CHAPTER 1. INTRODUCTION

1.1 BACKGROUND OF DIABETES MELLITUS IN ZIMBABWE AND IN DEVELOPING COUNTRIES

The current number of patients afflicted with diabetes in the world is 135 million and diabetes mellitus has now reached epidemic proportions [1]. Within the next 10 years the projected number will exceed 200 million. It is estimated that by the year 2025, three quarters of the world's 300 million adults with diabetes will be in industrialising countries, and almost a third of these will be in India and China alone. Africa, a developing continent with a population very similar to that of India will not escape this scourge. Zimbabwe is a developing country situated in southern-central Africa and comprises of a few ethnic groups with blacks making up the majority (98%) of the subjects [2].

Although there are ethnical, racial and geographical differences in the prevalence of type 2 diabetes mellitus (T2DM) in the world's population, the Zimbabwean black population can be considered to be in a transition from a phase of parasitic and infectious morbidity and mortality to that of chronic cardiovascular and degenerative diseases. This is attributed to urbanisation, a rise in socio-economic status, dietary habits and a decline in physical activity, all attributes of the modern Western lifestyle. It is thus expected that a significant percentage of its population will in the future be affected with diabetes mellitus. A pilot study conducted in Zimbabwe yielded a prevalence of 2% diabetes in both urban and rural communities with a glucose intolerance of 7% [3]. Recently the Minister of Health reported that if the HIV pandemic is removed from the number of deaths

in the country, then DM is the number one cause of all mortalities in the country (Herald report, 2001).

In recent decades, dramatically high rates of T2DM have been observed in historically undernourished, recently urbanized populations and this has reached epidemic proportions [4-6]. The increase in the incidence of T2DM in traditional populations, such as the Australian Aborigines, Native Americans, Pacific islanders and some migrant populations like the Asian Indians could be explained by rapid and profound changes in their environment and lifestyles, from those of hunters, food gatherers and subsistence agriculturists to a contemporary model whose fundamental features are more sedentary occupations (low energy expenditure), high energy fuel resources (access to more refined carbohydrates, i.e. less complex carbohydrates and high-calorie food) as well as overabundant food sources. Rapid and profound alterations in diet and lifestyle factors can transform genes that once bestowed selective biologic advantage to non infectious perturbations such as T2DM, hypertension and cardiovascular disease phenotypes, and these disorders will emerge as significant causes of morbidity and mortality in developing nations just as in developed ones.

A classic example of the devastating effects of rapid and profound changes of the environment in the evolution of metabolic disorders is that of the Pima Indians of America [7, 8]. The Pimas have the highest prevalence of diabetes in the world, attributable to numerous environmental as well as cultural, and psychosocial alterations, including a genetic susceptibility. The environmental factors mainly

involve dietary changes and the cultural effects result from increased incidence of obesity.

Thus predications indicate a potentially explosive increase in the prevalence of diabetes mellitus world-wide, especially in the developing countries.

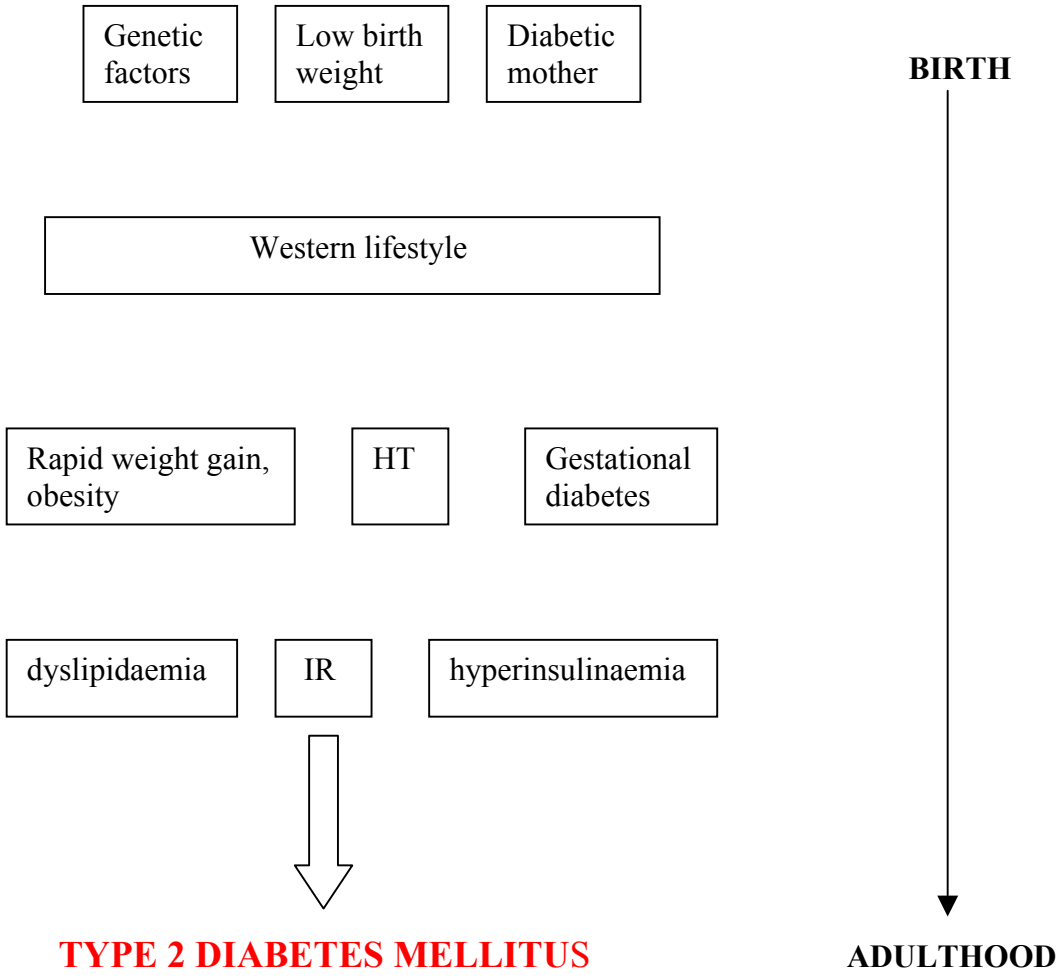
1.2 DIABETES MELLITUS

Diabetes mellitus (DM) is not a single disease entity but a heterogeneous group of metabolic syndromes of poorly understood aetiology, with varying severity, varying treatment regimes, and is characterised by glucose intolerance, hyperglycaemia as well as a relative or absolute deficiency of insulin. Diabetes mellitus is a major public health problem, consisting of two major forms, type 1 (T1DM) and type 2 (T2DM) and afflicts 3-5% of the Western Europe and North American Caucasian populations [1, 9]. However diabetes is also becoming an increasing problem in developing countries affecting about 10% of urban Indians, 10% of American Blacks, 10% of Brazilians and 2% of urban Tanzanians [9, 10]. Pima Indians have the highest prevalence of T2DM reported in the world, reaching 70% in the 55-64 year age group [7, 8]. Although the prevalence of T2DM, previously known as non-insulin-dependent-diabetes mellitus (NIDDM) differs greatly between the world populations, it is the most common form of diabetes afflicting about 90% of all diabetic subjects [11]. The incidence of T1DM varies greatly among populations, the Sardinians and Finns having the highest rates [12, 13].

T1DM, previously known as insulin-dependent diabetes mellitus (IDDM), develops predominantly in children and in young adults, although it appears in all age groups. It is characterised by absolute insulin deficiency through autoimmune destruction of the beta cells of the pancreatic cells. The major genetic susceptibility to T1DM is linked to the human leukocyte antigen (HLA) complex on chromosome 6 [14]. However the genetic background interacts with the environment, for example the food one eats, the climate one lives in and the microorganisms one is subjected to, resulting in beta cell destruction.

The development of T2DM, a heterogeneous disorder, which affects mostly middle-aged individuals, is due to a complicated interplay between genes and the environment on one side, and the interaction between metabolic defects in various tissues [15-17], (Figure 1.1). The metabolic dysfunction in various tissues includes the pancreatic beta cell (decreased insulin secretion), skeletal muscle (insulin resistance), liver (gluconeogenesis), adipose tissue (increased lipolysis) and these defects are worsened by the developing hyperglycaemia in the afflicted individuals. Thus the hyperglycaemia of T2DM from the interplay of genetic and environmental factors, influence a number of intermediate traits e.g. beta cell mass, insulin secretion, insulin action, fat distribution, and obesity. Before T2DM develops, several processes occur, including elevated blood insulin levels, disproportionate to the degree of glycaemia as well as obesity, thus reflecting insulin resistance. This insulin resistance plays a key role in the development of glucose intolerance and underpins the eventual development of T2DM in about 30% of affected individuals. The disorder of T2DM is associated with a cluster of

FIGURE 1.1. Theoretical framework of risk factors from childhood to adulthood that can lead to the development of T2DM.



