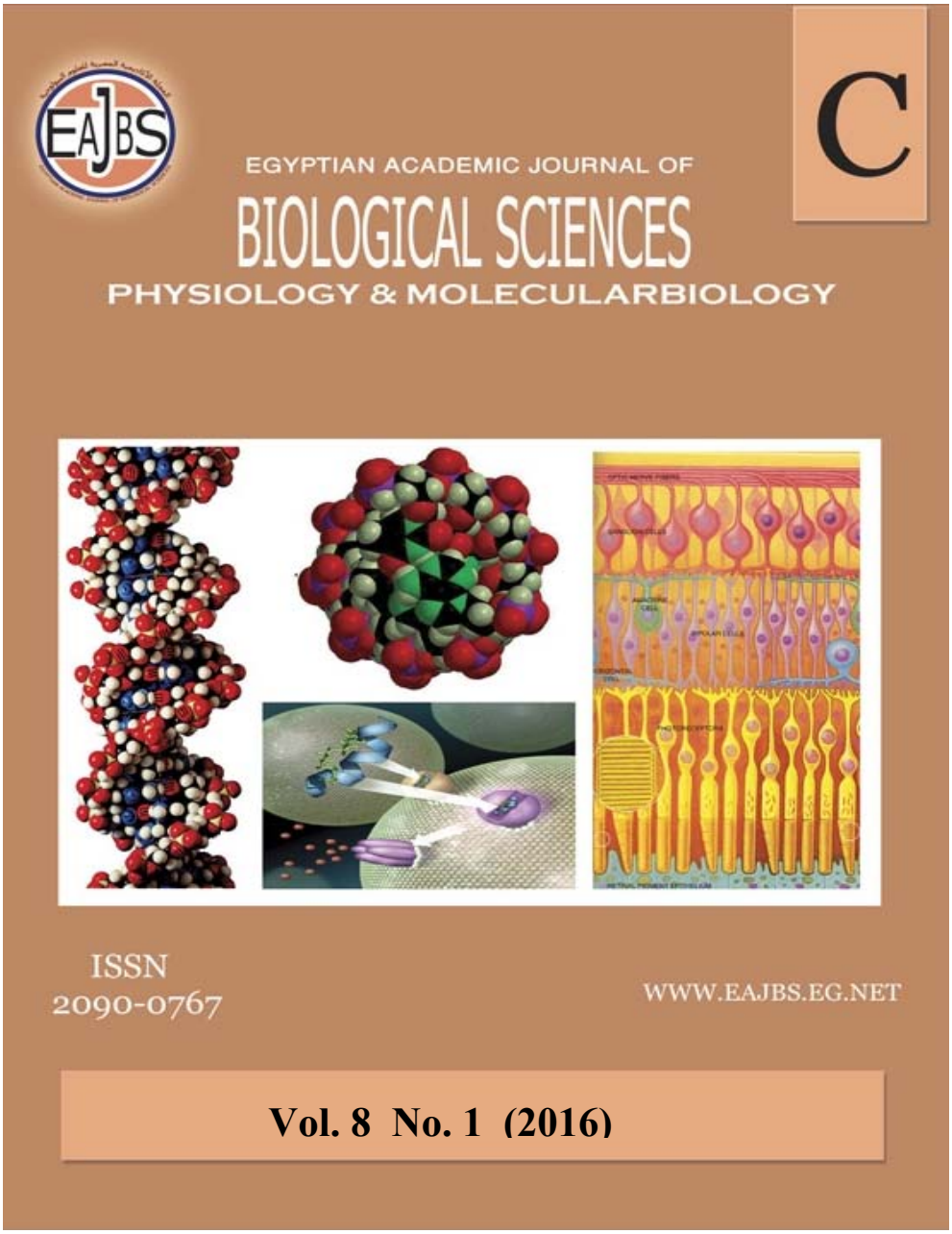

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Determination OF Apolipoprotein (E) Genotypes By PCR And Relation To Plasma Lipid In Coronary Heart Disease Patients

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ABSTRACT

Coronary artery disease (CAD) is the most prevalent type of cardiovascular disease (CVD) which, according to the American Heart Association accounts for 35% of all deaths in U.S.A. Apolipoprotein E (ApoE) is an apoprotein found in the chylomicrons and IDLs that binds to a specific receptor on liver cells and peripheral cells and it is essential for the normal catabolism of triglyceride-rich lipoprotein constituents .This study was carried out on 35 subjects divided into two groups. First : 25 CAD patients, the patients ages were ranged between 43 to 71 years. Second : 10 control were normal, the control ages were ranged between 29-54 years. All the patients and control were selected from Cairo Medical Center, cardiology department.

