SUPEROXIDE DISMUTASE 2 VAL16ALA POLYMORPHISM IS ASSOCIATED WITH AMIODARONE-ASSOCIATED LIVER INJURY

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ABSTRACT

Association of SOD2 V16A single-nucleotide polymorphism (rs4880) with drug hepatotoxicity were reported but relationships with amiodarone prescriptions remained unexplored. Research was an exploratory, controlled prospective clinical trial. Patients hospitalized and treated in Clinical Center in Kragujevac, Serbia (in year 2017) were divided into experimental (using amiodarone, having liver injury, n=29, 19 males, the mean age 66.8±10.4 years), control A (neither amiodarone use nor hepatotoxicity, n=29, 19, 66.1±10.3) and control B group (using amiodarone, not having hepatotoxicity, n=29, 19, 66.8±9.8). From blood samples, among other routine biochemistry, genotyping for SOD2 polymorphism Val16Ala was conducted using real-time PCR method with TaqMan® Genotyping Master Mix and TaqMan® DME Genotyping Assay for rs4880. Patients taking amiodarone and having liver injury were mostly carriers of Val/Val (TT) genotype (13 of 24 patients, 54.2%) while Val/Ala (TC) and Ala/Ala (CC) genotypes prevailed in control group A (19 of 40, 47.5%) and control group B (9 of 23, 39.1%), respectively (2=10.409, p=0.034). Frequency of Val (T) and Ala (C) alleles were 0.51 and 0.49, respectively in the whole study sample (Hardy Weinberg equilibrium, 2=0.56, p=0.454). Carriers of TT genotype had significantly higher ALT (437.0±1158.0 vs 81.9131.5 U/L), total bilirubin (28.320.5 vs 15.313.0 mol/L) and total bile acid concentrations (10.910.2 vs 6.45.3 mol/L) compared to carriers of TC genotype (U=2.331, p=0.020, U=3.204, p=0.001 and U=2.172, p=0.030, respectively). Higher incidence of 47T allele of SOD2 was inpatients with amiodarone-associated liver injury as compared to patients on amiodarone not experiencing hepatotoxic effects.

Keywords: *Superoxide dismutase; polymorphism, single nucleotide; amiodarone, inpatients, chemical and drug induced liver injury.*

INTRODUCTION

Nowadays, amiodarone represents one of the most important antiarrhythmic drugs. Yet, its prescriptions are rather restricted due to numerous adverse reactions (1): it accumulates in tissues and cells, damaging phospholipid membranes, lysozymes and mitochondria (2-4). It is well known that amiodarone inhibits phospholipases (enzymes targeting the membrane phospholipids), but the effect on other signaling molecules is less understood. It has been reported that amiodarone could also increase the synthesis of hydrogen peroxide, leading to a state of oxidative stress (5).

Several studies have demonstrated association between the toxic effects of amiodarone and the activity of superoxide dismutase (SOD), an important enzyme for defense against oxidative stress, such as the induction of pulmonary fibrosis (6) or the direct cytotoxicity (7).On the other hand, amiodarone had variable influence on SOD activity within the patients' erythrocytes, depending on the existence and the type of adverse reactions (8). Differences in the observed effects of amiodarone could be based on different tissue and/or cellular distribution of individual forms of this enzyme. Superoxide dismutases (SODs) are involved in the reaction which converts superoxide into oxygen and hydrogen peroxide, and they also control the concentrations of several other reactive molecules (9). There are tree known isozymes of SOD in mammals: SOD1 (cytoplasmic form), SOD2 (mitochondrial form) and SOD3 (extracellular form) (10).

