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ASSOCIATION OF *PvuII* POLYMORPHISM IN THE LIPOPROTEIN LIPASE GENE WITH THE CORONARY ARTERY DISEASE IN MACEDONIAN POPULATION

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A b s t r a c t: In the etiology of coronary artery disease there are many factors involved as a result of the complex interaction between genetic predisposition and environmental influences. The lipoprotein lipase (*LPL*) plays a very important role in lipid metabolism. It hydrolyzes the triglycerides in hylomicrones and very low density lipoproteins – VLDL. *PvuII* polymorphism in the *LPL* gene is a frequent variant and it increases triglyceride levels and the risk of the appearance of coronary arterial disease.

Aim: The aim of this work is to show *LPL-PvuII* polymorphism as an independent risk factor and also as a predictor of coronary arterial disease in the Macedonian population.

Material and Methods: The study included 109 randomized patients with coronary artery disease (CAD) (83 males, 26 females), treated at the Cardiology Clinic. The stenosis of coronary arteries greater than 70% of the artery lumen was angiographically documented in the CAD group. The control group consisted of 32 patients (25 males, 7 females) with documented normal coronarographic findings. The patients' age ranged from 50 to 59; the mean age in the CAD group was 59.4 and the mean age in the control group was 57.9. *LPL-PvuII* polymorphism in the intron 6 in the CAD and control group was detected by PCR amplification and restriction enzyme digestion.

Results: A statistically significant association between CAD and the control group was found regarding the presence of hyperlipidaemia ($p < 0.001$), diabetes ($p < 0.05$) and the use of antilipidaemic drugs ($p < 0.049$). The presence of *LPL-PvuII* polymorphism in both investigated groups does not represent a statistically significant risk factor for the appearance of coronary artery disease ($p = 0.816$).

The *PvuII* + allele frequency of 0.495 and 0,469 was obtained in both the angiographically confirmed CAD and the control groups, respectively. This finding indicates no significant differences between the prevalence of the *LPL-PvuII* genotypes in both study groups, suggesting a lack of association of *LPL-PvuII* polymorphism with CAD.

However, the homozygous genotype (*Pvu*II +/+) was more prevalent in the CAD group (22.9%) in comparison with the control group (15.6%).

Conclusion: In our study *LPL-PvuII* polymorphism was not identified as an independent risk factor for the appearance of CAD.

Key words: *LPL-PvuII* polymorphism, coronary artery disease.

Introduction

Coronary artery disease (CAD) is a leading cause of exceptionally high invalidity, mortality and morbidity in the world. Atherosclerosis is a complex process, and several major risk factors and a growing number of new risk markers have been reported which contribute to an understanding of the etiopathogenetic mechanisms of CAD [14]. Thus, when evaluating the individual risk concerning coronary artery disease, the determination of the genotype for certain mutations and polymorphism has an important role, besides the relevant phenotype measurable laboratory and clinical parameters. In the greatest number of cases of coronary artery disease, specific polymorphism or mutations in certain genes can be found [24, 28].

The association between polymorphisms and mutations in the lipoprotein lipase (*LPL*) gene has been the subject of numerous studies [6]. The *LPL* gene is located on the short branch of the 8p22 chromosome and extends to a length of 30 kb. It consists of 10 exons with high evolutionary conservation (homology between the species). The complementary DNA (cDNA) codes a protein with 475 amino acid residues including a 27 amino acids signal peptide. The protein product of this gene is the LPL enzyme. It is a multifunctional protein which hydrolyzes the triglycerides from the circulating hilomicrones and from the cholesterol complexes with very low density (VLDL). The liberated triglycerides are metabolized in hepathocytes or converted into LDL particles by the hepatic lipase. During this process, the free cholesterol and phospholipids transfer into HDL particles thus increasing the concentration of HDL-cholesterol. The catalytic centre of the enzyme is formed by three amino acid residuals, namely serin-132, asparagine acid-156 and histidin-241. About 100 mutations and single nucleotide polymorphisms (SNPs) in the human *LPL* gene have been described so far. The missense mutations dominate (61) by substitution of one into another amino acid residual. The greatest number of them is located in the

exons 5 and 6. The nonsense mutations (12) follow and they lead to a shortened protein product. At least ten mutations have been identified in the promoter region of the *LPL* gene. Small deletions or insertions lead to the frame shift. The frequency of mutations in the *LPL* gene varies significantly among different populations. In patients with a certain polymorphism of *LPL*, the gene effects are only faintly expressed and environmental factors determine the intensity and result of the clinically manifested arteriosclerosis [10].

