APOE Gene polymorphism among Jordanian Alzheimer's patients with relation to lipid profile

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ABSTRACT

الأهداف: قياس تكرارات الأليلات المختلفة لجين الإبوليبوبروتين (APOE) وأشكاله الجينية المختلفة لدى الأردنيين الذين يعانون من مرض الألزهايمر المتأخر ومقارنته مع أفراد المجموعة الضابطة، وكذلك دراسة مستويات شحوم الدم عند أفراد المجموعتين.

الطريقة: شملت هذه الدراسة المقطعية المرضى الأردنيين والمصابين بمرض الألزهايمر المتاخر (العدد=38) وكذلك المجموعة الضابطة (العدد=33)، وكانت أعمار المشاركين ≤65 سنة. تم استدعاء جميع المشاركين إما من بيوت الضيافة في مدينة عمان/الأردن أو من المرضى الذين يراجعون مستشفى الجامعة الأردنية. تم الدراسة وكذلك جمع عينات الدم في الفترة الممتدة ما بين يناير 2010م إلى ديسمبر 2013م. لكل عينة تم فحص الأشكال الثلاثة للجين (ε2, ε3, ε4) باستعمال طريقة (PCR)، أما شحوم الدم فقد تم قياس كل من مجمل الكولسترول (TC) والكولسترول عالي الكثافة (HDL) والكولسترول منخفض الكثافة (LDL) وكذلك الغليسريدات الثلاثية (TG).

النتائج: أظهرت الدراسة وجود بعض الاختلافات قي توزيع أليلات الجين عند كل من المرضى والمجموعة الضابطة. حيث لوحظ زيادة في توزيع كل من أليل ε4 والشكل الجيني ε3/ε4 لدى مرضى ألزهايمر مقارنة مع المجموعة الضابطة وكانت هذه الزيادة ذات دلالة احصائية. كما أظهرت النتائج لدى مرضى الألزهايمر وجود علاقة نمطية (Trend) بين كل من مستويات الكولسترول الكلي (TC) والكولسترول منخفض الكثافة (LDL) حيث لوحظ انخفاض مستوياتها لدى المرضى الحاملين للأليل ε2 وزيادة تلك المستويات لدى المرضى الحاملين للأليل ε4 ولم تكن هذه الفروقات ذات دلالة إحصائية.

الخاتمة: لوحظ زيادة في تكرار ε4 لدى مرضى الألزهايمر وصلت تقريبا إلى أربعة أضعاف المجموعة الضابطة (15.8% مقارنةً مع 4.5% على التوالي: p=0.03)، كما أن العلاقة النمطية بين مستويات شحومات الدم عند حاملي الـ ε2 و ε4 بحاجة إلى دراسة أوسع وذلك بزيادة حجم العينة.

Objectives: To investigate the frequencies of the apolipoprotein E (APOE) alleles and genotypes and study their relationship with the lipid profile in Jordanian patients with late-onset Alzheimer's disease (AD).

Methods: This case-control study was carried out on 71 Jordanian individuals: 38 patients with late-onset AD (age ≥65 years) and 33 age-matched healthy controls. All participants were recruited from senior homes and Jordan University Hospital, Amman, Jordan between January 2010 and December 2013. Each sample was examined for APOE's 3 major isoforms (ε2, ε3, ε4) using the polymerase chain reaction technique (PCR) followed by the sequencing technique. In addition, samples were screened for lipid profiles (total cholesterol (TC), high-density lipoprotein (HDL), lower-density lipoprotein (LDL), and triglyceride (TG) levels.

Results: The ε3/ε4 genotype and ε4 allele prevalence were higher in AD patients compared to healthy controls (26.3% vs. 3.0%, p=0.03 and 15.8% vs. 4.5%, p=0.03; respectively). In the AD group, the ε2 carriers showed the lowest levels of total and LDL cholesterol, and the ε4 carriers showed the highest levels of total and LDL cholesterol, although the difference was not statistically significant (p>0.05).

Conclusion: APOE-ε4 frequency was almost 4 times higher in the AD group compared to the control group, and this difference was statistically significant. A trend that was observed in the AD group regarding the lipid profile and ε2 and ε4 carriers requires further investigation using a larger sample size.

