STUDY ON APOLIPOPROTEIN E (APOE) GENE POLYMORPHISM AND CORONARY ARTERY DISEASE BIOMARKER IN DIABETIC PATIENTS

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2016

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by

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Dissertation Submitted in
Partial Fulfillment Of The Requirements
For The Degree Of
Master of Science

UNIVERSITI SAINS MALAYSIA
2016

ACKNOWLEDGEMENT

I thank Allah SWT for His blessing and mercy until I accomplish my dissertation as the requirement for the degree of Master Science. First and foremost I would like to express my very great appreciation to my supervisor, Dr. Shahrul Bariyah Sahul Hamid, who had supported me throughout my research and dissertation with her expertise and advice. One simply could not wish for a better or friendlier supervisor. I would also like to thank my co-supervisor, Dr. Nor Hazwani Ahmad for her guide and encouragement in carrying out this research. Without their support, this dissertation would not have been possible.

Secondly, the biggest thanks for my beloved parents Mr. Abdul Sani Ibat and Mrs. Nazriah Ahmad for their infinite supports, prayers and love. May both of them always blessed by Allah SWT. I am particularly grateful for the kindness of my classmates whose always gave moral support and for the assistance given by all the staffs and friends in Oncology Lab. I would like to acknowledge with gratitude to my beloved best friends whose always there for me whenever I need them, my colleagues and superiors for their thoughtful and to Ministry of Health for granting me study leave and scholarship for graduate studies.

I would also like to extend my thanks to AMDI Student Research Fund for providing the funding for this research. Last but not least, many thanks to all those who are indirectly involved in helping me and inspired me to complete this research. Indeed, their help means a lot to me.

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LIST OF ABBREVIATIONS AND SYMBOLS

APOE	Apolipoprotein E
BAS	Bile acid sequestrants
BMI	Body mass index
CAD	Coronary artery disease
CHD	Coronary heart disease
EDTA	Ethylenediaminetetraacetic acid
HbA1c	Haemoglobin A1c
HDL-C	High density lipoprotein cholesterol
HKL	Hospital Kuala Lumpur
hs-CRP	High sensitive C- reactive protein
IDF	International Diabetes Federation
LDL-C	Low density lipoprotein cholesterol
LRP	LDL-receptor related protein
MI	Myocardial infarction
NCD	Non-communicable disease
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
SD	Standard deviation

T2DM	Type 2 diabetes mellitus
TAE	Tris-acetate-EDTA
TC	Total cholesterol
TG	Triglycerides
VLDL-C	Very low density lipoprotein cholesterol
WHO	World Health Organization

KAJIAN TENTANG POLIMORFISME GEN APOLIPOPROTEIN E (APOE) DAN BIOMARKER PENYAKIT ARTERI KORONARI DI KALANGAN PESAKIT DIABETES

ABSTRAK

Gen apolipoprotein E (APOE) memainkan peranan yang penting dalam metabolisme lipoprotein dan pengangkutan lipid. Pelbagai kajian tentang kaitan antara polimorfisme gen APOE di kalangan pesakit diabetes yang menghidap arteri koronari (CAD) telah dijalankan dalam beberapa tahun kebelakangan ini. Walau bagaimanapun, tiada kajian yang dijalankan di kalangan populasi Malaysia berkenaan kaitan antara polimorfisme gen APOE di kalangan pesakit diabetes yang menghidap CAD. Oleh itu, objektif kajian ini dijalankan adalah untuk mengkaji peranan polimorfisme gen APOE dalam mempengaruhi CAD di kalangan pesakit diabetes mellitus jenis 2 (T2DM) di Hospital Kuala Lumpur (HKL). Sejumlah 45 orang pesakit T2DM (21 lelaki dan 23 wanita) telah dikaji yang mana 11 orang daripadanya adalah penghidap CAD. Analisis biokimia termasuk glukosa, HbA1c dan profil lipid yang merangkumi kolesterol (TC), trigliserida (TG), lipoprotein berketumpatan rendah (LDL-C) dan lipoprotein berketumpatan tinggi (HDL-C). Maklumat diperoleh dari pangkalan data Makmal Patologi Hospital Kuala Lumpur. Kajian penjenisan gen APOE telah dijalankan dengan menggunakan teknik polimerfisme panjang fragmen restriksi (RFLP). Produk PCR dicernakan dengan enzim restriksi. Fragmen dipisahkan dengan menggunakan elektroforesis gel agarosa. Pesakit T2DM dengan dan tanpa CAD mempunyai perbezaan signifikan terhadap glukosa dan TG dalam plasma (p = 0.048 and p = 0.019). Genotip APOE yang dikesan dalam kajian ini ialah 3/ε3, ε3/ε4, ε2/ε3, ε2/ε4 and ε4/ε4 kecuali

genotip ε2/ε2. Hasil kajian menunjukkan, frekuensi genotip ε3/ε4 adalah lebih tinggi di kalangan T2DM dengan CAD berbanding T2DM tanpa CAD. Walau bagaimanapun, tiada perbezaan signifikan dalam taburan genotip dan alel APOE antara kedua-dua kumpulan pesakit. Kajian menunjukkan perbezaan signifikan antara HDL-C dan genotip (p = 0.004) serta alel (p = 0.001) APOE. Didapati hanya pesakit T2DM tanpa CAD yang terkesan dengan variasi alel dan HDL-C. Analisis selanjutnya tentang kaitan antara polimorfisme gen APOE dan profil lipid menunjukkan tiada perbezaan signifikan terhadap TC, TG dan LDL-C untuk kedua-dua genotip dan alel. Konklusinya, alel ε4 adalah tinggi di kalangan CAD dan rendah HDL-C.