All subjects were submitted to the following:

1. Full history taking.
2. General clinical examination.
3. Coronary angiography (tarnsfemoral catheterization).
4. Laboratory investigation:

- Assessment of apolipoprotein E polymorphism by PCR and restriction enzymes.
- Measurement of serum cholesterol.
- Measurement of serum triacylglycerol.
- Measurement of serum low density lipoprotein cholesterol (LDLc).
- Measurement of serum high density lipoprotein cholesterol (HDLc).
- Measurement of serum cardiac enzymes and troponine.

INTRODUCTION

Coronary artery disease (CAD) also called coronary heart disease (CHD) is defined as a condition in which a plaque builds up inside the coronary arteries and this plaque narrows the arteries and reduces blood flow to the heart muscle (NHLPI, 2009).

Coronary artery disease (CAD) is the most prevalent type of cardiovascular disease (CVD) which, according to the American Heart Association accounts for 35% of all deaths in U.S.A (Corwin, 2008).

Apolipoprotein E (ApoE) is an apoprotein found in the chylomicrons and IDLs that binds to a specific receptor on liver cells and peripheral cells and it is essential for the normal catabolism of triglyceride-rich lipoprotein constituents (Entrez Gene, 2009).

The ApoE gene (ApoE) is mapped to chromosome 19 in a cluster with apolipoprotein C1 and apolipoprotein C2, ApoE consists of four exons and three introns, totaling 3597 base pairs (Hoek *et al*., 2008).

The genetic polymorphism of ApoE results from the existence of three common co-dominant alleles (E2, E3, and E4) that code for three apolipoprotein isoforms (E2, E3, and E4) resulting in six common genotypes (E2E2, E2E3, E2E4, E3E3, E3E4, and E4E4) (Tascilar *et al*., 2009).

These isoforms differ from each other only by single amino acid substitution at position 112 and 158, but have profound physiological consequences (Entrez Gene, 2009).

The most frequent isoform E3 contains a cystein at residue 112 and an arginine at residue 158. E2 and E4 differ from the E3 isoform in the fact that the E2 isoform contains a cystein at residue 158 and the E4 contains an arginine at residue 112 (Chaaba *et al*., 2008).

Plasma lipids are ApoE isoform dependent and it has been reported in association with atherosclerotic vascular disease (Graner *et al*., 2008; Tascilar *et al*., 2009).

Lipoproteins play a central role in development of atherosclerotic vascular disease, so, with their ability to affect lipid level, the ApoE polymorphism could be one of the factors influencing development of atherosclerosis (Chaaba *et al*., 2008).

The ApoE gene is one of the candidate genes for the risk of CHD, the E4 allele of the ApoE gene is maladaptive in the developed world leading to increased risk of CHD, consequently, the derived E3 allele is adaptive in the modern environment (Ding and Kullo, 2009).

The ApoE4 allele was found to be associated with high LDLc and CAD in type 2 diabetes (Chaaba *et al*., 2008).

AIM OF THE WORK

The aim of this work is to determine the relation between apolipoprotein E polymorphism with the lipid profile in atherosclerotic coronary artery diseases.

SUBJECTS and METHODS

Subjects:

This study was carried out on 35 subjects: 25 coronary artery disease patients (8 females and 17 males) who angiographically had stenosis in at least one of the major coronary vessels and 10 subjects as control group consisted of (6 females and 4 males) who underwent angiography procedures and were normal. All The patients and control were selected from Cairo Medical Center, Cardiology Department, The patient's ages were ranged between 43 to 71 years. The controls ages were ranged between 29 to 54 years. The excluding criteria for enrollment into the study included familial hypercholesterolemia, cancer, renal disease, and any other chronic illnesses. The genotypes were grouped in three groups: E2 group (E2E2, E2E3), E3 group (E3E3) and E4 group (E2E4, E3E4, E4E4) (Table 1).

