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PARAOXONASE 1 (PON 1) Q192R GENOTYPE DISTRIBUTION IN GHANAIANS WITH TYPE II DIABETES MELLITUS

BY

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THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF MPHIL MEDICAL BIOCHEMISTRY DEGREE

JULY 2013

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DECLARATION

I hereby declare that this project is my own research work carried out under punctilious supervision, at the Department of Medical Biochemistry, University of Ghana Medical School and the National Diabetes Management and Research Centre (NDMRC). This work has not been presented, neither in part or whole for the award of any degree elsewhere. The authors of other studies used in this work have been duly acknowledged. I am entirely responsible for any mistakes found in this report.

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ABSRACT

Background: Recent studies have indicated PON 1 polymorphism to be associated with cardiovascular-related diseases such as diabetes mellitus but varied reports have been provided from such association studies. In Ghana, there is no such information on PON 1 polymorphism in disease situations; except one instance reported in non-diseased Ghanaians. The present study has therefore provided baseline information on PON 1 Q192R polymorphism in Ghanaians with type II Diabetes Mellitus.

Main Objective: To determine the PON 1 Q192R genotypes in Ghanaian type II diabetes mellitus patients and its impact on clinical variables

Design: The study was located at the National Diabetes Management and Research Centre (NDMRC), Korle Bu Teaching Hospital, Accra. Subjects were recruited consecutively at the outpatients' clinic of the NDMRC; and interrogated through a questionnaire for demographic and clinical information. Fasting blood samples were collected from 112 Type II diabetes mellitus patients and 97 non-diabetic control participants. The fasting plasma glucose and lipid profiles were measured. DNA extracted from the diabetic and control buffy–coat samples was used in determining the PON 1 Q192R polymorphism, by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) analysis.

Results: The R allele was strongly associated with diabetes mellitus. All three PON 1 Q192R genotypes (QQ, RR, and QR) were present in Ghanaians with corresponding frequencies in Type II diabetic population being 18.18%, 74.55%, 7.27% and 33.33%, 57.14%, 9.52% in the control group respectively ($\chi^2 = 3.432$, $p = 0.1798$). The type II

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diabetics in all the PON 1 Q192R genotype groups recorded significantly higher TG and VLDL levels ($p < 0.05$).

Conclusion: The RR genotype was the most frequent in the populations studied, whereas the QR genotype was the least frequent. The R allele was significantly associated with type II diabetes mellitus. PON 1 Q192R RR and QQ genotypes were associated with high FPG levels in the diabetics.

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CITATION

Knowledge is gained by searching for it and sharing what you have gained.

Akuba-Muhyia Annan, 2013

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DEDICATION

I dedicate this work to God the Father Almighty, who by His mercies has seen me through the entire period of my study, and to my family for supporting me in every phase of my life.

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ACKNOWLEDGEMENT

This work has been done not by my strength but by the might and grace of God. I owe a debt of gratitude to Him and to all the people He used as His instruments to help me.

First, I wish to thank Professor S. Asante-Poku for his guidance and provision of equipment. I record my indebtedness to Dr. Nii Ayite Aryee for his immense help. Here I wish to mention his financial assistance, direction, tolerance, and silent criticism which kept me on my toes.

I render an appreciation to Professor A. G. B. Amoah of the Department of Medicine (U.G.M.S) for providing me with materials which helped me to start this work.

I am also grateful to Dr. Bartholomew Dzudzor, Head of Department, Medical Biochemistry (U. G. M. S), for the trouble he took to find out constantly how the work was progressing. Profound gratitude is expressed to Dr. Grace K. Ababio for the items she made available to help me undertake the laboratory analysis. Auntie Grace, I very much appreciate the knowledge you imparted indirectly to me through your comments and the pain you took to ensure that I presented the best.

Thanks are also due to Mr. Tagoe (School of Allied Health Sciences, U. G. M. S) for his dedicated support and guidance and to Mr. Asmah (School of Allied Health Sciences, U. G. M. S) for laboratory space and kind permission to use the School's laboratory equipment.

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I offer special appreciation to Dorothy Honny, Albert Amenyedor, Agnes Dorbu, Hope Azasu, Elizabeth Gyamfi, Mr. Adu, Mr. Alhassan and the entire staff of the Department of Medical Biochemistry (U. G. M. S.) for helping in diverse ways from the beginning of the study to its successful completion.

Finally I am grateful to my family and friends, especially Crosby, Awo Salo, Ama-Ekyi, Anyanzu, Nyamenlekpole, Caleb, Yehoda and Christian for offering me comfort and for supporting me with their prayers.

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ABBREVIATIONS

μL	Micro litre
3-DG	3-Deoxyglucosone
4-HDoHE	(±) 4-hydroxy-5, 7, 10, 13, 16, 19-docosahexaenoic acid
5-HETEL	(±) 5-hydroxy-6, 8, 11, 14-eicosatetraenoic acid 1, 5-lactone
A_{260}	Absorbance at 260nm
A_{280}	Absorbance at 280nm
ABCA1	ATP binding cassette transporter A1
AGE	Advanced glycation end product
ANOVA	Analysis of variance
Apo	Apolipoprotein/apoprotein
AR	Aldose reductase
Arg	Arginine
BMI	Body mass index
bp	base pair
bpm	beats per minute
CE	Cholesteryl ester
CHD	Coronary heart disease
CI	Confidence interval
CL-OOH	Cholesteryl linoleate hydroperoxides
DAG	Diacylglycerol
DBP	Diastolic blood pressure
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates

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EDTA	Ethylenediaminetetra acetic acid
EPF	Early pregnancy failure
F-3-P	Fructose-3- phosphate
Fig.	Figure
FPG	Fasting plasma glucose
Gln	Glutamine
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
HDL	High density lipoprotein
HDL-C	High density lipoprotein cholesterol
hrs	Hours
IDDM	Insulin dependent diabetes mellitus
kDa	kilodaltons
kg	kilogram
LADA	Latent autoimmune diabetes in adults
LCAT	Lecithin–cholesterol acyl transferase
LDL	Low density lipoprotein
LDL-C	Low density lipoprotein cholesterol
Leu	Leucine
LPC	Lysophosphatidylcholine
m^2	Square meter
mAmp	Milliamperes
Met	Methionine
$MgCl_2$	Magnesium chloride

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min	Minutes
mL	Millilitre
mM	Millimolar
mmHg	Millimeters of mercury
mol L-1	Mol per litre
MW	Molecular weight
NaCl	Sodium chloride
NAD(P)H	Nicotinamide adenine dinucleotide phosphate (reduced)
NADH	Nicotinamide adenine dinucleotide
NDMRC	National Diabetes Management and Research Centre
NF-κb	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIDDM	Non-insulin dependent diabetes mellitus
nm	Nano meters
nmol/L	Nano mol per litre
No.	Number
NOx	NADH oxidase
OGTT	Oral glucose tolerance test
ox-LDL	Oxidized low density lipoprotein
PAF-AH	Platelet activating factor acetylhydrolase
PCR	Polymerase chain reaction
PKC-α	Protein kinase C-α
PL	Phospholipids
PL-OOH	Phospholipid hydroperoxides
PON 1	Paraoxonase 1
PON 2	Paraoxonase 2

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PON 3	Paraoxonase 3
RAGE	Receptor of advanced glycation end products
RCC	Renal cell carcinoma
RFLP	Restriction fragment length polymorphism
ROS	Reactive oxygen species
rpm	Revolutions per minute
SBP	Systolic blood pressure
SD	Standard deviation
SDH	Sorbitol dehydrogenase
sec	Second
SNP	Single nucleotide polymorphism
SPSS	Statistical package for social sciences
TAE	Tris acetate diaminoethanetetra- acetic acid
Taq	*Thermus aquaticus*
TC	Total cholesterol
TG	Triglycerides
U. G. M. S	University of Ghana Medical School
UC	Unesterified cholesterol
VLDL	Very low density lipoprotein
WHO	World health organization
yrs	Years

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CHAPTER ONE

1.0 INTRODUCTION

Paraoxonase 1 (PON 1), a high density lipoprotein (HDL)-associated enzyme, has been linked with diabetes mellitus, coronary heart disease and cardiovascular related diseases in many studies (Hassett *et al*., 1991; Odawara *et al*., 1997; Mackness *et al*., 1998b; Mackness *et al*., 2002b; Graner *et al*., 2006). It is an anti-oxidant which is capable of inhibiting the oxidation of lipids as well as preventing the formation of their atherogenic forms (Watson *et al*., 1995; Tward *et al*., 2002; Rosenblat and Aviram, 2011). Due to its antioxidant properties, PON 1 has been related to the presence or absence of macrovascular and microvascular complications in type II diabetic patients (Mackness *et al*., 1998b; Mackness *et al*., 1998c; Mackness *et al*., 2002b). In these patients, the activity of the PON 1 enzyme has been reported to be low (Ruiz *et al*., 1995; Mackness *et al*., 1998b; Mackness *et al*., 2002b).

PON 1 activity in diabetic patients and normal control individuals has been demonstrated to be under the influence of the PON 1 gene (Mackness *et al*., 1998b; Mackness *et al*., 1998c; Abessolo *et al*., 2012). The gene exhibits polymorphisms in its coding region, which may result in amino acids substitutions in the protein product. Two of the PON 1 gene polymorphisms cause the substitutions of Glutamine (Gln) with Arginine (Arg) at amino acid position 192, and Methionine (Met) with Leucine (Leu) at position 55. The 192 and 55 polymorphisms have been reported to largely account for the variations in the activity and serum concentration of the enzyme (Mackness *et al*., 1998c; Leviev *et al*., 1997).

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PON 1 Q192R polymorphism produces two allozymes classified as allozyme-A and allozyme-B. Allozyme-A corresponds to the polymorph with Gln (Q) at position 192 of the PON 1 gene; whilst the polymorph substituted with Arg (R) gives the allozyme-B (Eckerson *et al*., 1983a; Eckerson *et al*., 1983b). Allozyme-A exhibits a low action against substrates like paraoxon and protects diabetic patients from cardiovascular diseases. On the other hand, the B-allozyme shows high *in vitro* activity and less protection from cardiovascular diseases (Ruiz *et al*., 1995). Some studies have however given different reports for the protective abilities of both allozymes (Cao *et al*., 1999, Flekac *et al*., 2008).

The alleles (Q and R) of the 192 polymorphism combine to give three genotypes – the QQ genotype, the RR genotype and the QR genotype. Many different allelic and genotypic frequencies have been reported in some populations (Pejin-Grubiša, 2012). The PON 1 Q192R genotypes and alleles have also been associated with type II diabetes and diabetic-complications (Odawara *et al*., 1997; Elattar *et al*., 2012). Although a relationship has been found between type II diabetes and the PON 1 activity, Q192R alleles and genotypes, in various studies elsewhere, no relationship has been established between PON 1 and the type II diabetic population in Ghana: this is the focus of the current study.

1.1 Problem Statement

PON 1 gene variants influence LDL oxidation, which is a potential risk factor for the development of cardiovascular diseases in diabetes mellitus (Mackness *et al*., 1998c; Rosenblat and Aviram, 2011). Diabetes mellitus, especially type II, is increasing worldwide; particularly, in developing countries like Ghana, due to urbanization and

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increase in sedentary lifestyle, changing dietary habits and obesity (Wild *et al*., 2004; Weinstein *et al*., 2004; Tuomilehto *et al*., 2001). Consequently since diabetes is an emerging disease, its incidence and associated complications continue to be prominent issues that are of public health concern.

Diabetes mellitus is one of the leading causes of mortality and morbidity (Jarrett *et al*., 1982; Stamler *et al*., 1993; Gu *et al*., 1998). About 4.8 million people aged between 20 – 70 years were estimated to have died from diabetes in 2011 (Diabetes Atlas, 2012a). Diabetic complications like retinopathy, nephropathy and peripheral neuropathy accounts for kidney disease, blindness, and limp amputations in many populations (http://www.kidney.niddk.nih.gov/KUDiseases/pubs/kdd/kdd_508.pdf; Young *et al*., 1993; Fong *et al*., 2004; WHO, 2005; http://www.diabetes.co.uk/diabetes-and-amputation.html). These complications also add to the mortality rates in diabetics. In a WHO multinational study, cardiovascular disease was attributed to 52 % deaths whereas renal disease accounted for 11% deaths in people with type II diabetes mellitus (Morrish *et al*., 2001).

Various factors (e.g. oxidative stress, low HDL levels, high levels of triglycerides and LDL, and genetics) contribute to the development of diabetic microvascular and macrovascular complications (Nishikawa *et al*., 2000; Libby *et al*., 2002; Margolis, 2005; National Cholesterol Education Program, 2002). PON 1 has also been shown to reduce the susceptibility of type II diabetes patients to develop these complications (Flekac *et al*., 2008; Poh and Muniandy, 2010).

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Studies have shown that the phenotypic expressions of PON 1 and the level of its enzyme activity are related to the Q192R genotypic variations (Flekac *et al*., 2008; Elattar *et al*., 2012; Abessolo *et al*., 2012). These studies and many others have established a relationship between type II diabetes mellitus and the PON 1 activity and Q192R polymorphism, but reports from such association studies have not been consistent. Furthermore, there is no such information in Ghana. Data is therefore needed in that regard. The current study thereby sought to investigate PON 1 Q192R polymorphism in Ghanaian diabetics and provide baseline information for disease management.

1.2 Justification

Recent studies have not been able to identify the particular PON 1 Q192R polymorphic form expressed in type II diabetic individuals only. The reports so far, are unique to the populations that were studied. This makes it imperative for data to be collated from analysis of PON 1 gene polymorphisms in more diabetic populations to find this polymorph.

Currently there is a single data on PON 1 192 allelic frequencies in a group of healthy Ghanaians (Fujihara *et al*., 2011); however, no data exists on the PON 1 genotypic distribution in Ghanaian type II diabetics. The current study was carried out to provide such information. Analysis of PON 1 gene polymorphisms which directs its activity and phenotypic expression will also shed more light on its physiological significance in diabetics.

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Furthermore, there is a clinical need for a marker of oxidative stress which could potentially identify diabetics at increased risk of type II diabetes-associated complications. Since the different polymorphic forms of PON 1 have been shown to be molecular markers which distinguish between people at risk of developing diabetic complications, identifying and understanding its contribution to the pool of risk factors would be beneficial for disease management.

1.3 Aim

To determine the PON 1 Q192R genotypes in Ghanaian type II diabetic patients and its impact on clinical and biochemical variables

1.4 Primary Objectives

1. To determine the distribution of the PON 1 Q192R genotypes in type II diabetics and a healthy control population.
2. To determine the Q and R allelic frequencies in type II diabetics and a healthy control population.
3. To identify the association between PON 1 Q192R genotypes and the clinical and biochemical variables in the studied populations.

1.5 Secondary Objectives

1. To examine clinical and biochemical variables (e.g. lipid profile, fasting plasma glucose levels) in the studied participants.
2. To determine the effect of body-mass index (BMI) and age on the measured clinical and biochemical variables in the diabetics.

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1.6 Testing Hypothesis (Alternate Hypothesis, H_i)

1.6.1 Primary

1. There is a significant difference between the PON 1 Q192R genotypic distributions in the type II diabetic patients and the healthy controls.
2. There is a difference in the Q and R allelic frequencies between the diabetics and the control population.
3. There is an association between PON 1 Q192R genotypes and the clinical and biochemical variables in the studied populations.

1.6.2 Secondary

1. There is an association between the BMI and ages of the diabetics and their clinical and biochemical variables.

1.7 Primary Endpoints

1. Distribution of the genotypes of the PON 1 Q192R polymorphism in the two populations;
2. Frequencies of PON 1 Q and R alleles in each population;
3. Association between the genotypic frequencies of PON 1 Q192R polymorphism and the clinical and biochemical variables.

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1.8 Secondary Endpoints

1. Estimated mean and standard deviations of the clinical and biochemical variables in the studied populations;
2. Significant effect of the BMI and age of diabetic participants on their clinical and biochemical variables.

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CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Paraoxonases (PON)

Paraoxonases are a family of lactonizing enzymes made up of three (3) isoforms namely: Paraoxonase 1 (PON 1), Paraoxonase 2 (PON 2) and Paraoxonase 3 (PON 3) (Draganov and La Du, 2004; Draganov *et al*., 2005). On the basis of the structural homology and evolutionary distance between the paraoxonases; PON 2 is apparently the oldest member of the family, followed by PON 3 and more recently PON 1 (Draganov and La Du, 2004). The genes of all three family members are clustered in tandem on the long arm of human chromosome 7 (Primo-Parmo *et al*., 1996) but in mice they can be found on chromosome 6 (Sorensen *et al*., 1995). Within a given mammalian species, the three isozymes share approximately 70% identity at the nucleotide level, and 60% identity at the amino acid level. However, between mammalian species, they share 81 – 90% identity at the nucleotide level and 79 – 90% identity at the amino acid level (Mackness *et al*., 2002b).

All three PON enzymes have been reported to exhibit antioxidant activities, and possess the ability to protect against oxidative stress (Carey *et al*., 2001, Li *et al*., 2003; Draganov and La Du, 2004). PON 3, like PON 1, attaches to high density lipoprotein (HDL) when secreted from cells which produce them. It is similarly expressed to a large extent in the liver, and can also degrade compounds like phenylacetate and organophosphates e.g. paraoxon found in insecticides (Reddy *et al*., 2001). PON 2 on the other hand, is not seen on HDL, but ubiquitously expressed in most tissues e.g., liver, lung, placenta, testis and heart (Carey *et al*., 2001). Although PON 3 is comparable to PON 1 in terms of expression, function and location, PON 1

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is more effective than PON 3; and it is by far the most-studied member of this family of enzymes. In addition, PON 1 has been shown to be involved in lipid metabolism and the occurrence of diabetic complications far more than PON 2 and PON 3 (Mackness *et al*., 1998b; Mastourika *et al*., 2006). PON 1 is also known for its role in hydrolyzing nerve gases, aromatic esters and many other substrates (Reddy *et al*., 2001). As an enzyme with multiple substrates, PON 1 exhibits both *paraoxonase* (EC 3.1.8.1) and *arylesterase* (EC 3.1.1.2) activities and requires calcium for both enzymatic actions (Mackness *et al*., 1998b; Harel *et al*., 2004).

2.1.1 PON 1 Gene Architecture and Function

2.1.1.1 Gene Architecture

PON 1 gene is found in a cluster of three related paraoxonase genes on the long arm of chromosome 7 at position 7q21.3 (http://www.genecards.org/cgi-bin/carddisp.pl?gene=PON1). The gene sequence is made of approximately 27kb, and spans the nucleotide positions 94,926,669 – 94,953,884 on the chromosome. The PON 1 gene codes for the PON 1 enzyme (http://www.ncbi.nlm.nih.gov/gene/5444).

2.1.1.2 PON 1 Protein Structure

PON 1 is a glycoprotein with an apparent mass between 43 kDa and 45 kDa. Each molecule of PON 1 contains about three sugar chains, which contributes 15.8% of its total weight, and 354 amino acid residues. Amino acids at positions 253 and 324 are suggested to play a role in the glycosylation of PON 1. The protein also has a high number of leucine residues and an isoelectric point of 5.1 (Gan *et al*., 1991; Mackness *et al*., 1998b; Harel *et al*., 2004). PON 1 has been shown to be a six-bladed β propeller

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protein having four β strands in each blade and two calcium ions embedded in its center (Fig. 2.1; Harel *et al*., 2004; Harel *et al*., 2007). One Ca^{2+} is involved in its catalytic function, and the other plays a structural role without which the enzyme is irreversibly denatured (Harel *et al*., 2004).