STUDY ON APOLIPOPROTEIN E (APOE) GENE POLYMORPHISM AND CORONARY ARTERY DISEASE BIOMARKER IN DIABETIC PATIENTS

ABSTRACT

Apolipoproteion E (*APOE*) gene plays a major role in lipoprotein metabolism and lipid transport. Various studies on the association of the *APOE* gene polymorphism among diabetes patients with coronary artery disease (CAD) also has been investigated in the last few years. However, there is no report among the Malaysian population in regard to the association of APOE gene polymorphism among patients with T2DM and CAD patients. Thus, the objective was to study role of *APOE* gene polymorphisms in development of CAD among type 2 diabetes mellitus (T2DM) patients in Hospital Kuala Lumpur (HKL). Total of 45 T2DM patients (21 men and 23 women) was investigated in which 11 of them had complications with CAD. Biochemical analyses included glucose, HbA1c and lipid profile which consists of total cholesterol (TC), triglycerides (TG), low density lipoprotein (LDL-C) and high density lipoprotein (HDL-C). Data were obtained from the Hospital Kuala Lumpur Pathology Laboratory database. Genotyping of APOE was done by using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The PCR product was digested with restriction enzymes. Fragments were separated using agarose gel electrophoresis. The T2DM with and without CAD subjects had significant differences in glucose and TG plasma levels (p = 0.048 and p = 0.019). The APOE genotypes detected in this study were of ε3/ε3, ε3/ε4, ε2/ε3, ε2/ε4 and ε4/ε4 except for ε2/ε2 genotype. Research finding showed, frequency of ε3/ε4 genotype was higher among T2DM with CAD compared to T2DM no CAD. However, there was no significant difference in distribution of the APOE genotype and alleles among both

groups. Study showed there was a significant difference of HDL-C level between APOE genotype (p = 0.004) and alleles (p = 0.001). It was noted only T2DM patients with no CAD were affected with allele's variation and HDL-C levels. Further analysis on the association of the *APOE* gene polymorphism and lipid profile showed there were no significant difference for TC, TG and LDL-C with genotypes and alleles. In conclusion ε4 higher in CAD and reduces HDL-C.

CHAPTER 1

INTRODUCTION

1.1 Diabetes and coronary artery disease

Non-communicable disease (NCD) such as diabetes, cardiovascular diseases, cancers and chronic respiratory diseases are responsible for 82 % of all deaths worldwide which are expected to increase to 52 million by 2030 (WHO, 2016). Diabetes mellitus is one of the most common metabolic disorders and the prevalence of diabetes in adults continue to increase due to the lifestyles which lead to reduced physical activity and increased obesity (Guariguata et al., 2014). This metabolic disorder affected 415 million people worldwide in 2015, which had been expected increase to 642 million in 2040 (International Diabetes Federation, 2016).

One of the main reasons of premature illness and death was due to diabetes with a higher chance of developing Coronary Artery Disease (CAD). CAD is a cause for around 50 % to 80 % of deaths among diabetics. The American Association Heart Association also reported that CAD is the one main reason of mortality among diabetes patients. Approximately between two to four-fold increase in risk of CAD compared to those without diabetes (Eckel et al., 2006). Type 2 Diabetes Mellitus (T2DM) patients with CAD have a worse prognosis for survival compared those CAD patient without diabetic (Grundy et al., 1999).

Previous studies showed that T2DM is also related to sudden cardiac death (Kucharska-Newton et al., 2010, Siscovick et al., 2010). Furthermore, cohort study done by Shah et al. (2015) concluded that the higher risk of cardiac arrest with T2DM is caused

by atherosclerotic coronary disease as studied on most common initial cardiovascular disease such as stable angina, heart failure or peripheral arterial disease. Besides that, the study showed there was an association between the Hemoglobin A1c (HbA1c) concentration and risk of cardiovascular diseases. Even though the concentration of HbA1c less than 48 mmol/mol (6.5 %), this major factor could resulting the person to develop peripheral arterial disease.

The risk of diabetic patients to have CAD is about 2 to 4 higher than non-diabetic patients. Moreover, diabetic patients may have different type and degree of the effect of CAD risk factors compared with non-diabetic patients (Chamnan et al., 2009). A retrospective cohort study among Chinese diabetic patients stated the incidence and predictors of CAD, which include smoking, body mass index (BMI), HbA1c, systolic blood pressure, diastolic blood pressure, Total Cholesterol-High Density Lipoprotein (TC-HDL) ratio and albumin/creatinine ratio. These factors also contributed to the development of CAD among T2DM patients. This can be used as a guideline for the tertiary preventive interventions (Wan et al., 2016).

T2DM patients that have a complication with cardiovascular were considered as experienced a severe complication since it was affected by their quality of life and survival. Garcia-Fontana et. al (2016) reported that phospholipids (PLs) are important metabolites involved in T2DM and CAD. They suggested that metabolomics approach was benefited in giving information about the changes in the metabolomics pathways involved in CAD associated with T2DM. This is beneficial in order to develop strategies for the new prevention of metabolic disorders.

1.2 Epidemiology of T2DM and CAD

Global estimates of diabetes prevalence have shown increases over the past 15 years. King et. al (1998) predicted the prevalence of diabetes would reach 300 million by 2025 whereas, WHO estimated that this would exceed to 366 million by the year of 2030. Furthermore, there were also an increase in the estimation of diabetes prevalence by International Diabetes Federation (IDF) from 151 million in 2000 (IDF, 2000); 194 million in 2003 (IDF, 2003); 246 million in 2006 (IDF, 2006); 285 million in 2010 (IDF, 2010); 366 million in 2011 (IDF, 2011) and most recently 415 in 2015 (IDF Diabetes Atlas, 2016).

