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ORIGINAL ARTICLE

Marked Differences of Haplotype Tagging SNP Distribution, Linkage, and Haplotype Profile of APOA5 Gene in Roma Population Samples

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Abstract Roma people are underprivileged, neglected population worldwide, with severe healthcare problems. They have significantly increased prevalence of cardiovascular morbidity, presumably related to their poor social status, alcohol consumption and smoking habits. Assuming that genetic background also plays a role in their susceptibility for cardiovascular diseases, we hypothesized that APOA5 gene polymorphisms, an important role-player in lipid metabolism and in the development of metabolic syndrome and cardio/cerebrovascular events, may also be involved. We examined four APOA5 polymorphisms in 363 Roma and 404 Hungarian DNA samples. For rs662799, rs2266788, rs207560 and rs3135506 we found elevated plasma triglyceride levels in the risk allele carriers compared to non-carriers in both populations. At least a two-fold significant increase was detected in minor allele frequencies in Roma when compared to Hungarians, except the rs2266788 variant. Haplotype analysis revealed significant increase of APOA5*2, APOA5*4 in Roma, as opposed to the higher levels of APOA5*5 found in Hungarians. Different linkage disequilibrium was found between rs207560 and rs3135506 variants in Roma compared to Hungarians. The profound differences observed in almost all APOA5 polymorphisms in Roma require special attention, since these variants are known to associate with cardio/cerebrovascular susceptibility.

Keywords APOA5 · Triglyceride · Haplotype · Linkage disequilibrium · Roma

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Background

Cardiovascular diseases (CVD) are the major cause of death worldwide. However, their exact prevalence is unknown [1, 2]. Several studies have established that elevated triglyceride (TG) levels are independent risk factors for vascular occlusions [3, 4]. Thus, research of the TG level modifier factors, especially genetic susceptibility variants, may have clinical importance. One of these factors is the APOA5 gene. Apolipoprotein AV (apoAV) protein is the latest identified member of the apolipoprotein family, which was discovered more than a decade ago [5, 6]. ApoA5 has been shown to play an important role in lipid metabolism via interacting with lipoprotein lipase (LPL) and the anchoring molecules [7–10]. This interaction accelerates the hydrolysis of triglyceride-rich lipoproteins by affecting LPL or promotes a receptor mediated endocytosis of lipoprotein [11, 12]. The presence of naturally existing polymorphisms of the

APOA5 gene modifies the effect of the protein on lipid metabolism, resulting in increased TG levels [5, 13, 14] as confirmed in several populations worldwide. The APOA5 polymorphisms may trigger the development of several diseases like metabolic syndrome, stroke and CVD [15–19].

In the past decade, several polymorphisms were identified in the protein encoding gene. Among these, the most extensively studied four single nucleotide polymorphisms (SNPs) are the following: rs662799, rs2266788, rs207560 and rs3135506. Epidemiological and clinical studies show that some of these SNPs are independent risk factors for CVD, metabolic syndrome and stroke [3, 20]. The most extensively studied rs662799 polymorphism has been associated with coronary artery disease in almost all populations [21]. Pennacchio et al. confirmed that the most common SNPs in the APOA5 gene are in strong linkage disequilibrium and constitute haplotype variants [13]. One of these haplotypes, APOA5*2, was found to be strongly associated with increased TG levels and confers susceptibility for metabolic and vascular events [18, 22].

Historical, linguistic and genetic studies suggest that the Roma people originate from South Asia, mainly Northwest India [23, 24]. The estimated size of the Roma population is 12–15 million globally. Most of them - approximately 10–12 million - live in Europe, with the highest population (70%) in Central and South-Eastern Europe [25]. Careful review of the available literature revealed that the Roma in Central-Eastern Europe often have a higher rate of cardiovascular events and morbidity than other European populations [26, 27]. This is related to the Roma population's poor socioeconomic status, social exclusion, and other risk factors like disturbed lipid metabolism, hypertension, obesity, drinking and smoking habits [28]. However, little information is available about the effects of genetic variants. Moreover, it is also known, that the Roma population has high prevalence of increased TG levels [28]. These considerations prompted us to examine TG level modifier APOA5 polymorphisms in Roma samples as susceptibility factor.