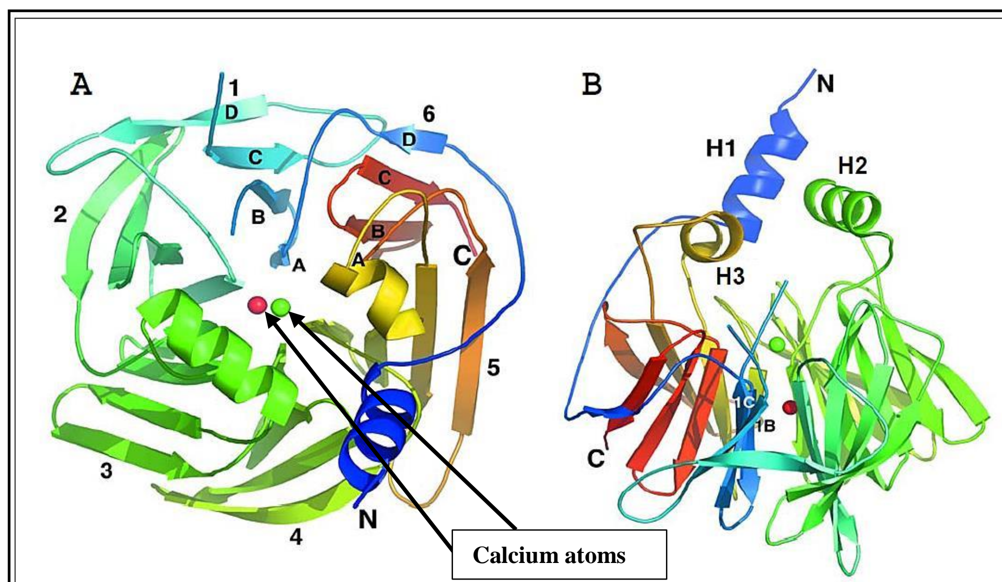

Fig. 2.1 Structure of PON 1 protein

A- Shown are the N and C termini, six blades, two calcium atoms (green and red spheres)

B- Three helices H1-H3

(Source: Harel *et al*., 2004)

2.1.1.3 PON 1 Synthesis and Transport

PON 1 is synthesized in the liver and secreted into the plasma where it closely associates with high density lipoprotein (HDL) (Hassett *et al*., 1991; Sorenson *et al*., 1999). Acting as the physiological acceptor of PON 1, HDL has been proposed to optimize the activity, and stability of the enzyme (Deakin *et al*., 2002; Deakin *et al*., 2007). The levels of HDL in plasma have been shown to correlate with the serum level and activity of PON 1 (Elkiran *et al*., 2007; Iqbal *et al*., 2007; Suvarna *et al*., 2011). This association between HDL and PON 1 is brought about by hydrophobic

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interactions between the HDL molecule and some amino acid residues in the N-terminal helix and the amphipathic helix-2 of the PON 1 protein (Harel *et al*., 2004). An amino acid found in helix-2, at position 192 of the protein, has been shown to be directly involved in the binding of PON 1 to HDL molecules which carry apolipoprotein A-1 (Deakin and James, 2004). This amino acid may undergo a substitution from glutamine to arginine (Q→R) which concomitantly affects the binding of PON 1 to the HDL molecule. A study undertaken by Gaidukov and others indicated that the Q isozyme of the PON 1 192 polymorphism has a significant lower binding affinity for HDL, and therefore a lower stability than the PON 1 192 R isozyme (Gaidukov *et al*., 2006). The PON 1-HDL association led to the assumption that PON 1 could have endogenous substrates related to lipids and/or may be involved in lipoprotein metabolism. PON 1 has actually been shown to metabolize some arachidonic acid derivatives and oxidized lipids in low density lipoprotein (LDL) and HDL particles (Mackness *et al*., 1998c; Draganov *et al*., 2005).

2.1.1.4 Biochemical Action and Properties of PON 1

PON 1 exerts its biochemical action by acting as a hydrolase or lactonase. Some studies have proposed strongly that it is more of a lactonase than a hydrolytic enzyme (Billecke *et al*., 2000; Draganov *et al*., 2005; Khersonsky and Tawfik, 2005). The enzymatic activities of PON 1 have been demonstrated with a range of organophosphates, aromatic esters, lactones, and hydroxycarboxylic acids. As compared to the other members of the paraoxonase family, organophosphatase activity is limited to PON 1 only. It is able to hydrolyze diazoxon, chlorpyrifos oxon and parathion, as well as nerve agents like sarin and soman (Draganov *et al*., 2005; Kanamori-Kataoka and Seto, 2009).

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PON 1 acts on aromatic esters e.g. phenylacetate, using its arylesterase activity. It has been reported that introduction of a nitro group to the para position of the aromatic ring in some aromatic esters dramatically decreases PON 1 activity (Draganov *et al.*, 2005).

PON 1 exhibits lactonase activity on aromatic and aliphatic lactones. It hydrolyses six-member ring lactones more efficiently than their five-member ring analogs; and the presence of a double bond within a lactone ring increases its rate of hydrolysis. It has also been observed that the hydroxyl group in homogentisic acid lactone promotes the lactonase activity of PON 1. Other compounds which are acted on via the lactonase activity of PON 1 include dihydrocoumarin, 2-coumaranone and other biological compounds (Billecke *et al.*, 2000; Draganov *et al.*, 2005).

PON 1 is also capable of hydrolysing two products of the enzymatic and non-enzymatic oxidation of arachidonic acid and docosahexaenoic acid, namely: (±)4-hydroxy-5,7,10,13,16,19-docosahexaenoic acid (4-HDoHE) and (±)5-hydroxy-6,8,11,14-eicosatetraenoic acid 1,5-lactone (5-HETEL). 5-HETEL is capable of inhibiting 5-lipoxygenase and thromboxanes and prostaglandin E2 synthesis in peritoneal macrophages. Both 4-HDoHE and 5-HETEL can trigger inflammatory responses; they therefore act as determinants of atherosclerotic disease (Draganov *et al.*, 2005; Pejin-Grubiša, 2012).

2.1.1.4.1 Anti-atherogenic activity of PON 1

Recent studies, focusing on the effects of the PON 1 antioxidant activity on plasma lipoproteins, have shown that PON 1 indeed plays an anti-atherogenic role (Watson *et*

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al., 1995; Tward *et al*., 2002). It inhibits macrophage-mediated oxidation of LDL (**A**) and its conversion into oxidized LDL (ox-LDL) [**B**]; it also hydrolyses oxidized lipids in the ox-LDL and converts it to the native LDL particle (**C**). PON 1 inhibits cholesterol biosynthesis in the macrophages (**D**) and macrophage-cholesterol influx (ox-LDL uptake via the CD-36 scavenger receptor), thus reducing macrophage oxidative stress (**E**). In addition it stimulates HDL-mediated cholesterol efflux (**F**) via the ATP binding cassette transporter A1 (ABCA1) (Figure 2.2; Rosenblat and Aviram, 2011). All these actions of PON 1 prevent macrophage foam cell formation and vascular impairment, hence attenuating the development of atherosclerosis.

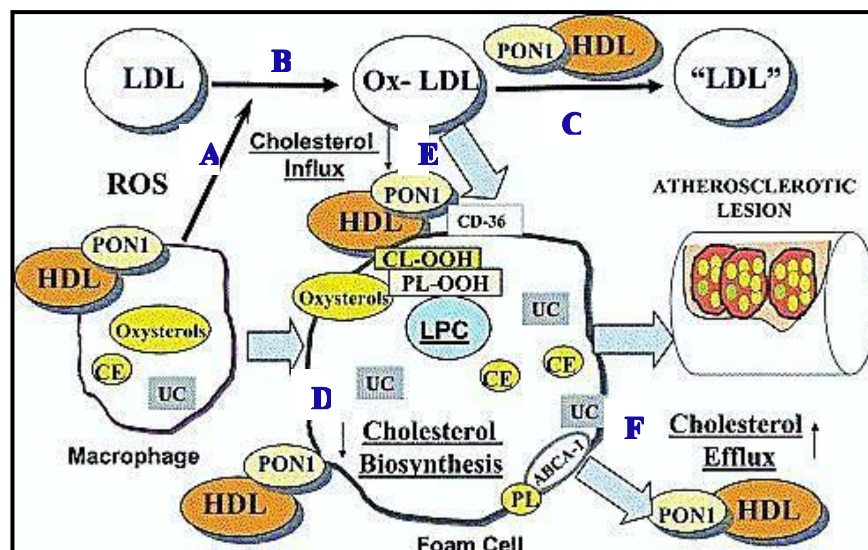

Fig. 2.2 Anti-atherogenic activity of HDL-associated PON 1

ATP binding cassette transporter A1 (ABCA1); cholesteryl ester (CE); unesterified cholesterol UC; phospholipids (PL); oxidized LDL (Ox-LDL); cholesteryl linoleate hydroperoxides (CL-OOH); phospholipid hydroperoxides (PL-OOH); lysophosphatidylcholine (LPC); reactive oxygen species (ROS); low density lipoprotein (LDL); high density lipoprotein (HDL).

(Source: Aviram and Rosenblat, 2004)

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Studies in mice have also demonstrated the protective effects of the PON 1 enzyme on native LDL and HDL. It was found that in paraoxonase-knockout mice, LDL was more susceptible to oxidation; these mice also exhibited atherosclerosis more than the control mice (Shih *et al*., 1998; Shih *et al*., 2000). On the contrary, it was shown that increasing the expression of PON 1 protein on HDL protected its integrity and function, and inhibited the formation of lipid hydroperoxides on the HDL (Oda *et al*., 2002). A study reported that HDL isolated from transgenic mice having human PON 1 gene in addition to the mouse PON 1 gene, protected LDL from oxidation better than HDL from non-transgenic mice. Furthermore, lipid peroxidation and sizes of atherosclerotic lesions was significantly reduced in the transgenic mice more than in their wild type counterparts (Tward *et al*., 2002).

The transformation of lipoproteins into biologically inactive compounds by PON 1 has been reported to vary due to its polymorphic forms, attributable to the 192 amino acid substitution (Mackness *et al*., 1998c). A study which investigated the effect of PON 1 Q192R polymorphism on HDL's ability to protect LDL from oxidative modification, indicated that HDL obtained from QQ homozygotes protected LDL from oxidation, better than HDL obtained from RR homozygotes (Mackness *et al*., 1998c). In another study however QQ homozygous patients were found to have higher levels of oxidized plasma fatty acids when compared with the RR homozygous patients (Bhattacharyya *et al*., 2008). Notwithstanding these reports, significant associations between PON 1 Q192R genotypes and LDL oxidation have not been reported elsewhere (Lakshmy *et al*., 2010).

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Additionally, PON 1 192 polymorphism may influence the levels of lipoproteins and lipids in plasma. In a healthy Hutterite population, it was found that PON 1 192QQ homozygotes had significantly lower levels of total cholesterol, non-high density lipoprotein cholesterol (HDL-C), triglycerides, and apoB, than the QR heterozygotes and RR homozygotes. But they had higher HDL-C and lower low density lipoprotein cholesterol (LDL-C) levels than the QR heterozygotes (Hegele *et al*., 1995).

2.1.2 PON 1 Gene Polymorphisms

It is reported that a total of 198 SNPs occur in the non-translated 5′-end, exonic regions, intronic regions and the non-translated 3′-end of the PON 1 gene (La Du, 2003). Sequencing amplified DNA upstream from the PON 1 open reading frame has also shown three polymorphic sites at nucleotide positions -107 (C or T), -824 (A or G) and -907 (C or G) in the PON 1 promoter binding region (Leviev and James, 2000). Most of these SNPs are of great importance because they affect the activity of the PON 1 enzyme to a large extent, although the PON 1 activity may also be influenced by the disease status of the populations being studied (Nevin *et al*., 1996; Eom *et al*., 2011; Pejin-Grubiša, 2012).

Among the exonic SNPs, the two most common and widely studied are the Q192R and L55M SNPs. The Q192R polymorphism occurs on exon 6 of the paraoxonase gene; and it results in the replacement of the codon C**A**A which codes for glutamine (Q) by codon C**G**A which codes for arginine (R) at position 192 of the amino acid sequence. On exon 3, the L55M polymorphism leads to an exchange of codon **A**UG for **U**UG [methionine (M) substituted with leucine (L)] at the 55th position on the PON 1 protein (Humbert *et al*., 1993). Some studies reported that these two

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polymorphisms occur at amino acid positions 191 and 54 respectively; and both are inherited by the simple Mendelian pattern of inheritance (Eckerson *et al.*, 1983b; Adkins *et al.*, 1993; Blatter-Garin *et al.*, 1997).

The Q192R substitution has been shown to affect the activity of PON 1 in an individual whilst the M55L substitution at position 55 was reported in an earlier study to be a determinant of the serum concentration of the enzyme (Humbert *et al.*, 1993; Blatter-Garin *et al.*, 1997). A later study, however, revealed that the 55 polymorphism also has a significant effect on the activity of PON 1 (Mackness *et al.*, 1998d).

2.1.3 Q192R Polymorphism and PON 1 Phenotypic Expression

The triplet codon that codes for glutamine (Q) or arginine (R), thereby rendering a unique polymorphic change at position 192, serves as the basis for classification of PON 1 enzyme into three phenotypes, represented as A, B and AB. The A phenotype (allozyme) corresponds to the polymorph with glutamine (Q) at position 192; whereas the polymorph substituted with arginine (R) gives the B phenotype (Adkins *et al.*, 1993). The AB phenotype has both Q and R substitutions as an allelic pair at the gene loci.

The PON 1 allozymes have different hydrolytic activities determined by their respective genotypes (Nevin *et al.*, 1996); and they can be distinguished from each other based on their individual rates of activity against substrates like paraoxon (Eckerson *et al.*, 1983b). The B-allozyme displays several-fold higher paraoxonase activity against paraoxon than the A-allozyme (Eckerson *et al.*, 1983a; Humbert *et al.*, 1993). The higher activity of the B-allozyme over the A-allozyme is stimulated to a greater extent in the presence of 1 mol L^{-1} NaCl (Eckerson *et al.*, 1983a). However, it

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has been reported that there is a difficulty in distinguishing between the activity of the AB-allozyme and that of the B-allozyme (Eckerson *et al*., 1983a). Although the activity of the allozymes on paraoxon can aid in the identification of the high activity and low activity phenotypes, genotype analysis gives a better mode of classification into the high activity homozygous (BB), intermediate activity heterozygous (AB), or low activity homozygous (AA) phenotypes (Humbert *et al*., 1993).

2.1.4 Distribution of the PON 1 Q192R Alleles

Studies conducted in many populations have shown varying distributions of the PON 1 Q192R genotypes and alleles. The results indicated that the variations may be dependent on the populations studied, that is their respective continents and countries of origin. It has also been observed that even within a particular country, many variations can be seen at the ethnic level (Pejin-Grubiša, 2012).

The R allele has been reported to be more frequent in African-Americans, some Southern, Central and West Africans; whereas, the Q allele is common in the temperate regions of Europe and North America (Draganov and La Du, 2004). Among the populations with higher frequency of the R-allele, some have extremely high R allelic frequency as compared to the others e.g., Yorubas and Ovambos (Table 2.1). Other populations in America and Africa such as the Mexicans, Peruvians, Xhosa, Mozabites and Ethiopians, are reported to have almost equal Q and R allelic frequencies (Table 2.1; Pejin-Grubiša, 2012).

In Asian populations however, many different frequencies of the Q and R alleles have been reported; some presented a high frequency of the Q allele and a corresponding

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low R allelic frequency and vice versa (Odawara *et al*., 1997; Pejin-Grubiša, 2012; http://alfred.med.yale.edu/alfred/SiteTable1A_working.asp?siteuid=SI001024H). Evolutionary influences might have contributed to the variations among the populations, but these need extensive investigation.

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Table 2.1 Frequencies of the PON 1 Q192R Alleles

POPULATION	**PON 1 Q192R ALLELE FREQUUENCY**	
	Q	**R**
English	0.780	0.220
Finnish	0.690	0.310
Dutch	0.680	0.320
Spanish	0.700	0.300
Italians	0.650	0.350
Turkish	0.690	0.310
Croatian	0.770	0.230
Czecs	0.540	0.460
Serbian	0.770	0.230
Asian Indians Punjabis	0.740	0.260
Japanese	0.400	0.600
Koreans	0.380	0.620
Chinese	0.420	0.580
Iranian	0.690	0.310
Caucasian-Americans	0.730	0.270
Canadians	0.730	0.270
African – Americans	0.370	0.630
Amazonian Amerindian tribes	0.270	0.730
Caribbean- Hispanics	0.540	0.460
Mexicans	0.510	0.490
Peruvians	0.539	0.461
Beninese	0.388	0.612
Ethiopians	0.592	0.408
Egyptians	0.670	0.330
Gabonese*	0.365	0.635
Ghanaian**	0.364	0.636
Hausa**	0.340	0.660
Yoruba**	0.170	0.830
Ovambos	0.321	0.679
Mozabite**	0.480	0.520
Xhosa**	0.485	0.515

Source: Lipoproteins - Role in health and disease (Pejin-Grubiša, 2012).

(**http://alfred.med.yale.edu/alfred/SiteTable1A_working.asp?siteuid=SI001024H;* Abessolo *et al.*, 2012)

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2.1.5 PON 1 Activity and the Q192R Polymorphism in Diseases

Some diseases and medical conditions such as pregnancy failure, cancer, cardiovascular diseases and diabetes, have been linked to PON 1 in terms of its activity and the Q192R polymorphism (Flekac *et al.*, 2008; Toy *et al.*, 2009; Uyar *et al.*, 2011).

Most studies have reported low levels of PON 1 activity in diseased persons. In a study which compared women who had experienced early pregnancy failure (EPF) to those without EPF, it was found that the basal and salt-stimulated paraoxonase/arylesterase activities in the EPF women were significantly lower; and lipid hydroperoxide levels were higher. The results suggested that PON 1 activity may play a role in disease development in EPF women, through increased susceptibility to lipid peroxidation (Toy *et al.*, 2009). Similarly, in patients with chronic renal failure and lung cancer, PON 1 activity (paraoxonase and arylesterase) were found to be lower than what was observed in their corresponding controls (Hasselwander *et al.*, 1998; Elkiran *et al.*, 2007). The activity of the PON 1 enzyme has also been reported to be low in coronary heart disease, and atherosclerosis (Mackness *et al.*, 1998b; Mackness *et al.*, 2001).

Generally, in diabetic patients the activity and concentration of PON 1 enzyme are lower than what is observed in the healthy control group (Mackness *et al.*, 1998a; Abbott *et al.*, 1995). Such trends have been observed in Gabonese, Egyptian and Japanese type II diabetic patients and some Caucasian populations (Mackness *et al.*, 1998a; Sakai *et al.*, 1998; Abessolo *et al.*, 2012; Elattar *et al.*, 2012). The high glucose levels in diabetics affect the activity of PON 1, its association with HDL, and also

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influences PON 1 dissociation from HDL (Rosenblat *et al*., 2008). It has been suggested that the PON 1 status (enzyme activity and concentration) in an individual is a risk marker to the development of microvascular and macrovascular complications (Abbott *et al*., 1995; Li *et al*., 2003; Mackness *et al*., 2003). In diabetics with vascular complications such as nephropathy, retinopathy, cardiovascular diseases and hypertension, PON 1 activity was found to be relatively lower than in patients without these complications (Abbott *et al*., 1995; Mackness *et al*., 2000; Tward *et al*., 2002; Suvarna *et al*., 2011; Abessolo *et al*., 2012). In addition, serum from type II diabetics and coronary heart disease (CHD) patients was analysed for HDL activity against ox-LDL due to the antioxidant activity of PON 1 on it. The results obtained showed that HDL from people with type II diabetes and CHD metabolized lower amounts of ox-LDL than HDL from the control subjects (Mastorikou *et al*., 2006).