The role of genetic polymorphism of variety of proteins associated with drug-induced liver injury (DILI) has recently been emphasized (11) and several studies reported significant association of *SOD2* V16A single-nucleotide polymorphism (rs4880) with hepatotoxic effects of drugs (12,13). However, in the studies evidencing causal relation between SOD2 genetic polymorphism and DILI, patients did not use amiodarone (12-14). As the knowledge about importance of the genetic polymorphism of SOD enzymes on the organtoxicity of amiodarone, including liver damage, is lacking, the aim of our study was to investigate the association between amiodarone use, acute hepatotoxic events and *SOD2* polymorphism rs4880 in patients hospitalized due to cardiovascular disease.

PATIENTS AND METHODS

The research was designed as an exploratory, controlled, three-arm prospective study, with cross-sectional approach, which included 87 patients hospitalized and treated in Clinical Center "Kragujevac" in Kragujevac, Serbia, during the year of 2017. Experimental group consisted of patients who were treated with amiodarone and experienced the symptoms and/or signs of liver injury. There were two groups: the first (A) included patients not treated with amiodarone and not having hepatotoxicity, while the second (B) consisted of patients treated with amiodarone and not experiencing any symptoms or signs of hepatotoxicity during the entire follow-up.

The inclusion criteria for the experimental group were: age between 17 and 75 years, amiodarone use and the presence of 3-fold elevated liver enzymes and/or 2-fold elevated bilirubin level (in comparison with the levels on admission day) and/or signs and symptoms of manifested liver injury (abdominal pain in upper right abdominal quadrant, nausea, vomiting, jaundice, hepatomegaly, disturbances in hemostasis) during the hospital stay. Patients in control groups (1:1 design) were gender- and age-matched (within 5-year interval) with the patients in experimental group. The exclusion criteria were: previous and/or existing liver illness (including hepatitis of various origins, primary biliary cirrhosis, gallstones, cholangitis, fatty liver), alcohol abuse, thrombocytopenia, hemochromatosis, Wilson's disease, porphyria, as well as any other disease disabling patient's participation in the study.

The study data were collected prospectively (retrieved from patient's hospital records and/or acquired during physical examinations) and included hematological parameters and serum biochemistry parameters that were routinely measured during the hospital stay. Two additional blood samples were also provided from each patient: one for detecting the level of ornithine carbamoyltransferase (OTC) and total bile acids (TBA) (hepatic injury biomarkers not used in routine practice, but exploited for research purposes), and the other for detecting the presence of *SOD2* polymorphism Val16Ala (47T>C, rs4880). The first blood sample (10 mL) was centrifuged and serum was stored at -20°C until OTC and TBA measurement, which was performed using the enzyme-linked immunosorbent assay (ELISA) and spectrophotometric analysis on standard automated biochemical analyzer, according to the previous studies (15,16).

From the second additional blood sample DNA was extracted, and the genotyping for *SOD2* polymorphism Val16Ala was conducted using previously described methods (12,17). In short, genomic DNA was prepared using PureLink Genomic DNA kit (Invitrogen™, Thermo Fisher Scientific). *SOD2* genotyping was performed using real-time PCR method (SacaceSa96 PCR System, Sacace, Italy), with TaqMan® Genotyping Master Mix and TaqMan® DME Genotyping Assay for rs4880, C___8709053_10 (Applied Biosystems, Waltham, MA). The reaction included initial DNA denaturation for 10 minutes at 95°C, followed by 40 cycles of 15 seconds long denaturation at 95°Cand 1 minute long annealing at 65°C and was completed by a final extension step at 4 °C for 10 min.

The additional patients' clinical variables were: the total score on CIOMS/RUCAM scale (Council for International Organizations of Medical Sciences/ Roussel Uclaf Causality Assessment Method), a survey for evaluation of xenobiotic-induced liver damage (18); the drug exposure expressed as the number of defining daily doses (DDD) per 100 patient's days (PD) of hospitalization according to the international ATC (Anatomical Therapeutic Chemical) classification system (http://www.whocc.no/atc_ddd_index); and the total

score of Charlson Comorbidity Index (CCI) used for assessment of comorbidities (19).

