INVESTIGATING THE MECHANISM OF INTERACTION BETWEEN GRAPHENE OXIDE AND REDUCED GRAPHENE OXIDE WITH MACROPHAGE-DERIVED FOAM CELLS

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SYMBOLS

μ	Micro
<	Less than
α	Alpha
β	Beta
γ	Gamma
TM	Trademark
®	Registered

LIST OF ABBREVIATIONS

ABCA-1	ATP-Binding Cassette Transporter-1
ABCG1	Atp-Binding Cassette Transporter G1
ACAT	Acyl-Coa: Cholesterol Acyltransferase
APCs	Antigen-Presenting Cells
Apo	Apolipoprotein
ANXA	Annexins
CD	Cluster Of Differentiation
cDNA	Complementary DNA
CM	Chylomicrons
CVD	Cardiovascular Disease
DCs	Dendritic Cells
DL	Delta-Like
ECs	Endothelial Cells
ECM	Extracellular Matrix
ELISA	Enzyme Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
GAGs	Glycosaminoglycans
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GBN	Graphene-Based Nanomaterials
HDL	High-Density Lipoproteins
HSPG	Heparan Sulfate Proteoglycan
IDL	Intermediate-Density Lipoprotein

IFN	Interferon
IL	Interleukin
LAL	Lysosomal Acid Lipase
LDL	Low-Density Lipoprotein
LDLR	Low-Density Lipoprotein Receptor
LOX-1	Lectin-Like Oxidized LDL Receptor-1
LRP1	Low-Density Lipoprotein Receptor-Related Protein 1
ORO	Oil Red O
NOX	NADPH oxidases
MerTK	Mer Tyrosine Kinase
MMPs	Matrix Metalloproteinases
MPO	Myeloperoxidase
NF-kB	Nuclear Factor Kappa B
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
qPCR	Quantitativereal Time-Polymerase Chain Reaction
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SR	Scavenger Receptors
SMCs	Smooth Muscle Cells
TC	Total Cholesterol
TCR	T-Cell Receptor
TGF-β	Transforming Growth Factor-Beta

Th	T-Helper
TLR	Toll Like Receptor
TNF	Tumor Nuclear Factor
Treg T	Regulatory Cells
VCAM 1	Vascular Adhesion Molecule 1
VSMCs	Vascular Smooth Muscle Cells
WHO	World Health Organization

LIST OF APPENDICES

MENYIASAT MEKANISME INTERAKSI ANTARA GRAFENA OKSIDA DAN GRAFENA OKSIDA TERTURUN DENGAN SEL BUIH TERBITAN MAKROFAJ

ABSTRAK

Aterosklerosis ialah penyakit keradangan kronik yang berlaku akibat pengumpulan lipid dan pembentukan sel buih yang berasal daripada makrofaj. Bahan nano berasaskan grafena termasuk grafena oksida (GO) dan grafena oksida terturun (rGO) menunjukkan potensi dalam mempengaruhi metabolisme lipid dan keradangan. Kajian ini meneliti kesan GO dan rGO terhadap protein yang berkaitan dengan aterosklerosis iaitu CD36, LOX1, SRA1, TLR4, ApoB dan LDLR menggunakan kaedah pengiraan komputer serta kajian makmal. Pendokongan molekul dan simulasi dinamik molekul menunjukkan bahawa rGO mempunyai keupayaan ikatan yang lebih tinggi terhadap protein berkaitan lipid melalui interaksi bersifat hidrofobik, manakala GO membentuk kompleks yang lebih stabil melalui ikatan hidrogen dan interaksi elektrostatik. GO dan rGO disintesis daripada batang kelapa sawit dan pencirian fungsi permukaan dilakukan menggunakan spektroskopi Raman, FTIR, XRD dan AFM. GO menunjukkan pengoksidaan permukaan dan sifat hidrofilik yang lebih tinggi, manakala rGO mengekalkan sebahagian sifat hidrofobik. Kajian dalam makmal menunjukkan GO lebih bersifat toksik kepada makrofaj berbanding rGO kerana mencetuskan tekanan oksidatif yang bergantung kepada dos dan masa. Kedua-duanya menghalang pengumpulan lipid akibat rangsangan oleh kolesterol teroksida, dengan GO memberi kesan yang lebih kuat. Ujian ELISA mengesahkan tiada penghasilan sitokin yang bersifat pro-radang. Analisis ekspresi gen mendapati GO meningkatkan penghasilan gen ABCA1

dan mengurangkan penghasilan beberapa reseptor penangkap serta gen keradangan, sekali gus menggalakkan pengaliran keluar kolesterol. rGO pula menunjukkan corak ekspresi berbeza dengan peningkatan penghasilan gen SRA1 dan IL-1 beta. Pemprofilan metabolomik menunjukkan pengubahsuaian metabolik yang khusus mengikut keadaan. Biosintesis glukosinolat didapati terganggu secara konsisten, dengan fenilalanina dan tirosina umumnya meningkat, manakala rGO yang digabungkan dengan oxLDL menghasilkan kesan yang lebih terhad kepada fenilalanina sahaja. Dalam metabolisme steroid, oxLDL dan GO+oxLDL meningkatkan sintesis aldosteron dengan pengurangan 11-deoksikortikosteron, manakala GO, rGO, dan rGO+oxLDL mengalihkan metabolisme ke arah penghasilan estron. Kesimpulannya, tahap kefungsian oksigen merupakan faktor penentu utama terhadap tindak balas biologi. GO menunjukkan kesan anti-aterogenik yang lebih luas dengan meningkatkan pengaliran keluar kolesterol serta mendorong pengubahsuaian metabolik yang menyeluruh, manakala rGO memberikan pengaruh yang lebih terpilih, khususnya melalui pengawalan SRA1 dan penghasilan estron. Penemuan ini menekankan potensi penyesuaian kimia permukaan grafena berasaskan nanomaterial untuk aplikasi terapeutik dalam aterosklerosis.

