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# Association of *UGT2B7, UGT1A9, ABCG2*, and *IL23R* polymorphisms with rejection risk in kidney transplant patients

Heloísa Lizotti Cilião[a], Rossana Batista Oliveira Camargo-Godoy[b], Marilesia Ferreira de Souza[a], Mariana Bisarro dos Reis[c]#, Lorena Iastrenski[a], Vinicius Daher Alvares Delfino[b], Silvia Regina Rogatto[c]#, and Ilce Mara de Syllos Cólus[a]

[a]Department of General Biology, Center of Biological Sciences, State University of Londrina, Londrina, Paraná, Brazil; [b]Center of Health Sciences, State University of Londrina, Londrina, Paraná, Brazil; [c]Faculty of Medicine, São Paulo State University (UNESP), Botucatu, São Paulo, Brazil

**ABSTRACT**

Despite advances in testing compatibility between donor and recipient, graft rejection remains a current concern. Single-nucleotide polymorphisms (SNPs) that codify altered enzymes of metabolism, drug transport, and the immune system may contribute to graft rejection in transplant patients. This study examined the association between SNPs present in genes of these processes and occurrence of graft rejection episodes in 246 kidney transplant patients, 35% of which were diagnosed with rejection. Genotype–gene expression associations were also assessed. Peripheral blood samples were used for genotyping of 24 SNPs on the following genes: *CYP3A4, CYP3A5, CYP2E1, POR, UGT2B7, UGT1A9, ABCB1, ABCC2, ABCG2, SLCO1B1, TNF, IL2, IRF5, TGFB1, NFKBIA, IL10, IL23R, NFAT*, and *CCR5* by real-time PCR. The analysis of gene expression was performed by RT-qPCR. The association between graft rejection episodes and polymorphic variants was assessed using odds ratios. Polymorphisms rs7662029 (*UGT2B7*) and rs6714486 (*UGT1A9*) were associated with occurrence of graft rejection episodes, rs7662029 (*UGT2B7*) exhibited a protective effect (1.85-fold), and rs6714486 (*UGT1A9*) an increased 1.6-fold increased risk of graft rejection. Among drug transporter genes, only rs2231142 (*ABCG2*) demonstrated an association with a 1.92-fold decrease in the risk of graft rejection. The immunological SNP rs10889677 (*IL23R*) was associated with a 1.9-fold enhanced risk of graft rejection. Association between genotypes and gene expression was not detected. Therefore, SNPs of *UGT2B7, UGT1A9, ABCG2*, and *IL23R* genes may be useful as candidate markers for screening of risk graft rejection in renal transplant patients. These markers may improve medical decisions, avoiding adverse effects.

## Introduction

In the absence of contraindications, kidney transplantation is considered the best option for restoring renal function in patients with advanced chronic kidney disease, not only for medical, social, and economic perspective, but also to increase survival of these patients (Wolfe et al., 1999). Following transplantation procedures, patients use immunosuppressive drugs to reduce the risk of organ rejection such as the triple immunosuppressive regimen consisting of corticosteroids, tacrolimus, and mycophenolate mofetil (MMF). However, prolonged use of these drugs triggers adverse effects (Chapman et al., 2005; Burckart & Amur, 2010), which limit the long-term benefits of transplantation. The adverse effects influence patients differently owing to inter-individual variability in the response to immunosuppressive drugs, a consequence that may arise, among other factors, from the presence of single-nucleotide polymorphisms (SNPs) in genes involved in the pharmacokinetics/pharmacodynamics of these drugs. These SNPs may be associated with rapid drug clearance, resulting in low plasma chemical concentrations, which initiate rejection episodes (Elens et al., 2011). In contrast, high concentrations of immunosuppressive drugs over a long period after transplantation might lead to (i) decline in renal function and/or graft loss due to nephrotoxicity (Fadili et al., 2013); (ii) loss of renal function due to immunological

**CONTACT** Ilce Mara de Syllos Cólus ilcecolus@gmail.com Departamento de Biologia Geral, Centro de Ciências Biológicas, Universidade Estadual de Londrina. Rodovia Celso Garcia Cid, Km 380, CEP: 86057-970, Londrina, Paraná, Brasil.

# Current address: Department of Clinical Genetics, Vejle Hospital, DK and University of Southern Denmark, Denmark.

damage as evidenced by inflammation and antibody-mediated injuries (Ong & Gaston, 2015), and (iii) development of neoplasia and inflammation (Apel et al., 2013).

Phase I metabolism genes, namely cytochrome P450 (CYPs) and drug-transporting P-glycoprotein genes, such as P-glycoprotein 1 also known as multidrug resistance protein 1 (MDR1, ABCB1 gene), have been reported as the most important genes involved in the pharmacokinetics/pharmacodynamics of calcineurin inhibitors (Hebert, 1997; MacPhee et al., 2005) and the immunosuppressant sirolimus (Sattler et al., 1992). Proteins encoded by *UGT2B7* and *UGT1A9* phase II metabolism genes are involved in mycophenolate mofetil (MMF) metabolism (Picard et al., 2004), and genes coding for efflux pumps, such as multidrug resistance protein 2 (*ABCC2*), breast cancer resistance protein (BCRP, *ABCG2* gene), and organic anion-transporting polypeptide (*SLCO1B1*), are responsible for the elimination of the major metabolites of MMF (Miura et al., 2008). Therefore, the presence of polymorphisms in these genes might affect the availability of the immunosuppressive drugs and consequently result in a poor correlation between dose, plasma concentration, and therapeutic response (Hesselink et al., 2003; Zununi Vahed et al., 2015).

