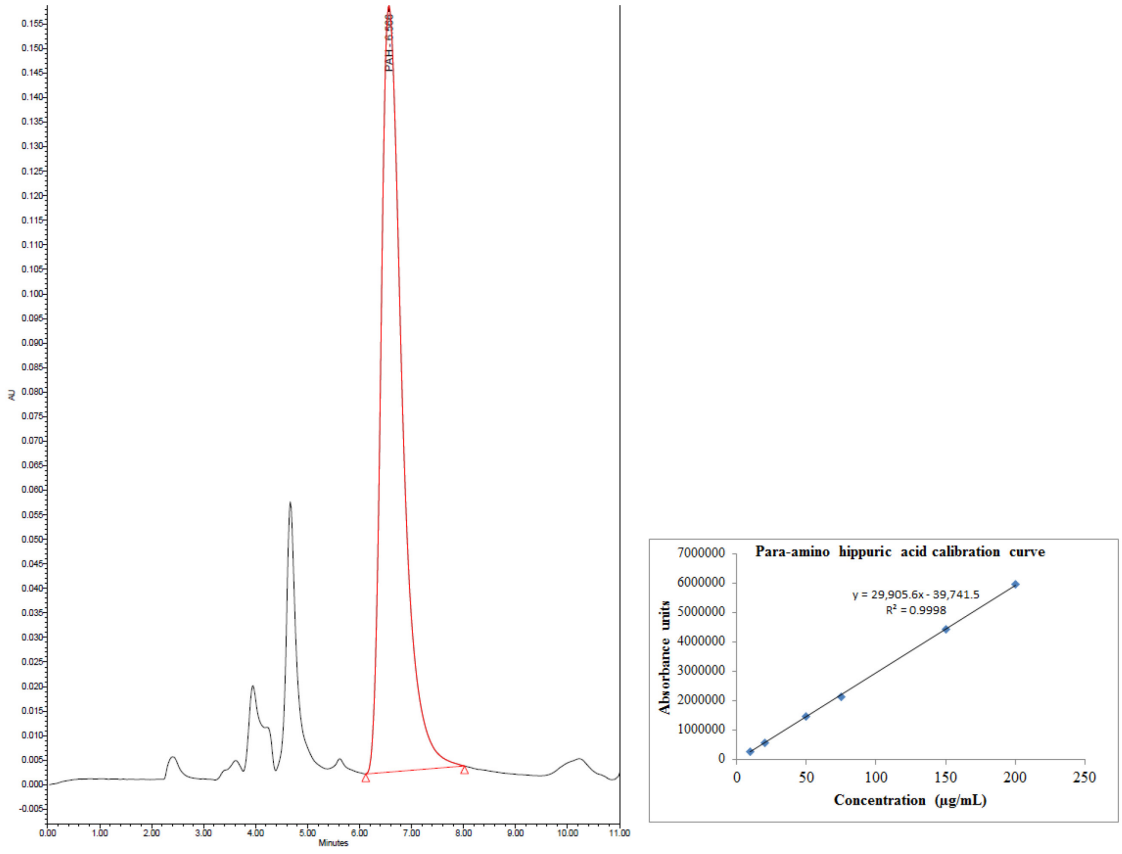


Figure 2.1. HPLC chromatogram and calibration curve of para-amino hippuric acid in perfusate and urine.

d) HPLC assay for metformin: HPLC analysis of metformin perfusate and urine was based on previously published method[170], with some modifications. 100 µls of perfusate or 50 µls of urine was mixed with 20 µls of 10% (w/v) TCA and shaken in a vortex for 30 secs, centrifuged at 13,000 rpm for 6 min at RT and 50 µls of supernatant was injected on to a Suplecosil LC-CN column (5 µm, 250 mm × 4.6 mm i.d.; Millipore Sigma, USA) maintained at 40 °C. An isocratic mobile phase comprising of 70 % (v/v) of 10 mM KH_2PO_4 with 1 mM sodium lauryl sulfate (pH 6.5) and 30 % (v/v) methanol was used at 1 mL/min. Standards ranged between 0.5-25 µg/mL in both matrices, as shown in Figure 2.2.

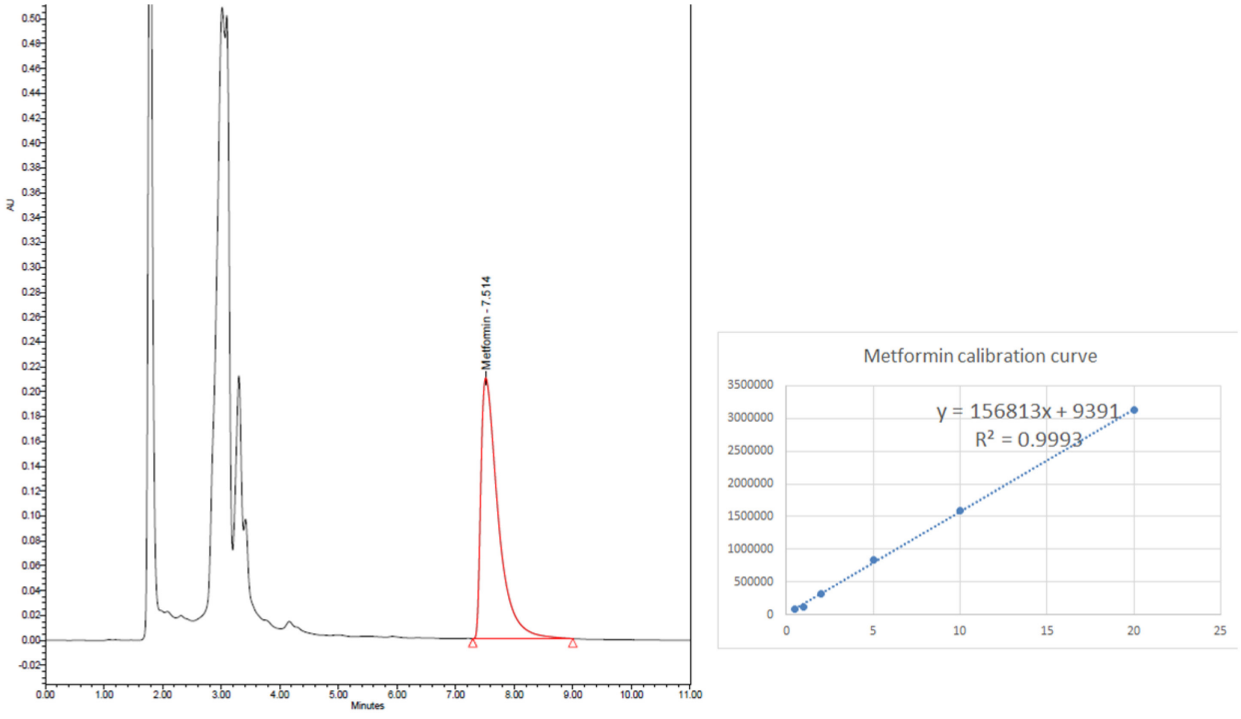


Figure 2.2. HPLC chromatogram and calibration curve of metformin in perfusate and urine

2.4.6 mRNA expression

The extraction was performed as per the instructions of the manufacturer and described briefly as follow: on ice, 25 mg or less of frozen kidney tissue was mixed with RNeasy RLT buffer (600 uL) and homogenized on ice with a conventional rotor–stator homogenizer. Lysate was transferred to QIA shredder column for removing tissue debris and further purified with a RNeasy column (Qiagen sciences, MD, US). The purity of isolated mRNA was assessed using a NanoDrop™ 2000c spectrophotometer.

2.4.7 Real time qPCR

Superscript IV reverse transcriptase (ThermoFisher scientific, US) was used to generate cDNA from purified mRNA samples. Samples had varying concentrations of mRNA, but all samples were diluted to 25 ng/µl starting concentration, using DPEC water. 250 ng of mRNA (10 µls) was used per reaction, in accordance to the Superscript IV protocol to obtain cDNA and final volume was adjusted to 20 µls with RNase free water.

RT-qPCR was performed in 96 well plates. Each well was loaded with 4 µL of cDNA, 1µL of TaqMan primer mix, 10 µls of TaqMan master mix, and 5 µls of RNase free water. TaqMan primers (assay ID) for rat slc22a6 (oat1) (4331182-Rn00568143_m1), slc22a2 (oct2) (4331182-Rn00580893_m1), slc47a1 (mate1) (4331182-Rn01460731_m1) and abcc4 (mrp4) (4331182-Rn01465702_m1) gene targets and actb (β-Actin) (431182-Rn00667869_m1), gapdh (431182-Rn99999916_s1) as housekeeping genes, were procured from Life Technologies (Carlsbad, CA, USA). Applied Biosystems® 7500 Real-Time PCR System was used to amplify and detect targeted genes. PCR amplification was performed in 40 cycles of 94°C for 15 s, then 50°C for 30 s, and

68°C for 60 s. The final elongation step was 68°C for 10 min. All samples were plated in duplicates to account for variability associated with technical errors and each target was restricted to a single 96 well plate to avoid inter-plate variability.

2.4.8 Histopathology

Kidney sections taken for histology were fixed in 10% formalin in phosphate buffered saline, embedded in paraffin and stained with hematoxylin-eosin. A pathologist unaware of the experimental conditions (blinded), reviewed the stained sections under light microscopy and provided a qualitative assessment for presence of lesions and other changes like interstitial edema, tubular dilatation, cell detachment for the CS and reperfused kidneys.

2.4.9 Calculations

The renal clearance (CL) of inulin, PAH and metformin were used as markers for glomerular filtration (GFR), anionic and cationic tubular secretion capacity of the kidney, respectively. Urine flow rate (UFR) was calculated from the ratio of volume of urine collected and corresponding duration.

$$\text{Renal CL of inulin} = \text{GFR} = \frac{[\text{Urine}]_{\text{inulin}} \times [\text{UFR}]}{[\text{Perfusate}]_{\text{inulin}}}$$

where [Urine] and [Perfusate] are concentrations of Inulin in urine and perfusate, respectively. Renal CL was calculated for each of the six collection periods (20 min) and mean CL was calculated for each perfused kidney. The filtration fraction (FF) was estimated from the ratio of GFR to the perfusate flow, both of which were normalized per gm of kidney weight.

Filtration fraction = [GFR (per g of kidney)/Perfusate flow (per g of kidney)]

In addition, the renal clearance of sodium and glucose using perfusate and urinary concentrations were estimated, in a pilot trial and calculated as above to determine viability (% TR = tubular reabsorption) of the perfused kidney.

$$\% \text{ TR} = \frac{1 - [R_{CL}] \quad X\ 100}{[\text{GFR}]}$$

where $[R_{CL}]$ is renal clearance determined for glucose or sodium, as applicable.

Renal CL of other markers PAH and metformin were determined as above, from their respective urinary and perfusate concentrations for each of six collection periods. In addition, their tubular secretory CL was determined from the difference of their renal CL and GFR.

Tubular CL = $[R_{CL} - \text{GFR}]$

where $[R_{CL}]$ is renal clearance determined for PAH or metformin, as applicable.

The relative levels of mRNA expression of target genes were normalized with the copy number of housekeeping genes (β–actin and gapdh). The relative levels of mRNA fold changes of all genes were quantified using the $2^{-\Delta\Delta CT}$ method[171,172].

2.4.10 Statistical methods

Analysis of variance (ANOVA) was used for comparison of mean parameters between treatment groups. Tukey HSD was used for post hoc analysis of variance between two groups. Comparison of parameters between treatment groups at different time points (repeated measures) was done by mixed model analysis. Statistical significance of $p<0.05$ was considered. ANOVA, mixed model analysis and plots were done using graph pad prism version 8 (GraphPad software,

CA, US) or SPSS® version 25 (IBM corporation, US). Gene expression results of groups were compared to controls utilizing analysis of variance (ANOVA) and Bonferroni post-hoc test. All data were expressed as mean ± standard deviation (SD).

2.5 RESULTS

2.5.1 Functional evaluation -filtration and viability

The donor parameters are presented in Table 2.1. There was no significant difference between the treatment groups in donor parameters. Though the larger right kidneys were perfused, and left kidneys were stored, an increase in kidney weights was observed after perfusion. The kidneys from control rats were not subject to cold storage and perfused immediately following isolation of kidney. The parameters obtained during isolated perfusion of kidneys are compared in Table 2.2. After 24 hours of CS, there was a significant loss of glomerular filtration and urine flow in reperfused CS kidneys, in comparison to control kidneys. CS-20 kidneys showed a significant ($p<0.01$) increase in urine flow compared to other CS kidneys.

Table 2.1. Parameters of donor rats used in IPRK

	Body weight (g)	**CS kidney weights (g)**	**Perfused kidney weights(g)**
Control	253 (24)	No CS	1.5 (0.1)
CS	287 (26)	1.1 (0.1)	1.5 (0.3)
CS-20	296 (34)	1.2 (0.1)	1.7 (0.4)
CS-50	312 (74)	1.2 (0.3)	1.8 (0.5)
CS-50-R	347 (54)	1.3 (0.1)	2.0 (0.1)

Data represented as Mean (SD) of n=4 rats per treatment group; CS- Cold Storage (24 h) in 30 mL of University of Wisconsin solution

However, the trend in improvement of GFR with CS-20 was not statistically different from other CS kidneys.

Table 2.2. Functional and viability parameters in IPRK

	Control	**CS**	**CS-20**	**CS-50**	**CS-50-R**
GFR (mL/min/g)	0.26 *** (0.1)	0.03 (0.01)	0.05 (0.02)	0.04 (0.03)	0.01 (0.004)
Urine flow rate (mL/min/g)	0.04 *** (0.01)	0.02 (0.01)	0.03 ** (0.02)	0.01 (0.01)	0.01 (0.001)
Urine pH	6.1 *** (0.5)	7.6 (0.1)	7.4# (0.2)	7.3# (0.2)	7.7 (0.1)
Perfusate flow rate (mL/min/g)	19.5 *** (1.1)	17.1# (1.6)	11.2 ¥ (2.5)	14.5 (1.4)	14.4 (2.9)
Filtration fraction	0.013 *** (0.005)	0.002 (0.001)	0.005 ** (0.004)	0.003** (0.002)	0.001 (0.0003)

Data reported as mean (S.D.) collected from n=4 perfusions per treatment group; CS- Cold Storage (24 h) in 30 mL of University of Wisconsin solution;
Statistically significant difference with ($p< 0.001$) represented by ***, #, ¥ and ($p<0.01$) represented by** in comparison to all other groups except control group; Tukey HSD post-hoc testing was used

Table 2.3 compares the tubular reabsorption capacity for glucose and sodium ion (viability), which were also significantly reduced during reperfusion of CS kidneys. No improvement in viability of CS kidneys in presence of treprostinil (5 ng/mL) was observed.

Table 2.3. Fractional reabsorption in IPRK (pilot trial)

	Control*	**CS**	**CS-5**
FR $_{glucose}$	98.2	7.4	6.8
FR $_{Na}$	91.0	6.3	5.0

FR-Fractional reabsorption of glucose or sodium by kidney; Pilot trial with n=2 perfusions in each group; CS-Cold storage for 72 h and Treprostinil 5 ng/mL added to CS solution; *Significantly different (p< 0.001) from other groups

Under identical perfusion conditions, the urine from control kidneys were more acidic and showed a further drop in pH with increase in duration of perfusion, as shown in Figure 2.3.

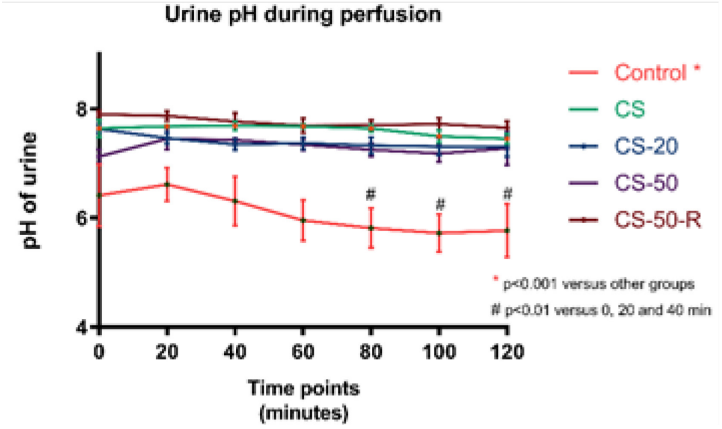


Figure 2.3. Urine pH at different timepoints during IPRK

The perfusate flow (normalized to kidney weight) was significantly higher in the control group kidneys than the CS and CS with treprostinil kidneys. Further, the kidney weight normalized perfusate flow during **r**eperfusion, was less in kidneys after addition of treprostinil during CS (CS-20, CS-50) or both CS and reperfusion (CS-50-R). However, treprostinil addition to CS (CS-20, CS-50) significantly improved the filtration fraction than the CS kidneys.

2.5.2 Functional evaluation- tubular secretion

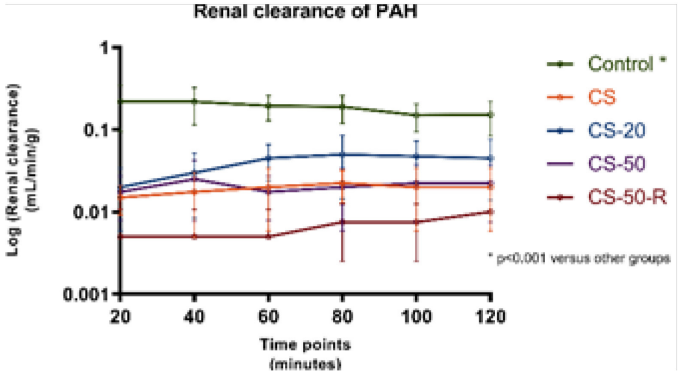


Figure 2.4. Renal Clearance of PAH at different timepoints during IPRK (µg/mL/g)

The clearance of PAH and metformin were used as markers for comparison of the tubular secretion by kidneys. Figures 2.4 and 2.6 compare the renal clearance, estimated at 20-minute intervals for PAH and metformin, respectively. The mean (SD) renal clearance is also summarized in Figures 2.5 and 2.7 for PAH and metformin, respectively.

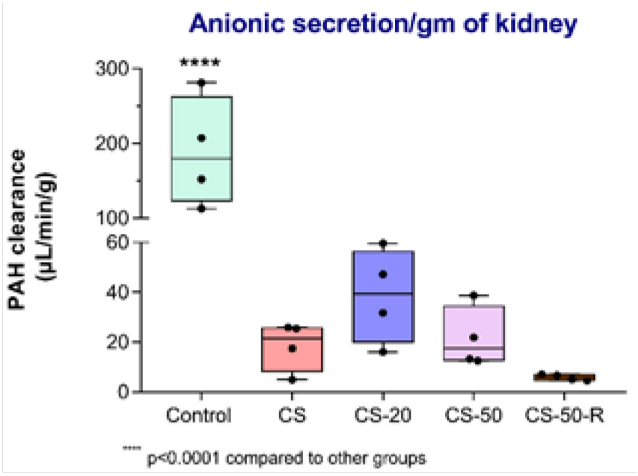


Figure 2.5. Anionic secretion based on renal excretion parameters of PAH

There was no drastic change in the tubular secretion capacity, which remained constant in all kidneys during the 2-hour perfusion period. Hence the mean values of the six individual clearance estimations (normalized to kidney weight) were used for further comparison and

interpretations. Like inulin clearance (GFR), the tubular secretion capacity of the CS kidneys dropped to less than 10% of control values.

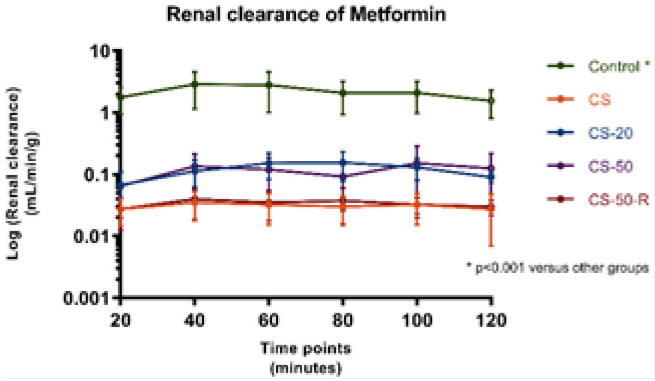


Figure 2.6. Renal Clearance of Metformin at different timepoints during IPRK (µg/mL/g)

There was no drastic change in the tubular secretion capacity, which remained constant in all kidneys during the 2-hour perfusion period. Hence the mean values of the six individual clearance estimations (normalized to kidney weight) were used for further comparison and interpretations. Like inulin clearance (GFR), the tubular secretion capacity of the CS kidneys dropped to less than 10% of control values.

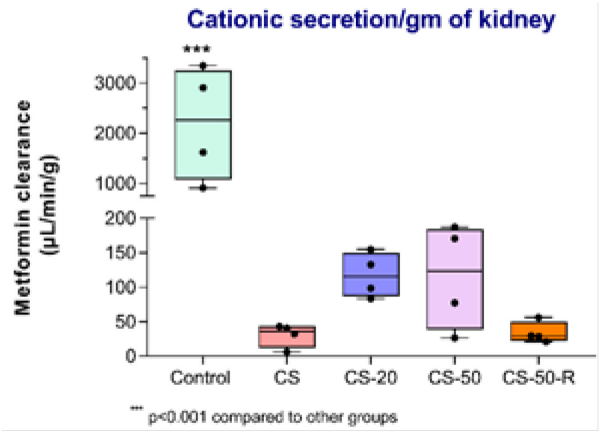
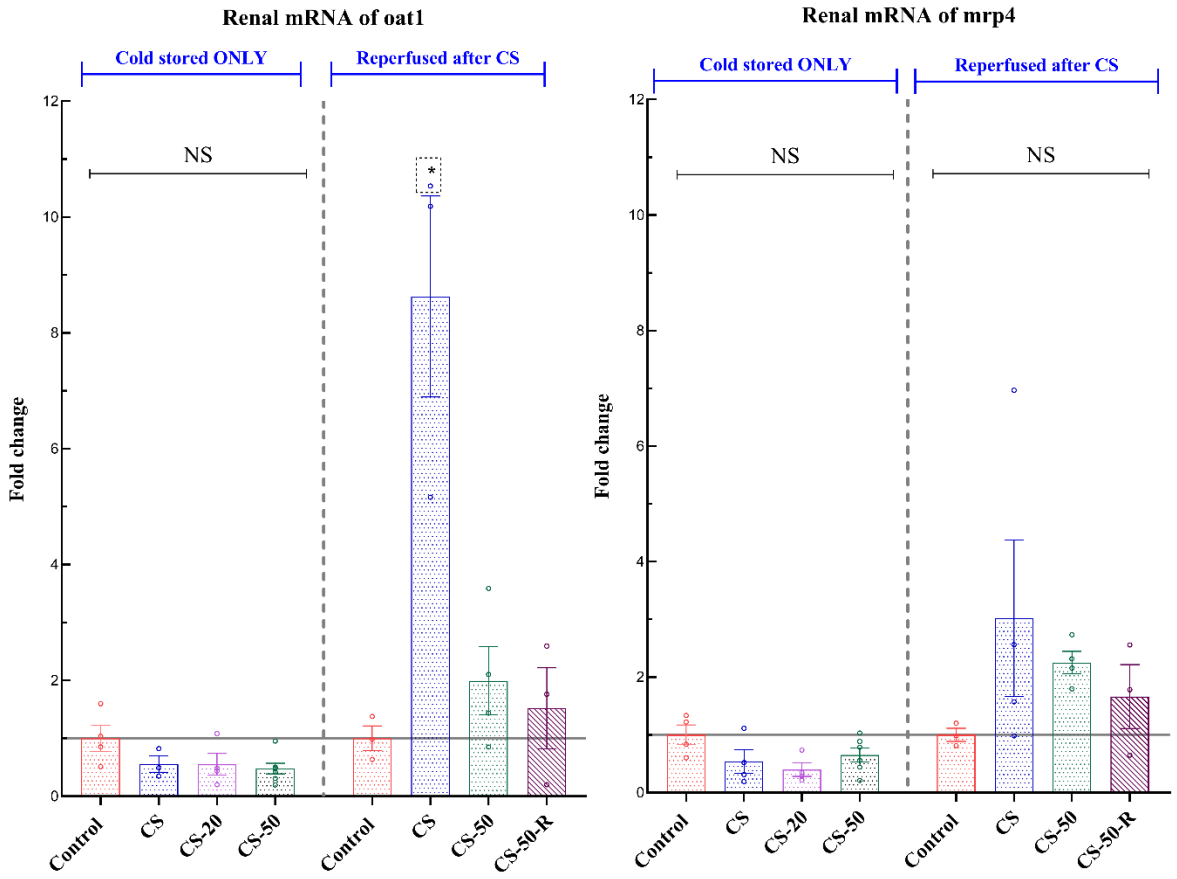


Figure 2.7. Cationic secretion based on renal excretion parameters of Metformin

Like GFR, a trend in improvement was observed with CS-20 kidneys for both anionic and cationic secretion and with CS-50 kidneys for cationic secretion only, however the effect was not statistically different in comparison to other CS kidneys.

2.5.3 mRNA expression

Gene expression for oat1/mrp4 and oct2/mate1, representing an anionic and cationic transporter system pair, respectively are compared in Figures 2.8 and 2.9. CS kidneys showed a significant increase ($p<0.05$) in oat1 after reperfusion, which was attenuated in CS-50 as well as CS-50-R (with 10 ng/mL added at reperfusion) kidneys, resulting in their post-reperfusion oat1 expression that was comparable to control kidneys (no CS). As shown in Figure 2.9, a significant increase in mRNA expression of oct2 and mate1 was observed due to 24 h CS, which was attenuated by both CS-20 or CS-50 and by CS-50, respectively.

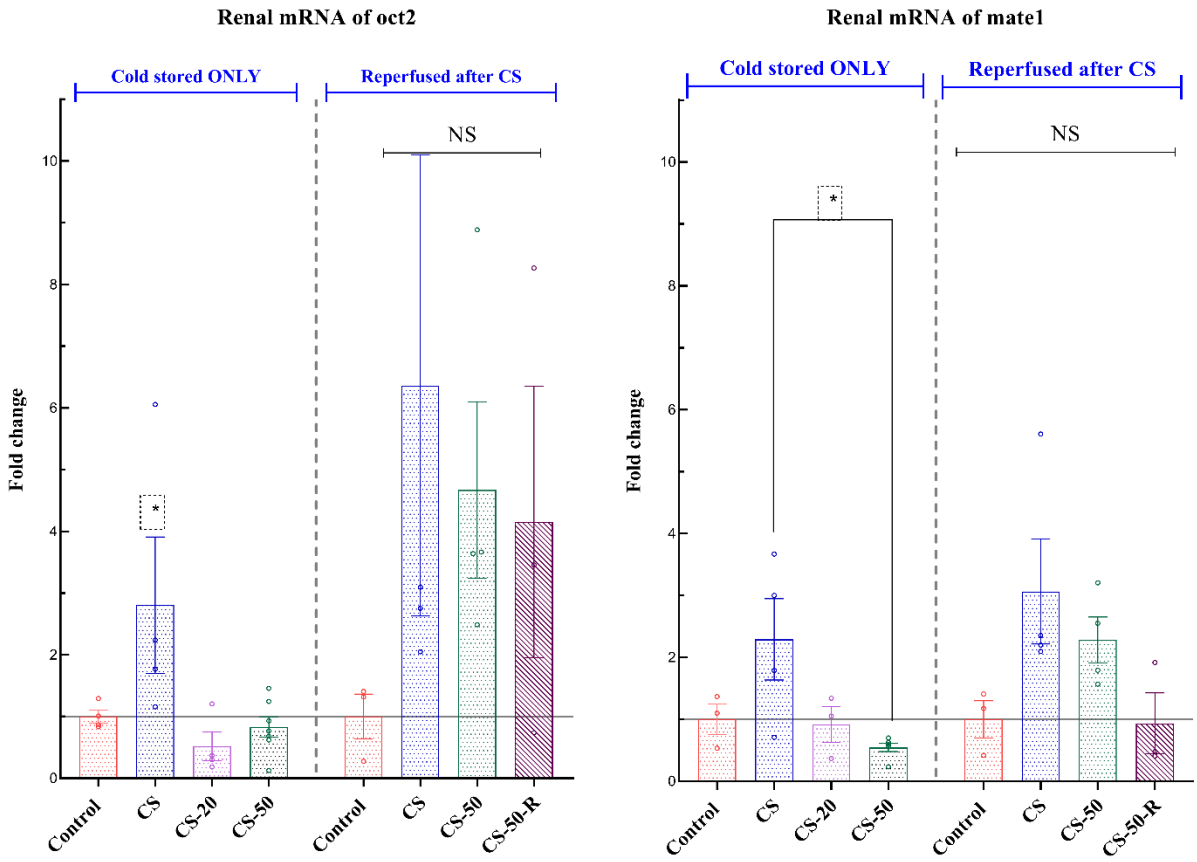


NS- No statistical difference between groups; * Significant ($p<0.05$) difference vs all other groups; ANOVA with Bonferroni post hoc test

Cold stored only (left kidneys) – mRNA expression studied in groups: control (no cold storage), CS (24 h cold storage), CS-20 (24 h cold storage + 20 ng/mL treprostinil), CS-50 (24 h cold storage + 50 ng/mL treprostinil);

Reperfused after CS (right kidneys) – mRNA expression studied after 2 h of reperfusion in groups: control (no cold storage), CS (24 h cold storage), CS-50 (24 h cold storage + 50 ng/mL treprostinil), CS-50-R (24 h cold storage + 50 ng/mL treprostinil) + 10 ng/mL treprostinil added during reperfusion

Figure 2.8. Expression of anionic transporters oat1 and mrp4 after cold storage or reperfusion after cold storage



NS- No statistical difference between groups; * Significant ($p<0.05$) difference vs all other groups; ANOVA with Bonferroni post hoc test

Cold stored only (left kidneys) – mRNA expression studied in groups: control (no cold storage), CS (24 h cold storage), CS-20 (24 h cold storage + 20 ng/mL treprostinil), CS-50 (24 h cold storage + 50 ng/mL treprostinil);

Reperfused after CS (right kidneys) – mRNA expression studied after 2 h of reperfusion in groups: control (no cold storage), CS (24 h cold storage), CS-50 (24 h cold storage + 50 ng/mL treprostinil), CS-50-R (24 h cold storage + 50 ng/mL treprostinil) + 10 ng/mL treprostinil added during reperfusion

Figure 2.9. Expression of cationic transporters oct2 and mate1 after cold storage or reperfusion after cold storage

2.5.4 Histopathology

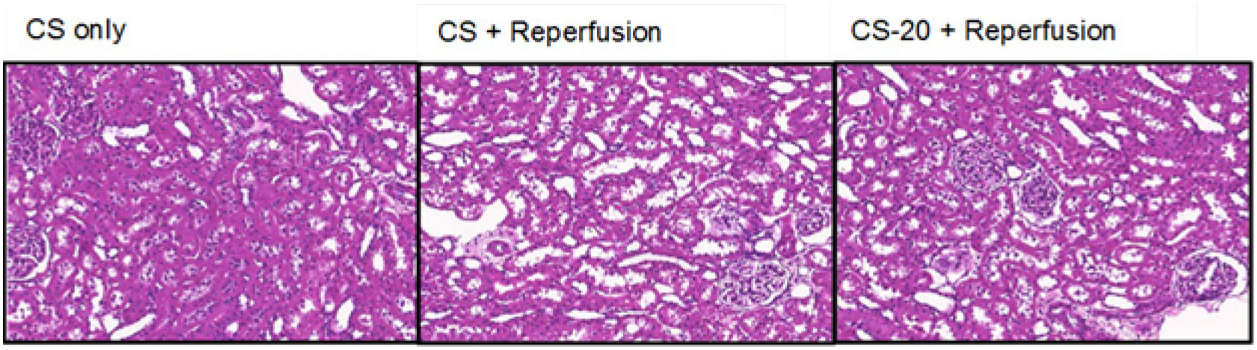


Figure 2.10. Sectional images of renal tissue representative of population after hematoxylin and eosin staining
(from Left to right- CS only, CS + reperfusion and CS-20 + reperfusion groups)

Histological evaluation revealed no damage with 24 h CS as well as CS-20 addition and they were both like fresh isolated kidneys. On the contrary, reperfusion of CS kidneys caused noticeable changes like tubular dilatation, epithelial blubs, sloughing, casts which was not prevented in CS-20 kidneys, as shown in Figure 2.10.

2.6 DISCUSSION

In this study, the protective effects of treprostinil on kidneys exposed to cold ischemia and reperfusion was evaluated using an isolated rat kidney perfusion system along with histology and analysis of selected transporter genes. The isolated kidney model is well established to study glomerular filtration, tubular secretion or the reabsorption function of kidneys, using various substrate compounds or under various experimental conditions. In our model, the cold stored kidneys were rewarmed by acellular perfusion under constant pressure which enabled easy urine

collection and simultaneous estimation of renal clearance for inulin, PAH and metformin, that represented filtration, anionic and cationic tubular secretion functions, respectively.

Results from the perfusion of fresh control kidneys for 2 hours, indicated stable renal function with good clearance of inulin (glomerular filtration) or PAH and metformin (tubular function) in addition to a stable urine flow, for the entire study duration. The values for tubular reabsorption of glucose and sodium indicated good viability of the control kidneys. Further the tubular secretion of marker compounds, PAH and metformin was also measured.

In contrast, a sub-optimal renal function was observed in 24 h cold stored kidneys that were re-perfused, under conditions identical to control kidneys. The constant pressure perfusion resulted in significantly lower perfusate flow for all the cold stored kidneys, indicative of a higher resistance within the renal system. A 10-40 % range in drop of perfusate flow however resulted in a greater than 80% drop in the glomerular filtration of CS kidneys, thereby decreasing their ratio (the filtration fraction). Cold storage and reperfusion are in fact sub-divided into four components, namely, ischemia with hypothermia in earlier part followed by rewarming with reperfusion (acellular), as described earlier[173]. Cold storage per se with hypoxia of kidney has been shown to induce the release of vasoconstrictors like angiotensin II and thromboxane[174,175] in addition to mechanical constriction of the peritubular capillaries and the vasa recta[176]. These factors could result in an increase of vascular resistance during reperfusion resulting in a drop of GFR, but it is further decreased on reperfusion, due to the tubular glomerular feedback activated by high Na^+ delivery to the macula densa[176,177].

Apart from the GFR, the tubular function of CS kidneys also showed a severe compromise in reabsorption of sodium and glucose as well as a decrease in tubular secretion of PAH and metformin. The decrease in energy-dependent tubular ion transporter functions are indicative of

deficiency in the availability and synthesis of ATP[178]. Cold storage can perturbate osmotic balance and aerobic metabolism resulting in many downstream effects on kidney. In particular, the disturbance of ATPase dependent ion homeostasis during cold ischemia have been linked to intracellular calcium and iron overload[163,179,180], leading to opening of permeability transition pore and mitochondrial swelling[163,173]. Reperfusion of CS kidneys has been shown to result in a rapid loss of adenine nucleotide content combined with loss in ATP re-synthesis due to mitochondrial swelling[181].

Histology of CS kidneys did not indicate lack of viability or injury, however the reperfused kidneys showed significant histological damages. These observations could be indicative of the damaging effects by reperfusion that are exacerbated after 24 hours of cold storage. Even in the absence of leukocytes during acellular reperfusion, oxygen free radicals can be produced from endothelial cells in kidney vessels[176,182] . The oxygen radicals can mediate cytotoxic actions including lipid peroxidation, protein oxidation, DNA damage, apoptosis, mediate inflammatory pathways and cause changes in expression and function of renal drug transporters[183]. mRNA expression of selected transporter pairs was evaluated at end of 24 hours CS as well as 2 h reperfusion, respectively. Oat1/mrp4 for anion transport and oct2/mate1 for cationic transport were selected as they are involved in transport of marker compounds PAH and metformin, respectively. Cold storage alone showed significant upregulation in mRNA expression of oct2/mate1, as highlighted in an earlier study[161]. The reperfusion however, caused a significant increase in expression of oat1 transporter in CS kidneys. Though mRNA data represents increased expression values for oat1 at the end of 2-hour perfusion, it was observed that the same kidneys showed severely impaired transport of PAH, during functional evaluation. Hence, the increased expression

of oat1 could be indicative of the regulatory response to mitigate the cold ischemia reperfusion mediated damage in CS kidneys.

Addition of treprostinil to storage solution at 20 ng/mL (CS-20) was associated with a modest but statistically insignificant improvement in GFR but a significant increase in urine flow compared to CS kidneys without addition of drug or CS kidneys exposed to higher concentrations of the drug. Notably CS-20 also increased the filtration fraction compared to all other treated or untreated CS kidneys, because CS-20 kidneys had the highest GFR in presence of lower perfusate flow per gram of kidney. A corresponding trend was also observed in the improvement of anionic and cationic tubular secretion with an increased renal clearance for PAH and metformin by the re-perfused CS-20 kidneys. The renal medulla can produce both thromboxane and prostacyclin, however during reperfusion and hypoxia, the former is favored resulting in increased vasoconstriction[176]. Treprostinil, a PGI2 analogue can mimic endothelium derived prostacyclins in producing cAMP mediated vasodilation and restore the balance in these eicosanoids during reperfusion. Further, by promoting cAMP mediated efflux of Ca^{2+} during ischemia reperfusion, treprostinil may ameliorate disruption in ion homeostasis and prevent ATP depletion[53,184]. Reperfusion induced increase in oat1 expression of CS kidneys was significantly prevented by both treprostinil addition to CS only and CS plus reperfusion. Even the CS induced increase in oct2/mate1 transporter expression was significantly reduced in kidneys stored with treprostinil. Even with addition of treprostinil to storage solution, a modest but statistically insignificant improvement was evident in functional evaluations when compared to kidneys stored without drug. Further, storage with treprostinil also negates cold ischemia reperfusion induced changes in the examined transporters. Addition of treprostinil to reperfusion and storage showed similar beneficial effects on mRNA expression, however it drastically affected glomerular and tubular

function without any impact on perfusate flow. This observation might be linked to a previous report showing a decrease in filtration coefficient of kidneys (kf) by direct infusion of PGI2. This decrease in kf was shown to be mediated by Angiotensin II induced mesangial cell contraction leading to a decrease in filtration fraction and GFR[185]. Direct PGI2 infusion to kidneys can also stimulate release of renin that leads to angiotensin II mediated decrease in GFR[186]. In this ex vivo setting, a higher exposure to treprostinil by addition to both storage and reperfusion in isolated kidney perfusion was counterintuitive by failing to show any advantage and points towards the need to select and optimize the time of addition as well as the concentration.

Though the isolated perfused kidney with cell free oxygenated medium supports kidney function, it failed to revive the cold stored kidneys, as unlike an in vivo setting, this model does not use blood for reperfusion. Hence, long-term evaluation of potential improvements associated with changes in protein expression or activity with treprostinil treatment, is also not possible using this ex-vivo model. Further examination of efficacy in a syngeneic renal transplant model might permit a longer follow-up and provide a more conclusive evidence on beneficial effects of treprostinil. Results of this study indicated the impact of 24 h CS and reperfusion on kidneys and treprostinil addition to CS improved filtration fraction as well as urine flow and a trend to improve the anionic and cationic tubular secretion as well as a protective effect on the mRNA expression of selected drug transporters. An extension of these observations using mRNA expression was conducted by investigating a comprehensive panel of genes and including a prolonged duration of CS for 72 h and will be presented in chapter 3.0.

3.0 ALTERATIONS IN GENE EXPRESSION OF ISOLATED RAT KIDNEYS FOLLOWING COLD ISCHEMIA AND WARM REPERFUSION INJURY

3.1 ABSTRACT

Ischemia-reperfusion (IR) is an inevitable consequence of renal transplantation. Prolonged duration of cold storage (CS) and warm reperfusion can be detrimental to post-transplant graft function and may also have unique effects on gene expression of kidneys. The purpose of this study was twofold: a) to determine alterations in the expression of important gene targets in kidney following 24 and 72 h of CS and after reperfusion following 24 h CS and b) to determine if treprostinil addition to cold storage (50 ng/mL) and reperfusion (10 ng/mL) could mitigate any of the unfavorable changes in gene expression associated with cold ischemia and warm reperfusion injury. Gene expression in cold stored or reperfused rat kidneys were quantified with a custom codeset of 52 selected gene targets using Nanostring ncounter® gene expression platform. Differential expression versus respective blank control kidneys were reported after statistical significance using t-test with FDR ($p<0.05$). CS for 72 h significantly upregulated cox-2 expression that was attenuated by addition of treprostinil. Perfused blank kidneys as well as the reperfused 24 h CS kidneys, all showed a significant upregulation in the expression of genes for Nfk-beta, Tnf-alpha, Il-1beta, Cxcl-10, Il-6, Il-8, Mcp-1, kim-1 and cox-2. The differentially expressed genes in reperfused 24 h CS kidneys were the transporter nhe3, MnSOD, Vitamin D receptor, which were downregulated, and ischemia reperfusion inducible protein (irip) that was upregulated, indicating IR induced injury. Treprostinil added to both storage and reperfusion attenuated the differential expression (DE) in all the four genes while addition to storage alone attenuated the DE of nhe3 and irip genes only. Results showed no major effect of CS until 72 h CS on the expression of selected genes whereas warm reperfusion for 2 h induced pronounced changes in gene expression of isolated kidneys. Treprostinil addition to storage and reperfusion showed protection against IR induced changes in select gene expression.

3.2 INTRODUCTION

Renal transplantation involves a set of procedures in transfer of the kidney from the donor to the recipient, resulting in an ischemic phase (cold ischemia and warm reperfusion injury). To minimize and prevent any deterioration due to warm ischemia, kidneys are mostly stored on ice (cold ischemia) until surgical implantation into the recipient. Warm reperfusion of the kidney with recipient blood results in the ischemia-reperfusion (IR) effects, which has been inevitable in every transplanted kidney. The IR effect on grafts is of much research interest and therapeutic importance as it could impact the post-transplant function of the kidney. During organ transplant, reoxygenation initiates production of reactive oxygen species in the hypoxic, ATP depleted kidney and activates a profound inflammatory response[39]. The nuclear transcription factors such as NF-κB are expressed in the resident renal cells including endothelium and tubular cells. During IR, NF-κB can activate the nucleus to produce and sustain a variety of inflammatory mediators such as chemokines, cytokines and further signal the migration of immune cells such as macrophages and neutrophils. The infiltrating immune cells can have immediate direct cytotoxic effects on the renal tissue but over a long term, probably play a major role in tissue repair[187]. The renal tubular cells are most sensitive to oxidative and inflammatory changes with a consequent downregulation of several transport proteins after IR[38,188]. Hence the net effect of IR on renal filtration as well as tubular secretion function has been associated with clinical consequences such as primary non function or delayed graft function, acute rejection and interstitial fibrosis of the graft[38]. Though renal injury after IR is an additive effect due to warm, cold ischemia and reperfusion, the individual cellular events during each are shown to differ[40], with unique opportunities for therapeutic interventions. The effects of IR have been widely studied, but clinically the contribution by cold storage or reperfusion is difficult to be isolated and has been rarely addressed. Previous reports

suggest a prolonged cold storage time to increase incidence of delayed graft function[189,190], presence of interstitial fibrosis and tubular atrophy[190]. During the seemingly quiescent phase of cold storage on ice (4°C), a slow metabolism and utilization of ATP is still ongoing at approximately 5% of the baseline[191], leading to a gradual depletion. The mitochondrial injury during CS and compromised electron transport are speculated to produce reactive oxygen species. Studies have revealed alteration in the expression of individual genes like endothelin-1[192] and icam-1[193] during CS, that are further hypothesized to increase vasoconstriction or immune cell migration during reperfusion. Apart from the scarce reports on individual genes, a systematic expression analysis of multiple genes, that may indicate transcriptional changes during prolonged duration of CS of kidney, is lacking. We proposed to utilize the quantitative determination of gene expression for comparative analysis of normal versus kidney tissue exposed to individual events of CS or perfusion or reperfusion after CS. Treprostinil, a stable PGI2 analogue was also evaluated by addition during cold storage or both during storage and reperfusion. Treprostinil had previously improved outcomes in hepatic rat transplant models[53] and has favorable pharmacology like endothelium relaxing effects, anti-inflammatory effects[194] and ability to activate PPAR-gamma receptors [195,196]. Consequent to lack of renal functional improvement by treprostinil in the short-term ex-vivo model, molecular effects of the drug are examined to guide a change in concentration or evaluation with an in vivo transplant model. Herein, it is hypothesized that prolonged cold storage and reperfusion may alter the expression of renal enzymes and transporters and pharmacologic manipulation by addition of treprostinil can show benefit against the storage or reperfusion induced alterations in gene expression. The relation between duration of cold storage, reperfusion and expression of multiple genes associated with renal function after cold

ischemia/reperfusion, such as mediators of inflammation and repair, receptors, enzymes and transporters were examined with a Nanostring assay.

3.3 MATERIALS

3.3.1 Chemicals

QIAshredder and RNeasy Plus mini kits were purchased from QIAGEN (Hilden, Germany). Custom codeset for nCounter® assays and nCounter Master Kit was purchased from NanoString Technologies (Seattle, WA, USA). Qubit RNA BR assay kit, RNAse free reagents and plastic were purchased from Invitrogen of ThermoFischer Scientific (Waltham, MA, USA). β-Mercaptoethanol was purchased from Millipore sigma (MO, USA). Remodulin® vials containing 20 mL of 1mg/mL was kindly provided by the manufacturer, United Therapeutics Corporation (Silver Spring, MD).

3.4 METHODS

3.4.1 Animals and grouping

Kidneys were isolated from male Sprague Dawley rats and were either cold stored (CS) or perfused for 2 h after cold storage, as described previously in Chapter 2. In addition, three groups of male rats (n=4 each) were assigned to a prolonged duration of cold storage (4° C for 72 h), with

or without addition of treprostinil and the third group served as naïve control. The experimental design is elaborated below.

3.4.2 Experimental design

3.4.2.1 Cold storage followed with reperfusion

In brief, right kidneys were cannulated, flushed with cold UW solution and isolated along with the ureter for storage in 30 mL of cold (4°C) University of Wisconsin (UW) solution. The cannula was used for reperfusion of kidney after CS. The reperfusion was at 37°C for 2 h using an oxygenated acellular buffer solution, as described earlier in chapter 2. The right kidneys of control group were perfused immediately without any CS. At the end of perfusion, kidneys were immediately flash frozen in liquid N_2 and stored at -80°C, until extraction of mRNA.

3.4.2.2 Cold storage only

The left kidneys were not cannulated but only flushed to remove blood followed by flushing with cold UW solution and then stored alongside the corresponding right kidneys. At the end of designated cold storage time, kidneys were flash frozen in liquid N_2 and stored at -80°C, until extraction of mRNA. The left kidneys of control group (fresh blank controls) were flushed with saline to remove blood, immediately flash frozen in liquid N_2 and stored at -80°C, until extraction of mRNA.

