UDC 575.
https://doi.org/10.2298/GENSR2201457P
Original scientific article

IMPACT OF THE FETUIN GENE POLYMORPHISMS IN CORONARY ARTERY CALCIFICATION AND MORTALITY OF PATIENTS WITH CHRONIC KIDNEY DISEASE AND RENAL TRANSPLANT

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Pavlović Jovičić S., S. Simić Ogrizović, Z. Bukumirić, M. Erić, N. Pavlović, B. Kotlica, I. Novaković (2022). *Impact of the fetuin gene polymorphisms in coronary artery calcification and mortality of patients with chronic kidney disease and renal transplant*. - Genetika, Vol 54, No.1, 457-472.

Fetuin A is a major systemic inhibitor of vascular calcifications. The aim of this study was to examine association of single nucleotide polymorphisms (SNP) in the gene for fetuin-A with fetuin-A serum levels, coronary arteries calcification (CAC) and mortality in renal transplant (RT) and chronic kidney (CKD) patients. This study included 88 patients (42 stable RT patients at least 6 months after transplantation and 46 CKD patients, stage 2-5 not requiring dialysis) followed five years. Detection and analysis of fetuin A gene polymorphisms in positions *C742T (Thr248Met; rs4917)* and *C766G (Thr256Ser; rs4918)* were performed using PCR method. Respondents with allele *742T* had at the same time *766G*. *Combined genotypes TT/GG* had lower serum fetuin A levels than *CT /CG* and *CC/CC*. Predictors of CAC in univariate analysis were age (p=0,000), serum fetuin-A levels (p=0.011) and *rs 4917* polymorphism (p=0.021) while multivariate determined age (p=0.001) and fetuin-A levels (p=0.031). Patients who were homozygous for variant *742T and 766G (combined genotype TT/GG)* had lowest survival rate. Our results suggest that allele *742T* and *766G* in gene for fetuin-A were associated with lower serum fetuin-A levels, higher CAC occurrence and higher mortality rate in RT and CKD patients.

Keywords: fetuin-A, gene polymorphism, vascular calcifications, chronic kidney disease, renal transplantation

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INTRODUCTION

Vascular calcifications (VC) are very common in chronic kidney disease (CKD) and are associated with high morbidity and mortality caused by cardiovascular (CV) events (RUSSO *et al*., 2004; KUMAR *et al*., 2014; KRAMER *et al*., 2005; KESTENBAUM *et al*., 2009; COVIC *et al*.,2010; SCHIFFRIN *et al*., 2007; ORTIZ *et al*., 2014). They are strong predictors of CV disease and all-cause mortality in hemodialysis (EDDINGTON *et al*., 2009; RAGGI *et al*., 2002; MATSUOKA *et al*., 2004) peritoneal dialysis and renal transplant (RT) patients (SEYAHI *et al*., 2011; LIEFELDT *et al*., 2010). VC progresses in patients undergoing any form of renal replacement therapy (JANSZ *et al*., 2018). Dialysis patients develop atherosclerotic vascular disease earlier than the general population (GOODMAN *et al*., 2000). Even though renal transplantation is the best treatment option for CKD patients, CV disease remains the leading cause of premature death in RT patients, with a 3.5 to 5% annual risk of a fatal or non-fatal event. ROSAS *et al*. (2005) detected VC in 65% of RT patients. Prevalence of VC in RT patients is higher than in CKD stage 3 but lower than in dialysis patients. Pre-existing VC in CKD is an independent predictor of CV and all-cause mortality following transplantation (HERNÁNDEZ *et al*., 2005). Successful kidney transplantation prevents de novo calcification in VC-free patients and slows (but does not halt) the progression of VC in comparison to hemodialysis patients (MEIER-KRIESCHE *et al*.,2004; MOE *et al*., 2005; MAZZAFERRO *et al*., 2009). Since some patients remain free of VC, it can be assumed that such patients are genetically protected or have high levels of calcification inhibitors, or both.

Various mechanisms are involved in the development of VC in CKD patients (MARTOLA *et al*., 2005; KRAŚNIAK *et al*., 2007). Two groups of vascular calcification risk factors need to be considered: the classic risk factors are age, sex, family history, smoking, obesity, hypertension, diabetes and dyslipidemia; uremia-associated risk factors are time on dialysis, uremic toxins, inflammation (advanced-glycation end products, oxidative stress and nitric oxide, asymmetric dimethylarginine, and homocysteine) (HAMIRANI *et al*., 2008), and increased serum levels of phosphate, calcium-phosphate product and parathyroid hormone (PTH). Recent studies showed that expression of bone morphogenic proteins was induced by uremic serum independently of phosphate concentrations. There is evidence that the VC process is an active process counterbalanced by circulating or local inhibitors that include fetuin-A, matrix Gla protein, and inorganic pyrophosphate. After successful renal transplantation uremia associated metabolic factors are partially or completely resolved, but RT patients are still exposed to several pro-calcifying stimuli that favor the progression of pre-existing vascular calcifications. Traditional risk factors, bone mineral disorders, inflammation, immunosuppressive drugs and deficiency of calcification inhibitors may all play a role (PODESTA *et al*., 2021)

