

**EFFEROCYTOSIS OF OXIDIZED
LOW-DENSITY LIPOPROTEIN
GENERATED APOPTOTIC BODIES BY
PRIMARY M1 AND M2 MACROPHAGES:
A COMPARISON BETWEEN
NON-OBSTRUCTIVE AND OBSTRUCTIVE
CORONARY ARTERY DISEASE PATIENTS**

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by

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LIST OF ABBREVIATIONS

ABCA1	Apolipoprotein A1/ ATP-binding cassette subfamily A member 1
ABCG1	Adenosine triphosphate (ATP)-binding cassette transporter G1
ACC	American College of Cardiology
ACCF	American College of Cardiology Foundation
AcLDL	Acetylated low density lipoprotein
ACS	Acute coronary syndrome
ADAM17	ADAM metalloproteinase domain 17
AHA	American Heart Association
ANOVA	Analysis of variance
APC	Allophycocyanin
APC/H7	Allophycocyanin/ hillite 7
Arg	Arginase
ATP	Adenosine triphosphate
AXL	AXL receptor tyrosine kinase
CAD	Coronary artery disease
cDNA	Complementary deoxyribonucleic acid
CFSE	Carboxyfluorescein succinimidyl ester
C _T	Cycle threshold
CVD	Cardiovascular disease
CXC3CL1	Chemokine C-X3-C motif ligand 1
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid

ELISA	Enzyme-linked immunoassay
FACS	Fluorescence-activated single-cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FNIII	Fibronectin type III
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GAS6	Growth arrest specific 6
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HbA1c	Glycated haemoglobin A1c
HDL	High-density lipoprotein
HRP	Horse radish peroxidase
IFN- γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IQR	Interquartile range
LDL	Low-density lipoprotein
LOX-1	Lectin-like oxLDL receptor-1
LPS	Lipopolysaccharide
M-CSF	Macrophage colony-stimulating factor
MERTK	MER proto-oncogene, tyrosine kinase
MFI	Mean fluorescence index
MPO	Myeloperoxidase
NLRP3	NLR family pyrin domain containing 3
oxLDL	Oxidised low-density lipoprotein

PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PE/CY7	Phycoerythrin/ cyanine 7
PFA	Paraformaldehyde
PI	Propidium iodide
qPCR	Quantitative polymerase chain reaction
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI-1640	Roswell Park Memorial Institute Medium-1640
SCAI	Society for Cardiovascular Angiography and Intervention
SD	Standard deviation
siRNA	Small interfering ribonucleic acid
SPSS	Statistical Product and Service Solutions
SR-A	Scavenger receptor-A
SR-B1	Scavenger receptor class B type 1
TAM	TYRO3, AXL, MERTK receptor
TBE	Tris/borate/EDTA buffer
TGF- β	Tumor growth factor beta
TMB	3,3',5,5'-tetramethylbenzidine
TNF- α	Tumor necrosis factor alpha

TYRO3	TYRO3 protein tyrosine kinase
VCAM1	Vascular cell adhesion protein 1
WHO	World Health Organization

LIST OF APPENDICES

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**EFFEROSITOSIS JASAD APOPTOTIK YANG DIJANA
LIPOPROTEIN KETUMPATAN RENDAH TEROKSIDA OLEH
MAKROFAJ PRIMER M1 DAN M2: PERBANDINGAN ANTARA
PESAKIT PENYAKIT ARTERI KORONARI TIDAK TERSUMBAT
DAN TERSUMBAT**

ABSTRAK

Efferositosis; proses imunologi pembersihan jasad apoptotik oleh makrofaj yang tidak tertandingi dalam fisiologi manusia. Namun, jasad apoptotik dijumpai dengan banyak pada plak aterosklerosis telah meletakkan plak pada risiko ketidakstabilan dan pecah. Disregulasi efferositosis dalam penyakit koronari arteri boleh berkait dengan fenotip makrofaj, peringkat apoptosis jasad apoptotik, atau kehadiran sitokin radang. Kajian ini telah mengukur kadar efferositosis makrofaj M1 dan M2 yang diperoleh daripada pesakit penyakit koronari arteri tidak tersumbat dan tersumbat terhadap jasad apoptotik peringkat awal dan akhir apoptosis. Penting sekali, implikasi sitokin pro-radang (TNF- α) dan sitokin anti-radang (TGF- β) terhadap disregulasi efferositosis telah disiasat. 27 orang pesakit penyakit koronari arteri tidak tersumbat dan 29 orang pesakit penyakit koronari arteri tersumbat telah direkrut, dan 30 mL darah periferi vena telah dikumpul untuk mendapatkan monosit daripada sel mono nukleus darah periferi. Makrofaj M1 dan M2 telah terpolarisasi daripada monosit menggunakan perangsang berikut (M1: 20 ng/ μ L GM-CSF, 20 ng/ μ L IFN- γ , dan 10 ng/ μ L LPS; M2: 10 ng/ μ L M-CSF, 20 ng/ μ L IL-4, dan 20 ng/ μ L IL-13). Jasad apoptotik peringkat awal dan akhir telah dihasilkan dengan merawat makrofaj bersama

100 µg/mL lipoprotein ketumpatan rendah teroksidasi selama masing-masing 72 dan 168 jam. Makrofaj M1 dan M2 kemudiannya telah ditransfeksi dengan siRNA TNF- α dan siRNA TGF- β untuk mengkaji implikasi paras sitokin terhadap efferositosis. Ekspresi mRNA reseptor efferositosis, MERTK, dan ADAM17 telah diukur menggunakan reaksi tindak balas rantai polimerase secara kuantitatif. Ujian “Independent t-test/Mann Whitney” atau “ANOVA/Kruskal Wallis” telah dilakukan untuk menentukan perbezaan ketara antara dua atau lebih parameter dengan sewajarnya. Seperti jangkaan berdasarkan kajian lepas, MERTK diekspresikan lebih tinggi oleh makrofaj M2 berbanding makrofaj M1 (11.88 ± 4.86 vs. 5.89 ± 1.69 , $p=0.002$). Kajian ini buat kali pertamanya menunjukkan efferositosis jasad apoptotik peringkat awal telah dilakukan dengan paling cekap oleh makrofaj M2 yang diperoleh daripada pesakit penyakit koronari arteri tidak tersumbat ($11.92\% \pm 5.54\%$, $p=0.049$) manakala jasad apoptotik peringkat akhir telah dibersihkan dengan paling cekap oleh makrofaj M2 yang diperoleh daripada pesakit penyakit koronari arteri tersumbat ($6.70\% \pm 3.68\%$, $p=0.036$). Menariknya, kajian ini menunjukkan apabila TNF- α disenyapkan, efferositosis telah bertambah baik dengan ketara pada kumpulan makrofaj M2 masing-masing (M2 tidak tersumbat-jasad apoptotik peringkat awal: $46.78\% \pm 0.76\%$, $p<0.001$; M2 tersumbat-jasad apoptotik peringkat akhir: $29.24\% \pm 2.46\%$, $p<0.001$). Kajian ini telah menunjukkan bahawa asal biologi makrofaj dan fenotipnya, peringkat jasad apoptotik, dan sitokin radang bertanggungjawab kepada disregulasi efferositosis. Maka, dengan mensasarkan faktor-faktor tersebut mungkin mampu membongkar potensi terapeutik dalam menangani kestabilan plak koronari untuk pesakit penyakit koronari arteri.

