INVESTIGATING THE ASSOCIATION OF APOLIPOPROTEIN E (APOE) GENE POLYMORPHISM AND ITS ASSOCIATED FACTORS TO CARDIOVASCULAR DISEASE (CVD) IN TYPE 2 DIABETES MELLITUS (T2DM) PATIENTS

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by

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LIST OF SYMBOLS

°	Degree
C	Celsius
%	Percent
Bpm	Beats per minute
kg/m^2	kilogram per square meter
mA	Milliampere
mmHg	Millimeter of mercury
mg/dL	Milligrams per deciliter
mmol/mol	Millimoles per mole
V	Voltage

LIST OF ABBREVIATIONS

ACS	Acute Coronary Syndrome
APOE	Apolipoprotein E
ARC	Animal Research Centre
BMI	Body Mass Index
BP	Blood Pressure
CAD	Coronary Artery Disease
CHD	Coronary Heart Disease
CVD	Cardiovascular Disease
DM	Diabetes Mellitus
EDTA	Ethylenediaminetetraacetic Acid
FBG	Fasting Blood Glucose
GDM	Gestation Diabetes Mellitus
HbA1c	Haemoglobin A1c
HDLC	High Density Lipoprotein Cholesterol
HTN	Hypertension
IDF	International Diabetes Federation
IDL	Intermediate Density Lipoprotein
IHD	Ischemic Heart Disease
IPPT	Institut Perubatan Pergigian Termaju
LDL	Low Density Lipoprotein Cholesterol
MLR	Multiple Logistic Regression
NCD	Non-communicable Disease
NDR	National Diabetes Registry
NLM	National Library of Medicine

OCP	Oral Contraceptive Pills
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
SD	Standard Deviation
SLR	Simple Logistic Regression
T1DM	Type 2 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TAE	Tris-Acetate-EDTA
TC	Total Cholesterol
TG	Triglycerides
ULR	Ultra-Low Range
USM	Universiti Sains Malaysia
VLDL	Very Low-Density Lipoprotein
WHO	World Health Organization

LIST OF APPENDICES

MENGKAJI HUBUNGKAIT POLIMORFISME GEN APOLIPOPROTEIN E (APOE) DAN FAKTOR BERKAITANNYA DENGAN PENYAKIT KARDIOVASKULAR DALAM KALANGAN PESAKIT DIABETES MELLITUS JENIS 2

ABSTRAK

Gen Apolipoprotein E (APOE) dikenali secara meluas kerana fungsi pentingnya sewaktu proses metabolisme lipid dijalankan dan ia dikaitkan dengan peningkatan risiko penyakit kardiovaskular (CVD) di kalangan pesakit diabetes mellitus jenis 2 (T2DM). Mengawal faktor risiko dapat membantu untuk mengurangkan sebarang kemungkinan menghidap penyakit ini. Kajian ini adalah untuk menganalisis kaitan di antara polimorfisme gen dengan faktor risiko terpilih dan profil lipid para pesakit yang didiagnosis dengan T2DM dan CVD. Sejumlah 101 subjek berumur antara 18 dan 80 tahun telah menyertai kajian ini dan dikelaskan kepada dua kumpulan; 59 pesakit T2DM dengan CVD dan 42 pesakit tanpa CVD. Kajian penjenisan gen APOE telah dijalankan menggunakan teknik polimorfisme panjang fragmen restriksi (RFLP). Genotip ε3/ε3 adalah yang paling tertinggi di kalangan kedua-dua kumpulan pesakit T2DM dengan dan tanpa CVD, diikuti oleh genotip ε2/ε4, ε3/ε4, ε2/ε3, dan ε2/ε2. Walau bagaimanapun, genotip ε4/ε4 tidak terdapat dalam kedua-dua kumpulan. Keputusan regresi logistik berganda (MLR) menunjukkan, genotip ε3/ε3 (0.052) (95% Cl 0.003, 0.792), alel ε3 (34.830) (95% Cl 1.118, 1085.134), SBP (1.046) (95% Cl 1.002, 1.091) dan HbA1c (2.286) (95% Cl 1.577, 3.314) adalah berkaitan dan signifikan (nilai-p <0.05). Di samping itu, perkaitan antara polimorfisme gen APOE dan lipid menunjukkan bahawa genotip ε3/ε3 adalah signifikan di lipoprotein berketumpatan rendah (LDLC). Sebagai kesimpulan, alel ε3

adalah faktor risiko dengan mempunyai risiko T2DM dan CVD yang lebih tinggi, serta profil lipid yang lebih tinggi.

