

Relationship between *ApoE* gene polymorphism and coronary heart disease in Gaza Strip

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ABSTRACT

Background: Apolipoprotein E (*ApoE*) plays a role in the regulation of lipid metabolism in humans. *ApoE*, a 229-amino-acid polypeptide, is classified into three major isoforms (E2, E3, and E4) according to the differences in amino acids at positions 112 and 158. In the normal population, *ApoE3* isoform is the most prevalent, and *ApoE2* or E4 is frequently associated with hyperlipoproteinemia. The objective of this work was to investigate the relationship between *ApoE* gene polymorphism and coronary heart disease (CHD) in Gaza Strip and investigate the association between serum lipid levels and CHD. **Material and Methods:** The study population consisted of 137 subjects including 69 CHD cases (45 male, 24 female) and 68 healthy subjects (33 male and 35 female). **Results:** The *ApoE3/E3* genotype was the most common in the control and the CHD groups. *ApoE2/E3* and *ApoE3/E4* were the next most common genotypes. The frequencies of *ApoE* alleles in the CHD subjects were 0.826 for E3, 0.137 for E4, and 0.0362 for E2. These frequencies are comparable to those found in the control group which were 0.875 for the E3, 0.073 for E4, and 0.0515 for E2. No statistically significant differences in *ApoE* genotypes were found between the patients and the control groups. Moreover, there was no significant difference between the mean of triglyceride (TG) and HDL levels among different *ApoE* genotypes. However, there was a significant difference in the mean of LDL and *ApoE* genotypes where the mean of LDL was 218.17 mg/dl in *ApoE4*, 149.67 mg/dl in *ApoE2*, and 184.52 mg/dl in *ApoE3*. A significant difference was also evident between the mean of LDL levels in the CHD and the control group where the mean of LDL was 126 mg/dl in CHD and 111.47 mg/dl in the control group. Our study indicated that there was no significant difference between the mean of cholesterol and TG levels of the CHD and the control groups. **Conclusions:** To our knowledge, this is the first study in Gaza Strip investigating the relation between *ApoE* genotypes and CHD. Further investigations are needed to link other genetic factors to CHD.

Key words: Apolipoprotein E, Polymerase chain reaction- restriction fragment length polymorphism, coronary heart disease, gene polymorphism

INTRODUCTION

Apolipoprotein E (*ApoE*) is a polymorphic protein codified

by three alleles in the same locus. Variability in *ApoE* binding to *ApoE* or LDL receptors results in different levels of lipids and lipoproteins and risk of coronary heart disease (CHD) among individuals predisposed to CHD.^[1] Cardiovascular diseases (CVDs), including CHD and stroke, are the most important causes of mortality and morbidity. The known risk factors affecting CHD will inevitably affect the relation of *ApoE* to CHD. Several studies have indicated that the classic cardiovascular risk factors including smoking, high blood pressure, low HDL cholesterol, and in most studies, also high total cholesterol

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are important predictors of CHD events in elderly as well as middle-aged subjects. Lately, new cardiovascular risk factors such as apolipoproteins, including *ApoE*, have been a focus of keen investigation.^[2] *ApoE* is an essential part of lipoprotein metabolism. It is present in lipoprotein particles and mediates lipoprotein binding to the LDL and lipoprotein remnant receptors. Defects in the *ApoE* protein could diminish its ability to bind to the receptors, which then leads to an elevated blood cholesterol level.^[3] The gene coding for *ApoE* is located on chromosome 19 near the genes for *ApoC-I* and *C-II*. Three different alleles, *E2*, *E3*, and *E4*, account for *ApoE* polymorphism and determine the six genotypes *E2/2*, *E2/3*, *E2/4*, *E3/3*, *E4/3*, and *E4/4*.^[2] *ApoE* polymorphisms have been associated with variations in the blood cholesterol level and with the risk of atherosclerosis and premature CVDs.^[4]

ApoE polymorphism has functional effects on lipoprotein metabolism mediated through the hepatic binding, uptake, and catabolism of lipid particles, i.e., chylomicrons, chylomicron remnants, VLDL, and HDL subspecies. *ApoE* contributes to variability in normal cholesterol levels in populations.^[5]