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Alzheimer's disease (AD) is a complex neurodegenerative disorder that is progressive in nature and has poor prognosis. Diagnosis of AD is made with certainty only by brain biopsy or autopsy. Today, the diagnosis of AD is possible by conducting a thorough medical history, mental status testing, and physical and neurological exams and tests (such as blood tests and brain imaging) to rule out other causes of dementia-like symptoms.[1] The incidence of AD in Jordan has not been reported yet. It has been estimated that 0.2-0.3% of Jordanians could be clinically classified as having AD.[2] Although the etiology of AD has yet to be elucidated, it is known to be a multifactorial disorder. Most likely, the development of the disease is a result of the interaction of several susceptible genes and environmental risk factors. Therefore, it is difficult to pinpoint a single gene polymorphism in the pathogenesis of AD. However, apolipoprotein E (APOE) gene polymorphism is the most studied gene in AD. This gene has three alleles: ε2, ε3, and ε4. These 3 single nucleotide polymorphisms (SNPs) differ from one another by the presence of either a C or a T nucleotide at codons 112 and 158.[3] These 3 alleles produce 6 different genotypes. Three of the genotypes are homozygous (ε2/ε2, ε3/ε3, and ε4/ε4), and the other 3 are heterozygous (ε2/ε3, ε2/ε4 and ε3/ε4). The distribution of these alleles varies among different ethnicities. Worldwide, the most common allele in all human groups studied up until now is ε3 78% (8.5-98%) followed by ε4 14.5% (0-49%) and ε2 being the least common 6.4% (0-37.5%).[4] The occurrence of APOE-ε4 is strongly linked with late-onset Alzheimer's disease and may be involved in its pathogenesis. For instance, it has been reported that the mean age of the onset of AD was 68 years in patients with 2 ε4 alleles, 76 years with one ε4 allele, and 84 years in individuals with no ε4 alleles. In contrast, APOE-ε2 appears to protect individuals from AD.[5]

Although the presence of APOE-ε4 increases the probability of the development of AD, it has been shown that the link between ε4 and AD is not necessarily one of cause and effect. In reality, the presence of ε4 is neither sufficient nor essential for the development of AD.[6] This fact emphasizes the importance of gene-environment interaction in the pathogenesis of AD. In this regard, dyslipidemia is believed to play a role in AD pathogenesis. Actually, APOE alleles have been shown to influence lipid levels. Carriers of ε4 showed higher plasma total and low density lipoprotein (LDL) cholesterol and lower high-density lipoprotein (HDL).[7] Hence, dyslipidemia and genetic susceptibility are among the different potential factors in the etiology of AD.

Therefore, this study aimed to elucidate the frequencies of the APOE alleles and genotypes in AD patients in the Jordanian population. The second aim was to examine the possible relationship between APOE gene polymorphism, lipid profiles, and the risk of developing AD.

Methods. ***Ethics statement.*** Informed consent was obtained from all participants or legal guardians in accordance with the Institutional Review Board for human study at the University of Jordan, Amman, Jordan.

Study population samples. This case-control study included 71 unrelated Jordanian participants: 38 patients with late-onset AD (age ≥65 years) and 33 age-matched healthy controls. All participants were recruited from senior homes and Jordan University Hospital, Amman, Jordan. All blood samples were collected between January 2010 and December 2013. Initially, all participants were screened with the Mini-Mental State Examination (MMSE) and the Clock Drawing Test (CDT). The screening methods were used exactly as they were in a previous study.[8]

The AD patients included in this study were diagnosed as having probable or possible AD according to the criteria of the National Institute of Neurological and Communicative Disorders and the Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA).[9] Familial AD patients were excluded from the study using patients' history.

Individuals in both the AD and control groups had no history of any relevant psychiatric disease or substance abuse and no systemic use of statins, other lipid-lowering agents, and psychotropic drugs.

All control participants failed to meet the diagnostic criteria for AD or dementia but fulfilled the rest of the inclusion criteria. They were screened using 2 scales, the MMSE and the CDT, and scored normally on both rating scales, were functionally independent, and were cognitively healthy Clinical dementia rating=0.[10]

Lipid profile measurement. Blood samples were routinely collected in the morning. Recruits were fasting for at least 12 hours (hrs) and less than 16 hrs taking all standard precautions. Serum was separated within

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30 min by centrifugation at 3500 rpm for 10 min and rapidly stored at 4°C until analysis.

