Apolipoprotein E polymorphism in a Romanian population

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Abstract

Background: Apolipoprotein E (ApoE) is a ubiquitous protein involved in maintaining cholesterol homeostasis and lipoprotein clearance from circulation. It is coded by three alleles (ε2, ε3, ε4) with six genotypes (ε3/ε3, ε3/ε4, ε2/ε3, ε4/ε4, ε2/ε4, and ε2/ε2). Several studies have shown a relationship between ApoE-specific isoforms and different diseases. There are few data about the prevalence of ApoE polymorphism in the Romanian population. We aimed to assess the prevalence of Apolipoprotein E alleles in a healthy population from Romania and compare it with the data found in other countries of Europe. **Methods**: ApoE genotype was examined in 187 unrelated healthy volunteers (131 males and 56 females) from the region of southwest Romania (mean age 48.04 ± 8.35 years). Analysis of ApoE genotype variants was done using the PCR method with allele-specific oligonucleotide primers. **Results**: The frequency of heterozygote genotypes ε2/ε3, ε2/ε4, and ε3/ε4 was 4.8%, 1.6%, and 24.73%, respectively, while the frequency of homozygote genotypes ε2/ε2, ε3/ε3, ε4/ε4 was 0.5%, 66.12%, and 2.1%, respectively, with a mean frequency of ε2, ε3 and ε4 alleles of 6.9%, 96.25%, and 28.49%, respectively. East European countries have the lowest mean frequency of the ε4 allele (9.96%), and the highest frequency of the ε4 allele (23.38%) is in the Nordic Countries. **Conclusions**: The ε4 allele prevalence in this Romanian population is higher in comparison with other European countries, similar to Nordic countries of Europe.

Keywords
apolipoprotein E, genotyping, allele variants, cardiovascular risk

Rezumat

Context: Apolipoproteina E (ApoE) este o proteină omniprezentă, implicată în menținerea homeostaziei colesterolului și a clearance-ului lipoproteinelor din circulație. Este codificată de trei alele (ε2, ε3, ε4) cu șase genotipuri (ε3/ε3, ε3/ε4, ε2/ε3, ε4/ε4, ε2/ε4 și ε2/ε2). Mai multe studii au arătat o relație între izoformele specifice ApoE și diferite boli. Sunt puține date despre prevalența polimorfismului ApoE în populația din România. Ne-am propus să evaluăm prevalența alelelor Apolipoproteinei E într-o populație sănătoasă din România și să o comparăm cu datele găsite în alte țări ale Europei. **Metode:** Genotipul ApoE a fost examinat la 187 de voluntari sănătoși neînrudiți (131 de bărbați și 56 de femei) din regiunea de sud-vest a României (vârsta medie 48,04 ± 8,35 ani). Analiza variantelor de genotip ApoE a fost realizată utilizând metoda PCR cu primeri oligonucleotidici specifici alelei. **Rezultate:** Frecvența genotipurilor heterozigote ε2/ε3, ε2/ε4 și ε3/ε4 a fost de 4,8%, 1,6% și, respectiv 24,73%, în timp ce frecvența genotipurilor homozigote ε2/ε2, ε3/ε3, ε4/ε4 a fost 0,5%, 66,12% și, respectiv 2,1%, cu o frecvență medie a alelelor ε2, ε3 și ε4 de 6,9%, 96,25% și, respectiv 28,49%. Țările din Europa de Est au cea mai mică frecvență medie a alelei ε4 (9,96%), iar cea mai mare frecvență a alelei ε4 (23,38%) este în țările nordice. **Concluzii:** Prevalența alelei ε4 la această populație românească este mai mare în comparație cu alte țări europene, similar țărilor nordice din Europa.

Cuvinte cheie
apolipoproteină E, genotipare, variante de alele, risc cardiovascular

Introduction

ApoE is involved in the metabolism of plasma lipoprotein and transport of lipids within tissues [1]. A 299-amino acid long protein, it belongs to a family of amphiphilic exchangeable apolipoproteins. Apolipoprotein E is synthesized in multiple cells, most abundantly in hepatocytes, followed by astrocytes, monocytes/macrophages, adipocytes, and kidney cells [2].

