# Differential expression of genes related to calcineurin and mTOR signaling and regulatory miRNAs in peripheral blood from kidney recipients under tacrolimus-based therapy

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**Background:** Genetic and epigenetics factors have been implicated in drug response, graft function and rejection in solid organ transplantation. Differential expression of genes involved in calcineurin and mTOR signaling pathway and regulatory miRNAs was analyzed in the peripheral blood of kidney recipient cohort (n=36) under tacrolimus-based therapy.

**Methods:** *PPP3CA*, *PPP3CB*, *MTOR*, *FKBP1A*, *FKBP1B* and *FKBP5* mRNA expression and polymorphisms in *PPP3CA* and *MTOR* were analyzed by qPCR. Expression of miRNAs targeting *PPP3CA* (miR-30a, miR-145), *PPP3CB* (miR-10b), *MTOR* (miR-99a, miR-100), and *FKBP1A* (miR-103a) was measured by qPCR array.

**Results:** *PPP3CA* and *MTOR* mRNA levels were reduced in the first three months of treatment compared to pre-transplant (P<0.05). *PPP3CB*, *FKBP1A*, *FKBP1B*, and *FKBP5* expression was not changed. In the 3rd month of treatment, the expression of miR-99a, which targets *MTOR*, increased compared to pre-transplant (P<0.05). *PPP3CA* c.249G>A (GG genotype) and *MTOR* c.2997C>T (TT genotype) were associated with reduced expression of *PPP3CA* mRNA and *MTOR*, respectively. *FKBP1B* mRNA levels were higher in patients with acute rejection (P=0.026).

**Conclusions:** The expression of *PPP3CA*, *MTOR* and miR-99a in the peripheral blood of renal recipients is influenced by tacrolimus-based therapy and by *PPP3CA* and *MTOR* variants. These molecules can be potential biomarkers for pharmacotherapy monitoring.

**Keywords:** Circulating miRNAs; gene expression; kidney transplant; pharmacogenomics

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## Introduction

Chronic kidney disease (CKD) is a major global health problem characterized by the gradual and irreversible loss of kidney function (1). The terminal stage, also known as end-stage renal disease (ESRD), requires renal replacement therapy—dialysis or transplantation—for the maintenance of life (2).

Kidney transplant provides better quality of life and longer survival, however to achieve good long-term outcomes, a life-long immunosuppression is required to prevent the allograft rejection (3). The immunosuppressive protocols usually include calcineurin inhibitors (e.g., tacrolimus) and/or mechanistic target of rapamycin (mTOR) inhibitors (e.g., everolimus), anti-proliferative drug (e.g., mycophenolate sodium) and corticosteroids (4,5).

Calcineurin, a key enzyme in T-cell activation, is a serine/threonine phosphatase composed of catalytic A (Isoforms encoded by *PPP3CA*, *PPP3CB* and *PPP3CC*) and regulatory B (Isoforms encoded by *PPP3R1* and *PPP3R2*) subunits (6). Tacrolimus or FK506 is able to block calcineurin signaling pathway and the production of pro-inflammatory cytokines, e.g., interleukin-2 (IL-2), and thereby attenuates cytokine receptor-dependent mTOR activation and lymphocyte proliferation (7). Tacrolimus immunosuppressive activity is mediated by the complex formed with FK506-binding proteins (FKBPs), best described for FKBP12 (encoded by *FKBP1A*) (8). It is known that the tacrolimus also binds with different affinities to FKBP12.6 (*FKBP1B*), the closest homolog of FKBP12, FKBP13 (*FKBP2*) and FKBP51 (*FKBP5*) (8,9).

Tacrolimus has high inter-individual variability of blood concentration, which requires therapeutic drug monitoring to maintain drug efficacy and safety. Genetic and non-genetic factors, such as age, gender, kidney and liver function, comorbidities, drug interactions, and others, may contribute to the variability of tacrolimus response (10,11).

Variants in pharmacokinetic-related genes, such as *CYP3A4*, *CYP3A5*, *ABCB1* and others, have been shown to influence the response to tacrolimus in kidney recipients from Brazil and other sample populations (12-17). However, polymorphisms in pharmacodynamics-related genes have been less explored in patients taking tacrolimus-based treatment (18,19). Our group investigated variants in genes related to calcineurin and mTOR signaling pathways and observed that *MTOR*, *FKBP1A*, *FKBP2* and *FOXP3* are associated with long-term impaired renal function, increased risk of acute rejection, and adverse events in kidney recipients treated with tacrolimus and everolimus-based immunosuppression (20,21).

Epigenetics factors, such as DNA methylation and histone acetylation, modulate the expression of genes and they have been suggested to influence allograft dysfunction and response to immunosuppressive therapies (22,23).

MicroRNAs (miRNAs) are short noncoding RNAs that bind the 3'UTR region of target mRNAs silencing gene expression. miRNAs regulate many biological processes including cell proliferation and differentiation (24). Differential expression of miRNAs has been implicated in CKD, and impaired graft function and rejection in kidney transplantation (25,26). Therefore, expression of miRNAs and target mRNAs in genes involved in pharmacodynamics may also contribute to the variability to immunosuppressive drugs response in kidney transplantation.

This study explored the expression of genes related to the calcineurin and mTOR signaling pathway (*PPP3CA*, *PPP3CB*, *MTOR*, *FKBP1A*, *FKBP1B* and *FKBP5*) and the miRNAs targeting *PPP3CA* (miR-30a, miR-145), *PPP3CB* (miR-10b), *MTOR* (miR-99a, miR-100), and *FKBP1A* (miR-103a) in the peripheral blood of kidney recipients in the initial phase of tacrolimus-based therapy. The influence of *PPP3CA* and *MTOR* polymorphisms on mRNA expression was also investigated.