Methods

Study Populations

DNA samples used in this study were selected from the Biobank of the Department of Medical Genetics at the University of Pécs, which is a member of the National Biobank Network of Hungary and Biomolecular Resources Research Infrastructure (BBMRI). The collection and analysis of the DNA samples were conducted according to the regulations of the Declaration of Helsinki Declaration in 1975 and the currently operative National regulations were followed. The study protocol was reviewed and approved by the Hungarian Scientific and Research Ethics Committee of the Medical Research Council (ETT TUKEB). Informed written consents were obtained from all subjects prior to study. During the sample collection samples were deposited from Roma minority who self-reported at least three past generations of Roma ancestry. Care was also taken during the selection of the already biobanked samples to minimize biased results coming from possible sampling errors; like exclusion of the known family members, selection from different living areas. The same also applied for the average Hungarians as well; none of them self-reported to belong to any known ethnic groups living in Hungary, they were apparently healthy and free from any know disease.

Sample size determination was based on our preliminary analyses of the prevalence of APOA5 SNPs. Based on the important significant difference in frequencies of the genetic alterations between Roma and Hungarian samples; we calculated how many samples we would need per group to be adequately small and large enough to detect a statistically significant difference and to exclude Type I and Type II errors (alpha = 0.05 and beta < 0.03, two tailed). Thus, a total of 363 (gender: 162 male, 201 female; age: 55.86 ± 0.99) Roma and 404 (gender: 141 male, 263 female; age: 61.51 ± 0.79) Hungarian samples. The fasting serum TG and total cholesterol levels of the subjects were measured right after taking blood samples, which was carried out morning up between 8:00 and 9:00 in the morning.

Genotyping

The genomic DNA was obtained from peripheral blood leukocytes. PCR-RFLP method was used for genotyping. GenBank reference sequence NM_001166598.1 and NG_015894.1 of APOA5 were used and variants were described in accordance with Human Genome Variation Society (HGVS) nomenclature guidelines. The APOA5 variants in this manuscript have been submitted to the Leiden Open Variation Database (http://databases.lovd.nl/shared/genes/APOA5, LOVD v.3.0, Screening ID: 000165133, Individual ID: 0016581, Variant ID: 0000036378). The APOA5 rs662799 variant was determined with the following primers: forward primer: 5′-CCCCAGGAACTGGAGCGACCTT-3′, reverse primer: 5′ TTCAAGCAGAGGGAAGCCTGTA 3′. PCR conditions were the following: 2 min of predenaturation at 96 °C then 35 cycles of denaturation at 96 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s followed by a final elongation period at 72 °C for 5 min. After amplification, the PCR products were digested by 1 U of *TruI* restriction enzyme (Fermentas, Burlington, ON, Canada). The digestion resulted in 109 and 289 bp long fragments in GG homozygous samples, 22, 109, 267 bp long fragments in AA homozygous samples, and 22, 109, 267, 289 bp long fragments in heterozygous samples. DNA fragments were

separated by agarose electrophoresis and visualized by ethidium bromide staining and UV illumination.

For genotyping the rs207560 variant the following primers were used: forward primer: 5′-CTCAAGGCTGTCTTCAG-3′, reverse primer: 5′-CCTTTGATTCTGGGGACTGG-3′. For amplification we used the following PCR conditions: 2 min of predenaturation at 96 °C then 35 cycles of denaturation at 96 °C for 30 s, annealing at 62 °C for 30 s and extension at 72 °C for 30 s followed by a final elongation period at 72 °C for 5 min. PCR products were digested with *MnlI* enzyme (Fermentas, Burlington, ON, Canada) resulting in 25, 41, 73, 141 bp long fragments in TT homozygous samples; 25, 114, 141 bp long fragments in CC homozygous samples and 25, 41, 73, 114, 141 bp long fragments if the sample was heterozygous for this variant. Separation and visualization were performed as written above.

For the rs2266788 polymorphism we used the following primers to amplify the region of interest: forward primer: 5′-TCAGTCCTTGAAAGTGGCCT-3′, reverse primer: 5′-ATGT AGTGGCACAGGCTTCC-3′. PCR conditions were the following: 2 min of predenaturation at 96 °C then 35 cycles of denaturation at 96 °C for 30 s, annealing at 62 °C for 30 s and extension at 72 °C for 30 s followed by a final elongation period at 72 °C for 5 min. After amplification, the PCR products were digested by 1 U of *BseGI* restriction enzyme (Fermentas, Burlington, ON, Canada). The digestion resulted in 35, 87 and 165 bp long fragments in GG homozygous samples, 122, 165 bp long fragments in AA homozygotes, and 35, 87, 122, 165 bp long fragments in the case of heterozygous samples. DNA fragments were separated by electrophoresis and visualized by ethidium bromide staining and UV illumination.

