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Association of Biochemical, Cytokine and Echocardiographic Markers of Cardiovascular Injuries with T894G Polymorphism of *Endothelial Nitric Oxide Synthase* Gene in Patients with Nonviral Liver Cirrhosis

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Abstract

Background & Aims: Alterations of vascular endothelium play an important role in the development of cardiovascular diseases, the occurrence of liver cirrhosis and the progression of its complications. Little is known about the impact of the *endothelial nitric oxide synthase* (*eNOS*) gene mutations on the development of cardiovascular disease in patients with liver cirrhosis. The aim of the study was to investigate the possible association of the T894G gene polymorphism of *eNOS* with biochemical, cytokine blood indices, structural and functional parameters of heart in patients with nonviral liver cirrhosis.

Methods: Investigation of *eNOS* gene polymorphism (T894G) was performed in 50 patients with nonviral liver cirrhosis and in 10 healthy volunteers. Furthermore, biochemical blood analysis, estimations of tumor necrosis factor-α, transforming growth factor-β1, interleukin-4, atrial natriuretic propeptide (proANP) plasma levels, echocardiographic studies were performed.

Results: It was established that the presence of T-allele of *eNOS* gene in patients with liver cirrhosis was associated with increased activity of aspartate aminotransferase, higher plasma content of proANP, larger left atrium diameter and increased left ventricular myocardium mass. In males with liver cirrhosis, T-allele was also associated with increased left ventricular myocardium mass index compared with patients with GG-genotype.

Conclusion: The presence of T-allele of *eNOS* gene in patients with nonviral liver cirrhosis is associated with occurrence of more severe cardiovascular alterations.

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Key words

liver cirrhosis; *endothelial nitric oxide synthase* gene; TNF-α; TGF-β1; IL-4; proANP

Introduction

Alterations of vascular endothelium play an important role in the development of cardiovascular diseases,[1,2] the occurrence of liver cirrhosis and the progression of its complications, such as portal hypertension.[3] Endothelial nitric oxide synthase (eNOS) producing nitric oxide (NO) plays a key role in the regulation of vascular tone, detoxification of superoxide anion radicals, inhibition of platelet aggregation, leukocyte adhesion and proliferation of smooth muscle fibers.[4]

The relationship between T894G, 4b/, T786C polymorphism of *eNOS* gene and the risk of ischemic heart disease have been actively investigated.[5] It has been postulated that the E298D polymorphism of the *eNOS* gene has an impact on the development of heart stroke in the Japanese, British, Germans and Americans. In other nationalities (Koreans, Austrians, French, Irish, Dutch and Chileans), this relationship is not fixed; Greek studies have shown controversial results.[6-9] It is also found that the E298D polymorphism of this gene is an independent risk factor for left ventricular hypertrophy in hypertensive patients.[10] In the Korean population, a significantly higher frequency of TT genotype of *eNOS* gene in patients with coronary atherosclerosis was revealed compared with healthy controls.[11] The relationship of the T894G polymorphism variants of the *eNOS* gene with hyperhomocysteinemia has been established, being one of the risk factors for ischemic heart disease. In particular, among the citizens of Tunisia, ischemic heart disease patients, TT genotype of the *eNOS* gene and hyperhomocysteinemia led to a more

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Table 1. Primer sequences, restriction enzyme and allele calling for *eNOS* SNPs

SNP	Restriction enzyme	Primers	Primer sequences (5'-3')	Allele calling (size of fragments, bp)
894T>G (rs1799983)	BanII	Forward	5–ATGAAGGCAGGAGACAGT GGATGG –3'	T: 250 bp; G: 160, 90 bp
		Reverse	5'– CCAGTCAATCCCTTTGGT GCTCA– 3'	

eNOS, endothelial oxide synthase.

progressive course of the disease.[12] In spite of fundamental investigation on how the *eNOS* gene polymorphism influences the development of various cardiovascular diseases, the association of gene polymorphism and liver pathology are not as yet fully elucidated.

