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Association of the apolipoprotein E ε4 allele in a Serbian population with Alzheimer's dementia

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Abstract: Although single nucleotide polymorphisms rs429358 and rs7412 in the apolipoprotein E gene (*APOE*) are widely investigated to analyze *APOE* alleles, there are no data on their association with Alzheimer's disease (AD) dementia in a Serbian population. This study aimed to investigate the distribution of *APOE* ε2/ε3/ε4 alleles in patients with dementia due to AD and cognitively unimpaired subjects and to assess the association of the *APOE* ε4 allele with disease risk in the Serbian population. A case-control study included patients with dementia due to AD and cognitively unimpaired individuals. *APOE* rs429358 and rs7412 were analyzed using the Real-Time PCR method with allele-specific TaqMan assays, followed by APOE ε2/ε3/ε4 allele carrier status analysis. Patients had a significantly higher frequency of the *APOE* ε4 allele than the control group (P<0.001). The *APOE* ε4 allele was found to be associated with a 3-fold higher risk of AD dementia compared to the reference ε3 allele (P<0.001). In conclusion, this is the first study to suggest that carriers of the *APOE* ε4 allele have a higher risk of developing dementia due to AD than those who carry the *APOE* ε3 allele in the Serbian population.

Keywords: apolipoprotein E ε4; Alzheimer's disease; dementia; rs429358; rs7412

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by the accumulation of protein fragments, specifically phosphorylated tau protein (p-tau) intracellularly and amyloid beta peptide (Aβ) extracellularly. According to the amyloid hypothesis, Aβ production occurs through beta and gamma-secretase action, resulting in Aβ42, which is insoluble and sticky, forming oligomers and plaques. This leads to a low concentration of Aβ42 in patients' cerebrospinal fluid (CSF), and oligomer accumulation results in tau protein hyperphosphorylation. The microtubule network and normal axonal transport are disrupted, ultimately causing degeneration, necroptosis, and an increase in the concentration of p-tau in the CSF. These pathohistological changes occur up to 20 years before the onset of symptoms during the asymptomatic phase, followed by mild cognitive impairment (MCI) and finally, dementia characterized by memory loss and the inability to perform daily activities [1-3].

AD is a significant cause of death among individuals over the age of 65. By 2050, it is predicted that there will be 131.5 million people who have dementia worldwide [2,4]. In Serbia, about 75,000 people were living with dementia in 2016, and this number is estimated to have increased to between 80,000 and 140,000 individuals today [5]. Age, genetics, and family history are all risk factors for developing AD, which has two forms: the rare familial or autosomal dominant form with early-onset caused by mutations in three genes, amyloid precursor protein gene (*APP*), and the *PSEN1* and *PSEN2* genes encoding for presenilin 1 and 2 proteins, respectively, and the more common sporadic or late-onset form (LOAD) associated with variations in the gene for apolipoprotein E (*APOE*) [2].

APOE is a 299 amino acid glycoprotein synthesized in the liver and brain that is rich in arginine. In the central nervous system (CNS), it is primarily produced by astrocytes and microglia and plays a role in complexing with Aβ to accelerate its clearance [6]. The *APOE* gene, located on chromosome 19q13.32, encodes a 22 kDa protein and has four exons. There are 1,638 variant alleles discovered so far, with two single nucleotide polymorphisms (SNPs), rs429358 and rs7412, that are crucial for determining whether an individual carries the *APOE* ε2, ε3, or ε4 allele and the corresponding APOE2, E3, or E4 protein isoforms. The rs429358 SNP (334T>C; Cys112Arg) is in exon 4 and causes the substitution of thymine with cytosine, substituting cysteine with arginine at position 112. The rs7412 SNP (472C>T; Arg158Cys) is characterized by the substitution of cytosine with thymine and the substitution of arginine with cysteine in position 158 [7]. The APOE2 isoform contains cysteine at both positions (112 and 158), the APOE3 isoform has cysteine at position 112 and arginine at position 158, and the APOE4 isoform contains arginine at both positions. Carrying the *APOE* ε4 allele has been associated with an increased risk of accumulating Aβ, the development of amyloid plaques, and sporadic AD [2,6]. Although *APOE* genotyping is vital in assessing the risk of developing sporadic AD and stratifying patients, there is currently no information on the influence of the *APOE* ε4 allele on AD dementia risk in the Serbian population.

This study aims to examine the distribution of *APOE* ε2/ε3/ε4 alleles and genotypes in patients with dementia due to AD and in cognitively unimpaired subjects, as well as to assess the association of carrying the *APOE* ε4 allele with the risk of developing the disease in the population being studied.