There is little data on fetuin-A in kidney transplant recipients. It has recently been demonstrated that fetuin-A levels increase after kidney transplantation along with the improvement of endothelial functions (MAZZAFERRO et al., 2007). Several studies have documented an inverse correlation between serum fetuin-A levels and the survival of dialysis patients (ZHOU, 2019). Stenvinkel et al. demonstrated that CKD patients with elevated inflammatory markers and *AHSG 256Ser (rs 4917 T)* allele had lower serum fetuin-A levels and higher all-cause and CV mortality rates (STENVINKEL *et al*., 2005). Studies of RT patients also

show that low fetuin-A levels are determined by variants in the ASHG gene and independently associated with VC and a higher risk of CV events and mortality (MARÉCHAL *et al*., 2011).

We studied the correlation of single nucleotide polymorphisms in gene for fetuin-A: *C742T (Thr248Met; rs4917)* and *C766G (Thr256Ser; rs4918)* with fetuin-A serum levels and we analyzed predictors of coronary artery calcification (CAC) and mortality in CKD and RT patients.

MATERIAL AND METHODS

Patients

A total of 88 patients were evaluated over a period of 72 months, including 42 stable renal transplant (RT) patients (25 males/17 females) at least 6 months after transplantation and 46 CKD patients (23 males/23 females) in stages 2-5 not requiring dialysis. Patients were selected from the population that was regularly monitored at the Clinic for Nephrology, Clinical Centre of Serbia, University in Belgrade. Diabetics and patients with acute inflammatory diseases and malignances were excluded.

Thirty one of the RT patients had received a kidney from a related living donor and 11 from a deceased donor. Average time from transplantation was 9.56 ± 5.27 years. Any history of hypertension, ischemic vascular disease (myocardial infarction, angina pectoris or cerebral stroke), heart failure and smoking habits was noted at the study entry interview.

The causes of end-stage kidney disease were: chronic glomerulonephritis (n = 26 in RT and n = 14 in CKD patients), chronic pyelonephritis and congenital urinary tract anomalies (n = 5 in RT and n = 4 in CKD patients), nephrosclerosis (n = 3 in RT and n = 14 in CKD patients), polycystic kidney disease (n = 2 in RT and n = 6 in CKD patients), other (n = 3 in RT and n = 8 in CKD patients) and unknown (n = 3 in RT and n = 0 in CKD patients). The immunosuppressive protocol for RT consisted of induction with antithymocite globulin or basiliximab and maintenance with calcineurin inhibitors (tacrolimus or cyclosporine), mycophenolate mofetile or azathioprine and prednisolone. CKD patients originally diagnosed with some form of glomerulonephritis received corticosteroids or other immunosuppressive therapy according to protocols.

The average age of RT patients was 49.18±10.58 years and CKD patients 38.21±15.56 years. The glomerular filtration rate in RT patients was 39.60±15.04 ml/min/1.73m^{2}and in CKD patients 30.86±22,15ml/min/1.73m^2.

Clinical and immunological observations were made laboratory parameters set and the coronary calcium score was determined using computed tomography on the same day.

All patients signed an informed written consent form before blood samples were taken. The study was approved by the Ethics Committee of the Medical School of the University in Belgrade (approval number approval number 29/XI-6).

Laboratory methods

One fasting blood sample was obtained from each patient in order to measure the following parameters: complete blood count, serum concentrations of uric acid, creatinine (s-creatinine), albumin, pre-albumin, transferrin, calcium, phosphate, intact parathyroid hormone (iPTH), cholesterol, triglycerides, high-sensitive CRP (hs-CRP), serum amyloid A (SAA),

fetuin-A, total homocysteine (tHcy) and interleukin-6 (IL-6). Hematological profiles were determined using an LH 750 hematology analyzer (Beckman Coulter Inc., California, USA). Creatinine, uric acid and albumin were analyzed employing routine methods (Olympus System Reagents using an AU 2700 Olympus analyzer, Hamburg, Germany). iPTH was determined by ELISA-PTH**, (**CIS bio international, GIF-sur-Yvette Cedex, France). hs-CRP, SAA, pre-albumin and transferrin were measured using immunonephelometric assays (Dade-Behring, BN II, Marburg, Germany). Serum IL-6 levels were determined with a highly sensitive colorimetric sandwich ELISA kit (Human IL-6 Quantikine HS ELISA kit; R&D Systems, GmbH, Germany). Serum fetuin-A was determined by ELISA (Epitope Diagnostics, Inc., San Diego, California, USA).