Analyzing the literature on the test questions, the role of the *eNOS* gene in the development and progression of liver disease[13,14] was found. Increased intrahepatic vascular resistance in patients with liver cirrhosis is accompanied by decreased activity of *eNOS* and is enhanced by the accession of inflammation.[15] In patients with liver cirrhosis, complicated with portal hypertension, a decrease in the activity of *eNOS* seems to cause decreased blood flow in the intrahepatic vessels.[13,15]

In this respect, little is known about the impact of genetic mutations, including *eNOS* gene polymorphism in the development of cardiovascular disease in patients with liver cirrhosis. Detailed and in-depth study of these issues is a precondition for optimizing therapeutic treatment in referred patients.

Considering the aforementioned data, the aim of the study was to investigate the possible association of T894G gene polymorphism of *eNOS* with the biochemical and cytokine blood parameters, structural and functional parameters of the heart in patients with nonviral liver cirrhosis.

Patients and methods

Investigation of *eNOS* gene polymorphism (T894G) was performed in 50 patients with nonviral liver cirrhosis and in 10 healthy volunteers. The predominant etiologic factor in the development of liver cirrhosis in 41 patients (82.0%) was alcohol abuse. In 9 patients (18.0%), the main etiologic cause of the liver cirrhosis was a long-term professional contact with industrial hepatotoxic agents, including gasoline, pesticides, varnish, etc.

Blood samples were obtained in the morning, before meal from antecubital vein in the first day of hospitalization until the appointment of treatment. As an anticoagulant, 5% solution of disodium salt ethylenediaminetetraacetate was used. The study protocol was in accordance with the revised Helsinki Declaration (2008) and was approved by the local ethics committee. Written informed consent was obtained by all participants.

The inclusion criterion for patients in the study was diagnosis of liver cirrhosis of toxic and alcoholic etiology.

Exclusion criteria comprised: hepatitis B- and C-associated liver cirrhosis; autoimmune liver cirrhosis; liver fibrosis as a result of cardio-vascular insufficiency; liver cirrhosis of C class severity due to Child-Pugh criteria; hemochromatosis; severe cardio-vascular diseases (heart stroke, atrial fibrillation, rheumatic or congenital heart disease, heart failure III-IV functional class (NYHA)); connective tissue diseases (rheumatoid arthritis, systemic lupus erythematosus, systemic sclerosis) and patients who refused to participate in the study protocol. Hepatitis B and C were excluded by polymerase chain reaction (PCR). Hemochromatosis was excluded by investigation of serum ferritin level and iron content in blood.

Diagnosis of liver cirrhosis was made by a combination of both clinical (patient history, signs of portal hypertension) and laboratory investigations (abnormal liver function tests, ultrasound findings of cirrhosis or endoscopic findings of varices).[16,17]

The degree of severity of liver cirrhosis was assessed by the Child-Pugh criteria.[18,19] Based on these criteria, 21 patients had class A and 29 patients class B severity of liver cirrhosis.

To study the alleles of polymorphic sites T894G in *eNOS* gene, genomic DNA was identified from peripheral blood leukocytes with subsequent amplification of polymorphic sites using PCR with the help of programmable amplifier "Amply-4L" ("Biokom", Russia), with individual temperature program primers for the studied gene. The calculated positions of primers on the chromosome and their oligonucleotide sequence are given in **table 1**.

In order to discriminate alleles of the *eNOS* gene restriction endonucleases Ban II (Eco241) "Fermentas®" (USA) were used. PCR products were analyzed by electrophoresis in 3% agarose gels in the presence of tris-borate buffer concentrated with ethidium bromide. Fragments were visualized with ultraviolet emitter in the presence of the molecular weights marker 100-1000 bp ("SybEnzym", Russia).

Electrophoregram amplification products are shown in **figure 1**. For *eNOS* gene in the presence of position 894 of exon 7-guanidine, amplifier, which contained 250 base pairs, was splintered by restrictase Ban II (Eco 241) into two fragments 90 and 160 nucleotide pairs. In case of replacement of G894 → T restriction site for Ban II was lost.

