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Original paper

The E4 allele of the apoE gene acts as a determinant of Metabolic syndrome in men

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Summary. Metabolic syndrome (MetS) is a cluster of conditions associated with increased blood pressure, blood sugar levels, excess body fat around the waist, and abnormal cholesterol or triglyceride levels. These conditions occur together, increasing the risk of heart disease, stroke and diabetes. It has been reported that genetic variability at several loci is associated with an increased risk of metabolic syndrome. The human apoE gene, coding apolipoprotein E, has three common polymorphisms in human populations: e2, e3 and e4, which are associated with impaired lipid metabolism. In the present study, apoE gene polymorphism was evaluated in relation to MetS in the male population of Vojvodina. A MetS patient group was examined for apoE variants in relation to biochemical and anthropometric parameters. Total genomic DNA was isolated from blood, and genotyping of apoE polymorphism was determined by PCR-RFLP. Regarding all examined parameters, significantly higher values were detected in men with MetS who were carriers of the e4 allele in their genotype comparing to other allele carriers. A positive correlation was found between the presence of the e4 allele and all measured parameters. These results suggest that the e4 allele may act as a determinant for the development of MetS.

Key words: apoE polymorphism, e4 allele, lipid metabolism, male population, metabolic syndrome.

INTRODUCTION

Metabolic syndrome (MetS) is a set of factors that increase systolic and diastolic blood pressure (SBP, DBP), blood sugar levels, cholesterol and triglycerides, and leads to an increase in the amount of visceral fat tissue. These specific set of factors occur together, due to a physiological cascade of events, increasing the risk of heart disease, stroke, and diabetes (Vučinić et al. 2017). Genetic variability within several loci has been shown to have a strong impact on the emergence and development of MetS. Over the past decade, MetS has become more common among men than women. The fact that fat accumulation, as a component of MetS, has a different obesity type between females and males contributes to differences in the prevalence of the syndrome between the sexes. The capacity for lipogenesis and lipolysis varies in relation to body region and gender, due to differences in secreted hormones. Pre-menopausal women are more prone to subcutaneous fat accumulation, while older women and men are more prone to so-called central obesity. The risk of cardiovascular disease (CVD) and the onset of diabetes mellitus type 2 (DMT2) should be considered in relation to sex, where greater attention should be given to elevated triglycerides (TG) in women and measures of waist circumference (WC) in males (Krotkiewski et al. 1983; Regitz-Zagrosek et al. 2006; Mosher et al. 2008; Vučinić et al. 2014).

So far, more than 70 genes that contribute to obesity have been described, and some polymorphisms have been found to have different effects in relation to sex, which suggests that inherited factors may have a different impact on men and women. The established facts indicate that pathophysiology differs in relation to gender, and is therefore also

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related to risk for CVD and DMT2. The above mentioned differences may be significant for the prevention, diagnosis and treatment of the syndrome (Power and Schulkin 2008; Vučinić et al. 2014).

The human apoE gene is 3.7kb long; it consists of four exons and three introns and is located on chromosome 19q13.2. The apoE gene belongs to a family that includes two other structural genes, apoC-I and apoC-II, as well as one pseudogene apoC-I '. To date, three alleles have been detected within this gene (e2, e3 and e4) that encode three isoforms of apolipoprotein E in plasma (E2, E3 and E4) as well as several rare variants of the sequence of this gene, which differ in binding affinity for the apo-B / E receptor. These three alleles define six possible genotypes in human populations. Existing genetic variability results in cysteine-to-arginine changes at positions 112 and 158 within the amino acid sequence of the ApoE protein. These changes have been shown to be due to base substitutions within the fourth exon of apoe gene. Allele e3 is the most common human allele and encodes a cysteine-like isoform at position 112 (112cys) and an arginine at position 158 (158arg). This isoform of ApoE is associated with elevated levels of total cholesterol and beta-lipoprotein. Isoform E2 of apolipoprotein E (112cys and 158cys) is correlated with reduced binding affinity for cellular receptors and reduced levels of cholesterol and beta-lipoprotein. Allele e4 is characterized by a combination of 112arg and 158arg and the presence of this allele in the genotype correlates with increased concentrations of low density lipoproteins (LDL) and lower concentrations of high density lipoprotein (HDL) (Djan 2009; Vučinić et al. 2014; Xu et al. 2014; Calvo et al. 2018).

