CHARACTERIZATION OF RENAL SECRETION IN RENAL TRANSPLANT PATIENTS

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The kidney is a vital organ in the human body. It conserves essential nutrients and eliminates endogenous and exogenous waste products by filtration and tubular secretion processes. Organic anionic and cationic transport systems expressed in the proximal tubular cells drive active renal secretion. Slow recovery and progressive graft function loss following renal transplantation due to prolonged cold ischemia (CI), calcineurin inhibitor (CNI) nephrotoxicity, BK virus nephropathy (BKVN) and varying grades of acute T-cell mediated rejection (TCMR) are hypothesized to affect the secretory function of renal allografts. This body of work is one of the first attempts to understand changes in expression and activity of renal transporters after renal transplantation. Pre-clinical studies in rats showed significant changes in gene expression of important transporters following prolonged-CI and renal transplantation, both in the presence and absence of tacrolimus treatment. Quantitative human gene expression studies showed significant differences in the expression of various transporters in kidney allografts that underwent prolonged-CI (CIT: 15.8±4.80 hrs) and in allografts with BKVN; allografts with acute TCMR and fibrosis had significantly compromised renal anionic transporter expression. Renal anionic secretory capacity was estimated in transplant patients using low-dose cefoxitin as a probe drug in 15 de-novo renal transplant recipients at two time-points post-transplantation. Results of this study suggest that anionic secretory capacity in living and deceased donor renal transplant recipients with no serious clinical complications is similar in the early post-transplant time-points. Renal transplant recipients, however, had a significantly higher cefoxitin exposure when

compared to historical healthy controls ($AUC_{0-\infty}$: 2.6-fold higher), indicating decreased (~60%) renal anionic secretory capacity. A robust physiologically based pharmacokinetic (PBPK) model of cefoxitin was built and validated in healthy subjects and renal transplant recipients. This PBPK model was used to predict the impact of changes in renal anionic transporter expression on anionic drug exposure. Overall this work shows that renal transplant recipients have altered expression of various renal transporters and altered activity of anionic transporters. Systematic characterization of changes in the activity of other transport systems and clinical monitoring of renal secretion is necessary to optimize pharmacotherapy of renally secreted drugs in renal transplant recipients.

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ABBREVIATIONS

A_e	Amount of drug recovered unchanged in the urine
ABC	Adenosine triphosphate binding cassette
AKI	Acute kidney injury
ANOVA	Analysis of variance
AP	Acidic phospholipids
ATP	Adenosine triphosphate
AUC	Area under the concentration-time curve
$AUC_{0-\infty}$	Area under the concentration-time curve between time of drug administration and infinite time
BCRP	Breast cancer resistance protein
BDCS	Biopharmaceutics drug classification scheme
BKVN	BK virus nephropathy
BSA	Body surface area
C_4	Plasma concentration at 4 hours
CAR	Constitutive androstane receptor
cDNA	Complementary deoxyribonucleic acid
CI	Cold ischemia
CIT	Cold ischemic time

CKD	Chronic kidney disease
$CL_{Filtration}$	Filtration clearance
CL_{int}	Intrinsic clearance
$CL_{int\ OAT}$	OAT transporter intrinsic clearance
$CL_{int\ MRP}$	MRP transporter intrinsic clearance
CL_{Renal}	Renal clearance
$CL_{Secretion}$	Secretion clearance
CL_{Total}	Total clearance
CMV	Cytomegalovirus
CNI	Calcineurin inhibitor
DDRT	Deceased donor renal transplant
DGF	Delayed graft function
DM-II	Type-2 diabetes mellitus
EDTA	Ethylenediaminetetraacetic acid
eGFR	estimated glomerular filtration rate
aE	Amplification efficiency
EMA	European Medicines Agency
E:P	Erythrocyte to plasma partitioning
ERCC	External RNA Control Consortium
ESI	Electron spray ionization
ESRD	End stage renal disease
ET1	Endothelin-1
EW	Extracellular water

FDA	Food and Drug Administration
FFPE	Formalin-fixed paraffin-embedded
f_u	Fraction unbound
FXR	Farnesoid X receptor
GEO	Gene expression omnibus
GFP	Green fluorescent protein
HLA	Human leukocyte antigen
HNF4α	Hepatocyte nuclear factor-4 α
HPLC-UV	High performance liquid chromatography with ultraviolet detection
HSV	Herpes simplex virus
HTN	Hypertension
IFNγ	interferon-gamma
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IM	Intramuscular
IRB	Institutional Review Board
IS	Internal standard
IV	Intravenous
IW	Intracellular water
k	Elimination rate constant
K_p	Tissue to plasma partition coefficient
$Kp,_{Alb}$	Tissue-plasma partition coefficient for serum

albumin

Kp,$_{LPP}$	Tissue-plasma partition coefficient for lipoproteins
LDRT	Living donor renal transplant
LEW	Lewis rats
LLOQ	Lower limit of quantification
MATE1	Multidrug and toxin extrusion protein-1
MATE2K	Multidrug and toxin extrusion protein-2K
MDR1	Multidrug resistance protein-1 or P-glycoprotein
MRM	Multiple reaction monitoring
mRNA	Messenger ribonucleic acid
MRP	Multidrug resistance protein
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
NHE3	Sodium-hydrogen exchanger-3
NL	Neutral lipids
NP	Neutral phospholipids
OAT	Organic anionic transporter
OAT1	Organic anionic transporter-1
OAT3	Organic anionic transporter-3
OCT	Organic cationic transporter
OCT2	Organic cationic transporter-2
PBPK	Physiologically based pharmacokinetics
P-gp	P-glycoprotein or multidrug resistance protein-1

PI	Percentile interval
PK	Pharmacokinetics
PXR	Pregnane X receptor
QC	Quality control
ROS	Reactive oxygen species
RT-qPCR	Real time quantitative polymerase chain reaction
RXRα	Retinoid X receptor-α
SD	Sprague-Dawley rats
SEM	Standard error of the mean
SLC	Solute carrier
SPE	Solid phase extraction
SrCr	Serum creatinine
$t_{1/2}$	Half-life
TCMR	T-cell mediated rejection
TNFα	Tumor necrosis factor-α
Tx	Transplantation
UPLC-MS/MS	Ultra-performance liquid chromatography with tandem mass spectrometry
UPMC	University of Pittsburgh Medical Center
UW	University of Wisconsin
V_e	Volume of erythrocyte
V_p	Volume of plasma
V_{ss}	Volume of distribution at steady state

V_t	Volume of each major tissue
V_z	Volume of distribution during terminal phase
VZV	Varicella zoster virus
WIT	Warm ischemic time
WT	Wild type

1.0   INTRODUCTION

1.1 RENAL DRUG TRANSPORTERS

The kidney is a vital organ in the human body that conserves essential nutrients and eliminates endogenous waste products, toxins, drugs and their metabolites. This is facilitated not only by renal filtration, but also by transporters that play an important role in the secretion and re-absorption of a wide range of endogenous and exogenous molecules [3-7]. More than 400 membrane transporters are encoded in the human genome. Drug transporters can be broadly classified into adenosine triphosphate (ATP)-binding cassette (ABC) superfamily and Solute Carrier (SLC) superfamily of integral membrane proteins. ABC transporters are active transporters which utilize energy generated from ATP hydrolysis to facilitate transport of substrates against their electrochemical gradients. Solute Carrier transporters, on the other hand, can facilitate both passive and active transport [4-6]. In the passive transport mode, substrates move down their electrochemical gradients and do not require an energy source. For active transport of substrates against their electrochemical gradient, they are coupled with a co-solute or ion in the direction or against the direction of transport. The transporters within each superfamily vary in their tissue localization and expression levels. A detailed list of important ABC and SLC drug transporters as identified by the United States Food and Drug Administration (FDA) and European Medicines Agency (EMA) for their relatively high abundancy, role in drug disposition and drug-drug interactions is provided in Table 1 [5-9].

Table 1. List of important ABC and SLC transporters and their relative expression in important tissues

Transporter Gene	Transporter Name	Relative Expression in Tissues
ABCB1	MDR1, P-gp	Kidney > small intestine > placenta > Liver > brain
ABCB4	MDR3	Liver > small intestine > kidney > placenta > brain
ABCB11	BSEP	Liver
ABCC1	MRP1	Placenta > small intestine > kidney > brain > liver
ABCC2	MRP2	Liver > kidney > small intestine > placenta > brain
ABCC3	MRP3	Liver > small intestine > kidney > placenta > brain
ABCC4	MRP4	Kidney > small intestine > brain > placenta > liver
ABCC5	MRP5	Brain > kidney > small intestine > placenta > liver
ABCC6	MRP6	Liver > kidney > small intestine > placenta > brain
ABCG2	BCRP	Placenta > small intestine > brain > liver > kidney
SLC10A1	NTCP	Liver > brain > placenta > kidney > small intestine
SLC10A2	ASBT	Small intestine > kidney > brain
SLC15A1	PEPT1	Small intestine > liver > kidney > placenta
SLC15A2	PEPT2	Kidney > brain > placenta > small intestine > liver
SLC22A1	OCT1	Liver > kidney > placenta > brain > small intestine
SLC22A2	OCT2	Kidney > placenta > brain > liver
SLC22A3	OCT3	Placenta > liver > kidney > small intestine > brain
SLC22A4	OCTN1	Kidney > small intestine > placenta > brain > liver
SLC22A5	OCTN2	Kidney > placenta > small intestine > brain > liver
SLC22A6	OAT1	Kidney > brain > liver
SLC22A7	OAT2	Liver > kidney > small intestine > brain > placenta
SLC22A8	OAT3	Kidney > brain > liver
SLC22A11	OAT4	Kidney > placenta
SLC22A12	URAT1	Kidney
SLC47A1	MATE1	Liver > kidney
SLC47A2	MATE2K	Kidney > liver
SLCO1A2	OATP1A2	Brain > liver > kidney > placenta > small intestine
SLCO1B1	OATP1B1	Liver > brain > kidney
SLCO1B3	OATP1B3	Liver > kidney
SLCO2B1	OATP2B1	Liver > placenta > small intestine > kidney > brain

Adapted from relative mRNA expression as reported by Nishimura et al. [7, 8, 10]

Several ABC or SLC uptake and efflux transporters are expressed in the renal tissue and translocate substrates across the epithelial cell membrane [6-8, 10, 11]. A cartoon showing directional orientation of important renal drug transporters in renal epithelial cells is provided in Figure 1.
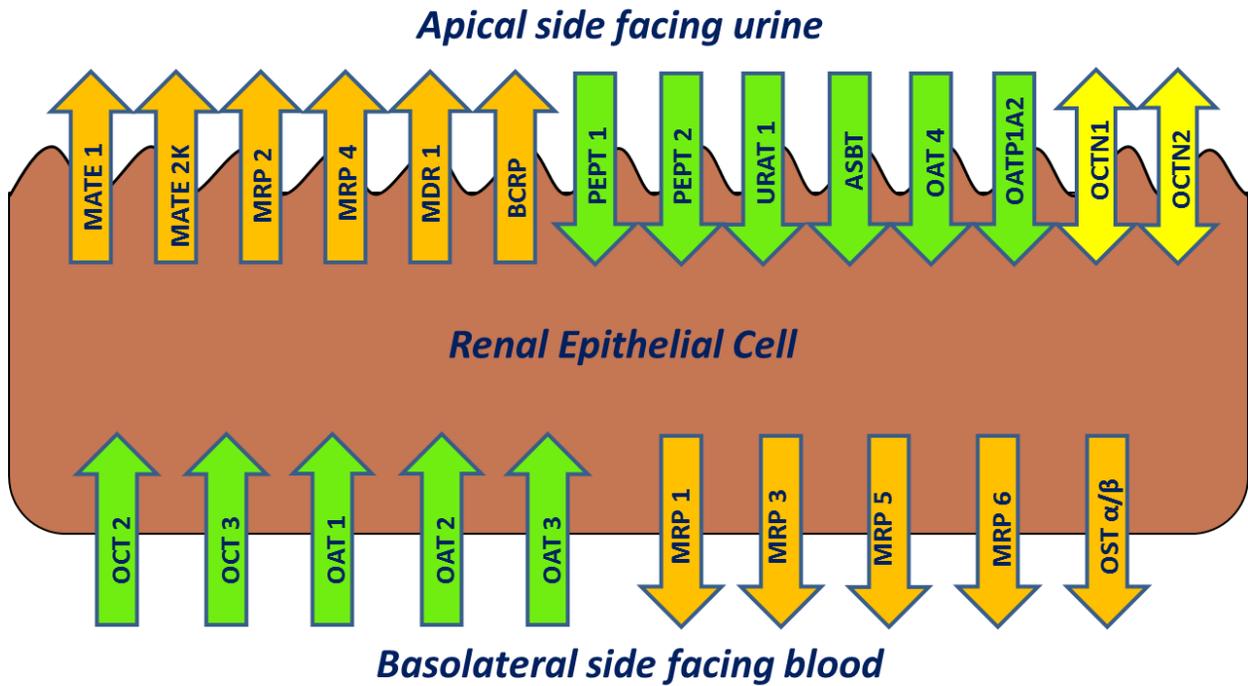
Figure 1. Orientation of important uptake (green), efflux (orange) and bi-directional (yellow) transporters expressed in renal proximal epithelial tubular cells [2]

Transporters often have overlapping substrates and function as transport systems to secrete or reabsorb endogenous and exogenous compounds. More than 90% of the prescribed drugs that are eliminated by the kidney are substrates of renal drug transporters which are primarily sequestered in the tubular epithelial cells of nephrons, the functional units of kidneys [12-14]. Proximal tubular cells are equipped with transport systems for organic anions and organic cations, each consisting of multiple transporters localized in the plasma membrane at both apical and basolateral sides of the cells. Organic cationic transporters (OCTs) and organic anion transporters (OATs) are examples of SLC uptake transporters that are primarily located on the basolateral membrane of renal proximal epithelial cells, and pump substrates from the blood side into the cells. Multi drug resistance proteins (MRPs), and breast cancer resistance protein (BCRP) are examples of ABC efflux transporters that are located on the apical side of the proximal epithelial tubular cells pumping specific substrates out of the cell and into the tubular lumen [4, 6, 12-14]. For a drug to be secreted it has to be a substrate of an uptake and efflux transporter pair. For example cefoxitin, an anionic cephalosporin antibiotic utilizes the OAT1/3 as the uptake transporters and the MRP2/4 as efflux transporters Figure 2 [1, 15, 16].

Expression of anionic and cationic transporters varies considerably with the high inter-subject variability associated with differences in genetic polymorphisms, dietary intake, disease conditions, age and ethnicity, among others. This variability directly affects the rate of secretion and re-absorption of administered drugs which may result in drug concentrations below or above the therapeutic range [15, 17-19]. Furthermore, the up- or down- regulation of renal transporters may result in altered drug exposure and undesired interactions of drugs administered as part of a therapeutic regimen. Alterations in renal transporter function can also alter the nephrotoxicity potential of certain drugs.
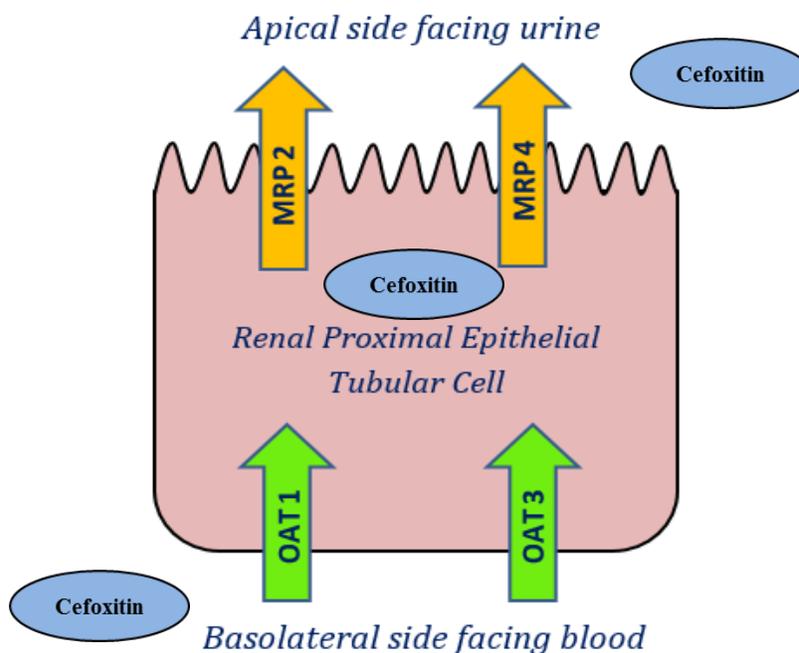
Figure 2. Orientation of OAT1/OAT3 uptake transporters and MRP2/MRP4 efflux transporters in renal proximal epithelial tubular cells [2]

1.1.1 Renal Organic Anion Transporters (OATs)

OAT transporters represent more than half of the SLC22 transporter family with seven important transmembrane proteins. OAT1 (*SLC22A6*), OAT2 (*SLC22A7*), OAT3 (*SLC22A8*), OAT4 (*SLC22A11*), OAT10 (*SLC22A13*) and URAT1 (*SLC22A12*) are the organic anionic transporters that are expressed in human renal proximal tubular epithelial cells whereas OAT7 (*SLC22A9*) is primarily expressed in the liver [16, 20, 21]. A list of common endogenous and exogenous substrates of renal OAT transporters is provided in Table 2 [8, 16]. Renal OAT1, OAT2 and OAT3 transporters are involved in taking up various substrate drugs and endogenous compounds from the basolateral side facing the blood into the renal epithelial cells. OAT4 is expressed on the apical side and helps efflux its substrates from the cell into the tubular lumen [10, 16, 22].

URAT1 is expressed in the apical side and is involved in reabsorption of uric acid from the proximal tubule, making it very important in the uric acid homeostasis [16, 23].

Table 2. Endogenous and exogenous substrates of OAT transporters

Transporter Name	Transporter Gene	Endogenous Substrates	Exogenous Substrates
OAT1	*SLC22A6*	Cyclic nucleotides, folates, prostaglandin E2/Fα, uric acid	Acyclovir, adefovir, apricitabine, cefaclor, cefonicid, cefoxitin, ceftriaxone, cephadrine, cidofovir, ciprofloxacin, dicloxacillin, furosemide, ganciclovir, ibuprofen, indomethacin, ketoprofen, methotrexate, olmesartan, para-aminohippurate, tenofovir, zalciabine, zidovudine
OAT2	*SLC22A7*	Deoxyguanosine, prostaglandin E2/Fα	5-fluorouracil, bumetanide, erythromycin, paclitaxel, tetracycline, theophylline, zidovudine
OAT3	*SLC22A8*	Conjugated hormones, carnitine, prostaglandin E2/Fα, uric acids	Bumetanide, cefaclor, ceftizoxime, cefonicid, cefoxitin, ceftriaxone, cephadrine, cidofovir, cimetidine, ciprofloxacin, dicloxacillin, fexofenadine, furosemide, ganciclovir, ibuprofen, indomethacin, ketoprofen, methotrexate, olmesartan, para-aminohippurate, pitavastatin, pravastatin, rosuvastatin, sitagliptin, tetracycline, zidovudine
OAT4	*SLC22A11*	Estrone sulfate, dehydropiandrosterone sulfate, prostaglandin E2/Fα, uric acid	Ochratoxin A
OAT10	*SLC22A13*	Uric acid	Nicotine
URAT1	*SLC22A12*	Uric acid, orotic acid, lactate, nicotinoate, acetoacetate, oxybutyrate	Oxypurinol

URAT1, OAT1 and OAT3 transporters have the highest relative expression in the renal tissue [7, 8, 10]. OAT1 and OAT3 transporters are considered to be the most important OAT transporters by the FDA and EMA for their role in drug disposition and drug-drug interactions [5-9]. OAT1 is selective for smaller amphiphilic anionic substrates, whereas OAT3 is selective for larger amphiphilic anionic substrates[24]. They transport organic anionic substrates against a negative membrane potential in exchange for α-ketoglutarate, which serves as the counter ion. A secondary active sodium-dicarboxylate co-transporter, which utilizes the sodium gradient maintained by the primary active Na+/K+ ATPase, maintains the α-ketoglutarate gradient [25]. BCRP, MRP2, MRP4 and OAT4 are thought to be potential efflux partners for OAT1 and OAT3 transporters [26-31]. A summary of clinically significant drug interactions involving OAT transporters and probenecid, a potent inhibitor of OAT and MRP transporters is provided in Table 3 [1, 32-39].

Table 3. Clinical drug-drug interaction with probenecid and anionic drugs disposed by the renal anionic transport system

Affected Drug	Fold Change in Clinical PK Parameters					References
	$AUC_{0-\infty}$	C_{max}	CL_R	CL/F	$t_{1/2}$	
Acyclovir	1.4	-	0.7	-	-	Laskin, 1982[36]
Cefaclor	2.1	1.5	-	-	1.6	Welling, 1979 [39]
Cefonicid	2.1	1.2	0.3	-	1.5	Pitkin, 1981[37]
Cefoxitin	2.4	-	0.4	-	2	Vlasses, 1980[1]
Cidofovir	-	-	0.5	0.6	-	Cundy, 1995[33]
Ciprofloxacin	1.7	-	0.4	0.6	1.5	Jaehde, 1995[35]
Dicloxacillin	1.9	1.8	0.3	0.5	-	Beringer, 2008 [32]
Famotidine	1.8	1.5	0.4	0.1	-	Inotsume, 1990[34]
Furosemide	2.7	1.5	0.3	0.4	1.7	Vree, 1995[38]

Summary of significant changes in clinical PK parameters ($p<0.05$) of anionic drug substrates when probenecid is used to inhibit OAT mediated secretory transport. AUC, area under the curve; C_{max}, maximum concentration; CL_R, renal clearance; CL/F, apparent clearance; $t_{1/2}$, half-life

'-': Not significant or not reported.

1.1.2 Renal Organic Cationic Transporters (OCTs)

OCT transporters represent the remaining SLC22 transporter family with six important transmembrane proteins [23, 40, 41]. OCT2 (*SLC22A2*), OCT3 (*SLC22A3*), OCTN1 (*SLC22A4*) and OCTN2 (*SLC22A5*) are organic cationic transporters that are expressed in human renal proximal tubular epithelial cells, whereas OCT1 (*SLC22A1*) is primarily expressed in the liver and OCT6 (*SLC22A16*) is expressed in the testes [23, 40, 41]. A list of common endogenous and exogenous substrates of renal OCT transporters is provided in Table 4 [8, 24, 41, 42]. Renal OCT2 and OCT3 transporters are primarily involved in transporting various substrate drugs and endogenous compounds from the basolateral side facing the blood into the renal epithelial cells [5, 8, 11]. However, OCT transporters facilitate passive diffusion of various organic cations down their electrochemical gradient in either direction. They are specific for small organic cations[41, 42]. Renal OCTN1 and OCTN2 are expressed on the apical side and help with bi-directional transport of its substrates to and from tubular lumen. OCTN1 transport activity can be affected by both sodium and proton gradients, depending on the substrate. OCTN2 also mediates both sodium dependent and sodium-independent uptake, depending on the substrate [5, 24, 41, 42].

Table 4. Exogenous and endogenous substrates of OCT transporters

Transporter Name	Transporter Gene	Endogenous Substrates	Exogenous Substrates
OCT1	*SLC22A1*	Choline, acetylcholine, agmatine, dopamine, norepinephrine, epinephrine, serotonin, histamine	Metformin, oxaliplatin, acyclovir, ganciclovir
OCT2	*SLC22A2*	Creatinine, bile acids, choline, acetylcholine, dopamine, norepinephrine, epinephrine, serotonin, histamine, putrescine, salsolinol, agmatine	Metformin, pindolol, procainamide, ranitidine, amantadine, amiloride, oxaliplatin, varenicline, cisplatin, debrisoquine, proplanolol, guanidine, D-tubocurarine, pancuronium, mematine, picoplatin, ifosfamide, cimetidine, famotidine, zalcitabine, lamivudine, berberine
OCT3	*SLC22A3*	Creatinine, carnitine, choline, guanidine, acetylcholine, dopamine, norepinephrine, epinephrine, serotonin, histamine, corticosterone, progesterone, testosterone, agmatine	Atropine, phenoxybenzamine, prazosin, diphenylhydramine, metformin, ranitidine, amantadine, ketamine, nicotine, phencyclidine, clonidine, etilefrine, o-methylisoprenaline dizocilpine, verapamil, procainamide, citalopram, desipramine, imipramine, granisetron, tropisetron, quinine, mitoxantrone, d-amphetamine, mematine, cimetidine
OCT6	*SLC22A16*	Carnitine, spermidine	Bleomycin, doxorubicin
OCTN1	*SLC22A4*	Ergothioneine, carnitine, acetylcholine	Tiotropium, ipratropium, pyrilamine, quinidine, quinine, verapamil, doxorubicin, mitoxantrone, gabapentin, oxaliplatin
OCTN2	*SLC22A5*	Carnitine	Etoposide, cephaloridine, ipratropium, tiotropium, mildronate, cephaloridine, emetine, verapamil, spironolactone

Among renal cationic transporters, OCT2 has the highest relative expression in renal tissues. It functions in conjunction with multidrug and toxin extrusion protein-1, MATE1 (*SLC47A1*) and MATE2-K (*SLC47A2*) which are involved in efflux of OCT2 substrates into the urine from the apical end of renal epithelial cells [42]. OCT2 along with MATE1/2-K are considered to be important OCT transporters by the FDA and EMA for their role in drug disposition and drug-drug interactions [5, 8, 9]. A summary of clinically significant OCT transporter level drug interactions involving various OCT inhibitors (cetirizine, cimetidine, and trimethoprim) is provided in Table 5 [43-52].

Table 5. Clinical drug-drug interaction of cationic drugs disposed by the renal cationic transport system

Affected Drug	Interacting Drug	Fold Change in Clinical PK Parameters					References
		$AUC_{0-\infty}$	C_{max}	CL_R	CL/F	$t_{1/2}$	
Apricitabine	Trimethoprim	1.7	1.3	0.6	0.6	1.4	Shiveley, 2008 [43]
Cephalexin	Cimetidine	-	-	0.8	0.8	-	van, 1986 [44]
Dofetilide	Cimetidine	1.5	1.3	0.7	0.7	1.3	Abel, 2000 [45]
Metformin	Cimetidine	1.5	1.7	0.7	-	-	Somogyi, 1987 [46]
Pilsicainide	Cimetidine	1.3	-	0.7	0.7	1.2	Shiga, 2000 [47]
Pilsicainide	Cetirizine	1.4	-	-	-	-	Tsuruoka, 2006 [48]
Procainamide	Cimetidine	1.4	-	0.6	-	1.3	Somogyi, 1983 [49]
Pindolol	Cimetidine	1.4	1.3	0.7	-	-	Somogyi, 1992 [50]
Ranitidine	Cimetidine	1.3	-	0.7	-	1.3	van, 1986 [44]
Varenicline	Cimetidine	1.3	-	0.8	0.8	-	Feng, 2008 [51]
Zidovudine	Trimethoprim	-	-	0.5	-	-	Chatton, 1992 [52]

Summary of significant changes in clinical PK parameters (p<0.05) of cationic drug substrates when inhibitors are used to inhibit OCT/MATE mediated secretory transport. AUC, area under the curve; C_{max}, maximum concentration; CL_R, renal clearance; CL/F, apparent clearance; $t_{1/2}$, half-life

'-': Not significant or not reported.

1.1.3 Effect of Renal Diseases on Renal Transporters

Research over the last decade has focused on elucidating expression and activity of renal transporters and their influence on the pharmacokinetic and pharmacodynamic response of renally secreted drugs. Administration of transporter inhibitors or diseases affecting renal function can alter the activity of specific renal transporters and ultimately alter exposure of drugs that are cleared by renal secretion. Renal dysfunction, acute kidney injury (AKI), chronic kidney disease (CKD), glomerulonephritis and diabetic nephropathy have been shown to differentially regulate renal OAT and OCT transporters [53-55].

Several studies in AKI animal models, have shown that Oat1 and Oat3 expression were significantly down regulated [56-60]. Erman et al. investigated the effects of lycopene on the expression of OATs, OCTs and MRPs in rats with cisplatin-induced nephrotoxicity. They observed a significant increase in Mrp2 and Mrp4 protein and a decrease in Oct1 and Oct2 protein levels as compared to controls. Another study evaluating the effect of AKI on P-gp showed that the function of P-gp was suppressed and this led to accumulation of P-gp substrates in plasma [61].

Effect of CKD on transporters has not been comprehensively studied. A pharmacokinetics study using fexofenadine as a probe drug for OATP and P-gp transporters exhibited significant increase in exposure (area under concentration–time curve) and decreased systemic clearance in CKD patients as compared to healthy adults [62]. A study conducted in the nephrectomized rat model showed an increase in Mrp2 expression which was proportional to the severity of CKD [63]. Other studies have characterized expression of P-gp and GLUT-1 in gloemerulonephritis and diabetic nephropathy, respectively. P-gp expression was significantly lower (0.33 ± 0.2 vs 1.0 ± 0.8; p-value < 0.05) in lupus nephritis patients compared with healthy

controls [64], while GLUT2 and SGLT2 expression was significantly higher in type-2 diabetes mellitus patients when compared to healthy control [65].

Preliminary preclinical and clinical evidence suggests that acute and chronic renal diseases differentially regulate expression and activity of renal transporters. A better characterization and understanding of the impact of renal diseases on renal secretory capacity is crucial to optimize pharmacotherapy in this patient population [66].

1.1.4 Regulation of transporters during inflammation

Complications associated with renal transplantation as well as acute and chronic renal diseases present with varying modalities of specific and non-specific inflammatory processes. Inflammation is known to activate of nuclear factor- κB (NF-κB) which decreases the expression of several nuclear receptors like pregnane X receptor (PXR), constitutive androstane receptor (CAR), and farnesoid X receptor (FXR). These receptors control the expression and activity of several drug metabolizing enzymes and transporters and a down regulation of these can downregulate the expression and activity of renal transporters [67].

Acute and chronic inflammation down regulate PXR and thereby downregulate several hepatic transporters [68]. However, the role of PXR during inflammation is still not clearly understood. Teng et al. evaluated the effect of endotoxins and cytokines on PXR in-vivo in a mouse model. It was observed that endotoxin and the inflammatory cytokine interleukin-6 (IL-6) caused a significant down regulation of PXR. IL-6 administration led to a significant downregulation of MRP2 protein levels; however, OATP2 down regulation did not reach statistical significance [68].

A recent study suggested that endotoxin activates NF-κB independent PXR activation and down regulates numerous ABC and SLC transporters in the liver [69]. There is no information available on the molecular mechanisms of regulation of transporters in kidney. Experiments in mice have explored the effect of inflammation on renal glucose transporters. Lipopolysaccaride induced inflammation led to decreased expression of Sglt2, Sglt3, Glut2, and Na^+-K^+-ATPase [70]. The same group also investigated the regulation of renal sodium [71], chloride [72] and uric acid[73] transporters in inflammation. All three studies concluded that down regulation was mediated by pro-inflammatory like tumor necrosis factor-α (TNFα), interleukin-1β (IL-1β), interferon-gamma (IFNγ), or IL-6. However, because of overlapping actions of different anti-inflammatory and pro-inflammatory cytokines, regulation of specific transporters cannot be attributed to a single cytokine.

The current body of work is an attempt to understand the effect of renal transplantation and associated complications on the secretory capacity of renal allografts using preclinical, clinical and PBPK modeling strategies.

1.2 RENAL DRUG TRANSPORTERS IN RENAL TRANSPLANT RECIPIENTS

Chronic kidney disease is the ninth leading cause of death in the United States affecting 26 million adults [74]. More than half-million of these patients are classified as having end stage renal disease (ESRD) with an estimated glomerular filtration rate (eGFR) of less than 15 mL/min/1.73 m2 [75]. Kidney transplantation is the treatment of choice for patients diagnosed with ESRD [3, 75]. In the year 2016, 19,060 kidney transplantations were performed in the USA with 13,431 kidneys coming from deceased donors and 5,629 kidneys coming from living donors [available from: www.unos.org]. Renal allografts are subjected to a unique set of injurious conditions such as prolonged CI before being transplanted into the recipient, warm reperfusion injury immediately after transplantation, exposure to nephrotoxic CNI based immunosuppression therapy, acute T-cell mediated rejection, interstitial fibrosis and bacterial/fungal/viral infections post-transplantation [76-86] (Figure 3).
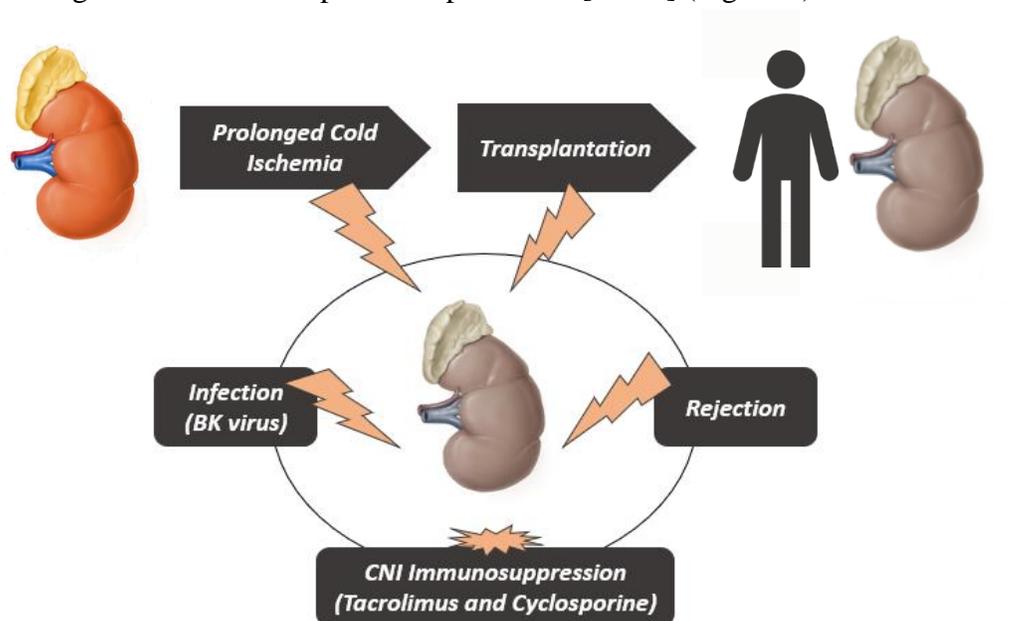
Figure 3. Conditions that can alter the function of renal allografts

Cold ischemic injury, CNI nephrotoxicity, BKVN and T-cell mediated rejection with fibrosis that a renal allograft may be subjected to or may have developed following transplantation, have been shown to lead to progressive loss of renal function. Inflammatory cytokines such as TNFα, IL-6, and IL-1β and the vasoactive hormones such as endothelin-1 (ET1) which are associated with these abuses are shown to be involved in regulation of drug transporters [87, 88]. All these abuses may lead to altered regulation or injury to the renal anionic and cationic transport systems and eventually affect the clearance of drugs that are predominantly cleared by renal secretion. It is important to characterize the anticipated changes in secretory capacity of renal allografts to ensure optimal pharmacotherapy in transplant recipients.

1.2.1 Cold Ischemic Injury

Patients with end stage renal disease undergoing kidney transplantation receive kidneys harvested from deceased or living donors. Kidneys from deceased donors are more readily available compared to living donors and are typically preserved in University of Wisconsin preservation solution at 4°C until a recipient is available for transplantation[89-91]. The average cold storage time has been reported to be around 20 hours over the last decade, and each additional hour can increase the risk of allograft failure and death [89-91]. The hypothermic conditions slow the degradative reactions and metabolism by a factor of 11-12. Prolonged hypothermic preservation causes vasoconstriction and endothelial damage leading to CI injury to the graft. This non-specific renal tissue injury is initiated by an inflammatory cascade including release of reactive oxygen species (ROS), cytosolic calcium, cytokines, chemokines, and leukocytes activation [85, 91, 92]. These effects are amplified by reperfusion and re-oxygenation

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resulting in delayed graft function (DGF), alloimmune reactivity and chronic damage, ultimately contributing to AKI where the kidney rapidly dysfunctions and leads to mortality [93].

The renal proximal epithelial cells, which are primarily involved in the secretion of various drugs, are also affected in a nonspecific manner and cold ischemic injury may alter the secretory capacity of the kidney.

1.2.2 Calcineurin Inhibitor Mediated Nephrotoxicity

Calcineurin inhibitors (CNI) are the most effective class of immunosuppressants available in transplant medicine for maintenance of immunosuppression and their use has dramatically improved short-term graft survival in solid organ transplant recipients [3]. Currently 94% of all renal transplant recipients are on cyclosporine or tacrolimus based CNI maintenance immunosuppressive regimen. However, CNI therapy is associated with acute and chronic nephrotoxicity and is a major contributing factor to allograft damage and graft loss beyond five years post-transplantation [84]. CNI nephrotoxicity is thought to involve a decrease in vasodilatory factors such as prostaglandin E2 and nitric oxide along with an increase in vasoconstrictive factors such as thromboxane, endothelin and renin-angiotensin system. CNI inhibition of prolyl isomerase is also thought to cause impairment of protein synthesis and accumulation of unfolded proteins, leading to enlargement of endoplasmic reticulum [79, 84]. Further, chronic renal allograft damage is associated with interstitial fibrosis, tubular atrophy, arteriolar hyalinosis, and glomerulosclerosis.

Association between the use of CNIs and nephrotoxicity has been observed from early on. For CNIs with narrow therapeutic windows, treatment should be carefully monitored to maintain a balance between efficacy and toxicity [3]. This intra- and inter-individual variability is associated with various factors including genetic polymorphisms, transplantation associated stress conditions, intestinal absorption, dietary regimen, ethnicity, diarrhea, etc. Pharmacokinetics of CNIs are also influenced by other therapeutics which are part of post-transplantation treatment regimen such as macrolide antibiotics, calcium channel blockers and antifungal drugs.

There are many local renal factors that may also play a role in chronic CNI toxicity, such as genetic polymorphisms in renal CYP3A4/5, age of the kidney, and salt depletion. It has been reported that decreased expression or activity of ABCB1 (MDR1, P-gp) efflux transporter protects proximal tubular cells against apoptotic stress induced by nephrotoxic agents; CNI exposure could further increase direct nephrotoxic effects by concentrating the drugs in the epithelial cells and thereby increasing the susceptibility to chronic tubulointerstitial damage of transplanted kidneys [84, 94]. Lower ABCB1 expression has also been linked to chronic histologic changes in kidney transplant patients treated with CNIs. The reduced expression of CYP3A5 in the renal tissue may contribute to nephrotoxicity in patients [84]. Conversely, other studies have failed to demonstrate a correlation between allograft survival and the ABCB1 genotype or the association of the CYP3A5 genotype and CNI-mediated nephrotoxicity. Renal expression of ABCB1 has been found to be less pronounced in renal specimens with calcineurin inhibitor-induced nephrotoxicity [95].

Hyperkalemia is another common complication in calcineurin inhibitor treated transplant patients. Cyclosporine and tacrolimus treatment is believed to inhibit potassium excretion by altering transporter activity and thereby increasing the paracellular chloride reabsorption. In-vitro studies have shown that CNIs can alter potassium secretion by three mechanism: (1) reduced activity of the Na^+K^+-ATPase pump [96], (2) inhibition of the apical secretory K^+ channels [97], and (3) increased reabsorption of chloride [98]. Increased chloride reabsorption via WNK kinases alteration prevents generation of lumen-negative potential. This further inhibits the potassium secretion.

Overall, the effect of CNI induced nephrotoxicity on expression of renal transporters may critically impact the survival of the transplanted allografts and may lead to other undesirable

effects. It is critical to understand their impact for better therapeutic management and long-term survival of the transplanted allograft.

1.2.3 Post-Transplant Infections and BK Virus Nephropathy (BKVN)

Over the last decade, there has been increased evidence demonstrating that the inflammatory responses alter the expression of several important drug transporters. [99] Proinflammatory cytokines such as IL-6, IL-1β, and TNFα have been shown to play an important role in the regulation of numerous drug transporters. NF-κB and nuclear factor-IL6 are up-regulated by these proinflammatory cytokines and are thought to be key transcription factors responsible for regulation of drug transporters during the acute-phase response to stresses such as infection and inflammation.[88]

Renal transplant recipients receive maintenance immunosuppressive therapeutics to reduce the rejection rates of the transplanted allograft. With the emergence of potent immunosuppressive agents, viral infections post-transplantation has emerged as a critical concern, which could result in increased morbidity and mortality. Transplant recipients are susceptible to various infections derived from the kidney donor and infectious complications of the surgical procedure in the immediate post-transplantation period (about 1-month post-transplantation).

BK virus infection is a common post-transplant viral infection, which affects about 15% of renal transplant recipients in the first-year post-transplantation. Current strategies for prophylactic management of this infection are not robust, and if unaddressed, it may progress to allograft dysfunction or loss [100-102]. BK virus nephropathy is therefore a critical concern and an evolving challenge in post-transplant management of patients. Currently there is no approved antiviral drug for treatment of BK virus. Leflunomide, cidofovir, fluoquinolones have been used

with varying degrees of success. Preliminary clinical observations in renal transplant recipients with BKVN involving cidofovir treatment in the presence and absence of probenecid suggest that renal anionic secretory capacity is compromised in allografts with BKVN [100].

Other donor derived infections in the immunosuppressed recipient include cytomegalovirus (CMV), herpes simplex virus (HSV), varicella zoster virus (VZV), meningococcus, syphilis, candida and aspergillus [77, 82]. Pneumocystic carinii, protozoal diseases, fungal infections, and mycobacteria (tuberculosis) are some other pathogens that may cause infections in the immunosuppressed recipients in the first 6 months post-transplantation [77, 82]. Post-transplant infections including BK virus infection have the potential to alter the expression of renal drug transporters. Prophylactic regimens of antibacterial, antifungal, and antiviral agents are routinely prescribed to renal transplant recipients to prevent these above-mentioned infections. Many of these drugs are secreted by various uptake and efflux renal transporters (OATs: acyclovir, cidofovir, cephalosporine antibiotics, ganiciclovir, fluoquinolone antibiotics) [3, 8].

1.2.4   Rejection of Renal Allografts

Transplanted kidneys can undergo rejection due to complex processes involving the cellular and molecular pathways resulting in a broad range of allograft injuries such as acute tubular injury, glomerulitis, capillaritis and fibrinoid necrosis. Allograft rejection can be classified as hyperacute (occurring within minutes after the vascular anastomosis), acute (occurring days to weeks after transplantation), late acute (occurring 3 months after transplantation), or chronic (occurring months to years after transplantation) [103, 104].

Acute T-cell mediated rejection (TCMR) is more prevalent and is mediated by human leukocyte antigens (HLA) and non-HLA antigens expressed on the allograft's endothelium. Post-transplantation, the recipient's immune system identifies these as foreign components and initiates an immune response (recruitment of leukocytes and facilitation of natural killer cell–mediated or monocyte/macrophage–mediated cytotoxicity) to attack the foreign invaders, thereby attacking the donor kidney leading to endothelial damage, loss of vascular integrity and increased coagulation [80, 83]. With immunosuppressive therapy, chances of acute TCMR can be significantly reduced. On the other hand, antibody mediated rejection is less common but can cause acute and chronic allograft dysfunction, and if left untreated can rapidly result in graft loss.

1.3   SUMMARY AND INTRODUCTION TO DISSERTATION

The kidney is a vital organ in the human body, which conserves essential nutrients and eliminates toxins drugs, and their metabolites. This is facilitated not only by renal filtration, but also by drug transporters that play an important role in the secretion and re-absorption of a wide range of endogenous and exogenous molecules. Anionic and cationic transport systems expressed in the renal tubular epithelial cells are involved in these critical processes. OAT1, OAT3 and OCT2 drug transporters along with their efflux transport partners (MRP2/4 and MATE1/2-K) are considered to be the most important renal anionic and cationic transporters by the United States FDA and EMA for their high renal abundance, role in disposition of most commonly prescribed drugs and clinically significant drug-drug interactions [5-9].

Renal transplantation is the treatment of choice for patients with end stage renal disease. Following transplantation, renal transplant recipients are left with one functioning kidney and the allografts are subjected to a unique set of injurious conditions such as prolonged CI, exposure to nephrotoxic CNIs, varying grades of allograft rejection (TCMR), and complications associated with infections (BKVN). Renal tubular injury due to each of these abuses may alter the expression of these transporters. Preliminary clinical observations in renal transplant recipients with BKVN involving cidofovir treatment in the presence and absence of probenecid suggest that renal anionic secretory activity may be compromised in allografts with BKVN [100]. 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 BKVN or TCMR [100].

To date, no information exists on the quantitative and comparative expression of important renal drug transporters in renal allografts. Currently there is very limited knowledge on changes in expression and activity of important transporters following renal transplantation and associated complications. The body of work described in this dissertation was performed to gain a better understanding of changes renal secretory capacity in renal transplant patients in order to improve pharmacotherapy in this patient population.

The overall hypothesis of this work is that injuries caused by prolonged cold ischemia, calcineurin inhibitor nephrotoxicity, varying grades of T-cell mediated rejection and BK virus nephropathy would significantly alter the renal anionic secretory capacity in renal transplant recipients. The effect of prolonged cold ischemia and tacrolimus treatment on the gene expression of five important transporters in a rat renal transplant model was evaluated first (Chapter 2.0). Quantitative and comparative gene expression of 36 drug transporters in renal biopsies collected from renal transplant recipients with no post-transplant complications, pathological findings consistent for BKVN, and varying grades of acute TCMR and interstitial fibrosis was then studied (Chapter 3.0).

Clinical evaluation of changes in renal anionic secretion following renal transplantation was performed by determining the pharmacokinetics of cefoxitin in living donor and deceased donor renal transplant recipients in order to assess the longitudinal changes in renal anionic secretory capacity, study the effect of prolonged cold ischemia on renal anionic secretory capacity and to compare renal anionic secretory capacity of renal transplant recipients with that of healthy volunteers. Cefoxitin was used as an ideal probe-drug to evaluate changes in renal anionic secretory capacity in renal transplant recipients due to its short half-life, limited protein binding, significant secretory clearance and safety profile.

Before clinical evaluation of cefoxitin pharmacokinetics, it was necessary to develop a rapid and sensitive assay for determination of cefoxitin in human plasma following minimal dose administration. Details of the analytical method are described in Chapter 4.0 . Details of this clinical pharmacokinetic study are described in Chapter 5.0

In order to gain a better understanding of the potential impact of physiological changes following renal transplantation on the disposition of drug substrates of renal anionic transport system, a physiologically based pharmacokinetic (PBPK) modeling approach was used. Cefoxitin physiochemical properties, physiological variables of healthy subjects and renal transplant recipients were used to build and validate a PBPK model for IV cefoxitin in healthy adults and renal transplant recipients to study the significance of changes in OAT transport system. Details of this novel modelling approach are described in Chapter 6.0 . Renal drug transporter expression data from renal biopsies can be combined with PBPK modeling strategies to optimize pharmacotherapy in renal transplant recipients. Summary and clinical implications of this work along with recommended future directions are discussed in Chapter 7.0 .

2.0   EXPRESSION OF RENAL DRUG TRANSPORTERS FOLLOWING RAT

KIDNEY TRANSPLANTATION

2.1   ABSTRACT

The kidney is an important excretory organ involved in the elimination of various endogenous and exogenous molecules. Changes in renal allograft's transporter mediated secretion capacity would significantly alter the clearance and thereby exposure to renally secreted drugs. Slow recovery and progressive graft function loss following renal transplantation due prolonged CI and CNI induced nephrotoxicity are expected to alter the secretory capacity renal allografts. This study is one of the first systematic attempts to understand the changes in expression of important renal drug transporters following prolonged CI, renal transplantation and tacrolimus based CNI treatment. Gene expression of five important renal drug transporters (Oat1, Oat3, Oct2, Mate1, and Mdr1a) was evaluated using renal tissues from a rat kidney transplant model. The mRNA expression of *Slc22a2* (Oct2) was significantly higher in rat kidneys that were subjected to 24 hours of CI. Expression of *Slc22a6* (Oat1)*, Slc22a8* (Oat3)*, Slc22a2* (Oct2) and *Slc47a1* (Mate1) were significantly lower immediately following syngeneic rat kidney transplantations at three hours and 12 hrs post-transplantation; the gene expression of Oat1, Oat3 and Mate1 recovered by four weeks post-transplantation, but Oct2 and Mdr1a did not. Among rats that were treated with tacrolimus following allogeneic or syngeneic transplantations, only those with allografts subjected to 24 hours of prolonged CI had a significantly lower expression of all five important drug transporters. The observations from this study suggests that renal transplantation, prolonged CI and their combination with CNI therapy may lead significant changes in gene expression of important renal drug transporter and this may lead to altered disposition of renally secreted drugs in renal transplant patients.

2.2 INTRODUCTION

Kidney transplantation is the treatment of choice for patients with end-stage renal diseases. Renal allografts are subjected to a unique set of injurious conditions such as prolonged CI before being transplanted into the recipients, warm reperfusion injury immediately after transplantation, and are also susceptible to progressive loss of graft function due to rejection, BK virus nephropathy, and CNI based immunosuppressive medication induced nephrotoxicity [3, 76, 85, 90]. Since kidney is a key excretory organ for drugs, their metabolites and various endogenous molecules, changes in renal allograft's filtration capacity, or transporter mediated secretion capacity would significantly alter the clearance and exposure (area under the concentration-time curve) of renally filtered or secreted compounds. Although creatinine clearance is used to estimate the functional capacity of the transplanted graft, there is currently very limited understanding on the effect of renal transplantation and associated complications on the expression of renal drug transporters that are primarily expressed in the renal tubular epithelial cells.

Transporter expression and activity can be influenced by drugs, disease states or tissue specific injuries. Vasoconstriction and endothelial damage in renal allografts during prolonged CI, non-specific pro-inflammatory cytokines released during warm allograft reperfusion and transplantation surgery, as well as the tubular damage secondary to tacrolimus treatment may all individually effect the expression of renal transporters and in turn the tubular secretory function of renal allografts [78, 84-86, 90, 105]. These physiological and pharmacological insults may affect the clearance of drugs that are predominantly cleared by renal secretion.

The objective of this study is to evaluate the expression of selected important renal drug transporters in renal allografts with transplantation associated complications by studying the effect of prolonged cold ischemia, transplantation surgery and tacrolimus treatment on the gene expression of 5 important transporters in a rat renal transplant model.

We hypothesize that renal allograft insults caused by prolonged CI, renal transplantation surgery and tacrolimus based CNI immunosuppressive treatment would alter the gene expression of Oat1, Oat3, Oct2, Mate1, and Mdr1a renal drug transporters that are involved in major clinical drug-drug interactions.

These drug transporters were selected based on their high relative expression in the renal tissue, homology in activity and relative expression between rats and humans, as well as their role in clinical significant renal drug-drug interactions as identified by US FDA and EMA.

2.3   METHODS

A rat kidney transplant model developed at University of Pittsburgh was used to understand the changes in mRNA expression of renal drug transporters in transplanted kidneys that undergo prolonged cold ischemia (CI) and treatment with a calcineurin inhibitor (tacrolimus) based immunosuppression. *Slc22a6* (Oat1)*, Slc22a8* (Oat3)*, Slc22a2* (Oct2)*, Slc47a1* (Mate1)*,* and *Abcb1a* (Mdr1a) were specifically selected as target transporters for this mRNA expression study as these are the five most expressed drug transporters in human renal tissue and their human analogues are involved in the secretion as well as drug-drug interactions with majority of the top 200 prescribed drugs in the United States [106].

