No Major Effect of Innate Immune Genetics on Acute Kidney Rejection in the First 2 Weeks Post-Transplantation

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Background: Innate immunity contributes to acute rejection after kidney transplantation. Genetic polymorphisms affecting innate immunity may therefore influence patients’ risk of rejection. IL2 -330T > G, IL10 -1082G > A, -819C > T, and -592C > A, and TNF -308G > A are not associated with acute rejection incidence in Caucasian kidney transplant recipients receiving a calcineurin inhibitor, ciclosporin or tacrolimus (TAC). However, other important innate immune genetic polymorphisms have not yet been extensively studied in recipients and donors. In addition, innate immunogenetics have not been investigated in kidney transplant cohorts receiving only TAC as the calcineurin inhibitor.

Objective: To investigate the effect of recipient and donor CASP1, CRP, IL1B, IL2, IL6, IL6R, IL10, MYD88, TGFβ, TLR2, TLR4, and TNF genetics on acute kidney rejection in the first 2 weeks post-transplant in TAC-treated kidney transplant recipients.

Methods: This study included 154 kidney transplant recipients and 81 donors successfully genotyped for 17 polymorphisms in these genes. All recipients were under triple immunosuppressant therapy of TAC, mycophenolate mofetil, and prednisolone. Recipient and donor genotype differences in acute rejection incidence within the first 2 weeks post-transplantation were assessed by logistic regression, adjusting for induction therapy, human leukocyte antigen mismatches, kidney transplant number, living donor, and peak panel-reactive antibody scores.

Results: A trend (Cochran-Armitage P = 0.031) of increasing acute rejection incidence was observed from recipient IL6 -6331 T/T (18%) to T/C (25%) to C/C (46%) genotype [C/C versus T/T odds ratio (95% confidence interval) = 6.6 (1.7 to 25.8) (point-wise P = 0.017)]. However, no genotype differences were significant after Bonferroni correction for multiple comparisons.

Conclusions: This study did not detect any statistically significant effects of recipient or donor innate immune genetics on acute rejection incidence in the first 2 weeks post-transplantation. However, the sample size was small, and future larger studies or
meta-analyses are required to demonstrate conclusively if innate immune genetics such as IL6 influence the risk of acute rejection after kidney transplantation.

**Keywords:** tacrolimus, immune genetics, kidney transplantation, acute rejection, IL6 -6331

**INTRODUCTION**

Acute rejection is the major short-term challenge following kidney transplantation and it also increases long-term graft loss (McDonald et al., 2007). Although induction therapy, human leukocyte antigen (HLA) mismatches, number of kidney transplants, living donor, and peak panel-reactive antibodies (PRAs) have been studied as potential acute rejection predictors (Hammond et al., 2010; Lim et al., 2012; Lim et al., 2015; Zhu et al., 2016), these factors only contribute partially to acute rejection incidence.

While the T-cell driven adaptive immune system is essential to acute rejection, the innate immune system also plays a key role. Extracellular damage-associated molecular patterns from transplantation surgery and ischemia/reperfusion injury can induce the translocation of nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) into T-cell nuclei via activation of the myeloid differentiation primary response 88 (MyD88)-dependent Toll-like receptor (TLR) signaling pathway (Li and Verma, 2002; Liew et al., 2005). Translocated NF-κB activates pro-inflammatory cytokine secretion [e.g. pro-interleukin (IL)-1β, IL-2, and tumor necrosis factor-α (TNF-α)] (Li and Verma, 2002). Caspase 1 (encoded by CASP1) is an inflammatory response initiator and converts pro-IL-1β into mature IL-1β (Kostura et al., 1989; Thornberry et al., 1992). These pro-inflammatory mediators can assist T-cell activation, proliferation, and differentiation, and intensify kidney tissue damage (Watson et al., 1980; Nankivel and Alexander, 2010; Anders and Schaefer, 2014). In contrast, anti-inflammatory cytokines (e.g. IL-10) can decrease pro-inflammatory cytokine release (Walsh et al., 2004) and therefore have the potential to attenuate rejection risk, whereas transforming growth factor β (TGF-β) and IL-6 have both pro- and anti-inflammatory action (Saxena et al., 2008; Scheller et al., 2011). Notably, IL-6 trans-signaling via soluble IL-6 receptor (IL-6R) is pro-inflammatory as it can enhance the expansion and activation of T- and B-cells and induce several acute phase reactants such as C-reactive protein (CRP) (Wolf et al., 2014).