INVESTIGATING THE MECHANISM OF INTERACTION BETWEEN GRAPHENE OXIDE AND REDUCED GRAPHENE OXIDE WITH MACROPHAGE-DERIVED FOAM CELLS

ABSTRACT

Atherosclerosis is a chronic inflammatory disease driven by lipid accumulation and macrophage-derived foam cell formation. Graphene-based nanomaterials, including graphene oxide (GO) and reduced graphene oxide (rGO), have shown potential in modulating lipid metabolism and inflammation. This study examined the effects of GO and rGO on atherosclerosis-related proteins (CD36, LOX1, SRA1, TLR4, ApoB, LDLR) using computational and experimental approaches. Molecular docking and molecular dynamics simulations revealed that rGO displayed higher binding affinity for lipid-associated proteins via hydrophobic interactions, while GO formed more stable complexes through hydrogen bonding and electrostatic interactions. GO and rGO were synthesised from oil palm trunk (OPT) and surface functionalisation was characterised by Raman spectroscopy, FTIR, XRD, and AFM. GO exhibited higher oxygen functionalisation and hydrophilicity, whereas rGO retained partial hydrophobicity. In vitro assays showed that GO was more cytotoxic to macrophages than rGO, inducing oxidative stress in a dose- and time-dependent manner. Both inhibited oxLDL-induced lipid accumulation, with GO demonstrating a stronger effect. ELISA confirmed no induction of pro-inflammatory cytokines. Gene expression analysis indicated that GO upregulated ABCA1 and downregulated multiple scavenger receptors and inflammatory genes, promoting cholesterol efflux, while rGO showed a distinct expression pattern with SRA1 and IL-1β upregulation. Metabolomic profiling demonstrated condition-specific

metabolic reprogramming. Glucosinolate biosynthesis was consistently perturbed, with phenylalanine and tyrosine generally elevated, while rGO combined with oxLDL produced a narrower effect limited to phenylalanine. In steroid metabolism, oxLDL and GO+oxLDL enhanced aldosterone synthesis with reduced 11-deoxycorticosterone, whereas GO, rGO, and rGO+oxLDL redirected metabolism toward estrone. In conclusion, the degree of oxygen functionalisation was a key determinant of biological responses. GO exerted broader anti-atherogenic effects by promoting cholesterol efflux and driving extensive metabolic reprogramming, while rGO induced a more selective influence, largely linked to SRA1 regulation and estrone production. These findings highlight the potential of tailoring graphene-based nanomaterials for therapeutic applications in atherosclerosis.

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Atherosclerosis is a chronic and progressive arterial condition characterised by lipid accumulation, inflammatory cell infiltration, and extracellular matrix deposition in the intima layer of the artery wall. This process results in the development of atherosclerotic plaques that may impede blood flow, causing significant cardiovascular issues such as myocardial infarction, stroke, and peripheral artery disease, which collectively account for around 17.3 million fatalities globally each year (Libby et al., 2019). The term "atherosclerosis" originates from the Greek terms "atherosis" (lipid-laden cells) and "sclerosis" (fibrotic layer), signifying the lipid and fibrotic elements of the condition (Ross, 1999; Rahimi, 2012).

The pathophysiology of atherosclerosis initiates with cholesterol accumulation in the artery intima and surrounding smooth muscle layers, resulting in the formation of fatty deposits termed atheromatous plaques. These plaques expand over time as fibrous tissue and smooth muscle cells multiply, protruding into the artery lumen and diminishing blood flow (Tavafi, 2013). Connective tissue, fibroblasts, and calcium contribute to arterial hardening, known as sclerosis, while thrombosis may develop on uneven plaque surfaces, significantly increasing the likelihood of abrupt blood flow restriction (Tavafi, 2013). Chronic inflammation and immunological responses are pivotal in both the initiation and progression of atherosclerosis, with intricate interactions

between the innate and adaptive immune systems perpetuating inflammation (Libby et al., 2019).

The primary lesions in atherosclerosis typically manifest as fatty streaks resulting from the accumulation of low-density lipoprotein (LDL) particles, which are abundant in cholesterol and triglycerides, within the intima. Under typical circumstances, a functional endothelium inhibits LDL penetration. Nevertheless, increased plasma lipid concentrations and endothelial injury permit LDL particles to permeate and undergo oxidation, resulting in oxidised LDL (oxLDL). OxLDL is essential for the recruitment of immune cells, such as monocytes and T cells, to the damage site (Orekhow, 2018). Monocytes develop into macrophages under the influence of macrophage colony-stimulating factor (M-CSF), which subsequently phagocytise oxLDL through scavenger receptors including scavenger receptor class A member 1 (SRA-1), cluster of differentiation 36 (CD36), and lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), leading to the generation of lipid-laden foam cells (Cochain&Zernecke, 2017).

Foam cells, characteristic of atherosclerosis, facilitate plaque development and inflammation by the secretion of cytokines that promote further lipid accumulation. Cholesterol ester (CE) deposition transpires due to an imbalance between cholesterol input and outflow, resulting in CE accumulation as cytoplasmic lipid droplets within macrophages (Remmerie& Scott, 2018). Foam cells eventually undergo apoptosis, discharging their lipid contents into the plaque core and forming a necrotic area. The degradation and rupture of the fibrous cap reveal the necrotic core to blood flow, triggering coagulation cascades that might result in thrombosis and, subsequently, life-threatening cardiovascular incidents (Orekhow, 2018). Atherosclerosis is a multifactorial

disease characterised by lipid accumulation, immune cell activation, and chronic inflammation, with foam cell production and plaque rupture as critical processes that result in arterial constriction and heightened cardiovascular risks. Macrophage-targeted therapeutics has acquired prominence for their crucial role in plaque formation, effectively lowering inflammatory responses and alleviating the course of atherosclerosis. Research indicates that macrophages internalise amphiphilic nanoparticles via scavenger receptors, suggesting that these nanoparticles may competitively obstruct oxLDL uptake and potentially impede plaque formation. This discovery has generated interest in compounds that can engage with macrophages to affect plaque development and diminish inflammation.

Graphene, isolated from graphite in 2004, has attracted significant attention because its distinctive features and several applications, including electronics and healthcare (Razaq et al., 2022). Graphene consists of a monolayer of carbon atoms organised in a hexagonal lattice, making it the thinnest and lightest material known, characterised by exceptional electrical and thermal conductivity, mechanical strength, optical transparency, and a substantial surface area (Khan et al., 2016; Razaq et al., 2022). The large-scale manufacturing of graphene is still problematic, and its absence of a band gap restricts certain applications, particularly in aquatic conditions (Geim & Novoselov, 2009). Graphene derivatives, including GO and rGO, have been created to address these constraints. GO is synthesised through the exfoliation of graphite oxide and comprises oxygen-containing functional groups, rendering it hydrophilic and readily dispersible in water (Priyadarsini et al., 2018; Trikkaliotis et al., 2021). This functionalisation allows GO to work as a versatile platform for multiple applications,

including biomedicine, owing to its non-toxic characteristics in normal cells (Trikkaliotis et al., 2021). The chemical reduction of GO yields rGO, which exhibits electrical conductivity characteristics akin to graphene, albeit with residual oxygen functional groups that affect its solubility and conductivity (Eda & Chhowalla, 2010).