MATERIALS AND METHODS

Ethics statement

The study was performed in compliance with the Declaration of Helsinki and the Ethical Committee of the Medical Faculty University of Niš that approved the study protocol (Decision No. 12-6422-2/3 dated July 23, 2020). All participants agreed to participate in the study, and informed consent was signed by healthy subjects and the patients or their legal guardians.

Participants

The study involved 53 patients diagnosed with probable dementia due to AD at the Neurology Clinic, University Clinical Center Niš; the control group consisted of 104 cognitively unimpaired individuals. The diagnosis of probable dementia due to AD was based on the National Institute on Aging-Alzheimer's Association diagnostic guidelines (NIA-AA) [8]. Patients underwent neurological examination and cognitive function assessments, including Mini-Mental State Examination (MMSE), Addenbrooke's Cognitive Examination Revised (ACE-R), Clinical Dementia Rating (CDR), and instrumental activities of daily living (IADL) using our recently validated Serbian version of the Amsterdam IADL questionnaire (A-IADL-Q) [9]. Brain magnetic resonance imaging (MRI) was performed according to the dementia protocol.

CSF sample preparation and biomarker analyses

CSF samples were obtained from all patients; the samples were placed in a polypropylene tube, centrifuged at 2000 ×g for 10 min at +4°C, and the supernatant was separated, aliquoted, and stored at -80 °C until analysis. Biomarkers used for AD diagnostics were determined in the CSF by the enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (EUROIMMUN, Germany, IVD). They included: Aβ42, Aβ40, total tau (t-tau), and phosphorylated tau (p-tau, 181P). The positivity of the selected CSF AD biomarkers was determined using the A/T/N classification (2016) [10]. The Aβ pathology (biomarker "A") was assessed with CSF Aβ42 and/or Aβ42/Aβ40 ratio, the tau pathology (biomarker "T") with CSF p-tau, and neurodegeneration (biomarker "N") with CSF t-tau. Individual CSF values were considered pathological ("positive") as follows: ≤570 pg/mL for Aβ42 and/or the Aβ42/Aβ40 ratio ≤ 0.095, >452 pg/mL for t-tau, and >61 pg/mL for p-tau.

Inclusion and exclusion criteria

Patients with ACE-R scores less than 88, MMSE scores less than or equal to 26, CDR scores greater than 0.5, A-IADL-Q scores less than or equal to 51.4, as well as positive MRI and CSF biomarkers (A+/T+/N+) were included in the study. The exclusion criteria were as follows: other brain pathology on MRI (significant

vascular changes, neoplasm), A/T/N negative patients, history of major psychiatric illnesses, history of non-AD dementia, stroke or other neurological illness, past serious head injury, significant motor impairment, vision and/or hearing impairment, heavy alcohol consumption, untreated obstructive sleep apnea, acute or chronic infection.

Blood sample preparation and genotyping

DNA isolation was performed using a commercial DNA isolation kit (Thermo Scientific GeneJET Whole Blood Genomic DNA Purification Mini Kit, Thermo Fisher Scientific, California, US). *APOE* rs429358 (334T>C; Cys112Arg) and rs7412 (472C>T; Arg158Cys) were determined by real-time PCR using allele-specific TaqMan assays (Thermo Fisher Scientific) in the Applied Biosystems™ 7500 Fast Real-Time PCR System. The 10 μL PCR reaction mixture contained 5 μL 2xUniversal Master Mix (Applied Biosystems, USA), 0.25 μL of specific 40xTaqMan SNP Genotyping Assay (rs429358: TaqMan Assy ID C___3084793_20; rs7412: TaqMan Assy ID C____904973_10) and 2 μL of DNA. Gene amplification was performed under the following conditions: pre-PCR at 60°C for 1 min, polymerase activation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing/extension at 60°C for 1 min, post-PCR at 60°C for 1 min. The analysis of both *APOE* SNPs (rs429358 and rs7412) was performed by 7500 Software v2.3, followed by determination of ε2/ε3/ε4 allele carriers and protein isoforms (E2 (cis112, cis158), E3 (cis112, arg158) or E4 (arg112, arg158); Supplementary Table S1). The study was conducted in the Laboratory for Medical Genetics at the Faculty of Medicine, University of Niš, Serbia.

