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ORIGINAL ARTICLE

The association of R47H variant in the *TREM2* gene and genetic susceptibility to Alzheimer's disease in Serbian population

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Summary

Introduction: Alzheimer's disease (AD) is a chronic neurodegenerative disease, which is clinically manifested by the development of dementia. Studies of genetic susceptibility to AD indicate a whole range of genes and their variants that can potentially influence an individual's susceptibility to develope the disease. AD17 represents a form of Alzheimer's disease associated with mutation(s) in the *TREM2* gene, encoding triggering receptor expressed on myeloid cells 2. The aim of this study was to determine the frequency of R47H variant of the *TREM2* gene in the population of AD patients, to compare the frequency of the variant in the population of AD patients and the control group, and to determine a possible association of a certain genotype with susceptibility to AD.

Material and Methods: The study included 168 consecutive patients with AD and 190 healthy controls. The clinical inerview, neurologic examination, and neuropsychological set of cognitive assessment were performed by neurologists and neuropsychologists in expertise with neurodegenerative deseases. Genotyping of rs75932628, R47H polymorphism of the *TREM2* gene was performed using Real-time Polymerase Chain Reaction and TaqMan® SNP genotyping assay (Applied Biosystem by Thermo Fisher Scientific, USA) according to the manufacturer's recommendations.

Results: In the group of AD patients the frequency of C allele was 98.8%, while the T allele was present in 1.2% of patients. The frequency of the T allele was statistically significantly higher among the AD population than among the control group ($p<0.05$). The frequency of homozygotes without mutation (CC genotype) was 97.62%, while the frequency of heterozygotes for the mutation (CT genotype) was 2.38% among patients with AD, and the frequency of homozygotes without mutation (CC genotype) was 100% among healthy controls.

Conclusion: Our study indicated a possible association of the heterozygous form of the R47H variant of *TREM2* gene with the susceptibility for the developement of AD in Serbian population.

Key words: Alzheimer's disease, polymorphism, prevalence, *TREM2*, R47H

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INTRODUCTION

Alzheimer's disease (AD) is a progressive chronic neurodegenerative disease, which is clinically manifested by the development of dementia (1). As a neurodegenerative disease, AD affects multiple brain functions, which causes a range of signs and symptoms that include a progressive loss of mental and intellectual functions that disrupt daily life, an early occurrence of disorders affecting executive functions, depression, insomnia, anxiety, agitation, and behavioral impairment (2). There is no simple and reliable test applicable for diagnosing AD, thus its course is followed based on clinical observations, cognitive testing, neuropsychological testing along with important neuroradiological procedures (e.g., CT, NMR, PET, PET-CT, etc.). The disease is classified as multifactorial, involving different causes, such as genetic predisposition, environmental factors, and lifestyle habits (3). The most important known risk factor for late onset Alzheimer's dementia is ageing (4). There are several etiopathogenetic mechanisms, and the most supported one is the amyloid hypothesis. Amyloid plaques represent extracellular formations, amyloid – (Aß) peptide being their major component. Aß peptide is produced through the proteolytic processing of amyloid precursor protein (APP) by β and γ secretases. Deposition of Aß peptide in extracellular space (ECS) results in neurotoxicity, it promotes apoptosis, and increases synthesis of oxidative stress mediators (5). On the other hand, neurofibrillary tangles (NFTs) are intracellular insoluble aggregates of hyperphosphorylated tau protein, that affect neurons in different regions of the brain. The result of intracellular deposition NFTs is axonal instability that impairs transport of nutrients along with cell signal communication, neurodegeneration, and apoptosis (6,7).

