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PHARMACOGENETICS MAY INFLUENCE TACROLIMUS DAILY DOSE, BUT NOT URINARY TUBULAR DAMAGE MARKERS IN THE LONG-TERM PERIOD AFTER RENAL TRANSPLANTATION

FARMAKOGENETIKA MOŽE IMATI UTICAJA NA DNEVNU DOZU TAKROLIMUSA, ALI NE I NA MARKERE TUBULARNOG OŠTEĆENJA U DUGOROČNOM PERIODU NAKON TRANSPLANTACIJE BUBREGA

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Summary

Background: The primary goal of this study was to evaluate the influence of cytochrome P450 (CYP) 3A5 (6986A>G) and ABCB1 (3435C>T) polymorphisms on tacrolimus (TAC) dosage regimen and exposure. Second, we evaluated the influence of TAC dosage regimen and the tested polymorphisms on renal oxidative injury, as well as the urinary activities of tubular ectoenzymes in a long-term period after transplantation. Also, we aimed to determine the association between renal oxidative stress and tubular damage markers in the renal transplant patients.

Methods: The study included 72 patients who were on TAC based immunosuppression. Allele-specific PCR was used for polymorphism determination. We measured the urinary thiobarbituric acid reactive substances (TBARS) and reactive carbonyl derivates (RCD) in order to evaluate oxidative injury, as well as the urinary activities of ectoenzymes (N-acetyl-β-D-glucosaminidase, aminopeptidase N and dipeptidyl peptidase IV) to evaluate tubular damage.

Results: The carriers of CYP 3A5*1 allele required statistically higher daily doses of TAC than CYP *3/*3 carriers, as well as the carriers of C allele of ABCB1 gene compared to those with TT genotype. Also, there were no differences in TBARS,

Kratak sadržaj

Uvod: Primarni cilj ovog rada bio je procena uticaja citohrom P450 (CYP) 3A5 (6986A>G) i ABCB1 (3435C>T) polimorfizama na dozni režim i izloženost takrolimusu (TAC). Sekundarni cilj bio je procena uticaja doznog režima TAC i ispitivanih polimorfizama na renalni oksidativni stres, kao i na urinarnu aktivnost tubularnih ektoenzima u dugoročnom periodu nakon transplantacije. Takođe, mi smo imali za cilj da odredimo povezanost između renalnog oksidativnog stresa i markera tubularnog oštećenja kod pacijenata sa transplantiranim bubregom.

Metode: Istraživanje je uključivalo 72 pacijenata, koji su bili na TAC imunosupresivnom režimu. Alel-specifični PCR metod je korišćen u cilju određivanja polimorfizama. Mi smo određivali nivo tiobarbituratna kiselina reaktivnih supstanci (TBARS) i reaktivnih karbonilnih grupa (RCD) u urinu u cilju procene oksidativnog stresa i aktivnosti ektoenzima (N-acetil-β-D-glukozaminidaza, aminopeptidaza N i dipeptidil peptidaza IV) u urinu, kao markere oštećenja tubula.

Rezultati: Nosioci CYP 3A5*1 alela imali su statistički veće dnevne doze TAC u poređenju sa nosiocima CYP *3/*3 genotipa, kao i nosioci C alela ABCB1 genskog polimorfizma u poređenju sa nosiocima TT genotipa. Takođe, nije

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RCD and the activities of ectoenzymes between the patients' genotypes. Our results showed significant correlations between urinary TBARS and RCD and the ectoenzymes' activities.

Conclusions: Our findings suggest that CYP 3A5 and ABCB1 3435 polymorphism may affect TAC daily doses, but not the drug's tubular toxicity. Furthermore, tubular damage may be associated with increased renal oxidative stress.

Keywords: ABCB1, CYP3A5, oxidative stress, renal transplantation, tacrolimus, tubular damage

bilo razlika u nivoima TBARS, RCD i aktivnostima ektoenzima između različitih genotipova pacijenata. Naši rezultati pokazali su značajne korelacije između urinarnog TBARS i RCD i aktivnosti ektoenzima.

Zaključak: Naši rezultati ukazuju na to da CYP 3A5 i ABCB1 3435 polimorfizam mogu uticati na dnevnu dozu TAC, ali ne i na tubularnu toksičnost izazvanu lekom. Pored toga, oštećenje tubula može biti udruženo sa povećanim renalnim oksidativnim stresom.

