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Tetyana Tsintar
Department of Pulmonology, Chernivtsi Regional Clinical Hospital Chernivtsi, Ukraine

Oleksandr Fediv
Department of Internal Medicine and Infectious Diseases, Bukovinian State Medical University Chernivtsi, Ukraine

Hanna Stupnytska
Department of Internal Medicine and Infectious Diseases, Bukovinian State Medical University Chernivtsi, Ukraine

Correspondence
Tetyana Tsintar
Department of Pulmonology, Chernivtsi Regional Clinical Hospital Chernivtsi, Ukraine

Some values of endothelial dysfunction, functional state of the liver, carbohydrate metabolism and lipid blood spectrum in patients with a combined course of non-alcoholic steatohepatitis and chronic obstructive pulmonary disease depending on the g894t gene polymorphism of the endothelial no-synthase

Tetyana Tsintar, Oleksandr Fediv and Hanna Stupnytska

Abstract
The purpose of the study was to analyze the incidence of alleles / genotypes of the G894T polymorphic variant of the eNOS gene in patients with non-alcoholic steatohepatitis (NASH) combined with chronic obstructive pulmonary disease (COPD) and their association with the functional state of the liver, endothelial dysfunction, carbohydrate metabolism and lipid blood spectrum. The polymorphism of the G894T gene of the eNOS was studied using a polymerase chain reaction in 40 patients with NASH in combination with COPD. It has been established that there is a more pronounced manifestation of endothelial dysfunction with the comorbidity of NASH and COPD and the presence of the T allele (as evidenced by higher endothelin-1 levels (by 30.5%) and sVCAM-1 (by 24.4%) with the simultaneous low level nitrates / nitrites in the blood) and more significant disorders in the blood lipid spectrum (especially regarding the level of low density lipoproteins and atherogenicity index) and the functional state of the liver with more pronounced cholestatic syndrome, cytolysis and decreased protein-synthetic liver function.

Keywords: eNOS G894T gene, non-alcoholic steatohepatitis, chronic obstructive pulmonary disease, endotelium.

1. Introduction
Endothelium plays an important role in regulating the vascular tone and maintaining the integrity of the vessels. Endothelial dysfunction is a fundamental component of the pathogenesis of cardiovascular disease. Despite the fact that many molecules that are involved in endothelial signaling are known, the genetic contribution to the endothelial function is still not fully elucidated [1]. At present, most studies aimed at the research of the genetic component of the endothelial vasomotor function have shown polymorphism in the genes of the angiotensin-converting enzyme, angiotensin II of the receptor type 1, cytochrome B-245 of the alpha chain (CYBA), NO-synthase 3 (NOS3), and GTP of cyclo-hydrolase 1 (GCH1) [1, 2, 3, 4, 5]. The most studied are two polymorphisms in NOS3, namely T-786 → C and G894 → T. G894 → T polymorphism in exon 7 NOS3 is the result of replacing aspartate with glutamate in codon 298. This polymorphism was to a large extent related to flow-mediated dilatation of the blood vessels. TT genotype carriers had higher flow-mediated dilatation than the carriers of genotype GG or GT [6].
At the same time, the Framingham Heart Study did not show significant associations between flow-mediated dilatation and 18 single-nucleotide polymorphisms (SNPs) in NOS3, including T-786 → C and Glu298Asp [7]. Consequently, the effect of NOS3 polymorphisms on the function of the endothelium is variable and may depend on a sample or other genetic or environmental factors [1]. Nitric oxide (NO) is a molecule that is involved in many physiological and pathological processes. It has been established that NO has a protective effect on the respiratory tract in humans, in particular, it promotes muscle relaxation, weakens respiratory hypersensitivity to bronchoconstrictor stimuli, and prevents microorganisms from getting into the airways [8]. NO is known to play an important role in the regulation of the functional state of the endothelium and the vascular tone [9]. NO is formed from L-arginine with NO synthase (NOS), which exists in several isoforms: the neuronal NOS (NOS1) induced