In Malaysia, there is also an increasing of the prevalence of T2DM among adult aged ≥ 18 years old over the past decade. The 5th Malaysian National Health and Morbidity Survey (NHMS V) in 2015, reported that the prevalence of T2DM increased to 17.5 % equal to 3.5 million, compared to previous National Health and Morbidity Survey (NHMS IV) in 2011, which is only 15.2 % reported (MOH, 2016).

As for comparison among Malaysian, the Indian ethnic group had the highest prevalence of T2DM (24.9 % in 2011 and 19.9 % in 2006), followed by Malays (16.9 % in 2011 and 11.9 % in 2006), and Chinese ethnic group (13.8 % in 2011 and 11.4 % in 2006) (Mafauzy et al., 2011, Mohamed, 2008). These statistics was correlated with the ongoing cohort patient registry by Audit of Diabetes Control and Management (ADCM) which concentrated on control and management of diabetes in Malaysia. It showed ethnic differences in glycemic control, whereby Chinese with T2DM had the lowest mean of HbA1c levels (7.8 %) compared to Indians had the highest (8.5 %) (Chew et al., 2011).

Fox et. al (2004) suggested that the increasing prevalence of T2DM is leading to an increasing rate of CAD because T2DM have similar risk factors of developing CAD. Cardiovascular is the leading global cause of NCD deaths in 2012 with approximately of 17.5 million deaths describing 31 % of global deaths or 46 % of NCD deaths. WHO reported that heart attack disease could cause an estimation about 7.4 million of deaths. The prevalence is expected to increase to more than 23.6 million by 2030. Statistics from WHO and United Nations stated that CAD mortality rates between countries were varied by more than twenty-fold. The previous study reported that the highest in the CAD mortality rate was in Eastern Europe and Central Asia. Furthermore, countries with the low and middle income were higher CAD mortality compared to countries with high income. More than 80 % of global CAD deaths came from the low and middle-income countries (Finegold et al., 2013).

In the year of 2010, CAD which is also the leading cause of death in Malaysia was estimated to cause almost 24.5 % of death in government hospitals (MOH, 2011). In Malaysia the prevalence of risk factors for cardiovascular are physical inactivity 60 %, smoking 26 %, obesity 49 %, hypertension 26 %, raised blood glucose 11 % and hypercholesterolemia 54 % (Chang et al., 2012, Song et al., 2004, Mustapha et al., 2014). Fifth National Health and Morbidity Survey in 2015 reported a prevalence of 47.7 % hypercholesterolemia adults (MOH, 2016).

Yussof K. (1996) reported that risk factors for CAD were noted among the rural and semirural population in Peninsular Malaysia. A study done in a rural population in Sarawak showed that there was a lower prevalence of risk factors which include smoking, hypercholesterolemia, elevated blood glucose and hypertension in the rural community but a higher prevalence of overweight (Ching Thon et al., 2012). Bloom et al. (2011) stated that cardiovascular disease is one of the diseases that is the dominant contributor to the global economic burden of NCDs.

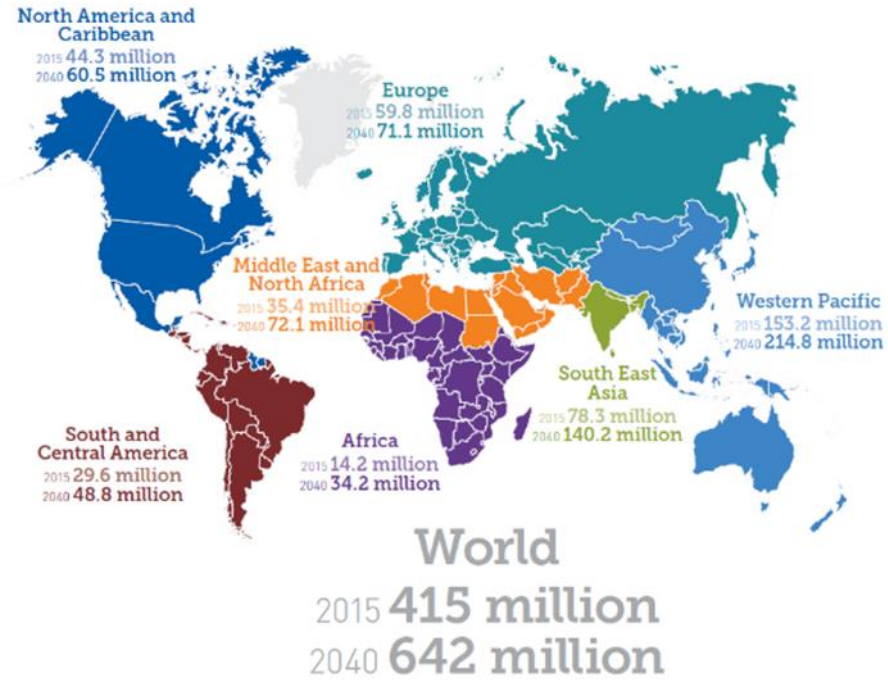


Figure 1.1: Estimated number of people with diabetes worldwide and per region in 2015 and 2040 (20-79 years).

