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ORIGINAL ARTICLE

Impact of APOE gene variants on risk of Alzheimer's disease, age at onset and cognitive decline in Slovaks

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Abstract

OBJECTIVE: The epsilon 4 allele of apolipoprotein E gene (*APOE* ε4) is a known genetic risk factor for late-onset Alzheimer's disease (LOAD). Our aim was to estimate the impact of *APOE* ε4 on LOAD risk, age at onset and degree of cognitive decline in the Slovak population.
DESIGN: Case-control association study.
SUBJECTS: 206 unrelated individuals with LOAD and 487 cognitively unaffected control subjects were included in the study. Montreal Cognitive Assessment (MoCA) test was used to evaluate cognitive performance in both study groups.
METHODS: *APOE* variants were genotyped by direct sequencing. Their association with LOAD risk was analysed by both crude chi-square test and logistic regression analysis adjusted for possible confounding variables, whereas unpaired t test and linear regression analysis were employed to evaluate the impact of *APOE* ε4 on age at LOAD onset and MoCA score.
RESULTS: Odds ratio (OR) for developing LOAD was significantly increased in *APOE* ε4 carriers compared to individuals with the reference *APOE* ε3/ε3 genotype (P <0.0001). Individuals carrying *APOE* ε4/ε4 had OR of 18.5 for LOAD risk, while carriers of ε3/ε4 had OR of 2.8. Age at disease onset was significantly reduced in a dose dependent manner from 78.1 years in patients with no ε4 allele to 76.8 in subjects carrying one ε4 copy and 73.7 in ε4/ε4 homozygotes (log-additive model: P = 0.0031). No significant correlation was observed between *APOE* ε4 and MoCA score.
CONCLUSION: *APOE* ε4 is a strong risk factor for LOAD in Slovaks, reducing the age at disease onset significantly.

Introduction

Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder in the world. It affects predominantly elderly people causing progressive decline of memory and cognitive skills. Based on the age at onset and type of heritability, two forms of AD exist: early-onset (EOAD) and late-onset Alzheimer's disease (LOAD). Early-onset AD accounts for approximately 5% of all cases and is characterised by the onset of symptomatology at around 40–60 years and the Mendelian pattern of inheritance. However, vast majority of AD cases is represented by the late-onset form in which symptoms become apparent at the age of mid-60s and later. The aetiology of LOAD is considered multifactorial, involving a strong genetic predisposition. Genetic component of LOAD is complex and heterogeneous, showing no single mode of disease transmission (Bird 2008; Gatz *et al.* 2006; van der Flier *et al.* 2011).

Numerous epidemiological studies have identified the apolipoprotein E gene (*APOE*) as the strongest genetic risk factor for Alzheimer's disease among people from various ethnic backgrounds (Bertram & Tanzi 2004; Coon *et al.* 2007; Roses *et al.* 1995). *APOE* is a 3.6 kb long gene located on chromosome 19q13.2 (Trask *et al.* 1993). It encodes for a multifunctional glycoprotein apolipoprotein E (ApoE) that occurs in three common isoforms, namely ApoE2, ApoE3, and ApoE4. ApoE isoforms differ from one another by single amino acid substitutions at positions 112 and 158. Apolipoprotein E is a major cholesterol carrier and functions as a key regulator to coordinate the mobilization of cholesterol between cells and to redistribute cholesterol within cells. These functions are critical for the nervous system, particularly for brain as other cholesterol transporters abundant in the plasma, such as ApoA1 and ApoB, are virtually absent in brain (Carrasquillo *et al.* 2013; Hatters *et al.* 2006; Leduc *et al.* 2010). Whether the pathological effects of ApoE4 are due to loss of protective function or due to gain of toxic function is not completely clear yet. It is possible that both mechanisms coexist, with certain constituents of the ApoE4 molecule and/or its downstream signaling mediating a toxic effect, while others are associated with a loss of protective function (Safieh *et al.* 2019).