In Malaysia, the oil palm industry generates significant biomass waste, such as oil palm trunks (OPT), which raises environmental concerns while also providing sustainable potential. Converting this biomass into high-value graphene-based materials, including GO and rGO, promotes environmental sustainability and fosters economic growth, especially in industries such as electronics, energy storage, and healthcare (Karim et al., 2019). The utilisation of oil palm-derived graphite as a precursor for GO and rGO demonstrates promise for novel applications in medical research, particularly in early diagnostics and therapies for atherosclerosis. This strategy corresponds with Malaysia's biomedical and environmental objectives, tackling the significant incidence of cardiovascular illnesses and the ecological impact of palm oil biomass.

Macrophages phagocytose diverse particles via scavenger receptors such as SRA-1, CD36, and LOX-1, hence starting immunological responses (Nakayama, 2018). Studies indicate that scavenger receptors facilitate the absorption of nanoparticles by macrophages, implying novel treatment strategies aimed at these pathways (Shannahan et al., 2015). Using docking software, our preliminary results indicate that graphene and rGO exhibit strong binding to ApoB after TLR4, while GO interacts more prominently with SRA1 following TLR4, potentially influencing oxLDL uptake. These interactions highlight the ability of graphene derivatives to modulate foam cell formation, either by binding to oxLDL or by interfering with scavenger receptor activity.Subsequent research

suggests that the toxicity of GO and rGO in vitro may fluctuate depending on variables such as dosage, particle size, and exposure circumstances (Rhazouani et al., 2021). GO is often internalised by macrophages more efficiently than rGO, but both substances exhibit promises as drug delivery systems (Cicuéndez et al., 2021). Furthermore, metabolomics analysis has demonstrated its utility in examining biological responses to nanomaterials, uncovering alterations in metabolic pathways in macrophages subjected to graphene nanoplatelets (Adamson et al., 2018). Currently, there is scant information on the global metabolome of macrophage-derived foam cells interacting with GO and rGO.

1.2 Problem Statement

Atherosclerosis remains a major contributor to cardiovascular disease, driven by the excessive uptake of oxLDL by macrophages through scavenger receptors such as SRA-1, LOX-1, and CD36. This uptake leads to foam cell formation, triggering inflammatory pathways that accelerate plaque development and vascular dysfunction. While macrophages possess mechanisms for cholesterol efflux through transporters like ABCA1, an imbalance between lipid uptake and clearance leads to excessive foam cell accumulation, exacerbating atherosclerosis progression.

Recent advancements in nanomedicine suggest that amphiphilic nanoparticles can be internalized by macrophages via scavenger receptors, potentially competing with oxLDL and mitigating foam cell formation. Among these materials, graphene-based nanomaterials, including GO and rGO, exhibit unique physicochemical properties that may influence lipid metabolism. However, their specific interactions with macrophage receptors and their role in cholesterol homeostasis require further investigation.

As one of the world's leading palm oil producers, Malaysia generates substantial biomass waste, particularly from oil palm trunks (OPT). The conversion of this agricultural waste into graphene-based nanomaterials through pyrolysis presents an environmentally sustainable approach while offering biomedical applications. Preliminary computational studies suggest that graphene-based materials interact strongly with scavenger receptors, indicating that GO and rGO, due to their functionalized surfaces, may exhibit superior binding affinities for these receptors and oxLDL, potentially reducing foam cell formation more effectively than pristine graphene.

While research has extensively explored oxLDL uptake by macrophages, the extent to which GO and rGO influence cholesterol efflux remains unclear. Given that lipid clearance via ABCA1 and ABCG1 transporters plays a crucial role in foam cell regulation, understanding whether GO and rGO promote lipid removal in addition to modulating oxLDL uptake is essential in evaluating their potential role in atherosclerosis intervention.

Additionally, foam cell formation is associated with significant metabolic shifts involving lipid metabolism, oxidative stress, and inflammatory pathways. However, the metabolic alterations induced by GO and rGO in macrophages remain insufficiently characterized. Metabolomic profiling of GO- and rGO-treated macrophages may provide critical insights into their biochemical effects, potentially identifying metabolic pathways that could be targeted for therapeutic intervention.

This study aims to investigate the molecular interactions of GO and rGO with scavenger receptors and oxLDL, assess their influence on foam cell formation, and

characterize their metabolic impact on macrophages. Through an integrative approach combining computational modeling, in vitro biological assays, and metabolomics analysis, this research will contribute to a deeper understanding of how graphene-based nanomaterials influence lipid metabolism and atherosclerosis progression. These findings may provide a foundation for the development of graphene-derived therapeutic strategies while promoting sustainable utilization of biomass waste

1.3 Research questions

1. How is the binding affinity of GO and rGO towards oxLDL and scavenger receptors?
2. How is the binding strength of GO and rGO that composed of functional groups effect macrophage derived foam cells formation?
3. What is the metabolomic profiling of GO and rGO-treated macrophage derived foam cells?

1.4 Research hypotheses

The stronger binding energy of GO and rGO due to their functional groups toward oxLDL and scavenger receptors may influence the acceleration or prevention of macrophage derived foam cells formation.

1.5 Research Objectives

The main aim of this study is to investigate the effect of graphene derivatives, GO and rGO derived from OPT on macrophage-derived foam cell formation. The study is organized around the following specific objectives:

1. To evaluate the binding affinity between GO and rGO with atherosclerosis-associated proteins such as CD36, LOX-1, SRA-1, TLR4, ApoB, and LDLR using computational approaches including molecular docking, molecular dynamics simulations, and MM-PBSA analysis.
2. To determine the effects of GO and rGO derived from oil palm trunk on macrophage-derived foam cell formation through synthesis and characterization, followed by in-vitro evaluation of cell viability, lipid accumulation, cholesterol levels, cytokine secretion, and gene expression in RAW 264.7 macrophages.
3. To characterize the metabolomic profile of GO and rGO-treated macrophage-derived foam cells using LC-MS/MS analysis and pathway enrichment via MetaboAnalyst 6.0.