We present the following article in accordance with the STROBE reporting checklist (available at http://dx.doi.org/10.21037/atm-20-1757).

## Methods

### *Patients and study protocol*

Thirty-six recipients of first kidney transplantation were selected at the at the Hospital do Rim, UNIFESP, Sao Paulo, Brazil. The allograft was ABO-compatible with a CDC-negative cross-match and a peak panel reactive antibody lower than 30%. The main exclusion criteria were significant hematologic or severe metabolic abnormalities; focal and segmental glomerulosclerosis; membrane proliferative glomerulonephritis; active infection or positivity for hepatitis B or C or human immunodeficiency viruses; and previous history of malignancy.

This investigation is part of a prospective core study (27). The individuals included in this study were selected based on the criteria of availability and quality of the biological samples for RNA studies. The study protocol was approved by the local ethics committees (UNIFESP protocol #

 

054/2008; FCF/USP protocol # 517), Sao Paulo, Brazil, and conducted according to good clinical practices and the Declaration of Helsinki guidelines (as revised in 2013). All subjects signed an approved written informed consent before enrollment.

Kidney recipients were treated with tacrolimus (0.1–0.2 mg/kg/day within 48 h post-transplant, and the doses were adjusted to maintain blood concentrations 8 to 15 ng/mL in the first month and afterwards 5 to 15 ng/mL). Treatment included enteric-coated mycophenolate sodium (1,440 mg/day) and prednisone (0.5 mg/kg initial dose, maximum of 30 mg, tapered to 20 mg at day 7, with a subsequent reduction of 5 mg/week to reach the dose of 5 mg/day by day 45). Some patients also received induction with basiliximab and methylprednisolone treatment before graft reperfusion in accordance with local practice.

### *Clinical data and laboratory tests*

Blood and urine samples were obtained in pre-defined times (before and one and three months after kidney transplantation) for laboratory tests and tacrolimus monitoring. Blood samples were also obtained in PAXgene™ blood RNA tubes (Qiagen GmbH, Hilden, Germany) for RNA extraction.

Creatinine, urea, glucose, total cholesterol, HDL cholesterol and triglycerides were measured by automated methods. The eGFR was calculated using the four-variable MDRD formula (28). Three months after the transplant, plasma levels of some cytokines such as interferon-γ (IFN-γ), interleukin (IL)-2, IL-4, IL-10 and IL-17 were measured by immunoassays Milliplex™ MAP for Luminex® xMAP™ technology (Merck Millipore, Massachusetts, USA).

Tacrolimus blood concentration was measured by an immunoassay using the Abbott Architect analyzer (Abbott Diagnostics, Illinois, USA). The tacrolimus C/D [ng/(mL·mg)] was calculated for each patient.

The biopsy-confirmed acute rejection (BCAR) was performed by the local pathologist according to the Banff 2003 classification (29). The delayed graft function (DGF) was considered to be the dialysis performed in 7 days after transplantation.

### *RNA extraction and quantification*

Total RNA was extracted from peripheral blood using the extraction system PAXgene™ Blood RNA Kit according to the manufacturer's protocol (Qiagen GmbH, Hilden, Germany). RNA concentration and purity were analyzed using NanoDrop ND-1000 photometer and Invitrogen Qubit® 2.0 fluorometer (Thermo Fisher Scientific Inc. Foster City, USA). RNA integrity was assessed by micro-capillary electrophoresis using RNA 6000 Nano Kit and Agilent Bioanalyzer 2100 (Agilent Technologies Inc., Santa Clara, USA). Samples with RNA integrity number (RIN) lower than 5.0 were not included. RNA samples were stored at –80 ℃.

### *Analysis of mRNA expression by real time PCR*

The cDNA was synthesized from total RNA using random primers and Invitrogen SuperScript II reverse transcriptase (Thermo Fisher Scientific Inc., Foster City, CA, USA) following the manufacturer protocol.

mRNA expression was measured by quantitative real-time PCR (qPCR) using predesigned TaqMan® Gene expression assays (*PPP3CA* Hs00174223_m1, *PPP3CB* Hs00917458_m1, *MTOR* Hs00234508_m1, *FKBP1A* Hs00356621_g1, *FKBP1B* Hs00997682_m1, and *FKBP5* Hs01561006_m) and Applied Biosystems 7500 Fast Real-Time PCR system (Thermo Fisher Scientific Inc., Foster City, USA). All cDNA samples were tested in duplicate.

Six reference genes were tested (*UBC*, *GAPD*, *B2M*, *HPRTI*, *SDHA* and *HMBS*) and analyzed using the GeNorm software (http://medgen.ugent.be/genorm). *UBC* and *B2M* were the most stable reference genes in the experimental conditions, and used further to normalize mRNA expression of the studied genes. The differential mRNA expression was calculated using the formula $2^{-\Delta CT}$.

### *Analysis of miRNA expression by PCR array*

Ingenuity Pathway Analysis (IPA) software (Qiagen, www.qiagenbioinformatics.com) was used to select miRNAs that target the mRNAs of genes involved in the mTOR and calcineurin pathways with validated results in human leukocytes experiments. Six miRNAs targeting *PPP3CA* (hsa-miR-30a-5p, hsa-miR-145-5p), *PPP3CB* (hsa-miR-10b-5p), *MTOR* (hsa-miR-99a-5p, hsa-miR-100-5p), and *FKBP1A* (hsa-miR-103a-3p) were selected. The predictive regulatory miRNAs were confirmed using the miRTarBase (http://mirtarbase.mbc.nctu.edu.tw) and TargetScan (www.targetscan.org) databases.

miRNAs expression of was measured using miScript miRNA PCR Array (Qiagen, Redwood City, USA) using Applied Biosystems 7500 Fast Real-Time PCR

