# PLAQUE INSTABILITY BIOMARKERS: A COMPARISON BETWEEN ACUTE CORONARY SYNDROME AND CHRONIC STABLE ANGINA PATIENTS

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# PLAQUE INSTABILITY BIOMARKERS: A COMPARISON BETWEEN ACUTE CORONARY SYNDROME AND CHRONIC STABLE ANGINA PATIENTS

**By**

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| ACS | Acute coronary syndrome |
| ACTB | Actin, beta |
| AMI | Acute myocardial infarction |
| AP-1 | Activator protein-1 |
| A.U | Arbitrary unit |
| AUC | Area under curve |
| B2M | Beta-2-microglobulin |
| β-ME | Beta-mercaptoethanol |
| BMI | Body mass index |
| BSA | Bovine serum albumin |
| CABG | Coronary artery bypass surgery |
| CAD | Coronary artery disease |
| CD40L | CD40 ligand |
| cDNA | Complementary DNA |
| CI | Confidence interval |
| CK | Creatine kinase |
| CK-MB | MB isoform of creatine kinase |
| CRP | C-reactive protein |
| CSA | Chronic stable angina |
| Ct | Cycle threshold |
| CV | Coefficient of variation |
| DNA | Deoxyribonucleic acid |
| EC | Endothelial cell |
| ECG | Electrocardiogram |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| eNOS | Endothelial nitric oxide synthase |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| GM-CSF | Granulocyte/macrophage colony-stimulating factor |
| GPBB | Glycogen phosphorylase isoenzyme BB |
| GUSB | Beta-glucuronidase |
| HCl | Hydrochloric acid |
| HDL | High-density lipoprotein |
| H-FABP | Heart-type fatty acid binding protein |
| HPRT 1 | Hypoxanthine-guanine phosphoribosyltransferase 1 |
| HRP | Horse radish peroxidase |
| IL | Interleukin |
| IL-1β | Interleukin-1 beta |
| IFN-γ | Interferon gamma |
| IQR | Interquartile range |
| IVUS | Intravascular ultrasound |
| KCl | Potassium chloride |
| LAD | Left anterior descending artery |
| LCA | Left coronary artery |
| LCx | Left circumflex artery |
| LDL | Low-density lipoprotein |
| LR− | Negative likelihood ratio |
| LR+ | Positive likelihood ratio |

| | |
|---|---|
| MACE | Major adverse cardiac events |
| MCP-1 | Monocyte chemoattractant protein-1 |
| M-CSF | Macrophage colony-stimulating factor |
| MMP | Matrix metalloproteinase |
| MPO | Myeloperoxidase |
| mRNA | Messenger ribonucleic acid |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| $NaHCO_3$ | Sodium bicarbonate |
| $Na_2CO_3$ | Sodium carbonate |
| NaCl | Sodium chloride |
| NaOH | Sodium hydroxide |
| NF-κB | Nuclear factor kappa B |
| NF | Normalization factor |
| $NH_4Cl$ | Ammonium chloride |
| NPV | Negative predictive values |
| NSTEMI | Non-ST-elevation myocardial infarction |
| OCT | Optical coherence tomography |
| OD | Optical density |
| PBS | Phosphate buffered saline |
| PCI | Percutaneous coronary intervention |
| PCR | Polymerase chain reaction |
| PlGF | Placental growth factor |
| PPAR | Peroxisome proliferator-activated receptor |
| PPAR-α | Peroxisome proliferator-activated receptor alpha |
| PPAR-β | Peroxisome proliferator-activated receptor beta |
| PPAR-γ | Peroxisome proliferator-activated receptor gamma |
| PPIA | Cyclophilin A |
| PPRE | Peroxisome proliferator response element |
| PPV | Positive predictive values |
| RCA | Right coronary artery |
| REVERSAL | Reversal of Atherosclerosis with Aggressive Lipid Lowering Trial |
| RNA | Ribonucleic acid |
| ROC | Receiver operating characteristic |
| ROS | Reactive oxygen species |
| RPLP | 60S acidic ribosomal protein P0 |
| RRN18S | 18S rRNA |
| RT-PCR | Reverse transcription polymerase chain reaction |
| RXR | Retinoic acid receptor |
| sCD40L | Soluble CD40 ligand |
| SD | Standard deviation |
| SDS | Sodium dodecyl sulfate |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| SPSS | Statistical Product and Service Solutions |
| STEMI | ST-elevation myocardial infarction |
| TAE | Tris-Acetate-EDTA |
| TBP | TATAA-box binding protein |
| TCA | Trichloroacetic acid |
| TEMED | Tetramethylethylenediamine |
| TMB | Tetramethylbenzidine |
| TNF-α | Tumor necrosis factor alpha |

| | |
|---|---|
| TNF-β | Tumor necrosis factor beta |
| Tris-HCl | Tris-hydrochloride |
| TUBB | Tubulin beta polypeptide |
| UA | Unstable angina |
| UBC | Ubiquitin C |
| v/v | Volume/volume |
| w/v | Weight/volume |
| WBC | White blood cell |
| YWHAZ | Tyrosine 3/tryptophan 5-monooxygenase activation protein, zeta |

**BIOPENANDA KETIDAKSTABILAN PLAK: PERBANDINGAN ANTARA SINDROM KORONARI AKUT DAN ANGINA STABIL YANG KRONIK**

## ABSTRAK

Biopenanda memainkan peranan yang penting dalam diagnosis dan pengurusan penyakit sindrom koronari akut (ACS). Biopenanda seperti, protein C-reaktif (CRP), ligan terlarut CD40 (sCD40L), faktor pertumbuhan plasenta (PlGF) dan mieloperoksida (MPO) telah dilaporkan terlibat dalam patogenesis penyahstabilan plak. Paras biopenanda tersebut dikaji dalam pesakit berumur 45 tahun dan ke bawah dan juga mereka yang berumur lebih daripada 45 tahun yang berpenyakit ACS dan penyakit angina stabil yang kronik (CSA). Hubungan antara paras biopenanda di peredaran koronari dan peredaran periferi juga dikaji bersama. Kajian ini merupakan penyelidikan pertama yang menyiasat pengekspresian reseptor pengaktif-proliferasi peroksisom (PPARs) dalam pesakit ACS. Sejumlah 79 pesakit (ACS: $n$ = 39, CSA: $n$ = 40) terlibat dalam kajian ini. Darah pesakit disampel daripada arteri koronari yang tersumbat (peredaran koronari) dan juga daripada vena kubital median (peredaran periferi). Paras protein CRP, sCD40L, PlGF dan MPO dalam sampel darah ditentukan dengan menggunakan teknik asai imunoerap terangkai enzim (ELISA). Paras PPARs intrasel pula diukur dengan menggunakan analisis pemendapan Western. Paras mRNA biopenanda tersebut diukur dengan menggunakan analisis masa sebenar tindak balas rantai polimerase (*real-time* PCR). Semua pesakit ACS yang menjalani susulan klinikal selama enam bulan dikenalpasti peristiwa mudarat kardiak major (MACE) berikutan peristiwa akut. Paras protein CRP, sCD40L, PlGF and MPO dalam peredaran periferi didapati meningkat dengan signifikan dalam pesakit ACS berbanding dengan pesakit CSA. Tambahan pula, hasil kajian