Single nucleotide polymorphisms (SNPs) in CASP1, CRP, IL1B, IL2, IL6, IL6R, IL10, TGFβ, and TNF can increase or decrease the protein production and/or function of these pro- and anti-inflammatory mediators in vitro (Kroeger et al., 1997; Turner et al., 1997; Awad et al., 1998; Hoffmann et al., 2001; Dunning et al., 2003; Hall et al., 2004; Trompet et al., 2008; Wang et al., 2009) or serum/plasma concentrations in vivo (Fishman et al., 1998; Grainger et al., 1999; Galicia et al., 2004; Smith et al., 2008; Lacruz-Guzmán et al., 2013). In addition, SNPs in the MyD88-dependent TLR signaling pathway affect innate immune responses to vaccines (Ovsyannikova et al., 2011) and susceptibility to infection or disease in vivo (Taniguchi et al., 2013; Santos-Martins et al., 2014). Therefore, these innate immunogenetic markers may serve as predictors of acute rejection post-kidney transplantation.

Meta-analyses have shown that recipient and/or donor IL2 -330T > G (rs2069762), IL10 -1082G > A (rs1800896), -819C > T (rs1800871), and -592C > A (rs1800872), and TNF -308G > A (rs1800629) SNPs do not affect acute rejection incidence in Caucasian kidney transplant recipients receiving immunosuppressive therapy (Hu et al., 2011; Hu et al., 2015; Xiong et al., 2015; Hu et al., 2016). However, none of the cross-sectional studies included in these meta-analyses was carried out in recipients treated with tacrolimus (TAC) as the sole calcineurin inhibitor (CNI). Since TAC has potent immunosuppression 100 times greater than ciclosporin (Kino et al., 1987), with fewer rejection complications (U.S. Multicenter FK506 Liver Study Group, 1994), most kidney transplant recipients in Europe and Australia have been treated with TAC as the first-choice CNI for immunosuppressive therapy since 2003 (Wadström et al., 2017) and 2009 (ANZDATARegistry, 2010), respectively. Therefore, it is worthwhile exploring the innate immunogenetic impact on kidney transplant recipients treated with only TAC as the CNI.

Only one study has investigated the impact of IL1B 3954C > T (rs1143634) on acute rejection incidence in kidney transplant recipients and found recipient 3954C/T genotype had higher rejection incidence than C/C genotype (point-wise P = 0.045) but without multiple comparison adjustment (Manchanda and Mittal, 2008). In terms of TLR4 896A > G (rs4986790) and 1196C > T (rs4986791), it is still controversial if these two SNPs affect acute rejection incidence in kidney transplant recipients (Ducloux et al., 2005; Palmer et al., 2006; Nogueira et al., 2007). Limited sample size, low minor allele frequency of the TLR4 SNPs, different criteria for acute rejection [biopsy-proven acute rejection (BPAR) versus clinical evidence, e.g. serum creatinine change], varied recipient/donor ethnicities, and different time of rejection post-transplantation between cross-sectional studies may contribute together to the inconsistent findings of TLR4 genetics on acute rejection incidence. In addition, adjustment for multiple statistical comparisons was not conducted. Notably, SNPs in CASP1, CRP, IL6R, MYD88, and TLR2 have not been examined for their impact on acute rejection in kidney transplant recipients.

To bridge these research gaps, this study aimed to explore the impact of recipient and donor CASP1, CRP, IL1B, IL2, IL6, IL6R, IL10, MYD88, TGFβ, TLR2, TLR4, and TNF genotypes on BPAR incidence in a cohort of predominantly Caucasian kidney transplant recipients treated with TAC as the only CNI (Hu et al., 2018; Hu et al., 2019a; Hu et al., 2019b). We hypothesized that these recipient and donor innate immunogenetics would affect BPAR incidence in kidney transplant recipients in the first 2 weeks post-transplantation.
MATERIALS AND METHODS

Study Participants and Data Collection

This study was approved by the Central Adelaide Local Health Network Human Research Ethics Committee (Protocol number 2008178). All procedures complied with the Declaration of Helsinki and/or institutional research committee ethical requirements.