The lipid profile measurements included serum total cholesterol (TC), triglycerides (TG), LDL, and high-density lipoprotein (HDL). All samples were measured in the National Center of Diabetes, Endocrinology and Genetics (NCDEG-Jordan) using the Roche Diagnostics COBAS INTEGRA 800 Biochemistry analyzer (USA), which employs an enzymatic colorimetric method.

DNA extraction. Venous blood samples (5 ml) were collected in tubes filled with ethylene diamine tetraacetic acid. Genomic DNA was extracted using Puregene Blood Core Kit A (Qiagen, Germany). Isolated DNA was stored at –20°C until use.

APOE genotyping. A polymerase chain reaction technique (PCR) was used to amplify APOE gene-exon 4. DNA was amplified using 2 PCR reactions in which 2 primer sets (Integrated DNA Technologies, USA) were used: 1. Set A: AD-F1(5'-ttgggtctctctggctcatc-'3; NC_000019.10: 44908437- 44908456) and AD-R1 (5'-ctgcccatctcctccatc-'3; NC_000019.10: 44909018-44909001) and 2. Set B: AD-F2 (5'-gccgatgacctgcagaag-'3; NC_000019.10:44908804-44908821) and AD-R2 (5'-gctggggcttagaggaaatc-'3; NC_000019.10: 44909432-44909413).

The PCR annealing temperature used for the 2 primer sets was 63^0C and 62^0C, respectively. The PCR products were analyzed on 1.5% agarose gel containing ethidium bromide. For set A, the PCR product size was 582 bp, while for set B, the product size was 629 bp.

Statistical Analysis Statistical analyses were performed using Statistical Package for Social Sciences (SPSS), Version 18.0. Data were represented as average and standard deviation (age, lipid parameters) or counts and percentage (genotypes and allelotypes). Deviation from the Hardy Weinberg Equilibrium was assessed using the Chi-square test with one degree of freedom.[11] The APOE allele frequencies were estimated by gene counting methods. The Fisher exact test or Chi-square test was used to assess genotype distribution between AD and control subjects. *P*-values <0.05 were considered statistically significant. Odds ratios (ORs) with 95% confidence intervals (95% CI) were used for categorical variables. Lipid profile variables were compared among different groups using an independent t-test, ANOVA, Man-Whitney test, or Kruskal-Wallis test as appropriate. Selection of parametric tests or non-parametric tests was based on a normality test (Kolmogorov-Smirnov or Shapiro-Wilk) and homogeneity of variance (Leven's test).

Results. ***Study population.*** This case-control study included 38 patients with AD and 33 healthy controls. The AD cases collected in this study were 65 to 85 years

Table 1 - Distribution of APOE genotypes frequencies in normal controls and AD patients.

Genotype	Control n=33	AD n=38	OR	CI	*P*-value	OR*	CI*	*P*-value*
	n (%)							
ε3/ε3	29 (87.9)	23 (60.5)	Ref			Ref		
ε2/ε3	1 (3.0)	4 (10.5)	5.0	0.53-48.3	0.18	4.8	0.48-48.6	0.18
ε2/ε2	0 (0)	0 (0)	†	†	†	†	†	†
ε2/ε4	2 (6.1)	0 (0)	†	†	†	†	†	†
ε3/ε4	1 (3.0)	10 (26.3)	12.6	1.5-105.8	0.0065	10.6	1.2-92.6	0.033
ε4/ε4	0 (0)	1 (2.6)	†	†	†	†	†	†

OR - odds ratio, CI - confidence interval, *values are adjusted to age and gender, †uncalculated due to empty cells, AD - Alzheimer disease, APOE - apolipoprotein E

Table 2 - Distribution of APOE alleles frequencies in normal controls and Alzheimer's disease patients.