HT = hypertension: IR = insulin resistance.

cardiovascular risk factors, which include obesity, dyslipidaemia, hypertension, atherosclerosis and a procoagulant state [18-21].

Iron overload is possible example of interplay of genetic and environmental factors that could result in the development of the T2DM phenotype. This could be explained by possible associations between various gene mutations of iron indices and enhanced absorption of non-haem iron from the diet, or high dietary iron content due to traditional beer (in Blacks of southern Africa) [22, 23]. Transferrin and haptoglobin could be candidate genes for iron overload in Blacks of sub-Saharan Africa, and in some Caucasians, the haemochromatosis gene on chromosome 6 has been associated with iron overload, and is a potential genetic marker for T2DM [24-26]. Some studies suggest that excessive tissue iron (iron overload) may contribute to the occurrence and complications of T2DM [27-29]. In man body iron is regulated primarily by gut absorption, as humans do not have a physiological mechanism of excreting excess iron. Although it is unlikely that prolonged high dietary iron intake could result in iron overload, it is possible that genetic defects could enhance absorption of dietary iron, resulting in iron overloading (excessive deposition or storage in parenchymal cells of organs). Accumulation of iron could affect insulin synthesis and secretion in the pancreas, could enhance free fatty acids oxidation through augmented production of free radicals, or could interfere with the insulin-extracting capacity of the liver [30-35]. Elevated free fatty acid oxidation diminishes glucose utilisation in muscle tissue and increases gluconeogenesis in the liver, a situation that could accelerate insulin resistance, glucose intolerance and eventually T2DM. As iron is a potent pro-oxidant, it is possible that it could interfere with insulin signalling at cellular

level. Thus abnormalities of iron homeostasis arising from environmental insults and genetic aberrations could result in a rare monogenic form of T2DM. Alternatively, the genetic aberrations could act as modifier genes that influence penetrance in T2DM by enhancing its phenotypic expression.

Almost all human diseases are influenced by genetic factors, which determine disease resistance or susceptibility and interrelations with environmental factors [36, 37]. T2DM is likely to have a major genetic component in view of the varied prevalence between ethnic groups, the familial clustering, the high concordance in monozygotic twins and the almost 100% concordance in type 2 diabetic pairs [38-40]. Intricate disorders and traits arise mainly from genetic variation that is abundant in the general population, rather than specific and comparatively rare mutations. Unfortunately, only a few of genetic regions and genes intrinsically associated with complex diseases have been unravelled. T2DM is likely to belong to this group of complex disorders and is still a genetic and metabolic nightmare [41-43]. Although the aetiology of T2DM is uncertain, it is hoped that metabolic profiling of diabetic subjects in this study will enable us to review our ideas on the molecular genetics of T2DM.

1.3 TYPE 2 DIABETES MELLITUS AND THE METABOLIC SYNDROME

The clustering of cardiovascular risk factors such as glucose intolerance, hypertension, hypertriglyceridaemia, hypo HDL-cholesterol, obesity, microalbuminuria and abnormalities in coagulation and fibrinolysis is known by several names such as the insulin resistance syndrome, the deadly quartet, the

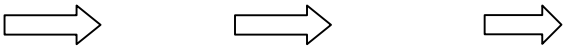
plurimetabolic syndrome, syndrome X and the metabolic syndrome [18-21]. The unifying name for these traits by the World Health Organization (WHO) is, metabolic syndrome, and the components of this syndrome are hypertension, dyslipidaemia, obesity and microalbuminuria [44]. According to WHO criteria, a person afflicted with T2DM has the syndrome if he or she fulfils 2 of the criteria in addition to being insulin resistant. The metabolic syndrome is a strong determinant of T2DM, as about 70% subjects afflicted by diabetes have the syndrome [45, 46]. Clustering of the syndrome in families suggests a genetic component, and environmental factors, due to affluence, may act as important modifiable risk factors to influence penetrance.

Table 1.1 attempts to illustrate how the environmental factors (acquired factors) can lead to the metabolic syndrome, diabetes mellitus and cardiovascular disease (CVD). The entire environmental insults initiate hyperinsulinaemia and/or insulin resistance and these can lead to hypertension or diabetes or CVD. Obesity on its own can directly precipitate hyperinsulinaemia and/or insulin resistance and dyslipidaemia can directly lead to CVD. However, it is important to note that diabetes mellitus, hypertension, obesity, dyslipidaemia and possibly CVD itself are under genetic control.

The metabolic syndrome is becoming a serious problem as populations age and become more obese. The acute and chronic complications of diabetes mellitus are major causes of hospital admissions, blindness, renal failure, amputations, stroke and CHD and substantially affect annual per capita health care expenditure for

TABLE 1.1. A model showing how acquired factors (environmental triggers) can lead to the metabolic syndrome, diabetes mellitus and cardiovascular disease (CVD).

ENVIRONMENTAL FACTORS	METABOLIC SYNDROME RISK FACTORS	CLINICAL SYNDROME	SEQUEL
Diet	hyperinsulinaemia		
	insulin resistance	hypertension	
Alcohol	hypertension		
	high WHR	**T2DM**	**CVD**
Smoking	glucose intolerance		
	dyslipidaemia	obesity	
sedentary lifestyle	microalbuminuria		



people with diabetes [47]. Cardiovascular disease, including both cardiac and cerebrovascular disease is the leading cause of death among persons with T2DM [48]. The major risk factors associated with mortality in a population with diabetes are, age, gender, current smoking, hypertension, dyslipidaemia and non-leisure physical inactivity. It is estimated that in developed countries, approximately 10% of the total health care expenditure is utilised for this purpose [49]. The financial burden brought about this disease and its secondary complications will put a tremendous strain on the limited economic resources of developing nations such as Zimbabwe, which are emerging as high-risk populations.

1.3.1 Insulin resistance and hyperinsulinaemia

The pancreatic beta cell hormone, insulin, plays a pivotal role in the regulation of glucose homeostasis and numerous cellular effects on metabolism, growth and differentiation. Insulin action is mediated through specific cell-surface (membrane) receptors and defects in insulin action and insulin secretion, which are both present in T2DM and are believed to be genetically predetermined [50].

Clinically, insulin resistance is defined by reduced insulin-mediated uptake of glucose in skeletal muscle, however studies show that the liver, fat cells and endothelial cells may also develop insulin resistance in subjects with the metabolic syndrome i.e. the physiologic antecedent is postulated to be tissue resistance to insulin [51]. Thus in such tissues as adipocytes, muscles or hepatocytes, the insulin resistance can be due to, reduced insulin receptor substrate-1 (IRS-1), reduced insulin binding to its receptor, reduced receptor

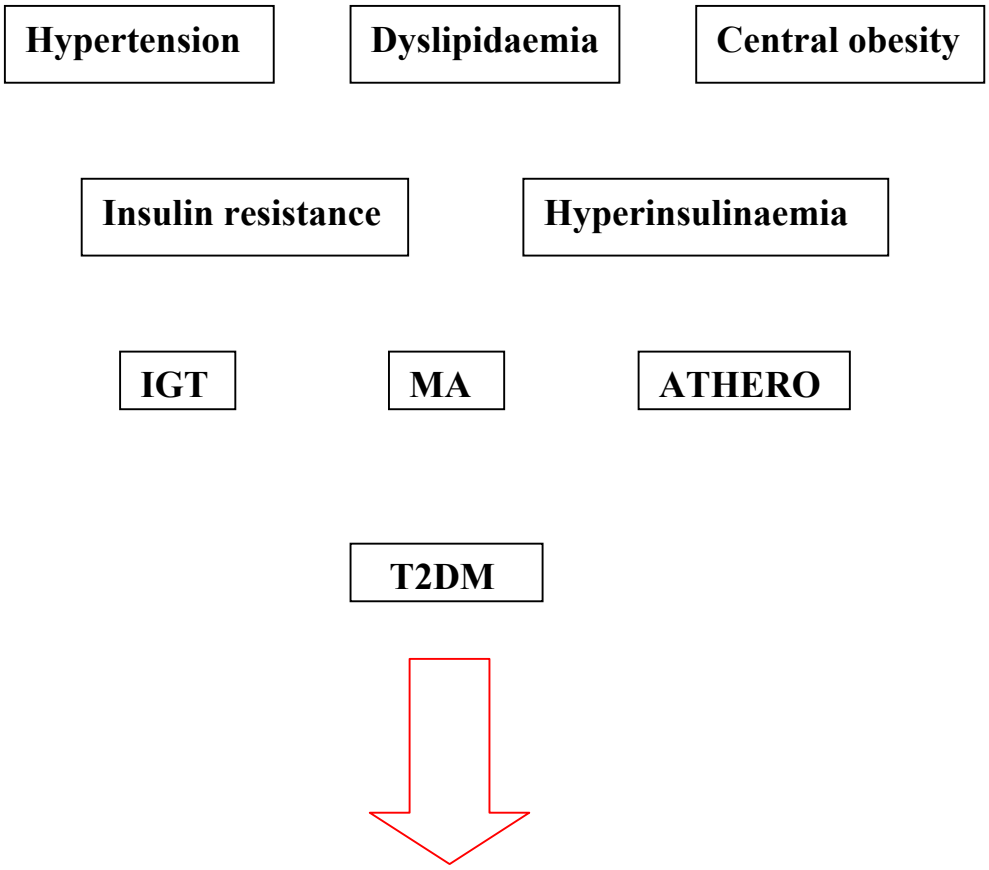
phosphorylation and tyrosine kinase activity, reduced phosphorylation of insulin receptor substrates or suppression of hepatic glucose output. Hyperglycaemia, dyslipidaemia and arterial hypertension may be induced by this resistance and these variables may provoke arteriosclerosis and "vascular ischaemic heart disease". Obesity in particular seems to be responsible for insulin resistance in subjects with the metabolic syndrome [52-54]. The mechanism could involve intracellular accumulation of Acyl CoA and triglyceride, increased production of peptides from the adipose tissue, such as TNF-alpha, and reduced production of adeponectin may also play a role.

