Study of Apolipoprotein E Polymorphism as a Biomarker in Coronary Artery Diseases

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ABSTRACT----***This study aims to evaluate the role of apolipoprotein (apo) E gene mutation of all potentially atherogenic lipoproteins, as predictors of Coronary Heart Diseases (CHD) and its association with other biochemical changes such as; total cholesterol,TG, HDL-c, LDL-c, VLDL-c, in absence and presence of diabetes mellitus to reflect the presence of diabetes mellitus (DM). Results: This study was conducted on 45 individualscategorised into, Control group, Cardiac patients without DM (Group I) and Cardiac patients with DM (Group II). In our studied population, the mean levels of cholesterol, triglycerides and the ratio of LDL/HDL didn't show any significant increase in group I compared to control group; whereas the mean levels of LDL and HDL showed significant increase compared to control group. The mean level of serum cholesterol, triglycerides, LDL, HDL and ratio of LDL/HDL showed significant increase in group II compared to control group.***

The major common allele in the control group was E2, which has represented 53.3 %, E3 has represented 33%, while E4 has contributed 14 %, in addition, the major common allele in the group I was E3 that formed 50 % of group I sample, followed by E2 with 33% and tailed by E4 with a 16 % contribution. In contrast to group I, group II major common allele was E3, which has represented 53% of samples examined, whereas E2 and E4 have represented 23.3 % for each.

Conclusion: ApoE expression is considered a good criteria to recognize patients suffering from coronary heart disease. Carriers of E2, E3, and E4 differ in their binding affinity to cholesterol. Carriers of the E3, E4 alleles are slower to clear dietary fat from the blood, whereas carriers E2 has high affinity for cholesterol and shows a protective effect against cardiovascular disease. This study clearly concludes that people who have alleles E2/E2, E2/E4 may not suffer from coronary heart disease.

Keywords--- Coronary artery diseases, apolipoprotein E, Atherosclerosis, triacylglycerol, HDL-c, LDL-c, Cholesterol

1. INTRODUCTION

Cardiovascular diseases (CVD) are one of the leading causes of mortality both in men and women. Unfortunately however, the risk of heart diseases in women is underestimated because of the perception that women are 'protected' against ischemic heart disease. What is not fully understood is that women during the fertile age have a lower risk of cardiac events, but this protection fades after menopause, thus leaving women with untreated risk factors vulnerable to develop myocardial infarction, heart failure, and sudden cardiac death [20]. Although cardiovascular disease is still the leading cause of death in both women and men in the United States, the mortality rates due to cardiovascular disease declined and then leveled off from 1980 to 2009. From 2000 to 2009, the crude mortality rate for ischemic heart disease-related deaths in women declined from 177.6 to 113.3 per 100,000 population. Whereas in men, the rate declined from 188.7 to 138.7 deaths per 100,000 population [4].

Cholesterol is a combination of a lipid (fat), and a sterol. Cholesterol is an important substance needed for the cellular functioning and synthesis of hormones. It has to be carried by transporters. Transporters, called lipoproteins (LPs), are able to mix with blood. Lipoproteins are made of lipid (fat) and protein. There are 4 main classes of lipoproteins [1]. Cholesterol has an essential physiological role in humans, but an excess of cholesterol leads to cardiovascular diseases. Its metabolism is regulated by various enzymes, receptors, and transfer proteins; present in the small intestine, liver, peripheral cells, and plasma. Cholesterol is secreted from the liver into plasma as very low density lipoprotein (VLDL), which gets converted to low density lipoprotein (LDL). LDL cholesterol is a risk factor for coronary heart disease, hence its called a bad cholesterol. High density lipoprotein (HDL) helps to carry cholesterol mobilized from peripheral cells and destined for disposal by the liver, a process termed reverse cholesterol transport. This role and epidemiological evidence that the concentration of HDL cholesterol in plasma correlates inversely with the risk of coronary heart disease have earned it the reputation of being good cholesterol [2,5].

Lipoproteins, VLDL or IDL or LDL, and HDL are responsible for carrying cholesterol and other fats through the bloodstream as little packages and are essential for the normal breakdown of these molecules. In particular, apolipoprotein E is a major component of specific lipoproteins called very low-density lipoproteins (VLDL). A major function of VLDL is to remove excess cholesterol from the blood and carry it to the liver for processing, LDL responsible for transporting cholesterol to peripheral cells (bad cholesterol), HDL is responsible for the cholesterol uptake from the cells- the process termed *Reverse Cholesterol Transport* (good cholesterol), Accumulation of cholesterol within the arterial wall is distinguished characteristic of atherosclerosis. So lipoproteins have relation with atherosclerosis and strongly associated with coronary heart disease (CHD) [13].