 

**Table 1** Characteristics of the kidney transplant recipients and donors

| Variables | Total (n=36) |
|---|---|
| Recipient | |
| Age, years | 48.0 (37.5–57.0) |
| Men, % | 66.7 [24] |
| Ethnicity | |
| Caucasian, % | 55.6 [20] |
| Intermediate, % | 30.6 [11] |
| African, % | 11.1 [4] |
| Asian, % | 2.8 [1] |
| Cause of end stage renal disease, % | |
| Hypertension | 11.1 [4] |
| Diabetes | 22.2 [8] |
| Other* | 27.8 [10] |
| Undetermined | 38.9 [14] |
| Cold ischemia time**, h | 19.6 (17.1–23.5) |
| Delayed graft function, % | 19.4 [7] |
| Biopsy-confirmed acute rejection | 16.7 [6] |
| Donor | |
| Age, years | 44.0 (35.5–50.5) |
| Men, % | 55.6 [20] |
| Ethnicity | |
| Caucasian, % | 66.7 [24] |
| Intermediate, % | 16.7 [6] |
| African, % | 11.1 [4] |
| Missing data, % | 5.5 [2] |
| Donor type (deceased), % | 47.2 [17] |

Number of individuals is in parentheses. Continuous variables are shown as median and interquartile range. Categorical variables are shown as percentage. *, Polycystic kidney disease, urinary tract malformation, lupus nephritis or nephrotic syndrome. **, For deceased donor only.

equipment (Thermo Fisher Scientific Inc., Foster City, USA). Briefly, cDNA was produced from total RNA by reverse transcription reaction using miScript II RT kit and amplified using QuantiTect SYBR Green PCR kit (Qiagen). The raw data were analyzed using the miScript miRNA PCR Array Web-based software (Qiagen). The expression values were normalized with four endogenous snRNAs: SNORD61, SNORD68, SNORD95, and RNU6B/RNU6-2. The differential miRNA expression was calculated using the formula $2^{-\Delta CT}$.

### *Analysis of gene polymorphisms*

The polymorphisms *PPP3CA* rs3730251 (c.249G>A) and *MTOR* rs1135172 (c.1437T>C), rs1064261 (c.2997C>T) and rs1057079 (c.4731G>A) were analyzed by qPCR using pre-designed TaqMan SNP genotyping assays (Thermo Fisher Scientific Inc., Foster City, USA). Details about DNA extraction, genotyping assays, and qPCR procedures and quality control were previously described (21).

### *Statistical analyses*

Categorical variables are shown as percentage and were compared by chi-square or Fisher's exact test. Continuous variables are shown as median and interquartile range and were compared by Mann-Whitney U test or Kruskal-Wallis test and Dunn's test for multiple comparisons. Paired data of metabolic profile and blood concentration of immunosuppressive drugs were analyzed by paired t-test or Friedman Repeated Measures ANOVA on Ranks and Dunn's test for multiple comparisons.

The correlation of laboratory variables (eGFR, serum creatinine, IFN-γ, IL-2, IL-4 and IL-17 plasma levels) with mRNA and miRNA expression, at 3-month post-transplant, was tested using Spearman correlation test.

Statistical analyses were carried out using SPSS for Windows version 22 (SPSS Inc., Illinois, USA) and GraphPad Prism® version 5.00 for Windows (GraphPad Software Inc., California, USA). Significance level was set at $P<0.05$.

## Results

### *Characteristics of the kidney recipients*

Demographic and clinical characteristics of the kidney transplant recipients are shown in the *Table 1*. Subjects were mainly men (66.7%) and Caucasians (55.6%), with a median age of 48.0 years old. Hypertension (11.1%) and diabetes (22.2%) were the main known causes of the ESRD. The donors were also mainly men (55.6%) and Caucasian (66.7%), with a median age of 44.0 years old. Transplantation of kidney from deceased donors was 47.2% in this study. The median cold ischemia time was 19.6 h,

**Table 2** Tacrolimus monitoring and laboratory data of kidney transplant recipients during the first 3 months post-transplant

| Variable | Follow-up | | | P value |
|---|---|---|---|---|
| | Day 7 | Month 1 | Month 3 | |
| Tacrolimus | | | | |
| Dose, mg/day | 11.5 (8.0–14.0) | 4.5 (4.0–6.0)* | 3.0 (2.5–5.0)§ | <0.001 |
| Concentration, ng/mL | 10.1 (6.3–13.2) | 7.6 (5.1–9.2) | 5.4 (4.4–6.8)§ | <0.001 |
| C/D, ng/(mL·mg) | 0.8 (0.5–1.5) | 1.7 (1.2–2.0)* | 1.7 (1.0–2.6)§ | <0.001 |
| Creatinine, mg/dL | 2.3 (1.4–5.9) | 1.4 (1.1–1.6)* | 1.2 (1.0–1.4)§ | <0.001 |
| eGFR, mL/min/1.73 $m^2$ | 35 (13–66) | 61 (49–77)* | 69 (56–84)§ | <0.001 |
| Glucose, mg/dL | 91 (78–155) | 95 (82–123) | 87 (79–102) | 0.436 |
| Total cholesterol, mg/dL | – | 197 (169–227) | 180 (138–206) | 0.007 |
| HDL cholesterol, mg/dL | – | 47 (39–58) | 39 (31–48) | <0.001 |
| LDL cholesterol, mg/dL | – | 118 (91–134) | 104 (76–124) | 0.097 |
| Triglycerides, mg/dL | – | 154 (110–206) | 151 (99–200) | 0.729 |
| Hemoglobin, g/dL | 11.8 (10.4–12.6) | 12.0 (11.3–13.6) | 12.6 (11.1–14.0)§ | 0.008 |
| Leucocytes $\times 10^3$, N/$mm^3$ | 10.0 (7.9–11.7) | 7.2 (6.1–8.2)* | 6.0 (4.8–7.9)§ | <0.001 |
| Platelets $\times 10^3$, N/$mm^3$ | 246.5 (177–286) | 252.5 (180–286) | 225.5 (194–278) | 0.423 |
| Interferon-γ, pg/mL | – | – | 9.1 (4.5–18.7) | |
| Interleukin-2, pg/mL | – | – | 1.1 (0.5–1.8) | |
| Interleukin-4, pg/mL | – | – | 7.2 (2.3–12.0) | |
| Interleukin-10, pg/mL | – | – | 4.8 (2.8–7.9) | |
| Interleukin-17, pg/mL | – | – | 3.6 (2.2–6.1) | |