The three ApoE isoforms are determined by three common alleles of the *APOE* gene (ε2, ε3 and ε4), defined by two single nucleotide polymorphisms in the fourth exon, namely rs7412:C>T and rs429358:T>C, showing different effects on lipid and neuronal homeostasis (Das *et al.* 1985; Phillips 2014). Globally, *APOE* ε3 is the most frequently occurring allele, constituting 60–90% of the allelic variation. In comparison to the common *APOE* ε3 allele, the less frequent *APOE* ε4 increases the risk and lowers the age at disease onset. *APOE* ε4 acts in a dose-dependent manner: carriers of two *APOE* ε4 alleles have a higher risk and earlier onset of AD than heterozygous subjects (Bekris *et al.* 2010; Corder *et al.* 1993). Some authors estimated that the *APOE* ε4 allele not only predisposes to Alzheimer's disease and modifies the age at onset but also influences promotion of cognitive decline (Caselli *et al.* 1999; Craft *et al.* 1998).

Although the association between the *APOE* ε4 allele and AD risk has been replicated by a number of studies, the frequencies of the three *APOE* alleles and the association of ε4 carriage status with LOAD occur to be highly variable among different populations (Crean *et al.* 2011; Xiao *et al.* 2017). Only few studies have examined the role of *APOE* in Alzheimer's disease patients in Slovakia (Trebunova *et al.* 2009; Shahpesandy *et al.* 2002). Therefore, the first goal of the current study was to establish the allele and genotype frequencies of the *APOE* gene in a group of Slovak patients with late-onset Alzheimer´s disease by a case-control study in order to estimate the effect of the *APOE* polymorphism on the risk of disease development. Furthermore, our aim was to estimate the effect of the *APOE* alleles on the age at disease onset and the degree of cognitive impairment reflected by the Montreal Cognitive Assessment (MoCA) score. To the best of our knowledge, there have been no such analyses performed in the Slovak population so far.

Methods

Study groups

A total number of 693 individuals were included in our study. The patient cohort consisted of 206 unrelated subjects meeting the NINCDS-ADRDA diagnostic criteria for probable late-onset Alzheimer's disease (McKhann *et al.* 1984). The mean age of patients at the time of examination performed for the purpose of the study was 79.30 ± 6.09 years, while the mean age at disease onset was 77.19 ± 6.39 years. The clinicians estimated age at onset (AAO) using standardized methodology; roughly, it is the age, at which the patient started to show significant symptoms of memory loss and cognitive impairment with a progressive clinical course. All LOAD patients have been recruited in the period from 2016 until 2019 by several psychiatric clinics throughout Slovakia.

The control group comprised 487 unrelated cognitively normal subjects aged ≥65 years without known family history of dementia and/or other neurodegenerative diseases among first-degree relatives. The Montreal Cognitive Assessment (MoCA) screening test was used to evaluate cognitive functions in both study groups (Nasreddine *et al.* 2005). MoCA is a widely used screening tool for assessing cognitive impairment in Alzheimer's disease and other forms of dementia with a score scale from zero to 30 points. Recommended cut-off MoCA score of 26 points and less was considered as clinically significant cognitive decline. Detailed demographic and clinical characteristics of both, LOAD patients and control individuals are summarised in Table 1.

Tab. 1. Demographic and clinical characteristics of the study groups

Parameter	LOAD patients (n = 206)	Controls (n = 487)	*P*
Age at examination, y; mean ± SD (age range)	79.30 ± 6.09 (65–95)	76.57 ± 7.82 (65–98)	<0.0001
Age at onset, y; mean ± SD (age range)	77.19 ± 6.39 (65–94)	-	-
Disease duration, y; mean ± SD (age range)	2.28 ± 1.96 (1–15)	-	-
Female/male, n (% female)	135/71 (65.53%)	293/194 (60.16%)	0.18
MoCA score; mean ± SD	14.55 ± 5.75	27.65 ± 1.39	< 0.0001

LOAD - Late-onset Alzheimer's disease; MoCA - Montreal Cognitive Assessment; SD - standard deviation

All participants in the study were Caucasians of Slovak descent. Blood samples for DNA extraction and genetic testing were obtained with informed written consent for enrolment in the study and for personal data management. Informed consent was signed either by the patients or by their legally authorized representatives as well as by all control subjects participating in the study in accordance with the International Ethical Guidelines and the Declaration of Helsinki. The study was approved by the Independent Ethical Committee of the Old Town Hospital of the University Hospital Bratislava and the Faculty of Medicine, Comenius University in Bratislava, Slovakia.

