EFFECT OF SMOKING AND WAIST CIRCUMFERENCE ON BIOCHEMICAL MARKERS OF OXIDATIVE STRESS IN SUBJECTS WITH IGT AND NEWLY DIAGNOSED DIABETICS FROM BELLVILLE SOUTH, WESTERN CAPE, SOUTH AFRICA.

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Effect of smoking and waist circumference on biochemical markers of oxidative stress in subjects with IGT and newly diagnosed diabetics from Bellville South, Western Cape, South Africa.

By

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DECLARATION

I, Timothy Ngatangwe Tjaronda, declare that the contents of this thesis represent my own work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

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Signed   **Date**

ABSTRACT

Studies have shown that oxidative stress (OS) is a major pathological risk factor in various diseases, including type 2 diabetes mellitus (T2DM). Hyperglycemia independently is a generator of free radicals, hence increases the level of OS in T2DM subjects. The oxidation of LDL is suggested to play a significant role in the pathogenesis of macrovascular complications observed in diabetic patients. In subjects with hyperglycemia or normoglycemia we investigated the relationship between MDA-protein adducts, HNE-protein adducts and auto-antibodies against oxLDL, and cardiovascular profile as measured by hs-CRP.

From an epidemiological study that screened a high risk urban population for diabetes using oral glucose tolerance test, 98 hyperglycaemic and 79 normoglycaemic individuals were selected for this study. Enzyme linked immuno-sorbent-assay methods were used to determine the levels of serum MDA-protein adducts, HNE-protein adducts or auto-antibodies against oxLDL. High sensitive CRP was measured by nephelometry.

The mean $\pm$ standard deviation age was not significantly different in female and male subjects, respectively, with a range of (48-51) and (49-53) years, $P<0.20$. No significant positive correlations differences were observed between the MDA- and HNE-protein adducts and glycaemic status of the subjects studied. On the other hand, anti-oxLDL antibodies were significantly lower in hyperglycaemic individuals ($p = 0.02$). Significant inverse correlations were observed between anti-oxLDL and hs-CRP ($r = -0.17$; $p=0.03$), HbA1c ($r = -0.21$; $p=0.004$), triglycerides ($r = -0.16$; $p=0.04$), serum cotinine ($r = - 0.15$), and fasting blood glucose
($r = -0.22$; $p=0.004$). The predictors of anti-oxLDL antibodies after multiple stepwise linear regression were post 2 hour blood glucose, serum cotinine, total cholesterol, triglycerides, age and waist circumference, the latter showed a positive association.

There is controversy regarding the role of these circulating antibodies in humans. In the present study we found IgG anti-oxLDL antibodies to be low in individuals with hyperglycaemia, especially in those with undiagnosed diabetes, perhaps due to the unmanaged glycaemic state. Furthermore, the low anti-oxLDL antibodies levels are associated with an increased risk of cardiovascular diseases as shown by the relationship between these antibodies and hs-CRP.

ACKNOWLEDGEMENTS

DEDICATION

To Ester, Mammie, Uetuzuvira, Rusuwo and Metutjindi, with love and in gratitude.

TABLE OF CONTENT

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ADA | American Diabetes Association
AGEs | advanced glycation end-products
ALA | alpha lipoic acid
ALB | albumin
ANOVA | Analysis of Variance
ATP | adenosine triphosphate
BG | blood glucose
BMI | Body Mass Index
CO | carbon monoxide
Co Q10 | Co-enzyme Q10
CRP | C-reactive protein
CVD | cardiovascular disease
DBP | diastolic blood pressure
DM | Diabetes mellitus
DNA | deoxyribonucleic acid
ELISA | enzyme linked immuno- sorbent assay
FBG | full blood glucose
FFA | free fatty acid
GGT | gamma glutamyl transferase
GSH | glutathione (reduced)
HbA1c | glycosylated haemoglobin
HDL | high density lipoprotein
HDL-C | high density lipoprotein cholesterol
HNE | 4-hydroxynonenal
HP | hip circumference
HsCRP | high sensitive C-reactive protein
H_2O_2 | hydrogen peroxide
IDDM | Insulin Dependent Diabetes Mellitus
IFG | Impaired fasting glucose
IGT | Impaired glucose tolerance
IgG | immunoglobulin G
LDL | low density lipoprotein
LDL-C | low density lipoprotein cholesterol
MDA | malondialdehyde
MnSOD | manganese superoxide dismutase

MODY   Maturity Onset Diabetes of the Young
NAD+   nicotinamide adenine dinucleotide
NADH   reduced nicotinamide adenine dinucleotide
NADPH   nicotinamide adenine dinucleotide phosphate
NF-κB   nuclear factor kappa-B
NIDDM   Non- Insulin Dependent Diabetes Mellitus
O_2 molecular oxygen
O^{2-} superoxide radical
OGTT   oral glucose tolerance test
-OH   hydroxyl radical
ONOO-   peroxynitrite
OS   oxidative stress
Ox LDL   oxidised low density lipoprotein
PAH   polycyclic aromatic hydro-compounds
RNS   reactive nitrogen species
ROS   reactive oxygen species
SBP   systolic blood pressure
SOD   superoxide dismutase
TC   total cholesterol
TG   triglycerides
TNF-a   tumor necrosis factor–alpha
UK   United Kingdom
USA   United States of America
VLDL   very low density lipoprotein
WC   waist circumference
WHO   World Health Organisation
XO   xanthine oxidase

CHAPTER ONE

LITERATURE REVIEW

1.1 Introduction

Oxidative stress (OS) is recognized as a condition which plays a prominent role in a number of pathological processes and diseases such as cancers, atherosclerosis, diabetes mellitus, rheumatoid arthritis, normal aging processes and neurodegenerative disorders (Dalle-Donne et al., 2006). The condition of oxidative stress is a result of the inability of the body's antioxidant capability to detoxify the productions of reactive oxygen species. Diabetes mellitus (DM) is consistently associated with increased OS, however there is uncertainty as to whether diabetes is the cause or the result of increased oxidative stress (Stephens et al., 2009). In subjects with impaired glucose tolerance (IGT) or DM, there is a state of persistent hyperglycaemia which is thought to be an enhancing factor for OS. Other factors include obesity and cigarette smoking (Benowitz, 2003; Stephen et al., 2009). Obesity, especially central obesity is responsible for the increase of small low density lipoprotein (LDL) particles, which are oxidized by free radicals and thereby increasing oxidative stress (Grundy, 2004). The gas and tar phases of cigarette smoke contain $>10^{15}$ and $>10^{17}$ free radicals per puff, respectively (Madamanchi et al., 2005).

The effects of smoking, overweight and obesity are understood to add to the toxic accumulation of reactive oxygen species (ROS) in the diabetic subject (Kenney, 2001). Elevated ROS may contribute to the production of free radicals inflicting injury to the components of the cells including proteins, deoxyribonucleic acid (DNA) and lipids (Lipinski, 2001). Lipid peroxidation ensues and produces more complex compounds such as oxidised low density lipoprotein (Ox LDL), malondialdehyde (MDA) and 4-hydroxynonenal (HNE) as by-products of proteins adducts (Baynes and Thorpe, 1999., Madamanchi et al., 2005, Soliman, 2008). MDA and HNE are physiological compounds produced by peroxidative decomposition of unsaturated lipids as by-products of arachidonic metabolism. This develops into MDA/HNE protein adducts accumulating in the arterial walls causing an autoimmune inflammation. Lipid peroxidation contributes to the development of atherosclerosis, consequently increased risk of cardiovascular diseases which can be assessed among other markers by plasma levels of high sensitive C-reactive protein (HsCRP).

1

1.2 Oxidative stress

Oxidative stress (OS) is a condition where antioxidants in the body cannot cope with excess free radicals generated during metabolic processes (Stephens et al, 2009). This state is characterized by an elevated production of free radicals creating an imbalance between the generation of reactive oxygen species / reactive nitrogen species (ROS/RNS), reactive chlorine species, reactive bromine species and the body's inability to produce an equal amount of antioxidants to counter-balance or neutralises the deleterious free radicals (Stephens, 2009). Previous studies confirmed that chronic and acute production of excessive ROS is central in the progression of cardiovascular diseases (Madamanchi et al., 2005). Furthermore, OS has been implicated as one of the major risk factors accelerating pathological processes and diseases such as diabetes mellitus, rheumatic arthritis, ageing, cancers, Alzheimer's disease and atherosclerosis (Goycheva et al., 2006).

Molecular oxygen is converted to superoxide radicals through the enzymatic and non-enzymatic pathways (Johansen et al., 2005). Enzymatic pathways may include xanthine oxidases and NADPH enzymes, and non-enzymatic pathways include mitochondrial respiratory chain and glucose autoxidation (Johansen et al., 2005). The production of ROS can be internal in origin, through metabolic processes and oxidative phosphorylation according to Madamanchi et al., (2005), or it may also originate from external sources like air pollutants, tobacco smoke, radiation and diets excessive of polyunsaturated fatty acids. In addition, internal sources of ROS may also come from drugs detoxification in the liver. Smooth muscle cells, endothelial cells and macrophages are all sites of ROS production (Madamanchi et al., 2005).

Hyperglycemia and free fatty acids (FFA) are stimuli directly linked to the generation of ROS at the mitochondrial level (Maier, 2008). Hyperglycemia and FFA act as catalysts during the Krebs cycle (Figure 1.3). This system incorporates mitochondrial oxidative phosphorylation and NADPH oxidase enzymes. The aerobic respiratory chain in the cell mitochondria is a major site of ROS production during adenosine triphosphate (ATP) synthesis (Stephens, 2009). At the initial stage of the Krebs cycle, glucose in the form of pyruvate, plus free fatty acid reacts with acetyl-CoA citrate and is converted to isocitrate (Stephens, 2009). Subsequently, nicotinamide adenine dinucleotide (NAD+) carries electrons in the process of oxidative phosphorylation to generate NADH. During the pathophysiological state of OS, the mitochondrial proton gradient increases, single electrons are transferred to molecular oxygen and a superoxide is formed (Chen et al, 2003). The mitochondrial respiratory complex I and

complex III are the major sites of ROS production during normal metabolism for the production of ATP (Young et al., 2002). In the study of Chen et al., (2003), it has been shown that complex I release $O_2^{\cdot-}$ into the matrix of the mitochondria when NADH is oxidised by glutamate, pyruvate/malate enzymes. Consequently, $O_2^{\cdot}$ released into the matrix is subjected to dismutation by manganese superoxide dismutase (MnSOD) to form hydrogen peroxide (H_2O_2), which in turn is catalysed by glutathione or catalase to water and molecular oxygen (Chen et al., 2003). However, in a state of antioxidant deficiency and elevated oxidative stress, H_2O_2 can also be converted into highly reactive hydroxyl radicals ($OH^{\cdot}$) by the Fenton reaction (Fig. 1.4), and may cause lipid peroxidation (Kyaw et al., 2004). In the study by Chen et al., (2003), complex III when blocked with antimycin caused the acceleration of ROS production in complex I and II. The Qo center located in complex III is in the vicinity of the intermembrane space, hence, $O_2^{\cdot-}$ generated at the Qo site is released into the outer membrane elevating oxidative stress in the cytosol. Contrary, superoxide released at the Qi center is likely to enter the matrix. The Qi center is a location in the complex III site of the mitochondria, which is responsible for superoxide generation (Chen et al., 2003). Complex III is the crucial site for ROS generation in the mitochondria, since the disposable amount of superoxide produced is released into the intermembrane space, and away from the matrix where the antioxidant enzymes are located (Chen et al., 2003). In addition, complex I superoxide production is mostly discharged in the matrix and neutralized by the defense enzymes MnSOD and catalase. Xanthine oxidases (XO) are enzymes responsible for the elevation of ROS by generating other free radicals through radical chain reactions (Johansen et al., 2005). Furthermore, XO is responsible with other oxidases for the electron reduction of oxygen and hence production of superoxide radicals.

1.2.1 Antioxidants system

It is well documented that hyperglycaemia exacerbates excess generation of reactive free radicals through autoxidation of glucose, increasing oxidative stress in diabetic subjects (Johansen et al., 2005). However, the body has the ability to endogenously produce its own defensive antioxidants to neutralize these free radicals. In addition, non-enzymatic antioxidants can be supplemented exogenously from external dietary sources (Johansen et al., 2005).