A total of 47 kidneys were assigned to 10 different groups as below:

CS only: (n=5 each)

Group 1:	Control -no CS – blank controls
Group 2:	24 h CS
Group 3:	24 h CS with treprostinil (50 ng/mL)

CS with reperfusion: (n=5 each)

Group 4:	Control- no CS only 2 h perfusion – perfused controls
Group 5:	24 h CS followed by 2 h perfusion
Group 6:	24 h CS with treprostinil (50 ng/mL) followed by 2 h perfusion
Group 7:	24 h CS with treprostinil (50 ng/mL) followed by 2 h perfusion with treprostinil (10 ng/mL)

CS only (longer duration): (n=4 each)

Group 8:	Control -no CS – blank controls
Group 9:	72 h CS
Group 10:	72 h CS with treprostinil (50 ng/mL)

3.4.3 Extraction of mRNA

The extraction and purification of mRNA was performed as per the manufacturer's protocol. Briefly, a section of frozen kidney tissue (approximately 30 mg) was placed on ice without thawing. About 600 µl of Buffer RLT (with β-Mercaptoethanol) was added to the solid tissue in a clean tube followed by tissue disruption using a rotor stator homogenizer. The lysate was transferred to a QIA shredder tube and debris free tissue homogenate was obtained after centrifugation at maximum speed for 2 minutes. The kidney homogenate was mixed with an equal volume of 70% v/v ethanol and transferred to a RNeasy spin column and centrifuged for 15 seconds at > 10,000 rpm. Further 3 steps were followed as below, to purify the RNA that was now bound to the spin column. 1. Addition of 700 µl RW1 buffer (15 seconds at >10,000 rpm) 2.

Addition of 500µL RPE buffer (15 seconds at >10,000 rpm) and 3. Addition of 500µL RPE buffer (2 minutes at >10,000 rpm). mRNA was eluted by addition of 30 µl of RNase-free water, equilibration for 1 minute followed by centrifugation (1 minute at >10,000 rpm). The yield of mRNA was obtained by quantification of 1 µl of solution with a NanoDrop™ 2000c Spectrophotometer (ThermoFisher, US). Further confirmation of yield with purity was verified with Qubit® RNA BR assay kit in a Qubit® 2.0 fluorometer (Life technologies, ThermoFisher, US).

3.4.4 Design of custom codeset (Nanostring)

A custom nCounter® assay codeset (Nanostring Technologies, WA, US) was initially designed with gene targets that were preidentified for quantitative assessment of expression in rat kidneys. These nCounter® assays are custom designed to provide a sensitive, reproducible, and direct method for detection of up to 800 gene targets in a single sample well, using molecular barcodes without the need for amplification of mRNA by reverse transcription. Some of the advantages of nCounter® technology include the ability to detect expression of gene targets in very low mRNA concentrations (0.1fM per copy per cell)[197], as well as ability to quantify expression despite significant degradation[198,199]. The custom codeset was developed with 48-wells each containing 57 preidentified gene targets that included 5 housekeeping genes (for correcting sample to sample variability in amount of mRNA used and for different degradation states), as listed in Table 3.1. The gene targets included important basolateral and luminal drug transporters, cyp, ugt enzymes and prostacyclin receptors that are endogenously expressed in rat kidney, along with targets like chemokines, cytokines and mediators of inflammation that show increased expression during ischemia and reperfusion.

Table 3.1. Details of target genes included in the Nanostring® custom codeset

Target class	*Description*	*HUGO gene name*	*Accession (position)*	*Tm CP*	*Tm RP*	*Total Isoforms*	*# Not Hit*
Anti-oxidant	superoxide dismutase 1 (CuSOD)	Sod1	NM_017050.1 (191-290)	82	79	1	0
	superoxide dismutase 2 (MnSOD)	Sod2	NM_017051.2 (1566-1665)	82	81	1	0
	superoxide dismutase 3 (extracellular sod3)	Sod3	NM_012880.1 (191-290)	83	83	2	0
BL Transporters	ATPase Na+/K+ transporting subunit alpha 1 (Nakatpase)	Atp1a1	NM_012504.1 (1886-1985)	82	80	1	0
	solute carrier family 13 member 3 (nadc3)	Slc13a3	NM_022866.2 (2186-2285)	83	79	1	0
	solute carrier family 22 member 2 (oct2)	Slc22a2	NM_031584.1 (746-845)	79	83	1	0
	solute carrier family 22 member 6 (oat1)	Slc22a6	NM_017224.2 (785-884)	79	82	2	0
	solute carrier family 22 member 8 (oat3)	Slc22a8	NM_031332.1 (1175-1274)	78	82	2	0
	solute carrier family 2 member 2 (Glut-2)	Slc2a2	NM_012879.2 (101-200)	79	80	2	0
	solute carrier family 9 member A3 (nhe3)	Slc9a3	NM_012654.1 (3871-3970)	83	81	2	0
Chemokines	C-C motif chemokine ligand 2 (MCP-1)	Ccl2	NM_031530.1 (191-290)	82	79	1	0
	C-X-C motif chemokine ligand 1 (IL8)	Cxcl1	NM_030845.1 (606-705)	83	80	1	0
	interleukin 12B (IL12)	Il12b	NM_022611.1 (365-464)	83	83	2	0
	C-X-C motif chemokine ligand 10 (CXCL-10)	Cxcl10	NM_139089.1 (586-685)	82	81	1	0
Cytokines	interferon gamma (IFN-gamma)	Ifng	NM_138880.2 (187-286)	80	82	1	0
	interleukin 10 (IL10)	Il10	NM_012854.2 (186-285)	81	79	3	0
	interleukin 1 beta (IL-1-beta)	Il1b	NM_031512.1 (441-540)	80	81	1	0
	interleukin 2 (IL2)	Il2	NM_053836.1 (6-105)	83	81	1	0
	interleukin 6 (IL6)	Il6	NM_012589.1 (56-155)	79	83	1	0
	nuclear factor kappa B subunit 1 (NFK-beta)	Nfkb1	XM_342346.3 (3561-3660)	83	82	3	0
	Tumor necrosis factor (TNF-alpha)	Tnf	NM_012675.2 (306-405)	82	79	1	0
Enzymes (UGT)	UDP glucuronosyltransferase family 1 member A1 (ugt-1a1)	Ugt1a1	NM_012683.2 (795-894)	79	82	1	0
	UDP glucuronosyltransferase family 1 member A6 (ugt-1a6)	Ugt1a6	NM_001039691.1 (791-890)	80	82	2	0
	UDP glucuronosyltransferase 2 family, polypeptide B1 (ugt-2b1)	Ugt2b1	NM_173295.1 (1504-1603)	76	78	1	0
Enzymes (Vitamin D)	cytochrome P450, family 24, subfamily a, polypeptide 1 (cyp24)	Cyp24a1	NM_201635.2 (817-916)	82	82	1	0
	cytochrome P450, family 27, subfamily b, polypeptide 1 (cyp27b1)	Cyp27b1	NM_053763.1 (2149-2248)	79	79	2	0
Housekeeping	actin, beta (Actin B)	Actb	NM_031144.2 (20-119)	83	83	1	0
	glyceraldehyde-3-phosphate dehydrogenase (gapd)	Gapdh	NM_017008.2 (851-950)	82	82	2	0
	mitogen activated protein kinase 14 (mapk14)	Mapk14	NM_031020.2 (738-837)	84	85	3	0
	Phosphoglycerate kinase 1 (pgk1)	Pgk1	NM_053291.3 (1566-1665)	80	81	1	0

Target class	*Description*	*HUGO gene name*	*Accession (position)*	*Tm CP*	*Tm RP*	*Total Isoforms*	*# Not Hit*
Luminal Transporters	Ring finger protein 1 (ring1)	Ring1	NM_212549.2 (345-444)	82	84	2	0
	ATP binding cassette subfamily C member 4 (mrp4)	Abcc4	NM_133411.1 (2626-2725)	78	83	2	0
	ATP binding cassette subfamily G member 2 (bcrp)	Abcg2	NM_181381.2 (1027-1126)	83	79	7	0
	Multidrug resistance protein 1 (mdr1a)	Abcb1	NM_001169152.1 (1971-2070)	79	83	1	0
	solute carrier family 13 member 2 (nadc1)	Slc13a2	NM_031746.1 (1641-1740)	82	81	3	0
	solute carrier family 15 member 1 (pept1)	Slc15a1	NM_001079838.1 (343-442)	82	78	3	0
	solute carrier family 15 member 2 (pept2)	Slc15a2	NM_031672.1 (931-1030)	83	79	1	0
	solute carrier family 16 member 1 (mct1)	Slc16a1	NM_012716.1 (1096-1195)	81	79	2	0
	solute carrier family 22 member 12 (urat1)	Slc22a12	NM_001034943.1 (1277-1376)	82	83	3	0
	solute carrier family 47 member 1 (mate1)	Slc47a1	NM_001014118.2 (1643-1742)	79	84	2	0
	solute carrier family 47 member 2 (mate-2k)	Slc47a2	NM_001191920.1 (1115-1214)	81	78	2	1[#]
	Solute carrier family 5 member 2 (SGLT-2)	Slc5a2	NM_022590.2 (605-704)	81	83	1	0
Inflammation	angiotensinogen (agt)	Agt	NM_134432.2 (874-973)	85	85	2	0
	hepatitis A virus cellular receptor 1 (kim-1)	Havcr1	NM_173149.1 (1186-1285)	82	81	5	0
	matrix metallopeptidase 2 (mmp-2)	Mmp2	NM_031054.2 (2436-2535)	83	83	1	0
	matrix metallopeptidase 9 (mmp-9)	Mmp9	NM_031055.1 (2566-2665)	79	78	1	0
	prostaglandin-endoperoxide synthase 1 (cox-1)	Ptgs1	NM_017043.3 (1881-1980)	81	83	2	0
	prostaglandin-endoperoxide synthase 2 (cox-2)	Ptgs2	NM_017232.3 (1606-1705)	80	78	1	0
receptors	Peroxisome proliferator activated receptor alpha (PPAR-alpha)	Ppara	NM_013196.1 (1011-1110)	80	78	9	0
	Peroxisome proliferator-activated receptor gamma (PPAR-gamma)	Pparg	NM_013124.1 (1291-1390)	79	82	4	0
	Prostaglandin D2 receptor-like	Ptgdr	NM_001135164.1 (286-385)	86	83	1	0
	Prostaglandin E receptor 2	Ptger2	NM_031088.1 (289-388)	83	84	2	0
	Prostaglandin I2 receptor	Ptgir	NM_001077644.1 (719-818)	84	83	1	0
	toll-like receptor 4	Tlr4	NM_019178.1 (2146-2245)	78	79	1	0
	Vitamin D receptor	Vdr	NM_017058.1 (1541-1640)	82	84	1	0
Regulator of transporter expression	hypoxia inducible factor 1 subunit alpha (HIF-1 alpha)	Hif1a	NM_024359.1 (363-462)	81	79	5	0
	yrdC N(6)-threonyl carbamoyl transferase domain containing protein (Ischemic reperfusion inducible protein)	Yrdc (irip)	NM_175604.2 (711-810)	83	83	1	0

[#] XM_017597448.1

3.4.5 Nanostring nCounter® assay

nCounter® Master kit was used to process, and load 100 ng of RNA obtained from kidney tissues (n=47) onto the custom codeset using manufacturer's instructions. nCounter® assays were processed on a fully automated PrepStation and data was collected and tabulated by the nCounter® Digital Analyzer at the Genomics Research Core, University of Pittsburgh, Pittsburgh, PA.

3.4.6 Internal and external controls

The technical performance of gene expression assay for each sample (each well) was assessed by the controls developed and tested by the External RNA Control Consortium (ERCC). The reporter probes designed against these ERCC transcript sequences are pre-mixed into every well of the codeset. Hence, each sample (well) has 14 internal controls tested by ERCC, that include 6 positive internal controls at varying concentrations (128 fM, 32 fM, 8 fM, 2 fM, 0.5 fM, and 0.125 fM) and 8 negative internal controls[200]. The range of internal concentrations corresponds to the expression levels of most mRNAs of interest present in 100 ng of total RNA[200,201]. Apart from the internal control, each well in this custom codeset was designed with 5 external controls (internal reference genes) that are otherwise called housekeeping genes (pgk1, ring1, actinβ, gapd and mapk14). The expression of five housekeeping genes that were chosen in this assay were used as internal reference for every sample, to adjust for the differences arising from amount and quality of mRNA across the samples. Normalization of target gene counts with the combined geometric means of these internal reference gene counts, permit comparison of observed gene expression levels when starting amounts for mRNA is not the same for samples and (or) even between samples with intact versus degraded mRNA.

3.4.7 Data Analysis

The nsolver® software (NanoString Technologies) was used for stepwise processing and data analysis of the results from nanostring ncounter® assays. The RCC files containing results are imported into nsovler and individual samples were visually inspected for any quality control flags. All samples selected for further data analysis were subjected to the nsolver® software-guided steps and described as follows. Expression counts of negative internal controls in each well were subtracted from the raw counts for each gene target in the corresponding sample lane to eliminate the non-specific background. Variability unrelated to the samples was eliminated by normalizing expression counts from each lane with the geometric mean of expression counts for the corresponding internal positive controls. Variability related to samples was minimized by calculating the average geometric means of housekeeping genes across all lanes and dividing this average by the geometric mean in each sample lane to get a lane-specific normalization factor. The normalized gene expression counts for each sample were finally obtained by multiplying the count of each gene by the lane (sample) specific factor. The nsolver® software was further used to build ratios in expression of every target gene across different treatment groups by using the normalized counts for that gene.

$$\text{Group 1 versus group 2} = \text{ratio} = \frac{\text{geometric mean of normalized gene count in group 1}}{\text{geometric mean of normalized gene count in group 2}}$$

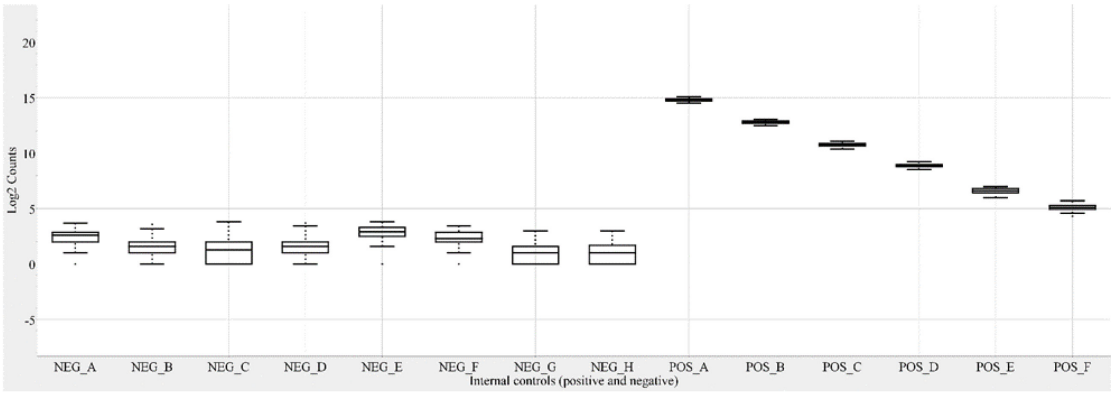
The differential expression of gene was finally expressed in fold-changes with 95% confidence interval, where a ratio >1 corresponds to the fold-change whereas, fold-change = (-1/ratio) when ratio <1. The positive and negative fold-changes were considered as relative over-expression and under-expression of the target gene, respectively.

Statistical testing was done on normalized log2 counts of each gene (log2 counts have normal distribution) by nsolver® using an in-built t-test with false discovery rate (FDR). nSolver performs a two-tailed t-test on the normalized log2 counts that assumes unequal variance and provides a p value. nSolver uses the Benjamini-Yekutieli procedure to calculate the FDR from the p-values returned by the t-test[200]. The 95 % CI for fold-change is also estimated from the distribution of the t-statistic calculated using the Welch-Satterthwaite equation. A p-value of <0.05 was considered as statistically significant difference in expression of target gene and the gene was reported to be differentially expressed between the compared groups.

3.5 RESULTS

3.5.1 Quality controls

The positive controls across all the 47 sample lanes, showed linear and consistent response without significant variability. As summarized in Figure 3.1, the approximately 1000-fold range (0.125 -128 fM) in positive control concentrations produced a matching linear response corresponding to a range of 10 units on the log2 scale. A positive control normalization flag was not observed in any of sample lane and was indicative of satisfactory assay efficiency across all sample lanes. The response of the lowest positive control was considered below the limit of detection of the system. Hence, response for eight negative controls across all sample lanes were below the limit of detection, as shown in Figure 3.1. This indicated that negative controls did not hybridize to any of targets within all samples, thus conclusively ruling out any non-specific binding of probes in the analyzed samples.



*n=47

Figure 3.1. Assay performance for positive and negative controls

3.5.2 Internal reference genes

All the five housekeeping genes were adequately detectable and showed a relatively stable expression across all the samples. As shown in Figure 3.2, the expression of housekeeping genes was separable as either high (actin beta, gapd), medium (pgk1) or low expression (mapk14, ring1) categories. Hence, the chosen housekeeping genes were satisfactory candidates for adequate minimization of sample variability, as they were representative of all genes in this assay, across the low to high expression range.

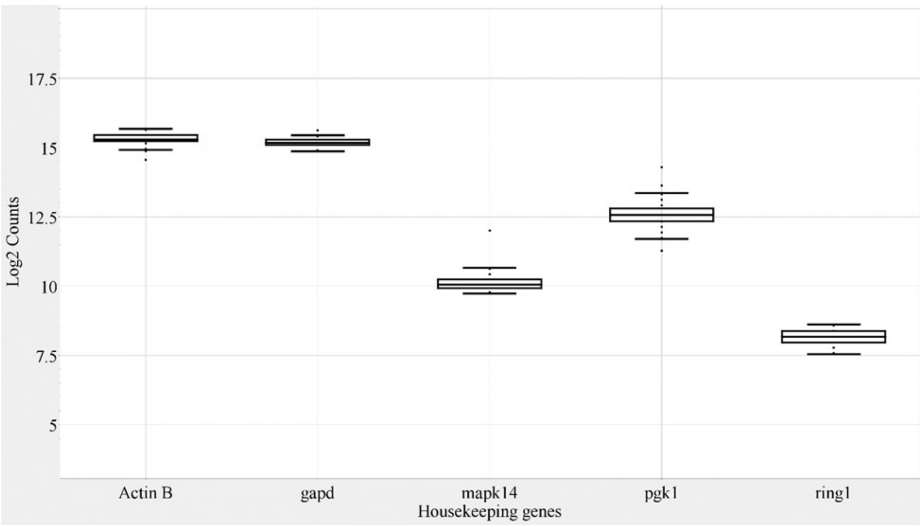


Figure 3.2. Expression of housekeeping genes (n=47 samples)

3.5.3 Expression of select gene targets in control (blank) rat kidneys

Blank frozen kidneys were used as controls for evaluating effects of 24 and 72 h cold storage on gene expression of selected targets. Results from blank tissues are shown as violin plots of normalized log 2 counts and are representative of the median and distribution in basal expression levels for the selected gene targets in 8-week-old Sprague Dawley rat kidneys.

a) Renal drug transporters:

Figure 3.3 shows the pattern in renal gene expression for seven basolateral and eleven apical (luminal) transporters. The Na/K-ATPase and glut-2 showed the highest and lowest relative expressions, respectively while oat-3 showed a higher range in expression count values when compared with other basolateral transporters. On the luminal side, mate1 and mate2k showed the highest and lowest relative expression. Pept1 and pgp had lower relative expression compared to other transporters that were evaluated, with the former showing a wide distribution in expression while the latter showed a very precise range for expression.

b) Renal cyp, antioxidant and ugt enzymes:

Figure 3.4 shows comparative expression levels in log2 counts for the chosen enzyme targets. Ugt2b1 had relatively poor expression in comparison to ugt1a1 and ugt1a6. Similarly, cyp27b1 had very low expression compared to cyp 24, while all the three antioxidant superoxide dismutase enzymes showed a relatively higher expression.

c) Cytokines, chemokines, markers of injury and inflammation:

As shown in Figures 3.5 and 3.6, the naïve kidneys showed a lower expression of target genes that are activated prior to or following inflammation and injury to an organ. The only exceptions with a moderately higher expression than other targets in this category were the cytokine Nfk-beta and hypoxia inducible factor-1.

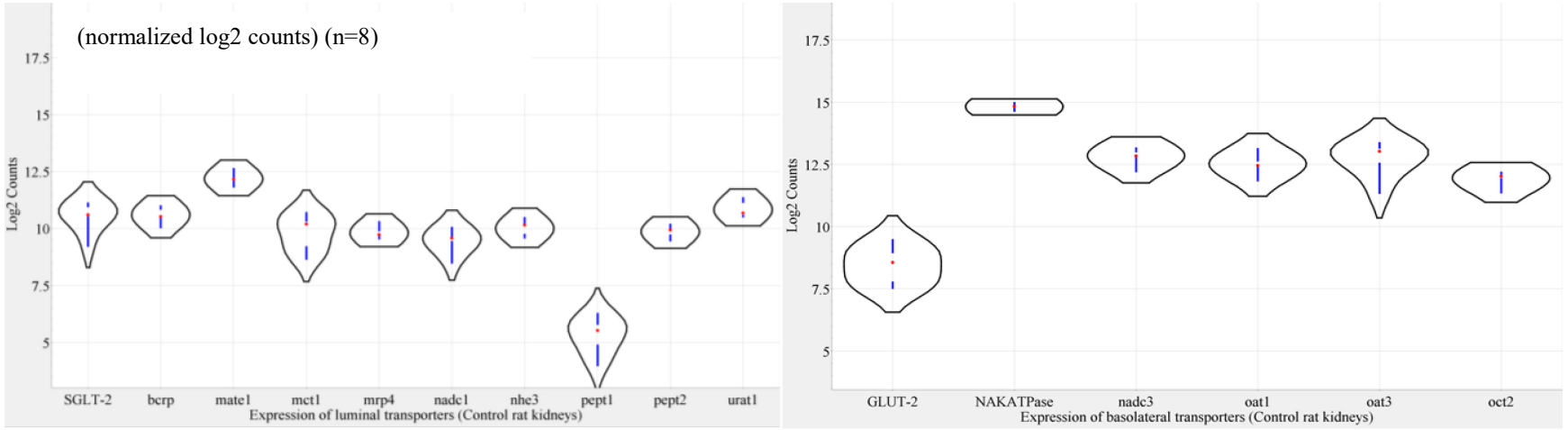


Figure 3.3. Relative expression of basolateral and apical transporters in blank control rat kidneys

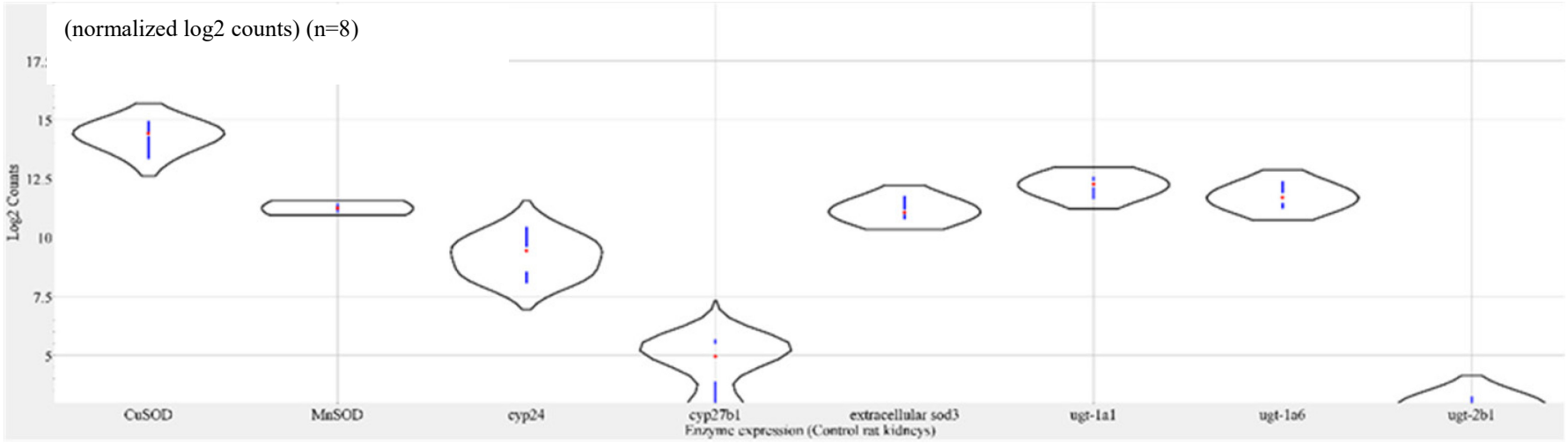


Figure 3.4. Relative expression of select antioxidant and metabolic enzymes in blank control rat kidneys

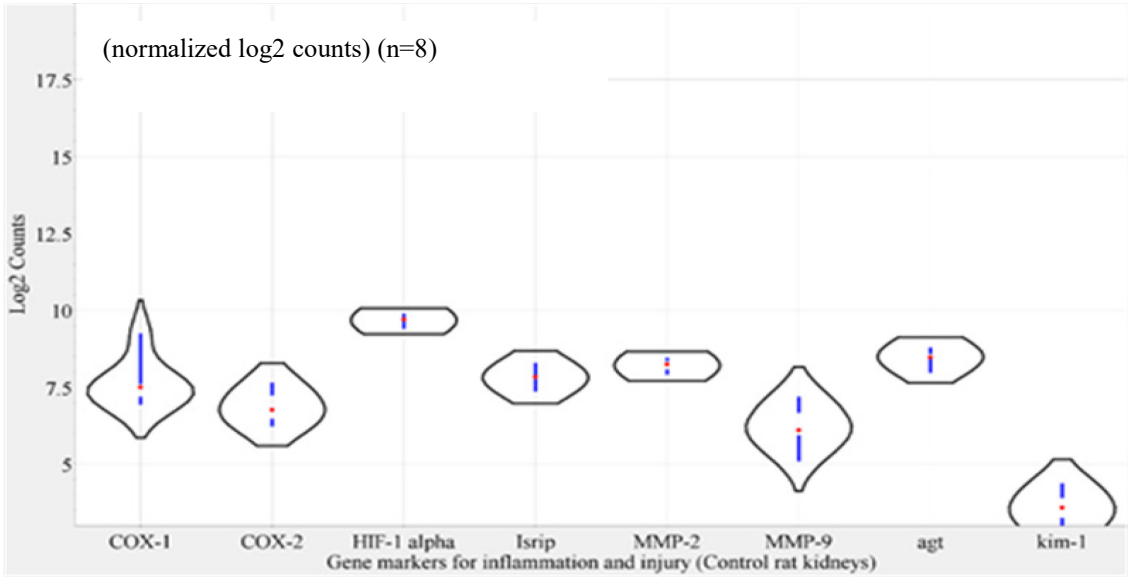


Figure 3.5. Relative expression of select markers for inflammation and injury in blank control rat kidneys

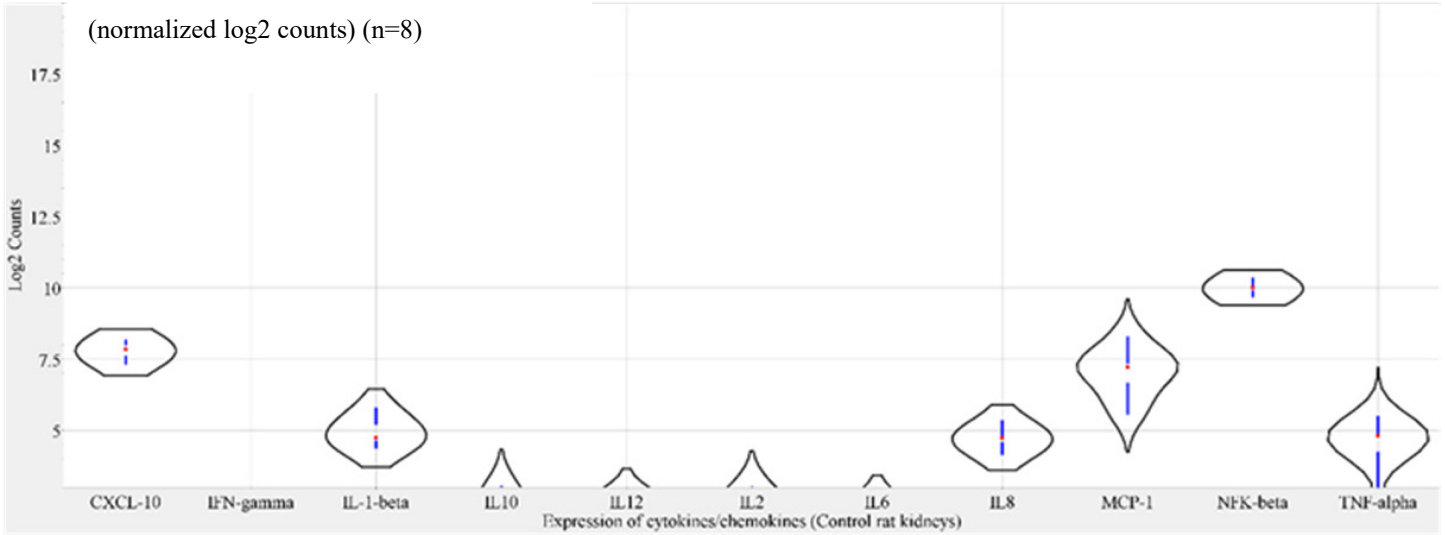


Figure 3.6. Relative expression of select cytokines and chemokines in blank control rat kidneys

3.5.4 Effect of CS (24 and 72 h CS- with or without treprostinil addition) on the expression of gene targets in rat kidneys

Results of gene expression were compared as fold changes (with 95% CI) between groups. Though the significance of fold-change was evaluated with FDR p-values, consideration was also given to the presence of an appreciable amount of gene target (at least > 100 counts) for the fold-change to be meaningful. Table 3.2 compares the expression between groups 1, 2 and 3 representing blank controls, 24 h CS and 24 h CS with 50 ng/mL of treprostinil, respectively. Overall, 24 h CS did gnot produce a statistically significant fold-change on expression of select target genes. A statistically significant upregulation in expression of bcrp and ugt1a1 genes was observed in 24 h CS with treprostinil addition when compared to controls.

Table 3.2. Renal gene expression after 24 h of CS (with or without treprostinil (50 ng/mL)) versus blank control

Class of gene	Gene name	24h-CS vs Ctrl Fold change (95 % CI)	p-value	24h-CS with treprostinil vs Ctrl Fold change (95 % CI)	p-value
BL Transporters	Nakatpase	1.08 (0.9 to 1.3)	0.303	1.06 (0.9 to 1.2)	0.237
	Oct2	-1.06 (-0.8 to -1.4)	0.465	1.16 (0.7 to 1.9)	0.318
	Glut-2	1.25 (0.2 to 6.7)	0.689	1.19 (0.3 to 5.1)	0.756
	Oat3	1.28 (0.5 to 3.7)	0.541	-1.13 (-0.2 to -5.6)	0.822
	Oat1	1.17 (0.5 to 3)	0.630	-1.07 (-0.4 to -3)	0.848
	Nadc3	1.01 (0.5 to 1.9)	0.981	-1.03 (-0.5 to -2)	0.919
Luminal Transporters	Bcrp	1.29 (1 to 1.7)	0.056	1.28 (1 to 1.6)	0.042
	Nadc1	1.27 (0.6 to 2.8)	0.431	1.32 (0.6 to 3)	0.387
	Abcb1	-1.07 (-0.6 to -2)	0.687	1.03 (0.9 to 1.2)	0.444
	Pept2	-1.11 (-0.8 to -1.5)	0.402	1.14 (0.7 to 1.8)	0.457
	Sglt-2	1.18 (0.4 to 3.2)	0.670	-1.26 (-0.4 to -3.8)	0.602
	Mct1	1.28 (0.5 to 3)	0.459	1.24 (0.3 to 5.2)	0.656
	Pept1	1.22 (0.3 to 5)	0.720	-1.26 (-0.1 to -11.1)	0.756
	Mate1	1.02 (0.7 to 1.6)	0.899	1.02 (0.7 to 1.6)	0.888
	Urat1	-1.07 (-0.6 to -1.8)	0.728	-1.02 (-0.6 to -1.8)	0.910

Class of gene	Gene name	24h-CS vs Ctrl Fold change (95 % CI)	p-value	24h-CS with treprostinil vs Ctrl Fold change (95 % CI)	p-value
	Mrp4	-1.03 (-0.7 to -1.5)	0.838	-1.01 (-0.7 to -1.4)	0.934
	Mate-2k	1.08 (0.3 to 4.2)	0.878	1.03 (0.3 to 3.6)	0.955
	Nhe3	-1.02 (-0.6 to -1.7)	0.921	-1.01 (-0.6 to -1.7)	0.962
Regulation of transporters	Hif-1 alpha	1.04 (0.8 to 1.4)	0.671	1.14 (0.9 to 1.4)	0.191
	Irip	1.02 (0.8 to 1.3)	0.791	1.02 (0.8 to 1.3)	0.842
Enzymes (UGT)	Ugt-1a1	1.11 (0.7 to 1.7)	0.520	1.65 (1.1 to 2.5)	0.024
	Ugt-2b1	-1.01 (-0.3 to -3.7)	0.984	1.61 (0.5 to 4.9)	0.285
	Ugt-1a6	-1 (-0.6 to -1.8)	0.997	1.08 (0.6 to 1.9)	0.720
Enzymes (vitamin D)	Cyp27b1	1.55 (0.3 to 8.8)	0.475	1.6 (0.1 to 27.7)	0.628
	Cyp24	1.66 (0.3 to 9.1)	0.407	1.24 (0.3 to 5.5)	0.667
Cytokines	Ifn-gamma	1.21 (0.7 to 2.1)	0.366	-1.7 (-1 to -2.9)	0.043
	Cxcl-10	1.14 (0.5 to 2.6)	0.644	1.38 (0.9 to 2.1)	0.104
	Tnf-alpha	1.34 (0.2 to 8.5)	0.642	1.94 (0.3 to 11.6)	0.352
	Il6	1.3 (0.1 to 20.4)	0.710	2.22 (0.1 to 46.2)	0.353
	Il10	1.53 (0.7 to 3.5)	0.216	1.61 (0.4 to 7.3)	0.378
	Il-1-beta	1.25 (0.6 to 2.6)	0.436	1.19 (0.6 to 2.3)	0.460
	Il2	1.96 (0.3 to 11.4)	0.299	1.31 (0.3 to 5)	0.603
	Nfk-beta	1.01 (0.8 to 1.3)	0.916	-1.02 (-0.9 to -1.2)	0.689
Chemokines	Mcp-1	2.07 (0.7 to 6)	0.114	2.25 (0.9 to 5.8)	0.072
	Il12	1.76 (0.5 to 5.8)	0.215	1.55 (0.5 to 5.2)	0.358
	Il8	1.39 (0.4 to 4.5)	0.443	1.44 (0.5 to 4.4)	0.369
Antioxidant	Cu sod	1.31 (0.7 to 2.3)	0.218	1.31 (0.8 to 2.3)	0.211
	extracellular sod3	1.04 (0.4 to 2.5)	0.845	-1.14 (-0.4 to -3.1)	0.643
	Mn sod	-1.11 (-1 to -1.3)	0.074	-1.02 (-0.9 to -1.1)	0.644
Inflammation	Cox-2	1.36 (0.9 to 2.1)	0.108	1.7 (0.8 to 3.6)	0.085
	Kim-1	1.34 (0.5 to 3.5)	0.438	1.34 (0.6 to 2.8)	0.330
	Mmp-9	1.52 (0.3 to 8.4)	0.495	1.8 (0.2 to 19.1)	0.362
	Agt	-1.22 (-0.9 to -1.7)	0.116	1.07 (0.9 to 1.3)	0.476
	Cox-1	-1 (-0.3 to -3.1)	0.993	1.26 (0.3 to 4.8)	0.661
	Mmp-2	-1.08 (-0.4 to -3)	0.776	1.03 (0.7 to 1.5)	0.852
Receptors	Pg I2 receptor	1.1 (0.5 to 2.5)	0.643	1.55 (0.9 to 2.6)	0.062
	Pg E receptor 2	1.17 (0.5 to 2.9)	0.644	1.51 (0.6 to 3.9)	0.278
	Pg receptor dp1	-1.04 (-0.3 to -3.1)	0.930	1.31 (0.4 to 4.2)	0.515
	Ppar-alpha	-1.17 (-0.7 to -2)	0.459	-1.12 (-0.6 to -2.1)	0.634
	Vit D receptor	-1.13 (-0.5 to -2.8)	0.605	-1.04 (-0.8 to -1.4)	0.654
	Toll-like receptor 4	1.03 (0.2 to 4.7)	0.935	-1.03 (-0.7 to -1.4)	0.836

Class of gene	Gene name	24h-CS vs Ctrl Fold change (95 % CI)	p-value	24h-CS with treprostinil vs Ctrl Fold change (95 % CI)	p-value
	Ppar-gamma	-1.8 (-0.8 to -4.2)	0.114	1.04 (0.5 to 2.1)	0.859

CI- Confidence Interval; significant differences are in bold font and p<0.05 after controlling for false discovery rate; genes with significant differences in fold-changes are highlighted in green (upregulated) or orange (downregulated) but genes with counts<100 are ignored; Pg- Prostaglandin; CS- Cold Storage

Similarly, comparison of groups 8, 9 and 10 did not indicate any major influence by 72 h CS, indicated by lack of significant changes in expression (fold-change), as shown in Table 3.3. Only 2 genes showed a statistically significant fold-change in comparison to blank control, bcrp was downregulated in 72 h CS with treprostinil addition and cox-2 was upregulated in the 72 h CS groups, respectively. Figure 3.7 shows attenuation of increased renal expression in cox-2 after 72 h CS with addition of treprostinil.

Table 3.3. Renal gene expression after 72 h of CS (with or without treprostinil (50 ng/mL)) versus blank control

Class of gene	Gene name	72h-CS vs Ctrl Fold change (95 % CI)	p-value	72h-CS with treprostinil vs Ctrl Fold change (95 % CI)	p-value
BL Transporters	Oat1	1.13 (0.8 to 1.6)	0.295	-1.34 (-0.9 to -2.1)	0.120
	Nadc3	1.07 (1 to 1.2)	0.082	-1.07 (-0.7 to -1.6)	0.561
	Nakatpase	1.1 (0.9 to 1.4)	0.190	1.07 (0.8 to 1.4)	0.314
	Glut-2	-1.06 (-0.3 to -3.6)	0.855	-1.24 (-0.3 to -5.6)	0.593
	Oct2	1.31 (0.8 to 2.2)	0.184	1.34 (0.8 to 2.3)	0.164
	Oat3	-1.14 (-0.7 to -1.7)	0.403	-1.08 (-0.3 to -4)	0.825
Luminal Transporters	Bcrp	-1.17 (-0.8 to -1.8)	0.211	-1.81 (1 to -3.2)	**0.034**
	Nadc1	1.02 (0.5 to 2)	0.928	-1.79 (-0.4 to -8.3)	0.246
	Pept2	1.08 (0.7 to 1.7)	0.615	-1.08 (-0.8 to -1.6)	0.532
	Mrp4	1.08 (0.9 to 1.3)	0.173	1.33 (0.7 to 2.5)	0.178
	Mate1	1.04 (0.9 to 1.2)	0.365	-1.16 (-0.6 to -2.1)	0.403
	Mate-2k	-1.21 (-0.2 to -7.1)	0.686	1.32 (0.6 to 2.7)	0.288
	Urat1	1.02 (0.6 to 1.8)	0.918	1.21 (0.8 to 1.9)	0.252
	Pept1	-1.06 (-0.3 to -4)	0.905	-1.46 (-0.4 to -4.8)	0.372
	Sglt-2	1.07 (0.5 to 2.1)	0.783	1.28 (0.6 to 2.6)	0.346
	Abcb1	1.07 (0.6 to 1.9)	0.631	-1.01 (-0.4 to -2.6)	0.976
	Mct1	-1.6 (-0.3 to -9.1)	0.350	-1.78 (-0.5 to -7.1)	0.195

Class of gene	Gene name	72h-CS vs Ctrl Fold change (95 % CI)	p-value	72h-CS with treprostinil vs Ctrl Fold change (95 % CI)	p-value
	Nhe3	1.03 (0.6 to 1.7)	0.854	1.04 (0.6 to 1.9)	0.817
Regulation of transporter	Irip	-1.07 (-0.7 to -1.7)	0.665	1.2 (0.8 to 1.8)	0.222
	Hif-1 alpha	1.05 (0.8 to 1.5)	0.548	1.2 (0.7 to 2.2)	0.316
Enzymes (UGT)	Ugt-1a1	-1.05 (-0.7 to -1.6)	0.758	-1.78 (-0.6 to -5.3)	0.118
	Ugt-2b1	1.21 (0.6 to 2.3)	0.332	1.16 (0.4 to 3.3)	0.591
	Ugt-1a6	-1.08 (-0.7 to -1.6)	0.566	-1.33 (-1 to -1.8)	0.050
Enzymes (vitamin D)	Cyp24	1.01 (0.2 to 4.6)	0.981	-1.32 (-0.2 to -7.1)	0.635
	Cyp27b1	-1.07 (-0.4 to -2.8)	0.827	1.68 (0.7 to 4.2)	0.158
Cytokines	Tnf-alpha	1.22 (0.6 to 2.7)	0.360	-1.45 (-0.6 to -3.7)	0.284
	Cxcl-10	1.26 (0.6 to 2.6)	0.283	-1.25 (-0.4 to -4.3)	0.507
	Il10	-1.28 (-0.3 to -6.3)	0.543	-1.05 (-0.3 to -3.6)	0.905
	Ifn-gamma	1.29 (0.4 to 3.9)	0.423	1.8 (0.4 to 8.5)	0.243
	Il6	-1.12 (-0.4 to -3.2)	0.748	-1.73 (-0.1 to -33.3)	0.464
	Il2	-1.23 (-0.3 to -5.6)	0.692	-1.35 (-0.3 to -5.9)	0.479
	Il-1-beta	1.39 (0.4 to 4.7)	0.335	1.48 (0.7 to 3.3)	0.222
	Nfk-beta	1.06 (0.8 to 1.4)	0.479	1.16 (1 to 1.4)	0.056
Chemokines	Il12	2.03 (0.6 to 6.9)	0.158	1.15 (0.2 to 7.1)	0.758
	Mcp-1	-1.02 (-0.3 to -3.3)	0.969	-1.31 (0 to -50)	0.789
	Il8	-1.3 (-0.4 to -3.8)	0.411	-1.29 (-0.5 to -3.2)	0.351
Anti-oxidant	extracellular sod3	-1.23 (-0.6 to -2.5)	0.412	1.47 (0.7 to 3.2)	0.210
	Cu sod	-1.27 (-0.8 to -2)	0.175	-1.7 (-0.5 to -6.3)	0.197
	Mn sod	-1.13 (-0.9 to -1.4)	0.139	-1.08 (-0.9 to -1.3)	0.269
Inflammation	agt	1.09 (0.7 to 1.8)	0.598	-1.26 (-0.8 to -1.9)	0.160
	Mmp-9	1.34 (0.4 to 4.8)	0.504	-1.45 (-0.4 to -5.3)	0.298
	Cox-2	1.96 (1.1 to 3.6)	**0.028**	1.03 (0.3 to 3.9)	0.923
	Cox-1	1.57 (0.6 to 3.9)	0.208	-1.06 (-0.5 to -2.3)	0.812
	Kim-1	1.74 (0.5 to 6)	0.175	-1.32 (-0.2 to -8.3)	0.660
	Mmp-2	1.05 (0.7 to 1.6)	0.642	1.04 (0.7 to 1.6)	0.807
Receptors	Ppar-alpha	-1.05 (-0.8 to -1.4)	0.631	1.07 (0.8 to 1.4)	0.480
	Pg E receptor 2	2.28 (0.2 to 24)	0.262	1.28 (0.1 to 16.5)	0.771
	Ppar-gamma	1.23 (0.8 to 2)	0.230	1.11 (0.7 to 1.8)	0.408
	Pg receptor dp1	1.27 (0.2 to 6.9)	0.598	1.44 (0.2 to 9.1)	0.459
	Pg I2 receptor	1.26 (0.4 to 4.6)	0.497	-1.03 (-0.1 to -7.1)	0.953
	Toll-like receptor 4	-1.13 (-0.7 to -1.9)	0.398	-1.11 (-0.4 to -3)	0.698
	Vit D receptor	1.04 (0.6 to 1.8)	0.809	1.03 (0.3 to 3.2)	0.914

CI- Confidence Interval; significant differences are in bold font and p<0.05 after controlling for false discovery rate; genes with significant differences in fold-changes are highlighted in green (upregulated) or orange (downregulated); Pg- Prostaglandin; CS- Cold Storage

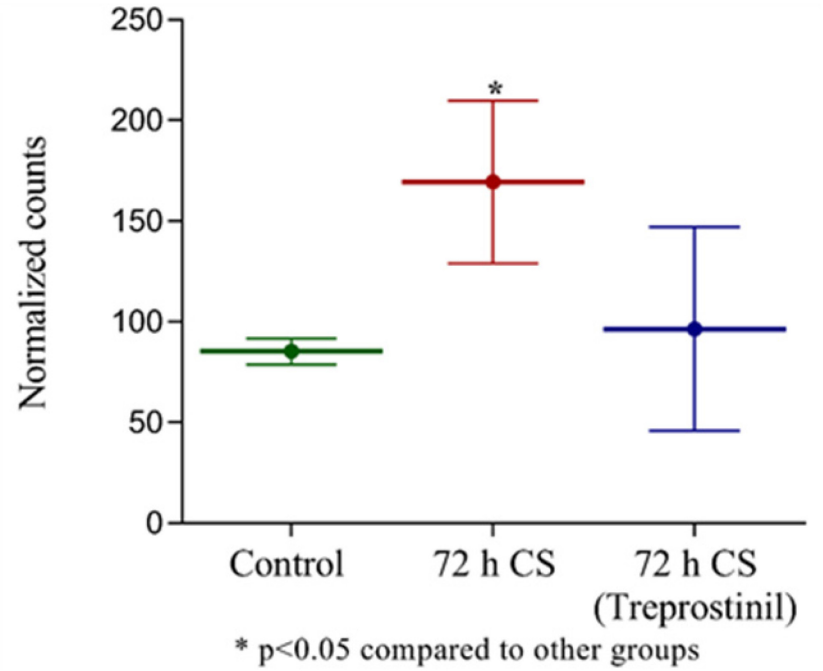


Figure 3.7. Cox-2 expression in rat kidneys after 72 h cold preservation
Control- no cold storage; 72 h CS- cold storage for 72 h in UW solution; 72 h CS (Treprostinil)- cold storage for 72 h in UW solution with 50 ng/mL treprostinil (n=4/group)

3.5.5 Effect of CS with reperfusion (24 h CS and 2 h reperfusion) on the expression of gene targets in rat kidneys

In contrast to CS only, CS with reperfusion for 2 hours, showed higher fold-changes in expression of several target genes. The control group was perfused for 2 hours immediately after isolation and without any cold storage. Figure 3.8 illustrates only the most differentially expressed genes in perfused control group versus blank kidneys and is highlighted by a several-fold change in expression for cytokines like Cxcl-10, Nfk-beta, Il-6, Il-1-beta, Tnf-alpha, chemokines like Mcp-1 and Il-8 and injury markers like kim-1 and cox-2. An equivalent fold-increase in expression of these genes was also observed in kidneys reperfused after CS, except for Cxcl-10 and kim-1,

that showed a significantly higher expression in the control perfusion group. Expression fold-changes for additional genes in kidneys reperfused after 24 h CS (groups 5, 6 and 7) are compared against the control perfused kidneys in Table 3.4 and significant differences are summarized in Figure 3.9. In reperfused kidneys, there was a significant decrease in expression of nhe3, MnSOD and Vit D receptor and an increase in expression of the gene for ischemia reperfusion injury protein (Irip). Treprostinil during CS was found to significantly attenuate the increase in Irip and the decrease in nhe3 expression. Similar effects were observed with addition of treprostinil to both CS and reperfusion in addition to preventing the decrease in anti-oxidant MnSOD, Vdr and an increase in urat1 transporter expression.

