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The Impact of Apo E Genetic Polymorphism on Carotid intimal-medial Thickness in Diabetic Elderly

KEYWORDS: Apolipoprotein E; Carotid intima-media thickness; Carotid plaques

Dr. Motassem Salah Amer
Professor of Geriatric Medicine and Internal Medicine, Faculty of medicine, Ain Shams University, Cairo, Egypt.

Dr. Laila Ahmad Abdurrahman
Professor of Radiology, Faculty of medicine, Ain Shams University, Cairo, Egypt.

Dr. Randa Reda Mabrouk
Professor of clinical pathology, Faculty of medicine, Ain Shams University, Cairo, Egypt.

Dr. Manar Mostafa Adel
Assistant professor of Geriatric medicine, Faculty of medicine, Ain Shams University, Cairo, Egypt.

Dr. Doaa Mohamed Abdel Aziz
Lecturer of clinical pathology, Faculty of medicine, Ain Shams University, Cairo, Egypt.

Dr. Sherine Mohamed Ibrahim Sharara
Lecturer of Radiology, Faculty of medicine, Ain Shams University, Cairo, Egypt.

Rasha Ahmed Mohammed Ali
Assistant lecturer of Geriatric Medicine and Gerontology, Faculty of medicine, Ain Shams University, Cairo, Egypt. (Corresponding author)

ABSTRACT ***Aim:*** *to investigate associations between ApoE polymorphisms and CIMT in elderly diabetics*

Methods: *This was a case- control study, ApoE genotyping was tested, total cholesterol, HDL-C, LDL-C, TG and bilateral carotid ultrasound was done in ninety elderly subjects who were divided into thirty elderly diabetic subjects with cardiovascular complications (group A), thirty elderly diabetic subjects without cardiovascular complications (group B) and thirty elderly subjects without DM (group C).*

Results: *The carotid plaques were significantly more frequent among those have E4 alleles in diabetic patients with cardiovascular complications while no significant association between different ApoE alleles and mean CIMT.*

Conclusions: *ApoE gene polymorphisms are significantly associated with increased risk of carotid plaque formation not intimal media thickness (IMT).*

INTRODUCTION

It is still unsettled whether ApoE4 genotype is associated [1] or not associated with carotid atherosclerotic plaques [2] and the relationship between ApoE and vascular disease has been the subject of a considerable amount of research. However, this relationship is far from clearly defined.

This deficiency appears to be due to a multitude of factors. Among these are differences in ethnicity, age (and possibly gender), diagnostic criteria, and environmental factors (eg, diet and smoking) that have contributed to the contradictory findings [3].

ApoE is a plasma glycoprotein of 34 kDa with 299-amino acids [4] human ApoE gene is located on the long arm of chromosome 19 (19q13.2) and consists of four exons and three introns spanning 3,597 nucleotides [5]. Genetic variations in the ApoE gene exon 4 lead to three common gene variants (alleles), E2, E3, and E4, which code for three major isoforms (E2, E3, and E4), resulting in six common genotypes : three homozygous genotypes E2/E2, E3/E3, E4/E4 and three heterozygous genotypes: E2/E3, E2/E4, E3/E4.

Carotid atherosclerosis was considered a surrogate marker for coronary atherosclerosis and thus, the measurement of IMT by ultrasound provides a quantitative basis for the extent of atherosclerosis [6], Although Apo E polymorphism is considered as a genetic risk factor for coronary heart disease [7,8] studies examining its association with different CIMT values have not been conclusive [9,10].

As a part of our ongoing study to evaluate the effect of ApoE polymorphisms on CIMT and abdominal aortic diameter in diabetic elderly Egyptian patients, the data represented in the current research article concerned with the effect of APOE polymorphisms on CIMT, while the other part which investigate the relation between APOE polymorphisms and abdominal aortic diameter will be discussed in another research article.

SUBJECTS and METHODS

Study population and design

This study was a case - control study involved 90 elderly participants were divided into 3 groups: **Group A:** Thirty elderly patients sixty years old and above, diagnosed to have type 2 diabetes mellitus with cardiovascular complications. **Group B**: Thirty elderly patients sixty years old and above diagnosed to have type 2 diabetes mellitus without cardiovascular complications. **Group C (control group):** Thirty elderly subjects sixty years old and above, without DM.

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All enrolled participants provided written informed consent prior to inclusion into the study

Laboratory Measurements
Venous blood (6 ml) was withdrawn aseptically into a sterile disposable syringe from each patient and control where, 5 ml were placed in EDTA tube for APOE genotyping and 2 ml were collected plain vacutainers to be clotted and centrifuged for measurement of total cholesterol, HDL-C, LDL-C ,TG and fasting glucose. On Synchron CX-9 autoanalyzer (Beckman Instruments Inc.; Scientific Instruments Division, Fullerton, CA 92634, 3100, USA). The laboratory work was conducted at Clinical Pathology Department, Ain shams university .