Polymorphisms in genes of the immune system may also influence the occurrence of graft rejection episodes (Karimi et al., 2014) including cytokines and/or their receptors (such as *IL2, IL10, TNF, IL23R*, and *CCR5*), growth factors (*TGFB1*) and regulatory factors (*IRF5*). Therefore, SNPs may prove to be of therapeutic use as biomarkers to identify appropriate doses, predict patient tolerance to treatment and risk of graft rejection (Burckart & Amur, 2010), and consequently have potential to be utilized for prognosis and/or diagnosis of graft rejection episodes.

Taking this into consideration, the aim of this study was to examine the association between incidence of graft rejection and the presence of SNPs in phase I (*CYP3A4, CYP3A5, CYP2E1*, and *POR*) and phase II (*UGT2B7, UGT1A9*) metabolism, drug-transporting (*ABCB1, ABCC2, ABCG2*, and *SLCO1B1*), and immune system genes (*TNF, IL2, IRF5, TGFB1, NFKBIA, IL10, IL23R, NFAT*, and *CCR5*).

## Methods

### Population studied

A total of 246 renal transplant patients undergoing post-transplant treatment at the Kidney Institute of Londrina (Londrina, PR, Brazil) were selected. These patients were classified into two groups, those who had graft rejection episodes (N = 86) and those with none (N = 160). Graft rejection episodes were confirmed by histological examination of the graft material obtained through biopsy.

The research protocol was approved by the Ethics Committee for Research in Human Beings at the State University of Londrina (CEP/UEL 153/2013 CAAE: 18263413.4.0000.5231). Patients signed a Free and Informed Consent Form, filled a questionnaire regarding lifestyle and history of environmental exposure, and each individual received a code. Patients were considered smokers and/or alcoholics when they in an interview responded to consuming any amount of cigarettes or alcohol. Patients were divided into two groups: descendant of Caucasian and descendant of Afro/Asian (African (N = 47) and Asian (N = 2) descendant). Information regarding the transplantation process, such as immunosuppressive therapy, graft rejection history, and diseases frequently developed after transplantation, was obtained from medical records of the patients. Peripheral blood samples (4 ml) were collected intravenously from each patient in vacuum blood collection tubes (EDTA 6%) (Labor Import, Osasco, Brazil).

### Analysis of polymorphic allelic variants

Genomic DNA was extracted from 200 µl blood, using the mini spin extraction kit (KASVI, Curitiba, Brazil; code K9-0250), following the manufacturer's recommendations. DNA samples were quantified using a NanoDrop 2000 spectrophotometer (ThermoScientific, Waltman, MA, USA).

The genes were selected based upon criteria previously associated with pharmacokinetics of immunosuppressive drugs (metabolism and drug transport genes) or with the immune response (Table 1). Genotyping was performed by real-time PCR in a Quantica thermocycler (TECHNE, Staffordshire, UK), using TaqMan® SNP Genotyping Assays (Applied Biosystems, Foster City, CA, USA), TaqMan Genotyping Master Mix (Applied

**Table 1.** Genes Evaluated in Renal Transplant Patients, Their Polymorphisms, and Type of Mutation That Originated Them.

| Gene | SNP_ID | Alternative nomenclature | Change | Chromosome location |
|---|---|---|---|---|
| *CYP3A4* | rs35599367 | 15389C > T (CYP3A4*22) | Intron | 7q22.1 |
| | rs4646437 | - | Intron | |
| *CYP3A5* | rs776746 | 6986A > G (CYP3A5*3) | Intron, splice acceptor variant | 7q21.1 |
| | rs4646450 | - | Intron | |
| *CYP2E1* | rs3813867 | −1295G > C *(CYP2E1*5B)* | Promoter | 10q24.3 |
| *POR* | rs1057868 | (POR*28) | Exon (Ala503Val) | 7q11.2 |
| *UGT1A9* | rs6714486 | −275T > A | Promoter | 2q37 |
| *UGT2B7* | rs7662029 | -327G > A | Promoter | 4q13 |
| | rs7438135 | −900A > G /-842A > G | Promoter | |
| *ABCB1* | rs1045642 | 3435C > T | Exon (Ile1145Ile) | 7q21.12 |
| *ABCC2/MRP2* | rs717620 | −24C > T | Promoter (5′UTR) | 10q24 |
| | rs2273697 | 1249G > A | Exon (Ile417Val) | |
| *ABCG2/BCRP* | rs2231142 | 421C > A | Exon (Lys141Gln) | 4q22 |
| *SLCO1B1* | rs4149056 | 521T > C (SLCO1B1*5) | Exon (Val174Ala) | 12p |
| *TNF* | rs1800629 | −308G > A | Promoter | 6p21.3 |
| *TGFB1* | rs1800470 | 29T > C | Exon (Leu10Pro) | 19q13.1-13.3 |
| | rs1800471 | 915G > C | Exon (Arg25Pro) | |
| *IL2* | rs2069762 | −330G > T | Promoter | 4q26-q27 |
| *IL10* | rs1800872 | −592C > A | Promoter (5′UTR) | 1q31-q32 |
| *IL23R* | rs10889677 | - | 3′UTR | 1p31.3 |
| *IRF5* | rs3757385 | - | Promoter | 7q32 |
| *NFAT* | rs10141896 | *NFATC4* | Intron | 14q11.2 |
| *NFKBIA* | rs696 | 2758A > G | 3′UTR | 14q13 |
| *CCR5* | rs333 | CCR5Δ32 | Intron deletion | 3p21 |

