UNIVERSITI TEKNOLOGI MARA

PREDOMINANT GENETIC VARIANTS OF FAMILIAL HYPERCHOLESTEROLAEMIA (*LDLR*, *APOB* AND *PCSK9*) IN MALAYSIAN PRIMARY CARE POPULATION USING NEXT-GENERATION SEQUENCING

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MSc

April 2026

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of the requirements for the degree of
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ABSTRACT

Familial hypercholesterolaemia (FH) is an autosomal dominant disorder which impairs low-density lipoprotein cholesterol (LDL-C) clearance and leads to development of atherosclerosis. Pathogenic variants (PVs) in *LDLR*, *APOB*, and *PCSK9* genes are predominantly associated with FH. For FH screening is usually based on the clinical rather than molecular diagnosis. In Malaysia, where first-line FH screening is usually conducted in primary care clinics, the screening of FH gene mutation is still under-reported. This study aimed to determine the PV's frequency of FH-causing gene (*LDLR*, *APOB*, and *PCSK9*) variants among those clinically diagnosed as FH based on Dutch Lipid Clinic Network (DLCN), Simon Broome (SB) and Familial Hypercholesterolaemia Case Ascertainment Tool (FAMCAT) criteria. A total of 310 patients (mean ± SD age: 45.3 ± 9.4 years; men = 51.6%) were recruited, and blood samples were collected. DNA samples were extracted and subjected using targeted next-generation sequencing (iSeq 100, Illumina). PVs were determined based on the American College of Medical Genetics and Genomics Guidelines. DLCN provides a structured scoring system with internationally validated cut-offs, enabling reliable stratification of patients into definite, probable, or possible FH. Hence, DLCN was considered the most appropriate standard for evaluating genetic findings in this population. Based on DLCN, 97.4% were classified as Definite and Probable (Potential FH) and Possible FH. Fifty PVs were identified [*LDLR* = 64.0%, *APOB* = 36.0%, and no *PCSK9*]. Among 245 variants reported, 13.1% were *LDLR* PVs and 7.3% were *APOB* PVs. Overall, 27.7% of clinically diagnosed FH patients has ≥1 PV (Monogenic *LDLR* = 47.7%, compound homozygous in *LDLR* = 1.2%, compound homo and hetero in *LDLR* = 1.2%, compound heterozygous in *LDLR* = 4.7%, double heterozygous in *LDLR* = 4.7% monogenic *APOB* = 34.9%, and compound heterozygous in *APOB* = 5.8%). In total, only 27.7% of clinically diagnosed FH patients were positive for FH-causing gene PVs, with *LDLR* as the predominant gene. The low detection rate of PVs among clinically diagnosed FH patients can be due to polygenic hypercholesterolaemia or the presence of novel PVs in genes other than FH.

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In the Name of Allah, The Most Gracious The Most Merciful.
Read in the name of your Lord Who created. He created man from a clot. Read and your Lord is Most Honourable, Who taught (to write) with the pen. Taught man what he knew not. (Surah Al Alaq 96: 1-5)

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LIST OF SYMBOLS

Symbols

&	And
≤	Less Than or Equal
≥	More Than or Equal
<	Less Than
>	More Than
%	Percent
mmol/L	Millimoles Per Litre
mg/dl	Milligrams Per Decilitre
β	Beta
kbp	Kilobase Pairs
kb	Kilobase
±	Plus Minus
Z	95% Confidence Interval (CI)
Δ	Maximum Acceptable Difference Between Sample and Population
p	Expected Proportion of Individuals
mL	Milliliter
rpm	Revolutions Per Minute

℃	Degree Celsius
µl	Microliter
G	G-Force
X	Concentration
M	Molar
KB	Kilobyte
V	Voltage
pg/µL	Picogram/Microliter
pg/L	Picogram/Liter
bp	Base Pair
nM	Nano Molar
pM	Pico Molar

LIST OF ABBREVIATIONS

Abbreviations

ABCG5	ATP Binding Cassette Subfamily G Member 5
ABCG8	ATP Binding Cassette Subfamily G Member 8
ACMG	American College of Medical Genetics
ADH	Autosomal Dominant Hypercholesterolaemia
AHA	American Heart Association
APOB	Apolipoprotein B
APOB100	Apolipoprotein B-100
APOE	Apolipoprotein E gene
BV	Benign Variant
CAD	Coronary Artery Disease
CHD	Coronary Heart Disease
CVD	Cardiovascular Disease
DALYs	Disability Adjusted Life Years
DLCN	Dutch Lipid Clinic Criteria
DNA	Deoxyribonucleic Acid
EGF	Epidermal Growth Factor
ExAC	Exome Aggregation Consortium
FAMCAT	Familial Hypercholesterolaemia Case Ascertainment

FDA	Food and Drug Administration
FDB	Familial Defective Apo B
FH	Familial Hypercholesterolaemia
FHBL	Familial Hypobetalipoproteinemia
GRCh37	Genome Reference Consortium Human Build 37
HC	Hypercholesterolaemia
HDL-c	High Density Lipoprotein Cholesterol
HeFH	Heterozygous FH
hg19	Human Genome version 19
HMG-CoA	Hydroxymethylglutaryl-CoA
HoFH	Homozygous FH
IDL	Intermediate-Density Lipoprotein
JFHMC	Japanese Familial Hypercholesterolaemia Management
LA	lipoprotein apheresis
LDL-c	Low-Density Lipoprotein cholesterol
LDLR	Low-Density Lipoprotein Receptor
LDLRAP1	Low-Density Lipoprotein Receptor Adaptor Protein 1
Lp(a)	Lipoprotein (a)
LP	Likely Pathogenic
MLPA	Multiplex Ligation-Dependent Probe Amplification
mRNA	Messenger Ribonucleic Acid
NGS	Next-Generation Sequencing

PCAD	Premature Coronary Artery Disease
PCR	Polymerase Chain Reaction
PCSK9	Proprotein Convertase Subtilisin/Kexin Type 9
PV	Pathogenic Variant
PVD	Peripheral vascular disease
SB	Simon Broome
SCORE	Systematic Coronary Risk Estimation
STAP1	Signal-Transducing Adaptor Protein 1
TC	Total Cholesterol
TG	Triglyceride
UiTM	Universiti Teknologi MARA
US MEDPED	United States Make Early Diagnosis to Prevent Early Deaths
VLDL	Very Low-Density Lipoprotein Cholesterol
VUS	Variant of Uncertain Significance
WHO	World Health Organization

CHAPTER 1
INTRODUCTION

1.1 Research Background

Familial hypercholesterolemia (FH) is a common genetic disease caused by gene mutations in the low-density lipoprotein receptor (*LDLR*)-mediated pathway for cellular uptake of low-density lipoprotein cholesterol (LDL-C) (Nohara et al., 2021; Razman et al., 2022). A lifelong elevation of LDL-C levels has been strongly linked to a higher risk of cardiovascular disease (CVD) in both men and women, premature coronary artery disease (CAD), and tendon and skin xanthomas (Bianconi et al., 2021; Bouhairie & Goldberg, 2015). In 2019, 17.9 million deaths were due to CVDs, accounting for 32% of all worldwide fatalities. Heart attacks and strokes were responsible for 85% of these deaths caused by CVDs (World Health Organization, 2021). FH may need early diagnosis, intensive treatment, and family screening (cascade screening) since it has a very high chance of developing into CAD (Harada-Shiba et al., 2023). Furthermore, premature CAD has been associated with FH. The prevalence of FH was eighteen times greater (1:17) in those with premature CAD than in the general population (Hu et al., 2020).

Classically, FH occurs due to a genetic mutation that shows an autosomal dominant inheritance pattern (Miroshnikova et al., 2021). *LDLR* are the most prevalent variants that cause FH (90–95%), with approximately 2000 *LDLR* variants documented in the Human Gene Mutation Database (Miroshnikova et al., 2021; Razman et al., 2022). While a variant in the gene for its ligand, apolipoprotein B100 (APOB100) (5-10%) and proprotein convertase subtilisin/kexin type 9 (PCSK9) genes are reported less than 10% and 3%, respectively (Razman et al., 2022; Vrablik et al., 2020). Globally, 90% to 95% of FH patients remain undiagnosed (Vallejo-Vaz & Ray, 2018).

Although FH is common in the general population, only about 15% to 20% of affected patients receive a clinical diagnosis. FH is classified as heterozygous (HeFH), homozygous (HoFH), compound heterozygous, or double heterozygous. HeFH is the most common of these variants. The prevalence of HoFH is estimated to be one to three in one million in the general population, whereas that of HeFH is higher, reaching 1 in 500 to 1 in 200 (Nohara et al., 2021; Wang et al., 2022). Malaysia has a

very high 1:100 prevalence of clinically confirmed FH, which is much higher than the expected global prevalence of 1:200–300 (Vallejo-Vaz & Ray, 2018; Chua et al., 2021). Early detection and initiation of lipid-lowering therapy are critical for preventing cardiovascular complications. Diagnosis relies on clinical characteristics, familial history, LDL-C levels, and the presence of tendon xanthomas, which are highly indicative of familial hypercholesterolaemia (Nohara et al., 2021). Individuals with HoFH have a more severe phenotype, with significantly higher LDL-C levels and a higher risk of premature CAD than those with HeFH (Lui et al., 2021).

The Dutch Lipid Clinic Network (DLCN) and the Simon Broome Register (SB) criteria are currently used by healthcare providers for FH diagnosis (Bagnasco, 2021). The DLCN FH criteria utilise a scoring system that incorporates the family history, clinical history, physical examination, LDL-C levels, and molecular diagnosis (*LDLR*, *APOB*, or *PCSK9*). Patients are classified as having definite FH (DLCN score >8), probable FH (DLCN score 6-8), possible FH (DLCN score 3-5), or unlikely FH (DLCN score 3) (Tada et al., 2021). SB criteria were developed in the United Kingdom in 2013 and are based on LDL-C levels, the presence of tendinous xanthoma in patients or first- or second-degree relatives, a family history of MI in less than 60 first-degree relatives or 50 second-degree relatives, and molecular diagnosis of FH-causing gene (*LDLR*, *APOB*, and *PCSK9*) mutations (Amerizadeh et al., 2022). Primary care clinic-based population screening, general population-based screening with FH diagnosed using DLCN and SB, patients' family histories with known premature CAD or severe hypercholesterolemia, and opportunistic screening at primary or secondary care clinics are all screening strategies. Ideally, FH should be diagnosed clinically and confirmed with DNA analysis, though genetic testing may be costly and less feasible in low-resource countries with high premature CAD burdens (Mach et al., 2020).

The familial hypercholesterolaemia case ascertainment tool (FAMCAT) was developed and validated using data from nearly three million primary care patients, including over five thousand cases of familial hypercholesterolaemia, from the Clinical Practice Research Datalink (CPRD) of 681 primary care practices in the United Kingdom. The algorithm was highly predictive in identifying primary care individuals who were most likely to have familial hypercholesterolaemia (S. Weng et al., 2019). The FAMCAT algorithm searches the patient's electronic health record (EHR) for the variables that are most likely to cause FH including triglycerides, secondary causes, statin prescribing, and family history. It concentrates on variables that are easily

extracted from routine entries. FAMCAT has begun to be included as a case-finding tool in primary care computer systems to determine who is qualified for additional evaluation, a specialist referral, and genetic testing for potential FH (Akyea et al., 2020; Qureshi et al., 2021; S. F. Weng et al., 2015).

Advances in targeted next-generation sequencing (NGS) methods allow for the simultaneous sequencing of multiple genes, resulting in genetic information in the FH-causing genes and other lipid metabolism-related genes in a clinical diagnostic setting. Nevertheless, it is debatable if these additional genes in NGS panels may raise the percentage of individuals with FH who had been molecularly identified (Reeskamp et al., 2020a). Genetic testing provided confirmation of FH in 28–80% over clinical criteria alone, depending on the diagnostic algorithm and the method of analysis (Lee et al., 2019). Numerous studies have shown that NGS is an exceptionally reliable technology for identifying FH mutations across a broad range of (Al-Khateeb et al., 2013; Chua et al., 2021b; Khoo et al., 2000; Lye et al., 2013; Razman et al., 2022; Reeskamp et al., 2020b).

1.2 Problem Statement

Malaysians have a significantly higher percentage of people with clinically diagnosed FH than most other populations globally (Chua et al., 2021). Most FH patients are misdiagnosed, untreated, or receive ineffective treatment, and many are discovered unexpectedly in primary care (Ramli et al., 2023). Hence, a targeted NGS approach was used to determine the percentage of patients attending primary care clinics in Malaysia that were referred for genetic testing who had a pathogenic variant (PV) in an established FH-causing gene (*LDLR*, *APOB100*, and *PCSK9*). Monitoring individuals who are at risk could be more informative if a PV is found in an affected individual. The Malaysian primary care population still underreports the genetic profiles of individuals with clinically diagnosed FH. Early detection and treatment aids ought to minimise the risk of FH Cascade screening is also suggested to be performed once the index patients are genetically confirmed, which enables early intervention and a precise diagnosis for affected family members.

In the Malaysian community, the overall frequency of genetically proven FH as defined by having potential FH was 1:427 (0.2%), but the prevalence of genetically confirmed FH as it included probable FH was greater, at 1:63 (1.6%) (Razman et al., 2022a). However, the primary care population still underreports the proportion of genetically confirmed FH among clinically diagnosed FH patients.

Prior research conducted in Malaysia demonstrated that the majority of FH patients are caused by monogenic FH, which is mostly caused by mutations in three genes: *LDLR*, *APOB100*, and *PCSK9* (AL-Khateeb & AL-Talib, 2016; Chua et al., 2021; Razman et al., 2022a). Approximately 25% of clinically diagnosed FH cases in the Malaysian community can be genetically validated by research that uses NGS. The study revealed that the majority of pathogenic variants (PV) were found in *LDLR* (45%) and *APOB* (37.5%), with both variants having nearly comparable frequencies, while 12.5% of patients had PV in *PCSK9* (Razman et al., 2022b). However, the status of the pathogenicity of FH-causing genes (*LDLR*, *APOB100*, and *PCSK9*) among clinically diagnosed FH patients attending Malaysia primary care clinics was not identified properly, as genetic testing is not routinely available in Malaysia. To confirm the significant proportion of clinically diagnosed FH in primary care settings, it is necessary to identify the genetic spectrum of FH in the Malaysian primary care population using

NGS. The results would also help in identifying the mutation pattern in Malaysian primary care patients, which may not be the same as in other parts of the world.

1.3 Research Questions of The Study

General research question:

1. What is the proportion of genetically confirmed FH amongst clinically diagnosed FH patients in the primary care population?
2. What are the genetic variants of FH-causing genes (*LDLR*, *APOB100*, and *PCSK9*) in patients with clinically diagnosed FH in the Malaysian primary care population?
3. What is the genetic spectrum and frequency of pathogenic variants in FH-causing genes among clinically diagnosed FH patients in the primary care population?

1.4 Objectives of The Study

General objective:

To investigate the genetic variants of FH-causing genes (*LDLR*, *APOB100*, and *PCSK9*) in patients with clinically diagnosed FH in the Malaysian primary care population.

Specific objectives:

1. To determine the proportion of genetically confirmed FH amongst clinically diagnosed FH patients in the primary care population by matching the grouping of DLCN (Possible FH, Probable FH, Definite FH) and SB (Possible FH and Definite FH) with the variants (PV, VUS, Benign) data from targeted next-generation sequencing.
2. To determine the genetic variants of FH-causing genes (*LDLR*, *APOB100*, and *PCSK9*) in patients with clinically diagnosed FH in the Malaysian primary care population by analysing PV, VUS and benign variants using targeted next-generation sequencing.
3. To determine the genetic spectrum and frequency of pathogenic variants in FH-causing genes amongst clinically diagnosed FH patients in the primary care population by analysing all variants of next-generation sequencing findings.

1.5 Hypothesis

1. The proportion of genetically diagnosed FH among primary care patients in Malaysia is significant, with most patients having Possible FH, followed by Probable FH and Definite FH according to DLCN and Possible FH and Definite FH according to SB.
2. The genetic spectrum and frequency of pathogenic variants of FH-causing genes, which are the *LDLR*, *APOB*, and *PCSK9* genes, are identified in clinically diagnosed FH patients in the primary care population.

1.6 Scopes and Limitations of The Study

1.6.1 Scope

The scope of this study focused on molecular analysis of PV for FH-causing genes (*LDLR*, *APOB* and *PCSK9*) using NGS in all blood samples of patients with clinically diagnosed of FH in the Malaysian primary care population. Genetic analysis was conducted to confirm pathogenic variants and determine the genetic spectrum of affected individuals.

1.6.2 Limitations

1. This study is limited to the recruitment of patients who were clinically diagnosed with FH according to DLCN (score ≥3), SB (Definite or Possible FH), and those who agreed/are available to participate in molecular analysis. The sample population confined to a certain area may not represent the whole Malaysian population, which was done only in the public health clinics in the area of the Klang Valley. The sample collection areas did not include public hospitals, private clinics, and private hospitals.
2. Genetic testing was done on FH-causing genes only and not included FH-associated genes such as apolipoprotein E (*APOE*), signal transducing adaptor family member 1 (*STAP1*), *LDLR* accessory protein 1 (*LDLRAP1*), lysosomal acid lipase (*LIPA*), ATP binding cassette sub-family G members 5 and 8 (*ABCG5/8*), and cholesterol 7 alpha hydroxylase (*CYP7A1*).

3. The study was limited by incomplete clinical and family history data, which may have influenced diagnostic accuracy. In addition, only 310 blood samples were subjected to NGS for the detection of variants in *LDLR*, *APOB*, and *PCSK9* genes.

1.7 Significance of The Study

This study provides an insight into the genetic spectrum of FH in Malaysian. The genetic spectrum refers to the range and types of gene variants identified within a population, including the distribution of mutations across genes such as *LDLR*, *APOB*, and *PCSK9*. In contrast, the frequency of pathogenic variants describes how often these clinically relevant mutations occur among individuals diagnosed with FH. Clarifying both aspects is important for understanding population-specific genetic characteristics and assessing the diagnostic yield of molecular testing. The result from this project may give further benefit to stakeholders to improve FH guidelines, particularly in Malaysia. It is essential to determine which targeted medicine may be administered to patients based on gene mutations discovered through genetic testing. Thus, the risk of mutations in any of the FH-causing genes leading to raised blood plasma cholesterol levels will be minimised by early measures to recognise FH diseases.

CHAPTER 2

LITERATURE REVIEW

2.1 Cardiovascular Disease

Cardiovascular disease (CVD) is a broad term that includes several conditions that affect the heart and blood vessels. Some examples are coronary artery disease (CAD), cerebrovascular disease (stroke), peripheral arterial disease, rheumatic heart disease, congenital heart disease, and venous thromboembolism. Globally, CVDs are the leading cause of death, responsible for 17.9 million deaths in 2021, representing 32% of all deaths in 2019. Heart attacks and strokes accounted for 85% of these fatalities, and more than three-quarters occurred in low- and middle-income countries. Early recognition of cardiovascular illnesses is essential to initiating treatment through counselling and medications. In 2019, CVDs accounted for 38% of the 17 million premature deaths (before age 70) caused by noncommunicable diseases (Roth et al., 2020).

Importantly, most cardiovascular diseases are preventable through the modification of behavioural risk factors such as smoking, unhealthy diets, physical inactivity, obesity, and excessive alcohol consumption. Despite this, the global burden of CVD continues to increase, with prevalence nearly doubling from 271 million in 1990 to 523 million in 2019. During the same period, deaths from CVD increased from 12.1 million to 18.6 million (Roth et al., 2020).

2.2 Coronary Artery Disease

CAD, the leading cause of death worldwide and a significant contributor to mortality and morbidity, is a concerning condition responsible for over 350,000 deaths annually (Khoja et al., 2021). Elevated serum low-density lipoprotein cholesterol (LDL-C) levels are known to contribute to coronary heart disease (CHD) (Packard et al., 2021). The effect of LDL-C on the artery wall, as well as the accumulation of lipids, calcification, and fibrous elements in endothelial cells, are crucial aspect including inactivity atherosclerosis formation (Jebari-Benslaiman et al., 2022). In addition to hypercholesterolaemia, several comorbidities significantly increase the risk of CAD,

inactivity and other hypertension, diabetes mellitus, obesity, metabolic syndrome, and chronic kidney disease, along with behavioural risk factors such as smoking and physical inactivity (Fuchs & Whelton, 2020). The risk of premature CAD through early diagnosis and treatment of hypercholesterolaemia is avoidable or reversible (Sawhney et al., 2019). Reducing risk factors for CAD is the main target for many health systems, including smoking cessation and drugs to manage diabetes, hypertension, and hyperlipidaemia (Brown et al., 2023).

2.2.1 Difference Between CVD and CAD

Cardiovascular disease (CVD) refers to all disorders of the heart and blood vessels, making it an umbrella term that includes a wide spectrum of diseases such as coronary artery disease (CAD), cerebrovascular disease (stroke), peripheral arterial disease, rheumatic heart disease, congenital heart disease, and venous thromboembolism (WHO, 2021; Benjamin et al., 2019). CAD, conversely, is a distinct form of CVD resulting from the constriction or obstruction of the coronary arteries that deliver blood to the cardiac muscle (Libby et al., 2019). Consequently, all cases of CAD are classified as CVD, whereas not all cases of CVD are classified as CAD.

In the context of Familial Hypercholesterolemia (FH), the stronger association is with CAD rather than with general CVD. FH is a genetic disorder characterised by lifelong elevated LDL-C levels due to mutations in genes such as LDLR, APOB, or PCSK9, which directly accelerate atherosclerosis in the coronary arteries (Nordestgaard et al., 2013; Cuchel et al., 2014). As a result, untreated FH patients are at a markedly higher risk of developing premature CAD, particularly myocardial infarction, often decades earlier than the general population (Mach et al., 2019). While FH can indirectly increase the risk of other CVD manifestations (e.g., stroke, peripheral artery disease), its primary and most severe clinical consequence is early-onset CAD (Nohara et al., 2021).

2.2.2 Prevalence of Coronary Artery Disease

The World Health Organisation (WHO) estimates that 17.9 million deaths worldwide in 2021 were due to cardiovascular disease (CVD), which accounted for 32% of all worldwide fatalities in 2019. Heart attacks and strokes were responsible for 85% of these fatalities. More than three-quarters of CVD fatalities occur in low- and middle-income nations. It is crucial to recognise cardiovascular illness as early as

possible in order to begin treatment with counselling and medications. In 2019, CVD was responsible for 38% of the 17 million premature deaths before the age of 70 that were caused by noncommunicable illnesses. Most cardiovascular illnesses may be avoided by addressing behavioural risk factors such as smoking, lack of nutrition and obesity, physical inactivity, and excessive alcohol use.

According to the Institute for Health Metrics and Evaluation, in 2019, the main cause of total and premature mortality in Iran, a country of approximately 85 million people in the Middle East, was ischaemic heart disease (Hosseini et al., 2021). The Ukraine and Russian Federation have the greatest mortality rates from coronary heart disease, with 718 and 654 fatalities per 100,000 population, respectively, while South Korea and Japan have the lowest, with 36.5 and 47.0 deaths per 100,000, respectively (Choon Seong & Kok Meng John, 2023). In collaboration with the National Institutes of Health (NIH) and other government institutions, the American Heart Association (AHA) publishes a Statistical Update providing the most recent information on cardiovascular disease and its contributing factors in the United States. The American Heart Association discusses how the coronavirus disease 2019 (COVID-19) impacted cardiovascular health, particularly among Black, Hispanic, and Asian Americans, many of whom were already at a higher risk of serious health consequences (Tsao et al., 2023).

Total cardiovascular disease prevalence nearly doubled from 271 million in 1990 to 523 million in 2019 (Figure 2.1), while cardiovascular disease fatalities continuously grew from 12.1 million in 1990 to 18.6 million in 2019. Cardiovascular disease was the primary cause of 9.6 million male fatalities and 8.9 million female deaths in 2019, accounting for around one-third of all deaths worldwide. Over 6 million of these fatalities occurred among adults aged 30 to 70 (Roth et al., 2020).

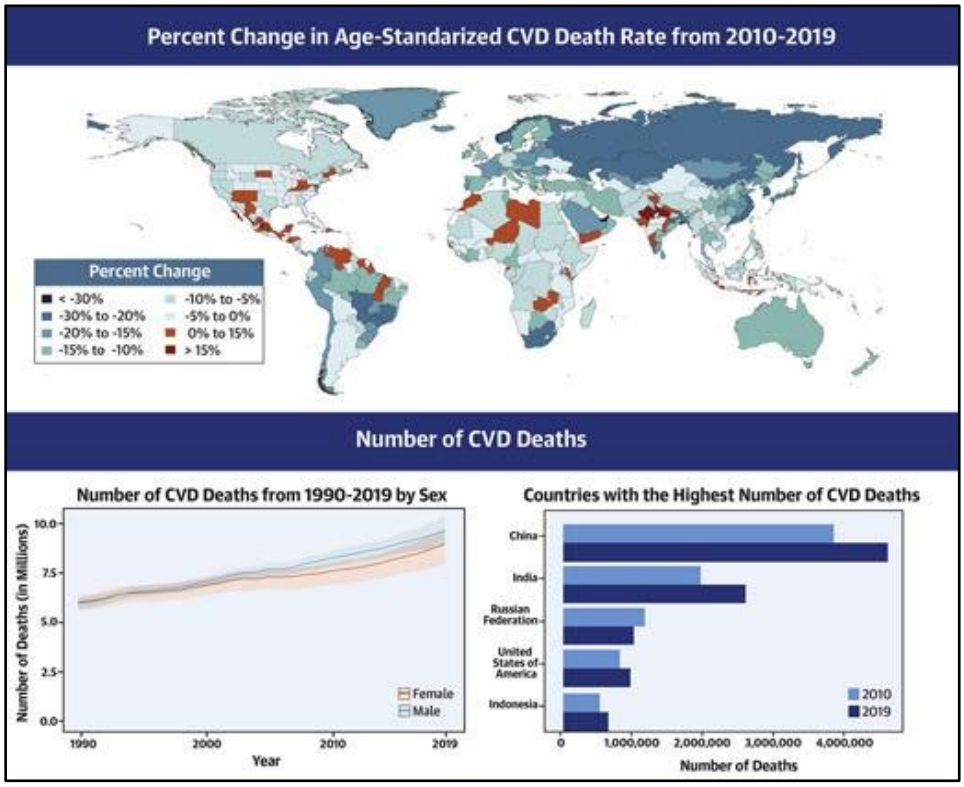

Figure 2.1 Map showing the percent change in age-standardized CVD death rate from 2010 to 2019 and number of CVD deaths. Adapted from Roth et al., 2020.

CHD remained the leading cause of death among all CVDs in Malaysia by 15.6% in 2019. According to the Malaysian Department of Statistics, ischemic heart disease remained the top cause of death in 2020, accounting for 17.0% of the 109,155 medically certified fatalities (Department of Statistics Malaysia, 2020). According to the Malaysian Ministry of Health (MOH), Disability-Adjusted Life Years (DALYs) lost from CVDs were RM59.85 billion in 2017, with CHD accounting for the biggest share of the illness at RM32.48 billion (Ministry of Health Malaysia, 2020). The top three and major causes of premature mortality of both men and women in Malaysia were ischaemic heart disease (29.5%) followed by cerebrovascular disease (stroke) (20.8%), lower respiratory infections (15.9%) (Chan et al., 2022).

2.3 Familial Hypercholesterolaemia

The gene coding for the low-density lipoprotein receptor (*LDLR*) protein is one of the most well-known for its relationship with CAD risk. Mutations in this gene induce familial hypercholesterolaemia (FH), an autosomal dominant condition characterised

by severe hypercholesterolemia and early CAD (Vrablik et al., 2020). FH is an inherited disease characterised by significantly elevated LDL-C and total cholesterol (TC) levels in the blood, resulting in atherosclerotic plaque deposition in the coronary arteries and proximal aorta at a young age and increasing the risk of premature cardiovascular events such as angina and myocardial infarction; stroke occurs less frequently (Vaezi & Amini 2023). The AHA reports that FH patients are exposed to high serum LDL-C levels from birth. However, LDL-C levels can vary significantly, depending on the type of variant but also on lifestyle or the presence of other types of disorder, making FH more difficult to detect, implying that the disease should always be investigated.

Over 1,700 various mutations in the *LDLR* gene have been described, each of which affects the structure and function of the *LDLR* in a different way; as a result, the level of LDL-C in individuals with FH caused by a variant in this gene can vary significantly (Al-Allaf et al., 2017). Meanwhile, just a few variants have been described in the apolipoprotein B (*APOB*) gene, with a large number of patients having only one type of mutation, which results in very uniform LDL-C levels (and on average lower than in the *LDLR* gene) (Vrablik et al., 2020). A pathogenic variation (PV) in the gene caused by gain-of-function mutations in the proprotein convertase subtilisin/kexin type 9 (*PCSK9*) gene may result in a clinical picture of FH, which has so far been detected in just one proband in the Czech Republic, making it an uncommon cause of FH (Vrablik et al., 2020; Xia et al., 2021a). Given that FH is an autosomal dominant disorder, there is a fifty percent likelihood that a heterozygous person will pass on the gene to any of their children. Compound heterozygotes with distinct PV in both alleles of the same gene, double heterozygotes with PV in two separate genes, and homozygous persons with identical mutations in both alleles would all inevitably have heterozygous offspring, provided their partner does not have FH (McGowan et al., 2019). Thus, cascade screening in families, which involves doing genetic testing on family members of a patient who has previously received a diagnosis, seems to be an effective strategy for finding FH patients (Vrablik et al., 2020).

2.3.1 Historical background

FH is an intriguing illness that was initially brought to the attention of a pathologist, Harbitz, by the occurrence of lipid deposits (xanthomata) in tendons and sudden mortality, before being defined as a medical condition by Muller in the first part of the previous century. Microscopically, he revealed how the so-called foam cells were more apparent and distinctive in those individuals than in those with senile arteriosclerosis (Marais, 2004). Carl Möller, a Norwegian health care provider, originally reported it in the late 1930s in an essential article by proposing that hypercholesterolaemia and tendinous xanthomas were correlated to cardiovascular (CV) disease by a single gene inheritance (Muller, 1939). He submitted his final report in 1939, based on his personal research of 17 households in Oslo over a brief period beginning in 1936. He stated that inherited heart disease caused by xanthomatosis and hypercholesterolaemia were rather frequent. It was shown to be a dominating trait in the families (Ose L., 2002). Less than fifty years later, research in patients and cultured cells revealed a defect in the LDL receptor, leading to Brown and Goldstein's Nobel prize-winning work on the regulation of the cholesterol synthesis pathway and a defect in its internalisation as the cause of familial hypercholesterolaemia (Marais, 2004).

2.3.2 Prevalence of Familial Hypercholesterolaemia

Numerous studies have been conducted to determine the prevalence of FH, and several studies have reported the ratio of 1:200 to 1:250 in the general population. Heterozygous FH (HeFH) prevalence was recently found to be 1 in 313 in the general population by a meta-analysis that included 11 million participants from 104 studies ((Beheshti et al., 2020). The European Atherosclerosis Society (EAS) Consensus Panel reported that the prevalence of HeFH is estimated to be 1 in 200 to 1 in 500 people depending on the community, although it might be as high as 1 in 31 in patients with atherosclerotic cardiovascular disease (ASCVD), and about 1 in 160 000 to 300 000 persons have homozygous FH (HoFH) (Sun et al., 2023).

However, FH is still underdiagnosed and undertreated, affecting up to 80% of individuals and leading to significant failures to avoid premature coronary artery disease (PCAD) (Qureshi et al., 2021). Fewer than 1% of FH patients are diagnosed in most countries (Truong et al., 2020). The CASCADE (Cascade Screening for Awareness and Detection) FH Registry stated that only 48% of FH patients attained LDL-C < 100 mg/dl and 22% obtained LDL-C < 70 mg/dl, indicating the limited effectiveness of therapy for FH (Duell et al., 2019). Due to founder effects, the adoption of various diagnostic criteria, and screening methods, the prevalence of FH differs between different nations (Toft-Nielsen et al., 2022). It is known that the founder effect contributes to the high prevalence of FH in some countries with a high percentage of non-native ethnic groups. These ethnic groups may have brought the PV of FH from their home countries and may have caused the founder effect for FH after thriving for several generations in the new countries (Chua et al., 2021).

Seventeen of the 195 nations on the globe (9%) have documented FH prevalence for the general population, while 4 more have reported FH prevalence specifically among founder populations (Figure 2.2). As a result, the prevalence of FH remains unknown in 178 of the world's nations (91%). Most of the studies that reported on the prevalence of FH were from Europe, North America, East Asia, and Australia, leaving out a substantial portion of Africa, South America, and Asia. The frequency of FH in the general population is lower in Asia (0.19%) than Europe and North America (0.32%). In 90% of the world's countries, the prevalence of FH in the general population remains unclear (Beheshti et al., 2020).