The major effect of genetic variation at this locus is its influence on cholesterol levels, one of the major risk factors for CVDs, particularly coronary artery disease. With reference to cholesterol effects from the *E3* allele, *E4* is associated with higher total and low density lipoprotein cholesterol levels and *E2* with lower levels. The cholesterol-lowering effect of *E2* tends to be greater than the cholesterol-raising effect of *E4*. In total, the contribution of this gene to cholesterol variability based on a variety of populations that have been studied is no more than 10%. Diet and other genes contribute to each individual's cholesterol level, as well as to the population's average level. Many studies have looked at interactions with variants of this gene as possible modifiers of other cardiovascular risks, such as high- and low-fat diets and active versus sedentary lifestyles. Interactions with lipid-lowering medications have also been investigated in relation to *ApoE*.^[6]

ApoE facilitates the binding of triglyceride (TG)-rich lipoprotein remnants to receptors that determine their clearance. The isoforms vary in their receptor-binding activity, with *ApoE4* having the greatest receptor binding and *ApoE2* having a decreased affinity. Individuals with *ApoE2* have higher levels of TGs, and *ApoE2* homozygotes have greatly increased concentrations of remnants, which frequently results in a form of dyslipidemia called type III or dysbetalipoproteinemia. Individuals with *E4*, conversely, tend to have higher concentrations of LDL cholesterol;

this increase is due, in part, to more efficient absorption of dietary cholesterol and perhaps down-regulation of the LDL receptor. Thus, *ApoE* is an important candidate gene for CVD.^[7]

Approximately, 1% of individuals in the general population are *ApoE2/2* homozygotes. However, only a small percentage (2–5%) of these *ApoE2/2* homozygotes develop type III hyperlipoproteinemia. Utermann *et al.* suggested that homozygosity for *ApoE2* is a necessary but insufficient genetic influence and that additional genetic or environmental factors are required for the expression of type III hyperlipoproteinemia in *ApoE2/2* homozygotes. Until 1979, no other abnormalities in other genes had been identified.^[8] High levels of total and LDL cholesterol and low levels of HDL cholesterol predispose individuals to the development of atherosclerosis. Serum levels of these lipids are partly determined by the *ApoE* genotype. With reference to *ApoE3* homozygotes, *ApoE2* is associated with lower levels of total and LDL cholesterol and with higher levels of HDL cholesterol, while *ApoE4* has opposite effects. *ApoE* plays a pivotal role in the transport of lipoproteins and is involved in numerous processes in the arterial wall.^[9]

The risk of atherosclerosis and coronary heart disease in middle age appears to be higher in the presence of the *ApoE4* allele, possibly because of altered lipid metabolism. Not all studies, especially those of elderly subjects or survivors of heart attack, however, find an association between *ApoE4* and CHD.^[10]

The *ApoE4* allele (Arg-112 and Arg-158) has been linked to atherosclerosis. Individuals with *E4/E4* genotype are at a higher risk of developing the disease.^[11]

The following were the objectives of this study

- To investigate the association between *ApoE* gene polymorphism and CHD
- To investigate the association between serum lipid levels and CHD
- To evaluate the effect of *ApoE* gene polymorphism on serum lipid levels.

MATERIALS AND METHODS

Study population

This study is a retrospective case–control study in which *ApoE* genotyping was performed on 137 individuals who were randomly selected. Sixty-nine subjects (24 female and 45 male) with coronary artery disease from Nasser Hospital

in Khan Younis and 68 normal subjects were included in this study (35 female and 33 male).

Sample collection

Blood samples were collected from the patients and the control subjects after a 12- to 16-h overnight fasting. Each sample was collected into two tubes, one EDTA tube and one glass tube; the sample in the glass tube was separated serum was used for lipid profiling. The EDTA sample kept at 4°C was used within 24 h for DNA extraction and subsequent PCR analysis.

Data analysis

The data was entered, stored, and analyzed by a personal computer using the SPSS 8.0 statistical package. Differences in proportions were assessed by ANOVA, *t*-test, and chi-square test; *P*-values < 0.05 were considered statistically significant.

