THE EVALUATION OF *Etlingera elatior* FLOWER (BUNGA KANTAN) AQUEOUS EXTRACT (EEAE) ON FATTY LIVER DISEASE IN HYPERCHOLESTEROLAEMIC RATS

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UNIVERSITI SAINS MALAYSIA
2025

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by

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Dissertation submitted in partial fulfillment
of the requirements for the degree
of Bachelor of Health Science (Honours)
(Biomedicine)

January 2025

CERTIFICATE

This is to certify that the dissertation entitled "The Evaluation of *Etlingera elatior* Flower (Bunga Kantan) Aqueous Extract (EEAE) on Fatty Liver Disease in Hypercholesterolaemic Rats" is the bona fide record of research work done by Ms. Betty Wong Shi Ting during the period from August 2024 to January 2025 under my supervision. I have read this dissertation and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation to be submitted in partial fulfillment for the degree of Bachelor of Health Science (Honours) (Biomedicine).

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DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated and duly acknowledged. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at Universiti Sains Malaysia or other institutions. I grant Universiti Sains Malaysia the right to use the dissertation for teaching, research and promotional purposes.

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ACKNOWLEDGEMENTS

First and foremost, I would like to thank and appreciate God for all the guidance, strength, and blessings throughout this journey to complete this research project at the School of Health Sciences, Universiti Sains Malaysia, Health Campus, Kelantan.

I would also like to express my deepest appreciation and gratitude to my supervisor, Associate Professor Dr. Wan Amir Nizam Wan Ahmad, and my co-supervisor, Dr. Liza Noordin and Ms. Noor Fadzlina Binti Hamid, for their continuous guidance, encouragement, support, and patience in helping and supervising me throughout this research project and thesis writing. I am also deeply grateful to the postgraduate students of my supervisor, Mr. Fazalda Annuar and Ms. Lee Yuen Shin, for their expertise, assistance, and advice during the research process, as their willingness to share knowledge and provide guidance helped and inspired me. Special thanks to my research team partners, Sharran A/L Selvapaandian, Toh Zeny, and Wee Lu Shuang, for their help, support, and motivation throughout the process of completing this study. I sincerely thank the staff of the Animal Research and Service Centre (ARASC) and all the laboratory staff of the School of Health Sciences (PPSK) Department for their support and cooperation during my study period.

Lastly, I am deeply thankful to my family and friends for their unconditional support, love, understanding, and encouragement throughout my academic journey. Their patience during challenges and unwavering belief in my abilities motivated me. Thanks to everyone involved, directly and indirectly, for their collective support, who played a crucial role in completing this research project. I am grateful for their contributions and support throughout this journey in helping me complete this research project.

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LIST OF SYMBOLS

-	To
%	Percentage
<	Less than
=	Equal
>	More than / Greater than
±	Plus-minus
×	Multiply
°C	Degree Celsius
cm	Centimetre
g	Gram
g/cm^2	Grams per square centimetre
g/L	Grams per litre
kg	Kilogram
L	Litre
mg	Milligrams
mg/dL	Milligrams per decilitre
mg/kg	Milligrams per kilogram
mg/mL	Milligrams per millilitre
mL	Millilitre
mL/min	Millilitres per minute
mm	Millimetre
mmol/L	Millimoles per litre
N	Normality

n	Sample Size
nm	Nanometre
ppm	Parts per million
U/L	Units per litre
µL	Microlitre
µm	Micrometre
µmol/L	Micromoles per litre

LIST OF ABBREVIATIONS

A/G	Albumin to Globulin
AASLD	American Association for the Study of Liver Diseases
ABCA1	Adenosine Triphosphate Binding Cassette Subfamily A Member 1
ABCB11	Bile Salt Export Pump
ABCG5/G8	Adenosine Triphosphate Binding Cassette Subfamily G Members 5/8
ABCG8	Adenosine Triphosphate Binding Cassette Subfamily G Members 8
ACAT2	Acetyl-Coa Acetyltransferase 2
ACS	Acute Coronary Syndrome
AFLD	Alcoholic Fatty Liver Disease
ALK1	Activin A Receptor-Like Type 1
ALP	Alkaline phosphatase
ALT	Alanine Aminotransferase
ANOVA	Analysis of Variance
Apo B	Apolipoprotein B
ARASC	Animal Research and Service Centre
AST	Aspartate Aminotransferase
BA	Bile Acid
BMI	Body Mass Index
CAT	Catalase
CE	Cholesterol Esters
CETP	Cholesteryl Ester Transfer Protein
CM	Chylomicrons
CREB	cAMP Regulatory Element-Binding Protein

CT	Computed Tomography
CTGF	Connective Tissue Growth Factor
CYP27A	Sterol 27-Hydroxylase
CYP7A1	Cholesterol 7α-Hydroxylase
EASD	European Association for the Study of Diabetes
EASL	European Association for the Study of Liver
EASO	European Association for the Study of Obesity
EEAE	*E. elatior* Flower Aqueous Extract
ER	Endoplasmic Reticulum
FC	Free Cholesterol
FDA	Food and Drug Administration
FGF19	Fibroblast Growth Factor 19
FH	Familial Hypercholesterolaemia
FRIM	Forest Research Institute Malaysia
FXR	Farnesoid X Receptor
GGT	Gamma-Glutamyl Transferase
GSH	Glutathione
H&E	Haematoxylin and Eosin
H_2SO_4	Sulphuric Acid
H_3BO_3	Boric Acid
HC	Hypercholesterolaemia
HCC	Hepatocellular Carcinoma
HCD	High-Cholesterol Diet
HCl	Hydrochloric Acid
HDL	High-Density Lipoprotein

HDL-C	HDL Cholesterol
HMGCR	3-Hydroxy-3-Methylglutaryl-Coa Reductase
HPLC	High-Performance Liquid Chromatography
HSCs	Hepatic Stellate Cells
IACUC	Institutional Animal Care and Use Committee
IDL	Intermediate-Density Lipoprotein
IIUM	International Islamic University of Malaysia
IL-6	Interleukin-6
IL-8	Interleukin-8
IP	Intraperitoneal
IR	Insulin Resistance
JNK	c-Jun N-terminal Kinase
KCs	Kupffer Cells
LDL	Low-Density Lipoprotein
LDL-C	LDL Cholesterol
LDLR	LDL Receptor
LFT	Liver Function Tests
LXR	Liver X Receptor
MDA	Malondialdehyde
MPO	Myeloperoxidase
MRI	Magnetic Resonance Imaging
MT	Masson’s Trichrome
NAFL	Non-Alcoholic Fatty Liver
NAFLD	Non-Alcoholic Fatty Liver Disease
NaOH	Sodium Hydroxide

