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Allelic Distribution of Genes for Apolipoprotein E and *MTHFR* in Patients with Alzheimer's Disease and Their Epistatic Interaction

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Abstract.
Background: Genetic risk factors play an important role in the pathogenesis of Alzheimer's disease (AD). However, the gene-gene interaction (epistasis) between specific allelic variants is only partially understood.
Objective: In our study, we examined the presence of the ε4 allele of apolipoprotein E (*APOE*) and the presence of C677T and A1298C (rs1801133 and rs1801131) polymorphisms in the methylenetetrahydrofolate reductase (*MTHFR*) gene in patients with AD and controls. We also evaluated the epistatic interaction between *MTHFR* and the *APOE* variants.
Methods: A total of 564 patients with AD and 534 cognitively unimpaired age-matched controls were involved in the study.
Results: The presence of the ε4 allele of *APOE* increases the risk of developing AD in a dose-dependent manner (OR 32.7: homozygotes, 15.6: homozygotes + heterozygotes, 14.3: heterozygotes). The combination of genotypes also increases the risk of developing AD in a dose-dependent manner: OR 18.3 (*APOE* 4/X and 4/4 + CT rs1801133), OR 19.4 (*APOE* 4/X and 4/4 + CT rs1801133 + AC rs1801131), OR 22.4 (*APOE* 4/X and 4/4 + TT rs1801133), and OR 21.2 (*APOE* 4/X and 4/4 + CC rs1801131). Homozygotes for variant alleles of *MTHFR* as well as patients with AD had significantly higher levels of homocysteine than homozygotes for standard alleles or controls.
Conclusion: Homozygotes for *APOE4* and carriers of *APOE4* with TT genotype of rs1801133 were found to be at the highest risk of developing AD. These findings suggest that the epistatic interaction of specific gene variants can have a significant effect on the development of AD.

Keywords: Alzheimer's disease, apolipoprotein E, epistasis, MTHFR

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INTRODUCTION

Human apolipoprotein E (*APOE*) is primarily expressed in three isoforms (*APOE2*, *APOE3*, and *APOE4*), which differ by only two residues. *APOE4* constitutes the most important genetic risk factor for Alzheimer's disease (AD), *APOE3* is neutral, and *APOE2* is protective [1]. The prevalence of *APOE* in European and North American populations is approximately 15–20%, with a significant interethnic variation. The prevalence of the ε4 allele is lower in the Mediterranean regions and increases toward the north [2, 3]. However, in patients with AD, the prevalence is 65–75% [4–7]. This percentage refers to the Caucasian populations of Western Europe, North America (USA and Canada), and Japan. Carriers of the ε4 allele in the homozygous or heterozygous state are at a considerably increased risk of developing AD with an earlier age of onset [4]. The earliest genetic and epidemiological studies revealed that the risk for AD is 2–3-fold higher in people with one *APOE* ε4 allele and approximately 12-fold higher in those with two *APOE* ε4 alleles [4, 7, 8]. Latter studies reported an odds ratio (OR) of three to five for heterozygotes and approximately fifteen for homozygotes [9, 10]. Some recent studies showed a much higher OR of more than 10 for heterozygotes and more than 20 for homozygotes [11, 12]. Remarkably, *APOE* ε4 prevalence and the consequent risk for AD vary significantly among different ethnic groups [12, 13]. Bertram et al. [11] reported 3.9 and 21.8 ORs among the Japanese population for heterozygotes and homozygotes, respectively. However, Raichlen et al. [12] reported 5.6 and 33.1 ORs for heterozygotes and homozygotes, respectively, among US Japanese. Thus, it appears that not only genotype but also the environment could influence the relative risk. In Slovakia as well as Central Europe (Czech Republic, Poland, and Hungary), an epidemiologic study focusing on *APOE* prevalence has not yet been performed. Therefore, one of the aims of this study was to investigate the prevalence of the *APOE4* isoform among patients with AD in comparison with a cognitively unimpaired elderly population group in this population.

APOE is highly expressed in the brain, primarily in the astrocytes, but its function is only partially understood [14]. *APOE* is a regulator of lipoprotein metabolism [15, 16]. *APOE* plays several important roles in the central nervous system such as: cholesterol transport, neuroplasticity, and inflammation [16–18]. Furthermore, *APOE* binds to Aβ and influences the clearance of soluble Aβ and the Aβ aggregation [16, 19]. Neuropathological and neuroimaging studies demonstrate that *APOE* ε4 carriers exhibit accelerated and more abundant Aβ deposition than *APOE* ε4 negative individuals [20–22]. Thus, genetic, cellular, animal, and human studies demonstrate that *APOE* is a risk factor for AD and modifies AD pathogenesis via an APP-dependent manner [20–23]. Apolipoprotein E4 has a detrimental effect on the brain in various pathological circumstances. Adult and pediatric carriers of the *APOE4* isoform have a significantly impaired regeneration process following a head trauma [24, 25]. *APOE ε4* allele could also be a risk factor for the cerebral palsy in children [26, 27]. According to study of Gümüş et al., ε4 allele increased the disease risk about three times and the *APOE 4/2* genotype appeared to be the most risky [27].

