

## GLUTATHIONE PEROXIDASE PRO200LEU GENE POLYMORPHISM AS A POTENTIAL PREDICTOR OF RENAL FUNCTION DECLINE IN RENAL TRANSPLANT RECIPIENTS

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Mutations in genes encoding antioxidant enzymes may reduce their activity and make organism more prone to oxidative damage. The objective of this study was to determine the distribution of glutathione peroxidase 1 (GPX1) Pro200Leu gene polymorphism in renal transplant recipients and healthy volunteers, as well as the association between investigated gene polymorphism and erythrocytes' antioxidative status and estimated GFR (eGFR) within a two-year follow-up after renal transplantation. A total of 85 patients with transplanted kidney and 110 healthy volunteers were genotyped for GPX1 Pro200Leu gene polymorphisms using the polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) method. Of all patients enrolled in genotyping analysis, only 72 patients on tacrolimus went into oxidative stress parameters research. We measured the erythrocytes' concentration of reduced glutathione (GSH) and the activities of GPX and glutathione reductase (GR). GFR was estimated by MDRD formula for creatinine clearance. There was no statistical difference in the distribution of GPX1 gene polymorphism between patients and controls. The obtained results demonstrated that renal transplant recipients with Leu/Leu genotype of tested GPX1 polymorphism had higher erythrocytes' activity of GR compared to the carriers of Pro/Pro genotype, but there were no differences in other oxidative stress parameters. The carriers of at least one Leu allele (Pro/Leu + Leu/Leu genotype) had significant decline in eGFR between the first and second year post-transplant. Genotyping of tested polymorphism in clinical practice may represent significant predictor of renal function decline and may provide identification of patients at high risk of graft loss. *Acta Medica Medianae* 2017;56(1):17-23.

**Key words:** gene polymorphism, glutathione peroxidase, oxidative stress, renal transplantation, renal function

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### Introduction

The introduction of calcineurin inhibitors (CNI), cyclosporine A and tacrolimus into renal transplantation therapy made a positive impact on short-term outcomes, especially in the incidence of the acute rejection and metabolic disorders associated with uremia (1, 2). However, increased oxidative stress and its role in the later periods

after renal transplantation are not completely understood (3, 4). The level of oxidative stress is reduced during the first week after transplantation and becomes smaller in comparison with patients undergoing hemodialysis, but remains elevated during the whole post-transplantation period compared to healthy volunteers (5). It has been shown that oxidative stress may contribute to the long-term adverse outcomes, such as cardiovascular (CV) morbidity and mortality, but chronic allograft nephropathy (CAN) as well. Chronic allograft nephropathy is a major cause of graft loss in a late period after transplantation (3). Oxidative injury may result from the end stage renal disease (ESRD)-associated oxidative stress, ischemia reperfusion injury, immune response to allograft, opportunistic infections and immunosuppressive therapy (3, 6, 7). Furthermore, mutations in genes encoding antioxidant enzymes may reduce their activities and make organism more prone to oxidative damage, which has been already shown in patients with breast cancer, diabetes, neurological, cardiovascular and chronic kidney disease (CKD) (8). Glutathione peroxidase

1 (GPX1) is a key enzyme in the endothelial protection against the harmful effects of the oxidative stress. However, the polymorphism present in GPX1 gene, substitution of cytosine (C) by thymine (T) at the position 599, leads to a substitution of proline (Pro) with leucine (Leu) at position 200 (depending on the database of the genome that is used, the position can be 197 or 198) and consequently influences decreased activity of the enzyme (9, 10). Hence, the objective of this study was to determine the distribution of GPX1 Pro200Leu gene polymorphism in renal transplant recipients and healthy volunteers, as well as the association between investigated gene polymorphism and erythrocytes' antioxidative status and estimated GFR (eGFR) within a two-year follow-up after renal transplantation.