Apolipoprotein E is a member of the apolipoprotein gene family. Apo E gene located on chromosome 19q13.2. It consists of four exons and three introns spanning 3,597 nucleotides and produces a 299 amino acid polypeptide. It is synthesized primarily in the liver, but other organs and tissues also synthesize apo E including brain, spleen, kidneys, gonads, adrenals, and macrophages. The structural gene is polymorphic with three common alleles, e2, e3 and e4 producing three isoforms of the protein E2, E3 and E4. These isoforms differ in amino acid sequence at position 112 and 158 .Apo E3 consists cysteine at 112 and arginine in 158. Apo E2 has cysteine at both position, and E4 has arginine at both sites .Polymorphism with its three alleles has been studied in relation to cardiovascular disease [12]. The plasma lipoproteins are complex macromolecular structures which play an essential role in fat transport and in the energy and membrane metabolism of higher organized organisms; their protein moieties, the so-called apolipoproteins [22].

The protein is involved in the efficient hepatic uptake of lipoprotein particles, stimulation of cholesterol efflux from macrophage foam cells in the atherosclerotic lesion, and the regulation of immune and inflammatory responses [14]. Apolipoprotein E plays a key role in the metabolism of cholesterol and triglycerides [6,21]. Apo E has different roles in some diseases: Abnormalities of blood lipids and cardiovascular disease, Immunological response, Alzheimer disease, Multiple sclerosis, Neurological disorder.

The efficiency of cholesterol transport from the mother to the embryo is affected by maternal APOE genotype, and that APOE plays a role in modulation of embryonic development and malformations [9]. Apo E is also important for myelin integrity. It is found in the brain plaques and neurofibrillary tangles that are characteristic of Alzheimer's disease (AD), but is present in normal brains as well [26,28].
There was a significant association between APOE4 genotype and decline in performance tests in women between 70 and 80 years, but not in men [24]. Previous study found that E4 carriers had a 6-fold increase in the relative risk of verbal learning deficits compared to non carriers. The effect was specific and not observed in other cognitive domains[16]. The E2 allele, but not the E4 allele, was positively associated with sporadic Parkinson disease (PD) [37]. The E4 allele increases disease risk for PD and is associated with earlier age at disease onset independent of cognitive impairment; however, the effect was not as strong as that observed in AD [18].

2. MATERIALS AND METHODS

This study was conducted on 45 individuals recruited from the Department of Cardiology, Al-Hussein University Hospital, Cairo, Egypt. Written consent was obtained from the participants after they were given an explanation of the study details. The individuals were categorized into, non-patients (Control group), Cardiac patients without DM (Group I) and Cardiac patients with DM (Group II). Approximately 4 ml of blood was drawn from the vein of each participant after informed consent and divided into two tubes (EDTA and heparin tubes). EDTA part was used for assay the plasma glucose, cholesterol, triglyceride, HDL and LDL by colorimetric assay (Human Gesellschaft for Biomedica and Diagnostics mph, Max-Planck-Ring 21, 65205 Wiesbaden, Germany). The heparinized tubes were used for Genomic DNA extraction [8].
DNA analysis: Genomic DNA was extracted from the whole blood using QIAamp Blood Kit (QIA) (Qiagen, Germany). APOE region was amplified by polymerase chain reaction (PCR) in a 50 µl reaction mixture consisting of 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM magnesium chloride, 0.1% Tween-20, 10% dimethyl sulfoxide, 0.2 mM of each dNTP, 5 pmol of each APOE primer.100 ng of genomic DNA, and 2.5 units of Taq polymerase. PCR was carried out in a the heating block in the DNA thermal cycler (Omnigene, Hybaid, 01747) with 35 cycles of denaturation for 1 min at 94ºC, annealing for 1 min at 65ºC, and extension for 1 min at 72ºC. To detect the APOE gene the PCR product was digested using *Hha*I endonuclease and separated on 4% agarose gel electrophoresis and Ultra - Violet Light Trans illumination was used to detect the APOE 244 bp fragment. For digestion, 10 µl of PCR product were added to 16-17 µl of nuclease-free water, 2 µl of 10X recommended buffer for restriction enzyme and 1-2 µl (10-20 u) of restriction enzyme *Hha*I and mixed well then incubated at 37ºC for 2 hours [8].

