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Carotid intima-media thickness in the first descendant of coronary artery disease patients with Apolipoprotein-E4 genotype

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ABSTRACT

Introduction: Carotid intima-media thickness (CIMT) is a predictor marker of subclinical atherosclerosis that can be measured by ultrasound technique. The clinical symptoms of atherosclerosis usually begin with the thickening of the carotid artery walls. Polymorphism in Apolipoprotein E4 (APO-E4) is considered to be an important genetic determinant of atherosclerosis and Coronary Artery Disease (CAD). The genetic role of APO-E4 on plasma lipid has shown that the APO-E4 allele is associated with increased plasma LDL and total lipid concentration. The main objectives of this study were to identify genotype polymorphisms of the APO-E gene and measure the thickness of the carotid intima in subjects with APO-E4 polymorphisms in the first descendant of coronary heart patients.

Patients and methods: A cross-sectional study design was used to determine the polymorphism of the APO-E4 genotype in the first descendant of CAD and non-CAD groups. There were 21 samples in each group. Gene polymorphism examination was carried out by PCR and the CIMT was measured with doppler ultrasound.

Results: The result showed that 21.4% of subjects had polymorphism of the APO-E4 genotype with a significant difference between CHD and non-CHD group. This data shows that the APO-E4 genotype is more dominant in the first descendant of patients with CHD when compared to non-CHD parents. The mean values of CIMT ini right and left-sided carotid arteries among APO-E4 genotype and non-APO-E4 genotype were 0.4743 mm vs. 0.4195 mm and bivariate analysis showed a significant difference with a P-value of 0.017. This shows that there is a significant difference in the thickness of the carotid intima in the first descendant of subjects who have the APO-E4 genotype and those who do not.

Conclusion: The APO-E4 gene polymorphism was more dominant in the first descendant of CAD patients with a higher CIMT mean value.

Keywords: CIMT, Apolipoprotein, PCR, Polymorphism, Ultrasonography.
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INTRODUCTION

Coronary heart disease (CHD) is a cardiovascular disease with high morbidity and mortality, contributing to a total of 379.559 deaths in America in 2010 with the percentage of CHD (43.8%), followed by stroke (16.8%), high blood pressure (9 .4%), heart failure (9.0%), arterial disease (3.1%), and other cardiovascular diseases (17.9%).[1] The 2013 Basic Health Research results show that the prevalence of CHD in Indonesia based on doctor's diagnosis population of all ages is 1.5% or about 883.447 people. Meanwhile, the prevalence of CHD in Bengkulu Province based on a doctor's diagnosis without symptoms is 0.3%, or about 3.748 people, while based on a doctor's diagnosis with symptoms, it is 0.6%, or 7.495 people.[2]

Coronary heart disease itself is a chronic, progressive and polygenic disease, and atherosclerosis appears to be the main pathophysiological process underlying coronary heart disease.[3] Most of the risk factors for CHD are associated with endothelial dysfunction, which is an atherosclerotic process.[4] In addition to environmental risk factors such as smoking, drinking, and a sedentary lifestyle, genetic factor, such as single nucleotide polymorphisms (SNPs), may play an important role in the development of CHD.[5]

Single nucleotide polymorphisms (SNPs) associated with CHD have been identified by genome-wide association studies (GWAS).[6]

Polymorphisms in apolipoprotein E (APO-E) are considered a genetic determinant of atherosclerosis. Serum lipoprotein levels are considered detrimental in the process of atherosclerosis. Apolipoprotein E occurs in 3 major isoforms, E2, E3 and E4, which are coded by the corresponding alleles, ε2, ε3, and ε4, and are associated with variation in blood lipid concentration. The genetic role of APO-E on plasma lipid suggests that the APO E-ε4 allele is associated with increased plasma LDL and total lipid concentrations.[7] Several studies have shown that the APO-E genotype modifies the relationship between omega-3 PUFAs and lipoprotein profiles because APO-E is central of lipid transport and metabolism.[8]

The frequency of the APO-E allele varies widely among geographic and ethnic populations in Asia.[9] The study shows that the frequency of the APO E-ε4 allele is found to be very high in the Iranian population compared to other populations in the region. In addition, the study reveals a strong association of the APO-E-ε4 allele as a significant contributor to increasing the risk of CHD or coronary artery disease in the heart and associated with Alzheimer's disease.[10]

However, a study conducted in Turkey reveals no association between the APO-E genotype and the risk of CHD.[11,12] A study from North-Western India (Punjab) reported a significant association of the APO-E genotype with high and low-density lipoprotein cholesterol. Subjects with genotype ε3/ε4 were three times more likely to develop CHD. In addition, APO-ε4 in Hong Kong China was associated with myocardial infarction. A study in Saudi Arabia also found an increased risk of CHD due to the ε4 allele.[13] Therefore, the APO-E polymorphism and its association with CHD risk vary from population to population in Asia.