Initially insulin resistance causes a compensatory hyperinsulinaemia and in individuals, who do not have any defect in beta cell function, compensate for insulin but this can eventually give way to pancreatic beta cell failure. Therefore, insulin resistance, a principal feature of T2DM precedes the clinical development of the disease by many years. Elevated insulin requirement, hyperglycaemia, hyperlipidaemia, and external factors such as obesity, sedentary lifestyle, and high-calorie food contribute to beta cell deterioration [55, 56]. Insulin resistance and its concomitant compensatory hyperinsulinaemia lead to the overproduction of VLDL particles [57]. A relative deficiency of lipoprotein lipase, an insulin-sensitive enzyme, is partly responsible for the decreased clearance of fasting and postprandial triglyceride-rich lipoproteins (TRLs) and the decreased production of HDL particles. The increased concentration of cholesteryl ester-rich fasting and postprandial TRLs is the central lipoprotein abnormality of the metabolic syndrome. These lipoprotein defects contribute largely to the increased cardiovascular risk in individuals with insulin resistance.

Insulin resistance and hyperinsulinaemia conspire together in the development of a diverse collection of risk factors for CAD, namely obesity, dyslipidaemia, hypertension, atherosclerosis and a procoagulant state [18-21]. This is commonly found in the T2DM insulin resistant state and is known as the metabolic syndrome. The components that conspire and lead to the development of the metabolic syndrome (insulin resistance syndrome) are illustrated in Figure 1.2.

Hyperinsulinaemia and insulin resistance are considered central components in the metabolic cardiovascular syndrome and as independent risk factors for CVD. Therefore, insulin resistance is a fundamental aspect of the pathophysiology of T2DM and a crucial factor in understanding the metabolic syndrome as insulin resistance and hyperinsulinaemia drive the atherogenic state. As insulin resistance is a complex process, it is likely that its inheritance involves a number of mutations in a variety of genes of the insulin-signalling network

FIGURE 1. 2. Schematic presentation of the varied components that can conspire and lead to the metabolic syndrome in T2DM.



MA = microalbuminuria, IGT = impaired glucose tolerance, athero = atherosclerosis.

1.3.2 Dyslipidaemia

Fatty acids and cholesterol are 2 simple lipids involved in human lipoprotein metabolism, whereas triglycerides and cholesteryl esters are the complex and nonpolar lipids. Triglycerides are the centre of lipid-rich inclusions in adipocytes and cholesterol the major component of inclusions in the macrophage foam cells in arterial lesions [58]. Lipoproteins are macromolecular complexes of lipids and proteins (apoliproteins) that contain free cholesterol, cholesteryl esters, triglycerides and phospholipids. These lipoproteins are defined according to their densities and their electrophoretic mobilities and comprise of high density lipoprotein (HDL), low density lipoprotein (LDL), intermediate density lipoprotein (IDL), very low density lipoprotein (VLDL) and chylomicrons (a triglyceride rich lipoprotein). HDL is bimodal and has 2 distinct subclasses, HDL2 and HDL3 with subclasses of HDL2a, HDL2b, HDL3a, HDL3b and HDL3c. HDL has another entity known as pre-beta-HDL which consists of Apo A-1, cholesterol and phospholipid that migrates with the pre-beta rather than the alpha mobility.

The classification and some properties of blood lipoproteins are shown in Table 1.2. The core of VLDL consists mostly of triglycerides and its surface has phospholipid, cholesterol, Apo B-100, Apo C as well as Apo E, the core of LDL consists mostly of cholesteryl ester and its surface has phospholipid, cholesterol as well as Apo B-100, whereas the core of HDL consists mostly of cholesteryl ester and some triglycerides and the surface has phospholipid, cholesterol, Apo A as well as Apo C.

TABLE 1.2. Classification, chief lipid components, major apoproteins, density and electrophoretic mobility of the key blood lipoproteins.

Lipoprotein	Lipid(s)	Apoproteins	Density	mobility
Chylomicron	TG	A-1, A-11, A-1V Cs, B-48, E	<0.950	origin
Chylomicron Remnant	CE, TG	B-48, E	<1.006	origin
VLDL	TG	B-100, Cs, E	<1.006	pre-beta
IDL	CE	B-100, Cs, E	1.006-1.019	broader beta
LDL	CE	B-100	1.0199-1.063	beta
HDL_2	CE, PL	A-1, A-11	1.063-1.125	alpha
HDL_3	PL	A-1, A11	1.125-1.210	alpha

TG = triglycerides, PL = phospholipids, CE = cholesteryl ester.

Blood lipids can be modified in structure and composition by various types of hyperlipidaemia and density can shift according to alterations in composition, whereas electrophoretic mobility is altered by changes in the charges on the proteins [59]. For instance LDL can be modified by a covalent attachment of Apo [a], to give Lp[a] and b-VLDL can be modified by enrichment with cholesteryl ester and ApoE. Human blood levels of lipids vary widely according to nutritional status and genetically determined differences [60]. Recently there has been a growing interest on the role of triglyceride as a cardiovascular risk factor as well as its role in the structure and metabolism of lipoprotein subclasses in the metabolic syndrome.

The function of apolipoproteins, which are synthesised in the liver or intestine, is to associate with lipids and mediate several biochemical steps involved in lipid metabolism. The apolipoproteins such as Apo A, Apo B, Apo C, Apo D and Apo E vary widely in their molecular masses ranging from 6 kDa to over 600 kDa. Most apolipoproteins are soluble and spontaneously associate with lipid surfaces. These proteins transport blood lipids, stimulate enzymes that degrade blood lipids and contain ligands that mediate the binding of apolipoproteins to cell surface receptors (a process responsible for the regulation of intracellular lipid synthesis) [61].

Apo A-I, A-II, A-IV, C-I, C-II, C-III and Apo E, are soluble apolipoproteins that reversibly associate with neutral lipid surfaces of lipoproteins [62]. Together with phospholipids, apolipoproteins form a surface film around the neutral lipids to make them soluble. The apolipoproteins, Apo B-100 and Apo B-48 are associated