Numerous studies have been conducted in which candidate genes were investigated for the putative association with CAD [19, 20]. Only a few gene polymorphisms and mutations were found to correlate with the risk of CAD or the clinically manifested disease, but the reports were often giving contradictory results. There are several reasons for these apparent inconsistencies that include, but are not restricted to, genetic heterogeneity, environmental factors, statistical methods, etc. [9, 13, 18]. It is believed that the presence of polymorphism in several genes at the same time (as those for the enzymes involved in the lipid metabolism, coagulation factors, inflammatory mediators and other proteins), is the basis for the multi-gene nature of arteriosclerosis and CAD [8, 34].

The *LPL* gene contains the polymorph sequence CAG↓CTG in intron 6 that can be recognized by the *PvuII* endonuclease restriction enzyme. *LPL-PvuII* polymorphism is one of the genetic variants that are described in the *LPL* gene that is associated with CAD appearance and progression in numerous studies [3, 5, 35–37]. On the other hand, other authors did not find a significant connection to CAD [1, 34, 26, 36].

Given the importance of LPL as a candidate gene for cardiovascular risk, we evaluated an independent, angiographically controlled Macedonian population to determine whether *LPL- PvuII* polymorphism was associated with defined CAD [2, 21, 27]. Therefore, the aim of this study is to show the possible associations of *LPL-PvuII* polymorphism and clinically verified coronary artery disease in the Macedonian population.

Aim

The aim of this study is to investigate the *LPL-PvuII* polymorphism as an independent risk factor, but also as a predictor of coronary artery disease in the Macedonian population.

Material and methods

The study is of a prospective character and it is randomized. Two institutions were involved, namely the Cardiology Clinic and the Molecular Biology

Laboratory at the Natural and Mathematical Sciences Faculty at the Ss Cyril and Methodius University in Skopje.

The study included 109 patients as the examined group (CAD group – 83 males, 26 females), treated at the Cardiology Clinic. It was angiographically documented that the patients suffered from stenosis of the coronary arteries over 70% of the artery lumen. The control group consisted of 32 patients (25 males, 7 females) and they had normal coronarographic findings, which were also angiographically documented.

The realization of the study was approved by the Ethical Committee of the Medical Doctors' Chamber of the Republic of Macedonia.

The following data on the patients were analyzed: demographic data: age, sex, place of living, and nationality; risk factors for CAD and their incidence (according to Joint Task Force for Cardiac Prevention, 1998 and ATP, 2001): profession, family predisposition to CAD, information on myocardial infarction, history of hyphercholesterolaemia, hypherlipidaemia, hypertension or diabetes mellitus, smoker or non-smoker, data on physical activity, education, use of antilipemics, use of alcohol, and BMI (Body-Mass Index).

The patients were chosen randomly, consecutively, according to the time of the angiographic examination performed at the Cardiology Clinic, and whether s/he satisfied the criteria for inclusion or exclusion from the study.

Sample Collection and DNA Extraction

Five millilitres of peripheral blood were aseptically collected in a test tube (Vaccutainer®) with anticoagulant (EDTA disodium salt), following informed consent, from all individuals who participated in this study. The standard isolation of genomic DNA was performed on nucleated cells, using DNA extraction with sodium chloride and chloroform, and ethanol-precipitation [23].

Detection of LPL-PvuII polymorphism

The *LPL-PvuII* polymorphism in intron 6 was identified by restriction enzyme digestion of the PCR-amplified segment of the *LPL* gene [2, 21]. The polymerase chain reaction (PCR) was performed with the primers: forward, 5'-ATC AGG CAA TGC GTA TGA GGT AA-3'; reverse, 5'-GAG ACA CAG ATC TCT TAA GAC-3'. Each PCR-amplification reaction was performed using 100–250 ng genomic DNA; 10 pmol of each primer; 200 mmol/L each of dATP, dCTP, dGTP, and dTTP; and 0.05 U of Taq polymerase in a total reaction volume of 20 :L. Amplification was performed in a GeneAmp PCR System 2400 (Perkin Elmer). The initial denaturation at 94°C for 5 minutes was followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 57°C for 1 minute, and extension at 72°C for 1 minute, with final extension at 72°C for 10 minutes.

