NOVEL CARDIOVASCULAR RISK MARKERS PARAOXONASE, APOLIPOPROTEIN A-1 (Apo A-I) AND GLUTATHIONE PEROXIDASE GENOTYPE-1 IN CORONARY ARTERY DISEASE

By

WICKRAMASINGHEGE DINUSHKA

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Thesis submitted to the University of Sri Jayewardenepura for the Degree of Master of Philosophy in Biochemistry on 18th August 2014.
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The work in this thesis was carried out by me under the supervision of Professor Hermantha Peiris (Professor of Biochemistry, Department of Biochemistry, Faculty of Medical Sciences, University of Sri Jayewardenepura), Professor Lal Chandrasena (Emeritus Professor, Department of Biochemistry, University of Kelaniya; Director, Clinical Laboratory, Nawaloka Hospitals PLC, Colombo), Dr. Vajira Senarathne (Consultant Cardiologist, Cardiology Unit, National Hospital, Colombo) and Dr. P. P. Rasika Perera (Senior Lecturer, Department of Biochemistry, Faculty of Medical Sciences, University of Sri Jayewardenepura) and a report on this has not been submitted in whole or in part to any University or any other institution for another Degree/Diploma.

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Dedication

I dedicate this thesis to

my wife Sugandika, parents and teachers.
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<td>kDa</td>
<td>kilo dalton</td>
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<tr>
<td>KO</td>
<td>Knockout mice</td>
</tr>
<tr>
<td>LDL-C</td>
<td>Low density lipoprotein cholesterol</td>
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<tr>
<td>Leu</td>
<td>Leucine</td>
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<tr>
<td>Lp (a)</td>
<td>Lipoprotein (a)</td>
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<tr>
<td>MI</td>
<td>Myocardial infarction</td>
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<tr>
<td>PAF</td>
<td>Platelet activate factor</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PON</td>
<td>Paraoxonase</td>
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<tr>
<td>PTCA</td>
<td>Percutaneous trans coronary angioplasty</td>
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<tr>
<td>RCT</td>
<td>Reverse cholesterol transport</td>
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<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>SBP</td>
<td>Systolic blood pressure</td>
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<td>Se</td>
<td>Selenium</td>
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<td>Acronym</td>
<td>Description</td>
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</tr>
<tr>
<td>SNP</td>
<td>Small Nucleotide Polymorphism</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
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Wickramasinghege Dinushka Wickramasinghe

ABSTRACT

Introduction: Coronary artery disease (CAD) is one of the major causes of mortality in both developed and developing countries. Oxidative stress has been demonstrated to have a role in pathogenesis of atherosclerosis. Reactive oxygen species (ROS) formed during oxidative stress result in oxidation of proteins and lipids of the cell membrane, leading to endothelial injury and microvascular dysfunction. Thus, the present study was designed to assess the relationship between severity of CAD as assessed by coronary angiography and Glutathione Peroxidase-1 (GPX-1), Paraoxonase-1 (PON-1), Apolipoprotein A-1 (Apo A-1) and GPX-1 genetic variants.

Objectives: This study was designed to investigate the relationship between GPX-1 variant, PON-1 and apoA-1 activity in healthy individuals and patients with CAD based on coronary angiographic severity scoring systems.

Methods: A case-control study of 75 patients (58 males, 17 females) with CAD (patients were selected from those awaiting coronary angiography) and age and sex matched 75 healthy volunteers as control subjects. Fasting venous blood samples were collected from all subjects for laboratory analysis of erythrocyte total GPX, erythrocyte GPX-1, serum PON-1 activity, Apo A-1 level and GPX-1 Pro198Leu polymorphisms.

Results: Data revealed that the serum PON-1 concentration, total erythrocyte GPX and erythrocyte GPX -1 activity were significantly ($p \leq 0.05$) low in patients when compared to controls. Paraoxonase-1 activity and Apolipoprotein A-1 levels did not show significant correlations with vessel, stenosis, and extent scores. Total erythrocyte GPX
and erythrocyte GPX-1 activities showed significantly strong inverse relationship with vessel, stenosis, and extent scores. Frequency distribution of GPX-1 Pro198leu (CT) genotype was significantly higher in patients (25.3%) when compared to controls (10.7%) ($\chi^2$ test =1.019). Results of genotype polymorphism in GPX-1 showed that the Leu198Leu (TT) genotype was not present in our study population. Interestingly, Pro198leu (CT) genotype showed a 2.84 fold risk for CAD [odds ratio 2.84 (95% CI 1.15 – 6.98), p = 0.019] in our study population. The Pro198Leu (CT) genotype carriers in subjects with age ≤ 50 years showed significantly higher (6.19 fold) risk for CAD compared to Pro198Pro (CC) genotype carriers in the same age group [odds ratio 6.19 (95% CI 1.1 – 34.3), p = 0.037]

Conclusion: Low serum PON-1 concentration, total erythrocyte GPX, and erythrocyte GPX-1 activity are independent risk factors for CAD. Decreased total GPX and GPX-1 activities are associated with increased severity of CAD. The Pro198Pro (CC) genotype is the most prevalent genetic variant of GPX-1 Pro198Leu polymorphism in the study population. However, Leu198Leu (TT) genotype not detected in our study population. The Pro198Leu (CT) genetic variant appeared to be the most significant predictor of CAD. Thus, this may have a future potential in early identification and management subjects with CAD.
1. INTRODUCTION

1.1. General introduction

Coronary artery disease (CAD) has been a major cause of death worldwide. World Health Organization (WHO) statistic has shown that 7.2 million deaths per year or 12% of all deaths occurring in the world are due to CAD (World Health Organization, 2008).

CAD has also become more of a burden in many developed and developing countries. Recently, CAD has contributed to the increasing percentage of deaths from total deaths in United Kingdom, United States of America and Australia (British Heart Foundation, 2010; Lloyd et al, 2009; Australian Institute of Health and Welfare, 2010).

Many developing countries in South Asia are also more susceptible to increasing CAD incidence (Iqbal et al, 2006; Joshi et al, 2007). WHO health statistics has predicted that CAD will be the major non-communicable disease in developing countries for another two decades or beyond (World Health Organization, 2011).

CAD mortality has shown two epidemiological patterns in the last three decades, which shows an initial rise in mortality rate followed by a descent in many developed nations. Yet, it has shown only a rise CAD mortality rate in developing countries. The possible reasons for this are fast development of sophisticated technology, changes of lifestyle or genetic factors leading to establishment of differences between developed and developing nations.
1.2. Atherosclerosis and coronary artery disease (CAD)

Atherosclerosis is a chronic disorder, which forms fatty streaks in intima of arteries and then gradually evolves into atherosclerotic plaques (Thompson et al, 2013; Abdelfattah et al, 2012). As a multifactorial disease, the development of atherosclerosis accelerates by several factors. Among them, most prominent factors are cigarette smoking, dyslipidemia, arterial hypertension, diabetes, obesity, physical inactivity, estrogen deficiency (menopause or male sex), chronic renal disease, genetic predisposition and aging (Barton, 2010).

Pathogenesis of atherosclerosis

CAD, defined as progressive narrowing or obstruction of coronary arteries typically caused by atherosclerosis. The reduction in coronary artery blood flow may be symptomatic or asymptomatic. It may occur with exertion or at rest and culminates in a myocardial infarction (MI). Severity of CAD depends on obstruction or progress of developing atheromatous plaque (Barton, 2013). Signs and symptoms of CAD are prominent in the advanced state of disease, most individuals with CAD does not show evidence of disease and the may progress even for decades before the first onset of symptoms and this often manifests as acute myocardial infarction (AMI) or even mortality.

CAD may begin when an individual is at a young age by gradually deposition of streaks of fat on interior coronary arterial walls. The fat build up gives rise to injury to endothelium with advancing age. An important concept in the pathogenesis of
Atherosclerosis is retained low-density lipoprotein cholesterol (LDL-C) particles in blood stream oxidized by reactive oxygen species (ROS) (Lankin et al, 2005) and invades the endothelium with endothelial damage. A complex series of inflammatory responses occurring in the injured site involve with variety of local vascular circulating factors such as monocytes, T-lymphocytes and other inflammatory substances take place. In this process, monocytes differentiate into macrophages. Macrophages ingest oxidized LDL and then slowly turn into large foam cells. The foam cells and platelets encourage the migration and proliferation of smooth muscle cells of the tunica media into the intima as a response to the secretion of cytokines from injured endothelial cells. Evidence also suggests that smooth muscle cells are also capable of uptake of oxidized lipids and become foam cells (Shih et al, 1996). Subsequently, the dead foam cells stimulate inflammatory responses and facilitate proliferation of more smooth muscle cells. Outer layer of plaque may consist with fibrous capsule or subsequently may become micro-calcified (Abedin et al, 2004).

Atheromatous plaques results to ischemia by decreased supply of oxygenate blood into the myocardium due to narrowing of coronary artery lumen. It is cause to ischemia. Ischemia may be short term if the blood supply to the myocardium is reestablished. However, long-term ischemia may lead to myocardial infarction by death of ischemic cardiac muscle.

Persons with atheromatous plaques have a greater risk of plaque rupturing at any occasion. Sudden rupture of plaque interferes with the oxygenated blood supply to the myocardium due to clot formation that leads to an acute myocardial infarction (AMI).
Most studies have reported that oxidative stress induces pathogenesis of atherosclerosis (Uysal, 2000; Sies, 1993). It has suggested that oxidative stress is the strongest hypothesis has been put-forward regarding the pathogenesis of atherosclerosis (Westhuyzen, 1997; Uysal, 2000).

1.3. **Risk factors associated with coronary artery disease**

Non-modifiable CAD risk factors associated with individuals cannot be change whereas, modifiable CAD risk factors can manipulate to decreases the risk of conventional risk factors.

1.3.1. Non modifiable coronary artery disease risk factors

1.3.1.1 Age

Humans are subject to multivariate changes in anatomically, physically and metabolically with increasing age. However, it has been revealed that males show early changes related to CAD (age 50–65 years) when compared to females (age 60–75 years) (National Institutes of Health, 2009). In most populations, elderly people are vulnerable to CAD death. Hence, CAD death rate also increases with the increase in the elderly population in a country and it have been suggested the elderly population contribute more towards CAD as a major cause of death in the world (World Health Organization, 2004). However, recent studies have shown that there had been an increasing CAD incidence or mortality in younger populations (Flaherty et al, 2009; Puymirat et al, 2012; Melanie et al, 2013).
1.3.1.2 Gender

Women in particularly are known to be at lower risk of having CAD mortality as compared to age-matched men. This difference not observed at all age groups, especially at after 70 years old the risk remains the same (Lloyd, 2010). However, studies have reported controversial findings in relation to differences of risk with gender in young populations (Pekka et al, 1999).

1.3.1.3 Family history

Persons with a family history of premature cardiovascular diseases in first-degree relatives have a 40-60% increased risk of CAD (Sivapalaratnam et al, 2010; Bachmann et al, 2012). According to definitions of premature CAD, studies have reported that CAD occurred in first-degree male relatives younger than 55 years of age or first-degree female relatives younger than 65 years of age.

1.3.1.4 Ethnicity

Prevalence of CAD varies among different ethnicities (Yancy et al, 2005). This variability may originate from ethnic differences or from exposure to risk factors, according to their cultural habits (Davis et al, 2007).
1.3.2. Modifiable CAD risk factors

1.3.2.1. Hyperlipidemia

Routinely measured lipid parameters are serum cholesterol, triglyceride, low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C). Several epidemiological studies have established a clear relationship between high levels of serum cholesterol and cardiovascular disease risk (Baigent et al, 2008). The primary role of serum cholesterol in CAD has been confirmed by clinical trials and it has been demonstrated that maintaining the serum cholesterol level in normal range help to reduce CAD mortality (Verschuren et al, 1995). A high level of low-density lipoprotein cholesterol (LDL-C) is a particularly important risk factor for atherosclerosis. Thus, LDL-C is the primary target of lipid lowering strategies (Caslake et al, 2004). In contrast, high-density lipoprotein cholesterol (HDL-C) broadly denoted as “good” cholesterol due to its inverse relationship with cardiovascular disease events (Boden, 2000; Wang et al, 2004).

1.3.2.2. Hypertension

Hypertension has identified as a well-known risk factor for CAD across the world. The high prevalence of hypertension among patients with CAD has confirmed by many studies (Bavry et al, 2010; Poole-Wilson et al, 2004). Relatively, different relationships have observed between systolic blood pressure (SBP) and diastolic blood pressure (DBP) for CAD with advancing age. Under 50 years of age, DBP is the major predictor of ischemic heart disease (IHD) risk, whereas SBP is the most important predictor for those above 60 years (Franklin et al, 2001). A meta-analysis has showed blood pressure...
over the range of 115/75 mmHg – 185/115 mmHg were associated with a high risk of developing fatal ischemic heart disease (Lewington et al, 2002). Furthermore, in those who are in the age group 40 – 60 years, an increase of SBP by 20 mmHg or an increase of DBP by 10 mmHg doubles the risk of fatal coronary disease. The relationship between CAD and blood pressure becomes strong with increasing age. Individuals with hypertension those who are at 80 years old have a 16 fold risk of CAD when compared with an individual who are at 40 years old (Clive et al, 2007).

1.3.2.3. Effect of diabetes mellitus

Diabetes mellitus is a well-recognized risk factor for coronary artery disease. Many studies have shown relationships between microvascular and macrovascular dysfunction and CAD due to diabetes (Yusuf et al, 2004). Individuals with uncontrolled diabetes have a greater risk for development of CAD.

1.3.2.4. Effect of smoking

Cigarette smoking is a major cause of CAD (Al-Nozha et al, 2009). Smokers have 2 - 4 times higher risk of developing CAD than nonsmokers. Many studies have shown that, smoking is a significant risk factor for enhancing CAD incidence (Anderson et al, 1991).
1.3.2.5. Effect of physical inactivity

Exercises provide number of benefits to an individual including decrease in obesity, burning of excess fat, improved blood circulation, better lung capacity, strengthening of muscles and maintenance of a healthy blood pressure (Thompson et al, 2003). In addition, regular exercises reduce myocardial oxygen demand and improve cardiac functions. Studies have shown that moderate-intensity exercises even 15 minutes per day or 90 minutes per week may be beneficial (Greenland et al, 2010). Chiuve et al (2011) has shown that a healthy lifestyle with proper exercises reduce coronary artery disease. Recently, studies have suggested that screen-based entertainment (television or other “screen time”) increases the risk of coronary artery disease (Stamatakis et al, 2011).

1.3.2.6. Obesity and overweight

Body mass index (BMI) is effortless method to measure health risk of peoples from weight in kilograms divided by the square of height in meters. WHO has defined overweight and obese as BMI value in 24 - 29 kg/m² and 30 kg/m² respectively (WHO expert consultation, 2004). However, this classification not compatible with the all Asian populations. Hence, WHO expert committee has defined new cutoff points for public health actions in Asian countries. According to that, many Asian populations, were classified as follows, less than 18.5 kg/m² underweight; 18.5 - 23 kg/m2 increasing but acceptable risk; 23 - 27.5 kg/m² increased risk, and 27.5 kg/m² or upper as high risk (WHO expert consultation, 2004).
People who have excess body fat, especially at the waist are more likely to develop heart disease, even in the absence of other risk factors. The risk of cardiovascular disease increases with the waist measurement of over 35 inches in women and over 40 inches in men (WHO expert consultation, 2004).

1.3.2.7. Alcohol

There is a lower risk of CAD among persons with a light to moderate alcohol intake compared with nondrinkers (Gaziano et al, 1993; Rimm et al, 1991). However, any publication have not been suggested that nondrinkers start using alcohol or that drinkers increases the amount they drink; too much alcohol consumption gives rise to adverse effects and lead to complicated diseases.
1.4. Reactive oxygen species (ROS)

1.4.1. Introduction

Reactive oxygen species (ROS) are produced as byproducts of aerobic respiration or during some oxidative reactions. Mitochondrial electron transport chain is the primary source of ROS production. ROS has both beneficial effects and harmful effects. ROS actively participate in cell signal transduction (Aruoma et al, 1991) and act as a cellular messenger in immune system and cell cycle (Trachootham et al, 2008).

Oxidative stress may result when the cellular antioxidant defense mechanisms are unable to keep pace with the detoxification of ROS (Dhalla et al, 2000). ROS cause damage to membrane lipids, proteins and DNA in cells (Sukru et al, 2012; Johnson et al, 2005). Various types of antioxidants are involved in counteracting harmful effects of ROS.

1.4.2. Varieties of ROS

ROS form as intermediates of various metabolic processes in cells. ROS can categorize as exogenous or endogenous according to their source of origin. Exogenous ROS are produce out of cell by different external sources, including drugs, radiation, tobacco smoke and pollutants. Endogenous ROS are form in various metabolic or catabolic pathways of cells (Chelikani et al, 2004). The main source of ROS synthesis mechanism is NADPH oxidative cycles in the cell, especially in the mitochondria or cell membrane (Van et al, 1997; Chelikani et al, 2004). Oxidative reduction pathways also create reactive oxygen atoms by adding electrons to atomic oxygen. They exist in
different reactive oxygen molecules according to the number of electrons added and the most known are superoxide, nitric oxide and hydrogen peroxide.

1.4.3. Adverse effects of ROS

Recently identified major harmful effects of ROS are

1). damage to DNA,

2). oxidase polyunsaturated fatty acids in lipids (lipid peroxidation),

3). oxidase amino acids in proteins,

4). oxidative inactivate specific enzymes by oxidation of co-factors.

1.4.4. Defense system against ROS

Cells have a variety of defense mechanisms to prevent harmful effects of ROS. Enzymatic systems are very important, due to cells necessity in maintaining these potentially damaging molecules within normal concentrations for normal cellular functions. The human body contains well-organized defense mechanisms to protect against oxidative stress by reducing ROS to alcohol or water.

GPX, PON, Superoxide dismutase (SOD) and Catalase are well known antioxidants against ROS. In the ROS eliminating process, harmful oxygen species are converts to hydrogen peroxide ($\text{H}_2\text{O}_2$) and oxygen ($\text{O}_2$) by antioxidant enzyme and then catalase enzyme removes the harmful $\text{H}_2\text{O}_2$ into water and oxygen in peroxisomes. However,
selenium containing GPX also catalyze the degradation of \( \text{H}_2\text{O}_2 \) and organic peroxides to alcohols (Forgione et al, 2002a; Arthur, 2000).

Lactoperoxidases, peroxiredoxins and small molecule antioxidants such as ascorbic acid (vitamin C), tocopherol (vitamin E), uric acid and glutathione also play important roles as cellular antioxidants.

1.4.5. Associations of ROS in oxidative stress

Oxidative stress initiates because of an imbalance between ROS and antioxidant defense. Chronically increased levels of ROS can contribute to varying pathological states (Thannickal et al, 2000). Acute increases of ROS in the body lead to reversible or irreversible massive damage to biomolecules in the body (De Haan et al, 1998; Cheng et al, 1997).

1.5. Oxidative stress and coronary artery disease

Oxidative stress is associated with over 100 human diseases (Aruoma et al, 1991). Increased ROS, demonstrates the role of oxidative stress in a wide array of disease states such as cancer (Halliwel, 2007), diabetes mellitus (Baynes, 1991), cardiovascular disease (Madamanch et al, 2005), pulmonary disease (Ryrfeldt et al, 1993) and neurodegenerative diseases including Alzheimer’s and Parkinson’s diseases (Simonian et al, 1996).

Increase of oxidative stress plays an important role in the onset of atherosclerosis in CAD with oxidative modification of LDL-C (Sukru et al, 2012). Oxidized LDL-C induces a wide variety of harmful cellular responses, such as induction of the
expression of adhesion molecules and pro-inflammatory cytokines and enhancing the progression of atherosclerotic CAD.

1.6. Antioxidants

There are many groups of antioxidant products in the human cell. There are enzymes (GPX, SOD, Catalase and PON etc.), proteins (GSH) and vitamins (vitamin A, C and E). Antioxidants play a vital role in the mechanisms involved in counteracting ROS. Physiological role of antioxidants is preventing ROS associated tissue damage by specific chemical reactions. This protection is carried out by non-enzymatic (metabolites) and enzymatic antioxidants. Amongst them, an important place is held by the glutathione system.

1.6.1. Non enzymatic antioxidants

1.6.1.1 Vitamin A

Vitamin A is a fat-soluble compound, which is structurally and metabolically equal to retinol. A well-known characteristic of vitamin A is its function in vision (Ross, 1999). Although, antioxidant role of vitamin A still controversial. Research studies have shown vitamin A has some possible antioxidant properties, especially in repairing damaged tissues (Ross, 1999).

1.6.1.2 Vitamin C (ascorbic acid)

Vitamin C is a water soluble, carbohydrate-like substance exists in both animals and plants. Vitamin C act as a catalyst for oxidative reduction processes (Dhalla et al, 2000). Hence, it is considered as an antioxidant in the body. Previous studies have
revealed that Vitamin C supplementation decreases vascular oxidative stress and improves the total antioxidant strength against ROS.

1.6.1.3 Vitamin E

Vitamin E is a fat-soluble compound having antioxidant properties. It has eight forms related to tocopherols and tocotrienols (Herrera and Barbas, 2001; Packer et al, 2001). Amongst them α-tocopherol is the most important compound acts against ROS (Traber et al, 2007). However, several studies have suggested contradictory roles regarding the antioxidant properties of vitamin E (Azzi, 2007; Zingg et al, 2004).

1.6.2. Enzymatic antioxidant

1.6.2.1. Catalase

Catalase is a protein found in peroxisomes of cells (Hiner et al, 2002). The main function of catalase enzyme is catalyzing the conversion of $\text{H}_2\text{O}_2$ to water and oxygen by using either iron or manganese as a cofactor (Chelikani et al, 2004).

1.6.2.2. Superoxide dismutase

Superoxide dismutase (SOD) is a potent antioxidant, which actively eliminates ROS from cells. SOD present in many cells of the body as well as extracellular fluids. It functions by breakdown of the superoxide anion into nontoxic oxygen and hydrogen peroxide (Zelko et al, 2002). SOD enzyme categorized according to metal ion cofactors in the compound. The metal ions may present as copper, zinc, manganese or
iron. In humans, it found in various parts of the body. The copper or zinc containing SOD isozyme is present in the cytosol, while manganese containing SOD isozyme is present in the mitochondria and third form of SOD exists in extracellular fluid consisting of both copper and zinc in its active site (Zelko et al, 2002). However, a study has shown lack of copper or zinc SOD isozyme in the body lead to numerous diseases and reduced lifespan in mice (Melov et al, 1998).

1.6.2.3. Glutathione Peroxidase

Glutathione Peroxidase (GPX) is a family of selenium containing antioxidant enzymes, which reduce oxidized lipids to their nontoxic metabolites. Biochemical function of GPX is reducing lipid hydroperoxides to their respective alcohols and free hydrogen peroxide to water (Blankenberg et al, 2003). GPX is an important intracellular antioxidant and found in very low concentrations in the extracellular space. GPX is one of the most important enzymes protecting intracellular lipids from peroxidation.

GPX has several isozymes expressed by different genes. These isozymes are varying in their cellular location and substrate specificity. At least eight isoforms of GPX has reported. Hence, it is called a GPX enzyme family than a single enzyme. GPX enzymes are present in almost every cell of animals with different isoforms showing different properties (Arthur, 2000). GPX activity is dependent on several factors, such as individual factors, different organs, different age groups and sex. Endocrine regulation also has an effect on the control of enzyme activity.

GPX-1 is the most prominent isozyme and it is the first identified selenoprotein in mammals. It is a key antioxidant enzyme in many cells, including vascular endothelium. GPX-1 activity is widespread in body with highest levels in liver, kidney,
and heart. It has identified as an important antiatherogenic enzyme (Blankenberg et al, 2003). In vitro data and studies in animal models have suggested that GPX-1 may protect arteries from atherosclerosis. However, available studies on the associations of GPX-1 activity, its genetic variants with severity of CAD in humans are limited.

1.6.2.4. Paraoxonase

Human serum paraoxonase (PON) circulate in blood associated with high-density lipoprotein cholesterols (HDL-C). PON is synthesized in the liver and it is a calcium dependent 45-kDa glycoprotein (Blatter et al, 1993). PON has the ability to hydrolyze organophosphate. It has used as an insecticide and as nerve gas. Hence, it has deeply studied in the toxicology field (Mackness et al, 1998a). The PON gene family consists of three isoforms, Paraoxonase-1 (PON-1), Paraoxonase-2 (PON-2) and Paraoxonase-3 (PON-3) (Draganov et al, 2005).

Recently, studies have shown that the serum PON-1 involved in preventing LDL oxidation and decreased PON-1 activity is associated with increased risk of CAD. In a study with PON-1 knockout mice, it has shown an essential protective factor in preventing oxidation of LDL cholesterol (Shih et al, 1998). Research studies suggest that paraoxonase (PON-1) has a protective effect against atherosclerosis based on experimental, transgenic, and prospective studies. However, research data are scanty regarding associations of PON-1 activity and severity of CAD.
1.7. Apolipoprotein A-I

Apolipoprotein A-I (Apo A-1) is HDL-C associated lipoprotein, which is responsible for functional activity of HDL-C against atherosclerosis. It promotes cholesterol efflux from cells and maintains cellular cholesterol homeostasis. Evidence exists that low HDL-C concentration is associated with CAD. Several studies have indicated that there is a positive correlation between serum APO A-1 and the pathogenesis of CAD (Wallidius et al, 2001). However, some studies have shown contradictory findings (Vander et al, 2007).

1.8. Novel risk markers of coronary artery disease

CAD is associated with various risk factors, including hypercholesterolemia, smoking, diabetes mellitus, hypertension and family history of premature CAD. Screening tests for CAD risk factors include evaluating total cholesterol, HDL-C and LDL-C levels and triglyceride levels. However, novel CAD risk factors have been developed to assess risk of CAD including homocysteine, C-reactive protein (CRP), fibrinogen, lipoprotein (a), coronary calcium score (Bhulani et al, 2013) and more sophisticated lipid analysis.