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ABSTRACT

Apolipoprotein E (APOE) gene is widely acknowledged for its crucial function in the process of lipid metabolism and it is associated with elevated likelihood of cardiovascular disease (CVD) among Type 2 diabetes mellitus (T2DM) subjects. Controlling the risk factors may help lower the likelihood of developing this disease. The current study has analysed the associations between the APOE gene polymorphism and selected risk factors, as well as lipid profiles among patients diagnosed with a combination of T2DM and CVD. A total of 101 subjects aged between 18 and 80 years old were recruited in this study and were classified into two groups; 59 T2DM subjects with CVD and 42 subjects without CVD. APOE genotyping was done using the polymerase chain reaction-restriction fragment length polymorphism (PCR-FRLP). The ε3/ε3 genotype was the most common among both groups of T2DM patients with and without CVD, followed by ε2/ε4, ε3/ε4, ε2/ε3 and ε2/ε2 genotypes. However, the ε4/ε4 genotype was not present in either group. Multiple logistic regression (MLR) results revealed; that the ε3/ε3 genotype (0.052) (95% Cl 0.003, 0.792), ε3/ allele (34.830) (95% Cl 1.118, 1085.134), SBP (1.046) (95% Cl 1.002, 1.091) and HbA1c (2.286) (95% Cl 1.577, 3.314) were relevant and significant (p-value <0.05). In addition, the association between APOE gene polymorphism and lipid profile has demonstrated that the ε3/ε3 genotype was significant at low-density lipoprotein cholesterol (LDLC). To sum up, the ε3 allele is

an independent risk factor, with a greater risk of T2DM and CVD, as well as higher lipid profiles.

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Over 80% of global deaths every year are related to cardiovascular disease (CVD). It is health issue, as indicated by the World Health Organization (WHO). CVD refers to cardiac complications caused by narrowed heart vessels that supply blood to the heart muscle. Plaque on the inside walls, or lining of the arteries stops the heart from receiving enough blood and oxygen, which can prompt a heart attack (1). Although lack of exercise and poor diet intake are related risk factors, certain genetic variants may also confer susceptibility to CVD. Early identification and efficient control of risk factors will prevent CVD complications. Consequently, numerous investigations have been conducted to evaluate the genetics of CVD, including the polymorphism of the Apolipoprotein E (APOE) gene.

The prevalence of diabetes mellitus (DM) among youth has increased dramatically each year. DM is the most common metabolic disease that refers to the hyperglycaemia state caused by abnormalities in either the body's secretion, or processing of insulin, or both (2). This condition does not have a cure, is progressive, and injurious at entire ages of the populace, but can be prevented (3). Type 1 Diabetes Mellitus (T1DM), which is insulin-dependent and Type 2 Diabetes Mellitus (T2DM), which is non-insulin-dependent, are the duo categories of diabetes. T1DM happens rapidly, since insulin production from the pancreas is destroyed, while T2DM occurs slowly, as insufficient insulin causes blood glucose levels to rise (4). Foreseeably, according to the International Diabetic Foundation (IDF), Malaysia would be a high-risk state for diabetics aged 20 to 79 by the end of 2030 (5).

The glycoprotein, APOE, is found in very-low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), high-density lipoprotein (HDL), chylomicron, and chylomicron remnants. Several Apolipoprotein gene families have been determined, including APO (A-I), APO (A-II), APO (A-IV), APO (C-1), APO (C-II), APO (C-III), and APOE (6). A total of 3,597 nucleotides are encoded by the APOE gene's four exons and three introns, which constitute 299 amino acid polypeptides (7). The ε2, ε3, and ε4 alleles are the most prevalent. APOE ε3 consists of cysteine and arginine at positions 112 and 158, respectively. APOE ε2 encloses cysteine at both positions, and ε4 has arginine at positions 112 and 158 (8). Among the general population, the ε3 allele is the most generally known and can be detected in every four out of five people, followed by ε4 and ε2 alleles, which are situated in the chromosome (9).

These alleles have been determined to be responsible for coding three isoforms, as well as ε2/ε2, ε4/ε2, ε3/ε2, ε3/ε3, ε4/ε3, and ε4/ε4 genotypes (10). Each APOE isoform interacts differently with these receptors (11). The ε3 is the most common form, encoded by the three alleles, and it binds to its receptors decently well. Compared to the ε3, the ε2 and ε4 forms have a lower and higher binding affinity, respectively. In consequence, ε2 carriers have shown low E-containing lipoprotein metabolism, which may lead to familial dysbetalipoproteinemia (12). APOE ε4 carriers have been linked to elevated cholesterol absorption, decreased sensitivity to statin treatment, and an increased possibility of atherosclerosis.

Although it is mostly secreted by the liver and intestine, certain tissues and cells, such as the brain and macrophages, can synthesise it locally (12). The APOE gene gives the instruction and guidance in the creation of protein. This protein interacts with fats

in the body to form lipoprotein, which are small molecules. Lipoproteins play an important role in transporting cholesterol along with other fats throughout the bloodstream (13). Thus, the APOE gene assumes a significant role in lipoprotein metabolism and lipid transport (14). Hence, this study was conducted to ascertain the association between APOE gene polymorphism, with selected risk factors, and its effect on lipid profile among diabetic patients with and without CVD.