For genotyping the rs3135506 variant we used the following primers: forward primer: 5′-AGAGCTAGCACCGC TCCTTT, reverse primer: 5′-TAGTCCCTCTCCAC AGCGTT-3′. For amplification we used the following PCR conditions: 2 min of predenaturation at 96 °C then 35 cycles of denaturation at 96 °C for 30 s, annealing at 62 °C for 30 s and extension at 72 °C for 30 s followed by a final elongation period at 72 °C for 5 min. PCR products were digested with *Cfr13I* enzyme (Fermentas, Burlington, ON, Canada) resulting 26, 79 and 151 bp long fragments in GG homozygous samples 79 and 177 bp long fragments in CC homozygous samples and 26, 79, 151 and 177 bp long fragments if the sample was heterozygous for this variant. Separation and visualization were performed as described above.

Statistical Analyses

All clinical data are represented as mean ± SEM. The variables showed non-normal distribution therefore we used non-parametric tests to assess the differences between the study groups. The χ^2 tests were used for discrete variables like sex of the participants, genotype- and allele count. Mann-Whitney tests were used for continuous variables like age and lipid levels. Linear regression analysis was used to identify any correlation between APOA5 variants and lipid levels. Variables were log-transformed to obtain approximate normal distribution for performing the analysis. Correlation coefficients (crude r) were derived from the analysis of lipid levels (dependent variable) and carriers of APOA5 risk alleles (independent variable) at 95% confidence intervals (CI). The regression models were adjusted for age and total cholesterol levels, which variables were significantly different between the study groups (adjusted r). Haploview 4.2 was used to study the linkage disequilibrium (LD) patterns. We required the minor allele frequency at each locus to be >0.05, with an r^2 value of <0.8 between pairs of loci, based on the default settings in Haploview [29]. We used HAPSTAT 3.0 (http://www.bios.unc.edu/wlin/hapstat/) for haplotype assignment. The value of $p \leq 0.05$ was considered as statistically significant. For statistical analysis of the data we used PASW statistics 20 software package (SPSS Inc., Chicago IL).

Results

All of the alleles and genotype frequencies of the studied APOA5 alterations are reported in Table 1. The allele and genotype frequencies were in Hardy-Weinberg equilibrium in all study groups. The number of alleles and genotypes of Roma subjects were compared to Hungarians and four other populations derived from two databases. For rs662799 APOA5 polymorphism we found that the frequency of the G allele was almost three times higher in the Roma population compared to Hungarian samples ($p = 0.0001$) and almost two times higher than in European population (1000Genomes; $p = 0.006$). There was also a significantly large difference in allele frequency between Roma and HapMap European population ($p = 0.0001$). The G allele was at least two-fold more frequent in Asian (1000Genomes) and HapMap Chinese population than in Roma subjects ($p = 0.002$; 0.001). Homozygous carriers of G allele were more frequent in Roma population than in Hungarians ($p = 0.037$) and in Europeans (1000Genomes; $p = 0.010$); however, it was less frequent in Romas than in Asian (1000Genomes; $p = 0.027$) population. There was no significant difference in GG genotype frequencies between Roma and HapMap Chinese populations. Results of rs207560 show the frequency of the T allele in Roma samples was almost double than in those of the Hungarian population ($p = 0.018$). The T allele was significantly frequent in Asian (1000Genomes; $p = 0.0001$) population than in Romas. There was no difference between Roma and European populations (1000Genomes; $p = 0.185$). Homozygous carriers of T allele were more frequent in Asian population (1000Genomes) than in Roma subjects ($p = 0.0001$); however, the frequency of TT genotypes was