The *LPL-PvuII* polymorphism was identified by restriction fragment length polymorphism (RFLP) analysis of the amplified fragments using *Pvu*II endonuclease (Sigma-Aldrich) digestion. The restriction products were resolved by agarose electrophoresis, visualized under UV-illumination (312 nm) following ethidium bromide staining and recorded by a digital camera (Canon A70). Images were analyzed by GelPro software.

Statistical data processing

The statistical tests were calculated using SPSS 8.0 for MS Windows. A *p*-value less than 0.05 was considered as significant.

Results

The study (CAD) group included 114 patients with angiographically confirmed stenosis of the coronary arteries greater than 70% of the artery lumen. The control group consisted of 35 patients with angiographically normal coronary arteries. PCR amplification was unsuccessful in 5 samples of the CAD group and in 3 of the control group. *LPL-PvuII* polymorphism was identified by restriction fragment length polymorphism (RFLP) of the amplified fragments using *PvuII* endonuclease digestion (Figure 1).

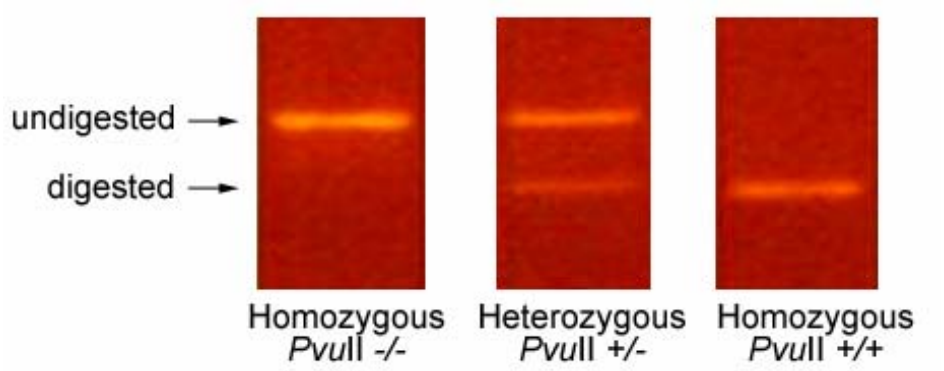

Figure 1 – Genotyping of PvuII polymorphism in intron 6 of LPL gene

Слика 1 – Генотипизација на PvuII полиморфизмот во интронот 6 од LPL генот

The *LPL-PvuII* genotype in this study was successfully determined in 109 patients (83 males and 26 females) of the CAD group included and 32 subjects (25 males and 7 females) of the control group.

Table 1 – Табела 1

Comparison of demographics and other parameters between CAD and the control groups

Поврзаност на демографските и другите параметри меѓу испитаниците со коронарна артериска болест и контролната група

Parameter		Study group n = 109	Control group n = 32	P value
Male/female		**83 (76%)/26 (24%)**	**25 (78%)/7 (22%)**	**p = 0.698**
Age (year)		**59.4 ± 9.2**	**57.9 ± 6.6**	**p < 0.4**
BMI (kg/m^2)		**27.26 ± 3.45**	**26.22 ± 3.11**	**p < 0.1**
Family history of CAD (%)		**42**	**7**	**p < 0.064**
Hiperlipidaemia (%)		**64**	**6**	**p < 0.001***
Diabetes (%)		**31**	**4**	**p < 0.05***
Hypertension (%)		**71**	**23**	**p < 0.713**
Use of antilipidaemic drug (%)		**47**	**8**	**p < 0.049***
Use of Alcohol		**29**	**6**	**p < 0.311**
Smoking (%)				
Never		**38**	**14**	
Ex-smoker		**29**	**8**	**p < 0.770**
Current smoker		**47**	**13**	
Physical activity (%)				
Low		**38**	**11**	**p < 0.740**
Moderate		**65**	**19**	
High		**11**	**5**	
Educational level				
Low (n)		**91**	**26**	**p < 0.485**
High (n)		**23**	**9**	
	***PvuII*$^{+/+}$ or *PvuII*$^{+/-}$**	**83**	**25**	
PvuII				**p = 0.816**
Polymorphism	***PvuII*-/-**	**26**	**7**	
	Unamplifible by PCR	**5**	**3**	

*****Significant *p*-value

The demographics, biochemical, risk factors: hypertension, diabetes, family history of CAD, physical activity, antilipidaemic drugs, alcohol consumption, BMI and other parameters between groups are summarized and compared in Table 1. There was no difference in the sex distribution and mean age between groups. A statistically significant association between the CAD and control

groups was found regarding the presence of hyperlipidaemia ($p < 0.001$), diabetes ($p < 0.05$) and the use of antilipidemic drugs ($p < 0.049$).