1.2.2 Endogenous antioxidants

Endogenous antioxidants are the body's synthesized own line of defences against ROS (Augustin et al., 1997). This antioxidant system includes superoxide dismutase (SOD), glutathione, Co-enzyme Q10 (Co Q10) and alpha lipoic acid (ALA) (Augustin et al., 1997). Additionally, for the mitochondria to maintain a high level of antioxidant capacity, they must always be in a high energized state. Glutathione is an essential water-soluble antioxidant

which is produced from three amino acids (glycine, glutamate and cysteine) in all human cells (Percival, 1998). Glutathione alone can neutralize the following free radicals, hydroxyl radical, superoxide radical, hydrogen peroxide and lipid peroxides (Percival, 1998). However, GSH (reduced), Co Q10, catalase and SOD are the first line of defence to neutralize ROS and for scavenging the free radicals (Sharma et al., 2004). Furthermore, the most important of these frontline antioxidants are SOD, glutathione and Co Q10, acting by absorbing an electron from a free radical. However, these antioxidants require co-factors such as selenium, copper, zinc and manganese to catalyze the free radicals effectively (Percival, 1998). Sharma (2004) also alluded to the fact that, small amounts of free radicals, which may escape the first line of defence, have the ability to initiate the autocatalytic chain of reaction. Consequently, this may produce a chain of lipid-peroxidation products forming the toxic electrophilic species like lipid hydroperoxides. Glutathione reductase is another enzyme that produces glutathione, used as a hydrogen donor through glutathione peroxidase to neutralize hydrogen peroxides (Johansen et al., 2005). In addition, catalase can also neutralize hydrogen peroxide (H_2O_2), converting it to water and oxygen (Chen et al., 2003). SOD is an antioxidant enzyme which depends on the cofactors, manganese, copper and zinc to have optimum catalytic activity (Percival, 1998). Furthermore, SOD mainly catalyses the most potent and very reactive radical, superoxide (O_2^-) to hydrogen peroxide, which is neutralized to water and oxygen by catalase or glutathione. Co Q10 is an endogenous lipid soluble antioxidant, which acts as an electron carrier in the complex II of the mitochondrial respiratory chain (Johansen et al., 2005). According to Madamanchi et al., (2005), complex II is a site of superoxide generation. This superoxide is then scavenged by Co Q10, improving endothelial dysfunction in diabetic subjects (Johansen et al., 2005). Furthermore, CoQ10 as an antioxidant of first line defense, transform $O_2^{\cdot-}$ into H_2O_2 (Johansen et al., 2005). Alpha lipoic acid is an endogenous antioxidant with a co-factor of sulphur (Percival, 1998). Additionally, it is able to absorb free radicals in both lipid and aqueous milieu. Hence, it can enter every part of the cell and exert its antioxidant property throughout the cell.

1.2.3 Exogenous antioxidants

Exogenous antioxidants are mainly non-enzymatic antioxidants which include vitamin C, vitamin E and beta carotene (vitamin A) (Johansen et al., 2005). Hence, these antioxidants are from external sources such as dietary supplements (Percival, 1998). Vitamin C (ascorbic acid), vitamin E (α-tocopherol) and beta-carotene act as effective antioxidants protecting lipoproteins against free radicals (Frei, 2004). Vitamin C protects oxidation of plasma lipids and proteins exposed to smoke. It also protects against certain pathophysiological conditions. Vitamin C is competent in neutralizing reactive oxygen species like hydroxyl radicals, superoxide radicals and hydrogen peroxide in the aqueous phase, before lipid peroxidation commences (Percival, 1998). Therefore, it is considered to be the most

4

essential water soluble exogenous antioxidant in extracellular fluids. Previous studies suggest that glutathione and vitamin C work interactively to neutralize free radicals (Percival, 1998). Furthermore, glutathione is in the first line of defence, whereas vitamin C is in the third line of defence, scavenging those radicals escaping from the second line of defence. Vitamin E is a lipid soluble antioxidant in human lipoproteins and acts as a neutralizing agent against free radicals such as hydrogen peroxide and lipid peroxides, preventing cell membrane lipid peroxidation (Frei, 2004). In addition to that, ß-carotene and other carotenoids are effective in overpowering singlet oxygen preventing OS mostly in lipid rich tissues (Percival, 1998). Vegetables and fruits are major sources of vitamin C and carotenoids. Whole grains with high quality vegetable oils are major sources of vitamin E.

1.3 Diabetes Mellitus

Diabetes mellitus (DM) is a metabolic disorder characterised by hyperglycaemia. The two major types of diabetes are Insulin Dependent Diabetes Mellitus (Type I DM) and Non-Insulin Dependent Diabetes Mellitus (Type 2). These two types account for 5% and 80% of DM, respectively (CDC, 2008). Type I DM occurs due to a complete lack of insulin secretion and it is thought to result from an autoimmune destruction of the pancreatic beta cells (Harrison *et al.,* 2008). On the other hand, Type 2 DM usually occurs in adulthood and is attributed to insulin resistance and is preceded by Impaired Fasting Glucose (IFG) and Impaired Glucose Tolerance (IGT) (Nichols *et al.,* 2007). The other rare types of diabetes include gestational and Maturity Onset Diabetes of the Young (MODY).

1.3.1 Impaired Fasting Glucose and Impaired Glucose Tolerance

Normal circulating levels of glucose are tightly under the control of two major hormones, namely insulin and glucagon. Insulin is the principal hormone that regulates uptake of glucose into most cells from the blood whilst glucagon is responsible for increasing blood glucose to desirable levels during prolonged fasting by facilitating conversion of stored glucose into available glucose (Le Roith *et al.*, 2004, Braunwald *et al.,* 2001). Hence, if insulin is not able to regulate the uptake of glucose after a carbohydrate containing meal, a state of hyperglycaemia will persist which will eventually lead to the development of diabetes mellitus. However, which may first lead to IFG or IGT. The measurement of plasma glucose levels can sufficiently be categorised as IGT after a glucose load, or IFG or diabetes at the elevated state of hyperglycaemia. The diagnostic values have been developed by the American Diabetes Association (ADA) (2004) and the World Health Organization (WHO1999) and are shown in Table 1.1.

Table 1.1 The ADA and WHO diabetes mellitus diagnostic criteria (Source ADA 2004; WHO 1999).

	ADA	WHO
Diabetes	Fasting plasma glucose = 7.0 mmol/L	Fasting plasma glucose = 7.0 mmol/L **and /or** 2 hr post glucose load = 11.1 mmol/L or both
IGT	Random plasma glucose = 11.1 mmol/L	Fasting plasma glucose < 7.0 mmol/L **and** 2 hr post glucose load =7.8 and <11.1 mmol/L
IFG	Fasting plasma glucose = 5.6 mmol/L	Fasting = 6.1 mmol/dl **and/ or** (If measured) 2 hr post glucose load <7.8mmol/L

1.3.2 Aetiology of Diabetes Mellitus

The major environmental factors that are associated with the development of DM, particularly NIDDM include a high fat diet, obesity, physical inactivity and family history of diabetes, which in turn are linked with urbanisation, demographic and epidemiological transitions (Mahler & Adler, 1999). In rare cases, benign and malignant tumors of the endocrine glands such as insulinomas and glucagonomas can also cause increased plasma glucose levels. Figure 1.1 shows the different factors that are associated with diabetes mellitus. Diabetes is a metabolic disorder which has its origin in lifestyle factors and characterised by aging, obesity, lack of physical activity, smoking and genetical factors (Colagiuri *et al.*, 2006).
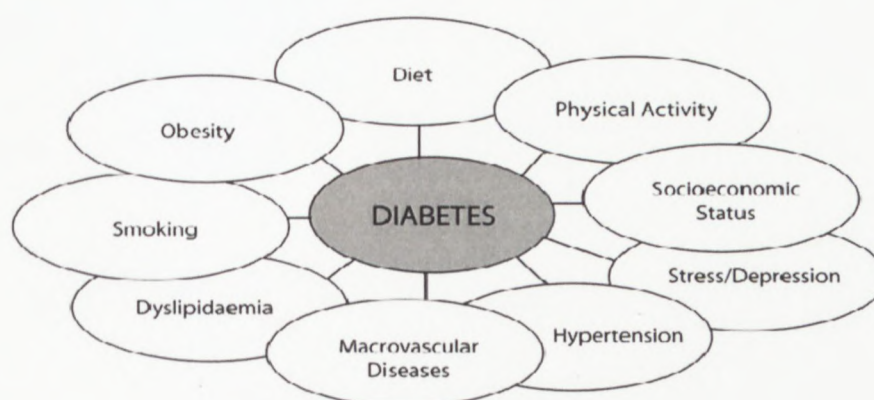

Figure 1.1 The risk factors for the development of type 2 diabetes mellitus. (Adapted from Colagiuri *et al.*, 2006).

1.3.3 Epidemiology of Diabetes Mellitus in South Africa

South Africa is a country that is undergoing major epidemiological transition and this has made DM one of the major non-communicable diseases currently enveloping the country.

6

Studies carried out in South Africa have shown marked geographical and ethnic variations in the prevalence of diabetes (Levitt *et al.*, 1993; Erasmus et al., 2001; Motala et al., 2003). The mixed ancestry population of South Africa has the second highest prevalence of diabetes preceded by that of the Indians, and the incidence is higher amongst those residing in urban areas. These variations are not limited to South Africa as such variations have been described in various population groups of the world (Schulz *et al.*, 2006; Ramachandran *et al.*, 1997). Diabetes mellitus, particularly Type 2 is highly associated with cardiovascular disease (CVD), because it aides in maintaining oxidative stress and progression of atherosclerosis (Ceriello & Motz, 2004). Mortality from CVD is two-fold to four-fold higher in those with the disease (Gu, 1998).

1.3.4 Diabetes Mellitus and Oxidative Stress

DM is consistently associated with increased OS, however there is uncertainty as to whether diabetes is the cause or the result of increased oxidative stress (Stephens *et al.*, 2009). Increased oxidative stress and impaired antioxidant defence have been suggested as contributory factors for initiation and progression of complications in diabetes mellitus (Atli *et al.*, 2004). This imbalance between pro-oxidants and antioxidants are thought to increase with age. On the other hand, the persistent state of hyperglycaemia is suggested to be an enhancing factor for oxidative stress due to the free radicals generated during auto-oxidation of glucose. The mechanisms by which hyperglycaemia can promote reactive oxygen species formation are illustrated in Figure 1.2. Hyperglycaemia may result in the glucose-mediated non-enzymatic glycosylation of proteins (the Maillard reaction) which will result in the formation of a covalent bond between the amine group of a protein and the aldehyde group of glucose. Further rearrangement and oxidation will result in the formation of glycosylation end-products (AGEs) which cause lipid peroxidation, platelet aggregation, O_2^{-} production and modified albumin (Stephens et al., 2009). Mitochondria is a generator of energy in the form of ATP (Madamanchi *et al.*, 2005). ATP is produced during oxidative phosphorylaion through the citric acid cycle (Krebs cycle). A sequence of electron transport carriers are confined to the interior membrane of the mitochondria (Madamanchi *et al.*, 2005).

   i)   Complex I NADH – ubiquinone oxidoreductase
   ii)   Complex II - succinate-ubiquinone oxidoreductase
   iii)   Complex III - ubiquinol-cytochrome c reductase
   iv)   Complex VI - cytochrome c reductase

Electrons leaking out of the Krebs cycle from these electron carriers are normally taken up by MnSOD (Madamanchi *et al.*, 2005). However, under pathophysiological conditions like hyperglycemia, increased triglycerides, elevated LDL and decreased high density lipoprotein (HDL), oxidative phosphorylation leads to increased production of reactive oxygen species, enhancing elevation of oxidative stress (OS) (Madamanchi *et al.*, 2005).