Variables are shown as median (interquartile range) and compared by paired *t*-test or Friedman Repeated Measures ANOVA on Ranks and Dunn's test for multiple comparisons (*, P<0.05, month 1 *vs*. day 7; §, P<0.05, month 2 *vs*. day 7). C/D, concentrations of tacrolimus for dose administered; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

for deceased donors only. The cumulative incidence of graft DGF was 19.4%. During the three-month of follow up, six patients (16.7%) had one episode of treated BCAR. The subjects who developed or not BCAR had similar demographic and clinical characteristics (P>0.05, *Table S1*).

Median tacrolimus dose decreased from the day 7 post-transplant (11.5 mg/day) to the first and third month (4.5 and 3.0 mg/day, respectively, P<0.001) (*Table 2*). The blood concentration of tacrolimus reduced from 10.1 ng/mL in the day 7 to 5.4 ng/mL in the third month (P<0.001). The concentration for dose-administered (C/D) of tacrolimus increased from the day 7 [0.8 ng/(mL·mg)] to the first and third months post-transplant [1.7 ng/(mL·mg), P<0.001]. The mycophenolate sodium doses did not change significantly with time (1,440 mg/day) but prednisone doses were reduced, as expected (30.0 to 5.0 mg/day from day 7 to month 3, P<0.001).

Kidney function and other laboratory variables of the kidney recipients improved from day 7 to month 3, as expected (*Table 2*). Serum creatinine and number of leucocytes were reduced, whereas the estimated glomerular filtration rate (eGFR) and hemoglobin were increased in the first and/or third month of therapy (P<0.05). Compared to month 1, total cholesterol and high-density lipoprotein (HDL) cholesterol levels were lower in the third month (P<0.05). Descriptive data of cytokines plasma levels at the third month post-transplant are also shown in the *Table 2*.

### *Circulating mRNAs differentially expressed*

mRNA expression of *PPP3CA* and *MTOR* in peripheral blood was reduced after one (*PPP3CA* 28%) and

 