By NOS (NOS2) and endothelial NOS (NOS3). In humans, NOS1 is found in neurons and endothelial cells of the lungs and NOS3 in the cells of the bronchial epithelium and in the endothelium [8]. The level and activity of NO depend on the allelic version of the NO synthase genes [10]. Some studies have shown that a missense mutation, which results in the replacement of aspartate with glutamate in the eNOS protein in the G894T position makes this molecular version more susceptible to proteolytic cleavage, while reducing enzymatic activity and production of basal levels of nitric oxide [11]. The NOS3 gene is positioned on the long shoulder of the 7th chromosome (7q36), consisting of 26 exons and 25 introns sized about 20 kb. More than 100 polymorphic markers of the NOS3 gene are known, the most studied of which are: VNTR, 894G / T, -691C / T, -788C / T, 774C / T, 1998C / G [12]. The polymorphism of the G894T eNOS gene is associated with a disorder in the endothelium-dependent vasodilatation, which may be due to either vasoconstrictive activity or to a decrease in the production of vasodilators, such as NO [11]. The results of many studies and meta-analyzes on the role of endothelial NO synthase polymorphism confirm their role in type 2 diabetes mellitus, COPD and obesity [9]. It has been established that one of the pathogenetic components of the development of nonalcoholic steatohepatitis is endothelial dysfunction, in which the level of NO in the blood is lowered. Functional state of the endothelium is also impaired in chronic hypoxia. The combined course of NASH and COPD is likely to exacerbate the imbalance in vasodilation and vasoconstriction regulation, which makes it advisable to use drugs that can reduce endothelial dysfunction. One of the methods to correct a low NO level is the administration of nitrogen oxide donators. However, their administration was carried out without taking into account the level of nitrates / nitrites in the blood and the polymorphism of the eNOS gene.

1.1. The aim of the research

to determine the incidence of the genotypes of the G894T polymorphic version of the eNOS gene for the combined course of non-alcoholic steatohepatitis and chronic obstructive pulmonary disease and to compare the values of the functional state of the liver and endothelium, the carbohydrate metabolism and the lipid blood spectrum, depending on the genotype of the indicated gene polymorphism.

2. Materials and Methods

2.1 Compliance with bioethics

The study was performed in compliance with the Council of Europe Convention on Human Rights and Biomedicine and recommendations of the Committee on Bioethics of the Ministry og Health of Ukraine. Patient's Examination Cards and Patient's Informed Consent Forms were approved by the Biomedical Ethics Comission of Bukovinian State Medical University (Chernivtsi, Ukraine). All enrolled patients were treated in the Regional Clinical Hospital (Chernivtsi, Ukraine) during 2013-2015. Genetic bench study performed at the laboratory of the State institution "Reference centre of molecular diagnostics of the Ministry of Health of Ukraine" (Kyiv, Ukraine). After screening (matching inclusion/exclusion criteria) 40 patients with NASH and COPD were selected for further examination.

2.2 Inclusion/Exclusion criteria

2.2.1 Inclusion criteria

Age of patients at the time of screening was over 40 years, patients with NASH, developed secondary to the 1st degree obesity, including COPD, signing a written informed consent form by the patient before participating in the study.

2.2.2 Exclusion criteria

Pregnancy, bronchial asthma, α1-antitrypsin deficiency, active tuberculosis, lung cancer, significant bronchiectasis, sarcoidosis, pulmonary fibrosis, interstitial lung disease; signs of clinically significant cardiovascular, neurological, psychiatric, renal, hepatic, immunological, gastrointestinal, urogenital disorders, lesions in the musculoskeletal system, skin, sensory organs, endocrine system (uncontrolled diabetes or thyroid disease) or hematologic illnesses that are uncontrolled, unstable liver disease, unstable or life-threatening heart disease, patients with malignant neoplasms who have not been in complete remission for at least 5 years, drug dependence, alcohol dependence, as well as viral, alcohol, drug-induced steatohepatitis, autoimmune hepatitis, liver cirrhosis of various etiologies.