According to Chen *et al*, (2003), complex III is the major site in the mitochondria, where ROS is generated. The production of ROS at complex III is directed away from the antioxidants and leak into the cell. In contrast to complex I where the oxidants are released in the vicinity of the antioxidants enzyme systems (Chen *et al.*, 2003).
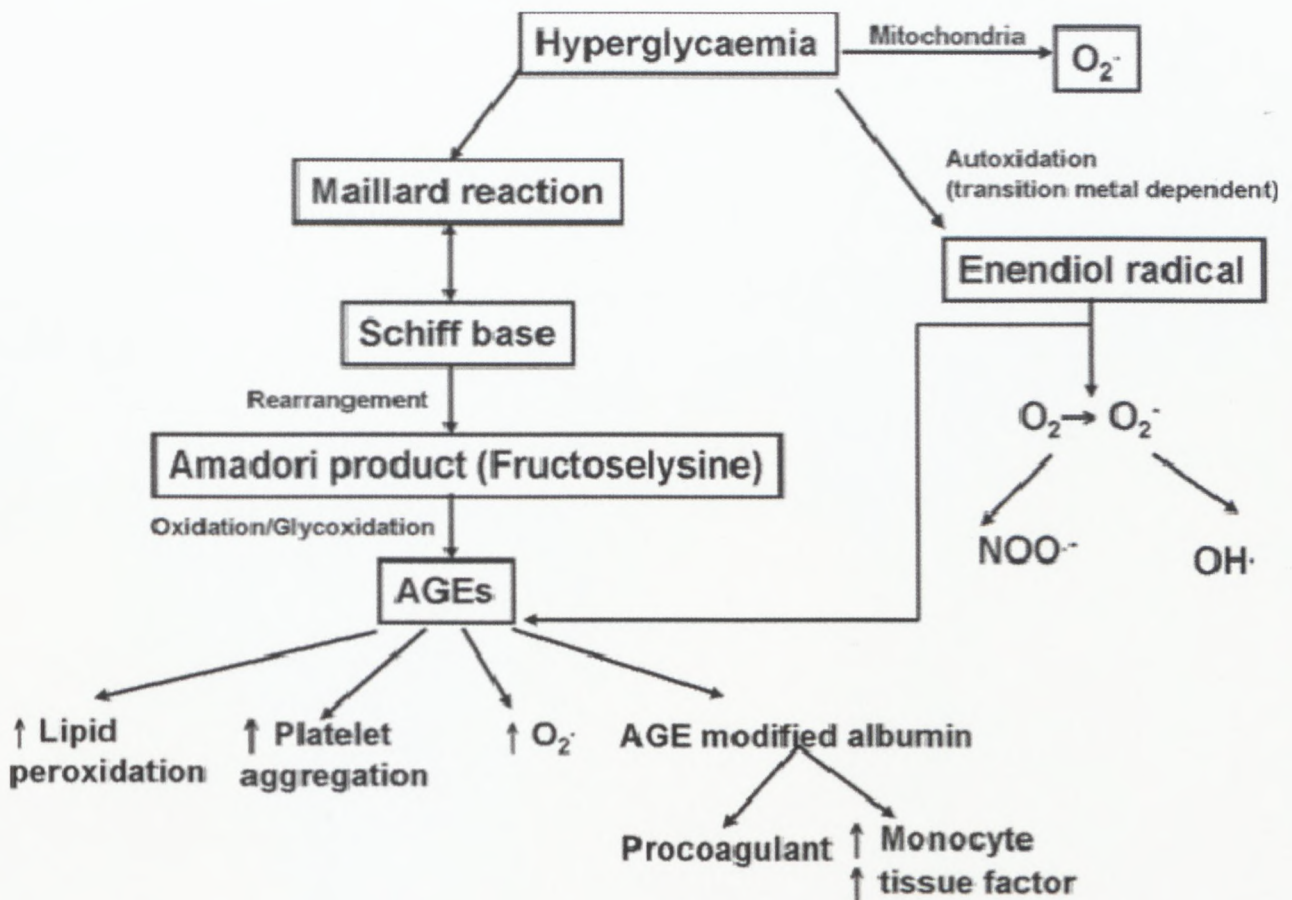
Figure 1.2: Hyperglycaemia induced oxidative stress. (Stephens *et al.*, 2009)

1.4 Obesity

Obesity is an internationally recognised condition that poses a health risk as it is associated with a large number of chronic disorders such as cardiovascular, pulmonary (such as sleep apnoea), metabolic (diabetes and dyslipidaemia), and osteoarticular diseases, common forms of cancer and serious psychological illness. Basically obesity develops when calorie intake exceeds energy expenditure (Krauss *et al.*, 1998). However, the epidemiological evidence for the role of food intake has shown only a modest association between BMI and calories or fat consumed (Kant & Graubard, 2006; Field *et al.*, 2004). Though there has been an overall decrease in the population's fat consumption in the United States of America, obesity rates have continued to rise (Heini and Weinsier, 1997). This could buttress the theory that obesity develops from an intricate interaction between genes and the

environment. The condition arises when an individual's genetic makeup is susceptible to an environment that promotes energy consumption over energy expenditure (Mutch and Clement, 2006). There is strong evidence to suggest that like height, weight is a highly heritable trait (40-70% heritability), (Farooqi and O'Rahilly, 2005; 2006), while cultural and societal factors may explain at least 30% of the variation (Marti *et al.,* 2004). The two major factors believed to contribute to the aetiology of obesity are diet and physical inactivity. In turn, each is influenced by genetic traits (Weinsier *et al.,* 1998).

1.4.1 Epidemiology of Obesity

According to the WHO predictions, 2.3 billion people will be overweight and 700 million will be obese by year 2015 world-wide (WHO, 2007). The prevalence of obesity varies with higher rates being observed in developed countries such as the United States of America (USA) and the United Kingdom (UK), with females having higher rates than males. With the exception of India and China, the prevalences of developing countries are approaching those in developed countries with South African women having similar rates as USA (WHO, 2007). In South Africa more than 29% men and 56% women are classified as being overweight or obese (Puoane *et al.,* 2002; Rocchini, 2002). In a sample of 7726 South African women aged 15-95 years old, black women had the highest prevalence of overweight and obesity (58,5%), followed by women of mixed ancestry (52%), white women (49,2%) and Indian women (48,9%). BMI was found to increase with age, while urban women were found to have a significantly higher BMI than their rural counterparts. In contrast, the prevalence of *overweight and obesity was the lowest in African men (25%) compared to that of white men* (54.5%) (Goedecke *et al.,* 2006). In the Western Cape, the prevalence of overweight and obesity among women and men is respectively 57.1% and 38.4% (Chopra *et al.,* 2007).

1.4.2 Obesity and Oxidative Stress

Though obesity is defined by a BMI of equal to or greater than $30kg/m^2$, it is the central obesity measured by waist circumference that is strongly associated with metabolic abnormalities. Waist circumference (WC), visceral fat or central obesity is the accumulation of fat predominantly in the intra abdominal cavity and is associated with high plasma triglycerides, elevated glucose levels, coronary risks factors, hypertension, glucose intolerance, dyslipoproteinemia and insulin resistance (Funanashi *et al.,* 1999). Visceral fat, through its adipose tissue, has increased lipogenic and lipolytic activity by which it secretes a substantial amount of free fatty acids (FFAS) into the liver. FFAs influence the synthesis of lipoprotein in the liver which translates in the synthesis and secretion of very low density lipoprotein (VLDL), a pathway which accordingly leads to hyperlipidaemia (Funahashi *et al.,* 1999). Furthermore, constant chronic exposure of elevated FFA has deleterious effects on pancreatic beta cells. This toxic effect of FFA on the beta cells induce them to overproduce

NO, interleukin-1B and ceramide, leading to apoptosis of fat-laden beta cells (Tataranni, 2002). Adipose tissue expresses inflammatory cytokines, interleukin-6 (IL-6) and tumor necrosis factor–a (TNF-a) which is a source of oxidative stress (Higdon & Frei, 2003). A relationship between abdominal fat and enhanced lipid peroxidation has been demonstrated by the direct proportional elevation of oxidative stress biomarkers, especially oxidized LDL (OxLDL) and waist circurmference (Weinbrenner et al., 2006).

Central obesity therefore elevates oxidative stress by supplying abundant FFAs which together with excess glucose as observed in DM subjects enter the citric acid cycle, resulting in the generation of excess mitochondrial NADH and reactive oxygen species as illustrated in Figure 1.3. The invasion of the citric acid cycle by a substrate increases the production of acetyl-coA and NADH (Maddux et al., 2001). Acetyl-coA is derived from either glucose via pyruvate or by the B-oxidation of FFA converted to isocitrate, and subsequently NAD+-dependent isocitrate dehydrogenase generates NADH. When excessive NADH cannot be dissipated by oxidative phosphorylation the mitochondrial proton gradient increases and single electrons are transferred to molecular oxygen, resulting in the formation of superoxide (Mahler et al, 1999).
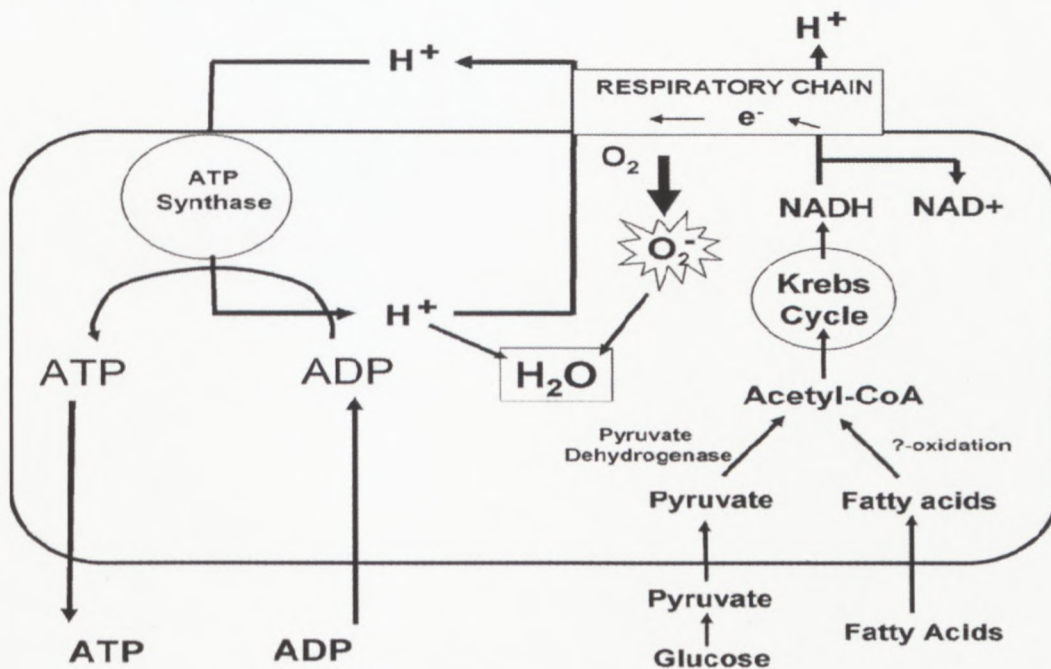
Figure 1.3 Glucose and fatty acid dependent generation of ROS in the mitochondria (Adapted from Kyaw et al., 2004).

Superoxide anion (O_2^-) is generated by key metabolic pathways through the induction of oxidative enzymes like NADH/NADPH oxidizes (Kyaw et al., 2004). Figure 1.3, NADPH oxidases transfer electrons across membranes to oxygen and generate superoxide anions

via the respiratory chain. Reactive oxygen species has been associated with an important role in the pathogenesis of endothelial cell damage, heart failure and eventually atherosclecrosis and other cardiovascular diseases (Cai & Harrison, 2000). The dominant enzymes which are capable of increasing O_2^- are NADH, xanthine oxidases and the enzymes of mitochondrial oxidative phosphorylation (Cai and Harrison, 2000). Nitric oxide reacts rapidly with superoxide anion radicals forming peroxynitrite (ONOO-). Peroxynitrate is implicated in the progress of atherosclerosis in the course of lipid peroxidation and protein nitrosylation (Kyaw *et al.*, 2004). Under normal conditions, O_2^- is converted to hydrogen peroxide by SOD and eventually to water and oxygen by catalase. However, if there is an increased activity of NADH, superoxide anion concentration elevation increases oxidative stress (Kyaw et al., 2004). This leads to the peroxidation of cell phospholipids membranes, oxidation of low density lipoprotein (LDL) and apoptosis of endothelial cells, aggravating the formation of atherosclerotic plaque (Kyaw *et al.*, 2004). ROS is the main cause of LDL oxidation leading to foam cell formation and atherosclerotic plaques (Johansen *et al.*, 2005). Previous research also showed that hyperglycemia is a generator of O_2^- radicals at the mitochondrial level, which trigger the elevation of oxidative stress (Johansen *et al.*, 2005). It has been suggested that superoxide is the instigator of more ROS and RNS production by activating the production of nuclear factor kappa-B (NF-κB) mediated cytokine and NADPH production (Johansen *et al.*, 2005).
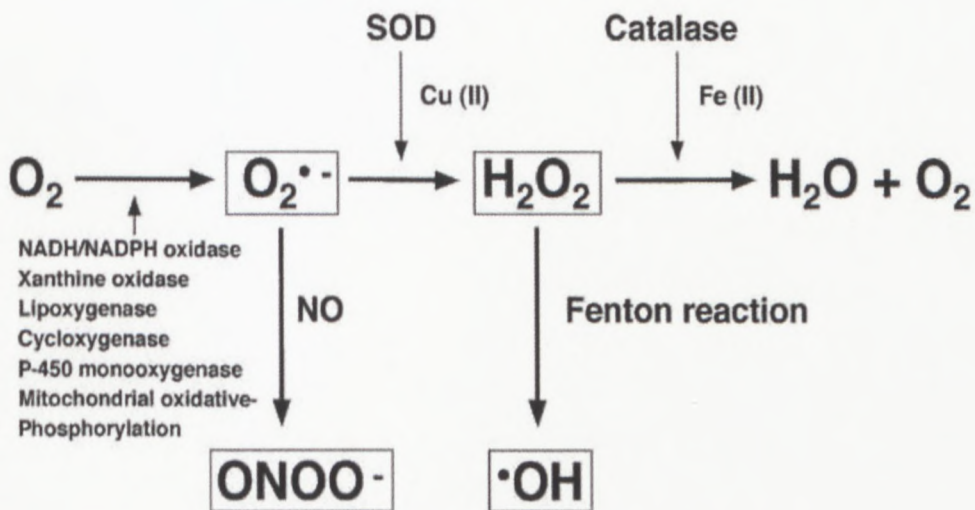
Fig. 1.4 Sources of reactive oxygen species (Kyaw *et al.*, 2004).