### Patients and methods

The study was conducted at the Research Centre for Biomedicine, Faculty of Medicine, University of Niš, Serbia and at the Clinic of Nephrology, Clinical Center Niš, Serbia during 2012-2013. The genotyping analysis included 85 renal transplant recipients (including patients on tacrolimus, cyclosporine A, sirolimus), who were monitored at the Clinic of Nephrology at the time of the beginning of the study. Of all patients enrolled in genotyping analysis, only 72 patients on tacrolimus went into oxidative stress parameters research. Renal transplant recipients were on triple immunosuppressive therapy that besides tacrolimus included prednisone (Pre), 10 mg/day (range: 5-20 mg/day), mycophenolate mofetil (MMF), 1.5 g/day (range: 0.5-2 g/day) or mycophenolic acid (MPA), 1080 mg/day (range: 360-1440 mg/day) orally. The first oral tacrolimus dose was administered on day 5 post-transplant at 8.00 hr before breakfast (0.05 mg/kg). Furthermore, tacrolimus was administered twice daily (08:00 h and 20:00 h), and the dose was adjusted according to the trough concentration of a drug in the blood in order to maintain drug trough concentration (C<sub>0</sub>) in the appropriate range (5 - 15 ng/ml). Besides standard immuno-suppressive therapy, patients also received antihypertensive drugs (beta-blockers, bisoprolol or metoprolol and calcium channel blockers, amlodipine in monotherapy as well as in the combination) and omeprazole as gastroprotective. The study was approved by Ethics Committee of Medical Faculty Niš and fully informed written consent was obtained from each patient (Number: 01 - 10204 - 13).

A fasting blood sample was taken from each patient during routine control at the Clinic of Nephrology. Of the whole blood sample, 200 µL was taken for DNA isolation and 150 µL for GSH determination. DNA was extracted from the whole blood with EDTA as an anticoagulant using Genomic DNA Purification Kit (Fermentas, Thermo Scientific, Lithuania), according to the manufacturer's instructions. After that, the rest of the

blood was used for erythrocytes isolation. Healthy volunteers gave their blood in the scheduled term at the clinic.

In order to evaluate erythrocytes' oxidative stress parameters, we measured the erythrocytes' concentration of reduced glutathione (GSH) and the activities of GPX and glutathione reductase (GR). The glutathione was determined according to the method of Beutler et al. (11). Reduced GSH was determined using Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB). After precipitation of the proteins, the sulfhydryl group of GSH reacts with added DTNB and generates 2-nitro-5-thiobenzoic acid (TNB) producing a yellow color that can be detected at absorbance 412 nm. Results are expressed as µmol/g HGB. The activity of GPX in erythrocytes was measured according to the method of Paglia and Valentine (12). We used the commercial kit RANSEL (Randox Labs, Crumlin, UK). Glutathione peroxidase catalyzes the oxidation of glutathione by Cumene hydroperoxide. In the presence of GR and NADPH the oxidized glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP<sup>+</sup>. The decrease in absorbance at 340 nm is measured. Results are expressed as U/g HGB.

The activity of GR in erythrocytes was measured using commercial kit of the RANDOX laboratories (Randox Labs, Crumlin, UK). The method is based on the reaction of GSSG reduction in the presence of the NADPH, which is oxidized afterwards to NADP<sup>+</sup>. The absorbance is measured at 340 nm and results are expressed as U/g HGB.

Serum urea (URE) and creatinine (KRE) concentration were measured by standard methods in Biochemical laboratory at the Clinic of Nephrology. Analyses were performed on automated random access clinical chemistry analyzer (ERBA XL - 600, ERBA Diagnostics Mannheim GmbH, Mannheim, Germany). GFR was estimated (eGFR) by MDRD formula. For the purposes of the analysis we used eGFR for a period from 6 up to 24 months after transplantation (follow-up period 18 months).