2.3 Diagnosis of Nonalcoholic Steatohepatitis and Chronic Obstructive Pulmonary Disease.

The diagnosis of NASH was based on anamnestic, clinical, laboratory (biochemical, serological, immunological) data, the determination of the serum markers of hepatitis B and C viruses, the results of the ultrasound examination in accordance with the Order of the Ministry of Health of Ukraine of June 13, 2005, No. «On approval of medical treatment protocols on the specialty «Gastroenterology»» taking into account ICD-10. The diagnosis of COPD was made in accordance with the Order of the Ministry of Health of Ukraine No. 128 of March 19, 2007 and by the Order No. 555 of the Ministry of Health of Ukraine of June 27, 2013. The degree of obstruction of the respiratory tract was determined according to the classification of GOLD, 2008: stage 1 (mild) - FEV1 ≥80%, stage 2 (moderate) - FEV1 50-79%, stage 3 (severe) - FEV1 30-49%, stage 4 (advanced) - FEV1 <30%. Obesity was diagnosed by the body mass index, which was calculated using the formula: weight (kg) / height2 (cm2). The assessment of body weight and the degree of obesity was performed according to WHO classification (1997): the normal body weight - BMI 19-24.9 kg / m2, overweight - BMI 25-29.9 kg / m2, the first degree obesity - BMI 30 -34.9 kg / m2, the second degree - BMI 35-39.9 kg / m2, the third degree - BMI ≥40 kg / m2. The type of obesity was determined by the ratio of the waist circumference (WC) to the circumference of the hip (HC) and the level of visceral fat (bio-impedantsometry). In the presence of abdominal obesity (AO) WC / HC exceeded 1.0 (in men) and 0.8 (in women), and the level of visceral fat was higher than 12.

2.4 Genotyping of the eNOS gene G-894T polymorphism

Because of the G on the 894th position of the 7th exon of the eNOS gene mutated to T (G894T), the Asp replaces Glu on the 298th position of the protein. The genomic DNA was amplified using PCR and digested by restriction enzyme. Then PCR-restriction fragment length polymorphism (RFLP) method was used to detect eNOS G894T gene polymorphism. This region was amplified by PCR using the forward primer 5′-AAGGCACAGGAGACAGTGGATGGA-3′ and the reverse primer 5′-CCCAGTCAATCCCTTTGGTGCT-3′. A

25-µl PCR mixture contained 50~200 ng of genomic DNA, 10 µl of 2.5× Taq buffer, 100 µmol/L of each dNTP, 0.4 µmol/L of each primer, and 0.1 U of Taq DNA polymerase. After the first 95 °C for 5 min, the PCR reaction was run at 95 °C for 1 min, 59 °C for 30 s, and 72 °C for 40 s for 30 cycles, and a final extension of 72 °C for 10 min. 12 µl of PCR product was mixed with 5 U of restriction enzyme BanII, 2 µl of 10× restriction enzyme digestion buffer, and 5 µl of deionized water, and incubated at 37 °C for 3 h. The digested PCR products were electrophoresed on 2.5% (w/v) agarose gels and visualized after ethidium bromide staining under ultraviolet transillumination. Finally, we randomly selected 10% of the samples to repeat the detection of eNOS G894T gene polymorphisms, and the results were fully confirmed.

2.5 Other Methods

The lipid blood spectrum was examined for the content of the total cholesterol, triglycerols (TG), LDL cholesterol, very low-density lipoproteins (VLDL) cholesterol and high-density lipoproteins (HDL) cholesterol using standard diagnostic sets (PZ Cormay, Poland).

Carbohydrate metabolism was studied on the level of glucose in the blood on an empty stomach with glycosylated hemoglobin (HbA1c), insulin and HOMA-IR insulin resistance index (glucose (mmol / l) × insulin ((µO / ml) / 22.5, QUICKI (1 / [(log (plasma glucose on an empty stomach) + log (insulin plasma on an empty stomach] and Caro (glucose (mmol / l) / insulin (µSO / ml).

The level of glycaemia was studied using glucose oxidase method by means of standard sets of reagents manufactured by NPP "Filsit Diagnostika" (Ukraine). Glycosylated hemoglobin was determined using a photocolorimetric method by means of a set of reagents from Erba Lachema s.r.o. (Czech Republic). The level of immunoreactive insulin (IRI) was studied by means of the immunogenic enzyme method using the reagents of DRG International Inc. (USA) on the STAT-Fax Plus-303 analyzer (USA).

The functional state of the endothelium was studied by the content of stable metabolites of nitrogen monoxide (nitrites / nitrates), ET-1, CCEE, with the content of sVCAM-1. The amount of blood circulating in the endothelial cells was determined according to the method of J. Hladovec (1978) modified by N.N. Petrischev *et al.* (1999). The content of stable NO (nitrite / nitrate) metabolites in the blood was investigated using L.C. Green *et al.* (1982), the level of ET-1 - by immuno-enzyme analysis using Biomedica Medizinprodukte GmbH and Co KG (Austria) reagents. sVCAM-1 was determined in the blood serum using ELISA by means of Bender MedSystems (Austria) reagents.