Homocysteine is an intermediate metabolite of methionine metabolism. In the general population, mild to moderate elevations are due to insufficient dietary intake of folic acid. Homocysteine levels may identify people at increased risk of heart disease (Veeranna et al, 2011).

Fibrinogen is a soluble protein that takes on a key role in platelet aggregation and blood viscosity, and it mediates the final step in clot formation. A study has been found the
significant association between fibrinogen level and risk of cardiovascular events (Ndrepepa et al, 2013).

An elevated lipoprotein (a) [Lp (a)] level also an important independent risk factor of premature CAD (Luc et al, 2002). Analysis of Lp (a) is more useful for young individuals with a family history of premature vascular disease and repeated coronary interventions. C-reactive protein (CRP) is a protein in the blood that demonstrates the presence of inflammation, which is a response to injury or infection. According to some studies, high levels of serum CRP may be associated with increased risk of developing CAD (Arroyo et al, 2004; Ridker et al, 2008).
1.9. Justification

Since the 20th century, humans have been expecting higher living standards along with advances in medical knowledge and health care. Health care improvement has led to development of better prevention strategies and treatment against communicable disease. Hence, it has contributed to a substantial gain in life expectancy. Recent findings have suggested that chronic non-communicable diseases have become the major cause of death among people (Sen and Bonita, 2000). This shift has named as the epidemiologic transition. Different societies belong to different levels of this transition; developed nations are at an advanced stage of this transition, whilst developing countries are undergoing the early phases of this transition (Gersh et al, 2010). Hence, CAD becomes the major cause of death in globally.

CAD mortality is reducing in developing countries due to the fast improving health technology, including new effective drugs, sophisticated surgical intervention and deep understanding of disease sources. CAD remains a global health problem and it is a main drawback for development of social and economic status. Several studies have shown CAD being accountable for one third of total young deaths in the world (Rosamond et al, 2008; Lloyd et al, 2010).

Currently, research has being conducted for acquire effective methods or technologies to diagnose CAD accurately in early stages to minimize morbidity and mortality rates. Thus, identification of antioxidant roles in reduce oxidative stress would help to minimize adverse effects of CAD. Over produced of ROS are neutralized by various antioxidants. Measurements of oxidative stress parameters and antioxidant activities are well-accepted methods to detect the extent of ROS generation. First line of defense
mechanism against ROS mediated cardiac injury comprises several antioxidant enzymes, including SOD, Catalase, GPX and PON. Among them, GPX and PON have reported to be sensitive cardio-protective enzymes. In vitro data and studies in animal models suggest that GPX-1 may protect arteries from atherosclerosis. However, information available on the association between severity of CAD and GPX-1 activity and genetic variants in humans is limited. PON-1 has found to play a significant role in preventing atherosclerosis based on experimental, transgenic, and prospective studies. Studies have been reported controversial findings regards to association of Apo A-1 and risk of CAD. Very few studies have done previously to assess relationship between PON-1 activity and severity of CAD.

Thus, it was decided to investigate the relationship between GPX-1, PON-1, Apo A-1 and GPX-1 genotype and severity of CAD. The present study was designed to evaluate the relationship between angiography findings of CAD, GPX-1, PON-1 and Apo A-1 activity between CAD patients and healthy individuals in Sri Lanka.
1.10. Objectives

1.10.1. General Objective

1. To investigate the relationship between GPX-1 variant, PON-1 and apoA-1 activity in healthy individuals and patients with CAD.

1.10.2. Specific Objectives

1. To study the genotype GPX-1 by Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP).

2. Assess the severity of CAD in relation to the presence of the GPX-1 variant and PON-1 activity.

3. To assess the relationship between PON-1 and apoA-1 in healthy individuals and patients with CAD.
2. LITERATURE REVIEW

2.1. Paraoxonase-1 and coronary artery disease

2.1.1. Human paraoxonase-1

2.1.1.1. Introduction of paraoxonase-1

Paraoxonase (PON) enzyme was identified initially in animal tissues and subsequent research was focused towards humans in 1950. The PON originated from its usage of organic phosphorous paraoxon as substrate. PON catalyze the hydrolysis of both organic phosphorous insecticides and nerve gases. Hydrolysis of paraoxon in human serum indicated that this esterase is capable of hydrolyzing organophosphates as well as aromatic esters. The PON is a calcium dependent glycoprotein enzyme (43-45 kDa) synthesized in the liver.

PON gene is located in chromosome 7 of human and consists of three isozymes namely PON-1, PON-2 and PON-3 respectively in discovering order (Draganov and La Du, 2004). All three isozymes show antioxidant characteristics (Deakin and James, 2004; Aviram and Rosenblat, 2004; Ekmekci et al, 2004). Although, those isozymes showing different expressions and dispersion patterns. PON-1 and PON-3 are the most abundant versions and both synthesized in the liver. Among three isozymes only PON-1 and PON-3 are associated with HDL-C in plasma (Aviram and Rosenblat, 2004).

All three PON enzymes play important roles in preventing oxidative stress and prevention of atherosclerosis (Precourt et al, 2011). PON-1 is the most focused enzyme in this family and well documented for its characteristic in protections against lipid
peroxidation, atherosclerosis and CAD. PON-2 and PON-3 unable hydrolyze paraoxon due to there is no lysine residue at 105 position of the amino acid structure.

2.1.1.2. Isozymes of Paraoxonase

2.1.1.2.i Paraoxonase-1

Human serum PON-1 is expressed on chromosome 7q21-22. It is a protein consisting of 354 amino acids. Recent studies have revealed that PON-1 has wide tissue distribution, including liver, blood and kidney (Draganov and La Du, 2004; Mackness et al, 2010). However, the main source of PON-1 synthesis is the liver and associated with HDL in circulation. The molecular weight of the PON-1 enzyme is between 43–45 kDa. It has a six-bladed β-propeller shape and two calcium ions are located in the central tunnel of the enzyme, it is believed to be important for both structural stability and catalytic function of enzyme (Harel et al, 2004).

HDL cholesterol has binding sites for PON-1. Calcium is an essential element for activation and stabilization of the PON-1 enzyme (Ali et al, 2003). Hence, PON-1 activity totally depends on calcium. Further, calcium chelate agents such as Ethylene DI-Amine Tetra Acetic acid (EDTA) irreversibly inhibit PON-1 activity in vitro. However, few studies have also suggested that calcium may not be required for the function of PON-1 in protection of LDL-C from the oxidation. Recent studies have revealed that serum PON-1 shows significant antioxidant characteristics and it is inversely associated with oxidation of LDL-C (Aviram and Rosenblot, 2004; Draganov and La Du, 2004).
2.1.2.ii Paraoxonase-2

PON-2 has a molecular weight of approximately 40 - 43 kDa, it is an intracellular enzyme expressed in all human tissues. PON-2 is especially located in the endothelial layer of liver, kidneys, heart, brain and testicular tissues. Fewer studies have done on PON-2 compared with PON-1. PON-2 is not present in blood and it is not associated with HDL-C particles in the circulation. However, it has also shown to reduce oxidative stress and provide protection against atherosclerosis.

2.1.2.iii Paraoxonase-3

PON-3 synthesizes in the liver and found in plasma associated with HDL-C. It is very similar to PON-1 in terms of gene expression, tissue distribution and physiological function. A study has shown that recombinant human PON-3 is able to delay LDL-C oxidation in vitro. However, the effectiveness of antioxidative function is lower than PON-1. Due to limited studies on PON-3 polymorphisms, the effect of PON-3 variant on human diseases remains questionable.

2.1.2. Metabolism of Paraoxonase-1

As previously described, human serum PON-1 mainly synthesized in the liver. It is initially synthesize as a 355 amino acid protein and during secretion and maturation becomes 354 amino acid compounds by removal of amino-terminal methionine residue. PON-1 is release from the liver by a binding process with HDL-C particles (Deakin et al, 2002). Hydrophobic N-terminal region of PON-1 is a barrier to optimal function of antioxidant. HDL-C provides an amphipathic environment to protect the PON-1 to
make effective action with its substrates (James & Deakin, 2004). In addition, the binding subunits of HDL-C with PON-1 contain Apolipoprotein A-1 (Apo A-1) and Apolipoprotein J (Apo J). HDL-C associated N-terminal signal peptide is a structural requirement for PON-1 (Sorenson et al, 1999) and PON-1 is entirely associated with HDL-C in human serum (Blatter et al, 1993). Several studies have shown an inverse association between HDL-C level and the development of coronary artery disease (Zuliani et al, 2007). It has also demonstrated that serum levels of HDL-C correlate with PON-1 enzyme levels. It has suggested that antioxidant function of HDL-C may preserve by the PON-1.

There are two calcium ions existing at the center of the PON-1 β-layers with 7.4 Å intervals. These two calcium ions are responsible for important functions of PON-1. If remove a structural calcium ion from enzyme, it may becomes irreversible denaturation. Additionally, calcium ion facilitates to preserve the catalytic ability of the enzyme.

PON-1 structure consists with three cysteine amino acids and two of them at 42 and 352 positions form a disulphide bond. This disulphide bond also has responsible to reserved PON-1 structure at stable. Another cysteine amino acid is free at the 284 position and facilitates substrate recognition and binding. The most important fact is this free cysteine amino acid prevents LDL-C oxidation (Ekmekci et al, 2004). Thus, it has suggested that free cysteine amino acid has a major role in antioxidant function (Aviram et al, 1998a).

Three-dimensional structure of PON-1 has provided multi substrate specificity for compounds including organophosphates, arylesters, carbamates, cyclic carbonate esters
(Draganov and La Du, 2004), lactones and possibly phospholipids. Almost all PON-1 substrates are hydrophobic and PON-1 provides deep active site for effective binding (Draganov and La Du, 2004; Harel et al, 2004). All substrates have the same affinity for binding with PON-1 active site, but binding strength differs according to the mode of binding (Harel et al, 2004).

Polymorphism of the PON-1 gene and its effects on susceptibility to organophosphate poisoning or prevention of atherosclerosis is interesting subjects of intensive research. The two most common PON-1 genetic variants are 192Q/R and 55L/M. However, commonly observed human PON-1 polymorphism is 192Q/R. The 55L/M polymorphism may considerably affect to PON-1 stability and thereby account for the lower enzymatic activity (Leviev et al, 2001; Harel et al, 2004).

2.1.3. Biochemical function of PON-1

2.1.3.1. Detoxification of toxic compounds

Initially, PON was identified as a hydrolase of organophosphate insecticides and nerve gases and paraoxon compound (Ekmekci et al, 2004). Hence, Organophosphate poisoning occurs in insects, birds and reptiles due to incapability of PON-1 synthesis. PON-1 enzyme activity varies from person to person due to genetic polymorphism. Hence, some studies have shown that some peoples are more sensitive to organophosphate poisoning due to low synthesis of PON. A study has reported that PON-1 protects against chlorpyrifos and diazinon poison exposure, but not parathion exposure. Hence, it has suggested that PON-1 may be use as an effective treatment
method for organophosphate poisoning. Furthermore, studies have also revealed that PON-1 has a protective function against inflammatory responses (Aviram et al, 1999).

2.1.3.2. Effect on oxidized LDL-C

Various oxidized lipid components of LDL-C are hydrolyzed by PON-1 (Mackness and Mackness, 2004). PON-1 neutralized reactive oxidative products by cooperation with platelet activating factor acetylhydrolase (PAF-AH) and GPX antioxidant (Norata et al, 2006). Increased amount of oxidized LDL-C levels probably reflect the decreased ability of modulating LDL-C lipid peroxidation by HDL-C and PON-1. Chronic increase of oxidized LDL-C may lead to develop premature CAD.

However, PON-1 may inactivated by high levels of oxidized LDL-C (Aviram et al, 1999). The free sulphhydryl group of PON-1 interacts with oxidized phospholipids of LDL-C, resulting in inactivation of PON-1 by oxidized cholesteryl esters or lysophosphatidylcholines formed during LDL-C oxidation. In addition, serum HDL-C levels have been shown to decrease during inflammation and change their composition by losing PON-1, Apo A-1 and platelet activating factors (van et al, 1995).

2.1.3.3. Prevention of accumulation of lipid peroxides

A direct role of PON-1 associated HDL-C is preventing atherosclerosis and it prevents the accumulation of lipid peroxides on LDL-C by an enzymatic process (Mackness et al, 1999; Mackness & Mackness, 2004). Hence, the degree of protection offered by HDL-C is related to its PON-1 activity (Mastorikou et al, 2006).
2.1.3.4. Inhibition of cholesterol influx

Cholesterol influx into cells is a key event in atherosclerosis. Monocyte chemotactic proteins (MCP-1) lead to enhanced cholesterol influx into monocytes. PON-1 actively inhibits MCP-1 secretion from oxidized LDL-C and prevents the monocyte transmigration and the cholesterol influx into cells (Barter et al, 2004).

2.1.3.5. Multiple antiatherogenic effects

HDL-C has multiple antiatherogenic effects including reduction of blood viscosity, regulation of prostaglandin and thromboxane synthesis and activation of fibrinolysis (Mackness and Mackness, 2000b). These antiatherogenic properties are attributed to various proteins, including PON-1 associated with HDL-C (Mackness & Durrington, 1995; Mackness & Mackness, 2004). PON-1 has shown inhibit the cholesterol influx by oxidized LDL-C into macrophages by different mechanisms: 1) hydrolysis of oxidized lipids in macrophage, 2) reducing macrophage-mediated formation of oxidized LDL-C, 3) increasing the breakdown of oxidized lipids including oxidized LDL-C, 4) decreasing lipid uptake into macrophages. Serum PON-1 activity is especially important in the protection of LDL-C phospholipids against oxidation (Deakin et al, 2002) and PON-1 has similar to peroxidase activity to enhance the protection ability. Hence, PON-1 reduces risks for CAD by destroying pro-inflammatory molecules that may promote atherosclerosis through oxidative damage.
2.1.4. Serum Paraoxonase-1 concentration

There is considerable variability in the serum concentration and activity of PON-1 among individuals. These variations appear due to several polymorphisms in the coding and promoter regions of the PON-1 gene (Adkins et al, 1993; Humbert et al, 1993; Leviev & James, 2000; Suehiro et al, 2000). PON-1 consists of a peptide chain with a highly hydrophobic N-terminal region (Sorenson et al, 1999). Therefore, it requires the proper vector to transport within the aqueous environment of the blood. Deakin et al (2002) has demonstrated that the most suitable vector for PON-1 is the lipid complex with HDL-C, which has the highest affinity for PON-1. HDL-C is transiently bound to the hepatocytes, possibly via scavenger receptor B1 (SR-B1) (Acton et al, 1996) whilst PON-1 is transferred to the apolipoproteins and this is very important for the enzymatic stability of PON-1 in the circulation (Deakin et al, 2002; James et al, 1998; Sorenson et al, 1999). Although, PON-1 in the circulation exclusively bound to HDL-C, evidence suggests that approximately 10% of circulating HDL-C bind to PON-1 (Blatter et al, 1993; Kelso et al, 1994).

2.1.5. Factors affecting Paraoxonase-1 activity

2.1.5.1. Genetic factors of Paraoxonase-1

Gene polymorphisms have an influence on enzyme activity through protein expression and substrate specificity (Precourt et al, 2011). More than 160 polymorphisms have been discovered to date in the human PON-1 gene, which are in the coding regions or promoter regions of the gene. Two major polymorphisms were found in the PON-1 coding region, which have been tested extensively in relation to CAD (Shih et al,
2000), resulting in a glutamine (Q)/arginine (R) substitution at position 192 (Q192R) and leucine (L)/methionine (M) substitution at position 55 (L55M) (Adkins et al, 1993). The PON-1 Q192R polymorphism does not affect PON-1 protein concentration; instead, it hydrolyzes paraoxon much less efficiently, however, it has high activity towards oxidized lipids. On the other hand, the L55M polymorphism has reported to have increased expression of PON-1 protein concentration (Leviev et al, 1997).

The frequencies of Q192R and L55M polymorphisms have analyzed in case-control studies in subjects with CAD. Many studies, have found significant associations between PON-1 genetic polymorphisms (Q192R, L55M) and PON-1 activities in CAD. Moreover, studies of the 192Q/R polymorphisms have shown that 192R allele is consistently associated with CAD compared with 192Q allele. Particularly, some studies have revealed that 192R was less effective in inhibiting in vitro LDL-C oxidation than the 192Q allele (Aviram et al, 1998b; Shih et al, 2000).

2.1.5.2. Oxidative stress and paraoxonase-1 activity

Research studies have suggested oxidative stress associated disease states may relate with low PON-1 activity. Precisely, how this relationship occurs is not clear. Increased levels of oxidized products could either down-regulate the gene expression of PON-1 or negatively affect PON-1 within the circulation. Aviram et al (1999) has demonstrated oxidized lipids including oxidized LDL-C cause to inactivate PON-1 activity after their co-incubation in mixture. Therefore, this information implies that PON-1 loses its functional ability in the bloodstream upon interaction and through subsequent hydrolysis of its active sites. This study has performed using long time incubation (18 hours), which additionally suggested that the degradation of PON-1 could be a chronic
effect of the oxidized lipids within the circulation (Aviram et al, 1999). Although, this mechanism not completely elucidated yet, it suggests that systemic degradation of PON-1 activity may relates to chronic exposure of oxidative stress in many pathophysiologies. Similarly, very little facts known about how oxidative stress affects to synthesis of PON-1, releasing, and binding to HDL-C in hepatocytes. Several studies have shown that incubation of oxidized LDL-C with hepatocytes reduced PON-1 mRNA by 3-fold. This limited evidence has suggested that constant exposure of oxidized LDL-C lead to loss of PON activity and reduce the expression of PON-1 in hepatic cells (Aviram et al, 1999).

2.1.5.3. Other contributory factors affecting paraoxonase-1 activity

Multiple factors affect to PON-1 activity in the population. The most important related factors are age, gender, diet, environmental chemicals, drugs and way of life. These factors modulate PON-1 activity to upward or downward.

2.1.5.3.i Age

Studies have revealed that age is one of the major determinants of PON-1 activity. Infants show low PON-1 activity and after 6 to 15 months adult levels are achieved. Adults PON-1 activity is constant for a long period (Karanth and Pope, 2000), but it progressively decreases with aging (Costa et al, 2005; Seres et al, 2004). Studies have suggested this decline may relate to the long exposure of oxidative stress conditions with aging (Seres et al, 2004).
2.1.5.3.ii  Gender

Studies conducted with animals have shown that females have relatively higher levels of serum PON-1 activity when compared with males (Ali et al, 2003). However, in humans a slightly higher mean value of serum PON-1 activity has been found in females compared to males, suggests that it was genetic heterogeneity in humans (Mueller et al, 1983; Ali et al, 2003).

2.1.5.3.iii  Smoking

Several studies have reported an association between cigarette smoking and low PON-1 activity (Ferre et al, 2003; Jarvik et al, 2002; Senti et al, 2003). It has suggested that the effect of smoking is apparently short-lived, because those studies have revealed PON-1 level return to control levels after 3 to 24 months after cessation of smoking (James et al, 2000). Studies have suggested that cigarette smoke directly leads to loss of PON-1 activity (Nishio and Watanabe, 1997).

2.1.5.3.iv  Alcohol

Studies have shown moderate consumption of beer, wine or spirits for three weeks increased PON-1 activity (Sierksma et al, 2002; Vander et al, 1999). Although, studies has shown heavy drinking cause to more adverse effects on PON-1 activity. Rao et al (2003) has found that heavy drinkers had a 20% lower, while moderate drinkers had a much higher PON-1 activity when compared to non-drinkers. Despite this evidence, researchers have suggested that moderate alcohol consumption can increase PON-1
activity. However, several studies have concluded that alcohol has no effect on the PON-1 activity (Ferre et al, 2003; Sarandol et al, 2003; Vincent et al, 2003).

2.1.5.3.v Diet

Many studies have carried out to investigate the effects of dietary and lifestyle habits on PON-1 activity. It has shown that a diet rich in saturated fats reduce serum PON-1 activity and a diet that is rich in unsaturated fats increase serum PON-1 activity. A fat-rich diet seems to be responsible for the decreased PON-1 in both animal and human studies. There was an interesting study on men where fat rich diet reduced PON-1 activity by 27% (Sutherland et al, 1999). Further, it has shown that ingestion of saturated fat instead of trans-fat reduces PON-1 activity by 6% in healthy peoples (Roos et al, 2002).

Antioxidant rich diets have yielded contrasting results for increase in PON-1 activity. Consumption of pomegranate juice for 2 weeks increased PON-1 activity by 18% in healthy individuals (Aviram et al, 2000a). Another study has also shown pomegranate juice being responsible for the increased binding ability of PON-1 to HDL-C in individuals with diabetes (Fuhrman et al, 2010). Jarvik et al (2002) has reported that Vitamin C and E appeared to be significant positive predictors of PON-1 activity. However, few studies have reported negative associations between Vitamin C and E rich fruit, vegetable intake and PON-1 activity (Kleemola et al, 2002; Rantala et al, 2002). In addition, Ferre et al (2003) found no association between Vitamins C and E, beta-carotene intake and serum PON-1 activity. Obviously, the interaction between dietary antioxidants and serum PON-1 activity necessitates further study (Ferre et al, 2003).
2.1.5.3.vi Effect of chemicals and metal ions on paraoxonase-1

PON-1 is a calcium dependent hydrolase, EDTA or other chelating agents have the ability to abolish its activity (Teiber et al, 2003). Other cations like barium, copper and mercury were found to inhibit the PON-1 activity. Further studies have indicated that these metal ions may attack to the free thiol group on the cys284 residue of PON-1 (Debord et al, 2003). Mice exposed to dichloroacetic acid (a by-product of water disinfection by chlorination) have shown a 50% decrease in PON-1 gene expression. A similar study has reported that carbon tetrachloride led to an 80% decrease of hepatic PON-1 in rats (Ferre et al, 2002).

2.1.5.3.vii Drugs affecting to Paraoxonase-1

Recently, pharmaceutical compounds such as lipid lowering drugs (statins and fibrates) have gained much attention for their capability to alter the PON-1 gene expression. Deakin et al (2003) found that simvastatin increased PON-1 promoter activity by increasing sterol regulatory element binding protein. However, another study has shown contradictory findings of simvastatin effects on PON-1 activity (Gouedard et al, 2003). Furthermore, some studies have shown lipid-lowering drugs, including ciprofibrate, bezafibrate, and gemfibrozil had no effect on PON-1 activity (Durrington et al, 1998; Turay et al, 2000). However, conflicting results were reported due to different concentrations of the drugs and the cell lines used. Several studies have indicated PON-1 activity increase in patients taking statins, gemfibrozil and fenofibrate (Balogh et al, 2001; Deakin et al, 2003; Paragh et al, 2003). In a cohort study of aspirin users, a significant increase of plasma PON-1 activity and concentration has been reported which may be due to the anti-inflammatory effect of aspirin (Blatter et al,

2.1.6. Paraoxonase-1 and its association with coronary artery disease

2.1.6.1. Antiatherogenic role of Paraoxonase 1

The mechanism of protection against atherosclerosis has traditionally focused on the role of HDL-C as a lipid transporter in reverse cholesterol transport (von et al, 2001). Currently oxidative modification of LDL-C in the artery wall is believed to be central role in the pathogenesis of atherosclerosis (Mackness et al, 1999). The capacity of HDL-C protection LDL-C against oxidative modification is mainly associated with PON-1 activity (Ng et al, 2005). PON-1 provides protection against atherosclerosis and lipid oxidation in vivo by reducing the formation of oxidized LDL-C and destroying the lipid hydroperoxides. Oxidized phospholipids activate inflammatory genes and promote the adhesion of monocytes to endothelium. Adhesion is a key event in monocyte recruitment into early atherosclerotic lesions. PON-1 removes oxidized phospholipids from LDL-C thus it plays a role in the decrease of premature atherosclerosis (Heinecke and Lusis, 1998). Recently, the role of PON-1 in protection against atherosclerosis has further investigated using a PON-1 transgenic mouse models. PON-1 knockout mice had an increased susceptibility to atherosclerosis due to low serum PON-1 enzyme (Shih et al, 1998). A human epidemiology study has revealed that persons in the highest quintile of serum PON-1 activity has a decreased risk of coronary artery disease.
compared with those in the lowest quintile (Mackness et al, 2003). All these evidences strongly supported to the role of PON-1 antioxidant enzyme in the prevention of CAD and atherosclerosis.

2.1.6.2. Low Paraoxonase-1 as a risk factor for coronary artery disease

There is a well-established negative relationship between HDL-C concentration and the incidence of CAD. Several prominent studies have shown that individuals with low HDL-C are more prone to CAD events than with high HDL-C (ATP-III, 2002). Whilst, the primary cardio-protective role of HDL-C is widely attribute by reverse cholesterol transport. Furthermore, HDL-C exhibits anti-inflammatory, antithrombotic and antioxidant properties that provide benefits to protect from CAD (Florentin et al, 2008). The antioxidant activity of HDL-C has mainly attributed by the activity of PON-1. Evidence has demonstrated that PON-1 disrupts many of the pro-oxidant processes involved in atherogenesis. There was established a negative association between HDL-C and atherosclerosis and evidence suggested that HDL-C could inhibit the oxidative modification of LDL-C (Parthasarathy et al, 1990). It has suggested HDL-C associated PON-1 enzyme play a part of the antioxidant properties displayed by the HDL-C. Some studies have reported that HDL-C and PON-1 prevents lipoperoxide generation to a similar extent, implying that the PON-1 enzyme was involved in the antioxidant effects observed by HDL-C. An in vitro experiment has conducted both with and without addition of purified PON-1 into LDL-C oxidation has been show that the addition of PON-1 enzyme greatly decreases LDL-C associated accumulation of oxidized lipids such as lipid peroxides (Mackness et al, 2004), lipid hydroperoxides (Navab et al, 2000) and aldehydes (Aviram et al, 1998a). Evidence has suggested that PON-1 can
reduce cholesteryl ester hydroperoxides and cholesteryl ester peroxides from the carotid and coronary artery atherosclerotic lesions (Aviram et al, 2000b).