1.6 Significance of the Study

This study is important since it examines the capability of GO and rGO, two promising nanomaterials, in influencing foam cell development. This research may offer innovative therapeutic methods for atherosclerosis by clarifying the molecular processes by which GO and rGO engage with scavenger receptors and influence lipid uptake. Furthermore, metabolomic analysis may uncover novel biomarkers or metabolic pathways associated with foam cell development, thereby aiding in the creation of targeted therapeutics. Furthermore, the utilisations of OPT-derived GO and rGO presents an environmentally friendly method for synthesising these materials, hence advancing the field of green nanotechnology. This study's findings may facilitate the advancement of

graphene-based materials as novel therapeutic agents for atherosclerosis and other lipid-related conditions.

1.7 Limitations of the Study

This research has several limitations that should be noted. To begin with, in-vitro studies conducted on RAW 264.7 macrophages cannot fully reproduce the complexity of the in-vivo atherosclerotic setting, where factors such as hemodynamics, vascular microenvironment, and systemic immune interactions play critical roles in foam cell development. In addition, the biological responses of GO and rGO are influenced by variables such as particle dimensions, surface chemistry, and administered dose, which may affect both reproducibility and clinical applicability. Lastly, while metabolomic profiling offers broad insights into pathway alterations, it does not fully explain underlying molecular mechanisms; therefore, complementary targeted validation is needed to substantiate the observed metabolic changes.

1.8 Thesis Organization

This thesis is organized based on the flow chart illustrated in Figure 1.1.

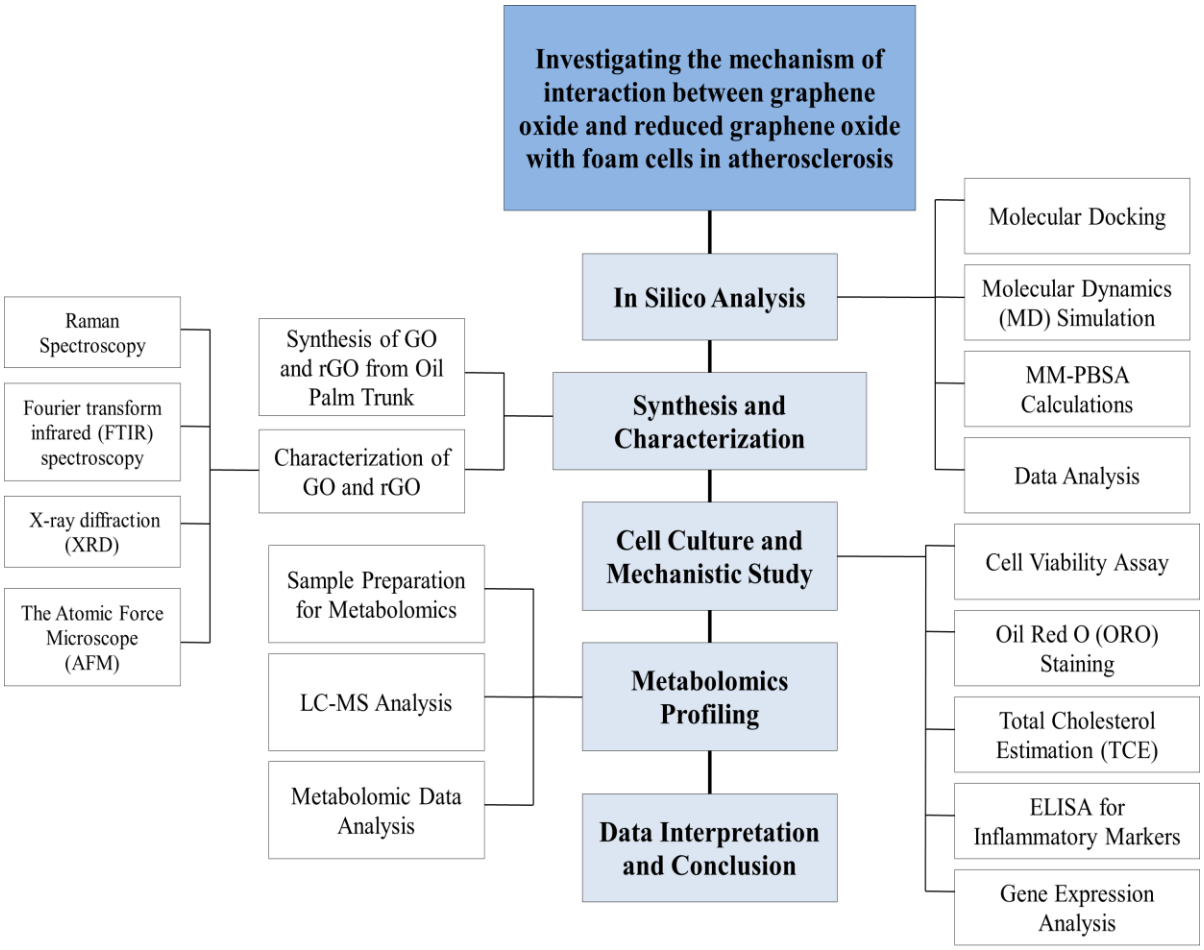

Figure 1.1: Overall workflow of the study, which also reflects the structure and organization of this thesis. Methodological overview for examining the interaction mechanism between GO and rGO with foam cells in atherosclerosis. The research has five primary elements: (1) *In silico* analysis encompassing molecular docking, molecular dynamics (MD) simulations, MM-PBSA calculations, and data analysis; (2) Synthesis and characterisation of GO and rGO derived from oil palm trunk, utilising Raman spectroscopy, FTIR spectroscopy, XRD, and AFM analysis; (3) Cell culture and mechanistic investigation concentrating on cell viability assays, Oil Red O (ORO) staining, total cholesterol estimation (TCE), ELISA for inflammatory markers, and gene expression analysis; (4) Metabolomics profiling involving sample preparation, LC-

MS/MS analysis, and metabolomic data interpretation; and (5) Data interpretation and conclusion synthesising findings from all phases.