An elevated level of homocysteine is considered to be a factor leading to impaired cognitive performance among an aging population and is also a risk factor for AD. Hyperhomocysteinemia is caused by the disruption of carbon monoxide transfer in methylenetetrahydrofolate reductase (*MTHFR*) polymorphisms, which catalyzes the conversion of homocysteine to methionine. Currently, two polymorphisms of *MTHFR*, i.e., C677T and A1298C, are described. The prevalence of polymorphisms varies widely in various studies and among different populations. For example, according to Stoccoro et al. [28], the frequency of the TT genotype of *MTHFR* C677T in Italian AD patients and a control group were 22.4% and 19.7%, respectively. According to the meta-analysis of Zhang et al. [29], the frequency of alleles and genotypes indicated inter-ethnic and intra-ethnic variability and also depended on the study's design and cohort definition. Overall, and despite the differences, Zhang's study confirmed C677T polymorphism as a mild risk factor for AD with OR ranging from 1.2–1.8 in individual studies.

Hyperhomocysteinemia resulting from polymorphisms of *MTHFR* may trigger the development of AD on at least two levels: biochemical and epigenetic. The biochemical level represents two main mechanisms. The first mechanism of the biochemical level is the direct toxic effect of homocysteine on the endothelium [30–32]. The second mechanism is the direct neurotoxic effect of homocysteine. Experimental tests on cell cultures and laboratory animals have shown that increased levels of homocysteine affect DNA repair, increase beta-amyloid formation, and sensitize neurons to amyloid toxicity [33]. Homocysteine also increases the secretion of inflammatory

mediators, especially NF-κ-B, and interleukins IL-1β, IL-6, and IL-8 [30–32].

The level of homocysteine in the blood is regulated by genetic, hormonal, and nutritional factors. The major genetic determinant affecting the level of homocysteine is the enzyme (*MTHFR*). Heterozygotes for these polymorphisms generally do not have increased homocysteine, but homozygotes typically show a slight increase (13–30 μmol/L). Epigenetic level refers to the mutual gene interaction (epistasis) between the *MTHFR* gene and other genes. In the AD context, the interaction between the following genes has been investigated: *MTHFR* and the IL-6 gene [34], *APOE* and the IL-6 gene [35], and *MTHFR* and the RFC-1 gene [36]. However, recent advances in our understanding of the human genome, namely technological advances in the methods to analyze millions of polymorphisms in thousands of subjects have revealed new genes associated with AD risk: *ABCA7, BIN1, CASS4, CD33, CD2AP, CELF1, CLU, CR1, DSG2, EPHA1, FERMT2, HLA-DRB5-DBR1, INPP5D, MS4A, MEF2C, NME8, PICALM, PTK2B, BDNF, ALDH2, SORL1,* and *ZCWPW1* [20, 37]. All these genes could also interfere with *APOE* or *MTHFR* and cause a summation of detrimental effect. We chose the interaction between the *MTHFR* gene and the *APOE* gene for this study as both genes are significantly involved in the pathogenesis of AD. There are few studies in the literature that analyzed the genetic interaction between *APOE* and *MTHFR*. According to a study by Stoccoro et al. 2017, the presence of the TT genotype rs1801133 increases the OR for AD in both *APOE4* carriers and non-carriers [28].

In our study, we analyzed 564 patients with AD and measured the frequency of individual alleles and combinations of genotypes to assess their risk. Our first aim was to analyze the frequency and distribution of *APOE* alleles in patients with AD and compare this data with age-matched controls without cognitive deficits. The second aim was to assess the occurrence of *MTHFR* C677T and A1298C polymorphisms in patients with AD and compare this data with age-matched controls without cognitive deficits. The third aim was to evaluate the gene interaction (epistasis) of *APOE* and *MTHFR* genes and evaluate the potential effect of each allele or genotype combination on the development of AD. Finally, the fourth aim was to evaluate and compare homocysteine levels in AD patients and controls; in particular, between specific genotype sets of rs1801133 and rs1801131 (homozygotes for standard alleles, heterozygotes and homozygotes for variant alleles).