Serum tHcy concentration was measured by high-performance liquid chromatography (HPLC) after reduction of the disulfide-bonds with dithiothreitol (normal range: 10-15 umol/l).

Blood samples were taken for genetic analysis and kept at minus 20 Celsius degrees until they were analyzed.

Genetic analysis: DNA was isolated from peripheral blood, sampled with EDTA, using the GeneJET whole blood genomic DNA purification mini-kit (Fermentas, Thermo Fisher Scientific Inc., St. Leon-Rot, Germany). Fetuin-A gene polymorphisms *C742T (Thr248Met; re4917)* and C766G (*Thr256Ser; rs4918)* were detected and analyzed using TaqMan® SNP Genotyping Assays (Applied Biosystems, FosterCity, CA, USA) on 7500Rt-PCR System with incorporated 7500 software (Applied Biosystems, Foster City, Ca, USA).

The glomerular filtration rate was estimated using the abbreviated Modification of Diet in Renal Disease (MDRD) formula study equation (LEVEY *et al*., 2006.).

Arterial hypertension was diagnosed when the systolic blood pressure was ≥140 mmHg and/or diastolic pressure was ≥90 mmHg, or if antihypertensive treatment had been prescribed.

Body mass index (BMI) was calculated according to the formula: weight (kg) /height (m).

The coronary artery calcification (CAC) score was determined using multi-detector row spiral computed tomography (MSCT) (General Electric Medical System, USA) operating with the following parameters: 64 slices with 912 detectors, 0.625 mm distance between slices, 0.35s rotation time and a tube current of 800 mA at 120 kV. Data were obtained during the diastolic phase of the heart cycle. The CAC score was calculated using formulae based on measurements of the total volume and area of calcified lesions, as well as the mean and maximum density. Individual CAC scores were calculated for the left main coronary artery, the descending branch of the left coronary artery, the circumflex branch of the left coronary artery and the right coronary artery. These scores then were added together to obtain the total coronary CAC score. The final score is expressed in modified Agatston units (AGATSTON *et al*., 1990). This calcium score was chosen in line with the latest guidelines from the American College of Cardiology and the American Society of Nuclear Cardiology, endorsed by the American Heart Association (ACC/ASNC) (GREENLAND *et al*., 2007).

The database included demographic (age and gender), clinical (CKD, dialysis and transplant duration, BMI and hypertension), the CAC score and hematological, biochemical and immunological variables.

Statistics

The primary data analysis was performed using descriptive statistical methods, statistical hypothesis testing and methods for analyzing the relationship between the outcome and potential predictors. We used the following descriptive statistical methods: measurements of the central tendency (arithmetic mean), variability (standard deviation) and relative numbers. Hypotheses on the difference between frequencies were tested using Chi-square Test and Fisher's test of exact probability. The T-test was used to test hypotheses on the arithmetic mean differences. The Kruskal–Wallis and Mann-Whitney tests were used to test the hypotheses on the median differences. Logistic regression was used to analyze the binary outcome and potential predictors. Patients survival according to serum fetuin-A and CRP levels were analyzed by the Kaplan-Meier method and the Log Rank test. Statistical hypotheses were analyzed at the level of significance of 0.05. Statistical data analysis was performed using IBM SPSS Statistics 22 (SPSS Inc., Chicago, IL, USA).

RESULTS

Serum fetuin-A levels averaged 0.432 (SD 0.125) g/L and ranged from 0.126 to 0.670 g/L. Prevalence of CAC was 31 %. The cut-off level of fetuin-A for occurrence of calcifications was 0.437 g/L and that was also the median of fetuin-A serum levels. Logistic univariate analysis of patients with fetuin-A serum levels below the cut-off level (< 0.437g/L) and patients with serum fetuin-A levels above that level (Table 1.) showed that there were no difference between the groups according to age, gender, body mass index and renal function, measured by glomerular filtration rate and serum creatinine levels.

Table 1. Demographic data, body mass index (BMI) and kidney function according to fetuin-A serum levels

Variables	fetuin <0.437 g/L	fetuin > 0.437 g/L	P
Age in years, mean (SD)	40.91 (14.52)	38.62 (13.20)	0.442
Gender male N (%)	22 (51.20)	27 (60.00)	0.404
BMI, mean (SD)	24.60 (4.11)	24.356 (2.777)	0.743
S. creatinine umol/l, median (range)	176.00 (80-946)	183.00 (99-500)	0.809
eGFR ml/min/1.73m2, median(range)	34.70 (4.8-110.0)	33.90(11.5-59.9)	0.848

eGFR, estimated glomerular filtration rate, S creatinin, serum creatinin concentration

There was significant inverse correlation of serum transferin, fibrinogen and C reactive protein levels with lower fetuin-A. Interleukin 6, serum amyloid A, free transferrin, total homocysteine serum levels did not differ between groups (Table 2.).