Table 1: Subjects included 25 patients and 10 matched apparently healthy controls:

No. of patients	E2E2	E2E3	E2E4	E3E3	E3E4	E4E4
25	1	17	-	2	5	-
No. of controls	E2E2	E2E3	E2E4	E3E3	E3E4	E4E4
10	-	6	-	2	2	-

Methods:

All subjects were submitted to the following:

5. Full history taking.
6. General clinical examination.
7. Coronary angiography (tarnsfemoral catheterization).
8. Laboratory investigation:
   - Assessment of apolipoprotein E polymorphism by PCR and restriction enzymes.
   - Measurement of serum cholesterol.
   - Measurement of serum triacylglycerol.
   - Measurement of serum low density lipoprotein cholesterol (LDLc).
   - Measurement of serum high density lipoprotein cholesterol (HDLc).

Blood sampling:

10 ml of venous blood were withdrawn from the cubital vein of every subject (the patients are fasting for 12 hours). 5ml was transferred slowly into vacunated EDTA tube for isolation of White Blood Cells. The remaining 5ml was transferred slowly into a plain tube for determination of serum lipid profile.

Detection of Apolipoprotein (E) Genotypes by PCR:

Materials used for Molecular studies:

Reagants used for DNA Extraction:

2X Sucrose triton (PH=7.6)
Nuclei Lysis buffer (NaCl + Na_2 EDTA) pH=8.2
20% SDS (Sodium dodecyle Sulfate)
20% Proteinase K Solution
Saturated NaCl
Absolute ethanol (95%)
70% ethanol
Double distilled water.

Apparatus used for Molecular studies:

Cooling centrifuge
Thermocycler (Biometra, Germany).
Gel electrophoresis apparatus
MD25K UV transilluminator

Reagents used for PCR and RFLP:

Primers for Apo E gene (Eurofins MWG GmbH, Germany):
Forward: 5′-TCCAAGGAGCTGCAGGCGGCGCA
Reverse: 5′-GCCCCGGCCTGGTACACTGCCA
In order to yield a 218-bp DNA fragment to be detected on a 2% Agarose gel.
DreamTaq™ DNA polymerase (5U/µl) (Thermo Fisher Scientific Co., USA)
10x DreamTaq™ buffer (Thermo Fisher Scientific Co., USA)
Q-Solution, 5X (Qiagen, Germany)
dNTP mix (dATP, dCTP, dGTP and dTTP, 2 mM each)
Nuclease free water for reconstitution of lypholized primers amd preparation of working primer solutions and dNTPs solution. Restriction Enzymes: *Afl*III and *Hae* II (2,000 units (20,000U/mL) (New England BioLabs, info@neb.com, www.neb.com)
Agarose,
Ethidium bromide (10 mg/ml),
Loading dye
DNA ladder (50bp and 100bp)

Determination of CAD severity

1. Cardiac enzymes:

- The patients with high range CK, CKMB, LDH.
- Cardiac enzymes are performed on chemistry instrument, (Beckman COULTER AU480).

2. Troponine test :

- The patients with high troponine I.
- Troponine I is measured by hormonal instruments (ARCHITECT PLUS ARC 1000).

3. Coronary Angiography and Cardiac Catheterization

Coronary angiography was the first available in vivo assessment of the coronary arteries. In this technique, an iodinated contrast agent is injected through a catheter placed at the ostium of the coronaries. The contrast agent is then visualized through radiographic

fluoroscopic examination of the heart (Chou, 2015).

RESULTS

Coronary artery disease (CAD) is the leading cause of death and premature disability. It is a complex disorder resulting from many risk factors and individuals with genetic predisposition to atherosclerosis have substantial risk for developing CAD, especially at early ages. While it is difficult to explore the relation between local vessel wall function and CAD severity, measuring DNA variants such as ApoE polymorphisms may provide a way to assess this link because of its known effect on endothelial cell proliferation (Demet *et al.*, 2010).

As shown in Table 2, statistically significant difference between groups as regard age was recorded, while gender had non-significant effect.

Table (3) shows statistically significant difference between groups as regard DM, the rest have insignificant.

A statistically significant difference between groups as regard lipid profile, using t-test was noticed (Table 4).

Table (5) shows statistically significant difference between patients and control as regard ApoEgenotyping, using Chi-square test, with p-value >0.05 NS.

Table 2: Sociodemographic criteria of CAD patients and control groups.