**EFFEROCYTOSIS OF OXIDIZED LOW-DENSITY LIPOPROTEIN
GENERATED APOPTOTIC BODIES BY PRIMARY M1 AND M2
MACROPHAGES: A COMPARISON BETWEEN NON-
OBSTRUCTIVE AND OBSTRUCTIVE CORONARY ARTERY
DISEASE PATIENTS**

ABSTRACT

Efferocytosis; an immunological process of macrophage clearing apoptotic bodies, is invincible in the human physiology. Nonetheless, unresolved apoptotic bodies are abundant in atherosclerotic plaque, putting it risky of instability and rupture. Dysregulated efferocytosis in coronary artery disease (CAD) may be linked to macrophage phenotypes, stages of apoptosis, or inflammatory cytokines. This study measured the efferocytosis rate of M1 and M2 macrophages from non-obstructive and obstructive CAD patients towards early and late apoptotic bodies. Importantly, the implication of the pro-inflammatory cytokine, tumor necrosis factor-alpha (TNF- α) and anti-inflammatory cytokine, tumor growth factor-beta (TGF- β) in dysregulating efferocytosis was investigated. 27 non-obstructive and 29 obstructive CAD patients were recruited, and 30 mL of peripheral venous blood was collected to isolate monocytes. M1 and M2 macrophages were polarized from the monocytes using respective stimulants (M1: 20 ng/ μ L GM-CSF, 20 ng/ μ L IFN- γ , and 10 ng/ μ L LPS; M2: 10 ng/ μ L M-CSF, 20 ng/ μ L IL-4, and 20 ng/ μ L IL-13). Early and late apoptotic bodies were generated by incubating macrophages with 100 μ g/mL oxidized low-density lipoprotein for 72 hours and 168 hours, respectively. M1 and M2 macrophages

were subsequently transfected with TNF- α siRNA and TGF- β siRNA to investigate the implication of cytokine level towards efferocytosis. The mRNA expression of the efferocytosis receptor, MER proto-oncogene, tyrosine kinase (MERTK), and its potential protease, ADAM metallopeptidase domain 17 (ADAM17) was measured using quantitative real-time polymerase chain reaction. Independent t-test/Mann Whitney or ANOVA/Kruskal Wallis test was performed to determine the significant difference between two or more than two parameters accordingly. As expected from previous research, the expression of MERTK in M2 was higher compared to M1 macrophages (11.88 ± 4.86 vs. 5.89 ± 1.69 , $p=0.002$). Adding to the depth of knowledge, this study for the first time showed that efferocytosis of early apoptotic bodies was most efficiently performed by M2 macrophages from non-obstructive CAD ($11.92\% \pm 5.54\%$, $p=0.049$), while late apoptotic bodies were most efficiently resolved by M2 macrophages from obstructive CAD ($6.70\% \pm 3.68\%$, $p=0.036$). Interestingly, this study revealed that upon the silencing of TNF- α , efferocytosis significantly improved in the respective group of M2 macrophages (M2 non-obstructive-early apoptotic bodies: $46.78\% \pm 0.76\%$, $p<0.001$; M2 obstructive-late apoptotic bodies: $29.24\% \pm 2.46\%$, $p<0.001$). This study suggests that macrophage origin and phenotype, stages of apoptotic bodies, and inflammatory cytokines contribute to efferocytosis dysregulation, revealing potential therapeutic targets for CAD patients.

CHAPTER 1

INTRODUCTION

1.1 Background of study

Coronary artery disease (CAD) lies under one of the umbrellas of cardiovascular diseases (CVD). It encompasses a diverse spectrum of patients with the syndromes of recurrent, transient episodes of angina pectoris attributed to the ischemia with a history of myocardial infarction and documented presence of plaque, assessed by catheterization (Libby, Ridker & Hansson, 2009; Braun & Stevens, 2018).

Under the hierarchical nomenclature of CAD, patients are further categorized into non-obstructive CAD or obstructive CAD according to the degree of stenosis assessed during coronary angiography (Ford, Corcoran & Berry, 2018). CAD is a dynamic and unpredictable disease, and thus, CAD patients remain at a high risk of manifesting major adverse cardiovascular events (Fox *et al.*, 2020). The unpredictable fate of CAD progressing into acute coronary syndrome (ACS) is highly likely to depend on atherosclerotic plaque's pathological environment, particularly plaque instability (Pizzi *et al.*, 2016).

Atherosclerotic plaque develops asymptotically over the years in CAD patients (Libby *et al.*, 2019). Plaque predominantly comprises oxidized low-density lipoprotein (oxLDL), which attracted monocyte recruitment into the intima-media (Salvayre, Negre-Salvayre & Camaré, 2016). The monocytes are then differentiated into macrophages and transformed into macrophage foam cells upon the scavenging of oxLDL and eventually undergo apoptosis, resulting in the accumulation of the apoptotic bodies and cell debris in the atherosclerotic plaque (Yang *et al.*, 2015). The

apoptotic bodies are supposedly cleared efficiently via an immunological process termed efferocytosis under normal physiological conditions (Doran, Arif Yurdagul & Tabas, 2020). However, efferocytosis is dysregulated in atherosclerotic plaque, leaving behind tons of unresolved apoptotic bodies that advance to secondary necrosis and worryingly increase the plaque burden (Tajbakhsh *et al.*, 2018).