In the presented study, we evaluated apoE gene polymorphism in relation to MetS in a male population from Vojvodina. MetS patient groups were examined for apoE variants in relation to tested biochemical and anthropometric parameters.

MATERIALS AND METHODS

The study included 50 males, 30-65 years of age, who were patients at the Clinical Center of Vojvodina, Novi Sad, Serbia. Signed informed consent was obtained from all participants, and the study was approved by the Institutional Ethics Review Committee. The research was performed according to the Declaration of Helsinki. Criteria for inclusion in the research were determined by the IDF (International Diabetes Federation) definition. Each respondent had a WC above 94 cm, and at least two of the following factors: TG ≥1.7 mmol, HDL ≤1.04 mmol, SBP ≥130 mmHg or DBP ≥85 mmHg, hyperglycemia defined as fasting plasma glucose of at least 5.6 mmol/L (Kassi et al. 2011).

Each person was physically examined for determination of systolic (SBP) and diastolic blood pressure (DBP) using a sphygmomanometer by Scipione Riva-Rocci in a seated position after 10-15 minutes of rest.

Anthropometric measurements

The following anthropometric measurements were performed: body mass (BM) 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. Body mass index (BMI) was calculated [BMI = BM/BH^2 (kg/m^2)]. Waist circumference (WC) was measured using a flexible measuring tape (measuring accuracy 0.1 cm) in the standing position, between the lowest point on the rib margin and the highest point on the iliac crest.

Determination of biochemical parameters

Using standard laboratory methods, the following parameters were measured: lipid and lipoprotein status (total cholesterol, HDL cholesterol, LDL cholesterol, TG, atherosclerosis index), fasting plasma glucose, insulinemia, C reactive protein (CRP). Assessment of the degree of insulin resistance was performed using the HOMA-IR (homeostasis model assessment of insulin resistance) according to the formula (glycemia (mmol / 1x insulinemia (uIU / I) / 22.5) (Mathews et al. 1984), which gives values of insulin sensitivity and functional capacity of β - pancreatic cells (percentage expressed in relation to normal). Total cholesterol and TG were determined using a commercial Boehringer Manheim GmbH kit. HDL cholesterol was determined by the precipitation method with sodium phospho-wolframate, while LDL cholesterol was calculated using the formula according to Friedewald et al. (1972). The plasma glucose level was determined using the Dialab glucose GOD-PAP method. The level of CRP was determined by the Latex immunoturbidimetric method. All blood samples were taken after a 12-hour fast.

Genotyping of the apoE gene

Total genomic DNA was extracted from full blood collected in EDTA tubes using phenol chloroform isoamylalcohol extraction (Kocher et al. 1989). Genotyping of each subject was done anonymously. Part of the fourth exon of apoE gene was cloned using a standard set of primers, F4 (5'A CAG AAT TCG CCC CGG CCT GGT ACA C 3 ') and F6 (5'TAA GCT TGG CAC GGC TGT CCA AGG A 3'), for each participant. Polymerase chain reaction (PCR) was performed according to a method modified from Hixon and Vernier (1990) using a Eppendorf Thermocycler. PCR reactions were conducted in a final volume of 25 μL, and contained approximately 100 ng of genomic DNA, 0.4 μM

of each primer, 200 μM dNTPs (dATP, dTTP, dCTP, dGTP), 1.5U Taq polymerase, 1 × Taq buffer, 2.5 mM $MgCl_2$ and 0.5 × Q solution. Initial denaturation was adjusted to 95 °C for 5 minutes, followed by 30 cycles of denaturation phase at 95 °C for 1 minute, annealing phase at 60 °C for 1 minute and elongation at 72 °C for 2 minutes. Final extension at 72 °C for 10 minutes was applied. The PCR products were digested with HhaI endonuclease for 3 hours at 37 °C. The apoE fragments obtained were separated by electrophoresis on a 4.0% MetaPhor agarose gel in 1 × TAE buffer and visualized under a UV transiluminator for each individual. The genotype of each person was determined from the restriction fragment length polymorphism (RFLP) profile.