The sample size calculation was based on data on *SOD2* rs4880 frequencies from 11 previously published studies (www.pharmgkb.org) and using appropriate software (20). Based on the imputed differences between frequencies of 13% and 47%, allocation ratio 1:1:1, study power 0.8, probability of alpha error 0.05 and two-sided analysis at least 27 subjects were required for each of three study groups, increasing the total study sample to 87 participants. The study data analysis included descriptive methods and hypothesis testing, with appropriate statistics according to the type and data distributions (Student's t-test or Man-Whitney U test, Chi-squared test, Fisher's exact test, Kruskal-Wallis test, one-way ANOVA - analysis of variance). To test for Hardy Weinberg equilibrium as well as to compare differences in *SOD2* allele frequencies and genotype distribution between patients with and without hepatotoxicity, chi-square test was used. The level of probability significance for differences was 5% (0.05) or less.

RESULTS

The patients' demographic and clinical characteristics between the study groups are presented in form of Table 1.

The patients having amiodarone-associated liver injury (experimental group) had been exposed to greater disease burden (both cardiovascular and non-cardiovascular) then the subjects in two other control groups, as measured by CCI. The median length of hospital stay was 2 days (range 2-7) for patients in all study groups (^{2}=0.0, p=1.0; Kruskal-Wallis test). The mean CIOMS RUCAM score in patients of experimental group was 8.48 points (standard deviation 1.20 points, minimal and maximal value 6 and 10 points, respectively) and 13 subjects had obvious symptoms and signs of hepatic disease on physical examination. Overall mortality rate was 3.4% and all 3 fatal outcomes were in patients of experimental group.

On the other hand, common hematological and serum biochemistry parameters, except those indicating hepatic damage, were fairly comparable between study groups. Statistically significant differences were found for C-reactive protein, urea and creatinine concentrations, but their magnitudes were mild-to-moderate and probably had no profound clinical importance (Table 2). The significant differences among the groups in terms of hepatocellular and cholestatic liver injury parameters (OTC and TBA, respectively) additionally confirmed the presence ofhepatic disease in the patients of experimental group comparing to the control ones.

Table 1. Demographic and clinical characteristics of study patients

Variable	Experimental group n=29; n (%)	Control group A n=29; n (%)	Control group B n=29; n (%)	Statistics*
Gender (male)	19	19	19	^{2}=0.0, p=1.0
Age (years)	66.8±10.4	66.1±10.3	66.8±9.8	F=0.040, p=0.961
Obesity	2 (6.9)	2 (6.9)	1 (3.4)	p=1.0
Fatty liver	14 (48.3)	0 (0)	2 (6.9)	^{2}=26.345, p<0.001
Hepatomegaly	18 (62.1)	1 (3.4)	4 (13.8)	^{2}=29.197, p<0.001
Heart failure	19 (65.5)	8 (27.6)	12 (41.4)	^{2}=8.644, p=0.013
Hypertension	13 (44.8)	17 (58.6)	15 (51.7)	^{2}=6.302, p=0.576
Coronary heart disease	25 (86.2)	5 (17.9)	20 (69.0)	^{2}=1.105, p<0.001
Arrhythmia	15 (51.7)	22 (75.9)	13 (44.8)	^{2}=29.452, p=0.043
Diabetes mellitus	6 (20.7)	8 (27.6)	7 (24.1)	^{2}=0.3766, p=0.828
Alcohol intake**	6 (20.7)	1 (3.4)	1 (3.4)	p=0.045
Smoking habit	5 (17.2)	4 (13.8)	1 (3.4)	p=0.326
CCI score (points)	6.8±0.6	3.6±1.3	3.9±1.1	^{2}=59.840, p<0.001

number represent the mean ± standard deviation (continuous variables), and number (percent) of patients (frequencies), as appropriate; p-probability for difference between the study groups; CCI- Charlson Comorbidity Index; *Chi-squared test, Fisher's exact test or Kruskal-Wallis test, depending on the data type and distribution; **-occasionally, not satisfying exclusion criteria (regular alcohol use was exclusion criterion, see methods)

