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# Apolipoprotein E polymorphism in Brazilian dyslipidemic individuals: Ouro Preto study

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## Abstract

The influence of apolipoprotein E alleles and genotypes on plasma lipid levels was determined in 185 individuals of mixed ethnicity living in Ouro Preto, Brazil. DNA was obtained from blood samples and the genotypes were determined by an RFLP-PCR procedure. The *3 allele was the most frequent (72%), followed by *4 (20%) and *2 (8%); *4 frequency was higher and *2 frequency was lower in the dyslipidemic group than in the normal control group. The *2 carriers presented lower LDL and total cholesterol levels compared to the *3 and *4 carriers. All six expected genotypes were observed in the individuals genotyped: E2/2 (2.1%), E4/4 (2.7%), E2/4 (3.7%), E2/3 (8.0%), E3/3 (53.3%), E3/4 (29.9%); no difference in genotype frequencies was found between the normal and dyslipidemic groups. Compared with *2, the presence of *3 increases more than two times the risk for dyslipidemia (OR = 2.31; P = 0.025; 95% CI = 1.06-5.06) and the presence of *4 increases it three times (OR = 3.31; P = 0.006; 95% CI = 1.36-8.04). The only significant effect of genotype was an increased risk for dyslipidemia in the *4 genotype carriers (E3/4 + E4/4) compared with the *2 genotype carriers (E2/2 + E2/3) with OR = 3.69 (95% CI = 1.25-10.88). The present study indicates that in the Ouro Preto admixed population the presence of APOE *2 can confer a protective effect, whereas the presence of APOE *4 implies an enhanced risk for dyslipidemia.

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**Key words**
- Apolipoprotein E polymorphism
- Lipids
- Dyslipidemia
- Brazilian population
- Atherosclerosis

## Introduction

Coronary artery disease (CAD) is the leading cause of mortality in many parts of the industrialized world (1). In Brazil, CAD is the most important cause of death for both genders in all regions of the country (2). CAD is the result of several risk factors including both biological and environmental factors. The role of genetic markers is considered to be of increasing importance in the evaluation of the risk for CAD (3).

Apolipoprotein E (APOE) polymorphism is one of the most important genetic markers for CAD and has also been related to some kinds of dementia (4,5) and resistance to

infections by some viruses (4-8). APOE is a glycoprotein related to the the metabolism of endogenous and dietary cholesterol and triglycerides (9). It is a component of very low-density lipoproteins (LDL) and is implicated in the transport and distribution of lipids between tissues, mainly acting in two different ways: by binding to the LDL receptor and to heparan sulfate proteoglycans present in hepatocytes (10). In addition to playing these roles, APOE influences the activity of lipoprotein lipase and enteric cholesterol absorption (11,12). APOE has three common alleles, APOE*2, APOE*3, and APOE*4, the frequencies of which differ significantly among diverse ethnic groups (13,14).

The most common allele is *3, which is present at a frequency close to 80% in Caucasian, Asiatic and Amerindian populations (15-17). In industrialized societies, individuals carrying the *4 allele have high serum levels of total cholesterol and LDL cholesterol, while individuals carrying the *3 allele have intermediate levels and those carrying the *2 allele have the lowest levels (18). It has been well established that the allele variation in the APOE gene has a significant effect on interindividual variation in plasma lipid and lipoprotein levels and on the risk of CAD in the general population (13,19), but most of this evidence comes from studies on adult populations of North American or Western European origin. Relatively few studies have been done on heterogeneous groups such as those living in Southeast Brazil.

Several studies have indicated that the population of the Southeast region of Brazil is a highly admixed group because of the intercrossing between Europeans, Africans and Amerindians, so that autosomal markers such as skin and hair colors are not reliable to infer individual ancestrality (20-23). Ouro Preto is a historical city in the Southeast region of Brazil that was founded during the "Gold Cycle" in the 18th century. Sub-Saharan African slaves made a large contribution to the formation of its population. As the *4 allele is found in up to 40% of some sub-Saharan African people, we genotyped the APOE gene in a sample of the population of Ouro Preto presenting normal serum lipids or dyslipidemia in order to assess the impact of the APOE alleles on the risk of dyslipidemia in this population.