3. RESULTS

The mean level of cholesterol, triglycerides and the ratio of LDL/HDL didn't show significant increase in group I compared with the control group; whereas the mean level of LDL and HDL show a significant increase compared with the control group.
On the other hand; the mean level of cholesterol, triglycerides, LDL, HDL and ratio of LDL/HDL showed a significanct increase in group II compared with the control group. [Figures (1-A) and (1-B)].

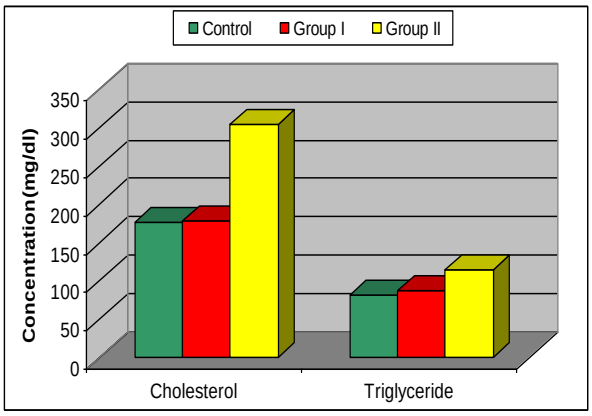

Figure (1-A): Concentration of total Cholesterol and Triacylglycerols among Control and Patient Groups

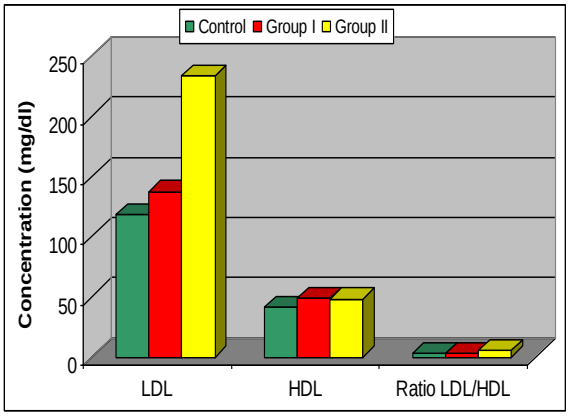

Figure (1-B): Concentration of HDL-c, LDL-c and Ratio LDL/HDL among Control and Patient Groups

The expression of APOE gene was evaluated in the control, group I, and group II uses the specific APOE primers, through amplification of the expected 244 bp (for wild type) single product by PCR from genomic DNA of the patients. The percentage of homozygous (Wild type) was expressed in subjects of controls 60% (9/15) while, in group I, and II APOE gene was expressed by 33.33% (5/15), and (33.33%) (5/15) respectively, and the percentage of heterozygous was expressed in subjects of controls as 40% (6/15) while in group I and II, APOE gene (Heterozygous type) was expressed by 66.66% (10/15), and 66.66% (10/15) **[Figure (2-A), (2-B)]**.

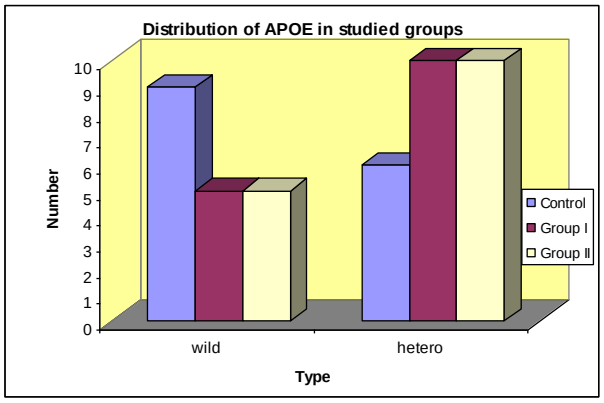

Figure (2-A): Distribution of APOE Gene Expression among Control and Patient Groups

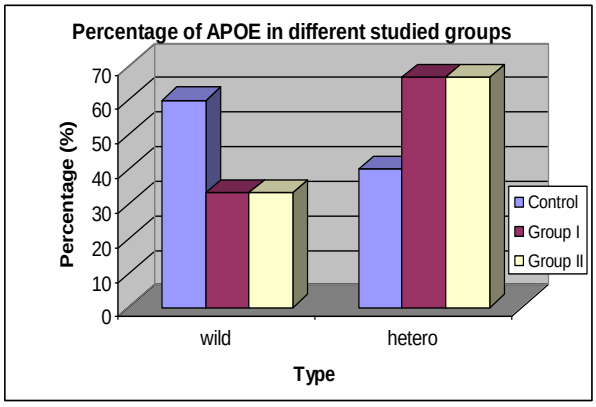

Figure (2-B): Percentage of APOE Gene Expression among Control and Patient Groups