1.2 Problem statement

DM is a chronic and debilitating illness that needs to be avoided, since cure is preferable to prevention, because this disease will lead to cardiovascular complications. Diabetes is a national health concern in Malaysia, and it is critical to raise awareness and work towards a solution, since this illness is spreading at an alarming pace. It is a result of obesity, greater life expectancy, and acceptance of sedentary lifestyles and physical inactivity. The metabolic syndrome is a collection of symptoms that are also acknowledged as dysmetabolic syndrome, or insulin resistance syndrome. Recognising these risk factors in T2DM patients is important for prescribing appropriate treatment regimens (15). Various studies regarding the association with the APOE gene polymorphism among patients with diabetes have been conducted in recent years. However, it is necessary to conduct a comprehensive study to implement a strong clarification of genetic variation and its impact on the occurrence of the disease, since studies in Malaysia are minimal and inconclusive.

1.3 Research Objectives

General objective

This study aimed to investigate the relationship of APOE gene polymorphism and its associated factors to CVD among diabetes patients.

Specific objective

This study aimed on a few objectives as the following:

i. To determine the association of APOE gene polymorphism among T2DM patients with and without CVD.

ii. To determine the associated factors for CVD among T2DM patients. The associated factors include APOE gene polymorphism and selected risk factors such as BMI, BP, glucose, TC, HbA1c, LDLC, HDLC, age, sex and family history.

iii. To assess the association of APOE gene polymorphism with lipid profile among T2DM patients with CVD.

1.4 Hypothesis

i. There are association of APOE gene polymorphism among T2DM patients with and without CVD.

ii. There are association between APOE gene polymorphism and selected risk factors (SBP, HbA1c) among T2DM patients with CVD.

iii. There are association between APOE gene polymorphism and lipid profiles.

CHAPTER 2

LITERATURE REVIEW

2.1 Diabetes Mellitus (DM)

Chronic diseases have grown increasingly common as a result of population increase and technological development. With one of the greatest critical instances of health issues humans currently face being DM, every nation around the globe is impacted. DM has become more common over time, with 422 million individuals being diagnosed in 2014, which is an increase from 380 million in 1980 (16). Many worldwide expert organisations believe that DM is a common metabolic disorder prompted through the failure of the pancreas to produce enough insulin hormone, otherwise known as hyperglycaemia, leading to a rise in blood glucose level. Insulin is an energy abundance-related hormone that has a variety of effects on carbohydrate, lipid, and protein metabolism (17).

Even though the pathological progression after the onset of the disease may be identical, numerous studies have revealed that DM may be divided into two types based on aetiologies (18), which are T1DM and T2DM. DM is a vascular disease, since it is associated with both macro- and microvascular problems, including retinopathy, nephropathy, peripheral neuropathy, dyslipidaemia, and cardiovascular diseases. The clinical signs and symptoms, as well as the diagnostic methods for these issues are well understood (19). The diabetes duration and level of metabolic control can affect the development of these chronic diseases (20).

T1DM was previously described as insulin-dependent, or a juvenile onset of diabetes mellitus. T1DM often appears during childhood or adolescence, although it may appear during any instance of an individual's life, including in their 80s and 90s (21). Due to dehydration and a catabolic state with low glycogen, proteins, and

triglycerides, T1DM patients will lose weight over time despite having a normal, or increased appetite. T2DM is related to several unknown environmental factors. T2DM may also be described as adult-onset DM, or non-insulin dependent DM. T2DM affects a significant number of people who are asymptomatic and may go undiagnosed for many years (22). In recent years, the incidence and prevalence of T2DM in teenagers have increased substantially. Obesity, age, genetic susceptibility, racial/ethnic origin, and women with a history of gestational diabetes mellitus (GDM) are all risk factors for T2DM (23). Another risk factor is comorbidity, with hypertension or dyslipidaemia. Table 2.1 lists the clinical characteristics of individuals who may have diabetes (24).

Table 2.1 Clinical Characteristics of Patients with Type 1 and Type 2 Diabetes Mellitus (DM) (24)

Features	**Type 1 diabetic mellitus (T1DM)**	**Type 2 diabetic mellitus (T2DM)**
Age at onset	Usually, <20 years	Usually, >30 years
Body mass	Low (wasted - normal)	Obese
Plasma insulin	Low or absent	Normal to high initially
Plasma glucagon	High, can be supressed	High, resistant to suppression
Plasma glucose	Increased	Increased
Insulin sensitivity	Normal	Reduced
Therapy	Insulin	Weight loss, thiazolidinediones, metformin, sulfonylureas, insulin

This chronic disease, which is reliant on blood sugar control, affects several organs (25). If left untreated, it may cause cardiovascular illness, renal failure, blindness, nerve damage, and other complications. Consequently, obesity and sedentary lifestyles may become more common (26). Despite being a serious health condition, diabetes can be significantly reduced by losing body weight, increasing physical activities, and adopting healthier eating habits consisting of whole grains, fruits, and

vegetables, as well as seafood. Health professionals should also be consulted regarding monitoring and managing blood glucose, cholesterol, and blood pressure.