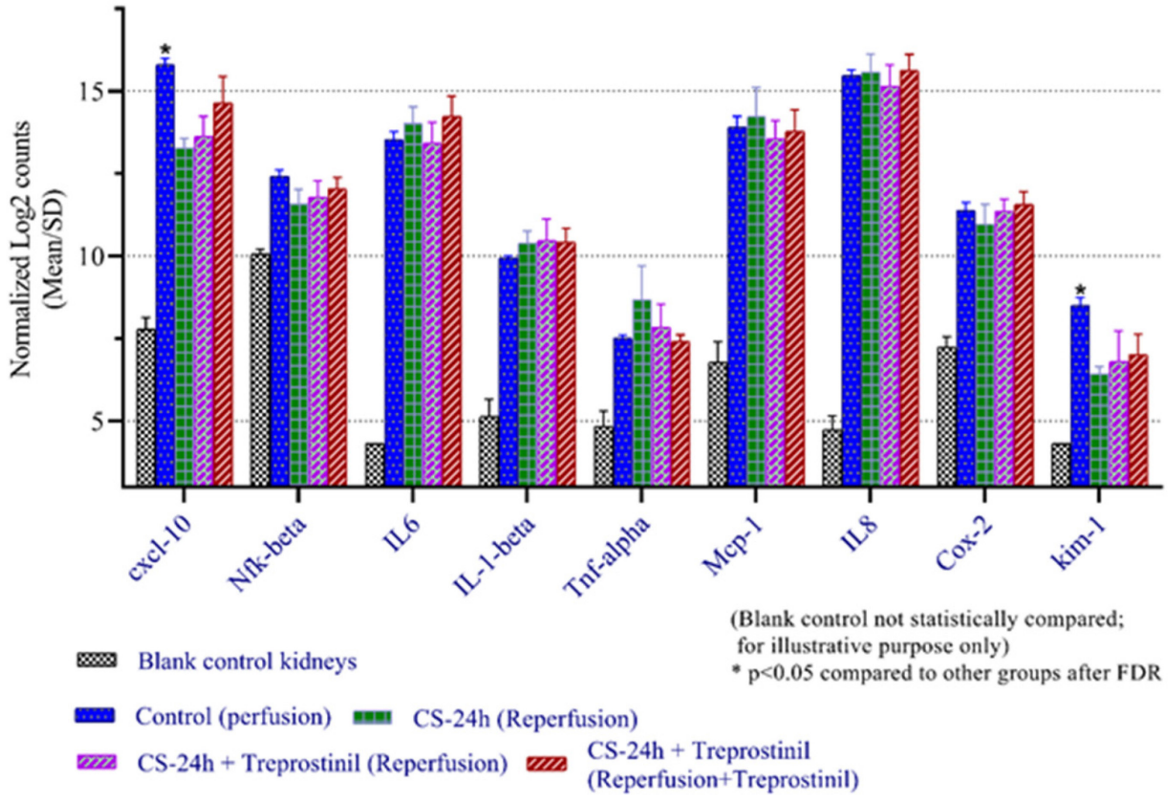


Figure 3.8. Differential expression of genes after isolated perfusion of fresh control rat kidneys (2 hours)

Table 3.4. Renal gene expression following 2-hour reperfusion after 24 h of CS and with treprostinil addition to only storage or both storage and reperfusion versus control kidneys (perfused without CS)

Class of gene	Gene name	**24h-CS + Reperfusion vs Ctrl perfusion** Fold change (CI)	p-value	**24h-CS (treprostinil) + Reperfusion vs Ctrl perfusion** Fold change (CI)	p-value	**24h-CS + Reperfusion (both treprostinil) vs Ctrl perfusion** Fold change (CI)	p-value
BL Transporters	Glut-2	1.04 (0.4 to 2.7)	0.913	1.21 (0.5 to 3)	0.540	1.01 (0.4 to 2.8)	0.979
	Nadc3	-1.1 (-0.7 to -1.7)	0.566	-1.06 (-0.8 to -1.4)	0.652	-1.09 (-0.5 to -2.6)	0.685
	Nakatpase	-1.35 (-0.7 to -2.5)	0.200	-1.1 (-0.8 to -1.5)	0.357	-1.11 (-0.8 to -1.6)	0.315
	Oat1	1 (0.5 to 2)	0.997	1.04 (0.6 to 2)	0.864	-1.2 (-0.4 to -3.6)	0.625
	Oat3	1.07 (0.5 to 2.4)	0.838	1.01 (0.6 to 1.6)	0.947	-1.15 (-0.5 to -2.6)	0.626
	Oct2	1.18 (0.7 to 2)	0.412	1.05 (0.5 to 2.3)	0.860	1.25 (0.8 to 1.9)	0.117
	Bcrp	1.61 (0.4 to 7)	0.411	1.65 (0.5 to 5.2)	0.250	1.45 (0.5 to 4.6)	0.374
Luminal Transporters	Mate-2k	-1.31 (-0.3 to -6.3)	0.603	-1.93 (-0.5 to -8.3)	0.262	-1.02 (-0.1 to -7.1)	0.972
	Mate1	1.03 (0.5 to 2)	0.907	-1.04 (-0.7 to -1.6)	0.790	1.03 (0.6 to 1.7)	0.875
	Mct1	1.31 (0.5 to 3.3)	0.464	1.16 (0.4 to 3.2)	0.711	1.15 (0.4 to 3.6)	0.652
	Mrp4	-1.49 (-0.9 to -2.6)	0.104	-1.18 (-0.8 to -1.7)	0.254	-1.07 (-0.8 to -1.4)	0.516
	Nadc1	-1.32 (-0.3 to -5.3)	0.612	1.18 (0.3 to 4.3)	0.710	-1.18 (-0.2 to -5.9)	0.763
	Nhe3	-1.65 (0.9 to -2.4)	0.014	-1.13 (-0.7 to -1.7)	0.455	-1.54 (-0.6 to -3.8)	0.145
	Pept1	-1.05 (-0.5 to -2.4)	0.876	-1.36 (-0.6 to -3.1)	0.358	-1.44 (-0.4 to -4.8)	0.398
	Pept2	1.22 (0.6 to 2.4)	0.409	1.18 (0.5 to 2.8)	0.570	1.35 (0.9 to 2.1)	0.090
	Abcb1	-1.26 (-0.9 to -1.7)	0.079	-1.06 (-0.9 to -1.2)	0.379	-1.04 (-0.6 to -1.8)	0.783
	Sglt-2	-1.15 (-0.6 to -2.2)	0.539	-1.17 (-0.7 to -1.9)	0.350	-1.2 (-0.6 to -2.3)	0.343
	Urat1	1.22 (0.5 to 2.8)	0.493	1.14 (0.6 to 2.2)	0.557	1.56 (1.2 to 2.1)	0.013
Regulation of transporter	Hif-1alpha	-1.04 (-0.7 to -1.6)	0.793	-1.27 (0.9 to -1.4)	0.004	-1.13 (-0.8 to -1.6)	0.265
	Irip	1.45 (1 to 2.2)	0.046	1.13 (0.9 to 1.5)	0.276	1.2 (0.9 to 1.6)	0.129
	Cyp24	-1.9 (-0.5 to -6.7)	0.220	-1.27 (-0.5 to -3.6)	0.511	-1.21 (-0.3 to -4.8)	0.595

Class of gene	Gene name	**24h-CS + Reperfusion vs Ctrl perfusion** Fold change (CI)	p-value	**24h-CS (treprostinil) + Reperfusion vs Ctrl perfusion** Fold change (CI)	p-value	**24h-CS + Reperfusion (both treprostinil) vs Ctrl perfusion** Fold change (CI)	p-value
Enzymes (vitamin D)	Cyp27b1	1.1 (0.8 to 1.5)	0.416	-1.13 (-0.8 to -1.5)	0.334	1.12 (0.7 to 1.9)	0.521
Enzymes (UGT)	Ugt-1a1	-1.06 (-0.5 to -2.1)	0.808	1.27 (0.7 to 2.4)	0.356	-1.02 (-0.4 to -2.4)	0.940
	Ugt-1a6	-1.17 (-0.4 to -3.3)	0.668	1.03 (0.3 to 3.5)	0.927	1.02 (0.3 to 3.4)	0.948
	Ugt-2b1	-1.36 (-0.6 to -3.2)	0.239	-1.3 (-0.6 to -2.9)	0.412	-1.79 (-0.7 to -4.8)	0.132
Cytokines	Cxcl-10	-5.8 (0.3 to -9.1)	0.000	-4.92 (0.5 to -11.1)	0.005	-2.65 (-0.4 to -16.7)	0.140
	Ifn-gamma	-5.46 (0.8 to -25)	0.028	-8.91 (0.3 to -25)	0.003	-5.35 (-0.4 to -100)	0.105
	Il-1-beta	1.31 (0.8 to 2.2)	0.186	1.28 (0.6 to 2.9)	0.409	1.33 (0.5 to 3.4)	0.328
	Il10	1.52 (0.4 to 5.7)	0.368	1.38 (0.8 to 2.4)	0.152	1.49 (0.7 to 3.1)	0.163
	Il2	1.76 (0.3 to 9.6)	0.398	1.26 (0.4 to 4.2)	0.621	1.89 (0.4 to 9.7)	0.287
	Il6	1.29 (0.5 to 3.4)	0.449	-1.21 (-0.5 to -3.2)	0.580	1.46 (0.3 to 7.4)	0.404
	Nfk-beta	-1.87 (0.9 to -3.1)	0.025	-1.66 (-0.8 to -3.4)	0.093	-1.33 (-0.6 to -2.9)	0.221
	Tnf-alpha	1.65 (0.4 to 6.3)	0.318	1.05 (0.3 to 3.3)	0.892	-1.08 (-0.7 to -1.7)	0.490
Chemokines	Il12	-2.36 (0.6 to -3)	0.001	-2 (0.8 to -3)	0.009	-1.38 (-0.6 to -3.3)	0.244
	Il8	-1.04 (-0.4 to -2.8)	0.915	-1.39 (-0.6 to -3.3)	0.308	1.05 (0.3 to 3.3)	0.871
	Mcp-1	-1.07 (-0.2 to -5)	0.896	-1.39 (-0.6 to -3.1)	0.317	-1.2 (-0.4 to -3.6)	0.640
Anti-oxidant	Cu sod	1.41 (0.5 to 3.8)	0.379	1.29 (0.6 to 2.9)	0.370	1.02 (0.4 to 2.8)	0.950
	extracellular sod3	1.21 (0.6 to 2.3)	0.457	-1.01 (-0.5 to -1.9)	0.972	1.37 (0.7 to 2.5)	0.203
	Mn sod	-1.75 (1 to -3)	0.043	-1.64 (0.8 to -2.3)	0.013	-1.34 (-0.7 to -2.6)	0.191
Inflammation	Agt	-1.2 (-0.6 to -2.4)	0.499	-1.09 (-0.5 to -2.4)	0.786	-1.21 (-0.6 to -2.3)	0.392
	Cox-1	-1.1 (-0.3 to -3.6)	0.811	-1.32 (-0.5 to -3.3)	0.395	-1.53 (-0.9 to -2.5)	0.063
	Cox-2	-1.48 (-0.6 to -3.6)	0.233	-1.06 (-0.6 to -1.8)	0.778	1.09 (0.4 to 2.8)	0.714
	Kim-1	-4.22 (0.4 to -6.7)	0.000	-4.5 (-1 to -20)	0.045	-3.22 (-0.5 to -20)	0.091
	Mmp-2	-2.58 (-0.4 to -14.3)	0.168	-1.15 (-0.5 to -2.5)	0.598	-1.04 (-0.5 to -2.4)	0.886
	Mmp-9	-2.44 (-0.3 to -25)	0.199	-1.74 (-0.3 to -10)	0.387	-1.18 (-0.2 to -6.7)	0.786

Class of gene	Gene name	**24h-CS + Reperfusion vs Ctrl perfusion** Fold change (CI)	p-value	**24h-CS (treprostinil) + Reperfusion vs Ctrl perfusion** Fold change (CI)	p-value	**24h-CS + Reperfusion (both treprostinil) vs Ctrl perfusion** Fold change (CI)	p-value
Receptors	Ppar-alpha	-1.3 (-0.8 to -2)	0.153	-1.33 (-0.9 to -2)	0.135	-1.27 (-0.7 to -2.3)	0.257
	Ppar-gamma	1.03 (0.2 to 4.5)	0.928	1.05 (0.3 to 4.1)	0.896	-1.36 (-0.5 to -4.2)	0.439
	Pg E receptor 2	1.51 (0.5 to 4.2)	0.316	1.22 (0.4 to 3.7)	0.637	1.71 (0.6 to 4.6)	0.167
	Pg I2 receptor	-2.28 (-0.6 to -8.3)	0.144	-1.56 (-0.6 to -4.2)	0.158	-1.36 (-0.5 to -3.8)	0.393
	Pg receptor dp1	1.54 (0.4 to 5.7)	0.403	1.71 (0.7 to 4.5)	0.185	1.91 (0.8 to 4.7)	0.087
	Toll-like receptor 4	-1.35 (-0.5 to -3.4)	0.423	-1.17 (-0.4 to -3.6)	0.599	-1.07 (-0.4 to -3)	0.852
	Vit D receptor	-2.39 (0.9 to -5)	0.028	-2.04 (0.6 to -2.6)	0.0003	-1.51 (-0.8 to -3)	0.104

CI- Confidence Interval; significant differences are in bold font and $p<0.05$ after controlling for false discovery rate; genes with significant differences are highlighted in green (upregulated) or orange (downregulated), but genes with counts<100 are ignored; Pg- Prostaglandin

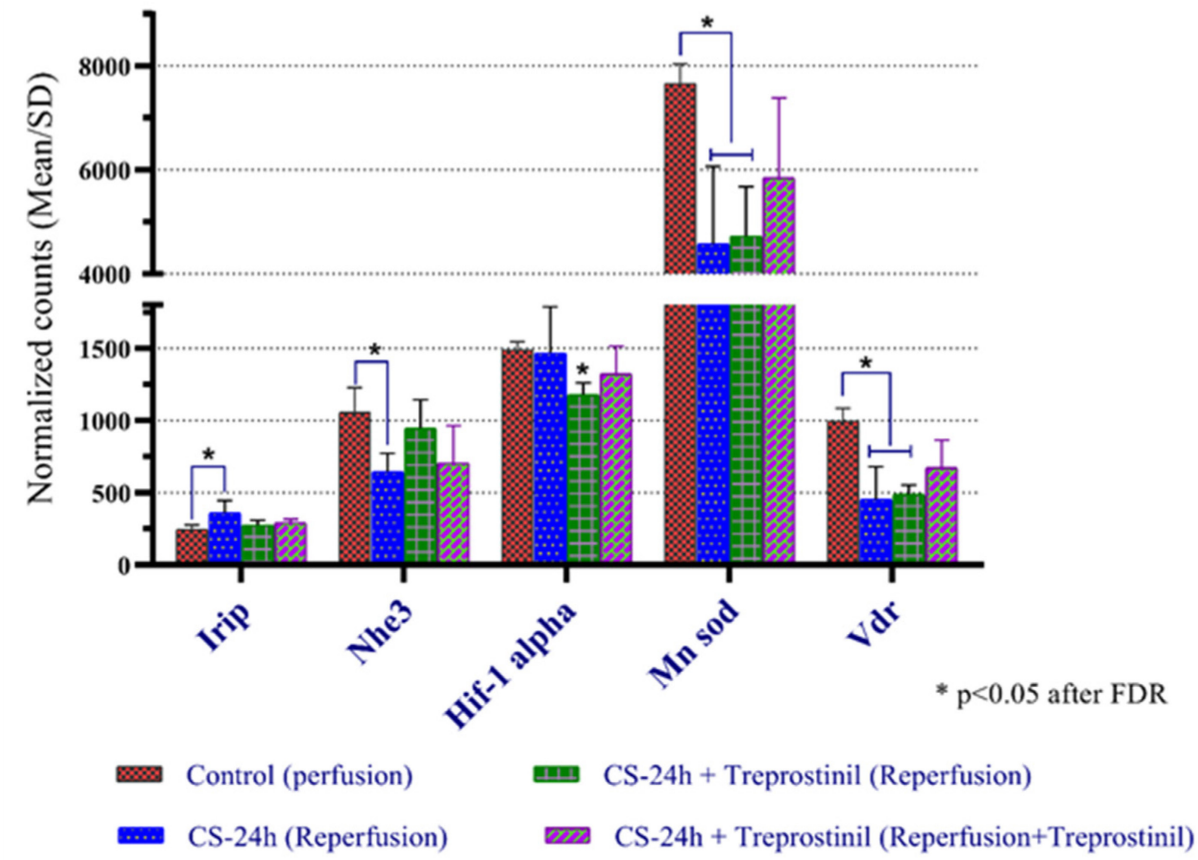


Figure 3.9. Differential gene expression and effect of treprostinil on kidneys reperfused after 24 h CS

3.6 DISCUSSION

Renal transplantation associated ischemia reperfusion (IR) not only originates from different cells of the renal, vascular or immune systems like endothelial, tubular or immune cells, but also results in activation of different injury mechanisms like inflammation or oxidative stress. In this ex-vivo model for IR during renal transplantation, gene expression changes in kidney were individually examined either after cold ischemia or following warm reperfusion. The panel of gene targets of renal importance were chosen in the context of IR injury such as mediators of inflammation, injury, antioxidants, enzymes and transporters. Simultaneous quantitative

comparison of expression in genes for various mechanisms was examined by application of nanostring assay. The nanostring® designed custom codeset for rat kidney contained target-specific oligonucleotide probes, capable of hybridizing directly to the single stranded RNA and are quantified after hybridizing with fluorescent tagsets . The nanostring based assay is also less stringent than PCR based method by using an enzyme free chemistry for quantification and is typically not affected by variability in degradation of mRNA between samples. In the current study design, left and right kidneys of same rat were utilized for separate evaluation of cold ischemia and warm reperfusion. A set of left kidneys were frozen immediately after isolation and right kidneys were frozen after 2 hours of perfusion and served as controls for the cold storage and reperfusion groups, respectively.

Results for the selected genes in blank controls are presented as comparison of absolute expression values in Sprague Dawley rat kidneys for the first time, using this technology. The results obtained in naïve kidneys are in concordance with that previously reported for drug transporter and enzyme expression in the SD rats[202]. Accordingly, a higher abundance was obtained for genes like oat1, oat 3, oct2 in comparison to others known to have lesser renal expression like pept-1. Further, as can be assumed during baseline condition, the expression of genes such as antioxidant enzymes was higher than those only activated during inflammation or injury such as interleukins, interferon gamma and Kim-1. For cold stored kidneys, the increasing duration of storage was hypothesized to be associated with transcriptional activations leading to a higher reperfusion mediated injury. The fold-change in gene expression was evaluated in kidneys that were cold stored for 24 and 72 hours to determine effect of cold storage and its duration on the organ, prior to reperfusion. Previous reports are limited to individual genes in rat kidney such as endothelin 1 and icam-1, that were shown to have increased expression with duration of cold

storage (ranging from 4- 72 h)[192,193]. An increased expression of the gene for antioxidant enzyme MnSOD was also observed following cold storage of human tubular cells[203], attributed to the production of free radicals in the mitochondria. Such changes during cold storage were hypothesized to be associated with greater damage during reperfusion leading to clinical effects such as delayed graft function due to enhancement of vasoconstriction[192], decreased antioxidant capacity[173], enhanced attraction of leukocytes[193] as well as increased allergenicity of the transplanted organ[204]. Hence, in this study we separately analyzed the effect of cold storage for 24 and 72 h on gene expression in kidney, in the absence of reperfusion effects.

However, among the panel of genes evaluated, there was no significant alteration in any of the gene at end of 24 and 72 h cold storage, except for a marginal upregulation in cox-2 at 72 h. Previous studies have examined upregulation of cox-2 only during reperfusion of rat kidneys or liver and report on protective effects of cox-2 inhibitors like indomethacin, parecoxib and meloxicam[205-207]. It is possible that cox-2 was activated during ischemia, but the increase in activity was more pronounced only with availability of oxygen during reperfusion[208]. However, in kidneys preserved with treprostinil (50 ng/mL), the cox-2 upregulation was not significantly different from controls. Overall, cold ischemia did not cause a significant fold-change in expression of selected genes in the kidney after 24 h storage as well as an adequately longer storage duration of 72 h.

Reperfusion of kidneys was carried out in an isolated kidney perfusion system (IPRK) for functional evaluation of 24 h CS groups, as presented in Chapter 2. This ex-vivo model of CS followed by reperfusion was an attempt to mimic the exposure of transplanted kidneys to cold ischemia followed by warm reperfusion and to evaluate immediate functional recovery as well as the changes in gene expression. Fresh control kidneys as well as 24 h CS kidneys were reperfused

at 37°C with an oxygenated acellular perfusate and the gene expression was compared at the end of 2 h perfusion.

The levels of gene expression for upstream response elements like NFK-beta, Ifn-gamma, Tnf-alpha, Il-1beta as well as other cytokines like cxcl-10, Il-6, Il-8, cox-2, Mcp-1 was significantly increased both in control as well as reperfused kidneys. Despite the presence of high gene expression for inflammatory cytokines, the renal function of perfused controls was superior and perfusate flow rate was significantly higher than other reperfused 24 h CS kidneys, as was presented in chapter-2. It is possible that the high perfusate flow rate observed in control kidneys during IPRK can result in vascular shear stress, endothelial detachment[209,210] and result in release of inflammatory mediators by damaged endothelium along with an increase in kim-1 (kidney injury molecule) expression. The significantly increased expression of Ifn-gamma and associated Cxcl-10 in control kidneys, could be suggestive of an early protective response, as previous studies have demonstrated beneficial effects of this pathway, including prevention of necrosis and thrombosis during the early post-transplantation period[52,211,212].

Kim-1 is a transmembrane protein of renal tubular epithelium and is a marker of renal injury or repair. Kim-1 upregulation in response to acute injury has been associated with tubular dedifferentiation and regeneration to rebuild a functional epithelium as well as phagocytose apoptotic or necrotic cells out of tubular lumen and thus is essential for graft recovery[213-215]. Unlike perfused control kidneys, the early activation of Cxcl-10 and kim-1 mediated response to reperfusion was significantly less in all 24 h CS groups, but the expression of other inflammatory mediators was not different.

However, in addition the 24 h CS kidneys also showed a significantly higher expression in irip gene and a lower expression of the transporter nhe3, the vitamin D receptor and the antioxidant

MnSOD at the end of 2 h reperfusion. The alteration in these genes in kidneys following ischemia reperfusion have been reported earlier and are discussed below. The function of irip is to regulate transporter activities [216,217] and the overexpression of irip during IR has been shown to negatively regulate the activities of transporters including oct1-2-3, Mate1, Oat1 and Pgp. It is interesting to note that irip may not affect the transcript levels of oct-1 or mate1 but the protein levels were downregulated after irip overexpression due to IR. Under baseline conditions low irip expression is thought to act on post-transcriptional level such as to decrease degradation of the transporter protein by proteasomes and thus increase the amounts of the functional transporter[217]. The apical Nhe3 transporter is the major isoform of Nhe (sodium hydrogen exchanger) that mediates one molecule of extracellular Na in exchange for one cytosolic proton. Nhe3 is responsible for sodium reabsorption in proximal tubules in addition to the indirect control of reabsorption for chloride, bicarbonate, amino acids, proteins and citrate[218].

Studies in rat model, have established severe reduction in mRNA and protein of nhe3 following IR and is associated with the natriuresis, acidosis and volume depletion following IR injury. Apart from the hypoperfusion during renal ischemia, the proinflammatory cytokines like TNF-alpha, IL-1-beta were established to mediate the nhe3 downregulation[219,220]. The IR can also lead to an increased production of reactive oxygen species that can overwhelm the antioxidant capacity leading to decrease in expression or activity of the antioxidant enzymes like SOD. Of the 3 isoforms, SOD2 (MnSOD) is located in the mitochondria, the organelle that has shown to be a main endogenous source of ROS during reperfusion[221]. Paradoxically, the ROS can trigger production of MnSOD by the mitochondria, however function of mitochondria also depends on ATP. Hence, during IR, the optimum expression of MnSOD could be indicative of preserved mitochondrial function and capacity to scavenge the ROS. Intravenous SOD has also been

clinically beneficial to prevent and treat kidney IR during transplantation[222]. Expression of Vitamin D receptor (Vit D) has also been shown to be downregulated following IR in a rat model[223]. Evidence points to the anti-inflammatory effect of Vitamin D to be mediated via the Vdr binding to p65 subunit of Nfk-beta and thereby disable its nuclear interaction and gene transcription[224]. In addition, paricalcitol, a vitamin D analogue has been shown to have protective effects in renal IR by increasing the expression of Vdr in a rat model[223].

Unlike the 24 h CS kidneys, 24 h CS kidneys with treprositnil (added to both storage and reperfusion) did not show any significant change in the expression of irip, nhe3, MnSOD or the Vdr in comparison to controls. The 24 h CS kidneys with treprostinil (added to storage only) did not differ from controls in expression of irip and nhe3 whereas the downregulation in MnSOD and Vdr were observed, as in the untreated 24 h CS kidneys. Taken together, the effects on expression of irip, nhe3, MnSOD, Vit D in kidneys after exposure to treprostinil indicate a decrease in IR effects at molecular level, either by its anti-inflammatory effects[225,226] or possible anti-oxidant effect. One of the limitations of this study was with the short duration of perfusion (2 h) and immediately following up with gene expression thereby limiting the observation changes to more immediate events after reperfusion and ignoring the possible downstream changes in expression. A longer follow up is possible with in vivo transplant model which would facilitate additional differentiation between CS groups based on effects on the downstream targets and/or on the adaptive and innate immune activation after IR.

3.7 CONCLUSION

The effect of cold storage and warm reperfusion on the renal gene expression was evaluated using nanostring assay for multiple genes. There was no impact on the duration of CS on the renal gene expression, until 72 h. Warm reperfusion for 2 h after 24 h CS was associated with inflammatory response and downregulation in expression of genes such as nhe3, MnSod, Vdr and an upregulation in irip, all of which are markers for renal IR effects. Treprostinil addition to both 24 h CS and reperfusion was able to reverse all the above expression changes due to IR, while treprostinil addition to storage could only reverse the expression of nhe3 and irip. Hence, even though renal functional improvement was not observed with treprostinil (chapter 2), changes in gene expression after treprostinil exposure indicate protection against the IR induced effects at a molecular level. The evaluation for potential beneficial effects needs to be further assessed in an *in vivo* setting of renal transplant model.

4.0 ESTIMATION OF 12-HOUR EXPOSURE TO TACROLIMUS DURING THE EARLY POST-TRANSPLANT PERIOD BY SPARSE SAMPLING

4.1 ABSTRACT

Tacrolimus and mycophenolate-based immunosuppression is the standard of care in renal transplantation. Protocol biopsies have revealed early subclinical rejections in patients on standard immunosuppressive therapy. The overall objective of this study was to evaluate the effect of early post-transplant exposure and response to mycophenolate and tacrolimus therapy on subclinical inflammation and acute rejections in the grafts of renal transplant patients. The specific objective in this chapter is to evaluate tacrolimus exposure during early post-transplant period (week-1=period I) and follow-up study on the day of the 3-month protocol biopsy (week-13=period II). Sparse samples were obtained at 1.5 and 3.5 h in addition to routine trough blood samples in 42 and 23 patients, during early and follow-up periods, respectively. $AUC_{0\text{-}12\ h}$ was estimated from a prior population model by maximum a posteriori bayesian estimation. The bias and imprecision of predictions were low and within acceptable limits. The predicted tacrolimus $AUC_{0\text{-}12\ h}$ ranged between 39-294 ng*h/mL and 101-302 ng*h/mL during periods I and II, respectively. The apparent CL/F of tacrolimus showed a trend to decrease from an average of 28.7 to 24.8 L/h from period I to II, associated with an increase in average hematocrit by 30%. CL/F was more than 2-fold higher in CYP3A5 expressers during period I, however none of the MDR1 genotypes (3435, 2677, 1236, 1199) showed significant differences. Trough concentrations were higher than 8 ng/mL in 30 % and 80 % of patients at period I and II, respectively and showed a good correlation with predicted AUC (r= 0.795 (0.679-0.872)). Only 55 and 60 % of the patients had exposure above the recommended 150 ng*h/mL during periods I and II, respectively. Hence, in conjunction with regular monitoring of trough levels, the more informative and ideal measure of exposure ($AUC_{0\text{-}12\ h}$) could be estimated by sparse sampling in an outpatient setting during early and stable post-transplant periods, for further improvements in outcomes.

4.2 INTRODUCTION

Renal transplantation is the treatment of choice for end stage renal diseases and has greatly improved the survival and quality of life in these patients. The current immunosuppressive regimen of tacrolimus along with the antimetabolite mycophenolate and glucocorticoids, has offered the best short-term outcomes in terms of decrease in acute kidney rejection episodes and a > 90 % allograft survival within the first-year post-transplant. Chronic rejection, however, causes late allograft failure in about 10-20 % of renal allograft recipients[227] and the presence of interstitial fibrosis with tubular atrophy (clinical and subclinical rejections SCR), remains the major factor in late allograft failure. Longitudinal analysis have also shown SCR to originate very early after transplantation and contribute to chronic damage of the kidney[228,229], irrespective of any immediate drop in renal function. As the rejection associated histological inflammation in graft is immunological in origin, the broad study objective was to determine the association of rejections (both clinical and subclinical) to immunosuppressant drug exposure and efficacy with the current standardized regimen of mycophenolate and tacrolimus during the early post-transplant period.

Tacrolimus is therapeutically monitored after transplantation to maintain therapeutic blood concentrations as well as to avoid the side effects due to overexposure. The target tacrolimus concentrations vary with time post-transplant and at this center, tacrolimus trough concentration (C_0) of 8-12 ng/mL is targeted during the initial 3-month study period. The use of trough (C_0) blood concentrations to adjust tacrolimus dose is based on observations on the association between a target C_0 and clinical outcome of acute rejections. With mycophenolate, steroids and anti-IL-2 induction therapy, a target C_0 between 5-10 ng/mL during the first year could be considered as a therapeutic range for tacrolimus[62,66]. In an early investigative trial for C_0 of tacrolimus, decreasing rejection was observed with increasing C_0 while increasing toxicity occurred beyond a limit,

thereby a range of 5-15 ng/mL was proposed during the first 7 weeks post-renal transplant[64]. Another study[67] derived an effective target C_0 of 9.3 ng/mL on the day 5 post-renal transplantation and C_0 values below the cut-off were associated with a shorter graft survival. In general, the target C_0 associated with avoiding rejections were comparatively lower in some studies performed at later post-transplant periods, with the lack of steroid co-therapy (after tapering of steroids). In contrast few other studies have failed to show any association of C_0 with rejection [69,70].

In the case of tacrolimus, C_0 are considered as surrogates for the AUC (area under the curve), which is the most reliable marker for drug exposure. However, the correlation of C_0 with AUC has been shown to be variable and questionable with a better relation during the first month post-transplant period than at later time periods [62,63].

The relationship of tacrolimus exposure to clinical outcome has also been investigated in several studies. It has been shown that patients experiencing rejections have lower tacrolimus AUC during the early post-transplant period [75]. An AUC exceeding 200 ng*h/mL was suggested for tacrolimus during the early post-transplant period for avoiding the risk of acute rejections[75,76]. Another study had shown exposures of 210 ng*h/mL and 125 ng*h/mL as optimal limit for tacrolimus, until the first 6- and 52-weeks post-transplant, respectively [73]. Recent consensus report suggests a post-transplant target exposure of 150 ng*h/mL to avoid rejections in recipients of kidney[230].

In addition to whole blood exposure, the tacrolimus intracellular concentrations were shown to be associated with histological rejections in renal transplant patients [231]. Further, the authors found polymorphisms in ABCB1 transporters may impact tacrolimus intracellular concentrations and association with rejections[231].

The importance of adequate tacrolimus exposure cannot be more emphasized as the current clinical needs are to minimize the exposure to tacrolimus but at the same time to prevent acute rejections, due to sub-optimal AUC (exposure). Further, expert panel discussions have recognized and pointed out the lack of controlled trials for determination of an appropriate tacrolimus target AUC[62,232] for a specific transplant population and hence target exposure values are based on observational studies in various patients at different post-transplant times. Hence, the purpose of this chapter is to determine individual patient exposures to tacrolimus on two separate occasions, during an early period and at 3-month follow-up after renal transplantation, in order to examine its association with occurrence of clinical and SCR. Determination of AUC by a sparse sampling procedure with bayesian estimation was utilized to determine 12 h exposure along with the genotyping of patients for ABCB1 transporter, as a surrogate for efflux of tacrolimus from its target site of action.

4.3 MATERIALS

Tacrolimus and internal standard (Ascomycin) were purchased from LC Laboratories (Woburn, Mass, US) and Calbiochem (San Diego, Calif, US), respectively. Ammonium acetate, formic acid, acetonitrile (Optima®), methanol and water were obtained from Fisher Scientific (Fair Lawn, NJ, USA). All other chemicals used for LCMS sample processing were purchased from Fisher Scientific (Fair Lawn, NJ). The QIAamp® DNA blood mini kit (cat no. 51104) was purchased from Qiagen (Qiagen Inc., MD, US). TaqPath ProAmp Master mix was purchased from ThermoFisher scientific Inc. (MA, US). The assays for genotyping of the selected target single

nucleotide polymorphisms were identified and purchased from TaqMan® (Applied Biosystems, CA, USA).

4.4 METHODS

4.4.1 Study approval

A review of the proposed study was conducted by the Institutional Review Board (IRB) at the University of Pittsburgh. The study was approved by IRB under the category of minimal risk to patients (titled "Exposure to Mycophenolic acid/Tacrolimus and outcomes in renal transplant recipients", Approval No. PRO16050030, dated 20 Oct 2016).

4.4.2 Screening

Potential renal transplantation candidates were screened with following inclusion and exclusion criterion.

Inclusion:

- Recipients of cadaveric or living donor kidney with proposed immunosuppressive regimen of MMF and immediate release tacrolimus
- Completed at least 2 days of therapy with same dose of tacrolimus and MMF
- Adults (18-70 years)

Exclusion:

- Combined renal-pancreas transplantation

- On pre-existing therapy with MMF or similar acting drugs like azathioprine
- On clopidogrel or heparin based anti-coagulant therapy (biopsy not anticipated)
- Therapy with enteric coated mycophenolate therapy
- Hemoglobin less than 8 g/dL
- Therapy with drugs that could interact with MMF like cholestyramine, metronidazole or ciprofloxacin
- Abnormal liver function tests with > 3 times of upper limit for enzymes ALT, AST or bilirubin.

4.4.3 Enrollment in study

As a part of the informed consenting procedure, patients that were deemed suitable for inclusion, were met by investigators and the study protocol was explained in detail. A printed copy of study procedures was also provided to the patients. Patients were informed that the participation in the study was voluntary and that they could withdraw their consent from study participation at any time. Once patients provided their signed consent, they were enrolled into the study.

4.4.4 Study design- sampling periods and time points

This was a prospective study design and the enrolled patients were to be studied on two separate occasions (periods) during their post-transplant follow-up visits (Figure 4.1). The period-I was scheduled to be completed within 14 days post-transplant and period-II was scheduled along with the 3-month protocol biopsy. In addition, a baseline blood sample was obtained prior to transplant surgery in all enrolled patients. This pre-transplant baseline sample was used for

genotyping as well as characterization of individual IMPDH enzyme activities in the absence of any immunosuppression.

During each study period, a limited sampling (LS) method (3.5 h) was proposed, with 3 blood samples obtained at trough (pre-dose), and at 1.5 and 3.5 h after combined dosing of MMF and tacrolimus. These LS time points were chosen for compatibility with the anticipated duration of outpatient visits (~4 h) as well as based on a previous study that identified the 1.5 and 3.5 h post-dosing time points using D-optimality criterion, to be the most informative for combined bayesian estimation of exposure to both tacrolimus and MPA[233].

4.4.5 Study conduct and sampling

Blood samples (approximately 10 mL) were drawn from a peripheral vein catheter at or around the designated time points into lithium heparin tubes and mixed well. The morning trough blood sample was initially obtained and the times of previous and current dosing for MMF and tacrolimus were individually documented. Following the morning dose of MMF and tacrolimus, patients were restricted to have water, juice or crackers until the first time point (1.5 h). The post-dose samples were obtained at or around 1.5 and 3.5 h. An aliquot of whole blood (~200 µls) was transferred into tubes coated with dried EDTA (2 % (w/v)) and stored at 4° C for not more than 2 weeks prior to quantification of tacrolimus by LCMS/MS.

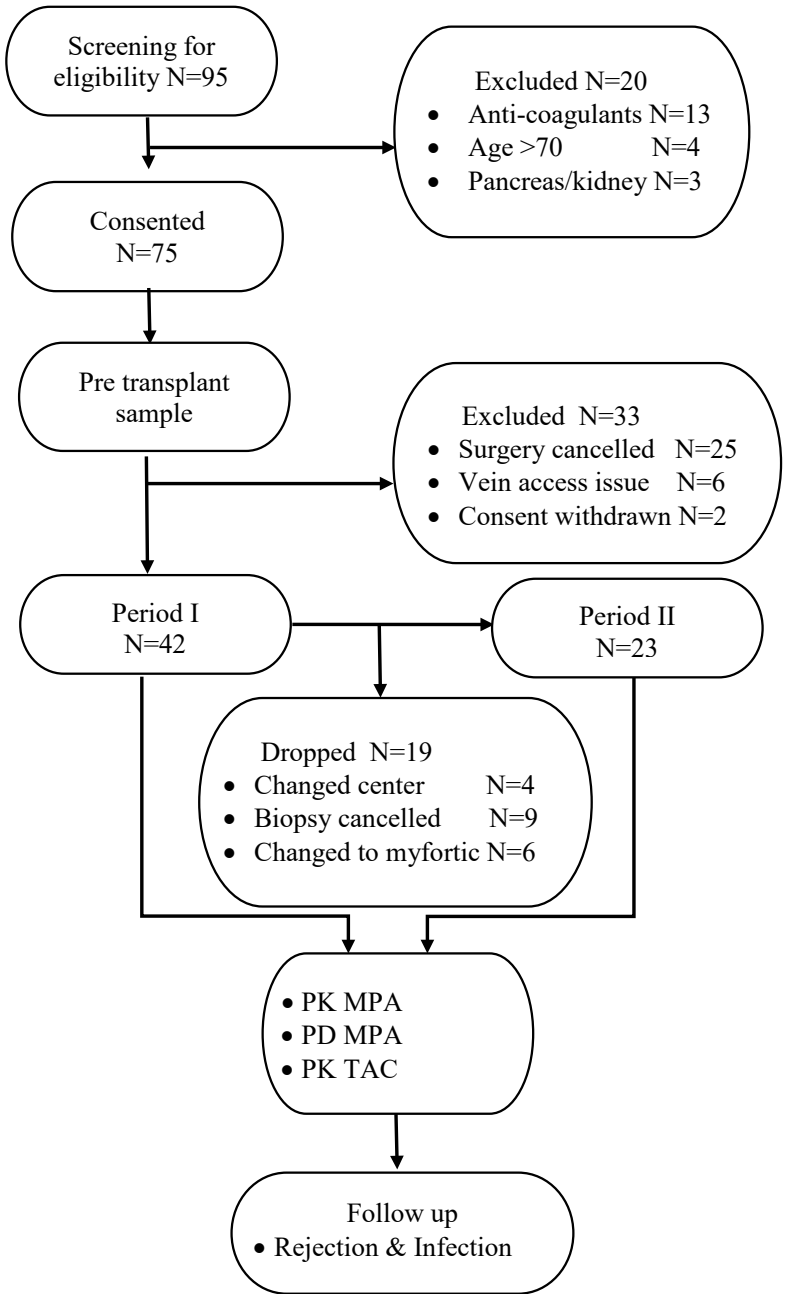
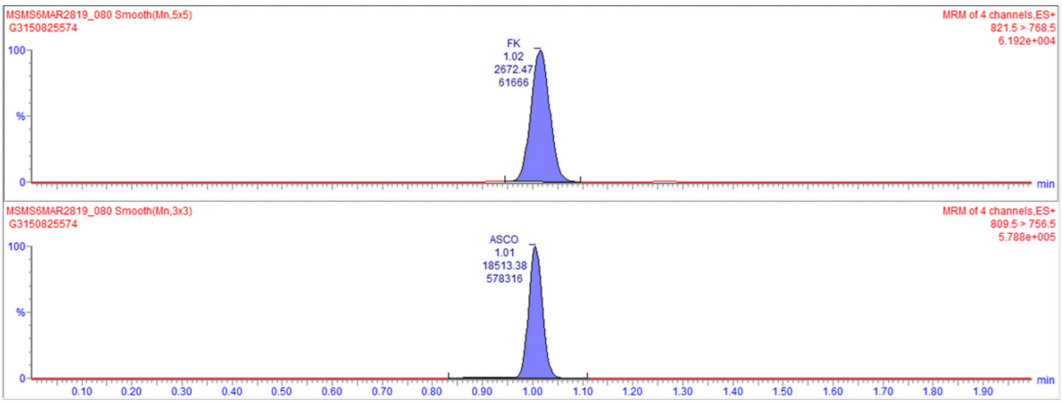


Figure 4.1. Cohort diagram

4.4.6 Processing of blood samples and quantification of tacrolimus

Tacrolimus concentrations in whole blood (ng/mL) were measured using a previously validated liquid chromatography tandem mass spectrometry method (LCMS/MS) in a Waters® Quattro micro API mass spectrometer. Processing of blood samples was done as follows: Fifty microliters of whole blood sample was mixed with two microliters of zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$) to precipitate the proteins. Acetonitrile (500 µls) spiked with internal standard (Ascomycin at concentration of 15 ng/ml) was then added and the mixture was vortexed for 1 min followed by centrifugation at 2000 g for 3 minutes. The supernatant was transferred into LCMS vials and 20 µls was injected on to a Nova-Pak® C18 2.1 x 10 mm cartridge (Waters # 186003523) maintained at 55° C. Analytes were effectively separated using a gradient elution consisting of an aqueous mobile phase (95% water / 5% methanol) with an organic mobile phase (100% methanol), at a flow rate of 0.6 mL/min. Both mobile phases also contained 0.1 % v/v formic acid and 2 mM ammonium acetate, in order to optimize ionization and chromatographic resolution. The MS/MS based multiple reaction monitoring was done in positive ion mode with m/z transition pairs of 821.5 → 768.5, 809.5 → 756.5 and retention times of 1.02, 1.01 min, both respectively for tacrolimus and ascomycin (Figure 4.2a). The validated method showed linearity over the assay range of 2 – 40 ng/mL (Figure 4.2b). Further, during each batch of analysis quality controls were verified at concentrations of 4, 16 and 25 ng/mL of tacrolimus in whole blood.



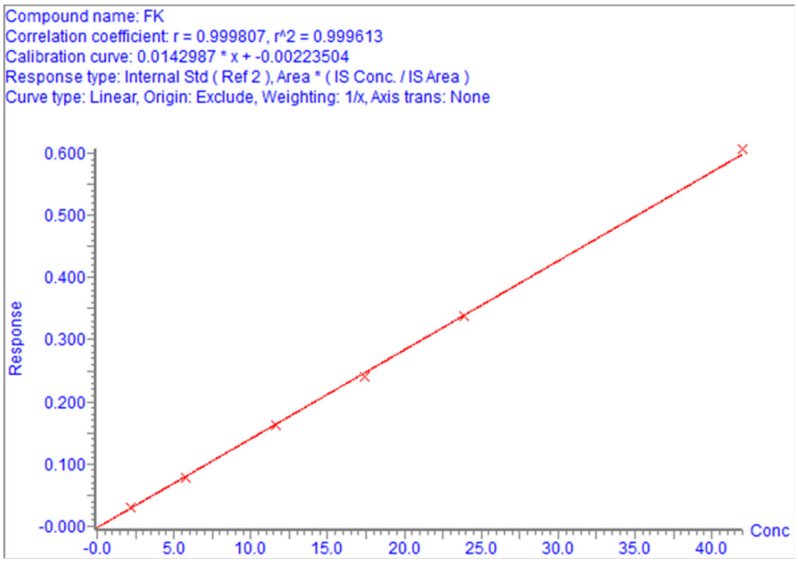


Figure 4.2. (a) Representative chromatogram of tacrolimus and internal standard (ascomycin) (b) Standard curve for tacrolimus (range: 2-40 ng/mL)

4.4.7 Data Analysis

4.4.7.1 Prediction of individual concentrations

Tacrolimus clearance in individual patients were determined by nonlinear mixed effect modeling of NONMEM® (version 7.4, Icon, Ireland) by using a maximum a posteriori (MAP) bayesian method with first order estimation. The MAP method utilized a prior population pharmacokinetic model for tacrolimus with parameters that were fixed to the median values under the subroutine ADVAN4 TRANS4. The individual blood concentrations from LS time points (obtained at trough, followed by 1.5 and 3.5 h post dosing), the exact clock timings and dosing history (mg dose and clock timings) of tacrolimus were available as prior information in every patient, that was subsequently used to estimate the apparent clearance (CL/F) of tacrolimus in each patient. The estimated individual parameters (such as CL/F, V/F) were output by specification of "posthoc". The maxeval=0 option was used to predict concentrations of tacrolimus at additional time points such as 0.25, 0.5, 1, 2, 3, 5, 8, 10 and until the next dose (12 h) on the day of study. For comparison, the total exposure (12 h) was also calculated from linear regression based equation by inputting the observed concentrations at 1.5 and 3.5 h into the published[233] equation 24.3 + (5.9 x $C_{1.5}$) + (12.2 x $C_{3.5}$), as ~90 % of sampling time points were within 10 minutes of 1.5 and 3.5 h.