menunjukkan bahawa kepekatan biopenanda tersebut dalam peredaran periferi berkait rapat dengan kepekatannya dalam peredaran koronari. Kajian ini juga menunjukkan bahawa golongan pesakit berumur di bawah 45 tahun mempunyai profil biopenanda yang serupa dengan mereka yang berumur lebih daripada 45 tahun. Paras PPAR-γ meningkat dengan signifikan dalam pesakit ACS dan parasnya berkait rapat dengan kepekatan sCD40L dan MPO. CRP dalam serum menunjukkan keluasan bawah lengkungan yang paling tinggi (0.79, $p < 0.001$) dalam pembezalayan penyakit ACS, diikuti oleh PlGF, MPO dan sCD40L. Tambahan pula, biopenanda tersebut juga menunjukkan keupayaan prognostik yang baik untuk meramal kejadian MACE dalam masa 30 hari dan enam bulan berikutan serangan akut ACS. Kesimpulannya, kajian ini memberi maklumat tambahan mengenai profil pengekspresian protein dan gen biopenanda ketidakstabilan plak dalam golongan ACS dan CSA. Biopenanda tersebut menyumbang kepada pembentukan plak tidak stabil dengan mencetuskan keradangan vaskular, penipisan topi serabut dan pembentukan teras lipid yang besar dalam plak koronari. Keupayaan mereka dalam membeza layan ACS dan meramal MACE juga menunjukkan keputusan yang memberansangkan.

**PLAQUE INSTABILITY BIOMARKERS: A COMPARISON BETWEEN ACUTE CORONARY SYNDROME AND CHRONIC STABLE ANGINA PATIENTS**

## ABSTRACT

Biomarkers play a pivotal role in the diagnosis and management of patients with acute coronary syndrome (ACS). Some biomarkers, such as C-reactive protein (CRP), soluble CD40 ligand (sCD40L), placental growth factor (PlGF) and myeloperoxidase (MPO) have been reported to be involved in plaque destabilization. The levels of these biomarkers were studied in ACS and chronic stable angina (CSA) patients aged ≤ 45 years and aged > 45 years. The relationship between these biomarkers in the coronary circulation and peripheral circulation was also investigated. This study was the first attempt to investigate the expression of peroxisome proliferator-activated receptors (PPARs) in ACS. A total of 79 patients (ACS: $n$ = 39, CSA: $n$ = 40) was recruited. The blood was sampled from the occluded coronary artery (coronary circulation) and also from the median cubital vein antecubital fossa (peripheral circulation). The serum protein levels of CRP, sCD40L and PlGF and plasma levels of MPO were measured using ELISA. The intracellular levels of PPARs were semi-quantified using Western blot. The mRNA levels of the biomarkers were measured by real-time PCR. All ACS patients that underwent six months clinical follow-up was assessed for major adverse cardiac events (MACE) after the acute event. The peripheral levels of CRP, MPO, sCD40L and PlGF were significantly increased in ACS compared to CSA patients. Furthermore, the peripheral concentrations of these biomarkers were significantly correlated with the concentrations found in the coronary circulation. The patients aged below 45 years and above 45 years shared

similar profiles of biomarkers. The expression of PPAR-γ was significantly increased in the ACS patients and correlated with both sCD40L and MPO. Serum CRP demonstrated the highest area under the curve value of 0.79 ($p < 0.001$) in discriminating ACS, followed by PlGF, MPO and sCD40L. In addition, the biomarkers also showed their promising prognostic abilities in predicting 30-day and six-month MACE in ACS patients. In conclusion, this study provided additional information on the proteins and gene expression profiles of plaque instability markers in both CSA and ACS patients. The biomarkers contribute to the formation of unstable plaque by triggering vascular inflammation, firous cap thinning and formation of large lipid core in coronary plaque. Their accuracies in discriminating ACS and predicting MACE also showed promising results.

# CHAPTER 1

# INTRODUCTION

## 1.1 Introduction of CAD

### 1.1.1 Burden of CAD

Despite dramatic advances in medicine, cardiovascular diseases persist the primary cause of death throughout the world and killed nearly 17.5 million people in 2012 (World Health Organization, 2012a). Of these 17.5 million deaths, an estimated 7.4 million were caused by coronary heart disease, 6.7 million due to stroke and the remaining 3.4 million due to other cardiovascular diseases such as hypertensive heart disease, inflammatory heart disease and rheumatic heart disease. According to World Health Organization (World Health Organization, 2012b), non-communicable diseases caused two-thirds of the total death worldwide in 2012 (Figure 1.1). From the total of 38 million deaths caused by non-communicable diseases in 2012, 46% or 17.5 million deaths were attributed to cardiovascular diseases.

In Malaysia, cardiovascular disease is the leading cause of death of non-communicable diseases. It caused a quarter of all deaths in Malaysia hospitals in 2013 (Figure 1.2) (Ministry of Health Malaysia, 2014). According to Ministry of Health Hospitals' Survey reported in year 2002, cardiovascular diseases persistently account for 15 to 16% of all hospital deaths annually from 1995 to 2002. Furthermore, ischemic heart disease was the primary cause of cardiovascular death and was responsible for 12.9% of total deaths in 2008 (Malaysia Department of Statistics, 2010).