T2DM, a big factor in developing CVD, is well-known for clustering with other symptoms, such as dys1ipidaemia, hypertension, and abdominal obesity (27). Each risk factor has a high CVD risk, and when they are combined, the person with T2DM faces a significantly higher CVD risk.

2.2 Cardiovascular Disease (CVD)

Cardiovascular disease (CVD) encompasses hypertension, atherosclerosis, transient ischemic attack (TIA), stroke, and peripheral vascular disease (PVD), and it has a complicated aetiology (28). In order to deliver blood and supply oxygen to the entire body, the heart is a self-adjusting two-stage muscle pump that operates in unison (29). Heart vessels take low-oxygenated blood from the body and carry it to the lungs for reoxygenation through the pulmonary trunk. The left-side pulmonary veins supply blood from the lungs to the aorta, where it is distributed throughout the body.

CVD is a chronic illness that advances over time and usually reaches an advanced stage by the time symptoms emerge (30). Atherosclerosis is the most frequent cause of CVD. The most well-known atherosclerotic CVD cases include ischaemic heart disease (IHD), cerebrovascular disease or stroke, hypertensive heart disease, peripheral vascular disease, and atherosclerotic aortic aneurysm (31). The prevalence of CVD is expected to rise, owing not only to growing obesity rate, and an epidemic of diabetes along with metabolic diseases, but also to population ageing (32). Since CVD risk factors are notable, including calorie-dense prepared food varieties, stress, an inactive lifestyle, and cigarette smoking, active preventative endeavours are required to battle this advancing scourge (33).

CVD is one of the dominant reasons for fatality and disability in most countries, including Malaysia, and it is projected to remain so until 2030 (34). Smoking, unhealthy diet, and physical inactivity are all significant risk conditions for CVD. In developed countries, several studies have shown that lifestyle risk factors exist in groups and are not equally distributed across populations. Throughout 2013, circulatory system disorder was the main cause of death in hospitals across Malaysia, accounting for 24.7% of all fatalities (35). Age, gender, dyslipidaemia, hypertension, T2DM, and smoking

have all been recognised as risk factors for CVD. CVD risk prevention and control are important aspects of CVD prevention. However, in order to enhance CVD prevention, lifestyle cardiovascular risks and cardiovascular hazards should be addressed (36).

According to the WHO, the age-adjusted CVD mortality rates in West Asian, South Asian, and South-East Asian nations were among the highest. Non-communicable diseases (NCDs) account for 73% of all fatalities in Malaysia, according to the WHO NCD national profile in 2014. In comparison to other NCDs, such as cancer, chronic respiratory diseases, and diabetes, CVD caused 36% of all fatalities in all ages and both sexes (37). This discovery may be useful in the future for creating, or formulating preventive measures, as well as increasing the information on cardiovascular risk reduction for everyone.

2.3 Epidemiology of DM and CVD

Numerous forms of CVD knowledge have been shared around the world, as well as between diverse communities and classes. The categories, symptoms, risk factors, diagnoses, and treatments of cardiovascular disorders were among the knowledge shared (38). However, literature on cardiovascular awareness has shown some surprising findings. The likelihood of developing CVD can be reduced by controlling the modifiable and unmodifiable risk factors. Modifiable risk factors include hypertension, dyslipidaemia, diabetes, prediabetes, overweight, obesity, smoking, inactivity, an irregular lifestyle, and tension. Meanwhile, the unmodifiable risk factors include age, ethnicity, and a family background of CVD (39). Malaysia has experienced significant socioeconomic growth in recent decades (40).

King et al. (41) predicted that the number of individuals living with DM will continue to rise over time. LDL is the primary cholesterol-bearing lipoprotein in diabetics and a crucial predictor of atherosclerosis. Due to altered lipoprotein metabolism, triglycerides, VLDL remnants, and IDL have been found to be extremely atherogenic. LDLC has been shown to be a CVD marker in diabetics. The risk of having the highest non-HDL cholesterol among diabetic women is greater than the risk of having the highest LDLC, or TG alone. Generally, LDL concentrations in diabetic patients should not be greater than their non-diabetic equivalent (42). Even normal LDL levels are extremely atherogenic owing to alterations in the structure of LDL particles, such as density, oxidation potential, and glycation (43).