4.4.7.2 Predictive performance

The predictive performance of the population model used for obtaining individual predicted concentrations were verified with diagnostic plots of the observed versus predicted (DV versus IPRED), weighted residuals (WRES) and normalized distribution of prediction errors (NPDE), using pirana® (Certara, NJ, US) and RStudio (version 1.1.453). Visual predictive check (prediction and variance corrected) was performed using pirana® and RStudio after 1000

simulations to derive replicates of model predicted concentrations. The median and 90 % prediction intervals (5th and 95th percentile) of model predicted concentrations were then compared against the corresponding distribution of observed values.

The bias in the predicted concentrations were estimated using mean prediction error (MPE) and the precision was estimated by median absolute prediction error (MDAPE) and by median percentage in absolute prediction error relative to the observed concentrations (%MDAPPE).

$$\text{MPE} = \frac{1}{N}\left[\text{Individual predicted concentration} - \text{Observed concentration}\right]$$

$$\text{MDAPE} = \text{Median}\left[\text{Abs}\left[\text{Individual predicted concentration} - \text{Observed concentration}\right]\right]$$

$$\%\ \text{MDAPPE} = \text{Median}\left[\text{Abs}\left[\frac{\text{Individual predicted concentration} - \text{Observed concentration}}{\text{Observed concentration}}\right] \times 100\right]$$

4.4.7.3 Non-compartmental pharmacokinetic analysis

The total exposures (predicted AUC $_{0\text{-}12\ h}$) were calculated from the predicted concentrations and corresponding time points using non-compartmental analysis (NCA) by Phoenix WinNonlin (Certara, NJ, US). In addition, the cumulative exposure (AUC $_{0\text{-}90\ days}$) from start of tacrolimus dosing until day 90 (representing 3-month protocol biopsy) was also calculated from trough blood concentrations obtained during therapeutic monitoring and corresponding day of blood sample, using NCA by Phoenix WinNonlin.

4.4.8 Genotyping

Whole blood samples (stored in -80°C) were used to genotype patients for select polymorphisms in enzymes *CYP3A4*, *CYP3A5* and the transporter *ABCB1*, which could impact metabolism and availability of tacrolimus into the lymphocyte compartment, respectively. The

genomic DNA was extracted and purified from whole blood using the QIAamp® DNA blood mini kit. The genotypes for *CYP3A4* (6 C>T), *CYP3A5* (6986A>G) and *ABCB1* (C3435T, G2677T/A, C1236T and G1199A) were determined by the allelic discrimination assays of Taqman® (Applied Biosystems, CA, US) and assays were performed according to manufacturer instructions. The genotypic variants and reaction details are presented in Table 4.1. In brief, 20 ng of genomic DNA was mixed with 1xTaqPath proamp master mix and 1xTaqman® allelic discrimination assay mix for the loci of interest. Following enzyme activation at 95 °C for 10 min, reactions were processed with forty cycles of 15 s at 95°C and 1 min at 60 °C. Data was collected in Realtime as well as with an endpoint read of fluorescent intensities. Genotypes were determined from the cluster analysis of the endpoint reads and further verification of cluster identification was done with Realtime data obtained using Taqman® method (7900 fast real time PCR system, Applied Biosystems, CA, USA).

Table 4.1. Assay details for identifying SNPs in select enzymes and transporters associated with tacrolimus disposition in patients

gene	SNP name	rs number	Assay ID (Taqman®)
CYP3A4	6 C>T (*22)	rs35599367	C__59013445_10
CYP3A5	6986A>G (*1, *3)	rs776746	C__26201809_30
ABCB1	C3435T	rs1045642	C___7586657_20
ABCB1	C1236T	rs1128503	C___7586662_10
ABCB1	G2677T/A	rs2032582 (assay 1/2)	C_11711720D_40/ C_11711720C_30
ABCB1	G1199A	rs2229109	C__15951365_20

4.4.9 Statistical analysis

The demographic and clinical variables in patients during both study periods were compared using Wilcoxon signed rank tests or paired t-test, as appropriate. The abbreviated, total exposure, dose normalized trough concentrations were statistically compared between the two occasions using paired t-test, for data from patients that participated in both periods. An independent t-test was used for statistical comparison of exposure and dose normalized trough concentrations among different genotype pairs. Statistical tests as well as correlation analysis were done with SPSS® version 25 (IBM corporation, US) and plotted using R software.

4.5 RESULTS

The demographic and clinical variables obtained during periods I and II of the study are summarized in Table 4.2. Median days corresponding to periods I and II were on 6^{th} and 91^{st} day post- transplant. The median age of patients was around 51 years with majority of patients being white (~75%) and recipients of cadaveric organs (~65-70%). All patients received thymoglobulin induction therapy and were started on 1000 mg dose (twice daily) of MMF from the day of surgery. Tacrolimus was usually started around the 48 h after transplant. The target trough levels for tacrolimus was 8- 12 ng/mL during the first 3 months followed by 6-10 ng/mL thereafter.

The liver function related values in Table 4.2 such as ALT, AST and bilirubin, indicate a stable hepatic function in the study population. After renal transplantation, an improvement in renal as well as hematological (hematocrit and hemoglobin) parameters are observed from period I to period II. With improved renal function, the significant drop in serum creatinine values and a

corresponding significant increase in the estimated glomerular filtration rate (calculated by the Chronic Kidney Disease Epidemiology Collaboration-CKD-EPI equation) are also evident, as in Table 4.2. The correlations between clinical and demographic variables are represented in Figure 4.3.

Table 4.2. Demographic and clinical characteristics of study population (Median (range))

Patient characteristic	All patients	Only patients that completed both periods		
	Period I n=42	Period I n=23	Period II n=23	p value*
Days post-transplant	6.00 [3.00, 9.00]	6.00 [4.00, 9.00]	91.0 [81.0, 117]	NA
Gender (male/female)	24/18	12/11	12/11	NA
Race (Caucasian/Afro-American /Others)	32/7/3	17/5/1	17/5/1	NA
Age	51.0 [23.0, 69.0]	50.0 [23.0, 66.0]	50.0 [23.0, 66.0]	NA
Cadaveric/ Living donor	29/13	15/8	15/8	NA
Weight (Kgs)	93 [52, 139]	97 [58, 136]	91 [58, 130]	<0.001

Patient characteristic	All patients	Only patients that completed both periods		
	Period I n=42	Period I n=23	Period II n=23	p value*
Albumin (g/dL)	3.80 [2.60, 4.70]	4.00 [2.60, 4.60]	4.10 [3.40, 4.60]	NS
ALT (IU/L)	14.0 [3.00, 375]	14.0 [3.00, 375]	16.5 [9.00, 68.0]	NS
AST (IU/L)	14.0 [7.00, 227]	13.0 [7.00, 227]	16.5 [9.00, 33.0]	NS
Total bilirubin (mg/dL)	0.5 [0.2, 1.4]	0.5 [0.2, 1.4]	0.4 [0.3, 0.9]	NS
Serum creatinine (mg/dL)	3.30 [0.700, 14.3]	3.30 [0.700, 13.0]	1.50 [0.900, 3.70]	< 0.001
eGFR (mL/min/1.73m^2)	22.0 [4.00, 100]	22.0 [4.00, 100]	51.0 [18.0, 90.0]	<0.001
Hematocrit (%)	27.5 [20.3, 38.3]	27.3 [20.3, 38.3]	35.0 [24.2, 47.2]	<0.001

Patient characteristic	All patients	Only patients that completed both periods		
	Period I n=42	Period I n=23	Period II n=23	p value*
Hemoglobin (g/dL)	9.15 [6.70, 13.0]	9.10 [6.70, 13.0]	11.5 [8.00, 15.5]	<0.001

*Wilcoxon signed rank test comparison in patients that completed both periods; NS- No significance ($p \geq 0.5$)

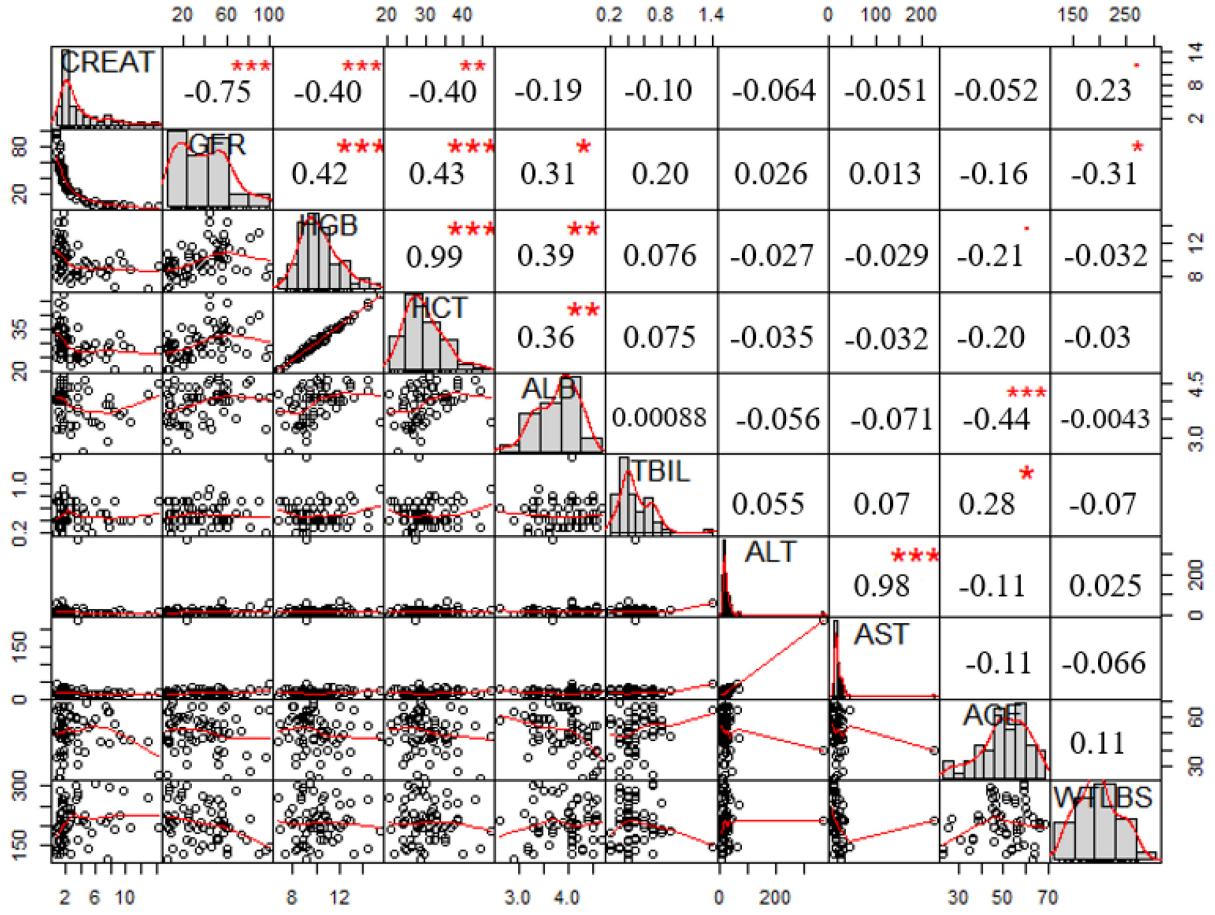


Figure 4.3. Evaluation of correlation between clinical and demographic parameters (Periods I & II)

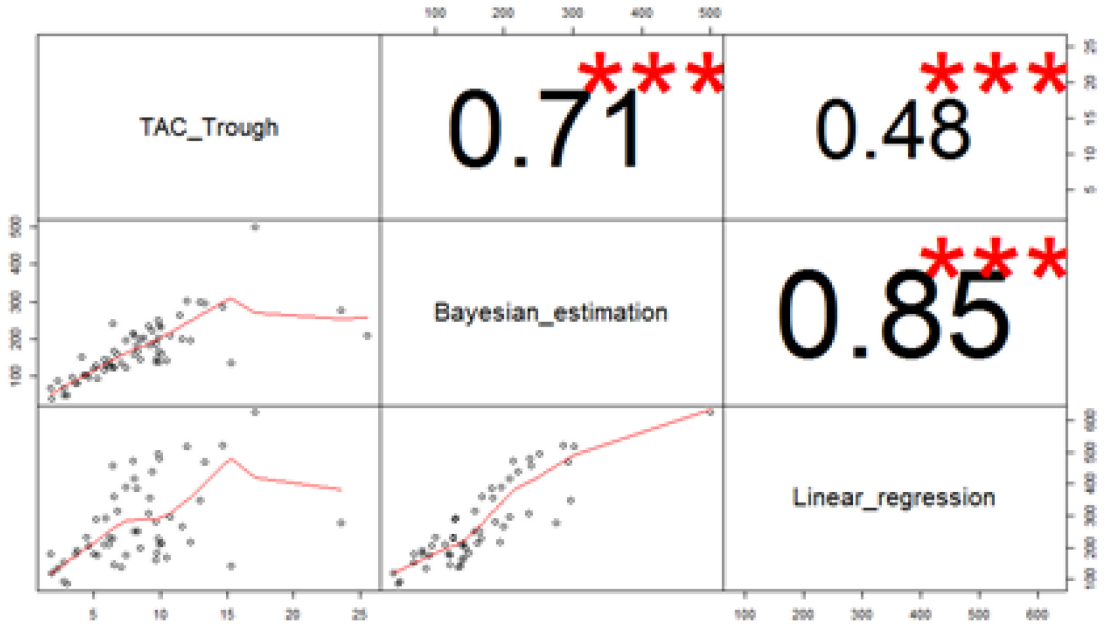
The population parameter estimates[233] used for maximum a posteriori Bayesian prediction of tacrolimus blood concentrations is shown in Table 4.3. The 2-compartment model with absorption lag time was used without fixing F (bioavailability) and hence clearance and volume terms are presented as apparent values (CL/F, Q/F and V1/F, V2/F).

The maximum a posteriori Bayesian estimation (MAP) showed superior predictions for $AUC_{0\text{-}12h}$ in comparison to linear regression (LR) equation-based estimation. As shown in Figure 4.4, there was a better correlation of MAP estimations with observed tacrolimus trough levels (r=0.71) compared to LR (r=0.48). Further, despite good correlation with MAP (r=0.85), a systematic overestimation in exposure occurred with LR method (slope = 1.34) compared to MAP estimation. The quality of Bayesian estimation from model were assessed from the bias and precision values that are shown in Table 4.4. The mean of prediction error with 95% CI was -0.09 (-0.30-0.12) ng/mL and was not significantly different from zero (p=0.38).

Table 4.3. Prior population parameters for tacrolimus in renal transplant recipients during early post-transplant period

PARAMETERS (2 compartment)[233]		THETAS	OMEGAS
Description	Units	Estimate	IIV –(% CV) *
Oral clearance (CL/F)	L/h	3.85	185
Central volume (V1/F)	L	221	133
Distributional Clearance (Q/F)	L/h	21.9	-
Peripheral volume (V2/F)	L	520.8	144
Absorption Rate (KA)	h^{-1}	1.68	199
Absorption Lag (TLAG)	h	0.045	350

* Exponential model used for all IIV terms; ε_{prop} (proportional term on residual error) was 0.01% CV; ε_{add} (additive term on residual error) was 0.97 ng/mL



Linear_regression = 1.337*(Bayesian_estimation) + 52.79

Figure 4.4. Correlation between tacrolimus exposure determined by Bayesian estimation, linear regression and trough blood levels

The scatter plot matrix shows individual panels examining correlation between tacrolimus exposure determined by two different methods (ng*h/mL) and correlation with the observed tacrolimus trough blood concentrations (ng/mL). Corresponding r values for correlations are in the diagonally opposite panel

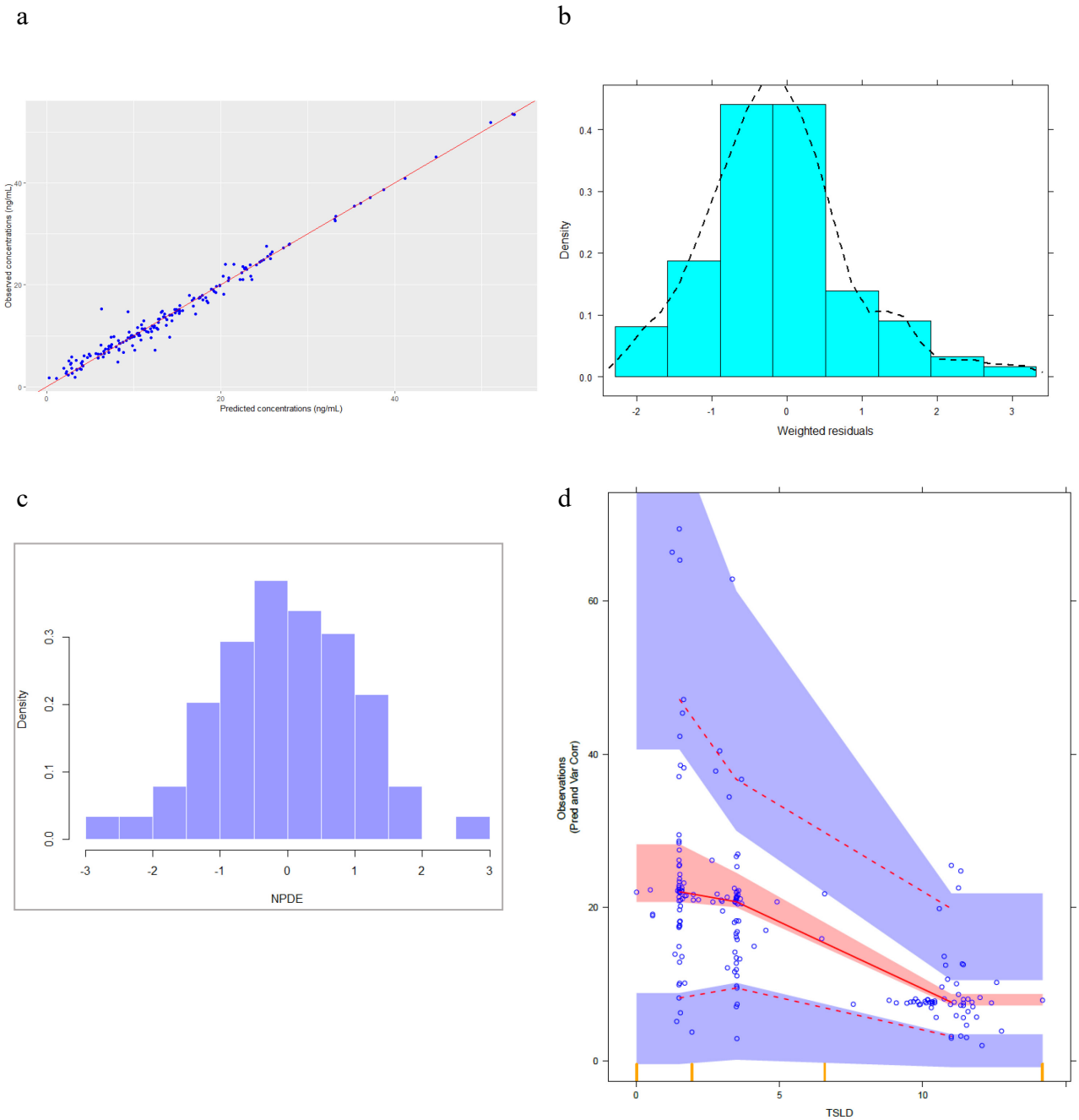
The imprecision was 0.47 (0.36-0.59) ng/mL, which was satisfactory and low in comparison to average trough concentrations (6-10 ng/mL). The median absolute percentage prediction error was 4.15 % (2.3-5.5) and was within acceptable limits. The model predictions were also visually evaluated for comparison with observed blood concentrations and are presented in panels a-d of Figure 4.5. As shown in figure 4.5a, the individual predicted concentrations agreed well with observed concentrations. The weighted residuals (after first order estimation) had values less than 3 units and both WRES and prediction errors showed an approximate normal distribution, as seen in panels b and c of Figure 4.5, respectively. The prediction and variability-corrected visual predictive check (pvcVPC) is presented in panel d of Figure 4.5. The pvc was done to correct for tacrolimus concentrations observed in patients taking different daily doses. The median as well as the 5th and 95th percentile of the prediction and variability-corrected observations overlapped with

the distribution of the simulated concentrations and indicated suitability of the model to predict tacrolimus concentrations in the study population.

Table 4.4. Predictive performance of Bayesian estimation for tacrolimus concentrations at sparse sampling time points

Measure	Estimate (95 % CI)
Mean prediction error (MPE, ng/mL)	-0.09 (-0.30 – 0.12)
Median absolute prediction error (MDAPE, ng/mL)	0.47 (0.36 – 0.59)
Median absolute percentage prediction error (MDAPPE, %)	4.15 (2.30 – 5.50)

The observed trough concentrations and predicted exposures ($AUC_{0\text{-}12\ h}$) are summarized in Table 4.5. The average dose was similar across both periods, suggesting absence of major dose changes after the PK study during period I. Though the mean trough concentrations during period I were slightly below the target 8 -12 ng/mL and significantly increased during period II, the mean predicted total exposures were close to the recommended 150 ng*h/mL and did not significantly differ between both periods (Table 4.5). However, only 55 % and 60 % of the patients had exposure exceeding 150 ng*h/mL during periods I and II, respectively.



a) Observed versus individual predictions (ng/mL) b) Histogram plot for weighted residuals (first order estimation) c) Plot of normalized prediction distribution errors (NPDE) d) pvc-VPC plot where blue dots represent the prediction and variance corrected observations, the solid and broken lines represent median and 90% prediction intervals. The shaded areas around lines represent 99% confidence intervals for median, 5th and 95th prediction intervals obtained from the simulations with IIV of V2/F and CL/F fixed at 80 and 125%, respectively

Figure 4.5. Evaluation of observations versus predictions with the tacrolimus population model

Table 4.5. Summary of observed and predicted pharmacokinetic parameters for tacrolimus

Mean ± SD Median (Min, max)	All patients	Only patients that completed both periods		
	Period I (n=40)	Period I (n=23)	Period II (n=23)	p value*
Dose (mg, bid)	4.0 [2.0, 6.0]	4.0 [2.0, 6.0]	4.0 [2.0, 10.0]	NS
Trough blood concentration (ng/mL)	7.0 ± 3.7	6.6 ± 3.4	9.9 ± 3.7	0.02
Dose-normalized trough (ng/mL/mg)	1.9 ± 1.2	1.8 ± 1.0	2.6 ± 1.3	0.009
Predicted $AUC_{(0\text{-}12\ h)}$ (ng*h/mL)	153.2 ± 69.5 142.3 [39, 294]	149.1 ± 63.8 143.1 [39, 264]	179.3 ± 55.9 165.3 [101, 302]	NS
Dose-normalized predicted $AUC_{(0\text{-}12\ h)}$ (ng*h/mL/mg)	40.8 ± 20.0	38.6 ± 16.7	47.4 ± 20.5	NS
Predicted CL/F (L/h)	28.7 ± 11.9	28.2 ± 9.9	24.8 ± 9.9	NS

*paired t-test comparison in patients that completed both periods; NS- No significance ($p \geq 0.05$)

A good correlation of tacrolimus trough concentrations to predicted $AUC_{0\text{-}12\ h}$ was observed with value for correlation coefficient r=0.795 (0.679-0.872), as shown in Figure 4.6. For fixed values of trough concentration, a range of corresponding predicted AUCs were observed. Figure 4.7 compares the change in apparent clearance of tacrolimus in relevance to that of hematocrit during periods I and II. An overall 12% decrease in CL/F was observed during the corresponding time period when hematocrit had increased in patients by a median of 28%.

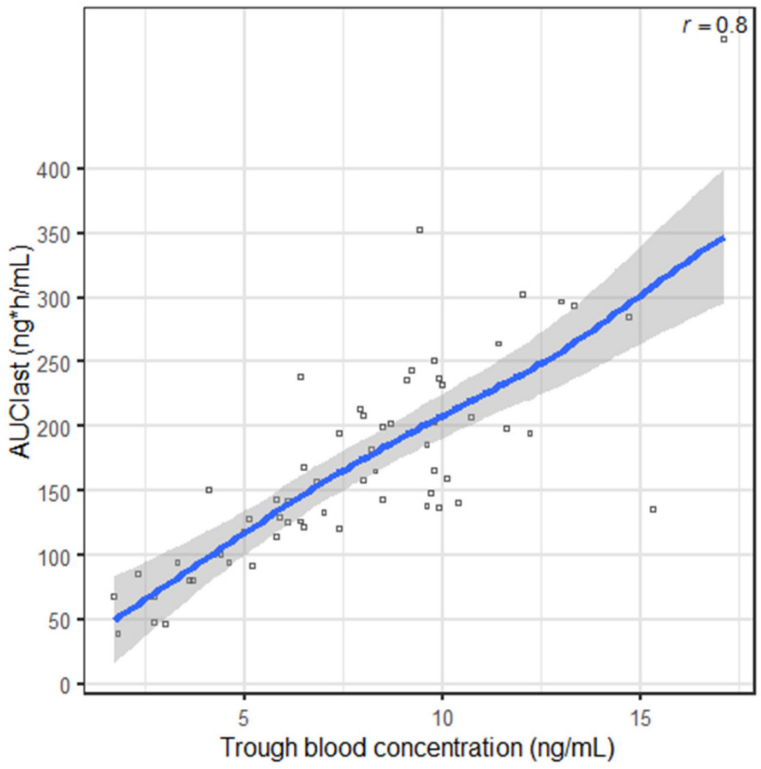


Figure 4.6. Correlation of observed trough blood concentration (ng/mL) and predicted 12 h AUC (ng*h/mL) for tacrolimus

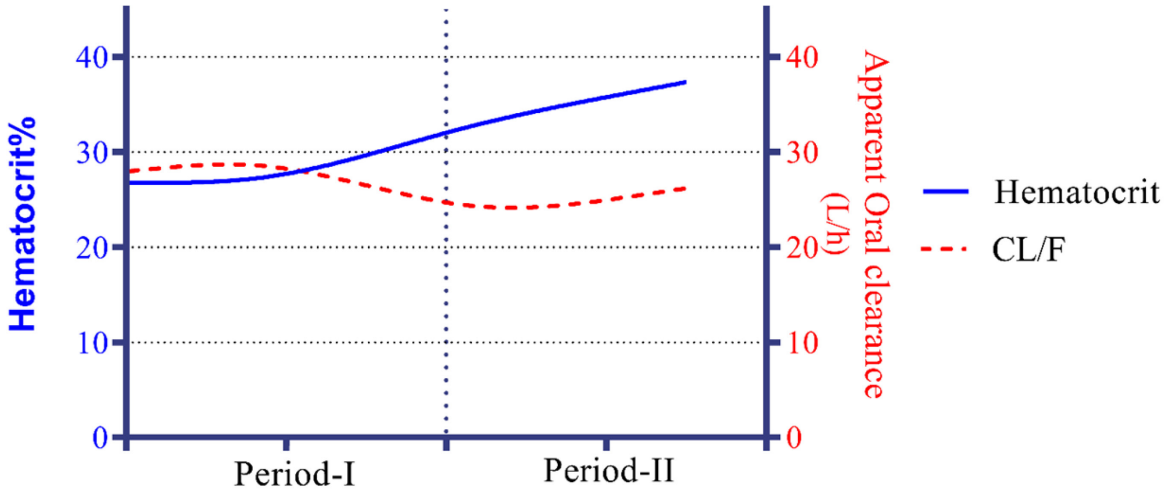


Figure 4.7. Comparison of hematocrit and tacrolimus Cl/F during early and intermediate post-transplant periods (I and II)

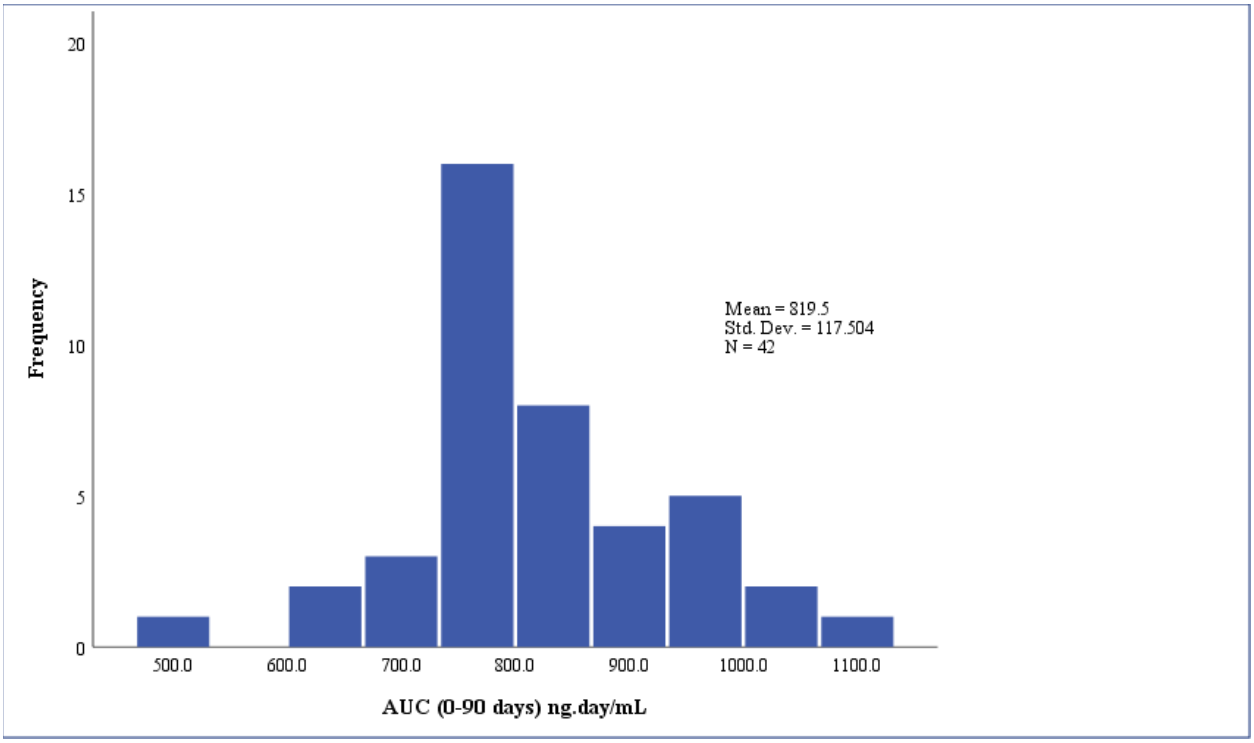


Figure 4.8. Cumulative exposure of tacrolimus ($AUC_{0\text{-}90\ days}$) during the first 90 days post renal transplantation

The distribution of cumulative exposure of tacrolimus calculated using periodic trough blood concentrations until 90th day post-transplant is represented in Figure 4.8. A 2-fold range of cumulative exposure was observed between the extreme values in patients and an average exposure of 820 ng.day/mL was observed.

The distribution of CYP 3A5, MDR1 genotypes in study population is shown as frequencies of alleles in Table 4.6. The frequencies for alleles were comparable to typical values reported for Caucasian population. The effect of genotypes on the tacrolimus dose normalized trough blood levels and predicted dose normalized $AUC_{0\text{-}12\ h}$ are presented in Figures 4.9- 4.10. The heterozygous CYP 3A5 expressers (*1/*3) and homozygous (*1/*1) were pooled for this analysis due to small sample size.

Table 4.6. Frequency (%) of the SNP variants for CYP 3A5 and MDR1 genes

SNP	Gene	Homozygous (wild type)	Heterozygous variant	Homozygous variant
rs776746	CYP3A5 6986A>G	3 (8.1)	5 (13.5)	28 (75.7)
rs1128503	MDR1 C1236T	12 (32.4)	22 (59.5)	3 (8.1)
rs2032582	MDR1 G2677T/A	13 (35.1)	21 (56.7)	3 (8.1)
rs1045642	MDR1 C3435T	6 (16.2)	24 (64.9)	7 (18.9)
rs2229109	MDR1 G1199A	34 (91.9)	3 (8.1)	0 (0)

N=37; difference in total for each genotype is due to genotype being "undeterminable" in few cases

As seen in Figure 4.9, expressers of CYP3A5 showed a significantly lower dose normalized trough and predicted dose normalized exposure for tacrolimus, during the early post-transplant period.

The period I values for mean dose normalized trough and predicted $AUC_{0\text{-}12\ h}$ in CYP3A5 expressers were (0.9 ± 0.4 vs 2.3 ± 1.2) ng/mL/mg, $p<0.05$ and (25.8 ± 16.7 vs 50.4 ± 25.7) ng*h/ml/mg, $p< 0.001$, respectively. The mean CL/F for tacrolimus during period I, observed in CYP3A5 expressors (*1/*1/*3) was more than 2-fold higher than non-expressors (51.5 ± 27.6 vs 23.7 ± 7.3) L/h. The differences between CYP3A5 genotypes in blood exposure of tacrolimus during period II were not significant, however the study sample sizes were also lower. In contrast to CYP3A5, the MDR1did not affect dose normalized trough blood levels or predicted $AUC_{0\text{-}12h}$, when the different genotypes were compared among patients, as shown in Figure 4.10.

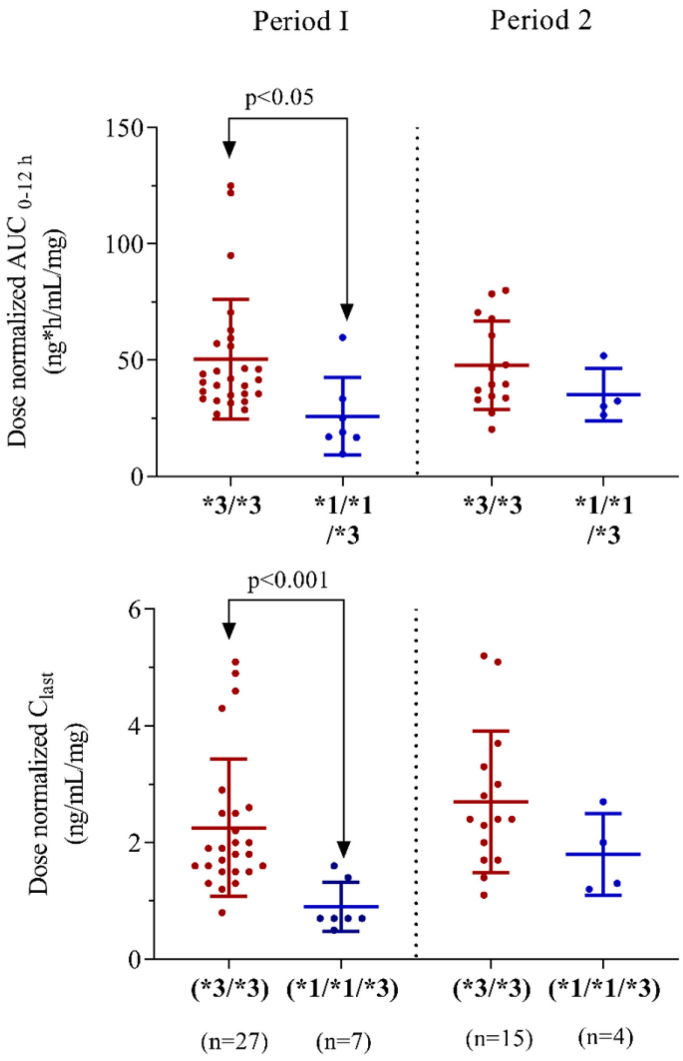


Figure 4.9. Tacrolimus dose normalized trough concentration (ng/mL) and predicted dose normalized $AUC_{0\text{-}12\ h}$ (ng*h/mL) according to CYP3A5 genotypic variants in Period I and II The heterozygous (*1/*3) and homozygous (*1/*1) CYP3A5 expressers were pooled for this analysis due to smaller frequency of the alleles and are indicated by (*1/*1/*3). (*3/*3) variants are considered as non-expressors of CYP3A5; Independent sample t-test used for statistical comparisons between genotypes with $p<0.05$ considered as significant difference

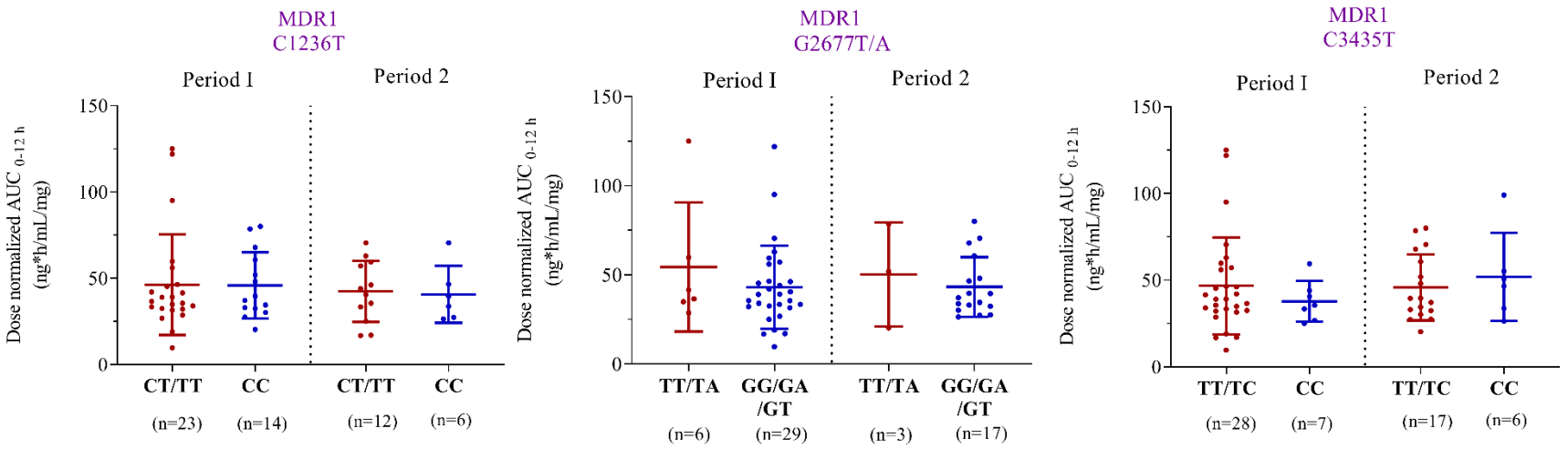


Figure 4.10. Tacrolimus predicted dose normalized $AUC_{0\text{-}12\ h}$ (ng*h/mL) according to MDR1 genotypic variants in Period I and II

Alleles with reported increase or decrease in MDR1 (p-glycoprotein) efflux activity were pooled together for comparisons; The variants with reported higher activity were CC for C1236T as well as C3435T and TT/TA for G2677T/A genotypes. G1199A was not compared due to absence of homozygous variant GG in study cohort; Independent sample t-test used for statistical comparisons between genotypes in each period, with $p<0.05$ considered as significant difference

4.6 DISCUSSION

Tacrolimus with mycophenolate therapy has been the preferred immunosuppressive regimen to avoid acute and chronic rejections after renal transplantation. Despite substantial reductions in acute clinical rejection rates, the presence of subclinical rejections (SCR) with clinical rejections (ACR) has been observed at 3 months after transplantation in our center, in patients on standard immunosuppression protocol with tacrolimus and mycophenolate[42,227,229]. Subclinical rejection is observed as tubulointerstitial infiltrates in the renal allograft without any functional deterioration but has been associated with progressive functional changes leading to long term graft loss[158]. Though not routinely performed at all transplant centers, the serial protocol biopsies obtained at 3 months and 12 months after kidney transplant enables monitoring of the early allograft inflammation and damage along with any detectable decline in the renal function. The causal relationship of SCR with the early post-transplant exposure to tacrolimus and mycophenolate-based immunosuppression remains unclear. Hence, the objective of the study in this chapter was to determine the exposure to tacrolimus at two separate occasions, one during the first few days after transplantation (1 week, early post-transplant) and one along with the 3-month protocol biopsy, for further comparison of association with outcomes.

After transplantation, mycophenolate therapy was administered as a fixed dose regimen (1000 mg of MMF twice daily) in the absence of TDM. For tacrolimus however, due to the high interpatient variability, TDM based dose adjustment was done using trough blood concentrations to avoid toxicity as well as minimize rejection. Though trough blood concentrations (C_0) of tacrolimus are routinely used in TDM for clinical practicality, the association of C_0 with the true

exposure ($AUC_{0\text{-}12\ h}$) as well as the clinical outcomes has been a matter of debate[62,63,73,74]. Tacrolimus trough targeting can also represent high variability in exposure as a fixed trough concentration can be associated with a 2-3-fold difference in corresponding 12 h exposure[234,235]. Historically, the evolution of tacrolimus target trough concentrations has slowly seen a shift from higher concentrations of around 20 ng/mL in earlier years of use to lower concentrations during recent times mainly after co-administration with mycophenolic acid[71]. However, incidences of toxicity and rejection are shown to occur in patients despite trough levels within acceptable therapeutic range[232], as patients might differ in $AUC_{0\text{-}12\ h}$ with under or over-exposures, despite the same observed C_0 in addition to the differences in exposures of co-administered MPA therapy. Further, lack of monitoring for MPA or trough based monitoring for tacrolimus, cannot account for the impact of high ADME changes are known to occur during periods such as early after transplant[236], on their respective total exposures. Hence, the determination of $AUC_{0\text{-}12\ h}$ is the best measure for exposure for the further study of relationship with clinical outcomes.

In routine clinical care, however, the determination of the full 12 h AUC is challenging due to patient burden and clinical feasibility, however obtaining sparse samples in outpatient clinic followed by bayesian estimation of the 12 h exposure to both tacrolimus and MPA, provides a flexible and more realistic alternative to the full AUC. Abbreviated AUCs or limited or sparse sampling (LS) followed by maximum a posteriori bayesian estimation (MAP) of exposure are not routinely employed in the clinic, as they require additional time from patients and clinical staff, increasing the cost of care. In addition, MAP bayesian estimation requires trained personal to implement or develop a population model with parameter estimates having adequate precision, in comparison to linear regression based (LR) based LS method. If resources can support MAP, determination of AUC from limited sampling followed by MAP estimation, has shown good

correlation with CNI inhibitor in prior studies in renal transplant patients[237]. Further, for tacrolimus, LS done at times 0, 1 and 3 h post-dose have shown bias and imprecision values of less than 15 % [77].

The LS followed by MAP estimation was chosen for its practicality in clinical usage, as it permits more variability around the blood sampling times and in addition, loss of a single time point will not invalidate the patient data, as it uses additional information obtained from individual patients, unlike the regression equation-based LS methods[234]. Using optimality criterion, a previous study had also shown the 1.5 and 3.5 h post dosing time points to be the most informative for combined bayesian estimation of exposure to both tacrolimus and MPA[233]. Further a less than 4- hour total study time is required to obtain patient compliance in the outpatient setting as well as to ensure minimal burden on routine clinical activities.

The LS protocol was implemented by obtaining two additional samples at 1.5 and 3.5 h post-dosing of immediate release tacrolimus and MMF, in addition to the trough samples collected during the clinical visits on two occasions, period-I and II around days 6 and 90 post-renal transplant, respectively. The post-dose LS sampling time points were obtained at 63 instances on an average of 1.57 ± 0.17 h and 3.47 ± 0.24 h, indicating the feasibility of LS in routine clinic and were well within the 30 min limit of deviation allowed for accurate bayesian estimation. The comparison of results from estimation by MAP Bayesian and LR, showed a systematic overprediction with LR and inability to calculate exposure in patients with missing time points. Further, as around 10 % of sampling time points had a deviation greater than 10 min from ideal sparse sampling time points, it could have impacted the accuracy of prediction by LR based calculations.

A two-compartment model with fixed absorption rate and time lag was used to describe the time profile after single dose of immediate release tacrolimus. Though the evaluated predictive performance for individual estimates were satisfactory, the higher values of estimates for interindividual variability of tacrolimus during the early post-transplant period resulted in a wide prediction interval around the median of the observed values, when applied to current study with smaller sample size. The MAP predicted $AUC_{0\text{-}12\ h}$ ranged from 39 – 294 ng*h/mL with median of 142.3 ng*h/mL during period I (day-6) and 101-302 ng*h/mL with median of 165.3 ng*h/mL on period II (day-90), though the median dose was around 4 mg on both occasions. The combined trough concentrations of both periods showed a good correlation with the predicted $AUC_{0\text{-}12\ h}$ as previously shown for tacrolimus[73,238]. However, it cannot be used for accurate estimation of exposure, as shown in the Figure 4.5, and in line with previous reports, a fixed trough concentration can sometimes have a 2-3-fold difference in corresponding 12 h exposure[234]. The predicted exposures during the two occasions were comparable with earlier observations in renal transplant patients. Previously, during initial 6 weeks of post-renal transplant period with basiliximab induction and MPA co-administration, a tacrolimus target of 210 ng*h/mL was suggested to be an ideal 12 h exposure which can be subsequently decreased to 150 ng*h/mL[73]. Recent consensus report identified 150 ng*h/mL to be an ideal target exposure during early post-transplant period in renal transplant recipients[230].

In comparison with desired target exposure, a less than optimal mean exposure was observed in our study patients, especially during the early post-transplant period around day-6 with only 55 % of patients having total exposure above 150 ng*h/mL. On period-II (day-90) around 60% of patients had exposure above 150 ng*h/mL. Trough concentrations were higher than the target (8 ng/mL) in 30 % and 80 % of patients at period I and II, respectively. The trough as well

as estimated exposure for tacrolimus were comparatively increased on continual therapy and hence the mean estimated exposure values in period II was within acceptable therapeutic range. This observation of a steady increase in tacrolimus exposure has been reported in earlier longitudinal studies with renal transplant patients on tacrolimus therapy[73,235,239]. Unlike earlier studies however, this study examines exposure in relevance to clinical and subclinical rejections.

The mean value of estimated apparent oral clearance was around 28 and 24 L/h respectively, during periods I and II. In fact, the trend in increase of estimated $AUC_{0\text{-}12h}$, should be due to the change in CL/F, as there was an absence of significant dose change. Tacrolimus has a highly variable oral bioavailability (F) mainly due to the first pass metabolism/efflux due to combined effect of CYP 3A enzymes and P-gp in the gut and liver[55]. In renal transplant patients on steroid tapering like our study population, the CL/F can be initially higher due to the induction effect of steroids on the CYP enzymes or P-glycoprotein in the gut and liver. It is also shown that the mean hematocrit and hemoglobin values in our study patients at period II were approximately higher by 30%, due to the normal post-transplant recovery. As tacrolimus shows high binding to RBCs, lower hematocrit during early post-transplant can increase the metabolism of unbound tacrolimus in the liver, leading to a higher CL/F during that time period. Previous studies and population models have also shown hematocrit to be a significant covariate that could determine CL and whole blood concentrations of tacrolimus[239]. The change in albumin however was marginal with time and hence its impact on CL/F due to changes in the binding of tacrolimus to plasma proteins could have been obscured.

