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CASE-CONTROL STUDY OF APOE GENE POLYMORPHISM IN YOUNG CHD PATIENTS AND CONTROLS IN THE SERBIAN POPULATION

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Abstract – Apolipoprotein E displays polymorphism with three common alleles, e2, e3, and e4. The aim of this research was to determine apoE gene polymorphism in a group of healthy patients and a group of patients with CHD, and to reveal the relation between anthropometric and biochemical parameters and the apoE genotype. In CHD group significantly higher values of blood pressure, waist circumference, BMI and fat %, triglycerides, insulin (HOMA IR) and CRP were found. A statistically significant higher presence of the e3e4 genotype and e4 allele was detected in the CHD group. Statistically significant differences between waist circumference, BMI, insulin and HOMA IR were found between subjects with e3e3 and e3e4 genotypes.

Key words: Apolipoprotein E, coronary heart disease, Serbia

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INTRODUCTION

Coronary heart disease (CHD) is a complex multifactorial state. Different exogenous and endogenous factors influence the development of CHD. Atherosclerosis is a major change in the development of CHD. Major attention in recent cardiovascular disease research has been given to understanding the molecular basis of atherosclerosis (Greenow et al., 2005). Apolipoprotein E (ApoE) is a structural component of both chylomicrons and very low-density lipoprotein (LDL) remnants. Apolipoprotein E is thought to play a central role in atherosclerosis by participating in overall plasma cholesterol homeostasis, mainly via the regulation of the hepatic uptake of remnant lipoproteins, by facilitating cholesterol efflux from macrophage foam cells within atherosclerotic lesions, and by modifying inflammatory responses (Dimitrios et al., 2006). The ApoE protein also carries out additional functions such as the prevention of platelet aggregation and the inhibition of the proliferation of T-lymphocytes and endothelial cells. ApoE therefore plays a key protective role in atherosclerosis (Rall and Mahley, 1992). In addition, ApoE participates in neuronal repair in the central nervous system (Weisgraber and Mahley, 1996; Al-Khedhairy, 2004).

The single polypeptide chain of ApoE (299 residues, 34.2 kDa molecular mass) contains two independently folded domains that are approximated by two thrombolytic fragments (residues 1–191 and

216–299), which are involved in different functions of the protein (Weisgraber, 1994; Segall et al., 2002). The 22 kDa N-terminal fragment contains the LDLR binding domain (residues 136–150) and the C-terminal 10 kDa fragment represents the major lipid-binding region.

Apolipoprotein E displays genetic polymorphism with three common alleles, e2, e3, and e4, in a single-gene locus on the chromosome 19q13.2 that gives rise to 3 homozygous (apoE2/2, apoE3/3, apoE4/4) and 3 heterozygous genotypes (apoE2/3, apoE2/4, apoE3/4) (Davignon et al., 1988; Dimitrios et al., 2006). A higher number of polymorphisms in the apoE gene have been identified, and the functional consequences of these changes along with their association with cardiovascular disease have been investigated. ApoE is a polymorphic protein with three common isoforms and more than 20 rare variants (Rall and Mahley, 1992). ApoE3 (Cys112, Arg158) is considered to be the parent form and ApoE4 (Arg112, Arg158) and ApoE2 (Cys112, Cys158) are variants. This polymorphism was found to be relevant for the ApoE plasma level, the receptor binding affinity of ApoE, plasma lipid and lipoprotein concentrations, and CAD (Koch et al., 2004).

Evidence exists to suggest that the variability of apoE has differential effects on the atheroprotective potential attributed to ApoE. The apoE allele e4 is associated with increased LDL cholesterol levels and decreased ApoE plasma concentrations (Davignon et al., 1988; Stokic et al., 2008). Conversely, the e2 allele is associated with reduced LDL cholesterol levels and higher ApoE plasma concentrations. Among patients with CAD, the e4 allele was related to more severe disease and the e2 allele to less severe disease (Koch et al., 2004). The presence of the e4 allele has been associated with increased death rates in patients with CAD. The cardiovascular risk attributed to the e4 allele may be related, at least in part, to a lower antioxidant activity of the ApoE4 isoform (112Arg/158Arg) compared to that of the ApoE2 isoform (112Cys/158Cys) or the ApoE3 isoform (112Cys/ 158Arg) (Koch et al., 2004). Another SNP of apoE, –219G/T, located in the promoter of apoE, was reported to be significantly associated with apoE promoter activity, ApoE plasma concentration, and CAD (Lambert et al., 2000; Hirashiki et al., 2003).