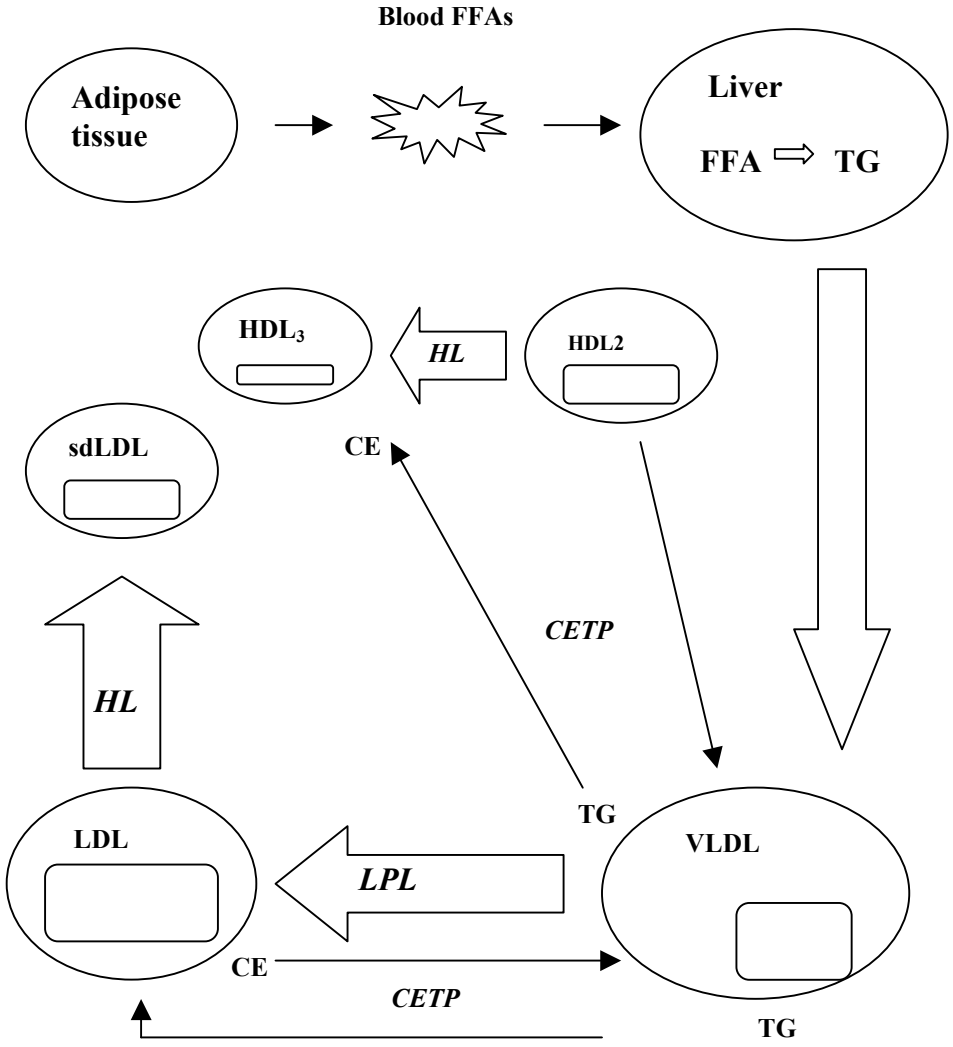
with cholesteryl ester-rich lipoproteins and triglyceride-rich lipoproteins. Apo B-100 has several lipid-binding domains such the heparin binding domain as well as numerous glycosylation sites. It also has numerous cysteines, some of which are located in a disulphide cluster and one of these cysteines forms a disulphide link with Apo[a] and this is the way Apo[a] is attached to LDL. Apo B-48 is the amino terminal 48% of Apo B-100, that contains some of the heparin binding sites, the glycosylation sites as well as the disulphide cluster of Apo B-100, although it lacks the receptor-binding domain of Apo B-100 responsible for targeting LDL to cell surface receptors. Apo D and Apo[a] do not seem to have any connection with lipid metabolism. Apo D belongs to a family proteins (e.g. alpha2-macroglobulin and retinol binding protein) that bind to and transport numerous ligands , whereas Apo[a] which is bound to a minority of LDL particles is involved in fibrolysis.

The glycoprotein lipoprotein lipase (LpL) is the major blood protein involved in hydrolysing triglycerides and diglycerides. This enzyme is synthesised in parenchymeal cells and secreted and transported into the capillary endothelium bound to heparin sulphate. The adipose tissue and striated muscle contain high concentrations of LpL. The active form of LpL is bound to Apo C-II and the active form of LpL in adipose tissue is induced by insulin [63]. A similar protein is hepatic lipase (HL) whose role is hydrolysis of triglycerides and phosphoglycerides of HDL and IDL, which normally does not possess the activator Apo C-II.

Lipogenesis is the synthesis of fatty acids from glucose and is highly regulated by hormones such as insulin, glucagon and somatostatin. Fatty acids are incorporated into triglycerides and secreted as VLDL particles. VLDL is further remodelled by hydrolysis in blood to form free fatty acids (FFAs). The FFAs are then incorporated into the adipose tissue. Elevated levels of FFAs augment hepatic extraction and production of TG and VLDL particles of increased size (Figure1.3). These VLDL particles furnish a pool of TG, which is exchanged for CE in LDL and HDL_2 in a process that is mediated by CETP [64]. The triglyceride-rich LDL and HDL_2 are acted upon by hepatic lipase (HL) to remove TG from these 2 particles to yield small dense LDL (sdLDL) and HDL_3 respectively. This remodelling process determines the metabolic fate and potential atherogenicity and cardioprotection of lipoproteins. The hypertriglyceridaemia of diabetes mellitus is partly due to the oversynthesis of fatty acids in the liver.

Diabetic subjects normally suffer from dyslipidaemia (diabetic dyslipidaemia) that is characterised by hypertriglyceridaemia, reduced HDL-cholesterol levels (as a result of decreases in the HDL2 subfraction), and conversion of LDL particles to smaller dense LDL, Figure 1.3, [65]. Small dense LDL micelles (< 25 nm), can be easily oxidised or glycated and subsequently taken by scavenger receptors into microphages to become foam cells which penetrate the vascular wall. Thus elevated levels of small dense LDL particles accelerate atherogenesis and subsequent manifestation of cardiovascular diseases.

Figure 1.3. A schematic representation showing the remodelling of the key blood lipoproteins by elevated FFAs, resulting in triglyceride-rich lipoproteins.



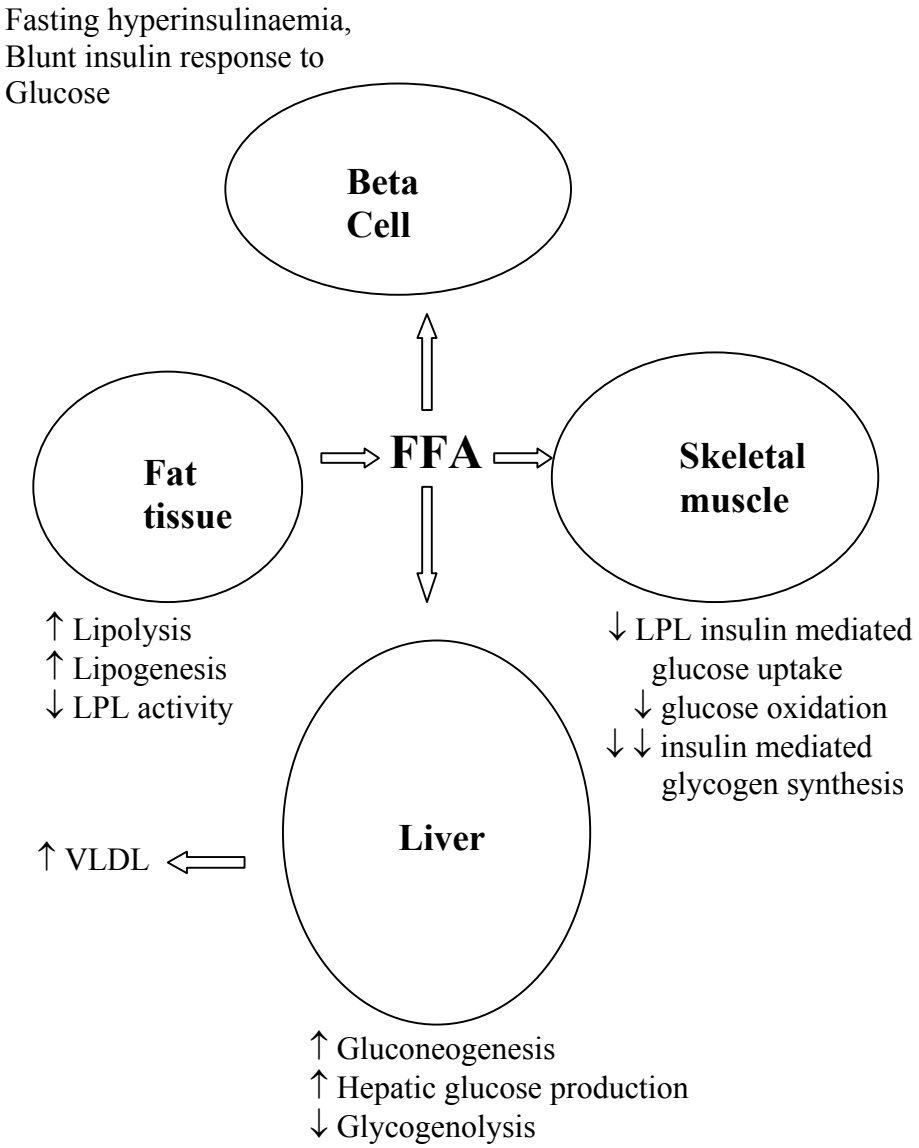
The rounded rectangular shapes in the lipoprotein ovals represent the TG content in the cores.