CYP3A5 is the single most influential covariate known to directly influence the dose requirement and blood concentrations of tacrolimus, as CYP3A5 has higher efficiency to metabolize tacrolimus when compared to CYP3A4[240]. Almost 76% of study patients were

CYP3A5 non-expressors and comparable to earlier reported range of 75 – 85 % in white population[62]. CYP3A5 is present in majority of the Caucasians as the homozygous *3/*3 genotype also called as non-expressers, due to the inherent loss of CYP3A5 expression due to *3 allele while those with *1 allele are called expressers. In our study patients, it was observed that the estimated $AUC_{0\text{-}12h}$ was halved and CL was more than doubled in CYP expressers having one *1 or both *1/*1 (homozygous) alleles. This effect has been widely reported in earlier studies associated with estimates of increased dose requirement by at least 50% in patients with *1 allele or even more so in homozygous expressers[241,242]. Hence, the Clinical Pharmacogenomic Implementation Consortium (CPIC) also recommends a 1.5 to 2-fold increase in starting dose of tacrolimus in CYP3A5 expressers[57]. Due to the lower sample size during period II, the comparison of estimated exposure between CYP3A5 genotypes was not statistically different.

Unlike CYP3A5, the blood exposure or dose of tacrolimus did not differ among patients with various MDR1 (P-gp) genotypes. However, it should also be noted that P-gp is expressed on lymphocytes and the genotypes can variably alter intracellular tacrolimus concentration and possibly be better representative of tacrolimus efficacy than the whole blood concentrations. Indeed an earlier study had shown higher dose adjusted tacrolimus concentration within PBMCs in carriers of 1199A and 3435T variants, both of which correspond to the lower activity of the MDRI protein[243]. Few studies have investigated associations of MDR1 gene polymorphisms in association with biopsy proven rejection but have been contradictory so far. Studies have shown association of variant alleles with rejection[243,244] in liver and kidney transplantation while another study reported no association[245]. In addition, a few practical challenges exist for the direct estimation of intracellular tacrolimus levels, such as the possible efflux of tacrolimus during PBMC isolation, requirement of sensitive LC-MS/MS method and obtaining adequate cell counts

during early post-transplant when other efficacy markers like IMPDH activity are also evaluated. Hence, the genotypic variants of MDR1 are currently treated as surrogates for intracellular tacrolimus concentrations and examined for association with biopsy proven outcomes (chapter 6). However, it is also acknowledged that a direct genotype-phenotype correlation of P-gp activity may be confounded by other non-genetic factors, such as changes in unbound tacrolimus caused by binding to alpha acid glycoprotein (AAG), as AAG can be induced during the immediate post-transplant periods[246].

In conclusion, the estimation of tacrolimus exposure from limited sampling was carried out at early post-transplant and followed up around day-90 along with routine monitoring of trough blood levels. The individual exposures to tacrolimus along as well as other influential covariates like hematocrit and genotypes of CYP3A5 and MDR1 genotypes were obtained. Genotyping revealed that the estimated exposure ($AUC_{0\text{-}12h}$)was halved and CL was more than doubled in CYP expressers having one *1 or both *1/*1 (homozygous) alleles while the blood exposure or dose of tacrolimus did not differ among patients with various MDR1 (P-gp) genotypes. The MAP Bayesian based approach was found to be better for estimation of exposure from limited sampling. The parameters for tacrolimus will be examined further by analysis along with efficacy and exposure to MPA, to determine cut-off values related to the occurrence of clinical/subclinical rejections and infections, in renal transplant patients.

5.0 ESTIMATION OF THE EXPOSURE TO MYCOPHENOLIC ACID AND ITS RELATIONSHIP TO INOSINE MONOPHOSPHATE DEHYDROGENASE ACTIVITY USING SPARSE SAMPLING IN RENAL TRANSPLANT PATIENTS

5.1 ABSTRACT

Mycophenolic acid and tacrolimus-based immunosuppression is the standard of care in renal transplant patients. Protocol biopsies have revealed early subclinical rejections in patients on standard immunosuppression therapy. The overall objective of this study was to determine the effect of early post-transplant exposure and response to therapy with mycophenolate and tacrolimus, on the evolution of biopsy proven acute rejections (clinical + subclinical) or episodes of infections in renal transplant patients. The specific objective of the study presented in this chapter is to evaluate individual exposure to MPA along with its effect on the inhibition of its target (IMPDH) during early post-transplant period (week-1=period I) and during follow up at the time of 3-month protocol biopsy (week-13=period II). Sparse samples for plasma and PBMC were obtained at predose, 1.5 and 3.5 h during the early and the follow-up periods, from 42 and 23 renal transplant patients, respectively. $AUC_{0\text{-}12\ h}$ was estimated from a prior population model by maximum a posteriori bayesian estimation. The mean MPA exposure (predicted $AUC_{0\text{-}12\ h}$) was comparable between Period I to Period II (48.3 ± 21.4 vs 58.2 ± 32.8, NS) with only 47 and 50 % of patients falling within the 30-60 µg*h/mL exposure window, respectively. The dose normalized exposure however showed an increase during Period II (0.05 ± 0.02 vs 0.07 ± 0.03 µg*h/mL/mg, p=0.002), while there was no change in the dose normalized free exposure (0.97 ± 0.78 vs 0.71 ± 0.36 ng*h/mL/mg, NS). IMPDH activity was significantly higher at early posttransplant with median values for predose (54.8 vs 36.3 µM XMP/M AMP/sec, p =0.04) and R_{min} (26.1 vs 19.0 µM XMP/M AMP/sec, p=0.01), during period I vs II, respectively. The IMPDH R_{min} was significantly higher while the predose activity tended to be higher in patients with the lowest MPA exposures (< 30 µg*h/mL). The individual estimates of exposure and IMPDH activity indicated time-dependent changes in MPA exposure and response during early post-transplant and the need

for post-transplant time-based assessments for MPA PK and PD, which could be enabled by sparse sampling in an outpatient setting.

5.2 INTRODUCTION

Mycophenolic acid (MPA), the active metabolite of the prodrug mycophenolate mofetil (MMF) is an immunosuppressant used in combination with tacrolimus for maintenance therapy in renal as well as other solid organ (liver, heart and lung) and hematopoietic stem cell transplant patients. Mycophenolic acid is a reversible inhibitor of the enzyme inosine monophosphate dehydrogenase [IMPDH] and the immunosuppression results due to the selective reduction in guanine nucleotide synthesis in lymphocytes, that depend on this de novo pathway[247]. Studies in renal transplant patients with MMF dosing have shown a significant inverse relationship between exposure or Area under the curve ($AUC_{0\text{-}12\ h}$) for MPA and biopsy proven rejection (BPAR)[72,109,131]. On the other hand, associations of trough (C_0) MPA levels are weak and inconsistent both with $AUC_{0\text{-}12\ h}$[248-250] as well as BPAR[72,109,118,131]. Hence, for the prevention of acute rejection in combination with tacrolimus, a MPA $AUC_{0\text{-}12\ h}$ of 30-60 μg*h/mL has been recommended [132,251].

Significant inter-patient variability in the pharmacokinetics of MPA is known[252] and with a fixed dosing of MMF, many patients do not reach or fall outside the intended 30-60 μg*h/mL target range[90,105,113,131] for MPA. Several patient covariates can impact pharmacokinetics of MPA leading to the between-patient variability in total exposure. MPA free fraction and clearance can decrease significantly with increasing albumin concentrations[88,253]. MPA clearance and variability are affected by plasma protein binding changes in presence of high concentrations of the metabolite MPAG or the uremic toxins, consequent to poor or delayed graft function. Hence, patients with creatinine clearance less than 25 mL/min showed a significantly higher mean MPA clearance on days 3, 7 and 21 post-transplantation[90]. A positive correlation between the clearance of MPA and body weight has also been shown in renal transplant patients[89]. Hence, the variability in MPA

pharmacokinetics are associated with changes in covariates following renal transplant (both within patient as well as between patients) and these are more profound and significant during early post-transplant period. Further, apart from MPA exposures, corresponding changes in biological effects of MPA (inhibition of IMPDH activity) may be an additional factor that needs to be taken into consideration.

The association of early post-transplant exposure of MPA as well as response to MPA with the diagnosis of acute rejection (clinical + subclinical) observed during early protocol biopsy in renal transplant patients on standard immunosuppression therapy, is not clear. Hence, the broad study objective was to determine the contributory role of individual exposures to MPA (along with efficacy) and tacrolimus during early post-transplant period, in renal transplant patients with diagnosis of biopsy proven rejections (clinical + subclinical) or infection (BK virus or CMV). However, as determination of full exposures ($AUC_{0\text{-}12\ h}$) is not clinically practical, estimation of AUC using sparse sampling provided a viable alternative. Population pharmacokinetic models with maximum a posteriori bayesian (MAP) estimation of $AUC_{0\text{-}12\ h}$ from sparse sample concentrations, are used in some clinics to predict dose as well as confirm exposures for MMF and MPA, respectively[134]. Hence, the objective of the study presented in this chapter is to determine individual patient exposures to MPA along with its association to IMPDH activity by application of sparse sampling on 2 occasions, once during early post-transplant (1-2 weeks) and followed up at 3rd month, in renal transplant patients with protocol biopsies performed at 3 months.

5.3 MATERIALS

Methanol (UPLC MS grade), acetonitrile and water (MS grade) were purchased from Fisher Scientific (Fair lawn, NJ, US). Formic acid and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO). MMF Cat No: M831450, mycophenolic acid (MPA) Cat No: M831500, deuterated mycophenolic acid (d3-MPA) Cat No: M831502 and MPAG Cat No: M831520, were purchased from Toronto Research Chemicals (ON, Canada). Blank plasma was obtained from central reference testing laboratory, Pittsburgh. The centrifree ultrafiltration device Cat No. 4104 MI was purchased from Fisher Scientific.

IMP, AMP, 8-bromoadenosine 5-monophosphate (Br-AMP) and Dulbecco's Phosphate Buffered saline without calcium were purchased from Sigma Aldrich. XMP was purchased from Toronto Research Chemicals, Ficoll-Paque solution was from GE Healthcare (QC, Canada) and Sepmate50® tubes were purchased from stem cell technologies (BC, Canada). Sodium dihydrogen phosphate, potassium chloride, ammonium acetate, potassium hydroxide was from Fisher Scientific. The details of kits and reagents related to genotyping were presented in chapter 4.

5.4 METHODS

5.4.1 Study approval and Screening

The study protocol was approved by IRB (Approval No. PRO16050030, dated 20 Oct 2016, titled "Exposure to Mycophenolic acid/Tacrolimus and outcomes in renal transplant

recipients"). The details of inclusion exclusion criterion, screening of patients, enrollment, study design and time points were as provided in chapter 4.

5.4.2 Blood sampling

The conduct of the study and blood sampling are as described in chapter 4. A baseline blood sample was obtained prior to transplant, for characterization of individual IMPDH enzyme activities in the absence of immunosuppression. On the study day, three blood samples of approximately 7-8 mL each were obtained from all subjects. The first morning tough blood sample was obtained on arrival of patient to the clinic. Following the oral dose of MMF with tacrolimus, two additional samples were drawn at around 1.5 and 3.5 h into lithium heparin tubes. The exact clock times of previous and current dosing and sampling were individually documented. An aliquot of 2 mL blood sample was centrifuged at 4°C for 4 min at 1500 x g. The supernatant plasma was then transferred to cryovials, labeled and stored as duplicates at −80°C, until analysis of MPA and MPAG concentrations by LCMS/MS. The remaining blood used for PBMC isolation.

5.4.3 PBMC isolation

Blood was diluted 1:3 by using Dulbecco's phosphate buffered saline (DPBS without Calcium) and layered on top of 15 mL of Ficoll reagent in a Sepmate® tube followed with immediate centrifugation at 1200 g for 20 min. The supernatant was decanted by quick flip over of tube for not more than 2 secs to avoid pouring out of the sedimented layer. The decanted supernatant was washed once with cold DPBS and made up to volume of 30 mL, followed by centrifugation at 300g for 8 min. After discarding supernatant, the pale white pellet was retained

along with approximately 0.5 mL of DPBS. The viable PBMC cells are counted by tryphan blue exclusion under 20x magnification using a hemocytometer. The PBMC cells were immediately lysed by adding 100 µls of ice-cold water and lysates were stored in duplicates at -80° C, until analysis of AMP and XMP concentrations by LCMS/MS.

5.4.4 Protein binding

The binding of MPA and MPAG in plasma was determined by ultrafiltration and using once sample for each patient per each occasion (period) of the study. Thawed plasma sample was incubated with mild shaking at 37° C for 30 min. Then about 250-300 µls was transferred to a centrifree® ultrafiltration tube, taking care to carefully add on sides of the tube to avoid formation of bubbles. The ultrafiltration tubes were centrifuged in a Sorvall RC-58 centrifuge at 1000 g for 30 min at 37° C and the obtained protein-free ultrafiltrate was retained and stored at -80°C, until analysis of MPA and MPAG that correspond to their unbound concentrations.

5.4.5 LCMS/MS analysis of plasma/ultrafiltrate samples

Prior to LCMS analysis, plasma samples (50 µls) were mixed with 200 µls of Acetonitrile containing 50 ng of internal standard (d3-MPA) and vortexed for 30 seconds followed by centrifugation at 15,000 rpm for 8 min at 12°C. Supernatant (25 µls) was taken into another tube and mixed with 475 µls of mixture containing water : methanol (1:1 v/v) and again centrifuged at 15,000 rpm for 8 min at 12°C. Supernatant (400 µls) was transferred into LCMS vials and 1 µl was injected on to a Acquity® UPLC HSS T3 column (1.8 µm, 100 mm × 2.1 mm i.d.) (Waters Corp., Milford, MA, USA). The mobile phase was 2 mM ammonium acetate containing 0.1% v/v

of formic acid and acetonitrile in a gradient mode at a constant flow rate of 0.3 mL/min for 8 minutes. The settings for MS were: Capillary voltage 2.5 kV, Cone voltage: 40 V, Desolvation temperature: 500 °C, Cone gas flow 150 l/hr and Desolvation gas flow 850 l/hr. The mass transitions for MMF, MPA, MPA d-3 and MPAG were 433.9>114, 320.96>206.97, 323.9>209.9 and 518.8> 343.1, respectively.

The assay range was linear for MPA between 0.01-25 µg/ml, while linear range for MMF and MPAG concentrations were from 0.1-100 µg/ml, as shown in Figure 5.1. The processing conditions for ultrafiltrate was similar except that 100 µls of acetonitrile was added to samples and 2 µls was injected on to the column. For ultrafiltrate samples, water was used for calibration standard preparation and the assay range for MPA and MMF were between 0.002-1 µg/ml, while the range for MPAG concentrations was from 0.02-10 µg/ml.

5.4.6 Incubation protocol for assessment of IMPDH activity

The protocol for evaluation of IMPDH activity in lysates of PBMC was adapted from Bemer et al.[254] after slight modifications, as described below. All activities were carried by incubation on ice. The frozen lysates were thawed and subjected to 15 secs of probe sonication for disrupting intact PBMC cells, if any. Next, the lysates were centrifuged for 1 minute at 1000g, to precipitate cellular debris. The clear supernatant lysates of PBMC were incubated in duplicate, one to assess XMP production from the externally added substrate IMP, and the other to assess AMP content as a surrogate for number of PBMCs used for incubations. For XMP incubations, 50 µls of lysate was mixed with 130 µls of ice-cold incubation buffer (composed of 40 mM sodium dihydrogen phosphate and 100 mM potassium chloride (pH 7.4) along with 1 mM of IMP and 0.25 mM of NAD^+). The incubation process was initiated by placing in a heated water bath at 37°C and

with gentle agitation for 180 min. In parallel, the duplicate of the lysate (50 µls) was mixed with 130 µls of ice-cold working buffer (composed of 40 mM sodium dihydrogen phosphate and 100 mM potassium chloride (pH 7.4) only) and incubated alongside the earlier sample, for the assessment of AMP content in PBMC. Unlike incubates to assess XMP production, the duplicate incubates for AMP estimation did not include NAD^+, because NAD^+ was an interference for AMP analysis, owing to the similarity in mass to charge ratio and retention times during LCMS/MS analysis.

Incubations were terminated with 1 mL of ice-cold methanol followed with addition of 20 µls of internal standard (10 µg/ml of Br-AMP in deionized water) and the mixture was vortexed for 30 seconds and centrifuged at 15,000 g for 10 min at 37° C. About 0.9 mL of supernatant was then transferred to 12 × 75 mm disposable culture tubes that were placed on ice and then evaporated to dryness under a stream of air at 30 °C. The dried samples were stored in -80°C after proper wrapping of the culture tubes and stored for up to 2 weeks until LCMS analysis. A requirement for greater than 2 weeks of storage did not arise, and no stability issues were encountered with up to 2 weeks of storage at -80°C.

5.4.7 LCMS analysis (XMP/AMP)

The dried samples were reconstituted with 100 µls of LCMS grade water, transferred into vials and 10 µls was injected onto a Thermo Scientific Hypercarb column (2.0mm × 100mm × 5µ, part no. 35005-102130; Thermo Scientific, Bellefonte, PA) for the analysis of XMP or AMP. The total sample run time was 10 min.

The chromatographic separation was performed on an Agilent HPLC/MS system series 1200 with a thermostatically controlled autosampler (Agilent Technologies, Palo Alto, CA) maintained at 4°

C. The mobile phase consisted of A: acetonitrile and B: 0.1 M ammonium acetate in LCMS water, adjusted to pH 8.5 with ammonium hydroxide and freshly prepared every time, just before analysis, failing which retention times were altered. Elution was achieved with a mobile phase gradient starting at 5% acetonitrile for first 0.5 min then increased to 30% at 4 min and held at 30% till 5 min and then switched back to 5% at 5.1 min. The first 3.5 minutes of eluate as well as the last 3 minutes were diverted to waste. The column temperature was set to 30 °C and the flow rate was maintained at 0.3 mL/min. The autosampler needle was washed between injections with 0.03 % v/v of ammonium hydroxide to avoid carryover with NAD^+ or AMP.

A SCIEX 4000 Q triple quadrupole ion trap mass spectrometer electron spray (ES) in positive ion mode was used. The temperature of the drying gas (nitrogen) was maintained at 350 °C at a flow of 11 L/min.

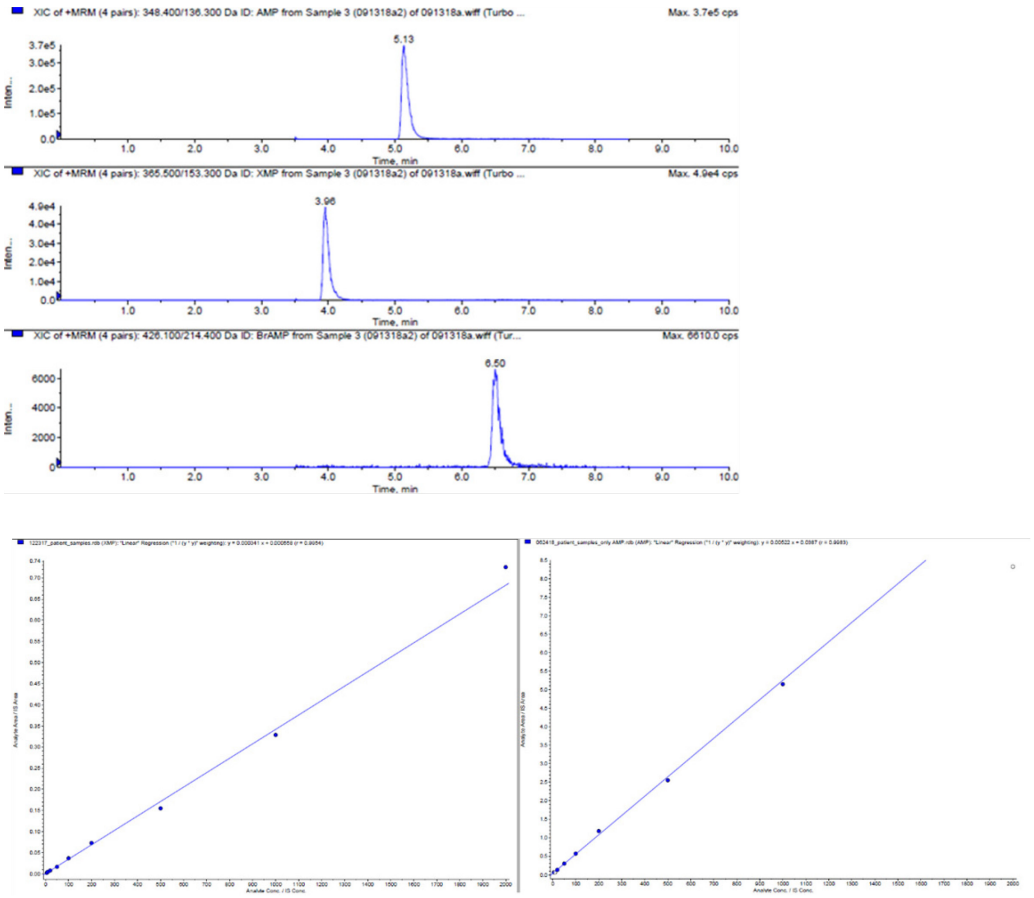


Figure 5.1. (a) Representative ion chromatograms of Adenosine monophosphate (AMP), Xanthosine monophosphate (XMP) and Internal standard (Br-AMP)
(b) Standard curve for XMP (range 5-2000 ng/mL) and AMP (5-1000 ng/mL)

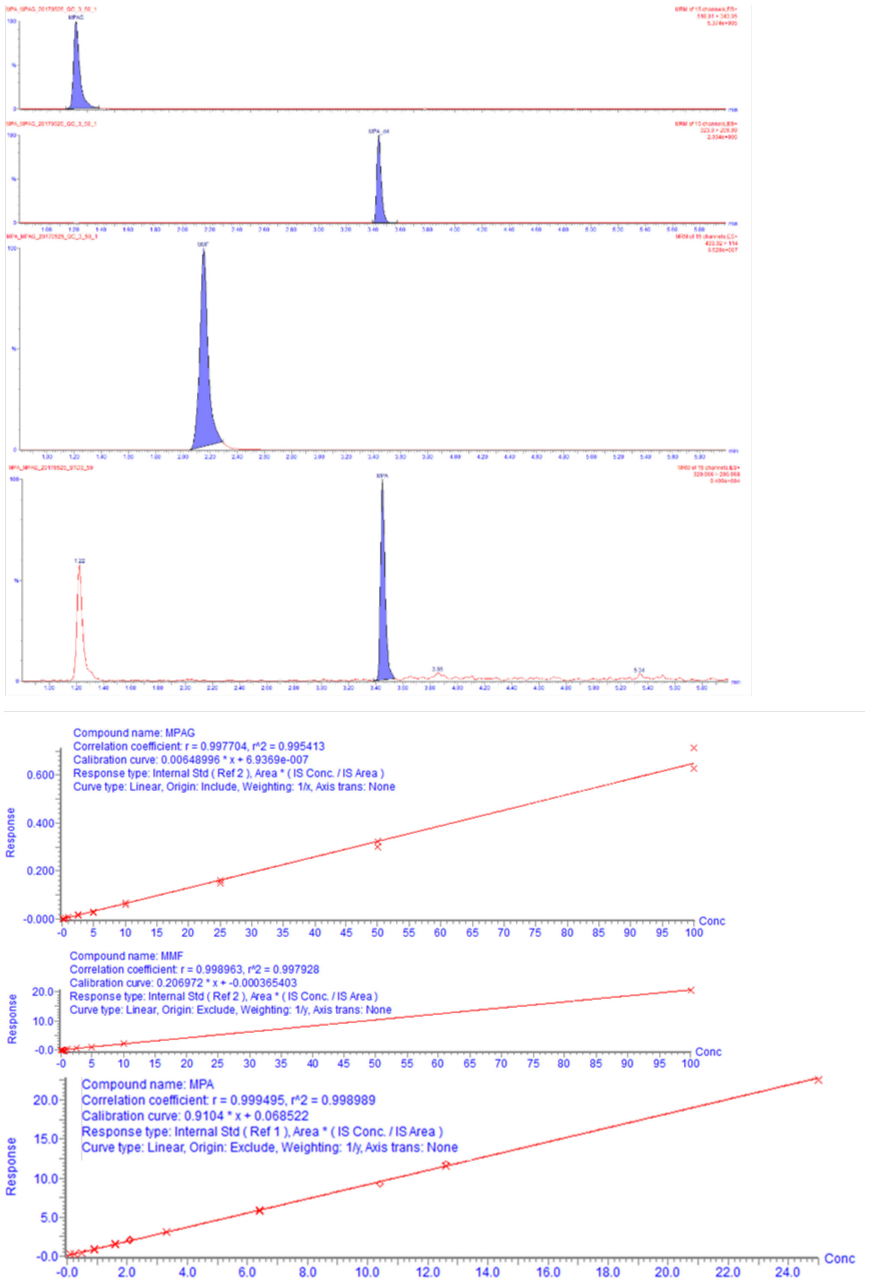


Figure 5.2. (a) Representative ion chromatograms of mycophenolic acid glucuronide (MPAG), Mycophenolate mofetil (MMF), Mycophenolic acid (MPA) and Internal standard (MPA-d4) at retention times 1.3, 2.2, 3.4 and 3.4 mins, respectively (b) Standard curve for MPAG (range 0.1-100 µg/mL), MMF (0.1-100 µg/mL) and MPA (0.01- 25 µg/mL)

The transition ion pairs under MRM mode were m/z 365.5> 153.3, 348.4> 136.3 and 426.1> 214.4 for XMP, AMP and Br-AMP, respectively. The typical retention times were 3.8 min for XMP, 5.5 min for AMP and 6.9 min for Br-AMP. As shown in Figure 5.2, the assay was linear for XMP and AMP in the range of 5-2000 and 5-1000 ng/mL, respectively.

5.4.8 Calculations and data analysis

The fraction unbound (f_u) of either analyte (MPA or MPAG) was determined as ratio of unbound (ultrafiltrate) to total (plasma) concentrations, as below

$$f_u = \frac{[\text{analyte}]_{\text{filtrate}}}{[\text{analyte}]_{\text{plasma}}}$$

% free = f_u x 100; where % free is the unbound free drug expressed in percentage.

5.4.9 Prediction of individual concentrations and predictive performance

Individual parameter estimates for CL were determined by nonlinear mixed effect modeling using NONMEM 7.4.3 (Icon, Ireland). The population values for MPA were fixed from a prior model [233] with subroutine ADVAN4 TRANS4 and maximum a posteriori (MAP) bayesian method with first order estimation was used. A two-compartment model with first order absorption (Ka=2.65 h^{-1}) was used to describe the time profile of MPA after single dose of mycophenolate mofetil (MMF). The CL/F was fixed at 6.83 L/h, central volume V1/F at 133 L, intercompartmental Q/F at 28.2 L/h and V2/F at 995 L. Exponential model was used for all the interindividual variability terms and a combined error model was used to describe the residual variability. The MMF dose and MPAG concentrations were converted into MPA equivalents by

multiplication with 0.739 and 0.645, respectively. The dosing history and clock timings were used as prior information for each patient for estimation of individual parameters and were output with specification of "posthoc" and maxeval=0. Individual concentrations of MPA were predicted until 12 h after MMF dosing on the day of study and compared with observed concentrations. Predicted MPA concentrations at desired time points were used to estimate the individual 12 h exposures ($AUC_{0\text{-}12\ h}$) for each period, as described later. Alternatively, a linear regression based equation was also used to estimate exposure, by using observed concentrations at 1.5 and 3.5 h in the published equation[233]: $16.5 + (4.9 \times C_{1.5}) + (6.7 \times C_{3.5})$, as ~90 % of sampling time points were within 10 minutes of 1.5 and 3.5 h.

Predictive performance of the population model was verified with diagnostic plots using pirana® (Certara, NJ, US) and RStudio (version 1.1.453), as described in chapter 4. The bias in predicted concentrations were estimated using mean prediction error (MPE) and precision was estimated by median absolute prediction error (MDAPE) and median percentage in absolute prediction error relative to the observed concentrations (% MDAPPE), as described earlier in chapter 4.

5.4.10 Non-compartmental analysis

The partial exposures ($AUC_{partial}$) for MPA and MPAG were calculated until 3.5 hours (or the actual time of last sample) on periods I and II, by using non-compartmental analysis (NCA) of Phoenix® WinNonlin (Certara, NJ, US). The partial exposures of MPA and MPAG were multiplied by their corresponding unbound fractions to obtain unbound partial exposures ($AUC_{p\text{-}free}$). The total exposures (predicted $AUC_{0\text{-}12\ h}$) were calculated from individual predicted concentrations

using NCA by Phoenix® WinNonlin. The total exposure of MPA was multiplied by corresponding unbound fractions to obtain unbound exposure (AUC_{free}).

5.4.11 IMPDH enzyme activity

Activity of IMPDH in PBMC lysates of patients was expressed as XMP (µmol) produced per unit time (sec) after normalization with AMP (mol), considering AMP content as a surrogate for the actual number of PBMC cells that were initially present in the lysate. The estimated AMP content was used for normalization, in lieu of cell counts or protein content, due to better accuracy and sensitivity in measuring AMP in comparison to cell counting or protein estimation by traditional methods.

IMPDH activity (µmolof XMP/second/mol of AMP)	=	[produced XMP] x 10^6 / (incubation time in secs x measured AMP)]

Parameters corresponding to description of individual IMPDH enzyme activities such as the predose activity, R_{min} and AUC_B were summarized using the drug effect module of NCA using Phoenix® WinNonlin. The AUC_B was the area under the IMPDH activity curve from baseline until the last observed time point of around 3.5 h and R_{min} corresponds to response minimum which is the least IMPDH activity during the observation interval and is associated with the maximum inhibition effect by MPA.

5.4.12 Genotyping

The protocol followed for exaction, purification of genomic DNA and genotyping by allelic discrimination assays, was as described in chapter 4. The genotypes associated with disposition of MPA and IMPDH activity such as *UGT 1A9* (98 T>C), *UGT 1A9* (275 T>A), *UGT 1A9* (2152 C>T), *IMPDH 1* (106 G>A), *IMPDH 1* (125 G>A) and *IMPDH 2* (3757 T>C) were considered for analysis, and details are as shown in Table 5.1.

Table 5.1. Assay details for identifying SNPs in select enzymes and transporters associated with mycophenolic acid disposition and IMPDH activity in patients

gene	SNP name	rs number	Assay ID (Taqman®)
UGT 1A9	98 T>C	rs72551330	C__64627083_10
UGT 1A9	275 T>A	rs6714486	C__27843087_10
UGT 1A9	2152 C>T	rs17868320	C__34418857_10
IMPDH 1	106 G>A	rs2278294	C__2830834_20
IMPDH 1	125 G>A	rs2278293	C__15965182_10
IMPDH 2	3757 T>C	rs11706052	C__1842928_10

5.4.13 Statistical analysis

Statistical tests were performed using SPSS® software version 25 (IBM corporation, US) and Prism version 8 (GraphPad software, CA, US). A paired t-test or Wilcoxon signed rank test

was used for comparison of values between period I and II within the same patient while overall comparisons between the patients in both periods were tested using unpaired t-test or Mann Whitney test, with significance of < 0.05. Ratio statistics of SPSS was used to obtain confidence interval around the median of predictive performance values. Graphical outputs were done using R (version 3.5), pirana® and Prism software.

5.5 RESULTS

A summary of the clinical variables and demographic information are described earlier (Table 4.2 of chapter 4). As noted earlier, a significant improvement in renal function (estimated glomerular filtration rate eGFR) with time was observed when comparing values from both periods, around the 6^{th} and 91^{st} day post-transplant, respectively. Though, median albumin levels did not differ between the 2 periods, there was a significant increase in percentage protein binding of MPA and MPAG in period II, in concordance with improvement in eGFR. As shown in Table 5.2 and Figure 5.3, the percentage free drug dropped from an average of 2.5 down to 1% and from 52 to 32% from period I to II for MPA and MPAG, respectively. The partial exposures (until 3.5 h after dosing of MMF) obtained during both periods for MPA and MPAG are compared in Table 5.2 and Figure 5.3. As MPAG is renally cleared, poor MPAG elimination was observed during period I, at around 6 days after transplantation of the graft, resulting in a significantly higher MPAG/MPA ratio (~ 50 vs 12, period I vs II). With time, there was a significant decrease in exposure to the renally eliminated MPAG, due to the improved function of the transplanted kidney, while the partial exposure to the hepatically cleared MPA was significantly increased from period I to period II. MPA is a low clearance drug and hence as ($CL_{total} = fu \times CL_{intrinsic}$), the CL of total

MPA is significantly higher when fu was higher (period I) followed by lower fu and decreased MPA clearance (period II). Again, despite lower fu during period II (increased protein binding of MPA), partial exposure to MPA_{free} was unchanged, while it significantly decreased for MPAG (Table 5.2 and Figure 5.3). The observations for unaltered MPA_{free} exposure was supported by predicted CL/F_{free} for MPA also being unaltered during the two periods (Table 5.4), as is usually the case with drugs having a low extraction ratio. This also indicated a normal conjugation activity by UGT enzymes involved in the metabolism of MPA during this post-transplant study period.

The maximum a posteriori Bayesian estimation (MAP) showed superior predictions for $AUC_{0\text{-}12h}$ in comparison to linear regression equation-based estimation (Figure 5.4). Figure 5.4 also shows that despite good correlation with MAP, a systematic overestimation in exposure occurred with the regression method. There was a better correlation of MAP estimations with observed MPA trough levels (r=0.89). The results with MAP estimation were more consistent with clinically reported exposures after 1000 mg MMF dosing .

Table 5.2. Protein binding and partial AUCs (total and free) of MPA and MPAG (Period I & II)

Mean ± SD Median (Min, max)	MPA				MPAG			
	All patients	Only patients that completed both periods			All patients	Only patients that completed both periods		
	Period I (n=42)	Period I (n=23)	Period II (n=23)	p value*	Period I (n=42)	Period I (n=23)	Period II (n=23)	p value*
$AUC_{partial}$ (µg*h/mL)#	13.8 ± 8.0 11.8 [2.5, 35]	15.0 ± 8.6 14.5 [2.5, 35]	22.4 ± 14.1 19.8 [5.8, 63.9]	0.03	432 ± 276 372.3 [87.2, 1234.5]	391.7 ± 252.5 300.5 [92.7, 966.6]	203 ± 62.7 198.8 [61.5, 334.9]	<0.005
fu (%)	2.5 ± 1.9 1.8 [0.4, 8.9]	2.5 ± 2.2 1.4 [0.4, 8.9]	1.0 ± 0.3 1.0 [0.4, 1.6]	<0.01	49.7 ± 21.4 52.8 [15.5, 90.0]	52.0 ± 24.3 53.7 [15.5, 90.0]	32.4 ± 16.7 26.5 [10.1, 73.1]	<0.001
$AUC_{partial.\ free}$ (µg*h/mL)	0.28 ± 0.21 0.23 [0.03, 1.1]	0.29 ± 0.2 0.23 [0.08, 0.9]	0.23 ± 0.17 0.19 [0.06, 0.8]	NS	239 ± 205 152.9 [22.6, 697.4]	225.5 ± 208.9 145.1 [30.2, 675.4]	69.2 ± 47.3 54.9 [16.5, 173.3]	<0.005
Ratio (MPAG/MPA)	49.6 ± 58 27.4 [3.5, 255]	46.1 ± 64.6 20.2 [5.5, 255]	12.0 ± 8.4 10.4 [3, 43.6]	<0.01	NA	NA	NA	NA

$AUC_{partial}$ = $AUC_{0\text{-}3.5\ h}$, * paired t-test comparison in patients that completed both periods; NS- No significance ($p \geq 0.5$)

Correlation to predicted $AUC_{0\text{-}12\ h}$ was r= 0.81 (0.66-0.89) and 0.98 (0.95-0.99) in period I (n=42) and II, respectively

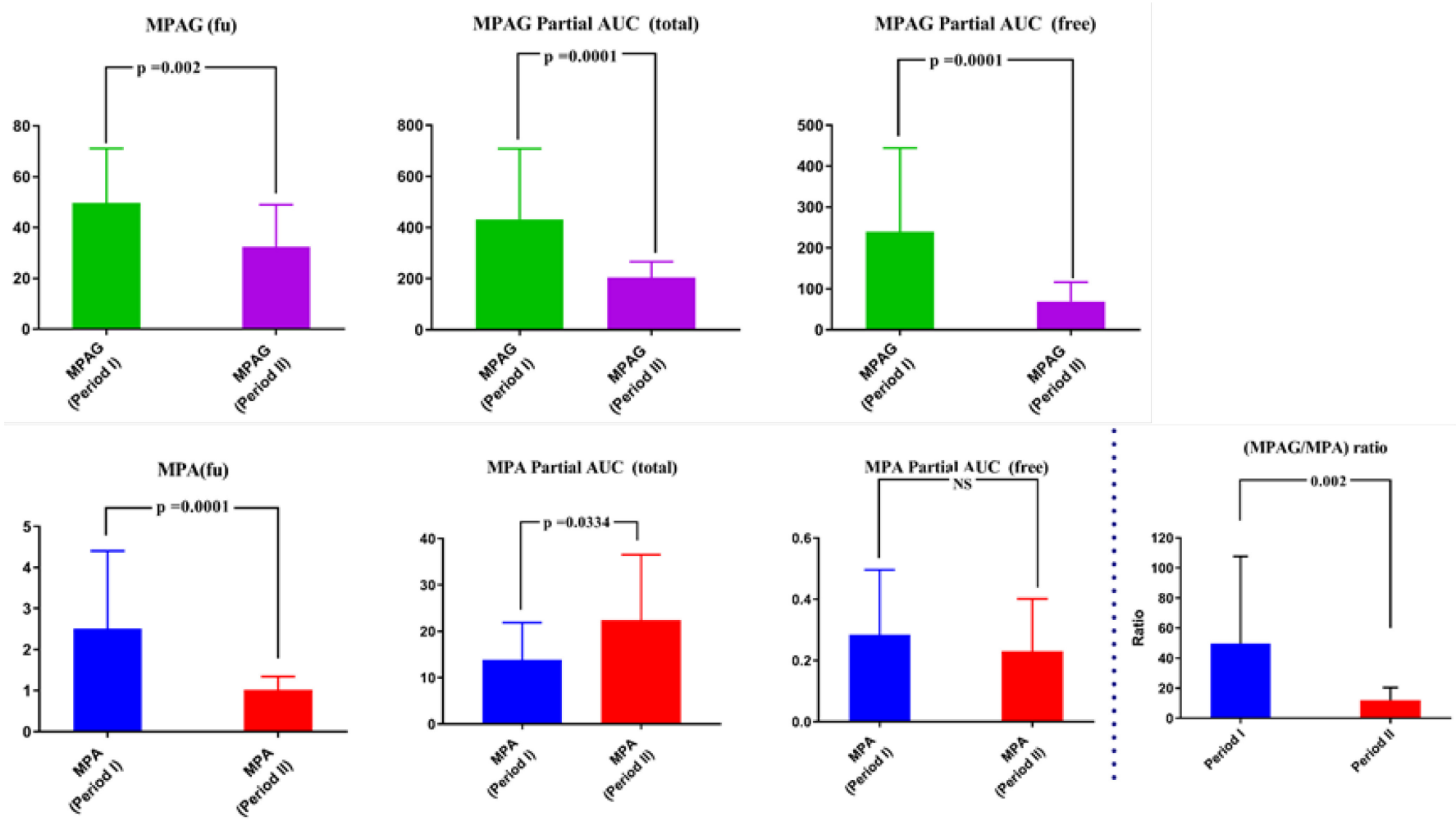
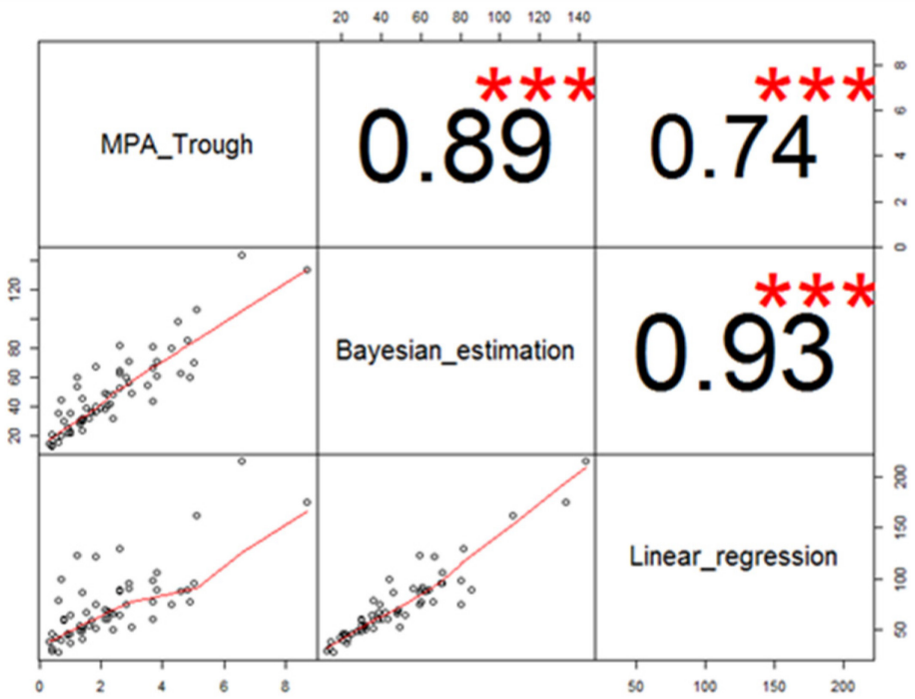


Figure 5.3. Summary of partial (total and unbound) Area Under Curves and fraction unbound (fu) for MPA and MPAG (Periods I & II)

Partial AUC of MPA or MPAG = $AUC_{0\text{-}3.5\ h}$



Linear_regression = 1.215*(Bayesian_estimation) + 15.41

Figure 5.4. Correlation between MPA exposure determined by Bayesian estimation, linear regression and trough plasma levels

The scatter plot matrix shows individual panels examining correlation between exposure determined by two different methods (µg*h/mL) and correlation with the observed MPA trough plasma concentrations (µg/mL). Corresponding r values for correlations are in the diagonally opposite panel

Table 5.3. Predictive performance of bayesian estimation for mycophenolic acid plasma concentrations

Measure	Estimate (95 % CI)
Mean prediction error (MPE, µg/mL)	0.04 (-0.21 – 0.29)
Median absolute prediction error (MDAPE, µg/mL)	0.80 (0.65 – 1.0)
Median absolute percentage prediction error (MDAPPE, %)	25 (21.8 – 27.5)

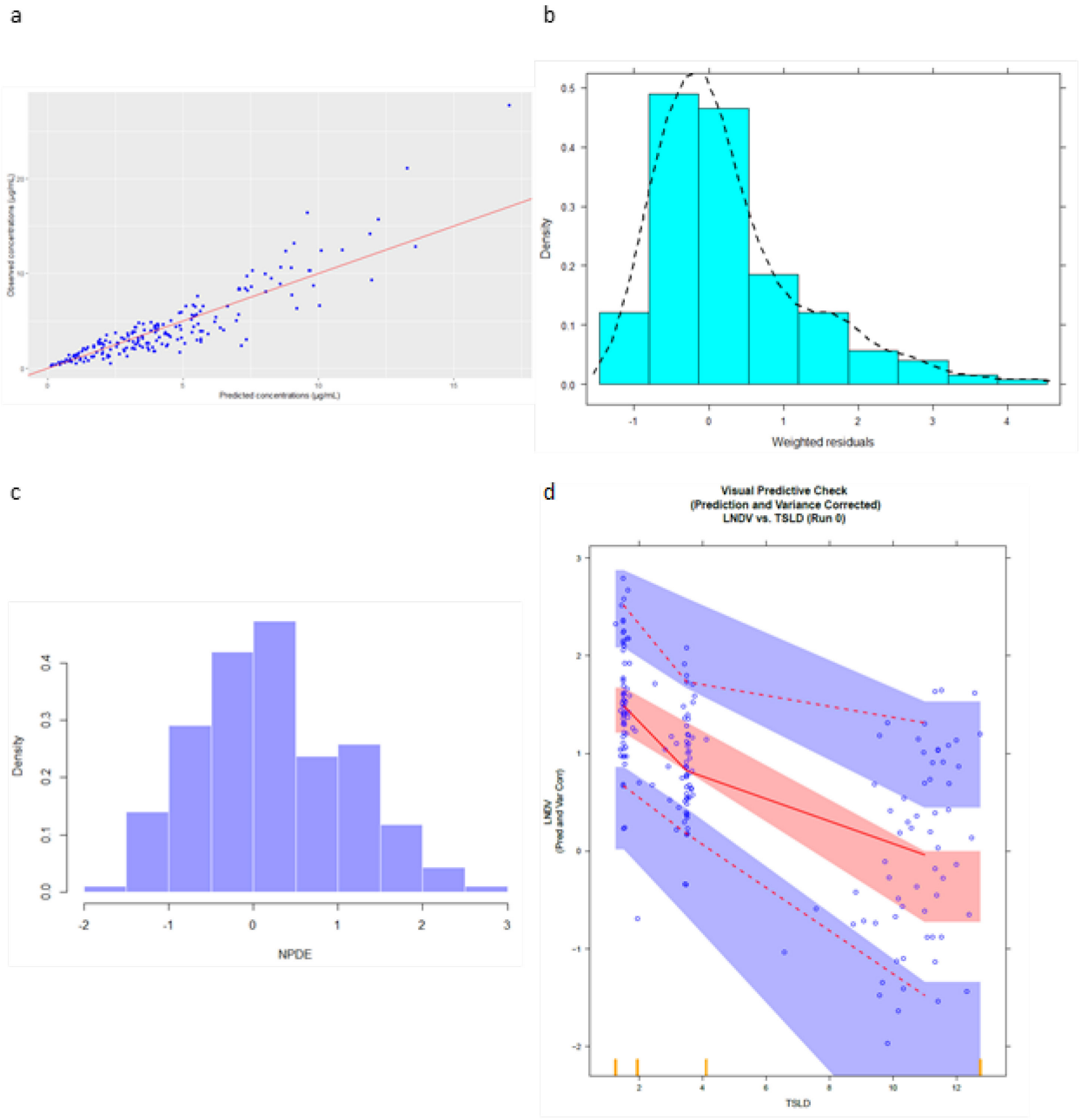
The quality of MPA concentration predictions using a prior model with maximum a posteriori bayesian estimation, were assessed from the bias and precision values, as shown in Table 5.3. The model predicted concentrations were also visually evaluated for comparison with observed plasma concentrations and are presented in panels a-d of Figure 5.5. The mean of prediction error (with 95% CI) was 0.04 (-0.21-0.29) µg/mL and was not significantly different from zero (p=0.733). The observed imprecision in prediction was 0.80 (0.7-1) µg/mL with an absolute prediction error of 25% (21.8 – 27.5). The predicted concentrations agreed well with observed concentrations, but the higher absolute prediction error was due to underpredictions at 1.5 h and slight overprediction at 3.5 h, observed in less than 5 % of samples. The weighted residuals (after first order estimation) and prediction errors showed an approximate normal distribution with a mild skew towards positive values. The prediction and variability-corrected visual predictive check (pvcVPC) is presented in panel d of Figure 5.5. The pvc was done to correct for MPA concentrations after different daily doses of MMF. As shown in the Figure, the median as well as the 5^{th} and 95^{th} percentile of the prediction and variability-corrected log transformed observations overlapped with the distribution of the simulated concentrations and indicated model acceptability in predicting MPA concentrations.

