A STUDY OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR (PPAR) ALPHA IN PATIENTS WITH ISCHEMIC HEART DISEASE AND HEALTHY VOLUNTEERS

by

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CERTIFICATE

This is to certify that the dissertation entitled

A Study of Peroxisome Proliferator-Activated Receptor (PPAR) Alpha in Patients with Ischemic Heart Disease and Healthy Volunteers

is the bona fide record of research work done by

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during the period from July 2008 to November 2008

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LIST OF ABBREVIATIONS

ABTS	2,2'-azino-di-[3-ethylbenzthiazoline sulfonate
ACS	Acute coronary syndrome
ACC	American College of Cardiology
AHA	American Heart Association
apo	Apolipoprotein
CK	Creatine kinase
CRP	C-reactive protein
CCRs	CC-Chemokine receptors
cDNA	Complementary deoxyribonucleic acid
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
dNTP	Deoxyribonucleotide triphosphate
DEPC	Diethylpyrocarbonate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ESC	European Society of Cardiology
ECM	Extracellular matrix
HRP	Horse radish peroxidase
H_2O_2	Hydrogen peroxide
HDL	High density lipoprotein
H_2SO_4	Sulphuric acid
IHD	Ischemic heart disease
Ig	Immunoglobulin
IGF-1	Insulin-like Growth Factor-1
ICAM	Intercellular adhesion molecules
IFNγ	Interferon gamma
IL	Interleukin
LPL	Lipoprotein lipase
LDL	Low density lipoprotein
MMP	Matrix metaloproteinases
M-CSF	Macrophage Colony Stimulating Factor
MMP	Matrix metalloproteinases
mRNA	Messenger ribonucleic acid
MI	Myocardial infarction
NaCl	Sodium chloride
NaN_3	Sodium azide
NO	Nitric oxide
PTCA	Percutaneous Transluminal Coronary Angioplasty
PPAR-α	Peroxisome proliferator-activated receptor alpha
PPREs	Peroxisome proliferator response elements
PBS	Phosphate buffered saline
PDGF	Platelet Derived Growth Factor

PCR	Polymerase Chain Reaction
ROS	Reactive oxygen species
RXR	Retinoic acid receptor
RCT	Reverse cholesterol transport
RT-PCR	Reverse transcription polymerase chain reaction
Rpm	Revolution per minute
RNA	Ribonucleic acid
SR-BI	Scavenger receptor class B type I
SAA	Serum amyloid A
SMC	Smooth muscle cells
S.D	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SBP	Systolic blood pressure
TEMED	Tetramethylethylenediamine
TGFβ	Transforming Growth Factor beta
TAE	Triacetic acid ethylenediaminetetraacetic acid
TF	Tissue factor
TNF α	Tumor Necrosis Factor alpha
VCAMs	Vascular cell adhesion molecule
VSMCs	Vascular smooth muscle cells
VLDL	Very low density lipoprotein
v/v	Volume to volume
w/v	Weight to volume
WHO	World Health Organization

KAJIAN TENTANG *PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR (PPAR) ALPHA* DALAM GOLONGAN PESAKIT PENYAKIT JANTUNG ISKEMIA DAN GOLONGAN INDIVIDU YANG SIHAT