Selection of primer sequences and PCR analysis were performed by the standard methods, used worldwide.[20,21]

All of the patients and healthy volunteers underwent general complex clinical, laboratory and instrumental diagnostic investigations. Biochemical studies were performed on blood biochemical analyzer "Accent-200" ("Cormay SA", Poland). The range of indicators of biochemical blood analysis included: total bilirubin and its fractions, cholesterol, triglycerides, uric acid, total protein and albumin, urea, creatinine, plasma enzyme activity [aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), gamma-glutamyl transferase (GGT), alkaline phosphatase (AP)]. Cytokine profile parameters were measured by ELISA analyzer "Statfax 303+" (USA). Tumor necrosis factor (TNF)-α was determined using enzyme immunoassay that involved a set of reagents (Diaclone); for transforming growth factor (TGF)-β_1 a commercial kit was used (Bender MedSystems GmbH), as also for interleukin

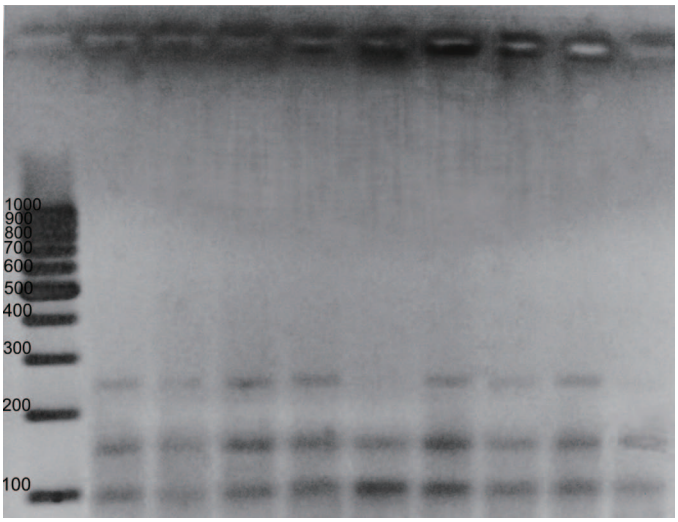

Figure 1. Electrophoregram of human DNA PCR products amplification of *endothelial nitric oxide synthase* (*eNOS*) 894T>G gene polymorphism. L: GeneRuler™ 100 bp DNA Ladder (1000-100 bp); G allele: 160, 90 bp; T allele: 250 bp; lines 1-4, 6-8: heterozygous TG variant; lines 5, 9: homozygous GG variant.

(IL)-4 (Diaclone), and atrial natriuretic propeptide (proANP) (Biomedica).

Echocardiographic studies were performed using ultrasound diagnostic system "En Visor HDS" ("Philips Ultrasound System", USA) with the definition of structural and functional parameters by the Asmi & Walsh method.[22] Calculation of cardiohemodynamics was conducted including: the left atrium (LA) diameter, the right ventricle diameter, the end-systolic and end-diastolic dimension of the left ventricle, interventricular septum thickness in systole and diastole, the thickness of the left ventricle posterior wall in systole and diastole. For each patient, values of ejection fraction, end-diastolic volume and end-systolic volume were calculated. Left ventricular myocardium mass (LVMM) was determined by the Devereux & Reichek formula as modified by the American Society of Echocardiography.[23,24]

LVMM index (LVMMI) was calculated using the formula: LVMMI (g/m²)=LVMM / BSA, where BSA, body surface area (m²).

In turn, BSA was calculated by the DuBois formula.[25] Left ventricular hypertrophy was diagnosed according to the guidelines for the management of arterial hypertension (2007).[26] Based on these recommendations, left ventricular hypertrophy was considered if LVMMI was increased; for men >125 g/m², while for women >110 g/m².

