A PILOT STUDY ON ASSOCIATION OF LIPOPROTEIN A (*LPA)* GENE COPY NUMBER VARIATION (CNV) AND APOLIPOPROTEIN E (*APOE)* GENE POLYMORPHISM WITH ACUTE MYOCARDIAL INFARCTION (AMI) IN YOUNG ADULTS

BY

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A thesis submitted in fulfillment of the requirement for the degree of Master of Health Sciences (Clinical Pathology)

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ABSTRACT

Acute myocardial infarction (AMI) is the most severe manifestation of coronary artery disease which is the leading cause of death globally. In Malaysia, there is an increasing number of AMI among young patients aged less than 45 years. The presentation of AMI at younger age suggests the possibility of genetic predisposition. Since *LPA* and *APOE* genes are known to be involved in lipid metabolism, they may contribute to the development of atherosclerosis leading to AMI. However, to date, there is no reported study on the association of *LPA* copy number variations (CNV) and *APOE* polymorphism with AMI in young patients. The aim of the current study is to assess the association of *LPA CNV* and *APOE* polymorphism with AMI in young adults. This study was conducted on 40 men grouped into 20 young acute myocardial infarction (YAMI) patients and 20 healthy controls. DNAs were purified from blood. The *APOE* genotyping was performed using multiplex PCR technique while *LPA CNV* was performed using digital PCR and the analysis of copy number was performed using QIAcuity Software Suite version 1.2 (QIAgen, Germany). Chi –square and Kruskall-wallis tests were used for data analysis. In order to improve the statistical power to signify the role of *APOE* polymorphism in CAD, meta-analyses were performed from this current result and selected studies among Asian populations using Comprehensive Meta-analysis version 3 software program. No significant association of *LPA* CNV with AMI was found (p = 0.459) in young adults. Although *APOE* genotypes and alleles were not significantly associated with AMI in young adults (p=0.484), this study found that E3/E3 genotypes and e3 allele were the commonest genotypes and allele. Lipid parameters were also found to be not significantly associated with *LPA* CNV status as well as *APOE* genotypes. However, from the current meta-analysis, *APOE* genotypes of E3/E3 was found to be protective whilst E3/E4 and e4 allele were found to increase the risk of CAD. In conclusion, this study found that there was no association of *LPA CNV* and *APOE* polymorphism with AMI in young adults, but the meta-analysis reaffirmed the role of *APOE* genotypes and e4 allele in the development of CAD. In pursuance of assessing the contribution of *LPA* gene CNV and *APOE* genotypes to our local population, a larger sample representation is recommended.

ملخص البحث

احتشاء عضلة القلب الحاد (AMI) هو أشد مظاهر مرض الشريان التاجي الذي هو السبب الرئيسي للوفاة على مستوى العالم. في ماليزيا، هناك عدد متزايد من AMI بين المرضى الشباب الذين تقل أعمارهم عن 45 عاما. عرض AMI في سن أصغر يشير إلى إمكانية الاستعداد الوراثي. بما أن جينات *LPA* و *APOE* معروفة بأنها تشارك في استقلاب الدهون، فقد تساهم في تطور تصلب الشرايين مما يؤدي إلى AMI. ومع ذلك، حتى الآن، لا توجد دراسة ذكرت عن ارتباط اختلافات عدد النسخ (CNV) *LPA* و *APOE* polymorphism مع AMI في المرضى الصغار. الهدف من الدراسة الحالية هو تقييم ارتبا *LPA* CNV و *APOE* polymorphism مع AMI في البالغين الشباب. أجريت هذه الدراسة على 40 رجلا تم تجميعهم في 20 مريضا من احتشاء عضلة القلب الحاد (YAMI) و 20 مريضا صحيا. تم تنقية DNAs من الدم. تم إجراء التنميط الجيني *APOE* باستخدام تقنية PCR متعددة بينما تم إجراء *LPA* CNV باستخدام PCR الرقمي وتم إجراء تحليل رقم النسخ باستخدام الإصدار 1.2 من QIAcuity Software Suite (Qiagen، ألمانيا). تم استخدام اختبارات Chi-square و Kruskall-Wallis لتحليل البيانات. من أجل تحسين القوة الإحصائية للدلالة على دور تعدد الأشكال *APOE* في CAD، تم إجراء التحليلات الوصفية من هذه النتيجة الحالية ودراسات مختارة بين السكان الآسيويين باستخدام برنامج التحليل التلوي الشامل 3. لم يتم العثور على ارتباط كبير بين *LPA* CNV و AMI ($p = 0.459$) في البالغين الشباب. على الرغم من أن الأنماط الجينية والأليلات *APOE* *لم تكن مرتبطة* بشكل كبير بـ AMI لدى البالغين الشباب ($p = 0.484$)، فقد وجدت هذه الدراسة أن الأنماط الجينية E3/ E3 وأليل E3 كانت أكثر الأنماط الجينية والأليل شيوعا. كما وجد أن بارامترات الدهون لا ترتبط ارتباطا كبيرا بحالة *LPA* CNV وكذلك الأنماط الجينية *APOE*. ومع ذلك، من التحليل التلوي الحالي، تم العثور على الأنماط الجينية *APOE* من E3/ E3 لتكون وقائية في حين تم العثور على أليل E4 /E3 و E4 لزيادة خطر CAD. في الختام، وجدت هذه الدراسة أنه لم يكن هناك ارتباط بين تعدد أشكال *LPA* CNV و *APOE* مع AMI في البالغين الشباب، لكن التحليل التلوي أعاد تأكيد دور الأنماط الجينية *APOE* وأليل E4 في تطوير CAD. سعيا إلى تقييم مساهمة الأنماط الجينية *LPA* GENE CNV و *APOE* لسكاننا المحليين، يوصى بتمثيل أكبر للعينات.

APPROVAL PAGE

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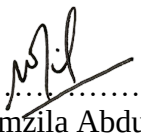

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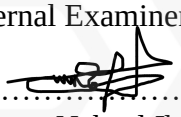

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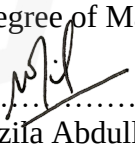

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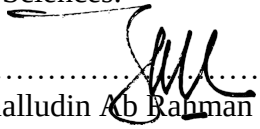

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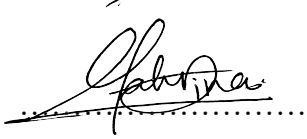

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LIST OF ABBREVIATIONS

°C	Degree Celsius
µl	Microlitre
4-AAP	4-aminoantipyrine
A	Ampere
ACS	Acute coronary syndrome
ADP	Adenosine-5'-diphosphate
AMI	Acute myocardial infarction
Apo(a)	Apolipoprotein a
ApoB	Apolipoprotein B
ApoB	Apolipoprotein B
APOE	Apolipoprotein E gene
Arg	Arginine
ATP	Adenosine triphosphate
BMI	Body mass index
Bp	Base pairs
CABG	Coronary artery bypass grafting
CAD	Coronary artery disease
CDC73	Cell Division Cycle 73
CDCP1	CUB domain containing protein 1
CDH13	Cadherin-13
CE	Cholesterol esterase
CHD	Coronary heart disease
CNV	Copy number variation
cTn	Cardiac troponins
CVD	Cardiovascular disease
Cys	Cysteine
DISC1	Disrupted-In-Schizophrenia-1

DNA	Deoxyribonucleic acid
dPCR	Digital polymerase chain reaction
DSP	Desmoplakin
ECG	Electrocardiogram
EDTA	Ethylenediamine tetraacetic acid
ELN	Elastin gene
Ɛ	Epsilon
g	Gram
gDNA	Genomic DNA
H2O2	Hydrogen peroxide
HDL	High density lipoprotein
HDL-C	High density lipoprotein cholesterol
HIC	High-income countries
HSPGs	heparan sulfate proteoglycans
IHD	Ischemic heart disease
IL-6ST	Interleukin-6 signal transducer
IQR	Interquartile range
Kb	Kilobase
KCNQ1	Potassium Voltage-Gated Channel Subfamily Q Member 1
kDa	Kilodalton
KIV-2	Kringle IV type 2
LDL	Low density lipoprotein
LDL-C	Low density lipoprotein cholesterol
LDLR	Low density lipoprotein receptor
LMICs	Low- and middle-income countries
Lp(a)	Lipoprotein a
LPA	Lipoprotein(a) gene
LQTS	Long QT syndrome
LXRs	Liver X Receptors
MI	Myocardial Infarction

ml	Millilitre
ng	Nanogram
NSTEMI	Non-ST segment elevation myocardial infarction
NTC	Non-template control
OxLDL	Oxidized LDL
PAI-1	Plasminogen activator inhibitor 1
PCI	Percutaneous coronary interventions
PCR	Polymerase chain reaction
PIK3C2G	Phosphatidylinositol-4-phosphate 3-kinase catalytic subunit type 2 gamma
PKP2	Plakophilin-2
PLG	Plasminogen
RCT	Reverse cholesterol transport
RET	Rearranged during transfection
rpm	Rotation per minute
SCD	Sudden cardiac death
SEAR	South-East Asia Region
SNP	Single nucleotide polymorphism
SREBP	Sterol regulatory element-binding protein
STEMI	ST- segment elevation myocardial infarction
TC	Total cholesterol
TG	Triglycerides
TLR-4	Toll-like receptor-4
t-PA	Tissue plasminogen activator
UV	Ultraviolet
V	Volt
VLDL	Very low-density lipoprotein
YAMI	Young acute myocardial infarction

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND AND JUSTIFICATION

Coronary heart disease (CHD) or ischaemic heart disease or coronary artery disease (CAD) is the primary cause of death in both developing and developed countries (Wang, 2020). Acute myocardial infarction (AMI) which is also known as the "classic heart attack" is grouped as coronary heart disease. It is defined as the necrosis of myocardial tissue caused by sudden loss of blood flow and ischemia to heart muscle due to the spasm or complete occlusion of the coronary artery by a thrombus (Wu, 2019). Mainly, AMI is caused by the disruption of a vulnerable atherosclerotic plaque. AMI is divided into ST- segment elevation myocardial infarction (STEMI) and non-ST segment elevation myocardial infarction (NSTEMI) (Reed et al., 2016).

In 2019, 17.9 million people died from cardiovascular disease (CVD) which accounts for 32% of global deaths. This number of total deaths is increased by 2% in the subsequent year. More than three quarters of CVD deaths were reported in low-and middle income-countries (WHO,2019). In Malaysia, ischemic heart disease remained as primary causes of death and over the last thirty years, there has been a continuous increase in the burden of cardiovascular disease (CVD) mortality and morbidity in Malaysia (Department of Statistic Malaysia (DOSM). The mortality rate from CAD is expected to soar in the coming decades due to worsening of metabolic risk factors (Ralapanawa & Sivakanesan, 2021). Data from Malaysian National Cardiovascular Disease Database (NCVD) – Acute Coronary Syndrome (ACS) registry showed that Malaysian are having myocardial infarction at younger age compared to the developed countries.

AMI may be caused by environmental, physiological factor and genetic predisposition. conventionally include modifiable and non-modifiable risk factors. Modifiable risk factors are those than can be controlled such as hypertension, dyslipidaemia and diabetes mellitus while the non-modifiable are those that cannot be

changed such as age, family history and sex (Yandrapalli et al., 2019). The genetic predisposition is classified as non-modifiable risk factors which is the most important risk factor that may explain younger onset of AMI patients in Malaysia.

Lipoprotein (a) *LPA* gene and Apolipoprotein E (*APOE*) gene are known to be involved in the regulation lipid metabolism which associated in the pathogenesis of atherosclerosis (Gudbjartsson et al., 2019; Hou et al., 2020) and hence AMI.

LPA gene is located at chromosome 6q25.3-q26. The variation in *LPA* locus encoding apo(a) is essential in the determination of circulation lipoprotein level known as lipoprotein a, Lp(a) (Enas et al., 2019b). Elevated Lp(a) levels have been linked to an elevated risk of coronary artery disease (CAD) and studies suggest that higher Lp(a) levels are linked to a greater risk of atherosclerosis, acute myocardial infarction, and ischemic stroke (Ugovšek & Šebeštjen, 2021) by accumulating in the intima of arteries and aortic valve leaflets, thereby contributing to the development of atherosclerotic lesions. In contrast to LDL cholesterol, Lp(a) infiltrate the intima independently and accumulates throughout the intima, showing a greater affinity for the vascular wall and promoting atherogenesis (Kiechl & Willeit, 2010).

The copy number variation (CNV) of the *LPA* gene, specifically the Kringle IV type 2 (KIV-2) CNV, is a major determinant of Lp(a) levels and explains 20-80% of the variance in Lp(a) levels (Kronenberg & Utermann, 2013). The KIV-2 CNV is transcribed into mRNA and translated into the apo(a) proteins of different sizes. More than 80% of all individuals carry two different-sized apo(a) isoforms, each inherited from one parent. Smaller apo(a) isoform up to and including 22 KIV-2 CNV is associated with higher Lp(a) levels and higher CAD risk independent of established risk factors. Therefore, the copy number of the *LPA* gene can play a significant role in determining Lp(a) levels and CAD risk (Enas et al., 2019; Nordestgaard & Langsted, 2016).

Meanwhile, apolipoprotein E (*APOE*) gene stands out as a consistent candidate associated with acute myocardial infarction (AMI), given its role in the development of atherosclerosis (Marais, 2021). *APOE* located at chromosome 19q13.2 and consist of 3 isoforms (ε2, ε3 and ε4) with 6 common genotypes which are E2E2, E2E3, E3E3,

E2E4, E3E4, and E4E4 (Zhang et al., 2015). Polymorphisms in *APOE* (rs7412 and rs429385) lead to modifications in the amino acid sequence at positions 130 and 176, giving rise to three distinct protein isoforms known as *APOE* 2, *APOE* 3, and *APOE* 4 (Ashiq & Ashiq, 2021).

APOE plays an important role in metabolism and transport of lipoprotein to the liver and other tissues through interactions with specific cell surface receptors (Gupta et al., 2018). *APOE* aids in clearing triglyceride-rich lipoproteins (TRLs) and cholesterol and the ε4 allele has been shown to impair this process, leading to the accumulation of TRLs and their remnants in the plasma. When *APOE* is deficient or dysfunctional, lipid build up in arterial walls can occur, leading to atherosclerotic plaque formation (Karahan et al., 2015; Lin et al., 2022).

AMI exhibits a high prevalence both in Malaysia and worldwide. Notably in Asia, including Malaysia, individuals are experiencing AMI at a younger age in comparison to well-developed countries. Genetic predisposition is identified as a significant factor contributing to the development of AMI in this younger population. Despite this, there are currently no reported studies investigating the association between *LPA* gene copy number variation (CNV) and *APOE* gene polymorphism in young individuals with AMI. This pilot study is essential to investigate this association, with a focus on young acute myocardial infarction (YAMI) patients. The pilot study is a preliminary study that usually have a smaller sample size, serving to evaluate the feasibility and potential for further research in this area. Thus, this study highlights the need for additional research to deepen our understanding of this relationship.

1.2 RESEARCH QUESTIONS

Is there any association between *LPA* gene CNV and *APOE* gene polymorphism in YAMI patients?

1.3 GENERAL HYPOTHESIS

LPA gene copy number variation and *APOE* gene polymorphisms is significantly associated in YAMI patients.

1.4 SPECIFIC HYPOTHESIS

i. There is significant association between *LPA* gene CNV and YAMI patients.
ii. There is significant association between *APOE* gene polymorphism and YAMI patients in the current study and in the combined meta-analysis studies.
iii. There is significant different in lipid parameters level of *LPA* gene CNV status in YAMI patients.
iv. There is significant different in lipid parameters level in *APOE* gene genotypes in YAMI patients.
v. There is a significant different in lipid parameters distribution and in both LPA gene and APOE gene polymorphism in YAMI patients.

1.5 GENERAL OBJECTIVES

To assess the association between *LPA* gene CNV and *APOE gene* polymorphism in YAMI patients in Kuantan, Pahang.

1.6 SPECIFIC OBJECTIVES

i. To determine the association of *LPA* gene CNV between YAMI patients and normal controls.

ii. To determine the association of *APOE* gene polymorphism between YAMI patients and normal controls using the current study population and combined meta-analysis population.

iii. To investigate the relationship between *LPA* gene CNV status and lipid parameters in YAMI patients.

iv. To investigate the relationship between *APOE* gene polymorphism and lipid parameters in YAMI patients.

v. To investigate the relationship between lipid parameters distribution and the presence of both LPA gene and APOE gene polymorphism in YAMI patients.

CHAPTER TWO

LITERATURE REVIEW

2.1 ACUTE MYOCARDIAL INFARCTION (AMI)

AMI is the most severe clinical presentation of coronary heart disease (CHD) considered one of the leading causes of death globally and is responsible for a significant burden of disease due to its impact on morbidity and mortality (Salari et al., 2023; Sanchis-Gomar et al., 2016). Therefore, early detection of AMI is essential to ensure appropriate and prompt management, prevent complications, decrease mortality rates, and preserve cardiac function.

2.1.1 Definition of AMI

A myocardial infarction, sometimes referred to as a "heart attack," is defined by a partial or total interruption of blood flow to a portion of the myocardium, which causes prolonged ischemia and ultimately myocardial cell death. This cell death predominantly occurs by oncosis and, to a lesser extent, apoptosis. Pathological manifestations of this cell death include contraction band necrosis, coagulation, or a mix of both. Within 10-15 minutes of the onset of ischemia, initial ultrastructural changes become apparent, such as reduced cellular glycogen, relaxed myofibrils, and the rupture of sarcolemmal fibers. The illness may manifest as a silent event that goes unnoticed or as a catastrophic event that might result in hemodynamic decline and unexpected death. Precise distinction between these pathological expressions requires a careful review of histologic sections by an experienced observer (Ferreira et al., 2018). Cardiac troponins (I and T) constitute integral parts of the contractile machinery within myocardial cells, and their expression is predominantly confined to the heart. Increased serum levels of

cardiac troponin do not provide specificity regarding the underlying injury mode, whether it be ischemic or tension-related (Ojha N et al, 2023). When symptoms of myocardial ischemia are present together with an ascending or falling trend in cardiac troponins (cTn) levels, and at least one value exceeds the 99th percentile of the upper reference limit (URL), this signals an acute myocardial infarction (MI). Testing for cTn values successively at 0 hours, 3 hours, and 6 hours offers a more thorough picture of the degree and temporal evolution of the myocardial damage (Goodman et al., 2006).

2.1.2 Prevalence, Morbidity and Mortality of AMI

In 2016, more than 17 million premature deaths were attributed to cardiovascular disease (CVD), making it a primary contributor to chronic disability and early mortality on a global scale (Chow et al., 2020; Hadley et al., 2018; Roth et al., 2017). In Europe, cardiovascular disease (CVD) accounts for 49% of female deaths and 40% of male deaths (Haider et al., 2020; Townsend et al., 2016)

Notably, the mortality rate associated with CVD has risen in low- and middle-income countries (LMICs), which constitute the majority of Southeast Asian nations, even as there has been a substantial decrease in high-income countries (Roth et al., 2020). It has been shown that implementing quality improvement programs for myocardial infarction (MI) care in high-income countries (HIC) can markedly enhance the adoption of therapies recommended in guidelines, leading to a subsequent decrease in mortality and disability (Ellrodt et al., 2013).

With a population exceeding 600 million, the majority of whom are under 65, the South-East Asia Region (SEAR) is currently undergoing a population surge concurrently with an increase in the poverty rate (Lam, 2015). The prevalence of cardiovascular risk factors is rapidly escalating in SEAR, leading to an earlier onset of cardiovascular disease (CVD) and a concerning rise in premature CVD-related fatalities (Dhillon et al., 2012; Peltzer & Pengpid, 2018).

Indonesia, as an example, has witnessed a significant improvement in life expectancy during the 1990s, with an increase of 8.0 years (95% CI 7.3-8.8). However, this rise in life expectancy has also resulted in a higher burden of chronic illnesses and an aging population (Mboi et al., 2018).

Institute for Public Health Malaysia (2020) reported CVD is the primary cause of both motility and morbidity, in Malaysia. Furthermore, in 2020, ischemic heart disease (IHD) was the leading cause of mortality, contributing to 17% of the total 109,155 medically certified deaths, showing a 2% increase from the previous year. Moreover, the incidence of AMI among individuals aged 41-59 is reported to be 20%, while for those over 60 years old, it is 18%. These rates have increased by 2% in each age group compared to the data from 2020. (Department of Statistics Malaysia, 2021).