Table 2. Laboratory parameters in patients of study groups

Variable	Experimental group n=29; n (%)	Control group A n=29; n (%)	Control group B n=29; n (%)	Statistics
ALT (U/L)	607.8±1047.6	29.3±17.8	23.5±11.6	^{2}=58.007, p<0.001
AST (U/L)	359.7±627.5	36.9±37.5	21.6±6.6	^{2}=34.677, p<0.001
GGT (U/L)	60.0±52.7	51.2±41.7	31.2±29.9	^{2}=6.826, p=0.033
Bilirubin total (mol/L)	35.1±19.4	12.9±6.5	11.7±4.5	F=34.375, p<0.001
ALP (U/L)	75.9±33.4	89.0±67.1	52.2±19.0	^{2}=9.345, p=0.009
LDH (U/L)	470.6±256.8	320.0±138.8	472.9±156.0	^{2}=9.291, p=0.010
CPK (U/L)	574.5±687.5	222.5±313.9	92.2±67.8	^{2}=7.863, p=0.020
OTC (ng/mL)	272.2±32.7	n.d.	241.4±28.8	t=3.800, p<0.001
TBA (mol/L)	11.9±10.2	6.5±3.7	5.7±4.2	^{2}=9.274, p=0.010
Amylase (U/L)	97.0±93.4	74.2±60.2	95.0±51.0	^{2}=2.843, p=0.241
Troponin (ng/mL)	1.2±1.8	1.5±3.7	0.1±0.1	^{2}=4.687, p=0.096
NT-proBNP (pg/mL)	8332.3±6488.2	1361.9±2323.3	2685.7±2142.7	^{2}=11.313, p=0.003
Proteins (g/L)	62.3±7.8	62.8±6.5	63.3±5.2	F=0.101, p=0.904
Albumin (g/L)	35.8±4.6	38.8±4.8	39.3±4.8	^{2}=9.337, p=0.009
Fibrinogen (g/L)	3.2±1.6	3.4±1.5	3.8±1.3	^{2}=4.032, p=0.122
C-reactive protein (mg/L)	36.9±42.9	17.7±20.2	16.8±27.8	^{2}=7.577, p=0.023
INR	1.7±0.8	1.3±0.3	2.0±1.6	^{2}=3.511, p=0.060
Glucose (mmol/L)	6.1±1.9	5.9±2.4	6.5±2.2	F=0.455, p=0.636
Cholesterol (mmol/L)	4.1±1.5	5.0±1.4	5.0±1.4	F=4.786, p=0.011
Triglycerides (mmol/L)	1.5±0.9	1.8±0.6	2.1±1.7	^{2}=0.936, p=0.626
Urea (mmol/L)	11.9±5.6	11.8±17.9	7.1±3.0	^{2}=13.595, p=0.001
Creatinine (mmol/L)	117.7±43.7	105.9±38.0	101.9±41.6	^{2}=2.679, p=0.009
Leukocytes (10^9/L)	11.2±5.3	9.7±2.9	8.8±3.0	F=2.150, p=0.124
Platelets (10^9/L)	215±86	228±80	202±46	F=0.735, p=0.483

numbers represent the mean ± standard deviation; p-probability for difference between study groups; ALT - alanine aminotransferase; AST - aspartate transaminase; GGT - gamma-glutamyl transferase; ALP - alkaline phosphatase; LDH - lactate dehydrogenase; CPK - creatine phosphokinase; OTC - ornithine carbamoyltransferase; TBA - total bile acids; NT-proBNP – N-terminal pro B-type natriuretic peptide; INR - international normalized ratio of prothrombin time; n.d. - not done; *Kruskal-Wallis test, one-way ANOVA (analysis of variance) or Student's t-test, depending on the data type and distribution

According to DDD analysis, no significant difference was observed in amiodarone use between experimental and control group B (195.5130.8 vs. 212.9171.8 DDD per 100 PD, U=415.0, p=0.932, Man-Whitney U test). Four patients in total used oral formulation of amiodarone, with no significant difference between the two groups in terms of amiodarone formulation (oral vs. parenteral; ^{2}=0.0, p=1.0).