The predicted individual exposures to MPA ($AUC_{0\text{-}12\ h}$) in patients are summarized in Table 5.4 and a graphical comparison of exposure versus the desired therapeutic range is presented in Figure 5.6. An overall increase in MPA exposures with higher dose of MMF was observed as shown in Figure 5.6. In addition, a trend to increase in mean predicted $AUC_{0\text{-}12\ h}$ was noted with time after transplant (48.3 vs 58.2 µg*h/mL), which was paralleled by a significant decrease in predicted CL/F from period I to II, as shown in Table 5.4.

Table 5.4. Predicted CL/F and exposure for MPA by maximum A Posteriori bayesian estimation

Mean ± SD	MPA			
	All patients	**Only patients that completed both periods**		
	Period I (n=42)	Period I (n=23)	Period II (n=23)	p value*
Predicted $AUC_{0\text{-}12\ h}$ (µg*h/mL)	43.9 ± 21.7	48.3 ± 21.4	58.2 ± 32.8	NS (0.1522)
Dose Normalized Predicted $AUC_{0\text{-}12\ h}$ (µg*h/mL/mg)	0.04 ± 0.02	0.05 ± 0.02	0.07 ± 0.03	0.0020
Predicted $AUC_{0\text{-}12\ h\ free}$ (µg*h/mL)	0.946 ± 0.736	0.847 ± 0.490	0.537 ± 0.392	0.0203
Dose Normalized Predicted $AUC_{0\text{-}12\ h\ free}$ (ng*h/mL/mg)	0.961 ± 0.733	0.974 ± 0.781	0.705 ± 0.362	NS (0.1029)
Predicted CL/F (L/h)	28.9 ± 16.0	25.9 ± 14.6	16.6 ± 6.3	0.002
Predicted $(CL/F)_{free}$ (L/h)	15.8 ± 10.4	16.8 ± 11.9	18.4 ± 11.5	0.697

*paired t-test comparison in patients that completed both periods; P<0.05 was significant difference; NS – not a statistically significant difference; AUC_{free}- unbound Area Under the Curve



a) Observed versus individual predictions (µg/mL) b) Histogram plot for weighted residuals (first order estimation) c) Plot of normalized prediction distribution errors (NPDE) d) pvc-VPC plot where blue dots represent the prediction and variance corrected natural log transformed observations, the solid and broken lines represent median and 90% prediction intervals. The shaded areas around lines represent 99% confidence intervals for median, 5th and 95th prediction intervals obtained from the simulations with IIV of V2/F and CL/F fixed at 50 and 100%, respectively

Figure 5.5. Evaluation of observations versus predictions with the Mycophenolic acid population model

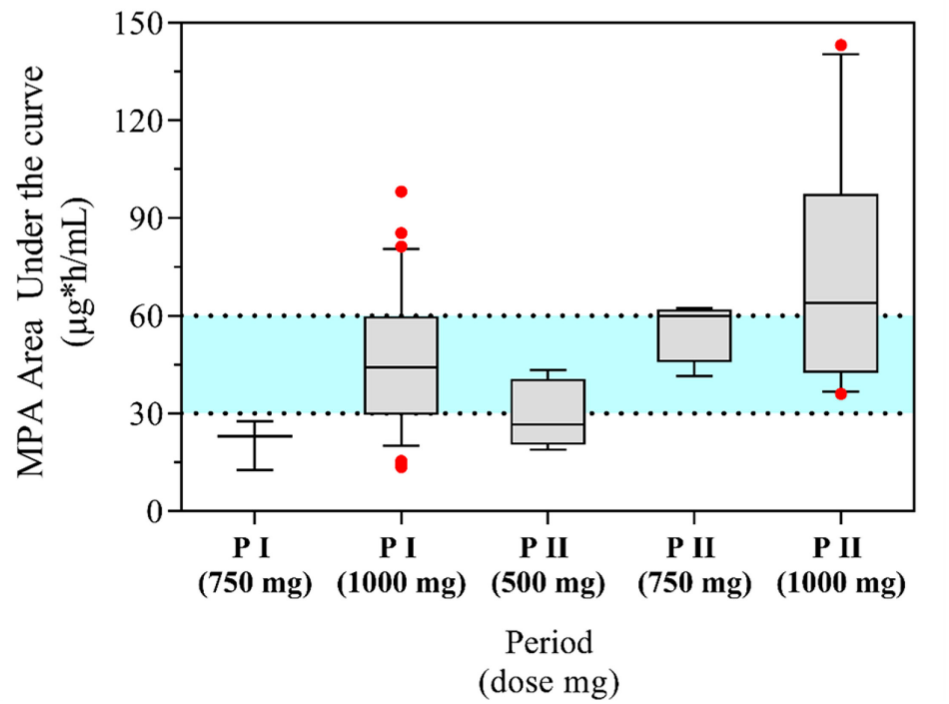


Figure 5.6. Summary of predicted area under the curve (AUC) values for Mycophenolic acid with MMF doses on Period I and II

The boxes represent median and inter-quartile range in predicted total MPA exposures achieved in patients on different doses of MMF (mg/ twice daily); The shaded between 30-60 µg*h/mL represents desired range of MPA exposure based on previous studies; The number of patients on each dose from left to right are n = 3 and 39 for 750 and 1000 mg bid in period I and n = 6, 4 and 13 for 500, 750 and 1000 mg bid in period II, respectively

The dose normalized predicted exposure was significantly increased during period II, while the dose normalized predicted free exposure showed no change (Table 5.4). The same pattern is also observed when exposures after similar MMF doses, were compared across both periods. Higher exposures occurred in period II after similar doses (Figure 5.6). Overall, only 47 and 50% of patients were within the 30-60 µg*h/mL range during periods I and II, respectively. Around 33 and 13% of patients were below and 13 and 37% of patients were above the AUC range, during periods I and II, respectively.

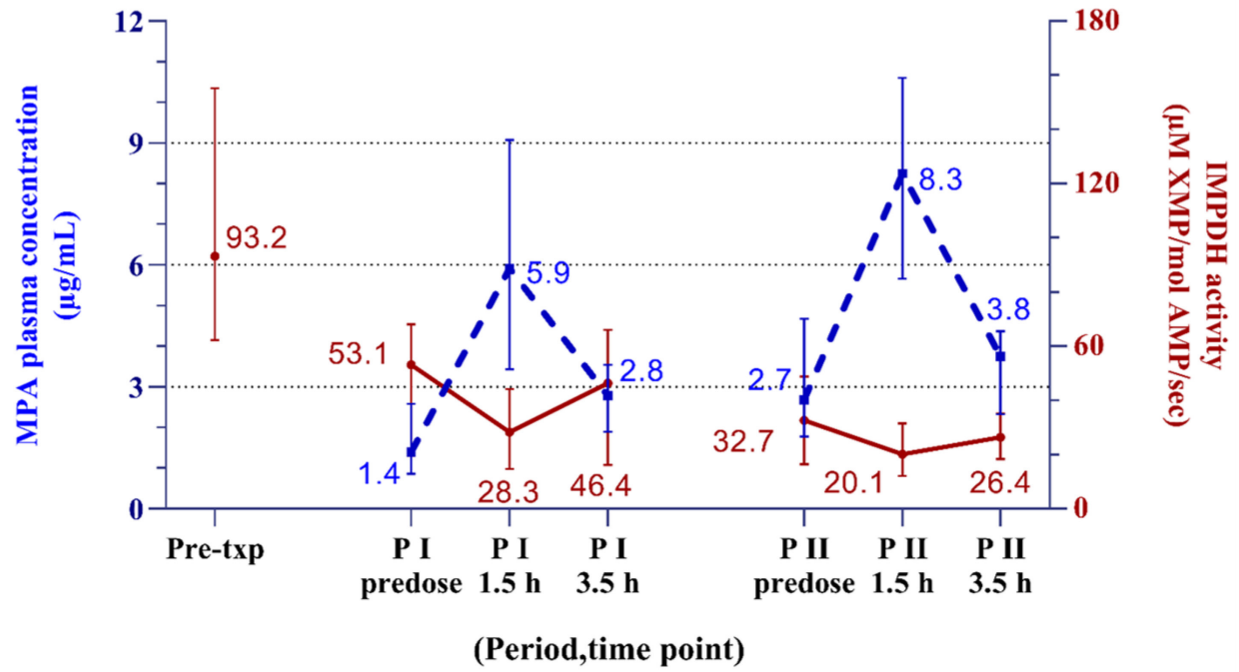


Figure 5.7. Summary of Mycophenolic acid concentrations versus IMPDH activity at sparse sampling time points (Periods I and II)

The median baseline pretransplant IMPDH activity was 93.2 (62.29, 155.2) µM XMP/M AMP/sec and variable between the patients. The IMPDH activity at the sparse sampling points in both periods along with corresponding MPA concentrations are illustrated in Figure 5.7. Overall, the IMPDH activity reached nadir values around 1.5 h at both periods corresponding to the peak values of MPA plasma concentrations. IMPDH activities at all time points in period II were lower than at similar time points in period I and the corresponding MPA concentrations were also higher. The higher inhibition of IMPDH activity in Period II was also evident on comparison of the pharmacodynamic parameters presented in Table 5.5. The average values of predose, R_{min} were significantly lower while those of maximum inhibition percentage, AUC_B were significantly higher, indicating better inhibition in IMPDH activity during Period II.

The pre-transplant IMPDH activity showed significant positive relationship with the predose activity as well as the R_{min} during Period I, so patients with higher pretransplant activity tend to

show higher predose activity and higher R_{min} during the early post-transplant period, as shown in Figure 5.8.

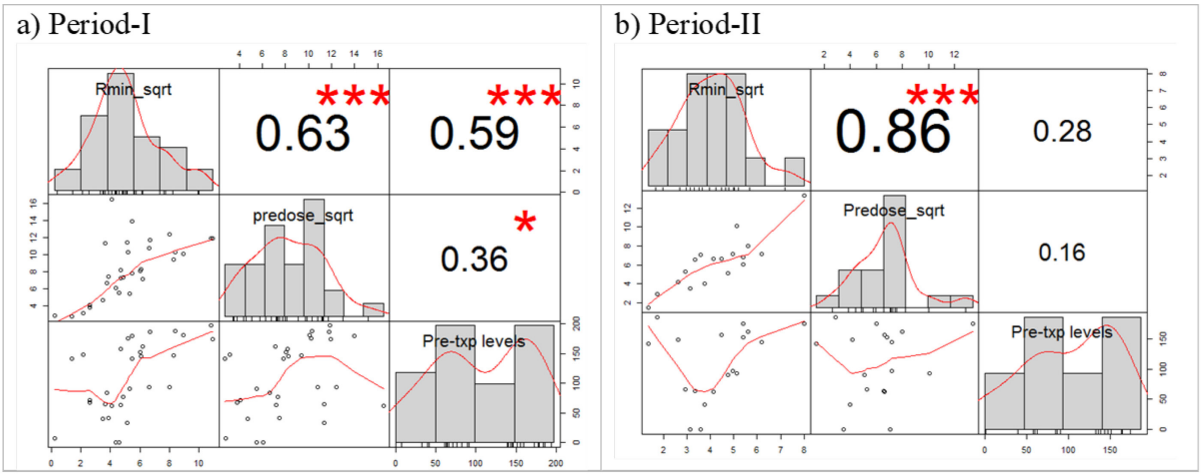


Figure 5.8. Relationship of pre-transplant IMPDH activity versus post-transplant IMPDH activity during trough (predose) and response minimum (Rmin) (Periods I and II)

The scatter plot matrix shows individual panels examining time-dependent correlation between parameters of IMPDH activity such as the R_{min} (Response minimum of IMPDH activity within the sparse sampling time points), predose (IMPDH activity at trough time point) and activity obtained at pretransplant; all IMPDH activity values were in µM of XMP/mol of AMP/sec; Square root transformation of R_{min} and predose values was done prior to evaluating correlation; Corresponding r values for correlations are in the diagonally opposite panel

This relationship was not significant during period II, after a prolonged exposure to MPA. IMPDH activity showed an inverse relationship with total MPA concentrations which was better than with the free MPA concentrations, examined during both periods. As shown in Figure 5.9, a negative relationship (r = - 0.28 – -0.38) existed for the IMPDH activity versus MPA concentrations in both periods, while comparable value was observed for free MPA, only in period II.

Table 5.5. Summary of response (PD) parameters related to IMPDH activity and corresponding exposure (PK) parameters for MPA on Periods I and II

	Period 1	**Period 1**	**Period II**	
	(n=34)	**Patients that finished both periods**		**p value (paired**
PD parameter				
Predose [μM XMP/M AMP/sec]	58.4 [7.7, 272]	54.8 [7.7, 272]	36.3 [2.1, 179]	0.04
R_{min} [μM XMP/M AMP/sec]	28.3 [13.9, 44.1]	26.1 [15.1, 39.1]	19.0 [10.2, 28.4]	0.01
AUC_B [h.μM XMP/M AMP/sec]	244.0 ± 157.0	248.6 ± 164.2	293.8 ± 166.6	0.009
PK parameter				
$AUC_{partial}$ **(μg*h/mL)**	14.5 ± 8.5	15.6 ± 8.7	21.4 ± 14.1	NS
$AUC_{p\text{-}free}$ **(μg*h/mL)**	0.3 [0.08, 1.0]	0.2 [0.08, 0.7]	0.2 [0.06, 0.8]	NS
Predicted $AUC_{0\text{-}12\ h}$ **(μg*h/mL)**	44.8 ± 23.5	46.6 ± 23.0	55.7 ± 33.5	NS
Predicted CL/F (L/h)	28.9 ± 16.0	25.9 ± 14.6	16.6 ± 6.3	0.002

Values are expressed as Mean (± SD) or Median (Range) ; Pre-dose- pre-dose trough values for IMPDH activity; AUC_B (AUC below baseline); R_{min}- Response minimum corresponds to the least IMPDH activity observed during the study duration; AUCpartial- partial 3.5 h AUC for MPA; AUCp-free- partial free AUC for MPA; $AUC_{0\text{-}12\ h}$- Individual estimated 12 h AUC for MPA

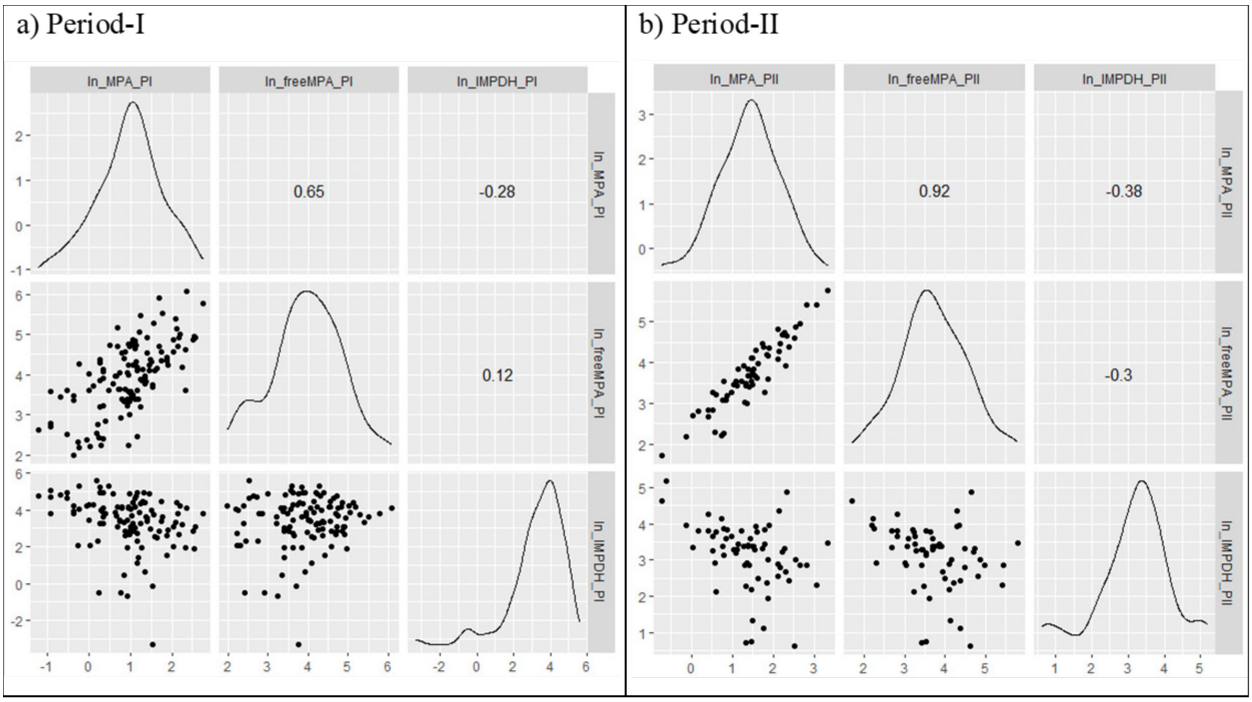


Figure 5.9. Correlations between IMPDH activity, MPA and free MPA concentrations (Periods I and II)

The overall comparison after dual monitoring of MPA and IMPDH activity, showed a significantly higher R_{min} (IMPDH activity at peak inhibition ~ 1.5 h post MMF dose) in patients with lower 12 h predicted exposure to MPA (< 30 µg*h/mL) when compared to patients with higher exposure (Figure 5.10). A similar trend was also observed with predose (trough) versus exposure (not shown) as R_{min} showed a good correlation with predose IMPDH activity, whereas the AUC_B was not significantly different between the categories of MPA exposure, as reported in an earlier study[255].

The distribution frequencies of *UGT* and *IMPDH* genotypes in study population are presented in Table 5.6. The frequencies for alleles were comparable to typical values reported for the Caucasian population. The effect of *UGT* genotypes on dose normalized exposure to MPA and MPAG are compared in Table 5.7 after pooling of heterozygous variants with homozygous variants, due to

low sample size. No significant differences were observed when MPA and MPAG exposures were compared between the genotype groups. Similarly, no significant differences in IMPDH activity and MPA exposure were observed on comparison of genotype groups for IMPDH I and II, except a higher pre-transplant activity with IMPDH II wild type, as in Table 5.8.

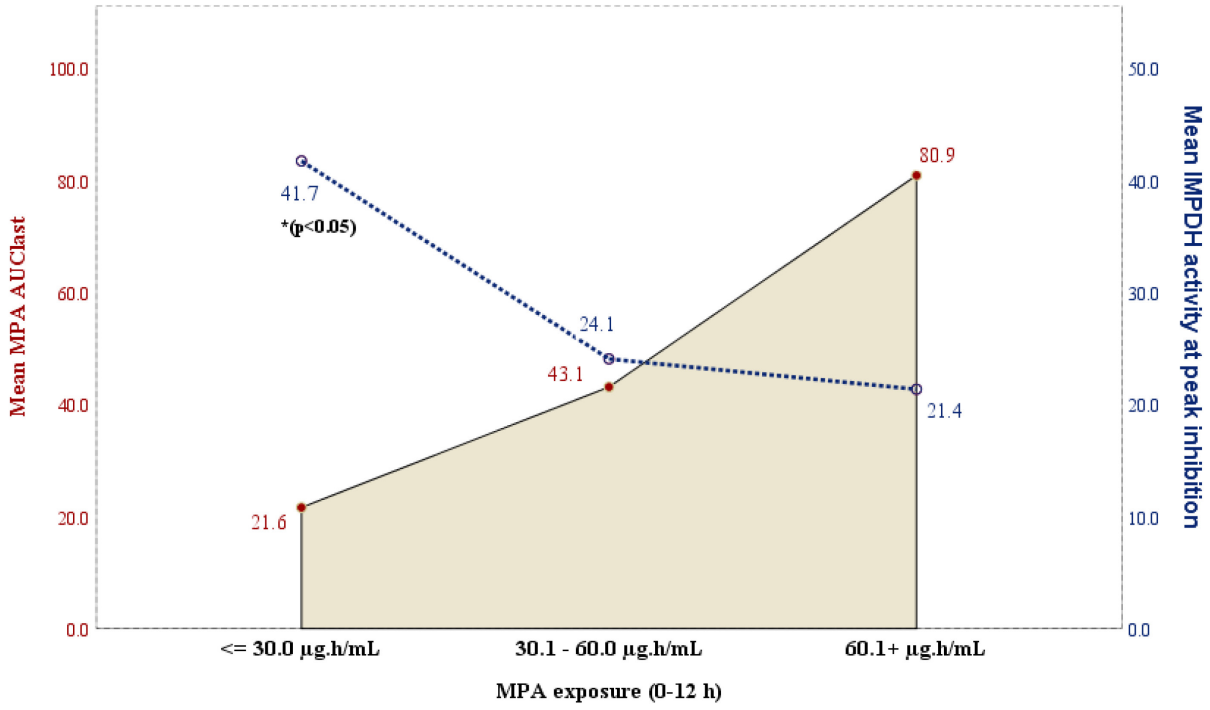


Figure 5.10. IMPDH activity in relation to corresponding MPA exposure

Table 5.6. Frequency (%) of the SNP variants for *UGT1A9* and *IMPDH* 1/2 genes

SNP	Gene	Homozygous (wild type)	Heterozygous variant	Homozygous variant
rs72551330	*UGT 1A9* 98 T>C	36 (97.3)	0 (0)	0 (0)
rs6714486	*UGT 1A9* 275 T>A	30 (81.1)	6 (16.2)	1 (2.7)
rs17868320	*UGT 1A9* 2152 C>T	33 (89.2)	3 (8.1)	0 (0)
rs2278294	*IMPDH 1* 106 G>A	18 (48.6)	16 (43.2)	2 (5.4)
rs2278293	*IMPDH 1* 125 G>A	11 (29.7)	18 (48.6)	8 (21.6)
rs11706052	*IMPDH 2* 3757 T>C	32 (86.5)	4 (10.8)	1 (2.7)

N=37; difference in total for each genotype is due to genotype not determinable in random patients

Table 5.7. Effects of SNP variants on MPA exposure

Parameter (Median)	Unit	*UGT1A9* 275 T>A Period I			*UGT1A9* 275 T>A Period II		
		Wild type	Variant (n =7)	p value	Wild type	Variant (n =7)	p value
Dose normalized $AUC_{partial}$ of MPA	(µg*h/mL)	0.020	0.010	ns	0.030	0.03	ns
Dose normalized $AUC_{partial}$ of MPAG		0.60	0.25	ns	0.35	0.32	ns
MPAG/MPA ratio		38.0	12.2	ns	12.3	9.8	ns
		UGT1A9 2152 C>T Period I			*UGT1A9 2152 C>T* Period II		
		Wild type (n =33)	Variant (n =3)	p value	Wild type (n =17)	Variant (n =3)	p value
Dose normalized $AUC_{partial}$ of MPA	(µg*h/mL)	0.020	0.030	ns	0.030	0.04	ns
Dose normalized $AUC_{partial}$ of MPAG		0.56	0.24	ns	0.35	0.32	ns
MPAG/MPA ratio		34.5	11.25	0.04	12.3	9.8	ns

MPAG- the glucuronide conjugate metabolite of MPA; $AUC_{partial}$ is partial area under the curve for mycophenolic acid or MPAG until approximately 3.5 h after dosing of MMF; UGT – Uridine glucuronyl transferase enzyme; Median data are presented, *Mann–Whitney U test, ns – not significant

Table 5.8. Effects of SNP variants on IMPDH activity

Parameter	Unit	*IMPDH 1 106 G>A*		p value*
		Wild type (n =18)	Variant carrier (n =18)	
Pre-transplant	[µM XMP/MAMP/sec]	72.1 [62.3, 148]	145 [90.5, 160]	0.088
Pre-dose		42.6 [18.3, 92.6]	40.6 [19.1, 63.1]	0.897
R_{min}		17.9 [11.3, 36.3]	25.2 [16.9, 35.5]	0.429
Dose normalized $AUC_{partial}$ of MPA	[µg*h/mL]	0.020 [0.01, 0.04]	0.020 [0.02, 0.03]	0.713
		IMPDH 1 125 G>A		
		Wild type (n =11)	Variant carrier (n =26)	
Pre-transplant	[µM XMP/MAMP/sec]	109 [63.2, 145]	142 [72.1, 162]	0.239
Pre-dose		44.0 [23.0, 83.4]	39.5 [18.6, 66.5]	0.664
R_{min}		19.6 [5.88, 31.4]	25.2 [15.5, 38.5]	0.221
Dose normalized $AUC_{partial}$ of MPA	[µg*h/mL]	0.020 [0.01, 0.04]	0.020 [0.02, 0.03]	0.706
		IMPDH II 3757 T>C		
		Wild type (n =32)	Variant carrier (n =5)	
Pre-transplant	[µM XMP/MAMP/sec]	142 [77.5, 158]	63.5 [32.2, 66.7]	**0.015**
Pre-dose		44.0 [22.3, 68.3]	35.6 [14.1, 50.3]	0.385
R_{min}		26.2 [14.8, 38.5]	11.3 [8.7, 22.0]	0.172

Dose normalized $AUC_{partial}$ of MPA	[µg*h/mL]	0.020 [0.01, 0.03]	0.030 [0.02, 0.03]	0.225

$AUC_{partial}$ is partial area under the curve for mycophenolic acid until approximately 3.5 h after dosing of MMF; IMPDH- Inosine monophosphate dehydrogenase enzyme; R_{min} – Minimum activity corresponding to maximum inhibition by MPA; data are expressed as median (interquartile range), XMP- Xanthosine monophosphate, AMP- Adenosine monophosphate, *Mann–Whitney U test.

5.6 DISCUSSION

Renal transplant patients have a greater risk for acute rejections during the first few weeks after transplantation. This includes an increased incidence of subclinical rejections (similar histological findings as clinical rejections but with no change in kidney function) during this time period[256]. Hence, post-transplant therapy is usually begun with a higher immunosuppression target for drugs like tacrolimus as well as steroids, which are subsequently tapered with time. On the contrary, a fixed dosing strategy is adopted for mycophenolate mofetil. In addition, though pharmacokinetic and pharmacodynamic approaches for monitoring response to MPA have been developed, they are not routinely employed in the clinic. As pointed out in several studies[252,257], a high inter-patient variability was observed for exposure of MPA after fixed dosing with MMF, especially during early post-transplant and this together with a higher initial risk for rejection, underscores the need for ensuring optimal exposure to MPA [251], at least during the early post-transplant period.

The higher between as well as within subject variability in MPA PK was thought to be related to changes in renal function as well as a low bioavailability[252], early after transplantation. Consequently, many patients on same starting dose of MMF do not reach the intended target MPA AUC of 30-60 µg*h/mL during early post-transplant period [90,105,113,131] and this indicates that early post-transplant monitoring of MPA could benefit renal transplant patients. Obviously, the

consensus guidelines of 2006[132] recommended MPA monitoring at least for specified time points including early post-transplant period to help optimize outcomes and more so in patients at high risk for rejection.

When pharmacokinetic monitoring is considered, the primary objective is to ensure appropriate patient exposure as total drug exposure is related with clinical endpoints or surrogate outcomes. In the case of MPA, the total area under the curve in a dosing interval ($AUC_{0–12\ h}$) has so far provided the most reliable measure for MPA exposure[132,258]. Previous studies had clearly established a good correlation of MPA total 12 h exposure with clinical efficacy in renal transplantation [251,259] and MPA exposure window, ranging between 30 to 60 µg*h/mL[113,132], has been suggested early after transplantation, to minimize rejections. The corresponding pre-dose (trough) concentrations determined in these studies ranged between 1 to 3.5 µg/mL. Maintaining the recommended AUC targets might not only provide adequate MPA concentrations during early post-transplant, but will also limit the adverse reactions like leukopenia[260].

However, determining $AUC_{0\text{-}12\ h}$ is impractical in the clinical setting and will also increase the cost of monitoring. Several alternatives exist to overcome full exposure measurement like determination of trough concentrations or abbreviated sampling strategies. But as trough (pre-dose) concentrations are near the low end of therapeutic range, reliance on this single measurement has shown to give misleading results on adequacy of exposure[251]. Trough MPA concentrations also show a wider range of % CV values in comparison to the corresponding AUCs, especially during the first few weeks after transplant[251]. C_0 also displays greater within-patient variability than $AUC_{0–12\ h}$, and correlations between MPA C_0 and $AUC_{0–12h}$ have been generally poor [248-250,261]. In addition, the MPA exposure has been shown to have better correlation to pharmacodynamic[85,139] or clinical outcome measures[72,109,118,131] than trough concentrations.

Abbreviated (limited or sparse sampling) strategies, on the other hand, may use between two to five blood samples and can estimate $AUC_{0\text{-}12\ h}$ of MPA with better accuracy[259]. The samples preferably should be within 4 hours post-dosing, to enable determinations in outpatient clinic. If a regression derived equation is used for estimation of AUC from sparse sampling, there is a need to follow exact sampling times in clinic. Alternatively, maximum a posteriori probability (MAP) bayesian method can be used to estimate the MPA $AUC_{0\text{-}12\ h}$[99] using a population based pharmacokinetic model (median drug clearance, volume of distribution) combined with data from individual patients (such as drug concentration, dose, other covariates). Advantages of MAP method includes, flexibility in timing of blood sampling and potential to improve estimations by adding more data to the model. For example, a bayesian estimation method for MPA $AUC_{0\text{-}12\ h}$ allowed a 10, 15- or 30-min window for sampling at 20, 60- and 180-minutes post-dosing, respectively[112]. However, the challenges limiting predictive performance for bayesian estimation of MPA during early post-transplant period include a high within and inter patient variability as well as aspects like food intake and co-morbidities[248,262]. Further, experiences with MPA that has complex PK profiles and high variability between patients, have indicated a need for compromise between practicality and acceptable accuracy in such estimation procedures during monitoring[263]. In this chapter, exposure to MPA ($AUC_{0\text{-}12\ h}$) along with corresponding inhibition in the IMPDH activity was determined in renal transplant patients, by using sparse sampling with MAP bayesian estimation on two different occasions. Determination of 12 h exposure to MPA after MMF dosing was the chosen measure, as we predict the Area Under the Curve to relate better with histological outcomes (clinical + subclinical rejections) determined based on protocol biopsies. Comparison of 12 h exposure determined using MAP and linear regression, indicated MAP estimations to be within clinically expected exposure limits while a systematic overprediction of exposure was noted

with regression equation method. Around 10% of sampling time points also had a deviation of greater than 10 min from ideal sparse sampling time points, that could have further impacted calculations with regression method. The results from MAP estimation indicated negligible bias and overall good agreement of predicted versus observed MPA concentrations, but with a higher absolute prediction error. This was predominantly due to mispredictions in ~ 5% of samples that was not systematic and were random across patients and time points. In the absence of systematic bias, values of CL/F, the primary parameter of interest, can be considered valid along with the estimated exposures. The mean predicted CL/F for this cohort of patients was 28.9 L/h during early post-transplant around day-6 and was decreased to 16.6 L/h during estimation at 3-month follow up. Values for CL/F were comparable to some previous studies[149] that were done around the same time period after renal transplantation.

PK studies with MPA have also shown that the clearance of MPA decreases during first year after transplantation and hence the exposure based on total MPA increases with time[264], as was observed in this study. Previous studies with fixed MMF dosing regimen have reported an approximately 30-50% lower exposure during early post-transplantation than the later post-transplantation period[118], that were attributed to changes in renal function, plasma protein binding due to changes in albumin concentrations or changes to co-administered drugs, such as steroid dose tapering. As albumin levels were not different, the lower MPA exposure during period I, was primarily due to the higher % free MPA as well as higher levels of the metabolite MPAG during the period I (early post-transplant period). With suboptimal renal function during period I, MPAG/MPA ratio was also significantly higher, with a high between patient variability. MPA is displaced from plasma protein binding sites by either MPAG or by uremic toxins, both of which are higher in concentration during early post-transplant period. Subsequently, with improvement

in renal function, MPAG levels decreased and % free MPA reached a normal range around 1% and hence MPA exposure was comparatively increased and MPAG/MPA ratio also decreased in period II. As MPA has low extraction ratio of 0.2[265], any increase in MPA_{free} is immediately subjected to glucuronidation and hence, MPA_{free} as well as CL/F_{free} of MPA was unchanged between the two periods, as expected for a low clearance drug.

The concentration-effect of MPA was shown by decrease in IMPDH activity with corresponding increase of total MPA exposure. The relationship of MPA_{free} with IMPDH activity at the sparse time points of pre-dose, 1.5 and 3.5 h was not superior in comparison to total MPA versus IMPDH activity. Hence, the total MPA was considered for further associations with IMPDH activity as well as for clinical outcomes, as established earlier for MPA therapy [266]. The nadir values for IMPDH inhibition occurred at 1.5 h post-dosing of MMF and started recovering by 3.5 h, as reported in few other studies. Median values around 1.5 h were approximately 50% of median trough values after 1000 mg dose and matched with 47% inhibition around 2 hours in another study with 1000 mg dose[140]. In comparison, only 26-29% inhibition at 1.5 h was observed after 500 mg dose[255]. Good correlation between the pretransplant IMPDH activity and R_{min} (r=0.59) and to a lesser extent with predose IMPDH activity (r=0.36) was found during the period I. However, during follow up at steady state the pretransplant activity was no longer correlated and only the relation between predose IMPDH activity and R_{min} persisted (r=0.86). In comparison to period I (day 6), the values for IMPDH predose and R_{min} were lower by an approximate 40 % after continuous MPA therapy, during period II (day 90) and corresponds with the higher values of trough as well as 12 h exposure for MPA during period II. Due to similar extent of decrease in Rmin and predose, they remained correlated, unlike the pre-transplant values that were collected once before start of MPA therapy and used the same values for comparisons during both periods.

The observations also suggests that the post-dose IMPDH activity corresponds with the post-transplant time dependent change in MPA pharmacokinetics.

Finally, a significant relation between the full 12 h exposure (PK) and IMPDH activity (PD) was also shown and patients with lower exposure to MPA showed a significantly higher R_{min} corresponding to less effective inhibition as well as a trend of higher predose IMPDH activity. An earlier study with long term follow up of patients[141], reported significantly higher predose IMPDH activity in patients who were rejecting the graft, though no significant differences occurred in the levels of immunosuppressant drugs. Therefore, our observations using sparse sampling, indicated both the pharmacodynamic markers, R_{min} obtained at 1.5 h post-dosing or predose IMPDH activity were responsive to changes in estimated exposures to MPA and the obtained values were also in agreement with earlier studies [141,255].

In conclusion, a practical sparse sampling strategy was used for estimation of exposure to total and unbound MPA as well as the metabolite MPAG in addition to determining the activity of its target IMPDH enzyme at around week 1 and at 3-month follow up in renal transplant patients. The utility of these measures in predicting outcomes of biopsy proven rejections and infections, will be evaluated further in chapter 6.

6.0 AN INTEGRATED ASSESSMENT OF PHARMACOLOGIC MEASURES OBTAINED DURING EARLY POST-TRANSPLANT PERIOD IN RELATION TO CLINICAL/SUBCLINICAL REJECTIONS AND INFECTIONS IN RENAL TRANSPLANT PATIENTS

6.1 ABSTRACT

The occurrence of subclinical and clinical rejections during early post-transplant period can affect long term graft survival in renal transplant patients. Currently, immunosuppressive therapy is individualized only for tacrolimus with routine TDM. Additional monitoring of patients on tacrolimus and MMF therapy, can provide a more reliable way to minimize clinical and subclinical rejections during the early post-transplant period and would be of great value. The study objective was to evaluate if additional monitoring with measures of total exposure to MPA, pre-transplant IMPDH activity, response minimum (R_{min}) of IMPDH activity and genotyping of p-gp along with the total exposure of tacrolimus can better reflect the incidences of subclinical and clinical rejections, during the early post-renal transplant period. A prospective PK study with limited sampling design was conducted in n=42 renal transplant recipients around post-transplant day 6 and followed up around day 90 (n=23) along with a protocol biopsy. There was a trend towards a lower total exposure to tacrolimus and MPA on day 6 in patients with subsequent rejection at 3 months, though differences were not statistically significant. Comparison of combined exposure to tacrolimus and MPA with respective AUC cut off values of 150 ng*h/mL and 45 µg*h/mL, revealed a two-fold higher incidence of rejection in patients with lower exposure to both drugs when compared to patients who had optimal exposure to both tacrolimus and MPA (46 vs 23 %, NS) with an odds ratio of 3.0 [95% CI: 0.31- 28.84; p =0.3414] for rejection. Patients with a higher pre-transplant IMPDH activity (>140 µM XMP/mol AMP/sec) and sub-optimal inhibition of IMPDH activity during therapy (Rmin >30 µM XMP/mol AMP/sec) had rejection despite optimal exposure to both tacrolimus and MPA. A composite scoring developed based on sum of poor prognostic factors like sub-optimal expsoures or high pre-trasnplant activity with sub-optimal R_{min} or high p-gp efflux activity, revealed that patients with higher scores around day 6 tend to have

higher incidence of rejection at 3 months. Hence, the combined monitoring of factors related to exposure and efficacy of MPA and exposure of tacrolimus by limited sampling during the early post-transplant period, can provide an additional opportunity for optimization of immunosuppression and improve the long-term graft outcomes in renal transplant patients.

6.2 INTRODUCTION

The true success in organ transplantation is realized by maintaining optimal function of the transplanted graft in the recipients. Though the short-term graft survival has improved since use of initial induction therapy followed by maintenance therapy with potent immunosuppression using tacrolimus and mycophenolate mofetil (MMF), a 10–20% of the renal transplant recipients still suffer from an acute rejection in the first 12 months after transplantation[267]. In addition, there is a higher prevalence of subclinical rejections (SCR) during the initial post-transplant period[268,269] which may decrease during the stable post-transplant phase[158]. Protocol biopsies obtained at fixed periods after transplantation provide histological evidence of SCR, which could include tubulo-interstitial mononuclear cell infiltration without detectable deterioration in renal function as evident with clinical monitoring based on serum creatinine levels. As several transplant centers do not perform routine protocol biopsies, the SCR is often overlooked, though longitudinal studies have shown SCR to originate very early after transplantation and to contribute to chronic damage to the kidney[228,229], irrespective of any immediate change in renal function. Taken together acute rejection (clinical and subclinical) can adversely affect the long term graft survival[270] by causing chronic allograft nephropathy[271,272], and lack of improvement in the long term graft survival for renal transplant patients[273].

Maintenance immunosuppression with tacrolimus/MMF combination in comparison to cyclosporine-based therapy is an important factor in the prevention or minimization of rejections (clinical and subclinical) [269]. Achieving optimal exposure to both tacrolimus and MPA[72,73,109,157], as measured by the 12 h Area Under the Curve (AUC)) of tacrolimus and MPA has shown better association with rejection outcomes. However, in therapeutic drug monitoring of (TDM) of renal transplant patients on tacrolimus/MMF therapy, only the trough concentrations for tacrolimus are

routinely monitored as a surrogate for tacrolimus exposure. For MMF, a fixed dosing strategy is currently adopted and MPA trough concentrations are not monitored due to its poor correlation with MPA exposure as measured by AUC.

The recommended target AUC for MPA is 30-60 µg*h/mL, but many patients on a fixed dose of MMF do not reach the intended target MPA AUC[90,105,113,131], due to the high interpatient variability especially during the early post-transplant period[252,257]. As early post-transplant period is associated with higher risk for rejections, monitoring of MPA exposure at specific time points during early post-transplant can improve outcomes with a reduction in rejection episodes. MPA is a non-competitive and reversible inhibitor of ionosine monophosphate-5′-dehydrogenase (IMPDH) types 1 and 2 activities and inhibits the proliferation of T and B-lymphocytes. A previous study showed an association between higher pre-transplant IMPDH activity and increased incidence of acute rejections during a 3-year follow up of renal transplant patients on MMF therapy [136]. It is also known that rejections may occur in recipients with MPA AUCs exceeding 30 µg*h/mL and it might be due to inadequate inhibition of IMPDH by MPA in such patients. Monitoring of IMPDH activity may improve outcomes in conjunction with MPA PK monitoring. The single time point (trough blood level) of tacrolimus has been used in TDM for convenience as well as to minimize cost. However, the prevalence of SCR and clinical rejections even when tacrolimus blood levels are within the therapeutic range and the association of intracellular concentrations to histological rejections in renal transplant patients [231] may suggest that whole blood levels may not accurately represent the concentration at the site of action. However, measurement of intracellular concentrations of tacrolimus is tedious, time consuming and not practical on a routine basis. Consideration of the activity of ABCB1 (p-gp) transporter that can

efflux tacrolimus out of the PBMCs might impact intracellular levels and may provide a more reasonable surrogate marker of tacrolimus exposure in PBMCs [69,274].

The 12 h exposure for both MPA and tacrolimus can be simultaneously estimated from limited sampling strategies combined with Bayesian estimation[77,99]. There is a lack of systematic evaluation of markers like pre-transplant IMDPH activity, response minimum of IMPDH activity, effect of efflux transporter genotype in lymphocytes in conjunction with the estimated 12 h exposures for both MPA and tacrolimus on the association with rejections (both clinical and subclinical) during early post-transplant period. Our hypothesis is that determination and inclusion of additional markers like pre and post-transplant IMPDH activity, genotyping of p-gp and combined exposure measurement of tacrolimus and MPA by limited sampling apart from the routine trough levels can improve the therapeutic outcomes post renal transplantation. The purpose of this chapter is to investigate the association of IMPDH activity and p-gp genotypes in conjunction with the total exposure to MPA and tacrolimus, on the incidence of early post-transplant rejections and infections in renal transplant recipients.

6.3 METHODS

The study protocol was approved by the IRB (Approval No. PRO16050030, dated 20 Oct 2016, titled "Exposure to Mycophenolic acid/Tacrolimus and outcomes in renal transplant recipients"). The details of IRB approval, inclusion exclusion criterion, screening of patients, enrollment, study design and time points are provided in chapter 4. As mentioned earlier, enrolled patients were studied on two separate occasions (periods) during their post-transplant follow-up visits. The period-I was scheduled to be completed within 14 days post-transplant and period-II

was scheduled along with the 3-month protocol biopsy. The exposures to tacrolimus and mycophenolic acid as well as inhibition of IMPDH activity were determined by sparse sampling at predose, 1.5 h and 3.5 h after oral dosing of the immunosuppressants.

6.3.1 Histology

Patients who participated in both periods had a protocol biopsy taken at 3rd month (period II), that was used for assessment of primary efficacy endpoint. Biopsy results were assessed by a transplant pathologist and the scores were defined as per the 2015 Banff classification system[275]. Acute (t, i, v, and g) and chronic (ct, ci, cv, and cg) scores were assessed and recorded for each biopsy. The chronic damage indices derived were interstitial fibrosis and tubular atrophy (IFTA 'ci'+'ct') and interstitial fibrosis with inflammation ('ci' + 'i' as 'ci' > 1 and i >1). Biopsy proven rejection was graded based on histological scores of 1 (mild), II (moderate) or III (severe). Sub-clinical rejection was based on protocol biopsy findings of borderline changes in the setting of stable graft function. Mild TCMR was treated with 3 doses of intravenous Solumedrol (250 mg each) with addition of maintenance Prednisone 5 mg daily. Moderate to severe (Banff grade >2A) rejections were treated with Thymoglobulin 1.5 mg/kg day for a total dose of 6 mg/kg.

Biopsy was also examined for the presence of BKV nephropathy. The assessment of BK viremia, viruria, CMV infections, presence of DSA-I and DSA-II were also carried out during the 3-month protocol biopsy and all results were obtained from the electronic records. The outcomes measures for rejection included both clinical, subclinical rejections at 3-month protocol biopsy and measures for infection included incidence of any infection (BKV or CMV) during the 12-month follow-up period.

6.3.2 Clinical information

Demographic, clinical data including the calculated panel reactive antibody values (cPRA) and estimated eGFR was obtained from the electronic records. The estimated GFR (eGFR) was calculated by CKD-EPI formula as described earlier in chapter 4.

6.3.3 Data analysis and statistics

The clinically recommended cut-off values for Area Under the curve (AUC) of tacrolimus and MPA were used for expression of exposure to tacrolimus and MPA as categorical variables (AUC above or below cut off for tacrolimus or MPA, expressed as values 0 and 1, respectively). Similarly, scores were assigned based on genotype of P-glycoprotein as 0, 1 and 2 corresponding to literature reports of low, medium and high efflux activities, respectively. The median values for response minimum (R_{min} <30 μM XMP/mol AMP/sec) and pre-transplant (baseline <140 μM XMP/mol AMP/sec) IMPDH activities were used as cut off for optimal inhibition or normal baseline, respectively and assigned a score of 0 or 1, if otherwise. The composite score (sum of scores based on sub-optimal exposure to MPA/ tacrolimus, high predicted p-gp efflux activity, high baseline IMPDH activity and high response minimum R_{min} of IMPDH activity) for every patient ranged between 0 to 6 and was used for comparisons with incidence of rejections or infections.

The distribution of data was examined for normality using Shapiro-Wilk test and results were summarized as mean (±) sd or median (range), as appropriate. The independent student t-test or Mann-whitney test were used for univariate comparison of the clinical characteristics, pharmacokinetic and pharmacodynamic parameters between patients without versus with outcome

events (rejection or infection). The Pearson chi-square test was used for comparisons between different categorical groups. The probability of rejection as a function of exposure to MPA and tacrolimus was analyzed using logistic regression. Exploratory plots were made to visualize the correlation between different categories of tacrolimus or MPA exposure or P-glycoprotein genotypes or IMPDH activities and the incidence of acute rejections or infections. The statistical analysis and graphical figures were performed using SAS® (version 9.4, SAS Institute, NC, US) and SPSS® (version 25, IBM corporation, US).

6.4 RESULTS

The demographic and clinical variables in study patients during periods I and II are summarized in Table 4.2 of chapter 4. Only the data from patients who had a protocol biopsy at 3 months were used for assessment of biopsy-proven rejection outcomes (n=33) while all patients were considered for assessment of infection outcomes, as it was obtained from electronic records corresponding to a follow-up duration of 12-months (n=42).

6.4.1 Rejections (clinical and subclinical)

The comparison of demographic variables between patients with rejection outcomes versus non-rejectors is shown in Table 6.1. The distribution of age, gender, genotype, dose of thymoglobulin (mg/kg) and incidence of infection were comparable between the two outcome groups. The Area under the curve (AUC) for MPA, AUC and trough concentrations for tacrolimus

and efficacy measures for MPA during period I (median 6 days), was compared between patients with rejection versus non-rejectors, as shown in Table 6.2.