Human PON-1 transgenic (Tg) mice, which displayed a significant increase in plasma PON-1 activity had reduced atherosclerotic lesions even after consuming a high fat diet (She et al, 2009; Tward et al, 2002). Plaques from the PON-1 Tg/ apoE KO mice also appeared to be more stable, as collagen and smooth muscle were increased and macrophage and lipid were decreased within the lesions (She et al, 2009). Similarly, macrophage and aortic-derived oxidative stress, as well as atherosclerotic lesion size has been reduced in human PON-1 Tg mice (Rozenberg et al, 2005). Over expression of human PON-1 has also shown to reduce plaque progression in mice (Mackness et al, 2006b). In a study which examined apoE KO older mice (18 months) with advanced atherosclerosis who were subjected to 13-15 fold increase in PON-1 activity did not have any effect on the lesion size, but decreased vascular oxidative stress and improved endothelial and smooth muscle cell functions. Furthermore, the study has demonstrated that PON-1 improves vasomotor function independently from the plaque size in older mice with preexists atherosclerosis (Guns et al, 2008).

Several epidemiological studies have investigated the relationship between PON-1 and the risk or incidence of CAD in humans. It has reported that the individuals with normal HDL-C concentration, but the low PON-1 activity were more susceptible to atherosclerosis compared to those individuals with normal HDL-C with significantly higher PON-1 activity (Navab et al, 1997). Several other case-control studies have established a significant inverse relationship between CAD or its risk factors and PON-1 activity (Jarvik et al, 2000; Mackness et al, 2001; Yildiz et al, 2008). Even so, none of
these subjects was able to indicate low PON-1 status is a cause or a result of CAD. These characteristics could be elucidated by longitudinal investigations. In a recent 3-4 year longitudinal study has assessed individuals who had undergone an elective diagnostic coronary angiography. This study revealed an inverse relationship between CAD prevalence and serum paraoxonase levels. Low levels of PON-1 activity were significantly associated with the presence of coronary artery disease. (Paraoxonase: OR (odds ratio) = 1.5; 95% CI = 1.03-2.3; p= 0.03) (Bhattacharyya et al, 2008). However, further studies may need to establish the causative relationships between PON-1 and CAD. Current evidence suggests that PON-1 activity may be an important factor in the prevention of atherosclerosis.

2.1.7 Association of Paraoxonase-1 and physiological or pathological conditions other than coronary artery disease

ROS induced oxidative stress is a known etiological factor for carcinogenesis. The human body has a number of endogenous free-radical scavenging systems, including PON-1. Therefore, its role in cancer etiology and prevention has been of great interest. Prostate cancer is one of the most commonly diagnosed cancers in developed nations. A study has revealed that a mutation in the coding region of PON-1 affected to the PON-1 expression level and increased risk of prostate cancer. Previous studies have reported that patients with cancer in lung, pancreas, stomach and breast had low serum PON-1 compared to healthy subjects (Stevens et al, 2006). These epidemiological studies provide evidence of the role of PON-1 in carcinogenesis. Thus, further studies need to investigate molecular mechanisms involved in these malignant transformations.
Evidence indicated that PON-1 activity suppressed in various nutritional and physiological conditions such as obesity and malnutrition. Low PON/HDL-C and PON/Apo AI ratios were reported in patients under high atherosclerosis risk (Azarsiz and Sonmez, 2000). Decreased levels of PON-1 activity have been found in patients with diabetes mellitus (Abbott et al, 1995; Ikeda et al, 1998) and neurological disorders such as Alzheimer’s and Parkinson’s diseases in several studies. Recently, it has suggested that PON-1 expressed in many tissues besides the liver, because it has shown a broad protective role in various human diseases (Marsillach et al, 2009). The hepatic PON-1 is located on the outside of the endoplasmic reticulum membrane with catalytic functions, which is believed to be protective against liver injuries. It was reported that transgenic expression of human PON-1 in mice protected from the liver injury (Zhang et al, 2008). A number of studies have been evaluated the role of PON-1 in entire spectrum of liver disorders and decreased PON-1 expression levels has linked to various liver diseases such as cirrhosis and hepatosteatosis (Ferre et al, 2002).

Low PON-1 enzymatic activity has been associated with several disease states that share the common theme of oxidative stress. These pathologies include types 1 and 2 diabetes (Abbott et al, 1995; Mackness et al, 2002a), chronic renal failure (Dirican et al, 2004), uremia (Biasioli et al, 2003), thyroid dysfunction (Azizi et al, 2003), rheumatoid arthritis (Tanimoto et al, 2003), multiple sclerosis (Ferretti et al, 2005), metabolic syndrome (Senti et al, 2003), hyperlipidemia (Paragh et al, 1998) and cerebrovascular diseases (Jarvik et al, 2003a).
Thus, evidence suggests that PON-1 has an overall protective role in inflammatory diseases. Based on these studies the role of PON-1 in human diseases opens up a new avenue for identification of the pathogenesis and management of disease in the future.

2.2. Glutathione Peroxidase and coronary artery disease

2.2.1. Human glutathione peroxidase

2.2.1.1. Introduction

Glutathione peroxidases (GPXs) are a family of tetrameric enzymes with characteristic actions in both first and second line antioxidant defenses. GPX is the largest single family among the selenoproteins. GPX is showing certain features due to its selenium (Se) ion presence with single selenocystine (SeCys) residue per monomer. It has an ability to reduce lipid peroxides using glutathione (reduced glutathione/ GSH) as a cofactor and produce a corresponding alcohol and glutathione disulfide (oxidized glutathione/ GSSG). The regeneration of GSH is carried out by glutathione reductase (GR), which use NADPH as reduced equivalents (Arthur, 2000; Chu et al, 2004). GPX reduces the H$_2$O$_2$ by transferring electrons from GSH to H$_2$O$_2$ with the subsequent formation of H$_2$O and O$_2$ meanwhile converting GSH to GSSG (Wu et al, 2004; Li and Shah, 2004; Hayes et al, 2005) (Figure 2.1). Several isozymes are encoding by different genes, which vary in cellular location and substrate specificity and each of them containing selenocysteine at the active site (Leopold and Loscalzo, 2005). The GPX family members have similar homotetrameric structure and function except GPX-4. However, GPX-4 is monomeric with a similar molecular weight (Toppo et al, 2008).
Substrate specificity varies among the GPXs family, such as efficient reduction of phospholipid hydroperoxides only done by GPX-4. Expression patterns also distinguish the GPX family. GPX-1 is ubiquitous, but especially high in the liver and kidney. The GPX-2 expression is primarily in the gastrointestinal (GI) tract and GPX-3 can found in extracellular as a glycoprotein. The expression of GPX-4 also ubiquitous, but expression is particularly high in the testes (Lei, 2001). The recently discovered GPX-6 is present in the adult olfactory epithelium (Kryukov et al, 2003). Furthermore, it has revealed that GPXs show an overlying characteristic on substrate specificity. Thus, some circumstances make it difficult to assay GPX activity and discriminate among various types of GPXs (Singh et al, 2006).

![Glutathione peroxidase reduction cycle](image)

Figure 2.1. Glutathione peroxidase reduction cycle.

Hydrogen peroxide is reduced to water, while glutathione (GSH) are oxidized in a reaction catalyzed by the selenoenzyme, glutathione peroxidase. Oxidized glutathione may be reduced by the flavin adenine dinucleotide (FAD)-dependent enzyme, glutathione reductase.
2.2.1.2 Isozymes of Glutathione peroxidase

2.2.1.2.1 Glutathione peroxidase-1

GPX-1 also known as cellular GPX, it was the first identified mammalian selenoprotein and the most abundant version in humans. Prediction of the function of GPX started with the work of Mills (1957), Schwarz and Folz (1957) who discovered GPX protection of hemoglobin against oxidation and the essentiality of selenium in prevents liver necrosis of rats. In addition to the above characteristic features, GPX-1 identified as a distinct peroxidase from others since its activity is not affected by azide or cyanide. Mammalian GPX-1 proteins are approximately 201 amino acids in length with a selenocysteine at position 47. GPX-1 is predominately a cytosolic protein and found localized in mitochondria (Esworthy et al, 1997). Entering mechanism of GPX-1 into the mitochondria has being elucidated and research findings indicate that it may play a role in protecting the mitochondria against high levels of oxidative stress or in the regulation of mitochondrial dependent cell signaling via H$_2$O$_2$ levels (Thu et al, 2010; Legault et al, 2000). Expression of GPX-1 varies widely and is dependent on changes of selenium status of the body (Cheng et al, 1997).

GPX-1 is an important antioxidant against oxidative stress. It is universally present, including cytosol, mitochondria (Esworthy et al, 2005; Li et al, 2000) and it has found more effective than catalase at removing intracellular accumulated hydrogen peroxides. Another important function of GPX-1 is ability of reduce lipid hydroperoxides and reduce phospholipid monoacylglycerol hydroperoxides.

GPX-1 protects against ROS (Esworthy et al, 1993) and GPX-1 knockout (KO) mice have shown an increased susceptibility to ROS induced oxidative stress (Fu et al,
2001), suggesting that GPX-1 has a protective effect against oxidative damage in endothelial cells (Zhang et al, 2005). Further evidence revealed that GPX-1 is essential for survival in the presence of excess levels of ROS (Legault et al, 2000; Thu et al, 2010; Duong et al, 2010).

2.2.1.2.11 Glutathione peroxidase-2

GPX-2 is a cytosolic GPX found primarily in the crypts of the intestinal epithelium cells of the gastrointestinal (GI) tract (Chu et al, 1993; Esworthy et al, 1997; Dreher et al, 1997). In addition, an important physiological feature of GPX-2 is the mRNA of GPX-2 is much more resistant to selenium deficiency than GPX-1 and GPX-3 (Chu et al, 1997; Wingler et al, 1999).

GPX-2 activity has been reported in other tissues in response to stress, such as in lung tissues when exposed to cigarette smoke and in breast during tumorogenesis (Singh et al, 2006). Several studies have suggested GPX-2 showing varied function as antioxidant and cellular protection. However, some studies have reported that GPX-2 in gastrointestinal tract epithelial cells provide an essential protection against gut pathogens (Chu et al, 2004b; Esworthy et al, 2005).

Low GPX-2 was a potential risk factor in the onset of precancerous lesions such as Barrett’s esophagus or in colorectal adenoma, where low GPX-2 expression was associated with increased cellular proliferation (Mork et al, 2000; Al-Taie et al, 2004). Initiation of malignant phenotype of these precancerous lesions decreases GPX-2 expression (Florian et al, 2001; Leier et al, 1996). GPX-1 and GPX-2 knockout mice
(GPX-1/2-KO) exhibit severe ileocolitis at early ages, when compared to those who had adequate activity of GPX-2 (Esworthy et al, 2001; Chu et al, 2004a).

2.2.1.2.iii Glutathione peroxidase-3

GPX-3 antioxidant protein synthesized in the proximal tubules of the kidney (Avissar et al, 1994). It has shown that decreased activity of GPX-3 was found in cancer when compared to normal tissue, due to hypermethylation of the gene (Peng et al, 2009). As GPXs family characteristics, it is tetramer and has a similar molecular weight and similar substrate specificity. GPX-3 was considered same as GPX-1 in beginning, but later found it has different biochemical characteristics and distinct regulations (Cohen, 1994). However, there has been reported 44% homology with human GPX-1. It encodes 226 amino acids with a SeCys at position 73 and has a molecular mass of 92kDa (Cohen, 1994). GPX-3 is an extracellular glycosylated enzyme involved in the reduction of H₂O₂, lipid hydroperoxides and phospholipid hydroperoxides.

2.2.1.2.iv Glutathione peroxidase-4

GPX-4 is structurally and functionally unique among GPXs family since it functions as a monomer and more resistant to selenium depletion than other selenoperoxidases. It also has reported that intracellular GPX-4 prevents lipid peroxidation, through antagonizing the actions of lipoxygenases and cyclooxygenase activity (Imai et al, 2003; Yant et al, 2003).
GPX-4 plays an important role in spermatogenesis and sperm function (Imai and Nakagawa, 2003). Many studies have established that the GPX-4 activity is high in testes. Ursini et al (1999) reported that GPX-4 is involved in sperm maturation and preserve structure of the sperm tailpiece in oxidative stress conditions. Furthermore, studies have shown that selenium deficiency leads to male infertility (Foresta et al, 2002).

GPX-4 has also shown to be involved in the inflammatory process, by inhibiting lipoxygenase activity through the availability of lipid hydroperoxides and thus limiting the production of the inflammatory mediator leukotrienes (Imai et al, 1998; Chen et al, 2003). Studies have shown that there is an inverse correlation between GPX-4 and tumor grades of breast cancer, individuals with higher-grade tumors have low GPX-4 levels (Cejas et al, 2007). Thus, it has been believed that GPX-4 show multiple important roles in the cells as an antioxidant in inflammation and cell death, as a structural protein in spermatogenesis and protection against tumor development.

2.2.2. Genetic variants of Glutathione peroxidase-1

Genetic variations of GPX-1 enzyme may alter its expression level and effective of function. GPX-1 is polymorphic and shows approximately 38 polymorphisms, although most polymorphisms have not found within the open reading frame in the 5' and 3' flanking regions (Foster et al, 2006). Most significant polymorphism is C > T alteration in codon 198, which results in a proline to leucine (Pro198Leu) alteration in the polypeptide chain. The complete functional consequence of Pro198Leu has not been elucidated. However, it has implicated as a risk factor in diseases including CAD. Leucine (Leu) allele has shown a decreased responsiveness to selenium supplementation. In a recombinant cell culture study, induction of GPX-1 activity was
lower in expressing Leu allele cells compared with expressing Pro allele cells with the equivalent selenium treatment (Hu and Diamond, 2003; Zhuo et al, 2009).

In-vivo studies have shown that Pro198Leu polymorphism directly affects to GPX-1 activity. Several studies have shown that Leu allele carriers of the GPX-1 showed lower GPX-1 activity when compared with Pro allele carriers and this effect was greater in males than females (Bastaki et al, 2006; Jablonska et al, 2009). Cell culture studies have shown that humans with similar plasma selenium levels but different GPX-1 genotypes had significantly different GPX-1 activity and different responses for selenium supplementation (Jablonska et al, 2009; Zhuo et al, 2009; Miller et al, 2012).

2.2.3. Biochemical mechanism of Glutathione peroxidase action

Glutathione are small molecules that act as cofactors for GPX in peroxide reduction. It is a thiol containing try-peptide, which helps in signal transduction and detoxification of drugs or toxins (Singh et al, 2012). However, GSH and other low molecular weight thiols have special characteristic, which is easily oxidate and regenerate. This beneficial characteristic makes it capable of participating in many biochemical and pharmacological reactions in the human body (Paolicchi et al, 2002). Furthermore, the glutathione system functions in redox balance, ROS detoxification and demonstrate as a potential target for genetic and therapeutic manipulation.

Most members of the GPX family are responsive to selenium status. Selenium is an essential trace element for GPX function. In 1978, the selenium molecules in GPX was identified as selenocysteine (Secys), which is the selenium analogue to serine (Ser) and
cysteine (Cys). It was confirmed that this amino acid was responsible for the catalytic action of the enzyme (Bastaki et al, 2006). However, different selenoproteins are preferentially translated and translations of GPX-1 and GPX-3 proteins were most sensitive to selenium deficiency (Low et al, 2000; Kipp et al, 2009). The effects of selenium deficiency were less in the translation of GPX-4 and GPX-2, whilst in some studies it have revealed that GPX-2 expression is increased during selenium deficiency (Lei et al, 1995; Wingler et al, 1999). The mechanism of GPX regulation was not been clearly elucidated. However, GPX-1 has shown that its mRNA is less stable during selenium deficiency (Moriarty et al, 1998; Sun et al, 2000).

Studies have also shown that selenium is an important integral component of GPX structure and influence increase the activity of GPX (Schwenke DC, 1998). Selenium is located in the structure of GPX enzyme assist to play an antioxidant role in the prevention of certain metabolic diseases such as atherosclerosis.

GPX antioxidant family consists of five Secys containing members and three non Secys containing members (Kryukov et al, 2003; Tosatto et al, 2008; Nguyen et al, 2011). These antioxidant enzymes detoxify H₂O₂ and lipid peroxides via the oxidation of GSH and found within multiple cellular compartments as well as in plasma.

Mechanism of peroxide reduction appears same for all of the isoforms. It is a multistep ping-pong type mechanism, which is GSH oxidized as a substrate to catalyze the reaction (Takebe et al, 2002). The selenol group of the selenocysteine is oxidized to selenenic acid following interaction with peroxides, which is subsequently reduced by GSH to form glutathiolated-selenol intermediates. Selenocysteine active site returns back to its reduced state by oxidizing an additional GSH molecule to convert the
glutathiolated-selenol to selen. The above is based on limited and inconsistent data on the role of GPX activity and selenium level in the CAD etiology despite more than 20 years of research.

2.2.4. Factors affecting glutathione peroxidase activity

2.2.4.1. Glutathione peroxidase-1 genetic variants and disease

Genetic polymorphism is an important factor for GPX-1 activity in the body. Most interesting polymorphism is Pro198Leu (CT) genotype. Recent studies have revealed that TT genotype is associated with low expression of GPX-1 activity in humans (Ye et al, 2013).

GPX-1 genotype studies have shown contradictory findings for selenium and dietary factor influences to its activity. Some studies have shown selenium can modulate GPX-1 expression in vivo (Jablonska et al, 2009) and at a certain level of plasma selenium maintain GPX-1 level at a steady level. However, some studies have also reported that GPX-1 activity does not relate to any plasma selenium level (Hansen et al, 2009; Ravn et al, 2006).

Several studies have shown a relationship between GPX-1 genetic polymorphism and the risk of developing CAD. It has been suggested that Leu (T) variant modulate the reduce GPX-1 activity. However, not all studies have found this association between genotype and GPX-1 activity in humans. Furthermore, it has not been found for GPX-1 polymorphic forms. Literatures have shown that GPX-1 Leu (T) allele is significantly associated with many diseases, including increased risk of CAD (Lei et al, 2009).
A study has reported that GPX-1 Pro198Leu polymorphism shows higher frequency of Leu/Leu (TT) genotype than Pro/Pro (CC) genotype in breast cancer (Hu et al, 2003, Ravn et al, 2006). However, there were conflicting findings about the relationship between the Leu allele (T) and breast cancer. Because of several studies failed to found an association between the GPX-1 Pro198Leu polymorphisms and risk of breast cancer (Ahn et al, 2005).

A study reported that Leu/Pro genotype provides a protective effect for prostate cancer when compared to Pro/Pro genotype (Arsova et al, 2009). Another study has found that Leu allele is related to increased risk of bladder cancer (Ichimura et al, 2004).

A study of male smokers has revealed a significant association of the Leu allele (T) with increased risk of lung cancer (Ratnasinghe et al, 2000). Another case control study has reported Leu allele carriers had increased risk for lung cancer when compared to Pro carriers (Lee et al, 2006). However, not all studies report increased lung cancer risk with the Leu allele (Raaschou et al, 2007; Yang et al, 2004).

Expression of GPX-1 activity also influences by other factors such as dietary selenium status, nearest gene and lifestyle. However, functional differences are still being investigating for associations between GPX-1 Pro and Leu alleles.

2.2.4.2. Oxidative stress

GPX-1 is a well-known antioxidant against oxidative stress. Although, it has suggested that in over expression of GPX-1 may remove essential oxidants from the body, which is necessary to signal transduction in cells. It may result in reduced cell proliferation and increased cellular apoptosis.
2.2.4.3 Effect of chemicals

It is expected that an increase of GPX concentration would allow the body to better cope with oxidative stress. Biological studies have shown that adding inorganic selenium salts, such as sodium selenite to the diet can lead to increased concentrations of selenoproteins and GPX activity. However, these salts can be toxic at high doses, hence not suitable as a source of intake of inorganic selenium into the body. A method was proposed to create organoselenium compounds, which mimic the reductive behavior of GPX in vivo and can act catalytically in the presence of thiols. A recent computational study showed that the calculated reaction rate in the reduction of hydrogen peroxide by free Selenocystein is smaller than the rate associated with GPX, which indicates that the enzyme is very important in antioxidant systems (Cardey et al., 2007). The organoselenium compound such as ebselen (2-phenyl-1,2-benziselenazol-3(2H)-one) is reported to exhibit GPX-like activity.

2.2.4.4 Effect of smoking

A study has done among young adult smokers and nonsmokers to investigate the association of GPX-1 activity and smoking. It revealed that GPX-1 activity was lower in smokers when compared to nonsmokers (Malling et al., 2009).

2.2.5 Glutathione peroxidase activity and coronary artery disease

When cells exposed to stressful stimuli, it generates ROS in response to the stress signals. ROS are inherently reactive and damaging to the cells, although cytoprotective enzymes such as GPX, SOD and catalase convert ROS into harmless products. Defects
in these antioxidant systems, may lead to subsequent accumulation of ROS in body including peroxides, which may contribute to oncogenesis by enhancing damage to macromolecules and DNA.

Recently, studies have suggested that GPX-1 negatively influences pro-atherogenic gene expression of endothelial cells in humans. Hence, low GPX-1 expression in these cells may improve expression of the vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemoattractant protein-1 (MCP-1) which facilitate to atherosclerosis. These findings have suggested a novel role of GPX-1 in preventing atherosclerosis. Similarly, GPX-1 protects against endotoxins induced by expression of inflammatory mediators in humans (Lubos et al, 2011).

Erythrocyte GPX-1 activity inversely correlates with atherosclerosis. A study has shown low GPX-1 activity affected to CAD at 2.3 times hazard ratio (95% CI: 1.4-4.0) (Espinola et al, 2007). These findings have suggested that GPX-1 has the ability to modify, enhance or reduce of atherogenesis.

Another prospective study has shown that GPX-1 activity a powerful predictor of CAD and confirmed the inverse relationship between CAD and GPX-1 activity (Blankenberg et al, 2003). In another prospective study, individuals having high GPX-1 activity provided more protection from high concentration of homocysteine (Schnabel et al, 2005).

2.2.6. Association of glutathione peroxidase with other diseases

Low GPX-1 is associated with susceptibility to develop several types of diseases, such as cancer, diabetes and CAD. Involvement of GPX-1 in these diseases may attributed to
alterations in the management of oxidative stress, such as increases ROS levels by environmental factors or from functional alterations of single nucleotide polymorphisms (SNPs) (Foster et al, 2006).

Decreased GPX-1 levels have been found in multiple cancer types with the loss in GPX-1 expression resulting from deletion of its chromosomal region (3p21) and these have been detected in cancers of the head and neck, breast, lung and colon (Hu and Diamond, 2003; Hu et al, 2005; Baliga et al, 2007; Hu et al, 2004). Altered regulation of GPX-1 has implicated as a potential factor in the development of metabolic diseases such as diabetes mellitus (DM). Diabetes mellitus is characterized by the presence of high blood glucose and poor tissue glucose uptake due to insulin resistance or hyperinsulinemia (Carey et al, 1996). One of the greatest risk factors for DM is obesity stemming from a high-fat diet (Savill et al, 2012). Excess fat cells have secretory factors that contribute to the desensitization of the insulin receptor and its response to insulin, thus negatively affecting glucose uptake (Hotta et al, 2001). Increased body fat levels also lead to an overall increase in ROS levels and oxidative stress levels, which can stimulate increased GPX-1 levels and influence insulin production in humans as well as mice (Yang et al, 2000; Faienza et al, 2012).

Increased GPX activity in pregnancy has correlated with an increase in insulin resistance over the course of gestation in humans (Chen et al, 2003). Pregnant mice fed with a high-selenium diet had increased GPX activity and ultimately developed gestational diabetes or diabetes shortly following delivery and offspring developed diabetes as adults indicating that increased GPX activity alters susceptibility to diabetes in both the mother and in offspring (Zeng et al, 2012). Furthermore, GPX-1 null mice
having decreased plasma insulin levels and increased islet β-cell mass in the pancreas as compared to wild-type controls, demonstrated how GPX-1 modifies insulin levels and affects the onset of DM (Wang et al, 2011). Mice fed on a high fat diet can develop insulin resistance, but in the absence of GPX-1, insulin resistance does not occur (Loh et al, 2009, Steinbrenner, 2010). However, another study has shown when GPX-1 over expressed exclusively in the pancreas; it has decreased hyperglycemia in mice with induced diabetes (Harmon et al, 2009).

Low GPX activity can alter susceptibility to developing severe cardiomyopathies, such as Keshan disease. Keshan disease is a severe cardiomyopathy endemic to regions of China, where soil selenium was low and cause to severe selenium deficiency in the population (Yang et al, 1988). In these regions, extremely low selenium intake results in decreased GPX-1 levels. There are existing selenium supplementation programs where disease incidence has decreased significantly (Alfthan et al, 2000). Further evidence of a protective effect of GPX-1 against cardiomyopathies was shown in GPX-1 over expressed mice that were treated with cardiotoxin compounds that showed a lower incidence of cardiomyopathies than wild-type control mice (Xiong et al, 2006).