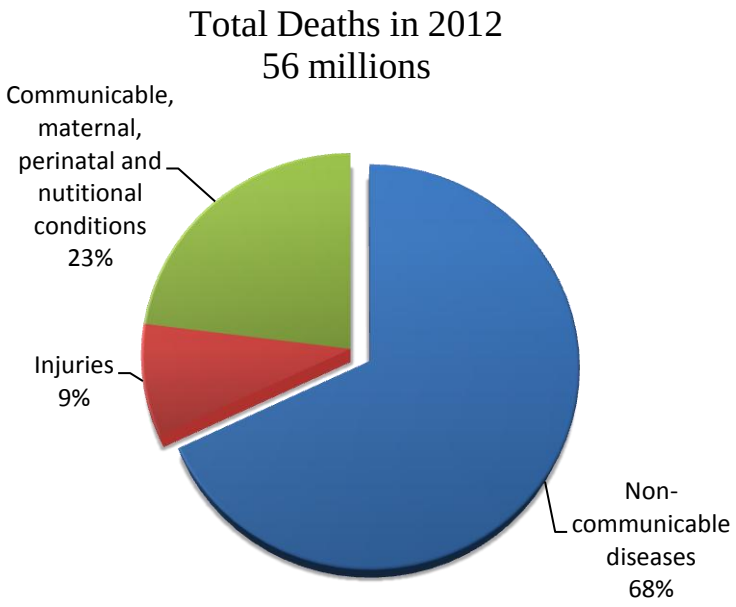


Figure 1.1 The main causes of death worldwide of all ages in year 2012 (World Health Organization, 2012b).

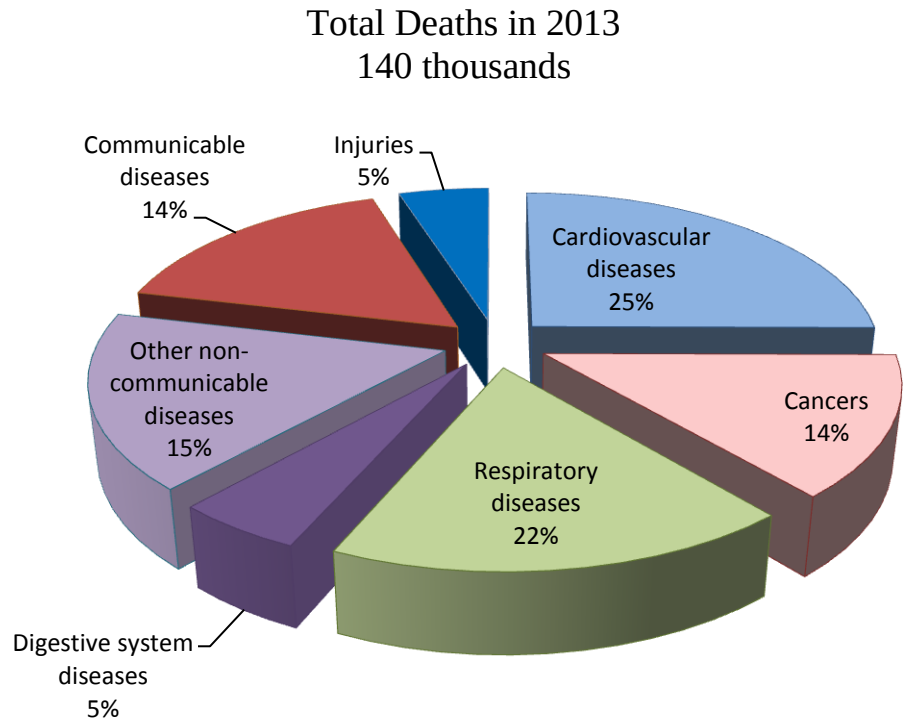


Figure 1.2 The main causes of death of all ages in Malaysia in year 2013 (Ministry of Health Malaysia, 2014).

### 1.1.2 Atherosclerosis and CAD

Synonymous with coronary artery disease (CAD), ischemic heart disease is the most common manifestation of myocardial ischemia attributed to critical narrowing (> 75% stenosis of the luminal diameter) of any of the three principal epicardial coronary arteries, *i.e.* the left anterior descending artery, left circumflex artery, and the right coronary artery. The heart muscle may not receive enough blood supply following the luminal narrowing, especially when workloads of the heart increase during exercise. This transient ischemia may cause chest pain called angina, which typically relieves on rest. The prolonged ischemia eventually leads to irreversible necrosis of the muscle cells located distal to the blockage.

In most cases, CAD results from atherosclerosis process. The pathogenesis of atherosclerosis has been studied extensively since the last few decades. Atherosclerosis is a chronic inflammatory process resulting from the interactions between the circulating components (extracellular matrix, plasma lipoproteins, leukocytes, platelets) and the arterial wall (Fan and Watanabe, 2003). It involves various highly interconnecting processes, including endothelial dysfunction (Topper *et al.*, 1996), inflammation (Libby *et al.*, 2002), vascular smooth cell activation (Lee *et al.*, 2001), platelets activation (Rauch *et al.*, 2001), altered matrix and lipid metabolism (Libby, 1995), thrombosis (Libby and Simon, 2001), remodeling and genetic factors (Faxon *et al.*, 2004).

#### 1.1.2.1 History of atherosclerosis research

The pathogenesis of atherosclerosis was a topic of interest among biological and medical scientists in the 18$^{th}$ century. In 1815, a London physician and surgeon

named Joseph Hodgson reported that inflammation was the underlying cause of atherosclerosis, and it occurred in the intima between the lumen and media of the diseased coronary vessels (Kaperonis *et al.*, 2006). In 1856, Rudolf Virchow studied the pathogenesis of atherosclerosis by investigating the histological characteristics of atherosclerotic lesion in all stages (Heidland *et al.*, 2006) and he came out with the term “endarteriitis deformans”, that refers to the formation of atheroma within intima resulted from inflammation and fibrous thickening attributed to a reactive change in intima (Heidland *et al.*, 2006). The report suggested that endarteriitis deformans was initiated by mechanical forces and the inflammation acted as a repair mechanism. Virchow also suggested in the same year that atherosclerosis occurred when plasma components induced an inflammatory response in the arterial wall and resulted in local intima injury (Zarifis, 2005). This report emphasizes the local intima injury as the earliest stimulus of inflammation.

The clarification on the role of cholesterol in the pathogenesis of atherosclerosis is one of the significant discoveries in the 20$^{th}$ century. In 1908, a Russian researcher named A.I. Ignatowski, described the relationship between cholesterol-rich food and experimental atherosclerosis by feeding rabbits with a diet of milks and eggs. The silky smooth lining of arteries of the rabbits turned into yellow cobble stone following the cholesterol-rich diet (Konstantinov and Jankovic, 2013). In 1910, Adolf Windaus who studied the structure of cholesterol showed that atheromatous lesions contained six times higher percentage of free cholesterol and 20 times higher amount of esterified cholesterol compared to a normal arterial wall (Konstantinov *et al.*, 2006). In 1913, a Russian doctoral student, Nikolai Nikolaevich Anichkov showed that cholesterol alone was responsible for the physical changes in the arterial

walls that led to the subsequent development of coronary or carotid plaque (Konstantinov *et al.*, 2006). These discoveries introduced a new era in the atherosclerosis research.