MATERIAL AND METHODS

Participants

The participants consisted of 564 patients with AD examined in the I. Neurological clinic, Bratislava between 2003 and 2018. AD diagnosis was determined based on a clinical picture and ancillary imaging methods following the DSM-IV and NINCDS-ADRDA criteria, and after 2010, as per the revised criteria and the new lexicon for AD [38]. All the patients underwent magnetic resonance imaging of the brain and were also screened for *APOE* and *MTHFR* genotype at the time of screening. From 2014, most of the patients underwent amyloid positron emission tomography (PET) or cerebrospinal fluid (CSF) biomarker testing. Subsequently, patients were stratified based on the *APOE* genotype, *APOE4* carriers, and *APOE4* non-carriers. The control set consisted of 534 age-matched participants without any cognitive deficit, who had all the tests performed as the patients with AD. The appropriate institutional review board of the Faculty of Medicine of Comenius University reviewed and approved all of the study procedures. Once an informed consent form was signed, the participants were examined thoroughly by a team consisting of qualified neurologists specialized in AD.

Determination of homocysteine

Homocysteine levels was estimated in the blood sample using ADVIA Centaur® HCY assay. The ADVIA Centaur HCY assay is a competitive immunoassay that uses direct chemiluminescence technology. Various forms of homocysteine in a patient sample are reduced to free HCY via a reducing reagent. Free homocysteine is then converted to S-adenosylhomocysteine (SAH) via an enzyme induced reaction. The altered SAH from the patient sample competes with the SAH, which is covalently linked to the solid phase paramagnetic particles, for a limited amount of the acridine ester-labeled anti-SAH antibody found in the Lite reaction. The physiological value of plasma homocysteine is in the range of 5–15 μmol/L.

DNA methods

DNA extraction

Genomic DNA was prepared from whole blood samples (0.2 ml) using the NucleoSpin Blood kit (Macherey-Nagel).

APOE

The four exons of *APOE* were amplified through the polymerase chain reaction (PCR) method using the following primers: *APOE* (length of PCR product is 227 bp) F-TCC AAG GAG CTG CAG GCG GCG CA; R-ACA GAA TTC GCC CCG GCC TGG TAC ACT GCCA. PCR fragments were performed in 20 μl reaction volumes containing 50 ng of DNA, 2X PCR MasterMix (Thermo-Fisher Scientific) 0.5 μM of each primer, and 10% of DMSO. The PCR programme for the *APOE* amplification was 4 min at 95°C, and then 35 cycles at 95°C for 30 s, 69°C for 20 s, and 72°C for 20 s, followed by 10 min at 72°C for the final extension. Genotyping was performed through restriction fragment length polymorphisms. A PCR fragment of the length 227 bp was digested by the restriction enzyme *HhaI* (5U) (Thermo Fisher Scientific) overnight at 37°C. Digestion pattern for possible *APOE* genotypes are: E2/E2-91 bp, 82 bp; E2/E3-91 bp, 82 bp, 48 bp, 34 bp; E3/E3-91 bp, 48 bp, 34 bp; E3/E4-91 bp, 72 bp, 48 bp, 34 bp, 19 bp; E4/E4-72 bp, 48 bp, 34 bp, 19 bp; E2/E4-91 bp, 82 bp, 72 bp, 48 bp, 34 bp, 19 bp.

MTHFR

Two single-nucleotide polymorphisms (C667T-rs 1801133 and A1298C- rs1801131) were analyzed through carrying out real-time PCR. The assays were performed using a Maxima 2X Probe Master Mix (Thermo Fisher Scientific) according to the manufacturer's instructions. The PCR primers and probes used in the assay (Taqman MGB Probes; Applied Biosystems, Foster City, CA), which were labelled with dyes (FAM or VIC), were as follows: for *MTHFR* C677T, forward primer 5′-GAAAAGCTGCGTGATGATG-3′, reverse primer 5′-TTGAAGGAGAAGGTGTC-3′, probe WT (VIC-dye labeled) AATCGGCTCCCGC, and probe MUT (FAM-dye labelled) AATCGACTCCCGC; for *MTHFR* A1298C, forward primer 5′-AAGAACGAAGACTTCAAA-3′, reverse primer 5′-TGGGGGGAGGAGCTGAC-3′, probe WT (FAM-dye labeled) ACACTTTCTTCACT, and probe MUT (VIC-dye labelled) ACACTTGCTTCACT. PCR using a 20 ng of genomic DNA was amplified in a real-time PCR thermal cycler (StepOne; Applied Biosystems, Foster City, CA). The amplifications consisted of an initial step of 95°C for 15 min, followed by 20 cycles of 92°C for 15 s and 60°C for 1 min. Genotyping was performed using the allelic discrimination method.