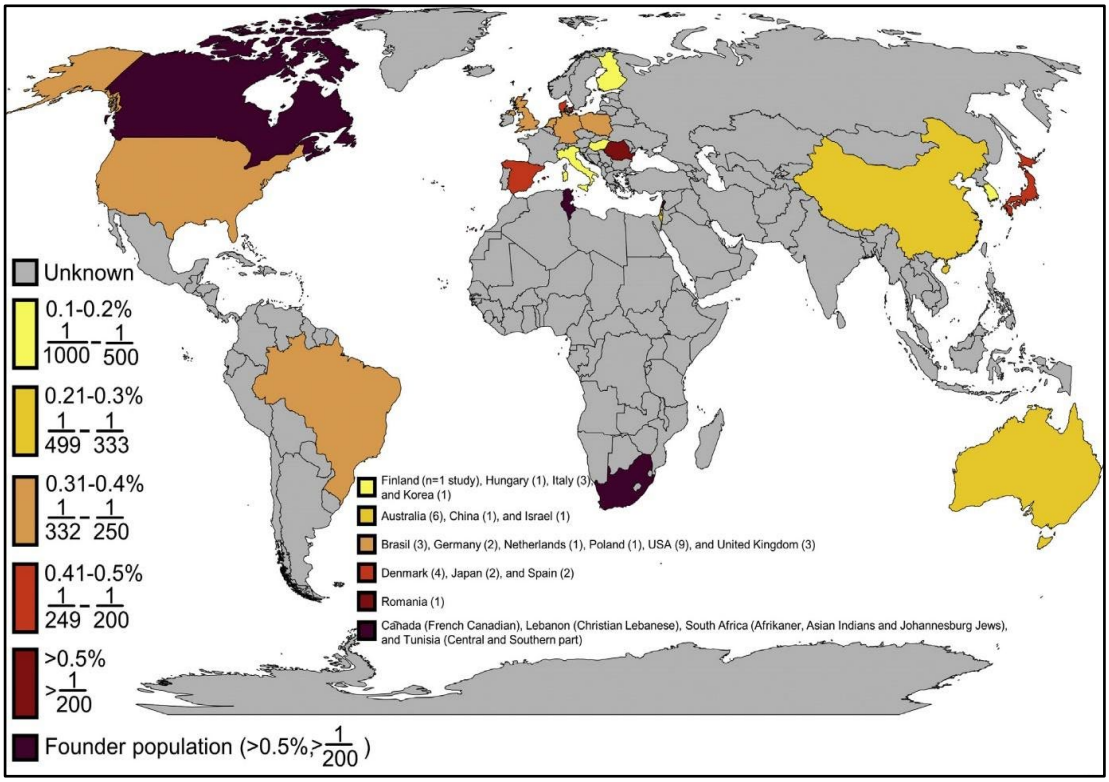

Figure 2.2 Worldwide FH Prevalence in the General Population (Beheshti et al., 2020).

Recent research in Malaysia estimated the frequency of clinically confirmed FH to be one in 100. Based on the current overall population of 32 million, an estimated 320,000 people have FH. Many of the cases had previously gone untreated, and while lipid-lowering drugs were administered to 30.5%-54.5% of these people, none of them met the therapeutic LDL-C threshold. The Malaysian population's failure to recognise and treat FH resulted in missed opportunities to avoid PCAD. Hence, improving FH detection in primary care has been identified as a low-cost option for reducing PCAD (Ramli et al., 2023).

2.4 Associated Factors of Familial Hypercholesterolaemia

2.4.1 Lipid Profile in Familial Hypercholesterolaemia

Cholesterol is a hydrophobic substance that circulates in the circulation of proteins known as "lipoproteins". John Gofman used an ultracentrifuge to extract lipoproteins from plasma. He also established that heart attacks were linked to elevated

blood cholesterol levels, particularly LDL-C. In contrast, increasing blood high-density lipoprotein (HDL) levels lowered the probability of heart attacks. Furthermore, the Framingham Heart Study, one of the most significant epidemiological studies in the cardiovascular arena, validated the positive benefits of HDL cholesterol (HDL-C) and the harmful effects of LDL-C on heart disorders (Duan et al., 2022).

2.4.2 Clinical Characteristics of Familial Hypercholesterolaemia

More studies are needed to better understand FH genetic testing implementation in various clinical settings and to develop solutions to minimise the amount of obstacles and utilise present facilitators for genetic testing (Alonso et al., 2020). A clinical diagnosis of FH is made based on a weighted combination of physical abnormalities, a personal or family history of hypercholesterolemia, early-onset PCAD, the level of circulating LDL-C, and the presence of tendon xanthomas and/or arcus cornealis before age 45 (Figure 2.3). Extensor tendon xanthomas, which generally occur in the Achilles, subpatellar, and hand tendons, are thought to be specific for FH. Atherosclerotic plaques' foam cell development and lipid buildup are histologically similar to severe extensor tendon xanthomas (Alonso et al., 2020; McGowan et al., 2019).

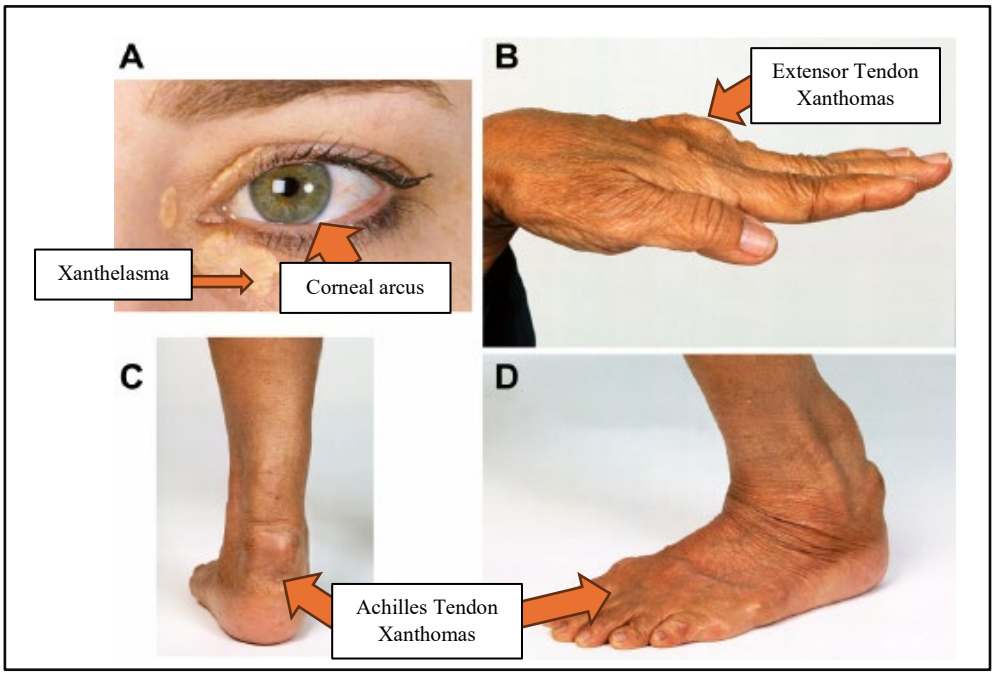

Figure 2.3 Clinical characteristics of familial hypercholesterolaemia. (A) Corneal arcus and xanthelasma; (B) extensor tendon xanthomas; (C and D) Achilles tendon xanthomas. Adapted from Bell et al., (2012)

2.4.2.1 Xanthomas

Xanthomas are lipid deposits that form within an organ system. Despite the fact that they are fundamentally harmless, they are frequently an important visual sign of systemic disorders. Xanthomas do not occur in individuals with hyperlipidaemia or hypercholesterolemia. Circulating lipoproteins in hyperlipidemic individuals infiltrate between vascular endothelial cells and deposit in the dermis, subcutaneous tissues, and tendons. Phagocytosis by tissue macrophages aids in the removal of the lipid components of these deposits from those locations. This process is thought to be the source of the distinctive "foam cells" present in xanthomas (Bell & Shreenath, 2023). On clinical examination, xanthomas are classified as eruptive, tuberoeruptive, tuberous, tendinous, or planar lesions. These lesions proclivity for specific anatomic locations, such as the elbows or palms, can be pathognomonic for specific kinds of hyperlipoproteinemia (Cruz et al., 1988). Tendinous xanthomas (TX) most typically affect the Achilles tendons, elbows, and hand extensor tendons (Carranza et al., 1999). These are slowly progressing lesions with a strong link to familial hypercholesterolemia. The heterozygous variant of this disease has more favourable outcomes than the homozygous type, in which people die at a young age (Wong et al., 2018). Early detection of FH may hinder delays in the management of patients with FH.

2.4.2.2 Corneal Arcus

Corneal arcus, also known as "arcus lipoides" or "arcus corneae," is a white discolouration of the cornea at the corneoscleral limbus. The lucid interval of Vogt is a narrow, transparent region that separates the arcus from the limbus. Arcus deposits often begin between 6 and 12 o'clock and fill in until they are totally circumferential. It arises as a result of lipid accumulation in the deep corneal stroma and the limbal sclera in combination with hyperlipidemia. Its occurrence rises with age. Corneal arcus should be distinguished from other lipid metabolic disorders that impact the cornea, such as lecithin-cholesterol acyltransferase deficiency, "fish eye" illness, and Tangier disease (Macchiaiolo et al., 2014).

2.3.2.3 Xanthelasma

Lesions of xanthelasma are yellowish and subcutaneous, appearing on the inner aspect of the upper and lower eyelids. These lesions often create an arc in both the upper and lower eyelids and are frequently related to elevated blood cholesterol levels. It has not been linked to ageing or hormonal changes. The onset age ranges from 15 to 73 years, with the peak occurring in the fourth and fifth decades. This disorder runs in one-third of sufferers' families. Xanthelasma has been linked to hyperlipidaemia, hypercholesterolemia, obesity, and cardiovascular abnormalities. Elevated cholesterol levels are found in around 33% of men and 40% of women with xanthelasma. Since xanthelasma lesions are non-invasive, they are only removed for cosmetic reasons. Despite removal, they tend to reappear. Smaller lesions can be eliminated using a chemical like trichloroacetic acid. Larger xanthelasma deposits necessitate surgical removal (Harold et al., 2018). The presence of TXs is an essential element for the diagnosis of HeFH, according to the Dutch Lipid Clinic Network (DLCN) criteria, since it is allocated 6 points (a score of 6 or 7 results in the diagnosis of HeFH probable). In accordance with the DLCN criteria, the presence of TXs is significant for HeFH diagnosis in various other clinical diagnostic methods for FH, such as the Simon Broome criteria and the Japan Atherosclerosis Society criteria, whereas corneal arcus is important in the DLCN. However, xanthelasma has been reported to be unrelated to FH (Rallidis et al., 2020).

2.5 Familial Hypercholesterolaemia Diagnostic Criteria

The Malaysian population's low therapeutic LDL-C goal accomplishment among people with FH might be attributed to a lack of understanding about FH among primary care physicians and patients. Less than half of the healthcare professionals involved in the management of acute coronary syndrome were found to have significant knowledge and awareness gaps about FH (Azraii et al., 2018; Chua et al., 2021; Mirzaee et al., 2019). Although primary care clinicians are well-positioned to detect FH patients, universal FH screening is not commonly adopted in practice (Zimmerman et al., 2019).

Multiple techniques are necessary for screening, diagnosing, and treating FH (Pang et al., 2020). Recent FH publications provide new data for developing clinical

models of care to improve the detection and identification of FH cases, such as the FH-case finding tool (FAMCAT), which was developed as a multivariate logistic regression model stratified by sex to calculate an individual's probability of having FH (Akyea et al., 2020; Pang et al., 2020). FH CatScreen© is a computer-based FH screening application that integrates four FH diagnostic criteria, such as Dutch Lipid Clinic Network (DLCN) criteria, Simon Broome's (SB) criteria, Japanese FH Management Criteria (JFHMC), and US Make Early Diagnosis to Prevent Early Deaths (MEDPED), into a one-stop web-based application (Rosli et al., 2020). There are three already well-known and widely used clinical diagnostic criteria for FH, which are the SB, which is widely used in the United Kingdom (Humphries et al., 2018), the DLCN, which is widely used by the European countries (Gidding et al., 2022), and the US MEDPED, which is widely used clinical diagnostic criteria in the United States (Maștaleru et al., 2022). Each of these criteria has its own unique characteristics and guidelines for diagnosing FH. The SB and DLCN criteria are the most widely used as a diagnostic tool for FH (Ruel et al., 2018).

The National Institute of Health and Care Excellence (NICE) FH guideline from 2008 suggests cascade screening for all individuals with a clinical diagnosis of FH. This would lead to the identification of many people with FH who are clinically asymptomatic. Subclinical coronary atherosclerosis risk assessment in asymptomatic individuals with FH who are at higher risk of advanced CAD is essential because these people may be eligible for intensive therapy at a younger age (Sharifi et al., 2016).

2.5.1 Dutch Lipid Clinic Network (DLCN) criteria

The DLCN for FH diagnosis is a modified version of the SB criteria. The DLCN included family and personal clinical history, clinical features, cholesterol levels, and DNA mutation analysis, but each criterion is given a score (Table 2.1). The DLCN defines FH patients into three groups: Definite FH, Probable FH, and Possible FH. The total points accumulated determine whether the individuals will be grouped under either Definite, Probable or Possible FH. But unlike Simon Broome criteria, the DLCN does not include total cholesterol as one of the criteria to be given point, instead DLCN rely only on LDL-C level of blood ≥4.0 mmol/L (Al-Rasadi et al., 2014).

The DLCN criteria use a scoring system that takes into account family history, clinical history, physical exam, and LDL cholesterol values. The scores gained in each category are then added. If a patient scores more than eight points, the individual is labelled as a 'definite' case of FH, while a 'likely' case of FH is identified if the patient scores between six and eight points, and any score between three and five points is called 'possible' FH, and a score less than three is considered to be unlikely FH (Mülverstedt et al., 2021). The preference of FH patients who are identified to be candidates for molecular testing is mostly determined by clinical FH criteria such as the DLCN criteria (Medeiros & Bourbon, 2023).

One of the main advantages of the Dutch criteria is that it addresses the molecular abnormality that causes FH, which will assist in giving a better therapeutic strategy or treatment to the identified FH patient (Al-Rasadi et al., 2014).

Table 2.1
Dutch Lipid Clinic Network criteria for FH

Dutch Lipid Clinic Network criteria for FH

Criteria	Points
Family history	
1) First degree relative with known premature (<55 years men; <60 years women) coronary disease and vascular disease OR LDL-C >95th percentile	1
2) First degree relative with tendon xanthomata and/or corneal arcus OR childhood (<18 years) with LDL-C >95th percentile	2
Clinical history	
1) Patient with premature CAD (men <55, women <60 years)	2
2) Patient with premature cerebral or PVD (men <55, women <60 years)	1
Physical examination	
1) Tendon xanthomas	6
2) Premature arcus	4
Lab analysis	
1) LDL-C > 8.5 mmol/L	8
2) LDL-C – 8.4 mmol/L	5
3) LDL-C – 6.4 mmol/L	3
4) LDL-C – 4.9 mmol/L	1
Functional DNA mutations	8
Definite FH: more than 8 points Probable FH: 6-8 points Possible FH: 3-5 points	

2.5.2 The Simon Broome's (SB) criteria

SB included cholesterol levels, clinical features, family history, and a molecular diagnosis via genetic testing (Table 2.2). The SB criterion classifies FH into two categories: Definite FH and Possible FH. The Definite FH by SB criteria is defined as a patient with a TC level greater than 7.5 mmol/L in adults or greater than 6.7 mmol/L in children under the age of 16, or an LDL-C level greater than 4.9 mmol/L in adults and/or greater than 4.0 mmol/L in children under the age of 16. The individual also must present with tendon xanthomata or gene analysis to confirm the presence of mutation on the related gene, such as *LDLR, APOB100*, or *PCSK9* genes. Possible FH by SB criteria is defined as having more than 7.5 mmol/L total cholesterol in adults and/or 6.7 mmol/L for those under the age of 16 or having an LDL level over 4.9 mmol/L and 4.0 mmol/L for adults and those under the age of 16. Furthermore, the patient must have a family history of early myocardial infarction before the age of 50 in second-degree relatives or before the age of 60 in first-degree relatives, or they must have a family history of elevated cholesterol levels greater than 7.5 mmol/L total cholesterol (Al-Rasadi et al., 2014).

According to the SB, a person is classified as having "definite FH" (DFH) if they have increased LDL-C, an early family history of heart disease, and tendon xanthomas; a person without these criteria is classed as having "possible FH" (PFH). CHD mortality is higher in DFH patients than in PFH patients, and we compare CHD death rates in people with "severe" FH (SFH) and DFH vs. PFH (Humphries et al., 2018).

Table 2.2
Simon Broome's criteria for FH

Simon Broome criteria for FH

Description	Yes	No
1) TC > 7.5 mmol/L in adults OR LDL levels > 4.9 mmol/L OR TC > 6.7 mmol/L OR LDL-C > 4.0 mmol/L (aged < 16 y/o)		
2) Tendon xanthomas in patient OR in 1st or 2nd degree relative		
3) DNA mutation in *LDLR*, *APOB* or *PCSK9*		
4) Family history of PCAD in: a) 1st degree relatives (age < 60 y/o) OR b) 2nd degree relatives (age < 50 y/o)		
5) Family history of hypercholesterolaemia in: a) Adult 1st or 2nd degree relatives (TC > 7.5 mmol/L) OR b) Children or siblings aged < 16 y/o: (TC > 6.7 mmol/L)		
Definite FH: YES in 1 & 2 or 1 & 3, Possible FH: YES in 1 & 4 or 1 & 5, Unlikely FH: None of the above		

2.5.3 Familial Hypercholesterolemia Case Ascertainment Tool (FAMCAT)

A team of UK researchers developed the Familial Hypercholesterolemia Case Ascertainment Tool (FAMCAT) (Ramli et al., 2023). The FAMCAT's dependability in the UK has been largely acknowledged (Qureshi et al., 2009, 2016; S. F. Weng et al., 2015a). The Clinical Practice Research Datalink (CPRD) of 681 primary care practices in the UK provided data on about 3 million primary care patients, including over 5000 instances of familial hypercholesterolemia, which were used to develop and verify FAMCAT (S. F. Weng et al., 2015a). Initially introduced in 2015 and later refined as FAMCAT2 in 2019, this tool has been widely employed in the UK to characterise and stratify individuals at risk of familial hypercholesterolaemia through electronic health record screening (S. F. Weng et al., 2019). However, its applicability and diagnostic performance within the Malaysian primary care population remain to be fully established.

A multivariable logistic regression model stratified by sex called FAMCAT was created to determine a person's likelihood of having familial hypercholesterolemia. FAMCAT was established as a multivariable logistic regression model stratified by gender to determine a person's likelihood of having familial hypercholesterolaemia using routinely collected data from electronic health records (EHRs). It incorporates ten predictors; including age, cholesterol concentrations, and triglycerides to generate a probability score for FH (S. Weng et al., 2019). The most current UK National Institute for Health and Care Excellence (NICE) lipid modification recommendations were used to classify statin potency. Diabetes and chronic renal disease were included as predictor factors for a decreased likelihood of familial hypercholesterolaemia, as they are secondary causes of elevated cholesterol (S. F. Weng et al., 2015b).

This web-based application includes three index tests: the FAMCAT, SB, and DLCN (S. F. Weng et al., 2015b). FAMCAT offers the advantage of automated case detection within primary care databases, enabling large-scale and early identification of potential FH cases without specialist input. In the present study, FAMCAT was evaluated alongside DLCN and SB to determine its predictive accuracy and applicability in the Malaysian healthcare setting. The characteristics needed to identify individuals with suspected FH using FAMCAT are listed in Table 2.3.

Table 2.3
The Clinical diagnostic variables for the FAMCAT (S. Weng et al., 2019)

Index tests	FAMCAT
Lipid levels	- The highest LDL-c ever measured - The highest total cholesterol ever measured - Triglycerides during LDL-c measurement - Age during LDL-c measurement - Lipid-lowering treatment during LDL-c measurement
Physical examination	- Nil
Personal history	- Personal history of premature myocardial infarction (<55 years in men, <60 years in women) - Diagnosis of diabetes - Diagnosis of chronic kidney disease
Family history	- Family history of premature myocardial infarction (<55 years in men, <60 years in women) - Family history of familial hypercholesterolemia

2.6 Pathophysiology and Pathology of Familial Hypercholesterolaemia

Lipoprotein uptake involves endocytosis pathways. First, ribosomes synthesise receptors, which are then folded in the endoplasmic reticulum (ER). The receptors are then glycosylated in the Golgi before being transferred to the cell surface. Lipoprotein ligands are bound by receptors in the plasma membrane. The receptors recycle back to the cell surface when the bound lipoproteins are released. Internalisation takes place through clathrin-coated pits, which eventually transfer the LDL particle-*LDLR* complex, which binds to *LDLR* clustered in clathrin-coated pits to become a coated vesicle. The LDL particle and its receptors are internalised via receptor-mediated endocytosis and destroyed in the lysozyme after endocytosis. This causes LDL to hydrolyse, releasing the unesterified cholesterol into the cytoplasm. As a result, LDL will be eliminated from the system; however, mutations in the cytoplasmic tail of the *LDLR* cause failure to cluster in clathrin-coated pits, resulting in high cholesterol levels and FH (Figure 2.4) (Redpath et al., 2022).

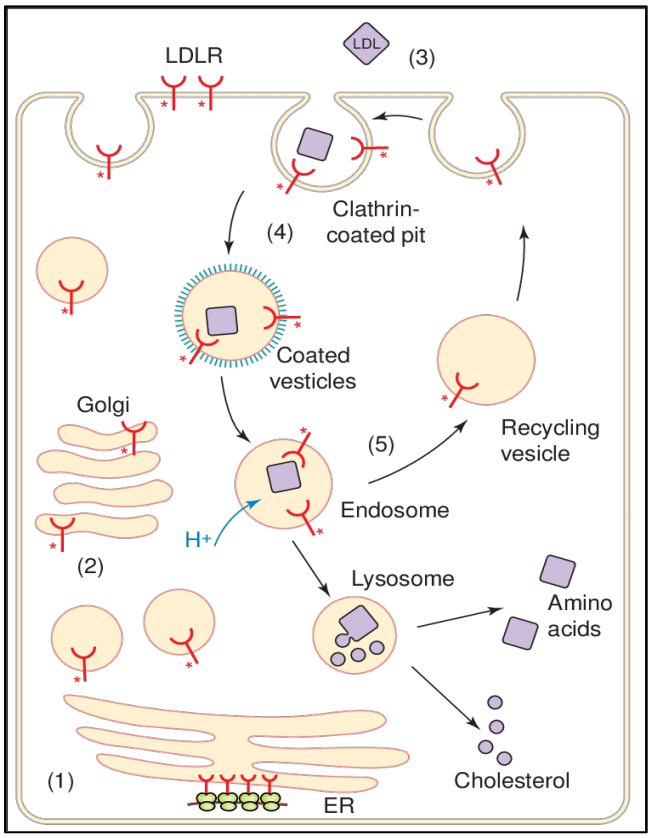

Figure 2.4 Cellular itinerary of the *LDLR* (Beglova & Blacklow, 2005)

FH is primarily autosomal dominant, with an occasional autosomal recessive component concentrated in areas with high levels of consanguinity (Figure 2.5). FH is classified as monogenic, oligogenic, or polygenic based on the frequency of alleles (specific DNA sequences) involved and the effect of the DNA variants on the protein's biological activity. Autosomal dominant hypercholesterolemia (ADH) is primarily caused by PV in the *LDLR*, *APOB*, or *PCSK9* genes. Clinical misunderstanding between the FH and ADH categories is explained by the PV in the *LDLR* gene that is present in the majority of patients (Abifadel & Boileau, 2023).

Clinical misunderstanding between FH and ADH arises because FH is generally considered a clinical diagnosis, whereas ADH is a genetic classification of monogenic hypercholesterolemia. FH is typically diagnosed based on lipid levels, family history, and clinical signs such as tendon xanthomas, while ADH is caused by pathogenic variants (PV) in major genes such as *LDLR*, *APOB*, or *PCSK9* (Santos et al., 2016; Defesche et al., 2017). Since the majority of patients with FH harbour PV in the *LDLR* gene, there is substantial overlap between FH and ADH, which can create diagnostic confusion (Nordestgaard et al., 2013). This overlap has important consequences for patient care. First, in regions where genetic testing is limited, individuals may be clinically diagnosed with FH without knowing whether their condition is due to monogenic ADH or polygenic hypercholesterolemia. Second, patients carrying *LDLR* variants tend to present with markedly elevated LDL-C levels and therefore require more aggressive therapy, including high-intensity statins, ezetimibe, and potentially PCSK9 inhibitors, to achieve target LDL-C reductions (Cuchel et al., 2014; Mach et al., 2019). Misclassification of such patients as having polygenic hypercholesterolemia rather than ADH may delay initiation of intensive therapy. Furthermore, confirmation of *LDLR*-related ADH through genetic testing facilitates cascade screening, enabling early identification and treatment of at-risk relatives, which is less reliable when only a clinical FH diagnosis is used (Sjouke et al., 2015).

Importantly, patients with *LDLR* mutations generally carry a higher lifetime risk of premature coronary artery disease (CAD) compared with patients whose hypercholesterolemia is polygenic in origin (Nohara et al., 2021). Thus, the clinical misunderstanding between FH and ADH categories, largely explained by the predominance of *LDLR* variants has significant implications for diagnosis, prognosis, treatment strategies, and family screening.

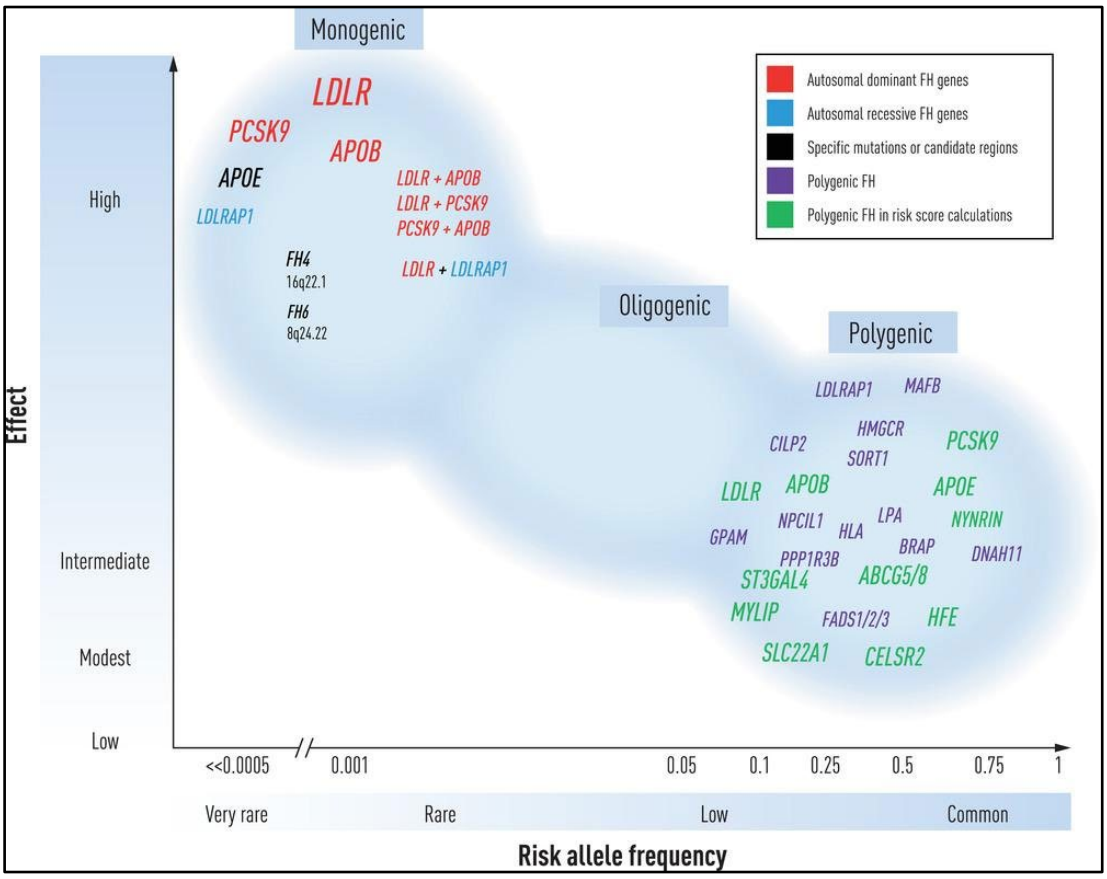

Figure 2.5 Genetic architecture and genes associated with FH (Abifadel & Boileau, 2023).

Autosomal recessive FH PV-containing genes are shown in blue, while autosomal dominant FH PV-causing genes are emphasised in red. All of the genes containing polymorphisms linked to polygenic FH are shown in purple or green for those taken into account when determining the polygenic risk score.

2.7 Familial Hypercholesterolaemia Genes

FH is often an autosomal dominant condition caused by mutations in one or more of three major genes: the *LDLR*, *APOB*, or *PCSK9* gene (Vrablik et al., 2020). Mutations in the *LDLR* are usually loss-of-function mutations, which induce high LDL-C levels and PCAD. Meanwhile, *LDLR* gain-of-function mutation with a major effect on LDL-C levels has yet to be documented (Bjornsson et al., 2021). The genetic variants in the *APOB* gene and the *PCSK9* gene give rise to the same functional deficiencies in

lipid homeostasis (Lye et al., 2013). *LDLR* mutations reduce the efficacy of the *LDLR* in clearing LDL particles, *APOB* mutations impair LDL particle binding to the *LDLR*, and *PCSK9* mutations reduce *LDLR* expression (fewer *LDLR*). Other factors (unrelated hereditary disorders, diet, and so forth) may also have an impact on LDL levels (Luo et al., 2022). The most frequent mutations are pathogenic variants of the *LDLR,* which are detectable in 90%–95% of FH cases. *APOB* mutations are the second most common genetics found in 5%–10% of patients where a molecular genetic basis of FH is identified. Mutations in the *PCSK9* gene are detectable in less than 3% of cases, which are less frequently involved in the FH phenotype. The Human Gene Mutation Database now has over 2000 *LDLR* variations, suggesting that PV in *LDLR* are the most common mutations that induce FH (Razman et al., 2022).

2.7.1 Familial Hypercholesterolemia Reflects Dysfunction of The *LDLR* Pathway

In genetically confirmed cases of FH, the mutations often impact the *LDLR gene.* Rarely, mutations are found in the *APOB* or *PCSK9* genes. *LDLR* function is essential for the absorption of LDL from the blood into hepatocytes. Defects in *LDLR* function can result in FH, known as an autosomal dominant condition. Because *LDLR* on the surface of hepatocytes is required for the binding and subsequent absorption of LDL molecules in the blood, a reduction in *LDLR* would result in a lower capacity of hepatocytes to absorb LDL and an increase in LDL in the blood (Pejic, 2014). *APOB* is responsible for LDL uptake in the liver. The single protein component of LDL, *APOB*, has been shown to attach to cell surface *LDLR* and to be internalised inside cells (Morita, 2016; Sakamoto & Rosenberg, 2011). Table 2.4 showed *LDLR* mutations are categorised into five classes based on their phenotypic behaviour. Small, dense LDL has a longer circulation time and a greater propensity to permeate the vessel wall and has been found to be more atherogenic than the bigger subfractions (Soppert et al., 2020).

Table 2.4:
Categorisation of *LDLR* mutations (Galicia-Garcia et al., 2020)

Class	Description
I	no protein synthesis
II	partial or complete retention of *LDLR* in the ER
III	defective binding to *APOB*
IV	defective endocytosis
V	diminished *LDLR* recycling capacity

The main protein associated with the circulating atherogenic LDL particle is *APOB*. When LDL attaches to the *LDLR*, it is normally removed; however, mutations in either the ligand *APOB* gene or the *LDLR* gene can disrupt binding. The *APOB* arginine-to-glutamine mutation at codon 3500 has been identified as a cause of *LDLR* failure and resultant hypercholesterolemia (Dairena Gaffney et al., 1995). Patients with *APOB* mutations often have a less severe phenotype than FH patients with *LDLR* mutations (Alves et al., 2014). *PCSK9* leads to *LDLR* degradation and is crucial in regulating plasma LDL-C levels (Gu et al., 2013). *PCSK9* lowers LDL absorption by binding to hepatic *LDLR* and stimulating their lysosomal breakdown, resulting in a rise in LDL-C concentrations. *PCSK9* gain-of-function mutations linked to elevated LDL-C and PCAD have been linked in the pathophysiology of autosomal dominant FH (Bergeron et al., 2015).