Biosystems). Genotyping of the polymorphism rs333 of the *CCR5* gene was performed by conventional polymerase chain reaction (PCR) in a Veriti 96-well thermocycler (Life Technologies of Brazil Ltda., São Paulo, Brazil), using 1.5 mM deoxynucleotide (dNTP), 30 mM $MgCl_2$, 2.5 μM of each primer, 0.5 U Taq DNA polymerase in 10X PCR buffer (Invitrogen-Life Technologies, São Paulo, Brazil), and 20 ng genomic DNA, in a final volume of 15 μl. The primers used were as follows: sense 5'-ACC AGA TCT CAA AAA GAA-3' and antisense 5'-CAT GAT GGT GAA GAT AAG CCT CA-3'; the PCR conditions were as follows: 94°C for 5 min, 30 cycles consisting of 94°C for 30 sec, 57°C for 30 sec, and 72°C for 30 sec, followed by a final annealing step of 10 min at 72°C. The PCR product had a different size owing to a 32-bp deletion. The fragment was 225-bp long when the prevalent allele was present and 193 bp in the presence of the allele with the deletion. Genotypes were determined by electrophoresis in 10% polyacrylamide gels stained with silver nitrate (Quimex, Brazil).

### Reverse transcription quantitative PCR (RT-qPCR)

Total RNA from 58 patients was extracted using TRIzol® Reagent (Ambion, Carlsbad, CA, USA) and PureLink® Total RNA Blood Kit (Ambion, Carlsbad, CA, USA); RNA samples were quantified in a Qubit 2.0 Fluorometer using the Qubit RNA HS Assay (Life Technologies, ref Q32855, Eugene, OR, USA), and its integrity was assessed using the Agilent 2100 Bioanalyzer RNA 6000 LabChip Kit (Agilent Technologies, Inc., Wilmington, DE, USA). For each sample, cDNA was synthesized using 500 ng total RNA and SuperScript® III (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's recommendations. Expression levels of *UGT2B7, UGT1A9, ABCG2*, and *IL23R* were evaluated by RT-qPCR. Predesigned oligonucleotide primers were purchased from Sigma-Aldrich (KiCqStart® SYBR® Green primers), and real-time thermocycler model 7900 (Applied Biosystems, USA) was employed. *GUSB* (fwd: CCTGCGTCCCACCTA GAATC, rev: ATACGGAGCCCCCTTGTCTG) and *PUM1* (fwd: CACAGACACCACCTCCTT CC, rev: CCATTCGTGAGTCCTCCCAG) genes were selected as a reference based on geNorm software analysis (http://medgen.ugent.be/~jvde somp /genorm/).

### Statistical analysis

The continuous variables age (years) and graft survival (years), and the categorical variables (gender,

degree of HLA compatibility, tobacco and alcohol consumption, ancestry, use of immunosuppressive drugs, development of cancer, diabetes and cardiovascular disease after transplantation) were compared in groups of patients according to graft rejection episodes, using the Student's t-test. Univariate logistic regression analysis was performed associating rejection episodes with each of the variables. Hardy–Weinberg equilibrium and linkage disequilibrium analysis were performed using HAPLOVIEW version 4.1 (Barrett et al., 2005). The haplotypes and their frequencies were determined using the Program PHASE version 2.1 (Stephens and Donnelly, 2003).

Associations between graft rejection episodes, and genotypes and haplotypes were performed using multivariate logistic regression analysis with SPSS version 20 (IBM, Armonk, NY, USA), and results presented as odds ratios (OR) with a 95% confidence interval (CI). The degree of HLA compatibility and ancestry ($p < 0.2$ in the univariate logistic regression analysis) were included to adjust the multivariate model. The relative expression of each gene was performed using the ΔΔCt method (Pfaffl, 2001). Association between transcripts expression levels and different genotypes was performed using the Student's t-test. For all statistical tests, the criterion used for significance was set at $p < 0.05$.