Carotid Intima-Media Thickness (CIMT) is an examination parameter that can be used to predict the incidence of atherosclerosis in the future. Previous studies reported that CIMT could predict the incidence of myocardial infarction or stroke in the next 10 years.[14] Identification of the genotype in APO-E gene polymorphism is carried out to determine the genetic variation in the first descendant of patients with CHD and non-CHD. This identification is important for detecting patients at risk for CHD and preventive steps can be taken.[15] Previous studies have reported the connection of APO-E polymorphisms with various parameters that lead to atherosclerosis events such as increased blood lipid concentrations and PUFA.[7,8] CIMT data in the first descendant with APO-E4 polymorphism has not been reported. Therefore, in this study, genotypes in APO-E gene polymorphisms and CIMT will be identified in the first descendant of patients with CHD. The APO-E gene polymorphism and CIMT data obtained can be used as a reference for carrying out preventive steps for atherosclerosis and CHD.

METHODS

Study and Sampling Design

This research was a descriptive observational study with a cross-sectional design, conducted in June 2020 - November 2021. The sample in this study was the first descendant of CHD and non-CHD patients in Bengkulu City. The inclusion criteria in this study were the first descendant of patients with CHD in Bengkulu City, the first descendant of non-CHD patients in Bengkulu City, and being willing to be research subjects and signing the informed consent. Exclusion criteria in this study were the subjects having a history of mental disorders, illiterate and deaf subjects, and refused to be the subjects of the study.

The sampling technique of this research was carried out by simple random sampling. The research sample for the first descendant of CHD patients was obtained through medical records from RSUD dr. M. Yunus Bengkulu City. The selection of candidates for the non-CHD first descendant sample in this study was carried out by filling out the WHO Rose Angina questionnaire through an online-based electronic. Respondents who had filled out the Google form and met the criteria would then be contacted for blood sampling and filling out an informed consent form. The numbers of samples used in this study were 42 subjects. Calculation of the number of samples is based on the prevalence of coronary heart disease in Bengkulu City. Measurement of CIMT was carried out with a pilot study and the sample size measured by the thickness of the carotid intima was based on subjects who had the APO-E4 polymorphism and those who did not.

Identification of Genotype APO-E Gene Polymorphism

Blood was drawn with a syringe and isolated with the ReliaPrep™ Blood gDNA Miniprep System kit. Identification of APO-E gene polymorphisms was carried out by polymerase chain reaction technique involving five pairs of primers. Table 1 shows the sequence of primary sequences used. The PCR process performs at Agilent Technologies Sure-Cycler 8900 with an initial denaturation temperature of 95°C for 3 minutes, denaturation of 95°C for 30 seconds, annealing (61°C for primers I and III, 63°C for primers II and IV) for 30 seconds, elongation 72°C for 1 minute, post elongation 72°C for 10 minutes.

DNA molecules from PCR results can be detected using 2% agarose gel electrophoresis 7x10 cm (0.39 gr agarose composition, 1 mL concentrated TAE buffer 50X, 49 mL distilled water and 1L ethidium bromide added). The PCR product was inserted into a well of as much as 9 L with a loading dye of 3 L and an electric current of 100 V was applied for 50 minutes. The results of the electrophoresis are then visualized with a Geldoc to see the DNA fragments that have been separated. The analysis between groups in this study was carried out using the ANOVA correlation test and independent t-test. The data were processed using Statistical Program for Social Science (SPSS) 25 version.

Table1. Primer sequences.