NASH	Non-Alcoholic Steatohepatitis
nCEH	Neutral Cholesterol Ester Hydrolase
NHMS	National Health and Morbidity Surveys
NICE	National Institute for Health and Care Excellence
NPC1L1	Niemann–Pick C1-Like 1
ox-LDL	Oxidised LDL
PAD	Peripheral Artery Disease
PCSK9	Proprotein Convertase Subtilisin/Kexin Type 9
PDA	Photodiode Array
PPARα	Peroxisome Proliferator-Activated Receptor Alpha
PPSK	School of Health Sciences
PTA	Phosphotungstic Acid
PUFAs	Polyunsaturated Fatty Acids
ROS	Reactive Oxygen Species
RXR	Retinoid X Receptor
SEM	Standard Error of the Mean
SCP-2	Sterol Carrier Protein 2
SD	Sprague-Dawley
SGOT	Serum Glutamic-Oxaloacetic Transaminase
SGPT	Serum Glutamic-Pyruvic Transaminase
SHP	Small Heterodimer Partner
SOD	Superoxide Dismutase
SR-BI	Scavenger Receptor Class B Type I
SREBP-2	Sterol Regulatory Element-Binding Protein 2
T2DM	Type 2 Diabetes

T-AOC	Total Antioxidant Capacity
TC	Total Cholesterol
TG	Triglycerides
TGF-β	Transforming Growth Factor-β
TLR-4	Toll-Like Receptor 4
TNF-α	Tumour Necrosis Factor-Alpha
UAE	Ultrasonic-Assisted Extraction
UPR	Unfolded Protein Response
US	Ultrasound
VLDL	Very-Low-Density Lipoprotein
WHO	World Health Organisation

PENILAIAN EKSTRAK AKUEUS BUNGA KANTAN (*Etlingera elatior*) (EEAE) TERHADAP PENYAKIT HATI BERLEMAK PADA TIKUS HIPERKOLESTEROLEMIK

ABSTRAK

Penyakit hati berlemak bukan alkohol (NAFLD) adalah penyebab utama penyakit hati kronik di seluruh dunia. Hiperkolesterolemia (HC) adalah penyebab utama NAFLD kerana pengambilan makanan kolesterol tinggi (HCD) yang berlebihan mengganggu homeostasis kolesterol. Ubat-ubatan sedia ada untuk rawatan NAFLD membawa pelbagai kesan sampingan, dan ubat tidak diluluskan oleh Pentadbiran Makanan dan Ubat-ubatan (FDA). Tumbuhan ubatan, bunga kantan (*Etlingera elatior*), menunjukkan sifat antioksidan dan hepatoprotektif. Kajian ini bertujuan untuk menilai kesan ekstrak akueus bunga kantan (EEAE) pada model tikus hiperkolesterolemik yang diinduksi HCD. Sebanyak lapan belas tikus jantan Sprague-Dawley dibahagikan secara rawak kepada tiga kumpulan (n = 6): tikus kawalan (Kumpulan 1), tikus HC tidak dirawat (Kumpulan 2), dan tikus HC dirawat dengan 1000 mg/kg EEAE (Kumpulan 3). Tikus diberi HCD selama enam minggu untuk mengaruh HC, diikuti dengan enam minggu rawatan oral EEAE. EEAE mengurangkan indeks jisim badan (BMI) dan tahap kolesterol darah dengan ketara. Pemeriksaan histopatologi hati menggunakan pewarnaan hematoksilin dan eosin (H&E) serta pewarnaan Masson Trichrome (MT) dinilai. Kumpulan HC tidak dirawat mengalami steatohepatitis bukan alkohol (NASH), yang dicirikan dengan steatosis makrovesikular, keradangan, degenerasi balon, dan fibrosis. Sebaliknya, tisu hati dalam kumpulan HC EEAE-terawat dapat dipulihkan dari NASH ke hati berlemak bukan alkohol (NAFL).

Selain itu, bunga kantan dinilai untuk komposisi nutrisinya, dan analisis HPLC menunjukkan bahawa EEAE mengandungi kandungan sianidin-3-O-glukosida klorida yang tinggi. Kesan EEAE pada nisbah berat hati terhadap berat badan memerlukan penilaian lanjut. Ujian fungsi hati (LFT) dan ujian profil lipid telah dijalankan untuk menilai kesan EEAE pada tikus hiperkolesterolemik. Secara keseluruhan, EEAE menunjukkan kesan antioksidan, anti-radang, dan antihiperkolesterolemia dalam hati.

THE EVALUATION OF *Etlingera elatior* FLOWER (BUNGA KANTAN) AQUEOUS EXTRACT (EEAE) ON FATTY LIVER DISEASE IN HYPERCHOLESTEROLAEMIC RATS

ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease with increasing prevalence worldwide. Hypercholesterolaemia (HC) is the leading cause of NAFLD because excessive high-cholesterol diet (HCD) intake disrupts cholesterol homeostasis. The current medications for NAFLD treatment bring various side effects, and there are no drugs approved by the Food and Drug Administration (FDA). A medicinal plant, *Etlingera elatior* flower, exhibits antioxidants and hepatoprotective properties. This study aims to evaluate the effects of *Etlingera elatior* flower (bunga kantan) aqueous extract (EEAE) on the HCD-induced hypercholesterolaemic rat model. Eighteen male Sprague-Dawley (SD) rats were randomly divided into three groups (n = 6): control rat (Group 1), HC-untreated rat (Group 2), and HC-treated 1000 mg/kg EEAE rat (Group 3). The rats were fed HCD for six weeks to induce HC, followed by six weeks of oral EEAE treatment. EEAE significantly reduced body mass index (BMI) and blood cholesterol levels. The histopathological examination of livers for Haematoxylin and Eosin (H&E) staining and Masson's Trichrome (MT) staining was evaluated. The HC-untreated group developed non-alcoholic steatohepatitis (NASH), characterised by macrovesicular steatosis, inflammation, ballooning degeneration, and fibrosis. In contrast, the liver tissue in the HC-treated EEAE group was reversible from NASH to non-alcoholic fatty liver (NAFL). Furthermore, *E. elatior* flower was evaluated for its nutritional composition, and HPLC analysis demonstrated that EEAE contains high levels

of cyanidin-3-O-glucoside chloride. The effect of EEAE on the liver weight to body weight ratio required further evaluation. Liver function tests (LFT) and lipid profile tests were conducted to evaluate the effect of EEAE in hypercholesterolaemic rats. Overall, EEAE exhibits antioxidant, anti-inflammatory, and antihypercholesterolaemia effects in the liver.

CHAPTER 1: INTRODUCTION

1.1 Background of Study

Fatty liver disease, also known as hepatic steatosis, is a condition characterised by the accumulation of excess fat in the liver cells. There are two main types of fatty liver disease, including alcoholic fatty liver disease (AFLD) and non-alcoholic fatty liver disease (NAFLD). AFLD is a condition caused by excessive alcohol consumption, whereas NAFLD is a condition that occurs in individuals who consume little to no alcohol (Avisena Specialist Hospital Shah Alam, 2023). In this study, NAFLD is conducted and induced in SD rats by feeding them a high-cholesterol diet (HCD). High cholesterol (hypercholesterolaemia) can transform fatty liver disease into a more serious and fatal condition known as non-alcoholic steatohepatitis (NASH). NAFLD is the accumulation of triglycerides in hepatocytes, occurring in people who drink little or no alcohol, the use of medications that cause steatosis, or the presence of other chronic liver diseases. NAFLD can be considered a disorder of fat metabolism associated with obesity, insulin resistance (IR), type 2 diabetes (T2DM), and hypercholesterolaemia (HC) or dyslipidaemia. This can occur due to an excessive influx of fat into the liver or a reduction in hepatic beta-oxidation (Kemas *et al.*, 2022).