1.5 Smoking

The WHO (2007) estimates that tobacco is solely responsible for the death of 5 million people each year globally. Cigarette smoking is classified as the fourth most common risk factor for disease worldwide. Nicotine is a strong addictive drug in cigarette smoke, which

11

addicts adults and juvenile alike. Cigarette smoking exposes an individual to a variety of highly oxidant gases and free radicals, thereby increasing oxidative stress in a diabetic individual (Benowitz, 2003). The effects of smoking are believed to add to the toxic accumulation of reactive oxygen species (ROS) in the diabetic subject (Kenney, 2001).

1.5.1 Epidemiology of smoking

The Western Cape Province, has the highest prevalence of smoking of all the provinces in South Africa at: 44.7% of men and 27% of women (Chopra *et al.*, 2007). Among the different population groups, the coloured population of the Western Cape has the highest smoking prevalence at 48% (Walbeek, 2001). In addition, smoking is a major factor which contribute to high mortality rate due to lung cancer in South Africa (Bello *et al.*, 2011) In the same province, the prevalence of overweight and obesity is also high among women with 57.1%, and highest of all provinces among men at 38.4% (Chopra *et al.*, 2007). According to Benowitz (2003), smoking causes 140,000 premature deaths due to CVD in the United States. Cigarette smoking is among other cardiovascular risk factors, which increases OS and enhances cardiovascular mortality. Cigarette smoking is implicated in ischaemic heart disease and stroke, characterized by diminished blood flow to the heart and brain (Benowitz, 2003). Cigarette smoke may accelerate atherogenesis which precipitates acute vascular events (Burns, 2003). However, oxidative stress biomarkers have not been researched and validated in diabetic subjects using cigarette smoking as a variable risk factor leading to CVD.

1.5.2 Cigarette smoking and Oxidative stress

A single cigarette smoke puff contains more than 10 billion free radicals (Ambrose and Barua, 2004). These toxic radicals are responsible for oxidation of structural and functional molecules of the cells (Carnavelli *et al.,* 2003). Among toxic oxidants and radicals of importance found in tobacco smoke are peroxynitrite, carbon monoxide, nicotine, polycyclic aromatic compounds (PAH), nitric oxide, nitrogen dioxide and perinitrate (Benowitz, 2003). Cigarette smoking is directly responsible for causing pulmonary emphysema, and may cause atherosclerosis indirectly in combination with risks factors such as obesity and diabetes (Carnavelli *et al.*, 2003; Steyn & Fourie, 2007). Carbon monoxide is rapidly absorbed in the bloodstream, displacing oxygen in a ratio of 2:1 (Benowitz, 2003). Nicotine is responsible for rapid heartbeats, during elevated levels of nicotine in the blood throughout the day (Benowitz, 2003). The reactive oxygen species in cigarette smoke include hydroxyl radicals, hydrogen peroxide and semiquinine (Singh *et al.*, 2004). These radicals react with molecular oxygen and produce superoxide radicals, and which in turn leads to a decreased capacity which is detected in smokers (Singh *et al.,* 2004).

More than 5000 compounds found in cigarette smoke and free radicals are associated or responsible for the following oxidative stress linked diseases:

o   Polycyclic aromatic hydrocarbons accelerate atherosclerosis

o   Carbon monoxide binds rapidly to haemoglobin displacing oxygen

o   Superoxide radicals and nicotine cause endothelial dysfunction initiating atherogenesis

o   Oxidant gases in the smoke elevate oxidative stress

o   Oxidant chemicals in cigarette smoke induce inflammatory reactions increasing fibrin levels

o   Nicotine acts as a chemotactic factor and increases leukocytes and their migration, elevating localized oxidative stress (Benowitz, 2003).

Cigarette smoking accelerates atherogenesis by adding more toxic reactive oxygen/nitrogen species to atherosclerotic plagues, thereby increasing oxidative stress. Continuous smoking is reported to lower the concentration of HDL-C, increases LDL-C and triglycerides levels (Lee *et al.*, 2004). The binding of carbon monoxide (CO) to haemoglobin has significant effects on the tissues:

o   Reduces the blood oxygen (O_2) carrying capacity

o   Increases serum carboxyhaemoglobin levels (Hassan *et al.*, 2011; Tesler, 2000).

The reduction of blood oxygen causes oxidative phosphorylative dysfunction in the cardiac cells (Hassan *et al.*, 2011). Diminished oxyhaemoglobin is also associated with dysfunction of oxidative phosphorylation in platelets, this leads to an increased production of mitochondrial reactive oxygen species.

When smoking is combined with other cardiovascular risk factors e.g. dyslipidaemia or hyperglycemia accelerate damage to the mitochondria and promote atherogenesis (Tesler, 2000).

Oxygen free radicals in cigarette smoke are associated with the breaking of DNA strands, hydroxyl radicals and peroxynitrite react with DNA and amino acids. This verifies a major reaction suggesting the development of carcinogenesis (Benowitz, 2003).

1.6 Aims and objectives of the present study

The state of hyperglycaemia is a risk factor for cardiovascular disease because it helps in maintaining oxidative stress and progression of atherosclerosis. Obesity and smoking are also associated with the accumulation of reactive oxygen species. Therefore, **the specific hypothesis of the present study was that the biochemical markers of oxidative stress**

will be significantly increased in hyperglycaemic subjects, and they would be exacerbated in smoking and/or obese subjects leading to an increased CVD risk as measured by hs-CRP. Thus the specific aims of this project were as follows:

1. Determine the differences in biochemical markers of oxidative stress between hyperglycaemia and control subjects.

2. Investigate the relationship between smoking, obesity and oxidative stress

3. Correlate oxidative stress with cardiovascular risk markers.

CHAPTER TWO

RESEARCH DESIGN AND METHODOLOGY

2.1 Ethical Considerations

The study was approved by the Cape Peninsula University of Technology, Faculty of Health and Wellness Sciences Ethics committee, and the study was conducted according to the Code of Ethics of the World Medical Association (Declaration of Helsinki). All participants signed written informed consent after all the procedures had been fully explained in the language of their choice.

2.2 Research Setting

Bellville-South is located within the northern suburbs of Cape Town, South Africa. It is traditionally a mixed ancestry township formed in the late 1950`s. In the South African context, 'township' usually refers to the often underdeveloped urban living areas that, under the Apartheid regime, were reserved for non-whites. According to the 2001 population census, its population stands at approximately 26 758 with those of mixed ancestry, making up 80.48% (21 536). About 46% are males while females represent 54% of which the target population, (coloureds aged 35 – 65 years) was about 6 500 (City of Cape Town Census 2001, accessed 2009. http://www.capetown.gov.za). Based on these statistics the sample size required for the pilot study was 650 (10%).

2.3 Recruitment Strategy

Information regarding the project was disseminated to the local residents through the local radio station (Radio Tygerberg), community newspaper, (The Tygerberger), brochures and fliers, the latter bearing information about the project and distributed through school children and taxis to the local residents by the recruitment team. This team consisted of unemployed matriculants and managed by a qualified retired nurse from the community. Three months into the study the response rate was very low with an average of 2.5 participants per day with Fridays having the least number of participant, hence data collections on this day were ceased and the day utilized for the weekly meetings. Subsequently, prayer and social groups were made use of. A research team member attended these prayer groups and handed out fliers that highlighted key aspects of the project. Additionally, the objectives and benefits of the study were comprehensively explained to the religious leaders for purposes of clarity in case the local community needed to know more during other prayer sessions. A "road show" strategy that involved a celebrity suffering from diabetes from the same community was also used, especially in the targeted streets. By use of a public address system (megaphone) on board a moving truck, the speaker used brief, but catching phrases "You could be diabetic

15

without knowing" and "Diabetes may lead to heart attack" to relay the message to the community members about diabetes. The celebrity was accompanied by the rest of the field team who alongside him issued fliers to the enthusiastic crowds that rushed to take a glimpse of the famous media actor. In this way, a big audience was reached.

2.4 Pre-participation counselling

Recruited subjects were visited by the recruitment team the evening before participation and reminded of all the survey instructions. The instructions included overnight fasting, abstinence from drinking alcohol or consumption of any fluids in the morning of participation. Since the participants were required to bring in early morning mid-stream urine samples, they were provided with a sterile container as well as instructed on how to collect the sample. Furthermore, participants were encouraged to bring along their medical/clinic cards and/or drugs they were currently using

2.5 Research Design and Study Population

This was a cross-sectional study aimed at establishing a cohort that can be followed up for insulin resistance and its sequel in randomly selected coloured subjects aged 35 – 65 years. The data presented here was collected mid January 2008 to March 2009. Using a map of Bellville South, random sampling was approached as follows: From a list of streets from each stratum, the streets were then classified as short, medium and long streets based on the number of houses. Streets with houses = 22 were classified as short, medium ; houses 23 – 40 and long streets were > 40 houses. A total of 16 short streets representing approximately 190 houses, 15 medium streets representing approximately 410 houses and 12 long streets representing approximately 400 houses were randomly selected across the different strata. From the selected streets, all household members meeting the selection criteria were eligible to participate in the study. Community authorities requested that participants outside the random selection area should benefit from the study; these were also included, but given a different code. A total of 956 subjects participated comprising random subjects between the ages 35 -65 years and 304 voluntary subjects, age range 16 – 95. The other 10 subjects were from other race groups and were excluded. From the database containing the 642 randomly selected subjects, a total of 177 (hyperglycaemia and normoglycaemia) individuals between 31 -65 years of age were randomly selected for this study.

2.6 Data Collection

2.6.1 Questionnaire

A detailed protocol describing data collection procedures (questionnaires and physical examination) was developed. The team members consisting of professional nurses and the recruitment team were trained. A pilot study in a neighboring community with similar

demographics was performed to validate the questionnaire and to synergise the workflow. A supervisor was allocated for each team who monitored the performance of the personnel and who was responsible for calibrating equipment according to a standard protocol. In addition, a weekly meeting was held to assess progress, solve problems and re-training of the research team. A questionnaire designed to retrospectively obtain information on lifestyle factors such as smoking and alcohol consumption, physical activity, diet, family history of CVD and DM, demographics etc. was administered by trained personnel. A detailed drug history was also obtained by interviews and by examining the clinic cards as well as the record of drugs that participants brought to the study site.

2.6.2 Anthropometric measurements and blood pressure

waist-hip ratio and skinfold thickness measurements), were performed on all subjects. BMI Anthropometric measurements (body weight and height; waist and hip circumference (HR); was calculated as mass per square meter of body height (kg/m^2). Blood pressure, waist and hip circumferences were measured. Blood pressure measurements were performed according to WHO guidelines (WHO, 1999b).

2.6.3 Biochemical analyses

All participants except the self reported diabetic subjects, confirmed by either medical card record or drugs in use, underwent a 75g oral glucose tolerance test (OGTT) as prescribed by the WHO, after fasting blood glucose was determined. Categories of glucose tolerance were defined applying the 1998 WHO criteria (Alberti, 2003). Blood samples were transported daily in an ice-pack box for processing at the Metropolis Private Pathology Laboratory (Century City, Cape Town). The blood was centrifuged for 10 minutes and the serum stored at below -80°C. Plasma glucose was measured by the enzymatic hexokinase method according to Cobas 6000, Roche Diagnostics kit. Glycosylated haemoglobin (HbA1c) was assessed by turbidimetric inhibition immunoassay (Cobas 6000, Roche Diagnostics). Total cholesterol (TC), high density lipoprotein cholesterol (HDL-C) and triglyceride (TG) concentration were determined by enzymatic colorimetric methods (Cobas 6000, Roche Diagnostics). Low density lipoprotein cholesterol (LDL-C) was calculated using Friedwald's formula. C-reactive protein (CRP) was measured by a high-sensitivity CRP assay, based on the highly sensitive Near Infrared Particle Immunoassay rate methodology (Immage® Immunochemistry System; Beckman Coulter), with a lower limit of detection of 0.2 mg/L. Participants with CRP concentrations <0.2 mg/L had a value assigned to them of 0.2 mg/L. To determine the serum antibodies against oxLDL we used the OLAB IgG anti oxidized low density lipoprotein ELISA kit (Biomedica Medizinprodukte GmbH and Co KG), as per manufacturer's instructions on serum that had be stored at − 80 degrees Celsius. MDA-protein adducts and HNE-protien adducts present in serum were probed by anti-MDA

antibody and anti-HNE antibody, respectively using Cell Biolabs Inc. Kits. Serum cotinine was measured using a chemiluminescent assay (Immulite 1000, Siemens). Urine microalbumin was measured by an immunoturbidimetric assay (Cobas 6000, Roche Diagnostics).

2.7 Statistical Analyses

Statistical analyses of the data were performed using STATISTICA (STATISTICA 9, StatSoft Inc 1984–2009). The continuous variables are presented as median, and (95% confidence interval). The Mann Whitney U test was used for independent data whilst Kruskal-Wallis Analysis of Variance (ANOVA) was used for multiple testing of independent variables. The relationship between anti-OxLDL antibodies or MDA or HNE or, hs-CRP and CVD risk factors was studied by means of Spearman's rank correlation coefficient, and multiple stepwise linear regression analysis. The dependent variables were log transformed prior to the regression analysis. Independent variables contained in the model were: Age, BMI, waist hip ratio or waist and hip circumferences, systolic and diastolic blood pressures, hs-CRP, HbA1c, total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, urine microalbumin, serum cotinine, fasting blood glucose and 2-hour post blood glucose.