Lipid profiling

Serum cholesterol determination

10 µl Sample /standard was mixed 1000 µl with reagent Cholesterol kit (DiaSys, Germany). The contents were mixed and incubated for 20 min at 20–25°C; then the absorbance of each tube was read at wavelength 500 nm against blank. The cholesterol content was then calculated by applying the following equation:

$$\text{Cholesterol (mg/dl)} = \frac{\text{Sample absorbance} \times \text{Standard concentration.}}{\text{Standard absorbance}}$$

And the Triglyceride the same method but used the triglyceride kit (DiaSys, Germany) and in both method used the normal and high Cholesterol control (DiaSys, Germany), normal and high Triglyceride control (DiaSys, Germany).

HDL-C measurement

HDL-cholesterol was measured after chylomicrons, VLDL, and LDL, were precipitated by adding phosphotungestic acid to the sample. Centrifugation afterward left only HDL in the supernatant. The HDL-C content was determined enzymatically using Ecoline S+ cholesterol as described below.

Assay procedure

The 200-µl serum samples were mixed with 500 µl of the precipitation reagent (phosphotungestic acid and magnesium chloride) and the mixture was incubated for 15

min at room temperature; then the mixture was centrifuged for 20 min at a speed of 2500 rpm; 0.1 ml of the clear supernatant was then transferred to the reaction solution for the determination of cholesterol.

The supernatant after the centrifugation should be clear; serum or plasma with TG contents ≥1000 mg/dl tends to produce a turbid supernatant or floating precipitates. In such case, the sample should be diluted 1:1 with the NaCl solution (0.9%) and then precipitation should be performed and the final result should be multiplied by 2.

LDL calculation

LDL can be calculated from the parameters determined above using the following formula:

$$\text{LDL} = \text{Total cholesterol} - (\text{HDL} + \text{Triglyceride}) \quad 5$$

ApoE genotyping

DNA extraction

The human genomic DNA was isolated from the human blood sample by using the Promega kit (Promega, WI, USA). Then the quality of the isolated DNA was determined by running each sample on ethidium bromide-stained 1.0% agarose gel.

PCR amplification of the ApoE gene

The primers used in the PCR forward Sequence 5' to 3' (TCCAAGGAGCTGCAGGCGGCGCA) reverse Sequence 5' to 3' (GCCCCGGCCTGGTACTACTGCCA)

Each PCR reaction mixture contained (~200 ng) of the prepared DNA template, 1× PCR buffer, 3.0 mM MgCl₂, 2.0 mM of each of the primers, and 200 mM of each deoxynucleotide triphosphate (dNTP); 1.25 U was the final concentration of Taq DNA polymerase (Promega) and DMSO (5%) in a thin-walled microfuge tube. The microfuge tubes were then placed in a thermal cycler and PCR amplification was done according to the following steps:

Step 1. Denaturation for 3 min at 95°C

Step 2. 40 cycles of

2.1. Melting for 60 s at 95°C

2.2. Annealing for 60 s at 58°C

2.3. Extension for 90 s at 72°C

Step 3. Final elongation for 10 min at 72°C.

Upon the completion of PCR, the products were analyzed by electrophoresis on 2% ethidium bromide-stained agarose gel; then the 218-bp amplicons were stored at 4°C until analysis and digestion.

Restriction fragment length polymorphism analysis

Genotyping for *ApoE* was performed by digesting the PCR product with restriction enzymes *AflIII* (5.000 U/ml) and *HaeII* (20.000 U/ml), 10× buffer, and 0.2 µl BSA. The contents were incubated for 24 h at 37°C. The digests were resolved on ethidium bromide-stained agarose gel and the results were documented by photography and separation of the resulting DNA fragments on agarose gel [Figure 1].

RESULTS

PCR results

The amplicon (PCR product) generated from the *ApoE* gene should yield a 218-bp-long double-stranded DNA fragment. A negative control (with water instead of the DNA template) was included in each reaction. The size of the amplicon was estimated by comparing it with a DNA molecular size marker (100 bp ladder DNA) run on the same gel.

Amplification product

A representative photograph of the *ApoE* PCR amplification product is illustrated in Figure 2. Lane 1 in the figure shows the 100 bp ladder, lane 4 contains a negative control, and the other lanes show the 218 bp *ApoE* amplicon.

RFLP result

Figure 3 illustrates a representative *ApoE* genotyping by *AflIII* (A) and *HaeII* (H) digestion of the 218 bp amplified fragment.