2.1.3 Aetiology and Risk Factors

The etiology of acute myocardial infarction (AMI) can be attributed to various factors, including a decrease or stoppage of blood flow to a portion of the heart, typically due to a blood clot in the epicardial artery that supplies that territory due to atherosclerosis. This can lead to necrosis of the heart muscle. Other causes of AMI include plaque rupture, erosion, or fissuring in the coronary artery, as well as rare causes such as coronary artery dissections, congenital anomalies of the coronary origins, and thrombosis in nonatherosclerotic normal coronary arteries due to hypercoagulable states (Krittanawong et al., 2023; Saleh & Ambrose, 2018)

Study by Hoo et al. (2016) outline acute coronary syndrome (ACS) is more prevalent in young male patients compared to females. It is theorized that estrogen, which has a cardioprotective effect, plays a crucial role in premenopausal women by reducing low-density lipoprotein (LDL) levels and inhibiting platelet aggregation, consequently lowering the risk of ACS (Rosano et al., 1996).

The risk factors for AMI can be classified into two main groups that are non-modifiable and modifiable. Non-modifiable factors are those that cannot be altered, such as gender, race, and having a family history of early heart disease (Yandrapalli et al., 2019). The most prominent risk factors for CVD in Malaysia are hypertension, hypercholesterolemia, and obesity, which have been increasing in prevalence. Other risk factors include diabetes, smoking, and physical inactivity. The prevalence of these risk factors increases with age, and they are more prevalent in rural areas. Malay ethnicity, unmarried status, and physical inactivity are associated with increased CVD risk. (Chang et al., 2012; Firus Khan et al., 2022; Mohd Nor et al., 2022; Thangiah et al., 2021).

2.1.4 Pathogenesis of AMI

The two subtypes of acute myocardial infarction type 1 are ST elevation myocardial infarction (STEMI) and non-ST-elevation myocardial infarction (NSTEMI) (Thygesen et al., 2012). NSTEMI is defined by elevated levels of cardiac biomarkers (such as cTn or creatine kinase) detected within 24 hours of the initial presentation. It can also be identified by ischemic symptoms at rest, resulting from a sudden rupture or erosion of a coronary plaque, lasting more than 10 minutes and occurring within 24 hours before admission to the hospital (Edmund Anstey et al., 2016).

Unstable angina falls under the category of acute coronary syndrome (ACS), serving as a warning sign for an imminent heart attack. Grouped together as non-ST-segment elevation ACS (NSTE-ACS), it is typically managed alongside NSTEMI and shares a similar underlying process. The primary causes of most myocardial infarctions involve the breakdown of the coronary artery endothelium (type 1) or the disruption of a vulnerable atherosclerotic plaque. While angina requires significant stenosis (70% diameter or more), type 1 myocardial infarction is less common due to the denser fibrotic caps, which are less prone to rupture, and the gradual development of collateral circulation. In contrast, plaques that are more susceptible to rupture have thin fibrous

caps, a higher concentration of inflammatory cells, and 30 to 50% stenosis. Upon plaque rupture, thrombogenic substances are discharged, initiating platelet activation, triggering the coagulation cascade, forming a thrombus on the arterial wall, and causing the embolization of atherosclerotic debris downstream. This heightened state of blood clotting may lead to the rupture of additional vulnerable fibroatheromas, potentially resulting in multiple culprit lesions (Libby, 2013).

The ultimate outcome is myocyte necrosis, which can be detected through an elevation in cardiac biomarkers in the bloodstream. The severity of ischemia is influenced by factors such as the extent of myocardium supplied, the duration of the blockage, the presence of collateral blood vessels, the degree of partial or complete artery occlusion, and the success of reperfusion during treatment (Reed et al., 2016). In Figure 2.1, it is depicted that there is no acute coronary thrombotic plaque disruption in type 2 myocardial infarction (Guimarães et al., 2017).

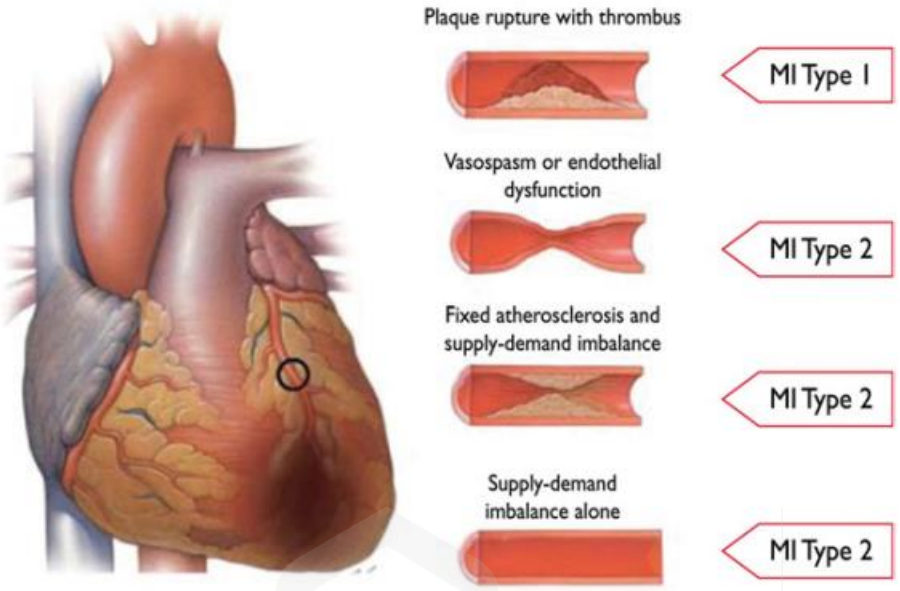


Figure 2.1 Differences in MI Type 1 and MI type 2 based on coronary arteries conditions.

Note: Adapted from (Thygesen et al., 2012).

Myocardial infarction type 2 results from an imbalance between the heart's oxygen supply and demand due to stressors unrelated to a coronary blockage. Type 3 occurs when a patient experiences cardiac death with symptoms suggesting a sudden lack of blood flow to the heart, as indicated by new ECG changes indicative of ischemia, prior to obtaining biomarker values. Acute myocardial infarction (MI) is typically associated with types 1, 2, and 3 classifications. Types 4a, 4b, and 4c involve myocardial ischemic injury related to specific circumstances such as percutaneous coronary interventions (PCI), stent thrombosis, and restenosis. Type 5 is associated with myocardial infarction related to coronary artery bypass grafting (CABG) (Thygesen, 2019)

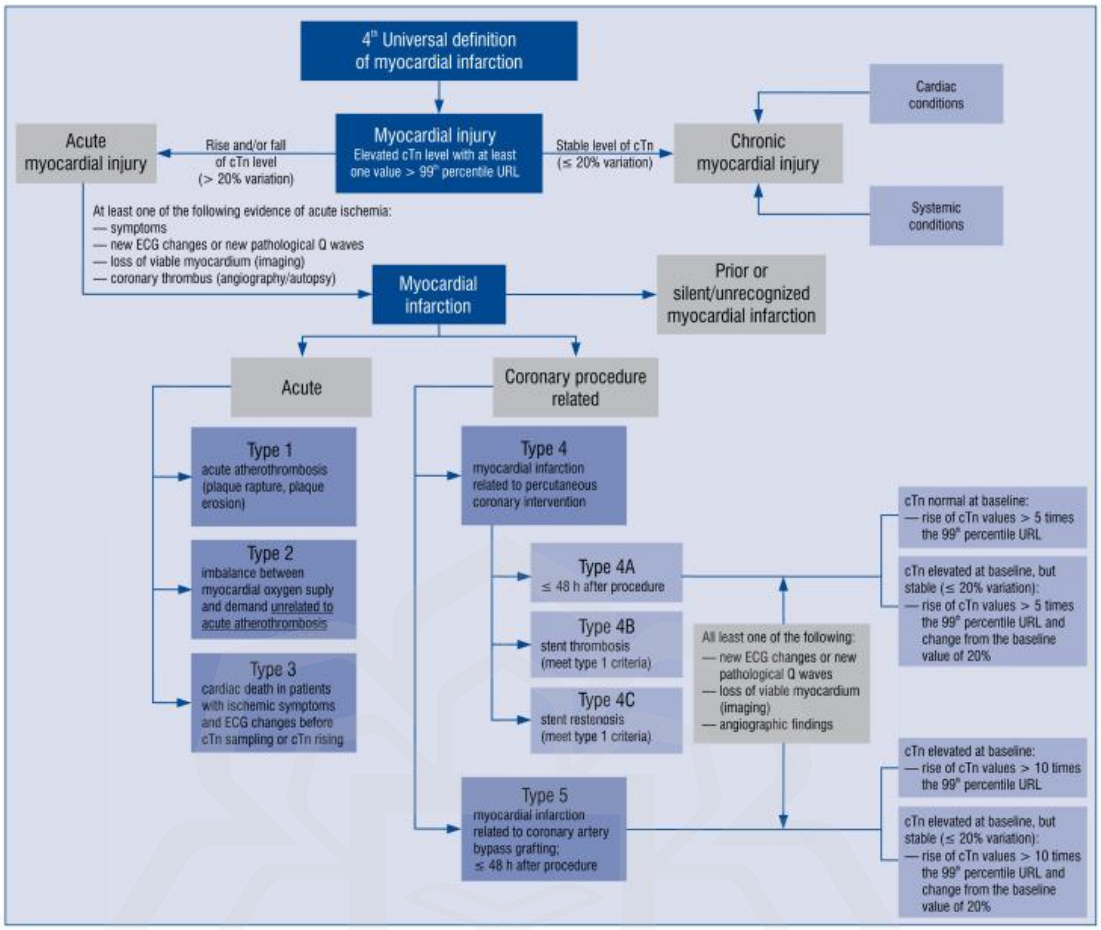


Figure 2.2 A method to differentiate between different forms of myocardial infarction and cardiac damage.

Note: Adapted from (Thygesen et al., 2018)

About 70% of fatal acute myocardial infarction (AMI) cases result from blockages caused by atherosclerotic plaques (Berg et al., 2018). As a chronic condition, atherosclerosis diminishes tissue perfusion and prompts the gradual development of collateral conduits. Thrombus formation or bleeding within an atherosclerotic plaque further narrows the artery lumen, as vulnerable atherosclerotic plaques are prone to ruptures, leading to the formation of blood clots that obstruct coronary arteries. The origin of unstable angina and acute coronary syndromes lies in thromboembolic events associated with atherosclerosis, originating from the rupture of unstable plaques, thereby reducing blood flow to the heart muscle, and elevating the risk of a heart attack (Libby, 2012).

2.1.5 Pathogenesis of Atherosclerosis

Atherosclerosis is characterized by the accumulation of cholesterol, infiltration of macrophages, proliferation of smooth muscle cells, accumulation of connective tissue components, and thrombus formation (Douglas & Channon, 2014; Rafieian-Kopaei et al., 2014). Despite generalized factors, this disease selectively affects specific circulatory regions, with lesion growth being abluminal in the early stages (Libby et al., 1998).

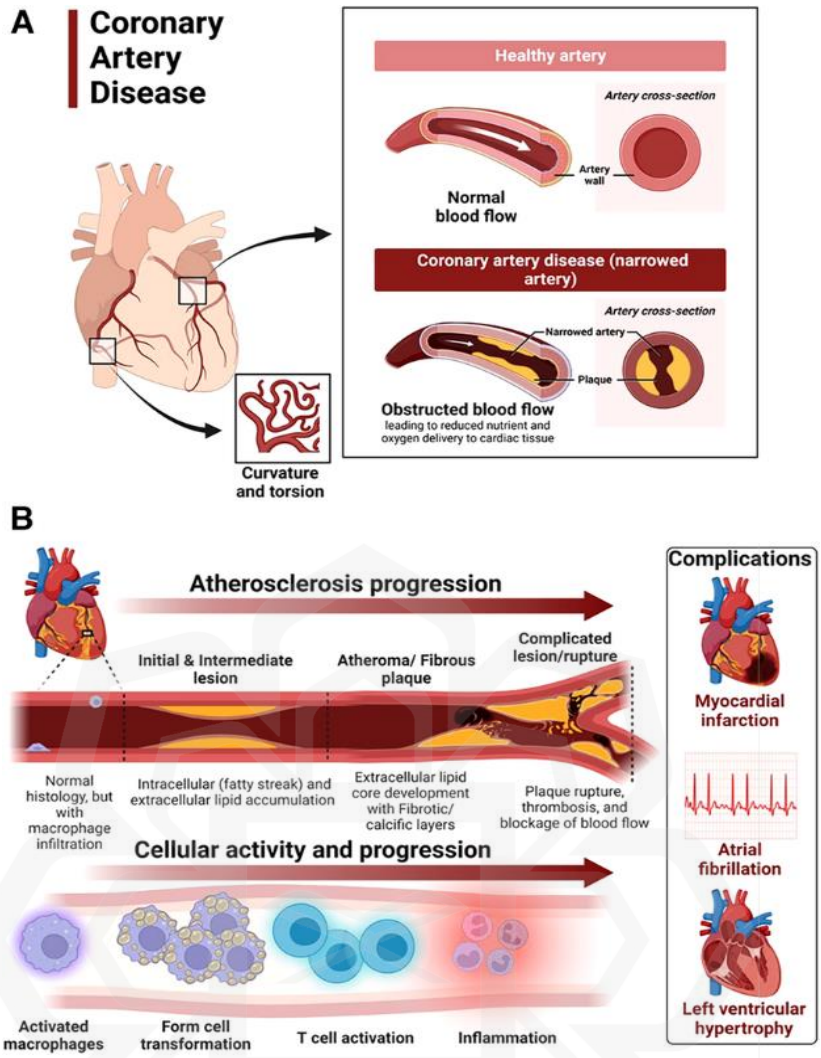


Figure 2.3 The advancement of atherosclerosis in coronary artery disease. (A) Narrowed coronary arteries as a result of advanced atherosclerotic fatty plaque are the hallmark of coronary artery disease. (B) Complications of atherosclerosis in coronary artery disease.

Note: Adapted from (Masenga & Kirabo, 2023)

The earliest visible manifestation is the fatty streak, resulting from lipid-laden foam cells in the artery's intimal layer (Crowther MA, Ginsburg D, 2005). Interestingly, atherosclerosis initiates early in life, and maternal hypercholesterolemia during pregnancy is linked to increased fetal fatty streak formation (Napoli et al., 1997; Palinski & Napoli, 1999).

As the fatty streak evolves, it transforms into fibrous plaques, ultimately containing large lipid amounts. Unstable plaques pose a risk of thrombotic artery occlusion (Crowther MA, Ginsburg D, 2005). High levels of low-density lipoprotein

cholesterol (LDL-C) are well-established as a major risk factor for atherosclerosis, but evidence suggests that elevated triglyceride (TG) levels are also an independent risk factor (Talayero & Sacks, 2011), with triglyceride-rich lipoproteins additionally implicated in the development of atherosclerosis (B. H. Zhang et al., 2022).

HDL plays a pivotal role in atherosclerosis, influencing reverse cholesterol transport (RTC) and countering proatherogenic LDL activities. RCT involves cholesterol efflux from cells to HDL particles, facilitated by ABC transporters like ABCA1 and ABCG1. HDL transports excess cholesterol to the liver for excretion, preventing build up in arterial walls. Liver X Receptors (LXRs) regulate cholesterol levels in macrophages by inducing expression of efflux genes.(Ouimet et al., 2019)

Furthermore, the retention of LDL in vessel walls and its subsequent oxidation are pivotal events in the early stages of atherosclerotic lesions. Lipid peroxidising enzymes, such as 15-lipoxygenase, play a role in atherogenesis, exhibiting both proatherogenic and antiatherogenic effects(Irani, 2000; R. B. Singh et al., 2002). Oxidation of LDL leads to the formation of aldehydic end products in the subendothelial space, and increased expression of 15-lipoxygenase in atherosclerotic lesions suggests its role in both proatherogenic and antiatherogenic effects (Levitan et al., 2010).

In later stages, minimally modified LDL, and oxidized LDL (oxLDL) activate cells, promoting vasoconstriction, thrombosis, and platelet aggregation. OxLDL uptake by macrophages leads to foam cell formation, a hallmark of atherosclerotic lesions. Endothelial cells mediate oxLDL uptake via lectin-like oxLDL receptor-1, induced by various factors, accelerating foam cell formation. This comprehensive understanding of atherosclerosis sheds light on the intricate processes involved in the development and progression of this cardiovascular disease (Jiang et al., 2022).

2.1.6 Cut-off Age of 'Young'

Most studies use an age cut-off of 40 to 45 years to define "young" patients with myocardial infarction (MI) (Ambroziak et al., 2020; Shah et al., 2016; Sood et al., 2023). The term "young" varies, with some studies suggesting ≤40, ≤45, or ≤55 years of age as a cut-off when defining "young" with respect to MI (Shah et al., 2016). Younger age at the time of MI is associated with a higher number of relatives with a history of premature atherosclerosis (Ambroziak et al., 2020). Patients under 45 years old who experience MI may have distinct risk factor profiles and prognosis compared to older individuals (W. Y. Wu et al., 2020). As per data from the Malaysian NCVD-PCI database, from 2007 to 2009, the incidence rate of young acute myocardial infarction (AMI) patients under the age of 45 was approximately 16% (Zuhdi et al., 2013).

Acute myocardial infarction (AMI) can result in severe repercussions, especially when occurring at a young age, affecting the patient's psychological well-being, work capacity, and imposing a significant socioeconomic burden. Since YAMI patients p may be the primary breadwinners in their families, the consequences of AMI can extend to numerous dependents. Moreover, a family history of myocardial infarction (MI) is an independent risk factor, with early occurrences of MI in relatives associated with the greatest risk, particularly among young individuals (Ranthe et al., 2015). Hence, for the purpose of this study, a YAMI patients was defined as individuals aged 45 years old or younger.

2.2 COPY NUMBER VARIATION (CNV)

2.2.1 Definition of Copy Number Variation

Copy Number Variation (CNV) refers to structural changes in the genome characterized by gains or losses of large DNA segments, such as deletions, duplications, insertions, or multiplications. These alterations result in changes in the number of copies of

particular genomic regions compared to the standard reference genome, with CNVs varying in size from small base pairs to megabases (Pollex & Hegele, 2007; Pös, Radvanszky, Styk, et al., 2021; A. K. Singh et al., 2021). Earlier definitions of copy number variations (CNVs) specified a DNA segment larger than 1000 bp, but this was later revised to sizes ranging from 50 bp to several Mb. CNVs are categorized as a subtype of structural genome variants, causing variations in the copy numbers of specific DNA sequences among individuals (Pös, Radvanszky, Buglyó, et al., 2021).

Copy Number Variations (CNVs) exhibit a dual nature with both harmful and beneficial effects on human health and disease. Harmful effects include disease susceptibility due to gene disruptions, dosage sensitivity leading to imbalances in gene expression, and position effects causing dysregulation of critical genes (Sismani et al., 2015). On the other hand, CNVs can confer adaptive traits, contribute to genetic diversity for adaptation to environmental changes, and drive evolutionary innovation through genetic variability (X. Shao et al., 2019).

2.2.2 Effect of Copy Number Variations on Gene Expression

Copy number variations (CNVs) can significantly impact gene expression by regulating flanking genes, influencing the expression of genes located near the CNV in a cis-acting manner. In other words, CNVs affect genes on the same chromosome where the variation occurs. An illustrative example of this mechanism is observed in Williams-Beuren syndrome, a genetic disorder caused by a deletion in chromosome 7q11.23. This deletion leads to the loss of several genes in that region, including the elastin gene (ELN). Consequently, the absence of ELN due to the CNV results in various symptoms associated with Williams-Beuren syndrome, such as cardiovascular issues and distinctive facial features (Merla et al., 2006).

Next, CNVs can influence gene expression through position effects by altering the spatial arrangement of chromatin and regulatory elements. The insertion or deletion

of sequences by CNVs can result in modifications to the proximity between regulatory elements and target genes, thereby affecting their expression levels (Gamazon et al., 2011).

Furthermore, CNVs can disrupt chromatin architecture, affecting the three-dimensional organization of chromatin and influencing gene expression globally. These alterations can impact the accessibility of regulatory elements to genes located at distant genomic sites, thereby modulating gene expression across the genome. This demonstrates how CNVs can have both local and long-range effects on gene expression through changes in chromatin structure (Gheldof et al., 2013).

Moreover, CNVs can impact gene expression by modifying dosage-sensitive genes through gene duplication or deletion events (Auwerx et al., 2022). Changes in gene dosage directly influence the expression levels of these genes, potentially contributing to phenotypic variations and disease susceptibility (Rice & McLysaght, 2017). Additionally, CNVs can regulate gene expression beyond co-localizing genes through distal regulatory mechanisms that extend over long genomic distances. By altering regulatory elements and chromatin structure, CNVs can influence gene expression patterns of genes located several hundred kilobases away, demonstrating the complex ways in which CNVs modulate genetic regulation and the transcriptome (Gamazon & Stranger, 2015).