Patients with lower fetuin-A levels (below the cut-off point of 0.437 g/L) showed a significantly higher presence of calcifications (51.2% vs. 24.4%, p=0.029) and they had a higher median calcification score than patients with fetuin-A levels above 0.437g/L (Table 3.).

Frequency of allele *T* in position *742 (rs4917)* was 0.34 as well as allele *G* in position *766 (rs 4918).*

Table 2. Inflammation parameters, cholesterol and homocysteine serum concentration according to fetuin-A serum levels

Variables	fetuin <0.437 g/L	fetuin > 0.437 g/L	P
Transferrin g/L, mean (SD)	1.98 (.53)	2.32(.46)	.007*
Fibrinogen g/L, mean(SD)	5.09 (1.51)	4.438 (.86)	.022*
Cholesterol mmol/L, mean (SD)	5.78 (1.20)	5.909 (1.19)	.607
hsCRP mg/L, median (range)	1.19 (.16-17.10)	.4400 (.15-14.40)	.046*
IL- 6 pg/ml , median (range)	2.80 (1.40-17.50)	3.1000 (.90-13.20)	.200
SAA mg/L, median (range)	6.10 (.80-91.10)	3.9000 (0.90-25.30)	.106
Homocysteine umol/L, mean (SD)	22.88 (10.65)	21.024(7.38)	.454

hs-CRP, high sensitive C-reactive protein, SAA, serum amyloid A, IL-6, interleukin 6

Table 3. Logistic regression analysis of fetuin-A serum levels and coronary artery calcifications

Variables	fetuin <0.437 g/L	fetuin > 0.437 g/L	P
CAC score group, median (range)	2.00 (0-4)	0 (0-4)	0.029*
Calcification presence N (%)	22 (51.2)	11 (24.4)	0.010*
CKD %	41.9	62.2	
RTRs %	58.1	37.8	

CAC, coronary artery calcification, CKD chronic kidney disease, RTRs renal transplant recipients

Polymorphisms *rs4917/rs4918* of fetuin gene is 100% linked. Analysis of combined genotype distribution showed *CC/CC* in 43 (44.3%) patients, *CT/CG* in 41 (42.3%) and *TT/GG* in 13 (13.4%) patients. The fetuin-A serum level was influenced by *rs4917/rs4918* polymorphic genotype, with dose dependent effect according to number of variant alleles (Fig1.). Genotypes *TT/GG* were associated with lower serum fetuin-A levels than *CT /CG* and *CC/CC* (Table 4).

Table 4. Rs 4917 genotype distribution among patients according to fetuin-A serum levels

Variables	fetuin <0.437 g/L	fetuin > 0.437 g/L
CC genotype N (%)	13(30.2)	27 (60)
CT genotype N (%)	22 (51.2)	14(31.1)
TT genotype N (%)	8 (18.6)	4 (8.9)

P=0.019

The logistic univariate model indicated that predictors of CAC were: age [Exp(B) 1.156, 95%CI for Exp (B) 1.093-1.223, p=0,000], serum fetuin-A levels [Exp (B) 3,238, 95%CI for Exp (B) 1,309-8,008, p=0,011] and *rs 4917* gene polymorphism [Exp (B) 2,801, 95%CI for Exp (B), p=0.021].

In the multivariate logistic model the significant predictors of calcifications were age and fetuin-A levels ≤0,437g/L. Patients had a 17% greater risk of presence of CAC for each year of age (OR=1,166). Patients with fetuin-A levels ≤0,437 g/L had a five-fold higher risk of development of CAC (OR=4,771) (Table 5).

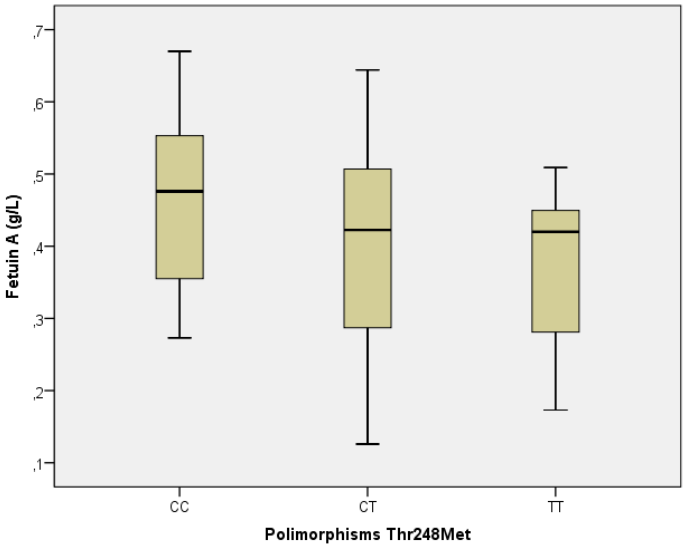

Fig 1. Mean Fetuin A level according to rs4917 genotypes

Table 5. The logistic multivariate model with CAC presence as a dependent variable