*Genotyping.* PCR-RFLP method was used to determine GPX1 Pro200Leu gene polymorphism (rs1050450), a 599C > T substitution, which leads to substitution of Pro (CCC) with Leu (CTC) in position 200 within GPX1 protein. The sequence of the primers, forward: 5'-GCCGCCGCTTCCAGACCAT-3' and reverse: 5'-CCCCCGAGACAGCAGCACT-3'. Each reaction mixture, in total volume of 12.5 mL, contained 6.25 µL of KAPA2G Readymix (KAPA2G Readymix FastHotStart, KapaBiosystems, Boston, USA), which already contains Hot Start DNA polymerase, dNTPs, MgCl<sub>2</sub> and stabilizers. In addition to the commercial mix, we added 0.25 µL of both primers (forward and reverse, concentration of 10 pmol/µL), 5.25 µL of deionized water and 0.5 µL of isolated DNA (average concentration 50 ng/µL). For the amplification of PCR product (128 bp), 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 sec at 68°C, elongation for 15 sec at 72°C with final elongation for 30 sec at 72°C. For RFLP assay, PCR product was incubated at 37°C for 12 h with *Apa*I (MBI Fermentas). Amplification products were detected on 8% polyacrylamide gel stained with ethidium bromide. Results were recorded with photographs of gels under UV light. The PCR product of the Pro allele was cleaved into 67 bp and 61 bp fragments. The Leu allele was identified by lack of *Apa*I restriction site.

**Statistical analysis.** The distribution of genotypes for each polymorphism was assessed for deviation from Hardy-Weinberg equilibrium (HWE), and differences in genotype frequency and in allele frequency between the groups were assessed using the  $\chi^2$  test. We used a Student's t-test for normally distributed data and Mann Whitney U test for data that were not normally distributed to compare biochemical data between patients and controls, and ANOVA, Post Hoc Tukey, for normally distributed data and Kurskal Wallis test, Post Hoc Mann Whitney U test for data that were not normally distributed to compare oxidative stress parameters between the groups of patients based on the GPX1 genotypes within same period after transplantation. The general linear model – repeated measures, with Bonferroni

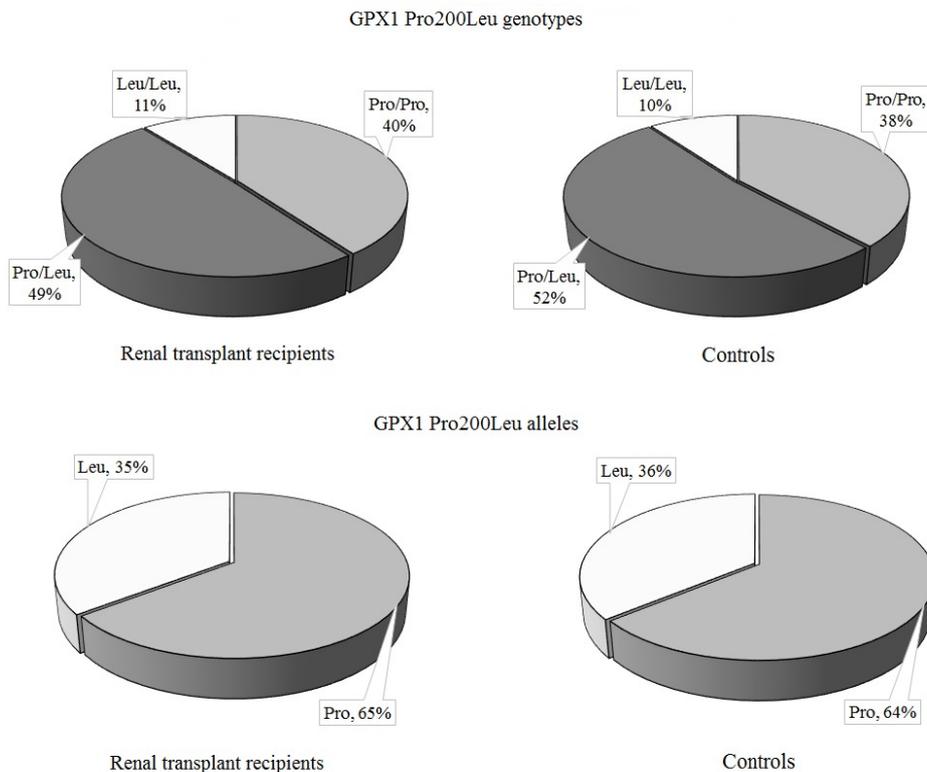
Post Hoc Test was performed to estimate the change in eGFR within patients with same GPX1 genotype between 6 and 24 months post-transplant. All analyses were performed with SPSS statistical analysis software, version 16.0 (SPSS, Chicago, IL, United States) at the significance level set at  $p < 0.05$ .