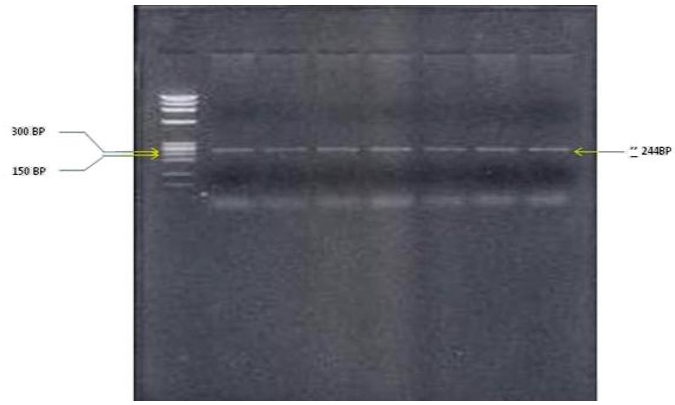

Figure (3): Agarose Gel Electrophoresis Analysis of PCR-Amplified Genomic DNA by using APOE primers.

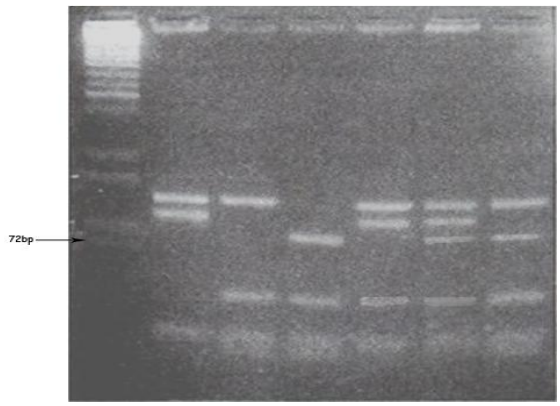

Figure (4): APOE genotyping: analysis of PCR amplified apo E, digested with the enzyme HhaI, on a 4 % agarose gel. Lane 1: represent PCR marker, Lanes (2-7) different genotypes

The Size of the Amplified Fragment is 244 bp.
Lane 1: PCR marker.
Lanes from 2 to 8 represent samples of PCR-Amplified Genomic DNA

Lane 1 : Ladder (Marker)
Lane 2: E2/E2…….5 bands (91,83,38,18 and16 bp)
Lane 3: E2/E3…….6 bands (91, 48,38,35,18 and 16 bp)
Lane 4: E3/E4…….7 bands (72, 48,38,35,19,18 and 16 bp)
Lane 5: E3/E3…….7 bands (91,83,48,38,35,18 and 16 bp)
Lane 6: E4/E4……. 9 bands (91,83,72,48,38,35,19,18 and 16 bp)
Lane 7: E2/E4…….8 bands (91,72, 48,38,35,19,18 and 16 bp)

Data depicted from this study show frequency of apoE alleles in all studied subjects, E2 represents 36.6%, E3 represents 45.53% and E4 comprises 17.73%.
It was clearly shown that the frequency of APOE alleles in the three different groups evaluated as follows; The major common allele in the control group was E2, which has represented 53.3 %, E3 has represented 33%, while E4 has contributed 14 %, in addition, the major common allele in the group I was E3 that formed 50 % of group I sample, followed by E2 with 33% and tailed by E4 with a 16% contribution. In contrast to group I, group II major common allele was E3 which has represented 53% of samples examined, while E2 and E4 both have represented 23.3 % for each [Figure (5-A), (5-B)].

