PARAOXONASE ACTIVITY IN TYPE 2 DIABETES MELLITUS MALAY PATIENTS IN HOSPITAL UNIVERSITI SAINS MALAYSIA (HUSM)

By

DR HANIM AFZAN BINTI IBRAHIM

A Dissertation Submitted In Partial Fulfillment of the Requirements For the Degree of Master of Pathology (Chemical Pathology)

UNIVERSITI SAINS MALAYSIA

2015

ACKNOWLEDGEMENTS

IN THE NAME OF ALLAH, THE BENEFICIENT, THE MERCIFUL.

I would like to express my appreciation to the following individuals for their support, guidance and help to bring forth this project to reality and praise be to ALLAH, without whose grace this dissertation would not have been completed. I am very grateful to my main supervisor, Assoc. Prof Dr. K.N.S Sirajudeen from Department of Chemical Pathology and co-supervisors, Dr. Julia Omar also the Head of Chemical Pathology Department and Dr. Wan Mohd Izani from Department of Internal Medicine for their guidance, support, patience and encouragement throughout the study and writing of this thesis.

Not forgetting my appreciation goes to the laboratory staffs who consistently and patiently helped me with the sample collection and analysis especially to En. Mohd Rafi, scientific officer from chemical pathology and Pn Jamaayah, Scientific officer from Sports Science Unit.

I wish to thank Assoc. Prof Dr Muhammad Rusli Abdullah from Department of Community Medicine and Pn. Anis, statistician from Institute Postgraduate Studies for their cooperation throughout the project and guidance on the statistical analysis of my data.

To my colleagues, Dr Noorazliyana Shafii, Dr Wan Ruhma Wan Hassan, Dr Noor Azlin Azraini Che Soh, Dr Siti Hajar Umar, Dr. Wan Norlina Wan Azman and Dr Nor Amani Ashari, I would like to thank them for their encouragement and support throughout the rough times that we have experienced together.

Last but not least, my deepest appreciation to my husband, Zulkhairi bin Othman, for all the love, support and encouragement that he has given me during this trying time and to my beloved children, Mohammad Abid Baihaqi and Nur Imanina Qaisara for their toleration of my preoccupation. I am also indebted to my parents, Ibrahim bin Wel and Naimah binti Omar for their great moral and spiritual support in making this thesis possible. Lastly, to everybody who has involved in this study either directly or indirectly.

An appreciation to the School of Dental Sciences for the financial support and this study was supported by USM short term grant (304/PPSG/61312123).

TABLE OF CONTENTS

10. APPENDICES

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABCA1	: ATP binding cassette A1
ADP	: Adenosine diphosphate
AGEs	: Advances glycated end products
Apo A-1	: Apolipoprotein A-1
AREase	: Arylesterase
ATP	: Adenosine triphosphate
$CaCl_2$	: Calcium chloride
CAD	: Coronary artery disease
CE	: Cholesteryl ester
CETP	: Cholesterol ester transfer protein
CKD	: Chonic kidney disease
CM	: Chylomicron
CSF	: Colony stimulating factor
CVA	: Cerebrovascular disease(stroke)
DR	: Diabetic retinopathy
EC	: Endothelial cell
ECM	: Extracellular matrix
FDAOS	: N-acetyl-N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxy-4-fluoroaniline
GSH	: Glutathione
HbA1c	: Glycated haemoglobin

HCl	: Hydrogen chloride
HDL	: High density lipoprotein
HDL-C	: High density lipoprotein cholesterol
HL	: Hepatic lipase
H_2O	: Water
H_2O_2	: Hydrogen peroxide
HRP	: Horse radish peroxidase
IHD	: Ischaemic heart disease
LCAT	: Lecithin cholesterol acyltransferase
LDL	: Low density lipoprotein
LDL-C	: Low density lipoprotein cholesterol
LPL	: Lipoprotein lipase
MØ	: Macrophage
MCP-1	: Monocyte chemoattractant protein 1
NaCl	: Sodium chloride
NAD^+	: Nicotinamide adenine dinucleotide
NADPH	: Nicotinamide adenine dinucleotide phosphate
NHMS1	: First National Health and Morbidity Survey
NHMS2	: Second National Health and Morbidity Survey
O_2	: Oxygen
oxLDL	: Oxidized LDL
PAFAH	: Platelet activating factor acyl hydrolase
PON	: Paraoxonase

PON1	: Paraoxonase 1
PON2	: Paraoxonase 2
PON3	: Paraoxonase 3
PR	: Peripheral neuropathy
RAGEs	: Receptor of advanced glycated end products
RCT	: Reverse cholesterol transport
ROS	: Reactive oxygen species
SMC	: Smooth muscle cells
TC	: Total cholesterol
T2 DM	: Type 2 Diabetes Mellitus
TG	: Triglyceride
TMB	: Tetramethylbenzidine
VLDL	: Very low density lipoprotein
VLDL-C	: Very low density lipoprotein cholesterol

ABSTRAK

AKTIVITI PARAOXANASE DALAM PESAKIT MELAYU DIABETES MELLITUS JENIS 2 DI HOSPITAL UNIVERSITI SAINS MALAYSIA (HUSM)

Pengenalan : Paraoxonase (PON) adalah enzim yang mempunyai kaitan dengan lipoprotein yang berketumpatan tinggi (HDL). PON berfungsi mengawal tindakbalas detoksifikasi peroksida lipid dan turut memainkan peranannya untuk mengurangkan tekanan oksidatif yang cenderung kepada risiko terhadap komplikasi diabetes mellitus jenis 2 (T2 DM) yang mempunyai hubungan yang rapat dengan kawalan diabetik pesakitnya. Keupayaan PON untuk melindungi lipoprotein berketumpatan rendah (LDL) keatas pengoksidaan adalah berbeza di antara tiga bentuk polimorfik (A, AB, B). PON dengan fenotip B adalah kurang berkesan dalam melindungi LDL daripada pengoksidaan. **Objektif:** Tujuan kajian ini adalah untuk mengetahui aktiviti PON1, PON1 fenotip polimorfik , profil lipid dan LDL teroksida (oxLDL) di kalangan pesakit diabetik Melayu dengan kumpulan kawalan bebas diabetik , hubungan mereka dengan komplikasi diabetik dan korelasi antara aktiviti PON dan lipid profil . **Kaedah** : Seramai 99 individu yang telah dipilih sebagai subjek dan mereka telah dibahagikan kepada tiga kumpulan [(kawalan bebas diabetik, diabetik terkawal (HbA1c $\leq$6.5 %) dan diabetik tidak terkawal (HbA1c > 6.5%)] (setiap kumpulan n = 33). Analisa sampel sera berpuasa telah dilakukan untuk aktiviti PON basal, aktiviti PON dengan rangsangan garam, aktiviti arylesterase (AREase), profil lipid dan oxLDL. Fenotip bagi PON adalah A, B dan AB dibuat berdasarkankan kepada nisbah aktiviti PON dengan rangsangan garam kepada aktiviti AREase. **Keputusan:** aktiviti PON basal dan aktiviti PON dengan rangsangan garam tidak berbeza di kalangan tiga kumpulan. Aktiviti AREase adalah lebih rendah pada pesakit T2 DM berbanding kumpulan kawalan bebas diabetik . Majoriti subjek mempunyai fenotip AB dan