The alterations caused by CNVs can have profound effects on gene expression regulation, protein function, and cellular processes, ultimately contributing to the development of various genetic disorders and diseases.

2.2.3 Copy Number Variation Studies in Coronary Heart Disease

The role of copy number variations (CNVs) in the diagnosis and treatment of coronary heart disease (CHD) is still under investigation, with a focus on researching their involvement in the development and progression of the condition. CNVs have been

identified as potent genetic variations that can regulate both genotype and phenotype in cardiovascular diseases (Vijay et al., 2018).

In the context of coronary heart disease (CHD), a gene-targeted analysis has uncovered three novel associations between copy number variants (CNVs) and CHD traits which are association between toll-like receptor-4 (TLR-4) CNVs and apolipoprotein AI, association between sterol regulatory element-binding protein (SREBP) CNVs and apolipoprotein AI and association between interleukin-6 signal transducer (IL-6ST) CNVs and apolipoprotein B. These studies have demonstrated that CNVs can impact pathways linked to lipid metabolism, inflammation, and vascular function, all of which are pivotal in the development and progression of CHD (Costelloe et al., 2012).

Previous studies have increasingly utilized CNV analysis in genetic diagnostics, identifying CNVs linked to cardiac diseases, including sudden cardiac death (SCD). In a study focusing on cardiac patients, significant CNVs were found: a DSP gene deletion in arrhythmogenic cardiomyopathy, KCNQ1 gene deletions in long QT syndrome, and PKP2 gene duplications in arrhythmogenic cardiomyopathy. These findings highlight CNVs' role in cardiac disorder pathogenesis and offer insights into genetic factors contributing to their development (Mates et al., 2018).

Study by Shia et al, (2011) identified significant CNV regions associated with hyperlipidemia and myocardial infarction. These regions, including 1p21.3, 1q31.2 (CDC73), 1q42.2 (DISC1), 3p21.31 (CDCP1), 10q11.21 (RET), 12p12.3 (PIK3C2G), and 16q23.3 (CDH13), harbor genes involved in lipid metabolism, inflammation, and cardiovascular function, suggesting their potential role in the pathogenesis of these conditions.

2.3 LIPOPROTEIN(A) (*LPA*) GENE AND ITS STRUCTURE

The *LPA* gene encodes for the apolipoprotein(a) component of lipoprotein(a) (Kronenberg, 2019). This gene is located on chromosome 6q26-27 and plays a crucial role in determining the plasma concentrations of Lp(a) (Kronenberg & Utermann, 2013). According to Clarke et al, (2009), the locus of the *LPA* gene at region 6q26–27 demonstrates the most pronounced association between elevated Lp(a) levels and the risk of coronary artery disease (CAD).

LPA seems to account for 91% of the variability in Lp(a) levels. Among this variability, 69% can be attributed to the number of KIV type 2 repetitions, with the remaining 22% attributable to other factors (Boerwinkle et al., 1992). However, Kronenberg (2016) reported that up to 90% of the variability in Lp(a) concentrations can be explained by genetic factors, with the *LPA* gene being the primary determinant. Another study found that the number of KIV type 2 repeats accounted for as much as 30% of the variability in Lp(a) levels (Berthold & Gouni-Berthold, 2013).

The *LPA* gene comprises 27 non-repetitive exons, starting with the 5'UTR, followed by one copy each of kringle (K) domains KIV-1, KIV-3 to KIV-10, and KV, each consisting of two exons. Subsequently, there are six exons encoding the protease-like domain, followed by the 3'UTR. Each KIV-2 repeat is approximately 5.5 kb long and comprises a first exon (160 bp), a long intron (4 kb), a second exon (182 bp), and a short intron (1.2 kb) and can be present in one to less than 40 copies per allele (Noureen et al., 2015).

2.3.1 Copy Number Variation of *LPA* gene

The Kringle IV-2 (KIV-2) domain is encoded by a copy number variation (CNV), resulting in the formation of over 30 gene alleles and protein isoforms (approximately 200–800 kDa) within the population (Kraft et al., 1992). The KIV-2 CNV gives rise to approximately 40 distinct alleles, leading to approximately 1600 potential genotypes

(Lackner et al., 1993). The combination of silent changes at specific positions within the first KIV-2 exon defines three KIV-2 subtypes such as the haplotypes, varying among ethnicities. The number of KIV-2 repeats explains Lp(a) variance nonlinearly, with low molecular weight isoforms associated with higher Lp(a) concentrations in Europeans. (Coassin et al., 2019).

The *LPA* gene, which encodes the apolipoprotein(a) [apo(a)] component of the Lp(a) particle, shares homology with the human plasminogen (PLG) gene, exhibiting significant sequence similarity, particularly in the coding regions of the kringle domains (Enas et al., 2019; Orsó & Schmitz, 2017). This homology can reach up to 70% (Maranhão et al., 2014).

Despite being homologous to plasminogen, apo(a) exhibits structural and functional differences. Unlike plasminogen that contains five kringles, apo(a) contains only two kringles (IV and V), with multiple subtypes of Kringle IV. This results in over 30 isoforms of apo(a), making it highly polymorphic. Additionally, the serine protease domain, active in plasminogen for clot dissolution, is inactive in apo(a), contributing to increased thrombus formation risk in individuals with elevated Lp(a) The homology between apo(a) and plasminogen results in the thrombogenic properties of Lp(a) (Rawther & Tabet, 2019).

Variations in the number of KIV type 2 (*KIV-2*) repeats in apo(a) lead to heterogeneity in Lp(a) isoform sizes, with more repeats resulting in larger isoforms. Larger apo(a) isoforms are less efficiently secreted from hepatocytes, leading to an inverse correlation between apo(a) isoform size and plasma Lp(a) levels (Cybulska et al., 2020; Hoogeveen, 2021). Individuals with smaller apo(a) isoforms exhibit higher Lp(a) levels and a significantly increased risk of coronary artery disease (CAD) (Saleheen et al., 2017). Notably, isoform size is associated with significant variations in Lp(a) concentrations across the general population, suggesting additional genetic factors influence Lp(a) levels (Coassin et al., 2019).

2.3.2 Proatherosclerotic and Prothrombotic Effects of Lipoprotein(a)

Lp(a) is a polymorphic lipoprotein produced by the liver, consisting of one molecule of the glycoprotein apo(a)), which is covalently linked to apoB-100-containing low-density lipoprotein (LDL)-like particles (Schmidt et al., 2016). Elevated levels of Lipoprotein(a) (Lp(a)) have been associated with proatherosclerotic and prothrombotic characteristics. Increased plasma concentrations of Lp(a) have been identified as a causative factor for coronary heart disease, atherosclerosis, and thrombotic incidents secondary to atherosclerosis (Boffa & Koschinsky, 2016; Spence & Koschinsky, 2012). Although, the mechanisms through which Lp(a) influences atherogenesis remain incompletely understood.

Study by Argraves et al (1997) proposed mechanism involves the direct deposition of Lp(a) onto arterial walls. Compared to LDL, Lp(a) is more prone to oxidation, which accelerates its uptake into macrophages through scavenger receptors. This process affects a fundamental mechanism of atherogenesis, wherein macrophages gradually transform into foam cells, the precursor of atherosclerosis. Lp(a) exhibits greater atherogenicity compared to LDL cholesterol. Apart from possessing all the proatherogenic characteristics of LDL cholesterol, apo(a) also functions as a reservoir for inflammatory molecules, such as oxidized phospholipids. These oxidized phospholipids can influence numerous pro-atherogenic properties of Lp(a), including its inflammatory activity (Rehberger Likozar et al., 2020).

Lp(a) and/or apo(a) have prothrombotic properties by interfering with fibrinolysis regulation. This interference includes inhibiting plasminogen binding to fibrinogen, fibrin, and tetranectin, blocking plasminogen activation by tissue plasminogen activator (t-PA), and enhancing the activity of plasminogen activator inhibitor-1 (PAI-1). These actions contribute to a procoagulant state, potentially increasing the risk of thrombotic events in individuals with elevated Lp(a) levels (Tada et al., 2019).

Lp(a) inhibits the binding of tissue plasminogen activator (t-PA) to fibrin and suppresses the enhancement of plasminogen activation by t-PA through fibrin and fibrinogen fragments. Additionally, Lp(a) inhibits plasminogen activation by

streptokinase by competing for plasminogen binding to monocytoid and epithelial cells. Tetranectin, a plasma protein, binds to plasminogen to enhance its activation by t-PA. Interestingly, Lp(a) has a higher affinity for binding to tetranectin compared to plasminogen (Singla et al., 2009).

Furthermore, Lp(a) induces platelet activation through multiple agonists, including platelet-activating factor and a thrombin-receptor-activating peptide, thereby exerting a prothrombotic effect. Additionally, Lp(a) facilitates coagulation by binding to and inhibiting tissue-factor pathway inhibitors (Rehberger Likozar et al., 2020; Tsironis et al., 2004). The proatherogenic and prothrombotic effects of Lp(a) underscore its role as a potential therapeutic target for the prevention and treatment of cardiovascular diseases.

2.4 SINGLE NUCLEOTIDE POLYMORPHISMS (SNPs)

Single nucleotide polymorphisms (SNPs) refer to a genetic variation where a single nucleotide is substituted at a specific position within the genome. SNPs are common genetic variations found at single base pair positions in genomic DNA, where different alleles exist in normal individuals within a population. The key distinction between SNPs and rare variations lies in their frequency and prevalence in the population. SNPs are characterized by the requirement for the least abundant allele to have a frequency of 1% or more, making them widespread and valuable markers for genetic studies and association analyses (Brookes, 1999).

Collins et al. (1998) stated that it is estimated that SNPs occur, on average, once every 250 to 1000 base pairs (bp) and constitute approximately 90% of DNA sequence variants in the human genome. SNPs can be found within genes' coding sequences, non-coding regions, or intergenic regions. Those within coding sequences may not always alter the resulting protein's amino acid sequence due to the redundancy of the genetic code (F. Zhang & Lupski, 2015; Zou et al., 2020) . These coding SNPs can be

categorized into two types: synonymous SNPs, which do not impact the protein sequence, and nonsynonymous SNPs, which do alter the amino acid sequence of the protein (Chu & Wei, 2019).

SNPs play a significant role in determining the genetic variations associated with various diseases. They are used in genetic and genome-wide association studies to identify genetic variations linked to disease susceptibility and progression. SNPs can affect gene transcription and protein activity, leading to differences between individuals and species. Besides, they are also used as biological markers to track the inheritance of disease-associated genetic variants within families and populations (Bell, 2002; Freitas et al., 2021).

2.5 APOLIPOPROTEIN E (*APOE)*

Apolipoprotein E (*APOE)* is a 34-kDa glycoprotein present in various tissues such as the liver and brain, belongs to the exchangeable apolipoprotein gene family. It serves as a structural constituent of lipoprotein particles and plays a crucial role in regulating both lipoprotein metabolism and cholesterol transport. *APOE* also mediates lipid transfer between circulating lipoproteins and tissues by binding to membrane receptors. Additionally, it exhibits high-affinity binding to lipophilic inflammatory components like amyloid beta, lipopolysaccharides, and beta-glucans, thereby contributing to pathogen clearance and bolstering the innate immune response (Chetty et al., 2017; Huebbe & Rimbach, 2017).

Moreover, *APOE* plays a crucial role in influencing cells within atherosclerotic lesions, particularly by promoting the removal of cholesterol from lipid-filled macrophages in the arterial wall, a process essential for averting atherosclerosis. Mostly originating from macrophages, *APOE* exerts protective effects against atherosclerosis, primarily by impeding the migration and growth of smooth muscle cells, which helps prevent the development of neointima in damaged arteries (Getz & Reardon, 2009).

APOE is a versatile plasma protein present in terrestrial and marine vertebrates, such as mammals, reptiles, and fish. While variations exist in size and amino acid sequences among different species, the general structure and function of *APOE* remain consistent. *APOE* is characterized by N- and C-terminal domains (NT and CT), whose interaction defines its tertiary structure. (Huebbe & Rimbach, 2017).

The N-terminal domain, which comprises a four-antiparallel helix bundle, includes the receptor-binding region (~136–150 and Arg172) and the binding region for heparan sulfate proteoglycans (HSPGs). It also possesses a limited lipid-binding capability (Wilson et al., 1991). The C-terminal domain contains amphipathic α-helices, including the high-affinity lipid-binding region located approximately at positions 244–272. Additionally, it encompasses the region from 267 to 299, which is responsible for *APOE* self-association (J. Chen et al., 2011).

Human *APOE* has three major isoforms distinguished by variations at two amino acid positions, 112 and 158: *APOE* 2 (Cys112; Cys158), *APOE* 3 (Cys112; Arg158), and *APOE* 4 (Arg112; Arg158), and the *APOE* gene is associated with various clinical conditions, including Alzheimer's disease and cardiovascular diseases (Y. Chen et al., 2021; Huang & Mahley, 2014).

2.5.1 Apolipoprotein E Polymorphisms in Coronary Heart Disease

The human *APOE* gene, found on chromosome 19q13.2, exhibits polymorphism with SNPs at positions 112 (rs429358) and 158 (rs7412), resulting in three distinct alleles epsilon2/epsilon3/epsilon4 (ε2, ε3, and ε4) that encode the primary *APOE* isoforms: *APOE* 2, *APOE* 3, and *APOE* 4, respectively. Various studies have suggested a link between these *APOE* gene variations and their impact on lipid metabolism and the clearance of lipoproteins, potentially influencing the development of coronary heart disease (CHD) (Dankner et al., 2020; Larifla et al., 2017; A. Shao et al., 2022).

These three alleles resulting in a total of six possible genotypes in humans: E2/E2, E2/E3, E2/E4, E3/E3, E3/E4, and E4/E4. These genotypes represent combinations of homozygous (E2/E2, E3/E3, E4/E4) and heterozygous (E2/E3, E2/E4, E3/E4) states (Sebastiani et al., 2019; Yousuf & Iqbal, 2015). Furthermore, frequencies of the ε2, ε3, and ε4 alleles are approximately 7%, 78%, and 14%, respectively (Phillips, 2014).

Polymorphisms in the *APOE* gene impact both the clearance of lipoproteins and lipid profiles, influencing the development of coronary artery disease (CAD). These genetic variations also affect lipid metabolism. Research has shown that individuals carrying the ε4 allele have an increased CAD risk by approximately 42%, likely due to elevated total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) levels (Luo et al., 2017). Moreover, ε4 carriers tend to show a poorer response to statin treatment compared to those with the ε2 allele (L. Zhang et al., 2019). *APOE* allele frequencies differ among racial groups, with Europeans and African Americans having higher ε4 frequencies, while Asians have lower frequencies of both ε2 and ε4 alleles (Ma et al., 2021).

Consistently, individuals carrying the *APOE* ε4 allele demonstrate increased risks of cardiovascular disease (CVD) morbidity and mortality, as well as all-cause mortality, according to several studies (Garatachea et al., 2015; Rajan et al., 2017). However, the *APOE* ε2 allele also linked with elevated risk (Xu et al., 2016). The different forms of the *APOE* protein vary in their ability to bind to lipoprotein receptors. *APOE* ε2 exhibits decreased affinity for low-density lipoprotein (LDL) receptors compared to *APOE* ε3 and *APOE* ε4, resulting in lower cholesterol levels. Conversely, both *APOE* ε3 and *APOE* ε4 show similar affinities for the LDL receptor, with only *APOE* ε4 associated with higher cholesterol levels (Dankner et al., 2020). Study by Rasmussen (2016) stated that individuals who carry the ε4 allele show elevated levels of total cholesterol and low-density lipoprotein cholesterol compared to those who do not carry this allele. As a result, the ε4 allele represents a significant genetic predisposition for heart disease within the general population.

The *APOE* protein possesses domains responsible for binding lipids and receptors, facilitating its function in guiding the uptake of chylomicrons and remnants of very low-density lipoprotein (VLDL) from the bloodstream through specific

receptors, notably the low-density lipoprotein receptor (LDLR). These polymorphisms influence the affinity of *APOE* for both lipids and the LDLR, leading to diverse impacts on lipid and cholesterol biomarkers and varying risks associated with different health outcomes (Lumsden et al., 2020).

2.6 RATIONALE OF THE STUDY

Acute Myocardial Infarction (AMI) stands as the leading cause of mortality worldwide. Notably, in Malaysia, individuals experience AMI at a younger age compared to individuals in more developed nations, with an age range of 55.9 to 59.5 years versus 63.4 to 68 years, as reported by Lee et al. (2021). According to Department of Statistics Malaysia (2021) the prevalence of AMI among individuals under the age of 45 in Malaysia is approximately 16%, with a noted increasing trend. Genetic predisposition is considered a significant contributing factor to the development of AMI in this younger population. Hence, it is crucial to comprehend the molecular-level pathological mechanisms underlying myocardial ischemic damage and infarction for both fundamental cardiovascular research and clinical studies.

Previous studies have revealed the involvement of *APOE* gene polymorphisms in the development of coronary artery disease (CAD)(Ashiq & Ashiq, 2021; Gu et al., 2013; Wang et al., 2015; M. D. Zhang et al., 2014). However, there is limited study reported the role of copy number variation of the Lipoprotein(a) (*LPA*) gene in CAD (Z. Wu et al., 2014). Additionally, there have been no reported studies on the association between *APOE* gene polymorphism and copy number variation of the *LPA* gene in AMI, especially in young patients. Hence, further investigation into the involvement of these genes in the pathogenesis of AMI in this young population is important. Therefore, the aim of the present study was to assess the association between *LPA* gene CNV and *APOE gene* polymorphism in YAMI patients in Kuantan, Pahang.

2.7 CONCEPTUAL FRAMEWORK

Figure 2.4 shows the conceptual framework of this study. Genetic predisposition has been attributed to be involved in the pathogenesis of atherosclerosis and AMI. This genetic predisposition also has an influence on lipid metabolism. The current study was performed to assess the relationship between *LPA* gene CNV and *APOE gene* polymorphism in YAMI patients in Kuantan, Pahang.

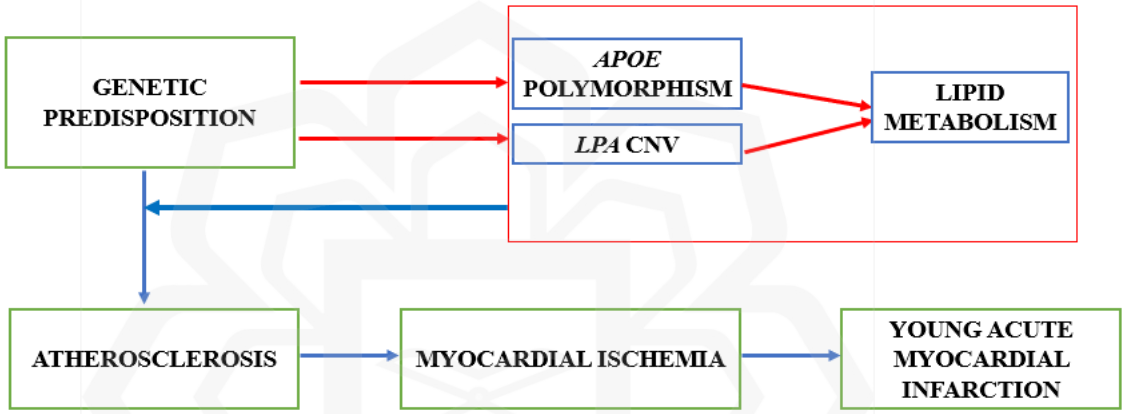


Figure 2.4 Conceptual Framework.

