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Association of Extrarenal Adverse Effects of Posttransplant Immunosuppression With Sex and *ABCB1* Haplotypes

Rocco C. Venuto, MD, Calvin J. Meaney, PharmD, Shirley Chang, MD, Nicolae Leca, MD, Joseph D. Consiglio, MA, Gregory E. Wilding, PhD, Daniel Brazeau, PhD, Aijaz Gundroo, MD, Neha Nainani, MD, Sarah E. Morse, PharmD, Louise M. Cooper, MS, and Kathleen M. Tornatore, PharmD

Abstract: Extrarenal adverse effects (AEs) associated with calcineurin inhibitor (CNI) and mycophenolic acid (MPA) occur frequently but are unpredictable posttransplant complications. AEs may result from intracellular CNI accumulation and low activity of P-glycoprotein, encoded by the *ABCB1* gene. Since *ABCB1* single nucleotide polymorphisms (SNPs) and sex influence P-glycoprotein, we investigated haplotypes and extrarenal AEs.

A prospective, cross-sectional study evaluated 149 patients receiving tacrolimus and enteric coated mycophenolate sodium or cyclosporine and mycophenolate mofetil. Immunosuppressive AE assessment determined individual and composite gastrointestinal, neurologic, aesthetic, and cumulative AEs. Lipids were quantitated after 12-hour fast. *ABCB1* SNPs: *c.1236C>T* (*rs1128503*), *c.2677G>T/A* (*rs2032582*), and *c.3435C>T* (*rs1045642*) were determined with haplotype associations computed using the THESIAS program, and evaluated by immunosuppression, sex and race using multivariate general linear models.

Tacrolimus patients exhibited more frequent and higher gastrointestinal AE scores compared with cyclosporine with association to CTT

($P = 0.018$) and sex ($P = 0.01$). Aesthetic AE score was 3 times greater for cyclosporine with TTC haplotype ($P = 0.005$). Females had higher gastrointestinal ($P = 0.022$), aesthetic ($P < 0.001$), neurologic ($P = 0.022$), and cumulative AE ratios ($P < 0.001$). Total cholesterol (TCHOL), low-density lipoproteins (LDL), and triglycerides were higher with cyclosporine. The TTC haplotype had higher TCHOL ($P < 0.001$) and LDL ($P = 0.005$). Higher triglyceride ($P = 0.034$) and lower high-density lipoproteins ($P = 0.057$) were associated with TTT with sex-adjusted analysis.

ABCB1 haplotypes and sex were associated with extrarenal AEs. Using haplotypes, certain female patients manifested more AEs regardless of CNI. Haplotype testing may identify patients with greater susceptibility to AEs and facilitate CNI individualization.

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Abbreviations: *ABCB1* = ATP binding cassette gene subfamily B member 1, AE = adverse effect, CI = 95% confidence interval, CNI = calcineurin inhibitor, EC-MPS = enteric coated mycophenolate sodium, eGFR = estimated glomerular filtration rate, GI = gastrointestinal, HDL = high-density lipoprotein, ISR = immunosuppressive regimen, LDL = low-density lipoprotein, MMF = mycophenolate mofetil, MPA = mycophenolic acid, MPAG = mycophenolic acid glucuronide, OR = Odds ratio, P-gp = P-glycoprotein, PM = phenotypic mean, SNP = single nucleotide polymorphism.

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Correspondence: Kathleen M. Tornatore, Pharmacy Practice, Immunosuppressive Pharmacology Research Program, Translational Pharmacology Research Core, New York State Center of Excellence in Bioinformatics and Life Sciences, University at Buffalo School of Pharmacy and Pharmaceutical Sciences, 701 Ellicott Street, Buffalo, NY 14203 (e-mail: tornator@buffalo.edu).

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INTRODUCTION

Calcineurin inhibitors (CNI), tacrolimus, or cyclosporine, combined with mycophenolic acid (MPA), are the recommended immunosuppressive regimens to prevent renal allograft rejection.^{1–5} Since these drugs exhibit notable interpatient and inpatient variability in pharmacokinetics and clinical response, therapeutic drug monitoring of trough concentrations is the standard of care.^{6–8} However, well defined trough concentration versus effect relationships are lacking among renal transplant populations.^{7,9,10} Despite CNI dose adjustments, extrarenal adverse effects (AEs), gastrointestinal, neurologic and aesthetic alterations, as well as hyperlipidemia occur in an unpredictable manner and contribute to decreased medication adherence, increased morbidity, and impact long-term allograft survival.^{11–22} AE assessment scales in transplant patients have focused on self-evaluation of symptoms or quality of life in contrast to validated AE assessments that are used for antiretroviral or antineoplastic therapies.^{18,23–25} We have recently reported a validated, standardized immunosuppressive AE scoring system that includes extrarenal toxicities.²⁶