Efferocytosis is defined as an anti-inflammatory immunological process of clearing the apoptotic bodies by efferocytes to maintain the homeostasis in the body, including in the coronary arteries (Cavaillon, 2013). Although many immune cells can carry out efferocytosis, the macrophage is regarded as the professional efferocytes with a rapid rate of apoptotic bodies uptake and a higher capacity to ingest multiple apoptotic bodies at once (Morioka, Maueröder & Ravichandran, 2019). The versatile macrophage, which not only responds to foreign materials and pathogen-associated molecular patterns (PAMPs), is also playing a critical role in keeping atherosclerosis at bay by ensuring the efficient elimination of apoptotic bodies promptly (Clark *et al.*, 2019).

There are two commonly distinguished subtypes of macrophages which are M1 (classically activated macrophages) and M2 (alternatively activated macrophages) named on the basis of fundamental function and activation, found in the atherosclerotic plaque at different loci (Yang *et al.*, 2020). M1 predominates in the advanced plaque characterized by the presence of pro-inflammatory cytokines, including tumor necrosis factor- α (TNF- α), interleukin (IL)-1 β , IL-6, IL-12, and reactive nitrogen intermediates (Stöger *et al.*, 2012). Meanwhile, the alternatively activated M2 macrophage is prominent in the early atherosclerotic lesion and fatty streak with an abundance of

anti-inflammatory cytokines such as tumor growth factor- β (TGF- β), IL-4 and, IL-10 (Brochériou *et al.*, 2011) (Bi *et al.*, 2019). Of interest, differences in the behavior of M1 and M2 macrophages may contribute to the dysregulation of efferocytosis.

Efferocytosis is an intricate process with four key steps, which are first, the release of find-me signals by apoptotic bodies; second, the attraction of efferocytes to apoptotic bodies after the release of eat-me signals; third, the ingestion of apoptotic bodies by efferocytes and finally, the processing of apoptotic bodies in the efferocytes and the production of anti-inflammatory mediators by the efferocytes (Morioka, Maueröder & Ravichandran, 2019). The faulty in any step of the efferocytosis process may lead to the detrimental consequences of the undefeated plaque progression, which will ultimately lead to plaque rupture, thrombosis, and fatal myocardial infarction in CAD patients.

This thesis focused on the third step of the efferocytosis process, which is the ingestion of apoptotic bodies by professional efferocytes- macrophages in CAD patients. Briefly, successful uptake of apoptotic bodies by macrophages requires a complete network of protein interaction whereby phosphatidylserine on the surface of apoptotic bodies is recognized by MER proto-oncogene, tyrosine kinase (MERTK) on the surface of macrophages, and their binding is mediated by a bridging molecule, growth arrest-specific 6 (GAS6) (Arif Yurdagul *et al.*, 2018). It should be noted that MERTK is bound to be the substrate of cleavage by ADAM metallopeptidase domain 17 (ADAM17) though how this is regulated is not well deciphered, particularly in atherosclerosis (Gooz, 2010). However, it is postulated that the imbalance in the combination of excessive pro-inflammatory mediators and the loss of anti-

inflammatory cytokines in the atherosclerotic plaque environment may be involved in activating ADAM17 (Newby, 2016).

Thus, this thesis was designed to investigate the specific role of pro-inflammatory cytokine (TNF- α) and anti-inflammatory cytokine (TGF- β) in the cleavage of MERTK by ADAM17. The selection of TNF- α and TGF- β was based on their pivotal roles in regulating macrophage phenotypes and inflammatory processes, which are central to atherosclerosis. TNF- α is a key pro-inflammatory cytokine involved in promoting macrophage polarization towards the M1 phenotype, leading to enhanced inflammation and plaque instability (de Queiroz, Lakkappa & Lazartigues, 2020). Conversely, TGF- β acts as a major anti-inflammatory cytokine associated with M2 macrophages, which are involved in resolving inflammation and stabilizing plaques (Edsfeldt *et al.*, 2023).

The focus on these two cytokines arises from their strong links to CAD progression and plaque dynamics, as highlighted in numerous studies. Preliminary evidence, such as observed changes in cytokine levels in CAD patients and their influence on macrophage activity, supports the hypothesis that TNF- α and TGF- β may play a significant role in the regulation of MERTK cleavage. This is particularly relevant because MERTK cleavage by ADAM17 is associated with defective efferocytosis, contributing to plaque growth and instability in CAD (Borén *et al.*, 2020).

To test this hypothesis, primary M1 and M2 macrophages were isolated from non-obstructive and obstructive CAD patients and subjected to in-vitro experiments.

This approach aims to provide mechanistic insights into how these cytokines modulate MERTK cleavage and contribute to plaque progression, as summarized in Figure 1.1. The findings are expected to enhance our understanding of the complex interactions between cytokines, macrophages, and plaque biology in CAD.