Statistical analysis

SPSS version 25.0 (Chicago, IL) was used for performing all the statistical analyses. Descriptive analysis was recorded in terms of mean and standard deviation. Kruskal-Wallis test was used to determine the significant difference in the age of the first clinical signs of AD among wild and variant genotypes. The *t*-test was used to determine significant differences in the homocysteine level among the specific genotype sets, as well as between the AD group and the control group. Pearson's chi-square test was used to determine the difference in the occurrence of individual genotypes between the AD group and the control group. A p-value of <0.05 was considered to be statistically significant. Regression analysis was also performed to find out interaction of ε4 allele of apolipoprotein E, rs1801133 and rs1801131.

RESULTS

A total of 564 patients with AD and 534 cognitively unimpaired age-matched control subjects were involved in the study.

Age of first clinical signs (further only AAO)

The mean AAO was 72.4 years. A significantly lower AAO (68.3 years) was for patients with the *APOE* 4/4 genotype. Patients that did not carry the ε4 allele had a significantly higher AAO (75.0 years). The patients carrying the ε4 allele and *MTHFR* polymorphisms were significantly younger at AAO (71.1 years). The highest AAO was for the *APOE4* non-carriers, who did not have any polymorphism of *MTHFR* (76.8 years) (Tables 1 and 2). The mean age of the control group was 71.5 ± 6.7 years.

Level of homocysteine

The average level of homocysteine was at a normal value in non-carriers of polymorphisms. In polymorphism carriers, the average level of homocysteine was significantly higher in comparison with those of non-carriers (Table 3). Patients with AD had a significantly higher level of homocysteine than controls (Table 4).

Table 1
Age of first clinical signs

Genotype *APOE*	X/X ($n=140$)	4/X ($n=364$)	4/4 ($n=60$)	4/X + 4/4 ($n=424$)	Total ($n=564$)	*P*
Age of first clinical signs (mean ± SD)	75.0 ± 7.4	72.1 ± 6.3	68.3 ± 8.1	71.6 ± 6.7	72.4 ± 7.0	<0.001[a,b,c] 0.002[d]

[a]X/X versus 4/X; [b]X/X versus 4/4; [c]X/X versus 4/X a 4/4; [d]4/X versus 4/4.

Table 2
Age of first clinical signs in a specific genotype set

APOE and *MTHFR* genotype set	X/X + CC + AA ($n=12$)	4/X + 4/4 + 677TT ($n=56$)	4/X + 4/4 + 1298 CC ($n=56$)	Overall AD ($n=564$)	*P*
Age of first clinical signs (mean ± SD)	76.8 ± 5.8	71.5 ± 5.5	73.9 ± 5.3	72.4 ± 7.0	0.009[a] 0.174[b]

[a](X/X and CC and AA) versus (4/X or 4/4) and 677 TT); [b](X/X and CC and AA) versus (4/X or 4/4) and 1298 CC).

Table 3
Plasma level of homocysteine (μmol/L) in specific genotype set

Genotype rs1801133/rs1801131	CC/AA ($n=175$)	CT/AC ($n=689$)	TT/CC ($n=234$)	*P*
Level of homocysteine (mean ± SD)	12.2 ± 3.8	13.2 ± 4.2	17.2 ± 3.7	0.003[a] <0.001[b,c]

[a]CC/AA versus CT/AC; [b]CC/AA versus TT/CC; [c]CT/AC versus TT/CC.

Table 4
Plasma level of homocysteine (μmol/L) in specific groups

Group	AD ($n=564$)	Controls ($n=534$)	*P*
Level of homocysteine (mean ± SD)	15.7 ± 4.2	13.7 ± 4.5	<0.001

Allelic and genotypic distribution of APOE and MTHFR

Apolipoprotein E

The frequency of the ε4 allele (genotype 4/3 and 4/4) was significantly higher in the AD group than in the control group (75.2% versus 16.3%, $p<0.001$, Table 5). The most common genotype in patients with AD was 4/3 (364 patients, 64.5%); 4/4 genotype had 60 patients (10.6%). Genotype 3/3 or 3/2 had 140 patients (24.8%, Table 5).

MTHFR

In the case of *MTHFR*, the presence of the two most common polymorphisms (C677T and A1298C) and their frequency were obtained for both studied patient groups: the AD and the control group. **Allele T** of the C677T polymorphism was significantly more frequent in the AD group (37.2%) than in the control group (28.2%). The genotype distribution showed a significantly higher **TT genotype** (homozygous polymorphism) in the AD group (14.2%) compared with the control group (7.3%, $p<0.001$). The frequency of the **CT genotype** (heterozygous polymorphism) was also significantly higher in the AD group (46.1%) compared with the control group (41.8%, $p=0.007$). A similar pattern was observed in the A1298C polymorphism. **Allele C** was significantly more frequent in the AD group (35.8%) than the control group (29.7%, $p=0.002$). The distribution of genotypes also showed a significantly higher percentage of genotype CC (homozygous polymorphism) in the AD group (13.5%) compared with the control group (8.8%, $p=0.004$). The frequency of the AC genotype (heterozygous polymorphism) was comparable between the AD group and the control group (Table 5).