Alterations in insulin action on the synthesis of apolipoproteins, action of insulin on adipocytes and muscles, action of cholesterol ester transfer protein (CETP), regulation of lipoprotein lipase (LpL) and the modification of blood lipoproteins by hyperglycaemia, are a few processes that have been proposed as causes of diabetic dyslipidaemia [66-68]. However obesity and insulin resistance without hyperglycaemia does also contribute to dyslipidaemia. Dyslipidaemia seems to play a central role in insulin resistance, a condition that unites various metabolic abnormalities. It is thus evident that glycaemic control, environmental factors and inherent genetic factors may potentially affect lipoprotein metabolism.

The adipose tissue is the largest storage organ for energy in form of triglycerides. Triglycerides are mobilised through the lipolysis process for the provision of fuel to other organs, for gluconeogenesis through glycerol and for lipoprotein synthesis through fatty acids. The metabolism to glycerol and free fatty acids is partially regulated by hormones such as insulin and catecholamines. Insulin increases the uptake of fatty acids from circulating lipoproteins by stimulating lipoprotein lipase activity in adipocytes. Adipocytes are highly insulin-sensitive cells and insulin is known to promote adipocyte glucose transport, triglyceride synthesis (lipogenesis) as well as inhibition of lipolysis [66-68]. However, physiological factors (exercise) and pathological conditions such as T2DM, dyslipidaemia and obesity, can modify the lipolysis process. Impaired lipolysis could be a pathogenic factor contributing to clustering of abdominal obesity and dyslipidaemia (components of the metabolic syndrome) and development of T2DM. High levels of serum free fatty acids, as a result of abdominal obesity, cause excessive tissue lipid accumulation, contributing to dyslipidaemia, and beta

cell dysfunction that leads to hepatic and peripheral insulin resistance (Figure 1.4).

FIGURE 1.4. The unifying hypothesis about the role of fatty acids (in various tissues) as a pathogenic factor in the development of T2DM.

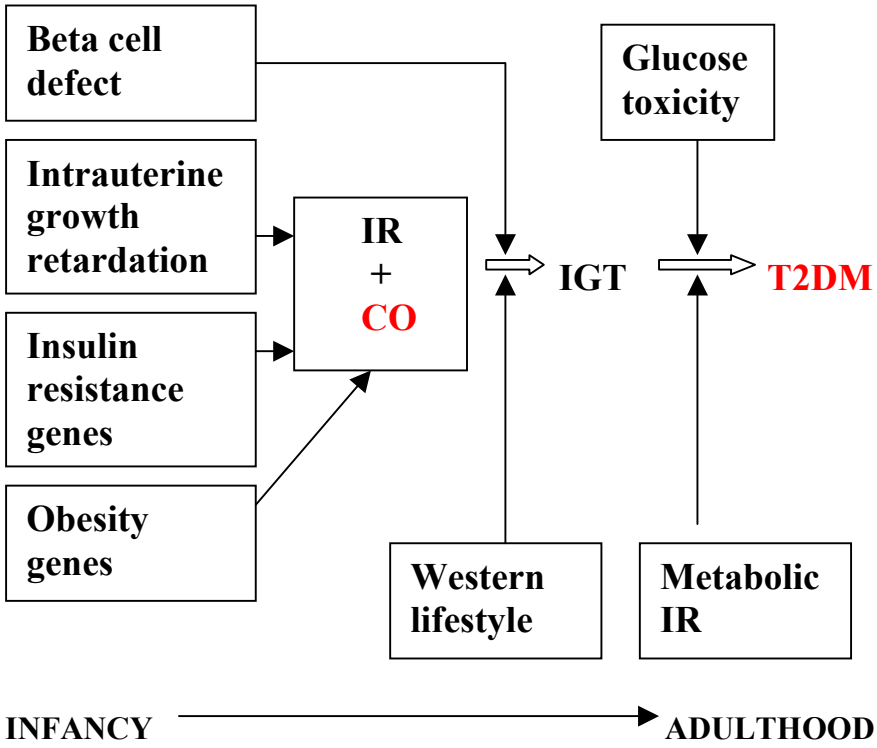


1.3.3 Obesity

The major environmental factors that have been linked to T2DM are obesity and a decline in physical activity [69]. Obesity is a common feature in patients with T2DM and obesity per se, without affiliation with a positive history of diabetes is often associated with hyperinsulinaemia and insulin resistance. Obesity is one of the major factors that induce insulin receptor (IR) dysfunction and together with prolonged hyperglycaemia (glucotoxicity), dyslipidaemia (lipotoxicity) and hypertension they are likely to aggravate the insulin resistance found in patients with T2DM. A progressive pathophysiologic model for the development of obesity and T2DM is depicted in Figure 1.5.

The expansion of the adipose depot, by for instance obesity, increases blood free fatty acids (FFAs) by increased release from expanded adipose mass and by impaired hepatic metabolism. Elevated FFAs impair the ability of insulin to suppress hepatic glucose production, reduce the ability of insulin to stimulate glucose uptake into skeletal muscle, and blunt insulin response to glucose in pancreatic beta cells (resulting in hyperinsulinaemia) (Figure 1.4). In muscle the FFAs may involve impaired activation of phoshatidylinositol-3-kinase [70]. The muscle triglyceride content correlates directly with insulin resistance.

FIGURE 1.5. A schematic model from infancy to adulthood that may lead to the development of obesity and Type 2 diabetes mellitus.

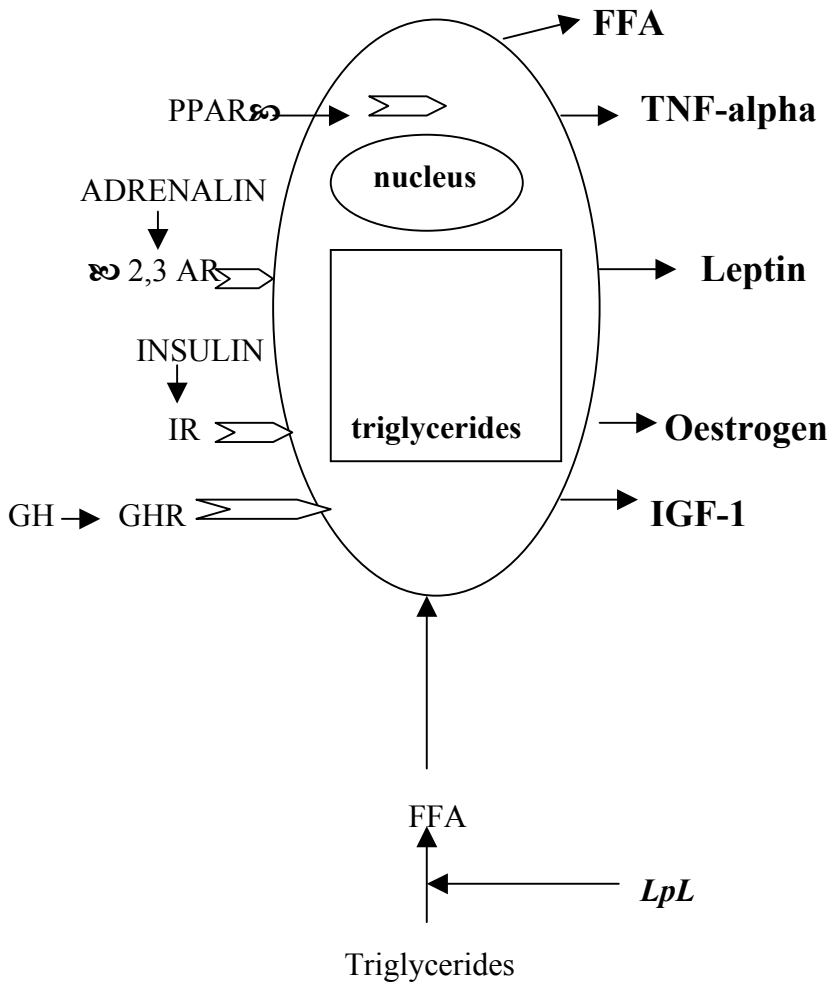


IR = insulin resistance; CO = central obesity.

The variability in body size is believed to be influenced by genetically determined responses to an environment. Individuals with a low metabolic rate are prone to weight gain and those with a high level of spontaneous physical activity are unlikely to become obese. Also individuals with a high 24-hour respiratory quotient are more likely to gain weight compared to those with a low respiratory quotient. Insulin sensitivity (not insulin resistance) is another metabolic predictor of obesity [71]. The level of obesity could be linked partly due to increases in calorie intake (overeating) and a decline in physical activity. As visceral fat deposits (unlike subcutaneous deposits) are directly drained into the liver and have a high turnover of triglycerides, portal free fatty acids are important in complications of obesity such as the metabolic syndrome. The activity of these free fatty acids is then likely to interfere with insulin action. It is vital to carry out studies in genetic polymorphisms to study proteins that regulate lipolysis to understand the genesis of obesity and its complications.