Allelic frequencies differ in different populations (Eichner et al., 2002). Furthermore, wide range population studies have indicated interethnic variations in apoE polymorphism. At a global level, higher frequencies of the e2 allele were observed in Africa and Oceania (0.099 and 0.083 and 0.111 and 0.052, respectively). Similarly, e4 allele averages were higher in Oceania (0.221 and 0.149) and Africa (0.209 and 0.090), while Indian and Asian populations showed the highest frequencies of e3 allele. The coefficient of gene differentiation was found to be highest in South America (9.6%), although the highest genetic diversity was observed in Oceania (48.7%) and Africa (46.3%). ApoE e2 revealed a statistically significant decreasing cline towards the north in Asia, which is not compatible with the coronary heart disease statistics in this continent. ApoE e4 showed a significant increasing cline in North European populations (Singh et al., 2006). It was shown that allele e4 was present in higher frequency in the Swedish population (20.3%), compared to other European populations (Eggertsen et al., 1993). In the healthy Serbian population the allele e3 was present with 73.6% frequency, and e2 and e4 with 14.9% and 11.5%, respectively (Stanković et al., 1999).

The investigation of apoE polymorphism in CHD patients from Serbia has not been done before. According to the MONICA study (Monitoring Trends and Determinants in Cardiovascular Diseases), the population of Vojvodina (northern Serbian Province) was in the worst position from the start of study in frequency of CHD. In the majority of other research centers included in the study, the research center in Vojvodina registered an increase of mortality rates due to CHD. The mean total cholesterol level registered in the beginning was 36th place for men and 31st for women (out of 38). At the end of the study the mean total cholesterol level increased and rose to 2nd place for men and 3rd for women (Jakovljevic and Planojevic, 2005).

The majority of studies dealing with the association of the apoE genotype and coronary heart disease development were performed either in general populations (Eggersten et al., 1993; Lahoz et al., 2001; Singh et al., 2006) or in the elderly (Al- Khedhairy, 2004; Stengård et al., 1999). Since inheritance can be considered to be an unchangeable risk factor, it seemed interesting to investigate the influence of the apoE genotype to the development and occurrence of CHD in the younger population.

Therefore, the aim of this research was to determine apoE gene polymorphism in a healthy control group and a group of patients with CHD, both comprising men from 18 to 45 years of age, and to reveal the relations between different anthropometric and biochemical parameters and the apoE genotype.

MATERIALS AND METHODS

Sixty individuals, aged from 18 to 45, from the northern Serbian province of Vojvodina were involved in the study. The control group consisted of 30 unrelated healthy men. In group of patients with CHD, 30 unrelated men were included who had had no hypolipidemic treatment. These patients underwent complete medical examination and CHD was determined by coronarography or MDCT (multi-slice detector computed tomography) coronarography. Cases with a previous myocardial infarction were included in this group. The study was performed according to the Declaration of Helsinki and informed consent was obtained from all participants.