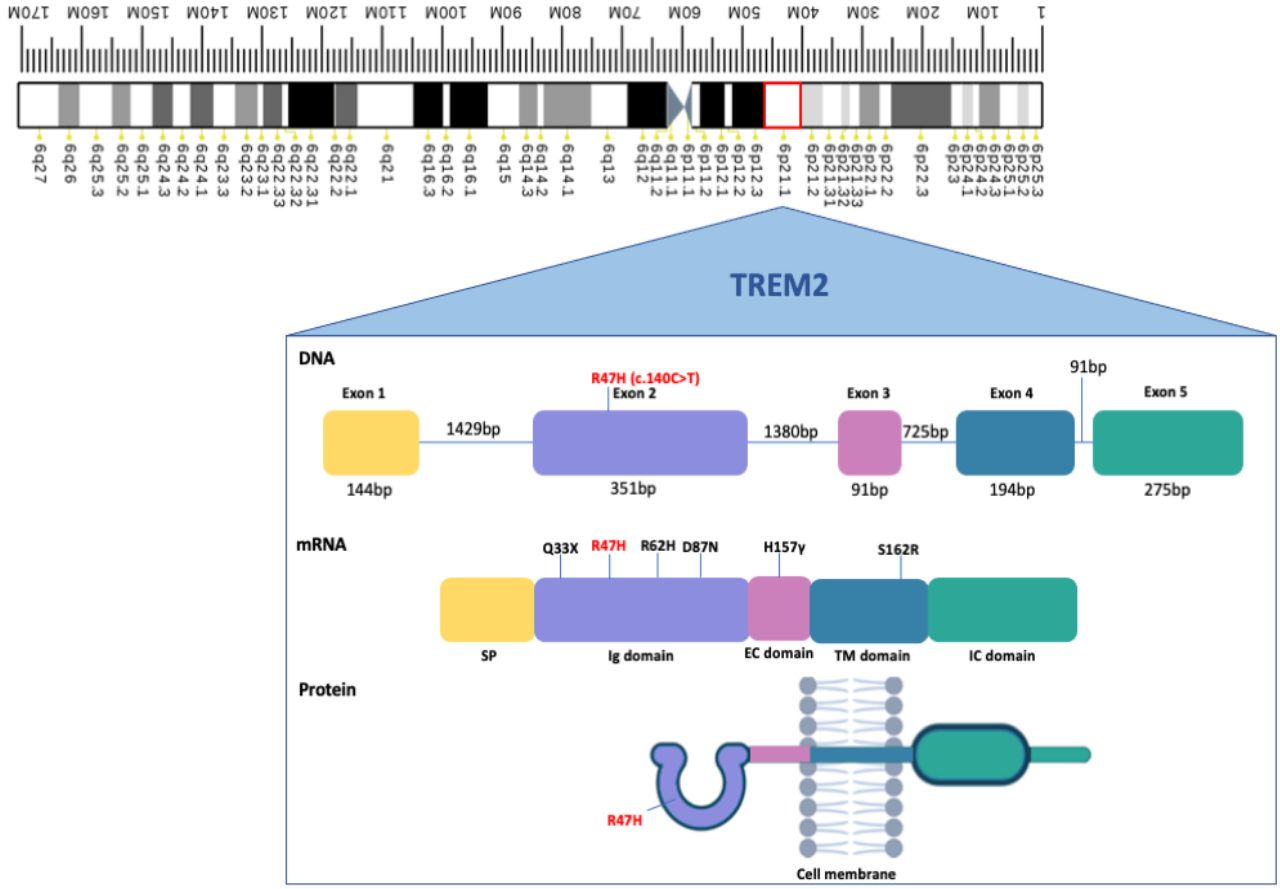

Image 1. Ideogram of chromosome 6 with schematic representation of the human TREM2 gene and protein structure, modified according to Yang et al (11).
The TREM2 gene is located on chromosome 6 in the region 6p21.1. It posses 5 exonic regions (boxes) and 4 intronic regions (lines). The mRNA is shown with transcribed regions, and different colors represent different exonic sequences of the transcription template. Most frequent mutations of the TREM2 gene occur within its exons, some of them being: R47H, Q33X, R62H, D87N, H157γ, S162R, described on the schematic representation of mRNA. Point mutation within the variable region of the TREM2 gene results in the loss of function, decreased ligand binding, disturbances in signal transmission and reduced expressions. The variant R47H of the TREM2 gene implies the existence of point mutation at position (c.140C-T), leading to a modification in the exon 2 domain. During translation, histidine is replaced by arginine at position 47 of the protein. The rs75932628 (R47H) shows an odds ratio (OR) range of 2.0-5.0, and it also has minor allele frequency (MAF) about < 1 % (26)
SP – signal peptide, Ig domain – immunoglobulin domain, EC domain – extracellular domain, IC domain – intracellular domain, TM – transmembrane domain.