The genotypes and alleles of the PON 1 Q192R polymorphism have been found in and linked to the risk of developing some diseases. In renal cell carcinoma (RCC) patients, the Q allele of the 192 polymorphism was found to be common, whereas the R allele was found in most of the controls. The investigators of the study proposed that the R allele may be a better protector from RCC; but they also indicated that PON 1 activity studies will have to be conducted in the patients to establish the actual relationship between PON 1 and RCC (Uyar *et al*., 2011). The Q 192 allele has also been associated with a significant reduction in the risk of ovarian cancer (Lurie *et al*., 2008). However, other PON 1 polymorphisms, apart from the PON 1 Q192R polymorphism have been marked as risk factors for the development of breast cancer

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(Stevens *et al*., 2006). No linkage has been found between colorectal cancer and the PON 1 Q192R polymorphism as well (Van Der Logt *et al*., 2005).

Associations between cardiovascular and coronary heart diseases and PON 1 192 polymorphism have been reported (Mackness *et al*., 1998d). In one study, an increased prevalence of coronary artery disease was observed more in participants who had the PON 1 192QQ genotype than those with the 192RR genotype. These homozygous QQ participants also had a history of coronary artery bypass graft surgery and percutaneous coronary intervention. The study also reported that individuals carrying the PON 1 192 QR genotype however, showed no differences in prevalent peripheral artery disease within the populations studied (Bhattacharyya *et al*., 2008). The R allele has been shown to be an independent risk factor for the development of coronary heart and coronary artery diseases in other studies (Ruiz *et al*., 1995; Odawara *et al*., 1997; Osei-Hyiaman *et al*., 2001).

An investigation conducted to identify the association between PON 1 Q192R polymorphism and clinical manifestations of atherosclerosis in a human population showed no significant differences between the allelic and genotypic frequencies of the Q192R polymorphism in the controls and cases with atherosclerosis. However, in a later study, the investigators found that the R allele of the Q192R polymorphism was a risk factor for the development of atherosclerosis in type II diabetes patients (Pejin-Grubiša, 2012).

In some diabetic populations, the RR genotype was associated with high PON 1 activity whereas the QQ genotype was an indicator of low PON 1 activity (Mackness

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et al., 1998a; Flekac *et al*., 2008). Since diabetics usually have low PON 1 activity, this observation may imply that it is not likely to encounter the R alleles in diabetics (Mackness *et al*., 1998a). A study supporting this assumption reported that the R allele of the Q192R polymorphism was infrequent in both type I and II diabetics; whereas the Q allelic frequency was higher in diabetic patients with macrovascular complications. In that study, plasma glycated hemoglobin levels was used to show that the RR homozygous patients exhibited better control of diabetes than their QQ homozygous counterparts. It may be concluded therefore that the R allele is associated with low risk for diabetes and diabetes-associated complications; whilst the Q allele may be linked to macrovascular complications (Flekac *et al*., 2008).

Conversely, other studies have linked the RR and QR genotypes more with type II diabetes mellitus and its associated vascular complications (Odawara *et al*., 1997; Ruiz *et al*., 1995) than the QQ genotype. The ability of HDL to metabolise ox-LDL was observed to be significantly lower in serum obtained from type II diabetic patients who had the PON 1-192 RR than those who had the PON 1-192 QQ or QR genotypes. Due to the low antioxidant activity of HDL observed in the PON 1-192 RR diabetic patients, their circulatory plasma ox-LDL concentration was found to be higher when compared with the other populations analysed (Mastorikou *et al*., 2006). Other studies also reported an increased risk of coronary artery disease in type II diabetic patients with the R allele (Pfohl *et al*., 1999; Osei-Hyiaman *et al*., 2001).

The QQ genotype may be absent in diabetic patients due to its association with individuals with low plasma LDL cholesterol levels, and its role in protecting LDL

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from oxidation (Hegele *et al*., 1995; Mastorikou *et al*., 2006) – two factors which may occur in the reverse in diabetics.

Contrary to the observation that PON 1 192 polymorphism is associated with type II diabetes, it was reported that in type I diabetics the activity and mass of PON 1 is not affected by the Q192R polymorphism. And this polymorphism did not cause any significant differences in the PON 1 activities between the type I diabetic patients with and without microvascular and macrovascular complications (Mackness *et al*., 2002a).

On the whole, the reports so far have suggested that PON 1 gene variants which influence PON 1 phenotypic expression in an individual are molecular markers for the development of diabetic microvascular and macrovascular complications (Ruiz *et al*., 1995; Odawara *et al*., 1997; Mackness *et al*., 2000; Osei-Hyiaman *et al*., 2001; Flekac *et al*., 2008).

2.2 Diabetes Mellitus (DM)

Diabetes mellitus is a metabolic disorder characterized by hyperglycaemia (Alberti and Zimmet, 1998; WHO, 2006). There are two main types of diabetes mellitus classified in the literature as type I [formerly called insulin dependent diabetes mellitus (IDDM)] and type II [also known as non-insulin dependent diabetes mellitus (NIDDM)] (Alberti and Zimmet, 1998). Other types of diabetes mellitus include gestational diabetes mellitus, latent autoimmune diabetes in adults (LADA); and some specific forms such as genetic defects of β-cell function, genetic defects in insulin

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action, diseases of the exocrine pancreas, endocrinopathies, and drug or chemical induced diabetes (WHO, 1999; American Diabetes Association, 2013a).

2.2.1 Etiology of Diabetes Mellitus

Type I diabetes mellitus is an immune mediated disease, it could be idiopathic or related to the destruction of the pancreas (Alberti and Zimmet, 1998; Weyer *et al*., 1999; Smiley, 2003; Donath *et al*, 2005). Type II diabetes mellitus develops from interplay between genetic, environmental, ethnic and behavioral factors (Modan *et al*., 1986; Weinstein *et al*., 2004; Tuomilehto *et al*., 2001; Weijnen *et al*., 2002; Oldroyd *et al*., 2005; Dreyer *et al*., 2009; Amoah, 2000; Pejin-Grubiša, 2012; International Federation of Diabetes, 2013).

The gender and ages of individuals are known predictors of diabetes mellitus. It has been observed that the prevalence of diabetes is higher in men than in women (Wild *et al*., 2004; Yang *et al*., 2010). The effect of age on diabetes however, renders type I diabetes mellitus to be seen in children and the youth; whilst type II being the most prevalent form of diabetes, occurs mostly in adults (Alberti and Zimmet, 1998; Maahs *et al*., 2010; American Diabetes Association, 2013a). In recent times however, more cases of type II diabetes have been reported in children aged between 6 and 11 years and in adolescents within the ages of 12 – 19 years (Copeland *et al*., 2005; National Diabetes Fact Sheet, 2011).

Considering the conditions which predispose persons to diabetes mellitus such as obesity, age and sex, significant incidence of undiagnosed diabetes have been reported in some populations. These cases of undiagnosed diabetes, were found to contribute to

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the overall burden of diabetes and diabetes-associated complications (Leiter *et al*., 2001; Gregg *et al*., 2004; Lauruschkat *et al*., 2005; Cowie *et al*., 2006; Bener *et al*., 2009; Diabetes Atlas, 2012b).

2.2.2 Diagnostic Criteria for Diabetes Mellitus

The diagnostic criteria for diabetes mellitus are usually based on the guidelines of the World Health Organization and the American Diabetes Association (WHO, 1999; WHO, 2006; American Diabetes Association, 2008). In healthy individuals, fasting plasma glucose (FPG) levels ranges from 4.2 – 6.1 mmol/L. Diabetes is diagnosed when the FPG levels are greater than or equal to 7.0 mmol/L (126 mg/dL), or 11.1 mmol/L (200 mg/dL) after a two hour glucose load (oral glucose tolerance test). For individuals with FPG levels between 6.1 and 6.9 mmol/L (110 – 125 mg/dL), it is requested that, oral glucose tolerance test is performed to establish their glucose tolerance status, and their risk to develop diabetes (WHO, 2006).

2.2.3 Epidemiology of Diabetes Mellitus

In Ghana, Amoah and others, in 2002, estimated the crude prevalence of diabetes for Ghanaians aged 25 years and above in the Greater Accra region to be 6.3% (Amoah *et al*., 2002). Worldwide, the number of people estimated to have diabetes in the year 2000 was 171 million. However, this figure is expected to rise to 366 million by the year 2030. With regard to the 2030 projection, a higher prevalence of the disease is expected to occur in the Middle East, sub-Saharan Africa and India, especially in the urban areas, than in other parts of the world (Wild *et al*., 2004).

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Diabetes is more common in individuals of certain age groups than others. A higher number of diabetic cases are reported in individuals aged 35 years and above, as compared to people younger than these ages (Oldroyd *et al*., 2005; Diabetes in Canada, 2011). It is expected that as a result of aging, the incidence of diabetes in individuals aged 65 years and above will increase in both developed and developing countries by the year 2030 (Wild *et al*., 2004).

2.2.4 Pathophysiological Changes in Plasma Lipoproteins and Type II Diabetes Mellitus

Many studies have reported changes in the nature (oxidative modification) and quantities of plasma lipoproteins in diabetes mellitus (Libby *et al*., 2002; Barter, 2005; Nakhjavani *et al*., 2006; Gilani *et al*., 2010). Plasma lipoproteins are a group of cholesterol, triacylglycerol and phospholipid transporting particles found in plasma. They are made up of lipids, and specific proteins called apolipoproteins or apoproteins (e.g. apo A-I – A-IV, apo A, apo B-48, apo B-100, apo C-I, apo C-II, apo D and apo E), which are synthesized by the liver and intestines (Elshourbagy *et al*., 1985; Glickman *et al*., 1986; Haddad *et al*., 1986; Kraft *et al*., 1989). The lipoproteins vary one from the other in size and density, due to their lipid and protein compositions. They include chylomicrons, very-low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL). They increase the solubility of the lipids they carry, and transport these lipids between tissues (Berg, 2002). An imbalance in the levels of plasma lipoproteins and their constituent lipids may lead to the development of disease conditions such as atherosclerosis, stroke and coronary heart disease (Sharett *et al*., 1994; Hodis *et al*., 1999).

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In type II diabetes mellitus, dyslipidemia (i.e. high plasma total cholesterol, high plasma LDL, low plasma HDL and elevated triglyceride levels) have been shown to contribute to the development of vascular problems (Nakhjavani *et al*., 2006; Gilani *et al*., 2010). Among the lipoproteins, changes in the levels and nature of HDL and LDL have been more associated with the onset of diabetes-associated complications (Francis *et al*., 1993; Robbesyn *et al*., 2003).

HDL possesses anti-atherogenic properties. It enhances cholesterol efflux, prevents the oxidation of LDL, and transports lipid peroxides from circulation to the liver (Barter, 2005). HDL's anti-atherogenic role is greatly improved when it is oxidatively tyrosylated *in vitro* (Francis *et al*., 1993; Pirillo *et al*., 2007), and more efficiently, when both apolipoprotein AI and AII are present on the HDL molecule (Wang *et al*., 1998). Furthermore the presence of enzymes such as paraoxonase 1 (PON 1), lecithin–cholesterol acyl transferase (LCAT), lipoprotein-associated phospholipase A2 and platelet activating factor acetylhydrolase (PAF-AH) on the HDL molecule enhances its protective activities appreciably (Mackness *et al*., 1998c; Forte *et al*., 2002; Tomas *et al*., 2004). Due to these properties of HDL, it has been established that low plasma HDL level is a risk factor for coronary artery disease (National Cholesterol Education Program, 2002). This risk gets increased in type II diabetic patients as a result of diabetic dyslipidemia (Mooradian, 2009).

On the other hand, LDL is considered safe when its level in plasma is considerably low (National Cholesterol Education Program, 2002). LDL molecules usually enter the wall of the artery and may return to the plasma. However, when the LDL level exceeds a certain threshold in the plasma, they accumulate in the artery faster than

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they can be removed. The accumulated LDL may then undergo oxidation (Barter, 2005). Oxidation of LDL initiates the inflammatory processes which lead to formation of atherosclerotic lesions in diabetes (Libby *et al*., 2002). Oxidized low density lipoprotein (ox-LDL) induces the expression of adhesion molecules, chemokines, pro-inflammatory cytokines, and other mediators of inflammation in macrophages and cells of the vascular wall. It also activates adaptive immune responses and the transcription factor NF-κB, which is involved in the expression of immune and inflammatory genes (Robbesyn *et al*., 2003).

Increases in plasma VLDL and triglycerides levels may result in LDL oxidation (Libby *et al*., 2002). Insulin-resistance in type II diabetes may lead to an increased release of free fatty acids from adipocytes into the plasma. The released fatty acids then cause the liver to synthesize more triglycerides and release triglyceride-rich VLDL into plasma. These VLDL molecules exchange their triglyceride molecules for cholesterol in LDL and HDL. Hydrolysis of the triglycerides in LDL and HDL reduces the sizes of the two lipoproteins and exposes them to oxidation (Barter, 2005; Mooradian, 2009).

2.2.5 Oxidative Stress and its Effects in Diabetes Mellitus

The hyperglycaemia associated with type II diabetes begins the processes that lead to vascular diseases. However, it has not been established that it is the direct cause of macrovascular and microvascular complications. It has been hypothesized that oxidative stress produced as a result of hyperglycaemia, is rather, a major contributor to the development of insulin resistance, impaired glucose tolerance, overt diabetes and diabetes-associated complications (Ceriello and Motz, 2004). And it has also been

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shown that oxidative stress increases remarkably in diabetic patients as compared to controls (Ramakrishna and Jailkhani, 2007).

Oxidative stress in diabetics may occur as a result of autoxidation of glucose accompanied by the production of hydroxyl radicals and other reactive oxygen species (ROS). Metabolism of glucose through the polyol and other pathways contributes to the increased levels of ROS in diabetics. Aldose reductase, the first enzyme in the polyol pathway which converts glucose to sorbitol (Fig. 2.3), has been shown to contribute immensely to oxidative stress production in the lenses and nerves of diabetic mice (Chung, *et al*., 2003). Other studies have however, reported that aldose reductase does not interact directly with glucose to produce an oxidative stress (Nishikawa *et al*., 2000; Giacco and Brownlee, 2010).

Another enzyme found in the polyol pathway which may contribute to increased ROS levels is sorbitol dehydrogenase (SDH). SDH converts sorbitol to fructose. Triose phosphate intermediates of the glycolytic pathway are produced from this fructose, and they play important roles in the generation of precursors of advanced glycation end products (AGEs), glycation of proteins and their associated ROS formation (Fig. 2.3; Amano *et al*., 2002; Nishikawa *et al*., 2000).

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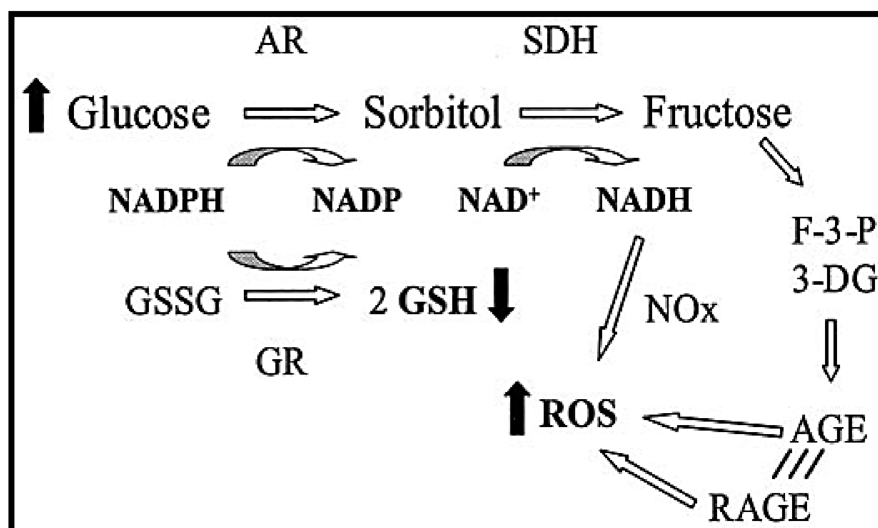

Figure 2.3 Role of the polyol pathway in ROS generation

Aldose reductase (AR); Sorbitol dehydrogenase (SDH); Fructose-3- phosphate (F-3-P); 3-deoxyglucosone (3-DG); Advanced glycation end products (AGE); Receptor of AGE (RAGE); Glutathione reductase (GR); Oxidised glutathione (GSSG); Reduced glutathione (GSH); NADH oxidase (NOx) Reactive oxygen species (ROS)

Source: Chung *et al*., 2003

Other enzyme systems which add to the production of ROS and subsequent oxidative stress in diabetics include xanthine oxidase, nitric oxide synthase and NAD(P)H oxidase (Guzik *et al*., 2002). Among these enzymes, NAD(P)H oxidase has been demonstrated to be a major mediator of superoxide anion production in the body, especially in disease states like diabetes (Channon and Guzik, 2002; Guzik *et al*., 2002; Ray and Shah, 2005; Newsholme *et al*., 2007). It was found that after inhibition of NAD(P)H oxidase and protein kinase C-α (PKC-α) activities in renal cells, factors which contributed to the generation of ROS via PKC-α and NAD(P)H oxidase pathway (such as expression of the receptor of AGE and the attendant generation of renal cytosolic superoxide and vascular endothelial growth factor) were attenuated and inhibited respectively (Thallas-Bonke *et al*., 2008).

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It has been observed that a number of enzymes involved in antioxidant processes exhibit low activities in diabetics. Examples of these enzymes are glutathione reductase, glutathione peroxidase, catalase, and superoxide dismutase. The activities of these enzymes decrease as oxidative stress increases (Ramakrishna and Jailkhani, 2007; Ramakrishna and Jailkhani, 2008).

Increased ROS production in diabetes may contribute to the reduced enzyme activity and the inability of antioxidant mechanisms to sufficiently combat the rising levels of ROS, further enhancing diabetic oxidative stress (Seidler, 2005). PON 1 which is also an antioxidant has been demonstrated to modulate systemic oxidative stress in humans (Bhattacharyya *et al*., 2008). However, PON 1 enzyme activity has been reported to be low in diabetics, in many studies (Mackness *et al*., 1998a; Sakai *et al*., 1998; Abessolo *et al*., 2012; Elattar *et al*., 2012). This low activity might be a result of diabetic associated oxidative stress. Subsequent to the incubation of serum obtained from healthy controls with hypochlorous acid (a potent ROS), it was observed that the activity of the PON 1 arylesterase in the hypochlorous acid-treated serum decreased with increasing concentration of the hypochlorous acid (Sutherland *et al*., 2004).
The changes observed in the activity of antioxidant enzymes may also be the result of mechanisms of protein glycation, and activities of glycation products and AGEs (Seidler, 2005).

2.2.6 Protein Glycation in Diabetes

Non-enzymatic glycation of proteins may be triggered by high ROS levels and hyperglycaemia (Nishikawa *et al*., 2000). Protein glycation usually occurs when amino acid residues on proteins interact with sugars such as glucose, fructose and

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mannose. This leads to the formation of Schiff base, Amadori products, and finally, advanced glycation end products (AGEs). The reactions that lead to the formation of Schiff bases and Amadori products can be reversed to reform the normal proteins, once the hyperglycaemic condition is controlled or removed. The AGEs formed however, are permanent; and proteins in this state remain so until they are finally destroyed. AGEs may further form cross links with amino acids on near-by proteins, leading to protein cross-links which can alter the half-life and normal activity of such proteins. Proteins modified by AGEs may bind to receptors of AGEs (RAGE) further elevating the ROS levels in the system (Nishikawa *et al*., 2000; Wautier and Schmidt, 2004).

In diabetics protein oxidation and glycation occur at a higher rate than in non-diabetic individuals (Ramakrishna and Jailkhani, 2007). The increased protein glycation and oxidative stress further exposes diabetics to diabetic-associated complications (Nishikawa *et al*., 2000). Protein glycation in diabetics causes the glycation of hemoglobin. The levels of glycated hemoglobin in the plasma can be used as a criterion for diagnosing diabetes and for monitoring the progress (glycemic control) of diabetic patients under treatment (WHO, 2006; American Diabetes Association, 2013b).