Anthropometric measurements (body weight, body height and waist circumference), body fat mass estimation and cardiovascular risk factors assessment (systolic and diastolic pressure, fasting serum lipids levels, fasting serum glucose levels, insulinemia, C-reactive protein) were done. With the subjects wearing light indoor clothes and no shoes body weight (BW) and body height (BH) were measured using a calibrated beam-type balance to the nearest 0.1 kg and a Harpenden anthropometer to the nearest 0.1 cm, respectively, and body mass index (BMI) was calculated [$BMI = BW/BH^2$ (kg/m^2)]. Waist circumference was measured using flexible tape to the nearest 0.1 cm at the level midway between the lowest point on the rib margin and the highest point on the iliac crest. Body fat mass was assessed with the bioelectrical impedance method using a Tanita TBF-310 Body Composition Analyzer (Tanita Corporation, Tokyo, Japan). Systolic (SBP) and diastolic (DBP) blood pressure were measured in the fasted state, early in the morning, using sphygmomanometer by Riva-Rocci, in sitting position after a 10-15 min rest period. As the most valid value, the mean of three measurements was taken. Total cholesterol and triglycerides were determined using the commercial kit Boehringer Manheim GmbH. HDL-cholesterol was estimated using the method of precipitation with Na-phosphorvolframate, while LDL-cholesterol was calculated using the formula by Friedewald et al. (1972). Fasting plasma glucose was measured using the Dialab glucose GOD-PAP method. All blood samples were drawn after an overnight 12-h fast. Estimation of insulin resistance was performed by HOMA IR (homeostasis model assessment of insulin resistance) according to the formula (glucose x insulin)/22.5 (Matthews et al., 1985). In order to ensure accuracy of measurement, subjects were told not to eat or drink within 4 h of the test, not to exercise within 12 h of the test, to urinate within 30 min of the test and not to consume alcohol within 48 h of the test.

DNA was extracted from blood by standard procedures utilizing the QIAGENE kit. Primers were designed to amplify the coding sequence of the fourth exon of the apoE gene. The following primers were used: F4 (5' CTA CAG AAT TCG CCC CGG CCT GGT ACA C 3') and F6 (5' TAA GCT TGG CAC GGC TGT CCA AGG A 3'). PCR amplification was done according to the modified method of Hixon and Vernier (1990). A sample of 100 ng of genomic DNA was used as a template in a 25 μL reaction solution with 0,4μM each primer, 200μM dNTPs (dATP, dTTP, dCTP, dGTP), 1x Buffer, 1,25U Taq polymerase, 10% DMSO and 2,5 mM $MgCl_2$. Genomic DNA was amplified for 30 cycles. Each cycle consisted of 1 min at 95°C, 1 min at 60°C and 1 min at 72°C. PCR products were digested with *HhaI* over a period of 3

h at 37°C. The fragments obtained were separated by electrophoresis on a 4% agarose gel in TAE buffer, and visualized by ethidium bromide fluorescence. The genotype of each person was determined from the RFLP-PCR profile.

All continuous variables are expressed as means ± standard deviation (SD). Genotype and allele frequencies were obtained by direct count. Student's t test, Principle Coordinate Analysis and χ^2 test were used in STATISTICA, version 8.0. (2008). Student's t test was used for testing differences between the means of anthropometric and biochemical parameters of the two groups. PCO analysis was applied in order to determine the parameters distinguishing the two analyzed groups. The differences in genotype and allelic frequencies between the two analyzed groups were determined using the χ^2 test. Probability values of less than 0.05 were regarded as statistically significant.

RESULTS

Differences in the average values of measured anthropometric and biochemical parameters between the group with CHD and control group were tested and only significant differences are presented in Table 1. In the CHD group, the apoE genotype was successfully determined for all subjects. In the control group, the apoE genotype could not be determined