Demographic Data	**CAD patients (N=25)**		**Control (N=10)**		**t-test**	**p-value**
Age (years)						
Range	43	71	29	54	52.266	**<0.001**
Mean±SD	55.8	7.2	41.4	8.3		
	No.	**%**	**No.**	**%**	**x2**	**p-value**
Gender						
Male	17	68.0%	4	40.0%	1.312	0.215
Female	8	32.0%	6	60.0%		

Table 3: Clinical parameters in CAD patients and control groups.

	CAD patients (N=25)		**Control (N=10)**		**x2**	**p-value**
Smoking						
Positive	10	40.0%	2	20.0%	0.536	0.464
Negative	15	60.0%	8	80.0%		
Hypertension						
Positive	20	80.0%	5	50.0%	1.851	0.174
Negative	5	20.0%	5	50.0%		
DM						
Positive	14	56.0%	0	0.0%	7.146	**0.007**
Negative	11	44.0%	10	100.0%		

Table 4: Statistical comparison between CAD patients and control groups as regards to lipid profile.

	CAD patients (N=25)		**Control (N=10)**		**t-test**	**p-value**
Total cholesterol (TC) mg/dl	258.08	110.74	165.60	18.85	3.698	**<0.001**
Triglyceride (TG) mg/dl	219.56	108.97	104.70	23.70	4.651	**<0.001**
HDLc (mg/dl)	50.38	15.89	46.69	6.71	2.000	**0.032**
LDLc (mg/dl)	163.79	106.70	97.97	19.52	2.729	**0.008**

Table 5: ApoE genotypes and alleles among CAD patients and control groups.

	CAD patients (N=25)		Control (N=10)		x2	p-value
ApoE genotyping						
E3/E3	2	8.0%	2	20.0%	0.176	0.674
E2/E3	17	68.0%	6	60.0%	0.003	0.955
E3/E4	5	20.0%	2	20.0%	0.219	0.640
E4/E4	0	0.0%	0	0.0%	0.000	1.000
E2/E4	0	0.0%	0	0.0%	0.000	1.000
E2/E2	1	4.0%	0	0.0%	0.232	0.630
ApoE genotyping	**(N=50)**		**(N=20)**			
E2	19	38.0%	6	30.0%	0.126	0.723
E3	26	52.0%	12	60.0%	0.117	0.723
E4	5	10.0%	2	10.0%	0.194	0.659

DISCUSSION

Coronary arteries supply blood to the heart muscle. Like all other tissues in the body, the heart muscle needs oxygen-rich blood to function, and oxygen-depleted blood must be carried away. The coronary arteries run along the outside of the heart and have small branches that dive into the heart muscle to bring it blood (Mazur *et al.,* 2015).

Apolipoprotein E is a plasma glycoprotein and a member of the apo gene family, it is located at chromosome 19q13.2, and consists of four exons and three introns spanning 3.597 nucleotides, and produces a 299 amino acid polypeptide with a molecular mass of about 34 kDa (Demet *et al.,* 2010).

Apolipoprotein E has a storied history as a lipid transport protein (Hauser *et al.,* 2010). Apo E is a constituent of triglyceride-rich lipoproteins and high density lipoproteins. Its major function is to mediate the binding of lipoprotein particles to cell surface receptors (Grammer *et al.,* 2011).

Apo E serves as a ligand for the apo B, E receptor, and the LDL receptor related protein and thus it plays a prominent role in the transportation and redistribution in both the influx and efflux of cholesterol in the body (Fallah *et al.,* 2011)

The receptor binding properties of ApoE are strongly influenced by isoform specific amino acid differences as well as the lipidation state of the protein (Hauser *et al.,* 2010).

These isoforms differ in amino acid sequence at positions 112 and 158, Apo E3 contains cysteine at 112 and arginine at 158, Apo E2 has cysteine at both positions, and E4 has arginine at both sites (Demet *et al.,* 2010).

While there are rare variants, among the variants of this gene, alleles E2, E3, and E4 constitute the common polymorphism found in most populations in relation to cardiovascular disease (Demet *et al.,* 2010).

Individuals with different apoE genotypes have different susceptibilities to CAD and studies have shown that the apo E4 allele is a genetic marker for CAD and an independent risk factor that predicts the incidence of cardiovascular disease (Li *et al.,* 2010).