Interaction between APOE4, C677T, and A1298C alleles

The following combinations were selected to assess the gene interactions:

1. *APOE* ε4 allele + T allele C677T
2. 4/X and 4/4 genotype of *APOE* (heterozygotes and homozygotes together) + CT genotype C677T and AC genotype A1298C (heterozygotes for both *MTHFR* alleles)
3. 4/X and 4/4 genotype of *APOE* + CT genotype C677T

Table 5
Frequency of alleles and genotypes in specific groups

Groups	Allele frequency			Genotype frequency			
WT	WT **n/%**	Variant **n/%**	*P*	WT **n/%**	Heterozygot **n/%**	Homozygot **n/%**	*P*
APOE	**Non-ε4**	**ε4**		**X/X**	**4/X**	**4/4**	
AD ($n = 564$)	140/24.8	424/75.2	<0.0001	140/24.8	364/64.5	60/10.6	<0.0001
HC ($n = 534$)	447/83.7	87/16.3		447/83.7	81/15.2	6/1.1	
	ref	OR = 15.6 CI 95% 11.5–21.0 $p < 0.001$		ref	OR = 14.3 CI 95% 10.6–19.5 $p < 0.001$	OR = 31.9 CI 95% 13.5–75.5 $p < 0.001$	
C677T	**C**	**T**		**C/C**	**C/T**	**T/T**	
AD ($n = 564$)	708/62.8	420/37.2	<0.0001[a]	224/39.7	260/46.1	80/14.2	<0.0001[b]
HC ($n = 534$)	767/71.8	301/28.2		272/50.9	223/41.8	39/7.3	
	ref	OR = 1.5 CI 95% 1.3–1.8 $p < 0.001$		ref	OR = 1.4 CI 95% 1.1–1.8 $p = 0.007$	OR = 2.5 CI 95% 1.6–3.8 $p < 0.001$	
A1298C	**A**	**C**		**A/A**	**A/C**	**C/C**	
AD ($n = 564$)	724/64.2	404/35.8	0.0026[c]	236/41.8	252/44.7	76/13.5	0.0093[d]
HC ($n = 534$)	751/70.3	317/29.7		264/49.4	223/41.7	47/8.8	
	ref	OR = 1.3 CI 95% 1.1–1.6 $p = 0.002$		ref	OR = 1.3 CI 95% 1.0–1.6 $p = 0.068$	OR = 1.8 CI 95% 1.2–2.7 $p = 0.004$	

[a]Comparison of T alleles between AD and HC; [b]Comparison of C677T genotypes between AD and HC; [c]Comparison of C alleles between AD and HC; [d]Comparison of A1298C genotypes between AD and HC.

4. 4/X and 4/4 genotype of *APOE* + TT genotype of C677T
5. 4/X or 4/4 genotype of *APOE* + CC genotype of A1298C

1. The combination of ε4 allele *APOE* and T allele C677T was significantly more frequent in the AD group compared with those of the control group (45.4% versus 10.5%). In addition, this combination was the most frequent among all the combinations in the AD group. The carriers of this combination had an OR that increased to 19.7 (Table 6A).
2. The combination of the *APOE* 4/X and 4/4 genotype and CT genotype of C677T were significantly more frequent in the AD group compared with those of the control group (35.5% versus 8.8%, $p < 0.001$). The carriers of this combination had an OR that increased to 18.3 (Table 6B).
3. The combination of the *APOE* 4/X and 4/4 genotype and both variant alleles in the heterozygous state were significantly more frequent in the AD group compared with those of the control group (17.7% versus 3.4%). The carriers of this combination had an OR that increased to 19.4 (Table 6C).
4. The combination of the *APOE* 4/X and 4/4 genotype and TT genotype of the C677T were also significantly more frequently found in the AD group compared with those of the control group (9.9% versus 1.7%). This combination appears to result in the highest risk for the development of AD in carriers. Its carriers had an OR that increased to 22.4 (Table 7).
5. The combination of the *APOE* 4/X and 4/4 genotype and CC genotype of A1298C were also significantly more frequent in the AD group compared with those of the control group (9.9% versus 1.7%) and increased the OR to 21.2; (Table 8).