Primer	Primer Sequences (5'-3')	PCR Product (bp)
I (Cys 158)	ATGCCGATGACCTGCAGAATT	588
II (Arg 158)	ATGCCGATGACCTGCAGAATC	588
III (Cys 112)	CGCGGACATGGAGGACGTTC	451
IV (Arg 112)	CGCGGACATGGAGGACGTTT	451
Common	GTTCAGTGATTGTCGCTGGGCA	-

Measurement of CIMT

Subjects were examined for their body conditions such as blood pressure and then the thickness of the Carotid Intima-Media was checked. The examination was carried out using B-Mode Ultrasonography Siemens Diagnostic Ultrasound System Acuson P300 at Dr. Hospital. M. Yunus Bengkulu City. Measurement of the thickness of the carotid-intima media was carried out by a heart and blood vessel specialist at M. Yunus Hospital. The examination was carried out on two right and left carotid arteries by taking an image angle on one parameter, namely mid-far lateral during the diastolic phase, thickness measurements were carried out in automatic mode directly by a computer for precise results. The data obtained will be analyzed by bivariate analysis. Bivariate comparison analysis of interval data and the interval between the independent variable and the dependent variable will use the independent T-test. The Mann-Whitney test is performed if the data is not normally distributed. Data were analyzed using SPSS software for windows version 25.

RESULTS

Characteristics of Research Subject

This study was approved by the Bengkulu University Health Research Ethics Committee with ethical exemption number 345/UN.30.14.9/LT/2020 for polymorphism examination and 261/UN30.14.9/LT/2021 for CIMT measurement. The distribution of data on the characteristics of research subjects including age, gender, and history of CHD in parents is listed in Table 2.[15] The data shows that the research subjects had characteristics with an age range of 20 – 30 years (81%). More women (66.7%) were research subjects than men (33.3 %). In this study, the subject's parents were also found with a history of coronary heart disease (CHD) (50%) and without a history of CHD (50%).

Genotype Polymorphism of Apolipoprotein E Gene

Amplification of the APO-E gene showed that samples 1 to 19 had 13 bands with a size of 588 bp. Different findings were shown by samples number 1, 2, 3, 7, 12, 18, that there was no visible band (Figure 1). Samples 20, 21, C, G, H showed bands of 588 bp, while samples I, K, L revealed bands of 588 bp and 451 bp (Figure 2). Samples M, N, O, S, T, U showed bands of 588 bp and 451 bp, while samples P, Q, R had bands of 588 bp (Figure 3).

Figure 4 shows the frequency of APOE genotypes found in this study. The data were tested using the Chi-Square test. The results of the polymorphisms of the E2, E3 and E4 genotypes of the Apolipoprotein E gene showed no significant difference between the E2 and E3 genotypes of the first descendant of patients with CHD and non-CHD with p values: 0.116 and p: 0.116. This is different from the E4 genotype which showed a significant difference with p value: 0.009 between the first descendant of CHD and non-

Table 2. Characteristics of Research Subjects.[15]

Characteristics	N	%
Age		
<20	3	7,1
20 – 30	34	81
30 – 40	3	7,1
>40	2	4,8
Gender		
Male	14	33,3
Female	28	66,7
History of Disease in Parents		
CHD	21	50
Non-CHD	21	50

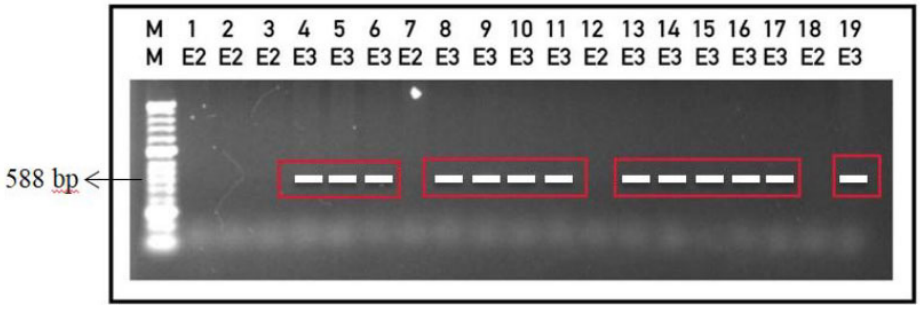


Figure 1. Electrophoresis of Arginine PCR Results of Non-CHD First Descendant Samples.