CHAPTER THREE

METHODOLOGY

3.1 MATERIAL

3.1.1 Equipment

1. Micropipettes
2. Vortex (Cole-Parmer™ Stuart™ Variable Speed SA8 Vortex Mixer, UK)
3. Microcentrifuge (Micro-6, Hanil Science Industrial, Korea)
4. Microcentrifuge (Rose Scintific, USA)
5. Water bath incubator (Memmert, Germany)
6. Centrifuge
7. Microvolume Fluorospectrophotometer Nanodrop (Simplinano, Ge Healthcare Life Science, USA)
8. Freezer (Sanyo Biomedical Freezer MDF-436, Japan)
9. PCR chamber
10. Clinical Chemistry Analyzer (BECKMAN COULTER AU680, USA)
11. Thermocycler (Eppendorf Vapo.protect Mastercycler Pro)
12. Analytical balance (Nimbus®, Adam Equipment USA)
13. Gel electrophoresis system (Bio-Rad, USA)
14. Gel documentation system (Enduro™ GDS Labnet)
15. Digital PCR (QIAcuity One 2plex, QIAgen, Germany)

3.1.2 Disposable Materials and Reagents

1. Disposable tips
2. Disposable gloves
3. Sterile pipette tips

4. Eppendorf tubes
5. Falcon tubes
6. Microcentifuge tube
7. QIAamp DNA Blood Midi Kit (QIAGEN, HILDEN, GERMANY)
8. Seeplex® *APOE* ACE Genotyping Kit (Seegene
9. 100% ethanol
10. RNase free water (QIAGEN, HILDEN, GERMANY)
11. 3X EvaGreen PCR Master Mix (green channel)
12. 25X dPCR Copy Number Assay (*LPA*)
13. 25X dPCR Copy Number Assay (Human Multicopy Reference R6)
14. Restriction Enzyme (EcoR1- Thermo Fisher Scientific)
15. 50 bp DNA Ladder DM1100 ExcelBand
16. FloroSafe DNA Stain 1st Base
17. Tris-borate-EDTA (TBE) Buffer

3.2 STUDY DESIGN

The study design that is used is a comparative cross-sectional study in which the subjects and controls who fulfil the study criteria were compared. There are two groups in this study comprising young acute myocardial infarction (YAMI) and healthy controls.

3.3 STUDY POPULATION

The AMI patients were recruited from the Emergency Department (ED) of Hospital Tengku Ampuan Afzan (HTAA), Kuantan, Pahang, and ED of Sultan Ahmad Shah Medical Centre @ IIUM (SASMEC@IIUM), Kuantan, Pahang. Volunteers from Klinik Kesihatan Bandar Kuantan and IIUM staff at IIUM Kuantan Campus were recruited as control subjects.

3.4 SAMPLE SIZE

OpenEpi Software version 3.01 by Dean et al. (2017) was used to calculate the sample size for the rest of the objectives using the comparison of two means. For this study, a power of 80% was chosen for it to have a relevant effect with a confidence interval of 95%. Objective one of this study was used to calculate the sample size. A study by Wu et al, (2014), was selected as a reference. The calculated sample size was 280 for each group. However, based on the data on hospital admission and the prevalence of acute myocardial infarction, it was impossible to collect the entire sample during this study period. Therefore, this study was conducted as a pilot study in which the targeted sample size was 20 samples for each group (Birkett & Day ,1994).

3.5 SELECTIONS OF SUBJECTS

In this study, we divided participants into two groups which were the Healthy Control group and the YAMI patients. Participants who met the inclusion and exclusion criteria were enrolled in the study after informed consents (Appendix 1) were obtained according to the Helsinki declaration (World Medical, 2013). A total of 40 participants were involved, including 20 healthy controls aged 18-45 years and 20 YAMI patients aged below 45 years.

3.5.1 Acute Myocardial Infarction Patients

The inclusion and exclusion criteria for YAMI follows the previous study designed which has been conducted in HTAA and SASMEC (Musa et al., 2022).

3.5.1.1 Inclusion Criteria

1. Malaysia citizens who are able to give written or inform consent.
2. Patients aged below 45 years old for YAMI.
3. Patients need to be presented with acute myocardial infarction (STEMI/NSTEMI) at the emergency department.

3.5.1.2 Exclusion Criteria

1. Patients who have received streptokinase or percutaneous coronary intervention (PCI).

3.5.2 Control Subjects

3.5.2.1 Inclusion Criteria

1. Healthy Malaysian who are able to give written and inform consent.
2. Participants aged 18-45 years old.

3.5.2.2 Exclusion Criteria

1. Regular alcohol consumer.
2. Participants aged above 45 years old.

3. Participants who have any known chronic illness as diabetes mellitus, hypertension, renal disease, liver disease and stroke.
4. Participants who are on medications.

3.5 SAMPLE AND DATA COLLECTION

3.6.1 Data from Case Report Form

1. Biodata of the patients comprising name, age, gender race and religion.
2. Information of the patients regarding the education level, occupation, family history of hypertension and medical history.
3. Information of the patients concerning risk factors including smoking and alcohol intake.
4. Blood pressure.
5. Pulse rate.
6. Fasting lipid profile.
7. Fasting blood sugar.
8. Weight.
9. Height.
10. Body mass index (BMI).

3.6.2 Blood and Sample Collection

AMI patients were recruited at the ED of HTAA or ED of SASMEC@IIUM. A blood sample was taken from a patient who met the study criteria and consented. For the healthy control group, all participants were asked to fast overnight before blood

collection at Klinik Kesihatan Bandar Kuantan and IIUM Kuantan Campus. A total volume of 5 milliliters (5 ml) of blood were drawn by venepuncture. Two milliliters (2 ml) of blood were transferred into a plain tube, followed by three milliliters (3 ml) into an ethylenediamine tetraacetic acid (EDTA) tube.

For the isolation of serum, the blood sample in the plain tube was centrifuged at 1300 x g for 10 minutes at room temperature and then was aliquoted into 1.5 ml microcentrifuge tube. The serum was kept in a labelled cryogenic box and stored at -80°C freezer until further biochemical analysis.

The buffy coat was isolated from EDTA tube after the centrifugation at 1300 x g for 10 minutes at room temperature. After centrifugation, the buffy coat was aliquoted into 1.5 ml microcentrifuge tube and kept in a labelled cryogenic box and stored at -80°C freezer until further genetic analysis.

The procedures from DNA extraction to *APOE* genotyping and detection of *LPA* gene copy number were conducted in Medical Human Genetics Laboratory located at Department of Pathology and Laboratory Medicine, Sultan Ahmad Shah Medical Centre (SASMEC) @ IIUM.

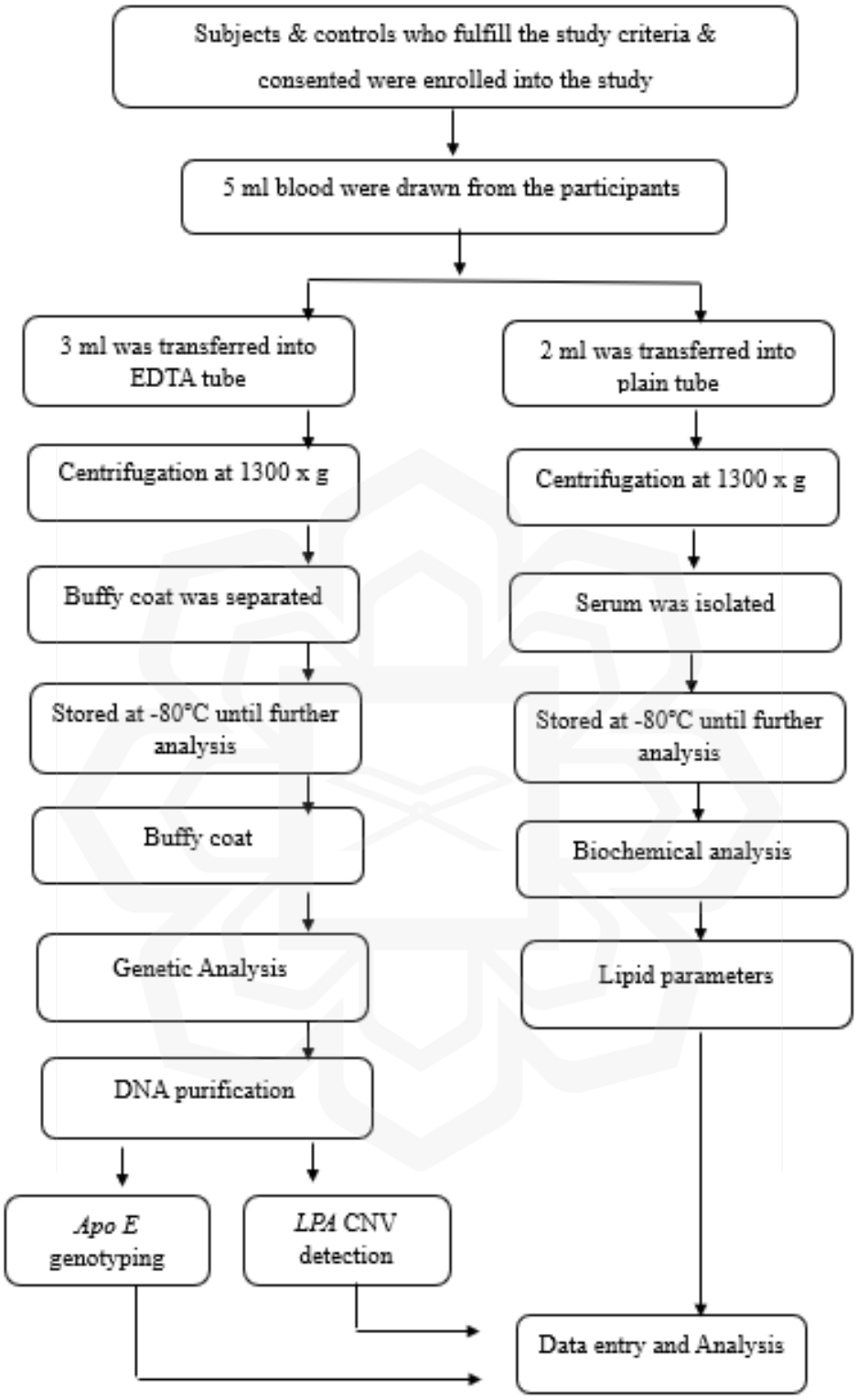


Figure 3.1 The study flow

3.7 PHASES OF STUDY

This study consists of five (5) phases. The first phase comprises the detection of *LPA* gene CNV which determine the association of *LPA* gene CNV between YAMI patients and healthy controls. The second phase comprises of *APOE* genotyping which determine the association of *APOE* gene polymorphism between YAMI patients and healthy controls followed by the investigation of the relationship between *LPA* gene CNV status and lipid parameters in YAMI patients. The fourth phase involve investigation of relationship between *APOE* polymorphism and lipid parameters in YAMI patients. The investigation of lipid parameters and the presence of both *LPA* gene CNV & *APOE* polymorphism in YAMI patients was done in last phase.

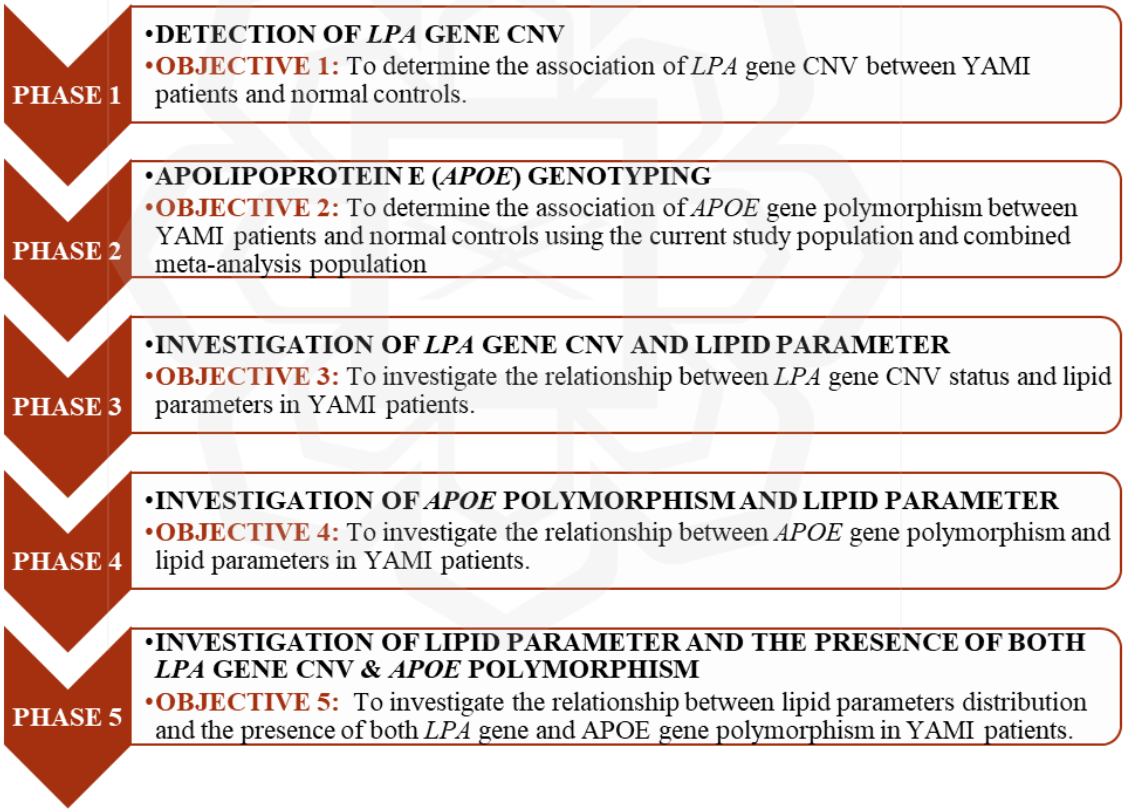


Figure 3.2 Phases of study.

3.8 DETERMINATION OF LIPID PARAMETERS

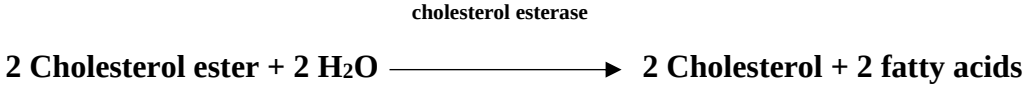
The lipid parameters include total cholesterol (TC), high-density lipoprotein (HDL) cholesterol (HDL-C), low-density lipoprotein (LDL) cholesterol (LDL-C), and triglycerides (TG). An AU680 Clinical Chemistry Analyzer (Beckman Coulter) with Beckman Coulter reagents was used to analyse TC, HDL-C, and TG. The Friedewald formula was used to calculate LDL (William T Friedewald, Robert I Levy, 1972)

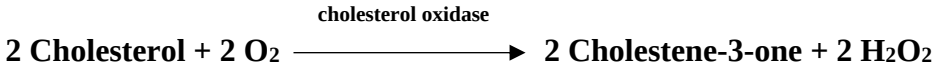
3.8.1 Total Cholesterol (TC)

Cholesterol reagent OSR6116 was used to analyse total cholesterol (TC). This reagent doesn't need any preparation beforehand. The analytical range for TC measurement is from 0.5 to 18.0 mmol/L.

3.8.1.1 Principle

The assay operates on the principle of utilizing enzyme cholesterol oxidase (Allain et al., 1974). In this process, cholesterol ester is broken down by cholesterol esterase (CE) into cholesterol and fatty acids. Subsequently, cholesterol is oxidized by cholesterol oxidase to form cholestene-3-one, accompanied by the generation of hydrogen peroxide (H2O2). Later, the hydrogen peroxide reacts with 4-aminoantipyrine and phenol to produce a red quinoneimine dye complex, facilitated by the peroxidase enzyme. This resulting quinoneimine dye complex is then measured as an endpoint reaction at 540/600 nm, which is directly proportional to the concentration of total cholesterol (TC).





3.8.1.2 Procedure

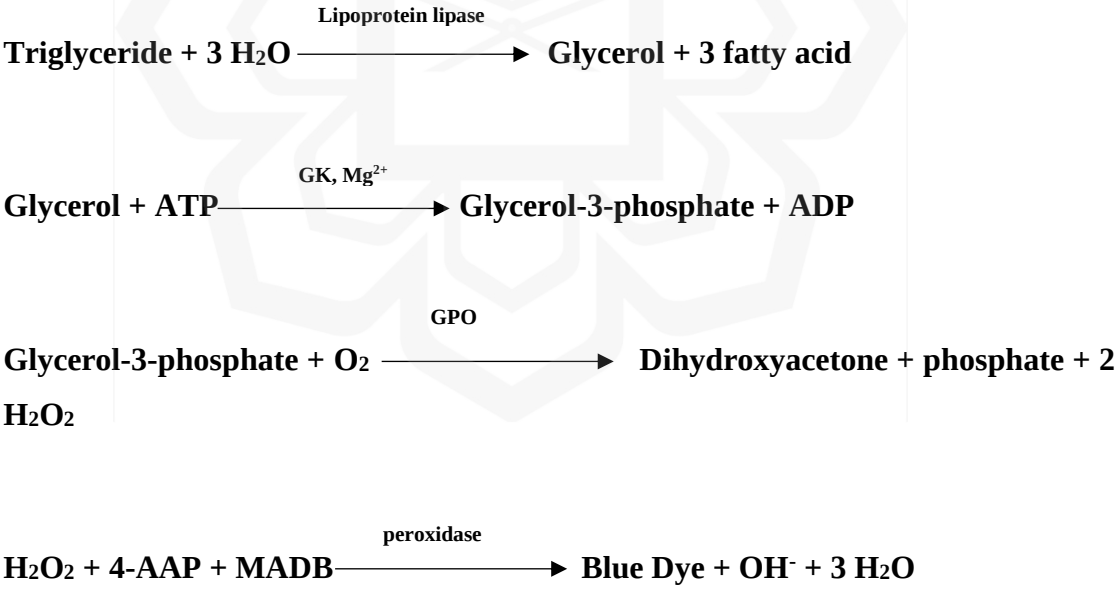
1. The frozen was thawed and about 0.50 ml was used per sample cup.
2. Each sample was measured in duplicate.
3. The sample cups containing serum were labelled and placed on the sample tray mounted on the Chemistry Analyzer.
4. The labels were also entered into the computer unit according to the label on each sample cup.
5. The test was then run at room temperature and the printed results were double-checked with the one on the computer. The average for each duplicate was taken and recorded.

3.8.2 Triglyceride (TG)

The analysis used Reagent OSR61118, which is ready-to-use and does not need any preparation. The analytical range for triglycerides (TG) measurement is from 0.1 to 11.3 mmol/L.

3.8.2.1 Principle

The assay operates through a series of enzymatic reactions (Jacobs & Van-Denmark, 1960; Koditschek & Umbreit, 1969; Trinder, 1969). Serum triglycerides (TGs) undergo hydrolysis by lipoprotein lipase to form glycerol and fatty acids. The released glycerol is then phosphorylated by adenosine triphosphate (ATP) to create glycerol-3-phosphate and adenosine-5'-diphosphate (ADP), catalyzed by the enzyme glycerol kinase (GK). Glycerol-3-phosphate undergoes oxidation by glycerol phosphate oxidase (GPO) to produce dihydroxyacetone phosphate and hydrogen peroxide (H_2O_2). The generated H_2O_2 reacts with 4-aminophenazone and N, N-bis(4-sulfobutyl)-3,5-dimethylaniline disodium salt (MADB) in the presence of peroxidase to generate a blue-coloured chromophore (blue dye). The intensity of the blue dye is then measured as an endpoint reaction at 660/800 nm, which is directly proportional to the concentration of triglycerides (TGs).



3.8.2.2 Procedure

1. The frozen serum was thawed and about 0.5 ml was used per sample cup.
2. Each sample was made in duplicate.
3. The labels were also entered into the computer unit according to the label of each cup.
4. The test was then run at room temperature and the printed results were double checked with the one on the computer. The average of the duplicate was taken as a final result.

3.8.3 Low Density Lipoprotein Cholesterol (LDL-C)

The calculation of LDL level was done using the Friedewald formula (Friedewald et al., 1972).

LDL-C = TG – (TG/2.2 + HDL-C) [units in mmol/l]

The formula operates under the assumption that the majority of circulating triglycerides (TGs) are carried in very low-density lipoproteins (VLDL) when chylomicrons are not detectable in the plasma. Consequently, the molar ratio of TG to cholesterol in VLDL remains constant at 2.2:1. However, the formula is not suitable for non-fasting blood samples due to elevated concentrations of chylomicrons or TG-rich particles typically found in non-fasting individuals (Cohen et al., 1988). Additionally, the formula may yield inaccurate results for LDL levels in cases of dysbetalipoproteinemia, such as type III hyperlipoproteinemia, where high cholesterol levels in VLDL can lead to an overestimation of LDL (Friedewald et al., 1972). Furthermore, the formula becomes invalid if the sample's TG concentration more than 4.5 mmol/l.

3.8.4 High Density Lipoprotein Cholesterol (HDL-C)

The analysis used Reagent OSR6187, comprising reagent R1 and R2, which is ready to use without requiring any preparation. The analytical range for high-density lipoprotein cholesterol (HDL-C) measurement is from 0.05 to 4.65 mmol/L.

3.8.4.1 Principle

The fundamental principle of this assay is derived from a direct method for measuring high-density lipoprotein (HDL) cholesterol, as described by Nauck et al. (1998).

1. The Anti human-b-lipoprotein antibody in R1 binds with non-HDL lipoproteins (LDL, very low-density lipoprotein (VLDL), and chylomicrons) to create Antigen-Antibody complexes. This renders them unreactive upon addition of the enzymatic cholesterol reagent during the assay. Consequently, non-HDL lipoproteins are effectively removed from the assay, allowing only HDL-C to be detected under these conditions.

