Risk factors and Blood borne-biochemical markers in type 2 diabetes mellitus

By

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"The Fear of the Lord is the beginning of wisdom"

Proverbs 1:7

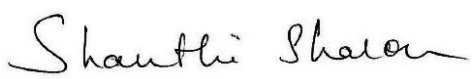
Dedication

I dedicate this thesis to The Lord Almighty and my parents Suneeth Kumar John Kappala and Kalpana Kappala for their boundless love, support and faith in me which made me what I am today. Mom and Dad, I love you and thank you for trusting in me. Your prayers kept me alive and never let me to give up on my dreams. This thesis would have been incomplete without you.

Declaration

I declare that while registered as a candidate for this degree, I have not registered as a candidate for any other award from an academic institution. The work present in this thesis, except where otherwise stated, is based on my own research and has not been submitted for any other award in this or any other University.

Signed



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Abstract

The burden of Diabetes Mellitus (DM) is increasing worldwide and it is estimated to reach indefinite proportions of about 450 million by year 2030. Patients with type 2 diabetes mellitus (T2DM) have a significantly increased risk of developing cardiovascular diseases (CVD). Moreover, CVD is the major cause of mortality and morbidity (75%) in T2DM patients. DM itself has been long recognised as an independent risk factor for several forms of CVD including coronary heart disease (CHD), peripheral arterial disease, cardiomyopathy and congestive heart failure in both men and women. It is well-known that T2DM is associated with several factors including hyperglycaemia, hypertension, dyslipidemia, obesity all of which contribute to CVD. In order to prevent CVD, early intervention on cardiovascular risk factors is vital during clinical assessment of T2DM patients. A major role of inflammation has been well described in the development of CVD in T2DM patients. Inflammatory process and factors which contribute to CVD in T2DM patients have recently become a focus in diabetic research. Elucidation of common patho-physiological mechanisms among T2DM patients might emphasize the role of inflammation in CVD.

The main purpose of this study was to investigate any patho-physiological changes in red blood cells (RBC), white blood cells (neutrophils and lymphocytes) and plasma, measuring RBC membrane fragility and proteins, intracellular free calcium concentrations $[Ca^{2+}]_i$ and several cations including $Na^{+,}$ Mg^{2+}, Ca^{2+}, Fe^{2+}, Zn^{2+} and Cu^{2+}, biochemical parameters and inflammatory mediators which normally serve as independent predisposing risk factors for CVD among T2DM patients compared to age-match healthy controls.

The results have shown that fura-2 loaded neutrophils and lymphocytes in blood from T2DM patients contain significantly ($p<0.05$) less $[Ca^{2+}]_i$ than neutrophils and

lymphocytes from healthy subjects upon stimulation with physiological doses of either fMLP or thapsigargin indicating a derangement in cellular calcium homeostasis during T2DM. Similarly, RBC membranes from T2DM patients contained significantly ($p<0.05$) more spectrin, ankyrin, band 3, band 4.1, glycophorin etc compared to RBC membranes from age-matched healthy control subjects. The results also show that the RBCs from T2DM patients were more fragile compared to RBC from healthy controls. Measurement of protein glycation in plasma have revealed significantly ($p<0.05$) more fluorescence in proteins form T2DM patients compared to control. In relation to plasma cations and intracellular markers and mediators, the results show that plasma from T2DM patients contain significantly ($p<0.05$) more $Na^{+,}$ Mg^{2+} , Ca^{2+}, Fe^{2+}, Zn^{2+} and Cu^{2+} compared to plasma levels from age-match healthy controls. Similarly, the concentrations of kidney and liver function markers such as urea, creatinine, alkaline phosphatase, ALT, AST, GGT, total protein and albumin increased significantly ($p<0.05$) compared to healthy controls. The same is also true for glucose, total cholesterol, triglycerides, CRP, HbA1c, WBC where the blood from T2DM patients contained elevated concentrations compared to blood from healthy age-matched control patients.

Together, the results of this study have clearly demonstrated marked and significant changes in cellular calcium homeostasis in white blood cells, RBC membrane proteins and fragility, plasma protein glycation and in plasma levels of cations, intracellular markers and mediators of T2DM patients compared to healthy controls. Therefore, it is proposed that an early integrated and multi-factorial intervention of risk factors and inflammatory markers must be done in order to reduce the risk of CVD and possible mortality of T2DM patients.

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Abbreviations

µg	microgram
µl	microlitre
µM	micromolar
A_{350}	absorbance at 350 nm
A_{420}	absorbance at 420 nm
A_{505}	absorbance at 505 nm
A_{595}	absorbance at 595 nm
AGEs	Advanced Glycation End-products
LMW AGEs	Low molecular weight AGEs
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BMI	Body Mass Index
CVD	Cardiovascular disease
CAD	Coronary artery disease
CHD	Coronary heart disease
CRP	C-reactive protein
DNA	deoxyribonucleic acid
ED	Endothelial dysfunction
ETOH	ethanol
g	gram
GFR	Glomerular filtration rate
GGT	Gamma-glutamyl transferase

GP	Glycophorin
HbA1c	Glycated haemoglobin A1c
HCT	Hematocrit
HDL-C	High density lipoprotein cholesterol
IL-6	Interleukine-6
IR	Insulin Resistance
kDa	Kilo Daltons
l	litre
LDL-C	Low density lipoprotein cholesterol
M	Molar
MCH	Mean corpuscular hemoglobin
MCV	Mean corpuscular volume
mg	milligram
$MgCl_2$	Magnesium Chloride
min	minute(s)
ml	millilitre
mmol	millimolar
MW	Molecular weight
NO	Nitric oxide
OS	Oxidative Stress
PAS	Periodic acid-Schiff
PKC	Protein kinase C
PLT	Platelets

RAGE	Receptor for AGEs
RBC	Red blood cells
WBC	White blood cells
ROS	Reactive Oxygen Species
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TAS	Total anti-oxidant Status
TC	Total cholesterol
TNF-α	Tumour necrosis factor-α
VLDL-C	Very low density lipoprotein cholesterol
SOCE	Store operated calcium entry
CRAC	Calcium release activated calcium
DAG	Di Acyl Glycerol
fMLP	N-formyl Methionyl Leucyl Phenyl Alanine
SMCE	Store mediated calcium entry
Fura 2 AM	Fura-2 Acetoxy methyl ester
AU	Arbitrary units
RPMI	Rosewell park memorial Institute
RPM	Rotations per minute
G	gravitational force
EDTA	Ethylenediaminetetraacetic acid

EGTA	Ethylene glycol tetraacetic acid
IGF	Insulin-like growth factor
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
ICP-MS	Inductively coupled plasma mass spectrometry
ABI	Ankle brachial index
ER	Endoplasmic reticulum
WHO	World Health Organisation
ADA	American Diabetes Association

Chapter 1

Introduction

1.1 Scope of this study

Diabetes mellitus (DM) is the most widespread complex metabolic disorder among the world's population currently affecting around 250 million people globally (Srinivasan *et al.,* 2008). In early 1930's, Himsworth documented that two types of diabetes existed (Himsworth, 1936). This complicated metabolic syndrome is due to either insulin insufficiency or impaired action of the insulin hormone or both (American Diabetes Association, 2004). This disease is classified as insulin- dependent or type 1 diabetes mellitus (T1DM) and non-insulin dependent diabetes mellitus or type 2 diabetes mellitus (T2DM) of which both types of are associated with high levels of morbidity and mortality (Alberti *et al.,* 2004). The epidemic of diabetes mellitus is accompanied by an increase in the incidence of diabetic complications including cardiovascular disease (CVD), retinopathy, nephropathy and microangiopathy, neuropathy and several other complications (Kumar and Clark, 2007).

It is well understood that CVD are the leading cause of mortality and morbidity in T2DM patients as there is an increased risk of CVD in this particular population. Some of the types of cardiovascular risk encountered in diabetic patients include coronary heart disease (CHD), small vessel coronary artery disease (CAD), cerebrovascular disease, peripheral vascular disease, hypertension and congestive heart failure (Grundy *et al.,* 1999). Diabetes-induced CVD is due to several conditions which are associated with increased concentration of glucose in the blood often referred to as hyperglycaemia. Dyslipidemia, hyperglycaemia and inflammation could also lead to insulin resistance (DSouza *et al.,* 2009; Mooradian, 2009).

There is strong evidence that inflammation forms the basis in the pathophysiology of both insulin resistance and CVD among T2DM patients (Sobel and Schneider, 2005) CVD has several underlying risk factors including the conventional hypertension,

hyperglycemia, increased levels of total cholesterol and low density cholesterol (LDL) and several other factors like race, origin, gender and smoking, lack of exercise, DM, genetics (Al-Ozairi *et al*.,2006) as well as the non-conventional risk factors like considerable variations in the levels of inflammatory markers including C-reactive protein (CRP), Interleukine-6 (L-6), Tumour necrosis factor (TNF-a), C-peptide and fatty acid binding protein (FABP) are all involved in the pathogenesis of insulin resistance (Erdmann, 2005; DSouza *et al.*, 2009).

The main aim of this study was to identify and evaluate the levels of various biochemical parameters and inflammatory markers, which serve as predisposition factors and govern the susceptibility to CVD in T2DM patients of different ages compared to healthy age-matched controls.

Chapter 1 of this study contains a general introduction of the subject area. Chapter 2 summarizes materials and methods involved in tackling the scientific problems. Chapter 3 of this thesis describes measurement of intracellular free calcium $[Ca^{2+}]_i$ in neutrophils and lymphocytes of T2DM patients and healthy controls. Chapter 4 is an analysis of red blood cell membrane proteins in T2DM patients compared to healthy controls using SDS-PAGE and Chapter 5 of this thesis is an analysis of various biochemical parameters in T2DM patients and healthy controls. Chapter 6 involves analysis of low molecular weight advanced glycation end products (AGEs), anti-oxidant status and osmotic fragility of red blood cells (RBC) in T2DM patients compared with healthy age-matched controls. Chapter 7 is a general discussion of this study followed by conclusion, the scope for future studies, the references, appendix and presentations.

1.2 Type 2 Diabetes Mellitus (T2DM)

Diabetes mellitus (DM) can be described as a metabolic disorder which is caused by the deficiency of insulin hormone secreted by the pancreatic beta cells (Rosenbloom *et al.,*

2008). The hormone is responsible for the uptake of glucose from the blood mainly into muscle and fat cells of the body and it also inhibits hepatic glucose production, and therefore regulates the concentration of blood glucose at normal levels of 4-6 mmol/l (Kumar and Clark, 2007).

1.2.1 Classification of diabetes

Generally, there are two types of DM namely, T1DM and T2DM. T1DM is also called insulin dependent or early onset diabetes, which is diagnosed during childhood and accounts for about 5-10% of DM. T1DM is due to autoimmune destruction of pancreatic β cells in islets of Langerhans resulting in a decrease of insulin production. T1DM is commonly associated with microvascular complications including nephropathy and retinopathy and it also predisposes to CHD (Adler *et al*., 2003).

T2DM is also called non-insulin dependent DM (NIDDM) or late onset diabetes and it is the most prevalent form of diabetes which usually makes its appearance later in life. It is due to a combination of defective secretion of insulin by pancreatic β cells and impairment of insulin- mediated glucose disposal, which is called insulin resistance. This accounts for 90-95% of DM (Kumar and Clark, 2007; DSouza *et al.*, 2009; Ripsin *et al.*, 2009; Ahmed, 2010).

The World Health Organisation classified diabetes in 1980 and revised it in 1985 into the two most common types which was insulin-dependent diabetes mellitus (IDDM) and non-insulin-dependent diabetes mellitus (NIDDM). Depending on the nutrition status, the WHO also categorized DM into other types like malnutrion-related diabetes, which is now omitted from the new classification due to its unknown aetiology, and diabetes, which is diagnosed during pregnancy as gestational diabetes. The WHO ultimately authorized the classification proposed by the American Diabetes Association

(ADA), which was based on aetiology in 1997 (Holt, 2004; Kumar and Clark, 2007; Ahmed, 2010).

1.2.2 Symptoms

Both types of diabetes have similar symptoms, but for T1DM the symptoms develop more rapidly in days or weeks. They include polyuria, polydipsia, unexplained weight loss, muscle wasting, fatigue, cramps, constipation, blurred vision and skin infections. Diabetes is often diagnosed from those complications of untreated patients like foot ulcers and eye problems. The various long term complications due to DM are classified as macrovascular, microvascular and nephropathic which cause different dysfunctions and are the major causes of morbidity and mortality in Diabetics (Kumar and Clark, 2007; Song and Hardisty, 2008; Ahmed, 2010).

1.2.2.1 Hypoglycaemia

Hypoglycaemia occurs due to low blood glucose levels. It is caused by over medication, excessive exogenous insulin, and strenuous exercise. Sometimes it may even lead to seizures, coma and irreversible brain death as blood glucose regulates the functioning of body and organs including the brain. Symptoms due to hypoglycaemia include irritability, sweating, tingling lips, weakness, hunger and nausea (Kumar and Clark, 2007; Ahmed, 2010).

1.2.2.2 Hyperglycaemia

Hyperglycaemia is due to an increase in blood glucose levels. It is due to many factors including not taking medication or exogenous insulin, or by not controlling the diet or taking exercise. It may also occur due to autoimmune destruction of β-cells of pancreas. Symptoms include increased thirst and urination, blurred vision, lethargy, weight loss, cramps and dehydration. Lack of insulin also releases ketones into the blood which in turn raise the acidity of the blood leading to a state called ketoacidosis. Both hypo and

hyperglycemia are reversible and can be treated (Kumar and Clark, 2007; DSouza *et al*, 2009; Ahmed, 2010).

1.2.2.3 Diet, physical activity and diabetes

Since this study is concerned mainly with T2DM, much emphasis will be placed on this type of DM. T2DM results from an interaction between genetic and environmental factors. The rapidly changing incidence rates, however, suggest a predominantly significant role for the latter as well as a potential for stemming the tide of the global epidemic of the disease. The most remarkable increases in T2DM are occurring in societies in which there have been major changes in the type of diet consumed, reductions in physical activity, and increases in overweight and obesity (Aronne and Segal, 2002).

The diets concerned are typically energy-dense, high in saturated fatty acids and depleted in non-starch polysaccharides (NSP). In all societies, overweight and obesity are associated with an increased risk of T2DM, especially when the excess adiposity is centrally distributed (Parker *et al.*, 1993). Conventional body mass index (BMI) categories may not be an appropriate means of determining the risk of developing T2DM in individuals of all population groups because of ethnic differences in the composition of the body and because of the importance of the distribution of excess adiposity. While all life style-related and environmental factors, which contribute to excess weight gain, thus may be regarded as contributing factors to T2DM. The evidence that an individual's dietary factors have an effect, which is independent of their obesity promoting effect, is unconvincing. Evidence that saturated fatty acids increase risk of T2DM and that NSP are protective is more convincing than the evidence for several other nutrients, which have been implicated (Lindgarde *et al.*, 2006). The presence of maternal diabetes, including gestational diabetes and intrauterine

growth retardation appears to increase the risk of developing diabetes in future (Dabelea, 2007).

The association between excessive weight gain, central adiposity and the development of T2DM is convincing. The relationship has been frequently demonstrated in longitudinal studies in diverse populations, with a striking gradient of risk apparent with increasing levels of BMI, adult weight gain, waist circumference and waist-to-hip ratio (Despres *et al.,* 2001). Indeed, either waist circumference or waist-to-hip ratio (reflecting abdominal or visceral adiposity) is a more powerful determinant of subsequent risk of T2DM than BMI (Colditz, 1990).

Central adiposity is also a vital determinant of insulin resistance, the underlying abnormality in most cases of T2DM (Hayashi *et al.,* 2003). Voluntary weight loss improves insulin sensitivity (McAuley *et al.,* 2002) and several randomized controlled trials have been shown to reduce the risk of progression from impaired glucose tolerance to T2DM (Tuomilehto *et al.*, 2005).

Longitudinal studies have clearly indicated that increased physical activity reduces the risk of developing T2DM regardless of the degree of adiposity (Myers *et al.,* 2003). Vigorous exercise (i.e. training to an intensity of 80--90% of age-predicted maximum heart rate for at least 20 minutes, at least five times per week) has the potential to substantially enhance insulin sensitivity. Physical activity might decrease T2DM by increasing insulin sensitivity although the intensity and duration of physical activity required to improve insulin sensitivity has not been established (Jeon *et al.,* 2007). Insulin sensitivity also increases with physical activity according to a study based on questionnaires (Mayer-Davis *et al.,* 1998). Offspring of diabetic pregnancies (including gestational diabetes) are often large and heavier at birth, tend to develop obesity in childhood and are at high risk of developing T2DM at an early age in life (Cook &

Hurley, 1998). Those born to mothers after they have developed diabetes have a three-fold higher risk of developing diabetes than those born before (Momiyama *et al.,* 1999).

A number of studies, mostly in developing countries, have suggested that intrauterine growth retardation and low birth weight are associated with subsequent development of insulin resistance (McCance *et al.,* 1994). In countries where there has been malnutrition, insulin resistance may have been selectively advantageous in terms of surviving famine. In populations where energy intake has increased and lifestyles have become more sedentary, however, insulin resistance and the consequent risk of T2DM have been enhanced. In particular, rapid postnatal catch-up growth appears to further increase the risk of T2DM in later life (Cook and Hurley, 1998). Appropriate strategies, which may help to reduce the risk of T2DM in this situation, include improving the nutrition of young children, promoting linear growth and preventing energy excess by limiting intake of energy-dense foods, controlling the quality of fat supply, and facilitating physical activity. At a population level, foetal growth may remain restricted until maternal height improves. This may take several generations to correct (Lindsay, 2009). The prevention of T2DM in infants and young children may be facilitated by the promotion of exclusive breast feeding, avoiding overweight and obesity, and thus, promoting optimum linear growth.

1.3 Epidemiology of T2DM

T2DM is a multifaceted disorder due to both genetic, lifestyle and environmental factors and it accounts for up to 90-95% of total diabetes cases. In the United Kingdom, around 2.4 million people have T2DM. Globally, over 250 million people have DM. The prevalence of diabetes has been increasing day by day and is expected to reach indefinite proportions by 2030 (Zimmet *et al.,* 2003). The worldwide distribution and the prevalence of T2DM are increasing rapidly. It has been envisaged by the World

Health Organisation (WHO) that the number of people with diabetes will be doubled by the year 2030 reaching almost 500 million (Holt, 2004).

The incidence of T2DM varies enormously from one population to the other. A major discrepancy in the frequency of this disease globally has been very well established by several prospective population-based studies. Much information on the incidence of T2DM is unavailable though it is the most common form of diabetes, which has no genetic factors associated with it. The Pima Native Americans of Arizona and those from South Pacific Island of Nauru are found to have the maximum rates of people with diabetes (Barnett, 2005).

In the present world, diabetes has become one of the most expensive health conditions to treat. In the United Kingdom, for example, it costs the NHS £5 billion to diagnose, treat and care for diabetic patients and UK only has 2.5 million cases. If these numbers are extrapolated to the current 250 million people globally, then it will cost the Governments of the world £500 billion annually to diagnose, treat and care for diabetic patients. Its prevalence worldwide has been growing in most populations (King *et al.*, 1998). Due to its insidious onset and asymptomatic nature, diabetes remains undiagnosed in many people which makes them susceptible to life-threatening complications, and thus reducing the quality of life.

1.4 Pathogenesis of T2DM

The two main reasons underlying the pathophysiology of T2DM are impaired insulin secretion due to the dysfunction of pancreatic beta cells and impaired insulin action because of insulin resistance (Kumar and Clark, 2007). A breakdown of these two mechanisms consequently results in abnormal high levels of plasma glucose concentrations that are usually maintained at a definite range irrespective of the various

changes for the requirement and supply which is regulated by a dynamic process between tissue sensitivity and insulin (Kasim-Karakas, 2000).

Inflammation and inflammatory cytokines have been very recently suggested to be the reason for the development of insulin resistance and T2DM. Insulin resistance develops from physical inactivity, obesity along with a substrate of genetic susceptibility. It is commonly accompanied by other cardiovascular risk factors like dyslipidemia, hypertension and pro-thrombotic factors (Al-Ozairi *et al*., 2006). These factors together in an individual are called the metabolic syndrome, which precedes T2DM (Kumar and Clark 2007, Ahmed, 2010).

1.5 Diagnosis

There was major perplexity regarding the criteria for diagnosis of diabetes mellitus before the 1970s. The WHO has put forward its first statement on the diagnosis of diabetes in 1965, which was later tailored and made simple. The ADA proposed its first diagnostic criteria following WHO, which emphasised on the concentration of fasting glucose. Consequently, WHO revised its old version. Figure 1.1 shows a flow diagram for the diagnosis and screening of T2DM including IGTT (Jonathan and Donald, 2003).

Since the diagnosis of diabetes mellitus has major implications on social, legal and medical aspects of an individual, security for diagnosis is indispensable. The test for diagnosing diabetes should include a venous plasma sample that can be tested in an accredited laboratory. A recent report by WHO recommends the acceptability of glycated haemoglobin (HbA1c) as an additional test to diagnose diabetes.

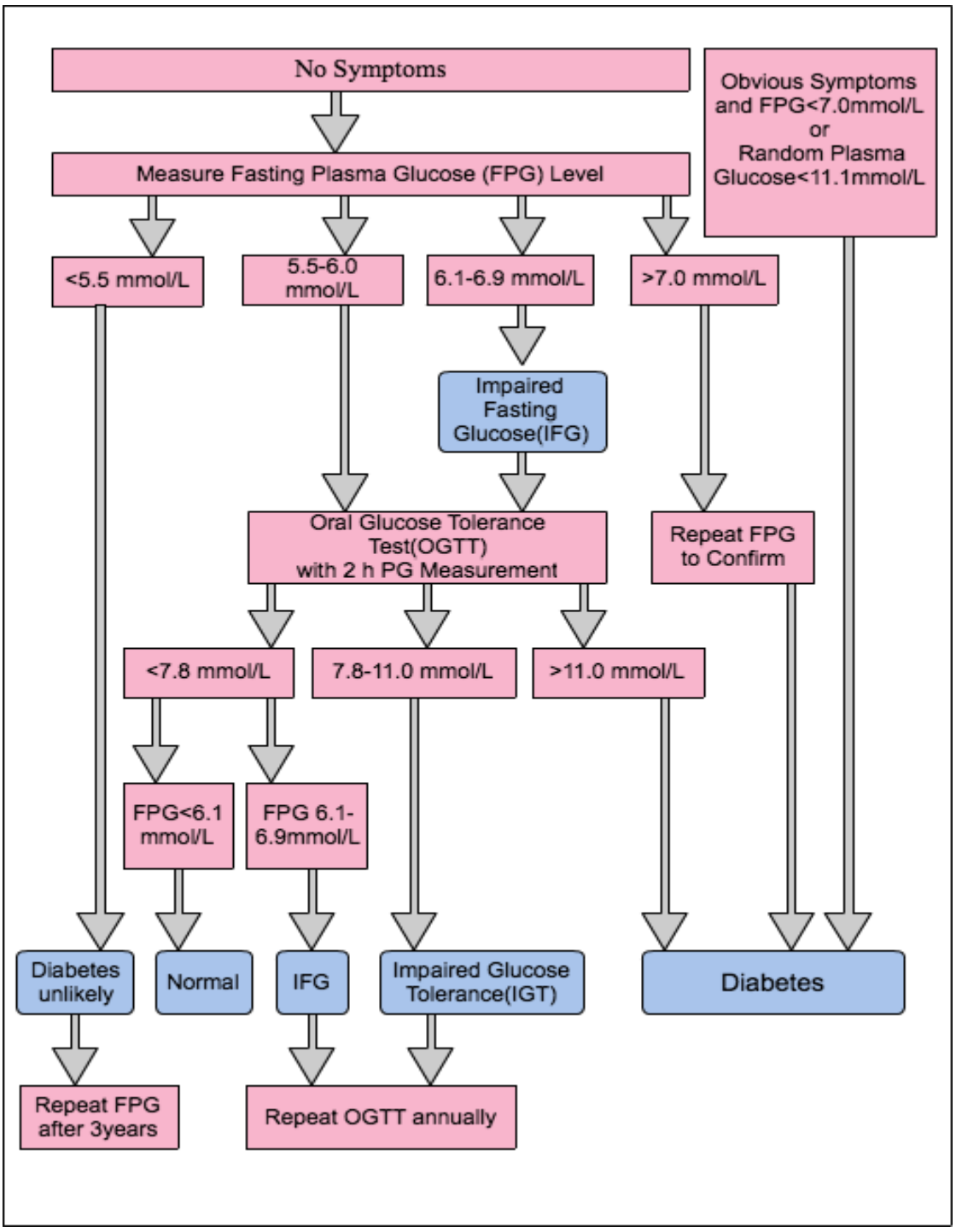

Figure 1.1: Diagnosis and screening for T2DM and impaired glucose metabolism (Taken from Jonathan and Donald, 2003).

1.6 T2DM: genetic and environmental factors.

T2DM is a multifactorial disease caused by the interaction of both genetic and environmental factors. Its strong genetic component is suggested by its familial aggregation and transmission patterns. Other factors include its high concordance rate in twins (60-100%), and its high prevalence in certain ethnic populations after controlling for lifestyle factors. The role of environmental factors is established in populations that are genetically similar, but have different living conditions (Kraine and Tisch, 1999).

Predisposition to develop T2DM following a change in lifestyle of an individual can be explained by the "thrifty genotype" hypothesis. According to this theory, factors in early humans that favoured fat deposition during periods of abundant food conferred survival advantage during subsequent periods of hardship and perhaps starvation, leading to the selection of genes for insulin resistance (James, 1962). However, these factors may be unfavourable to the individual post-urbanisation, resulting in the development of diabetes during the normal ageing process or with the onset of obesity (McDermott, 1998; Lee, 2001). Consequently, relative resistance to glucose uptake in skeletal muscle leads to increased adiposity contributing to the pathogenesis of diabetes and other metabolic abnormalities (Dube *et al.*, 2006).

In contrast, the "thrifty phenotype" theory proposes that the excess modem living is the major cause for insulin resistance (Hales and Barker, 2001). These excesses lead to reprogramming of carbohydrate metabolism and subsequent β cell dysfunction and insulin resistant tissues. Disparities in T2DM and its complications between ethnic groups suggest that T2DM may represent a selection of particular genotypes in response to different evolutionary pressures (Speakman, 2008).

1.7 Cellular basis for type 2 diabetes

1.7.1 Introduction

Insulin resistance and a deficiency in insulin secretion are the major cellular basis of T2DM (Mahler and Adler, 1999). The action of insulin is to lower the glucose levels in the blood and to stimulate the uptake of glucose principally in muscle and liver cells, thus involved in promoting glucose oxidation and glycogenesis (Johnson *et al.,* 2008). Insulin also plays a major role in controlling the production of hepatic glucose and inhibits lipolysis, which is the process of breakdown of fat in adipose tissue (Barbetti, 1996). The initiation of the insulin action takes place by its binding to the extracellular domain of the β subunit in the insulin receptor, leading to auto phosphorylation of several tyrosine residues in the intracellular domain of the β subunit. Activation of tyrosine kinase of the insulin receptor, by insulin, then directs to phosphorylation of tyrosine residues on the insulin receptor substrates 1 and 2 (IRS-I and IRS-2), shc, and various other intracellular proteins which are uncharacterised (Polonsky *et al.*, 1996).

Consequently, the insulin-signalling cascade is triggered when the phosphotyrosines of these proteins bind to SH2 domains on the other signal kinases. IRS proteins preferentially involve in different signal pathways for glucose uptake as well as glycogen synthesis. They also involve in stimulating protein synthesis by transferring both the growth promoting and mitogenic signals of insulin to the nucleus (Polonsky *et al.,* 1996; Ahmed, 2010).

1.7.2 Insulin resistance

The presence of a very high and unusual amount of endogenous or exogenous insulin to obtain a normal biologic response is the state of insulin resistance (IR). In muscle, fat and liver cells, the resistance to endogenous insulin is compensated by the high levels of serum insulin in association with normal or high blood glucose concentrations. The

main cause underlying diabetes, hypertension and coronary heart disease is insulin resistance (Nosadini et al., 1993; Ahmed and Thornalley, 2007; DSouza et al., 2009).

Different environmental factors and the diverse patterns of lifestyle habits are involved in the development of insulin resistance in a person. Glucose intolerance, hyperinsulinemia, dyslipidemia and hypertension are included in the insulin resistance syndrome. Insulin resistance may occur due to many reasons involved in the functioning of insulin which can be either at the stage of the binding of insulin to the receptor or after the level of downstream signalling (Kumar and Clark, 2007). These defects caused by mutations in the insulin receptor gene which may include alterations in the receptor number, structure and function in signalling resulting in insulin resistance (Nigro *et al.*, 2006). The high levels of circulating free fatty acids increase the release of glucose from the liver reducing the disposal of glucose in the skeletal muscle leading to insulin resistance. Substantial information have established that the increased levels of inflammatory markers in the insulin resistance state helps in predicting the progress of T2DM and cardiovascular diseases (Festa *et al.*, 2003).

Adipose tissue plays a major role in the state of insulin resistance by producing the complement factor B, adipsin, acylation-stimulating protein. In addition, the adipose tissue also help in triglyceride synthesis and they are also involved in the increase of paracrine signalling (DeFronzo, 2006).

In T2DM, the inability of insulin to maintain normal glycaemic levels is caused by a combination of both insulin resistance and a fault in glucose-stimulated secretion. Insulin resistance has been a strong predictor of T2DM, which is identified as hyperinsulinemia 10 to 20 years preceding the diagnosis of the disease.

The major causes of insulin resistance in T2DM are defects in glucose oxidation and glycogen synthesis in skeletal muscle. It is observed that when adipocytes are taken from T2DM, they are found to have impaired insulin-stimulated glucose uptake and moreover, lipolysis is increased. By reducing glucose oxidation through the glucose fatty acid cycle in the muscle, it is possible that the elevated levels of circulating free fatty acids will induce insulin resistance. Greater levels of free fatty acids can also induce insulin resistance by reducing hepatic clearance of insulin and by enhancing gluconeogenesis (Tang *et al.*, 2001).

It has been previously suggested that long chain fatty acids are actively involved in directly affecting the glycogen synthase activity and can also modulate the transcription of pancreatic β cell transcription factor HNF-4α. Increased expression of cytokines may also result in insulin resistance. When tumour necrosis factor (TNFα) is over expressed in obese people, it inhibits the phophorylation of insulin receptor and insulin receptor substrate (IRS-1), which in turn blocks the insulin-signalling cascade in the adipose tissue (Federici, 2004). In T2DM, the isoform of low affinity insulin receptor and hybrid receptors when over expressed are also proposed to contribute to insulin resistance (Kroder *et al.*, 1996).

Hybrid receptor is formed when fusion occurs in between the insulin and insulin growth factor (IGF) receptors (Sesti *et al.*, 2005). The insulin receptor is found to be negatively correlated with insulin sensitivity *in vivo* as it has greater affinity for insulin like growth factor (ILGF) than insulin (Yamauchi *et al.*, 1996). Recent studies have also suggested that insulin resistance is caused by poor foetal and postnatal nutrition, leading to β cell dysfunction and insulin resistant tissues and thus can be detected by low birth weight of the child. According to "thrifty phenotype" hypothesis, these individuals have a greater chance of developing diabetes with the onset of diabetes during the later stages of life

(Hales & Barker, 2001). In contrast, the "thrifty genotype" theory also proposed that smaller birth weight would enable the foetus to store energy and fat more efficiently in an unfavourable intrauterine environment and this can be genetically determined (Dunger *et al.,* 2007).

1.7.3 Insulin secretion

In response to increasing plasma glucose concentrations, pancreatic β cells are involved in the secretion of insulin. In subjects with T2DM, several abnormalities in β cell insulin secretion have been detected. Insulin response to intravenous glucose is decreased in patients with T2DM, with loss of the first phase insulin secretion response, defective pulsatile pattems, and hyper-proinsulinaemia (Gumbiner *et al.,* 1996).

T2DM is characterised by a 20-50% reduction in β cell mass and this could be an impulsive factor in defective insulin secretion. Progressive loss of β cell function is associated with insulin resistance in skeletal muscle, and in the formation of islet myeloid deposits in the pancreas. In turn, this can induce pancreatic β cell death *in vitro* (Westermark & Wilander, 1978). In early or first-phase insulin secretion, the pattern of loss is the initial defect followed by a reduction in glucose to potentiate non-glucose signals, and finally β cell failure that requires insulin treatment (Lorenzo *et al.,* 1994). In mice, the development of islet amyloid has been attributed to a high fat diet and it is associated with hyperglycaemia. However, a 50% reduction in β cell mass alone is not enough to explain the fasting hyperglycaemia observed in these animals, suggesting that the amyloidogenic process may impair the function of islet cells prior to cell death and reduction in islet mass (Verchere *et al.,* 1996).

Mitochondrial DNA (mtDNA) may also affect insulin action. Mitochondria are the major site for Krebs cycle function, ATP synthesis and transfer of fatty acids (Ahmed, 2010). Elevated ATP in the cytoplasm is necessary for the membrane-dependent

increase in cytosolic Ca^{2+}, which in turn triggers insulin exocytosis by the β cell. Patients with T2DM are found to have approximately 35% less mtDNA in their peripheral blood leukocytes (Lee *et al.,* 1998). This reduction has been observed to precede the onset of diabetes. Low copy numbers of mtDNA are inversely correlated to fasting plasma glucose levels in patients with T2DM, and are associated with insulin resistance and defective insulin secretion in the offspring of diabetic mice. Mothers with low mtDNA in their peripheral blood leukocytes, tend to have offspring's with low birth weight indicating that mtDNA content may be an significant characteristic of the heritable "thrifty phenotype" (Lee, 2001).

The relationship between low levels of mtDNA and diabetes is unknown. *In vitro*, β cell depletion of mitochondrial DNA results in altered mitochondrial morphology and inhibition of glucose-stimulated ATP production that is required for downstream insulin release by the islet β cell (Lee *et al.,* 2005). Glucose toxicity may be another mechanism for defective insulin secretion. When β cells are exposed to high glucose levels for prolonged period, impaired insulin gene transcription can result in decreased insulin secretion. Chronic exposure to high levels of glucose can also cause defective K^+ channel function and a reduced expression of GLUT-2, which is responsible for glucose storage and transportation to the cell membrane (Maechler & Wollheim, 2000).

Severe hyperglycaemia is correlated with increased insulin deficiency (DSouza *et al*., 2009). Once hyperglycaemia has developed, glucose toxicity can induce insulin resistance and decreased pancreatic β cell function. It has been predicted that a 50% decrease in β cell function (i.e. decreased β cell function) in the presence of insulin resistance may induce hyperglycaemia (Chen *et al.,* 1998).

In addition, glucose responsive tissue resistance to insulin will increase as β cell function decreases (Gerich, 2003). Hyperglycaemia can be considerably improved by

insulin therapy or by sulfonylureas and treatment with diet, resulting in reduction of insulin resistance and glucose toxicity (Cook *et al.,* 1993). Insulin secretion and resistance are hence interrelated at several levels; however, it is unclear which defect is primary in the aetiology of diabetes (Kahn and Porte, 1988). It has been envisaged that a 50% decrease in β cell function in the presence of insulin resistance results in significant levels of hyperglycaemia (Halter *et al.,* 1985). Once hyperglycaemia develops, glucose itself leads directly to a loss of glucose induced-insulin release and impairment in glucose disposal (glucose toxicity), resulting in exacerbation of the disease state (Meece, 2007).

1.8 Genetics of type 2 diabetes mellitus

Both the types of diabetes mellitus have a characteristic feature to run in a family, which indicates a genetic predisposition to the disease. Different genes are likely to be involved in each type of DM as they have major difference in their underlying aetiology. The wide spread distribution and the ever growing prevalence of T2DM in the modern society has urged the research community to investigate the underlying genetic defects of the disease. The statement that T2DM is principally caused by genetic factors is known for many years. Until recently, the identification of genetic variants, contributing to the difference among individual susceptibility to T2DM is characterised by very slow progress in spite of strenuous efforts (Kahn *et al.,* 1996).

With the advances in the techniques involved in gene expression, like genome-wide association analysis, there has been a remarkable transformation in the ability to mark the common genetic variants, which play a major role in contribution to the predisposition of complex metabolic disorders such as T2DM during the last few years. Around 20 common variants have been implicated in the susceptibility of T2DM through related studies (Shu *et al.,* 2010).

The two main candidate gene associations claimed for T2DM are Pro12Al variant in the peroxisome proliferator-activated receptor gamma (PPARG) gene, which encodes the target for thiazolidinedione class of drugs and the Glu23Lys variant in KCNJ11, which encodes the target for sulphonylureas (Gouda *et al.,* 2010). These are the two most common polymorphisms involved in influencing the risk of T2DM. Some rare mutations in both of these genes are also involved in other syndromes, which have severe metabolic disturbances as their characteristic feature (Gouda *et al.,* 2010).

It has been reviewed earlier that the variants in the PPARG, KCNJ11 and TCF7L2 genes are confirmed as carriers of established T2DM susceptibility loci and studies have led to further identification and replication of novel T2DM-susceptibility loci (Chauhan *et al.,* 2010). The discovery that the variants in the Transcription factor 7-like 2 gene (TCF7L2) have a great effect on the susceptibility to T2DM has paved the way to identification of genes related to T2DM including gene association studies. After several replication studies, it has been finally proven that, the variants in TCF7L2 have a powerful role than those of PPARG and KCNJ11. It has been reported that almost 10% of Europeans carrying this homozygous allele have a greater risk of developing T2DM than those without it (Zeggini and McCarthy, 2007). Alterations in either TCF7L2 gene function or expression causes disturbance in the function of pancreatic islet cells thereby dysregulating the gene expression of proglucagon, which decreases the secretion of insulin and results in T2DM. The effort towards discovering the genes responsible for a disease is driven by expectations to generate key insights into its various mechanisms giving a chance for clinical translation (Zeggini and McCarthy, 2007; Chauhan *et al.,* 2010). Further knowledge of the various biochemical pathways involved in T2DM is required for future research to understand the genetic variation in populations.

1.8.1 Candidate genes for type 2 diabetes

The aetiology and pathogenesis of insulin resistance involve multiple signalling proceedings and pathways, and therefore a number of target genes. See (Table 1.1). Numerous researchers have sought to identify a genetic determinant that may be sufficient to considerably impair one or more of the major transduction pathways. Linkage of diabetes to different genetic loci in diverse populations has been well demonstrated by whole genome studies (Kahn, 1988; Mahtani *et al.*, 1996).

On the other hand, mutations in individual genes resulting in defective insulin secretion and signalling leading to T2DM have been unidentified by the candidate gene approach, which tests the association of a particular gene to diabetes. However, a single gene mutation is yet to be identified, with exception of monogenic form of diabetes (Hart *et al.*, 1999).

Table 1.1: Candidate genes for insulin resistance and insulin secretion defects.

Insulin resistance genes	
Insulin receptor	
Insulin receptor substrate- I	IRS-1
Glucose transporter-4	GLUT-4
Glycogen synthase	GYS
Amylin	IAPP
β -3-adrenergic receptor	β-3AR
Fatty acid binding protein 2	FABP2
Obesity	OB
Prohormone convertase-2	PC-2
Tumour necrosis factor- α	TNF- α
Ras associated with diabetes	Rad
Insulin secretion defects	
Glucose transporter-2	GLUT-2
Maturity onset diabetes of the young	MODY1, 2,3,4,5
NeuroD1/ β 2	β 2
Mitochondrial DNA	mtDNA

1.9 Cardiovascular diseases (CVD)

The major cause of mortality and morbidity worldwide among diabetics is due to CVD which are expected to reach indefinite proportions very soon (Murray and Lopez, 1997). The burden of CVD is a major challenge for community health worldwide and this has been especially noticeable in developing countries. The WHO placed CHD in the sixth place for morbidity and mortality and it is believed that it which would reach first place in future (Hoang *et al.*, 2003). CHD is caused because of accumulation of atheromatous plaques inside the arterial walls which supply the myocardium (Mensah, 2007). It has been suggested that healthy men and women have a chance of developing CVD at age 40 and above (Rosamond *et al.*, 2007).

According to WHO, CVD had affected around 15 million people in 1990, which increased to 17 million in 1999 and this figure is expected to reach 25 million in 2020 (Murray and Lopez, 1997). The fact that CVD is associated with the lifestyle habits of an individual including high fatty diet, smoking, lack of exercise has been well known for years. Increased urbanisation in developing world has brought about a major difference in the various aspects of life, ultimately making it sedentary. The prevention of CVD is an important social and economic issue as it costs the UK Government, £26 billion per year (BHF, 2007). It is of paramount importance to identify individuals at higher risk of a cardiovascular event and to manage their lifestyle. Understanding the molecular mechanisms in the progression of CVD and developing novel approaches for prevention and treatment of CVD is urgently required at this moment in time.

1.9.1 Development of atherosclerosis

The presence of atherosclerotic lesions within the vasculature is virtually a pre-requisite for acute ischaemic events. Complex lesions within the coronary arteries can cause luminal occlusion leading to stable and unstable angina pectoris (Dsouza *et al.*, 2009).

Either erosion or rupture of vulnerable lesions can cause to atherothrombotic complications leading to myocardial infarction (MI), stroke, renal failure, heart failure, sudden death and peripheral vascular disease (PVD) (Cohn, 2004). Indeed, atherosclerosis and atherothrombosis are generalised processes, although vascular pathology is usually discussed and managed on an organ-by-organ basis. Patients may only clinically present with arterial pathology in a specific area, but the disease is almost invariably ubiquitous, and it is the root cause of the associated morbidity and mortality. For example, in a study of 1500 patients with leg artery atherosclerosis (intermittent claudication), it was found that in a 4-10 year follow-up, 60% of deaths were cardiac, 17% were cerebrovascular, and 8% were due to other vascular causes and complications such as ruptured aortic aneurysm (Kahn, 1988; Laing S. *et al.*, 1991). In addition, a recent study has established that the ankle brachial blood pressure index (ABI) (a marker of peripheral atherosclerosis and subclinical stenosis) is associated with the site and number of arterial beds affected by atherosclerosis in vascular patients and to a lesser extent with the patient risk factor profile. These studies show that the atherosclerosis underlying the claudication pathology and this is also by far the main cause of mortality in this patient group.

It is therefore advantageous to fully comprehend the complex cellular and molecular interplay involved in the development of atherosclerosis, considering the ubiquitous nature of atherosclerosis in the Westernised population, and its diffused nature in the vasculature. The rationale is that individuals who are at risk of progressing to an event may be identified at an early stage. Equally, it is also possible to identify pathways of therapeutic intervention for these individuals.