Table 1 Genotype and allele frequencies of APOA5 gene variants

APOA5 variant	Population	N	Alleles N		Risk allele frequency (%)	p-value[a]	Genotypes N (%)			p-value[b]
g.116792991G > A			A	G	G		AA	AG	GG	
	Roma from Hungary (this study)	363	628	98	13.50		278 (76.58)	72 (19.84)	13 (3.58)	
	non-Roma from Hungary (this study)	404	765	43	5.32	0.0001	366 (90.60)	33 (8.17)	5 (1.24)	0.037
	Asian (1000Genomes)	504	718	290	28.80	0.002	251 (49.80)	216 (42.86)	37 (7.34)	0.027
	European (1000Genomes)	503	922	84	8.35	0.006	424 (84.30)	74 (14.71)	5 (0.99)	0.010
	HapMap Chinese	45	66	24	26.70	0.001	23 (51.11)	20 (44.44)	2 (4.44)	0.780
	HapMap European	60	118	2	1.67	0.0001	58 (96.67)	2 (3.33)	0	-
g.116791110 T > C			C	T	T		CC	CT	TT	
	Roma from Hungary (this study)	363	680	46	6.34		318 (87.60)	44 (12.12)	1 (0.28)	
	non-Roma from Hungary (this study)	404	779	29	3.59	0.018	376 (93.07)	27 (6.68)	1 (0.25)	0.940
	Asian (1000Genomes)	504	768	240	23.81	0.0001	290 (57.54)	188 (37.3)	26 (5.16)	0.0001
	European (1000Genomes)	503	924	82	8.15	0.185	426 (84.70)	72 (14.31)	5 (0.99)	0.211
g.116791691G > C			C	G	G		CC	CG	GG	
	Roma from Hungary (this study)	363	658	68	9.37		300 (82.64)	58 (15.98)	5 (1.38)	
	non-Roma from Hungary (this study)	404	770	38	4.70	0.001	367 (90.84)	36 (8.91)	1 (0.25)	0.079
	Asian (1000Genomes)	504	1008	0	0	-	504 (100)	0	0	-
	European (1000Genomes)	503	938	68	6.76	0.066	438 (87.08)	62 (12.33)	3 (0.59)	0.240
g.116789970G > A			A	G	G		AA	AG	GG	
	Roma from Hungary (this study)	363	679	47	6.47		317 (87.33)	45 (12.40)	1 (0.27)	
	non-Roma from Hungary (this study)	404	747	61	7.55	0.473	344 (85.15)	59 (14.60)	1 (0.25)	0.940
	Asian (1000Genomes)	504	768	240	23.81	0.0001	290 (57.54)	188 (37.30)	26 (5.16)	0.0001
	European (1000Genomes)	503	914	92	9.15	0.062	416 (82.70)	82 (16.30)	5 (0.99)	0.211
	HapMap Chinese	45	68	22	24.40	0.0001	24 (53.33)	20 (44.44)	1 (2.22)	0.082
	HapMap European	59	113	5	4.24	0.375	54 (91.53)	5 (8.47)	0	-

Ancestral alleles are underlined

[a] indicates significance of the differences between Roma and other population risk alleles

[b] indicates significance of the differences between Roma and other population homozygous carriers

similar in Hungarians and in Europeans (1000 Genomes; $p = 0.940$; 0.211). Data of rs3135506 show that the G allele frequency in Roma's was more than two times higher compared to the Hungarian population ($p = 0.001$); however, it does not differ significantly from the European population (1000Genomes; $p = 0.066$). There was no significant difference in the frequency of GG genotype of Hungarians and of Europeans (1000 Genomes; $p = 0.079$; 0.240) compared to Roma subjects. Allele- and genotype frequency data of rs207560 and rs3135506 in European and Chinese populations are not available in HapMap database, thus the analyses were not executed in these cases. We also analyzed the rs2266788 variant, where we did not find any difference between G allele frequencies of Hungarian and European populations (1000Genomes, HapMap) compared to Roma subjects ($p = 0.473$; 0.062; 0.375). We found the frequency of the G allele was more than three times higher in Asian populations (1000Genomes and HapMap Chinese) compared to Roma samples ($p = 0.0001$). The frequency of GG genotype was significantly different only in Asian population (1000Genomes) compared to Roma ($p = 0.0001$). Cases with $n = 0$ were not analyzed statistically.

Table 2. summarizes the lipid parameters of the populations according to genotypes. The plasma triglyceride levels were significantly elevated in the carriers of the risk alleles when compared to non-carriers for all SNPs in both populations. Significantly higher triglyceride levels were found in heterozygous carriers of rs207560, rs3135506 and rs2266788 variants compared to non-carriers in both study groups. Homozygous carriers of rs662799 variant have higher triglyceride levels than non-carriers in Roma subjects. In Hungarians, we did not find any difference in triglyceride levels between homo- or heterozygous carriers and non-carriers. Comparison of the cholesterol levels did not show any difference.