Genotyping

APOE genotyping was performed by direct sequencing based on the determination of two single nucleotide polymorphisms: rs429358:T>C and rs7412:C>T located in the fourth exon of the *APOE* gene. The minor alleles of rs429358 and rs7412 generally indicate the presence of ε4 and ε2, respectively, while the combination of rs429358 T with rs7412 C defines the most common *APOE* ε3 allele.

Briefly, PCR amplification was performed with a pair of flanking primers (5'-ACTGACCCCGGTGGCGGAGGAGACGCGGGC-3' as forward and the 5'-TGTTCCACCAGGGGCCCCAGGCGCTCGCGG-3' as reverse) using genomic template DNA extracted from EDTA-treated blood samples as described previously in detail by Durmanova *et al.* (2018). PCR conditions consisted of denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 68°C for 30 s and elongation at 72°C for 30 s, completed by final elongation at 72°C for 7 min. The 318 bp PCR products were subsequently sequenced with both primers using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) and Applied Biosystems 3130xl Genetic Analyzer (Life Technologies). Sequence data were evaluated by Finch TV Version 1.4.0 software (Geospiza, Inc, Washington, USA) that enabled to definitely determine the *APOE* genotypes.

Statistical analysis

Differences in clinical and demographic variables between the study groups were evaluated by the Pearson chi-square test for categorical variables (sex, *APOE* allele and genotype frequencies) and by the Welch's corrected t test for continuous variables (age, MoCA score). Allele and genotype frequencies were determined by direct counting and differences between the patient and control group were assessed by the chi-square test for a 2x3 and 2x6 contingency table, respectively. Association between *APOE* genotypes and LOAD risk was analysed by both crude chi-square test and logistic regression analysis, with the latter

Tab. 2. Distribution of *APOE* alleles and genotypes in LOAD subjects and controls

***APOE* gene**	**LOAD (n = 206)**	**Controls (n = 487)**	**χ^2**	***P***
Alleles			**74.05**	**< 0.0001**
ε2	20 (4.85%)	80 (8.21%)		
ε3	278 (67.48%)	799 (82.03%)		
ε4	114 (27.67%)	95 (9.75%)		
Genotypes			**69.71**	**< 0.0001**
ε2/ε2	0 (0%)	6 (1.23%)		
ε2/ε3	16 (7.77%)	60 (12.32%)		
ε3/ε3	98 (47.57%)	330 (67.76%)		
ε2/ε4	4 (1.94%)	8 (1.64%)		
ε3/ε4	66 (32.04%)	79 (16.22%)		
ε4/ε4	22 (10.68%)	4 (0.82%)		

Tab. 3. Association of *APOE* genotypes with LOAD risk

APOE genotypes	LOAD (n = 206)	Controls (n = 487)	Crude analysis		Adjusted analysis*	
			P	OR (95% CI)	*P*	OR (95% CI)
ε3/ε3	98 (47.57%)	330 (67.76%)		reference		reference
ε2/ε2 + ε2/ε3	16 (7.77%)	66 (12.32%)	0.50	0.82 (0.45–1.47)	0.52	0.83 (0.46–1.50)
ε2/ε4	4 (1.94%)	8 (1.64%)	0.40	1.68 (0.50–5.71)	0.45	1.62 (0.48–5.53)
ε3/ε4	66 (32.04%)	79 (16.22%)	< 0.0001	2.81 (1.89–4.19)	< 0.0001	3.03 (2.02–4.54)
ε4/ε4	22 (10.68%)	4 (0.82%)	< 0.0001	18.52 (6.23–55.04)	< 0.0001	21.54 (7.16–64.76)

LOAD - Late-onset Alzheimer's disease; MoCA - Montreal Cognitive Assessment; OR - odds ratio; CI - confidence interval
Genotypes ε2/ε2 and ε2/ε3 were pooled because of their low numbers; *Analysis adjusted for age and sex

adjusted for age (AAO in patients and age at examination in controls) and sex as possible confounding covariates. Calculations were done for each genotype using the *APOE* ε3/ε3 genotype as reference value. The effects of *APOE* ε4 allele carriage status on AAO and MoCA score, respectively, were analysed by both crude unpaired t test and covariate-adjusted linear regression analysis under codominant, dominant, recessive and log-additive inheritance models. The level of statistical significance was set at $P<0.05$.