A study has suggested that GPX-1 has an antitumorogeneses action in some cancers by eliminating ROS (Liu et al, 2004). Furthermore, it has shown that GPX-1 facilitates to reduce development of some cancer cell lines in culture. After administering this cell lines into healthy mice, it has shown a decrease in their tumorogencity (Liu et al, 2004).
2.3. Apolipoprotein A-I and coronary artery disease

2.3.1. Human apolipoprotein A-I

2.3.1.1. Introduction

Apo A-I is synthesized in the liver and small intestine in mammals and contributes to approximately 70% of the HDL mass. Apo A-I consists of 243 amino acids in a single polypeptide chain of 28 kDa molecular weight. Brewer et al 1978 determined the primary structure of Apo A-I. Apo A-I is the main lipoprotein amongst associated lipoproteins with HDL cholesterol. Several studies have shown that serum concentration of Apo A-I positively correlated with serum HDL-C concentration (Srivastava et al, 2000). Apo A-I is a cofactor for lecithin cholesterol acyl transferase (LCAT) (Brouillette et al, 2001) which is an important enzyme in transport of excess cholesterol from tissues via reverse cholesterol transport (RCT) (Waildius and Jungner, 2004). Furthermore, Apo A-I is a ligand for the ATP-binding cassette protein A-I (ABCA1) that is involved in the binding procedure with cholesterol in RCT either directly or indirectly (Wailidus and Jungner, 2004).

HDL-C exists in five forms namely HDL-2a, HDL-2b, HDL-3a, HDL-3b and HDL-3c. Furthermore, HDL comprises of two types of lipoproteins such as Apo A-I and Apo A-II (HDL A-I: HDL A-II). Studies have shown that HDL A-I is more effective than HDL A-I: A-II in promoting cholesterol efflux, and it is involved in the atheroprotective mechanism of LCAT.
2.3.1.2. Structure and metabolism of apolipoprotein A-I

Human Apo A-I gene is located on chromosome 11. The gene encodes propeptide containing 267 amino acids (residues), it is cleaved intracellularly to a 249 amino acid propeptides (Davidson et al, 2007). After release into plasma, the propeptides are cleaved to yield a 243 amino acid polypeptide protein (28.1 kDa) that lacks glycosylation and disulfide linkages (Davidson et al, 2007) due to the absence of cysteine residues. The primary structure of Apo A-I has been extensively analyzed using computer programs developed to identify and characterize the signature pattern of all apolipoproteins and amphipathic helices (Martin et al, 2006). Residues 44-243 contain ten amphipathic α-helices containing either 11 or 22 residues belonging to class A or class Y. The class A amphipathic helices has been shown to be associated with strong lipid-binding properties and it is defined by positively charged residues at the polar-non polar interface and negatively charged residues at the center of the polar face.

HDL particles enclosed with two or four molecules of Apo A-I that present as anti-parallel dimers. Due to the unique configuration of Apo A-I, it is thought to consist primarily of amphipathic alpha helices punctuated by links at regularly spaced proline (Martin et al, 2006; Frank et al, 2000).

Recent studies have proposed three models of Apo A-I structure in discoidal HDL. The “picket fence” model was the most commonly presented. In this model, the 22 amino acid repeats in amphipathic alpha helices aligned in parallel to the phospholipids acyl chains in the bilayer discs and connected by hairpin loops (Wang et al, 2002). Whereas in the “double belt” model, each of two ring-shaped Apo A-I molecules were oriented in anti-parallel formation and comprised a small segment of phospholipids each in its
own leaflet. The helical “hairpin” model shows two pairs of Apo A-1 molecules resembles “head-to-head” or “head-to-tail” orientation (Davidson et al, 2007).

Since, Apo A-1 has been a primary factor in reverse cholesterol transport (RCT), it is important to understand its structure at various points along the RCT pathway. In human plasma, Apo A-I has been shown to exist in multiple lipid-bound and lipid-free forms. It has indicated 5-10% of Apo A-1 exists in lipid-free state in circulating plasma, while the rest of Apo A-1 is distributed among various lipid-bound states (Davidson et al, 2007).

The beneficial effect of HDL-C on atherosclerosis has been well established. It has been shown that elevated levels of HDL-C enhance the efflux of cholesterol from arterial walls, increase reverse transport or excretion. The major steps in the RCT pathway is the active efflux of cholesterol and phospholipids from cells by ATP-binding cassette A1, binding of cholesterol to apolipoproteins, forming pre-beta-HDL, the esterification of HDL-bound cholesterol by LCAT. This RCT model has used to explain both HDL-C’s role in lipid metabolism and the inverse association between HDL-C plasma concentrations and risk of CAD. Hence, Apo A-1 was subsequently used for therapeutic intervention.

2.3.1.3. Biochemical function of Apolipoprotein A-I

Primarily, HDL consists of two apolipoproteins namely Apo A-I and Apo A-II; and the other one Apo A-I alone (Schultz et al, 1993). The significant cardioprotective effect has been shown by the form of Apo A-I alone. In addition, it has shown that Apo A-1
also shows anti-inflammatory, anti-oxidative, anti-apoptotic, anti-thrombotic, vasodilatory and anti-infective functions.

2.3.1.3.i Stimulation of reverse cholesterol transport

Many clinical and epidemiological studies have demonstrated an inverse relationship between HDL-C and the risk of CAD. This antiatherogenic activity is believed depend on the function of HDL as a carrier of excessive cholesterol from peripheral tissues to the liver, this process referred as RCT. In RCT, generally excessive cholesterol in peripheral tissues removed and transported to the liver. Excess cholesterol in the liver subsequently catabolized or excreted into bile (Lewis and Rader, 2005). During this process, the adenosine triphosphate-binding cassette transporter A1 (ABCA1) (Oram and Heinecke, 2005) located on arterial macrophages, promotes the transport of cholesterol to the extracellular compartments or as a nascent pre-beta-HDL. Subsequently, LCAT esterifies cholesterol within nascent pre-beta-HDL to produce cholesteryl esters and converts it into mature alpha-HDL. Thereafter, Mature alpha-HDL takes further cholesterol via the macrophage adenosine triphosphate-binding cassette transporter G1 (ABCG1) (Singh et al, 2007).

Cholesterol efflux mechanism via macrophages occurs as a passive diffusion by interacting with scavenger receptor BI (SR-BI) or binding with ABCA1 transporter pathways (Yancey et al, 2003; Brewer, 2004). This, process involves the exchange of cholesterol between mature spherical alpha-HDL and cellular membranes. During RCT, Apo A-I interacts with other cellular receptors, including ABCA1, SR-BI and activation of LCAT (Brouillette et al, 2001; Zheng et al, 2004; Bergt et al, 2004).
Evidence suggests that plasma level of Apo A-I is one of strongest predictive risk marker of CAD.

2.3.1.3.ii Scavenging effect on toxic phospholipids

Apo A-I prevents toxic accumulation of lysophosphatidylcholine (Lyso-PC), which form as a byproduct during LDL oxidation (Matsuda et al, 1993). It has been reported that smooth muscle cell cytotoxicity is decreased by Apo A-I using Lyso-PC and platelet activating factor (PAF). Thus, it is involved in protection of smooth muscle cells from the apoptotic effects.

2.3.1.3.iii Antioxidant effect of HDL and apolipoprotein A-I

Antioxidant effects of HDL have recognized as the result of consisting metal ions and arylesterase enzyme, which predominantly carried by Apo A-I and Apo J (clusterin) (Stein et al, 1999).

2.3.1.3.iv Anti-inflammatory effects of HDL and apolipoprotein A-I

HDL and Apo A-I are capable of neutralizing lipopolysaccharide and endotoxins and thereby preventing release of lipopolysaccharide-induced tumor necrosis factor and inhibit complement activation. This leads to reduced cytokine induced endothelial vascular cell adhesion molecules and reduce macrophage infiltration (Shah et al, 1998). HDL-C and Apo A-I have anti-inflammatory effects and its results in inhibition of LDL oxidation.

2.3.1.3.v Effect of HDL and Apolipoprotein A-I in autoimmunity

Autoimmune disorders occur when the immune system loses tolerance to self-antigens. Activated T cells stimulate B cells to produce antibodies against antigens. If T cells
activated against self-antigens stimulate produced antibodies, which referred as autoantibodies. Humans with autoimmune disorders such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) have been shown increased atherosclerosis and decreased HDL-C levels compared to controls. Previous studies have shown that HDL associated Apo A-1 inhibits the production of inflammatory cytokines such as interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) by binding to T cells. Apo A-1 has shown protective actions by inhibiting the contact between stimulated T cells and monocytes under normal conditions to prevent the development of inflammatory diseases, including autoimmune diseases and atherosclerosis (Barter et al, 2004; Negre et al, 2006).

2.3.2. Factors affecting Apolipoprotein A-1 levels

2.3.2.1. Medicinal drugs

2.3.2.1.1 Niacin (Vitamin B3)

Niacin is the most effective drug currently available to raise HDL-C levels. At higher pharmacological doses, niacin can reduce LDL-C, triglycerides and Apo B, while increasing HDL-C and Apo A-1 (Chapman, 2005). These changes have been shown to translate into clinical benefits, decreasing morbidity and mortality from CAD (McCormack and Keating, 2005).
2.3.2.1.ii Statins

Statins are the most effective pharmacological drugs lowering LDL-C. They inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the enzyme that catalyzes the rate-limiting step in cholesterol synthesis (Slater and MacDonald, 1988). By inhibiting cholesterol synthesis in the liver, statins activate hepatocyte LDL receptors and produce significant reductions in circulating LDL-C (Slater and MacDonald, 1988). Several large randomized clinical trials have shown statins reduced LDL-C levels in body, thereby reducing the risk of cardiovascular events and decreasing mortality (Nicholls et al, 2007). Studies have reported statin treatment increased Apo A-I levels in human (Bonn et al, 2002; Maejima et al, 2004). Thus, statin therapy reduces the risk of coronary atherosclerosis by significantly reducing LDL-C and increasing HDL-C levels (Nicholls et al, 2007).

2.3.3. Apolipoprotein A-I and association with coronary artery disease

2.3.3.1. Antiatherogenic role of Apolipoprotein A-I

The lipid hypotheses were proposed more than a century ago and it has been based on the idea that dyslipidemia is the major cause for atherosclerosis (Steinberg, 2005; 2006a; 2006b). Ultimately, the validity of the hypothesis was established by major epidemiological studies, such as the “Seven Countries Study” with a 25-year follow-up (Verschuren et al, 1995). Studies have shown an inverse relationship between blood levels of HDL-C and the development of atherosclerosis (Boden, 2000). This inverse association between HDL-C concentrations and cardiovascular risk has shown that each
1 mg/dl increment in serum HDL-C represents a 2%-3% reduction in CAD risk, while a 1% reduction in LDL-C decreases the risk of CAD by 1%-2% (Boden, 2000).

Atherosclerosis is an inflammatory disease characterized by the formation of cholesterol containing lesions in the vascular endothelium (Libby et al, 2002). These atherosclerotic plaques enhance inflammation, cell death and fibrosis (Hansson, 2005). Apo A-1 associated HDL-C has reported as an anti-inflammatory, anti-atherogenic lipoprotein that contribute to cholesterol efflux from tissues (Rader, 2006).

Framingham Heart Study reported that high serum cholesterol was a major risk factor for coronary heart disease (Kannel et al, 1961). It has been a widely accepted belief that dietary saturated fats and dietary cholesterol result in increased serum total cholesterol, as well as LDL-C and thereby increases the risk of heart disease. Subsequently, it has shown that high levels of circulating LDL-C in the blood are susceptible to lipid peroxidation, which increases oxidized LDL-C, promotes scavenging effect by macrophages on lining of coronary arteries to lead atherosclerosis (Steinberg, 2005).

Most of the Apo A-I in the plasma has been associated with HDL-C, although Apo A-I may be dissociated from the major HDL-C sub fraction (alpha-HDL) and up to 13% of Apo A-1 is present in lipid-poor or as pre-beta-HDL fraction. Apo A-1 activates LCAT, and it is required for binding of phospholipids transfer protein of HDL. It has shown translocation of intracellular cholesterol to the plasma and promotes efflux of intracellular cholesterol regulate by Apo A-1 (Oikawa et al, 1993). Furthermore, Apo A-1 initiates signaling pathways in cholesterol efflux (Garver et al, 1997) and regulate the expression of adhesion molecules.
2.3.3.2. Low apolipoprotein A-1 and coronary artery disease

Prospective epidemiological studies have shown that incidence of CAD and several HDL related parameters including serum HDL-C, serum Apo A-1, HDL A-I and HDL A-I: A-II related to CAD risk (Luc et al, 2002). However, Apo A-1 was the strongest predictor for CAD, suggesting that low levels of serum HDL-C associated Apo A-1 may influences to CAD risk (Waldius and Jungner et al, 2004; Francis and Frohlich et al, 2001; Rader, 2006).
3. MATERIALS AND METHODS

3.1. Chemicals and reagents

3.1.1. Water

Distilled water was used in all experiments and deionized water was used when necessary.

3.1.2. Chemicals

Analytical grade chemicals were used for the experiments except for gene analysis, where used molecular biology grade.

3.1.3. Special chemicals/ reagents

Following reagents were used in the study. Paraoxonase-1 ELISA assay kits (USCN life sciences Inc, Wuhan, China), Glutathione Peroxidase-1 ELISA assay kits (Northwest Life Sciences Specialities (NWLSS) LLC, Vancouver, USA), Ransel Glutathione Peroxidase assay kits (Randox Laboratories Limited, United Kingdom), Apolipoprotein A-1 assay kits (Biosystems S.A, Barcelona, Spain) and Molecular biology enzymes and reagents (Promega Corporation, USA).
3.2. Methods

3.2.1. General laboratory practice

A laboratory coat and latex powder-free gloves were worn during all procedures. Hazardous reagents and infectious samples were handled in accordance with clinical laboratory safety procedures utilizing facemasks and fume hoods where necessary. Laboratory glassware were cleaned by using detergent after that rinsed with deionized water and dried in a 37 °C cabinet. Sterile, disposable plastic-ware utilized included 1.5 ml micro-centrifugal tubes (Tarsons products, India).

Volumes 2 - 1000 µl were dispensed using appropriate Plus-Sed micropipettes (Linear chemicals S.L, Spain) for general purposes and Accupipette micropipettes in the genetic laboratory.

3.2.2. Selection of subjects

3.2.2.i Patients

Seventy-five patients (n = 75) were selected from those who were awaiting coronary angiography at the National Hospital, Colombo and Nawaloka Hospitals PLC, Colombo.

3.2.2.ii Controls

Seventy-five (n = 75) apparently healthy persons matched for age (± 1 year) and sex with the patients were recruited from those who attended for their health-screening programme at the Family Practice Centre, University of Sri Jayewardenepura.
3.2.3. Sample size calculation

Sample size was calculated for a matched case control study with a power of 80%; ratio of cases to controls 1:1; exposure in controls 30%; expected odds ratio of 2.6 and an alpha error of 5%.

3.2.4. Criteria of subject selection

3.2.4.1. Inclusion criteria for patients:

The study included all male and female patients, who were awaiting Percutaneous Trans Coronary Angioplasty (PTCA) or Coronary Artery Bypass Grafting (CABG) within the age group of 26 - 72 years with no history of diabetes mellitus, renal diseases, liver dysfunction and cancer.

3.2.4.2 Inclusion criteria for control group:

Healthy volunteer's age and sex matched with the patient group. None of them had clinical or diagnostic evidence of any diseases, including past or present history of heart disease, diabetes mellitus, renal disease, liver dysfunction or cancer.

3.2.5. Study design

An analytical case control study
3.2.6. Study settings

3.2.6.i Recruitment of patients

- Cardiology Unit, National Hospital, Colombo, Sri Lanka.
- Nawaloka Hospitals PLC, Colombo, Sri Lanka.

3.2.6.ii Recruitment of controls

- Family Practice Centre, Faculty of Medical Sciences, University of Sri Jayewardenepura, Gangodawila, Nugegoda, Sri Lanka.

3.2.6.iii Clinical laboratories

- Clinical Biochemistry Laboratory, Department of Biochemistry, Faculty of Medical Sciences, University of Sri Jayewardenepura, Gangodawila, Nugegoda, Sri Lanka.
- Clinical Laboratory, Nawaloka Hospitals PLC, Colombo, Sri Lanka.
- GENETECH, Molecular Diagnostics and School of Gene Technology, Kitulwatta Road, Colombo 08, Sri Lanka.

3.2.7.1 Collection of blood samples

Overnight fasting (10 hours) blood samples were obtained from the patients who were awaiting coronary angiography, whilst the blood samples were drawn from healthy volunteers (controls) with 10 hours fasting in order to provide similar experimental conditions as in patients.
Approximately 5 ml of venous blood was collected under aseptic conditions from the patients and controls. Aliquots of Blood samples were transferred carefully into three vacutainer tubes containing K$_3$ EDTA and Li-Heparin as anticoagulant and into a plain tube without an anticoagulant respectively.

Immediately after collection, Li-Heparinized blood samples were subdivided into aliquots for total GPX assay and the remaining aliquots for preparation of red blood cell hemolysate for GPX-1 assay. Samples were stored for a maximum of one month at -80 °C pending analysis.

EDTA blood samples were used for a genetic study of DNA analysis in Genotyping Glutathione Peroxidase-1 locus by PCR/ RELP. Samples were stored provisionally at a temperature of 4 °C in the refrigerator pending DNA extraction.

Blood samples collected into plain tubes were allowed to clot 20 minutes at room temperature and serum was separated after centrifuge at 2500 rpm for 10 minutes. Serum samples were stored at -80 °C for pending analysis of PON-1 and Apo-A1.

3.2.7.2 Data collection

An interviewer-administered questionnaire was administered to all subjects recruited to the study (Appendix 2).

Summary of collected data and definitions
1. Age (to the nearest completed year)
2. Sex - Male / Female
3. Height in meters
4. Weight in kilograms
5. Smoking

Current smokers were defined as those individuals who currently smoke any tobacco in every day or on some days and included those who had stopped smoking within the past 12 months. Former smokers were defined as those individuals who had quit smoking more than a year earlier, whilst non-smokers were defined as individuals who had not smoked any tobacco at any stage of their life (Centers for Disease Control and Prevention (CDC), 1994).

6. Family history

Heart disease

A family history of heart disease was defined as a premature CAD had been reported in first-degree male relatives younger than 55 years of age or first-degree female relatives younger than 65 years of age.

Diabetes

Kidney disease

Liver dysfunction

Cancer

7. Hypercholesterolemia

(12 hr fasting total cholesterol level ≥ 240 mg/dl or taking lipid-lowering therapy)

8. Hypertension

(Individuals who had Systolic blood pressure ≥ 140 mmHg and diastolic blood pressure ≥ 90 mmHg or taking antihypertensive medication.)
9. History of vascular diseases

Deep vein thrombosis

Peripheral vascular disease

10. Physical activities

Regularly involved in moderate (walking, cycling) or strenuous exercise (jogging, football, vigorous swimming) for 4 hours or more per week considered as physically active.

11. Alcoholism

Regular alcoholism was defined as consumption of any form of alcohol at least three times per week and former users were defined as those who quit use of alcohol more than a year earlier.

12. Dietary habits

Non-Vegetarian

Vegetarian

Type of vegetarian

1. Ovo vegetarianism - Consumption eggs, but not dairy products
2. Lacto vegetarianism - Consumes dairy products but not eggs
3. Ovo-lacto vegetarianism - Consumption animals' products such as eggs, milk and honey.
4. Veganism – Avoid from all animal flesh and animal
products, including milk, honey, eggs and may also omit any products tested on animals or any clothing from animals.

Height, weight and Body Mass Index

Height and weight of the subjects were recorded using standard procedures. An analytical digital balance was used to weigh all subjects with an accuracy of 0.1 kg and a standard measuring tape was used to measure the subject’s standing height. The body mass index (BMI) was calculated by using height and weight measurements, to determine the nutritional status of subjects as follow

\[
\text{Body Mass Index} = \frac{\text{Weight (kg)}}{[\text{Height (m)}]^2}
\]
3.2.8. Quantitative analysis of erythrocyte total Glutathione peroxidase activity

Erythrocyte total Glutathione Peroxidase assay was carried out with Li-heparinized whole blood samples using Randox reagents (U.K). Heparinized whole blood 0.05 ml was mixed with 1 ml of diluting agent in a 1.5 ml eppendorf tube and incubated at room temperature for 5 minutes. Drabkin’s solution 1 ml was added and then mixed well. The addition of cyanide serves to inhibit positive interference of presence peroxidases in human blood, which may give falsely elevated results. Pretreated samples were analyzed within 20 minutes of adding the drabkin’s solution in the Konelab 20XT auto analyzer. The analyzer was programmed to compare the decrease in sample absorbance with a reagent blank prepared automatically inside the analyzer and given the glutathione concentration in Units/litre (U/L) of hemolysate after correcting for the dilution factor.

3.2.8.1 Reaction principle (Randox Ransel assay kit)

Glutathione Peroxidase (GPX) catalysis the oxidation of Glutathione (GSH) by Cumene Hydroperoxide. In the presence of Glutathione Reductase (GR) and NADPH the oxidized Glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP\(^+\). The decrease in absorbance at 340 nm is measured.
3.2.8.2 Calibration

Calibration of GPX was carried out using Ransel GPX controls (Cat no. SC692). The Konelab 20XT auto analyzer generates a calibration curve from the measured calibrators for the calculation of GPX levels in the samples.

3.2.8.3 Quality control

The GPX control sample provided by the manufacturer (Ransel control cat no. SC 692) were used before beginning of each batch of samples to ensure that the assay control values were within ± 1SD of the specified concentration.
3.2.9. Quantitative analysis of erythrocyte Glutathione peroxidase-1

Erythrocyte Glutathione Peroxidase-1 assay was carried out with Li-heparinized red blood cell hemolysate using NWLSS ELISA assay kit. Ice-cold isotonic sodium chloride (0.9 %) was used to wash red cells three times and then the washed packed red cells were used to prepare hemolysate by suspending 4 X pack cell volumes of cold, deionized water. Subsequently 50 μL of diluted sample, 50 μL of working NADPH, 50 μL of working H₂O₂ were added to micro plate well. After one minute, absorbance was measured at 340 nm for 5 minutes with a recording interval of every 30 seconds by using Bio Rad 680 microplate reader. GPX-1 activity was calculated from the net rate.

Calculation of GPX-1 concentration using the NADPH absorption coefficient:

\[
\text{GPX 1 concentration} = \frac{2(\text{mRate}_s - \text{mRate}_b) \times V_{Rxn}}{2.74 \times V_s} \times df
\]

\[m Rates = -1000 \times \Delta A_{340}/\text{min of sample}\]

\[m Rate_b = -1000 \times \Delta A_{340}/\text{min of blank}\]

\[2.74 = \text{NADPH 340 nm millimolar absorption coefficient at 1 cm path length.}\]

\[V_{Rxn} = \text{Volume of Reaction Mixture}\]

\[V_s = \text{Volume of Sample}\]

\[2 = \text{Correction for 2 moles GSH oxidized to 1 mole GSSG per mole NADPH oxidized.}\]

\[df = \text{Sample dilution factor}\]
3.2.9.1 Reaction principle (North West Life Science assay kit)

The NWLSSTM Glutathione Peroxidase Assay is an adaptation of the method of Paglia and Valentine (1967). Glutathione Peroxidase catalyzes the reduction of hydrogen peroxide (H$_2$O$_2$) by oxidizing reduced glutathione (GSH) to form oxidized glutathione (GSSG). GSSG is then reduced by glutathione reductase (GR) and β-nicotinamide adenine dinucleotide phosphate (NADPH) forming NADP$^+$ (resulting in decreased absorbance at 340 nm) and recycling the GSH. Because GPX is limiting, the decrease in absorbance at 340 nm is directly proportional to the GPX concentration.

*GPX activity is reported as units based on the definition:*

One unit of GPX-1 = the amount of enzyme necessary to catalyze the oxidation (by H$_2$O$_2$) of 1.0 mole GSH to GSSG, per minute at 25 °C, at pH 7.0.
3.2.10. Quantitative analysis of serum Paraoxonase-1 concentration

Paraoxonase 1 assay was carried out using an ELISA assay kit form USCN Life Sciences (China). The 100 μL serum sample was added into each well. Incubated at 37 °C for 2 hours; aspirated and added 100 μL of prepared Detection Reagent A. Incubated at 37 °C for 1 hour; aspirated and washed thrice; added 100 μL of prepared Detection Reagent B. Incubated at 37 °C for 30 minutes; aspirated and washed 5 times. The 90μL substrate solution was added into each well. Incubated 15-25 minutes at 37 °C; added 50μL Stop Solution. Then Bio Rad 680 microplate reader was used to measure at 450 nm immediately. A standard curve was used to calculate the PON-1 concentration.

3.2.10.1 Reaction principle (USCN life sciences ELISA assay kit)

The ELISA assay kit was provided with specific pre-coated antibody consists in microtiter plate wells. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated antibody specific to PON-1. Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. After that 3,3’5,5’- Tetramethylbenzidine (TMB) substrate solution is added, only those wells that contain PON-1, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a colour change. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the colour change is measured spectrophotometrically at a wavelength of 450 nm ± 10 nm. The concentration of PON-1 in the samples is then determined by comparing the O.D. of the samples to the standard curve.
3.2.10.2 Calibration

Average of the duplicate readings for each standard was taken and the average zero standard optical density was subtracted. A standard curve was constructed by plotting the mean PON-1 concentration for each standard and best-fit curve was drawn through the points on the graph.

3.2.11. Quantitative analysis of serum Apolipoprotein A-I

Apolipoprotein A-I assay was carried out by turbidimetry method using Biosystems S.A (Spain) assay kit. Thawed Serum samples were mixed thoroughly by low speed vortex. Sample cups were pipetted 200 µl volume of serum sample and concentration was determined on a Konelab 20XT auto analyzer.

3.2.11.1 Reaction principle

Apolipoprotein A-I in the sample will precipitates when presence of anti-human apolipoprotein A-I antibodies. The light scattering of the antigen-antibody complexes are proportional to the apolipoprotein A-I concentration and can be measured by turbidimetry at 340 nm (Marcovina et al, 1991; Price et al, 1983).