We analyzed associations among the four APOA5 variants in both study groups. (Table 3) Strong correlations were found among rs662799, rs207560 and rs2266788 variants. However, rs3135506 variant did not show significant correlation with other APOA5 variants in Roma samples as well as in Hungarians. The same associations were detected after inclusion of the adjustment parameters like age and total cholesterol levels.

The associations between APOA5 variants and triglyceride/cholesterol levels are summarized in Table 4. Significant correlations were found between all of the APOA5 variants and triglycerides in both populations. After inclusion of the adjustment parameters, such as age and total cholesterol levels, the association became even stronger. We did not observe any significant correlation between allelic variants and cholesterol levels in both populations (data not shown).

Furthermore, we examined the linkage disequilibrium among the APOA5 major polymorphisms in both populations. We found moderate association between the rs2266788 and the rs3135506 variants ($r^2 = 0.56$), likewise between rs207560 and rs3135506 ($r^2 = 0.42$) in Hungarian population. In Roma population we found strong association between the rs207560 and the rs3135506 variants ($r^2 = 0.97$).

Table 2 Lipid parameters (mmol/l) of the Roma and Hungarian population samples according to APOA5 gene variants

APOA5 variant	Parameter	Roma				Hungarian			
rs662799		AA	AG	GG	AG + GG	AA	AG	GG	AG + GG
	Triglyceride	1.59 ± 0.04	1.72 ± 0.10 $p = 0.245$	2.00 ± 0.16 $p = 0.009$	1.76 ± 0.08 $p = 0.049$	1.51 ± 0.02	1.79 ± 0.13 $p = 0.060$	1.88 ± 0.31 $p = 0.133$	1.81 ± 0.12 $p = 0.024$
	Cholesterol	4.66 ± 0.07	4.77 ± 0.13 $p = 0.352$	4.91 ± 0.39 $p = 0.494$	4.79 ± 0.12 $p = 0.280$	5.57 ± 0.06	5.43 ± 0.22 $p = 0.234$	6.14 ± 1.16 $p = 0.928$	5.52 ± 0.24 $p = 0.257$
rs207560		CC	CT	TT	CT + TT	CC	CT	TT	CT + TT
	Triglyceride	1.59 ± 0.04	1.92 ± 0.12 $p = 0.009$	1.27 ± 0 $p = 0.668$	1.91 ± 0.12 $p = 0.011$	1.51 ± 0.02	1.84 ± 0.13 $p = 0.009$	1.60 ± 0 $p = 0.602$	1.84 ± 0.12 $p = 0.008$
	Cholesterol	4.67 ± 0.06	4.85 ± 0.19 $p = 0.242$	3.90 ± 0 $p = 0.371$	4.83 ± 0.19 $p = 0.302$	5.57 ± 0.06	5.50 ± 0.29 $p = 0.377$	4.70 ± 0 $p = 0.318$	5.47 ± 0.28 $p = 0.295$
rs3135506		CC	CG	GG	CG + GG	CC	CG	GG	CG + GG
	Triglyceride	1.59 ± 0.04	1.81 ± 0.10 $p = 0.028$	1.96 ± 0.50 $p = 0.586$	1.82 ± 0.10 $p = 0.025$	1.52 ± 0.03	1.71 ± 0.07 $p = 0.001$	1.50 ± 0 $p = 0.943$	1.71 ± 0.06 $p = 0.001$
	Cholesterol	4.67 ± 0.06	4.88 ± 0.14 $p = 0.131$	4.14 ± 0.43 $p = 0.269$	4.82 ± 0.13 $p = 0.251$	5.55 ± 0.06	5.67 ± 0.17 $p = 0.419$	5.50 ± 0 $p = 0.862$	5.66 ± 0.17 $p = 0.442$
rs2266788		AA	AG	GG	AG + GG	AA	AG	GG	AG + GG
	Triglyceride	1.59 ± 0.04	1.92 ± 0.12 $p = 0.008$	1.27 ± 0 $p = 0.671$	1.90 ± 0.12 $p = 0.010$	1.51 ± 0.03	1.69 ± 0.07 $p = 0.004$	1.60 ± 0 $p = 0.575$	1.69 ± 0.07 $p = 0.004$
	Cholesterol	4.67 ± 0.06	4.84 ± 0.19 $p = 0.282$	3.90 ± 0 $p = 0.373$	4.82 ± 0.19 $p = 0.347$	5.57 ± 0.06	5.52 ± 0.18 $p = 0.384$	4.70 ± 0 $p = 0.322$	5.50 ± 0.17 $p = 0.327$