Studies of genetic susceptibility to AD indicate a whole range of genes and their variants that can potentially influence an individual's susceptibility to develop the disease. Certain protein-coding genes, such as *TREM2, CD33, CR1, ABCAT, SHIP1, PSENI, PSEN2*, are in close relation with microglia, and they influence immune regulation and inflammatory response. Mutations in these genes are also linked with increased susceptibility for AD (8–10).

Triggering receptor expressed on myeloid cells 2 (TREM2) is a protein coded by *TREM2* gene. The *TREM2* gene is located on an autosomal locus, and it is mapped on the region 6p21.1 (11). TREM2 is a transmembrane glycoprotein which includes 227 amino acids, and consists of an extracellular region, the membrane-traversing segment, and an intracellular region. TREM2 is expressed on the myeloid cells' membrane, granulocytes, macrophages, immature monocyte-derived dendritic cells, osteoclasts, alveolar macrophages, Kupffer cells, and microglia, which are immune cells in the central nervous system (CNS). The ectodomain of TREM2 includes an Ig-like V-type domain. The intracellular region has signal function via DNAX activator proteins. Ectodomain shows affinity to binding ligands such as glycoproteins, lipids, lipopolysaccharides (LPS), high density lipoprotein (HDL), low density lipoprotein (LDL), apolipoprotein A1 (ApoA1), apolipoprotein A2 (ApoA2). apolipoprotein B (ApoB), clusterin or apolipoprotein J (ApoJ), and apolipoprotein E (ApoE) (12).

A large number of frameshift, and missense mutations with different changes in the expression in the *TREM2* gene have been described in association with neurodegenerative diseases (13). Most of mutations were located in a coding sequence, yet those in non-coding regions have also been described. It has been observed that certain polymorphisms of *TREM2* gene can increase the risk of some neurological diseases in particular AD, posterior cortical atrophy (PCA), essential tremor (ET), multiple sclerosis (MS), progressive non-fluent aphasia (PNFA), Parkinson's disease, and Lewy body dementia (14). AB17 is another recognizable entity of Alzheimer's disease that is solely associated with mutations in the *TREM2* gene.

The aim of this study was to determine the genotype of R47H variant of the *TREM2* gene in the population of AD patients, and to compare the frequency of the variant in the population of AD patients and healthy controls, as well as to determine the possible association of a certain genotype with susceptibility to develop the disease.

MATERIALS AND METHODS

Patients and methods

The study comprised 168 consecutive patients with Alzheimer's disease, and 190 healthy controls. Clinical diagnosis of AD was assesed according to the valid diagnostic criteria (15). A clinical interview, a neurological examination, and neuropsychological testing were performed by a neurologist, with subspecialization in neurodegenerative dementia, and a clinical neuropsychologist, respectively. All subjects fulfilled the same examination protocol. For the purposes of our study global cognitive assesement by Mini Mental State Examination Test (MMSET) and Goldman score were investigated.

The MMSET systematically assesses the cognitive status of a patient. This test provides an insight into eleven domains of cognitive functioning, including temporal orientation, spatial orientation, immediate orientation, attention/concentration, calculation, delayed recall, naming, verbal repetition, verbal comprehension, writing, reading a sentence, and constructional praxis. The maximum number of points is 30, and a score of 23 or less indicates a cognitive impairment.

A Goldman score (GS) was assigned to each subject according to family medical history in order to indicate the pattern of inheritance. Goldman score 1 denotes the presence of at least three affected individuals in two generations on the genealogical tree (where one person is a first-degree relative to the other two affected individuals); Goldman score 2 is a family aggregation of three or more family members with dementia who do not meet the criteria for score 1; Goldman score 3 refers to the existence of one affected family member with early-onset dementia; Goldman score 3.5 is assigned if the disease has occurred after the age of 65 in the affected family member (late-onset dementia); Goldman score 4 is in case of unknown medical history or in families with insufficient data (16).