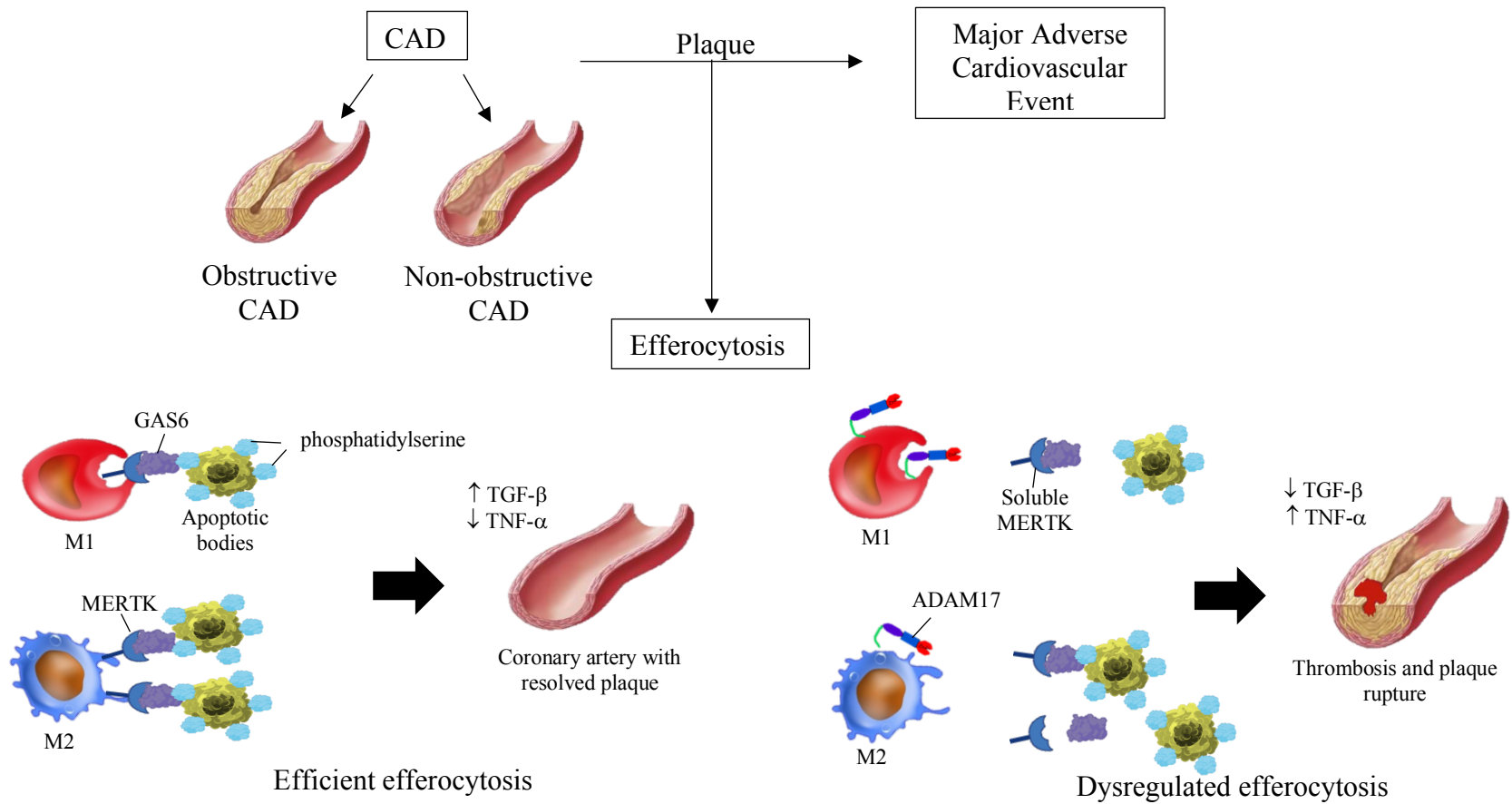


Figure 1.1 Study background of elucidating the mechanism behind pro-inflammatory (TNF- α) and anti-inflammatory (TGF- β) interference on the cleavage of MERTK by ADAM17 investigated in primary M1 and M2 macrophages isolated from non-obstructive and obstructive CAD patients. Adapted from (Thorp, 2010; Thorp *et al.*, 2011; Kojima, Weissman & Leeper, 2017).

1.2 Problem statement and study rationales

Efferocytosis, a crucial anti-inflammatory process, plays a vital role in maintaining cellular homeostasis by efficiently clearing apoptotic bodies, including dead red blood cells, at an astounding rate of 7,000 cells per second (Morioka, Maueröder & Ravichandran, 2019). This process ensures that cellular debris does not accumulate, preventing inflammation and the potential development of pathological conditions. However, emerging evidence highlights a significant dysregulation of efferocytosis in coronary artery disease (CAD), where the failure to clear apoptotic cells in atherosclerotic plaques contributes to plaque instability and thrombosis, which can lead to adverse cardiovascular events (Ramachandra *et al.*, 2020). Despite the fact that CAD patients often present similar systemic lipid profiles, a striking difference exists in their plaque burden. Some individuals exhibit non-obstructive plaques with relatively stable disease progression, while others suffer from obstructive plaques that predispose them to higher risks of life-threatening cardiovascular complications. This paradox suggests that factors beyond lipid levels, particularly cellular and molecular disturbances, may be responsible for the varying progression of CAD. In particular, macrophages, which play an essential role in efferocytosis, may be dysregulated in obstructive CAD patients, impairing their ability to efficiently clear apoptotic cells.

A key factor that may underlie this dysfunction is the cytokine milieu surrounding the plaques. Pro-inflammatory cytokine TNF- α and anti-inflammatory cytokine TGF- β are two such cytokines that have been extensively studied for their roles in macrophage polarization and the regulation of inflammatory responses (Chen *et al.*, 2023). TNF- α is a critical cytokine that drives macrophage polarization towards

the M1 phenotype, associated with enhanced inflammation, plaque destabilization, and the progression of atherosclerosis (Theofilis *et al.*, 2023). In contrast, TGF- β promotes M2 macrophage polarization, which is linked to the resolution of inflammation, tissue repair, and plaque stabilization (Edsfeldt *et al.*, 2023). These contrasting roles suggest that a shift in the balance of TNF- α and TGF- β may significantly impact the functionality of macrophages in CAD patients. Furthermore, both TNF- α and TGF- β have been implicated in regulating MERTK cleavage by ADAM17, a critical process for macrophage-mediated efferocytosis (Waterborg *et al.*, 2018). MERTK, a receptor essential for the engulfment of apoptotic cells, is cleaved by ADAM17, impairing the macrophage's ability to perform efferocytosis effectively. This process of MERTK cleavage has been shown to be influenced by the surrounding inflammatory environment, with elevated levels of TNF- α potentially exacerbating the cleavage of MERTK and inhibiting efferocytosis, while TGF- β may have a protective effect in modulating this process (de Queiroz, Lakkappa & Lazartigues, 2020).

This thesis aims to investigate the dysregulation of efferocytosis in CAD by focusing on how cytokines TNF- α and TGF- β contribute to the cleavage of MERTK by ADAM17 in primary M1 and M2 macrophages isolated from non-obstructive and obstructive CAD patients. By examining these macrophages, this study will explore the uncertainty of whether macrophages from obstructive CAD patients are less efficient in clearing apoptotic cells compared to those from non-obstructive CAD patients, potentially due to differences in macrophage phenotype and inflammatory cytokine regulation. The focus on TNF- α and TGF- β is critical, as they are well-established mediators of macrophage polarization and efferocytosis. Understanding how these cytokines influence the cleavage of MERTK and the subsequent impact on

macrophage efferocytosis will provide valuable insights into the mechanisms underlying plaque instability in CAD. The findings could open new avenues for therapeutic strategies targeting the restoration of macrophage efferocytosis in CAD patients, offering the potential to prevent plaque progression and reduce the risk of thrombosis.