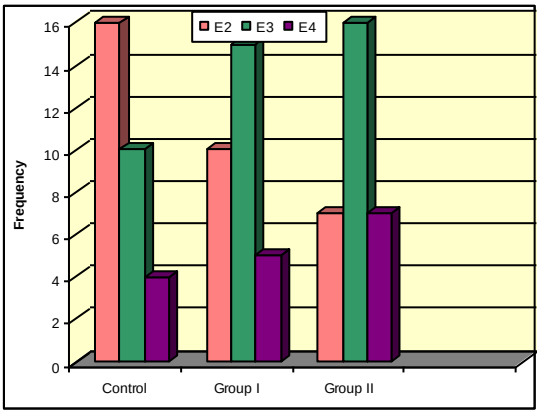

Figure (5-A): Frequency of Different Isoforms among Control and Patient Groups

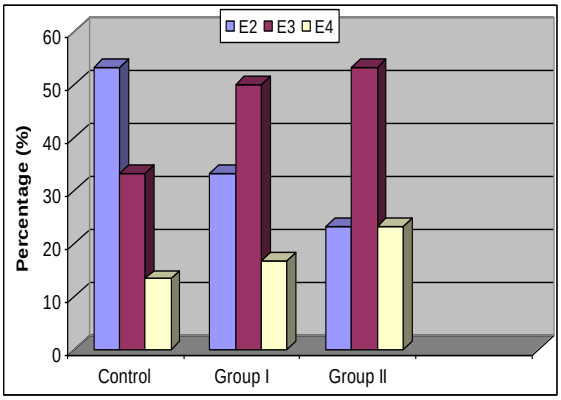

Figure (5-B): Percentage of Different Isoforms among Control and Patient Groups

From Table (3) and Figure (6-A), Figure (6-B) it was observed that:

As shown in figure (6) it was observed that: Control Group show the absence of E2/E4 and E4/E4 genotypes, while E2/E2 genotype represent 46.7 % (7/15), E2/E3 and E3/E3 alleles have the same frequency 13.3 % (2/15), whereas E3/E4 represents 26.7 % (4/15) but in In Group I: it was observed that E2/E3 was most common genotype that represents 46.7 % (7/15) followed by E3/E3 which represents 20 % (3/15), but the least common genotypes were E2/E2, E2/E4 and E4/E4 which represent 6.7 % (1/15) and in Group II: E2/E3 represents 46% followed by E3/E3 and E3/E4 which represent 20% then E4/E4 13 % (2/15). While E2/E2, E2/E4 is the least common genotypes in group II.

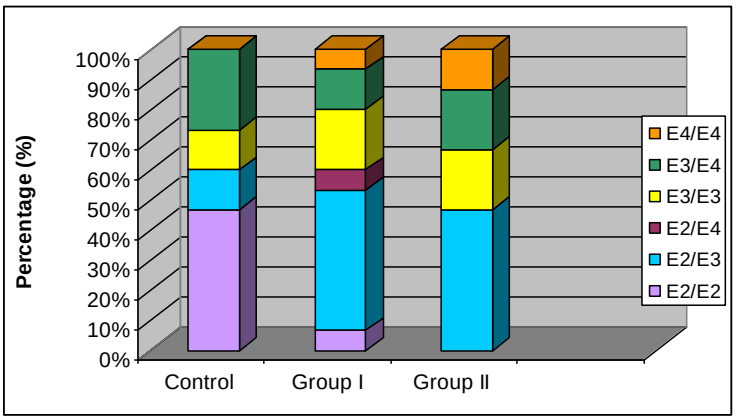

Figure (6): Statistical Analysis of Distribution of Different Genotypes in Control and Patient Group

Lipid Profile among Different Genotypes

1- Cholesterol:

There was no significant difference in cholesterol level (P > 0.05) in group I compared to the control group. On vice verse a highly significant difference (P < 0.05) in group II compared to the control group. whereas no significant difference in cholesterol level (P>0.05) in group I compared to the control group which all carring the E2/E2, E2/E4 & E4/E4 alleles; whereas there was highly significant difference in E2/E3, E3/E3 and E3/E4 compared to the control group, but in group II carrying E2/E3, E3/E3, E3/E4, and E4/E4 alleles showed a significant increase (P< 0.05) in cholesterol level compared to the control group [Figure (7)].

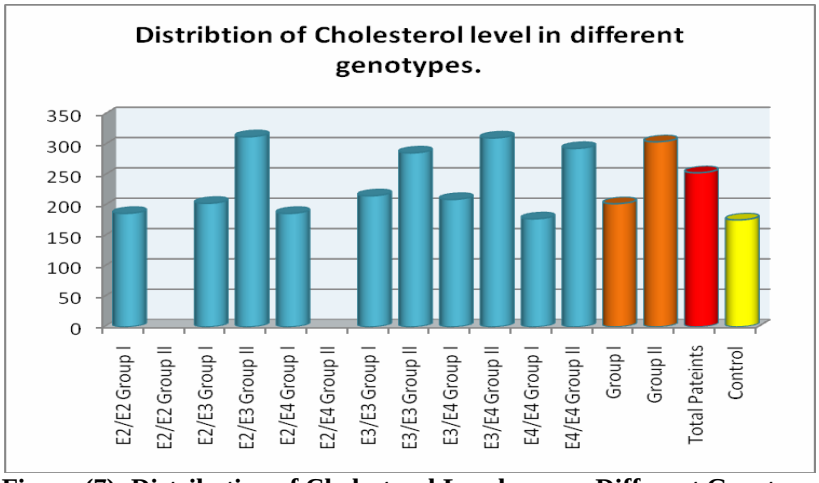

Figure (7): Distribution of Cholesterol Level among Different Genotype

2- Triglycerides (TG):