2.7.2 Genetic Mutations

FH is caused by inherited mutations in the *LDLR*, *APOB*, and *PCSK9* genes, which impact homeostasis of cholesterol metabolism in the human system. A mutation in one of these three genes is detected in 60–80% of persons with FH. There is genetic testing available to look for mutations in these genes. However, there are certainly additional FH-related undiscovered genes. Most FH patients have only one FH-causing mutation. A person can have two FH-causing mutations in both copies of the same gene in extremely rare circumstances, resulting in a far more serious and uncommon type of FH (Rutkowska et al., 2022).

2.7.1.1 Low Density Lipoprotein-Receptor

The *LDLR* gene, located at 19p13.2, is made up of 18 exons and 17 introns that span 45 kilobases. This region is translated into 5.3 kb mRNA, which encodes the 860 amino acid *LDLR* proprotein and contains a 21 amino acid signal peptide (Figure 2.6). The mature 160 kDa (839 amino acid) glycoprotein is produced by cleaving the 21 amino acid signal peptide of the *LDLR* proprotein, which is synthesised in the ER, and then glycosylating the protein. Nearly all cell types have this transmembrane receptor on their surface, which is in charge of internalising LDL into cells through a process known as receptor-mediated endocytosis (Abifadel & Boileau, 2023). The ligand binding domain of the *LDLR* gene, which is encoded by exons 2 to 6, mediates the interaction of the receptor with lipoproteins containing *APOB* and *APOE*, as well as the epidermal growth factor (EGF) precursor homology domain, which is encoded by exons 7 to 14, followed by the O-linked sugar domain (encoded by exon 15), transmembrane domain, and finally a short 55 amino acid part (cytoplasmic tail of *LDLR*) is encoded by the remaining exons 17 and 18, which is internalised into clathrin-coated pits (Abifadel & Boileau, 2023; Gabcova-Balaziova et al., 2015; Henderson et al., 2016).

Common PV are found in some ethnic/geographic groups due to founder effects. For example, 81.5% of Lebanese FH patients had the p. (Cys681*) variant; 76% of French-Canadian FH patients have five unique variants; Ashkenazi Jews have G197del (FH Lithuania, now p. [Gly219del]); Finns, Afrikaners, and Druze have specific variants (Abifadel & Boileau, 2023). By utilising the Leiden Open Variation Database (LOVD), 1741 mutations were discovered. There are 1295 different variants among them, 1064 of which are anticipated to be PV, 143 to be non-PV, and 88 to be of unknown significance. A recent study has discovered a greater number of PV in the *LDLR* gene related to FH. The *LDLR* gene has undergone extensive research, with the finding of over 2900 variations in the LOVD. *LDLR* mutations have been divided into "null" and "defective" mutations based on functional alterations (D. Sun et al., 2018). ClinVar has discovered about 1307 pathogenic and 831 potential pathogenic variants. Exon 4 variants are typically linked to a more severe phenotype, despite the fact that the *LDLR* gene lacks a specific mutation hotspot. This is most likely because exon 4 encodes three of the seven repeats of the "ligand binding domain," a region that is necessary for LDL binding via its *APOB* (Figure 2.7) (Abifadel & Boileau, 2023). Exon 4 mutations have been linked to a more severe FH phenotype and an increased risk of

congenital heart disease; PV in this area is thus primarily damaging to receptor function (Moradi et al., 2021).

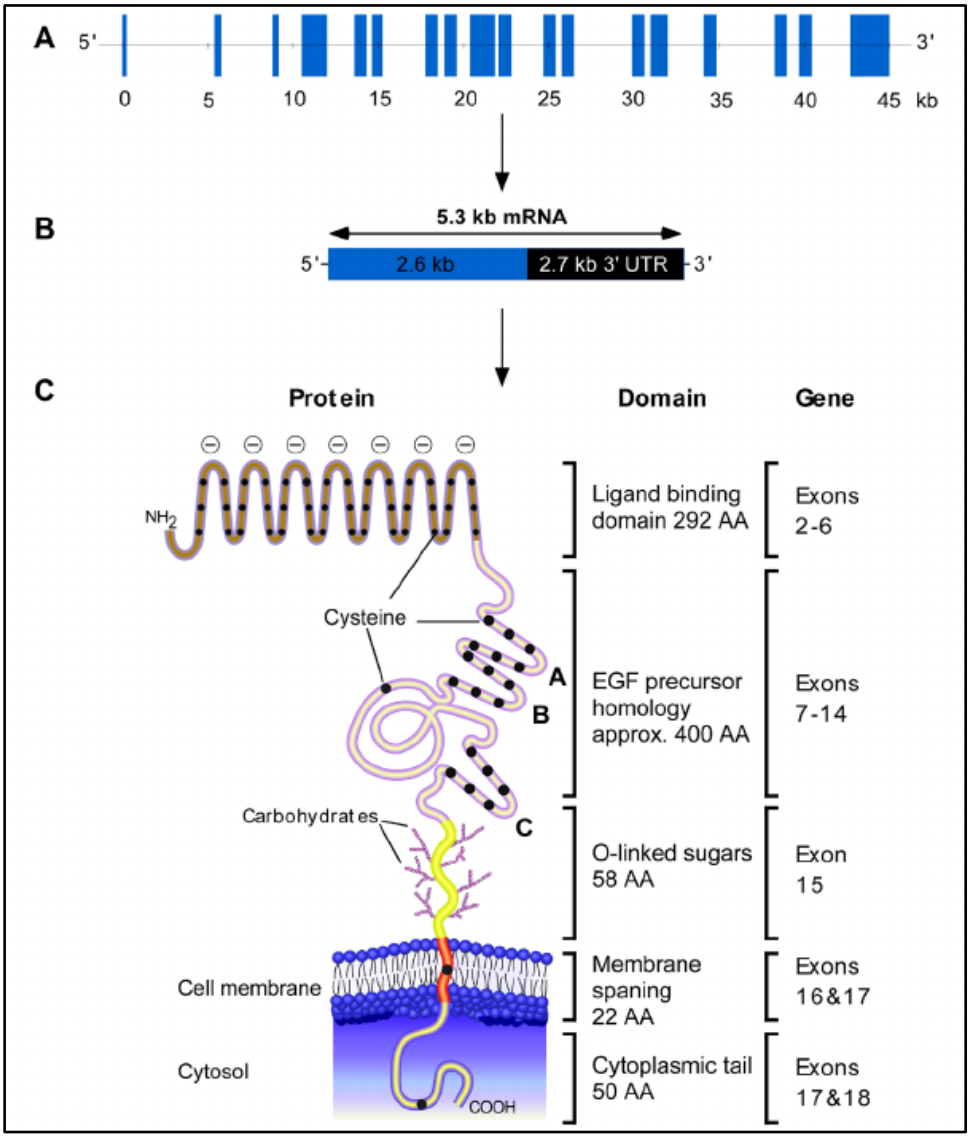

Figure 2.6 The LDL receptor structure. A) A schematic illustration of the human *LDLR* gene, with exons highlighted in blue and the gene's size in kb given below the picture; B) mRNA 3'UTR; C) LDL receptor domains. Adapted from Gabcova-Balaziova et al. (2015)

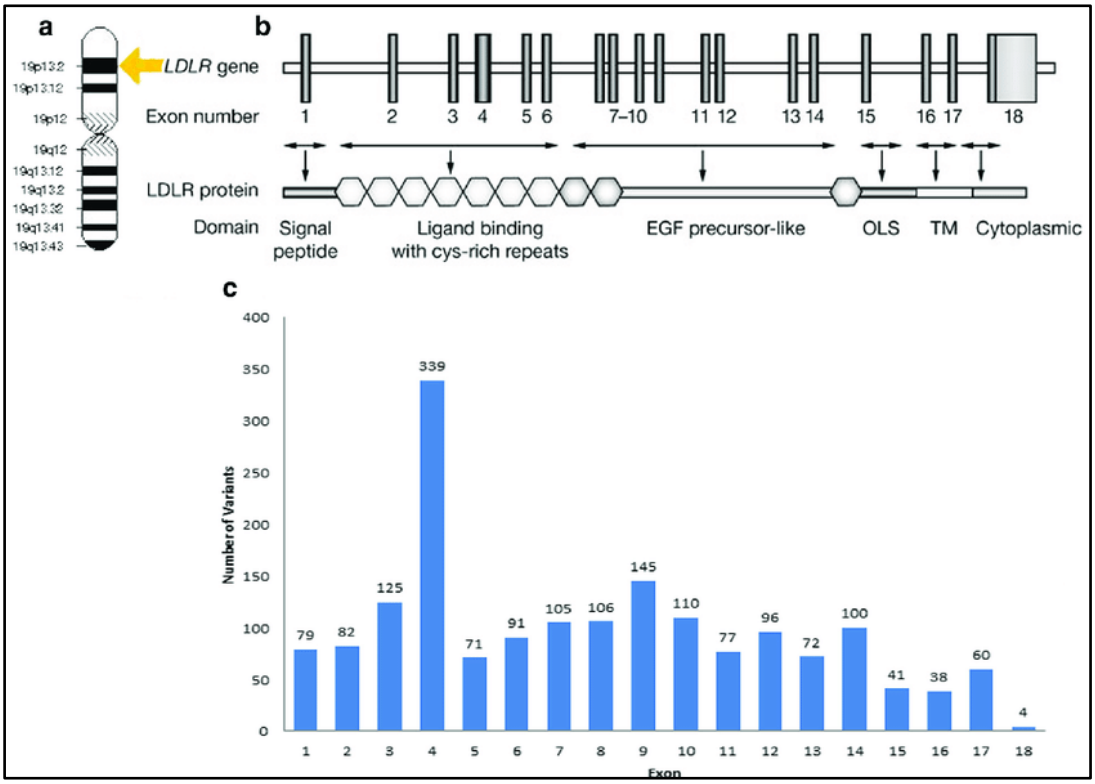

Figure 2.7 The *LDLR* gene. A) The *LDLR* gene is located on the short (p) arm of chromosome 19 at location 13.2. B) Numbered vertical bars indicate exons, lone exons, or groups of exons that encode the *LDLR* protein's multiple domains. C) 1,741 mutations in the *LDLR* gene exons (Henderson et al., 2016)

2.7.1.2 Apolipoprotein B-100

Lipoproteins high in TG and cholesterol, such as chylomicrons, VLDL, IDL, and LDL particles, include the quantitatively dominant and irreplaceable apolipoprotein known as *APOB*. These lipoproteins cannot be produced or secreted without *APOB*. Exon 26 is the main site of APOB variants (Figure 2.8). The human APOB gene resides on the short arm of the second chromosome, which is 43 kb long (2p24-23). This gene is mostly expressed in hepatocytes and enterocytes. The 14-kb mRNA transcript has a half-life of 16 hours and is made up of 29 exons and 28 introns (Gabcova-Balaziova et al., 2015). Humans have two primary isoforms: *APOB48* and *APOB100*. *APOB48* shares 48% of its N-terminal sequence with *APOB* (Innerarity et al., 1987). *APOB100* is 4536 amino acids long and weighs 517 to 550 kDa, making it one of the biggest known monomers. *APOB100* has five domains: βα1, β1, α2, β2, and α3, which are designated after the -helix and -pleated sheet structures (Gabcova-Balaziova et al.,

2015). Patients with familial ligand-defective *APOB* may manifest with FH in a milder manner than those with *LDLR* mutations (Henderson et al., 2016).

The R3500Q mutation (c.10580C>T or c.10580G>A), p. (Arg3527Gln), is the most frequent in *APOB (Abifadel & Boileau, 2023)*. The majority of identified *APOB* gene variations have been found to cause familial hypobetalipoproteinemia (FHBL) (Ayoub et al., 2021). On the other hand, ADH is caused by certain missense variants. In 1987, Innerarity and the research team reported the earliest examples of "familial defective apolipoprotein B cases" (FDB). Exons 26 and 29 of the *APOB100* gene include mutations that cause FDB. These mutations are located in the LDL binding domain 29 (AL-Khateeb & AL-Talib, 2016). This autosomal dominant genetic condition was identified by high LDL-C levels induced by impaired hepatocyte clearance of *APOB*-containing particles (Abifadel & Boileau, 2023). In the US, around 0.1% of Caucasians and Northern Europeans have the R3500Q mutation. German-speaking Switzerland has the greatest frequency of carriers in Europe, while the Amish community has the highest frequency globally, with 12% carrying the variation (Shen et al., 2010). An increased incidence of ischaemic heart disease and an early onset of coronary artery disease have been associated with FDB. FDB carriers died about ten years sooner than noncarriers and were at a higher risk of coronary artery calcification, even at LDL-C levels equivalent to noncarriers (Abifadel & Boileau, 2023).

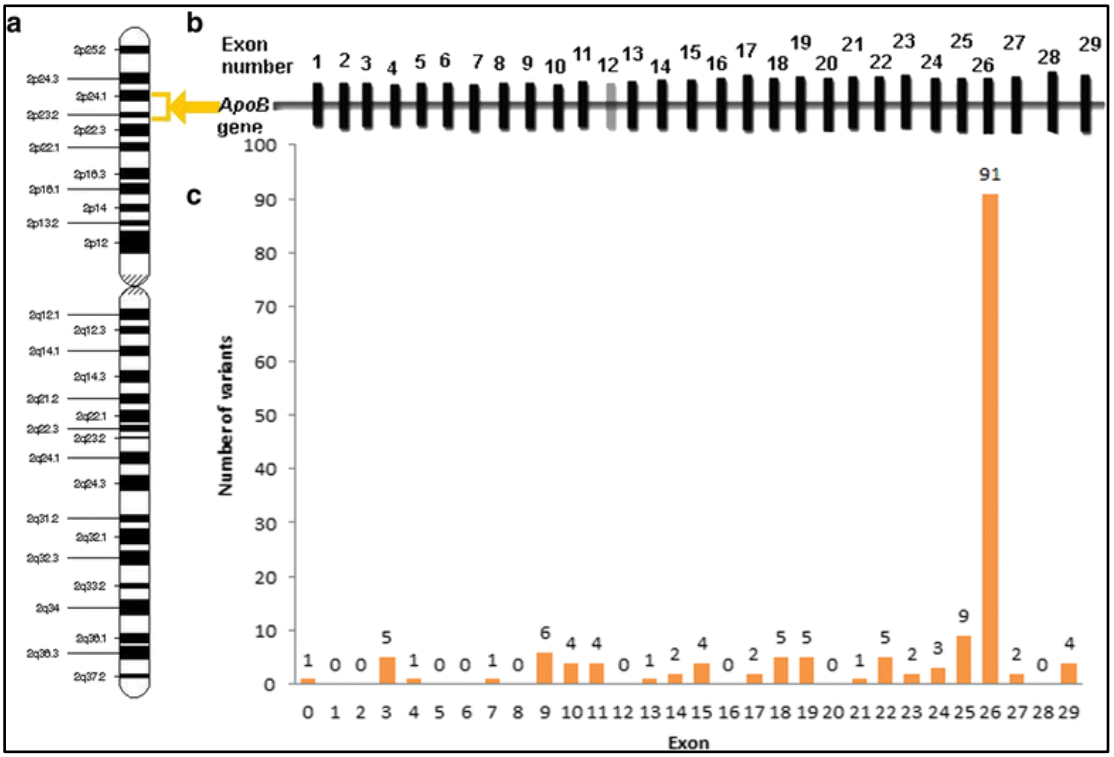

Figure 2.8 *APOB* gene. A) The *APOB* gene's location on the short arm of 2nd chromosome. B) Vertical bars represent the exons. C) The majority of disease-associated sequence variations have been discovered at the mutational hotspot exon 26 (Henderson et al., 2016).

2.7.1.3 Proprotein Convertase Subtilisin/Kexin Type 9

The human gene *PCSK9* is found on the first chromosome (1p32.3) and consists of 12 exons spanning 3617 bp (Figure 2.9). The *PCSK9* gene codes for a 692 amino acid-long glycoprotein. *PCSK9* is produced as an inactive proenzyme with 692 amino acids. *PCSK9* has the same structure as other members of the subtilisin convertase family: a signal sequence (1-30 AA), a prodomain (31-152 AA), and a catalytic domain that consists of the D-H-S catalytic triad, followed by a C-terminal domain (residues 452-692) (Lambert et al., 2012). The prodomain (14 kDa) is being cleaved off the mature protein (63 kDa) and passes through the ER. The prosegment functions as an inhibitor and intramolecular chaperone of the catalytic domain, which is required for *PCSK9* folding and release from the ER (Gabcova-Balaziova et al., 2015). Autocatalytic cleavage in the presence of calcium is essential for *PCSK9* maturation and secretion (Lambert et al., 2012).

This gene is found on chromosome 1 and is 25.3 kbp long, with 12 exons (Figure 2.10) (Henderson et al., 2016). *PCSK9* protein is a circulatory protein that interacts with the LDL-LDL receptor complex and promotes cytoplasmic degradation and cellular recycling of the *LDLR* (Xia et al., 2021). *PCSK9* missense mutations that result in a gain of function (GOF) produce a rare form of FH. In the *PCSK9* gene associated with ADH, GOF mutations enhance the affinity of *PCSK9* for the receptor and raise plasma LDL-C levels. Loss of function has been proven to reduce LDL-C levels and protect against CHD in some ethnic communities (Varret & Rabes, 2012).

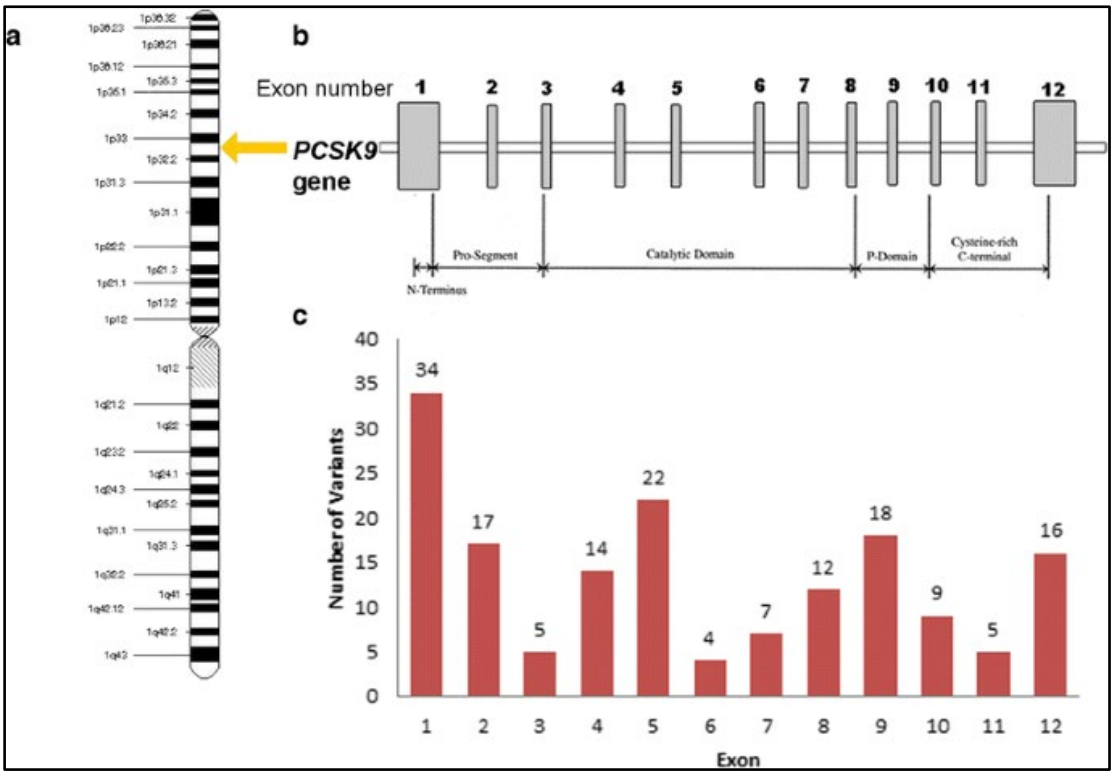

Figure 2.9 *PCSK9* gene. A) Location of the *PCSK9* gene on the short arm of chromosome 1 at position 32.3. B) Vertical bars represent exons. C) 163 mutations seen in the *PCSK9* gene (Henderson et al., 2016).

2.8 Next Generation Sequencing

FH genetic testing provides prognostic information, as well as the opportunity for enhanced risk categorisation. Advances in targeted next-generation sequencing (NGS) technologies have enabled the inclusion of genetic information in classical FH genes, minor FH genes, and other lipid metabolism-related genes in clinical diagnostic settings (Sturm et al., 2018). Traditionally, clinicians have used microarray, PCR, or

DNA sequencing, including Sanger sequencing of all coding areas in LDLR and one or ;8two specific exons in APOB, to diagnose FH (Wang et al., 2005). Multiplex ligation-dependent probe amplification (MLPA) can identify large *LDLR* rearrangements (Reeskamp et al., 2020).

The principle of NGS is based on massively parallel sequencing, where millions of DNA fragments are simultaneously amplified and sequenced in a single run. Compared with Sanger sequencing, which reads one DNA fragment at a time, NGS allows high-throughput analysis by fragmenting DNA into small pieces, attaching adapters, and sequencing them in parallel using sequencing-by-synthesis chemistry. Fluorescently labelled nucleotides or ion-detection systems are commonly employed to capture incorporation events, which are then translated into digital sequence data by bioinformatics pipelines (Goodwin, McPherson, & McCombie, 2016; van Dijk, Jaszczyszyn, Naquin, & Thermes, 2018). This enables comprehensive coverage of large genes such as *LDLR*, as well as simultaneous testing of multiple FH-related genes, with greater efficiency and lower cost than traditional methods.

NGS provides for rapid and low-cost parallel sequencing with excellent specificity and sensitivity, and numerous NGS gene panels are currently available to diagnose FH (Faiz et al., 2013; Vandrovcova et al., 2013; Johansen et al., 2014; Maglio et al., 2014). NGS is effective for detecting FH mutations in patients visiting lipid clinics, with sensitivity and specificity comparable to traditional capillary sequencing (Vandrovcova et al., 2013). Furthermore, NGS mutation detection is well suited to high-throughput testing of large numbers of samples, which is not always possible with traditional capillary sequencing systems (Norsworthy et al., 2014).

However, establishing high-quality NGS and bioinformatics capacity remains challenging. Key considerations include selecting appropriate sequencing platforms, bioinformatics pipelines, skilled personnel, and sufficient computational infrastructure (Gargis et al., 2016). The use of DNASeq library preparation, with a minimal-bias fragmentation phase and frequently updated primer sets, has consistently produced good sequencing data across a wide range of sample types (Ogorzaly et al., 2015; Maljkovic et al., 2016; Faria et al., 2016; Salje et al., 2017; Fernandez-Garcia et al., 2018). Raw sequencing data are typically cleaned, trimmed, and filtered to remove low-quality and duplicate reads, and host genome sequences are excluded to reduce background noise (Grard et al., 2012; LaBreck et al., 2018).

2.9 Treatment Options for Familial Hypercholesterolaemia

Lipid-lowering drugs are beneficial for patients who have a higher risk of cardiovascular events, such as unstable angina, nonfatal MI, nonfatal stroke, or cardiovascular mortality. It is critical to understand that these medications can be helpful when combined with lifestyle changes. These drugs can be used to prevent cardiovascular events, either primary or secondary (Al-Rasadi et al., 2014).

Three-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, bile acid sequestrants, a cholesterol-absorption inhibitor, fibrates, nicotinic acid, and omega-3 fatty acids are currently available medications for lipid-lowering therapy. These medications have different mechanisms of action and pharmacokinetic properties. The HMG-CoA reductase inhibitors, or "statins," are the lipid-lowering medications that are most frequently administered. By binding to the enzyme that produces cholesterol, these substances decrease its synthesis in the liver. Statins may potentially improve endothelial dysfunction, reduce inflammation, and contribute to the regression of atherosclerosis and plaque stabilisation, in addition to their effects on lipids (Ridker et al., 1999; Corti et al., 2001; Schartl et al., 2001). Bile acids are bound by bile acid sequestrants (BAS) in the gut, inhibiting the reabsorption of bile from the intestine. Since there is less intrahepatic cholesterol, *LDLR* that binds plasma LDL is produced (Guyton & Goldberg, 2009).

In 2013, the Agency for Healthcare Research and Quality (AHRQ) stated that fibrates do not alter lipid production but rather lower fatty acid levels in the blood. They cut triglycerides, raise HDL, and lower LDL. Niacin (nicotinic acid) suppresses the formation of very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL). Although the mechanism of omega-3 fatty acids is unknown, they may block acyl-CoA:1,2 diacylglycerol acyltransferase, boost hepatic beta-oxidation, decrease hepatic triglyceride production, or increase plasma lipoprotein lipase activity (Egashira et al., 1994; Backes et al., 2016; Krupa et al.,2023). The hypocholesterolemic medication ezetimibe has been shown to bind Niemann-Pick C1-Like 1 (NPC1L1) and prevent cholesterol absorption (Ge et al., 2008). It has been discovered that ezetimibe can lower LDL-C levels and enhance CV outcomes when used in combination with statin therapy. However, there are minimal benefits, and there is very little overall outcome information about ezetimibe monotherapy (Cannon et al., 2015). The Food and Drug Administration (FDA) has authorised alirocumab and evolocumab, the two currently

available antibodies, as fully human immunoglobulin-G (IgG) subtypes4 for the treatment of HeFH and the prevention of cardiovascular events in those with pre-existing CVD. For HoFH, evolocumab is also advised (Kaddoura et al., 2020; Santos et al., 2020).

In clinical settings such as FH, familial *APOB100* defect, polygenic hypercholesterolemia, familial combined hypercholesterolemia, and isolated lipoprotein (a) [Lp(a)] elevation, lipoprotein apheresis (LA) therapy is primarily indicated for the elimination of severely elevated *APOB*-containing lipoproteins (Bambauer et al., 2012). LA is not a recommended extracorporeal elimination technique for high TG. Large-particle TG causes haemolysis by producing clots during the procedure by raising the transmembrane pressure. As a result, plasmapheresis should be carried out in cases of extreme hypertriglyceridemia that pose a risk to life (Joglekar et al., 2017; Thompson & Parhofer, 2019; Kayikcioglu, 2021). The combination of plasma LDL-cholesterol levels and response to medication treatment determines the indications for LA. The main reason for initiating LA is the presence and severity of atherosclerotic CVD. Since there are not enough outcome studies, the current guidelines' standardised recommendations for beginning LA therapy are still lacking. However, all relevant recommendations classify LA as a life-saving last resort treatment for HoFH-related refractory severe hypercholesterolemia (Kayikcioglu, 2021). Early identification and LDL-C reduction are still required to prevent ASCVD morbidity and death in FH patients. Otherwise, FH may remain underdiagnosed and undertreated despite substantial advances in our understanding of the illness and effective medications.

CHAPTER 3

METHODOLOGY

3.1 Study Design

As shown in flowchart Figure 3.1, this cross-sectional study recruited 310 clinically suspected FH patients from Ministry of Health of Malaysia (MOH) primary care clinics that are located in Selangor, Kuala Lumpur and Putrajaya. This study involved 11 primary care clinics as shown in Figure 3.2.

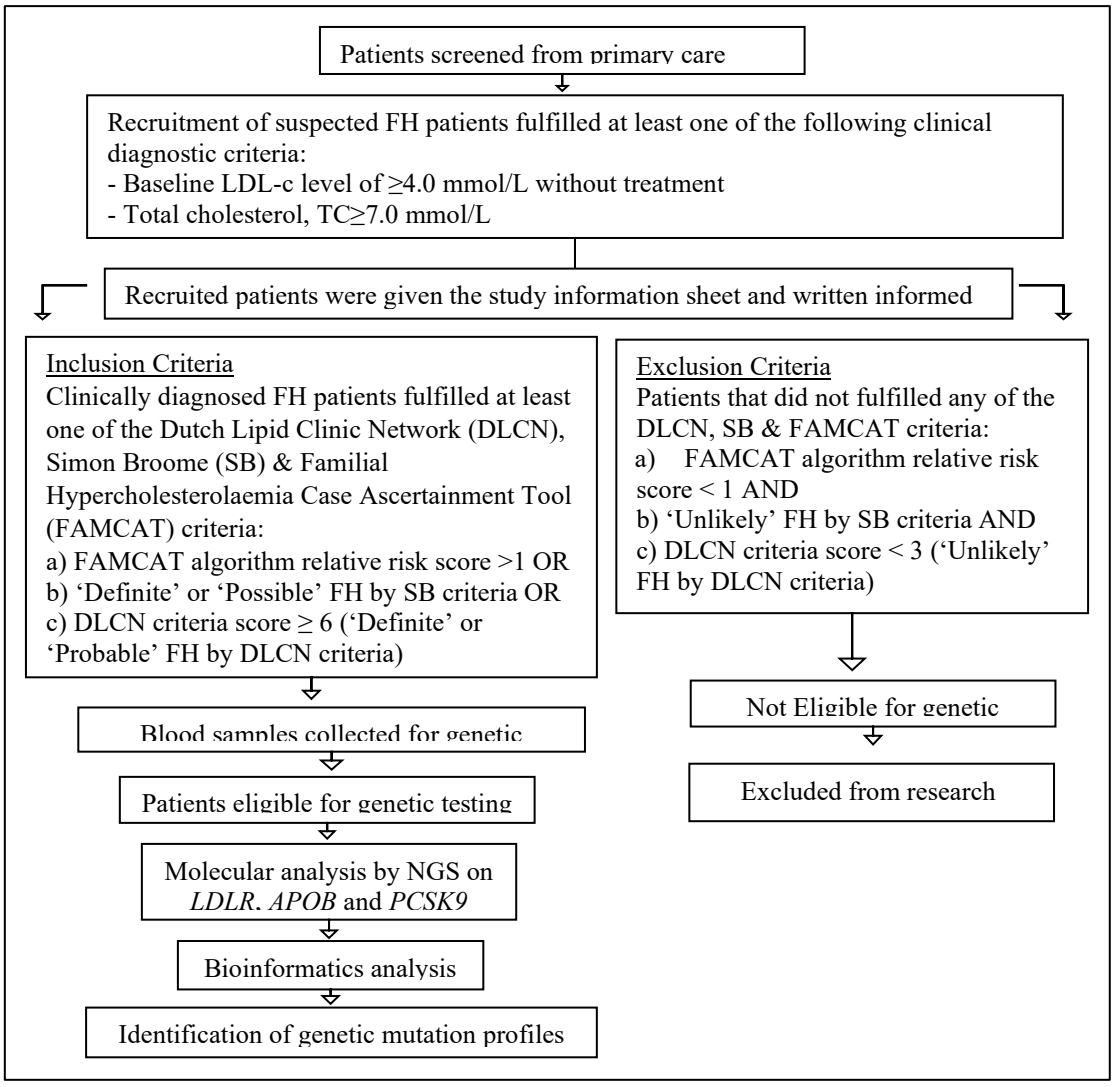

Figure 3.1 Flowchart of the study and patients recruitment

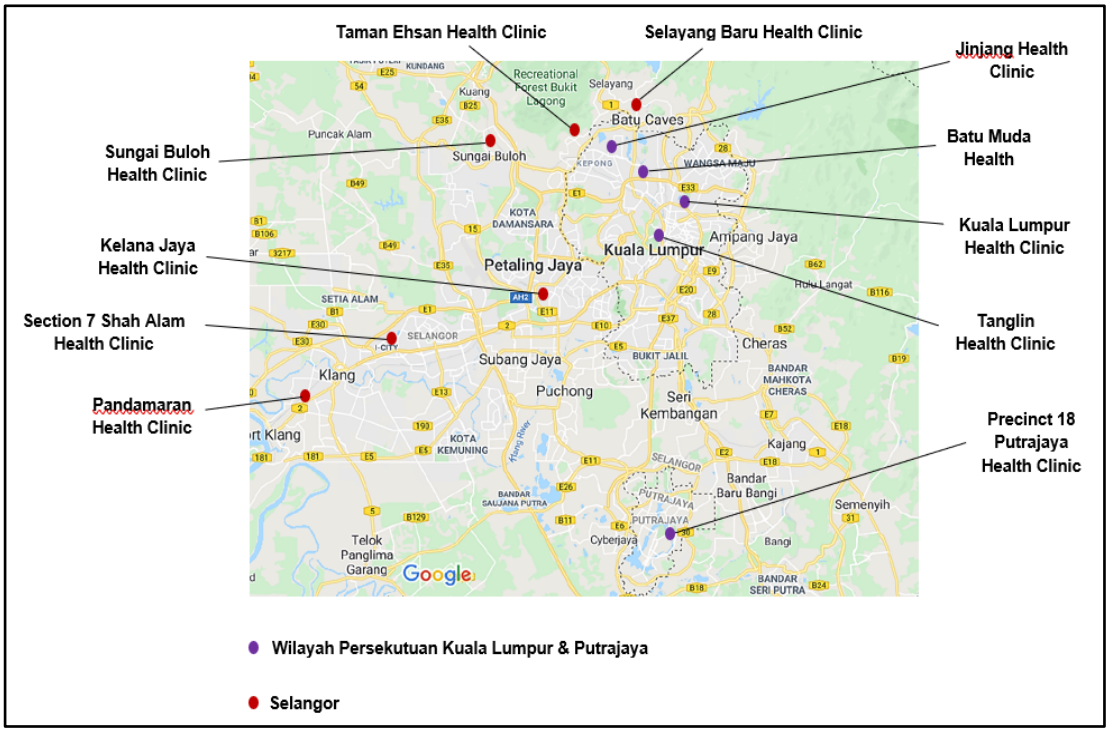

Figure 3.2 The Study Site of 11 MOH Primary Care Clinics in Selangor, Kuala Lumpur and Putrajaya

3.2 Ethical Approval

This study has obtained ethical approval from UiTM Research Ethical Committee [Reference number: 600-TNCPI (5/1/6)] (Appendix 1). This study was carried out in accordance with the ethical guidelines for medical research involving human subjects outlined in the International Conference for Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and Good Clinical Practice Guidelines, Malaysia Good Clinical Practice (GCP) Guidelines, and the Declaration of Helsinki.