Allele	Control n=33	AD n=38	OR	CI	*P*-value
	n (%)				
ε3	60 (90.9)	60 (78.9)	Ref	-	-
ε2	3 (4.5)	4 (5.3)	1.3	0.29-6.2	0.99
ε4	3 (4.5)	12 (15.8)	4.0	1.07-14.9	0.03

OR - odds ratio (not adjusted), CI - Confidence Interval, AD - Alzheimer disease, APOE - apolipoprotein E

Table 3 - Plasma lipid levels in normal controls and Alzheimer's disease patients.

Groups	Control n=33	AD n=38	*P*-value	*P*-value*
TC	187.1±34.6	183.7±41.4	0.7	0.5
TG	159.6±66.8	161.3±113.7	0.9	0.8
LDL	113.6±26.4	103.9±32.7	0.2	0.048 [a]
HDL	39.1±12.0	43.8±12.3	0.1	0.06

TC - total cholesterol, TG - triglycerides, LDL - low density lipoprotein, HDL - high-density lipoprotein, AD - Alzheimer disease

Table 4 - Lipid profile in AD patients with respect to ε2 and ε4 carriers and non-carriers.

Groups	ε2(+) n=4	ε2(-) n=34	*P*-value	*P*-value*	ε4(+) n=11	ε4(-) n=27	*P*-value	*P*-value*
TC	170±50	185.3±40.9	0.7	0.4	189.7±30.9	181.2±45.3	0.6	0.6
TG	147.2±28.9	162.9±119.9	0.5	0.8	125.7±33.0	175.8±131.2	0.4	0.2
LDL	98±39.7	104.5±32.4	0.9	0.8	111.0±22.3	100.9±36.0	0.6	0.99
HDL	47.3±16.4	43.4±11.9	0.8	0.4	46.5±11.1	42.8±12.8	0.6	0.7

*Values are adjusted to age and gender, only one patient showed ε3(-) which is not enough to achieve sufficient statistical power. Thus, we only reported ε2(±) and ε4(±), the results are expressed as mean±standard deviation, mg/dL, AD - Alzheimer disease, TC - total cholesterol, TG - triglycerides, LDL - low density lipoprotein, HDL - high-density lipoprotein

old with a mean age of 74.2±5.4 years and included 24 females (63.2%) and 14 males (36.8%).

Control samples selected in this study were 65 to 88 years old with a mean age of 72.4±6.3 years and included 11 females (33.3%) and 22 males (66.7%). An independent t-test showed no significant difference between the AD group and the control group for age (p=0.2), while a Chi-square test showed a significant difference between the groups in gender (p=0.012).

APOE genotyping

The genotype distribution of the SNPs was in the Hardy-Weinberg equilibrium for both cases and controls [(SNP112: Alzheimer Disease: X^2=0.01, p=0.99; Control: X^2=0.07, p=0.8), (SNP158: Alzheimer Disease: X^2=0.12, p=0.7; Control: X^2=0.07, p=0.8)]. The APOE genotype distribution and allele frequencies of the AD patients and the controls are given in **Tables 1 and 2**. The most common genotype in AD patients was the ε3/ε3 homozygote, followed by the ε3/ε4 heterozygote. The ε3/ε3 genotype was higher in control subjects when compared to AD patients (87.9% vs. 60.5%, p=0.02). The ε3/ε4 genotypes were higher in AD patients compared to control subjects (ε3/ε4: 26.3% vs. 3.0%, p=0.03). Similarly, the ε4 allele showed a higher incidence in the AD group compared to the control group (15.8% vs. 4.5% respectively; p=0.03).

Lipid profile measurement. The mean lipid profile tests, including TC, TG, LDL, and HDL, of AD patients showed no significant difference compared to controls (**Table 3**).

The relationship between APOE alleles and serum lipid concentrations in AD patients is shown in **Table 4**. The ε2 allele carriers (ε2+) showed lower total and LDL-cholesterol levels compared to the ε2 non-carriers (ε2-). The opposite effect was noticed with regards to ε4. The total and LDL-cholesterol levels in the ε4 carrier were higher compared to those in ε4 non-carriers. However, none of these differences between the compared groups i.e. ε4 carriers and non-carriers and ε2 carriers and non-carriers were statistically significant (p>0.05).

Discussion. Currently, there are no recognized blood biomarkers that facilitate the diagnosis of AD. Therefore, research interest has focused on the identification of asymptomatic individuals with increased risk of AD. Efforts are underway to discover such biomarkers.[12] Dyslipidemia and APOE-ε4 are considered among the potential predictors of AD.