2. Upon the addition of reagent R2, cholesterol esterase and cholesterol oxidase enzymes convert HDL-cholesterol into choles-4-en-3-one, fatty acids, and hydrogen peroxide. The action of cholesterol oxidase on HDL-C results in the production of hydrogen peroxide, which then reacts with 4-aminoantipyrine (4-AAP) and N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxy-4 fluoroaniline (F-DAOS) in the presence of peroxidase, forming a blue quinoneimine dye. The intensity of the quinoneimine dye, measured at a wavelength of 600/700 nm, is directly proportional to the concentration of HDL cholesterol.

$$\text{HDL-cholesterol} + H_2O + O_2 \xrightarrow{\text{CHE and CHO}} \text{Cholest-4-en-3-one} + \text{fatty acids} + H_2O_2$$

$$H_2O_2 + \text{4-AAP} + \text{F-DAOS} \xrightarrow{\text{peroxidase}} \text{Blue dye}^{+} + F^{-} + 2\,H_2O$$

F-DAOS = N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-3.5-dimethoxy-4-fluoroaniline

CHE = Cholesterol esterase

CHO = Cholesterol oxisase

3.8.4.2 Procedure

The reagents are pre-prepared and do not need any additional preparation. The protocol followed was identical to that of the total cholesterol (TC) assay.

3.9 PURIFICATION OF GENOMIC DNA FROM BUFFY COAT

The genomic DNA were purified using commercially QIAmp DNA Blood Midi Kit (Qiagen) according to the manufacturer's protocol. This spin protocol is used to purify the genomic DNA (gDNA) from up to 2 ml of blood sample.

The purification of gDNA started by adding 200 µl of QIAGEN Protease which is the optimal enzyme for use with Buffer AL for sample lysis into the bottom of a 15 ml centrifuge tube. Next, 2 ml of buffy coat was added into the centrifuge tube and mixed briefly. After that, 2.4 ml of lysis buffer, Buffer AL (provided in the kit) was added and mixed thoroughly by inverting the tube 15 times, followed by additional vigorous shaking for 1 minute to yield a homogenous solution. Then, the mixture was

incubated at 70°C for 10 minutes in water bath to lyse the cells in order to obtain maximum DNA yield.

After incubation, 2 ml of absolute ethanol was added into the sample and the mixture was mixed by inverting the tube 10 times, followed by vigorous shaking for 20 seconds. One half of the solution was then transferred onto spin column (QIAmp Midi column) that was placed in a 15 ml centrifuge tube and then was centrifuged at 3000 rpm for 5 minutes.

After the centrifugation, the spin column was removed, and the filtrate was discarded. The spin column was placed back into the 15 ml centrifuge tube. The remainder of the solution was loaded onto the spin column and then centrifuged at 3000 rpm for 5 minutes. After that, the spin column was removed, and the filtrate was discarded. The spin column was then placed back into the tube. Without moistening the rim, 2 ml of Buffer AW1 (wash buffer) was added to the spin column and then centrifuged at 4400 rpm for 4 minutes. Next, 2 ml of Buffer AW2 was added to the spin column and centrifuged at 4400 rpm for 20 minutes.

The spin column was placed in a clean 15 ml collection tube after the centrifugation. 300 µl of Buffer AE was added directly onto the membrane of spin column and incubated at room temperature for 5 minutes followed by centrifugation at 4400 rpm for 5 minutes. The Buffer AE was functioned to elute the DNA as well as for the long-term storage in the freezer.

To obtain the high concentration of DNA, the elute containing DNA was reloaded onto the membrane of spin column and incubated at room temperature for 5 minutes and then centrifugated at 4400 rpm for 5 minutes. Once the procedures were completed, the DNA was aliquoted into 1.5 ml microcentrifuge tube and stored in -30°C freezer.

3.9.1 Quantification of Purified Genomic DNA Using Simplinano®

The concentration of the purified DNA was measured by using SimpliNano spectrophotometer according to the manufacturer's protocol. The concentration of the DNA is measured when UV/Visible spectrophotometers shine light through a liquid sample and measure its absorbance. The steps began with switching on the instrument by pressing the power button. The application mode for DNA was selected by pressing 1. After that, 2 µl of elution buffer (Buffer AE) was pipetted into the indicated sampling port. The BLANK button was pressed, and the expected zero concentration was displayed on the screen. The sampling port was wiped with lint-free tissue. Next, 2 µl of purified DNA sample was pipetted into the sampling port. The SAMPLE button was pressed, and the reading displayed on the screen. The ratio of absorbance A260/A280 was recorded together with the concentration of DNA. The sampling port was then cleaned with lint-free tissue and 70% alcohol. The procedures were repeated for other purified DNA samples. The A260/A280 ratio should be 1.8-1.9 to be considered pure for DNA.

3.9.2 Dilution of Genomic DNA Stock

There are two concentrations of genomic DNA used in this study. 6 ng/µl of gDNA was used in *APOE* genotyping and 15 ng/µl was used in detection *LPA* gene copy number using dPCR. The procedure started with preparing the sterilized and labelled 1.5 ml microcentrifuge tube, 200 µl PCR tube and RNAse free water. To obtain 6 ng/µl gDNA in 100 µl of working stock, the prepared DNA stock with the concentration of 370 ng/µl as for example, was diluted with 98.4 µl RNAse free water. The formula used and the calculation are as follows:

Volume of DNA stock needed:

$$M1V1 = M2V2$$

$$(370\ ng/\mu l)\ (V1) = (6ng/\mu l)\ (100\ \mu l)$$

$$V1 = \frac{(6ng/\mu l)\ (100\ \mu l)}{(370\ ng/\mu l)}$$

$$V1 = 1.6\mu l$$

Volume of RNAse free water needed.

$$100\ \mu l - 1.6\mu l = 98.4\mu l$$

1.6µl of DNA was pipetted into the 1.5 ml microcentrifuge followed by 98.4µl of RNAse free water. The solution was mixed by pipetting up and down. Next, the diluted DNA solution was transferred into 200 µl PCR tube and then stored in -30°C freezer. By using the same formula, 15 ng/µl DNA was prepared.

3.10 GENOTYPE ANALYSIS

3.10.1 Polymerase Chain Reaction (PCR) Principle

Polymerase chain reaction (PCR) is an enzymatic assay that allows for the amplification of a specific DNA fragment from a large pool of DNA. Source of DNA from a range of tissues and organisms, including peripheral blood, skin, hair, saliva, and microorganism can be used in PCR. For the PCR to make enough copies to be examined using conventional laboratory procedures, only trace quantities of DNA are required since PCR is a sensitive assay (Garibyan & Avashia, 2013)

Template DNA, primers, nucleotides, and DNA polymerase are essential for this assay. The DNA polymerase is an enzyme that link the individual nucleotides to generate the PCR product. The nucleotides are the four bases that contained in DNA: adenine, thymine cytosine and guanine (A, T, C, G). These serve as building blocks that utilized by the DNA polymerase to produce PCR product. The reaction's primers indicate which DNA product should be amplified. Short DNA fragments with a

specified sequence complementary to the target DNA to be detected and amplified are used as primers (Garibyan & Avashia, 2013)

PCR involves three discrete steps which are denaturation, annealing, and extension (Ahrberg et al., 2016). Temperature cycling are used in PCR to initiate and bursts of enzyme-catalyzed DNA synthesis. PCR cycles start with denaturation. In the denaturation step, double stranded DNA denatures into two single strands by heating the template approximately 96°C. During the annealing step, the temperature is decreases approximately 56°C. Two synthetic oligonucleotide primers are annealed to the denatured template DNA. These primers length 20-25 nucleotides and complementary to the sequence of opposite strand of the target DNA. The temperature is raised approximately 72°C in extension step to increase the activity of the enzyme. In this step, the DNA synthesis is initiated at the 3' ends of the bound primers. All the steps are taken place in a programmed thermal cycler which are repeated about 25-35 times (Green & Sambrook, 2019)

3.10.2 Detection of *APOE* Genotypes

The identification of six *APOE* genotypes was performed using commercially available kit, Seeplex APOE ACE Genotyping which is a qualitative *in vitro* test. The polymerase chain reaction (PCR) DNA amplification technique which displays high sensitivity and specificity is used to conduct this test. This kit uses the proprietary oligo technology called DPO (Dual Priming Oligonucleotide) which provides flexibility in primer design and PCR optimization and maximizes PCR specificity and sensitivity through the inhibition of non-specific priming. The DPO – SNP PCR also has the high possibility to attain mutant discrimination in one PCR step. All the procedures that have been done are in accordance with manufacturer protocols.

Before performing the test, the reagents were taken out from the -35°C freezer and need to be completely thawed on ice. The reagent tubes were then centrifuged using minicentrifuge and vortexed thoroughly to mix the solution and remove drops from inside the cap and to. The detection of the genotypes begun with the preparation of PCR

Mastermix. The necessary amount of each reagent needed based on the number of reactions (sample + control) was calculated prior to the preparation of PCR Mastermix. The reagents used to prepare the PCR Mastermix were 5X AP PM, 8-MOP Solution, 2X Multiplex Master Mix. One-time reaction of PCR Mastermix was prepared by adding 2.5uL of 5X AP PM, 2uL of 8-MOP Solution and 5.5uL of 2X Multiplex Master Mix into the 1.5mL appendorf tube. The 5X AP PM is the primer mixture that pairs for *APOE* genotyping and internal control whereas the 8-methoxypsoralen, 8-MOP Solution is used to extinguish the template activity of contaminated DNAs with the purpose of preventing the carry-over contamination.

Next, the solution was mixed by inverting the tube 5 times and followed by brief centrifugation. After that, 10ul of the PCR Mastermix was aliquoted into 0.2 mL PCR tubes. Next, 5uL of (6ng/ µl) sample's nucleic acid was added into the tubes containing PCR Mastermix. For the negative control (NC), 5uL of RNAse free water was added into the tube and for the positive control (PC), 5uL of AP PC was added into the tube instead of gDNA. Thpositive control (PC) of AP PC is the mixture of *APOE* and internal control clones. Lastly, the tubes were placed in the preheated (94°C) thermal cycler. All the preparation of reaction mixture was conducted in an ultraviolet (UV) PCR cabinet.

Table 3.1 Preparation of *APOE* genotyping PCR reaction mix using optimized protocol.

Reagent	Volume per reaction (µl)
5X AP PM	2.5
8-MOP Solution	2.0
2X Multiplex Master Mix	5.5
Template gDNA (6ng/µl)	5.0
Final volume	10.0

The program of the thermocycler used for the amplification of *APOE* gene was based on the Seeplex *APOE* ACE Genotyping procedures. The table below shows the program used for the PCR reaction.

Table 3.2 PCR program used for amplification of *APOE* gene.

Initial duration	94°C	15 min	1 cycle
Denaturation	94°C	30 sec	
Annealing	65°C	30 sec	35 cycles
Extension	72°C	1 min	
Final extension	72°C	10 min	1 cycle

After the cycles of the PCR were completed, the amplification products were analyzed on 2.0% agarose gel.

3.10.3 Agarose Gel Electrophoresis Principle

Electrophoresis is a technique that uses an electric field to separate and analyse charged molecules. An electrophoretic system is comprised of two electrodes with opposite charges (anode and cathode) that are connected by electrolyte which is known as conducting medium. The negatively charged molecules migrates toward positive pole and the migration flow is determined by molecular weight whereby small molecules migrate faster than the large molecule (Sambrook & Russel 2001). Gel electrophoresis is commonly used to separate and purify proteins and nucleic acids of different sizes, charges, or conformation. Agarose gel electrophoresis is usually performed after amplification of DNA by the polymerase chain reaction (PCR) to separate restricted genomic DNA or RNA prior to Southern or Northern analysis. (Tisbir, 2013).

3.10.4 *APOE* Genotyping Using Gel Electrophoresis

In this study, 2.0% of agarose gel was used to perform the test. Prior to the preparation of agarose gel, the gel tray and comb were cleaned using 70% ethanol. The gel tray was placed and adjusted on the gel caster and the followed by the comb.

The preparation of 2% agarose gel started with adding 1.6 g of agarose into then Erlenmeyer flask containing 80 ml 1 x TBE buffer, and the flask was swirled to mix. Next, the flask was heated in the microwave for about 120 seconds until the agarose was dissolved and became a thick solution. After that, the flask was taken out and 4ul of flourosafe was pipetted immediately into the melted gel and was swirled to mix the solution. The agarose solution was then poured into the gel mould. If there were any air bubbles, it will be removed using pipette tip. The agarose allowed to set completely in the room temperature for approximately 30-40 minutes.

The comb was gently removed from the solidified gel and the gel tray was then inserted in the electrophoresis tank filled with 1 x TBE buffer that covers the surface of the gel, with the wells on the gel at the negative pole. Next, 4ul of DNA ladder (50 bp) was loaded into the first well. After that, 5ul of amplification products were carefully

loaded into the additional wells of the gel. The lid was placed on top of the tank and the electrodes were double checked to make sure they were plugged into the correct slots in the power supply.

Next, the power supply was turned on and was set to the constant current and voltage of 385 A and 90 V for 60 minutes until the dye migrated to the appropriate distance. After the electrophoresis has completed, the power supply was turned off and the lid was removed from the tank. The gel tray was then removed from the tank and placed on the paper towels to drain off the excess buffer. The gel was examined using the gel documentation system (gel doc) by removing the gel from the gel tray and exposing the gel to the UV light. The bands were displayed on the PC screen and the pictures were taken as the results.

3.11 DIGITAL POLYMERASE CHAIN REACTION (DPCR)

3.11.1 DPCR Principle

In DPCR, the term “digital” represents the signal switching in single entities which include on or off, activated or not and clotted or not clotted as well as fluorescent or non-fluorescent (Writters et al., 2014). The DNA is divided into the numerous small volume compartment also called partition whereby the molecules are randomly distributed. Each partition consists of zero, one or many molecules (Zhu G et al., 2015) and the number of positive and negative reaction will be counted by fluorescence probes in each partition that shows the presence or absence of the target DNA. Moreover, in the DPCR, the background signal is reduced when evaluation is done in individual partition by increasing the signal-to-noise ratio. Therefore, it is significantly improving the detection sensitivity (Belmonte et al., 2016; Bhat and Emslie 2016; Svobodova et al., 2015; Taylor et al., 2015). In addition, DPCR is non reliant on the kinetics of the PCR rewaction and eliminates the dependency on standard curves, thus allowing

absolute quantification. This DPCR instrument adopt the Poisson distribution (Debski at al.,2015: Dong ET AL., 2015: Majumdar et al., 2015).

3.11.2 Detection of *LPA* gene Copy Number Variation

The detection of copy number variation of *LPA* gene was performed using the commercially available kit. The QIAcuity Nanoplate 8.5K 24-well was used as it is recommended for the applications using small number of samples and low input volumes. One reaction mix was separated into approximately 8500 partitions in each well. Before starting the test, the QIAcuity EG PCR Master Mix, template DNA, dPCR Copy Number Assay were thawed. The individual solution was mixed by vortexing and followed by brief centrifugation. Next, the reaction mix was prepared by pipetting 4µl of 3x EvaGreen PCR Master Mix (green channel), 0.48 µl 25x dPCR Copy Number Assay (*LPA*), 0.25 EcoR1, and 2.27 RNase-Free Water into 1.5 ml appendorf tube. The reaction mix was prepared for 11 times reactions. The same procedure was used to prepare reaction mix for the reference assay by substituting 25x dPCR Copy Number Assay (*LPA*) with 25x dPCR Copy Number Assay (Human Multicopy Reference R6).

Next, 7 µl of the reaction mix were dispensed into the 24-well Nanoplate 8.5 k. The procedures proceed with the addition of 5 µl of (15ng/µl) template DNA into each well containing the reaction mix except for the 2 wells that were used for the non-template control (NTC). For NTC, 5 µl of RNase-Free Water were dispensed into the well. The nanoplate was sealed using the QIAcuity Nanoplate Seal consist of a plate seal and 2 protective foils. The bottom white protective foil was carefully removed, and the seal was aligned properly on the plate. The plate seal was fixed with the Nanoplate roller in the vertical and horizontal direction. Afterwards, the upper protective foil was removed and the Nanoplate roller was used with high force to fix the seal. The plate seal was rolled over at least 3 times forwards and backwards in horizontal and 3 times forwards and backwards in vertical direction. It is important to seal the plate properly to prevent evaporation and contamination as well bad filling of the wells.

As the restriction enzyme has been included in the reaction, the sealed plate was left for 10 min at room temperature (15–25°C) for the digestion of DNA. After 10 min, the dPCR program started by placing the nanoplate into the QIAcuity instrument. During the procedure, the plate needs to be handled properly and not to touch the bottom of the plate which is covered with foil as the QIAcuity reads fluorescence from the bottom of the plate.

The table shows the reagent preparation of dPCR reaction mix using the optimized protocol.

Table 3.3 Preparation of dPCR reaction mix (1x) for Nanoplate 8.5 k (24-well).

Reagent	Volume per reaction (µl)
3x EvaGreen PCR Master Mix (green channel)	4.00
25x dPCR Copy Number Assay (*LPA*)	0.48
Restriction Enzyme (EcoR1)	0.25
RNase-Free Water	2.27
Template gDNA (15ng/µl)	5.00
Final volume	12.00

3.11.3 Operating The QIAcuity Instrument and QIAcuity Software Suite

Before starting a run, the plate was created and its name, plate type and dPCR parameter were defined using QIAcuity Software Suite. The plate was created using the plate configurator in the instrument software. The plate configuration started with entering the plate's name and plate type was automatically selected based on the scanned barcode. Next, the description for the plate was included. Then, DPCR parameter was

tapped to proceed with the next step. The dPCR parameter tab consists of Priming, Cycling and Imaging tabs. The applicable priming profile was selected in the Priming Tab. In the Cycling tab, the temperature and duration were entered based on the optimized protocol used for dPCR program in current study. In the **Imaging tab**, the applicable channel was selected and the exposure duration as well as gain was entered in **Exposure duration** and **Gain fields**.

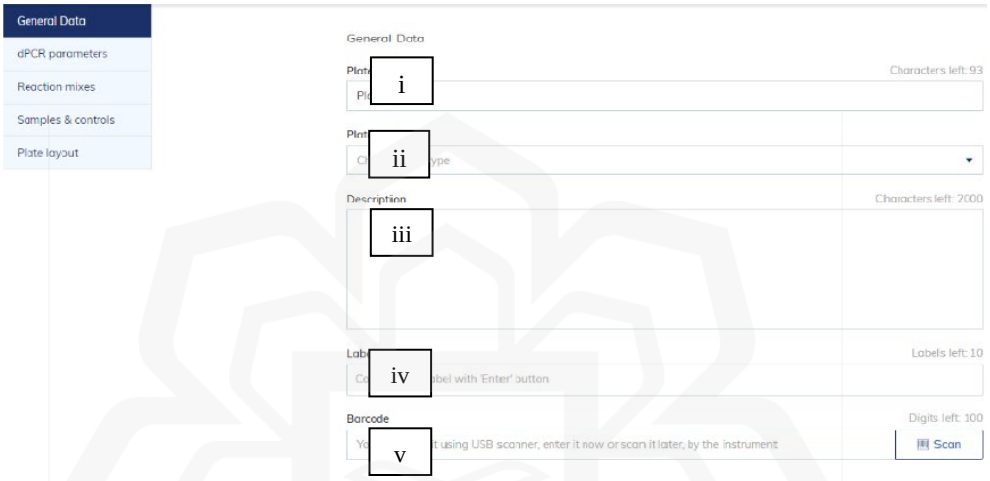


Figure 3.3 General Data interface in QIAcuity Software Suite. (i) Plate name, (ii) Plate type, (iii) Description, (iv) Labels, (v) Barcodes

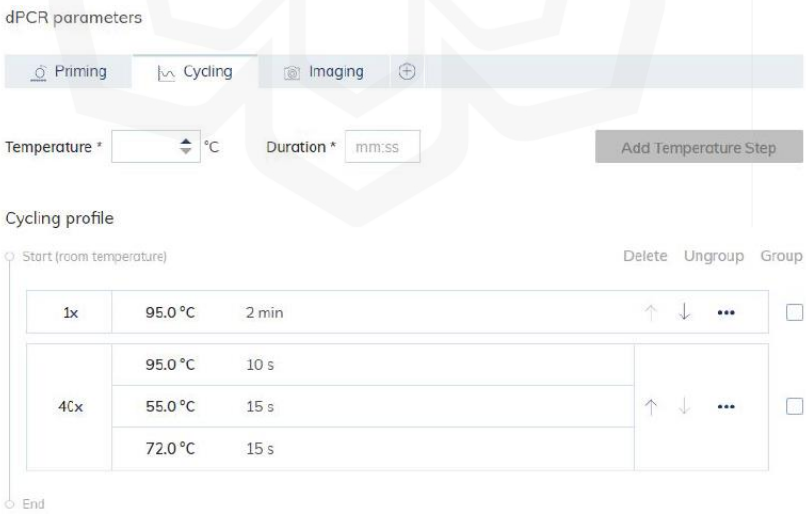


Figure 3.4 The cycling interface of QIAcuity Software Suite.