## Results

The main characteristics of the 246 patients included in this study (145 were men and 101 women) are showed in Table 2. Transplant patients displayed a

**Table 2.** General and Clinical Characteristics of Kidney Transplant Patients (246) Who Developed and Who Did Not Develop Rejection Episodes.

| | General (n = 246) | With rejection episode(s) (n = 86) | Without rejection episode(s) (n = 160) |
|---|---|---|---|
| Characteristics of patients | N (%) | N (%) | N (%) |
| **Gender** | | | |
| Male | 145 (58.9) | 53 (61.6) | 92 (57.5) |
| Female | 101 (41.1) | 33 (38.4) | 68 (42.5) |
| **Degree of HLA[a] compatibility** | | | |
| Live HLA-identical | 54 (22.0) | 11 (12.8) | 43 (26.9)* |
| Live HLA-haploidentical | 109 (44.3) | 39 (45.3) | 70 (43.8) |
| Deceased unrelated donor | 83 (33.7) | 36 (41.9) | 47 (29.4) |
| **Smoker** | | | |
| Yes | 18 (7.30) | 5 (5.80) | 13 (8.10) |
| No | 228 (92.7) | 81 (94.2) | 147 (91.9) |
| **Alcoholic** | | | |
| Yes | 35 (14.2) | 13 (15.1) | 22 (13.8) |
| No | 211 (85.8) | 73 (84.9) | 138 (86.2) |
| **Ancestry** | | | |
| Caucasian | 197 (80.1) | 62 (72.1) | 135 (84.4)* |
| Afro or Asian descendant | 49 (19.9) | 24 (27.9) | 25 (15.6) |
| **Use of immunosuppressive** | | | |
| Cyclosporine | 47 (19.1) | 20 (23.3) | 27 (16.9) |
| Tacrolimus | 112 (45.5) | 35 (40.7) | 77 (48.1) |
| Sirolimus | 16 (6.50) | 6 (7.00) | 10 (6.30) |
| AZA[b] | 61 (24.8) | 20 (23.3) | 41 (25.6) |
| MMF[c] | 144 (58.5) | 49 (57.0) | 95 (59.4) |
| Steroids | 244 (99.2) | 86 (100) | 158 (98.8) |
| MMF+Tacrolimus +Steroids | 87 (35.4) | 24 (27.9) | 63 (39.4) |
| **Development of disease** | | | |
| Cancer | 25 (10.2) | 7 (8.10) | 18 (11.3) |
| Diabetes | 53 (21.5) | 19 (22.1) | 34 (21.3) |
| Cardiovascular | 29 (11.8) | 18 (20.9) | 11 (6.90)* |
| Warts | 91 (37.0) | 35 (40.7) | 56 (35.0) |

[a]HLA-Human Leukocyte Antigen, [b]AZA-Azathioprine, [c]MMF-mycophenolate mofetil;
*$p < 0.05$ Student's t-test comparing patients with rejection and without rejection.

mean age of 48.6 ± 12.6 years and duration of transplant ranging from six months to 34 years (10.6 ± 8.3 years). The comparison of these variables showed no significant differences in cases with or without graft rejection episodes

Most grafts were obtained from living donors, 22% from identical living donors, 44.3% from haploidentical living donors, and 33.7% from deceased donors. Most patients were Caucasian (80.1%), followed by 19.9% Afro or Asian descendant. Eighteen patients (7.3%) reported being smokers, and thirty-five (14.2%) consumed alcohol.

Triple immunosuppressive regimen (corticosteroids, tacrolimus, and MMF) was reported in 87 patients (35.4%); only two subjects (0.80%) did not use steroids, and MMF was the second immunosuppressant more frequently utilized by individuals (58.5%). Among the most prevalent diseases presented after transplantation, the development of diabetes (21.5%), cardiovascular diseases (11.8%), and cancer (10.2%) were predominant (Table 2).

A total of 86 patients (34.9%) displayed graft rejection episodes. Multivariate regression analysis demonstrated that the degree of human leukocyte antigen (HLA) compatibility and ancestry is risk factors for graft rejection, and therefore, data were adjusted for these variables. Receiving the graft from an HLA-haploidentical living donor or from a deceased donor resulted in a marked greater than 2-fold increase in graft rejection risk. Afro or Asian descendant patients also were found to show a significant 2-fold rise in organ rejection risk.

All examined SNPs were in Hardy–Weinberg equilibrium. The multivariate logistic regression analysis revealed four SNP *UGT2B7* (rs7662029), *UGT1A9* (rs6714486), *ABCG2* (rs2231142), and *IL23R* (rs10889677) with a significant association with rejection episodes (Table 3). Protection against organ rejection episodes was observed among carriers of the rare alleles of either the rs7662029 (UGT2B7) and rs2231142 (ABCG2) SNP. The association of the genotypes A/A and A/G in the polymorphism rs7662029 represented a significant 1.85-fold higher protective factor, while genotypes C/A and A/A at SNP rs2231142 resulted in a 1.92-fold marked decrease in rejection risk

The presence of the polymorphisms rs6714486 (*UGT1A9*) and rs10889677 (*IL23R*) resulted in significant elevated risk of organ rejection, with the association of genotypes T/A and A/A at SNP rs6714486 showing a 1.6-fold significant rise in risk of rejection, while subjects with genotypes A/A and A/C at SNP rs10889677 of the *IL23R* gene displayed a 1.9-fold higher risk of developing rejection.