NAFLD encompasses a spectrum of liver conditions ranging from simple hepatic steatosis to NASH, which can progress to liver fibrosis and cirrhosis. The study of NAFLD is crucial due to its increasing incidence and prevalence worldwide. According to Teng *et al*. (2022), the incidence of NAFLD in males was 70.8 cases per 1,000 person-years, while the incidence of NAFLD in females was 26.9 cases per 1,000 person-years. Globally, the estimated incidence of NAFLD from 1994 to 2018 was 47.0 cases per 1,000 population and was higher in males than in females. Besides, a meta-analysis focused on

NAFLD in Asia reported an incidence of 50.9 cases per 1,000 person-years between 2002 and 2017, with the highest incidence observed in Mainland China at 63.0 cases per 1,000 person-years and the lowest in Japan at 29.0 cases per 1,000 person-years. Based on Riazi *et al*. (2022), the estimated global prevalence of NAFLD in adults was 32.4% (1,030,160 individuals) from 1994 to 2019, with a higher prevalence in males (39.7%) than in females (25.6%). The prevalence of NAFLD increased from 25.5% in studies conducted before 2005 to 37.8% in studies conducted after 2016. In Asia, the prevalence of NAFLD was reported as 29.6% by Li *et al*. (2019). The prevalence of NAFLD in Southeast Asia was 38.5% in Malaysia, 40.43% in Singapore, and 51.04% in Indonesia. The prevalence increased over time, rising from 25.3% between 1995 and 2005 to 28.5% between 2006 and 2011 and 33.9% between 2012 and 2017. This occurred due to a higher prevalence of metabolic risk factors, including hypertension, HC, and hyperglycaemia. Mathematical modelling studies predict that the burden of NAFLD and NASH will continue to increase globally over the next 10 years. The global prevalence of NAFLD is expected to reach 55.4% by 2040 (Teng *et al.*, 2022).

NAFLD is now recognised as the most common chronic liver disease worldwide and is prevalent alongside metabolic syndrome and obesity. Patients with NAFLD are at increased risk of end-stage liver disease, hepatocellular carcinoma (HCC), and liver-related mortality. However, the most significant cause of death in patients with NAFLD is cardiovascular disease, followed by extrahepatic malignancies, while liver-related mortality is only the third leading cause of death (Tomeno *et al.*, 2020). Besides, NAFLD is strongly linked to oxidative stress. Oxidative stress is a condition characterised by an imbalance between the production of free radicals and antioxidant protection in the body (Arroyave-Ospina *et al.*, 2021). This imbalance occurs because of fat accumulation triggers metabolic disturbances, producing excessive mitochondrial reactive oxygen

species (ROS) and endoplasmic reticulum (ER) stress. These disturbances lead to inflammation, cellular dysfunction, injury, and cell death, with impaired antioxidant defences in the liver significantly contributing to the development and progression of NAFLD (Arroyave-Ospina *et al.*, 2021). The clinical and experimental evidence indicates that oxidative stress is a key factor in the pathophysiology of NAFLD. Therefore, various antioxidants are proposed as potential therapeutic agents for treating NAFLD (Arroyave-Ospina *et al.*, 2021). Medicinal plants are rich in natural products containing secondary metabolites with diverse pharmacological activities, including chemicals and antioxidants capable of stabilising free radicals, thereby delaying or inhibiting the oxidation process (Noordin *et al.*, 2022).

Etlingera elatior, commonly known as torch ginger or locally called "bunga kantan" in Malaysia, is one of the medicinal plants belonging to the ginger family, Zingiberaceae. It is native to Southeast Asia, including Malaysia (bunga kantan), Indonesia (bunga siantan), and Thailand (kaa laa). It is also widely cultivated for ornamental and culinary purposes (Ismail & Ridzuan, 2023). It is an essential ingredient in Malaysian cuisine and is widely used as a spice and fresh vegetable. It is known for its strikingly large inflorescences, which resemble torches, hence the name torch ginger. The flowers are bright and colourful, usually pink or red, and grow on tall, sturdy stems that can reach 1.8 to 7.5 meters in height (Gardenia.net, 2024). Due to its pharmaceutical properties, *E. elatior* plant is traditionally used as an alternative medicine to reduce diabetes and hypertension. This plant contains various bioactive compounds, including phenolics and flavonoids, with significant health benefits such as antioxidant, anticancer, antimicrobial, antidiabetic, and anti-inflammatory properties. Extensive research has recently demonstrated that EEAE possesses antioxidant activity, which helps to protect cells from oxidative stress, improves lipid metabolism, and reduces the risk of developing

NAFLD (Nor *et al.*, 2020). This study aims to evaluate the potential of EEAE in treating fatty liver disease by inducing HC in rats.

1.2 Problem Statement

NAFLD is the most common cause of chronic liver disease in industrialised countries, which poses a serious public health challenge. The incidence and prevalence of NAFLD are estimated to increase continuously over the next decade due to global obesity. NAFLD has a complex pathophysiology and is associated with multiple complications. If NAFLD is left untreated, it can progress to NASH, which can lead to cirrhosis and HCC. Currently, the treatment for NAFLD involves lifestyle changes such as diet and exercise, but some patients may struggle to maintain these improvements. In addition, some medications used to treat NAFLD can bring various side effects. This is because the FDA has approved no drugs for the treatment of NAFLD, so there is currently a lack of consensus on the most valuable and appropriate pharmacological therapy. Therefore, *E. elatior*, a medicinal plant, has been explored in previous findings to have potential anti-hypercholesterolaemic activity. This plant can lower HC, thereby reducing the risk of NAFLD. However, there is insufficient knowledge about EEAE as an alternative treatment for fatty liver disease in hypercholesterolaemic patients. Thus, this study aims to evaluate the effects of EEAE on fatty liver disease in rats with HC.

1.3 Research Questions

1. What are the nutritional compounds in the *E. elatior* flower?
2. Are there any effects of EEAE on cholesterol levels in hypercholesterolaemic rats?
3. What are the effects of EEAE on liver histology in hypercholesterolaemic rats?

1.4 Study Objectives

1.4.1 General Objective

To evaluate the effects of *Etlingera elatior* flower (bunga kantan) aqueous extract (EEAE) on fatty liver disease in hypercholesterolaemic rats.

1.4.2 Specific Objectives

1. To evaluate the nutritional compounds in the *E. elatior* flower.
2. To evaluate the effects of EEAE on cholesterol levels in hypercholesterolaemic rats.
3. To evaluate the liver histology between hypercholesterolaemic rats treated with EEAE and untreated hypercholesterolaemic rats.

1.5 Study Hypothesis

1.5.1 Null Hypothesis (H_0)

1. The *E. elatior* flower did not contain significant nutritional compounds.
2. The EEAE did not significantly affect cholesterol levels in hypercholesterolaemic rats.
3. There was no significant difference in liver histology between hypercholesterolaemic rats treated with EEAE and untreated hypercholesterolaemic rats.