Furthermore, protein glycation reactions greatly affect enzymes and other soluble proteins by causing them to be inactive or to exhibit reduced activities (Wautier and Schmidt, 2004; Piconi and Ceriello, 2007). It has been shown that PON 1 enzyme can be negatively affected by protein glycation reactions (Mastorikou *et al*., 2008). A study demonstrated that human HDL and purified paraoxonase protein incubated with

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glucose showed high levels of glycation and advanced glycation end products. The glycated HDL and paraoxonase showed reduced paraoxonase activity, and they both did not inhibit adhesion of monocytes to human aortic endothelia cells *in-vitro* (Hedrick *et al*., 2000; Ferretti *et al*., 2001).

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CHAPTER THREE

3.0 METHODOLOGY

3.1.0 Study Participants

A total of one hundred and twenty (120) Ghanaian participants with type II diabetes mellitus were recruited from the outpatients' clinic of the National Diabetes Management and Research Centre (NDMRC), Korle Bu, Accra. However, eight diabetics withdrew from the study, leaving a total of 112 diabetics. The control group was made up of a hundred and five (105) non-diabetic individuals, from the Methodist Church of Ghana at Avenor, a suburb in Accra, staff of the College of Health Sciences, University of Ghana Medical School (U.G.M.S) and Korle Bu Teaching Hospital. Of these controls eight again withdrew from the study leaving 97 participants. The participant enrolment for the controls was based on accessibility and proximity to the researcher.

3.1.1 Inclusion/ Exclusion Criteria

3.1.2.1 Inclusion

The diabetic patients included all persons diagnosed of type II diabetes mellitus. The control group was made up of healthy non-diabetic individuals living in a similar socioeconomic environment as the diabetics.

3.1.2.2 Exclusion

Participants who took in excessive alcohol or smoked heavily were eliminated. To successfully match participants, all those with ages below the cut-off of 30 years were excluded from the study.

3.1.2 Sample Size

The minimum sample size was determined using the equation below;

$Sample\ size, n; prevalence, P; standard\ score, Z$(95% confidence level),

$$n = \frac{Z^2(P)(1-P)}{Error^2}$$

[$Z = 1.96$; $P = 0.063$ (Amoah *et al.*, 2002); Error = 0.05]

$$n = \frac{1.96^2(0.063)(1-0.063)}{0.05^2}$$

$$n = 90.71$$

The minimum sample size was approximated to 91 participants each for the type II diabetic and control populations. Priori confounders (age, BMI and sex) were adjusted for by stratification. Age was compensated for by eliminating control participants below 30yrs. For the genetic association studies, a multiplicative genetic model was used. Although the sample size for the genotype analysis was small, it was justified by power analysis using CaTS software (http://www.sph.umich.edu/csg/abecasis/CaTS/index.html).

3.1.3 Demographics

The ages, sex, weight, height, educational status and occupation of all the participants (i.e. type II diabetics and controls) were obtained through the administration of questionnaires (Appendix B). The questions were read out to participants who couldn't read and write; whilst the literate participants filled out the questionnaires themselves. The questions for patient baseline demographics and clinical information were pretested with 7 participants (5 type II diabetics and 2 controls) at the National

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Diabetes Management and Research Centre (NDMRC) and the Department of Medical Biochemistry, U.G.M.S., Korle Bu, Accra.

3.1.4 Anthropometry

The waist circumference was taken by placing a tape measure, approximately between the iliac crest and the last rib of the participants. It was held tightly to the body without compressing the contents of the stomach. The tape measure was placed around the widest area of the buttocks for the hip measurement. A ratio of the waist and hip measurements was determined. Height was measured with a stadiometer and the weight was measured with a heavy duty Seca 770 floor scale (Hamburg, Germany). The BMI was calculated using the Quetelet index (Eknoyan, 2008); i.e., the ratio of the weight in kilograms (kg) to the square of the height in meters (m^2). BMI groupings were based on WHO recommendations (WHO, 2008).

The blood pressure and pulse rates were measured with a blood pressure monitoring kit (Omron Digital blood pressure monitor, Japan) after the participants had rested 10-15min prior to the measurement. Participants were made to sit in a relaxed position, with feet flat on the ground, one arm resting on the thighs and the other on a table. No communication was allowed during the period of the measurement. The measurements were taken three times and an average of the last two readings was used in the analysis.

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3.2 Sample Processing

3.2.1 Blood Collection

Blood samples were collected by venipuncture in the fasting state with adequate precautions. From each participant, 10 mL of fasting blood was drawn and distributed into fluoride and EDTA tubes. Plasma was obtained from fluoride tubes after centrifugation at 4000 rpm for 10 min; this was used for fasting plasma glucose (FPG) analysis. After centrifugation of the blood in the EDTA tubes, buffy coat was obtained and stored at -30°C for DNA extraction. The plasma from the EDTA tubes were stored at -80°C until analysis of the total cholesterol (TC), triglycerides (TG), high density lipoprotein (HDL) and low density lipoprotein (LDL) levels.

3.2.2 Oral Glucose Tolerance Test (OGTT)

Oral glucose tolerance test was performed for all the controls to ascertain that their tolerance to glucose was not impaired (WHO, 2006). To 250 ml clean boiled water, 75g of anhydrous glucose was added; the solution was mixed thoroughly to obtain a uniform mixture. Each control participant was given a glucose solution after the fasting blood sample had been collected. Each participant was timed for two (2)-hours; the 2 hour period begun immediately the participant took the first sip: the solution was consumed within 5 minutes. Immediately after 2 hours, two (2) ml of the participant's blood was drawn into a fluoride tube for two-hour plasma glucose measurement.

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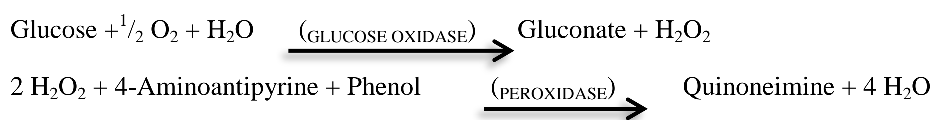
3.3 Sample Analysis

3.3.1 Fasting Plasma Glucose (FPG) Determination

Fasting plasma glucose and two-hour plasma glucose measurements were determined using the BS-400 Chemistry analyzer (Mindray, China). The analysis was done based on the principle that glucose is oxidized by glucose oxidase to produce gluconate and hydrogen peroxide. The hydrogen peroxide reacts with 4 - aminoantipyrine and phenol in the presence of peroxidase to yield a pink quinoneimine dye. The concentration of the dye was measured colorimetrically at 500 nm. The reaction is illustrated below (Equation 3.1):



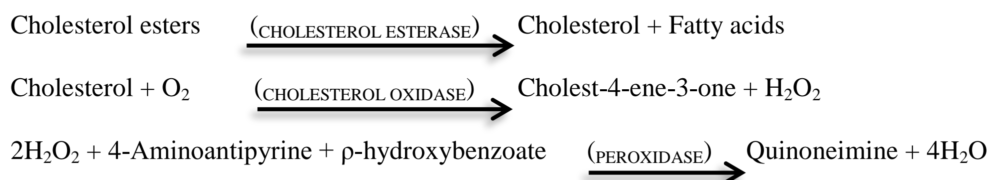
$$\text{Glucose} + {}^{1}/_{2}\,O_2 + H_2O \xrightarrow{(\text{GLUCOSE OXIDASE})} \text{Gluconate} + H_2O_2$$

$$2\,H_2O_2 + \text{4-Aminoantipyrine} + \text{Phenol} \xrightarrow{(\text{PEROXIDASE})} \text{Quinoneimine} + 4\,H_2O$$

Equation 3.1 Chemical reactions for the colorimetric determination of plasma glucose

3.3.2 Total Cholesterol (TC) Determination

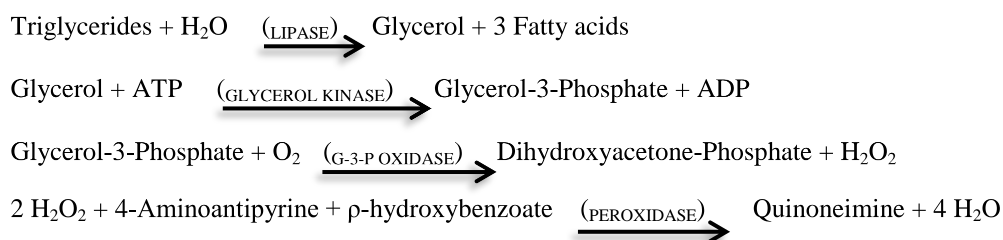
Total plasma cholesterol was determined quantitatively using enzymatic colorimetric method. To 10 µL of the EDTA plasma, 1000 µL of cholesterol reagent was added. The cholesterol esterase present in the reagent enzymatically hydrolyzed all cholesterol esters to cholesterol and free-fatty acids. The already available and released free cholesterol were then oxidized to choles-4-en-3-one and hydrogen peroxide by cholesterol oxidase. A peroxidase present in the test reagent catalyzed the reaction combining hydrogen peroxide, phenol and 4-aminoantipyrine to form a chromophore (quinoneimine dye). The absorbance of the dye was measured at 505 nm. The reactions involved are described below (Equation 3.2):



Equation 3.2 Chemical reactions for the colorimetric determination of plasma cholesterol

3.3.3 Determination of Plasma Triglycerides (TG)

The amount of triglycerides in 10 μL of the EDTA plasma was quantified colorimetrically using 1000 μL of the triglyceride reagent. The triglycerides were cleaved to glycerol and free fatty acids using a lipase. Glycerol was then converted to glycerol-3-phosphate and ADP in the presence of ATP, by the action of glycerol kinase. Glycerol-3-phosphate oxidase oxidized the glycerol-3-phosphate to dihydroxyacetone phosphate and hydrogen peroxide. A quinoneimine dye was formed from the reaction between the hydrogen peroxide, phenol and 4-aminoantipyrine, catalyzed by peroxidase in the test reagent. The absorbance of the dye was measured at 500 nm. The coupled reactions are illustrated in equation 3.3:

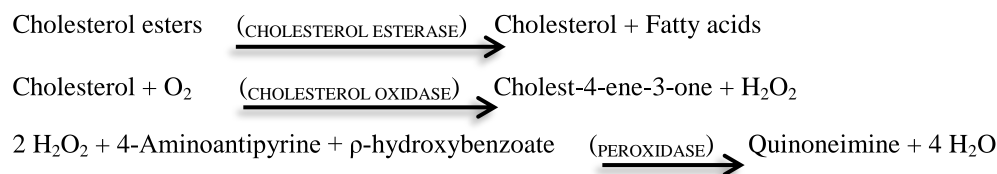


Equation 3.3 Chemical reactions for the colorimetric determination of plasma triglycerides

3.3.4 High Density Lipoprotein Cholesterol (HDL-C) Determination

Very low density lipoproteins (VLDL) and low density lipoproteins (LDL) were precipitated from 0.2 mL plasma with phosphotungstate and magnesium ions leaving

HDL in the solution. After centrifugation, the HDL cholesterol esters in the supernatant were determined spectrophotometrically as shown in coupled reactions below (Equation 3.4):



Equation 3.4 Chemical reactions for the colorimetric determination of plasma HDL-C

3.3.5 Low Density Lipoprotein Cholesterol (LDL-C) Determination

The LDL level in the plasma was calculated using the Friedewald Formula shown below:

$$LDL - C = TC - (HDL - C) - \frac{TG}{2.17}$$

The LDL values were recorded in mmol/L

3.3.6 Calculation of Very Low Density Lipoprotein (VLDL) Level

Very low density lipoprotein levels were estimated using the equation below:

$$VLDL = \frac{Triglycerides(mmol/L)}{2.2}$$

(Triglyceride < 4.5 mmol/L)

3.3.7 DNA Extraction

DNA was extracted from leukocytes in the buffy coat samples using the Quick-gDNA™ Blood MiniPrep DNA extraction kit (Epigenetics Company, USA). The

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concentration and purity of the extracted DNA samples were also determined using the NanoDrop 2000/2000C (Thermo Scientific, USA). The DNA samples were stored at -80°C until needed for further analysis.

3.3.8 Polymerase Chain Reaction (PCR)

The extracted DNA samples were amplified by PCR with the PON 1 192 primers. The primer sequences were 5' TATTGTTGCTGTGGGACCTGAG 3' for the forward primer and 5' CACGCTAAACCCAAATACATCTC 3' for the reverse primer respectively. The PCR conditions were as described by (Mackness *et al*., 1998a) with a few modifications (Appendix D 1.0).

The PCR was performed in heat block PCR tubes to ensure that the mixture did not evaporate in the course of the reaction. The constituents of each PCR tube in their respective quantities are shown below (Table 3.1). Positive and negative controls were set up. The tubes for the two controls had the same materials as the test PCR tubes, but the 2 μL of DNA was replaced with 2 μL PCR water in the negative control tube. The reaction was performed for 46 cycles in a Gene Pro thermal cycler (Hanghou Bioer Technology Co. Ltd., China). The conditions for the PCR were as follows: initial denaturation was done at 95 °C for 5 min, denaturation at 94 °C for 1 min, annealing at 61 °C for 30 sec, extension at 72 °C for 1 min and final extension at 72 °C for 10 min. The thermal cycler was set to hold the products at 4 °C until they were retrieved at the end of the 46 cycles. The products were then resolved on 3% agarose gel.

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Table 3.1 Constituents of the PCR Mixture

Item	**Volume (µL)**
DNA	2.0
Deoxyribonucleotides (dNTP) Mixture	0.4
PON 1 192 forward primer	0.1
PON 1 192 reverse primer	0.1
Dream Taq	0.1
$MgCl_2$	0.8
PCR coral load (15mM $MgCl_2$)	2.0
Q solution	4.0
PCR water	10.5
Total volume	**20.0**

3.3.9 Restriction Fragment Length Polymorphism (RFLP) Analysis

Restriction fragment length polymorphism (RFLP) analysis was done on DNA samples of 59 type II diabetics and 45 controls. To 5 µL of the PCR product in an eppendorf tube, nine µL of nuclease-free water, one µL of 10x buffer Tango (Thermo Fisher Scientific Inc.) and 0.5 µL of 2u/ µL BspPI (AlwI) restriction enzyme (Thermo Fisher Scientific Inc.) were added. The mixture was tapped gently and spun down for 5 seconds. It was then incubated for 2hrs at 55°C in a water bath (U Clear, England).

The RFLP products obtained after the restriction enzyme digestion were resolved on 3% agarose gel. The RR genotype of PON 1 192 polymorphism contains a unique BspPI restriction site, which was expected to give fragment sizes of 66 bp and 33 bp; the QQ genotype has only one fragment with a size of 99 bp. The QR genotype was expected to give three fragments with sizes 99 bp, 66bp and 33bp (Table 3.2).

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Table 3.2 Expected DNA Fragment Sizes after Restriction Enzyme Digestion

RESTRICTION ENZYME	GENOTYPE	NUMBER OF FRAGMENTS	FRAGMENT SIZES (bp)
BspPI (AlwI)	PON 1 192 QQ	1	99
	PON 1 192 RR	2	66, 33
	PON 1 192 QR	3	99, 66, 33

3.3.10 Agarose Gel Electrophoresis

The PCR and RFLP products were separated on 3% agarose gel (Thermo Fisher Scientific Inc.) which was prepared with 1 X tris acetate diaminoethanetetra- acetic acid (TAE) buffer (Appendix C 1.1, C 1.2). The gel was poured and allowed to set. It was placed in an electrophoretic chamber (Owl Separation System Inc., USA) filled with 750 ml of 1 X TAE buffer containing 20 µL of 10 mg/mL ethidium bromide solution (Sigma Chemical, USA; Appendix C 1.3). An O'Gene ruler 50 bp DNA ladder (Thermo Fisher Scientific Inc.) was ran alongside the PCR products in the first well. The electrophoresis was performed using 56 mAmp electric current at a potential difference of 80 volts for 90 min. The gel was visualized with an ultraviolet transilluminator (Uvitec, Cambridge, UK) and photographed with a photoman (Uvitec, Cambridge, UK). The images were printed with a video copy processor (Mitsubishi, Malaysia)

3.4 Ethical Issues

The present study was approved by the Ethical and Protocol Review Committee of the University of Ghana Medical School (MS-Et/M.3 – P 5.6/2011-12).

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3.5 Statistical Analysis

All data obtained was captured and cleaned in Microsoft excel (Microsoft office, 2007). The data was analyzed with SPSS statistical software version 16.0. For descriptive statistics, mean and standard deviations were used to summarize quantitative and or continuous variables (e.g. age, BMI, hip-waist ratio), and percentages were used to summarize all qualitative variables. Inferential statistics i.e. *p*-value, ANOVA, student *t*-test (www.graphpad.com) and chi-square was performed. Differences between frequencies of the PON 1 Q192R genotypes and alleles were established by chi-square analysis. A significance level of $p < 0.05$ was set.

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CHAPTER FOUR

4.0 RESULTS

4.1 General Characteristics and Demographic Data of the Studied Populations

A total of 225 participants were recruited for the study. Some of them however withdrew leaving 209 participants (112 type II diabetics and 97 controls). A total of 104 participants (59 type II diabetics and 45 controls) were selected randomly for PON 1 Q192R genotype determination due to limited logistics. The Demographics of the populations studied are presented in Table 4.1. There were more females in the diabetic population than in the control population ($\chi^2 = 7.761$, $p < 0.05$); while the number of males between the two populations was not statistically different ($\chi^2 = 2.176$, $p > 0.05$). There was not much difference in the educational background between the populations studied ($p > 0.05$). Alcohol intake was found to be higher in the controls ($\chi^2 = 5.743$, $p < 0.025$), while smoking status was similar in both populations ($p > 0.05$). The percentage of participants with family history of diabetes was very high in the diabetic population than in the control population ($\chi^2 = 66.659$, $p < 0.001$). The average duration of diabetes in the cases was 10.00 ± 7.31 years (Table 4.1).

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Table 4.1 Demographics of the Studied Populations

Variable	Type II diabetics (n = 112)	Controls (n = 97)	χ^2	*p*-value
Gender				
Males	32 (28.57)	58 (59.79)	2.176	> 0.05
Females	80 (71.43)	39 (40.29)	7.761	< 0.05
Educational background				
Basic	51 (38.14)	37 (38.14)	0.021	> 0.05
Secondary	27 (24.11)	33 (34.02)	1.925	> 0.05
Tertiary	23 (20.54)	20 (20.62)	0.026	> 0.05
No formal education	11 (9.82)	7 (7.22)	0.164	>0.05
Alcohol intake	23 (20.54)	43 (44.30)	5.743	< 0.025
Smoking history	2 (1.79)	2 (2.06)	0.141	> 0.05
Family history of diabetes	69 (80.36)	21 (21.64)	66.659	< 0.001
Duration of diabetes (years)*	10 .00 ±7.31	-	-	-
Therapy				
Metformin	73 (65.18)	-	-	-
Other glycaemic control drugs	25 (22.12)	-	-	-
Others (insulin, hypertensive drugs)	26 (23.21)	-	-	-

Table 4.1 Categorical values are presented as number (%). *Values are presented as means ± standard deviation; $p < 0.05$ was considered statistically significant.

4.2 Clinical and Biochemical Variables of the Studied Populations

There was no significant difference ($p > 0.05$) in the mean age and BMI of the diabetics when compared with the controls. However, the systolic blood pressure (SBP), diastolic blood pressure (DBP) and pulse rates were significantly higher in the type II diabetics than the controls ($p < 0.05$). The fasting plasma glucose (FPG), total cholesterol (TC), triglycerides (TG), low density lipoprotein cholesterol (LDL-C) and very low density lipoprotein (VLDL) levels were also significantly elevated in the diabetics more than in the controls ($p < 0.05$). Atherogenic index and cardiovascular

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risk values were higher in the type II diabetic patients than the controls ($p < 0.05$). The HDL-C levels and waist/hip ratios between the two populations showed no significant differences ($p > 0.05$). The oral glucose tolerance test (OGTT) results [6.09 ± 2.01 (95% CI $_{mean}$ = 5.75 – 6.43)] in the control group was found to be in the recommended range (WHO, 2006).