We also investigated the haplotypes with statistical probes in Roma and Hungarian populations. The structure of the probable haplotypes is summarized in Table 5. With the applied methods, we identified seven haplotypes in each population. Six of these haplotypes, APOA5*1, APOA5*2, APOA5*3, APOA5*4, APOA5*5 and ht7 were found to occur most frequently in both populations. The frequencies of the haplotypes are shown in Table 5. Significant differences were found in the presence of APOA5*2, APOA5*4, APOA5*5 and ht7 haplotypes between the Roma and Hungarian populations. However, we did not identify differences in the presence of APOA5*1 and APOA5*3 haplotypes between these populations. Ht5 haplotype in Roma and ht4 haplotype in Hungarian population could not be detected.

Discussion

In the present study, we examined the effect of the major APOA5 polymorphisms (rs662799; rs2266788; rs3135506; rs207560) on lipids, especially triglyceride levels. In our results, heterozygous carriers of rs2266788; rs3135506; rs207560 variants had higher triglyceride levels than non-carriers. Homozygous carriers of rs662799 variant have higher triglyceride levels than non-carriers in Roma subjects. To our best knowledge, such association has not yet been described. However, most of the studies do not present the homozygous and heterozygous samples separately because of the low risk allele frequencies of the APOA5 variants. In our study, after combining the homozygous and heterozygous samples, significantly elevated plasma TG levels were found in carriers of risk alleles of the APOA5 variants when compared to the non-carriers in both Hungarian and Roma populations. All four APOA5 variants showed correlation to triglyceride levels with or without adjustment factors like age and cholesterol levels. These findings correspond to the results of previous studies [16–19, 30].

Health status is a major problem for health care systems of those countries where Romas are in significant minority [28]. In Central-Eastern Europe, Romas are likely more susceptible to metabolic syndrome and stroke, have higher morbidity rates, and lower life expectancy than other European populations [26, 31]. CVD among Romas is one of the most common causes of death [27, 32, 33] reportedly with 2.5-fold higher premature CVD mortality compared to the overall population [34] and increased prevalence of various CVD risks factors [35–38]. The situation is not restricted to the Eastern European countries [36, 37].

There are several classic risk factors that increase the development of CVD e.g.: smoking, family history with CVD, hypertension, lipid and lipoprotein abnormalities (elevated total cholesterol and low density lipoprotein (LDL)-cholesterol, high density lipoprotein (HDL)-cholesterol, hypertriglyceridemia and increased lipoprotein(a) (Lp(a)), diabetes (insulin resistance, hyperinsulinemia and elevated blood glucose level), obesity and metabolic syndromes. According to various clinical studies in Central-Eastern Europe [28], the two most common risk factors contributing to the development of CVD presented in most of the Roma populations are obesity and smoking. These are followed by diabetes, metabolic syndrome, hypertension, and increase of triglycerides. Higher prevalence of obesity may result from decreased physical activity and unhealthy dietary habits of the Romas. Smoking is strongly supported as cultural, ethnical, and individual