Ključne reči: ABCB1, CYP3A5, oksidativni stres, transplantacija bubrega, takrolimus, oštećenje tubula

Introduction

Renal transplantation is the chosen treatment for patients suffering from chronic kidney disease (CKD), but progress in the long-term survival of transplanted organs has not gone in parallel with the short-term outcomes in a sense of complications that can occur (1). Immunosuppressive treatment significantly affects the survival of transplanted organs in the post-transplantation period, but also may exert adverse effects and toxicity. Furthermore, clinical use of tacrolimus (TAC), part of the most immunosuppressive regimens, is complicated by its narrow therapeutic range and large inter-individual variability in pharmacokinetics (2, 3). Partly, this variability can be explained by gene polymorphisms in cytochrome P450 3A5 (CYP3A5) and P-glycoprotein (P-gp). These proteins are assumed to be the most relevant in the absorption, distribution and elimination of TAC. The significance of CYP 3A5 6986A>G (rs776746) gene polymorphism in TAC pharmacokinetics is well documented, while the role of ABCB1 3435C>T (rs1045642) gene polymorphism (encoding P-glycoprotein) is still controversial (4, 5).

Besides variability in pharmacokinetics that may complicate post-transplantation immunosuppressive treatment, chronic nephrotoxicity as well may lead to undesirable outcomes, foremost chronic allograft nephropathy (CAN), the main cause of late kidney graft loss. Both calcineurin inhibitors (CNI), TAC and cyclosporine A (CsA) may exert toxic effects on proximal tubular cells and endothelial function (6, 7). Furthermore, oxidative stress is one of the pathophysiological mechanisms that underlie tubular damage as well as CAN and may be associated with CKD and the process of transplantation, but also generated through immunosuppressive treatment (1). Although oxidative injury appears to decrease in the first week after transplantation, it stays elevated during the post-transplantation period in comparison to healthy individuals (8). Additionaly, reactive oxygen species (ROS) may be generated through activation of cytochrome P450 3A (CYP3A4/5) and NADPH oxidase in the liver. Still, the implication of TAC dosage regimen, CYP 3A5 and ABCB1 polymorphism in post-transplant oxidative stress, as well as in chronic nephrotoxicity, is still controversial and the subject of ongoing researches (7, 9). Oxidative damage leads to the lipid peroxidation process, forming the end products, primarily malondialdehyde (MDA), which can react with thiobarbituric acid (TBA) and form thiobarbituric acid reactive substances (TBARS). Urinary levels of TBARS and reactive carbonyl derivates (RCD) were used as parameters of the renal oxidative injury, as well as tubular damage markers in different renal diseases, such as diabetic nephropathy (10). Also, increased urinary activities of tubular ectoenzymes, N-acetyl-β-D-glucosaminidase (NAG), aminopeptidase N (APN) and dipeptidyl peptidase IV (DPP IV) may be useful markers of proximal tubular injury (11).

The primary goal of this study was to evaluate the influence of CYP 3A5 (6986A>G) and ABCB1 (3435C>T) polymorphisms on TAC dosage regimen and exposure. Secondary, we evaluated the influence of TAC dosage regimen and the tested polymorphisms on renal oxidative injury, as well as the urinary activities of tubular ectoenzymes in a long-term period after transplantation. Also, we aimed to determine the association between renal oxidative stress and the tubular damage markers in renal transplant patients.

Materials and Methods

The study was conducted during 2013 at the Clinic of Nephrology, Clinical Center Niš, Serbia and at the Research Centre for Biomedicine, Faculty of Medicine, University of Niš, Serbia. Our research included 72 renal transplant patients who were monitored at the Clinic of Nephrology and met some exclusion criteria before they were enrolled into this study. We excluded patients with the time period after transplantation less than 12 months, with any sign of chronic graft rejection and with concomitant disease or state that can enhance oxidative stress. Also, patients with variable TAC dosage regimen six months prior to the research were excluded. The study was approved by the Ethics Committee of Medical Faculty Niš and fully informed written consent was obtained from each patient. Of the patients, 49 were men and 23 were women, mean age 41.62 ± 11.03. Time period after transplantation was 27 (range 13–52)