Source: International Diabetes Federation (2016)

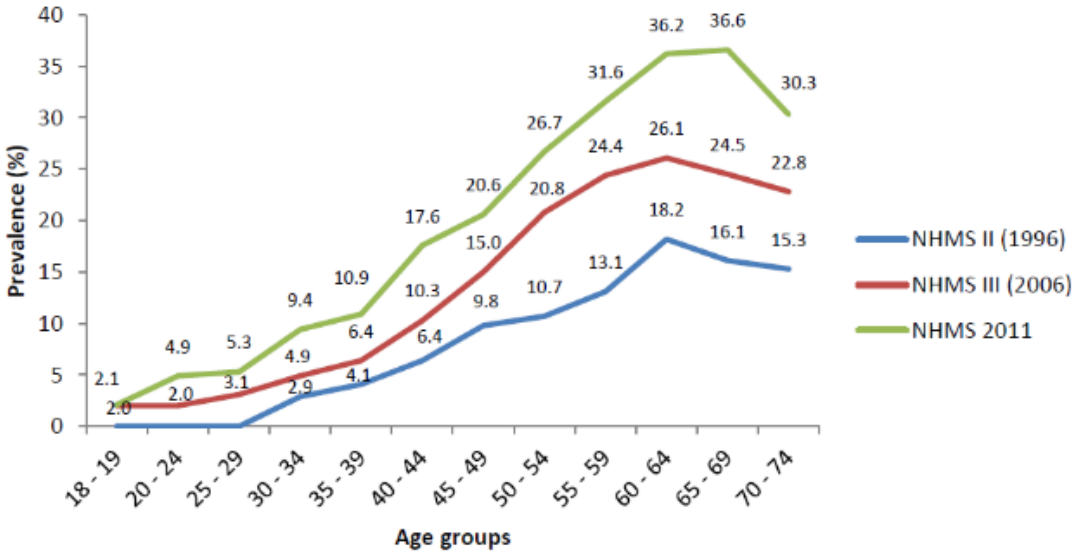


Figure 1.2: Prevalence of diabetes ≥ 18 years old in Malaysia, by age groups (1996, 2006, 2011).

Source: National Diabetes Registry (2012)

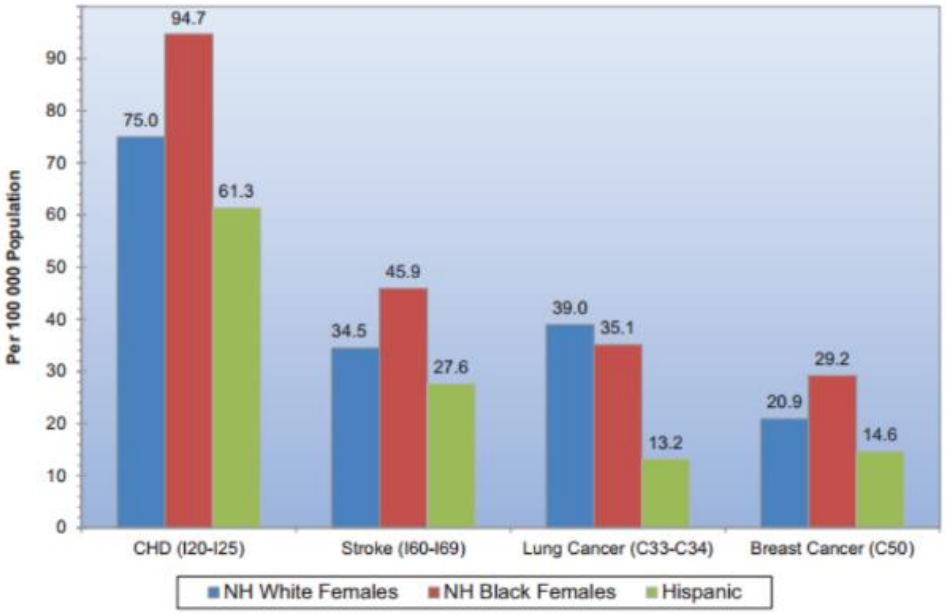
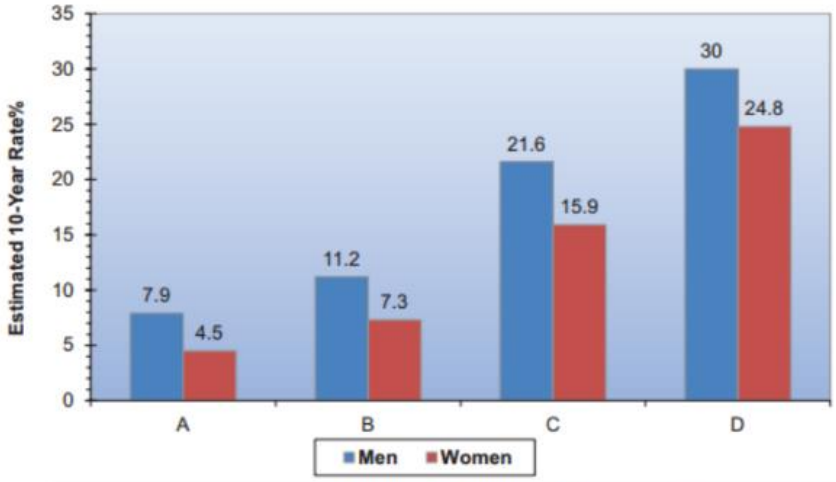


Figure 1.3: Age-adjusted death rates for coronary heart disease (CHD), stroke, lung and breast cancer for white and black females (United States: 2013)

Source: National Centre for Health Statistics and National Heart, Lung, and Blood Institute.



	A	B	C	D
Age	50-54	50-54	50-54	50-54
HDL Cholesterol, mg/dL	45-49	45-49	35-44	35-44
Total Cholesterol, mg/dL	160-199	200-239	200-239	200-239
Systolic BP mm/Hg, no treatment	120-129	130-139	130-139	130-139
Smoker	No	No	No	Yes
Diabetes	No	No	Yes	Yes

Figure 1.4: Estimated average 10-year cardiovascular disease risk in adults 50 to 54 years of age according to levels of various risk factors (Framingham Heart Study).