## Results

The genotypes and alleles distribution of the GPX1 Pro200Leu polymorphism is shown in Figure 1. This part of the study included 85 patients on tacrolimus, cyclosporine A and sirolimus and 110 controls. Genotype and allele frequencies did not deviate from Hardy-Weinberg equilibrium for tested polymorphism. Also, there was no statistical difference in distribution of GPX1 gene polymorphism between patients and controls.

The investigation of erythrocytes' oxidative stress parameters in relation to GPX1 genotype included 72 patients on tacrolimus-based immunosuppressive treatment (Table 1). The data reported that patients and controls are gender- and age-matched. Also, there was no difference in body mass and BMI between these two groups.

The results of our study demonstrated that renal transplant recipients with Leu/Leu genotype of tested GPX1 polymorphism had higher erythrocytes' activity of GR compared to the carriers of



**Figure 1.** Genotype and allelic frequencies of the GPX1 Pro200Leu polymorphism

**Table 1.** Characteristics of the patients and controls

	Renal transplant recipients	Controls	t/Z*/ $\chi^2$	p
Gender (M/F)	48/24	36/26	1.054**	0.305
Age (years)	41.83 ± 10.89 41.00(34.50-51.00)	42.63 ± 12.59 44.00(32.00-49.00)	- 0.060*	0.952
Body mass (kg)	76.26 ± 14.28 73.00(67.00-83.50)	77.13 ± 18.82 82.00(64.50-91.00)	- 0.596*	0.551
BMI (kg/m <sup>2</sup> )	25.98 ± 4.03 25.56(23.14-29.09)	24.72 ± 4.15 24.10(21.62-28.08)	1.269	0.208
KRE (μmol/L)	137.97 ± 47.52 122.00 (105.55-158.00)	90.92 ± 11.23 92.90 (83.55-98.00)	- 6.804*	< 0.001
eGFR (mL/min/ 1,73m <sup>2</sup> )	53.18 ± 16.80 54.69(41.43-62.61)	72.49 ± 10.05 72.23(65.00-73.00)	- 6.497	< 0.001
URE (mmol/L)	8.59 ± 3.79 8.20 (5.60-10.10)	4.53 ± 1.13 4.45 (3.90-5.30)	- 7.169*	< 0.001
Type of transplantation (LDRT/DDRT)	56 / 16	/		
Period after transplantation (years)	3.45 ± 2.48 3.00 (1.50-4.00)	/		

M – male; F – female; BMI – body mass index; LDRT – living donor renal transplantation; DDRT – deceased donor renal transplantation; KRE – serum creatinine; URE – serum urea  
Data are shown as mean ± standard deviation and median (interquartile range) or number.

**Table 2.** Comparison of the glutathione peroxidase and glutathione reductase activities and reduced glutathione concentration in erythrocytes of renal transplant patients with respect to GPX1 genotype.