This research holds significant clinical potential, as improving the efficiency of efferocytosis in CAD patients could be a key strategy for enhancing plaque stability and preventing cardiovascular events. Given that cardiovascular disease remains one of the leading causes of death worldwide (WHO, 2020), understanding the molecular and cellular mechanisms that underlie CAD progression is critical for the development of more effective diagnostic and therapeutic interventions. By investigating the role of inflammatory cytokines in macrophage dysfunction, this study could contribute to the development of targeted therapies that restore the ability of macrophages to efficiently clear apoptotic cells, thereby improving the prognosis for CAD patients. Additionally, this research is in line with the World Health Organization's vision of ensuring that patients with cardiovascular diseases receive appropriate care and treatment to prevent premature deaths, especially in the context of the increasing global burden of cardiovascular diseases.

1.3 Objectives of study

In order to shed light on several research questions stated above, this study aimed to:

1.3.1 General objective:

To elucidate the dysregulation of efferocytosis induced by the interference from pro-inflammatory (TNF- α) and anti-inflammatory (TGF- β) cytokines indicated by the cleavage of MERTK by ADAM17 in primary M1 and M2 macrophages isolated from non-obstructive and obstructive CAD patients.

1.3.2 Specific objectives:

1. To examine the differences between M1 and M2 phenotypic markers in primary macrophages isolated from non-obstructive and obstructive CAD patients.
2. To determine the differences in the percentage of efferocytosis performed by M1 and M2 macrophages isolated from non-obstructive and obstructive CAD patients towards oxLDL generated apoptotic bodies.
3. To investigate the effect of TNF- α and TGF- β downregulation on the percentage of efferocytosis of oxLDL generated apoptotic bodies by M1 and M2 macrophages isolated from non-obstructive and obstructive CAD patients.
4. To determine the regulation of MERTK and ADAM17 expression following the downregulation of TNF- α and TGF- β on M1 and M2 macrophages isolated from non-obstructive and obstructive CAD patients.

1.4 Hypothesis

It was postulated that macrophages isolated from obstructive CAD patients perform poorer efficiency of efferocytosis than macrophages isolated from non-obstructive CAD patients, resulting in a high degree of stenosis in coronary arteries.

Importantly, TNF- α presumably promotes the cleavage of MERTK by ADAM17, while TGF- β may preserve the intact membrane-bound MERTK to mediate successful efferocytosis.

Specifically, this study hypothesized:

1. M1 express higher CD80 and CD86 cell surface markers than M2 macrophages, while M2 express higher CD206 and CD200R cell surface markers than M1 macrophages.
2. M2 macrophages isolated from non-obstructive CAD patients have higher percentages of efferocytosis efficiency towards oxLDL generated apoptotic bodies than macrophages isolated from obstructive CAD patients.
3. The efficiency of efferocytosis performed by macrophages isolated from non-obstructive and obstructive CAD patients increases following the TNF- α downregulation and decreases after TGF- β downregulation.
4. TNF- α is directly proportional to ADAM17 but inversely proportional to MERTK. Meanwhile, TGF- β is directly proportional to MERTK but inversely proportional to ADAM17.

1.5 Study flow chart

The flow chart of this study is shown in Figure 1.2.