Statistical analysis

Statistical processing of the data was performed by a computer program PAST Version 2.05.[27] To determine the type of data distribution, comparing the arithmetic mean, median and mode, and Wilcoxon-Shapiro test were used. To determine the statistical differences between two independent groups Mann-Whitney test and in case of three or more independent groups the Kruskal-Wallis test were applied. Analysis of qualitative data (categorical variables) was assessed using chi-square test (χ^2). Hardy-Weinberg equilibrium was calculated to describe the relationship between gene frequency and genotype frequency, comparison of allele frequencies with 1 degree of freedom and genotypes between the groups and control with 2 degrees of freedom. Multiple linear regression analysis was performed. The association between LVMMI, proANP, age and AST, gender, LA, T allele, LVMMI, LVMM were tested using odds ratio (OR), risk ratio (RR) with 95% confidence interval (CI). Correlation between variables was calculated by means of linear regression analysis. Reliable probability of error was considered less than 5% ($p<0.05$).[28,29]

Results

Polymorphism (T894G) of *eNOS* gene was studied in 50 patients (34 men; mean age 54.1±9.9 years) with nonviral liver cirrhosis and 10 healthy volunteers (control group) (7 men; mean age 52.4±10.4 years). The distribution of genotypes of the *eNOS* gene polymorphism is shown in **table 2**.

Among patients with liver cirrhosis 2 patients (4.0%) had TT genotype; 32 patients (64.0%) had TG genotype; and 16 patients (32.0%) had GG genotype. T-allele carriers were 18 persons (36.0%), and G-allele carriers were 32 persons (64.0%). In the group of healthy volunteers, homozygous carriers T-allele were none, 70.0% of surveyed individuals in this group were heterozygotes, 30.0% homozygous carriers of G-allele, which probably is not different from the distribution of genotypes among patients with liver cirrhosis. The distribution of all alleles and genotypes of analyzed genes were determined within the Hardy-Weinberg equilibrium.

The association of biochemical blood parameters of patients with liver cirrhosis with T894G polymorphism of the *eNOS* gene are presented in **table 3**. Due to the fact that the number of carriers of the unfavorable TT-genotype was limited (n=2), there was a need to analyze indicators for the presence of abnormal T-allele. In patients with T-allele, AST activity was significantly higher at 27.4% ($p<0.05$) than in patients with the GG-genotype. Also, in patients with T-allele a significantly greater urea plasma level by 33.3% ($p<0.05$) and creatinine plasma level by 22.2% ($p<0.05$) than in patients with GG-genotype was revealed. For the rest of the blood biochemical parameters differences between genotypes were not statistically significant.

In patients with T-allele, the concentration of proANP in plasma by 89.2% ($p<0.05$) was significantly higher compared with the cytokine level in patients with liver cirrhosis with GG-genotype, which was associated with more severe manifestations of cardiovascular alterations: more frequent complaints of chest pain, discomfort in epicardium, shortness of breath, and in some patients the development of heart failure of IIA-IIB stage and edema (**Table 4**).

Heart rate, systolic and diastolic blood pressure between genotypes of the *eNOS* gene carriers did not differ significantly (**Table 5**). Only a tendency to tachycardia was noted in patients with liver cirrhosis compared with healthy volunteers.

Structural and functional parameters of the heart in patients with liver cirrhosis depending on the T894G polymorphism of

Table 2. Distribution of the *eNOS* gene polymorphism (T894G) in patients with liver cirrhosis and healthy individuals

***eNOS* gene genotype**	**Patients with liver cirrhosis (n=50)**		**Healthy volunteers (n=10)**	
	Absolute quantity, n	**Percentage**	**Absolute quantity, n**	**Percentage**
TT	2	4.0%	0	0.0%
TG	32	64.0%	7	70.0%
GG	16	32.0%	3	30.0%

eNOS, endothelial oxide synthase.