Table 4.2 Clinical and Biochemical Variables of the Type II Diabetic and Non-diabetic Populations

Variables	Type II Diabetics (n = 112)	Non-Diabetics (n = 97)	95 % CI of mean difference	*p*-value$_{(t\text{-test})}$
Age (years)	55.27 ± 11.89	50.04 ± 10.46	-2.85 – 3.31	0.8830
BMI (kg/m^2)	28.72 ± 7.18	27.05 ± 7.60	-0.35 – 3.69	0.1042
SBP (mmHg)	127.07 ± 19.51	120.94 ± 24.22	0.16 – 12.10	0.0441
DBP (mmHg)	75.00 ± 10.27	69.86 ± 15.42	1.61 – 8.67	0.0045
Pulse rate (bpm)	77.72 ± 11.78	71.19 ± 10.21	3.50 – 9.56	0.0001
Waist-Hip ratio	0.90 ± 0.20	0.90 ± 0.10	-0.04 – 0.04	1.0000
FPG (mmol/L)	9.96 ± 4.75	5.27 ± 1.19	3.71 – 5.67	0.0001
TC (mmol/L)	5.38 ± 1.88	4.35 ± 1.25	0.59 – 1.47	0.0001
TG (mmol/L)	1.40 ± 0.44	0.90 ± 0.49	0.37 – 0.63	0.0001
HDL-C (mmol/L)	1.27 ± 0.38	1.28 ± 0.41	-0.12 – 0.10	0.8551
LDL-C (mmol/L)	3.48 ± 1.84	2.74 ± 1.43	0.29 – 1.19	0.0015
VLDL (mmol/L)	0.64 ± 0.20	0.41 ± 0.22	0.17 – 0.29	0.0001
HDL-C/LDL-C	0.65 ± 0.23	0.96 ± 0.30	0.17 – 0.31	0.0001
Cardiovascular Risk	4.68 ± 2.46	3.89 ± 2.09	0.16 – 1.42	0.0139
Atherogenic Index	3.68 ± 2.46	2.89 ± 2.09	0.16 – 1.42	0.0139
OGTT (mmol/L)	-	6.09 ± 2.01	-	-

Table 4.2 Values are presented as means ± standard deviation; $p < 0.05$ was considered statistically significant. BMI = Body mass index; SBP = Systolic blood pressure; DBP = Diastolic blood pressure; FPG= Fasting plasma glucose; TC = Total cholesterol; TG = Triglycerides; HDL-C = High density lipoprotein cholesterol; LDL-C = Low density lipoprotein cholesterol; VLDL = Very low density lipoprotein; Cardiovascular risk = [TC/HDL-C]; Atherogenic index = [(TC-HDL-C)/HDL-C].

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4.3 Clinical and Biochemical Variables of Type II Diabetics and Non-diabetics According to Gender

The age, SBP, DBP, FPG, TC, TG, LDL-C and VLDL, HDL-C/LDL-C, cardiovascular risk and atherogenic index were significantly higher in the diabetic males ($p < 0.05$) than the control males (Table 4.3). The type II diabetic females had higher pulse rates and elevated FPG, TG and VLDL levels ($p < 0.05$) than the non-diabetic females in the study (Table 4.3). Gender stratification was also done with an equal number of males and females from both populations (Appendix E, Table E.1)

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Table 4.3 Gender Stratification of Clinical and Biochemical Variables in the Diabetic and Non-diabetic Populations

Variables	Male Type II diabetics (n = 32)	Male Non-diabetics (n = 58)	*p*-value (*t*-test)	Female Type II diabetics (n = 80)	Female Non-diabetics (n = 39)	*p*-value (*t*-test)
Age (years)	55.03 ± 10.95	49.29 ± 10.75	0.0181	55.36 ± 12.32	51.15 ± 10.06	0.0664
BMI (kg/m^2)	23.92 ± 4.64	24.90 ± 6.81	0.4700	30.64 ± 7.14	30.25 ± 7.66	0.7853
SBP (mmHg)	130.38 ± 21.96	118.63 ± 23.63	0.0230	125.74 ± 18.42	124.39 ± 24.98	0.7400
DBP (mmHg)	76.72 ± 12.04	66.66 ± 14.41	0.0012	74.31 ± 9.46	74.62 ± 15.82	0.8942
Pulse rate (bpm)	74.05 ± 11.78	70.78 ± 10.82	0.1871	79.19 ± 11.53	71.81 ± 9.30	0.0007
Waist-Hip Ratio	0.91 ± 0.30	0.87 ± 0.23	0.4813	0.92 ± 0.11	0.90 ± 0.12	0.3681
FPG (mmol/L)	10.94 ± 5.40	5.25 ± 0.66	0.0001	9.57 ± 4.44	5.31 ± 1.72	0.0001
TC (mmol/L)	5.78 ± 1.17	4.06 ± 1.15	0.0001	5.34 ± 1.79	4.77 ± 1.29	0.0785
TG (mmol/L)	1.38 ± 0.54	0.84 ± 0.45	0.0001	1.41 ± 0.40	0.98 ± 0.54	0.0001
HDL-C (mmol/L)	1.23 ± 0.30	1.39 ± 0.40	0.0514	1.29 ± 0.42	1.11 ± 0.38	0.0255
LDL-C (mmol/L)	3.63 ± 2.13	2.36 ± 1.29	0.0007	3.41 ± 1.72	3.32 ± 1.45	0.7788
VLDL (mmol/L)	0.63 ± 0.25	0.38 ± 0.20	0.0001	0.64 ± 0.18	0.45 ± 0.25	0.0001
HDL-C/LDL-C	0.56 ± 0.27	1.32 ± 0.52	0.0001	0.53 ± 0.37	0.42 ± 0.27	0.1010
Cardiovascular Risk	4.92 ± 2.93	3.21 ± 1.44	0.0004	4.58 ± 2.26	4.91 ± 2.49	0.4711
Atherogenic Index	3.92 ± 2.93	2.21 ± 1.44	0.0004	3.58 ± 2.26	3.91 ± 2.49	0.4711

Table 4.3 Values are presented as means ± standard deviation; $p < 0.05$ was considered statistically significant. BMI = Body mass index; SBP = Systolic blood pressure; DBP = Diastolic blood pressure; FPG= Fasting plasma glucose; TC = Total cholesterol; TG = Triglycerides; HDL-C = High density lipoprotein cholesterol; LDL-C = Low density lipoprotein cholesterol; VLDL = Very low density lipoprotein; Cardiovascular risk = [TC/HDL-C]; Atherogenic index = [(TC – HDL-C)/HDL-C].

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4.4 Stratification of Clinical and Biochemical Variables the Type II Diabetics by Age

The hypothesis that there would be no difference in the clinical variables of diabetics with age was tested (Table 4.4). It was observed that the FPG levels did not vary with age ($p > 0.05$) as expected in diabetics (Blunt *et al*., 1991). However, the SBP of the type II diabetics was significantly elevated in categories C, D, E ($p < 0.05$); Table 4.4) more than in category A. The Category E participants had higher SBP values than those in categories B, C and D respectively ($p < 0.05$). They also had significantly higher BMI and lower pulse rates when compared with participants in category A.

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Table 4.4 Clinical and Biochemical Variables of Type II Diabetics According to Age

Variable	Age categories (years)						
	A (30 – 39) $_{14*}$	**B** (40 – 49) $_{21*}$	**C** (50 – 59) $_{42*}$	**D** (60 – 69) $_{22*}$	**E** (70 – 79) $_{10*}$	**F** (80 – 89) $_{2*}$	p-value $_{(ANOVA)}$
BMI (kg/m^2)	24.50 ± 6.01	29.10 ± 5.65	28.64 ± 7.04	27.41 ± 5.79	32.70 ± 12.41	28.00 ± 2.83	0.134
SBP (mmHg)	112.57 ± 12.56	123.52 ± 22.12	129.62 ± 19.14	127.27 ± 15.06	143.50 ± 22.01	117.00 ± 2.83	0.004
DBP (mmHg)	74.43 ± 9.84	72.81 ± 10.60	76.83 ± 12.27	73.68 ± 7.03	72.20 ± 8.18	80.50 ± 2.12	0.568
Pulse rate (bpm)	84.26 ± 14.21	76.71 ± 12.16	77.86 ± 11.13	77.32 ± 11.18	71.10 ± 9.76	71.75 ± 0.35	0.145
Waist-Hip Ratio	0.87 ± 0.09	0.97 ± 0.20	0.96 ± 0.16	0.92 ± 0.13	0.89 ± 0.07	0.97 ± 0.02	0.304
FPG (mmol/L)	11.56 ± 7.50	9.39 ± 3.83	10.33 ± 4.92	9.12 ± 2.77	8.11 ± 2.87	7.90 ± 2.97	0.441
TC (mmol/L)	5.51 ± 1.35	5.34 ± 2.63	5.53 ± 1.75	5.09 ± 1.95	5.68 ± 1.37	4.23 ± 1.38	0.878
TG (mmol/L)	1.14 ± 0.53	0.90 ± 0.53	0.98 ± 0.52	0.91 ± 0.29	0.80 ± 0.42	1.00 ± 0.00	0.586
HDL-C (mmol/L)	1.26 ± 0.47	1.18 ± 0.40	1.19 ± 0.38	1.22 ± 0.29	1.41 ± 0.538	1.30 ± 0.00	0.711
LDL-C (mmol/L)	3.49 ± 1.56	3.41 ± 2.42	3.61 ± 1.74	3.15 ± 1.85	3.57 ± 1.5	2.15 ± 1.34	0.862
VLDL (mmol/L)	0.68 ± 0.32	0.64 ± 0.21	0.62 ± 0.19	0.625 ± 0.13	0.582 ± 0.16	0.66 ± 0.02	0.906
HDL-C/LDL-C	0.53 ± 0.55	1.160 ± 2.79	0.48 ± 0.43	0.63 ± 0.63	0.48 ± 0.31	0.76 ± 0.48	0.516
Cardiovascular Risk	5.05 ± 3.25	4.65 ± 2.21	5.06 ± 2.87	4.15 ± 1.61	4.23 ± 1.48	3.13 ± 1.06	0.641
Atherogenic Index	4.05 ± 3.25	3.65 ± 2.21	4.03 ± 2.87	3.15 ± 1.61	3.23 ± 1.48	2.13 ± 1.06	0.641

Table 4.4 Values are presented as means ± standard deviation; * population size; $p < 0.05$ was considered statistically significant. BMI = Body mass index; SBP = Systolic blood pressure; DBP = Diastolic blood pressure; FPG= Fasting plasma glucose; TC = Total cholesterol; TG = Triglycerides; HDL-C = High density lipoprotein cholesterol; LDL-C = Low density lipoprotein cholesterol; VLDL = Very low density lipoprotein; Cardiovascular risk = [TC/HDL-C]; Atherogenic index = [(TC – HDL-C)/HDL-C].

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4.4.1 Stratification of Clinical Variables of Controls According to Age

SBP and pulse rates were significantly different (Table 4.4.1) within the control population after age-stratification ($p < 0.05$). Participants in category F recorded higher SBP values than categories A, B, C, D, and E. On the other hand, individuals in category A had a significantly ($p<0.05$) lower SBP than those in categories C and D. In addition, category A controls had higher pulse rates than those in categories B, C and D. Their HDL levels were also higher than categories D, E and F. However, they recorded lower levels of TC and LDL when compared with the category C controls.

Some clinical and biochemical variables (SBP, FPG, TG, TC and LDL-C) of an equal number of diabetics and controls selected at random were matched according the age categories (Appendix E, Table E.2). The diabetics in some of the age categories recorded comparatively higher values for certain variables ($p < 0.05$) than their respective controls.

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Table 4.4.1 Clinical and Biochemical Variables According to Age categories in the Control Population

Variable	Age categories (years)						
	A(30-40)$_{14}$*	**B**(40-50)$_{21}$*	**C**(50-60)$_{42}$*	**D**(60-70)$_{22}$*	**E**(70-80)$_{10}$*	**F**(80-90)$_{2}$*	*p*-value $_{(ANOVA)}$
SBP (mmHg)	110.11 ± 17.24	119.43 ± 25.97	123.96 ± 21.85	131.17 ± 22.40	126.50 ±12.58	175.25 ± 29.34	0.003
DBP (mmHg)	65.59 ± 9.80	72.07 ± 19.58	70.50 ± 13.15	66.61 ± 12.71	70.17 ± 13.36	83.00 ± 24.04	0.513
Pulse rate(bpm)	76.59 ± 9.77	70.71 ± 9.62	69.36 ± 9.21	66.00 ± 7.44	67.50 ± 24.64	72.25 ± 6.72	0.077
BMI (kg/m^2)	24.47 ± 8.35	27.81 ± 6.87	28.53 ± 6.22	29.20 ± 11.62	21.77 ± 0.43	21.44 ± 3.03	0.212
Waist-Hip Ratio	0.86 ± 0.07	0.91 ± 0.07	0.92 ± 0.09	0.86 ± 0.20	0.92 ± 0.05	0.90 ± 0.03	0.150
FPG (mmol/L)	5.09 ± 0.66	5.59 ± 1.76	5.20 ± 0.57	4.88 ± 0.78	4.87 ± 0.31	4.95 ± 0.35	0.465
TC (mmol/L)	3.96 ± 1.38	4.36 ± 1.18	4.71 ± 1.27	4.44 ± 1.24	4.32 ± 0.81	3.37 ± 0.84	0.370
TG (mmol/L)	0.85 ± 0.59	0.95 ± 0.51	0.91 ± 0.50	0.86 ± 0.17	0.71 ± 0.24	0.76 ± 0.14	0.940
HDL-C (mmol/L)	1.46 ± 0.39	1.27 ± 0.41	1.28 ± 0.40	1.13 ± 0.38	0.81 ± 0.21	0.78 ± 0.11	0.028
LDL-C (mmol/L)	2.16 ± 1.46	2.72 ± 1.38	3.14 ± 1.46	3.06 ± 1.44	3.26 ± 0.75	2.24 ± 1.01	0.238
VLDL (mmol/L)	0.39 ± 0.27	0.43 ± 0.23	0.41 ± 0.23	0.39 ± 0.08	0.32 ± 0.11	0.35 ± 0.06	0.940
HDL-C/ LDL-C	1.49 ± 2.27	1.08 ± 2.16	0.60 ± 0.61	0.51 ± 0.42	0.25 ± 0.06	0.40 ± 0.23	0.491
Cardiovascular Risk	3.09 ± 2.30	3.89 ± 1.93	4.18 ± 2.14	4.45 ± 2.13	5.43 ± 0.92	4.46 ± 1.70	0.296
Atherogenic Index	2.09 ± 2.30	2.89 ± 1.93	3.18 ± 2.14	3.45 ± 2.13	4.43 ± 0.92	3.46 ± 1.70	0.296

Table 4.4.1 Values are presented as means ± standard deviation; * population size; $p < 0.05$ was considered statistically significant. BMI = Body mass index; SBP = Systolic blood pressure; DBP = Diastolic blood pressure; FPG= Fasting plasma glucose; TC = Total cholesterol; TG = Triglycerides; HDL-C = High density lipoprotein cholesterol; LDL-C = Low density lipoprotein cholesterol; VLDL = Very low density lipoprotein; Cardiovascular risk = [TC/HDL-C]; Atherogenic index = [(TC – HDL-C)/HDL-C].

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4.5 Stratification of Clinical and Biochemical Variables of the Type II Diabetics According to BMI

The hypothesis that there is no association between BMI and the clinical variables was tested. It was observed that in conformity with the hypothesis, the values for DBP, FPG, TG, VLDL and atherogenic index between the diabetic BMI groups (Table 4.5; Appendix F) were not statistically significant ($p < 0.05$). Post-hoc analysis however showed that the FPG levels were higher ($p < 0.05$) in underweight diabetics than in the normal, the overweight and obese diabetics. Comparing the underweight diabetics with the normal, the overweight and obese diabetic patients, TG and VLDL levels were found to be higher ($p < 0.05$). The overweight diabetics were older than the underweight and normal diabetics respectively ($p < 0.05$).

In the control population, BMI stratification (Table 4.5.1) showed that the underweight controls were significantly younger ($p<0.05$) than the normal controls. DSP values were lower in the underweight and normal controls as compared to the overweight and obese controls respectively. The controls with normal BMI values had lower TC levels than their overweight and obese counterparts. HDL levels were higher in the obese group than in the underweight, normal and overweight groups. These obese controls also recorded higher LDL, TG and VLDL values than the normal controls ($p<0.05$).

The following clinical and biochemical variables; SBP, FPG, TG, TC and LDL-C were matched between an equal number of diabetics and controls according to the BMI groupings (Appendix E, Table E.3). The normal, overweight and obese diabetics recorded higher levels of FPG and TG than the corresponding controls ($p < 0.05$)

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Table 4.5 Clinical and Biochemical Variables in the Type II Diabetic Population by BMI Stratification

Variable	BMI categories				
	Underweight$_{3*}$	Normal$_{31*}$	Overweight$_{47*}$	Obese$_{31*}$	*p*-value (ANOVA)
Age (years)	42.00 ± 13.89	51.58 ± 12.32	58.23 ± 11.86	55.74 ± 9.87	0.019
SBP (mmHg)	117.33 ± 6.35	127.32 ± 25.26	126.64 ± 18.49	127.48 ± 15.46	0.674
DBP (mmHg)	69.67 ± 5.51	74.87 ± 13.95	74.79 ± 9.37	75.07 ± 7.62	0.608
Pulse rate (bpm)	71.00 ± 10.21	76.36 ± 12.06	79.10 ± 12.79	77.65 ± 10.05	0.861
Waist-Hip Ratio	0.87 ± 0.06	0.94 ± 0.19	0.92 ± 0.11	0.96 ± 0.17	0.608
FPG (mmol/L)	12.67 ± 8.02	11.42 ± 6.56	9.24 ± 2.90	8.78 ± 3.70	0.065
TC (mmol/L)	6.12 ± 0.87	5.53 ± 1.88	5.25 ± 1.64	5.37 ± 2.30	0.834
TG (mmol/L)	1.67 ± 1.15	0.97 ± 0.48	0.94 ± 0.38	0.90 ± 0.47	0.061
HDL-C (mmol/L)	0.97 ± 0.49	1.19 ± 0.33	1.28 ± 0.33	1.22 ± 0.50	0.472
LDL-C (mmol/L)	4.13 ± 1.86	3.64 ± 1.91	3.23 ± 1.65	3.45 ± 2.07	0.708
VLDL (mmol/L)	0.97 ± 0.68	0.61 ± 0.15	0.64 ± 0.16	0.61 ± 0.21	0.025
HDL-C /LDL-C	0.30 ± 0.20	0.57 ± 0.64	0.79 ± 1.87	0.54 ± 0.54	0.771
Cardiovascular Risk	8.34 ± 6.79	4.99 ± 2.77	4.24 ± 1.78	4.68 ± 2.26	0.033
Atherogenic Index	7.34 ± 6.79	3.99 ± 2.77	3.24 ± 1.78	3.68 ± 2.26	0.033

Table 4.5 Values are presented as means ± standard deviation; *population size; $p < 0.05$ was considered statistically significant. BMI = Body mass index; SBP = Systolic blood pressure; DBP = Diastolic blood pressure; FPG= Fasting plasma glucose; TC = Total cholesterol; TG = Triglycerides; HDL-C = High density lipoprotein cholesterol; LDL-C = Low density lipoprotein cholesterol; VLDL = Very low density lipoprotein; Cardiovascular risk = [TC/HDL-C]; Atherogenic index = [(TC-HDL-C)/HDL-C].