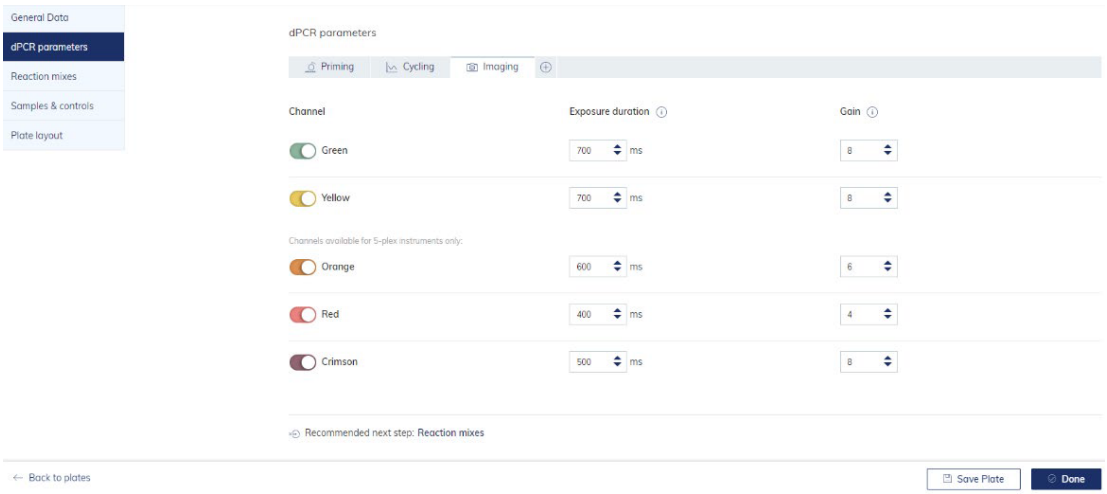


Figure 3.5 Imaging interface QIAcuity Software Suite.

For the defining reaction mixes, at the Reaction mixes section, the New Reaction Mix was selected, and the window appeared on the screen. At the Reaction mix field, the name of the reaction mix was entered without any special characters. Next, the color of reaction mix and the target name was entered in the **Edit reaction mix color** and **Target name field**. After that, the channel was selected from the channel list and the button **Create** was clicked to add the reaction mix to the database. In the current study, the Green channel was selected based on the target that need to be detected. The button Save was tapped to save the progress and the button Done was tapped to save the run and then went back to the Running Status Window.

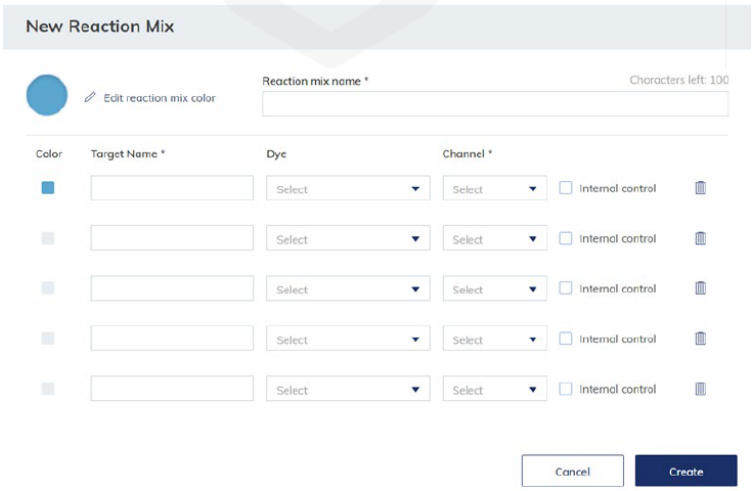


Figure 3.6 Reaction Mix interface QIAcuity Software Suite.

After reaction mixes tab was completed, the samples and control database were entered in the software. Firstly, the New Sample was clicked in the Sample tab. Secondly, the information such as sample name, label, amount, and description were entered in this filed according to the number and type of samples used in the study. Lastly, the button Create was clicked to add the database in the software. For the Controls and Non Template Control (NTC) the information were entered in Controls and Non Template Controls tab.

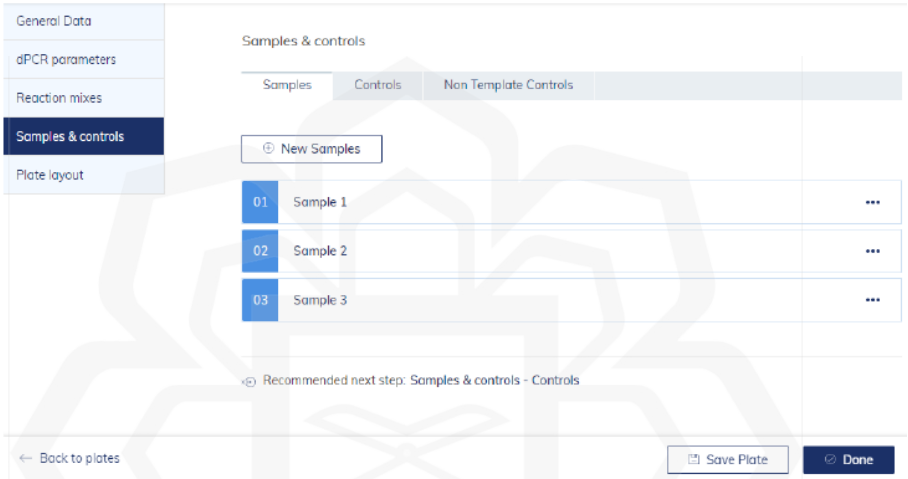


Figure 3.7 Samples interface QIAcuity Software Suite.

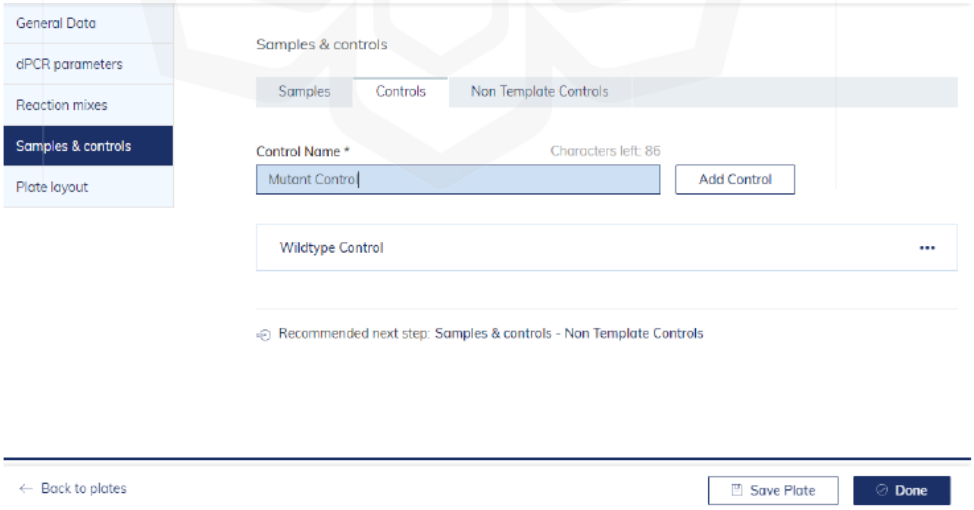


Figure 3.8 Controls interface QIAcuity Software Suite.

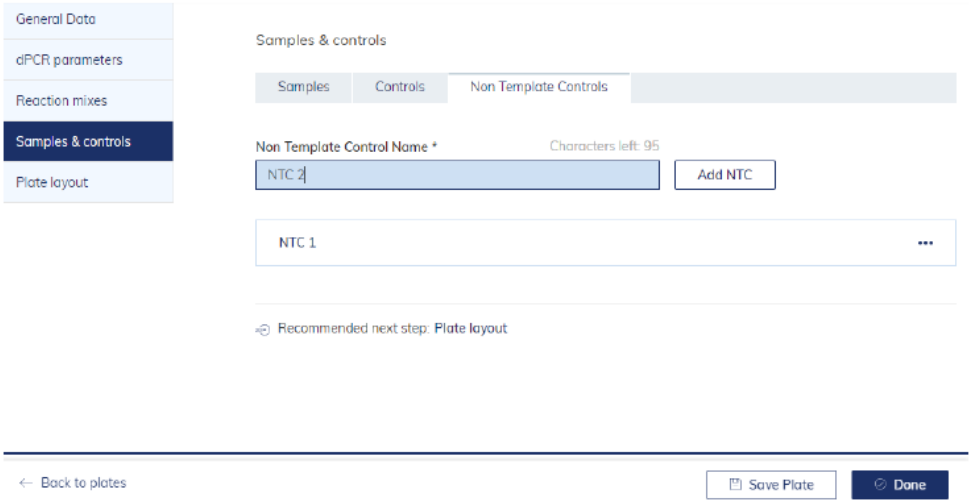


Figure 3.9 Non Template Controls interface QIAcuity Software Suite.

Afterward, the plate layouts were created by adding the reaction mix, sample, and controls to the plate. Firstly, the well was marked by left-click and continued with left-click the + icon. Then, **Add reaction mix** was selected and the window was appeared. Secondly, the reaction mix was selected, and the button Assign was clicked to add the reaction mix to the well. Thirdly, the samples were added by right-click the marked wells. Fourthly, the controls were added by right-click on the marked well and **Add control** was selected. When the **Add Control window** appeared, the controls were selected form the controls list and were added to the plate. In the Control type list, the positive or negative control was selected based on the specific target and the well were marked with a C after clicking the **Assign** button. Lastly, for the NTC, the marked well was right-click and **Add NTC** was selected. At the Non Template Control window, the NTC from **Non Template Control name** list was selected and the Assign button was clicked and the well was marked with NTC.

After inserting all the information of the plate in the QIAcuity Software Suite, the QIAcuity instrument was turned on by pressing the power button. This instrument was operated through touchscreen. The control software was logged in after filling in the credentials in Username and Password fields. Then, the tray was placed in the slot correctly and the **Close Tray** button was pressed to close the tray. The barcodes on the plate were scanned and the availability of the plate was detected. The label of the

corresponding pane was then changed to **Plate is detected**. After that, the **Run** icon was tapped to start the run when the plate was correctly labelled, and the corresponding date was received from the QIAcuity Software Suite.

QIAcuity Software Suite calculated the copy number by calculating the concentration of target copies divided by concentration of reference copies and multiplied by 6.

$$\text{CNV}=\frac{Concentration\ of\ copies\ \mu L\ (LPA)}{Concentration\ of\ copies\ \mu L\ (R6)}\text{x6}$$

Table 3.4 The standard protocol for dPCR program.

Initial heat activation	95°C	2 min	1 cycle
Denaturation	94°C	15 sec	40 cycles
Annealing	60°C	15 sec	
Extension	72°C	15 sec	
Cooling down	42°C	5 min	1 cycle

3.12 META ANALYSIS OF GENETIC ASSOCIATION STUDIES

3.12.1 Meta Analysis Is Performed in This Study For The Following Justifications:

a. To combine results from independent studies.
b. To minimize false positive results due to type 1 error or false negative results.
c. To increase data for combined genetic association analysis that is required for analysis of candidate genes with small effects.

3.12.2 Relevant Studies Pertaining Genetic Association Between APOE and CAD

All Relevant studies pertaining genetic association between APOE and CAD were retrieved from Medline. The inclusion of studies selected for the meta-analysis is according to the flow chart as in Figure 3.10.

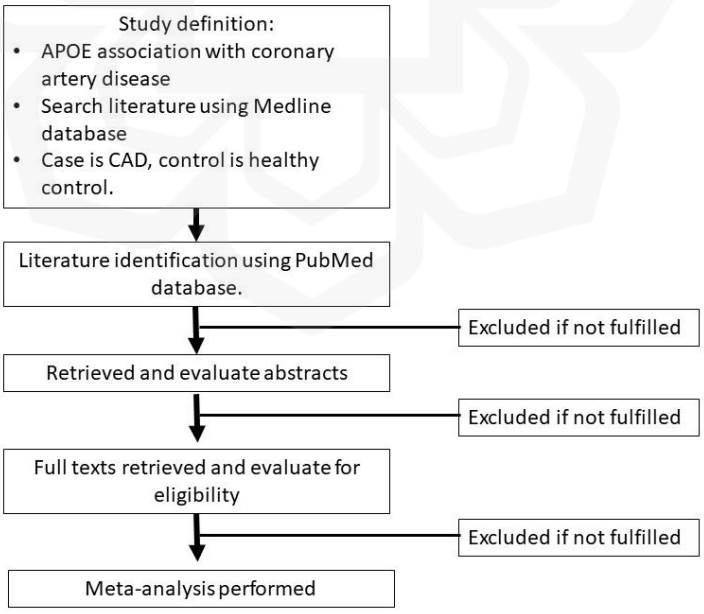


Figure 3.10: Flow chart of the study meta-analysis.

3.13 STATISTICAL ANALYSES

The copy numbers were analysed using QIAcuity Software Suite version 1.2 (QIAgen, Germany). The statistical analysis was performed using SPSS software version 27.0 and online SHEsis program (Shi et al., 2015). The Hardy-Weinberg equilibrium for the genetic distribution of SNP was tested in cases and controls by Chi Square (X^2) test. The association of *LPA* CNV between YAMI patients and controls was tested by Chi Square (X^2) test. The allele frequencies and genotypes distribution of *APOE* were assessed between YAMI patients and control subjects by Chi Square (*X2*) test. The comparison between *LPA* CNV status and lipid parameters in YAMI patients was tested by Kruskall-wallis test. Moreover, the comparison between *APOE* gene polymorphism and lipid parameters as well the distribution of both *LPA* CNV status and *APOE* gene polymorphism with lipid parameters in YAMI patients were also tested by Kruskall-wallis test. Meta-analyses were performed using the Comprehensive Meta-analysis Version 3 software (Borenstein et al., 2003). The p-value <0.05 is considered as statistically significant.

CHAPTER FOUR

RESULTS

4.1 STUDY POPULATION

The study population comprised 20 YAMI patients (YAMI) patients and 20 healthy controls. The clinical characteristics and demographic data of the study participants are depicted in Table 4.1. In order to maintain the homogeneity of the data, only Malay males were included in the study population. Therefore, there was an equal sex and race distribution within these two groups. Hence there is no statistical test was performed for this variable because gender is a constant.

The age of the population ranged between 28 to 43 years old in YAMI patients and 19 to 44 years old in healthy controls respectively. There was a statistically significant difference in the median age between these two groups ($p < 0.001$). The median (IQR) age for YAMI patients and controls were 39.50 (8) and 27.50 (12) respectively (Table 4.1).

The median (IQR) of BMI, total cholesterol (TC), triglyceride (TG) and low-density lipoprotein cholesterol (LDL-C) were higher in YAMI patients [28.705 (5.48), 5.775 (2.36), 1.575 (0.92), and 4.015 (1.72) mmol/L] than in healthy controls [24.531 (6.09), 5.150 (1.25), 1.070 (0.52), and 3.235 (1.35) mmol/L] respectively. However, high-density lipoprotein cholesterol was reported to be higher in healthy controls [1.160 (0.44) mmol/L] than YAMI patients [0.990 (0.33) mmol/L]. There was a statistically significant difference in BMI between the YAMI patients and healthy controls ($p < 0.001$) (Table 4.1). YAMI patients also had significantly higher frequencies of smoking compared with healthy controls [17(85%) and 6 (30%)], $p=0.001$. Noticeably, YAMI patients had significantly higher concentrations of TC ($p=0.041$) and TG ($p=0.002$) and insignificantly lower concentrations of HDL-C ($p=0.040$) than healthy controls group. However, there is no significant differences in LDL-C between YAMI and healthy controls.

Table 4.1 Baseline clinical characteristics of cases and controls.

Variable	YAMI (n=20)	Healthy controls (n=20)	p-value
Age (years)	39.50 (8)	27.50 (12)	<0.001*
Smoking (yes); number (%)	17(85%)	6 (30%)	0.001*
BMI (kg/m^2); median (IQR)	28.705 (5.48)	24.531 (6.09)	0.002*
TC (mmol/L) median (IQR)	5.775 (2.36)	5.150 (1.25)	0.041*
TG (mmol/L) median (IQR)	1.575 (0.92)	1.070 (0.52)	0.002*
HDL-C (mmol/L) median (IQR)	0.990 (0.33)	1.160 (0.44)	0.040*
LDL-C (mmol/L) median (IQR)	4.015 (1.72)	3.235 (1.35)	0.113

Data are presented as median (IQR), except for smoking, which is presented as number of samples (percentage). Kruskall-Wallis (for mean differences analysis of not normally distributed numerical data) and $\chi 2$-test (for association analysis of categorical parameters) were used to analyse the data. $p < 0.05$ is taken as statistically significant at 95% confidence interval *significant difference.

4.2 DETECTION OF *LPA* GENE COPY NUMBER VARIATION (CNV)

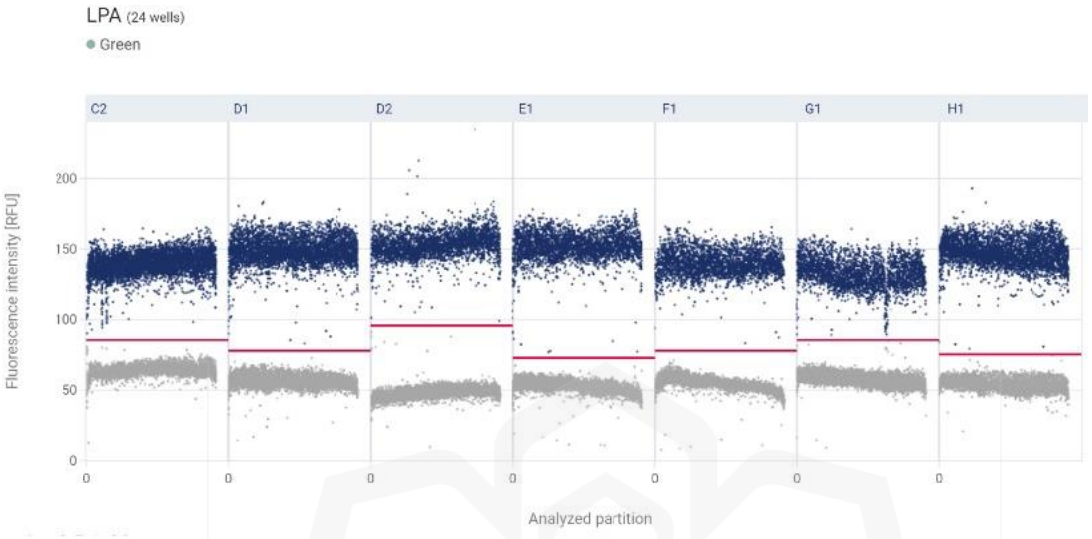


Figure 4.1 1D scatterplot view for *LPA* probe and green channel which concentrates the diagrams for each selected well in a horizontal way separated by a vertical line (column indicator). A header indicates well coordination on each well diagram. Red line indicates the current threshold intensity value (decimal value) to distinguish positive/negative partitions. Fluorescence values below the threshold are shown in grey, and above the threshold in blue.

4.2.1 ASSOCIATION BETWEEN COPY NUMBER VARIATION (CNV) OF *LPA* GENE WITH YAMI PATIENTS AND HEALTHY CONTROLS

The copy number variations (CNV) of *LPA* gene were measured using QIAcuity instrument and QIAcuity software suit. The results were acquired by analyzing 40 gDNA samples from all two groups of subjects. Table 4.2 shows the association between CNV of *LPA* gene with YAMI and healthy controls. The copy number of *LPA* gene were grouped into normal (copy number = 2), loss (copy number < 2) and gain (copy number > 2). The percentage of gain in CNV *LPA* was showed higher in YAMI compared to healthy controls subjects with 25%. Moreover, healthy controls, as expected displayed the highest percentage of normal copy number compared to YAMI subjects. Nevertheless, the percentage of loss in copy number was slightly higher in healthy controls subjects (30%) compared to YAMI subjects (25%). Although

gain *LPA* CNV was found higher in YAMI [n=5 (25%)], no significant difference was found (p = 0.459).