Table 3. Biochemical blood parameters in patients with liver cirrhosis according to T894G polymorphism of the *eNOS* gene

Plasma level	**Healthy volunteers, n=10**	**Patients with liver cirrhosis, n=50**	
		T-allele carriers, n=34	**GG-genotype carriers, n=16**
Total bilirubin, mkmol/L (N=5.0-20.5)	12.38±2.89	50.80±9.58*	54.62±8.51*
Direct bilirubin, mkmol/L (N=0.5-5.0)	4.90±0.54	14.30±2.90*	15.56±2.43*
Cholesterol, mmol/L (N=3.1-6.8)	4.51±0.24	4.13±0.39	3.75±0.23*
Triglycerides, mmol/L (N=0.4-1.8)	1.44±0.18	1.29±0.18	1.11±0.10*
Albumin, g/L (N=35-50)	43.43±1.36	32.26±1.45*	34.56±1.81*
Total protein, g/L (N=65-85)	68.67±1.02	69.39±1.35	69.40±1.32
Urea, mmol/L (N=2.4-8.3)	5.13±0.43	6.65±0.64**	4.99±0.37
Creatinine, mkmol/L (N=40-110)	89.20±2.96	99.74±9.48**	81.63±6.57
Aspartate aminotransferase, units of action/L (N<37)	18.00±0.90	74.48±8.96*/**	58.44±4.68*
Alanine aminotransferase, units of action/l (N<32)	21.17±2.57	43.16±5.00*	38.31±5.46*
Lactate dehydrogenase, units of action/L (N=210-420)	273.33±25.05	430.78±27.28*	401.88±32.11*
Alkaline phosphatase, units of action/L (N=42-141)	62.29±5.75	129.88±8.71*	108.69±7.30*
Gamma-glutamyl transferase, units of action/L (N=10-50)	29.57±6.13	126.00±27.61*	110.94±25.45*

*: significance of differences (p<0.05) compared with the figures in the group of healthy people; **: significance of differences (p<0.05) compared with rates in patients with the GG-genotype. *eNOS, endothelial oxide synthase.*

Table 4. Cytokine plasma content in patients with liver cirrhosis depending on the T894G polymorphism of the *eNOS* gene

Plasma level	**Healthy volunteers, n=10**	**Patients with liver cirrhosis, n=50**	
		T-allele carriers, n=34	**GG-genotype carriers, n=16**
Interleukin-4, pg/mL	1.85±0.17	0.84±0.14	0.76±0.14
Tumor necrosis factor-α, pg/mL	32.82±2.40	62.41±7.00*	70.49±10.08*
Transforming growth factor-β_1, pg/mL	323.18±83.94	406.29±47.73	477.85±82.10
Atrial natriuretic propeptide, nmol/L	0.32±0.04	2.27±0.34*/**	1.20±0.15*

*: significance of differences (p<0.05) compared with the figures in the group of healthy people; **: significance of differences (p<0.05) compared with rates in patients with the GG-genotype. *eNOS, endothelial oxide synthase.*

Table 5. Heart rate, systolic and diastolic blood pressure depending T894G polymorphism of the *eNOS* gene

Investigated parameter	**Healthy volunteers, n=10**	**Patients with liver cirrhosis, n=50**	
		T-allele carriers, n=34	**GG-genotype carriers, n=16**
Heart rate	72.29±0.52	84.50±1.64*	84.00±3.41*
Systolic arterial blood pressure	123.57±1.80	123.38±2.45	122.19±3.56
Diastolic arterial blood pressure	77.14±1.84	77.94±1.25	73.75±1.80

*: significance of differences (p<0.01) compared to rates in the group of healthy people. *eNOS, endothelial oxide synthase.*

the *eNOS* gene are presented in **table 6**.

The LA diameter was larger significantly in T allele carriers with liver cirrhosis, than in homozygous G allele patients by 13.6% (p<0.01). Also, in T-allele carriers, there was a higher left LVMM by 19.5% (p<0.01) and LVMMI in males at 12.2% (p<0.05), than in patients with liver cirrhosis with GG-genotype.