CHAPTER THREE

RESULTS

3.1 Characteristics of study population according to gender

The study group consisted of 177 subjects of whom 114 were females and 63 were males. The subjects were categorized, into those with hyperglycaemia (29 undiagnosed DM, 25 known DM, 15 IFG and 29 IGT) and 79 normoglycaemia (normal) and their characteristics are presented as median (95% confidence interval) in Table 3.1. In females, BMI, hip circumference, and hs-CRP levels were significantly higher ($p < 0.05$), whilst in males GGT, systolic and diastolic blood pressures were significantly higher ($p < 0.05$).

Table 3.1 Characteristics of the 177 participants according to gender. (Median (95%CI)

	Females: N = 114	Males: N = 63	P-value
Age (years)	48 (48, 51)	50 (49, 53)	0.20
BMI (kg/m^2)	31.3 (29.8, 32.7)	26.6 (25.8, 28.4)	**<0.001**
WC (cm)	100 (95, 101)	95 (92, 99)	0.29
HP (cm)	112 (111, 117)	101 (99, 103)	**<0.0001**
SBP	117 (115, 121)	125 (122, 131)	**0.02**
DBP	74 (72, 76)	77 (76, 82)	**0.007**
FBG (mmol/L)	6.0 (6.3, 7.3)	5.5 (6.0, 7.9)	0.15
Post BG	7.5 (13.0, 23.7)	6.8 (17.9, 37.8)	0.26
HbA1c (%)	6.0 (6.2, 6.7)	5.7 (6.0, 6.7)	0.19
TC (mmol/L)	5.6 (5.3, 5.7)	5.6 (5.2, 5.8)	0.89
TG (mmol/L)	1.3 (1.3, 1.6)	1.4 (1.4, 1.8)	0.42
HDL-C (mmol/L)	1.1 (1.2, 1.3)	1.2 (1.1, 1.3)	0.71
LDL-C (mmol/L)	3.5 (3.5, 3.8)	3.4 (3.2, 3.8)	0.29
Urine ALB (mg/L)	3.1 (6.0, 10.2)	5.7 (7.0, 15.1)	0.17
GGT (IU/L)	28 (26, 36)	32 (34, 48)	**0.001**
Serum Cotinine (ng/mL)	9 (98, 166)	9 (90, 178)	0.81
Hs-CRP (mg/L)	6.2 (6.4, 9.0)	3.5 (3.7, 6.3)	**0.005**
OxLDL (Mu/mL)	1246 (1738, 2514)	1585 (1616, 2372)	0.43
HNE (µg/mL)	6.0 (5.8, 6.6)	6.4 (5.7, 6.9)	0.78
MDA (pmol/mg)	593 (582, 680)	559 (540, 683)	0.42

Age (years);BMI, body mass index; WC, waist circumference, HP, hip circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; FBG, fasting blood glucose; Post BG; HbA1c, Glycated haemoglobin;TC, total cholesterol; TG, triglycerides; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; Urine ALB, urine microalbumin,GGT, gammaglutamyl transferase; Serum cotinine; Hs-CRP, high sensitivity C-reactive protein; OxLDL, anti oxidised low density lipoprotein; HNE, 4-Hydroxynonenal; MDA, malondialdehyde.

3.2 Characteristics of study population according to glycaemic state

Glycaemic status was assessed according to the WHO criteria and the categories are presented in Figure 3.1. The subjects were first grouped into two categories, hyperglycaemia (DM, IFG and IGT) and normoglycaemia (normal). The Mann-Whitney U test was used to compare the groups and the results are summarized in Table 3.2. By selection, glycated heamoglobin (HbA1c), fasting plasma glucose and 2-hour post 75g glucose load blood glucose were significantly higher in the hyperglycaemic group. Obesity as measured by BMI, WC and HP were significantly more prevalent in hyperglycaemic individuals. However, smoking as assessed by serum cotinine was more common in the normoglycaemic subjects. Anti-OxLDL antibody concentrations and HDL cholesterol were significantly lower in hyperglycaemic individuals. Anti-OxLDL and HDL were further investigated according to the individual glycaemic states (normal, IGT, IFG, undiagnosed diabetics and self-reported diabetics using ANOVA (Figures 3.2 and 3.3).
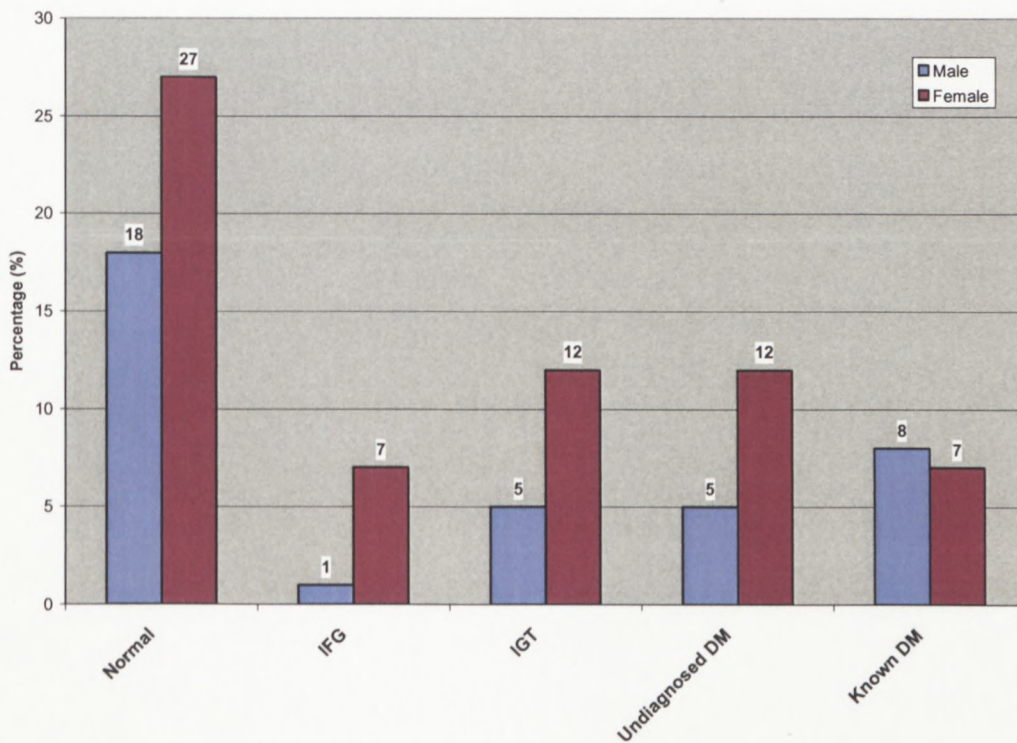
Figure 3.1 WHO categories of glycaemic state of participants.

Table 3.2: Characteristics of the 177 participants according to glycaemic state.

	Normal N = 79	Hyperglycaemia N = 98	P-value
Age (years)	47 (46, 50)	53 (50, 53)	0.006
BMI (kg/m^2)	24.3 (24.7, 27.5)	32.2 (31.4, 34.1)	<0.0001
WC (cm)	87 (86, 93)	103 (101, 106)	<0.0001
HP (cm)	101 (101, 106)	111 (111, 117)	<0.0001
SBP	105 (114, 122)	112 (121, 127)	0.02
DBP	75 (72, 78)	75 (74, 78)	0.92
FBG (mmol/L)	5.0 (4.9, 5.2)	6.6 (7.6, 9.0)	<0.0001
Post BG	5.7 (5.6, 6.0)	8.8 (9.4, 11.7)	<0.0001
HbA1c (%)	5.6 (5.5, 5.7)	6.3 (6.7, 7.4)	<0.0001
TC (mmol/L)	5.5 (5.2, 5.7)	5.6 (5.3, 5.8)	0.45
TG (mmol/L)	1.1 (1.2, 1.5)	1.5 (1.5, 1.8)	<0.001
HDL-C (mmol/L)	1.2 (1.2, 1.4)	1.1 (1.1, 1.2)	0.03
LDL-C (mmol/L)	3.3 (3.3, 3.7)	3.6 (3.5, 3.9)	0.28
Urine ALB (mg/L)	2.9 (5.2, 8.2)	4.6 (7.9, 14.6)	0.09
GGT (IU/L)	23 (27, 36)	31 (34, 43)	0.005
Serum Cotinine (ng/mL)	346 (120, 206)	174 (75, 142)	0.06
Hs-CRP (mg/L)	3.7 (3.9, 6.3)	6.6 (6.7, 9.5)	0.001
OxLDL (Mu/mL)	1699 (1842, 2670)	1119 (1551, 2328)	0.02
HNE (µg/mL)	6.4 (5.8, 6.9)	5.9 (5.7, 6.6)	0.69
MDA (pmol/mg)	612 (586, 712)	426 (552, 656)	0.22
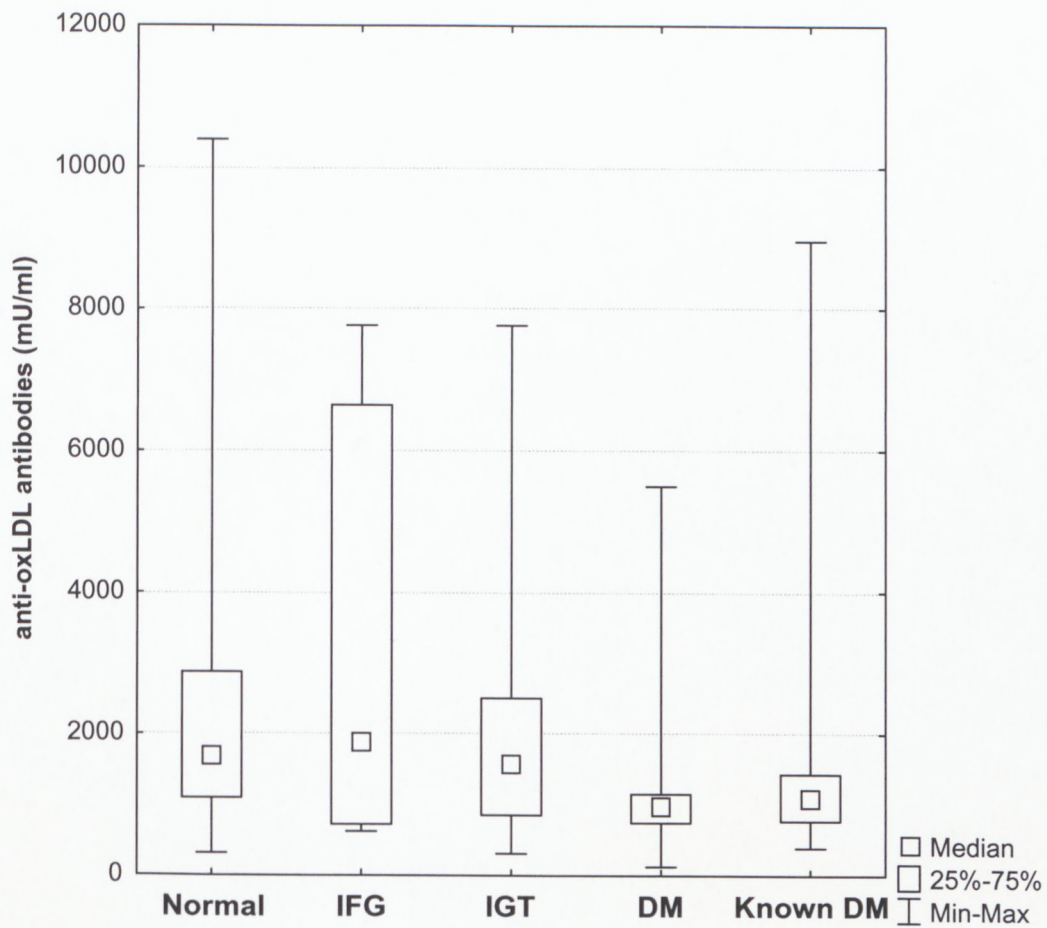
Figure 3.2 Box plot representing Anti-OxLDL antibodies in Normal, normoglycaemia; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; DM, undiagnosed diabetes and Known DM, self-reported diabetes. Undiagnosed diabetes significantly lower, $p = 0.01$, Kruskal-Wallis ANOVA.
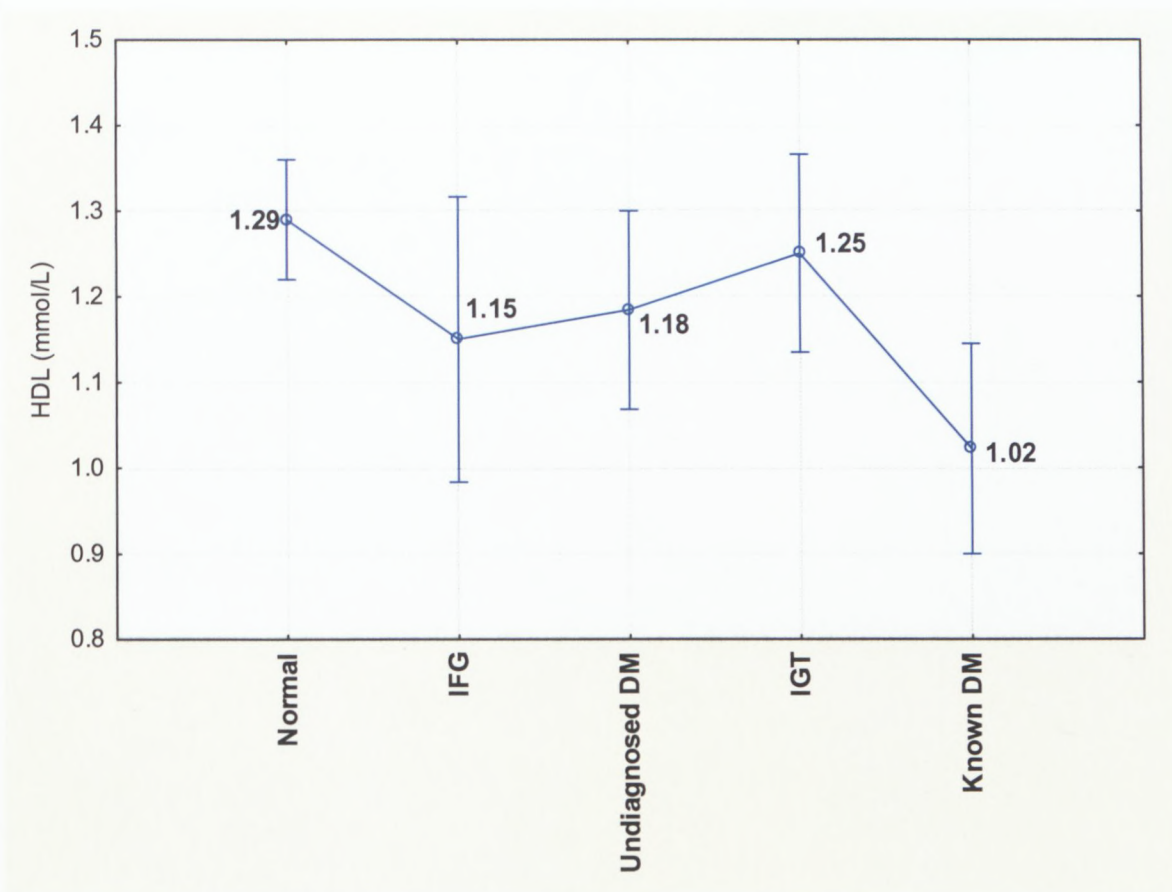
Figure 3.3 HDL cholesterol according to glycemic state.