P-glycoprotein serves as an adenosine triphosphate (ATP)-dependent efflux pump for substrates, such as CNI, resulting in reduction of systemic exposure and lower intracellular drug

accumulation. Extensive tissue distribution of this efflux transporter reinforces the functional contribution of P-gp in the development of AEs.^{27–31} Alterations in P-gp expression or function have been attributed to genetics, sex, environment, or endogenous inhibitors.^{27–31} Reports regarding the influence of common *ABCB1* single-nucleotide polymorphisms (SNPs): *c.1236C>T* (*rs1128503*), *c.2677G>T/A* (*rs2032582*), and *c.3435C>T* (*rs1045642*) have focused on renal pharmacodynamics including acute rejection and nephrotoxicity postrenal transplant.^{7,10,32–34} However, the association between *ABCB1* SNPs and extrarenal adverse effects related to CNI is not well described, possibly due to the lack of a standardized AE assessment criteria, retrospective analysis, and uncontrolled patient inclusion criteria.^{3,15,26,35} Some reports have described individual SNPs, an approach that may not include the effect of multiple *ABCB1* SNPs and their interrelationship to AEs.^{33,36} These commonly evaluated *ABCB1* SNPs are inherited as a haplotype.^{10,33,34,36} Due to linkage disequilibrium, the 1236T-2677T-3435T (TTT) haplotype is the most prevalent variant, which has been associated with 80–100% reduced P-gp activity compared with wild type.^{28,37} Therefore, this haplotype variant is postulated to decrease P-gp activity and subsequently increase intracellular drug exposure with the potential for increased CNI AEs.^{7,33,38} It has been suggested that inclusion of *ABCB1* haplotypes may provide more insightful associations to AE phenotypes during CNI immunosuppression.^{33,34,39–42}

With regard to sex, hepatic and intestinal P-gp is significantly less in females compared with males and may contribute to increased AEs.^{29–31,43–45} These gender findings are often overlooked in pre- and/or postapproval studies in spite of the recognized increase in adverse effects that are manifested in women.^{43–46} Despite these recognized pharmacologic differences, limited sex-related studies of CNI pharmacokinetics have been reported and no sex-specific pharmacodynamic evaluations focused on AEs have been conducted.^{47–51} In a recent report, *ABCB1* expression in peripheral blood mononuclear cells (PBMC), the site of CNI pharmacologic action, was reduced and cyclosporine clearance was decreased in Caucasian female transplant recipients.⁵¹ These findings coupled with sex differences in drug metabolism, pharmacologic response, and physiology support further sex-specific evaluations of adverse drug effects related to CNI-based immunosuppression.^{31,43,46,52}

The objective of this study was to investigate extrarenal adverse effects and their association with *ABCB1* polymorphisms, haplotypes, and demographic factors including sex and race of stable renal transplant recipients receiving CNI and MPA immunosuppressive regimens.

METHODS

Ethics Statement

The study was approved by the University at Buffalo Health Sciences Institutional Review Board before enrollment. All patients provided informed consent with adherence to Declaration of Helsinki. The clinical research reported was consistent with the Principles of the Declaration of Istanbul as outlined in the 'Declaration of Istanbul on Organ Trafficking and Transplant Tourism'.

Study Participants and Protocol

Stable renal transplant recipients from the University at Buffalo (UB) transplant program at the Erie County Medical

Center were enrolled in prospective cross-sectional clinical pharmacology studies for assessment of nonrenal immunosuppressive AE. Patients receiving maintenance CNI and MPA immunosuppression were enrolled using standardized inclusion and exclusion criteria to verify clinical stability. Patients were stratified between Caucasians and African Americans with ethnicity verified for two generations. Inclusion criteria included the following: first, age 25–65 years; second, minimum 6 months postrenal transplantation; third, immunosuppressive regimen of either cyclosporine (*Neoral* microemulsion capsules, Novartis) with mycophenolate mofetil (MMF; *Cell-Cept*, Roche) or tacrolimus (*Prograf*, Astellas) with enteric-coated mycophenolate sodium (EC-MPS; *Myfortic*, Novartis) for ≥ 3 months; fourth, no change in immunosuppressive doses ≥ 7 days before study; fifth, no change in serum creatinine ≥ 0.25 mg/dL in prior 2 clinic visits; sixth, white blood cell count $\geq 3000/\text{mm}^3$; and hematocrit $\geq 30\%$. Exclusion criteria included the following: first, serum creatinine ≥ 3.5 mg/dL; second, active infection or acute rejection within 2 weeks; third, significant comorbid diseases limiting participation including gastrointestinal, cardiovascular, hematologic, psychiatric, neurologic, or oncologic disease; fourth, medication nonadherence; fifth, cytochrome P4503A4 or P-glycoprotein inhibitors or inducers within 4 weeks; sixth, drugs that interfere with absorption of CNI or MPA. Physical examinations included comprehensive metabolic panels. Complete blood counts were completed after enrollment and repeated with lipid panels after overnight fast on study morning. Medication history and compliance assessment were performed upon enrollment and 7 days before study. Routine therapeutic drug monitoring for CNI was ongoing. Targeted tacrolimus troughs were 5–10 ng/mL and 90–150 ng/mL for cyclosporine. Estimated glomerular filtration rate (e-GFR) was calculated using the 4-factor MDRD equation.⁵³

A 0 hour blood sample (~25 mL) was collected before oral immunosuppressive administration for CNI and MPA troughs with baseline laboratory tests. Standardized low fat, low sodium meals were provided over study. Insulin, antihypertensive and antilipidemic medications were administered at least 3 hours after immunosuppressive medications. Blood (20 mL) was collected in Cell Preparation Tubes (CPT- BD Vacutainer) with sodium citrate pre-dose with separation of peripheral blood mononuclear cells (PBMCs). Tubes were immediately inverted 10 times and processed with centrifugation for 25 minutes at 1600 RCF at 25 °C. Plasma was aspirated leaving PBMC which were harvested; immediately frozen in liquid nitrogen and stored at -70°C until genotype analysis.