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Table 4.5.1 Clinical and Biochemical Variables of Controls According to BMI Groups

Variable	BMI categories				
	Underweight$_{(7)}$*	Normal$_{(38)}$*	Overweight$_{(27)}$*	Obese$_{(25)}$*	p-value$_{(ANOVA)}$
Age (years)	43.00±7.05	52.55±13.73	48.15±7.76	50.24±6.40	0.098
SBP (mmHg)	106.93±19.64	120.29±24.08	123.54±24.62	123.06±25.07	0.417
DBP (mmHg)	59.00±11.64	64.82±13.57	72.46±13.37	77.74±17.12	0.001
Pulse rate (bpm)	71.00±9.45	71.33±11.21	70.80±10.37	71.46±9.14	0.100
Waist-Hip Ratio	0.83± 0.09	0.87±0.10	0.92±0.07	0.93±0.09	0.004
FPG (mmol/L)	4.93±0.66	5.13±0.40	5.27±0.77	5.60±2.13	0.391
TC (mmol/L)	4.03±1.08	3.95±1.06	4.61±1.26	4.75±1.41	0.039
TG (mmol/L)	0.72±0.60	0.79±0.40	0.91±0.54	1.10±0.49	0.067
HDL-C (mmol/L)	1.43±0.42	1.34±0.41	1.38±0.38	1.04±0.36	0.007
LDL-C (mmol/L)	2.27±1.29	2.28±1.18	2.92±1.43	3.38±1.58	0.016
VLDL (mmol/L)	0.33±0.27	0.36±0.18	0.41±0.24	0.50±0.22	0.067
HDL-C/LDL-C	2.02±3.86	0.97±1.20	0.91±1.74	0.69±1.63	0.369
Cardiovascular Risk	3.06±1.24	3.30±1.54	3.65±1.60	5.30±2.79	0.001
Atherogenic Index	2.06±1.24	2.30±1.54	2.65±1.60	4.30±2.79	0.001

Table 4.5.1 Values are presented as means ± standard deviation; *population size; $p < 0.05$ was considered statistically significant. BMI = Body mass index; SBP = Systolic blood pressure; DBP = Diastolic blood pressure; FPG= Fasting plasma glucose; TC = Total cholesterol; TG = Triglycerides; HDL-C = High density lipoprotein cholesterol; LDL-C = Low density lipoprotein cholesterol; VLDL = Very low density lipoprotein; Cardiovascular risk = [TC/HDL-C]; Atherogenic index = [(TC-HDL-C)/HDL-C].

4.6.1 DNA Yield and Purity

It was observed that DNA extracted from type II diabetic patients was one and half times (1.53:1) more than that of the controls. All extracted DNA samples had absorbance ratio (A_{260}/A_{280}) between 1.74 and 1.90.

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4.6.2 Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP)

Amplification of DNA by PCR generated a 99 bp amplicon (Fig 4.1) which yielded a 67.01 ± 3.47 bp (95 % CI 66.29 – 67.73) DNA fragment upon digestion with BspPI (Fig 4.2; Appendix D 2.0). The smaller fragment (33 bp) expected after the restriction enzyme digestion did not resolve on the gel. PON 1 Q192R genotypes were therefore assigned based on the presence or absence of a restriction site in the PCR amplicon (Fig. 4.2; Table 4.6).

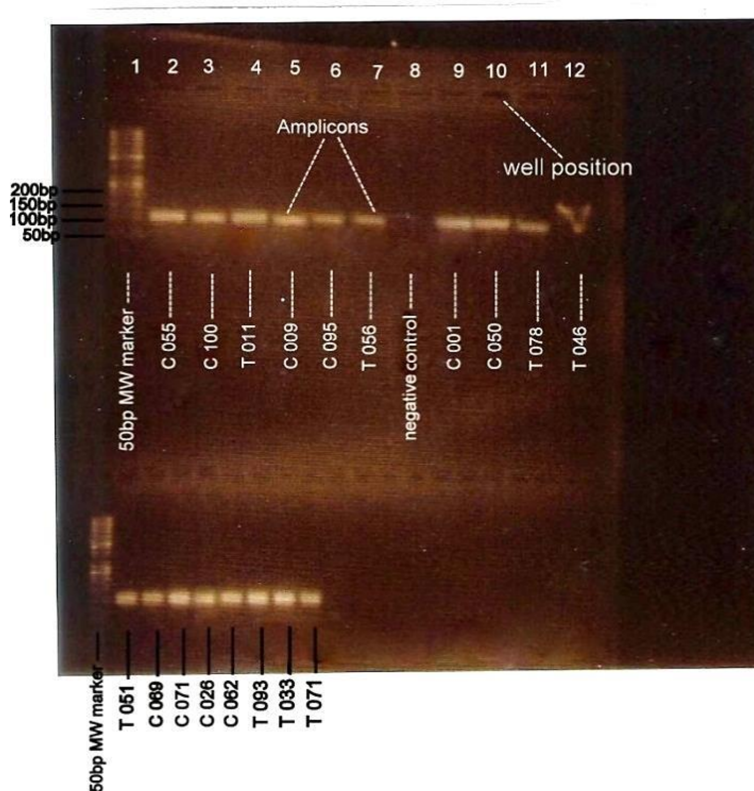

Fig 4.1 Agarose gel electrophoretogram of PON 1 Q192R PCR amplicons

Lane 1: 50 bp molecular weight marker, Lane 8: negative control, Lanes: 2, 3, 4, 5, 6, 7, 9, 10, 11, 12 PON 1 Q192R PCR products

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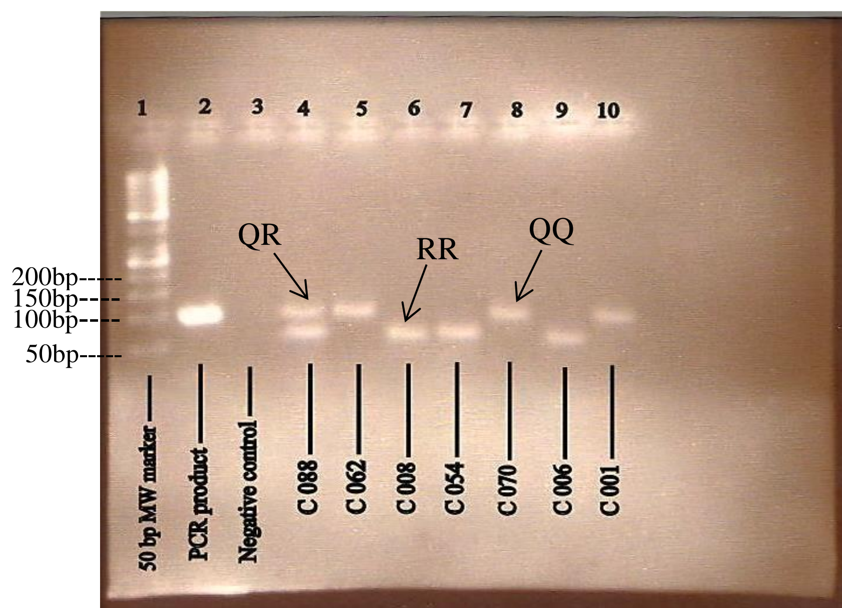

Fig. 4.2 Agarose gel electrophoretogram of PON 1 Q192R RFLP products

Lane 1: 50 bp molecular weight marker, Lane 2: PON 1 Q192R PCR product, Lane 3: Negative control, Lanes 4, 5, 6, 7, 8, 9, 10: PON 1 Q192R RFLP products

Table 4.6 Classification of PON 1 Q192R Genotypes upon Interrogation with BspPI

RFLP site	Primer sequence	Product size	After cut		GENOTYPE
			—RFLP	+RFLP	
PON1 Q192R Primer	5'TATTGTTGCTGTGGGACCTGAG3' 5'CACGCTAAACCCAAATACATCT3'	99bp	99bp	—	QQ
		99bp	—	99bp, 67bp	QR
		99bp	—	67bp	RR

(—) corresponds to no digestion, (+) corresponds to successful digestion

4.7 PON 1 Q192R Allelic and Genotypic Distributions in the Populations Studied

The distribution pattern of the PON 1 Q192R genotypes (QQ, RR and QR) in the type II diabetic population was similar to what was observed in the control group (χ^2 = 3.432, p = 0.1798) (Table 4.7.1). The number of RR homozygotes observed in both the type II diabetic and control populations was higher than the QQ homozygotes. The

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heterozygous QR genotype was the least frequent in the two populations (Table 4.7.1).

The distribution of the Q and R alleles was significantly different in the two populations ($\chi^2 = 5.357$, $p = 0.0206$). In the diabetic population, the frequency of the R allele was higher than what was observed in the control population; whereas the Q allele was more frequent in the control population. The R allele was significantly associated with type II diabetics (OR = 2.21; 95% CI = 1.17 – 4.14; $p = 0.0141$) whilst the Q allele was weakly associated with them (OR = 0.45; 95% CI = 0.24 – 0.85; $p = 0.0141$) (Table 4.7.2).

Table 4.7.1 Distribution of PON 1 Q192R Genotypes and Alleles

	Type II Diabetics (n = 55)	**Controls (n = 42)**	χ^2	***p*-value**
	Number (%)	**Number (%)**		
PON 1 192 Genotype				
QQ	10 (18.18)	14 (33.33)	3.432	0.1798
RR	41 (74.55)	24 (57.14)		
QR	4 (7.27)	4 (9.52)		
PON 1 192 Allele				
Q	24 (21.80)	32 (38.00)	5.357	0.0206*
R	86 (78.20)	52 (62.00)		

*χ^2 with Yates correction; $p < 0.05$ was considered statistically significant

Table 4.7.2 Odds of Association between PON 1 Q192R Alleles and Type II Diabetes Mellitus

PON 1 Q192R Allele	**Odds Ratio**	**95% CI**	***p*-value**
R	2.2051	1.17 – 4.14	0.0141
Q	0.4535	0.24 – 0.85	0.0141

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4.8 Departure from Hardy-Weinberg Equilibrium

In the type II diabetic and control populations the observed and expected PON 1 Q192R genotypic frequencies (Tables 4.8) were found to be statistically different ($p < 0.00001$).

Table 4.8 Observed and Expected PON 1 Q192R Genotypic Frequencies in the Type II Diabetic and Control Populations

PON 1 Q192R Genotype	Type II Diabetics (n = 55)		*p*-value (χ^2)	Controls (n = 42)		*p*-value (χ^2)
	Observed Number	Expected Number		Observed Number	Expected Number	
QQ	10	2.62		14	6.10	
RR	41	33.62	< 0.00001	24	16.10	< 0.00001
QR	4	18.76		4	19.81	

$p < 0.05$ was considered statistically significant

4.9 Clinical Variables of Type II Diabetes Patients and Controls According to the PON 1 Q192R Genotype Classes

The type II diabetics in the QR and RR groups recorded higher pulse rates than their corresponding controls (Table 4.9). QQ and RR homozygous diabetics were found to have higher FPG levels and lower waist-hip ratios ($p < 0.05$) than the respective control counterparts. In the type II diabetic population, QQ, QR and RR genotype groups recorded significantly higher values for TG and VLDL ($p < 0.05$) than the controls in these groups. A higher total cholesterol level was observed in QR type II diabetics only (Table 4.9).

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Table 4.9 Comparison of Clinical and Biochemical Variables between Type II Diabetics and Controls According to PON 1 Q192R Genotypes

Variable	QQ			RR			QR		
	Diabetics$_{10*}$	Control$_{14*}$	*p*-value$_{(t\text{-test})}$	Diabetics$_{41*}$	Control$_{24*}$	*p*-value$_{(t\text{-test})}$	Diabetics$_{4*}$	Control$_{4*}$	*p*-value $_{(t\text{-test})}$
BMI (kg/m^2)	32.17 ± 5.67	29.16 ± 5.48	0.2044	27.48 ± 6.04	28.55 ± 9.54	0.5813	31.53 ± 4.83	24.76 ± 7.46	0.1785
SBP (mmHg)	120.90± 12.53	121.50±26.74	0.9482	131.50± 22.93	125.13±27.79	0.3217	127.13± 8.44	108.38± 6.22	0.0117
DBP (mmHg)	74.30 ± 9.30	75.36 ± 19.20	0.9092	74.98 ± 11.38	72.06 ± 17.69	0.4205	73.50 ± 4.36	63.38 ± 7.78	0.0637
Pulse rate (bpm)	73.35 ± 12.37	67.86 ± 8.56	0.2110	76.01 ± 11.60	68.04 ± 8.09	0.0043	88.00 ± 9.26	65.63 ± 1.31	0.0031
Waist-Hip Ratio	1.02 ± 0.16	0.89 ± 0.14	0.0461	0.93 ± 0.08	0.88 ± 0.09	0.0235	0.96 ± 0.04	0.86±0.10	0.1127
FPG (mmol/L)	8.98 ± 2.38	5.04 ± 0.73	0.0001	10.02 ± 4.97	5.13 ± 0.67	0.0001	8.63 ± 3.75	5.35 ± 0.75	0.1371
TC (mmol/L)	4.90 ± 1.45	4.32 ± 0.82	0.1584	5.68 ± 2.14	4.82 ± 1.45	0.0858	5.32 ± 0.82	3.50 ± 0.97	0.0286
TG (mmol/L)	1.46 ± 0.25	0.93 ± 0.60	0.0156	1.39 ± 0.45	1.00 ± 0.62	0.0048	1.63 ± 0.30	0.91 ± 0.28	0.0127
HDL-C (mmol/L)	1.19 ± 0.21	1.17 ± 0.44	0.8956	1.31 ± 0.41	1.24 ± 0.39	0.5014	1.11 ± 0.25	1.02 ± 0.29	0.6549
LDL-C (mmol/L)	3.04 ± 1.25	2.73 ± 0.99	0.5046	3.75 ± 2.05	3.19 ± 1.62	0.2569	3.46 ± 0.72	2.07 ± 1.25	0.1022
VLDL (mmol/L)	0.67 ± 0.11	0.42 ± 0.27	0.0115	0.63 ± 0.21	0.45 ± 0.28	0.0045	0.74 ± 0.14	0.41 ± 0.13	0.0136
HDL-C/LDL-C	0.46 ± 0.24	1.00 ± 0.80	0.0517	0.81 ± 0.60	0.55 ± 0.44	0.0691	0.34 ± 0.12	0.77 ± 0.66	0.2471

Table 4.9 Values are presented as means ± standard deviation; * population size; $p < 0.05$ was considered statistically significant. BMI = Body mass index; SBP = Systolic blood pressure; DBP = Diastolic blood pressure; FPG= Fasting plasma glucose; TC = Total cholesterol; TG = Triglycerides; HDL-C = High density lipoprotein cholesterol; LDL-C = Low density lipoprotein cholesterol; VLDL = Very low density lipoprotein; Cardiovascular risk = [TC/HDL-C]; Atherogenic index = [(TC – HDL-C)/HDL-C].

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4.10 PON 1 Q192R Genotypes and the Clinical Variables within the Diabetic and Control Populations

It was observed that within the diabetic and control populations RR homozygotes recorded higher values for SBP, TC, TG, HDL-C and LDL-C than the QQ and QR participants; however these differences were not statistically significant (Table 4.10).

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Table 4.10 PON1 Q192R Genotypes and the Clinical and Biochemical Variables within the Type II Diabetic and Control Populations

Variable	Type II diabetic patients			*p*-value	Controls			*p*-value
	QQ (n = 10)	RR (n = 41)	QR (n = 4)		QQ (n = 14)	RR (n = 24)	QR (n = 4)	
SBP (mmHg)	120.90 ± 12.53	131.50 ±22.93	127.13 ± 8.44	0.354	121.50 ±26.74	125.13 ±27.79	108.38 ± 6.22	0.502
DBP (mmHg)	74.30 ± 9.30	74.98 ±11.38	73.50 ± 4.36	0.956	75.36 ±19.20	72.06 ±17.69	63.38 ± 7.78	0.490
Pulse rate (bpm)	73.35 ± 12.37	76.01 ±11.60	88.00 ±19.26	0.132	67.86 ± 8.56	68.04 ± 8.09	65.63 ± 1.31	0.853
BMI (kg/m^2)	32.17 ± 5.67	27.48 ± 6.04	31.53 ± 4.83	0.056	29.16 ± 5.48	28.55 ± 9.54	24.76 ± 7.46	0.639
Waist-Hip Ratio	1.02 ± 0.16	0.93 ± 0.08	0.96 ± 0.04	0.018	0.89 ± 0.14	0.88 ± 0.09	0.86 ± 0.10	0.814
FPG (mmol/L)	8.98 ± 2.38	10.02 ± 4.97	8.63 ± 3.75	0.717	5.04 ± 0.73	5.13 ± 0.67	5.35 ± 0.75	0.733
TC (mmol/L)	4.90 ± 1.45	5.68 ± 2.14	5.32 ± 0.82	0.530	4.32 ± 0.82	4.82 ± 1.45	3.50 ± 0.97	0.120
HDL-C (mmol/L)	1.19 ± 0.21	1.31± 0.41	1.11 ± 0.25	0.445	1.17 ± 0.44	1.24 ± 0.39	1.02 ± 0.29	0.576
LDL-C (mmol/L)	3.04 ± 1.25	3.75 ± 2.05	3.46 ± 0.72	0.560	2.73 ± 0.99	3.19 ± 1.62	2.07 ± 1.25	0.287
TG (mmol/L)	1.46 ± 0.25	1.39 ± 0.45	1.63 ± 0.30	0.517	0.93 ± 0.60	1.00 ± 0.62	0.91 ± 0.28	0.921
VLDL (mmol/L)	0.67 ± 0.11	0.63 ± 0.21	0.74 ± 0.14	0.504	0.42 ± 0.27	0.45 ± 0.28	0.41 ± 0.13	0.926
HDL-C/LDL-C	0.46 ± 0.24	0.81 ± 0.60	0.34 ± 0.12	0.073	1.00 ± 0.80	0.55 ± 0.44	0.77 ± 0.66	0.096
Cardiovascular Risk	4.12 ± 0.89	4.72 ± 2.14	4.95 ± 1.30	0.643	4.22 ± 1.65	4.48 ± 2.66	3.91 ± 2.20	0.879
Atherogenic Index	3.12 ± 0.89	3.72 ± 2.14	3.95 ± 1.30	0.643	3.22 ± 1.65	3.48 ± 2.66	2.91 ± 2.20	0.879

Table 4.10 Values are presented as means ± standard deviation; $p < 0.05$ was considered statistically significant. BMI = Body mass index; SBP = Systolic blood pressure; DBP = Diastolic blood pressure; FPG= Fasting plasma glucose; TC = Total cholesterol; TG = Triglycerides; HDL-C = High density lipoprotein cholesterol; LDL-C = Low density lipoprotein cholesterol; VLDL = Very low density lipoprotein; Cardiovascular risk = [TC/HDL-C]; Atherogenic index = [(TC – HDL-C)/HDL-C].

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4.11 Association between PON 1 Q192R Genotypes and the Clinical Variables in the Type II Diabetes Population

No association was found between the PON 1 Q192R genotypes and BMI, HDL-C, LDL-C, TG and TC in the type II diabetics ($p > 0.05$) (Table 4.11). The RR and QQ genotypes were associated with high FPG levels in the type II diabetics ($p < 0.004$).

Table 4.11 Association between PON 1 Q192R Genotypes and the Clinical and Biochemical Variables of the Type II Diabetics

Variables	PON 1 Q192R Genotypes		
	RR	QQ	QR
BMI	1.16 (0.41 – 3.30)	9.00(0.43 – 188.98)	21.00 (0.64 – 690.03)
FPG	53.43 (10.15 – 281.11) *	43.33 (3.89 – 481.84) *	9.00 (0.366 –220.94)
HDL-C	0.93 (0.32 – 2.72)	0.45(0.06 – 3.00)	0.33 (0.02 – 0.65)
LDL-C	1.57 (0.55 – 4.47)	5.57(0.48 –64.09)	9.00 (0.298 – 271.67)
TC	1.62 (0.59 – 4.48)	6.00 (0.87 – 41.90)	21.00 (0.64 – 690.03)
TG	1.83 (0.51 – 6.57)	1.44 (0.08 – 26.23)	0.11 (0.00 – 3.35)

*$p < 0.05$, Odds ratio (95% CI)

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CHAPTER FIVE

5.0 DISCUSSION

Paraoxonase 1 (PON 1) is a typical HDL - related enzyme that is associated with type II diabetes and diabetes-associated microvascular and macrovascular diseases (Mackness *et al.*, 1998b; Flekac *et al.*, 2008; Elatter *et al.*, 2012; Abessolo *et al.*, 2012). Here, a report on PON 1 Q192R polymorphism in a Ghanaian diabetic population and its impact on clinical and biochemical variables are presented. The study provides for the first time in Ghana information on the PON 1 Q192R polymorphism in a diabetic population. In addition, the odds of associating PON 1 polymorphs with diabetes have also been examined.