Genotype frequencies

The *ApoE* allele frequencies in the control subjects were 5.15% for the *E2* allele, 87.5% for the *E3* allele, and 7.3% for the *E4* allele. The frequencies in the CHD group were 3.62% for the *E2* allele, 82.6% for the *E3* allele, and 13.77% for the *E4* allele [Table 1].

Distribution of subjects according to *ApoE* genotypes

The distribution of the subjects according to *ApoE* genotypes was as follows: 70.1% *E3/E3*, 21.2% *E3/E4*,

and 8.8% *E3/E2*

Relationship between CHD and *ApoE* gene polymorphism

The results showed that there was no statistically significant

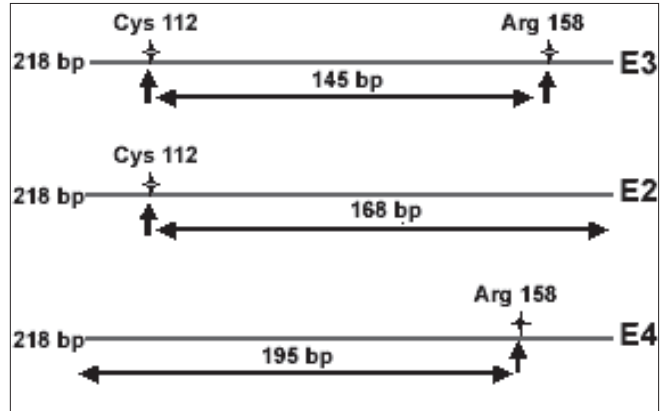


Figure 1: A schematic diagram illustrating *ApoE* RFLP analysis

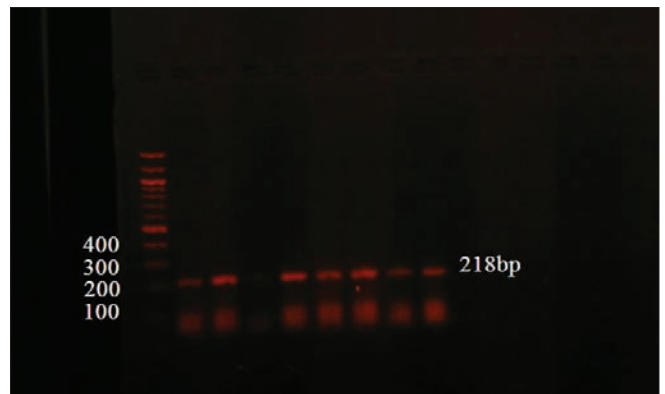


Figure 2: A photograph of the *ApoE* amplification product. Lane 1 shows the 100 bp ladder, lane 4 negative control, and the rest of lanes show the 218 bp *ApoE* PCR product

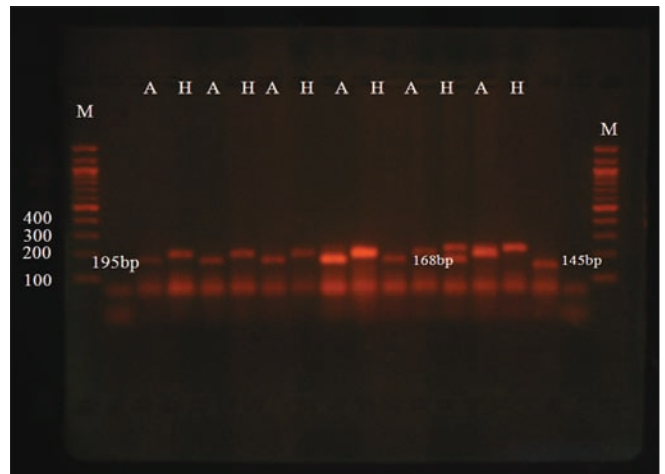


Figure 3: A representative photograph of *AflIII* (A) and *HaeII* (H) digestion of the *ApoE* 218 bp amplified fragment. M indicates the 100 bp size marker. The digests were run on ethidium bromide-stained 3% agarose gel; *E3* = 145 bp, *E2* = 168 bp, *E4* = 195 bp

Table 1: Frequency of *ApoE* alleles among the case and the control groups

Alleles	CHD group		Control group	
	n	Percentage	n	Percentage
E3	114	82.60	119	87.5
E2	5	3.62	7	5.15
E4	19	13.77	10	7.35
Total	138	100	136	100

relation between CHD and *ApoE* gene polymorphism ($\chi^2 = 3.4$, P -value = 0.17), where the presence of the *E3/E3* genotype was the most frequent among cases and controls (65.2% and 75%, respectively) as shown in Table 2.