Genetic investigation

During hospitalization, every patient was venepuctured to obtain 10 ml of peripheral blood. After sampling, sodium citrate was added to the blood at a concentration of 0.38% (w/v), and then the samples were stored at -20°C until the analysis. Genomic DNA was isolated from the peripheral blood leukocytes using the *PureLink™ Genomic DNA Mini Kit (Life Technologies, USA)*. Genotyping of rs75932628, R47H polymorphism of the TREM2 gene was performed using *Real-time Polymerase Chain Reaction (qPCR)* and *TaqMan® SNP genotyping assay (Applied Biosystem by Thermo Fisher Scientific, USA*). The reaction mix in the total volume of 15 µl consisted of 5 µl Taqman genotyping master mix, 0.75 µl Taqman SNP assay (20x), a total of 1 µl DNA sample and 5.75 µl water.

PCR temperature profile was as follows: one cycle of initial step of 10 min at 60°C to activate the chemically modified *Taq DNA polymerase,* followed by 40 cycles of denaturation for 15 s at 95°C, and subsequent hybridization and extension at 60°C for 1 minute. The initial phase of data processing from molecular analysis was

performed by Life Technologies Real Time PCR software, which shows allelic discrimination data results as a scatter plot of allele 1 (VIC® stain) versus allele 2 (FAM® stain). Each reaction well of the plate is represented as a single point on the graph, which allows the identification for the following genotypes of the R47H *TREM2* gene variant: CC – homozygous without mutation, CT – heterozygous for mutation, TT – homozygous for mutation.

Statistical analyses

For data analysis, selective methods of descriptive statistics were used: measures of central tendency (arithmetic mean, median), variability measures (standard deviation, range), and structure indicators (absolute and relative numbers). In order to compare differences in allele frequency, Pearson's chi-squared test with Yates correction or Fisher's exact test were employed. To test the statistical significance of the association between most frequent alleles and clinical presentation, an odds ratio (OR) was calculated with 95% confidence interval (CI). The Mann-Whitney U Test was used to evaluate statistically significant differences among MMSET and Goldman score. Spearman correlation coefficient was computed to assess the relationship between the MMSET score and the level of education. The criteria for the significance of the statistical differences were $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

RESULTS

Demographic and clinical characteristics

The study included 168 consecutive patients with the Alzheimer's disease (mean age of onset 57.6±6.7 years, 54.8% for women and 45.2% for men), and 190 healthy controls. The youngest patient was diagnosed with AD at the age of 35, while the oldest patient was diagnosed with AD at the age of 83. The duration of the disease from the time of diagnosis to the inclusion in the study was 3.6±2.1 years. The early onset form of AD occurred in 91.7% of patients, while the late onset form occurred in 8.3% of patients. The average years of education of our patients are shown by class intervals. Among patients, 33% had 0-8 years of education, 42.9% had 8-12 years of education, and 23.2% had more than 12 years of education. The presence of neurological and non-neurological comorbidities occurred in 39.9%, while 60.1% of respondents had only cognitive issues as clinical presentation.

Analyzing the Goldman score, we determined that 4.2% of patients had GS 1; 1.8% had GS 2; 8.3% had GS 3; 22.6% of patients had GS 3.5; and finally, 63.1% of patients received score 4 **(Table 1)**.

Table 1. Demographic and clinical characteristics of AD patients

Variable		AD
The size of the sample (n)		168
[a] Age (years)		57.63±6.71
[a] Time elapsed from onset to diagnosis (years)		3.64±2.12
[b] Gender	Male	76 (45.2%)
	Female	92 (54.8%)
[b] Level of education	0-8 years	57 (33.9%)
	8-12 years	74 (42.9%)
	>12 years	39 (23.2%)
[b] Goldman score	1	7 (4.2%)
	2	3 (1.8%)
	3	14 (8.3%)
	3.5	38 (22.6%)
	4	106 (63.1%)
[b] Comorbidities	Positive	67 (39.9%)
	Negative	101 (60.1%)
[b] Clinical presentation	Early onset (Early AD)	154 (91.7%)
	Late onset (Late AD)	14 (8.3%)

[a] Mean (± SD)
[b] absolute frequency (relative frequency in %)

MMSE test scores varied greatly among patients. The MMSE test score ranged from 3/30 to 28/30, and the average value of the score was 15.43±6.60. About 50.6% of patients had the MMSE test score <15 **(Table 2)**.