2.3.1   Chemicals

QIAshredder, and RNeasy Mini kits were purchased from QIAGEN (Hilden, Germany). iScript™ Reverse Transcription Supermix for RT-qPCR was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). TaqMan primers for drug transporters and housekeeping genes were purchased from Life Technologies (Carlsbad, CA, USA).   All chemicals and reagents were purchased from Fischer Scientific (Fair Lawn, NJ, USA) unless otherwise noted.

2.3.2 Animals

Male Lewis (LEW) rats were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN, USA). A breeding colony of green fluorescent protein (GFP)-transgenic and wildtype (WT) Sprague-Dawley (SD) rats, originally generated by Masaru Okabe (University of Osaka, Osaka, Japan) [107] was maintained at the University of Pittsburgh [108]. All rats weighed 200-250 grams and were maintained in a 12-hour light-dark cycle at the University of Pittsburgh Animal Center in laminar flow cages. Standard diet and water *ad libitum* was provided to the rats. The University of Pittsburgh Guidelines of the Council on Animal Care and the National Research Council's Guide for the Humane Care and Use of Laboratory Animals were followed for all procedures.

2.3.3 Rat Kidney Transplantation

Orthotopic kidney transplantations were performed using GFP-transgenic SD rats as recipients. Syngeneic transplantations were performed using WT SD rats as donors and allogeneic transplantations were performed using LEW rats as donors. All surgeries were performed using techniques previously described by Neto et al [109]. Left kidney of the donor rats were nephrectomized and flushed with 3 ml University of Wisconsin (UW) solution (Du Pont, Wilmington, DE, USA) and transplanted either immediately or after 24 hrs preservation in UW at 4°C into the recipient by end-to-side anastomoses to recipient infra-renal abdominal aorta and infra-renal vena cava. Left native kidney of the recipient rats were removed, and end-to-end ureteral anastomosis was performed [110].

2.3.4   Experimental Design

The rat transplant study was designed with two specific aims: (1) to study the effect of prolonged cold ischemia and renal transplantation on the mRNA expression of selected renal drug transporters and (2) To study the effect of tacrolimus treatment in the presence and absence of cold prolonged ischemia on the mRNA expression of selected renal drug transporters.

2.3.4.1 Effect of Prolonged Cold Ischemia and Renal Transplantation

Kidneys from WT SD rats were either nephrectomized and stored immediately as controls at -80℃ (n=3), nephrectomized, maintained in UW solution at 4℃ for 24 hours and stored -80℃ as cold ischemic kidneys (n=4), or nephrectomized, maintained in UW solution at 4℃ for 24 hours and orthotopically transplanted to GFP-SD rats (n=9). Transplant recipients were sacrificed at 3 hrs (n=3), 12 hrs (n=3) and 4 weeks (n=3) post transplantation and renal tissues were stored. These timepoints were chosen to study the effect of transplantation and prolonged cold ischemia immediately after transplantation (3 hrs and 12 hrs) and at 4 weeks after transplantation. All samples collected in this experiment were immediately flash frozen in liquid nitrogen and stored at -80℃ until analysis. All SD rats in this experiment underwent syngeneic transplantation and no immunosuppressants were used.

2.3.4.2 Effect of Tacrolimus Treatment in the Presence and Absence of Prolonged CI

This experiment was divided into two parts:

- **Part 1:** WT SD rat kidneys were nephrectomized, maintained in UW solution at 4℃ for 30 min, and orthotopically transplanted to GFP-SD recipient rats in a syngeneic fashion. Transplanted rats were sacrificed and renal tissues stored after 4 weeks of either no

tacrolimus treatment (n=3), or with oral tacrolimus treatment (n=3) at 0.5 mg/ day dose for 2 weeks followed by 1 mg/ week dose for 2 weeks.

- **Part 2:** WT SD and LEW rat kidneys were nephrectomized, maintained in UW solution at 4℃ for 24 hrs, and orthotopically transplanted to GFP-SD recipient rats in a syngeneic (WT SD as donor) and allogeneic (LEW as donor) manner. Transplanted rats were sacrificed and renal tissues stored after 4 weeks of either no tacrolimus treatment (n=3, syngeneic only), or with oral tacrolimus treatment at 0.5 mg/day dose for 2 weeks followed by 1 mg/week dose for 2 weeks (n=3, syngeneic; n=3, allogeneic).

All samples collected in this experiment were immediately flash frozen in liquid nitrogen and stored at -80℃ until analysis.

2.3.5 Sample Preparation and Complementary Deoxyribonucleic Acid (cDNA) Preparation

Rat kidney tissue samples stored at -80℃ were dipped in liquid nitrogen and ground using a chilled motar and pestle to create a homogeneous tissue sample. About 30mg tissue was added to Eppendorf tubes dipped in liquid nitrogen to prevent thawing. Six hundred µL of *Buffer RLT* from the RNeasy Mini Kit® was added to the pulverized tissue and homogenized. The lysate was transferred to QIAshredder homogenizer and rinsed with additional 100µL *Buffer RLT*. QIAshredder was centrifuged in a micro centrifuge at full speed for 2min. Following additional centrifugation, the supernatant was transferred and mixed with an equal volume of 70% ethanol. This mix was further transferred into the RNeasy Mini Kit column in a 2mL collection tube, centrifuged for 15sec at ≥8000g and filtrate was discarded. Three hundred and fifty µL *Buffer*

RW1 was added to the RNeasy column, centrifuged for 15sec at ≥8000g and filtrate was discarded. Ten μL of DNase1 incubation mix was added to RNeasy column membrane, and placed on benchtop (20-30°C) for 15min. The RNeasy column is further treated with 350μL Buffer RW1 followed by centrifugation for 15 sec at ≥8000g and filtrate was discarded. This step was repeated with 500μL Buffer RPE (2min at ≥8000g) two times to wash the column and once without buffer for 1 min to dry the membrane. The RNeasy spin column was placed in a new 1.5ml collection tube and 30μL RNase-free water was added and centrifuged for 1min at ≥8000g to elute the mRNA. This step was repeated for 2 more times to collect remaining mRNA in lower concentrations.

mRNA yield was quantified using NanoDrop 2000c Spectrophotometer. Onto the spectrophotometer pedestal, 2μL of RNase free water was loaded to blank the instrument followed by 2μL of purified mRNA sample to measure total mRNA concentrations and purity of eluted mRNA. All samples were stored at -80°C for further analysis.

iScript™ Reverse Transcription Supermix for RT-qPCR was used to generate cDNA from purified mRNA samples. Samples had varying concentrations of mRNA, but 1 μg of mRNA was mixed with 4μL of iScript RT supermix and the volume of the contents was adjusted to 20 μL with RNase free water. The mix was incubated at 25°C for 5 min, at 42°C for 30 min to initiate reverse transcriptase activity, and at 85°C for 5 minutes to inactivate the transcriptase enzyme.

2.3.6   Real Time Quantitative Polymerase Chain Reaction (RT-qPCR)

RT-qPCR was performed in 96 well plates. Each well was loaded with 4μL of sample cDNA, 1μL of TaqMan primer mix, 10 μL of TaqMan master mix, and 5 μL of RNase free water. All

plates were sealed and centrifuged for 3 minutes at 3000 rpm and 4C to bring the contents to bottom of the wells. 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. TaqMan primers against rat *Slc22a6* (Oat1)*, Slc22a8* (Oat3)*, Slc22a2* (Oct2)*, Slc47a1* (Mate1)*,* and *Abcb1a* (Mdr1a) for transporter targets and *Actb* (β-Actin) for housekeeping were procured from Life Technologies (Carlsbad, CA, USA). Table 6 provides details of the primers used for the experiment.

Table 6. TaqMan® primers used for RT-qPCR experiment

Gene	Assay ID	Accession Number	Assay Location	Amplicon length
Slc22a6 (Oat1)	Rn00568143_m1	NM017224.2	1083	66
Slc22a8 (Oat3)	Rn00580082_m1	NM031332.1	879	64
Slc22a2 (Oct2)	Rn00580893_m1	NM031584.2	1592	55
Slc47a1 (Mate1)	Rn01460731_m1	NM001014118.2	1309	66
Abcb1a (Mdr1a)	Rn01639253_m1	NM133401.1	2122	79
Actb (β-Actin)	Rn00667869_m1	NM031144.3	881	91

Actb was used as the housekeeping gene

All samples were plated in triplicates to avoid variability associated with technical errors and each target was restricted to a single 96 well plate to avoid inter-plate variability.

2.3.7   Data Analysis and Statistical Analysis

Standard curves were generated for each target gene including the housekeeping gene by serial dilution of target cDNA and running RT-qPCR in technical triplicates. Standard curves were drawn with the C_t plotted against the log of the quantity of a target for each dilution prior to

PCR. The amplification efficiency (aE) was calculated from slope the curve using the following formula:

$$aE = 10^{-1/\text{slope}}$$

Target specific standard curves were used to estimate the concentration of each target mRNA in all the experimental samples. mRNA expression of each sample was normalized to β-actin expression in the corresponding sample and reported in relative actin units. A standard concentration of β-actin was used as an inter-plate variability marker and its expression was used to normalize expression across all PCR plates. Groups were compared to controls utilizing analysis of variance (ANOVA) and Bonferroni post-hoc test were run. Differences were considered statistically significant when $p < 0.05$. A minimum of 3 rats were included in each experimental group and RT-qPCR runs were carried out in technical triplicates. All data were expressed as mean ± standard error of the mean (SEM). Data was analyzed using GraphPad Prism 7 statistical software for windows (GraphPad Software, La Jolla, CA, USA). A p-value of <0.05 was considered as statistically significant difference.

2.4   RESULTS

2.4.1   Effect of Prolonged Cold Ischemia and Transplantation on mRNA Expression of Selected Renal Transporters in Transplanted Rat Kidneys

Subjecting rat kidneys to cold ischemia for 24 hours significantly up-regulated expression of *Slc22a2* (Oct2). Orthotopic syngeneic renal transplantation following 24 hours CI had down-regulatory effect on the mRNA expression of *Slc22a2* (Oct2), *Slc22a6* (Oat1), *Slc22a8* (Oat3) and *Slc47a1* (Mate1) at 3 hours and 12 hours post-transplantation. By 4 weeks post-transplantation, *Slc22a6* (Oat1) mRNA expression recovered to baseline, *Slc22a8* (Oat3) mRNA levels recovered to less than baseline where as *Slc22a2* (Oct2), *Slc47a1* (Mate1), and *Abcb1a* (Mdr1a/Pgp) expression did not recover. The timecourse of changes in expression of these transporters were different. Results of this experiment are shown in Figure 4. These results validated prior work in a rat model and provides evidence that prolonged cold ischemia and renal transplantation significantly and differentialy alters the mRNA expression of the five most expressed and important renal drug transporters.
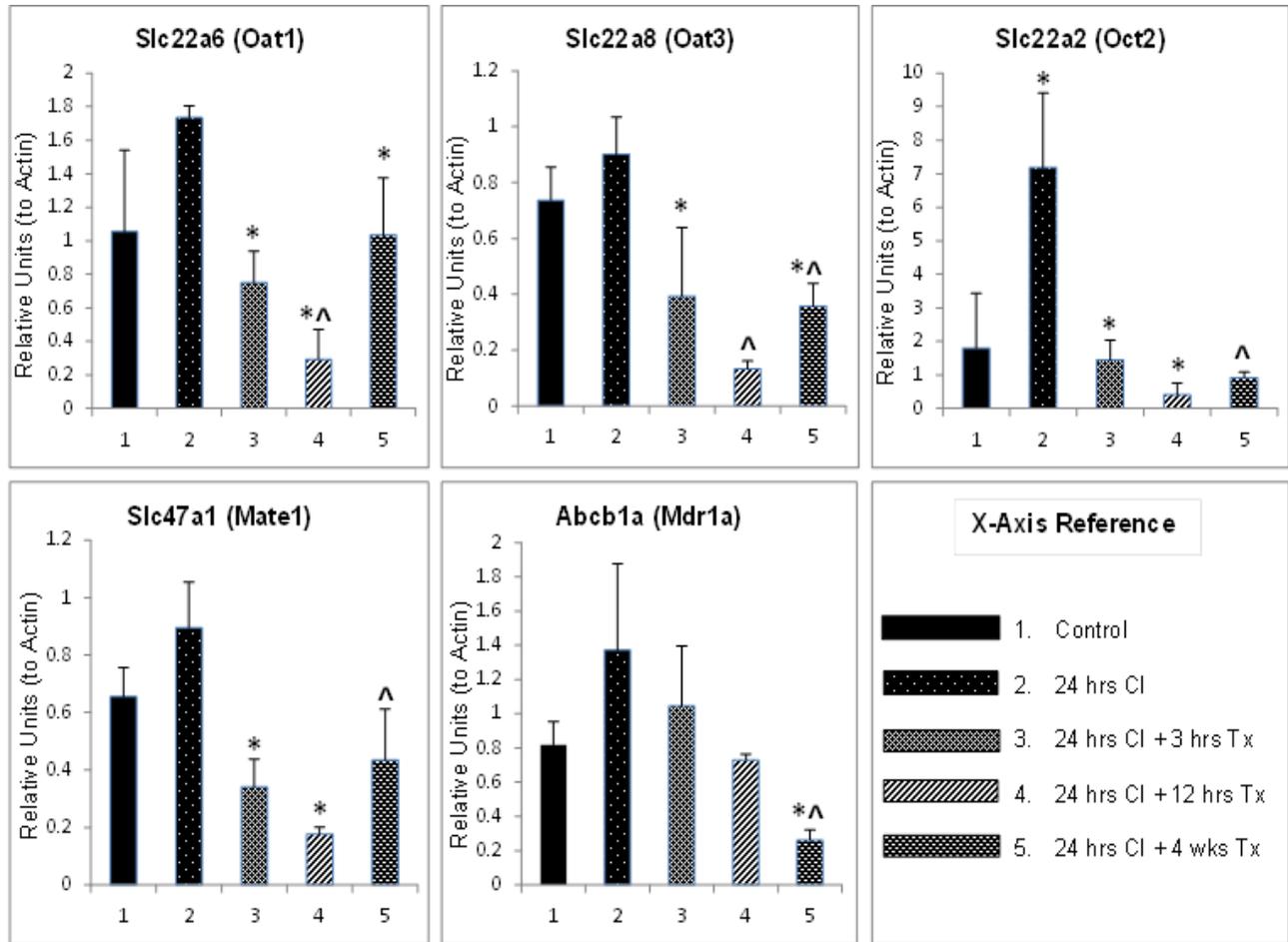
Figure 4. Effect of prolonged cold ischemia, early and long-term effects of renal transplantation on the mRNA expression of 5 selected renal drug transporters in rat renal allografts.

CI: cold ischemia; Tx: post-transplantation; *p<0.05 compared to previous time point; ^ p<0.05 compared to 24hrs CI group

2.4.2 Effect of Tacrolimus Treatment in the Absence of Prolonged Cold Ischemia on mRNA Expression of Selected Renal Transporters in Transplanted Rat Kidneys

The results of this experiment suggest that tacrolimus treatment at 0.5 mg per day for 2 weeks followed by 1 mg per week for two weeks to GFP-SD rats that underwent orthotropic syngeneic transplantation had no significant impact on the expression of the selected 5 renal drug transporters when the allografts were not subjected to CI (30 min cold ischemia). Results of this experiment are shown in Figure 5.
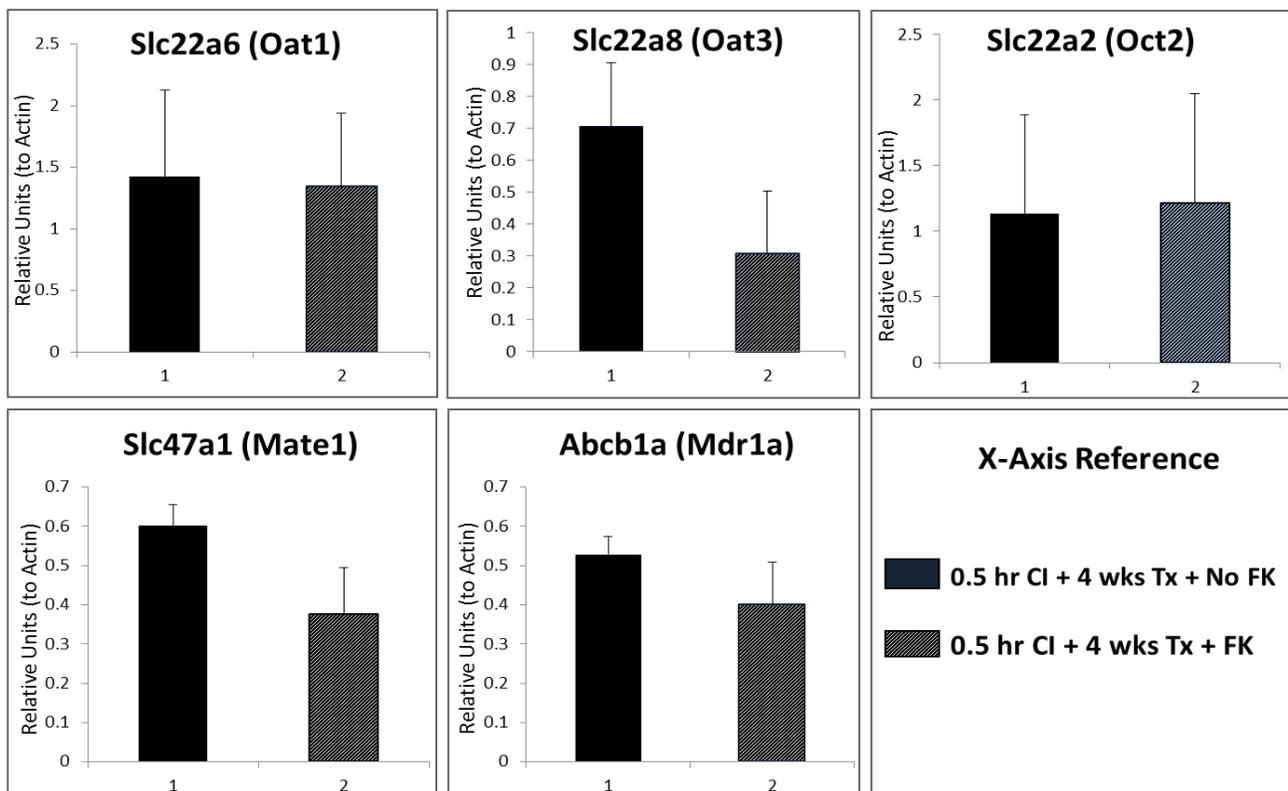
Figure 5. Effect of 4 weeks tacrolimus treatment in the absence of prolonged cold ischemia on the mRNA expression of 5 selected renal drug transporters in rat renal allografts.

CI: cold ischemia; Tx: post-transplantation

2.4.3 Effect of tacrolimus treatment in the presence of prolonged cold ischemia on mRNA expression of selected renal transporters in transplanted rat kidneys

The results of this experiment suggest that tacrolimus treatment at 0.5 mg per day for 2 weeks followed by 1 mg per week for two weeks to GFP-SD rats that underwent orthotropic syngeneic transplantation had significant down-regulatory impact on the expression of the selected 5 renal drug transporters when the allografts were subjected to 24 hrs CI. This effect was reproduced in rats that underwent orthotopic allogeneic transplantations and received similar tacrolimus treatment. Results of this experiment are shown in Figure 6.
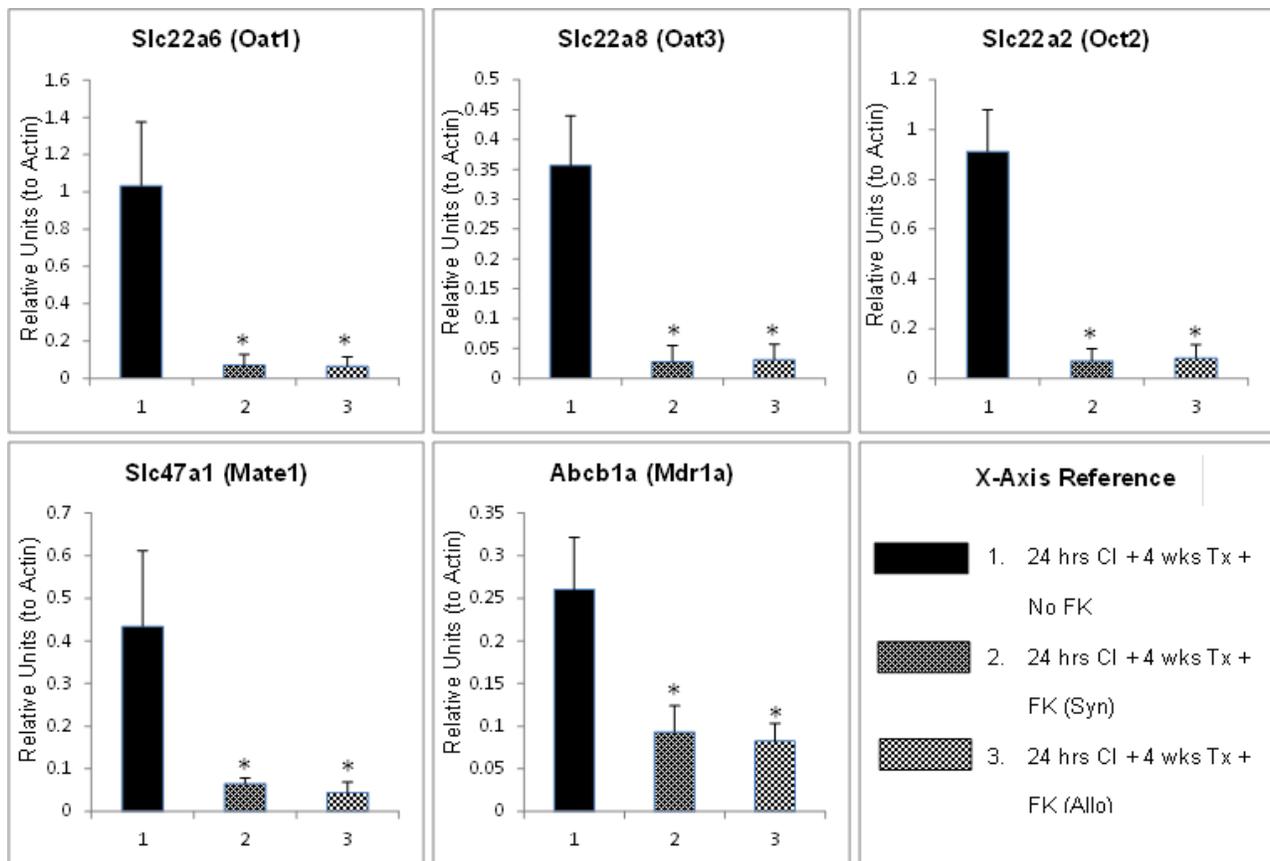
Figure 6. Effect of 4 weeks tacrolimus treatment in the presence of prolonged cold ischemia on the mRNA expression of 5 selected renal drug transporters in rat renal allografts.

CI: cold ischemia; Tx: post-transplantation; FK: tacrolimus treatment

*$p < 0.05$ compared to no tacrolimus treatment group

2.5 DISCUSSION

The kidney is a key excretory organ for various endogenous and exogenous molecules, and any changes in renal allograft's transporter mediated secretion capacity would significantly alter the clearance and exposure of renally secreted drugs. The slow recovery and progressive graft function loss following renal transplantation due to injuries such as cold ischemic injury and drug induced nephrotoxicity are thought to affect the secretory capacity of a transplanted kidney. There is currently very limited understanding on renal secretion in renal transplant recipients and this study is one of the first attempts to understand the changes in expression of important renal drug transporters in renal allografts.

In the current rat kidney transplant study, the effect of prolonged cold ischemia, transplantation, and tacrolimus treatment on mRNA expression of *Slc22a6* (Oat1)*, Slc22a8* (Oat3)*, Slc22a2* (Oct2)*, Slc47a1* (Mate1)*, and *Abcb1a* (Mdr1a) was systematically evaluated. The selected drug transporters are five of the highest expressed renal drug transporters. They are involved in the disposition and drug-drug interactions of the most commonly prescribed medications in the United States as identified by the US FDA and EMA.

Cold ischemic injury results from vasoconstriction and endothelial damage during prolonged hypothermic preservation of renal allografts. This nonspecific renal tissue injury is initiated by an inflammatory cascade including release of reactive oxygen species, cytosolic calcium, cytokines, chemokines, and leukocytes activation [85, 91, 92]. These effects are amplified by warm reperfusion and re-oxygenation after transplantation contributing to acute kidney injury. The renal proximal epithelial cells, which are primarily involved in the secretion

of various drugs, are also affected in a nonspecific manner and we would expect this cold ischemic injury to alter the secretory capacity of the kidney.

Work in rat liver ischemic injury models by several investigators showed that hepatic ischemia reperfusion injury significantly changes expression of drug transporters. Kudo et al. reported reduction of transporter mediated biliary excretion in rat liver allografts that were subjected to 8 hrs of CI at 4℃ in UW solution [111]. This was attributed to decline in expression and activity of Mrp2 transporter. Hypoxia treatment to hepatocytes was shown to decrease the mRNA and protein expression of Ntcp, Bsep, and Mrp2 transporters as well as the nuclear factors involved in the transactivation of these proteins such as hepatocyte nuclear factor-4 α (HNF4α), retinoid X receptor-α (RXRα) and FXR [112]. Ikemura et al. studied the effect of rat liver ischemia (60 min) and reperfusion (12 hr) injury on the expression and activity of renal cationic transporters (Oct2/Mate1) using cimetidine as a substrate drug for this cationic transport system. The results of this study showed that oxidative stress induced by liver ischemia/reperfusion injury significantly decreased renal Oct2 expression leading to altered cimetidine pharmacokinetics [113]. Oxidative stress following ischemia and reperfusion has been shown to result in oxidative damage to mitochondria and ATPase activity in mice cardiac ischemia/reperfusion model. These changes would lead to lower functional activity of ABC transporters. [114]These observations suggest that expression of drug transporters is sensitive to tissue level insults caused by ischemia reperfusion injuries and there is differential regulation to various drug transport systems.

In the current study, rat kidneys treated with 24 hours of cold ischemia had a significantly higher expression of *Slc22a2* (Oct2) and the expression of other transporters were unchanged. However, the expression of all 5 transporters was significantly lower immediately after

orthotopic syngeneic transplantation following 24 hours of CI. Some of the transporters' expressions recovered by 4 weeks post-transplantation. These results suggest that renal transplantation recipients with prolonged cold ischemia may have altered renal secretory capacity immediately post-transplantation and the recovery of transporter expression is not uniform. Proinflammatory cytokine mediated nuclear receptor level regulation of these transporters may be different leading to different expression profiles. Observations from this study are consistent with results from previous unpublished work on Oat1/3 expression in orthotopic syngeneic rat kidney transplant model as well as results from liver ischemia/reperfusion studies.

Limitations of this study include possible inherent differences in regulation of drug transporters between rats and humans, variability in the expression of housekeeping gene at different allograft conditions, use of only one housekeeping gene and use of very high dosage tacrolimus regimen. Changes in mRNA expression do not always translate into changes in protein expression and transporter activity.

Calcineurin inhibitors are the most effective class of immunosuppressants available in transplant medicine for maintenance of immunosuppression with tacrolimus being the most commonly used CNI. However, tacrolimus treatment is riddled with acute and chronic nephrotoxicity profiles which lead to allograft damage and graft loss [84]. CNI nephrotoxicity involves decrease in vasodilation factors such as prostaglandin E2 and nitric oxide along with an increase in vasoconstriction factors such as thromboxane, endothelin and renin-angiotensin system. CNI inhibition of prolyl isomerase is also thought to cause protein synthesis impairment and accumulation of unfolded proteins, leading to endoplasmic reticulum enlargement [79, 84]. Since renal drug transporters are primarily expressed in renal tubular region, CNI mediated

nephrotoxicity was hypothesized to deleteriously affect renal secretion. In the current study, rats that were treated with tacrolimus following syngeneic or allogeneic transplantation of allografts subjected to 24 hours of prolonged cold ischemia had a significantly lower expression of all selected renal drug transporters. This effect was absent in rat allografts that were not subjected to 24 hrs CI but exposed to tacrolimus treatment. The renal allografts may have increased susceptibility to nephrotoxic profile of high dose tacrolimus following injury due to prolonged cold ischemia.

The results of this study show differential expression of various important renal drug transporters in rat renal allografts. A systematic evaluation of changes in expression of other important renal transporters in human tissues is presented in Chapter 3.0 . A prospective study to assess the renal anionic secretory capacity in renal transplant recipients was planned and conducted following the observations of this study (Chapter 5.0).

3.0   EXPRESSION OF RENAL DRUG TRANSPORTERS IN RENAL TRANSPLANT

PATIENTS

3.1 ABSTRACT

Renal transplantation is the treatment of choice for patients with ESRD. Since kidney is a major excretory organ for various endogenous and exogenous molecules, changes in renal graft function would significantly alter the clearance, and thereby exposure of renally secreted drugs. Slow recovery and progressive graft function loss following transplantation due to injuries and complications such as prolonged CI, CNI nephrotoxicity, BKVN and acute TCMR with interstitial fibrosis are thought to affect the secretory capacity renal allografts. This study is the first comprehensive attempt to understand changes in expression of important renal transporters in renal transplant patients with normal allografts and those with transplant associated complications. Gene expression of important renal transporters was evaluated using formalin-fixed paraffin-embedded (FFPE) renal biopsies procured from living donor and deceased donor renal transplant patients (LDRT and DDRT). In renal biopsies of renal transplant patients, gene expression of 36 renal transporters were quantified by NanoString nCounter® gene expression. DDRT recipients had significantly higher expression of *SLC5A1* (SGLT1; 2.7-fold), when compared to LDRT recipients. Biopsies from patients with BKVN had a significantly lower expression of *SLC9A3* (NHE3; 5.3-fold low) when compared to controls. Expression of several transporters involved in the renal anionic transport system was significantly compromised in allografts with acute TCMR and fibrosis (OAT1: 11-fold lower; OAT3: 4.4-fold lower). Results of this study suggest that renal transplant recipients may experience significant changes in renal transporter mediated disposition of various endogenous and exogenous compounds and systematic evaluation of renal secretory activity is warranted in this patient population.

3.2   INTRODUCTION

Kidney transplantation is the treatment of choice for patients with end-stage renal disease. Renal allografts are subjected to a unique set of injurious conditions such as prolonged cold ischemia before being transplanted into the recipients, warm reperfusion injury immediately after transplantation and are also susceptible to progressive loss of graft function due to graft rejection, BKVN, and CNI induced nephrotoxicity [3, 76, 85, 90]. Optimal therapy with various immunosuppressive, anti-bacterial, antifungal and antiviral medications is necessary for long term graft and patient survival in renal transplant recipients. In a recent report on functional recovery of renal graft following kidney transplantation (Tx) in 310 living kidney transplant patients, 77.1% had an immediate recovery post-Tx as evidenced by a mean pre-Tx serum creatinine of 7.1 mg/dL that decreased to 1.4 mg/dL by day 1 post-Tx and 0.7 mg/dL by day 14 post-Tx whereas 22.9% had a much slower recovery of the renal graft function [105]. The slow recovery and progressive graft function loss is even more pronounced in kidneys transplanted from deceased donors.

Since kidney is a key excretory organ for drugs, their metabolites and various endogenous molecules, changes in renal allograft's filtration capacity, or transporter mediated secretion capacity would significantly alter the clearance and exposure of renally filtered or secreted compounds. Although creatinine clearance is used to estimate the functional capacity of the transplanted graft, there is currently very limited understanding of the effect of renal transplantation and associated complications on the expression of renal drug transporters that are primarily expressed in the renal tubular epithelial cells.

Transporter expression and activity can be influenced by various disease or tissue specific injuries. About 40-80% of transplant recipients experience at least one infection during the first year after transplantation [115]. BK virus is a polyomavirus that replicates in the nuclei of renal tubular epithelial cells [116] where majority of renal drug transporters are expressed. BK viral infection leads to inflammation and ultimately BK virus nephropathy (BKVN) in about 10% of renal transplant recipients [117] and this may lead to compromised renal secretion. Decreased renal secretion of cidofovir, a substrate of anionic transport system, has been reported in kidney transplant recipients with BK viral infection [100]. Vasoconstriction and endothelial damage during prolonged CI, pro-inflammatory cytokines released during warm reperfusion and transplantation surgery, acute TCMR as well as the tubular damage secondary to tacrolimus treatment may all individually effect the tubular secretory capacity of renal allografts [78, 79, 84-86, 90, 105]. These physiological and pharmacological insults to the renal allograft may eventually affect the clearance of drugs that are predominantly cleared by renal secretion.

We hypothesize that renal allograft insults caused by tacrolimus treatment following prolonged CI, BKVN and TCMR with interstitial fibrosis would differentially alter the expression of important ABC and SLC renal drug transporters involved in the disposition of various endogenous and exogenous compounds.

The objective of this study is to evaluate the gene expression of 36 important drug transporters in renal biopsies collected from LDRT and DDRT patients with no post-transplant complications, pathological findings consistent for BKVN, and varying grades of acute TCMR and interstitial fibrosis.

3.3   METHODS

Effect of prolonged cold ischemia, BKVN and allograft rejection on the gene expression of various important renal drug transporters was studied by directly quantitating RNA that was purified from FFPE tissues collected from renal biopsies of living donor and deceased donor renal transplant recipients.

3.3.1   Chemicals

QIAshredder and RNeasy FFPE kits were purchased from QIAGEN (Hilden, Germany). Custom code-set for nCounter® assays and nCounter Master Kit was purchased from NanoString Technologies (Seattle, WA, USA). All chemicals and reagents were purchased from Fischer Scientific (Fair Lawn, NJ, USA) unless otherwise noted.

3.3.2   Study Subjects

This study was performed using renal biopsies procured from adult renal transplant recipients. Adult living donor and deceased donor renal transplant recipients who underwent renal transplantation procedure and follow-up transplant care at the University of Pittsburgh Medical Center (UPMC) Montefiore hospital were considered for this study. The renal biopsies used in this study were accessed under a retrospective study protocol (IRB# PRO14040523) and a prospective study protocol (IRB# PRO15010155) which were granted approval by the

Institutional Review Board (IRB) at the University of Pittsburgh. Written informed consent was obtained from all patients prior to participation in the prospective study. Written informed consent requirement was waived for the retrospective study since all patient information and biopsy samples were de-identified and retrospectively collected (exempt status). All biopsies used for this study were collected in the routine standard of care for the transplant recipients.

All patients included in this study received anti-thymocyte globulin based induction therapy based induction regimen and tacrolimus based maintenance immunosuppressive therapy. A detailed description of patient who participated in the prospective study is provided in Chapter 5.0 (Section 5.3.2).

3.3.3   Renal Biopsies

Needle renal biopsies that were FFPE for preservation were used for this study. All biopsies were collected either during 3 months post-transplant care visit or when clinically deemed necessary as a part of standard of care (median: 99.5 days post-Tx; range: 14 to 392 days post-Tx). Biopsy samples used in this study were accessed from the biopsy repository at the University of Pittsburgh Medical Center, Department of Pathology. They were de-identified and provided for gene expression analysis by IRB approved honest broker.

3.3.4   Study Design

This renal biopsy study was designed with two specific aims: (1) to study the effect of prolonged cold ischemia and tacrolimus treatment on gene expression of important renal drug transporters

in renal allografts and (2) To study the effect of BKVN and allograft rejection in renal transplant recipients on gene expression of important renal drug transporters.

3.3.4.1 Effect of prolonged cold ischemia and tacrolimus treatment

Renal biopsies collected from living donor and deceased donor renal transplant recipients who were a part of the prospective study described in Chapter 5 were used for this experiment. Drug transporter gene expressions in biopsies collected from deceased donor renal transplant recipients (n=8) were compared with those collected from living donor renal transplant recipients (n=8) to evaluate the effect of prolonged cold ischemia in the presence of tacrolimus treatment.

3.3.4.2 Effect of BKVN, interstitial fibrosis with tubular atrophy and acute TCMR

Renal biopsies (n=23) collected from living donor and deceased donor renal transplant recipients who were a part of retrospective and prospective protocols were used for this experiment. Based on the pathology findings, biopsies were grouped into controls (N=6), BKVN (N=4), borderline acute TCMR with varying grades of interstitial fibrosis (N=7), and acute TCMR with varying grades of fibrosis (N=6). Each group description is provided in Table 7.

Table 7. Study group characteristics

Groups	Biopsy Characteristics
Control (N=6)	No significant pathological finding for rejection or BKVN
BKVN (N=4)	Significant pathological findings for BK virus nephropathy
Borderline Acute TCMR w/fibrosis (N=7)	Intermediate interstitial inflammation and foci of mild tubulitis with varying degrees of interstitial fibrosis
Acute TCMR w/fibrosis (N=6)	Significant interstitial inflammation and foci of severe tubulitis with varying degrees of interstitial fibrosis; Banff IA and IB acute TCMR

Banff IA and Banff IB as defined by Banff 2013 classification was used [80, 83]; TCMR: T-cell mediated acute rejection

Drug transporter gene expressions in biopsies from BKVN, borderline acute TCMR with fibrosis and acute TCMR with fibrosis groups were compared with those in control group to evaluate the effect of BKVN and acute TCMR with interstitial fibrosis.

3.3.5   Renal Biopsy Sample Preparation

About 15-20 μm tissue of the FFPE blocks was discarded before cutting sections for RNA extraction. RNA was purified from at least four, 5μm thick tissue sections containing more than 75% renal tissue using RNeasy FFPE kit according to manufacturer's instructions (similar to sample processing described in Section 2.3.1.5). mRNA yield was quantified using NanoDrop 2000c Spectrophotometer. Onto the spectrophotometer pedestal, 2μL of RNase free water was loaded to blank the instrument followed by 2μL of purified mRNA sample to measure total mRNA concentrations and purity of eluted mRNA. All samples were stored at -80℃ for further analysis.

3.3.6   NanoString nCounter® Assay

NanoString Technologies nCounter assays (NanoString Technologies, Seattle, WA, USA) are designed to provide a single-tube, sensitive, reproducible, and provide a method for direct detection of targets with molecular barcodes without the use of reverse transcription or amplification. nCounter® is a multiplexed assay that can detect expression of gene targets in very low mRNA concentrations (0.1fM per copy per cell) [118]. This technology has shown to detect quantitative expression even in samples with significantly degradation, where at least 20% of the sample has RNA fragments of greater than 300 base pairs [119, 120]. A custom

nCounter® assay code-set was developed with 75 gene targets for quantitative assessment of expression. The gene targets include 36 renal ABC and SLC drug transporters, 4 housekeeping genes (*ACTB*, *B2M*, *GAPDH* and *PGK1*) and 35 additional targets representing CYP and UGT enzymes as well as various inflammation markers. The custom code-set was designed for various types of human tissues. A detailed list of genes and their corresponding target sequences included in our custom code-set is provided in Table 8. **List of drug transporter genes and their corresponding target sequences included in the custom code-set**

nCounter® Master kit was used to process and load 300 ng of RNA purified from FFPE samples (n=26) onto the custom code-sets 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 in University of Pittsburgh, Pittsburgh, PA.

Table 8. List of drug transporter genes and their corresponding target sequences included in the custom code-set

Class	Target Genes	Position	Target Sequence
ABC Transporters	ABCA1	871-970	GGTTCCTGTATCACAACCTCTCTCTCCCAAAGTCTACTGTGGACAAGATGCTGAGGGCTGATGTCATTCTCCACAAGGTATTTTTGCAAGGCTACCAGTT
	ABCB1	784-883	TATCAATGATACAGGGTTCTTCATGAATCTGGAGGAAGACATGACCAGGTATGCCTATTATTACAGTGGAATTGGTGCTGGGGTGCTGGTTGCTGCTTAC
	ABCB4	2092-2191	AAATTCACAAATGTGTCAGAAGAGCCTTGATGTGGAAACCGATGGACTTGAAGCAAATGTGCCACCAGTGTCCTTTCTGAAGGTCCTGAAACTGAATAAA
	ABCB11	2357-2456	TTCTGAAATTCAGTGCTCCAGAATGGCCCTACATGCTGGTAGGGTCTGTGGGTGCAGCTGTGAACGGGACAGTCACACCCTTGTATGCCTTTTTATTCAG
	ABCC1	326-425	CCCTTCTACTTCCTCTATCTCTCCCGACATGACCGAGGCTACATTCAGATGACACCTCTCAACAAAACCAAAACTGCCTTGGGATTTTTGCTGTGGATCG
	ABCC2	1381-1480	TCAGTGTTATTTCCAACTGTGCTTCAAGCTGGGTGTAAAAGTACGGACAGCTATCATGGCTTCTGTATATAAGAAGGCATTGACCCTATCCAACTTGGCC
	ABCC3	1213-1312	GGGTGAAGTTTCGTACTGGGATCATGGGTGTCATCTACAGGAAGGCTCTGGTTATCACCAACTCAGTCAAACGTGCGTCCACTGTGGGGGAAATTGTCAA
	ABCC4	1775-1874	CAGTGTATCAAGATGCTGACATCTATCTCCTGGACGATCCTCTCAGTGCAGTAGATGCGGAAGTTAGCAGACACTTGTTCGAACTGTGTATTTGTCAAAT
	ABCC5	228-327	GGAGAGAACCAGCACTTCTGGGACGCACAGAGACCGTGAAGATTCCAAGTTCAGGAGAACTCGACCGTTGGAATGCCAAGATGCCTTGGAAACAGCAGCC
	ABCC6	1661-1760	TCTACATTTCTGGTCGCACTGGTGGTGTTTGCTGTCCACACTCTGGTGGCCGAGAATGCTATGAATGCAGAGAAAGCCTTTGTGACTCTCACAGTTCTCA
	ABCG1	936-1035	GCGCCAAACTCTTCGAGCTGTTCGACCAGCTTTACGTCCTGAGTCAAGGACAATGTGTGTACCGGGGAAAAGTCTGCAATCTTGTGCCATATTTGAGGGA
	ABCG2	2021-2120	TGCATCTTGGCTGTCATGGCTTCAGTACTTCAGCATTCCACGATATGGATTTACGGCTTTGCAGCATAATGAATTTTTGGGACAAAACTTCTGCCCAGGA
SLC Transporters	SLC2A1	2501-2600	AGGCTCCATTAGGATTTGCCCCTTCCCATCTCTTCCTACCCAACCACTCAAATTAATCTTTCTTTACCTGAGACCAGTTGGGAGCACTGGAGTGCAGGGA
	SLC2A2	3181-3280	CAATTATGGAAATATAGTTCTGATGGGTCCCAAAAGCTTAGCAGGGTGCTAACGTATCTCTAGGCTGTTTTCTCCACCAACTGGAGCACTGATCAATCCT
	SLC5A1	617-716	GCTGGGGTGGTGACAATGCCAGAGTACCTGAGGAAGCGGTTTGGAGGCCAGCGGATCCAGGTCTACCTTTCCCTTCTGTCCCTGCTGCTCTACATTTTCA
	SLC5A2	481-580	GATCTCAGTGGACATGTTCTCCGGAGCTGTATTCATCCAGCAGGCTCTGGGCTGGAACATCTATGCCTCCGTCATCGCGCTTCTGGGCATCACCATGATT
	SLC8A1	441-540	GAGACCACCAAGACAACTGTGAGGATCTGGAATGAAACAGTTTCTAACCTGACCTTGATGGCCCTGGGATCTTCTGCTCCTGAGATTCTCCTTTCAGTAA
	SLC9A1	2313-2412	TGGCTGTGAAGAAAAAGCAAGAGACGAAGCGCTCCATCAACGAAGAGATCCACACACAGTTCCTGGACCACCTTCTGACAGGCATCGAAGACATCTGTGG
	SLC9A3	736-835	CCTGTTCATCATCGTCTTCGGGGAGTCGCTGCTGAACGACGCAGTCACCGTGGTTCTGTACAATGTGTTTGAATCTTTCGTGGCGCTGGGAGGTGACAAC
	SLC10A1	1241-1340	TAAACTAGAGAGAGCAGCAAAAACACCAGTCTTGCCTGAGTCTTTCTCCAGCATTTCCAGTACATCTATCAGAATCATCAAGTCTTGGCCGGGAACACAG
	SLC19A1	1206-1305	TGGCTGTGCTATGCGGCCTTCGTGCTGTTCCGCGGCTCCTACCAGTTCCTCGTGCCCATCGCCACCTTTCAGATTGCATCTTCTCTGTCTAAAGAGCTCT
	SLC22A1	1021-1120	GCTCAAAAGAATGGGAAGTTGCCTCCTGCTGATTTAAAGATGCTTTCCCTCGAAGAGGATGTCACCGAAAAGCTGAGCCCTTCATTTGCAGACCTGTTCC
	SLC22A12	965-1064	AGGCCGACACGGAGCCGTGTGTGGATGGCTGGGTCTATGACCGCAGCATCTTCACCTCCACAATCGTGGCCAAGTGGAACCTCGTGTGTGACTCTCATGC
	SLC22A2	1636-1735	CTAACATCTGGCTTGAGCTCCCGCTGATGGTTTTCGGCGTGCTTGGCTTGGTTGCTGGAGGTCTGGTGCTGTTGCTTCCAGAAACTAAAGGGAAAGCTTT
	SLC22A3	661-760	TCCCTGTGTTTGTGATCTTCCGCTTCCTGCAAGGTGTATTTGGAAAGGGGACGTGGATGACTTGCTACGTGATTGTGACAGAAATAGTAGGTTCGAAACA
	SLC22A6	901-1000	TCTGGCTGGCATCTCCCTCAACTGCATGACACTGAATGTGGAGTGGATGCCCATTCACACACGGGCCTGCGTGGGCACCTTGATTGGCTATGTCTACAGC
	SLC22A7	647-746	TGGGCCTGGCATCTGCAGCCTCCGTCAGCTATGTAATGTTTGCCATCACCCGCACCCTTACTGGCTCAGCCCTGGCTGGTTTTACCATCATCGTGATGCC
	SLC22A8	1171-1270	TTTGGCTATGGGTGTGGAAGAATTTGGAGTCAACCTCTACATCCTCCAGATCATCTTTGGTGGGGTCGATGTCCCAGCCAAGTTCATCACCATCCTCTCC
	SLC44A1	1809-1908	CCTTTGTCATTCTGGTGGAGAATGCTTTGCGAGTGGCTACCATCAACACAGTAGGAGATTTTATGTTATTCCTTGGCAAGGTGCTGATAGTCTGCAGCAC
	SLC44A2	123-222	CCACAGAAGTATGATCCCACTTTCAAAGGACCCATTTACAATAGGGGCTGCACGGATATCATATGCTGTGTGTTCCTGCTCCTGGCCATTGTGGGCTACG
	SLC47A1	1181-1280	TACTACCGACCGAGACATCATTAATCTGGTGGCTCAGGTGGTTCCAATTTATGCTGTTTCCCACCTCTTTGAAGCTCTTGCTTGCACGAGTGGTGGTGTT
	SLC47A2	383-482	GTGACCCTCGCCGGTGGCCTTTGTCAATGTCTGCGGAGTTTCTGTAGGAGTTGGTTTGTCTTCGGCATGTGACACCTTGATGTCTCAGAGCTTCGGCAGCC
	SLCO1A2	915-1014	GTTGACACTGGATTTGTGAACACAGATGATCTGATCATAACTCCCACTGACACTCGTTGGGTCGGTGCATGGTGGTTTGGCTTTCTGATTTGTGCAGGAG
	SLCO1B1	1173-1272	ACTTATGTCTTCAAATACGTAGAGCAACAGTATGGTCAGCCTTCATCTAAGGCTAACATCTTATTGGGAGTCATAACCATACCTATTTTTGCAAGTGGAA
	SLCO1B3	71-170	AACAGCAGAGTCAGCATCTTCAGAGAAAAAGAAAACAAGACGCTGCAATGGATTCAAGATGTTCTTGGCAGCCCTGTCATTCAGCTATATTGCTAAAGCA
	SLCO2B1	2141-2240	CTCCTCTGAGTCCTTTGCCCAAGATTGGGTGTCAAGAGCCCTGTGTTCCATTCTGGCTCCTCCACTAAATTGCTGTGTGACTTCAGGCAAGACATTGATC
Housekeeping	ACTB	1011-1110	TGCAGAAGGAGATCACTGCCCTGGCACCCAGCACAATGAAGATCAAGATCATTGCTCCTCCTGAGCGCAAGTACTCCGTGTGGATCGGCGGCTCCATCCT
	B2M	26-125	CGGGCATTCCTGAAGCTGACAGCATTCGGGCCGAGATGTCTCGCTCCGTGGCCTTAGCTGTGCTCGCGCTACTCTCTCTTTCTGGCCTGGAGGCTATCCA
	GAPDH	973-1072	CACTCCTCCACCTTTGACGCTGGGGCTGGCATTGCCCTCAACGACCACTTTGTCAAGCTCATTTCCTGGTATGACAACGAATTTGGCTACAGCAACAGGG
	PGK1	1031-1130	GCAAGAAGTATGCTGAGGCTGTCACTCGGGCTAAGCAGATTGTGTGGAATGGTCCTGTGGGGGTATTTGAATGGGAAGCTTTTGCCCGGGGAACCAAAGC

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3.3.7 Code-set Internal and External Controls

The codeset was designed to contain 4 external controls (housekeeping genes: *ACTB*, *B2M*, *GAPDH* and *PGK1*), 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 in each assay well [121]. NanoString has adopted positive and negative control sequences developed and tested by the External RNA Control Consortium (ERCC). Reporter probes designed against ERCC transcript sequences are pre-mixed into every code-set. The range of internal concentrations corresponds to the expression levels of most mRNAs of interest present in 100 ng of total RNA [121, 122].

3.3.8 Bioinformatics and Statistical Data Analysis

Geometric mean of expression counts of negative internal controls in each well were subtracted from the expression data in the corresponding well to eliminate the non-specific background. Variability unrelated to the samples was eliminated by normalizing expression counts from each well with expression counts of the internal positive controls. Variability related to samples was minimized by calculating the average of the geometric means of housekeeping genes across all wells and dividing this average by the geometric mean in each well to get a lane-specific normalization factor. Each well specific factor was multiplied by all the target expression counts to get normalized gene expression counts.

nSolver® software (NanoString Technologies) was used to perform Student's t-tests to compare the mean expression between comparator groups of samples. For assessment of fold changes in target genes between two groups, the numerator samples were compared to the

denominator samples to determine the statistical significance of the calculated fold change value. A two-tailed t-test was used for comparisons of log-transformed normalized data. The distribution of the t-statistic was calculated by using the Welch-Satterthwaite equation for the degrees of freedom and in the estimation of the 95% confidence limits for observed differential expression between groups. The statistical significance was set at $p<0.05$.