Relationship between the *ApoE* genotype and gender

The results showed that there was no statistically significant relation between the *ApoE* genotype and gender ($\chi^2 = 3.39$, P -value = 0.18; Table 3).

Relationship between CHD and lipid profile

There was no statistically significant difference between the means of cholesterol and TG levels between the CHD and the control groups ($t = 1.3$, P -value = 0.19 and $t = 1.6$, P -value = 0.1, respectively). On the other hand, there were significant differences between the mean of LDL and HDL levels between cases and controls (P -value = 0.02 and $P < 0.05$, respectively).

Relationship between the lipid profile and *ApoE* genotypes

The relationship between the lipid profile and *ApoE* is provided in Table 4.

As shown in the table, there was no significant difference between the mean of cholesterol, TGs, and HDL regardless of the *ApoE* genotype. On the other hand, there was a

significant difference between the *ApoE* genotype and the mean of LDL.

CHD and age

The results of the study showed that there was a statistically significant difference between the means of age of cases and controls (P -value < 0.05) where the mean age of cases was 57.65 years, while that of the controls was 44.01.

CHD and smoking

The results of the study showed that there was a statistically significant difference between the smokers and nonsmokers in cases and controls ($\chi^2 = 4.56$, P -value = 0.049) as shown in Table 6.

DISCUSSION

According to the results obtained from PCR–RFLP, the *ApoE* allele frequencies in the control group were 0.0515 for the *E2* allele, 0.875 for the *E3* allele, and 0.073 for the *E4* allele. This distribution is in agreement with the distribution in the control group reported in a Saudi study: 0.888 for *E3*, 0.050 for *E2*, and 0.062 for *E4*.^[12] The frequencies of the *ApoE* alleles in the CHD subjects were 0.0362 for the *E2* allele, 0.826 for the *E3* allele, and 0.1377 for the *E4*.

Table 2: Distribution of the *ApoE* genotypes among the cases and the control groups

Genotype	Case		Control		Total		P-value
	N	Percentage	n	Percentage	n	Percentage	
E3/E3	45	65.2	51	75	96	70.1	0.17
E3/E2	5	7.2	7	10.3	12	8.8	
E3/E4	19	27.5	10	14.7	29	21.2	
Total	69	100	68	100	137	100	

Table 3: Relationship between the *ApoE* gene and gender

Genotype	Male (n)	Female (n)	Total	P-value
E3/E3	50	46	96	0.18
E3/E4	19	10	29	
E3/E2	9	3	12	
Total	78	59	137	

Table 4: Relationship between CHD and lipid profile

Lipid profiles	Mean			t-value	P-value
	CHD group	Control group			
Cholesterol (mg/dl)	193.6	183.49		1.3	0.19
Triglyceride (mg/dl)	133.86	116.94		1.6	0.1
LDL (mg/dl)	126	111.47		2.3	0.02
HDL (mg/dl)	39.70	49.94		4.11	0.000

Relation between *ApoE* gene polymorphism and CHD

According to our results, it was found that 27.5% (19 of 69) had *E3/E4* in the CHD group as compared to 14.7% (10/68) of the control group. When compared to other studies, this percentage is close to that reported in Saudi Arabia where 20.8% of the CHD subjects had *E3/E4* as compared to 12.5% from the control group.^[12] Kolovou has shown that 21.8% of the CHD and 16.3% of the control group had the *E3/E4* genotype.^[13] The present study however indicated that there is no statistically significant relation between CHD and *ApoE* genes ($\chi^2 = 3.4$, P -value = 0.17). These results are in agreement with different studies such as the Saudi and the Taiwanese studies which recorded that no statistical difference in genotype or allelic distribution was found between the patients of CHD and the control groups.^[12,13] These results can be explained by the fact that other genetic or environmental risk factors may interact with *ApoE4* in determining the CHD risk. We did not find a significant difference for the *ApoE* gene and genotype frequencies between patients and controls, suggesting that the *E4* allele is not a strong risk factor alone for CHD in Gaza Strip. For the *E4* allele, a significantly increased frequency in patients compared to healthy controls has been described in some but not all studies and the *E4* allele could be a risk factor in association with other genetic or environmental factors.^[14]