months. Regarding the type of transplantation, 54 out of 72 patients got the transplanted kidney from a living (L) donor, and 18 out of 72 got their organ from deceased (D) donors. All of the patients were on a triple immunosuppressive regimen, which included TAC in the dose of 0.04 mg per day (range 0.02–1.4 mg/kg per day), mycophenolate mofetil (MMF) or mycophenolate acid (MPA) in the dose of 720 mg per day (range 360–1440 mg per day, calculated on a dose of MPA) and prednisone (PRE) in the dose of 7.5 mg per day (range 5–10 mg per day). TAC was administered twice daily (08:00 h and 20:00 h), and the dose was adjusted according to the trough concentration of the drug in the blood, in order to maintain drug concentration in the appropriate range (5–15 ng/mL). The trough concentration of TAC in the blood was measured by an immunoassay method according to the manufacturer's instructions (Architect, Abbott, Abbott Park, IL, USA). The dose-adjusted concentration was calculated as a trough concentration divided by a corresponding dose of TAC and it is considered as the index of the drug exposure.

A fasting blood sample was taken from each patient during routine control at the Clinic. Of the whole blood sample, 200 μL was taken for DNA isolation. DNA was extracted from the whole blood with EDTA as an anticoagulant using a Genomic DNA Purification Kit (Fermentas, Thermo Scientific, Lithuania), according to the manufacturer's instructions. Urine (24 h collected) was also taken from each patient during routine control. Urine samples were stored at – 80 °C.

Hematological and routine biochemical parameters, such as the level of hemoglobin (HGB), number of erythrocytes (RBC) and leukocytes (WBC), plasma level of albumin (ALB), glucose (GLU), cholesterol (CHOL), triglycerides (TG), urea (URE), creatinine (CRE) and the activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by standard methods in the Biochemical Laboratory at the Clinic of Nephrology. Analyses were performed on an automated random access clinical chemistry analyzer (ERBA XL – 600, ERBA Diagnostics Mannheim GmbH, Mannheim, Germany). GFR was estimated by the MDRD formula. We also calculated Body Mass Index (BMI).

As one of the parameters of the OS, we determined thiobarbituric acid reactive substances (TBARS), which are formed in the reaction of thiobarbituric acid (TBA) with the end product of the lipid peroxidation process, foremost malondialdehyde (MDA). TBARS content, a measure of lipid peroxidation, was assayed in urine according to the methods of Siciarz et al. (12) and expressed in μmol/mmol creatinine to rule out the influence of urinary dilution or concentration. The protein oxidation level was monitored by a spectrophotometric determination of carbonyl content by the method of Levine et al. (13) using 2,4-dinitrophenylhydrazine (DNPH) as a classic carbonyl reagent. Spectrophotometric measurement of reactive carbonyl derivates (RCD) values was performed and calculated using the extinction coefficient of DNPH-reactive carbonyl derivates at 370 nm and expressed as μmol/mmol creatinine (10). Urinary N-acetyl-β-d-glucosaminidase (NAG) activity was determined according to the method of Horak et al. (14). The activity of aminopeptidase N (APN) and dipeptidyl peptidase IV (DPP IV) activity were determined by a method previously described in the article of Stefanović et al. (11). Urinary NAG, APN and DPP IV excretions were expressed as U/mmol creatinine. Analytical recovery for the applied methods was 90–95%.