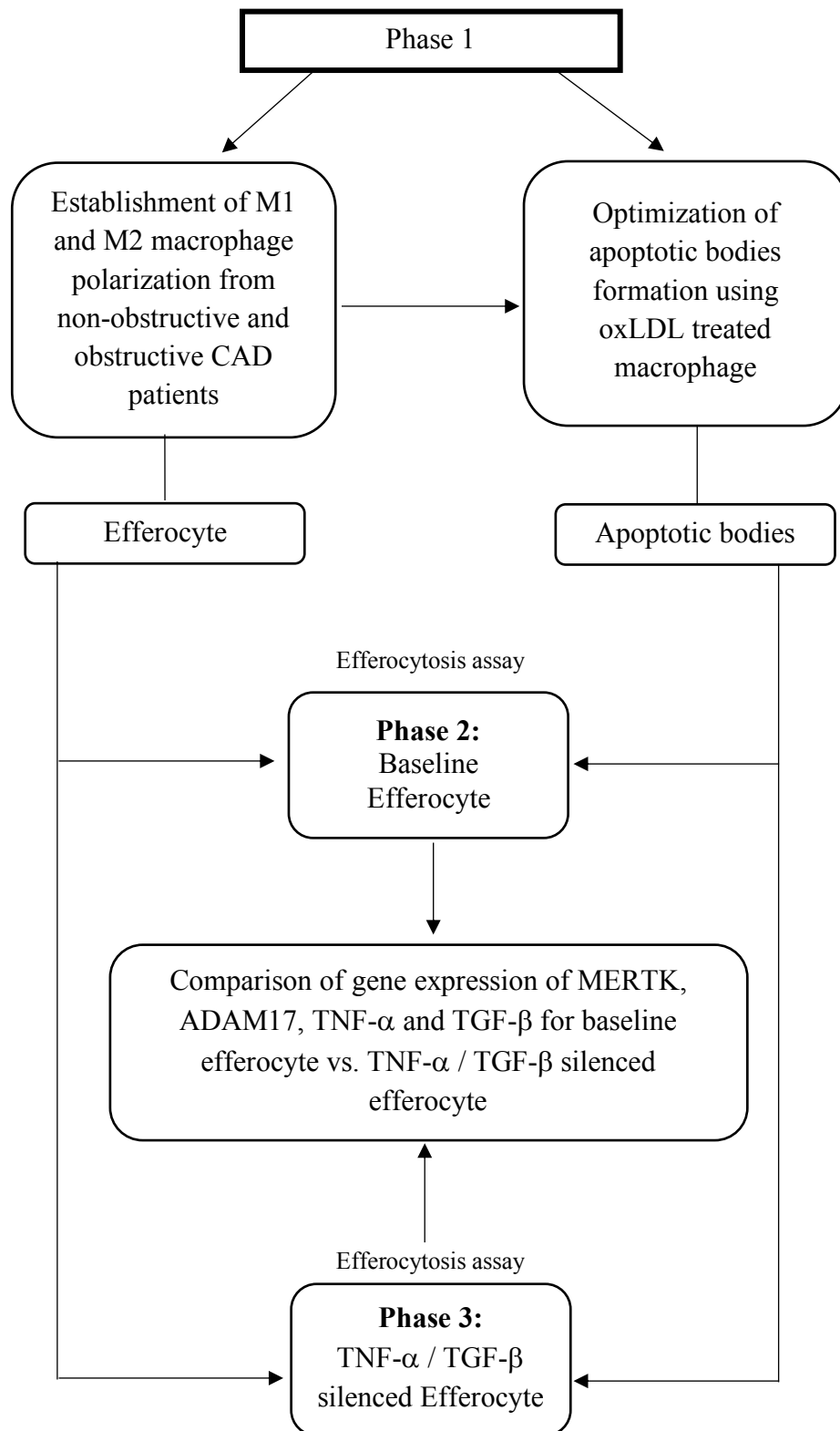


Figure 1.2 Flow chart of the study

CHAPTER 2

LITERATURE REVIEW

2.1 Background of coronary artery disease

2.1.1 Coronary artery disease in the umbrella of cardiovascular disease

The terms “cardiovascular disease” and “coronary artery disease” are often mistakenly regarded as referring to the similar pathology of the heart. In actual fact, cardiovascular disease is the big umbrella for all types of diseases of the heart (cardio) and the blood vessels (vascular) including CAD, cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease, deep vein thrombosis and pulmonary embolism (WHO, 2020). In this thesis, we focused on one of the most prevalence cardiovascular diseases which is CAD.

2.1.2 History of coronary artery disease

Around two centuries ago, CAD was first described as a disorder of the breast by Heberden while John Warren characterized chest pain as “angina pectoris” that means “infection of throat” in Latin or “strangling sensation” in Greek while pectoris is referring to the chest (John, 1812). The biopsy of a dying dog with quivering ventricle and the blood clot in its coronary artery was the eureka moment for the physiologist to understand that thrombotic occlusion in the coronary arteries is related to the myocardial ischemia (McWilliam, 1889). The thrombotic occlusion is currently understood as the fatal manifestation of the disease of the heart vessel which is termed as CAD. Since then, the discovery of the complex and multi-factorial CAD is growing and expanding exponentially.

2.1.3 Global burden of coronary artery disease

CAD is the leading cause of death globally including in the low-income and middle-income countries. It contributes to the major proportion of death from the overall amount of 17.9 millions of cardiovascular deaths equivalent to 31% of all deaths worldwide (WHO, 2020). In Malaysia, ischemic heart disease arises from CAD is the number one cause of mortality among five principal causes of death which accounted for 15.6% from 109,164 medically certified deaths in 2019 (Mahidin, 2020) (Figure 2.1). This remains as the number one cause of death when stratified into demographic factor of major ethnics in Malaysia; Bumiputera (14.5%), Chinese (14%) and, India (20.4%) and population in the age group of 41-59 years (18%) and above 60 years (16%) (Mahidin, 2020). The statistics showed that premature death from CAD is high in Malaysia and this may give harmful impact to the socioeconomic status of our country.

The burden of CAD arises from the risk factors not limited to longer average life span, tobacco use, sedentary lifestyles, and consumption of unhealthy diets (Nabel & Braunwald, 2012). In South Asian countries, higher rates of major adverse cardiovascular events and greater risk of cardiovascular deaths are found to be associated with poor knowledge of the disease, lack of access to healthcare, low affordability of medications, easier access to cheap cigarettes and low prevalence of healthy eating and physical activity especially in the low income countries compared to middle and high income countries (Rehman *et al.*, 2018).

Aside from the modifiable risk factors mentioned above which put CAD as a preventable disease, CAD is also associated with non-modifiable risk factors such as

genetic familial hypercholesterolemia, autoimmunity and inflammation in the blood vessels which further increase the global burden of CAD (Agrawal *et al.*, 2020). The gender differences also contribute to the prevalence of CAD as a higher number of male (79.44%) was reported to develop acute coronary syndrome compared to female (20.56%) in Malaysia (Lee *et al.*, 2021). Therefore, strategic prevention that incorporates multi-factorial risk factors is necessary to reduce the mortality and morbidity related to CAD.

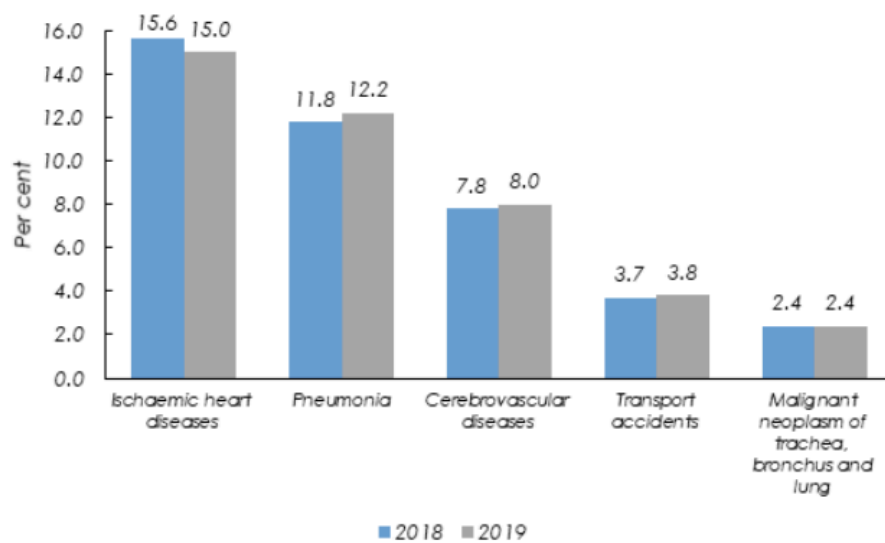


Figure 2.1 Statistics on five principal causes of deaths in Malaysia. Ischemic heart disease remained as the number one cause of deaths in Malaysia followed by pneumonia, cerebrovascular disease, transport accident and malignant neoplasm of trachea, bronchus and lung.

Image source:(Mahidin, 2020). Department of Statistics Malaysia Press Release: Statistics on Causes of Death, Malaysia, 2020.

2.1.4 Definition and classification of coronary artery disease

CAD is defined as the disease of the coronary vasculature which is manifested from the disruption in the hemodynamic flow of oxygenated blood in the coronary arteries supplying the heart muscle due to the deposition and accumulation of lipid plaque (Libby, Ridker & Maseri, 2002). The clinical conundrum of CAD has classified patients with angina pectoris into non-obstructive and obstructive CAD according to the degree of stenosis in the coronary arteries (Ford, Corcoran & Berry, 2018).

While there are a few terms have been proposed in the taxonomy of CAD to put non-obstructive and obstructive CAD under the category of “stable coronary artery disease” or “stable coronary syndromes”, both non-obstructive and obstructive CAD are described as a group of patients who have not had a recent acute event but the risk of cardiovascular event is continue to exist and should not be underestimated as a stable condition (Braun & Stevens, 2018; Fox *et al.*, 2020).