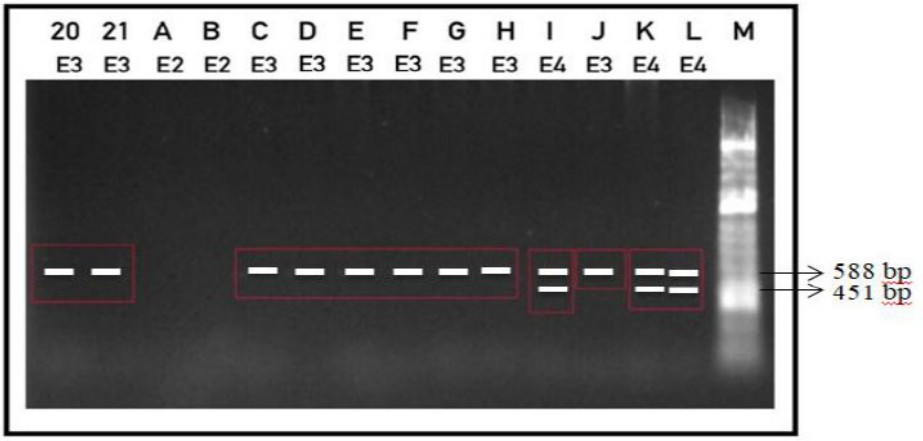


Figure 2. Electrophoresis of Arginine PCR Results for Non-CHD (20.21) and CHD (A-L) First Descendant Samples.

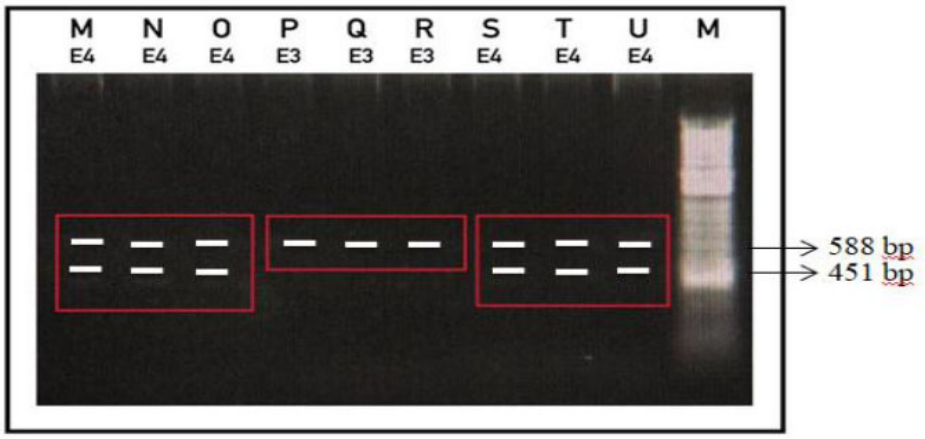
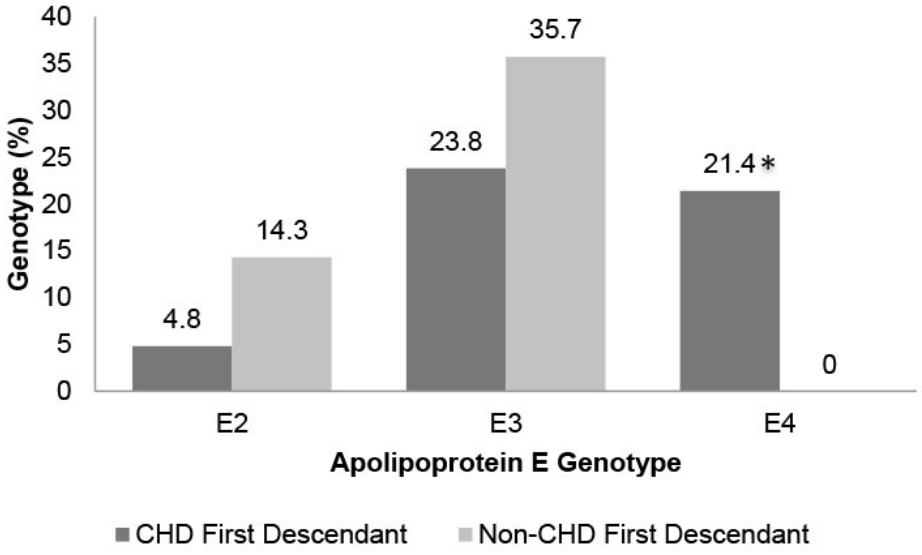


Figure 3. Electrophoresis of Arginine PCR Results for CHD First Descendant Samples (M-U).



Note: * = Significantly different in CHD and Non-CHD first descendant.

Figure 4. Persentations of Apolipoprotein E Genotype among Subjects.

CHD. Thus, it can be interpreted that the Apolipoprotein E gene polymorphisms in the CHD and Non-CHD first descendant samples were statistically significant. This indicates that the APO-E4 polymorphism was found to be more dominant in the first descendant of patients with coronary heart disease compared to the first descendant of parents who did not suffer from coronary heart disease.