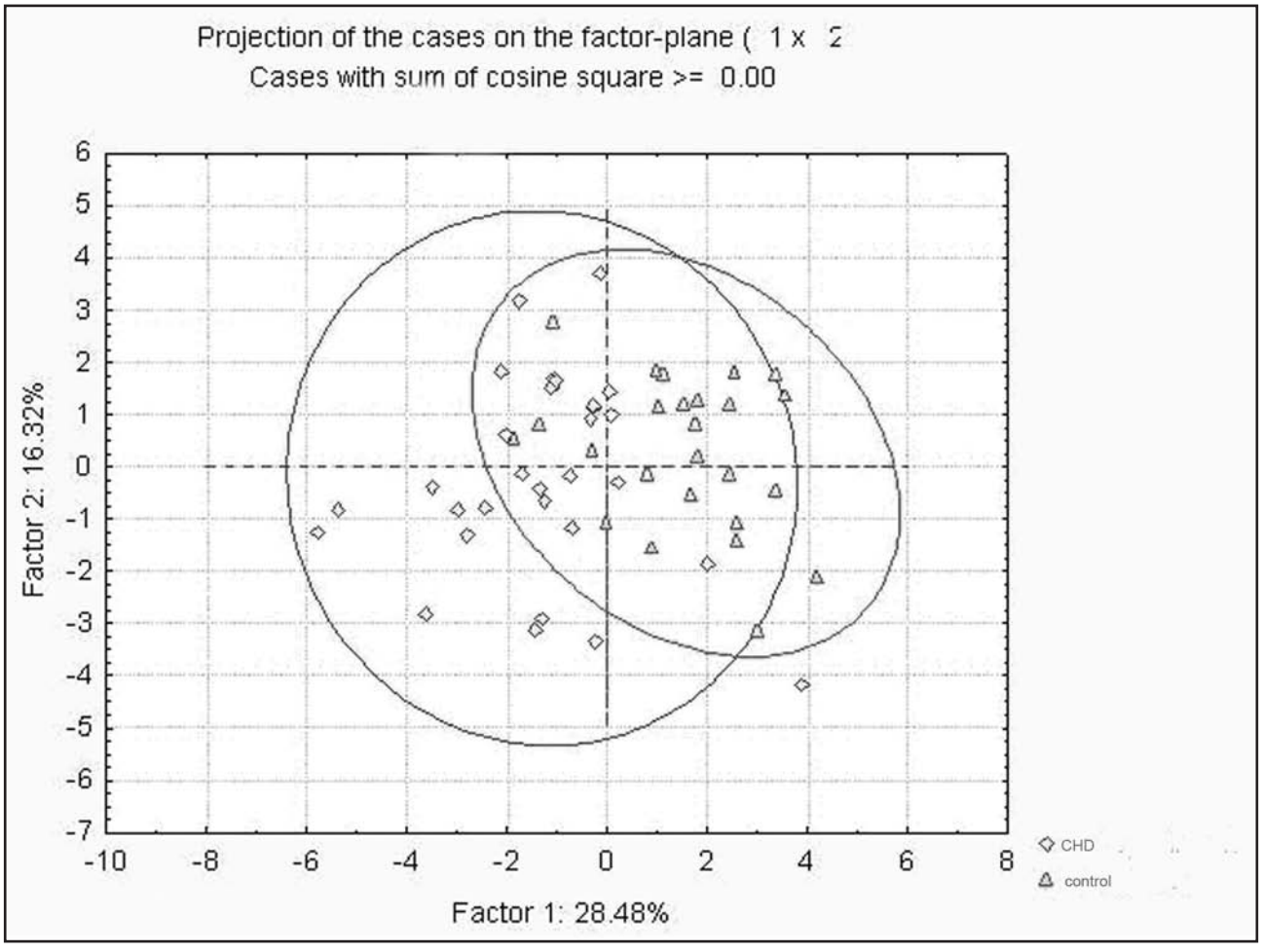

Fig. 1. PCO analysis of measured parameters between CHD and control groups

Table 1. Differences in anthropometric and biochemical parameters and in allelic and genotype distribution between CHD and control group. For each continuous variable the results are presented as mean ±SD, median and range.

Parameter		CHD	Control	p
		138.2±9.7	125.8±8.9	0.00001
Systolic pressure** (mmHg)		140	125	
		110 – 160	110 – 140	
		89.5±9.0	84±7.3	0.017
Diastolic pressure* (mmHg)		90	80	
		70 – 100	70 – 100	
		105.2±9.1	96.5±8.9	0.0008
waist circumference (cm)**		106	96	
		80 – 123	80 – 119	
		29.3±2.8	27.3±3.6	0.032
BMI (kg/m^2)*		29.2	27	
		21.5 – 36.5	22.5 – 36.3	
		25.9±3.8	21.1±5.2	0.0002
Fat %*		25.5	20.6	
		14.9 – 36.5	12.5 – 30.4	
		30.1±26.5	9.5±4.2	0.0003
Insulin (μlU/ml)**		21.5	9	
		2.11 – 103	3.4 – 21.1	
		6.5±5.8	2.1±1.1	0.0004
HOMA IR **		4.42	2	
		0.43 – 22.37	0.7 – 5.06	
		5.1±5.3	1.5±2	0.003
CRP (U/ml)**		3.95	0.6	
		0.36 - 31	0.1 – 8.8	
	e3e3	16(53.33%)	19 (76%)	0.038*
	e3e4	13(43.33%)	4 (16%)	
Genotype distribution	e2e4	1(3.34%)	0	
	e4e4	0	1 (4%)	
	e2e3	0	1 (4%)	
	e3	45 (75%)	43 (86%)	0.011*
Allele distribution	e4	14(23.33%)	6 (12%)	
	e2	1 (1.67%)	1 (2%)	