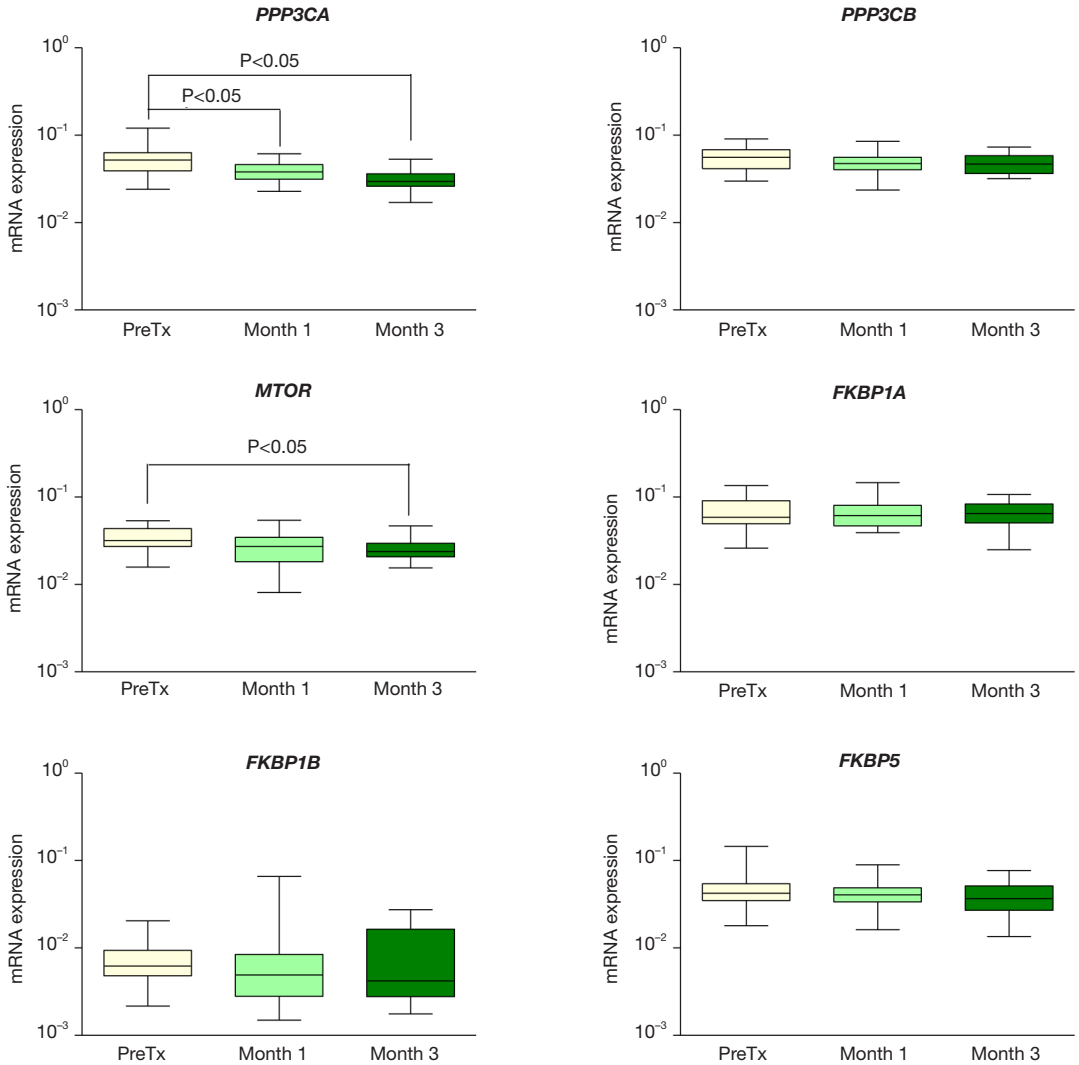


**Figure 1** Expression of genes in peripheral blood of kidney recipients (n=36). The mRNA expression was measured by qPCR and normalized with *UBC* and *B2M*. The relative mRNA expression was calculated by $2^{-\Delta CT}$ formula. Box plots with median and the minimum and maximum values. Data compared by Kruskal-Wallis test and Dunn's test for multiple comparisons. PreTx, pre-transplant.

three (*PPP3CA* 41% and *MTOR* 25%) months of immunosuppressive therapy, compared to pre-transplant (PreTx) (P<0.05) (*Figure 1*). The expression of the genes encoding calcineurin beta (*PPP3CB*) and FK506-binding proteins (*FKBP1A*, *FKBP1B* and *FKBP5*) did not change during treatment (P>0.05).

As an exploratory analysis, the deregulation of *PPP3CA* and *MTOR* expression was independent on the BCAR condition (P>0.05, *Figure S1*). However, at the first month, BCAR patients showed higher *FKBP1B* expression than non-BCAR group (median normalized mRNA expression of 0.009 versus 0.004, respectively, P=0.026).

### *Circulating miRNAs differentially expressed*

Expression of miRNAs targeting *PPP3CA* (miR-30a, miR-145), *PPP3CB* (miR-10b) *MTOR* (miR-99a, miR-100), and *FKBP1A* (miR-103a) was analyzed in peripheral blood of kidney recipients. The results of this analysis showed that miR-99a levels were about two-fold increase in the third month of therapy compared to PreTx (P<0.05), whereas the miR-100, miR-145, miR-30a, miR-10b, and miR-103a expression remained unchanged (P>0.05) (*Figure 2*). The expression of the miRNAs did not differ between BCAR and non-BCAR groups (P>0.05) (data not shown).