There was no significant difference (P > 0.05) in group I compared to the control group. On vice verse there was a highly significant difference (P < 0.05) in group II compared to the control group. There was a significant difference in TG level (P < 0.05) in group I carry E2/E2 and E3/E3 genotypes compared to the control group. On the other hand, there was no significant difference in TG level (P>0.05) in group I carry E2/E3, E2/E4, E3/E4, and E4/E4 genotypes compared to that obtained from the control group. Other wisely, there was a highly significant difference in TG level (P<0.05) obtained from group II carry E2/E3, E3/E3, E3/E4, and E4/E4 genotypes compared to that obtained from the control group **[Figure (8)]**.

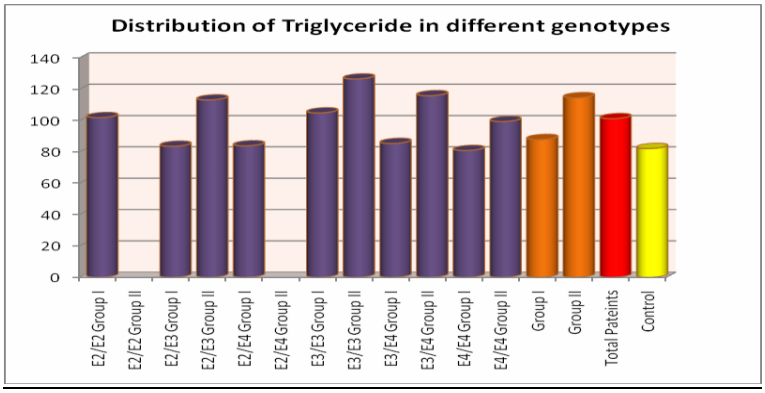

Figure (8): Distribution of Triglyceride Level of Different Genotypes among studied groups

3- Low Density Lipoprotein (LDL):

There was a significant difference in LDL level (P > 0.05) in group I compared to the control group, and highly significant difference in group II compared to the control group. No significant difference in LDL concentration obtained from E2/E2, E2/E4, E3/E4 and E4/E4 in group I compared with the control group. There was a significant difference in LDL level (P<0.05) in group I compared to group II. There was a significant difference in LDL levels (P<0.05) in group I carrying E2/E2, E3/E3 compared to the control group. There was high significant difference in concentration of E2E3, E3E3, E3/E4 and E4/E4 genotypes in group II compared to the control group [Figure (9)].

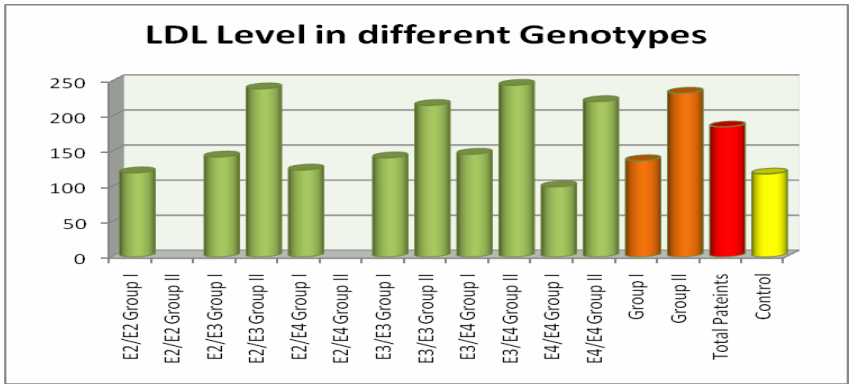

Figure (9): Distribution of LDL Level of Different Genotypes among studied groups

4- High Density Lipoprotein (HDL):

It was observed that a slight increase in HDL level ($P > 0.05$) obtained from group I and II compared with that obtained from the control group and highly significant difference in concentration of HDL of E3/E3 and E4/E4 group I. Also, highly significant difference in concentration of HDL of E2/E3 and E4E4 of group II [Figure (10)].