ABSTRAK

Penyakit jantung iskemia merupakan penyebab utama kematian dalam Malaysia dan juga seluruh dunia. Oleh itu, satu penanda diagnosis baru untuk mengesan penyakit jantung pada peringkat awal, *peroxisome proliferator-activated receptors-alpha* (PPAR-α) diperkenalkan dalam kajian ini. Tujuan kajian ini ialah untuk mengkaji perbezaan kandungan mRNA PPAR-α dan protein PPAR-α dalam pesakit jantung iskemia berbanding dengan individu yang sihat. Kandungan mRNA untuk gen PPAR-α dan gen Protein C-Reaktif (CRP) dikaji dengan cara tindak balas rantaian polimeras transkripsi balik (RT-PCR). Kandungan protein untuk PPAR-α dan CRP dalam kumpulan kajian ditentukan dengan analisis *Western blot* dan *Enzyme linked immunosorbent assay* (ELISA). Dalam kajian ini, kandungan mRNA dan protein untuk serum CRP didapati meningkat dengan signifikannya ($p<0.05$) dalam pesakit penyakit jantung iskemia. Selain itu, protein PPAR-α variasi asli (*native*) dan terkupas (*truncated*) didapati terkandung dalam sel darah putih manusia. Kandungan mRNA untuk PPAR-α variasi asli (*native*) dan terkupas (*truncated*) didapati menurun dengan signifikannya ($p<0.05$) dalam pesakit jantung iskemia. Walaubagaimanapun, kandungan protein PPAR-α pula didapati hampir sama ($p>0.05$) dalam kedua-dua kumpulan kajian. Kajian ini mendapati bahawa kandungan mRNA

untuk kedua-dua PPAR-α variasi berkurangan dalam pesakit penyakit jantung iskemia. Kajian seterusnya akan dijalani untuk menerangkan fenomena ini. Dalam kajian ini, ekspresi-terlampau protein PPAR-α variasi terkupas (*truncated*) berkemungkinan terlibat dalam patogenesis penyakit jantung iskemia.

A STUDY OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR (PPAR) ALPHA IN PATIENTS WITH ISCHEMIC HEART DISEASE AND HEALTHY VOLUNTEERS

ABSTRACT

Ischemic heart disease (IHD) is the most common cause of cardiovascular mortality in Malaysia and around the globe. New novel biomarker, Peroxisome Proliferator-Activated Receptors-Alpha (PPAR-α) was investigated in this study to give significant contribution to early detection of IHD. In this study, we investigated the mRNA expression and protein levels of both PPAR-α and C-Reactive Protein (CRP) genes in the peripheral blood of IHD patients (n=10) and apparently healthy volunteers (n=10). The mRNA levels of PPAR-α (native and truncated) and CRP were determined by reverse transcription–polymerase chain reaction. The protein expression was evaluated by Western blot and measured by Enzyme linked immunosorbent assay (ELISA). The CRP mRNA expression and serum levels increased significantly ($p<0.05$) in IHD patients compared to normal volunteers. There was presence of both native active and truncated inhibitory PPAR-α protein in human blood leukocytes. The mRNA expressions of both native and truncated variants of PPAR-α were lower ($p<0.05$) in IHD patients group than those in the normal volunteers group. However, there was no significant difference ($p>0.05$) in PPAR-α protein level between IHD patients and normal volunteers. These findings suggest that mRNA expression level of both PPAR-α variants is decreased in IHD patients.

An over-expression of the truncated inhibitory isoform of PPAR-α protein may be involved in the pathogenesis of IHD. Further study will be conducted to elucidate this matter.

1.0 INTRODUCTION

Despite dramatic advances in medicine, cardiovascular disease remains a major healthcare problem in Malaysia and around the globe. According to World Health Organization (WHO, 2005), cardiovascular disease encountered 30% of the total death worldwide in 2005 (Figure 1.1). In Malaysia, cardiovascular disease is the leading cause of death of non-communicable diseases. It encountered 30% of the all deaths in Malaysia in 2002 (Figure 1.2). According to Ministry of Health Hospitals' Survey reported in year 2002, cardiovascular disease persistently accounts for 15-16% of all hospital deaths annually since 1995 to 2002. Furthermore, ischemic heart disease was the most common cause of cardiovascular mortality and accounted for 2,556 deaths in 2002 with a further 896 deaths due to heart failure of ischemic origin in Malaysia.