Table 2. MMSET score in AD patients

Variable			Value
[b] The size of the sample (n)			168
[a] MMSET score			15.43±6.60
[b] MMSET score	28-30	Normal	4 (2.4%)
	25-27	Minimal cognitive impairment	11 (6.5%)
	21-24	Mild cognitive impairment	32 (19.1%)
	16-20	Moderate cognitive impairment	36 (21.4%)
	<15	Severe cognitive impairment	85 (50.6%)

[a] Mean (± SD)
[b] absolute frequency (relative frequency in %)

The difference in MMSE test score values among different genotypes of the R47H variant of AB patients did not reach statistical significance (p=0.3786). The comparison of the Goldman score among different genotypes of the R47H variant did not reveal any statistically significant differences (p=1) **(Table 3)**.

A higher score of the MMSE test score at the beginning of the disease was statistically significantly associated with a higher level of education in patients (r=0.3144; p=0.00003; r2=0.0988). Patients with higher educational level exhibited significantly higher MMSE test score values at the beginning of the disease **(Image 2)**.

Table 3. The MMSET score and Goldman score among different genotypes of the R47H variant of AD patients

Score	AD (genotype CC)	AD (genotype CT)	p
	Mean	Mean	
MMSET	**The size of the sample, n=164**	**The size of the sample, n=4**	
	15.5	12.5	**0.3786**
Goldman	**Sthe sze of the sample, n=164**	**The size of the sample, n=4**	
	4	4	**1**

For comparing statistical significance in difference of MMSET and Goldman score *Mann-Whitney U Test* was used (*p<0.05, **p<0.01, ***p<0.001).

Molecular genetics analysis

In the group of AD patients, the frequency of the C allele was 98.8%, while the T allele was present in 1.2% of patients. Within the control group, the frequency of the C allele was 100%, while the T allele was not found in any of the healthy subjects. The frequency of heterozygous carriers of the R47H variant of the TREM2 gene in the group of patients differed significantly from the frequency in the control healthy population of Serbia (p<0.05; OR 0.10; 95% CI 0.01-1.81) **(Table 4)**.

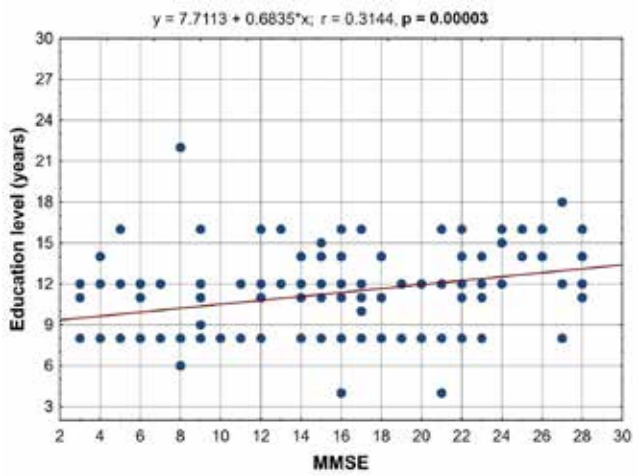

Image 2. Correlation between MMSET score and years of education in AD patients
A bivariate scatterplot with a smooth regression line shows the interrelationship between the MMSET score and the level of education expressed in years of education. Correlation analysis was performed using the Spearman Rank test, which shows the existence of a statistically significant positive relationship between the examined variables. The level of statistical significance was p<0.001.

The frequency of homozygotes without mutation (CC genotype) was 97.62%, the frequency of heterozygotes for the mutation (CT genotype) was 2.38% **(Image 3)**.