This study was funded by the Newton-Ungku Omar Fund (NUOF): The United Kingdom's Medical Research Council (MRC) [MRC Grant Ref: MR/T017384/1] under the UK-Malaysia Joint Partnership Call on Non-communicable Diseases-Reducing Premature Coronary Artery Disease by Early Identification of Familial Hypercholesterolemia (Appendix 2). The funding bodies did not play any role in the design of the study or in data collection, analysis or interpretation.

3.3 Sample size

The sample size was calculated using single proportion formula as below:

$$n = \left(\frac{z}{d}\right)^2 \times p\,(1-p) \tag{3.1}$$

n = Sample size
z = 95% confidence interval (CI)
d = Precision (if the precision is 5%, then d=0.05)
p = Expected proportion of individuals

Based on previous study, the proportion of positive detection rate observed with next-generation sequencing was 27.0% (Sturm et al., 2021). To achieve a 95% confidence interval that is within 5% of the true value. The calculated sample size required was 332; however, 310 cases were successfully recruited in this study.

$$n = \left(\frac{1.96}{0.05}\right)^2 \times 0.27\,(0.73) \tag{3.2}$$

$$n = 302$$

Considering a drop-out rate of 10%, the sample size required for this study was n=332. A 10% dropout rate was applied to account for potential participant withdrawal, incomplete data, or inadequate blood samples for NGS analysis. This study formed the genetic component of the NUOF project, for which only 310 samples were available for this research team. Although the calculated sample size was 332, the target could not be fully achieved due to time limitations and the impact of the COVID-19 pandemic on sample collection and processing.

3.3.1 Data Collection

The patients were assisted by the healthcare providers in filling out standard validated questionnaires and written informed consent forms. Age, gender, ethnicity, gross household income, education, marital status, personal and family history of illnesses like hypertension, hypercholesterolemia, CVD, and other lifestyle behaviours like smoking and drinking status are among the socio-demographic factors that make

up the parameters, also Family History Questionnaire (Qureshi et al., 2005). The electronic medical record (EMR) was accessed while the patients were in attendance in order to obtain clinical data, such as significant medical history, medication history, and prior lipid profile results (total cholesterol, triglycerides, and LDL-C).

For subsequent study, all the data collected from the questionnaire were stored in a Microsoft Excel spreadsheet. In order to ensure accurate clinical assessment of the patients , clinical examinations were obtained under the supervision of the clinicians who were involved in the research project. These patients were recruited if they had fulfilled either one of the inclusion criteria (Table 3.1) presented with or without clinical features indicative of cholesterol deposition, such as corneal arcus, xanthelasma, Achilles tendon xanthomata, or tendon xanthomas.

The Edinburgh Claudication and the WHO Rose Angina Questionnaire were used to determine which individuals need urgent medical intervention (Rabia K & Khoo EM, 2007; Hassan et al., 2007). Patients with suspected FH who were advised to undergo genetic testing were given an additional set of patient information sheets and an informed consent form (Appendix 3). It offers vital information on genetic testing. Participants were advised about their risk of having FH, the pattern of inheritance, the genetic testing process, confirmation of diagnosis following genetic testing, future treatment, and the necessity for cascade screening of family members if genetically diagnosed. Participants who agreed to participate were given written informed consent (Appendix 4).

3.3.2 Inclusion and Exclusion Criteria

Table 3.1
The Inclusion and Exclusion Criteria Used in Recruiting Patients for Genetic Testing

Inclusion Criteria	Exclusion Criteria
- Malaysian males and females AND - Patients ≥18 years old which either with: 1) Baseline LDL-C level of ≥4.0 mmol/L without treatment OR 2) Total cholesterol, TC 7.0 mmol/L OR 3) DLCN: Possible FH or ((Probable or Definite FH) (Potential FH)) (Score ≥ 3) OR	- Patients who had undergone previous genetic testing for FH with a confirmed diagnosis. - Pregnant or lactating women. - Individuals who lack the mental capacity to provide informed consent. - Non-Malaysian citizens.

4) SB: Definite and Possible FH OR **5)** FAMCAT algorithm relative risk score >1	- Patients with incomplete clinical or biochemical data required for FH assessment.

3.3.2.1 Inclusion Criteria

Based on the inclusion criteria Table 3.1 above, this study included patients with suspected FH aged 18 years old and above. The DLCN criteria score which was also used to provide a clinical diagnosis of FH was calculated based on the patients' significant medical history, medication history, and prior blood results, such as lipid profile, that were retrieved from the EMR. The study included individuals diagnosed with hypercholesterolemia, comprising both patients who had commenced lipid-lowering therapy and those who had not yet started treatment at the time of sampling. This inclusive approach was adopted to represent the genetic diversity and clinical spectrum within the study cohort. Suspected FH is defined by DLCN, SB and FAMCAT. Potential FH was characterised as having a DLCN score of 6 for Probable FH and greater than 8 for Definite FH and those with a DLCN score of 3 to 5 as Possible FH (Tada et al., 2021). SB categorises those individuals with elevated LDL-C, an early family history of heart disease, and tendon xanthomas are classified as having Definite FH while those without these conditions are classified as having Possible FH (Humphries et al., 2018). FAMCAT algorithm relative risk score > 1 were classified as possibly having FH (Ramli et al., 2023). In addition, individuals with a family history of premature coronary artery disease (PCAD) and/or physical stigmata of FH were considered to have a higher likelihood of FH and were prioritised for further genetic evaluation. Those that fulfilled all those inclusion criteria were eligible for genetic testing.

3.3.2.2 Exclusion Criteria

The exclusion criteria were the one that had previously been diagnosed with FH through genetic testing, pregnant, or lacked mental ability to provide informed consent, and did not fulfil either one of the diagnostic criteria tools of DLCN, SB and FAMCAT.

3.3.3 Patients Recruitment

Patients were initially screened in participating primary care clinics using routine lipid profile data retrieved from the EMR system. Individuals aged 18 years and above with LDL-C values of 4.0 mmol/L or above that were obtained from the EMR were divided into four groups: ≥7.0 mmol/L, 6.0-6.9 mmol/L, 5.0-5.9 mmol/L, and 4.0-4.9 mmol/L. Patients identified were further evaluated using established FH diagnostic tools, including the DLCN SB, and FAMCAT. These assessments were used to classify patients into possible, probable, or definite FH categories for recruitment in the genetic study. Patients from these four categories were contacted to participate through a social media messaging service. Patients were contacted, and if the patient was unresponsive to the invitation message given through the social media messaging service.

Those who consented to participate were scheduled to be screened at the primary care clinic based on the inclusion and exclusion criteria as in Table 3.1. Participants were given a research information sheet and provided signed informed consent (Appendix 5) at the clinic. Those who voluntarily accepted to participate were evaluated for eligibility based on the inclusion and exclusion criteria (Table 3.1). Systematic interviews were carried out with patients to assess eligibility requirements. Those who met the eligibility requirements and consented to participate were recruited, and written informed consent were filled in. Patients were recruited until the sample size was attained.

3.4 Next Generation Sequencing Process

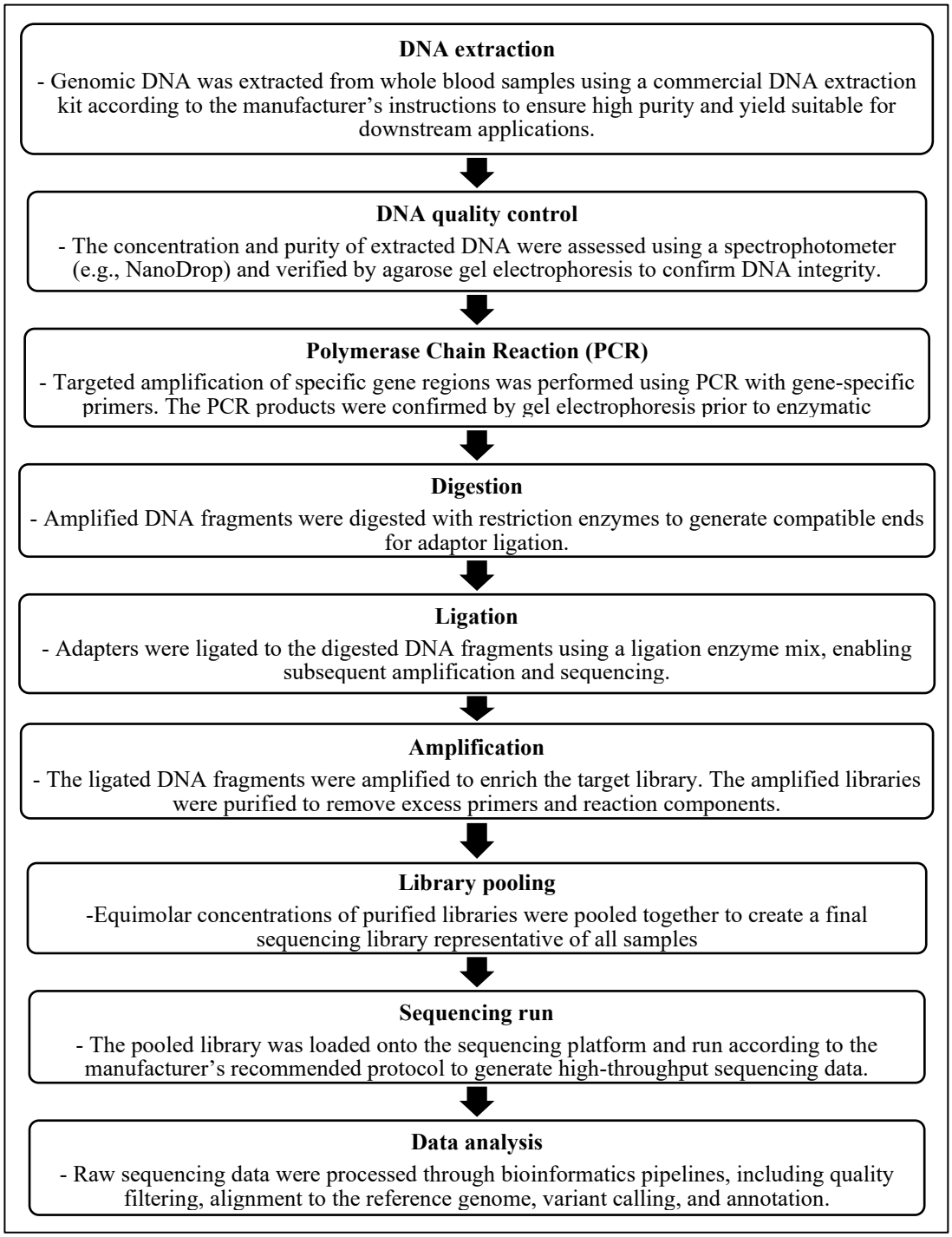

Figure 3.3 Summary of Next Generation Sequencing Process

3.4.1 Sample Collection

The participants blood samples were collected around 6 mL. The blood was drawn into blood collection tubes which were EDTA tubes. On the same day, these

samples were delivered to the Institute of Pathology, Laboratory and Forensic Medicine (I-PPerForM) Genetic Laboratory within 3 hours and were stored in a -20°C freezer until analysis. DNA samples were extracted from whole blood using a MasterPure™ DNA Purification Kit for Blood Version II, which was used to eliminate contaminated macromolecules, avoiding hazardous organic solvents. In order to purify 200 µl of whole blood, the extraction was carried out in accordance with the instructions provided in the manual insert of the extraction kit manufactured by Lucigen Corporation (USA). Table 3.2 showed the description of the kit components and reagents. All reagents and buffers used in this study, including TBE Buffer, loading dye, and staining solutions, were commercially obtained in ready-made form and used according to the manufacturer's instructions, unless otherwise stated. All of the preparation of research materials were shown in Appendix 6.

Table 3.2
Components and reagents of DNA extraction kit

Product	Component	Reagent specification
MasterPure™ DNA Purification Kit for Blood Version II	TE Buffer	Tris-EDTA (TE) buffer is used to solubilize and stabilize DNA or RNA while preventing degradation. Tris maintains a stable pH, and EDTA chelates divalent metal ions (e.g., Mg^{2+}), thereby inhibiting DNase activity (Panda et al., 2019).
	RNase A	RNase A is used to degrade contaminating RNA during DNA purification. It is typically added after DNA precipitation, washing, and resuspension to ensure removal of RNA and obtain high-quality genomic DNA (Healey et al., 2014).
	MPC Protein Precipitation Reagent	Used to precipitate and remove proteins from cell lysates prior to nucleic acid purification, ensuring a clean DNA sample.
	Red Cell Lysis Solution	Selectively lyses erythrocytes (red blood cells) to release and remove them, leaving leukocytes (white blood cells) intact for subsequent DNA extraction.
	Tissue & Cell Lysis Solution	Breaks open cells and tissues to release nucleic acids and proteins. It facilitates efficient lysis for downstream molecular biology applications and helps maintain protein stability.

3.4.2 DNA Extraction

The estimated yield of DNA from the extraction was 3-9 µg. Firstly, a microcentrifuge tube was filled with 200 µl of whole blood using a pipette. Then, 600 µl of Red Cell Lysis Solution was added. The tube was turned upside down three times to be mixed. The residual substance was then suspended by flicking the bottom of the tube. The tube was incubated for five minutes at room temperature (RT) before being vortexed. Then, the tube was quickly vortexed. Then the cells were pelleted by centrifugation for 25 seconds in a microcentrifuge. About 25 µl of liquid were left after the majority of the supernatant was removed, and I proceeded with suspending the pellet by vortexing it. The white blood cell was resuspended in 300 µl of ready-made tissue and cell lysis solution. 1µl of RNase A was added and was thoroughly mixed. Following that, the samples were incubated at 37°C for 30 minutes.

Total DNA precipitated after the samples were put on ice for three to five minutes. The 300 µl lysate sample was added, followed by 175 µl of MPC (MasterPure™ Precipitation Cocktail) Protein Precipitation Reagent, and vigorously vortexed for 10 seconds. The debris was centrifuged in a microcentrifuge for 10 minutes at ≥10,000 x g to form pellets. The pellet was discarded after the supernatant had been transferred into another autoclaved centrifuge tube. Then, 500 µl of isopropanol was added into the tube and was inverted for 30 to 40 times to mix the tube content.

The tube was centrifuged at 4 °C for 10 minutes to form the pellet. Without moving the DNA pellet, the isopropanol was gently drained away using a pipet. The DNA pellet was rinsed twice with 70% ethanol, without dislodging the pellet. A pipet was used to remove all of the remaining ethanol. Then, 35 µl of TE Buffer was used to resuspend the DNA. The DNA was then quantified. To maintain the DNA integrity, the extracted DNA was kept in a freezer at -20°C.

3.4.3 Quantification and quality control of DNA

3.4.3.1 QuickDrop Quantification

The concentration (ng/µl) and purity of the components were examined by SpectraMax® QuickDrop™ Micro-Volume Spectrophotometer (Molecular Devices, San Jose, CA, USA). On the microvolume port, 1 µl of TE buffer was measured as a blank, followed by 1 µl of DNA sample. Electrophoresis was performed on samples with concentrations of greater than or equal to 20 ng/µl and purity ratios of 1.70–2.00 for A260/A280 (Figure 3.4). After each read, the micro-volume port was cleaned clean with a lint-free wipe.

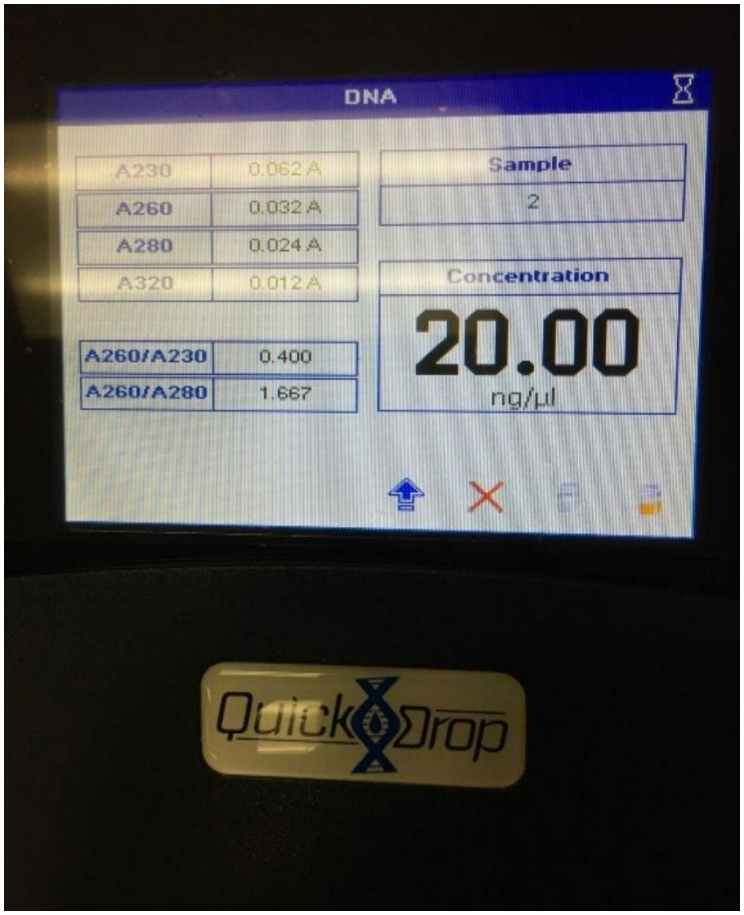

Figure 3.4 Data display on QuickDrop. The QuickDrop touchscreen displays the computed and provided parameters which quantifies nucleic acids based on absorbance at 260 nm. The A260/A280 ratio indicates sample purity.

3.4.3.2 Agarose gel electrophoresis

Agarose gel electrophoresis was used to assess the quality of the DNA. In a conical flask, 0.5 grams of 1% agarose powder and 50 ml of 1X Tris-Borate-EDTA (TBE) buffer were mixed. The mixture was microwaved for roughly 2 minutes and left on a bench till it reached RT. Then, 1µl of SYBR Safe DNA Gel Stain (Thermo Fisher Scientific, USA) was added to the mixture and was then poured into mould. The comb was then put into the mould to create wells, and the gel was allowed to solidify at RT. After about 30 minutes, the comb was removed, and the gel was ready to be put in a buffer tank full of running 1X TBE buffers.

The first well was loaded with 1 µl of 6X TriTrack DNA Loading Dye (Thermo Fisher Scientific, USA) and 1 µl of 1 kB DNA Ladder (SMOBIO Technology). Then, 1 µl of DNA samples with 1 µl of DNA loading dye were pipetted into the sample wells. The lid and power leads were attached to the equipment, and a 90V voltage current was applied for 40 minutes. DNA travelled from negative (cathode) to the positive (anode) electrode (Figure 3.4). Gel DocTM XR+ System with Image Lab^{TM} Software (Bio-Rad Laboratories, Malaysia) was used to view the samples in the gel. The samples that showed clear intactness (Figure 3.5) were then subjected to further concentration clarification using a Qubit™ fluorometer.

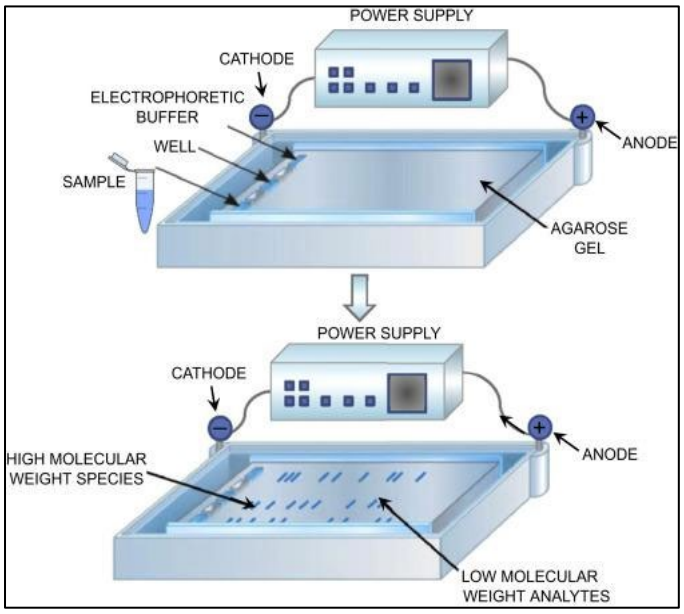

Figure 3.5 Illustration of an agarose gel electrophoresis setup (Drabik et al., 2013).

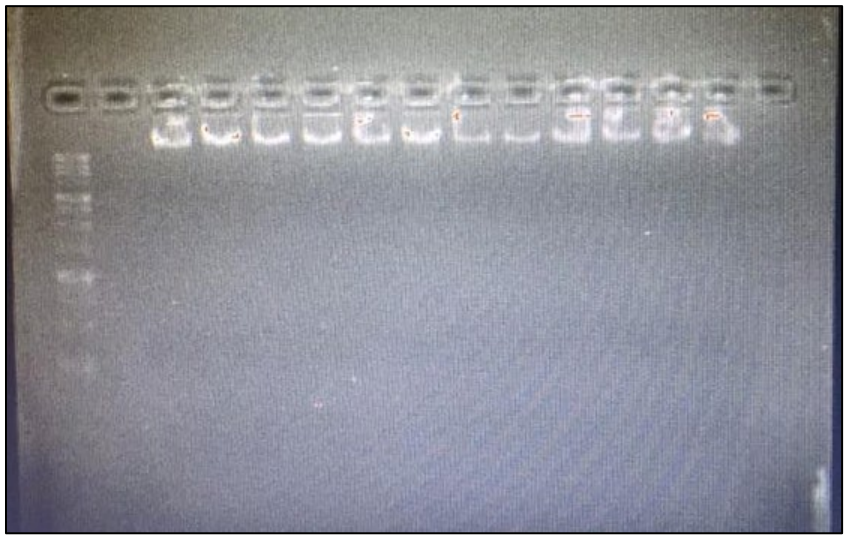

Figure 3.6 Typical result of DNA electrophoresis

3.4.3.3 Qubit Quantification

Qubit® dsDNA HS (High Sensitivity) Assay Kit (Thermo Fisher Scientific, USA) was used for a more accurate DNA quantification. The test kit has been manufactured to be accurate for initial DNA sample concentrations ranging from 5 pg/L to 120 ng/µl, yielding a detection range of 0.1-120 ng. Each kit included a ready-to-use working solution as well as DNA standards. According to the manufacturer instructions, samples having a concentration of ≥ 50 ng/µl based on the Quickdrop readings were diluted to any volume ranging from 1 to 20 µl to run the assay, and the concentration was measured using a Qubit™ Fluorometer (Thermo Fisher Scientific, Wilmington, DE, USA) (Figure 3.6). However, solely for this study, DNA samples were diluted in any volume range of 18–30 ng/µl using autoclaved distilled water (ddH2O) in case the reading was underestimated before proceeding to Qubit quantification (Thermo Fisher Scientific, USA). The assay is precise for initial sample concentrations ranging from 10 pg/µl to 100 ng/µl. The test was done at RT. Table 3.3 shows the descriptions of the kit components and reagents.

Table 3.3
The components of Qubit® dsDNA HS (High Sensitivity) Assay Kit

Material	Amount	Storage	Stability
Qubit® dsDNA HS Standard #1	1 ml or 5 ml	Freezer	Kits are
Qubit® dsDNA HS Standard #2		≤ 4°C	stable
Qubit® dsDNA HS Buffer	50 ml or 250 ml	RT	for 6
Qubit® dsDNA HS Reagent	250 µl or 1.25 ml		months

Two standards are required for the Qubit® dsDNA HS assay. The necessary number of 0.5 ml PCR tubes were prepared for Standard #1 and Standard #2 alongside number of samples to be tested. The Qubit® working solution was prepared by mixing the Qubit® dsDNA HS Reagent and Qubit® dsDNA HS Buffer (ratio 1:200) to a final volume of 200 µl in each tube. Each tube that used for standards was added with 190 µl of Qubit® working solution followed by 10 µl of each Qubit® Standard #1 and Standard #2. Meanwhile, each tube that was used for sample tubes was added with 198 µl of Qubit® working solution and 2 µl of DNA samples that needed to be measured. The PCR tubes contained 2 standard solutions, and all samples were then vortexed for 2-3 seconds. The tubes were incubated at room temperature for 2 minutes before being read by the Qubit™ Fluorometer to allow the Qubit® assay to achieve optimum fluorescence.

The dsDNA High Sensitivity was selected as the assay type by pressing “DNA” on the Qubit™ Fluorometer's Home Screen. The Standards Screen was automatically appearing. New calibration was launched by pressing “Yes” on the Standards Screen. Then, tube containing Standard #1 was calibrated by inserting into the Qubit™ Fluorometer by pressing “Read”. The reading took approximately 3 seconds. Standard #1 was then removed and replaced with Standard #2 using the same steps as previous. The result was displayed on the screen upon the completion of the measurement. The sample was then inserted into the Qubit™ Fluorometer by pressing “Read Next Sample” on the screen. Sample readings were repeated until all samples have been read. All of the readings were then recorded into a single database.

Figure 3.7 Reading on Qubit ™ Fluorometer

3.5 Workflow for DNA Library Preparation

<table>
<tr><td colspan="2">Quantify and Dilute DNA</td></tr>
<tr><td>• Total hands on: 10 min
Reagents used: Low TE</td><td>The concentration of extracted genomic DNA was measured using a fluorometric assay (e.g., Qubit). DNA samples were then diluted to the required working concentration using Low TE buffer to ensure uniform input for amplification.</td></tr>
<tr><td colspan="2">▼</td></tr>
<tr><td colspan="2"><u>Amplify Targets</u></td></tr>
<tr><td>• Total hands on: 1.5-4 hours
Reagents: 2X AmpliSeq Custom DNA Panel (3 primer pools), 5X AmpliSeq HiFi Mix</td><td>Target regions were amplified using the AmpliSeq Custom DNA Panel containing three primer pools and AmpliSeq HiFi Mix. The amplification reaction was performed according to the manufacturer’s recommended thermal cycling conditions to generate target-specific amplicons.</td></tr>
<tr><td colspan="2">▼</td></tr>
<tr><td colspan="2"><u>Partially Digest Amplicons</u></td></tr>
<tr><td>• Total hands on: 50 min
Reagents: FuPa Reagent</td><td>The amplified DNA products were treated with FuPa reagent to partially digest primer sequences and prepare amplicons for adapter ligation.</td></tr>
<tr><td colspan="2">▼</td></tr>
<tr><td colspan="2"><u>Ligate Indexes</u></td></tr>
<tr><td>• Total hands on: 55 min
Reagents: DNA Ligase, AmpliSeq CD indexes, Switch Solution</td><td>DNA ligase, AmpliSeq CD indexes, and Switch Solution were added to attach barcoded adapters (indexes) to the digested amplicons, enabling sample identification during sequencing.</td></tr>
<tr><td colspan="2">▼</td></tr>
<tr><td colspan="2"><u>Clean Up Library</u></td></tr>
<tr><td>• Total hands on: 25 min
Reagents: 70% EtOH, AMPure XP Beads</td><td>The ligated libraries were purified using AMPure XP magnetic beads and 70% ethanol to remove unincorporated nucleotides, enzymes, and other impurities.</td></tr>
<tr><td colspan="2">▼</td></tr>
</table>

Amplify Library	
• Total hands on: 45 mins Reagents: 1X Library Amp Mix, 10X Library Amp Primers	The purified adapter-ligated libraries were further amplified using Library Amp Mix and Library Amp Primers to enrich fragments containing correct adapter sequences.
▼	
Perform Second Cleanup	
• Total hands on: 45 min Reagents: 70% EtOH, AMPure XP Beads, Low TE	A second purification step using AMPure XP beads and Low TE buffer was performed to remove residual primers, enzymes, and short DNA fragments, ensuring high-quality libraries.
▼	
Check Libraries	
Total hands on: 1-1.5 hours	Library size distribution and concentration were evaluated using a fragment analyzer or equivalent system to confirm library quality prior to sequencing.
▼	
Dilute to Starting Concentration	
• Total hands on: 20 min Reagents: Low TE, Elution Buffer, Tween 20	Final libraries were diluted to the required starting concentration using Low TE buffer, Elution Buffer, and Tween 20 before pooling for sequencing.

Figure 3.8 The summary of library preparation

3.5.1 Quantification and Dilution of DNA

The input DNA was quantified and diluted in this step to the appropriate concentration for subsequent steps. Table 3.4 shows a list of consumables used for quantification and dilution of DNA. The volume of diluted DNA required depends on the number of primer pools (Table 3.5).

Table 3.4
Consumables for Quantification and Dilution of DNA

Reagent	Storage
DNA	-25°C to -15°C (long-term) 2°C to 8°C (short-term)
Low TE	-25°C to -15°C

The DNA samples that were previously quantified using Qubit™ fluorometer were thawed at RT and diluted to an intermediate concentration of ~20-50 ng/µl using Low TE (Target Efficiency) and followed by re-quantification using the same fluorometer method. The samples were thawed at RT for 45 minutes and then diluted into the desired final concentration of 3.5 ng/µl using Low TE. The final concentration depends on the number of primer pools, which were designed and recommended by the manufacturer. This research required 3 primer pools. Target-specific primers were included in a AmpliSeq™ custom DNA (Thermo Fisher Scientific) designed to amplify all coding regions and exon–intron boundaries of the *LDLR, APOB,* and *PCSK9* genes associated with FH *PCSK9* was included to capture less common FH-causing mutations that increase LDL-C by promoting LDL receptor degradation. Although rarer than *LDLR* or *APOB* variants, including *PCSK9* ensures comprehensive genetic screening and maximises diagnostic yield in this study. In targeted amplicon sequencing, multiple primer pools are used to amplify overlapping gene regions. Although four pools were initially designed for full coverage, one was excluded due to low target enrichment efficiency and poor amplification performance. Using three optimised pools maintained sufficient coverage and sequencing quality while reducing non-specific amplification, cost, and workflow complexity. The primer sequences are proprietary to the manufacturer and were not disclosed.

Table 3.5
Lists example dilutions to result in standard DNA input per pool

Number of Pools	DNA Concentration (ng/µl)	Diluted DNA Volume (µl)	Total DNA Input (ng)
1	2	5	10
2	4	5	20
3	6	5	30

3.5.2 Amplification of DNA Targets

In this stage, DNA sample target regions were amplified using PCR. Table 3.6 shows a list of consumables for amplification of DNA targets.

Table 3.6
Consumables for Amplification of DNA Targets

Reagent	Storage
5X AmpliSeq HiFi Mix	-25°C to -15°C
2X AmpliSeq Custom DNA (Pool 1, 2 & 3)	
DNA	

7 µl of 5X Ampliseq HiFi Mix is a specialized PCR (Polymerase Chain Reaction) master mix (thawed on ice) and 10.5 µl of DNA samples were added into a microcentrifuge tube which totalled 17.5 µl of working solution for each 24 samples per every run. Ampliseq HiFi Mix was designed to amplify specific regions of DNA (called amplicons) with high accuracy and efficiency. The working solution was then pipetted to be mixed and centrifuged. Each of the solutions were transferred into 72 PCR tubes and were properly labelled as '1', '2' and '3'. Working solution was then allotted to 5 µl per every 72 PCR tubes, which was divided into three sections (pool 1, 2 and 3). Followed by each 24 tubes with the label "1" received 5 µl of 2X Ampliseq Custom DNA Panel Pool 1, then each 24 tubes with the label "2" received 2X Ampliseq Custom DNA Panel Pool 2, and finally other 24 tubes with the label "3" received 2X Ampliseq Custom DNA Panel Pool 3 which totalled 10 µl in each 72 tubes which containing the combination of 5 µl working solution and 5 µl of primer pool. The mixture was then pipetted and was briefly centrifuged. All tubes were then transferred on preprogrammed thermal cycler with a heated lid and ran based on AMP_DNA program which was saved according to manufacturer's instruction as follows:

AMP_DNA

- Preheated lid option was set to 105°C
- The reaction volume was set to 10 µl, 99°C – Two minutes
- 19 cycles of:
  - 99°C for 15 seconds, 60°C for 4 minutes
- Hold at 10°C for up to 24 hours

3.5.3 Partially Digest Amplicons

FuPa (Fused Primer and Adapter removal) Reagent is a consumable (Table 3.7) that was used in this phase to partly digest amplicons and digest primer dimers.