In the present study it was observed that fasting plasma glucose (FPG), total cholesterol (TC), triglycerides (TG) and low density lipoprotein cholesterol (LDL-C) levels were elevated in the type II diabetics ($p < 0.05$) and most of them had a family history of diabetes (Table 4.2). These findings were consistent with similar studies elsewhere (Alberti and Zimmet, 1998; Libby *et al.*, 2002; Gilani *et al.*, 2010; Van't Reit *et al.*, 2010).

Hyperglycaemia (increased FPG levels) (Table 4.2) might lead to oxidative stress (Witztum and Steinberg, 1991; Ceriello and Motz, 2004) in diabetics which could adversely affect the activity of PON 1 enzyme (Mastorikou *et al.*, 2008). Also, the dyslipidaemic state (i.e. high LDL-C) observed in the diabetic population (Tables 4.2; 4.3) could be attributed to poor glycemic control (Gatti *et al.*, 2009). This state could predispose individuals to inflammation (Witztum and Steinberg, 1991; Hanachi *et al.*,

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2009) which could be a significant threat on health (Stratton *et al*., 2000; Barter, 2005; Koro *et al*., 2006).

Compared to other reports, the data presented no influence of gender on the lipid profiles of the participants (Tables 4.3; Gilani *et al*., 2010; Nakhjavani *et al*., 2006). Also, the high density lipoprotein cholesterol (HDL-C) levels in the study populations were similar, unlike other studies reported (Gatti *et al*., 2009; Gilani *et al*., 2010). However, the diabetics had higher cardiovascular risk (TC/HDL-C) values and atherogenic indices [(TC-HDL-C)/HDL-C] than the controls (Table 4.2) as expected (Kannel and McGee, 1979). The physiological roles of HDL may lead to a reversal of the atherogenic effects of oxidized LDL in plasma (Barter, 2005; Koro *et al*., 2006; Pirillo *et al*., 2007), which is also enhanced by the presence of the PON 1 protein (antioxidant enzyme) (Aviram *et al*., 1998, Elkiran *et al*., 2007).

In the present study both female diabetics and controls were found to be obese (BMI $\geq$ 30 kg/m^2; Table 4.3) unlike their male counterparts. Some studies have also reported a higher incidence of obesity in females (Santos and Barros, 2003; Wahab *et al*., 2011). Diabetics aged between 70 and 79 years were also obese (Table 4.4). BMI has been shown to increase with age (Santos and Barros, 2003; Vittal *et al*., 2010). Obesity and sedentary lifestyle are factors which increase the risk of type II diabetes (Liu *et al*., 2012; Resnick *et al*., 2000; Mokdad *et al*., 2003). However, increased physical activity reduces these risks (Hu *et al*., 2001; Laaksonen *et al*., 2002), but physical activity declines with age (Sallis, 2000; Trost *et al*., 2002). The data presented therefore showed that individuals in the higher age categories (Table 4.4) might have experienced reduced physical activity, a strong indication of higher BMI values.

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Underweight diabetics in the present study recorded higher FPG values when compared with the overweight and obese diabetics (Table 4.5) and this conformed to similar findings in India (Dudekula *et al*., 2012). Contrary to the findings by Vittal and others, BMI had an irregular pattern of variation with FPG levels (Table 4.5; Vittal *et al*., 2010). The underweight diabetic patients with such states might experience poorer glycemic control with a comorbid dyslipidaemic state (Stratton *et al*., 2000; Gatti *et al*., 2009).

The ages of the participants did have a significant influence on the systolic blood pressure in both the type II diabetic and control populations (Tables 4.4; 4.4.1) and these were in congruence with another study in which both systolic and diastolic blood pressures were observed to increase with increasing age (Mungreiphy *et al*., 2011). A prospective diabetes research group and findings from Adler and others have indicated that high systolic blood pressure may be associated with the risk of diabetic complications in type II diabetes (UK Prospective Diabetes Study Group, 1998; Adler *et al*., 2000).

Individual genotypic classes of PON 1 Q192R polymorphism identified in the study population (Fig. 4.2; Tables 4.7; 4.9) likewise exerted a positive influence on some of the clinical variables within the study population (Table 4.11). Recent reports have indicated that PON 1 polymorphisms may have a significant influence on glycemic control, BMI, and levels of lipoproteins and lipids (Hegele *et al*., 1995; Mackness *et al*., 2000; Bajnok *et al*., 2007). The results obtained in the current study showed a significant association between high FPG levels and the QQ and RR genotypes in diabetics (Table 4.11). Both genotypes have been associated with poorer glycemic

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control (high glycated hemoglobin levels) in type II diabetics (Flekac *et al*., 2008; Mackness *et al*., 2000). In the current study, glycated hemoglobin levels of the participants were not determined; however hyperglycaemia may lead to glycation of proteins e.g. hemoglobin (Nishikawa *et al*., 2000; Ramakrishna and Jailkhani, 2007). Therefore a strong association between high FPG levels and the QQ and RR genotypes may indicate poorer glycemic control in the participants having these genotypes.

It was observed that under the PON 1 Q192R genotype groups the type II diabetics in the current study recorded significantly higher TG and VLDL values when compared with the controls (Table 4.9). However, within the type II diabetic population of the current study, no association was found between PON 1 Q192R polymorphism and the plasma lipid and lipoprotein levels (Table 4.11). Several studies have also not found any significant or extensive effect of the PON 1 Q192R polymorphism on plasma lipids and lipoprotein levels in type II diabetics (Abbott *et al*., 1995; Antikainen *et al*., 1996; Mackness *et al*., 2000; Lueresen *et al*., 2011). However, in a population of Hutterites the PON 1 Q192R polymorphism did influence the lipoprotein and lipid levels (Hegele *et al*., 1995).

Also, contrary to the report from Wang and others (Wang *et al*., 2012) the diabetics with QQ genotype appeared to have higher waist-hip ratios than their RR and QR counterparts but this difference was not statistically significant (Table 4.10). High waist-hip ratios are indicative of increased risk of hypertension, cardiovascular disease and metabolic complications such as diabetes and mortality (WHO, 2008). The waist-hip ratios of the diabetics in all the genotype groups (Table 4.10) were as

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expected for persons with metabolic complications such as diabetes, and this may put these individuals at risk of developing cardiovascular disease (WHO, 2008).

In both the type II diabetic and control populations, the QQ, RR and QR genotypes of the PON 1 Q192R polymorphism were distributed in a similar pattern (Table 4.7.1; $p > 0.05$). Other studies have reported no differences in the PON 1 Q192R genotype distributions between populations of coronary artery disease, type II diabetic, ischeamic stroke patients and their respective controls (Antikainen *et al.*, 1996; Mackness *et al.*, 2001; Pasdar *et al.*, 2006). In the present study the RR genotype was the most frequent among the diabetics and the controls, followed by QQ genotype. The QR was the least frequent genotype in the two populations. Similar frequencies of the RR genotype have been reported in diabetic and non-diabetic Gabonese and Japanese populations; but contrary to our results the frequency of their QR genotype was higher than QQ in these populations (Kuremoto *et al.*, 2003; Abessolo *et al.*, 2012). However, in Caucasians and Egyptians the QQ genotype is highly presented over the RR genotype (Flekac *et al.*, 2008; Elattar *et al.*, 2012).

With regard to the Q and R allele distributions, the type II diabetics had a higher frequency of the R allele (Table 4.7.1, $p < 0.05$) than the controls and this allele was strongly associated with the disease (OR = 2.21, $p = 0.0141$), (Table 4.7.2). The R allele has been reported to be common in other type II diabetic populations (Odawara *et al.*, 1997, Juretic *et al.*, 2006; Abessolo *et al.*, 2012) but not in all studies (Osei-Hyiaman *et al.*, 2001; Elattar *et al.*, 2012). The frequency of R allele in the control population was supported by a previous study in healthy Ghanaians (Fujihara *et al.*, 2011). In populations of African origin such as the African–Americans, Beninese and

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Gabonese higher frequencies of the R allele have been reported as compared to the Q allele (Table 2.1; Pejin-Grubiša, 2012; Abessolo *et al.*, 2012 (http://alfred.med.yale.edu/alfred/SiteTable1A_working.asp?siteuid=SI001024H). Previous studies in Korean and Japanese populations in Asia also presented similar reports (Pejin-Grubiša, 2012; Kuremoto *et al.*, 2003). The Q allele on the other hand is more frequent in European, Egyptian and some Asian populations (Pejin-Grubiša, 2012). The variations in the Q and R allelic frequencies may be attributed to differences in the geographical locations and ancestral backgrounds of participants in these studies and other evolutionary factors (Pejin-Grubiša, 2012).

The allelic frequencies were used to estimate the expected PON 1 Q192R genotypic frequencies in the next generation based on the Hardy-Weinberg equilibrium principle (http://www.nfstc.org/pdi/Subject07/pdi_s07_m01_02.p.htm; Tamarin, 2001; Nussbaum *et al.*, 2007). However, the estimated (expected) PON 1 Q192R genotypic frequencies in the diabetic and control populations were significantly different from the observed genotypic frequencies ($p < 0.00001$; Table 4.8). The studied populations were therefore not in Hardy-Weinberg equilibrium at the PON 1 Q192R gene locus. Contrary to these findings, most case-control studies reported that the PON 1 Q192R genotypic frequencies in either cases and/or controls were in Hardy-Weinberg equilibrium (Serrato and Marian, 1995; Odawara *et al.*, 1997; Kuremoto *et al.*, 2003; Fujihara *et al.*, 2011; Abessolo *et al.*, 2012). Deviations from Hardy-Weinberg equilibrium may be due to a small population size, non-random mating, migration, mutation and selection for particular alleles in a population (Tamarin, 2001; Nussbaum *et al.*, 2007). When selection for an allele occurs in a diseased population,

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an association between the gene locus (where the allele is located) and the disease may be assumed (Nielsen *et al*., 1998).

One unique finding in this study was the PCR-RFLP product size of 67.01±3.47 bp (CI = 66.29 - 67.73; Session 4.6.2; Table 4.6). The size of the product was comparable with other reports (Mackness *et al*., 1998a; Mackness *et al*., 2000; Elattar *et al*., 2012). However, the 33 bp fragment was not resolved on the 3% agarose gel; this was also experienced in an earlier study (Browne *et al.*, 2007). The running conditions were varied (concentration of agarose gel was increased, polyacrylamide gel was used, and the voltage and duration for the electrophoresis were decreased) to enable the resolution of smaller DNA fragments (Barril and Nates, 2012) yet the fragment was not seen. The explanation for this observation is not immediately known, but may be attributed to the presence of mutant Ghanaian strains that repetitively gave only 67 bp fragments. Notwithstanding the absence of the 33 bp fragment, the Q192R genotype of each individual was assigned based on the presence or absence of a restriction site, determined by the number and positions of the bands on the electrophoretogram (Fig. 4.2; Table 4.6).

This is the first report of PON 1 Q192R polymorphism in the Ghanaian diabetic population, much work is therefore needed in this regard. Also, considering the long term complications associated with type II diabetes, it will be of significant interest to investigate extensively on PON 1 polymorphism and its phenotypic contribution to the diabetes disease phenotype. When this is done, it would aid in the management of type II diabetes mellitus.

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5.1 CONCLUSION

In Ghanaian type II diabetics the RR, QQ and QR genotypes of the PON 1 Q192R polymorphism are present. The RR genotype was the most frequent in the populations studied, whereas the QR genotype was the least frequent. In the Ghanaian diabetics, the R allele was more frequent (78.2%) than the Q allele (21.8%). The R allele was also significantly associated with type II diabetes mellitus, and it may suggest a higher risk of developing type II diabetes.

Type II diabetics in all the PON 1 Q192R genotype groups had higher TG and VLDL levels than the controls; however in the diabetic population the Q192R polymorphism had no significant influence on the lipids and lipoprotein levels. In the diabetic population the QQ and RR genotypes but not the QR were associated with high fasting plasma glucose levels.

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5.2 RECOMMENDATIONS

- Further work is required in a larger population of Ghanaians to confirm the PON 1 Q192R genotypic frequencies in Ghanaian diabetics.
- Family studies: Diabetics with a family history of type II diabetes would have to be examined together with other family members.
- PON 1 activity and concentration measurements should be added to genotype analysis to identify the impact of the genotype on the enzyme's physiological action, activity, plasma lipids and lipoproteins and its ability to protect against diabetic complications in Ghanaians.

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5.3 LIMITATIONS

- Limited logistics e.g. financial constraints
- Some participants withdrew from the study.

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APPENDICES

APPENDIX A

CONSENT FORM

Tel: 0302661311

Department of Medical Biochemistry

P. O. Box 4236

Accra- Ghana

Patient Informed Consent Form

Participant's ID No.: ……………………………..

Participant's Name: ……………………………….

Title of Study: Paraoxonase 1 Gene Polymorphism and Enzyme Activity in Ghanaian Subjects with Type II Diabetes

This research is on a blood substance Paraoxonase 1 (PON 1). Its antioxidant properties help reduce the oxidative stress associated with diabetes and it deleterious effects.

The purpose of this study is to determine PON 1 activity, polymorphism, phenotype distribution and their association with type II diabetes mellitus in Ghanaians.

You were asked to partake in this research because you may or may not have diabetes. Please, taking part in this research is entirely voluntary. You can refuse or withdraw with no penalty or loss of any benefits to which you were otherwise entitled to. Should you stop your participation at any time and for any reason, there will be no objection from anyone and your relationship with the National Diabetes Management and Research Centre will not be affected.

We will take your weight, height, hip, waist circumference, blood pressure, pulse rate and a little blood.

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Should we find early stages of adverse conditions, we will offer you care normally given to patients with such conditions.

Do you have any questions?

CONSENT

I have explained to ………………………………………………………………

the purpose of the above study and the risks involved. I have answered and intended to answer all questions to the best of my knowledge.

---------------------- --- --------------

Signature Name of Staff Date

Dr. Nii Ayite Aryee (Supervisor), Prof. A. G. B. Amoah (Tel: 0202012224), Dr. Grace K. Ababio, (E-mail: graceababio2002@yahoo.com) will be available to answer any questions you may have. You may contact Dr. Aryee at naaryeek16@yahoo.com; and the master student, Dorcas Akuba-Muhyia Annan (Tel: 0242269515).

When this study is reported anywhere, you will not be identified by name or any other identifying information. Your signature or thumbprint below indicates that you fully wish to participate. You do not give up any of your legal rights in signing this consent form.

-- ----------------------

Signature / Thumbprint Date

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APPENDIX B

QUESTIONNAIRE FOR PROJECT PARTICIPANTS

Date …………………… Participant's code……………………..

SECTION A: DEMOGRAPHIC DATA

1. (a) Name ……………………………………………………………...

   (b) Age ………………………..

   (c) Sex: Male/Female (Please underline)

2. Nationality (*please tick*) a) Ghanaian ☐ b) Other (*please specify*) ……………..

3. Educational level (*Please tick and/ or underline specific occupation*)

   (a) Elementary ☐ (Primary school/Middle school)

   (b) Secondary/ Vocational/ Commercial

   (c) Tertiary ☐

   (d) Other (*please specify*) ………………………………………

4. Employment status (*Please tick*)

   (a) Unemployed ☐

   (b) Part-time employment ☐

   (c) Full employment ☐

   (d) Retired and not working ☐

   (e) Retired but working ☐

5. Occupation (*Tick and/ or underline specific occupation*)

   (a) Executive/Professional/Managerial/Administrative

   (b) Paraprofessional ☐ (Teacher, Nurse, Technologist, Technician)

   (c) Business person ☐

   (d) Hawker/Trader

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(e) Clerical staff /Accounts clerk/Messenger

(f) Skilled manual worker/Labourer/ Unskilled labourer

(g) Housewife ☐

(h) Student ☐

(i) Other (*Please specify*) …………………………………..

SECTION B: RISK FACTOR DATA

1. (a) Do you smoke cigarettes currently? Yes/ No

   (b) If yes, how many sticks do you averagely smoke in a day? …………………..

   (c) At what age did you start smoking? ……………………….……………

2. (a) Do you currently take in alcohol? Yes/ No

   (b) If yes, how many drinks on average do you have in a week? ....

   (c) If not that frequent, how many drinks in a month? …………………………

3. (a) Do you have diabetes? Yes/ No

   (b) If yes, what type of diabetes? Type I diabetes mellitus (T1DM)/ Type II diabetes mellitus (T2DM)/undiagnosed by doctors

   (c) How long have you had diabetes………………..years……….months

SECTION C: FAMILY HISTORY

1. (a) Does anyone in your family(not a spouse) have diabetes?

   Yes/ No/ Do not know

   (b) If yes, who? ………………………………………………………………..

2. Does anyone in your family have any of the following conditions?

   (a) Hypertension? Yes/ No/ Do not know

   (b) Stroke? Yes/ No/ Do not know

   (c) High blood lipids (cholesterol, triglycerides, LDL, HDL)?

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Yes/ No/ Do not know

(d) Limb amputation that is not through an accident ? Yes/ No/ Do not know

(e) Coronary artery by-pass surgery? Yes/ No/ Do not know

(f) Heart disease? Yes/ No/ Do not know

(g) Angina? Yes/ No/ Do not know

(h) Heart attack? Yes/ No/ Do not know

SECTION D: HISTORY

1. Has any healthcare personnel told you that you have any of the following conditions?

(a) Hypertension? Yes/ No/ Cannot remember

(b) Heart attack/ heart disease? Yes/ No/ Cannot remember

(c) Stroke? Yes/ No/ Cannot remember

2. Please underline the appropriate answer for the following questions

(a) Do your legs/ feet feel numb? Yes/ No

(b) Have you any burning sensation in your legs and/or feet? Yes/ No

(c) Are your feet sensitive to touch? Yes/ No

(d) Do you experience muscle cramps in your legs and/or feet? Yes/ No

(e) Do you experience prickling feelings (pins in the legs) in your legs or feet?

Yes/ No

(f) Do you have persisting sores on your foot? Yes/ No

(j) Do you feel weak all over most of the time? Yes/ No

(l) Do your legs hurt when you walk? Yes/ No

(m) Are you able to sense your feet when you walk? Yes/ No

(n) Is the skin on your feet so dry that it cracks open? Yes/ No

(i) Have you ever being diagnosed of diabetic neuropathy? Yes/ No

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SECTION E: MEDICATIONS

1. Which of the following medications do you take regularly? (*Tick all that apply*)

(a) Metformin ☐

(b) Glibenclamide ☐

(c) Glimepiricide ☐

(d) Glipizide ☐

(e) Others (*please specify all*) ……………………………………………………

CLINICAL EXAMINATION (Anthropometry/Physical Measurements)

(a) Height (m) ………………..

(b) Weight (kg) …………………

(c) Waist girth (cm) ………………

(d) Hip (cm) ……………………

(e) Blood Pleasure…………………………………………… (After 10 mins rest)

(f) Pulse/Heart rate beats/min (after 10 minutes rest)…………………..

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APPENDIX C

SOLUTION PREPARATION, SOURCE OF REAGENTS AND EQUIPMENT

C 1.0 Solution Preparation

C 1.1 Tris acetate diaminoethanetetra- acetic acid (TAE) Buffer (50X)

Ethylenediaminetetra acetic acid (EDTA) solution of concentration 0.5M was prepared by weighing 29.23 g of the EDTA powder (FW= 292.25) and dissolving in 200 ml distilled water. The pH of this solution was adjusted to 8.03 using concentrated NaOH solution.