Variable	B	P	OR	OR 95% CI
Age	0.15	0.001	1.17	1.09-1.24
Fetuin-A level	1.56	0.03	4.77	1.15-19.74
Polymorphism	0.15	0.82	1.16	0.30-4.47
CRP nominal	-0.59	0.55	0.55	0.71-3.89

Eleven patients died during the observation period. Kaplan –Meier survival analysis revealed that patients with low fetuin-A levels (<0.437g/L) and CRP> 5mg/L had the lowest survival rate of only 42%. Patients with low fetuin-A levels (<0.437g/L) and CRP <5mg/L had a survival rate of 75%, and patients with fetuin-A serum levels ≥0.437g/L had a survival rate of 100% (p<0.001) (Fig. 2).

In addition, patients with *T* allele in *rs4917* and CRP ≥5mg/L had a survival rate of 50 %, patients with mutant allele *T* in r*s4917* gene and CRP< 5mg/L -81% and patients without mutant alleles and CRP> 5mg/L had a survival rate of 96.9%, but for patients with CRP< 5mg/L the survival rate was 100% p=0.001 (Fig. 3).

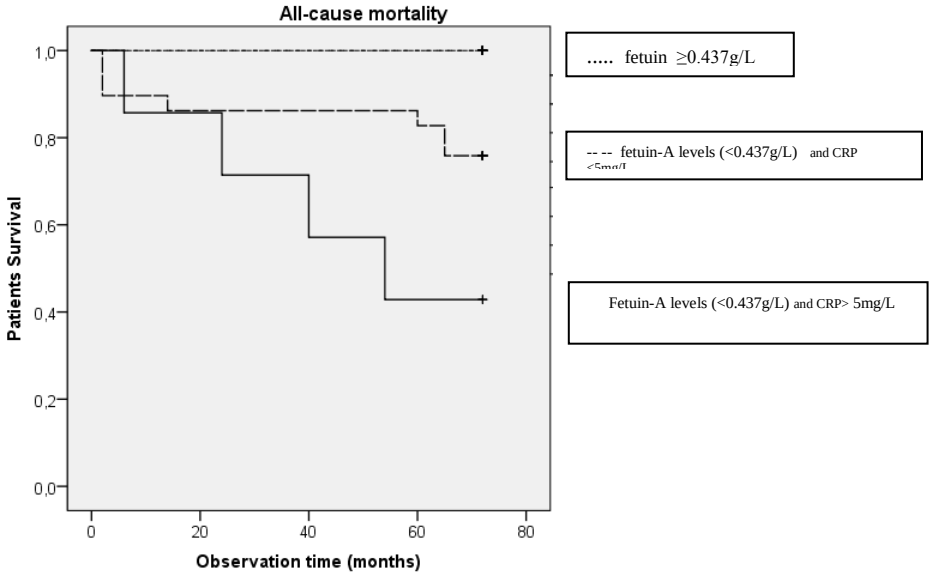

Fig. 2. Kaplan-Meier analysis of the fetuin-A level and inflammation influence on all-cause mortality

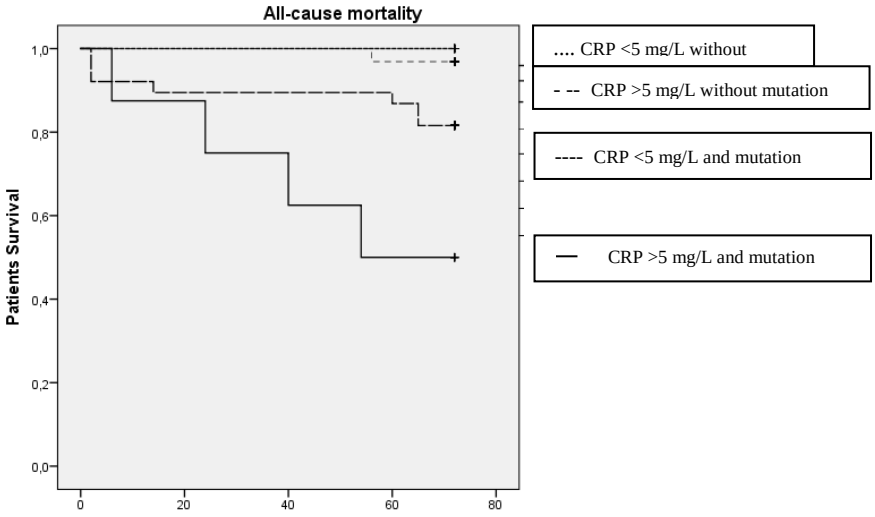

Fig.3.Kaplan-Meier analysis of the ASHG rs 4917 variant and inflammation influence on all- cause mortality