	GPX (U/g HGB)	GR (U/g HGB)	GSH (μmol/g HGB)
Pro/Pro	61.02 ± 21.17 59.59 (45.84-76.52)	13.76 ± 4.45 <sup>§</sup> 13.46 (10.52-16.31)	14.78 ± 3.40 14.20 (12.53-16.11)
Pro/Leu	58.38 ± 17.37 58.89 (44.05-74.11)	16.07 ± 4.76 15.92 (12.15-19.32)	15.05 ± 3.27 14.12 (12.79-16.74)
Leu/Leu	63.19 ± 11.23 65.27 (61.60-71.12)	19.39 ± 4.61 18.09 (15.82-20.46)	15.56 ± 4.41 16.24 (10.62-18.14)
F/ $\chi^2$	0.204	3.529	0.322*
p value	0.816	0.037	0.851

GPX - glutathione peroxidase; GR - glutathione reductase; GSH - reduced glutathione; HGB - hemoglobin  
Data are expressed as mean ± SD and median (interquartile range). §: Pro/Pro vs. Leu/Leu, p = 0.045

**Table 3.** eGFR following 24 months after renal transplantation with respect to GPX1 genotype

	eGFR (mL/min/1.73m <sup>2</sup> )	
	GPX1 Pro/Pro	GPX1 Pro/Leu + Leu/Leu
6 months	47.61 ± 11.25	49.83 ± 12.79
12 months	47.68 ± 10.45	54.26 ± 18.84
24 months	44.29 ± 10.64	48.78 ± 14.78
F value	1.976	5.127
P value	0.178	0.013
Post Hoc	/	12 months vs. 24 months, p = 0.009

eGFR – estimated glomerular filtration rate

Pro/Pro genotype (Table 2).

Table 3 shows that carriers of at least one Leu allele (Pro/Leu + Leu/Leu genotype) had significant decline in eGFR in a two year follow-up. Post Hoc analysis demonstrated that significant difference in eGFR was found between the 12<sup>th</sup> month and 24<sup>th</sup> month post-transplant (54.26 ± 18.84 vs. 48.78 ± 14.78; p = 0.009).

## Discussion

Oxidative stress is associated with the progression of many diseases, including cancers, diabetes mellitus, CV diseases, neurodegenerative diseases and CKD (13, 14). Increased oxidative stress is a result of increased production of free radicals and/or reduced antioxidant capacity of the

organism. Numerous factors may contribute to diminished efficacy of antioxidants, including changes in antioxidative enzymes, toxins or underlying disease, leading to their degradation (8, 15). The investigation of association between mutations, present in genes encoding antioxidative enzymes, and development and/or progression of disease is of particular importance (16). These kinds of researches could provide rational background for identification and selection of patients towards risk for development and/or progression of disease associated with increased oxidative stress. Previous studies showed that GPX1 Pro200Leu gene polymorphism may have been associated with some diseases, including CKD (8,16). The obtained results showed that genotypes and alleles of GPX1 gene polymorphism did not differ between patients and controls. This may indicate that particular polymorphism do not contribute to the development of chronic renal failure, which consequently leads to renal transplantation. Crawford et al. showed in CKD patients that carriers of Leu/Leu genotype had reduced GFR (17).

The system glutathione/glutathione peroxidase is a major mechanism against elevated oxidative damage. Glutathione peroxidase 1 is a cytosol enzyme, mainly present in erythrocytes, kidneys and liver (9). The results of conducted research indicated that patients with Leu/Leu genotype had increased activity of GR in erythrocytes compared to the carriers of Pro/Pro genotype. The obtained result may be explained by the compensatory response of increased activity of GR on potentially low values of GPX. There was no difference in the activity of GPX and the concentration of reduced glutathione among patients with different GPX1 genotypes (Table 2).

The previous study showed that GPX1 Pro200Leu gene polymorphism may have affected eGFR within CKD patients, but had no influence on progression of disease (8). Conversely, our study showed that investigated polymorphism has been associated with the eGFR worsening among renal transplant recipients. In the carriers of at least one Leu allele, there was a significant decline in eGFR

between the first and second year post-transplant compared to patients with Pro/Pro genotype. It is assumed that GPX1 gene polymorphism contributes to the renal function impairment through the process of atherosclerosis, as GPX1 is the key enzyme in blood vessels protection against oxidative injury (18).