2.6 Statistical analysis

The mathematical processing of the data was carried out using BioStat 2009 Professional, version 5.8.4.3 (AnalystSoft Inc.), SPSS (Statistical Package for Social Science Statistics) 16.0, Statistics 10.0 StatSoft Inc., Microsoft Excel 2010. The statistics of the hypothesis were tested by the coefficients of asymmetry and an excess using the Khan-Shapiro-Wilky criterion to analyze the normality of the distribution of variables in randomized samples. Student's t-criterion was only used in the case of a normal distribution with the equality of the general dispersion of the comparable samples, which was checked by the Fisher (F-criterion). In other cases, Mann-Whitney's non-parametric rank test was used to compare the results. To compare several groups, a dispersion analysis was used. Differences were considered to be reliable at the level of significance $p < 0,05$. The probability of changes in variations in the dynamics of treatment with normal distribution in the samples was determined on the basis of the Student's paired test, in other cases, on the nonparametric paired T-criterion of Wilcoxon. The correlation analysis was performed by determining the linear parametric Pearson correlation coefficient and the nonparametric Spirman ranks correlation coefficient.

3. Results and Discussions

The first stage of the study was to determine the incidence of genotypes of the polymorphic version of the G894T of the eNOS gene, which is shown in Table. 1. The fincidence of the genotype GG in patients with non-alcoholic steatohepatitis combined with chronic obstructive pulmonary disease was 55%, genotype GT - 37,5% and genotype TT - 7,5%.

One of the stages of the research was studying the features of the development of endothelial dysfunction, depending on the polymorphism of the eNOS gene (G894T / G1917T) with the combined course of non-alcoholic steatohepatitis and chronic obstructive pulmonary disease.

Table 1: The incidence of genotypes of the polymorphic version of the eNOS gene in a combined course of non-alcoholic steatohepatitis and chronic obstructive pulmonary disease

N	eNOS (G894T/ G1917T)					
	GG		GT		TT	
	n	%	n	%	n	%
40	22	55	15	37,5	3	7,5

Among healthy individuals in the Ukrainian population (n = 83), the genotype GG was in 29%, GT – in 67%, and TT was in 4% [14]. According to the results of another study, the incidence of genotypes among healthy individuals was: GG - 42%, GT - 54% and TT - 4% [15]. Depending on the gender, among healthy women the incidence of genotypes was as follows: GG - 46.08%, GT - 40.20%, and TT - 13.73% [16]. In other populations, including the Egyptians, according to Hany Younan *et al.* [17] there was no difference in the incidence of genotypes between the cohort of patients with cardiovascular diseases and healthy individuals (normal angiography). The incidence of genotypes among healthy people was 53.1% (n = 17) with the genotype GG, 43.8% (n = 14) - genotype GT and 3.1% (n = 1) - TT genotype. Many studies are related to the research of the polymorphic version in the seventh exon of the eNOS gene in hypertension, CHD and associated endothelial dysfunction. There is controversial data on the effect of this polymorphism on endothelium-dependent vasodilatation. For instance, one of the studies showed that with GG genotype endothelium-dependent vasodilatation was less pronounced according to the sample with reactive hyperemia among patients with CHD and congestive HF [18]. On the other hand, the study of MS Joshi *et al.* [19] found less endothelium-dependent vasodilatation in carriers of the T allele of the G894T of the eNOS gene. The study of Voronkova L. G. *et al.* noticed a tendency towards its deterioration in the carriers of the allele T [20]. Another study also revealed the dependence of endothelial function values in patients with hypertension on the G894T polymorphism of the endothelial NO synthase gene. For instance, the content of NO metabolites was reliably lower than those of the TT genotype, however, the level of sVCAM-1 for this genotype was reliably higher than that of carriers of the GG genotype [15].

The values of the endothelium function depending on the polymorphism of the eNOS gene (G894T) are shown in Table 2.