3.3 Characteristics of study population according to obesity

Obesity/overweight status was determined according to the WHO criteria, and central obesity was assessed using the waist circumference cut-offs recommended by the International Diabetes Federation (IDF), = 94cm in males and =80cm in females. Obesity was more common in females using either the WHO or IDF criteria, respectively Figures 3.4 and 3.5. The characteristics of normal weight and obese subjects are summarized in Table 3.3. All subjects with BMI > 25 kg/m^2 were classified as obese/overweight. Age, total cholesterol, anti-OxLDL, Urine Albumin and HNE were not significantly different between normal weight and obese subjects, $p > 0.05$. MDA and serum cotinine were significantly lower in normal weight subjects whilst serum cotinine was significantly higher when using BMI as a measure of obesity. On the other hand, LDL and GGT were significantly lower in normal weight subject when using the waist circumference for obesity. The characteristics of BMI, WC, HP, SBP, DBP, FBG, Post BG, and HbA1c showed all a significant difference between the normal and obese subjects. These values are both valid for the WHO criteria and the IDF criteria.
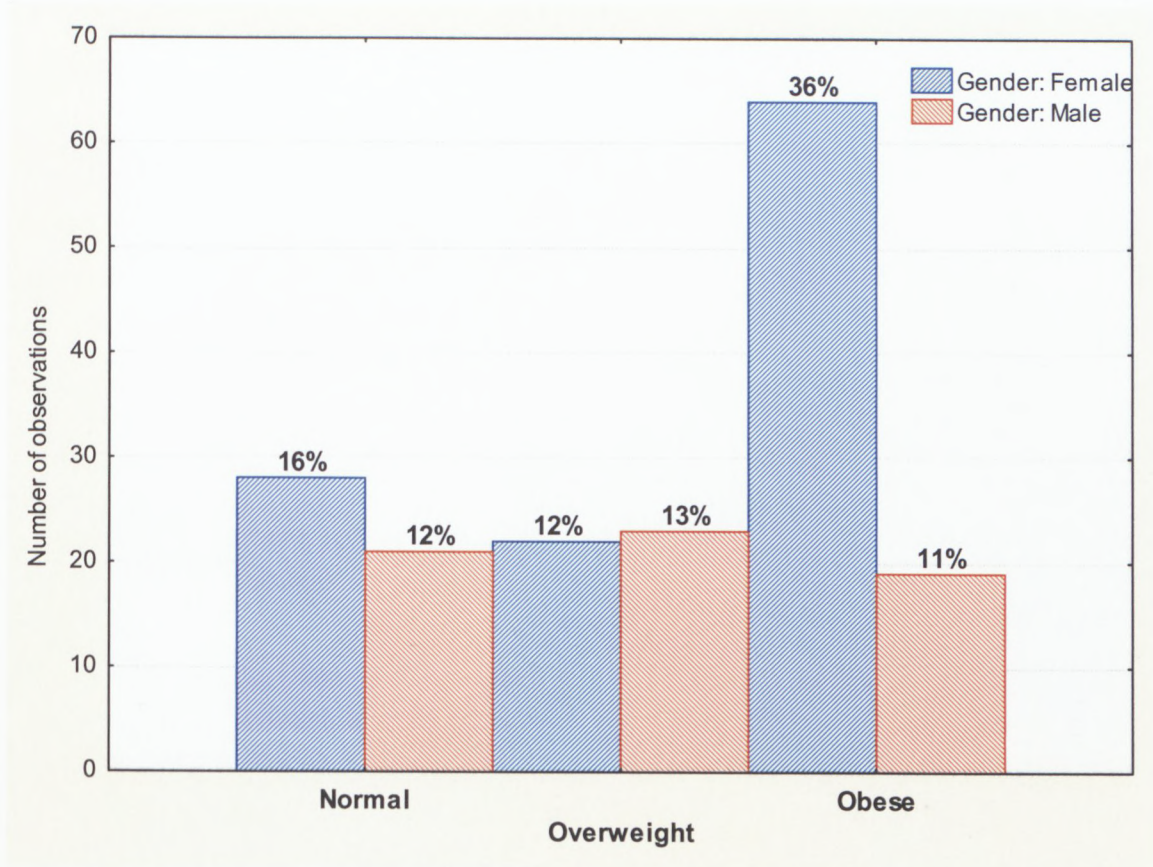
Figure 3.4 Subjects were categorised according to the WHO criterion, whereby BMI < 25 kg/m^2 is considered normal, overweight is BMI > 25 kg/m^2 and < 30 kg/m^2, and obese = BMI = 30 kg/m^2.

Figure 3.5. Central obesity was categorised using the International Diabetes Federation, obese is waist circumference = 94 cm in males and = 80 cm in females

Table 3.3 Characteristics of the 177 participants according to obesity using WHO BMI cut-off values and IDF waist circumference cut-off values

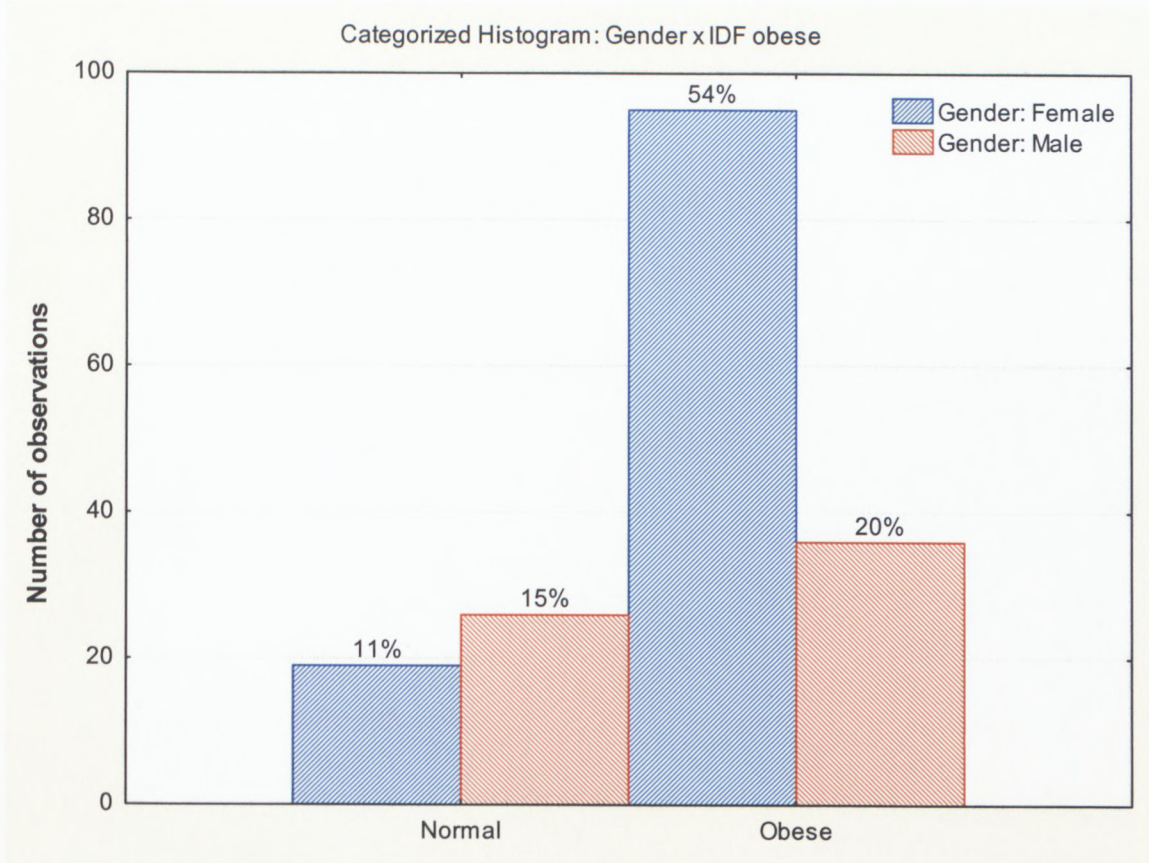|	WHO criteria			IDF criteria		
	Normal N = 49	Obese N = 128	P-value	Normal N= 46	Obese N = 131	P-value
Age (years)	46 (46, 51)	50 (49, 52)	0.13	48 (46, 51)	50 (49, 52)	0.27
BMI (kg/m^2)	22 (21, 22)	32 (32, 34)	**<0.0001**	22 (21, 23)	32 (31, 34)	**<0.0001**
WC (cm)	79 (78, 82)	103 (102, 106)	**<0.0001**	78 (77, 81)	103 (101, 105)	**<0.0001**
HP (cm)	95 (93, 96)	111 (112, 117)	**<0.0001**	94 (92, 96)	111 (112, 117)	**<0.0001**
SBP	109 (106, 116)	124 (122, 128)	**<0.0001**	113 (108, 119)	123 (121, 127)	**<0.001**
DBP	68 (67, 73)	76 (76, 80)	**<0.0001**	71 (67, 74)	76 (75, 79)	**0.003**
FBG (mmol/L)	5.0 (4.8, 5.1)	6.3 (7.0, 8.2)	**<0.0001**	5.0 (4.7, 5.7)	6.1 (6.8, 8.0)	**<0.0001**
Post BG	6.1 (5.7, 6.7)	7.5 (8.1, 9.9)	**<0.0001**	5.4 (5.3, 6.2)	7.5 (8.1, 9.9)	**<0.0001**
HbA1c (%)	5.5 (5.4, 5.6)	6.1 (6.5, 7.0)	**<0.0001**	5.5 (5.5, 5.9)	6.0 (6.4, 6.9)	**<0.0001**
TC (mmol/L)	5.2 (5.0, 5.8)	5.6 (5.4, 5.7)	0.20	5.2 (5.0, 5.6)	5.6 (5.4, 5.8)	0.15
TG (mmol/L)	1.0 (1.0, 1.3)	1.4 (1.5, 1.7)	**<0.0001**	1.0 (1.0, 1.4)	1.4 (1.5, 1.7)	**<0.0001**
HDL-C (mmol/L)	1.4 (1.3, 1.5)	1.1 (1.1, 1.2)	**<0.0001**	1.3 (1.3, 1.5)	1.1 (1.1, 1.2)	**0.001**
LDL-C (mmol/L)	3.3 (3.1, 3.8)	3.6 (3.5, 3.8)	**0.09**	3.1 (3.1, 3.7)	3.6 (3.5, 3.8)	**0.04**
Urine ALB (mg/L)	3.3 (4.6, 8.7)	3.7 (7.6, 12.8)	0.39	3.5 (4.3, 8.5)	3.5 (7.3, 12.3)	0.32
GGT (IU/L)	25 (25, 38)	30 (33, 41)	**0.06**	22 (23, 37)	31 (33, 40)	**0.01**
Serum Cotinine (ng/mL)	85 (130, 247)	9 (83, 141)	**0.04**	34 (118, 241)	9 (88, 147)	**0.09**
Hs-CRP (mg/L)	3.0 (3.2, 6.9)	6.0 (6.3, 8.5)	**<0.001**	3.5 (3.0, 6.9)	6.2 (6.3, 8.5)	**0.001**
Anti-OxLDL (Mu/mL)	1728 (1610, 2564)	1205 (1729, 2425)	0.33	1772 (1633, 2645)	1205 (1699, 2377)	0.20
HNE (µg/mL)	6.2 (5.5, 6.8)	6.1 (5.8, 6.7)	0.86	6.6 (5.7, 7.2)	5.8 (5.8, 6.5)	0.34
MDA (pmol/mg)	656 (604, 763)	549 (554, 648)	**0.04**	654 (591, 757)	559 (561, 654)	0.11