Source: American Heart Association (2016)

1.3 Lipid profile

T2DM patients who had tight control of glycemia and CAD with hypertension and dyslipidemia were reported to have less CAD morbidity and mortality (Turnbull et al., 2005). To monitor the association of cardiovascular risk and lipid-lowering therapies in patients with and without diabetes, serum concentration of lipids are used. Lipid profile consist of total cholesterol (TC), high density lipoprotein (HDL-C), low density lipoprotein (LDL-C) and triglyceride (TG). The ratios of TC/HDL-C and LDL-C/HDL-C also are referred. Besides that, recent guidelines have recommended that non-HDL-C as one of the parameter to predict the cardiovascular risk (Perk et al., 2013). T2DM patients have abnormal levels of serum lipid profile including a lower concentration of HDL-C, elevated LDL-C as well as TG. Individual with no diabetes but had an abnormality in their lipid profile also potentially to develop CAD (Grundy et al., 2002).

Metabolic syndrome with obesity, diabetes and history of insulin resistance commonly have high TG level and low HDL-C level (Kannel and Vasan, 2009). Since cholesterol is transferred from HDL-C to TG-rich very low density lipoprotein (VLDL), TG and HDL-C have a close contrary relationship (Feingold and Grunfeld, 2000). The lipid profile is a marker for the presence of the high atherogenic small dense LDL-C particles that penetrate the intima (Rajman et al., 1999) and are subject to oxidation (Tribble et al., 1992). The latest study by Hirakawa et. al (2016) also reported the positive association of risk of CAD with increased TC and decreased HDLC. They reported although BMI does not affect total and HDL cholesterol, but high BMI exacerbates the effects of TG on CAD. There was an association in the effects of TG on CHD with BMI, which was still evident after adjusting for HDL-C and TC. Furthermore, their study indicates that one of the factors to reduce the burden of CAD is by the body weight control together with management of dyslipidemia.

For the treatment of hyperlipidemia, statin had been used since their introduction in the 1980s (Endo, 2008). Besides that, bile acid sequestrants (BASs) also have been used to reduce the cholesterol level. The second generation of BAS, colesevelam was introduced in the early 2000s and was proven had a higher potency in binding bile acids and better tolerability (Sandhu et al., 2016). Colesevelam benefit for those statin intolerance and needed the second-line LDL-lowering agents. It also effective in lowering glucose level especially among adults older than 65 years (Gavin et al., 2014).

1.4 Apolipoprotein E gene

Apolipoprotein gene family consists of Apolipoprotein E (Apo E) gene apart of others Apo (A-I), Apo (A-II), Apo (A-IV), Apo (C-1), Apo (C-II) and Apo (C-III) (Luo et al., 1986). Apo E gene has four exons and three introns involving 3597 nucleotides that encode 299 amino acid polypeptides. It is present on chromosome 19q13.2 and related to the Apo C-I/C-II gene complex. It is a type of plasma glycoprotein of 34 kDa that is linked to HDL, VLDL and chylomicrons. Apo E is synthesized and secreted from different organs and cells including liver, brain, spleen, kidneys, gonads, adrenals and macrophages. It is also rich in the plasma, interstitial fluid and lymph (Huang and Mahley, 2014).

Apo E also functioned as a ligand for the removal of the VLDL and HDL from the circulation (Winkler et al., 2010). Apo E is important for the plasma lipid levels and involves in the regulation of plasma and tissue lipid content because Apo E has binding affinity for lipoprotein receptors. It interacts with the Apo E-containing lipoproteins to the LDL receptor, the LDL receptor-related protein (LRP), the VLDL receptor, the Apo E receptor-2, and glycoprotein 330. However, each isoform of the Apo E has differents in their interaction with these receptors (Mahley and Huang, 1999).

Besides that, each Apo E isoforms also differ in binding affinity to cell surface heparin sulfate proteoglycans (HSPGs). The interaction between Apo E and HSPGs could attract and sequester Apo E-containing lipoproteins and assist their interaction with the LRP. HSPGs mediate the internationalization of the Apo E-containing lipoprotein (Mahley and Huang, 2007). The differences in structural and binding of Apo E2, Apo E3 and Apo E4 to the lipid is associated with the distribution of isoform-specific Apo E among the different lipoprotein (Mahley and Huang, 1999).

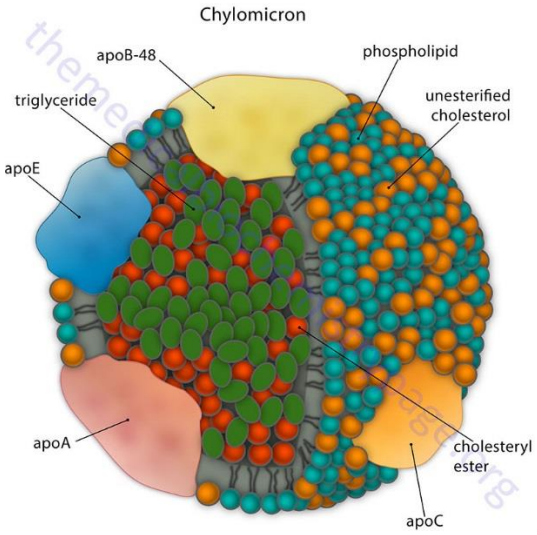


Figure 1.5: Structure of a chylomicron as a representative structure of a typical lipoprotein particle.