Table 3.7
Consumable for Partially Digest Amplicons

Reagent	Storage
FuPa	-25°C to -15°C

All tubes were then briefly centrifuged after AMP_DNA PCR was completed. 10 µl target amplification reactions in the 76 tubes labelled with '1', '2' and '3' were combined into 24 tubes containing pool 1 for a final volume of 30 µl for each sample. 3 µl of the FuPa reagent (thawed on ice) were added to each target amplification process, making a total of 33 µl per sample. The tubes were then briefly centrifuged at 280 g for 1 minute after being briefly vortexed at 1600 RPM for 1 minute. Then, all 24 tubes were set up on a thermal cycler that was preprogrammed and ran as follows:

FuPA

- Preheated lid option and was set to 105°C
- Reaction volume – 33 µl
- 50°C for 10 minutes
- 55°C for 10 minutes
- 62°C for 20 minutes
- Hold at 10°C for up to one hour

3.5.4 Ligate Indexes

The following consumables based on were prepared for this phase (Table 3.8):

Table 3.8
Consumables for Ligation of DNA

Reagent	Storage
AmpliSeq CD Indexes	-25°C to -15°C
DNA Ligase	
Switch Solution	

After the FuPa program was completed, the tubes were briefly centrifuged, and the contents were collected. 6 µl Switch Solution; has a chemical role in enabling adapter ligation (thawed at RT), 3 µl Ampliseq CD (Combinatorial Dual) Indexes (thawed at RT); CD Indexes was used to label each DNA library with a unique identifier (index) which enables multiple samples pool and sequenced together in a single sequencing run and finally 3µl DNA Ligase (thaw on ice) were added to each tube orderly to each tubes containing the 33 µl digested amplicons, making a total of 45 µl each sample. The tubes were then briefly vortexed at 1600 RPM for a minute, followed by centrifugation at 280 g for a minute. The 24 tubes were placed on preprogrammed thermal cycler and ran as follows:

LIGATE

- Preheated lid option and was set to 105°C
- Reaction volume – 45 µl
- 22°C for 30 minutes, 68°C for five minutes, 72°C for five minutes
- Hold at 10°C for up to 24 hours

3.5.5 Clean Up Library

Agencourt AMPure XP beads (Beckman Coulter, USA) were used to clean up the library in this phase and the beads were carried over for the next phase. Magnetic beads that bind DNA to help purify, clean, and concentrate DNA during NGS library preparation. List of consumables were prepared based on Table 3.9.

Table 3.9
Consumables for Clean Up Library

Reagent	Storage
1X Lib Amp Mix	-25°C to -15°C
10X Library Amp Primers	
Agencourt AMPure XP beads	2°C to 8°C

After the Ligate program was completed, the tubes were briefly centrifuged. 45 µl of AMPure XP beads (thawed at RT) was added to each library and was vortexed. After a brief centrifugation, each well was inspected to ensure the mixture was homogeneous. The tubes were then incubated at RT for five minutes. Then, all tubes

were placed on a magnetic stand for approximately 2 minutes until the mixture was clear. The supernatant was carefully removed from each tube while it was still placed on the magnetic platform. 150 µl of freshly prepared 70% ethanol was added into each tube to wash the beads and the mixture was incubated at RT for about 30 seconds until it became clear. Without disturbing the pellet, the supernatant was discarded from each well. The beads were washed once again using the prior procedure and were allowed to air-dry until EtOH was no longer visible.

3.5.6 Amplify Library

This second amplification step amplifies libraries to ensure there are enough for sequencing on Illumina systems. The beads, which were left over from the prior phase, were used during this amplification reaction. List of consumables were prepared based on Table 3.10.

Table 3.10
Consumables for Amplification of DNA

Reagent	Storage
1X Lib Amp Mix	-25°C to -15°C
10X Library Amp Primers	
Agencourt AMPure XP beads	2°C to 8°C

The amplification master mix was prepared by combining 1.125 ml of 1X Lib Amp Mix (thawed on ice) and 125 µl of 10X Library Amp Primers (thawed at RT). The master mix was vortexed and centrifuged briefly. After that, all tubes were then removed from the magnetic stand and 50 µl of the master mix was added to each tube. The tubes were placed on preprogrammed thermal cycler and ran as follows:

AMP_7

- Preheated lid option and was set to 105°C
- Reaction volume – 50 µl
- 98°C for two minutes
- 7 cycles of: 98°C for 15 seconds, 64°C for one minute
- Hold at 10°C for up to 24 hours

3.5.7 Second Cleanup

Agencourt AMPure XP beads were used in this second cleanup step to perform two rounds of purification in which high molecular-weight DNA was captured by the beads and discarded in the first round. Then, the retained libraries and primers in the supernatant were transferred to new tubes. For the second round, the libraries that were captured by the beads pellet were saved and eluted from the beads while primers remained in the supernatant. Table 3.11 shows the list of consumables for second clean up.

Table 3.11
Consumables for Second Cleanup

Reagent	Storage
Agencourt AMPure XP beads	2°C to 8°C
Low TE	-25°C to -15°C

After the second clean-up library phase was done, the tubes were briefly centrifuged, and the contents were collected. The library was vortexed and briefly centrifuged after 25 µl AMPure XP beads were added to each well containing the library (~50 µl). The tubes were then incubated at RT for 5 minutes and were placed on the magnetic stand until the solution cleared. The whole supernatant that contained approximately 75 µl amplicon library was transferred to another new tube, and each tube was added with 60 µl of AMPure XP beads. The tubes were then incubated at RT for 5 minutes after briefly vortexed and centrifuging. The tubes were then placed on a magnetic stand for another 5 minutes until the liquid cleared. The supernatant was discarded from each tube. The beads that remained were washed by adding 150 µl newly made 70% ethanol to each well and were incubated at RT approximately 30 seconds until the solution cleared. The supernatant was then discarded from each tube without disturbing the pellet and the washing procedure was repeated. After removing all remaining EtOH, the tubes were air dried for 5 minutes on the magnetic stand. 30 µl Low TE was added to each tube and was mixed well by briefly vortexed and centrifuged to disperse the beads. The tubes were placed again on the magnetic stand until the solution cleared. After that, about 25 µl of the supernatant that contains the amplicon libraries were transferred to new 24 PCR tubes.

3.5.8 Libraries Checking

Each library underwent Qubit quantification. In this phase, the Qubit reading ranged from 1 to 10 ng/µl. Each of the library Qubit reading was recorded in excel sheet, 390 bp average library size was used to measure the qualification and the molarity of the library was calculated using the following formula (nM):

$$\frac{ng/\mu l\ X\ 10^6}{660\ g/mol\ X\ 390} X\ Molarity\ (nM)$$

3.5.9 Dilution of library to the starting concentration

This procedure was necessary as part of the preparation for the sequencing system's final loading concentration, as specified in the manufacturer's manual. The libraries were combined into a single tube in this phase. Firstly, the master mix which consists of Elution buffer (EB) that combined with 0.1% Tween20 was prepared based on the total molarity that was calculated for each sample. Then, 5 µl was allotted from each of origin library samples along with the volume of the master mix varying according to the calculation of the molarity of each sample were transferred into another new microcentrifuge tube. A vortex was used to mix the solution. The following step involved pooling 5 µl of each library into one microcentrifuge tube. The molarities average of pooled libraries must be ranging from 4 nM to 6 nM. The library pool was then quantified using Qubit™ fluorometer to get the concentration reading. The average reading from three readings was recorded in the excel sheet. The reading was used to calculate the molarity of the library pool prior sequencing using Illumina iSeq 100 sequencers (the next-generation sequencing (NGS) platform). All reagents used in this procedure were ready-made and provided by the manufacturer.

3.5.10 Sequencing

The libraries were prepared using Illumina Ampliseq Custom Amplicon kit (Illumina, CA, USA) according to the manufacturer's protocols. The DNA samples that

passed the quality control procedures were proceeded to the NGS step. The sequencing was carried out using Illumina iSeq 100 System (Figure 3.9), to identify FH causing

mutations covering whole coding sequence and intron-exon junctions (up to 50 base pairs), 5′ and 3′ untranslated regions of *LDLR*, *APOB* and *PCSK9* genes. Pooled libraries were sequenced for the targeted genes using Illumina iSeq 100 sequencers. In order to accommodate the demands of modern workflows, the iSeq 100 System offers incremental sequencing capacity while preserving the reliable Illumina data quality. The iSeq 100 System ran less than 5 minutes and the sequencing was completed between 9.5 to 19 hours pending run size. Sequencing runs can be analysed using the on-instrument software (Illumina BaseSpace) and from any web browser to review real-time data and performance metrics (Figure 3.10). The iSeq 100 System is ideal for targeted sequencing of a set of genes or gene regions analysis. The final design allowed the analysis of up to 275 bp of targeted DNA insert within three reaction pools with an overall coverage of approximately 98.61%. The iSeq 100 System enables virtually any lab to acquire powerful NGS technology. By providing both higher data resolution and deeper coverage compared to qPCR and Sanger sequencing, the iSeq 100 System allows greater statistical confidence for calling variants or low-frequency alleles.

Figure 3.9 Illumina AmpliSeq iSeq 100 System

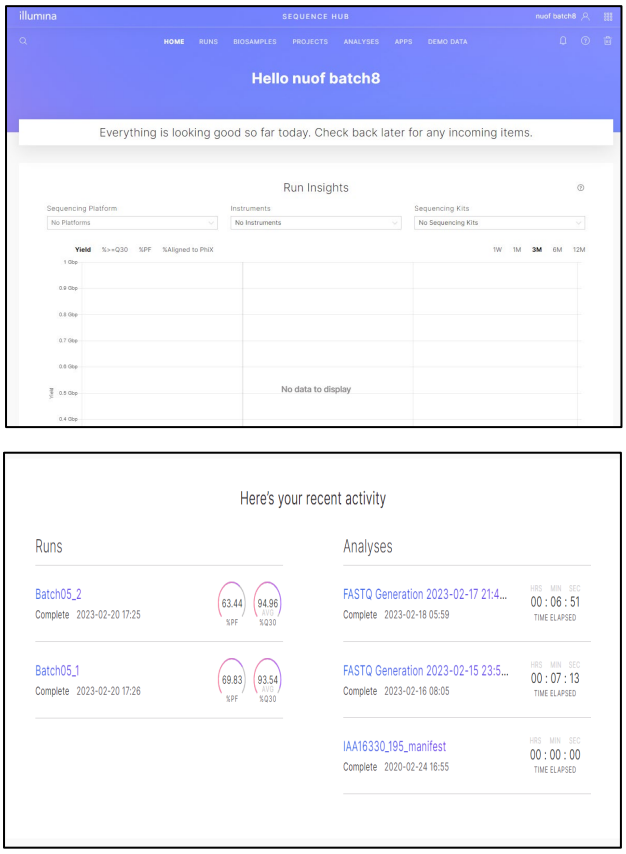

Figure 3.10 Interface of Illumina BaseSpace Sequence Hub Software

3.5.11 NGS Sample Preparation

PhiX was used in this phase. PhiX is a well-known adapter-ligated library that is used as a control for Illumina sequencing runs. PhiX Control can also be used as a high-concentration spike-in control for unbalanced samples, a low-concentration spike-in control for alignment calculations and quantification efficiency, a dedicated control lane alongside low-diversity samples, and a control for troubleshooting cluster generation problems, assisting in determining whether an error is related to library preparation.

The 4 nM - 6 nM library pool was diluted to 1 nM in a final volume of 15-20 µl with EB. By adding 95 µl EB to 5 µl of the 1 nM library pool, the pool was further diluted to 50 pM loading concentration. In the meanwhile, 10 nM PhiX was diluted with EB to 1 nM in a final volume of 15-20 l. By adding 95 µl EB to 5 µl of the 1 nM PhiX, it was diluted to 50 pM too. 2 µl of 50 pM PhiX was then added to a 98 µl of 50 pM library pool for a 2% PhiX spike-in, making a total of 100 µl NGS sample.

3.5.12 NGS Sample Loading Procedure

The cartridge (Figure 3.11) and flow cell were thawed before the sample was loaded. Firstly, the cartridge was thawed in a 25°C water bath for 6 to 18 hours. The cartridge bag was positioned face up and ~2 kg weight was placed above it to prevent floating and ensured the cartridge to be completely submerged.

Figure 3.11 Illumina iSeq 100 Single-Use Cartridge

The flow cell was ready to be thawed once the cartridge had been totally thawed and the NGS sample was ready to be loaded. To avoid condensation, the flow cell was thawed for 10 to 15 minutes before loading the NGS sample into the cartridge. To mix reagents within the cartridge, the iSeq 100 cartridge was inverted five times. After that, the cartridge was tapped five times on the benchtop to dislodge any air bubbles that may have developed during the inversion process. The loading reservoir's lid was then teared using a pipette tip. The bottom reservoir was then pipetted with a 20 µl library (Figure 3.12). Figure 3.13 shows how the flow cell was placed into a cartridge with its upward facing side.

Figure 3.12 NGS sample was loaded into cartridge

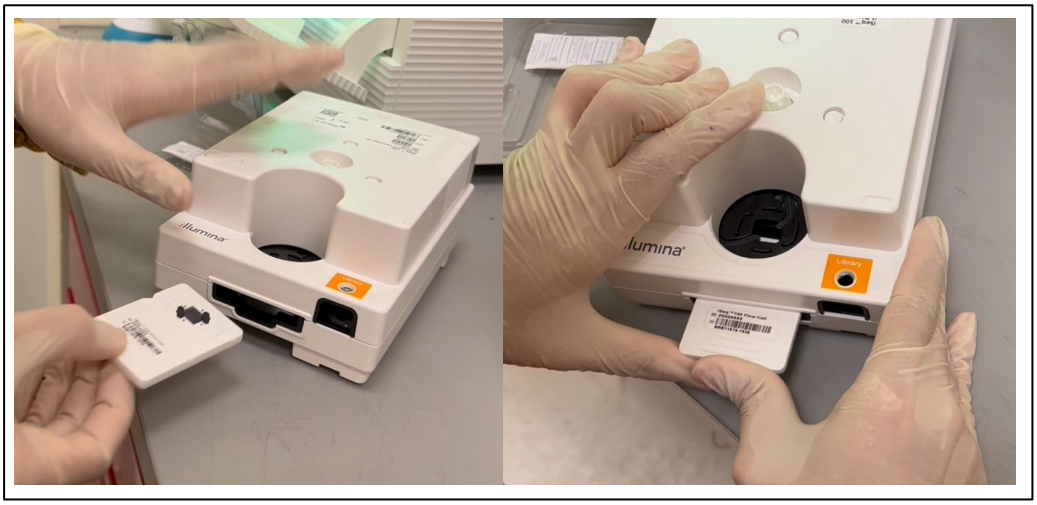

Figure 3.13 Flow cell was inserted into the cartridge prior to NGS sequencing

The cartridge was then ready for NGS sequencing run which was then placed on the iSeq 100 doc. Run was initiated using instrument control software as in Figure 3.14. The run time break down are as following:

Run time break down

- 9 to 17.5 hours
- 15 min pre run check
- 80 min mixing (ExAmp)
- 180 min – denaturation and dilution; cluster generation
- 2min/cycle – read 1 sequencing
- 110 min – indexing
- 2 min/cycle – reads 2 sequencing
- 25 min – post run purge

Figure 3.14 The NGS sequencing process began, and the screen displayed the estimated completion time

3.5.13 Bioinformatics analysis

Raw sequencing data were converted into FASTQ format [Figure 3.15 (a)] and analysed using a custom pipeline. The sequences were aligned to GRCh37/hg19 human reference genome and sequence alignment and conversion to BAM file were conducted using BaseSpace Sequence Hub application (Illumina, San Diego, CA, USA) for identification of LDLR, APOB and PCSK9 exons. Variant calling for LDLR, APOB and PCSK9 genes was performed with HaplotypeCaller and GenotypeGVCFs (GATK tools), where the minimum Phred-scale confidence for a variant call was set as Q20.

The Variant Call Format (VCF) files were extracted and downloaded from BaseSpace Sequence Hub (Illumina) [Figure 3.15 (b)]. The pathogenicity of annotated variants were interpreted based on, Global Minor Allele Frequency (GMAF), in silico applications, ClinVar, LOVD database. The frequency of the global minor allele based

on 1000 Genomes values were annotated using BCFtools which at >1% and intronic variants outside exon-intron boundaries will be considered non-pathogenic and will be excluded from further analysis. Variants with minor allele frequency (GMAF) of ≤ 0.05 based on the Exome Aggregation Consortium (ExAC) browser and 1000 Genomes were considered as mutants (Mohd Nor et al., 2019). The VCF files were outsourced (BioEasy Sdn. Bhd. Malaysia) for data cleaning and bioinformatics interpretation.

Clinical significance of the variants referred from ClinVar (human reference genome (GRCh37)) were annotated using BCFtools. In silico variant effects were annotated using REVEL, SIFT and PolyPhen-2. Annotated variants were filtered for variants with any sample having MAF of 5% or below. Updated versions of the software and database used were reported. Results were annotated in a single excel file. The pathogenicity was then determined based on the American College of Medical Genetics and Genomics (ACMG) guidelines, which classified variants as pathogenic, likely pathogenic, benign, likely benign or variants of uncertain significance (VUS). Likely pathogenic and pathogenic variants were collectively referred to as PV.

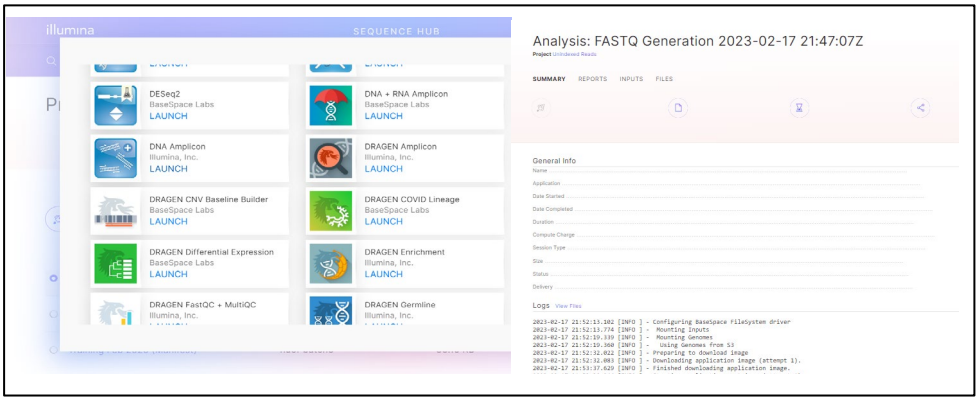

Figure 3.15 (a) FASTQ Analysed using Illumina BaseSpace

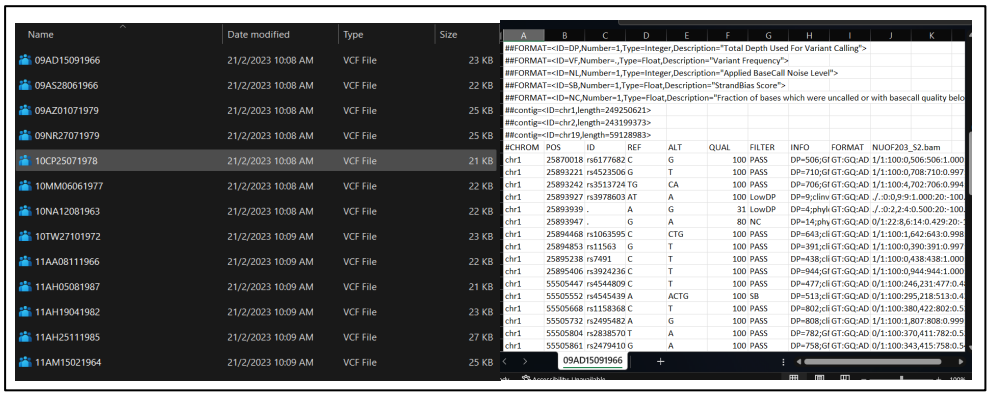

Figure 3.15 (b) VCF files were extracted using Illumina BaseSpace

3.6 Clinical Diagnostic Tools and Selection Rationale

Three established clinical diagnostic tools; DLCN, SB, and FAMCAT were applied to the study cohort to compare their diagnostic performance in identifying FH patients. The inclusion of multiple tools allowed assessment of their sensitivity and concordance with genetic findings, thereby strengthening the evaluation of FH diagnosis within the Malaysian primary care population. This approach was aligned with the overall study objectives, which aimed to investigate the genetic variants of FH-causing genes (*LDLR, APOB*, and *PCSK9*) and to determine the proportion and spectrum of genetically confirmed FH among clinically diagnosed individuals.

Comparing several clinical algorithms provided a broader understanding of their diagnostic utility and relevance to local clinical settings. However, subsequent analyses primarily focused on the DLCN criteria, as it has been widely validated, integrates both clinical and biochemical parameters, and shows a strong correlation with molecular diagnosis. Its established role as an internationally recognised standard also ensures consistency and comparability with previous studies. Therefore, the DLCN score was used as the primary clinical reference for subsequent analyses in this study.

Following the clinical classification of patients utilising these diagnostic instruments, genetic analyses were performed to identify and elucidate variants in the principal genes responsible for familial hypercholesterolaemia (FH). The following section outlines the methods used for variant detection, annotation, and interpretation based on internationally recognized guidelines.

3.7 Variant Interpretations

The cleaned and compiled VCF data were then assessed for variant pathogenicity using modified American College of Medical Genetics (ACMG) criteria . The ACMG guideline categorises variations based on evidence categories such as medical history, demographic, computational, functional, and segregation data. Using the ClinVar and Leiden Open-source Variation Database (LOVD) websites, functional studies and previously published data were located and evaluated. In silico prediction was performed using SIFT, REVEL and Polyphen2. Table 3.12 showed each accessible evidence was classified as “Very Strong”, “Strong”, “Moderate”, or “Supporting evidence” and Table 3.13 showed the variations were categorised as 'pathogenic' (PV), ‘likely pathogenic’ (LP), 'benign variant' (BV), ‘likely benign’ (LB) or 'variant of

undetermined significance' (VUS) based on the quantity, strength of the evidence gathered and evaluations' consensus from all of the researchers involved.

Table 3.12
Classification criteria for PV. Source: Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the ACMG (Richards et al., 2015).

Evidence	Category
Very strong	**PVS1** - null variant (nonsense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single or multiexon deletion) in a gene where LOF is a known mechanism of disease
Strong	**PS1** - Same amino acid change as a previously established PV regardless of nucleotide change Example: Val→Leu caused by either G>C or G>T in the same codon **PS2** - De novo (both maternity and paternity confirmed) in a patient with the disease and no family history **PS3** - Well-established in vitro or in vivo functional studies supportive of a damaging effect on the gene or gene product **PS4** - The prevalence of the variant in affected individuals is significantly increased compared with the prevalence in controls
Moderate	**PM1** - Located in a mutational hot spot and/or critical and well-established functional domain (e.g., active site of an enzyme) without benign variation **PM2** - Absent from controls (or at extremely low frequency if recessive) in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium **PM3** - For recessive disorders, detected in *trans* with a PV **PM4** - Protein length changes as a result of in-frame deletions/insertions in a nonrepeat region or stop-loss variants **PM5** - Novel missense change at an amino acid residue where a different missense change determined to be PV has been seen before

Supporting	**PP1** - Cosegregation with disease in multiple affected family members in a gene definitively known to cause the disease **PP2** - Missense variant in a gene that has a low rate of benign missense variation and in which missense variants are a common mechanism of disease **PP3** - Multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact, etc.) **PP4** - Patient's phenotype or family history is highly specific for a disease with a single genetic etiology **PP5** - Reputable source recently reports variant as pathogenic, but the evidence is not available to the laboratory to perform an independent evaluation

Table 3.13
Combination rules for classifying sequence variants. Source: Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the ACMG (Richards et al., 2015).

Evidence	Category
Pathogenic (PV)	1) 1 Very strong (PVS1) AND a) ≥1 Strong (PS1–PS4) OR b) ≥2 Moderate (PM1–PM6) OR c) 1 Moderate (PM1–PM6) and 1 supporting (PP1–PP5) OR d) ≥2 Supporting (PP1–PP5) 2) ≥2 Strong (PS1–PS4) OR 3) 1 Strong (PS1–PS4) AND a) ≥3 Moderate (PM1–PM6) OR b) 2 Moderate (PM1–PM6) AND ≥2 supporting (PP1–PP5) OR c) 1 Moderate (PM1–PM6) AND ≥4 supporting (PP1–PP5)
Likely pathogenic (LP)	1) 1 Very strong (PVS1) AND 1 moderate (PM1–PM6) OR 2) 1 Strong (PS1–PS4) AND 1–2 moderate (PM1–PM6) OR 3) 1 Strong (PS1–PS4) AND ≥2 supporting (PP1–PP5) OR 4) ≥3 Moderate (PM1–PM6) OR 5) 2 Moderate (PM1–PM6) AND ≥2 supporting (PP1–PP5) OR 6) 1 Moderate (PM1–PM6) AND ≥4 supporting (PP1–PP5)
Benign (BV)	1) 1 Stand-alone (BA1) OR 2) ≥2 Strong (BS1–BS4)
Likely benign (LB)	1) 1 Strong (BS1–BS4) and 1 supporting (BP1–BP7) OR 2) ≥2 Supporting (BP1–BP7)
Uncertain Significance (VUS)	1) Other criteria shown above are not met OR 2) The criteria for benign and pathogenic are contradictory

3.7.1 Clinical Variation Database (ClinVar)

ClinVar, (https://www.ncbi.nlm.nih.gov/clinvar/) is kept in a National Center for Biotechnology Information (NCBI) of the National Institutes of Health's National Library of Medicine (NLM) (NIH)-funded resource is a freely available archive for interpretations of clinically significant variants (Figure 3.16) (Landrum et al., 2014; 2016). ClinVar has a ranking system to indicate the level of quality associated with each database contribution. ClinVar is a publicly accessible public archive of human genetic variations and assessments of their disease relevance. Clinical testing laboratories, research laboratories, locus-specific databases, expert panels, and other entities make claims about the clinical significance of variants to ClinVar. Submissions must contain a description of the variation(s), the disease for which the variant was interpreted, an interpretation of the variant's clinical significance, with the option of providing mode of inheritance, and evidence for that interpretation (Landrum et al., 2018). More than two-star submission, for example, comes from ‘‘expert panel" and ‘‘practice guidelines" submitters, the most ClinGentrusted sites for variant interpretation (Shah et al., 2018).

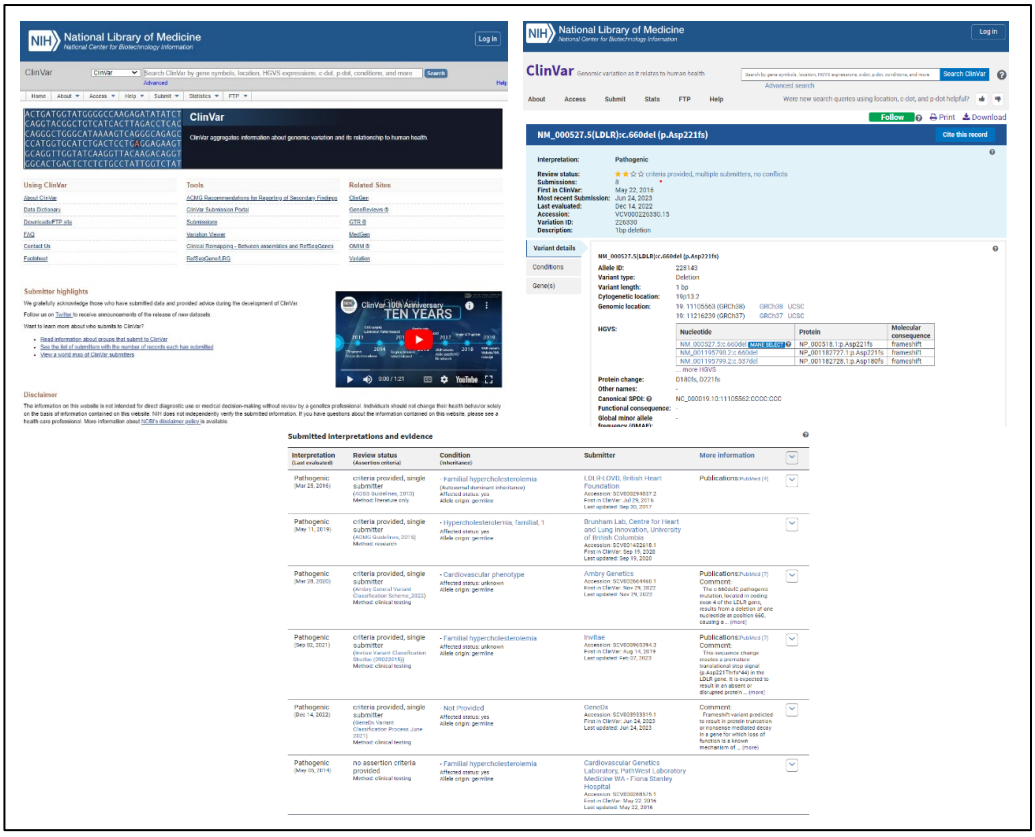

Figure 3.16 Interface of ClinVar

3.7.2 Leiden Open-source Variation Database (LOVD)

The Leiden Open-source Variation Database (LOVD) is a free web-based software (https://www.lovd.nl/) for collecting, displaying, and curating DNA variations in locus-specific databases (LSDBs) (Figure 3.17). LOVD software allows anybody to store and distribute gene variation data in a standardised database installation at their facility (Fokkema et al., 2011, 2021).

Figure 3.17 Interface of LOVD

3.7.3 Sorting Intolerant from Tolerant (SIFT)

Sorting Intolerant From Tolerant (SIFT) is a web-based tool that classifies amino acid changes based on sequence homology (AASs) and may be found at (https://sift.bii.astar.edu.sg/www/SIFT_seq_submit2.html) (Ng & Henikoff, 2001). This technique's basic assumption is based on the evolutionary conservation of amino acids within protein families. Positions with a high degree of conservation are usually intolerant to substitution, whereas those with a low degree of conservation may accept most substitutes. SIFT predicts that a replacement will have an effect on protein function if the scaled likelihood, commonly known as the SIFTscore, is less than a particular threshold number and summarizes the data into probability score resulting in the outcome of either ‘tolerated’ or ‘deleterious’. A highly conserved position is often intolerant to most replacements, whereas a weakly conserved position can accept most changes (Kumar, P., Henikoff, S., & Ng, P. C., 2009). SIFT successfully predicted 69 percent of the substitutions linked with the disease to impact protein function when applied to a dataset of mutations discovered in disease-affected people (Ng & Henikoff, 2002). When applied to a second dataset of nsSNPs in healthy individuals, SIFT predicted that only 19% of variations would alter protein function (Cargill et al., 1999).

3.7.4 The Rare Exome Variant Ensemble Learner (REVEL)

The Rare Exome Variant Ensemble Learner (REVEL) is an algorithm for predicting the pathogenicity of uncommon missense variants using an ensemble method. REVEL is an ensemble method for estimating the pathogenicity of missense variants that uses scores from 13 different tools: MutPred, FATHMM v2.3, VEST 3.0, PolyPhen-2, SIFT, PROVEAN, MutationAssessor, MutationTaster, LRT, GERP++, SiPhy, phyloP, and phastConsult are some of the tools available (Ioannidis et al., 2016). All other meta-predictors (cADD, MetaSVM, eigen) were surpassed by REVEL, with higher positive predictive value, equivalent negative predictive value, larger yield, and better overall prediction performance (Tian et al., 2019). The REVEL score for a single missense variant can vary from 0 to 1, with higher values indicating that the variant is more likely to cause illness. Variants with a REVEL score greater than 0.5 were judged to be 'likely disease causing' (Ioannidis et al., 2016).

3.7.5 Polymorphism Phenotyping 2 (PolyPhen2)

PolyPhen-2 is a computer programme that predicts the effect of an amino acid change on the structure and function of a human protein. This type of automated prediction is critical for understanding huge datasets of uncommon genetic variations, which have several uses in current human genetics research (Adzhubei et al., 2010). The PolyPhen-2 prediction pipeline produces a prediction of probably detrimental, perhaps destructive, or benign, as well as a numerical score ranging from 0.0 (benign) to 1.0 (harmful) (damaging). It is difficult to assess the accuracy of PolyPhen-2 predictions in a systematic manner, but we anticipate that they will be relatively accurate in general (Adzhubei et., 2013).

3.8 Data Analysis

IBM SPSS Statistics Version 26 was used to analyse the data (IBM, NY, USA). For continuous variables, the data were shown as means ± standard deviation (SD), and for categorical variables, as percentages. Chi-square test was used to assess the significance of differences between categorical variables. Every final analysis that had a p-value less than 0.05 was deemed statistically significant.