Until the mid-1970s, atherosclerosis was considered a bland lipid storage disease where lesion swelling and stenosis were thought to be the primary cause of luminal occlusion

leading to ischemic heart disease and stroke (Ross & Harker, 1976). Indeed, much historical and current evidence supports the role of plasma lipids (cholesterol and triglycerides) and lipoproteins (low-density lipoprotein [LDL] and very low density lipoprotein [VLDL]) in atherogenesis (Kannel, 1979; Martin *et al.*, 1986). To suggest that lipid storage alone is the cause of atherogenesis is, however, an over-simplification. With the extent of atherosclerosis being proportional to sustained blood lipid concentration in most cases, it would be a reasonable assumption that the main driving force would be hyperlipidaemia, if atherosclerosis was purely a lipid storage disease (Lloyd-Jones *et al.,* 2001). Though hyperlipidaemia is a major risk factor for future cardiovascular death, almost half of all coronary events occur in individuals without clinical hyperlipidaemia (Braunwald, 1997) and other risk factors such as smoking and blood pressure are of similar importance. Furthermore, hyperlipidaemia is only weakly related to ischemic stroke (Shahar *et al.,* 2003).

Adipocytes play a very important role in the process of development of atherosclerosis by producing inflammatory factors like tumour necrosis factor, interleukine-6, interleukine-8, interleukine-10, monocyte chemoattractant protein-1, plasminogen activator inhibitor-1 and also adipokines such as adiponectin, leptin, angiotensinogen and resistin all of which accelerate the progression of atherosclerotic events (Hattori *et al.,* 2006). It has been shown very recently that leptin enhances cellular immune responses and it also takes part in the increase of blood pressure. It has been observed that leptin tends to decrease the sensitivity to insulin when injected in obese rats. In the presence of high levels of glucose, leptin is also involved in the accumulation of cholesterol by the macrophage (Piemonti *et al.,* 2003).

The development of atherosclerosis *in vivo* is currently thought to be a more complex process, requiring persistent lipid presence, endothelial dysfunction and inflammation

(Libby *et al.*, 2002; Szmitko *et al.*, 2003; Willerson and Ridker, 2004; Hansson, 2005) (See Figure 1.2). This model in Figure 1.2 gives inflammatory cells and cytokines a pivotal role in the development of the lesion.

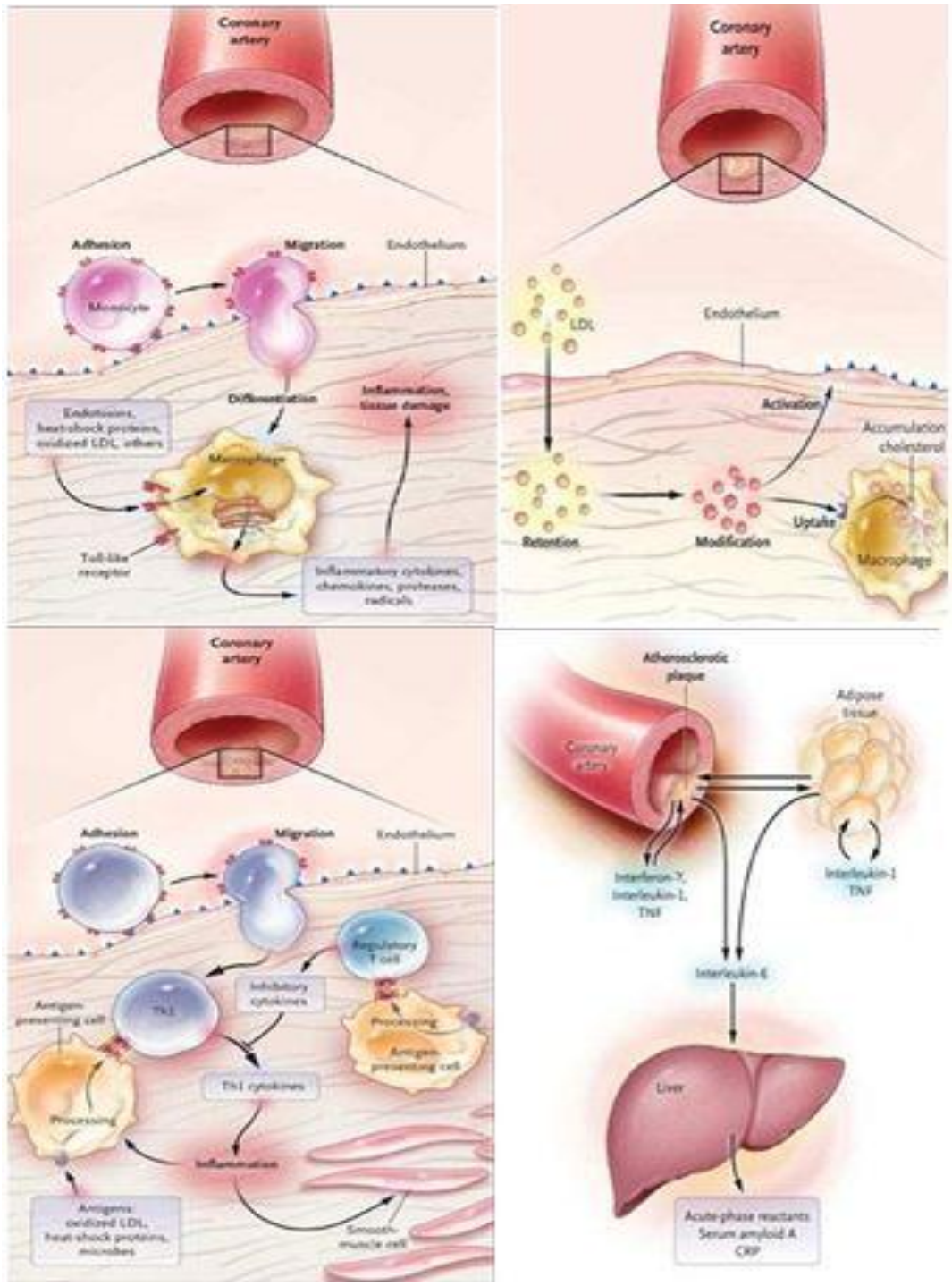

Figure 1.2: Schematic diagram showing the development of atherosclerosis

Low density-lipoprotein (LDL) infiltrates the arterial wall and is modified by oxidation processes. It is then phagocytosed by macrophages which become active inflammatory lipid-laden foam cells characteristic of early atherosclerotic fatty streaks. Activated local endothelium starts to produce P- and E- selectins and integrins such as vascular cell adhesion molecule-1 (VCAM-1). Monocytes and activated lymphocytes from the periphery roll adhere and extravasate into the tissue. Macrophages activated via toll like receptors (TLRs) produce inflammatory cytokines, chemokines, proteases, and free radicals which perpetuate inflammation and damage tissue. A Th1 type adaptive response becomes established and interferon- (IFN-) γ is produced. This stimulates further activation of macrophages and proliferation of smooth muscle cells (SMCs). A stable fatty lesion develops with the collagenous cap manufactured by the SMCs. Production of IL-6 and other cytokines (interleukin-1, TNFα) leads to stimulation of the hepatic acute phase response (APR) releasing acute phase reactants such as fibrinogen, serum amyloid A, and C-reactive protein (CRP) (Taken from Hansson, 2005).

1.9.2. Role of inflammation in the pathogenesis of atherosclerosis

Accumulating evidence from both basic and applied laboratories indicates that inflammation plays a critical role in several stages of atherogenesis, including acute plaque rupture as well as early initiation of foam cell deposits (Libby *et al.*, 2002). The plaque is typically an advanced atherosclerotic lesion covered by a fibrous cap consisting of smooth muscle cells and connective tissue containing macrophages and T lymphocytes. Under the fibrous cap, the lipid-rich core is filled with macrophages, lipids, calcium and other materials. Unstable plaques often have activated macrophages and leukocytes on their "shoulders". Certain characteristics may predispose a plaque to rupture. These characteristics include a softer lipid core, the presence of macrophages,

fewer smooth muscle cells, a thinner fibrous cap and increased inflammatory activity (Adams III and Vickie, 2001). Cytokines, which cause the *de novo* hepatic production of acute phase reactants, such as C-reactive protein, have been shown to increase in acute coronary syndromes (Liuzzo *et al.,* 1994).

1.9.3 Endothelial dysfunction

Endothelial dysfunction is commonly described as a diminished ability of the endothelium to produce the vasorelaxing compound nitric oxide (NO), or a profound imbalance in the relative amounts of other vasoactive compounds such as angiotensin II and oxidants (Verma and Anderson, 2002). Using this definition, endothelial dysfunction is confined to unfolding inappropriate vasomotor constriction of the vasculature, consequently aggravating luminal occlusion caused by conventional atherosclerotic lesions.

In atherosclerotic development, however, diminished NO production by the endothelium is an intricate player in the development of patho-physiological inflammatory and molecular processes in the vasculature (Szmitko *et al.,* 2003).

In addition to vasodilatory effects, NO also generates an anti-thrombotic environment by limiting platelet aggregation at the endothelium (Freedman *et al.,* 1997), In turn, this prevents leukocyte adhesion to the endothelium by suppressing expression of adhesion molecules (Gauthier *et al.,* 1995) and it maintains vascular smooth muscle in a non-proliferative state (Cornwell *et al.,* 1994). This is a delicate balance, and local changes in vascular environments (such as LDL cholesterol, free radicals, infectious microorganisms, low shear stress, and angiotensin II-induced hypertension) can cause endothelial activation, in part by reducing the intracellular concentration of NO (Sloop *et al.,* 1999).

1.9.3.1 Initiation of the atherosclerotic lesion; where inflammation meets endothelial dysfunction

Systemic risk factors such as elevated LDL, hypertension, high blood sugar, and by-products of tobacco smoking help in the process of initiation and exacerbation of atherosclerosis. Atherosclerotic lesions develop preferentially at bifurcations, branch points, and inner curvatures of arteries, although the entire vascular endothelium is exposed to these stimuli, which suggests that local factors contribute to the susceptibility of the disease. It is widely established that the complex patterns of blood flow in these regions expose the endothelium to "disturbed" hemodynamic forces low shear stress (Lowe, 2004), which may in turn cause physical disruption of the endothelium and concomitant inflammation (Gimbrone *et al.,* 1997).

1.10 Diabetes-induced changes in myocardial fuel selection

Remarkable changes occur in the concentration of various energy substrates within the circulation in T2DM, which in turn can control cardiac metabolism. The rate of substrate uptake and oxidation in cardiomyocytes is significantly affected due to an increase in the levels of both plasma glucose and fatty acids in diabetes. Fatty acid-induced inhibition of sarcolemmal GLUT4 expression impairs the uptake of glucose in the diabetic heart. Increased supply of FA can also result in the activation of cardiac PPARα, which promotes the expression of genes involved in uptake of FA and oxidation. In diabetes mellitus, an abundance of fatty acid causes an increase in the uptake of fatty acid in the myocardium increasing the oxidative capacity of the heart. This in turn leads to an accumulation of triglycerols, fatty acid intermediates, and ceramides resulting in a state of lipotoxicity within the cardiomyocytes (DSouza *et al.,* 2009; Lopaschuk *et al.,* 2010).

1.11 Metabolic syndrome

WHO defined metabolic syndrome as a state, which includes central obesity, dyslipidemia, impaired glucose tolerance, high blood pressure and T2DM. An individual with a BMI of >30 kg/m^2 (obese) carrying a minimum of two other risk factors like dyslipidemia and hypertension is required to meet the criteria for diagnosis of metabolic syndrome.

1.12 Obesity- a major health concern

One of the major health concerns in the 21^{st} Century is obesity. This is when a person has a BMI in excess of 25-30 and moreover, it can be defined as excess body weight with an abnormally high proportion of body fat. An imbalance between energy intake and energy expenditure can lead to obesity.

Obesity occurs due to interplay of sedentary lifestyle habits, genetic factors, medical illness, microbiological aspects and social factors. Figure 1.3 depicts the relationship between obesity, diabetes and the development of heart diseases.

1.12.1 Measurement of obesity

Obesity is commonly defined as body mass index (BMI, weight in kilograms divided by height in meters squared) of 30 kg/m^2 or higher. BMI (or Quetelet index) was introduced by the Belgian polymath Adolphe Quetelet between 1830 and 1850. The classification of body weight according to WHO is shown in table 1.2.

Table 1.2: Classification of overweight in adults according to BMI[a]

Classification	BMI (kg/m^2)	Risk of comorbidities
Underweight	<18.5	Low
Normal range	18.5--24.9	Average
Overweight	≥25.0	
Pre-obese	25.0-29.9	Increased
Obese class I	30.0--34.9	Moderate
Obese class II	35.0--39.9	Severe
Obese class III	≥40.0	Very severe

[a]These BMI values are age-independent and the same for both sexes. However, BMI may not correspond to the same degree of fatness in different populations due, in part, to differences in body proportions. The table shows a simplistic relationship between BMI and the risk of comorbidity, which can be affected by a range of factors, including the nature and the risk of comorbidity. In turn this can be affected by a range of factors, including the nature of the diet, ethnic group and activity level. The risks associated with increasing BMI are continuous and graded and begin at a BMI below 25. The interpretation of BMI gradings in relation to risk may differ for different populations. Both BMI and a measure of fat distribution (waist circumference or waist: hip ratio (WHR)) are important in calculating the risk of obesity comorbidities.

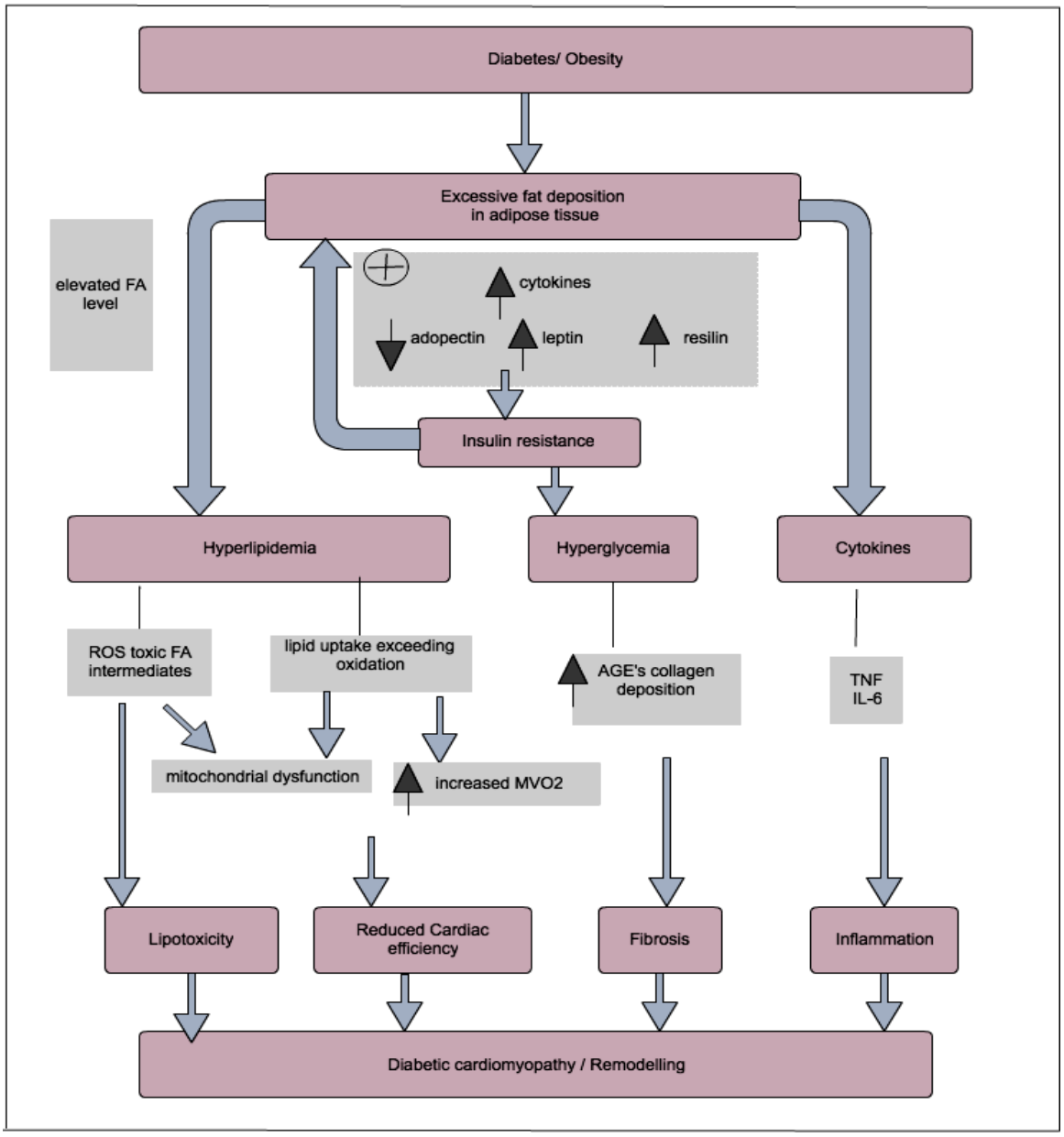

Figure 1.3: Flow chart showing potential mechanisms for the development of diabetic cardiomyopathy: ROS, reactive oxygen species; TNFα, tumor necrosis factor alpha; IL-6, interleukin-6; AGE, advanced glycation end products.

1.13 Diabetes mellitus and cardiovascular disease

As mentioned earlier that DM is classified as type 1 and type 2 DM. Lifestyle, dietary habits, obesity, fat distribution, and age are the main pre-disposition factors for DM. T1DM is mostly caused by genetic predisposition with a combination of autoimmune disorders and viral infections. The prevalence of type 2 diabetes is growing globally and is established to be related to morbidity and mortality due to cardiovascular events. T2DM patients are more prone to acute myocardial infarction and angina and their mortality in terms of cardiac events is found to be higher when compared to non-diabetics. Ischaemic heart disease and diabetic cardiomyopathy are the two important patho-physiological processes leading to diabetes-related cardiovascular disease. Metabolic disturbances like hyperglycemia, hyperlipidemia, insulin resistance, myocardial fibrosis, oxidative stress, and pro-inflammatory cytokines are the various contributing factors to this complex process (DSouza *et al.,* 2009).

1.13.1 Epidemiology of CVD

1.13.1.1 The concept of "risk factors"

Epidemiological studies trying to determine the various factors responsible for chronic diseases associated with infection and nutritional deficiency in the past have put forward the concept of "risk factors". Research focussing on this type of disorders has been subsequently tailored with the improval of public health sector and when chronic diseases related to aging such as cancer and cardiovascular disease have become very common (Kannel *et al.*,, 1961).

Therefore, the diverse factors linked to a disease have been statistically evaluated using epidemiological methods. The concept of 'risk factors' in cardiovascular disease was initially popularised by the Framingham Heart Study (Kannel *et al.,* 1961) which originated from the latin verb "facere" meaning "to make". According to the definition,

a risk factor has to be the fundamental behind the progression of a disease and the term should be employed where it has been associated with increasing the risk of a disease but yet to be proved as casual even though they are often interchangeably used. In some cases like infection (e.g. HIV virus infection giving rise to the acquired immunodeficiency syndrome, AIDS.), which have particular pathogens that lead to clinical symptoms and disease states, the cause is very clear whereas in cardiovascular disease, however, the root cause is very unclear (Black, 1992).

Development of atherosclerosis involves the interplay of complex molecular and cellular pathways and several factors that contribute to the disease before clinical presentation of an end event. The major concern is the determination of these factors in terms of major influence over the progression of the disease for use as a clinical marker in the prediction of the disease or for development of therapeutic interventions.

Table 1.3: Some major conventional risk factors for CVD

Classification of factor	**Examples of risk**
Non-modifiable	Sex (♂>♀), Age, Diabetes, Family history, Ethnic background, Gestational diabetes.
Modifiable	Smoking, Blood pressure, sedentary lifestyle, adiposity (BMI or waist circumference), cholesterol (total or total: HDL), salt intake, hypertension, lack of exercise.

1.13.1.2 Current cardiovascular risk factors

All conventional risk factors that are recognized by most lay people and healthcare professionals today came from epidemiological work including Framingham Study. Some general risk factors include sex, age, family history, lifestyle, smoking, obesity, hypertension and hyperlipidemia, which interrelate in several ways both directly and indirectly. Age, sex and family history are obviously non-modifiable, so the five main risk factors usually considered clinically are smoking, diabetes, hyperlipidaemia, adiposity, and blood pressure (Kannel *et al*, 1961, Hu *et al*., 2001).

These major risk factors are currently combined in scores or charts, which predict risk of CVD, e.g. the Joint British Societies, chart (See Figure 1.4). This current chart utilises the total HDL: cholesterol ratio as a measure of atherogenic blood lipids and hence takes into account the fact that not all cholesterol is equal in terms of CHD risk. VLDL and LDL cholesterol play direct roles in atherogenesis, but HDL cholesterol, in contrast, plays a role in reverse lipid transport (and other anti-atherogenic processes) and hence reduces risk of coronary heart disease (Barter *et al.*, 2004). So although in general hyperlipidaemia is a risk factor for CHD, this can be tempered if a high part of the total circulating cholesterol is HDL (Haffner and Ashraf, 2000).

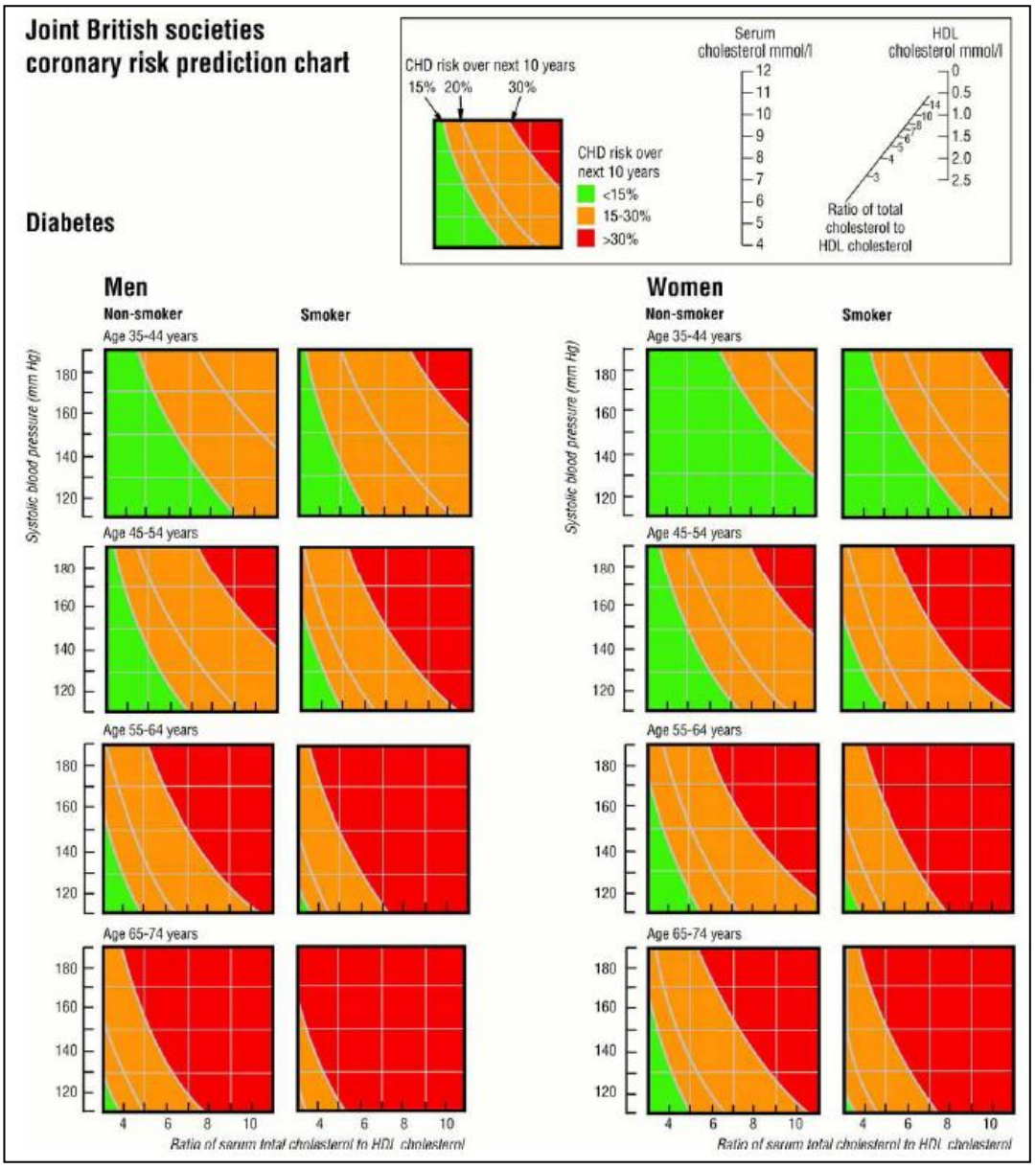

Figure 1.4: Charts showing Clinical Joint British Societies risk assessment for CHD in diabetic patients. (Adapted from: BMJ, 2000).

1.13.1.3 Emerging CVD risk markers

Recently, considerable interest has been shown in novel metabolic and dietary markers. The metabolic syndrome (The WHO criteria, 1999; defined as presence of diabetes mellitus, impaired glucose tolerance, impaired fasting glucose or insulin resistance, plus any two of hypertension, dyslipidaemia, obesity, and microalbuminuria – although there

are several proposed clinical definitions) has attracted considerable attention as a predictor of T2DM as well as cardiovascular disease.

Recent meta-analysis shows T2DM as a predictor of CVD (Galassi *et al.,* 2006), although the clinical utility in diagnosing the syndrome as a CHD risk marker over and above the Framingham risk assessment is extremely uncertain despite enthusiasm for its use particularly in the USA (Wannamethee *et al.,* 2005). In other examples, of individual dietary hormones, lipids and triglycerides have recently been confirmed to be moderately associated with the incidence of CHD during meta-analysis (Sarwar *et al.,* 2007). This is believed to have lipoprotein (a) (Danesh *et al.,* 2000) and apolipoprotein B:A ratios (Thompson and Danesh, 2006). In contrast, the hormone adiponectin is often reported to be inversely associated with CHD, but any association was recently shown to be weak on meta-analysis (Sattar *et al.,* 2006). Some haemostatic markers have also been shown to be associated with the incidence of CVD, perhaps due to their roles in thrombus formation, longevity and degradation (Lowe, 2004).

In addition to these, measurements of subclinical plaque progression and stenosis have also been shown to have associations with incident CVD. The simplest measure of peripheral arterial resistance is the ankle brachial index (ABI), which shows a low sensitivity, but high specificity association with CVD and CHD risk. More recent computer imaging shows both non-invasive, and invasive procedures can predict CHD risk (Ottolini *et al.,* 2005). Typically, all of these markers have risk associations with CVD and CHD that can be described as moderate, but are clearly not as useful as the Framingham-based tests in clinical practice.

1.14 Inflammatory risk markers

As mentioned above, the role of inflammation in atherogenesis would suggest, a great deal of work has been done looking for inflammatory markers that are risk factors for

CHD (Libby *et al.,* 2002). The most widely examined markers are fibrinogen, C-reactive protein (CRP), white cell count (WCC) and IL-6. All of these markers are associated with acute phase response and hence circulating levels reflecting the current inflammatory state of the individual with WCC and CRP being the routine clinical measures of inflammation.

These have been used in epidemiological studies to examine the hypothesis "elevated baseline inflammation predicts risk of CVD." Meta-analysis of prospective studies in generally healthy populations has shown that WCC (Wheeler *et al.,* 2004), CRP (Danesh *et al.,* 2004), and fibrinogen (FSC, 2005) are risk predictors for CHD. CRP has been promoted as a risk marker in cardiovascular disease, and much publication space has been dedicated to this, particularly from the USA (Verma *et al.,* 2005). Publication bias is an important confounding factor in the epidemiological study of newer risk markers.

In the USA, the measurement of CRP level has been suggested to add to established risk factors for individual risk prediction for cardiovascular prevention (Pearson *et al.,* 2003), but not in Europe. Several other markers were considered potential candidates as inflammatory risk markers in this debate (including WCC, fibrinogen and IL-6), but it was decided that CRP best fitted the above criteria, and was generally the most comprehensively studied of the markers (Pearson *et al.,* 2003).

1.14.1 Inflammation and T2DM

T2DM is a progressive disorder emerging as a global epidemic and imposes a tremendous burden on the health economy of many countries due to its fatal micro and macrovasular complications (Zimmet *et al*., 2003). T2DM patients have an increased risk for CVD due to many conventional risk factors which cannot fully account for the disease. There is increasing evidence that atherosclerosis is accompanied by

inflammation in several research studies. Full blood cell count including RBC, WBC cell counts, C- reactive protein (CRP), HbA1c, lipid profile including HDL, LDL, triglycerides are all positively associated with increasing CVD risk in T2DM (Kumar and Clark, 2007).

1.14.2 Inflammation

Inflammation is the local protective response to tissue injury. The word inflammation means "setting on fire" (16^{th} century), and the process has been known since Egyptian times (c. 2500 B.C.). The cardinal signs of redness, swelling, heat, and pain were described by Celsus (first century A.D.), and loss of function was added by Galen (130–200 A.D.) (Rather, 1971). Microscopically, these features are due to vasodilation, accumulation of leukocytes, increased capillary permeability and interstitial fluid, and stimulation of nerve endings by mediators such as substance P.

Cells such as monocytes, macrophages, T-cells, endothelial cells, vascular smooth muscle cells, release inflammatory mediators including cytokines and chemokines during the process of plaque development, which develop an inflammatory response in the vessel (Ross, 1999). Potential epidemiological studies have found increased cardiovascular risk with elevated basal levels of some of these same mediators particularly, cytokines such as interleukin (IL)-6 or tumor necrosis factor-α (TNF-α), soluble adhesion molecules, and downstream acute phase reactants such as C- reactive protein (CRP), fibrinogen, and serum amyloid A (SAA) which have shown to predict the risk of cardiovascular events (Libby *et al.*, 2002).

It has been proposed that inflammatory cytokines secreted by the adipose tissue exert an endocrine effect which is responsible for insulin resistance in liver, skeletal muscle and vascular endothelial tissue and ultimately results in clinical expression of both T2DM and CVD (Yudkin *et al.*, 1999; Hu and Stampfer, 2003). An elevated production of

adipocyte cytokines particularly, tumor necrosis factor (TNF-α) and interleukin-6 (IL-6) produces an acute phase response that leads to an increased production of C-reactive protein (CRP), a sensitive marker of low-grade systemic inflammation (Hotamisligil & Spiegelman, 1994; Caballero *et al.*, 1999). Apart from promoting insulin resistance, TNF-α, IL-6 and CRP also stimulate endothelial production of adhesion molecules including E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) which are crucial mediators of endothelial dysfunction in capillary and arteriolar endothelium (Gabay and Kushner, 1999; Targher *et al*., 2005).

Interleukine 6 is a major proinflammatory cytokine produced in tissues including activated leukocytes, adipocytes and endothelial cells. C- reactive protein is the main downstream mediator of acute phase response and is primarily derived from IL-6-dependent haepatic biosynthesis (Sattar *et al*., 2007). IL-6 has been shown to induce gluconeogenesis, subsequent hyperglycemia, and compensatory insulinemia in rodent models of glucose metabolism. Similar metabolic responses have been observed in humans after subcutaneous administration of recombinant IL-6 (Tsigos *et al.*, 1997). Cross–sectional studies have shown that inflammation plays a major role in the aetiology of T2DM and also demonstrated that elevated levels of IL-6 and CRP have been observed in individuals with T2DM (Sattar *et al*., 2004).

Markers of inflammation are associated with T2DM and features of the metabolic syndrome in cross-sectional studies (Thorand *et al*., 2007). Several cross-sectional studies in non-T2DM subjects, the general population or in individuals with impaired glucose tolerance (IGT)/impaired fasting glucose (IFG) have confirmed that acute-phase reactants such as CRP (and sometimes the cytokines IL-6 and TNF-α) are positively correlated with measures of insulin resistance/plasma insulin concentration, BMI/waist

circumference, and circulating triglycerides and negatively correlated with HDL cholesterol concentration.

In general, increasing components of the metabolic syndrome in individuals are associated with higher levels of inflammatory markers. In subjects with IGT or IFG, IL-6 but not TNF-α appears to be elevated compared with individuals with normal glucose tolerance and in one study, inflammatory markers were related to insulin resistance but not to insulin secretion.

Additional cross-sectional studies in newly diagnosed or established T2DM patients have confirmed that acute-phase markers such as CRP and IL-6 are elevated in these subjects compared with non-diabetic control subjects. In the study by Leinonen *et al*., 2003, all markers of inflammation, including CRP, serum amyloid A, secretory phospholipase A2, and IL-6, and endothelial dysfunction (soluble cell adhesion molecules) correlated with the homeostasis model–measured insulin resistance.

1.15 Cations in T2DM

Several studies were published during the last century evaluating the various cations and trace elements in T2DM. However, significant changes were observed within similar reports and these differences were shown to be a result of various factors including age, genetic factors, dietary habits, medication and glycaemic control of patients. Alterations in the levels of various cations could also result due to chronic hyperglycaemia and possibly modulation of glucose homeostasis by micronutrients themselves. Blood plasma is considered to be a tissue pool for various cations and single trace elements. However, recently, it became evident that micronutrient level of plasma is a reflection of cations and trace elements only in cases of severe deficiency or in excess. Several underlying factors for excess and deficiency of these various cations

in plasma include infection, regulation of hormones, stress, circadian rhythm, impaired homeostatic regulation.

1.15.1 Sodium (Na^+)

Hyperglycaemia in patients with T2DM is a common clinical problem in which variable changes in plasma sodium (Na^+) concentration can occur. Acute studies suggest that hyperinsulinemia may cause sodium retention and increased sympathetic activity, which will be an important cause of hypertension (Reaven & Hoffman, 1987). Much evidence from epidemiological, migration, intervention, animal and genetic studies suggest that salt intake plays an vital role in regulation of blood pressure (He and MacGregor, 2003). Several other studies have described significant effects of dietary salt intake on blood pressure in normotensive and hypertensive patients (Morgan *et al.*, 1978).

Recently, it has been predicted that through its effects on blood pressure, the cardiovascular benefits of reduced salt intake are comparable with the benefits of population-wide reductions in tobacco use, obesity and cholesterol levels (Bibbins-Domingo *et al.*, 2010). However, dietary salt intake may play a more important role in diabetes than in the community at large, as it has been shown that patients with diabetes have an increased total body exchangeable sodium (Beretta-Piccoli & Weidmann, 1982) and moreover, they have higher blood pressure levels and higher aggregate cardiovascular risk than the general population (Sowers *et al.*, 2001).

1.15.2 Potassium (K^+)

The results from the Atherosclerosis Risk in Communities (ARIC) Study, suggest that serum potassium is an independent predictor of incident T2DM. Essentially, maintenance of normal potassium homeostasis is important limiting factor in the therapy of cardiovascular disease.

A diet deficient in potassium intake has a critical role in regulating blood pressure in primary hypertension (Horacio and Nicolaos, 2007) and may potentially increase the risk of stroke (Green *et al.*, 2002). Several studies have shown that hypokalemia could be a possible risk factor for T2DM. Serum potassium levels affect insulin secretion by pancreatic β-cells. Serum potassium in randomized controlled trials of thiazide diuretics was found to be inversely associated to glucose which might possibly be a result of oral potassium supplementation (Zillich *et al.*, 2006). Experimental evidence also suggests that thiazide-induced hypokalemia can lead to reduced insulin secretion (Rowe *et al.*, 1980, Adrogue and Madias, 2007).

1.15.3 Calcium (Ca^{2+})

Calcium is the most important physiological cation in the body (Berridge *et al.*, 2000). It is the mediator and promoter of several physiological processes. Calcium is an essential component for insulin secretion as it is a calcium-dependent process (Hellman *et al*., 1994). Therefore, fluctuations in calcium levels might intervene and effect β-cell secretory function. Inadequate calcium intake might alter the balance between extracellular and intracellular cell calcium pools which interfere with normal insulin release, particularly when there is glucose overload (Berridge, 1994). In insulin-responsive tissues such as skeletal muscle and adipose tissue calcium are essential for insulin-mediated intracellular processes (Ojuka, 2004; Wright *et al.*, 2004). A very narrow range of $[Ca^{2+}]_i$ is needed for optimal insulin-mediated functions (Draznin *et al.*, 1987). Changes in $[Ca^{2+}]_i$ in primary insulin target tissues possibly contribute to peripheral insulin resistance via impaired insulin signal transduction, resulting in a decreased glucose transporter-activity (Draznin *et al.*, 1989).

1.15.4 Magnesium (Mg^{2+})

Magnesium (Mg^{2+}) is the fourth most abundant cation in the human body and the second most abundant intracellular cation, which is an important cofactor for many enzymes, particularly those involved in phosphate transfer reactions (Wacker, 1969). Mg^{2+} is therefore essential in the regulation of metabolism of other ions and cell functions. Recently, interest has been focused on the clinical importance of altered Mg^{2+} status (Whang, 1987). It has been shown that Mg^{2+} deficiency is associated with serious cardiovascular diseases, such as cardiac arrhythmia and coronary heart disease, as well as with risk factors including hypertension, hypercholesterolemia and diabetes mellitus and moreover, a deficiency of serum magnesium has been reported in T2DM (Altura and Altura, 1991).

1.15.5 Zinc (Zn^{2+})

Zinc (Zn^{2+}) forms part of many protein domains, such as zinc metaloenzymes, and are involved in the synthesis, storage and secretion of insulin monomers (Maret & Sandstead, 2006; Kazi *et al.*, 2008). It is considered to be deficient if serum zinc is less than 60 μg/dl. Diabetes is usually accompanied by zincuria and consumption of nutritional supplements can substantially increase zinc absorption by the gastrointestinal tract (Cunningham *et al.*, 1994). Nevertheless, the effects of zinc supplement, if dietary intake is adequate, are incompletely understood. More clinical data on diabetic patients who are at increased risk of zinc deficiency would be helpful as zinc has an insulinomimetic effect and moreover, it also protects against the oxidative damage associated with the disease (Chen *et al.*, 1998). Additionally, oxidative damage in diabetic patients may result in lower antioxidant micronutrient status, especially trace elements (Wolff *et al.*, 1991).

Therefore, abnormal serum Zn^{2+} status, can promote the progression of diabetes (Ding *et al.*, 1998). Microalbuminuria is a major factor which predicts the onset of overt renal disease and reflects glomerular dysfunction in diabetic patients (O'Brien *et al.*, 1997). Zinc supplementation has beneficial effects on microalbuminuria and serum lipid profile in T2DM with microalbuminuria (Mahmoud *et al.*, 2008).

1.15.6 Copper (Cu^{2+})

Copper is one of the essential trace elements and it plays an important role in cytochrome oxidase function at the terminal end of the mitochondrial electron transport chain. Loss of this activity might contribute to the characteristic swelling and distortion of mitochondria which can be observed in copper deficiency, particularly in metabolically active tissues such as pancreatic acinar cells, enterocytes, and hepatocytes (Aggett, 1985). In subjects with insulin dependent diabetes mellitus (IDDM), zinc concentrations have been demonstrated to be lower in leucocytes and erythrocytes than in serum, while no such alteration has been found with copper (Razz & Haivivi, 1989). No definite association has been explained between copper concentrations and the clinical status of patients with diabetes mellitus (Fujimoto, 1987).

1.15.7 Iron (Fe^{2+})

Iron is the most abundant transitional metal in the body and it has been recognized that an increase of total body iron stores is associated with an increased risk for the development of T2DM (Fernández-Real *et al.*, 2002). T2DM is a common manifestation of haemochromatosis, a disease of iron overload. Iron is a catalyst involved in the formation of hydroxyl radicals, which are powerful pro-oxidants that attack cellular membrane lipids, proteins, and nucleic acids (Beard, 2001; Andrews, 1999). Formation of hydroxyl radicals catalyzed by iron contributes initially to insulin resistance and subsequently to decreased insulin secretion which leads to the

development of T2DM (Larry, 1988). Thus, iron plays a major role in several steps of insulin action and glucose metabolism.

1.16 Biochemical markers

1.16.1 Glucose

T2DM is a disease associated with abnormal carbohydrate metabolism which arises due to insulin deficiency as insulin is the key hormone responsible for glucose homeostasis in blood (Kumar *et al.*, 2005). Consequently, elevation of blood glucose predominantly affects RBC's, vascular endothelial cells and walls of capillaries which often leads to microvascular complications in T2DM including retinopathy, nephropathy and neuropathy (Pirat, 1978). Hyperglycaemia can lead to vascular complications through various mechanisms (DSouza *et al*, 2009). High blood glucose concentration or hyperglycaemia can activate several factors including nuclear factor kB, which in turn increases the expression of various genes in endothelial cells, monocyte-derived macrophages and vascular smooth-muscle cells (DSouza *et al*., 2009). Various mechanisms have been proposed to explain how hyperglycaemia causes vascular complications in T2DM. An increase in glucose can lead to an increase in the fluctuation of glucose to sorbitol through the polyol pathway as well as an increase in glucosamine -6-phosphate via the hexosamine pathway, activation of PKC (protein kinase C) via *de novo* synthesis of diacylglycerol DAG (Ahmed, 2010). The first study which reported glucose as a risk factor in the UK was the Bedford Study in 1965 and the Tecumseh Study in the U.S which suggested that elevation of glucose could be associated with CHD mortality and verified this hypothesis.

The results from several clinical trials have proven that intensive glucose control can reduce the risk of microvascular complications among T2DM patients although its effect on CVD is uncertain. One of the most crucial features of T2DM is hyperglycaemia that affects haemoglobin and membrane proteins of RBC as a result of

abnormal glycation which is shown to be positively correlated with reduced membrane fluidity.

1.16.2 Liver function tests (LFT's)

Human liver plays an important role in maintaining blood glucose concentration during both fasting and postprandial states. An increase in haepatic glucose production following glycogenolysis is due to loss of insulin effect on the liver. Early manifestations of conditions that are characterized by insulin resistance and detected earlier than fasting hyperglycaemia include abnormalities of triglyceride storage and lipolysis in insulin-sensitive tissues such as liver. The sequence of various events and factors leading to underlying insulin resistance include genetic, environmental and metabolic factors. However, the mechanism how these factors induce insulin resistance is not clearly understood. Individuals with T2DM have a higher incidence of liver function test abnormalities when compared with healthy age-matched controls (Lewis *et al.*, 2002; Harris, 2005).

Serum aminotransferases, alkaline phosphatase, bilirubin, albumin are the most common LFT. Aminotransferases including alanine aminotransferase (ALT) and aspartate aminotransferase (AST) serve as markers of hepatocyte injury by measuring the concentration of intracellular hepatic enzymes that leaked into the circulation. Albumin and prothrombin reflect liver synthetic function while alkaline phosphatase (AP) and γ-glutamyl transpeptidase (GGT) act as markers of biliary function and cholestasis.