According to the Malaysian Department of Statistics in 2019 the top cause of deaths among Malaysians was IHD. IHD stood its ground as the primary cause of death, comprising of 15.0% of all 109,164 medically certified fatalities. Pneumonia was the second largest cause of death (12.2%), along with cerebrovascular diseases (8.0%),

transportation accidents (3.8%), and malignant neoplasm of the trachea, bronchus, and lungs (2.4%) (44). These data are graphically represented in Figure 2.1.

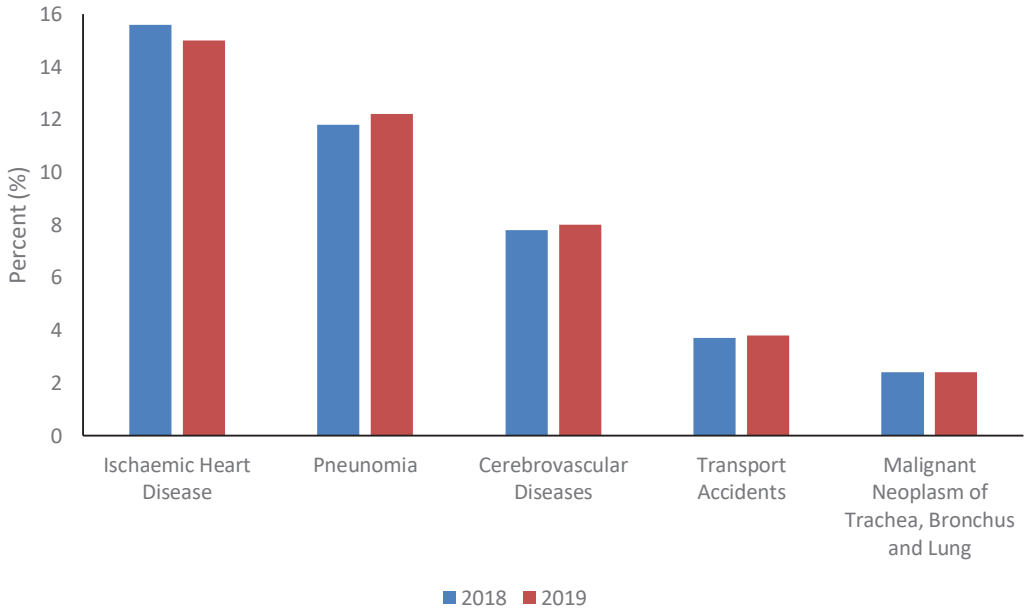

Figure 2.1 Principal causes of death (44)

According to the major causes of death by gender in 2019, IHD has prevailed as the most common cause of mortality among the males in Malaysia, accounting for 69.4% compared to females. Most people who died under the age of 40 were in transport accidents, whereas those over the age of 41 mostly died because of IHD. The risk of CVD increased significantly with age in both genders. CVD continues to be the major cause of death among Indians, Bumiputera, and Chinese, accounting for 20.4%, 14.5%, and 14.0%, respectively (44).

2.4 Lipid profile

The risk of CVD increases significantly with age in both sexes. Furthermore, the total serum cholesterol levels will rise as people become older. The growth rates of serum cholesterol levels among women increase dramatically between the ages of 60 and 65 (45). Blood pressure will also rise with age, with women suffering more than men. The reduction in oestrogen synthesis after menopause shifts female lipid metabolism into an atherogenic state, lowering high-density lipoprotein cholesterol (HDLC), while increasing low-density lipoprotein cholesterol (LDLC), total cholesterol (TC), triglycerides (TG), and lipoprotein (a) levels (46).

T2DM patients with tight glycaemic and CVD function, as well as hypertension and dyslipidaemia have lower CVD morbidity and mortality. Serum lipid concentrations are used to monitor the relationship between CVD and cholesterol-lowering therapies among people with and without diabetes. The TC, HDLC, LDLC, and TG comprise the lipid profile. People who do not have diabetes, but have an abnormal lipid profile are nonetheless still at risk of developing CVD. Such polymorphisms occur with the pathogenesis of atherosclerosis, which leads to CVD, and this genetic mutation has the potential to change plasma lipid levels (47).

Cigarette smoking, asthma, low HDLC, a family history of early CVD, and age are all significant risk factors for CVD, as oppose to LDLC. The interrelation between LDLC levels and the chances of CVD remains consistent along a broad range of LDLC levels, from low to high (48). Table 2.2 shows the classification of LDLC (a), as well as the HDLC (b), TG (c), and TC (d) classifications (49).