Calculations were performed with the InStat statistical software package (GraphPad Software, Inc., San Diego, CA, USA) and the SNPstats web software freely available at http.//bioinfo.iconcologia.net/snpstats/start.htm.

Results

We have analysed 206 individuals suffering from late-onset Alzheimer's disease and 487 cognitively normal subjects in our study. Their demographic and clinical parameters are displayed in detail in Table 1. Comparison of sex distribution in patients and controls showed no statistically significant difference between the two groups ($P = 0.18$). As all subjects meeting criteria for participation in the study have been selected randomly, the mean age at examination happened to be higher in the patient group than in controls (79.30 vs 76.57 years; $P <0.0001$). On the other hand, from the merits of the matter, MoCA score reflecting the global cognitive performance was significantly lower in LOAD individuals when compared to the controls (14.6 vs 27.8 points; $P <0.0001$).

Observed *APOE* allele and genotype frequencies in the investigated individuals are shown in Table 2. The most frequent *APOE* allele in both, LOAD patients and controls was the ε3 allele (67.5 % vs 82.0 %), followed by ε4 (27.7 % vs 9.8 %) and ε2 (4.9 % vs 8.2 %). Chi-square analysis indicated statistically significant differences in allele and genotype distribution between the LOAD and control groups ($P <0.0001$).

Analysis of association between individual *APOE* genotypes and LOAD risk revealed that odds ratio (OR) for developing LOAD was statistically significantly increased in *APOE* ε4 carriers compared to subjects with the most common ε3/ε3 genotype (Table 3). Specifically, individuals bearing the *APOE* ε4/ε4 genotype had OR of 18.5 for developing LOAD ($P <0.0001$), while carriers of ε3/ε4 had OR of 2.8 ($P <0.0001$), respectively. Conversely, carriage of the *APOE* ε2 allele (ε2/ε2 or ε2/ε3 genotype) tended to correlate with decreased risk of LOAD development; however, this association was not statistically significant ($P = 0.50$). Logistic regression enabling adjusting for sex and age (AAO in patients and age at examination in controls) had only little effect on the ORs obtained by crude analyses (Table 3).

When we looked at the age at onset in LOAD patients with different *APOE* ε4 carriage status, we noticed a tendency towards the earlier disease onset among the carriers of ε4 allele (73.7 ± 4.6 years for ε4/ε4 homozygotes and 76.7 ± 5.7 for carriers of one ε4 copy) when compared to patients negative for ε4 (78.1

Tab. 4. Association of *APOE* ε4 allele with age at LOAD onset

APOE ε4	AAO, mean ± SD	Genetic model	Mean difference (95% CI)	Crude *P*	Mean difference (95% CI)*	Adjusted *P**
		Codominant (1 vs. 0)	-1.37 (-3.24 – +0.49)	0.16	-1.38 (-3.25 – +0.49)	0.16
0 copies	78.13 ± 6.85	Codominant (2 vs. 0)	-4.40 (-7.27 – -1.54)	0.0045	-4.31 (-7.19 – -1.44)	0.0054
1 copy	76.76 ± 5.71	Dominant (1 + 2 vs. 0)	-2.10 (-3.84 – -0.36)	0.019	-2.08 (-3.81 – -0.34)	0.020
2 copies	73.73 ± 4.63	Recessive (2 vs. 0 + 1)	-3.88 (-6.66 – -1.10)	0.0068	-3.79 (-6.58 – -1.00)	0.0085
		Log-additive	-1.93 (-3.19 – -0.67)	0.0031	-1.90 (-3.16 – -0.63)	0.0036