3.4   RESULTS

3.4.1   Quality Control

The overall expression of 6 positive controls at 128 fM, 32 fM, 8 fM, 2 fM, 0.5 fM, and 0.125 fM concentrations loaded in each nCounter code-set well was consistently reproducible with limited variability (Figure 7).
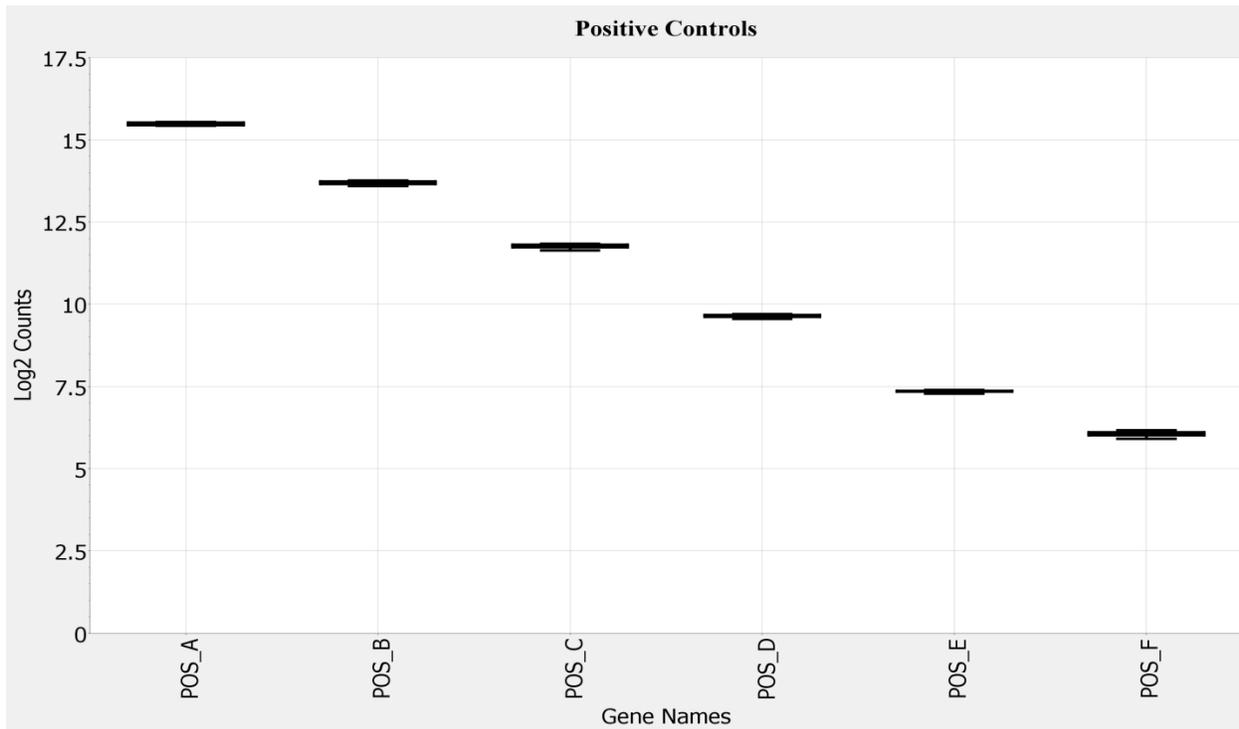
Figure 7. Expression of positive controls at 6 different concentrations in 26 renal biopsy samples.

Among the 8 negative controls, 4 controls were consistently not detectable across all assays and the other 4 controls had very small mRNA counts (mean counts < 12.5 per assay) indicating that there was very minimal background noise (Figure 8). This negative control signal was used to eliminate the background noise in gene expression counts.
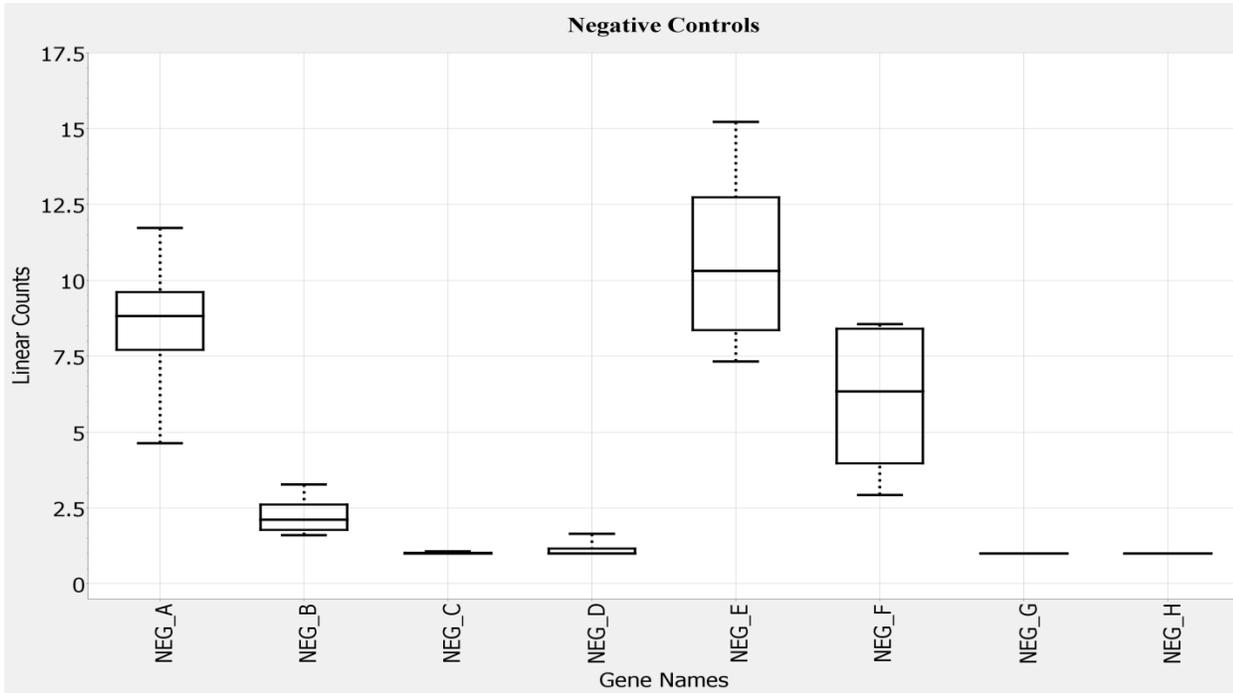
Figure 8. Expression of 8 negative controls in 26 renal biopsy samples.

All four housekeeping genes were expressed in all the samples tested. The relative expression of *B2M* was highest and that of *PGK1* being the lowest. There was more variability in the expression of *B2M* gene among the renal transplant biopsies. Using the average of the geometric means of all four housekeeping gene expression would reduce the variability when normalizing target gene expressions. Figure 9 shows the relative expression of housekeeping genes in the assayed biopsy samples.
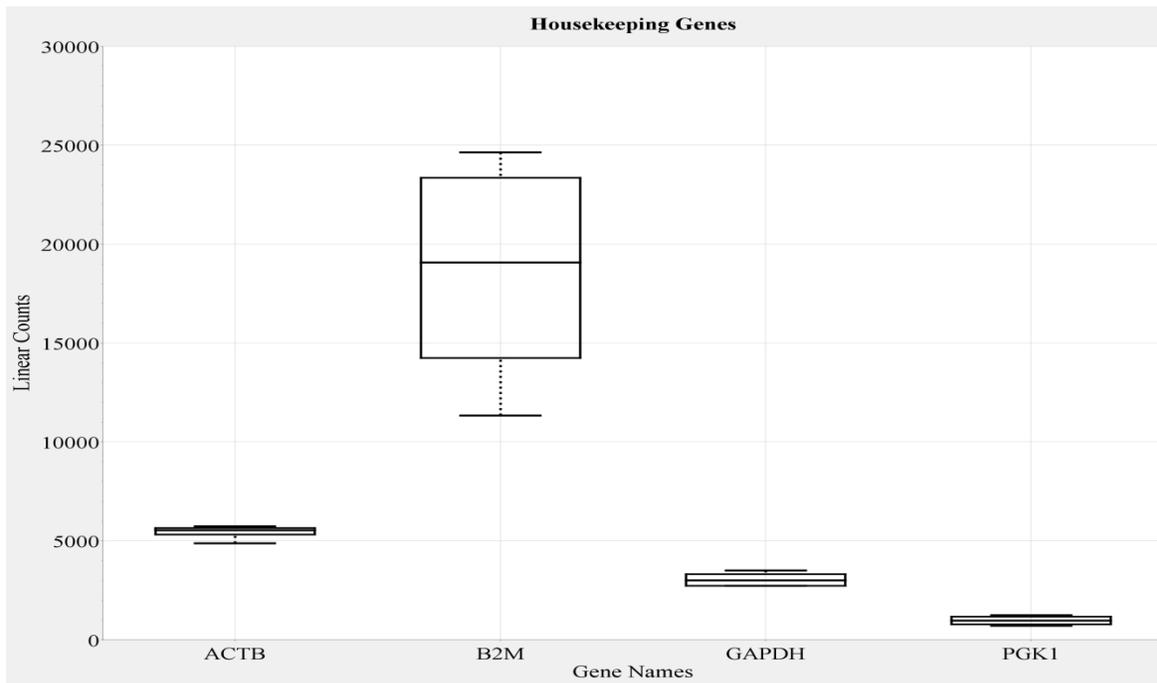
Figure 9. Relative expression of 4 housekeeping genes in 26 renal biopsy.

3.4.2   Relative Expression of Renal Drug Transporters

A total of 12 ABC and 24 SLC transporters were included in the nCounter code-set. The relative gene expression of important ABC transporters in all 26 renal transplant biopsies is presented in Figure 10. Among the genes quantitated, *ABCB1* had the highest median gene expression and *ABCB4* had the lowest median expression. There was a wide variability in expression of *ABCC2*, *ABCB11* and *ABCB1* when all 26 biopsies from renal transplant recipients were pooled together.
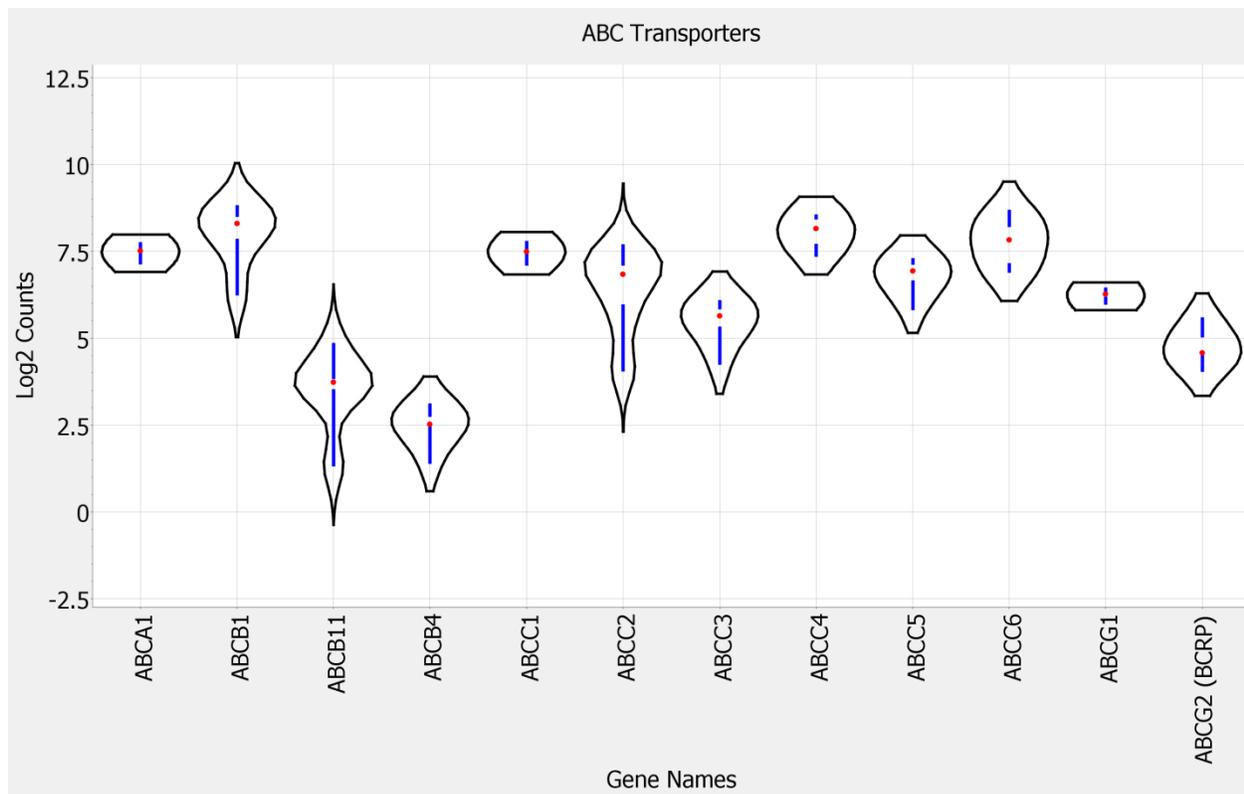
Figure 10. Relative expression of ABC transporters in 26 renal biopsy samples.

The blue line represents lower adjuster value, 1st quartile, 3rd quartile and upper adjusted value when following from bottom to top. Red dot depicts the median and curve around the plot shows distribution.

Of the 24 SLC transporters present in the code-set, 12 are primarily involved in disposition of endogenous compounds and the other 12 are involved in disposition of exogenous compounds (drugs and their metabolites). In the assayed renal biopsies *SLC8A1* had the highest median expression and *SLC10A1* had the lowest median expression among the SLC transporters involved in disposition of endogenous compounds. *SLC22A8* had the highest median gene expression among SLC transporters involved in disposition of exogenous compounds. *SLCO1B1* and *SLCO1B3* had negligible expression. The relative gene expression of important SLC transporters in all 26 renal transplant biopsies is presented in Figure 11 and Figure 12.
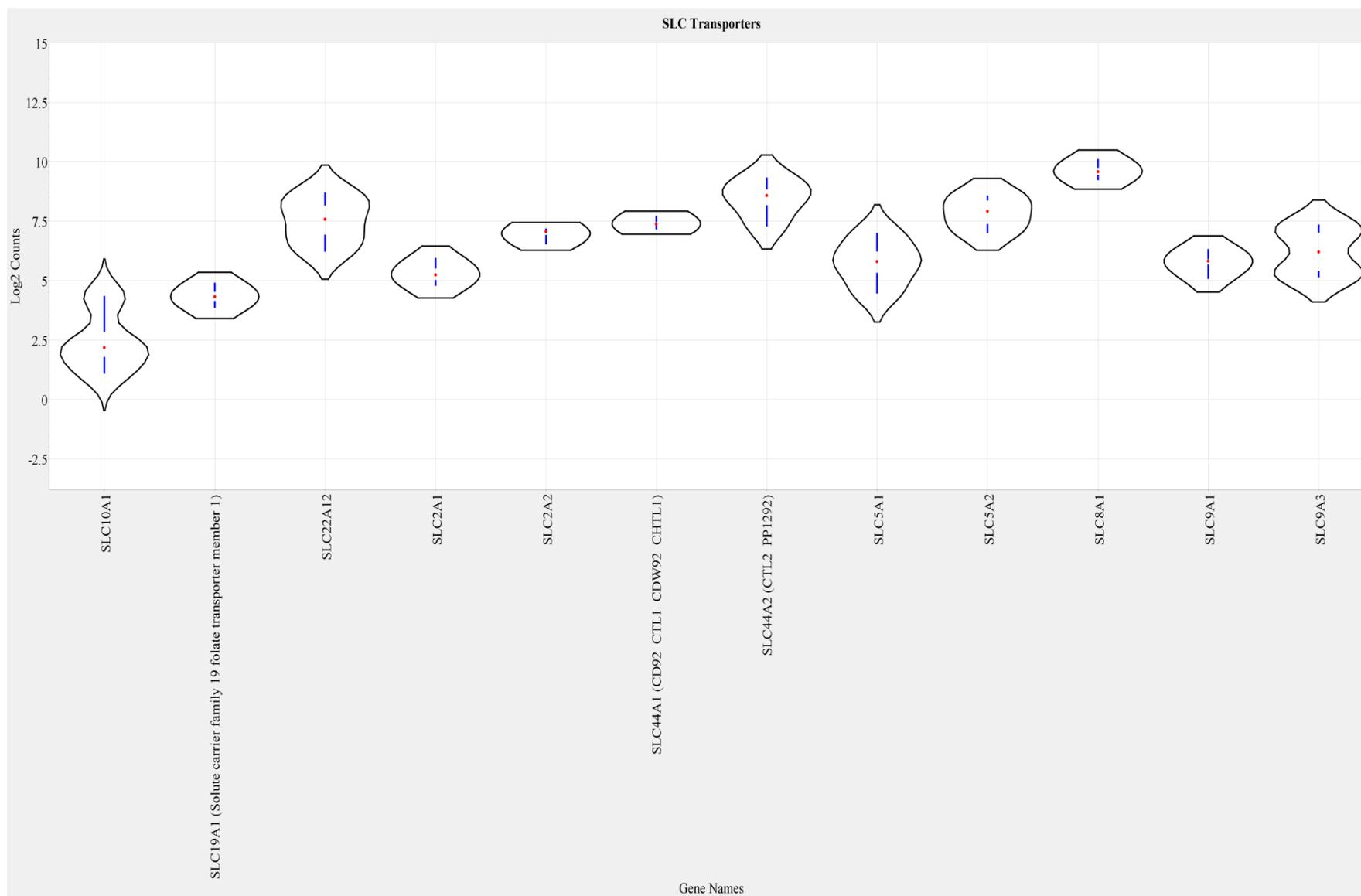
Figure 11. Relative expression of SLC transporters primarily involved in disposition of endogenous compounds.

The blue line represents lower adjuster value, 1st quartile, 3rd quartile and upper adjusted value when following from bottom to top. Red dot depicts the median and curve around the plot shows distribution.

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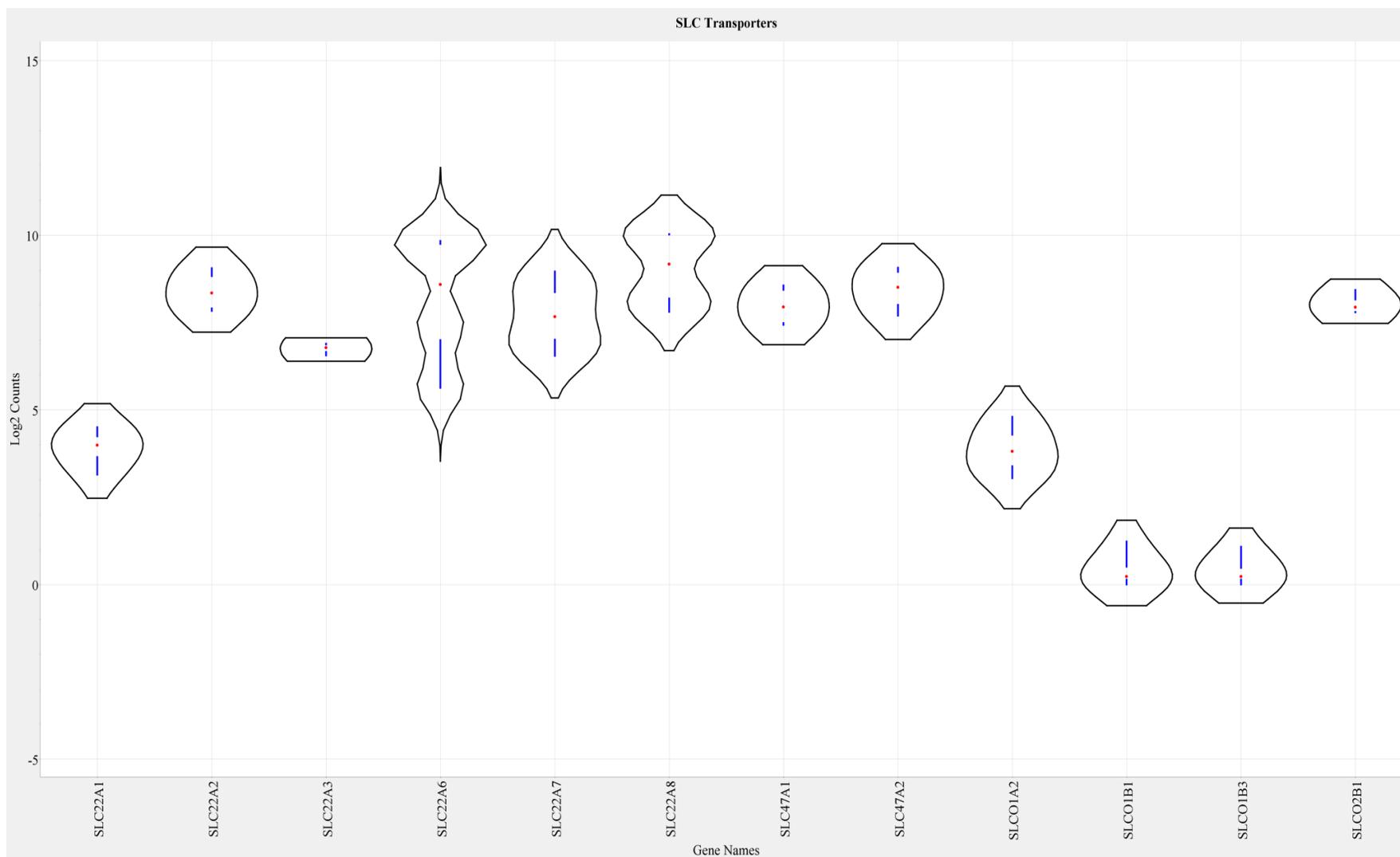
Figure 12. Relative expression of SLC transporters primarily involved in disposition of exogenous compounds.

The blue line represents lower adjuster value, 1st quartile, 3rd quartile and upper adjusted value when following from bottom to top. Red dot depicts the median and curve around the plot shows distribution.

3.4.3 Effect of Prolonged Cold Ischemia and Tacrolimus Treatment

The effect of prolonged cold ischemia and tacrolimus treatment on gene expression of 36 renal drug transporters was evaluated by comparing results of renal biopsies collected from living donor renal transplant, LDRT recipients (n=8) and decease donor renal transplant, DDRT recipients (n=8). *SLC8A1*, *SLC22A6*, *SLC22A8*, *ABCB1*, *ABCC4* and *ABCC6* had the highest relative expression among ABC and SLC transporters in all renal allografts. Most of these transporters are primarily involved in the transport anionic drugs (Chapter 1.0). Among ABC transporters, *ABCB1*, *ABCC3*, *ABCC4*, and *ABCG1* were significantly higher in DDRT recipients (1.2 to 1.87-fold higher; *p*-value<0.05) when compared to LDRT recipients. The relative expression of ABC transporters between LDRT and DDRT recipients is shown in Figure 13. Among SLC transporters, *SLC2A1*, *SLC5A1*, SLC44A1, *SLC44A2*, and *SLC22A7* were significantly higher in DDRT recipients (1.30 to 2.66-fold higher; *p*-value<0.05) when compared to LDRT recipients. The relative expression of SLC transporters between LDRT and DDRT recipients is provided in Figure 14 and Figure 15. A detailed statistical comparison of gene target expressions between DDRT and LDRT groups is provided in Table 9.
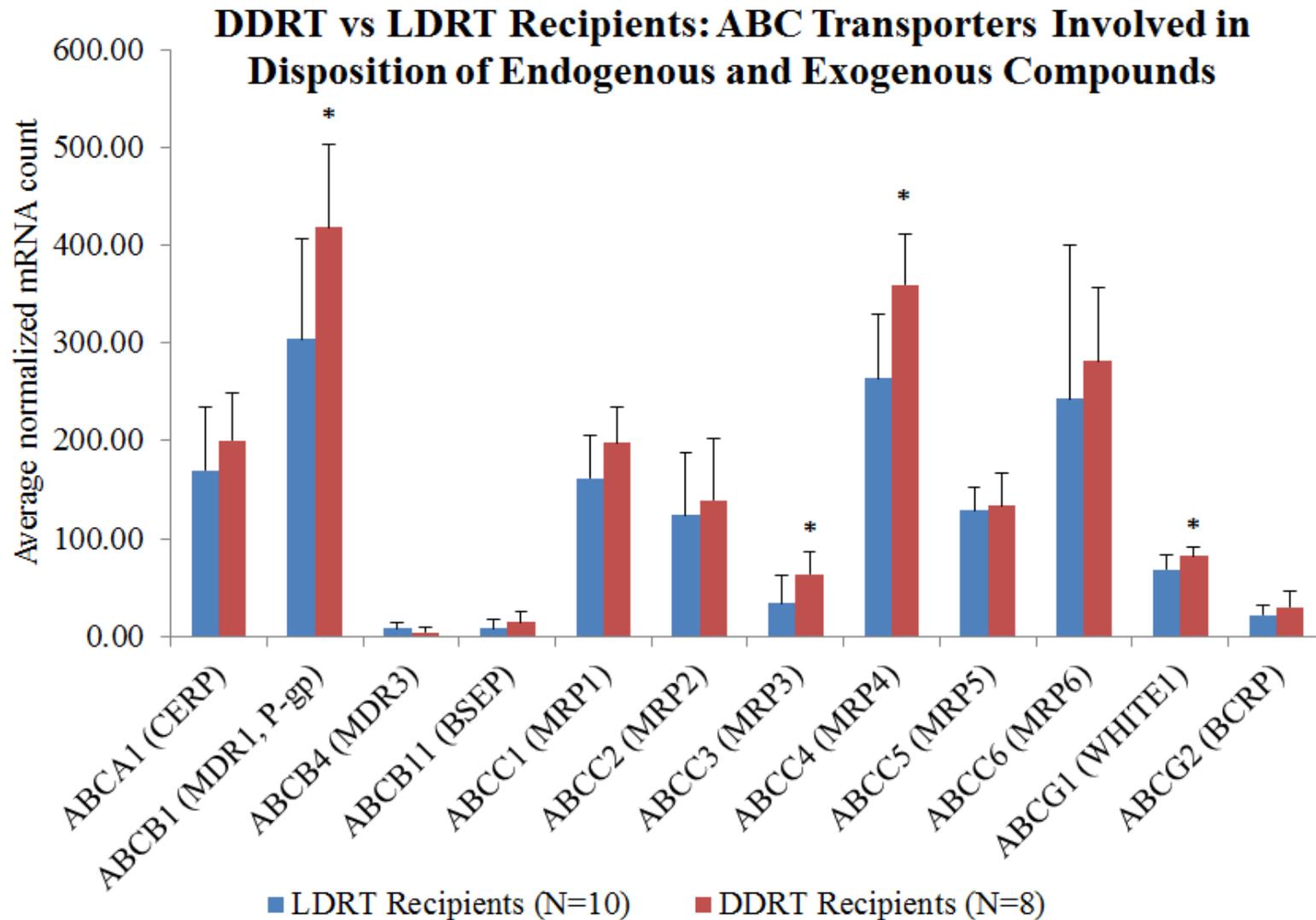
Figure 13. Bar chart comparing gene expression of ABC transporters in renal allograft biopsies in LDRT and DDRT recipients.

*p-value <0.05 when comparing with corresponding LDRT expression; error bars represent standard deviation.
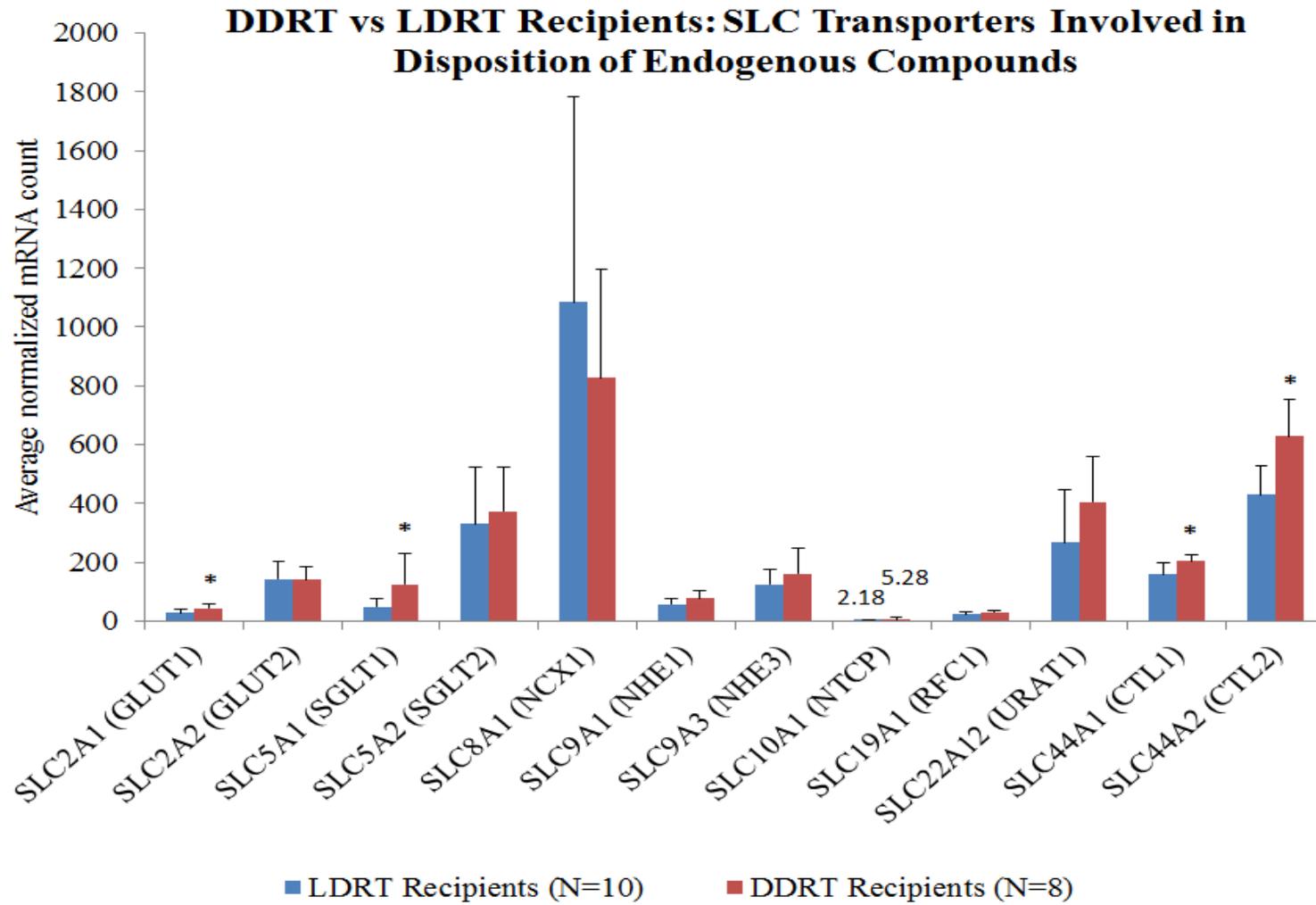
Figure 14. Bar chart comparing gene expression of SLC transporters involved in disposition of endogenous compounds in renal allograft biopsies from LDRT and DDRT recipients.

*p-value <0.05 when comparing with corresponding LDRT expression; error bars represent standard deviation.
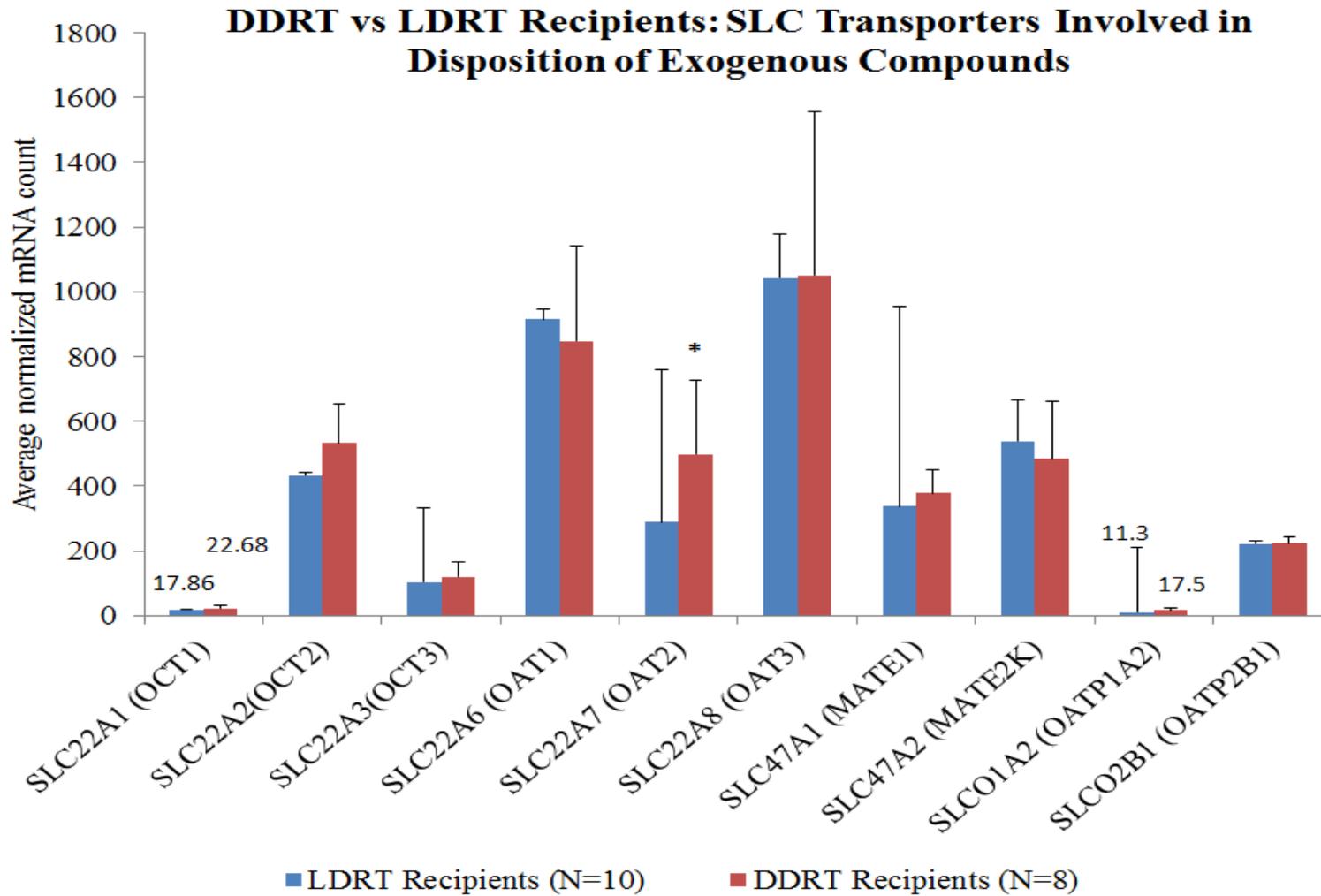
Figure 15. Bar chart comparing gene expression of SLC transporters involved in disposition of exogenous compounds in renal allograft biopsies from LDRT and DDRT recipients.

*p-value <0.05 when comparing with corresponding LDRT expression; error bars represent standard deviation.

3.4.4 Effect of BKVN and Acute TCMR with Interstitial Fibrosis

The effect of BKVN and acute TCMR in the presence of interstitial fibrosis on gene expression of 36 renal drug transporters was evaluated by comparing assay results of renal biopsies collected from allografts with BKVN (n=4), borderline acute TCMR with fibrosis (N=7) and acute TCMR with fibrosis (N=6) groups with renal biopsies collected from healthy allografts (N=6).

Biopsies with significant findings consistent for BKVN had significantly lower expression of *ABCB1*, *ABCC5*, *SLC9A3*, *SLC44A2* and *SLC47A2* targets, when compared to controls (1.65 to 5.25-fold lower; *p*-value<0.05). A comparison of relative expression of the selected transporters between control and BKVN groups is presented in Figure 16, Figure 17, Figure 18 and a detailed statistical comparison of gene target expressions between BKVN and control groups is provided in Table 9.
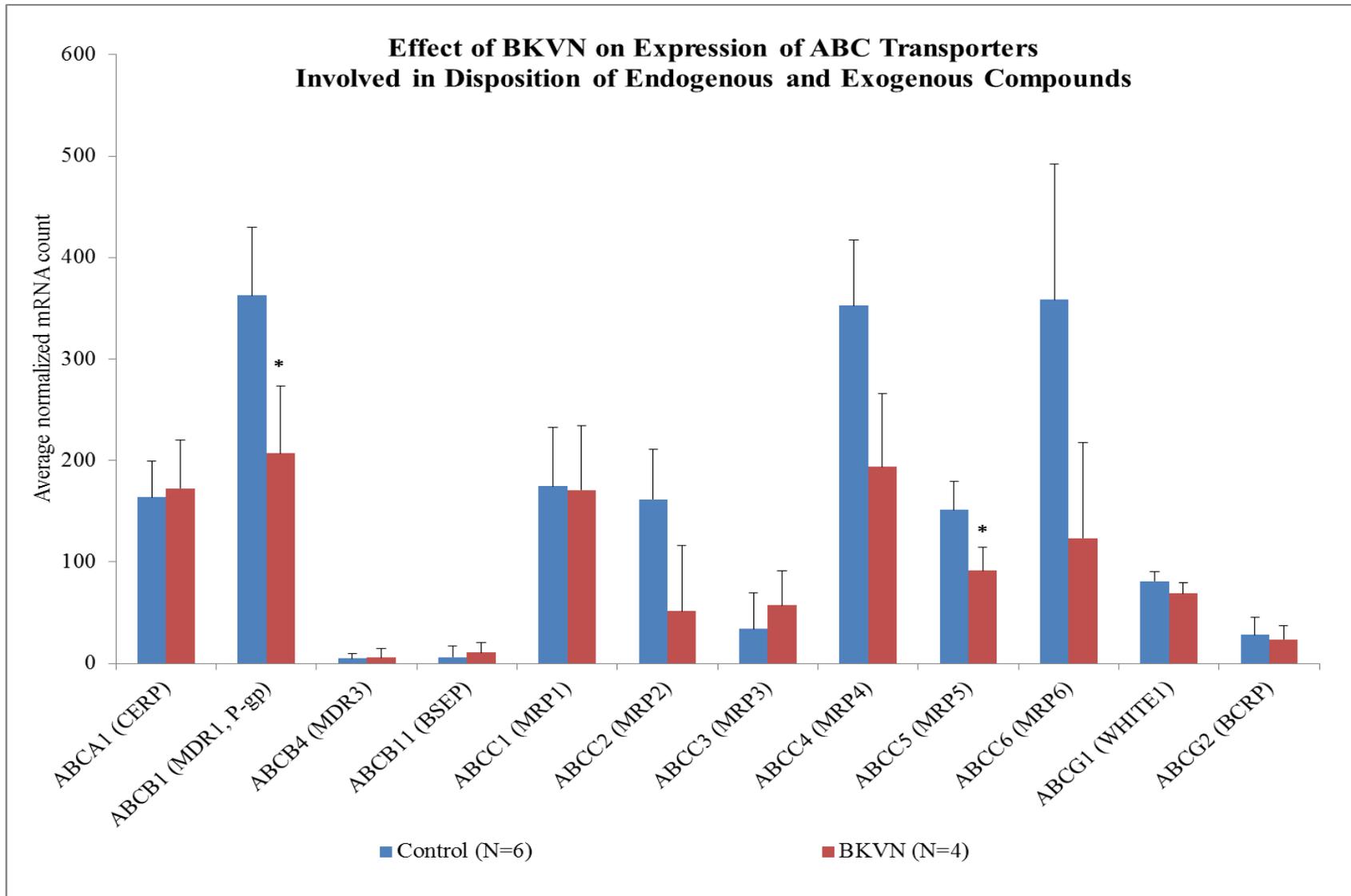
Figure 16. Bar chart comparing quantitative gene expression of ABC transporters from renal allograft biopsies in BKVN and control groups.

*p-value <0.05 when comparing with corresponding control group expression; error bars represent standard deviation.
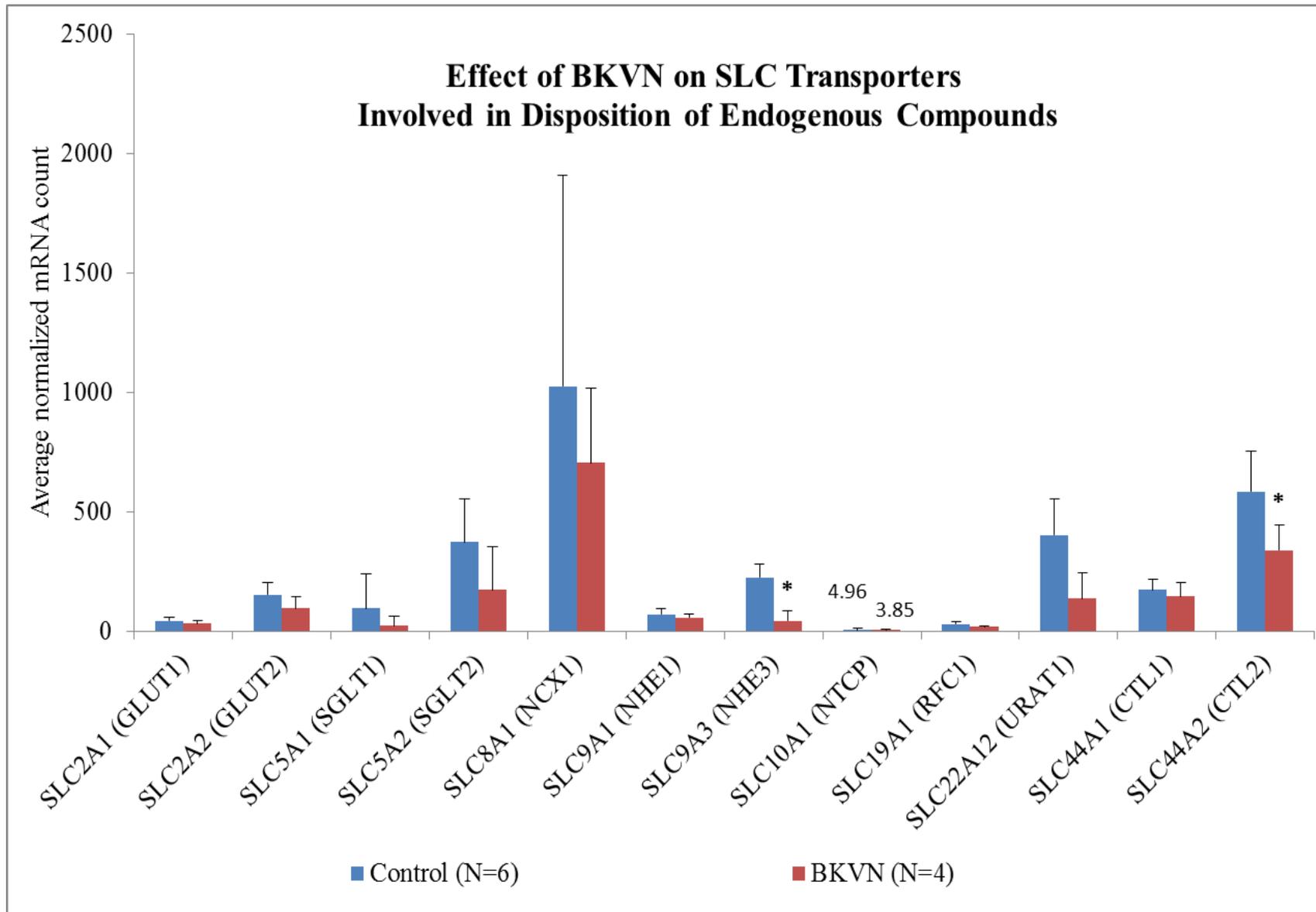
Figure 17. Bar chart comparing quantitative gene expression of SLC transporters involved in disposition of endogenous compounds from renal allograft biopsies in BKVN and control groups.

*p-value <0.05 when comparing with corresponding control group expression; error bars represent standard deviation.
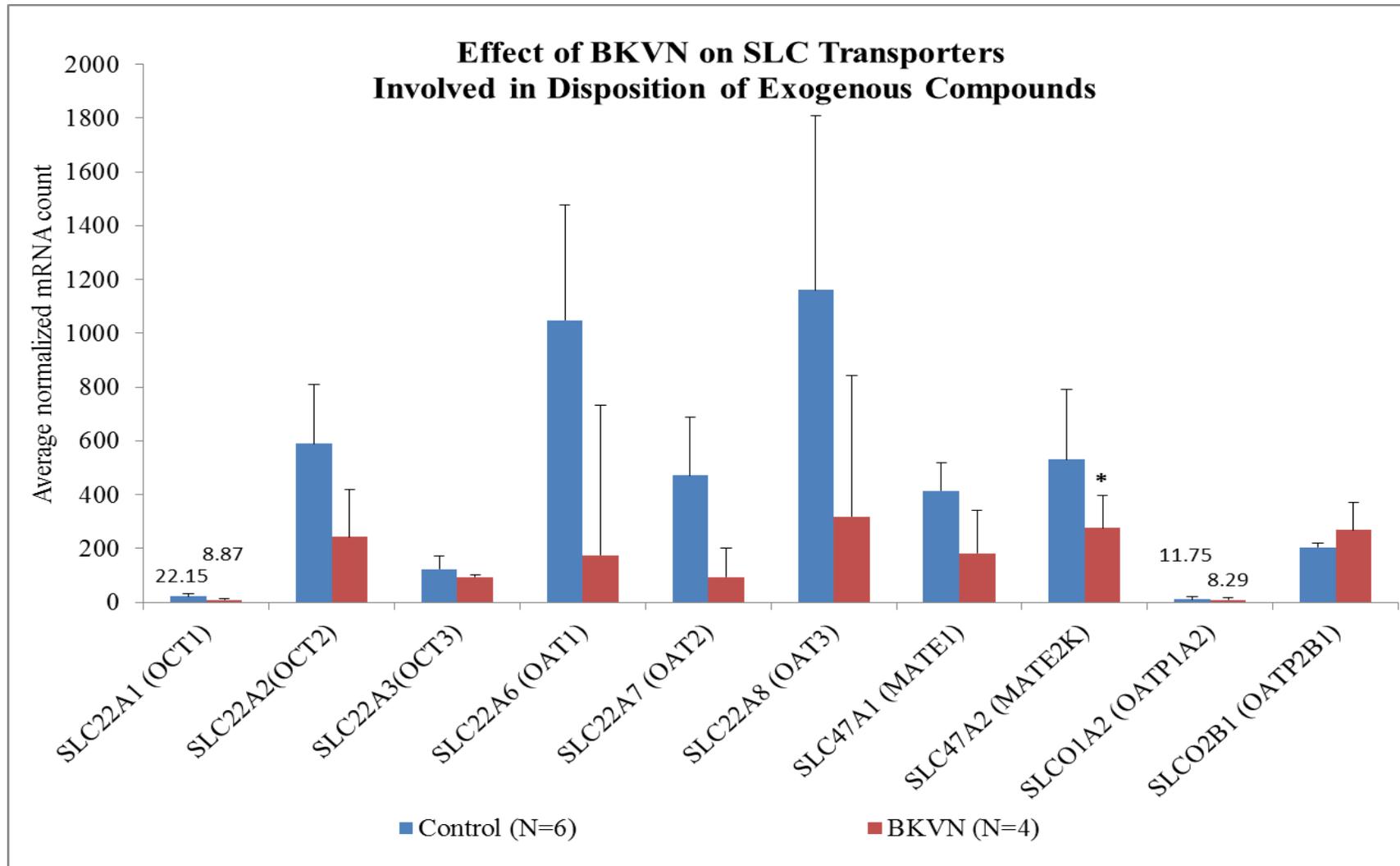
Figure 18. Bar chart comparing quantitative gene expression of SLC transporters involved in disposition of exogenous compounds from renal allograft biopsies in BKVN and control groups.

*p-value <0.05 when comparing with corresponding control group expression; error bars represent standard deviation.

Biopsies with intermediate interstitial inflammation and foci of mild tubulitis with varying degrees of interstitial fibrosis (borderline acute TCMR w/fibrosis) had significantly lower expression of *SLC9A3* (1.93-fold lower; *p*-value: 0.001) targets when compared to controls. Biopsies with findings consistent for Banff IA or Banff IB acute TCMR with varying grades of interstitial fibrosis (acute TCMR w/fibrosis) had significantly lower expression of *ABCC2*, *ABCC4*, *ABCC6*, *SLC5A2*, *SLC9A3*, *SLC19A1*, *SLC22A6*, *SLC22A7*, *SLC22A8*, *SLC22A12*, and *SLC47A1* (1.97 to 11.02-fold lower; *p*-value<0.05) targets when compared to controls. *SLCO2B1* expression was significantly higher for both TCMR groups when compared to controls. Comparison of relative expression of the selected 36 transporters between the comparator TCMR groups is presented in Figure 19, Figure 20, and Figure 21. A detailed statistical comparison of gene target expressions between all study groups is provided in Table 9 and Table 10. Most of the transporters with compromised quantitative expression in acute TCMR group belonged to the organic anionic transport system.
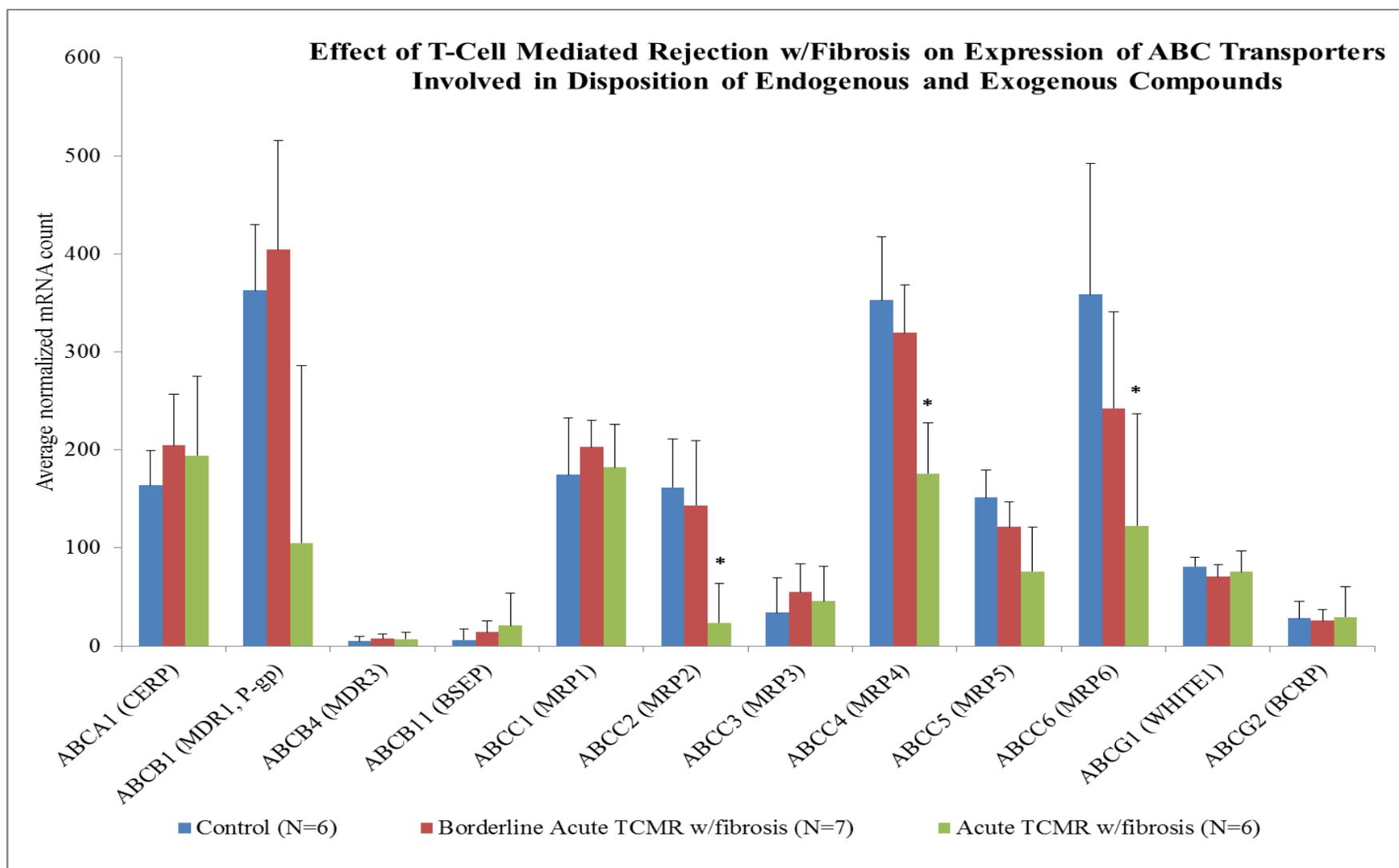
Figure 19. Bar chart comparing quantitative gene expression of ABC transporters from renal allograft biopsies in control, borderline acute TCMR with fibrosis and Acute TCMR with fibrosis groups.