tiada satu pun subjek kawalan bebas diabetic mempunyai fenotip B. Pesakit diabetik dengan komplikasi penyakit buah pinggang kronik (CKD) menunjukkan aktiviti PON dengan rangsangan garam adalah lebih rendah berbanding dengan mereka yang tidak mempunyai CKD. Analisa profil lipid bagi kumpulan diabetik tidak terkawal menunjukkan rendah nilai jumlah kolesterol (TC), HDL dan LDL serta lebih tinggi nilai trigliceride (TG) berbanding kumpulan kawalan bebas diabetik . Korelasi positif telah diperolehi antara HDL dan aktiviti PON basal bagi kumpulan diabetik tidak terkawal dan korelasi positif antara TC dan HDL dengan aktiviti PON rangsangan garam juga dalam kumpulan diabetik tidak terkawal .Tahap OxLDL tidak memberi perbezaan ketara antara tiga kumpulan namun nisbah PON kepada oxLDL (PON/oxLDL) menunjukkan nilai lebih tinggi dalam kumpulan kawalan bebas diabetik berbanding dengan kumpulan diabetik tidak terkawal. **Kesimpulan:** Aktiviti AREase adalah rendah dalam kumpulan T2 DM berbanding kumpulan kawalan bebas diabetic dan majoriti daripada subjek(populasi Melayu) mempunyai fenotip AB.Nisbah PON/oxLDL bagi kumpulan diabetik tidak terkawal lebih kecil berbanding dengan kumpulan kawalan bebas diabetik dan kumpulan diabetik terkawal walaupun nilai PON dan oxLDL tidak signifikasi. Oleh itu, kumpulan diabetik tidak terkawal masih cenderung kepada risiko komplikasi.

ABSTRACT

PARAOXONASE ACTIVITY IN TYPE 2 DIABETES MELLITUS MALAY PATIENTS IN HOSPITAL UNIVERSITI SAINS MALAYSIA (HUSM)

Introduction: Paraoxonase (PON) is an enzyme associated with high-density lipoprotein (HDL). PON is involved in the detoxification of lipid peroxides and play a role in decreasing oxidative stress which are related with the risk for complication in diabetes mellitus type 2(T2 DM) patients that is strongly associated with their diabetic control. The ability of PON to protect low density lipoprotein (LDL) against oxidation has been shown to vary between the three polymorphic forms (A, AB, B). PON with phenotype B is less efficient in protecting LDL against oxidation. **Objectives:** The purpose of this study was to determine the PON1 activities, PON1 phenotypic polymorphism, lipid profile and oxidized LDL (oxLDL) in T2 DM among Malay population and healthy control group, their relation with diabetic complications and the correlation between PON activities and lipid profile. **Methods:** A total of 99 subjects were chosen for three groups (healthy control, good control diabetes mellitus (HbA1c $\leq$ 6.5%) and poor control diabetes mellitus(HbA1c > 6.5%) (each group n=33). Fasting serum were analysed for PON, salt stimulated PON, arylesterase (AREase) activities, lipid profile and oxLDL. The phenotypes assesment for PON can be A, B, AB based on the ratio of salt stimulated PON to the AREase activity. **Results:** The basal PON and salt stimulated PON activities were not statistically significant among the three groups. AREase activity was lower in T2 DM patients compared to healthy control.Majority of subjects were AB phenotype and none of the healthy control subjects with B phenotype. Diabetic patients with chronic kidney disease (CKD) showed significantly lower salt stimulated PON activity when compared to those without CKD.

Lipid profile analyses for poor control diabetic group showed significantly lower in total cholesterol (TC), HDL and LDL level and higher in triglyceride (TG) level compared to healthy control. A positive correlation between the HDL levels and basal PON activity in poor control diabetic and a positive correlation between the TC and HDL with salt stimulated PON activity in poor control group. OxLDL level was not statistically significant among the three groups however the PON to oxLDL ratio (PON/oxLDL) shows significantly higher in healthy control compared to poor control diabetic group. **Conclusion**: AREase activity was lower in T2 DM patients compared to healthy control and majority of subjects (Malay population) were AB phenotype. Poor control T2 DM group had lesser PON/oxLDL ratio when compared with healthy control and good control T2DM although PON and oxLDL levels not significant. Thus it could relate with the risk of complications in poor control T2 DM**.**

1. INTRODUCTION

1.1 TYPE 2 DIABETES MELLITUS

Type 2 diabetes mellitus (T2 DM) is a disease of metabolic dysregulation, involving the impaired uptake and the utilization of glucose, altered lipid metabolism, the accumulation of various lipid species in the circulation and in the tissues, and the disruption of metabolic signaling pathways that regulate insulin secretion from the pancreatic beta-cells (Mafauzy, 2006). Previous studies have shown that increased levels of oxidative damage to lipids in diabetes, and their presence correlated with the development of complications. Several studies have demonstrated that the increased susceptibility of low density lipoprotein (LDL) to oxidation and higher levels of oxidized LDL in diabetes mellitus correlated with an increased risk of cardiovascular complications (Syvänne and Taskinen, 1997).

T2 DM is a common disease causing significant mortality and morbidity. In Malaysia, the First National Health and Morbidity Survey (NHMS 1) conducted in 1986 reported a prevalence of diabetes mellitus of 6.3% and in the Second National Health and Morbidity Survey (NHMS 2) in 1996, the prevalence had risen to 8.2%. A study in Kelantan, the prevalence was reported to be higher at 10.5% (Syvänne and Taskinen, 1997).

1.2 COMPLICATIONS OF TYPE 2 DIABETES MELLITUS

Plasma lipids alteration in patients with T2 DM were related to coronary artery disease, cerebrovascular disease and nephropathy (Syvänne and Taskinen, 1997). Every one mmol/L reduction of low-density lipoprotein- cholesterol (LDL-C) decreased 36% coronary artery diseases (CAD) risk (Syvänne and Taskinen, 1997). Glycated and oxidized LDL-C contribute to the progression of atherosclerosis by promoting vascular smooth muscle cells migration and proliferation (Chew *et al.*, 2012).

Lipoproteins in diabetes are subjected to increased oxidative stress, which may make it a more critical factor in diabetic atherosclerosis than in atherogenesis from other causes (Abbott *et al.*, 1995). The mechanism by which high density lipoprotein (HDL) renders its protective effect against atherosclerosis was focus on its role of HDL in reverse-cholesterol transport (RCT) (Ayub *et al.*, 1999). However, recent studies have suggested more diversity in the role of HDL in atherogenesis that HDL has reported to have protection against LDL oxidative modification (Mackness *et al.*, 1993) which is believed to be central to the initiation and progression of atherosclerosis.