Adverse Effect Assessment

Patients were evaluated using the validated immunosuppressive AE rating system to document 17 nonrenal adverse drug responses and severity (Table 1).²⁶ Nephrologists used this AE evaluation in patients with physical examination, review of systems, recent laboratory results, and medication adherence assessment. Adverse effects were evaluated as a change from pretransplant status. Patients received a ranked score of 0 (no AE), +1, +2, +3 (severe AE) based upon each manifestation (Table 1).²⁶ To incorporate AE severity and interpatient comparisons, a ratio of the sum of AE scores divided by the maximum possible score was generated as the adverse effect ratio. Individual AE were combined into four composite categories: gastrointestinal (GI) (vomiting, diarrhea, dyspepsia, and acid suppressive therapy); neurologic (headache, tremor,

TABLE 1. Immunosuppressive Adverse Effects Scoring System²⁶

<u>Gastrointestinal Adverse Effects*</u>	
0:	None
1+:	Minimal vomiting
2+:	Excessive vomiting requiring symptomatic treatment
Diarrhea	
0:	None
1+:	One loose bowel movement per day
2+:	Two to five loose bowel movements per day
Dyspepsia	
0:	None
1+:	Episode of indigestion within one hour after taking immunosuppressive medication
2+:	Indigestion for at least half the day
3+:	Indigestion the majority of the day requiring symptomatic treatment
Acid suppressive therapy	
0:	None
1+:	Daily use of a histamine-2 receptor antagonist OR a proton pump inhibitor
2+:	Daily use of a histamine-2 receptor antagonist AND a proton pump inhibitor
<u>Aesthetic adverse effects*</u>	
Acne	
0:	None
1+:	Lesion restricted to face, no specific treatment
2+:	Numerous facial lesions; lesions of upper trunk
3+:	Grade II and topical treatment
4+:	Grade II and systemic treatment
Skin changes	
0:	No change
1+:	No gross change but easy bruising
2+:	Obvious thinning multiple or frequent ecchymoses
3+:	Grade II and easy sloughing and/or striae; lacerations
Hirsutism	
0:	None
1+:	New hair growth anywhere
2+:	Marked increase in hair growth with noticeable change in appearance
3+:	Grade II and use of depilatories or shaves
Gingival hyperplasia	
0:	Absent
1+:	Some hyperplasia
2+:	Severe hyperplasia and bleeding
<u>Neurologic adverse effects*</u>	
Tremor	
0:	None
1+:	Noticeable movement of paper suspended over outstretched hands with fingers spread
2+:	Prominent movement of suspended paper
3+:	Resting tremor and/or tremor that impairs function
Headache	
0:	Absent
1+:	Present
Insomnia	
0:	None
1+:	Difficulty sleeping
2+:	Noticeable loss of sleep; requires pharmacotherapy to achieve adequate sleep
3+:	Severe sleep deprivation; difficulty sleeping despite pharmacotherapy
<u>Miscellaneous adverse effects*</u>	
Myopathy	
0:	None
1+:	Complaints of muscle weakness or decreased strength

2+:	Clinically apparent weakness difficulty rising from chair or squatting position
3+:	Muscle wasting + severe limitation of exercise capacity
Posttransplant	Symptoms of diabetes plus casual plasma glucose ≥ 200 mg/dl OR
Diabetes mellitus (2 of the criteria are required)	Fasting plasma glucose ≥ 126 mg/dL OR
0: absent	2 hour plasma glucose ≥ 200 mg/dL during an oral glucose tolerance test OR
1+: present	Glycated hemoglobin A1C $> 7\%$ OR
	Use of anti-diabetic agents

Lipid panel

Total cholesterol, mg/dL	_____
High density lipoprotein, mg/dL	_____
Low density lipoprotein, mg/dL	_____
Triglycerides, mg/dL	_____

* The scoring system presents a quantitative severity rating of the common adverse effects of maintenance immunosuppressive therapy consisting of a calcineurin inhibitor, mycophenolic acid formulation, with or without corticosteroids. Each adverse effect was assessed as a change from pretransplant status to document that the manifestation was attributed to immunosuppressive therapy or a change related to therapy in the posttransplant period.

and insomnia); aesthetic (acne, skin changes, hirsutism, and gingival hyperplasia); and cumulative AE that represented the summation of GI, neurologic, and aesthetic AE plus myopathy and posttransplant diabetes. Hyperlipidemia was defined from fasting lipid profiles with total cholesterol ≥ 200 mg/dL or antilipidemic pharmacotherapy. Posttransplant diabetes was defined in Table 1 with no pretransplant hyperglycemia.

Genetic Analysis

The PBMC samples were stored at -70°C until analysis. All specimens from 149 patients were viable and analyzed in a genomics laboratory. Laboratory personnel with no knowledge of clinical data performed the genotyping.

DNA isolation from PBMC of patients was completed per manufacturers' protocol (Wizard Genomic DNA Purification. Promega, Madison, WI). Ten ng of DNA was used to characterize the following *ABCB1* SNPs: *c.1236C>T*, (*rs1128503*), *c.2677G>T/A* (*rs2032582*), and *c.3435C>T* (*rs1045642*) using validated TaqMan allelic discrimination assays (Applied Biosystems, Foster City, CA) with Bio-Rad Laboratories CFX96 Real-Time Polymerase Chain Reaction Detection System (Hercules, CA). Each sample was analyzed in duplicate for each SNP. Allele frequencies were confirmed in Hardy-Weinberg equilibrium. Linkage disequilibrium (LD) among the 3 *ABCB1* SNPs was found to be significant and ranged from 0.89 (2677–3435) to 0.72 (1236–3435). Given the linkage among all 3 SNPs, haplotype analysis was conducted. In addition, haplotype analysis provides greater power to detect potential unknown functional variants than SNPs alone.⁴² *ABCB1* haplotype estimation was determined using the THESIAS program.⁴² THESIAS uses maximum likelihood algorithm for the simultaneous estimation of haplotype frequencies and their associated effects on single or multiple phenotypic measures. Significant AE associations were reported relative to phenotypic means for each haplotype (single chromosome). Total patient phenotypic means such as individual AEs, AE ratios, and lipid profiles represented the sum of contributions from haplotypes from both chromosomes.