## Material and Methods

Ouro Preto is located in a metallurgic zone in Central Minas Gerais, Southeast Brazil. The town has approximately 9,287 homes and 37,603 inhabitants (24). In a cross-sectional study (Ouro Preto Study), 930 homes were selected by simple random sampling, stratified by the density of homes of the 33 census enumeration districts of the town of Ouro Preto. In each home sampled, the individual aged 15 years or more whose birthday was closest to the day of the interview was selected to participate in the study. The study was approved by the Research Ethics Committee of the Federal University of Ouro Preto (CEP/UFOP). Participation in the study was voluntary, and all participants gave written informed consent.

Blood pressure was measured at home and sociodemographic information (gender, age, skin color, education, and economic class) and behavior (smoking, drinking habits and physical activity) were obtained by face-to-face interview. Soon after, the volunteers were invited to attend the health service of the Federal University of Ouro Preto for collection of serum samples and anthropometrics measurements. Body weight was measured once to the nearest 200 g on a Tanita BF542 scale (Tanita Corporation of America, Inc., Arlington Heights, IL, USA) and height was measured with a stadiometer fixed to a wall. Body mass index (BMI) was calculated by dividing body weight (kg) by height squared (m) and classified according to the criteria of the World Health Organization (25).

All biochemical analyses were performed with an Airone 200 automatic analyzer (Crony Instruments, Rome, Italy). Total cholesterol, high-density lipoprotein (HDL) cholesterol and triglyceride levels were determined by the enzymatic-colorimetric test (*In Vitro* Diagnóstica S/A, Itabira, MG, Brazil). LDL cholesterol was determined by the Friedwald equation. Lipid levels were classified according to the III Brazilian Guidelines on Dyslipidemia (26). Total cholesterol was defined as normal (<200 mg/dL), moderate (200-239 mg/dL) and high (≥240 mg/dL). For LDL cholesterol, the following ranges were considered: normal (<129 mg/dL), moderate (130-159 mg/dL) and high (>160 mg/dL). For HDL cholesterol, the following ranges were considered: high (>60 mg/dL), moderate (40-60 mg/dL) and low (<40 mg/dL). For triglycerides, values above 200 mg/dL were considered high, values from 150 to 200 mg/dL were considered moderate and values below 150 mg/dL were considered normal. Individuals who presented at least one alteration in these values were considered to be dyslipidemic.

From the data base of the Ouro Preto Study, we randomly selected 185 individuals with normal or altered serum lipid levels, matched for gender and age. We obtained genomic DNA and performed the genotyping of APOE polymorphism. Briefly, blood samples were collected into EDTA or mouth swabs were collected in absolute ethanol and used to obtain genomic DNA by an alkaline lysis and proteinase K digestion procedure modified in our laboratory (27). DNA from swab samples was precipitated with NaCl and isopropanol after digestion, while DNA from blood samples was extracted with phenol:chloroform before precipitation (27). APOE polymorphisms were determined after DNA amplification by the polymerase chain reaction using oligonucleotide primers F4(5'-ACAGAATTCGCCCC GGCCTGGTACAC-3') and F6(5'-TAAG CTTGGCACGGCTGTCCAAGGA-3') described by Emi et al. (28). The reaction tubes contained 100 ng DNA, 200 mM Tris-HCl, pH 8.4, 500 mM KCl, 1.5 mM $MgCl_2$, 10% dimethylsulfoxide, 20 pmol of each primer, and 1 unit of *Taq* DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA) in a final volume of 30 µL. After an initial denaturation for 5 min at 94ºC, the amplifications were done for 35 cycles of 1 min at 50ºC, 1 min at 72ºC and 1 min at 94ºC, followed by a final extension at 72ºC for 10 min. After amplification, 15 µL of the product was digested with 5 units *Hha*I (Invitrogen Life Technologies) in the presence of the buffer provided with the enzyme, for 4 h at 37ºC. The restriction fragment length polymorphism analysis was done by separating the digestion products on 6% polyacrylamide nondenaturing gel electrophoresis, silver stained, and the genotype was determined for each individual as described by Hixson and Vernier (29).