Statistical analysis of the obtained data was performed using STATISTICA package version 10.0 (StatSoft 2011). For all parameters descriptive statistics was performed, and absolute frequencies were calculated for alleles and genotypes. A T-test for independent samples was used to investigate the significance of differences among arithmetic means of anthropometric and biochemical parameters between genotypic groups of tested individuals. For all statistical tests the significance threshold value was 0.05.

RESULTS

A fragment of the fourth exon of the apoE gene was cloned successfully for each individual and a product of 245 bp length was obtained. Using the RFLP method, the genotype of each individual was determined according to visualized band patterns on the gel. Four genotypes were determined: e3e4 (28 subjects), e3e3 (20 subjects), e2e4 (1 subject) and e2e3 (1 subject). The e2e2 and e4e4 genotype was not detected. Alleles e2, e3 and e4 were detected with the following frequencies: e2 3.97%, e3 40.48%, e4 55.56%.

Student's t-test examined the differences between the arithmetic means of anthropometric and biochemical parameters among the e3e3 and e3e4 genotype groups of males with MetS (Table 1). Carriers of the e2 allele (e2e3 and e2e4) were excluded from the study because of the small number of subjects, therefore only e3e3 and e3e4 genotypes were compared for differences in anthropometric and biochemical parameters.

Results of the t-test revealed significant differences ($p < 0.05$) in the means of biochemical and anthropometric measurements between e3e3 and e3e4 genotype groups for systolic blood pressure (SBP), diastolic blood pressure (DBP), C-reactive protein (CRP), insulin level (IRI) and homeostasis model assessment of insulin resistance (HOMA IR) , and highly significant differences for body mass (BM), body mass index (BMI), waist circumference (WC), high density lipoproteins (HDL) and low density lipoprotein cholesterol level (LDL). Our observed correlation between the presence of the e4 allele and elevated CRP values, as well as an association with obesity parameters, is a novel finding in the field of apoE polymorphism research.

DISCUSSION

Analysis of apoE polymorphism in a healthy Serbian population by Stankovic et al. (2004) showed the following frequencies: e3 (73.6%), e2 (14.9%), e4 (11.5%). The study confirmed a similar distribution of apoE alleles as in other European populations (Stankovic et al. 2004). In the present study, alleles e2, e3 and e4 were detected with the following frequencies: e2 3.97%, e3 40.48%, e4 55.56%, which is different in comparison with the research of Djan et al. (2011) for the frequencies of e3 and e4 alleles. The explanation for these deviations may be differences between the investigated groups, as in the research of Djan et al. (2011) their group consisted exclusively of men under 45 years old, who had ischemic heart disease, while the present study included males aged 30-65 years with MetS, where the significance of impaired lipid metabolism is crucial.

Olivieri et al. (2007) have established a significant relationship ($p < 0.05$) between the presence of the e4 allele and the onset of MetS, as well as the severity of the syndrome in the MetS group of both sexes. The US National Prevention Program and IDF criteria were used by Tao et al. (2011), they found a significant correlation between e4 allele presence and the prevalence of MetS. Vučinić et al. (2014) have shown that carriers of e4 alleles have an 11.25-fold chance of metabolic syndrome onset, related to carriers of the e3 and e2 allele of the apoE gene in the MetS group that includes both sexes.

Numerous population studies have demonstrated the effect of apoE alleles on plasma lipid levels (Eggertsen et al. 1993; Djan et al. 2011; Chaudhary et al. 2012; Vučinić et al. 2014; Shatwan et al. 2018; Tang and Yang 2018). The association of the e4 allele with a significant increase in total cholesterol concentrations among healthy populations has been reported, and this allele has been designated as a predisposing allele for onset of atherosclerosis (Eggertsen et al. 1993; Guerra et al. 2003; Djan et al. 2011; Chaudhary et al. 2012; Vučinić et al. 2014; Shatwan et al. 2018; Tang and Yang 2018; Marais 2019). Guerra et al. (2003) found significantly higher total cholesterol, HDL and LDL / HDL levels in e3e4 carriers compared to e2e3 and e3e3 carriers, so our results for HDL and LDL cholesterol (Table 1) are in accordance with the above mentioned studies, although our sample included only males. In a review paper, Regitz-Zagrosek et al. (2006) stated that there are significant differences in the pathophysiology of MetS between males and females, and that the prevalence of MetS in Europe is greater in men than in women, especially among younger members of the population (20-39 years). It was noted that central obesity is more prevalent in men

Table 1. Differences in the means of anthropometric and biochemical parameters between e3e3 and e3e4 genotype groups.