CHAPTER 4

RESULTS

4.1 Proportion of clinically diagnosed Familial Hypercholesterolaemia

Based on 310 recruited patients, the proportion of clinically diagnosed with Familial Hypercholesterolaemia (FH) definite, probable, and possible FH was 97.4% by Dutch Lipid Clinic Network (DLCN), 83.9% definite and possible FH by Simon Broome (SB), 47.4% yes FH by Familial Hypercholesterolaemia Case Ascertainment Tool (FAMCAT). In this cohort (n=310), 51.6% were males and 48.4% were females and the average age of patients with FH was 45.3±9.4. The presence of existing personal premature coronary artery disease (PCAD) (10.5%) and a family history of PCAD (6.3%) were in higher proportions in the; potential FH by DLCN (57.6%) compared to the Possible FH (58.6%) by SB and Yes FH by FAMCAT (65.3%) respectively (Table 4.1). Based on DLCN, Potential FH (Definite and Probable FH) cases were reported among 67.7%, Possible FH 29.7% and Unlikely FH 2.6%. Definite FH, Possible FH and Unlikely FH by SB cases were reported among 6.8%, 77.1% and 16.1%. Meanwhile, according to FAMCAT, Yes FH and No FH were reported about 47.4% and 52.6%. All these recruited patients (n=310) were referred for targeted Next-Generation Sequencing (NGS) (Figure 4.1).

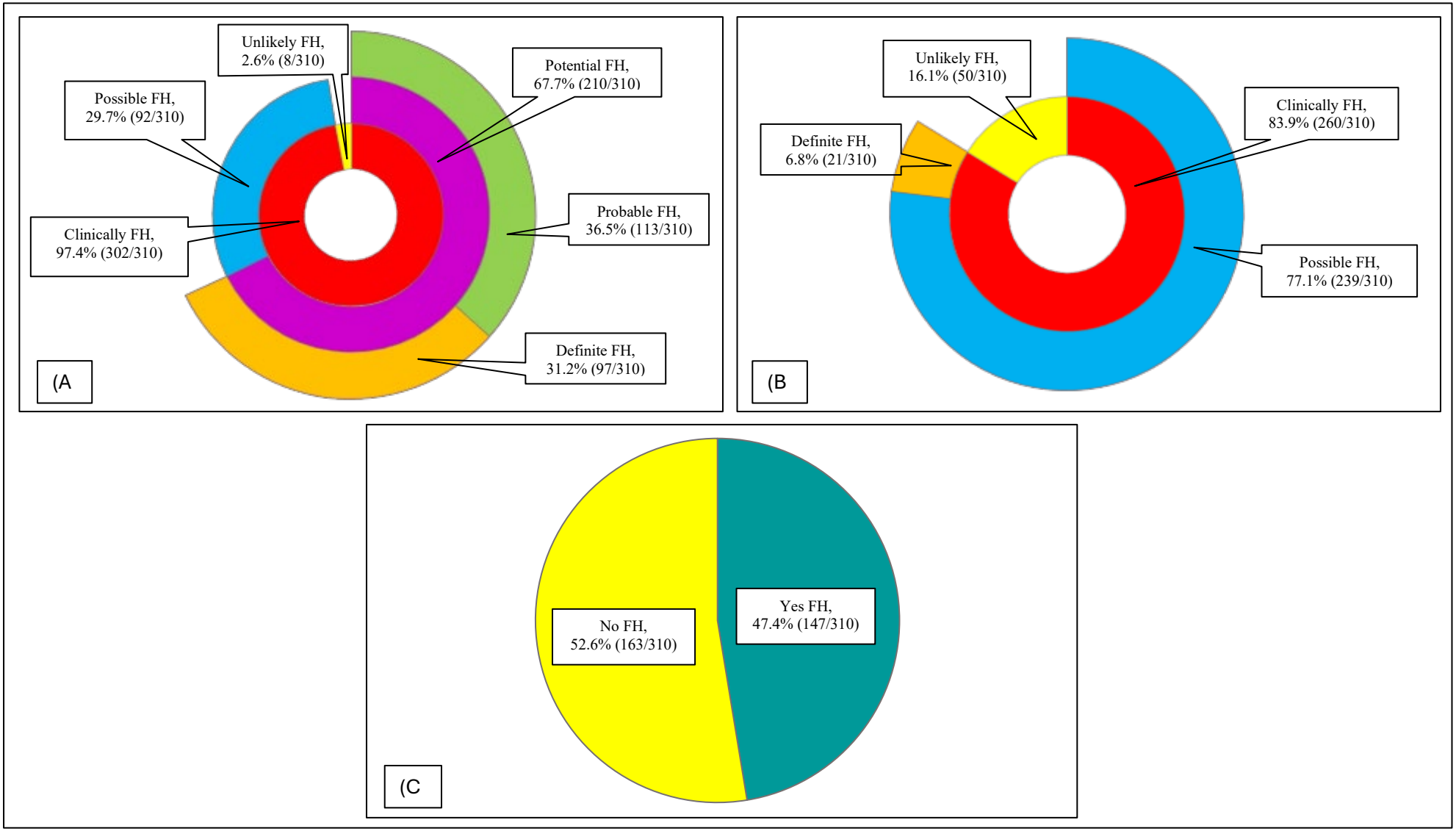

Figure 4.1 Proportion of clinically FH according to (A) DLCN, (B) SB and (C) FAMCAT (n=310)

Table 4.1
Demographic characteristics of patients according to each of category DLCN, SB and FAMCAT criteria (n=310)

Variables	All clinically diagnosed FH patients (n=310)	DLCN (n=310)			SB (n=310)			FAMCAT (n=310)	
		Potential FH (n=210)	Possible FH (n=92)	Unlikely FH (n=8)	Definite FH (n=21)	Possible FH (n=239)	Unlikely FH (n=50)	Yes FH (n=147)	No FH (n=163)
Age (years old)	45.3±9.4	44.6±9.2	46.9±9.6	47.0±10.8	45.4±9.0	46.0±9.5	42.2±8.5	43.9±10.0	46.6±8.7
Gender, % (n)									
Male	51.6% (160)	57.1% (120)	39.1% (36)	50.0% (4)	33.3% (7)	49.4% (118)	70.0% (35)	49.7% (73)	53.4% (87)
Female	48.4% (150)	42.9% (90)	60.9% (56)	50.0% (4)	66.7% (14)	50.6% (121)	30.0% (15)	50.3% (74)	46.6% (76)
Ethnicity, % (n)									
Malay	74.5% (231)	75.7% (159)	71.7% (66)	75.0% (6)	76.2% (16)	74.5% (178)	74.0% (37)	76.9% (113)	72.4% (118)
Chinese	11.3% (35)	10.0% (21)	14.1% (13)	12.5% (1)	19.0% (4)	11.3% (27)	8.0% (4)	10.2% (15)	12.3%20)
Indian & Others	14.2% (44)	14.3% (30)	14.1% (13)	12.5% (1)	4.8% (1)	14.2% (34)	19.0% (9)	13.0% (19)	15.3% (25)
Smoker, % (n)									
Current smoker	14.9% (46)	16.6% (35)	10.9% (10)	12.5% (1)	4.8% (1)	13.4% (32)	26.0% (13)	15.7% (23)	14.1% (23)
Ex-smoker	17.1% (53)	18.1% (38)	16.3% (15)	0.0% (0)	14.3% (3)	18.4% (44)	12.0% (6)	11.6% (17)	22.1% (36)
Non-smoker	68.1% (211)	65.2% (137)	72.8% (67)	87.5% (7)	81.0% (17)	68.2% (163)	62.0% (31)	72.8% (107)	63.8% (104)
Alcohol drinker, % (n)									
Current alcohol drinker	8.4% (26)	9.0% (19)	7.6% (7)	0.0% (0)	9.5% (2)	8.8% (21)	6.0% (3)	5.4% (8)	11.0% (18)
Ex-alcohol drinker	7.4% (23)	7.1% (15)	7.6% (7)	12.5% (1)	4.8% (1)	7.9% (19)	6.0% (3)	5.4% (8)	9.2% (15)
Never drink alcohol	84.2% (261)	83.8% (176)	84.8% (78)	87.5% (7)	85.7% (18)	83.3% (199)	88.0% (44)	89.1% (131)	79.8% (130)
PCAD, % (n)	7.7% (24)	10.5% (22)	8.7% (2)	0.0% (0)	14.3% (3)	6.3% (15)	12.0% (6)	9.5% (14)	6.1% (10)
Diabetes, % (n)	29.4% (91)	31.9% (67)	22.8% (21)	37.5% (3)	47.6% (10)	26.8% (64)	34.0% (17)	27.9% (41)	30.7% (50)
Hypertension, % (n)	39.4% (122)	35.7% (75)	48.9% (45)	25.0% (2)	23.8% (5)	41.0% (98)	38.0% (19)	32.0% (47)	46.0% (75)
Family history of PCAD, % (n)	55.2% (171)	57.6% (121)	51.1% (47)	37.5% (3)	61.9% (13)	58.6% (140)	24.0% (12)	65.3% (96)	42.3% (69)

Family history of HC, % (n)	30.3% (94)	30.5% (64)	29.3% (27)	37.5% (3)	19.0% (4)	29.7% (71)	38.0% (19)	26.5% (39)	33.7% (55)
Tendon xanthomata, % (n)	5.5% (17)	8.1% (17)	0.0% (0)	0.0% (0)	71.4% (15)	0.4% (1)	2.0% (1)	6.1% (9)	4.9% (8)
Corneal arcus (<45 y/o), % (n)	39.4% (122)	57.6% (121)	1.1% (1)	0.0% (0)	52.4% (11)	34.3% (82)	58.0% (29)	36.7% (54)	41.7% (68)
Baseline Lipid Profiles									
TC (mmol/L) (±SD)	8.4±1.5	8.7±1.6	7.9±0.9	6.6±0.5	9.4±1.4	8.4±1.3	8.3±2.2	8.6±1.7	8.2±1.3
TG (mmol/L) (±SD)	1.8±1.0	1.8±1.2	1.6±0.7	1.4±0.8	1.5±1.1	1.8±0.9	1.8±1.4	1.5±0.9	2.0±1.1
LDL-c (mmol/L) (±SD)	7.1±2.4	7.7±2.7	5.9±0.7	4.6±0.3	9.6±3.2	7.0±2.3	6.7±2.2	7.8±2.7	6.5±2.0
HDL (mmol/L) (±SD)	1.4±1.0	1.4±1.2	1.3±0.5	1.5±0.3	1.4±0.4	1.4±1.2	1.3±0.4	1.4±1.4	1.3±0.5

Data presented as percentage (%) and number (n) for categorical data and mean ± standard deviation for continuous data.
SD = Standard deviation; PCAD = Premature coronary artery disease; HC = Hypercholesterolaemia; TC = Total cholesterol; TG = Triglyceride; LDL-C = Low-density lipoprotein cholesterol; HDL-C = High-density lipoprotein cholesterol

4.2 Patients with pathogenic variants (PV)

Overall, 27.7% of FH patients found to carry at least one ≥1 PV (*LDLR*, *APOB* and *PCSK9*), and 72.3% have no PV (Table 4.2). As shown in Table 4.3, variants in *LDLR, APOB*, and *PCSK9* were identified among clinically diagnosed FH patients. Of the total cohort (n = 310), 8.1% had PV only, 53.9% had VUS only, 19.7% carried both PV and VUS, and 18.4% had no variants detected. Most PVs occurred in *LDLR*, consistent with its role as the predominant FH-causing gene, followed by fewer variants in *APOB* and *PCSK9*. These results highlight the genetic heterogeneity of the cohort and the value of multigene testing for precise molecular diagnosis.

Table 4.2
Summary of Clinically FH Patients According to the Presence of PV (n=310)

Pathogenic variants	**All clinically diagnosed FH patients (n=310)**
At least 1 PV	27.7% (86/310)
No PV at all	72.3% (224/310)
Total	100.0% (310/310)

Table 4.3
Summary of clinically FH patients (n=310) according to the presence of all gene variants (PV/ VUS)

Patients with PV/VUS	All clinically diagnosed FH patients (n=310)	DLCN (n=310)			SB (n=310)			FAMCAT (n=310)	
		Potential FH (n=210)	Possible FH (n=92)	Unlikely FH (n=8)	Definite FH (n=21)	Possible FH (n=239)	Unlikely FH (n=50)	Yes FH (n=147)	No FH (n=163)
PV only	8.1% (25/310)	8.6% (18/210)	6.5% (6/92)	12.5% (1/8)	23.8% (5/21)	7.9% (19/239)	2.0% (1/50)	10.9% (16/147)	5.5% (9/163)
VUS only	53.9% (167/310)	51.9% (109/210)	60.9% (56/92)	25.0% (2/8)	28.6% (6/21)	54.0% (129/239)	64.0% (32/50)	47.6% (70/147)	59.5% (97/163)
Have both PV/VUS	19.7% (61/310)	21.9% (46/210)	14.1% (13/92)	25.0% (2/8)	33.3% (7/21)	20.0% (47/239)	14.0% (7/50)	25.2% (37/147)	14.8% (24/163)
No PV/VUS	18.4% (57/310)	17.6% (37/210)	18.5% (17/92)	37.5% (3/8)	14.3% (3/21)	18.4% (44/239)	20.0% (10/50)	16.3% (24/147)	20.2% (33/163)
TOTAL	100.0% (310/310)	100.0% (210/210)	100.0% (92/92)	100.0% (8/8)	100.0% (21/21)	100.0% (239/239)	100.0% (50/50)	100.0% (147/147)	100.0% (163/163)

As shown in Table 4.4 the DLCN criteria identified the highest proportion of individuals within the Potential and Possible FH categories (97.4%), with only a small fraction classified as Unlikely FH (2.6%). Notably, most PV carriers were concentrated within the Potential and Possible FH groups, reinforcing the diagnostic strength of DLCN in distinguishing genetically confirmed cases. Although PVs were predominantly detected among [Definite and Probable DLCN (Potential FH)] classifications, a subset was unexpectedly present in the Unlikely group of DLCN, suggesting a small possible underestimation of FH risk by this tool. In contrast, SB classified a smaller proportion as Definite or Possible FH (83.9%) and a higher number as Unlikely FH (16.1%), indicating rather reduced sensitivity compared to DLCN. FAMCAT demonstrated the lowest alignment with genetic findings, with nearly equal distribution between FH (47.4%) and non-FH (52.6%) classifications. Collectively, these findings highlight the superior discriminative ability of DLCN in detecting genetically confirmed FH within the study population, compared with SB and FAMCAT.

Table 4.4
Percentage of patients with PV and without PV among subgroup of patients with clinical FH based on DLCN, SB, and FAMCAT.

Diagnostic criteria Tools	All clinically diagnosed FH patients, %(N)	Total of patients with FH PV, %(N)	Total of patients without FH PV, %(N)
Potential FH	67.7% (210/310)	62.2% (64/210)	137.7% (146/210)
Possible FH	29.7% (92/310)	20.7% (19/92)	79.3% (73/92)
Unlikely FH	2.6% (8/310)	37.5% (3/8)	62.5% (5/8)
Definite FH	6.8% (21/310)	60.0% (12/20)	45.0% (9/20)
Possible FH	77.1% (239/310)	27.6% (66/239)	72.4% (173/239)
Unlikely FH	16.1% (50/310)	16.0% (8/50)	84.0% (42/50)
Yes FH	47.4% (147/310)	35.4% (52/147)	64.6% (95/147)
No FH	52.6% (163/310)	20.9% (34/163)	79.1% (129/163)

FH: Familial hypercholesterolaemia; PV: Pathogenic variants; DLCN: Dutch Lipid Clinic Network criteria; SB: Simon Broome's criteria; FAMCAT: Familial Hypercholesterolemia Case Ascertainment Tool

Table 4.5
Total of Positive Clinically FH Patients with PV According to DLCN, SB and FAMCAT

Diagnostic Criteria Tools	Total of Positive FH Patients with PV, % (N)
DLCN	26.8% (83/310)
SB	25.2% (78/310)
FAMCAT	16.8% (52/310)

FH: Familial hypercholesterolaemia; PV: Pathogenic variants; DLCN: Dutch Lipid Clinic Network criteria; SB: Simon Broome's criteria; FAMCAT: Familial Hypercholesterolemia Case Ascertainment Tool

Table 4.5 illustrates the percentage of positive FH patients, according to each diagnostic tool, who were identified as carrying a PV. Patients were classified as positive for FH based on classification; DLCN score of ≥6 (corresponding to "Definite" or "Probable" FH, collectively referred to as "Potential FH"), those categorized as "Definite" or "Possible" FH by the SB criteria, or those with a FAMCAT relative risk score >1, indicating an increased likelihood of FH. Conversely, patients who did not meet any of these thresholds were considered not positive for FH, defined as having a

FAMCAT relative risk score <1, “Unlikely FH” by SB criteria, and a DLCN score <3 (classified as “Unlikely FH” by DLCN criteria).

This represents the rate of genetic confirmation among clinically positive FH cases, with DLCN showing 26.8%, SB showing 25.2%, and FAMCAT showing 16.8%. The figure demonstrates that the DLCN tool identified the highest proportion of genetically confirmed FH patients with pathogenic variants, followed closely by the SB tool, while the FAMCAT tool showed the lowest detection rate. This finding suggests that the DLCN and SB criteria have a stronger correlation with genetic confirmation compared to FAMCAT in identifying true FH cases.

Table 4.6
Number of genetically confirmed FH patients with PV in *LDLR* and/or *APOB* genes related to FH.

PV	All clinically diagnosed FH patients (n=310)	DLCN (n=310)			SB (n=310)			FAMCAT (n=310)	
		Potential FH (n=210)	Possible FH (n=92)	Unlikely FH (n=8)	Definite FH (n=21)	Possible FH (n=239)	Unlikely FH (n=50)	Yes FH (n=147)	No FH (n=163)
***LDLR* (hetero)**	13.2% (41/310)	15.2% (32/210)	9.8% (9/92)	0.0% (0/8)	38.1% (8/21)	13.0% (31/239)	4.0% (2/50)	20.4% (30/147)	6.7% (11/163)
***LDLR* (homo) + *LDLR* (homo)**	0.3% (1/310)	0.5% (1/210)	0.0% (0/92)	0.0% (0/8)	4.8% (1/21)	0.0% (0/239)	0.0% (0/50)	0.7% (1/147)	0.0% (0/163)
***LDLR* (homo) + *LDLR* (hetero)**	0.3% (1/310)	0.0% (0/210)	1.1% (1/92)	0.0% (0/8)	0.0% (0/21)	0.4% (1/239)	0.0% (0/50)	0.0% (0/147)	0.6% (1/163)
***LDLR* (hetero) + *LDLR* (hetero)**	1.3% (4/310)	1.9% (4/210)	0.0% (0/92)	0.0% (0/8)	0.0% (0/21)	1.3% (3/239)	2.0% (1/50)	1.4% (2/147)	1.2% (2/163)
***LDLR* (hetero) + *APOB* (hetero)**	1.3% (4/310)	1.4% (3/210)	1.1% (1/92)	0.0% (0/8)	4.8% (1/21)	1.3% (3/239)	0.0% (0/50)	1.4% (2/147)	1.2% (2/163)
***APOB* (hetero)**	9.4% (29/310)	10.0% (21/210)	5.4% (5/92)	37.5% (3/ 8)	9.5% (2/21)	9.2% (22/239)	10.0% (5/50)	10.2% (15/147)	8.6% (14/163)
***APOB* (homo)**	0.3% (1/310)	0.0% (0/210)	1.1% (1/92)	0.0% (0/8)	0.0% (0/21)	0.4% (1/239)	0.0% (0/50)	1.4% (2/147)	0.0% (0/163)
***APOB* (hetero) + *APOB* (hetero)**	1.6% (5/310)	1.4% (3/210)	2.2% (2/92)	0.0% (0/8)	0.0% (0/21)	2.1% (5/239)	0.0% (0/50)	0.7% (1/147)	1.8% (3/163)
Total individuals	27.7% (86/310)	30.5% (64/210)	20.7% (19/92)	37.5% (3/8)	57.1% (12/21)	27.6% (66/239)	16.0% (8/50)	36.1% (53/147)	20.2% (33/163)

FH: Familial hypercholesterolaemia; PV: Pathogenic variants; *LDLR*: Low-density lipoprotein receptor gene; *APOB*: Apolipoprotein B gene; *PCSK9*: Proprotein convertase subtilisin/kexin type 9 gen

Table 4.6 shows that there were eighty-six patients that have PV in *LDLR* and *APOB* among 310 FH patients. This cohort did not have any *PCSK9* mutation, hence the gene mutation is not included in Table 4.5. More than half of the patients have PV in *LDLR* gene which is about 51 patients (59.3%) and including four of them have double heterozygous PV of *LDLR* and *APOB* gene. Followed by another 40.7% patients (thirty-five) have PV in *APOB* gene consisting of *APOB* alone (heterozygote/homozygote) and *APOB* compound heterozygote.

4.3 Frequency of Variants among 310 patients with complete analysis

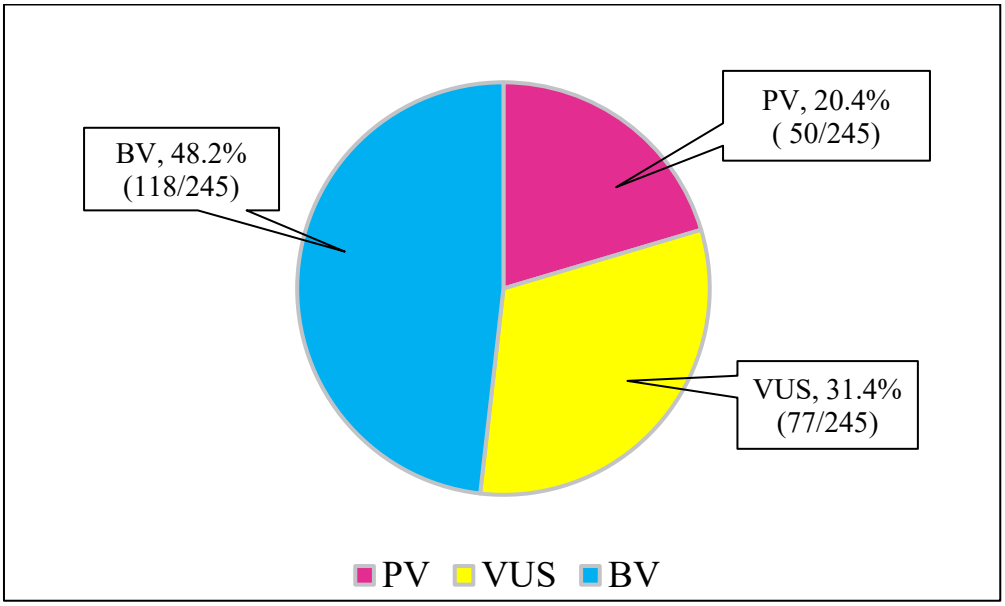

Figure 4.2 Variant Distribution Among 310 clinically FH Patients (n=245)

Based on Figure 4.2, PV of *LDLR, APOB*, and *PCSK9* were sequenced among all 310 clinically diagnosed FH patients. A total of 245 different variants were identified but only 50 of them were classified as PV while 77 and 118 were classified as VUS and benign, respectively, according to American College of Medical Genetics and Genomics (ACMG) criteria. The ACMG criteria were applied to ensure standardized and evidence-based classification of genetic variants. This guideline was chosen because it provides a globally recognised framework that integrates multiple lines of evidence, such as population data, computational prediction, and functional studies, allowing consistent and clinically relevant interpretation of variant pathogenicity. Among the PV, 32 (64.0%), and 18 (36.0%) were from *LDLR*, *APOB* and none were detected for *PCSK9* PV respectively. Table 4.6 shows the most frequent variants that

were identified among overall variants were *APOB*, 44.1% followed by *LDLR,* 33.1% and *PCSK9*, 22.9% .

Table 4.7 Distribution of variants identified in 310 clinically diagnosed FH patients according to the genes.

Genes	PV	VUS	PV+VUS	BV
LDLR	64.0% (32/50)	19.5% (15/77)	37.0% (47/127)	28.8% (34/118)
APOB	36.0% (18/50)	54.5% (42/77)	47.2% (60/127)	40.7% (48/118)
PCSK9	0.0% (0/50)	26.0% (20/77)	15.7% (20/127)	30.5% (36/118)
Total	100.0% (50/50)	100.0% (77/77)	100.0% (127/127)	100.0% (118/118)

LDLR: Low-density lipoprotein receptor gene; *APOB*: Apolipoprotein B gene; *PCSK9*: Proprotein convertase subtilisin/kexin type 9 gene; PV = Pathogenic variant; VUS = Variant of unknown significance; BV = Benign Variant.

Table 4.7 shows the details of each PV in *LDLR* and *APOB* which were identified among clinically diagnosed FH patients in this study. No PV in *PCSK9* gene was identified. A total of 50 PVs were identified overall, 32 were from *LDLR* and 18 from *APOB*. Among the 32 *LDLR* PV, 22 were nonsynonymous variants, one was start-lost variant, one was stop-gain variant, four were splicing site variant, two were frameshift variants, one was codon-insertion variant and one was intronic variant while in *APOB,* 17 were missense PV and one was frameshift deletion/insertion PV. Additionally, 77 variants were classified as variants of uncertain significance (VUS), and when combined, PV and VUS accounted for 127 variants, while 118 variants were classified as benign variants (BV).

Table 4.8
List of pathogenic variants identified among genetically confirmed FH patients (n=310).

	Variant			Database			*In silico* predicted effects			ACMG	Novel variant
No	**HGVS**	**Exon/ Intron**	**No of patients**	**dbSNP**	**LOVD**	**ClinVar**	**PolyPhen**	**SIFT**	**REVEL**		
LDLR nonsynonymous variants											
1	c.148G>A (A50T)	2	1	rs137853960	LB	VUS, LB	Benign	Tolerated	Likely disease causing	LP (PM1, PM2, PP2, PP4)	No
2	c.226G>C (G76R)	3	1	rs574337291	NR	VUS	Probably damaging	Deleterious	Likely disease causing	PV (PS3, PM1, PM2, PP2, PP3)	No
3	c.241C>T (R81C)	3	1	rs730882078	PV, LP	PV, LP, VUS	Probably damaging	Deleterious	Likely disease causing	LP (PM1, PM2, PP2, PP3, PP4, PP5)	No
4	c.268G>A (D90N)	3	2	rs749038326	PV, LP	PV, LP, LB	Probably damaging	Deleterious	Likely disease causing	PV (PS3, PM1, PM2, PP2, PP3, PP4, PP5)	No
5	c.580A>G (S194G)	4	2	rs373488885	NR	VUS	Benign	Tolerated	Likely benign	LP (PM1, PM2, PP2, PP4)	No

Variant				Database			*In silico* predicted effects			ACMG	Novel variant
No	**HGVS**	**Exon/ Intron**	**No of patients**	**dbSNP**	**LOVD**	**ClinVar**	**PolyPhen**	**SIFT**	**REVEL**		
6	c.622G>A (E208K)	4	1	rs879254597	PV, LP	PV, LP	Probably damaging	Deleterious	Likely disease causing	PV (PS4, PM1, PM2, PP2, PP3, PP4, PP5)	No
7	c.763T>A (C255S)	5	3	rs879254668	LP	LP	Possibly damaging	Deleterious	Likely disease causing	LP (PM1, PM2, PP3, PP4, PP5)	No
8	c.769C>T (R257W)	5	2	rs200990725	LP, VUS, LB	PV, VUS, LB	Probably damaging	Deleterious	Likely disease causing	LP (PM1, PM2, PP1, PP2, PP3, PP4, PP5)	No
9	c.910G>A (D304N)	6	1	rs121908030	PV, LP	PV, LP	Possibly damaging	Tolerated	Likely disease causing	PV (PS3, PM1, PM2, PP2, PP3, PP4, PP5)	No
10	c.938G>A (C313Y)	6	1	rs875989911	PV, LP	PV, LP	Probably damaging	Deleterious	Likely disease causing	LP (PM1, PM2, PP2, PP3, PP4, PP5)	No
11	c.1066G>A (D356N)	8	1	rs767767730	LP, LB	VUS, LB	Possibly damaging	Tolerated	Likely benign	LP (PM1, PM2, PP2, PP4, PP5)	No

Variant					Database					ACMG	Novel variant
No	**HGVS**	**Exon/ Intron**	**No of patients**	**dbSNP**	No	**HGVS**	**Exon/ Intron**	**No of patients**	**dbSNP**	No	**HGVS**
12	c.1246C>T (R416W)	9	1	rs570942190	PV	PV, LP	Probably damaging	Deleterious	Likely disease causing	PV (PS3, PM1, PM2, PP2, PP3, PP4, PP5)	No
13	c.1247G>A (R416Q)	9	1	rs773658037	PV, LP	PV, LP, VUS	Probably damaging	Deleterious	Likely disease causing	LP (PM1, PM2, PP1, PP2, PP3, PP4, PP5)	No
14	c.1284C>G (N428K)	9	7	rs368708058	PV, VUS	VUS	Probably damaging	Deleterious	Likely disease causing	LP (PM1, PM2, PP2, PP3, PP4, PP5)	No
15	c.1462A>G (I488V)	10	1	-	NR	NR	Benign	Deleterious	Likely disease causing	LP (PM2, PM5, PP2, PP4)	Yes
16	c.1720C>T (R574C)	12	1	rs185098634	LP	VUS	Probably damaging	Deleterious	Likely disease causing	LP (PM2, PP2, PP3, PP4, PP5)	No
17	c.1747C>T (H583Y)	12	2	rs730882109	LP, P	PV, LP, VUS, LB	Probably damaging	Deleterious	Likely disease causing	LP (PM2, PP2, PP3, PP4, PP5)	No
18	c.1765G>A (D589N)	12	2	rs201971888	VUS, LP	VUS	Probably damaging	Tolerated	Likely benign	LP (PM2, PP1, PP2, PP4, PP5)	No

	Variant				Database		*In silico* predicted effects			ACMG	Novel variant
No	**HGVS**	**Exon/ Intron**	**No of patients**	**dbSNP**	**LOVD**	**ClinVar**	**PolyPhen**	**SIFT**	**REVEL**		
19	c.1867A>G (I623V)	13	1	rs555292896	LB	VUS, BV, LB	Benign	Tolerated	Likely disease causing	LP (PM1, PM2, PP2, PP4)	No
20	c.1946C>T (P649L)	13	1	rs879255081	LP, VUS	LP, VUS	Benign	Deleterious	Likely disease causing	LP (PM1, PM2, PP2, PP3, PP4, PP5)	No
21	c.1855T>C (F619L)	13	1	rs747134711	LP	LP	Benign	Deleterious	Likely disease causing	PV (PS3, PS4, PM2, PP2, PP3, PP4, PP5)	No
22	c.862G>A (E288K)	6	1	rs368657165	LP	PV	Probably damaging	Deleterious	Likely disease causing	PV (PS3, PS4, PM1, PM2, PP2, PP3, PP4, PP5)	No
LDLR splice site variant											
23	c.1187-2A>G	8	3	rs879254823	LP, P	LP, P	NA	NA	NA	PV (PVS1, PM1, PM2, PP4, PP5)	No

	Variant				Database		*In silico* predicted effects			ACMG	Novel variant
No	**HGVS**	**Exon/ Intron**	**No of patients**	**dbSNP**	**LOVD**	**ClinVar**	**PolyPhen**	**SIFT**	**REVEL**		
24	c.1358+2T>G	9	1	-	NR	NR	NA	NA	NA	PV (PVS1, PM1, PM2, PP2, PP4)	Yes
25	c.2548-1_2548delGAinsTC	18	2	-	NR	NR	NA	NA	NA	PV (PVS1, PM2, PP4)	Yes
26	c.313+2dup	3	1	rs875989897	NR	PV	NA	NA	NA	PV (PVS1, PS3, PS4, PM2, PP4, PP5)	No
LDLR intron variants											
27	c.190+4A>T	2	1	rs769446356	VUS, P	LP	NA	NA	NA	PV (PS3, PM2, PP4, PP5)	No

	Variant				Database		*In silico* predicted effects			ACMG	Novel variant
No	**HGVS**	**Exon/ Intron**	**No of patients**	**dbSNP**	**LOVD**	**ClinVar**	**PolyPhen**	**SIFT**	**REVEL**		
LDLR frameshift variant											
28	c.660del (G219)	4	4	rs875989905	NR	PV	NA	NA	NA	PV (PVS1, PM1, PM2, PP2, PP4, PP5)	No
29	c.416A>C (D139D?)	4	1	-	NR	NR	NA	NA	NA	PV (PVS1, PM1, PM2, PP4)	Yes
LDLR start loss variant											
30	c.2T>A (M1K)	1	2	-	NR	NR	Possibly damaging	Deleterious	Likely disease causing	PV (PVS1, PP3, PP4)	Yes
LDLR stop gained variant											