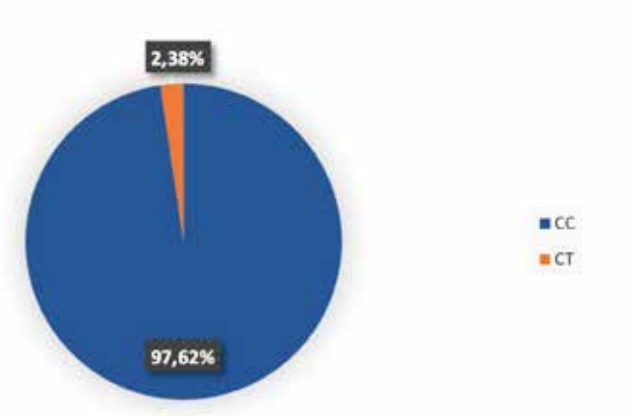

Image 3. Frequency of CC, CT genotype of variant R47H among patients with AD

DISCUSSION

According to our knowledge, this research is the first original investigation which analyzed distribution of alleles of R47H variant of the *TREM2* gene in patients with AD.

Recent clinical studies have shown that certain variants of the TREM2 gene could have a significant impact on the occurrence of dementia, in addition to the development of clinical presentation typical for AD (1,8,10).

In our study group, cognitive performance on the MMSET among subjects with a low level of education was shown to be worse compared to subjects with a higher level of education. These findings could be explained by cognitive reserve theory. Interestingly, as brain development

Table 4. Frequencies of C and T alleles of the R47H *TREM2* gene variant among AD patients and controls

Allele	AB	Control group*	Odds ratio (95% confidence interval)	p
	Allele frequency (%)	Allele frequency (%)		
C				
c.140C	98.8	100	0.10 (0.01-1.81)	***0.0329**
T				
c.140C>T	1.2	0	0.10 (0.01-1.81)	***0.0329**

* Healthy population of Serbia, 190 persons
In order to assess the statistical significance of the difference in relative allele frequency (%) Fisher's exact probability test was used (*p<0.05, **p<0.01, ***p<0.001).

proceeds, cognitive reserve tends to increase primarily early in life through young adulthood, and then tends to deplete later in life. However, as formal education affects cognitive abilities, and consequently the achievement of certain values on the MMSET, people with a lower level of education can potentially be misidentified as being at risk of developing dementia (17,18). An early onset of AD (between 30–50 years of age) is associated with the existence of autosomal dominant mutations within the genes encoding amyloid precursor protein (APP), presenilin-1 (PSEN1) and presenilin-2 (PSEN2) (19–21). Palonev and colleagues (Palonev J, et al. 2002) showed that the mutated homozygous form of the R47H variant of the *TREM2* gene led to the dysfunction of the TREM2 protein, which is associated with the development of various forms of dementia, indicating a great importance of the TREM2 signaling molecule in brain immune homeostasis (22). A meta-analysis by Lu Y, et al. pointed out that the R47H variant of the TREM2 gene increased the risk of developing AD up to 2.70 times (23). Research of Cruchaga et al, which examined the association of the R47H variant with the progression of AD, showed that there was an association of this variant with a faster progression of AD along with the findings of high levels of hyperphosphorylated tau in the cerebrospinal fluid of AD patients. Later studies reported similar results (24, 25). Furthermore, two independent studies indicated that there was an association between the rare heterozygous form of the R47H variant of the TREM2 gene with the development of AD, and subsequent studies confirmed this observation in the population of Spain and France. Benitez et al. also analyzed the frequency of the R47H heterozygous variant among AD patients of the Spanish population, and it was estimated to be 7/550 (1.27%) (26,27). In one genome-wide association study conducted on 3550 AD patients and a large number of controls in Iceland, Jonsson et al. found a significant association between the T allele of the R47H variant of the TREM2 gene (rs75932628) and the risk of developing the disease (p= 3.42×10(-10); OR 2.92; 95% CI 2.09-4.09); this association was stronger when healthy control subjects were older than 85 yrs; there were four subjects who were homozygous for the TT genotype mutation, while AD was confirmed *postmortem* in the two of them (28). Yet, one association study of Ma J et al. conducted in 279 patients with late-onset AD and 346 controls in Chinese population concluded that no T allele of rs75932628T was found neither in patients nor in controls (29). High-throughput sequencing of the TREM2 gene in 988 patients with late-onset AD and 1354 healthy controls of Chinese origin by Jiang et al. identified four rare coding variants, and then showed that the H157Y variant (rs22342555) represented a risk factor for the development of the disease (30).