Oxidative stress and oxidative damage to tissues are common end points of chronic diseases, such as atherosclerosis, diabetes, and rheumatoid arthritis. Increased oxidative stress has a primary role in the pathogenesis of diabetic complications (Baynes and Thorpe, 1999). Increased oxidative stress is a widely accepted participant in the development and progression of diabetes and its complications (Baynes and Thorpe, 1999).

1.3 OXIDATIVE STRESS AND TYPE 2 DIABETES MELLITUS

The imbalance between the systemic manifestation of free radicals and a biological system's ability to detoxify the reactive intermediates has emerged as one of the principal causes of atherogenic modifications in low-density lipoproteins (LDL) and, consequently, of atherosclerotic disease (Steinberg, 1997). The increase in glycoxidation and lipoxidation products in plasma and tissue proteins suggests that oxidative stress is increased in diabetes (Baynes and Thorpe, 1999). Diabetes is usually accompanied by increased production of free radical or impaired antioxidant defenses (Baynes and Thorpe, 1999). Mechanisms by which increased oxidative stress is involved in the diabetic complications including activation of transcription factors, advanced glycated end products (AGEs), and protein kinase C (Maritim *et al.*, 2003).

Excessively high levels of free radicals cause damage to the cellular proteins, membrane lipids and nucleic acids, and cell death (Maritim *et al.*, 2003). Various mechanisms have been suggested to contribute to the formation of these reactive oxygen-free radicals (Maritim *et al.*, 2003). Glucose oxidation is believed to be the main source of free radicals. In its enediol form, glucose is oxidized in a transition-metal dependent reaction to an enediol radical anion that is converted into reactive ketoaldehydes and to superoxide anion radicals. The superoxide anion radicals undergo dismutation to hydrogen peroxide and in the presence of transition metals, can lead to production of extremely reactive hydroxyl radicals (Wolff and Dean, 1987).

Hyperglycemia is also found to promote lipid peroxidation of LDL by a superoxide-dependent pathway resulting in the generation of free radicals (Wolff and Dean, 1987). Another important source of free radicals in diabetes is the interaction of glucose with proteins leading to the formation of an Amadori product and then AGEs

(Hori *et al.*, 1996). These AGEs, via their receptors (RAGEs), inactivate enzymes and alter their structures and functions, promote free radical formation (Vlassara, 1997). By increasing intracellular oxidative stress, AGEs activate the transcription factor that enhances the production of nitric oxide, which is believed to be a mediator of islet beta cell damage (Maritim *et al.*, 2003).

Activation of the sorbitol pathway by glucose as a component in the pathogenesis of diabetic complications, for example, in lens cataract formation and peripheral neuropathy (Kador and Kinoshita, 1984; Greene *et al.*, 1992). Various hypotheses were made for the formation of the cataract and in the aldose reductase osmotic hypothesis, accumulation of polyols can initiates lenticular osmotic changes. In addition, oxidative stress is linked to decreased glutathione levels and depletion of nicotinamide adenine dinucleotide phosphate (NADPH) levels(Cheng and González, 1986). Increased sorbitol dehydrogenase activity is associated with altered nicotinamide adenine dinucleotide (NAD+) levels, which results in protein modification by nonenzymatic glycosylation of lens proteins (Williamson *et al.*, 1993). Mechanisms linking the changes in diabetic neuropathy and induced sorbitol pathway are not well delineated. One possible mechanism, metabolic imbalances in the neural tissues, has been implicated neurotransmission changes, Schwann cell injury, and axonopathy (Kalichman *et al.*, 1997).

Diabetic hyperglycemia results in an increase in free-radical production by the mechanism involving glucose oxidation followed by protein glycation and oxidative degeneration. When molecules have slow turnover rates, these Amadori products undergo multiple dehydration reactions and rearrangements to irreversibly form AGEs (Maritim *et al.*, 2003). AGEs are involved in the production of many of the irreversible

complications of diabetes, including expanded extracellular matrix, cellular hypertrophy, hyperplasia, and vascular complications (Brownlee *et al.*, 1988).

Markers that used for estimating the degree of protein glycation in diabetes include fructosamine and glycated hemoglobin (HbA1c) levels. Nonenzymatic glycation may alter the structure and function of antioxidant as they are unable to detoxify free radicals, exacerbating oxidative stress in diabetes (Maritim *et al.*, 2003).

1.4 OXIDIZED LDL IN TYPE 2 DIABETES MELLITUS

Atherosclerotic vascular diseases are more common in T2 DM than nondiabetic populations. Furthermore, these diseases constitute the principal cause of mortality among diabetic patients (Uusitupa *et al.*, 1996). Oxidized LDL (oxLDL) may play a fundamental role in the pathogenesis of atherosclerosis (Witztum and Steinberg, 1991). Chronic hyperglycemia and its complications may increase oxidative stress in diabetes (Baynes and Thorpe, 1999). In addition to their role in the formation and accumulation of foam cells in the arterial intima during the development of early atherosclerosis, oxLDL particles may accelerate the progression of more advanced atherosclerotic lesions (Witztum and Steinberg, 1991).

Atherosclerosis involves LDL oxidation and platelet activation and both of these processes can affect each other. Platelet activation is increased by oxidative stress, and oxidized LDL was shown to enhance platelet activation. Activated platelets, in turn can increase LDL oxidizability (Figure 1.1).

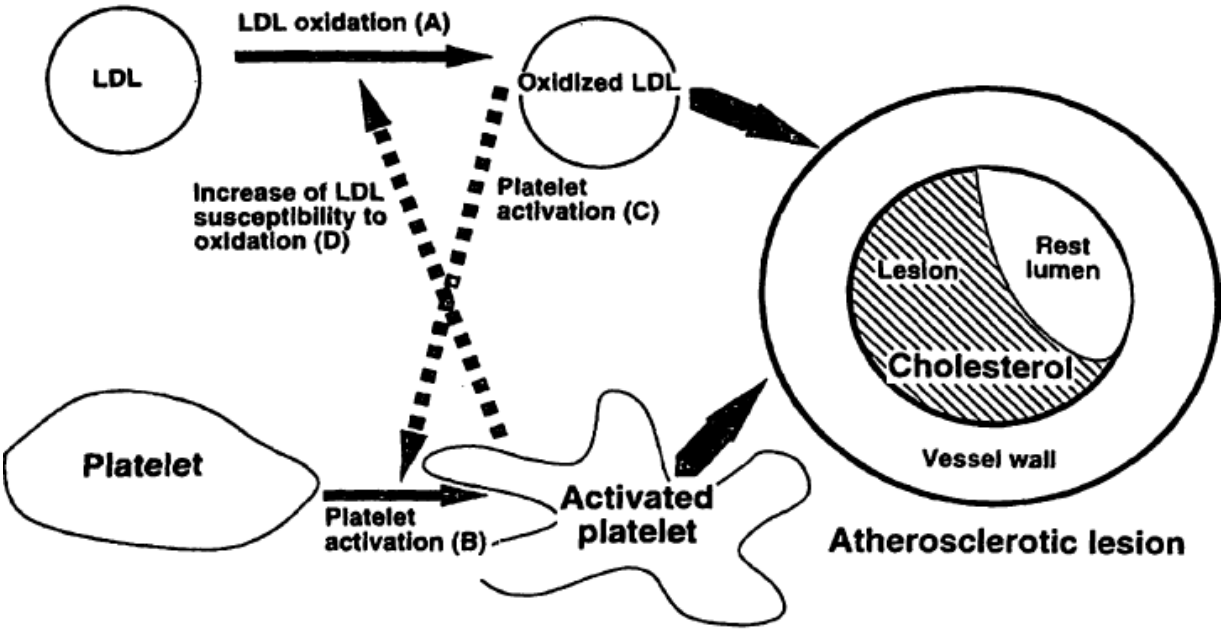

Figure1.1: Oxidized LDL, platelet activation and atherosclerosis.