Statistical analysis

The differences between demographic and clinical parameters in the patients and controls were evaluated by the t-test and the chi-squared ($\chi 2$) test, as appropriate. The study analyzed the frequency of alleles and genotypes in both patients and controls. The $\chi 2$ test or Fisher's exact test was used to compare frequencies and detect any deviation from the expected values of the Hardy-Weinberg equilibrium. Univariate binary logistic regression was applied to analyze the association of genetic variations with AD. The odds ratio (OR) and the 95% confidence interval (CI) were calculated to evaluate the risk of developing AD. Statistical significance was set at a $P<0.05$. SPSS version 20.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses.

RESULTS

The subjects' demographic, clinical, and CSF laboratory parameters are presented in Table 1. There was no statistically significant difference in gender frequency between patients and cognitively unimpaired individuals ($P=0.581$), while age and MMSE score differences were observed between these groups ($P<0.001$, $P<0.001$, respectively).

Genotype and allele frequencies of the rs429358 *APOE* gene polymorphism

Genotype frequencies of *APOE* rs429358 and rs7412 SNPs did not deviate from the normal distribution of Hardy-Weinberg equilibrium in the patients and control group ($P>0.05$). Patients with dementia due to AD had a statistically significant difference in the genotype distribution of rs429358 ($\chi 2=15.879$, df=2, $P<0.001$), as well as a higher frequency of the minor C allele compared to the control group ($\chi 2=15.303$, df=1, $P<0.001$; Table 2).

Table 1. Demographic, clinical, and CSF laboratory parameters of the subjects

	Control N=104	AD dementia N=53	P
Gender N (%)			
Male	46 (44.2)	21 (39.6)	0.581[a]
Female	58 (55.8)	32 (60.4)	
Age (M±SD)	60.82±4.41	70.77±7.97	<0.001[b]
MMSE (M±SD)	28.33±1.02	16.96±6.62	<0.001[b]
CSF biomarkers (Median (IQR))			
Aβ42/Aβ40 ratio		0.06 (0.05-0.08)	
t-Tau (pg/mL)		651.50 (495.75-825.20)	
p-Tau (pg/mL)		135.70 (101.56-187.44)	

AD – Alzheimer's disease, CSF – cerebrospinal fluid, IQR – interquartile range (25%-75%), MMSE – Mini-mental State Examination, N – number of subjects, [a] Pearson's $\chi 2$ test, [b] t-test, P – probability value

Table 2. Genotype and allele frequencies of rs429358 in the studied groups

Genotype (rs429358)	Control N=104	AD dementia N=53	P
TT	84 (80.8%)	29 (54.7%)	<0.001
TC	20 (19.2%)	20 (37.7%)	
CC	0 (0.0%)	4 (7.6%)	
Allele			
T	188 (90.4%)	78 (73.6%)	<0.001
C	20 (9.6%)	28 (26.4%)	

AD – Alzheimer's disease, N – number of subjects

Table 3. Genotype and allele frequencies of rs7412 in the studied groups

Genotype (rs7412)	Control N=104	AD dementia N=53	P
CC	96 (95.8%)	50 (94.3%)	0.751
CT	8 (4.2%)	3 (5.7%)	
TT	0 (0.0%)	0 (0.0%)	
Allele			
C	200 (96.2%)	103 (97.2%)	0.756
T	8 (3.8%)	3 (2.8%)	

AD – Alzheimer's disease, N – number of subjects

Table 4. Genotype frequencies of *APOE* in the studied groups

Genotype	Control N=104	AD dementia N=53	P
ε2/ε2	0 (0.0%)	0 (0.0%)	0.001
ε2/ε3	8 (7.7%)	3 (5.7%)	
ε3/ε3	76 (73.1%)	26 (49.1%)	
ε2/ε4	0 (0.0%)	0 (0.0%)	
ε3/ε4	20 (19.2%)	20 (37.7%)	
ε4/ε4	0 (0.0%)	4 (7.5%)	

AD – Alzheimer's disease, N – number of subjects

Table 5. Allele (isoform) frequencies of *APOE* in the studied groups

Isoform (allele)	Control 2N=208	AD dementia 2N=106	P	OR (95% CI)	P
E2 (ε2)	8 (3.9%)	3 (2.8%)	<0.001	1.096 (0.270-4.443)	0.898
E3 (ε3)	180 (86.5%)	75 (70.8%)		1	1.000
E4 (ε4)	20 (9.6%)	28 (26.4%)		2.923 (1.363-6.270)	0.006

AD – Alzheimer's disease, N – number of subjects, OR – odds ratio, 95% CI 95% – confidence interval

Genotype and allele frequencies of the rs7412 *APOE* gene polymorphism

The distribution of rs7412 SNP genotypes and alleles did not differ significantly between patients with AD dementia and the controls (P=0.751 and P=0.756, respectively; Table 3).