Source: The Medical Biochemistry Page (2016)

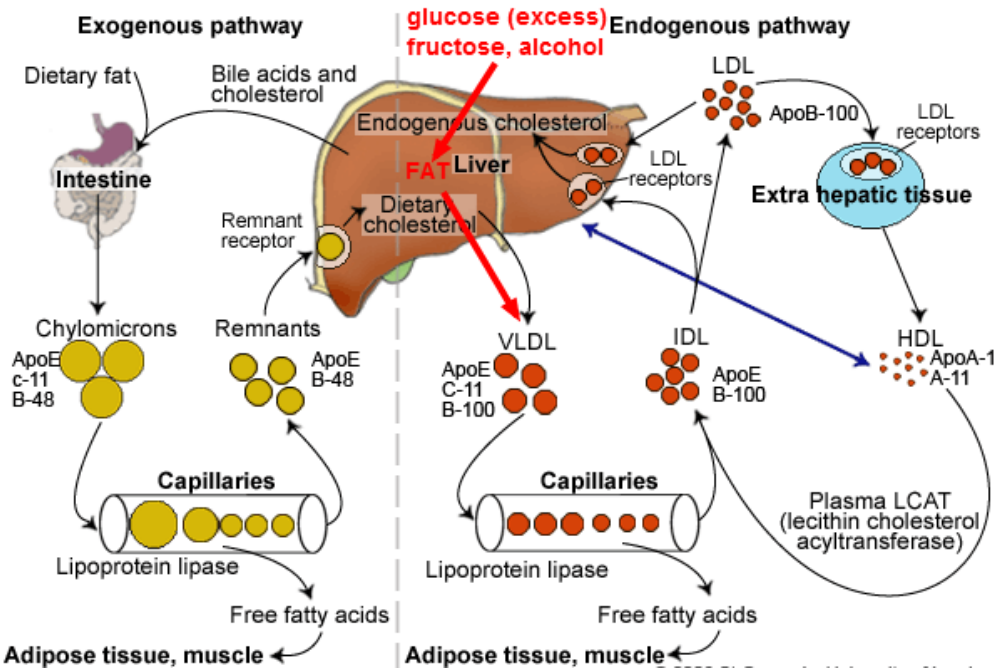


Figure 1.6: Summary of the general pathway of lipoprotein metabolism.

Source: Journal of Internal Medicine

1.5 Apo E polymorphism and disease

There is an association between the lipoprotein-related mechanisms with an abnormality of the cardiovascular system among diabetic patients (Jenkins et al., 2004). A study conducted among the 53 Turkish patients also suggested that Apo E gene polymorphism is linked with atherosclerosis and play a critical role in lipid metabolism (Arslan Ince et al., 2010). According to Grundy et. al (2006), various Apo E gene studies were conducted in regard to determine the development of CAD since it is important for lipoprotein transportation and metabolism. Polymorphism of the Apo E also responsible for about 7 % of the cholesterol variation in the population (Davignon et al., 1988).

Single nucleotide polymorphisms (SNPs) of the Apo E gene at position 112 and 158 of the gene resulting in three major alleles which are known as ε2, ε3 and ε4. These alleles are coding for three isoforms which are Apo E2 consists of (Cys112/Cys158), Apo E3 consists of (Cys112/Arg158) and Apo E4 consists of (Arg112/Arg158). There are 6 possible genotypes of Apo E that are of ε2/ε2, ε2/ε3, ε3/ε3, ε4/ε4 and ε2/ε4 (El-Lebedy et al., 2016b).

Apo E alleles affect the lipid metabolism. Apo ε2 allele is associated with elevated levels of Apo E, reduction in LDL-C and lowering risk of CAD. Apo ε4 is associated with low level of Apo E but higher levels of TC, LDL-C, VLDL-C and greater risk of CAD compared to Apo E3 homozygotes (Siest et al., 1995). Populations studies have constantly shown that ε4 subjects have higher serum concentration of TC and LDL-C, ε3 subjects have an average concentration of TC and LDL-C while ε2 subjects have the lowest concentration of TC and LDL-C (Corella et al., 2001, Wilson et al., 1994). Since Apo ε4 has a higher affinity towards LDL-R it contributes to the impaired lipid clearance and causes to increase of LDL particles. This condition will inhibit the LDL-R synthesis.

This will result in delayed clearance of lipoproteins (Knouff et al., 1999). Guang-da et. al (2004) also reported that Apo ε4 allele is associated with CAD.

The cardiovascular role of Apo ε2 is, however, uncertain according to meta-analysis study by Wilson et. al (1996). It has been related to high TG concentration (Dallongeville et al., 1992). The combination of Apo ε2 homozygote with other disorders may develop type III familial hyperlipidaemia and premature atherosclerosis. While Apo ε3 and Apo ε4 binding affinity are similar, Apo ε2 has only 2 % of this binding affinity resulting in dysfunctional lipoprotein metabolism producing atherosclerosis (Clark et al., 2009).

Since Apo E gene is able to regulate lipid levels it is not surprising that the mutations and polymorphisms of the Apo E gene can affect protein function owing to the complexity of the Apo E structure-function. This is because the receptor binding properties of Apo E are strongly influenced by isoform-specific amino acid differences as well as the state of the protein modification by lipid (Hauser et al., 2011).