The presence of *LPL-PvuII* polymorphism in both investigated groups does not represent a statistically significant risk factor for the appearance of coronary artery disease ($p = 0.816$).

LPL-PvuII genotype distribution and allelic frequencies of *LPL-PvuII* polymorphism among patients with coronary artery disease (CAD) and the control group are presented in Table 2.

Table 2 – Табела 2

Genotype distribution and allelic frequencies of LPL-PvuII polymorphism among patients with coronary artery disease (CAD) and control groups.

Дистрибуција на генотиповите и фреквенциите на алелите за LPL-PvuII полиморфизмот кај испитаниците со коронарна артериска болест и контролната група

Group:	Genotype +/+	Genotype +/-	Genotype -/-	Allele frequency *Pvu*II +	Allele frequency *Pvu*II -
CAD	**n = 25 (22.9%)**	**n = 58 (53.2%)**	**n = 26 (23.9%)**	**0.495**	**0.505**
Control	**n = 5 (15.6%)**	**n = 20 (62.5%)**	**n = 7 (21.9%)**	**0.469**	**0.531**
p-value	**< 0.522**	**< 0.466**	**< 0.823**		

Discussion

In our study the accent was placed on the examination of *LPL* as a gene-candidate for coronary artery disease. A total of 109 Macedonian patients with angiographically confirmed CAD and a control group consisting of 32 patients with coronarographic findings were analysed for the possible connection of *LPL-PvuII* polymorphism with coronary artery disease. In addition, we examined whether *LPL-PvuII* polymorphism could be separated as an independent risk factor for the appearance of CAD, as well as the mutual influence of this polymorphism with the risk factors for the appearance of the disease.

It was reported that the presence of *LPL-PvuII* polymorphism varies among different populations. [9] In our study the analysis showed that the difference in the distribution of *LPL-PvuII* polymorphism between the examined and the control group of patients, does not represent a significant risk factor for appearance of CAD ($p = 0.816$; table 1).

The genotype *LPL-PvuII*+/+ has been found to be associated with hypertrigliceridaemia, arteriosclerosis and pancreatitis, while the *LPL-PvuII*-/- genotype is dominant in the healthy population. Population-based, long-term pros-

pective studies and large-scale clinical trials have incontrovertibly demonstrated the association of lipid abnormalities with the appearance of CAD. According to these trials at least half of the variation in serum cholesterol and other lipids can be explained by genetic variation [9, 22, 30]. In the study of Wang, *et al*., [35] as well as in the Chinese population [15], no connection of *LPL-PvuII* polymorphism to increased values of triglycerides was found, while examinations of the Japanese [7] and French [11] population showed a significant association of triglyceride level with *LPL-PvuII* polymorphism. In the study of the Turkish population, *LPL-PvuII* polymorphism was identified as being associated with increased values in total lipids. [4]

In this study, we did not evaluate the *LPL-PvuII* polymorphism association with altered plasma lipids or elevated triglyceride levels since previous studies had failed to do so or had led to inconsistent results [16, 29, 32]. Instead, we investigated the possible association of this *LPL* polymorphism to angiographically verified CAD in patients. In addition, we evaluated whether this polymorphism can be used as an independent genetic risk factor for CAD in the Macedonian population. In the available literature to date, among the known *LPL-PvuII* genotypes, a very modest association between individuals who are homozygous *LPL-PvuII* genotype and CAD was reported [3].

Among the angiographically confirmed CAD and the control groups tested for the *LPL-PvuII* genotypes, an *LPL-PvuII* + allele frequency of 0.495 and 0.469 was obtained, respectively, obeying the Hardy-Weinberg equilibrium. This finding indicates no significant differences between the prevalence of the *LPL-PvuII* genotypes in both study groups, suggesting a lack of association of *LPL-PvuII* polymorphism and CAD. The *LPL-PvuII* + allele frequency rates observed in our population were comparable to the rates reported for other populations, which ranged from 0.49 to 0.64 [3, 14, 25, 33].