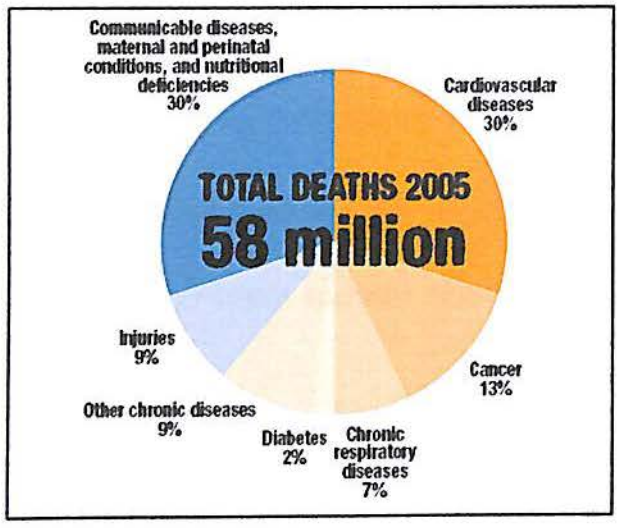

Figure 1.1. The main causes of death worldwide in all ages in year 2005 (WHO, 2005)

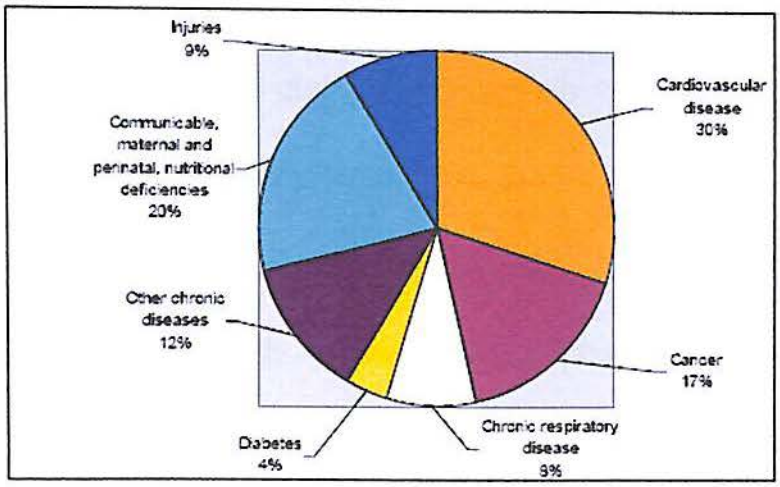

Figure 1.2. Deaths by cause in all ages in Malaysia in year 2002. (WHO, 2002)

Ischemic heart disease is synonymous with coronary heart disease. The term coronary heart disease is used to describe a disorder of the heart muscle resulting from narrowing of the coronary arteries. Because of the narrowing, the heart muscle may not receive sufficient blood. Often the blood supply is only insufficient when demands on the heart are increased as on exercise. This transient ischemia may cause a chest discomfort called angina, which characteristically disappears on rest. When the reduction in blood supply is so severe as to cause death of the muscle cells beyond obstruction, this is known as 'myocardial' referring to the heart muscle and 'infarction' to the death cells (Julian and Marley, 1991).

In the vast majority of cases, coronary heart disease is the result of a process known as atherosclerosis (Julian and Marley, 1991). Atherosclerosis is a specific form of chronic inflammatory process resulting from interactions between

plasma lipoproteins, cellular components (monocyte or macrophages, T lymphocytes, endothelial cells and smooth muscle cells) and the extracellular matrix of the arterial wall (Fan and Watanabe, 2003). The atherosclerotic plaque is composed of fibrous cap that covers the surface. Beneath the cap is the soft portion of the plaque filled with lipid-laden macrophages, oxidized low-density lipoprotein cholesterol and other cellular waste products possibly including calcium deposits. The plaque narrows the lumen of the coronary arteries, reduces the volume of blood that can flow through them, and leads to myocardial ischemia. Coronary atherosclerosis begins in early life but it required decades to develop mature plaques responsible for ischemic heart disease (Pasterkamp and Falk, 2000). Plaque formation also predisposes to thrombosis, which can provoke myocardial infarction.