	Mean values		
Tested parameters	e3e3 genotype group	e3e4 genotype group	p-value
Age	35.62	36.95	0.75
BM (kg)**	90.54	127.96	**0.00**
BMI (kg/m^2)**	27.45	38.87	**0.00**
WC (cm)**	94.38	123.09	**0.00**
SBP (mmHg)*	125	139.76	**0.02**
DBP (mmHg)*	81.54	91.43	**0.02**
TC (mmol/L)	5.59	5.03	0.17
TG (mmol/L)	1.24	1.87	0.09
HDL (mmol/L)**	1.13	0.89	**0.00**
LDL (mmol/L)**	1.23	3.07	**0.00**
glucose (mmol/L)	3.52	3.91	0.22
HOMA IR1*	9.35	15.75	**0.03**
CRP (10 mg/L)*	1.99	3.37	**0.03**

BM = body mass, BMI = body mass index, WC = waist circumference, SBP = systolic blood pressure, DBP = diastolic blood pressure, TC = total cholesterol, TG = triglycerides, HDL = high density lipoproteins, LDL = low density lipoproteins, HOMA IR = homeostasis model assessment of insulin resistance, CRP = C-reactive protein.
Paired Student's t-test: **$p < 0.01$; *$p < 0.05$.

and represents a major source of free fatty acids and adipocytokins for the liver and therefore leads to the development of insulin resistance, dyslipidemia, and elevated blood pressure, while subcutaneous (peripheral) obesity that occurs in women, allows for a higher degree of obesity that can cause the same metabolic disorders in women. In the present study, a highly significant link between the presence of e4 alleles and WC measures in men with MetS was found (Table 1), and indicates the strong influence of this allele on central male obesity. In their previous study of obesity, Krotkiewski et al. (1983) found an association between the e4 allele and higher blood pressure, TG levels, glucose levels and insulin in males, which was also found in our present study: with the exception of elevated glucose levels in carriers of the e4 allele compared to e2 and e3 alleles in the genotype. According to Mosher et al. (2008), understanding gender differences with respect to the effects of the apoE gene on HDL cholesterol levels during increased carbohydrate intake is of great importance. The association between the effects of carbohydrate intake and apoE polymorphism were significantly influenced by gender. In all males, a significant decrease in HDL levels after increased intake of carbohydrates was found. In the present study, the e4 allele also showed a significant association with low levels of HDL cholesterol in men (Table 1) Ilveskoski et al. (2000) did not find a correlation between apoE genotype and HDL cholesterol levels and blood pressure; thus, our results are not in agreement with the aforementioned study, because they show a significant effect of the e4 allele on reduced levels of HDL cholesterol and on increased systolic and diastolic blood pressure (Table 1). Chen et al. (2003) have shown a correlation between the e4 allele and vascular disease, as well as a link between e4 and increased levels of total and LDL cholesterol, which was also confirmed by the results of the present study for LDL cholesterol levels in the presence of e4 alleles. A study of the risk of atherosclerosis in society (ARIC) (Volcik et al. 2006) on 15,000 subjects showed an association between ApoE isoforms and BMI: BMI ApoE4 <ApoE3 <ApoE2. The results obtained in the present study strongly suggest a significant link between the presence of the e4 allele and BMI in males with MetS.

CONCLUSIONS

Based on important role of the apoE gene in lipid and lipoprotein metabolism, apoE polymorphism can serve as a potential determinant of MetS, which is characterized by a set of metabolic disorders, including impaired lipid and lipoprotein metabolism.

In the present research, apoE allele polymorphism was shown to affect the occurrence and development of MetS, most likely by its effects on lipid metabolism, which causes obesity and insulin resistance. Our study confirmed that the e4 allele can be considered to be a genetic determinant for the development of MetS. This research included only males, and further research should be done on a larger number of subjects, as well as in addition to genotyping additional genes involved in lipid metabolism.

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