ApoE has a genetic polymorphism represented by three alleles, ε2, ε3, and ε4, with six distinct genotypes (ε3/ε3, ε3/ε4, ε2/ε3, ε4/ε4, ε2/ε4, and ε2/ε2). The structure seems to be the main determinant of ApoE function [3]. All isoforms are derived from ApoE3, considered the normal variant (wildtype allele). The ε3/ε3 genotype is the most common, with a frequency of 62% in the entire population [1]. Several studies have suggested an association between ApoE specific isoforms and different diseases [4]. The presence of different isoforms could affect the development of cardiovascular disease. Important risk factors related to cardiovascular disease are high low-density lipoprotein cholesterol (LDL) and low high-density lipoprotein cholesterol (HDL) [5]. The ε2 carriers have generally lower LDL levels but high levels of triglycerides [6–8].

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Subjects with ε2/ε3 genotype have low LDL levels and elevated very-low-density lipoprotein cholesterol (VLDL), while patients with ε3/ε4 show increased LDL [9]. Numerous studies have suggested that the homozygous genotype ε2/ε2 may be one of the causes of type III hyperlipoproteinemia [10]. Even though the ε2/ε2 genotype is associated with hyperlipoproteinemia type III, atherosclerosis progression is mild [3, 8]. Instead, patients with ApoE4 have elevated levels of LDL with more severe atherosclerotic disease [11]. Also, ε4 alleles carriers have an increased risk of Alzheimer's and coronary artery disease compared with ε3 or ε2 carriers [12–15].

The prevalence of ApoE genotypes varies widely across populations. In most populations, the commonest allele is ε3, while ε4 is more frequent in African Americans and Northern Europeans, and Asian individuals have low prevalence of ε2 and ε4 [16–18].

Romania is an emerging nation with a high predominance of cardiovascular diseases (CVD), and the risk factors are likewise expanding due to industrialization and lifestyle changes [19]. Under these conditions, the ApoE alleles frequency may have clinical implications. Even if there are a lot of data in the literature on the frequency of these alleles in other countries, there is little information about the prevalence of ApoE polymorphism in the Romanian population [20]. We aimed to assess the prevalence of Apolipoprotein E alleles in a healthy population in Romania and compare it with the data found in other countries of Europe.

Materials and Methods

1. Subjects

This was a cross-sectional study that included healthy volunteers selected randomly from southwestern Romania (Oltenia region). Inclusion criteria were the lack of symptoms of diseases, passing a normal clinical examination, normal electrocardiogram (ECG), and a lack of history of chronic diseases. Participants with any chronic prescriptions, including lipid lowering drugs, were excluded from the study. Clinical parameters and ECGs were collected for each participant. The study included 187 unrelated healthy volunteers (131 males and 56 females) from the southwest region of Romania (mean age 48.04 ± 8.35 years). The study was approved by the University of Medicine and Pharmacy of Craiova Ethics Committee (registration number 20/26.02.2016) and conducted in compliance with the ethical guidelines of the Declaration of Helsinki. A signed consent form was obtained from each participant before his or her enrollment in the study.

2. DNA extraction and genotyping

A 4 mL venous blood sample was drawn from each participant into an EDTA sample tube and preserved at –20°C. The extraction of genomic DNA was performed using a commercial kit Wizard® Genomic DNA Purification Kit from Promega. Genotyping of polymorphic ApoE alleles was done after polymerase chain reaction (PCR) amplification. Allele-specific oligonucleotide primers were designed and synthesized according to previous literature [21, 22]. The polymerase chain reaction (PCR) parameters were as follows: DNA was denatured for 10 min at 96°C; forty cycles of denaturation at 96°C for 10 sec; annealing at 58°C for 30 sec; and extension at 65°C for 1 min. After that, the samples were run on a horizontal electrophoresis system for 40 min at 150w/150A over 2% agarose gel stained with ethidium bromide visualized under UV light. Each of the six possible ApoE genotypes corresponds to a unique combination of bands. For quality control purposes, some of the samples were reanalyzed for its genotype in a blinded fashion, and the same results were obtained. Thus, the ApoE genotypes identified, ranking from most to least common, were ε3/ε3, ε3/ε4, ε2/ε3, ε4/ε4, ε2/ε4, and ε2/ε2.

3. Statistical analysis

Statistical analysis was performed using SPSS version 19.0 (IBM Inc., Armonk, NY, USA). Continuous variables were expressed as mean ± standard deviation and were analyzed with Student's t-test or ANOVA. Categorical variables were expressed as numbers and percentages and were analyzed with the chi-square test. Hardy–Weinberg equilibrium was evaluated by chi-square test. A level of $p < 0.05$ was considered to represent statistical significance.