Multiple linear regression analysis, RRs, and ORs estimated for analyzed variables are presented in **table 7**. LVMMI was significantly related to AST (RR=1.70, 95% CI=1.17-2.47, p=0.002), LA (RR=2.53, 95% CI=0.94-5.85, p=0.02) and proANP (RR=2.0, 95% CI=1.08-3.72, p=0.002), whereas proANP was reliably related to LVMMI (RR=5.16, 95% CI=1.46-18.18, p=0.0001) and T allele presence (RR=2.35, 95% CI=0.95-5.85, p=0.02). Age and gender however were not related to either LVMMI or proANP in liver cirrhosis patients. T allele of *eNOS* gene is considered as independent additional predictor

Table 6. Structural and functional indices of the cardiovascular system in patients with liver cirrhosis depending on the T894G polymorphism of the *eNOS* gene

Investigated parameter		Healthy volunteers, n=10	Patients with liver cirrhosis, n=50	
			T-allele carriers, n=34	GG-genotype carriers, n=16
Aorta diameter (cm)		3.39±0.12	3.44±0.10	3.57±0.17
Left atrium diameter (cm)		3.92±0.14	4.52±0.09*/**	3.98±0.08
End diastolic dimension (cm)		5.26±0.12	5.48±0.15	5.12±0.21
End systolic dimension (cm)		3.41±0.09	3.63±0.11*	3.50±0.19
End diastolic volume (mL)		133.14±7.03	147.60±8.90	125.83±11.04
End systolic volume (mL)		51.71±3.34	56.27±4.04	51.67±6.52
Thickness of the posterior wall of the left ventricle in diastole (cm)		1.07±0.06	1.24±0.02*	1.21±0.04*
Thickness of the posterior wall of the left ventricle in systole (cm)		1.54±0.08	1.64±0.04	1.72±0.05
The thickness of the intraventricular septum in diastole (cm)		1.11±0.06	1.26±0.04*	1.22±0.04*
The thickness of the intraventricular septum in systole (cm)		1.25±0.04	1.39±0.04*	1.33±0.06*
Right ventricular diameter (cm)		2.11±0.04	2.35±0.07*	2.31±0.14*
Ejection fraction (%)		63.33±0.40	60.21±1.06*	60.60±1.17*
Left ventricular myocardium mass (mg)		213.94±20.94	302.86±20.57*/**	253.40±21.58*
Left ventricular myocardium mass index (mg/m²)	male	123.81±12.00	155.33±11.41*/**	138.39±7.06
	female	103.97±10.08	135.79±8.79*	120.29±11.09*

*: significance of differences ($p<0.05$) compared to rates in the group of healthy people; **: significance of differences ($p<0.05$) compared with rates in patients with the GG-genotype. *eNOS, endothelial oxide synthase.*

Table 7. Association of diagnostic variables with left ventricle hypertrophy, ProANP and age in liver cirrhosis patients

Variables		β	p	RR	OR	95% CI RR	p
LVMMI	Age	0.05	0.58	1.04	1.08	0.80-1.28	0.65
	Sex	-0.09	0.41	0.91	0.53	0.73-1.13	0.49
	AST	0.22	0.03	1.70	26.25	1.17-2.47	0.002
	LA	-0.28	0.02	2.53	12.5	0.94-5.85	0.02
	T allele	-0.18	0.08	2.92	12.5	0.88-9.67	0.04
	ProANP	0.61	0.00	2.0	–	1.08-3.72	0.002
R^2=0.8711736; ε=18.395; F=18.89042; df =5.30; p<0.0000							
ProANP	Age	-0.07	0.52	0.98	0.92	0.78-1.25	0.59
	Sex	-0.10	0.38	1.10	1.88	0.88-1.37	0.36
	AST	-0.21	0.07	0.59	0.04	0.40-0.85	0.02
	LA	0.21	0.07	0.42	0.08	0.17-1.06	0.02
	T allele	0.30	0.05	2.35	12.5	0.95-5.85	0.02
	LVMMI	0.77	0.00	5.16	67.5	1.46-18.18	0.0001
	LVMM	0.01	0.94	1.97	8.75	0.93-4.18	0.058
R^2=0.83485435; ε=0.45014; F=17.82602; df=4.31; p<0.0000							
Age	AST	-0.08	0.86	1.35	7.92	0.97-1.86	0.075
	LA	0.37	0.027	2.96	13.75	0.90-9.77	0.02
	T allele	0.03	0.86	1.05	1.22	0.74-1.48	0.53
	LVMMI	0.10	0.53	1.92	6.75	0.88-4.67	0.055
	LVMM	0.19	0.36	1.93	7.50	0.90-4.13	0.05
	ProANP	0.04	0.81	–	–	–	–
R^2=0.37; ε=12.06; F=5.35; df=1.34; p=0.027							