In order to determine the polymorphisms of CYP 3A5 and ABCB1 gene, we used the allele-specific PCR method of Ashavaid et al. (15). Each reaction mixture (for a single patient) was prepared in duplicate, one for determination of the wild-type allele (CYP3A5*1 and ABCB1 3435C) and the second for determination of the mutant-type allele (CYP3A5*3 and ABCB1 3435T) of both polymorphisms. Hence, to identify each of the tested polymorphisms, we used one forward and two reverse primers (wild-type and mutant-type reverse primer) for a single polymorphism (16). Forward primer is mutual for both reaction mixtures while the reverse primer is different. The sequence of the forward primers: CYP3A5: 5′-CAC TTG ATG ATT TAC CTG CCT TC – 3′, ABCB1: 5′-ACT ATA GGC CA GAGA GGC TGC – 3′. The sequence of the reverse primers: CYP3A5 (wild-type): 5′-GGT CCA AAC AGG GAA GAG ATA T – 3′, CYP3A5 (mutant-type): 5′-GGT CCA AAC AGG GAA GAG ATA C – 3′, ABCB1 (wild-type): 5′-GTG GTG TCA CAG GAA GAG CTC – 3′, ABCB1 (mutant-type): 5′-GTG GTG TCA CAG GAA GAG CTT – 3′. In a total volume of 25 μL, each reaction mixture contained 12.5 μL of KAPA2G Readymix (KAPA2G Readymix FastHotStart, KapaBiosystems, Boston, USA), which already contains Hot Start DNA polymerase, dNTPs, $MgCl_2$ and stabilizers. In addition to the commercial mix, we added 0.5 μL of both primers (forward and reverse, concentration of 10 pmol/μL), 10.5 μL of deionized water and 1 μL of isolated DNA (average concentration 50 ng/μL). For the amplification of PCR products, we followed the program: initial denaturation for 2 min at 95 °C, followed with 35 cycles of denaturation for 15 sec at 95 °C, annealing for 15 s at 60 °C, elongation for 15 s at 72 °C with final elongation for 30 s at 72 °C. Amplification products were detected on 3% agarose gel. The length of an amplified product in the determination of CYP3A5 is 218 base pairs (bp) as well as for ABCB1, 134 bp.

Statistical analyses

Characteristics of the study group were expressed as mean ± SD and median (interquartile range, IQR). We used Student t-test for normally distributed

data (expressed as mean ± SD) and Mann Whitney U test for data that were not normally distributed (expressed as Median and IQR in brackets) to compare the investigated parameters between CYP3A5 and ABCB1 genotypes, as well as between patient groups formed based on the dose of TAC. Correlation analyses were performed using Pearson correlation test (normally distributed data) and Spearman test (not normally distributed data). All analyses were performed with SPSS statistical analysis software, version 16.0 (SPSS, Chicago, IL, United States) at the significance level set at $p<0.05$.

Results

Clinical and biochemical data of the renal transplant recipients are given in *Table I*.

Genotyping analysis showed that 11 of the 72 patients were carriers of the CYP 3A5 *1/*3 genotype and 61 were homozygote for CYP 3A5 *3 allele. Furthermore, 16 out of 72 patients were homozygous for ABCB1 CC and 15 out of 72 were homozygous for ABCB1 TT genotype. Forty-one patients enrolled into the study had the ABCB1 CT genotype. It has been shown that CYP 3A5 *1 carriers required statistically higher doses of TAC to maintain the optimal concentration of the drug than CYP 3A5 *3/*3 carriers. Furthermore, CYP 3A5 *1 carriers were less exposed to the drug than CYP 3A5 *3/*3 carriers (*Table II*). Also, carriers of the C allele of ABCB 1 gene (including both CC and CT genotypes) required higher doses of TAC than the carriers of TT genotype ($p<0.05$), but there was no difference between CT and TT genotypes or CC and CT genotypes.

Table I Clinical and biochemical data of renal transplant recipients.

	Kidney Transplant Recipients
Age (years)	42±11
Period after transplantation (months)	27 (17)
Tx type (L donor/D donor)	49/23
Sex (M/F)	34/18
Body mass (kg)	76.40±14.04
BMI (kg/m^2)	25.63±3.49
CRE (μmol/L)	134±33
URE (mmol/L)	7.44±2.33
eGFR (mL/min/1.73 m^2)	51.17±14.87
GLU (mmol/L)	5.04 (1.08)
CHOL (mmol/L)	5.90±0.96
TG (mmol/L)	2.20 (1.00)
RBC × 10^{12}/L	4.86±0.72
HGB (g/L)	136±22
WBC × 10^9/L	8.40 (2.50)
ALB (g/L)	42.64±3.10
ALT (U/L)	21.00 (17.50)
AST (U/L)	18.00 (8.13)

Data are expressed as mean ± SD – for normally distributed data or median (IQR) – for not normally distributed data. eGFR was calculated by MDRD formula.

Based on a median value of the daily dose of TAC, the patients were divided into two groups, those with a dose of TAC lower than 0.04 mg/kg per day and those with a dose of the drug higher than 0.04 mg/kg per day (including patients with a dose of 0.04 mg/kg per day). Beside this, the patients were divided into groups based on a genotype of CYP3A5 and ABCB1. Furthermore, we investigated the potential influence of TAC dosage regimen and the tested polymorphisms on the serum creatinine level and clearance of creatinine (*Figure 1*).