In the 20$^{th}$ century, the lipid theory dominated the field of atherogenesis research until Russell Ross reopened the discussion on the inflammatory nature of atherosclerosis in 1976. Ross, Glomset and Harker produced a response-to-injury hypothesis, which suggested that the atherosclerosis lesion is attributed to arterial endothelial cell injury (Ross *et al.*, 1977). The endothelial injury is subsequently followed by a series of events, including endothelial desquamation, platelets adherence and aggregation at the injured sites and intimal smooth muscle proliferative response, which are the essential changes prior to the atherosclerotic lesion formation (Ross *et al.*, 1977). Moore *et. al.* (1976) and Bowie *et. al.* (1975) stressed on the vital role of platelets in the promotion of intimal smooth muscle proliferation that contributes to the progression of atherosclerosis lesion. The response-to-injury hypothesis of atherosclerosis is still valid today with minor alterations. The revised response-to-injury hypothesis focused on the endothelial dysfunction rather than the endothelial denudation as the trigger of the inflammatory response and progression of atherosclerosis (Ross, 1999, Endemann and Schiffrin, 2004).

#### 1.1.2.2 Progression of atherosclerosis in relation to coronary plaque formation

Atherosclerosis is a multifactorial and multistep process that usually occurs many years before any clinical symptom manifests. According to the response-to-injury theory, endothelial dysfunction is the first step in atherosclerosis (Ross *et al.*, 1977)

and from this point on, an inflammatory response is triggered that leads to the progression of atherosclerotic plaque (Figure 1.3).

Endothelium maintains the normal vascular tone and blood fluidity under normal homeostatic conditions. However, endothelial dysfunction can occur in response to a variety of stimuli, such as oxidized low-density lipoprotein (LDL), free radicals caused by smoking, hypertension, diabetes, genetic alterations, elevated plasma homocysteine concentrations and infectious microorganisms (Kaperonis *et al.*, 2006).

Healthy endothelium maintains the non-thrombogenic surface of the vascular system. However, activated endothelial cells begin to express adhesion molecules, such as selectins, vascular cell adhesion molecules and intercellular adhesion molecules, on their surface soon after exposure to atherogenic stimuli. These adhesion molecules act as receptors for glycoconjugates and integrins present on monocytes and T-cells (Kaperonis *et al.*, 2006). Li *et. al.* (1993) showed an increased expression of vascular cell adhesion molecule-1 on endothelial cells activated by proinflammatory cytokines.

Increased cellular adhesion and associated endothelial dysfunction eventually trigger the release of cytokines, recruitment of inflammatory cells and uptake of lipid into the atherosclerotic plaque. The vascular cell adhesion molecule-1, intercellular adhesion molecule-1 and some of the beta-chemokine receptors assist on the adherence of circulating monocytes to the endothelium. Monocytes adhere to the endothelium and migrate into the intima with the help of locally activated matrix metalloproteinases (MMPs).

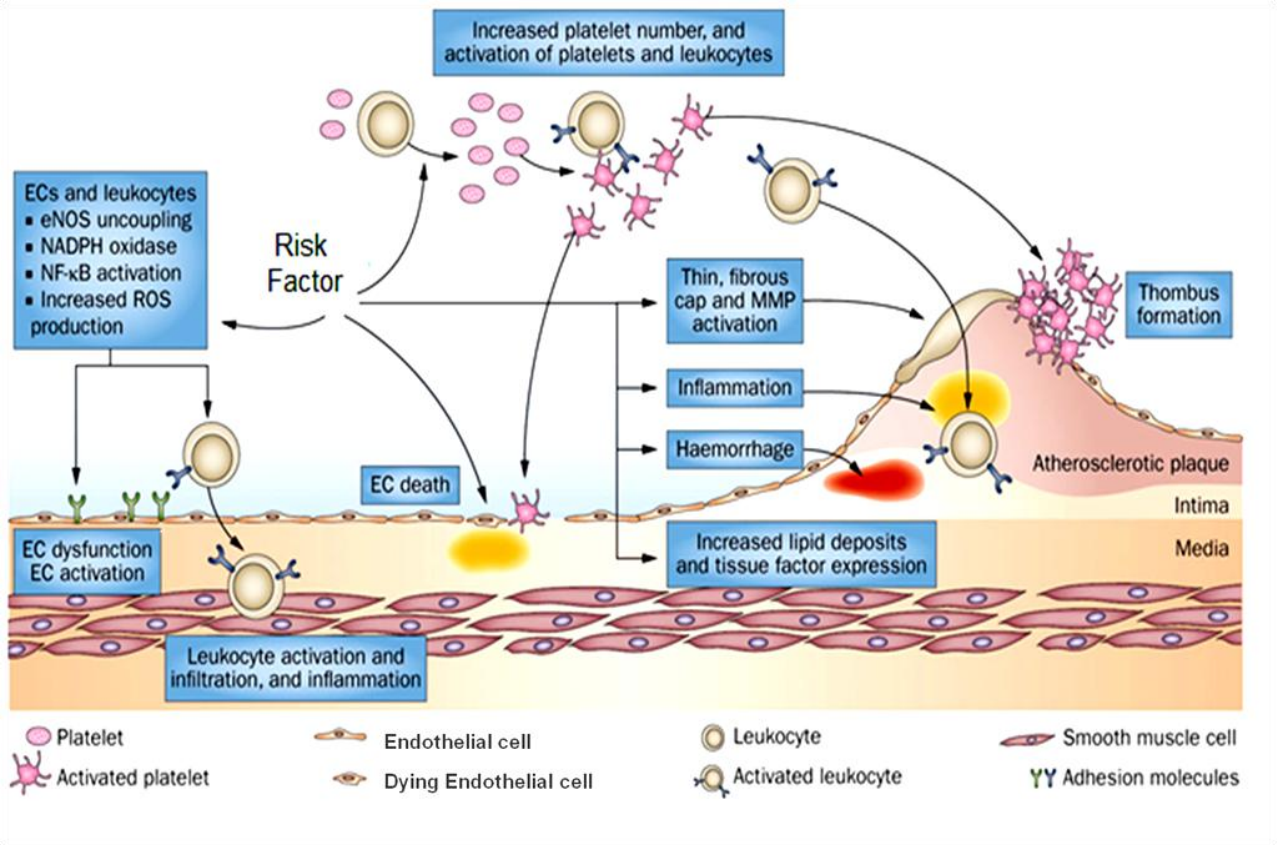


Figure 1.3 A schematic diagram of processes in the development and progression of atherosclerosis, plaque destabilization and rupture and thrombus formation (Csordas and Bernhard, 2013).
Abbreviations: EC: endothelial cell, eNOS: endothelial nitric oxide synthase, NADPH: nicotinamide adenine dinucleotide phosphate, NF- κB: Nuclear factor-κB, ROS: reactive oxygen species, MMP: matrix metalloproteinase.