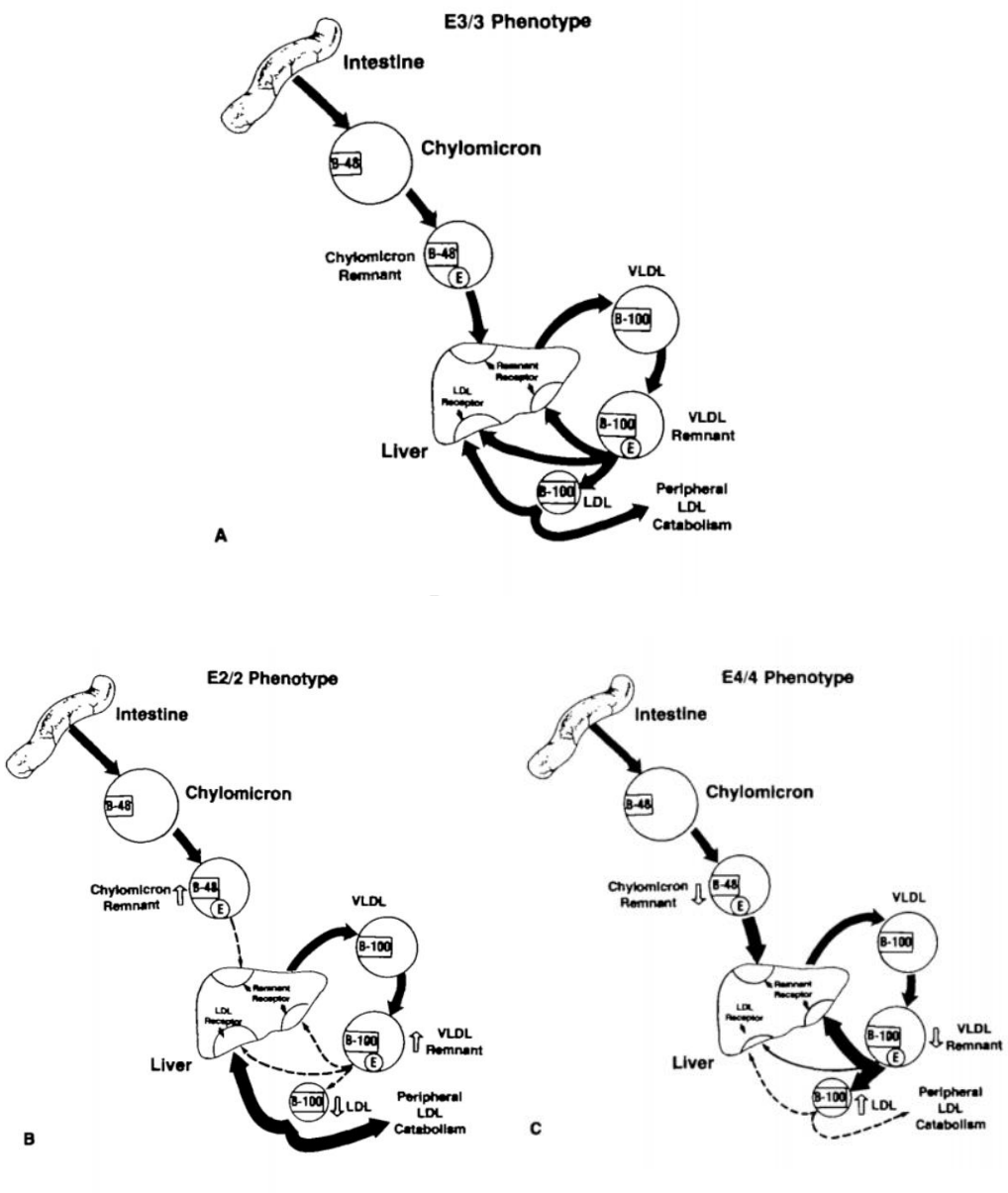


Figure 1.7: Schematic representation of the metabolism of Apo E-containing lipoproteins in humans with different genotypes.

Source: (Davignon et al., 1988)

The width of the **solid arrows** is proportional to the rate of conversion or binding and degradation of the respective lipoprotein fraction, while the size of the receptor in the liver is proportional to its binding activity. An **open arrow** beside a lipoprotein particle indicates the change in the concentration of that particle in an individual with the indicated APOE genotype when compared to the ε3/3 genotype.

1.6 Frequency of APOE polymorphism

Frequency of the common polymorphism at the Apo E locus (ε2, ε3 and ε4) are different between populations and these can effect on plasma lipids and cardiovascular disease in the populations (Hallman et al., 1991), thus several studies on Apo E gene polymorphisms was conducted in varies of populations particularly (Bennet et al., 2007). A cardiovascular cohort study in Singapore showed the highest rate of CAD among Asian Indians and followed by Malays and Chinese (Lee et al., 2001). Furthermore, Asian Indians also exhibited the lowest concentration of HDL-C and highest concentration of LDL-C compared to those observed in Chinese (Tan et al., 1999). Both Apo E genotypes and environmental factors can influence the lipid profile and CAD risks among all the ethnic groups in Singapore (Tan et al., 2003). Another study carried out by Seet et. al (2004) showed the most frequent genotype and allele among Malaysians were ε3/ε3 and ε3 respectively. Indians had a high frequency of the ε4 and ε3 allele compared to the other two ethnic groups. Chinese had a high frequency of the ε2 allele and lowest frequency of ε4 allele. Their finding also similar to (Hallman et al., 1991) that Indians subjects had significantly higher frequency of ε4 and lower frequency of ε2 compared to other ethnic groups. It is corresponds to which Indian diabetes patients are more possibility to have CAD complication.

A previous study among South Asian population by Sapkota et. al (2015) suggests a modest impact of Apo E genetic variation for increasing cardiometabolic susceptibility in patients with and without T2DM. Their results also suggest significantly improved cardiometabolic outcomes among high-risk Apo ε4 carriers in response to antidiabetic therapy. In general, Apo E gene is associated with lipoprotein concentration neither in diabetic nor in non-diabetic individuals (Kataoka et al., 1996). Apo E polymorphism and differences in serum lipid level are also dependent on ethnicity factor (Jemaa et al., 2006).

