



## Apolipoprotein E gene polymorphisms as risk factors for carotid atherosclerosis

Polimorfizmi u genu za apolipoprotein E kao faktori rizika od ateroskleroze karotidnih arterija

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### Abstract

**Background/Aim.** Atherosclerosis is still the leading cause of death in Western world. Development of atherosclerotic plaque involves accumulation of inflammatory cells, lipids, smooth muscle cells and extracellular matrix proteins in the intima of the vascular wall. Apolipoprotein E participates in the transport of exogenous cholesterol, endogenously synthesized lipids and triglycerides in the organism. Apolipoprotein E gene has been identified as one of the candidate genes for atherosclerosis. Previous studies in different populations have clearly implicated apolipoprotein E genetic variation ( $\epsilon$  polymorphisms) as a major modulator of low density lipoprotein cholesterol levels. Data considering apolipoprotein E polymorphisms in relation to carotid atherosclerosis gave results that are not in full compliance. The aim of present study was to investigate the apolipoprotein E polymorphisms in association with carotid plaque presence, apolipoprotein E and lipid serum levels in patients with carotid atherosclerosis from Serbia. **Methods.** The study group enrolled 495 participants: 285 controls and 210 con-

secutive patients with carotid atherosclerosis who underwent carotid endarterectomy. Genotyping of apolipoprotein E polymorphisms were done using polymerase chain reaction and restriction fragment length polymorphism methods. **Results.** Patients had significantly decreased frequency of the  $\epsilon 2$  allele compared to controls. Patients who carry at least one  $\epsilon 2$  allele had a significantly higher level of serum apolipoprotein E and significantly lower low density lipoprotein cholesterol levels compared to those who do not carry this allele. **Conclusion.** Our results suggest protective effect of apolipoprotein E  $\epsilon 2$  allele on susceptibility for carotid plaque presence as well as low density lipoprotein cholesterol lowering effect in Serbian patients with carotid atherosclerosis. Further research of multiple gene and environmental factors that contribute to the appearance and the progression of atherosclerosis should be continued with respect to different populations.

**Key words:** genetic predisposition to disease; carotid artery disease; polymorphism, genetic; apolipoproteins e.

### Apstrakt

**Uvod/Cilj.** Ateroskleroza je vodeći uzročnik smrtnosti u zapadnom svetu. Proces formiranja aterosklerotskog plaka podrazumeva akumulaciju ćelija inflamatornog odgovora, lipida, glatkih mišićnih ćelija i proteina vanćelijskog matriksa u intimi zida krvnog suda. Apolipoprotein E učestvuje u transportu egzogenog holesterola, endogeno sintetisanih lipida i triglicerida u organizmu. Gen koji kodira apolipoprotein E smatra se jednim od gena kandidata u izučavanju ateroskleroze. Rezultati prethodnih studija, urađenih na različitim populacijama, jasno ukazuju da su varijacije u genu za

apolipoprotein E glavni modulatori nivoa lipoproteina male gustine u serumu. Do sada objavljeni rezultati koji se tiču povezanosti polimorfizama u genu za apolipoprotein E sa aterosklerozom karotidnih arterija su kontradiktorni. Zato je cilj ove studije bio da se ispita povezanost polimorfizama u genu za apolipoprotein E sa rizikom od nastanka aterosklerotskog plaka u karotidnim arterijama kao i sa nivoom proteina apolipoprotein E i lipida u serumu bolesnika sa aterosklerozom karotidnih arterija iz Srbije. **Metode.** Ovom studijom bilo je obuhvaćeno ukupno 495 ispitanika: 285 zdravih ispitanika u kontrolnoj grupi i 210 bolesnika sa aterosklerozom karotidnih arterija koji su bili upućeni na endarte-