The enzymes aid on the migration of monocytes into the intima through degrading the connective tissue matrix (Crowther, 2005). The migrated monocytes subsequently differentiate into tissue macrophages in the presence of various cytokines such as macrophage colony-stimulating factor (M-CSF), tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), proinflammatory interleukins (*e.g.* interleukins-1 and -2; IL-1, -2) and growth factors (such as transforming growth factor beta, platelet derived growth factor and insulin-like growth factor-1) (Ross, 1999).

The M-CSF gives rise to the ingestion of lipids and multiplication and differentiation of monocytes into macrophage foam cells. The macrophage foam cell produces cytokines and growth factors, further promoting atherosclerosis. It serves as a source of MMPs that further promoting the weakening and rupture of the fibrous cap (Zandbergen and Plutzky, 2007). The lesion that formed underneath a monolayer of endothelial cells, is the first lesion of atherosclerosis and it is named as fatty-streak lesion (Kaperonis *et al.*, 2006).

In the fatty-streak lesion, activated T-cells and native vascular wall cells secrete cytokines [tumor necrosis factor beta (TNF-β) and IFN-γ)], fibrogenic mediators and growth factors that can promote the migration and proliferation of smooth muscle cells (Kaperonis *et al.*, 2006). The smooth muscle cells in the intima produce extracellular matrix components, including elastin, collagen and proteoglycans, which form the fibrous cap and contribute to the stability of the plaque. This fibrous cap surrounds a lipid rich core in advanced plaques. The smooth muscle cells also synthesize proinflammatory cytokines, including IL-1 and TNF-α. Gradually, an

advanced atherosclerotic lesion is formed, with the characteristic of large core of lipids and necrotic tissue that is covered with a thin fibrous cap. The atherosclerotic lesion that built up in the lining coronary artery is called coronary plaque, which blocks blood flow to cardiac muscles.

### 1.1.3 Coronary plaque composition, morphology and clinical outcome

#### 1.1.3.1 Stable plaque and CSA

Coronary plaques can be classified into two types, *i.e.* white plaques and yellow plaques, based on their color and appearance under coronary angioscopy. The white plaques have a small lipid core surrounded by thick fibrous cap (Thieme *et al.*, 1996). These plaques are rich in collagen, which makes the plaque more stable in structure. Most of these lesions grow slowly over several decades in coronary arteries and remain clinically silent, or on a long term, may lead to chronic stable angina (CSA) (Davies, 1990).

Angina episodes in patients with CSA are typically precipitated by an increase in myocardial oxygen demand in the setting of a consistent decrease in blood supply due to long-standing and well-developed atherosclerotic white plaques. Coronary plaques that contribute to exertional angina symptoms usually obstruct 70% of the epicardial coronary vessel lumen. The white plaques in CSA patients are more stable, have a reduced lipid pool, and they seldom rupture. Since their geometry does not typically change acutely, they provide a relatively fixed reduction in myocardial oxygen supply.

### 1.1.3.2 Unstable plaque and ACS

In comparison to white plaque, the yellow plaques have thinner fibrous caps with larger amount of lipids accumulate in the plaque (Thieme *et al.*, 1996). The lipid core expands and the thick fibrous cap begins to erode to form a weak fibrous cap, which is highly susceptible to rupture (Virmani *et al.*, 2000). Therefore, the yellow plaques are always referred as the unstable plaques. The unstable plaque refers to a plaque that is likely to rupture and subsequently leads to thrombus formation.

Unstable plaques are responsible for the clinical manifestation of acute coronary syndrome (ACS). ST-elevation myocardial infarction (STEMI), non-ST-elevation myocardial infarction (NSTEMI) and unstable angina (UA) are a range of clinical presentations with a similar underlying pathological mechanism of ACS (Falk, 1985, Fuster, 1994). Morphologically, an unstable plaque is characterized by (a) a large lipid core (40% of the entire plaque); (b) a thin fibrous cap with thickness less than 65 mm; (c) with increased macrophage and proteoglycan content; (d) presence of calcified nodules (Kolodgie *et al.*, 2001, Virmani *et al.*, 2003).

#### 1.1.3.2 (a) Mechanism of coronary plaque destabilization

Atherosclerotic plaque undergoes destabilization process to form unstable plaque, which is susceptible to rupture and subsequently leads to thrombus formation. During atherosclerosis, plaque stability is influenced by the inflammatory cells that destabilize the plaque and the reparative function of smooth muscle cells that promote plaque stability. Cheng *et. al.* (1993) hypothesized that the plaque rupture occurs following an imbalance between synthesis and breakdown of extracellular matrix in the fibrous cap, resulting in depletion of collagen and other matrix

components, which in turn leads to cap thinning (Figure 1.4). A thick fibrous cap prevents contact between the lipid core and circulating blood. It stabilizes the plaque by reducing circumferential tensile stress. In contrast, a thin fibrous cap increases the risk of plaque rupture due to its increased tensile stress (Loree *et al.*, 1992).

Vascular smooth muscle cell is the only cell type in the human body that is able to synthesize essential extracellular matrix proteins, such as collagen and elastin from amino acids. The extracellular matrix is essential in forming the skeleton and enhancing stability of the atherosclerotic plaque. Therefore, a decrease in smooth muscle cells also results in matrix depletion (Felton *et al.*, 1997, Burleigh *et al.*, 1992). Inflammatory cells, such as T cells, have been shown to inhibit the matrix synthesis by smooth muscle cells. An *in vitro* study using human smooth muscle cells has demonstrated that activated T cells produce IFN-γ, which eventually resulted in the down regulation of collagen synthesis by smooth muscle cells (Amento *et al.*, 1991).