AAO - age at onset; CI - confidence interval; SD - standard deviation; *Analysis adjusted for sex

Tab. 5. Association of *APOE* ε4 allele with MoCA score

APOE ε4	MoCA, mean ± SD	Genetic model	Mean diff. (95% CI)	Crude *P*	Mean diff. (95% CI)*	Adjusted *P**
		Codominant (1 vs. 0)	+0.14 (-2.10 – +2.38)	0.90	+0.11 (-2.18 – +2.40)	0.92
0 copies	14.65 ± 5.94	Codominant (2 vs. 0)	-1.55 (-5.35 – +2.26)	0.44	-1.39 (-5.31 – +2.54)	0.48
1 copy	14.78 ± 5.27	Dominant (1 + 2 vs. 0)	-0.22 (-2.29 – +1.84)	0.83	-0.20 (-2.32 – +1.92)	0.85
2 copies	13.10 ± 6.51	Recessive (2 vs. 0 + 1)	-1.59 (-5.32 – +2.14)	0.41	-1.42 (-5.27 – +2.43)	0.47
		Log-additive	-0.41 (-1.98 – +1.16)	0.61	-0.37 (-1.98 – +1.25)	0.66

MoCA - Montreal Cognitive Assessment; CI - confidence interval; SD - standard deviation; *Analysis adjusted for sex, disease duration and age at onset

± 6.9 years), as shown in Table 4. Therefore, we further evaluated the effect of *APOE* ε4 on age at LOAD onset under different inheritance models. Crude unpaired t test revealed that each copy of *APOE* ε4 significantly reduced AAO by approximately 2 years (log-additive model: $P = 0.0031$; dominant model: $P = 0.019$) and that individuals with two copies of *APOE* ε4 had the onset of the disease more than 4 years earlier than those with no ε4 copies (codominant model: $P = 0.0045$). These associations were also confirmed by the linear regression analysis adjusted for sex as a possible confounding covariate (Table 4).

Furthermore, we also analysed the specific effect of the *APOE* ε4 dose on the degree of cognitive dysfunction expressed as the MoCA score in our group of LOAD patients. The MoCA score was recorded as a continuous variable ranging from 0 to 30 points, with the mean value of 14.5 ± 5.8 in LOAD patients. As indicated in Table 5, there was a tendency towards a decreased MoCA score at the time of examination in *APOE* ε4/ε4 homozygotes (13.1 ± 6.5) when compared to subjects with one (14.8 ± 5.3) or no ε4 copies (14.7 ± 5.9), respectively; however, this difference was not significant in any of the genetic models. Adjusting for sex, disease duration and AAO had almost no effect on the results of these analyses (Table 5).

Discussion

Late-onset Alzheimer's disease is defined by age at onset of 65 years or older and characterised by genetic predisposition of non-Mendelian fashion, with a heritability estimate of 58 – 79 % (Gatz *et al.* 2006). The role of *APOE* gene in AD development was initially suggested by Strittmatter (1993), who showed that, of the three polymorphic variants, carriers of ε4 are more likely to develop the disease. Since then, numerous studies have confirmed that possession of the ε4 allele is the strongest genetic risk factor for late-onset as well as early familial forms of Alzheimer's disease. The risk effects of *APOE* ε4 in AD were linked to pleiotropic functions of apolipoprotein E that lead to reduced cholesterol transport, less efficient Aβ clearance and more aggregation, triggering neurotoxicity through Tau phosphorylation, increased brain neuronal atrophy, reduced synaptic plasticity, and greater neuroinflammation (Di Battista *et al.* 2016; Holtzman *et al.* 2012). However, in certain ethnic groups, the *APOE* ε4 allele seems to have either a weaker effect or no clear effect on AD development. These results suggest that additional genetic factors as well as medical comorbidities, lifestyle choices, and other environmental factors also contribute to LOAD risk (Baumgart *et al.* 2015; Rabinovici 2019). In addition to the well-established effects of *APOE*, numerous genome-wide association studies have identified more than 30 genomic loci that are associated with LOAD risk. Unlike the *APOE* variants, the majority of other identified risk polymorphisms do not alter the protein sequence and are not necessarily the actual causal variants (Karch & Goate 2015; Lambert *et al.* 2013; Van Cauwenberghe *et al.* 2016).