rektomiju. Genotipizacija polimorfizama u genu za apolipoprotein E je urađena metodama lančane reakcije polimeraze i analizom fragmenata dobijenih digestijom sa restrikcionim enzimima. **Rezultati.** Bolesnici su imali statistički značajno manju frekvenciju alela  $\epsilon 2$  u odnosu na kontrolnu grupu. Ispitanici koji su bili nosioci genotipa sa bar jednom kopijom alela  $\epsilon 2$  imali su statistički značajno viši nivo proteina apolipoprotein E i statistički značajno niži nivo lipoproteina male gustine u serumu u odnosu na bolesnike koji su bili nosioci genotipova bez prisustva alela  $\epsilon 2$ . **Zaključak.** Rezultati ove

studije ukazuju na zaštitni efekat alela  $\epsilon 2$  od nastanka aterosklerotskog plaka u karotidnim arterijama, kao i povezanost sa nižim nivoom lipoproteina male gustine u serumu bolesnika sa njihovom aterosklerozom. Neophodna su dalja istraživanja gena i faktora rizika iz spoljašnje sredine uključenih u nastanak ateroskleroze u različitim populacijama.

#### Ključne reči:

**bolest, genetska, predispozicija; a.carotis, bolesti; polimorfizam, genetički; apolipoproteini e.**

## Introduction

As a multifactorial vascular disease, atherosclerosis is one of the most frequently occurring illnesses of the modern world. According to the theory given by Ross and Glomset<sup>1</sup>, which is still among the most common, atherosclerosis is initiated by a blood vessel injury accompanied by inflammatory processes. Plaque, an accumulation of cells with different content (including lipids), forming on the inside of blood vessels in the area of injury can lead to a large lumen narrowing (stenosis) of the vessel<sup>2</sup>. The consequences of atherosclerotic lesions rupture may be heart attack or stroke, which eventually could lead to death.

Taking into account the causes and consequences associated with the formation of atherosclerotic plaques, effective prevention of this disease including genetic data and epidemiological studies could provide the only permanent solution to combat this disease. Many genes can be considered as candidates whose polymorphisms in interaction with certain risk factors such as: dyslipidemia, hypertension, obesity, stress, smoking, diabetes and physical inactivity, can lead to the development and/or progression of atherosclerosis<sup>3-5</sup>.

Apolipoprotein E gene (APOE) has been identified as one of the candidate genes for atherosclerosis. Apolipoprotein E (apoE) is apolipoprotein which plays a major role in regulating the metabolism of chylomicrons, very low density lipoproteins (VLDL), and high density lipoproteins (HDL) via the apoE receptor and by the low density lipoprotein (LDL) receptors<sup>6</sup>. It is responsible, in part, for uptake of dietary cholesterol in the form of chylomicron remnants, clearance of VLDL remnants, and removal of the excess cholesterol from peripheral tissues through hepatic clearance of HDL containing apoE<sup>6</sup>. Thus, ApoE participates in the transport of exogenous cholesterol, endogenously synthesized lipids and triglycerides (TG) in the organism<sup>7</sup>. This protein has two domains: the C-terminal, which is associated with lipoprotein particles and the N-terminal, which binds to the lipoprotein receptor<sup>8,9</sup>.

Polymorphisms in the APOE analyzed in this study are located in a region that encodes the N-terminal domain of apoE protein. Depending on whether the Arg or Cys are present in two polymorphic positions (codons 112 and 158), the possible alleles are:  $\epsilon 2$  (112 Cys, 158 Cys),  $\epsilon 3$  (112 Cys, 158 Arg) and  $\epsilon 4$  (112 Arg, 158 Arg)<sup>10</sup>. Previous studies in different populations have clearly implicated APOE genetic variation as a major modulator of LDL cholesterol levels<sup>11-13</sup>. The

$\epsilon 2$  allele carriers have decreased, whereas  $\epsilon 4$  carriers have increased the level of cholesterol compared to the  $\epsilon 3$  allele carriers<sup>14</sup>. A meta analysis by Dallongeville et al.<sup>15</sup> indicated that subjects with the  $\epsilon 2$  and  $\epsilon 4$  alleles had higher triglyceride levels than subjects with the  $\epsilon 3$  allele. Blood lipid level has been recognized as risk factor for carotid artery plaque formation, so variation at APOE locus could be a major determinant of atherosclerosis risk in the general population.