β: standard regression coefficient; R^2: multiple coefficient of determination; ε: Standard error of estimate; RR, risk ratio; OR, Odds Ratio; df, degrees of freedom; 95% CI RR, confidence interval of Risk Ratio; LVMM, left ventricular myocardium mass; LVMMI, left ventricular myocardium mass index; proANP, atrial natriuretic propeptide.

of left ventricle hypertrophy (RR=2.92, 95% CI=0.88-9.67, p=0.04) and heart failure appearance (RR=2.35, 95% CI=0.95-5.85, p=0.02). Age was associated in liver cirrhosis patients only with an increased risk for LA diameter (RR=2.96, 95% CI=0.90-9.77, p=0.02).

Discussion

We conducted a detailed analysis of the relation of biochemical blood parameters, cytokine blood profile, structural and functional parameters of the heart with the T894G polymorphism *eNOS*

gene. In our own patients with liver cirrhosis, T-allele carriers showed a significantly higher activity of AST and increased concentrations of proANP, which are markers of cytolytic process in myocardium and the development of cardiovascular insufficiency compared with GG-genotype carriers.[30,31] Thus, the above might indicate a greater tendency of these processes to develop in patients with T-allele of *eNOS* gene. In patients with T-allele such changes were associated with a significant increase in the LA diameter by 13.6% ($p<0.01$) and LVMM by 19.5% ($p<0.01$). In males T-allele carriers LVMMI was also increased by 12.2% ($p<0.05$) compared with males with GG-genotype. In patients with liver cirrhosis, T-allele carriers had significantly higher urea plasma level by 33.3% ($p<0.05$) and creatinine plasma level by 22.2% ($p<0.05$) compared with patients with the GG-genotype. This may indicate that in T-allele carrier cirrhotic patients, kidney and cardiovascular function is more severely impaired[32,33] than in GG-genotype patients. Thus, we hypothesized that T allele presence in liver cirrhosis patients may predispose to more severe damage of the target-organs of cardiovascular system with development of latent heart failure and can be considered as an early diagnostic marker of vascular injury in these patients.

Gene *eNOS*, discovered in 1993, is localized on chromosome 7q35-36, occupying interval 4.4 kB genomic DNA, and consists of 26 exons encoding the 135-kDa protein containing 1203 amino acids. Three clinically important polymorphisms of *eNOS* have been studied, with an established association with a number of cardiovascular diseases: single nucleotide polymorphisms, one in the promoter region (T-786C), and the other in exon 7 (T894G), with variable number tandem repeats in intron 4.[34-36] It was found that the only mutation of the *eNOS* gene, which leads to a change in the amino acid sequence of protein structure, is T894G (rs1799983) variant in which guanine is replaced by thymine in exon 7, which leads to the substitution of glutamate (E) to aspartate (D) (E298D) at codon 298.[37]