Allele frequencies were estimated by gene counting. Both deviation from Hardy-Weinberg equilibrium and the comparison of each variable with the allele frequencies and genotype distribution were assessed by chi-square tests. Mean lipid levels were compared among APOE allele and genotype groups by analysis of variance (ANOVA) followed by a *post hoc* Student test for multiple comparisons. The odds ratio (OR) with 95% confidence intervals (CI) was adjusted for gender, age, BMI, and skin color using multiple logistic regression. To evaluate the reproducibility of the assays, a different observer repeated the genotyping of 12% of the samples and the kappa test was done. The level of significance was set at 5% in all tests. Statistical analysis was performed using the SPSS software, version 12.0, 2003 (Chicago, IL, USA).

## Results

Most of the individuals self-reported their skin color as non-black and less than 12%

Table 1. Apolipoprotein E (APOE) allele distribution and genotype frequencies in normal and dyslipidemic individuals from Ouro Preto, Brazil.

| | All | Normal | Dyslipidemics |
|---|---|---|---|
| APOE allele+ | | | |
| *2 | 0.08 | 0.12 | 0.05 |
| *3 | 0.72 | 0.72 | 0.72 |
| *4 | 0.20 | 0.16 | 0.23 |
| APOE genotype | | | |
| E2/2 | 4 (2.1%) | 4 (5.10%) | 0 (0%) |
| E2/3 | 15 (8.0%) | 8 (10.1%) | 7 (6.6%) |
| E2/4 | 7 (3.7%) | 3 (3.8%) | 4 (3.8%) |
| E3/3 | 98 (53.3%) | 45 (57.0%) | 53 (50.0%) |
| E3/4 | 56 (29.9%) | 16 (20.3%) | 40 (37.7%) |
| E4/4 | 5 (2.7%) | 3 (3.8%) | 2 (1.9%) |

+P = 0.025 for comparisons of the allele frequencies between normal and dislipidemic individuals (chi-square test).

Table 2. Clinical characteristics of individuals from Ouro Preto, Brazil.

| Variable | Normal (N = 79) | Dyslipidemics (N = 106) |
|---|---|---|
| Age (years) | 54.74 ± 15.08 | 53.13 ± 13.85 |
| Total cholesterol (mg/dL) | 169.85 ± 23.78 | 222.83 ± 39.75* |
| HDL cholesterol (mg/dL) | 61.25 ± 9.51 | 71.30 ± 16.46* |
| LDL cholesterol (mg/dL) | 89.25 ± 20.28 | 111.65 ± 37.80* |
| Total/HDL cholesterol ratio | 2.81 ± 0.48 | 3.23 ± 0.85 |
| Triglycerides (mg/dL) | 96.71 ± 39.53 | 191.99 ± 87.06* |
| BMI (kg/m$^2$) | 26.22 ± 4.25 | 27.90 ± 5.55* |

Data are reported as means ± SD. HDL and LDL cholesterol = high- and low-density lipoprotein cholesterol, respectively; BMI = body mass index.
*P < 0.05 compared to normal individuals (ANOVA).