Table 3 Correlations among APOA5 variants in the study groups

Population	Correlation coefficient (R)	APOA5 variant	APOA5 variant			
			rs662799	rs207560	rs3135506	rs2266788
Roma	Crude	rs662799	-	0.562 $p < 0.001$ CI = 0.612–0.832	0.021 $p = 0.684$ CI = −0.092–0.140	0.552 $p < 0.001$ CI = 0.593–0.813
		rs207560	0.562 $p < 0.001$ CI = 0.612–0.832	-	0.084 $p = 0.110$ CI = −0.163–0.017	0.988 $p < 0.001$ CI = 0.980–1.013
		rs3135506	0.021 $p = 0.684$ CI = −0.092–0.140	0.084 $p = 0.110$ CI = −0.163–0.017	-	0.087 $p = 0.098$ CI = −0.167–0.014
		rs2266788	0.552 $p < 0.001$ CI = 0.593–0.813	0.988 $p < 0.001$ CI = 0.980–1.013	0.087 $p = 0.098$ CI = −0.167–0.014	-
	Adjusted†	rs662799	-	0.636 $p < 0.001$ CI = 0.665–0.883	0.119 $p = 0.913$ CI = −0.119–0.133	0.625 $p < 0.001$ CI = 0.642–0.860
		rs207560	0.636 $p < 0.001$ CI = 0.665–0.883	-	0.138 $p = 0.110$ CI = −0.185–0.019	0.985 $p < 0.001$ CI = 0.977–1.016
		rs3135506	0.119 $p = 0.913$ CI = −0.119–0.133	0.138 $p = 0.110$ CI = −0.185–0.019	-	0.137 $p = 0.099$ CI = −0.190–0.016
		rs2266788	0.625 $p < 0.001$ CI = 0.642–0.860	0.985 $p < 0.001$ CI = 0.977–1.016	0.137 $p = 0.099$ CI = −0.190–0.016	-
Hungarian	Crude	rs662799	-	0.780 $p < 0.001$ CI = 0.826–0.967	0.073 $p = 0.143$ CI = −0.173–0.025	0.485 $p < 0.001$ CI = 0.328–0.469
		rs207560	0.780 $p < 0.001$ CI = 0.826–0.967	-	0.019 $p = 0.702$ CI = −0.133–0.090	0.626 $p < 0.001$ CI = 0.769–0.984
		rs3135506	0.073 $p = 0.143$ CI = −0.173–0.025	0.019 $p = 0.702$ CI = −0.133–0.090	-	0.084 $p = 0.090$ CI = −0.224–0.016
		rs2266788	0.485 $p < 0.001$ CI = 0.328–0.469	0.626 $p < 0.001$ CI = 0.769–0.984	0.084 $p = 0.090$ CI = −0.224–0.016	-
	Adjusted†	rs662799	-	0.803 $p < 0.001$ CI = 0.833–0.970	0.076 $p = 0.160$ CI = −0.177–0.029	0.512 $p < 0.001$ CI = 0.345–0.488
		rs207560	0.803 $p < 0.001$ CI = 0.833–0.970	-	0.061 $p = 0.703$ CI = −0.110–0.074	0.637 $p < 0.001$ CI = 0.769–0.986
		rs3135506	0.076 $p = 0.160$ CI = −0.177–0.029	0.061 $p = 0.703$ CI = −0.110–0.074	-	0.090 $p = 0.098$ CI = −0.233–0.020
		rs2266788	0.512 $p < 0.001$ CI = 0.345–0.488	0.637 $p < 0.001$ CI = 0.769–0.986	0.090 $p = 0.098$ CI = −0.233–0.020	-

† Adjusted for differences in age and cholesterol levels

identity, thus, they usually start smoking in their early teens. Most of the population is exposed to these risk factors in their direct family environment [28].

Previous studies of some susceptibility genetic variants revealed Roma are a genetically special population [39–42] and their genetic constitution can differ from other populations (we supposed susceptibility alleles in the background also as genetic factors) [43, 44]. In our study, the minor allele frequencies of the studied APOA5 variants were collected in 1000Genomes and HapMap databases, to compare those found in the Roma population with those in Europeans and Asians. For rs662799, significantly different risk allele frequencies were detected between Roma and other studied populations. The allele frequencies in Roma subjects were in between those of Europeans and Asians. We found Roma risk allele frequencies differed significantly from those of the Asian populations for rs662799, rs207560, rs2266788 variants. We found, that the risk allele frequencies were significantly higher in Roma than in Hungarian population for rs662799; rs3135506 and rs207560 variants. It is important to emphasize, that the frequencies found in Hungarians correspond with those detected in European populations [13, 45, 46]. The reasons for these differences have not yet been cleared. At the same time, the already confirmed definitive

Table 4 Correlations between carrying APOA5 risk alleles and triglyceride levels in Roma and Hungarian population samples