Significant differences in the frequency of drug prescription (other than amiodarone) between the three study groups have been found for proton pump inhibitors (used by 21 patients in experimental group, 21 patients in control group A and 11 patients in control group B, ^{2}=9.656, p=0.008) and high-ceiling diuretics (22 vs. 11 vs. 13, ^{2}=9.503, p=0.009). The prescription of other drugs did not differ significantly between the study groups (p>0.05), including: acetylsalicylic acid, selective beta blockers, angiotensin converting enzyme (ACE) inhibitors, atorvastatin, enoxaparin sodium, organic nitrates, clopidogrel, xanthines, trimetazidine, H_2 receptor antagonists, spironolactone, benzodiazepines, metforminand dihydropyridines, which were prescribed to 55, 50, 49, 43, 40, 37, 36, 23, 22, 17, 15, 14, 10 and 9 patients in total, respectively.

Study patients had also taken some other drugs with possible hepatotoxicity but their uses was sporadic, precluding analysis of individual drug influence. Taking into account these drugs as a class, there were significant differences in their prescription between study groups (^{2}=10.963; p=0.004).

The distribution of *SOD2* genotypes was statistically different between study groups (Table 3). The patients taking amiodarone and having liver injury were mostly carriers of Val/Val (TT) genotype, while Val/Ala (TC) genotype prevailed in control group of patients without hepatic disease who did not use amiodarone, and Ala/Ala (CC) genotype was most frequent in patients using amiodarone experiencing no hepatoxicity. The frequency of Val (T) and Ala (C) alleles were 0.51 and 0.49, respectively in the whole study sample, with no deviation from Hardy Weinberg equilibrium (^{2}=0.56, p=0.454).

In addition, we found statistically significant differences in serum ALT and bilirubin concentrations among different SOD2 genotype subgroups (Table 4).

Table 3. *SOD2* genotype distribution in patients according to the study groups

SOD2 genotype Val16Ala (47T>C)	Experimental group n=29; n (%)	Control group A n=29; n (%)	Control group B n=29; n (%)	Statistics*
Val/Val (TT)	13 (44.8)	4 (13.8)	7 (24.1)	^{2}=10.409, p=0.034
Val/Ala (TC)	8 (27.6)	19 (65.5)	13 (44.8)	
Ala/Ala (CC)	8 (27.6)	6 (20.7)	9 (31.0)	
Total	29 (100)	29 (100)	29 (100)	

*Chi-square test

Table 4. The hepatic parameter values of patients according to *SOD2* genotypes

Variable	Genotype TT (n=24)	Genotype TC (n=40)	Genotype CC (n=23)	Statistics*
ALT (U/L)	437.01158.0	81.9131.5	234.4419.1	^{2}=6.698, p=0.035
AST (U/L)	224.2653.8	81.0180.2	152.5283.7	^{2}=2.592, 0.274
Bilirubin total (mol/L)	28.320.5	15.313.0	19.312.8	^{2}=10.635, p=0.005
OCT** (ng/mL)	250.535.1	257.834.6	263.033.6	F=0.623, p=0.540
TBA (mol/L)	10.910.2	6.45.3	7.75.4	^{2}=4.658, p=0.097

*Kruskal-Wallis test or one-way ANOVA (analysis of variance), depending on the data distribution;
**not measured in control group A (without both amiodarone and liver injury), data from 20, 21 and 17 patients in respective genotype subgroups

The carriers of TT genotype had significantly higher ALT, total bilirubin and total bile acid concentrations compared to carriers of TC genotype (U=2.331, p=0.020, U=3.204, p=0.001 and U=2.172, p=0.030, respectively; Man-Whitney U test), but not compared to carriers of CC genotype (p>0.05; Man-Whitney U test) (Figures 1 and 2).