Table 6.1. Demographics of patients with and without rejection (clinical and subclinical) at 3 months post-renal transplantation

	All Patients	**Rejection**	**No rejection**	p-value
	n=33	**n=14**	**n=19**	
Age (years), mean ± sd		50.1 ± 10.5	47.5 ± 13.2	0.52
Male, n (%)	19 (57.6)	8 (57.1)	11 (57.9)	1.0
Race, n (%)				0.14
White	23 (69.7)	8 (57.1)	15 (79.0)	
African American	7 (21.2)	3 (21.4)	4 (21.1)	
Other	3 (9.1)	3 (21.4)	0 (0.0)	
Living donor	12 (36.4)	4 (28.6)	8 (42.1)	0.42
Thymoglobulin (mg/kg)	5.17 (0.6)	5.21 (0.5)	5.16 (0.7)	0.84
Genotype missing, n (%)	5 (15.2)	2 (14.3)	3 (15.8)	1.00
PGP_C3435T	4 (12.1)	1 (7.1)	3 (15.8)	0.86
PGP_C1236T	8 (24.2)	4 (28.6)	4 (21.1)	0.89
PGP_G2677T_A	23 (69.7)	12 (85.7)	11 (57.9)	0.14
PGP_G1199A	27 (81.8)	12 (85.7)	15 (79.0)	1.00
Genotype score, n (%)				0.47
Low efflux score	1 (3.0)	0 (0.0)	1 (5.3)	
Medium efflux score	23 (69.7)	11 (78.6)	12 (63.2)	
High efflux score	4 (12.1)	1 (7.1)	3 (15.8)	
Infection outcomes				
any infection, n (%)	10 (30.3)	4 (28.6)	6 (31.6)	1.00
CMV	2 (6.1)	1 (7.1)	1 (5.3)	0.37
BK virus urine (3 month)	6 (18.2)	3 (21.4)	3 (15.8)	1.00
BK virus urine (12 month)	7 (21.2)	2 (14.3)	5 (26.3)	0.43

No significant difference in total AUC for tacrolimus and MPA as well as measures of IMPDH activity was observed between rejection versus non-rejection groups. In addition, Figure 6.1 shows no significant difference in the incidence of rejections between patients who had optimal

tacrolimus or MPA exposure versus patients with sub-optimal exposure, less than the recommended 150 ng*h/mL or 45 µg*h/mL for tacrolimus and MPA, respectively.

Table 6.2. Pharmacokinetic and efficacy endpoints for tacrolimus and MPA in recipients with versus without rejection (clinical and subclinical) at 3 months post-renal transplantation

	All Patients	**Rejection**	**No rejection**	p-value
	n=33	**n=14**	**n=19**	
Tacrolimus trough (ng/mL)				
missing, n	2	1	1	
Mean ± SD	7.1 ± 4.1	7.2 ± 4.8	7.1 ± 3.6	0.92
median [IQR]	6.4 [4.1, 9.4]	5.8 [3.3, 10.0]	6.6 [5.1, 8.5]	
Tacrolimus AUC (ng*h/mL)				
missing, n	1	1	0	
Mean ± SD	167.8 ± 94.0	146.2 ± 80.7	182.5 ± 101.5	0.29
median [IQR]	147.1 [98.0, 217.2]	126.4 [95.0, 182.8]	194.9 [126.1, 220.8]	0.43
MPA AUC (µg*h/mL)				
missing, n	0	0	0	
Mean ± SD	45.1 ± 2.1	42.7 ± 28.8	46.9 ± 18.4	0.64
median [IQR]	40.7 [29.7, 59.6]	34.1 [15.4, 54.3]	44.2 [31.5, 62.6]	0.47
Exposure Group, n (%)				0.036
Low/Low	8 (25.0)	6 (46.2)	2 (10.5)	
Low/ High	18 (56.3)	4 (30.8)	14 (73.7)	
High / High	6 (18.8)	3 (23.1)	3 (15.8)	
	IMPDH activity			
Pretransplant IMPDH (Baseline) (µm XMP/mol AMP/sec)				
Mean ± SD	120.8 ± 51.0	135.2 ± 43.1	109.3 ± 55.2	0.20
median [IQR]	141.7 [77.5, 162.0]	144.7 [93.1, 175.3]	96.6 [64.4, 157.3]	0.20
R_{min} (Response minimum of IMPDH) (µm XMP/mol AMP/sec)				
Mean ± SD	35.1 ± 27.5	44.9 ± 33.4	27.2 ±19.4	0.10
median [IQR]	30.0 [14.7, 44.5]	43.0 [18.0, 66.6]	23.9 [14.7, 36.8]	0.12
Pre-dose IMPDH				

	All Patients	Rejection	No rejection	p-value
	n=33	n=14	n=19	
(µm XMP/mol AMP/sec)				
Mean ± SD	73.5 ± 58.4	70.8 ± 49.4	75.7 ± 66.4	0.83
median [IQR]	55.4 [39.4, 106.6]	49.4 [40.7, 111.8]	61.4 [30.1, 101.1]	0.97
% INHIBITION (Pre-dose to R_{min})				
Mean ± SD	46.2 ± 30.4	37.3 ± 28.8	53.3 ± 30.6	0.18
median [IQR]	54.9 [20.5, 67.8]	44.3 [7.6, 60.4]	55.9 [24.3, 73.4]	0.20
IMPDH, n miss	6	2	4	

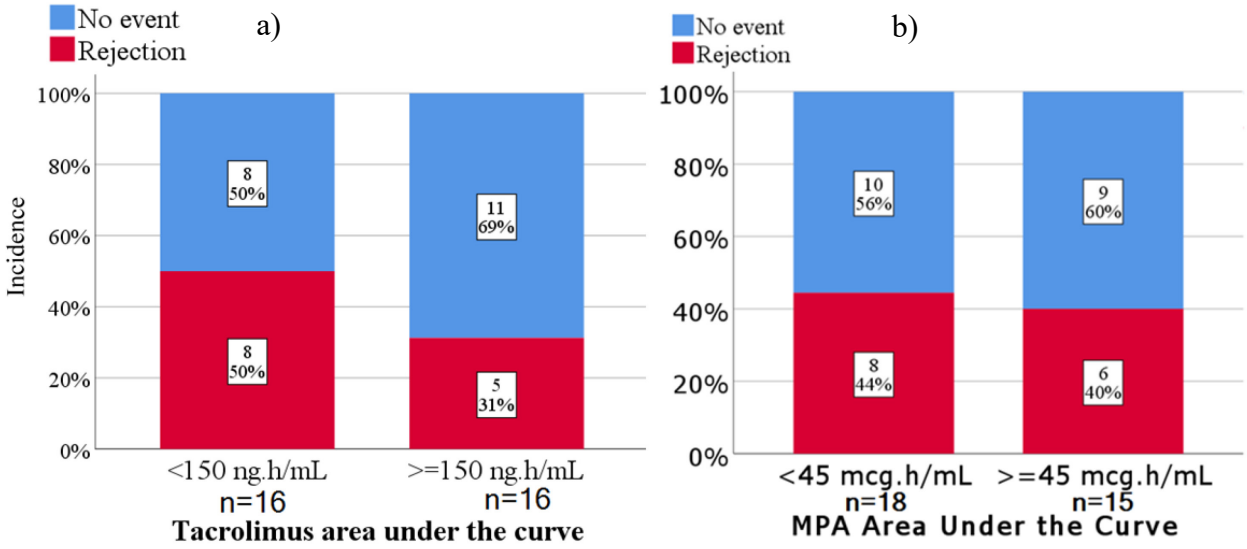
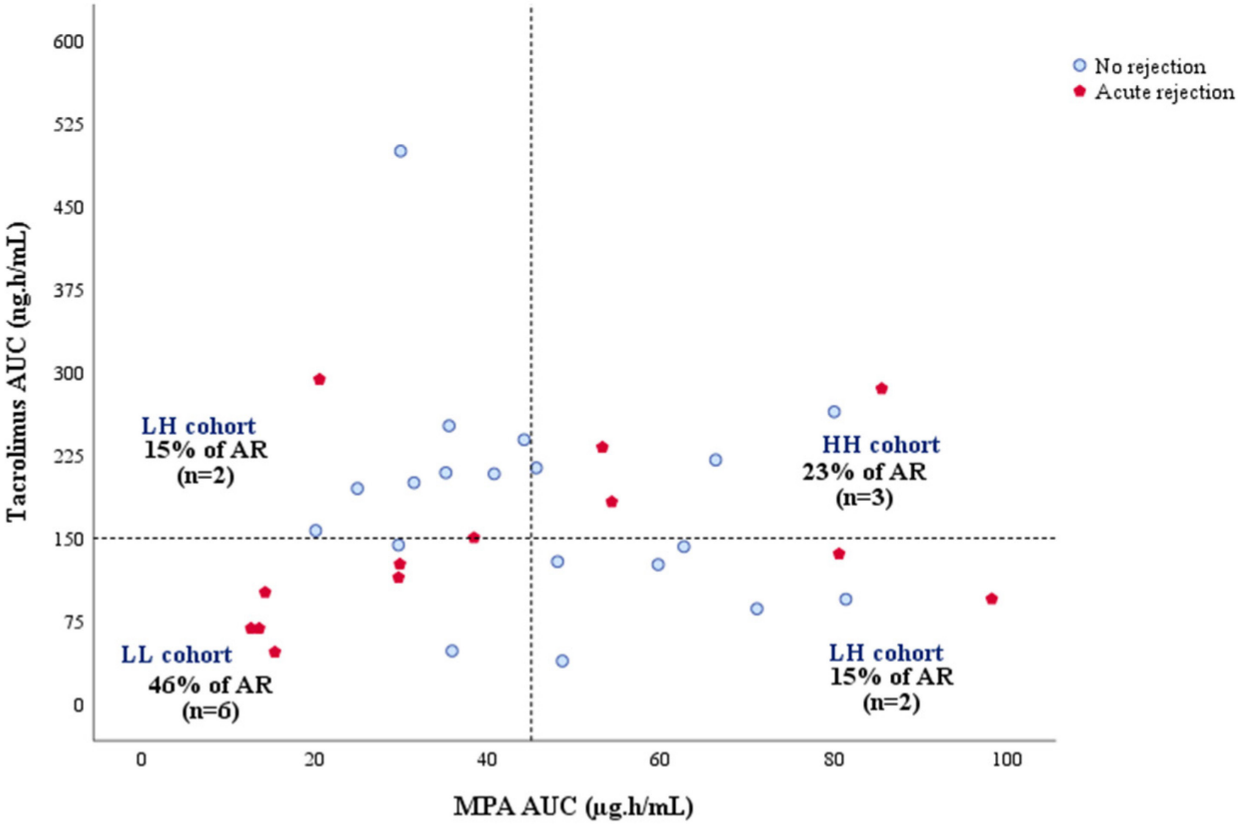


Figure 6.1. Incidence of rejections (acute and subclinical) during 3-month protocol biopsy in relation to total AUC of a) Tacrolimus and b) MPA (Period I, median day 6 post-renal transplant)

The cumulative 90-day exposure of tacrolimus did not differ between patients with versus without rejections (794.3 vs 837.9 ng.day/mL, NS).

For further analysis, categorical scores were given to patient exposures based on clinically recommended cut-off values for tacrolimus and MPA AUCs, for the simultaneous comparison of both drug exposures between rejection versus non-rejector groups. Hence, patients having low (sub-optimal) exposure to both immunosuppressants were defined as LL (tacrolimus AUC<=150

ng*h/mL; MPA AUC<= 45 µg*h/mL). HH was defined as optimal exposure to both tacrolimus and MPA (>150 ng*h/mL and >45 µg*h/mL, respectively).



Dotted lines indicate 45 µg*h/mL and 150 ng*h/mL cut-off for total 12 h AUC for MPA and tacrolimus, respectively

Figure 6.2. Incidence of rejections (acute and subclinical) during 3-month protocol biopsy in relation to total AUC of a) Tacrolimus and b) MPA (Period I, median day 6 post-renal transplant)

LH was defined as optimal exposure to either of the immunosuppressant (tacrolimus AUC<=150 ng*h/mL; MPA AUC >45 µg*h/mL or tacrolimus AUC>150 ng*h/mL; MPA AUC<45 µg*h/mL). Overall, 25, 19 and 56 % of patients were categorized under the LL, HH and LH groups, respectively. As shown in Figure 6.2, there was a trend towards higher incidence in acute rejections in the LL cohort when compared with HH cohort (46 vs 23 %).

Table 6.3. Estimates of odds ratio for acute rejection among 3 cohorts based on combined total exposure to MPA and Tacrolimus

Effect	Point Estimate	95% Wald Confidence Limits		p value
LL group vs HH	3	0.312	28.841	0.3414
LH group vs HH	0.286	0.041	2.005	0.2076

LL (group with low exposure to both MPA and Tacrolimus);

HH (group with optimal exposure to both MPA and Tacrolimus);

LH (group with low exposure only to either MPA or Tacrolimus)

The odds of acute rejection in LL and LH were compared to HH as the reference group with logistic regression analysis and results are summarized in Table 6.3. The odds of rejection were higher in the LL group versus the HH group [odds ratio 3.0 (95% CI:0.31, 28.84)], but this was not statistically significant (p = 0.3414). The odds of rejection in the LH group did not differ significantly from the HH group [odds ratio 0.286 (95% CI:0.041, 2.00), p= 0.2076)]. Based on individual patient genotypes for p-gp efflux pump, a low, medium or high efflux of Tacrolimus from lymphocytes was predicted in 1 (3.0 %), 23 (69.7 %), and 4 (12.1 %) of the 33 patients with biopsy outcomes, respectively. All the rejectors were predicted to have either medium or high efflux activities and majority of such patients were in the LL cohort with low exposure to both tacrolimus as well as MPA. Owing to low sample size, no further distinctive pattern with p-gp genotype on rejection outcomes were observed.

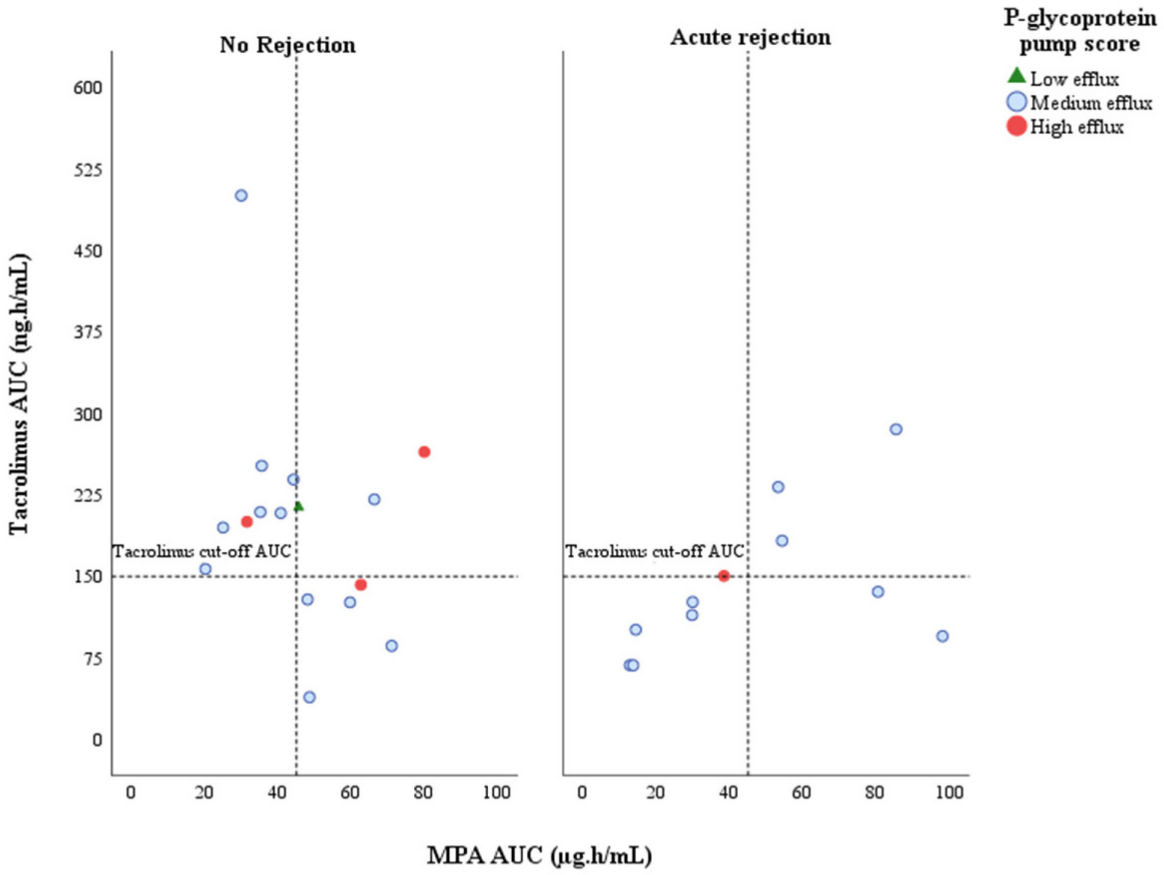
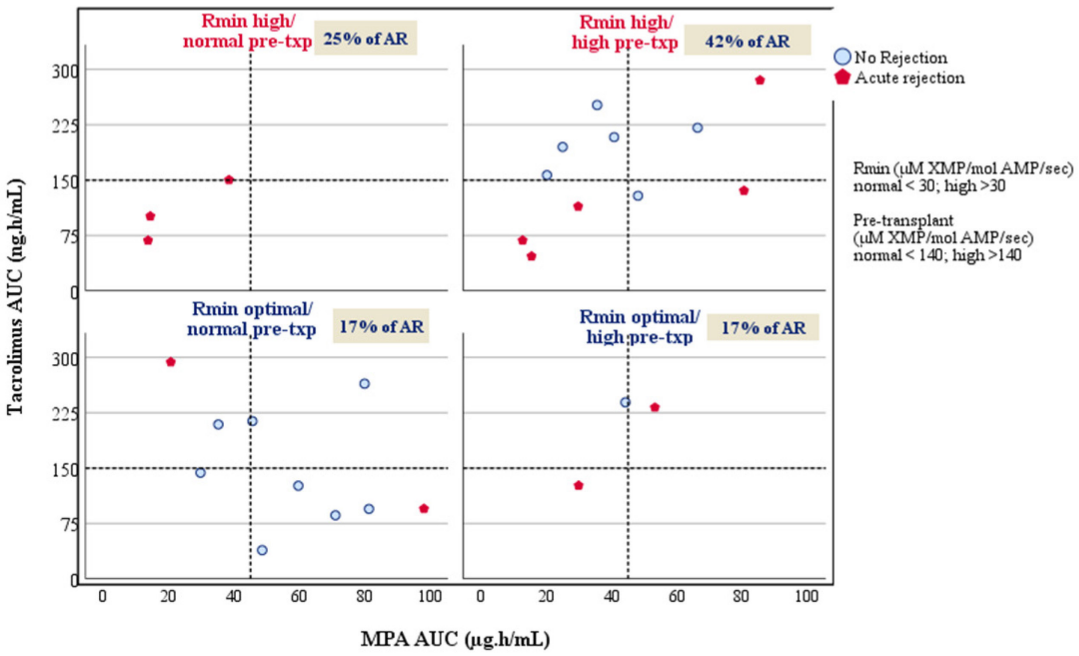


Figure 6.3. Explorative analysis of rejection, combined exposure to MPA and Tacrolimus and predicted efflux activity based on genotypes of ABCB1 (p-glycoprotein)

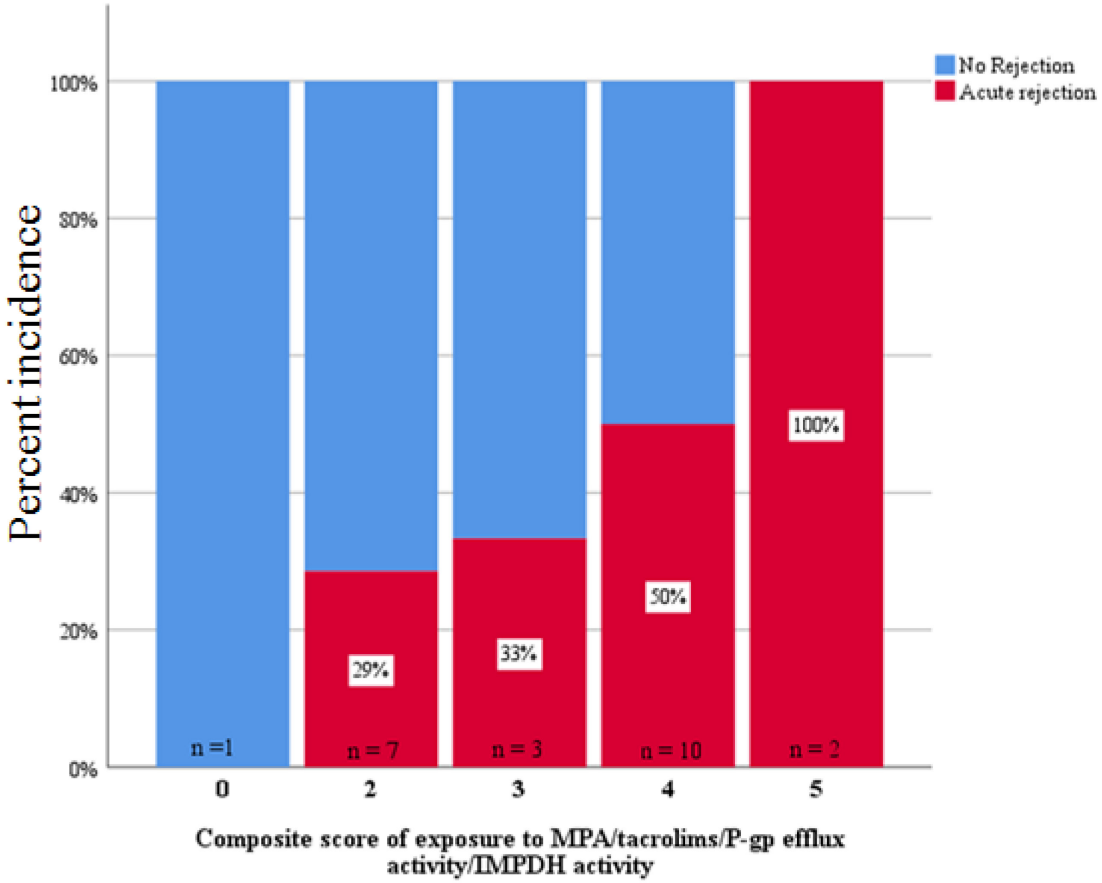
The IMPDH activity (both pre-transplant baseline and response minimum R_{min} in response to MPA therapy) and combined exposure to MPA and tacrolimus with corresponding incidence of rejections, were examined in scatter plot shown in Figure 6.4. The plot has 16 quadrants each representing a unique combination of exposure to tacrolimus/MPA, pre-transplant IMPDH and R_{min} for IMPDH with corresponding incidence of rejections. As seen in the figure, almost 50% of rejections were in two quadrants that had low exposures to both MPA and tacrolimus with a corresponding high R_{min} for IMPDH. When different cohort of patients were compared, the majority of rejection incidence (42 %) was observed in the cohort of patients with high pre-transplant and high R_{min} (sub-optimal inhibition of IMPDH activity).



Dotted lines indicate 45 µg*h/mL and 150 ng*h/mL cut-off for total 12 h AUC for MPA and tacrolimus, respectively

Figure 6.4. Scatter plot of IMPDH activity in PBMCs and combined total AUCs for MPA and tacrolimus (AUC0-12 h) during Period I with corresponding incidence of rejections (3 month) after renal transplantation

Among patients who were rejecting their kidney with higher than cut-off values for R_{min}, sub-optimal MPA exposure was the main factor (6 out of 8 patients), while despite optimal MPA exposure the remaining 2 patients also had higher than cut-off values for pre-transplant IMPDH. Hence, in addition to monitoring optimal exposure to MPA/ tacrolimus, IMPDH activity provided an additional dimension for examining or predicting rejection outcomes. Examination of cohort of patients rejecting their transplanted kidney with normal pre-transplant and optimal R_{min}, however, revealed sub-optimal exposure to MPA and occasionally tacrolimus, as the sole discriminating factor.



Patient gets score 1 each for MPA AUC < 45 µg*h/mL, Tacrolimus AUC < 150ng*h/mL, predicted p-gp medium efflux activity (2 for high efflux activity), IMPDH R_{min} > 30 µM XMP/mol AMP/sec, baseline IMPDH > 140 µM XMP/mol AMP/sec and score 0 each, if otherwise. Patient was excluded if values for any one parameter was missing; (maximum possible score =6, minimum =0)

Figure 6.5. Prevalence of acute rejection in patients according to the composite score (higher scores for sub-optimal exposures to MPA/Tacrolimus or higher IMPDH activities or higher predicted p-gp efflux activity)

A composite scoring for individual patients was generated from the sum of scores assigned for poor exposure to MPA, tacrolimus, a high predicted activity of p-gp to efflux tacrolimus out of lymphocytes and a high pre-transplant IMPDH activity with sub-optimal inhibition of IMPDH activity in response to MPA therapy, as indicated in Figure 6.5 and was compared with incidence of biopsy proven rejection. Non-availability of any one of the parameters, led to exclusion of patient from this comparison and hence only 23 out of the 33 patients with biopsy outcomes were included in the analysis. Majority of patients (44 %) received a score of 4 out of possible 6. As

shown in Figure 6.5, a trend to an increased incidence of rejection was observed in patients with a higher composite score, obtained from the monitoring of PK and PD parameters.

6.4.2 Infections (CMV or BK virus)

During the one year follow up after renal transplantation, about one third of patients (n=14, 33.3 %) developed at least one incidence of infection with BK virus or CMV or both, as shown in Table 6.4. Comparison of demographic factors between patients developing infections versus infection-free patients did not reveal any significant differences.

Table 6.4. Demographics of patients with and without infections during the 12-month follow-up post-renal transplantation

	All Patients	**Any Infection**	**Infection Free**	p-value
	n=42	**n=14**	**n=28**	
Age (years), Mean ± SD	50.4 ± 12.1	53.5 ± 9.9	48.9 ± 12.9	0.21
Male, n (%)	24 (57.1)	6 (42.9)	18 (64.3)	0.19
Race, n (%)				0.26
White	32 (76.2)	11 (78.6)	21 (75.0)	
African American	7 (16.7)	1 (7.1)	6 (21.4)	
Other	3 (7.1)	2 (14.3)	1 (3.6)	
Living donor	13 (31.0)	3 (21.4)	10 (35.7)	0.49
Genotype missing, n (%)	5 (11.9)	5 (17.9)	0 (0.0)	0.15
PGP_C3435T	7 (16.7)	1 (7.1)	6 (26.1)	0.22
PGP_C1236T	12 (28.6)	3 (21.4)	9 (39.1)	0.31
PGP_G2677T_A	31 (73.8)	12 (85.7)	19 (82.6)	1.00
PGP_G1199A	34 (81.0)	14 (100.0)	20 (87.0)	0.27
Genotype copies, n (%)				0.23
Low efflux score	2 (4.8)	0 (0)	2 (7.1)	
Medium efflux score	29 (69.0)	13 (92.9)	16 (57.1)	
High efflux score	6 (14.3)	1 (7.1)	5 (17.9)	
Outcomes				
Acute rejection	14 (33.3)	4 (28.6)	10 (35.7)	0.74

	All Patients	Any Infection	Infection Free	p-value
	n=42	n=14	n=28	
any CMV	2 (4.8)	2 (14.3)	-	
CMV 3 month	1 (2.3)	1 (3.6)	-	
CMV 12 month	0 (0.0)	0 (0.0)	-	
BK virus urine (3 month)	10 (23.8)	10 (71.4)	-	
BK virus urine (12 month)	8 (19.0)	8 (57.1)	-	

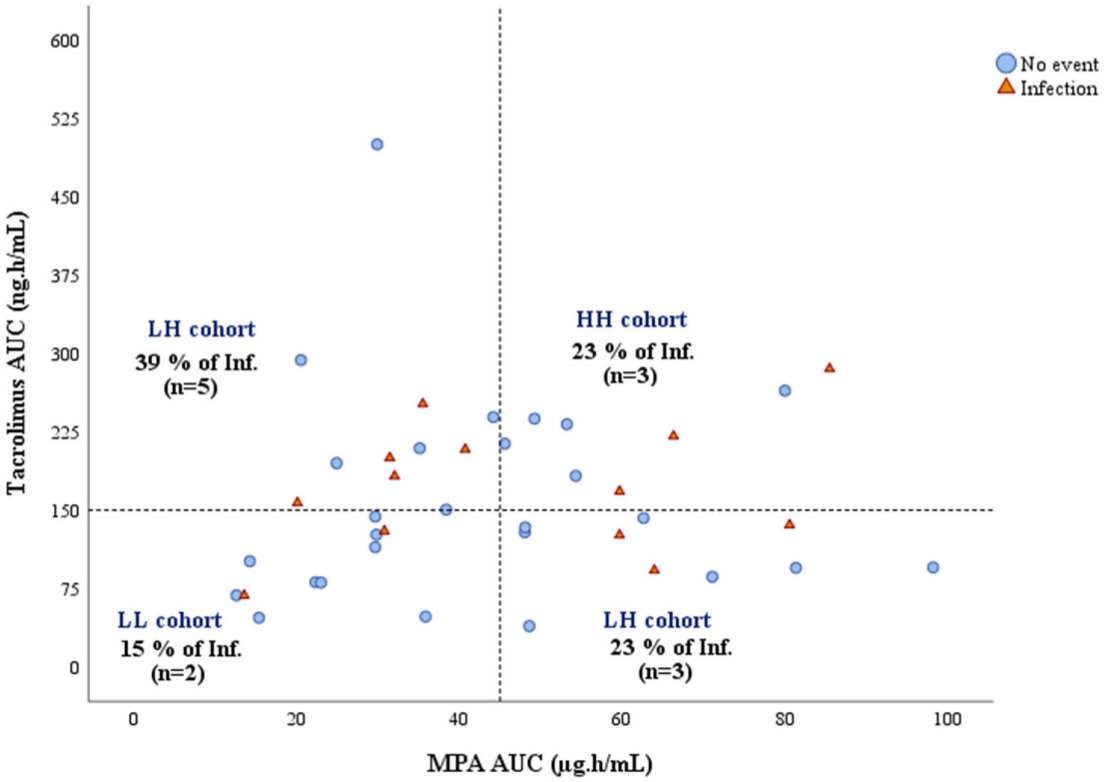
The comparison of pharmacokinetic parameters for tacrolimus and MPA as well as the efficacy measures for MPA during period I (median 6 days), between patients with infections versus infection-free patients, showed a trend for higher tacrolimus exposure in patients with infections, although none of the differences were statistically significant (Table 6.5).

Table 6.5. Pharmacokinetic and efficacy endpoints for tacrolimus and MPA in recipients with versus without infections during the 12 months follow-up post-renal transplantation

	All Patients	Any Infection	Infection Free	p-value
	n=42	n=14	n=28	
Tacrolimus trough (ng/mL)				
Mean ± SD	7.0 ± 3.7	8.2 ± 3.7	6.4 ± 3.7	0.16
median [IQR]	6.4 [4.1, 9.2]	8.0 [6.0, 9.6]	5.8 [3.5, 8.2]	0.09
Tacrolimus AUC (ng*h/mL)				
Mean ± SD	161.8 ± 87.9	171.2 ± 62.1	157.3 ± 98.6	0.59
median [IQR]	143.1 [94.9, 211.4]	168.0 [128.0, 214.5]	133.4 [86.1, 213.6]	0.36
MPA AUC (µg*h/mL)				
Mean ± SD	43.9 ± 21.7	48.0 ± 22.1	41.9 ± 21.6	0.40
median [IQR]	39.6 [29.7, 59.6]	46.5 [31.5, 63.9]	37.1 [26.2, 51.2]	0.29
Exposure Group, n (%)				0.49
Low/Low	11 (27.5)	2 (15.4)	9 (33.3)	
Low/ High	21 (52.5)	8 (61.5)	13 (48.2)	

	All Patients	Any Infection	Infection Free	p-value
	n=42	n=14	n=28	
High / High	8 (20.0)	3 (23.1)	5 (18.5)	
Pretransplant IMPDH (Baseline) (µm XMP/mol AMP/sec)				
Mean ± SD	119.7 ± 55.3	132.7 ± 56.0	113.2 ± 55.0	0.35
median [IQR]	141.7 [72.1, 174.8]	153.1 [66.7, 181.9]	111.2 [72.1, 157.3]	0.30
R_{min} (Response minimum of IMPDH) (µm XMP/mol AMP/sec)				
Mean ± SD	47.2 ± 28.8	38.3 ± 23.5	31.5± 32.3	0.4838
median [IQR]	27.0 [13.9, 44.1]	37.4 [17.8, 54.3]	22.0 [13.3, 39.4]	
Pre-dose IMPDH (µm XMP/mol AMP/sec)				
Mean ± SD	74.4 ± 59.8	98.4 ± 78.2	61.4 ± 43.7	0.1505
median [IQR]	54.8 [30.6, 106.6]	76.7 [38.5, 146.2]	53.1 [30.1, 105.2]	
% INHIBITION (Pre-dose to R_{min})				
Mean ± SD	33.8 ± 29.4	47.1 ± 27.5	47.2 ± 30.2	0.988
median [IQR]	52.5 [21.9, 67.8]	47.7 [23.1, 63.1]	55.4 [16.4, 68.3]	
IMPDH, n miss	8	2	6	

As defined earlier, categorical classification based on the optimal combined exposure to tacrolimus and MPA was done for further examination with corresponding incidence of infections, as shown in the scatter plot (Figure 6.6). Around 62 % of infections (8 out of 13) occurred in patients with higher than optimal exposure for tacrolimus corresponding to the trend for higher tacrolimus exposure in patients with infections, that was noted earlier. On the contrary, the LL cohort showed least incidence of infections in contrast to the highest incidence of rejections observed in this cohort.



Dotted lines indicate 45 µg*h/mL and 150 ng*h/mL cut-off for total 12 h AUC for MPA and tacrolimus, respectively

Figure 6.6. Scatter plot of combined total AUCs for MPA and tacrolimus ($AUC_{0\text{-}12\ h}$) during Period I and incidence of infection (BK virus and CMV) over 12 months post-renal transplantation

Based on p-gp genotype information available in 37 patients, a low, medium or high efflux of tacrolimus from lymphocytes was predicted in 2 (4.8 %), 29 (69.0 %), and 6 (14.3 %) of the patients, respectively and compared with respective incidence of infection. High efflux of tacrolimus by p-gp could decrease intracellular concentrations within lymphocytes and might counteract the risk of infection by avoiding over immunosuppression. Figure 6.7 showed majority of the predicted high effluxers to be infection-free, though further statistical inference was not done owing to low sample size.

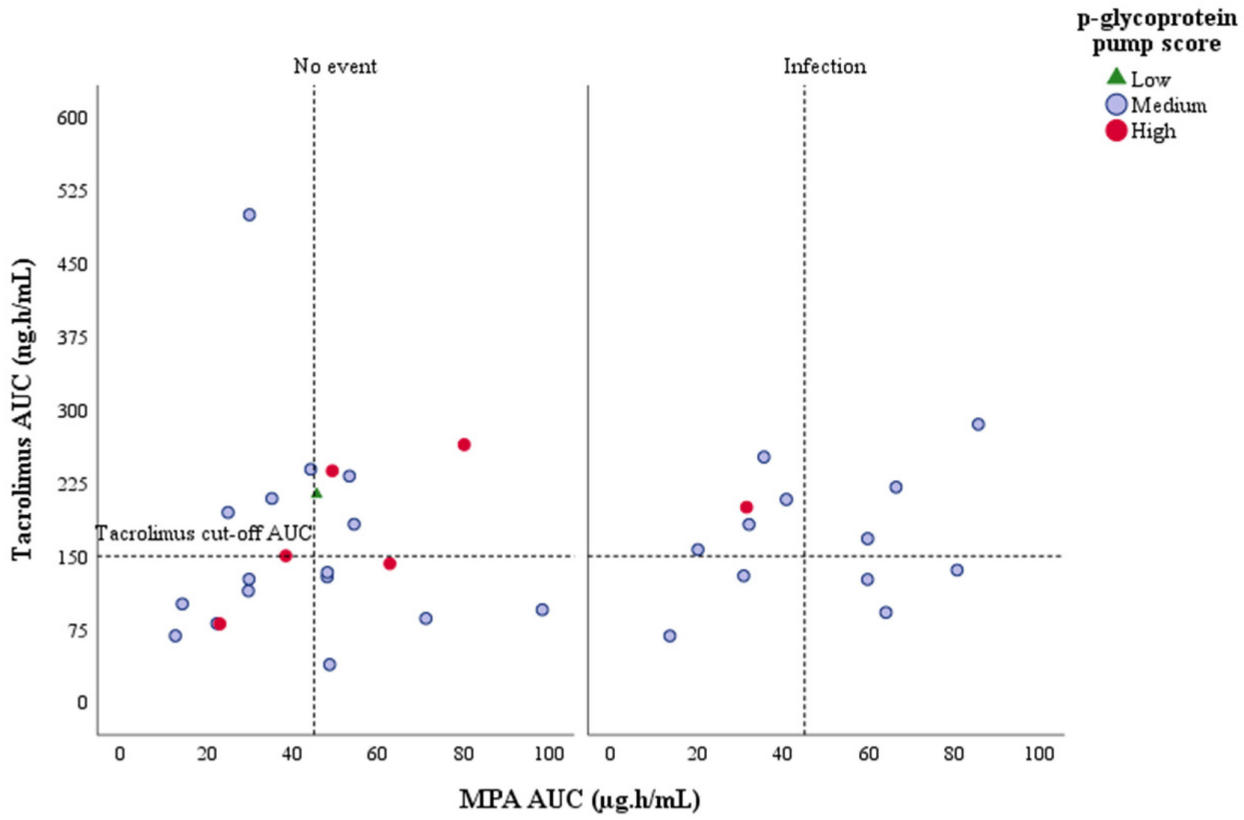


Figure 6.7. Explorative analysis of infections, combined exposure to MPA and Tacrolimus and predicted efflux activity based on genotypes of ABCB1 (p-glycoprotein)

The composite score generated earlier in individual patients was compared with incidence of infections in Figure 6.8. The scores ranged between all the possible values from 0-6, however majority of patients received a score of 4. Again, the exploratory analysis revealed a sharp contrast in observations with infection compared to rejections, as patients with highest scores (low exposures) were found to have the lowest incidence of infections.

Patient gets score 1 each for MPA AUC < 45 µg*h/mL, Tacrolimus AUC < 150ng*h/mL, predicted p-gp medium efflux activity (2 for high efflux activity), IMPDH R_{min} > 30 µM XMP/mol AMP/sec, baseline IMPDH > 140 µM XMP/mol AMP/sec and score 0 each, if otherwise. Patient was excluded if values for any one parameter was missing; (maximum possible score =6, minimum =0).

Figure 6.8. Prevalence of infections in patients according to the composite score (higher scores for sub-optimal exposures to MPA/Tacrolimus or higher IMPDH activities or higher predicted p-gp efflux activity)

6.5 DISCUSSION

In the present chapter, a comprehensive analysis was performed to determine the effect of combined exposure to both tacrolimus and MPA including markers like predicted efflux activity by p-glycoprotein (p-gp) along with IMPDH activity on the early post-transplant incidence of biopsy proven rejections (clinical and subclinical) as well as infections. The biopsy proven outcome in this study were obtained after systematic evaluation of 3-month protocol biopsies using

BANFF 2015 criterion and included both subclinical and clinical rejections. Though trough blood level-based dose adjustment for tacrolimus was done in this study, patients were on a fixed dose of MMF. The Area under curve ($AUC_{0\text{-}12\ h}$) or total exposure is generally considered as the ideal marker of drug exposure. Hence, total exposure to both tacrolimus as well as MPA was estimated from limited sampling strategy and were used along with IMPDH activity, to reveal associations with early post-transplant outcomes. The genotype based predicted efflux activity of MDR1 (p-gp) was also considered for analysis as the intracellular tacrolimus concentrations at the site of action within PBMCs may be affected by p-gp mediated efflux of tacrolimus resulting in lack of correlation with whole blood levels.

In this prospective evaluation, early protocol biopsy done at 3rd month post-transplant revealed subclinical changes in renal allograft in addition to clinical rejections in around 40% of the patients. The routinely determined tacrolimus trough concentrations (period I, median day 6) were comparable between patients with and without rejections during the 3rd month post-transplant. The total exposure to tacrolimus was determined by limited sampling strategy (LSS) during period I and was not found to be significantly different in patients with rejections versus non-rejectors. Undre et al had previously shown tacrolimus AUC on day-2 post-transplantation was significantly less in patients with rejection (157 vs 215 ng*h/mL) and also indicated lower incidence of rejection above 200 ng*h/mL[75]. The study had higher number of patients (n=56) but examined different doses of MMF as co-therapy and was only focused on acute rejections without a clear specification of the time for evaluation of rejections. In another study, tacrolimus AUC was however not different when compared at 3 as well as 12 months in patients with subclinical rejection versus non-rejectors in 125 renal transplant patients [228]. In the current study, an empirical cut-off value for AUC with 150 ng*h/mL was used for comparison of rejections, in line with the

current consensus on the TDM of tacrolimus[230] which was based on previous observational studies. Though majority (8/13) patients with rejection had tacrolimus exposure below the cut-off values, the differences were not statistically significant. It is possible that further lower levels of tacrolimus than that are targeted during the initial 3 months could be differentiative of rejections or a higher sample size is needed for differentiation between rejection versus non-rejectors.

Apart from the whole blood exposure, few studies have suggested that intracellular concentrations of tacrolimus within lymphocytes or the kidney could be affected by the p-gp efflux pump variants, thereby altering outcomes like rejection or nephrotoxicity, respectively[231,274]. Efflux activities of p-gp were predicted from genotyping based on prior reports of activity using rhodamine 123 efflux assays. For example, all the TT carriers in variants for C3435T or G2677T or C1236T were shown to have lower p-gp efflux activity than non-carriers[276]. In the case of G2677T/A at exon 21, two different amino acid changes may occur leading to drastic decrease in the ability of p-gp to drive the efflux of tacrolimus[277,278]. Interestingly, synergism in the case of mutated alleles at 2 different SNPs of 3435 T and 1199 A was also shown to result in a very low activity which might be predicted to result in higher intracellular concentrations and possibly higher efficacy for tacrolimus[231]. It was also shown that the expression of p-gp in lymphocytes had high inter-patient variability in renal transplant patients but did not differ during rejection or in comparison to healthy controls[279]. The distribution of genotypic variants or the predicted scores for efflux activity did not differ between patients with rejection versus non-rejectors. However, majority of patients were assigned a medium efflux score and less than 20% of patients had low or high efflux scores. Exploratory charts showed high efflux in combination with poor exposure to both tacrolimus as well as MPA had a rejection while other patients with high efflux score and optimal exposure were rejection free.

In addition to these possibilities that focus only on tacrolimus exposure versus rejections, other factors like the exposure and efficacy of MPA were also considered in this study. The LSS based total 12 h exposure determination for MPA has been shown to be superior measure of MPA exposure than single time point based monitoring. Like tacrolimus there was no significant difference in the total MPA exposure on period I (median day 6) when compared between rejectors versus non-rejectors. Again, analyzing MPA exposure as a dichotomous variable with cut off values above and below the recommended cut-off value of 45 µg*h/mL also failed to reveal a significant difference. In the study by Undre et al[75], the day-2 AUC of MPA following 1 g or 2g of MMF did not differ in patients with versus without rejections, but the timeline for evaluation of rejections were not provided. A similar finding was reported in an earlier study with a smaller sample size of n=10 patients on tacrolimus co-therapy[280]. In addition to the smaller sample size, the study however focused only on BPAR without considering subclinical rejections within first month and also used 6 h AUC in lieu of the total MPA exposure.

The main objective of this study, however, was to demonstrate the benefit of examining the combined exposure to both tacrolimus and MPA as both tacrolimus and MPA are involved in the prevention of rejections. The combined exposures to both the drugs in patients during period I were expressed using previously defined cut-off values of 150 ng*h/mL and 45 µg*h/mL for tacrolimus and MPA, respectively. The resulting four quadrants (patient cohorts) with low-low (LL) or high-high (HH) or high-low (HL) or low-high (LH) were found to differ in the incidence of rejections, with a 2-fold increase in LL cohort compared to HH cohort, reinforcing the importance for achieving target exposure for both drugs. In addition, the incidence of rejection was also less in LH/ HL cohorts, when exposure only to either tacrolimus or MPA was sub-optimal when compared to LL cohort, with sub-optimal exposure to both drugs. As the number of patients

with sub-optimal exposure considering tacrolimus or MPA alone were comparable during period I, so were the incidence of rejections in the LH and HL cohorts. There was 3 times higher odds of rejection in the LL cohort versus HH cohort, though was not statistically significant probably due to the decrease in power with dichotomous variables as well as due to scattered incidences of rejection across all cohorts. However, the results demonstrate the importance of ensuring optimal exposure to both tacrolimus and MPA during the early post-transplant period.

In the prospective study by Kuypers et al[72,133], exposure to MPA or tacrolimus in relation to very early acute rejections on day 7 was evaluated. There was no relation of MPA or tacrolimus exposure with acute rejection when considered individually, however, a tendency for lower acute rejections were demonstrated when adequate AUCs for both tacrolimus (>150 ng*h/mL) and MPA (>45 µg*h/mL) were achieved on day 7. In addition to general early post-transplant period, the implications of adequate MPA exposure will also be important to consider both in the case of nephrotoxicity associated dose reduction required for tacrolimus or tapering of tacrolimus dose beyond 3 months post-transplant. As previously shown for early cyclosporine withdrawal in patients, the risk for acute rejections was significantly increased in patients manifesting subclinical rejections along with an inadequate exposure to MPA[114].

The comparison of combined exposure to tacrolimus and MPA in rejection versus non-rejectors also revealed incidence of rejections in few patients despite optimal exposure to both drugs. In line with our hypothesis for the benefit in determination of pretransplant IMPDH activity, the rejecting patients with optimal exposures were also found to have a high pre-transplant activity as well as sub-optimal inhibition of IMPDH activity during MPA therapy (period I). Hence, it is possible that high IMPDH activity at both pretransplant as well as at R_{min} (response minimum), could indicate poor responders to MPA therapy. Alternately, higher IMPDH activity ($R_{min,}$ if not

pretransplant) might also be a response to the ongoing rejection process, as was reported in an earlier study[141]. It should also be noted however, that PD values were obtained around day-6 post-transplant, in comparison to rejections that were observed at 3rd month. However, this exploratory observation needs to be confirmed in larger number of such patients and alternate dosing or treatment strategies may need to be explored.

Next, the effect of exposures to tacrolimus and MPA were examined in relation to the incidence of infections (including BV viral and CMV infections). Though exposures during period I were not statistically different between patients with or without infections, majority of patients had higher total as well as trough exposure to tacrolimus during early post-transplant irrespective of the associated exposure in MPA. In the study by Kuypers et al, the tacrolimus AUC but not the trough levels were significantly higher during day 7 as well as week 6, only in patients with infections while the MPA AUC was similar in both groups[72]. Examination of the effect of combined exposure revealed the lowest number of infections in the LL cohort in contrast to the observations with exposure-rejection relationship, while the cohort with higher exposure to tacrolimus showed an increased incidence of infections. The p-gp driven efflux of tacrolimus can decrease intracellular concentrations within lymphocytes and may counteract the potential overimmunosuppression. When the predicted p-gp efflux was explored against the incidence of infection, 5 out of 6 patients with high efflux scores were found to be infection free and it could suggest that higher p-gp activity may counteract the over-immunosuppressive effects of tacrolimus, although confirmation with intracellular tacrolimus concentrations are required in future studies.