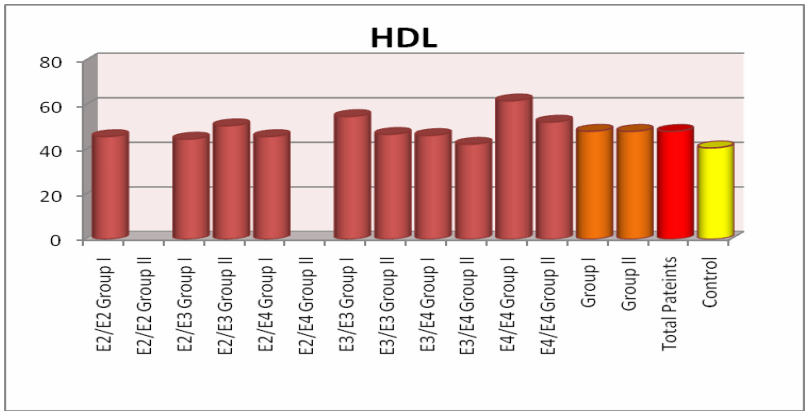

Figure (10): Distribution of HDL Level of Different Genotypes among studies group

5- LDL/HDL Ratio:

There are no significant difference ($P > 0.05$) in the ratio calculated from group I compared to that obtained from the control group, On vice versa there was a highly significant difference in the ratio obtained from group II compared to that obtained from the control group; ratio obtained from group II significantly higher ($P < 0.05$) than that obtained from group I and there was a significant increase ($P < 0.05$) in ratio calculated in group I carrying E4/E4 alleles comparing to same allele in group II [Figure (11)].

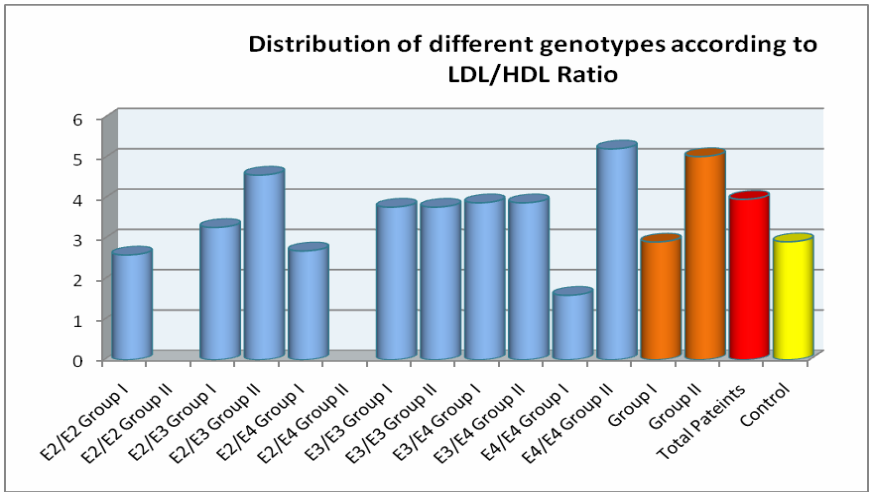

Figure (11): Distribution of LDL/HDL Ratio of Different Genotypes among studied groups

4. DISCUSSION

Apolipoprotein E (apoE) plays a key role in the metabolism of cholesterol and triglycerides. Apolipoprotein E is a multifunctional protein that is synthesized by the liver and several peripheral tissues and cell types, including macrophages. The protein is involved in the efficient hepatic uptake of lipoprotein particles, stimulation of cholesterol efflux from macrophage foam cells in the atherosclerotic lesion, and the regulation of immune and inflammatory responses [3,14]**.** ApoE expression considered a good criterion to recognize patients suffering from coronary heart disease [15]. Carriers of the E3, E4 allele may be advised to pay particular attention to prevent and control other CHD risk factors. Some studies have shown highly significant associations between E4 and CHD [32]. The risk of coronary heart disease (CHD) may be related to genetic mutations in the production of apolipoprotein E via alterations to the metabolism of CHD-related blood lipids such as low-density lipoprotein cholesterol and triglycerides [35].