Main conclusions

1. The presence of the ε4 allele of *APOE* increases the risk of developing AD in a dose-dependent manner (OR 31.9 for homozygotes, 15.6 for homozygotes + heterozygotes, and 14.3 for heterozygotes).
2. The presence of homozygous *MTHFR* polymorphisms independently increases the risk of developing AD by 2.5-fold (genotype TT C677T) and 1.8-fold (genotype CC A1298C).

Table 6A
Combination of *APOE* ε4 allele and T allele C677T

APOE ε4	T allele C677T	AD ($n = 564$) n/%	HC ($n = 534$) n/%	OR (95% CI)	*P* (AD versus HC)
–	–	56/9.9	241/45.1	1 (reference)	
+	+	**256/45.4**	**56/10.5**	**19.7 (13.1–29.6)**	**<0.001**

Table 6B
Combination of *APOE* 4/X and 4/4 genotype and CT genotype of C677T

APOE ε4	CT genotype of C677T	AD ($n = 564$) n/%	HC ($n = 534$) n/%	OR (95% CI)	*P* (AD versus HC)
–	–	56/9.9	241/45.1	1 (reference)	
+	+	**200/35.5**	**47/8.8**	**18.3 (11.9–28.2)**	**<0.001**

Table 6C
Combination of *APOE* ε4 allele and CT genotype of C677T simultaneous with AC genotype of A1298C (heterozygotes for both *MTHFR* variants)

APOE ε4	Heterozygotes for both *MTHFR* variants	AD ($n = 564$) n/%	HC ($n = 534$) n/%	OR (95% CI)	*P* (AD versus HC)
–	–	112/19.8	392/73.4	1 (reference)	
+	+	**100/17.7**	**18/3.4**	**19.4 (11.3–33.5)**	**<0.001**

Table 7
Combination of *APOE* 4/X and 4/4 genotype and TT genotype of C677T

APOE 4/X and 4/4 genotype	TT genotype C677T	AD ($n = 564$) n/%	HC ($n = 534$) n/%	OR (95% CI)	*P* (AD versus HC)
–	–	116/20.6	417/78.1	1 (reference)	
+	+	**56/9.9**	**9/1.7**	**22.4 (10.7–46.6)**	**<0.001**

Table 8
Combination of *APOE* 4/X and 4/4 genotype and CC genotype of A1298C

APOE 4/X and 4/4 genotype	CC genotype A1298C	AD ($n = 564$) n/%	HC ($n = 534$) n/%	OR (95% CI)	*P* (AD versus HC)
–	–	120/21.2	409/76.6	1 (reference)	
+	+	**56/9.9**	**9/1.7**	**21.2 (10.2–44.1)**	**<0.001**

3. The combination of genotypes also increases the risk of developing AD in a dose-dependent manner:

   OR 18.3 – 4/X and 4/4 *APOE* + CT C677T,
   OR19.4 – 4/X and 4/4 *APOE* + CT C677T + AC A1298C
   OR 22.4 – 4/X and 4/4 *APOE* + TT C677T
   OR 21.2 – 4/X and 4/4 *APOE* + CC A1298C

DISCUSSION

Apolipoprotein E is considered to be the most critical risk factor for AD. In our dataset, we confirmed a high prevalence of ε4 alleles in AD patients (75.2%) compared with the prevalence in controls at 16.3%. Our results of the prevalence of ε4 allele in the control subjects are in agreement with the overall population prevalence of the ε4 allele, regarded to be approximately 15–20% in the normal population [4, 5, 7]. However, the prevalence of the ε4 allele among AD patients (75.2%) is higher than reported in earlier studies (60–65%) [4–7]. These proportions, however, vary widely and may depend on different characteristics of the study population, including ethnicity [2, 3, 39] and geographical location [39]. In more recent and more extensive studies, there is a remarkable trend supporting the higher prevalence (65–70%) of the ε4 allele among AD patients [40]. Similarly, our preliminary results in 2010 on a sample of 150 AD patients showed a lower prevalence of the ε4 allele (73%; unpublished data). These observations suggest a substantial difference in the prevalence rates over time. The majority of recent studies reporting a higher prevalence of the ε4 allele used PET or CSF biomarkers for confirming the underlying AD

pathology and thus, achieved a higher accuracy of AD diagnosis [40]. Similarly, in our study, the majority of AD or MCI patients since 2014, underwent PET or CSF biomarker investigations for the confirmation of underlying AD pathology. The ε4 allele carriers (homozygous and heterozygous together) had an OR that increased to 15.6 compared to non-carriers, whereas carriers of genotype 4/X (heterozygous) had an OR of 14.3 and the carriers of genotype 4/4 (homozygous) had an OR of 31.9 (in the control group, only six patients had genotype 4/4). Nevertheless, genotype 4/4 is considered the highest risk for the development of AD. One of the highest OR reported so far for the 4/4 genotype was 33.1 among the US-Japanese population [12]. We also confirmed a significantly lower age of onset of AD in the ε4 allele carriers in the homozygous state (genotype 4/4) at 68.3 years, compared with the average age of onset of all AD patients studied in our dataset, which was 72.4 years. Patients that did not carry the ε4 alleles had a significantly higher mean age of onset (75.0 years). Also, when the relationship between patients' clinical findings and genotypes were compared, we did not observe significantly different clinical course in *APOE4/4* carriers or a faster progression compared to *APOE4* heterozygotes or *APOE4* non-carriers.