Table 2.2 Classification of low-density lipoprotein cholesterol (LDLC), high-density lipoprotein cholesterol (HDLC), triglycerides (TG), and total cholesterol (TC) (49)

a) LDL cholesterol (LDLC)

Lipoproteins levels (mg/dL)	**Classifications**
<100	Optimal
100-129	Near optimal/above optimal
130-159	Borderline high
160-189	High
≥190	Very high

b) HDL cholesterol (HDLC)

Lipoproteins levels (mg/dL)	**Classifications**
<40	Low
≥60	High

c) Triglycerides (TG)

Lipoproteins levels (mg/dL)	**Classifications**
<150	Normal
150-199	Borderline high
200-499	High
≥500	Very high

d) Total cholesterol (TC)

Lipoproteins levels (mg/dL)	**Classifications**
<200	Desirable
200-239	Border high
≥240	High

Pharmacological therapy of blood pressure, blood lipids, and levels of glucose only addresses a portion of the negative effects of poor lifestyle choices. Patients who are taking medication for hypertension, high cholesterol, or DM are more prone to having a heart attack, or stroke than people who do not have risky lifestyle behaviours. Diabetes patients have greater TG and LDLC levels, but lower HDLC levels than non-diabetic people. Dyslipidaemia is defined in T2DM patients as high TG levels, especially triglyceride-rich VLDL, and low HDLC levels (46). LDLC levels in diabetics are often comparable to those in non-diabetics. Meanwhile, patients with T2DM have elevated proportions of smaller, denser, oxidised LDLC particles, which may contribute

to atherogenicity, even when LDLC levels are not elevated. As a consequence, high TG levels and low HDLC levels are the best predictors of CVD among patients with T2DM.

Thus, the TC/HDLC and LDLC/HDLC ratios are utilised to predict CVD. Several lipid indicators have been linked to residual cardiovascular risk. LDLC is a huge factor that may lead to CVD. Arsenault et al. (51) conducted a research study in 2010 to determine if other lipid indicators, such as non-HDLC levels, TG levels, and the TC/HDLC ratio are still associated with an increased risk of CVD, irrespective of LDLC levels. Although LDLC is the primary goal of lipid-lowering treatment, other aspects of the lipoprotein-lipid profile may be more directly related to the risk of CVD. According to this prospective study, participants with high non-HDLC levels, high TG levels, or an elevated TC/HDLC ratio faced a greater CVD risk, regardless of their plasma LDLC levels. CVD risk management algorithms and lipid objectives in lipid-lowering trials must incorporate readily accessible measures such as treatment of non-HDLC (52). In 2010, Alvim et al. (53) found that the ε4 is related to a higher level of LDLC and a higher risk of CVD, while the ε2 is associated with a lower level of LDLC and a reduced risk of CVD. Because the APOE gene is associated with lipid levels in the body, it is critical to comprehend how lipid levels may increase the chances of CVD (53).

2.5 Apolipoprotein E (APOE) gene

The proteinaceous component of lipoprotein complexes is called apolipoproteins, or apoproteins. Plasma apolipoproteins are polypeptide chains that form the structural elements of lipoprotein particles (54). Many of these apolipoproteins have a helical domain that is unconcerned at binding and transporting lipids. They exist in different forms and are labelled as “APO” and trailed by letters A, B, C, and so on for each form. Each lipoprotein class has one or more types, which contribute in various proportions to the composition, ranging from 70% in some HDLC types to just about 1% in some chylomicrons (55). Table 2.3 lists numerous factors, such as molecular weight (MW), chromosomal position, movability, primary source, lipoprotein interaction, and structure to distinguish these apolipoprotein forms.

Table 2.3 Apolipoprotein forms and factors included molecular weight (MW), chromosomal location, movability, primary source, lipoprotein association and function (11)

Apolipoprotein	Apo A-I	Apo A-II	Apo A-IV	Apo B-48	Apo B-100	Apo C-I	Apo C-II	Apo C-III	Apo E
Molecular Weight (MW)	29016	17414	44465	240800	512723	6600	8900	8800	34000
Chromosomal Location	11	1	11	2	2	19	19	11	19
Movability	√	√	√	X	X	√	√	√	√
Primary Source	Liver, Intestine	Liver	Intestine	Intestine	Liver	Liver	Liver	Liver	Liver
Lipoprotein Association	HDL, chylomicrons	HDL, chylomicrons	HDL, chylomicrons	Chylomicrons	VLDL, IDL, LDL, Lp (a)	Chylomicrons, VLDL, HDL	Chylomicrons, VLDL, HDL	Chylomicrons, VLDL, HDL	Chylomicron remnants, IDL, HDL
General Function	Structural protein for HDL, Activates LCAT	Structural protein for HDL, Activates hepatic lipase	Activates LCAT	Structural protein for chylomicrons, Secretion of triglyceride from the intestine	Structural protein, Ligand for LDL receptor, Secretion of triglyceride from liver	Activates LCAT, Inhibits clearance of chylomicrons	Co-factor for LPL	Inhibits LPL, Inhibits clearance of chylomicrons	Ligand for LDL receptor, Facilitates uptake of chylomicron remnant and IDL