1.16.3 HbA1c

Haemoglobin A_{1c} is a marker of cumulative glycaemic exposure over the preceding two to three months in diabetic patients. Measurement of glycated haemoglobin was the first clinically used method around 30 years ago to assess the levels of hyperglycaemia in

T2DM patients (Koenig *et al.*, 1976). The values of HbA1c in patients are a reflection of weighted mean glucose levels over the past 3-month period (Tahara and Shima, 1995). Elevated HbA1c is strongly associated with long-term microvascular complications and assessment of HbA1c is used for monitoring effective glycaemic levels.

In recent years, several research studies have made an effort to expand the role of HbA1c as an index of cumulative glycaemic exposure in both diabetes and cardiovascular risk assessment (Ko *et al.*, 2000). Several studies have also tried to evaluate the function of HbA1c in predicting future T2DM in patients with high risk pre-diabetes. Recent studies also have suggested that HbA1c can be used to predict cardiovascular risk in T2DM patients. During the past three decades, elevated HbA1c has been associated with long-term risk of microvascular complications and currently, HbA1c assessment is being used ubiquitously for monitoring effective glycaemic control as a keystone of diabetes care (Little, 2003). In one of a recent case-control study, it has been shown that elevated levels of HbA1c could predict the incidence of cardiovascular events. However, this similar effect was not observed when some of the other cardiovascular risk factors were adjusted (Blake, 2004).

1.16.4 Full blood count

A number of studies have reported significant associations of routine haematological parameters with T2DM and CVD. Results suggest that these abnormalities might form the common soil for both T2DM and CVD. In this study, the levels of various haematological parameters including haemoglobin, RBC, WBC, glucose concentration were evaluated and compared with non-diabetics. A strong association between insulin resistance syndrome and cardiovascular diseases has been demonstrated. High RBC

count is a strong independent predictor of acute cardiovascular events including stroke and myocardial infarction.

Haemorheological parameters including haematocrit, plasma proteins, erythrocyte aggregation, and erythrocyte deformability in T2DM patients are often disturbed. These abnormalities lead to an increase of both plasma and whole blood viscosity (WBV). Deformability of RBC's is one of the haemorheological parameters which is altered in T2DM patients. RBCs of T2DM patients tend to aggregate more easily when compared with healthy subjects. Excessive aggregation of RBC is one of the most important features in T2DM patients with poor glycaemic control. This has a direct effect on the WBV (Grigoleit *et al.*, 1973).

1.16.5 Lipid profiles

A significant percentage of T2DM patients have abnormal serum lipid. Recent studies have revealed that insulin resistance is not only associated with hyperglycaemia alone, but also with several other disorders which are associated with the concentrations of lipoproteins (Grundy, 1997). In T2DM patients, typical abnormalities frequently observed in lipid profile are elevated total and VLDL cholesterol, triglyceride, low levels of HDL, and a large number of dense LDL particles (Lamarche *et al.*, 1997). It is well understood that diabetic dyslipidemia is a major hallmark of metabolic syndrome and found to play an extensive role in the pathogenesis of CVD (Wannamethee *et al*., 2007). Moreover, it has become the main reason responsible for cardiovascular morbidity and mortality in T2DM patients. An increased risk of coronary heart disease has been observed in both T2DM patients and non-T2DM subjects having triglyceridemia (Fontbonne, 1989). The central characteristic of dyslipidemia in T2DM patients is an elevated triglyceride level, particularly triglyceride-rich VLDL and decreased HDL cholesterol levels, though concentration of LDL cholesterol does not

significantly differ from non-diabetic subjects. This characteristic lipid triad is often referred to as atherogenic dyslipidemia which is commonly observed in people having premature CAD and considered as diabetic dyslipidemia when observed in T2DM patients and which leads to CVD risk. According to American Diabetes Association (ADA), increased levels of triglyceride and decreased levels of HDL is the best predictor of CVD in T2DM patients.

1.16.6 C- reactive protein (CRP)

Multiple organs and organ systems as the heart, brain, kidneys and peripheral arteries are typically affected by the risk factors for CVD. T2DM and hypertension are two major candidates which are associated with CVD risk and other organ damage. Recently, novel CV risk markers have been introduced as predictors of cardiovascular morbidity and mortality. One such factor is C-reactive protein (CRP). CRP is the prototypic acute-phase protein which can predict cardiovascular (CV) morbidity and mortality in high-risk patient groups such as T2DM and the general population. CRP is a sensitive marker of inflammation (King *et al*., 2003; Lloyd *et al*., 2006). Inflammation, either systemically or at local sites may underlie the atherosclerotic process resulting in vascular end-organ damage or applies to multiple vascular beds. Indeed, slight increases in serum CRP levels are associated with an increased risk for damage in different vascular beds (Ridker *et al.*, 1997) including the heart, brain, kidneys and lower extremities. However, CRP is not an independent risk marker for CV disease in some reports.

CRP, an acute phase biomarker in systemic inflammation plays an important role in the patho-physiology of T2DM (Dehghan *et al*., 2007). It is also one of the emerging independent risk factors for cardiovascular disease and it can be used as a predictor in some healthy populations (King *et al*., 2002). CRP is an oligomeric protein belonging to

the family of pentraxin and is known to engage in innate immunity in recognition of patterns (see Table 1.4).

CRP actively participates in complement fixation, modulation of platelet activation, in enhancement of activity of leukocytes and in the elimination of cellular debris from the sites of active inflammation (King, 2003).

CRP is produced by the liver through the stimulation of other cytokines like Interleukin 1 and 6 and tumour necrosis factor. Many prospective recent studies have suggested that CRP carries a higher risk for the development of T2DM (Doi *et al.,* 2005). Numerous cross sectional studies stated that high levels of CRP in serum are linked with obesity, insulin resistance and glucose intolerance (Ford, 1999). A high level of C-reactive protein is associated with an increase in the risk of thrombotic events and later development of diabetes (Ridker *et al.,* 1997). There is an established relation between CRP and insulin resistance (Festa *et al.,* 2003). It is widely assumed that CRP represents the inflammatory component of the process of atherosclerosis in the arterial vessel wall (Table 1.4).

Table 1.4: Direct role of C-reactive protein in atherogenesis

Localisation
Localises in shoulder regions of (vulnerable) plaques
Depositions with inflammatory cells and lipid-rich areas
Co-localisation with complement in infarcted tissue
CRP ligand binding
Binds selectively to LDL, especially oxLDL
Aggregated and/or ligand-complexed CRP activates complement
Phagocytic function
Enhances LDL uptake by macrophages
Vascular function
Decreases NO synthase expression in endothelial cells
Decreases prostacyclin release from human aortic cells
Relaxes human vessels
Leukocyte adhesion
Induces expression of leukocyte adhesion molecules
Induces secretion of MCP-1
Thrombosis
Increased rate of thrombosis in CRP-transgenic mice
Tissue factor production by macrophages

1.16.7 Interleukin-6 (IL-6)

Interleukin-6 is a pleiotropic cytokine belonging to the IL-6 family which include IL-11, oncostatin M, ciliary neurotrophic factor, cardiotrophin-1, cardiotrophin like cytokine and leukemia inhibitory factor (Giraud *et al.*, 2007). It is a 26 kDa pleiotropic cytokine mainly produced by endothelial cells, macrophages, adipocytes and lymphocytes (Kerr *et al.*, 2001). As mentioned previously, IL-6 is implicated as a pivotal acute phase response (APR)-inducing cytokine in IL-6 knock out (KO) mice, although there is evidence that it is not exclusively responsible for this. In addition to its role in the APR, it is thought to stimulate haemostasis and influence T- and B-cell differentiation (Kerr *et al.*, 2001) as well as stimulating endothelial cells to produce chemokines (in complex with soluble IL-6 receptor [sIL-6R]). Moreover, it also plays a major role in the transition between acute and chronic inflammatory responses (Gabay, 2006). Mouse models indicate that exogenous addition of IL-6 exacerbates atherogenesis, indicating a role for IL-6 (Huber *et al.*, 1999) through the mechanisms described above. Despite this, IL-6 KO models indicate that basal physiological levels of IL-6 may be required to maintain plaque integrity. This observation may be consistent with some of the condition-specific pro- versus anti-inflammatory actions of IL-6 (Kerr *et al.*, 2001; Gabay, 2006).

In epidemiology, major correlates of IL-6 are age, smoking and acute phase response markers. A recent meta-analysis of 10 available prospective studies (Ridker *et al.*, 1997) including 2089 cases of non-fatal MI or CHD deaths, demonstrated that men in the top third of the population for IL-6 expression had an OR 1.67 (95% confidence intervals [CI] 1.35 –2.05) independent of conventional risk markers. This does not include more recent data, such as that from the Edinburgh Artery Study (Tzoulaki *et al.*, 2007).

Although the data are again in broad agreement with the meta-analysis; OR 1.85 (1.33 to 2.58) in the top versus bottom tertile in a fully adjusted model.

In the same study (Mann *et al.,* 2011) a meta-analysis of the well-studied IL-6 promoter polymorphism -174 G/C was performed, since this allele is generally thought to be associated with elevated circulating concentrations of IL-6. In this analysis, and in contrast with some published literature, no association between the single nucleotide polymorphism (SNP) and circulating levels of IL-6 was observed (in 9 studies with 5871 participants) and unsurprisingly nor was there any association with CHD risk. Hence, IL-6, like fibrinogen and CRP, is associated with atherogenesis in some molecular and animal models, and moreover, it is associated with CHD risk in epidemiological studies to a similar extent.

IL-6 impinges on the immunoregulation and non-immune processes in various cells of the body. Several studies have revealed the relation between IL-6 and pathogenesis of type 2 diabetes (Yudkin *et al*., 2000; Kristiansen and Mandrup, 2005). IL-6 is produced by many types of cells counting endothelial cells, skeletal and smooth muscle cells, islet-cells, microglial cells, hepatocytes, astrocytes, adipocytes and most of the cells of immune system.

1.16.8 Fatty acid binding protein (FABP)

H-FABP is a protein of low -molecular weight concerning the uptake of free fatty acids in the myocardium (de Windt *et al.*, 2002). It is abundantly found to be in the cytoplasm of cardiomyocytes and it gets elevated when there is myocardial injury. FABP assists in the transportation of fatty acids in cardiomyocytes and moreover, it holds a key role in the mitochondrial beta oxidative system.

Heart type fatty acid binding protein (HFABP) is known to play a major role in myocardial infarction (Naraoka *et al.*, 2005). FABP is found to be a new biomarker predicting the development of T2DM. It has been recognized that there is an augmented level of circulating FABP in people with glucose dysregulation.

It has been shown earlier that FABP levels are associated with metabolic syndrome leading to an increased risk of T2DM (Tso *et al.*, 2007). FABP alters the production of inflammatory cytokines and the accretion of cholesterol esters in the macrophages which are quite similar to adipocytes (Xu *et al.*, 2006).

1.16.9 Homocysteine

Homocysteine is recognized as a possible independent risk factor for atherosclerotic disease (Selhub *et al.*, 1995). It a sulphur containing amino acid produced by the metabolism of amino acid methionine (Cronin *et al.*, 1998) which is reversibly converted into homocysteine by the transmethylation process. It is identified as an independent cardiovascular risk factor among diabetic patients and is quite often linked to insulin resistance syndrome (Tessari *et al.*, 2005) by numerous potential clinical studies. Prospective studies have also suggested a possible association between an increased level of plasma homocysteine and insulin resistance (Malinow *et al.*, 1995, Cornelius *et al.*, 1998).

An array of risk factors for cardiovascular disease such as obesity, hypertension, and hyperlipidemia are related to insulin resistance which consequently can result in atherosclerotic events in diabetic population (Anan *et al.*, 2007). One of the possible means by which elevated levels of plasma homocysteine can lead to atherosclerosis is through inflammatory processes (Zhang *et al.*, 2004).

1.16.10 Tumour Necrosis Factor- α (TNF- α)

TNFα is now acknowledged as being a pluripotent cytokine, and the mechanisms of many of its biological activities are still not clearly understood. It seems to be capable of inducing diverse, and at times, contradictory physiological effects depending on the experimental setting. It is known that TNFα can cause apoptosis, septic shock, inflammation and cachexia systemically (Beutler and Ceramic, 1988). Yet, in contrast, it can induce growth through mitosis, it immunologically protects the host, and may also help to induce obesity by metabolic mechanisms (Ferrari, 1999).

TNFα has a long scientific history associated with CVD. For more than 2.5 millennia, it has been recognised that patients with end-stage chronic heart failure (CHF) share clinical features with individuals with neoplasia or inflammatory disorders (Ferrari, 1999).

1.17 Cellular Calcium (Ca^{2+}) homeostasis and Type 2 diabetes (T2DM)

Moreover, complications of T2DM can also affect vascular endothelium, vascular smooth muscle cells, platelets, monocytes and several other cells and tissues. Ca^{2+} homeostasis and Ca^{2+} signalling play a very crucial role in the regular functioning of these cells as they are actively involved in cell-proliferation, cell-death and secretion in many cells (Kumar and Clark, 2007). Numerous studies have investigated the diverse aspects of Ca^{2+} signalling in β-cells and homeostasis in T2DM. It is well known that Ca^{2+} plays a very active physiological role in insulin secretion and there is also evidence that Ca^{2+} is involved in mediating actions of insulin contributing to the pathogenesis of insulin resistance in T2DM. It is observed that basal Ca^{2+} is elevated and Ca^{2+} signalling is impaired in many cells including platelets, endothelial cells and vascular smooth muscle cells in diabetes and hypertension (Resnick *et al*., 1983). Insulin decreases vascular resistance by stimulating nitric oxide production in endothelial cells and

reduces Ca^{2+} in vascular smooth muscle cells. High concentration of Ca^{2+} in endothelial cells increases secretion of basement membrane proteins and contributes to thickening of basement membrane which leads to narrowing of blood vessels.

There is increasing evidence which reveals that T2DM is a state in which calcium homeostasis is impaired (Marhoffer *et al.*, 1992; Levy *et al.*, 1994b). Dysfunctioning of calcium regulation is found in various body cells including erythrocytes, platelets, lymphocytes and neutrophils which confirm the basic pathology associated with diabetic condition. An increase in the calcium level is the most common finding which was described in diabetes among the various other problems associated with the dysregulation of calcium homeostasis.

The primary defect of these multiple functions involved in Ca^{2+} regulation is still unknown. Ca^{2+} metabolism plays a very important role in the vascular complications associated with T2DM such as atherosclerosis, hypertension and angiopathy and also in the pathogenesis of some of the microvascular complications. Therefore, Ca^{2+} homeostasis could be a common pathway which links most of features of T2DM.

1.17.1 Cell Ca^{2+} regulation

Even though there underlies tissue specificity, most of the mechanisms for the maintenance of Ca^{2+} homeostasis are shared by tissues (Howard, 1989; Okamoto *et al.*, 1992; Antony, 1994; Levy *et al.*, 1994a). Intracellular free Ca^{2+} concentration $[Ca^{2+}]i$ is 10^4 times lower than extracellular Ca^{2+} levels which is maintained by a complex interplay of various Ca^{2+} transporters responsible for the influx, extrusion and release of Ca^{2+} in different cells. These include the Na^+/Ca^{2+} exchanger, the calmodulin- (CaM) dependent Ca^{2+}- ATPase, the sarcoplasmic endoplasmic ATPase pump (SERCA) which induces calcium release from the ER or SR and the different Ca^{2+} channels that regulate Ca^{2+} entry into the cell (Rasmussen, 1986; Petersen *et al.*, 1994).

Ca^{2+} is mainly stored in cell organelles such as the endoplasmic sarcoplasmic reticulum (ER/SR), and the mitochondria within the cell (Ann, 1993). Regulation of $[Ca^{2+}]i$ is more active in the ER than the mitochondria. Release of Ca^{2+} from the ER has several mediators which include inositol triphosphate (Ip3), Ca^{2+} via ryanodine receptors and also cyclic adenosine triphosphateribose (cADP-ribose) (Takasawa *et al.*, 1993). The entry of Ca^{2+} into the ER is mediated by a CaM-independent Ca^{2+}-ATPase.

Flooding of excess Ca^{2+} into the cytoplasm after its release from the cellular stores is mediated by several cellular responses (Clapham, 1993). Excess Ca^{2+} is pumped out of the cell and back to the ER which is mediated by Ca^{2+}-ATPase pumps. To prevent depletion of Ca^{2+} stores due to the repetition of such cycles, there are Ca^{2+} specific Ca^{2+} channels in the plasma membrane which allow a small amount of Ca^{2+} into the cell and pumped into the Ca^{2+} stores to replenish the ER. The rate of this Ca^{2+} entry is determined by Ca^{2+} influx factor and cytoplasmic Ca^{2+} levels. $(Na^{+} + K^{+})$-ATPase and the Na^{+}/H^{+} and Na^{+}/ Ca^{2+} exchanger are some of the other mechanisms that regulate $[Ca^{2+}]_i$ which affect $[Na^{+}]_i$. The stimulation or inhibition of Na^{+}/Ca^{2+} exchanger might result in the increase or decrease of Ca^{2+} levels which ultimately leads to the disruption of $[Ca^{2+}]_i$ homeostasis and damage Ca^{2+}-regulated ligand functions (Levy *et al.*, 1994a). However, it is important to emphasize that the calcium signal is very specific to the particular stimuli involved. This specificity of calcium signal is accomplished since two factors determine the "calcium language": the amplitude of the change in $[Ca^{2+}]_i$ level, and the distance that this change spreads. A specific pattern of change is created by these two determinants in the level and distribution of the cation in the cell, and make each Ca^{2+} signal unique to the specific stimuli (Allbritton *et al.*, 1992; Petersen *et al.*, 1994).

1.17.1.1 Abnormal-cell Ca^{2+} regulation in diabetes

Although $[Ca^{2+}]_i$ is important for vital functions in all cells, some of its effects such as contraction in muscle and secretion in glands are tissue-specific (Boris, 1988; Levy *et al.*, 1989; Levy *et al.*, 1994a).Therefore, defects in $[Ca^{2+}]_i$ homeostasis might be expressed differently in the different tissues involved. Further, since the mechanisms that regulate $[Ca^{2+}]_i$ are tightly interrelated, a defect in one mechanism may impair the function of the other mechanisms, making the identification of the primary defect a complicated task. Various abnormalities in $[Ca^{2+}]_i$ regulation were described in animal models of diabetes and obesity and in obese and diabetic patients (Kato *et al*., 1994; Levy *et al.*, 1994b). These include pathologies of function of the plasma membrane and the ER/SR Ca^{2+}-ATPase, $(Na^+ + K^+)$-ATPase, Na^+- Ca^{2+} exchanger, Ca^{2+}-induced Ca^{2+} release from the ER, the tissue total calcium content, and the actual $[Ca^{2+}]_i$ levels (Juntti *et al*, 1993; Roe *et al.*, 1994).

Defects in cell Ca^{2+} homeostasis were described in erythrocytes, cardiac muscle, platelets, skeletal muscle, kidney, aorta, adipocytes, liver, osteoblasts, arteries, lens, peripheral nerves, brain synaptosomes, retinal tissue, and in pancreatic β cells (Tomizawa *et al.*, 1993; Roe *et al.*, 1994; Janicki *et al.*, 1994; Levy *et al.*, 1998). However, there is noticeable variation with regard to the described specific defect in each of the $[Ca^{2+}]_i$ regulatory functions. For example, basal $[Ca^{2+}]_i$ levels have been described to be either high, low, or normal in cardiomyocytes in diabetes (Schaffer & Mozaffari, 1996; Hayashi & Noda, 1997; Yu *et al.*, 1997). Similarly, basal activity of the plasma membrane Ca^{2+}-ATPase was described to be either high, low, or normal (Levy *et al.*, 1994b). However, even when basal functions were normal, dynamic studies that tested responses of the $[Ca^{2+}]_i$ regulatory function to an agonist were abnormal, suggesting that dynamic studies might be more sensitive in identifying early defects in cell Ca^{2+} homeostasis in diabetes and obesity. Since the functional state of the

$[Ca^{2+}]_i$ regulatory mechanisms, such as the ATPase's, depends on many factors including membrane phospholipid content, level of Mg^{2+}, free fatty acids, cholesterol, and glucose and these conditions were not standardized in the different studies, it is expected to observe contradictory results obtained in the different reports. However, an increase in the level of $[Ca^{2+}]_i$ has been described most frequently as a defect in cell $[Ca^{2+}]_i$ homeostasis in diabetes and obesity (Levy *et al.*, 1988; Gonzalez Flecha *et al.*, 1990; Mene *et al.*, 1993; Williams & Schrier, 1993, Espino *et al*., 2010).

1.17.1.2 Impaired-Cell Ca^{2+} Homeostasis and Insulin Secretion

So far, defects in β-cell $[Ca^{2+}]_i$ homeostasis have been described only in animal models of diabetes and in islet cells exposed to high glucose for a long time, but it is expected that similar defects will appear in human diabetes (Roe *et al.*, 1994). Islets from db/db mice lack the initial reduction of $[Ca^{2+}]_i$ and the subsequent Ca^{2+} oscillations in response to glucose, and islets from neonatal induced T2DM rats lacking insulin response to glucose, but respond to amino acid stimulation have selective defect in the increase of $[Ca^{2+}]_i$ in response to glucose (Giroix *et al.*, 1983; Tsuji *et al.*, 1993). L-type voltage-dependent Ca^{2+} channel activities are increased in β cells from these T2DM rats and their Ca^{2+}-ATPase activity is decreased. Since oscillations of $[Ca^{2+}]_i$ are essential for the pulsatile insulin secretion, loss of these oscillations may play a significant role in the loss of pulsatile insulin secretion observed early in T2DM (Leahy, 1990). Finally, there is evidence that the "glucose toxicity," which selectively impairs insulin secretion in response to glucose (and may play a significant role in the β-cell dysfunction in diabetes) may be mediated in part by a glucose-induced impairment in the β cells' $[Ca^{2+}]_i$ homeostasis (Dunbar *et al.*, 1989; Okamoto *et al.*, 1992). Culture of rat islet cells in media that contain high glucose concentration cause a progressive, dose- and time-dependent decrease in the membrane Ca^{2+}-ATPase activity of the islet cells (Levy *et al.*,

1998). In turn, the significance of the Ca^{2+} ATPase for normal insulin secretion has recently been considered (Gagliardino & Rossi, 1994).

1.17.2 Calcium and cardiovascular disease

T2DM is a well-established and independent risk factor for the development and progression of atherosclerosis. The mechanisms that predispose T2DM subjects to organ injury and atherosclerosis are multifactorial. Abnormalities of endothelium function and morphology appear to play a central role in the pathogenesis of T2DM-related atherosclerosis. Among the mechanisms causing endothelial dysfunction that have been recently implicated in essential T2DM are insulin resistance and its accompanying compensatory hyperinsulinemia, oxidative stress (OS), and inflammation. Recent evidence from various studies suggests that Ca^{2+} might play a major role in hypertension, atherosclerosis and cardiomyopathy (Levy *et al.*, 1994a; Schaffer & Mozaffari, 1996).

It has been suggested that diabetes and cardiovascular disease are associated with a common defect of divalent cation metabolism, which also includes calcium (Lawrence, 1989). $[Ca^{2+}]_i$ has a role as a key mediator in the production of oxidants by PMN and in their degranulation, resulting in OS, inflammation, and damage to endothelial cells, vasoconstriction, and hypertension. Elevated $[Ca^{2+}]_i$ has been described in various cells in insulin-resistant states such as uremia, diabetes, and hypertension. An elevation in $[Ca^{2+}]_i$ in PMN from hemodialysis patients, diabetic patients, and diabetic rats has been previously described as playing an important role in the pathogenesis of their impaired phagocytosis (Espino *et al*., 2011).

1.18 Structure of red blood cell (RBC) or Erythrocyte membrane

Structure of RBC membrane is studied comprehensively as a model for the membrane of many other complex cells. Due to their availability in large numbers and relatively

simple membranes which can be purified easily, erythrocytes have attracted such investigations for many years including medical conditions like haemolytic anaemia, transfusion reactions and so on. Erythrocyte skeleton plays a major role in determining the shape and the deformability of the cell. Disruption of the interaction between the various components of the red cell membrane skeleton may cause loss of structural and functional integrity of the membrane.

The erythrocyte holds a unique, biconcave shape which allows an excess of about 40% membrane surface to cell volume (Mayhew *et al.,* 1994). This shape provides plasticity to the cell and contributes to its deformation, which is an essential component to traverse capillary beds containing vessels smaller than a diameter of 8 μm. A dynamic interaction between the cytoskeleton and the membrane enables these morphological changes to take place in the RBC (Sivilotti, 2004). The membrane skeleton is made of a protein network which consists of structural proteins on the inner surface of the erythrocyte membrane and is bound, through linking proteins, to integral proteins of the erythrocyte membrane (Delaunay, 2007). Hence, the membrane is considered as a structure that extends into the cytoplasm as well as the extracellular space (Bruce *et al.*, 2003).

Erythrocyte membrane belongs to the bilayer membrane composed of various proteins and lipids. The nomenclatures and ordinal numbers of the erythrocyte membrane proteins are assigned according to the mobility of their bands during separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Petropoulos *et al.,* 2007). In general, erythrocyte membrane proteins can be divided into two groups. One group includes peripheral proteins such as spectrin, ankyrin, protein 4.1, actin, protein 4.2 and the other group includes integral proteins such as band 3 and glycophorin (Figure. 4.1).

1.18.1 RBC membrane proteins

1.18.1.1 Spectrin

Spectrin is the major components of the RBC membrane skeleton and it comprises of 25%–30% of total membrane proteins. It is a heterodimer consisting of alpha- and beta-subunits that are encoded by different genes and associate head-to-head to form tetramers. The molecular weight of α-spectrin is 240 kDa, which contains 20 homologous 106 amino acid repeat segments and two non-homologous segments. The molecular weight of β-spectrin is 220 kDa, which contains 17 homologous 106 amino acid repeat segments, non-homologous amino terminal anti-parallel segments, connected by short non-helical segments. Spectrin heterodimer is a rod-like flexible filament, measuring approximately 100 nm in length (Gallagher and Forget, 1993). Each erythrocyte contains about 200,000 copies of α and β Spectrin and these chains interlink in an anti-parallel fashion, forming the heterodimer. Spectrin tetramers link together in a hexagonal array which is connected to the plasma membrane at junctional complexes by interacting with actin, protein 4.1 and GPC. Self-association of spectrin dimers into tetramers is possibly the best-characterised interaction of membrane proteins, allowing the erythrocyte to acquire its mechanical properties (Van Zwieten *et al.*, 1995).

The functions of spectrin include maintenance of erythrocyte shape, regulation of lateral motion of integral proteins and to provide structural support to the lipid bilayer and are dependent on its interaction with membrane proteins. Spectrin also plays an essential role in maintaining local concentration of plasma membrane proteins. It is also involved in early stages of cell junction formation and regulation of entrance of secretory vesicles into the plasma membrane in non-erythroid cells (Schwartz *et al.*, 1991).

1.18.1.2 Ankyrin

Human ankyrin is an asymmetrical polar protein composed of 1881 amino acid residues, with a molecular weight of 206 kDa. It is a mixture of proteins with different sizes and contains three functional domains defined by chymotrypsin cleavage. These include the 89 kDa N-terminal domain extending from Pro-2 to Phe-827 containing the binding site for band 3 and tubulin, the central 62 kDa domain which starts at Lys-828 and ends at either Leu-1382 or Tyr-1386 or both. It contains the binding sites for β-Spectrin and the intermediate filament proteins vimentin and desmin. Spectrin is anchored on the cell membrane using a functional 55 kDa domain formed from the remaining 495 th−499 th C-terminal amino acids. It participates in the widely selective processing of mRNA and modulates the interaction of ankyrin with Sp and band 3. This domain can affect the hydrodynamic properties of ankyrin and is extremely sensitive to proteases, suggesting that it may be more loosely assembled and its last 20 kDa segment possibly extends from the ankyrin molecule like a tail.

The binding protein, ankyrin, creates a second linkage by linking of spectrin to the erythrocyte anion exchanger, band 3 through high affinity (Bruce *et al.*, 2003). Recently, two erythrocyte membrane multiprotein complexes namely band 3 complex, positioned close to the center of the spectrin tetramer, and protein 4.1 located at the membrane skeletal junctions have been described elaborately (Salomao *et al.*, 2008).

1.18.1.3 Protein 4.1

Protein 4.1 accounts for about 6% of the total membrane proteins and can be separated by PAGE into two isoforms (4.1a and 4.1b) with molecular weights of 80 kDa and 78 kDa, respectively. Protein 4.1 gene is located on chromosome 1 adjacent to Rh protein locus, and usually encounters alternative mRNA processing that shows cell differentiation and tissue-specific nature. Protein 4.1 possesses two important functions

which include interaction of its 10 kDa Spectrin-binding domain with the N-terminal of β-Sp which promotes the binding of spectrin with actin and binding of its N-terminal domain with GPC. These negatively charged lipids which are present on the inner hemi-leaflets of lipid bilayer allow the distal ends of spectrin tetramer to bind to erythrocyte membrane.

1.18.1.4 Actin

Actin is one of the important proteins which is required for muscular contraction and motion of the cell. Each erythrocyte consists of appropriately 40~50 $x10^4$ actin monomers, arranged into short protofilaments of F-actin composing of 12–14 monomers within erythrocyte membrane, 30–40 nm in length. β-actin participates in the formation of junctional complex (JC). Protein 4.2 is present in almost same number of copies as Spectrin or ankyrin. It is associated with the cytoplasmic domain of band 3, ankyrin, and protein 4.1, but its function is unknown. A complete deficiency of protein 4.2 may cause spheroovalocytosis and hemolytic anemia, suggesting that it plays a certain role in the maintenance of erythrocyte membrane integrity.

1.18.1.5 Band 3

Band 3 is the major trans-membrane protein of erythrocytes, comprising of about 25% of the total membrane proteins. Each erythrocyte contains 106 copies of band 3 protomer with a molecular weight of 95 kDa. Various forms of band 3 exist in erythrocyte membrane including homodimer, tetramer or oligomer; and its polymorphism appears approximately in 6% of population. This is a multifunctional protein consisting of 3 domains, a membrane-spanning domain that carries out chloride/bicarbonate exchange, a short C-terminal cytoplasmic domain, and a large N-terminal cytoplasmic domain (Tanner, 1997). The C-terminal cytoplasmic domain of band 3 binds carbonic anhydrase II (CAII), forming a metabolon that channels HCO^-_3 at

the cytoplasmic face of band 3 and facilitates its interaction with CAII and band 3.3. The N-terminal cytoplasmic domain of band 3 binds glycolytic enzymes, haemoglobin (Hb), and hemichromes that may induce the aggregation of band 3 and cell turnover. However, a major function of the N-terminal domain of band 3 is to anchor the RBC membrane to the underlying cytoskeleton. About 60% of Band 3 exists as dimers and 40% as tetramers in the RBC membrane. The tetrameric form binds ankyrin and protein 4.2 (Michaely and Bennett, 1995), and it is the major attachment site of the RBC membrane to the cytoskeleton. The 40 kDa N-terminal ankyrin-binding domain is located within the cytoplasm, whereas the C-terminal 55 kDa anion transport portion spans across the erythrocyte membrane for 13 to 14 times (Figure 4. 1). The N-terminus of band 3 is embedded deeply inside the cytoplasm, with its C-terminus extended outside the membrane. Band 3 is glycosylated at a single external site (Asn-642).

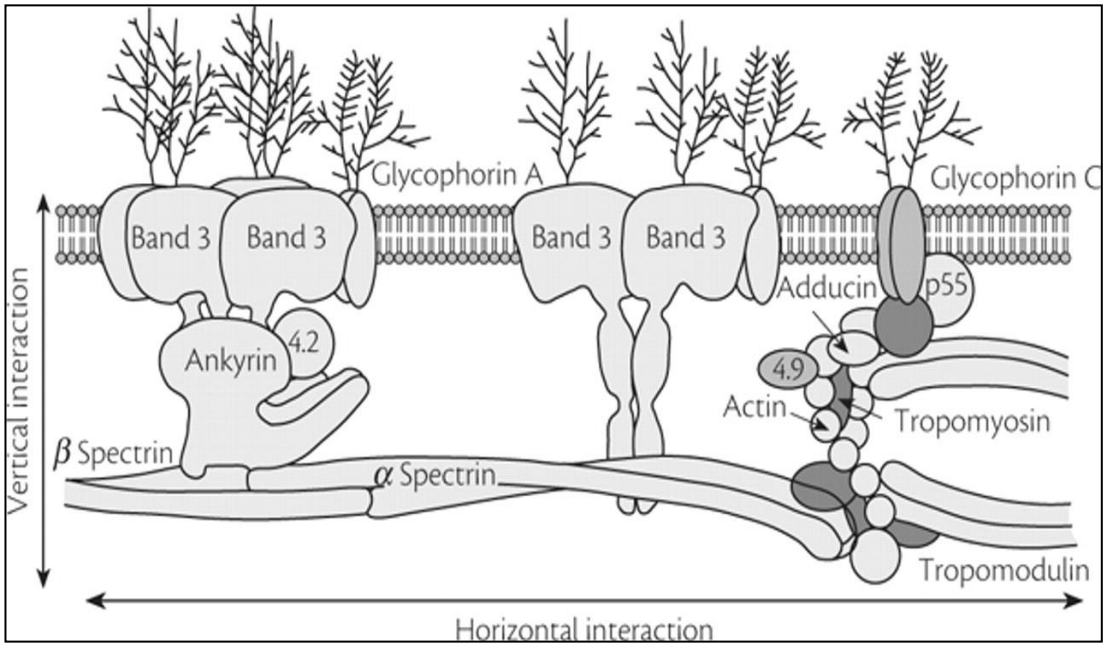

Figure 1.5: Diagram showing the structure of erythrocyte membrane; The model depicting the interactions and associations of the major human erythrocyte membrane proteins which include α and ß spectrin, ankyrin, band 3, 4.1 (protein 4.1), 4.2 (protein 4.2), actin, and GP (glycophorin) (Adapted from Tse and Lux, 1999).

1.18.1.6 Glycophorin

Glycophorin (GP) molecules consist of about 60% saccharides. The N-terminus of its polypeptide chain extends beyond the erythrocyte membrane. The majority of 16 scattered oligosaccharides hold trioses and tetroses on their side chains, with sialic acid as end sugar residues. Erythrocyte membrane of healthy subjects display 4-5 GP bands after electrophoretic separation using periodic acid-Schiff (PAS) staining Human GPs belong to transmembrane sialoglycoprotein families, including GPs A, B, C, D (α, δ, β, γ GPs), and GP E. GPs A, B, and E carry the MNSs blood group antigens: while GPs C and D carry the Gerbich (Ge) blood group antigens. The structures of all five GPs and their encoding genes are clearly explained, with molecular weights ranging in between 17–36 kDa. High degree of homology is found in the amino acid sequences of primary structures among GPs A, B, and E. The α, δ and ε genes encoding GPs A, B, and E are located on q31-34 of chromosome 4. They relate to the autosomes of codominant heredity, which are organized in clusters, with spanning sequence of approximate 330 kb in order of 5p−GP A−GP B−GP E−3p. Previous research studies provided information that about 25 variants were detected in GP families, most of them which originated as a result of unequal homologous recombination or micro-conversion between α GP genes and δ GP genes. Five types of GP variants were also identified in Southern China (Lu and Liu, 1999).

1.19 Osmotic fragility

Osmotic fragility of Red blood corpuscles (RBCs) can be defined as the rate at which the cells burst in hypotonic solutions. Several factors including disease, age, and drugs can alter the fragility of red blood cell membrane which plays a major role in the survival and function of the RBCs. Cell membranes are semi- permeable barriers and there are certain established osmotic gradients between intracellular and extracellular

fluids which allow water to flow in and out of these membranes. The concentration of non-diffused ions on either side of the cell membrane determines the amount of osmotic pressure.

Prolonged exposure of RBC membrane to elevated concentrations of oxygen and iron in haemoglobin (Hb), and its high polyunsaturated fatty acid contents are some of the factors which make RBCs a very sensitive model to study injury caused by oxidative stress (Kusmic *et al.,* 2000). Elevated levels of glucose (hyperglycaemia) in blood is known to cause damage to cell membrane and cell death in various cells including cultured pericytes, endothelial cells, kidney cells, retinal cells and RBC's (Li *et al.*, 1984).

Anaemia is a highly prevalent condition in T2DM patients. According to World Health Organisation (WHO), nearly 30% of T2DM patients have anaemia, especially those having kidney problems (Almoznino-Sarafian *et al.*, 2010). People with T2DM have anaemia with a complex and multifactorial etiopathogenesis and is associated with an increased risk of cardiovascular mortality (Zoppini *et al.,* 2010). Recent epidemiological studies predominantly performed on Asian and African populations reported that patients with T2DM are characterised by increased erythrocyte osmotic fragility (Ramana Devi *et al.,* 1997; Ibanga *et al.,* 2005; Osuntokl *et al.,* 2007; Kung *et al.,* 2009).

1.20 Advanced Glycation End-products (AGEs)

T2DM is greatly associated with an increased risk of CVD which cannot be explained by known risk factors. Diabetes is regarded as a CHD risk equivalent by the adult Treatment Panel III. Some of the factors involved in determining atherosclerotic disease are formation of advanced glycation end-products (AGEs). There is increasing evidence that AGEs play a major role in atherosclerosis in diabetics. Accumulation of AGEs is

considered as a measure of hyperglycaemia and also represents the increasing metabolic burden, oxidative stress and inflammation (Baynes and Thorpe, 2000). Inflammatory reactions and endothelial dysfunction occur as a result of interactions between AGEs and AGE-specific receptors (Ahmed, 2005). AGEs cause damage through the formation of unusual cross-links in collagen which might contribute to vascular stiffening (Vlassara and Palace, 2002). Recent studies have reported that there is an increased serum level of AGEs in T2DM patients with Coronary Heart Disease (CHD). It has also been shown that AGEs tend to accumulate in coronary atherosclerotic plaques and cardiac tissue of T2DM patients in several immune-histochemical studies (Nakamura *et al.*, 1993, Grillo and Colombatto, 2008).

1.20.1 Formation of AGEs

Glucose reacts non-enzymatically and chemically with proteins *in vivo*, to form covalently attached glucose-addition products and cross-links between proteins. Advanced glycation end-products are a class of complex products formed as a result of Maillard's reaction which occurs between carbohydrates and free amino group of proteins. Intermediate products during this reaction are a result of dehydration, molecular rearrangement which leads to carboxyl residue as 3-deoxyglucosone which in turn reacts with free amino groups and form cross linked proteins. Maillard's reaction is a succession of chemical reactions linked in a complicated network. The first step results in the formation of a Schiff base after a condensation reaction between amino group and a carboxyl group. The second step includes a molecular rearrangement that results in the formation of N-substituted glycosylamine which leads to Amadori's products. Finally, Amadori's products undergo further rearrangements to form AGEs (Ahmed, 2005; Diamanti *et al.*, 2007; DSouza *et al.*, 2009).

AGEs are very unstable, reactive compounds and their end products are often complicated to be completely analysed. Pentosidine and carboxymethylysine (CML) are the best chemically characterised AGEs in humans. Excessive accumulation of these products is believed to contribute to chronic complications of DM. Chemical modification of proteins by reducing sugars in diabetes alters the structure and function of tissue proteins leading to further diabetic complications (Monnier, 1990). Glycation involves formation of chemically reversible early glycosylation products with proteins namely Schiff bases and Amadori's products. These products undergo slow and complex rearrangements and form AGEs.

The significance of oxidizing conditions and reactive oxygen species involved in the formation of glycoxidation products which are a major class of AGEs that accumulate in diabetic tissues was studies by Baynes and colleagues (Baynes and Thorpe, 2000). Some of the other pathways which lead to the formation of AGEs are autoxidation of glucose by reactive oxygen species and through carbonyl compounds (Kilhovd *et al.*, 2003). Predominantly, methylglyoxal which is a reactive dicarbonyl metabolite of glucose has received substantial consideration as the most reactive AGE precursor in endothelial cells (Ahmed, 2010). Curcumin diminishes the impact of hyperglycemia. This is affected through abrogating GLUT2 gene expression. It also induces expression of PPARγ to reduce oxidative stress (Lin and Chen, 2011). Curcumin also eliminates oxidised LDL by suppressing Lectin like oxidised LDL receptor-1 (LOX-1) (Kang and Chen, 2009).

1.20.2 AGEs in Diabetes

Some of the most common features of diabetic microvascular complications are capillary basement membrane thickening and hypertrophy of extracellular matrix. High plasma levels of glucose lead to an accumulation of AGEs in tissues which further

cause tissue damage and moreover, several studies showed a significant correlation between AGE deposits and diabetic complications (Hammes *et al.*, 1999, Kaji *et al*., 2006; Meerwaldt *et al*., 2008).

A state of exaggerated oxidative stress in diabetic patients is because of excessive production of free radicals including free oxygen species, increased oxidation of substrates and auto-oxidation of glucose. In several animal studies, it has been demonstrated that excess production of free radicals via a decrease in production of nitric oxide (NO) leads to endothelial dysfunction which further restricts vaso-relaxation of smooth muscle cells (Testafamarian, 1994).

1.20.3 AGE's in cardiovascular disease

There is an increased risk of cardiovascular morbidity and mortality in patients with T2DM. AGEs play a significant role in the development and progression of CVD in diabetic patients (Jakus and Rietbrock, 2003). Serum level of AGEs are higher in diabetic patients with CHD when compared with those without CHD (Kiuchi *et al.*, 2001). Serum AGE levels are found to be significantly associated with CHD even after other CVD risk factors have been adjusted and several studies have demonstrated deposits of AGEs in atherosclerotic plaques and also in myocardium fibres (Sakata *et al.*, 1995; Schalkwijk *et al.*, 2004).

AGEs also increase the level of NF-Kappa B, a transcription factor which is considered to be involved in the development of atherosclerosis and also in apoptosis (Bierhaus *et al.*, 1997). AGEs promote atherogenesis by oxidizing low density lipoproteins and cause changes in the intimal collagen. Some studies have led to isolation of a receptor for AGEs on cell surface which is termed as RAGE. This functions as a signal transduction receptor and in binding non-AGE-related pro-inflammatory molecules such as S100/calgranulins and amphoterins. Elevated levels of AGEs and CML have

been reported in T2DM patients with CHD (Kilhovd *et al.*, 1999). Analyses of human atherosclerotic lesions have demonstrated extracellular as well as intracellular deposition of AGEs in macrophages and vascular smooth muscle cells (Nakamura *et al.*, 1993; Vlassara *et al.*, 1996). Concentration of AGEs in tissues is associated with the severity of atherosclerotic lesions and accumulation of plasma proteins, lipids and lipoproteins in the vessel wall (Sims *et al.*, 1996). AGEs can highly affect the integrity and function of blood vessel wall through various mechanisms. The first method to promote atherogenesis includes formation of AGE cross bridges within the vessel wall macromolecules causing mechanical dysfunction and the second is adherence of circulating blood cells to the vessel wall due to excessive accumulation of AGEs (Sell and Monnier, 1989).