Adipocytes secrete cytokines such as TNF-alpha, hormones such as oestrogen, cortisol and leptin, prohormones such as angiotensinogen and enzymes from adipocytes and act in an endocrine, autocrine or paracrine fashion (Figure 1.6). The production of several of these proteins is affected by obesity. Therefore adipocyte secretions have a potential capacity to influence numerous organs such as the adipocyte itself, the liver, muscle and the systemic biochemistry. TNF-alpha has numerous effects on adipocytes, including inhibition of lipogenesis and elevation of lipolysis [72]. It is possible that TNF-alpha could induce a decrease in IRS-1 phosphorylation in adipocytes, thus decreasing the tyrosine kinase activity of the insulin receptor and impairing glucose utilisation. Another

FIGURE 1.6. A schematic illustration of an adipocyte (fat cell) and some adipocyte-related secretions.



PPAR = peroxisome proliferator-activated receptor; AR = adrenaline receptor; IR =insulin receptor; GHR = growth hormone receptor; IGF = Insulin growth factor.

possibility is that TNF-alpha may stimulate the release of leptin from human mature adipocytes, which may contribute to obesity-linked hyperleptinemia.

Weight gain is a strong predictor of the metabolic syndrome and overweight predisposes to hypertension, a condition that exacerbates cardiovascular disorders. Several segregation studies suggest that obesity and obesity-associated hypertension may share some genetic determinants [73] and one such candidate gene is the TNF-alpha.

Therefore, it is vital to study the relationship between adipose tissue, insulin action and glucose homeostasis as these evolved out of survival needs to maintain fuel supplies when food was scarce, a situation that has now changed in affluent societies. In such societies, obesity is reaching epidemic proportions and the genetic aberrations that influence these environmental changes should be identified. The evolution from obesity to diabetes, may represent a continuum, that progresses through different phases, in which defects in both insulin action and insulin secretion play a critical interaction, and must be looked at in concert.

1.3.4 Microalbuminuria (MA)

Microalbuminuria is an abnormally elevated urinary albumin excretion less than the level of clinical albuminuria (20-200 ③g/min) [74]. It can independently predict cardiovascular disease in type 2 diabetes mellitus. Epidemiological studies indicate that there is an association between MA and insulin resistance, dyslipidaemia, as well as obesity in patients with diabetes and hypertension. The concept of MA as marker of endothelial dysfunction or general vasculopathy,

which might lead to an elevated atherogenic state, is currently under investigation [75]. The reasons for endothelial dysfunction could be numerous, including (a) insulin resistance, (b) penetration of atherogenic lipoprotein particles possibly arising from peripheral hyperinsulinaemia/insulin resistance in the arterial wall, (c) hyperglycaemia and (d) a procoagulant state.

Microalbuminuria is an important predictor of overt nephropathy in patients with T1DM and of cardiovascular mortality in patients with T2DM [76]. Diabetic nephropathy is an essential factor that contributes to the development of hypertension in T1DM, whereas hyperinsulinaemia, insulin resistance and obesity are thought to be responsible for HT in T2DM. The nephropathy of T1DM is mainly due to poor glycaemic control. In both types of diabetes, hypertension accelerates diabetic renal disease. There is accumulating evidence to suggest that diabetic patients with MA may have more advanced retinopathy, higher blood pressure and worse dyslipidaemia than patients with normal albumin excretion rate (AER) [77]. Diabetic nephropathy is a clinical syndrome characterised by chronic albuminuria, a resolute decline in glomerular filtration rate, elevated arterial blood pressure, increased relative mortality for cardiovascular diseases and is the prime cause of end-stage renal failure. Patients with end-stage renal disease are on the whole invariably hypertensive. The pathogenesis of diabetic nephropathy is assumed to be multifactorial, with contributions from metabolic abnormalities, haemodynamic aberrations and genetic factors [78].

Genetic susceptibility may contribute to the risk of diabetic nephropathy and it is possible that hypertension and lipid disorders may instigate diabetic nephropathy.

Polymorphisms in lipid metabolism and components of blood pressure regulation are candidates for the genetic component of risk for diabetic nephropathy. Therefore is important to investigate the genetic factors that predispose diabetic patients initially to microalbuminuria and subsequently the initiation of diabetic nephropathy. Candidate genes could be the angiotensin converting enzyme gene and the apolipoprotein E gene, genes linked to hypertension and lipid disorders, respectively.

1.3.5 Hypertension (HT)

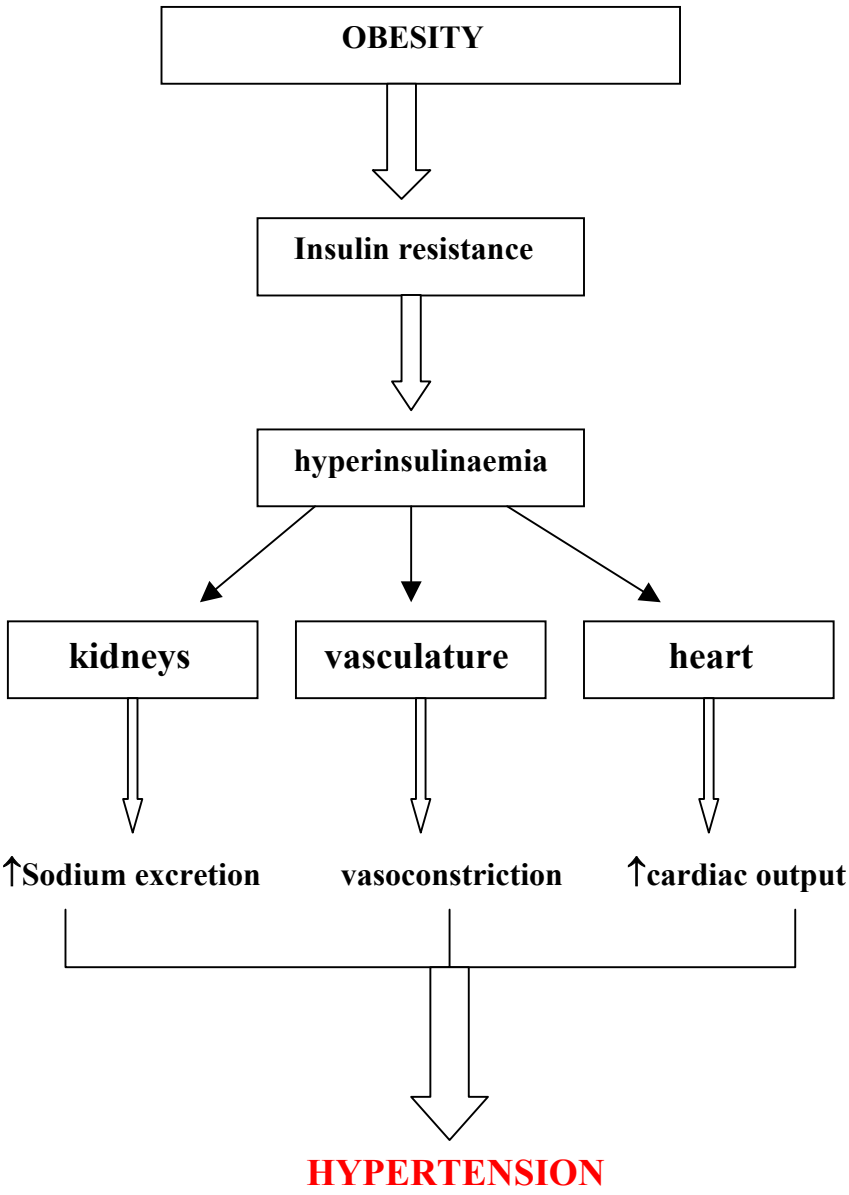
In diabetic patients, HT is twice as frequent than in the age-matched non-diabetic individuals in the general population [79, 80]. T2DM patients exhibit a higher prevalence of hypertension than their T1DM counterparts. The coexistence of hypertension with diabetes mellitus is at a greater frequency than chance alone would predict. Hypertension affects up to 50% of European adult, diabetic patients and people from Afro-Caribbean as well as Afro-American ancestry are particularly at a higher risk of HT than their caucasian counterparts [81-83]. The HT could be secondary to atherosclerosis, or could be renal, secondary to diabetic nephropathy or could be essential hypertension. In T1DM, the onset is generally before the diagnosis of diabetes and in T2DM, it is strictly related to the presence of nephropathy.