Two *UGT2B7* polymorphisms were in linkage disequilibrium (D′ = 0.99; $r^2$ = 0.97). Linkage disequilibrium was also observed between three other SNPs: rs4646450 (*CYP3A5*) and rs776746 (*CYP3A5*) (D′ = 0.92; $r^2$ = 0.51), rs4646450 (*CYP3A5*) and rs4646437 (*CYP3A4*) (D′ = 0.90; $r^2$ = 0.45), and rs4646437 (*CYP3A4*) and rs776746 (*CYP3A5*) (D′ = 0.86; $r^2$ = 0.68). Using multivariate logistic regression analysis, the presence of these haplotypes did not show marked association with rejection episodes (data not shown).

*ABCG2* and *IL23R* gene expression analysis demonstrated no marked association with the SNPs mapped in these genes (rs2231142 and rs10889677, respectively) (Figure 1). Due to the low number of patients with genotype AA (rs10889677), analysis of association between

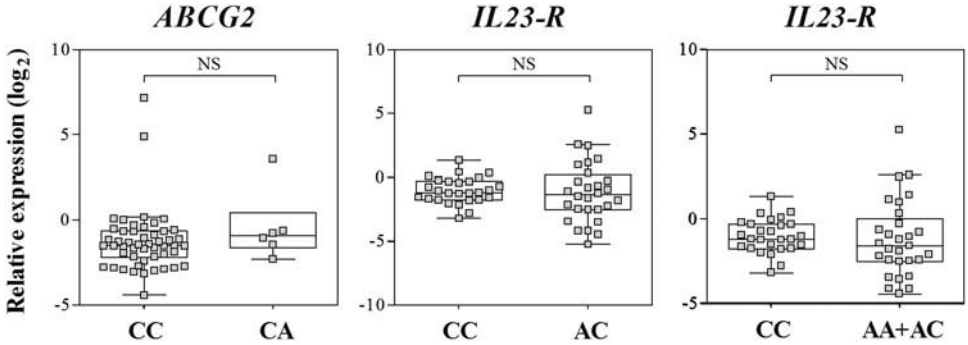


**Figure 1.** Relative expression of *ABCG2* and *IL23R* detected by RT-qPCR in different genotypes of each gene. The transcript expression values are shown in log scale.*: *P* value < 0.05.

**Table 3.** Genotypic Frequencies of Genes *CYP3A4, CYP3A5, CYP2E1, POR, UGT1A9, UGT2B7, ABCB1, ABCC2, ABCG2, SLCO1B1, TNF, TGFB1, IL2, IL10, IL23R, IRF5, NFAT, NFKBIA* and *CCR5* in 246 Kidney Transplant Patients, and the Association between Gene Polymorphisms and Rejection Episodes.