The final mechanism is through alterations of cellular function of various cells including macrophages, endothelial cells, smooth muscle cells, renal and neuronal cells by binding to a variety of receptors (Hori *et al.*, 1995; Miyata *et al.*, 1996;Yan *et al.*, 1996). Formation of AGEs alters functional properties of several important matrix molecules. Collagen within the blood vessel has a comparatively long biological half-life and undergoes non-enzymatic glycation to play a significant role in atherosclerosis (Brownlee *et al.*, 1986). AGEs also entrap soluble plasma proteins like LDL and immunoglobulin (IgG) and form covalent cross links (Meng *et al.*, 1998). Furthermore, the mechanism through which AGEs could lead to the development of CVD is unclear. Some of these mechanisms including endothelial and renal dysfunction, low-grade inflammation and arterial stiffness could contribute to the progression of CVD in T2DM patients (Suliman *et al.*, 2003; Goldin *et al.*, 2006; Semba *et al.*, 2009).

1.21 Total Antioxidant Status

In recent years, several studies have investigated the possible role of reactive oxygen species (ROS) in the pathogenesis of T2DM (Galli *et al.*, 2005). In disease mechanisms, where increased oxidative stress might play a major role, ROS are considered to be actively involved in a large variety of physiological and patho-physiological processes. Oxidative stress can be defined as excessive formation of highly reactive molecules (free radicals) including ROS and reactive nitrogen species. Oxidation reactions produce free radicals which start chains of reactions that damage cells (DSouza *et a.l*, 2009; Ahmed, 2010).

Antioxidants serve as reducing agents by terminating these chain reactions through removal of free radical intermediates and inhibition of other oxidation reactions (Sies, 1997). There are several other potential sources of free radical production in diabetes which include autooxidation of glucose, activation of leukocytes and increased bioavailability of transition metal which could perturb the natural antioxidant defence in the body (Wolff *et al.*, 1991). These highly reactive molecules quench nitric oxide (NO), which is the main endothelial vasodilatation factor and impairs NO-mediated vasodilation by reducing its bioavailability (Honing *et al.*, 1998). Serum of diabetic patients is found to contain increased levels of lipid peroxides produced due to oxidative damage caused to lipids and proteins which shows an association with the development of diabetic complications (Nishigaki *et al.*, 1981; Jones *et al.*, 1988; Stringer *et al.*, 1989). Research has exposed that free radicals have an important role to play in aetiology of T2DM and a strong association exists between oxidative stress and secondary complications of diabetes (Singal *et al.*, 2001; Rolo and Palmeira, 2006).

Patients with T2DM have chronic oxidative stress (Ghiselli *et al.*, 1995). It has been demonstrated that increased levels of intracellular ROS play a significant role in chronic

inflammatory responses to atherosclerosis (Berliner *et al.*, 1995; Kojda & Harrison, 1999; Chisolm & Steinberg, 2000). In T2DM patients, LDL oxidation contributes to LDL accumulation and therefore leads to an accelerated development of atherosclerosis (Panasenko *et al.*, 1991). Recently, much attention has been given to the possibility that T2DM patients might be subjected to severe oxidative stresses. Oxidative modification of LDL particles is presently recognized as a vital step in the atherogenic process (Steinberg *et al.*, 1989).

Higher levels of oxidative stress in T2DM patients might be a result of prolonged periods of hyperglycaemia which cause non-enzymatic glycation of plasma proteins including LDL (Tames *et al.*, 1992). Persistent hyperglycaemia increases the production of ROS within the aortic endothelial cells. Some of the recognized biochemical mechanisms of hyperglycaemia induced cell and tissue damage are ROS-induced activation of protein kinase-C isoforms, increased formation of glucose–derived AGEs, increased glucose flux via aldose reductase pathways and activation of cytokines (Brownlee, 1995). Reaction of ROS on cell membranes leads to the formation of lipid peroxidation products such as malondialdehyde (MDA).

Patients with T2DM suffer from an increased risk of vascular disease when compared with those without T2DM (Deckert *et al.*, 1978). Vascular complications take the form of premature and accelerated atherosclerosis of large arteries or cause increased permeability of capillaries which is associated with thickening of the basement membrane. Increased prevalence of dyslipidemia and hypertension are the most important predisposing factors for the development of vascular disease in diabetes (Bierman, 1992).

Several low molecular weight antioxidant molecules such as vitamin C, vitamin E, urate, thiols and bilirubin act as primary defence against oxidative stress in extracellular

fluids (Frei *et al.,* 1988). A wide range of natural antioxidants including superoxide dismutase, glutathione-glutathione peroxidase system are present to scavenge free radicals in order to prevent oxidative damage. Superoxide dismutase becomes inactive due to prolonged hyperglycaemia in diabetic patients (Arai *et al.*, 1987).

Antioxidant therapy may be useful to relieve symptoms and complications in T2DM patients. Many traditional medicinal plants are found to possess substantial amount of anti-oxidant properties that were effective against diabetes and its related complications (Bailey and Day, 1989). Correspondingly, it has been proven that anti-oxidants including vitamin C and E improve endothelium-dependent vasodilation in DM (Keegan *et al.*, 1995; Ting *et al.*, 1996; Timimi *et al.*, 1998).

1.22 Working hypothesis

Endogenous inflammatory markers and mediators can predict the risk for cardiovascular diseases in T2DM patients.

1.22.1 Main Aim

The main aim of this study was to investigate the relationship between endogenous inflammatory markers and mediators and the risk of CVD in T2DM patients.

1.22.2 Objectives

The objectives of this study are the following

- To carry out an epidemiological study of some risk factors among T2DM patients.
- To measure intracellular free calcium concentrations in lymphocytes and neutrophils of T2DM patients compared to healthy age-matched controls.

- To measure cations, inflammatory markers and mediators in blood of T2DM compared to healthy age-matched controls.

- To investigate the osmotic fragility and different membrane proteins in RBC of T2DM patients compared to healthy age-matched control.

- To measure low molecular weight advanced glycation end products, anti-oxidant status in the blood of T2DM patients compared to healthy age-matched controls.

Chapter 2

Materials and Methods

2.1 Subjects and samples

2.1.1 Recruitment of subjects

This project recruited both male and female T2DM patients and healthy age-matched control subjects from Royal Preston Hospital, Chorley General Hospital, and Christina Infant hospital in Badajoz, Spain, University of Central Lancashire. Type 2 diabetic patients who attended the Diabetic Clinics at Royal Preston Hospital, Chorley General Hospital were recruited for this study. The project had relevant ethical clearance from LREC (LTHTR Ethics Committee), UCLan Ethics Committee and the University of Extremadura in Spain.

Since each chapter of this thesis comprised of different cohort of patients, the recruitment process including venipuncture techniques, handling of the samples, and patient follow up were discussed individually in each chapter.

2.2 Blood collection

Samples of blood were collected by venipuncture technique from both T2DM patients and healthy controls following consent. The patients were also given a questionnaire to fill in which contained several questions regarding their lifestyle habits and medical history for epidemiological study.

2.3 Sample handling and storage

The collection, handling and storage of blood samples were considered a vital component in order to minimise any inter-sample differences. The results obtained from these studies were definitely a reflection of the care taken during these procedures. Therefore, blood samples were only collected from patients who were diagnosed with T2DM and healthy age-matched controls.

2.3.1 Blood handling and preservation

Universal precautions were followed while handling the blood samples. Plasma and serum tubes were labelled properly with an ID number and collection date. The samples were centrifuged depending on the requirements followed by storage at appropriate temperatures.

2.3.2 Blood storage

The blood (5 ml) was collected by the candidate in EDTA tubes, centrifuged at 3500 rpm for 10 minutes to separate the red blood cells from the plasma, buffy coat and was stored at -80 ºC for later analysis. The candidate has obtained relevant training at Royal Preston Hospital, Preston to collect blood from patients and age matched control subjects by venipuncture technique.

2.4 Comparison and analysis of various biochemical parameters and inflammatory mediators in plasma of T2DM and healthy controls

2.4.1 Measurement of various cations in plasma

Measurement of various cations including sodium, potassium, calcium, magnesium, zinc, copper, iron and selenium was performed using inductively coupled plasma mass spectrometry, (ICP-MS) technique with plasma of both T2DM and healthy controls. Blood was collected in an EDTA tube by venipuncture technique and it was centrifuged at 3500 rpm for 10 min to separate plasma from the red blood cells. A volume of 0.2 ml of plasma was diluted with 9.8 ml deionised water to make 10 ml solution in each tube. A volume of 4 ml of nitric acid was added to 1 ml of the above solution in each tube and 10 µl of internal standard was added to each tube. The HNO_3 was used to denature any proteins in the plasma in order to facilitate the measurement of the ions. Cations were measured using ICP-MS according to standard protocol of the instrument. Values

were expressed as parts per billion which were subsequently converted into mM/L values.

2.4.2 Measurement of various biochemical parameters

Various biochemical parameters in blood including glucose, insulin, full blood count, cholesterol, serum lipid profile, HbA1c, U and E, and C - reactive protein were measured according to standard protocols in both T2DM and healthy controls using Pre-modular analytics by Roche/Hitachi in the Biochemistry Laboratory, at the Pathology Department at Royal Preston Hospital.

2.4.3 Measurement of inflammatory mediators

Inflammatory mediators were measured using microarray kits. Quantitative measurement of 10 human cytokines in the serum samples was done using Quantibody® Human Inflammation Array 1 from Ray Biotech, Inc., USA. The cytokines including IL-1α, IL-1β, IL-4, IL-6, IL-8, Il-10, IL-13, MCP-1, IFNγ, and TNFα were detected by fluorescence measurement of samples using laser scanner with Cy3 equivalent dye.

2.4.3.1 Experimental procedure

The glass chip was removed from the box and air dried by leaving at room temperature for 20-30 min inside the sealed plastic bag. Thereafter, the slide was removed from the plastic bag, the cover film was peeled off and air dried again for 1-2 hours at room temperature as incomplete air drying of the slide would result in the formation of comet tails.

2.4.3.2 Preparation of standard dilutions of cytokines

The cytokine standard mix was provided in lyophilized form and then was reconstituted by adding 500 µl of sample diluents to the tube. The powder was dissolved thoroughly by mixing gently and labelled as standard 1. This was done within one hour of usage. A

total of 6 clean microcentrifuge tubes were labelled as Std 2 to Std 7 and 200 µl of sample diluent was added to each tube. A volume of 100 µl of Std 1 was pipetted into Std 2 tube and mixed gently. A serial dilution was performed by carrying on to Standard 3 up to Standard 7. A volume of 100 µl of sample diluent was added to another tube labelled as control. This tube was used as negative control. Either standard cytokines or sample was not added to this tube.

2.4.3.3 Blocking and incubation

A volume of 100 µl of sample diluent was added into each well and incubated at room temperature for 30 min to block slides. The buffer was decanted from each well and 100 µl of standard of each cytokine and samples were added to each well. The array was incubated at room temperature for 2 hours. This step was performed overnight at 4 ºC for best results.

2.4.3.4 Wash

The samples were decanted from each well and washed 5 times per min for 5 min each time with 150 µl of 1 x wash buffer I at room temperature with gentle shaking. The wash buffer was completely removed in each wash step. 20 x wash buffer 1 was diluted with distilled water. The glass chip with frame was placed in a box with 1x wash buffer and washed at room temperature with gentle shaking for 20 min. The 1 x wash buffer was decanted from each well and washed with 150 µl of wash buffer II for minimum time of 5 min each at room temperature with gentle shaking. The wash buffer was completely removed in each wash step. 20 x wash buffer II was diluted with distilled water.

2.4.3.5 Incubation with detection antibody and washing

The detection antibody was reconstituted by adding 1.4 ml of sample diluent to the tube and spinned briefly. A volume of 80 µl of detection antibody cocktail was added to each

well and incubated at room temperature. The samples were decanted from each well and washed 5 times with 150 µl of 1 x wash buffer I and then 2 times with 150 µl of 1 x wash buffer II at room temperature with gentle shaking. The wash buffer was completely removed in each wash step.

2.4.3.6 Incubation with Cytochrome 3 (Cy3) equivalent dye- Streptavidin and wash

A volume of 1.4 ml of sample diluent was added to Cy3 equivalent dye-conjugated streptavidin tube after briefly spinning down and mixed gently. An aliquot or a volume of 80 µl of Cy3 equivalent dye- conjugated streptavidin was added to each well. The device was covered with aluminium foil to avoid exposure to light and incubated in a dark room at room temperature for one hour. The samples were decanted from each well and washed 5 times with 150 µl of 1x wash buffer I at room temperature with gentle shaking. The wash buffer was removed completely in each wash step.

2.4.3.7 Detection of fluorescence

The device was disassembled by pushing the clips outward from the side of the slide and the slide was carefully removed from the gasket. The slide was placed in a slide washer and enough amount of 1 x wash buffer was added to cover the whole slide and gently shaken at room temperature for 15 min. The wash buffer I was decanted and washed with about 30 ml of 1 x wash buffer II by gently shaking at room temperature for 5 min. The water droplets were completely removed by placing the slide in a washer and drying the glass chip by centrifugation at 1000 rpm for 3 min without cap.

2.4.3.8 Imaging

The imaging of the signals was visualised using a laser scanner equipped with a Cy3 wavelength Axon GenePix. Data extraction was done using microarray analysis software GenePix and quantitative analysis was done using Quantibody® Q-Analyser software.

2.5 Analysis of Red blood cell (RBC) membrane proteins using SDS-PAGE

In this series of experiments, the analysis of human RBC membrane proteins was done using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Membrane proteins including spectrin, ankyrin, band 3, band 4.1 and glycophorin were analysed and compared between T2DM patients and healthy controls. Isolation of membrane proteins from human RBC was performed using a kit method from Fermentas, UK and native extraction method.

2.5.1 Extraction of RBC membrane proteins by native method

2.5.1.1 Purification of RBC from whole blood

Fresh whole blood was obtained using EDTA tubes and stored at 4 °C. RBC were purified within 48 hours of withdrawal from the human donors. Human RBC were initially isolated by centrifugation at 600 x g for 20 min at 4 °C. After the careful removal of plasma and buffy coat layers by careful suction, the RBC pellet was resuspended in cold isotonic buffer (145 mM NaCl, 5 mM KCl, and 5 mM Hepes, (pH 7.4) by trituration and then recentrifuged at 2000 x g for 10 min at 4 °C. The RBC were washed with the isotonic buffer four times to completely remove the plasma and buffy layers. To remove white blood cells from the RBC suspensions, the samples were passed through the white blood cell filters (Plasmodipur, Euro-diagnostica B. V.) a total of two times.

At each step in the purification process, the upper RBC layer was removed. The final preparation of washed RBC was resuspended in cold isotonic buffer at pH 7.4 to an approximate hematocrit 50% and stored at 4 °C. All RBC samples were stored at 4 °C and used for analysis within 24 h of purification.

2.5.1.2 Purification of human RBC open ghosts

A volume of 1 ml of packed RBC was lysed by trituration and stirring on ice for 10 min in 100 ml of hypotonic lysing solution containing 15 mM KCl, 0.01 mM EDTA, 1 mM EGTA, and 5 mM Hepes solution, pH 6.0 at 4 °C to reduce premature, spontaneous resealing of the erythrocyte ghosts. The membranes were then centrifuged at 12,000 x g for 10 min at 4 °C. After discarding the supernatant, the membranes were then washed in 100 ml of hypotonic lysing solution without EGTA by trituration and recentrifuged at 12,000 x g for 10 min at 4 °C. Once again, after discarding the supernatant, the membranes were washed in 100 ml of EGTA –free hypotonic lysing solution containing 2 mM Mg^{2+} by trituration and re-centrifuged at 12,000 x g for 10 min at 4 °C. After removing the supernatant a final time, the erythrocyte ghost membranes were concentrated in 4 ml of the EGTA-free lysing solution containing 2 mM Mg^{2+} by centrifugation at 12,000 X g for 10 min at 4°C. This method has been shown to dilute most cytosolic components of RBC by 4 million fold.

2.5.1.3 RBC plasma membrane washes

Purified RBC open ghost membranes (300 µl) were suspended in 10 mM NaOH (30 ml) at 4 °C**.** The membrane suspensions were then placed on a nutator and were rotated at medium speed at 4 °C for 30 min. Washed membrane samples were then pelleted at 12,000 x g for 10 min and resuspended in fresh 10 mM NaOH (1 ml) at 4 °C. The washed membranes were then immediately subjected to proteolytic digestion with Proteinase K.

2.5.1.4 Proteolytic digestion of RBC plasma membrane

On purifying erythrocyte plasma membranes from human ghosts and washing the membrane samples with NaOH, the RBC ghost membranes were shaved by using Proteinase K, a serine protease with broad cleavage specificity. In this study, Proteinase

K was active over the pH range 7.5-12.0 but was most often used in the pH range 7.5-9.0. The activity of the enzyme was at a maximum at 37 ºC, but the activity was >80% of its maximum between 20 ºC and 60 ºC. Washed RBC ghost membranes were pelleted in a microcentrifuge at 12,000 x g at room temperature for 10 min. The supernatant was then removed and discarded and the pellet was resuspended in 10 mM NaOH.

Proteinase K was then added to the membrane suspension at a final concentration of 3.0 mg/ml per 100 µl of washed membranes for each shaving reaction and the samples were left on a nutator at medium speed for varying durations of time (up to 48 h) at room temperature. The digestions with Proteinase K were conducted at room temperature rather than at 37 °C where protease activity is maximised to reduce the amount of resealing of the erythrocyte open ghost membranes that occurred completely within 6 min at 37 °C.

At designated time points, PMSF, a protease inhibitor specific for Proteinase K, was added at a final concentration of 5 mM and the samples were left on a nutator at medium speed for 30 min to be sure that all of the Proteinase K activity was inhibited. The membranes were then pelleted in a microcentrifuge at 12,000 x g at room temperature for 10 min. The supernatant was then removed and discarded and the pellet was resuspended in 10 mM NaOH once again.

This washing procedure of the fully washed and shaved RBC open ghost membranes was repeated 2 more times for a total of four washes. In the final round, the membrane samples were resuspended in buffer K (15 mM KCl, 5 mM Hepes, 2 mM Mg^{2+}, 0.01 mM EDTA, pH 8.0). To be sure that Proteinase K and PMSF were removed from the membrane samples with the series of four wash steps, samples of the supernatants and membrane pellets from each wash step were run on 1D SDS/PAGE gels and silver-stained. By the fourth wash step, all the Proteinase K and PMSF were removed from the

membrane pellet. This particular gel system was chosen because it provided a much lower pH environment than traditional SDS/PAGE systems. The advantages of the lower pH include sharper band resolution and higher accuracy of results.

2.5.2 Extraction of RBC membrane proteins from suspension cells

Membrane proteins from human RBC were extracted using ProteoJET^{TM} Membrane Protein Extraction Kit (Fermentas Life Sciences, UK). The kit was designed for efficient isolation of membrane proteins from mammalian cells. This kit isolated high quality membrane and cytoplasmic protein extracts without cross-contamination between the two fractions. Both fractions contained pure, non-denatured functional proteins. Membrane protein fraction was used directly in SDS-PAGE analysis. Cells were first permeabilized with cell permeabilization buffer to release the cytoplasmic protein fraction. The cell debris was then treated with the membrane protein extraction buffer which selectively solubilized the majority of integral and membrane-associated proteins and isolated them as a separate fraction. The extraction procedure was performed on ice and ice-cooled buffers were used throughout this process. A protease inhibitor cocktail (Fermentas Life Sciences, UK) was added to cell permeabilization and membrane protein extraction buffers to minimize proteolysis. The detailed procedure included the following:

2.5.2.1 Cell harvesting and permeabilization

Approximately, 5 x 10^6 cells were pelleted by centrifugation for 5 min at 250 × g and the supernatant was discarded. The cells were resuspended in 3 ml of ice-cold cell wash solution, centrifugation was repeated for 5 min at 250 × g and the supernatant was discarded. A volume of 1.5 ml of ice-cold cell wash solution was added and transferred into a 2 ml Eppendorf tube. The cells were centrifuged for 7 min at 250 x g and the supernatant was discarded. A volume of 1.5 ml of ice-cold cell permeabilization buffer

was added to the solution and vortexed briefly to eliminate cell clumps. This solution was incubated for 10 min at 4 ºC while shaking continuously in a shaking water bath in the cold room.

2.5.2.2 Extraction of cytoplasmic proteins

The permeabilized cells were centrifuged at 16000 x g for 15 min at 4 ºC. After centrifugation, the supernatant was carefully removed (cytoplasmic protein extract) and was transferred into a new tube. This was stored as aliquots at -70 ºC. The cell debris pellet which contains membrane proteins was set on ice. All the liquid, which contained cytoplasmic proteins, was carefully removed.

2.5.2.3 Extraction of membrane proteins

A volume of 1 ml of ice-cold membrane protein extraction buffer was added to the cell debris pellet and pipetted thoroughly. This was incubated for 30 min at 4 ºC in the thermomixer, shaking at 1400 rpm. Clear membrane protein was extracted by centrifugation at 16000 x g for 15 min at 4 ºC. The supernatant (membrane protein fraction) was transferred into a new tube. This was used directly or stored as aliquots at -70 ºC for later analysis.

2.6 Protein quantification by Bradford Assay

Membrane proteins isolated from RBC were quantified using Bradford assay (Bradford, 1976) kit produced by Fermentas, UK.

2.6.1 Bovine Serum Albumin (BSA)

BSA standard set, ready-to-use, (Fermentas, UK), contained seven pre-diluted protein concentrations in the following standard solutions 2.0 mg/ml, 1.5 mg/ml, 1.0 mg/ml, 0.75 mg/ml, 0.50 mg/ml, 0.25 mg/ml and 0.125 mg/ml. The standard assay was performed in glass test tubes. The different concentrations of BSA mentioned above

were provided in 0.15 M NaCl solution containing 0.05% sodium azide as preservative. The Bradford reagent was gently mixed by inverting the bottle a few times before it was used, and the amount of reagent needed was aliquoted and equilibrated to room temperature. A volume of 20 µl of either each pre-diluted standard or sample was pipetted to be assayed into appropriately labelled test tubes. An aliquot of 1 ml of Bradford reagent was added to each tube and mixed well. The solution was then incubated at room temperature for 5 min and transferred into a cuvette. The spectrophotometer was set to 595 nm. The instrument was set to zero with the blank sample. The absorbance of either standard or the sample solution was measured. A standard curve was created by plotting the absorbance at 595 nm vs protein concentration of each protein standard (mg/ml). The protein concentration of unknown samples was determined by comparing their absorbance values against the standard curve.

2.6.2 SDS-PAGE Electrophoresis

Analysis of membrane proteins was performed using SDS-PAGE electrophoresis using a mini protean system with 12% Ready gel- Tris HCl gel (Bio-Rad Laboratories, UK). 10 x Tris- glycine-SDS electrophoresis buffer was used as running buffer (Fermentas, UK).

Protein sample was loaded into SDS-PAGE gels according to manufacturer's protocol. Dual Color Protein Loading Buffer (Fermentas, UK) was used for the preparation of protein samples for SDS-PAGE. Some of the proteins were sensitive to pH changes and temperature fluctuations during electrophoresis in Tris buffers. As such, the optimised composition of this loading buffer prevented protein degradation during sample heating prior to SDS-PAGE and during the electrophoresis run. PageRuler^{TM} Plus Prestained Protein Ladder (Fermentas, UK) was used as it was a mixture of 9 recombinant, highly

purified individually prestained proteins with apparent molecular weights of 10-250 kDa and contained two coloured reference bands orange and green. This ladder was provided in a loading buffer for direct use. The ladder was thawed to room temperature or at 37-40 ºC for a few min to dissolve any precipitated solids and mixed gently and thoroughly, to ensure that the solution was homogeneous. A volume of 5 µl of the ladder was directly loaded on the SDS-PAGE gel (Table 2.1).

Table 2.1: Step by step procedure for SDS-PAGE.

Step	**Procedure**
Thawing	The reducing agent was thawed at room temperature. Any precipitated solids in the loading buffer were dissolved at 37 ºC.
Mixing	The components of the pack were vortexed gently to ensure that solutions are homogeneous. A volume of 2 µl of 20 x reducing agent was placed into a clean micro centrifuge tube and 10 µl of 4 x DualColor protein loading buffer was added to it. Each protein sample was added and the total volume was made up to 40 µl using nuclease free water.
Denaturing	Samples were heated at 100 ºC for 3-5 min
Loading	Loading of the samples was done by applying directly to SDS-polyacrylamide gel after centrifuging the samples briefly.

2.6.3 Silver Staining Method

PageSilver™ staining kit from Fermentas was used to stain SDS-Page gels after the electrophoresis. This kit facilitated rapid and sensitive staining of proteins in polyacrylamide gels. This procedure was better than traditional Coomassie Blue staining and allowed detection of very low abundance proteins. In this process, the protein was bound to silver nitrate at a weakly acidic pH followed by subsequent reduction of silver ions to metallic silver by formaldehyde at an alkaline pH. Silver staining included the following steps:

2.6.3.1 Preparation of Fixative Solutions

Fixative solutions in volumes sufficient to stain one gel were prepared and stored in tightly closed bottles at room temperature (Table 2.2).

Table 2.2: Preparation of gel fixing solutions for Silver Staining.

Reagent	**Gel Fixing Solution 1**		**Gel Fixing Solution 2**		
	Volume	Final Concentration	Volume 1	Volume 2	Final Concentration
Ethanol	50 ml	50% (v/v)	60 ml	90 ml	30% (v/v)
Glacial acetic acid	10 ml	10% (v/v)	-	-	-
Water (ultrapure)	40 ml	-	140 ml	210 ml	-
Total volume	**100 ml**	**-**	**200 ml**	**300 ml**	**-**

2.6.3.2 Preparation of staining solutions

The following four staining solutions were prepared using the reagents provided in the kit. The staining solutions were prepared on the same day prior to staining. Formaldehyde was added only immediately prior to staining. Staining solutions sufficient for staining one gel were prepared and stored in marked clean glass bottles and indicated volumes of reagents.

Table 2.3: Preparations of reagents/staining solutions for Silver Staining.

Reagent	**Sensitizing Solution**	**Staining Solution**	**Developing Solution**	**Stop Solution**
Sensitizer concentrate	0.4 ml	-	10 µl	-
Staining reagent	-	4 ml	-	-
Developing reagent	-	-	10 ml	-
Stop reagent	-	-	-	8 ml
Water (ultrapure)	to 100 ml	to 100 ml	to 100 ml	92 ml
Formaldehyde	-	54 µl	27 µl	-
Total volume	**100 ml**	**100 ml**	**100 ml**	**100 ml**

Staining protocol for maximum sensitivity of protein staining was used which enables visualisation of 0.05 ng of protein per band in approximately 2 hour 40 min. This protocol did not involve any microwave steps (Table 2.3 and 2.4).

Table 2.4: Silver Staining protocol for maximum staining.

Step	Procedure
1	**Gel fixing 1** The gel was placed into a staining tray and rinsed briefly with deionised water. A volume of 100 ml of gel fixing solution 1 was added and gently agitated for 60 min. Gel fixing solution 1 was discarded.
2	**Gel fixing solution 2 and Washing** The gel fixing 2 procedure was performed three times for maximum sensitivity. A volume of 100 ml of gel fixing solution 2 was added to the gel and gently agitated for 20 min. The gel fixing solution 2 was discarded after agitation. A volume of 100 ml of deionised water was added and gently agitated for 20 seconds. Deionised water was discarded and washing procedure was repeated.
3	**Sensitising and Washing** A volume of 100 ml of sensitising solution was added to the gel and gently agitated for 1 min. The sensitising solution was discarded after agitation. A volume of 100 ml of deionised was added and gently agitated for 20 seconds. Deionised water was discarded and washing procedure was repeated.
4	**Staining and Washing** A volume of 100 ml of staining solution was added to the gel and gently agitated for 20 min. The staining solution was discarded after agitation. A volume of 100 ml of deionised water was added and gently agitated for 20 seconds. Deionised water was discarded and washing procedure was repeated.
5	**Developing** A volume of 100 ml of developing solution was added to the gel and

	gently agitated for approximately 4 min until bands are well-developed and the required intensity was reached. The developing solution was discarded.
6	**Terminating** A volume of 100 ml of stop solution was added to the gel and gently agitated for 10 min. The stop solution was discarded and the gel was stored in deionised water or dried according to recommendations.

2.6.3.3 Gel imaging

Gel imaging was performed using ChemiDoc^{TM} XRS (Bio-Rad, UK). Lanes and protein bands in each lane were automatically detected and expressed in terms of intensity, arbitrary units (AU). The bands were compared between T2DM and healthy controls for analysis.

2.7 Measurement of cytosolic free calcium concentration ($[Ca^{2+}]_i$) in human lymphocytes and neutrophils of T2DM and healthy controls

2.7.1 Preparation of cells

Lymphocytes and neutrophils were prepared from blood samples of both T2DM and healthy control blood subjects using the following protocol.

2.7.2 Preparation of lymphocytes

Blood was collected in an EDTA tube by venipuncture technique after informed consent. Ficoll Histopaque from Sigma Aldrich was used as separating medium. Cells were isolated after Density Gradient centrifugation at 600 g for 30 min at room temperature. The upper phase of lymphocytes was transferred into polypropylene tubes and washed with PBS which was stored in a water bath at 37 ºC. The cells were

centrifuged at 480 x g for 10 min at room temperature. The supernatant was discarded and each pellet was resuspended in 4 ml of RPMI (Sigma Aldrich, UK).

A volume of 16 µl of 4 µM Fura-2 AM (fura-2 acetoxymethyl ester) was added to each tube and incubated in dark at room temperature. After incubation, the cells were centrifuged at 480 x g for 10 min at room temperature and the supernatant was discarded. The cells were resuspended by adding 1 ml of Na-HEPES (1 x) per cuvette and left for 10-15 min for cleaving.

2.7.3 Preparation of neutrophils

Blood was collected in an EDTA tube by venipuncture technique after informed consent. Ficoll Histopaque from Sigma Aldrich was used as separating medium. Cells were isolated after Density Gradient centrifugation at 600 x g for 30 min at room temperature. The lower interphase of neutrophils were transferred into polypropylene tubes and washed with PBS which was stored in a water bath at 37 ºC. The cells were centrifuged at 480 x g for 10 min at room temperature. After centrifugation, the supernatant was discarded and 3-4 ml of cold ammonium chloride was added to each tube and stored at 4 ºC for 5 min to lyse the red blood cells in the pellet. The tubes were centrifuged at 480 x g for 10 min at room temperature and the supernatant was discarded. Each pellet was resuspended in 4 ml of RPMI (Sigma Aldrich). A volume of 16 µl of 4 µM Fura-2 AM was added to each tube and incubated for 30 min in dark at room temperature. After incubation, the cells were centrifuged at 480 x g for 10 min at room temperature and the supernatant was discarded. The cells were resuspended by adding 1 ml of Na-HEPES (1 x) per cuvette and left for 10-15 min for cleaving.

2.7.4 Measurement of intracellular free calcium concentration $[Ca^{2+}]_i$ in lymphocytes

Fura-2-loaded human lymphocytes from healthy and T2DM patients were stimulated with 1 µM thapsigargin (TG) in normal calcium solution (1.2 mM $[Ca^{2+}]_0$). A volume of 1 ml of suspended lymphocytes was taken in a cuvette and 10 µl thapsigargin was added to the cells to measure the level of intracellular Ca^{2+} release as a result of the thapsigargin stimulation. For calibration of the curve, a volume of 6 µl of triton was added, left for 50 seconds and then 200 µl of 0.5 M EGTA was added. Fluorescence of the sample was measured from the above 1 ml aliquots of magnetically stirred lymphocyte suspensions were observed using a Shimadzu spectrofluorophotometer with excitation at 340/380 nm and emission at 505 nm and changes in $[Ca^{2+}]_c$ were calculated using the fura-2 340/380 ratio and were calibrated according to the method previously described by Espino *et al.* (2009).

2.7.5 Measurement of intracellular free calcium concentration $[Ca^{2+}]_i$ in neutrophils

A volume of 1 ml of suspended neutrophils was taken in a cuvette and 10 µl of fMLP Formyl-Methionyl-Leucyl-Phenylalanine (final concentration 10 mM) was added to the cells to measure the level of intracellular Ca^{2+} release induced by fMLP. For calibration, a volume of 6 µl of triton was added, left for 50 seconds and then a volume of 200 µl of 0.5 M EGTA was added. Fluorescence of the sample was measured from the above 1 ml aliquot of magnetically stirred neutrophil suspensions using a Shimadzu spectrofluorophotometer with excitation at 340/380 nm and emission at 505 nm. Changes in $[Ca^{2+}]_c$ were calculated using the fura-2 340/380 ratio and values were calculated. Calcium release were estimated using the integral of the rise in $[Ca^{2+}]_c$ for 2.5 min after addition of thapsigargin or fMLP. Calcium release were expressed as

nanomolar taking a sample every second (nM • s), as previously described by Espino *et al.* (2009).

2.8 Measurement of low molecular weight AGEs in serum of T2DM and healthy control samples

Low molecular weight AGEs in serum samples of T2DM and healthy controls were measured using the following procedure:

Plasma samples were thawed to room temperature and 0.2 ml of serum sample was diluted with 1.8 ml distilled water. Fluorescence of 10 fold diluted serum samples was measured in a luminesecence spectrometer at a wavelength of 420 nm after excitation at 350 nm. The measurement was performed in triplicate. Haemolytic samples were excluded. Fluorescence intensity standards were used to calibrate and monitor the performance of the instrument and calibrated against quinine sulphate (1 mg/ml) in HSO. Deionised water was used as blank to zero the instrument. The fluorescence signals of samples were expressed as the relative fluorescence intensity in arbitrary unit (A.U) and multiplied by the dilution factor.

2.9 Antioxidant assay

Antioxidant assay was performed on plasma samples from both T2DM and healthy control samples using Antioxidant assay kit method (Cayman Chemical Company, USA). This kit was used to measure the total anti-oxidant capacity of plasma. This kit gave the combined anti-oxidant activities of all its constituents including vitamins, proteins, lipids, glutathione, uric acid, etc. The assay relied on the ability of anti-oxidants in the sample to inhibit the oxidation of ABTS® (2, 2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) to $ABTS^{®•+}$ by metmyoglobin. The amount of $ABTS^{®•+}$ produced was monitored by reading the absorbance at 405 nm. The capacity

of the anti-oxidants in the sample to prevent ABTS® oxidation was compared with that of Trolox, a water soluble tocopherol analogue, and was quantified as millimolar Trolox equivalents.

2.9.1 Reagent Preparation

Some of the kit components were provided in lyophilized (under vacuum) or concentrated form and were reconstituted prior to use.

2.9.1.1 Antioxidant Assay Buffer

A volume of 3 ml of concentrated assay buffer was diluted with 27 ml of HPLC-grade water. This diluted assay buffer (5 mM potassium phosphate, pH 7.4, containing 0.9% sodium chloride and 0.1% glucose) was used to reconstitute the metmyoglobin.

2.9.1.2 Antioxidant Assay Chromogen

Chromogen was supplied as lyophilised powder of ABTS®. This was reconstituted by adding 6 ml of HPLC-grade water to the vial and vortexed well. The reconstituted reagent was stable for 24 h at 4 ºC.

2.9.1.3 Antioxidant Assay Metmyoglobin

This was provided in lyophilised powder of metmyoglobin. The content was reconstituted by adding 600 µl of assay buffer and vortexed well. The reconstituted reagent was stable for at least one month at -20 ºC.

2.9.1.4 Antioxidant Assay Trolox

Lyophilized powder of Trolox (6-Hydroxy-2,5,7,8-tetramethylchouroman-2-carboxylic acid) was reconstituted by adding 1 ml of HPLC-grade water and was vortexed well. The reconstituted reagent was used to prepare the Trolox standard curve. The reconstituted reagent was stable for 24 h at 4 ºC.

2.9.1.5 Antioxidant Assay Hydrogen Peroxide

Hydrogen peroxide was supplied as a stock solution of 8.82 M. A volume of 10 µl of this solution was diluted with 990 µl of HPLC-grade water. It was further diluted by removing 20 µl and added to 3.98 ml of HPLC-grade water to yield a 441 µM working solution. The hydrogen peroxide working solution was stable for 4 hours at room temperature.

2.9.2 Sample Preparation (Serum)

Blood sample was collected without using an anticoagulant and then allowed to clot for 30 min at 25 ºC. Blood was then centrifuged at 2000 x g for 15 min at 4 ºC. After centrifugation, the top yellow serum layer was pipetted off without disturbing the white buffy layer. Serum was then stored on ice. Before assaying serum was diluted to 1:20 and 1:30.

2.9.3 Performing the Assay

The assay was performed using 96 well microtitre plate. Trolox standards were prepared in seven clean glass tubes. Amount of reconstituted Trolox and assay buffer was added to each tube as described in table 2.5.

Table 2.5: Preparation of Trolox standard for making standard curve.

Tubes	Reconstituted Trolox (µl)	Assay Buffer (µl)	Final Concentration (mM Trolox)
A	0	10000	0
B	30	970	0.045
C	60	940	0.090
D	90	910	0.135
E	120	880	0.18
F	150	850	0.225
G	220	780	0.330

Master mix was prepared for Trolox standard wells using 10 µl of Trolox standard (tubes A-G), 10 µl of metmyoglobin and 150 µl of chromogen per well. For each sample well, 10 µl of sample was added in instead of Trolox standards along with 10 µl of metmyoglobin and 150 µl of chromogen per well. The reaction was then initiated by adding 40 µl of hydrogen peroxide working solution to all the wells that were used. Hydrogen peroxide was added as quickly as possible according to the protocol. After adding all the reagents, the 96 well plate was covered with a plate cover and incubated on a shaker for 5 min at room temperature. Absorbance of the samples in each well was measured in a Tecan plate reader at 405 nm.

2.9.4 Analysis of results

Average absorbance of each standard and sample was calculated and the standard graph was plotted as a function of the final Trolox concentration (mM) from the table 2.5. Antioxidant concentration of the samples was then calculated using the equation obtained from the linear regression of the standard curve by substituting the average absorbance values for each sample into the equation:

$$\text{Antioxidant (mM)} = \frac{\text{Sample average absorbance} - \text{y intercept}}{\text{Slope}} \times \text{Dilution}$$

2.10 Comparison of osmotic fragility of RBC's of T2DM and healthy controls.

Erythrocytes placed in hypotonic solutions, absorbed water osmotically to reach a critical volume which resulted in their lysis due to the fragility of their membranes. This phenomenon of osmotic fragility was originally described by Parpart *et. al*., in 1946 (Parpart *et al.*, 1946). In this study, the osmotic fragility of RBC was compared between T2DM patients and healthy controls. Blood was collected in EDTA tubes by a venipuncture technique after obtaining consent from both T2DM patients and healthy

controls. This experiment was performed within 2 h after the withdrawal of blood. Blood tubes were centrifuged at 3500 rpm for 10 min to separate the plasma from the buffy coat and RBC. A volume of 50 µl of RBC was added to each solution of NaCl in increasing concentrations and incubated for 30 min at room temperature. After incubation, the tubes were centrifuged at 1500 rpm for 10 min and absorbance of the supernatant in each tube was measured in a spectrophotometer at 540 nm.

The result of osmotic fragility test (OFT) was expressed as the concentration of NaCl causing 50% haemolysis and the median corpuscular fragility for fresh normal samples was taken between 4.0 and 4.45 g/L. Percentage of haemolysis for each solution was plotted against NaCl concentrations. The osmotic fragility curve was then compared between T2DM and healthy controls. This experiment required preparation of a series of hypotonic solutions with NaCl ranging from 0.1% to 0.9%. Dilutions of different concentrations of buffered NaCl (100g/L) were made from the stock solution as given in the table 2.6 below:

Table 2.6: Preparation of dilutions using different concentration of NaCl for measurement of osmotic fragility.

Tubes	Concentrations (g/L)	1% NaCl (ml)	Deionised water (ml)
1	0	0	5.0
2	2.8	1.4	3.6
3	3.2	1.6	3.4
4	3.6	1.8	3.2
5	4.0	2.0	3.0
6	4.4	2.2	2.8
7	4.8	2.4	2.6
8	5.2	2.6	2.4
9	5.6	2.8	2.2
10	6.0	3.0	2.0
11	6.4	3.2	1.8
12	6.8	3.4	1.6
13	7.2	3.6	1.4
14	10	5	0

2.11 Statistical analysis

All the data from T2DM patients and age-matched controls from different experiments were analysed and compared using Student's t-test and ANOVA test. Data obtained are presented as mean ± standard error of the mean (SEM) A value of $p<0.05$ was taken as significant. A value of $p<0.001$ was taken as highly significant.

Chapter 3

Measurement of intracellular calcium in neutrophils and lymphocytes of T2DM patients and healthy controls

3.1 Objectives of this study

To measure basal $[Ca^{2+}]_i$ and to investigate the effect of either thapsigargin or fMLP on $[Ca^{2+}]_i$ in neutrophils and lymphocytes from T2DM patients and healthy age-matched control subjects for comparison.

3.2 Introduction

It is known well that in T2DM condition where the host defence mechanism is highly compromised (Kumar and Clark, 2007). Impaired functions of granulocytes and lymphocytes and several other immunological dysfunctions have been reported in studies relating to diabetic patients and animals (Marhoffer *et al.*, 1992; Alexiewicz *et al.*, 1995). There are many on-going studies trying to elucidate the association between diabetes and leukocyte functions. Calcium plays a very important role as a second messenger in mediating cellular regulation (Berridge, 1995). A disturbance in the intracellular free calcium concentration $[Ca^{2+}]_i$ is a major cause of metabolic disorders and there is much evidence that Ca^{2+} plays an important regulatory role in the cascade of insulin-generated signals and β-cell function. An elevated or sustained level of calcium is the major reason to diminish cellular sensitivity to insulin and contributes to the pathogenesis of T2DM. In this chapter, $[Ca^{2+}]_i$ was measured in lymphocytes and neutrophils of T2DM subjects and compared with healthy controls. Formyl-methionyl-leucyl-phenylalanine (fMLP) and thapsigargin-induced changes in $[Ca^{2+}]_i$ of both lymphocytes and neutrophils were evaluated using spectrophotometric methods (Espino *et al*, 2009).

3.2.1 Role of calcium in lymphocytes

Calcium signals are crucial for various functions of all immune cells including differentiation, effector function, and gene transcription. $[Ca^{2+}]_i$ ions are increased

through the sequential operation of two interdependent processes: depletion of endoplasmic reticulum Ca^{2+} stores as a result of binding of inositol trisphosphate (IP_3) to IP_3 receptors, followed by 'store-operated' Ca^{2+} entry through plasma membrane Ca^{2+} channels (Berridge, 1995; Peterson *et al*, 1994).