Statistical Analysis

Continuous data were reported as means and standard deviations and categorical data using frequencies and

percentages. Differences in demographic variables for groups defined by sex, race, and immunosuppressive regimen were statistically assessed with Student's *t* test, ANOVA, or Fishers exact tests as appropriate. Individual AE were dichotomized as present or absent and modeled using logistic regression. General linear models were used to determine association of predictors: race, sex, CNI, and the race-sex interaction with AE ratios. Groups defined by the predictors were compared in a pair-wise fashion with Bonferroni adjusted *P*-values for multiple testing when appropriate. Model assumptions were assessed using diagnostic plots and data transformations were applied as needed. In order to examine the effect of the predictors, independent of confounding variables, separate models were fit incorporating single demographic, clinical, or genetic covariates including the following: time posttransplant, cyclosporine or tacrolimus troughs, weight-adjusted MPA and CNI doses, MPA and MPA-glucuronide 12-hour troughs, estimated glomerular filtration rate (eGFR), hemoglobin, *ABCB1* genotypes and presence or absence of diabetes, statin or prednisone use. Nominal 2-sided significance level of 0.05 was used throughout analysis. Statistical analyses, which were based on all available data, were conducted using SAS Statistical Software (version 9.3, SAS Institute Inc, Cary, NC). THESIAS was used to estimate haplotype frequencies and phenotypic means (with 95% confidence intervals) for each AE and ratios.⁴² Association of haplotype frequencies with individual AEs and respective scored ratios were analyzed using standard goodness-of-fit tests. Pairwise comparisons between race-sex groups were associated with a minimum detectable difference of 1.2 standard deviations at 80% power with the study sample sizes.

RESULTS**Demographics**

From January 2004 through June 2011, 149 stable renal transplant recipients were enrolled with AE evaluation completed using validated criteria outlined in Table 1.²⁶ Demographic and laboratory parameters are summarized in Table 2. The daily MPA dose was lower with the tacrolimus regimen compared with the cyclosporine treatment ($P < 0.001$). During tacrolimus therapy, the MPA troughs were twice those reported with cyclosporine regimens ($P < 0.001$). Lower MPAG troughs

TABLE 2. Demographic and Laboratory Parameters for RTR Treated With Calcineurin Inhibitors and Mycophenolic Acid

Parameter*	Cyclosporine and MMF (n = 82)	Tacrolimus and EC-MPS (n = 67)	P Value [†]
Sex, n (%)			<0.001 [‡]
Male	68 (82.9%)	38 (56.7%)	
Female	14 (17.1%)	29 (43.3%)	
Race, n (%)			0.078 [‡]
African-American	31 (37.8%)	35 (52.2%)	
Caucasian	51 (62.2%)	32 (47.8%)	
Age, yr	52.5 (10.2)	49.0 (11.5)	0.046
Time post-transplant, yr	4.77 (3.58)	3.01 (2.53)	0.001
Body mass index, kg/m ²	30.0 (6.26)	30.1 (6.02)	0.891
Serum creatinine, mg/dL	1.72 (0.56)	1.45 (0.46)	0.002
eGFR (mL/min/1.73 m ²)	47.8 (17.8)	50.5 (15.6)	0.024
White blood cells (× 10 ³ /mm ³)	6.12 (1.91)	5.27 (1.92)	0.004
Albumin, g/dL	3.95 (0.43)	4.13 (0.29)	0.007
Total cholesterol, mg/dL	189 (51.1)	157 (40.9)	<0.001
LDL, mg/dL	99.6 (38.9)	79.1 (27.8)	<0.001
HDL, mg/dL	44.3 (18.1)	49.9 (17.1)	0.019
Triglycerides, mg/dL	227 (210)	141 (121)	<0.001
Prednisone use [§] n, %	40 (48.8%)	14 (20.9%)	<0.001 [‡]
Statin use n, %	41 (50.0%)	23 (34.3%)	<0.001 [‡]
Calcineurin inhibitor daily dose, mg/day	240 (103)	6.7 (3.5)	NA
Calcineurin inhibitor trough, ng/mL	130.3 (46.9)	7.2 (1.9)	NA
MPA daily dose, mg	1910 (633)	1206 (343)	<0.001
MPA trough concentration, mg/L	2.09 (1.42)	4.07 (2.22)	<0.001
MPAG trough concentration, mg/L	98.5 (57.1)	72.7 (46.0)	0.006

EC-MPS = enteric coated mycophenolate sodium; eGFR = estimated glomerular filtration rate; HDL = high-density lipoprotein; LDL = low-density lipoprotein; MMF = mycophenolate mofetil; MPA = mycophenolic acid; MPAG = mycophenolic acid glucuronide; NA = not applicable, RTR = renal transplant recipients.

* Data represented as mean (standard deviation) or frequency (percentage).