Besides extracellular matrix depletion, the increased in extracellular matrix breakdown also attributes to plaque rupture. Macrophages within the fibrous cap can be activated by the expression of CD40 ligand on T cells (Phipps, 2000). The activated macrophages secrete MMPs, such as collagenases, gelatinases and stromelysin that support the breakdown of collagen and elastin to peptides and amino acids. The reduction of extracellular matrix content in fibrous cap of a coronary plaque thereby increases its likelihood to rupture (Libby, 1995).

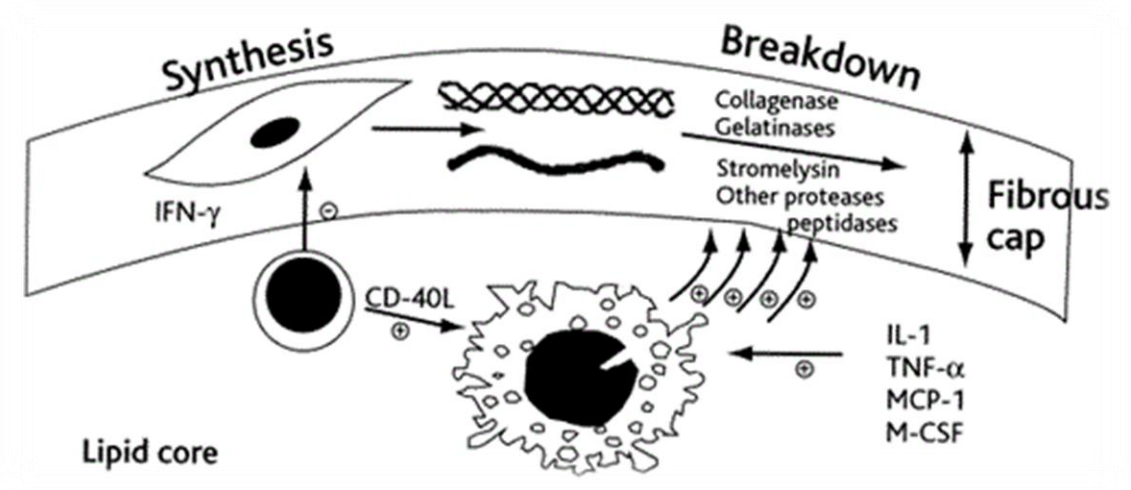


Figure 1.4 The importance of collagen synthesis and breakdown in the maintenance of the integrity of the fibrous cap (Finn *et al.*, 2010). Abbreviations: IFN-γ: interferon gamma, CD-40L: CD40 ligand, IL-1: interleukin 1, TNF-α: tumor necrosis factor alpha, MCP-1: monocyte chemoattractant protein -1, M-CSF: macrophage colony stimulating factor

#### 1.1.3.2 (b) Clinical presentations following plaque destabilization and rupture

The rupture of an unstable plaque may occur spontaneously or in response to a variety of mechanical and hemodynamic forces (MacIsaac *et al.*, 1993, Lee and Kamm, 1994). Plaque rupture is defined as a lesion that consists of a necrotic core with an overlying thin ruptured fibrous cap, which subsequently leads to luminal thrombosis following the interaction between circulating platelets with the thrombogenic necrotic core (Virmani *et al.*, 2006). The rupture usually occurs at the shoulder regions of the fibrous cap, as this region withstands the highest tensile stress and increased MMP production (Galis *et al.*, 1994). Although plaque rupture often leads to thrombosis with clinical manifestations of ACS, but it may also occur without clinical consequences.

The most common clinical manifestation of plaque rupture is ACS. ACS refers to any group of clinical symptoms compatible with acute myocardial ischemia and covers a wide spectrum of clinical conditions, ranging from UA to NSTEMI to STEMI (Kumar and Cannon, 2009). STEMI is the most severe condition that involves complete blockage of coronary artery. As a result, all the surrounding heart muscles at the blockage region become ischemic and eventually infarction happens. The infarction causes a characteristic elevation in the ST segment on electrocardiogram (ECG). The conditions and clinical presentations of UA and NSTEMI are similar, but they differ in severity. A diagnosis of NSTEMI can be made when the ischemia is sufficiently severe to cause myocardial damage, that results in the release of biomarkers of myocardial necrosis into the circulation. The biomarkers include muscle and brain fraction of creatine kinase [CK-MB] and cardiac-specific troponins

T or I. In contrast, the patient is considered to have experienced UA if there is no such biomarker detected in the bloodstream within 12 hours after the initial onset of ischemic chest pain.

## 1.2 Identification of unstable plaque

The formation of unstable plaque could lead to the occurrence of ACS. Therefore, it has been a growing interest in the identification and treatment of unstable plaques and unstable patients, which can eventually reduce the mortality and morbidity of the disease. During the past decades, enormous efforts have been addressed by scientist to identify the unstable plaques using various methods.

### 1.2.1 Identification of unstable plaque using imaging techniques

Imaging techniques, such as coronary angiography and echocardiogram, are widely used to visualize the coronary arteries. The coronary angiography is an invasive imaging test that uses contrast dye and special x-rays to visualize the inner sides of coronary arteries. It is the gold standard for the evaluation of coronary plaque. However, the unstable plaque is often asymptomatic and it cannot be reliably visualized by routine coronary angiography (Cademartiri *et al.*, 2007). To date, there is no potential technology that can effectively identify the unstable plaques. However, several invasive and non-invasive imaging modalities are currently being developed.

The invasive modalities used to identify unstable plaques include coronary intravascular ultrasound (IVUS) and optical coherence tomography (OCT). The IVUS views the coronary arteries using a miniaturized ultrasound transducer placed at the tip of a catheter (DeMaria *et al.*, 2006). In contrast to coronary angiography

that can only visualize the vessel lumen, IVUS visualizes both the lumen and the vessel wall. Areas of fibrosis and calcification, as well as plaque ruptures or ulcerations can also be visualized. Similar to IVUS, the OCT also uses catheter to visualize the coronary arteries (Stamper *et al.,* 2006). It uses back reflection of waves to characterize arterial wall and plaque structures. However, OCT differs from IVUS as it uses infrared light instead of ultrasound.

Besides morphological visualization, the activity of the coronary plaque can be reflected in temperature changes. Plaque with high levels of inflammatory activity produces heat (Casscells *et al.,* 1996). The heat production could be due to leukocyte metabolic activity, ineffective metabolism or increased neoangiogenesis (Madjid *et al.,* 2006). Several coronary temperature mapping methods using specialized catheters like Volcano catheter and ThermoCoil Guidewire are currently under development. These thermo-sensitive catheters measure temperature at the plaque and compare the recording to temperature within healthy vascular walls or the bloodstream. The "hot spot" detected is hypothesized to represent region prone to plaque rupture and local thrombosis.