In lymphocytes, mast cells and other immune cell types, store-operated Ca^{2+} entry through specialized Ca^{2+} release-activated calcium (CRAC) channels constitutes the major pathway of intracellular Ca^{2+} increase. In lymphocytes, the main mechanism for the entry of extracellular Ca^{2+} across the plasma membrane is store-operated Ca^{2+} entry (SOCE) through Ca^{2+} release-activated calcium (CRAC) channels. Opening of CRAC channels leads directly to the sustained increase of intracellular Ca^{2+} concentrations resulting in both short-term and long-term functional consequences (Berridge 1995; Peterson *et al*, 1994).

3.2.2 Role of calcium in neutrophils

Neutrophils play a significant role in immune response during the initial stages of host response to infection via a series of processes which include chemotaxis, phagocytosis and the production of reaction oxygen species (ROS) (Roitt *et al.*, 1996; Vlahos *et al.*, 1995). This leads to an activation of phosphatidylinositol cascade, resulting in inositol triphosphate (IP3) and diacylglycerol (DAG) production which is an activator of protein kinase C (PKC) (Hu *et al.*, 1999). These processes are calcium dependent in which $[Ca^{2+}]_i$ acts as a second messenger in most of signal transduction pathways. It is also shown that $[Ca^{2+}]_i$ is actively involved in insulin signalling where it actively depends on intracellular calcium swings rather than large calcium swings resulting by influx from intracellular compartments (Clausen *et al.*, 1974; Jakubczak *et al.*, 2006).

In T2DM, neutrophil dysfunction is considered to augment bacterial infection (Repine *et al.*, 1980; Marhoffer *et al.*, 1992) and cardiovascular disease (Berliner *et al.*, 2000; McDonagh *et al.*, 1997). Neutrophils from patients with T2DM also show signs of impaired phagocytosis, chemotaxis, adhesion and oxidase activity. Alterations in the level of $[Ca^{2+}]_i$ is responsible for many functions of neutrophils. $[Ca^{2+}]_i$ transients are essential for the release of neutrophil granule contents and oxidase activity as well as neutrophil adherence and chemotaxis (Jaconi *et al.*, 1988; Lew, 1989; Levy *et al.*, 1994a; Alteraifi & Zhelev, 1997) .There is also increasing evidence that $[Ca^{2+}]_i$ levels are higher in most tissues in T2DM (Levy *et al.*, 1994b). In T2DM, normalization of hyperglycaemia is associated with a lowering of $[Ca^{2+}]_i$ to non-diabetic levels and an associated improvement in neutrophil function (Alexiewicz *et al.*, 1995).

The initial mechanism involved in the response of leukocytes to inflammatory stimuli is mobilization of $[Ca^{2+}]_i$. Activation of Polymorphonuclear leukocytes (PMNs) by chemoattractants like N-formyl-,methionyl-leucyl-phenylalanine (fMLP) induce changes in $[Ca^{2+}]_i$. This process is characterised by three major steps: a rapid increase, which is followed by a small sustained increase which returns to the initial levels in the final stage. Calcium release from internal stores is mediated by inositol 1,4,5-triphosphate (IP). Response of $[Ca^{2+}]_i$ is biphasic with a transient release of Ca^{2+} from intracellular stores (calciosomes) which is followed by a delayed influx of extracellular Ca^{2+} across the plasma membrane as it requires extracellular calcium $[Ca^{2+}]_o$. The principal second messengers for Ca^{2+} influx across the plasma membrane is possibly Ca^{2+} release from intracellular stores and Ins(1,4,5)P3 and inositol 1,3,4,5-tetrakisphosphate (Ins1,3,4,5)P4 as neutrophils do not express voltage-operated Ca^{2+} channels (Kelly *et al.*, 1989; Popko *et al.*, 2003).

The mechanism of store-mediated calcium entry (SMCE) is not very well understood and several hypotheses have been proposed (Alvarez *et al.*, 1992; Fasolato *et al.*, 1993; Randriamampita & Tsien, 1993; Sargeant *et al.*, 1993). An integrated secretion-like coupling model involves trafficking of the Ca^{2+} stores towards the cell surface and a physical interaction with the plasma membrane (Patterson *et al.*, 1999; Rosado & Sage, 2000a). The actin cytoskeleton plays a major role in intracellular trafficking, however, cortical actin filaments hinder coupling of Ca^{2+} stores with the plasma membrane. Therefore, cytoskeletal remodelling is a key event in SMCE (Rosado & Sage, 2000b). It has been shown earlier that neutrophil actin polymerization is impaired in T2DM (Advani *et al.*, 2002). As SMCE is dependent on cytoskeletal remodelling it is hypothesized that, in response to stimulation, Ca^{2+} influx across the plasma membrane would be impaired in neutrophils from patients with T2DM.

Calcium plays a central role in signal transduction for many responses in neutrophils (Sha'afi *et al.*, 1981). An early increase in $[Ca^{2+}]_i$ ~ has been recorded after stimulation of neutrophils with a variety of agents (Bengtsson *et al.*, 1986; Pozzan *et al.*, 1983). Neutrophil adhesion is a major event of the early inflammatory response that is characterized by the recruitment of neutrophils into areas of inflammation, which begins with the binding of these cells to endothelium followed by their transmigration into tissues (Albelda *et al.*, 1994).

Distinct phases have been characterized during neutrophil binding, including rolling, activation, and firm adhesion (Ley, 1996). The molecular basis for these phases is the up-regulation of various cell adhesion molecules that belong to three major families. These include the selectins (e.g., P-selectin, L-selectin, and Eselectin), the b2

integrins (e.g., CD11/CD18), and the immmunoglobulin super family (e.g., intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and platelet endothelial cell adhesion molecule-1) (Albelda *et al*., 1994).

The main aim of this chapter of the study was to measure basal $[Ca^{2+}]_i$ and to investigate the effect of either thapsigargin or fMLP on $[Ca^{2+}]$ homeostasis in neutrophils and lymphocytes of T2DM patients and healthy age-matched control subjects for comparison especially since derangement in $[Ca^{2+}]_i$ homeostasis is associated with several diseases.

3.3 Methods

As described in chapter 2 of this thesis.

Table 3.1 Subjects recruited for this study

	Healthy controls	**T2DM**
No of subjects	6	6
Age group	30-60 years	30-60 years
Gender	4 Male & 2 Female	5 Male & 1 Female
Diabetic status	Non-T2DM	T2DM
Treatment	-	Insulin
Ethnicity	European	European

3.4 Results

3.4.1 Effect of fMLP on calcium release in neutrophils of T2DM and healthy controls.

Basal $[Ca^{2+}]_i$ in lymphocytes and neutrophils of age-matched control subjects was 100±0.01 nM and 75.0±0.01 nM, respectively. Basal $[Ca^{2+}]_i$ in lymphocytes and neutrophils of T2DM patients was 0.92±0.01 nM (n=16) and 0.87±0.01 nM (n=16), respectively. These values show no significant differences between basal $[Ca^{2+}]_i$ for control and T2DM patients. Graphs were plotted to compare the difference of calcium release from neutrophils of T2DM patients and healthy controls and the results are presented in Figure 3.1.

Figure 3.1 shows original time course chart recordings of $[Ca^{2+}]_i$ level in neutrophil of T2DM patients and healthy age-matched controls following stimulation with fMLP. The data show that fMLP can elicit a large and transient increases in $[Ca^{2+}]_i$ from neutrophils of both control and diabetic subjects. However, the release of Ca^{2+} was much more prominent in neutrophils of control subjects compared to T2DM subjects. The results also show that $[Ca^{2+}]_i$ reach a maximum level after 20-30 sec of fMLP stimulation followed by a rapid decline but remained slightly above the basal level. These experiments were repeated over 24 times for neutrophils of 6 T2DM patients and 6 control subjects and the peak responses for each were analysed and plotted as bar charts in Figure 3.1B. The results show that neutrophils of T2DM patients contain significantly ($p<0.05$) less $[Ca^{2+}]_i$ compared to neutrophils from healthy age-matched controls.

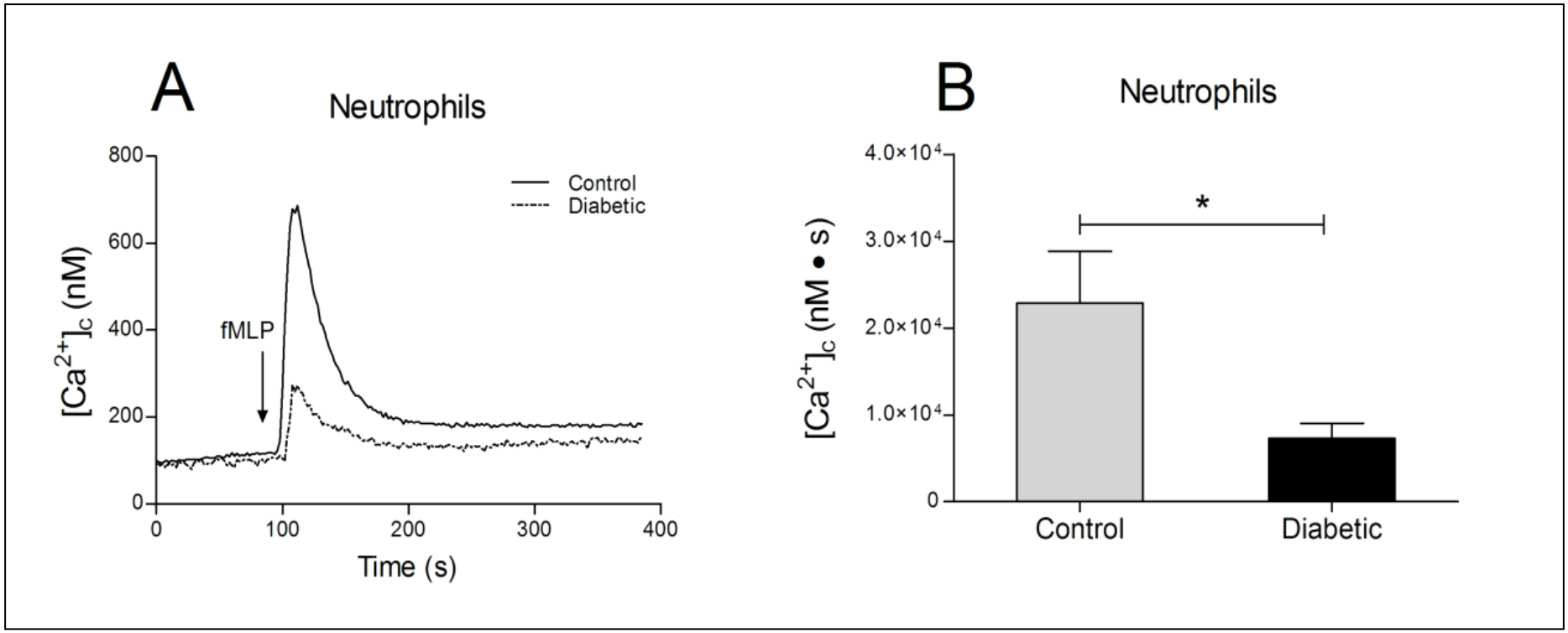

Figure 3.1 – (A) Original time course chart readings showing $[Ca^{2+}]_i$ transients in human neutrophils of age-matched controls and T2DM patients following fMLP stimulation. (B) Bar charts showing mean ±SD data for $[Ca^{2+}]_i$ from fMLP stimulated neutrophils of age-matched controls and T2DM human subjects (n=6), $^{*}p<0.001$ for diabetic compared to control. Note the significant elevation in $[Ca^{2+}]_i$ for T2DM patients compared to control.

3.4.2 Effect of thapsigargin (TG) on calcium release in neutrophils of T2DM patients and healthy controls.

Thapsigargin is a major Ca^{2+} mobilising secretagogue (Berridge 1995; Peterson *et al*, 1994). It specifically acts on the endoplasmic reticulum (ER) in epithelial cells and release Ca^{2+}. As such, TG was employed in this study to analyse $[Ca^{2+}]_i$ release from WBC.

Similarly, calculations were performed and graphs were plotted to compare the difference of calcium release between T2DM patients and healthy controls. A significant difference was observed when neutrophils were stimulated with thapsigargin under presence of normal calcium conditions. A decreased response to thapsigargin was observed in T2DM patients when compared with healthy controls (Figure 3.2).

Original traces are representative of six independent experiments (Figure 3.2 A). Histogram represents the integral for 2.5 min of the calcium release, in healthy controls and T2DM patients, calculated as described in Methods section. Values are mean ± SD of six independent experiments $^{*}P < 0.05$ (Figure 3.2 B). The results clearly show that thapsigargin can evoke significant and sustained increases in $[Ca^{2+}]_i$ in neutrophils from T2DM patients and age-matched controls compared to basal $[Ca^{2+}]_i$. Moreover, the results also show that neutrophils from T2DM patients produced significant ($P<0.01$) less $[Ca^{2+}]_i$ compare to neutrophils from age-matched control subjects following thapsigargin stimulation.

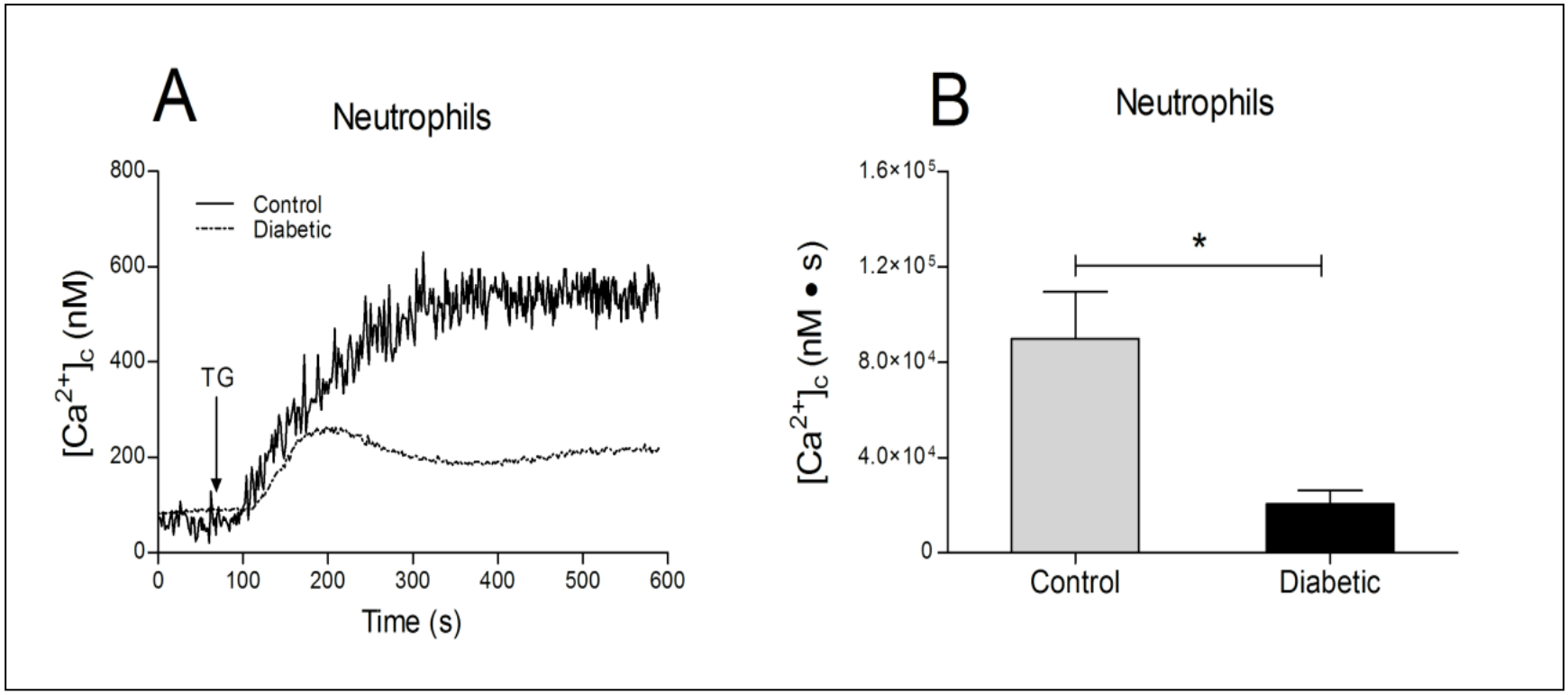

Figure 3.2 - (A) Original time course chart readings showing $[Ca^{2+}]_i$ transients in human neutrophils of age-matched controls and T2DM patients following thapsigargin stimulation. (B) Bar charts showing mean ±SD data for $[Ca^{2+}]_i$ from thapsigargin stimulated neutrophils of age-matched controls and T2DM human subjects (n=6), $^{*}p<0.001$ for diabetic compared to control. Note the significant elevation in $[Ca^{2+}]_i$ for T2DM patients compared to control.

3.4.3 Effect of thapsigargin on calcium release in lymphocytes of T2DM patients and healthy controls.

Figure 3.3 (A) shows the effect of thapsigargin (TG) on $[Ca^{2+}]_i$ from lymphocytes of T2DM patients and age-matched control subjects. The results show (A) time course original chart readings of Ca^{2+} release and (B) the mean±SD peak responses following TG stimulation.

The graphs in Figure 3.3B were plotted to compare the difference of calcium release between T2DM patients and healthy controls. The results show that thapsigargin can evoke significant ($p<0.05$) and sustained increase in from neutrophils of healthy age-matched control subjects compare to basal release. Analysis of the data show a significant ($p<0.05$) difference when lymphocytes from T2DM patients were stimulated with thapsigargin under presence of normal calcium conditions compared to healthy controls (see Figure 3.3 B).

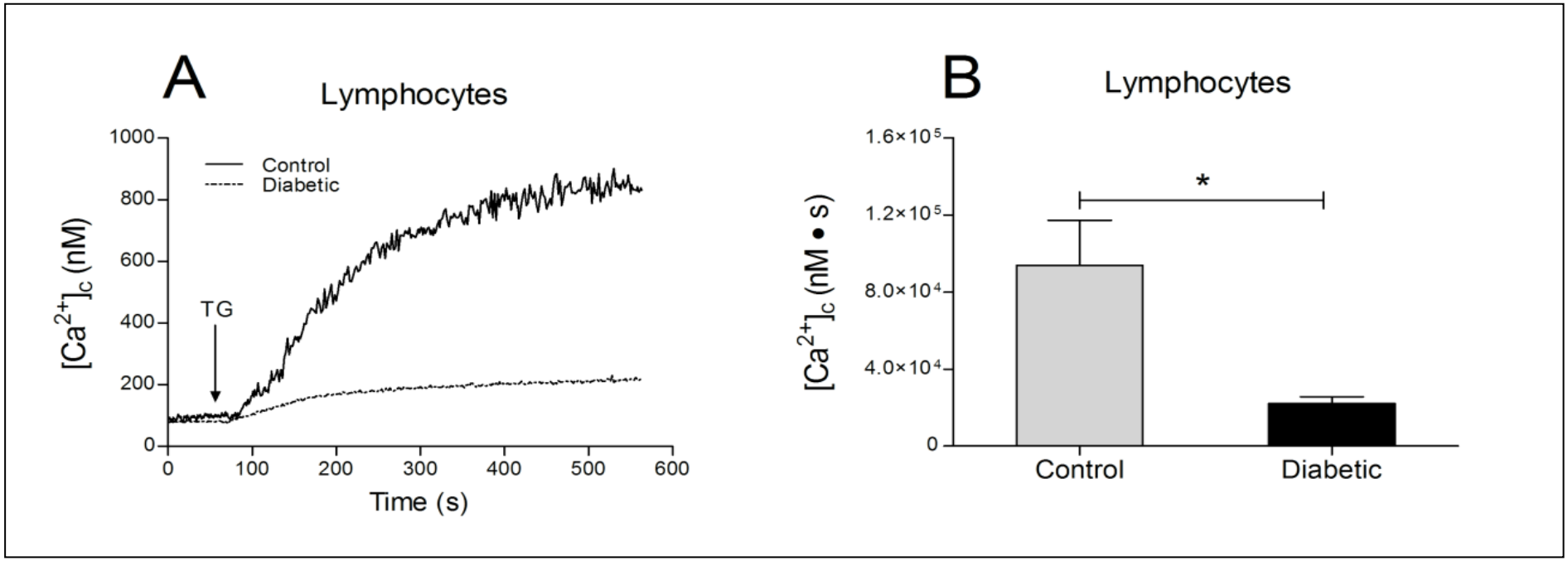

Figure 3.3 – (A) Original time course chart readings showing $[Ca^{2+}]_i$ transients in human lymphocytes of age-matched controls and T2DM patients following thapsigargin stimulation. (B) Bar charts showing mean ±SD data for $[Ca^{2+}]_i$ from thapsigargin stimulated lymphocytes of age-matched controls and T2DM human subjects (n=6), $^{*}p<0.001$ for diabetic compared to control. Note the significant elevation in $[Ca^{2+}]_i$ for T2DM patients compared to control.

3.5 Discussion

The common links among several clinical disorders including T2DM and cardiovascular disease have been suggested in various studies (Gonzalez Flecha *et al.*, 1990; Levy *et al.*, 1994a; Schaffer & Mozaffari, 1996). One of the most common reported clinical manifestations is insulin resistance. However, there is limited data available about the underlying molecular basis for insulin resistance and cardiovascular dysfunction. The major findings made in the present study are the significant and marked changes in $[Ca^{2+}]_i$ in neutrophils and lymphocytes from T2DM patients compared to healthy controls. The measurement of Ca^{2+} concentration was determined by detection of the fluorescent dyes Fura 2. Bacterial peptide fMLP and thapsigargin were used for stimulation of lymphocyte and neutrophil. The results of this study show a significant decrease in peak $[Ca^{2+}]_i$ after stimulation with either fMLP or thapsigargin, reflecting impaired activity of neutrophils and lymphocytes from T2DM patients when compared with healthy controls.

Insulin secretion is a calcium-dependent biological process and an elevation of calcium is required for both first and second-phase insulin secretions. T2DM is associated with a shift from biphasic to monophasic insulin release, which might result from a functional impairment of $Ca_v2.3$ Ca^{2+} channels (Berridge, 1993). The threshold of blood glucose, which normally causes sustained second phase insulin release is increased. Elevated level of serum calcium is the reflection of extracellular calcium possibly in conjunction with impairments of voltage-gated Ca^{2+} channels (Berridge, 1993). There is also evidence of a link between serum calcium levels and cellular function relating to cytosolic calcium (Boris, 1988) .

This altered metabolic response detected in granulocytes (neutrophil and lymphocytes) from T2DM patients under stimulation with fMLP may be a reflection of a disease-

induced adaptive metabolic response. Defects in intracellular Ca^{2+} homeostasis have been implicated in the impaired mechanical performance of the diabetic heart (Roe *et al.*, 1994). Deranged $[Ca^{2+}]_i$ may also play a role in the development of other diabetic complications, such as nephropathy and cardiomyopathy (Pierce & Russell, 1997). It is suggested that albuminuria in T2DM patients is associated with a primary defect in $[Ca^{2+}]_i$ homeostasis (Volzke *et al.*, 2006). The results of the present study show an appreciable impairment of Ca^{2+} kinetics in neutrophils and lymphocytes from T2DM patients compared to control. Thus, decreased $[Ca^{2+}]_i$ level in immune cells may be one of the mechanisms of impaired immunity in diabetic patients. Secondly, this may also be associated with constant infection and delayed wound healings normally associated with T2DM patients (Kumar and Clark, 2007). Lymphocytes play a major role in the development and production of antibodies in the body. Neutrophils are responsible for killing or engulfing foreign organisms (bacteria etc.) once they enter the body (Kumar and Clark, 2007).

From the present results, it can be speculated that a correct response of granulocytes to extrinsic stimuli depends on the proper function of cell signalling pathways. A reduction in neutrophilic functional activity may contribute to higher susceptibility to and severity of infections in T2DM (Kumar & Clark, 2007). Such alterations are strongly associated with cytosolic calcium disturbances. It is concluded that patients with T2DM have a decreased cytosolic calcium level in polymorphonuclear granulocytes after thapsigargin and fMLP stimulation. This abnormality is possibly responsible for impaired neutrophilic function seen in these patients (McManus *et al.*, 2001).

The present study also shows that in neutrophils from patients with T2DM, despite elevated resting cytosolic-free calcium $[Ca^{2+}]_i$, stimulation gives rise to a significantly subnormal $[Ca^{2+}]_i$ signal. The defect in neutrophil $[Ca^{2+}]_i$ rise in T2DM is evident when

cells are incubated with either fMLP or thapsigargin. This suggests the presence of an abnormality that may affect more than one signalling pathway. Thus, in T2DM, the impaired $[Ca^{2+}]_i$ signal results from a defect in influx of extracellular calcium across the plasma membrane and is likely owing to an abnormal store mediated calcium entry, which is a cytoskeletal-dependent process. There was a significantly greater response to fMLP in neutrophils from the controls than from the patient's with T2DM. Thapsigargin caused a similar change in $[Ca^{2+}]_i$ as fMLP, with a significantly greater increase in control neutrophils and lymphocytes (Popko *et al.*, 2003).

The concentration (10^{-6} M) of thapsigargin used has been suggested to cause inhibition of Ca^{2+} entry (Geiszt *et al.*, 1995). However, it is unlikely that the response in neutrophils from patients is owing to a differential sensitivity to thapsigargin, as the finding could be reproduced with fMLP. Previous work has shown that incubation of neutrophils, from healthy controls, with 10 $nmolL^{-1}$ fMLP is associated with a significant increase in $[Ca^{2+}]_i$ and that this can occur when cells are incubated in calcium-free medium (Espino *et al*, 2009).

The demonstration of increased $[Ca^{2+}]_i$ in resting neutrophils from patients with T2DM is consistent with previous findings in neutrophils and other tissues (Lew, 1989). Increased resting $[Ca^{2+}]_i$ may be owing to reduced activity of membrane Ca^{2+}-ATPase activity in T2DM (Levy *et al.*, 1986). In contrast to the present study, in platelets and fibroblasts from patients with T2DM, increased Ca^{2+} influx has been observed (Ishii *et al.*, 1991). However, the present observations in leucocytes are consistent with reports of a reduced $[Ca^{2+}]_i$ response to increased glucose concentrations in endothelial cells (Salameh and Dhein, 1998) and cardiomyocytes (Yu *et al.*, 1997b). Intriguingly, increased $[Ca^{2+}]_i$ is associated with platelet hyper function in diabetes whereas the defect in neutrophil behaviour is one of impaired function (Ishii *et al.*, 1991).

It is unclear whether abnormal calcium homeostasis in T2DM is primarily genetic or metabolic. Improvement in glycaemic control is associated with normalization of resting $[Ca^{2+}]_i$ (Alexiewicz *et al.*, 1995). On the other hand, a defect in Ca^{2+} handling by fibroblasts is most commonly observed in a subgroup of patients with T2DM with hypertension and microalbuminuria, suggesting a basic cellular defect (Solini *et al.*, 1996). In this study, subject groups differed in terms of BMI, blood pressure, lipids and insulin levels as well as level of glycaemia. There was, however, no correlation done between any of these parameters and the response of neutrophils to stimulation. The cause of the defect that was observed in patients with T2DM might be a reduced Ca^{2+} influx across the plasma membrane secondary to impaired neutrophil cytoskeletal remodelling (Advani *et al.*, 2002). Alternative hypothesis for the defect in Ca^{2+} homeostasis in neutrophils and lymphocytes may be due to lipid abnormalities affecting membrane composition and possible insulin resistance (Pierce & Russell, 1997). Similar results have been obtained in cardiomyocytes from T2DM rats (Bracken *et al*, 2004).

In conclusion, the results of this study, have clearly demonstrated a derangement in calcium homeostasis in both neutrophils and lymphocytes of T2DM patients compare to healthy age-matched control subjects. Either fMLP or thapsigargin can elicit marked and significant increases in $[Ca^{2+}]_i$ from either neutrophils or lymphocytes of age-matched control subjects. In contrast, in T2DM patients, either fMLP or thapsigargin elicited significantly less $[Ca^{2+}]_i$ from either neutrophils or lymphocytes compared to controls. The decreases in $[Ca^{2+}]_i$ from neutrophils and lymphocytes in T2DM patients may be responsible for constant infections and delayed wound-healing, normally seen in T2DM patients. Therefore, altered intracellular calcium metabolism may represent a common, underlying abnormality linking the metabolic and cardiovascular manifestations of the diabetic disease process. Further studies are required to determine the molecular mechanisms responsible for this effect.

These include the measurement of Ca^{2+} from extracellular medium and its release from intracellular organelles including the endoplasmic reticulum and the mitochondria. Additionally, it is worthy to measure gene expressions for the different calcium transporting proteins in lymphocytes and neutrophils.

Chapter 4

Analysis of red blood cell membrane proteins in T2DM patients compared to healthy controls using SDS-PAGE

4.1 Objectives of this study

To analyse RBC cell membrane proteins from T2DM patients and healthy age-matched controls using SDS-PAGE electrophoresis and silver staining techniques.

4.2 Introduction

T2DM is a multifaceted syndrome which leads to the development of cardiovascular diseases (CVD) (Nishikawa *et al.*, 2000). Significant evidence has demonstrated that T2DM is responsible for the production of reactive oxygen species (ROS) which contributes for the initiation and progression of atherosclerosis and other related complications (Saha *et al.*, 2005). In recent years, there has been growing interest in characterising red blood corpuscle (RBC) membrane since structural and functional alterations of the membrane might also contribute to patho-physiology of the disease (Kahane *et al.*, 1978). Cell membranes are fluid membrane structures which actively participate in the central regulation of different functions of the cell. Moreover, they are responsible for the maintenance of fluidity which is a prerequisite for their function, viability and growth. Zeta potential (ZP) is a characteristic signature for the diagnosis of haemolytic diseases, studies of membrane permeability, and other alterations leading to destruction of erythrocytes (Ohno *et al.*, 1985).

In this chapter, alterations of erythrocyte membrane proteins including spectrin, ankyrin, band 3, band 4.1 and glycophorin in T2DM patients and healthy controls were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and quantitative estimation of protein bands was performed using scanning densitometry as described in chapter 2

4.3 Methods

Methods for this chapter as described in chapter 2.

Table 4.1 Subjects recruited for this study

	Healthy controls	**T2DM**
No of subjects	9	9
Age group	20-60 years	20-60 years
Gender	5 Male & 4 Female	7 Male & 2 Female
Diabetic status	Non-T2DM	T2DM
Treatment	-	Medication & Insulin
Ethnicity	Caucasian	Caucasian

4.4 Results

Figure 4.2 shows the overall electrophoretic mobility and relative intensity of stained bands. The results reveal that the bands for proteins spectrin, ankyrin, band 3, band 4.1, glycophorin were significantly ($p<0.05$) different between the two groups (Figure 4.4).

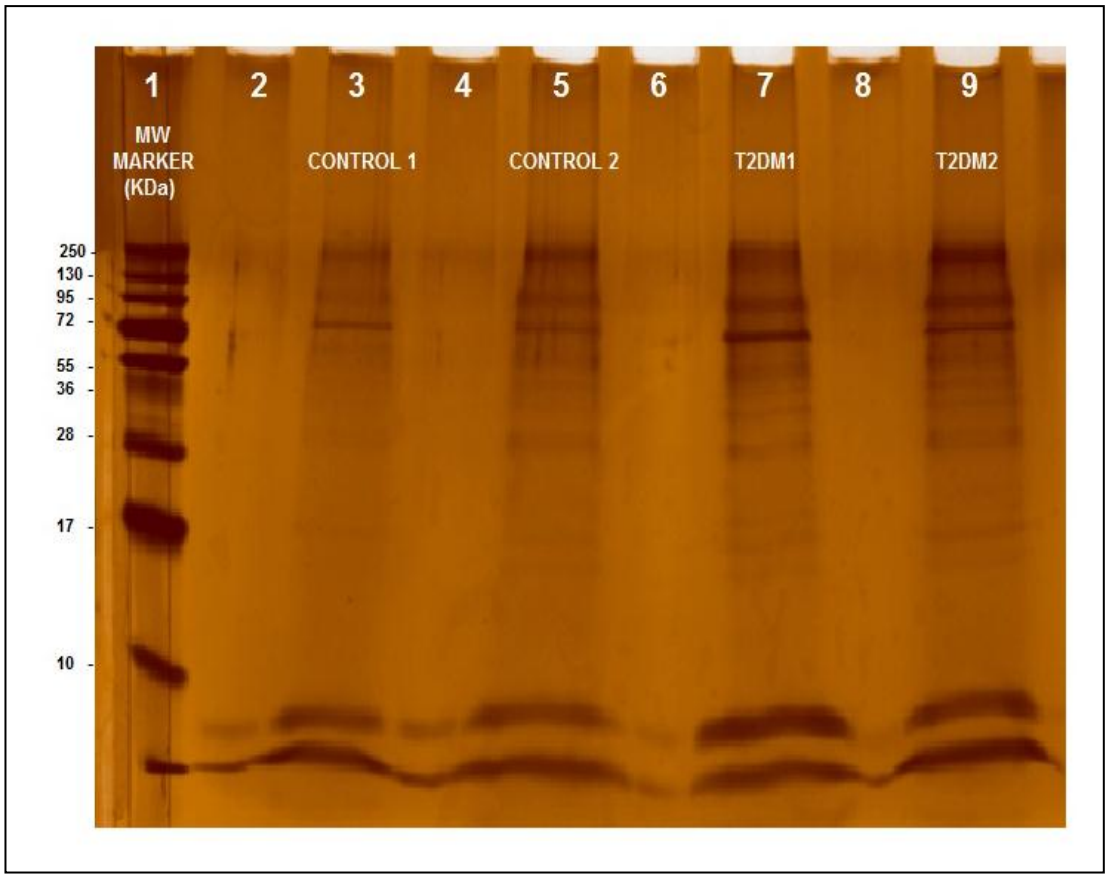

Figure 4.1: SDS-PAGE protein patterns of T2DM patients following silver staining procedure using Fermentas Page Silver Staining Kit. Lane 1 represents molecular weight marker. Lane 3 and 5 represent controls and lanes 7 and 9 represent T2DM samples. These images are typical of 9 such experiments.

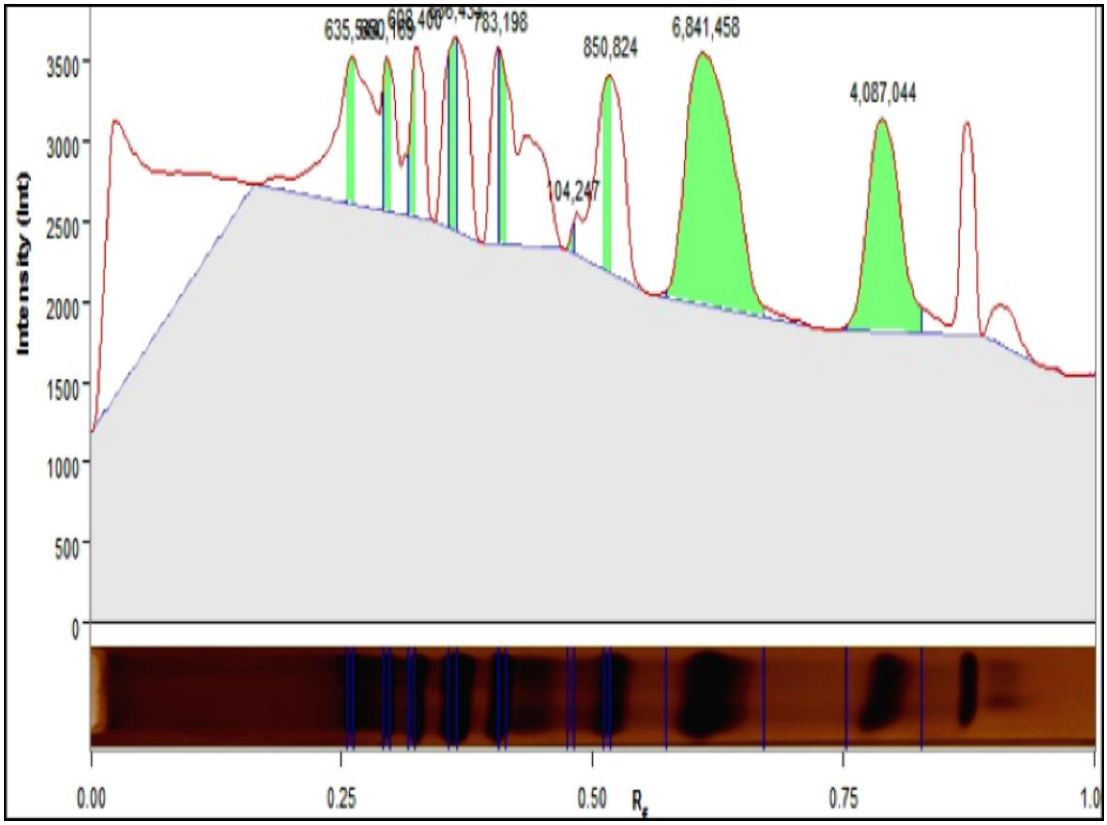

Figure 4.2: Analysis of protein bands performed by Bio-Rad Gel Doc analyser. Each electrophoretic protein band is expressed in terms of intensity (arbitrary units, AU). The peaks represent the intensity of each band in terms of AU units in this figure. Typical of 9 such experiments.

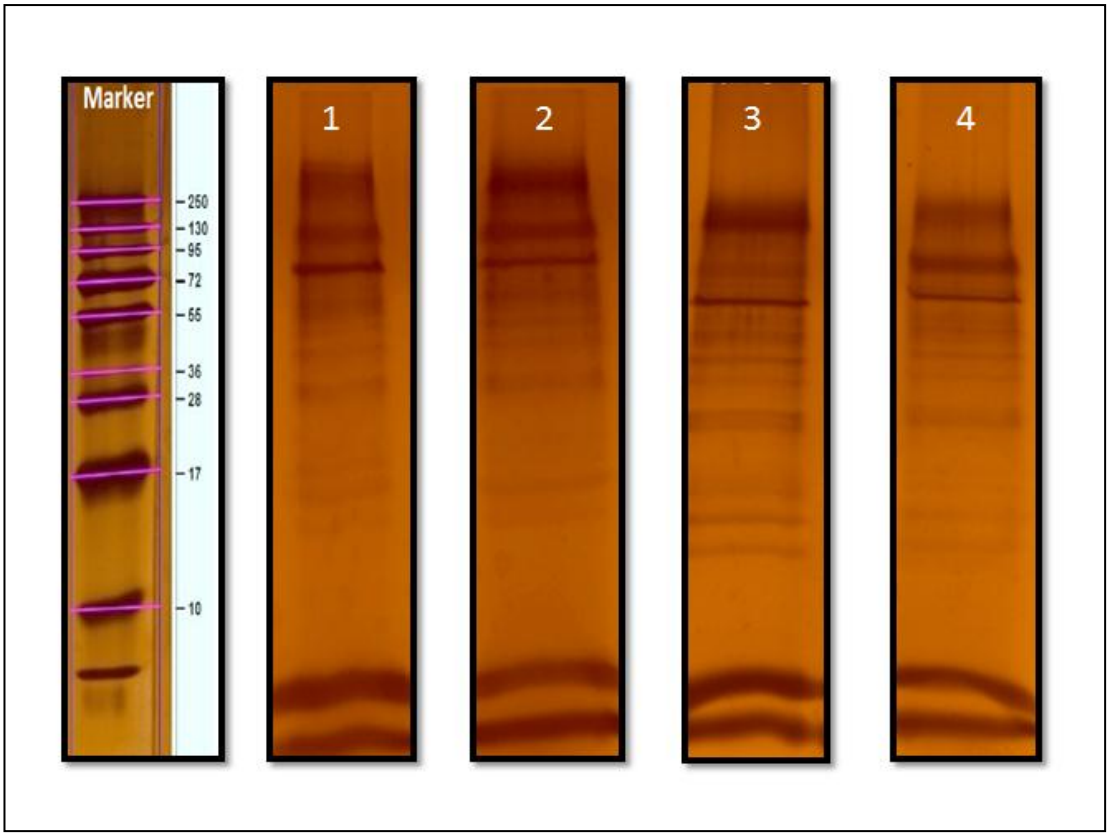

Figure 4.3: Sodium dodecyl sulfate–polyacrylamide gel (SDS-PAGE) electrophoresis patterns of protein extractions performed on Bio-Rad mini-gel system using Bio-Rad 12% Tris-HCl gel following silver staining technique. The first column on the left hand side represents molecular weight marker in kDa. Lanes 1 and 2 represent healthy controls and lanes 3 and 4 represent T2DM patients. Typical of 9 such different experiments.

The data presented in Figure 4.3 showed significant ($p<0.05$) increases in the intensity of bands of all proteins in T2DM patients compared to healthy controls which might indicate a genetic problem underlying the excess production of these proteins in T2DM. Following quantification of the different bands, the data are presented as a family of bar charts in Figure 4.5. The results have indicated significant (Student's t-test; $p<0.05$; $n=9$) increases in the amounts of all five proteins in erythrocyte membranes of T2DM patients compared to healthy controls.

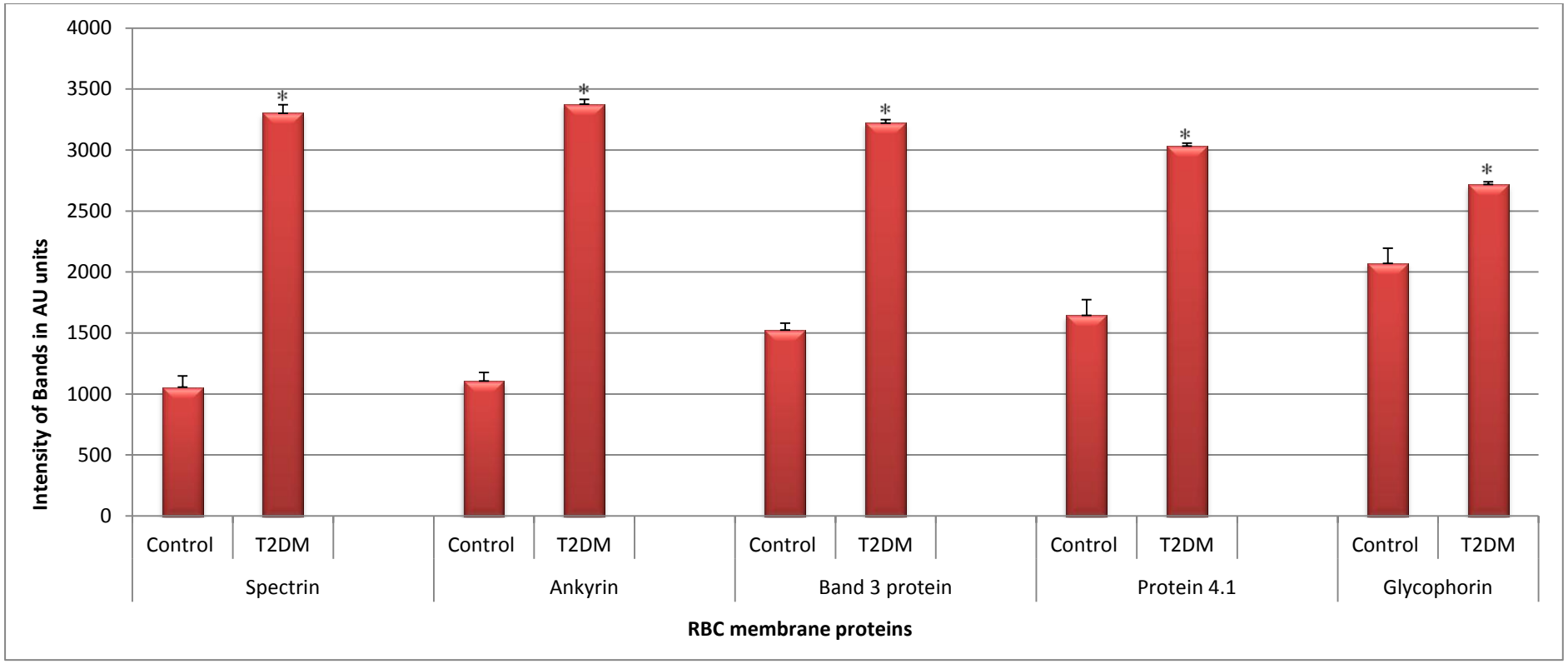

Figure 4.4: Bar charts showing the levels of 5 different proteins in RBC membranes of healthy age-matched controls and T2DM patients. Data are mean±SEM, n=9, *p<0.05 for T2DM compare to controls. Note the significant increases for spectrin, ankyrin, band 3, band 4.1 and glycophorin compared to healthy controls

.