Figure 1A shows that there is no significant difference between CRE in relation to TAC doses and CYP3A5 and ABCB1 3435 genotype. Conversely, *Figure 1B* shows that patients with higher daily doses of TAC had statistically lower GFR (47.36±11.45 vs. 55.84±13.76, $p<0.05$). Also, the patients carrying an ABCB1 TT genotype had significantly higher GFR than patients with the C allele (59.27 ± 11.55 vs. 49.00 ± 12.72, $p<0.05$). There was no difference in GFR between CYP3A5 *1/*3 and CYP3A5 *3/*3 genotype (43.06±12.21 vs. 52.71±12.68, $p<0.057$).

Table III shows a comparison between the tested oxidative stress parameters and the activities of ectoenzymes in relation to the TAC dosage regimen, CYP3A5 and ABCB1 genotypes of the renal transplant recipients. Our study showed that urinary TBARS differed in relation to TAC daily doses, but there were no differences in other parameters in relation to the patients' dosage regimens or tested polymorphisms.

The correlation analysis including dose, plasma trough concentration as well as dose-adjusted concentration of TAC and oxidative stress parameters, ectoenzymes activities and renal function parameters is shown in *Table IV*. There is a positive correlation between the dose of TAC and serum creatinine level ($p<0.01$), as well as a negative correlation between the drug dose and GFR ($p<0.01$). Oppositely, the dose-adjusted concentration of TAC negatively correlates with creatinine level ($p<0.05$) and shows a positive correlation with GFR ($p<0.05$). The oxidative stress parameters and the enzyme markers do not express any significant association in relation to dose, level and dose-adjusted concentration of TAC.

Table II Dose, trough concentration and dose-adjusted concentration of TAC in relation to patients' genotype for CYP3A5 and ABCB1 gene.

CYP3A5 genotype	*1 / *3	*3 / *3	p
Daily dose (mg)	4.75 (3.75)	3.00 (2.00)	0.039*
Daily Dose (mg/kg)	0.06 (0.06)	0.04 (0.03)	0.044*
Trough concentration (ng/mL)	6.28±1.26	6.74±1.55	0.428
Dose-adjusted concentration (ng mL^{-1}/mg $kg^{-1}day^{-1}$)	113.57±50.68	179.88±76.07	0.025*
ABCB1 3435 genotype	CC+CT	TT	p
Daily dose (mg)	3.00 (2.00)	2.25 (1.25)	0.042*
Daily Dose (mg/kg)	0.04 (0.03)	0.03 (0.01)	0.036*
Trough concentration (ng/mL)	6.58±1.52	7.03±1.40	0.155
Dose-adjusted concentration (ng mL^{-1}/mg $kg^{-1}day^{-1}$)	157.22±68.82	207.04±95.58	0.097

Data are expressed as mean ± SD – compared with Student t-test or median (IQR) – compared with Mann Whitney U test.