LDL oxidation (A) and platelet activation (B) can lead to the formation of the atherosclerotic lesion. These processes are interrelated, in that oxidized LDL can activate the platelets (C), and activated platelets increase the susceptibility of LDL to oxidation (D).

Source:(Aviram, 1996)

Oxidized LDL possesses several atherogenic properties such as increase uptake by macrophages for the foam cell formation, act as chemoattractant for circulating monocytes, inhibit the movement of tissue macrophages back to the circulation, act as chemoattractant for T-lymphocytes, alter gene expression of neighbouring cells [monocyte chemoattractant protein 1 (MCP-1), colony stimulating factors(CSF)], can elicit autoantibody formation, inhibit nitric oxide-stimulation of vasodilation, enhance coagulation processes and increase platelet activation which can also contribute to the development of the atherosclerotic lesion (Aviram 1996).

Cholesterol in the atherosclerotic lesion accumulates as cholesteryl ester droplets and as unesterified cholesterol (Kruth, 1984). This is due to the uptake of oxidized LDL via scavenger receptors (Sparrow *et al.*, 1989). Arterial wall macrophages were shown to accumulate oxidized lipids in areas of the atherosclerotic lesion and these cells can oxidize LDL in the vascular interstitial space. Macrophage-mediated oxidation of LDL is a key event in the early atherosclerosis and under certain oxidative conditions, LDL bind to the cellular LDL receptor (Aviram and Rosenblat, 1994). The susceptibility of LDL to oxidation depends both on the activities of cellular oxygenases and on the cellular antioxidants (Figure 1.2).

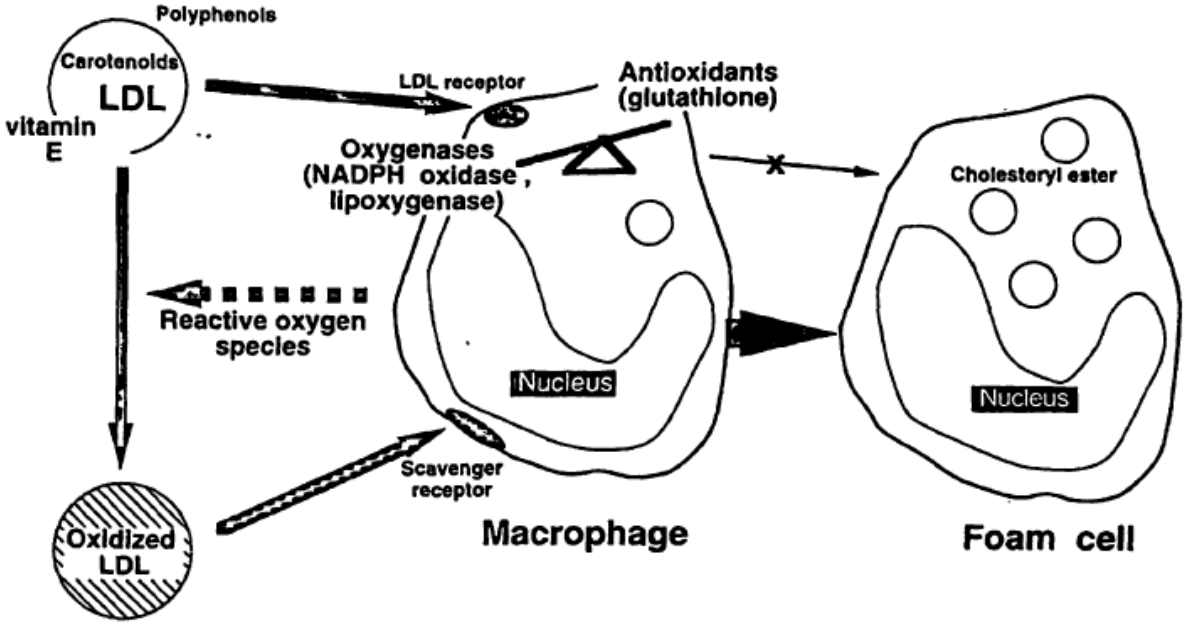

Figure 1.2: Lipoproteins and antioxidants.

LDL oxidation by macrophages is affected by the antioxidants and by the balance between cellular antioxidants [such as glutathione (GSH)] and cellular oxygenases [such as NADPH oxidase and lipoxygenases]. oxLDL can lead to the conversion of macrophages to foam cells.

Source:(Aviram, 1996)

1.5 ANTIOXIDANTS

While on the one hand hyperglycemia promotes free radicals, on the other hand it also impairs the endogenous antioxidant defense system in many ways in diabetes (Saxena *et al.*, 1993) . Antioxidant defense mechanisms involve both enzymatic and nonenzymatic strategies. Common antioxidants include the vitamins A, C, and E, glutathione, and the enzymes superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and paraoxonase (Maritim *et al.*, 2003; Sentí *et al.*, 2003). The other antioxidants include α-lipoic acid, mixed carotenoids, coenzyme Q10, several bioflavonoids, antioxidant minerals (copper, zinc, manganese, and selenium), and the cofactors (folic acid, vitamins B1, B2, B6, B12) (Maritim *et al.*, 2003). Each antioxidant has different mechanism of reducing oxidative stress and they work in synergy with each other and against different types of free radicals (Rani and Mythili, 2014). Vitamin E suppresses the propagation of lipid peroxidation; vitamin C, with vitamin E, inhibits hydroperoxide formation; metal complexing agents, such as penicillamine, bind transition metals involved in some reactions in lipid enzyme and the PON 1 enzyme closely associated with HDL and it helps in preventing and reverting LDL oxidation (Aviram *et al.*, 1998b) (Figure 1.3).