Variations in interactions between the N- and C-terminal domains appear to be a major contributing factor in lipoprotein-binding preference; apoE2 and apoE3 bind preferentially to HDL, whereas apoE4 prefers VLDL. These variations resulted in different metabolic properties of apoE isoforms, which are linked to an increased risk for the development of atherosclerosis and Alzheimer's disease [27]**.** ApoE4 has a much higher lipid affinity compared with the other isoforms regardless of particle size [29]. The apoE4 variant has been associated with increased LDL production from VLDL, increased uptake of postprandial lipoproteins, increased intestinal absorption of cholesterol, decreased bile acid synthesis, and faster LDL clearance from plasma compared with the apoE3 or apoE2 variants [23]**.**

The distribution of the apo-E alleles is highly variable among the different populations throughout the world. The E3 is the most common form of the gene in most of the population followed by E4 and E2 alleles [7]**.** The frequency of APOE alleles in the three studied groups evaluated as follows; the major common allele in the control group was E2, which has represented 53.3 %, E3 has represented 33%, while E4 has contributed 14 %, in addition, the major common allele in the group I was E3 that formed 50 % of group I sample, followed by E2 with 33% and tailed by E4 with a 16% contribution. In contrast to group I, group II major common allele was E3 which has represented 53% of samples examined, while E2 and E4 both have represented 23.3 % for each. Overall, the frequency of apoE alleles in all studied subjects, E2 represents 36.6%, E3 represents 45.53% and E4 comprises 17.73%. The frequency of apoE alleles in all studied subjects was similar to those of the Sudanese, African Americans and Nigerian [30,35]**.**

In this study the cholesterol in group I ranged from 159 to 200 mg/dl, in group II, from 261 to 380 mg/dl and in the control group, from 156 to 210 mg/dl there was a highly significant increase in cholesterol level in group II in comparing with the control group but there was no significant difference in cholesterol level in group I compared to the control group. In agreement with previous studies whom stated that there was a higher risk occurrence of microangiopathy with hypercholesterolemia, and the familial hypercholesterolemia (FH) is an autosomal dominant disorder associated with a high risk of CHD [10,34].

As regard the TG level in group I ranged from 59 to 132 mg/dl with mean value of ± 88, in group II, from 69 to 156 mg/dl with mean value of ± 114.66, in control group, from 66 to 111 mg/dl with a mean value of ± 82.26 , there was a highly significant increase in TG level among cases in comparison to the control group. This in agreement with previously studies [11,17], whom stated that, there was a gradient increase in rates of microangiopathy as TG levels increased, even after adjustment for major microangiopathy risk factors, including high LDL cholesterol levels. Thus, evidence is strong that hypertriglyceridemia is a risk factor for microangiopathy. Subjects with E3/E4 and E4/E4 genotypes absorb cholesterol effectively and have higher serum triglyceride values than E4 negative individuals [33]**.**

The level of HDL-C in group I ranged from 34 to 62 mg/dl, in group II, from 32 to 60 mg/dl and in control group, from 34 to 50 mg/dl, there was a significant decrease in HDL-C level in cases in comparison to the control group.

High-density lipoprotein (HDL) level has been reported to be significantly lower in diabetic than non-diabetics. These results are consistent with high incidence and natural history of atherosclerotic coronary heart disease in diabetic and with a higher death rate from myocardial infarction. These incidences are two to four times higher than those of persons without diabetes [17,25].

In relation to LDL-C in group I ranged from 92 to 138 mg/dl with a mean value of ± 137.26, in group II ranged from 179 to 300 mg/dl with a mean value of ± 233.53, in the control group ranged from 92 to 147 mg/dl with mean value of ± 118.52, there was a significant elevation of LDL-C level in patients in comparison to the control group. So it is suggested that, there is a higher risk of microangiopathy with high level of LDL-C which was approved by other study [13].

Data depicted from the present study showed that E2/E2 alleles and E2/E4 alleles not founded in group I. While significant difference (P<0.05) in distribution E2/E3, E3/E3, E3/E4 and E4/E4 allele; the higher probability of E4/E4 in group II comparing with E4/E4 in control and group I genotyping E4/E4. This was confermed by the previous data [35,36], who proved that the comparable frequencies were observed in the CHD group and apoE4 allele is a significant risk factor for CHD. In both APOE2 carrier and APOE3 groups, the CHD patients expressed abnormal lipid profiles while the control group expressed normal lipid profiles. The APOE4 carriers, however, expressed abnormal lipid profiles in both normal control and CHD groups, in the control group exhibited frequencies of 84.6% APOE3, 7.9% APOE4, and 7.5% APOE2.

CONCLUSION: ApoE expression considered a good criterion to recognize patients suffering from coronary heart disease. Carriers of E2, E3, and E4 differ in their binding affinity to cholesterol. Carriers of the E3, E4 alleles are slower to clear dietary fat from the blood, whereas carriers E2 has high affinity for cholesterol, so carriers E2 have a protective effect against cardiovascular disease.

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