Genotype and allele frequencies of the *APOE* ε2/ε3/ε4

The distribution of *APOE* ε2/ε2, ε2/ε3, ε3/ε3, ε2/ε4, ε3/ε4, ε4/ε4 genotypes showed a significant difference between patients with AD dementia and controls ($\chi2=15.893$, df=3, P=0.001; Table 4). Patients had a significantly higher frequency of ε3/ε4 genotype compared to cognitively unimpaired individuals (37.7% vs 19.2%, $\chi2=6.332$, df=1, P=0.012). The frequency of ε3/ε3 genotype was significantly lower in AD dementia patients compared to the control group (49.1% vs. 73.1%, $\chi2=8.900$, df=1, P=0.003), while the ε2/ε3 genotype frequency did not differ between patients and controls (5.7% vs. 7.7%, P=0.751)

The distribution of *APOE* ε2, ε3, and ε4 alleles showed a statistically significant difference between patients with AD dementia and the control group ($\chi2=15.325$, df=2, P<0.001). Patients with AD dementia had a higher frequency of the *APOE* ε4 allele than cognitively unimpaired individuals (26.4% vs. 9.6%, $\chi2=15.303$, df=1, P<0.001), and the ε4 allele was associated with a 3-fold higher risk of developing dementia due to AD according to univariate logistic regression analysis, compared to the reference ε3 allele (OR=2.923, 95% CI: 1.363-6.270, P=0.006; Table 5). The frequency of the *APOE* ε3 allele was significantly lower in patients in comparison to the control (70.8% vs. 86.5%, $\chi2=11.464$, df=1, P=0.001), while no difference in *APOE* ε2 allele frequency was observed between patients and controls (P=0.756).

DISCUSSION

Although rs429358 and rs7412 SNPs are the most investigated polymorphisms of the *APOE* gene, there are no data on the association of the *APOE* ε4 allele with the risk of developing dementia due to AD in the Serbian population, to the best of our knowledge based on the reference search we conducted. This study's

results show a statistically significant difference in the distribution of *APOE* rs429358 SNP genotypes between patients with AD dementia compared to controls and that patients had a significantly higher frequency of the minor C allele compared to cognitively unimpaired individuals. On the other hand, the distribution of genotypes and alleles of the *APOE* rs7412 SNP did not show a significant difference between the patients and the control group. *APOE* genotyping for these two polymorphisms is crucial in determining the carrier status of *APOE* ε2, ε3, or ε4 alleles and assessing the risk of developing the disease. It is also essential for stratifying patients into *APOE* ε4+ and *APOE* ε4-. The distribution of six possible *APOE* genotypes (ε2/ ε2, ε2/ ε3, ε3/ ε3, ε2/ ε4, ε3/ ε4, ε4/ ε4) was significantly different in patients with AD dementia compared to controls in our study. The frequencies of genotypes ε3/ε4 and ε4 allele were higher in patients compared to cognitively unimpaired individuals, and the *APOE* ε4 allele was associated with a 3-fold higher risk of developing dementia due to AD compared to the reference allele *APOE* ε3.

APOE plays a crucial role in the clearance of Aβ, which is removed from the brain through the blood-brain barrier (BBB) or cellular/enzymatic degradation. APOE is synthesized in microglia and astrocytes, as well as in neurons under stress conditions. After lipidation, it binds soluble Aβ and accelerates its clearance, including uptake via the LDL receptor (LDLR) and LDL Receptor-Related Protein 1 (LRP1) (cellular clearance), as well as transit through the BBB (perivascular clearance). The APO E4 isoform has a lower affinity for and binds to Aβ more weakly than APO E3, which slows cellular and perivascular clearance, leading to the accumulation of Aβ in the brain and further progression of the disease [6,11]. Research has also shown that microglia in *APOE* ε4 carriers have reduced phagocytic activity and reduced expression of enzymes (neprilysin and insulin-degrading enzyme (IDE)) that participate in removing Aβ. Furthermore, *APOE* ε4 carrier state is associated with the deposition of tau, α-synuclein, and other proteins involved in neurodegeneration, independently of Aβ [2,6,12,13].