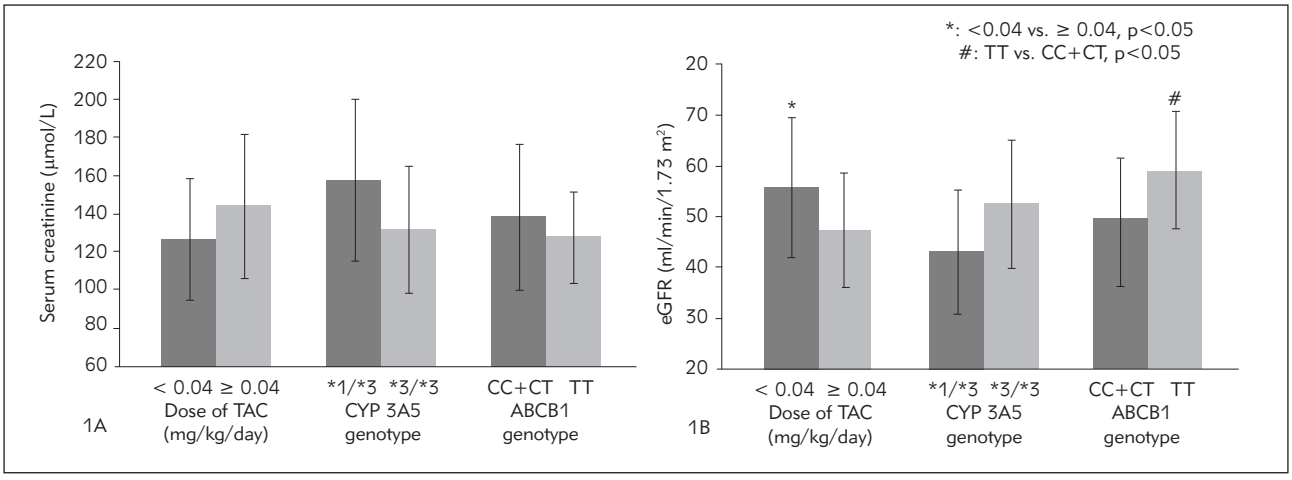

Figure 1 Serum creatinine level (A) and GFR (B) in relation to dosage regimen of TAC, CYP 3A5 and ABCB1 polymorphism.

Table III Plasma and urine oxidative stress parameters in relation to dose of TAC, CYP 3A5 and ABCB1 3435 genotype.

	TBARS (µmol/mmol CRE)	RCD (µmol/mmol CRE)	NAG (U/mmol CRE)	DPP IV (U/mmol CRE)	APN (U/mmol CRE)
Dose of TAC					
< 0.04 mg/kg/d	1.57 (0.36)*	30.29 (12.42)	1.19 (1.21)	0.52 ± 0.28	0.24 (0.20)
≥ 0.04 mg/kg/d	1.23 (0.37)	27.15 (13.81)	1.32 (1.08)	0.45 ± 0.21	0.28 (0.14)
CYP3A5 genotype					
*1 / *3	1.37 (0.66)	29.19 (14.70)	0.95 (0.31)	0.42 ± 0.24	0.22 (0.17)
*3 / *3	1.33 (0.51)	27.75 (12.73)	1.36 (1.16)	0.50 ± 0.24	0.26 (0.15)
ABCB1 3435 genotype					
CC + CT	1.33 (0.50)	28.81 (14.72)	1.32 (1.28)	0.49 ± 0.25	0.25 (0.16)
TT	1.42 (0.55)	25.51 (8.79)	1.29 (0.68)	0.45 ± 0.20	0.25 (0.14)

Data are expressed as mean ± SD – compared with Student-t test or median (IQR) – compared with Mann Whitney U test.
*: p<0.05

Table IV Dose, trough concentration and dose-adjusted concentration of TAC in relation to patients' genotype for CYP3A5 and ABCB1 gene.

	TBARS	RCD	NAG	DPP IV	APN	CRE	eGFR
Dose (mg/day)	–0.172	0.157	0.114	0.033	0.112	0.410**	–0.510**
Trough concentration (ng/mL)	–0.107	–0.071	–0.143	–0159	–0.029	0.186	–0.264
Dose-adjusted concentration (ng mL^{-1}/mg day^{-1})	0.097	–0.205	–0.130	–0.120	–0.121	–0.302*	0.343*

Data is expressed as correlation, Pearson's coefficient – r or Spearman's coefficient – rho. eGFR was calculated by MDRD formula. *: $p<0.05$, **: $p<0.01$

Table V Correlation analysis between the tested oxidative stress parameters and enzyme activities in the urine of renal transplant recipients.

	RCD	NAG	DPP IV	APN	CRE	eGFR
TBARS	0.658**	0.312*	0.750**	0.798**	–0.355*	0.315*
RCD		0.395*	0.783**	0.667**	0.043	–0.030
NAG			0.324*	0.302*	0.157	–0.126
DPP IV				0.828**	–0.280	–0.266
APN					–0.308*	0.236
CRE						–0.819**

Data is expressed as correlation, Pearson's coefficient – r or Spearman's coefficient – rho. eGFR was calculated by MDRD formula. *: $p<0.05$, **: $p<0.01$

Also, Pearson and Spearman correlation analyses showed significant correlations between the tested oxidative stress parameters and the enzyme activities in urine of the patients (*Table V*).