1.5.2 Alternative Hypothesis (H_A)

1. The *E. elatior* flower contained significant nutritional compounds.
2. The EEAE significantly affected cholesterol levels in hypercholesterolaemic rats.
3. There was a significant difference in liver histology between hypercholesterolaemic rats treated with EEAE and untreated hypercholesterolaemic rats.

1.6 Significance of Study

The findings of this study will contribute to the development of accessible and affordable treatments that can be incorporated into public health strategies to reduce the incidence and prevalence of NAFLD worldwide. This study also makes a contribution to a research proof on *E. elatior* flower as a medicinal plant through the investigation of its hepatoprotective effect that contains natural antioxidant compounds neutralising excess free radicals to lower cholesterol and prevent fatty tissue peroxidation. The potent antioxidant potential and reducing activity of these flowers are beneficial in preventing oxidative damage and protecting liver cells and tissues. The phytochemical properties of *E. elatior* have gained global attention for their biological effects, including antioxidants, anticancer, and antimicrobial activities. In addition, these natural compounds will provide a safer alternative to synthetic drugs by reducing side effects commonly associated with current medicines used for NAFLD treatment. This medicinal plant can widely promote its medicinal and nutritional values by facilitating sustainable cultivation. This approach will create economic opportunities for the agricultural and nutraceutical industries, especially in its native Southeast Asian regions. Using hypercholesterolaemic rats as a model, researchers can gain insight into the progression of fatty liver disease in conditions that mimic human HC, leading to potential benefits for patients with metabolic symptoms.

CHAPTER 2: LITERATURE REVIEW

2.1 High-Cholesterol Diet (HCD)

2.1.1 Overview of Cholesterol

Cholesterol is a waxy, fat-like substance that plays an important role in the cellular structure and function of the body. It is an essential component of cell membranes, contributing to their structural composition and regulating their fluidity. It serves as a precursor for the biosynthesis of essential molecules, including vitamin D, steroid hormones such as cortisol, aldosterone, and adrenal androgens, and sex hormones such as testosterone, estrogen, and progesterone. It is also a component of bile salts, which aids in the digestion and absorption of fat-soluble vitamins A, D, E, and K. In addition, it can be found in animal fats, usually in its free form. Its primary food sources include egg yolks, milk and its derivatives, beef, shrimp, poultry skin, and organ meats (Cunha *et al.*, 2021; Huff *et al.*, 2023).

Furthermore, cholesterol, a lipophilic molecule, is transported in the bloodstream along with triglycerides (TG) within lipoprotein particles, including high-density lipoprotein (HDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL), very-low-density lipoprotein (VLDL), and chylomicrons (CM). The lipid profile can be measured in clinical settings to determine HDL, LDL, TG, and total cholesterol (TC) levels in the blood. HDL is considered “good” cholesterol as it helps remove cholesterol from the bloodstream. In contrast, LDL is known as “bad” cholesterol because it is associated with the development of atherosclerosis, which occurs when plaque (fatty deposits) builds up in the arteries (National Library of Medicine, 2024).

The blood cholesterol levels are measured in millimoles per litre (mmol/L). In humans, the Malaysian Clinical Practice Guideline states that normal cholesterol levels

are defined as TC levels below 5.2 mmol/L, HDL levels should exceed 1.0 mmol/L for males and 1.2 mmol/L for females, LDL levels below 2.6 mmol/L, and TG levels below 1.7 mmol/L (Baharudin *et al.*, 2022). Meanwhile, rats' normal blood cholesterol level is between 0.6 and 3.0 mmol/L (10 – 54 mg/dL) (Harini & Astirin, 2009). The body helps to maintain cholesterol homeostasis through dietary intake (absorption), endogenous synthesis in the liver, and elimination (Huff *et al.*, 2023). The dysregulation of cholesterol homeostasis can affect various cellular functions, leading to serious health problems such as HC and atherosclerosis (Yu & Han, 2024).

2.1.2 Impact of HCD on Humans

A diet high in cholesterol is characterised by excessive consumption of cholesterol-rich foods, including egg yolks, fatty meats, and full-fat dairy products, which can seriously affect human health. These diets can lead to the accumulation of cholesterol in the arteries. This can cause plaque formation, narrowing and hardening of the arteries, known as atherosclerosis. This condition increases the risk of hypertension, heart attack, and stroke. The consumption of HCD can disrupt lipid metabolism by raising LDL cholesterol (LDL-C) and TC levels in the bloodstream. High LDL-C levels contribute to plaque buildup on the arterial walls. This imbalance may lower HDL cholesterol (HDL-C), which helps to remove excess cholesterol by transporting it to the liver for excretion. In addition, HCD significantly affects liver health because the liver is central to cholesterol metabolism. Excess dietary cholesterol can overwhelm the liver, potentially leading to liver damage over time. This includes the accumulation of fat and cholesterol in liver cells, known as hepatic steatosis, a key feature of NAFLD (Huff *et al.*, 2023). Furthermore, high cholesterol levels increase the production of ROS, causing oxidative stress, cell damage and inflammation. In severe cases, this progresses to NASH, marked by liver inflammation, damage and fibrosis. Diets high in cholesterol are also usually high in calories, especially those containing processed or fried foods. Over time, this condition can lead to weight gain and obesity, which is another risk factor for NAFLD, heart disease, T2DM and other chronic diseases (Clemente-Suárez *et al.*, 2023).

2.1.3 Impact of HCD on Rats

In experimental studies, HCD in rats is commonly used to mimic HC in humans. Studies showed that prolonged exposure to high levels of cholesterol can lead to serious health problems in rats, closely mirroring human disease patterns. Inducing HC through HCD can significantly impact the liver of rats, leading to weight gain and structural damage. This damage is due to the accumulation of cholesterol in the liver. Rats fed with HCD can elevate serum cholesterol levels, especially LDL-C, a major risk factor for atherosclerosis. This disruption mimics dyslipidaemia in humans, resulting in an imbalance between LDL and HDL. In addition, high cholesterol levels can reduce the activity of antioxidant enzymes in the liver, such as superoxide dismutase (SOD) and catalase (CAT), weakening the antioxidant defences of the liver. These enzymes are essential in neutralising ROS, and their decreased activity exacerbates oxidative damage. The HCD can also reduce vitamin C levels in rats, further impairing their ability to combat oxidative stress (Cunha *et al.*, 2021).

2.2 Hypercholesterolaemia (HC)

2.2.1 Overview of HC

HC is defined as high cholesterol levels in the blood caused by an increase in cholesterol and apolipoprotein B (apoB)-rich lipoproteins, also commonly known as LDL (Hassan *et al.*, 2023). HC can be divided into two main types: primary and secondary HC. Primary HC, also called familial hypercholesterolaemia (FH), is a genetic disorder of lipoprotein metabolism that leads to elevated serum LDL-C levels, which increases the risk of premature cardiovascular diseases. FH usually manifests early, often in childhood, due to a genetic disorder inherited from family members (Varghese, 2014). In about 85% of cases, FH is caused by a mutation in the LDL receptor (LDLR) gene, resulting in LDLR deficiency or dysfunction (Yu & Han, 2024). Due to a decrease in LDLR activity in the liver, the rate of clearance of LDL from the bloodstream slows down. According to previous studies, the mutations in the LDLR gene can cause LDL-C levels greater than 190 mg/dL in heterozygotes and greater than 450 mg/dL in homozygotes. In some cases, mutations in other genes, including apoB and proprotein convertase subtilisin/kexin type 9 (PCSK9), can also contribute to FH. ApoB mutations affect the ability of LDL particles to bind to the LDLR, whereas PCSK9 mutations increase LDLR degradation, leading to elevated LDL-C levels (Ibrahim *et al.*, 2023).