The collective impact of individual patient measures obtained during monitoring in period I on the incidence of rejections and infections were examined by assigning scores (with equal

weight) to each of the measures. The unfavorable aspects for each measure in consideration to rejections, as for example, sub-optimal exposure to MPA, tacrolimus or high efflux by p-gp or poor inhibition of IMPDH activity were each assigned a score of 1, versus 0, if otherwise.

The composite score was the sum of individual scores with higher scores hypothesized to result in rejections. Majority of patients received a high score of 4 out of possible 6, highlighting the increased risks during early post-transplant period. In addition, a trend towards higher incidence of rejections (week 13) was noted in patients with high composite scores, which considered several measures that were obtained during the pre-transplant or early post-transplant (week 1) monitoring of renal transplant patients.

Hence, in addition to the TDM of tacrolimus, consideration of total exposure and response to MPA therapy along with an estimate of the genotyping based or direct intracellular tacrolimus concentrations, during the early post-transplant period can help identify patients at a risk for higher rejections (subclinical and clinical). Further exploration is required on the impact of individualized preemptive dose adjustment in patients based on the respective pharmacologic measures in decreasing the incidence of subclinical and clinical rejections during the follow up period. In conclusion, monitoring of the combined exposure to both tacrolimus and MPA in addition to determination of IMPDH activity or p-gp genotyping, during the early post-transplant period can provide a composite measure for improved prediction of clinical/subclinical rejections and an opportunity for individual optimization of immunosuppression in renal transplant patients.

7.0 SUMMARY AND FUTURE DIRECTIONS

7.1 SUMMARY AND CONCLUSIONS

Renal transplantation provides the best treatment option for patients with end-stage renal diseases (ESRD). Ever since the first successful transplantation in 1954, a variety of improvements in surgical techniques as well as introduction of potent immunosuppression drugs have increased the number of transplants performed and improved the short-term outcomes by decreasing rejections. The benefits of renal transplantation are however limited by the availability of kidneys for transplantation in comparison to the number of patients with ESRD. Renal transplant data for 2018 showed that, out of an estimated active waiting list of around 65,500 patients with ESRD, only around 21,167 received a kidney transplant[8,9] and almost 70% of these transplanted kidneys were from cadaveric donors. Hence, to meet the high demand, better utilization of kidneys from marginal donors as well as reducing the number of discarded kidneys are being pursued as viable alternatives[10]. As cadaveric kidneys are more susceptible to ischemia reperfusion injury (IRI), strategies aimed at preventing or minimizing IRI would be beneficial for post-transplant graft and patient survival. The pharmacologic approach to minimize IRI is one such strategy that can target and attenuate different IRI associated pathways by supplementation of preservation solution with appropriate agents. This approach can increase the utility of kidneys from the finite donor pool and improve the short term and long-term outcomes after renal transplantation.

The first part of the dissertation focused on evaluation of treprostinil as a pharmacological agent to improve the preservation of renal grafts. Treprostinil is a prostacyclin (PGI2) analogue approved by the U.S. FDA for the treatment of pulmonary artery hypertension (PAH). The pharmacological properties of treprostinil are expected to decrease ischemic damage by restoring blood flow and also to minimize damaging effects of the reactive oxygen species and inflammatory mediators during reperfusion. Treprostinil was shown to attenuate the decrease in ATP content

during 24 h cold preservation of livers and decrease hepatic IR injury in an orthotopic rat liver transplant model[53]. Due to the beneficial effects in liver transplantation, treprostinil was chosen as the pharmacological agent for ex vivo addition to the kidneys that are subjected to cold preservation as well as for protective treatment during the reperfusion of cold preserved kidneys. The overall hypothesis is that the ex-vivo addition of treprostinil to cold storage solution can attenuate the IR injury and preserve the function of the kidney graft by its vasodilatory action on renal endothelial cells and by the decrease of reperfusion induced activation of inflammatory and coagulation pathways.

In chapter 2, a model for ischemia reperfusion injury was established in isolated rat kidney perfusion system. Isolated rat kidneys were cold stored (CS) at 4 °C for 24 h with or without treprostinil and reperfused with an oxygenated acellular perfusion solution to evaluate renal function as well as changes in mRNA expression. During the 2 h reperfusion, CS kidneys showed a significant loss in recovery of renal function as assessed by the fall in GFR, reabsorption of sodium and glucose and tubular secretion, to less than 10% of control values. Treprostinil addition (20 ng/mL) to CS kidneys significantly improved the filtration fraction as well as urine flow and showed a trend to increase the anionic and cationic tubular secretion. Future evaluations of treprostinil with a longer follow-up duration (beyond the 2 h used in the ex vivo perfusion system) using an in vivo rat transplant model, might allow for evaluation of the changes in protein expression and activity.

Renal injury after IR is an additive effect due to warm/ cold ischemia and reperfusion, but the individual cellular events during each are shown to differ and offer unique opportunities for therapeutic interventions[40]. The effects of IR have been widely studied, but clinically the contribution by cold storage or reperfusion is difficult to isolate and has been rarely addressed. In

chapter 3, the changes in mRNA expression following cold storage (24, 72 h) or reperfusion of the kidneys were individually examined, using a quantitative determination of gene expression using Nanostring® technology. The gene targets included important basolateral and luminal drug transporters, cyp, ugt enzymes and prostacyclin receptors that are endogenously expressed in rat kidney, along with targets like chemokines, cytokines and mediators of inflammation that are upregulated during ischemia and reperfusion. The expression of drug transporters and enzymes in the naive Sprague Dawley rat kidneys using Nanostring® were found to be in concordance with a prior study using microarrays. CS for 72 h significantly upregulated cox-2 expression that was attenuated by the addition of treprostinil. In comparison to 24 h CS only, the warm reperfusion for 2 h after 24 h of CS induced the most pronounced changes in gene expression in the isolated rat kidneys. The differentially expressed genes in reperfused 24 h CS kidneys were the transporter nhe3, MnSOD, Vitamin D receptor, which were downregulated, and ischemia reperfusion inducible protein (irip) that was upregulated, indicating IR induced injury. Treprostinil addition to both storage and reperfusion attenuated the differential expression (DE) in all the four genes while addition to storage alone attenuated the DE of nhe3 and irip genes. Hence, despite a lack of a significant renal functional improvement in the short-term IPRK model, changes in gene expression after treprostinil exposure indicated protection against the IR induced effects at a molecular level, further suggesting a longer duration of follow up in a rat renal transplant model.

The second part of the dissertation focused on minimizing incidences of subclinical and clinical rejections during early post-transplant period in renal transplant patients. The success of renal transplantation is dependent on long duration of graft survival with an optimal renal function in the recipient. Renal transplant recipients receive maintenance immunosuppression with tacrolimus and mycophenolate mofetil (MMF) for prevention of graft rejection. Monitoring of the renal grafts

using early post-transplant protocol biopsies have indicated the occurrence of acute cellular rejections (clinical/subclinical) in patients on tacrolimus/MMF which are known to be associated with progression of fibrosis at 1 year[1], chronic allograft injury[14,15] and reduced long term graft survival[14-16]. The initial post-transplant graft conditions can impact the long-term graft survival. However, the occurrence of early post-transplant inflammatory changes can be minimized by individualization of immunosuppression in the patients using clinical pharmacologic approaches encompassing therapeutic monitoring techniques in combination with other genotyping and pharmacodynamic assessments. Individualization of immunosuppression is therefore essential for improving the long-term outcomes in renal transplant patients.

The overall hypotheses were (a) the relationship of tacrolimus exposure and outcome (clinical/subclinical rejections) will be influenced by genotype of the MDR1 gene expressed on the lymphocytes (PBMC) (b) personalizing MMF dosing based on MPA exposure (abbreviated area under the curve (AUC) and monitoring of the inhibition of IMPDH activity will improve outcomes. These early post-transplant pharmacologic measures were obtained in patients and evaluated for association with incidence of rejections (clinical and subclinical) and infection in renal transplant patients.

In chapter 4, tacrolimus exposure was evaluated during early post-transplant period (Period I; week 1) and follow-up study on the day of the 3-month protocol biopsy (Period II; week 13). Patients were also genotyped for CYP3A5 and MDR1 polymorphisms, for further evaluation of their associations with rejections due to potential impact on tacrolimus blood and lymphocyte concentrations, respectively. The total exposure ($AUC_{0\text{-}12\ h}$) was estimated with a population model by maximum a posteriori bayesian estimation, using limited sampling time points using 3 blood samples viz., predose and at 1.5 and 3.5 h in 42 and 23 patients, during the early and follow-

up periods, respectively. The predicted tacrolimus $AUC_{0\text{-}12\ h}$ ranged between 39-294 ng*h/mL and 101-302 ng*h/mL during periods I and II, respectively. Only 55 % and 60 % of the patients had exposure above the recommended 150 ng*h/mL during periods I and II, respectively. CYP3A5 expressers had more than 2-fold higher clearance than non-expressors. However, none of the MDR1 genotypes (3435, 2677, 1236, 1199) showed significant differences in tacrolimus exposure. Trough concentrations showed a good correlation with predicted AUC (r= 0.8 (0.7-0.9)). In this part of the study, the more informative and ideal measure of exposure ($AUC_{0\text{-}12\ h}$) for tacrolimus was obtained by sparse sampling along with genotype of MDR1 for further evaluations against the incidence of subclinical/clinical rejections and infections.

The therapeutic monitoring is currently performed only for tacrolimus trough levels, at our transplant center. The present study however, included estimation of the exposure to MPA in parallel with tacrolimus exposure. In chapter 5, the individual exposure to MPA along with its effect on the inhibition of its target (IMPDH enzyme) was determined by obtaining plasma and PBMCs, respectively in parallel to the whole blood samples obtained for the tacrolimus study. The partial exposures to MPA, MPAG and MPA_{free} were obtained from limited sampling points. The total MPA exposure ($AUC_{0\text{-}12\ h}$) was estimated with a population model by maximum a posteriori bayesian estimation. The mean MPA exposure (predicted $AUC_{0\text{-}12\ h}$) was comparable between Period I to Period II (48.3 ± 21.4 vs 58.2 ± 32.8, NS) with only 47 and 50 % of patients falling within the 30-60 µg*h/mL exposure window, respectively. The partial as well as dose normalized exposure of MPA_{free} as well as the CL/F_{free} was unchanged between the two periods, as MPA is a low clearance drug. MPAG exposure was inversely related to renal function and hence lower exposure was observed in Period II and a similar MPAG/MPA ratio was also suggestive that the conjugation activity of UGT enzymes was unaffected during early post-transplant period.

The dose normalized total MPA exposure showed an increase during Period II and the corresponding IMPDH activity was significantly lowered during period II versus period I, as shown by lower values for predose activity (54.8 vs 36.3 μM XMP/M AMP/sec, p =0.04) and the response minimum, R_{min} (26.1 vs 19.0 μM XMP/M AMP/sec, p=0.01). Pooled analysis showed the subset of patients with the lowest MPA exposures (< 30 μg*h/mL) to have significantly higher R_{min} for IMPDH as well as a trend for higher predose IMPDH activity. The results of this part of the study showed a change in MPA exposure and response during early post-transplant and the need for post-transplant time-based assessments for MPA PK and PD, which could be enabled by sparse sampling in an outpatient setting.

Chapter 6 examined if the combined measures of total exposure to MPA, pre-transplant IMPDH activity, response minimum (R_{min}) and genotyping of p-gp along with the total exposure of tacrolimus can better reflect the incidences of subclinical and clinical rejections, during the early post-renal transplant period. Individual comparisons of total exposure to tacrolimus or MPA did not reveal significant differences in relation to rejections at 3rd month. However, comparison of the combined exposure to tacrolimus and MPA with respective AUC cut off values of 150 ng*h/mL and 45 μg*h/mL, revealed a two-fold higher incidence of rejection in patients with lower exposure to both drugs when compared to patients that had optimal exposure to both tacrolimus and MPA (46 vs 23 %, NS) with an odds ratio of 3.0 [95% CI: 0.31- 28.84; p =0.3414] for rejections. A few rejecting patients with optimal exposure to both tacrolimus and MPA were found to have higher IMPDH activities both at pre-transplant (>140 μM XMP/mol AMP/sec) and at Rmin (>30 μM XMP/mol AMP/sec) indicating a sub-optimal inhibition of IMPDH activity. A composite scoring was developed from the sum of poor prognostic factors such as sub-optimal exposures or high pre-transplant activity with sub-optimal R_{min} or high p-gp efflux activity.

Patients with higher composite scores on week 1 (Period I), tended to have higher incidence of rejection at 3 months post-transplant. In contrast to increased rejections with low exposure, the highest incidence of infections occurred in patients with higher than optimal exposure for tacrolimus (62 % of infections). A trend was observed in predicted p-gp high effluxers to be infection-free as higher p-gp activity could decrease intracellular concentrations within lymphocytes and might counteract the risk of overimmunosuppression. However, determination of intracellular tacrolimus concentrations is needed to provide confirmatory evidence.

Overall, based on the study results, it is concluded that the combined monitoring of pharmacological factors related to exposure and efficacy of MPA (IMPDH activity) and tacrolimus by limited sampling during the early post-transplant period, provides an opportunity for optimization of immunosuppression and will enable physicians to provide individualized treatment to transplant patients.

7.2 LIMITATIONS

1. Isolated rat kidney perfusion (IPRK) model (Chapter 2)

Reperfusion in IPRK was performed for a duration of 2 hours, beyond which there could be depletion of the perfusate components leading to decrease in renal function. Hence long-term evaluation of renal function recovery and characterization of drug effect is not feasible with this model. In addition, though the oxygenated buffer solution used for reperfusion of kidneys after cold storage is optimized to sustain the function of isolated kidneys, use of rat blood might be ideal to mimic the in vivo conditions that are available for recovery of a kidney allograft, however it is difficult to obtain rat blood in high volumes to perform in vitro studies.

2. Measurement of ATP in kidneys (Chapter 2)

ATP content in CS and treprostinil treated CS kidneys were not evaluated or compared, which might have enabled verification of the hypothesis on the beneficial effects of treprostinil at cellular level during CS and reperfusion.

3. Gene expression study (Chapter 3)

The changes in mRNA expression may not be translatable into protein expression or functional changes. Further this model using rat kidney might not account for differences that may exist in regulation of gene or protein targets in humans. Nanostring based analysis also showed less differentiation or lesser fold-change in differences between groups in relation to the fold-changes observed with RT-PCR technique.

4. Population model (Chapter 4,5)

There was no reference $AUC_{(0\text{-}12\ h)}$ available to validate the model with the current study population, as a published model developed in renal transplant patients was used. Further, population models tend to differ with respect to post-transplant period of study and are also specific for either MMF or EC-MPS.

5. Cut-off values for IMPDH activity (Chapter 6)

There are no prior reports on cut-off values for IMPDH activity associated with transplant outcomes like rejections or infections in renal transplant patients. Hence, arbitrary cut-off values such as median values were used for pre-transplant and R_{min} of IMPDH activity. Further, the units for IMPDH activity in literature are different in recent versus older studies and are not interconvertible.

6. Genotyping and P-gp efflux activity (Chapter 6)

The predicted efflux activity was obtained from literature-based reports on rhodamine efflux capacity by the different MDR1 (p-gp) genotypes. In addition, the combined effects of all genotypes in individual patients cannot be established. Hence, the direct determination of intracellular tacrolimus concentrations is better alternative.

7. Sample size (Chapter 6)

The sample size in period II was reduced following loss of follow-up in patients, primarily due to the cancellation of biopsy or change in the formulation from MMF to EC-MPS. Hence, evaluation of the pharmacological measures obtained in period II in relation to outcomes were not performed.

7.3 FUTURE DIRECTIONS

1. Rat kidney transplant model:

The trends in the improvement observed with treprostinil addition to the cold storage solution at 20 ng/mL in ex vivo and gene expression studies, needs to be further evaluated in a rat kidney transplant model. The transplant model will enable re-perfusion in recipient animal with whole blood and as well permit a longer duration (days-weeks) of follow-up in the recipients to allow for recovery and differentiation of renal graft function between untreated versus treprostinil treated groups. The model can further be expanded to include other pharmacological treatment options such as pre-treatment of donor or treatment of recipient with treprostinil during reperfusion, along with addition to cold storage solution. Evaluation of mitochondrial function and ATP content in tissues exposed to treprostinil should also be possible.

2. Increment of sample size:

The trends shown in association of exposures and IMPDH activity with rejection outcomes (subclinical and clinical) need to be evaluated further, by developing a receiver operating characteristic curve (ROC) for each of the pharmacologic measure in relation to the subclinical and clinical rejections, which is possible after obtaining data in additional number of patients.

3. Intracellular tacrolimus concentrations:

The concentrations of tacrolimus within PBMCs will provide the true target site concentrations for tacrolimus and can account for the differences in efficacy despite identical whole blood concentrations. The methodology to isolate PBMCs from whole blood needs to be optimized further and validated to ensure prevention of tacrolimus efflux out of lymphocytes during the processing of blood for isolation of PBMCs.

APPENDIX A

A.1 IRB PROTOCOL PRO16050030

TITLE : Exposure to Mycophenolic acid/Tacrolimus and outcomes in renal transplant recipients

A.1.1 ABSTRACT

Renal transplantation is the best treatment option for patients with endstage renal diseases. Renal transplantation has significantly improved patient survival and quality of life. Though the short- and long-term patient and graft survival have improved, around 25% of grafts are lost at 5 years due to chronic rejection. Prior episodes of acute rejection and/or the presence of subclinical rejection are suggested to contribute to chronic rejection. Unlike the histological changes associated with kidney graft dysfunction in acute cellular rejection (ACR), the subclinical rejection (SCR) is characterized by histologic findings that are associated with local inflammation in the absence of any graft dysfunction (like decreased creatinine clearance). The pathogenesis of SCR is not clear, however, there is an undisputed involvement of immune activation and inflammation in spite of standard maintenance immunosuppression. Hence, there is a vital need to characterize the adequacy of maintenance therapy in renal transplant recipients, receiving tacrolimus and mycophenolate mofetil (MMF). We hypothesize that in patients treated with tacrolimus and MMF, the incidence of ACR/SCR is mainly due to low exposure at site of action (PBMC) and low response to immunosuppressive therapy. We predict that patients with ACR/SCR have inadequate

exposure to tacrolimus at the target site and inadequate inhibition of IMPDH, the target of mycophenolic acid (active component of MMF).

A.1.2 AIMS

Aim 1: To determine plasma and peripheral blood mononuclear cells (PBMC) exposure to MPA using limited sampling in renal transplant recipients on MMF. To measure exposure in whole blood and PBMC in renal transplant patients on tacrolimus.

Aim 2: To determine the efficacy of MPA by measuring exvivo IMPDH (target of MPA) activity in PBMC of renal transplant recipients on MMF therapy. IMPDH activity will be measured in PBMC at pretransplant (baseline), early posttransplant (within first two weeks), around month 3, as well as during incidence of subacute or acute rejection or infectious episodes.

Aim 3: To develop a pharmacodynamic range for IMPDH inhibition in renal transplant recipients and to determine associations of the key study parameters like IMPDH baseline activity, IMPDH inhibition, measures of MPA and tacrolimus exposure with outcomes like subclinical acute rejection, acute cellular rejection, opportunistic infections. The results of this study will associate efficacy of MPA using IMPDH inhibition and the exposure to tacrolimus at its target site of action, with the short-term clinical outcomes in renal transplant recipients.

A.1.3 INVESTIGATORS

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Kalluri Hari Varun	School of Pharmacy
Venkataramanan Raman	School of Pharmacy

A.1.4 BACKGROUND

Renal transplantation is the best treatment option for patients with end stage renal disease. Renal transplantation has significantly improved patient survival and quality of life. Though the short- and long-term patient and graft survival have improved, around 25% of grafts are lost at 5 years due to chronic rejection. Prior episodes of acute rejection and/or the presence of subclinical rejection are suggested to contribute to chronic rejection[281]. Unlike the histological changes associated with kidney graft dysfunction in acute clinical rejection (ACR), the subclinical rejection (SCR) is characterized by histologic findings that are associated with local inflammation in the absence of any graft dysfunction (like decreased creatinine clearance). The pathogenesis of SCR is not clear, however, there is an undisputed involvement of immune activation and inflammation leading to downstream effects on the long-term allograft function[269].

Immunosuppression in renal transplantation:

At UPMC, the preferred maintenance immunosuppression regimen in renal transplant recipients consists of tacrolimus, steroids, and mycophenolate mofetil. Tacrolimus inhibits the early phase of T-cell activation by inhibiting calcineurin and its dose is targeted to maintain trough levels of 510 ng/mL. Mycophenolate mofetil (MMF) is dosed along with tacrolimus, twice daily at a fixed dose of 2000 mg/day. MMF is an ester prodrug with highly variable absorption profile and is hydrolyzed by esterases in the body to mycophenolic acid (MPA). MPA is a selective noncompetitive and reversible inhibitor of the Inosine monophosphate dehydrogenase (IMPDH), a key enzyme in the exnovo synthesis of GTP[247]. The immunosuppressant activity ensues as lymphocytes depend on this exnovo GTP synthesis significane for their clonal expansion.

Clinical experience with MPA dose and exposure (AUC):

MPA is administered at a fixed dose without a personalized dosing approach. After fixed dose of MMF, there is a high variability among patients in the exposure to MPA. Trough concentrations of MPA do not correlate with MPA exposure. Several studies have shown association of MPA exposure over a dosing interval (AUC) with decreased rejection outcome resulting in a consensus document that proposed to target MPA exposure at 30–60 mg*h/L[282]. But, in clinical practice, monitoring of MPA exposure for 12 h is impractical. Herein, we propose to implement a limited sampling algorithm reported previously in renal transplant recipients[149]. They have been validated to represent 12 h MPA exposure in adult de-novo recipients and more importantly provide a practical option for personalizing MMF dosage. In this study we will evaluate MPA exposure based on limited sampling strategy.

Clinical experience with tacrolimus dosing and exposure (AUC):

Tacrolimus dosing is personalized using therapeutic drug monitoring in whole blood. However, the concentration of tacrolimus at the site of action (T-cell) is controlled by the activity of p-

glycoprotein in PBMC. Tacrolimus trough concentration and occurrence of ACR has been studied with a focus on Pgp efflux transporter expressed in PBMCs. Pgp expression in PBMCs of adult liver transplant patients was marginally higher and significantly lower in acute rejection and severe infections, respectively than in those with an uneventful evolution[283]. As tacrolimus is a substrate for Pgp, the change in its expression can alter PBMC concentrations of tacrolimus and modulate its efficacy. The results of Capron et al[231] showed significant differences in tacrolimus concentrations attained within PBMCs depending on the genotypic changes in Pgp. In this study we will measure tacrolimus levels in whole blood, PBMC and determine the expression of Pgp in PBMC.We hypothesize that the incidence of subclinical and clinical acute rejection will be associated with high Pgp expression and low tacrolimus concentrations at the target site of action viz., within PBMC.

Efficacy of MPA:

MPA acts by inhibition of IMPDH. Measurement of IMPDH activity offers a direct means to adjust MPA dosing based on response to MPA. Studies with IMPDH monitoring[136,284,285] have consistently indicated an inverse relationship between MPA concentration and IMPDH activity. A wide interindividual pharmacodynamic activity was also observed with comparable MPA concentrations exhibiting different degrees of IMPDH inhibition. Glander et al[136] have shown dose reduction in patients with higher pretransplant IMPDH activity (> 8.53 nmol/mg protein/h) was associated with significantly higher incidence of rejection (81.8% vs. 36.4%, $p<0.01$) over a 3year followup of renal transplants. In another study[143] using ECMPS formulation, IMPDH inhibition was significantly lower in renal transplant patients with BPAR at pretransplant, 1 and 2week time points. Further, IMPDH inhibition but not the MPA AUC correlated with acute rejection. In the present study we will evaluate pre transplant baseline IMPDH activity and IMPDH activity during

early posttransplant period (about 1 week) and at around 1 month and at the time of SCR/AR or infections.

Rejection and Infection phenotypes in renal transplantation:

Overall it is realized that drug therapy has to be individualized to avoid suboptimal or excess immunosuppression leading to ACR/SCR and infections, respectively. As shown in various studies, IMPDH inhibition can be a reliable marker for optimizing therapy. However, there is lack of a study designed to monitor IMPDH activity changes corresponding with clinical phenotypes like infection or rejection as well as to define a level of IMPDH inhibition required during such episodes. We hypothesize that the incidence of subclinical and acute rejection in patients on MMF dosing will be associated with higher basal IMPDH activity and/or minimal inhibition of IMPDH activity. The study will determine quantitative differences in basal IMPDH activity and magnitude of IMPDH inhibition among patients with sub clinical rejection, clinical rejection and no rejection. We will also evaluate if the magnitude of IMPDH inhibition is associated with MPA exposure as measured by abbreviated MPA AUC (total and unbound) in these patients. We also hypothesize that incidence of infection (BK virus), will be higher in patients with low basal IMPDH activity and/or high inhibition of IMPDH activity. The study will determine quantitative differences in basal IMPDH activity and magnitude of IMPDH inhibition among patients with and without infections. Results from this study will be used to associate efficacy of MPA using IMPDH inhibition and significance to the exposure to tacrolimus at its target site of action, with the short-term clinical outcomes in renal transplant recipients

A.1.5 SIGNIFICANCE

There are several studies in transplant patients that have related MPA exposure/dose to clinical rejections[72,286]. Studies have also evaluated IMPDH activity as a biomarker in response to MPA exposure. Some of these have correlated IMPDH genotypes or pretransplant IMPDH activity to outcomes like rejection and adverse events[137,139,141]. We identified 3 studies so far, that prospectively looked into IMPDH activity changes during immediate posttransplant in conjunction with acute rejection[143,144,285].

We believe that they all have specific limitations, as listed below:

1. Previous studies were performed to relate IMPDH activity or genotype to clinical acute rejection. Our study, in addition, includes sub clinical rejection, which is increasingly being recognized as an important surrogate end point in kidney transplantation[227].

2. Previous studies had used ECMPS based therapy along with cyclosporine. Our protocol will use tacrolimus with MMF based therapy which is now the accepted standard of care for majority of the institutions worldwide. Moreover, the results of previous studies were contradictory, which atleast in part could be related to methodological limitations of the older studies.

3. Many of the studies were performed several months after transplantation and not early post transplantation. Performing our study in early posttransplant period is a strength for our study, as it will give us insights into mechanisms of early graft injury and rejection.

4. So far, none of the previous studies have quantified IMPDH inhibition in renal transplant patients with subclinical or acute clinical rejection while on tacrolimus with MMF dosing.

5. Previous studies had not evaluated and simultaneously taken into account, both total and unbound MPA exposure and IMPDH activity, to study the MPA exposure adequately.

Following are the knowledge gaps that this study intends to fill:

I. Previous studies have utilized ECMPS with cyclosporine-based dosing. This study will generate information with patients on Tacrolimus regimen, which is more commonly used in renal transplant patients along with MPA, currently. This study will utilize MMF based dosing, which has not been the case in previous studies.

II. This study will evaluate a 4-hour abbreviated MPA/tacrolimus exposure with limited blood sampling using only three time points and has a potential for adoption in other centers.

III. Existing results on inhibition of IMPDH activity during acute rejection are contradictory to each other. Further, no study has so far reported on subclinical rejection which is an outcome of major interest under the current maintenance immunosuppression. Our study has access to protocol biopsy which permits detection of subclinical rejection as an outcome. Unlike any other study before, this study will test our hypothesis in the context of this outcome which will be of major interest to the transplant community.

IV. Unlike other studies so far, results from this study will be unique to consider both IMPDH inhibition and PBMC levels of tacrolimus in the same patient as contributory factors to the clinical outcome.

A.1.6 RESEARCH DESIGN AND METHODS

Renal transplant recipients in this study would be on Tacrolimus and MMF therapy as their usual standard of care and dosing of these drugs would not be done for research purposes. All kidney transplant patients are potentially eligible to participate in this study.

Research activities on subjects:

After eligible renal transplant recipients are identified and enrolled, the following research activities would be carried out on three occasions (as below), at the outpatient transplant clinic in

7th floor of Montefiore hospital. The subjects will be monitored by the nursing staff, physician investigator and or study staff during the period corresponding to this study.

a) Pretransplant (baseline value)

Fifteen mL of blood would be drawn to determine the baseline IMPDH activity as well as to genotype the subjects for enzymes and transporters involved in the disposition of immunosuppressant drugs like MMF and tacrolimus. This procedure will take around five minutes. This will be performed by nursing staff or the physician investigators in the Montefiore hospital or transplant clinic. Whenever possible, this pretransplant blood will be drawn on the day of transplant before the patients receive a dose of MPA. However, if it was not possible to draw blood on the day of transplant, any leftover blood collected at a prior time point will be utilized.

b) Initial posttransplant (between Days 4-10)

Subjects will be required to hold their regular tacrolimus and MMF dose and take it in the clinic in the presence of investigators. To ensure this, one of the study personnel would call or send reminder to participating subjects to hold only their morning dose of immunosuppressants (Tacrolimus and MMF only) and bring them to the clinic. They will also be reminded that they should continue taking their other drugs as usual. On the study day, subjects will be advised to refrain from eating for a period of at least one hour prior to dosing until about 2 hours after dosing. They will be notified that drinking water is permitted. Ten ml of blood will be collected for trough blood and PBMC tacrolimus levels. Ten ml blood will be collected at least 2 and not more than 3 time points during the 4 hours following MMF administration. This phase will last approximately 4 hours and will be coordinated during the time these subjects wait for their standard of care clinical tests and results, at the 7th floor clinics of Montefiore hospital by nursing staff or physician investigators. Total blood volume drawn will be 40 ml.

c) Posttransplant (Around 3 months or routine biopsy visit and episode of acute rejection)

This phase will be coordinated with the standard of care clinical visit. This approach has been successfully practiced in previous studies. Similar to earlier time period (section b), this phase will last approximately 4 hours and follow the same fasting, dosing and sampling schedule, as described earlier and will take place on the 7th floor of Montefiore hospital by nursing staff or the physician investigators. Apart from the above 3month blood draw, if there is a scheduled visit for treatment of infection or rejection, for a one-year period, there will be blood draws. This will be required to establish any association between posttransplant rejection/infection and therapeutic activity of MPA/ tacrolimus. Total blood volume drawn will be 40 ml for 3-month visit and another 40 mL if visiting during episode of acute rejection or infection.

Research activity on the blood samples obtained:

1. Drug levels: MMF, MPA (total and unbound) and its metabolites will be quantified in plasma by a validated LCMSMS methodology. Tacrolimus will be measured in whole blood using a validated liquid chromatography mass spectrometry (LCMSMS) method.

2. PBMC isolation: PBMCs will be isolated using Ficoll-Paque Plus gradient centrifugation and lysed after being counted. Cell lysate samples will be frozen until analysis. A lysate containing approximately 510 million cells will be used for quantifying the IMPDH activity. The IMPDH activity will be determined from the conversion of inosine monophosphate (IMP) to xanthosine monophosphate (XMP) and normalizing it to intracellular Adenosine Monophosphate (AMP) concentration. PBMC count will be used to standardize the IMPDH activity measurement. The PBMCs obtained from blood samples will be used to quantify the IMPDH activity and if available, to quantify the intracellular tacrolimus levels using a validated LCMSMS methodology.

3. Genotyping and other biomarkers: The pretransplant blood sample will be used to genotype patients using a DNA extraction kit and DNA stored at −80°C until further analysis. Genotyping will be done for enzymes CYP3A5, UGT1A9, UGT2B7 and transporters MRP2, ABCB1. In addition, the left-over blood sample from clinical samples drawn for routine monitoring will be saved for measurement of cytokines and other biomarkers.

Other data collected from Standard of care Procedures:

Clinical, demographic and miscellaneous data generated as a part of routine clinical care for renal transplant recipients will be utilized to better characterize the relation of the IMPDH activity, MPA exposure to outcomes like rejection, infections, hematological or gastrointestinal side effects. Data to be utilized will include but not limited to the following demographic, clinical and outcome data. Demographic data for recipients, donors, conditioning and immunosuppression regimens, HLA matching and source of graft will be recorded. Clinical details of recipients like age, weight, body surface area, gender, ethnicity, concomitant medications, serum albumin levels, renal, liver function tests and other relevant laboratory values will be recorded in standardized data collection forms. Posttransplant clinical outcomes, including transplant procedure related complications, occurrence and severity of delayed graft function, subacute, acute and chronic rejection, gastrointestinal and other adverse effects, infection status, recipient survival and other clinical endpoints of interest will also be captured using standardized data collection forms.

APPENDIX B

CONSENT TO ACT AS PARTICIPANT IN A RESEARCH STUDY

TITLE: Exposure to Mycophenolic acid/Tacrolimus and outcomes in renal transplant recipients

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SOURCE OF SUPPORT: Starzl Transplant Institute (STI) and Clinical Pharmacokinetics Laboratory Funds, University of Pittsburgh School of Pharmacy

Why is this research being done?
After kidney transplantation, you will receive drugs called mycophenolate mofetil or mycophenolic acid and tacrolimus to prevent your body from rejection of the transplanted kidney. Mycophenolate and tacrolimus are Food and Drug Administration (US FDA) approved for use in kidney transplantation. The primary objective of this study is to develop a simple strategy to simultaneously measure the level and effect of these drugs in kidney transplant patients, in order to facilitate acceptance of the transplanted kidney. The results of this study may provide valuable information to the clinicians on how these measurements can better improve efficient dosing of these drugs. In the future, results from this study may be used to guide dosing of mycophenolate mofetil/tacrolimus after transplant to better prevent rejection, infection and increase patient survival.

Who is being asked to take part in this research study?
You are being invited to take part in this research study because you are between the ages of 18 and 75 years and will be receiving a kidney transplant. You will participate in this study only if you receive mycophenolate mofetil and tacrolimus as part of your clinical treatment as a medication to prevent rejection of your transplanted kidney. You will not be given any medications for the sake of this research study. This study will take place at the University of Pittsburgh Medical Center, Pittsburgh, PA, and will include approximately 90 participants.

How will the study be done?
You will participate in this study only if you receive mycophenolate mofetil and tacrolimus as part of your treatment. If you decide to participate in this study, you will undergo a screening procedure and participate in the study for at least three time points: Before your transplant; 4-14 days following transplantation; at follow-up around 3 months following transplantation. In addition, if you make a visit at a time of rejection or infection, you will be seen for a fourth time point. The duration of your participation in this study will be around five minutes before transplant and around 4 hours each on the other time points. The entire period of your participation would be about one year following your transplantation. We will be collecting information on your medical and surgical history from your hospital, as well as surgical and clinic records during your study participation, and for up to 1 year after transplantation. We may also be conducting biochemical tests such as assessing the levels of certain enzymes and proteins, on the left-over blood samples from routine clinical testing that were taken from the time of your transplant for up to one year. This may include genetic testing. Genetic testing is inherited information (a blueprint) about the structure and functions of cells in the human body that make up the color of our hair and eyes and may influence the way our bodies respond to certain stimuli such as an illness or infections. Any and all information collected will be confidentially held and used only for the purposes of the research study.

Research Procedures
Before any study-related tests and procedures are performed, you will be asked to read and sign this consent document.

Screening
Recruitment for this study shall begin with initial review of some of your information to determine if you are qualified to take part in this clinical study. This will be done following your selection as a recipient for kidney transplantation. To determine if you meet the criteria for participation in this study, your doctor will review and collect information about your medical history including but not limited to your age, gender, weight, height, medical history, infection status as well as clinical laboratory test results indicative of your liver and kidney function. If you are a woman of child-bearing age, pregnancy testing is normally performed as part of the standard of care. Once you meet all the study participation criteria, you will be approached by the investigators to determine your interest in participating in this research study. If you express interest, the study will be explained to you by one of the coordinators and you will be allowed sufficient time to read and understand the consent form and sign the consent. No study procedures will be performed until after you sign the informed consent.

Pre-transplant
Prior to your transplant, 15 mL (one tablespoon) of blood will be drawn for lab and genetic testing related to the proteins in your body that handle mycophenolate mofetil/tacrolimus and mediate their effect. This activity will take about 5 minutes.

Post-transplant
After your transplantation, there will be two or in some cases three time points that will coincide with your scheduled hospital visits.

a) The early post-transplant time point will occur anytime between 4-14 days following transplantation along with your early follow-up hospital visit

b) The 3-month post-transplant time point will occur during your biopsy visit around 3 months.

c) In addition, if you are scheduled to visit the hospital anytime for one year following transplantation, for the treatment of rejection or infections, you will participate in an additional time point.

At each of these post-transplant time points, the following will take place.

- Prior to study day, you will get a reminder to hold the morning dose of immunosuppressant drugs (tacrolimus and mycophenolate) and bring them with you to the clinic. You will be advised to continue taking other drugs as usual.
- You will also be reminded about the need to refrain from eating or drinking fluids for a period of at least one hour prior to dosing until about 2 hours after dosing, on the day of study. However, drinking water will be permissible.
- On the day of study, you will be asked to take your morning dose of mycophenolate mofetil and tacrolimus in the clinic. As reminded earlier, please bring them with you to the clinic.
- During study, you will be asked to refrain from eating or drinking fluids except water for a period of at least one hour prior to dosing with mycophenolate mofetil and tacrolimus until about 2 hours after dosing.
- Blood samples for the study will be drawn from an intravenous catheter inserted in your arm.
- One blood sample (10 ml or approximately 2 teaspoons) will be collected before you take your drugs, along with your scheduled clinical sample for tacrolimus monitoring.
- You will be asked to take your morning dose of mycophenolate mofetil and tacrolimus in the clinic.
- Blood samples (10mL) will be collected for not more than 3 time points during a period of 4 hours after taking the dose. A total of 40 mL (approx. 3 tablespoons) of blood will be collected over a duration of 4 hours.

The total volume of blood that will be drawn for entire study including the possible visit at the time of infection/rejection will be 135 mL (approximately 9 tablespoons). In addition, if you are making adequate urine, you may be requested to void and provide the total urine excreted during the study period.

Biological Samples for Future Research

All biological samples collected during your clinical visits may also be used for future testing related to this study or related to the use of medications in transplant recipients. The samples that will be collected in this study include blood, plasma and a type of cell within the blood known as peripheral blood mononuclear cells (PBMCs) as well as urine. The samples will be stored indefinitely in the Clinical Pharmacokinetics Laboratory at the University of Pittsburgh under the direct supervision of the research investigator Dr. Raman Venkataramanan. Only members of the

research team or laboratory personnel conducting the laboratory tests will have access to the samples. Samples will be labeled with the numerical code assigned to each subject in order to correlate with clinical data obtained during the study and only the authorized study team members will know to whom the sample belongs. You will not be notified about pending results on these tests, as they have no bearing on your medical management. These samples may be shared with other investigators who are interested in transplant, but none of the samples shared will contain your identifiers.

What are the possible risks, side effects, and discomforts of this research study?
There may be certain possible risks associated with participation in this study. These may include the following:

I. **Blood draws or Intravenous (IV) catheter insertion:**

Bruising, bleeding, swelling and pain. Subjects (participants) may also be subject to the rare risk of infectious contamination associated with the blood draw. To minimize these risks, subjects will be monitored after blood draws for bleeding or signs of fainting, and blood will not be drawn if hemoglobin is less than 7 g/dL (low blood count). Blood sampling will only be done by experienced individuals.

II. **Breach of Confidentiality:**

There is also a remote risk associated with breach of confidentiality related to genetic testing. There is a possibility that, if the results of the research studies involving your biologic materials were to become generally known, this information could impact future insurability, employability, or reproduction plans, or have a negative impact on family relationships, and/or result in stigmatization. To minimize this risk, any information about you obtained from this research will be kept as confidential (private) as possible. All records or samples related to your involvement in this research study will be stored in a secure, double-locked area or password-protected computer database that is accessible only by members of the research team. Your identity on these records and samples will be indicated by your subject identification number rather than by your name, and the information linking your subject identification number with your identity will be kept separate from the research records.

What are possible benefits from taking part in this study?
There is no direct benefit from participating in this study. However, the information learned from this study may help others who undergo transplantation in the future.

What treatment or procedures are available if I decide not to take part in this research study?
If you decide not to take part in this research study, you will undergo normal procedures associated with the living or deceased donor kidney transplantation. This study will not affect your chance to receive a kidney transplant and associated treatment procedures.

If I agree to take part in this research study, will I be told of any new risks that may be found during the course of the study?
You will be promptly notified if, during the conduct of this research study, any new information develops which may cause you to change your mind about continuing to participate in this study.

Will my insurance provider or I be charged for the costs of any procedures performed as part of this research study?
Some of the services you will receive during this time are "research only services" that are being done only because you are in the study. These services will be paid for by the study and will not be billed to your health insurance company or you. Examples are certain blood draws, biochemical measurements, and genetic testing.

Some of the procedures that you will undergo during this study are considered to be "routine clinical services" that you would have even if you were not in the study. Examples are the actual kidney transplant surgery, other transplant-related tests, use of immunosuppressive medications and routine care medications, hospitalization and all associated costs. These services will be billed to your health insurance company or you, if you do not have health insurance.

Will I be paid if I take part in this research study?
You will not receive any payment for taking part in this clinical research study. Your biological sample or genetic material may lead, in the future, to new inventions or products. If the research investigators are able to develop new products from the use of your biological sample or genetic material, there are currently no plans to share with you any money or other rewards that may result from the development of the new product.

Who will pay if I am injured as a result of taking part in this study?
University of Pittsburgh investigators and their associates who provide services at UPMC recognize the importance of your voluntary participation in their research studies. These individuals and their staff will make every reasonable effort to minimize, control and treat any injuries that may arise as a result of this research. If you believe that you are injured as the result of the research procedures being performed, please contact the principal investigator or one of the investigators listed on the first page of this form.

Emergency medical treatment for injuries solely and directly related to your participation in this research study will be provided to you by the hospitals of UPMC. Your insurance provider may be billed for the costs of this emergency treatment, but none of those costs will be charged directly to you. If your research-related injury requires medical care beyond this emergency treatment, you will be responsible for the costs of this follow-up care. At this time, there is no plan for any additional financial compensation.

Who will know about my participation in this research study?
Any information about you obtained from this research will be kept as confidential (private) as possible. All records related to your involvement in this research study will be stored in a locked file cabinet. Your identity on these records will be indicated by a case number rather than by your name, and the information linking these case numbers with your identity will be kept separate from the research records. You will not be identified by name in any publication of the research results.

Will this research study involve the use or disclosure of my identifiable medical information?
This research study will involve the recording of past, current and/or future identifiable (pertaining to only you) medical information from your hospital and/or other (e.g., physician office) records. The information that will be recorded will be limited to information concerning your transplant

(e.g., diagnostic information, lab results, medications, medical history). This information will be used to determine your eligibility for this study and to follow your response once you are enrolled in the study.

This research study will not result in identifiable information that will be placed into your medical records held at UPMC. If the results of this study are published or presented at meetings, you will not be identified.

Who will have access to identifiable information related to my participation in this research study?

In addition to the investigators listed on the first page of this authorization (consent) form and their research staff, the following individuals will or may have access to identifiable information (which may include your identifiable medical information) related to your participation in this research study:

I. Authorized representatives of the University of Pittsburgh Research Conduct and Compliance Office may review your identifiable research information (which may include your identifiable medical information) for the purpose of monitoring the appropriate conduct of this research study.

II. Authorized representatives of UPMC hospitals or other affiliated health care providers may have access to identifiable information (which may include your identifiable medical information) related to your participation in this research study for the purpose of (1) fulfilling orders, made by the investigators, for hospital and health care services (e.g., laboratory tests, diagnostic procedures) associated with research study participation; (2) addressing correct payment for tests and procedures ordered by the investigators; and/or (3) for internal hospital operations (i.e. quality assurance).

III. In unusual cases, the investigators may be required to release identifiable information (which may include your identifiable medical information) related to your participation in this research study in response to an order from a court of law. If the investigators learn that you or someone with whom you are involved is in serious danger or potential harm, they will need to inform, as required by Pennsylvania law, the appropriate agencies.

Records of your participation in this study will be held confidential except as disclosure is required by law or as described in this informed consent document.

For how long will the investigators be permitted to use and disclose identifiable information related to my participation in this research study?

The investigators may continue to use and disclose, for the purposes described above, identifiable information (which may include your identifiable medical information) related to your participation in this research study for a minimum of seven years after final completion or publication of the project and for as long (indefinite) as it may take to complete this research study.

Is my participation in this research study voluntary?

Your participation in this research study, to include the use and disclosure of your identifiable information for the purposes described above, is completely voluntary. (Note, however, that if you

do not provide your consent for the use and disclosure of your identifiable information for the purposes described above, you will not be allowed to participate in the research study). Whether or not you provide your consent for participation in this research study will have no effect on your current and future care at a UPMC hospital or affiliated health care provider or your current or future relationship with a health care insurance provider. If you choose not to participate in this study, it will not impact your care or your chances of receiving a kidney transplant.

Your doctor may be an investigator in this research study, and as an investigator, is interested both in your medical care and in the conduct of this research. Before entering this study or at any time during the research, you may discuss your care with another doctor who is in no way associated with this research project. You are not under any obligation to participate in any research study offered by your doctor.

May I withdraw, at a future date, my consent for participation in this research study?
You may withdraw, at any time, your consent for participation in this research study, to include the use and disclosure of your identifiable information for the purposes described above. (Note, however, that if you withdraw your consent for the use and disclosure of your identifiable medical record information for the purposes described above, you will also be withdrawn, in general, from further participation in this research study). Any identifiable research or medical information recorded for, or resulting from, your participation in this research study or blood samples collected prior to the date that you formally withdrew your consent may continue to be used and disclosed by the investigators for the purposes described above.
To formally withdraw your consent for participation in this research study you will be requested to provide a written and dated notice of this decision to the principal investigator of this research study at the address listed on the first page of this form.

If I agree to take part in this research study, can I be removed from the study without my consent?
It is possible that you may be removed from the research study by the researchers if you experience unexpected conditions and in the opinion of the investigators that it is in your best interest. If your mycophenolate mofetil or tacrolimus is stopped at the recommendation of your physician, you will also be removed from the study. If this happens, your data may still be kept and used depending on when your mycophenolate mofetil or tacrolimus is discontinued.

VOLUNTARY CONSENT

All of the above has been explained to me and all of my current questions have been answered. I understand that I am encouraged to ask questions about any aspect of this research study during the course of this study, and that such future questions will be answered by the researchers listed on the first page of this form.

Any questions which I have about my rights as a research participant will be answered by the Human Subject Protection Advocate of the IRB Office, University of Pittsburgh (1-866-212-2668). By signing this form, I consent to participate in this research study and provide my authorization to share my medical records with the research team. A copy of this consent form will be given to me.

_____ _____

Participant's Signature Date/Time

CERTIFICATION OF INFORMED CONSENT

I certify that I have explained the nature and purpose of this research study to the above-named individual(s), and I have discussed the potential benefits and possible risks of study participation. Any questions the individual(s) have about this study have been answered, and we will always be available to address future questions as they arise. I further certify that no research component of this protocol was begun until after this consent form was signed.

_____ _____

Printed Name of Person Obtaining Consent Role in Research Study

_____ _____

Signature of Person Obtaining Consent Date/Time

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