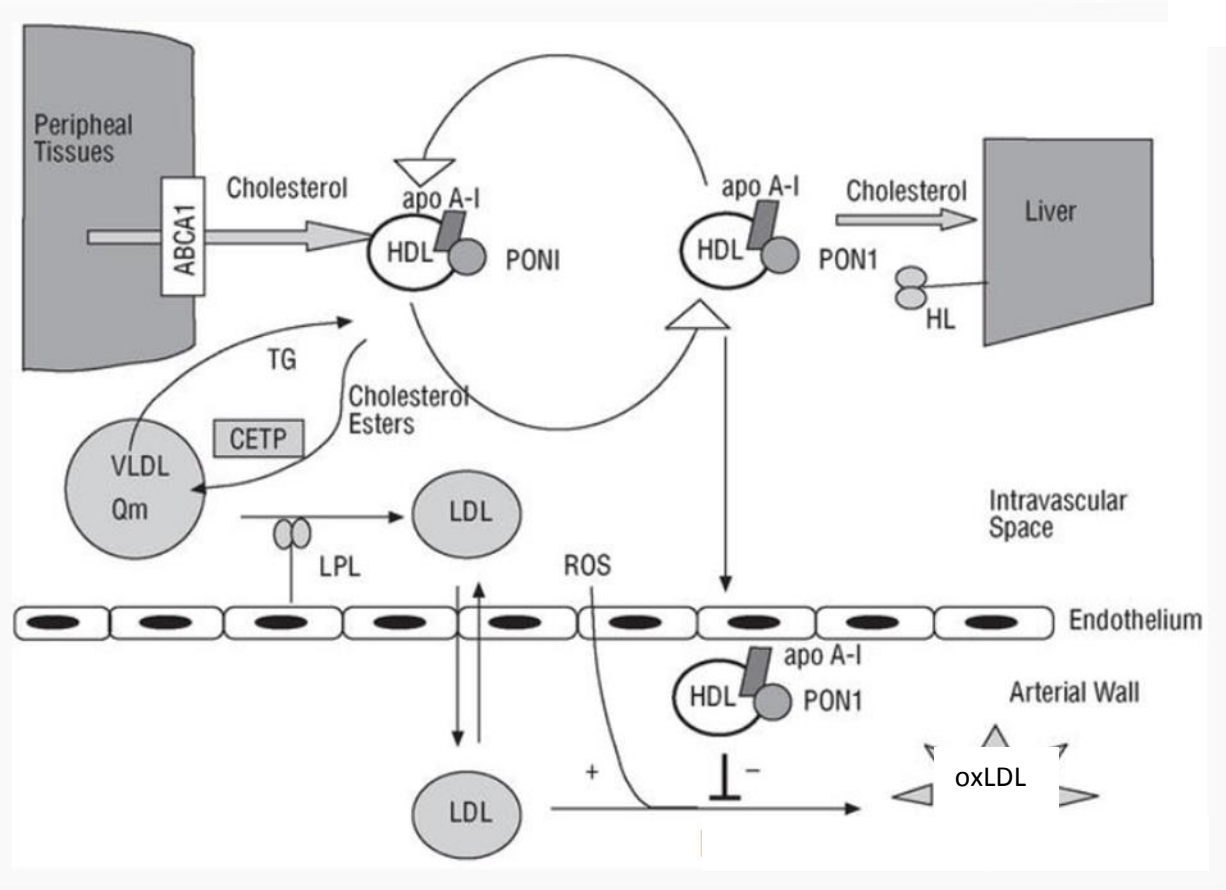

Figure 1.3: The antioxidant role of PON1 in arteriosclerotic plaque formation.

ABCA1 indicates ATP-binding cassette A1; apo A-I, apolipoprotein A-I; CETP, cholesterol ester transfer protein; HL, hepatic lipase; LPL, lipoprotein lipase; PON1, paraoxonase-1; Cm, chylomicron; ROS, reactive oxygen species; TG, triglycerides; VLDL, very-low-density lipoprotein.

Source:(Tomás *et al.*, 2004)

The antioxidant activity of high-density lipoprotein is largely due to its PON1 content. Experiments with transgenic PON1 knock-out mice indicate the potential for this enzyme to protect against atherogenesis. PON1 hydrolyzes lipid hydroperoxides and is a potent lactonase. One of its natural substrates is homocysteine thiolactone, a mediator in hyperhomocysteinemia-associated LDL damage. PON1 activity could be a target for pharmacological intervention. PON1 also decreases during inflammation and is atheroprotective in animal models of hypercholesterolemia (Gugliucci *et al.*, 2006).

Lipid peroxides inhibit the paraoxonase, arylesterase and antioxidant activities of PON1, probably via interactions with a sulfur group on the enzyme (Aviram *et al.*, 1998a). One important consequence of this phenomenon is that, if HDL is oxidized, there will be an accompanying reduction in paraoxonase activity and, therefore, also a reduction in the enzyme's protective activity against LDL oxidation (Aviram *et al.*, 1998a).

1.6 PARAOXONASE ENZYME

Paraoxonase is an aryldialkylphosphatase synthesized in the liver and transported in the systemic circulation exclusively in association with a HDL subfraction containing apolipoprotein (apo A-1)(Abbott *et al.*, 1995). PON are a family of enzymes comprising 3 members, PON1, PON2 and PON3, whose genes are located adjacent to each other on chromosome 7 (Primo-Parmo *et al.*, 1996). They all possess antioxidant properties, share 65% similarity at the amino acid level(Cabana *et al.*, 2003). In humans, PON1 and PON3 genes are produced in many cell types and their protein products are found in the circulation bound to HDL. PON2 is an intracellular enzyme which is not found in plasma but particularly in the cells associated with the artery wall and in macrophages, and may not be associated with lipoproteins (Ng *et al.*, 2001). Among the three members of PON, PON1 is the most widely studied. All these enzymes are able to prevent LDL oxidation and cellular oxidative stress (Abbott *et al.*, 1995).

PON1 circulates in plasma tightly bound to HDL using its unprocessed hydrophobic signal sequence as an anchor into the lipoprotein particles (Sorenson *et al.*, 1999). Some authors have reported PON1 protein expression in kidney and aorta (Mackness *et al.*, 1997). PON1 also was found in chondrocytes, enterocytes, eye lens and retinal layers, skin epidermis, stomach, tongue and trachea. Since PON1 metabolizes toxic agents such as oxidized lipids, it would be logical to find the protein where its function is needed. In this vein, PON1 was also found in the muscle fibers of both skeletal and cardiac muscle, areas where free radicals are produced as a consequence of energy metabolism. PON1 also plays a protective role against lipid peroxidation, so it is not surprising to find this protein in adipocytes, and acini from exocrine pancreas, submandibular gland and sebaceous gland. Consistent with this

concept, PON1 is also expressed in cells where oxidative stress occurs and lack of detoxication could result in significant disease in liver, kidney proximal tubules, fiber tracts of the encephalon and the spinal cord. Finally, inability to modulate oxidative stress could contribute to infertility, emphasizing the importance of finding PON1 in ovary follicular fluid, seminiferous tubules and spermatozoa (Furlong *et al.*, 2010).

PON1 is a calcium-dependent esterase that is known to catalyze hydrolysis of organophosphates, and is widely distributed among tissues such as liver, kidney, intestine, and also serum (La Du *et al.*, 1993). PON specificity towards endogenous serum and tissue substrates is not well-characterized, and therefore synthetic substrates are used to monitor the enzyme's activity (Aviram *et al.*, 1998b). Serum PON activity was shown to be reduced in patients after myocardial infarction (McElveen *et al.*, 1986), in patients with familial hypercholesterolemia (Mackness *et al.*, 1991b), and in patients with diabetes mellitus (Mackness *et al.*, 1991b; Abbott *et al.*, 1995) in comparison to healthy subjects. Although PON1 can offer protection against the toxicity of some organophosphates, its physiological role is still not known; however, evidence exists for a protective effect of PON against oxidative damage (Mackness *et al.*, 1993). PON was suggested to contribute to the antioxidant protection conferred by HDL on LDL oxidation (Mackness *et al.*, 1993).