It has been found that in patients with GT and TT genotype, the level of endothelin-1 was reliably higher than in patients with the GG genotype (by 30.5%). The level of sVCAM was also reliably higher by 24.4% when patients had genotype GT and TT. The number of circulating cast-off endothelial cells did not differ reliably between the first and second groups of patients. However, the level of nitrates / nitrites in patients with GT and TT genotype was by 27.3% lower than in the first group of patients. It has been also noted that the genotype GG corresponds to the highest level of catalytic activity of eNOS [21]. Polymorphisms of the eNOS Glu298Asp gene (G894T) and -786T> C have also been analyzed in several meta-studies. The Glu298Asp polymorphism in exon 7 is associated with low levels of NO and reduced vascular reactivity, and some researchers associate it with a dose-dependent decrease in the enzymic activity of eNOS and a decrease in NO production [10]. Thus, in patients with non-alcoholic steatohepatitis combined with chronic obstructive pulmonary disease and the T allele, the manifestations of endothelial dysfunction are more pronounced, as indicated by higher endothelin-1 and sVCAM levels with a simultaneous low level of nitrates / nitrites in the blood.

Table 2: The values of endothelium functional state in patients with non-alcoholic steatohepatitis combined with chronic obstructive pulmonary disease depending on the polymorphic version of the eNOS (G894T) gene

Values	Genotypes of the eNOS gene	
	GG, n=22 (group1)	GT, n=15+TT, n=3 (group 2)
Endothelin-1, pmol/l	0,185±0,03	0,266±0,07 p<0,05
sVCAM, ng/ml	2423,14±346,64	3206,47±341,52 p<0,05
The number of circulating cast-off endothelial cells 10^4/l	20,06±0,83	20,67±1,09 p>0,05
Level of nitrates / nitrites, μmol / l	15,35±1,28 n=11	12,06±1,02 p<0,05

Notes: p – the difference between the group of patients with the genotype GT + TT and the group of patients with genotype GG.

One of the stages of the research was studying the functional state of the liver in patients with a combined course of non-alcoholic steatohepatitis and chronic obstructive pulmonary disease depending on the genotype of the polymorphous version of the eNOS (G894T) gene.

The analysis of values of the liver functional state depending on the genotype of the eNOS gene found that in the patients from the second group the total bilirubin was by 19.5% higher than in those with genotype GG (Table 3). The level of conjugated and unconjugated bilirubin was reliably higher in the group of patients with genotype GT and TT (16 and 21.6% respectively) as well.

The activity of Alat and Asat in patients with genotype GT and TT was reliably different from those in patients in the first group (it was by 32.0 and 20.8% higher respectively).

Accordingly, the patients from the second group had a lower coefficient of de Richtis (Asat / Alat) (by 29.9%). GGPP was by 19% higher in this group than in the patients with the genotype GG as well. The thymol (turbidity) test and alkaline phosphatase activity in the patients with genotype GT and TT were reliably higher (by 18.7% and 21.3% respectively) compared with the first group. The total protein in patients with combined non-alcoholic steatohepatitis and chronic obstructive pulmonary disease decreased, but it was reliably lower in the patients with the genotype GT and TT. However, there was no intergroup difference in serum globulin levels.

Therefore, in the combined course of non-alcoholic steatohepatitis and chronic obstructive pulmonary disease, depending on the genotype of the eNOS gene, the liver function values were characterized by more pronounced changes in the genotypes GT and TT, shown by more significant manifestations of cholestasis, cytolysis and decreased protein-synthetic liver function.

4. Conclusions

The patients with non-alcoholic steatohepatitis combined with chronic obstructive pulmonary disease, in the presence of the T allele, have more pronounced manifestations of endothelial dysfunction, as shown by higher endothelin-1 levels (by 30.5%) and sVCAM-1 (by 24.4%) with the simultaneous low level of nitrates / nitrites in the blood and more significant disorders in the functional state of the liver with a cholestatic syndrome, cytolysis and a decrease in the protein-synthetic function of the liver, disorders in the lipid blood spectrum, especially in relation to the level of the low density lipoproteins and atherogenic index.