As described previously, 165 kidney transplant recipients and 129 donors were recruited (Hu et al., 2018; Hu et al., 2019a; Hu et al., 2019b). All recipients and living donors gave written informed consent before participation. For deceased donors, their respective recipients gave informed consent to use excess donor tissue blood vessels for genotyping. Recipient inclusion and exclusion criteria, demographics, anti-CD-25 induction therapy, immunosuppressant regimen (TAC, mycophenolate mofetil, and prednisolone), the number of HLA mismatches (HLA-A, -B, and -DR antigens) between recipients and donors, number of kidney transplants, donor type (living or deceased), PRA scores (%), and BPAR based on Banff classification of Solez et al., 2008 (as the transplants were performed between 2005 and 2011) have been described previously (Hu et al., 2018; Hu et al., 2019a; Hu et al., 2019b).

Genotyping

Genomic DNA was extracted from blood, buccal swab, and kidney tissue (Hu et al., 2018; Hu et al., 2019a). A panel of 21 SNPs in 15 genes described previously (Mulolland et al., 2014; Barratt et al., 2015; Coller et al., 2015; Somogyi et al., 2016; Coller et al., 2019) were assayed using Agena Bioscience (formerly known as Sequenom) MassARRAY at the Australian Genome Research Facility (Brisbane, Australia). This panel included SNPs in the MyD88-dependent TLR signaling pathway—TLR2 1350T > C (rs38041000), TLR4 896A > G and 1196C > T, and MYD88 1593A > G (rs6853); pro- and anti-inflammatory mediators—CASP1 5352G > A (rs580253) and 10643G > C (rs554344), CRP -717T > C (rs2794521), IL1B -511C > T (rs16944), -31T > C (rs1143627), and -954C > T, IL2 -330T > C, IL6 -6331T > C (rs10499563), IL6R -48892A > C (rs8192284), IL10 -1082G > A and -819C > T, TGFβ -509C > T (rs1800469), and TNF -308G > A. The panel also included BDNF 196G > A (rs6265) and OPRM1 118A > G (rs1799971) that were considered outside the scope of this study, and TGFβ -1287G > A (rs11466314) and LY96 379C > T (rs11466004) that are known to be of very low frequency in Caucasians; these four SNPs were therefore not included in the analyses described below.

Statistical Analyses

Hardy-Weinberg Equilibrium (HWE) tests for all genotypes, linkage disequilibrium (LD) between SNPs and haplotype inference within genes, and logistic regression analyses, were as described previously (Hu et al., 2018; Hu et al., 2019a). Due to the relatively limited sample size, only SNPs with minor allele frequencies >5% were included in logistic regression analyses. For SNPs in perfect or near-perfect (r² > 0.9) LD, only 1 of the linked SNPs in that gene, instead of haplotypes/diplotypes, was analyzed in logistic regression analysis.

Genotype differences in BPAR incidence were analyzed for each SNP separately by logistic regression, adjusting for induction therapy [yes/no (Y/N)], living donor (Y/N), HLA mismatches (<3 or ≥3), kidney transplant number (1 or ≥2), and peak PRA scores (≤10% or >10%). Statistical significance was assessed by the likelihood-ratio test, and effects described by odds ratios (OR) with 95% confidence intervals (CI). Genotype differences in BPAR without adjusting for non-genetic variables were tested by Cochran-Armitage test for trend in GraphPad Prism v8 (GraphPad Software, San Diego, CA, USA), or Fisher’s exact test for SNPs with rare homozygous genotypes (n < 5) combined with heterozygotes, and OR with 95% CI.

P-value thresholds for significance were corrected for multiple testing by Bonferroni-adjustment (α = 0.05/N, where N is the number of SNPs analyzed in the recipient or donor cohort, respectively).

RESULTS

One hundred and fifty-four recipients and 81 (57 living, 24 deceased) donors had sufficient DNA for genotyping. In total, 23% (n = 35) of recipients with genotype data developed BPAR in the first 2 weeks post-transplantation. The impact of induction therapy, HLA mismatches, kidney transplant number, living donor, and peak PRA scores on BPAR incidence has been reported (Hu et al., 2019a); none were statistically significant (likelihood-ratio test P-value > 0.1).