The lipid profile was also examined and linked to APOE genotype. The distribution of the APOE allele in Jordanians was comparable to that of Levant region populations such as Lebanon (ε2 4.3%, ε3 85.9%, and ε4 9.8%) and the Gaza Strip (ε2 5.1%, ε3 87.5%, and ε4 7.3%).[13-14] We did not find similar studies from other Levant countries such as Syria and Iraq. The Levant region shares geographic location, cuisine, and a probable gene pool, which may explain such similarities in APOE genotype. Comparing our results with non-Levant Arab communities, three different studies from Saudi Arabia showed the total absence of the ε2 allele in healthy Saudis.[15-17] Kuwaiti, Omani, and Iranians showed almost similar APOE genotype and allele distribution as the Jordanian volunteers in this study.[18-20] In addition, data from populations of the Mediterranean basin such as Turks,[21] Greeks,[22] and Sardinians[23] also showed a similar distribution of APOE alleles to that of Jordanians.

The high APOE ε3/ε4 genotype and the ε4 allele frequency among Jordanians with AD is in agreement with other studies that reported similar observations,[24-25] but still remain lower than that reported in Caucasians, African Americans, and Japanese.[26] Contrary to our findings, others have shown that the possession of the ε4 allele did not increase the risk of AD.[27] Actually, a debate in the literature focused on whether it is the presence of ε4 or the absence of ε2 and ε3 that places individuals at risk for AD.[28] It is generally accepted that

the APOE-ε4 allele accounts for the overall genetic risk for AD; however, other genes may also be involved on the pathogenesis of the disease.[27]

Dyslipidemia is neither specific nor sensitive in predicting the development of AD. Up to date, there is no definite link between high serum cholesterol level and AD; data are not consistent. Nevertheless, few studies have shown that hypercholesterolemia is considered as a risk factor for developing AD, and this risk may be significantly reduced with the use of statins or other lipid-lowering agents.[29] In addition, Sabbagh et al[30] reported an increase in TC and LDL-C in AD patients, but the cholesterol levels were not linked to the degree of cognitive impairment among those patients. An association between AD pathology and lipid profile has been reported, and this differs in patients with different levels of neurotic plaques in their brain.[31] On the other hand, Reitz et al[32] concluded that lipid levels and the use of lipid-lowering agents do not seem to be associated with the risk of AD. In addition, Mielke et al[33] showed that high cholesterol in late life was even associated with decreased dementia risk. Numerous studies showed a link between the APOE-ε4 allele and dyslipidemia.[34-37] Mendez et al[38] reported an inverse correlation between plasma triglyceride levels and the number of ε4. Similar to our results, Isbir et al[37] also observed high total serum cholesterol in ε4 carriers and low TC in ε2 carriers among AD patients. However, none of these differences were statistically significant (p>0.05). Others have shown that hypercholesterolemic ε4 non-carriers, but not ε4 carriers, are at high risk of developing AD.[39] Romas et al[40] showed that the link between dyslipidemia and AD was independent of APOE genotype.

Besides the ε4 and lipid profile, AD may be influenced by other factors, such as atherosclerosis, metals such as copper and aluminum, and other unidentified environmental factors.[8]

Despite its useful findings, this study had a number of limitations. Regarding the distribution of gene polymorphism, this study included a small sample size, especially when only individuals ≥65 years old were selected to participate. The control group was not gender-matched, which could explain the lack of significant data in the subgroup analysis. Thus, future research should use a larger and more representative sample of the general population.

In conclusion, The APOE allele frequency distribution in Jordanians found in this study was similar to the APOE allele frequency distribution in most Arab populations. Our results demonstrated an increased frequency of the APOE-ε4 allele in AD patients versus controls. There was no significant difference in the frequency of the ε2 allele between AD patients and controls. These results support the previous assumption that APOE-ε4 can be considered, at least partly, as a predisposing risk factor for AD susceptibility, and APOE-ε2 may not play a protective role in the development of AD in Jordanians. Lipid profile did not differ between the AD patients and controls. Future studies should involve a larger sample and proper gender matching.

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