3.2.11.2 Calibration

Apo A-I Standard (code: 31100) 274 mg/dl is traceable to the WHO/ IFCC SP1-01 reference standard (Center for Disease Control and Prevention, USA). Standard vial reconstituted with 1.0 ml of distilled water. Dilution series of the standards were
prepared by using of 9 g/L saline as diluent. Dilutions series were as 0, 34.3, 68.5, 137.0, 205.5, 274.0 mg/dl were made to draw the calibration curve using the spline fit method for the calculation of apolipoprotein A-I levels in the samples.

3.2.11.3 Quality control

The manufacturer has recommends to use the Lipid Control Serum level I (Cod. 18040) and Lipid Control Serum level II (Cod. 18041) for verifications of the performance of the procedure. Lipid Control Serum level I apolipoprotein A-I concentration was 163 mg/dl (114-212 mg/dl) and Lipid Control Serum level II apolipoprotein A-I concentration was 74.9 mg/dl (52.5-97.4 mg/dl).

Lyophilized materials quality control vials were reconstituted carefully with 1.00 ml distilled water and allowed to stand 20 minutes at room temperature until resolved. Swirled carefully avoid foam formation and reconstituted contents use as a quality control sample. The quality control serum sample of Apo A-I was run before start study samples and in between every 50 study samples to verify the accuracy and precision of the analysis.

3.2.12. Genotyping to detect Pro198Leu polymorphisms of Glutathione peroxidase-1

3.2.12.1. Polymerase chain reaction / restriction fragment length polymorphism

Genomic DNA was isolated from peripheral blood lymphocytes by using DNA extraction kit, followed by isopropanol extraction and ethanol precipitation method. Next, target DNA sequence amplified by polymerase chain reaction (PCR) and analyzed with restriction fragment length polymorphism (RFLP).
The amplified PCR product was digested with the restriction enzyme 12 U of *ApaI* (Promega Corporation, Madison, USA) overnight at 37 °C and resolved for 2 hours at 50 V on 2% agarose gel stained with ethidium bromide. Restriction fragments were visualized under UV light and photographs made (Elite camera systems). The created patterns of bands were interpreted to identify respective genotypes.

3.2.12.2 DNA extraction

EDTA anticoagulated venous blood samples were used to isolate DNA from leukocytes. DNA extraction was carried out using Wizard® Genomic DNA Purification Kit (Promega Corporation, USA). DNA extraction was performed according to manufacturer protocol. Blood samples were mixed thoroughly by inverting the tube 5-6 times. EDTA whole blood 200 μl volume was added to a sterile 1.5 ml microcentrifuge tube containing with 900 μl of Cell Lysis Solution. Allowed to incubate for 10 minutes at room temperature until complete lysis of red blood cells. The mixture was centrifuged at 12000 X g for 30 seconds at room temperature. Removed and discarded as much supernatant as possible without disturbing the visible white pellet. The tube was vigorously vortexes 10-15 seconds until the white blood cells resuspended. Nuclei Lysis Solution (300 μl volume) was added to the tube containing the resuspended cells. It was pipetted 5-6 times back and forth to break the leukocytes without forming air bubbles in the solution until the solution become viscous. The mixture was incubated at 65 °C for 15 minutes in a dry bath. RNAse Solution (1.5 μl volume) was added into the tube, and the sample mixed by inverting the tube 2-5 times. The mixture was incubated at 37 °C for 15 minutes and then allowed to cool at room temperature. Then 100 μl volume of Protein Precipitation Solution was mixed with nuclear lysate and vortex
thoroughly for 10-20 seconds. Small protein clumps were visible after vortex. The mixture was centrifuged at 13,000 X g for 3 minutes at room temperature. A dark brown protein pellet was visible. The supernatant was transferred to a new sterile 1.5 ml microcentrifuge tube containing 300 µl isopropanol at room temperature. The tube was gently mixed by inversion until the white threadlike strands of DNA formed a visible mass. The tube was centrifuged at 12,000 X g for 1 minute at room temperature. The DNA was visible as a small white pellet. Supernatant was decanted and 300µl volume of 70% ethanol was added to the DNA (at room temperature). The tube was gently inverted several times to wash the DNA pellet and the sides of the microcentrifuge tube. The tube was centrifuged at 12,000 X g for 1 minute at room temperature. The DNA was visible as a small white pellet. Carefully aspirated the ethanol using a micropipette. Inverted the tube on clean absorbent paper and air-dried the pellet for 10-15 minutes. DNA Rehydration Solution (75 µl volume) was added to the tube and rehydrated the DNA by incubating at 65 °C for 1 hour. The solution was periodically mixed by gently tapping the tube. DNA was stored at +2 °C to +8 °C until analysis.

3.2.12.3. Polymerase Chain Reaction (PCR)

3.2.12.3.i  Principle of the PCR

The polymerase chain reaction (PCR) is a DNA amplification method involves with specific primers and enzyme. Taq DNA polymerase is the enzyme that catalyzes the DNA amplification reaction. In this reaction, millions of target sequence multiplies by using oligonucleotide primer pair and deoxynucleoside triphosphates (dNTPs).
Genomic DNA was isolated from leukocytes using the Wizard® Genomic DNA Purification Kit (Promega Corporation, USA). All the primers used in this study were purchased from Integrated DNA Technologies, USA. Target fragments were amplified using polymerase chain reaction (PCR).

Two primers, forward 5'-AGCCCAACTTCATGCTCTTC-3' and reverse 5'-CAGGTGTTCCTCCCTCGTAG- 3' (Integrated DNA Technologies, USA) were used to amplify the 400-base pair (bp) fragment containing the C/T polymorphic site. Lyophilized primer containing tubes were briefly centrifuged prior to opening as some of the product may have been dislodged during shipping. Primers were rehydrated using DNA rehydration solution (pH 8.0) to prepare 100 μM solutions. Volume of 415 μl and 323 μl rehydration solution was added into forward (41.5 nmol) and reverse (32.3 nmol) primers respectively. Then 5 μM volume of working primer solution was prepared by 25 μl solution of each primer adding in to a 1.5 ml microcentrifuged containing 450 μl of sterile H₂O to make a total volume of 500 μl.

The PCR reaction was performed in a 25 μL of reaction mixture containing; 2.5 μl of 10 X PCR buffer (500 mM KCl, 100 mM Tris-HCl, 1.0% Triton X-100; Promega), 2.5 mM MgCl₂, 2.5 μl 3.5 mM dNTPs, 2.5 μl 5 μM of each primer, 0.4 μl of 2 U of Taq polymerase in storage buffer B [20 mM Tris-HCl, 100 mM KCl, 0.1 mM ethylene diamine tetra acetic acid, 1 mM dithiothreitol (DTT), 50% glycerol, 0.5% Nonidet-P40, and 0.5% Tween 20; Promega] and 3 μl of isolated DNA. Finally, 14.1 μl of sterile H₂O was added to make 25 μL of total volume of PCR mixture.
PCR was carried out in Applied Biosystem verity® thermo cycler (USA). The PCR cycling conditions of the assay were 94°C for 5 minutes, followed by 35 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 45 seconds, with a final extension step at 72°C for 7 minutes. Amplicons of size 400 bp was verified by running 5 μl volume of PCR product on a 2% agarose gel.

3.2.12.4. Restriction fragment length polymorphism (RFLP)

A mixture was prepared for restriction enzyme digestion containing; 2 μl of 10 X buffer, 0.2 μl of bovine serum albumin (BSA), 0.5 μl of *ApaI* enzyme (Promega Corporation, Madison, USA), 6 μl of amplified PCR product, and added 11.3 μl of sterile H2O for made up final volume 20 μl. Mixture was incubated for overnight at 37°C. Digested DNA was resolved in 2% agarose gel stained with ethidium bromide at 50 V for 2 hours. Number of DNA fragments as well as the size of these fragments allowed to genotype determination. Band patterns of DNA fragments were visualized under ultra violet light and photograph taken. The Pro198Pro (CC) genotype yielded 195 bp, 117 bp and 88 bp fragments; the Pro198Leu (CT) genotype yielded 205 bp, 195 bp, 117 bp and 88 bp fragments. Leu198Leu (TT) genotype was not found in any subjects in the study. The presence of Leu198Leu (TT) would have given 205 bp and 195 bp fragments.
3.2.12.5. Agarose gel electrophoresis

Agarose gel (2%) was prepared as follows: A gel mould was prepared by sealing the edges of clean, dry Perspex plate with tape to make a tray. A comb was positioned at 0.5 - 1 mm above the plate, so that a well will be formed when added the agarose. Meanwhile, the plate kept in a horizontal position on the bench. Agarose gels were weighted in 1 g of agarose (Sigma-Aldrich Corporation U.S.A.) into a 200 ml conical flask and adding 1X TBE buffer (containing 40 mM Tris-borate and 1 mM EDTA) to make 50 ml. The flask was loosely capped and the slurry heated in a microwave until the agarose dissolved. The solution was allowed to cool until 60 °C temperature. The solution was poured into the mold and the mold inspected to make sure that air bubbles were not formed under or between the teeth of the comb. The gel was left to set for 30 - 45 minutes at room temperature. After the gel was completely set, the comb and the tape were carefully removed and the gel transferred to the electrophoresis tank. After that, 1X TBE electrophoresis buffer was added to cover the gel to a depth of about 1 mm and 5 μl volume of ethidium bromide was added to the buffer.

3.2.12.6. Quality control

In PCR, DNA free controls were used to detect whether they formed non-specific amplified products. DNA free sample were used in Apal restriction enzyme digestion to detect the non-specific reactions. The procedure was repeated if any of the above incidents were found. Two independent observers interpreted gel images and if it was difficult to make a clear decision in any case, those samples were regenotyped.
3.2.13. Assessment of severity of coronary artery disease

3.2.13.1. Coronary angiography

Coronary angiography provides clear images of the coronary arteries for diagnose disease stage accurately. Iodinated contrast agent is used to visualize coronary arteries by radiographic fluoroscopic examination.

3.2.13.2. Severity of coronary artery disease

Two experienced cardiologists, who was blinded to the patients’ data including clinical and laboratory findings, independently reviewed coronary angiograms. Angiogram findings were then evaluated using three different score systems as vessel score, stenosis score and extent score. The angiograms were scored according to a method described by Sullivan et al in 1990. Identified coronary arteries were Main Left Coronary Artery, Left Anterior Descending Artery, Main Diagonal Branch, First Septal Perforator, Left Circumflex Artery, Obtuse Marginal and Posterolateral Vessels, Right Coronary Artery, Main Posterior Descending Branch.

3.2.13.2.i Calculation of vessel score

Left main coronary artery, left anterior descending artery, left circumflex artery and right coronary artery were considered in scoring the vessels score. If $\geq 70\%$ stenosis in lumen gave score 1, while left main coronary artery with $\geq 70\%$ stenosis it was considered as single vessel disease (score 1). Therefore, vessel score ranged from 0 to 3 depending on the number of vessels involved.
3.2.13.2.ii Calculation of stenosis score

Stenosis score was calculated by a modified Gensini score as described by Reardon et al in 1985 and Hamsten et al in 1986, which places emphasis on the severity of stenosis while including some measure of the extent of coronary artery disease. The maximum diameter reduction of eight coronary segments (left main coronary artery, left anterior descending artery, main diagonal branch, first septal perforator, left circumflex artery, obtuse marginal and postero-lateral vessels, right coronary artery, main posterior descending branch) were scored according to the luminal narrowing, that is; a score 1 for 1 - 49% reduction in luminal diameter, 2 for 50 - 74%, 3 for 75-99% and 4 for total occlusion. The scores for each of the eight segments (mentioned above) were provided a sum of theoretical maximum 32.

3.2.13.2.iii Calculation of extent score

This score was developed by Sullivan et al in 1990. According to the proportional length of each vessel segment in the coronary artery tree, segments are graded with different numbers of maximum points: 5 points for the left main stem, 20 for the left anterior descending artery, 10 for the main diagonal branch, 5 for the first septal branch, 20 for the left circumflex artery, 10 for the marginal and posterolateral vessels, 20 for the right coronary artery and 10 for the main posterior descending branch. If the major vessel supplying the lateral ventricular wall was a large obtuse marginal or intermediate vessel, a factor of 20 was applied to the marginal and postero-lateral vessels and 10 to the left circumflex artery. The scores for each vessel or branch were
added to give a total score up to a maximum of 100, representing the estimated percent of atheromatous involvement of the intimal coronary surface.

3.2.14. Data processing and statistical analysis

Data were entered into Microsoft Excel and analyzed using SPSS 16.0 for Windows (Chicago, Illinois). Continuous variables were analyzed using independent sample t test, Analysis of variance (ANOVA), Spearman’s correlation. Categorical variables were presented as absolute frequencies and percentages, whilst analyzed using chi square, Fisher’s exact test, where appropriate. Multivariate logistic regression was performed and Odds Ratio was calculated to assess risk of CAD in population. A p value < 0.05 was considered statistically significant.

3.2.15. Ethical considerations

Ethical approval was obtained from the Ethics Review Committee of the Faculty of Medical Sciences, University of Sri Jayewardenepura (Appendix 3). The study objectives were explained to all participants verbally in Sinhala or English and an information sheet given (Appendix 4 - 7). Informed written consent was obtained afterwards from all participants by way of an approved consent form (Appendix 8 - 11). Data was secured in a password protected computer, only accessible to the investigators of the study.
4. RESULTS

4.1. Association between coronary artery disease and serum paraoxonase levels

4.1.1. Characteristics of subjects

Table 4.1 and 4.2 show the demographics and anthropometric characteristics and
distribution of classical risk factors of the study population. A total of 150 subjects
were recruited for the study, which comprised of 75 volunteers diagnosed with CAD
and 75 age and sex matched healthy subjects. Each group consisted of 17 females and
58 males (Table 4.2).

Age ranged from 26 - 72 years for both groups and the mean age of patients and
controls were 55 ± 9 years and 54 ± 9 years respectively (p = 0.620). In addition,
patients and controls had a mean BMI of 25.2 ± 4.7 kg/m² and 24.7 ± 4.6 kg/m²
respectively, and there was no significant difference in BMI between the patients and
controls (p = 0.441) (Table 4.1).

In distribution of classical risk factors, family history of heart disease showed a
statistically significant association with CAD when compared to controls [Odds ratio
5.15 (95% CI 2.0-12.8), p = 0.000]. However, smoking [Odds ratio 1.62 (0.85-3.09)],
alcoholism [Odds ratio 1.81 (0.94-3.48)] and veganism [Odds ratio 0.845 (0.27-2.64)]
did not show a significant association with CAD (Table 4.2).

Subjects with age ≤ 50 years group showed a higher mean BMI level when compared to
the control group (27.5 ± 5.7 kg/m², 24.5 ± 5.2 kg/m² respectively; p = 0.073). In
contrast, there were approximately similar mean BMI values observed in subjects with
age > 50 years group of patients and controls (24.4 ± 4.1 kg/m², 24.7 ± 4.3 kg/m²
respectively; p= 0.711) (Table 4.3).
Table 4.1. Demographics and Anthropometric characteristics of patients and controls.

<table>
<thead>
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<th>Variable</th>
<th>Patients</th>
<th>Control</th>
<th>p value&lt;sup&gt;#&lt;/sup&gt;</th>
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<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
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<tr>
<td>Age</td>
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<td>24.7 ± 4.6</td>
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<sup>#</sup>Independent sample t test comparing patients and controls
Table 4.2. Demographic features and distribution of classical risk factors of patients and controls

<table>
<thead>
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</tr>
<tr>
<td>Yes</td>
<td>7</td>
<td>9.3</td>
<td>6</td>
<td>8.0</td>
</tr>
<tr>
<td>No</td>
<td>68</td>
<td>90.7</td>
<td>69</td>
<td>92.0</td>
</tr>
</tbody>
</table>

# Pearson chi square test comparing patients and controls.

*Significant at p ≤ 0.05
Table 4.3. Distribution of body mass index among two age groups of the study population

<table>
<thead>
<tr>
<th>Age</th>
<th>n</th>
<th>Mean</th>
<th>± SD</th>
<th>p value#</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 50 years</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>25</td>
<td>24.5</td>
<td>5.2</td>
<td>0.073</td>
</tr>
<tr>
<td>Patients</td>
<td>20</td>
<td>27.5</td>
<td>5.7</td>
<td></td>
</tr>
<tr>
<td>&gt; 50 years</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>50</td>
<td>24.7</td>
<td>4.3</td>
<td>0.711</td>
</tr>
<tr>
<td>Patients</td>
<td>55</td>
<td>24.4</td>
<td>4.1</td>
<td></td>
</tr>
</tbody>
</table>

#Independent sample t test comparing patients and controls
4.1.2. Association between serum paraoxonase-1 level and coronary artery disease

Analysis of data revealed that serum Paraoxonase-1 concentration in patients and controls were ranged from 7.9 - 114.0 μg/ml and 14.9 - 395.2 μg/ml respectively. The mean ± SD values of PON-1 concentration were 46.2 ± 23.9μg/ml and 111.7 ± 64.0 μg/ml for the case and control groups respectively (p = 0.000). There was a significant moderate strength inverse relationship observed between age and Paraoxonase-1 concentration in patients. However, no significant relationship was found in the control group (Table 4.4, Table 4.5) (Fig. 4.1, Fig. 4.2).

PON-1 concentration was significantly lower subjects with age > 50 years group (42.6 ± 21.1 μg/ml) when compared to subjects with age ≤ 50 years group in patients (56.1 ± 28.7 μg/ml; p = 0.030). Although control group did not show a significant difference between subjects with age ≤ 50 years group and subjects with age > 50 years group (116.2 ± 65.1 μg/ml, 109.5 ± 63.9 μg/ml respectively; p = 0.675) (Table 4.6).
Table 4.4. Paraoxonase 1 concentrations between patients and controls

<table>
<thead>
<tr>
<th></th>
<th>Patients (µg/ml)</th>
<th>Control (µg/ml)</th>
<th>p value#</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>46.2</td>
<td>111.7</td>
<td>0.000*</td>
</tr>
<tr>
<td>± SD</td>
<td>23.9</td>
<td>64.0</td>
<td></td>
</tr>
<tr>
<td>Minimum</td>
<td>7.9</td>
<td>14.9</td>
<td></td>
</tr>
<tr>
<td>Maximum</td>
<td>114.0</td>
<td>395.2</td>
<td></td>
</tr>
</tbody>
</table>

\# Independent sample t test comparing patients and controls

* Significant at p \leq 0.05

Table 4.5. Correlation between paraoxonase-1 concentration and age of the study population

<table>
<thead>
<tr>
<th></th>
<th>Correlation coefficient#</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>-0.251</td>
<td>0.030*</td>
</tr>
<tr>
<td>Control</td>
<td>-0.158</td>
<td>0.175</td>
</tr>
</tbody>
</table>

\# Spearman's correlation

* Significant at p \leq 0.05
Figure 4.1. Distribution of paraoxonase 1 concentration with age in patients with CAD.

Figure 4.2. Distribution of paraoxonase 1 concentration with age in controls.
## Table 4.6. PON-1 concentrations among two age groups of study population

<table>
<thead>
<tr>
<th>Age Group</th>
<th>n</th>
<th>Mean (µg/ml)</th>
<th>± SD</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patients</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age ≤ 50 years group</td>
<td>20</td>
<td>56.1</td>
<td>28.7</td>
<td>0.030</td>
</tr>
<tr>
<td>Age &gt; 50 years group</td>
<td>55</td>
<td>42.6</td>
<td>21.1</td>
<td></td>
</tr>
<tr>
<td><strong>Controls</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age ≤ 50 years group</td>
<td>25</td>
<td>116.2</td>
<td>65.1</td>
<td>0.675</td>
</tr>
<tr>
<td>Age &gt; 50 years group</td>
<td>50</td>
<td>109.5</td>
<td>63.9</td>
<td></td>
</tr>
</tbody>
</table>

*Independent sample t test comparing patients and controls

*Significant at p ≤ 0.05
4.2. Associations between coronary artery disease and erythrocyte Glutathione peroxidase activity.

Results revealed that the total glutathione peroxidase activity was significantly (p ≤ 0.05) low in patients when compared to controls with a range of 119.1 U/L to 484.8 U/L for patients and 225.5 U/L to 480.3 U/L for controls. The mean ± SD value of GPX for patients and controls were 290.6 ± 84.8 U/L and 380.5 ± 50.5 U/L respectively (p = 0.000). Interestingly, the activity of glutathione peroxidase-1 was significantly low (121.6 ± 32.4 U/L) in patients when compared to controls (179.1 ± 46.0 U/L; p = 0.000) (Table 4.7).

Total GPX and GPX-1 isozyme showed a strong positive relationship for patients (r = 0.736; p = 0.000) as well as controls (r = 0.243; p = 0.000) (Table 4.8; Figure 4.3 and Figure 4.4).

However, there was no statistically significant relationship for GPX and GPX-1 with age (Table 4.9; Figure 4.5 and Figure 4.6).
Table 4.7. Glutathione peroxidase activities between patients and controls

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>±SD</th>
<th>Minimum</th>
<th>Maximum</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Glutathione peroxidase activity (U/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients</td>
<td>290.6</td>
<td>84.8</td>
<td>119.1</td>
<td>484.8</td>
<td>0.000*</td>
</tr>
<tr>
<td>Controls</td>
<td>380.5</td>
<td>50.5</td>
<td>225.5</td>
<td>480.3</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Glutathione peroxidase-1 activity (U/L)</strong></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>121.6</td>
<td>32.4</td>
<td>39.4</td>
<td>192.0</td>
<td>0.000*</td>
</tr>
<tr>
<td>Controls</td>
<td>179.1</td>
<td>46.0</td>
<td>92.0</td>
<td>302.2</td>
<td></td>
</tr>
</tbody>
</table>

* Independent sample t test comparing patients and controls

* Significant at p ≤ 0.05
Table 4.8. Relationships of total GPX Vs GPX-1 in patients and controls

<table>
<thead>
<tr>
<th></th>
<th>Correlation coefficient</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>0.736</td>
<td>0.000*</td>
</tr>
<tr>
<td>Controls</td>
<td>0.243</td>
<td>0.000*</td>
</tr>
</tbody>
</table>

*Pearson correlation

*significant at p ≤ 0.05
Figure 4.3. Relationship between total GPX and GPX-1 activity in patients

Figure 4.4. Relationship between total GPX and GPX-1 activity in controls
Table 4.9. Correlation between glutathione peroxidase activity and age of the study population

<table>
<thead>
<tr>
<th></th>
<th>Correlation coefficient</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Glutathione peroxidase activity (U/L)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients</td>
<td>-0.113</td>
<td>0.334</td>
</tr>
<tr>
<td>Control</td>
<td>0.081</td>
<td>0.488</td>
</tr>
<tr>
<td><strong>Glutathione peroxidase 1 activity (U/L)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients</td>
<td>-0.113</td>
<td>0.336</td>
</tr>
<tr>
<td>Control</td>
<td>0.014</td>
<td>0.908</td>
</tr>
</tbody>
</table>

*Spearman's correlation*
Figure 4.5. Distribution of total glutathione peroxidase activity with age in patients and controls.

Figure 4.6. Distribution of glutathione peroxidase-1 activity with age in patients and controls.
4.3. The association between coronary artery disease and Apolipoprotein A-I.

There was no significant difference in apolipoprotein A-I levels between patients and controls. The mean ± SD of apolipoprotein A-I levels were 110.7 ± 19.8 mg/dl and 114.5 ± 20.5 mg/dl for patients and controls respectively (p = 0.229). In addition, there was no significant relationship observed with age (Table 4.10, Table 4.11).

Ratio of Apo A-I to PON-1 showed a significant increase in patients (3.19 ± 2.3) when compared to controls (1.31 ± 0.9; p = 0.000).
Table 4.10. Apolipoprotein A-I concentrations in patients and controls

<table>
<thead>
<tr>
<th></th>
<th>Patients (mg/dl)</th>
<th>Control (mg/dl)</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>110.7</td>
<td>114.5</td>
<td>0.229</td>
</tr>
<tr>
<td>± SD</td>
<td>19.8</td>
<td>20.5</td>
<td></td>
</tr>
<tr>
<td>Minimum</td>
<td>71.0</td>
<td>79.0</td>
<td></td>
</tr>
<tr>
<td>Maximum</td>
<td>181.0</td>
<td>185.0</td>
<td></td>
</tr>
</tbody>
</table>

*Independent sample t test comparing patients and controls.

Table 4.11. Correlation between apolipoprotein A-I concentrations and age in the study population

<table>
<thead>
<tr>
<th></th>
<th>Correlation coefficient</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>0.092</td>
<td>0.430</td>
</tr>
<tr>
<td>Control</td>
<td>0.069</td>
<td>0.556</td>
</tr>
</tbody>
</table>

*Spearman's correlation
Table 4.12. Ratio of apolipoprotein A-1 to Paraoxonase 1 concentration in patients and controls

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>± SD</th>
<th>p value&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>1.31</td>
<td>0.9</td>
<td>0.000*</td>
</tr>
<tr>
<td>Patients</td>
<td>3.19</td>
<td>2.3</td>
<td></td>
</tr>
</tbody>
</table>

"Independent sample t test comparing patients and controls

*Significant at p ≤ 0.05
4.4. Severity of coronary artery disease in the patients.

Severity of coronary artery disease was determined by three scoring systems based on angiogram findings, using a scoring system involving vessels score, stenosis score and extent score.

A consultant cardiologist evaluated coronary angiograms. Severity of CAD was assessed by the extent of stenosis in coronary arteries based on the method described by Sullivan et al 1990.

Left main coronary artery, left anterior descending artery, left circumflex artery and right coronary artery were considered in scoring the vessels score and if \( \geq 70\% \) stenosis in lumen given score 1 while left main coronary artery with \( \geq 70\% \) stenosis was considered as single vessel disease (score 1). Therefore, vessel score ranged from 0 to 3. Frequency distribution of vessel score was higher for single vessel disease (48%) when compared to double and triple vessel disease in patients with CAD (Table 4.13).