	Variant				Database		*In silico* predicted effects			ACMG	Novel variant
No	**HGVS**	**Exon/ Intron**	**No of patients**	**dbSNP**	**LOVD**	**ClinVar**	**PolyPhen**	**SIFT**	**REVEL**		
31	c.460C>T (Q154*)	3	1	rs879254534	PV	PV	NA	NA	NA	PV (PVS1, PS1, PM1, PM2, PP4, PP5)	No
LDLR codon insertion variant											
32	c.2556_2557ins TCAGTCTGG (M852MSVW)	18	2	-	NR	NR	NA	NA	NA	LP (PM1, PM2, PM4, PP4)	Yes
APOB nonsynonymous variants											
1	c.1400C>G (A467G)	11	6	rs376602710	LB	VUS	Probably damaging	Deleterious	Likely disease causing	LP (PM2, PP1, PP3, PP4, PP5)	No
2	c.1559C>T (S520L)	12	5	-	NR	NR	Probably damaging	Deleterious	Likely disease causing	LP (PM2, PP3, PP4)	Yes

	Variant			Database			*In silico* predicted effects		ACMG	Novel variant
No.	**HGVS**	**Exon/ Intron**	**No of patients**	**dbSNP**	**LOVD**	**ClinVar**	**PolyPhen**	**SIFT**	**REVEL**	
3	c.11383G>A (D3795N)	26	11	rs759284932	NR	NR	Possibly damaging	Deleterious	Likely benign	LP (PM1, PM2, PP3, PP4)	No
4	c.11356C>T (L3786F)	26	1	rs571485213	VUS	VUS, LB	Probably damaging	Deleterious	Likely benign	LP (PM1, PM2, PP3, PP4	No
5	c.10978G>A (D3660N)	26	2	-	NR	NR	Probably damaging	Deleterious	Likely benign	LP (PM1, PM2, PP3, PP4)	Yes
6	c.10579C>T (R3527W)	26	3	rs144467873	PV	PV, LP, VUS	Probably damaging	Deleterious	Likely disease causing	PV (PS3, PM1, PM2, PP3, PP4, PP5)	No
7	c.8462C>T (P2821L)	26	4	rs72653095	B, VUS	PV, LP, VUS	Probably damaging	Deleterious	Likely benign	LP (PM1, PM2, PP3, PP4)	No
8	c.4951G>A (G1651R)	26	3	rs748424949	VUS	NR	Possibly damaging	Deleterious	Likely benign	LP (PM1, PM2, PP1, PP3, PP4)	No

	Variant			Database			*In silico* predicted effects			ACMG	Novel variant
No.	**HGVS**	**Exon/ Intron**	**No of patients**	**dbSNP**	**LOVD**	**ClinVar**	**PolyPhen**	**SIFT**	**REVEL**		
9	c.7619G>T (G2540V)	26	1	rs571626569	B	B, LB, VUS	Possibly damaging	Deleterious	Likely benign	LP (PM1, PM2, PP3, PP4)	No
10	c.6655C>T (R2219C)	26	1	rs141641980	PV, LB	VUS	Benign	Deleterious	Likely benign	LP (PM1, PM2, PP4, PP5)	No
11	c.11953G>A (D3985N)	28	1	rs772544842	LP	VUS	Possibly damaging	Deleterious	Likely benign	LP (PM2, PP3, PP4, PP5)	No
12	c.12814C>G (L4272V)	29	1	-	NR	NR	Benign	Tolerated	Likely benign	LP (PM1, PM2, PP4)	Yes
13	c.10094A>T (H3365L)	26	1	-	VUS	NR	Probably damaging	Deleterious	Likely benign	LP (PM1, PM2, PP3, PP4)	Yes
14	c.9937C>G (L3313V)	26	1	-	VUS	NR	Probably damaging	Deleterious	Likely benign	LP (PM1, PM2, PP3, PP4)	Yes

Variant				Database			*In silico* predicted effects			ACMG	Novel variant
No.	**HGVS**	**Exon/ Intron**	**No of patients**	**dbSNP**	**LOVD**	**ClinVar**	**PolyPhen**	**SIFT**	**REVEL**		
15	c.7684A>C (T2562P)	26	1	-	NR	NR	Possibly damaging	Deleterious	Likely benign	LP (PM1, PM2, PP3, PP4)	Yes
16	c.10093C>T (H3365Y)	26	1	-	NR	NR	Probably damaging	Deleterious	Likely benign	LP (PM1, PM2, PP3, PP4)	Yes
17	c.9337G>A (A3113T)	26	1	-	NR	NR	Possibly damaging	Deleterious	Likely benign	LP (PM1, PM2, PP3, PP4)	Yes
APOB frameshift variants											
1	c.13028_13029del (Y4343)	29	1	rs760832994	NR	VUS	NA	NA	NA	PV (PVS1, PS4, PM1, PM2, PM5, PP4, PP5)	No

LDLR: Low-density lipoprotein receptor gene; *APOB:* Apolipoprotein B gene; *PCSK9:* Proprotein convertase subtilisin/kexin type 9 gene; *LDLRAP1:* Low-density lipoprotein receptor adaptor protein 1 gene; P = Pathogenic; LP = Likely pathogenic; VUS = Variant of unknown significance; B = Benign; LB = Likely benign; NR = Not reported; NA = Not available; PVS = Very strong evidence; PS = Strong evidence; PM = Moderate evidence; PP = Supporting evidence

Table 4.8 contains a summary of novel variants. In all, fourteen novel PVs were discovered across the three major genes in this research. One new *LDLR* PV (c.1462A>G), a nonsynonymous mutation at exon 10, was discovered in one person. Furthermore, one patient has a novel splice site mutation (c.1358+2T>G) at exon 9. Another novel splice site mutation (c.2548-1_2548delGAinsTC) in exon 18 has been identified in two patients. There are two patients with a novel start loss mutation (c.2T>A) at exon 1. Then, in exon 18, this novel *LDLR* codon insertion c.2556_2557insTCAGTCTGG was discovered in two patients. Only one patient was found to have this novel *LDLR* frameshift (c.416A>C) exon 4 variant. This novel nonsynonymous *APOB* exon 12 mutation (c.1559C>T) was found in five people. In addition, two patients carry this novel nonsynonymous *APOB* mutation (c.10978G>A exon 26). Only one person carries one of these six novel nonsynonymous PVs of *APOB* (c.12814C>G, c.10094A>T, c.9937C>G, c.7684A>C, c.10093C>T, and c.9337G>A) at exon 26.

CHAPTER 5

DISCUSSION

5.1 Proportion of genetically confirmed FH amongst clinically diagnosed FH patients in primary care population

This study aimed to further assess clinically diagnosed FH patients by different clinical diagnostic criteria Dutch Lipid Clinic Network (DLCN), Simon Broome (SB), and Familial Hypercholesterolaemia Case Ascertainment Tool (FAMCAT) and determine the proportion of genetically confirmed FH in the Malaysian primary care population using NGS targeting the three FH-causing genes (*LDLR, APOB-100*, and *PCSK9*).

Clinically diagnosed FH patients were identified based on clinical features, lipid profiles, and family history of premature cardiovascular disease (PCAD). These standard clinical tools, which combine LDL-C levels, physical signs, and family history to figure out the likelihood of FH, were used to make the diagnosis. Individuals classified as Possible, Probable, or Definite FH were considered clinically diagnosed and subsequently included for genetic analysis. Although this study did not directly assess diagnostic sensitivity or specificity, it evaluated the genetic confirmation rate, defined as the proportion of clinically positive FH cases carrying pathogenic variants (PVs), serving as an indirect indicator of the diagnostic performance of each tool in a primary care setting. Comparable diagnostic systems such as DLCN, SB, FAMCAT, and MEDPED have been widely used internationally, and differences in demographics, screening techniques, and diagnostic criteria can significantly influence PV detection rates (Abdul-Razak et al., 2017; Eishwarya Liyanage, 2009; Mata et al., 2014; Razman et al., 2022; Sun et al., 2018).

Among FH patients who consented to participate in the genetic study (n = 310), at least one or more pathogenic (PV) variants were identified in 27.7%. Using DLCN as the primary diagnostic framework, 67.7 % (210/310) were classified as Potential FH, of which 30.5 % (64/210) were genetically confirmed. The DLCN system was selected for its validated, structured scoring algorithm that incorporates biochemical, clinical,

and familial indicators, providing strong correlation with molecular confirmation and making it a reliable benchmark for FH identification in population-based studies.

The relatively high proportion of clinically diagnosed Potential FH in this study, compared with the global FH prevalence of approximately 1 in 300 (0.33%) (Beheshti et al., 2020; Toft-Nielsen et al., 2022), may reflect the inclusion of participants exhibiting visible lipid stigmata and a positive family history of PCAD, which contribute to higher DLCN scores (Migliara et al., 2017). Specifically, 39.4% of participants presented with corneal arcus, while 5.5% exhibited tendon xanthomata. These clinical signs are well-recognised features of FH and substantially influence DLCN classification, often elevating individuals into the "Probable" or "Definite" FH categories. However, extra care was taken throughout the screening process to minimise overscoring—only corneal arcus diagnosed before the age of 45 was considered a positive criterion to ensure specificity, as senile corneal arcus, unrelated to lipid disorders, is common in older adults. Although tendon xanthomata were rare, their presence is highly specific for FH, and their inclusion strengthens the diagnostic accuracy of DLCN in this cohort. Due to the high cost of genetic testing, it remains uncommon as a frontline diagnostic tool in many healthcare systems (Chua et al., 2021; Pang et al., 2019; Razman et al., 2022). The inclusion of hyperlipidaemia stigmata may be the reason for the high rate of clinically diagnosed Potential FH observed in this study, such as corneal arcus or tendon xanthomata, as well as personal and family history of hypercholesterolemia and PCAD. The inclusion of these clinical signs also helps to achieve high scores in the DLCN scoring system (Migliara et al., 2017). The results of those studies showed that the likelihood of receiving a diagnosis of Potential FH was higher in those with corneal arcus and a family history of PCAD. The majority of potential FH in this study were probable people, whose DLCN scores were primarily influenced by the presence of corneal arcus and a family history of PCAD. Moreover, tendon xanthomata were present in only two of the Probable FH by DLCN patients in this present study, which increases the risk of overscoring because of corneal arcus. However, extra care was taken throughout the screening, and only patients with premature corneal arcus diagnosed before the age of 45 were considered to have positive corneal arcus. Due to the high expense of genetic testing, it is not a common diagnostic approach for identifying FH individuals in the majority of nations (Chua et al., 2021; Pang et al., 2019; Razman et al., 2022). Nonetheless, a number of investigations have

demonstrated that next-generation sequencing (NGS) is a highly dependable method to screen FH mutations (Alex et al., 2012; Al-Khateeb et al., 2013; Chua et al., 2021; Iacocca et al., 2017; Khoo et al., 2000; Razman et al., 2022; Vandrovcova et al., 2013). Millions of DNA fragments may be sequenced at once using NGS, providing detailed information on gene activity, genetic variation, genome structure, and changes in gene behaviour. NGS offers clear benefits in the diagnosis of birth abnormalities and genetic illnesses involving several genes, various variant types, and uncommon variants. It is also highly successful in discovering novel genes that cause disease (Bamshad et al., 2019; Boycott et al., 2013; Tong et al., 2022). However, NGS needs to sustain a high throughput of samples, processing a substantial number of samples daily and keeping their sequencing systems occupied with producing data to be cost-effective and efficient (Pei et al., 2023; Satam et al., 2023).

Nonetheless, next-generation sequencing (NGS) has demonstrated itself as a dependable, scalable, and sensitive method for identifying FH mutations (Alex et al., 2012; Al-Khateeb et al., 2013; Chua et al., 2021; Iacocca et al., 2017; Khoo et al., 2000; Razman et al., 2022; Vandrovcova et al., 2013). NGS allows for the simultaneous sequencing of millions of DNA fragments, giving us a complete picture of enablable gene activity, variant types, and structural changes (Bamshad et al., 2019; Boycott et al., 2013; Tong et al., 2022). However, to maintain cost-effectiveness, NGS platforms require high-throughput sample processing and consistent sequencing workloads (Pei et al., 2023; Satam et al., 2023).

In this study, a total of 245 distinct variants were detected in the three major FH genes. Of these, 50 were classified as pathogenic, 77 as variants of uncertain significance (VUS), and 118 as benign variants (BV) according to ACMG guidelines. In line with regional and global findings, LDLR and APOB PVs were the most common among clinically diagnosed FH cases (Chua et al., 2021). 13.2 % of genetically confirmed FH patients carried PVs in *LDLR*, with c.1284C>G as the most common *LDLR* mutation (found in seven patients) and c.11383G>A as the most frequent *APOB* PV (found in eleven patients). These results align with previous studies reporting *LDLR* as the most frequently mutated gene, followed by *APOB* and rarely PCSK9 (Graça et al., 2022; Huang & Charng, 2020a). In Malaysia, similar distributions were observed in Kelantan, where 29 *LDLR* variants were detected in 117 of 154 FH patients (76.0%) (Huang & Charng, 2020b), and in another cohort reporting 18 *LDLR*, 15 *APOB*, and 5 *PCSK9* PVs (Razman et al., 2022). In China, 285 unrelated FH patients revealed 84

LDLR, 31 *APOB*, and 4 *PCSK9* PVs, with an overall detection rate of 51.9 % (Sun et al., 2018). These inter-study variations highlight how population genetics, inclusion criteria, and diagnostic protocols affect variant detection.

In comparison with international data, the present study's PV detection rate of 27.7 % among DLCN-defined FH patients was lower than in other countries, including China (51.9 %) (Sun et al., 2018), Hong Kong (57.4 %) (Tan et al., 2018), Malaysia (76.6 %) (Lye et al., 2013), and India (47.0 %) (Setia et al., 2020). The discrepancy is primarily due to differences in cohort characteristics and study settings—earlier studies recruited from tertiary referral or lipid clinics, which typically involve patients with severe phenotypes, whereas the present study involved community-based primary care patients, representing a broader and less severe clinical spectrum. This distinction underscores the significance of the current study as it provides insight into FH detection within the real-world Malaysian primary care context, reflecting the genetic and clinical characteristics of the general population rather than highly selected hospital cohorts.

The PV detection rates using other diagnostic criteria, 25.2 % for SB and 16.8 % for FAMCAT, were similarly lower than those reported in Taiwan (62.5 %) (Chiou & Charng, 2012), Singapore (56.2 %) (Pek et al., 2018), and the United Kingdom (28.0 %) (Qureshi et al., 2021). However, the current proportion exceeded that of Sri Lanka, where 18.5 % of 27 clinically confirmed FH patients analysed using Sanger sequencing were found to carry PVs (Paththinige et al., 2018), suggesting that NGS provides greater detection sensitivity than conventional sequencing.

The relatively low PV detection rate in this study may also be explained by large gene rearrangements, copy-number variants, or deep-intronic mutations that are not captured by standard NGS panels, which primarily target coding regions. Such structural variants require complementary molecular assays like multiplex ligation-dependent probe amplification (MLPA) or long-read sequencing to achieve comprehensive variant detection (Iacocca et al., 2017). Despite these limitations, this study demonstrates that NGS is a powerful, high-throughput, and scalable platform for FH diagnosis, supporting its integration into Malaysia's national FH detection initiatives and contributing valuable data on the genetic epidemiology of FH in the primary care population.

5.2 Genetic spectrum and frequency of PV in FH genes amongst clinically diagnosed FH patients in primary care population

A reduction in the number or function of *LDLR* decreases the ability of hepatocytes to internalise LDL, resulting in elevated plasma LDL levels. The *LDLR* gene is essential for binding and internalising LDL particles in cells (Goldstein & Brown, 2009; Pirahanchi et al., 2023). *APOB-100*, the ligand for *LDLR*, plays a complementary role in LDL clearance (N & Feingold, 2000). The identification of gain-of-function mutations in *PCSK9* established it as the third gene responsible for autosomal dominant familial hypercholesterolaemia (FH) (Abifadel et al., 2003).

In the present study, 245 distinct variants were identified across *LDLR, APOB*, and *PCSK9* among clinically diagnosed FH participants. Of these, 50 (18.1%) were classified as pathogenic variants (PVs), 77 (30.9%) as variants of uncertain significance (VUS), and 118 (51.0%) as benign variants (BV), according to the American College of Medical Genetics (ACMG) classification criteria. Of the PVs, 64.0% were found in LDLR and 36.0% were found in APOB. No PVs were found in PCSK9. This distribution mirrors global trends where *LDLR* mutations remain the predominant genetic cause of FH. Most *LDLR* PVs detected in this study occurred within ligand-binding and EGF precursor homology domains, which are known mutation hotspots responsible for receptor function and LDL clearance efficiency. This aligns with the molecular spectrum reported in previous Malaysian and regional studies (Abdul Murad et al., 2023; Alicezah et al., 2021; Al-Khateeb et al., 2011b; Kamal et al., 2023; Nawawi et al., 2014; Razman et al., 2022), reinforcing the central pathogenic role of *LDLR* variants in Southeast Asian populations.

Several novel *LDLR* and *APOB* variants were identified, suggesting possible population-specific mutations or underexplored regional founder effects in Malaysia. Their presence among clinically diagnosed FH individuals, often with markedly elevated LDL-C and physical stigmata such as corneal arcus and tendon xanthomata, supports their pathogenic potential. Collectively, these findings highlight that the Malaysian FH genetic profile largely overlaps with established global patterns while also contributing new, locally relevant variant data to the international literature. *APOB* represented the second most common gene with PVs in this cohort, consistent with other Asian and Western studies (Sun et al., 2018). Detected *APOB* variants were predominantly located in exons 26 and 29, regions critical for *LDLR* binding and LDL

particle formation. Patients harbouring APOB PVs often presented with elevated LDL-C levels, a corneal arc before age 45, or a family history of premature coronary artery disease (PCAD), reflecting phenotypic parallels with *LDLR* mutation carriers. In contrast, no *PCSK9* PVs were detected, consistent with their global rarity and lower contribution to FH prevalence (Abifadel et al., 2012). This absence may also reflect the limited penetrance or ethnicity-specific frequency of *PCSK9* mutations in Asian populations.

Of the 310 clinically diagnosed FH cases, 86 (27.7%) carried at least one PV, while 224 (72.3%) tested negative for PVs in known FH genes. The absence of identifiable mutations in the majority of patients may indicate polygenic hypercholesterolaemia, non-coding or structural variants, or yet-undiscovered genes involved in lipid metabolism (Sharifi et al., 2019). Polygenic forms can phenotypically overlap with monogenic FHs, emphasising the need for comprehensive clinical-genetic correlation. Cascade screening remains a key cost-effective public health strategy for identifying FH carriers and improving detection rates (Marks et al., 2002; Ned & Sijbrands, 2011). The Centers for Disease Control and Prevention (CDC) recognises FH as one of three "Tier 1" genomic disorders where genetic testing provides high clinical and preventive value (George et al., 2015; Khera & Hegele, 2020). Integrating next-generation sequencing (NGS) into primary care settings can therefore enhance early identification, clarify disease burden, and reveal the unique mutational landscape of FH within the Malaysian population.

In the present cohort, 5.5% of genetically confirmed cases exhibited tendon xanthomata, while 39.4% displayed corneal arcus, both hallmark phenotypic indicators of FH. However, extra care was taken during clinical screening, and only patients with premature corneal arcus (diagnosed before age 45) were considered positive for this feature. Despite the demonstrated accuracy of NGS in FH detection (Alex et al., 2012; Al-Khateeb et al., 2013; Chua et al., 2021; Iacocca et al., 2017; Razman et al., 2022; Vandrovcova et al., 2013), the high cost of genetic testing continues to limit its routine diagnostic use in many countries (Chua et al., 2021; Pang et al., 2019; Razman et al., 2022). Nevertheless, as sequencing throughput improves, NGS offers substantial advantages for the detection of rare variants, polygenic contributions, and novel FH genes. When implemented efficiently, it provides detailed insight into gene function and population-level disease architecture, strengthening precision medicine approaches in lipid disorders.

5.3 Strengths and Limitations

Each participant who consented to take part in this study underwent a comprehensive medical examination conducted by trained primary care physicians. This ensured that the clinical evaluation process, including lipid profiling and the assessment of physical signs, was systematic and recognisable across all sites. Nonetheless, various limitations must be recognised.

Firstly, not all baseline LDL-C values were measured under fasting or treatment-naïve conditions. Some values were obtained from the highest recorded LDL-C levels, which may have contributed to an overestimation of clinical FH probability scores when applying the DLCN, Simon Broome, and FAMCAT criteria. Consequently, these limitations could have led to a higher proportion of clinically diagnosed FH cases compared to genetically confirmed ones.

Secondly, the study population was limited to patients attending public primary care clinics within the Klang Valley area. As such, the findings may not be fully representative of the entire Malaysian population, as regional differences in healthcare accessibility, ethnicity distribution, and screening practices could influence both the prevalence and presentation of FH.

Thirdly, family cascade screening was not conducted among relatives of genetically confirmed index cases. Previous studies have shown that cascade screening guided by genetic testing of known FH mutations in index patients is a more effective approach to identifying affected individuals (Sturm et al., 2018). Future research could benefit from incorporating next-generation sequencing (NGS)–based cascade screening to identify additional carriers of FH-causing variants and strengthen the genetic mapping of FH in Malaysia.

Fourthly, functional validation of the identified variants was not performed in this study. While the classification of variants was guided by the American College of Medical Genetics (ACMG) criteria and supported by existing literature, functional assays would provide stronger evidence of their biological effects on protein function and lipid metabolism.

Lastly, because the participants were recruited through primary care screening were based on self-reporting. The questionnaire may have introduced recall bias or underreporting of clinical features such as tendon xanthomata or premature coronary

artery disease, which could affect the accuracy of clinical FH classification. Despite these limitations, this study provides useful information regarding the genetic landscape of familial hypercholesterolaemia among Malaysia's primary care population and demonstrates the feasibility of implementing genetic testing within a community-based healthcare setting.

CHAPTER 6

CONCLUSION

Familial hypercholesterolaemia (FH) is the most prevalent hereditary lipid disorder that, if left untreated, can lead to premature and potentially fatal cardiovascular disease. Despite advances in molecular diagnostics and the availability of potent lipid-lowering therapies, FH remains substantially underdiagnosed and undertreated worldwide. This study represents one of the first comprehensive attempts to characterise the genetic landscape of FH among clinically diagnosed patients attending primary care clinics in Malaysia. A total of 310 clinically diagnosed FH patients were recruited and categorised into Definite, Probable (Potential FH), and Possible FH according to internationally recognised diagnostic criteria—DLCN, Simon Broome, and FAMCAT. Among these, pathogenic variants (PVs) were detected in 27.7% of patients, representing genetically confirmed FH cases. The LDLR gene was the most affected (64%), followed by the APOB gene (36%). There were no PVs found in the PCSK9 gene. These findings, consistent with global trends, suggest possible population-specific variant patterns within Malaysia. Clinically, 5.5% of FH patients presented with tendon xanthomata and 39.4% with corneal arcus, demonstrating notable phenotypic heterogeneity.

Diagnosis was established using standard clinical tools incorporating LDL-C levels, clinical manifestations, and family history of premature cardiovascular disease. Although diagnostic sensitivity and specificity were not directly evaluated, the rate of genetic confirmation reflected each tool's predictive performance in primary care. Among the criteria used, the Dutch Lipid Clinic Network (DLCN) appeared the most promising, showing better concordance with genetic confirmation than Simon Broome or FAMCAT. Only patients with corneal arcus diagnosed before age 45 were considered positive, ensuring a more accurate clinical characterisation. Collectively, this study provides the first primary care–based genetic insight into FH in Malaysia and demonstrates that next-generation sequencing (NGS) can be effectively integrated into community-level screening programmes. The integration of genetic testing into the public primary care system could strengthen early diagnosis, targeted therapy, and family-based cascade screening. The relatively low number of PVs found could be due to gene rearrangements that the current platform can't find, or it could be because the

patients were chosen differently, the lipid cut-off levels were different, or the diagnostic methods were different than in previous studies.

Future research should include family-based cascade screening and large genomic rearrangement analyses to identify additional FH variant carriers, as well as evaluation of the cost-effectiveness and diagnostic accuracy of tools such as DLCN, Simon Broome, and FAMCAT. Broader sampling across Malaysian regions would help validate these genetic and phenotypic findings. Further investigation of polygenic hypercholesterolaemia in patients lacking identifiable monogenic pathogenic variants would improve the accuracy of lipid management. It is essential that all clinically diagnosed FH patients, irrespective of genetic validation, receive guideline-recommended lipid-lowering therapy to guarantee equitable, risk-based care. In conclusion, this study successfully characterised the genetic spectrum of FH in Malaysia's primary care population and quantified the proportion of genetically confirmed cases. The predominance of LDLR and APOB variants aligns with global observations while contributing novel insights to the local population. Integrating genetic testing and validated diagnostic frameworks such as the DLCN into primary care can significantly improve early detection, cascade screening, and treatment outcomes for families affected by FH. Continued research and policy development are essential to bridge the gap between clinical diagnosis, genetic confirmation, and optimal patient management in Malaysia.

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APPENDICES

APPENDIX 1

www.uitm.edu.my

UNIVERSITI TEKNOLOGI MARA | Pejabat Timbalan Naib Canselor (Penyelidikan dan Inovasi)

Reference : 600-TNCPI (5/1/6)
Our reference : REC/03/2020) (FB/48)
Date : 5th May 2020

Prof. Dr Anis Safura Ramli
Faculty of Medinice
Sungai Buloh Campus
Jalan Hospital
47000 Sungai Buloh
Selangor

Dear Prof. Dr Anis,

ETHICS APPROVAL BY UiTM RESEARCH ETHICS COMMITTEE

Title: Reducing Premature Coronary Artery Disease in Malaysia by Early Identification of Familial Hypercholesterolaemia

Trial Site: 1. 12 Ministry of Health Primary care clinics in Selangor, Wilayah Persekutuan Kuala Lumpur & Putrajaya.
2. Lipid Specialist Clinic, UiTM Specialist Medical Centre, Sungai Buloh Campus

Thank you for your research ethics application on 17th March 2020 and your amendment for the above study on 4th April 2020. We would like to inform that the UiTM Research Ethics Committee had deliberated your proposal on the 21st April 2020.

It is our pleasure to inform you that the Research Ethics Committee has agreed to grant an ethics approval for the said study. The approval code for the study is REC/03/2020) (FB/48), and validity period is from 21st April 2020 until 31st December 2021.

Please submit progress report of the study to the REC Secretariat 12 months after the date of approval letter and annually until the study has completed. Please kindly notify the REC for any amendments to the relevant documents for this study. Notification should be made to the REC for any protocol deviation and in case of serious adverse event, please notify the REC within 48 hours from the time known. A summary of the final report should be submitted at the end of your study.

The UiTM Research Ethics Committee operates in accordance to the ICH Good Clinical Practice Guidelines, Malaysia Good Clinical Practice Guidelines and the Declaration of Helsinki.

Thank you.

Yours truly,

[redacted]

PROFESSOR DR. NOR ASHIKIN MOHAMED NOOR KHAN
Chair
UiTM Research Ethics Committee

Universiti Teknologi MARA
Aras 3, Bangunan Wawasan
40450 Shah Alam, Selangor, MALAYSIA
Tel: (+603) 5544 2004/2255
Faks: (+603) 5544 2070

ISO 9001:2015 No. Sijil: 10120150

UiTM di hatiku

APPENDIX 2

JAWATANKUASA ETIKA & PENYELIDIKAN PERUBATAN
(*Medical Research & Ethics Committee*)
KEMENTERIAN KESIHATAN MALAYSIA
d/a Kompleks Institut Kesihatan Negara
Blok A, No 1, Jalan Setia Murni U13/52,
Seksyen U13, Bandar Setia Alam,
40170 Shah Alam, Selangor.
Tel: 03-3362 8888/8205

Ref : KKM/NIHSEC/ P20-431 (12)
Date: **25-Mar-2020**

Prof Nadeem Qureshi
School of Medicine, University of Nottingham

Professor Dr. Anis Safura Ramli
UNIVERSITY TEKNOLOGI MARA (UITM) - SUNGAI BULOH CAMPUS

Dear Sir / Mdm,

ETHICS INITIAL APPROVAL: NMRR-20-272-52797 (IIR)
Protocol No: MR/T017384/1
Reducing Premature Coronary Artery Disease in Malaysia by Early Identification of Familial Hypercholesterolaemia using FAMCAT Primary Care Screening Tool

This letter is made in reference to the above matter.

2. The Medical Research and Ethics Committee (MREC), Ministry of Health Malaysia (MOH) has provided ethical approval for this study. Please take note that all records and data are to be kept strictly **CONFIDENTIAL** and can only be used for the purpose of this study. All precautions are to be taken to maintain data confidentiality. Permission from the District Health Officer / Hospital Administrator / Hospital Director and all relevant heads of departments / units where the study will be carried out must be obtained prior to the study. You are required to follow and comply with their decision and all other relevant regulations, including the Access to Biological and Benefit Sharing Act 2017.

3. The investigators and study sites involved in this study are:

KLINIK KESIHATAN BATU
Dr MOHAMED SYARIF BIN MOHAMED YASSIN
Dr VALARMATHI A/P MASILAMANI

KLINIK KESIHATAN BATU 9 CHERAS
Dr AZNIDA FIRZAH ABDUL AZIZ
Dr Nor Hazlin binti Talib

KLINIK KESIHATAN JINJANG
Dr Noorhida Binti Baharudin
Dr. Siti Rohani bt. Mohamed Alias

KLINIK KESIHATAN KELANA JAYA
Dr Nik Mazlina Mohammad
Dr Siti Fatimah binti Badlishah Sham

Klinik Kesihatan Kuala Lumpur
Dr Azira Baharuddin
Dr Baizury Bashah
Dr Nagammai A/P Thiagarajan
Dr Noorhida Binti Baharudin

KLINIK KESIHATAN PANDAMARAN

APPENDIX 2

Ref : KKM/NIHSEC/ P20-431 (12)

Dr Noor Harzana Binti Harrun
Dr Suraya Abdul Razak

Klinik Kesihatan Putrajaya Presint 18
Dr Hazira Hanum binti Mohd Yusof
Dr Nazrila Hairizan Bt Nasir
Dr Nurzaitil Aswani bt Zainuddin
Dr Rozita Zakaria
Professor Dr. Anis Safura Ramli (Penyelidik Utama)

KLINIK KESIHATAN SELAYANG BARU
Dr MOHAMED SYARIF BIN MOHAMED YASSIN
LEE YEOW SIONG

Klinik Kesihatan Shah Alam Seksyen 7
Dr MOHD KHAIRI BIN MOHD NOOR
Professor Dr. Anis Safura Ramli (Penyelidik Utama)

KLINIK KESIHATAN SUNGAI BULOH
Dr Fauziah Ahmad
Dr Suraya Abdul Razak

KLINIK KESIHATAN TAMAN EHSAN
Dr Siti Fatimah binti Badlishah Sham
Dr. Rosnah bt Mat Isa

KLINIK KESIHATAN TANGLIN
Dr AZNIDA FIRZAH ABDUL AZIZ
Dr. Nor Faizah Ghazali

UNIVERSITY TEKNOLOGI MARA (UITM) - SUNGAI BULOH CAMPUS
Dr Alyaa Rajih Mahmood Al-kateeb
Dr Hasidah Abdul Hamid
Dr. Stephen Weng
Prof Joe Kai
Prof Nadeem Qureshi (Penyelidik Utama)
Prof. Dr. Hapizah Binti Mohd Nawawi
Professor Dr. Anis Safura Ramli (Penyelidik Utama)
Professor Dr. Jo Leonardi-Bee

4. The following study documents have been received and reviewed with reference to the above study:

Documents received and reviewed with reference to the above study:

1. Study Protocol_Version 1.2, dated 24-Mar-2020
2. Information Sheet & Parental Consent Form_English_Version 1.2, dated 24-Mar-2020
3. Information Sheet & Parental Consent Form_Malay_Version 1.2, dated 24-Mar-2020
4. Questionnaire_Version 1.2, dated 24-Mar-2020
5. Data Collection Form_Version 1.2, dated 24-Mar-2020
6. Investigator's documents : Declaration of Conflict of Interest (COI), IAHOD, and CV:
   a) Dr Alyaa Rajih Mahmood Al-kateeb
   b) Dr Hasidah Abdul Hamid
   c) Dr. Stephen Weng
   d) Prof Joe Kai
   e) Prof Nadeem Qureshi (Penyelidik Utama)
   f) Prof. Dr. Hapizah Binti Mohd Nawawi
   g) Professor Dr. Anis Safura Ramli (Penyelidik Utama)
   h) Professor Dr. Jo Leonardi-Bee
   i) Dr AZNIDA FIRZAH ABDUL AZIZ

APPENDIX 2

Ref : KKM/NIHSEC/ P20-431 (12)

j) Dr. Nor Faizah Ghazali
k) Dr Siti Fatimah binti Badlishah Sham
l) Dr. Rosnah bt Mat Isa
m) Dr Fauziah Ahmad
n) Dr Suraya Abdul Razak
o) Dr MOHD KHAIRI BIN MOHD NOOR
p) Dr MOHAMED SYARIF BIN MOHAMED YASSIN
q) Dr LEE YEOW SIONG
r) Dr Hazira Hanum binti Mohd Yusof
s) Dr Nazrila Hairizan Bt Nasir
t) Dr Nurzaitil Aswani bt Zainuddin
u) Dr Rozita Zakaria
v) Dr Noor Harzana Binti Harrun
w) Dr Azira Baharuddin
x) Dr Baizury Bashah
y) Dr Nagammai A/P Thiagarajan
z) Dr Noorhida Binti Baharudin
aa) Dr Nik Mazlina Mohammad
bb) Dr. Siti Rohani bt. Mohamed Alias
cc) Dr Nor Hazlin binti Talib
dd) Dr VALARMATHI A/P MASILAMANI

5. Please note that ethical approval is valid until **24-Mar-2021**. The following are to be reported upon receiving ethical approval. Required forms can be obtained from the Medical Research Ethics Committee (MREC) website (http://www.nih.gov.my/mrec).

i. **Continuing Review Form** has to be submitted to MREC within 2 month (60 days) prior to the expiry of ethical approval.
ii. **Study Final Report** upon study completion to the MREC.
iii. Ethical approval is required in the case of **amendments / changes** to the **study documents/ study sites/ study team**. MREC reserves the right to withdraw ethical approval if changes to study documents are not completely declared.
iv. Applicable for Clinical interventional Studies only: Report occurrences of all Serious Adverse Events (SAEs), Suspected Unexpected Serious Adverse Reaction (SUSARs) and Protocol Deviation/Violation at all MREC approved sites to MREC. SAEs are to be reported within 15 calendar days from awareness of event by investigator. Initial report of SUSARs are to be reported as soon as possible but not later than 7 calendar days from awareness of event by investigator, followed by a complete report within 8 additional calendar days.