Biochemical markers play a vital role in the diagnosis and management of patients with ischemic heart disease. The World Health Organization (WHO) has traditionally defined myocardial infarction as requiring the presence of at least two of three diagnostic criteria, namely, an appropriate clinical presentation, typical changes in the electrocardiogram and raised "cardiac" enzymes, essentially total creatine kinase (CK) or its MB iso-enzyme (CK-MB) activities (Panteghini, 2004). Enzymatic assays for CK and lactate dehydrogenase catalytic activities were developed 20 years ago for the retrospective detection of cardiac tissue necrosis (Panteghini, 2004). In the latter part of the 20th century,

highly sensitive and specific assays for the detection of myocardial damage, such as cardiac troponins have become available, assigning to the laboratory a pivotal role in the diagnosis and follow-up of patients with cardiac disease (Panteghini, 2004). The weakness for all of these biochemical markers is that they only can detect myocardial ischemia or IHD at later stage (necrosis stage) with irreversible myocyte injury which will then complicate treatment. Therefore, there is an urgent necessecity for the development of early markers that can reliably detect coronary atherosclerosis at early presentation and also detect myocardial ischemia in the absence of irreversible myocyte injury.

Substantial experimental and clinical evidence links local and systemic inflammation to atherosclerosis (Kaperonis *et al.*, 2006) and supports a central role for inflammation in early atherogenesis, in the progression of lesions, and finally in the thrombotic complications of the atherosclerotic process (Libby *et al.*, 2002). In the last few years, there has been an increasing interest in specific serum markers that could reflect the severity of systemic inflammation. C-reactive protein (CRP), serum amyloid A (SAA), proinflammatory cytokines and adhesion molecules have all been linked to increased risk of future cardiovascular events (Kaperonis *et al.*, 2006) but CRP has been the most widely studied and much has been written and discussed regarding its relationship to inflammation, coronary artery pathology and coronary disease outcome (Panteghini, 2004).

CRP is an acute-phase protein synthesized by the liver in response to microbial infection, tissue injury, and autoimmune disorders. It has been shown to be an excellent predictor of future cardiovascular events in apparently healthy men and women due to its biological properties such as its stability, lack of diurnal variation and lack of influence of gender and age (Calabró *et al.*, 2003). CRP has also been found in human atherosclerotic plaques which could be the result of indirect deposit from circulating cells or direct production by human coronary artery smooth muscle cells after stimulation by inflammatory cytokines (Calabró *et al.*, 2003). In clinical laboratories, normal cut-off values of either 5 or 8 µg/ml of serum CRP are used to assess whether the presence or absence of inflammation (Wu *et al.*, 2002). During the acute phase reaction, serum CRP levels often increase up to 100 or 200 µg/ml (Calabró *et al.*, 2003). However, the level of serum CRP that is useful for predicting cardiovascular risk is 1 to 3 µg/ml because CRP production by human coronary artery smooth muscle cells is less robust than those produced by liver (Calabró *et al.*, 2003). In fact, patients with serum CRP levels more than 10 µg/ml should have the test repeated at a later date to exclude infection, autoimmune diseases, or malignancy (Calabró *et al.*, 2003).

A multimarker paradigm using a combination of both established and new markers for risk assessment and clinical decision-making has the potential to improve substantially the outcomes in patients with acute coronary syndrome caused by ischemic heart disease (Morrow and Braunwald, 2003). Therefore there is an urgent need to search for a new and novel biomarker to give further significant contribution to multi-marker strategy in early diagnostic of IHD. The emergence of Peroxisome Proliferator-Activated Receptor Alpha (PPAR-α) as transcriptional regulators involved in lipid metabolism, inflammation and atherosclerosis has directed attention towards the advent of a new biomarker for early detection of IHD.