* p<0.05; **p<0.01

for 5 of the subjects, even though the analysis was repeated. ApoE gene amplification was unsuccessful for 2 subjects, and genotype determination could not be performed for 3 subjects. Statistical analysis showed significant differences in genotype distribution between the two groups (Tab. 1). A statistically significant higher presence of the e3e4 genotype was detected in the CHD group. Allele distribution analysis revealed differences in allelic frequencies as well (Tab. 1). A significantly higher frequency of the allele e4 was found in the CHD group (p=0.019).

PCO analysis defined the parameters distinguishing between the CHD and control group in two dimensions and these are: waist circumference, BMI, fat %, index of atherosclerosis, insulin and HOMA IR (Fig. 1.).

In both groups five genotypes were detected. Genotypes e2e4, e4e4 and e2e3 were found in only

Table 2. Differences in anthropometric and biochemical parameters between e3e3 and e3e4 genotype groups. For each parameter the results are presented as mean ±SD, median and range.

Parameter	e3e3	e3e4	p
Waist circumference (cm)*	99.1±9.9	105.5±8.8	0.028
	100	106	
	80-118	91-123	
BMI (kg/m²)*	27.9±3.08	29.7±3.2	0.048
	27.8	28.4	
	21.5-36.3	24.5-36.5	
Fat %*	23.0±5.0	25.5±4.3	0.087
	24.5	25.2	
	12.5-30.6	16.1-36.5	
Insulin (μlU/ml)**	15.7±19.3	33.3±25.0	0.007
	9	23.1	
	2.11-103	4.72-79.9	
HOMA IR **	3.4±3.8	7.3±6.0	0.006
	2	4.62	
	0.43-17.85	1.05-22.37	

* $p<0.05$; ** $p<0.01$

one person each. The remaining 52 subjects with e3e3 and e3e4 genotypes were divided into two groups according to their genotype, and regardless of their belonging to the CHD or control group. Average values of anthropometric and biochemical parameters were tested for differences and significant results are presented in Table 2.

PCO analysis in two dimensions defined waist circumference, BMI, fat %, index of atherosclerosis (IA) (LDL/HDL), insulin and HOMA IR as factors distinguishing the e3e3 and e3e4 groups (Fig. 2).

DISCUSSION

ApoE gene polymorphism was examined in 60 subjects, divided into two groups: one of 30 patients with CHD and the other a healthy control group. A detailed medical examination, with anthropometric and biochemical parameter measurements, was performed for each person. The ApoE genotype was successfully determined in 55 (91.7%) subjects. Unsuccessful PCR amplification in 2 subjects might be due to a mutation in the primer binding region, and the unsuccessful determination of the apoE genotype in 3 subjects might be due to a novel mutation in exon 4 that created new restriction sites and unusual RFLP-PCR profiles. However, these presumptions must be further evaluated.

In this research the apoE allelic frequencies in the healthy control group were: e3 - 86%, e4 - 12% and e2 -2%. In the CHD group the allelic distribution was the following: e3 - 75%, e4 - 23.33%, and e2 - 1.67%. Compared to previous analysis of a healthy Serbian population (Stanković et al., 1999), the higher frequency of the e3 allele in the control group and the significantly higher frequency of the e4 allele in the CHD group compared to the control, were detected.