reported it as black (P < 0.05). The APOE genotype and allele frequencies observed are presented in Table 1. The genotype frequency distribution observed did not differ significantly from that expected according to Hardy-Weinberg equilibrium (data not shown). The kappa test showed 100% concordance between the genetic analyses performed by two different observers on 12% of the samples. The *3 allele was the most frequent both in normal and dyslipidemic individuals, followed by the *4 and *2 alleles. The alleles and genotype frequencies were compared between dyslipidemic and normal individuals (Table 1). The frequency of the *4 allele was higher while the frequency of the *2 allele was lower in dyslipidemic individuals (P = 0.025). All six expected genotypes were observed in the individuals genotyped: E2/2 (2.1%), E4/4 (2.7%), E2/4 (3.7%), E2/3 (8.0%), E3/3 (53.3%), E3/4 (29.9%). As expected, the frequency of individuals homozygous for the *2 and *4 alleles was low, while heterozygous E3/4 and homozygous E3/3 individuals were the most frequent in both the normal and dyslipidemic groups. Due to the low number of homozygous individuals for the *2 and *4 alleles, these genotypes were excluded from

Table 3. Clinical characteristics according to apolipoprotein E alleles and genotype in individuals from Ouro Preto, Brazil.

| | N | Total cholesterol (mg/dL) | HDL cholesterol (mg/dL) | LDL cholesterol (mg/dL) | Total/HDL colesterol ratio | Triglycerides (mg/dL) | BMI (kg/m$^2$) |
|---|---|---|---|---|---|---|---|
| Allele | | | | | | | |
| *2 | 30 | 183.20 ± 44.59$^{a,b}$ | 67.23 ± 16.45 | 84.17 ± 27.69$^{a,b}$ | 2.77 ± 0.50$^{a,b}$ | 159.23 ± 85.61 | 26.67 ± 4.40 |
| *3 | 267 | 202.61 ± 43.50$^{a}$ | 67.12 ± 15.17 | 103.76 ± 31.96$^{a}$ | 3.07 ± 0.69$^{a}$ | 147.03 ± 83.49 | 27.24 ± 5.31 |
| *4 | 73 | 201.75 ± 42.93$^{b}$ | 66.00 ± 12.50 | 102.57 ± 37.22$^{b}$ | 3.13 ± 0.96$^{b}$ | 163.94 ± 89.37 | 26.96 ± 4.33 |
| P | | 0.068 | 0.842 | 0.008 | 0.070 | 0.283 | 0.804 |
| Genotype | | | | | | | |
| E2/2 + E2/3 | 19 | 188.58 ± 52.97 | 67.84 ± 15.43 | 88.68 ± 27.78$^{e}$ | 2.82 ± 0.49 | 160.42 ± 91.08 | 26.90 ± 4.31 |
| E2/4 | 7 | 177.00 ± 26.24 | 68.57 ± 10.24 | 70.71 ± 28.96$^{c,d}$ | 2.61 ± 0.44 | 188.43 ± 81.08 | 25.24 ± 4.73 |
| E3/3 | 98 | 201.45 ± 43.34 | 67.24 ± 15.43 | 103.77 ± 30.66$^{c}$ | 3.03 ± 0.56 | 136.96 ± 78.39$^{a}$ | 27.16 ± 5.67 |
| E3/4 + E4/4 | 61 | 206.52 ± 42.09 | 65.89 ± 23.63 | 106.75 ± 36.52$^{d,e}$ | 3.22 ± 1.02 | 167.43 ± 91.13$^{a}$ | 27.37 ± 4.28 |
| P | | 0.202 | 0.919 | 0.012 | 0.057 | 0.090 | 0.802 |

Data are reported as mean ± SD. HDL and LDL cholesterol = high- and low-density lipoprotein cholesterol, respectively; BMI = body mass index. Same letters indicate a statistically significant difference (P < 0.05) between values in the multivariate analysis (*post hoc* Student test). P values at the bottom of each column refer to ANOVA results comparing the values of each clinical variable between all allele or genotype groups.

the statistical analysis (chi-square test). Although dyslipidemic individuals tended to present a higher frequency of the E3/4 genotype compared with normal individuals, the difference in these frequencies was not statistically significant (Table 1).