CVD risk is influenced by several well-established risk factors, such as body mass index (BMI), an indicator of overweight and obesity, blood lipids, diabetes, and blood pressure. These are intermediate phenotypes, correlated among themselves and having their own genetic and

environmental determinants, including diet, nutrition, hormones, smoking, alcohol intake, and physical activity.^[15]

Serum lipid profile and the *ApoE* genotypes

As we noted from our results, the mean of cholesterol level (218.17 mg/dl) was higher in subjects with the *E4* genotype as compared to those with *E3* (184.5 mg/dl), while the *E2* genotype had the lowest level (149.6 mg/dl). These results confirm the effect of variation in the *ApoE* genotype on lipoprotein levels. The *E4* allele has been shown to increase intestinal cholesterol absorption, to affect LDL synthesis in the liver, and to be associated with higher levels of total cholesterol and LDL-C and a higher prevalence of atherosclerosis.

CHD and lipid profile

Our study results showed that there is a significant increase in LDL in the case group as compared to the control group. No statistically significant differences were evident between the mean of cholesterol between the case group and the control group. It has been suggested that LDL-C indicates the cardiovascular risk in individuals better than total cholesterol. This result is in agreement with other authors who reported that the most common lipid disorder was high TC and high LDL-C in CHD, which was much more prevalent than high TG. A high concentration of LDL must be considered the principal risk factor because in the absence of elevation of LDL-C, atherosclerosis develops slowly and usually remains subclinical. This is true even when the other independent risk factors are present.

CONCLUSION AND RECOMMENDATIONS

The present study focused on the detection of *ApoE* genotypes in Gaza Strip and the relationship between those genotypes and lipid profile in 69 cases of CHD from Nasser and AlShifa Hospitals as compared to 68 healthy subjects. The results of this study can be summarized as follows:

- In Gaza Strip, the *ApoE3/E3* genotype was the most common in the control and the CHD groups. *ApoE2/E3* and *ApoE4/E3* were the next most common

Table 5: Plasma lipid profile according to the *ApoE* genotype

Lipid (mg/dl)	Genotype			P-value
	<i>E3/E3</i>	<i>E3/E4</i>	<i>E3/E2</i>	
TC	184.52	218.17	149.67	0.8
TG	128.36	128.31	95.33	0.3
LDL	112.94	149.31	93.67	0.02
HDL	45.66	43.97	37.08	0.2

Table 6: Relationship between CHD and smokers

Subject	Smokers						P-value
	Smokers		Nonsmokers		Total		
	n	Percentage	n	Percentage	n	Percentage	
CHD	18	69.2	51	45.9	69	50.4	0.049
Control	8	30.8	60	54.1	68	49.6	
Total	26	100	111	100	137	100	

genotypes. Other genotypes such as *ApoE2/E2*, *ApoE4/E4*, or *ApoE2/E4* were not encountered in the examined subjects.

- The frequencies of *ApoE* alleles in the CHD subjects were as follows: 0.826 for *E3*, 0.137 for *E4*, and 0.0362 for *E2*. These frequencies are comparable to those found in the control group where we obtained the following results: 0.875 for *E3*, 0.073 for *E4*, and 0.0515 for *E2*.
- No statistically significant differences in *ApoE* genotypes were found between the patients and the control groups.
- The distribution of the *ApoE* genotypes in the Gaza Strip population was similar to that of other Asian populations.
- Our study indicated that there was no significant difference between the mean of cholesterol and TG levels of the CHD and control groups.
- There was a significant difference between the mean of LDL and HDL levels between the CHD and the control group.
- There was no significant difference between the mean of TGs and HDL between the different *ApoE* genotypes. However, there was a significant difference in the mean of LDL and *ApoE* genotypes.
- Smoking, hypertension, and advanced age are important risk factors for CHD.

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