Our study analyzed younger men (less than 45 years old) and during the two year period of research, 30 patients could be included in this study (mean age 35.2±4.5). Considering the age limitation, we found this number quite satisfactory. First, it was expected that coronary heart disease is present in lower frequency in younger populations. Furthermore, some registered cases couldn't be included in the research due to either the hypolipidemic medication treatment they were taking in a previous time period or

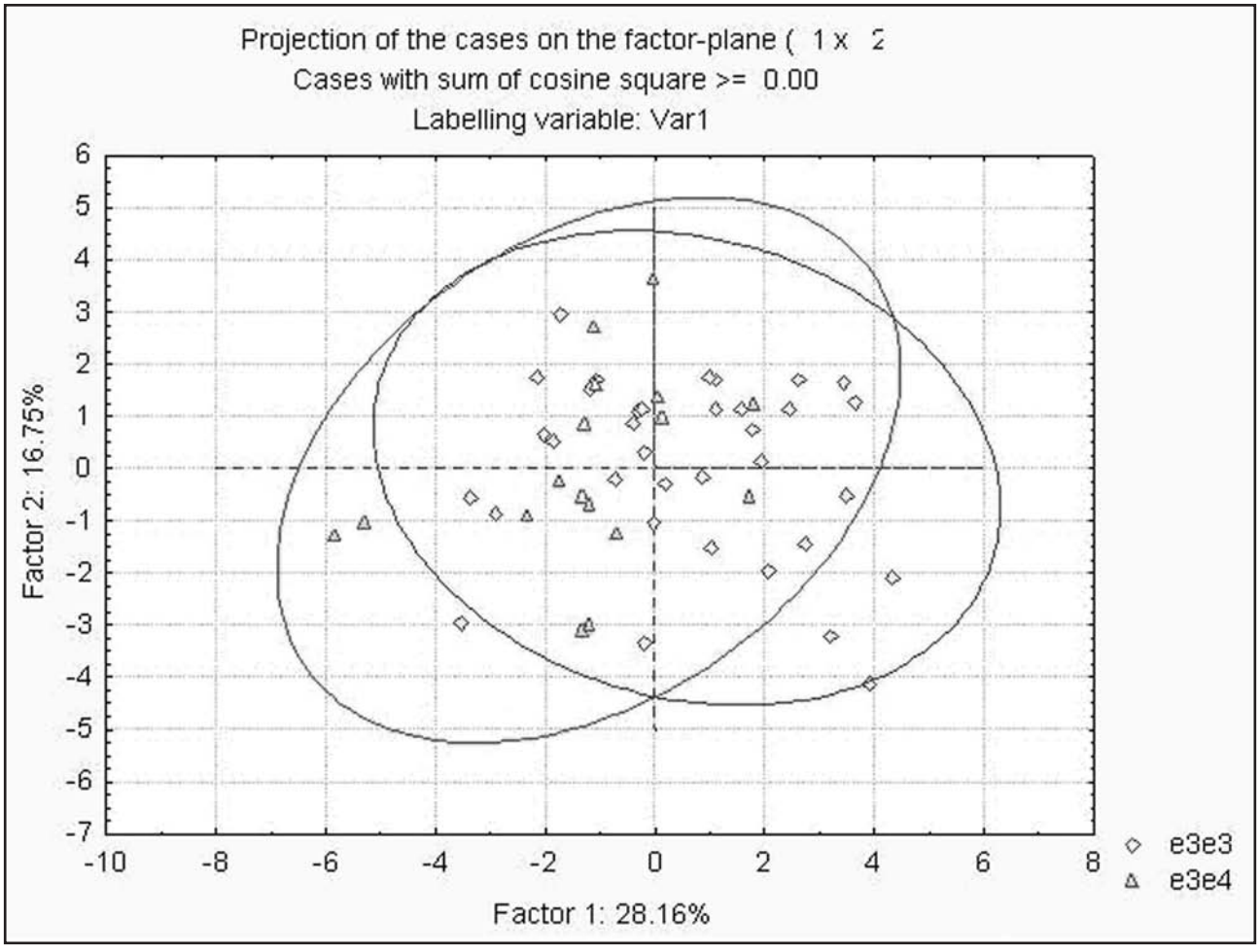

Fig. 2. PCO analysis of measured parameters between e3e3 and e3e4 groups.

due to the fact that some patients didn't give signed informed consent. Nonetheless, this small sample size revealed expected differences in several anthropometric and biochemical parameters.