Secondary HC, or acquired HC, develops due to non-genetic factors that disrupt cholesterol metabolism. These factors include unhealthy lifestyle habits, disease conditions, and the use of certain medications (Yu & Han, 2024). Unhealthy lifestyle habits, including a sedentary lifestyle and increased intake of saturated and trans fats, can significantly raise LDL-C levels. Besides, disease conditions such as diabetes mellitus, obesity, hypothyroidism, chronic kidney disease, and liver disease can cause cholesterol and lipid imbalance. For example, liver disease is associated with impaired lipoprotein

and bile acid (BA) synthesis and secretion, affecting the transport and excretion of cholesterol and TG from the liver. Depending on the type and severity of the liver disease, cholesterol and TG levels in the blood can be increased or decreased. Obesity is also linked to increased production of VLDL and decreased liver clearance of CM. This results in high TG and low HDL-C levels. Furthermore, certain drugs, including cyclosporine, thiazide, and diuretics, can alter lipid and lipoprotein metabolism, depending on the type of drug and dosage (Ibrahim *et al.*, 2023; Pappan *et al.*, 2024).

The World Health Organisation (WHO) reported that Europe (53.7%) and America (47.7%) had the highest prevalence of HC, whereas Southeast Asia (30.3%) and Africa (23.1%) had a much lower prevalence. In Malaysia, the National Health and Morbidity Surveys (NHMS) showed an increasing trend for HC in 2006, 2011 and 2015, reporting 20.7% (2006), 35.1% (2011) and 47.7% (2015). The NHMS also stated that the prevalence of overall raised blood cholesterol among Malaysians in 2019 was 38.1%. In addition, Chalitsios *et al.* (2023) found that HC was highly prevalent in 11,288 adults, with 64.0% having elevated total cholesterol (> 5.2 mmol/L) and 56.7% having elevated LDL-C (> 3.4 mmol/L). The broader factors contributing to the risk of HC include food insecurity, low health literacy, poverty, income disparities, rapid ageing societies, and rapid urbanisation (Ganapathy *et al.*, 2020; Mohamed-Yassin *et al.*, 2021; Chalitsios *et al.*, 2023).

2.2.2 Clinical Significance of HC

HC is a major risk factor for the formation of atherosclerotic plaques. These plaques increase the risk of serious conditions such as coronary artery disease, peripheral artery disease (PAD), aortic aneurysms, and stroke. High LDL levels in the blood are a major cause of atherosclerotic lesions. The development of atherosclerotic plaques begins with endothelial damage. Endothelial damage causes endothelial cell dysfunction, an early indicator of atherogenesis, allowing more LDL particles to permeate the vascular wall. The accumulation of LDL particles promotes transendothelial infiltration of circulating LDL into the vascular intima by transcytosis. LDL transcytosis is mediated by the endothelial scavenger receptor class B type I (SR-BI) and activin A receptor-like type 1 (ALK1) receptors (Jebari-Benslaiman *et al.*, 2022; Huff *et al.*, 2023).

Trapped LDL particles are oxidised in the subendothelial space by free radicals present in the extracellular media. LDL oxidation involves the depletion of antioxidants and the breakdown of polyunsaturated fatty acids (PUFAs), generating more reactive short-chain aldehydes. LDL can be modified to oxidised LDL (ox-LDL) during oxidative stress (Khatana *et al.*, 2020). Ox-LDL acts as a pro-inflammatory antigen, triggering an immune response by attracting monocyte-derived macrophages to combat it. These macrophages enter the activated endothelium to uptake the ox-LDL in the intima and secrete pro-inflammatory cytokines and ROS, which cause local inflammation. Due to the lack of negative feedback control, macrophage scavenger receptors continuously take up ox-LDL, resulting in excessive lipoprotein phagocytosis and the formation of foam cells (Liu *et al.*, 2023). The foam cells accumulate within the arterial intima to form fatty streaks, the earliest visible lesions in atherosclerosis (Yancey *et al.*, 2024).

As the inflammatory process continues, vascular smooth muscle cells from the media migrate to the intima and proliferate in response to growth factors released by foam

cells. These proliferating vascular smooth muscle cells produce extracellular matrix components such as collagen, elastin, and proteoglycans, forming a fibrous cap that encapsulates the necrotic core consisting of dead cells and lipid-rich debris and protects it from exposure to the vessel lumen (Jebari-Benslaiman *et al.*, 2022; Huff *et al.*, 2023). However, continuous exposure to an atherogenic environment makes plaques vulnerable when a large necrotic core, a thin fibrous cap, and an increased inflammatory response are present at the lesion site, making the fibrous cap prone to rupture. This rupture exposes the subendothelial space to the bloodstream, triggering a coagulation process that leads to fibrin formation. Fibrin covers the lesion, forming a stable and well-arranged structure known as the thrombus (Jebari-Benslaiman *et al.*, 2022). As a result, the thrombus formation can block the blood vessels, leading to ischemia and myocardial infarction or stroke (Huff *et al.*, 2023). Figure 2.1 illustrates the progression of atherosclerotic plaque formation.

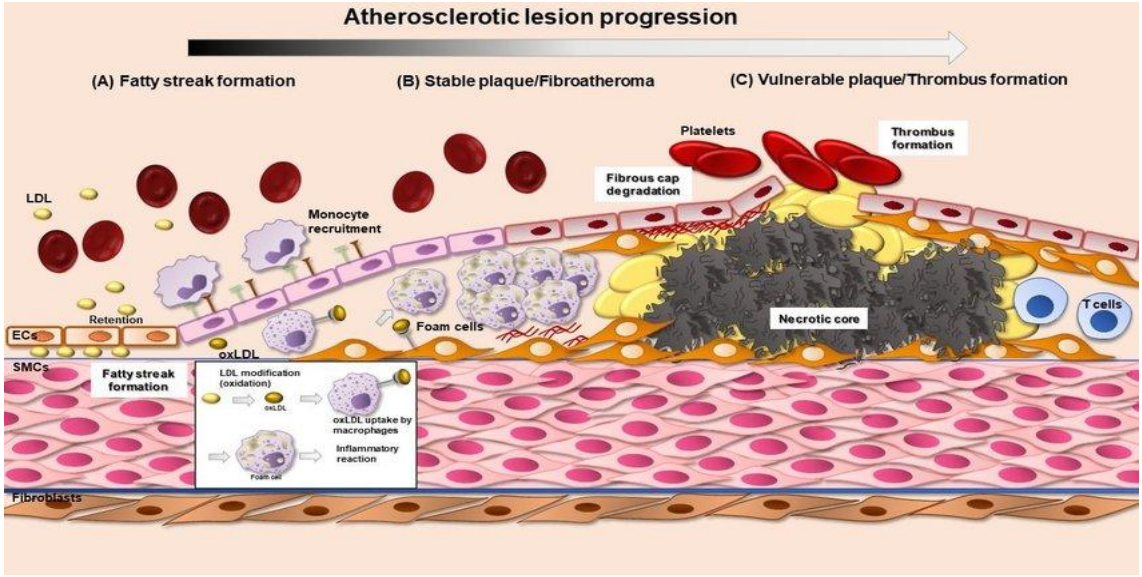

Figure 2.1: Progression of atherosclerotic plaque formation (adapted from Charla *et al.*, 2020).