In result interpretation, stenosis score was stratified into 3 groups as 1-7 mild disease, 8-15 moderate disease, and more than 16 severe disease. Frequency distribution was higher for stenosis score \(< 7\) mild disease group (63%) when compared to the other two groups (Table 4.13).

Further, extent score was stratified into three groups as less than 25 % mild disease, 26 - 49 % moderate disease, and more than 50% severe disease. Results showed that frequency distribution of extent score was higher in \( \geq 50\% \) severe disease score group (55%) in the study population when compared to the other two groups (Table 4.13).
A mean vessel score 1.72 from a possible maximum of 3 was observed in patients with CAD and the mean stenosis score was 8.43 from a theoretical maximum of 32, whilst the mean extent score was 52.13% from the total of 100% in the study group. The results revealed that the severity of CAD is high in the study population (Table 4.14).

Table 4.13. Frequency distributions of three severity-score systems in patients with CAD.

<table>
<thead>
<tr>
<th></th>
<th>Patients (n)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vessel score</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single vessel disease</td>
<td>36</td>
<td>48</td>
</tr>
<tr>
<td>Double vessel disease</td>
<td>24</td>
<td>32</td>
</tr>
<tr>
<td>Triple vessel disease</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td><strong>Stenosis score</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1-7) Mild disease</td>
<td>45</td>
<td>63</td>
</tr>
<tr>
<td>(8-15) Moderate disease</td>
<td>21</td>
<td>25</td>
</tr>
<tr>
<td>(16≤) Severe disease</td>
<td>9</td>
<td>12</td>
</tr>
<tr>
<td><strong>Extent score</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(≤ 25%) Mild disease</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>(26-49%) Moderate disease</td>
<td>29</td>
<td>39</td>
</tr>
<tr>
<td>(≥ 50%) Severe disease</td>
<td>41</td>
<td>55</td>
</tr>
</tbody>
</table>
Table 4.14. Severity of CAD in patient group.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vessel score</td>
<td>1.72</td>
<td>0.78</td>
</tr>
<tr>
<td>Stenosis score</td>
<td>8.43</td>
<td>4.76</td>
</tr>
<tr>
<td>Extent score</td>
<td>52.13</td>
<td>18.56</td>
</tr>
</tbody>
</table>
4.4.1. Summary of associations between severity of coronary artery disease and paraoxonase-1, glutathione peroxidase and apolipoprotein A-I.

Paraoxonase-1 concentration did not show a significant correlation for vessel, stenosis, and extent scores. However, there was a trend where a decreased of PON-1 activity was observed when the severity of CAD increases.

Total glutathione peroxidase activity showed significant strong inverse relationship for vessel, stenosis and extent scores. Similarly, glutathione peroxidase-1 activity also showed a significant inverse relationship for vessel, stenosis, and extent scores. Although, apolipoprotein A-1 did not show a significant correlation for vessel, stenosis, and extent scores it showed an inverse relationship with the severity of CAD (Table 4.15).
Table 4.15. Association of Paraoxonase 1, Glutathione peroxidase and apolipoprotein A-1 concentrations with severity of CAD.

<table>
<thead>
<tr>
<th></th>
<th>Vessel score</th>
<th></th>
<th></th>
<th>Stenosis score</th>
<th></th>
<th></th>
<th>Extent score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Correlation</td>
<td>p</td>
<td>Correlation</td>
<td>p</td>
<td>Correlation</td>
<td>p</td>
<td>Correlation</td>
</tr>
<tr>
<td></td>
<td>coefficient</td>
<td>value</td>
<td>coefficient</td>
<td>value</td>
<td>coefficient</td>
<td>value</td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td>0.076</td>
<td>0.514</td>
<td>-0.027</td>
<td>0.817</td>
<td>-0.037</td>
<td>0.754</td>
<td></td>
</tr>
<tr>
<td>Paraoxonase-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Glutathione</td>
<td>-0.799</td>
<td>0.000*</td>
<td>-0.559</td>
<td>0.000*</td>
<td>-0.508</td>
<td>0.000*</td>
<td></td>
</tr>
<tr>
<td>Glutathione peroxidase-1</td>
<td>-0.773</td>
<td>0.000*</td>
<td>-0.482</td>
<td>0.000*</td>
<td>-0.435</td>
<td>0.000*</td>
<td></td>
</tr>
<tr>
<td>Apolipoprotein A-1</td>
<td>-0.201</td>
<td>0.084</td>
<td>-0.210</td>
<td>0.071</td>
<td>-0.196</td>
<td>0.093</td>
<td></td>
</tr>
</tbody>
</table>

*Spearman’s correlation

*Significant at p ≤ 0.05
Mean distribution of paraoxonase-1, glutathione peroxidase and apolipoprotein A-1 levels in subgroups of three scoring systems of severity of CAD was determined by using analysis of variance (ANOVA) statistical test.

Result showed that paraoxonase-1 concentration did not significantly differ in subgroups of vessel, stenosis and extent score groups. Interestingly, total glutathione peroxidase activity showed significant differences in subgroups of vessel, stenosis and extent score groups. In addition, there was a trend towards reduced GPX activity when the severity of CAD increases. Similar to total GPX, glutathione peroxidase-1 activity also showed significant differences in subgroups of vessel, stenosis and extent score groups and there was a trend towards reduced GPX-1 activity when the severity of CAD increases. However, apolipoprotein A-1 levels did not show significant differences in subgroups of vessel, stenosis and extent score groups (Table 4.16.1; Table 4.16.2; Table 4.16.3).
Table 4.16.1. Differences of PON-1, GPX, GPX1, Apo A-i levels in subgroups of vessel score with severity of CAD.

<table>
<thead>
<tr>
<th>Vessel score subgroups</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SVD</td>
</tr>
<tr>
<td>Paraoxonase 1 (μg/ml)</td>
<td>41.0 ± 18.2</td>
</tr>
<tr>
<td>Total Glutathione peroxidase (U/l)</td>
<td>357.2 ± 66.9</td>
</tr>
<tr>
<td>Glutathione peroxidase 1 (U/l)</td>
<td>145.4 ± 28.1</td>
</tr>
<tr>
<td>Apolipoprotein A-1 (mg/dl)</td>
<td>114.1 ± 21.6</td>
</tr>
</tbody>
</table>

SVD-Single Vessel Disease, DVD-Double Vessel Disease, TVD-Triple Vessel Disease

*One way Analysis of variance (ANOVA) test

*aSignificant at p≤0.05 level
Table 4.16.2. Differences of PON-1, GPX, GPX1, Apo A-1 levels in subgroups of stenosis score with severity of CAD.

<table>
<thead>
<tr>
<th>Stenosis score subgroups</th>
<th>p value#</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1-7) Mild disease</td>
<td>(8-15) Moderate disease</td>
</tr>
<tr>
<td>Paraoxonase 1 (μg/ml)</td>
<td>43.7 ± 19.2</td>
</tr>
<tr>
<td>Total Glutathione</td>
<td>322.6 ± 76.4</td>
</tr>
<tr>
<td>Peroxidase (U/l)</td>
<td>130.9 ± 31.1</td>
</tr>
<tr>
<td>Glutathione Peroxidase 1 (U/l)</td>
<td>113.4 ± 21.6</td>
</tr>
</tbody>
</table>

#One way Analysis of variance (ANOVA) test

*Significant at p≤0.05 level
Table 4.16.3. Differences of PON-1, GPX, GPX1 and Apo A-1 levels in subgroups of extent score with severity of CAD.

<table>
<thead>
<tr>
<th>Extent score subgroups</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(≤25%)</td>
<td>(26-49%)</td>
</tr>
<tr>
<td>Mild disease</td>
<td>Moderate</td>
</tr>
<tr>
<td>Paraoxonase 1 (µg/ml)</td>
<td>41.9 ± 21.9</td>
</tr>
<tr>
<td>Total Glutathione peroxidase (U/l)</td>
<td>375.5 ± 54.1</td>
</tr>
<tr>
<td>Glutathione peroxidase 1 (U/l)</td>
<td>147.7 ± 29.1</td>
</tr>
<tr>
<td>Apolipoprotein A-1 (mg/dl)</td>
<td>127.2 ± 37.5</td>
</tr>
</tbody>
</table>

*One way Analysis of variance (ANOVA) test

*Significant at p≤0.05 level
Patients were recommended to Percutaneous Trans Coronary Angioplasty (PTCA) or Coronary Artery Bypass Grafting (CABG) according their severity of angiogram findings by a consultant cardiologist.

Total glutathione peroxidase activity was significantly low in the CABG group (242.3 ± 77.2 U/L) when compared to PTCA group (309.3 ± 80.7 U/L) (p = 0.002) (Table 4.17).

Glutathione peroxidase-1 activity was also significantly low in the CABG (104.9 ± 30.2 U/L) group when compared to PTCA group (128.0 ± 31.1 U/L) (p = 0.005) (Table 4.17). However, both paraoxonase-1 and apolipoprotein A-1 levels did not show significant difference between CABG and PTCA groups (Table 4.17).
Table 4.17. Paraoxonase-1, Glutathione peroxidases, and Apolipoprotein A-1 levels between patients awaiting PTCA (n = 54) and CABG (n = 21).

<table>
<thead>
<tr>
<th></th>
<th>Mean ±SD</th>
<th>Range</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Paraoxonase 1 (µg/ml)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTCA</td>
<td>46.3 21.7</td>
<td>(14.9 - 105.2)</td>
<td>0.978</td>
</tr>
<tr>
<td>CABG</td>
<td>46.1 29.4</td>
<td>(7.9 - 114.0)</td>
<td></td>
</tr>
<tr>
<td><strong>Total Glutathione peroxidase activity (U/L)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTCA</td>
<td>309.3 80.7</td>
<td>(187.4 - 484.8)</td>
<td>0.002**</td>
</tr>
<tr>
<td>CABG</td>
<td>242.3 77.2</td>
<td>(119.1 - 454.3)</td>
<td></td>
</tr>
<tr>
<td><strong>Glutathione peroxidase 1 activity (U/L)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTCA</td>
<td>128.0 31.1</td>
<td>(78.8 - 192.0)</td>
<td>0.005**</td>
</tr>
<tr>
<td>CABG</td>
<td>104.9 30.2</td>
<td>(39.4 - 178.8)</td>
<td></td>
</tr>
<tr>
<td><strong>Apolipoprotein A-1 (mg/dl)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTCA</td>
<td>110.6 20.9</td>
<td>(71 - 181)</td>
<td>0.951</td>
</tr>
<tr>
<td>CABG</td>
<td>111.0 17.1</td>
<td>(73 - 143)</td>
<td></td>
</tr>
</tbody>
</table>

*Independent sample t test comparing patients and controls

**Significant at p ≤ 0.05
4.5. Association of glutathione peroxidase-1 genotype (Pro198Leu polymorphism) and coronary artery disease

The study involved determination of genotype distribution of the GPX-1 Pro198Leu polymorphism in the study population. Plate 4.2 shows the gel electrophoresis images of the PCR products after digestion by the respective restriction enzymes. Summary of genotyping results has shown in Table 4.18. In overall results for the Pro198Leu polymorphism, the CC genotype was the most frequent followed by CT. However, TT genotype was not isolated from the whole study population.

Frequency distribution of Pro198Pro (CC) genotype was significantly higher in controls (89.3%) when compared to patients (74.7%). However, Pro198Leu (CT) was significantly low in controls (10.7%) when compared with patients (25.3%) (χ² test = 1.019). Interestingly, Pro198Leu (CT) genotype showed a 2.84 fold risk for CAD [OR 2.84 (95% CI 1.15 - 6.98), p = 0.019] (Table 4.18).

When the allelic frequency in CAD patients and controls were analyzed the common Pro198 (C) encoding variant was higher in controls (94.6 %) when compared to patients (87.3 %) and, on the other hand, Leu198 (T), encoding variant was 12.7 % and 5.4 % respectively in patients and controls (Tables 4.19).

Age was stratified into two groups as age ≤ 50 years and age > 50 years. GPX-1 Pro198Leu (CT) genotype distribution was higher in subjects with age ≤ 50 years group patients (35 %) when compared to respective control groups (8 %) (Table 4.20).
Plate 4.1. Amplified DNA by PCR

Extracted DNA amplified by PCR and resolved in 2% agarose gel stained with ethidium bromide at 50 V for 2 hours. Gel electrophoresis given 400 bp bands as target sequence.
Plate 4.2. Agarose gel electrophoresis image of restriction fragment length polymorphism.

Amplified DNA by PCR was digested with the restriction enzyme 12 U of *ApaI* overnight at 37 °C and resolved in 2% agarose gel stained with ethidium bromide at 50 V for 2 hours. Resolved band patterns were photographed under ultra violet light by the Elite camera systems.
Table 4.18. Frequency distribution of Glutathione peroxidase-1 variants in study subjects

<table>
<thead>
<tr>
<th></th>
<th>Patients</th>
<th>Controls</th>
<th>Odds ratio</th>
<th>(95% CI)</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro198Pro</td>
<td>56</td>
<td>67</td>
<td>2.84</td>
<td>1.15 – 6.98</td>
<td>0.019*</td>
</tr>
<tr>
<td>(CC)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro198Leu</td>
<td>19</td>
<td>8</td>
<td>10.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(CT)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu198Leu</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(TT)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Pearson’s chi square test

* Significant at p ≤ 0.05
Table 4.19a. Allelic frequency of GPX1 Pro198Leu polymorphism in patients and controls

<table>
<thead>
<tr>
<th></th>
<th>Patients</th>
<th></th>
<th>Controls</th>
<th></th>
<th>All study population</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>GPX-1 Pro198Leu</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro198 (C)</td>
<td>131</td>
<td>87.3</td>
<td>142</td>
<td>94.6</td>
<td>273</td>
</tr>
<tr>
<td>Leu198 (T)</td>
<td>19</td>
<td>12.7</td>
<td>8</td>
<td>5.4</td>
<td>27</td>
</tr>
</tbody>
</table>
Table 4.19b. Calculation of Hardy Weinberg Equilibrium (HWE) for GPX-1 Pro198Leu polymorphism frequency distribution.

<table>
<thead>
<tr>
<th></th>
<th>Pro198Pro</th>
<th>Pro198Leu</th>
<th>Leu198Leu</th>
<th>Chi-square value ($X^2$)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Observed frequency</td>
<td>56</td>
<td>19</td>
<td>0</td>
<td>1.575</td>
<td>0.209</td>
</tr>
<tr>
<td>Expected H.W frequency</td>
<td>57.2</td>
<td>16.59</td>
<td>1.2</td>
<td>(76.27%)</td>
<td>(22.12%) (1.6%)</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Observed frequency</td>
<td>67</td>
<td>8</td>
<td>0</td>
<td>0.235</td>
<td>0.625</td>
</tr>
<tr>
<td>Expected H.W frequency</td>
<td>67.21</td>
<td>7.57</td>
<td>0.21</td>
<td>(89.62%)</td>
<td>(10.10%) (0.28%)</td>
</tr>
</tbody>
</table>

Degree of freedom = 1

Critical value of chi-square for $p=0.05 = 3.841$

Observed and expected frequencies not significantly different in patients with CAD ($X^2 < 3.841$) as well as controls ($X^2 < 3.841$). Hence, study population at Hardy Weinberg equilibrium.
Table 4.20. Association of GPX-1 Pro198Leu polymorphism in two age groups

<table>
<thead>
<tr>
<th></th>
<th>Patients</th>
<th>Controls</th>
<th>Odds ratio</th>
<th>(95% CI)</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td><strong>Age ≤ 50 years group</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro198Pro (CC)</td>
<td>13</td>
<td>65</td>
<td>23</td>
<td>92</td>
<td>6.19</td>
</tr>
<tr>
<td>Pro198Leu (CT)</td>
<td>7</td>
<td>35</td>
<td>2</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td><strong>Age &gt; 50 years group</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro198Pro (CC)</td>
<td>43</td>
<td>78</td>
<td>44</td>
<td>88</td>
<td>2.04</td>
</tr>
<tr>
<td>Pro198Leu (CT)</td>
<td>12</td>
<td>22</td>
<td>6</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>

*Pearson’s chi square test

*Significant at p ≤ 0.05
4.5.1 Association of glutathione peroxidase-1 activity with GPX-1 Pro198Leu polymorphism.

Since GPX-1 activity has been shown to vary according to gene expression. This study investigated the effect of GPX-1 Pro198Leu polymorphism on GPX-1 activity. Table 4.21 shows the distribution of GPX-1 activity, according to genotypes in the study population.

GPX-1 activity was significantly low in Pro198Pro (CT) genotype (116.7 ± 28.6 U/L) when compared to Pro198Leu (CC) genotype (136.0 ± 38.9 U/L) in patients with CAD (p = 0.023). Similar result was observed in control groups, as GPX-1 activity high in Pro198Pro (CC) genotype (181.5 ± 46.1U/L) when compared with Pro198Leu (CT) genotype (158.4 ± 41.6 U/L), although this difference was not statistically significant (p = 0.182) (Table 4.21).
Table 4.21. Association of glutathione peroxidase-1 activity with GPX-1 Pro198Leu polymorphism.

<table>
<thead>
<tr>
<th></th>
<th>Glutathione peroxidase-1 activity (U/L)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>± SD</td>
<td>P value#</td>
</tr>
<tr>
<td><strong>Patients</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro198Pro (CC)</td>
<td></td>
<td>136.0</td>
<td>38.9</td>
<td>0.023*</td>
</tr>
<tr>
<td>Pro198Leu (CT)</td>
<td></td>
<td>116.7</td>
<td>28.6</td>
<td></td>
</tr>
<tr>
<td><strong>Controls</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro198Pro (CC)</td>
<td></td>
<td>181.5</td>
<td>46.1</td>
<td>0.182</td>
</tr>
<tr>
<td>Pro198Leu (CT)</td>
<td></td>
<td>158.4</td>
<td>41.6</td>
<td></td>
</tr>
</tbody>
</table>

#Independent sample t test

*Significant at p ≤ 0.05
4.5.2. Association of scoring systems of CAD severity with GPX-1 Pro198Leu genotypes.

Mean stenosis score was significantly high in patients with Pro198Leu (CT) genotype when compared to Pro198Pro (CC) genotype of patients with CAD (p = 0.018). Although, mean vessel score and mean extent score did not show a significant difference between two genotypes in patients with CAD. However, Pro198Leu (CT) genotype showed a non-significant trend towards increase severity of CAD (Table 4.22).
Table 4.22. Association of GPX-1 Pro198Leu polymorphism with severity of coronary artery disease

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>±SD</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vessel score</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro198Pro (CC)</td>
<td>1.47</td>
<td>0.69</td>
<td>0.112</td>
</tr>
<tr>
<td>Pro198Leu (CT)</td>
<td>1.80</td>
<td>0.79</td>
<td></td>
</tr>
<tr>
<td><strong>Stenosis score</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro198Pro (CC)</td>
<td>6.21</td>
<td>2.3</td>
<td>0.018*</td>
</tr>
<tr>
<td>Pro198Leu (CT)</td>
<td>9.18</td>
<td>5.1</td>
<td></td>
</tr>
<tr>
<td><strong>Extent score</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro198Pro (CC)</td>
<td>46.32</td>
<td>15.6</td>
<td>0.115</td>
</tr>
<tr>
<td>Pro198Leu (CT)</td>
<td>54.1</td>
<td>19.1</td>
<td></td>
</tr>
</tbody>
</table>

*Independent sample t test

*Significant at p ≤ 0.05
4.6. Associations of risk models for coronary artery disease in study population by multivariate logistic regression analysis

Multivariate logistic regression analysis was performed to eliminate the influences of confounding factors for CAD. Result showed that a positive family history has an odds ratio of 3.29 [95% CI (0.60 - 18.0) p = 0.170] after adjusting for age, sex, BMI, GPX-1 CT genotype, PON-1, GPX and GPX-1. Similarly, GPX-1 Pro198Leu (CT) genotype also showed an odds ratio of 3.19 [95% CI 0.63 - 16.0] p = 0.159) after adjusting age, sex, BMI, positive family history, PON-1, total GPX and GPX-1 (Table 4.23).

Furthermore, multivariate logistic regression was performed to find the risk factors for CAD in subjects with age ≤ 50 years group of the study population. GPX-1 Pro198Leu (CT) genotype showed an odds ratio of 4.87 [95% CI 0.65 - 36.24] p = 0.122] after adjusting for age, sex, BMI and positive family history.
Table 4.23. Associations of risk models for coronary artery disease in study population by multivariate logistic regression analysis

<table>
<thead>
<tr>
<th>Variable</th>
<th>Odds ratio</th>
<th>95% confidence interval</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>Positive Family history of CAD</td>
<td>5.15</td>
<td>2.0 - 12.8</td>
</tr>
<tr>
<td></td>
<td>GPX 1 Pro198Leu (CT) variant</td>
<td>2.84</td>
<td>1.15 - 6.98</td>
</tr>
<tr>
<td>b</td>
<td>Positive Family history of CAD</td>
<td>5.22</td>
<td>2.0 - 13.1</td>
</tr>
<tr>
<td></td>
<td>GPX 1 Pro198Leu (CT) variant</td>
<td>2.90</td>
<td>1.1 - 7.3</td>
</tr>
<tr>
<td>d</td>
<td>Age (Years)#</td>
<td>1.00</td>
<td>0.97 - 1.04</td>
</tr>
<tr>
<td></td>
<td>Gender (Male)</td>
<td>1.10</td>
<td>0.48 - 2.54</td>
</tr>
<tr>
<td></td>
<td>Body mass index (kg/m²)#</td>
<td>1.02</td>
<td>0.95 - 1.10</td>
</tr>
<tr>
<td></td>
<td>Positive Family history of CAD</td>
<td>5.19</td>
<td>2.05 - 13.0</td>
</tr>
<tr>
<td></td>
<td>GPX 1 Pro198Leu (CT) variant</td>
<td>2.89</td>
<td>1.13 - 7.3</td>
</tr>
<tr>
<td>e</td>
<td>Age (Years)#</td>
<td>0.97</td>
<td>0.90 - 1.04</td>
</tr>
<tr>
<td></td>
<td>Gender (Male)</td>
<td>0.91</td>
<td>0.21 - 3.98</td>
</tr>
<tr>
<td></td>
<td>Body mass index (kg/m²)#</td>
<td>1.04</td>
<td>0.92 - 1.18</td>
</tr>
<tr>
<td></td>
<td>Positive Family history of CAD</td>
<td>3.29</td>
<td>0.60 - 18.0</td>
</tr>
<tr>
<td>Parameter</td>
<td>Mean</td>
<td>95% CI</td>
<td>p-value</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>------</td>
<td>----------</td>
<td>---------</td>
</tr>
<tr>
<td>GPX 1 Pro198Leu (CT) variant</td>
<td>3.19</td>
<td>0.63 - 16.0</td>
<td>0.159</td>
</tr>
<tr>
<td>Paraoxonase 1 (µg/ml)#</td>
<td>0.93</td>
<td>0.90 - 0.95</td>
<td>0.000*</td>
</tr>
<tr>
<td>Glutathione peroxidase (U/l)#</td>
<td>0.99</td>
<td>0.98 - 1.00</td>
<td>0.134</td>
</tr>
<tr>
<td>Glutathione peroxidase 1 (U/l)#</td>
<td>0.95</td>
<td>0.93 - 0.98</td>
<td>0.001*</td>
</tr>
</tbody>
</table>

# Consider as continuous variable.

* Significant at p ≤ 0.05.
Table 4.24. Associations of risk models for CAD in age ≤ 50 years group in the study population by multivariate logistic regression analysis

<table>
<thead>
<tr>
<th>Variable</th>
<th>Odds ratio</th>
<th>95% confidence interval</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>a GPX 1 Pro198Leu (CT) variant</td>
<td>6.19</td>
<td>1.1 - 34.3</td>
<td>0.037*</td>
</tr>
<tr>
<td>b Positive Family history of CAD</td>
<td>12.6</td>
<td>1.35 - 118.9</td>
<td>0.026*</td>
</tr>
<tr>
<td>GPX 1 Pro198Leu (CT) variant</td>
<td>4.36</td>
<td>0.68 - 27.9</td>
<td>0.120</td>
</tr>
<tr>
<td>c Age (Years)#</td>
<td>0.91</td>
<td>0.80 - 1.04</td>
<td>0.174</td>
</tr>
<tr>
<td>Gender (Male)</td>
<td>0.70</td>
<td>0.13 - 3.78</td>
<td>0.687</td>
</tr>
<tr>
<td>Body mass index (kg/m²)#</td>
<td>1.10</td>
<td>0.95 - 1.26</td>
<td>0.188</td>
</tr>
<tr>
<td>Positive Family history of CAD</td>
<td>12.9</td>
<td>1.14 - 145.7</td>
<td>0.039*</td>
</tr>
<tr>
<td>GPX 1 Pro198Leu (CT) variant</td>
<td>4.87</td>
<td>0.65 - 36.24</td>
<td>0.122</td>
</tr>
</tbody>
</table>

# Consider as continuous variables.

* Significant at p ≤ 0.05.
5. DISCUSSION

Coronary artery disease (CAD) is proceeding to cause significant morbidity and mortality despite the advent of major therapeutic strategies. Although the precise mechanisms involved in CAD is not clear, current researches predict that it caused by an inflammatory responses of the arterial wall. There are many risk factors cause to accelerate or modify the complex, chronic inflammatory processes and ultimately, manifest as a fibrous atherosclerotic plaque (Thom et al, 2006). Subsequently, this process progresses to narrowing and occlusion of coronary arteries and interruption oxygenated blood supply to heart muscles. Furthermore, coronary artery occlusion leads to angina, arrhythmia, myocardial infarction, heart failure and even death (Haft et al, 2010).