In the CHD group significantly higher values of blood pressure, waist circumference, BMI and fat %, as well as, levels of triglycerides, insulin (HOMA IR) and CRP, were found. Even though no statistically significant difference was found for some expected measurements, such as LDL cholesterol or total cholesterol, PCO analysis detected the index of atherosclerosis (IA) as a distinguishing factor between the two groups. Since IA directly depends on LDL cholesterol, it can be concluded that this parameter also differs between these groups.

Frikke-Schmidt et al. (2004) showed significant differences between CHD patients and healthy control (men and women) in total cholesterol, LDL cholesterol, HDL cholesterol and triglyceride levels, and BMI and blood pressure. In our research we confirmed findings for blood pressure, BMI and triglycerides. McCarthy et al. (2004) also confirmed significant differences in BMI and blood pressure between CHD patients and controls. Furthermore, März et al. (2004) detected significant differences in blood pressure between patients with coronary artery disease

and controls, and no differences in LDL cholesterol and HDL cholesterol, similar to our research. Also, Kolovou et al. (2002) did not register differences in cholesterol levels between CHD and control groups. However, studies in which significant differences in cholesterol levels were reported, analyzed larger numbers of subjects (1309 (März et al., 2004) and 9238 (Frikke-Schmidt et al., 2004)). Therefore it can be concluded that detected differences partially depend on the size of the analyzed population and the origin of the population. In our research the Student t test revealed p=0.06 for testing difference in the LDL cholesterol level between the CHD and control groups, indicating that a larger number of subjects would lead to a statistically significant difference for this parameter.

As was expected, in the CHD group a statistically significant higher percentage of the e3e4 genotype and e4 allele was found. Other studies revealed a similar correlation between e4 allele presence and CHD. In Finnish men older than 65 a higher CHD mortality was found in those with an e3e4 genotype compared to those with an e3e3 genotype (Stengård et al., 1999). The Framingham Heart Study found that the presence of the e4 allele in men shows a positive correlation with CHD development, and no such correlation was found in women (Lahoz et al., 2001). Furthermore, it was detected in the Framingham Heart Study that apoE gene polymorphism and CHD risk are not correlated in non-smoking subjects, while this relation was positive in smoking subjects (Talmud et al., 2005). A significant correlation between the apoE gene and CHD was confirmed in the analysis of 111 candidate genes (McCarthy et al., 2004). It was also shown that allele e4 of the apoE gene has an additive effect in myocardial infarction (Yamada et al., 2002). On the other hand, several studies did not confirm a correlation between e4 allele and CHD (Kolovou et al., 2002).

Statistically significant differences in waist circumference, BMI, insulin and HOMA IR were found in our research between subjects with e3e3 and e3e4 genotypes. Numerous population studies have detected the effect of apoE gene polymorphism on lipid levels. Eggertsen et al. (1993) and Chen et al. (2003) showed a positive correlation between e3 and e4 alleles and total cholesterol level, as well as between e4 allele and LDL cholesterol level. On the other hand, Guerra et al. (2003) did not find a difference in lipid concentration between e3e3 and e3e4 genotype groups. In our research the level of LDL cholesterol in the e3e3 genotype group was 3.0±0.8 mmol/l, and in the e3e4 genotype group, 3.5±1.0 mmol/l (p=0.098). The confidence interval in our research is quite wide (OR 2.23, CI 0.78 – 6.33), indicating that a total of 60 subjects is not sufficient for detecting a difference for this parameter. In other studies that have found this difference the number of analyzed subjects was from 250 to 10000.

As previously mentioned, the lower number of subjects was partially defined by the study design (male patients younger than 45). Since only 30 men could be included in the CHD group, the control group was created to match the CHD patients group (the mean age in the control group was 32.8±6.1). Statistical analysis showed that a difference in the LDL cholesterol level can be revealed with a higher number of patients.

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