Discussion

Chronic nephrotoxicity is the major long-term adverse effect of both CsA and TAC, and may lead to CAN, the main cause of late kidney graft loss (17). Beside differences in their structures, TAC has similar mechanisms with CsA underlying chronic nephrotoxicity, but lower potential to cause these effects (18). Adverse effects affect the tubulo-interstitium, as well as vessels and glomeruli (7). Among the rest, TAC toxic effects may be caused by an increased oxidative stress, suggesting it may underlie long-term tubular damage.

Toxicity associated with CNI therapy can occur at drug concentrations similar to that required for a beneficial effect. Additionally, the narrow therapeutic range and between patient variability in the pharmacokinetics of TAC complicate achieving the optimal post-transplantational goals. The previous studies showed that CYP3A5 gene polymorphism is one of the main determinants, along with demographic factors and drug–drug interactions, that contribute to the patients' variability in TAC pharmacokinetics (4, 19). Also, the frequency of CYP3A5 * 1 allele is largely dependent on ethnic origin (4). In Caucasians, the frequency of CYP3A5 *1 allele is approximately 5–15% (4), which is consistent with our results. Frequency of this allele varies among other ethnic groups: in African Americans it is 45–73%, 15% in the Japanese, 27–35% in the Chinese, 30% in Koreans, 25% in Mexicans and 27% in Moroccans (4, 20–22). Patients carrying at least one CYP3A5*1 allele, A at the position 6986 (expressers) expressed larger amounts of CYP3A5 protein, and also required a higher dose of TAC to maintain optimal blood concentration than homozygotes for the CYP3A5*3 allele (non -expressers) (23, 24). Accordingly, our findings showed that individuals with CYP3A5 *1/*3 required a higher daily dose of TAC than CYP3A5 *3 / *3 carriers ($p<0.05$). Also, there is significant difference in the dose-adjusted concentration between the patients with different genotypes for CYP 3A5 (*1/*3 < *3/*3, $p<0.01$). Furthermore, the patients enrolled in our study had undergone transplantation surgery at least 12 months earlier, suggesting that CYP 3A5 polymorphism is not only significant in a short-term

period after transplantation, but it is still a considerable determinant of long-term drug exposure.

The most studied ABCB1 polymorphism refers to the C to T transition at the position 3435 within exon 26. Previous researches suggested that individuals with ABCB1 3435 TT genotype expressed lower intestinal P-gp activity and therefore eliminated lower amounts of TAC from the intestinal cells, which led to a better absorption of the drug and a lower daily dose requirement in these patients (4, 25). Our findings are consistent with these, since the patients in our study carrying the TT genotype had significantly lower daily doses of TAC than the carriers of at least one C allele (CC and CT genotype). But, ABCB1 polymorphism did not affect the dose-adjusted level of the drug (*Table II*).

In order to evaluate the potential influence of CYP3A5 and ABCB1 polymorphism, as well as TAC daily dose on renal function parameters and urinary nephrotoxicity markers, we chose the stable renal transplant patients, who had no change in daily dose of the drug or the change was less than 0.5 mg per day, 6 months prior to our research. Our findings showed that patients with TAC doses of 0.04 mg/kg per day, or higher, might have had lower GFR (*Figure 1B*), which led us to assume that lower doses of TAC in the long-term period after transplantation led to better preservation of the renal function. Besides, we found that patients carrying the TT genotype of ABCB1 polymorphism had higher GFR, which is in accordance with the previous finding that lower doses of TAC in the maintenance period led to better renal function (*Figure 1B*). This is consistent with the finding of Zheng et al. (26) in lung transplantation, but opposite to Hasselink et al. (27). There was no such finding for the CYP3A5 polymorphism, regardless the fact that 75% of the *1 allele carriers in our study had TAC doses higher than 0.04 mg/kg per day. Also, the patients with the higher daily doses of TAC had lower urinary TBARS levels (*Table III*). This result is contradictory to the previous one (*Figure 1B*) and may be explained by our finding that patients with better renal function had increased glomerular filtration of the systemic MDA into the urine (*Table V*). Furthermore, there were no other significant differences between the parameters of oxidative injury and tubular damage measured in the urine with respect to TAC dosage regimen or CYP 3A5 and ABCB1 polymorphisms, which was in accordance with the previous studies (28, 29). Alternatively, Kuypers et al. showed that patients on TAC daily doses of 0.20 mg/kg might develop calcineurin inhibitor associated nephrotoxicity (CNIT) (29). The same authors suggested that CYP 3A5 *1 allele contributed to CNIT, but this finding was not supported by our study as well. However, these doses were two-fold higher than the doses administered to the patients in our study (0.07 ± 0.04 mg/kg per day in patients with CYP 3A5 *1/*3 genotype), which might explain why we did not find any difference in nephrotoxicity markers with respect to dosage regimen. Nevertheless, our results show that the patients on a higher daily dose of TAC in the long-term period after transplantation may also have deteriorated renal function. This finding suggests that even without proven tubular damage (expressed as elevation in urinary nephrotoxicity markers), dosage regimen may affect renal function (*Table IV*). Moreover, a possible explaination for such a finding may affirm the recent studies that toxic effects of TAC may actually be explained by its metabolites' effect (7, 30, 31).