3.4 Characteristics of study population according to smoking

Cigarette smoking was assessed by means of a questionnaire and validated by measuring serum cotinine levels of the respondents. Smoking status was classified according to whether an individual was a current smoker, smoked in the past or never smoked. No significant differences were observed in smoking patterns between males and females, Figure 3.6 Furthermore, serum cotinine levels were significantly lower ($p < 0.0001$) in subjects that had never smoked compared to current and past smokers, as well as between past smokers and current smokers ($p < 0.0001$) (Figure 3.7) Table 3.4 shows the characteristics of participants according to smoking status. There were only 22 past smokers

and these subjects were excluded in the analysis of general characteristics. Measures of BMI, waist and hip circumferences were lower in smoking individuals. Although total cholesterol and LDL cholesterol were significantly higher in non smoking subjects, HDL cholesterol and anti-OXLDL were higher in these subjects.

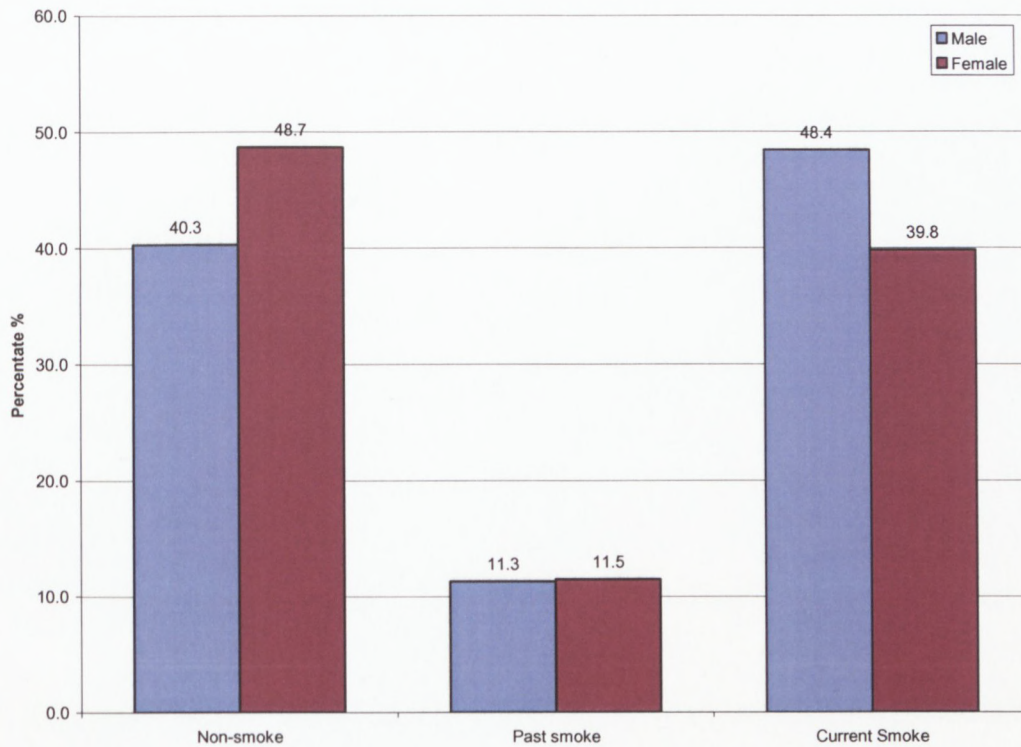**Figure 3.6.** Smoking status in males and females. No significant differences were observed in smoking patterns between males and females.
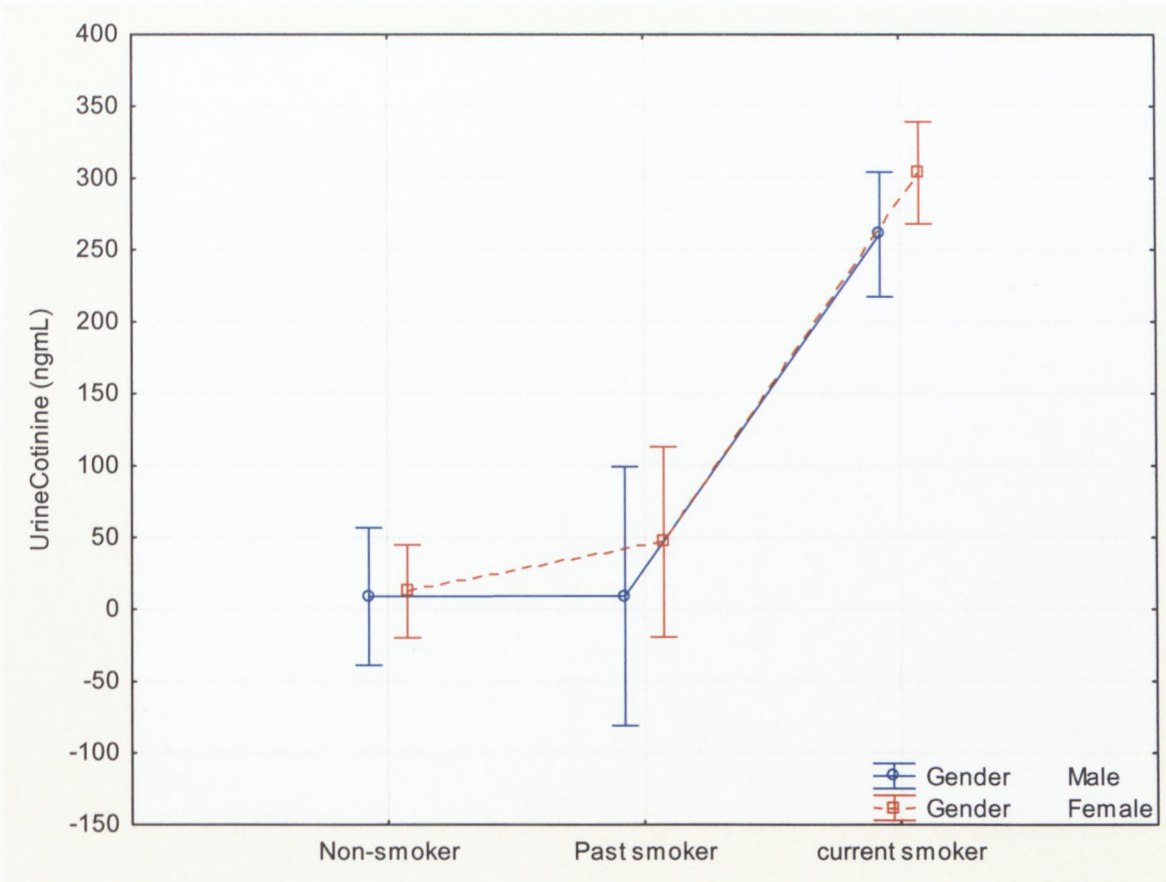
Figure 3.7. Serum cotinine levels according to smoking status and gender. Serum cotinine levels were significantly lower (p < 0.0001) in subjects that have never smoked compared to current and past smokers, as well as between past smokers and current smokers (p < 0.0001).

Table 3.4 Characteristics of non-smokers and current smokers

	Non-smoke N = 80	Current smoke N = 75	P-value
Age (years)	52 (50, 54)	47 (46, 49)	**0.002**
BMI (kg/m^2)	30.1 (29.2, 32.1)	27.5 (26.7, 30.1)	**0.07**
WC (cm)	98 (95, 101)	95 (92, 99)	0.29
HP (cm)	109 (108, 114)	103 (102, 109)	**0.006**
SBP	123 (120, 128)	117 (114, 123)	**0.08**
DBP	75 (74, 79)	77 (72, 78)	0.63
FBG (mmol/L)	6.1 (6.1, 7.5)	5.7 (6.1, 7.8)	0.13
Post BG	7.2 (7.1, 9.4)	6.8 (6.9, 8.8)	0.52
HbA1c (%)	6.0 (6.1, 6.8)	5.6 (6.0, 6.7)	0.31
TC (mmol/L)	5.7 (5.5, 6.0)	5.1 (5.0, 5.5)	**0.003**
TG (mmol/L)	1.4 (1.3, 1.6)	1.3 (1.3, 1.7)	0.71
HDL-C (mmol/L)	1.2 (1.2, 1.3)	1.1 (1.1, 1.3)	**0.04**
LDL-C (mmol/L)	3.8 (3.6, 4.0)	3.2 (3.2, 3.6)	**0.003**
Urine ALB (mg/L)	3.1 (5.4, 10.4)	3.8 (6.8, 13.0)	0.33
GGT (IU/L)	27 (28, 37)	31 (33, 44)	**0.07**
Serum Cotinine (ng/mL)	9 (8.3, 14.7)	308 (246, 327)	**<0.0001**
Hs-CRP (mg/L)	4.9 (5.1, 8.0)	4.9 (5.7, 8.7)	0.45
OxLDL (Mu/mL)	1482 (1858, 2732)	1232 (1480, 2363)	**0.09**
HNE (µg/mL)	6.2 (5.9, 6.9)	6.2 (5.5, 6.5)	0.45
MDA (pmol/mg)	529 (541, 659)	593 (573, 698)	0.31

3.5 Association tests

3.5.1 Correlations

The relationship within and between markers of oxidative stress and cardiovascular risk factors were investigated using spearman's correlation and linear regression analysis. When a significant correlation was observed between concentrations of MDA, HNE or anti-OxLDL antibodies and cardiovascular disease biomarkers, it was negative. MDA and HNE strongly correlated with each other and the correlation was positive. The results of correlations studies are summarized in tables 3.5 and 3.6. The association between FBG, Post BG and HbA1c was negatively correlated. Anti-OxLDL antibodies correlated negatively with serum cotinine, hsCRP, TG and showed a weak negative association with total cholesterol. HNE showed a significant correlation with the other markers LDL-C, GGT and total cholesterol which were negative. MDA correlated significantly negative with LDL-C, GGT, TG, TC, HbA1c, FBG, DBP and SBP.

3.5.2 Regression analysis

Stepwise linear regression analysis was used to study association between markers of oxidative stress and the other variables. The dependent variables, that is hs-CRP or markers of oxidative stress were log transformed prior to the regression analysis. Independent variables contained in the model were: hs-CRP, anti-OxLDL, MDA, HNE, age, BMI, waist and hip circumferences, systolic and diastolic blood pressure, HbA1c, total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, urine microalbumin, serum cotinine, GGT, fasting blood glucose and 2-hour post blood glucose. Whenever a variable was tested as an independent variable it was removed from the independent variable list. The contributors to all models and the percent contribution (R^2) are summarized in Tables 3.9 to 3.12. MDA associated significantly positive with urine albumin and with HNE in the linear regression analysis. However, MDA also associated significantly with SBP and Post BG, though negatively. Anti-OxLDL continued to exhibit the same tendency of association with TC, Post BG and serum cotinine significantly, but negatively. HsCRP associated negatively with FBG, but associated positively with hip circumference.