| Genes (SNP_ID) | Genotypes | With rejection episode(s) n (%) | Without rejection episode(s) n (%) | *Odds Ratio*[a] (CI95%) |
|---|---|---|---|---|
| *CYP3A4* (rs35599367) | C/C | 81 (94.2) | 153 (95.6) | Ref.[b] |
| | C/T | 5 (5.80) | 7 (4.40) | 1.5 (0.58–4.0) |
| *CYP3A4* (rs4646437) | G/G | 54 (62.8) | 106 (66.3) | Ref. |
| | G/A | 25 (29.1) | 45 (28.1) | 0.95 (0.56–1.6) |
| | A/A | 7 (8.10) | 9 (5.60) | 1.1 (0.41–3.0) |
| | A/G and A/A | 32 (37.2) | 54 (33.7) | 0.98 (0.66–1.4) |
| *CYP3A5* (rs776746) | G/G | 53 (61.6) | 102 (63.8) | Ref. |
| | A/G | 25 (29.1) | 49 (30.6) | 0.87 (0.47–1.6) |
| | A/A | 8 (9.30) | 9 (5.60) | 1.3 (0.41–4.0) |
| | A/A and A/G | 33 (38.4) | 58 (36.2) | 0.92 (0.57–1.5) |
| *CYP3A5* (rs4646450) | C/C | 33 (38.4) | 85 (53.1) | Ref. |
| | C/T | 41 (47.7) | 58 (36.2) | 1.5 (0.78–3.1) |
| | T/T | 12 (13.9) | 17 (10.6) | 1.6 (0.54–4.7) |
| | C/T and T/T | 53 (61.6) | 75 (46.8) | 1.6 (0.77–3.2) |
| *CYP2E1* (rs3813867) | G/G | 77 (89.5) | 136 (85.0) | Ref. |
| | G/C and C/C | 9 (10.5) | 24 (15.0) | 0.69 (0.24–2.0) |
| *POR* (rs1057868) | C/C | 42 (48.8) | 91 (56.9) | Ref. |
| | C/T | 36 (41.9) | 59 (36.9) | 1.4 (0.71–2.7) |
| | T/T | 8 (9.30) | 10 (6.20) | 2.4 (0.72–8.4) |
| | C/T and T/T | 44 (51.2) | 69 (43.1) | 1.5 (0.78–3.0) |
| ***UGT1A9* (rs6714486)** | T/T | 69 (80.2) | 142 (88.7) | Ref. |
| | T/A and A/A | 17 (19.8) | 18 (11.3) | 1.6 (1.0–2.5)* |
| ***UGT2B7* (*rs7662029*)** | G/G | 40 (46.5) | 48 (30.0) | Ref. |
| | A/G | 39 (45.4) | 89 (55.6) | 0.57 (0.32–1.0) |
| | A/A | 7 (8.10) | 23 (14.4) | 0.42 (0.16–1.1) |
| | A/A and A/G | 46 (53.5) | 112 (70.0) | 0.54 (0.3–1.0)* |
| *UGT2B7* (*rs7438135*) | A/A | 39 (45.3) | 49 (30.6) | Ref. |
| | A/G | 39 (45.3) | 87 (54.4) | 0.61 (0.35–1.1) |
| | G/G | 8 (9.30) | 24 (15.0) | 0.48 (0.19–1.2) |
| | A/G and G/G | 47 (54.6) | 111 (69.4) | 0.59 (0.33–1.0) |
| *ABCB1* (rs1045642) | C/C | 34 (39.5) | 56 (35.0) | Ref. |
| | T/C | 39 (45.4) | 72 (45.0) | 0.96 (0.61–1.5) |
| | T/T | 13 (15.1) | 32 (20.0) | 0.70 (0.38–1.3) |
| | T/C and T/T | 52 (60.5) | 104 (65.0) | 0.88 (0.60–1.3) |
| *ABCC2* (rs717620) | C/C | 61 (71.0) | 104 (65.0) | Ref. |
| | C/T | 23 (26.7) | 51 (31.9) | 0.82 (0.43–1.6) |
| | T/T | 2 (2.30) | 5 (3.10) | 0.65 (0.10–4.1) |
| | C/T and T/T | 25 (29.0) | 56 (35.0) | 0.81 (0.50–1.3) |
| *ABCC2* (rs2273697) | G/G | 54 (62.8) | 103 (64.4) | Ref. |
| | A/G | 30 (34.9) | 50 (31.2) | 1.1 (0.74–1.7) |
| | A/A | 2 (2.30) | 7 (4.40) | 0.52 (1.6–1.7) |
| | A/G and A/A | 32 (37.2) | 57 (35.6) | 1.0 (0.75–1.5) |
| ***ABCG2* (rs2231142)** | C/C | 76 (88.4) | 129 (80.6) | Ref. |
| | C/A and A/A | 10 (11.6) | 31 (19.4) | 0.52 (0.28–0.96)* |
| *SLCO1B1* (rs4149056) | T/T | 63 (73.2) | 107 (66.9) | Ref. |
| | T/C | 20 (23.3) | 48 (30.0) | 0.75 (0.31–1.8) |
| | C/C | 3 (3.50) | 5 (3.10) | 1.0 (0.13–8.2) |

(*Continued*)

**Table 3.** (Continued).

| Genes (SNP_ID) | Genotypes | With rejection episode(s) n (%) | Without rejection episode(s) n (%) | *Odds Ratio*[a] (CI95%) |
|---|---|---|---|---|
| | T/C and C/C | 23 (26.8) | 53 (33.1) | 0.77 (0.40–1.5) |
| *TNF* (rs1800629) | G/G | 63 (73.3) | 116 (72.5) | Ref. |
| | G/A and A/A | 23 (26.7) | 44 (27.7) | 0.94 (0.41–2.1) |
| *TGFB1* (rs1800470) | C/C | 26 (30.2) | 39 (24.4) | Ref. |
| | C/T | 34 (39.6) | 83 (51.9) | 0.72 (0.35–1.5) |
| | T/T | 26 (30.2) | 38 (23.7) | 1.1 (0.50–2.5) |
| | C/T and T/T | 60 (69.8) | 121 (75.6) | 0.85 (0.56–1.3) |
| *TGFB1* (rs1800471) | G/G | 73 (84.9) | 137 (85.6) | Ref. |
| | G/C and C/C | 13 (15.1) | 23 (14.4) | 1.1 (0.46–2.5) |
| *IL2* *(rs2069762)* | T/T | 48 (55.8) | 91 (56.9) | Ref. |
| | T/G | 32 (37.2) | 58 (36.2) | 1.2 (0.64–2.1) |
| | G/G | 6 (7.00) | 11 (6.90) | 1.0 (0.34–3.2) |
| | T/G and G/G | 38 (44.2) | 69 (43.1) | 1.1 (0.65–2.0) |
| *IL10* (rs1800872) | C/C | 32 (37.2) | 63 (39.4) | Ref. |
| | C/A | 44 (51.2) | 77 (48.1) | 1.1 (0.76–1.6) |
| | A/A | 10 (11.6) | 20 (12.5) | 0.91 (0.51–1.6) |
| | C/A and A/A | 54 (62.8) | 97 (60.6) | 1.1 (0.81–1.4) |
| ***IL23R* (rs10889677)** | C/C | 33 (38.4) | 85 (53.1) | Ref. |
| | A/C | 43 (50.0) | 66 (41.3) | 1.7 (0.98–3.1) |
| | A/A | 10 (11.6) | 9 (5.60) | 3.0 (1.1–8.5)* |
| | A/A and A/C | 53 (61.6) | 75 (46.9) | 1.9 (1.3–2.7)* |
| *IRF5* (rs3757385) | T/T | 17 (19.8) | 29 (18.1) | Ref. |
| | T/G | 43 (50.0) | 79 (49.4) | 1.1 (0.48–2.6) |
| | G/G | 26 (30.2) | 52 (32.5) | 0.94 (0.38–2.3) |
| | T/G and G/G | 69 (80.2) | 131 (81.9) | 1.0 (0.62–1.8) |
| *NFAT* (rs10141894) | G/G | 81 (94.2) | 150 (93.7) | Ref. |
| | G/T and T/T | 5 (5.80) | 10 (6.30) | 0.92 (0.22–3.8) |
| *NFKBIA* (rs696) | G/G | 30 (34.9) | 59 (36.9) | Ref. |
| | G/A | 42 (48.8) | 73 (45.6) | 0.97 (0.41–2.3) |
| | A/A | 14 (16.3) | 28 (17.5) | 0.81 (0.26–2.6) |
| | G/A and A/A | 56 (65.1) | 101 (63.1) | 0.92 (0.63–1.4) |
| *CCR5* *(rs333)* | wt/wt | 77 (91.7) | 150 (93.7) | Ref. |
| n = 244 | wt/Δ32 | 7 (8.30) | 10 (6.30) | 1.2 (0.48–3.0) |