† Statistical comparisons between groups analyzed by T-test, unless noted otherwise.

‡ P-values determined by a χ^2 test.

§ Prednisone use was ≤ 5 mg as daily doses.

($P < 0.006$) were found in the tacrolimus treated patients. No sex differences were noted between CNI and MPA troughs within each regimen.

Extrarenal Adverse Effects

Table 3 summarizes the individual extrarenal AEs according to immunosuppressive regimen and severity rating. Gingival hyperplasia (OR: 28.1; CI 9.20–85.7, $P < 0.001$); hirsutism (OR: 11.6; CI 3.92–34.2, $P < 0.001$), skin changes (OR: 4.10; CI 1.57–10.7, $P = 0.004$), and tremor (OR: 2.22; CI 1.07–4.57, $P = 0.031$) were more common with cyclosporine and MMF combination. More frequent insomnia (OR: 2.34; CI 1.10–5.00, $P = 0.027$); diarrhea (OR: 4.17; CI 1.69–10.2, $P = 0.002$); and dyspepsia (OR: 2.93; CI 1.41–6.10, $P = 0.004$) were noted with tacrolimus and EC-MPS. Females experienced more dyspepsia (OR: 2.68; CI 1.02–7.06, $P = 0.046$), skin changes (OR: 4.93; CI 1.82–13.4, $P = 0.002$), hirsutism (OR: 4.08; CI 1.41–11.8, $P = 0.01$), and myopathy (OR: 3.65; CI 1.35–9.82, $P = 0.01$). A race–sex interaction was noted with myopathy ($P = 0.01$). Post-transplant diabetes was present in 10% of patients with no difference between regimens. No covariate was significant for individual AE. Hyperlipidemia was observed in 58% of combined patients. Higher total cholesterol ($P < 0.001$), low-density lipoprotein (LDL) ($P < 0.001$), and triglycerides ($P < 0.001$) were found during cyclosporine treatment with higher HDL with tacrolimus regimen ($P = 0.019$) as summarized in Table 2.

Statin or prednisone therapies were not significant covariates associated with lipids.

Table 4 summarizes the AE ratios and lipid parameters relative to sex and immunosuppressive regimen. All AE ratios ranged from 1.4 to 2.0-fold greater in females. The tacrolimus and EC-MPS group exhibited GI AE ratio twice the cyclosporine regimen. Cyclosporine and MMF patients had an aesthetic AE ratio 3 times greater than the tacrolimus regimen ($P < 0.001$). Sex differences were found with higher GI ($P < = 0.022$) and neurologic AE scores ($P < = 0.022$) in females on a tacrolimus-based regimen. Tacrolimus and cyclosporine troughs were significant covariates with GI AE ratio. Race–sex interaction was found with higher neurologic AE ratios in 77% of African American females compared with 32% for other groups ($P = 0.04$). The lipid parameters are further categorized according to sex, race, and immunosuppressive regimen in Table 4 with higher total cholesterol, LDL, and triglycerides found in patients on cyclosporine and MMF. Sex differences were found with higher total cholesterol ($P = 0.026$) and high-density lipoproteins ($P < 0.001$) in females. No associations with CNI and MPA troughs and individual AE were detected.

ABCBI SNPs and Associations With Extrarenal Adverse Effects

Distribution of ABCBI genotypes (n = 149) for SNPs is summarized in Table 5. Hardy–Weinberg equilibrium was

TABLE 3. Frequency of Severity Scores for Immunosuppressive Adverse Effects^{*,†}

Adverse Effects	0	1+	2+	3+	Overall Frequency (as %)	P-Value
Vomiting	77 / 60	2 / 7	1/0	NA	3.75/10.4	0.190
Diarrhea	71/44	7/15	2/8	NA	11.3/34.3	0.002
Dyspepsia	55/25	21/3	4/34	0/5	31.3/62.7	0.004
Acid suppressive therapy	32/28	47/35	1/4	NA	60.0/58.2	0.968
Acne [‡]	64/56	13/9	3/2	0/0	20.0/16.4	0.532
Skin changes	51/54	24/11	5/1	0/1	36.3/19.4	0.004
Hirsutism	41/58	18/8	15/0	6/1	48.8/13.4	<0.001
Gingival hyperplasia	24/62	54/5	2/0	0/0	70.0/7.46	<0.001
Tremor	25/33	48/31	7/3	0/0	68.8/50.7	0.031
Headache [§]	73/55	7/12	NA	NA	8.75/17.9	0.281
Insomnia	61/34	13/16	6/16	0/1	23.8/49.3	0.027
Myopathy	61/54	16/12	2/1	1/0	23.8/19.4	0.073
Posttransplant diabetes mellitus [§]	68/64	12/3	NA	NA	15.0/4.48	0.063

NA = nonapplicable since no rating.

* Data displayed as cyclosporine and MMF regimen (n = 80; 2 patients did not have adverse effects assessment completed)/tacrolimus and EC-MPS regimen (n = 67).

† Severity score determined using Figure 1.

‡ No patients received a 4+ for acne.

§ Rated as present or absent.

|| P-values for comparison of cyclosporine + MMF regimen to tacrolimus + EC-MPS regimen using logistic regression.