Figure 1. The ALT values (U/L) in patients with different rs4880 SOD2 polymorphism (CC, TC and TT subgroups with 23, 40 and 24 patients, respectively); for the sake of clarity outliers are removed; asterix - p=0.020 comparing to carriers of TC genotype

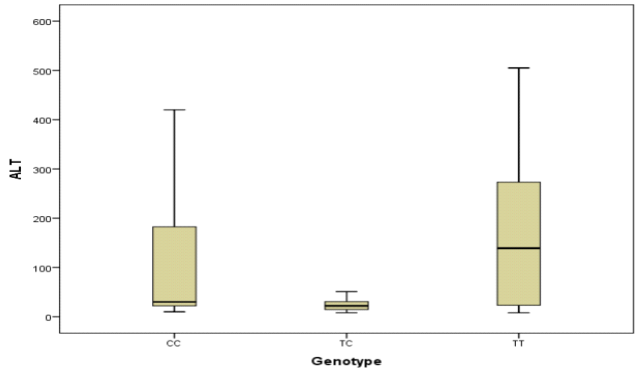
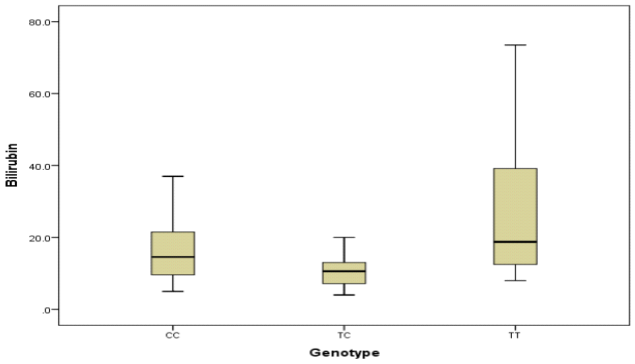


Figure 2. The total bilirubin values (mol/L) in patients with different rs4880 SOD2 polymorphism (CC, TC and TT subgroups with 23, 40 and 24 patients, respectively); for the sake of clarity outliers are removed; asterix - p=0.001 comparing to carriers of TC genotype



There were no significant differences of the ALT, AST, total bilirubin, OCT and TBA concentrations between the two control groups (p>0.05).

DISCUSSION

Our study reveals significant association between different SOD2 Val16Ala genotypes and amiodarone-associated hepatotoxicity in hospitalized patients. Carriers of Val/Val (TT) genotype who used amiodarone experienced acute liver injury more frequently than the carriers of Val/Ala (TC) or Ala/Ala (CC) genotypes. To the best of our knowledge, this is the first study investigating and reporting relation among

amiodarone use, rs4880 SOD2 polymorphism and hepatic damage in the clinical setting.

Within the family of superoxide dismutase enzymes, which is known to modulate oxidative stress response, manganese-dependent isoform (MnSOD or SOD2) is particularly important due to its presence in mitochondria (21). Decades of research conducted so far have evidenced the role of SOD2 in different metabolic, cardiovascular and neoplastic diseases, which is affected by SOD2 genetic polymorphism and epigenetic control mechanisms, as well as by life-style habits such as diet and exercise (22). The published data support our finding related to the importance of Val16Ala polymorphism in amiodarone-associated hepatotoxic symptoms and signs. In addition, there are evidence of rs4880 SOD2 polymorphism contribution to liver injury associated with anti-tuberculosis drugs (12), modification of methotrexate cytotoxic effects (23) or attenuation of rosuvastatine cholesterol-lowering action (24).