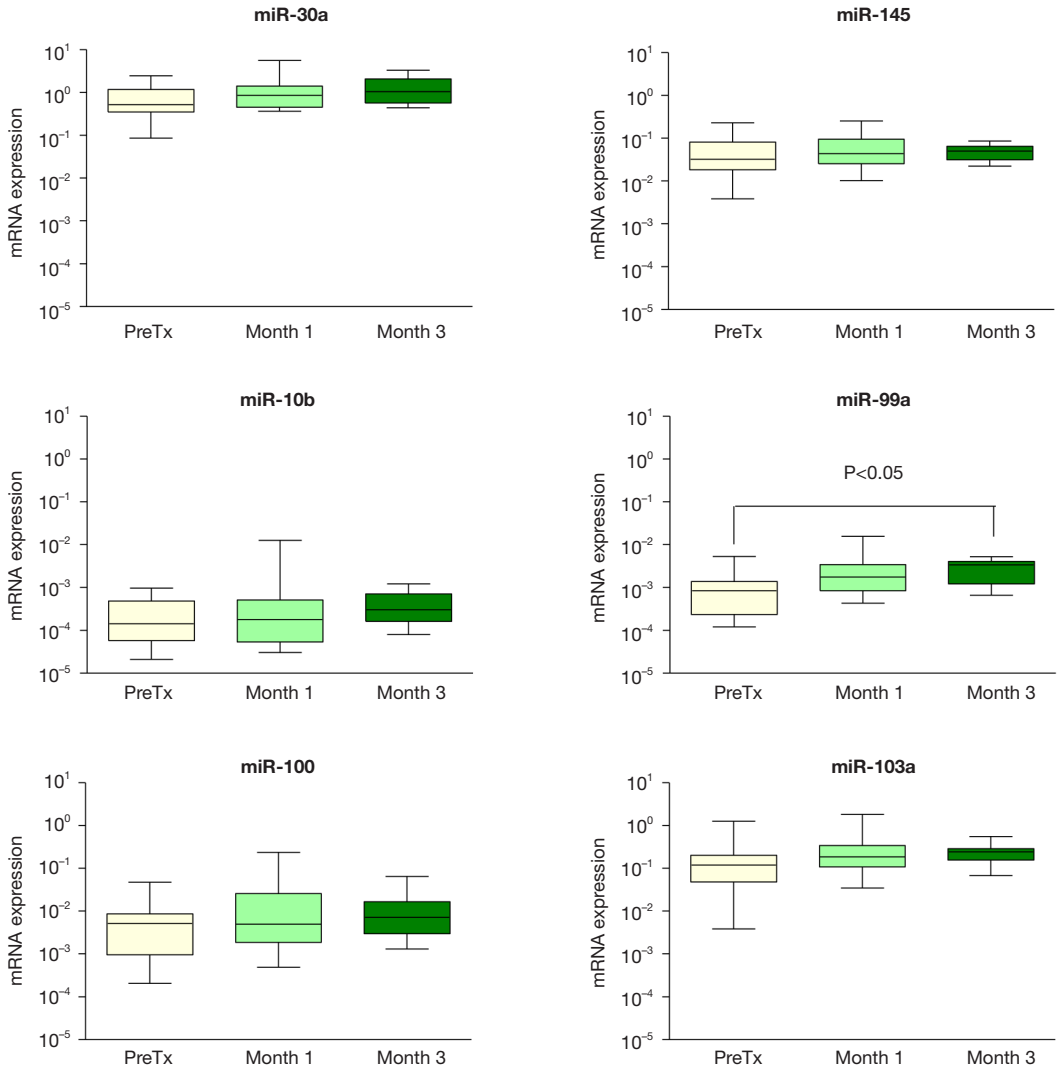


**Figure 2** Expression of miRNAs in peripheral blood of kidney recipients (n=22*). The miRNA expression was measured by qPCR array and normalized with RNU6-2, SNORD61, SNORD68, and SNORD95. The relative miRNA expression was calculated by $2^{-\Delta CT}$ formula. Box plots with median and the minimum and maximum values. Data compared by Kruskal-Wallis test and Dunn's test for multiple comparisons. PreTx, pre-transplant. *, only 22 individuals had no missing data in evaluated times.

### *Influence of PPP3CA and MTOR variants on mRNA expression*

As shown in the *Figure 3*, *PPP3CA* expression was downregulated at 1 and 3 months post-transplant in patients carrying *PPP3CA* c.249GG genotype (P<0.05) but not in GA genotype carriers (P>0.05, *Figure 3*). However, this result should be analyzed with caution, because only five kidney recipients were carriers of *PPP3CA* c.249GA genotype in this sample. Analysis of *MTOR* c.2997C>T showed that patients carrying the TT genotype had lower *MTOR* mRNA expression than the C allele carriers (TC+CC genotype) at month 1 post-transplant (median 0.023 versus 0.031, respectively, P=0.041) (*Figure 3*).

### *Correlation of mRNA or miRNA expression with laboratory variables*

The main results of the correlation analyses at the 3rd month post-transplant are shown in *Table 3*. Positive correlations were found between eGFR and miR-30a, and serum

 

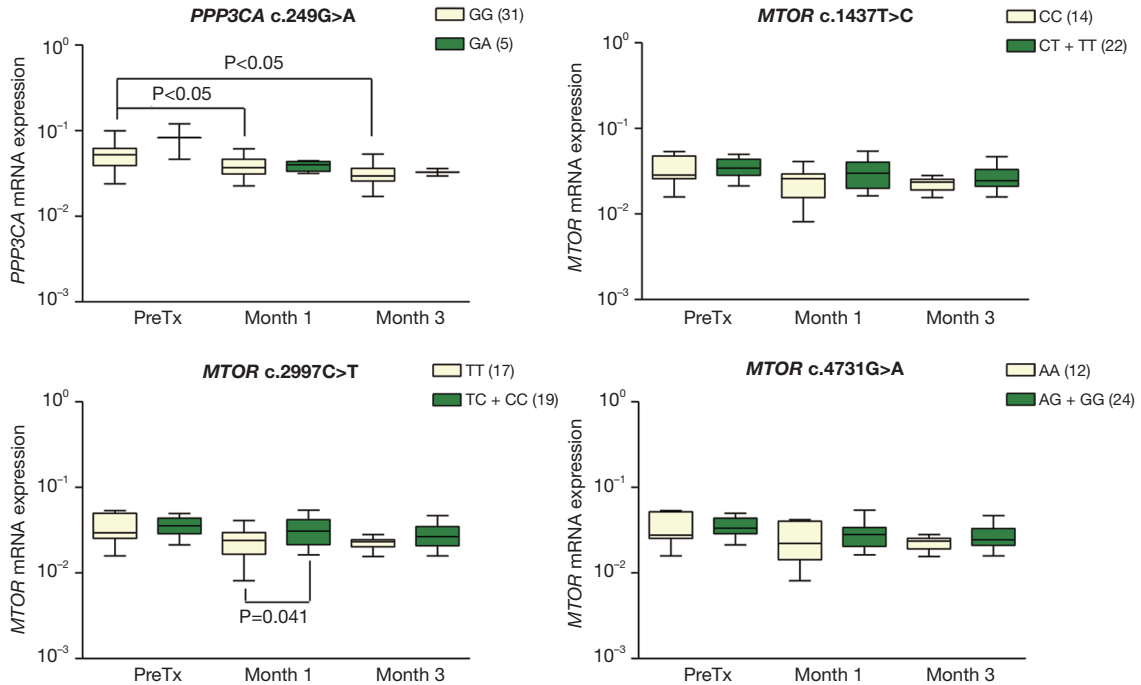


**Figure 3** Association of *PPP3CA* and *MTOR* polymorphisms with mRNA expression in peripheral blood of kidney recipients (n=36). The mRNA expression was measured by qPCR and normalized with *UBC* and *B2M*. The relative mRNA expression was calculated by $2^{-\Delta CT}$ formula. Box plots with median and the minimum and maximum values. Data compared by Mann-Whitney U test and Kruskal-Wallis test and Dunn's test for multiple comparisons. PreTx, pre-transplant.

**Table 3** Correlation of laboratory variables with mRNA and miRNA expression at 3rd month post-transplant

| Variables | R | P value |
|---|---|---|
| Creatinine (mg/dL) versus *FKBP1A* mRNA expression | 0.494 | 0.044 |
| eGFR, (mL/min/1.73 $m^2$) versus miR-30a expression | 0.692 | 0.013 |
| IFN-γ (pg/mL) versus *PPP3CA* mRNA expression | 0.524 | 0.037 |
| IL-17 (pg/mL) versus *PPP3CB* mRNA expression | 0.571 | 0.026 |
| IL-2 (pg/mL) versus miR-10b expression | 0.709 | 0.022 |
| IL-2 (pg/mL) versus miR-100 expression | 0.697 | 0.025 |
| IL-4 (pg/mL) versus miR-10b expression | −0.770 | 0.009 |

R, Spearman correlation coefficient. eGFR, estimated glomerular filtration rate; IFN, interferon; IL, interleukin.

creatinine and *FKBP1A* mRNA (P<0.05). Moreover, plasma IFN-γ was positively correlated with *PPP3CA* mRNA, IL-2 with miR-10b and miR-100 expression, and IL-17 with *PPP3CB* mRNA (P<0.05). On the other hand, IL-4 levels were inversely correlated with miR-10b expression (P=0.009).