Results

Demographic characteristics of the study population are presented in Table 1. The ApoE allele frequencies in Romanian population are shown in Table 2. The prevalence of ε2/ε3, ε2/ε4 and ε3/ε4 (heterozygotes) was 4.8%, 1.6%, and 24.73%, respectively, while the frequency of ε2/ε2, ε3/ε3, and ε4/ε 4 (homozygotes) was 0.5%, 66.12%, and 2.1%, respectively, with a mean frequency for the ε2, ε3, and ε4 alleles of 6.9%, 96.25%, and 28.49%, respectively. The allele ε3 was the most frequent (0.9625 relative frequency), and ε4 was the next most common (0.2849 relative frequency), followed by ε2 (0.069 relative frequency).

Table 1 - Characteristics of the population.

Variables	
Age (years)	48.04 ± 8.35
Sex (% male)	131 (70.05%)
Alcohol, *n* (%)	109 (58.28%)
Smoking, *n* (%)	96 (51.33%)
BMI, kg/m2	26.59 ± 3.88

Table 2 - ApoE alleles frequency in Romanian population

Allele	Allelic count (%)
ε3	180 (96.25%)
ε4	53 (28.49%)
ε2	13 (6.9%)
Genotype	***n* (%)**
ε3/ε3	123 (66.12%)
ε3/ε4	46 (24.73%)
ε2/ε3	9 (4.8%)
ε2/ε2	1 (0.5%)
ε2/ε4	3 (1.6%)
ε4/ε4	4 (2.1%)

Table 3 - The frequencies of ApoE alleles in European countries

Population, no. of individuals	Allele count (%)			Genotype (%)						Reference
	ε2	ε3	ε4	ε2/ε2	ε2/ε3	ε2/ε4	ε3/ε3	ε3/ε4	ε4/ε4	
Romania, 187	6.9	96.25	**28.49**	0.5	4.8	1.6	66.12	24.73	2.1	
East European Countries										
Mean	7.74	82.28	**9.96**	0.85	13.24	1.25	71.7	17	1.5	
Serbia, 93	6.67	83.33	10.0	0	13.33	0	70	13.33	3.33	[23]
Hungary, 757	7.8	81.9	10.3	0.7	12.8	1.6	66.9	16.9	1.1	[24, 25]
Ukraine, 96	13.5	77.1	9.4	2.1	20.8	2.1	59.4	14.6	1	[26]
Slovakia, 351	6,9	85.5	7.6	0.6	11.7	1.1	72.9	13.1	0.6	[27]
Czechia, 175	6.3	83.4	10.3	1.7	8.0	1.1	72.0	14.9	2.3	[28]
Poland, 137	5.5	83.9	10.6	0	13.81	1.81	96.67	25.32	1.66	[29]
Russia, 350	7.52	80.86	11.57	0.9	12.3	1.1	64.3	20.9	0.6	[26]
North European Countries										
Mean	11.29	87.49	**23.38**	0.71	11.36	2.62	58.92	24.11	2.54	
Norway, 395	8.7	78.1	13.2	0.8	12.7	2.3	61	20.5	1.7	[30]
Sweden, 750	14.8	89.7	32.6	0.65	11.1	3.05	55.9	22.7	1.9	[31, 32]
Estonia, 297	8.3	75.4	16.3	0.67	12.46	2.69	56.90	24.58	2.69	[33]
Lithuania, 1,035	10	78	11	0.4	16.6	3.3	61.3	17.3	1.1	[34]
Denmark, 466	8.5	74.1	17.4	1.7	11.6	1.9	55.8	25.1	3.9	[35]
Finland, 203	11.9	95.2	42.9	0.5	9.9	1.5	46.8	35.5	5.9	[24]
Iceland, 185	13.5	93.6	29.7	0	10.3	3.2	60.0	23.2	3.2	[24]
Ireland, 100	12	95	24	1	9	2	66	20	2	[36]
South European Countries										
Mean	11.4	88.63	**11.29**	0.9	13.68	1	69.2	14.18	0.98	
Bosnia, 170	12.6	78	9.4	1.8	19.4	2.4	60.5	14.7	1.2	[37]
Croatia, 456	5.7	85.5	8.8	0.2	10.5	0.5	71.9	16.7	0.2	[38]
Greece, 140	17.4	97.15	13.57	0.71	15.72	0.71	70.0	11.43	1.43	[39]
Portugal, 607	12.5	97.5	17.3	0.3	11.3	0.9	71.1	15.1	1.3	[40]
Spain, 399	7.5	85	7.4	1.5	11.5	0.5	72.5	13	0.8	[41]
West European Countries										
Mean	9.67	78.17	**17.8**	0.9	12.12	2.1	58.87	23.45	2.35	
Austria, 749	8.3	74.3	17.4	0.7	12.6	2.9	55.0	25.8	3.0	[24, 42]
Germany, 2,031	7.7	77.8	14.5	0.6	12.0	2.2	60.5	22.6	2.1	[43]
France, 498	14.5	85.5	22.5	1.6	11.9	1	64	20.1	1.4	[44]
Netherlands, 2,000	8.2	75.1	16.8	0.7	12.0	2.3	56.0	25.3	2.9	[45]