Genetic Variability in Kidney Transplant Recipients and Donors

All recipient and donor allele and genotype frequencies are summarized in Table 1. Six recipients each received a kidney from three deceased donors (two kidneys per donor), therefore, these three donors were counted only once for HWE tests but were treated independently for logistic regression analyses. For some SNPs, one to four recipients and/or donors had missing genotypes due to genotyping failure. All recipient and donor genotypes were in HWE (P ≥ 0.2). CASP1, IL1B, IL10, and TLR4 haplotype and diplotype frequencies are summarized in Supplementary Table 1. Recipient and donor CASP1 10643G and 5352G, IL1B -511C and -31T, and TLR4 896A and 1196C were in perfect or near-perfect LD (D’ > 0.99; r² ≥ 0.96) while IL10 -1082G and -819C were in complete but not perfect LD (D’ = 1.0; r² = 0.30; resulting in six observed diplotypes [Supplementary Table 1]). Therefore, only 5352G > A in CASP1, -511C > T and 3954C > T in IL1B, and 896A > G in TLR4, along with all SNPs (including IL10 -1082G > A and -819C > T separately) in other innate immune genes, were included in the subsequent analyses.

Rare homozygous genotypes (n < 5) were combined with heterozygous genotypes for logistic regression and Fisher’s exact test as follows: recipient MYD88 rs6853 A/A genotype versus G allele carriers (A/G + G/G), TLR4 896A/A genotype versus G allele carriers (A/G + G/G); donor IL6 -6331T/T genotype versus C allele carriers (T/C + C/C); recipient and donor CASP1 5352G/G genotype versus A allele carriers (G/A + A/A), TLR2 1350T/T
genotype versus C allele carriers (T/C + C/C), TNF -308G/G genotype versus A allele carriers (G/A + A/A).

Consequently, a multiple testing-adjusted P-value threshold for significance was determined at 0.0036 ($\alpha = 0.05/14$).

**Table 2** summarizes the associations between recipient and donor genotypes and BPAR incidence in the first 2 weeks.
post-transplantation, adjusting for induction therapy, HLA mismatches, kidney transplant number, living donor, and peak PRA scores. Although recipients with IL6-6331C/C genotype had a higher incidence of BPAR compared to T/T genotype recipients [OR (95% CI) = 6.6 (1.7–25.8), likelihood-ratio test P-value = 0.017], none of the genetic factors (including IL6 -6331T > C) statistically significantly affected BPAR incidence after correction for multiple comparisons (P-value threshold = 0.0036).

In univariate analysis, there was a trend of increasing BPAR incidence for recipient IL6 -6331T > C (18% in T/T, 25% in T/C, and 46% in C/C; Cochran-Armitage P = 0.031), although it was non-statistically significant after correcting for multiple comparisons (P-value threshold = 0.0036). Similar trends of increasing BPAR incidence were observed in recipient CRP -7117T > C (16% in T/T, 30% in T/C, and 31% in C/C; Cochran-Armitage P = 0.048), recipient CASP1 55342G > A (18% in G/G, 34% in G/A, and 33% in A/A; Cochran-Armitage P = 0.033) and donor IL6R -8892A > C (15% in A/ A, 28% in A/C, and 47% in C/C; Cochran-Armitage P = 0.019). Point-wise Cochran-Armitage and Fisher’s exact test P-values were > 0.05 for all other recipient and donor SNPs.

Supplementary Table 2 summarizes recipient and donor genotype differences in BPAR incidence in the first 2 weeks post-transplantation for all 21 SNPs included in the genotyping panel.

**DISCUSSION**

To our knowledge, this is the first innate immunogenetic study retrospectively investigating both recipient and donor genetics of pro- and anti-inflammatory mediators for their impact on BPAR incidence in kidney transplant recipients receiving only TAC as the CNI.

The IL6-6331 T/T genotype was associated with up to 6-fold higher plasma IL-6 concentrations than C allele carriers in acute inflammatory-status patients post-coronary artery bypass grafting surgery and in patients requiring intensive periodontal therapy, whereas no significant impact was found in healthy volunteers (Smith et al., 2008). However, the relationship between -6331T > C genotypes and plasma IL-6 concentration has not previously been examined post-kidney transplantation, nor the impact of these genotypes on BPAR incidence in kidney transplant recipients. Our results indicate that recipient C/C genotype is associated with 6.6-fold higher incidence in kidney transplant recipients, nor the impact of these genotypes on BPAR incidence in kidney transplant recipients receiving only TAC as the CNI.