It has been observed that SOD2 polymorphism rs4880 could influence the protein trafficking across mitochondrial membranes and, consequently, the oxidative stress response based on this enzyme activity. Namely, during experimental conditions SOD2 protein that contains alanine (Ala) was freely transported into the mitochondria matrix, while the valine (Val) containing form is confined within the inner mitochondrial membrane (25).The cellular consequences of these events are much higher concentrations of mature protein and SOD2 activity in the presence of Ala than Val (26). In addition, the Ala variant of rs4880 SOD2 gene polymorphism, together with the diet rich in antioxidant substances, decrease DNA damage in healthy, young people despite high inter-individual variations in basal and oxidant-triggered responses (27). Other researchers also reported importance of essential nutrients for antioxidative defense system and body function in both experimental and clinical conditions (28, 29). Finally, the augmentation of inflammatory signaling pathways could contribute to the effects of adverse, prooxidative state in the presence of SOD2 polymorphism rs4880. It has been recently reported that the human peripheral blood mononuclear cells from the carriers of Val/Val SOD2 genotype produced more proinflammatory cytokines (e.g. IL-1, IL-6,TNF-α, IFN-γ) than their Ala/Ala counterparts (30).

The majority of studies so far suggested that Val at position 16 of SOD2 reduced activity of the enzyme and increased oxidative stress, but there were opposite findings as well - higher enzyme activity in the carriers of the variant in comparisons with homozygous Ala/Ala (CC) carriers (31). In addition, the effects of the polymorphism on drug actions were not absolutely consistent. For example, patients with SOD2 rs4880 T allele taking dopaminergic anti-parkinson drugs had less nausea and vomiting, but the motor adverse reactions remained unchanged (32). Further, clozapine response in patients with schizophrenia had not been affected with Val16Ala SOD2 polymorphism (33). In our study, patients experiencing amiodarone-associated liver damage (the group where Val16Val (TT) SOD2 genotype was the most frequent) had, in general, more disease burden and somewhat more frequent use of other drugs known to have hepatic adverse effects. In addition, there were no gradual increase in serum concentration of hepatic damage biomarkers (ALT, AST, bilirubin total) from the CC, across the TC to the TT polymorphism carriers, and the levels of OTC and TBA did not differ among the groups with different rs4880 SOD2 genotypes. Therefore, the contribution of other factors to hepatic injury in our patients cannot be excluded (34).

Our study reports Val16Ala SOD2 gene polymorphism distribution in Serbian population and our results correspond well to the distributions across the subjects participating in two clinical trials, which have been recently conducted by two different research groups in Belgrade (35,36). Another study that included patients with bronchial asthma from the area of Nis City reported larger differences between allele frequencies (37). Such divergence seems rather a random event than the consequence of true genetic diversity of this single nucleotide polymorphism in Serbian population, as it does not conform to results of the abovementioned studies, nor to the data available from The International Genome Sample Resource 1000 Genomes Project with reference to rs4880 SOD2 gene allele distributions for other Europeans(https://www.pharmgkb.org/variant/PA166156900).

The relatively small sample size represents the major limitation of our study, as it precludes multivariable analysis of all factors contributing to acute liver disease, such as heart failure, occasional alcohol intake, burden of comorbidities and additional hepatotoxic drugs. Supplementary control sample, including patients with hepatic injury not taking amiodarone, could possibly increase final performance of statistical modeling. Detection of other important polymorphisms of genes coding for cytohromes, transferases, transporters, cytokines and other gene products associated with drug-induced hepatotoxicity would provide data for more comprehensive pharmacogenomic approach.

In conclusion, our study reports higher incidence of 47T allele of SOD2 in patients with amiodarone-associated liver injury as compared to patients on amiodarone not experiencing hepatotoxic effects. In order to evaluate SOD2rs4880 polymorphism as a potential and clinically meaningful predictive marker for DILI in patients on amiodarone treatment, additional studies involving larger number of patients and investigating additional genetic markers should be conducted.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national) and the Helsinki Declaration of 1975, as revised in 2013. The study was approvedby the Ethic Committee of Clinical Center Kragujevac, the number of Ethical Approval 01/3518. Voluntary written and informed consent was obtained from each participant prior to enrollment in the study.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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None.

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