Previously published studies, which investigated APOE polymorphisms in relation to carotid atherosclerosis, gave results that are not in full compliance. The  $\epsilon 2$  allele was associated with a reduced risk for carotid plaque presence, compared with the presence of allele  $\epsilon 3$ <sup>16</sup>. In other study the same was suggested for the carriers of  $\epsilon 4$  allele<sup>17</sup>.

Since the formation of a plaque via lipid accumulation on the inside of the vessel is an important factor for the initiation of atherosclerosis, and there is no clear conclusion about which of the APOE alleles or genotypes are associated with the carotid plaque (CP) presence the present study examined the APOE polymorphisms in association with CP presence, apoE and lipid serum levels in carotid atherosclerosis (CA) patients from Serbia who underwent carotid endarterectomy.

## Methods

### Subjects

The study group enrolled 495 participants: 285 controls and 210 consecutive patients with CA who underwent carotid endarterectomy. All the participants were Caucasians of European descent from Serbia. From all of them medical history was collected including smoking and drinking habits, presence of diabetes, peripheral arterial occlusive disease, coronary artery disease and drug treatment. Exclusion criteria for all patients were carotid kinking, carotid aneurism, history of previous carotid endarterectomy (possible restenosis), tumors, autoimmune disease, chronic inflammatory diseases or renal failure. The patients already diagnosed with diabetes mellitus, having a fasting glucose  $\geq 7.0$  mmol/L, or taking insulin or hypoglycemic drugs were classified as having diabetes mellitus. Those with previous myocardial infarction or stable angina pectoris evaluated by selective coronography that confirmed coronary artery disease were classified as having coronary heart disease. Peripheral artery disease was diagnosed in those with an ankle-brachial index  $< 0.90$ . Hypertension was defined as systolic blood pressure

$\geq 140$  mmHg, a diastolic blood pressure  $\geq 90$  mmHg, or current treatment with antihypertensive drugs<sup>18</sup>. From the individuals undergoing annual medical check-up at Occupational Medical Center, Belgrade, Serbia, who underwent clinical and ECG examination, 285 that were without evidence of CA, cerebrovascular or cardiovascular disease, any chronic inflammatory disease, renal failure or diabetes mellitus were recruited as controls<sup>19</sup>.

A total of 210 patients were recruited from individuals consecutively admitted for carotid endarterectomy to the Clinic for Vascular and Endovascular Surgery, Clinical Center of Serbia, Belgrade, Serbia, during 2008 with evidence of carotid plaque in the internal carotid artery (ICA) or common carotid artery (CCA). Ultrasound assessment of the bilateral carotid arteries was performed as previously described<sup>18</sup>.

Atherosclerotic plaques were defined as focal widening relative to adjacent segments as evidenced by protrusion into the lumen and/or localized roughness with increased echogenicity. For ultrasound carotid measurements, intraclass correlation coefficients for inter-rater and intra-rater reliability were 0.916 and 0.968, respectively. Carotid atherosclerosis was defined as the presence of atherosclerotic plaques in internal or common carotid artery<sup>18</sup>.

All biochemical analyses were performed at the hospital laboratory by standard procedure.

The study was approved by Ethic Committee of the participating medical center with written informed consent given by each participant in this study.

#### Genetic analysis

Genomic DNA was isolated from the whole blood samples collected with ethylenediamine tetraacetic acid (EDTA) by standardized BloodPrep<sup>®</sup> DNA Chemistry isolation kit (Applied Biosystems, Forester City, CA) on the ABI PRISM<sup>™</sup> 6100 Nucleic Acid PrepStation (Applied Biosystems, Forester City, CA). Genotyping of APOE polymorphisms was done by polymerase chain reaction (PCR) using the following primers: forward 5'-TAAGCTTGGCACGGCTGTCCAAGGA-3' and reverse 5'-ACAGAATTCGCCCCGGCCT GGTACAC-3', designed according to the previously described protocol<sup>20</sup>. PCR mixture component concentrations were as follows: 1X PCR buffer (10X), 1.5 mM MgCl<sub>2</sub>, 1% DMSO, 0.2 mM dNTP, 0.45 mM of each primer, 0.25 U/ $\mu$ l Taq polymerase. Temperature conditions of the PCR reaction were as follows: 95 °C 7 min., and 33 cycles (95 °C 40s, 65 °C 30s, 72 °C 60s) without a fi-