It is well known that vascular endothelial dysfunction plays an important role in the development of cardiovascular diseases,[1,2] the occurrence of liver cirrhosis and progression of its complications, such as portal hypertension.[3] The background for endothelial dysfunction serves an imbalance between local generation and decay of vasoactive substances, among which the NO plays the key role.[4,7,37,38] In experimental models of chronic parenchymal liver disease complicated by portal hypertension, increased activity of nitric oxide synthase and increased concentrations of NO were observed,[39] but the mechanisms of these changes remain vaguely understood. *eNOS* gene modulates the activity of the enzyme *eNOS*, that, in turn, synthesizes NO by reaction converting L-arginine to L-citrulline, which involves the transfer of five electrons by NAD(P)H. *eNOS* utilizes L-arginine and molecular oxygen and requires the cofactors NAD(P)H, flavin adenine dinucleotide, flavin mononucleotide, and (6R-) 5,6,7,8-tetrahydrobiopterin.[40] Along with the mentioned mechanism of *eNOS* activity regulation, calmodulin and calcium intracellular level play an important role in these processes.[41,42] Bradykinin, histamine, serotonin are agents that increase intracellular calcium level by increasing calcium input from the outside or by stimulating calcium mobilization from intracellular depots, can activate *eNOS*, and respectively NO synthesis.[43,44] In addition to direct regulation of *eNOS*, NO availability is also dependent on the activity of oxygen free radicals generation, and an increase in superoxide anion can be determinant in reducing NO availability.[45,46] *eNOS,* mostly expressed in endothelial cells, induces vasodilation, controls blood pressure, and has various vasoprotective and anti-atherosclerotic effects.[40,45] Thus, the production of NO is regulated by direct modeling of *eNOS* gene expression and enzyme activity of *eNOS* or indirectly through changes in the activity of endogenous cofactors and inhibitory molecules.[47,48]

Therefore, our study confirms the results of other authors[13,14] which postulate the influence of *eNOS* genotype to the liver cirrhosis, moreover we investigate that polymorphism of *eNOS* gene is involved in the development of cardiovascular complications in such patients. It was shown that in patients with liver cirrhosis with portal hypertension, a decreased activity of *eNOS* was recorded, which led to disturbances of intrahepatic bloodflow.[13,15] However, there is a little information about the changes of central blood flow and condition of cardiovascular system in such patients. Together with echocardiac findings, plasma levels of proANP, a molecule which is one of the most undisputable markers of cardiovascular insufficiency,[49,50] were increased in patients with liver cirrhosis. Moreover, it was found that T-allele carriers had significantly higher proANP plasma level compared with GG-genotype carriers, which correlated with structural changes of the heart: increased LA diameter and LVMM and LVMMI in males. To our knowledge, no relative clinical data exist, but similar changes were mentioned by Inserte et al. on rats, who found that portal hypertension associated to biliary cirrhosis induces marked left ventricle hypertrophy and increased myocardial NO synthesis due to increased *eNOS* expression.[51]

Potential limitations of the study are the small number of enrolled subjects together with the need for additional studies to determine the pathogenetic mechanisms by which the *eNOS* (T894G) genotypes influence the NO system.

Conflicts of interest

The authors declared no conflicts of interest.

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Summary Box

What is already known:

- The polymorphism of the *endothelial nitric oxide synthase* (*eNOS*) gene is related with a higher risk of cardiovascular diseases.
- TT genotype of the *eNOS* gene is associated with more frequent development of coronary atherosclerosis, heart stroke and higher plasma content of homocysteine, which leads to a more severe course of ischemic heart disease.
- Activity of *eNOS* enzyme is associated with changes in the intrahepatic blood flow and the severity of inflammation in liver parenchyma. Increased intrahepatic vascular resistance in patients with liver cirrhosis is accompanied by decreased activity of *eNOS* and is enhanced by the accession of inflammation. In patients with liver cirrhosis, complicated with portal hypertension, a decrease in the activity of *eNOS* causes decreased blood flow in the intrahepatic vessels.

What the new findings are:

- The presence of T-allele of *eNOS* gene in patients with liver cirrhosis is associated with increased activity of aspartate aminotransferase, higher content of atrial natriuretic propeptide in blood, increased left atrium diameter and left ventricular myocardium mass compared with patients with the GG-genotype.
- In male patients with liver cirrhosis, presence of T-allele was also associated with increased left ventricular myocardium mass index compared with patients with the GG-genotype.
- The presence of T-allele of *eNOS* gene in patients with nonviral liver cirrhosis is associated with more severe cardiovascular alterations.

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