TAE buffer (50X) was prepared by dissolving 12.11 g of Trizma base (FW = 121.14) in 37.5 ml distilled water, 5 mL of 0.5 M EDTA (pH 8.03) and 2.9 mL glacial acetic acid (99+%, FW = 60.05) solutions were added and made up to the 50 mL mark with distilled water.

To prepare the working solution of 1X TAE buffer, 20 ml of the 50X TAE buffer was measured and diluted with distilled water to a total volume of 1 L. The pH of this solution was 8.0.

C 1.2 Agarose Gel (3%)

To prepare 3% agarose solution, 6 g of agarose was weighed and dissolved in 200mL 1X TAE buffer.

C 1.3 Ethidium bromide solution (10 mg/mL)

To make 10 mg/mL ethidium bromide solution, 0.1 g ethidium bromide tablet was weighed and dissolved in 10mL 1X TAE buffer.

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C 2.0 Source of Reagents

Sodium hydroxide (Analysis # 297484 890, R00298) was obtained from Fluka Chemie AG CH-9470 Buchs, Switzerland.

EDTA (Cas 24288W 34082, Art # 7538.5) was supplied by Hopkin & Williams LTD. Chadwell Health. Essex., England.

Glacial acetic acid (Lot 36H3713, EEC # 200-580-7, A-6283), Ethidium bromide tablets (Lot 50K0871, 1239-45-8, EC # 214-984-6, E- 2515) and Trizma base (Cas 77-86-1, batch # 103K5423, EC 201-064-4) were from Sigma Chemical and Sigma-Aldrich Companies.

DNA was extracted from buffy coat using Quick- gDNA^{TM} Blood MiniPrep (Catlog No. D3072, Lot ZRC173110) from Zymo Research Corporation, the Epigenetis Company, USA.

Taq PCR core kits (dNTP, Q, $MgCl_2$, 10x Cl) (Lot 139301969, Cas No. 201223, Mat No. 1054462) were from Qiagen, Hilden - Germany

Top vision agarose (Lot 00099683 # R0491), Dream Taq DNA polymerase (Lot 00110141 # EP0701), O'Gene ruler 50bp DNA ladder (Lot 00109725 # SM1133) and BspPI (AlwI) (Lot 00107342 # ER1321) were from Thermo Fisher Scientific Inc.

Paraoxonase 1 (PON 1) 192 forward and reverse primers were obtained from Eurogentec North America Inc., USA.

Polymerase chain reaction (PCR) water (Lot 12K2377, W4502) was obtained from Sigma, USA.

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Accucare Cholesterol reagent set (Lot LCHOL 1206) was from Lab care diagnostics (India) Pvt. Ltd.

Triglyceride (Lot 230AB, REF 11528) and HDL cholesterol (Lot 067AA, REF 11523) reagents were from Biosystems S.A Costa Brava, Barcelona (Spain).

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C 3.0 Source of Equipment

Digital Blood Pressure Monitor (Model HEM-907XL Lot 6Y00280AF/ 4X00098A), Omron Healthcare Inc., Japan

Heavy duty Seca 770 floor scale; Hamburg, Germany

EDTA tubes (6 mL); (Lot 5153089 REF 367863) BD Vacutainer, USA

Gel separation tubes (5 mL); (Lot 8157255 REF 367954) BD Vacutainer, UK

Fluoride tubes (5 mL); (Lot 20111120) Surgifiled Medical, England

Pipette (0.5- 10 μL) (Cat. No. 19110L), Human Diagnostics Worldwide, Germany

Pipette (20- 200 μL) (Cat. No. 19130L), Human Diagnostics Worldwide, Germany

Pipette (100- 1000 μL) (Cat. No. 19140L), Human Diagnostics Worldwide, Germany

Pipette tips (200- 1000μL) (Lot 100715 Code E 1007), EREZ Medical, Johannesburg, RSA

Eppendorf tube (2 mL) (Lot 110901 Code E 1009), EREZ Medical, Johannesburg, RSA

Flat top Microfuge tube (0.6 mL) (No. 120895), Fisher Scientific, USA

Fasting plasma glucose measurement; (FPG) BS-400 Chemistry analyzer (SN WD-93100390) Shenzhen Mindray Bio-Medical Electronics Co., Ltd. China

Absorbance measurement (triglycerides, total cholesterol and HDL cholesterol analysis); Spectrophotometer, (SN 830027567) Axiom, Germany

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pH meter (SN A11003) Thermo Fisher Scientific, USA

Electronic balance (No. 009025) U-Therm International (H.K) Ltd., England

Water bath (Model DK-420 Lot 1007336) U Clear, England

Whirli Mixer (SN 61424) Fisons Scientifice Equipment, England

Eppendorf Centrifuge (No. 5425 26240) Eppendorf AG, Germany

DNA quantification; NanoDrop 2000C Thermo Scientific, USA

PCR; Gene Pro Thermal Cycler Model TC- E*, version -2009-1.3, Hanghou Bioer Technology Co. Ltd., China

Agarose gel electrophoresis; Owl Easycast (Model B1, SN 349501), Owl Separation System Inc., USA

Agarose gel electrophoresis; Owl Easycast (Model B2, SN 346965), Owl Separation System Inc., USA

Ultravoilet transilluminator (SN 0721904), Uvitec, Cambridge, UK

Photoman (SN 0710317) Uvitec, Cambridge, UK

Video copy processor (Model P93E), Mitsubishi, Malaysia

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APPENDIX D

POLYMERASE CHAIN REACTION (PCR) CONDITIONS AND ESTIMATION OF DNA FRAGMENT SIZES

D 1.0 PCR Conditions

Table D 1.1 Preparation of PCR Reaction Mixture

Item	Final concentration, C_2	Initial concentration, C_1	Initial volume, V_1 (µL)
DNA			2.0
Deoxyribonucleotides (dNTP)	0.2 Mm	10 mM	0.4
PON 192 (forward primer	0.5 µM	100 µM	0.1
PON 192 (reverse primer)	0.5 µM	100 µM	0.1
Dream Taq	1 unit	200 units	0.1
$MgCl_2$	1 Mm	25 mM	0.8
PCR coral load (15mM $MgCl_2$)	1 x	10 x	2.0
Q solution	1 x	5 x	4.0
PCR water			10.5
Total volume	-	-	20.0

Table D 1.2 PCR Conditions

ACTIVITY	TEMPERATURE (°C)	TIME
Initial denaturation	95	5 minutes
Denaturation	94	1 minutes*
Amplification	61	30 seconds*
Extension	72	1 minutes*
Final extension	72	10 minutes
Holding	4	∞

*** repeated for 46 cycles**

D 2.0 Estimation of the Sizes of PCR Amplicons and RFLP Products

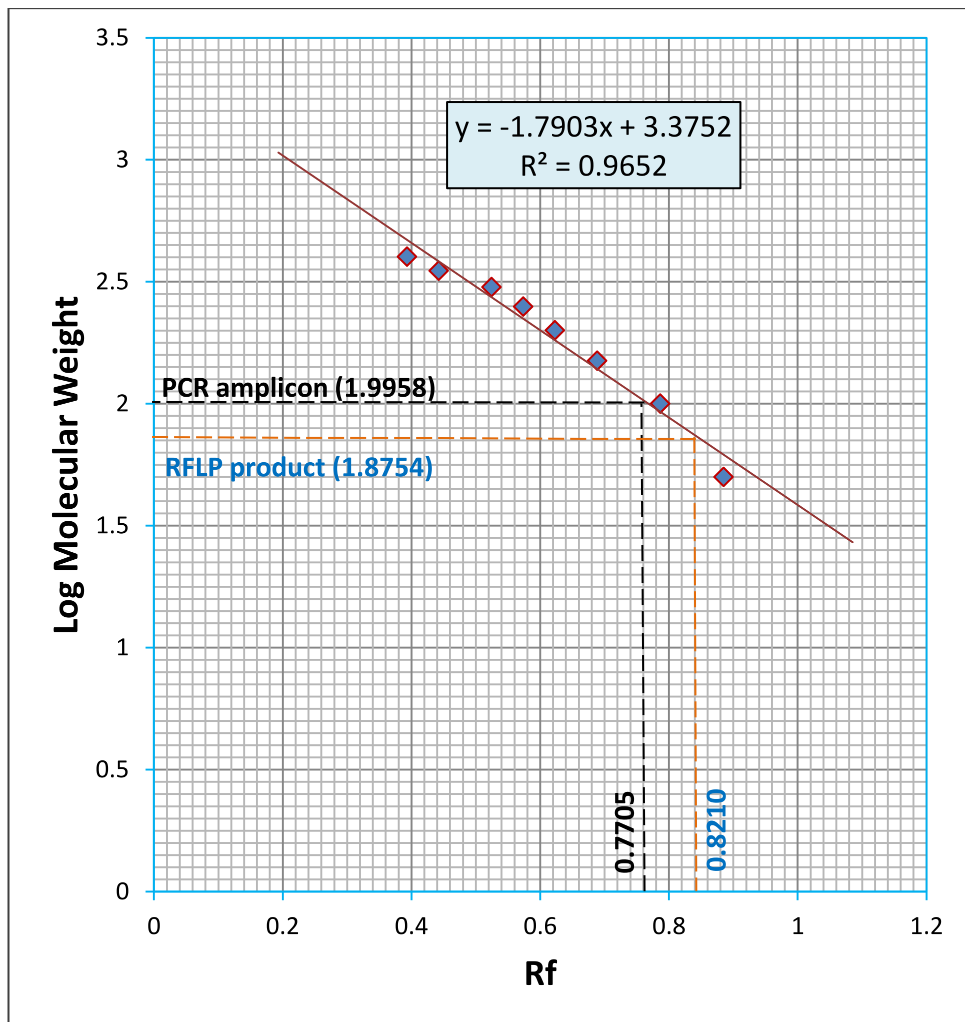

Figure B.2: Standard curve of Log molecular weight verses Rf

$$Rf = \frac{\text{distance travelled by fragment}}{\text{Distance travelled by coral load } (0.35+ x)}$$

(x = distance travelled by 50bp fragment in molecular marker)

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APPENDIX E

E.1 Individual Matching of Diabetic and Controls by Equal Gender

Individual matching of controls and diabetics by equal gender indicated that the FPG, TG and VLDL were significantly ($p < 0.005$) higher in diabetic males and females than in the controls (Table 4.3.1). Age, DBP, TC, LDL, HDL/LDL, atherogenic index and cardiovascular risk were higher in diabetic males only when compared with the control males.

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Table E.1 Individual matching of participants by equal gender

Variables	Males		p-value $_{(t\text{-test})}$	Females		p-value$_{(t\text{-test})}$
	Diabetics$_{(32)}$*	Controls$_{(32)}$*		Diabetics$_{(39)}$*	Controls$_{(39)}$*	
Age (years)	55.03±10.95	49.28±11.18	0.0418	53.73±13.03	51.15±10.06	0.3308
SBP(mmHg)	130.38±21.96	120.91±28.06	0.1374	127.19±28.26	124.38±24.98	0.6431
DBP(mmHg)	76.72±12.04	67.98±16.10	0.0167	74.82±16.10	74.62±15.82	0.9560
Pulse rate(bpm)	74.05±11.78	70.30±11.26	0.1978	72.22±15.63	71.81±9.30	0.8884
BMI(kg/m^2)	23.92±4.63	26.20±8.46	0.1860	31.35±4.66	30.35±5.43	0.3855
Waist-Hip ratio	0.95±0.21	0.91±0.07	0.3107	0.93±0.24	0.91±0.09	0.6275
FPG (mmol/L)	10.94±5.40	5.20±0.66	0.0001	10.78±5.32	5.31±1.72	0.0001
TC(mmol/L)	5.49±2.12	4.10±1.39	0.0002	5.39±2.14	4.77±1.29	0.1254
TG(mmol/L)	1.38±0.54	0.89±0.45	0.0029	1.36±0.54	0.98±0.54	0.0027
HDL-C (mmol/L)	1.23±0.30	1.39±0.38	0.0663	1.20±0.33	1.11±0.38	0.2676
LDL-C (mmol/L)	3.63±2.13	2.42±1.57	0.0120	3.59±2.08	3.32±1.44	0.5071
VLDL(mmol/L)	0.63±0.25	0.40±0.20	0.0001	0.62±0.25	0.45±0.25	0.0036
HDL-C/LDL-C	0.56±0.57	1.75±2.85	0.0061	0.56±0.55	0.42±0.27	0.1577
Atherogenic index	3.92±2.93	2.24±1.61	0.0238	3.89±2.85	3.91±2.49	0.9738
Cardiovascular risk	4.92±2.93	4.24±1.61	0.0238	4.86±2.86	4.91±2.49	0.9346

Table E.1 Values are presented as means ± standard deviation; *population size; $p < 0.05$ was considered statistically significant. BMI = Body mass index;; DBP = Diastolic blood pressure; FPG= Fasting plasma glucose; TC = Total cholesterol; TG = Triglycerides; HDL-C = High density lipoprotein cholesterol; LDL-C = Low density lipoprotein cholesterol; VLDL = Very low density lipoprotein; Cardiovascular risk = [TC/HDL-C]; Atherogenic index = [(TC-HDL-C)/HDL-C].

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E.2 Matching of Diabetics and Controls by Age

After matching the same number of individuals from each study cohort by age (Table 4.4.2), it was observed that FPG levels were higher ($p<0.001$) in all diabetic groups as compared to the controls except in the 70- 80 and 80-90 year group. Higher levels of TC and LDL were seen in diabetics aged between 30-40 and 50-60 years. The diabetics in the following age categories (40-50, 50-60, 60-70, and 80-90 years) also showed higher TG levels than the corresponding controls.

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Table E.2 Individual Matching of Some Clinical and Biochemical Variables in the Studied Populations by Age

Age categories (years)		FPG(mmol/L)	TC(mmol/L)	TG(mmol/L)	LDL-C(mmol/L)	SBP(mmHg)
$30\text{-}40_{(14)*}$	Diabetics	11.67±7.61	5.51±1.35	1.51±0.70	3.53±1.56	112.57±12.56
	Controls	5.06±0.45	3.88±1.61	1.04±0.65	2.14±1.74	107.18±16.44
	p-value (*t*-test)	0.0032	0.0074	0.0771	0.0349	0.3386
$40\text{-}50_{(22)*}$	Diabetics	9.48±3.95	5.40±2.58	1.42±0.45	3.53±2.38	123.52±22.12
	Controls	5.55±2.25	4.65±1.41	1.02±0.41	3.08±1.61	121.76±22.43
	p-value (*t*-test)	0.0002	0.2382	0.0036	0.4667	0.7992
$50\text{-}60_{(45)*}$	Diabetics	10.26±5.00	5.58±1.71	1.38±0.43	3.72±1.70	129.62±19.14
	Controls	5.24±0.57	4.63±1.24	0.98±0.55	3.06±1.38	122.20±26.49
	p-value (*t*-test)	0.0001	0.0033	0.0002	0.0462	0.1450
$60\text{-}70_{(11)*}$	Diabetics	8.16±2.67	4.58±1.37	1.49±0.37	2.70±1.39	127.27±15.06
	Controls	4.92±0.70	4.73±1.40	1.00±0.38	3.27±1.59	135.72±22.51
	p-value (*t*-test)	0.0009	0.8021	0.0061	0.3814	0.3132
$70\text{-}80_{(3)*}$	Diabetics	10.30±4.56	4.94±1.02	1.40±0.45	3.21±0.68	125.57±16.91
	Controls	4.87±0.31	4.32±0.81	0.71±0.24	3.26±0.75	126.50±12.58
	p-value (*t*-test)	0.1087	0.7939	0.0791	0.9359	0.9427
$80\text{-}90_{(2)*}$	Diabetics	8.00±3.11	4.23±1.38	1.46±0.07	2.21±1.37	120.66±13.08
	Controls	4.95±0.35	3.37±0.84	0.76±0.14	2.24±1.01	175.25±29.34
	p-value (*t*-test)	0.3021	0.5301	0.0241	0.9824	0.1381

Table E.2 Values are presented as means ± standard deviation; *population size; $p < 0.05$ was considered statistically significant. FPG= Fasting plasma glucose; TC = Total cholesterol; TG = Triglycerides; LDL-C = Low density lipoprotein cholesterol; SBP = Systolic blood pressure.

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E.3 Matching of Clinical and Biochemical Variables in Diabetic and Control Populations According to BMI

Individual matching of same number of diabetics and controls using BMI (Table 4.5.3) showed that diabetics with normal BMI values had higher levels of FPG, TC, TG and LDL than the corresponding controls. Overweight and obese diabetics recorded significantly higher FPG and TG values.

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Table E. 3 Individual matching of some clinical and biochemical variables in the two populations according to BMI

Variables		FPG (mmol/L)	TC (mmol/L)	TG (mmol/L)	LDL (mmol/L)	Cardiovascular Risk	Atherogenic index
Normal	Diabetics	$11.94 \pm 6.66_{(33)*}$	$5.58 \pm 1.84_{(33)*}$	$1.41 \pm 0.55_{(33)*}$	$3.73 \pm 1.91_{(33)*}$	$4.99 \pm 2.77_{(31)*}$	$3.99 \pm 2.77_{(31)*}$
	Controls	$5.14 \pm 0.40_{(33)*}$	$4.04 \pm 1.07_{(33)*}$	$0.82 \pm 0.42_{(33)*}$	$2.35 \pm 1.19_{(33)*}$	$3.28 \pm 1.58_{(31)*}$	$2.28 \pm 1.58_{(31)*}$
	p- value$_{(t\text{-test})}$	0.0001	0.0001	0.0001	0.0001	0.0041	0.0041
Overweight	Diabetics	$9.08 \pm 3.65_{(27)*}$	$5.36 \pm 2.08_{(27)*}$	$1.43 \pm 0.50_{(27)*}$	$3.49 \pm 1.83_{(27)*}$	$4.09 \pm 1.99_{(47)*}$	$3.09 \pm 1.65_{(47)*}$
	Controls	$5.27 \pm 0.77_{(27)*}$	$4.61 \pm 1.26_{(27)*}$	$0.91 \pm 0.54_{(27)*}$	$2.92 \pm 1.43_{(27)*}$	$3.65 \pm 1.60_{(47)*}$	$2.65 \pm 1.60_{(47)*}$
	p- value $_{(t\text{-test})}$	0.0001	0.1151	0.0006	0.2079	0.2405	0.1926
Obese	Diabetics	$8.99 \pm 2.29_{(25)*}$	$4.98 \pm 1.75_{(25)*}$	$1.45 \pm 0.35_{(25)*}$	$3.03 \pm 1.72_{(25)*}$	$4.66 \pm 1.58_{(31)*}$	$3.66 \pm 2.34_{(31)*}$
	Controls	$5.60 \pm 2.08_{(25)*}$	$4.75 \pm 1.38_{(25)*}$	$1.10 \pm 0.48_{(25)*}$	$3.38 \pm 1.55_{(25)*}$	$5.30 \pm 2.79_{(31)*}$	$4.30 \pm 2.79_{(31)*}$
	p- value$_{(t\text{-test})}$	0.0001	0.6082	0.0050	0.4534	0.2708	0.3317

Table E.3 Values are presented as means ± standard deviation; *population size; $p < 0.05$ was considered statistically significant. FPG= Fasting plasma glucose; TC = Total cholesterol; TG = Triglycerides; LDL-C = Low density lipoprotein cholesterol; SBP = Systolic blood pressure

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APPENDIX F

Table F.1 Classifications of Body Mass Index (BMI)

Classes/Groups	**BMI (kg/m^2)**
Underweight	<18.5
Normal weight	18.5–24.9
Overweight	25–29.9
Obesity (Class 1)*	30–34.9
Obesity (Class 2)*	35–39.9
Extreme obesity (Class 3)*	≥40

*Classified as obese

Source: NHLBI Obesity Education Initiative (2000)