Table 3: The values of the liver functional state in patients with non-alcoholic steatohepatitis combined with chronic obstructive pulmonary disease depending on the polymorphic version of the eNOS (G894T/ G1917T) gene

Values	Genotypes of the eNOS gene	
	GG, n=22 (group 1)	GT, n=15+TT, n=3 (group 2)
Total bilirubin, μmol / l	26,42±0,76	32,82±2,26 p<0,05
Conjugated bilirubin, μmol / l	10,09±0,36	11,97±1,02 p<0,05
unconjugated bilirubin μmol / l	16,33±0,52	20,84±1,40 p<0,05

Alat, μmol / h × l	1,21±0,59	1,78±0,17 p<0,05
Asat, μmol / h × l	0,80±0,04	1,01±0,06 p1<0,05
Asat/Alat	0,87±0,12	0,67±0,08 p<0,05
GGTP, μmol / h × l	5,38±0,34	6,64±0,27 p<0,05
thymol (turbidity) test (units)	3,14±0,19	3,86±0,20 p<0,05
Alkaline phosphatase, μmol / h × l	1,22±0,04	1,55±0,05 p<0,05
Total protein, g/l	69,30±1,60	65,17±1,98 p<0,05
Albumines, %	44,50±0,88 p<0,05	41,44±1,02 p<0,05
Globulines, %	55,50±0,88 p<0,05	58,56±1,02 p>0,05

Notes: p – the difference between the group of patients with the genotype GT + TT and those with genotype GG.

One of the studies shows the association of the homozygous genotype TT polymorphism of G894T of the eNOS gene with a higher level of immunoreactive insulin, an insulin resistance index and glycosylated hemoglobin [22].

According to the results of our study on the analysis of carbohydrate metabolism, depending on the genotype of the eNOS gene, it was found that there was no reliable difference between the groups of patients with the genotype GG and the genotype GT and TT, except for glycosylated hemoglobin (it was by 8.9% lower in the second group) (table 4).

The lipid spectrum of the blood was characterized by the fact that in the patients with genotypes of GT and TT changes in the eNOS gene were more pronounced than in patients from the first group. This is especially true for values such as cholesterol low density lipoprotein and an atherogenicity index that were by 8.6 and 20.4% higher than in the GG genotype (Table 5).

Table 4: The values of carbohydrate metabolism in patients with non-alcoholic steatohepatitis combined with chronic obstructive pulmonary disease, depending on the polymorphous version of the eNOS (G894T/ G1917T) gene

Values	**Genotype of the eNOS gene**	
	GG, n=22 (group 1)	GT, n=15+TT, n=3 (group 2)
Glucose on an empty stomach, μmol / l	6,31±0,56	5,91±0,31 p>0,05
Glucose after 2 hours, μmol / l	9,46±1,06	7,91±0,85 p>0,05
HbA1c, %	6,51±0,39	5,98±0,41 p<0,05
IRI, μUn \ ml	17,24±4,44	14,23±4,58 p>0,05
HOMA-IR	5,60±1,61	3,76±1,19 p>0,05

Notes: p – the difference between the group of patients with the genotype GT + TT and those with genotype GG.

Table 5: The values of the lipid blood spectrum of in patients with non-alcoholic steatohepatitis combined with chronic obstructive pulmonary disease, depending on the polymorphous version of the eNOS (G894T / G1917T) gene

Values	**Genotype of the eNOS gene**	
	GG, n=22 (group 1)	GT, n=15+TT, n=3 (group 2)
Total cholesterol, μmol / l	6,28±0,23	6,66±0,30 p<0,05
Triglycerols, μmol / l	2,24±0,12	2,32±0,15 p<0,05
Cholesterol of low density lipoproteins, mg / dl	64,73±2,73	70,61±3,06 p<0,05
Cholesterol of high density lipoproteins, μmol / l	1,02±0,05	0,96±0,06 p1<0,05
Cholesterol of a very low density lipoproteins, μmol / l	1,04±0,05	1,20±0,08 p>0,05
Atherogenic index	4,73±0,45	5,98±0,80 p<0,05

Notes p – the difference between the group of patients with the genotype GT + TT and those with genotype GG.

Therefore, there was not any particular dependence of carbohydrate metabolism values on the genotype of the gene polymorphism in patients with non-alcoholic steatohepatitis combined with chronic obstructive pulmonary disease. However, regarding the lipid blood spectrum, especially regarding the level of low density lipoproteins and the atherogenic index, a reliable dependence on the genotype of the polymorphic version of the eNOS gene (G894T) has been established - in the presence of the T allele the lipid blood spectrum values were worse.

4.1 Limitations of the Study: The present study was limited by a number of enrolled subjects.

4.2 Conflict of Interest: None declared.

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