*p-value <0.05 when comparing with corresponding control group expression; error bars represent standard deviation.
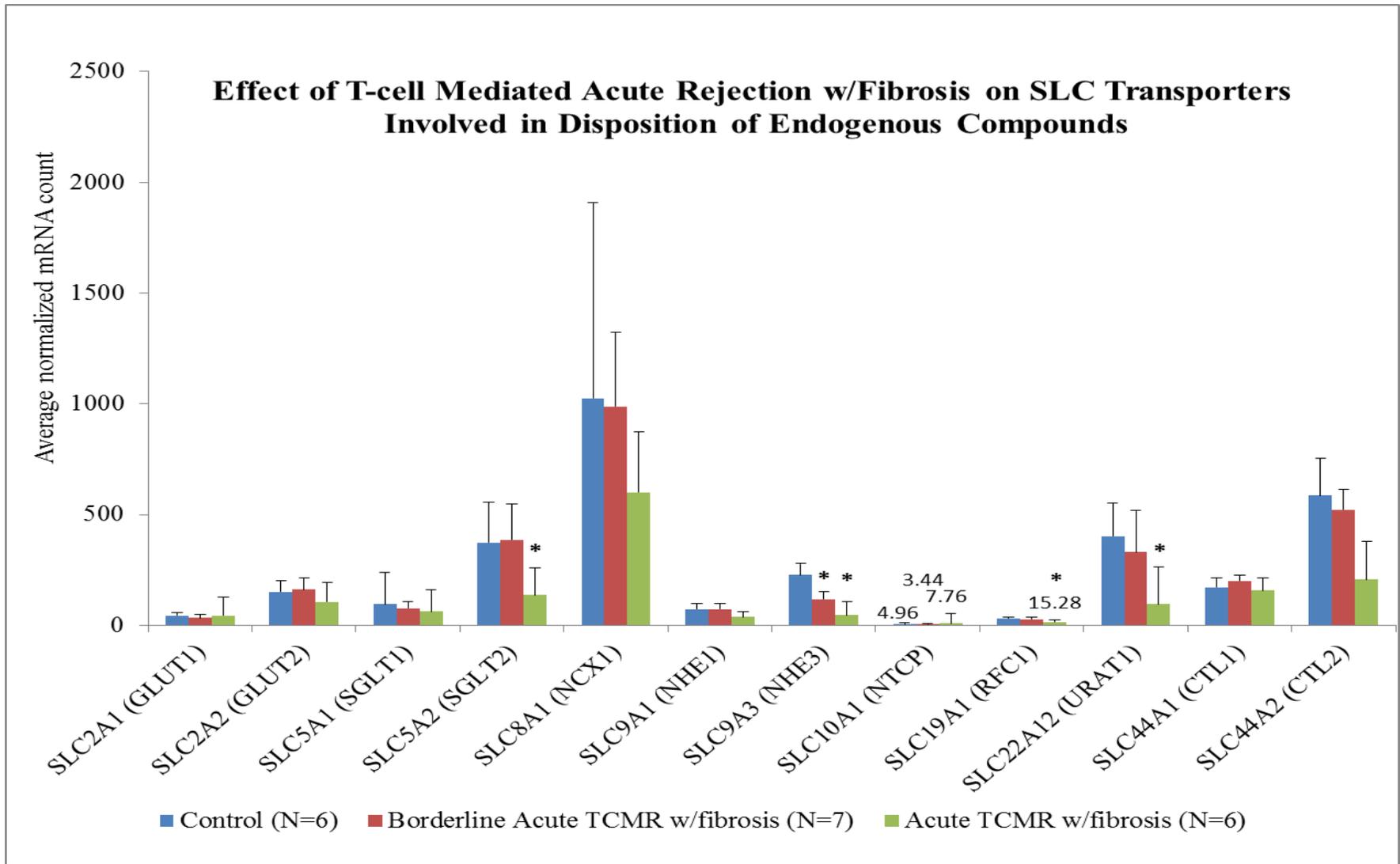
Figure 20. Bar chart comparing quantitative gene expression of SLC transporters involved in the disposition of endogenous compounds from renal allograft biopsies in control, borderline acute TCMR with fibrosis and Acute TCMR with fibrosis groups.

*p-value <0.05 when comparing with corresponding control group expression; error bars represent standard deviation.
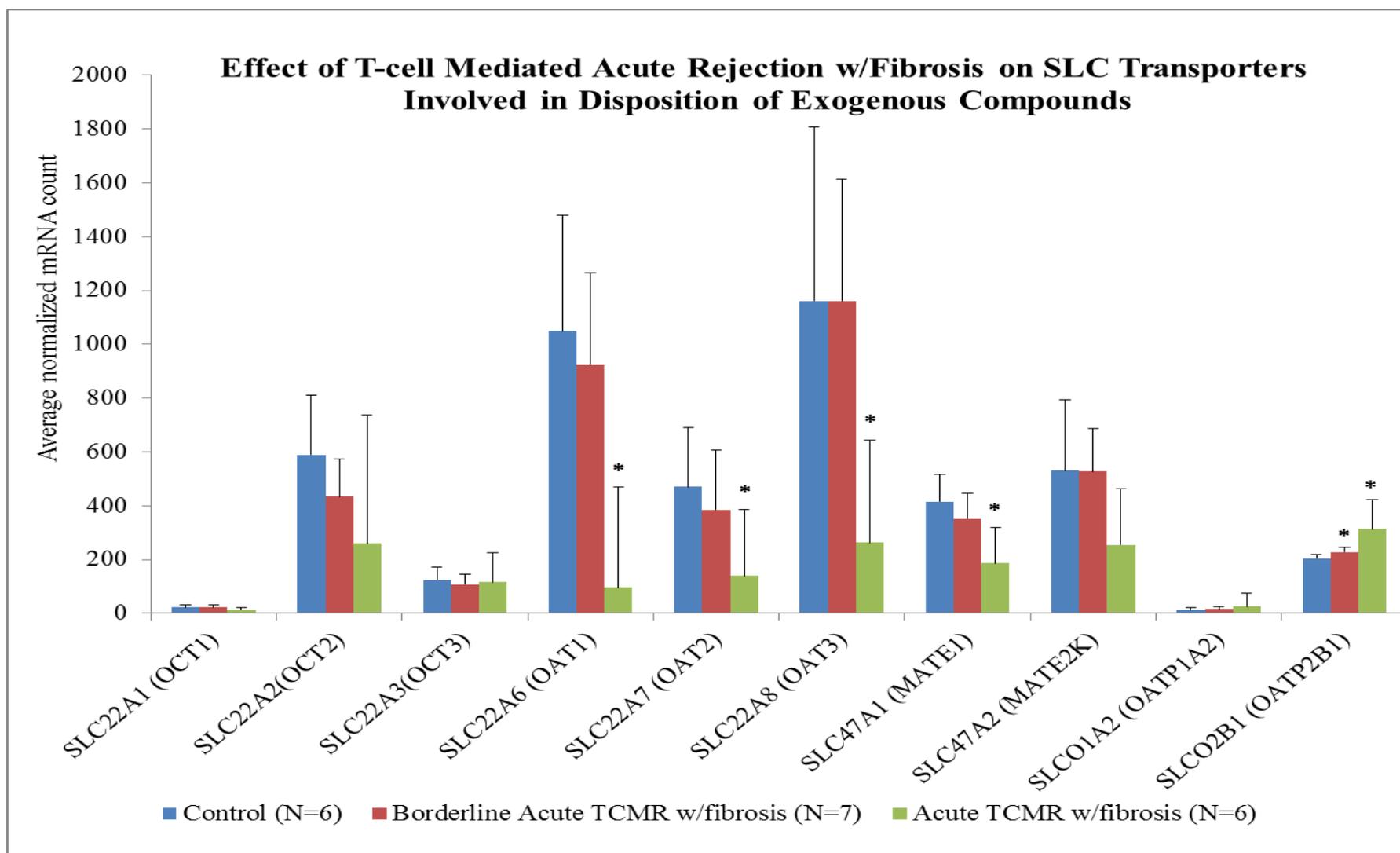
Figure 21. Bar chart comparing quantitative gene expression of SLC transporters involved in the disposition of exogenous compounds from renal allograft biopsies in control, borderline acute TCMR with fibrosis and Acute TCMR with fibrosis groups.

*p-value <0.05 when comparing with corresponding control group expression; error bars represent standard deviation.

Table 9. Statistical comparison of ABC and SLC gene target expressions between DDRT vs LDRT and BKVN vs Control groups

Gene Name (Protein)	DDRT vs LDRT		BKVN vs Control	
	Fold Change (95% CI)	p-value	Fold Change (95% CI)	p-value
ABCA1 (CERP)	1.18 (-1.11 to 1.55)	0.216	1.06 (-1.42 to 1.58)	0.745
ABCB1 (MDR1, P-gp)	**1.37 (1.05 to 1.80)**	**0.023**	**-1.75 (-3.00 to -1.02)**	**0.044**
ABCB4 (MDR3)	-1.81 (-4.56 to 1.39)	0.187	1.19 (-5.84 to 8.32)	0.823
ABCB11 (BSEP)	1.89 (-1.19 to 4.24)	0.114	1.80 (-3.09 to 9.98)	0.444
ABCC1 (MRP1)	1.22 (-1.02 to 1.53)	0.074	-1.02 (-1.85 to 1.78)	0.933
ABCC2 (MRP2)	1.12 (-1.48 to 1.88)	0.633	-3.14 (-12.77 to 1.30)	0.066
ABCC3 (MRP3)	**1.87 (1.11 to 3.14)**	**0.021**	1.69 (-1.63 to 4.64)	0.257
ABCC4 (MRP4)	**1.36 (1.11 to 1.67)**	**0.006**	-1.82 (-3.56 to 1.07)	0.051
ABCC5 (MRP5)	1.04 (-1.21 to 1.30)	0.737	**-1.65 (-2.44 to -1.12)**	**0.019**
ABCC6 (MRP6)	1.16 (-1.35 to 1.81)	0.492	-2.91 (-10.00 to 1.18)	0.057
ABCG1 (WHITE1)	**1.20 (1.02 to 1.42)**	**0.029**	-1.18 (-1.49 to 1.07)	0.119
ABCG2 (BCRP)	1.39 (-1.18 to 2.30)	0.175	-1.19 (-2.92 to 2.07)	0.662
SLC2A1 (GLUT1)	**1.53 (1.06 to 2.22)**	**0.026**	-1.29 (-2.21 to 1.33)	0.297
SLC2A2 (GLUT2)	-1.00 (-1.47 to 1.46)	0.979	-1.60 (-3.29 to 1.28)	0.149
SLC5A1 (SGLT1)	**2.66 (1.47 to 4.80)**	**0.003**	-4.26 (-66.79 to 3.69)	0.213
SLC5A2 (SGLT2)	1.13 (-1.45 to 1.84)	0.611	-2.14 (-7.89 to 1.72)	0.171
SLC8A1 (NCX1)	-1.31 (-2.10 to 1.22)	0.237	-1.45 (-3.17 to 1.50)	0.289
SLC9A1 (NHE1)	1.39 (-1.05 to 2.02)	0.084	-1.26 (-2.06 to 1.29)	0.289
SLC9A3 (NHE3)	1.27 (-1.31 to 2.12)	0.324	**-5.25 (-21.96 to -1.26)**	**0.030**
SLC10A1 (NTCP)	2.42 (-1.05 to 6.11)	0.059	-1.29 (-6.32 to 3.81)	0.709
SLC19A1 (RFC1)	1.31 (-1.04 to 1.79)	0.078	-1.67 (-2.31 to -1.21)	0.006
SLC22A1 (OCT1)	1.27 (-1.16 to 1.88)	0.211	-2.50 (-6.49 to 1.04)	0.051
SLC22A2(OCT2)	1.23 (-1.15 to 1.73)	0.223	-2.42 (-6.13 to 1.05)	0.055
SLC22A3(OCT3)	1.16 (-1.17 to 1.57)	0.319	-1.31 (-1.89 to 1.11)	0.116
SLC22A6 (OAT1)	-1.08 (-1.77 to 1.52)	0.749	-5.98 (-88.63 to 2.47)	0.119
SLC22A7 (OAT2)	**1.73 (1.03 to 2.89)**	**0.039**	-5.02 (-30.27 to 1.20)	0.054
SLC22A8 (OAT3)	1.01 (-1.79 to 1.82)	0.978	-3.65 (-18.53 to 1.39)	0.084
SLC22A12 (URAT1)	1.52 (-1.12 to 2.59)	0.111	-2.94 (-10.01 to 1.16)	0.049
SLC44A1 (CTL1)	**1.30 (1.05 to 1.61)**	**0.021**	-1.18 (-2.10 to 1.51)	0.496
SLC44A2 (CTL2)	**1.47 (1.19 to 1.81)**	**0.001**	**-1.73 (-2.82 to -1.06)**	**0.033**
SLC47A1 (MATE1)	1.13 (-1.18 to 1.50)	0.386	-2.29 (-7.41 to 1.41)	0.098
SLC47A2 (MATE2K)	-1.12 (-1.63 to 1.31)	0.549	**-1.92 (-3.68 to -1.01)**	**0.046**
SLCO1A2 (OATP1A2)	1.55 (-1.21 to 2.91)	0.156	-1.42 (-8.66 to 4.31)	0.638
SLCO1B1 (OATP1B1)	-1.07 (-1.31 to 1.15)	0.511	-1.20 (-1.59 to 1.10)	0.156
SLCO1B3 (OATP1B3)	-1.07 (-1.31 to 1.15)	0.511	-1.20 (-1.59 to 1.10)	0.156
SLCO2B1 (OATP2B1)	1.00 (-1.14 to 1.15)	0.950	1.32 (-1.43 to 2.47)	0.258

CI: confidence interval; DDRT: deceased donor renal transplant recipient; LDRT: living donor renal
Transplant recipient; BKVN: BK virus nephropathy
Statistically significant changes (*p*-value <0.05) are bolded

Table 10. Statistical comparison of ABC and SLC gene target expressions between T-cell mediated acute rejection groups with varying grades of fibrosis and control group

Gene Name (Protein)	Borderline Acute TCMR w/fibrosis vs. Control		Acute TCMR w/fibrosis vs. Control	
	Fold Change (95% CI)	p-value	Fold Change (95% CI)	p-value
ABCA1 (CERP)	1.25 (-1.05 to 1.64)	0.098	1.19 (-1.26 to 1.78)	0.347
ABCB1 (MDR1, P-gp)	1.11 (-1.2 to 1.49)	0.425	-3.45 (-14.59 to 1.23)	0.077
ABCB4 (MDR3)	1.58 (-2.01 to 5.00)	0.388	1.47 (-2.16 to 4.66)	0.463
ABCB11 (BSEP)	2.34 (-1.63 to 8.90)	0.171	3.44 (-1.16 to 13.81)	0.071
ABCC1 (MRP1)	1.16 (-1.22 to 1.65)	0.332	1.04 (-1.40 to 1.53)	0.804
ABCC2 (MRP2)	-1.13 (-1.84 to 1.44)	0.595	**-6.78 (-22.87 to -2.01)**	**0.007**
ABCC3 (MRP3)	1.61 (-1.47 to 3.80)	0.236	1.34 (-2.07 to 3.74)	0.529
ABCC4 (MRP4)	-1.10 (-1.35 to 1.11)	0.294	**-2.01 (-2.78 to -1.45)**	**0.001**
ABCC5 (MRP5)	-1.24 (-1.60 to 1.03)	0.079	-1.99 (-4.09 to 1.04)	0.051
ABCC6 (MRP6)	-1.48 (-2.27 to 1.04)	0.067	**-2.92 (-6.69 to -1.28)**	**0.018**
ABCG1 (WHITE1)	-1.15 (-1.38 to 1.05)	0.120	-1.07 (-1.42 to 1.24)	0.576
ABCG2 (BCRP)	-1.10 (-2.30 to 1.89)	0.764	1.05 (-2.32 to 2.54)	0.909
SLC2A1 (GLUT1)	-1.28 (-2.20 to 1.34)	0.328	1.02 (-2.57 to 2.68)	0.960
SLC2A2 (GLUT2)	1.07 (-1.45 to 1.65)	0.753	-1.45 (-3.05 to 1.45)	0.273
SLC5A1 (SGLT1)	-1.26 (-3.76 to 2.37)	0.624	-1.54 (-5.22 to 2.21)	0.445
SLC5A2 (SGLT2)	1.03 (-1.73 to 1.84)	0.909	**-2.75 (-6.58 to -1.15)**	**0.026**
SLC8A1 (NCX1)	-1.04 (-2.08 to 1.93)	0.905	-1.71 (-3.56 to 1.22)	0.128
SLC9A1 (NHE1)	1.02 (-1.60 to 1.66)	0.932	-1.95 (-4.53 to 1.19)	0.099
SLC9A3 (NHE3)	**-1.93 (-2.69 to -1.38)**	**0.001**	**-4.94 (-13.23 to -1.85)**	**0.007**
SLC10A1 (NTCP)	-1.44 (-4.82 to 2.32)	0.514	1.56 (-4.27 to 10.44)	0.602
SLC19A1 (RFC1)	-1.12 (-1.61 to 1.28)	0.492	**-1.97 (-3.38 to -1.14)**	**0.019**
SLC22A1 (OCT1)	-1.02 (-1.64 to 1.59)	0.943	-1.66 (-3.17 to 1.15)	0.108
SLC22A2(OCT2)	-1.36 (-2.01 to 1.09)	0.112	-2.27 (-7.34 to 1.43)	0.124
SLC22A3(OCT3)	-1.15 (-1.74 to 1.31)	0.458	-1.07 (-2.19 to 1.92)	0.830
SLC22A6 (OAT1)	-1.14 (-1.90 to 1.47)	0.587	**-11.02 (-124.62 to 1.03)**	**0.049**
SLC22A7 (OAT2)	-1.22 (-2.23 to 1.49)	0.471	**-3.37 (-9.19 to -1.24)**	**0.022**
SLC22A8 (OAT3)	1.00 (-2.01 to 2.01)	1.000	**-4.41 (-13.88 to -1.40)**	**0.017**
SLC22A12 (URAT1)	-1.21 (-1.97 to 1.34)	0.392	**-4.25 (-15.9 to -1.13)**	**0.032**
SLC44A1 (CTL1)	1.16 (-1.18 to 1.60)	0.277	-1.10 (-1.67 to 1.38)	0.617
SLC44A2 (CTL2)	-1.12 (-1.53 to 1.22)	0.418	-2.84 (-9.55 to 1.18)	0.071
SLC47A1 (MATE1)	-1.18 (-1.62 to 1.16)	0.269	**-2.23 (-4.55 to -1.09)**	**0.031**
SLC47A2 (MATE2K)	-1.01 (-1.71 to 1.69)	0.981	-2.08 (-4.65 to 1.08)	0.066
SLCO1A2 (OATP1A2)	1.33 (-2.2 to 3.90)	0.537	2.13 (-1.73 to 7.82)	0.219
SLCO1B1 (OATP1B1)	-1.00 (-1.36 to 1.35)	0.981	1.55 (-1.51 to 3.64)	0.255
SLCO1B3 (OATP1B3)	-1.00 (-1.36 to 1.35)	0.981	1.45 (-1.63 to 3.41)	0.333
SLCO2B1 (OATP2B1)	**1.11 (1.02 to 1.22)**	**0.022**	**1.53 (1.08 to 2.16)**	**0.022**

CI: confidence interval; TCMR: T-cell mediated rejection
Statistically significant changes (p-value <0.05) are bolded

3.5   DISCUSSION

The kidney is an important excretory organ, which is involved in the elimination of various endogenous and exogenous molecules. Changes in transporter mediated secretion capacity of the renal allograft would significantly alter the clearance and exposure of renally secreted drugs. The slow recovery and progressive graft function loss following renal transplantation due to injuries and complications such as cold ischemic injury, drug induced nephrotoxicity, BKVN, interstitial fibrosis and varying grades of TCMR are thought to affect the secretory capacity of a transplanted kidney. Although creatinine clearance is used to estimate the GFR, there is currently very limited understanding of the renal secretion capacity in renal transplant recipients. The effect of prolonged CI, renal transplantation, and tacrolimus treatment on the gene expression of five important renal drug transporters (Oat1, Oat3, Oct2, Mate1, and Mdr1a) was studied in a rat kidney transplant model (Chapter 2.0). Significantly lower expression of the selected transporters was observed in rat kidneys, which were subjected to 24 hrs CI before transplantation and where recipient rats were treated with tacrolimus post-transplantation. Observations of this study prompted us to systematically evaluate the effect of prolonged CI with tacrolimus treatment, BKVN and TCMR with fibrosis on the gene expression of renal drug transporters in transplant patients.

Gene expression of 36 important drug transporters in renal transplant recipients were quantitated using a novel NanoString nCounter® gene expression assay. nCounter® gene expression assay offers high sensitivity and reproducibility in a single-tube without the necessity for a cDNA creation or mRNA replication steps like in the case of qPCR-based quantitation. This method also gives us the ability to measure absolute expression as compared to relative

expression with traditional methods. The nCounter® assay has been successfully used to quantitate expression of gene from FFPE samples [123].

FFPE biopsies from 26 renal allografts were used to extract the mRNA for quantitation of drug transporter gene expression. This work is the first comprehensive attempt to understand changes in expression of important renal drug transporters in renal transplant patients with normal allografts and those with transplant associated complications. The relative expressions of important ABC and SLC transporters in all renal allografts were similar to the relative expressions observed in non-transplanted kidneys reported by Nishimuta et al with the exception of *SLC22A12* (URAT1) which was shown to have the highest relative expression in non-transplanted healthy renal tissues [7]. OAT1, OAT3, OCT2, MATE1/2K and MDR1 had highest relative gene expressions in all sampled renal allografts and URAT1 gene expression was less than half the expression of OAT1/3. These comparisons suggest that renal transplant patients may have compromised uric acid reabsorption capacity compared to healthy subjects. Hyperuricemia has been linked to renal disease and the progression of CKD by various animal and human experimental studies [124]. The findings of this study is supported by various observations reporting compromised uric acid regulation in renal transplant recipients [125]. There was also a wide variability in the expression of OAT1, OAT3, BSEP and MRP2 gene expression when samples from renal allografts with and without transplantation associated complications were pooled together. This comparison suggests that organic anionic transport system (OAT/MRP) is sensitive to allograft insults associated with renal transplantation.

FFPE renal biopsies from 16 LDRT and DDRT recipients who were on tacrolimus treatment were used to study the effect of CI and tacrolimus treatment on gene expression of the selected renal transporters. DDRT patients had allografts with 15.8±4.8 hrs CI whereas LDRT

recipients had allografts with 1.3±0.4 hrs CI and all patients were on tacrolimus-based immunosuppression therapy as per UPMC protocols. DDRT recipients have a significantly higher expression of several ABC and SLC transporters when compared to LDRT recipients suggesting differential regulation of specific renal drug transporters in the presence of prolonged cold ischemia and tacrolimus treatment. However, the magnitude of change in expression of transporters may not be clinically significant except for *SLC5A1* (SGLT1) which was significantly increased by 2.66-fold. SGLT1 is involved in the active tubular reabsorption of glucose in the kidneys [126]. An increase in SGLT1 expression in DDRT allografts suggests a protective mechanism to conserve glucose.

Renal allografts with pathological findings consistent for BKVN had significantly lower expression of several ABC and SLC transporters when compared to healthy allografts suggesting a loss of function. *SLC9A3*, which codes for sodium-hydrogen exchanger 3 (NHE3) had 5.25-fold lower expression in BKVN group, suggesting renal transplant recipients with BKVN may have a compromised sodium homeostasis. Preliminary clinical observations in renal transplant recipients with BKVN involving cidofovir treatment in the presence and absence of probenecid suggested that renal anionic secretory capacity (OAT1/3 and MRP2/4) is compromised in allografts with BKVN [100]. In the current study, allografts with BKVN had lower mean and median gene expression of OAT1/3 and MRP2/4 transporters when compared to control allografts but this decline was not statistically significant (Table 11). A structured study with more biopsies in each group is warranted to better understand the effect of BKVN on organic anionic transport system.

Table 11. Comparison of OAT1/3 and MRP2/4 gene target expressions between BKVN and Control groups

Gene Name (Protein)	BKVN vs Control	
	Fold Change (95% CI)	p-value
SLC22A6 (OAT1)	-5.98 (-88.63 to 2.47)	0.119
SLC22A8 (OAT3)	-3.65 (-18.53 to 1.39)	0.084
ABCC2 (MRP2)	-3.14 (-12.77 to 1.30)	0.066
ABCC4 (MRP4)	-1.82 (-3.56 to 1.07)	0.051

CI: confidence interval

Several transporter targets involved in the renal anionic transport system were significantly lower in the acute TCMR with fibrosis group with the expression of *SLC22A6* (OAT1) being 11.0-fold lower and *SLC22A8* (OAT3) being 4.41-fold lower when compared to controls. The expression of OAT1/3 counterparts on the apical side, *ABCC2* (MRP2) and *ABCC4* (MRP4) were also significantly lower by 6.78-fold and 2.01-fold respectively. These results suggest that renal transplant recipients with Banff IA or higher acute TCMR and interstitial fibrosis may have severely compromised renal anionic secretory capacity. Gene expression of NHE3, SGLT2 and URAT1 were significantly lower in acute TCMR with interstitial fibrosis group, suggesting that renal transplant recipients with Banff IA or higher acute TCMR may have compromised sodium, glucose, and uric acid homeostasis.

The custom code-set used for this assay was designed to facilitate transporter expression studies for various tissue types; some of the transporter targets on the code-set were not functionally relevant for renal tissue due to low relative expression levels (example: *SLCO1B1* and *SLCO1B3*). Changes in mRNA expression do not always translate into changes in protein expression and transporter activity and this is a limitation to directly relate these changes to transporter activity changes.

The results of this study show differential expression of various important renal drug transporters in renal allografts. A systematic evaluation of changes in regulation of endogenous substances such as sodium, glucose and uric acid in renal transplant patients is warranted. Involvement of changes in expression of proinflammatory cytokines and their regulatory mediators in altered expression and activity of renal transporters should also be evaluated. A prospective study to assess the renal anionic secretory capacity in renal transplant recipients was planned and conducted following the observations of this study (Chapter 5.0).

4.0 RAPID AND SENSITIVE ULTRA-PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRIC ASSAY FOR DETERMINATION OF CEFOXITIN IN HUMAN PLASMA FOLLOWING LOW DOSE DRUG ADMINISTRATION

4.1   ABSTRACT

A rapid, sensitive, and selective method for the determination of cefoxitin in human plasma using ultra performance liquid chromatography with tandem mass spectrometry (UPLC–MS/MS) was developed and validated. Plasma samples were processed by a solid-phase extraction (SPE) using Oasis® HLB 1cc extraction cartridges prior to chromatography. Cefuroxime was used as the internal standard (IS). Chromatographic separation was performed using Acquity UPLC HSS T3 1.8 μm column (2.1x100 mm), which was combined with a 1.8 μm Vanguard Pre-column (2.1x 5 mm) using a gradient elution with a mobile phase consisting of [A] 5% Acetonitrile in water containing ammonium acetate (2 mM) and formic acid (0.1%), and [B] Acetonitrile containing ammonium acetate (2 mM) and formic acid (0.1%) at a flow rate of 0.3 mL/min. The total run-time was 6 min, with cefoxitin and cefuroxime eluting at 1.56 min and 1.40 min, respectively. The analytes were detected by a XEVO TQS mass spectrometer in negative electron spray ionization (ESI) mode using multiple reaction monitoring (MRM). The extracted ions monitored following MRM transitions were m/z 426.16 → 156.05 for cefoxitin and m/z 423.10 → 207.13 for cefuroxime (IS). The assay was linear over the range of 25–50,000 ng/mL. Inter-day accuracy (% bias: -0.7 to 10.7%), intra-day accuracy (% bias: -3.2 to 2.2%), inter-day precision (% CV: 2.3 to 3.4%), and intra-day precision (% CV: 3.4 to 5.7%). This assay has a short run time (6 min), uses limited sample volume (20 μL), and gives us the ability to perform quantitative assessment of plasma cefoxitin in the range of 25-50,000 ng/mL, enabling us to perform low dosing and limited volume sampling studies for estimation of renal secretory changes in renal transplant patients for academic research purposes.

4.2   INTRODUCTION

Cefoxitin is a second-generation cephalosporin antibiotic. It is a semisynthetic anionic compound derived by chemical modification of cephamycin C, a naturally occurring substance produced by *Streptomyces lactamdurans* [127, 128]. This modification enables cefoxitin to have a high degree of resistance towards inactivation by cephalosporinases and penicillinases of Gram-negative and Gram-positive bacteria. It has been shown to be active against various Gram-positive, Gram-negative and Anaerobic bacteria (Table 12) [129-131].

Table 12. Cefoxitin Antibacterial Activity

Gram-positive bacteria	Gram-negative bacteria	Anaerobic bacteria
*Staphylococcus aureus**	*Escherichia coli*	*Clostridium spp.*
*Staphylococcus epidermidis**	*Haemophilus influenzae*	*Peptococcus niger*
Streptococcus agalactiae	*Klebsiella spp.*	*Peptostreptococcus spp.*
Streptococcus pneumoniae	*Morganella morganii*	*Bacteroides spp.*
Streptococcus pyogenes	*Neisseria gonorrhoeae*	
	Proteus mirabilis	
	Proteus vulgaris	
	Providencia spp.	

*methicillin-susceptible isolates only

Intravenous (IV) and intramuscularly (IM) administered cefoxitin is approved by the United States FDA for systemic treatment of various infections and for prophylaxis of infection in patients undergoing various surgeries [129]. Cefoxitin is not readily metabolized and about 85% of the administered dose is excreted unchanged in the urine. Probenecid, a non-specific

inhibitor of organic anionic transporters, increased cefoxitin exposure ($AUC_{0-\infty}$) by 2.4-fold indicating that cefoxitin is primarily secreted by the renal anionic transport system into urine [1]. Cefoxitin has a very short half-life of about 40 minutes in healthy volunteers with an acceptable safety profile [1, 129]. Given its short half-life, safety profile, being a substrate of anionic transport system, and the feasibility to conduct pharmacokinetic studies with low IV/IM doses within a short study duration, it is an ideal probe drug to study renal anionic secretory clearance in various patient populations, including renal transplant recipients.

Although cefoxitin pharmacokinetics have been described in subjects with normal renal function and with impaired renal function [1, 132-135], no data is available regarding its pharmacokinetics in patients with single transplanted kidneys. In order to evaluate the pharmacokinetics of cefoxitin for the assessment of renal anionic secretory capacity in renal transplant recipients, it was necessary to develop a sensitive and specific assay method for the determination of cefoxitin in human plasma. To date, several high performance liquid chromatography techniques with ultraviolet detection (HPLC-UV) have been developed for quantification of cefoxitin in biological fluids and limited literature articles with LC-MS/MS methods [128, 131, 136-145].

All published methods require a large blood volume making intensive sampling difficult. Most of these methods have used protein precipitation using different organic solvents like trichloroacetic acid [128, 131, 143], methanol [130] and acetonitrile [146]. This is the first report of a method using solid phase extraction for sample preparation and UPLC for separation with tandem mass spectrometric detection.

The objective of this study was to develop a rapid, highly sensitive and reproducible UPLC-MS/MS analytical method to quantify cefoxitin concentrations in human plasma following administration of low doses in kidney transplant recipients.

4.3 MATERIALS AND METHODS

4.3.1 Chemicals and Reagents

Chemical structures of Cefoxitin and the internal standard, cefuroxime are shown in Figure 22 and Figure 23, respectively. Cefoxitin and cefuroxime was purchased from Toronto Research Chemicals (Toronto, ON, Canada). Oasis® HLB 1cc extraction cartridges with 30 mg sorbent per cartridge and 30 µm particle size were purchased from Waters (Milford, MA, USA). Ammonium acetate (99.999 trace metals basis) and Optima™ LC/MS grade acetonitrile, formic acid, methanol and water were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Human plasma was procured from central blood bank of Pittsburgh (Pittsburgh, PA, USA).
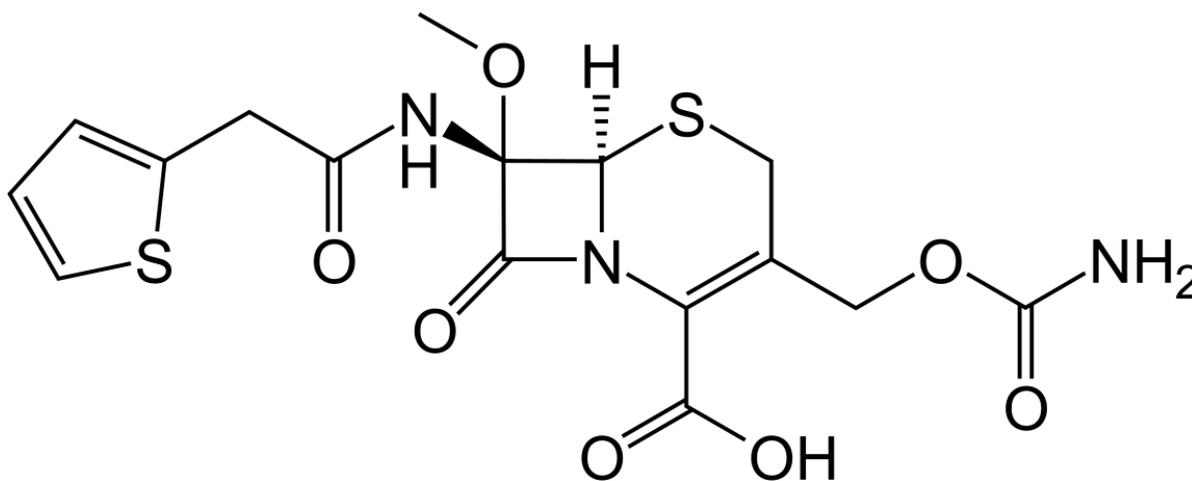
Figure 22. Chemical structure of cefoxitin (molecular weight: 427.45 g/mol)
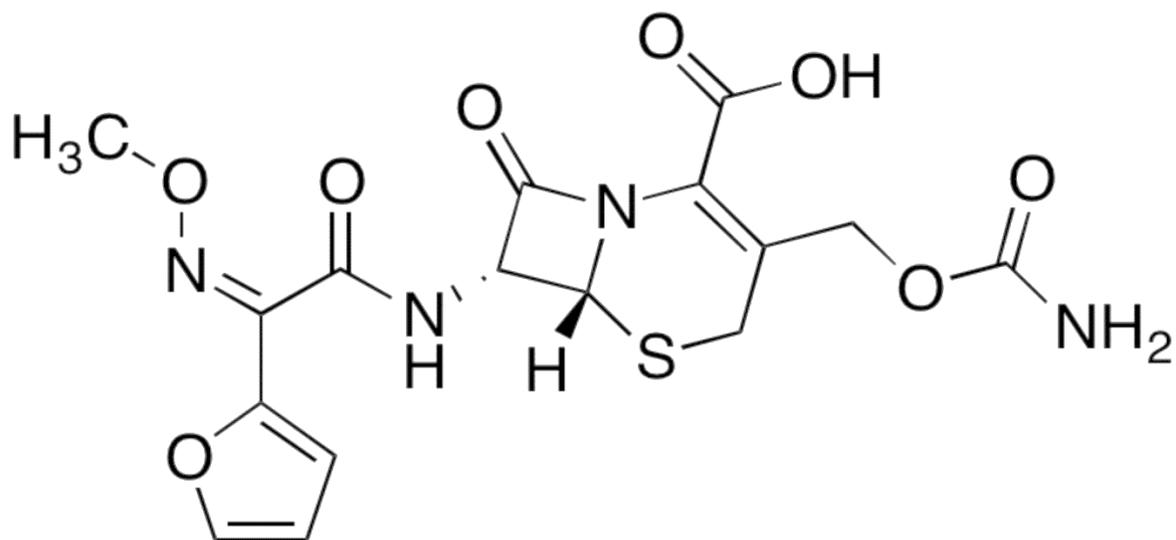
Figure 23. Chemical structure of cefuroxime (molecular weight: 424.39 g/mol)

4.3.2 Chromatographic Conditions

The UPLC system used for the analysis of Cefoxitin was a Waters Acquity H class model (Waters Corporation, MA, USA). Separation was achieved on Acquity UPLC HSS T3 1.8 μm, 2.1 x 100 mm column with a Acquity UPLC HSS T3 1.8 μm Vanguard Pre-column (2.1 x 5 mm). The mobile phase used consisted of [A] 5% Acetonitrile in water containing ammonium acetate (2 mM) and formic acid (0.1%) and [B] Acetonitrile containing ammonium acetate (2 mM) and formic acid (0.1%). A sample volume of 20 μL was injected and a 6 min gradient method at a flow rate of 0.3 mL/min was developed for elution of the compounds. The gradient started at 30% 'B', maintained for 0.5 min, then increasing to 90% 'B' from 0.51 min to 2.5 min, maintaining for 0.5 min, then decreasing to 30% 'B' from 3.01 to 3.6 min and maintained at 30% 'B'.

4.3.3 Mass Spectrometric Conditions

Analysis was performed on a XEVO TQS mass spectrometer (Waters, Milford, MA, USA) with negative electro spray ionization mode using multiple reaction monitoring (MRM). MRM settings for cefoxitin and cefuroxime (internal standard) are provided in Table 13.

Table 13. Multiple reaction monitoring settings

Setting	Value
Capillary Voltage	3.5 kV
Source Temperature	150 °C
Desolvation temperature	500 °C
Cone Gas flow	150 L/hr
Desolvation Gas flow	1000 L/hr
Collision Gas flow	20 mL/min
Argon pressure	20±10 psig
Nitrogen pressure	100±20 psig
Dwell time	0.025 sec

The extracted ions following MRM transitions monitored were m/z 426.16→156.05 for Cefoxitin, m/z 423.10→207.1 for Cefuroxime (IS). The cone and collision energy for Cefoxitin was 28 V and 8 V respectively; cone and collision energy for Cefuroxime was 2 V and 16 V respectively. Dwell times are 0.025 s for both compounds. The LC–MS system was controlled by Masslynx® software version 4.1, and data were collected with the same software.

4.3.4 Standards and Quality Controls

Primary stock solutions for standards and quality control samples were prepared in methanol (1 mg/mL). Working standards and quality control samples were diluted from primary stock

solution with 50% MeOH : 50% water. The working internal standard solution (1000 ng/mL) was prepared from the primary stock in 50% methanol and 50% water. Routine daily calibration curves and controls were prepared by spiking human plasma with working solution. The concentrations of the spiked plasma standards were 25, 50, 100, 250, 500, 1000, 2500, 5000, 25,000 and 50,000 ng/mL. The concentrations of quality control (QC) samples were initially planned to be either targeting a lower range (Low quality control: 40 ng/mL; Mid quality control: 2,000 ng/mL; High quality control: 4000 ng/mL) or a higher range (Low quality control: 400 ng/mL; Mid quality control: 8,000 ng/mL; High quality control: 40,000 ng/mL) depending on the concentration of cefoxitin in patient samples. Working standard solutions, spiked plasma standard, quality control samples and internal standard solutions were stored at −80°C.

4.3.5   Sample Preparation and Assay Methodology

Routine daily calibration curves, controls and the clinical samples were thawed at room temperature.

4.3.5.1 Plasma samples

Twenty μL of plasma was diluted 50 times with 1 mL of LC/MS grade water containing 0.2% formic acid. To this, 10 μL of internal standard (Cefuroxime,1000 ng/mL) was added. Samples were extracted using solid phase extraction by passing the solution through Oasis® HLB 1cc (30 mg) extraction cartridges. The cartridges were previously conditioned with methanol and LC/MS grade water (0.2% formic acid), under vacuum. After washing with 1 mL of LC/MS grade water (0.2% formic acid), sample was eluted with 2 mL of methanol and the eluent was evaporated to dryness under air. The residue was reconstituted in 100 μL of 50% MeOH:50%

H$_2$O (0.2 % formic acid) of starting mobile phase and 10 µL was injected into the UPLC system connected to the mass spectrometer.

4.3.6 Assay Validation

4.3.6.1 Calibration curve and lower limit of quantitation

Human plasma spiked with working solutions of standard cefoxitin in 50% methanol and 50% water were used in establishing the standard curve. The final concentrations ranged from 25–50,000 ng/mL for plasma samples. Calibration curves were constructed by plotting the peak area ratio of analyte to the internal standard (Y) against the analyte concentration (X). A linear regression analysis with weighing (1/X) was used to determine slopes, intercepts, and correlation coefficients. Concentration of analyte in the unknown samples were calculated from their peak area ratios and the calibration curve. The acceptance criterion for each back-calculated concentration of QC standards (low range QCs: 40, 2000, 4000 ng/ml or high range QCs: 400, 8000, and 40,000 ng/mL) and standards was set at ≤15% deviation from the nominal value, except at the lower limits of quantification, where it was ≤20%. Lower limit of quantification (LLOQ) was determined as the lowest concentration of the test compound that gave a signal-to-noise ratio of at least 5:1 and had deviation from nominal concentration of less than 20%.

4.3.6.2 Accuracy and precision

The accuracy and precision of the developed method were determined by analyzing plasma samples with cefoxitin at the 400, 8000, and 40,000 ng/mL concentrations since all patient samples were more than 400 ng/mL. Accuracy, expressed as percent bias, was measured as the percentage difference from theoretical value according to the equation:

$$\text{Bias}(\%) = \left[\frac{(Con_M - Con_T)}{(Con_T)} \right] \times 100$$

where Con_M is measured concentration and Con_T is theoretical concentration.

Precision was calculated as the percent coefficient of variance (%CV) where %CV is standard deviation/mean x 100. A deviation and precision within ±15% of the nominal value is considered acceptable. The precision and accuracy of the developed method were determined by analysis of QC samples following injecting three samples at each concentration on the same day assessed intra-day variation of the assay. Inter-day variation was assessed by injecting six samples of each concentration on 5 days.

4.3.6.3 Extraction recovery and matrix effect

The extraction recovery of cefoxitin from human plasma was determined by comparing the absolute response of an extract of control plasma to which cefoxitin had been added after extraction, with respect to the absolute response of an extract of plasma to which the same amount of cefoxitin had been added before extraction. To evaluate the effect of endogenous matrix constituents on the assay, responses of cefoxitin at the 3 QC concentrations in triplicate were evaluated. The matrix effect of plasma on cefoxitin was defined as the effect on the signal when comparing the absolute response of an extract of control plasma to which cefoxitin had been added after the extraction with the absolute response of reconstitution solvent to which the same amount of cefoxitin had been added.

4.3.6.4 Stability

The stability of cefoxitin in plasma was evaluated at the three QC concentrations in triplicates following room temperature storage for 24 hr, storage at 4°C for 7 days, storage at -80°C for

1 month. Three freeze-thaw cycles of plasma samples prior to extraction were assessed. Stability was expressed in terms of percentage of nominal concentration. The acceptance criterion for percent relative recovery was set at $100 \pm 10\%$. The assay was developed to support academic studies and requires further qualification and validation to support further development through commercialization which is beyond the scope of this work.

4.4   RESULTS

4.4.1   Mass Spectrometry and Chromatography

Following injection of cefoxitin and cefuroxime (IS) into the mass spectrometer with the negative ion electrospray ionization interface, the mass to charge transition from parent to product ions were observed to have m/z 426.16 → 156.05 for cefoxitin and m/z 423.10 → 207.13 for cefuroxime. Instrument parameters were selected to optimize specificity and selectivity of both parent and daughter ions. The final instrument parameters were, capillary voltage: 3.5 kV, source temperature: 150°C, desolvation temperature of 500 °C, cone gas flow of 150 L/hr, desolvation gas flow of 1000 L/hr, argon pressure of 20±10 psig, and nitrogen pressure of 100±20 psig and dwell time of 0.025 seconds. The optimization of collision energy for cefoxitin and cefuroxime are shown in Figure 24 and Figure 25 respectively.
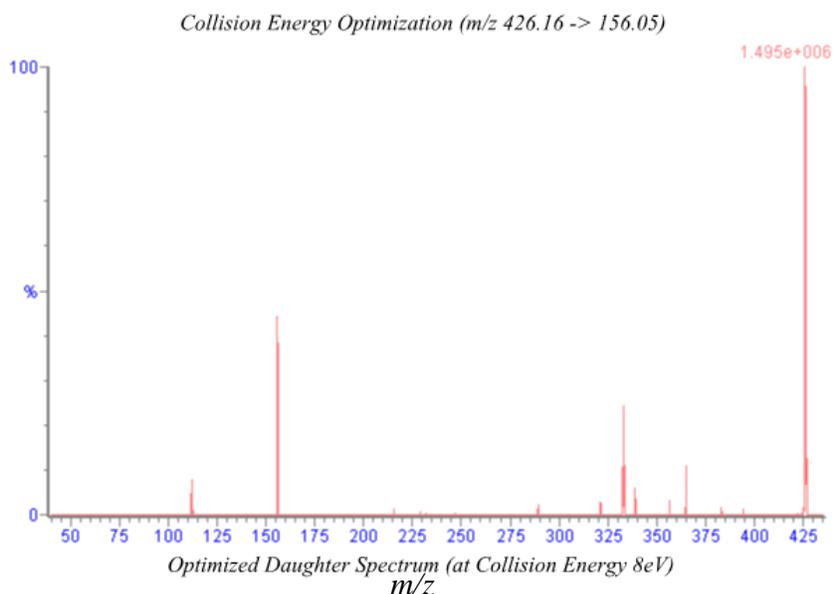
Figure 24. Optimization of collision energy in mass spectrometry for cefoxitin.

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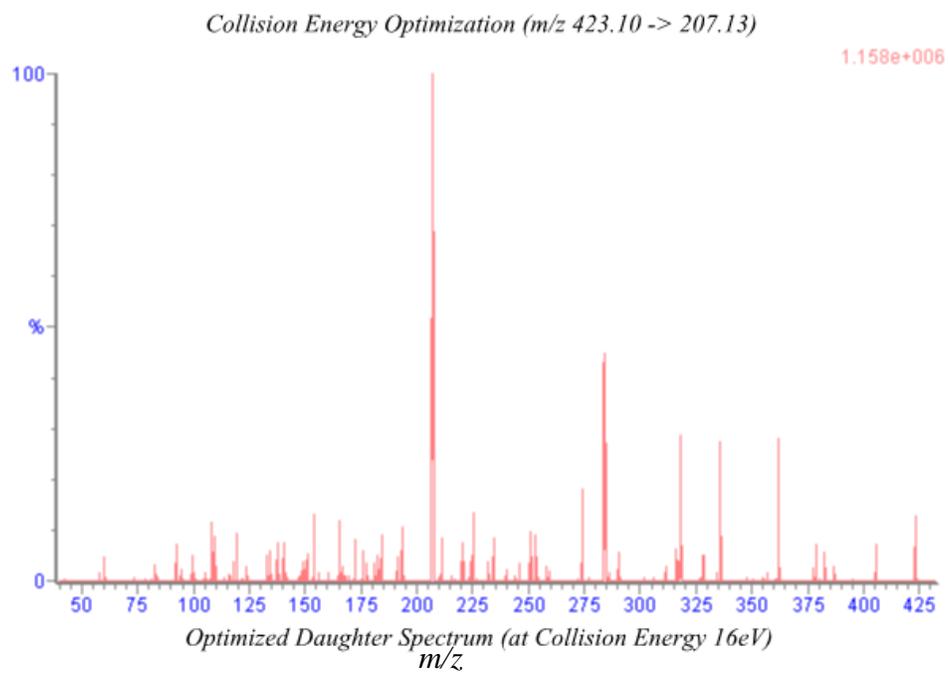
Figure 25. Optimization of collision energy in mass spectrometry for cefuroxime.

The retention times for cefoxitin and cefuroxime were 1.56 min and 1.40 mins respectively with a total run time of 6 minutes. Representative chromatograms of cefoxitin and cefuroxime spiked in plasma at 500 ng/mL concentrations is provided in Figure 26 and Figure 27.
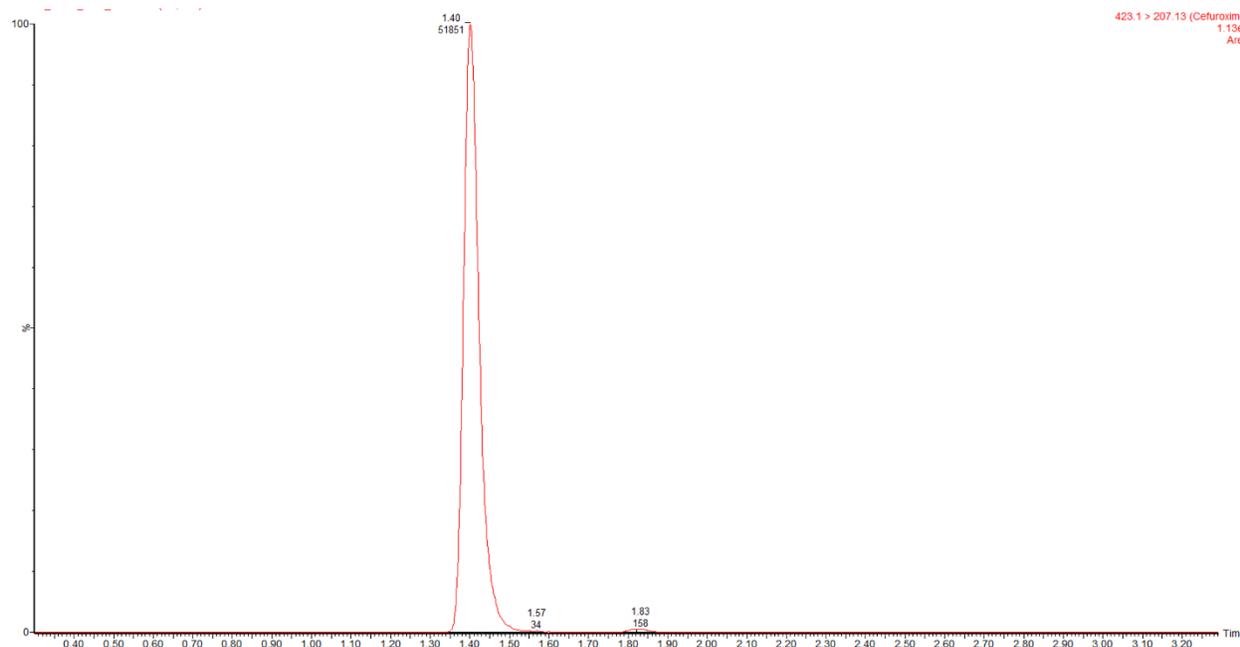
Figure 26. Representative chromatogram of cefoxitin in plasma (500 ng/mL).
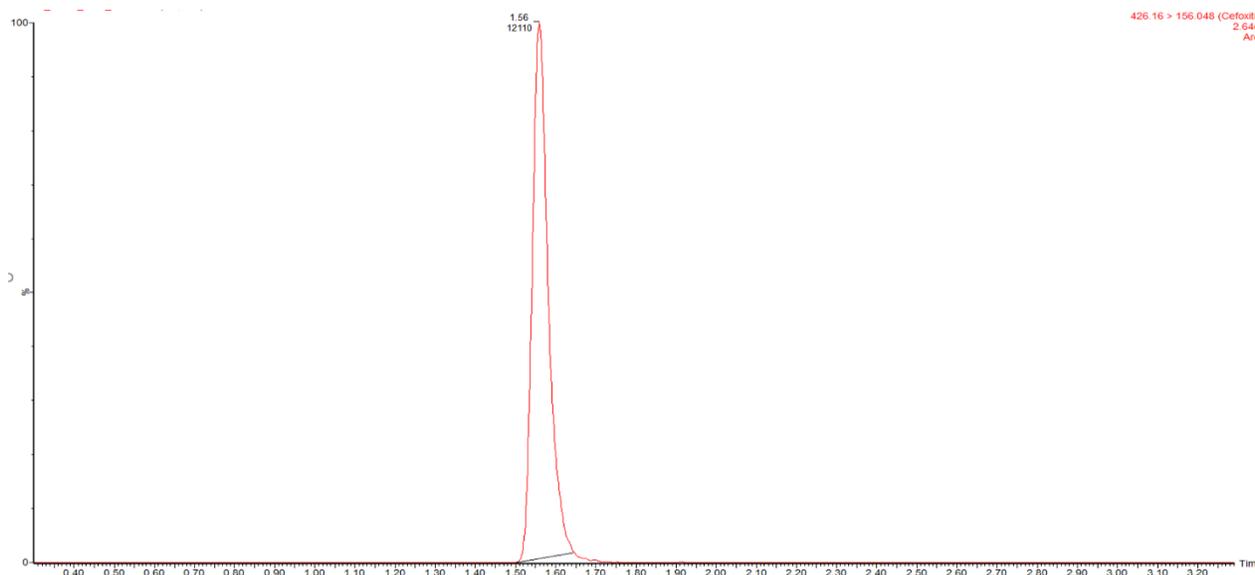
Figure 27. Representative chromatogram of cefuroxime in plasma (500 ng/mL).