Hypercholesterolemia, hypertension, obesity and diabetes have identified as major risk factors of CAD (Rosamond et al, 2008). However, CAD is perceived primarily as a lifestyle disease, as both the incidence and negative outcome of CAD are associated with lifestyle factors such as physical inactivity, unhealthy diet and smoking (Boekholdt et al, 2006). Evidence suggests that hereditary factors show a strong positive relationship with CAD (Juonala et al, 2007).

The present study demonstrates the association of paraoxonase-1, apolipoprotein A-1, glutathione peroxidase activity and the GPX-1 Pro198Leu polymorphism with severity of coronary artery disease. Furthermore, this study is the first to report on the relationship between GPX-1 genotypes and severity of CAD in Sri Lanka.
5.1. Association of traditional risk factors and coronary artery disease.

Several studies have suggested that there was more than one chromosome involved in the genetic link and that the multi-factorial risk profile of CAD makes it difficult to identify a single genetic causal relationship (Lloyd et al, 2004). However, the mechanism or the relationship between hereditary CAD and families with positive premature CAD history is not yet completely elucidated. Evidence has suggested that strong family history is an independent risk factor associated with CAD (Lloyd et al, 2004; Andresdottir et al, 2002). Recent guidelines on risk stratification of CAD suggest that incorporation of family history of premature heart disease would have a greater assistance in clinical decision making and management of patients (Qureshi et al, 2012; Sivapalaratnam et al, 2010; Andresdottir et al, 2002). However, association between family history and risk of CAD is not clearly explained by the traditional risk factors (Andresdottir et al, 2002). Hence, it has suggested that apart from these risk factors, other factors also contribute to increased risk associated with CAD. The present study revealed that family history of heart disease had a significantly positive correlation with CAD when compared to controls [odds ratio 5.15 (95% CI 2.0 - 12.8), p = 0.000]. Despite the age, gender and BMI adjusted model ‘d’ showed a significant association for CAD [odds ratio 5.19 (95% CI 2.0 – 13.0), p = 0.000], it showed a 3.29 fold risk for CAD adjusted with age, gender, BMI, PON-1, total GPX and GPX-1 in model ‘e’ in non significantly [odds ratio 3.29 (95% CI 0.6 – 18.0), p = 0.170]. Our findings are consistent with previous studies reported on the association of family history and CAD (Sivapalaratnam et al, 2010; Qureshi et al, 2012; Yuka et al, 2012; Yuka et al, 2013).
Body mass index (BMI) is routinely used in the assessment of nutritional status, which has a direct impact on non-communicable diseases including CAD. Although the BMI reflects both fat and lean mass, it does not indicate the extent of distribution of visceral fat (Mason et al., 2008).

The present study indicated that there was no significant difference in BMI between patients and controls ($p = 0.441$). However, when stratified by age for two groups as adults less than 50 years and more than 50 years, the subjects with age $\leq 50$ years group with CAD had higher BMI ($27.5 \text{ kg/m}^2$) compared to the control subjects ($24.5 \text{ kg/m}^2$; $p = 0.070$). At the same age group, recent studies have shown that subjects with age $\leq 50$ years group with higher BMI are more susceptible to development of CAD (Arzamendi et al., 2011). These data suggest that high BMI appeared to be an important factor in risk stratification of subjects with age $\leq 50$ years group in the Sri Lankan population.

### 5.2. Association of Paraoxonase-1 concentration with coronary artery disease

Studies have been reported that PON-1 activity is dependent on the number of PON-1 molecules in HDL-C rather than the serum HDL-C concentration. The present study also showed that a significant increase in Apo A-1 to PON-1 ratio in patients with CAD (Apo A-1/PON-1 = 3.19) when compared to controls (Apo A-1/PON-1 = 1.31; $p = 0.000$). It is suggested that patients with CAD had less protection against oxidative stress and PON-1 concentration is independent from Apo A-1 concentration.

Results revealed by serum PON-1 activity in our study are in accordance with previous studies on CAD (Maharudra et al., 2013; Nabatchian et al., 2008; Sarkar et al., 2006; 131
Tward et al, 2002; Marit et al, 2006; James et al, 2000; Senti et al, 2001; Michael et al, 2002).

Chiyan et al (2013) has suggested that many covariates affect PON-1 activity when bound with the HDL-C. However, precise system of PON-1 modulation has not been completely elucidated yet. Evidence shows that PON-1 activity is influenced by many factors, including genetic and environmental (Balcerzyk et al, 2008; Rainwater et al, 2009). Several epidemiological studies have also suggested that low PON-1 activity is a reliable risk marker for the CAD (Senti et al, 2003; Nabatchian et al, 2008; Sarkar et al, 2006; Mackness et al, 2006a).

Serum PON-1 activity is rather constant over time, once it reaches adult values. However, recent investigations have reported a progressive decrease in PON-1 activity in elderly subjects (Jarvik et al, 2002; Milochevitch et al, 2001; Seres et al, 2004). Another study has revealed such decline of PON-1 activity levels with advancing age, it is suggested that it may be related to the development of oxidative stress conditions with aging, and would have an impact on the increased incidence of atherosclerosis with age (Seres et al, 2004).

In the present study there was a significant inverse relationship observed between age and Paraoxonase-1 concentration in patients ($\rho = -0.251, p = 0.030$). Although, PON-1 level in the control group showed a non-significant inverse relationship with age ($\rho = -0.158, p = 0.175$). The PON-1 concentration appeared to be significantly low in patients more than 50 years of age group compared to patients less than 50 years of age. These differences may attribute in CAD patients being exposed to other confounding factors.
Oxidized LDL is one of the important atherogenic substances involved in the pathogenesis of cardiovascular disease. Serum PON-1 is an HDL-C associated enzyme, and appears to have a function in the protection against LDL from oxidation (Senti et al, 2001). Hence, PON-1 activity is directly involved in lowering the risk incidence of development of CAD. Furthermore, studies have suggesting that low serum PON-1 concentration appeared to be a potentially good predictor of CAD (Aamir et al, 1999).

5.3. Association of Apolipoprotein A-i concentration with coronary artery disease

Apolipoprotein Al (Apo A-I) is a HDL-C associated protein, which plays an important role in lipid metabolism by removal of excess lipids from tissues to liver. Hence, serum Apo A-1 concentration is believed to have a strong correlation with the serum HDL-C concentration (Kuyl et al, 1992). Many studies have reported that low levels of HDL and Apo A-1 could increase the risk of development of CAD (Wilson et al, 1998; Manninen et al, 1992; Wallidius et al, 2001). Further, prospective studies such as AMORIS (Walldius et al, 2001), INTERHEART (McQueen et al, 2008) and PRIME (Canoui et al, 2010) have also confirmed that Apo A-1 concentration may have a beneficial predictive value for risk evaluation of CAD. However, according to a recent study by Navid et al, in 2014 and other prospectives studies, reported by Quebec Cardiovascular (Lamarche et al 1996) and ARIC (Sharrett et al, 2001) studies have shown that serum Apo A-1 concentration may not be a significant predictive marker of risk evaluation for CAD. Similarly, our study also did not show a significant difference in Apo A-1 levels between patients with CAD compared to controls (p = 0.229).
Although, it has been shown a significant correlation between Apo A-I and severity or extent of coronary artery stenosis in the patients with CAD (Habib et al, 2009). The present study did not show a significant correlation with the severity of CAD suggesting that Apo A-I may not be a sensitive indicator for assessment of severity of CAD.

It has also reported that Apo A-I concentration increases with age (Jungner et al, 1998). In contrast, some studies have not reported any such association between Apo A-I and age (Sharma et al, 2006; Connelly et al, 1999) which are consistent with our present findings.

The relationship between Apo A-I and PON-I has been well documented. It has suggested that as a HDL-C subfraction, Apo A-I bound to PON and act as an antioxidant to prevents oxidation of LDL. Although, in our study no significant association shown between Apo A-I, PON-I and the severity of CAD. However, Apo-A-I to PON-I ratio was high in patients’ group compared to control subjects and this may be one of the possible causes for failing protection against LDL oxidation, which probably would have directed to development of CAD.

5.4. Association of Glutathione peroxidase activity with coronary artery disease

A meta-analysis has reported a strong, inverse association of GPX levels with CAD (Gemma et al, 2009) and high levels of GPX contribute to cellular defense mechanism against increased oxidative stress during acute MI (Mral et al, 2012).
GPX-1 antioxidant has many functional effects in the human body such as antioxidant defense mechanism against oxidation, cellular metabolism and signal transduction. Oxidative stress resulting lipid peroxidation and vascular endothelial injury. Thus, oxidative hypothesis directed to study association of GPX-1 and severity of CAD in this study.

Blankenberg et al (2003) has shown that a high level of GPX-1 activity had a hazard ratio of 0.29 (95% CI, 0.15 to 0.58) when compared with those in the lowest level. Thereby low GPX activity could be used as a reliable marker for coronary artery disease (Blankenberg et al, 2003).

In the present study, erythrocyte total GPX and GPX-1 activities were significantly decreased in CAD patients compared to controls. These findings are in accordance with the earlier studies of Blankenberg et al, 2003; Mral et al 2012 and Pawan et al, 2010.

Present study shows a strong significant positive correlation between total GPX and GPX-1 for patients (r = 0.736, p = 0.000) and controls (r = 0.243, p = 0.000), suggesting decrease of total GPX possibly due to decreased GPX-1 isoenzyme activity in patients. Therefore, GPX-1 could be considered as a sensitive risk marker for coronary artery disease in our study population. On the other hand, results have shown that GPX-1 was closely associated with its genetic variants for risk of coronary artery disease, which is discuss in more detail under GPX-1 gene polymorphism in the thesis.

Previous population studies have offered contradictory results on how GPX activity alters with age (Junqueira et al, 2004; Habdous et al, 2003). It has shown that low GPX activity leads to direct tissue damage due to increased hydrogen peroxide and activation of inflammatory pathways. Mulholland et al (1999), Artur et al (1992) and Sara et al
(2008) are few written reports providing additional evidence for GPX activity decreasing with age.

The present study did not show a significant relationship between total GPX activities and age in both patients and controls. GPX-1 also followed a similar pattern; there was no significant relationship between GPX-1 activity and age in both patients and controls. However, patients showed a non-significant inverse relationship between both GPX and GPX-1 with age. Suggesting that both GPX and GPX-1 could be reduced due to increased oxidative stress associated with chronic exposure to conventional risk factors.

5.5. Associations of Glutathione peroxidase-1 activity, GPX-1 Pro198Leu polymorphism with coronary artery disease

GPX-1 Pro198Leu polymorphism is located in exon 2 of the GPX-1 gene with proline to leucine transition at codon 198 (Moscow et al, 1994). To date, very few studies have linked the GPX-1 Pro198Leu polymorphism to CAD (Nemoto et al, 2007; Hamanishi et al, 2004; Sergeeva et al, 2001).

GPX-1 polymorphism study revealed that the Pro198Pro (CC) genotype was the most frequent in the control group compared to patients, being 89.3 % and 74.7 % respectively. The Pro198Leu (CT) genotype was less frequent in controls compared to patients (10.7 % and 25.3 % respectively). Distributions of GPX-1 Pro198Leu polymorphism in homozygotes or heterozygotes are in Hardy-Weinberg equilibrium in both groups (Table 4.19b). However, Leu198Leu (TT) genotype was not identified in the study population, making it one of the most important findings of this study. This
finding was consistent with several population studies in which the percentage of Leu198Leu (TT) genotype was present in a very small percentage of their study population, suggesting that these subjects were at very high risk of CAD. Thus, these findings of Pro198Leu (CT) have opened up a new window for detailed clinical analysis.

Present study also observed allelic frequency in CAD patients and controls with varying erythrocyte GPX-1 activity. As shown in Tables 4.19, the allele frequency of the most common Pro198 (C) encoding variant was 87.3% and 94.6% for patients and controls respectively. On the other hand, Leu198 (T) encoding variant was observed for patients (12.7%) and controls (5.4%) respectively.

The present study also showed a significant difference in GPX-1 Pro198Leu genotype distribution between patients with CAD compared to controls. In order to eliminate the influence caused by CAD risk factors a multivariate logistic regression analysis was performed when assessing the impact of the GPX-1 Pro198Leu polymorphism on the risk of development of CAD. There was a 2.84 fold risk in Pro198Leu carriers for susceptibility of CAD in the study population [odds ratio 2.84 (95% CI 1.15 - 6.98), p = 0.019]. This risk also remained in age, gender and BMI adjusted model ‘d’ in multivariate logistic regression analysis [odds ratio 2.89 (95% CI 1.13 - 7.3), p = 0.026]. Multivariate logistic regression model ‘e’ shows that Pro198Leu (CT) genotype was 3.19 fold risk for CAD [odds ratio 3.19 (95% CI 0.63 - 16.0), p = 0.159] which was not significance suggesting further investigations may be required for confirmation of CAD risk in relation Pro198Leu (CT) genotype.
Present findings of GPX-1 Pro198Leu polymorphism are in consistent with previous studies reported on GPX-1 Pro198Leu polymorphism and CAD. Hamanishi et al (2004) have shown that in a Japanese population those with Pro198leu (CT) genotype had significantly higher mean intima media thickness of common carotid arteries (1.04 ± 0.23 vs. 0.91 ± 0.18mm, P = 0.0028), prevalence of CAD (24.2% vs. 10.6%, P=0.035) and peripheral vascular disease (15.2% vs. 7.9%, P = 0.027) than those with Pro198Pro (CC) genotype. Nemoto et al (2007) has also reported that Pro198leu (CT) genotype is associated with significantly higher coronary artery calcium score among the Japanese population. However, Sergeeva et al (2001) have reported that there was no significant association between the GPX1 Pro198leu polymorphism and myocardial infarction in hypertensive non-insulin-dependent diabetes mellitus patients in Russia. Further, it has been shown that a Chinese population with Pro198Leu (CT) genotype were at high risk for CAD than those with Pro198Pro (CC) genotype (Tang et al, 2008) suggesting that the genotype of GPX-1 is important in assessing the risk of CAD in Asian population.

A study has reported increased death rate in the young age group due to CAD directly associated with a genetic factor (Marenberg et al, 1994). Present study investigated the association of GPX-1 Pro198Leu variants with the CAD risk in subjects with age ≤ 50 years and age > 50 years. The findings revealed Pro198Leu (CT) genotype in subjects with age ≤ 50 years had 6.19 fold significant risk for CAD than Pro198Pro (CC) genotypes in the same group [OR 6.19 (95% CI 1.1 - 34.3), p = 0.037). Thus, these findings will open up a new window of research, screening, and management of that age group who have vulnerable family risk of CAD.
Multivariate logistic regression analysis was performed to eliminate the influences caused for CAD risk factors to assess the impact of the GPX-1 Pro198Leu polymorphism on the susceptibility to CAD in subjects with age ≤ 50 years. Pro198Leu (CT) genotype shows 4.87 fold risk (95% CI 0.65 - 36.24) for CAD which was not significant even after adjusting for age, gender, BMI and positive family history of CAD suggesting further investigations confirming these findings.

Thus, screening of GPX-1 Pro198Leu polymorphism would help to identify a young population with risk factors of Pro198Leu (CT) to minimize the incidence of morbidity and mortality due to CAD in Sri Lanka.

Present study also examined the associations between erythrocyte GPX-1 activity, GPX-1 genotypes and CAD. GPX-1 is the most abundant isoform of the GPXs family. GPX-1 is a key antioxidant enzyme within many cells, including vascular endothelial cells and in function converts hydrogen peroxide to water and lipid peroxides to their respective alcohols (Arthur, 2000). Previous studies have indicated the biological relevance for GPX-1 in atherogenesis. Further, Forgione et al (2002b) also demonstrated that GPX-1 deficiency was accompanied by increased inflammatory complications surrounding the coronary arteries. Thus, the deficiency of GPX-1 could result in endothelial dysfunction and the progression of atherosclerosis ( Forgione et al, 2002b; Torzewski et al, 2007). Furthermore, decreased GPX-1 activity was observed in patients with CAD and those with acute myocardial infarction (Loeper et al, 1991; Blankenberg et al, 2003).

This study also showed that GPX-1 activity is significantly low in Pro198Leu (CT) genotypes compared to Pro198Pro (CC) genotypes in CAD patients (p = 0.023).
Similarly, GPX-1 activity was low in Pro198Leu (CT) genotypes when compared to Pro198Pro (CC) genotype in the control group (p = 0.182) (Table 4.21). These new findings clearly indicate the association between the GPX-1 genotypes and GPX-1 activity in risk of CAD.

Several studies have reported the effect of GPX-1 Pro198Leu polymorphism on the function of GPX-1 enzyme activity. However, Ravn et al (2006) reported that the variant allele of Pro198Leu (CT) correlates with lower GPX-1 activity. According to Hu and Diamond (2003) the 198Leu (T) allele was less responsive to the stimulation of GPX-1 enzyme activity compared with 198Pro (C) allele. Furthermore, it has been shown that Hamanishi et al (2004) the subjects with 198Leu (T) allele significantly lower GPX-1 activity than those with the 198Pro (C) (whereas 20.1 ± 10.6 U/mg protein and 32.9 ± 9.5 U/mg protein respectively, P = 0.023).

Forsberg et al (2000) also showed the association between the 198Leu (T) variant and erythrocyte GPX-1 activity in 66 subjects from the Finnish/Swedish population and no significant association was observed. However, the allele frequency of 198Leu (T) was higher (41%) than the present study.

Previous studies have reported that low GPX-1 activity was associated with the increased risk of CAD (Blankenberg et al, 2003). This may be due to its antioxidant functions associated with the vascular endothelium and exists in many cell types including macrophages. Subjects with Pro198Leu (CT) genotype may prone to having low level of GPX-1 activity especially in vascular endothelium.

Concerning Pro198Leu (CT) genotype, it has been suggested that modification of the GPX-1 activity and expressions, such as nutrition (dietary selenium), nearby genes or...
other lifestyle changes (fitness, alcohol) may influence the effects of GPX-1 activity in different polymorphic genotypes for CAD.

5.6. Summary of the associations of Paraoxonase-1, Glutathione peroxidase and Apolipoprotein A-1 levels with severity of Coronary artery disease

The present study did not find a significant correlation between Paraoxonase-1 concentration and vessel, stenosis and extent scores. However, the PON-1 activity was showing an inverse relationship with the single vessel disease compared to double and triple vessel disease. As far as the stenosis score was concerned, a low PON-1 activity was observed in severe disease group (16 ≤ stenosis score). On the other hand, extent score showed low PON-1 activity in moderate group (≤ 25% extent score). However, these three scoring systems did not show a significant difference in PON-1 levels.

A recent report on the Chinese population regarding the association of PON-1 activity and angiographically detected CAD (Chiyan et al, 2013), based on method of Gensini score system (Gensini, 1983) revealed that PON-1 activity was significantly lower in CAD patients compared to non-CAD patients. Furthermore, the regression analysis in the same study showed a strong inverse relation between PON-1 activity and the severity of CAD. However, only limited information is available on the association between PON-1 activities in angiographically confirmed CAD. Present study opens up new research area on the assessment of CAD.

This study has generated clinically important findings in relation to GPX activity and the severity of CAD with special emphasis on vessel, stenosis and extent score which has not reported before. This is the first study to report the clinical association of GPX
and severity of CAD in Sri Lanka and South East Asian countries. Thus, the total GPX/GPX-1 activity may be more useful marker in clinical assessment and management of patients with proven risk for CAD.

Although the serum apolipoprotein A-I concentration did not show a significant correlation with the vessel, stenosis and extent scores, Apo A-I levels showed an inverse relationship with the severity of CAD.

Overall antioxidant enzyme levels (total GPX, GPX-1) appeared to be more sensitive marker of CAD. Thus, the GPX-1 could be used as a sensitive marker in assessing the severity of CAD.

This study also showed the GPX-1 Pro198Leu polymorphism was significantly associated with stenosis score, which has not been reported before. Thus, genotyping of GPX-1 Pro198Leu polymorphism could be used as an early screening method to identify subjects vulnerable to CAD.
6. CONCLUSIONS

Findings of this study indicate that,

1. Serum Paraoxonase-1 concentration decreases with advancing age and low PON-1 activity is observed in patients with CAD.

2. Serum Paraoxonase-1 concentration was not associated with severity of coronary artery disease as assessed by the vessel, stenosis and extent scores. Thus PON-1 may not be a sensitive marker of assess CAD.

3. Apolipoprotein A-1 concentration may not be a sensitive marker of CAD.

4. Decreased total erythrocyte Glutathione Peroxidase activity and erythrocyte Glutathione Peroxidase-1 activities are independent risk factors for CAD.

5. Erythrocyte Glutathione Peroxidase-1 activity correlates with total erythrocyte Glutathione Peroxidase activity. Hence, GPX-1 is a potential antioxidant marker of CAD.

6. Total erythrocyte Glutathione Peroxidase activity and erythrocyte Glutathione Peroxidase-1 activities are not associated with age.

7. Decreased total erythrocyte Glutathione Peroxidase activity and erythrocyte Glutathione Peroxidase-1 activities were associated with increased severity of coronary artery disease as assessed by vessel, stenosis and extent scores. Suggesting that GPX-1 was appeared to be more sensitive marker of CAD.

8. Pro198Pro (CC) genotype and Pro198 (C) allele are the most prevalent genetic variant of GPX-1 Pro198Leu polymorphism in the study population. Leu198Leu (TT) genotype was not isolated in the study population.
9. Pro198Leu (CT) genotype is a significant predictor of CAD. Pro198Leu (CT) genotype carriers were 2.84 times more likely to develop CAD as compared to those with Pro198Pro (CC) in the study population.

10. Pro198Leu (CT) genotype carriers in subjects with age ≤ 50 years group were 6.19 times more likely to develop CAD as compared to those with Pro198Pro (CC) genotype in the same age group. This may have a clinical importance in the screening and management of patients with CAD.

**Recommendation**

Based on the results of this study, could be recommend the following:

1. Glutathione peroxidase 1 (GPX-1) activity may be value in screening of subjects with high risk for CAD.

2. Introducing new screening test for young population to recognize Pro198Leu (CT) carriers for risk assessment for CAD and it may help early identification of and clinical management of patients with CAD.

**Limitations/ short comes from the study**

1. Paraoxonase-1 assay and Glutathione peroxidase-1 assay have not been studied/ reported in Sri Lanka before and thus cannot be compared.

2. The findings regarding the frequency of GPX-1 Pro198Leu polymorphism have not been studied / reported in Sri Lanka before and thus, cannot be compared.
3. A larger sample would have been more useful, although this was not included in the research proposal and the cost and time factor had to be considered. Nevertheless, the present study provides valuable insights and interesting information and may serve to guide future studies in this area.

4. Control subjects were selected from apparently healthy individuals who had no history of angina pectoris and no symptoms or signs of other atherosclerotic vascular diseases based on routine laboratory investigations and Electrocardiogram examinations; yet, we cannot exclude the possibility of CAD in any of them without performed coronary angiography. However, it cannot be done due to ethical reasons.

5. The GPX-1 Pro198Leu polymorphism associated with CAD in this study may be in linkage disequilibrium with polymorphisms of other nearby genes that actually led to the evolution of CAD.

Proposals for future studies

1. Population studies to define reference intervals for Paraoxonase-1 concentration.

2. Studies to assess the influence of age on Glutathione peroxidase activities to define age specific reference intervals for Glutathione peroxidase.

3. Studies to confirm and postulate mechanisms on the influence of Glutathione peroxidase activity on the severity of CAD.

4. Associations in this study are difficult to interpret in mechanistic or etiologic terms; hence, high-quality prospective studies evaluating the associations
between low antioxidant enzyme activity levels (PON-1, GPX, GPX-1) and CAD endpoints are required.

5. Future studies should be focused to evaluate the selenium status of patients with CAD to rule out selenium deficiency.

6. An association between Pro198Leu and GPX-1 activity requires further research with larger number of samples. Deep investigations into functional differences of the polymorphism remains to be further examined. Further work is also required to identify novel polymorphisms located in potential regulatory regions of the GPX-1 gene, which may modify gene expression and thus associated with CAD risk.
7. REFERENCES


Chen, CJ, Huang, HS and Chang, WC, (2003). Depletion of phospholipid hydroperoxide glutathione peroxidase up-regulates arachidonate metabolism by


*Arterioscler Thromb Vasc Biol.* 25(7): 1332-1340.


HDL The Role of ‘Paraoxonase-1 Activity’ as an Antioxidant in Coronary Artery Diseases. *Journal of Clinical and Diagnostic Research.* 7(7): 1284-1287.


Light, but not heavy alcohol drinking, stimulates paraoxonase by upregulating liver mRNA in rats and humans. *Metabolism.* 52(10): 1287-1294.


APPENDIX 1

List of publications and communications from this thesis.

1. WD Wickramasinghe, H Peiris, LG Chandrasena, V Senaratne, PPR Perera. Low serum paraoxonase; A risk factor for Coronary Artery Diseases?. *Annual Scientific Session-2012*, Faculty of Medical Sciences, University of Sri Jayewardenepura. OP 12, pp 24.