1.7 Restriction fragment length polymorphism (RFLP)

There are a variety of methods used in the analysis of APOE polymorphism (Davignon et al., 1988). Earlier isoelectric focusing technique on genotyping analysis is based on charge differences between proteins limits was used for the detection of rare variants that can present the same charge as common isoforms. Although there have been improvements in this technique, false results may occur due to the variability of sialylation. This happened especially in abnormal conditions such as diabetes mellitus (Snowden et al., 1991). Several polymerase chain reaction (PCR) techniques can be used for Apo E genotyping. The weakness of PCR and allele-specific oligonucleotide (ASO) hybridization are involved numerous and very cautious hybridization procedures. Moreover, sometimes false genotype was produced. Because of that the molecular technique of Apo E genotyping has been developed (Richard et al., 1994)

In the clinical laboratory, Apo E genotyping was done by digestion of polymerase chain reaction (PCR) product by restriction endonucleases and separation of the fragmented genomic product by electrophoresis analysis. This is referred as restriction fragment length polymorphism (RFLP) (Bolla et al., 1995, Guo et al., 1993, Hixson and Vernier, 1990, Kontula et al., 1990, Zivelin et al., 1997). This technique has advantages such as can be performed on multiple samples simultaneously and the fragment patterns can be visualized. It is time-consuming without hybridization and sequencing steps. Besides that, in vitro amplified DNA is not methylated, thus a wide variety of restriction enzymes can be used (Hixson and Vernier, 1990). The major advantage of this technique is the simplicity of the detection method by using polyacrylamide gel electrophoresis, which is no radioactive materials are required (Kontula et al., 1990). In addition, it is less costly because it does not involve use of advanced instruments and extensive staff training.

However, some disadvantages of this technique are not suitable for high-throughput analysis and the exact genotyping cannot be achieved when there is more than one nucleotide variation in a restriction enzyme recognition site. Several variants of RFLP-PCR have been developed including the techniques using a gel-free method such as PCR combined with restriction fragment melting temperature (PCR-RFMT), amplified fragment length polymorphism (AFLP), terminal restriction fragment length polymorphism (T-RFLP) and inverse PCR-based amplified restriction fragment length polymorphism (iFLP) (Henrik 2012).

1.8 Problem statement

APOE gene polymorphism is associated with atherosclerosis and plays critical roles in lipid metabolism. The effects of the APOE polymorphism on lipid profile has been shown among healthy individuals and diabetes population. Various studies on the association of the *APOE* gene polymorphism among diabetes patients with coronary artery disease (CAD) also has been investigated in the last few years.

However, there is no report among the Malaysian population in regard to the association of *APOE* gene polymorphism among patients with T2DM and CAD patients. Thus, this study was conducted in order to determine the distribution of APOE polymorphism among T2DM and CAD patients and the association of APOE polymorphism with lipid profile.

1.9 General objective

To study the association of *APOE* gene polymorphisms with lipid profile among diabetic patient with and without coronary artery disease.

1.10 Specific objectives

- To determine the *APOE* gene polymorphism among diabetic patients with and without CAD.
- To correlate the *APOE* gene polymorphism with lipid profile.

CHAPTER 2

MATERIALS AND METHODS

2.1 Sample

2.1.1 Sampel size determination

Sample size was determined by dichotomous test and calculated using PS Software as shown below:

Level of significance, $\alpha = 0.05$

Power of the study = 0.8

Probability of exposure among controls, $P_0 = 0.69$

Probability of exposure among cases, $P_1 = 0.95$ (probability in cases is higher by 25%).

Ratio of control group to T2DM patient, m = 2

Thus, sample size:

= 58 + 25% drop out of T2DM patients without CAD = 78 control patients

= 29 + 25% drop out of T2DM patients with CAD = 38 case-patients

From this calculation, a total of 78 subjects were needed for T2DM without CAD group and 38 patients were needed in T2DM with CAD group. Total samples that had been collected were 159 samples.

2.1.2 Subjects

A total of 45 adult patients aged 30 – 60 years from 159 samples collected, were selected among the T2DM patients attending Diabetic Clinic at the Hospital Kuala Lumpur (HKL). The patients that fulfil the inclusion and exclusion criteria were selected from the patient's record. The inclusion criteria for the patient with T2DM is either fasting glucose serum level > 7.0 mmol/L, modified oral glucose tolerance test > 11.1 mmol/L or HbA1c > 6.5%. The second group studied was those with T2DM and had any of the following conditions: (1) stable angina (2) acute coronary syndromes (ACS) with unstable angina, (3) non-ST elevation myocardial infarction (NSTEMI) and (4) ST elevation myocardial infarction (STEMI). These symptoms were diagnosed by clinician and recorded. Patients who smoke, consume alcohol, pregnant and with hyperthyroidism were excluded from the study. Informed written consent was obtained from each individual before participation. The study was approved by the Ethics Committee of Ministry of Health Malaysia and Universiti Sains Malaysia.

2.2 Biochemical analysis

A total of 3 ml blood sample was collected in an EDTA tube for genotyping analysis. The biochemical analyses results were obtained from the Laboratory Database at HKL. They include: (1) fasting glucose (2) glycosylated haemoglobin (HbA1c) (3) fasting lipid profile (total cholesterol, triglycerides, low density lipoprotein cholesterol and high density lipoprotein cholesterol). Biochemistry assays were performed on Cobas 8000 modular analyser (Roche Diagnostic, USA). Serum glucose was measured with the enzymatic colorimetric assay (Glucose GOD-PAP, Roche Diagnostic, USA). Total serum cholesterol was measured with the enzymatic colorimetric assay (Cholesterol Chod-PAP, Roche Diagnostic, USA). HDL-C was determined enzymatically by cholesterol esterase