The baseline clinical and biological characteristics of the individuals are given in Table 2. Dyslipidemic individuals presented significantly higher serum levels of triglycerides, total cholesterol, LDL cholesterol and HDL cholesterol than normal individuals. No differences in age or total cholesterol/HDL cholesterol ratio were observed between groups. Individuals classified as dyslipidemic presented a significantly higher BMI than normal individuals although both groups were classified as non-obese according to this parameter.

Table 3 presents the clinical characteristics of the individuals according to APOE alleles and genotypes. ANOVA showed a significant difference in LDL cholesterol levels according to the alleles present, with the *2 allele carriers presenting lower levels compared with the *3 and *4 allele carriers. On the other hand, total cholesterol, HDL cholesterol and triglyceride levels as well as the total cholesterol/HDL cholesterol ratio and the BMI were not affected by the allele present. Multivariate analysis showed that the *2 allele carriers presented lower levels of total cholesterol compared with the *3 allele (P = 0.021) and *4 allele (P = 0.05) carriers. The LDL cholesterol levels was also lower in the *2 allele carriers compared with the *3 allele (P = 0.002) and *4 allele (P = 0.01) carriers.

For the purpose of this statistical analysis, the sample was subdivided into four APOE genotype groups: individuals carrying the E2/2 and E2/3 genotypes, individuals carrying the E3/4 and E4/4 genotypes, individuals carrying the E3/3 genotype, and individuals carrying the E2/4 genotype. ANOVA showed a significant difference in LDL cholesterol levels among groups, with the *2 allele carriers showing the lowest values. Lower triglycerides were also observed in the E3/3 individuals, although this difference was not significant. Multivariate analysis showed that the E3/4 + E4/4 genotype group presented higher LDL cholesterol levels than the E2/4 group (P = 0.007) and the E2/2 + E2/3 group (P = 0.033). E3/3 individuals presented higher LDL cholesterol levels than E2/4 individuals (P = 0.012) and lower triglyceride levels than the E3/4 + E4/4 genotype group (P = 0.02).

Table 4 presents the risk of dyslipidemia adjusted for gender, age, BMI, and skin color, regarding the APOE alleles. Dyslipidemia was two times more frequent in the presence of the *3 allele (OR = 2.31; 95% CI = 1.06-5.06; P = 0.025) and three times more frequent in the presence of the *4 allele (OR = 3.31; 95% CI = 1.36-8.04; P = 0.006) compared to the presence of the *2 allele.

The risk of dyslipidemia adjusted for gender, age, BMI, and skin color, regarding genotype groups is presented in Table 5. As the E2/4 genotype group had few individuals compared with the other groups, it was

Table 4. Relationship between apolipoprotein E alleles and incidence of dyslipidemia assessed by simple logistic regression.

| | $\chi^2$ | OR | 95% CI | P |
|---|---|---|---|---|
| *3 x *2 | 4.645 | 2.31 | (1.06-5.06) | 0.025 |
| *4 x *2 | 7.351 | 3.31 | (1.36-8.04) | 0.006 |
| *4 x *3 | 1.694 | 1.43 | (0.83-2.45) | 0.121 |

OR = odds ratio; 95% CI = confidence interval at 95%.

Table 5. Relationship between apolipoprotein E genotypes and incidence of dyslipidemia assessed by simple logistic regression.

| | $\chi^2$ | OR | 95% CI | P |
|---|---|---|---|---|
| (E2/2 + E2/3) x (E3/4 + E4/4) | 6.002 | 3.69 | (1.25-10.88) | 0.015 |
| E3/3 x (E3/4 + E4/4) | 2.953 | 1.79 | (0.91-3.52) | 0.060 |
| E3/3 x (E2/2 + E2/3) | 2.001 | 0.48 | (0.11-1.33) | 0.122 |

OR = odds ratio; 95% CI = confidence interval at 95%.

not considered in this analysis. The only significant effect seen was an increased risk for dyslipidemia in the genotype group of *4 allele carriers (E3/4 + E4/4) compared with the genotype group of *2 allele carriers (E2/2 + E2/3) with an OR = 3.69 (95% CI = 1.25-10.88; P = 0.015).