2.3 Non-Alcoholic Fatty Liver Disease (NAFLD)

2.3.1 Overview of NAFLD

NAFLD has become the most common cause of chronic liver disease worldwide, evolving from a relatively unknown condition to a significant global health problem. NAFLD refers to a range of liver diseases characterised by excessive fat accumulation, with steatosis affecting more than 5% of hepatocytes, which can be identified through imaging or histological examination (Lujan *et al.*, 2021; Guo *et al.*, 2022). This condition is primarily linked to metabolic risk factors, including obesity, T2DM, metabolic syndrome, and dyslipidaemia, while excluding alcohol consumption and other chronic liver diseases as potential causes. The increase in NAFLD is strongly associated with a global economy and a Westernised lifestyle, presenting a significant yet often neglected public health challenge (Lujan *et al.*, 2021).

NAFLD can be classified into two types based on histological characteristics, including NAFL and NASH. NAFL is characterised by the buildup of liver fat (steatosis), with or without mild lobular inflammation. In contrast, NASH is associated with more severe liver damage, which includes hepatocyte ballooning degeneration, diffuse lobular inflammation, and fibrosis. Initially, healthy livers can develop into NAFL with hepatocellular steatosis. If left untreated, NAFL can progress to NASH, which may eventually lead to cirrhosis and potentially to HCC over time. The NAFL and NASH are reversible with lifestyle modifications and bariatric surgery. However, progression to cirrhosis and HCC marks stages where the damage becomes largely irreversible, requiring liver transplantation (Wang *et al.*, 2022). Although simple steatosis is often regarded as a "benign" condition, this progression highlights the significant role of NAFLD in liver disease-related mortality (Guo *et al.*, 2022). Figure 2.2 illustrates the progression of NAFLD.

Hyperlipidaemias, including hypertriglyceridaemia and HC, are associated with the development of NAFLD. Recent studies have explored the link between NAFLD and HC, as cholesterol can accumulate in the arteries and liver (Kim *et al.*, 2014). Elevated cholesterol levels can lead to liver damage through mechanisms such as lipotoxicity, oxidative stress, and inflammation, which exacerbate lipid imbalances and facilitate the progression from simple steatosis to NASH (Ipsen *et al.*, 2018).

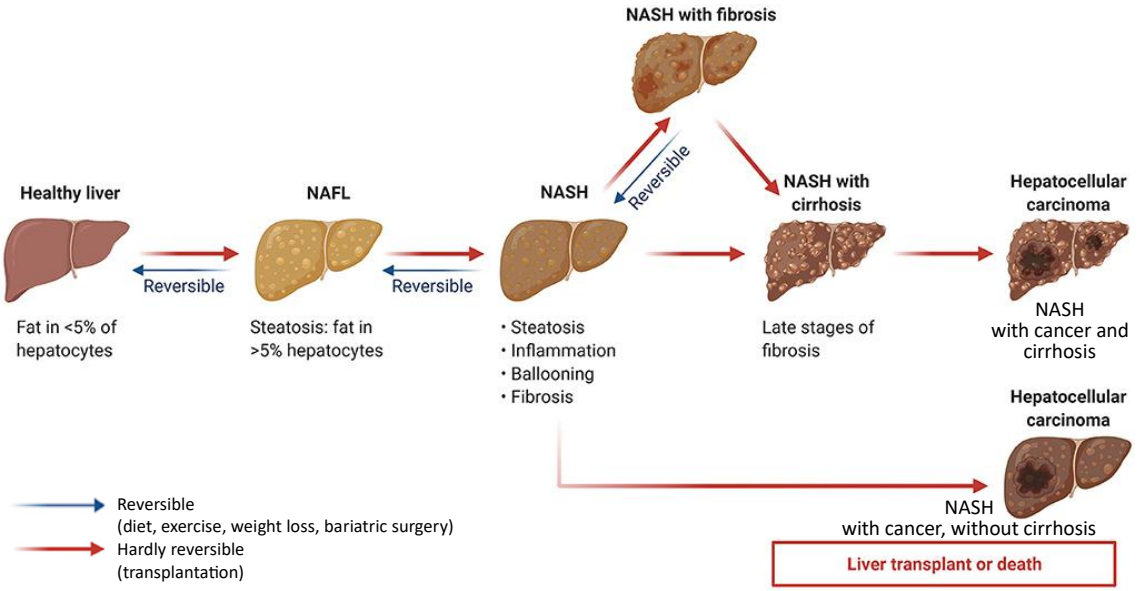

Figure 2.2: Progression of NAFLD (adapted from Wang *et al.*, 2022).

2.3.2 Pathophysiology of NAFLD

The exact pathophysiology of NAFLD remains incompletely understood, and current concepts regarding its disease pathogenesis are evolving with new findings from both experimental models and human studies (Arguello *et al.*, 2015). The hallmark of NAFLD is the excessive accumulation of neutral lipids in the liver due to an imbalance between lipid availability and lipid removal. Recent findings indicate that disruptions in cholesterol homeostasis and accumulation of free cholesterol (FC) in the liver are important factors in the development of NAFLD (Figure 2.3) (Perla *et al.*, 2017). Hepatic FC accumulation in NAFLD arises from disruptions in intracellular cholesterol transport and imbalances in cellular cholesterol regulation. These imbalances are marked by increased activation of cholesterol biosynthesis, enhanced cholesterol de-esterification, and reduced cholesterol export and BA synthesis pathways (Arguello *et al.*, 2015).

In plasma, patients with NAFLD often exhibit atherogenic dyslipidaemia, a lipid profile abnormality characterised by elevated plasma TG, CM, VLDL, and LDL levels, as well as decreased HDL and apoA-I levels (Li *et al.*, 2021). Decreased lipid-free or lipid-poor apoA-I levels produced by hepatocytes interact with decreased adenosine triphosphate binding cassette subfamily A member 1 (ABCA1) protein levels to take up FC, forming nascent HDL particles. Hepatic SR-BI selectively absorbs circulating HDL cholesterol esters (CE). Hepatic FC can be esterified by acetyl-CoA acetyltransferase 2 (ACAT2) enzyme. This enzyme facilitates the incorporation of CE into VLDL particles for secretion into the bloodstream. Excess TG in the liver can increase the secretion of VLDL into plasma. In circulation, VLDL is further converted into smaller LDL particles, subsequently raising LDL levels through cholesteryl ester transfer protein (CETP)-mediated exchange (Tacer & Rozman, 2011). The liver absorbs LDL particles through LDLR-mediated endocytosis on its hepatocyte membrane. In addition, the increased

expression of hepatic neutral cholesterol ester hydrolase (nCEH) results in elevated hepatic FC levels. This occurs because nCEH facilitates the de-esterification of CE, which releases cholesterol back into the intrahepatic free cholesterol pool (Min *et al.*, 2012). Furthermore, excess cholesterol secreted by the liver can be reabsorbed in the intestine to form CM, which enters the intestinal lymph. These CM particles are then transported back to the liver, facilitating cholesterol recycling through the enterohepatic circulation (Li *et al.*, 2021).