4.4.2 Assay Validation

Triplicate standard curves were performed in the plasma on five sequential days. The ratio of peak cefoxitin area to peak cefuroxime concentration was linear for cefoxitin between the concentration range of 25 to 50,000 ng/mL. The 1/x weighted correlation coefficient of calibration curve was in the range of 0.995-0.999. The LLOQ was 25 ng/mL using a plasma volume of 20 µL. The calibration curves for cefoxitin in plasma are presented in Figure 28.

Compound name: Cefoxitin 1
Correlation coefficient: r = 0.998812, r^2 = 0.997626
Calibration curve: 0.000112432 * x + -0.00158429
Response type: Internal Std (Ref 2), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Include, Weighting: 1/x, Axis trans: None
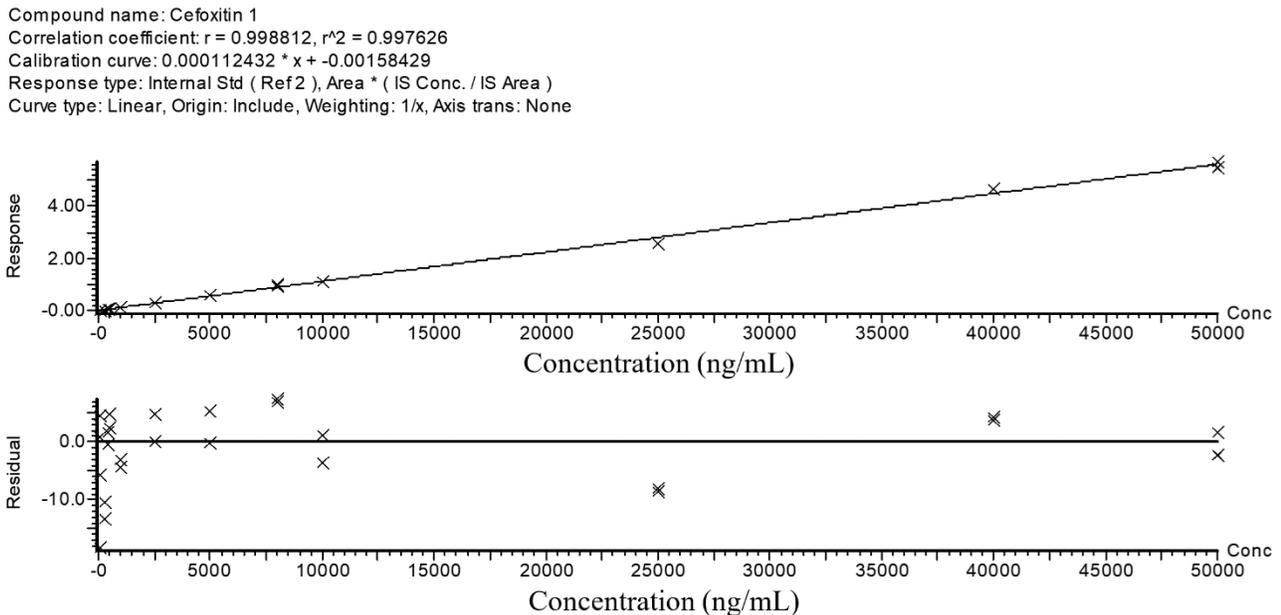
Figure 28. Standard curve of cefoxitin in plasma over a concentration range of 25-50000 ng/mL along with a plot of residuals.

4.4.3 Accuracy and Precision

The inter-day and intra-day accuracy and precision for cefoxitin were performed with the higher range of QCs (400, 8,000, and 40,000 ng/mL) since all the patient plasma samples quantitated were greater than 400 ng/mL. The inter-day and intra-day accuracy and precision for cefoxitin were within 10.7%. Results of the assay precision and accuracy are presented in Table 14 and Table 15 respectively.

Table 14. Inter-day and Intra-day precision of cefoxitin assay

Inter-day	QC concentration (ng/mL)		
Test Day	**400 (N=3)**	**8000 (N=3)**	**40,000 (N=3)**
Day 1	404	7708	45436
Day 2	383	8156	42967
Day 3	392	7692	45175
Day 4	390	8260	43949
Day 5	417	8144	43967
Mean ± SD	397±14	7992±270	44299±1008
%CV	3.4	3.4	2.3
Intra-day	**QC concentration (ng/mL)**		
Occasion	**400 (N=3)**	**8000 (N=3)**	**40000 (N=3)**
1	388	8273	39151
2	355	7903	36383
3	378	8224	38140
4	407	8562	41519
5	408	7920	38698
Mean ± SD	387±22	8176±274	38778±1857
%CV	5.7	3.4	4.8

QC: Quality control; SD: standard deviation; %CV: percent coefficient of variation

Table 15. Inter-day and Intra-day accuracy of cefoxitin assay

Inter-day	QC concentration (ng/mL)		
Test Day	**400 (N=3)**	**8000 (N=3)**	**40,000 (N=3)**
Day 1	404	7708	45436
Day 2	383	8156	42967
Day 3	392	7692	45175
Day 4	390	8260	43949
Day 5	417	8144	43967
Mean	397	7992	44299
%Bias	-0.7	-0.1	10.7
Intra-day	**QC concentration (ng/mL)**		
Occasion	**400 (N=3)**	**8000 (N=3)**	**40000 (N=3)**
1	388	82734	39151
2	355	7903	36383
3	379	8224	38140
4	407	8562	41519
5	408	7920	38698
Mean	387	8176	38778
%Bias	-3.2	2.2	-3.1

QC: Quality control; %CV: percent coefficient of variation

4.4.4 Stability Data

Samples were stable on the bench-top at room temperature for 24 hours, in the autosampler (4℃) for 7 days and following 3 freeze-thaw cycles from -80℃ for up to 3 months. The average percent change of peak areas were ≤10.6%. Results of stability studies at each QC level and shown in Table 16.

Table 16. Results of stability studies

	QC concentration (ng/mL), mean		
	400 (N=3)	**8000 (N=3)**	**40000 (N=3)**
RT	412	8374	41478
% Bias	3	4.7	3.7
4C	443	8191	37111
% Bias	10.6	2.4	-7.2
-80 C	401	8629	38177
% Bias	0.1	7.9	-4.6

QC: Quality control; RT: room temperature

4.4.5 Recovery and Ion Suppression

The SPE recovery of cefoxitin was determined by comparing the absolute response of an extract of control plasma to which cefoxitin was added at three QC concentrations after SPE with the absolute response of an extract of plasma to which the same QC concentrations of cefoxitin was added before SPE. Recovery data and relative response when tested from matrix effects are shown in Table 17. There was minimal matrix effect and the total recovery was 83.6% across the three QC concentrations.

Table 17. Total and ion suppression recovery of cefoxitin in human plasma

QC Concentrations (ng/ml), N=3	Ion Suppression Relative Recovery Mean ± SD (%)	Total Recovery Mean ± SD (%)
400	88.6 ± 0.8	84.1 ± 4.6
8000	97.6 ± 4.1	80.2 ±1.2
40000	104 ± 1.1	86.5 ± 1.1

4.4.6   Cefoxitin Detection in Renal Transplant Patients

The developed cefoxitin assay was used to quantitate cefoxitin plasma concentrations in 15 renal transplant recipients following administration of a single dose of 200 mg IV cefoxitin over 1-2 minutes as an IV push. The concentration versus time plot of cefoxitin in renal transplant recipients is provided in Figure 29. Very few plasma samples had concentrations above range of the standard curve. These samples were diluted to fall within range of quantitation. Further details of the clinical study are discussed in Chapter 5.0 .
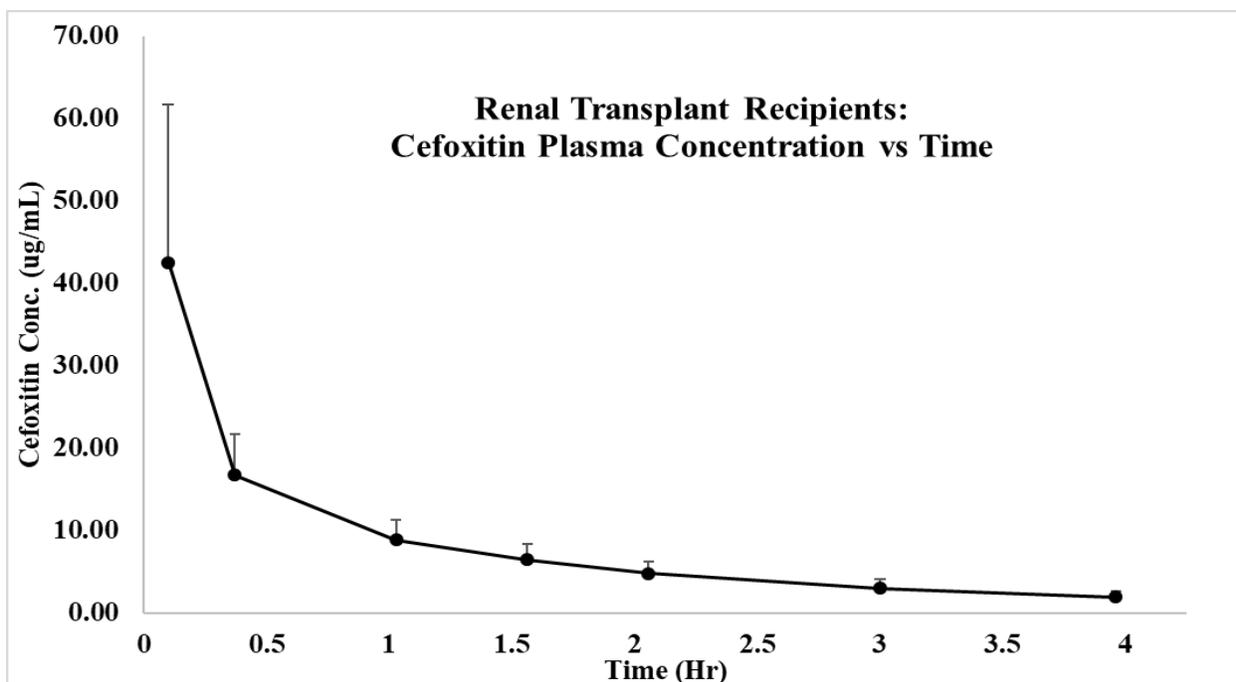
Figure 29. Concentration versus time cure showing cefoxitin exposure in renal transplant recipients following administration of 200 mg IV cefoxitin over 1-2 min. Error bars represent standard deviation.

4.5 DISCUSSION

Cefoxitin is a second-generation cephalosporin antibiotic with activity against various Gram-positive, Gram-negative and Anaerobic bacteria (Table 12) [129-131]. Given its short half-life, acceptable safety profile, its properties as a substrate of anionic transport system, and feasibility to conduct pharmacokinetic studies with low IV/IM doses within a short study duration, it is an ideal probe drug to study renal anionic secretory clearance in renal transplant recipients. To date, several HPLC-UV chromatographic techniques have been developed for quantification of cefoxitin in biological fluids, but limited LC-MS/MS methods have been described in the literature. All currently published methods are limited by their requirement for relatively large volume of serum or plasma (100-1000 µL), longer run-times (elution time: 5.30 to 12.9 min), and higher LLOQ (1.00 to 100 µg/mL) [128, 131, 136-143].

Here we describe the development and validation of a rapid and sensitive UPLC-MS/MS assay to detect cefoxitin in human plasma following administration of low doses of cefoxitin for the evaluation of renal anionic secretion in renal transplant recipients. During initial assay development, higher sensitivity was observed with negative ionization mode. An anion exchange solid phase extraction procedure was used to process plasma samples. Separation of cefoxitin from other components in plasma was performed using an analytical column with a gradient profile. The selected column and mobile phase provided well separated and sharp peaks. Cefoxitin has been successfully used as an internal standard for cefuroxime LC-MS/MS assay [140] and so cefuroxime was used as an internal standard in this cefoxitin LC-MS/MS assay. Cefuroxime provided consistent response under the conditions utilized in this method. It also eluted close to the analyte of interest and facilitated a short run-time (6 minutes).

In clinical pharmacokinetic studies plasma samples are handled at room temperature or 4°C and stored at -20 to -80 °C until analysis and exposed to various temperatures during assay procedures. As a result, it was necessary to understand the stability of cefoxitin at these varying conditions. Stability was determined by the comparison of QC concentrations of fresh samples to those left for 24 h at room temperature, 7 days at 4°C, and 3 months at −80°C. Additionally cefoxitin stability for up to 3 freeze-thaw cycles was tested. These different sample handling and storage conditions did not affect estimated cefoxitin concentrations indicating stability under the conditions evaluated. There was minimal matrix effect and the total recovery was 83.6% across the three QC concentrations.

It is necessary to determine cefoxitin concentration in urine samples in order to evaluate cefoxitin pharmacokinetics from urine data. Since majority of cefoxitin is excreted unchanged in the urine, its concentrations in urine are expected to be very high, especially in renal transplant recipients with limited urine output [147]. For these samples, sensitivity in the nanograms/mL is not necessary and so similar methodology described above was used to determine cefoxitin concentration in urine with some minor modifications. Urine samples underwent direct serial dilutions (1000 fold) in water containing 0.2% formic acid without solid phase extraction.

We successfully evaluated cefoxitin exposure in renal transplant recipients using this assay. A detailed report on this clinical study is provided in Chapter 5.0 . This rapid and sensitive UPLC–MS/MS method for quantitative assessment of cefoxitin in human plasma has a short run time (6 min), uses limited sample volume (20μL) and gives us the ability to perform cefoxitin quantitative assessment in the range of 25-50,000 ng/mL. This assay enables us to perform low dosing and limited volume sampling studies.

5.0 CLINICAL EVALUATION OF CHANGES IN RENAL ANIONIC SECRETION FOLLOWING LIVING DONOR AND DECEASED DONOR RENAL TRANSPLANTATION: CLINICAL PHARMACOKINETICS OF MINIMAL DOSING CEFOXITIN

5.1 ABSTRACT

Renal transplantation is the treatment of choice for patients with ESRD. Since kidney is the primary excretory organ for various drugs and their metabolites, changes in renal graft function would significantly alter the clearance and therefore exposure of renally secreted drugs. Kidneys from living and deceased donors that are transplanted into recipients normally undergo numerous insults including CI injury and are also subjected to nephrotoxicity due to CNI. These physiological and pharmacological stresses can alter the expression and functional capacity of renal anionic drug transporters. The objectives of this study were to (1) assess the longitudinal changes in renal anionic secretory capacity, (2) study the effect of prolonged CI on renal anionic secretory capacity in kidney transplant patients on tacrolimus therapy, and (3) and to compare renal anionic secretory capacity of renal transplant recipients with healthy volunteers. Cefoxitin was used as a probe drug to assess renal anionic secretory capacity. Cefoxitin plasma and urine pharmacokinetic studies were performed in 15 de-novo renal transplant recipients following administration of 200 mg IV cefoxitin within 14 days post-transplantation, and beyond 90 days post-transplantation. The concentrations of cefoxitin in plasma and urine were measured using a validated LC-MS/MS method. Historical data from cefoxitin pharmacokinetic in healthy volunteers was used to compare results. There were no differences in renal anionic secretory capacity in de-novo LDRT and DDRT recipients during the early post-transplant period. Renal anionic secretory capacity in renal transplant recipients was reduced by 60% compared to historical healthy controls. This study shows that renal transplant recipients would need

significantly lower dosage of drugs that are renally secreted via organic anionic transport system despite having eGFR in the normal range post-transplantation.

5.2 INTRODUCTION

Chronic kidney disease (CKD) is the ninth leading cause of deaths in the United States. An estimated 26 million adults or 13% of the US population is expected to have CKD [74]. About 500,000 CKD patients are classified as having ESRD with an eGFR of less than 15 mL/min/1.73 m^2 [75]. Kidney transplantation is the treatment of choice for the patients diagnosed with ESRD. In the year 2016, 19,060 kidney transplantations were performed in the USA with 13,431 kidneys coming from deceased donors and 5,629 kidneys coming from living donors (available from: www.unos.org) [3, 76]. Renal allografts are subjected to a unique set of injurious conditions such as prolonged CI before being transplanted into the recipient, warm reperfusion injury immediately after transplantation, exposure to nephrotoxic CNI based immunosuppression therapy, varying grades of allograft rejection, and bacterial/fungal/viral infections post-transplantation [3, 76, 78, 79, 84-86]. The cold ischemic injury and nephrotoxic CNI therapy that the renal transplant recipients receive have been shown to lead to progressive loss of renal function with a five-year recipient survival of 84% for deceased donor kidney transplantations as compared to 91% for living donor kidney transplantations (available from: www.unos.org) [105]. The tubular damage caused by CI and CNI could lead to alteration in the expression and activity of renal drug transporters, which primarily reside in renal tubular epithelial cells. This damage may eventually affect the clearance of drugs that are predominantly cleared by renal secretion.

Drugs that are eliminated by tubular secretion primarily undergo active transport into the lumen of the proximal tubule. For a drug to be successfully cleared it is usually a substrate of an uptake and efflux pair of transporters such as in the case of cefoxitin where OAT1/3 and MRP2/4

are thought to be involved in the uptake and efflux activities respectively in the renal epithelial cells [26, 27, 29-31].

Renal organic anionic transporters are specifically of interest in the context of renal transplant recipients as they are involved in the clearance of various medications (acyclovir, cidofovir, fluoqionolone antibiotics, several cephalosporine antibiotics and many others) prescribed to renal transplant recipients. Some of these medications such as cidofovir is nephrotoxic and is considered a narrow therapeutic index drug. OAT1 and OAT3 renal uptake transporters are considered to be the most important renal organic anionic transporters by the US FDA and EMA for their role in drug disposition and drug-drug interactions [9]. For the disposition of various anti-infective medications, MRP2 and MRP4 are thought to be the efflux partners for OAT1 and OAT3 [26, 27, 29-31] (Figure 30).
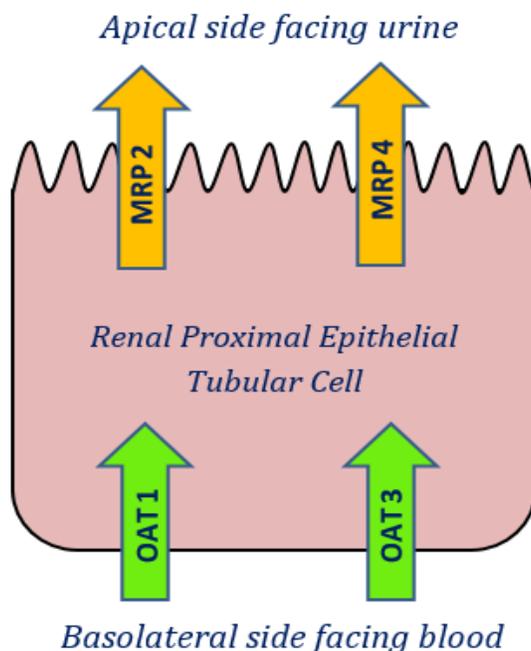
Figure 30. Orientation of OAT1 and OAT3 uptake transporters and MRP2 and MRP4 efflux transporters in renal proximal epithelial tubular cells.

As described in Chapter 2.0 , our work on the mRNA expression of Slc22a6 (Oat1), Slc22a8 (Oat3), Slc22a2 (Oct2), Slc47a1 (Mate1), and Abcb1a (Mdr1a/P-gp) renal transporters in rat transplant model showed that rat kidneys exposed to 24 hrs of CI in the presence of tacrolimus treatment for 4 weeks following transplantation, has significant lower mRNA expression (3 to 14.5-fold decline) of the above mentioned 5 important renal transporters that are highly expressed in human renal tissues. This effect was reproduced after allogeneic rat kidney transplantations. Interestingly, tacrolimus treatment mediated downregulation of the selected transporters was absent in kidneys that were not subjected to 24 hrs of CI.

These results show that tacrolimus mediated down regulation of renal Oat1/3 mRNA occurs only in the presence of prolonged CI. Since renal allografts undergo prolonged CI, warm reperfusion injury and nephrotoxic CNI therapy, it is important to characterize the anticipated change in renal organic anionic secretory capacity in living donor and deceased donor renal transplant recipients to ensure optimal pharmacotherapy.

The present study was conducted to determine the pharmacokinetics of cefoxitin in living donor and deceased donor renal transplant recipients in order to (1) assess the longitudinal changes in renal anionic secretory capacity, (2) study the effect of prolonged cold ischemia on renal anionic secretory capacity in kidney transplant patients on tacrolimus based maintenance immunosuppression therapy, and (3) and to compare renal anionic secretory capacity of renal transplant recipients with that of healthy volunteers.

5.3   MATERIALS AND METHODS

5.3.1   Selection of Substrate to Assess Organic Anionic Secretory Capacity:

Probenecid, a non-specific potent OAT inhibitor has been clinically used to successfully show the involvement of OATs in the renal secretion of several drugs [1, 32-39]. A systematic literature search was performed to identify renally cleared drugs which have been shown to have altered clinical pharmacokinetics (PK) with the administration of probenecid in healthy volunteers. Table 18 summarizes the observed significant changes in the clinical PK parameters reported in literature [1, 32-39].

Table 18. Clinical Drug-Drug Interaction with Probenecid and Anionic Drugs

Affected Drug	Fold Change in Clinical PK Parameters					References
	$AUC_{0-\infty}$	C_{max}	CL_R	CL/F	$t_{1/2}$	
Acyclovir	1.4	-	0.7	-	-	Laskin, 1982[36]
Cefaclor	2.1	1.5	-	-	1.6	Welling, 1979 [39]
Cefonicid	2.1	1.2	0.3	-	1.5	Pitkin, 1981[37]
Cefoxitin	2.4	-	0.4	-	2	Vlasses, 1980[1]
Cidofovir	-	-	0.5	0.6	-	Cundy, 1995[33]
Ciprofloxacin	1.7	-	0.4	0.6	1.5	Jaehde, 1995[35]
Dicloxacillin	1.9	1.8	0.3	0.5	-	Beringer, 2008 [32]
Famotidine	1.8	1.5	0.4	0.1	-	Inotsume, 1990[34]
Furosemide	2.7	1.5	0.3	0.4	1.7	Vree, 1995[38]

Summary of significant changes in clinical PK parameters ($p<0.05$) of anionic drug substrates when probenecid is used to inhibit OAT mediated secretory transport. AUC, area under the curve; C_{max}, maximum concentration; CL_R, renal clearance; CL/F, apparent clearance; $t_{1/2}$, half-life

'-': Not significant or not reported.

Among the drugs identified in Table 18, we excluded nephrotoxic agents as well as the drugs transplant clinicians were not comfortable administering to their patients for research purposes and ones without a clinical need. Of the remaining drugs, cefoxitin had a good safety profile when given at low doses as an intravenous push administration, a short half-life and highest change in exposure when co-administered with oral probenecid as compared to cefoxitin alone ($AUC_{0-\infty}$ was 2.4-fold higher) [1]. The pharmacokinetic properties of cefoxitin are summarized in Table 19. With a short half-life of 40 minutes, we have the ability to perform a complete PK study of this drug during scheduled clinic visits of renal transplant patients and avoid having a separate study visit.

Table 19. Pharmacokinetic properties of cefoxitin in healthy volunteers [1]

Drug	Cefoxitin
Dosage Form	IV
Half-Life (Hrs)	0.8
Clearance (ml/min/1.73 m^2)	329
Renal Clearance (ml/min/1.73 m2)	280
% unchanged in Urine	85%
Protein Binding	74%
AUC Fold Change with Probenecid	2.4

Based on safety profile, pharmacokinetic properties, and feasibility of conducting a pharmacokinetic study in renal transplant recipients in an academic hospital, cefoxitin was selected as an ideal drug substrate to evaluate changes in renal organic anionic transport in renal transplant recipients.

5.3.2 Patients

5.3.2.1 Renal transplant recipients

This study was performed in adult renal transplant recipients. Living donor and deceased donor renal transplant recipients who were going to undergo their renal transplantation procedure and follow-up transplant care at the UPMC Montefiore hospital were approached to participate in the study. The study protocol was approved by the Institutional Review Board of the University of Pittsburgh (IRB# PRO15010155) and written consent was obtained from all patients prior to participation in this study. Kidney transplant recipients were routinely screened clinically and evaluated for participation in the study.

Inclusion criteria included:

1. Have been scheduled for living or deceased donor renal allograft transplantation at UPMC

2. Men and women aged between 18 and 65 years

3. Subjects who are scheduled to receive de novo kidney transplant

4. Subjects willing to sign informed consent form

5. Be treated in accordance with the standard care protocols currently in effect for living and deceased donor renal transplant patients including immunosuppressants use and other elements of pre- and post-surgery

Exclusion criteria included:

1. Subjects receiving UNOS extended criteria donor organs

2. Pregnant or breastfeeding women

3. Re-transplantation

4. Subjects with HIV or Hepatitis B/C

5. Active tuberculosis

6. Body mass index > 35 kg/m^2

7. Subjects who have developed malignancy or any medical condition that, in the investigator's opinion, should not be treated with cefoxitin

8. Subjects who can't undergo anti-thymocyte globulin based induction therapy

9. Subjects allergic to tacrolimus or cefoxitin

10. Subjects with unresolved delayed graft function by 14 days post-transplantation

11. Subjects with a hemoglobin of 8 g/dL or less.

Patients with the above-mentioned characteristics were excluded as patients with these factors would add additional variability to the transporter activity study. The listed criteria such as hepatitis, greater BMI, etc. were not exclusion criteria for renal transplantation at UPMC, but are exclusion criteria specifically for this study.

Once an evaluation has been performed if the patient meets the inclusion/exclusion criteria, they were further screened before being included in the study.

Screening procedures included:

1. Ability to understand the informed consent and provide consent to participate willingly in the study

2. Medical history

3. Medication reconciliation, medication allergy and dietary history

4. Baseline clinical laboratory measurements: renal function tests including serum and urine creatinine, creatinine clearance, BUN and urine pH; liver function tests including ALT, AST, alkaline phosphatase, bilirubin, albumin, α1 acid glycoprotein collected as part of the standard of care.

Investigators discussed the study with each patient face-to-face and introduced every detail of the study. The patients were informed at the very first time that their participation was voluntary, and they could withdraw from the study at any time. They were encouraged to ask any questions and take sufficient time to think about the study.

5.3.2.2 Historical controls/healthy volunteers

Six healthy volunteers who participated in cefoxitin pharmacokinetic studies conducted in the presence and absence of orally administered probenecid by Vlasses et al. were used as historical controls [1]. Since this is a pilot study to assess the effect of renal transplantation, historical controls were utilized instead of prospective controls to minimize resource utilization and minimize drug exposure in healthy volunteers. All subjects gave written consent and the protocol of this study was approved by the Thomas Jefferson University Committee on Research.

5.3.3 Study Design

A prospective longitudinal single center pharmacokinetic study was performed on two separate occasions in living donor and deceased donor renal transplant recipients who met the study criteria. Part 1 was conducted approximately 1-2 weeks post-transplantation, when the serum creatinine level stabilized as determined by the transplant clinician. Part 2 was conducted after 3 months following transplantation only in subjects who underwent part-1 of the study. In both parts, the pharmacokinetic parameters of cefoxitin were evaluated following administration of a single dose of 200 mg Cefoxitin administered intravenously over 1-2 minutes (IV push). Cefoxitin powder was reconstituted with sterile water and 2 ml of 100 mg/mL concentration cefoxitin was drawn-up into a 5-ml syringe for the study by the Investigational Drug Services pharmacist at University of Pittsburgh Medical Center. The study design is outlined in Figure 31.

For comparison of this study results with healthy volunteer data, cefoxitin concentration data from a historical crossover pharmacokinetic study performed in the presence and absence of orally administered probenecid was used [1]. In this study, the investigators administered 2 grams of cefoxitin as an IV push over 3 minutes to perform the first PK study for 10 hours. After a 1-week washout period the investigators administered 2 grams of cefoxitin as an IV push over 3 minutes administered with 1 gram of probenecid given orally 1 hour before the cefoxitin dose to perform the second PK study in the same study subjects.
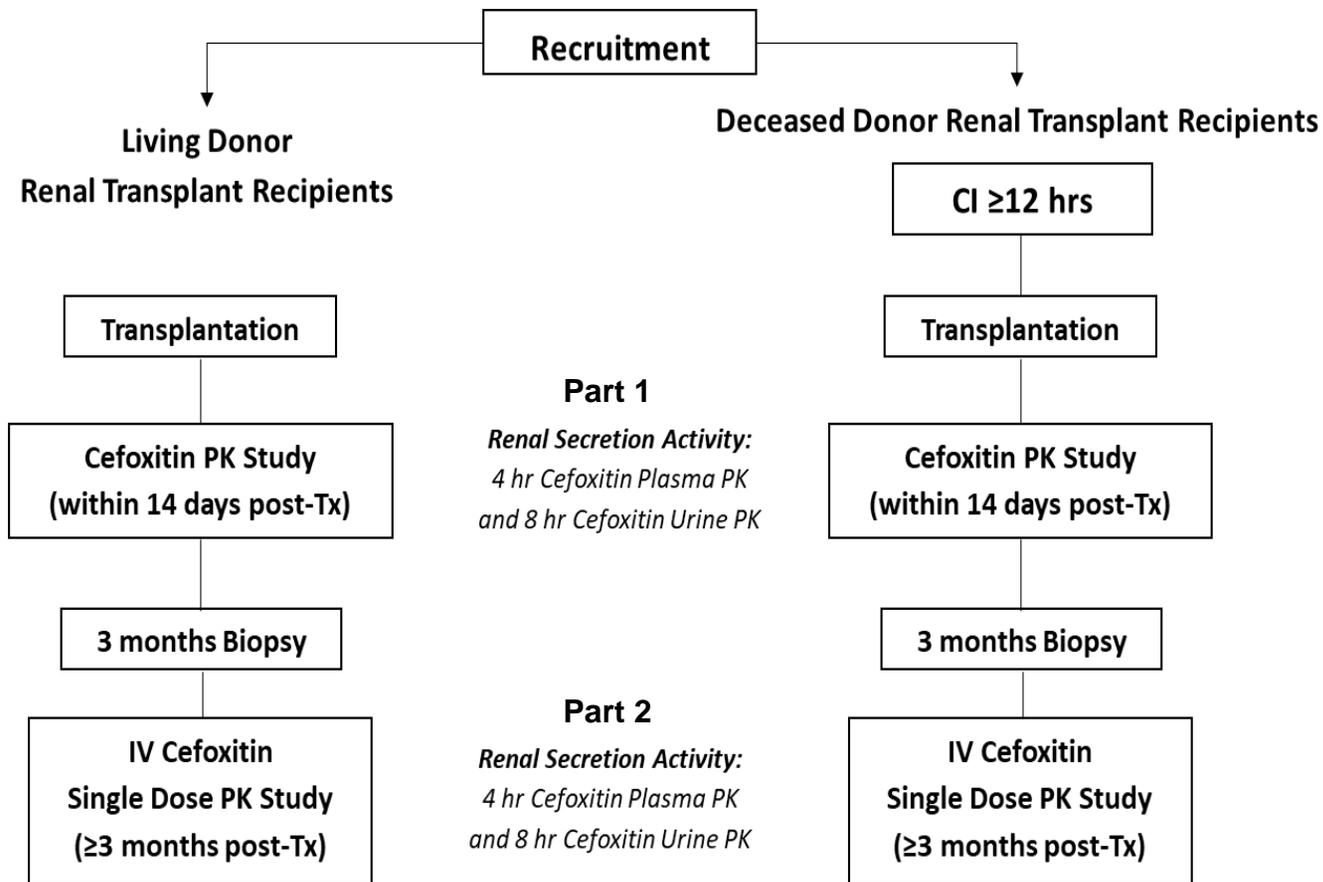
Figure 31. Schematic of cefoxitin pharmacokinetic study design

5.3.4 Blood and Urine Sampling

For the cefoxitin plasma pharmacokinetic assessment, a peripheral venous catheter was placed in left or right hand. The IV line was used for administration of a single dose of 200 mg cefoxitin as well as collection of blood samples. 4 ml of blood were collected into lavender capped K2-EDTA coated vacutainers at approximately 0, 15 min, 30 min, 1hr, 1.5hr, 2hr, 3hr and 4hrs post-administration of cefoxitin. The IV line was flushed with 0.9% sodium chloride solution prior to and after cefoxitin administration and prior to each blood sample draw. Plasma was separated within 30 minutes of blood collection and frozen at -80℃ until analysis.

Urine was collected in aliquots from 0-1, 1-2, 2-4, and 4-8 hours or when voided by the patient. The total volume of urine voided by each subject in each interval is noted and aliquots of urine are labeled to match the time-period. Urine samples were frozen at -80℃ until analysis.

5.3.5 Analytical Methodology

Cefoxitin concentrations in plasma were determined by the liquid chromatographic-mass spectrometric method described in Chapter 4.0 . Briefly, plasma samples were processed by an anion exchange solid phase extraction procedure. Chromatography was performed using a Acquity UPLC HSS T3 analytical column, 1.8 μm, 2.1 x 100 mm, with isocratic elution. Cefoxitin was detected by a triple quadrupole mass spectrometer in negative electron spray ionization mode using multiple reaction monitoring with cefuroxime as the internal standard. With 10μL injections of samples the LLOQ for the cefoxitin in plasma assay was 50 ng/mL.

Since majority of cefoxitin is excreted unchanged in the urine, its concentrations in urine are expected to be very high, especially in renal transplant recipients with limited urine output [147]. For these samples, sensitivity in the nanograms/mL is not necessary and so similar methodology described in Chapter 4.0 was used to determine cefoxitin concentration in urine with some minor modifications in sample processing. Urine samples underwent direct serial dilutions (1000 fold) in water containing 0.2% formic acid. With 10μL injections of samples, the LLOQ for the cefoxitin in urine assay was 10 µg/mL. Representative chromatogram of a blank urine samples spiked with cefoxitin is displayed in Figure 32.
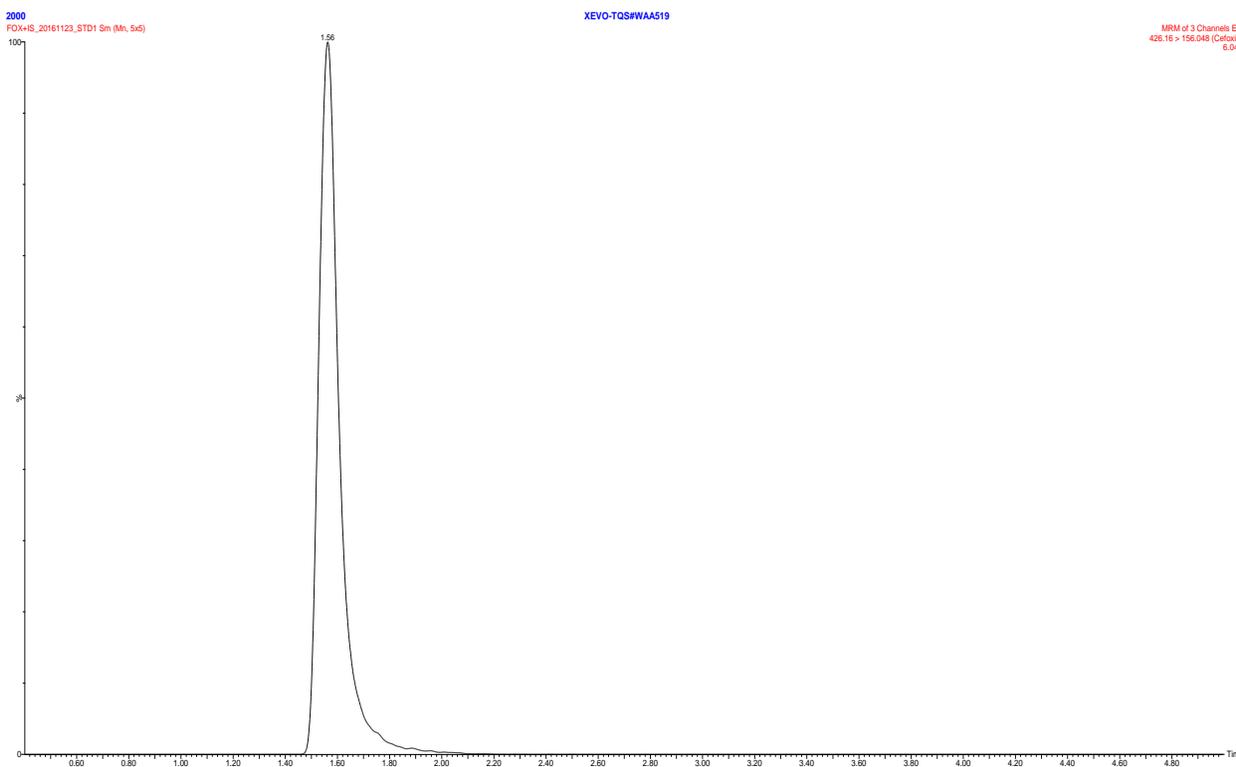
Figure 32. Representative chromatogram of blank human urine spiked with cefoxitin

The ratio of peak cefoxitin area to peak cefuroxime coincentration was linear for cefoxitin between the concentration range of 25 to 2,000 ng/mL. The 1/x weighted correlation coefficient fo calibration curve was in the range of 0.995-0.999. Equation of linearity was $y = 0.000890x – 0.000250$, where x = cefoxitin concentration in µg/mL and y = cefoxitin area/cefuroxime area. The LLOQ was 10 µg/mL using a urine volume of 20 µL. The calibration curves for cefoxitin in urine are presented in Figure 33.

Compound name: Cefoxitin 1
Correlation coefficient: r = 0.999764, r^2 = 0.999528
Calibration curve: 0.000889947 * x + -0.000250007
Response type: Internal Std (Ref 2), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Include, Weighting: 1/x, Axis trans: None
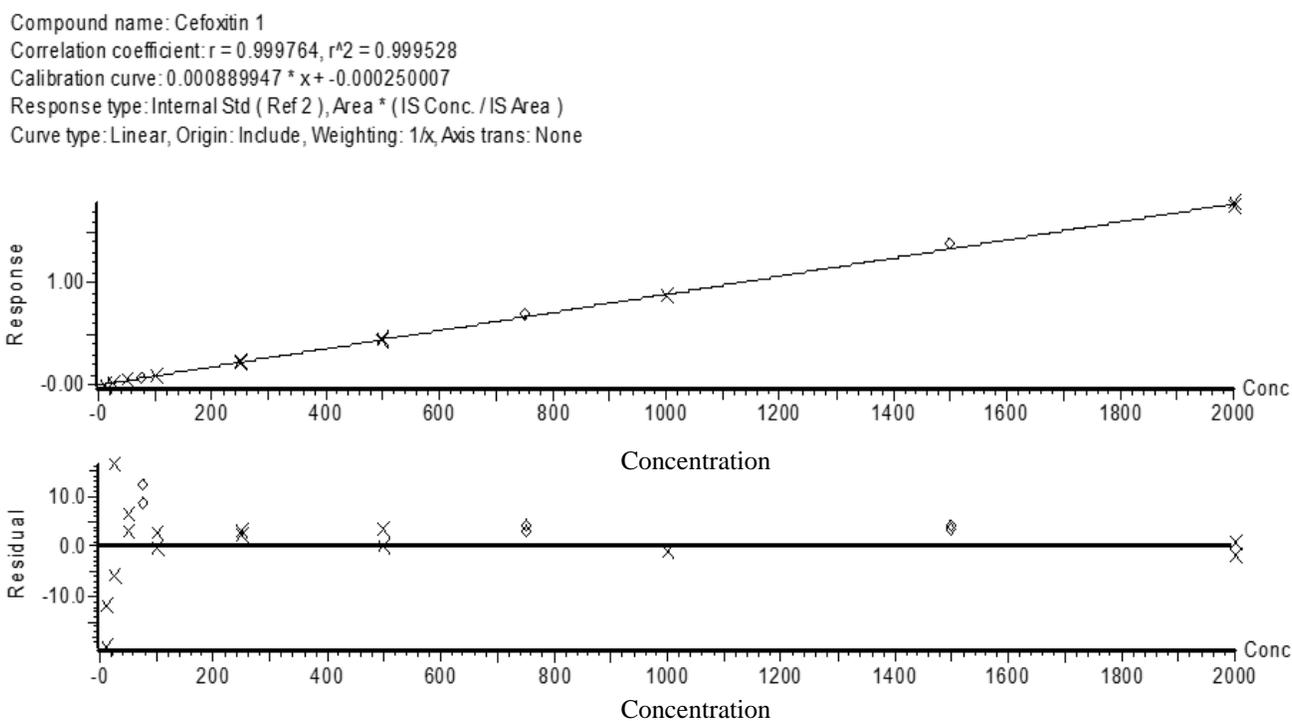
Figure 33. Standard curve of cefoxitin in urine over a concentration range of 5-2000 µg/mL along with a plot of residuals.

5.3.6 Noncompartmental Pharmacokinetic Analysis

Plasma cefoxitin concentrations assayed in blood samples collected for part-1 and part-2 of the study were used for pharmacokinetic analysis. Descriptive pharmacokinetic parameters for cefoxitin were estimated by noncompartmental analysis Phoenix WinNonlin® (Certara, St. Louis, MO). The terminal disposition rate constant (k) was obtained by linear regression of at least the last 3 data points, and half-life ($t_{1/2}$) was calculated by dividing 0.693 by k. The area under the plasma concentration-time profile from the time of dosing until infinity was calculated by the log-linear trapezoidal method with extrapolation beyond the last measured concentration, according to:

$$AUC_{0-\infty} = AUC_{0-4} + C_4/k$$

Total body clearance (CL_{Total}) and the volume of distribution during terminal phase (V_z) were determined using the following equations:

$$CL_{Total} = Dose / AUC_{0-\infty}$$

$$V_z = Dose /[(AUC_{0-\infty}) \times k]$$

Urine cefoxitin concentration in samples collected following IV dose was with the volume of urine collected for that particular time interval to estimate the amount of cefoxitin renally eliminated in a given time depending on last urine collection. Sum of amounts of cefoxitin eliminated for all urine collections was used to estimate the total amount of cefoxitin renally eliminated in 4 hours ($A_{e(0-4)}$). Renal clearance (CL_{Renal}) was estimated using the following equation:

$$CL_{Renal} = (A_{e(0-4)})/ AUC_{0-4}$$

Cefoxitin tubular reabsorption was assumed to be negligible (0 mL/min), cefoxitin filtration clearance ($CL_{Filtration}$) and tubular secretion clearance ($CL_{Secretion}$) were estimated using the following equations:

$$CL_{Filtration} = fu \times CL_{Cr}$$

$$CL_{Secretion} = CL_{Renal} - CL_{Filtration}$$

Where fu is the fraction of cefoxitin unbound (0.26) [129] and CL_{Cr} is the creatinine clearance based estimate of the glomerular filtration rate which is calculated using the Cockcroft Gault equation:

$$CL_{Cr} = (140\text{-age})(\text{weight kg}) / (72 \times SrCr) \text{ in mL/min}$$

$$\text{multiplied by 0.85 for female subjects}$$

Plasma and urine cefoxitin concentration data reported by Vlasses et al [1] in the presence and absence of 1 gram probenecid given orally 1 hour before cefoxitin administration were considered in calculations. Non-compartmental PK analysis and derived PK parameters described above were employed for estimating PK parameters of cefoxitin in healthy volunteers.

5.3.7 Statistical Analysis

All data were expressed as mean ± SD (standard deviation). Student's paired t-tests will be used to statistically compare patient demographic parameters and PK parameters such as $t_{1/2}$, $AUC_{0-\infty}$, CL_{Total}, CL_{Renal}, $CL_{Filtration}$ and $CL_{Secretion}$ between living donor and deceased donor renal transplant recipients at both time-points and also within living donor renal transplant recipients comparing results for both time-points and within deceased donor renal transplant recipients comparing results for both time-points. Dose normalized PK parameters were used when comparing PK results from renal transplant recipients and PK results from healthy volunteers

(historical controls). Data was analyzed using GraphPad Prism 7 statistical software for windows (GraphPad Software, La Jolla, CA, USA). A p-value of <0.05 was considered as statistically significant difference.

5.4   RESULTS

5.4.1   Patient Demographics

Patient characteristics for subjects who completed at least one pharmacokinetic study are provided in Table 20. Forty seven renal transplant recipients who met the inclusion/exclusion criteria for the study were approached and 15 of them consented to participate in the study and underwent part 1 (PK study $\leq$ 14 days post-transplantation) and 9 of the 15 subjects who underwent Part 1 of also completed Part 2 of the study (PK study $\geq$ 90 days post-transplantation).

Table 20. Patient characteristics

Patient Characteristics	All Subjects (n=15)	LDRT (n=8)	DDRT (n=7)	*p - value
Age (years) (mean ± SD)	47.5 ± 12.7	50.3 ± 15.6	44.3 ± 8.6	0.39
Weight (kg) (mean ± SD)	86.6 ± 27.2	92.1 ± 25.9	80.3 ± 29.2	0.42
BSA (m²) (mean ± SD)	1.9 ± 0.3	2.1 ± 0.3	1.8 ± 0.2	0.05
Sex	M= 6 ; F= 9	M= 4 ; F= 4	M= 2 ; F= 5	-
African American	6	1	5	-
Caucasian	9	7	2	-
CIT (hrs) (mean ± SD)	8.1 ± 8.1	1.3 ± 0.4	15.8 ± 4.8	<0.05
WIT (hrs) (mean ± SD)	0.8 ± 0.3	0.8 ± 0.3	0.7 ± 0.4	0.95
Transplant Reason				
IgA Nephropathy	5	2	3	-
Hypertension	5	3	2	-
DM-II/HTN	2	2	0	-
Other	3	1	2	-
Donor Information				
Age (years) (mean ± SD)	43.4 ± 15.8	47.0 ± 17.5	38.5 ± 12.9	0.31
Cadaveric =7 ; Living-Related = 7; Living unrelated =1				

LDRT: living donor renal transplant recipient; DDRT: deceased donor renal transplant recipient; BSA: body surface area; CIT: cold ischemic time; WIT: warm ischemic time; DM-II: type-2 diabetes mellitus; HTN: Hypertension; * comparing LDRT vs DDRT

Difficulty obtaining IV access and scheduling conflict were the reasons for the 6 subjects to not complete Part 2 of the study. On average, the study participants were 47.5 ± 12.7 years of age and weighed 86.6 ± 27.2 kgs. Of the 15 study participants 8 underwent LDRT and 7 underwent DDRT. Majority of LDRT recipients were Caucasian (7/8, 87.5%) and majority of DDRT recipients were African American (5/7, 71.4%). The average cold ischemic time experienced by allografts transplanted to LDRT recipients (1.3 ± 0.4 hrs) was significantly shorter compared to that of DDRT recipients (15.8 ± 4.8 hrs). Majority of the living donors were related to recipients (7/8, 87.5%) and living donors were relatively older than deceased donors but their age was not significantly different.

All subjects underwent rabbit anti-thymocyte globulin based induction therapy and received tacrolimus and mycophenolic acid based maintenance immunosuppression. Prophylactic anti-infective regimens taken by all patients included valganciclovir and sulfamethoxazole-trimethoprim. None of the patients were taking any other medications that are known to be renally eliminated by the OAT transport system.

Additional details on patient characteristics before starting Part 1 and Part 2 of the study are provided in Table 21. On average all study subjects were 7.1 ± 2.3 days post transplantation before starting Part 1 of the study and 115.6 ± 20.0 days before starting Part 2 of the study with 114.3 ± 21.0 days between both the PK studies. All patients had stable renal function during both the PK studies and the tacrolimus tough levels were within their target therapeutic ranges. Although the serum creatinine for DDRT recipients (1.2 ± 0.1 mg/dL) was significantly lower than that of LDRT recipients (1.4 ± 0.1 mg/dL), this was not a clinically significant difference.

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Table 21. Patient characteristics comparing LDRT vs DDRT and Part 1 vs Part 2

Patient Characteristics Comparing LDRT vs DDRT			
	LDRT Recipient (mean±SD), N=8	**DDRT Recipient** (mean±SD), N=7	***p - value**
Part 1: Days Since Tx	6.9 ± 1.8	7.3 ± 3.0	0.75
Part 2: Days Since Tx	112.8 ± 10.6	121.50 ± 27.1	0.41
Days Between Part 1 & 2	110.8 ± 13.7	118.8 ± 29.7	0.51
SrCr (mg/dL)	1.4 ± 0.1	1.2 ± 0.08	<0.05
CrCL (mL/min)	55.9 ± 19.5	52.5 ± 8.4	0.68
Blood Concentrations of FK (ng/mL)	8.2 ± 1.9	9.9 ± 3.2	0.21
Patient Characteristics Comparing all Subjects at Part 1 vs Part 2			
	Part 1: ≤ 14 Days Post-Tx (mean±SD), N=15	**Part 2: ≥ 90 days Post-Tx** (mean±SD), N=9	***p - value**
Days Since Tx	7.1 ± 2.3	115.6 ± 20.0	-
Days Between Part 1 & 2	114.3 ± 21.0		-
SrCr (mg/dL)	1.5 ± 0.7	1.3 ± 0.1	0.30
CrCL (mL/min)	49.8 ±17.4	54.4 ± 14.8	0.52
Blood Concentrations of FK (ng/mL)	8.8 ± 2.9	9.1 ± 2.1	0.78

LDRT: living donor renal transplant recipient; DDRT: deceased donor renal transplant recipient; SrCr: serum

creatinine; CrCL: creatinine clearance calculated by Cockcroft Gault equation; FK: tacrolimus trough level

* comparing LDRT vs DDRT and Part 1 vs Part 2

In the historical control study, 6 male volunteers between the ages of 21 and 35 years

participated in the crossover study. All subjects were within ±10% of ideal body weight and

evaluated to be healthy based on physical examinations and results of laboratory screening

studies as determined by the investigators [1].