TABLE 4. Adverse Effect Ratios and Lipids by Immunosuppressive Regimen and Sex (n = 149)

Adverse Effect Ratio [*]	Cyclosporine and MMF (n = 82)		Tacrolimus and EC-MPS (n = 67)		P Value [†]
	Male (n = 68)	Female (n = 14)	Male (n = 38)	Female (n = 29)	
Cumulative	0.134 ± 0.0813	0.192 ± 0.073	0.107 ± 0.062	0.180 ± 0.078	ISR: 0.037 Sex: < 0.001 Race: 0.168
GI	0.121 ± 0.110	0.143 ± 0.111	0.231 ± 0.166	0.310 ± 0.235	ISR: < 0.001 Sex: 0.022 Race: 0.293
Neurologic [‡] frequency (%) of “high” score (AE ratio >0.25)	0.164 ± 0.149	0.163 ± 0.124	0.169 ± 0.148	0.256 ± 0.159	ISR: 0.200 [‡] Sex: 0.022 [‡] Race: 0.282 [‡]
Aesthetic	27.9%	28.6%	34.2%	62.1%	ISR: < 0.001 Sex: < 0.001 Race: 0.271
Lipid Parameters					
Total cholesterol, mg/dL	182 ± 47	223 ± 57	152 ± 48	163 ± 28	ISR: < 0.001 Sex: 0.026 Race: 0.717
LDL, mg/dL	117 ± 46	96 ± 37	76 ± 29	83 ± 26	ISR: < 0.001 Sex: 0.106 Race: 0.532
HDL, mg/dL	43 ± 18	50 ± 15	44 ± 13	58 ± 19	ISR: 0.569 Sex: < 0.001 Race: 0.123
Triglycerides, mg/dL	232 ± 217	203 ± 177	164 ± 153	112 ± 43	ISR: 0.027 Sex: < 0.215 Race: 0.522

AE = adverse effect; EC-MPS = enteric coated mycophenolate sodium; GI = gastrointestinal; MMF = mycophenolate mofetil.

* Data represented as mean (standard deviation).

† P values derived from general linear model.

‡ Logistic regression model for dichotomized neurologic AE ratio.

TABLE 5. ABCBI Genotype Frequencies

	Homozygous Wild Type n (%)	Heterozygous n (%)	Homozygous Variant n (%)
Allele position			
1236 C/T	63 (42.3)	61 (40.9)	25 (16.8)
2677 G/T	75 (50.7)	55 (37.2)	18 (12.2)
3435 C/T	57 (38.3)	68 (45.6)	24 (16.1)

Note: for 2677 position n = 148, all other positions n = 149. Hardy–Weinberg equilibrium was confirmed for allele frequencies at each position.

confirmed for allele frequencies at each position. Significant linkage disequilibrium existed among the 3 loci (0.72 to 0.89). No genotype associations were observed with individual AE or ratios, except for lower acne scores with 2677TT genotype compared with 2677GG (OR: 0.115; CI 0.017–0.768; $P=0.02$) and 3435CT genotype compared with 3435CC (OR: 0.320; CI 0.116–0.881; $P=0.04$). Estimated ABCBI haplotype frequencies (n = 148) are summarized in Figure 1 and do not vary significantly compared with previously reported estimated frequencies.^{54–56}

ABCBI haplotype and phenotypic AE associations are summarized in Table 6. The aesthetic AE ratio associated with TTC ($P=0.005$) was 3 times greater than observed with wild-type haplotype with significant covariates of tacrolimus

($P<0.0001$) or cyclosporine troughs ($P<0.0001$). The GI AE ratio associated with CTT haplotype was twice the wild-type haplotype ($P=0.018$) with sex ($P=0.01$), tacrolimus ($P<0.0001$), and cyclosporine troughs ($P<0.0001$) as significant covariates. TTT haplotype had no association with AE ratios. There were no ABCBI haplotype associations with individual ranked AEs.

As summarized in Table 6, total cholesterol phenotypic mean associated with TTC was approximately 1.7 times higher than the wild-type CGC ($P<0.001$). The LDL phenotypic mean associated with TTC haplotype ($P=0.005$) was 1.75 times higher than wild type. These haplotype associations for total cholesterol and LDL were not altered by covariate analysis including sex, race, e-GFR, statin, or prednisone therapy with tacrolimus ($P<0.001$) and cyclosporine ($P<0.001$) troughs as significant covariates. The HDL phenotypic mean associated with TTT haplotype ($P=0.057$) was 25% lower than wild type with sex-adjusted analysis. The triglyceride phenotypic mean associated with TTT ($P=0.034$) was approximately 35% higher than wild type with sex-adjusted analysis. Race, e-GFR, statin, and prednisone therapy were not significant covariates influencing HDL or triglyceride phenotypic mean. Tacrolimus ($P=0.04$) and cyclosporine ($P<0.0001$) troughs were significant covariates relating to triglyceride. Gender subanalysis was conducted. In this analysis, females with the TTT haplotype had higher cholesterol ($P=0.048$) and LDL ($P=0.056$) compared with the wild-type phenotype. This was not seen in males with TTT haplotype. Females with TTT haplotype had 17% lower HDL ($P=0.089$) and 60% higher triglycerides ($P=0.089$) compared with wild type. Counterpart males with TTT haplotype had 21% lower HDL ($P=0.065$) and 49 % higher triglycerides ($P=0.102$) compared with wild-type phenotype.