Table 4.2 Analysis of the association of *LPA* CNV between YAMI patients and healthy controls

LPA CNV	YAMI n=20 (%)	Controls n=20 (%)	Chi-square	p-value
Loss	5 (25%)	6 (30%)		
Normal	10 (50%)	12 (60%)	1.558	0.459
Gain	5 (25%)	2 (10%)		

Chi-square test (for association analysis of categorical parameters), $p < 0.05$ is taken as statistically significant at 95% confidence interval *significant difference.

4.3 GENOTYPES ANALYSIS

4.3.1 Amplification of *APOE*

The target region of *APOE* gene involving codon 112 and 158 SNPs was successfully amplified following the manufacturer's protocol as described before. Figure 4.1 – 4.3 show the representative photographs of the gel electrophoresis.

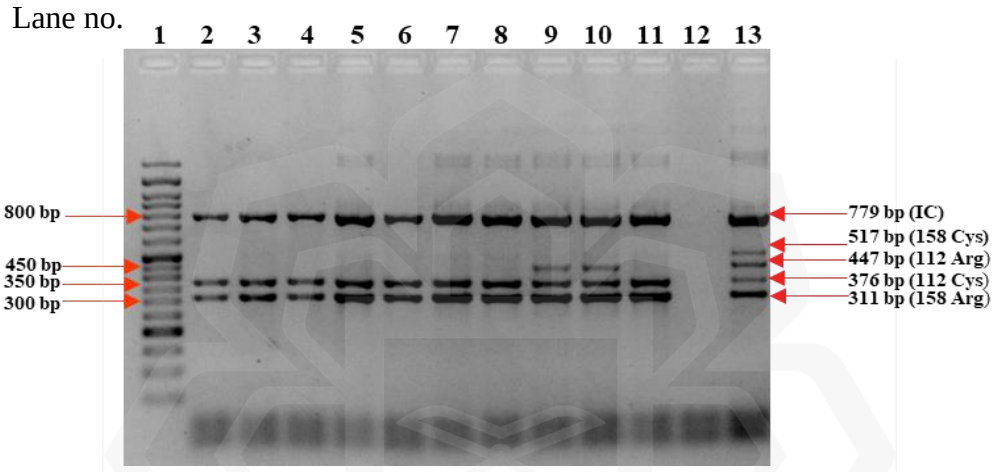


Figure 4. 2 Agarose gel electrophoresis of PCR products of *APOE* amplicons (Lane 1; DNA ladder 50 bp, Lane 2-8; E3/E3 genotypes, Lane 9&10; E3/E4 genotypes, Lane 11; E3/E3 genotypes, Lane 12; NC, Lane 13; AP Marker)

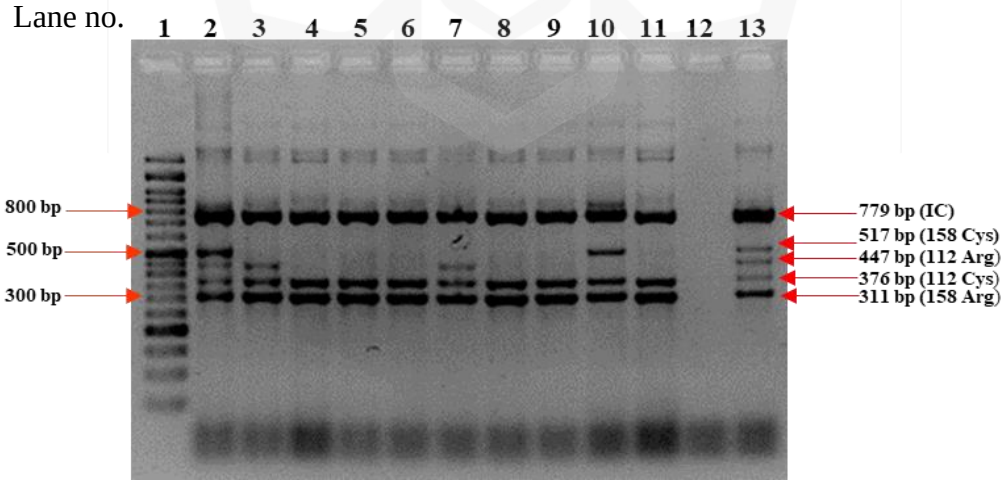


Figure 4. 3 Agarose gel electrophoresis of PCR products of *APOE* amplicons (Lane 1; DNA ladder 50 bp, Lane 2; E2/E4 genotypes, Lane 3&7; E3/E4 genotypes, Lane 4,5,6,8,9&11; E3/E3 genotypes, Lane 12; NC, Lane 13; AP Marker)

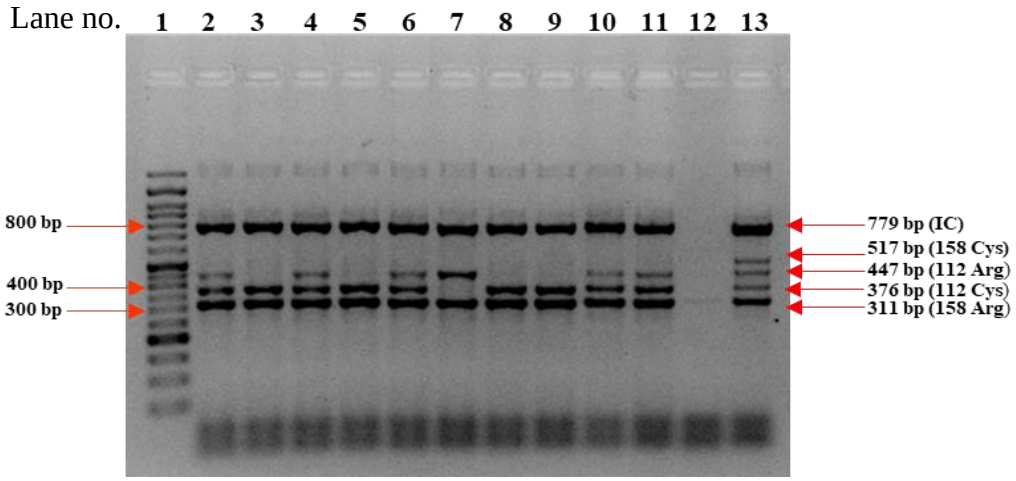


Figure 4. 4 Agarose gel electrophoresis of PCR products of *APOE* amplicons (Lane 1; DNA ladder 50 bp, Lane 2,4,6,10&11; E3/E4 genotypes, Lane 3,5,8&9; E3/E3 genotypes, Lane 7; E4/E4 genotypes, Lane 12; NC, Lane 13; AP Marker)

4.4 GENOTYPES AND ALLELE FREQUENCIES

4.4.1 The Association of *APO* Gene Polymorphism Between YAMI Patients and Controls.

Tables 4.5 and 4.6 showed the distribution of *APOE* genotypes and alleles among YAMI and healthy controls. There was no deviation of genotype frequencies from the Hardy-Weinberg equilibrium in the cases and control groups.

Table 4.3 *APOE* genotypes and alleles in YAMI and Healthy control

APOE genotypes	YAMI (n=20) N (freq)	Controls (n=20) N (freq)	Chi-square	p-value
E2/E2	1 (0.05)	0 (0)		
E2/E3	0 (0)	2 (0.1)		
E2/E4	1 (0.05)	1 (0.05)	4.471	0.484
E3/E3	14 (0.7)	11 (0.55)		
E3/E4	4 (0.2)	5 (0.25)		
E4/E4	0 (0)	1 (0.05)		
Alleles				
Ɛ2	3 (0.075)	3 (0.075)		
Ɛ3	32 (0.8)	29 (0.725)	0.8398	0.657
Ɛ4	5 (0.125)	8 (0.2)		

The chi-square test (for association statistical analysis of categorical parameters), $p < 0.05$ is taken as statistically significant at 95% confidence interval *significant difference.

E3/E3 and Ɛ3 were the most frequent *APOE* genotypes and alleles in both study groups respectively. However, there were no significant differences in *APOE* genotypes and alleles between YAMI and healthy controls.

The distribution of *APOE* genotypes and alleles insignificantly differed between YAMI and control (p=0.484 and p= 0.657). As shown in Table 4.3, E3/E3 genotypes were the most common in both groups with frequencies of (0.7) and (0.55), followed by E3/E4 genotypes with frequencies of (0.2) in YAMI and (0.25) in healthy control subjects.

Ɛ4 allele in YAMI subjects was more frequent (0.125) than Ɛ2 allele (0.075). However, Ɛ4 allele is higher in healthy controls (n = 8) compared to YAMI (n = 5). On the other hand, the wild type Ɛ3 allele showed the highest frequency and the most common allele in this study.

Table 4.4 shows the genotypic and allelic differences between YAMI and healthy control subjects. The genotype distribution of the two SNPs conformed to the Hardy-Weinberg equilibrium in the control sample. There were no significant differences in genotypes and allele frequencies between YAMI and controls for both SNPs.

Table 4.4 Association of genotypes and alleles distribution of two *APOE* -related SNPs with YAMI and control subjects

Marker	Genotype			p-value (2df)	HWE	Allele		p-value (1df)	OR (95% CI)
	CC	TC	TT			C	T		
rs429358									
YAMI	0 (0)	5 (0.250)	15 (0.750)	0.540	0.523	5 (0.125)	35 (0.875)	0.363	0.57 (0.17-1.93)
Control	1 (0.050)	6 (0.300)	13 (0.650)		0.780	8 (0.200)	32 (0.800)		
rs7412									
YAMI	18 (0.900)	2 (0.100)		0.632	0.814	38 (0.950)	2 (0.050)	0.644	1.54 (0.24-9.75)
Control	17 (0.850)	3 (0.150)			0.717	37 (0.925)	3 (0.075)		

P > 0.05 for HWE in both cases and controls indicates no violation of population stratification. The chi-square test (for association statistical analysis of categorical parameters) , p < 0.05 is taken as statistically significant at 95% confidence interval *significant difference.

4.4.2 Meta-Analyses of *APOE* Gene In Coronary Artery Disease

The meta-analyses were subsequently performed to improve the statistical power of this study due to the insignificant findings in the results. A total of 25 studies on association of *APOE* genotypes with coronary artery disease were found on the literature search. Eight (8) studies from Asian populations with similar methodology were selected for the meta-analysis with total samples of 4,369 and 3,196 for CAD and healthy controls respectively. This meta-analysis discovered that individuals with E3/E4 genotype [p=0.000, OR= 1. 60 (95% CI: 1.41-1.83) had a significantly increased the risk of CAD while individuals with E3/E3 genotype [p=0.000, OR= 0.73 (95% CI: 0.66-0.81) were protective against CAD. The E2/E3 genotype [p=0.206, OR= 0.91 (95% CI: 0.78-1.06) was not found to be protective against CAD.

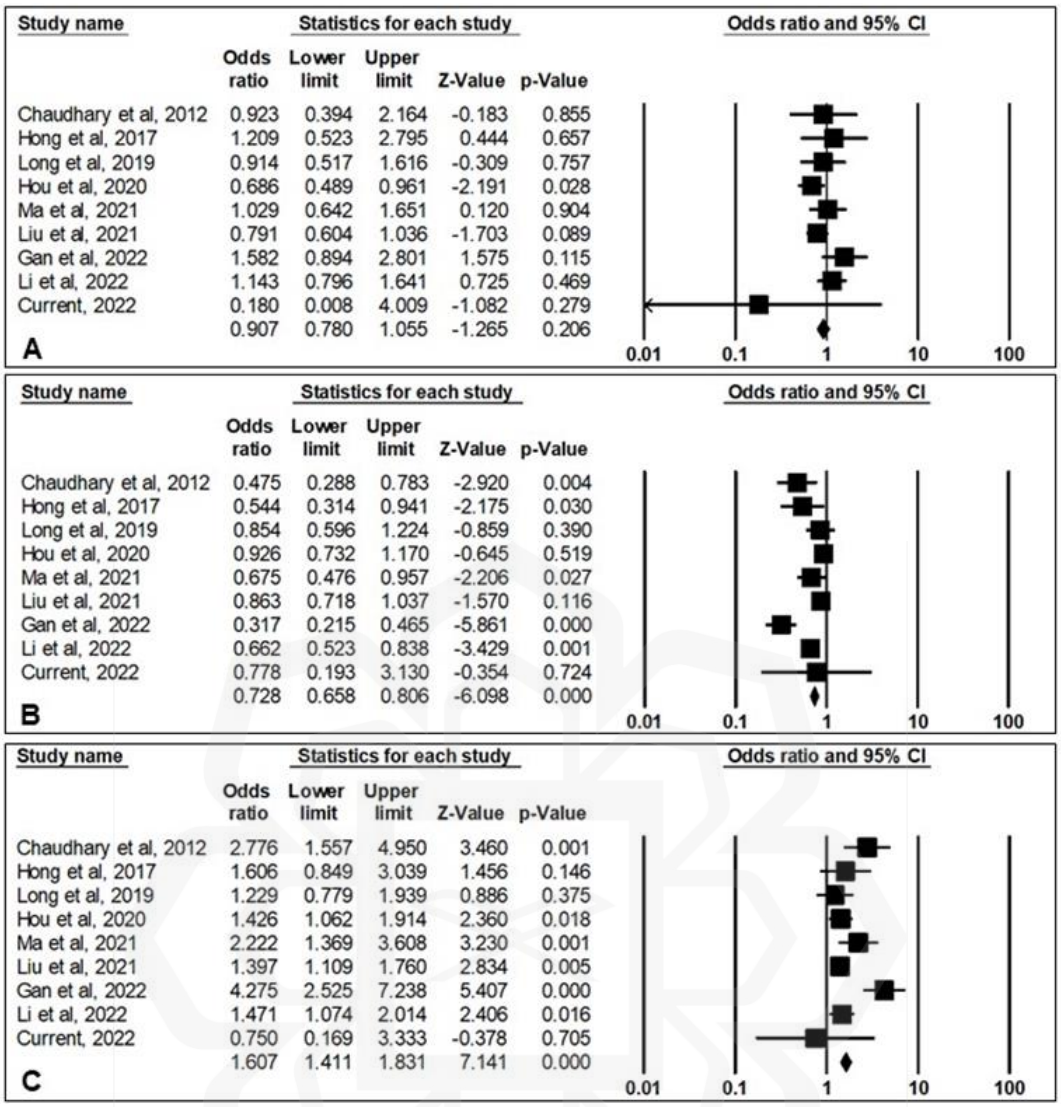


Figure 4.5 Meta-analysis findings *APOE* genotypes of E2/E3 (A), E3/E3 (B) and E3/E4 (C)

A subsequent of meta-analysis on *APOE* alleles confirmed the CAD risk of Ɛ4 allele [p=0.000, OR= 1.56 (95% CI: 1.40-1.74)], [p=0.000, OR=1.60 (95% CI: 1.36-1.89)] as compared to Ɛ3 and Ɛ2 alleles respectively. The Ɛ2 allele was not found to be protective against CAD [p=0.76, OR= 0.98 (95% CI: 0.86-1.118)] as compared to Ɛ3 allele.

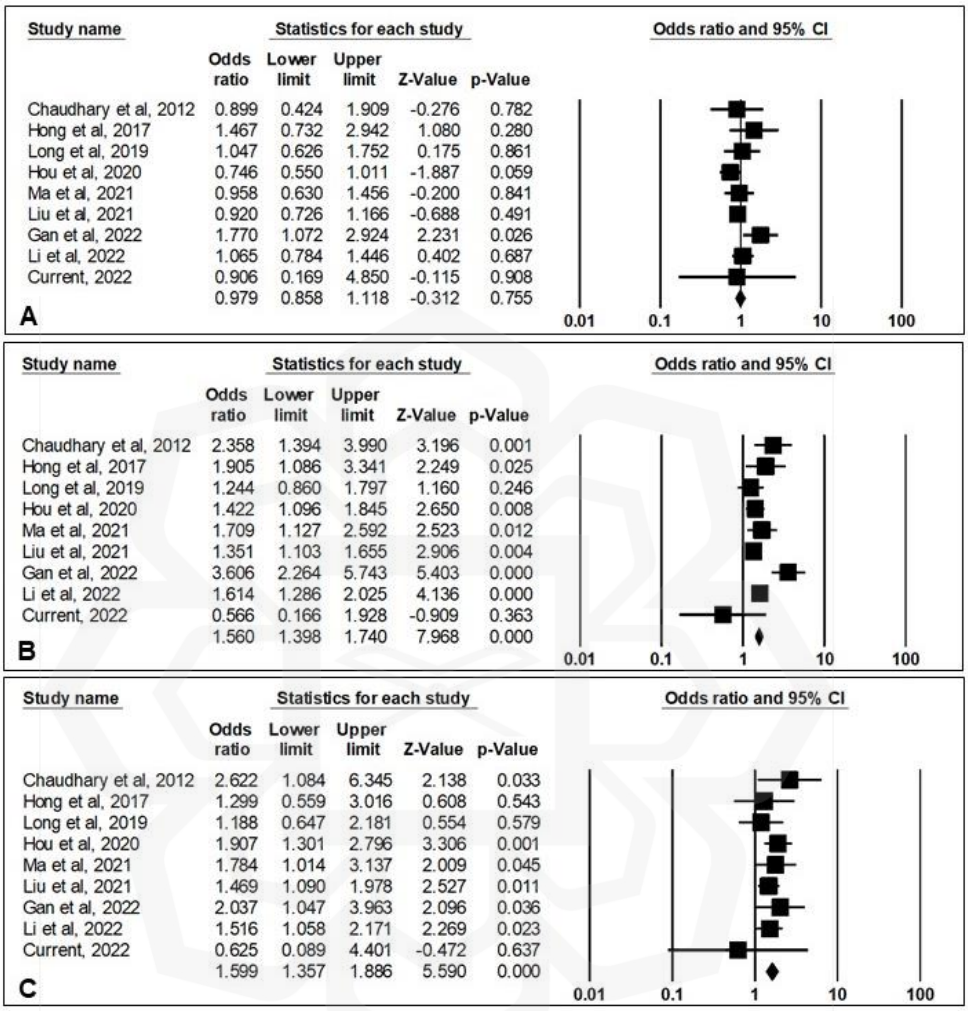


Figure 4.6 Meta-analysis findings of *APOE* alleles of Ɛ2 vs Ɛ3 A), Ɛ4 vs Ɛ3 (B) and Ɛ4 vs Ɛ2 (C)

4.5 LIPID PARAMETERS DISTRIBUTION IN *LPA* CNV STATUS IN YAMI PATIENTS

Table 4.5 shows the distribution of lipid parameters in *LPA* CNV status in YAMI patients. However, there is not statistically significant differences between lipid parameter and *LPA* CNV status.

Table 4.5 Relationship between lipid parameters and *LPA* CNV status in YAMI.

Lipid parameter	*LPA* CNV	N	Median	IQR	P value
Total cholesterol (TC) mmol/L	Loss	5	5.8	2.84	0.076
	Normal	10	4.94	2.13	
	Gain	5	6.24	1.57	
	Total	20			
Triglycerides (TG) mmol/L	Loss	5	1.4	2.43	0.149
	Normal	10	1.83	0.73	
	Gain	5	1.3	1.58	
	Total	20			
HDL cholesterol (HDL-C) mmol/L	Loss	5	1.17	0.20	0.058
	Normal	10	0.895	0.24	
	Gain	5	1.16	0.23	
	Total	20			
LDL cholesterol (LDL-C) mmol/L	Loss	5	4.39	1.89	0.197
	Normal	10	3.35	1.83	
	Gain	5	4.07	1.49	
	Total	20			

Kruskal Wallis test, $p < 0.05$ is taken as statistically significant at 95% confidence interval *significant difference.

4.6 LIPID PARAMETERS DISTRIBUTION OF *APOE* GENE POLYMORPHISM IN YAMI PATIENTS

There are 6 possible genotypes of *APOE.* However, only 4 genotypes were discovered in the current study. The analysis of the relationship between lipid parameter and *APOE* genotypes only tested on E3/E3 and E3/E4. However, there was no statistically significant differences between lipid parameter and *APOE* genotypes.

Table 4. 6 Relationship between lipid parameters and *APOE* genotypes in YAMI

Lipid parameters	*APOE* genotypes	N	Median	IQR	p-value
Total cholesterol (TC) mmol/L	E2/E2	1			
	E2/E4	1			
	E3/E3	14	5.7	1.69	0.282
	E3/E4	4	7.03	3.83	
Triglycerides (TG) mmol/L	E2/E2	1			
	E2/E4	1			
	E3/E3	14	1.47	0.62	0.218
	E3/E4	4	1.985	3.33	
HDL cholesterol (HDL-C) mmol/L	E2/E2	1			
	E2/E4	1			
	E3/E3	14	0.99	0.37	0.473
	E3/E4	4	1.10	0.28	
LDL cholesterol (LDL-C) mmol/L	E2/E2	1			
	E2/E4	1			
	E3/E3	14	3.89	1.22	0.310
	E3/E4	4	5.045	2.81	

Kruskal Wallis test, $p < 0.05$ is taken as statistically significant at 95% confidence interval *significant difference.