## Discussion

APOE polymorphism has been widely studied mainly in European populations. One of the most important findings of these investigations was a clear North to South decreasing gradient of the *4 allele frequency, with an opposite direction for the *3 allele. The prevalence of the *4 allele is higher than 20% in Finland and Sweden and less than 8% in the Iberian Peninsula (19,30,31). Much less information is available for African populations, who have a higher frequency of the *4 allele (24-30%) and a lower prevalence of the *3 allele compared to European populations (13,14,32,33). Few studies are available regarding the APOE allele and genotype frequencies in the general Brazilian population. Although most of the individuals in our study self-reported their ethnicity as non-black, the Ouro Preto population had a large contribution of African people in its formation. As can be observed in Table 1, considering all individuals, the prevalence of the *4 allele (20%) was similar to that found in Southern Afro-Brazilians (23%) and higher than that found in Brazilians of European descent (11%) (34,35). In a northeastern admixed Brazilian population, the prevalence of the *4 was 17% (36), a finding similar to our results. The genotypes most frequently found in the normal group were E3/3 (57%) and E3/4 (20%). The E3/3 prevalence was lower than that found in Brazilians of European descent (70%) and in a Caucasian population of Southeast Brazil (72%), but was similar to that found in a northeastern admixed Brazilian population (59%) (35,36).

APOE polymorphism is plastic and depends on environmental factors to determine a risk for CAD. Thus, a better understanding of the role of this common genetic variation in the risk of atherosclerosis in specific populations may permit the identification of subgroups that are at increased risk to develop such diseases. According to our data, there was a higher prevalence of the *4 allele in the dyslipidemic group. We also found a lower prevalence of the *2 allele in the dyslipidemic group compared with the normal group (Table 1). These results confirm that, as observed for other populations of different genetic backgrounds, the presence of these alleles can be important markers for the risk assessment of CAD in admixed people living in Southeast Brazil. Regarding the genotypes, we did not find differences in their prevalence between the normal and dyslipidemic groups.

In the present study, although the levels of all lipid fractions analyzed were significantly higher in the dyslipidemic group (Table 2), only the total cholesterol and triglyceride levels could be classified as being above normal values according to the III Brazilian Guidelines on Dyslipidemia (26). Associations between the *4 allele and increased total and LDL cholesterol levels and between the *2 allele and lower levels of these lipids have been documented in many studies and were independent of ethnic group (13). In our study, we found a significant association between the *2 allele and lower levels of total and LDL cholesterol and a lower total/HDL cholesterol ratio (Table 3). On the other hand, we did not find differences in these parameters between *4 and *3 individuals. When the genotype groups are considered, significant differences between groups can be observed only in LDL cholesterol and triglyceride levels. The E3/3 genotype group presented lower LDL cholesterol and triglyceride levels compared to the E3/4 + E4/4 group. The E3/3 and E3/4 + E4/4 genotype groups also presented higher LDL

cholesterol levels than the E2/4 genotype group. The same was observed when the E3/3 genotype group was compared to the E2/2 + E2/3 genotype group, suggesting a protective effect of the *2 allele. This protective effect was confirmed by the 2- and 3-fold increase in relative risk when the presence of the *2 allele is compared with the presence of the *3 and *4 alleles, respectively (Table 4). However, considering the genotype groups, an almost four times higher risk was observed only when the genotype of the *4 allele carrier group (E3/4 + E4/4) was compared with the genotype of the *2 allele carrier group (E2/2 + E2/3). Taken together and confirming previous findings, the present data indicate that in the Ouro Preto population the presence of the APOE *2 allele can confer a protective effect, whereas the presence of the APOE *4 allele implies an enhanced risk for dyslipidemia.

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