[a]Odds Ratio (calculated by logistic regression using degree of HLA compatibility and ancestry as covariates);
[b]Ref – genotype used as reference;
*$p < 0.05$.

gene expression and genotype was not performed. Furthermore, no detectable transcripts levels were found in peripheral blood samples for *UGT1A9* and *UGT2B7* genes.

## Discussion

In our cohort of cases, 34.9% of the patients presented rejection episodes and the average duration of transplants was 10.6 ± 8.3 years. This rejection frequency is higher than described by Ro et al. (2012) in subjects where mean duration of kidney transplant of about 4.2 years had an organ rejection rate of 20.5%, and Karimi et al. (2014) reported 28% rejection in patients after 3 months of transplant. One of the main risk factors for graft dysfunction and rejection is the compatibility between donor and recipient in the genes involved in human leukocyte antigen (HLA). Lack of compatibility reduces the long-term survival of the graft; therefore, molecular typing of HLA improves clinical outcomes

(Tiercy, 2002). In this study, patients who did not receive a graft from a live donor with identical HLA showed a 2-fold increased risk of graft rejection indicating that HLA compatibility enhancing organ graft survival.

Data also showed that Afro or Asian descendants patients displayed a higher than 2-fold risk of developing rejection than Caucasian descendants patients. This corroborates the findings of Palanisamy et al. (2015), which demonstrated that African-American (AA) patients displayed an 8% rise in graft loss after 5 years of transplantation compared with non-AA patients. Palanisamy et al. (2015) also noted that AA patients demonstrated a higher prevalence of hypertension, diabetes mellitus, acute rejection, delayed graft function, and elevated incidence of cardiovascular diseases, when compared with non-AA patients. In contrast, in the present study, a significant difference between incidence of these diseases and the two ethnic groups examined was not detected (data not shown).

SNPs in genes involved in the phase I metabolism analyzed in this study were not markedly associated with graft rejection episodes. However, SNPs in phase II metabolism genes (*UGT1A9* and *UGT2B7*) were associated with organ rejection episodes. After oral administration, the MMF prodrug is usually hydrolyzed to mycophenolic acid (MPA), its active metabolite. The immunosuppressive effect of MPA is inactivated by UGT1A9 enzyme through glucuronidation producing its major metabolite, the mycophenolic acid glucuronide (MPAG) (Picard et al., 2004). However, MPA is also inactivated by the UGT2B7 enzyme, generating mycophenolic acyl-glucuronide acid (AcMPAG) (Shipkova et al., 1999). This metabolite, although produced in lower amounts, may induce pro-inflammatory responses that subsequently lead to various adverse effects (Wieland et al., 2000).

In this study, it was observed that the association between genotypes (T/A and A/A) of SNPs rs6714486 (*UGT1A9)* enhanced the risk of graft rejection 1.6-fold. Our results corroborate those of Van Schaik et al. (2009), which also found a higher risk of rejection in transplant patients with the same allelic variants. The increased risk of rejection and its association with the *UGT1A9* gene was explained by Girard et al. (2004), who demonstrated that the polymorphism rs6714486 was associated with a 1.4-fold elevation in hepatic levels of the protein and a 1.9-fold increase in the glucuronidation of MPA. The enhanced expression and activity of UGT1A9 enzyme reduced the concentration of MPA and therefore diminished its immunosuppressive activity (Picard et al., 2004), promoting the initiation of organ rejection episodes.