In conclusion, this study did not detect any statistically significant impact of recipient and donor innate immune genetics on BPAR incidence in the first 2 weeks post-kidney transplantation. However, due to the limited sample size, future immunogenetic studies and/or meta-analyses are still required to demonstrate conclusively if innate immune genetics such
as IL6 -6331T > C influence the risk of BPAR incidence post-kidney transplantation.

**DATA AVAILABILITY STATEMENT**

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservations, to any qualified researcher.

**ETHICS STATEMENT**

This study was approved by the Central Adelaide Local Health Network Human Research Ethics Committee (Protocol number 2008178). The patients provided their written informed consent to participate in this study.

**AUTHOR CONTRIBUTIONS**

AS, BS, and JC contributed to the conception and design of the study. JC performed the DNA extraction and collation of genotyping results for the panel. RH and BS performed the statistical analyses. RH wrote the first draft of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

**TABLE 2 |** Recipient and Donor Innate Immune Genotype Differences in BPAR Incidence in the first 2 Weeks Post-Transplantation, Adjusting for HLA Mismatches, Induction Therapy, Kidney Transplant Number, Living Donor and Peak PRA Scores.

| Genes & SNPs | Recipients* (n = 153-154) | Donors* (n = 83-84) |
|-------------|------------------------------|---------------------|
| **Genotypes (n)** | **BPAR (n, %)** | **OR [95% CI]** | **P** | **Genotypes (n)** | **BPAR (n, %)** | **OR [95% CI]** | **P** |
| **CASP1** | G/G (107) | 19, 18 | Ref | 0.07 | G/G (60) | 16, 27 | Ref | 0.9 |
| | G/A + A/A (47) | 16, 34 | 2.2 [0.9–5.2] | 0.12 | G/A + A/A (24) | 7, 29 | 1.0 [0.3–3.9] | 0.3 |
| **CRP** | T/T (77) | 12, 16 | Ref | 0.05 | T/T (34) | 6, 18 | Ref | 0.1 |
| | T/C (61) | 18, 30 | 3.0 [1.2–7.6] | 0.04 | T/C (39) | 15, 38 | 3.1 [1.0–10.5] | 0.1 |
| | C/C (16) | 5, 31 | 2.1 [0.5–7.8] | 0.03 | C/C (10) | 2, 20 | 1.3 [0.2–7.5] | 0.05 |
| **IL1B** | C/C (76) | 18, 24 | Ref | 0.9 | C/C (41) | 13, 32 | Ref | 0.5 |
| | C/T (63) | 13, 21 | 0.8 [0.3–1.9] | 0.75 | C/T (34) | 9, 26 | 0.7 [0.2–2.2] | 0.3 |
| | T/T (15) | 4, 27 | 0.9 [0.2–3.6] | 0.8 | T/T (9) | 1, 11 | 0.1 [0.01–1.9] | 0.05 |
| **3954C > T** | C/C (84) | 16, 19 | Ref | 0.2 | C/C (54) | 13, 24 | Ref | 0.07 |
| | C/T (61) | 18, 30 | 2.0 [0.9–4.6] | 0.05 | C/T (25) | 10, 40 | 2.3 [0.8–6.6] | 0.03 |
| | T/T (9) | 1, 11 | 0.6 [0.03–4.1] | 0.6 | T/T (5) | 0, 0 | NA | 0.05 |
| **IL2** | T/T (70) | 12, 17 | Ref | 0.3 | T/T (41) | 10, 24 | Ref | 0.09 |
| | T/G (63) | 16, 25 | 1.5 [0.6–3.6] | 0.07 | T/G (37) | 9, 24 | 1.1 [0.4–3.2] | 0.05 |
| | G/G (21) | 7, 33 | 2.4 [0.7–7.2] | 0.02 | G/G (6) | 4, 67 | 8.1 [1.2–78.5] | 0.05 |
| **IL6** | T/T (80) | 14, 18 | Ref | 0.02 | T/T (52) | 11, 21 | Ref | 0.09 |
| | T/C (61) | 15, 25 | 1.6 [0.7–4.0] | 0.05 | T/C + C/C (32) | 12, 38 | 2.4 [0.9–6.9] | 0.05 |
| | C/C (13) | 6, 46 | 6.6 [1.7–25.8] | 0.01 | |
| **IL6R** | A/A (50) | 12, 24 | Ref | 0.9 | A/A (29) | 4, 14 | Ref | 0.09 |
| | A/C (78) | 16, 21 | 0.8 [0.3–2.1] | 0.3 | A/C (39) | 11, 28 | 2.3 [0.6–10.1] | 0.05 |
| | C/C (25) | 6, 24 | 0.9 [0.3–3.2] | 0.07 | C/C (15) | 7, 47 | 5.4 [1.2–27.5] | 0.05 |
| **IL10** | G/G (31) | 8, 26 | Ref | 0.7 | G/G (18) | 3, 17 | Ref | 0.4 |
| | G/A (79) | 19, 24 | 1.0 [0.4–2.9] | 0.01 | G/A (37) | 11, 30 | 2.3 [0.6–11.8] | 0.05 |
| | A/A (43) | 8, 19 | 0.7 [0.2–2.3] | 0.02 | A/A (29) | 9, 31 | 2.5 [0.6–13.9] | 0.05 |
| **MYD88** | C/C (88) | 22, 25 | Ref | 0.4 | C/C (44) | 9, 20 | Ref | 0.05 |
| | C/T (54) | 10, 19 | 0.6 [0.2–1.4] | 0.02 | C/T (36) | 14, 39 | 2.7 [1.0–7.9] | 0.05 |
| | T/T (11) | 3, 17 | 1.2 [0.4–4.6] | 0.01 | T/T (4) | 0, 0 | NA | 0.05 |
| **TGFβ** | A/A (123) | 28, 23 | Ref | 0.6 | A/A (66) | 17, 26 | Ref | 0.5 |
| | A/G + G/G (31) | 7, 23 | 0.7 [0.2–2.0] | 0.05 | A/G (18) | 6, 33 | 1.5 [0.4–4.7] | 0.05 |
| **TLR2** | C/C (81) | 18, 22 | Ref | 0.7 | C/C (47) | 14, 30 | Ref | 0.5 |
| | C/T (80) | 13, 22 | 1.0 [0.4–2.3] | 0.05 | C/T (29) | 6, 21 | 0.5 [0.2–1.7] | 0.05 |
| | T/T (13) | 4, 31 | 1.7 [0.4–6.1] | 0.2 | T/T (8) | 3, 18 | 1.3 [0.2–6.2] | 0.05 |
| **TLR4** | A/A (137) | 31, 23 | Ref | 0.7 | A/A (74) | 20, 27 | Ref | 0.9 |
| | A/G + G/G (17) | 4, 24 | 1.3 [0.3–4.3] | 0.04 | A/G (10) | 3, 30 | 0.9 [0.2–3.8] | 0.05 |
| | G/G (113) | 21, 19 | Ref | 0.04 | G/G (53) | 13, 25 | Ref | 0.5 |
| | G/A + A/A (41) | 14, 34 | 2.4 [1.0–5.7] | 0.01 | G/A + A/A (31) | 10, 32 | 1.4 [0.5–3.8] | 0.05 |