Carotid Intima-Media Thickness

Measurements of weight, height and body mass index were carried out before measuring the thickness of the carotid arteries. Table 3 shows the data on the weight, height and BMI characteristics in the subjects to be examined for the thickness of the carotid intima. The dominant body weight range of the research subjects was 61-70 kg (35.3%) and the dominant height was 156-160 cm (29.4%). Other demographic data is body mass index which is mostly in the overweight category (35.3%).

The mean reference value of CIMT in subjects under 30 years of age was 0.43 mm. The data from the measurement of carotid artery thickness showed that the mean value of CIMT obtained in the group with the APO-E4 genotype was 0.47 mm (medium risk category). In addition, subjects with the APO-E4 genotype also had a significantly different mean CIMT value with study subjects who did not have this genotype (Figure 5).

DISCUSSION

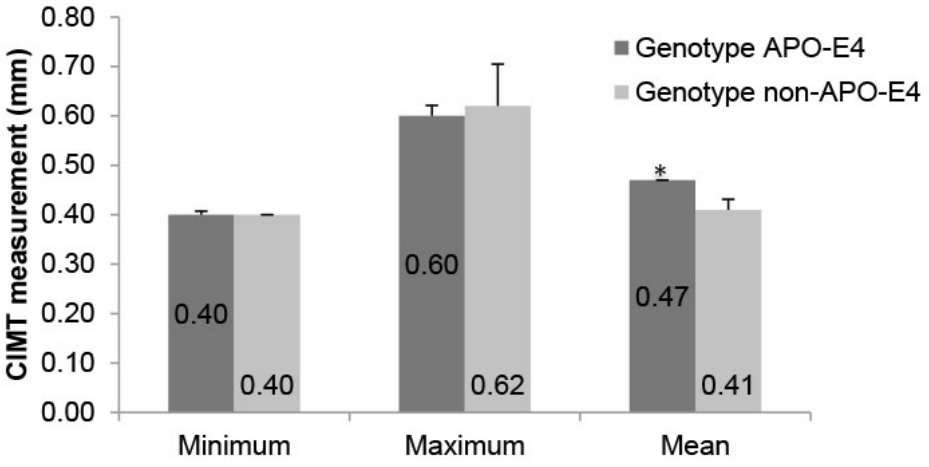
General Characteristics of Research Subjects

This study was followed by 42 research subjects who met the criteria. The age range of the sample in this study was found to be the largest, namely the age of 20 – 30 years (81%) followed by the age of < 20 years and of 30 – 40 years (7.1%) and of > 40 years (4.8%). South Asian population tend to be at high risk of developing CHD even at a young age.[16] Asians tend to be at risk of atherosclerosis with the highest mortality due to complications compared to other ethnic groups studied. It has been documented that younger age groups may also develop more progressive disease in recent years.[16]

Most of the gender characteristics in this study were female (66.7%) and male (33.3%). In some studies, the incidence of CHD in men compared to women is 3 times higher and the mortality is 5 times higher. Most of the risk factors are more favorable in women, but the sex difference in risk factor level diminished with age. Differences in risk factors between sex, particularly in HDL cholesterol and smoking, account for nearly half of the difference in CHD risk between men and women. Differences in serum total cholesterol level, blood pressure, body mass index, and diabetes prevalence explain about one-third of the increased risk of age-related CHD among men and 50% to 60% among women.[17]

Polymorphism of APO E Genotype

The E2 and E3 genotype polymorphisms showed no significant difference in the first descendant of patients with CHD and non-CHD. In this study, E2 and E3 polymorphisms were dominant in the first non-CHD descendant. These results are consistent with other studies which state that genotypes E2 and E3 are not associated with the incidence of severe CHD.[18] Therefore, in this study, this type of gene polymorphism was not significantly different in the first descendant of CHD compared to non-CHD. These results may be explained by the fact that other genetic or environmental risk factors may interact with Apo-E in determining CHD risk. Thus, this polymorphism is not a single risk factor. In this study, we did not find a



Note: * = Significantly different in genotype APO-E4 and Non-APO-E4

Figure 5. Mean of CIMT value among the subjects.

Table 3. Characteristic Subjects.