The pathogenesis of HT in diabetes is linked to overactivity of the sympathetic nerves due to the activation of the renin-angiotensin-aldosterone (RAA) system in T1DM with chronic renal failure or to insulin resistance/hyperinsulinaemia in T2DM patients with the metabolic syndrome [84, 85]. Hyperinsulinaemia could

be an important factor as it stimulates the sympathetic nervous system, promotes sodium retention, inhibits the sodium pump and disturbs membrane ion transport, resulting in essential HT. Certain changes that occur in the kidney of the diabetic patient include, mesangial matrix expansion, altered charge and size, selectivity of the glomerular basement membrane and elevated intraglomerular pressure. Sympathetic overactivity promotes sodium reabsorption, stimulates the RAA system, elevates the heart rate, elevates the stroke volume and increases peripheral vascular resistance, therefore inducing HT and promoting cardiovascular risk (Figure 1.7).

The most common type of HT is probably a consequence of insulin resistance and hyperinsulinaemia. Overally, hypertension and hyperglycaemia are major risk factors for both small and large vessel diseases, contributing to accelerated atherogenesis and progression of diabetic nephropathy and retinopathy [86]. The increased risk of hypertension in T2DM may relate to insulin resistance and its sequelae. This process could follow this sequence, **obesity** → insulin resistance → hyperinsulinaemia → dyslipidaemia → impaired glucose tolerance, T2DM, hypertension. Thus in T2DM, hypertension can be seen in close association with obesity, insulin resistance, hyperinsulinaemia, lipid changes, and hyperglycaemia within the framework of the metabolic syndrome.

Figure 1.7. Hypothetical framework showing how hyperinsulinaemia can generate obesity-related hypertension. The kidneys, vasculature and the heart are stimulated by the sympathetic nervous system, which is mediated by hyperinsulinaemia.



1.4 METABOLIC GENES IN TYPE 2 DIABETES MELLITUS

The T2DM complex phenotypes are a product of the action and interaction of multiple genes and environmental factors, unlike those of simple Mendelian disorders that result from a mutation in a single gene [15-17]. One can deduce 4 hypothetical stages in the development of diabetes comprising of genetics, the environment, impaired glucose tolerance (IGT) and T2DM. Stage one (genetics) is composed of insulin resistance, hyperinsulinaemia, glucose resistance and dyslipidaemia. Stage 2 is a combination of genetic and environmental factors and is made up of dietary factors, physical inactivity and obesity which all lead to beta cell dysfunction. Stage 3 and 4 consist of IGT and T2DM respectively.

The most challenging biomedical research now and in the future is to unravel the latent genetic architecture of complex disorders such T2DM. Genes are involved in determining enzymes, receptors as well as cofactors, structural components involved for instance in, the regulation of blood pressure, the metabolism of lipids, lipoproteins, inflammatory and coagulation factors. A mutation in genetic material can lead to missing information, or to too much information or to interchange of information. The defective genetic material so produced is then passed on from one generation to another generation, and could be responsible for causing inherited disorders such as diabetes mellitus. Evidence for a genetic component for T2DM includes the finding of a variety of metabolic defects in various tissues in non-diabetic subjects with a genetic predisposition to T2DM, higher concordance rates for abnormal glucose tolerance including T2DM in monozygotic compared to dizygotic twins, and familial clustering [87-91].

In this study, the apolipoprotein E gene (apoE) polymorphism at codons 112 and 158, the angiotensin converting enzyme gene (ACE) 287-bp insertion/deletion polymorphism, and the tumour necrosis factor gene (TNF) 308G⌋A polymorphism will be investigated in an attempt to understand the T2DM metabolic phenotype [92-95]. The ApoE gene locus influences the blood levels of lipoproteins but may also modulate the association between obesity and dyslipidaemias. ACE catalyses cleavage of angiotensin I to angiotensin II, an active component of the renin-angiotensin system (RAS) which affects vasoconstriction and sodium retention and as a result alters the haemodynamics of the kidney and elevates systemic blood pressure. The cytokine TNF-alpha that is produced by the adipocytes, has an effect on lipid metabolism and therefore may mediate insulin resistance.

1.4.1 Genetic polymorphism

Genetic polymorphism is the occurrence together in the same habitat of two or more discontinous forms of a species (alleles) in such proportions that the rarest of them cannot be maintained merely by recurrent mutation. Normally a genetic locus is considered to be polymorphic if the rare allele or alleles has or have a frequency of at least 0.01 and the frequency of the heterozygotes carrying the allele is greater than 2% [95]. Thus genetic polymorphism is a sequence of genetic material within a population resulting in a number of variants. Differences among individuals in the base pairs at particular locations throughout the whole human genome are notably common and DNA in such positions is said to be polymorphic. Over one-third of human genetic loci that has been studied so far is polymorphic [96].

If the nucleotide differences are in the non-coding sequences (introns), the coded protein product is not altered, but if the differences occur in the coding regions (exons), this can lead to amino acid differences and serious clinical consequences. Other effects of nucleotide differences in introns and exons are creation or abolition of cutting site for a particular restriction enzyme. This type of polymorphism is termed restriction fragment length polymorphisms (RFLPs) and these create differences in the DNA fragments produced by restriction endonucleases [97]. RFLPs are useful as markers for known or unknown loci that can be associated with disease.

There are two types of polymorphisms, namely, balanced and transient polymorphism. In balanced polymorphism, two or more forms are maintained by equilibrium of selective advantages, mainly when a heterozygote is favoured compared to the homozygote. A classic example in Africa is the sickle cell gene (Hb S gene), where in some populations the incidence is as high as 40% and approximately one person in three is a carrier [98]. A situation like this could only have arisen because of an advantage conferred on the heterozygote. Carriers of the sickle cell gene are more resistant to infection by the *Plasmodium falciparum* malarial species than normal persons. The explanation could be that in the heterozygote, the parasites in the infested red blood cells are removed successfully from circulation by phagocytosis. Since heterozygotes are more resistant than the normal individuals, they have a superior chance of survival as well as reproductive prowess. Homozygotes for the mutant sickle cell allele are affected by sickle cell anaemia, a severe haemolytic anaemia and normally die before they reach their reproductive age.

Thalassaemia and glucose-6-phosphate dehydrogenase (G6PD) deficiency in the Mediterranean region, are examples of transient polymorphism, which is explained by the change in the environment [99]. This results in the decrease in the allele frequency of the ℘-thalassaemia and the G6PD deficiency genes to a new equilibrium at a lower level in each generation, as people who carry the insulting genes are no longer exposed to the disease, for instance, to malaria.

Therefore it is reasonable to assume that the genetic diversity in the population and individual genetic uniqueness can be responsible for differences in genetic susceptibility to a variety of disease traits. In this study DNA polymorphisms in various genes will be investigated by electrophoretic resolution, after performing the polymerase chain reaction, to determine whether particular allelic variants may be associated with T2DM metabolic traits.

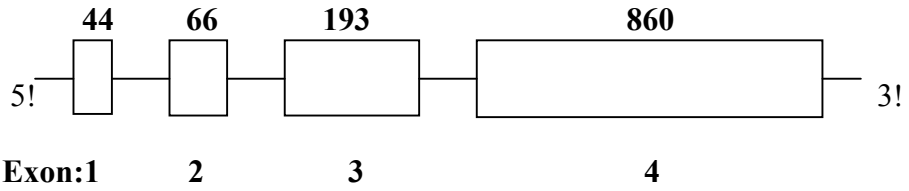
1.4.2 Apolipoprotein E (ApoE) gene polymorphism

The structural gene locus of apolipoprotein E (ApoE) gene is polymorphic and the normal physiology of this gene is to regulate the metabolism of lipoproteins[100-102]. The gene is located in chromosome 19 (19q13.2) and spans on 3,7 KB and is made up of 4 exons and 3 introns [103, 104]. Exon 1 codes for part of the 5'-untranslated region of the apolipoprotein, exon 2 codes for the remainder of the 5'-untranslated region and a portion of the leader sequence that usually characterises secreted proteins, exon 3 codes for the remainder of leader sequence and about 40 residues of the primary polypeptide structure and exon 4 codes for the region that confers the distinct physiological character of the apolipoprotein (Figure 1.8).