5.4.2   Safety and Tolerability

Cefoxitin given at a low-dose of 200 mg as an IV push over 1-2 minutes was well tolerated. There were no injection site reactions in any of the patients, and none of the patients were allergic to cefoxitin. No changes were observed in biochemical indices of kidney or liver function after administration. Two patients experienced metallic taste following cefoxitin administration and this was resolved within 5 minutes. The resolution of this effect is consistent with the observed rapid disposition of cefoxitin.

5.4.3   Pharmacokinetics of Cefoxitin: Non-Compartmental Analysis

Cefoxitin was used as a surrogate marker to assess the renal anionic secretory capacity in renal transplant recipients. Cefoxitin pharmacokinetics studies were performed in 8 LDRT and 7 DDRT recipients following administration of a single dose of 200 mg cefoxitin as an IV push over 1-2 minutes, cefoxitin 4 hr plasma and urine PK studies were conducted within 14 days post-transplantation and beyond 3 months post transplantation. This study was performed to investigate longitudinal changes in renal anionic secretory capacity in LDRT and DDRT recipients, to investigate the effect of prolonged cold ischemia on renal anionic secretory capacity immediately and beyond 90 days post renal transplantation, and to compare renal anionic secretory capacity of renal transplant recipients to that of healthy volunteers.

5.4.3.1 Assessment of longitudinal changes in renal anionic secretory capacity in renal transplant recipients

Post-transplant changes in renal anionic secretory capacity among LDRT and DDRT recipients were evaluated by assessing cefoxitin pharmacokinetics at two early post-transplant time-points (≤14 days and ≥ 90 days post transplantation). Linear plots of cefoxitin plasma concentration versus time at both time-points are shown in Figure 34. Concentration vs Time plot of 200 mg cefoxitin given as IV push in renal transplant recipients at ≤ 14 Days (blue) and ≥ 90 days (orange) post transplantation.. The concentration-time curves were virtually superimposable, suggesting no difference in cefoxitin clearance in renal transplant recipients by at least 90 days post transplantation when compared to immediately after transplantation. A summary of pharmacokinetic parameters for IV cefoxitin at these two time-points is presented in Table 22.
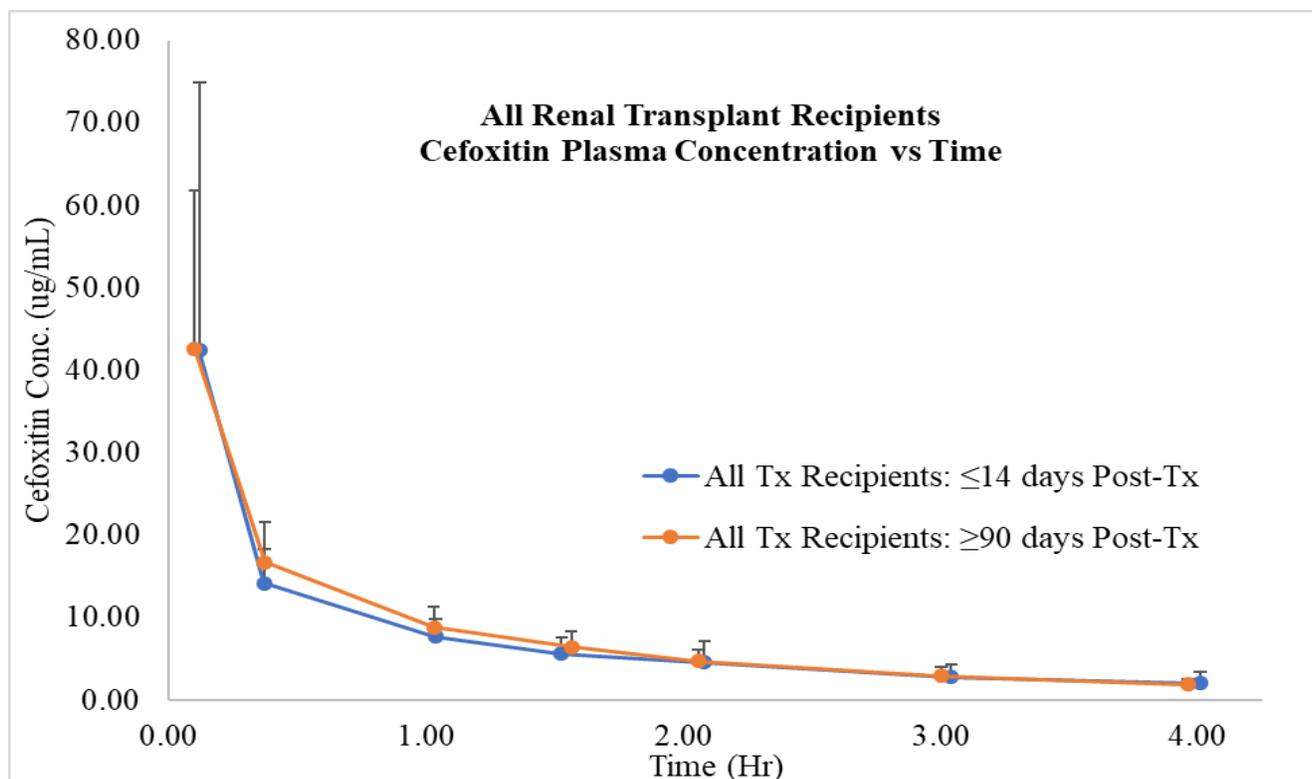
Figure 34. Concentration vs Time plot of 200 mg cefoxitin given as IV push in renal transplant recipients at ≤ 14 Days (blue) and ≥ 90 days (orange) post transplantation.

Tx: transplantation

Table 22. Summary of pharmacokinetic parameters of 200 mg cefoxitin in renal transplant recipients at ≤ 14 Days and ≥ 90 days post transplantation

PK Parameters	Part 1 ≤ 14 Days Post-Tx (N=15), mean±SD	Part 2 ≥ 90 days Post-Tx (N=9), mean±SD	Combined (N=15), mean±SD	*p - value
$AUC_{0-\infty}$ (mg*hr/L)	35.0 ± 13.1	35.6 ± 9.3	35.2 ± 11.6	0.91
$t_{1/2}$ (Hrs)	1.4 ± 0.7	1.1 ± 0.2	1.3 ±0.6	0.25
V_z (L)	15.0 ± 4.6	12.1 ± 3.4	13.9 ± 4.4	0.10
CL_{Total} (mL/min)	108.1 ± 40.0	99.3 ± 24.7	104.8 ± 34.7	0.56
CL_{Renal} (mL/min)	90.2 ± 33.4	82.9 ± 20.6	87.5 ± 29.0	0.56
$CL_{Filtration}$ (ml/min)	13.0 ± 4.5	14.1 ± 3.4	13.4 ± 4.2	0.52
$CL_{Secretion}$ (mL/min)	77.3 ± 28.9	73.3 ± 25.1	74.1 ± 24.8	0.74

$AUC_{0-\infty}$: area under the concentration-time curve from time dose administration to infinite time; $t_{1/2}$: half-life; Vz: terminal volume of distribution; CL_{Total}: cefoxitin total clearance; CL_{Renal}: cefoxitin renal clearance; $CL_{Filtration}$: cefoxitin filtration clearance; $CL_{Secretion}$: cefoxitin secretion clearance;* comparing Part 1 and Part 2.

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Cefoxitin exposure ($AUC_{0-\infty}$), total clearance (CL_{Total}), renal clearance (CL_{Renal}), filtration clearance ($CL_{Filtration}$) and secretion clearance ($CL_{Secretion}$) were statistically similar during Part 1 and Part 2 of the study. The majority of CL_{Total} was attributed to its $CL_{Secretion}$ (~71%). Half-life of cefoxitin in renal transplant patients is about 1.3 ±0.6 hrs in both periods.

Linear plots of cefoxitin plasma concentration versus time at both time-points among LDRT and DDRT are shown in Figure 35 and Figure 36 respectively. The concentration-time curves were virtually superimposable when looking at LDRT and DDRT recipients separately, suggesting no difference in cefoxitin clearance in renal transplant recipients by at least 90 days post transplantation when compared to immediately after transplantation in LDRT and DDRT recipients. Summaries of pharmacokinetic parameters for IV cefoxitin at these two time-points in LDRT and DDRT recipients are presented in Table 23 and Table 24 respectively.
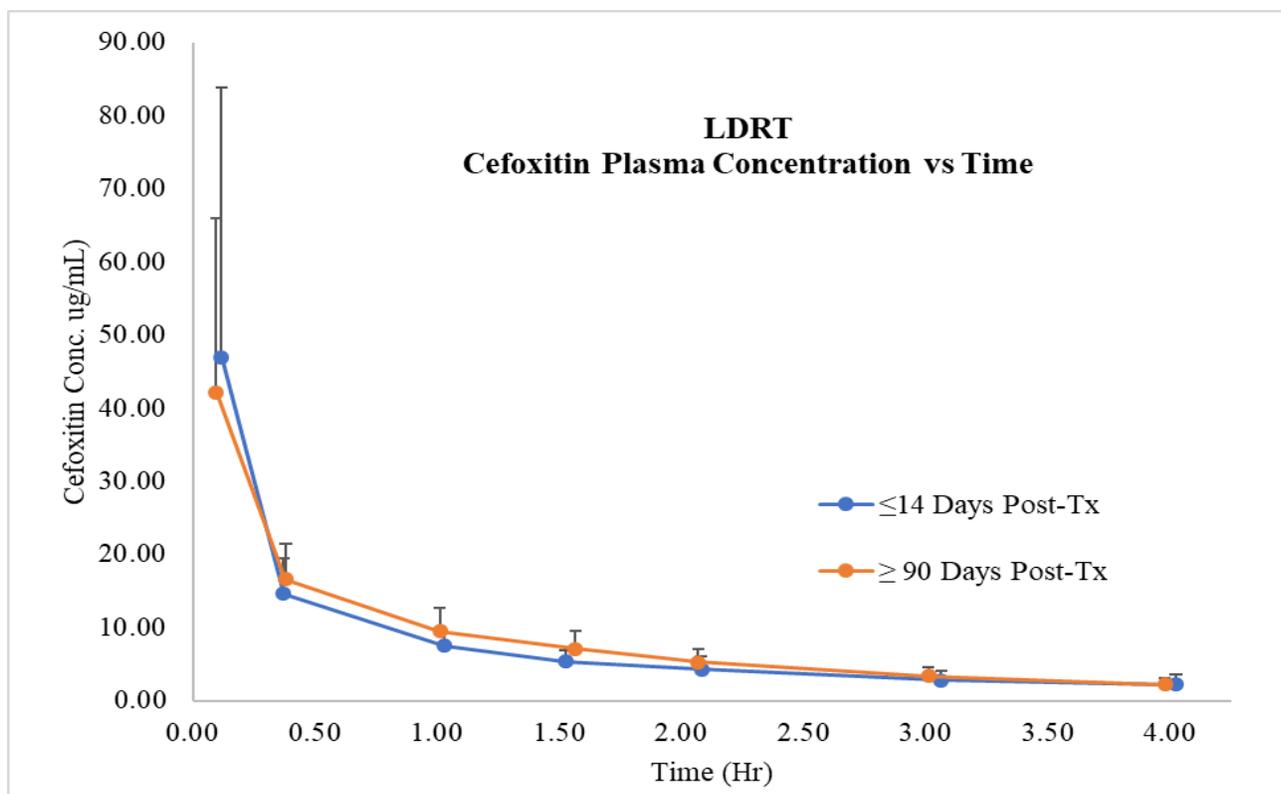
Figure 35. Concentration vs Time plot of 200 mg cefoxitin given as IV push in LDRT renal transplant recipients at ≤ 14 Days (blue) and ≥ 90 days (orange) post transplantation.

LDRT: living donor renal transplant recipients; Tx: transplantation

Table 23. Summary of pharmacokinetic parameters of 200 mg cefoxitin in LDRT recipients at ≤ 14 Days and

≥ 90 days post transplantation

PK Parameters	Part 1 ≤ 14 Days Post-Tx (N=8), mean±SD	Part 2 ≥ 90 days Post-Tx (N=5), mean±SD	Combined LDRT (N=8), mean±SD	*p - value
$AUC_{0-\infty}$ (mg*hr/L)	36.3 ± 9.7	38.0 ± 11.3	37.0 ± 9.9	0.77
$t_{1/2}$ (Hr)	1.5 ± 0.8	1.2 ± 0.2	1.4 ± 0.7	0.47
Vz (L)	15.2 ± 4.70	12.3 ± 4.3	14.1 ± 4.60	0.28
CL_{Total} (mL/min)	97.4 ± 24.3	94.9 ± 30.5	96.5 ± 25.6	0.87
CL_{Renal} (mL/min)	81.4 ± 20.3	79.2 ± 25.5	80.6 ± 21.4	0.88
$CL_{Filtration}$ (ml/min)	13.2 ± 5.3	14.5 ± 5.1	13.7 ± 5.03	0.66
$CL_{Secretion}$ (mL/min)	68.2 ± 15.0	64.7 ± 20.4	66.8 ± 16.4	0.73

$AUC_{0-\infty}$: area under the concentration-time curve from time dose administration to infinite time; $t_{1/2}$: half-life; Vz: terminal volume of distribution; CL_{Total}: cefoxitin total clearance; CL_{Renal}: cefoxitin renal clearance; $CL_{Filtration}$: cefoxitin filtration clearance; $CL_{Secretion}$: cefoxitin secretion clearance;* comparing Part 1 and Part 2.
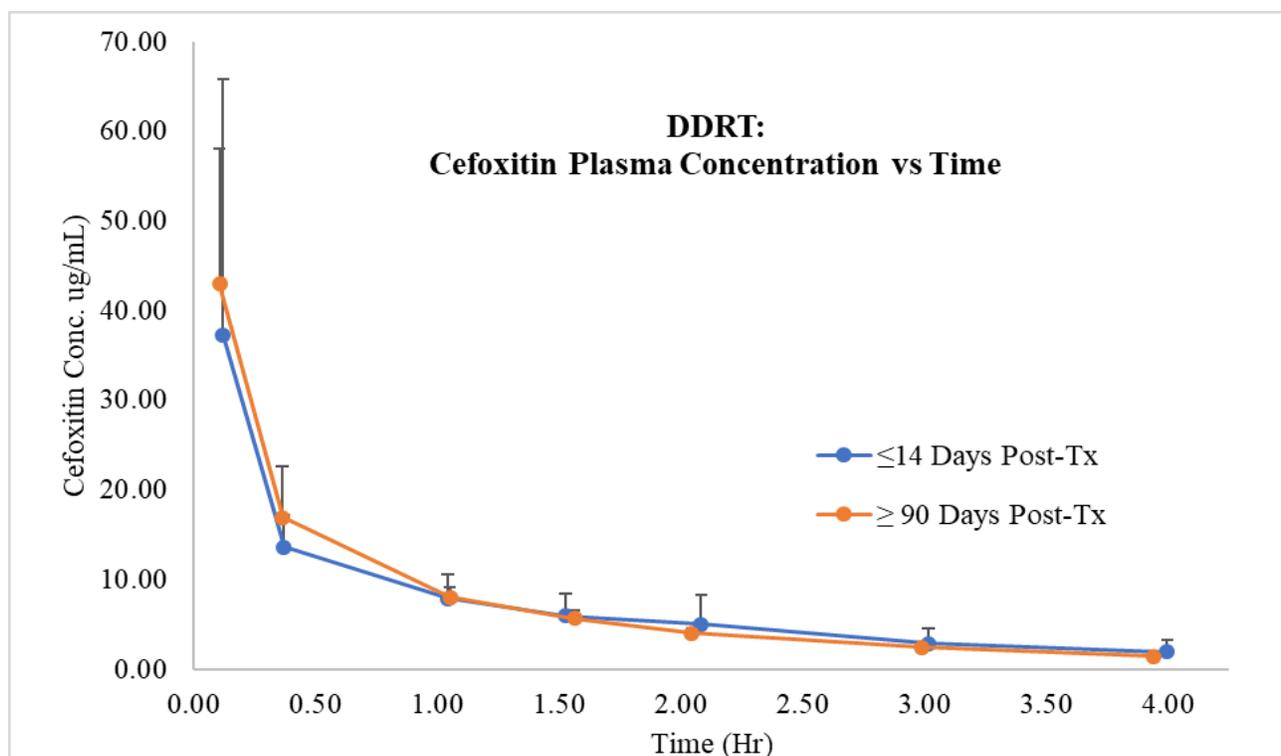
Figure 36. Concentration vs Time plot of 200 mg cefoxitin given as IV push in DDRT renal transplant recipients at ≤ 14 Days (blue) and ≥ 90 days (orange) post transplantation.

DDRT: deceased donor renal transplant recipients; Tx: transplantation

Table 24. Summary of pharmacokinetic parameters of 200 mg cefoxitin in DDRT recipients at ≤ 14 Days and ≥ 90 days post transplantation

PK Parameters	Part 1 ≤ 14 Days Post-Tx (N=7), mean±SD	Part 2 ≥ 90 days Post-Tx (N=4), mean±SD	Combined DDRT (N=7), mean±SD	*p - value
$AUC_{0-\infty}$ (mg*hr/L)	33.6 ± 16.9	32.6 ± 6.1	33.2 ± 13.6	0.92
$t_{1/2}$ (Hr)	1.3 ± 0.5	1.0 ± 0.2	1.2 ± 0.4	0.30
V_z (L)	14.8 ± 4.8	11.8 ± 2.6	13.7 ± 4.3	0.28
CL_{Total} (mL/min)	120.2 ± 52.2	104.7 ± 17.7	114.6 ± 42.3	0.60
CL_{Renal} (mL/min)	100.3 ± 43.6	87.5 ± 14.8	95.7 ± 35.3	0.58
$CL_{Filtration}$ (mL/min)	12.7 ± 3.8	13.6 ± 2.18	13.0 ± 3.2	0.66
$CL_{Secretion}$ (mL/min)	87.7 ± 39.7	73.8 ± 12.6	82.6 ± 32.1	0.52

$AUC_{0-\infty}$: area under the concentration-time curve from time dose administration to infinite time; $t_{1/2}$: half-life; Vz: terminal volume of distribution; CL_{Total}: cefoxitin total clearance; CL_{Renal}: cefoxitin renal clearance; $CL_{Filtration}$: cefoxitin filtration clearance; $CL_{Secretion}$: cefoxitin secretion clearance;* comparing Part 1 and Part 2.

Cefoxitin exposure ($AUC_{0-\infty}$), total clearance (CL_{Total}) , renal clearance (CL_{Renal}), filtration clearance ($CL_{Filtration}$) and secretion clearance ($CL_{Secretion}$) were statistically similar during Part 1 and Part 2 of the study for LDRT and DDRT recipients when compared separately. Majority of CL_{Total} was attributed to its $CL_{Secretion}$ (~72%). Average half-life of cefoxitin in LDRT and DDRT were similar (1.4 ± 0.67 hrs and 1.2 ± 0.41 hrs respectively).

5.4.3.2 Effect of prolonged cold ischemia on renal anionic secretory capacity in kidney transplant recipients on tacrolimus based maintenance immunosuppression therapy

Renal anionic secretory capacity between LDRT recipients with allografts that underwent an average of 1.3 ± 0.4 hrs of cold ischemia and DDRT recipients with allografts that underwent an average of 15.8 ± 4.8 hrs of cold ischemia were compared to study the effect of prolonged cold ischemia at two time-points post transplantation (≤14 days and ≥90 days post transplantation). Cefoxitin pharmacokinetics among LDRT and DDRT recipients were compared at both early time-points and the linear plots of cefoxitin plasma concentration versus time during Part 1 (≤14 days post transplantation) and Part 2 (≥ 90 days post transplantation) are shown in Figure 37 and Figure 38 respectively.
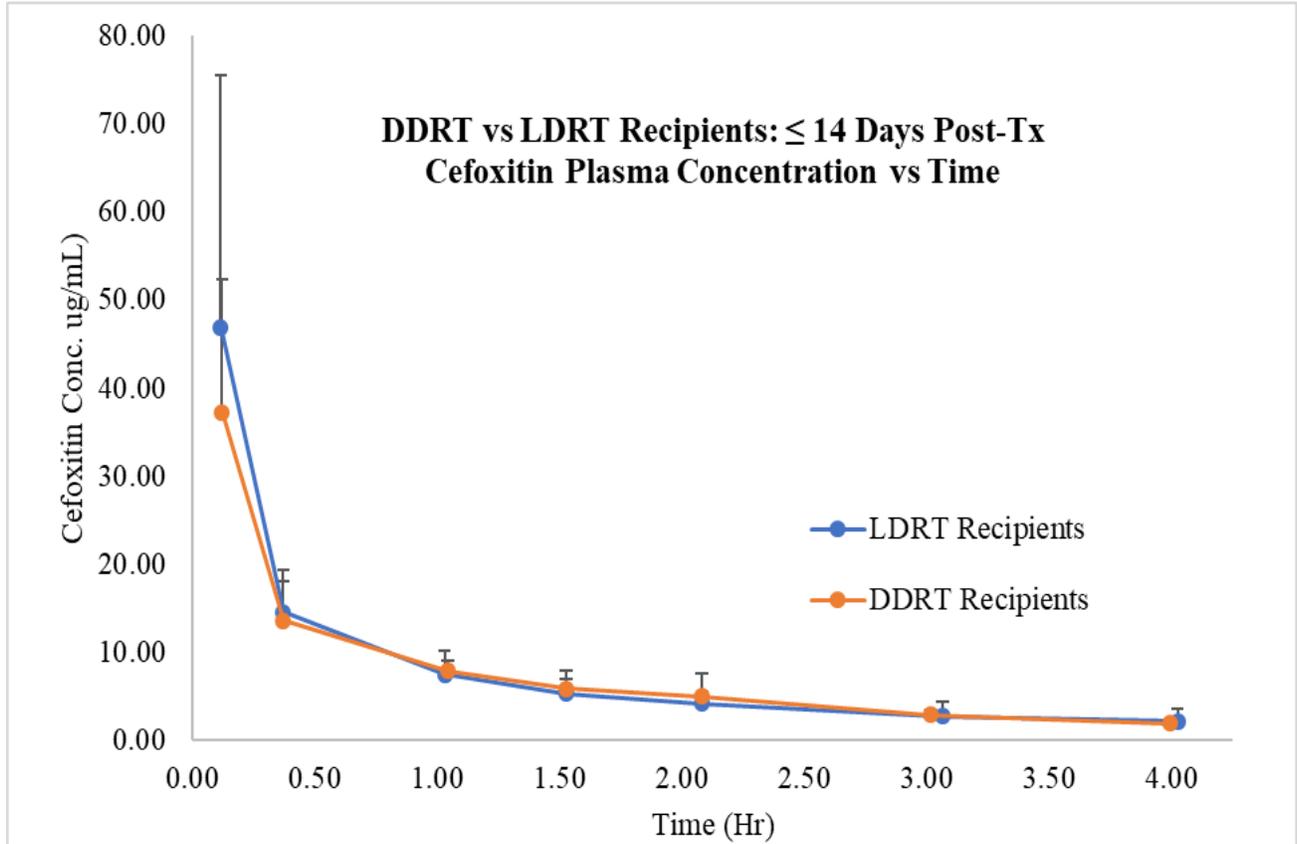
Figure 37. Concentration vs Time plot of 200 mg cefoxitin given as IV push in LDRT (blue) and DDRT (orange)

renal transplant recipients at ≤ 14 days post transplantation.

LDRT: living donor renal transplant; DDRT: deceased donor renal transplant; Tx: transplantation
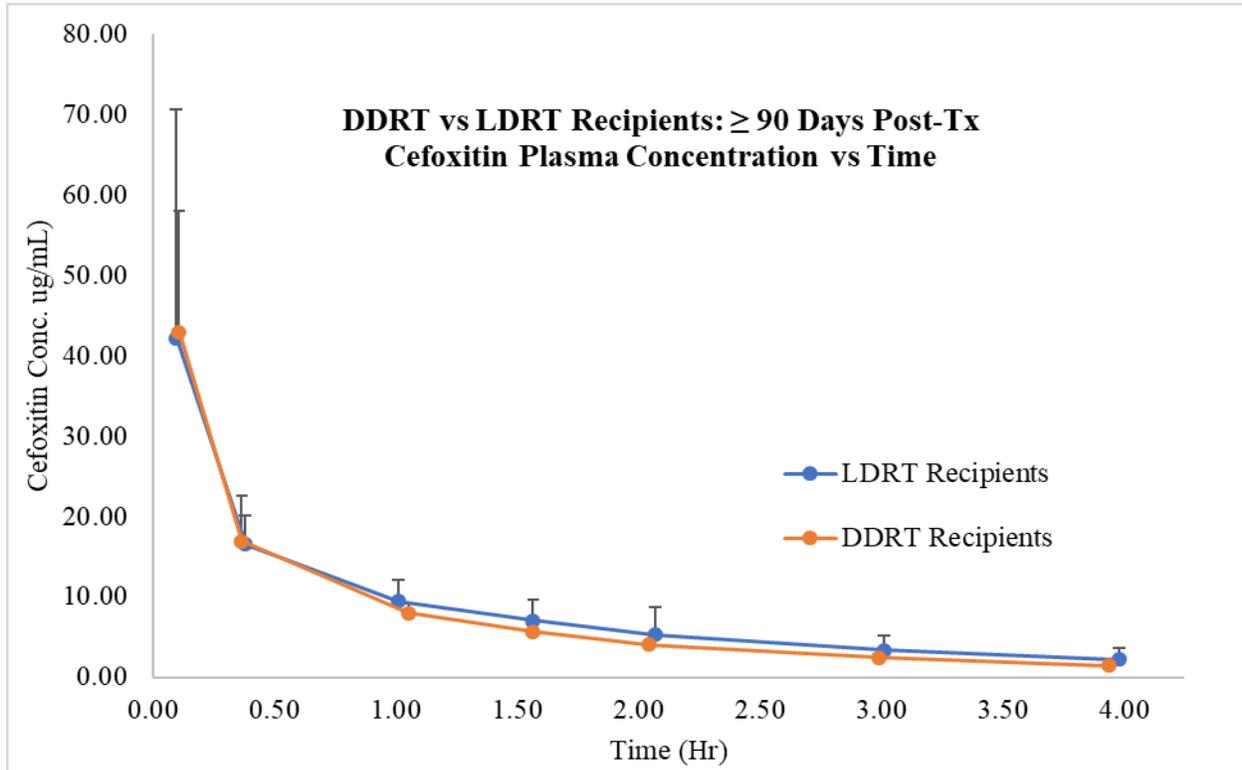
Figure 38. Concentration vs Time plot of 200 mg cefoxitin given as IV push in LDRT (blue) and DDRT (orange) renal transplant recipients at ≥ 90 days post transplantation.

LDRT: living donor renal transplant; DDRT: deceased donor renal transplant; Tx: transplantation

Cefoxitin exposure ($AUC_{0-\infty}$), total clearance (CL_{Total}), renal clearance (CL_{Renal}), filtration clearance ($CL_{Filtration}$) and secretion clearance ($CL_{Secretion}$) were statistically similar between LDRT and DDRT recipients during Part 1 and Part 2 of the study when compared separately. There was no significant impact of prolonged cold ischemia (15.8 ± 4.8 hrs for DDRT vs 1.3 ± 0.4 hrs for LDRT recipients) on renal anionic secretion of cefoxition immediately after transplantation and beyond 90 days post transplantation (Table 23 and Table 24).

5.4.3.3 Comparing renal anionic secretory capacity of renal transplant recipients with that of healthy volunteers

Healthy volunteer data from a crossover cefoxitin pharmacokinetic study performed in the presence and absence of orally administered probenecid (anionic secretion blocker) was used to compare renal anionic secretory capacity of renal transplant recipients [1]. In the first part of the healthy volunteer crossover study, subjects underwent a 4 hr cefoxitin PK study following IV administration of 2 grams cefoxitin over 3 min. Following a 1-week washout period, they underwent a 4 hr cefoxitin PK study following IV administration of 2 grams cefoxitin over 3 min and oral administration of 1 gram probenecid 1 hour prior to cefoxitin administration. Summarized linear plots of dose normalized cefoxitin concentration versus time in renal transplant patients (15 patients; 24 PK studies) and in historical healthy controls (6 patients) with and without probenecid treatment are shown in Figure 39.
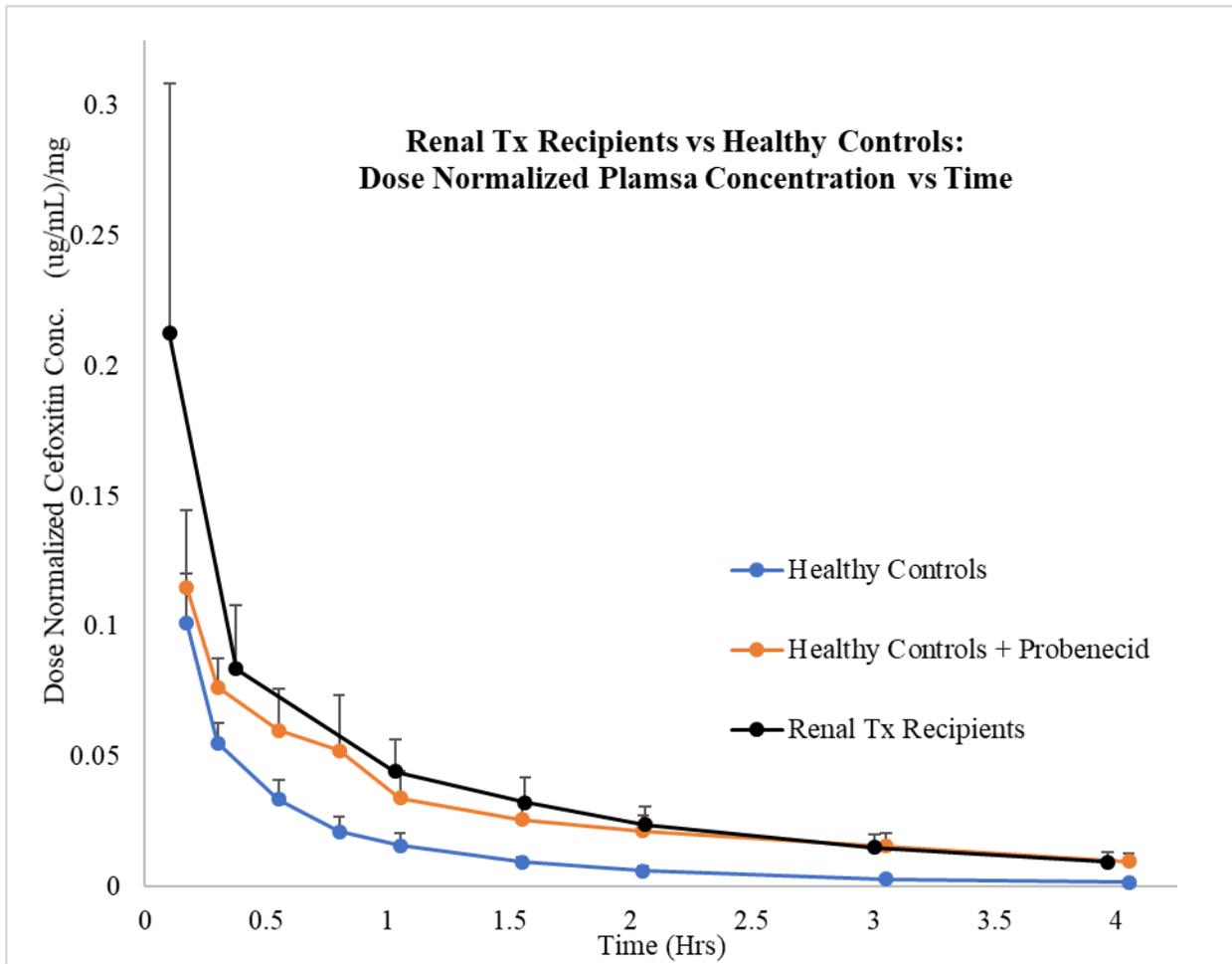
Figure 39. Dose normalized concentration vs time plot following administration of IV cefoxitin in renal transplant recipients in early post-transplant period (black), historical healthy controls without probenecid treatment (blue), and historical healthy controls

Cefoxitin concentration-time data in healthy volunteers reported by Vlasses et al [1] was used as historical healthy controls.

Visually, the dose normalized concentration-time curves suggest that renal transplant recipients experience a higher exposure of cefoxitin when compared to healthy volunteers not treated with probenecid. There was no statistically significant difference in cefoxitin exposure when comparing renal transplant recipients and healthy volunteers who are treated with probenecid. A summary of pharmacokinetic parameters for IV cefoxitin in these subjects is provided in Table 25.

Table 25. Comparison of dose normalized cefoxitin PK parameters between healthy controls ± 1 g probenecid administered orally 1 hr prior to cefoxitin administration and in renal transplant recipients

PK Parameters	Historical Healthy Controls (mean±SD)	Historical Healthy Controls + 1g Probenecid (mean±SD)	Renal Tx Recipients (mean±SD)	*p*- value
$AUC_{0-\infty}$/Dose (mg*hr/L)/g	68.5 ± 8.1	$170.1 \pm 43.9^{\text{ᵎ}}$	176.2 ± 58.0	0.0001
$t_{1/2}$ (Hr)	0.6 ± 0.1	$1.5 \pm 0.2^{\text{ᵎ}}$	1.3 ± 0.6	0.0070
V_z (L)	17.5 ± 5.1	16.1 ± 5.2	13.9 ± 4.4	0.090
CL_{Total} (mL/min)	246.2 ± 29.8	$105.8 \pm 37.5^{\text{ᵎ}}$	104.8 ± 34.7	0.0001
CL_{Renal} (mL/min)	205.6 ± 24.9	$88.3 \pm 31.3^{\text{ᵎ}}$	87.5 ± 30.0	0.0001
$CL_{Secretion}$ (mL/min)	~117	-	74.1 ± 24.8	-

Cefoxitin concentration-time data in healthy volunteers reported by Vlasses et al [1] was used as historical healthy controls. $AUC_{0-\infty}$: area under the concentration-time curve from time dose administration to infinite time; $t_{1/2}$: half-life; Vz: terminal volume of distribution; CL_{Total}: cefoxitin total clearance; CL_{Renal}: cefoxitin renal clearance; $CL_{Filtration}$: cefoxitin filtration clearance; $CL_{Secretion}$: cefoxitin secretion clearance; Tx: transplantation

* comparing healthy controls and renal Tx recipients; ᵎ comparing healthy controls and healthy controls + probenecid

Renal transplant recipients had significantly higher dose normalized exposures of cefoxitin when compared to healthy volunteers who were not administered probenecid (176.2±58.0 vs 68.5±8.10 mg*hr/L/g). Total clearance (CL_{Total}) and renal clearance (CL_{Renal}) were significantly lower in renal transplant recipients when compared to healthy volunteers who were not administered probenecid. $CL_{secretion}$ in healthy controls was estimated to be about 117

mL/min by subtracting CL_{Renal} in probenecid treated arm from CL_{Renal} in the control arm. For this estimate probenecid was assumed to have blocked all the anionic secretion in healthy volunteers. Contribution of $CL_{Secretion}$ when compared to CL_{Total} was considerably higher in renal transplant recipients (71% vs 48%). Cefoxitin exposure, CL_{Total}, CL_{Renal}, and $t_{1/2}$ were statistically similar between renal transplant recipients and healthy volunteers who were administered 1 gram of probenecid 1 hr prior to cefoxitin administration.

5.5   DISCUSSION

Following transplantation, renal transplant patients have only one functioning kidney that is subjected to various insults such as prolonged CI, CNI exposure, opportunistic infections, BKVN and acute TCMR. Clinicians routinely monitor changes in filtration capacity to evaluate allograft function and adjust dose/frequency of renally cleared drugs, including those that are primarily secreted. A better understanding of changes in secretory capacity following renal transplantation is needed to optimize pharmacotherapy of renally secreted drugs. This study is one of the first attempts to systematically assess renal anionic secretory capacity in LDRT and DDRT recipients. Longitudinal changes in cefoxitin exposure and renal secretory clearance in early post-transplant period was studied to understand the effect of renal transplantation on renal anionic secretory function; differences in cefoxitin exposure and renal secretory clearance between DDRT and LDRT recipients was studied to assess the effect of prolonged cold ischemia on renal anionic secretory capacity; dose normalized cefoxitin exposure and renal clearance in renal transplant recipients was compared with that of historical healthy controls to identify differences in renal anionic secretory capacity in renal transplant recipients.

Cefoxitin is a suitable probe drug to assess the renal anionic secretion as more than 85% of the drug is eliminated unchanged in urine, with majority of renal clearance attributed to secretion. Probenecid is a potent inhibitor of organic anionic transporters and probenecid treatment was shown to increase IV cefoxitin exposure by 2.4-fold in healthy volunteers [1]. Based on this clinical observation and relative abundance of OAT transporters in renal epithelial cells, cefoxitin is thought to be primarily taken up into renal tubular epithelial cells by OAT1 and

OAT3 transporters. The drug transporters responsible for cefoxitin efflux across the apical membrane has not been identified yet, but MRP2 and MRP4 are thought to be involved in this process since they act as efflux transporter pairs on apical side for drugs that are taken up by OAT1 and OAT3 on the basolateral side of renal tubular epithelial cells (Figure 40). Probenecid was also shown to inhibit MRP2 and MRP4 [26, 27, 29-31].
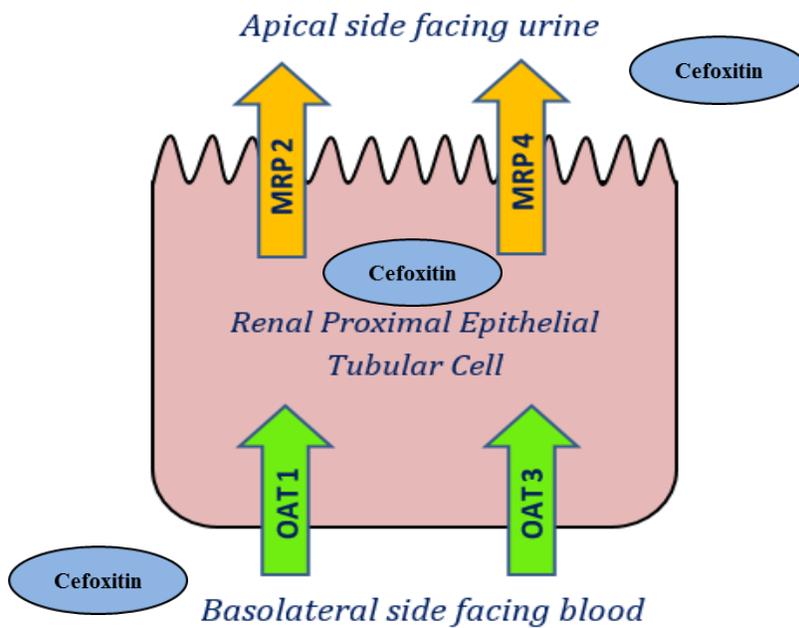
Figure 40. Orientation of OAT1/OAT3 uptake transporters and MRP2/MRP4 efflux transporters in renal proximal epithelial tubular cells [1]

Results of the longitudinal study shows that cefoxitin exposure and renal secretory clearance in renal transplant patients remains unchanged in the early post-transplant period when comparing cefoxitin PK within 14 days post-transplantation and that beyond 90 days post-transplantation (Table 22). There were no longitudinal differences in cefoxitin pharmacokinetics when LDRT and DDRT recipients were compared separately (Table 23 and Table 24). This suggests that there are no clinically significant changes in function of renal anionic transporters

in the early post-transplant period among patients without BKVN or Banff1A or higher acute TCMR. Half-life of cefoxitin in renal transplant patients was 1.3 ±0.6 hrs for both periods.

The effect of prolonged CI in the presence of tacrolimus treatment was studied by comparing cefoxitin pharmacokinetics between DDRT recipients with 15.8±4.8 hrs CIT and LDRT recipients with 1.3±0.4 hrs CIT. There was no significant difference in cefoxitin exposure between DDRT and LDRT recipients during Part-1 ($\leq$ 14 Days Post-Tx) and Part-2 ($\geq$ 90 days Post-Tx) of the study (Table 23 and Table 24). These results show that renal anionic secretory capacity of allografts that are subjected to prolonged cold ischemia (in DDRT) is similar to that of allografts that are not subjected to prolonged cold ischemia (in LDRT). However, the LDRT group was primarily Caucasian and DDRT group was primarily African American.

Although 47 de-novo renal transplant recipients were approached, only 15 consented to participate in the study. Some of the limitations in this prospective pilot study include limited number of study subjects in LDRT (n=7) and DDRT (n=8) groups. Of the 15 patients who participated in Part 1 of the study, only 9 (5 LDRT recipients and 4 DDRT recipients) completed Part 2 of the study. Difficulty obtaining IV access and scheduling conflict were the reasons for the 6 subjects to not complete Part 2 of the study.

Cefoxitin renal clearance was estimated in 21 of the 24 PK studies as patients accidentally flushed-down the urine samples in 3 instances. Most urine samples were measured in urine collection jugs with the exception of a few instances where the study nurse recorded urine volumes using a urine hat. The average duration of urine collection was 4.3 ± 1.0 hrs. The amount of cefoxitin excreted unchanged into the urine for both study periods is presented in Table 26. There was no statistically significant difference in the amount of drug excreted into the urine between all four groups.

Table 26. Cefoxitin urine data

Study grouping	Duration of Urine Collection (Hr)	Amount of Drug Excreted into Urine, Ae (mg)	Percent of Drug Excreted into Urine (%)
LDRT	4.0 ± 0.3	164.5 ± 25.6	82.1 ± 12.6
DDRT	5.0 ± 1.8	189.1 ± 13.6	94.1 ± 7.7
≤ 14 Days Post-Tx	4.5 ± 1.2	166.3 ± 24.3	83.2 ± 12.2
≥ 90 days Post-Tx	4.5 ± 1.6	192.4 ± 11.5	95.4 ± 7.1

LDRT: living donor renal transplant recipients; DDRT: deceased donor renal transplant recipients; Tx: transplantation

Cefoxitin pharmacokinetics in renal transplant recipients in early post-transplant period was compared to that in historical healthy volunteers (with and without probenecid treatment) to understand differences in renal anionic secretory capacity between these two populations. Dose normalized cefoxitin exposure in renal transplant recipients was significantly higher ($AUC_{0-\infty}$/Dose: 176.2 ± 58.0 mg*hr/L/g) when compared to healthy controls who were not treated with probenecid ($AUC_{0-\infty}$/Dose: 68.5 ± 8.1 mg*hr/L/g). Cefoxitin renal clearance was 57.3% lower (renal-Tx: 87.5 ± 29.0 mL/min; healthy volunteers: 205.6 ± 24.9 mL/min) and half-life was 2.2-fold higher (renal-Tx: 1.3 ± 0.6 hrs; healthy volunteers: 0.6 ± 0.1 hrs) in renal transplant recipients when compared to healthy volunteers. Contribution of $CL_{Secretion}$ when compared to CL_{Total} was considerably higher in renal transplant recipients (71% vs 48%). There was no statistically significant difference in cefoxitin pharmacokinetic parameters when comparing renal transplant recipients (1 functioning kidney) and healthy volunteers (2 functioning kidneys) who were treated with 1 gram of oral probenecid 1 hour prior to cefoxitin therapy (Table 25). These findings suggest that renal anionic tubular secretion in renal transplant recipients is significantly lower compared to healthy volunteers. The findings of this study are consistent with the observations in the rat renal anionic transporter expressions in allografts that were subject to prolonged CI and tacrolimus treatment (Chapter 2.0). Although absolute gene

expression of OAT1, OAT3, MRP2 and MRP4 transporters in renal allografts has been quantitated using sensitive nCounter® assay (Chapter 3.0), a comparative quantitation in healthy renal tissue is currently not available. Currently published gene expression studies reported relative expression of renal transporters using semi-quantitative approaches (RT-qPCR and Microarray) [7, 8, 10]. In comparison to these findings, transporter gene expression studies in renal biopsies from patients with Banff IA or higher acute TCMR with interstitial fibrosis show that OAT system is significantly compromised in these patients (OAT1: 11-fold lower; OAT3: 4.4-fold lower; MRP2: 6.78-fold lower; MRP4: 2.01-fold lower) when compared to biopsies from healthy allografts (Table 10). Preliminary clinical observations in renal transplant recipients with BKVN involving cidofovir treatment in the presence and absence of probenecid suggest that renal anionic secretory function is decreased in allografts with BKVN [100].

Historical healthy controls were used to compare the results of this prospective study in renal transplant recipients. The healthy volunteer study was conducted several decades ago with less sensitive bioanalytical methods in human serum. All healthy volunteers were young male subjects between the ages of 21 and 35 years. In this study the investigators did not estimate cefoxitin filtration clearance and so cefoxitin secretory clearance could not be accurately estimated in healthy volunteers. Assuming 1 g probenecid blocked all the anionic secretion in healthy volunteers, $CL_{secretion}$ in healthy controls was estimated to be about 117 mL/min by subtracting CL_{Renal} in probenecid treated arm from CL_{Renal} in the control arm.

Renal anionic secretory function in renal transplant recipients is reduced by 61% as evidenced by differences in cefoxitin pharmacokinetics between renal transplant recipients and healthy volunteers (no probenecid treatment). There were no differences in renal anionic

secretory capacity within de-novo renal transplant recipients who had no BKVN or acute TCMR and underwent rabbit anti-thymocyte globulin based induction regimen and tacrolimus based maintenance immunosuppression. Results of this study suggest that cefoxitin secretion per functioning kidney is higher in renal allografts when compared to healthy kidneys.

Low-dose cefoxitin was well tolerated by study subjects with no adverse events. Four-hour PK study was sufficient to characterize cefoxitin secretion in this patient population. Currently transplant clinicians adjust dosage and frequency of all renally excreted anionic drugs based on CL_{Cr}. This study shows that renal transplant recipients would need significantly lower dosage of drugs that are renally secreted via organic anionic transport system despite having normal CL_{Cr} (LDRT recipients: 55.9 ± 19.5 mL/min; DDRT recipients: 52.5 ± 8.4 mL/min) post-transplantation for dosing renally cleared drugs.

A prospective study evaluating renal anionic secretion in renal transplant recipients with no complications, BKVN, acute TCMR and in healthy volunteers is warranted. A cefoxitin micro-dosing pharmacokinetic study with limited sampling and dried-blood-spot based sample collection should be conducted in renal transplant patients in order to validate cefoxitin micro-dosing, limited and minimally-invasive sampling strategy in this population. A validated study will give us the ability to evaluate renal anionic secretion in more renal transplant patients and would give clinicians the opportunity to optimize pharmacotherapy of renally secreted drugs.

6.0 PHYSIOLOGICALLY BASED PHARMACOKINETIC MODELING OF OAT/MRP TRANSPORT SYSTEM IN RENAL TRANSPLANT RECIPIENTS

6.1   ABSTRACT

Renal allografts are subjected to a unique set of injurious conditions such as prolonged CI, CNI induced nephrotoxicity, BKVN and varying grades of acute TCMR. Tubular damage caused by these factors could lead to alteration in the expression and activity of renal drug transporters which may eventually affect the clearance of drugs that are predominantly cleared by renal secretion. Renal OATs are specifically of interest in the context of renal transplant recipients as they are involved in the clearance of various medications prescribed to renal transplant recipients. OAT1/OAT3 renal uptake transporters and MRP2/MRP4 efflux transporter are thought to work as a paired transport system to secrete anionic drugs. Cefoxitin is a suitable probe drug to assess the renal anionic secretion as it primarily undergoes anionic secretion, it has a short half-life, good safety profile, and offers feasibility to conduct PK studies with low doses within a short study duration. In the present study, PBPK modeling approach was used to help us gain a better understanding of the impact of physiological changes following renal transplantation on the disposition of cefoxitin. Full-PBPK model of IV cefoxitin in healthy subjects was built and validated. Virtual renal transplant population was developed in SimCyp® PBPK software and cefoxitin PBPK model was validated in this population. The PBPK model incorporated organic anionic transport system based disposition of cefoxitin and its disposition into 12 major tissues in the body. The model was robust in predicting cefoxitin exposure in 3 independent studies (healthy subjects and renal transplant recipients) across a dose range of 200-2000 mg. All predicted IV cefoxitin PK parameters fell well within ±14% range of the corresponding PK parameters calculated from observed studies. With this PBPK model we have the ability to predict drug exposure of cefoxitin and other anionic drugs that are primarily disposed by renal OAT systems in renal transplant recipients.

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6.2   INTRODUCTION

Kidney transplantation is the treatment of choice for the patients diagnosed with ESRD with more than 16,000 kidney transplants performed in the US every year [3, 76]. Renal allografts are subjected to a unique set of injurious conditions such as prolonged CI before being transplanted into the recipient, warm reperfusion injury immediately after transplantation, exposure to nephrotoxic CNI based immunosuppression therapy, acute/chronic rejection of the organ, and bacterial/fungal/viral infections post-transplantation [3, 76, 78, 79, 84-86]. The tubular damage caused by these factors could lead to alteration in the expression and activity of renal drug transporters which primarily reside in renal tubular epithelial cells. This damage may eventually affect the clearance of drugs that are predominantly cleared by renal secretion. As described in the prospective cefoxitin clinical pharmacokinetic study in renal transplant recipients (Chapter 5.0), anionic secretory capacity of renal allografts is reduced by 61% as evidenced by differences in cefoxitin pharmacokinetics between renal transplant recipients and historical healthy controls. This study showed that renal transplant recipients would need significantly lower dosage of drugs that are renally secreted via organic anionic transport system despite having normal eGFR for dosing drugs (LDRT recipients: 55.9 ± 19.5 mL/min; DDRT recipients: 52.48 ± 8.39 mL/min) post-transplantation.

Renal organic anionic transporters are specifically of interest in the context of renal transplant recipients as they are involved in the clearance of various medications prescribed to renal transplant recipients. OAT1 and OAT3 renal uptake transporters are considered to be the most important renal organic anionic transporters by the US FDA and EMA for their relative

abundance, role in drug disposition and drug-drug interactions [9]. For the disposition of various anti-infective medications, MRP2 and MRP4 are thought to be the efflux partners for OAT1 and OAT3 [26, 27, 29-31].

Cefoxitin is a suitable probe drug to assess the renal anionic secretion as more than 85% of the drug is eliminated unchanged in urine, with majority of renal clearance attributed to anionic secretion, a short half-life (0.59 ± 0.13 hrs in healthy volunteers; 1.3 ± 0.6 hrs in renal transplant recipients), good safety profile [1, 129] and offers feasibility to conduct pharmacokinetic studies with low IV/IM doses within a short study duration. Based on clinical observations and relative abundance of OAT transporters in renal epithelial cells, cefoxitin is thought to be primarily taken up into renal tubular epithelial cells by OAT1 and OAT3 transporters. The drug transporters responsible for cefoxitin efflux across the apical membrane has not been identified yet, but MRP2 and MRP4 are thought to be involved in this process.

The present study was conducted to gain a better understanding of the impact of physiological changes following renal transplantation on the disposition of drug substrates of renal anionic transport system. Physiologically based pharmacokinetic (PBPK) modeling approach was used to help us gain this understanding. PBPK modeling is a very comprehensive and relatively inexpensive strategy to address the impact of various clinical pharmacotherapeutic and physiological factors that impact drug dosing. PBPK modeling approach incorporates a drug's physiochemical properties, human physiological variables and population variability estimates to predict drug exposure [9, 29, 85, 129, 148]. Because population PBPK models incorporate anatomical, physiological, and drug transporter attributes, any physiological alterations induced due to disease, age, gender, genetic polymorphism, and other pathophysiologic conditions can be captured by such a model. To the best of our knowledge, the

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use of PBPK modeling in predicting cefoxitin exposure has not been explored in healthy and renal transplant populations. The objective of this work was to build and validate a PBPK model for IV cefoxitin in healthy adults and renal transplant recipients to study the significance of changes in OAT transport system.