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APPENDIX 2
Questionnaire/ Data sheet

Date [ ] [ ] Code [ ] [ ]

7. Age [ ] [ ]
8. Sex [ ] Male [ ] [ ] [ ] Female [ ] [ ]
9. Height [ ] [ ] m
10. Weight [ ] [ ] kg BMI: .................kgm⁻²

Coronary artery diseases risk exposure

1) Smoking
* Current smokers are defined as individuals who smoked any tobacco in the previous 12 months.
** Former smokers are defined as individuals who had quit smoking more than a year earlier.
- Current smoker *? [ ] Yes [ ] No [ ]
- Former smoker **? [ ] Yes [ ] No [ ]
- Never smoked? [ ] Yes [ ] No [ ]
2) Family history

1. Heart disease
   - Mother [ ]
   - Father [ ]
   - Sibling [ ]

2. Diabetes mellitus [ ]

3. Kidney disease [ ]

4. Liver dysfunction [ ]

5. Cancer [ ]

3) Hypercholesterolemia
   ± 12 hr fasting total cholesterol level ≥ 240 mg/dl or on lipid lowering therapy.
   - Yes [ ]
   - No [ ]

4) Hypertension
   »Blood pressure ≥140/90 mmHg, or on antihypertensive medication.
   - Yes [ ]
   - No [ ]

5) History of vascular diseases
   - Deep vein thrombosis
     - Yes [ ]
     - No [ ]
   - Peripheral vascular disease
     - Yes [ ]
     - No [ ]

   Yes [200] No [ ]
6) Physically active

Moderate (walking, cycling) or strenuous exercise (jogging, football, vigorous swimming)
For 4 or more hours per week

7) Alcoholism

Regular ☐ Former ☐ Non ☐

! Consumption of alcohol at least three times a week.
# Quit use of alcohol more than a year earlier.

8) Dietary habits

Vegetarian ☐ Non Vegetarian ☐

Type of vegetarian

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<th>Includes eggs, but not dairy products.</th>
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</thead>
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<td>2. Lacto vegetarianism</td>
<td>Includes dairy products but not eggs.</td>
</tr>
<tr>
<td>3. Ovo-lacto vegetarianism</td>
<td>Includes animal/dairy products such as eggs, milk, and honey.</td>
</tr>
<tr>
<td>4. Veganism / vegans</td>
<td>Excludes all animal flesh and animal products, including milk, honey, and eggs, and may also exclude any products tested on animals, or any clothing from animals.</td>
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- **BMI and classification**

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<td>25.0–29.9</td>
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</tr>
<tr>
<td>04</td>
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</tr>
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<td>35.0–39.9</td>
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</tr>
<tr>
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<td>Class III obesity</td>
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APPENDIX 3

Ethical clearance

Ethics Review Committee
Faculty of Medical Sciences, University of Sri Jayewardenepura
Gangodawila, Nugegoda, Sri Lanka

ERC meeting date: 23rd February 2012

Mr. WD Wickramasinghe
301, Habaraluwewa
Sevanagala

Application No: 615/11
Novel cardiovascular risk markers Paroxonase Apolipoprotein A1 and Glutathione Peroxidase genotype – 1 in coronary artery disease

We are pleased to inform you that the FMS/JUSJP ERC at its meeting held on 23rd February 2012 has granted ethical approval for your project as per details given below:

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<tr>
<th>Document</th>
<th>Version No</th>
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</table>

The ethical approval is effective from 28th February 2012. Please note that ethical approval would be withdrawn if any alteration is made to the project without obtaining prior written consent from the ethics review committee.

Dr. C. A. Wanigatunge
Chairperson

Dr. V. Jayasuriya
Secretary

Address all correspondence to: Secretary, Ethics Review Committee, Department of Community Medicine, Faculty of Medical Sciences, University of Sri Jayewardenepura, Gangodawila, Nugegoda, Sri Lanka. Tel: 94-11-2795988, Fax 94-11-2811480, erc.fms.sjup@gmail.com
APPENDIX 4
Information sheet used for patients (English)

I am Mr. W. D. Wickramasinghe, a graduate in B. Sc (Hons) in Medical Laboratory Sciences, Faculty of Medical Sciences, University of Sri Jayewardenepura. This research project is conducted by me as a postgraduate study.

This research is conducted to assess “Novel cardiovascular risk markers Paraoxonase, Apolipoprotien A-I (apoA- 1) and Glutathione Peroxidase genotype -1 in Coronary Artery Disease”.

I conduct this research under the supervision of

**Principal Supervisor:-**
Prof. Hemantha Peiris (BV.Sc., M.Phil, PhD) Professor of Biochemistry, Faculty of Medical Sciences, University of Sri Jayewardenepura.

**Supervisors:-**
Prof. L. G. Chandrasena (BSc, PhD, FRSC, Fl C Chem, FACB, FNASSL) Emeritus Professor, Department of Biochemistry, University of Kelaniya; Director, Clinical Laboratory, Nawaloka Hospitals PLC, Colombo 02.
Dr. Vajira Senaratne (MBBS, MD, FRCP), Consultant Cardiologist, National Hospital, Colombo.
Dr. P. P. Rasika Perera (MBBS, PhD) Senior Lecturer, Department of Biochemistry, Faculty of Medical Sciences, University of Sri Jayewardenepura.

The sample of this research includes 240 Participants (80 awaiting Coronary Artery Bypass Grafting (CABG) / Percutaneous Trans Coronary Angioplasty (PTCA) and 160 healthy people from routine health screening) at Nawaloka Hospitals PLC, Colombo 02.

We are conducting a research to assess the risk factors affected with Coronary Artery Disease (CAD). The outcome of this study would help in early diagnosis and improve the management of CAD patients. No additional cost or blood samples will be taken
from you as a participant of this study. Your information is kept privacy and confidentiality by the responsible research team.

If you volunteer for the study you will be required to give 5ml of blood to conduct the study to assess Paraoxonase, Apolipoprotein A-1 (apoA-1) and Glutathione Peroxidase genotype -1 (testing for a gene) in Coronary Artery Disease.

If any new findings come out of the study, we would like to use your blood samples to do genetic testing for other associated genes besides Glutathione Peroxidase genotype -1. For this, we need your specific consent. Blood samples will not be used for any other purposes.

The privacy of the research participants and the confidentiality of data provided by them are completely protected. Participants have the right to refrain from participating in the study and or leave the study at any point.

Participants are entirely free to clarify about the problems they have regarding the study at any time.

We would like to participate you our study as a volunteer case.
APPENDIX 5
Information sheet used for patients (Sinhala)

මහාමු. දැයින් විය මට ප්‍ර යි සැමාවානි ආශ්‍රයුතයේ නිර්දේශයක් නොමැති විය සෙසු විට මහාමු දැයින් දමුමක් දිය (උෂ්ණියක් ගැබෙන් යිය). මෙම ආරාමීය ආශ්‍රයුතයේ මෙම ප්‍රධාන ආශ්‍රයුතයේ මව විස්තර සැමාවානි ප්‍රථම ලිපියක් අයි. මෙය පිළිතික පිළිතුරු පරිණාමව මෙම ආශ්‍රයුතයේ මව විස්තර සැමාවානි ප්‍රථම ලිපියක් අයි.

මෙම ආශ්‍රයුතය ප්‍රධාන ස්තූෂ දෙකුට,

මතික විස්තර

මතික විස්තරයේ අවසන් ස්ථාන

මතික ලාංකික කාර්ය, කතා බෝගිය මෙය

මතික විස්තරයේ අවසන් ස්ථාන

මතික ලාංකික කාර්ය, කතා බෝගිය මෙය

විවිධ

විවිධ විස්තරයේ අවසන් ස්ථාන

විවිධ ලාංකික කාර්ය, කතා බෝගිය මෙය

විවිධ

විවිධ විස්තරයේ අවසන් ස්ථාන

විවිධ ලාංකික කාර්ය, කතා බෝගිය මෙය

210 පවුලුවල 240 පවුලුවල 105. උජෝජතා කාර්ය (0 CABG/PTCA කාර්ය පැවිත්තු මෙයි, 160 - ඉඨනාගන්නගන්න)

3 පසු පමණක් පසු පමණ පැවිත්තු රාජ බෝගිය මෙය මෙම මෙම බෝගිය කාර්ය සහ මෙම මෙම බෝගිය කාර්ය සහ මෙම මෙම බෝගිය කාර්ය සහ මෙම මෙම බෝගිය කාර්ය සහ මෙම මෙම බෝගිය කාර්ය සහ මෙම මෙම බෝගිය කාර්ය.

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වම් සහිතික ප්‍රශ්ඨාංගකාරී මාළිග පිළිතුරු අමාත්‍යික සමාජයේ සොයා සැමා (05ml) යින් කිරීමට නො සිටී පොකු නිසා සැමා අමාත්‍යික සමාජයේ සොයා සැමා. 

වෙනස්වාරාමා මාළිග පිළිතුරු සොයා සැමා අමාත්‍යික සමාජයේ සොයා සැමාවේවිට-1 සොයා සැමා අමාත්‍යික සමාජයේ සොයා සැමා සොයා සැමාවේවිට-1 සොයා සැමා අමාත්‍යික සමාජයේ සොයා සැමා. 

වෙනස්වාරාමා මාළිග පිළිතුරු සොයා සැමාවේක් අමාත්‍යික සමාජයේ සොයා සැමාවේක් අමාත්‍යික සමාජයේ සොයා සැමාවේ සොයා සැමාවේක් අමාත්‍යික සමාජයේ සොයා සැමාවේ සොයා සැමාවේ. 

මාළිග පිළිතුරු සොයා සැමාවේ අමාත්‍යික සමාජයේ සොයා සැමාවේ අමාත්‍යික සමාජයේ සොයා සැමාවේ අමාත්‍යික සමාජයේ සොයා සැමාවේ අමාත්‍යික සමාජයේ සොයා සැමාවේ.

වෙනස්වාරාමා මාළිග පිළිතුරු සොයා සැමාවේ අමාත්‍යික සමාජයේ සොයා සැමාවේ අමාත්‍යික සමාජයේ සොයා සැමාවේ අමාත්‍යික සමාජයේ සොයා සැමාවේ අමාත්‍යික සමාජයේ සොයා සැමාවේ.
APPENDIX 6
Information sheet used for controls (English)

I am Mr. W. D. Wickramasinghe, a graduate in B.Sc (Hons) in Medical Laboratory Sciences, Faculty of Medical Sciences, University of Sri Jayewardenepura. This research project is conducted by me as a postgraduate study.

This research is conducted to assess “Novel cardiovascular risk markers Paraoxonase, Apolipoprotien A-1 (apoA-1) and Glutathione Peroxidase genotype -1 in Coronary Artery Disease”.

I conduct this research under the supervision of

Principal Supervisor:-
Prof. Hemantha Peiris (BV.Sc., M.Phil, PhD) Professor of Biochemistry, Faculty of Medical Sciences, University of Sri Jayewardenepura.

Supervisors:-
Prof. L. G. Chandrasena (BSc, PhD, FRSC, FI C Chem, FACB, FNASSL) Emeritus Professor, Department of Biochemistry, University of Kelaniya; Director, Clinical Laboratory, Nawaloka Hospitals PLC, Colombo 02.
Dr. Vajira Senaratne (MBBS, MD, FRCP), Consultant Cardiologist, National Hospital, Colombo.
Dr. P. P. Rasika Perera (MBBS, PhD) Senior Lecturer, Department of Biochemistry, Faculty of Medical Sciences, University of Sri Jayewardenepura.

The sample of this research includes 240 Participants (80 awaiting Coronary Artery Bypass Grafting (CABG) / Percutaneous Trans Coronary Angioplasty (PTCA) and 160 healthy people from routine health screening) at Nawaloka Hospitals PLC, Colombo 02.

We are conducting a research to assess the risk factors affected with Coronary Artery Disease (CAD). The outcome of this study would help in early diagnosis and improve the management of CAD patients. No additional cost or blood samples will be taken...
from you as a participant of this study. Your information is kept privacy and confidentiality by the responsible research team.

If you volunteer for the study you will be required to give 5ml of blood to conduct the study to assess Paraoxonase, Apolipoprotein A-1 (apoA-1) and Glutathione Peroxidase genotype -1 (testing for a gene) in Coronary Artery Disease.

If any new findings come out of the study, we would like to use your blood samples to do genetic testing for other associated genes besides Glutathione Peroxidase genotype -1. For this, we need your specific consent. Blood samples will not be used for any other purposes.

The privacy of the research participants and the confidentiality of data provided by them are completely protected. Participants have the right to refrain from participating in the study and or leave the study at any point.

Participants are entirely free to clarify about the problems they have regarding the study at any time.

We would like to participate you our study as a volunteer control if as you do not have any past history of heart disease or chronic diseases.
APPENDIX 7
Information sheet used for controls (Sinhala)

අඩු පිවිශේෂණ අත් මැනුණක ආරම්භ

මායිමේ. පිවිශේෂණ පෙළ මො සැමෙන්නාවට පිළිකාර්මක නැතීමෙන් එකිනෙකුත් පිවිශේෂණ පෙළ පිළිකාර්මක නැතීමෙන් එකිනෙකුත් පිවිශේෂණ පෙළ (සියුසි) ගාණනයට අවශ්‍ය. එකිනෙකුත් පිවිශේෂණ පෙළ පිළිකාර්මක කලාප නැතීමෙන් ගැනීමට අවශ්‍ය.

වැඩ පිවිශේෂණේ වැඩයේ මිටියක් අද වෘක්‍රාමිකේ මිටියක් පිළිකරනු ලබන්නේ ගැටුම පිවිශේෂණේ ලෙස A1 සහ පිවිශේෂණේ නැතිමෙන් 1 මෙටරික් පිළිකාර්මක නැතී.

කේන්ද්‍ර විශේෂයක තානම,

කේන්ද්‍ර විශේෂය

විශේෂ වියවරණවත් මිටිය

විශේෂ වියවරණවත් මිටිය, පිවිශේෂණ කෝලා, පිවිශේෂණ කෝලා

යෝජනාව ප්‍රධාන

යෝජනාව විශේෂයේ විශේෂය, පිවිශේෂණ කෝලා, පිවිශේෂණ කෝලා

240 එක්වලක් විශේෂයේ විශේෂයේ විශේෂයම. (80 CABG/PTCA විකල්ප විකල්ප විකල්ප, 160 - පිවිශේෂණක්)

240 එක්වලක් විශේෂයේ විශේෂයම. (80 CABG/PTCA විකල්ප විකල්ප විකල්ප, 160 - පිවිශේෂණක්)
ම කාලාභාවිත්තමව අති කොටස් නිර්මාණ සඳහා උදාහරණම පාලනය කළේ (05ml) මෙමින් පැනෙන විට නිශ්චිත විශේෂ ශිල්පි සමඟ පුළුත් සමග සිදුකොටවීමේදී, 
කාලාභාවිත්තමවේ A-1 සහ ප්‍රධාන සමඟාභාවිත්තමවේ-1 උදාහරණ පැහැදිලි ප්‍රධාන සිනමාවේ සමඟා සාමාන්යික විශේෂීය විද්‍යාවේදී පිළිබඳ උදාහරණ දක්නට ලැබේ.

විශේෂීය විද්‍යාවේ පරේරණ අතරින් සාමාන්යික උදාහරණ විශේෂීය විද්‍යාවේ පරේරණ වසරින් ආරක්ෂා කරන විද්‍යාව සමඟ ප්‍රධාන විද්‍යාව මටින් පිළිබඳ උදාහරණ දක්නට ලැබේ. උදාහරණ අතරින් පරේරණ උදාහරණ විද්‍යාව මටින් පිළිබඳ උදාහරණ දක්නට ලැබේ.

කාලාභාවිත්තමවේ A-1 සහ ප්‍රධාන ශිල්පිවල් විශේෂීය විද්‍යාව මටින් පිළිබඳ උදාහරණ දක්නට ලැබේ. උදාහරණ අතරින් පරේරණ උදාහරණ විද්‍යාව මටින් පිළිබඳ උදාහරණ දක්නට ලැබේ.

ම අතරින් පරේරණ උදාහරණ විද්‍යාව මටින් පිළිබඳ උදාහරණ දක්නට ලැබේ.
APPENDIX 8

Research consent form used for patients (English)

Name and address of the investigator:

Mr. W. D. Wickramasinghe (B.Sc.(Hons) in MLS)
Faculty of Graduate Sciences
University of Sri Jayawardanepura, Gangodawila, Nugegoda.

Under the supervision of,

Principal Supervisor:-
Prof. Hemantha Peiris (BV.Sc., M.Phil, PhD) Professor of Biochemistry,
Faculty of Medical Sciences, University of Sri Jayewardenepura.

Supervisors:-
Prof. L. G. Chandrasena (BSc, PhD, FRSC, FI C Chem, FACB, FNASSL) Emeritus Professor, Department of Biochemistry, University of Kelaniya; Director, Clinical Laboratory, Nawaloka Hospitals PLC, Colombo 02.
Dr. Vajira Senaratne (MBBS, MD, FRCP), Consultant Cardiologist, National Hospital, Colombo.
Dr. P. P. Rasika Perera (MBBS, PhD) Senior Lecturer, Department of Biochemistry, Faculty of Medical Sciences, University of Sri Jayewardenepura.

Address of the institution where the study is to be carried out
Nawaloka Hospitals PLC, Colombo 02

The purpose of the study (with a brief description of the procedure to be carried out):

The main purpose of this study is “Novel cardiovascular risk markers Paraoxonase, Apolipoprotein A-1 (apoA-1) and Glutathione Peroxidase genotype -1 in Coronary Artery Disease”. This study, which will include 240 participants (80 Patients and 160 healthy) selected according to convenience sampling method.
Glutathione Peroxidase -1 (GPX-1) genotype is known to be a cardiovascular protector and the underlying post genetic susceptibility in Coronary Artery Disease (CAD) may be of importance in risk assessment of patients with CAD. The Paraoxonase is believed to be a potent antioxidant by inhibiting the oxidation of LDL with apoA-1 and thereby reducing the risk of Atherosclerosis. Hence, the antioxidant properties of the above markers may have future benefits in the management of patients with CAD in Sri Lanka.

The study has been explained to me and I understood,
   a. What the study involves
   b. That refusal to participate in the study will not affect my private life anyway.
   c. That I may withdraw at any time and it will not affect me adversely in any manner

I therefore agree to participate in the study as a case and give consent to use my blood for further studies, including genetic analysis, on the topic if necessary in the future.

Signature of the participant

Full name

Date

Postal address

I have been present while the procedure has been explained to the participant and I have witnessed his/her consent to take part in the study.

Signature of witness

(The witness should be a person NOT connected with the study)

Full name

Date

Postal address
APPENDIX 9
Research consent form used for patients (Sinhala)

research consent form used for patients (Sinhala)

We are conducting a study to investigate the relationship between dietary factors and certain health outcomes. This study is being carried out in the Department of Nutrition at the University of Colombo. The study involves collecting detailed information about participants' dietary habits and other relevant factors.

The study involves a comprehensive examination of participants, including medical history, dietary assessment, and various health-related tests. Participants will be followed up for a period of two years to monitor changes in their health status.

Participants will be divided into two groups: a control group and an intervention group. The intervention group will receive personalized dietary counseling and support to encourage healthier eating habits.

The study will be conducted in compliance with all relevant ethical guidelines and regulations. Participants will be informed about the study's purpose, methods, and potential risks and benefits. The confidentiality of all information collected will be strictly maintained.

Participation is voluntary, and participants may withdraw from the study at any time without any consequences. Participants will be reimbursed for their time and effort.

Your participation is crucial to the success of this study. We appreciate your willingness to contribute to this important research.
විධායකයේ ප්‍රශ්න මෙන් ගැටලුවේ ප්‍රශ්නය මෙන් මෑලක්වේදීමින් නැහැඳියේ.

1. මෑලක්වේදීමින්
2. ක්‍රමයේ ප්‍රශ්නයේදීමින් මෙන් ගැටලුවේ ප්‍රශ්නය මෙන් මෑලක්වේදීමින්
3. ක්‍රමයේ ප්‍රශ්නයේදීමින්

විධායකයන් ප්‍රශ්න මෑලක්වේදීමින් ප්‍රශ්නය මෑලක්වේදීමින් ප්‍රශ්නය මෑලක්වේදීමින්


c:…………………………………………………………

මෑලක්වේදීමින් ක්‍රමයේදීමින්

මෑලක්වේදීමින් ක්‍රමයේදීමින්

c:…………………………………………………………

මෑලක්වේදීමින් ක්‍රමයේදීමින්

මෑලක්වේදීමින් ක්‍රමයේදීමින්
APPENDIX 10
Research consent form used for controls (English)

Name and address of the investigator:

Mr. W. D. Wickramasinghe (B.Sc.(Hons) in MLS)
Faculty of Graduate Sciences
University of Sri Jayawardanepura, Gangodawila, Nugegoda.
Contact No.: - 0783816027

Under the supervision of,

Principal Supervisor:-
Prof. Hemantha Peiris (BV.Sc., M.Phil, PhD) Professor of Biochemistry,
Faculty of Medical Sciences, University of Sri Jayewardenepura.

Supervisors:-
Prof. L. G. Chandrasena (BSc, PhD, FRSC, FI C Chem, FACB, FNASSL) Emeritus Professor, Department of Biochemistry, University of Kelaniya; Director, Clinical Laboratory, Nawaloka Hospitals PLC, Colombo 02.
Dr. Vajira Senaratne (MBBS, MD, FRCP), Consultant Cardiologist, National Hospital, Colombo.
Dr. P. P. Rasika Perera (MBBS, PhD) Senior Lecturer, Department of Biochemistry, Faculty of Medical Sciences, University of Sri Jayewardenepura.

Address of the institution where the study is to be carried out
Nawaloka Hospitals PLC, Colombo 02.

The purpose of the study (with a brief description of the procedure to be carried out):
The main purpose of this study is “Novel cardiovascular risk markers Paraoxonase, Apolipoprotien A-1 (apoA- 1) and Glutathione Peroxidase genotype -1 in Coronary Artery Disease”. This study, which will include 240 participants (80 Patients and 160 healthy) selected according to convenience sampling method.
Glutathione Peroxidase -1 (GPX-1) genotype is known to be a cardiovascular protector and the underlying post genetic susceptibility in Coronary Artery Disease (CAD) may be of importance in risk assessment of patients with CAD. The Paraoxonase is believed to be a potent antioxidant by inhibiting the oxidation of LDL with apoA-1 and thereby reducing the risk of Atherosclerosis. Hence, the antioxidant properties of the above markers may have future benefits in the management of patients with CAD in Sri Lanka.

The study has been explained to me and I understood,

a. What the study involves
b. That refusal to participate in the study will not affect my private life anyway.
c. That I may withdraw at any time and it will not affect me adversely in any manner

I therefore agree to participate in the study as a control and give consent to use my blood for further studies, including genetic analysis, on the topic if necessary in the future.

Signature of the participant
Full name
Date
Postal address

I have been present while the procedure has been explained to the participant and I have witnessed his/her consent to take part in the study.

Signature of witness
Full name
Date
Postal address

(The witness should be a person NOT connected with the study)
APPENDIX 11
Research consent form used for controls (Sinhala)

මෙම අමතරයින් ශ්‍රීංග්‍රහණය කළා දෙන්න වඩා වඩාත්ම ආදර්ශනය විය යි.

මුහුදම් කිසිවකම් පැරණි හා හොඳම පිරිසිරා පැවා අවන්නාව විය වශයෙන්, මුකු ආ-1 යන අතරින් එක්සත්මතික පවත්කම් -1 ලෙසින් පුළුළු මැටි විය යි.

මුහුදම් කිසිවකම් පැරණි හා හොඳම

පැවා අවන්නාව විය වශයෙන්, මූලාංග කිසිකම්, මුකු ආ-1 යන අතරින් එක්සත්මතික පවත්කම්.

මුහුදම්

පැවා අවන්නාව විය වශයෙන්, මූලාංග කිසිකම්, මුකු ආ-2 යන අතරින් එක්සත්මතික පවත්කම්, මෙහෙය ප්‍රදේශයද, මුකු ආ-1 යන අතරින් එක්සත්මතික පවත්කම්.

මුහුදම් කිසිකම්

පැවා අවන්නාව විය වශයෙන්, මූලාංග කිසිකම්, මූලාංග කිසිකම්, මූලාංග කිසිකම්, මූලාංග කිසිකම්, මුකු ආ-2 යන අතරින් එක්සත්මතික පවත්කම්.

මුහුදම් කිසිකම්

පැවා අවන්නාව විය වශයෙන්, මූලාංග කිසිකම්, මූලාංග කිසිකම්, මූලාංග කිසිකම්, මූලාංග කිසිකම්, මූලාංග කිසිකම්, මූලාංග කිසිකම්, මූලාංග කිසිකම්, මූලාංග කිසිකම්, මූලාංග කිසිකම්, මූලාංග කිසිකම්, මියිනිස්සීම් කිසිකම්, මූලාංග කිසිකම්.
මෙහෙළ විශේෂ අංකයන් සහ අදහස්වත් ඔබේ විශේෂිතතාවේ නාමයේ යොදා කරන විය. මෙය පෙළ කළ ගැටේක් පිළිබලිකම පෙර, මෙමෙක් ඉතික්කම අංකය පෙන්වූ විට අතර පිළිබලී විය. මෙමෙක් ආභිදි නිලධාරී විය නිසා දක්වා ලබා ගනන්නේ බිම්කොට්ඨ විය අතර.

1. අභිජනනයක අක්කර

2. අභිජනනය කාලයේ අයුරින් අතර පිළිබලී පුභක් සාමාන්‍ය පිළිබලී නාමයන්නේ පිළිබලී යර්ක කෙනෙකු.

3. අභිජනනය විශේෂිත විශේෂිත මාර්ගය දක්වා ගන්නේ මෙහෙ Sri Lanka එක් මෙන් ඛාපු දක්වා තුළ පවසී විට.

විශේෂිත කාලය දෙදවට, මෙහෙප්දේෂිතයන් පුභක් කාලයේ කොටසය ගැන පිළිබලීය තැන්නේ අභිජනනය නාමයන්නේ ඇති අදහස්වත්ට ආභිදි විය. මෙහෙප්දේෂිත අංකයට කුඩා විශේෂිත විශේෂිත පුභක් නොමැතියන්නේ ආභිදි විය. මෙහෙප්දේෂිත පුභක් විශේෂිත කාලයට අංකයන්නේ ඇති අදහස්වත්ට ආභිදි විය.

ඒකජනත්ව නම් අදහස්වත්

ඒකජනත්ව අදහස්වත්

ඒකජනත්ව අදහස්වත්