4.5 Discussion

The membrane of the RBC plays a significant role in the structural and functional aspects of the cell and therefore any defects in the blood cell membrane could lead to various diseases including severe haemolytic anaemias. Unfortunately, present diagnostic tools are limited and the cause of these membrane defects is still unknown. The analysis of membrane proteins is challenging, as they are complicated to solubilise and also have the tendency to precipitate during analysis while the dynamic interaction between membrane and cytoskeleton of the RBC membrane makes it more difficult to analyse the proteins in its membrane. Therefore, for future analysis of patient with unknown deficiencies, detection of new potential biomarkers should be always confirmed by analysis including ELISA/radio immuno assays, immunohistochemistry or confocal immuno-fluorescence microscopy.

Hyperglycaemia which results from uncontrolled glucose regulation is generally documented as an underlying link between diabetes and diabetic complications (Rolo & Palmeira, 2006). Chronic hyperglycaemia causes oxidation and non-enzymatic glycosylation (Maillard reaction) of proteins which also affects the erythrocyte membrane proteins (Bunn, 1981). Particularly, the slow turnover of the erythrocyte membrane proteins makes them more susceptible to hyperglycaemia. Glycosylation of erythrocyte membrane decreases the negative surface electric charge due to cleavage of terminal sialic acid components of glycophorin A, leading to accelerated ageing of erythrocytes (Raz *et al.*, 1988). It also leads to increased erythrocyte aggregation and accumulation of advanced glycation end products (AGEs), which are associated with erythrocyte deformability defects and microvascular complications of diabetes (Rogers *et al.*, 1992). The present study was able to show some of the erythrocyte membrane

protein alterations that may result from altered glucose metabolism to produce diabetic complications.

Erythrocyte membrane proteins perform various specific functions. For example, band 3 is the most abundant erythrocyte trans-membrane protein to carry out anion exchange at the plasma membrane level (Kay, 1991). Other trans-membrane proteins serve as pumps or channels for the movement of ions and assist in the transport of glucose and other small molecules. Furthermore, cytoskeletal proteins provide structural integrity and are also important for the maintenance of the biconcave shape of the erythrocyte.

Therefore, disorders of the erythrocyte membrane proteins may alter the surface electric charge and the mechanical properties of plasma membrane, including shape and deformability, which in turn reflect the elastic reserve of the erythrocyte when it receives shear stress forces while passing through either small vessels or capillaries. In conclusion, erythrocyte membrane protein alterations are linked to altered erythrocyte rheology. Several studies provide evidence of the association of haemorrheological abnormalities with diabetes mellitus which possibly participate in the pathogenesis of diabetic microangiopathy. Altered rheology of erythrocytes leads to increased aggregation, endothelial damage and capillary closure. This also applies on diabetic retinopathy, the most common of diabetic vascular microangiopathies (Babu and Singh, 2004; Ernst and Matrai, 1986).

There has been improved emphasis on the pathogenic mechanisms linking erythrocyte membrane protein alterations following altered erythrocyte rheology and further development of diabetic complications. Several studies described a variety of erythrocyte membrane protein alterations in diabetic humans or animals. Oxidation of spectrin has been demonstrated in diabetic erythrocytes (Schwartz *et al.*, 1991).

Absence of spectrin has been reported in T1DM and increased ankyrin and band 6 has been reported in both T1DM and T2DM (Adewoye *et al.*, 2011).

In the present study, electrophoretic alterations of the erythrocyte membrane proteins are shown in T2DM patients compared to healthy controls. Detection of such alterations may help to clarify the pathogenic steps that contribute to the development of CVD in T2DM patients and they also might serve as a blood marker for the existence of diabetic microangiopathy or as a sign of increased risk to develop diabetic complications. In recent studies, alterations in the ultrastructure of red blood cell have been considered as a possible progression marker in T2DM (Straface *et al.*, 2002). There are several possible interpretations to these alterations in the RBC structure. Alterations of the red blood cell membrane might lead to accelerated ageing of erythrocytes in T2DM (Mazzanti *et al.*, 1992). It has been reported that the proteins present in erythrocyte membrane have an increased susceptibility to lysis by endogenous enzymes in diabetes (Jones, 1984).

However, in this study it has not been possible to find out whether the increased percentage of protein bands in T2DM patients is attributed to a glycosylation defect or to increased proteolysis. Abnormal low MW bands possibly arise due to increased proteolysis of original membrane proteins. The high MW band of above 255 KDa appeared due to a cross linkage event between spectrin and haemoglobin and has been previously found in membranes of oxidatively stressed erythrocytes. The formation of spectrin-haemoglobin cross linking represents a well-known oxidant damage of the senescent erythrocyte membrane (Snyder *et al.*, 1983) and is reproduced *in vitro* by hydrogen peroxide treatment which is associated with echinocyte transformation, membrane rigidity, adherence and phagocytosis (Snyder *et al.*, 1985).

In conclusion, the results presented in this study have shown that structural alterations of the erythrocyte membrane proteins, that occur as a result of oxidation and non-enzymatic glycosylation of those proteins in chronic diabetes and which in turn affect the erythrocyte rheology, possibly are associated with the development of microvascular complications of diabetes, in particular diabetic cardiovascular disease. A lot of such protein alterations are shown exclusively in the present study. Their detection may serve as a blood marker for the development of diabetic microangiopathy or as a sign of increased risk to develop diabetic microangiopathy. Further studies are needed to assess whether pharmaceutical intervention to the rheology of erythrocytes can prevent or alleviate microvascular diabetic complications. Future research might explore the biochemical foundation of this difference and, possibly, its clinical significance.

Therefore, an approach to study erythrocyte membrane proteins by SDS PAGE, quantification by scanning densitometry was used in this study which allowed identification and quantification of proteins in the RBC membrane. This may lead to a new proteomic approach to discover the unknown defects in erythrocyte membrane proteins and to obtain potential biomarkers, which may lead to an improved understanding of erythrocyte functioning in T2DM patients. Therefore, a more profound analysis of the erythrocyte membrane proteins is required, which can lead to the discovery of novel biomarkers and also provide an extension of the existing diagnostic tools. Proteomics technology is most suited for this research as it can provide simultaneous detection and quantification of proteins.

Chapter 5

Analysis of various biochemical parameters in blood of T2DM patients and healthy controls

5.1 Objectives of this study

To measure the concentrations of cations, biochemical parameters and inflammatory markers in plasma and serum of T2DM patients and healthy age-matched controls for comparison.

5.2 Introduction

T2DM is a metabolic disease associated with a group of abnormalities including hyperglycemia, dyslipidemia, hypertension, elevated levels of biochemical and inflammatory markers in circulation. This condition predisposes an individual to a number of adverse consequences which include atherosclerotic cardiovascular disease, neuropathy, nephropathy and retinopathy (Kumar and Clark, 2007). T2DM and atherosclerotic CVD have many common antecedent factors that frequently coexist, which has given rise to the concept of "common soil" (Jarrett & Shipley, 1988; Stern, 1995). These factors are associated with increased concentrations of inflammatory markers in people with T2DM (Pickup *et al.*, 1997; Nilsson *et al.*, 1998). Inflammatory processes play a part in the cause of atherosclerotic CVD (Ross, 1999). Concentrations of acute-phase response markers and mediators of inflammation–cytokines such as tumour necrosis factor α (TNF-α) and interleukin-6 are increased in people with T2DM. This finding has led to the suggestion that raised concentrations of proinflammatory cytokines and the resultant acute-phase response may underlie much of the metabolic disease including glucose intolerance (Pickup and Crook, 1998).

Moreover, patients with T2DM have an increased risk of developing extensive atherosclerosis and its sequelae including unstable angina pectoris, and acute myocardial infarction (Beckman *et al.*, 2002). During the last few years, experimental data have emphasised the role of inflammation in atherogenesis. Clinical studies have revealed that this upcoming idea of the role of inflammation in atherosclerosis applies

directly to patients (Libby *et al.*, 2002). Therefore the aim of this chapter of the study is to measure the concentrations of blood plasma cations and other biochemical parameters and inflammatory markers of T2DM patients compared to healthy age - matched controls for comparison.

5.3 Methods

Materials and methods of this study are as described in Chapter 2 of this thesis.

Table 5.1 Subjects recruited for this study

	Healthy controls	**T2DM**
No of subjects	22	22
Age group	30-60 years	30-60 years
Gender	14 Male & 8 Female	12 Male & 10 Female
Diabetic status	Non-T2DM	T2DM
Treatment	-	Medication & Insulin
Ethnicity	Caucasian & Asian	Caucasian & Asian

5.4 Results

In this cationic study, sodium, potassium, calcium, iron, zinc and magnesium were measured in plasma of T2DM and healthy controls by a method described in chapter 2. A total of 22 T2DM patients and 19 healthy controls were involved in this particular study. The results in table 5.1 show that all the trace elements were observed to be significantly ($p<0.05$) higher in concentrations in T2DM patients when compared with healthy controls except for potassium which remains more or less the same for each group. The values are also expressed graphically in figures 5.1 to 5.7 for comparison.

The results for the measurements of the different cations are presented in figure 5.1 to figure 5.7. The results show that the plasma of T2DM patients contains significant ($p<0.05$) more sodium, calcium, copper, zinc, and magnesium compared to plasma of age healthy age-matched controls. The results also showed that there was no change in potassium levels comparing control with T2DM patients. However, T2DM patients had significantly ($p<0.05$) less iron compared to age-matched healthy controls.

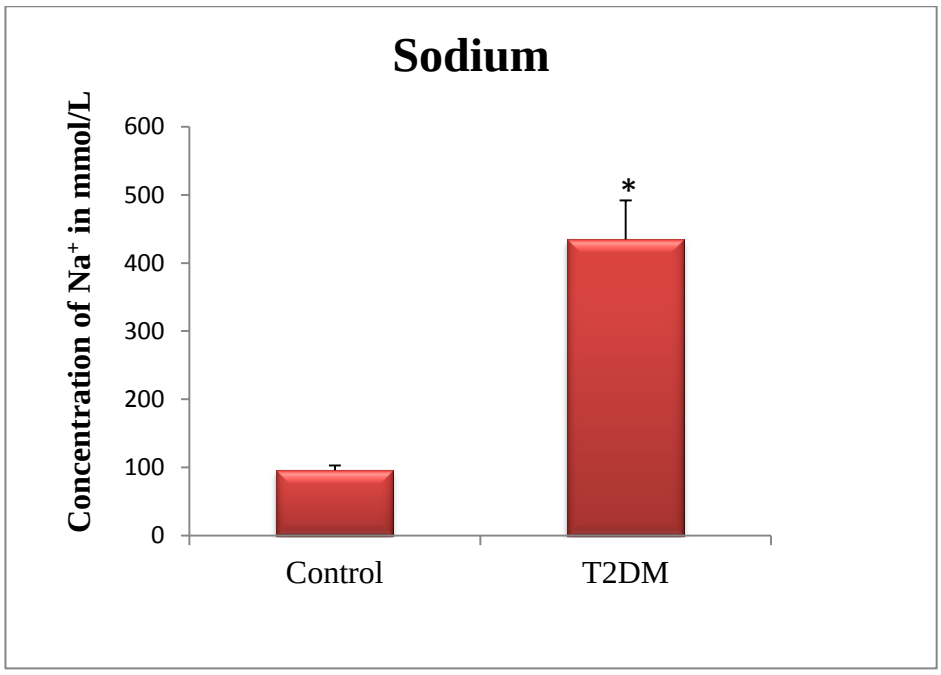

Figure 5.1: Bar chart showing plasma sodium (Na^+) concentrations (mmol/L) in T2DM compared to controls. Values are expressed in terms of mean ± SEM, n=22, *p<0.05 for T2DM compared to control group.

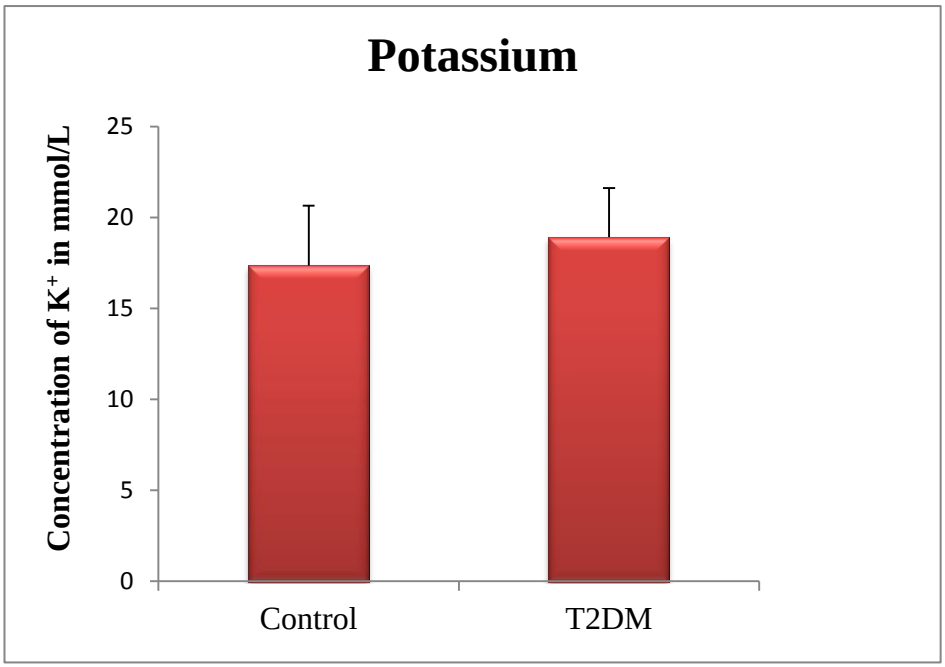

Figure 5.2: Bar chart showing plasma potassium (K^+) concentrations (mmol/L) in T2DM compared to controls. Values are mean ± SEM, n=22. Note that there is no significant difference in K^+ levels in both control and diabetic groups.

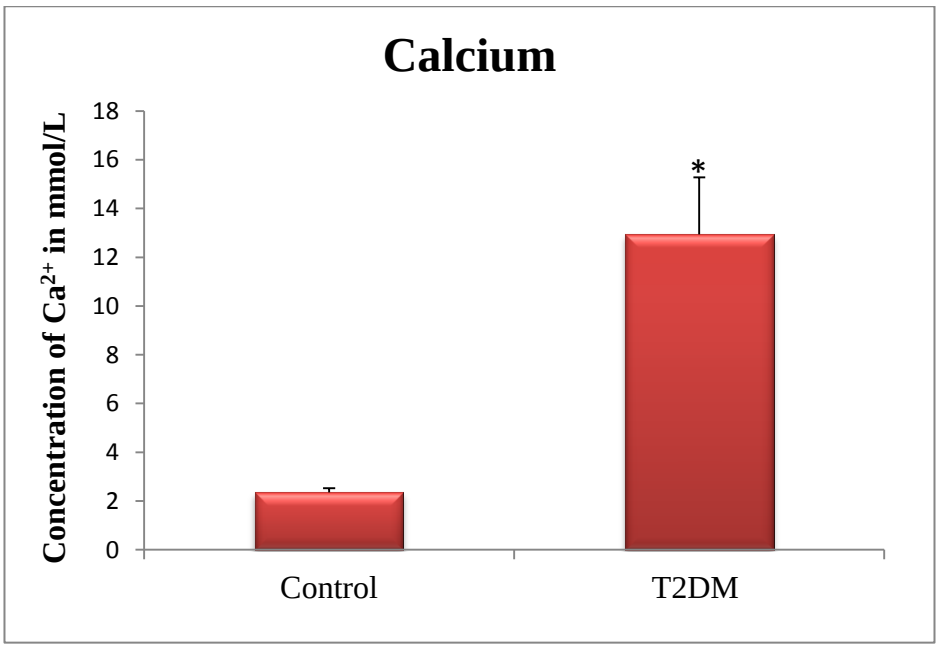

Figure 5.3: Bar chart showing plasma calcium (Ca^{2+}) concentrations (mmol/L) in T2DM compared to controls. Values are mean ± SEM, n=22, *p<0.05 for T2DM compared to control group.

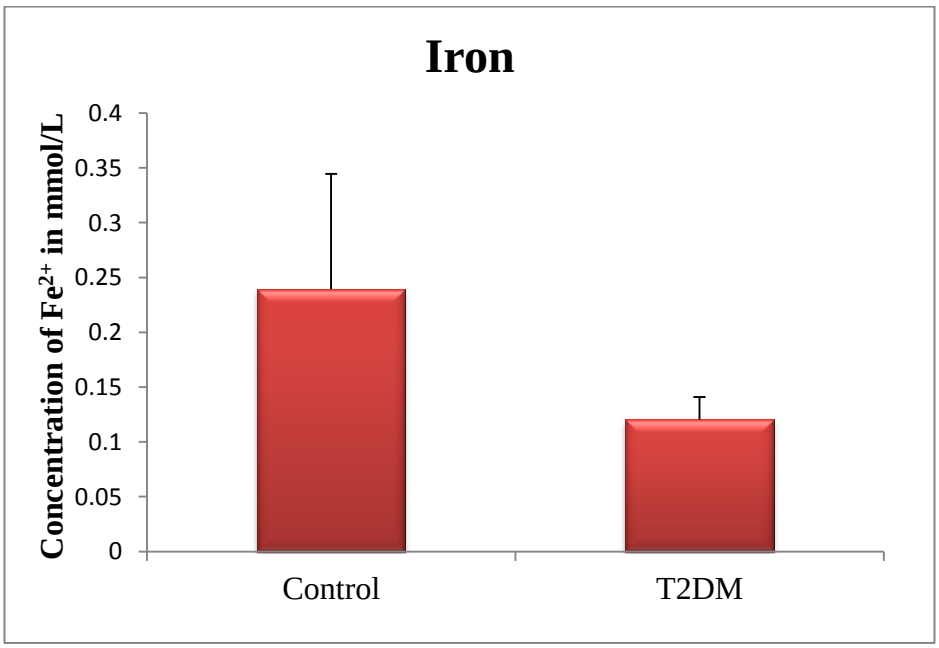

Figure 5.4: Bar chart showing iron (Fe^{2+}) concentrations (mmol/L) in T2DM compared to controls. Values are mean ± SEM, n=22, *p<0.001 for T2DM compared to control group.

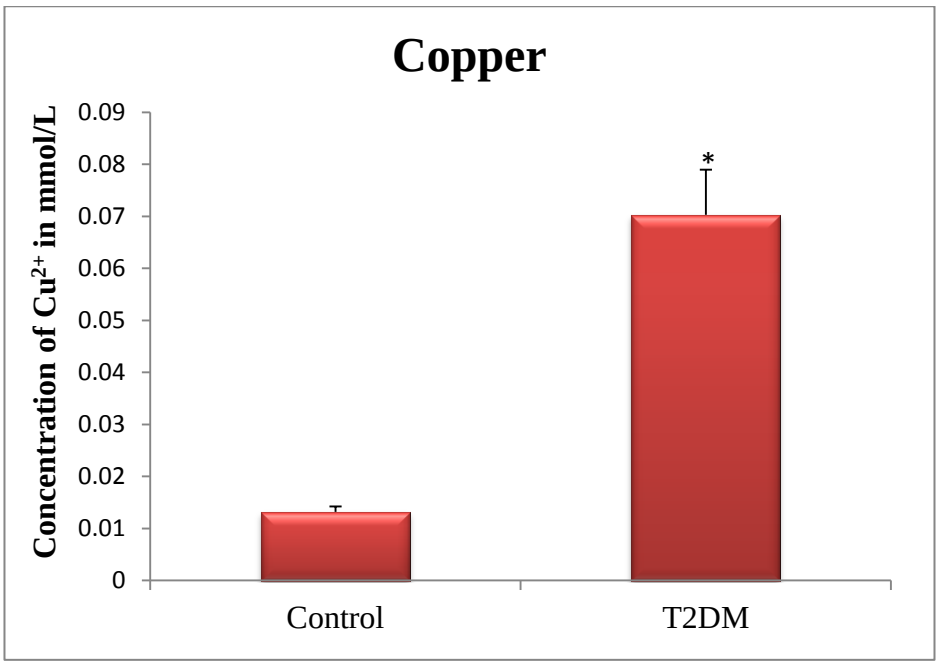

Figure 5.5: Bar chart showing plasma copper (Cu^{2+}) concentrations (mmol/L) in T2DM compared to controls. Values are mean ± SEM, n=22, *p<0.001 for T2DM compared to control group.

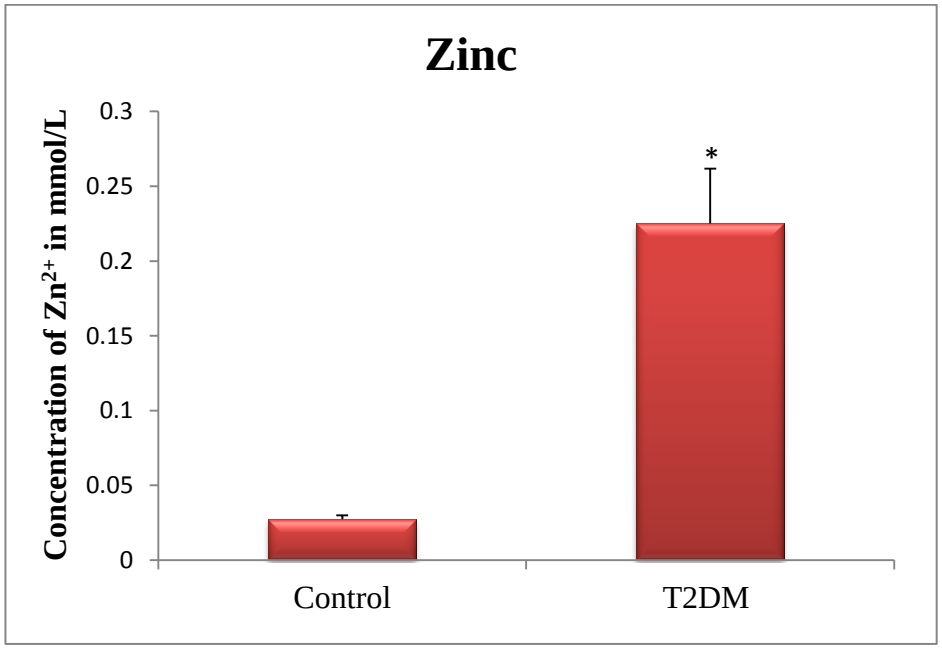

Figure 5.6: Bar chart showing plasma zinc (Zn^{2+}) concentrations (mmol/L) in T2DM compared to controls. Values are mean ± SEM, n=22, *p<0.001 for T2DM compared to control group.

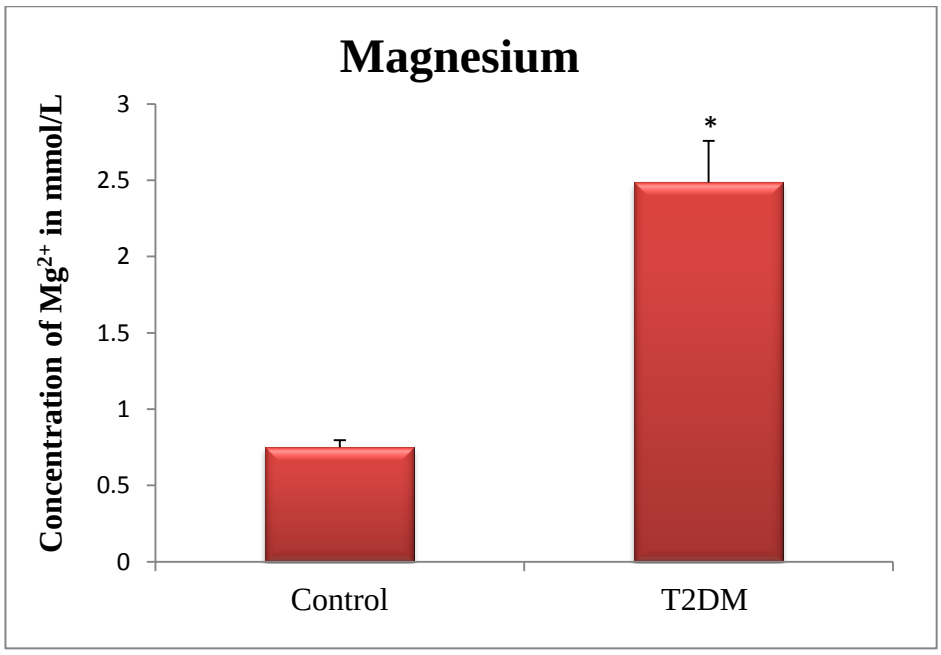

Figure 5.7: Bar chart showing plasma magnesium (Mg^{2+}) concentrations (mmol/L) in T2DM compared to controls. Values are mean ± SEM, n=22, *p<0.001 for T2DM compared to control group.

5.4.1 Biochemical parameters

Table 5.1 and figures 5.8–5.13 show the results of full blood count in plasma of T2DM patients and healthy age-matched controls. The results show higher concentrations of RBC, haemoglobin, HCT and lymphocytes in healthy controls when compared with T2DM patients and lower concentrations of WBC, platelets, MCV, MCH, neutrophils, monocytes and eosinophils in healthy controls when compared with T2DM patients (See Table 5.2).

Figures 5.14-5.16 show the serum lipid profiles in plasma of control and T2DM patients. The results show high levels of serum lipid profiles including cholesterol, HDL-C, LDL-C and total cholesterol ratio in healthy age-matched controls when compared with T2DM patients. In contrast, the levels of triglycerides were found to be lower in healthy controls when compared with T2DM patients.

Figures 5.17-5.23 show the levels of plasma glucose, CRP, HbA1c, urea, creatinine, alkaline phosphatise and GTT in healthy controls and T2DM patients. The results show that levels of plasma glucose, C-reactive protein, HbA1c were significantly ($p<0.05$) higher in T2DM patients when compared with those without T2DM (healthy controls).

The results also show significantly ($p<0.05$) high levels of urea, creatinine, alkaline phosphatase, GGT in plasma of T2DM patients when compared to plasma from age-matched healthy controls. There were also significantly ($p<0.01$) low levels of total bilirubin, ALT, total protein, albumin, total calcium in plasma of TDM patients when compared with plasma from healthy age matched controls.

Table 5.2: Table showing the results of all the biochemical parameters measured in plasma of healthy controls and T2DM patients. All results are expressed as mean±SEM,* $p<0.05$ for T2DM compared to control group.

Haematological parameters **Full blood count**	**Healthy control (n=19)**	**T2DM (n=21)**	***p* values**
Red blood cells (10*12/L)	5.142±0.145	4.511±0.140	
White blood cells (10*9/L)	6.425±0.360	8.542±0.380	0.05
Platelets (10*9/L)	222.8±13.091	245.388±15.260	-
Lymphocytes (10*9/L)	2.284±0.131	2.040±0.168	-
Monocytes (10*9/L)	0.412±0.029	0.635±0.038	0.05
Neutrophils (10*9/L)	3.4785±0.296	5.464±0.307	0.05
Eosinophils (10*9/L)	0.2295 ± 0.0421	0.2311±0330	-
Haemoglobin (g/Dl)	14.83±0.333	13.311±0.317	-
HCT (ratio)	0.434±0.008	0.402±0.008	-
MCV (fL)	85.07±1.318	89.72±1.311	-
MCH (pg)	28.98±0.473	29.64±0.464	-
Biochemical/Lipid parameters **Serum lipid profile**	**Healthy control (n=19)**	**T2DM (n=21)**	
Total Cholesterol (ratio)	4.480±0.184	3.965±0.181	-
HDL-C (mmol/L)	1.268±0.094,	1.030±0.079	-
LDL-C (mmol/L)	2.584±0.188	1.921±0.200	0.05
Triglycerides (mmol/L)	1.418±0.123	2.223±0.266	0.05

Total Cholesterol ratio	3.913±0.316	3.805±0.232	-
Kidney function tests	**Healthy control (n=19)**	**T2DM (n=21)**	
Urea (mmol/L)	4.278±0.266	8.616±0.881	0.05
Creatinine (umol/L)	74.695±2.770	106.66±12.004	0.05
Liver function tests	**Healthy control (n=19)**	**T2DM (n=21)**	
Total bilirubin (umol/L)	11.565±1.449	18, 8±1.275	0.05
Alkaline Phosphatase (u/L)	64.347±3.198	89.722±10.497	0.05
ALT (U/L)	31.090±8.359	20.785±2.727	0.05
GGT (u/L)	20.217±1.506	69.111±17.683	0.05
Total Protein (g/L)	79.304±0.723	74.444±0.682	0.05
Albumin (g/L)	48.565±0.543	45.111±0.675	-
Total Calcium (mmol/L)	2.375±0.016	2.374±0.02	-
Plasma Glucose (mmol/L)	4.726±0.189	11.23±1.337	0.05
HbA1c (DCCT %)	5.547±0.084	8.277±0.407	0.05
HbA1c (IFCCmmol/mol)	37.238±0.940	67.111±4.469	0.05
CRP (mg/L)	1.421±0.209	8.441±2.438	0.05

5.4.1.1 Full blood count

Analysis of full blood counts in plasma included measurements of RBC, WBC, platelets, lymphocytes, neutrophils, eosinophils, monocytes, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and haematocrit (HCT) in both T2DM patients and healthy age-matched controls (Figures 5.8-5.13).

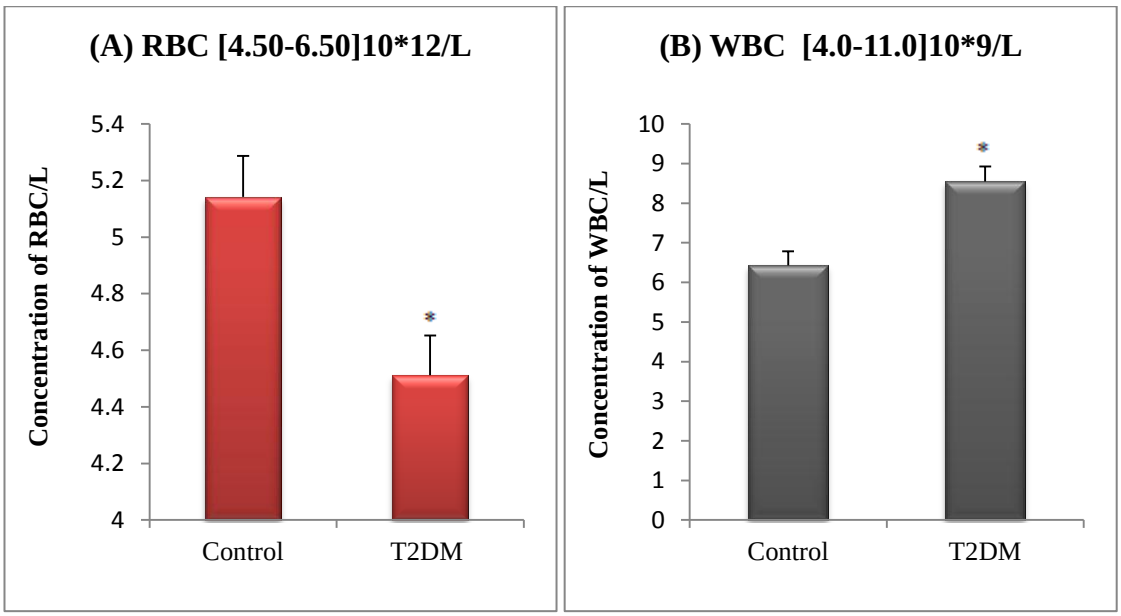

Figure 5.8: Bar charts A and B showing the concentrations of RBC and WBC in T2DM patients compared to controls, respectively. Data are mean ± SEM, n=22; *p<0.05 for T2DM compared with control group. Range of normal values is also shown in the figure for comparison.

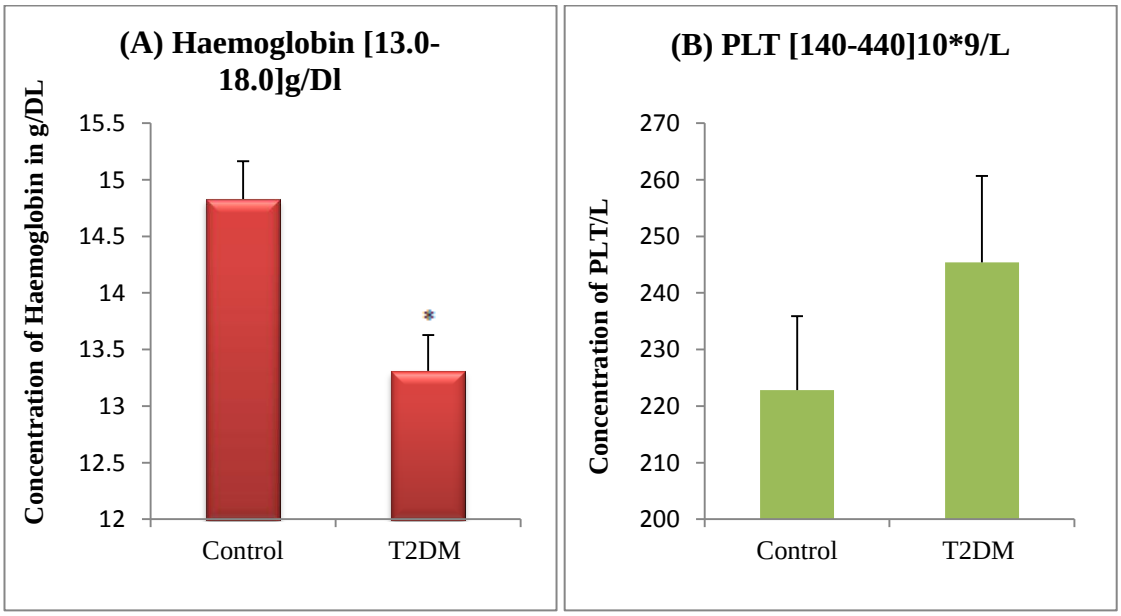

Figure 5.9: Bar charts A and B showing the concentrations of haemoglobin and platelets (PLT) in T2DM patients compared to controls, respectively. Data are mean ± SEM, n=22; *p<0.05 for T2DM compared with control group. Range of normal values is also shown in the figure for comparison.

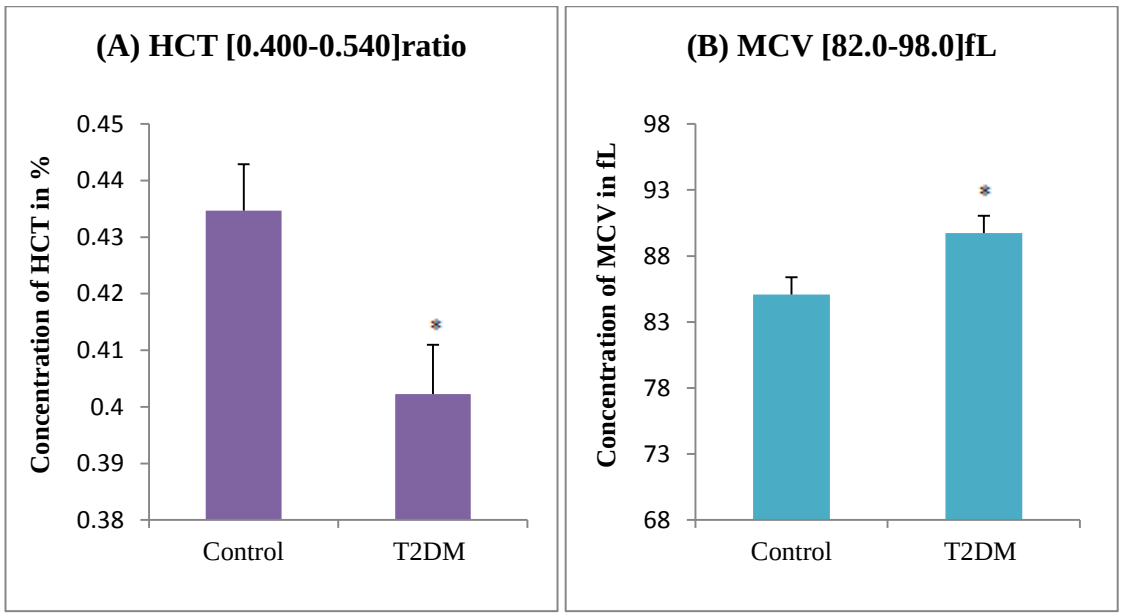

Figure 5.10: Bar charts A and B showing the concentrations of HCT and MCV in T2DM patients compared to controls, respectively. Data are mean ± SEM; n=22; *p<0.05 for T2DM compared with control group. Range of normal values is also shown in the figure for comparison.

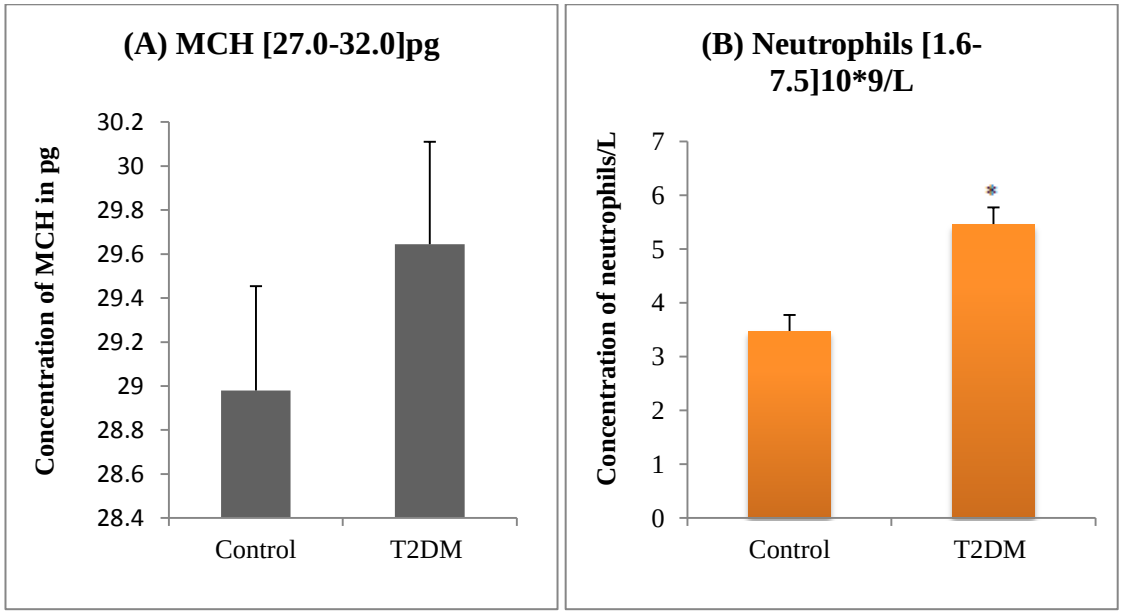

Figure 5.11: Bar charts A and B showing the concentrations of MCH and neutrophils in T2DM patients compared to controls, respectively. Data are mean ± SEM; n=22; *p<0.05 for T2DM compared with control group. Range of normal values is also shown in the figure for comparison.

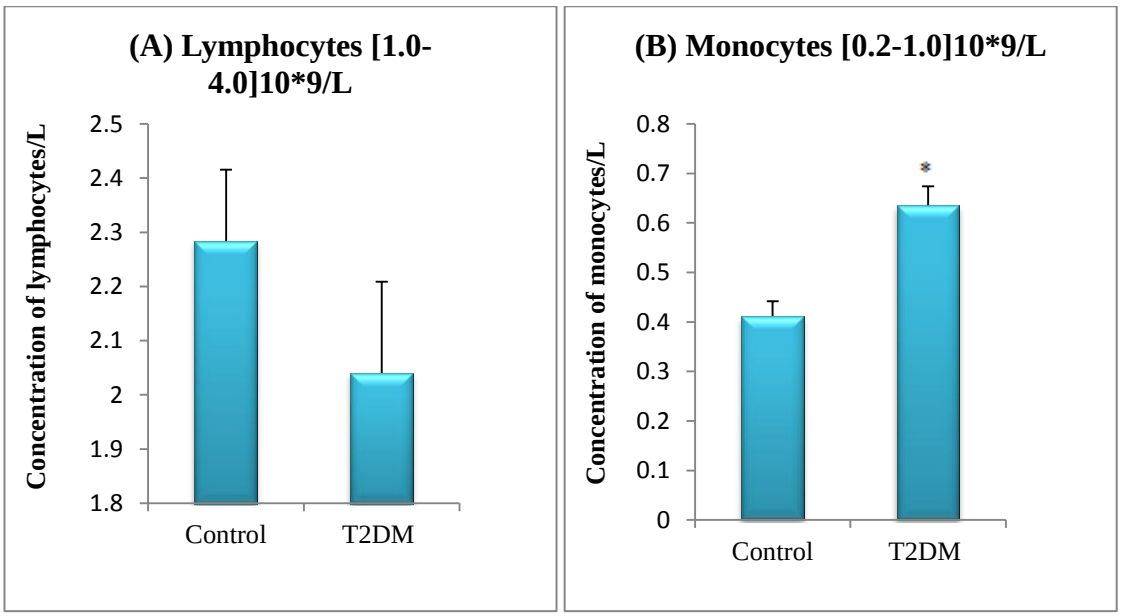

Figure 5.12: Bar charts A and B showing the concentrations of lymphocytes and monocytes in T2DM patients compared to controls, respectively. Data are mean ± SEM; n=22; *p<0.05 for T2DM compared with control group. Range of normal values is also shown in the figure for comparison.