Apolipoproteins assume three significant roles in the digestion of lipoproteins, including maintaining structural stability as a hydrophilic factor, actuating, or restraining certain essential catalysts engaged with lipoprotein metabolism, and intervening lipoprotein recognition by acting as ligands for specific cell 34 surface receptors (56). APOE gene acts as a ligand for several receptors, including the chylomicron remnant receptor, the LDLC receptor, and the LDLC receptor with antigen to regulate the absorption of different lipoproteins. Thus, this will enhance the interaction between lipoproteins and proteoglycans. Instead of concentrating on APOA, APOB, APOC, and other proteins, the current research has concentrated on the APOE gene, which is one of the most typical ones. It has the greatest effect on plasma lipid levels of any single gene polymorphism on a populace level. The APOE gene is polymorphic (57), while the ε2, ε3, and ε4 are three common alleles that each code for three significant isoforms to produce six common phenotypes.

2.6 APOE and Diabetes Mellitus (DM)

In 2014, a case-control study was conducted in Saudi Arabia, with 898 genetically unrelated Saudis involving 438 subjects with T2DM and 460 subjects without T2DM (58). The statistical package for the social sciences (SPSS IBM Inc., Chicago, IL) software was applied to analyse background details and family history of all participants. The direct counting method was utilised to assess the genotype and allele frequency distributions of the case and control groups. Their findings showed an association between APOE ε2 allele in both lipid profile and T2DM. The ε4 allele was also proven to be a strong predictor of lipid status, with a connection between ε4, T2DM, and obesity. Overall, the study results showed an association between APOE polymorphisms and elevated incidences of T2DM in Saudi Arabia. As a consequence, if it goes untreated, it may cause CVD, renal failure, blindness, nerve damage, and other complications.

Rahman et al. (59) selected 102 Malaysian aged 40 years old and older for an analytic investigation; 51 individuals with diabetes and 51 without diabetes. Blood samples were collected from fasting individuals during an initial period and placed in two vacutainers overnight for 8 hr or more. Then, plasma was extracted from the blood samples soon after it was drawn and kept at -80 °C until further analysis. Additionally, EDTA vacutainers holding whole blood samples were likewise kept under identical temperatures. The blood sample of fasting individuals was analysed for fasting blood glucose (FBG), and their APOE genotype was established using restriction fragment length polymorphism (RFLP) genotyping. Statistical analyses were conducted using version 18.0 of the Predictive Analytics Software (PASW). This study aimed to find the correlation between the frequency of APOE allele and FBG fixation among T2DM patients.

Luo et al. (60) examined prior research to determine the relationship between APOE gene polymorphisms (ε2/ε3/ε4) and CVD risk among individuals with T2DM. They searched the Embase, PubMed, China National Knowledge Infrastructure (CNKI), and Wanfang databases for all published research starting from August 2, 2017. Stata 12.0 was used for all statistical analyses in their study. According to their findings, the APOE gene was more strongly associated with diabetics than with healthy individuals. Furthermore, the APOE gene ε4 mutation was linked to an elevated risk of coronary artery disease (CAD) among T2DM patients, while the ε2 variation was unrelated to the disease. Thus, they recommended further study with a bigger sample size to evaluate the gene-environment interaction. Their aim was to demonstrate a definitive connection between APOE gene ε2/ε3/ε4 mutations and CAD risk development among T2DM patients. Their study results were supported by the results obtained by Irie et al. (61).

Peila et al. (62) conducted a study with 2,574 people; 70% were non-diabetic and 30% were diabetic patients. Their population-based study was focused on a large number of people. The FBG test was used to assess the fasting lipid profile, which comprised TC, TG, HDLC, and LDLC. Diabetes was shown to have a slightly greater risk of cardiovascular illness, according to the results. Diabetes and the APOE polymorphism were also linked to APOE ε2 allele carriers.

According to previous studies (58–62), those with diabetes have a higher risk of CVD. Continued research in this area will help establish an association between the APOE polymorphism and CVD risk factors.

2.7 APOE and Cardiovascular Disease (CVD)

More than 80% of fatalities worldwide are attributed to CVD and this percentage is expect to increase in future years according to the WHO. CVD damages the arteries that provide oxygen and nutrients to the heart muscles through blood circulation. Thus, the APOE ε2 and APOE ε3 alleles may elevate the risk of heart disease (63).