Among our AD patients there were 4 heterozygous patients (genotype CT), as well as 164 homozygotes without mutation (genotype CC), while homozygotes for a mutation (genotype TT) were absent. The frequency of allele C was 97.62%, and the frequency of allele T was 2.38% among patients with AD **(Image 3)**. Within the control group, the frequency of allele C was 100%, while the T allele was absent. Compared to the healthy population, the heterozygous form of this mutation was found significantly more often among patients with AD **(table 4.)**.

CONCLUSION

Currently, neurologist dealing with AD lack a causal therapeutic intervention for their patients. The etiological cause of AD is notably diverse, encompassing both early-onset familial and late-onset sporadic variants, each bearing a distinct genetic basis. Early-onset familial AD pathogenesis is tangled and intertwined with an autosomal dominant inheritance pattern governed by three salient genes: *APP*, *PSEN1*, and *PSEN2*. The most ubiquitous sporadic form of AD, typically manifesting subsequent to the age of 60, has consistently, across a multitude of empirical investigations, been linked to the involvement of a single gene, the apolipoprotein E (*APOE*) gene. While extensive investigations have probed the mechanistic roles played by these genes in the pathogenesis of Alzheimer's disease (AD), the biological basis governing the progression of AD has yet to be definitively characterized. Notably, a hypomorphic variant identified within the microglial receptor *TREM2*, denoted as R47H, has demonstrated a marked escalation in the risk for AD. This study which was conducted on Serbian peatients indicated this possible influence of the heterozygous R47H variant of the *TREM2* gene in susceptibility to develop the disease.

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STUDIJA POVEZANOSTI VARIJANTE R47H GENA *TREM2* SA GENETIČKOM PODLOŽNOŠĆU ZA ALCHAJMEROVU BOLEST U POPULACIJI SRBIJE

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Sažetak

Uvod: Alchajmerova bolest (AB) je hronično neurodegenerativno oboljenje, koje se klinički ispoljava razvojem demencije. Zastupljenost genetičkih faktora rizika kao i njihova uloga u patogenezi AB nisu do kraja razjašnjeni. Istraživanja u oblasti genetičke podložnosti za AB ukazuju na postojanje čitavog niza gena i njihovih varijanti, koje potencijalno mogu uticati na sklonost pojedinca da razvije AB. AB17 je entitet Alchajmerove bolesti koji se povezuje sa mutacijama u genu *TREM2* koji kodira okidački receptor mijeloidnih ćelija tip 2.

Cilj ovog istraživanja bilo je ispitivanje učestalosti varijante R47H gena *TREM2* u populaciji obolelih od AB, poređenje učestalosti ispitivane varijante u populaciji ispitanika i zdravoj populaciji, kao i utvrđivanje rizika za oboljevanje kod nosilaca određenog genotipa.

Materijal i metode: U studiju je uključeno 168 konsekutivnih ispitanika sa dijagnozom AB i 190 zdravih kontrola. Klinički intervju, neurološki pregled i neuropsihološki set testiranja obavljeni su od strane neurologa i neuropsihologa koji se bave neurodegenerativnim bolestima. Genotipizacija rs75932628, R47H polimorfizma *TREM2* gena vršena je korišćenjem metode *PCR* u realnom vremenu (engl. *Real-time Polymerase Chain Reaction, qPCR*) i *TaqMan® SNP genotyping* eseja (*Applied Biosistem by Thermo Fisher Scientific, USA*).

Rezultati: U grupi ispitanika učestalost alela C iznosila je 98,8%, dok je T alel bio zastupljen kod 1,2% pacijenata. Učestalost T alela bila je statistički značajno veća u populaciji obolelih nego u kontrolnoj grupi ($p<0,05$). Učestalost homozigota bez mutacije (genotip CC) iznosila je 97,62%, učestalost heterozigota za mutaciju (CT genotip) iznosila je 2,38% unutar grupe obolelih od AB, dok je učestalost homozigota bez mutacije (genotip CC) iznosila 100% unutar zdrave kontrolne grupe.

Zaključak: Naša studija ukazala je na mogući uticaj ređeg alela varijante R47H gena *TREM2* na razvoj AB u našoj populaciji.

Ključne reči: Alchajmerova bolest, polimorfizam, prevalenca, *TREM2*, R47H

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