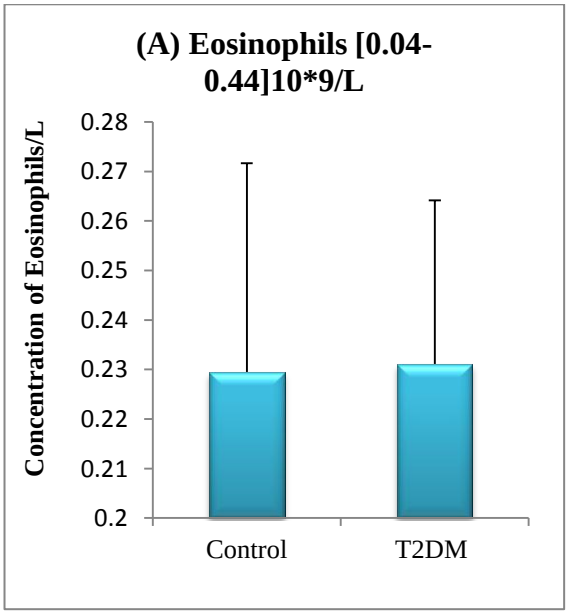

Figure 5.13: Bar chart A showing the concentrations of eosinophils in T2DM patients compared to controls. Data are mean ± SEM; n=22. Note that there was no significant difference between the values. Range of normal values is also shown in the figure for comparison.

5.4.1.2 Serum Lipid Profile

Analysis of serum lipid profiles included measurements of such parameters as cholesterol, triglycerides, HDL-C, LDL-C and total cholesterol ratio in both T2DM patients and healthy controls. The data are shown in figures 5.14-5.16.

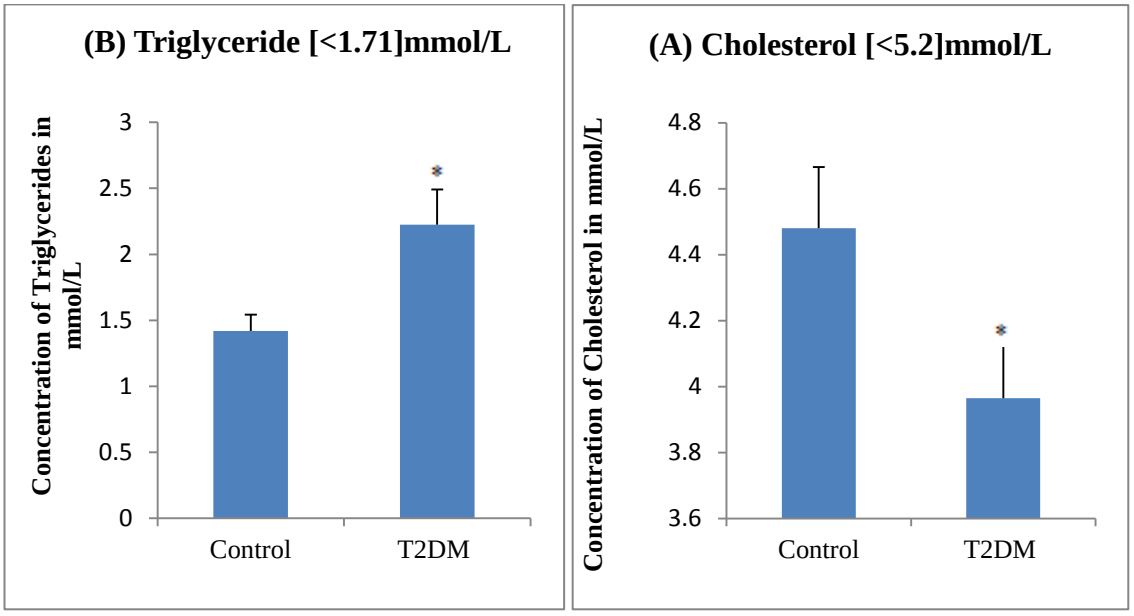

Figure 5.14: Bar charts A and B showing the concentrations of cholesterol and triglyceride in T2DM patients compared to controls, respectively. Data are mean ± SEM; n=22; *p<0.05 for T2DM compared with control group. Range of normal values is also shown in the figure for comparison.

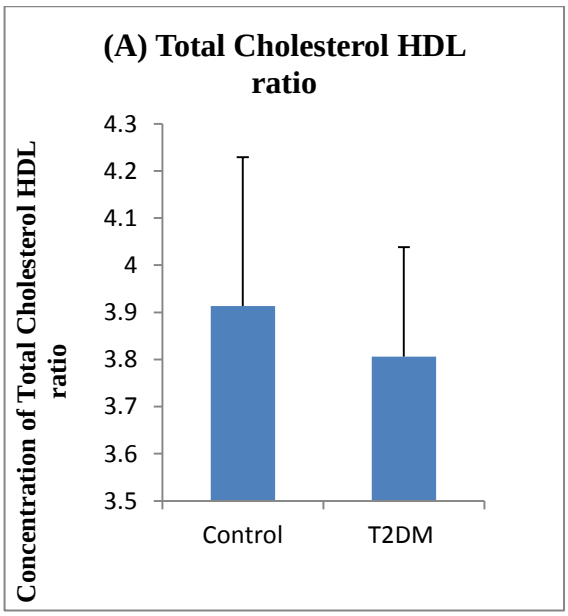

Figure 5.15: Bar chart A showing the concentrations of total cholesterol in T2DM patients compared to controls. Data are mean ± SEM; n=22; *p<0.05 for T2DM compared with control group.

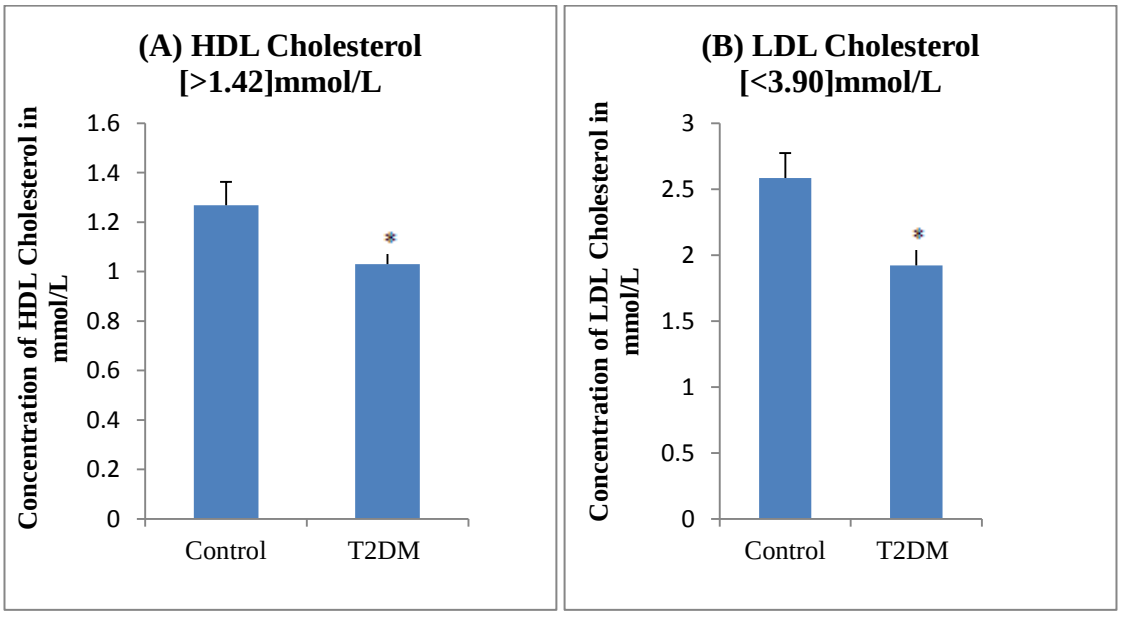

Figure 5.16: Bar charts A and B showing the concentrations of HDL cholesterol and LDL cholesterol in T2DM patients compared to controls, respectively. Data are mean ± SEM; n=22; *p<0.05 for T2DM compared with control group. Range of normal values is also shown in the figure for comparison.

5.4.1.3 Glucose, CRP and HbA1c

This analysis included measurement of plasma glucose, C-reactive protein and HbA1c in both T2DM patients and healthy controls. The data are shown in Figures 5.17-5.18. The results show significant ($p<0.05$) increases in the levels of glucose, CRP and HbA1c in plasma of T2DM compared to control.

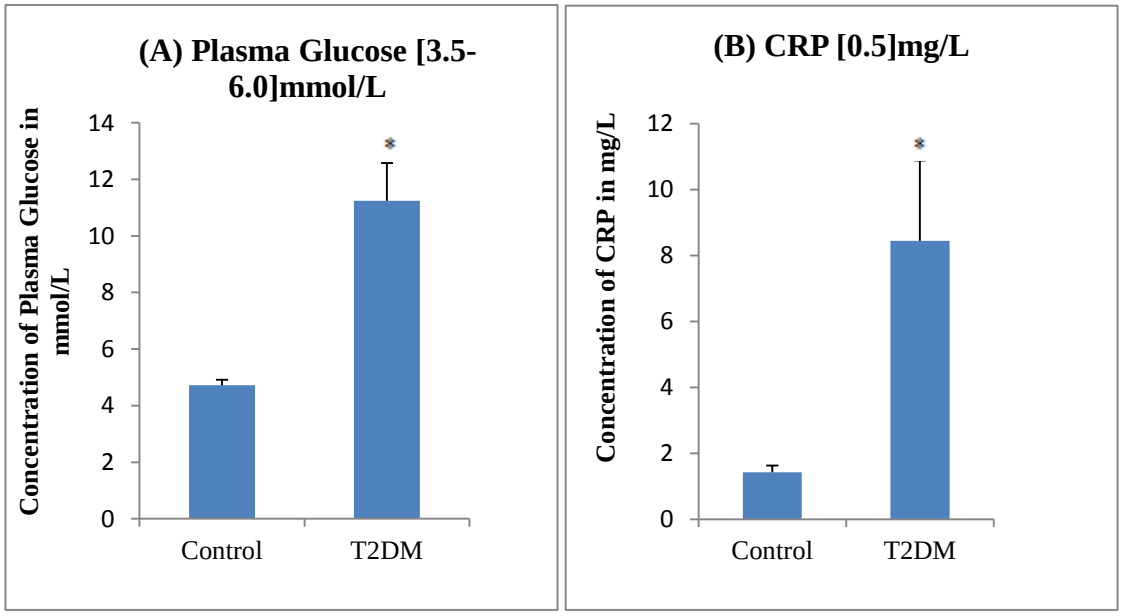

Figure 5.17: Bar charts A and B showing the concentrations of plasma glucose and CRP in T2DM patients compared to controls, respectively. Data are mean ± SEM; n=22; *$p<0.05$ for T2DM compared with control group. Range of normal values is also shown in the figure for comparison.

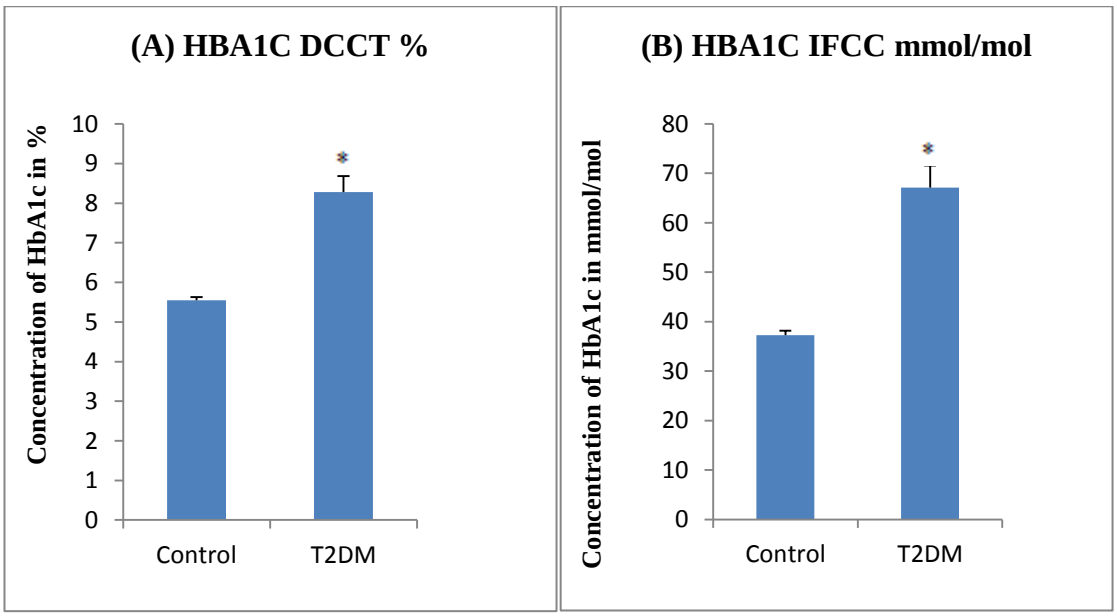

Figure 5.18: Bar charts A and B showing the concentrations of HbA1c (% and mmol/mol) in T2DM patients compared to controls, respectively. Data are mean ± SEM; n=22; *p<0.05 for T2DM compared with control group. Range of normal values is also shown in the figure for comparison.

5.4.1.4 Kidney function tests

Figure 5.19 shows the results for kidney function tests. The measurements included urea and creatinine in T2DM and healthy controls. The results have shown significant ($^{*}p<0.05$) increases of both urea and creatinine in T2DM patients compared to controls.

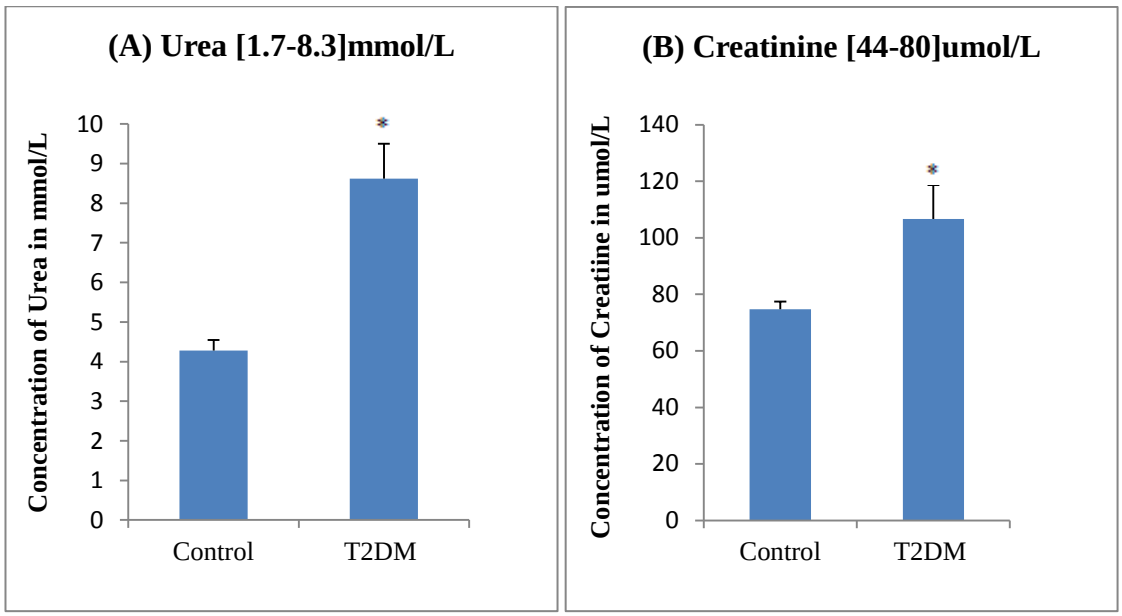

Figure 5.19: Bar charts A and B showing the concentrations of urea and creatinine in T2DM patients compared to controls, respectively. Data are mean ± SEM; n=22; *p<0.05 for T2DM compared with control group. Range of normal values is also shown in the figure for comparison.

5.4.1.5 Liver function tests (LFT's)

Figures 5.20-5.23 show analysis of Liver function tests and the measurements included total calcium, total bilirubin, alkaline phosphatase, ALT, GGT, total protein and albumin in both T2DM and healthy controls. The results show no significant change in total calcium between T2DM and control. However, the results also show significant ($p<0.05$) increases in alkaline phosphatase and GGT in T2DM patients compared to control.

In contrast, there were significant ($p<0.05$) decreases in total bilirubin, ALT, AST, total protein and albumin in T2DM patients compared to age-matched controls.

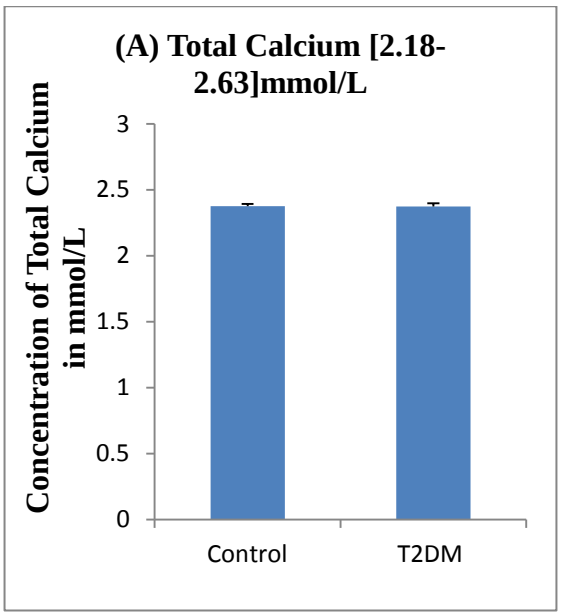

Figure 5.20: Bar chart A showing the concentration of total calcium in T2DM patients compared to controls. Data are mean ± SEM; n=22. Note that there is no significant difference in the data. Range of normal values is also shown in the figure for comparison.

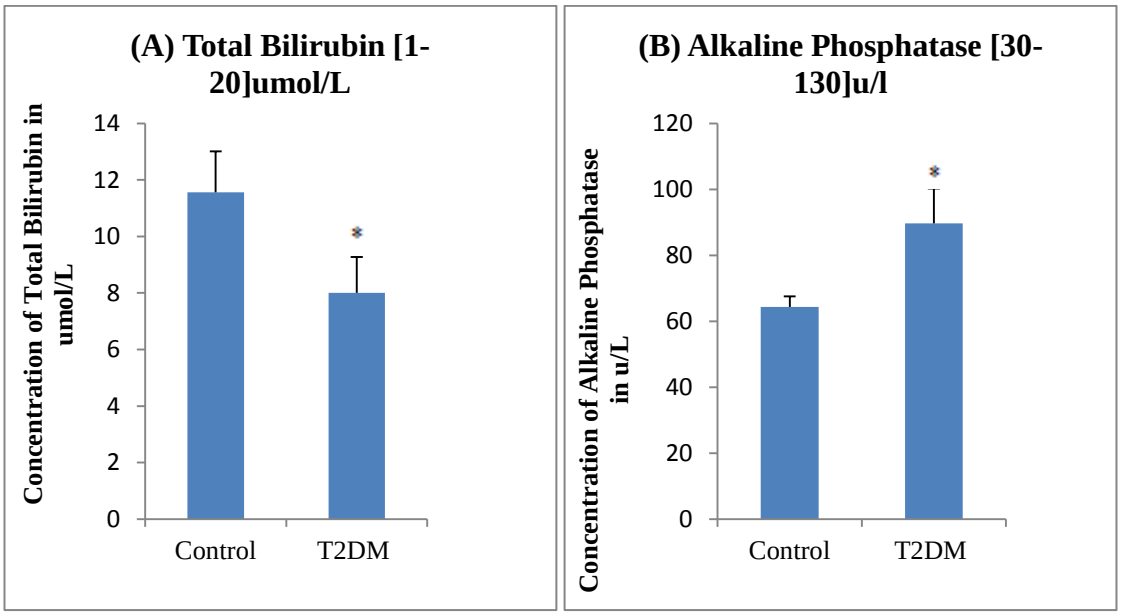

Figure 5.21: Bar charts A and B showing the concentrations of total bilirubin and alkaline phosphatase in T2DM patients compared to controls, respectively. Data are mean ± SEM; n=22; *p<0.05 for T2DM compared with control group. Range of normal values is also shown in the figure for comparison.

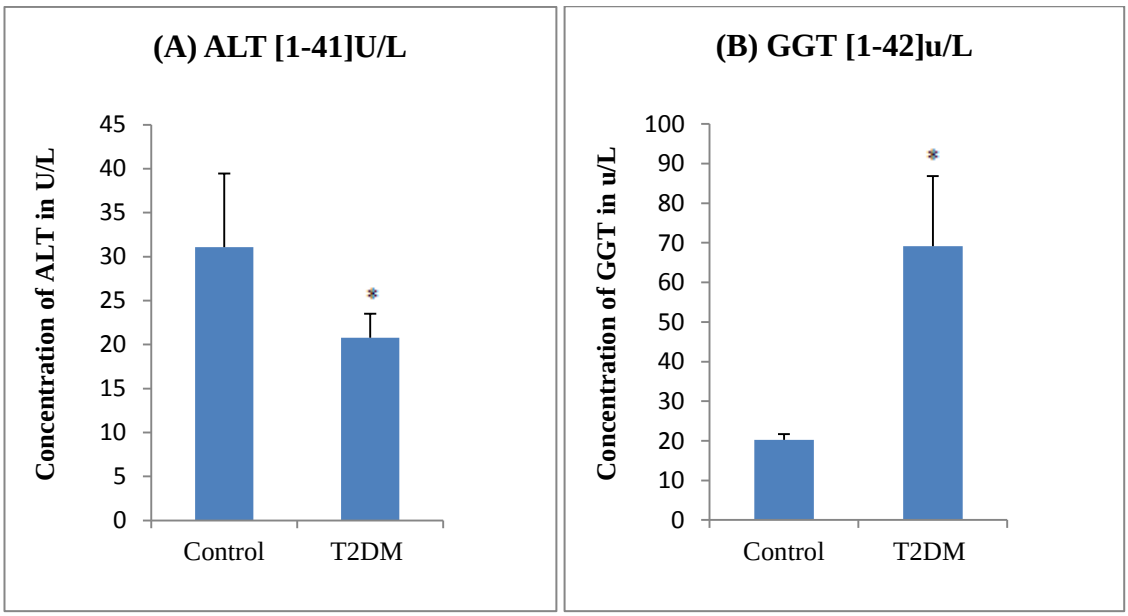

Figure 5.22: Bar charts A and B showing the concentrations of ALT and GGT in T2DM patients compared to controls, respectively. Data are mean ± SEM; n=22; *p<0.05 for T2DM compared with control group. Range of normal values is also shown in the figure for comparison.

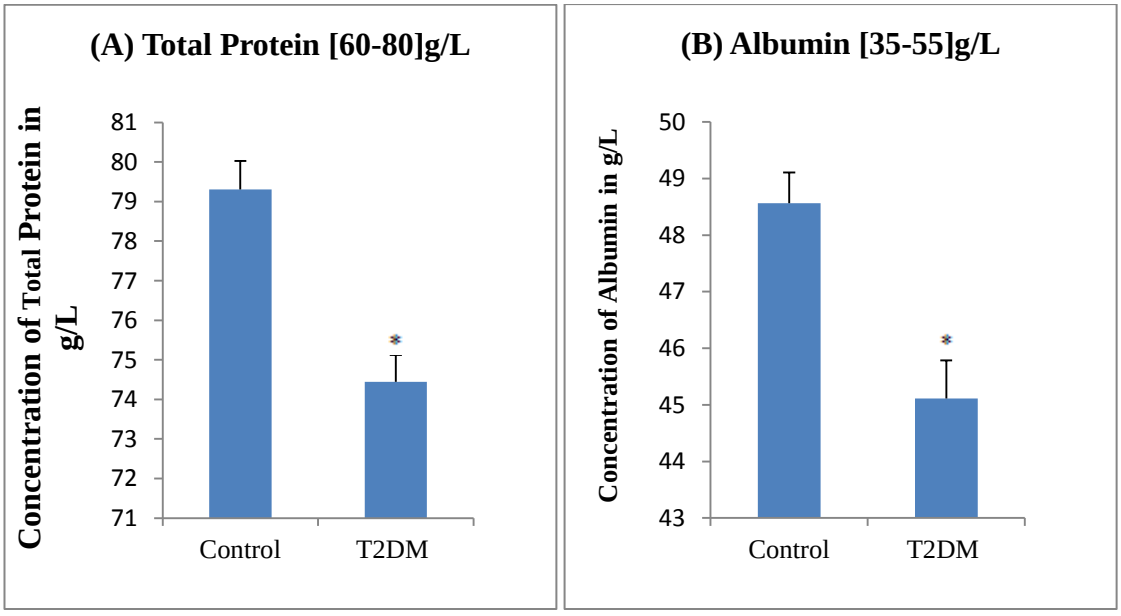

Figure 5.23: Bar charts A and B showing the concentrations of total protein and albumin of T2DM patients compared to controls, respectively. Data are mean ± SEM; n=22; *p<0.05 for T2DM compared with control group. Range of normal values is also shown in the figure for comparison.

5.5 Discussion

There is now much evidence that risks for CVD are higher among T2DM patients compared to age-matched healthy control subjects. Other well-known risk factors including dyslipidaemia, hypertension, sedentary lifestyle, smoking and obesity add to this risk. In fact, 75-80% of mortality rate in T2DM patients are due to a combination of coronary heart disease, peripheral vascular disease and cerebrovascular diseases. Several research studies have provided evidence that patients with T2DM have several abnormalities in their blood and lipid profiles including U and E's, CRP, HbA1c, Glucose and various other biochemical parameters (Wilson *et al*., 2005).

The results of this study have revealed marked and significant elevation in all the cations (except potassium) in plasma of T2DM patients compared to control. The results of cationic studies (Table 5.1) provide evidence that there is an alteration in the plasma concentrations of various cations including sodium, magnesium, calcium, zinc, copper, and iron in plasma of T2DM patients when compared with healthy age-matched controls. These interesting findings suggest that perturbations in cationic metabolism in T2DM are more pronounced and might lead to further complications. Further exploration of these data may provide fundamental new insights as to whether these differences are a cause or a consequence of T2DM.

The present results are in total agreement with current evidence regarding the association of inflammatory markers with the development of metabolic disease, CVD and causes of mortality (Table 5.2). These prospective results support a possible role for inflammation in T2DM and they go with the hypothesis that T2DM might possibly be an expression of ongoing cytokine-mediated acute phase response which is initiated by the innate system of our body.

The present results also show that an elevation of plasma glucose (hyperglycaemia) is observed in T2DM patients when compared with those without T2DM. Glucose- related complications such as formation of advanced glycation end-products might be important in the pathogenesis of diabetic-late complications. In most of the previous studies, glycaemia and the duration of clinical T2DM did not appear to be very strong risk factors CHD. However, the reason for this has not been considerably understood.

Elevated WBC count in T2DM patients might contribute to the development of vascular complications. Chronic inflammation might play a crucial role in the pathogenesis of CVD in T2DM patients. The close association between WBC and various complications of T2DM provides evidence that inflammation might be a common linking factor and consequently documented as a major risk factor for atherosclerosis (DSouza *et al.*, 2009). Mononuclear leukocytes are recruited to the site of endothelial injury and form foam cells in the plaque. Inflammatory markers including various cytokines like IL-6, TNF-α are released from activated leukocytes and cause endothelial dysfunction. An interaction between various risk factors and inflammatory responses lead to widespread vascular damage, endothelial dysfunction, and cause complications in T2DM patients. Further research is required to establish the relation between these factors and underlying mechanisms of diabetic complications.

Abnormalities in lipid profile might be a result of unbalanced metabolic states of diabetic patients that include hyperglycaemia and insulin resistance (Haffner, 2000). Aggressive therapy of diabetic dyslipidemia can reduce the risk of CVD in T2DM patients. Primary therapy should be directed to lower the levels of LDL. Patients with T2DM have many lipid abnormalities including elevated levels of Low-density lipoprotein cholesterol (LDL-C), very low density lipoprotein cholesterol (VLDL-C)

and low levels of high-density lipoprotein cholesterol (HDL-C). The results of this study are found to be consistent with the literature (Kumar and Clark, 2007).

The results of this study also reveal high prevalence of hypercholesterolemia, hypertriglyceridemia, high LDL-C, and low HDL-C levels which are well-known risk factors for CVD. Insulin affects apolipoprotein production in the liver and regulates enzymatic activity of lipoprotein lipase (LpL) and cholesterol ester transport protein. All the above factors are responsible for dyslipidemia in T2DM. Insulin deficiency reduces the activity of haepatic lipase and therefore, several steps involved in the production of biologically active LpL might be altered in T2DM.

This study shows that there is an elevated level of LFT's in T2DM patients when compared with those without T2DM. Possible explanations for elevated transaminases in insulin resistant states include oxidant stress as a result of lipid peroxidation, peroxisomal beta-oxidation, and inflammatory cells (Salmela *et al*, 1984; Erbey *et al*., 2000).

GGT is a nonspecific marker which is known to rise in patients with T2DM. In a cohort study of 7,458 non-T2DM men which was conducted for 12 years. It was determined that elevated GGT could predict development of T2DM (Perry *et al.*, 1998). Similarly, the study in 1988 Ohlson et al have found elevated ALT levels in non-T2DM Swedish men to be an independent risk factor (Ohlson *et al.*, 1988).

Prospectively, increasing ALT concentrations were associated with a decrease in insulin sensitivity and T2DM risk. In this study ALT concentrations were found to be higher in healthy controls when compared with T2DM patients.

The results for urea and creatinine prove to be significantly higher in T2DM patients when compared with healthy controls. This observation is in agreement with the study

conducted by Alder et al, 2003 who showed that elevated levels of creatinine and urea in T2DM patients might signify a pre-renal problem such as volume depletion. In T2DM patients, impairment of renal function is assessed through measurement of plasma concentrations of urea and creatinine. Plasma creatinine and urea are established markers of Glomerular Filtration Rate (GFR). Plasma creatinine is a more sensitive index of kidney function compared to plasma urea level. Creatinine fulfils most of the requirements of a perfect filtration marker. Plasma creatinine is also helpful in recognising when there is a drop in kidney function (Perrone et al., 1992).

Previous studies measuring HbA1c as a predictor of CVD in T2DM patients have been largely confined to high-risk populations (Peters, 1997). Findings from earlier studies including Pima Indians, Japanese, and Chinese suggest that in pre-diabetic individuals, elevated levels of HbA1c predicts the progression to biochemical diabetes as determined by oral glucose tolerance testing (Little, 1994; Narayan *et al.*, 1996; Yoshinaga and Kosaka, 1996). Association of HbA1c with incidence of cardiovascular events is largely attributed to coexistent risk factors. The findings of this particular study demonstrate the potential prognostic importance of this biomarker in clinical care.

Measurement of CRP, a specific marker of inflammation, is an additional finding in this study. Levels of CRP have been observed to be significantly higher in T2DM patients when compared with healthy controls. CRP is a member of the pentraxin family of oligomeric proteins involved with pattern recognition in innate immunity. The biological mechanism through which CRP increases the risk of T2DM is not well understood. CRP is a marker of low-grade inflammation and influences both insulin resistance and insulin secretion indirectly as a result of heightened systemic inflammation. Positive association between CRP and T2DM reflects underlying endothelial dysfunction and sub-clinical atherosclerosis. Studies should be directed at

confirming these findings in animal models. It can be concluded that CRP is a risk marker for cardiovascular disease and could emerge as a mediator in atherogenesis.

5.6 Conclusion

The best approach to prevent potential CVD in T2DM patients is early recognition of risk factors and aggressive therapy. In this study, it is found that elevated plasma levels of several biochemical parameters and inflammatory markers were independent predictors of CVD in T2DM patients. The findings in this study support the hypothesis that low-grade systemic inflammation is an underlying factor in the pathogenesis of T2DM and also a common antecedent for both T2DM and CVD. The data from this particular study also provide further evidence that inflammatory markers might provide a method for early detection of CVD risk. These data might have many significant implications for the prevention and treatment of T2DM. Modification of lifestyle habits and management of systemic inflammation should be the major targets for prevention and treatment of CVD in T2DM patients. Pharmacological therapies with anti-inflammatory properties might also play an essential role in T2DM induced CVD. Healthy diet, regular exercise, yoga and meditation, may also help in both reduction and prevention of T2DM-induced long term cardiac complications.

Chapter 6

Analysis of osmotic fragility and advanced glycation end-products (AGEs) in RBC of T2DM patients and healthy age-matched controls

6.1 Objectives of this study

To measure osmotic fragility of RBC, low molecular weight AGEs in seum and anti-oxidant status in serum of T2DM patients compared to healthy age-matched controls.

6.2 Introduction

Biophysical features of red blood corpuscles (RBCs) play a crucial role in cell behaviour during blood flow. RBCs determine blood viscosity and blood flow through processes like cell aggregation and deformation and hence, they maintain effectiveness of blood circulation and normal physiological conditions in an organism.

In this chapter, the biophysical and biochemical changes of erythrocytes including osmotic fragility, formation of advanced glycation end-products (AGEs) and anti-oxidant status in T2DM patients are analysed and compared to healthy age-matched controls.

In light of these different endogenous results of T2DM patients, this chapter of the thesis investigated the osmotic fragility of the RBC and low molecular weight AGEs and anti-oxidant status in serum of T2DM patients compared to healthy age-matched controls.

6.3 Methods

Materials and methods of this chapter are as described in chapter 2 of the thesis.

Table 6.1 Subjects recruited for this study

	Healthy controls	**T2DM**
No of subjects	22	22
Age group	30-60 years	30-60 years
Gender	14 Male & 8 Female	12 Male & 10 Female
Diabetic status	Non-T2DM	T2DM
Treatment	-	Medication & Insulin
Ethnicity	Caucasian & Asian	Caucasian & Asian

6.4 Results

6.4.1 Osmotic fragility

Figure 6.1 shows the osmotic fragility curves of RBC in 5 T2DM patients compared with 5 healthy age -matched controls. The results show that there were increases in osmotic fragility (haemolysis) in the RBC of all the 5 T2DM patients as compared with healthy controls with increased concentration of NaCl up to 4.8 mM. Thereafter, the fragility increased in the RBC as NaCl concentration increased to 10 mM. Median corpuscular fragility (MCF) is calculated from the graph at which 50% of RBCs were haemolysed. The MCF in 2 T2DM patients was found to be present between 2.8-3.6 g/L NaCl concentration and 3 T2DM patients the values were found to be present between 4.8-5.6 g/L NaCl concentration while control remained at 4.4-5.2 g/L NaCl concentration.

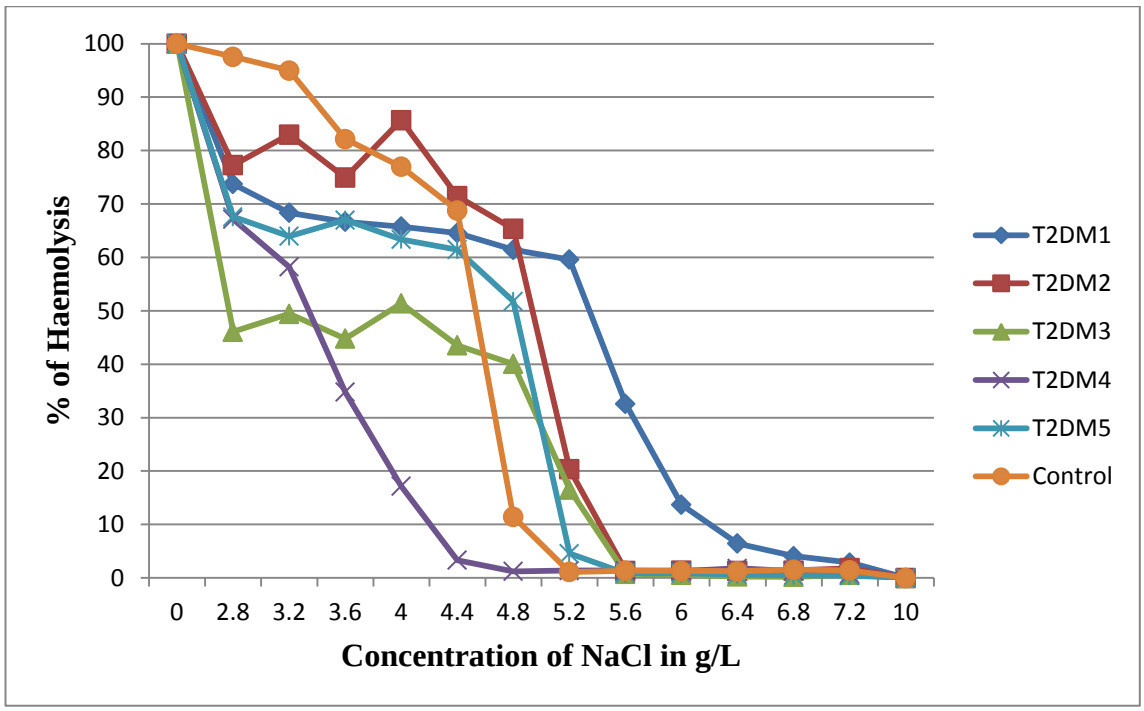

Figure 6.1: Graph represents the osmotic fragility curve of RBC in T2DM patients compared with healthy control, n=5 for T2DM patients and 5 for controls.

6.4.2 Advanced glycation end-products

Low molecular weight AGEs in 50-fold diluted plasma were measured in triplicate on luminescence spectrometer. The fluorescence signals of samples were expressed as the relative fluorescence intensity in arbitrary unit (A.U) and multiplied by dilution factor and the data are presented in Figure 6.2. The results are expressed as mean (±SEM) for T2DM patients (368.93±66.14), n=22 and healthy controls (236.67 ±29.05), n=10. The data clearly show that the serum of T2DM patients contain significantly ($p<0.05$) more LMW AGEs compared to serum from healthy age-matched control subjects.

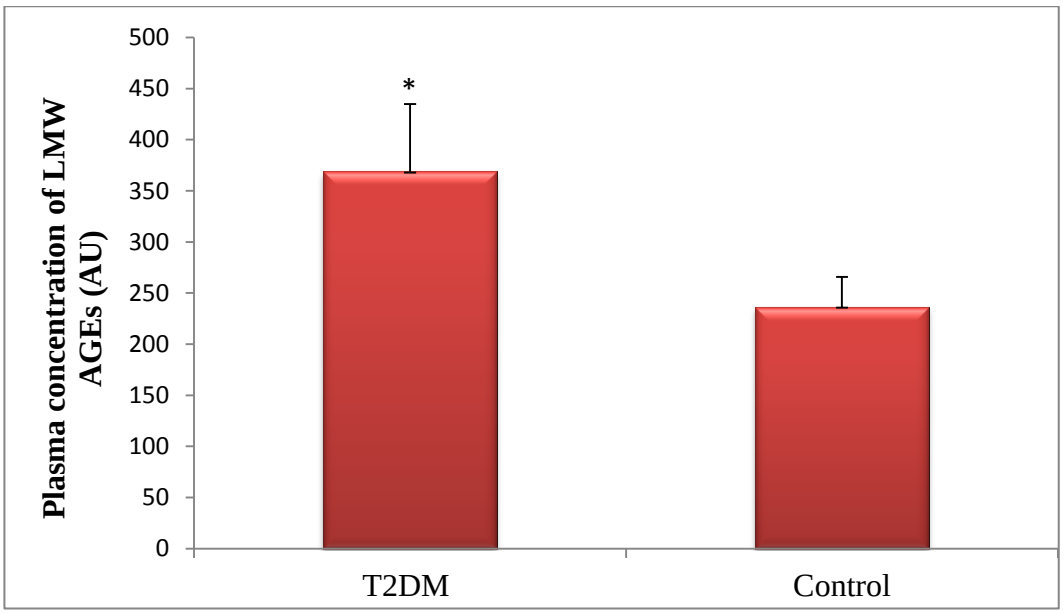

Figure 6.2: Bar charts showing low molecular weight plasma AGEs in T2DM and healthy controls. The results are expressed as mean (±SEM) of the samples (n=22 for T2DM and n=10 for controls); * $p<0.05$ for T2DM compared to control. Note the significant increases in LMW AGEs in the serum of T2DM patients compared to healthy age-matched controls.

6.4.3 Antioxidant status

Analysis of anti-oxidant assay was done according to method described in chapter 2 of this thesis. This series of experiments was performed at several dilutions of trolox standards in order to obtain a standard graph and absorbance was measured at 750 nm.

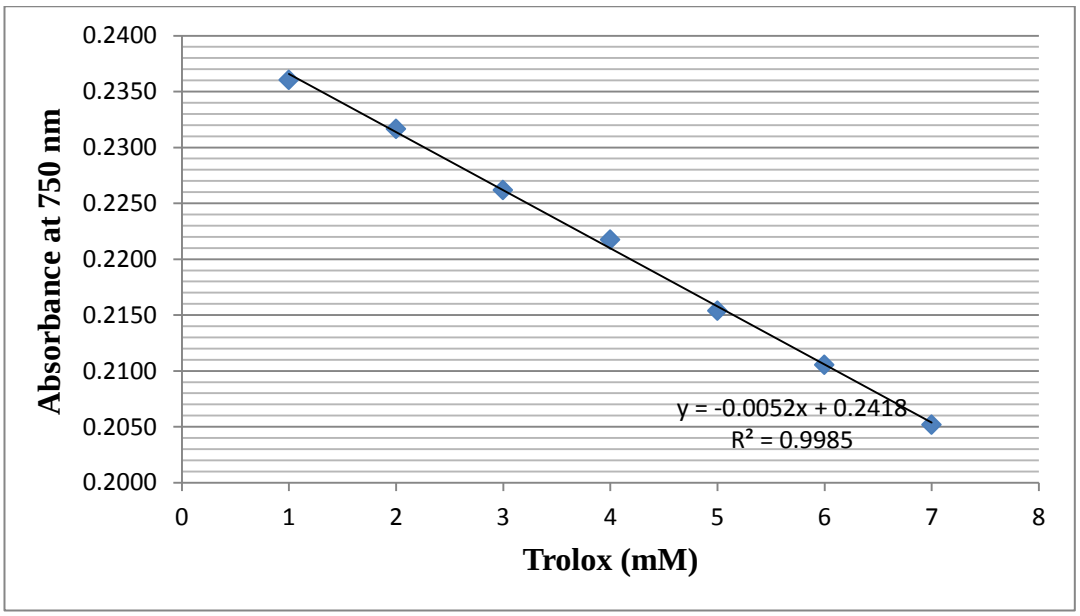

Figure 6.3: Standard curve for different concentrations of trolox at absorbance of 750 nm. This curve is typical of 4-5 such different experiments.