DISCUSSION

Fetuin-A, also known as 2-Heremans-Schmid glycoprotein (AHSG human fetuin), is an important inhibitor of extra skeletal calcification and it accounts for about 50% of all potential inhibitory effects. In vitro, fetuin-A inhibits the de novo formation and precipitation of calcium phosphate. Massive ectopic calcification occurs in fetuin-A knockout mice receiving a diet with a high content of calcium and vitamin D. Ketteler et al. suggest that fetuin-A plays a role in preventing the accelerated extra skeletal calcification observed in CKD patients (KETTELER *et al.*, 2005). PRICE *et al.* (2004). found that vitamin D–induced vascular calcification is associated with a 70% reduction of serum fetuin-A level. Therefore, loss of serum and local fetuin-A could directly promote vascular calcification in uremic patients.

In our previous study we found a higher prevalence of coronary artery calcifications (CAC) in RTR compared to chronic kidney disease (CKD) grade 2-5 patients, despite better parameters of renal function and bone mineral metabolism. RT patients also had longer CKD duration, lower levels of serum fetuin-A and higher levels of inflammation parameters (high sensitivity CRP -hsCRP, interleukin 6 and serum amiloid A- SAA) than CKD patients. The observation period was 30 months and independent predictors of mortality were age, SAA and CAC score (SIMIC-OGRIZOVIC *et al.*, 2012.). The possible modulating effect of polymorphisms in ASGH gene on serum fetiun–A levels in patients with inflammation was analyzed in some studies.

The median fetuin-A serum level was 0.437 g/L, which was similar to a previous study of RT patients (MARÉCHAL *et al.*, 2011), while the mean fetuin-A serum level was 0.58+-0.13g/L. In the general population older than 65 years (HERMANS *et al.*, 2007) average serum fetuin-A levels were 0.64+-0.17g/L, but and in the dialysis population the median fetuin–A was 0,225g/L (STENVINKEL *et al.*, 2005). Hemodialysis patients have lower levels of fetuin-A than age- and sex-matched controls and lower fetuin-A concentrations are associated with increased vascular calcifications and enhanced mortality from cardiovascular disease in patients with end-stage renal disease (KETTELER *et al.*, 2005.). However, little data exists on fetuin-A in kidney transplant recipients. Recently, it was demonstrated that fetuin-A levels increased after kidney transplantation along with the improvement of endothelial functions (CAGLAR *et al.*, 2007). Some other authors have found that despite a correlation of fetuin-A levels with creatinine clearance and estimated GFR, there was no significant increase in fetuin-A levels over the first three months after renal transplantation. Moreover, a significant decrease in serum fetuin-A levels was noted at 2 weeks ($p < 0.001$). Fetuin-A levels subsequently increased ($p < 0.001$), reaching pre-transplant values after three months (URBANOVÁ *et al.*, 2009).

The present study showed that low serum fetuin-A levels in RT and CKD patients were influenced by ASHG gene polymorphisms. Frequency of polymorphic *T* allele in *rs4917* gene and allele *G* in *rs4918* gene was 0.34, which is similar to that observed in the European Caucasian population. Patients who are heterozygotes or homozygotes for mutation in position *rs 4917* were at the same time heterozygotes or homozygotes for mutation in position *rs4918*, so they appeared as linked genes which were reported in two previous studies (STENVINKEL *et al.*, 2005). Upon further analysis, we therefore described data on combined genotypes. Serum fetuin-A levels were inversely correlated with each additional minor allele of genotype (mutant *T* allele in *ASHG rs4917* and allele *G* in position *rs 4918*). Our findings accorded with the findings of

Stenvinkel, who demonstrated the effects of variations in ASHG genes on circulating fetuin-A levels in Swedish dialysis patients. Verdujin confirmed in a study of 549 Dutch dialysis patients that serine allele carriers have lower circulating fetuin-A levels (VERDUIJN *et al*., 2011). Circulating fetuin-A levels were significantly different for each of the three genotype groups, with lower levels for serine allele carriers. In the general population (as described in metaanalysis by Luagsand), mutant *rs 4917* and *rs 4918* were closely associated with lower fetuin-A serum levels (LAUGSAND *et al*., 2015). In contrast to previous reports, COZZOLINO *et al*., (2007) suggests that CKD patients on HD treatment have a similar polymorphism distribution of the AHSG gene compared to the overall population and that the reduction in serum fetuin-A levels in Italian HD patients is not associated with a change in the distribution of *AHSG T256S* polymorphisms.