Population	Correlation coefficients		APOA5 variant			
			rs662799	rs207560	rs3135506	rs2266788
Roma	Crude	R/Beta	0.102	0.149	0.124	0.149
		R^2	0.011	0.022	0.015	0.022
		95% CI	0.000–0.092	0.027–0.144	0.011–0.113	0.027–0.143
		p	0.051	0.004	0.018	0.004
	Adjusted†	R	0.360	0.351	0.362	0.351
		R^2	0.129	0.123	0.131	0.123
		Beta	0.138	0.111	0.142	0.113
		95% CI	0.014–0.115	0.002–0.126	0.018–0.129	0.003–0.126
		p	0.012	0.043	0.009	0.040
Hungarian	Crude	R/Beta	0.155	0.161	0.142	0.137
		R^2	0.024	0.026	0.020	0.019
		95% CI	0.024–0.106	0.031–0.125	0.019–0.102	0.014–0.081
		p	0.002	0.001	0.004	0.006
	Adjusted†	R	0.200	0.214	0.182	0.199
		R^2	0.040	0.046	0.033	0.039
		Beta	0.152	0.171	0.129	0.151
		95% CI	0.022–0.106	0.033–0.128	0.012–0.098	0.018–0.086
		p	0.003	0.001	0.012	0.003

† Adjusted for differences in age and cholesterol levels

association of susceptibility variants and the development of diseases mean that increased minor allele frequencies obligatory lead to increased vascular events [21]. Thus, the data of the present study support that elevated rates of susceptibility alleles are in relationship- at least in part- with increased prevalence of CVDs in Roma minority.

The most common APOA5 variants, like rs662799, rs207560, rs2266788 and rs3135506 are in strong linkage disequilibrium and create two major haplotype variants (APOA5*2 and *3). These two haplotypes together with wild type haplotype (APOA5*1–3) constitute approximately 98% of the average population [13]. Five common haplotypes were identified this far (APOA5*1–5) [47–49]. Additional possible haplotypes are also known (ht4, 5, 7) composing only a small portion of the population. In our study, we prospected linkage disequilibrium among the APOA5 polymorphisms in both populations and attempted to investigate the haplotypes in Roma and Hungarian samples. Different linkage was found between the rs207560 and rs3135506 in Roma and Hungarian populations. In Roma the linkage between the variants was strong, while in Hungarians moderate, possibly because of the distinct origin of the two populations. To our best knowledge, such association has not yet been described.

The haplotype analyses for APOA5 revealed eight theoretical haplotypes, but only seven occurred in both populations. Five of these (APOA5*1, APOA5*2, APOA5*3, APOA5*4 and APOA5*5) are the most extensively studied haplotypes of the gene. Ht7 haplotype has not yet been investigated in susceptibility studies, so far. Comparison of the prevalence's in Roma and Hungarian populations revealed APOA5*2, APOA5*4 and ht7 haplotypes were significantly prevalent in Roma population, whereas APOA5*5 haplotype was more frequent in Hungarians.

Table 5 The structure of the individual APOA5 haplotype variants with percentage of Romas and Hungarians

Haplotypes	APOA5 variant				Population
	rs662799	rs207560	rs3135506	rs2266788	Roma/Hungarian (%)
APOA5*1/ ht1	A	C	C	A	77.8/85.6
APOA5*2/ ht8	G	T	C	G	5.4†/3.2
APOA5*3/ ht3	A	C	G	A	7.6/4.7
APOA5*4/ ht2	G	C	C	A	6.3†/2.0
APOA5*5/ ht6	A	C	C	G	0.1†/4.1
ht4	G	C	G	A	1.8/–
ht5	G	T	C	A	–/0.1
ht7	A	T	C	G	1.0†/0.2

† $p < 0.05$ vs Hungarian samples

Results of previous studies describe APOA5*2 haplotype is associated with elevated TG levels and might confer as susceptibility for metabolic syndrome and ischemic stroke in the Hungarian population [18, 22]. Our results suggest, Roma people have higher risk for hypertriglyceridemia and for vascular events because of increased prevalence of the APOA5 susceptibility alleles. Previously, Kisfali et al. did not confirm connection between the presence of APOA5*4 and the risk for metabolic syndrome in Hungarians. Moreover, APOA5*5 haplotype was found to have a protective effect against metabolic syndrome, and associated with decreased TG levels [22]. Our findings on APOA5*5 reported here also provide indirect support for APOA5 variant's having a role in the Roma susceptibility to CVD.

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Compliance with Ethical Standards

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Conflict of Interest The authors declare no conflict of interest.

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