CHAPTER 2

LITERATURE REVIEW

2.1 Cardiovascular disease

Cardiovascular disease (CVD) remains the foremost cause of death across the globe, accounting for approximately 19.8 million deaths in 2022, which represents close to one third of all global mortality (World Health Organization, 2025). Evidence indicates that about four out of five of these deaths occur in low and middle-income countries, reflecting persistent inequalities in healthcare provision and access to preventive measures (Di Cesare et al., 2024). In Malaysia, the burden of CVD is equally significant. Recent health statistics identify ischaemic heart disease as the principal medically certified cause of death in 2022, contributing to nearly 16.1 percent of all registered deaths (Hasani et al., 2024). These findings emphasize the urgent need for effective community-based prevention programs and improved therapeutic approaches within the healthcare system. Atherosclerosis is the central pathological mechanism driving CVD. It is defined by the progressive build-up of lipids in the arterial wall together with persistent inflammation, both of which promote narrowing of the vessel lumen and heighten the risk of cardiovascular complications (Frostegård, 2013; Gistera & Hansson, 2017). Disease progression results from complex interactions between vascular cells and lipoproteins that stimulate immune activation and chronic inflammatory responses (Cekici et al., 2014). The involvement of both innate and adaptive immunity plays a pivotal role, leading to plaque growth and, in advanced cases, rupture that can provoke thrombosis and life-threatening events such as myocardial infarction and stroke (Hansson & Hermansson, 2011; Miteva et al., 2018). The World Heart Report 2023 further

underscores the scale of this challenge, estimating more than 523 million prevalent cases and 19.8 million deaths in 2022. The report also reveals striking geographical disparities, with the highest age-standardised mortality rates observed in Eastern Europe, Central Asia, and Oceania, while lower rates are seen in high-income regions where access to prevention and treatment is more widespread (World Heart Federation, 2023). As shown in Figure 2.1, the global distribution of age-standardised mortality reveals substantial geographical variation, which further emphasizes the urgent need for region-specific preventive strategies.

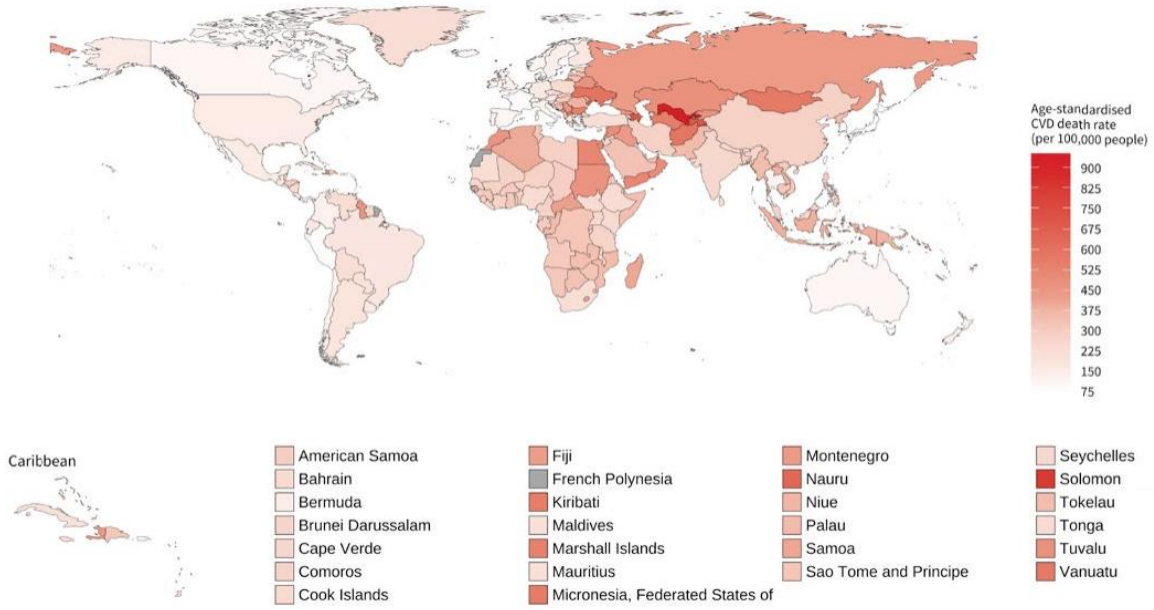

Figure 2.1: Global age-standardised cardiovascular disease mortality per 100,000 populations. Darker shades indicate countries with higher death rates. Source: Adopted from World Heart Report 2023 (World Heart Federation, 2023).

2.1.1 Atherosclerosis

Atherosclerosis is a major contributor to CVD and remains asymptomatic until plaque rupture or arterial blockage occurs, drastically impacting global health (Malekmohammad et al., 2021; Ajoolabady et al., 2024). It is an inflammatory disease marked by intense immunological activity, with immune cells, lipoproteins, and vascular cells interacting to promote chronic inflammation (Malekmohammad et al., 2021; Ajoolabady et al., 2024). The World Health Organization (WHO) classifies atherosclerosis progression into distinct stages, including fatty streaks, atheroma, fibrous plaques, and complex lesions (Malekmohammad et al., 2021). Atherosclerotic plaques consist of necrotic cores, calcified regions, oxidized lipoproteins (oxLDL), inflamed smooth muscle cells (SMCs), endothelial cells (ECs), leukocytes, and foam cells, all of which contribute to disease progression (Malekmohammad et al., 2021; Ajoolabady et al., 2024). These plaques are structurally complex and are often protected by fibrous caps of varying thickness. The shoulder regions of the fibrous cap are infiltrated by activated T cells, macrophages, and mast cells that secrete various pro-inflammatory mediators and enzymes, exacerbating the inflammatory response. As the plaque progresses, arterial stenosis (narrowing of the lumen) occurs, leading to ischemia in surrounding tissues, ultimately increasing the risk of cardiovascular complications (Hansson & Hermansson, 2011; Ajoolabady et al., 2024). Understanding the underlying mechanisms of CVD and atherosclerosis, including their inflammatory nature and immune-mediated progression, is critical in developing effective therapeutic interventions to mitigate disease onset and progression.

2.1.2 Pathogenesis of atherosclerosis

Atherosclerosis is a chronic cardiovascular disease characterized by the accumulation of lipids, persistent inflammation, and fibrotic changes in the arterial walls, ultimately leading to plaque formation and vascular obstruction. The process is initiated by endothelial dysfunction, which can result from factors such as hypertension, smoking, diabetes, and elevated low-density lipoprotein (LDL) cholesterol levels. Under normal physiological conditions, endothelial cells regulate vascular homeostasis; however, when these cells are damaged, their permeability increases, allowing LDL and very-low-density lipoprotein (VLDL) to infiltrate the intima. Within the arterial wall, these lipoproteins undergo oxidative modifications, forming oxidized LDL (oxLDL), which stimulates the expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1). These molecules facilitate monocyte adhesion to the endothelium, marking the onset of an inflammatory cascade (Libby et al., 2019; Ajoolabady et al., 2024).