Furthermore, Dutkiewicz et al. evaluated the influence of GPX1 gene polymorphism on the development of delayed graft function, acute rejection and development of CAN. There was no significant association between concrete gene polymorphism and these post-transplant complications (19). Still, another study showed that variability in GPX1 gene may be important for the development of post-transplant DM (PTDM). Authors reported that carriers of the Leu allele were more prone to the development of PTDM (18). This is a very interesting finding considering that tacrolimus has stronger diabetic potential compared to cyclosporine A (20).

In conclusion, there was no difference in GPX1 Pro200Leu distribution between renal transplant recipients and healthy volunteers. This may suggest that investigated polymorphism is not associated with the development of CKD, which led to dialysis and/or transplantation. Regarding our results, GPX1 gene polymorphism may contribute to the progression of renal function decline in late period after renal transplantation. Therefore, genotyping of tested polymorphism in clinical practice may represent a significant predictor of renal function decline and may provide identification of patients at high risk of graft loss.

#### **Acknowledgment**

This study was supported by grant of Ministry of Science and Technological Development of Serbia – project number 41018.

#### **Conflict of interest statement**

The results presented in this paper have not been published previously in whole or part, except in abstract form. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Originalan rad

UDC: 575.224:577.152.1]:616.61-089.843-071

doi:10.5633/amm.2017.0103

## GLUTATION PEROKSIDAZA PRO200LEU GENSKI POLIMORFIZAM KAO POTENCIJALNI PREDIKTOR SMANJENJA BUBREŽNE FUNKCIJE KOD BOLESNIKA SA TRANSPLANTIRANIM BUBREGOM

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Mutacije prisutne u genima koji kodiraju antioksidativne enzime mogu smanjiti njihovu aktivnost i povećati sklonost organizma ka oksidativnom oštećenju. Cilj ovog rada bio je da utvrdi distribuciju glutation peroksidaze (GPX1) Pro200Leu genskog polimorfizma kod bolesnika sa transplantiranim bubregom i kod zdravih dobrovoljaca. Dodatno, ispitana je povezanost između pomenutog genskog polimorfizma sa eritrocitnim antioksidativnim statusom i procenjenom GFR (eGFR) u dvogodišnjem periodu nakon transplantacije bubrega. Metodom lančane reakcije polimeraze analiza dužine restrikcionih fragmenata (PCR-RFLP), izvršena je genotipizacija GPX1 Pro200Leu polimorfizma kod 85 bolesnika sa transplantiranim bubregom i 110 zdravih dobrovoljaca. Od ukupnog broja, 72 bolesnika na takrolimus-baziranoj imunosupresiji je uključeno u ispitivanje parametara oksidativnog stresa. Odredili smo koncentraciju redukovano glutation (GSH), aktivnost GPX i glutation reduktaze (GR) u eritrocitima bolesnika. GFR je procenjena na osnovu MDRD formule za klirens kreatinina. Nije bilo statistički značajne razlike u pogledu distribucije GPX1 genskog polimorfizma između bolesnika i kontrola. Dobijeni rezultati su pokazali da bolesnika sa Leu/Leu genotipom imaju veću aktivnost GR u poređenju sa nosiocima Pro/Pro genotipa, dok nije bilo razlike u drugim parametrima oksidativnog stresa. Nosioци najmanje jednog Leu alela (Pro/Leu + Leu/Leu) imali su značajno smanjenje bubrežne funkcije između prve i druge godine nakon transplantacije. Genotipizacija ispitivanog polimorfizma u kliničkoj praksi može predstavljati značajan prediktor smanjenja bubrežne funkcije i može doprineti identifikaciji bolesnika sa povećanim rizikom od gubitka grafta. *Acta Medica Medianae 2017;56(1):17-23.*

**Ključne reči:** genski polimorfizam, glutation peroksidaza, oksidativni stres, transplantacija bubrega, bubrežna funkcija