PPAR-α activation interferes with early stage of atherosclerosis by reducing leukocyte adhesion to activated endothelial cells of the arterial vessel wall and inhibiting subsequent transendothelial leukocyte migration. Besides, PPAR-α activation also inhibits the formation of macrophage foam cells in the later stage of atherosclerosis. Furthermore it also increases the stability of atherosclerotic plaques and limits plaque thrombogenicity (Zandbergen and Plutzky, 2007). The purpose of this study is to develop and optimize methods for measurement of human PPAR-α in human blood sample and also to investigate the difference of PPAR-α level in blood sample between apparently healthy volunteers and IHD patient with ischemic heart disease.

2.0 OBJECTIVE

The objectives of this present study are:

1. To optimize reverse transcription polymerase chain reaction (RT-PCR) method for measurement of human PPAR-α mRNA expression in human blood sample.

2. To optimize Enzyme-linked immunosorbent assay (ELISA) method for measurement of human PPAR-α protein level in human blood sample.

3. To investigate the difference of PPAR-α mRNA expression level between apparently healthy volunteer and IHD patient with RT-PCR.

4. To investigate the difference of PPAR-α protein level between apparently healthy volunteer and IHD patient with ELISA.

3.0 LITERATURE REVIEW

3.1 Atherosclerosis (Underlying Cause of IHD)

(A) History of Atherosclerosis

In 1815, London surgeon Joseph Hodgson observed the inflammatory characteristics of atherosclerotic lesions. He published an important monograph on vascular disease, claiming that inflammation was the underlying cause of atherosclerosis and it is not a natural degenerative occurrence of the aging process. He also identified that this disease process occurred in the intima, between the lumen and the media of the diseased vessels (Kaperonis *et al.*, 2006).

In 1856, Rudolf Virchow's description of the pathogenesis of atherosclerosis was an in-depth investigation of the histologic characteristics of the atherosclerotic lesion in all its stages (Heidland *et al.*, 2006). He was the first to coin the term of "endarteriitis deformans". By this he meant that the atheroma was a product of an inflammatory process within the intima and that the fibrous thickening evolved as a consequence of a reactive fibrosis induced by proliferating connective tissue cells within the intima (Heidland *et al.*, 2006). He maintained that mechanical forces initiated the irritative stimulus and that the endarteritis was part of a repair mechanism. He proposed in the same year that atherosclerosis was caused when plasma components elicited an inflammatory response in the arterial wall and resulted local intima injury (Zarifis, 2005). His theory has

elements that are acceptable to current thinking, but it also has features that have been invalidated. However, suffice it to say that Virchow's concept of local intima injury as the initiating "irritative" stimulus is still accepted and it has been extended to include other factors besides mechanical factors. His hypothesis formed the basis of response-to-injury hypothesis of Russel Ross more than a century later.

For the major part of the 20th century, the lipid theory dominated the field of atherogenesis until R.Ross reopened the discussion on the inflammatory nature of the atherosclerosis in 1976. Ross, Glomset and Harker produced a response-to-injury hypothesis which postulates that the lesions of atherosclerosis result as a response to some form of injury to arterial endothelial cells. It is followed by endothelial desquamation and platelet adherence, aggregation, and release at the sites of exposed subendothlial connective tissue. These events precede the intimal smooth muscle proliferative response that is a prerequisite of atherosclerotic lesion formation. These studies pointed to the key role of the platelet in the stimulation of intimal smooth muscle proliferation that leads to the development of lesions of atherosclerosis (Ross *et al.*, 1977)
The response-to-injury theory of atherogenesis is still valid until now with minor alterations.