HDL-associated enzymes other than PON, such as lecithin/cholesterol acyltransferase (LCAT) and platelet-activating factor acyl hydrolase (PAFAH) were also implicated in the antioxidative properties of HDL (Mackness *et al.*, 1993). In fact, HDL has been shown to be the major carrier of lipid hydroperoxides in human serum (Hahn and Subbiah, 1994). In this context it is of interest that HDL-associated cholesteryl ester hydroperoxides are more rapidly reduced to their less reactive hydroxides than are those associated with LDL (Christison *et al.*, 1995). Oxidative

modification of HDL has also been shown to impair the ability of the lipoprotein to promote cholesterol efflux (Nagano *et al.*, 1991). Thus, inhibition of HDL oxidation by PON may preserve the anti atherogenic functions of HDL in reverse cholesterol transport, as well as its protection of LDL from oxidation.

PON1 has an extremely hydrophobic N-terminal end that is stabilized by apolipoprotein A-1.The retention of the N-terminal hydrophobic leader sequence may facilitate the transfer of the enzyme between phospholipids surfaces and to the site of oxidative and inflammatory injury in order to inactive toxic lipid products resulting from oxidative damage as illustrated in figure 1.4. PON1 transits the default secretory pathway and is bound to the hepatocyte plasma membrane, as expected for a protein with a retained hydrophobic N-terminal signal peptide. PON1's N-terminus associates with HDL phospholipids and is stabilized by apoA-I. Movement of HDL particles away from hepatocytes prevents diffusion back to the hepatocyte plasma membrane. PON1 is able to enter the intravascular space with HDL. PON1 transfers to phospholipids in plasma membranes through its N-terminus under more static conditions favoring diffusion, perhaps during apoA-I–mediated recruitment of cholesterol from endothelial or smooth muscle cells. PON1 may therefore have access to the interstitium and areas of LDL accumulation and oxidative damage.

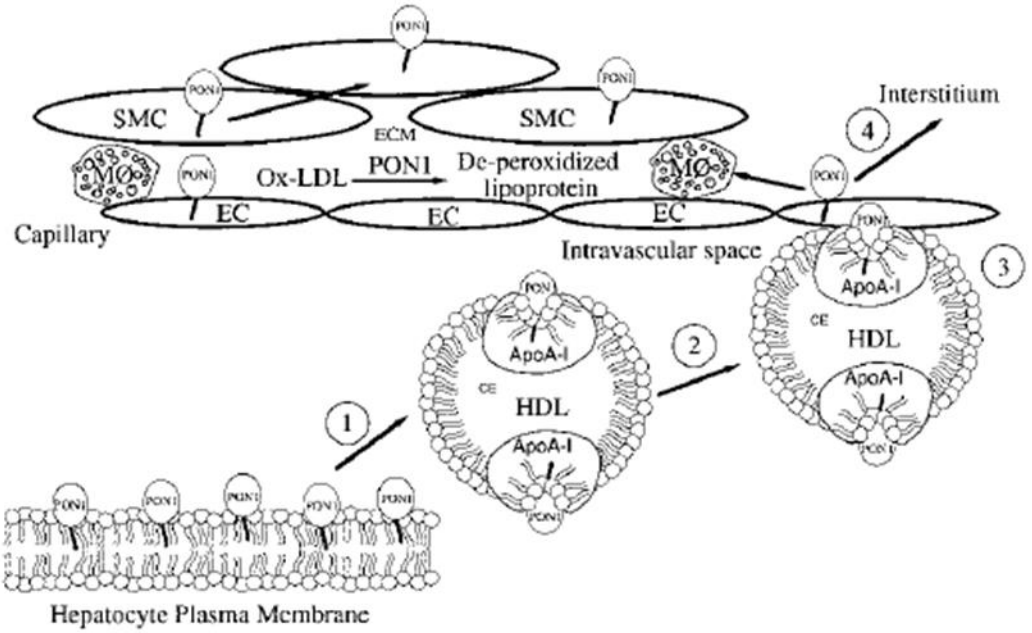

Figure 1.4: Potential role for the retained hydrophobic N-terminal peptide in PON1's association with HDL and its transfer to sites of oxidative and inflammatory injury.

The retained hydrophobic N-terminal signal peptide is represented by a thick, black line. EC indicates endothelial cells; SMC, smooth muscle cells; MØ, macrophages; ECM, extracellular matrix; Ox-LDL, products of LDL oxidation; and CE, cholesteryl esters.

Source: (Sorenson *et al.*, 1999)

In human serum, paraoxonase is in association with HDL which acts as its carrier and site of action. Several studies have shown serum HDL concentration to be inversely related to the risk of developing atherosclerosis. HDL was shown to be effective in preventing the oxidative modification of LDL. Paraoxonase isolated from human HDL in liposomes has also been shown to decrease the susceptibility of LDL to lipid peroxidation(Mackness *et al.*, 1993). This suggests a potential role for paraoxonase in the detoxification of lipid peroxides and suggests that individuals with a low paraoxonase activity may have a greater risk of developing a disease such as atherosclerosis, which may involve lipid peroxidation, than high-activity individuals(Abbott *et al.*, 1995).

In 1953, Aldridge divided esterases into two categories, those that catalytically hydrolyzed organophosphate substrates (A-esterases) and those that were inhibited by organophosphates(B-esterases) (Aldridge, 1953).PON1 is an A-esterase .It has three known enzymatic molecules, including PON, arylesterase (AREase), and diazoxonase. PON1 hydrolyzes organophosphates such as paraoxon, hydrolyzes aromatic esters such as phenylacetate, and also decreases the accumulation of lipid peroxidation products(Erdem *et al.*, 2010).

AREase, a thiol enzyme, is reactivated by 2-mercaptoethanol and cysteine but not by reduced glutathione. ARE acts on phenyl acetate to release phenol, which can produce a stable indophenol dye with 4-aminoantipyrine and potassium ferricyanide (Erdem *et al.*, 2010). Human serum PON1 and AREase are both esterase enzymes that have lipophilic antioxidant characteristics (Elkiran *et al.*, 2007). These enzymes play a role in decreasing oxidative stress. PON1 in particular is an important endogenous free radical scavenging system in the human body (Elkiran *et al.*, 2007). Serum PON1 acts in conjunction with AREase to function as a single enzyme (Gan *et al.*, 1991).