In the hepatocyte, the elevated expression and activity of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) in NAFLD patients, are associated with increased miR-34a levels, a microRNA that suppresses sirtuin-1 expression, which is a protein with histone deacetylase activity and reduces phosphorylated HMGCR levels (Min *et al.*, 2012). HMGCR is a membrane protein in the ER that plays a crucial role in cholesterol *de novo* synthesis in the liver (Shi *et al.*, 2022). It catalyses the rate-limiting reaction of this process, utilising acetyl-CoA, NADPH, and ATP to produce mevalonate, a key precursor in cholesterol biosynthesis (Li *et al.*, 2021). In the hepatocyte nucleus, the sterol regulatory element-binding protein 2 (SREBP-2), an intracellular transcription factor regulating cholesterol synthesis, is disrupted and increased to stimulate the expression of the HMGCR gene, enhancing cholesterol synthesis and FC accumulation. Furthermore, the down-regulation of the farnesoid X receptor (FXR) is associated with a decreased expression of the adenosine triphosphate binding cassette subfamily G members 8 (ABCG8) gene in the livers of patients with NAFLD. This gene encodes a half-transporter that facilitates biliary excretion and reduces the intestinal absorption of cholesterol. The down-regulation of ABCG8 contributes to FC accumulation in the liver (Arguello *et al.*, 2015). Besides, the co-regulatory interaction between FXR and liver X receptor (LXR) with retinoid X receptor (RXR) is crucial for their function as

transcription factors. In FC accumulation, the formation of heterodimers between these nuclear receptors and RXR may be hindered. This compromise weakens their ability to bind to DNA and effectively regulate gene expression (Arguello *et al.*, 2015; Calkin & Tontonoz, 2012). The activation of a small heterodimer partner (SHP) by FXR inhibits the expression of cholesterol 7α-hydroxylase (CYP7A1), which is the key enzyme in BA synthesis (Fuchs *et al.*, 2024).

The synthesis of BAs occurs via two main biosynthetic pathways, which are the classical pathway involving microsomal CYP7A1 and the alternative pathway involving mitochondrial sterol 27-hydroxylase (CYP27A). In NAFLD patients, the expression of CYP7A1 and CYP27A in the liver is decreased. This decrease limits the degradation of cholesterol and hinders the secretion of BA into the small intestine via the bile salt export pump (ABCB11), leading to intrahepatic cholesterol accumulation (Min *et al.*, 2012; Henkel *et al.*, 2013). Insulin appears to have dual effects on the transcription of the CYP7A1 gene. While physiological insulin concentrations can upregulate the human CYP7A1 gene, prolonged insulin treatment leads to the induction of SREBP-1c, inhibiting CYP7A1 gene transcription (Vargas-Alarcón *et al.*, 2024). As a result, worsening IR and hyperinsulinaemia may reduce CYP7A1 activity, leading to decreased BA synthesis, which subsequently raises cholesterol plasma levels and increases the risk of acute coronary syndrome (ACS) (Arguello *et al.*, 2015; Vargas-Alarcón *et al.*, 2024).

In addition, fibroblast growth factor 19 (FGF19) is a hormone or enterokine derived from the ileum that plays a significant role in hepatic lipid metabolism and glucose and protein homeostasis (Byrne & Targher, 2015). The expression of FGF19 is initiated by intestinal FXR signalling and is secreted into the portal circulation, where it binds to the FGFR4/β-klotho receptor complex located in hepatocytes. Once bound to its receptor, FGF19 regulates hepatic metabolism by inhibiting BA production and glucose

synthesis. It suppresses BA synthesis by inhibiting CYP7A1 and reduces gluconeogenesis by inactivating the transcription factor cAMP regulatory element-binding protein (CREB). In patients with NAFLD, FGF19 levels are reduced, resulting in elevated BA production, dysfunction of FXR-FGF19 feedback signalling, increased gluconeogenesis, and enhanced hepatic *de novo* lipogenesis pathways (Arguello *et al.*, 2015; Fuchs *et al.*, 2024).

Moreover, sterol carrier protein 2 (SCP-2) facilitates the transport of cholesterol to the canalicular membrane, where it is excreted into the bile through the adenosine triphosphate binding cassette subfamily G members 5/8 (ABCG5/G8), which functions as a heterodimeric complex under normal conditions (Arguello *et al.*, 2015; Zein *et al.*, 2019). Studies in humans and animals demonstrate that ABCG5/G8 play essential roles in regulating cholesterol secretion from the liver and the intestinal absorption of cholesterol and plant sterols (Wang *et al.*, 2020). In patients with NAFLD, biliary cholesterol excretion is reduced due to impaired ABCG5/G8, contributing to hepatic cholesterol accumulation and hepatic IR. Besides, the Niemann–Pick C1-Like 1 (NPC1L1) protein also plays an important role in NAFLD. It is found in the apical membrane of enterocytes and the canalicular membrane of hepatocytes. It acts as a cholesterol importer responsible for intestinal cholesterol absorption and prevents excessive cholesterol loss by transporting biliary cholesterol into hepatocytes (Park, 2013). In the enterocyte, NPC1L1 serves as the molecular target of ezetimibe, a clinically approved cholesterol absorption inhibitor used to manage dyslipidaemia, including HC (Yamanashi *et al.*, 2022). In NAFLD patients, the hepatic NPC1L1 on the bile canalicular membrane reabsorbs more cholesterol from bile into the liver. Therefore, recent studies have shown that the hepatic NPC1L1 expression exacerbates NAFLD (Toyoda *et al.*, 2019).

Cholesterol accumulation, especially elevated FC levels, significantly leads to liver injury in NAFLD (Figure 2.4) (Arguello *et al.*, 2015). The accumulation of FC in NAFLD causes liver damage by activating intracellular signalling pathways in non-parenchymal cells, such as Kupffer cells (KCs), hepatic stellate cells (HSCs), and hepatocytes. In the activated KCs, FC accumulation can trigger the release of pro-inflammatory cytokines, including interleukin-6 (IL-6), interleukin-8 (IL-8), and tumour necrosis factor-alpha (TNF-α), as well as pro-fibrotic factors like transforming growth factor-β (TGF-β), which amplify inflammation and fibrosis (Rafaqat *et al.*, 2024). In the activated HSCs, FC overload upregulated Toll-like receptor 4 (TLR-4), increasing sensitivity to TGF-β and promoting HSC activation and fibrogenesis. FC accumulation in hepatocytes induces lipotoxicity and cellular dysfunction, primarily through mitochondrial and ER damage. Within mitochondria, FC disrupts membrane dynamics, impairs glutathione (GSH) transport, and increases ROS production, leading to oxidative stress (Perla *et al.*, 2017). At the same time, FC in the ER triggers the unfolded protein response (UPR), causing ER stress, apoptosis, and activation of stress pathways like c-Jun N-terminal Kinase (JNK) signalling. These processes create a feedback loop where oxidative stress and lipid peroxidation products exacerbate damage to KCs, HSCs, and hepatocytes, perpetuating inflammation, steatosis, and fibrosis. This vicious cycle accelerates disease progression in NAFLD and NASH (Arguello *et al.*, 2015).