Besides the temperature difference, arterial walls with unstable plaques have different mechanical properties compared to the healthy vascular tissue (Schaar *et al.*, 2006). Thin fibrous layers, large lipid cores, and inflammatory cells can alter the stress-strain relationship of coronary vessels. Intravascular ultrasound palpography is developed to assess the mechanical properties of the vascular wall in order to identify the unstable plaques. It is a technique that depicts the distribution of the mechanical strain over the luminal surface of coronary arteries. It utilizes conventional radio

frequency signals to detect the microscopic tissue displacements, which can be directly translated into local strain of the vessel wall (de Korte *et al.*, 1998). The local strain of the tissue is displayed as a color-coded palpogram on the luminal boundaries of IVUS echogram (Doyley *et al.*, 2001).

Magnetic resonance imaging is the most advanced diagnostic modality for cardiac structure and function. It is non-invasive and does not utilize nephrotoxic agents as in coronary angiography. In addition, the ability of magnetic resonance imaging to characterize soft tissue allows it to give a clear picture of various components of the atherothrombotic plaque. Magnetic resonance imaging can distinguish between adventitial and medial layers, as well as fibrous caps and lipid cores (Wilensky *et al.*, 2006). Its ability to visualize plaques in the coronary arteries, however, is limited by a reduced spatial resolution for deep structures and small size coronary vessels, and artefacts due to motion and movement of the patients (Wilensky *et al.*, 2006).

The major drawbacks of these cardiac imaging techniques to be used routinely as a point of care test for diagnosis of plaque instability are high cost, time consuming and IVUS also carries risk of complications, such as spasm, embolism, thrombus formation and dissection (Bourantas *et al.*, 2011). Another limitation of these techniques is they are very operator-dependent as they have to be performed by trained specialists only (Gani *et al.*, 2007). Besides, the accuracy and precision of imaging technique such as magnetic resonance imaging are highly rely on the patient's cooperation, making it not suitable for unstable patients (Shah *et al.*, 2009).

### 1.2.2 Identification of unstable plaque using biomarkers

The genesis and progression of unstable plaque are accompanied by the release of a series of proteomic mediators and these mediators have the potential to be utilized as biomarkers. The National Institutes of Health Biomarkers Definitions Working Group (2001) defined a biomarker as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. A biomarker can be measured on a biological sample (as blood, urine, or tissue test), a recording obtained from a person (blood pressure, heart rate or ECG) or it may be an imaging test (echocardiogram or computed tomography scan).

According to Biomarkers Definitions Working Group (2001), biomarkers can be classified as antecedent biomarkers (identifying the risk of developing an illness), screening biomarkers (screening for subclinical disease), diagnostic biomarkers (recognizing overt disease), staging biomarkers (categorizing disease severity) or prognostic biomarkers (predicting future disease course, including recurrence and response to therapy, and monitoring efficacy of therapy). An ideal biomarker has the potential to enhance the ability of a clinician to manage the patient optimally. The biomarker will be of clinical value only if it is accurate, can be reproduced in a standardized fashion, acceptable to the patient, easy to be interpreted by clinicians, has high sensitivity and specificity for the outcome. It should also consistently explain a reasonable proportion of the outcome independent of established predictors (Vasan, 2006).

The major advantage of biomarker over imaging technique in identifying unstable plaque is the ease of performing the test on blood sample to detect the presence of a biomarker. Another advantage of the use of biomarker is that it is more economical and less time consuming compared with the use of imaging techniques. Therefore, the detection of biomarkers in blood has been gaining cardiologists' interest in recent years.

Biomarkers play a pivotal role in the diagnosis and management of patients with ACS. Early recognition of a cardiac ischemic event and a proper placement of patients in the risk spectrum of ACS are crucial in improving the efficiency of clinical care of patients. The World Health Organization has traditionally defined myocardial infarction as requiring the presence of at least two of three diagnostic criteria, namely, (i) the appropriate clinical presentation; (ii) typical changes in the ECG; (iii) raised cardiac enzymes, essentially total CK or MB isoform of creatine kinase (CK-MB) activities (Panteghini, 2004). Consensus documents published by the European Society of Cardiology, the American College of Cardiology, and the American Heart Association are to make recommendations on the use of biomarkers for the detection of myocardial infarction. The redefined criteria used to classify ACS patients presenting with ischemic symptoms as acute, evolving, or recent myocardial infarction are heavily predicated on the increased serum or plasma cardiac troponin concentration (The Joint European Society of Cardiology/American College of Cardiology Committee, 2000).

Troponin is a complex of three contractile regulatory proteins, troponin C, T and I. It regulates the interactions between actin and myosin in skeletal and cardiac muscles.

Troponin I and T are specific to cardiac muscles while troponin C is expressed in both cardiac and skeletal muscles. Thus, troponin C is not appropriate to be used in the diagnosis of myocardial damage (Babuin and Jaffe, 2005). Myocardial necrosis causes the release of cardiac troponins into circulation. Hence, cardiac troponins, mainly cardiac troponin I, become the ideal biomarkers for the detection of cardiac injury (Babuin and Jaffe, 2005).

Both cardiac troponin I and cardiac troponin T have similar kinetics. They are detectable in the serum within 4 to 12 hours after the onset of myocardial infarction, and depending on the duration of ischemia and reperfusion status, the peak values of cardiac troponins occur between 12 to 48 hours after symptom onset (Boden *et al.*, 2008). Therefore, serial sampling, including a baseline sample and follow-up examination at 8 to 12 hours after symptom onset is recommended (Newby *et al.*, 2003). Due to the tissue specificity of cardiac troponins, any reliably detected concentration of cardiac troponins in the peripheral circulation is regarded as abnormal and indicative of myocardial injury (Jaffe *et al.*, 2000).

Sudden coronary death is the major outcome of ACS and even those who survive from it remain at high risk of recurrence. Therefore, despite the success of cardiac troponins that can rule out ACS from the emergency room at present, there is still a need for the development of early biomarkers that can reliably detect myocardial ischemia in the absence of irreversible myocyte injury and thus predict risk for developing ACS in asymptomatic patients. There are two categories of biomarkers under investigation nowadays, namely markers of early injury or ischemia and

markers of inflammation or coronary plaque instability and disruption (Panteghini, 2004).