4.7 LIPID PARAMETERS DISTRIBUTION IN THE PRESENCE OF BOTH *LPA* GENE AND *APOE* GENE POLYMORPHISM IN YAMI PATIENTS

Table 4.7 shows the results obtained from the Kruskal Wallis analysis of the relationship between lipid parameters and the presence of both *LPA* gene and *APOE* gene polymorphism in YAMI and control subjects. There are statistically significant differences in lipid parameter distribution of triglycerides mmol/L (p=0.025), and HDL cholesterol mmol/L (p=0.020) in presence of both *LPA* gene and *APOE* gene polymorphism.

Table 4.7 Relationship between lipid parameters and the presence of both *LPA* gene and *APOE* gene polymorphism in YAMI

Lipid parameters	*LPA* CNV status & *APOE* allele	N	Median	IQR	p-value
Total Cholesterol mmol/L	Ɛ4/ Gain	1			
	Ɛ4/ Loss	1			
	Ɛ4/ Normal	3	6.42	-	
	Non Ɛ4 / Gain	4	6.11	1.33	0.0076
	Non Ɛ4 / Loss	4	5.75	1.52	
	Non Ɛ4 / Normal	7	4.67	1.69	
	Total	20			
Triglycerides mmol/L	Ɛ4/ Gain	1			
	Ɛ4/ Loss	1			
	Ɛ4/ Normal	3	1.68		
	Non Ɛ4 / Gain	4	1.11	0.60	0.025
	Non Ɛ4 / Loss	4	1.3	0.44	
	Non Ɛ4 / Normal	7	1.86	0.68	
	Total	20			
HDL cholesterol mmol/L	Ɛ4/ Gain	1			
	Ɛ4/ Loss	1			
	Ɛ4/ Normal	3	1.03		
	Non Ɛ4 / Gain	4	1.18	0.07	0.020
	Non Ɛ4 / Loss	4	1.09	0.21	
	Non Ɛ4 / Normal	7	0.84	0.21	
	Total	20			
LDL cholesterol mmol/L	Ɛ4/ Gain	1			
	Ɛ4/ Loss	1			
	Ɛ4/ Normal	3	4.4		
	Non Ɛ4 / Gain	4	4.105	1.42	0.226
	Non Ɛ4 / Loss	4	4.245	1.69	
	Non Ɛ4 / Normal	7	3.20	1.24	
	Total	20			

Kruskal Wallis test, $p < 0.05$ is taken as statistically significant at 95% confidence interval *significant difference.

CHAPTER FIVE

DISCUSSION

5.1 OVERVIEW OF THE STUDY

APOE is a serum glycoprotein that serves as a ligand for cell-surface receptor uptake of chylomicrons and very low-density lipoproteins (VLDL) in the liver and controls intestinal cholesterol absorption which is essential in lipid metabolism (Ashiq & Ashiq, 2021; Bennet et al., 2007; Gupta et al., 2018; Karahan et al., 2015). Lipoprotein (a) [Lp(a)] is a protein consisting of low-density lipoprotein particles which covalently attached to apolipoprotein (a) [apo(a)] and can interfere with plasminogen activation. It has atherogenic potential as a lipoprotein particle after receptor-mediated uptake (Wu et al., 2014). This highly atherogenic Lp(a) is primarily controlled by the *LPA* gene locus (Kronenberg, 2016). *APOE* polymorphism and *LPA* CNV are the prominent genetic determinants of AMI. The role of *APOE* and its polymorphism in AMI have been widely studied in various populations. However, there was a limited reported study on the role of copy number variation in AMI and no published data on this study in Malaysia. Moreover, the involvement of both *APOE* polymorphism and *LPA* CNV in the pathogenesis of AMI in young populations have not been extensively explored. The present study was undertaken to investigate the association of *LPA* gene copy number variation and *APOE* polymorphism with AMI in young adults in comparison to healthy controls in Kuantan, Pahang.

5.2 GENERAL CHARACTERISTICS OF THE STUDY POPULATION

The study population comprised of twenty (20) YAMI patients and twenty (20) healthy controls. There are many confounding factors such as age, gender, disease states and past interventions for AMI and medications that might influence the outcome of the study. Therefore, measures were taken to minimize the effects of these factors, including setting up the inclusion and exclusion criteria for the participants and the comparative groups in this study. In order to maintain the homogeneity of the data, this study only includes Malay males as the participants. However, the demographic and clinical characteristics showed differences in age, BMI, smoking status and lipid profile. The significant differences observed in these aspects could have potentially influenced the results of our study, acting as a confounding factor. These distinctions are relevant because they can have a notable impact on the levels of *LPA* and *APOE,* crucial elements in our research. Despite our efforts to enhance the similarity of age among groups, these observed distinctions in factors may still exert an influence on our findings, potentially altering the outcomes. These parameters must be taken into consideration as the previous study demonstrated the age (Kolovou & Anagnostopoulou, 2007; Li et al., 2022), smoking (Dai et al., 2018), BMI (Teng et al., 2020) and lipid metabolism(Nawabi et al., 2019) are the risk factors for CAD which affect the apolipoprotein E gene polymorphism and Lp(a) levels.

In this study, the majority of YAMI patients are active smokers and have higher BMI, TC, TG, and LDL-C with lower HDL-C as compared to healthy controls. There were significant differences in BMI, TC, TG, HDL-C and smoking status which reveals that active smokers and abnormal lipid profiles are important risk factors for AMI in our study population. Similarly, a study by (Biery et al., 2020) reported that 2072 individuals developed their first MI at the age of 50 or younger and half of them were smokers at the time of presentation. Moreover, (Gao et al., 2021) highlighted in their study that a total of 375 patients aged less than 50 years old were more often to be smokers, obese and dyslipidaemia.

5.3 DETECTION OF *LPA* GENE COPY NUMBER VARIATION

This study discovered that the gain in copy number of *LPA* (>2 copies) was higher in YAMI patients than in healthy controls. Moreover, the loss in copy number of *LPA* (<2 copy) was shown to be higher in healthy controls. However, there were no significant associations reported in the current study. Similarly, a study by (Wu et al., 2014) investigated the association between CNV of the *LPA* gene and CAD in the Southern Han Chinese population. In this study, they reported that a gain in copy number (3 copies) of *LPA was* associated with the increased risk of CAD whilst a loss in copy number of *LPA* (1 copy) reduced the risk. The low copy number of *LPA* alters its binding affinity for fibronectin and glycosaminoglycans which reduces the impairment of fibrinolytic function and binding to pro-inflammatory oxidized phospholipids as well as inhibits the progression of atherosclerosis.

The *LPA* gene Kringle IV type 2 (KIV-2) repeat copy number is reported to be a major gene in determining the variations and concentration of Lp(a) that is associated with CAD in different populations (Nordestgaard & Langsted, 2016). A small Lp(a) lipoprotein size and the *LPA* gene expression were highly associated with elevated levels of Lp(a) lipoprotein in which high levels of Lp(a) are related to an increased risk of cardiovascular disease due to its atherosclerotic and prothrombotic properties. This influences coronary plaque composition (Li et al., 2021). Despite this, our study failed to demonstrate the relationship between the copy number of *LPA* and the concentration level of circulating Lp(a).

The association between copy number variation (CNV) of the *LPA* gene and the level of Lp(a) has been investigated in several studies. In the context of the Chinese Han population, a study by (Sun et al., 2018) explored the correlation of Lp(a) mass or particle concentration with KIV-2 repeat copy number and its application for coronary atherosclerotic heart disease (CAHD) risk assessment 1. This study found an association between low *LPA* gene Kringle IV-2 repeat copy number and elevated Lp(a) concentration, suggesting that KIV-2 copy number variation is linked to Lp(a) levels and may serve as an independent risk factor for CAHD in the Chinese Han population. Individuals with a lower number of Kringle IV-2 (KIV-2) repeats in the *LPA* gene tend to have higher concentrations of Lp(a), whereas those with a higher number of KIV-2

repeats exhibit lower levels of Lp(a). This indicates an inverse correlation between the KIV-2 repeat copy number variation of the *LPA* gene and the concentration of Lp(a) in individuals. This genetic diversity within the *LPA* gene substantially contributes to the variations in Lp(a) concentrations among individuals and populations (Schmidt et al., 2016).

5.4 ASSOCIATION OF *APOE* GENE POLYMORPHISM BETWEEN YAMI PATIENTS AND CONTROLS

In the present investigation, the frequency of *APOE* genotypes E3/E3 and Ɛ3 allele was the highest in AMI patients and healthy controls. These findings were similar to a study by (Lin et al., 2022) where the most common *APOE* genotype observed in patients with CHD was ε3/ε3, which is associated with normal lipid concentrations. Similarly, in the healthy control group, the predominant *APOE* genotype was also ε3/ε3. Furthermore, the ε3 allele was identified as the most prevalent allele in both patients with CHD and healthy controls.

A study by Elmadbouh et al, (2013) reported *APOE* genotype frequencies in patients with coronary artery disease (CAD) compared to controls. The study found the following distribution of genotypes among CAD patients: E3/E3 (62.50%), E2/E3 (18.75%), E3/E4 (17.50%), E2/E4 (1.25%), E4/E4 (0), and E2/E2 (0). This indicates that the E3/E3 genotype was the most common among CAD patients, followed by E2/E3 and E3/E4 genotypes, while E4/E4 and E2/E2 genotypes were not present in the CAD patient group.

Furthermore, in other studies, the Ɛ4 allele, particularly the E3/E4 and E4/E4 genotypes, has been consistently linked to an elevated risk of (CAD) across diverse populations (Lin et al., 2022; Ma et al., 2021a; Prakash D. Zende, 2020). Moreover, studies have indicated that individuals carrying the e4 allele of the *APOE* gene have a 42% higher risk of CHD, suggesting a potential link between *APOE* gene polymorphism and CHD risk in Asians (Yousuf & Iqbal, 2015).

A meta-analysis of 22 studies revealed that the *APOE* Ɛ2 allele was associated with a reduced risk of myocardial infarction (MI), while the Ɛ4 allele was linked to an

increased risk of MI, especially in Caucasian and Asian populations. Additionally, the E2/E3 genotype was associated with a decreased risk of MI, whereas the E3/E4 and E4/E4 genotypes were associated with an elevated risk of MI. These findings indicate a substantial association between *APOE* genotypes and the risk of myocardial infarction (Wang et al., 2015).

In contrast, the current study found that the frequencies of *APOE* Ɛ4 allele, E3/E4 genotype and E4/E4 genotype were noted to be higher in healthy controls than in YAMI patients. When *APOE* genotypes and alleles were analysed for their association between YAMI and healthy controls, no associations were found.

APOE gene contains two main polymorphisms, rs429358 and rs7412, located on its fourth exon (Najd-Hassan-Bonab et al., 2023). These rs429358 and rs7412 are associated with the amino acid positions 112 and 158 in the *APOE* protein, where the variations at these sites are labelled as C and T alleles. The presence of the C allele at rs429358 corresponds to the E4 isoform. Correspondingly, the rs7412 SNP is associated with the amino acid change from arginine to cysteine at position 158 in the *APOE* protein. The presence of the T allele at rs7412 corresponds to the E3 isoform and the C allele to the E4 isoform. Carriers of the ε4 allele have been reported to have a higher risk of MI compared to non-carriers (Shao et al., 2022)

The current study showed no significant differences in genotypes and allele frequencies between YAMI and controls for rs429358 and rs7412. Nevertheless, research conducted by Rahman et al., (2023) reported that the E4 allele and E3/E4 genotype of *APOE* variant rs429358 with an odds ratio (OR) of 2.13 (1.32–2.65) and a p-value of 0.031 showed a significant association with MI patients. However, this study did not find a significant association between rs7412 and the risk of MI.

5.5 RELATIONSHIP BETWEEN *LPA* CNV STATUS AND LIPID PARAMETERS IN YAMI PATIENTS

The present study also observed the relationship between *LPA* CNV status and lipid parameters in YAMI patients. This study found that there are no statistically significant between the level of lipid parameters and *LPA* CNV status. However, there is limited study that investigate the association between *LPA* copy number variation (CNV) status and lipid parameters in patients with MI.

The study by Wu et al., (2014) showed the stratified analyses of the *LPA* 1 copy number with the risk of coronary artery disease (CAD) in cases and controls. In high Lp(a) (≥ 0.3 g/L) population, one copy number carriers had a 77% reduced risk of CAD compared to two copy number carriers ($P < 10^{-3}$, 95% CI 0.10-0.55) and in low Lp(a) (< 0.3 g/L) population, no significant association was found between one copy number carriers and reduced risk of CAD (P = 0.06, OR = 0.52, 95% CI 0.26-1.02). In non-hyperlipidemia (HLP) population one copy number carriers had a significantly reduced risk of CAD ($P < 10^{-3}$, OR = 0.36, 95% CI 0.18-0.71). These analyses suggest that the impact of *LPA* one copy number on the risk of CAD may vary based on Lp(a) levels and the presence of hyperlipidemia, with a more pronounced protective effect observed in individuals with high Lp(a) levels and non-HLP.

The majority of previous studies have primarily focused on comparing lipid parameters with risk factors among individuals with coronary artery heart disease (CAHD) and those without.

5.6 RELATIONSHIP BETWEEN *APOE* GENOTYPES AND LIPID PARAMETERS IN YAMI PATIENTS

Out of the six potential *APOE* genotypes, only four were identified in the present study. The investigation of the relationship between lipid parameters and *APOE* genotypes specifically focused on testing for E3/E3 and E3/E4. However, no statistically significant differences were found between lipid parameters and *APOE* genotypes.

The previous study investigated the relationship between serum lipid profiles and *APOE* alleles in coronary artery disease (CAD) patients. Among CAD patients with different *APOE* alleles (ε2, ε3, and ε4), the study analyzed various lipid levels. The results indicated no significant differences in total cholesterol, high-density lipoprotein, low-density lipoprotein cholesterol, triglycerides, creatinine, or apolipoprotein A-I levels among carriers of different *APOE* alleles. However, ApoB levels and the ApoB/ApoA-I ratio was significantly lower in ε2 carriers compared to ε3 or ε4 carriers. Additionally, *APOE* levels were significantly higher in ε2 carriers compared to ε3 or ε4 carriers (Ma et al., 2021).

Moreover, a review of the existing study by (Nawabi et al., 2019) reported Han Chinese individuals with the *APOE* 4 allele demonstrate elevated levels of low-density lipoprotein (LDL) cholesterol, which can contribute to dyslipidemia, a recognized risk factor for cardiovascular diseases.

5.7 META-ANALYSES OF *APOE* GENE IN CORONARY ARTERY DISEASE

Additionally, a meta-analysis was conducted to enhance the statistical strength of this study by combining data from several prior studies conducted on Asian populations. In this meta-analysis, we examined the allele frequencies of Ɛ2, Ɛ3, and Ɛ4 in comparison to those reported in previously published studies on other Asian populations.

Our meta-analysis confirmed the association between the Ɛ4 allele and the risk of coronary artery disease (CAD), while there is no indication that the Ɛ2 allele provides protection against CAD. This finding regarding the Ɛ4 allele's association with CAD risk aligns with previous meta-analyses (Ashiq & Ashiq, 2021; M. D. Zhang et al., 2014; Y. Zhang et al., 2015). Moreover, a meta-analysis conducted by Xu et al, (2016) revealed that individuals carrying the Ɛ4 allele had a 46% increased risk of coronary artery disease (CAD), with (OR = 1.46, 95% CI = 1.28– 1.66). The Ɛ4 allele elevates cholesterol levels by facilitating the transfer of cholesterol ester from high-density lipoproteins (HDL) to triglyceride-rich lipoproteins. This process promotes hepatic remnant clearance via *APOE* receptors while concurrently reducing low-density lipoprotein receptor (LDLR) activity (Wang et al., 2015).

In numerous studies and meta-analyses, the Ɛ2 allele has consistently been identified as having a protective effect against coronary artery disease (CAD) (Shao et al., 2022; H. Xu et al., 2014). This is attributed to the poor binding of *APOE* Ɛ2 to LDL receptors (LDLR), resulting in an increase in LDLR numbers and subsequently lowering cholesterol levels (Wang et al., 2015). However, our meta-analysis findings contradict this trend. This discrepancy could potentially be due to a higher number of coronary artery disease (CAD) cases compared to controls in the studies included (Gan et al., 2022; Li et al., 2022; Liu et al., 2021; Long et al., 2019).

5.8 STUDY LIMITATIONS AND FUTURE RECOMMENDATION

The present study did not include similar age groups for both YAMI patients and healthy controls. It is important to match participants based on age groups between both groups to reduce the impact of age-related differences on the results. This can be achieved by either selecting controls within similar age ranges as the patients or by categorizing the analysis into separate age brackets. By doing so, any potential effects of age-related differences on the outcomes can be minimized, thus enhancing the validity and reliability of the study findings.

Furthermore, in the present study, the insignificant results reported could be due to the small sample size. Gathering samples for YAMI patients within the designated timeframe proved to be challenging, as such cases are not common occurrences at the hospital. It is suggested that additional research with larger sample sizes to confirm the identified associations, particularly concerning *LPA* gene copy number variations and their interaction with *APOE* gene polymorphisms.

Moreover, in future studies, it's important to consider gene-environment interactions to better evaluate the causal role of genes in the development of coronary artery disease (CAD). This involves examining how genetic factors interact with environmental influences, such as lifestyle choices, diet, and exposure to pollutants or toxins. Understanding these interactions can provide valuable insights into the mechanisms underlying CAD and help disease prevention and treatment plans accordingly.

CONCLUSION

In conclusion, the present study showed that:

1. There is no significant association of *LPA* gene CNV between YAMI patients and normal controls.
2. There is no significant difference between *LPA* gene CNV status and lipid parameters in YAMI patients.
3. There is no significant difference between *APOE* gene polymorphism and lipid parameters in YAMI patients.
4. There is no association between the copy number of the *LPA* gene and the presence of *APOE* polymorphism in young acute myocardial infarction.
5. Current meta-analysis supports the notion that the Ɛ4 allele significantly increases the risk of CAD, whereas the Ɛ2 allele neither increases nor decreases the risk of CAD.

Apart from larger sample size, other factors such as gene-environment interactions need to be considered in future studies in order to evaluate the etiological role of the genes in the development of CAD.

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APPENDIX I: LIST OF PUBLICATIONS AND AWARDS

1. Iffah Irdhina Mohd Zamri, Nor Zamzila Abdullah, Norlelawati A. Talib, Nurul Ashikin Muhammad Musa, Aszrin Abdullah. The Association of *LPA* Gene Copy Number Variation and Apolipoprotein E (*APOE)* Gene Polymorphism in Young Acute Myocardial Infarction. Winner. Poster presented at International Virtual Medical Research Symposium 2022 –15th December 2022.

1. Iffah Irdhina Mohd Zamri, Nor Zamzila Abdullah, Norlelawati A. Talib, Nurul Ashikin Muhammad Musa, Aszrin Abdullah. The Association of *LPA* Gene Copy Number Variation and Apolipoprotein E (*APOE)* Gene Polymorphism in Young Acute Myocardial Infarction. Participant. Poster presented at 46th Annual Conference of The Malaysian Society for Biochemistry and Molecular Biology - 24-25 August 2022.


MSBMB 46

This certificate is presented to

IFFAH IRDHINA BINTI MOHD ZAMRI

INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA

in recognition of his/her participation as

POSTER PRESENTER

for the paper entitled

THE ASSOCIATION OF LPA GENE COPY NUMBER VARIATION (CNV) AND APOLIPOPROTEIN E (APOE) GENE POLYMORPHISM IN YOUNG ACUTE MYOCARDIAL INFARCTION (AMI)

at the

46TH ANNUAL CONFERENCE OF THE MALAYSIAN SOCIETY FOR BIOCHEMISTRY AND MOLECULAR BIOLOGY

"ADVANCEMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY IN THE DIGITAL ERA"

held on the

24-25 AUGUST 2022

BANGI RESORT HOTEL, SELANGOR, MALAYSIA

ASSOCIATE PROFESSOR
DR. NG CHYAN LEONG
CHAIRMAN
46th Annual Conference of the Malaysian Society for Biochemistry and Molecular Biology

ASSOCIATE PROFESSOR
DR. ANTHONY HO SIONG HOCK
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