The results obtained in this study were not found to be accurate due to several reasons which might include the dilution of the samples, age of the samples, concentrations of trolox standards, high or low concentration of anti-oxidants in the samples. However, there was no significant difference observed in the absorbance readings between T2DM patients and healthy controls in this particular experiment.

6.5 Discussion

T2DM patients have a higher incidence of CVD compared with non-T2DM individuals. Atherosclerosis is considered as an inflammatory disease since the presence of humoral and cellular components of the immune response has been detected within the atherosclerotic lesion. Increased production of activated leukocyte-derived products such as ROS, enzymes, and pro-inflammatory mediators contribute to the development of atherosclerotic process (Ross, 1999; Libby, 2002).

The results of RBC membrane fragility experiments in this chapter of the study have indicated both an increase as well as decrease in the membrane fragility of T2DM patients when compared with those without T2DM. These reciprocal changes might be a result of disturbances in ions through the membrane and changes in the properties of membrane which leads to further changes in metabolic functions. Enhancement of glycosylation of membrane protein in DM might be one of the possible reasons for reduced membrane fluidity (Lux *et al.,* 1978). The degree of squeezing of RBCs depends on the elasticity of their membranes as they have to pass through blood capillaries of diameters smaller than themselves.

Decreased RBC membrane elasticity, can lead to an increase in blood capillary resistance for RBC passage to carry out normal metabolism due to which toxicity occurs in few organs of the body (Ali *et al.,* 2003). However, it is necessary to emphasize the urgent need for increasing investigations on the pathogenic mechanisms involved in the process of RBC fragility in order to prevent CVD in T2DM patients.

Several animal and human studies have shown that AGEs have been implicated in the pathogenesis of long- term complications in T2DM patients (Peppa *et al*., 2003). AGEs react with extra cellular proteins and activate production of cytokines and transcription factors through binding to AGE receptors (Tan *et al.,* 2004). Accumulation of AGEs predicts future development of cardiovascular complications in T2DM patients. Experimental evidence to date demonstrates that AGEs can alter the walls of the vessel through various mechanisms including modifications in permeability of extracellular matrix, release of inflammatory cytokines and growth factors, alterations of antithrombotic properties of vascular endothelium along with increased expression of adhesion molecules and chemokines on vascular cells (Chappey *et al*., 1997; Singh *et al*., 2001).

In this study, it was observed that there was an increase in the concentration of low molecular weight AGEs in T2DM patients when compared with healthy controls. Therefore, AGEs might constitute a specific target for treatment of CVD in patients with T2DM. The findings of this study are in agreement with some of the previous studies which have shown positive associations between plasma AGEs particularly non-CML AGE, pentosidine, Amadori-albumin and microvascular complications but were limited in size of their population and cross-sectional designs (Salman *et al.*, 2009; Miura *et al.*, 2001; Schalkwijk *et al.*, 2002).

Several studies have demonstrated that increased levels of LMW AGEs were associated with macrovascular disease and suggested that fluorescent AGEs are not independently associated with presence of diabetic complications in T2DM patients. Other smaller studies have established an association between fluorescent serum AGEs and CAD in T2DM.

It is clear that while AGEs appear to be capable of contributing to diabetic complications individually, advanced glycation is only one pathway by which injury may be induced in diabetes. LMW AGEs form through the incomplete digestion of AGE-modified protein and incorporation of AGE modifications. These accumulate in the circulation of T2DM patients. Indeed, LMW AGEs might have a higher toxic potential than those of larger AGE-modified proteins and react with AGE-receptors through circulation (Thomas *et al*., 2005). Hence, LMW fluorescence could be considered as a biomarker of measurement of toxic molecules with the ability to activate specific AGE receptors. Several factors including diet, inflammation, metabolic acidosis and dialysis modality might affect the levels of circulating AGEs in diabetic patients (Uribarri *et al.*, 2003).

In diabetes, LMW fluorescence is induced as result of hyperglycemia and oxidative stress, but not exclusively via the Maillard reaction. Drugs that hinder the formation of AGEs also reduce LMW fluorescence, which suggests a close relation between the two. LMW fluorescence is elevated in experiment models only within a few weeks of disease and progressively accumulates in proportion to the duration of diabetes and with normal aging in control animals (Sharp *et al*., 2003). LMW fluorescence is also elevated in patients with diabetes, particularly those with impaired renal function or anemia (Thomas *et al*., 2005). These findings provide support for the association between LMW fluorescence and diabetes. The identification of the molecular source of LMW fluorescence remains a priority to elucidate the associations demonstrated in this research.

Increased oxidative stress is observed in a system where the production of free radicals is higher and anti-oxidant mechanisms are impaired. In recent times, free radicals produced through oxidative stress have been implicated in the pathology of T2DM (Irshad and Chaudhuri, 2002; Tian *et al.*, 2005; American Diabetes Association, 2006; Ashour *et al.*, 1999). The most crucial factor for the increased production of free radicals is the condition of hypercholesterolemia which might induce damage through overproduction of superoxide radicals in mitochondria. Lipid peroxidation is initiated when superoxide is converted into hydro-peroxyls and diffuses through membranes (Baynes, 2000). The most important risk factors and crucial steps involved in the pathogenesis of atherosclerosis are hypercholesterolemia and abnormalities in lipoprotein metabolism (Mallika *et al.*, 2007; Maxfield and Tabas, 2005). In DM, hyperglycaemia leads to production of oxygen-free radicals through various processes including auto-oxidation of glucose, non-enzymatic glycosylation of proteins, and through activation of enzymes that produce oxidant species and subsequently oxidative stress (Atamer *et al.*, 1998).

In conclusion, these preliminary results presented in this study have demonstrated that oxidative stress can be significantly increased in T2DM patients compared with non-T2DM controls. These initial experiments could lead to further investigation of anti-oxidant status in diabetic patients.

CHAPTER 7

General discussion and scope for future studies

7.1 General Discussion

Within recent years, there has been an increasing interest in research on inflammation involving T2DM patients who have developed cardiovascular diseases (CVD) (Kumar and Clark, 2007). This project was originally designed to investigate the effect of age on different cardiovascular risk and inflammatory markers in T2DM patients compared to healthy controls. However, as the project developed and expanded in a logical manner and it was of paramount importance to measure the parameters associated with T2DM. Second, it was very difficult to obtain enough samples of blood from different age groups. It was only possible to measure blood samples from age-matched control and T2DM patients pooling all the data. Blood was obtained mainly from age-groups 40 years and over in the study compared to the respective age–matched controls.

The results in this study are presented in four different chapters focussing on measurement of intracellular free Ca^{2+} concentration $[Ca^{2+}]_i$ in lymphocytes and neutrophils in blood of T2DM patients compared to healthy controls, an analysis of RBC membrane proteins, an analysis of haematological and biochemical parameters in blood and finally measurement of low molecular weight advanced glycation end-products (LMW-AGEs) in T2DM compared with healthy controls. Collectively, the results have demonstrated marked and significant changes in all the measured parameters in this thesis. Therefore, the general discussion of this thesis will be focussed on the four measured parameters.

The results of Chapter 3 are significant as $[Ca^{2+}]_i$ are found to be affected in T2DM patients compared to healthy controls. Several previous studies have demonstrated elevated levels of $[Ca^{2+}]_i$ in cells from diabetic individuals (Levy *et al*., 1989; Levy *et al*., 1994; Takaya et al., 1997). In this study, a decreased response to both fMLP and thapsigargin was observed in both neutrophils and lymphocytes of T2DM patients when

compared with healthy controls in normal calcium conditions. These findings suggest the presence of an abnormality in cellular calcium homeostasis probably affecting more than one calcium transport pathways in neutrophils and lymphocytes in blood of T2DM patients.

The results of this study have demonstrated a significant lower response to both fMLP and thapsigargin for $[Ca^{2+}]_i$ in neutrophils and lymphocytes of T2DM patients compared to controls suggesting either a deficit in influx of extracellular calcium across the plasma membrane or a decrease in the release of calcium from intracellular stores or both. It is still not clear whether abnormal calcium homeostasis in T2DM is primarily genetic or metabolic. The findings of this study have indicated a possible role for Na^+/Ca^{2+} exchanges in Ca^{2+} regulation in neutrophils and lymphocytes and their modulation in neutrophils and lymphocytes of blood of T2DM patients compared to controls. The original basis behind cellular Ca^{2+} shifts in T2DM is not obvious. Genetic defects involved in the transport of Ca^{2+} across cell membrane might contribute to alter cellular Ca^{2+} homeostasis. Altered Ca^{2+} homeostasis at both cellular and sub-cellular levels can cause abnormal insulin secretion, increased vascular resistance, reduced muscle contraction, reduced enzyme secretion and altered response of vascular smooth muscle cells to Ca^{2+} mobilising vasoactive hormones (Fleischhacker *et al.*, 1999; Levy, 1999; Berridge *et al.*, 2000). Other metabolites including free fatty acids, triglycerides and cholesterol can also affect Ca^{2+}-ATPase activity (Levy *et al.*, 1994; Levy *et al.*, 1986).

Evaluation of Ca^{2+} homeostasis in populations that are genetically susceptible to development of T2DM should be performed to determine the primary defect of impairment in $[Ca^{2+}]_i$ regulation. This study is more complicated as there are a number of similar abnormalities including ageing, hypertension and obesity that are associated

with impaired insulin action and insulin resistance (Boris, 1988; Draznin, 1993; Levy & Gavin, 1995; Orlov *et al.*, 1995). Further studies in future on the molecular and genetic levels of altered Ca^{2+} homeostasis in WBC might reveal the initial defect and its genetic component.

The results presented in Chapter 4 of thesis are either contradictory or very interesting compared to previous findings. Several workers have found either less or more of the different RBC membrane proteins in their studies (Petropoulos *et al*., 2007, Adewoye *et al*., 2011). Nevertheless, the result of the present study have clearly demonstrated that the RBC membrane proteins including spectrin, ankyrin, band 3, band 4.1, and glycophorin were found to be present in significantly higher concentrations in T2DM patients when compared with healthy controls. Blood vessel is considered as a major target organ in T2DM. It is therefore, practical to assume that properties of RBC membrane might have certain abnormalities during disease progression such as CVD in T2DM patients (Kahane *et al*., 1978).

Many studies have demonstrated erythrocyte deformability in several diseases including T2DM and CVD (Kahane *et al*., 1978; Saha *et al*., 2005; Ohno *et al*., 1985). However, very little is known about the content and quality of membrane proteins which contribute to this. SDS-PAGE analysis of RBC membrane proteins from T2DM showed weaker bands compared to healthy controls. These results suggest that the observed abnormalities might be a result of reduced erythrocyte membrane deformability which is associated with T2DM.

RBC membrane proteins perform several functions including maintaining the membrane integrity, anion exchange, serve as channels for movement of cations, involved in transport of glucose, carbon dioxide and oxygen and moreover, they confer blood groupings (Kumar and Clark, 2007). Cytoskeletal proteins are crucial for the

maintenance of structure and integrity of the erythrocyte membrane. Thus, defects in RBC membrane proteins might result in alteration of surface electric charge, and mechanical properties of the membrane thus affecting erythrocyte rheology (Baba *et al*., 1979; Petropoulos *et al*., 2007).

There is increasing evidence regarding association of haemorheological abnormalities in T2DM patients leading to increased aggregation, endothelial damage, and capillary closure (Adak *et al*., 2008). These results are consistent with findings by Schwartz *et al* (1991) who demonstrated oxidation of spectrin in diabetic erythrocytes (Schwartz *et al.*, 1991). A study by Adewoye *et al*., (2001) showed the absence of spectrin in RBC of T2DM patients and increased ankyrin and band-6 in both T1DM and T2DM patients (Adewoye *et al.*, 2011; Kaymaz *et al*., (2005) showed weaker actin and the absence of band 4.5 and band 4.9 in diabetic cats (Kaymaz *et al.*, 2005). Several possible interpretations/explanations can be drawn to these alterations in RBC proteins of T2DM patients. These include ageing of erythrocytes in diabetes (Mazzanti *et al.*, 1992), the relation of AGEs with macrophages (Vlassara *et al.*, 1987) and increased susceptibility of membrane proteins to lysis by endogenous enzymes in diabetes (Gaczynska *et al.*, 1993; Graham Lloyd, 1984).

Therefore, it can be hypothesised that detection of such alterations in RBC membrane proteins of T2DM patients might contribute to the development of CVD and that the results of this study might serve as a blood marker for the existence of CVD risk in T2DM patients.

In chapter 5, the results show that markers of inflammation in T2DM patients were clearly elevated when compared with healthy controls. This study has also shown a significant increase in several other factors in T2DM patients when compared with healthy controls. These findings support the hypothesis that low-grade systemic

inflammation is an underlying factor in the pathogenesis of T2DM. Furthermore, these findings might have important implications for the prevention and treatment of T2DM (Libby *et al*., 2002; Festa *et al*., 2003; Zozulinska and Wierusz, 2006). Recently, C-reactive protein has emerged as an important independent predictor of CVD in both men and women (Ridker *et al.*, 1997; Ridker *et al.*, 2000). It is observed to be present in higher levels in diabetic subjects when compared with non-diabetic control subjects which suggests that inflammation might contribute to accelerated atherosclerosis in diabetes (Ford, 1999).

Recent cross sectional data have also established that elevated levels of CRP are associated with obesity, insulin resistance and glucose intolerance. CRP is a marker of low-grade inflammation and it is involved both in insulin secretion and resistance through altered innate immune response due to heightened systemic inflammation (Pradhan *et al*., 2001; Pickup, 2004). Diabetic vascular complications induce tissue injuries which stimulate cytokine secretions from cells like macrophages and endothelium. The biological mechanisms behind this are not very well understood (Pickup *et al.*, 1997; Pickup and Crook, 1998). In this study, it was observed that there was a higher level of HbA1c in T2DM patients when compared with healthy controls which suggests hyperglycaemia.

High increase in triglyceride level was observed in T2DM patients when compared with healthy controls, which is a common feature of DM. Several research studies have suggested that this is due to an inhibition of lipolysis as a result of reduced action of insulin on adipocytes. This in turn leads to great increase in non-esterified fatty acids due to reduced hydrolysis of stored triglycerides. Increased triglycerides and LDL levels are risk factors for CVD (Garber, 2000; Nesto, 2008).

Impairment of renal function was observed in T2DM patients, which was assessed by the measurement of plasma concentrations of creatinine and urea in both T2DM and healthy controls. The results of this study were consistent with reports of several other workers (Alder 2003; Judykay 2007; Wagle 2010). Plasma creatinine and urea are recognized markers of Glomerular Filtration Rate (GFR) and in nephropathy, it is suggested that high levels of creatinine which is observed in diabetic patients might be due to impairment in the function of nephrons. It is well known that one long term complication of T2DM is nephropathy (Kumar and Clark, 2007). Similarly, high levels of urea could be a result of reduced filtering capacity of kidneys which leads to accumulation of waste products within the system of T2DM patients. Hyperuricemia is commonly associated with DM, obesity, hypertension and in the development of CAD (Kelley & Palella, 1991; Madsen *et al.*, 2005; Short *et al.*, 2005).

Plasma glucose was found to be significantly higher in T2DM patients compared with healthy controls. One of the earlier studies showed that a higher concentration of glucose was observed in T2DM patients when compared with non-diabetic controls (D'Souza *et al*., 2009; Wagle, 2010). Increased liver function test (LFT) abnormalities were observed in this study in T2DM patients compared to controls. There was a higher incidence of LFT abnormalities in T2DM patients when compared with healthy controls. One of the most common marker, is elevated levels of ALT, a gluconeogenic enzyme which specifies impairment of insulin signalling rather than merely hepatocyte injury (O'Brien and Granner, 1991). Chronic elevations of transaminases in T2DM patients reflect underlying insulin resistance.

Levels of trace elements and cations were observed to be higher in T2DM patients compared with healthy controls. Cations and trace elements are required especially for the maintenance of healthy metabolism in the body and either the lack or excess of these

elements can lead to numerous diseases in the body (Kumar and Clark, 2007). The results of this study have shown that the levels of various cations including calcium, magnesium, zinc, copper, and sodium were significantly elevated in T2DM patients compared to controls. These findings are in agreement with previous studies which support alteration of these cationic levels in T2DM patients when compared with healthy controls (Kazi *et al*., 2008; Mahmoud *et al*., 2008). The results of Zn levels in the present study are consistent with the findings of Mateo *et al* (1975) who showed that levels of Zn are equal or higher in diabetic patients compared to that of controls which might be due to heterogenecity of patients (Mateo *et al.,* 1975; Melchior *et al.,* 1989; Zargar *et al.,* 1998). Various studied have shown similar results that diabetic patients have higher plasma levels of Zn (Golik *et al.,* 1993; Rohn *et al.,* 1993; Ruiz *et al.,* 1998; Zargar *et al.,* 1998). Plasma Zn varies according to metabolic status and hormonal changes and hence it is a poor indicator of zinc status. Hyperzincuria is consistent with other studies and has been associated with hyperglycaemia (Ekmekcioglu *et al.,* 2001).

The results of Cu levels are consistent with those reported by Noto *et al.,* (1984) who showed high levels of Cu in older subjects (Noto *et al.,* 1984). These results also support the findings by Walter *et al.,* 1991 and Zargar *et al.,* 1998 who demonstrated high levels of Cu in diabetic women on oral contraceptives and with retinopathy and hypertension (Walter *et al.,* 1991; Zargar *et al.,* 1998). Increased concentration of Cu is associated with several disorders related to structure of arterial walls, stress, infection and diabetes mellitus (Beshgetoor and Hambidge, 1998).

The results of Mg levels are inconsistent with those findings of several other research studies which demonstrated that plasma levels of Mg are lower in diabetic patients than controls Mg levels are observed to be higher in T2DM patients compared with healthy

controls in this particular study which are consistent with other studies that already have been published (Fujii *et al.*, 1982; McNair *et al.*, 1978; Sjögren *et al.*, 1988).

The results of iron levels are consistent with those of other research studies. DM is associated with altered iron homeostasis in both human and animal models. Iron serves as a catalyst for lipid and protein oxidation and it is involved in generation of reactive oxygen species. It also contributes to diabetic nephropathy. Excessive iron is also implicated in the pathogenesis of T2DM and its complications (Thomas *et al.*, 2004).

Even though the data of this study are observed to be consistent with those of other research studies, it is very important to obtain more quantitative data from larger groups in order to fully elucidate the relationship between T2DM and various cations in the body. Analyses could also be performed between various cations in plasma and compare their levels in cells including erythrocytes and lymphocytes.

Levels of total protein were observed to be lower in T2DM patients when compared with healthy controls which are characteristic of nephrotic syndrome that occurs due to long standing diabetes and is also the primary indication for dialysis. One of the common causes of hypoproteinemia is excessive protein discharge in urine (Hanoune *et al.*, 1972; Ahmed, 2010).

The results presented in Chapter 6 of this study have shown an increased level of low molecular weight AGEs in plasma of T2DM patients when compared with healthy controls. Incomplete digestion of AGEs-modified protein can result in the formation of low-molecular weight AGEs. These LMW-AGEs have a high toxic potential and LMW fluorescence increases with duration of disease which appears to be higher in patients with T2DM. LMW fluorescence is a marker of tissue fluorescence which is induced in DM due to a result of hyperglycaemia and oxidative stress, but not only because of

Maillard's reaction (Ahmed, 2005; Ahmed and Thornalley, 2007; DSouza *et al*., 2009; Ahmed, 2010). Excessive accumulation of AGEs underlies various complications in patients including diabetic neuropathy, nephropathy, vasculopathy and retinopathy (Peppa *et al.*, 2003; Ahmed, 2005). AGEs have been implicated in the initiation and progression of CVD (Thomas *et al.*, 2005; DSouza et al., 2009). LMW-AGEs can predict mortality in patients receiving chronic haemodialysis. (Roberts *et al.*, 2006). Therefore, the possible involvement of LMW-AGEs in the mechanisms leading to atherosclerosis and inflammation in T2DM patients may need further investigation.

The main findings of this study show that individual risk factors for both T2DM and CVD provide a common soil for the development of either of them or both. This study supports a positive association between various markers and T2DM and moreover, it supports the hypothesis that inflammation is closely involved in the pathogenesis of T2DM. Clearly, T2DM is more than a risk factor for CVD in T2DM patients. The elevated levels of various risk factors and blood borne markers in T2DM patients lead to development of CVD. Further studies are needed to establish pathophysiological relationship between inflammation and T2DM.

7.2 Conclusion

This study was originally designed to investigate how T2DM can lead to CVD and subsequent to morbidity and mortality of the patients compared to healthy age-matched controls. In doing so, the project employed blood to measure a number of biochemical markers and mediators including cations in plasma, intracellular free calcium concentrations in WBC, osmotic fragility of RBC and proteins in RBC membrane which are associated with the development of T2DM. The results show that inflammation is very strongly associated with micro-vascular complications and CVD in diabetes. Measurement of these inflammatory markers in T2DM patients is very

important for risk assessment of diabetic complications. Prevalence of T2DM is increasing worldwide. There is also an increase in the number of people with CVD among T2DM patients. In order to prevent and to reduce the incidence of cardiovascular morbidity and mortality in high-risk diabetic patients, it is imperative to emphasize on routine measurements of these blood borne biochemical markers and mediators. Although this appears to be a challenging task, strict screening for an underlying sub-clinical CVD and aggressive prevention programmes would possibly achieve the target in T2DM patients.

7.3 Limitations

There are several limitations for this study. The sample size of this study could have been larger in terms of T2DM patients and healthy controls in all chapters. T2DM patients with diabetic complications including diabetic retinopathy, neuropathy, and nephropathy could also have been included in this study. Measurement of $[Ca^{2+}]_i$ could also have been performed in platelets of T2DM patients and controls.

7.4 Scope for future studies

- Future studies can involve the measurement of $[Ca^{2+}]_i$ in different blood cells including basophils, eosinophils and platelets of both T1DM and T2DM patients and compare them with healthy controls. In addition, it is also important to characterize the different transporters which are associated with cellular Ca^{2+} homeostasis using specific agents.

- Studies can also include the measurement of biochemical parameters and more inflammatory markers in large population groups and comparison can be done with various other populations. These include both T1DM and T2DM patients and diabetic patients who suffer from heart failure, coronary heart disease,

hypertension, arrhythmias, nephropathy, retinopathy and neuropathy compared to controls.

- Further studies can involve an investigation of age-related changes on various measured biochemical parameters concentrating more on different age-group (eg 15-39, 40-59, 60 and above) taking also into account factors like gender and ethnicity.

- A study of how chronic T1DM and T2DM affect blood vessels and heart at both cellular and sub-cellular levels including development of hypertrophy and fibrosis could be performed. This will involve the use of animal models for both T1DM and T2DM to analyse fibrosis, remodelling and contraction.

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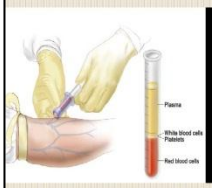
Appendices

Appendix 1

Poster presentation at "Physiology 2011", Physiological Society Meeting, University of Oxford, 11-14 July, 2011.



A comparative study of erythrocyte membrane proteins in type 2 diabetic patients and healthy controls

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Introduction

Type 2 Diabetes mellitus (T2DM) is a major metabolic disorder affecting more than 200 million people worldwide (1). Altered structural and functional properties of erythrocyte membrane proteins have been widely implicated in T2DM patients (2). The main aim of this study is to analyse the proteins in red blood cell membrane of T2DM patients compared to healthy controls which included spectrin, ankyrin, band 3, protein 4.1 and glycophorin (Fig. 1) in a total of 9 T2DM patients and healthy controls.

Material and methods

1. T2DM patients and healthy control subjects were recruited from the local hospital diabetic clinic and the University.
2. Blood samples (10 ml per subject) were obtained from each subject through venipuncture technique and erythrocyte membrane proteins were isolated separately from each sample.
3. The total protein concentration of membrane proteins was determined by Bradford assay using bovine serum albumin (BSA) as standard.
4. Gel electrophoresis (SDS-PAGE) was performed on a Bio-Rad mini gel system using 12% ready Tris-Hcl gel.
5. The protein quantification of each electrophoretic band was accomplished through scanning densitometry using Bio-Rad Gel Doc analyser and each protein was expressed in terms of intensity of each band (Fig. 3).

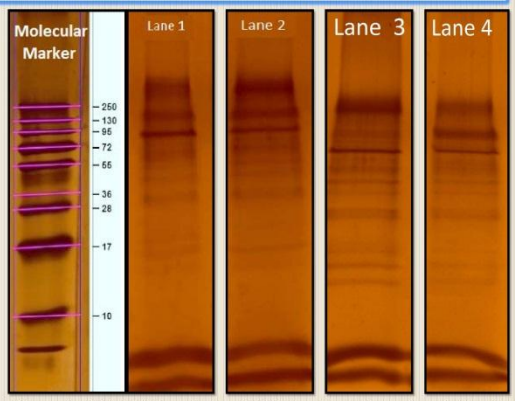

Figure 2: SDS-PAGE analysis of T2DM patients (Lane 3 and 4) and healthy controls (Lane 1 and 2). Molecular weight marker range 10-250 KDa was used .

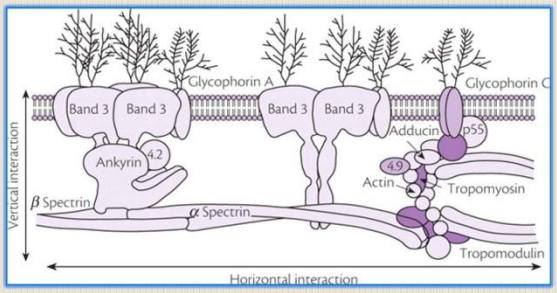

Figure 1: The erythrocyte membrane; A model depicting the major proteins of the erythrocyte membrane which include α and ß spectrin, ankyrin, band 3, 4.1 (protein 4.1), 4.2 (protein 4.2), actin, and GP (glycophorin) (3).

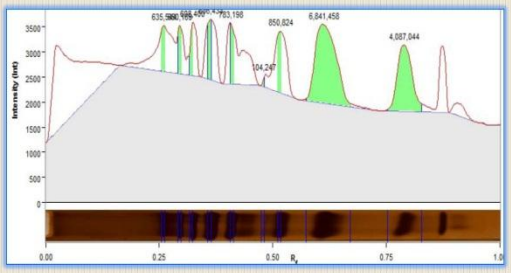

Figure 3: Graphical representation of molecular weight marker in terms of intensity of each band, analysed by Bio-Rad Gel Doc analyser.

Results

Initial experiments have indicated significant (Student's t-test; $p<0.05$; $n=9$) increases in the volumes of all five proteins in erythrocyte membranes of T2DM patients compared to healthy controls (Fig. 2). Typically, the values (mean± SEM) are (A) spectrin – control 1,056 ± 92 and T2DM 3,302 ± 70, (B) ankyrin – control 1,107 ± 69 and T2DM 3,376 ± 40, (C) band 3 protein- control 1,525 ± 56 and T2DM 3,221 ± 28, (D) protein 4.1- control 1,646 ± 128 and T2DM 3,033 ± 23 and (E) glycophorin - control 2,072 ± 124 and T2DM 2,719 ± 20.

Discussion

These results provide evidence of profound quantitative and qualitative alteration of the erythrocyte membrane proteins in T2DM patients compared to healthy controls indicating functional implications of the diabetic patients.

References

1. Zimmet P, Alberti KGMM, & Shaw J (2001). Global and societal implications of the diabetes epidemic. Nature 414, 782-787.
2. McMillan DE, Utterback NG, La Puma J (1978). Reduced erythrocyte deformality in diabetes 27(9), 895-901.
3. Tse WT, Lux SE (1999). Red blood cell membrane disorders. Br J Haematol.104, 2–13.

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Appendix 2

Poster presentation at "The 3rd International Conference on Advanced Technologies and Treatments for Diabetes", Basel, Switzerland, Feb 10-13, 2010.

Cardiovascular Risk Markers and Type 2 Diabetes Mellitus

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Introduction

Type 2 Diabetes mellitus (T2DM) is a complex metabolic disorder with major epidemiological impacts affecting over 200 million people worldwide. This number is expected to reach indefinite proportions by the year 2030 (Fig 1) (1). Individuals with T2DM have an increased risk of cardiovascular diseases (CVD). British Medical Association has reported that CVD accounts for up to 80% mortality associated with T2DM in the United Kingdom. Type 2 diabetics (T2DPs) carry an array of risk factors including dyslipidemia, hyperglycaemia, insulin resistance and elevated concentrations of various biomarkers in their circulation which lead to an accelerated probability of CVD (2). Consequently, rates of cardiovascular mortality and morbidity are very high in T2DPs. Since the accelerated atherosclerosis and cardiovascular diseases in diabetes are likely to be multifactorial, there is an urgent need for consideration of different therapeutic approaches.

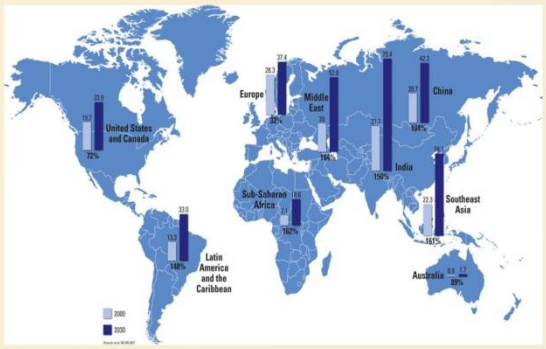

Figure 1:World prevalence of Diabetes mellitus 2000-2030 (3)

Aims and Objectives

• The main aim of this study is to identify and compare a number of risk factors and biochemical parameters which serve as predisposition factors and govern susceptibility to CVD in T2DM and to evaluate their possible roles as cardiovascular risk markers among type 2 diabetic patients.

• To measure the levels of glucose, insulin, lipid profile, inflammatory markers like C-reactive protein (CRP), interleukine-6 (IL-6), tumour necrosis factor (TNF), fatty acid binding protein (FABP), HbA1c, homocysteine in T2DPs and to compare them with controls.

• To analyse the various cations in the blood plasma of T2DPs and compare their levels with healthy age-matched controls.

Materials and Methods

• Blood from T2DPs was collected from the Lancashire Teaching Hospitals (NHS), UK. A total of 20 T2DPs and 25 healthy age-matched controls were screened.

• Analysis of plasma glucose, insulin, lipid profile, C-reactive Protein (CRP), HbA1C was done using Modular Pre-analytics® (Roche Diagnostics) at the Royal Preston Hospital, United Kingdom.

Results

Initial observations indicate a highly significant ($P<0.01$) increase in the levels of glucose, triglycerides, Hba1c, urea, CRP, Low-density lipoprotein (LDL) in T2DPs when compared with healthy age-matched controls.

These results clearly indicate that different biochemical markers may be used for the assessment of CVD risk in T2DM patients. Further studies following these preliminary findings are currently in development and are focussed on age-related changes that would probably predispose T2DPs to Cardiovascular disease in future.

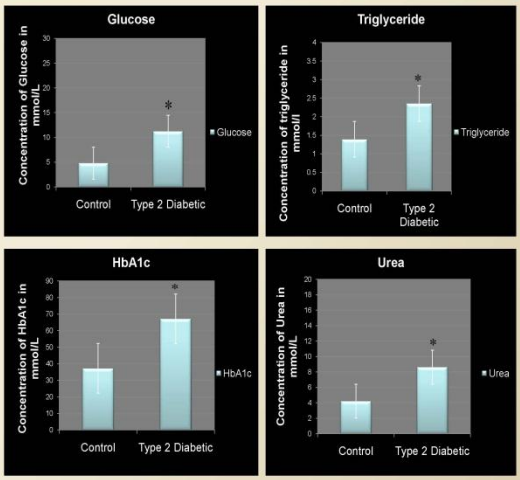

Figure 2: Graphical representation of selective biochemical parameters in T2DPs and controls

References

1. Zimmet P., K.G.M.M. Alberti & J.Shaw (2001). Global and societal implications of the diabetes epidemic. Nature **414**:782-787.
2. Amos, A., D. Mccarty & P. Zimmet (1997). The rising global burden of diabetes and its complications; estimates and projections to the year. Diabetic Med. **14**:S1-S85.
3. Hossain et al. NEJM 2007.

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Appendix 3

Poster presentation at “Annual Graduate Conference”, University of Central Lancashire, Preston, United Kingdom, June 2009.

Cardiovascular Risk Markers in Type 2 Diabetes Mellitus

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Introduction

Diabetes mellitus (DM) is a metabolic disorder caused by insulin deficiency which results in abnormal levels of glucose in the bloodstream (1) (Fig. 1). It is a disease of major epidemiological impact affecting over 200 million people worldwide.There are two types of diabetes mellitus, type 1 and type 2. Patients with type 2 diabetes mellitus have an increased risk of cardiovascular disease (CVD) (2). British Medical Association has reported that CVD accounts for up to 80% mortality associated with type 2 DM in the UK. Cardiovascular risk markers have not been well understood in type 2 diabetes. People with type 2 DM carry an array of risk factors for CVD. A number of unfavourable conditions predisposing to cardiovascular disease (CVD) (Fig. 2) co-exist with diabetic status including hyperglycemia and its immediate biochemical sequelae, insulin resistance and dyslipidemia that are associated with elevated concentrations of various biomarkers in the circulation (Fig. 3). Consequently, rates of mortality and morbidity are very high in patients with type 2 diabetes mellitus.

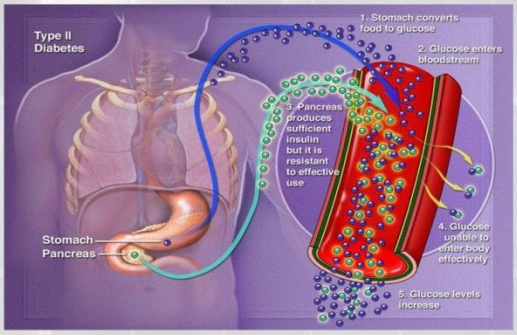

Figure 1: Illustration of Type 2 diabetes (3)

Aims and Objectives

The main aim of this study is to identify and compare a number of risk factors and biochemical parameters which serve as predisposition factors and govern susceptibility to CVD in type 2 DM and to evaluate their possible roles as cardiovascular risk markers among type 2 diabetic patients.

To measure the levels of glucose, insulin, lipid profile, inflammatory markers like C-Reactive protein (CRP), Interleukine-6 (IL-6), Tumour necrosis factor (TNF), Fatty acid binding protein (FABP), HbA1c, homocysteine in patients with type 2 DM and to compare them with controls.

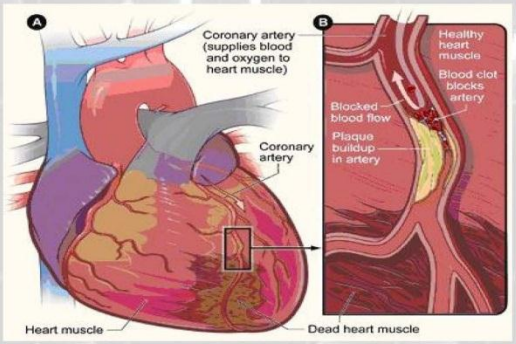

Figure 2: Formation of Plaque in diabetic heart (3)

Materials and Methods

Patients of different ages will be screened for body mass index (BMI), glucose, insulin, lipid profile, and inflammatory markers including C-Reactive Protein (CRP), Interleukine, Homocysteine, Fatty acid binding protein (FABP), HbA1C and analysis of cations in blood plasma using Inductively coupled plasma mass spectroscopy (ICP-MS) and their levels are compared with healthy age-matched controls.

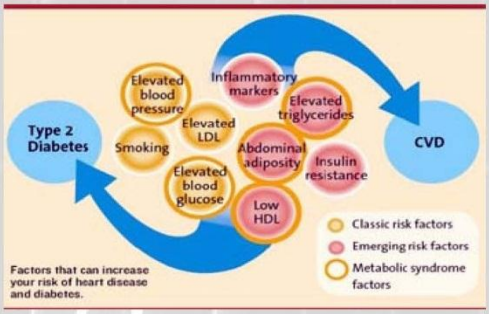

Figure 3: Risk factors for Type 2 diabetes (3)

Expected outcomes

The results of this particular study will provide an evidence for a better understanding of Cardiovascular risk markers in type 2 Diabetes mellitus. These results will have potentially far reaching implications in the development of more effective clinical strategies and therapeutic interventions to prevent, manage or delay onset of Cardiovascular disease in type 2 diabetic patients.

References

1.Zimmet P., K.G.M.M. Alberti & J.Shaw (2001). Global and societal implications of the diabetes epidemic. Nature **414**:782-787.
2. Amos, A., D. Mccarty & P. Zimmet (1997). The rising global burden of diabetes and its complications; estimates and projections to the year. Diabetic Med. **14**:S1-S85.
3. www.nlm.nih.gov

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Appendix 4

Chemicals and Reagents

Chemicals and Reagents

1) Cold isotonic buffer

145 mM NaCl

5 mM KCl

5 mM Hepes

2) Hypotonic lysing solution

15 mM KCl

0.01 mM EDTA

1 mM EGTA

5 mM Hepes (4-2-hydroxyethyl-1-piperazine-ethanesulphonic acid)

pH = 6.0

3) RBC incubating solution

Tris ATP - 5 mM

KCl - 25 mM

NaCl - 74 mM

$MgCl_2$ - 5mM

EGTA - 0.1 mM

Tris-HCl - 25 mM

Appendix 5

Conference Proceedings

Proceedings of The Physiological Society

- Human Physiology Abstracts

Physiology 2012 (Edinburgh) (2012) **Proc Physiol Soc 27, PC145**

Poster Communications

Effect of type 2 diabetes mellitus on intracellular free calcium concentration in human neutrophils and lymphocytes

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Medline articles by:

- Kappala, S
- Espino, J
- Rodriguez, A
- Pariente, J
- Singh, J

Type 2 diabetes mellitus (T2DM) is a major global health problem currently affecting more than 220 million people. T2DM is associated with a number of symptoms and long term complications including neuropathy, cardiomyopathy, nephropathy, retinopathy, delayed wound healing and constant infections. This study investigated the effect of T2DM on intracellular free calcium concentration [Ca2+]i in neutrophils and lymphocytes from T2DM patients compared to healthy age-matched controls. The study had the relevant ethical clearance from LREC in UK and UNEX in Spain. Both neutrophils and lymphocytes were isolated by density gradient centrifugation using Ficoll histopaque and loaded with fura 2-AM. [Ca2+]i was measured using established fluorescent method. Changes in [Ca2+]c were calculated using the fura-2 340/380 ratio. Mean (± SD) basal [Ca2+]i was 100±11 nM (n=6) and 75.0±10 nM, (n=6) in healthy age-matched control neutrophils and lymphocytes, respectively. In T2DM patients [Ca2+]i was 92±10 nM (n=6) and 87±10 nM (n=6) in neutrophils and lymphocytes, respectively. These results show no significant difference in basal [Ca2+]i comparing control with T2DM in either neutrophils or lymphocytes. Stimulation of human neutrophils with 10-8 M fMLP resulted in transient and marked increases in [Ca2+]i above basal level reaching maximum within 15-25 sec, followed by a rapid decline in both age-matched control and T2DM cells. Peak [Ca2+]i was significantly (Student's t-test; $p<0.05$) decreased in neutrophils from T2DM patients compared to age-matched controls. Typically, [Ca2+]i was 2.3×104 nM (n=6) and 0.08×104 nM (n=6) for control and T2DM neutrophils, respectively. Similarly, stimulation of neutrophils with 10-6 M thapsigargin resulted in a gradual increase in [Ca2+]i reaching maximum within 4-5 min. There was a significant ($p<0.05$) decrease in [Ca2+]i in T2DM human neutrophils compared to age-matched controls. [Ca2+]i was 9.0×104 nM (n=6) and 2.0×104 nM (n=6), in control and T2DM neutrophils, respectively. Stimulation of fura-2 loaded lymphocytes with 10-6 M thapsigargin resulted in gradual increases in [Ca2+]i in both age-matched control and T2DM. However, [Ca2+]i was significantly ($p<0.05$) less in lymphocytes from T2DM compared to age-matched control. Typically, [Ca2+]i was 9.0×104 nM (n=6) and 2.0×104 nM (n=6) in control and T2DM lymphocytes, respectively. Together, the present results have demonstrated that [Ca2+] homeostasis seemed to be deranged in both neutrophils and lymphocytes of T2DM patients compared to age-matched controls suggesting a relationship between cellular calcium and frequent infections normally associated with T2DM patients.

Where applicable, experiments conform with Society ethical requirements

Proceedings of The Physiological Society

- Human Physiology Abstracts

University of Oxford (2011) **Proc Physiol Soc 23, PC97**

Poster Communications

A comparative study of erythrocyte membrane proteins in type 2 diabetic patients and healthy controls

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Medline articles by:

- Singh, J
- Kappala, S
- Raj bhandari, S
- Abel, P

Type 2 Diabetes mellitus (T2DM) is a major metabolic disorder affecting more than 200 million people worldwide (Zimmet et al., 2001). Altered structural and functional properties of erythrocyte membrane proteins have been widely implicated in T2DM patients (McMillan et al., 1978). This study analysed the specific proteins of the erythrocyte membrane in T2DM patients compared to healthy controls. T2DM patients and healthy control subjects were recruited from the local hospital diabetic clinic and the University. Blood samples (10 ml per subject) were obtained from each subject and erythrocyte ghost membranes were isolated separately from each sample. Total erythrocyte membrane protein concentration of each sample was determined using bovine serum albumin (BSA) as standard. The protein components of the erythrocyte ghost membranes were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) which was performed on a Bio-Rad mini gel system using 12 % ready Tris-HCl gel. The gels were stained using silver staining method and identification of protein bands was done using molecular weight marker on a Bio-Rad gel documentation system. The project had the relevant ethical clearance from LREC and the University Ethics Committee to undertake the study. This investigation analysed five proteins namely spectrin, ankyrin, band 3, protein 4.1 and glycophorin in 9 T2DM patients and 9 healthy controls for comparison. The protein quantification of each electrophoretic band was accomplished through scanning densitometry and each protein was expressed in terms of intensity of each band. Initial experiments have indicated significant (Student's t-test; $p<0.05$; $n=9$) increases in the volumes of all five proteins in erythrocyte membranes of T2DM patients compared to healthy controls. Typically, the values (mean ± SEM) were - (A) spectrin- control 1,056 ± 92 and T2DM 3,302 ± 70, (B) ankyrin- control 1,107 ± 69 and T2DM 3,376 ± 40, (C) band 3 protein- control 1,525 ± 56 and T2DM 3,221 ± 28, (D) protein 4.1- control 1,646 ± 128 and T2DM 3,033 ± 23 and (E) glycophorin - control 2,072 ± 124 and T2DM 2,719 ± 20. These results provide evidence of profound quantitative and qualitative alteration of the erythrocyte membrane proteins in T2DM patients compared to healthy controls indicating functional implications of the diabetic patients.

Where applicable, experiments conform with Society ethical requirements