Analysis of SNPs rs7662029 (*UGT2B7* gene) showed that association of A/G and A/A genotypes produced a 1.85-fold greater protection against graft rejection. The effect of this polymorphism on gene expression has not been elucidated. This polymorphism is in strong linkage disequilibrium (LD) with the polymorphism rs7438135. According to Hu et al. (2014), 23 SNPs mapped in the *UGT2B7* promoter region are in LD. These authors also observed LD between SNPs rs7662029 and rs7438135 and reported that the haplotype carrying the G allele for the rs7438135 showed a 50% decrease in promoter activity of the gene and enzyme compared with the haplotype carrying the A allele (wild-type), thus identifying this polymorphism as functional. In the present study, the association of genotypes A/G and G/G demonstrated a non-significant trend toward protection, with an elevated 1.69-fold rise in protection against organ rejection episodes. This finding suggests that the presence of the G allele may contribute to a lower activity of the enzyme and consequently reduced inactivation of MPA into AcMPAG, resulting in a lower risk of rejection.

As the rs7662029 polymorphism (genotype AA) is in LD with rs7438135 (genotype GG), it is possible that patients with a combination of these genotypes possess lower levels of enzymatic activity, eliminating immunosuppressive drugs more slowly. Univariate logistic regression analysis of the haplotypes formed between these two genotypes showed marked association with protection against rejection of almost 2-fold. However, multivariate analysis, which considered the degree of HLA compatibility and ancestry, found no significant association (data not shown).

MPA metabolites (MPAG and AcMPAG) are eliminated through the bile by drug efflux transporter proteins ABCC2 and ABCG2 (Kobayashi et al., 2004; Miura et al., 2008). Subsequently, some metabolites undergo deconjugation by bacteria and are

resorbed in the gastrointestinal tract through enterohepatic recirculation. This occurs in 10–60% of all MPAG, producing a second peak in MPA drug concentration (Bullingham et al., 1988). In this study, the association of C/A and A/A genotypes of SNP rs2231142 of the *ABCG2* gene was associated with a 1.92-fold increase in protection against organ rejection episodes. The presence of this SNP leads to an amino acid change, from lysine to glutamine, at codon 141, which decreases expression of the protein by 50% compared with the prevalent genotype (Tamura et al., 2007). Functionally, this SNP affects stability of the ABCG2 protein in the endoplasmic reticulum and raises susceptibility to ubiquitin-mediated degradation by the proteasome (Furukawa et al., 2009). Patients with the C/A and A/A genotypes, compared with those with C/C genotype, demonstrated an elevation in the concentration of plasmatic MPAG (Miura et al., 2008), possibly as a result of a reduced efflux of MPAG into the bile ducts. This may account for protective effect in carriers of the rs2231142 minor allele, because reduced expression of the ABCG2 transporter for rs2231142 is in agreement with findings of Miura et al., (2008). This study demonstrated for the first time the association between SNP rs2231142 and diminished risk of graft rejection. However, additional studies are required to clarify the effect of this SNP on pharmacokinetics of immunosuppressive MMF.

Of the 10 immune system genes analyzed in this study, only IL23 receptor (*IL23R*) showed association with graft rejection episodes. This gene is involved in the inflammatory process mediated by T helper 17 cells and plays an important role in autoimmune inflammation (Zhou et al., 2013). A study by Tsai et al. (2011) with 422 Chinese renal transplant patients showed that the C allele of the rs10889677 polymorphism leads to a 1.79-fold increase in the risk of developing interstitial fibrosis and tubular atrophy in the graft. In the present study, the association of A/A and A/C genotypes of this SNP resulted in a 1.83-fold rise in graft rejection risk. Karimi et al. (2014) also observed an increase in the incidence of acute rejection in renal transplant male patients with the A/A genotype.

According Zheng et al. (2012) and Zhou et al. (2013), the presence of the polymorphic variant A of rs10889677 in the 3'-UTR of the mRNA prevents binding of the microRNA let-7f and consequently increases transcription of *IL23R*. On the other hand, the C allele enhances the binding affinity of the microRNA let-7f and thus negatively regulates *IL23R* expression (Zheng et al, 2012; Zhou et al., 2013). Vanden Eijnden et al. (2005) found elevated expression of *IL23R*, and amount of receptors present on the cell surface might assist in activation of IL23 interleukins, production of interferon-γ, and differentiation of T helper 1 cells, leading to increased inflammation and stimulation of pro-inflammatory cytokines levels. The findings cited above might account for the observed increased risk of rejection shown in our study.

In conclusion, this study demonstrated an association between SNPs rs7662029 (*UGT2B7*), rs6714486 (*UGT1A9*), rs2231142 (*ABCG2*), and rs10889677 (*IL23R*), and the incidence of graft rejection episodes in 246 Brazilian kidney transplant patients. Although transcription studies in cell lines to determine the functionality of SNPs were not conducted, these findings may contribute in the future to be used as candidate markers for screening kidney transplant patients with higher or lower rejection risk and thus help in medical management.

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## Declaration of interest

The authors declare no conflicts of interest.

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