### 1.3 Recommendation on the use of new biomarkers in ACS

The main etiology of ACS involves rupture of unstable plaque. Several factors such as inflammation, impaired endothelial function, impaired plaque stabilization and thrombus formation, can lead to plaque instability and a subsequently acute coronary event (Libby, 1995, Falk *et al.*, 1995, Davies, 1996, Brown *et al.*, 1993). The biomarkers like C-reactive protein (CRP), soluble CD40 ligand (sCD40L), placental growth factor (PlGF) and myeloperoxidase (MPO), were chosen as targets in this study, due to their significant roles in the pathogenesis of unstable plaque.

#### 1.3.1 CRP and vascular inflammation

CRP, a biomarker of inflammation, is a valuable predictor of future cardiovascular events in apparently healthy men and women (Ridker *et al.*, 2001, Ridker, 2003). The CRP could directly participate in the pathogenesis of atherosclerosis through the activation of endothelial cells (Calabro *et al.*, 2003).

CRP, named for its capacity to bind to the C-polysaccharide of *Streptococcus pneumoniae*, was the first acute-phase protein described in Yeh and Palusinski (2003). It is synthesized by the hepatocytes in response to microbial infection, tissue injury, and autoimmune disorders. It had been shown that Interleukin-1 beta (IL-1β) and IL-6 actively induced the expression of CRP in human hepatocytes and hepatoma cells (Calabro *et al.*, 2003). Human neuronal cells were found to produce CRP in Alzheimer's disease (Yasojima *et al.*, 2000). In addition, human renal

cortical tubular epithelial cells were shown to produce CRP after inflammatory stimulation (Jabs *et al.*, 2003). Interestingly, CRP has been detected in human atherosclerotic plaques and it could be resulted from indirect deposition of circulating cells or direct production by cells in the arterial wall (Yasojima *et al.*, 2001). Furthermore, it was demonstrated to be secreted by other cell types such as smooth muscle cells (Calabro *et al.*, 2003), macrophages (Ciubotaru *et al.*, 2005), and endothelial cells (Venugopal *et al.*, 2005).

The exact function of CRP is unclear. However, it is thought to stimulate tissue factor production and activate complement when aggregated (Volanakis, 1982). Tissue factor may be the main stimulus for initiating blood coagulation. An *in vitro* study have shown that aggregated CRP binds to LDL and very-LDL, which in turn activates complement, stimulates tissue factor production by macrophages and eventually initiates blood coagulation cascade (de Ferranti and Rifai, 2002).

Another theory sees CRP as a culprit in atherogenesis. It acts as pro-coagulant and increases opsonization (Tracy, 1998). CRP in the presence of serum mediates the uptake of LDL into macrophages, which then become foam cells (Zwaka *et al.*, 2001). It also destabilizes plaques (Lagrand *et al.*, 1999). Furthermore, CRP is hypothesized as a marker of vascular inflammation. This hypothesis is supported by the finding of decreased forearm vascular responsiveness in patients with increased CRP (Fichtlscherer *et al.*, 2000). Then, a work has contributed further to the theory of CRP as a marker of vascular inflammation. Pasceri *et al.* (Pasceri *et al.*, 2000) found that CRP caused an increase in the expression of intercellular adhesion molecule-1, vascular cell adhesion molecule-1 and E-selectin, and promotes local

infiltration by monocytes and lymphocytes. Further, Yoo and colleagues (2011) measured vascular inflammation in their study using F-fluorodeoxyglucose positron emission tomography. They found that the vascular inflammation increased in healthy individuals without hyperlipidemia but with elevated CRP.

### 1.3.2 sCD40L and platelets activation

CD40 is a cell membrane-spanning protein of 50 kDa that is found originally on B lymphocytes. Its ligand, CD40 ligand (CD40L) or termed as CD 154, is a trimeric transmembrane protein with a molecular mass of 36 kDa. CD40L is expressed in hematopoietic cell types such as T lymphocytes, monocytes, or platelets and nonhematopoietic cells such as endothelial and smooth muscle cells (Mach *et al.*, 1997b, Foy *et al.*, 1996).

Ligation of CD40 on various vascular cells plays a role in the pathogenesis of atherosclerosis, thrombosis, and inflammatory processes (Henn *et al.*, 1998, Mach *et al.*, 1998). The engagement of CD40 on endothelial cells or monocytes leads to the synthesis of reactive oxygen species, production of chemokine and cytokine as well as the expression of adhesion molecules, such as E-selectin, intercellular adhesion molecule 1 and vascular cell adhesion molecule 1, which further promotes the recruitment of leukocytes and enhances atherogenesis.

Beside present in the form of membrane bound protein, the CD40L is also present in soluble form, termed as sCD40L (Graf *et al.*, 1995). This biologically active sCD40L is shed from stimulated lymphocytes and released into circulation following platelets stimulation (Lee *et al.*, 1999, Henn *et al.*, 2001). It triggers inflammatory response in

vascular endothelial cells by secretion of cytokines and chemokines (Andre *et al.*, 2002). sCD40L is also able to inhibit the endothelial cell migration (Urbich *et al.*, 2002), activate platelets aggregation (Chakrabarti *et al.*, 2005) and enhance the monocyte tissue factor expression and thrombin generation (Sanguigni *et al.*, 2005).

Membrane bound CD40L and sCD40L interact with the CD40 receptor molecules, which are located not only on B cells but also on monocytes, macrophages, endothelial and smooth muscle cells in atheroma. The interaction leads to release of matrix MMPs and subsequently plaque destabilization (Schonbeck and Libby, 2001). The CD40/CD40L interactions also play an important role in thrombotic events after plaque rupture. The CD40/CD40L interactions induce tissue factor expression on macrophages and endothelial cells as well as diminish the expression of thrombomodulin, which favoring coagulation and thrombosis (Aukrust *et al.*, 2004). Gavins and colleagues (2010) showed that the CD40-CD40L system significantly enhanced microvascular thrombosis in both mouse models of chronic and acute inflammation.

### 1.3.3 PlGF and inflammatory instigation

PlGF is a member of the vascular endothelial growth factor family, encoding for a 50-kDa angiogenic protein with approximately 50% identical to vascular endothelial growth factor in the platelet-derived growth factor–like domain (Maglione *et al.*, 1991). The PlGF was initially discovered in the placenta and it has been proposed to control the growth and differentiation of trophoblasts (Khaliq *et al.*, 1996). Besides the placenta, PlGF is also expressed in the heart, lungs, goiter and thyroid tissues