However, the homozygous genotype (*LPL-Pvu*II +/+) was more prevalent in the CAD group (22.9%) in comparison with the control group (15.6%), respectively.

MI and other forms of atherosclerotic CAD are the leading causes of death in men and women [12]. In domestic and foreign literature, authors and studies emphasize that in the etiology of CAD, many risk factors are involved and result in a complex interaction between genetic predisposition and environmental influences [1, 3, 8, 29]. The environmental factors determine the intensity and result of the clinically manifested CAD. In our study we analysed the connection of the major risk factors between groups and we found a significant influence of hyperlipidaemia ($p < 0.001$), diabetes ($p < 0.05$) and the use of antilipidaemic drugs ($p < 0.049$; table 1).

These preliminary observations with a small size sample need to be verified in a larger study group, including additional gene mutations and polymorphisms. Ethnic differences cannot be excluded, either.

Conclusion

The presence of *LPL-PvuII* polymorphism in CAD and the control groups does not represent a statistically significant risk factor for coronary artery disease in the investigated Macedonian population.

It remains for other gene polymorphisms and mutations to be researched by contemporary molecular, biological, and genetic techniques in order to shed light on the etiopathogenetic mechanisms of the heart and vascular diseases.

Acknowledgment

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Резиме

ПОВРЗАНОСТ НА *PvuII* ПОЛИМОРФИЗМОТ ВО ГЕНОТ ЗА ЛИПОПРОТЕИН ЛИПАЗА СО КОРОНАРНА АРТЕРИСКА БОЛЕСТ КАЈ МАКЕДОНСКАТА ПОПУЛАЦИЈА

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Вовед: Во етиологијата на коронарната артериска болест се инволвирани повеќе фактори кои се резултат на комплексна интеракција меѓу генетската предиспозиција и енвиронменталните влијанија. Липопротеин липазата (*LPL*) игра многу важна улога во липидниот метаболизам, хидролизирајќи ги триглицеридите во хиломикрони и липопротеини со многу ниска густина VLDL. *PvuII* полиморфизмот на *LPL* генот е честа варијанта и го зголемува нивото на триглицеридите и ризикот од појава на коронарна артериска болест.

Цел: приказ на *LPL-PvuII* полиморфизмот како независен ризик фактор и како предиктор на коронарната артериска болест кај македонската популација.

Материјал и методи: Во студијата рандомизирано се вклучени 109 испитаници со коронарна артериска болест (КАБ), и тоа 83 мажи и 26 жени, кои се лекувани на Клиниката за кардиологија и кај кои ангиографски беше документирана стеноза на коронарните артерии над 70% од луменот на артеријата. Контролната група ја сочинуваат 32 испитаници (25 мажи, 7 жени), кај кои ангиографски беше документиран уреден коронарографски наод. Најголем број од испитаниците се на возраст од 50–59 години, при што возраста на испитаниците кај КАБ групата е во просек 59,4, а додека кај контролната група 57,9 год. Во студијата е применета PCR амплификација со последователна рестрикциска дигестија за да се детектира *PvuII* полиморфизмот во интронот 6 од *LPL* генот кај испитуваната и контролната група.

Резултати: Статистички сигнификантна разлика меѓу групата со КАБ и контролната група беше најдена во однос на следните ризик-фактори: хиперлипидемија ($p < 0,001$), дијабетес ($p < 0,05$) и користење на антилипидемици ($p < 0,049$). Присуството на *LPL-PvuII* полиморфизмот и кај испитуваната и кај контролната група не претставуваше статистички сигнификантен ризик-фактор за појава на КАБ ($p = 0,816$).

Алелните фреквенции за *PvuII*+ алелата изнесуваа 0,495 и 0,469 кај ангиографски потврдените пациенти КАБ и кај контролната група испита-

ници, соодветно. Ваквиот наод не индицира сигнификантни разлики во преваленцијата на *LPL-PvuII* генотиповите кај двете испитувани групи, што упатува на отсуство на поврзаност на *LPL-PvuII* полиморфизмот со КАБ.

Сепак, хомозиготниот генотип (*Pvu*II +/+) беше позастапен кај КАБ групата (22,9%), во споредба со контролната група (15,6%).

Заклучок: Во нашата студија *PvuII* полиморфизмот во *LPL* генот не беше идентифициран како независен ризик-фактор за појава на КАБ.

Клучни зборови: *LPL-PvuII* полиморфизам, коронарна артериска болест.

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