6. There will be **6800 subjects/ patients/ respondents** targeted to be enrolled in this study within Malaysia.

7. Please take note that the reference number for this letter must be stated in all correspondence related to this study to facilitate the process.

Comments (if any): Suggest to have Research Agreement that includes all parties.

Project Sites:
KLINIK KESIHATAN BATU
KLINIK KESIHATAN BATU 9 CHERAS
KLINIK KESIHATAN JINJANG
KLINIK KESIHATAN KELANA JAYA
KLINIK KESIHATAN KUALA LUMPUR
KLINIK KESIHATAN PANDAMARAN
KLINIK KESIHATAN PUTRAJAYA PRESINT 18
KLINIK KESIHATAN SELAYANG BARU
KLINIK KESIHATAN SHAH ALAM SEKSYEN 7
KLINIK KESIHATAN SUNGAI BULOH
KLINIK KESIHATAN TAMAN EHSAN
KLINIK KESIHATAN TANGLIN

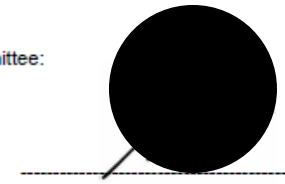
APPENDIX 2

Ref : KKM/NIHSEC/ P20-431 (12)

UNIVERSITY TEKNOLOGI MARA (UITM) - SUNGAI BULOH CAMPUS

Decision by Medical Research & Ethics Committee:
(√) Approved
( ) Disapproved

Date of Approval : **25-Mar-2020**



DR HJH SALINA ABDUL AZIZ
Chairperson
Medical Research & Ethics Committee
Ministry of Health Malaysia
MMC No: 27117

\MREC_Share\Approval 2020\Expedited by Primary Reviewer\March 2020\52797

APPENDIX 3

PARTICIPANT INFORMATION SHEET & CONSENT FORM: REDUCING PREMATURE CORONARY ARTERY DISEASE IN MALAYSIA BY EARLY IDENTIFICATION OF FAMILIAL HYPERCHOLESTEROLAEMIA

Work Stream 1: Optimising the FH Case Identification Tools in the Malaysian Primary Care Setting

Sponsors

The UK-Malaysia Joint Partnership Call on Non-Communicable Diseases

1. Ministry of Higher Education, Malaysia
   2, Jalan P5/6, Presint 5, 62200 Wilayah Persekutuan Putrajaya
   MOE/UiTM Grant Ref: 100-TNCPI/GOV 16/6/2 (002/2020)

2. Medical Research Council, United Kingdom
   58 Victoria Embankment
   London EC4Y 0DS
   MRC Grant Ref: MR/T017384/1

Introduction

This study is designed to identify individuals with high risk of Familial Hypercholesterolaemia (FH). FH is a genetic condition characterized by high plasma level of low density lipoprotein-cholesterol (LDL) i.e. the bad cholesterol. FH is associated with 22-fold increase in death caused by coronary artery diseases (CAD) i.e. heart attack, if it is left untreated. This contributes to the premature CAD epidemic in Malaysia, resulting in a younger age at first heart attack compared to developed countries.

Purpose of the study

The purpose of this study is to systematically identify individuals with suspected FH in the Malaysian primary care setting using the Familial Hypercholesterolaemia Case Ascertainment Tool (FAMCAT), Simon Broome (SB) and Dutch Lipid Clinic Criteria (DLCC). Approximately 6800 participants will be invited to be screened. Those who are suspected to have FH by either of these criteria will be offered genetic testing, which is the gold standard in diagnosing FH. You are invited to participate in this study because you have high cholesterol and may have FH.

Participation in the study

It is important that you understand why the study is being done and what it will involve. Please take your time to read through and consider this information carefully before you decide if you are willing to participate. Ask the study staff if anything is unclear or if you would like to have more information. After you are properly satisfied that you understand this study, and that you wish to participate, you must sign this informed consent form. To participate in this study, you will be required to provide your doctor with information on your health history; you may harm yourself if you are not truthful with the information provided.

Your participation in this study is voluntary. You do not have to be in this study if you do not want to. You may also refuse to answer any questions you do not want to answer. If you volunteer to be in this study, you may withdraw from it at any time. If you withdraw, any data collected from you up to your withdrawal will still be used for the study. Your refusal to

APPENDIX 3

participate or withdrawal will not affect any medical or health benefits to which you are otherwise entitled. You will not be paid for your participation in this study.

This study has been approved by the Medical Research and Ethics Committee, Ministry of Health Malaysia.

Study procedures and your responsibilities when taking part in this study

First of all, you will be screened whether you are eligible to participate. If you are eligible, have agreed to participate and signed the consent form, you will be given a Data Collection Form and a Family History Questionnaire to be answered. The Data Collection Form contains 3 sections: i) Sociodemographic & Clinical Data ii) WHO Rose Angina Questionnaire (to screen whether you have heart disease) iii) Edinburgh Claudication Questionnaire (to screen whether you have pain in the leg caused by blocked blood vessels). The Family History Questionnaire contains 5 sections asking detail questions on history of raised cholesterol and heart disease among your family members.

This study will involve you filling in your personal and family history information into these forms. It is important that you answer all of the questions asked by the study staff honestly and completely which will take about 30 minutes of your time.

The study team will also access your medical records for the following information:

- Previous cholesterol results
- Medication list
- Previous history of heart attack
- Family history of heart attack
- Family history of raised cholesterol
- Family history of FH
- Diagnosis of diabetes
- Diagnosis of chronic kidney disease

You will be examined by a graduate medical research assistant to look for possible signs of FH in your eyes, ankles and joints. All of this information will be used to determine whether you are suspected to have FH. If you are identified to have suspected FH, you will be offered to have genetic test and referral to the Lipid Specialist Clinic in UiTM Sungai Buloh Campus for further management of your condition.

Benefit of the study

Information obtained from this study will benefit you as a participant, doctors and researchers for the advancement of knowledge and practice of medicine in future.

If you are found to have suspected FH, you will be offered a free genetic test to confirm the diagnosis by the UiTM Lipid Specialist Clinic. Currently, this test is not freely available in the public healthcare service.

It is also beneficial to your family if you are genetically diagnosed to have FH, as they will also be offered screening of the condition by the UiTM Lipid Specialist Clinic. This will lead to early diagnosis and treatment of FH, which will help to prevent premature heart attack.

APPENDIX 3

Treatment you will receive after your participation in this study

No study product will be given to you at the end of your participation in the study as this is not a clinical trial or interventional study. Whether you complete the study or withdraw early, your doctor will discuss the best alternatives for your future treatment with you.

You and your family are encouraged to continue long term follow-up treatment at the UiTM Lipid Specialist Clinic if you are diagnosed to have FH. The cost of the treatment is subjected to the university regulation. If you are a civil servant or pensioner, you may be entitled for free treatment at UiTM.

Study risk

This study is minimal risk at this stage as it does not involve collection of blood sample. If you are found to have suspected FH and blood taking is required for genetic testing, you will be requested to sign another consent form.

Early termination of the study

The study doctor or the sponsor may due to concerns for your safety, stop the study or your participation at any time. If the study is stopped early for any reason you will be informed and arrangements will be made for your future care. You may be asked to attend a final follow-up visit.

Confidentiality

All information obtained in this study will be kept and handled in a confidential manner, in accordance with applicable laws and/or regulations. The data will be stored securely and anonymously in electronic databases at both UiTM and the University of Nottingham, United Kingdom. When analyzing, publishing or presenting the study results, your identity will not be revealed without your expressed consent. Individuals involved in this study, qualified monitors and auditors, and governmental or regulatory authorities may inspect the study data, where appropriate and necessary. Data will be destroyed after the maximum data storage period of 10 years.

By signing this consent form, you will authorize the review of records, analysis and use of the data arising from this study. You will be informed if new information relevant to consent becomes available and you will be asked to re-consent if the needs arise.

Contact information

If you have any questions about your rights as a participant in this study, please contact:

1. Professor Dr. Anis Safura Ramli, the Project Leader at 019 384 4503.
2. The Secretary, Medical Research & Ethics Committee, Ministry of Health Malaysia, at telephone number 03-3362 8407/8205/8888.

APPENDIX 3

PARTICIPANT INFORMATION SHEET & CONSENT FORM: REDUCING PREMATURE CORONARY ARTERY DISEASE IN MALAYSIA BY EARLY IDENTIFICATION OF FAMILIAL HYPERCHOLESTEROLAEMIA

Consent Form

I herewith confirm that I have met the requirement of age and am capable of acting on behalf of myself as follows:

1. I understand the nature and scope of the research being undertaken.
2. I have read and understood all the terms and conditions of my participation in the research.
3. All my questions relating to this research and my participation therein have been answered to my satisfaction.
4. I voluntarily agree to take part in this research, to follow the study procedures and to provide all necessary information to the researcher(s) as requested.
5. I agree to be contacted by the researcher(s) if there is additional information needed for the research.
6. I may at any time choose to withdraw from this research without giving reasons.
7. I understand that study staff, qualified monitors and auditors, the sponsor or its affiliates, and governmental or regulatory authorities, have direct access to my medical record in order to make sure that the study is conducted correctly and the data are recorded correctly. All personal details will be treated as STRICTLY CONFIDENTIAL.
8. I have received a copy of the Participant Information Sheet, have read and understood its content.
9. I agree/disagree* for my family doctor to be informed of my participation in this study. (*delete which is not applicable)
10. Except for damages resulting from negligent or malicious conduct of the researcher(s), I hereby release and discharge UiTM and University of Nottingham, UK and all participating researchers from all liability associated with, arising out of, or related to my participation and agree to hold them harmless from any harm or loss that may be incurred by me due to my participation in the research.

Participant:

Signature: I/C number:

Name: Date:

Investigator conducting informed consent:

Signature: I/C number:

Name: Date:

Impartial witness: *(Required if participant is illiterate and contents of participant information sheet is orally communicated to participant)*

Signature: I/C number:

Name: Date:

Participant Information Sheet and Consent Form – English version 1.2, dated 24 March 2020

APPENDIX 4

PARTICIPANT INFORMATION SHEET & CONSENT FORM: REDUCING PREMATURE CORONARY ARTERY DISEASE IN MALAYSIA BY EARLY IDENTIFICATION OF FAMILIAL HYPERCHOLESTEROLAEMIA

Work Stream 2: Identifying Genetic Mutation Profiles of Individuals with Suspected FH

Sponsors

The UK-Malaysia Joint Partnership Call on Non-Communicable Diseases

3. Ministry of Higher Education, Malaysia
   2, Jalan P5/6, Presint 5, 62200 Wilayah Persekutuan Putrajaya
   MOE/UiTM Grant Ref: 100-TNCPI/GOV 16/6/2 (002/2020)

4. Medical Research Council, United Kingdom
   58 Victoria Embankment
   London EC4Y 0DS
   MRC Grant Ref: MR/T017384/1

Introduction

This study is designed to identify individuals with high risk of Familial Hypercholesterolaemia (FH). FH is a genetic condition characterized by high plasma level of low density lipoprotein-cholesterol (LDL) i.e. the bad cholesterol. FH is associated with 22-fold increase in death caused by coronary artery diseases (CAD) i.e. heart attack, if it is left untreated. This contributes to the premature CAD epidemic in Malaysia, resulting in a younger age at first heart attack compared to developed countries.

Purpose of the study

Approximately 310 participants with suspected FH will be invited to participate in this study. You are offered to participate in this study because you are suspected to have FH by Familial Hypercholesterolaemia Case Ascertainment Tool (FAMCAT), Simon Broome (SB) or Dutch Lipid Clinic Criteria (DLCC). The purpose of this study is to confirm whether you have FH by genetic testing, which is the gold standard in diagnosing FH. You will be informed of the genetic findings once the results are available.

Participation in the study

It is important that you understand why the study is being done and what it will involve. Please take your time to read through and consider this information carefully before you decide if you are willing to participate. Ask the study staff if anything is unclear or if you would like to have more information. After you are properly satisfied that you understand this study, and that you wish to participate, you must sign this informed consent form. To participate in this study, you may be required to provide your doctor with information on your health history; you may harm yourself if you are not truthful with the information provided.

Your participation in this study is voluntary. You do not have to be in this study if you do not want to. You may also refuse to answer any questions you do not want to answer. If you volunteer to be in this study, you may withdraw from it at any time. If you withdraw, any data collected from you up to your withdrawal will still be used for the study. Your refusal to participate or withdrawal will not affect any medical or health benefits to which you are otherwise entitled. You will be paid a one-off honorarium of RM50.00 for your participation in this study, to cover your transportation cost to the UiTM Lipid Specialist Clinic in Sungai Buloh.

Participant Information Sheet and Consent Form – English version 1.2, dated 24 March 2020

APPENDIX 4

PARTICIPANT INFORMATION SHEET & CONSENT FORM: REDUCING PREMATURE CORONARY ARTERY DISEASE IN MALAYSIA BY EARLY IDENTIFICATION OF FAMILIAL HYPERCHOLESTEROLAEMIA

This study has been approved by the Medical Research and Ethics Committee, Ministry of Health Malaysia.

Study procedures and your responsibilities when taking part in this study

You will be referred to the Lipid Specialist Clinic in UiTM Sungai Buloh Campus to confirm the diagnosis of FH and for further management of your condition. The doctor at the Lipid Specialist Clinic will reconfirm your medical history and will perform physical examinations as necessary. It is important that you answer all of the questions asked by the doctor honestly and completely.

At the Lipid Specialist Clinic, you will undergo a session on genetic counselling regarding FH, which will last between 15 – 30 minutes. After the counseling session, approximately 15 ml (1 tablespoon) of blood will be taken by a trained personnel e.g. nurse or research assistant. The blood will be sent to the research laboratory at UiTM for genetic analysis (10 ml) and fasting serum lipid profile (5 ml). Since FH is a genetic condition that runs in a family, your close family members i.e. first degree relatives will also be invited to be screened for FH at UiTM Lipid Specialist Clinic.

Benefit of the study

Information obtained from this study will benefit you as a participant, doctors and researchers for the advancement of knowledge and practice of medicine in future.

You will get a free genetic test to confirm the diagnosis of FH as the cost of this test will be funded by the research grant. Currently, this test is not freely available in the public healthcare service.

You will be informed of the genetic findings by the specialist at UiTM Lipid Specialist Clinic once the results are available. You will be offered further management and long term follow-up of your condition by the specialist at UiTM Lipid Specialist Clinic.

It is also beneficial to your family if you are genetically diagnosed to have FH, as they will also be offered screening of the condition by the UiTM Lipid Specialist Clinic. This will lead to early diagnosis and treatment of FH, which will help to prevent premature heart attack.

Treatment you will receive after your participation in this study

No study product will be given to you at the end of your participation in the study as this is not a clinical trial or interventional study. Whether you complete the study or withdraw early, your doctor will discuss the best alternatives for your future treatment with you.

You and your family are encouraged to continue long term follow-up treatment at the UiTM Lipid Specialist Clinic if you are diagnosed to have FH. The cost of the treatment is subjected to the university regulation. If you are a civil servant or pensioner, you may be entitled for free treatment at UiTM.

Study risk

This study is medium risk as it involves collection of blood sample and also genetic testing. You may experience discomfort or bruising during and after the process of blood taking. Otherwise, there is no other direct risk associated with this study.

Participant Information Sheet and Consent Form – English version 1.2, dated 24 March 2020

APPENDIX 4

Injury which may incur during the study

If you are injured as a result of being in this study, you should contact your study doctor. In the event of a bodily injury or illness directly resulting from the blood taking required for this study, the sponsor will pay for reasonable and necessary treatment. The sponsor is not responsible for medical expenses due to pre-existing medical conditions, any underlying diseases, any ongoing treatment process, your negligence or willful misconduct, the negligence or willful misconduct of your study doctor or the study site or any third parties. You do not lose any of your legal rights to seek compensation by signing this form.

Early termination of the study

The study doctor or the sponsor may due to concerns for your safety, stop the study or your participation at any time. If the study is stopped early for any reason you will be informed and arrangements will be made for your future care. You may be asked to attend a final follow-up visit.

Confidentiality

All information obtained in this study will be kept and handled in a confidential manner, in accordance with applicable laws and/or regulations.

Your blood specimen will be sent to research laboratory in UiTM for testing. The specimen will be coded and information that can identify you will be removed. Only your study doctor and study staff will be able to link the code with you. The blood sample will be stored for a maximum period of 1 year and will only be analysed to check for your lipid profile and to confirm whether you have FH by genetic testing. The researchers will not store the excess/leftover blood specimens or use your blood sample for future research which is beyond the scope of this study. The blood specimen WILL NOT be sent to the University of Nottingham, UK or any foreign country.

The genetic analysis results will be stored securely in electronic databases at both UiTM and the University of Nottingham, United Kingdom. It will be coded and information that can identify you will be removed. Only your study doctor and study staff will be able to link the code with you. When analyzing, publishing or presenting the study results, your identity will not be revealed without your expressed consent. Individuals involved in this study, qualified monitors and auditors, and governmental or regulatory authorities may inspect the study data, where appropriate and necessary. Data will be destroyed after the maximum data storage period of 10 years.

By signing this consent form, you will authorize the review of records, analysis and use of the data arising from this study. You will be informed if new information relevant to consent becomes available and you will be asked to re-consent if the needs arise.

Contact information

If you have any questions about your rights as a participant in this study, please contact:

1. Professor Dr. Anis Safura Ramli, the Project Leader at [redacted].
2. The Secretary, Medical Research & Ethics Committee, Ministry of Health Malaysia, at telephone number 03-3362 8407/8205/8888.

Participant Information Sheet and Consent Form – English version 1.2, dated 24 March 2020

APPENDIX 4

PARTICIPANT INFORMATION SHEET & CONSENT FORM: REDUCING PREMATURE CORONARY ARTERY DISEASE IN MALAYSIA BY EARLY IDENTIFICATION OF FAMILIAL HYPERCHOLESTEROLAEMIA

Consent Form

I herewith confirm that I have met the requirement of age and am capable of acting on behalf of myself as follows:

1. I understand the nature and scope of the research being undertaken.
2. I have read and understood all the terms and conditions of my participation in the research.
3. All my questions relating to this research and my participation therein have been answered to my satisfaction.
4. I voluntarily agree to take part in this research, to follow the study procedures and to provide all necessary information to the researcher(s) as requested.
5. I agree to be contacted by the researcher(s) if there is additional information needed for the research.
6. I may at any time choose to withdraw from this research without giving reasons.
7. I understand that study staff, qualified monitors and auditors, the sponsor or its affiliates, and governmental or regulatory authorities, have direct access to my medical record in order to make sure that the study is conducted correctly and the data are recorded correctly. All personal details will be treated as STRICTLY CONFIDENTIAL.
8. I have received a copy of the Participant Information Sheet, have read and understood its content.
9. I agree/disagree* for my family doctor to be informed of my participation in this study. (*delete which is not applicable)
10. Except for damages resulting from negligent or malicious conduct of the researcher(s), I hereby release and discharge UiTM and University of Nottingham, UK and all participating researchers from all liability associated with, arising out of, or related to my participation and agree to hold them harmless from any harm or loss that may be incurred by me due to my participation in the research.

Participant:

Signature: I/C number:

Name: Date:

Investigator conducting informed consent:

Signature: I/C number:

Name: Date:

Impartial witness: *(Required if participant is illiterate and contents of participant information sheet is orally communicated to participant)*

Signature: I/C number:

Name: Date:

Participant Information Sheet and Consent Form – English version 1.2, dated 24 March 2020

APPENDIX 5

PARTICIPANT INFORMATION SHEET & CONSENT FORM: REDUCING PREMATURE CORONARY ARTERY DISEASE IN MALAYSIA BY EARLY IDENTIFICATION OF FAMILIAL HYPERCHOLESTEROLAEMIA

Work Stream 3: Improving Implementation of FH Identification Tools in the Malaysian Primary Care Setting – Patient Information Sheet

Sponsors

The UK-Malaysia Joint Partnership Call on Non-Communicable Diseases

3. Ministry of Higher Education, Malaysia
   2, Jalan P5/6, Presint 5, 62200 Wilayah Persekutuan Putrajaya
   MOE/UiTM Grant Ref: 100-TNCPI/GOV 16/6/2 (002/2020)

4. Medical Research Council, United Kingdom
   58 Victoria Embankment
   London EC4Y 0DS
   MRC Grant Ref: MR/T017384/1

Introduction

This study is designed to identify individuals with high risk of Familial Hypercholesterolaemia (FH). FH is a genetic condition characterized by high plasma level of low density lipoprotein-cholesterol (LDL) i.e. the bad cholesterol. FH is associated with 22-fold increase in death caused by coronary artery diseases (CAD) i.e. heart attack, if it is left untreated. This contributes to the premature CAD epidemic in Malaysia, resulting in a younger age at first heart attack compared to developed countries.

Purpose of the study

Approximately 25 participants who have undergone genetic testing will be invited to participate in this study. You are offered to participate in this study because you have undergone the genetic testing. The purpose of this study is to explore your experience, concerns and expectations of undergoing genetic testing for FH.

Participation in the study

It is important that you understand why the study is being done and what it will involve. Please take your time to read through and consider this information carefully before you decide if you are willing to participate. Ask the study staff if anything is unclear or if you would like to have more information. After you are properly satisfied that you understand this study, and that you wish to participate, you must sign this informed consent form. To participate in this study, you may be required to provide your doctor with information on your health history; you may harm yourself if you are not truthful with the information provided.

Your participation in this study is voluntary. You do not have to be in this study if you do not want to. You may also refuse to answer any questions you do not want to answer. If you volunteer to be in this study, you may withdraw from it at any time. If you withdraw, any data collected from you up to your withdrawal will still be used for the study. Your refusal to participate or withdrawal will not affect any medical or health benefits to which you are otherwise entitled. You will be paid a one-off honorarium of RM50.00 for your participation in this study, to cover your transportation cost to the UiTM Lipid Specialist Clinic in Sungai Buloh.

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This study has been approved by the Medical Research and Ethics Committee, Ministry of Health Malaysia.

Study procedures and your responsibilities when taking part in this study

You will be invited to a semi-structured in-depth interview with the researcher for about 45 minutes. The interview will be audio-recorded to ensure the correct information is captured. It is important that you answer all of the questions asked during the interview honestly and completely.

Benefit of the study

Information obtained from this study will benefit you as a participant, doctors and researchers for the advancement of knowledge and practice of medicine in future.

It is important to explore your views and experience, as this information will be used to improve the referral process and genetic testing procedure.

Treatment you will receive after your participation in this study

No study product will be given to you at the end of your participation in the study as this is not a clinical trial or interventional study. Whether you complete the study or withdraw early, your doctor will discuss the best alternatives for your future treatment with you.

You and your family are encouraged to continue long term follow-up treatment at the UiTM Lipid Specialist Clinic if you are diagnosed to have FH. The cost of the treatment is subjected to the university regulation. If you are a civil servant or pensioner, you may be entitled for free treatment at UiTM.

Study risk

This study is minimum risk as it does not involve collection of biological specimen e.g. blood. There is no direct risk associated with this study.

Confidentiality

All information obtained in this study will be kept and handled in a confidential manner, in accordance with applicable laws and/or regulations. The audio recording will be de-identified, and there will be no mention of your personal identifying information such as names, IC number, etc. during the interview. The audio recording is for transcription purposes and will not be copied/sent to any other individual or used for any other purpose. After transcription, the audio recording will be disposed of securely.

The information collected will be analysed anonymously and will be stored at both UiTM and the University of Nottingham, United Kingdom. It will be coded and information that can identify you will be removed. Only your study doctor and study staff will be able to link the code with you. When analyzing, publishing or presenting the study results, your identity will not be revealed without your expressed consent. Individuals involved in this study, qualified monitors and auditors, and governmental or regulatory authorities may inspect the study data, where appropriate and necessary. Data will be destroyed after the maximum data storage period of 10 years.

Participant Information Sheet and Consent Form – English version 1.2, dated 24 March 2020

APPENDIX 5

By signing this consent form, you will authorize the audio-recording of the session, review of medical records, analysis and use of the data arising from this study. You will be informed if new information relevant to consent becomes available and you will be asked to re-consent if the needs arise.

Contact information

If you have any questions about your rights as a participant in this study, please contact:

1. Professor Dr. Anis Safura Ramli, the Project Leader at [redacted].
2. The Secretary, Medical Research & Ethics Committee, Ministry of Health Malaysia, at telephone number 03-3362 8407/8205/8888.

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PARTICIPANT INFORMATION SHEET & CONSENT FORM: REDUCING PREMATURE CORONARY ARTERY DISEASE IN MALAYSIA BY EARLY IDENTIFICATION OF FAMILIAL HYPERCHOLESTEROLAEMIA

Consent Form

I herewith confirm that I have met the requirement of age and am capable of acting on behalf of myself as follows:

1. I understand the nature and scope of the research being undertaken.
2. I have read and understood all the terms and conditions of my participation in the research.
3. All my questions relating to this research and my participation therein have been answered to my satisfaction.
4. I voluntarily agree to take part in this research, to follow the study procedures and to provide all necessary information to the researcher(s) as requested.
5. I agree to be contacted by the researcher(s) if there is additional information needed for the research.
6. I may at any time choose to withdraw from this research without giving reasons.
7. I understand that study staff, qualified monitors and auditors, the sponsor or its affiliates, and governmental or regulatory authorities, have direct access to my medical record in order to make sure that the study is conducted correctly and the data are recorded correctly. All personal details will be treated as STRICTLY CONFIDENTIAL.
8. I have received a copy of the Participant Information Sheet, have read and understood its content.
9. I agree/disagree* for my family doctor to be informed of my participation in this study. (*delete which is not applicable)
10. Except for damages resulting from negligent or malicious conduct of the researcher(s), I hereby release and discharge UiTM and University of Nottingham, UK and all participating researchers from all liability associated with, arising out of, or related to my participation and agree to hold them harmless from any harm or loss that may be incurred by me due to my participation in the research.

Participant:

Signature: I/C number:

Name: Date:

Investigator conducting informed consent:

Signature: I/C number:

Name: Date:

Impartial witness: *(Required if participant is illiterate and contents of participant information sheet is orally communicated to participant)*

Signature: I/C number:

Name: Date:

Participant Information Sheet and Consent Form – English version 1.2, dated 24 March 2020

APPENDIX 6

RESEARCH MATERIALS | MANUFACTURER

Chemicals and Reagents

TE buffer	Lucigen, USA
RNase A	Lucigen, USA
MPC Protein Precipitation Reagent	Lucigen, USA
Red Cell Lysis Solution	Lucigen, USA
Tissue & Cell Lysis Solution	Lucigen, USA
Ethanol	R&M Chemical, UK
Isopropyl Alcohol (2-Propanol)	R&M Chemical, UK
1% of agarose powder	Choice-Care Sdn. Bhd.
1X Tris-Borate-EDTA (TBE) buffer	Biobasic, Singapore
SYBR Safe DNA Gel Stain	Thermo Fisher Scientific, USA
6X TriTrack DNA Loading Dye	Thermo Fisher Scientific, USA
DNA Ladder	SMOBIO Technology, Taiwan
DNA loading dye	SMOBIO Technology, Taiwan
Qubit® dsDNA HS Reagent (Component A)	Thermo Fisher Scientific, USA
Qubit® dsDNA HS Buffer (Component B)	Thermo Fisher Scientific, USA
Qubit® dsDNA HS Standard #1 (Component C)	Thermo Fisher Scientific, USA
Qubit® dsDNA HS Standard #2 (Component D)	Thermo Fisher Scientific, USA
Low TE	Illumina, US
2X Ampliseq Custom DNA Panel	Illumina, US
5X Ampliseq HiFi Mix	Illumina, US
FuPa Reagent	Illumina, US
DNA Ligase	Illumina, US
Ampliseq CD Indexes	Illumina, US
Switch Solution	Illumina, US
AMPure XP Beads	Illumina, US
1X Library Amp Mix	Illumina, US
10X Library Amp Primers	Illumina, US
PhiX	Illumina, US

APPENDIX 6

Kits

MasterPure™ DNA Purification Kit for Blood Version II	Lucigen, USA
Qubit® dsDNA HS (High Sensitivity) Assay Kit	Thermo Fisher Scientific, USA
Ampliseq Custom DNA Panel Kit	Illumina, US

Apparatus

EDTA Vacutainer Tubes	Becton Dickinson, USA
Centrifuge Tubes	Thermo Fisher Scientific, USA
Microcentrifuge Tubes	Eppendorf, Germany
Micropipette Tips	Eppendorf, Germany
PCR Tubes	Eppendorf, Germany
Invitrogen™ DynaMag™-96 Side Magnet On demand	Thermo Fisher Scientific, USA

Laboratory Equipment

Micropipette	Eppendorf, Germany
Biomedical freezer: -20 and -80°C	Sanyo, Japan
Single Vortex mixer	Grant Instruments, UK
Thermo Shaker	Grant Instruments, UK
Centrifuge; Minispin, 5430R and 5810R	Eppendorf, Germany
Gel Doc™ XR+ System with Image Lab™ Software	Bio-Rad Laboratories, Malaysia
SpectraMax® QuickDrop™ Micro-Volume Spectrophotometer	Molecular Devices, California
Qubit fluorometer	Thermo Fisher Scientific, USA
Shaker	
Thermal Cycler A1, 96 Well, 384 well	Antylia Scientific, US
Illumina iSeq 100	Illumina, US

AUTHOR'S PROFILE

Nur Syahirah Binti Shahuri graduated from the University of Malaysia Terengganu in October 2020 with a Bachelor of Science (Hons.) in Biology. She is now studying her Master of Science (Medicine) at Universiti Teknologi MARA. Her MSc thesis is on identifying familial hypercholesterolaemia in Malaysian primary care patients using targeted next generation sequencing (NGS).

List of Publication:

Shahuri, N. S., Kasim, N., Nawawi, H., Chua, Y.-A., Al-Khateeb, A., & Abdul Kadir, S. H. (2024). Familial Hypercholesterolaemia Patients with *LDLR* Mutation Among Asian Population in Southeast Asian Countries: Systematic Review. *Scientific Journal of King Faisal University: Basic and Applied Sciences*, 15–21.https://doi.org/10.37575/b/med/240022

Kamal, A., Kanchau, J. D., Shahuri, N. S., Mohamed-Yassin, M. S., Baharudin, N., Razak, S. A., ... & Ramli, A. S. (2023). Case Series of Genetically Confirmed Index Cases of Familial Hypercholesterolemia in Primary Care. The American Journal of Case Reports, 24, e939489-1.

Ramli, A. S., Qureshi, N., Abdul-Hamid, H., Kamal, A.,Kanchau, J. D., Shahuri, N. S., ... & Nawawi, H. (2023). Reducing Premature Coronary Artery Disease in Malaysia by Early Identification of Familial Hypercholesterolemia Using the Familial Hypercholesterolemia Case Ascertainment Tool (FAMCAT): Protocol for a Mixed Sim, S.F., T.Y. Ling, S. Lau, and M.Z. Jaafar, A novel computer-

aided multivariate water quality index. Environ Monit Assess, 2015. 187(4): p. 181.