The prevalence of the *APOE* ε4 allele in the healthy population varies by ethnicity, with a frequency of 40% in Central Africa, 37% in Oceania, 26% in Australia, about 25% in Northern Europe and Asia, while in southern China and Mediterranean countries, the frequency is below 10% [14-16], which is similar to our results (9.6%). The *APOE* ε4 allele is considered the leading risk factor for sporadic AD and its earlier onset, depending on carrier status. Carriers of the *APOE* ε4 allele (*APOE* ε4+) are estimated to have an average onset of the disease 12 years earlier than *APOE* ε4- individuals [17]. Additionally, Aβ positivity and *APOE* ε4 carrier status were shown to individually increase the risk of disease progression from MCI to dementia in a similar manner. The combination of Aβ+ and *APOE* ε4+ increases the risk of disease progression 4.5-fold, while the risk for Aβ+ and *APOE* ε4- is the same as for Aβ- and *APOE* ε4- patients [2]. Research has also shown that carriers of one *APOE* ε4 allele have a 2-3 times higher risk, and carriers of two such alleles have an even higher risk of 10-15-fold for developing AD [6].

Although numerous studies have shown the association of the *APOE* ε4 allele with a higher risk of developing AD, which is supported by our results, data from the literature on the distribution of the *APOE* ε4 allele and its association with the risk of developing the disease show differences depending on ethnicity. Caucasians, Tunisians, and Japanese carrying the *APOE* ε4 allele are associated with a higher risk of developing AD dementia compared to African Americans and Hispanics [18,19]. Results obtained on the Venezuelan population indicate a higher frequency of the *APOE* ε4 allele in patients with AD [20]. On the other hand, the association of the *APOE* ε2 allele with a higher risk of developing AD, and a higher frequency of ε2/ε2 and ε2/ ε4 genotypes in patients with AD compared to healthy subjects, was shown in the Moroccan population [21].

Considering the role of APOE in amyloid clearance, the higher frequency of the *APOE* ε4 allele in patients with AD dementia in our study supports the research data indicating the association of the *APOE* ε4 allele and APO E4 isoform with slowed clearance of Aβ, leading to its further accumulation, stimulation of tau protein hyperphosphorylation, accumulation of neurofibrillary tangles, disruption of the cytoskeleton network, neurodegeneration, and disease progression to the terminal stage of dementia. Additionally, it was revealed that in patients carrying the *APOE* ε4 allele, different regions of the brain are affected by the pathohistological process compared to *APOE* ε4- patients. As a result, these two groups of patients have different clinical presentations. Namely, *APOE*

ε4 negative patients have a cognitive decline in other domains (language, behavior, attention) compared to *APOE* ε4 positive patients, who first experience memory disorders (amnestic AD) [19,22]. Given that our sample included patients with an amnestic form of the disease, a higher frequency of the *APOE* ε4 allele in patients compared to controls can be observed in this context as well. Based on numerous studies, the latest probabilistic model of AD indicates that there are two sporadic forms of AD, *APOE* ε4-related AD and *APOE* ε4-unrelated AD, and that the determination of *APOE* ε4 status, except for the risk assessment, is of key importance for the stratification of patients and is recommended to be included in the guidelines for the diagnosis and treatment of AD [2].

The limitations of our study are primarily related to the relatively small sample size. Furthermore, the study focused exclusively on A+/T+/N+ AD dementia patients using the A/T/N classification. As a result, the findings may not represent other subgroups or different stages of AD. Additionally, the study did not include individuals with MCI due to AD. This omission hinders our understanding of the potential influence of the APOE ε4 allele on disease risk in the early stages of AD.

CONCLUSIONS

The results of this study demonstrate that patients with dementia due to AD exhibit a significantly higher frequency of the *APOE* ε4 allele compared to the group of cognitively unimpaired individuals in a Serbian population. These results support previous research indicating that carriers of the *APOE* ε4 allele are at a greater risk of developing AD dementia when compared to those with the *APOE* ε3 allele. Future research involving a large sample size, consideration of the different stages of AD, and correlation with neuroimaging, particularly with the results of amyloid and tau PET scans, will be necessary to confirm the role of this biomarker in both the diagnosis and stratification of patients for further examination and assessment of their risk for disease progression in the Serbian population.

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Data availability: Data underlying the reported findings have been provided with the submitted article and are available here: https://www.serbiosoc.org.rs/NewUploads/Uploads/Basic%20et%20al_APOE%20e4%20allele_Dataset.pdf

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SUPPLEMENTARY MATERIAL

Supplementary Table S1. APOE allele and isoform analyses

Allele	rs429358	rs7412
ε2	TGC	TGC
ε3	TGC	CGC
ε4	CGC	CGC
Protein	112	158
E2	Cys	Cys
E3	Cys	Arg
E4	Arg	Arg