## Discussion

This study investigated the expression of genes related to calcineurin and mTOR signaling pathways and their regulatory miRNAs in peripheral blood of kidney

recipients. The association of *MTOR* and *PPP3CA* variants with variability in mRNA expression was also analyzed.

In the first three months post-transplant, the mRNA expression of *PPP3CA* and *MTOR* reduced in peripheral blood. *PPP3CA* encodes the A subunit of the calcineurin, a calcium and calmodulin-dependent phosphatase, which binds the immunophilin FKBP12 forming a complex that activates signal transduction and transcription of *IL2* and other cytokines genes involved in T-cell proliferation and differentiation. Tacrolimus inhibits the formation of the complex calcineurin-FKBP12. *MTOR* encodes a serine/threonine kinase of the PI3K/AKT signaling pathway, which also binds FKBP12. Activation of this pathway triggers a series of intracellular processes resulting in cell growth, proliferation and differentiation (14).

Reduction of calcineurin activity in peripheral blood by tacrolimus-based therapy was observed previously in renal transplant recipients within the first 15 days of therapy (30). Calcineurin activity measured by NFAT1 translocation to the nuclei of peripheral blood mononuclear cells (PBMC) was also reduced by tacrolimus in patients with end-stage liver disease waiting for transplantation (31).

Downregulation of *PPP3CA* and *MTOR* mRNA may be caused by genetic or epigenetic mechanisms which can contribute to the immunosuppressive effect of tacrolimus on cell proliferation and differentiation (16,25). Moreover, other drugs contained in the immunosuppressive regimen may be also modulating this effect. In rats treated with mycophenolate mofetil, the hippocampal mRNA and protein levels of mTOR was found to be decreased (32).

The synonymous variant *PPP3CA* c.249G>A and the missense variant *MTOR* c.2997C>T were associated with variability of gene expression in peripheral blood of the kidney recipients. Besides their influence on gene expression, these polymorphisms may alter the interactions of tacrolimus with molecules within the calcineurin and mTOR signaling pathway. The role of these variants in the regulation of gene expression and in the interaction of these molecules with tacrolimus has yet to be investigated by functional studies.

In this study, *FKBP1A*, *FKBP1B* and *FKBP5* mRNA expression has not changed in the early phase after transplantation. Interestingly *FKBP1A* mRNA expression was positively correlated with serum creatinine at the 3-month therapy. In addition, *FKBP1B* was upregulated in patients with acute rejection. These results are suggestive that increased mRNA levels of *FKBP1A* and *FKBP1B* are associated with to worse kidney allograft function and increased risk of acute rejection.

Expression of six miRNAs was also evaluated in peripheral blood of kidney recipients within three months after transplantation. miR-30a, miR-145, miR-10b and others miRNAs bind to the 3'UTR of the *PPP3CA* and *PPP3CB* mRNA regulating their expression post-transcriptionally. Previous studies reported that reduced expression of miR-30a was associated with acute rejection and poor prognosis of graft function in kidney transplantation (33,34). Downregulation of miR-145 in biopsy samples was shown to be associated with the risk for acute kidney transplant rejection (35). miR-145 was also shown to be downregulated in blood cells of kidney recipients with interstitial fibrosis/tubular atrophy, antibody-mediated graft rejection, and reduced allograft survival (36).

The expression of mir-30a and miR-145 in peripheral blood remained stable and it was not associated with BCAR within the 3-first months of treatment. These miRNAs are likely to be more informative as biomarkers for long-term allograft rejection. Interestingly, miR-30a expression was positively correlated with eGFR values, which is suggestive that increased levels of this miRNA in peripheral blood maybe associated with better allograft renal function at early stage post-transplant.

miR-10b is considered a potentially oncogenic miRNA and increased expression of miR-10b was reported in oral, pancreatic and other neoplasms (37,38). Urinary expression of miR-10b and miR-210 was reported to be downregulated in renal allograft recipients with acute rejection (39,40). However, the expression of miR-10b or the target *PPP3CB* mRNA was not altered by exposure to immunosuppressive therapy or BCAR in this sample.

miR-99a, which targets *MTOR* mRNA, was upregulated in this study. This result is consistent with the downregulation of the *MTOR* expression at 3-month post-transplant. It has been reported that miR-99a and miR-100 have inhibitory effects on proliferation of breast and cervical cancer cells and on bladder carcinogenesis by targeting mTOR signaling pathway (41-43). Overexpression of miR-99a and miR-125b was shown to downregulate the activation and cytotoxicity of human circulating γδ T cells, which express T-cell receptors composed of γ and δ chains (44). Despite of small subset of T cells in peripheral blood, the γδ T cells display broad functional abilities and constitute an active and dynamic component of the solid organ transplant (45).

Expression of miR-99a, miR-100 and other miRNAs in serum was reported to be upregulated in kidney

 

recipients with acute rejection compared to patients with continuous stable kidney function (46). Despite of the positive correlation between miRNA-99 and miR-100 expression and plasma IL-2, which has been associated with immunosuppressive monitoring and response in kidney recipients (47), the deregulation of these miRNAs was not associated with acute rejection in this sample.