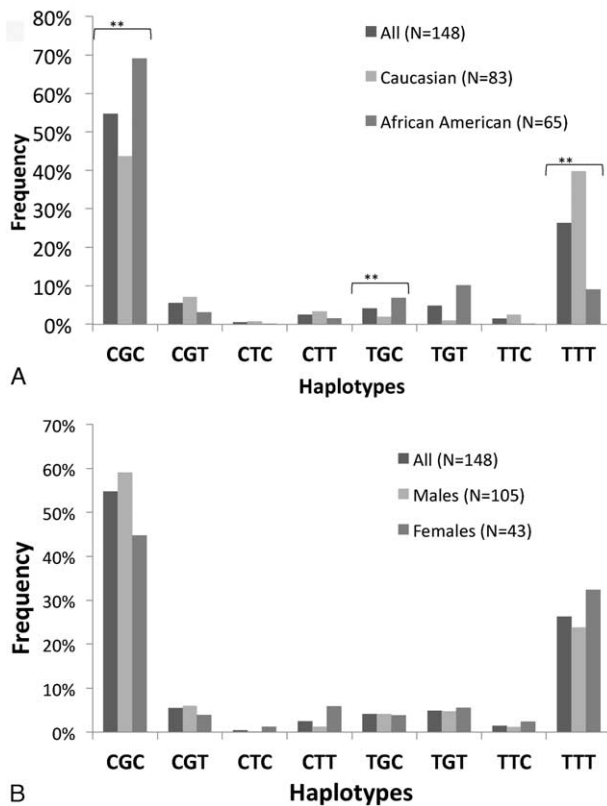


FIGURE 1. ABCBI haplotype frequencies by race (A) and sex (B). There were significant differences (** $P<0.001$) in haplotype frequencies between Caucasians and African Americans for CGC, TGC, and TTT. There were no significant differences in haplotype frequencies between sexes.

DISCUSSION

This is the first report of extrarenal immunosuppressive AE associations to sex and ABCBI haplotypes in stable renal transplant recipients using a validated assessment approach.²⁶ Since extrarenal AEs have been linked to medication nonadherence with implications for renal allograft survival, use of standardized AE assessment may provide a clinically feasible approach to document these manifestations longitudinally and contribute to approaches that individualize immunosuppression. As 60–70% of renal transplant patients develop hyperlipidemia post-transplant attributed to CNI immunosuppression, our sex-specific findings with TTT haplotype associations to fasting lipid parameters may have important clinical implications.^{57–59} Premature cardiovascular morbidity is the primary cause of death post-transplant necessitating aggressive intervention and monitoring of lipid parameters.^{57–59} Identification

TABLE 6. *ABCB1* Haplotype Associations With Adverse Effect Phenotype

Adverse Effect Measure	Variant Haplotype* and Phenotypic Mean† (95% CI)			
	Wild Type Haplotype* (CGC) Association to Phenotypic Mean† (95% CI)	Variant Haplotype	Phenotypic Mean (95% CI)	P Value‡
GI AE ratio	0.092 (0.70–0.114)	CTT	0.243 (0.122–0.364)	0.018
Neurologic AE ratio	0.100 (0.080–0.119)	No associations	NS	
Aesthetic AE ratio	0.058 (0.041–0.076)	TTC	0.187 (0.100–0.273)	0.005
Cumulative AE ratio	0.069 (0.058–0.079)	No associations	NS	
Lipid panel				
Total cholesterol, mg/dL	84 (78–91)	TTC	144 (120–169)	<0.001
LDL, mg/dL	43 (38–48)	TTC	76 (53–98)	0.005
HDL, mg/dL	16 (12–20)	TTT	12 (5–18)	0.057§
Triglycerides, mg/dL	134 (78–190)	TTT	181 (109–254)	0.034§

AE = adverse effect; CI = confidence interval; GI = gastrointestinal; HDL = high-density lipoprotein; LDL = low-density lipoprotein; NS = no significant associations.

* Haplotypes displayed at positions 1236–2677–3435. Wild-type is CGC.

† Phenotypic mean estimated from maximum likelihood methods by THESIAS; phenotype reported as contribution of single haplotype (one chromosome). For example, if a patient is homozygous wild-type, their total cholesterol would be estimated as 168 (84 × 2) or LDL estimated as 86 (43 × 2).

‡ P value represents variant haplotype comparison to wild type by THESIAS.

§ Reflects sex-adjusted analysis.

of stable patients with *ABCB1* variants and higher fasting lipid parameters in our study despite monitoring and antilipidemic therapy may identify high-risk patients needing preemptive therapy in the early post-transplant period. Our study reinforces different AE profiles between CNI-based regimens and this is the first AE evaluation that include sex and race associations. Existing postapproval drug evaluations for CNI lack this type of assessment.^{16,17,22,60–63}

P-gp interrelates with cytochrome P450 3A isoenzymes, CYP3A4 and CYP3A5, to modulate CNI pharmacokinetics that impact systemic and cellular distribution.^{6,7,21,33,34,36} The sex-specific difference in AE may be associated, in part, with the different function and expression of these isoenzymes and P-gp in liver and intestine between males and females. This sex difference may account for greater systemic CNI exposure in women.^{29,31,64,65} P-gp is encoded by the ATP-binding cassette, subfamily B, member 1 transporter (*ABCB1*) gene and is present on hepatocytes, enterocytes, renal tubular cells, blood brain barrier, and lymphocytes.^{33,34,36,66} Therefore, P-gp plays an essential role in CNI intracellular pharmacokinetics due to modulation of CNI concentration at the site of pharmacologic action and/or the site for organ-specific adverse effects.^{35,36,67} Limited clinical evaluation of the role of P-gp in drug-related AE has been conducted in stable renal transplant recipients as it is difficult to quantitate the transporter activity at tissue sites.²⁸ We observed sex differences in P-gp expression in peripheral mononuclear cells (PBMC) in recipients receiving cyclosporine and MMF.⁵¹ Specifically, Caucasian females were found to have 25–70 times less *ABCB1* gene expression in PBMC, suggesting that females have reduced P-gp expression and substrate efflux leading to higher intracellular cyclosporine concentrations.⁵¹ In subsequent studies, stable female and male renal transplant recipients receiving tacrolimus and EC-MPS had P-gp function in PBMC that was reduced in African American and Caucasian women.⁶⁸ These data provide

additional evidence for greater intracellular drug accumulation and increased AEs in females⁶⁸ and indirect support for sex-specific AEs with CNI immunosuppression.⁴⁴