	Characteristics	%
Body Weight (kg)	40 – 50	17.6
	51 - 60	17.6
	61 - 70	35.3
	71 - 80	11.8
	81 – 90	11.8
	91 - 95	5.9
Body Height (cm)	145 – 150	11.8
	151 – 155	5.9
	156 – 160	29.4
	161 – 165	11.8
	166 – 170	11.8
	171 – 175	17.6
	176 - 180	11.8
Body Mass Index	< 18	11.8
	18.5 – 22.9	23.5
	23 – 24.9	17.6
	25 – 29.9	35.3
	> 30	11.8

significant difference between patients and controls for the Apo-E gene and genotype frequency, indicating that the E2 and E3 alleles were not strong risk factors for coronary heart disease in Bengkulu.

A different case was shown in the E4 genotype analysis. This study shows that in Bengkulu province, children with coronary heart disease were more at risk of developing CHD if they had the type of APO-E gene polymorphism. This genotype was significantly different in the first descendant of patients with CHD and non-CHD. In the first-descendant group of parents with coronary heart disease, the E4 genotype was more common than in the first-descendant group parents who were not affected by CHD. These findings suggest that inherited risk factors may be associated with the APO-E4 gene polymorphism. Many other studies have linked the E4 polymorphism with coronary heart disease and blood lipid.[19–21] In addition, the presence of the APO-E4 genotype also affects the treatment given.[19]

These results are in accordance with research reported that the variation of amino acid E3 is the most common isoform in the world population with a prevalence of almost 75%.[22] Then isoform E4 with a prevalence of 15% and isoform E2 which is at least 10%. The result of this study is consistent with the Hardy-Weinberg equilibrium. In addition, it has also been reported that modification of the structure of Apo-E E4 at the genetic level can lead to the development of atherosclerosis. Previous studies have shown that E4 causes an increase in total plasma VLDL and cholesterol, leading to an increased risk of coronary heart disease, whereas E2 has been found to be associated with a decrease in cholesterol level and remnants of VLDL. This indicates that E4 can be considered as a positive factor and E2 as a negative factor for the development of atherosclerosis.[23]

Analysis of CIMT among Subjects

Body weight and height are characteristics commonly studied in patients with APOE4 gene polymorphisms, especially concerning body mass index. In this study, the research subjects had the highest proportion of BMI in the overweight range (35.3%). This is in line with the research that showed the baseline characteristics of the population subject with the genotype having the APO-E4 polymorphism had an average of 24.2 ± 2.9 of the total number of subjects 1,516 participants in the cohort study.[24]

In statistical calculations, the results of the average right and left CIMT values in subjects who have APO-E4 gene polymorphisms where in subjects who have APO-E4 gene polymorphisms have a thicker thickness than subjects who do not have APO-E4 gene polymorphisms. Previous studies have shown an association between APO-E4 and CIMT where the carriers of the APO-E4 gene have CCA wall thickness and carotid bifurcation. This study also mentions that there is a meta-analysis of the genetic contribution to CIMT where it was found that the APO-E4 gene was one of the genes most closely associated with CIMT in studies with large populations.[25]

Previous studies reported no association between APOE-E4 and the presence of carotid plaque or plaque areas was found. Plaque is formed as a result of chronic inflammation and infiltration of immune cells into the arterial wall, whereas CIMT shows pathological hypertrophy of the medial layer. CIMT functions more as a surrogate marker of early atherosclerosis than a manifestation of atherosclerosis. Carotid plaque and CIMT both had significant heritability, but genetic influences appeared to be stronger in CIMT, whereas environmental factors played a larger role in plaque. Many

studies have confirmed the association between APOE-ε4 and total CIMT whereas others have not. Differences between these studies may be influenced by race, ethnic composition of the study population, sample size, and inclusion of lipid parameters in the regression analysis. However, a meta-analysis of genetic contributions to CIMT found that, among many candidate genes, only APOE-ε4 had a convincing association with CIMT with larger study populations.[25]

CONCLUSION

The APO-E2 and APO-E3 genotype were more dominant in non-CHD first descendant. The APO-E4 gene polymorphism was more dominant in the first descendant of CAD patients with a higher CIMT mean value.

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ETHICAL STATEMENT

Ethical Comitte Faculty have approved this study of Medicine, Universitas Bengkulu with Ethical Clearance Reference Number 261/UN30.14.9/LT/2021.

CONFLICT OF INTEREST

All author declares there is no conflict of interest regarding publication of this study.

AUTHOR CONTRIBUTION

All author has contributed in manuscript writing and agreed for the final version of manuscript for publication.

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