Expression of miR-103a in peripheral blood, as its target *FKBP1A* mRNA, did not differ during the initial 3-month of the immunosuppressive therapy. Other studies have indicated that increased miR-103a expression was associated with suppression of cell proliferation, migration, and invasion in gastric cancer and malignant mesothelioma (48,49). Expression of miR-103a and other four miRNAs in blood cells were downregulated in kidney recipients with severe T cell-mediated vascular rejection (50).

The expression of *MTOR* and *PPP3CA* mRNA can also be modulated by other miRNAs during the early phase of immunosuppressive therapy. Therefore, further studies with larger samples and wider range miRNAs are necessary to investigate the modulators of the genes involved in the calcineurin and mTOR signaling pathways in kidney transplant models.

The main limitations of this study were the number of the highly selected kidney transplant recipients with a low immunological risk for rejection and the time of follow up. However, this work presents innovative data on the pharmacogenomics and epigenomics of kidney transplantation, contributing to the knowledge of possible mechanisms capable of influencing the therapeutic response in renal transplant patients.

In conclusion, the expression of *MTOR* and *PPP3CA* mRNA is downregulated and miR-99a is upregulated in peripheral blood of kidney recipients in the early phase of tacrolimus-based therapy, and *PPP3CA* and *MTOR* variants influenced mRNA expression. These molecules may have potential application as biomarker candidates for pharmacotherapy follow up.

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## Footnote

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*Conflicts of Interest:* All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/atm-20-1757). The authors have no conflicts of interest to declare.

*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study conducted according to good clinical practices and the Declaration of Helsinki guidelines (as revised in 2013). The study protocol was approved by the ethics committees of the Federal University of Sao Paulo (UNIFESP, # 054/2008) and School of Pharmaceutical Sciences of the University of Sao Paulo (FCF/USP, # 517), Sao Paulo, Brazil. Written informed consent was obtained from each participant for the collection of clinical data.



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## Supplementary

**Table S1** Characteristics of the kidney transplant recipients and donors according to biopsy-confirmed acute rejection (BCAR) status

| Variables | BCAR (n=6) | Non-BCAR (n=30) | P value |
|---|---|---|---|
| Recipient | | | |
| Age, years | 43.5 (39.0–52.0) | 49.0 (37.0–59.0) | 0.663 |
| Men, % | 50.0 [3] | 70.0 [21] | 0.378 |
| Ethnicity | | | |
| Caucasian, % | 66.6 [4] | 53.4 [16] | 0.798 |
| Intermediate, % | 16.7 [1] | 33.3 [10] | |
| African, % | 16.7 [1] | 10 [3] | |
| Asian, % | 0 [0] | 3.3 [1] | |
| Cause of end stage renal disease, % | | | |
| Hypertension | 0.0 [0] | 13.3 [4] | 0.751 |
| Diabetes | 16.7 [1] | 23.3 [7] | |
| Other* | 33.3 [2] | 26.7 [8] | |
| Undetermined | 50.0 [3] | 36.7 [11] | |
| Cold ischemia time**, h | 11.5 (1.3–21.8) | 19.6 (17.5–26.6) | 0.08 |
| Delayed graft function, % | 16.7 [1] | 20.0 [6] | 1.000 |
| Donor | | | |
| Age, years | 38.0 (33.0–48.0) | 45.0 (37.0–51.0) | 0.574 |
| Men, % | 66.6 [4] | 53.4 [16] | 0.672 |
| Ethnicity | | | |
| Caucasian, % | 83.3 [5] | 63.3 [19] | 0.552 |
| Intermediate, % | 0 [0] | 20.0 [6] | |
| African, % | 16.7 [1] | 10.0 [3] | |
| Missing data, % | 0 [0] | 6.7 [2] | |
| Donor type (deceased), % | 33.3 [2] | 50.0 [15] | 0.750 |

Number of individuals is in parentheses. Continuous variables are shown as median and interquartile range. Categorical variables are shown as percentage. *, Polycystic kidney disease, urinary tract malformation, lupus nephritis or nephrotic syndrome. **, For deceased donor only.

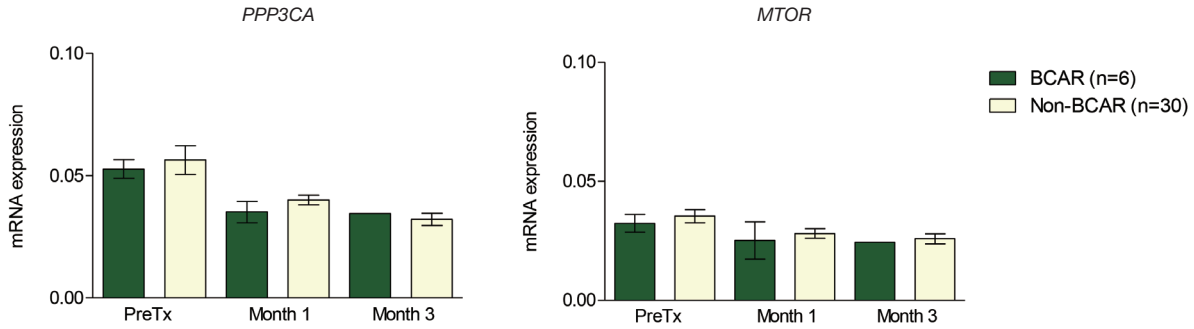


**Figure S1** Relationship of *PPP3CA* and *MTOR* mRNA expression and acute rejection of kidney recipients. The mRNA expression was measured by qPCR and normalized with *UBC* and *B2M*. The relative mRNA expression was calculated by $2^{-\Delta CT}$ formula. The data are shown as mean ± SEM. BCAR, biopsy confirming acute rejection; PreTx, pre-transplant.