The present study was carried out to determine the relation of ApoE gene polymorphisms and lipid profile in CAD defined by coronary angiography and assesses the findings in relation to the severity of disease in Egyptian patients. The current study showed significant higher age, male gender, smoking, hypertension and diabetes in CAD patients as compared with control.

These results agree with Peixoto *et al.,* (2007); Dias *et al.,* (2009) and Fallah *et al.,* (2011) who reported a predominance of male gender and mean

age in CAD patients. Bhanushali and Das, (2010) showed a significant difference in smoking and hypertension between CAD patients and control.

Grammer *et al.,* (2011) reported that patients with CAD were significantly older than controls. Current or past smoking, diabetes mellitus, and hypertension were more prevalent in CAD compared to controls. The current study showed significant higher total cholesterol (TC), triglycerides (TG), LDLc while lower HDLc in CAD patients as compared with control.

These results agree with those reported by Kharrazi *et al.,* (2006) and Bahri *et al.,* (2008) and explained this dyslipidemia as a classic risk factor for CAD.

The present study showed that the distribution of ApoE genotypes in CAD patients are as follow: E3E3, E2E3, E3E4, E4E4, E2E4 and E2E2 are 8.0%, 68%, 20%, 0.0%, 0.0% and 4.0% while in control are 20.0%, 60.0%, 20.0%, 0.0%, 0.0% and 0.0%. The frequency of E2, E3 and E4 alleles in CAD patients are as follow: 38.0%, 52.0% and 10.0% while in controls are 30.0%, 60.0% and 10.0% respectively.

The current study showed that E3E4 genotype is significantly higher in CAD patients as compared with controls while there was no significant difference as regards other genotypes. The study also showed that E2E3 genotype increased risk of CAD (p-value 0.955). These results are in agreement with Kharrazi *et al.,* (2006) and Singh *et al*., (2008).

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ARABIC SUMMERY

تحديد الأنماط الجينية للبروتين حامل الدهون (هـ) بواسطة التفاعل المتسلسل بالبوليميريز وعلاقته بدهنيات الدم فى مرضى الشريان التاجى

سعيد السيد إبراهيم حسن [1] ، نجلاء خلوصي [1] ، رندا محمد طلعت [2]

1- قسم الوراثة الجزيئية المناعية ـ المركز القومى للبحوث .

1- قسم المشخصات الجزيئية - معهد بحوث الهندسة الوراثية والتكنولوجيا الحيوية - جامعة المنوفية

تعتبر أمراض الشرايين التاجية هي الأكثر شيوعاً في أمراض القلب ، وهي السبب في 35% من الوفيات في الولايات المتحدة الأمريكية حسب إحصائيات جمعية القلب الأمريكية وهي تنتج عن تراكم الجلطات في جدار الشريان التاجي الذي يمد عضلة القلب بالأوكسجين والتغذية . الأبوليبوبروتين (هـ) هو بروتين موجود في حاملات الدهون والدهون منخفضة الكثافة وهو يتحد مع مستقبلات على خلايا الكبد والخلايا الأخرى وهو ضروري لتكسير الليبوبروتين الغنية بالدهون الثلاثية .

قد أجريت هذه الدراسة على 35 من المرضى وقد تم تقسيمهم إلى مجموعتين :-

المجموعة الأولى : وتشمل 25 من مرضى تصلب الشريان التاجي وتتراوح أعمارهم بين 43-71 عام .

المجموعة الثانية : وتشمل 10 من الأصحاء تتراوح أعمارهم بين 29-54 عام .

كل المرضى تم إختيارهم من قسم القلب وحدة الرعاية المركزة بمستشفى القاهرة التخصصي وقد تم قياس التالي لكلاً من المرضى والمجموعة الضابطة .

1- أخذ التاريخ المرضي بالكامل .
2- إجراء الفحص الإكلينيكي الشامل .
3- رسم الشريان التاجي لمرضى القلب (القسطرة التاجية .
4- الفحوصات المعملية .

- تحديد تعدد أشكال الأبوليبوبروتين (هـ) جين باستخدام تفاعل البوليميراز التسلسلي وإنزيمات التحديد .
- قياس نسبة الكوليسترول في الدم .
- قياس نسبة الترايجلسرايد في الدم .
- قياس نسبة الدهون منخفضة الكثافة في الدم .
- قياس نسبة الدهون عالية الكثافة في الدم .
- قياس نسبة إنزيمات القلب والتروبونين .