OxLDL, along with subclass B of LDL, functions as a damage-associated molecular pattern (DAMP), activating endothelial cells and triggering an inflammatory response. This leads to the secretion of pro-inflammatory cytokines that further enhance leukocyte adhesion by upregulating E- and P-selectins, ICAM-1, and VCAM-1 (Habas & Shang, 2018; Ajoolabady et al., 2024). Simultaneously, monocyte chemoattractant protein-1 (MCP-1) is produced, attracting circulating monocytes that migrate into the intima. Under the influence of growth factors such as macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF), these monocytes differentiate into macrophages, which play a central role in foam cell

formation (Minelli et al., 2020). Figure 2.2 by Minelli et al., (2020) illustrates this process, highlighting the sequential events of endothelial activation, monocyte adhesion, and transmigration leading to foam cell development.

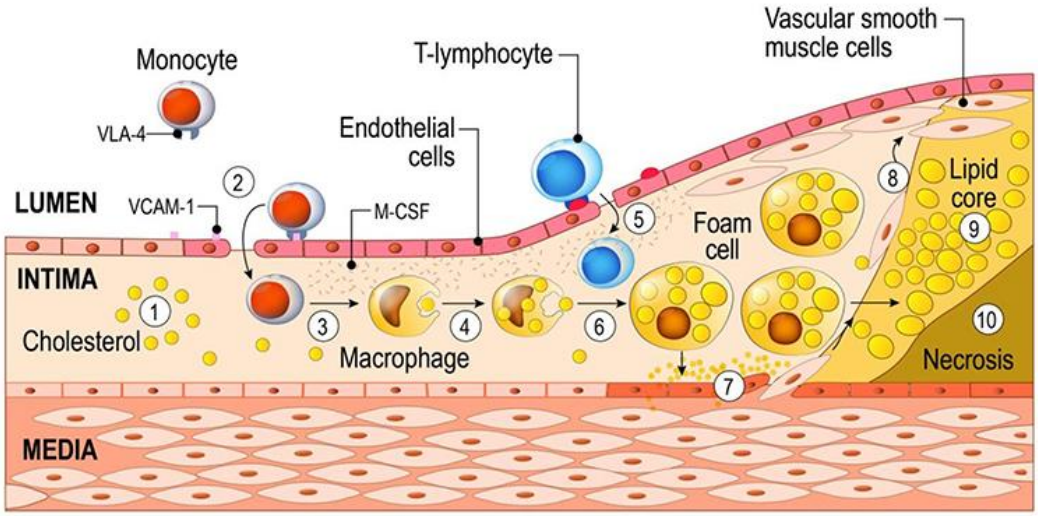

Figure 2.2: Schematic representation of atherogenesis. The presence of oxidized low-density lipoproteins (oxLDLs) triggers endothelial cell activation, prompting the expression of leukocyte adhesion molecules such as VCAM-1, which facilitates monocyte attachment to the endothelium (1,2). These monocytes then migrate into the intima and differentiate into macrophages (3,4). As plaque formation progresses, T lymphocytes also infiltrate the intima, further contributing to inflammation (5). Macrophages engulf modified lipoproteins, transforming into lipid-laden foam cells (6). The inflammatory response promotes vascular smooth muscle cell migration and proliferation, leading to fibroproliferative plaque development (7,8). Additionally, macrophages within the plaque exhibit altered lipid metabolism, leading to reduced cholesterol efflux (9). This dysfunction results in the accumulation of apoptotic cells and

necrotic debris, ultimately forming a necrotic core (10). Figure adopted from Minelli et al. (2020).

Upon entering the subendothelial space, macrophages engulf oxLDL through scavenger receptors, leading to their transformation into lipid-laden foam cells, which are a hallmark of early atherosclerotic lesions, commonly known as fatty streaks (Woollard & Geissmann, 2010). These foam cells secrete inflammatory cytokines and chemokines, perpetuating immune cell recruitment and promoting chronic inflammation. In response, vascular smooth muscle cells (SMCs) migrate from the media to the intima, proliferating and synthesizing extracellular matrix (ECM) proteins, including collagen and elastin. This process results in the development of a fibrous cap, which stabilizes the plaque and separates it from the circulating blood (Basatemur et al., 2019). However, SMCs can undergo phenotypic switching, acquiring macrophage-like characteristics that contribute to foam cell accumulation and plaque progression.

As atherosclerosis progresses, macrophages within plaques exhibit altered lipid metabolism, leading to reduced cholesterol efflux and an increase in apoptotic cells and necrotic debris. Over time, this accumulation contributes to the formation of a necrotic core, covered by a fibrous cap that can weaken due to continuous matrix remodeling. Persistent inflammation and the activity of matrix metalloproteinases (MMPs), particularly MMP-1, MMP-8, and MMP-13, degrade the extracellular matrix, resulting in the thinning of the fibrous cap. This creates a vulnerable plaque that is highly prone to rupture. Once the fibrous cap ruptures, the thrombogenic contents of the plaque come into direct contact with circulating blood, triggering thrombus formation. This event significantly increases the risk of acute cardiovascular complications, including

myocardial infarction and stroke (Moore & Tabas, 2011). The stages of atherosclerotic progression, from fatty streak formation to plaque rupture, are illustrated in Figure 2.3.