Genotyping of Apo E was performed using polymerase chain reaction and restriction fragment length polymorphism. Genotyping of three common alleles (E2, E3, and E4) of the Apo E gene was performed. Genomic DNA was extracted from human blood cells using QIAamp DNA Mini Kit (Qiagen, USA) the extracted DNA was stored at -20°C until processed. The primers used were 5'-ACAGAATTCGCCCCGGCCTGGTACAC-3' and 5'-TAAGCTTGGCACGGCTGTCCAAGGA-3' as described by Emi et al. (1988) (11) and prepared by Promega (Madison,USA). PCR product were carried out in volume of 50 µl containing 5 µl genomic DNA, 25 µl of the ready to use master mix supplied by Qiagen, 2.5µl (25 pmol) of forward primer, 2.5 µl (25 pmol) of reverse primer and 15 µl of deionized water. The PCR conditions were an initial denaturation at 95°C for 3 minutes and 40 cycles consisting of denaturation at 95°C for 15 seconds, annealing at 62°C for 15 seconds, extension at 72°C for 45 seconds before a final extension at 72°C for 4 minutes. Restriction digestion of the amplified product was performed using 0.5 µl (10 units/ µl) Hha1 restriction enzyme supplied by promega. Site of restriction: (5´GCG¯C 3´) (3´ C¯GCG 5´). The enzyme was added to 5 µl of PCR amplified product, 12.3 µl deionized water, 2 µl of buffer (10 x of 60mM tris-Hcl) and 0.2of µl acetylated bovine serum albumin (BSA)(10ug/ µl), followed by gentle mix. Then the tubes were placed on a heat block for 3 hours at 37°C. The enzyme was inactivated at 65°C for 15 minutes. As experimental control: no-enzyme "mock" digest was used

Amplified product of DNA samples and restriction fragments were run on 8% polyacrylamide gel and stained with ethidium bromide. DNA molecular weight marker was also run to identify the site of bands (20 bp DNA ladder supplied by (Promega, USA). Digestion of the amplified product (270 bp) by the restriction enzyme Hha1 resulted in the following different individual genotypes that were categorized based on the following band length criteria:E2/2=91 ,83,38;E3/3:91,48,38,35;E4/4:72,48,38,35;E2/3:91,83,48,38 ,35;E2/4:91,83,72,48,38,35;E3/4:91,72,48,38,35. (figure 1)

Carotid Ultrasound
Both left and right CCAs were depicted. With the aid of an Alpinion-Korae- E-CUBE-9 ultrasound unit equipped with a 7.5-MHz transducer. The sonographers measured CIMT of the posterior wall of the right and left common carotid arteries, 1.5 cm proximal to the bifurcation. The mean value of the right and left common carotid IMT was used for analysis.

RESULTS
The study population characteristics are described in Table 1. There is no significant difference between study groups regarding age and sex, the mean HDL level is significantly higher in control group than other groups. As regard the frequency of ApoE allele among study groups, ApoE2 allele is significantly more frequent in-group B, C compared to group A while ApoE3 allele is significantly more frequent in group A and group C compared to group B. In addition, ApoE4 allele is more frequent in group A, B comparing to group C with no statistical significance. Both CIMT and presence of carotid plaques are significantly higher in group A in comparison to control group. After pooling the overall subjects, the results showed higher mean LDL among ApoE4 carrier subjects compared to E3 and E2 and the difference is significant statistically (P=0.02). The results revealed that there was no significant association was observed between ApoE alleles and mean CIMT in the whole study population while the presence of carotid plaques are significantly more frequent among those have E4 allele compared to those have E3 allele in group A.

DISCUSSION
Most studies of the ApoE polymorphism have shown carotid IMT to be lower in carriers of the E2 allele and higher in carriers of the E4 allele, compared with E3/E3 homozygotes [12-14].These associations have not been totally consistent however, with de Andrade et al. and Hanon et al. showing higher CIMT in E2 carriers [15,16] Zannad et al showing higher CIMT in E3/E3 homozygotes compared with E2 or E4 carriers but on the right side only [17] and several studies [18-26], showing no association. The results are also varied depending on the country, gender, and age subgroups. CIMT values increased in the North American and Australian Apo E4/ E4 men in ARIC and CUDAS studies [14, 27]. However, no such differences were found among the genotype subgroups in Rotterdam study conducted on Central European elderly participants [28].

The current study results were consistent with those found in a Calmarza et al., (2015) study who assessed the CIMT and ApoE genotypes in 171 individuals older than 40 years. Their results showed no significant difference among E3, E4 and E2 groups regarding CIMT, however ApoE4 allele was associated with higher levels of LDL-C but not with early atherosclerosis as assessed by CIMT [26]. Similarly, the studies carried out by Beilby et al., (2003) who perform a cross-sectional study of 1111 randomly selected community subjects. The multivariate modeling had suggested that, in men there was a log-additive trend in the risk of carotid plaque with increasing numbers of E4 alleles while, In women the E2 allele was associated with a lower risk for plaque development relative to the E3 allele. Also their results showed that ApoE genotypes were not associated with CIMT in the whole study population [14].

The controversy of results of relation between ApoE polymorphism and CIMT, can be explained as there are other environmental and genetic factors are involved in the pathogenesis of carotid atherosclerosis in diabetic patients.