Evaluation of *ABCB1* genotypes or haplotypes as indirect markers of cellular P-gp has been used to identify patients at higher risk for CNI-associated AE including nephrotoxicity, gingival hyperplasia, and neurotoxicity with variable success.^{69–75} This is the first prospective study to incorporate *ABCB1* haplotype analysis with standardized extra-renal AE assessment and fasting lipid parameters using pre-established patient inclusion criteria.²⁶ The validity of haplotype-based analysis has been widely accepted in association studies of unrelated individuals.⁴⁰ Haplotype analyses using maximum likelihood methods combined with stochastic expectation-maximization (SEM) algorithms have recently been used to assess candidate genes with specific phenotypes.^{41,42} Haplotypic data provide greater power to detect associations compared with single genotypes, especially when analyzed in conjunction with demographic and clinical covariates as conducted in this study.^{39,76} Inclusion of 3 loci as *ABCB1* haplotypes improves detection probability relative to the composite AE ratios and fasting lipid parameters. The TTC haplotype was associated with a 3.2-fold increase in aesthetic AE ratio regardless of immunosuppressive regimen. Interestingly, the significant association of GI AE ratio with CTT haplotype was not present when sex was included as a covariate, suggesting that being female was the predominate influence. In addition, tacrolimus and cyclosporine troughs were significant covariates when evaluating *ABCB1* associations with GI and Aesthetic AE scores.

ABCB1 polymorphisms have been associated with the lipid-lowering effect of statins and an inter-relationship of P-gp function with cholesterol transport.^{66,77,78} These findings have important implications in the transplant population as well as the general population considering the association with the

primary TTT variant with reduced function of P-gp and sex associations shown in our report.^{33,34,36} Clinical trials for treatment of hyperlipidemia and their implications on risk reductions of coronary artery disease have focused primarily on males enrolled.^{44,79,80} In addition, sex differences have been found in metabolism of high-density lipoproteins and triglycerides. Increased cardiovascular risks in females have been attributed to hormonal differences and sex-specific variants in *ABCB1*.^{44,77,81} Current treatment guidelines for hyperlipidemia have focused on low-density lipoproteins resulting in notable changes in males after intervention. However, this may not be a valid biomarker for females with this disease.^{44,77,81} The sex-specific TTT haplotype associations with HDL and triglycerides found in stable renal transplant patients in our study suggest evaluation in larger patient populations. The TTC haplotype was associated with higher total cholesterol and LDL concentrations. Greater triglycerides and higher HDL were found in patients with the reduced function TTT haplotype and this association remained significant when adjusted for statin or prednisone therapies. Our findings are consistent with reports of heart transplant recipients and TTT haplotypes.⁸² Although haplotype association studies provide a comprehensive view of genetic variants, limitations do exist. These can be a consequence of retrospective evaluation, small sample size, inconsistent use of haplotype analyses, multiple testing, and poorly defined phenotypic endpoints (eg, AEs).⁷⁶ Due to our sample size, some of the rare haplotypes are poorly represented and may limit our ability to detect significant effects for those very rare haplotypes. Individual *ABCB1* SNPs: c.2677G > T/A (rs2032582), and c.3435C > T (rs1045642) have variable outcomes as pharmacogenomic predictors with different drug substrates; thus, the haplotype approach may improve detection ability for phenotypic differences as we have employed.^{83,84} However, our study was designed as prospective evaluation of stable patients with pre-established inclusion criteria using validated AE assessment as the phenotypes of interest.

The advantages of our study include the prospective enrollment of stable recipients using pre-established inclusion criteria and incorporation of validated, scored extra-renal AE with severity quantification, as well as fasting lipid profiles. Another advantage is the inclusion of *ABCB1* haplotype analysis to minimize the limitations of multiple testing with use of individual variants. Multivariate analysis also incorporated common clinical covariates to further identify patients at risk for AEs. Reporting bias of AEs may occur with females due to increased body awareness and conscientious medical follow-up.⁸⁵ However, this was minimized by using a physician administered standardized, objective AE that incorporated physical findings, updated laboratory results, concomitant medications, and adherence assessment.

This adverse effect rating approach used with *ABCB1* haplotypes may be incorporated into baseline medical evaluation and monitoring postrenal transplant. This report has identified patients who may be at higher risk to develop specific immunosuppressive adverse effects.

CONCLUSION

This is the first report of *ABCB1* haplotype and sex associations with extrarenal AEs in stable transplant patients receiving CNI and MPA immunosuppression. Significant associations of *ABCB1* haplotype variants were observed with gastrointestinal and aesthetic AE ratios as well as with lipid profiles. Using the haplotype approach, female patients were

identified who manifested more AEs regardless of the CNI regimen. These results indicate that *ABCB1* haplotype testing in conjunction with sex will likely identify patients at risk for extrarenal adverse effects.

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