nal extension step. After PCR, the synthesized fragment was 244 bp in length. Restriction analysis of synthesized DNA fragments (RFLP) was carried out by a Hin6I-mediated digestion of amplified PCR fragments. The lengths of the fragments obtained by digestion with the enzyme Hin6I are as follows: E2: 36bp, 16bp, 91bp, 18bp, 83bp; E3: 36bp, 16bp, 91bp, 18bp, 48bp, 35bp; E4: 36bp, 16bp, 19bp, 72bp, 18bp, 48bp, 35bp. The digestion products were loaded on an 8% polyacrilamide gel for genotyping and run for 2 h in electric field of 12 V/cm. Gels were stained with silver nitrate and visualized using a GDS8000 gel documentation system (Ultra Violet Products Inc, Upland, USA).

#### Statistical analysis

The allelic frequencies and genotype distribution were estimated by gene counting method. Differences in allele frequencies and genotype distribution between the cases and controls as well as deviation from Hardy-Weinberg equilibrium were estimated by  $\chi^2$ . Normal distribution of continuous variables was tested by Kolmogorov-Smirnov test with Lilliefors's correction. The influence of genotype on the variability of biochemical parameters was analyzed using ANOVA and appropriate *post-hoc* test or Kruskal Wallis ANOVA as a nonparametric test. The results are presented as mean  $\pm$  standard deviation (SD). Statistical analysis was performed using Statistica Version 8, software package (StatSoft Inc, 2008). In all tests, differences with two-tailed alpha-probability  $p < 0.05$  were considered significant.

#### Results

This study examined the association of APOE polymorphisms with the carotid atherosclerotic plaque presence. The study included 285 subjects in the control (healthy) population and 210 patients with CA. None of the genders was significantly prevalent in any of the groups (49.64% of women and 50.36% of men in the control population and 42.58% women and 57.42% men in the atherosclerotic population,  $p > 0.05$ ). However, the group of patients was significantly older than the control population (age of the control population was  $51.50 \pm 9.30$  years and of the patients' group it was  $60.02 \pm 10.08$  years,  $p < 0.01$ ). The relative frequencies of APOE genotypes did not deviate significantly from Hardy-Weinberg equilibrium and differences in their distribution between the studied groups were not statistically significant (Table 1).

**Table 1**  
Relative frequencies of apolipoprotein E (APOE) genotypes in the controls and the group of patients with carotid atherosclerosis

Genotype	Control group n (%)	Patient group n (%)	<i>p</i>
E2/2	6 (2.11)	3 (1.43)	NS
E2/3	44 (15.44)	17 (8.10)	
E2/4	5 (1.75)	0 (0.00)	
E3/3	188 (65.96)	156 (74.29)	
E3/4	39 (13.68)	32 (15.24)	
E4/4	3 (1.05)	2 (0.95)	
Total	285 (100)	210 (100)	

*p* – Pearson  $\chi^2$  test; NS – not statistically significant.

Allele frequency distribution was significantly different between the patients and the controls (Table 2). The  $\epsilon 2$  allele had significantly decreased frequency in the patient group compared to the controls ( $\chi^2 = 11.5$ ,  $df = 1$ ,  $p < 0.01$ ).

**Table 2**  
Relative frequencies of apolipoprotein (APOE) alleles in the controls and the patients with carotid atherosclerosis

Allele	Controls (%)	Patients (%)	$p^*$
$\epsilon 2$	0.11	0.05	< 0.05
$\epsilon 3$	0.80	0.86	
$\epsilon 4$	0.09	0.09	

\*Pearson  $\chi^2$  test.

We also measured concentrations of apoE in the serum of patients with carotid atherosclerosis. Concentrations of apoE, represented as mean  $\pm$  SD, were not significantly different according to the APOE genotypes (Table 3).