As the genetic and environmental factors interact to influence a person's biological makeup, including the predisposition to different diseases. There are several biological mechanisms that affect expression of genes ("switched" on or off) can be affected positively and negatively by environmental factors, such as exercise, diet, chemicals, or smoking, alcohol, to

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which an individual may be exposed.

CONCLUSIONS

There was an association between ApoE4 and dyslipidemia in the form of higher mean LDL among ApoE4 carrier subjects. The mean carotid intimal thickness is considered as an early marker of atherosclerotic disease, it was the highest in diabetic patients with cardiovascular complications compared to diabetic patient without cardiovascular complications and also healthy control .Carotid plaques were significantly higher in diabetic patients with cardiovascular complications compared to controls. Moreover, Carotid plaques were significantly more frequent among those have E4 alleles while there was no significant association was between ApoE genotypes and mean CIMT.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

Table (1): Comparison between study groups regarding age (years) and sex, hypertension, laboratory findings and CIMT

Variable	Group A (N=30)	Group B (N=30)	Control (N=30)	$P_{A/B}$	$P_{A/C}$	$P_{B/C}$
Age **Mean±SD** **Range**	65.4±6.6 60.0–85.0	65.3±6.6 60.0–86.0	67.2±6.4 60.0–82.0	0.969^	0.285^	0.268^
Sex (n, %) **Female** **Male**	 21 (70.0%) 9 (30.0%)	 21 (70.0%) 9 (30.0%)	 17 (56.7%) 13 (43.3%)	1.000#	0.284#	0.284#
Cholesterol **Mean±SD** **Range**	 161.7±56.9 62.0–300	 191.0±62.3 93.0–350	 190.9±55.1 95.0–299	0.062	0.048*	0.991
TG (mg/dL) **Mean±SD** **Range**	 135.8±70.8 35.0–335	 151.6±76.6 68.0–400	 146.4±76.2 38.0–391	0.411	0.580	0.793
LDL (mg/dL) **Mean±SD** **Range**	 92.0±36.6 21.0–196	 112.0±47.8 35.0–219	 108.2±43.9 47.0–230	0.075	0.128	0.750
HDL (mg/dL) **Mean±SD** **Range**	 33.6±13.1 8.0–71.0	 38.0±11.1 11.0–65.0	 46.8±14.3 16.0–73.0	0.165	^0.001*	0.009*
E2 allele	0 (0.0%)	16 (53.3%)	14 (46.7%)	^0.001*	^0.001*	0.606
E3 allele	18 (60.0%)	3 (10.0%)	7 (23.3%)	^0.001*	0.166	0.004*
E4 allele	12 (40.0%)	11 (36.7%)	9 (30.0%)	0.791	0.417	0.584
CIMT (mm) **Mean±SD** **Range**	 1.2±0.4 0.8–1.9	 1.1±0.2 0.8–1.7	 1.0±0.2 0.6–1.6	0.060	0.040*	0.755
Carotid plaques	14(46.7%)	11(36.7%)	5 (16.7%)	0.432	0.012*	0.080

^Independent t-test, #Chi square test, #Chi square test, &Fisher's Exact test

Table (2): Comparison between different APOE genotypes regarding Total cholesterol, Triglyceride, HDL and LDL among studied groups

	E2 allele N=30	E3 allele N=28	E4 allele N=32	F	P	
Total cholesterol	172.6±60.2	178.2±67.0	191.7±50.0	0.8	0.4	
Triglyceride	158.4±78.0	145.9±85.3	130.3±57.4	1.1	0.3	
HDL	40.3±14.1	35.4±13.8	42.1±13.3	1.8	0.1	
LDL cholesterol	97.4±45.1	93.8±34.3	**120.8±**44.7	3.7	0.02*	**E4 vs E2,E3**

Comparison of CIMT and presence of carotid plaques as regard APOE alleles within group A

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Variable	E3 (N=18)	E4 (N=12)	P
CIMT (mm)	1.1±0.3	1.3±0.4	^0.079
Carotid plaques	5 (27.8%)	9 (75.0%)	**#0.011***

^Independent t-test, #Chi square test,

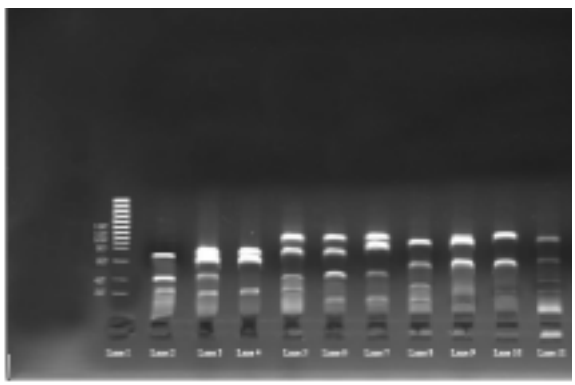

Figure (1): APO E genotypes by polyacrylamide gel electrophoresis: lane 1 shows ladder 20 bp, lane 5,9,10 shows E2/E2 genotype, lane 6,7,11 shows E3/E4 genotype, lane 2, 4,3,12 shows E4/E4 genotype and finally lane 8, 13 shows E3/E2 genotype.

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