Recently, the American College of Cardiology (ACC), the American Heart Association (AHA) and the Society for Cardiovascular Angiography and Intervention (SCAI) have produced the latest guideline for coronary artery revascularization describing that coronary angiography remains the default method to define coronary anatomy and to characterize the severity of coronary arterial stenosis in order to categorize patients into non-obstructive and obstructive CAD for further optimized treatment strategy (Lawton *et al.*, 2022).

2.1.4(a) Non-obstructive coronary artery disease

In the 2011 practice guidelines for percutaneous coronary intervention by the American College of Cardiology Foundation (ACCF), AHA and SCAI, non-obstructive CAD is defined as a coronary artery stenosis of more than 20% but less than 50% in the left main coronary artery or less than 70% in any other epicardial coronary artery (Levine *et al.*, 2011). The prevalence of non-obstructive lesion finding during coronary angiogram is relatively high (10% - 25%) but was once considered “insignificant” in the medical literature (Maddox *et al.*, 2014).

However, an increasing number of non-obstructive CAD patients with evidence of ischemia has challenged the conventional concept of “insignificant” and warranted further research to discover the pathology of non-obstructive CAD despite the heterogeneity of patients under this group (Herscovici *et al.*, 2018). Strikingly, more than 50% of women undergoing coronary angiography are diagnosed with non-obstructive CAD and are often undertreated due to the lack of proper diagnosis (Makarovic *et al.*, 2018). As a result, non-obstructive CAD patients are three times more likely to suffer from a cardiac event within the first year following cardiac catheterization (Wittekoek & Piek, 2018).

2.1.4(b) Obstructive coronary artery disease

Meanwhile, the evident of obstructive CAD is based on the presence of more than 70% stenosis in non-left main coronary arteries and more than 50% stenosis in the left main coronary artery during coronary angiogram (Lawton *et al.*, 2022). As of now, there is no standard cut offs for the length of lesion to classify stenosis severity although it may contribute to the physiological lesion severity in which, longer

moderate lesion may result in more ischemia compared to focal severe lesion (Lawton *et al.*, 2022).

Obstructive CAD patients are more likely to be male and older with higher risk ratio of diabetic, hypertensive and dyslipidemic (Reeh *et al.*, 2019). In addition, meta analyses of 33 studies comparing the cardiovascular outcomes revealed that obstructive CAD patients have higher rates of myocardial infarction, cardiac death and major adverse cardiovascular events (5.9 %, 6.0%, 16.8%) compared to non-obstructive CAD patients (1.4%, 1.2%, 9.2%) (Pizzi *et al.*, 2016). This explains the priority given to the obstructive CAD patients in receiving revascularization treatment as their first-line therapeutic regime.

However, with the presence of lipid plaque irrespective of the degree of stenosis, all CAD patients have the likelihood to suffer from acute coronary syndrome most likely due to the plaque instability.

2.2 Atherosclerosis and plaque instability

2.2.1 Definition of atherosclerosis

Atherosclerosis is a chronic and progressive narrowing of the artery lumen due to the deposition of lipid which accumulate over the years forming a calcified plaque which consequently vulnerable to rupture and form thrombus (Libby, 2002). The atherosclerotic plaque is an active biological environment with vital players such as lipid, immune cells, smooth muscle cells and, endothelial cells that contribute to the ongoing inflammatory reaction in the plaque (Wang *et al.*, 2012). In the recent article

by Libby, pathogenesis of atherosclerosis is conveniently divided into three phases which are initiation, progression and complication (Libby *et al.*, 2019).

2.2.2 Initiation of atherosclerosis

Low-density lipoprotein (LDL) is the agent that initiate atherosclerosis in the coronary artery (Libby *et al.*, 2019) (Figure 2.2). The causal relationship between LDL and atherosclerosis was established in a study that revealed patients with familial hypercholesterolemia achieve cumulative LDL burden threshold at early ages and develop premature CAD (Nordestgaard *et al.*, 2013). Although the mechanism behind how excessive LDL initiate atherosclerosis is ambiguous, it is clear that LDL is deposited in the arterial wall and remains within the intimal layer and form aggregates by binding to the intimal proteoglycans (Libby *et al.*, 2019). In addition to LDL, triglyceride and lipoprotein(a) also contribute to the initiation of atherosclerotic plaque in the coronary artery (Agrawal *et al.*, 2020).

Apart from deposited in the intimal layer of coronary artery, LDL also participates in the activation of inflammatory pathway and drive the function of cell wall towards atherosclerosis by governing the master transcriptional regulator nuclear factor- κ B pathway (Hopkins, 2013). Furthermore, the cardiovascular diseases risk factor such as cigarette smoking and diabetic comorbidity can elicit inflammatory response in the coronary artery via the production of soluble inflammatory mediators such as cytokines that later activate cells in the intima (Libby, Ridker & Hansson, 2009).

Besides, the endothelium exposed to the atherogenic risk factors as described above is altered during the initiation of atherosclerosis. Expression of adhesion

molecule such as vascular cell adhesion protein 1 (VCAM-1) and chemoattractant are upregulated and thus, promote the uptake of leukocyte into the intima (Libby *et al.*, 2019) (Figure 2.2). Altogether, excessive LDL, triglyceride, lipoprotein(a), inflammatory cytokine, and dysregulated endothelium which recruit leukocyte into the coronary artery are responsible in atheroma formation at the early stage of atherosclerosis.