**Table 3**  
Mean serum concentration of apolipoprotein E (apoE) with the reference to apolipoprotein E (APOE) genotypes

Genotype	n	ApoE (mg/L)
		mean $\pm$ SD
E2/2	1	103.20 $\pm$ 0.00
E2/3	11	69.14 $\pm$ 36.37
E2/4	0	0.00 $\pm$ 0.00
E3/3	76	46.98 $\pm$ 11.54
E3/4	15	42.75 $\pm$ 10.75
E4/4	2	35.30 $\pm$ 15.13
Total	105	49.01 $\pm$ 18.01

Nevertheless, when we grouped APOE genotypes in those containing  $\epsilon 2$  allele (E2/E2 + E2/E3),  $\epsilon 4$  allele (E3/E4 + E4/E4) and homozygotes for the  $\epsilon 3$  allele (E3/E3) (not including the genotype E2/4), Kruskal-Wallis ANOVA test showed a statistically significant difference ( $p < 0.01$ ) in serum apoE levels among these groups of genotypes. The patients who carried at least one  $\epsilon 2$  allele (E2/E2 + E2/E3) had a significantly higher level of serum apoE compared to those with E3/3 and E3/E4 + E4/4 genotypes (Table 4).

**Table 4**  
Mean serum concentrations of apolipoprotein E (apoE) in the serum of patients by the grouped genotype

Genotype	n	ApoE (mg/L)	$p^*$
		mean $\pm$ SD	
E2/2 + E2/3	12	71.98 $\pm$ 36.05	< 0.01
E3/3	76	46.98 $\pm$ 11.54	
E3/E4 + E4/4	17	41.88 $\pm$ 11.03	
Total	105	49.01 $\pm$ 18.01	

\*Kruskal-Wallis ANOVA.

We also examined values of lipid parameters measured in the serum of patients with atherosclerosis according to the grouped apoE genotypes, excluding E2/E4 genotype (Table 5). ANOVA LSD *post-hoc* test revealed that the carriers of at least one  $\epsilon 2$  allele had significantly lower LDL cholesterol levels compared to those who did not carry this allele. The concentrations of HDL cholesterol and triglyceride levels were not significantly different ( $p > 0.05$ ) according to the grouped genotypes.

## Discussion

This study investigated a possible association of APOE polymorphisms with the occurrence of carotid plaque as well as with apoE and lipid serum values in Serbian patients with CA. There were no statistically significant differences in the frequencies of APOE genotypes between the two groups, but there was a statistically significant decrease in the frequency of allele  $\epsilon 2$  in the group of patients compared to the controls. This result indicates the protective effect of the  $\epsilon 2$  allele for carotid plaque presence and is consistent with previous studies<sup>16,21</sup>. Some studies have shown that the  $\epsilon 2$  allele contributes to the reduced risk of atherosclerosis occurrence only if subjects are of normal weight and younger than 80<sup>22</sup>. The presence of  $\epsilon 2$  allele has also been shown to lead to the smallest intima media thickness<sup>16,23</sup>.

This study, however did not demonstrate compliance with some of the results from previous studies in respect to the  $\epsilon 4$  allele. It was characterized as atherogenic in French and Thai population<sup>21,24</sup>, but not in the Caucasians from The Rotterdam study<sup>16</sup>. Concordantly with our results, they did not find any significant association of the  $\epsilon 4$  allele with carotid plaques presence in a large sample of more than 4,000 Caucasians<sup>16</sup>. The majority of studies showed contradictory results regarding the impact of different APOE alleles on the development of atherosclerosis<sup>16,17,21,23-25</sup>. Potential reasons for the conflicting results in these studies may be: a non-representative sample, analysis of different age groups of persons and/or different stages of the illness, different environmental conditions, and different ethnic origin. Frequency of the  $\epsilon 4$  allele in the Serbian population was in concordance with the frequency obtained from Italian population and the study group of Caucasians investigated in The Rotterdam study<sup>14,16</sup>.