The correlation analysis showed positive correlation between urinary level of TBARS and RCD with the activities of ectoenzymes, NAG, DPP IV and APN, in the urine of the renal transplant patients (*Table V*). Hence, this indicates that patients with a higher level of renal oxidative stress also might have more pronounced tubular damage, suggesting oxidative stress underlies tubular injury. Accordingly, Ha et al. (32) showed that products of intracellular lipid and protein oxidation, such as MDA and carbonylated proteins, may be increased in CAN. Contradictory, our study showed a significant negative correlation between urinary TBARS and CRE ($r=-0.4$), as well as a positive relationship between TBARS and GFR ($r=0.3$). Still, the APN activity correlated negatively with CRE ($r=-0.3$). These findings may be explained by the assumption that improvement in renal function leads to increased filtration of TBARS through the glomeruli into the urine. The other explanation for such a phenomenon can be found in the work of Locatelli et al. (33) in which urinary TBARS levels may represent only oxidative injury that occurred locally in the kidney, independent from systemic oxidative stress. Hence, the positive correlation between urinary TBARS and GFR was indirect and associated to the previous finding that TAC higher doses led to lower urinary TBARS.

The urinary TBARS and RCD levels may refer to an oxidative damage occurring in the kidney (10). Previous studies as well indicated that renal transplant recipients had higher TBARS levels than healthy individuals (8, 34, 35). Increased urinary TBARS may be the result of lipid peroxidation occurring locally in the tubules and/or may represent systemic exposure to oxidative damage. Tada et al. suggest that TAC may increase MDA production in kidneys (36). TAC may decrease the activity of catalase as well as the activity of other antioxidant enzymes (37, 38), but also it may contribute to ROS generation through activation of cytochrome P450 3A (CYP3A4/5) and NADPH oxidase (7, 38). The process of membrane lipid peroxidation may cause proximal tubular damage and lead to elevated activities of the tubular membrane enzymes (NAG, APN, DPP IV) in the urine of the patients.

NAG, a lysosomal enzyme present in renal proximal tubular cells, as well as APN and DPP IV, two

brush border enzymes of proximal tubular cells had higher urinary activities in patients compared to controls (6, 17, 39). Also, Bone et al. (40) showed that urinary activity of NAG was higher in patients taking CNI than in patients with native nephropathies or healthy volunteers, suggesting it as one of the most sensitive markers of renal proximal tubular cells injury. Urinary activity of DPP IV was studied before as a marker of tubular damage, but there have been no recent researches dealing with DPP IV as a marker of CNI toxicity, while the results regarding APN are discrepant. Marchewka et al. (6) showed that APN did not differ between renal transplant recipients and healthy volunteers.

The study population was chosen carefully so consequently the number of patients involved in the study was limited.

In conclusion, our findings confirmed the influence of CYP 3A5 polymorphism on TAC dosage regimen and exposure, but also showed that ABCB1 3435 polymorphism may affect the daily dose of the drug. In this study, we did not find an association between TAC dosage regimen, CYP 3A5 and ABCB1 genotype and the nephrotoxicity markers. In comparison with previous studies, our study population was administered lower daily doses of the drug, which may be the reason for the linkage absence. Furthermore, tubular damage may be associated with renal oxidative stress. Nevertheless, TAC dose rather than CYP 3A5 genotype may influence the renal function and potentially cause nephrotoxicity.

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Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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