(B) Progression of Atherosclerosis

Atherosclerosis involves several highly interrelated processes, including lipid disturbances, platelet activation, thrombosis, endothelial dysfunction, inflammation, oxidative stress, vascular smooth cell activation, altered matrix metabolism, remodeling, and genetic factors (Faxon *et al.*, 2004). Endothelial dysfunction seems to be the first step in atherogenesis and from this point on, an inflammatory response is triggered that leads to the development of atherosclerotic plaque. Endothelial dysfunction can occur in response to a variety of stimuli such as oxidized LDL, free radicals caused by smoking, hypertension, diabetes, genetic alterations, elevated plasma homocysteine concentrations and infectious microorganisms (Kaperonis *et al.*, 2006).

Healthy endothelium, does not normally bind white blood cells. Soon after activation by atherogenic stimuli, many endothelial cells begin to express on their surface adhesion molecules (selectins, intercellular adhesion molecules ICAMs, vascular cell adhesion molecule VCAMs) that act as receptors for glycoconjugates and integrins present on monocytes and T-cells (Kaperonis *et al.*, 2006). Vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1) and some of the CC-Chemokine Receptors (CCRs) enable the subsequent adherence of circulating leukocytes to the endothelium. In response to signals generated within the early plaque, leukocytes adhere to the endothelium and then migrate through the endothelium and basement

membrane into the intima by elaborating enzymes, including locally activated matrix metaloproteinases (MMP) that responsible for the migration of monocytes into intima through degrading the connective tissue matrix (Crowther, 2005). The migrated leukocytes will differentiate into tissue macrophages in the presence of different cytokines such as Macrophage Colony Stimulating Factor (M-CSF), Tumor Necrosis Factor α (TNF α), Interferon γ (IFNγ), proinflammatory interleukins (e.g. Interleukins-1 and -2; IL-1, -2) and growth factors (like Transforming Growth Factor β (TGFβ), Platelet Derived Growth Factor (PDGF) and Insulin-like Growth Factor-1 (IGF-1) (Ross, 1999). M-CSF leads to the ingestion of lipids, and to the multiplication and differentiation of monocytes into macrophage foam cells. Foam cells also produce cytokines and growth factors, further promoting atherosclerosis and are also postulated to serve as a source of matrix metalloproteinases, enzymes implicated in the weakening and rupture of the fibrous cap, the process thought to underlie most myocardial infarctions (Zandbergen and Plutzky, 2007). This characteristic lesion which consists of macrophage foam cells and T-cells, situated under a monolayer of endothelial cells, is the first lesion of atherosclerosis, the so called fatty-streak (Kaperonis *et al.*, 2006).

In the fatty-streak lesion, T-cells are activated and together with native vascular wall cells, secrete cytokines (tumor necrosis factor-b, γ-interferon), fibrogenic mediators and growth factors that can promote the migration and proliferation of

smooth muscle cells (SMC) (Kaperonis *et al.*, 2006). SMC in the intima produce extracellular matrix components, including collagen, elastin and proteoglycans, which forms the fibrous cap and contributes to the stability of the plaque. This fibrous cap surrounds a lipid rich core in advanced plaques. SMC also synthesise proinflammatory cytokines including interleukin-1 (IL-1) and tumour necrosis factor-alpha (TNF-α). Gradually, the so called advanced atherosclerotic lesion is formed, with the characteristic core of lipids and necrotic tissue which is covered by a fibrous cap.

3.2 Role of Biochemical Markers

Biochemical markers play a pivotal role in the diagnosis and management of patients with ischemic heart disease (IHD). The World Health Organization (WHO) has traditionally defined Myocardial infarction as requiring the presence of at least two of three diagnostic criteria, namely, an appropriate clinical presentation, typical changes in the electrocardiogram and raised "cardiac" enzymes, essentially total creatine kinase (CK) or its MB iso-enzyme (CK-MB) activities (Panteghini, 2004).

Consensus documents recently published by the European Society of Cardiology (ESC), the American College of Cardiology (ACC), and the American Heart Association (AHA) make specific recommendations on the use of biomarkers for the detection of myocardial infarction (MI). The redefined criteria