While smaller studies in this field have already been published, to our knowledge this is the first report of *APOE* allele frequencies and *APOE* genotypes in a larger LOAD sample of Slovak population. Determination of the *APOE* allele distribution in our study groups revealed, as expected, significantly higher frequency of the *APOE* ε4 carriers in the LOAD cohort as compared to the controls. The analysis of *APOE* genotypes showed that carrying the *APOE* ε4 allele was associated with an significant increase in LOAD risk when compared to the most common ε3/ε3 genotype, while the carriage of ε2 allele showed an opposite, albeit non-significant, trend. Notably, allele and genotype frequencies observed in our study are consistent with previous large-scale meta-analyses of the Caucasian AD patients (Farrer *et al.* 1997; Ward *et al.* 2012)

Possession of the *APOE* ε4 allele is also associated with an earlier age of AD onset across multiple populations (Kwon 2016; Sando *et al.* 2008). In our study, we were examining the effect of ε4 on age at disease onset and cognitive decline. We could notice a clear correlation of the reduction of mean AAO with *APOE* ε4 in a dose dependent manner.

While the effect of ε4 on the risk and age at onset is generally consistent in most studies, there have been numerous conflicting reports regarding whether *APOE* polymorphism influences the rate and degree of cognitive decline following disease onset (Asada *et al.* 1996; Fan *et al.* 2017; Frisoni *et al.* 1995; Hoyt *et al.* 2005). Some

studies suggest that the effect of the ε4 allele on cognitive impairment is stronger in the earlier clinical stages of disease and weaker during the later and more severe clinical stages (Cosentino *et al.* 2008; Juva *et al.* 2000; Rawle *et al.* 2018). To our knowledge, we were the first to examine genetic association of *APOE* gene variants with the MoCA score in Slovak Alzheimer's disease patients. Our findings show that carriers of the ε4/ε4 genotype have only slightly lower overall MoCA score than patients with one or no copies of ε4 and that this difference is not statistically significant. Hence, the results of our study do not suggest a gene dose-dependent effect of *APOE* ε4 on promoting the decline of cognitive performance, as was the case with the age of disease onset.

We are aware of several limitations of the current study. First, the relatively small sample sizes may reduce the power of the study to detect associations between *APOE* genotype and age at onset as well as *APOE* genotype and MoCA score. A larger number of LOAD patients would be desirable for replication, especially with regard to low prevalence of some of the genotypes such as *APOE* ε2/ε2 or ε2/ε4. On the other hand, our findings are in keeping with previous assessments in Caucasian populations, supporting the reliability of the current study. We assume our present-day results as useful for sample size planning in future investigations on this topic. Secondly, the estimation of age at onset may be biased by different factors such as the significance of clinical symptoms as experienced by patients. Moreover, other genetic or environmental factors may directly affect age at disease onset and modify the effect of *APOE* genetic variation. Third, score obtained by the Montreal Cognitive Assessment used to evaluate multiple cognitive domains could have been influenced by different factors, which may limit the conclusions that can be drawn from our findings. To these factors belong demographic characteristics of the tested person such as education and/or clinical expertise of the investigator performing the testing, etc.

Nevertheless, our results undoubtedly confirm *APOE* ε4 as a strong LOAD-associated genetic factor also in the Slovak population. Replication of genetic association findings in independent studies represents an important validation tool in the search for susceptibility genes playing roles in complex diseases such as Alzheimer's disease. Genetic association studies in different populations have significant contribution to a better understanding of the pathophysiology and possible treatment options of this multifactorial neurological disorder.

Conclusion

In summary, our findings confirm the *APOE* ε4 allele as a significant genetic risk factor for late-onset Alzheimer's disease in the population from Slovakia. *APOE* ε4 dose is associated with earlier age at onset and lower MoCA score reflecting promotion of cognitive decline in LOAD individuals.

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Disclosure

The authors declare no potential conflicts of interest.

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