The obtained values of serum apoE concentrations indicate a declining trend in the direction: E2/2 > E2/3 > E3/3 > E3/4 > E4/4. After grouping of the genotypes, it was found

**Table 5**  
Mean serum values of lipid parameters according to the grouped apolipoprotein E (APOE) genotypes

Genotype	n	HDLC (mmol/L)	LDLC (mmol/L)	TG (mmol/L)
		mean $\pm$ SD	mean $\pm$ SD	mean $\pm$ SD
E2/E2 + E2/E3	16	1.09 $\pm$ 0.37	3.09 $\pm$ 1.17*	2.08 $\pm$ 0.92
E3/E3	126	1.17 $\pm$ 0.35	3.79 $\pm$ 1.06	1.80 $\pm$ 0.78
E3/E4 + E4/E4	26	1.20 $\pm$ 0.32	3.88 $\pm$ 1.07	1.58 $\pm$ 0.68
Total	168	1.17 $\pm$ 0.35	3.74 $\pm$ 1.09	1.79 $\pm$ 0.78

\* $p < 0.05$ ; [ANOVA, Fisher's Least Significant Difference (LSD)] *post hoc* test; HDLC- HDL cholesterol; LDLC- LDL cholesterol; TG - triglycerides.

that carriers of at least one  $\epsilon 2$  allele had significantly increased, and the carriers of at least one  $\epsilon 4$  allele significantly decreased apoE levels in serum, compared to the carriers of genotype E3/3. These results are consistent with previous studies<sup>16,26</sup>. According to Weisgraber et al.<sup>8</sup>, ApoE3 and ApoE4 isoforms have normal receptor-binding activity because arginine is at the position 158 in the protein, unlike ApoE2 isoforms, which at this position contains cysteine and binds to the receptor with reduced efficiency.

Among patients with CA, the carriers of at least one  $\epsilon 2$  allele showed a significant reduction in LDL cholesterol levels compared to the carriers of any other genotype, while no significant differences in the level of HDL cholesterol was noted between genotypes. Previously published results for the random population from Serbia<sup>27</sup> have shown the same pattern. The results we got for the LDL cholesterol levels correspond to previous studies<sup>16,28,29</sup>. From the obtained results it can be concluded that the  $\epsilon 2$  allele has atheroprotective function, but the exact mechanism of ApoE2 and ApoE4 isoforms' influences on LDL cholesterol remains unknown.

This study found no association of APOE genotypes and HDL cholesterol levels, which is consistent with the results of some previous studies<sup>30</sup> but differs from others<sup>31</sup>. HDL participates in reverse cholesterol transport from peripheral tissues to the liver, thereby performing the atheroprotective role. Also, our study showed no statistically significant association of APOE genotype with serum triglyceride levels. Analyzing the results from numerous studies there is no evidence of a consistent relationship between the APOE genotypes and triglyceride or HDL levels<sup>32</sup>. Thus, we can assume that the effect APOE genotypes could have on

variability of HDL or triglycerides is rather small or population specific.

## Conclusion

This study suggests a protective effect of APO  $\epsilon 2$  allele on carotid plaque presence as well as LDL cholesterol lowering effect. A considerable number of contradictory results regarding the exact role of APOE polymorphisms in the development and progression of atherosclerosis are a consequence of the very nature of atherosclerosis as a disease. The development of atherosclerosis depends on a large number of both genetic (other apolipoprotein isoforms, polymorphisms in genes for proteins involved in inflammatory processes), and environmental factors (lifestyle, therapy, diet). In order to get the most reliable results on the genetic causes of atherosclerosis, it is certainly necessary to do analysis of multiple genes in a representative sample of healthy and diseased individuals and to estimate the risk accurately by adjusting for confounding factors. Also, it is possible that ApoE performs a dual function in apoE-related transport of lipids in the body and so the balance between processes that it performs determines its atherogenic or atheroprotective role in atherosclerosis. Further research of multiple gene and environmental factors that contribute to the appearance and the progression of atherosclerosis should be continued with respect to different populations.

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