6.3   METHODS

Cefoxitin PBPK modeling and simulations were conducted using SimCyp® population-based simulator v15.1 (SimCyp limited, Sheffield, UK). Systematic and extensive literature search in MEDLINE through PubMed was performed to identify published physicochemical properties (Table 27), plasma protein binding of cefoxitin, and clinical trials using IV cefoxitin in healthy volunteers. These data were tabulated and digitized where necessary for PBPK model building or model validation. GetData Graph Digitizer V.2.26 [149] was used to digitize published cefoxitin clinical pharmacokinetic data.

Table 27. Summary of cefoxitin physiochemical parameters

Parameter	Value
MW (g/mol)	427.45
Log $P_{o:w}$	-0.020
Compound type	Monoprotic Acid
pKa1	2.2
B/P	0.55*
f_u	0.25*

Source: Pubchem/ DrugBank and cefoxitin package insert[129, 150]

MW: molecular weight; logP: logarithm of the octanol to water partition coefficient, pKa: negative logarithm of the acid dissociation constant, B/P: blood to plasma partition coefficient; fu: Plasma fraction unbound; *parameters were fitted by non-linear mixed effect modeling strategy using parameter estimation module of SimCyp®. Unity and 0.26 were used as initial estimates for B/P and f_u.

6.3.1 General Workflow for Model Building and Model Validation

A full PBPK model was initially developed for IV cefoxitin using physiochemical properties (Table 27) [129, 150] and published IV cefoxitin clinical PK data in healthy subjects [1]. Following IV administration, cefoxitin was modeled to enter the systemic circulation through venous blood (Figure 41).
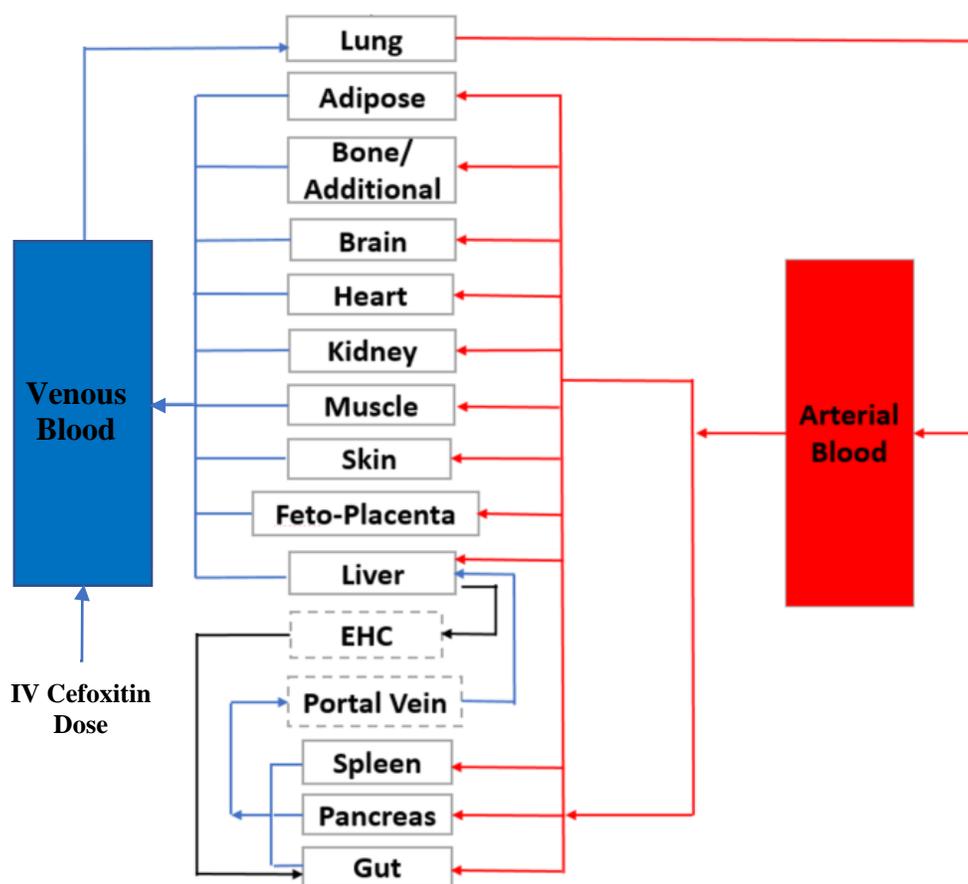
Figure 41. Compartmental structure of the full IV cefoxitin PBPK model in healthy volunteers

A model naïve IV cefoxitin clinical PK dataset was used to perform model validation in healthy volunteers by comparing mean $AUC_{0-\infty}$, CL_{Total}, CL_{Renal} and $t_{1/2}$ values between the

observed and predicted data. $CL_{Secretion}$ was not compared for healthy volunteer model since eGFR was not available in the healthy volunteer datasets used to build and validate the model. After establishing a validated IV cefoxitin PBPK model in healthy volunteers, a cefoxitin PBPK model in renal transplant recipients was built and validated by creating a virtual renal transplant recipient population in SimCyp® and validating cefoxitin PBPK model using the clinical PK data obtained from cefoxitin PK study in renal transplant population (Chapter 5.0). Cefoxitin $AUC_{0-\infty}$, CL_{Total}, CL_{Renal}, $CL_{Secretion}$ and $t_{1/2}$ pharmacokinetic parameters between the observed and predicted renal transplant data was compared.

For the validations, we performed visual predictive checks by using plots of fitted and predicted against the observed mean concentration-time profiles. Fifth to 95th percentile intervals (PI) were calculated to show the overall inter-patient variability. The goal was to use IV cefoxitin PBPK model to predict $AUC_{0-\infty}$, CL_{Total}, CL_{Renal} and $t_{1/2}$, and compare it to observed data. The criterion for model validation is the difference of the mean predicted and observed $AUC_{0-\infty}$, CL_{Total}, CL_{Renal} and $t_{1/2}$ in 100 virtual subjects should fall $\pm$ 25%. There is no established FDA guidance regarding an acceptable error range that should be used for the evaluation of predicted data by PBPK models. Other investigators in this field have used up to a 2-fold error criterion for model validation. [151, 152]

AUC_{0-t} is the drug exposure between time zero and t hours (the last blood collection time point) and this was estimated using trapezoidal method. $AUC_{0-\infty}$ is the drug exposure between zero hours and infinite time and this was estimated by the summation of AUC_{0-t} and extrapolated exposure from C_{last} to infinite time ($AUC_{last-\infty} = C_{last}/k$), where k is the terminal disposition rate constant. CL_{Total} was calculated per the following equation:

$$CL_{Total} = dose/AUC_{0-\infty}$$

C_{max} is the observed maximus concentration after administration of a dose. CL_{Renal} was calculated per the following equation:

$$CL_{Renal}= (A_{e(0-t)})/ AUC_{0-t}$$

Where $A_{e(0-t)}$ is the amount of drug eliminated in the urine in time 't'.

In renal transplant recipients Cefoxitin tubular reabsorption was assumed to be negligible (0 mL/min), cefoxitin filtration clearance ($CL_{Filtration}$) and tubular secretion clearance ($CL_{Secretion}$) were estimated using the following equations:

$$CL_{Filtration} = fu \times CL_{Cr}$$

$$CL_{Secretion} = CL_{Renal} - CL_{Filtration}$$

Where fu is the fraction of cefoxitin unbound (0.26) [129] and CL_{Cr} is the creatinine clearance which is estimated using the Cockcroft Gault equation:

$$CL_{Cr} = (140\text{-age})(weight\ kg) / (72 \times SrCr)\ in\ mL/min$$

$$multiplied\ by\ 0.85\ for\ female\ subjects$$

6.3.2   IV Cefoxitin PBPK Model Development in Healthy Volunteers

6.3.2.1 Absorption component

IV cefoxitin PBPK model was developed and so absorption specific parameters were not estimated and incorporated into the model.

6.3.2.2 Distribution component

Tissue composition and blood flow rates outlined by SimCyp® for the virtual healthy volunteer population was used for building IV cefoxitin PBPK model (Figure 42and Figure 43).

| | Relative Volume of Wet Tissue (%) | | | | AP (mg/g) | Binding Proteins | |
	EW	IW	NL	NP		$Kp_{,ALB}$	$Kp_{,LPP}$
Adipose	14.1	3.9	79	0.2	0.4	0.037	0.068
Bone	9.8	34.1	7.4	0.11	0.67	0.1	0.05
Brain	9.2	67.8	5.1	5.65	0.4	0.048	0.041
Gut	26.7	45.1	4.87	1.63	2.84	0.158	0.141
Heart	31.3	44.5	1.15	1.66	3.07	0.157	0.16
Kidney	28.3	50	2.07	1.62	2.48	0.13	0.137
Liver	16.5	58.6	3.48	2.52	5.09	0.086	0.161
Lung	34.8	46.3	0.3	0.9	0.5	0.212	0.168
Muscle	9.1	66.9	2.38	0.72	2.49	0.034	0.059
Pancreas	12	66.4	4.1	0.93	1.67	0.06	0.06
Skin	62.3	9.47	2.84	1.11	1.32	0.277	0.096
Spleen	20.8	57.9	2.01	1.98	2.81	0.097	0.207
Plasma	94.5	0	0.35	0.22	0.04		
RBC	0	66.6	0.17	0.29	0.44		

Figure 42. Tissue composition parameters used for building IV cefoxitin PBPK model in healthy volunteer population.

Water; IW : % Intracellular Water; NL : % Neutral Lipids; NP : % Neutral Phospholipids; AP : % Acidic Phospholipids; $Kp_{,Alb}$: tissue-plasma partition coefficient for serum albumin; $Kp_{,LPP}$: tissue-plasma partition coefficient for lipoproteins

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Tissue Blood Flow Rates
(% cardiac output)

	Male	Female
Adipose	5	8.5
Bone	5	5
Brain	12	12
Stomach and Oesophagus	1	1
Small Intestine	10	11
Villi	6	6
Large Intestine	4	5
Heart	4	5
Kidney	19	17
Liver (Arterial)	6.5	6.5
Liver (Portal)	19	21.5
Lung	100	100
Muscle	17	12
Pancreas	1	1
Skin	5	5
Spleen	2	3

Figure 43. Blood flow rates associated with each physiological compartment that were used for building IV cefoxitin PBPK model in healthy volunteer population.

Screenshot from SimCyp® population-based simulator v15.1 (SimCyp limited, Sheffield, UK).

Cefoxitin reference volume of distribution at steady state (V_{ss} = 0.167 L/kg) was

estimated from the data reported by Vlasses et al [1] using the following equation:

$$V_{ss} = (Dose \times AUMC_{0-\infty}) / (AUC_{0-\infty})^2$$

Where $AUMC_{0-\infty}$ is the areas under the moment curve from time of administration to infinite time. PBPK distribution component was built using cefoxitin physicochemical properties tissue to plasma partition coefficients (K_p) for all major tissue specific physiological compartments for cefoxitin were estimated. Predicted V_{ss} was estimated by serial addition of plasma volume (V_p), erythrocyte volume (V_e) and volumes associated with each major tissue (V_t) [153].

$$V_{ss} = V_p + V_e \times (E{:}P) + \sum V_t \times K_p$$

Where E:P represents erythrocyte to plasma partitioning. The E:P is estimated using the SimCyp® parameter estimation modules based on the information of blood to plasma ratio and hematocrit.

Tissue specific K_p values of cefoxitin for the full-PBPK model were estimated using the Rogers and Rowland method [154, 155]. This model accounts for tissue water volume to be split into intra- and extra cellular components, addition of the tissue acidic phospholipid fraction and takes account of the extent of ionization of a compound at the pH of the concerned compartment. Rogers and Rowland method of estimating K_p values enable us to predict transporter mediated disposition of drug compounds [154, 155].

Cefoxitin tissue specific K_p values for 12 major physiological tissues estimated using Rogers and Rowland method are presented in Table 28.

Table 28. Distribution parameters for cefoxitin drug profile

Parameter	Value
Model	Full PBPK
Tissue Partition Coefficients (K_p)	
Adipose	0.067
Bone	0.133
Brain	0.148
Gut	0.230
Heart	0.240
Kidney	0.247
Liver	0.164
Lung	0.292
Pancreas	0.141
Muscle	0.030
Skin	0.100
Spleen	0.182

Predicted K_p values for all tissues using

Rodgers and Rowland method [154, 155]

6.3.2.3 Metabolism component

Since cefoxitin is primarily excreted into the urine as unchanged drug (85% unchanged) and there is lack of evidence of specific drug metabolism enzymes involved in clearance of cefoxitin, enzymatic metabolism of cefoxitin was not incorporated into this model [129] and the remainder clearance (~15%) was attributed to the default additional systemic clearance in SimCyp® Simulator.

6.3.2.4 Excretion component:

Cefoxitin is extensively secreted into the urine as unchanged drug. Clinical pharmacokinetic studies in the presence and absence of probenecid, suggest a significant involvement of renal organic anionic transport system in its disposition. In order to model cefoxitin renal transporter mediated disposition, the default renal OAT uptake transporter in the SimCyp® healthy volunteer population was considered to be a combined OAT1/OAT3 active renal uptake transport entity and the default renal MRP efflux transporter was considered to be a combined MRP2/MRP4 active efflux transport entity. A full-PBPK permeability-limited kidney model (Mech-KiM) was considered for cefoxitin PBPK modeling.

Initial estimates of intrinsic clearance (CL_{int}) attributed to uptake and efflux transport system was estimated from cefoxitin $CL_{secretion}$ which was calculated from clinical pharmacokinetic data used to build the cefoxitin PBPK model [1]. Intrinsic clearance attributed to uptake and efflux transport system is expressed in 'µL/min/millions of proximal tubular cells' and this was estimated by normalizing cefoxitin $CL_{Secretion}$ in the absence of probenecid with number of proximal tubular cells in an average kidney (7980 million cells; [156, 157]). In all modeling exercises, CL_{int} associated with passive permeability was assumed to be negligible.

Initial estimates of $CL_{int\ OAT}$ and $CL_{int\ MRP}$ were calculated to be 26 µL/min/millions of proximal tubular cells based on $CL_{secretion}$ of 205.6 ± 24.9 mL/min (Table 25). The final model estimates of $CL_{int\ OAT}$ and $CL_{int\ MRP}$ were estimated using the parameter estimation module within SimCyp®. $CL_{int\ OAT}$ value of 65 µL/min/millions of proximal tubular cells and $CL_{int\ MRP}$ value of 50 µL/min/millions of proximal tubular cells yielded the best final model fit when comparing model predictions to the observed data based on the validation criteria set forth.

After the final IV cefoxitin PBPK model was built, cefoxitin PK parameters in 100 age-matched virtual healthy volunteers (21-35 years old) were compared to those calculated from PK data reported by Vlasses et al. [1]. The prediction model dosing administration was matched to that of the observed clinical study and a PK sample every 10 seconds was considered for the model predicted PK profile.

6.3.3 IV Cefoxitin PBPK Model Validation in Healthy Volunteers

Cefoxitin clinical pharmacokinetic data in healthy volunteers reported by Ko et al [2] was used for model validation. Cefoxitin clinical pharmacokinetic data following IV administration of 2 grams cefoxitin over 5 min in healthy volunteers was used for validating the built IV cefoxitin PBPK model. One hundred virtual healthy subjects spread over 10 trials were used for the PBPK simulation.

As mentioned above, visual predictive checks were performed by using plots of fitted and predicted against the observed mean concentration-time profiles. The goal was to use IV cefoxitin PBPK model to predict $AUC_{0-\infty}$, CL_{Total}, CL_{Renal} and $t_{1/2}$, and compare it to observed data. The criterion for model validation is the difference of the mean predicted and observed $AUC_{0-\infty}$, CL_{Total}, CL_{Renal} and $t_{1/2}$ in 100 virtual healthy subjects should fall $\pm 25\%$.

6.3.4 Virtual Renal Transplant Population

Virtual renal transplant population was built by modifying existing SimCyp® healthy volunteer population [156]. Since renal transplant recipients have only one functioning kidney, the number of functioning nephrons was halved in the custom-built virtual renal transplant population to

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0.807 million nephrons per subject from 1.615 million nephrons in healthy adults. Since the cefoxitin exposure increased by 2.6-fold and renal secretory capacity went down by about 60% in renal transplant recipients (Chapter 5.0), the abundance of OAT and MRP transport proteins was lowered by ~60% from the individual baseline values. Although OAT and MRP quantitative gene expression in renal allografts was investigated (Chapter 3.0), their quantitative expression in healthy kidney tissues is currently not available and so change in anionic transport activity was used to build this model. Changes in albumin level and hematocrit levels were not sensitive to cefoxitin exposure and clearance and so these parameters were unchanged in the virtual renal transplant population.

6.3.5   IV Cefoxitin PBPK Model Validation in Renal Transplant Population

Cefoxitin clinical pharmacokinetic data in renal transplant recipients presented in Chapter 5.0 which involved IV administration of 200 mg cefoxitin over 2-3 min in renal transplant recipients was used for validating the built IV cefoxitin PBPK model in virtual renal transplant population. One hundred virtual renal transplant subjects spread over 10 trials were used for the PBPK simulation.

As mentioned above, visual predictive checks were performed by using plots of fitted and predicted against the observed mean concentration-time profiles. The goal was to use IV cefoxitin PBPK model to predict $AUC_{0-\infty}$, CL_{Total}, CL_{Renal}, $CL_{Secretion}$ and $t_{1/2}$, and compare it to observed data. The criterion for model validation is the difference of the mean predicted and observed $AUC_{0-\infty}$, CL_{Total}, CL_{Renal}, $CL_{Secretion}$ and $t_{1/2}$ in 100 virtual subjects should fall $\pm 25\%$.

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6.4   RESULTS

Six healthy volunteers who participated in cefoxitin pharmacokinetic studies conducted in the absence of orally administered probenecid by Vlasses et al. were used as historical controls [1]. All subjects were between 21 and 35 years of age and within ±10% of ideal body weight and evaluated to be healthy based on physical examinations and results of laboratory screening studies as determined by the investigators [1]. In this study, the investigators administered 2 grams of cefoxitin as an IV push over 3 minutes. Linear plots of cefoxitin serum concentration versus time for these 6 subjects is shown Figure 44. Cefoxitin concentration-time data in these subjects was used to build the cefoxitin PBPK model.
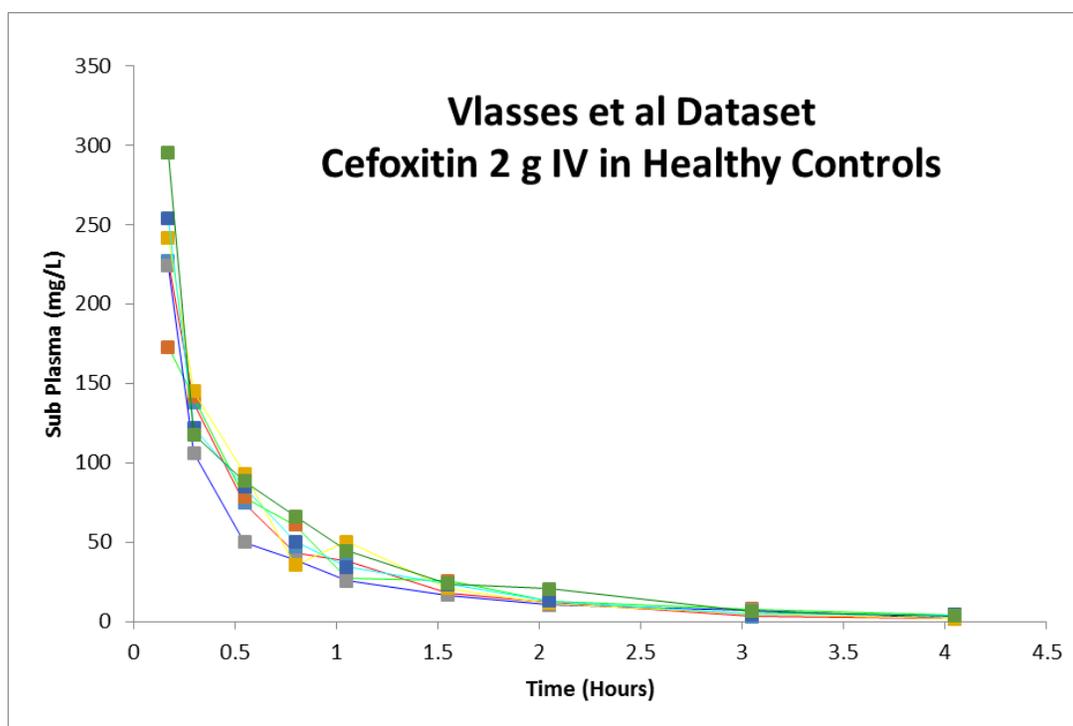
Figure 44. Concentration vs Time plot of 2000 mg cefoxitin given as IV push over 3 min to healthy subjects.

Data was reported by Vlasses et al and was used to build cefoxitin PBPK model [1].

6.4.1   IV Cefoxitin Final PBPK Model-Build in Healthy Volunteers

The final IV cefoxitin PBPK model in age matched healthy volunteers with predicted means of
concentration-time profile and 90% PI overlaid with the observed data reported by Vlasses et al
is provided in Figure 45 [1]. As shown, the observed data was within the 90% PI of the
variability observed around the predicted mean exposure. The predicted and observed mean
concentration-time profiles were visually similar.
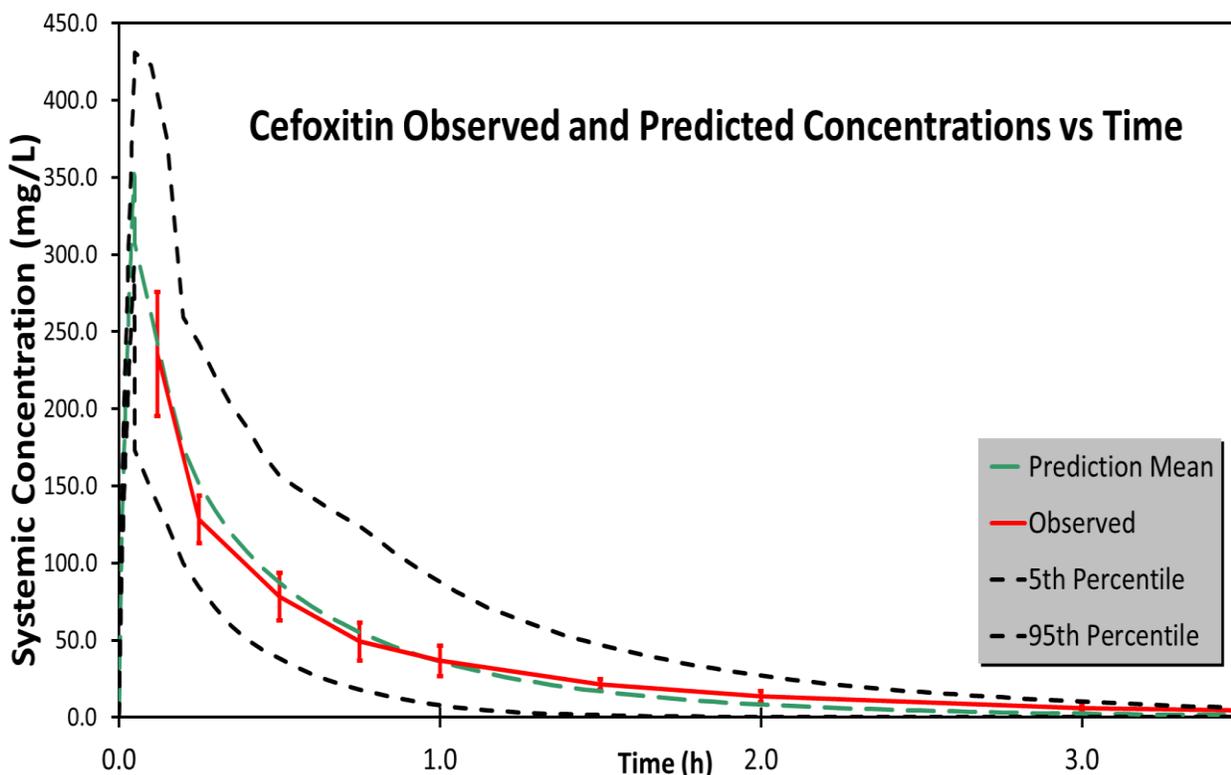
Figure 45. Concentration-time profiles of final-model prediction and observed data in healthy volunteers.
Exposure profiles following administration of 2000 mg cefoxitin given as IV push over 3 min to 100 virtual healthy
subjects and 6 historical healthy subjects along with 5th and 95th percentile population variability limits. Data was
reported by Vlasses et al [1] and was used to build cefoxitin PBPK model.

The accuracy of the predicted means of $AUC_{0-\infty}$, CL_{Total}, CL_{Renal} and $t_{1/2}$ were within ±5%
of the observed means (Table 29).

Table 29. IV cefoxitin PBPK model prediction vs observed parent dataset

PK parameters	Vlasses et al. [1] Observed mean (SD)	Predicted mean (SD)	% Change
CL_{Total} (L/hr)	16.72 (3.28)	16.9 (6.08)	1.08
CL_{Renal} (L/hr)	13.86 (1.53)	14.22 (5.66)	2.60
$AUC_{0-\infty}$ (mg.hr/L)	136 (16.7)	137.18 (58.97)	0.87
$t_{1/2}$ (hr)	0.8 (0.2)	0.82	2.50

SD: Standard deviation; % change is the percentage change between predicted and observed

parameters

6.4.2 IV Cefoxitin Final PBPK Model -Validation in Healthy Volunteers

The cefoxitin PBPK model in healthy volunteers was validated using a model-naïve clinical PK

dataset reported by Ko et al [2] where 2000 mg of IV cefoxitin was administered over 5 min to

16 healthy male volunteers between the ages of 20 and 49 years. The model prediction in age

matched virtual healthy subjects with predicted means of concentration-time profile and 90% PI

overlaid with the observed data reported by Ko et al is provided in Figure 46 [2]. The predicted

and observed mean concentration-time profiles were visually similar. The accuracy of the

predicted means of $AUC_{0-\infty}$, CL_{Total}, CL_{Renal} and $t_{1/2}$ were within ±8% of the observed means

(Table 30).
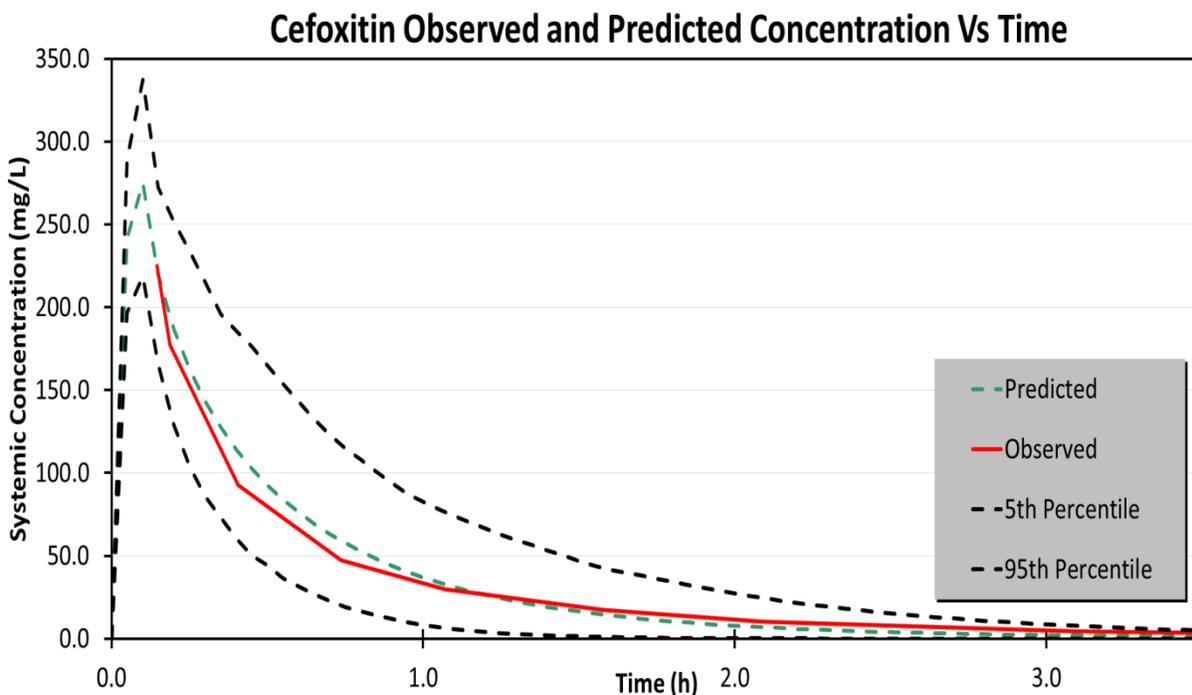
Figure 46. Concentration-time profiles of model predicted and observed data in healthy volunteers

(Validation).

Exposure profiles following administration of 2000 mg cefoxitin given as IV push over 5 min to 100 virtual healthy

subjects and 16 historical healthy subjects along with 5th and 95th percentile population variability limits. Data was

reported by Ko et al [2] and was used to validate cefoxitin PBPK model.

Table 30. IV cefoxitin PBPK model prediction vs observed model naïve dataset

PK parameters	Ko et al. [2] Observed mean (SD)	Predicted mean (SD)	% Change
CL_{Total} (L/hr)	16.74 (1.76)	16.93 (5.98)	-1.14
CL_{Renal} (L/hr)	13.26 (2.05)	14.28 (5.54)	-7.69
$AUC_{0-\infty}$ (mg.hr/L)	129 (13.23)	135.44 (54.89)	-4.99
$t_{1/2}$ (hr)	0.81 (0.1)	0.825	-1.85

SD: Standard deviation; % change is the percentage change between predicted and observed

parameters

6.4.3 IV Cefoxitin PBPK Model in Renal Transplant Patients

The cefoxitin PBPK model in renal transplant recipients was validated using a model-naïve clinical PK dataset reported in Chapter 4, where 200 mg of IV cefoxitin was administered over 2-3 min to 15 living donor and deceased donor renal transplant recipients. The model prediction in age matched virtual renal transplant subjects with predicted means of concentration-time profile and 90% PI overlaid with the observed is provided in Figure 47.
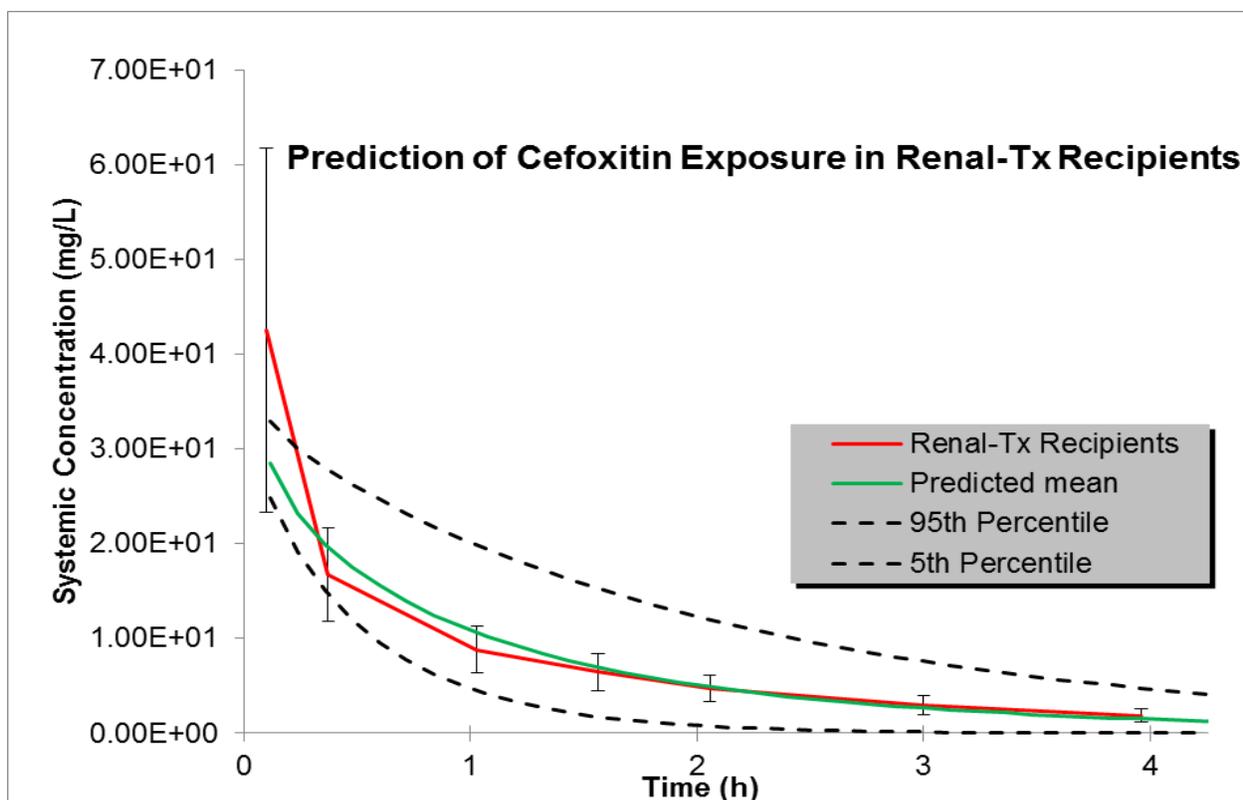
Figure 47. Concentration-time profiles of model prediction and observed data in renal transplant patients.
Exposure profiles following administration of 200 mg cefoxitin given as IV push over 3 min to 100 virtual renal transplant subjects and 15 renal transplant recipients along with 5th and 95th percentile population variability limits. Observed clinical PK data reported in detail in Chapter 5.0 was used to validate cefoxitin PBPK model in renal transplant recipients.

The predicted and observed mean concentration-time profiles were visually similar with the exception of the first time-point. Since cefoxitin undergoes rapid disposition following administration as an IV push, collecting a very earlier first cefoxitin sampling time-point immediately after dose administration is critical to better capture the true exposure of cefoxitin. The earliest data-point in the renal transplant patients was earlier (3-5 min post dose administration) than the earliest data-point in the healthy subject datasets (10 min) used to build and validate the base model. The accuracy of the predicted means of $AUC_{0-\infty}$, CL_{Total}, CL_{Renal}, $CL_{Secretion}$ and $t_{1/2}$ were within ±19% of the observed means (Table 31).

Table 31. IV cefoxitin Transplant PBPK model prediction vs observed model naïve dataset

PK parameters	Renal-Tx: Observed mean (SD)	PBPK-Tx: Predicted mean (SD)	% Change
CL_{Total} (L/hr)	104.8 (34.7)	96.9 (35.5)	-7.5
CL_{Renal} (L/hr)	87.5 (29.0)	76.8 (38.7)	-12.2
$AUC_{0-\infty}$ (mg.hr/L)	35.2 (11.6)	34.7 (18.8)	-1.4
$t_{1/2}$ (hr)	1.3 (0.6)	1.5 (0.3)	13.3

SD: Standard deviation; Tx: Transplantation; % change is the percentage change between predicted and observed parameters

6.5   DISCUSSION

Most drugs that are eliminated by tubular secretion primarily undergo active transport into the lumen of the proximal tubule. For a drug to be successfully cleared it is usually a substrate of an uptake and efflux pair of transporters such as in the case of cefoxitin where OAT1/3 and MRP2/4 are thought to be involved in the uptake and efflux activities respectively in the renal epithelial cells [26, 27, 29-31]. Renal organic anionic transporters are specifically of interest in the context of renal transplant recipients as they are involved in the clearance of various medications (acyclovir, cidofovir, fluoqionolone antibiotics, several cephalosporine antibiotics, etc.) prescribed to renal transplant recipients. OAT1 and OAT3 renal uptake transporters are considered to be the most important renal organic anionic transporters by the US FDA and EMA for their role in drug disposition and drug-drug interactions [9]. For the disposition of various anti-infective medications, MRP2 and MRP4 are thought to be the efflux partners for OAT1 and OAT3.

Cefoxitin is a suitable probe drug to assess the renal anionic secretion as more than 85% of the drug is eliminated unchanged in urine, with majority of renal clearance attributed to anionic secretion, it also has a short half-life (0.6 ± 0.1 hrs in healthy volunteers); 1.3 ±0.6 hrs in renal transplant recipients), good safety profile [1, 129], and offers feasibility to conduct pharmacokinetic studies with low IV/IM doses within a short study duration. A prospective clinical PK study using cefoxitin as a probe drug was conducted in renal transplant patients to assess the effect of renal transplantation on anionic secretory capacity in this population (Chapter 5.0). There were no differences in renal anionic secretory capacity in de-novo LDRT and DDRT

recipients during the early post-transplant period. However, renal anionic secretory capacity in renal transplant recipients was reduced by 60%.

In this study, we built and validated full-PBPK model of IV cefoxitin in healthy subjects and renal transplant recipients. The full-PBPK model incorporates organic anionic transport system based disposition of cefoxitin and its disposition into 12 major tissues in the body. The model was robust in representing the multi-compartment first order disposition of cefoxitin. The predicted concentration-time profiles in the study-matched virtual patient populations are consistent with observed data across 3 independent studies among healthy subjects and renal transplant recipients across a dosage range of 200 mg to 2000 mg. The predicted IV cefoxitin PK parameters fell well within ±25% validation range of the corresponding PK parameters calculated from the IV cefoxitin observed studies.

Cefoxitin was modeled as a substrate of OAT/MRP transporter system based on clinical evidence of increased cefoxitin exposure (2.4-fold increase) in the presence of high dose probenecid, a known potent organic anionic transport blocker [1]. Since there is currently no in-vitro transporter data available on the disposition kinetics of cefoxitin by OAT1/OAT3 uptake transporters and MRP2/MRP4 efflux transporters, uptake transporters were modeled together as OAT transport system and efflux transporters were modeled together as paired MRP transport system. Renal OAT1 and MRP2 abundancies incorporated in SimCyp® were used to model their contributions in virtual healthy subjects. Cefoxitin intrinsic clearance for OAT uptake and MRP efflux was initially estimated by normalizing cefoxitin secretory clearance to the number of proximal epithelial cells assuming uniform distribution of transport proteins in all tubular cells. Parameter estimation module within SimCyp® was used to further optimize CL_{int} values for

OAT and MRP transport systems [156]. On performing exploratory sensitivity analysis, changes in cefoxitin exposure was found to be sensitive to OAT intrinsic clearance value when compared to MRP intrinsic clearance values. $CL_{int\ OAT}$ of 65 µL/min/millions of proximal tubular cells and $CL_{int\ MRP}$ value of 50 µL/min/millions of proximal tubular cells yielded the best final model fit when comparing model predictions to the observed data based on the validation criteria set forth. Due to lack of specific in-vitro data of MRP2/4 mediated cefoxitin disposition, this model cannot differentiate the percent contribution of MRPs and OATs. In future, with availability of in-vitro data this model can be updated and used to tease out these differences.

Renal allografts undergo abuses such as prolonged cold ischemic injury, calcineurin inhibitor based nephrotoxicity, BKVN and various grades of rejection. Each of these injuries or a combination of varying degrees of these injuries could compromise the renal anionic transport system. Transporter gene expression studies in renal biopsies from patients with Banff IA or higher acute TCMR with interstitial fibrosis show that OAT system is significantly reduced in these patients (OAT1: 11.0-fold lower; OAT3: 4.41-fold lower; MRP2: 6.78-fold lower; MRP4: 2.01-fold lower) when compared to biopsies from healthy allografts (Table 10). This PBPK modelling work was conducted to gain a better understanding of the impact of such physiological changes following renal transplantation on the disposition of drug substrates of renal anionic transport system. Figure 48 outlines the predicted concentration-time plots of cefoxitin in subjects with varying functional abundancies of renal OAT1/3 transporters.
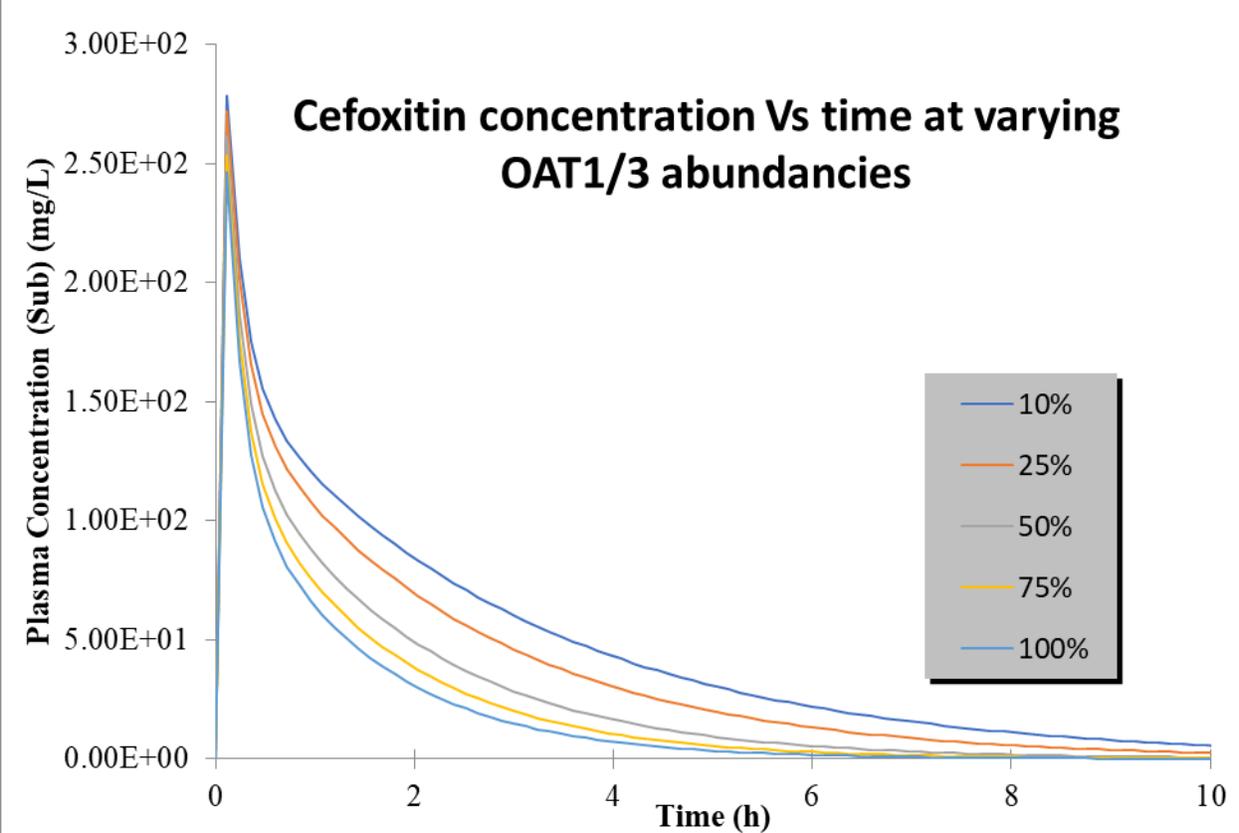
Figure 48. Predicted concentration-time profiles following administration of 2000 mg cefoxitin given as IV push over 3 min to virtual population representative with varying functional abundancies of OAT1/3 uptake transporters.

Table 32 outlines projected fold changes in cefoxitin exposure ($AUC_{0-\infty}$) in healthy subjects and renal transplant recipients with varying abundancies of OAT1/3 uptake transporters in renal proximal epithelial cells of renal allografts. Based on gene expression data and these projections, renal transplant recipients with Banff 1A or higher acute TCMR and interstitial may experience up to 4-fold higher exposure ($AUC_{0-\infty}$) of cefoxitin when compared to healthy volunteers.

Table 32. Projected fold changes in cefoxitin AUC0-∞ among renal transplant recipients when compared to healthy volunteers with two functioning kidneys

Percent Abundance of OAT1/3	Fold Change in cefoxitin $AUC_{0-\infty}$ Relative to Healthy Volunteers	
	Healthy Volunteers With 2 Functioning Kidneys	Renal-Tx Recipients with 1 Functioning Kidney
100% OAT1/3 abundance	1.0	1.7
75% OAT1/3 abundance	1.2	2.0
50% OAT1/3 abundance	1.4	2.3
25% OAT1/3 abundance	1.5	2.6
10% OAT1/3 abundance	2.4	4.0

Using the developed PBPK model we have the ability to predict drug exposure of cefoxitin and other narrow therapeutic index drugs (such as cidofovir) that are primarily disposed by renal OAT systems in renal transplant recipients. Renal drug transporter expression data from renal biopsies can be combined with PBPK modeling strategies to optimize pharmacotherapy in renal transplant recipients.

7.0   SUMMARY AND FUTURE DIRECTIONS

7.1   SUMMARY AND CLINICAL INFERENCE

The kidney is a vital organ in the human body. It conserves essential nutrients and eliminates toxins, drugs and their metabolites by filtration, tubular secretion and re-absorption processes. Organic anionic and organic cationic transport systems expressed in the proximal tubular cells are primarily responsible for renal secretion of endogenous and exogenous compounds. OAT1, OAT3 and OCT2 renal up-take transporters along with their efflux transport partners (MRP2/4 and MATE1/2-K) are considered to be the most important renal anionic and cationic transporters by the United States FDA and EMA for their high renal abundance, role in drug disposition of most commonly prescribed drugs and clinically significant drug-drug interactions [5-9].

Administration of transporter inhibitors, changes in physiology and renal diseases affect renal function and can alter the activity of specific renal transporters and ultimately alter exposure of drugs that are cleared by renal secretion. Research over the last decade has focused on elucidating expression and activity of renal transporters and their influence on the pharmacokinetic and pharmacodynamic response of renally secreted drug. Renal dysfunction, AKI, CKD, glomerulonephritis and diabetic nephropathy have been shown to differentially regulate renal OAT and OCT transporters [53-55].

Kidney transplantation is the treatment of choice for patients diagnosed with ESRD [3, 75] with over 19,000 kidney transplantations performed in the USA in 2016 alone [available from: www.unos.org]. Progressive loss of renal function following transplantation is shown to be caused by various allograft insults to the allograft associated with renal transplantation such as, cold ischemic injury, CNI nephrotoxicity, BKVN and T-cell mediated rejection with fibrosis.

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Inflammatory cytokines such as TNFα, IL-6, and IL-1β and the vasoactive hormones such as endothelin-1 (ET1), which are associated with these insults are known to be involved in the regulation of drug transporters [87, 88]. These insults may lead to altered regulation or injury to the renal anionic and cationic transport systems including counter transport systems that contribute to SLC transporter activity and ATPase activity. The overall hypothesis of this work is that injuries caused by prolonged cold ischemia, CNI nephrotoxicity, varying grades of TCMR and BKVN would significantly alter the renal anionic secretory function in renal transplant recipients. Additionally, clinical observations in renal transplant recipients with BKVN involving cidofovir treatment in the presence and absence of probenecid suggest that renal anionic secretory activity may be compromised in allografts with BKVN [100].

It is important to systematically characterize the anticipated changes in secretory function of renal allografts to ensure optimal pharmacotherapy in transplant recipients. This body of work is one of the first attempts to understand changes in expression and functional activity of renal transporters after renal transplantation.

In chapter 2.0 , we described the pre-clinical evaluation of changes in expression of important renal drug transporters in renal allografts with transplantation associated complications by studying the effect of prolonged CI, transplantation surgery and tacrolimus treatment on the gene expression of Oat1, Oat3, Oct2, Mate1, and Mdr1a transporters in a rat renal transplant model using TaqMan RT-qPCR assays. The mRNA expression of *Slc22a2* (Oct2) was significantly higher in rat kidneys that were subjected to 24 hours of CI. Expression of *Slc22a6* (Oat1)*, Slc22a8* (Oat3)*, Slc22a2* (Oct2) and *Slc47a1* (Mate1) were significantly lower immediately following syngeneic rat kidney transplantations at 3 hrs and 12 hrs post-transplantation; the gene expression of Oat1 and Oat3 recovered by 4 weeks post-transplantation,

but Oct2 and Mdr1a did not. This suggests that Oct2 transporter is more susceptible to insults by CI and transplantation when compared to Oat1/3 transporters. Observations from this study are consistent with results from previous unpublished work on Oat1/3 expression in orthotopic syngeneic rat kidney transplant model as well as results from liver ischemia/reperfusion studies. Among rats that were treated with tacrolimus following allogeneic or syngeneic transplantations, only those with allografts subjected to 24 hours of prolonged CI had a significantly lower expression of all 5 important drug transporters. The renal allografts may have increased susceptibility to nephrotoxic profile of high dose tacrolimus following injury due to prolonged cold ischemia. Rat kidney transplant model captures changes in renal transporter expression following transplantation and serves as a good model for future renal transporter studies.

In the next part of the study (Chapter 3.0), we evaluated the effect of prolonged CI with tacrolimus treatment, BKVN and acute TCMR with fibrosis on the gene expression of 36 important drug transporters in renal biopsies collected from renal transplant patients. FFPE renal tissue biopsies procured from LDRT and DDRT recipients were utilized and gene expression of transporters were quantified by NanoString nCounter® gene expression assay. nCounter® gene expression assay offers high sensitivity and reproducibility in a single-tube without the necessity for a cDNA creation or mRNA replication steps like in the case of qPCR based quantitation. This method also gives us the ability to measure absolute expression as compared to relative expression with traditional methods. The nCounter® assay has been successfully used to quantitate expression of gene from FFPE samples [123].

This work showed that relative expressions of important ABC and SLC transporters in all renal allografts were similar to the relative expressions observed in non-transplanted kidneys reported by Nishimuta et al with the exception of *SLC22A12* (URAT1) which was shown to have

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the highest relative expression in non-transplanted healthy renal tissues [7]. DDRT recipients had significantly higher expression of SGLT (2.7-fold) when compared to LDRT recipients suggesting that prolonged CI may have a protective mechanism in renal allografts to conserve glucose. Biopsies from patients with BKVN had a significantly lower expression of NHE3 (5.25-fold lower) when compared to controls, suggesting that renal transplant recipients with BKVN may have a compromised sodium homeostasis. Allografts with BKVN also had lower mean and median gene expression counts for OAT1, OAT3, MRP2 and MRP4 transporters when compared to control allografts but this decline was not statistically significant. Expression of several transporters involved in the renal OAT system was significantly compromised in allografts with acute TCMR and fibrosis (OAT1: 11-fold lower; OAT3: 4.4-fold lower). Results of this study suggest that renal transplant recipients may experience significant changes in renal transporter mediated disposition of various endogenous and exogenous compounds and systematic evaluation of renal secretory activity is warranted in this patient population.

For the evaluation of anionic transport capacity in renal transplant patients, Cefoxitin, a second-generation cephalosporin antibiotic was selected as a probe drug due to its short half-life, acceptable safety profile, its properties as a substrate of anionic transport system, and feasibility to conduct pharmacokinetic studies with low IV/IM doses within a short study duration. To date, several HPLC-UV chromatographic techniques have been developed for quantification of cefoxitin in biological fluids. All currently published methods are limited by their requirement for relatively large volume of serum or plasma (100-1000 μL), longer run-times (elution time: 5.30 to 12.9 min), and higher LLOQ (1.00 to 100 μg/mL) [128, 131, 136-143]. In chapter 4.0 , we describe the development and validation of a rapid and sensitive UPLC-MS/MS assay to detect cefoxitin in human plasma following administration of low doses of cefoxitin. This assay

uses cefuroxime as the IS, requires SPE for sample processing, has a short run time (6 min), uses limited sample volume (20µL) and gives us the ability to perform cefoxitin quantitative assessment in the range of 25-50,000 ng/mL enabling us to perform low dosing and limited volume sampling studies for estimation of renal secretory changes in renal transplant patients. Similar strategy was used for quantitation of cefoxitin in human urine.