The second gene of interest was the *MTHFR* gene encoding the *MTHFR* enzyme. We confirmed that there is a high frequency of C677T (41.8% CT, 7.3% TT) and A1298C polymorphisms (41.8% AC, 8.8% CC) in Slovak seniors without cognitive deficit (control group). This finding is consistent with a larger study conducted by Petrovič et al. where similar allele and genotype frequencies were found on a sample of 1180 people – C677T (44.3% CT, 11.0% TT) and A1298C (41.9% AC, 8.0% CC) (unpublished data). Interestingly, similar frequencies of CC genotype of A1298C (9.4%) in Caucasians were reported by Kummar et al. [41]. However, among different ethnic groups, different results in the frequencies of the CC genotype of A1298C have been reported: a rate of 16.8% reported for India is much higher than that observed among the Chinese (3.3%) and Japanese (1.6%) populations [41]. Likewise, there is no consensus in the literature about the effect of A1298C polymorphism in the development of AD. In the Mansouri's study, CC genotype of A1298C polymorphism significantly increased the risk for the development of AD [34], while in the Liu 's study, there was no significant association between A1298C polymorphism and increased risk of AD [42].

The frequency of variant alleles T of C677T polymorphism and C of A1298C polymorphism, as well as the frequency of the CT genotype of C677T (polymorphism in heterozygous state), was significantly higher in the AD group compared with the control group. However, a more critical result is the significantly higher frequency of both polymorphisms in the homozygous state in patients with AD versus the controls (TT genotype C677T 14.2% versus 7.3%, and CC genotype A1298C 13.5% versus 8.8 %). The presence of the TT genotype in C677T increased the OR to 2.5, and that of the CC genotype in A1298C polymorphism increased the OR to 1.8. Regression analysis confirmed ε4 allele of *APOE* as strongest risk factor for AD followed by TT genotype of rs1801133 and CC genotype of rs1801131. The OR for AD of homozygous carriers of both polymorphisms was slightly higher compared to those of previous studies, e.g., Mansoori et al. [34].

We also found that carriers of single homozygous polymorphisms have significantly higher levels of homocysteine compared with heterozygous and nullizygous carriers (17.2 ± 3.7 versus 13.2 ± 4.2 and 12.2 ± 3.8, respectively; $p < 0.001$ in all the cases). Likewise, we discovered that patients with AD had a significantly higher levels of homocysteine than controls (15.7 ± 4.2 versus 13.7 ± 4.5, $p < 0.001$). In individuals with homozygous variant alleles, this result is expected. In the case of patients with AD, we attribute a significantly higher level of homocysteine to a higher proportion of variant homozygotes for *MTHFR* in this cohort.

In the next analysis that was performed, we examined the frequency of the combination of alleles ε4 + T of C677T and ε4 + C alleles of A1298C polymorphism, as well as the frequency of the combination of individual genotypes. We found that the combination of the ε4 allele with the T allele of C677T polymorphism was significantly more frequent in the AD group than the combination of ε4 with the C allele of C677T polymorphism (45.4% versus 29.8%, $p < 0.001$). Furthermore, this combination was significantly more frequent in the AD group than in the controls (45.4% versus 10.4%, $p < 0.001$) and increased the OR to 19.7 (Table 6A).

In summary, the ε4 allele of *APOE* is the most significant genetic risk factor for the development of late-onset AD (OR 31.9 for homozygotes, 15.6 for homozygotes + heterozygotes, 14.4 for heterozygotes). The presence of homozygous polymorphisms of *MTHFR* without the *APOE* ε4 allele represents a significantly lower risk factor for the development of

AD (OR 2.5 for genotype TT C677T, OR 1.8 for genotype CC A1298C). The combination of the ε4 allele of *APOE* and *MTHFR* polymorphisms increased the risk for AD in a dose-dependent manner, whereas the most risky was the combination of *APOE* 4/X and 4/4 + TT C677T, which increased the OR to 22.4.