PON1 is an important component of HDL responsible in part for the ability of HDL to prevent LDL lipid peroxidation (Mackness *et al.*, 1991a). PAFAH has also been shown to inhibit the formation of lipid peroxides on LDL, and it is possible that HDL-associated PON and PAFAH act in concert to inhibit LDL lipid peroxidation and that a number of other HDL-associated proteins, such as apoA-1 and lecithin-cholesterol acyltransferase, may also aid this process(Watson *et al.*, 1995a). LDL modification by lipid peroxides might thus be accelerated in diabetes because of low paraoxonase activity, and this has been implicated in the genesis of atherosclerosis, the risk of which is increased in diabetes (Abbott *et al.*, 1995).

HDL has a well-established inverse correlation with the incidence of coronary disease. Several studies have shown that PON1 protects LDL and HDL against oxidative modification. It has been demonstrated that PON1 deficiency is related to increased susceptibility to LDL oxidation and development of atherosclerosis (Laplaud *et al.*, 1998).

PON1 endows HDL with its antioxidant properties and is probably responsible for the principal mechanism inhibiting the oxidation of both LDL and HDL itself, a process that is directly involved in the initial phases of arteriosclerosis (Tomás *et al.*, 2004). In vitro, PON1 neutralizes hydrogen peroxide (H_2O_2) and peroxidized lipids that are either free or present in atherosclerotic lesions or in minimally oxidized LDL (Aviram *et al.*, 1998b). PON1 may be able to activate platelet activating factor acetylhydrolase, an enzyme that hydrolyzes this well-known proinflammatory factor. This process could give PON1 its anti-inflammatory properties (Rodrigo *et al.*, 2001).

T2 DM is characterized by a raised blood glucose level, hypertriglyceridemia, increased oxidative metabolism, reduced HDL cholesterol concentration, high

prevalence of obesity, and accelerated arteriosclerosis (Malin *et al.*, 1999). T2 DM is also associated with the occurrence of cardiovascular events, which are probably linked to low HDL cholesterol concentrations rather than to elevated LDL cholesterol concentrations (Cao *et al.*, 1999). Moreover, T2 DM present with a lower paraoxonase activity level and a lower ratio of paraoxonase activity to HDL cholesterol level than those found in health control subjects (Ikeda *et al.*, 1998). In addition, diabetic patients who present with complications such as coronary heart disease, retinopathy or neuropathy have lower paraoxonase activity levels than diabetics without these complications (Abbott *et al.*, 1995; Garin *et al.*, 1997; Ikeda *et al.*, 1998). Paraoxonase activity decreases as the number of metabolic alterations linked to the metabolic syndrome rises and that this decrease is accompanied by increasingly high concentrations of lipid peroxides (Sentí *et al.*, 2003).

Apparently, PON1 activity declines as diabetes progresses and reaches a particularly low level in advanced stages of the disease (Tomás *et al.*, 2004). In patients with obesity associated with an elevated leptin concentration, reduced PON1, AREase and lactonase activities and increased oxidative stress have been observed (Tomás *et al.*, 2004). It has also been observed that paraoxonase activity and HDL concentrations are lower in diabetic patients undergoing hemodialysis than in non-diabetic patients undergoing hemodialysis (Zhang *et al.*, 2003). In vitro, an elevated glucose concentration reduces HDL's antioxidant capacity, partly because both paraoxonase activity and the ratio of paraoxonase activity to HDL cholesterol level decrease while the concentration of oxidation markers simultaneously increases (Hedrick *et al.*, 2000). In rats with streptozocin-induced diabetes, serum paraoxonase activity declines progressively with time (Patel *et al.*, 1990).

Previous studies have shown that increased levels of oxidative damage to lipids in diabetes, and their presence correlated with the development of complications and monitoring the trends in cardiovascular complications via PON1 is of critical importance in managing patients with T2 DM (Mackness *et al.*, 2000).

1.6.1 Paraoxonase Genotype

Many studies have indicated the existence of a genetic polymorphism of paraoxonase is a determinant of its activity . This polymorphism is due to an amino acid substitution in the active site of the enzyme, giving rise to low- and high-activity isoenzymes (Adkins *et al.*, 1993). The genetic basis of the paraoxonase polymorphism was first carefully investigated by Playfer *et al*. (1976). They concluded that high and low serum paraoxonase activities were controlled by two alleles at a single autosomal locus (Playfer *et al.*, 1976). The two alloenzymes are presumed to be products of a gene located on the long arm of chromosome 7 (Primo-Parmo *et al.*, 1996) . Molecular studies on human paraoxonase genes had been studied to determine the DNA basis for the paraoxonase polymorphism. Protein sequencing has revealed the possible occurrence of an intramolecular disulfide bond, the location of one asparagine-linked carbohydrate, and the first amino acids in the mature enzyme protein (Adkins *et al.*, 1993). Two common polymorphic sites at amino acids “55” and “192” have been identified (Hassett *et al.*, 1991).

The first polymorphism involves the substitution of amino acid glutamine (Q allele) for arginine (R allele) that affects amino acid 192 whereas the second polymorphism of the PON1 due to amino acid 55 that involves the substitution of

leucine (L allele) to methionine (M-allele) (Adkins *et al.*, 1993) (Figure 1.5). It has been shown that the PON1 genetic polymorphisms may be an independent risk factor for coronary artery disease (CAD); both the 55L and 192Q genotype have been shown to be associated with increased susceptibility to CAD (Ruiz *et al.*, 1995).

Between the two polymorphisms, PON1 192 polymorphism is a commonly occurring polymorphism that strongly modulates PON1 activity. Protection against organophosphates will depend not only on the level of the enzyme in blood and tissues but on the particular isozymes present as well (La Du *et al.*, 1999). The B-type, now called the R-isozyme, is several times more efficient than the A-type (Q-isozyme) in hydrolyzing paraoxon, but most organophosphates are hydrolyzed appreciably better by the Q- than the R-isoenzyme (La Du *et al.*, 1999). Thus, both the level and the type of PON1 must be taken into consideration in evaluating the protective role of PON1 against such compounds that may be substrates catalytically inactivated by the enzyme (La Du *et al.*, 1999).