Table 3.5. Correlation between anti-OXLDL and cardiovascular diseases biomarkers

	Spearman R	P-value
Age (years)	-0.15	0.05
BMI (kg/m^2)	-0.10	0.16
WC (cm)	-0.12	0.10
HP (cm)	-0.10	0.20
SBP	-0.002	0.97
DBP	0.04	0.64
FBG (mmol/L)	-0.22	0.004
Post BG	-0.22	0.006
HbA1c (%)	-0.21	0.004
TC (mmol/L)	-0.14	0.06
TG (mmol/L)	-0.16	0.04
HDL-C (mmol/L)	-0.003	0.96
LDL-C (mmol/L)	-0.10	0.15
Urine ALB (mg/L)	-0.03	0.71
GGT (IU/L)	-0.03	0.71
Serum Cotinine (ng/mL)	-0.15	0.05
Hs-CRP (mg/L)	-0.17	0.03
HNE (µg/mL)	0.04	0.57
MDA (pmol/mg)	0.08	0.23

Table 3.6. Correlation between HNE and cardiovascular diseases biomarkers

	Spearman R	P-value
Age (years)	0.002	0.98
BMI (kg/m^2)	0.02	0.83
WC (cm)	-0.02	0.76
HP (cm)	0.05	0.54
SBP	-0.05	0.50
DBP	-0.05	0.52
FBG (mmol/L)	-0.05	0.49
Post BG	0.002	0.97
HbA1c (%)	-0.03	0.74
TC (mmol/L)	-0.18	0.03
TG (mmol/L)	-0.10	0.17
HDL-C (mmol/L)	0.04	0.53
LDL-C (mmol/L)	-0.18	0.02
Urine ALB (mg/L)	-0.19	0.01
GGT (IU/L)	-0.13	0.09
Serum Cotinine (ng/mL)	-0.07	0.29
Hs-CRP (mg/L)	-0.002	0.97
OxLDL (Mu/mL)	0.04	0.57
MDA (pmol/mg)	0.48	<0.0001

Table 3.7 Correlation between MDA and cardiovascular diseases biomarkers

	Spearman R	P-value
Age (years)	-0.01	0.89
BMI (kg/m^2)	-0.05	0.48
WC (cm)	-0.08	0.32
HP (cm)	0.02	0.86
SBP	-0.22	0.004
DBP	-0.18	0.02
FBG (mmol/L)	-0.20	0.008
Post BG	-0.04	0.67
HbA1c (%)	-0.22	0.003
TC (mmol/L)	-0.22	0.004
TG (mmol/L)	-0.16	0.03
HDL-C (mmol/L)	-0.11	0.13
LDL-C (mmol/L)	-0.25	<0.001
Urine ALB (mg/L)	0.01	0.89
GGT (IU/L)	-0.19	0.01
Serum Cotinine (ng/mL)	-0.002	0.97
Hs-CRP (mg/L)	0.03	0.72
OxLDL (Mu/mL)	0.08	0.28
HNE (µg/mL)	0.48	<0.0001
MDA (pmol/mg)		

Table 3.8. Correlation between hs-CRP and cardiovascular disease biomarkers

	Spearman R	P-value
Age (years)	0.06	0.42
BMI (kg/m^2)	0.26	**<0.001**
WC (cm)	0.23	**0.002**
HP (cm)	0.28	**<0.001**
SBP	0.07	0.39
DBP	0.03	0.66
FBG (mmol/L)	0.18	**0.02**
Post BG	0.30	**<0.001**
HbA1c (%)	0.21	**0.005**
TC (mmol/L)	0.07	0.37
TG (mmol/L)	0.14	**0.06**
HDL-C (mmol/L)	-0.06	0.44
LDL-C (mmol/L)	0.05	0.51
Urine ALB (mg/L)	0.07	0.32
GGT (IU/L)	0.22	**0.004**
Serum Cotinine (ng/mL)	0.08	0.29
Hs-CRP (mg/L)	0.03	0.72
OxLDL (Mu/mL)	0.08	0.28
HNE (µg/mL)	0.48	**<0.0001**
MDA (pmol/mg)		

Table 3.9. Anti-OXLDL model: stepwise linear regression analysis

Predictors	B	P-value
Post BG (mmol/L)	-0.241	**0.008**
Serum Cotinine (ng/mL)	-0.267	**0.003**
TC (mmol/L)	-0.206	**0.02**
HP (cm)	-0.117	0.2

$R^2 = 0.1422$

Table 3.10. MDA model: stepwise linear regression analysis

Predictors	B	P-value
HNE (µg/mL)	0.393	**<0.001**
SBP (mmHg)	-0.251	**0.004**
Post BG (mmol/L)	-0.253	**0.006**
Urine ALB (mg/L	0.235	**0.009**
WC (cm)	0.135	0.12
LDL-C (mmol/L)	-0.159	**0.06**
Age (years)	0.113	0.19
HbA1c (%)	-0.09	0.27

$R^2 = 0.315$

Table 3.11. HNE model: stepwise linear regression analysis

Predictors	B	P-value
MDA (pmol/mg)	0.417	**<0.0001**
Urine ALB (mg/L	-0.187	**0.02**
Serum Cotinine (ng/mL)	-0.178	**0.03**
LDL-C (mmol/L)	-0.120	0.16
OxLDL (Mu/mL)	-0.088	0.30
GGT (IU/L)	-0.082	0.31

$R^2 = 0.254$

Table 3.12. hs-CRP model: stepwise linear regression analysis

Predictors	B	P-value
HP (cm)	0.187	0.05
HbA1c (%)	0.249	0.13
FBG (mmol/L)	-0.401	0.02
Post BG (mmol/L)	0.205	0.24

$R^2 = 0.056$

CHAPTER FOUR

DISCUSSION AND CONCLUSION

The specific hypothesis of the present study was that the biochemical markers of oxidative stress would be significantly increased in hyperglycaemic subjects, and would be exacerbated by smoking and/or obesity leading to an increased CVD risk as measured by hs-CRP. In this study, oxidative stress was indirectly assessed by measuring levels of MDA-protein adducts, HNE-protein adducts and antibodies against OxLDL, and only the latter showed significant differences between normoglycaemic and hyperglycaemic subjects. Hence the discussion is focused on the antibodies against oxLDL.

The inflammatory nature of atherosclerosis is well established. Key to this is the oxidation of LDL resulting in the formation of immune complexes and production of antibodies against OxLDL (Steinbrecher et al, 1984; Steinberg, 1997; Witztum & Steinberg, 2001). It is well recognized that diabetes mellitus increases the risk of developing cardiovascular diseases and the oxidation of LDL is suggested to play a significant role in the pathogenesis of macrovascular complications observed in diabetic patients (Ceriello et al., 2002). Antibodies against oxidised LDL have been detected in healthy and diseased individuals (Virella et al., 1993; Bellomo et al., 1995; Lughetti et al., 1999; Karabinos et al., 2003), however their role in disease is poorly understood. In the present study we found IgG anti-OxLDL antibodies to be low in individuals with hyperglycaemia, especially in those with undiagnosed diabetes, perhaps due to the unmanaged glycaemic state. Furthermore, after the multiple stepwise linear regression analysis, 2 hour post blood glucose showed a negative association with anti-OxLDL antibodies. Although there are several reports on the levels of anti-OxLDL antibodies in patients with diabetes, there is considerable inconsistency in reports from several investigators. In a longitudinal study, Garrido-Sánchez et al (2008) demonstrated that low levels of anti-oxLDL antibodies in subjects with normal glucose tolerance, impaired fasting glucose or impaired glucose tolerance were a significant predictor of type 2 diabetes onset. Furthermore, another report had previously shown a distinct fall of these antibodies after the age of 35 years and increasing age is strongly associated with the development of Type 2 diabetes. In contrast, other studies have found higher levels (Bellomo et al., 1995; Festa et al., 1998) or comparable levels of anti-OxLDL antibodies in both type 1 and type 2 diabetes (Uusitupa et al.,1996; Korpinen et al., 1997; Mironova et al., 1997).

Anti-oxidised LDL antibodies have been found in patients with advanced atherosclerotic lesions (Inoue, 2001) however, their role in atherosclerosis is also controversial. Salonen et al. (1992), reported high levels of autoantibodies to malonyldialdehyde-LDL particles in

subjects with carotid atherosclerosis, but the Framingham Offspring Study failed to show a relation between baseline levels of IgG anti-OxLDL antibodies and the development of CVD or coronary heart diseases (Wilson, 2006). Furthermore, another study found an inverse relationship between antibody levels and carotid intima-medial thickness after adjusting for several CVD risk factors, such as age, blood pressure, BMI and lipids (Fukumoto *et al.,* 2000). We also observed a negative association with cardiovascular disease risk factors, as shown by the inverse association between anti-oxLDL antibodies and several markers of cardiovascular diseases. After multiple stepwise linear regression, an increase in total cholesterol, triglycerides and age was associated with a reduction in the levels of anti-OxLDL antibodies.

Furthermore, HbA1c and hs-CRP correlated negatively with anti-OxLDL antibodies. Similarly, *Santos et al.,* (2009), showed a negative correlation between anti-OxLDL antibodies and hs-CRP and an inverse association after performing a multiple linear regression analysis. CRP and HbA1c have recently gained popularity in the assessment of cardiovascular disease risk. However, the range of CRP associated with vascular risk is decreased below the reference range used to assess inflammation, and high sensitivity assays were developed to measure CRP associated with CVD risk (Ridker, 2009). Recent prospective studies have shown that elevated HbA1c levels are associated with risks of CVD and death (Selvin *et al.,* 2010). This association has recently been extended to non diabetic subjects, where in non-diabetic adults with HbA1c levels of 6.0% or higher were found to be at an increased risk of developing CVD and diabetes mellitus (Selvin *et al.,* 2010).

It has been suggested that measurement of antibodies against OxLDL may serve as an index of *in vivo* LDL oxidation, however this remains to be elucidated. Lipid peroxidation is a well characterised mechanism of cellular damage and lipid peroxides are unstable indicators of oxidative stress in cells that decompose to form more complex and reactive compounds such as MDA and HNE. Therefore, measuring these natural by-products (MDA and HNE) of lipid peroxidation is one of the most widely accepted methods of assessing oxidative damage. In this study, MDA-protein adducts were shown to be positively associated with the waist circumference in the stepwise linear regression analysis. Central obesity is the main perpetrator increasing LDL particles into the blood circulation, amplifying the capacity of free radicals to oxidize polyunsaturated fatty acids, consequently, oxidative stress is raised (Grundy *et al.,* 2004). Obesity is regarded as an independent risk factor for atherosclerosis because it is linked to oxidative stress and inflammation with profound effects on cardiovascular disease, one of which is hypertension (Krauss *et al.,* 1998; Weinbrenner *et al.,* 2006). Anthropometric measurements BMI, WC and HP were significantly elevated in hyperglycaemic subjects, with increased triglycerides, compared to the normal subjects

36

(Table 3.2). This is consistent with the results of Tataranni (2002) that obesity enhances dyslipidemia, accelerates oxidative stress and has an essential role in the transformation of type 1 DM into type 2 DM. In addition, central obesity through its adipose tissue has increased lipogenic and lipolytic activities leading to the accumulation of free radicals (Funanashi *et al.*, 1999; Matzuzawa *et al.*, 1994).

Cigarette smoking exposes an individual to a variety of highly oxidant gases and free radicals, and is believed to add to the toxic accumulation of ROS in the diabetic subject (Benowitz, 2003). Thus, our results partially support the role of these antibodies as an indirect assessment of oxidation whereby lower antibody levels may indicate increased oxidative stress with a consequent increase in LDL oxidation. It is however, pertinent to mention that waist circumference was retained after multiple stepwise linear regression analysis and showed a positive association with anti-OxLDL antibodies. Though obesity is defined by the body mass index it is central obesity as measured by the waist circumference that is strongly associated with metabolic abnormalities. Adipose tissue expresses inflammatory cytokines, interleukin-6 (IL-6) and tumor necrosis factor–a (TNF-a) which are a source of oxidative stress (Higdon & Frei, 2003). A relationship between abdominal fat and enhanced lipid peroxidation has been demonstrated by the direct proportional elevation of oxidative stress biomarkers, especially oxidized LDL (OxLDL) and waist circumference (Weinbrenner *et al.*, 2006). The discrepancies in the role of antibodies against oxidised LDL reported in this study and other studies could be due to the heterogeneous effect of these antibodies. Human IgG antibodies have different subclasses including IgA, IgG1, IgG2, and IgG3 (Wu *et al.*, 2003). Whilst IgG2 is the dominant subclass to respond to epitopes containing phospholipids, it is the IgG3 subclass that is more pathogenic as it fixes complement better and binds Fc receptors more ardently (Devey & Voak, 1974).

The major limitation in this study was the measurement of total IgG anti-oxLDL antibodies as quantification of the specific antibody subclasses may elucidate the role and usefulness of these antibodies in various disease states. The other limitation in this study was the inclusion of only one ethnic group, the mixed ancestry population of South Africa with a reportedly high incidence of diabetes (Levitt *et al.*, 1993) as these antibodies have been shown to differ between South Asian individuals with an increased risk of atherosclerosis compared to whites (Miller *et al.*, 2009). In conclusion, our findings demonstrated that anti-OxLDL antibodies are reduced in subjects with hyperglycaemia, and that these low levels are associated with an increased risk of cardiovascular diseases as measured by the hs-CRP. Furthermore, our results also demonstrated that obesity and cigarette smoking contribute to oxidative damage in both normoglycaemic and hyperglycaemic individuals. However, these

findings should be corroborated by further studies that take into account the different subclasses of human IgG anti-OxLDL antibodies as well as different ethnic groups.

CHAPTER FIVE

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