Our findings could be explained by a synergistic and potentiating mechanism for the presence of *APOE4* and homozygous *MTHFR* polymorphisms as an AD-promoting factor. In addition to the mechanisms outlined in the introduction, disease-associated microglia (DAM) is likely to play a role. *APOE4*, in the context of the aging process, affects the microglia and promotes its conversion to DAM [43]. DAM possesses specific-disease signatures, which are characterized by the downregulation of homeostatic genes and the upregulation of proinflammatory genes. *APOE4* has proinflammatory potential, and there is an ongoing debate regarding its potential in promoting the development of DAM [43]. Homocysteine also has a pro-inflammatory potential by increasing the secretion of inflammatory mediators, especially NF-κ-B and interleukins IL-1β, IL-6, and IL-8. Elevated levels of homocysteine also increase the gene expression of IL-6, which subsequently upregulates the APP and tau protein genes. Furthermore, homocysteine affects DNA repair, increase beta-amyloid formation, and sensitizes neurons to amyloid toxicity [16, 21]. Therefore, the common denominator of the two disadvantageous variants (*APOE4* and homozygous *MTHFR* variants carriers) would be: the activation of pro-inflammatory processes, including DAM, the promotion of beta amyloid deposition, and the promotion of neurodegeneration.

The findings of our study build on the current trend in AD research, which is the study of gene interaction on disease development. In recent literature, studies on the potentiating effect of the combination of polymorphisms of *MTHFR* and *IL-6* have been reported, where the presence of CC genotype of A1298C polymorphism of *MTHFR* and CC genotype of G174C polymorphism of *IL-6* increases the risk of developing AD by 2.8 times [34]. Several studies assessed the potentiating effect of *MTHFR* polymorphisms on AD development. The OR values range from 1.15–1.60 [29]. Polito et al. published a study that presented the epistatic interaction of *APOE* and *MTHFR* genes in a sample of elderly patients without dementia. The study monitored the qualitative and quantitative impairment of cognitive performance. In summary, high homocysteine, per se, or *MTHFR* C677T TT in combination with the *APOE* ε4 allele, might be primarily associated with executive dysfunctions rather than memory loss according to this study [29]. There is consensus in the literature, from both relatively large case-control studies and meta-analyses [29, 36, 45, 46], indicating that the C677T *MTHFR* polymorphism increases AD risk in *APOE4* carriers, particularly for homozygous 677 TT carriers [28].

Conclusions

Currently, there is a trend to shift the pathogenesis of AD to the genetic and epigenetic levels. In the pathogenesis of AD, multiple genes are involved, where each gene may individually have only a slight effect. Therefore, it is essential to study gene epistasis to achieve a better understanding of the pathogenesis of the disease, because a gene does not function by itself but instead acts with other genes, similar to a choreography built up by a functional orchestra. It is also known that this epistasis exhibits a more significant effect than the effect of polymorphism at any individual locus [34]. The findings of this study strongly support this fact. The *APOE4* isoform is a significant risk factor for Alzheimer's disease. Its pathophysiological activity involves the activation of multiple pathological pathways that is disadvantageous to neurons. The result is increased amyloid-β production, its decreased clearance, tau protein hyperphosphorylation, deterioration of synaptic plasticity, and the disruption of regenerative processes. Increased homocysteine causes an indirect (via endothelial dysfunction) and direct neurotoxic effect. *APOE4* and *MTHFR* polymorphisms in homozygous state can probably synergize in their ability to activate multiple proinflammatory pathways which finally increases the risk for neurodegeneration. This risk, as has been mentioned several times in the preceding text, may not be the same for all individuals. The interaction of multiple polymorphisms in a particular individual is likely to create an individual pattern of susceptibility which may combine with other epigenetic factors to trigger a pathological process. These processes are likely to include multiple levels of complexity that requires detailed elucidation. Genetic-epigenetic interactions are probably the common denominator of these processes. Epistasis (gene interaction) as a part of epigenetics (factors affecting gene expression) helps to explain the inter-individual differences in patients with AD (such as age of disease onset, progression rate, and clinical picture). Currently, the study

of epigenetics is rapidly progressing. New markers and disease determinants are continually being sought. The gene interaction of susceptible genes, which undoubtedly includes *APOE* and *MTHFR*, is one of the main subjects of the study on epigenetics on AD. Our study, though on a relatively limited set of patients, adds to the body of knowledge in this research direction.

Study limitations

The calculation of the odds ratio is always loaded with errors due to the abundance of a given trait in patients and controls. The frequency of a given trait or combination of traits, although reflecting the actual incidence in the affected or control population, is not necessarily for the pathological manifestation in each patient. Accordingly, an individual risk assessment of the particular trait is required for each particular patient in a complex context.

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