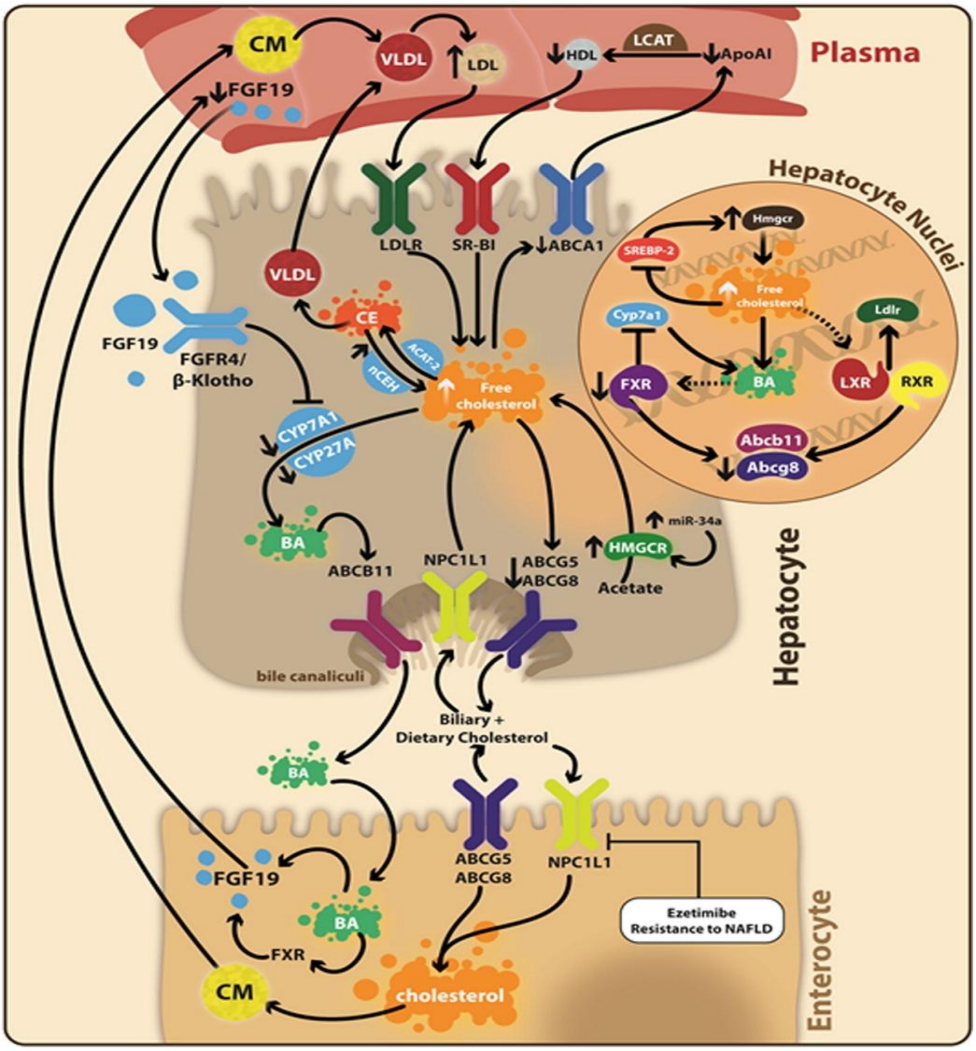

Figure 2.3: Accumulation of FC due to the dysregulation of cholesterol homeostasis in NAFLD (adapted from Arguello *et al.*, 2015).

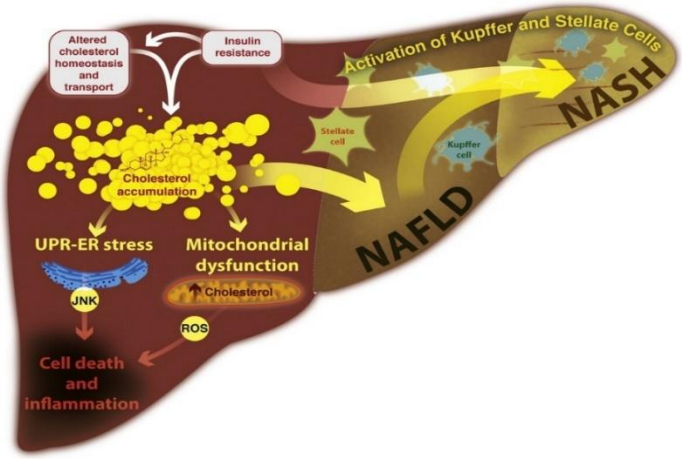

Figure 2.4: FC accumulation to liver damage in NAFLD and NASH (adapted from Arguello *et al.*, 2015).

2.3.3 Clinical Presentation of NAFLD

NAFLD is often asymptomatic in its early stages. Many individuals with NAFLD are often unaware of their condition, which usually remains undetected until it progresses to advanced stages, such as cirrhosis. However, some individuals may experience symptoms, including fatigue, pain or discomfort in the right upper quadrant abdomen where the liver is located, unexplained weight loss and weakness, or present with clinical signs such as hepatomegaly (enlarged liver), acanthosis nigricans (darkened and thickened skin), and lipomatosis (abnormal fat deposition) (Pouwels *et al.*, 2022). When NAFLD progresses to NASH or cirrhosis, more severe symptoms may develop. For example, abdominal swelling (ascites) is caused by fluid accumulation in the abdominal cavity, which results from decreased liver function and portal hypertension. Spider-like blood vessels, also known as spider angiomas, are small red or purple spots that may appear on the skin. In addition, an enlarged spleen (splenomegaly) may occur due to increased portal vein blood pressure. The yellowing of the skin and eye (jaundice) is caused by elevated bilirubin levels, indicating severe liver dysfunction (MedNotes, 2024). Therefore, most of the signs and symptoms of NAFLD are non-specific, making it difficult to identify the condition.

Most patients with NAFLD are diagnosed incidentally during routine health check-ups when elevated liver enzymes, including aspartate aminotransferase (AST) and alanine aminotransferase (ALT), which act as serum markers of hepatocellular injury, are detected. The elevations of ALT are more common than of AST (ALT > AST). ALT levels are typically higher in patients with NASH compared to those with simple steatosis. In addition, gamma-glutamyl transferase (GGT) levels can be elevated in NAFLD patients (Basaranoglu & Neuschwander-Tetri, 2006). Alkaline phosphatase (ALP) can also be elevated two to three times the upper normal limits, indicating elevated intrahepatic obstruction. Besides, imaging studies, such as abdominal ultrasound (US), computed