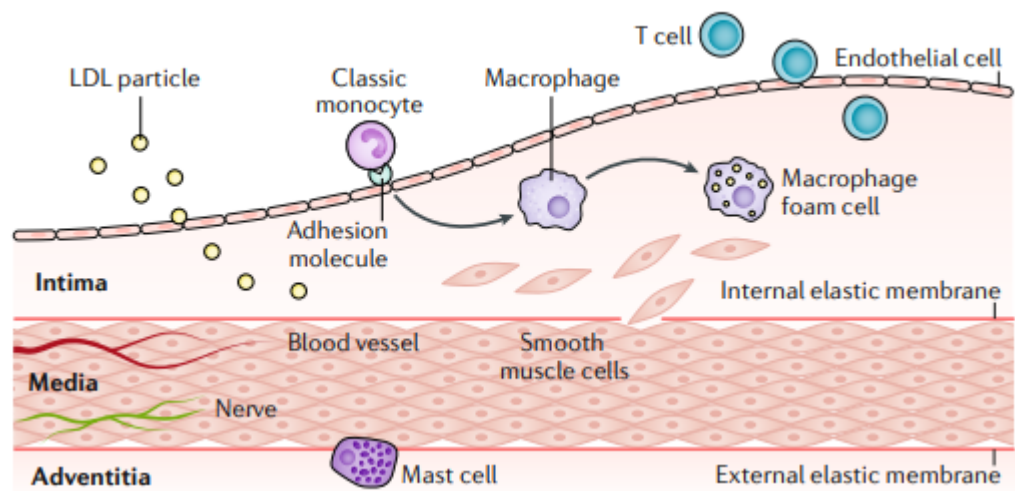
2.2.3 Progression and complication of atherosclerosis

Infiltration of leukocyte which is mainly consist of monocyte into the coronary artery lumen is followed by the proliferation and differentiation of monocyte into macrophage (Libby *et al.*, 2019) (Figure 2.2). Lipid-engorged cells are accumulated in the second stage of atherosclerosis resulted from the interaction between macrophage and excessive LDL in the atheroma which ultimately increase the plaque burden (Yu *et al.*, 2013). Furthermore, smooth muscle cells developed at the atheroma site tend to migrate into the coronary intima and proliferate over the years together with extracellular matrix macromolecules that adds to the bulk of the established atherosclerotic plaque (Stone, Libby & Boden, 2023).

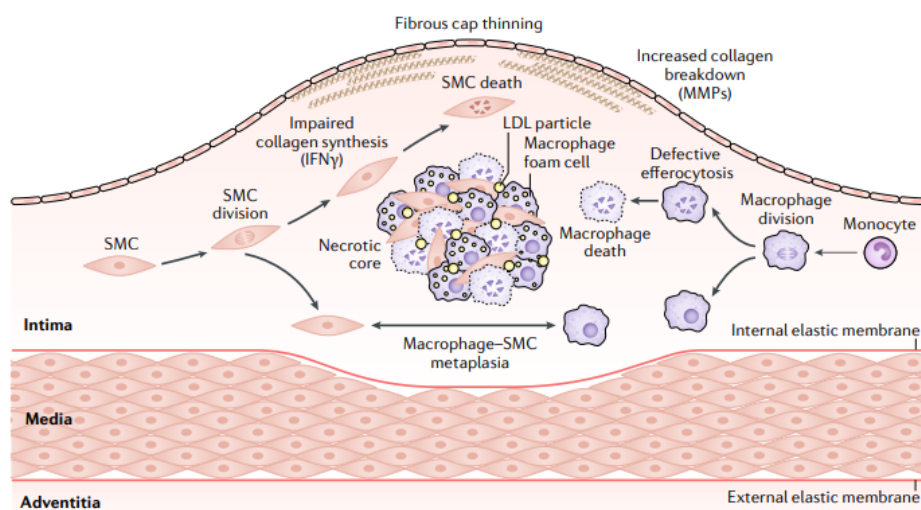
Moreover, the extracellular matrix which consists of interstitial collagen, elastins, proteoglycans and glycosaminoglycans is capable to entrap more lipoprotein and further promote the lipid accumulation in the atherosclerotic plaque (Libby *et al.*, 2019) (Figure 2.2). At this stage, more inflammatory leukocytes are present not only due to the infiltration at the endothelium but also due to the proliferation within the atherosclerotic plaque without the tendency to egress (Robbins *et al.*, 2014). The accumulation of LDL, foam cells and, extracellular matrix has evolved the atherosclerotic plaque and subsequently promote the deposition of calcium mineral in

the atheroma due to the dysregulation of calcium mineral deposition and impaired clearance (Ruiz, Hutcheson & Aikawa, 2015). Calcified plaque with mechanical instability resulted in the complications of plaque stability at the advanced stage of atherosclerosis.

At this point, the growing atherosclerotic plaque begins to significantly obstruct the arterial lumen leading to the formation of flow-limiting lesions. As a consequence, the complication of atherosclerosis is clinically developed due to the impairment of coronary arterial blood flow especially during the increase myocardial oxygen demands which can lead to ischemia and angina pectoris symptoms (Rahman *et al.*, 2019). Commonly, atherosclerotic plaque with large lipid cores and covered with thin fibrous cap ($<60\text{ }\mu\text{m}$) is highly susceptible to plaque rupture (Bentzon *et al.*, 2014). The rupture of atherosclerotic plaque leads to acute thrombosis of coronary arteries resulting in fatal myocardial infarction.



Initiation and progression of atherosclerosis



Progression and complication of atherosclerosis

Figure 2.2 Initiation, progression, and complication of atherosclerosis

Image source: (Libby *et al.*, 2019). *Atherosclerosis*. Nature Reviews.

2.2.4 The culprit behind plaque instability and plaque rupture

The complex and alarming biological environment in the atherosclerotic plaque with abundance of inflammatory immune cells, excessive apoptotic cells, and soluble cytokines that induce further inflammatory response have threatened the plaque stability. It is crucial to understand the culprit behind the plaque instability in order to exploit ways for the therapeutic potential to impede plaque rupture.

From the pathogenesis of atherosclerosis, the main culprit of plaque instability emerged during the second stage of atherosclerosis which is the formation of macrophage foam cells. Within the cytoplasm of the macrophage in the atherosclerotic plaque, lipoprotein engulfed by the macrophage was processed in the endoplasmic reticulum resulting in the re-esterification of free cholesterol and formation of lipid droplets which is the hallmark of macrophage foam cell formation (Moore & Tabas, 2011). However, impairment of free cholesterol re-esterification due to the accumulation of free cholesterol has trigger cell apoptosis via unfolded protein response as the adaptive mechanism to maintain the homeostasis of endoplasmic reticulum (Silvestre-Roig *et al.*, 2014).

The apoptosis of macrophage foam cells has been in debate as to whether it brings benefit or causes harm to the atherosclerotic lesion. At the early stage of atherosclerosis, the apoptosis of macrophage foam cells may reduce atherosclerotic lesion severity and plaque progression (Martinet *et al.*, 2019). This notion is supported by a few animal studies that suggested an inverse relationship between macrophage foam cells apoptosis and early atherosclerotic lesion. For instance, LDLR^{-/-} mice reconstituted with bone marrow derived hematopoietic cells without the pro-apoptotic