In our study lower fetuin-A serum levels (< 0,437 g/L) correlated significantly with higher parameters of inflammation **(**fibrinogen, CRP, transferrin), which accords with the findings in other studies of dialysis patients (STENVINKEL *et al*., 2005; KETTELER *et al*., 2005; HERMANS *et al*., 2007.). Fetuin-A is a negative acute phase glycoprotein and levels of serum fetuin-A fall during the cellular immunity phase of inflammation (VERDUIJN *et al*., 2011). The negative correlation between serum fetuin-A levels and concentration of the inflammatory cytokines IL-1, IL-6 and tumor necrosis factor in chronic kidney disease and hemodialysis patients has recently been demonstrated (DERVISOGLU *et al*., 2008.). Inflammation-dependent down-regulation of fetuin-A mRNA expression was also found. Moreover, it has been shown that fetuin-A administration inhibits the synthesis of tumor necrosis factor in a model of acute inflammation (ABEDINI *et al*., 2009). Inflammation is a significant risk factor for cardiovascular events and mortality in both general and ESRD populations (CARRERO *et al*., 2009). Inflammation may facilitate CAC due to its effect on circulating factors such as fetuin-A (DERICI *et al*., 2006.). One part of the relation between low fetuin-A levels and increased mortality appeared to be explained by the potential down-regulation of fetuin-A in inflammatory states (MAZZAFERRO *et al*., 2007.). However, some studies have not found any relationship between CACs and inflammation parameters such as TNF-α, CRP, and IL-6 in PD and HD patients (TURKMEN *et al*., 2011.). The role of serum fetuin-A levels in vascular calcification may thus be far more complex than previously thought (HERRMANN *et al*., 2012.). In addition to inflammation-dependent fetuin-A down regulation, other putative factors such as uremic toxins and genetic predisposition may be involved (FLOEGE *et al*., 2004.).

In our study patients with lower fetuin-A levels (below the cut-off of 0.437 g/L) showed a significantly higher presence of calcifications (51.2% vs. 24.4%) and they had a greater median calcification score than patients with fetuin-A levels above 0.437g/L. Patients with lower fetuin A levels had a five 5 times greater risk of developing CAC. In a multivariate analysis independent predictors of CAC were age and serum fetuin-A level. Our findings showed that lower fetuin-A serum levels are inversely correlated to the presence and severities of CAC (measured by CAC score) are partly in concordance with studies of the general population. In MESA study of the general population each SD (0,10g/L) decrease in the fetuin-A level was associated with a 12% increase in the severity of CAC, although no significant correlation was found with the incidence of CAC (JH *et al*., 2012.). Several studies focusing on CAC progression in RT patients have shown varying correlations between both traditional (blood

pressure, smoking, dyslipidemia, age, body mass index and history of cardiovascular events) and non-traditional risk factors (CAC at baseline, inflammation, hyperparathyroidism and dialysis duration) (ROSAS *et al*., 2005; MAZZAFERRO *et al*., 2007; SEYAHI *et al*., 2012). MCCULLOUGH *et al*. (2009) found a strong correlation between baseline CAC score and CAC progression and confirmed previous studies on non-renal and hemodialysis patients. The patients with CAC at baseline probably came from a worse metabolic and inflammatory situation and had a more severe history of vascular disease during the pre-transplant period. Mazzaferro et al. detected a significant improvement in the biochemical parameters of secondary hyperparathyroidism in transplant patients after an interval of two years, which, as expected, favorably affected the progression of CAC(18). Some authors (MAZZAFERRO *et al*., 2009; MOE *et al*., 2004) have reported a trend towards normalization of serum levels of calcification inhibitors after transplantation: fetuin-A, matrix Gla protein (MGP) and osteoprotegerin (OPG). Since fetuin-A serum levels are responsible for approximately 50% of the calcification inhibitory capacity of normal human plasma, higher values can be expected to protect against calcification. Despite the powerful pro-calcification effects of numerous factors, some patients do not develop CAC. In previous studies on non-renal patients with no CAC at baseline (Min), no CAC progression developed in 62-75% of patients. In a study on RT patients, 65% of 97 patients with no baseline CAC did not convert to a positive score after 2.8 years (SEYAHI *et al*., 2012). MARÉCHAL *et al*. (2011) found a CAC score of 0 in 24.4% and an AoC score of 0 in 18% of patients, while 83.3% (DERICI *et al*., 2006) and 75% (GREENLAND *et al*., 2007) of the respective groups still had a score of 0 after 4 years of follow-up. There are no exact explanations for patients who remain free of VC in the follow-up, but we can assume that such patients may be genetically protected or have high levels of calcification inhibitors or both.