In chapter 5.0 , we (1) evaluated the longitudinal changes in renal anionic secretory capacity of a renal allograft, (2) studied the effect of prolonged CI on renal anionic secretory capacity in kidney transplant patients on tacrolimus therapy, and (3) and compared renal anionic secretory capacity of renal transplant recipients with historical healthy volunteers. Cefoxitin pharmacokinetic studies were performed in 15 de-novo renal transplant recipients following administration of 200 mg IV cefoxitin within 14 days post-transplantation, and beyond 90 days post-transplantation. Historical data from cefoxitin pharmacokinetic in healthy volunteers was used to compare results.

Results of this study show that renal anionic secretory capacity of allografts that are subjected to prolonged cold ischemia (in DDRT) is similar to that of allografts that are not subjected to prolonged cold ischemia (in LDRT). Dose normalized cefoxitin exposure in renal transplant recipients was significantly higher ($AUC_{0-\infty}$/Dose: 176.2 ± 58.0 mg*hr/L/g) when compared to healthy controls who were not treated with probenecid ($AUC_{0-\infty}$/Dose: 68.5 ± 8.1 mg*hr/L/g). Cefoxitin renal clearance was 57.3% lower and half-life was 2.2-fold higher in renal transplant recipients when compared to historical healthy controls. These findings suggest that renal anionic transport function in renal transplant recipients is significantly lower compared to healthy volunteers. However, percent contribution of renal secretion per kidney is higher for renal allografts. The findings of this clinical study are in accordance with the findings of renal

anionic transporter expression changes observed in the renal allograft transplant model (Chapter 2.0).

In order to gain a better understanding of the impact of physiological changes following renal transplantation, PBPK modeling approach was used to predict and study the impact of changes in OAT transport system on disposition of anionic drug substrates in renal transplant patients. In Chapter 6.0 , we described the model building and validation of IV cefoxitin PBPK models in healthy adults and renal transplant recipients. PBPK modeling is a relatively inexpensive strategy to address the impact of various clinical pharmacotherapeutic and physiological factors that impact drug dosing. PBPK modeling approach incorporates a drug's physiochemical properties, human physiological variables and population variability estimates to predict drug exposure [9, 29, 85, 129, 148]. To the best of our knowledge, the use of PBPK modeling in predicting cefoxitin exposure has not been explored in healthy and renal transplant populations.

Cefoxitin is a class-3 drug in the biopharmaceutical drug classification scheme. It was modeled as a substrate of OAT/MRP transporter system based on clinical evidence of increased cefoxitin exposure (2.4-fold increase) in the presence of high dose probenecid, a known potent organic anionic transport blocker [1]. Since there is currently no in-vitro transporter data available on the disposition kinetics of cefoxitin by OAT1/OAT3 uptake transporters and MRP2/MRP4 efflux transporters, uptake transporters were modeled together as OAT transport system and efflux transporters were modeled together as paired MRP transport system. Cefoxitin intrinsic clearance for OAT uptake and MRP efflux was initially estimated by normalizing cefoxitin secretory clearance to the number of proximal epithelial cells assuming uniform

distribution of transport proteins in all tubular cells. Parameter estimation module within SimCyp® was used to further optimize CL_{int} values for OAT and MRP transport systems [156]. Cefoxitin exposure was relatively sensitive to OAT intrinsic clearance value when compared to MRP intrinsic clearance values. $CL_{int\ OAT}$ of 65 µL/min/millions of proximal tubular cells and $CL_{int\ MRP}$ value of 50 µL/min/millions of proximal tubular cells yielded the best final model fit when comparing model predictions to the observed data based on the validation criteria set forth.

The final models were robust in representing the multi-compartment first order disposition of cefoxitin. The predicted concentration-time profiles in the study-matched virtual patient populations are consistent with observed data across 3 independent studies among healthy subjects and renal transplant recipients across a dosage range of 200 mg to 2000 mg. The predicted IV cefoxitin PK parameters fell well within ±25% validation range of the corresponding PK parameters calculated from the IV cefoxitin observed studies.

FDA and EMA require drugs to be studied in renally compromised patients and alternate dosing schedules are provided for drugs that are significantly renally cleared. However, all FDA and EMA approved renal dosing schedules are based on changing filtration function (CL_{Cr}) of the patients and do not account for changing secretory function. Currently, all renally cleared drugs, even those that are significantly renally secreted are routinely dose and dose-frequency adjusted by transplant clinicians based on their regulatory labeling. Renal transplant recipients who have sufficient renal filtration for dosing renally cleared drugs ($CL_{Cr} \geq 50$ mL/min), but significantly reduced secretory function, would be at risk for over exposure.

The results of this study make a convincing argument to monitor renal secretory function among renal transplant recipients in order to optimize their pharmacotherapy.

7.2   LIMITATIONS

Rat gene expression study (Chapter 2.0):

- Limitations include possible inherent differences in regulation of drug transporters between rats and humans, variability in the expression of housekeeping gene at different allograft conditions, use of only one housekeeping gene and use of very high dosage tacrolimus regimen. Changes in mRNA expression do not always translate into changes in protein expression and transporter activity.

Human gene expression study (Chapter 3.0):

- Custom code-set used for this assay was designed to facilitate transporter expression studies for various tissue types; some of the transporter targets on the code-set were not functionally relevant for renal tissue due to low relative expression levels (example: *SLCO1B1* and *SLCO1B3*). Changes in mRNA expression do not always translate into changes in protein expression and transporter activity and this is a limitation to directly relate these changes to transporter activity changes. Use of FFPE tissues may have non-specific mRNA degradation compared to frozen tissues. The number of biopsies evaluated in BKVN group were not sufficient to see a statistically significant difference in expression of OAT/MRP transporters. Predictions based on expression studies assume no change in co-transporter and/or ATPase activity.

Cefoxitin clinical PK study (Chapter 4.0):

- Limited number of study subjects were evaluated in LDRT (n=7) and DDRT (n=8) groups. The LDRT group was primarily Caucasian and DDRT group was primarily African American. Of the 15 patients who participated in Part 1 of the study, only 9 (5 LDRT recipients and 4 DDRT recipients) completed Part 2 of the study. Difficulty in obtaining IV access and scheduling conflict were the reasons for the 6 subjects to not complete Part 2 of the study.

- Cefoxitin renal clearance was estimated in 21 of the 24 PK studies as patients accidentally flushed-down the urine samples in 3 instances. Most urine samples were measured in urine collection jugs with the exception of a few instances where the study nurse recorded urine volumes using a urine hat. Variability in measurement of volume by these two methods is a limitation of this study. Fraction of drug unbound to plasma proteins was assumed to be 0.26 for the purposes of calculating $CL_{Filtration}$. There may be variability in protein binding in renal transplant patient population and this is a limitation. Biopsy samples from the study patients were analyzed for transporter expression but expression-activity relationship could not be established since there was a lack of transporter expression data in healthy volunteers.

- The historical healthy volunteers were studied and reported several decades ago with less sensitive bioanalytical methods in human serum. All healthy volunteers were young male subjects between the ages of 21 and 35 years. In this study the investigators did not estimate cefoxitin filtration clearance and so cefoxitin

secretory clearance could not be accurately estimated in healthy volunteers. Assuming 1 g probenecid blocked all the anionic secretion in healthy volunteers, $CL_{secretion}$ in healthy controls was estimated to be about 117 mL/min by subtracting CL_{Renal} in probenecid treated arm from CL_{Renal} in the control arm.

Cefoxitin PBPK study (Chapter 6.0):

- Uptake transporters (OAT1/3) were modeled together as OAT transport system and efflux transporters (MRP2/4) were modeled together as paired MRP transport system. Due to lack of in-vitro cefoxitin disposition data in OAT1/3 and MRP2/4 transporter, we are not able to discriminate the percent contribution of each of these transporters in the elimination of cefoxitin. Parameter estimation module within SimCyp® was used to further optimize CL_{int} values for OAT and MRP transport systems.

7.3   FUTURE DIRECTIONS

1) Quantitative gene expression of important renal transporters should be systematically evaluated in larger cohorts of renal allografts with BKVN, CNI nephrotoxicity, and varying grades of acute and chronic TCMR and they should be compared to corresponding expressions in healthy non-transplanted renal tissues or tissues from transplant patients with normal biopsies. This work will give us the ability to correlate transporter expression changes to activity changes and enable us to enhance PBPK models in renal transplant patients.

2) Flash frozen renal biopsy samples should be tested for co-transporter expression or ATP content.

3) A cefoxitin micro-dosing pharmacokinetic study with limited sampling and dried-blood-spot based sample collection should be conducted in renal transplant patients in order to validate cefoxitin micro-dosing, limited and minimally-invasive sampling strategy in this population. A validated study will give us the ability to evaluate renal anionic secretion in more renal transplant patients and would give clinicians the opportunity to optimize pharmacotherapy of renally secreted drugs.

4) Renal anionic secretory capacity of renal transplant patients with BKVN and varying grades of acute TCMR should be evaluated using a validated cefoxitin micro-dosing, limited and minimally-invasive sampling strategy.

5) Renal cationic secretory capacity of renal transplant patients with transplantation associated complications should be evaluated by prospectively studying plasma and urine pharmacokinetics of metformin (probe for OCT2 and MATE1/2K transporters).

6) Longitudinal studies should be performed pre- and post-transplantation in order to minimize inter-subject variability in transporter expression.

7) Robust PBPK models should be built and validated for renally secreted anionic and cationic drugs in renal transplant patients by incorporating post-transplantation physiological changes in this patient population.

Currently there is very limited knowledge on changes in expression and activity of important transporters following renal transplantation and associated complications. The proposed future research direction will help us gain a better understanding of changes renal secretory capacity in renal transplant patients and help clinicians improve pharmacotherapy in this patient population.

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A.1 ABSTRACT

Title: Effect of Prolonged Cold Ischemia in the Presence of Tacrolimus Therapy on Renal Secretory and Vitamin-D Metabolic Capacity of Transplanted Kidneys

Renal transplantation (Tx) is the treatment of choice for patients with various end-stage renal diseases. Since kidney is the primary excretory organ for endogenous compounds, drugs and their metabolites, changes in renal graft function (filtration or secretion by transporter or reabsorption) would significantly alter the clearance and exposure (AUC) of endogenous compounds as well as renally filtered or secreted drugs. Kidney also contributes significantly to the metabolism of various endogenous compounds including Vitamin-D. Kidneys from living and deceased donors that are transplanted into recipients normally undergo numerous insults including cold ischemia and warm-reperfusion injury, and are also subjected to nephrotoxicity due to calcineurin inhibitors (CNI). These physiological and pharmacological stresses can alter the expression and functional capacity of renal drug transporters and endogenous metabolic enzymes. The main objectives of this study are to improve dosing of prescription medications such as organic anionic drugs (Acyclovir, Cefoxitin, Cidofovir, Ciprofloxacin and others) that are renally secreted in deceased donor renal transplant (DDRT) and living donor renal transplant (LDRT) recipients, and to better understand Vitamin-D metabolism in these patients.

Our studies were prompted by our recent observations in animal models of kidney transplantation. After syngeneic rat kidney-Tx we have observed a significant down-regulation (3-14.5-fold decline) of m-RNA of several renal transporters (Oat1, Oat3, Oct2, Mdr1a, and Mate1) following 24hr cold ischemic time (CIT) and 4 weeks of tacrolimus treatment post-Tx.

However, this effect was not observed in rats that did not undergo 24hrs of CIT, indicating greater susceptibility of kidneys to tacrolimus with prolonged CIT. Reduced expression and activity of organic anionic secretion (renal OAT1/3 and MRP2 transporters) is expected to increase the systemic exposure of several anionic drugs that are primarily secreted in to the urine through this pathway. We hypothesize that endothelial damage and vasoconstriction associated with prolonged CIT in combination with CNI induced renal tubular damage will significantly alter the expression and activity of OAT1, OAT3 and MRP2 transporters, and vitamin D metabolizing enzymes in a transplanted kidney. In this study, we propose to evaluate the function of renal OAT1/3 and MRP2 transporters using cefoxitin, a second generation cephalosporin antibiotic, that is primarily cleared by renal tubular secretion. Calcitriol to calcidiol ratio will serve as a measure of vitamin-D metabolic capacity. Living donor and deceased donor renal transplant recipients (LDRT: n=10 with cold ischemic time of 12 hrs) will be recruited for this study. All of them will be treated with tacrolimus as part of standard of care. Within 1-week post-Tx and approximately 3 months post-Tx, a single dose of 200 mg of Cefoxitin will be administered iv and 4 ml of blood will be collected at approximately 0, 15 min, 30 min, 1hr, 1.5hr, 2hr, 3hr and 4hrs. Total urine voided over 8 hours will also be collected. The concentrations of cefoxitin in plasma and urine will be measured using a validated HPLC-UV method. Renal clearance and renal secretory clearance of cefoxitin will be calculated. Calcitriol to calcidiol ratio in plasma samples collected prior to transplantation, at approximately 1-week post-Tx and at 3 months post-Tx will provide a measure of Vitamin-D metabolic capacity of the kidney in LDRT and DDRT recipients. Due to increased damage to the tubular secretory transporters, DDRT recipients with longer CIT (>12 hr) would have a significantly lower secretion and metabolic capacity when compared to recipients with shorter CIT.

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A.3   STUDY OBJECTIVE AND SPECIFIC AIMS

Objective:
The primary objectives of this study is to evaluate changes in OAT1/3 and MRP2 mediated renal secretory clearance in deceased donor and living donor kidney transplant recipients.

The Secondary objective of this study is to evaluate changes in Vitamin-D metabolism (conversion of calcidiol to calcitriol) in deceased donor and living donor kidney transplant recipients.

Specific Aims:
Hypothesis:
Endothelial damage and vasoconstriction associated with prolonged cold ischemia in combination with calcineurin inhibitor mediated renal tubular damage will significantly down-regulate the expression and activity of OAT1/3 and MRP2 transporters and Vitamin-D metabolizing enzyme (CYP27B1) in deceased donor kidney transplant recipients.

Specific Aim 1:
To study the longitudinal changes in the activity of OAT1/3 and MRP2 renal transporters as measured by cefoxitin renal tubular clearance in living donor renal transplant (LDRT) and deceased donor renal transplant (DDRT) recipients on tacrolimus therapy.

Specific Aim 2:
To study the longitudinal changes in Vitamin-D metabolic capacity by comparing calcitriol (1,25-dihydroxycholecalciferol) to calcidiol (25-hydroxycholecalciferolin) ratio in LDRT and DDRT recipients on tacrolimus therapy.

Secondary Aims:
1. To study the longitudinal changes in mRNA and protein expression of OAT1/3 and MRP2 transporters in LDRT and DDRT recipients on tacrolimus therapy.
2. To study changes in FGF23 levels as a surrogate marker for decompensated renal secretory and metabolic function due to prolonged CI and tacrolimus therapy in DDRT.
3. To study the longitudinal changes in mRNA and protein expression of Vitamin-D receptor (VDR), and 25-hydroxyvitamin D-1 alpha hydroxylase (CYP27B1) in LDRT and DDRT recipients on tacrolimus therapy.
4. To associate serum cytokine concentrations to changes in activity of transporters studied.

A.4   BACKGROUND

Chronic kidney disease (CKD) is the ninth leading cause of death in the United States. An estimated 26 million adults or 13% of the US population is expected to have CKD. About 500,000 CKD patients are classified as having end stage renal disease (ESRD) with an estimated glomerular filtration rate (eGFR) of less than 15 mL/min/1.73 m^2. Kidney transplantation is the treatment of choice for the patients diagnosed with ESRD. About 16,000 kidney transplants are performed in the US every year. Renal allografts are subjected to a unique set of injurious conditions such as prolonged cold ischemia (CI) before being transplanted into the recipient, warm-reperfusion injury immediately after transplantation, exposure to nephrotoxic calcineurin inhibitor (CNI) based immunosuppression therapy, acute/chronic rejection of the organ, and bacterial/fungal/viral infections post-transplantation. The cold ischemic injury and nephrotoxic CNI therapy that the renal transplant recipients receive have been shown to lead to progressive loss of renal function with a five-year recipient survival of 84% for deceased donor kidney transplantations as compared to 91% for living donor kidney transplantations.

Cold Ischemic Injury:

Kidneys from deceased donors are typically preserved in University of Wisconsin preservation solution at 4C until a recipient is available for transplantation. This process of hypothermic preservation causes vasoconstriction and endothelial damage leading to cold ischemic injury to the graft. Cold ischemic injury is a non-specific renal tissue injury mediated by the release of hydroxyl radicals and cytosolic calcium. The renal proximal epithelial cells, which are primarily involved in the secretion of various drugs are also affected in a non-specific manner and we would expect this cold ischemic injury to alter the secretory capacity of the kidney.

Calcineurin Inhibitor Mediated Nephrotoxicity:

Calcineurin inhibitors (CNI) are the most effective class of immunosuppressants available in transplant medicine for maintenance of immunosuppression and their use has dramatically improved short-term graft survival in solid organ transplant recipients. Currently 94% of all renal transplant recipients are on CNI based maintenance immunosuppressive regimen. However CNI therapy is riddled with an acute and chronic nephrotoxicity profile and is a major contributing factor in allograft damage and graft loss beyond 5 years post-transplantation. CNI nephrotoxicity is thought to involve a decrease in vasodilation factors such as prostaglandin E2 and nitric oxide along with an increase in vasoconstriction factors such as thromboxane, endothelin and renin-angiotensin system. CNI inhibition of prolyl isomerase is also thought to cause protein synthesis impairment and accumulation of unfolded proteins, leading to endoplasmic reticulum enlargement.

Change in Secretory Capacity of a Kidney Alters Drug Exposure:

The tubular damage caused by cold ischemia and CNI could lead to alteration in the expression and activity of renal drug transporters and eventually affect the clearance of drugs that are predominantly cleared by renal secretion.

Total renal excretion function of a drug is a combination of glomerular filtration, tubular secretion and tubular reabsorption. Most drugs that are eliminated by tubular secretion primarily undergo active transport into the lumen of the proximal tubule. Different adenosine triphosphate binding cassette (ABC) or solute carrier (SLC) uptake and efflux transporters are located in the proximal tubules of the kidneys. Organic cationic transporters (OCTs) and organic anion transporters (OATs) are examples of SLC uptake transporters that are located on the basolateral membrane of renal proximal epithelial cells pumping drug substrates from the blood side into the

cells. Multi-drug resistance proteins (MRPs), and multidrug and toxin extrusion proteins (MATEs) are examples of efflux transporters that are located on the apical side of the proximal epithelial tubular cells pumping drugs specific for them out of the cell and into the tubular lumen. For a drug to be successfully cleared it has to be a substrate of an uptake and efflux pair (eg. Cefoxitin: OAT1/3 and MRP2, respectively). Progressive kidney disease, acute kidney injury, or administration of transporter inhibitors can alter the activity of specific renal transporters and ultimately alter exposure of drugs that are cleared by those renal transporters.

Renal dysfunction and CKD have also been shown to decrease rat Oct2, Oat1, Oat3 and Mate1 mRNA and protein expression levels. MRP2 (part of ABC superfamily) efflux transporter levels have been shown to be regulated by inflammatory cytokines such as TNF-α, IL-6, and IL-1β and the vasoactive hormone endothelin-1 (ET-1). Since renal allografts undergo prolonged CI, warm reperfusion injury and nephrotoxic CNI therapy, it is important to characterize the anticipated change in secretory capacity of renal allografts to ensure an optimal exposure of the drugs in transplant recipients.

Tacrolimus therapy in Combination with 24 hrs prolonged Cold Ischemia Reduces Selected Renal Transporter mRNA Expression in Transplanted Rat Kidneys:

Our recent work on the mRNA expression of Slc22a6 (Oat1), Slc22a8 (Oat3), Slc22a2 (Oct2), Slc47a1 (Mate1), and Abcb1a (Mdr1a/Pgp) renal transporters in the male Lewis rats showed that 24 hr Cold Ischemia in the presence of tacrolimus treatment has a significant down-regulatory effect on mRNA expression (3-14.5 fold decline) of the above mentioned 5 important renal transporters that are highly expressed in human renal tissues. This effect was reproduced after allogeneic transplantation. Interestingly, tacrolimus treatment mediated down-regulation of the selected transporters was absent in kidneys that were not subjected to 24 hrs of CI. These results

show that the tacrolimus mediated down regulation of renal transporter expression ooccurs only in the presence of prolonged cold ischemia. We expect to see similar results in the deceased donor renal transplant recipients who are also on tacrolimus based nephrotoxic calcineurin inhibitor maintenance therapy.

<u>Renal Function and Calcidiol metabolism:</u>

Vitamin-D is an essential steroidal hormone that has been associated with various functions including, calcium absorption, calcium, phosphate, and PTH homeostasis, modulation of cell growth, and immunomodulation. Recently Vitamin D has been studied for its potential utility in conditions that affect the immune system such as multiple sclerosis, type 1 diabetes, systemic lupus erythematosus, and solid organ transplantation. In animal kidney transplant models, supplementation with active vitamin D has shown beneficial with prolonged allograft survival. Kidneys are responsible for metabolizing majority of systemic calcidiol or inactive vitamin-D to its biologically active form Calcitriol (1,25-dihydroxycholecalciferol) by renal CYP27B1 enzymes in renal proximal epithelial cells. Clinically plasma concentrations of calcidiol are normally measured for monitoring vitamin-D levels due to its prolonged half-life compared to calcitriol. However, in renal transplant recipients the metabolic capacity of a transplanted kidney might be significantly reduced secondary to prolonged cold ischemic injury and tacrolimus mediated nephrotoxicity. Therefore, by studying calcitriol to calcidiol concentration ratio at different time-points post-LDRT and DDRT we can get a better understanding of changes in vitamin-D metabolic capacity of the transplanted kidney.

A.5 SIGNIFICANCE

<u>Need for Optimal Pharmacotherapy of Secreted Drugs in Renal Transplant Recipients:</u>

Renal transplant recipients are susceptible to various infections derived from the kidney donor and infectious complications of the surgical procedure in the immediate post-transplantation period (about 1-month post-Tx). Donor derived infections in the immunosuppressed host are cytomegalovirus (CMV), herpes simplex virus (HSV), varicella zoster virus (VZV), meningococcus, syphilis, candida, and aspergillus. Pneumocystic carinii, protozoal diseases, fungal infections, and mycobacteria (tuberculosis) are some other pathogens that may cause infections in the immunosuppressed recipients in the first 6 months post-transplantation. Prophylactic regimens of anti-bacterial, anti-fungal, and anti-viral agents are routinely prescribed to renal transplant recipients to prevent these above mentioned infections. Most of these drugs such as acyclovir (OAT1), valacyclovir (PEPT1), cidofovir (OAT1), trimethoprim/sulfamethoxazole(OATs/OCTs), ciprofloxacin (OATs/MRPs) are secreted by various uptake and efflux renal transporters in addition to being filtered. Currently, the dose and frequency of administration of these drugs are adjusted based on the filtration capacity rather than taking in to consideration the secretory capacity of the transplanted kidney. CI, warm reperfusion injury, stress due to transplantation surgery, and nephrotoxic immunosuppressive drugs (tacrolimus) may affect the renal secretory capacity and possibly leave these patients at an increased risk for anti-infective drug toxicity or sub-therapeutic anti-infective coverage. Additionally, transplant recipients may also see significant pharmacodynamic changes or drug toxicities due to increased exposure of drugs prescribed to treat co-morbidities. For example, diabetic transplant recipients may experience increased exposure to metformin due to OCT2 and MATE1 inhibition leading to abnormal decline of blood glucose levels.

Importance of Studying the Activity of Organic Anion Transporters (OATs) in Renal Transplant Recipients:

OAT1, OAT2, OAT3, and OAT4 are the 4 organic anionic transporters of the solute carrier superfamily expressed in human renal proximal tubular epithelial cells (RPTEC). OAT1, OAT2 and OAT3 are involved in taking-up substrate drugs and endogenous compounds from the basolateral side facing the blood into the RPTEC cells. OAT4 is expressed on the apical side of the RPTEC cells and helps efflux its substrates from the cell into the tubular lumen. OAT1 and OAT3 renal uptake transporters are considered to be the most important by the Federal Drug Administration (FDA) and European Medicines Agency (EMA) for their role in drug disposition and drug-drug interactions. OAT1 is selective for smaller amphiphilic substrates whereas OAT3 is selective for larger amphiphilic substrates. BCRP, MRP2, MRP4 and OAT4 are thought to be the efflux partners for OAT1 and OAT3. Since prolonged cold ischemia, transplantation, and calcineurin inhibitor therapy have been shown to cause considerable damage to the overall function of a transplanted kidney, we expect that the OAT1, OAT3 and MRP2 mediated organic anion substrate (eg. Cefoxitin) clearance would be altered. Studying the mRNA and protein expression as well as the secretory activity in renal transplant recipients would give us a better understanding to optimally dose OAT/MRP substrate drugs in renal transplant recipients.

A.6   RESEARCH DESIGN AND METHODS

This is longitudinal, prospective, open-label, non-randomized, pharmacokinetic study in deceased donor and living donor renal transplant recipients.

Subjects who have provided consent to participate in this study will undergo the following research related activities:

Renal transplant recipients will participate in the study at 3 different time-points: immediately prior to transplantation (baseline), approximately '1-2 week post-transplantation' (when the Serum Creatinine level stabilizes) and at approximately '3 months follow-up visit following transplantation'.

During the pre-transplantation time-point:

Medical history, physical examination, biochemical parameters that are collected in the routine standard care of the subjects will be collected. A single blood sample collected from the patient for routine clinical care (biochemical parameters) will be used to assay for proinflammatory cytokines, calcidiol, calcitriol and FGF23 levels. A protocol needle biopsy will be obtained as per UPMC renal transplant guidelines prior to transplantation for routine clinical monitoring. A part of the biopsy that is not used for clinical monitoring will be used to quantitate mRNA and protein expression of OAT1, OAT3 and MRP2 transporters as well as VDR and CYP27B1.

During the 1-2 week and 3 months post-transplantation time-points:

Biochemical parameters that are collected in the routine standard care of the subjects will be collected. For the cefoxitin pharmacokinetic assessment, a single dose of 200 mg of Cefoxitin will be administered intravenously and 4 ml of blood will be collected at approximately 0, 15 min, 30 min, 1hr, 1.5hr, 2hr, 3hr and 4hrs time-points post administration. Total urine voided over 8 hours from the time of administration of the drug will also be collected in 2 hour intervals.

The concentrations of cefoxitin in plasma and urine will be measured using a HPLC method. Proinflammatory cytokines, Calcidiol, calcitriol and FGF23 levels will also be quantitated from the 0 min time point blood sample. A total of 36 ml (about seven teaspoonful) of blood will be collected for each study. A total of 72 ml (about 14 teaspoonful) of blood will be collected during the entire study.

A protocol needle biopsy will be obtained as per UPMC renal transplant follow-up care guidelines at the 3 months follow-up visit. A part of the biopsy that is not used for clinical monitoring will be used to quantitate mRNA and protein expression of OAT1, OAT3 and MRP2 transporters as well as VDR and CYP27B1.

In patients with unresolved delayed graft function as determined by the transplant care team, the cefoxitin pharmacokinetic study will be rescheduled to another date/time within the first 2 weeks post-transplantation when the serum createnine improves and stabilizes as determined by the clinicials. If the renal function remains to be unstable or is not improving beyond 2 weeks post-transplantation, we will not be performing cefoxitin pharmacokinetic studies in these patients.

Patients who experienced an acute rejection episode in the first 3 months following transplantation will not undergo cefoxitin pharmacokinetic studies as acute rejection may alter the expression and activity of renal OAT/MRP transporters and may interfere with the interpretation of the study results.

The baseline blood samples from these patients will still be used for proinflammatory cytokines, calcitriol, calcidiol, and FGF-23 concentration measurements and baseline biopsy sample will still be used for mRNA and protein expression of OAT1, OAT3 and MRP2 transporters as well as VDR and CYP27B1 in these patients.

Personnel performing the procedures:

Study staff and staff at clinic or physician's office will perform the procedures.

Location of procedures: Montefiore Hospital and clinics.

Duration of procedures: Each study period will last a maximum of 8 hrs. The total duration of the entire study will be about 2 year.

Blood samples collected for quantitation of cefoxitin, calcitriol and calcidiol (before and during pharmacokinetic studies) may be used to quantitate other biomarker and drug levels.

A.7   HUMAN SUBJECTS

Subgroups	Number to undergo research procedures	Number to undergo screening procedures
Deceased donor renal transplant recipients with kidneys that underwent more than 12 hours of cold ischemia	10	20
Deceased Donor Renal Transplant Recipients with Kidneys that underwent less than 12 hours of cold ischemia	10	20
Living Donor Renal Transplant Recipients	10	20

Statistical justification:

The KDIGO 2012 CKD classification and corresponding cefoxitin secretory clearance+/- standard deviation reported by Kampf el al following IV Cefoxitin administration are G1: 237+/- 108 ml/min; G3a: 171+/-51 ml/min; G4: 97+/-72 ml/min; G5: 29+/-13 ml/min. We performed our sample size calculation based on magnitude and variability observed in cefoxitin tubular secretory clearance in the above patients with varying degrees of renal function. We need 10 renal transplant recipients in each of the 3 groups (1 LDRT control and 2 DDRT groups) with 2 measurement time-points in each group to detect a change of at least 30% in tubular secretory clearance of cefoxitin between the deceased donor comparator groups and corresponding living donor control group. The Alpha and beta errors are set at 5% and 20% respectively. This calculation was based on ANOVA: Repeated measures, within-between interaction tests performed on G*Power software.

Inclusion Criteria:

1. Have been scheduled for living or deceased donor renal allograft transplantation at UPMC

2. Men and women aged between 18-65 years

3. Subjects who are scheduled to receive de novo kidney transplant

4. Subjects willing to sign informed consent form

5. Be treated in accordance with the standard care protocols currently in effect for living and deceased donor renal transplant patients including immunosuppressants use and other elements of pre and post surgery

Exclusion Criteria:

1. Subjects receiving UNOS ECD organs

2. Pregnant or breastfeeding women

3. Re-transplantation

4. Subjects with HIV or Hepatitis B/C

5. Active tuberculosis

6. Body mass index > 35 kg/m2

7. Subjects who have developed malignancy or any medical condition that, in the investigator's opinion, should not be treated with cefoxitin

8. Subjects who can't undergo anti-thymocyte globulin based induction therapy

9. Subjects allergic to tacrolimus or cefoxitin

10. Subjects with unresolved delayed graft function by 14 days post-transplantation

11. Subjects with a hemoglobin of 8 g/dl or less.

We want to exclude patients with the above-mentioned characteristics as we think patients with these factors would add additional variability to the transporter activity and Vitamin D metabolic capacity studies. The above-mentioned criteria such as hepatitis, greater BMI, etc are not exclusion criteria for renal transplantation at UPMC but are exclusion criteria specifically for this study.

A.8   POTENTIAL RISKS AND BENEFITS OF STUDY PARTICIPATION

Research Activity:	Cefoxitin
Common Risks:	Cefoxitin will be administered at 1/5th FDA approved dose (200 mg) and so the risk of common and serious adverse effects associated with cefoxitin is anticipated to be much lower. Diarrhea is most common adverse reaction reported with cefoxitin use (1-10%).
Infrequent Risks:	Hypersensitivity reaction to cefoxitin or any components of the formulation may lead to an allergic reaction.
Other Risks:	*No Value Entered*

Research Activity:	Collection of Medical Record Information
Common Risks:	*No Value Entered*
Infrequent Risks:	*No Value Entered*
Other Risks:	Breach of confidentiality

Research Activity:	Intravenous Blood Draws
Common Risks:	Bruising, bleeding, swelling, pain associated with intravenous blood draws.
Infrequent Risks:	Fainting, infection
Other Risks:	*No Value Entered*

Research Activity:	Intravenous Drug Administration
Common Risks:	Bruising, bleeding, swelling, pain associated with intravenous route of administration
Infrequent Risks:	Fainting, infection
Other Risks:	*No Value Entered*

Steps taken to prevent or to minimize the severity of the potential risks:

Study involves administration of cefoxitin intravenously and obtaining blood and urine samples.

Collection of blood samples may pose minimal risk in-terms of bearable pain and risk for infection at the blood drawing site. Collection of urine poses no risk.

Biopsy sample will be obtained only as per standard clinical protocol outlines in the UPMC renal transplant guidelines.

[CONSENT FORMS AND STUDY SHEETS]

University of Pittsburgh

School of Pharmacy
Department of Pharmaceutical Sciences

711 Salk Hall
3501 Terrace Street
Pittsburgh, PA 15261
412-624-3330
Fax: 412-383-7436

TITLE: Effect of Prolonged Cold Ischemia in the Presence of Tacrolimus Therapy on the Renal Secretory and Vitamin-D Metabolic Capacity of Transplanted Kidneys

PRINCIPAL INVESTIGATOR:
Raman Venkataramanan, Ph.D. Professor of Pharmaceutical Sciences and Pathology
718 Salk Hall, University of Pittsburgh School of Pharmacy, 3501 Terrace Street, Pittsburgh, PA 15261, Phone: 412-400-7027

Co-investigators:

Department of Pharmaceutical Sciences:
Hari Varun Kalluri,
731 Salk Hall, School of Pharmacy, 3501 Terrace Street, Pittsburgh, PA 15261
Phone: 816-223-9360

Thomas Starzl Transplantation Institute:
Sundaram Hariharan; Abhinav Humar, Puneet Sood, Parmjeet Randhawa, Amit Tevar,
Miah Md Kowser; Thanukrishnan Harisudan
Montefiore Hospital, 3459 Fifth Avenue, Pittsburgh, PA 15213
Common Phone: 412-647-9966

SOURCE OF SUPPORT: Clinical Pharmacokinetics Laboratory Funds

Who is being asked to take part in this research study?
You are being invited to take part in this research study because you will receive either a kidney from a living donor or deceased donor. Female and male subjects, between the ages of 18 and 65 who are going to undergo living or deceased donor kidney transplantations are being asked to participate in this clinical study. This study will take place at the University of Pittsburgh Medical Center- Montefiore Hospital, Pittsburgh, PA, and will include approximately 30 kidney transplant recipients.

Why is this research being done?
Since kidney is the primary organ that clears various drugs from the body, changes in function of the kidney would significantly alter how drugs are removed from the body. A decrease in the ability of the

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kidney to remove medications from the body may cause increased drug related adverse effects. Kidneys that are transplanted into the recipients normally undergo numerous insults due to storage in cold preservation solution and use of drugs such as tacrolimus (Prograf®), which is necessary to prevent rejection of the transplanted kidney.

The primary objective of this study is to determine the effect of storing the kidney in cold preservation solution and use of a drug called tacroimus on the ability of the kidney to remove drugs that are normally removed by the kidney. We plan to study this by administering a FDA approved antibiotic called Cefoxitin and looking at how fast it is removed from the body at two different time points following your kidney transplantation. The secondary objective of this study is to determine changes in a transplanted kidney's ability to chemically change Vitamin D in the body. The results of the study will provide valuable information to the physicians and pharmacists to help improve proper dosing of drugs prescribed to future kidney transplant recipients.

How will the study be done?
If you decide to participate in this study, you will undergo a screening procedure and participate in the study at two times (at about 7 -14 days following transplantation and at 3 months following transplantation). The duration of the study will be a maximum of 8 hours at each time point. The entire duration of your participation would be about 100 days 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. All information collected will be confidentially held and used only for the purposes of the research study.

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

Screening Visit
The Screening Visit will determine if you are qualified to take part in this study. The screening visit will occur after you sign the informed consent following your selection as a recipient for living donor or deceased donor kidney transplantation. The doctor will review and collect information about your medical history including but not limited to your age, gender, weight, height, medical history, as well as clinical laboratory test results indicative of your liver and kidney function and infection status. You will also have a physical examination and your vital signs will be measured. If you meet all the study participation conditions, you are eligible to enter the study. Pregnancy testing is normally performed as part of the standard of care. This information will be used to make sure that only female subjects, who are not pregnant, to participate in this study.

Baseline Study (pre-transplantation): On the day of transplantation
- At this time, informed consent will be confirmed by your doctor. Your doctor will make a final decision if you are still eligible to participate in this study.
- A small piece of the kidney tissue (biopsy) is normally collected prior to transplantation as a part of routine clinical practice. Following the use of this tissue for clinical monitoring, if available a small part of the tissue that is no longer needed for clinical evaluation or decision-making will be stored for analysis as part of this study.
- Blood sample collected for your routine clinical care prior to transplantation will be used for additional analysis as part of this study.

Early Post-transplant study: around week 1 or 2 post-transplantation
During the first week or two following transplantation, a 200mg or $1/5_{th}$ regular dose of cefoxitin, an FDA approved antibiotic will be administered through a small tube inserted into your vein.

- Blood samples (4 ml or approximately 1 teaspoonful) will be collected at approximately 0 min, 15 min, 30 min, 1hr, 1.5hr, 2hr, 3hr and 4hr following administration of the drug. You will be asked to collect urine for 8 hours following the administration of the drug. Levels of cefoxitin and creatinine (a normal maker of your kidney function) will be measured.
- Additionally 1 drop of blood following a finger-stick (similar to finger-stick obtained during blood glucose monitoring) at the identical time points mentioned above (0, 15 min, 30 min, 1hr, 1.5hr, 2hr, 3hr and 4hrs) will be collected (total 8 blood drops).

Late Post-transplant study: around 3 month post-transplantation
This study will take place around your 3 month follow-up visit day. On this day the transplant clinical team will perform routine work-up. Female subjects will undergo a pregnancy test during this visit. Female subject will proceed with the study only if she is not pregnant.

- As per UPMC renal transplant guidelines, a small piece of kidney tissue (biopsy) will be obtained for routine clinical care at 90 days post-transplantation. Following the use of the tissue for clinical monitoring, if available a small part of the biopsy that is no longer needed for clinical evaluation or decision-making will be stored for analysis for the purposes of this study.

- Similar to the 'early post-transplant study', a 200mg dose of cefoxitin will be administered through a small tube inserted in to your vein.

- Blood samples (4 ml or approximately 1 teaspoonful) will be collected at approximately 0 min, 15 min, 30 min, 1hr, 1.5hr, 2hr, 3hr and 4hr following administration of the drug. You will be asked to collect urine for 8 hours following the administration of the drug. Levels of cefoxitin and creatinine (a normal maker of your kidney function) will be measured.

- Additionally 1 drop of blood following a finger-stick (similar to finger-stick obtained during blood glucose monitoring) at the identical time points mentioned above (0, 15 min, 30 min, 1hr, 1.5hr, 2hr, 3hr and 4hrs) will be collected (total 8 blood drops).

The entire duration of the study is going to be about 100 days from the day of your transplantation.

Blood samples collected during the study may be used for additional analysis as part of this study.

Follow-up Procedures:
The study staff will contact you via telephone within 24 hours of your discharge from the hospital (after the early post-transplant study). Study staff will also contact you within 24 hours following the 3 months follow-up visit (after the late post-transplant study). The purpose of the follow-up calls is to monitor and document any side effect that may occur due to cefoxitin (study drug). Diarrhea is the most commonly reported adverse effect. Allergic response, seizure, etc. are some of the rare but serious adverse reactions documented with cefoxitin use. Due to the nature of the drug, we do not anticipate any adverse-effects to persist beyond the follow-up call time frame.

Biological Samples for Future Research
All biological samples already collected during your clinical visits may also be stored and used for future testing related to the study. The samples will be stored for a maximum of ten years in the Clinical Pharmacokinetics Laboratory at the University of Pittsburgh under the direct supervision of 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 the subject in order to correlate with clinical data obtained during the study and only the study investigators will know to whom the sample belongs to. You will not be notified about any results from these tests, as they have no bearing on your medical management at this time.

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

Risk associated with intravenous (through a catheter inserted into the vein of your arm) blood draws and drug administration:
Bruising, bleeding, swelling, pain

Risks associated with Cefoxitin:
Cefoxitin will be administered at $1/5_{th}$ FDA approved dose (200 mg) and so the risk of common and serious adverse effects associated with cefoxitin is anticipated to be much lower. Diarrhea is most common adverse reaction reported with cefoxitin use (1-10%). Allergic reactions to cefoxitin or any components of the formulation may occur. An allergic reaction may range from a rash to shortness of breath depending on the severity of the reaction. Your medical team at UPMC will be prepared appropriately treat any allergic episode.

There is also a remote risk associated with breach of confidentiality

What are possible benefits from taking part in this study?
There is no direct benefit from participating in this study. However, your participation may help others in the future by what the doctors learn from your involvement in this study.

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 donor or deceased donor kidney transplantation.

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 procedures that you will undergo during this time are "research only services" that are being done only because you are in this study. These services will be paid for by the study and will not be billed to your health insurance company or you. Examples are measurement of cefoxitin in your blood and urine samples, biopsy analysis, etc.

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, immunosuppressive medications and routine care

medications, hospitalization and all associated care costs. These services will be billed to your health insurance company or you, if you do not have health insurance.

You will be responsible for paying any deductibles, co-payments or co-insurance that are a normal part of your health insurance plan. If you have the Medicare Advantage Plan you could be billed as if you were a Fee-for Service patient. You may also be responsible for the total cost of the transplant under a 3rd party Medicare plan. You may want to get more detailed information about what "routine clinical services" your health insurance is likely to pay for. You may want to talk to a member of the study staff and/or a UPMC financial counselor to get more information.

Will I be paid if I take part in this research study?
For each study visit you will receive a sum of $50. For the two study visits you will receive a total of $100.

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 staffs 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 (Dr. Venkataramanan) or one of the co-investigator (Drs. Kalluri, Harihara, Humar, Sood) 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.

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 recording of current and/or future identifiable medical information from your hospital and/or other (e.g., physician office) records. This research study will result in identifiable information that will be placed into your medical records held at UPMC Presbyterian and Montefiore. 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 (under "Confidentiality" or "Authorization to Use and Disclose Protected Health Information"). The study doctor, the sponsor or persons working on behalf of the sponsor, and under certain circumstances, the United States Food and Drug Administration (FDA) will be able to inspect and copy confidential study-related records, which identify you by name. Therefore, absolute confidentiality cannot be guaranteed. 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:

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.

Authorized representatives from the Food and Drug Administration may review and or obtain your identifiable (which may include your identifiable medical information) related to your participation in this research study for the purposes of monitoring the accuracy and completeness of the research data. While the U.S. Food and Drug Administration understands the importance of maintaining the confidentiality of your identifiable research and medical information, the UPMC and University of Pittsburgh cannot guarantee the confidentiality of this information after it has been obtained by the U.S. Food and Drug Administration.

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).

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.

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 7 years.

May I have access to my medical information that results from my participation in this research study?
In accordance with UPMC Notices of Privacy Practices document that you have been given, you are permitted access to information (including information resulting from your participation in this research study) contained within your medical records filed with your health care provider. A description of this clinical trial will be available on http://www.ClinicalTrials.gov as required by US Law. This website will not identify you. At most the Web site will include a summary of the results. You can search this site at any time.

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 University or Pittsburgh or UPMC hospital or affiliated health care provider or your current or future relationship with a health care insurance provider.

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 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 should 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, for example, your pregnancy test proves to be positive. You may be removed from the study if you experience unexpected conditions and in the opinion of the investigators that it is in your best interest. The study may also be stopped by the investigators or the sponsor if they felt that it is in the best interest of the patients.

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 Printed Name

_____   _____
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

Appendix section's first paragraph.

        Second paragraph.

UPMC
LIFECHANGINGMEDICINE

PHYSICIAN'S ORDER SHEET
PRO15010155

Subject Name:_____

Subject ID:_____

Date/Time Due:_____

Deliver to:_____

AUTHORIZATION IS GIVEN TO PHARMACY TO DISPENSE AND
TO THE NURSE TO ADMINISTER THE GENERIC OR CHEMICAL
EQUIVALENT WHEN THE DRUG IS FILLED BY THE PHARMACY
OF PRESBYTERIAN UNIV HOSPITAL OR MONTEFIORE UNIV
HOSPITAL - UNLESS NAME IS CIRCLED

DATE	TIME	***IDS*** PRO15010155
		TITLE: Effect of Prolonged Cold Ischemia in the Presence of Tacrolimus Therapy on the Renal Secretory and Vitamin-D Metabolic Capacity of Transplanted Kidneys
		IRB#: PRO15010155
		PRINCIPAL INVESTIGATOR: Raman Venkataramanan, Ph. D.
		CO-INVESTIGATORS: Sundaram Hariharan, MD; Abhinav Humar, MD; Hari Varun Kalluri, Pharm D; Parmjeet Randhawa, MD; Bodhisatwa Sengupta, MD; Ilango Sethu, MD; Puneet Sood, MD; Amit Tevar, MD; Raman Venkataramanan, Ph.D.
		COORDINATOR: Hari Varun Kalluri, Pharm D.
		FAX ORDER TO IDS @ (412)647-9651 M-F 6:30AM to 3PM
		PATIENT CONSENT SIGNED: YES or NO (circle one)
		DISPENSE: Cefoxitin 200mg/2ml for injection in syringe
		DIRECTIONS: Inject Cefoxitin 200 mg (2ml) intravenously as bolus.
		PHYSICIAN SIGNATURE:_____DATE:_____

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INVESTIGATIONAL DRUG SERVICE PATIENT ENROLLMENT SHEET

Study Title: Effect of Prolonged Cold Ischemia in the Presence of Tacrolimus Therapy on the Renal Secretory and Vitamin-D Metabolic Capacity of Transplanted Kidneys

IRB#: PRO15010155

PI: Raman Venkataramanan, Ph. D.

Patient Name:_____ ____ _____
                      first   mi   last

Patient Address:_____

          _____

          _____

Patient Phone number: (_____) _____

Social Security Number: _____

Date of birth:_____

Allergies:_____

Patient signed informed consent on file:YES / NO(circle one)

Scheduled first day of therapy:_____

Enrollment completed by:_____

Phone number for questions:_____

Fax completed form to the IDS Office at 647-9651
Please call to IDS Office with any questions at
  647-4958 or 647-3178 (pharmacists) or 647-9065 (technician)

DOSE MODIFICATION
 Date   DRUG   MODIFICATION   REASON
1)_____ _____ _____ _____

2)_____ _____ _____ _____

A.11   CEFOXITIN PHARMACOKINETIC SAMPLING FORM

IRB #:   PRO15010155   PI: Dr. Raman Venkataramanan

Title: Effect of Prolonged Cold Ischemia in the Presence of Tacrolimus Therpay on Renal Secretory and Vitamin-D Metabolic Capacity of Transplanted Kidneys

CEFOXITIN– PHARMACOKINETIC SAMPLING

Subject I.D. **FOX**	Date:	
Time of 200 mg iv Cefoxitin Administration:		
Volume of blood samples: 4 ml		
Expected time of collection – Before and after iv Cefoxitin Administration	Actual time of collection (hour)	Status
0 min/hr		
5-10 min		
20-30 min		
1 hr		
1.5 hr		
2 hr		
3 hr		
4 hr		
Expected time of Urine Sample collection (hours) – Before and after iv Cefoxitin Administration	Actual time of collection (hour)	Status
0 min/hr		
End of 2 hrs post iv cefoxitin (1-2 hrs period)		
End of 4hrs post iv cefoxitin (2-4 hrs period)		
End of 8hrs post iv cefoxitin (4-8 hrs period)		

Drug Administration:
Name:_____

Designation:_____

Date/Time:_____

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IRB #:   PRO15010155

Title: Effect of Prolonged Cold Ischemia in the Presence of Tacrolimus Therapy on Renal Secretory and Vitamin-D Metabolic Capacity of Transplanted Kidneys

PI: Dr. Raman Venkataramanan
Co-PI: Dr. Puneet Sood
Co-PI: Dr. Hari V Kalluri (816-223-9360)
Research Coordinator: Megan Basch

CEFOXITIN– PLASMA PHARMACOKINETIC SAMPLING

Subject ID: **FOX**

Date:

Sample for Cefoxitin: **Blood**

Time sample to be taken:

Actual time sample taken:

Please collect sample (4 ml) and keep it in the refrigerator for pick-up by investigators

Sample Collection:

Name:_____

For Research Investigators:

Time blood sample is centrifuged:

Duration of centrifugation:

Triple aliquot of plasma samples:

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IRB #:   PRO15010155

Title: Effect of Prolonged Cold Ischemia in the Presence of Tacrolimus Therapy on Renal Secretory and Vitamin-D Metabolic Capacity of Transplanted Kidneys

PI: Dr. Raman Venkataramanan

Co-PI: Dr. Puneet Sood

Co-PI: Dr. Hari V Kalluri (816-223-9360)

Research Coordinator: Megan Basch

CEFOXITIN– URINE PHARMACOKINETIC SAMPLING

Subject ID: **FOX**

Date:

Sample for Cefoxitin: **Urine**

Immediately before iv cefoxitin   2 hrs after iv cefoxitin 4 hrs after iv cefoxitin

Volume of Urine:

Time sample to be taken:

Actual time sample taken:

Please collect sample (8 ml) and keep it in the refrigerator for pick-up by investigators

Sample Collection:

Name:_____

For Research Investigators:

Triple aliquot of Urine samples:

A.12   CONCOMITANT MEDICATIONS LIST

IRB #: PRO15010155   PI: Dr. Raman Venkataramanan

Title: Effect of Prolonged Cold Ischemia in the Presence of Tacrolimus Therapy on Renal Secretory and Vitamin-D Metabolic Capacity of Transplanted Kidneys

Subject ID: FOX

CONCOMITANT MEDICATIONS

Circle one: Donor / recipient (pre-transplant) / recipient (post-transplant)

Medication	Indication for Use	Dosage/Regimen	Start Date	Stop Date	Comments

A.13 PRE-TRANSPLANTATION CHECKLIST

CHECK LIST - BASELINE PHASE (Pre-Transplantation)
IRB: PRO15010155
Title: Effect of Prolonged Cold Ischemia in the Presence of Tacrolimus Therapy on the Renal
  Secretory and Vitamin-D Metabolic Capacity of Transplanted Kidneys

Check the box below	CHECKLIST
	Informed Consent
	Assessment of eligibility criteria for enrollment
	Complete medical history
	Physical examination / vital signs
	Availability of biopsy sample
	Clinical laboratories
	Part of routine monitoring blood sample set aside for Vitamin D quantitation
	Recipient demographics (including indication for transplantation)
	Review current medications
	Cadaver donor demographics
	Cytotoxic cross match

Signature: Date:

A.14 ADVERSE EVENTS/ UNANTICIPATED PROBLEMS LOG

IRB #: PRO15010155   Title: Effect of Prolonged Cold Ischemia in the Presence of Tacrolimus Therapy on Renal PI: Dr. Raman
Venkataramanan   Secretory and Vitamin-D Metabolic Capacity of Transplanted Kidneys

Subject ID	Description	Start Date	Stop Date	Is the AE also an Unanticipated Problem YES/NO (see footnote)	Causality 1 - Related 2 - Possibly Related 3 - Not Related	Treatment / Comments	Sponsor Notification Date or NA	IRB Notification Date or NA
FOX								

Unanticipated Problem Criteria:
- Is the adverse event unexpected?
- Is the adverse event related or possibly related to participation in the research?
- Does the adverse event suggest that the research places subjects or others at a greater risk of harm than was previously known or recognized?

If the answer to **all three questions** is *yes,* then the adverse event is an unanticipated problem and must be reported to appropriate entities under the HHS regulations at 45 CFR 46.103(a) and 46.103(b)(5)
NOTE: Some sponsors may require reporting of all adverse event.