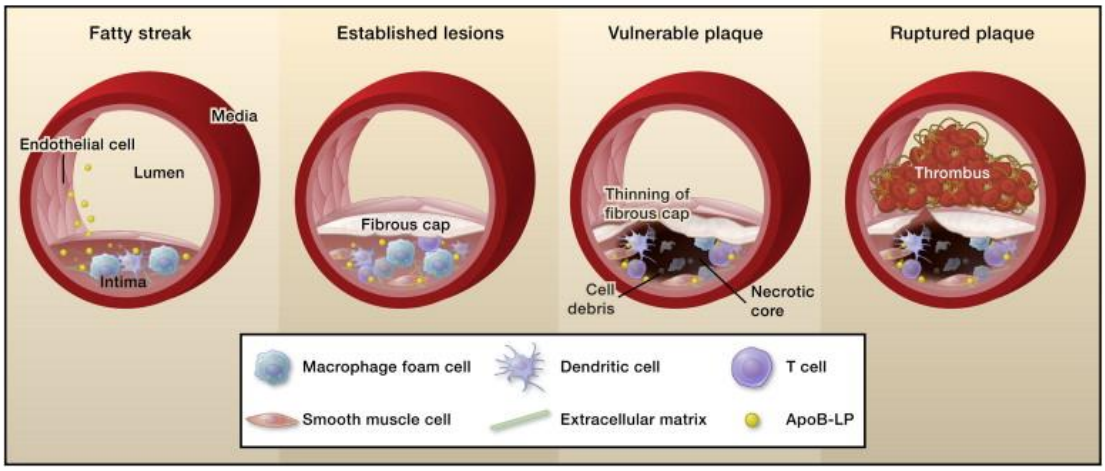

Figure 2.3: Progression of an atherosclerotic lesion. This schematic highlights the sequential phases of atherosclerosis, beginning with lipoprotein infiltration and oxidation, leading to foam cell formation and chronic inflammation. Over time, a fibrous cap forms as smooth muscle cells migrate and produce extracellular matrix components. However, persistent inflammation weakens this cap, resulting in plaque vulnerability and an increased risk of thrombus formation, which can cause acute ischemic events. Figure adopted from Moore & Tabas, (2011).

The immune system plays a crucial role in atherosclerosis, with both B and T lymphocytes contributing to plaque progression (Ammirati et al., 2015). Neutrophils exacerbate the disease by promoting endothelial activation and monocyte infiltration, increasing oxidative stress in resident macrophages and enhancing foam cell formation. Additionally, neutrophils release inflammatory mediators such as TNF-α, IL-1, IL-6, IL-8, GM-CSF, and myeloperoxidase, further amplifying inflammation and destabilizing plaques (Kolaczkowska & Kubes, 2013; Silvestre-Roig et al., 2020).

As atherosclerosis advances, plaques may develop additional complications, including calcification and cholesterol crystal formation, which further contribute to plaque instability. In severe cases, plaque rupture or erosion exposes thrombogenic material, leading to platelet aggregation and thrombus formation, which can result in life-threatening ischemic events (Loftus, 2011; Meng et al., 2016; Mehu et al., 2022).

2.1.2(a) Fatty streak development

Fatty streak formation is the earliest stage of atherosclerosis and is marked by the accumulation of lipids within macrophages and vascular smooth muscle cells (VSMCs) in the arterial intima. This process is initiated by endothelial dysfunction, which disrupts vascular homeostasis and promotes lipid infiltration, monocyte adhesion, oxidative stress, and an inflammatory response, all of which contribute to the development of fatty streaks (Esper et al., 2006; Sun et al., 2020). One of the critical events in this process is the recruitment of monocytes into the arterial wall. Activated endothelial cells (ECs) express adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), which facilitate monocyte adhesion and migration into the intima (Yu et al., 2015; Liu et al., 2017). Once inside, monocytes differentiate into macrophages, which engulf oxidized low-density lipoproteins (oxLDLs) via scavenger receptors such as CD36, scavenger receptor-A1 (SRA1), and lectin-like oxidized LDL receptor-1 (LOX-1), leading to the formation of foam cells (Chistiakov et al., 2016). These foam cells accumulate cholesterol esters and further contribute to inflammation by secreting cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6), which amplify monocyte recruitment

and foam cell formation, thereby accelerating fatty streak development (Takahashi at al., 2021).

As illustrated in Figure 2.4, adapted from Yuan et al. (2012), monocyte recruitment is triggered by chemotactic signals, leading to their adhesion and diapedesis into the intima. Once in the subendothelial space, monocytes differentiate into macrophages and engulf modified LDL via scavenger receptors (SRs) and LDL receptors (LDLR), resulting in the formation of lipid-laden foam cells. These foam cells, through the accumulation of cytoplasmic lipid droplets, sustain inflammation and contribute to the early development of atherosclerotic plaques. The formation of foam cells marks the transition from initial fatty streaks to more advanced atherosclerotic lesions, highlighting its significance in disease progression (Yuan et al., 2012)

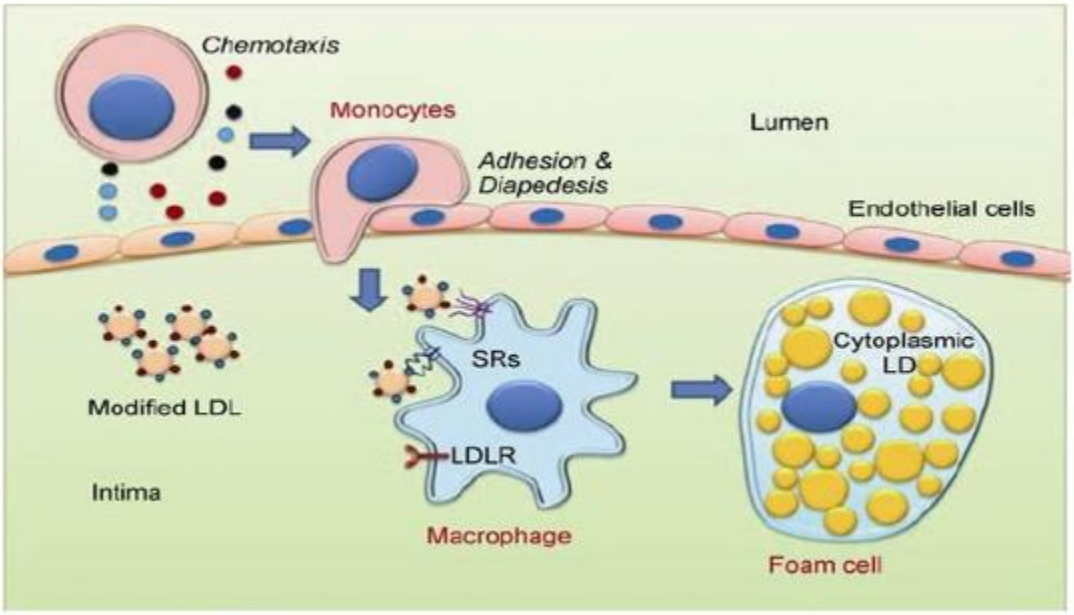

Figure 2.4: Schematic representation of fatty streak formation in atherosclerosis. The process begins with monocyte recruitment in response to chemotactic signals, followed

by adhesion and diapedesis into the intima. Once inside, monocytes differentiate into macrophages, which engulf modified low-density lipoproteins (LDL) via scavenger receptors (SRs) and LDL receptors (LDLR), leading to foam cell formation. The accumulation of cholesterol-laden foam cells contributes to inflammation, sustaining the progression of atherosclerotic plaques and marking the early stage of atherosclerosis development. Figure adopted from Yuan et al., (2012).