BPAR, biopsy-proven acute rejection; HLA, human leukocyte antigens; HLA-A, -B, and -DR mismatches; n, number; NA, not available; OR, odds ratio; P, likelihood-ratio P-value; peak PRA, peak panel-reactive antibodies scores assessed by serum lymphocytotoxicity assay; Ref, reference group; SNP, single nucleotide polymorphism; 95% CI, 95% confidence interval. Donors*, donor numbers may differ from those in Table 1, as each of the 3 deceased donors provided kidneys for 6 different recipients, these 3 donors were counted only once for HWE tests but they were treated independently when associated with BPAR for the individual recipients. In addition, donor numbers may differ within Table 2 due to genotyping failure. Recipients*, recipient numbers may differ within Table 1 due to genotyping failure.
FUNDING

This study was supported by a project grant (#565038) from the National Health and Medical Research Council, Australia.

ACKNOWLEDGMENTS

The authors acknowledge Prof. Graeme Russ and the Central and Northern Adelaide Renal and Transplantation Service for their assistance with kidney transplant recipient recruitment.

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Lacruz-Guzmán, D., Torres-Moreno, D., Pedrero, F., García-Carrión, P., García-Rubio, D., and Northern Adelaide Renal and Transplantation Service for their assistance with kidney transplant recipient recruitment.

Sincere thanks also to Mr. Ben Noll for assistance with study sample collection. The authors acknowledge use of the services and facilities of AGRF.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2019.01686/full#supplementary-material
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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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