The results of ApoE allele frequencies were in Hardy–Weinberg equilibrium ($p > 0.05$).

To assess the differences between the frequencies of ApoE alleles in the Romanian population and those in other European nations, we scanned the PubMed database for papers related to ApoE prevalence in different populations. Only data from population-based studies and separate groups of healthy subjects were included; cohorts with a particular disease were omitted. In the Romanian population, our data showed that ε4 and ε3 alleles appear to be more common compared with other European communities, while ε2 is lower (Table 3).

Discussion

Our study provides an insight into ApoE polymorphism in a healthy group from Romania. Representative data from Romania are missing: until now, only one study has been published with 279 subjects (152 females and 127 males), and only 91 healthy subjects were included in the study. The results matched those reported for other Caucasian individuals. The distribution of ApoE alleles in this population showed that the ε4 allele was more frequent in groups of obese patients that had coronary heart disease or metabolic syndrome [20].

In our study, ε4 and ε3 appear to be more common compared with other European populations while ε2 is less common.

Like many other genetic polymorphisms, the ε4 allele's prevalence in Europeans exhibits a remarkably interesting gradient that is oriented from south to north. The proportion of ε4 carriers increases from 10%–15% in southern Europe to 40%–50% in the northern part of the continent [46]. The frequency of the ε4 allele in our population of Romania does not respect the geographical pattern of ApoE alleles' frequencies.

This European hereditary variety has been molded by factors of demography and evolution such as the demic expansion of agriculture from the Middle East that started around 10,000 years

ago [47]. Consequently, the ApoE4 gradient could be the result of the intermixture of migrating populations of farmers with low ApoE4 frequency, with populations of hunters/gatherers that came from the north, that had high ApoE4 frequency [48]. Romania is located in southeastern Europe, between northeastern and southern European countries. As a major crossroads between Asia and Europe, Romania has experienced continuous migration and invasion episodes. The precise routes may have been shaped by the topology of the territory and had a diverse impact on Romanians' genetic diversity.

In the Neolithic period, Romania was at a crossroads between the Eastern European steppes (Ukraine), Pontic area (Turkey and Balkans), the Baltics (Poland, Lithuania), and Western Europe (Germany, Austria). During this period the Cucuteni culture was spread on the territory of the country, a civilization represented by farmers. At the end of the Neolithic period came Indo-European tribes of hunters from the Northern Pontic steppes [49].

Over the centuries the Romanian population has suffered many changes, being a mixture of local and surrounding populations. Although they lived in different areas, the Viking warriors had contacts with the Romanians. Archaeologists attest to the existence of Scandinavian ships on the shores of the Black Sea, and other material evidence even shows the existence of conflicts between the Vikings and the Romanians. Also, the Vikings held a monopoly on the eastern routes. The partial conquest of Dacia by the Romans was followed by a period of colonization by various groups from the Roman Empire (Italians, Illyrians, Thracians, Greeks, Celts, Germans, and Eastern or North Africans) [50].

This population movement could have resulted in genetic variation in the present-day Romanian population. According to Hellenthal et al. the population of Romania is estimated to have similar DNA to the Lithuanian (37.7%), Finnish (4.7%), Greek (18.1%), Cypriot (13.4%), and Southern Italian (8.6%) populations [51].

Interactions between genes and environment could influence disease patterns in European populations. ApoE is an intensely studied gene involved in multiple processes, mainly lipid metabolism and neurobiology. ApoE4 is associated with hyperlipidemia and hypercholesterolemia [2]. The higher prevalence of ε4 in this population could affect the lipid profile. The main cause of death in Romania (59.3% of all deaths) is cardiovascular disease. Romania is fifth according to a recent statistical report and is listed among the high CVD risk nations according to the ESC [52].