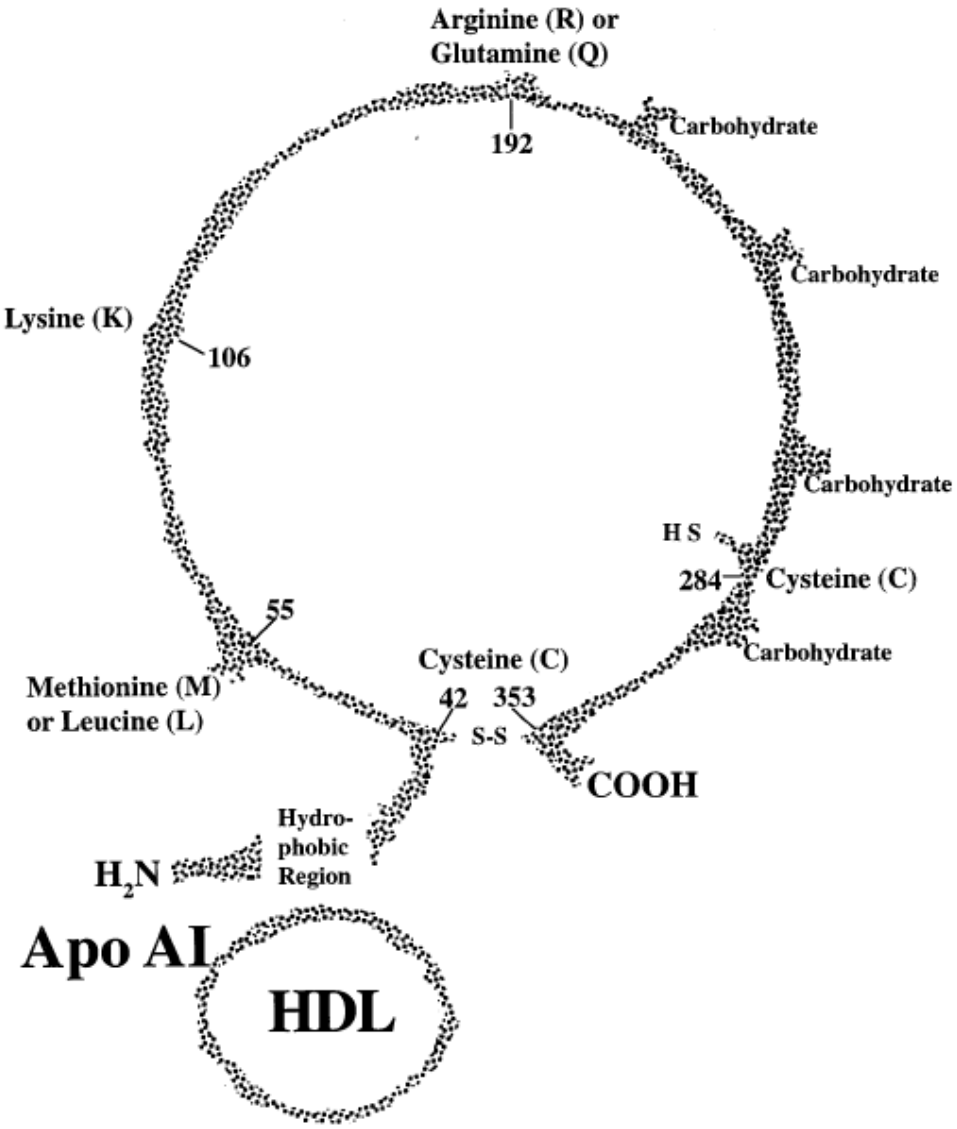

Figure 1.5: Human PON1 structure

Human PON1 structure illustrating the two polymorphic sites at positions 55 and 192, the internal disulfide bond between cysteine residues 42 and 353, the free cysteine at position 284, the location of potential sugar chains, and the hydrophobic retained leader sequence at the amino terminal end.

Source: (La Du *et al.*, 1999)

1.6.2 Paraoxonase Phenotypes

PON is an aromatic esterase that requires calcium for activity and it is not irreversibly inhibited by organophosphates, unlike the serum cholinesterase(Aldridge and Reiner, 1972). In Caucasian populations, serum paraoxonase activity is bimodally distributed and the level of activity appears to be inherited as a simple dominant Mendelian trait determined by two alleles at one autosomal locus (Playfer *et al.*, 1976).

The "high" and "low" alleles (Eiberg and Mohr, 1981), called esterase B and esterase A to emphasize the qualitatively different properties of the isozymes. The B isozyme is more highly stimulated by NaCl and usually has greater activity than the A isozyme (Eckerson *et al.*, 1983a); the two isozymes also have different apparent Km values, calcium requirements, and pH optima (Eiberg and Mohr, 1981).

AREase of human serum is also designated aromatic esterase (Aldridge, 1953). AREase activity has most often been measured with phenylacetate as the substrate (Eckerson *et al.*, 1983b). AREase activity is greatest for aromatic substrates with an acetate moiety (Eckerson *et al.*, 1983b). AREase, like PON, requires calcium for activity and is not inhibited by cholinesterase inhibitors such as eserine and organophosphates (La Du and Snady, 1971).

In spite of these similarities, there has been some doubt whether human serum PON and AREase activities are properties of the same enzyme or are two distinct enzymes. The distribution of aAREase in a Caucasian population has a single mode, whereas PON activity is bimodal (Eckerson *et al.*, 1983b).

A study within a Caucasian population from the United States shows the ratio of serum PON activity to AREase activity was distributed trimodally. All three paraoxonase phenotypes could be clearly identified by the ratio of paraoxonase/arylesterase activities. The paraoxonase/arylesterase ratio characteristic was inherited as a simple, autosomal Mendelian trait, without dominance, whereas, previously, the paraoxonase activity polymorphism was bimodally distributed. Furthermore, phenylacetate was an inhibitor of both the A- and B-type paraoxonase activity. These results are consistent with the hypothesis that a single gene locus determines the arylesterase/paraoxonase phenotypes of human serum, and, most likely, different isozymic forms of the enzyme accounts for the paraoxonase polymorphism and the different ratio of paraoxonase/arylesterase activities with the two substrates (Eckerson *et al.,* 1983b).

The phenotypes for PON1 192 polymorphism can be assigned as A,AB, or B based on the ratio of paraoxonase activity in the presence of 1 M sodium chloride (NaCl) to the arylesterase activity (Adkins *et al.,* 1993). Phenotype A has low activity towards paraoxon, phenotype B has high activity towards paraoxon and phenotype AB has intermediate level of activity (Adkins *et al.*, 1993).

The phenotypes for PON1 192 polymorphism can be assigned as A, AB and B based on the ratio of PON activity in the presence of 1 M NaCl to AREase activity. Phenotype ranges are 0.9 – 2.5 , 2.6 – 7.5 and 7.6 – 12.0 for A, AB and B- type alloenzyme (Adkins *et al.*, 1993).

HDL from PON-BB homozygotes is less efficient at protecting LDL against oxidation than HDL from PON-AA homozygotes, suggesting that the activity of the B alloenzyme of PON1 in metabolizing lipid peroxides is less than that of the A alloenzyme (Ayub *et al.*, 1999).

1.7 RATIONALE OF THE STUDY

Malays have predominant PON phenotype AB (44%) in a study of the healthy population done in Kelantan (Nor Zamzila Abdullah, 2004) . A reduction in activity towards paraoxon was associated with a significant increase in risk for cardiovascular disease complications in diabetic patients. Variation in PON1 activity towards paraoxon was observed with ethnicity. Malay ethnic origin have significantly higher than expected activity towards paraoxon than subjects of Chinese and Indian subjects (Poh and Muniandy, 2007). However in another study shows Chinese has the higher paraoxanose activity compare to Malay and Indian subjects (Nor Zamzila Abdullah, 2004).

PON1 study in T2 DM was done all over the world and it is differs among ethnicity. However among Malay T2 DM patients it is not well studied. Therefore, this study will be carried out to find out the PON1 activities and the distribution of PON1 phenotypic polymorphism among Malay population with T2 DM with and without complication. PON1 activity can also be a marker and monitoring tools in assessing the complication of T2 DM.