VSMCs also play a crucial role in fatty streak formation by internalizing oxLDLs through scavenger receptors, similar to macrophages, and undergoing phenotypic transformation into foam cells (Sheedy et al., 2013). Notably, research has indicated that a significant proportion of foam cells in atherosclerotic plaques originate from VSMCs rather than monocytes, emphasizing their contribution to early atherosclerotic lesion development (Choi et al., 2009). Additionally, VSMCs in the intima express lower levels of ATP-binding cassette transporters such as ABCA1, which impairs cholesterol efflux, leading to excessive lipid accumulation (Choi et al., 2009). The buildup of cholesterol within foam cells further exacerbates the inflammatory response by triggering the activation of the NLRP3 inflammasome, which leads to the release of pro-inflammatory cytokines such as IL-1β and IL-18, reinforcing the atherogenic environment (Sheedy et al., 2013; Guo et al., 2015). The presence of cholesterol crystals within plaques also contributes to inflammasome activation and serves as an indicator of early atherosclerotic progression (Abela, 2010).

The formation of fatty streaks is driven by a combination of endothelial dysfunction, monocyte recruitment, foam cell formation, and sustained inflammatory signaling. The imbalance between cholesterol uptake and efflux, particularly in

macrophages and VSMCs, results in lipid accumulation, which is a defining feature of early-stage atherosclerosis (Nasiri et al., 2015). A deeper understanding of these mechanisms can provide valuable insights into potential therapeutic strategies for preventing or slowing the progression of atherosclerosis in its initial stages.

2.1.2(b) Fibrous cap formation and its role in atherosclerotic plaque stability

The development of the fibrous cap is a key process in the progression of atherosclerotic plaques, as it functions as a protective barrier that stabilizes lesions. This structure is formed when vascular smooth muscle cells (VSMCs) migrate from the tunica media into the intima in response to various growth factors, including platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), and vascular endothelial growth factor (VEGF), which are secreted by endothelial cells (ECs) and foam cells (Louis & Zahradka, 2010; Watson et al., 2019). Upon reaching the intima, VSMCs transition from their usual contractile phenotype to a synthetic state, characterized by enhanced proliferation and extracellular matrix (ECM) production. This matrix, composed of components such as interstitial collagen, elastin, and proteoglycans, strengthens the fibrous cap, thereby reducing the risk of thrombosis by preventing direct exposure of prothrombotic material from the necrotic core (Bentzon et al., 2014; Sorokin et al., 2020). However, prolonged exposure to mitogens can prevent VSMCs from returning to their contractile state, further contributing to plaque expansion (Mack, 2011; Zhang et al., 2012). The structural integrity of the fibrous cap, including its thickness, cellular makeup, and ECM composition, plays a crucial role in determining plaque stability and vulnerability to rupture (Anlamlert et al., 2017).

Beneath this fibrous layer, the necrotic core forms the central lipid-rich, cell-depleted region of atherosclerotic plaques, significantly influencing disease progression. This core enlarges as a result of continuous macrophage apoptosis combined with impaired efferocytosis, the process responsible for clearing apoptotic cells (Linton et al., 2016). In healthy conditions, programmed macrophage death is regulated by caspases, and efferocytosis ensures the removal of dead cells to prevent inflammation (Cabrera & Makino, 2021). However, as atherosclerosis advances, increased oxidative stress, the activation of cell death pathways, and reduced nutrient availability promote excessive apoptosis in macrophages and VSMCs (Gonzalez & Trigatti, 2017). Additionally, phagocytes lose their ability to clear apoptotic debris due to impaired receptor function, including MerTK, LRP1, CD36, and SR-B1, leading to an accumulation of cholesterol crystals and further aggravation of inflammation (Coornaert et al., 2018; Wagage et al., 2021). The persistent presence of apoptotic and necrotic cells results in the release of matrix metalloproteinases (MMPs), which degrade the fibrous cap and heighten the risk of plaque rupture (Wagage et al., 2021).

Another significant feature of advanced atherosclerosis is plaque calcification, a process that occurs predominantly in regions experiencing chronic inflammation and cell death (Nakahara et al., 2017; Shi et al., 2020). This process begins with the release of matrix vesicles from dying macrophages and synthetic VSMCs, which act as sites for calcium phosphate accumulation (Kapustin et al., 2015). Over time, these deposits transition from amorphous calcium phosphate to crystalline structures (Nakahara et al., 2017). Additionally, VSMCs and pericytes may undergo osteogenic transdifferentiation, acquiring osteoblast-like properties that enable them to generate a mineralized matrix,

leading to calcium deposition within the plaque (Otsuka et al., 2014). Although calcification is often associated with larger plaques, its impact on plaque vulnerability is determined more by the location and type of calcification rather than the overall amount of calcium present (Nakahara et al., 2017; Shi et al., 2020).

2.1.2(c) Advanced plaque and rupture

In advanced atherosclerosis, plaque instability often leads to rupture and subsequent thrombus formation, significantly increasing the risk of cardiovascular complications. As plaques progress, they develop a large necrotic core, a thin fibrous cap, and heightened inflammatory activity, all of which contribute to their vulnerability (Stefanadis et al., 2017). The fibrous cap, primarily composed of extracellular matrix (ECM) produced by vascular smooth muscle cells (VSMCs), acts as a protective barrier between the thrombogenic core and circulating blood. However, in advanced stages, VSMC apoptosis reduces ECM synthesis, while increased matrix metalloproteinase (MMP) activity weakens the cap by degrading its structural components (Sodhi & Brown, 2019). Pro-inflammatory cytokines, such as IFN-γ, IL-1β, TNF-α, and CD40 ligand (CD154), further contribute to plaque instability by suppressing collagen synthesis in VSMCs and enhancing MMP activity, particularly at the plaque shoulders and fibrous cap (Libby, 2002). The combination of these factors weakens the plaque, making it highly susceptible to rupture when exposed to hemodynamic forces (Groen et al., 2007; Badimon et al., 2017).

Plaque rupture exposes the underlying tissue to circulating blood, triggering a coagulation cascade that results in thrombus formation. The necrotic core releases tissue