Mooijaart et al. (64) studied the association between APOE genotypes and CVD risk among elderly individuals. A total of 546 participants were randomly selected for this study. Unrelated to APOE genotype or plasma lipids, they found that high plasma APOE levels preceded an increase in circulating C-reactive protein (CRP), which was significantly associated with CVD mortality among the elderly. The mortality rates for the carriers of ε2 and ε4 alleles were found to be identical. Risk factors for CVD were also found to change with age, whether due to a change in lifestyle, selective survival, or physiologic changes caused by ageing (65). Further research on the role of APOE alleles in gender-specific survival in old age is required according to Rosvall et al. (66).

Marrzoq et al. (67) conducted a study, with 137 participants selected randomly among Gaza populations, with 69 CHD patients and 68 as control. The goal of their research was to determine the link between APOE gene polymorphism, lipid levels, and CHD. Blood samples from the patients and controls were kept at 4 °C for APOE genotyping and lipid profile investigations. Their findings showed that the APOE ε3/ε3 genotype was the most common genotype in both the control and CHD groups. APOE ε2/ε3 and APOE ε4/ε3 were the next most common genotypes. Other genotypes, such as APOE ε2/ε2, APOE ε4/ε4, and APOE ε2/ε4 were not found in the tested patients. There were no statistically significant variations in APOE genotypes between the patient and control groups. Thus, more research into the genetic origins of CHD is required, since this was the first study of its kind in the Gaza Strip.

2.8 APOE, Diabetes Mellitus (DM), and Cardiovascular Disease (CVD)

An association between cardiovascular system abnormalities and lipoprotein-related processes was found among diabetics. Sudong Liu et al. (68) studied the association between APOE gene polymorphism and CVD risk among people with T2DM. This cross-sectional study involved 924 participants that were separated into four groups. There were 211 participants in the control group, also known as the healthy group without diabetes or CVD. Then, there were 247 T2DM patients who did not have CVD, 232 non-T2DM patients who did have CVD, and 234 T2DM patients who did have CVD. To summarise the results, T2DM and CVD patients showed higher levels of ε3/ε4. Patients with non-T2DM, or T2DM with CVD have more ε4 alleles than those in the control group.

In Malaysia, Ashari et al. (69) conducted clinical research to investigate the relationship between APOE gene polymorphism and CHD risk prediction among diabetic patients. A total of 115 T2DM patients who met the criteria were chosen; 78 patients without CAD and 37 patients with CAD. The collected data were analysed using SPSS 22.0 and the ε3 allele was the most frequent in both populations. These findings showed that T2DM patients with CAD who carried the ε4 allele have higher levels of LDLC and HDLC alleles. To establish the occurrence of the ε4 allele as a risk factor for CAD among T2DM patients, future study should include a bigger sample size.

Apart from that, 714 people participated in a study conducted by Vaisi-Raygani et al. (70) in Iran. There were 152 T2DM patients with CAD, 262 non-T2DM patients with CAD, and 300 healthy volunteers who served as controls. According to their study, T2DM patients with the APOE ε2 and ε4 alleles showed a higher risk of developing

CAD than non-diabetic individuals in Iran's western population. Furthermore, the ε4 allele has higher chances of being associated with CAD than the ε2 allele.

Participants at the Siriraj Hospital in Bangkok were recently diagnosed with T2DM. This study by Chaudhary et al. (71) enlisted the participation of 451 people, with 149 healthy subjects as controls, 155 T2DM subjects without CAD, and 147 T2DM subjects with CAD as case-patients. T2DM risk factors and CAD were identified using univariate and multivariable logistic regression analysis. Their results were supported by the results obtained by (70), which identified the ε4 allele as an independent risk factor in the link between T2DM and CAD.

2.9 Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP)

The PCR-RFLP technique was selected for analysing the APOE genotype in this current study. This technique has several advantages, such as able to analyse multiple samples simultaneously and offer visualisation of the fragment patterns. However, it is time-consuming without hybridisation and sequencing steps. Additionally, in vitro amplified DNA is not methylated, thus, a wide variety of restriction enzymes can be used (72). The major advantage of this technique is the simplicity of the detection method, because it uses polyacrylamide gel electrophoresis, which does not require radioactive materials (73). It is also less costly because it does not require advanced instruments and extensive staff training.

The 5' part of exon 4 of the human APOE gene at chromosome 19q13.13-19q13.32 coding for amino acids 61-174 was amplified using polymerase chain reaction (PCR) (74). Figure 2.2 shows that the human APOE gene exhibits three common variants (ε2, ε3, and ε4) that differ from each other at residues 112 and 158 in the mature protein. The ε4 has arginine and the ε2 has cysteine at both sites, whereas ε3 has cysteine at site 112 and arginine at site 158 (75). A section of the APOE gene that contains the genotype differentiating sites was amplified using PCR. The primer sequences were as follows:

F6	upstream primer	5'-TAAGCTTGGCACGGCTGTCCAAGGA-3'
F4	downstream primer	5'-ACAGAATTCGCCCCGGCCTGGTACAC-3'