During the six-year observation period 11 patients died, six of them from cardiovascular disease. Kaplan Meyer analysis showed that patients with lower fetuin-A serum levels had a lower chance of survival, especially if they had elevated parameters of inflammation. The observed association between fetuin-A levels and mortality was comparable with the findings of HERMANS *et al***.** **(**2007) based on data from 987 NECOSAD patients, that HR of 0.91 (95% CI 0.84–0.99) per 0.1-g/L increase in fetuin-A levels was found. Recent meta analysis showed that lower fetuin-A levels are associated with an increased risk of all-cause mortality independent of diabetes and inflammation in dialysis patients, and there may be a dose-response relationship between them (ZHOU *et al*., 2019).

In our study patients with mutant allele T (Met) in rs4917 had a lower chance of survival, and that effect was worsened if they had elevated parameters of inflammation. VERDUJIN investigated whether the *Thr256Ser rs 4918* polymorphism in the gene for fetuin is associated with mortality in a large cohort of incident dialysis patients from the Netherlands Cooperative Study on the Adequacy of Dialysis (NECOSAD) and found that the risk of mortality for individuals genetically predisposed to decreased serum fetuin-A levels was up to 10% higher (VERDUIJN *et al*.2011). The mortality risk increased by 4% for each serine allele (HR 1.04, 95% CI 0.90–1.22). Cumulative mortality curves show increased mortality risks for inflamed patients, independent of their genotype. Carriers of a serine allele, both in inflamed and in non-inflamed patients, had no increased mortality risk compared with non-serine carriers.

These findings indicate that other (non-genetic) risk factors, including inflammation and diabetes, may be influencing fetuin-A levels in ESRD patients, potentially explaining the reported correlation between low circulating fetuin-A levels and increased mortality.

Our study produced a different finding: carriers of mutant allele had a worse survival rate and the presence of inflammation additionally increased the existing negative effect on survival. Patients without mutations had a significantly better chance of survival, with minor effect of inflammation status. We confirmed that genetic variants in the gene encoding fetuin-A were associated with mortality. Observed data increases the likelihood that the fetuin-A protein is a causal risk factor for mortality.

CONCLUSION

The present study confirms that single nucleotide polymorphisms in gene for fetuin-A: *C742T (Thr248Met; rs4917)* and *C766G (Thr256Ser; rs4918)* were associated with low serum fetuin-A levels in RT and CKD patients. The best predictors of CAC were age and low serum fetuin-A levels, and patients with mutant alleles in gene for fetuin-A (*rs 4917* and *rs 4918)* had a higher mortality rate.

Received, September 20th, 2021
Accepted January 10th, 2022

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UTICAJ POLIMORFIZMA U GENU ZA FETUIN NA KALCIFIKACIJE KORNARNIH ARTERIJA I MORTALITET BOLESNIKA SA HRONIČNOM BUBREŽNOM SLABOSTI I SA TRANSPLANTIRANIM BUBREGOM

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Izvod

Fetuin A je glavni sistemski inhibitor vaskularnih kalcifikacija. Cilj ove studije bio je da se ispita povezanost polimorfizama pojedinačnih nukleotida (SNP) u genu za fetuin-A sa nivoima fetuina-A u serumu, kalcifikacijama koronarnih arterija (KKA) i mortalitetom kod bolesnika nakon transplantacije bubrega (TB) i bolesnika hroničnom bubrežnom slabosti (HBS). Studija je obuhvatila 88 bolesnika (42 bolesnika sa stabilnom funkcijom alografta i 46 bolesnika sa bolesnika sa HBS, stadijum 2-5 koji ne zahtevaju dijalizu) praćenih pet godina. Detekcija i analiza polimorfizama u genu za fetuin A na pozicijama *C742T (Thr248Met; rs4917)* i *C766G (Thr256Ser; rs4918)* izvršena je metodom PCR u realnom vremenu. Ispitanici sa alelom *742T* imali su istovremeno *766G*. Kombinovani genotipovi *TT/GG* su imali niže nivoe fetuina-A u serumu od *CT/CG* i *CC/CC*. Prediktori KKA u univarijantnoj analizi bili su starost (p=0,000), nivo fetuina-A u serumu (p=0,011) i polimorfizam rs 4917 (p=0,021), a u multivarijantnoj analizi starost (p=0,001) i nivo fetuina-A (p= 0,031). Bolesnici homozigoti za varijante *742T* i *766G (TT/GG)* su imali najnižu stopu preživljavanja. Naši rezultati sugerišu da su alel 742T i 766G u genu za fetuin-A povezani sa nižim nivoima fetuina-A u serumu, većom pojavom KKA i većom stopom mortaliteta kod pacijenata sa TB i HBS.

Primljeno 20.IX.2021.
Odobreno 10. I. 2022.