Studies suggest that individuals carrying the ε4 allele have a greater digestive retention of fats than others. Exposure to the contemporary environmental conditions (Western eating regimen, longer life expectancies) might have delivered a vulnerability allele for Alzheimer's disease (AD) and coronary artery disease [43]. ApoE4 is one of the strongest genetic risk factors for Alzheimer's disease. It is associated with both beta-amyloid (Aβ) and tau pathology. Studies have shown that E4 carrier who eat saturated fat are more likely to get AD than non-carriers who eat the least saturated fat. ApoE may offer novel treatment targets for atherosclerosis and neurological dysfunction. Different genetic therapies are being developed based on the switch between ε4 to ε3 or ε2 but the transition to human trials is still difficult to achieve [53]. The highest ε4 frequency is in Northern Europe, and the lowest is in Asia and Southern Europe, but substantial heterogeneity of these prevalence estimates was observed. ApoE2 generally is the most favorable and apoE4 the least favorable for cardiovascular and neurological health. It is also worth mentioning that new studies suggest an increased risk of developing a severe form of COVID-19 in ε4 carriers [54].

Although ApoE genotyping is usually done for research purposes to understand the role of genetic factors in cardiovascular disease, there is an established clinical utility in confirming the diagnosis of type III hyperlipoproteinemia. Assessment of the ApoE genotype might help guide medical decisions in clinical practice. For example, ApoE genotyping has the potential to help guide lipid treatment and give insight into disease prevention [55]. In hyperlipidemia and hypercholesterolemia, statins are the treatment of choice to decrease the risk of developing cardiovascular disease, but there is wide variability in the response to these drugs, which could in part be influenced by the ApoE genotype [56]. ApoE4 is known to be associated with higher cholesterol levels and has been implicated in AD-related processes such as Aβ burden and inflammation, and it may function as a biomarker for subjects who would benefit to a greater extent from the use of statins. Studies have shown that the reduction rate of TC, LDL-C, and the increased rate of HDL-C in non-ApoE4 carriers were all higher than in ApoE4 carriers, preliminarily indicating that ApoE4 affects the clinical effect of statins [57]. In view of the current evidence, the combined effect of statins and the Mediterranean diet reduces CVD risk in ApoE4 carriers [58]. Also, statins have shown beneficial effects on cognitive function in APOE ε4 homozygotes, as they are associated with a reduction in AD risk [59].

Our study has a number of limitations. First, we have included a relatively small number of subjects from a specific geographical area; therefore, we cannot draw conclusions about the country distribution of ApoE polymorphism. Also, the study included only healthy young subjects, and we lack data on their lipid profiles. Consequently, we cannot infer the impact of the ApoE alleles on their health and cardiovascular risk due to alterations in the lipid profile.

Conclusions

In this southwestern Romanian population, the ApoE4 allele was found to be the most common. Since the ApoE4 polymorphism increases the risk of atherosclerosis, the high frequency of the ApoE4 allele in the Romanian population may increase its susceptibility to cardiovascular diseases. These observations support the increased need for personalized treatment on the basis not only of mass observations from clinical trials but also in view of the genetic characteristics of each individual. Genotyping ApoE may have applications in disorders of lipoprotein metabolism and may be relevant to personalized medicine in understanding cardiovascular risk and the outcome of nutritional and therapeutic interventions.

Availability of Data and Materials

The data presented in this study are available upon request from the corresponding author. The data are not publicly available due to institutional research policies.

Author Contributions

Conceptualization: RD, EL, and ID; Data curation: RD, ET, AP, and DA; Formal analysis: EL, AP, and DA; Investigation: RD, A-RD, and AP; Methodology: RD, A-RD, EL, ET, T-AB, and ID; Project administration: EL, MI, T-AB, and ID; Resources: EL, MI, and T-AB; Software: DA; Supervision: ID; Writing original draft: RD and A-RD; Writing, review, and editing: EL, AP, MI, DA, ET, T-AB, and ID. All authors have contributed to editorial changes in the manuscript. All authors have read and approved the final manuscript.

Ethics Approval and Consent to Participate

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of University of Medicine and Pharmacy of Craiova (decision number 20/26.02.2016). Informed consent was obtained from all subjects involved in the study.

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Conflict of Interest

The authors declare no conflict of interest.

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