The product of the Apo E gene, the mature apolipoprotein E glycoprotein has a molecular weight of 34-kDa consisting of 299 amino acids, and its plasma concentration varies from 2 to 6 mg/dl [105]. The polymorphism of human ApoE is determined by 2 autosomal codominant alleles, ApoE(n) and ApoE(d) and homozygosity of ApoEd leads to primary dysbetalipoproteinaemia [106]. ApoE4 phenotype inheritance is autosomal and compatible with 3 common alleles acting at a single gene locus [107]. The ApoE gene has 3 common alleles, epsilon 2 (ε2), epsilon 3 (ε3) and epsilon 4 (ε4) and these code for 3 main isoforms, resulting in 6 common genotypes, E2E2, E2E3, E2E4, E3E3, E3E4 and E4E4. E2, E3 and E4 isoforms differ in their primary structure (amino acid sequence) at two substitution sites, site A, residue 112 and site B, residue 158 (codons 112 and 158), [108, 109]. At sites A/B, ApoE2, E3 and E4 contain cysteine/cysteine, cysteine/arginine, and arginine/arginine residues respectively. The 3 forms have 0, + and ++ electric charges respectively and these are responsible for their different electrophoretic movement.

The polypeptide Apo E is predominantly synthesised in the liver, and plays a crucial role in regulation of the metabolism of lipoproteins by facilitating efficient hepatic uptake of lipoprotein remnants rich in cholesterol and triglyceride. This is brought about since ApoE has the ability to bind to LDL receptors, lipoprotein receptor related protein (LRP) or heparan sulphate proteoglycans (HSPGs) [110-112]. ApoE a main component of chylomicrons, plays a crucial role in lipid metabolism through its ability to bind to receptors on the liver and peripheral

Figure 1.8. Schematic representation of the Apolipoprotein E gene showing the 4 exons and the number of amino acids each exon encodes.



cells as a ligand. ApoE plays a basic role in degradation of particles rich in cholesterol and triglycerides, and it is also able to bind to LDL and chylomicron receptors. The physiological function of ApoE is to facilitate the binding of triglyceride-rich lipoprotein remnants to the receptors that determine their clearance. Variation in human apolipoprotein genes is a major source of phenotypic differences in lipid metabolism and there is also variation at apolipoprotein alleles in different populations [113]. Thus the 3 different alleles code for proteins that differ in lipoprotein receptor binding activity or in their catabolism. The variability of apolipoprotein genes relates to the variance of lipoprotein concentrations and is a major factor in the multifactorial forms of hyperlipidaemia [114].

These variations in ApoE genes provide possibilities of studying processes involved in lipid metabolism and the associated disorders such as diabetes mellitus. One study found that approximately 6% of the total variation of LDL-C could be accounted for by the ApoE locus in normolipidaemic and hypercholesterolaemic subjects alike [115]. Another study on Italian children aged between 8-11 found that the variation of the ApoE gene locus explained 5.1% of the sample variance in serum total cholesterol levels, 7.6% in LDL-C levels, 7,3% in ApoB levels and 14,1% in HDL-ApoE levels [116]. The variation in lipid levels was found to be gender specific. Thus the 3 allelic variants of ApoE determine, in concert with other gene products the levels of plasma lipoproteins.

Several studies have demonstrated heterogeneity of ApoE phenotype frequency in different ethnic populations. North European populations have significantly

increased frequencies of the ε4 allele, whereas certain Asian populations have lower ε2 and ε3 alleles [117, 118]. However a study of the Yanomani Indians of north-western Brazil discovered only two alleles, ε3 and ε4 than the common alleles found in Caucasians [119]. Also the Mayans of the Yucatan Peninsula from Mexico lack the ε2 allele and a relatively low incidence (9%) of the ε4 allele, yielding the lowest average heterozygosity at ApoE locus to date [120]. The ε4 allele was associated with approximately 4% decrease in mean serum cholesterol levels and this is contrary to the majority of the studies carried out so far. In univariate analysis the ε4 allele was significantly associated with elevated blood glucose, glycated haemoglobin and uric acid levels. Similar results were obtained for the Evenki herders of Siberia, as the well-established association between ε4 allele and LDL-cholesterol was absent as the Evenki have significantly lower levels of cholesterol [121].

The importance of the above results is that there may be a gene and diet interaction that modulates the effect of ApoE polymorphism and that apolipoprotein distributions and their associations with lipid and carbohydrate metabolism may show ethnic variability. Since diabetes mellitus is associated with dyslipidaemia it is crucial to investigate the extent of gene/environment relationships between ApoE polymorphism and plasma lipids and lipoproteins among different ethnic groups. This is supported by a study that demonstrated significant differences among ApoE phenotypes for total cholesterol and LDL-C in black and white children as well as significant effects for HDL-C only in blacks [122]. Excess of ε2 allele showed higher LDL-C concentrations in both races although more so in the whites. ApoE3 is the most common (wildtype)

isoform that affects the metabolism of lipids in a standard way, the ε2 allele has four mutations, arg158 → cys (most common), lys146 → gly, arg145 → cys, arg136 → ser and has a protective role, whilst ε4 allele, believed to be the ancestral gene, is associated with a high risk of lipid metabolic disorders [123]. The ε4 allele confers the greatest receptor-binding affinity and individuals with the ε4 allele are prone to high levels of LDL-cholesterol as a result of the efficient absorption of dietary cholesterol and also possibly as a result of the downregulation of the LDL receptor. The ε4 allele has been associated with atherogenic lipid profile that is linked to increased risk of CAD and may also be associated with peripheral arterial disease [124, 125]. The accelerated atherogenesis in diabetic patients may be due to the augmented glycation of ApoE. This glycation significantly reduces ApoE heparin-binding activity [126, 127]. ApoE glycation can then impair lipoprotein-cell interactions, which are mediated via the heparan sulphate proteoglycans and results in enhanced lipid dysfunction.

The ε2 allele has the least great receptor-binding affinity and individuals with ε2 allele have higher levels of triglycerides and lower levels of total plasma cholesterol and LDL-C levels when compared to the ε4 and ε3 allele. ApoE2 homozygotes present with greatly elevated levels of triglyceride-rich lipoprotein remnants and this results in Type III dyslipidaemia (dysbetalipoproteinaemia) [128, 129]. The ε2 isoform has defective binding of remnants to hepatic lipoprotein receptors and delayed clearance from plasma. However other genetic and environmental factors are required for this disorder to manifest itself. As this disorder involves the exogenous cholesterol transport system the degree of

hypercholesterolaemia is sensitive to dietary cholesterol levels. The subjects exhibit elevated plasma cholesterol levels and an abnormal lipoprotein, beta-VLDL. The blood levels of VLDL are raised, LDL is reduced and carbohydrates exacerbate the hyperlipidaemia. In addition this ε2 allele has the effect of raising HDL-cholesterol.

The evolution of diabetic nephropathy in T1DM is brought about mainly by chronic hyperglycaemia, but its progression may be influenced by such factors as glomerular hypertension due to capillary vasodilation and dietary protein intake [130]. In contrast, in T2DM, it presents with a different natural history as well as atherosclerosis, and can precede diabetes diagnosis as well as the onset of patent hyperglycaemia [131]. A recent study has shown that the ε2 allele of exon 4 of ApoE is significantly higher in the diabetic nephropathy of T1DM subjects than in control subjects [132]. The risk of diabetic nephropathy was 3,1 times higher in subjects carrying this allele than in non-carriers. A study of T2DM subjects found a 2-fold increase of nephropathy in E2 non-carriers [133]. LDL-C levels were lower in ε2 allele carriers and this study suggests a role of LDL in the development of human diabetic nephropathy. Similarly, a Korean study found out that ApoE2 and E2 carrier frequencies were significantly higher in microalbuminuric T2DM subjects [134].

The above studies are supported by a previous study which reported that subjects with a stable renal function without overt proteinuria had a higher cholesterol level, lower incidences of hypertension and proliferative diabetic retinopathy and a higher frequency of the ε4 allele than subjects with a decline in renal function

in both Type 2 diabetic and non diabetic patients [135]. Also the diabetic patients with the ε4 allele had higher cholesterol level than the non-carriers of this allele and the authors concluded that the ε4 allele has aprotective role in nephropathy and the other alleles are a risk factor. Thus ApoE polymorphism may be associated with the progression of diabetic nephropathy.

The ApoE gene locus does not only influence the blood levels of lipoproteins but it also modulates the association between obesity and dyslipidaemias [136]. For these reasons ApoE is an important candidate gene in the development of cardiovascular disease, obesity and diabetes mellitus. ApoE can be used as a genetic factor that may interact with the environment to modulate blood cholesterol and triglyceride levels and secondarily influence the individual tendencies to develop atherosclerosis. The ε2 allele is associated with lower levels and the ε4 allele with higher levels of cholesterol than the ε3 allele [137]. The ε4 is a risk factor, which predisposes to coronary atherosclerosis [138] and it is possible that the ε2 allele may have protective powers, provided no other factor, ecological or hereditary, intervenes to incite the development of an atherogenic hypertriglyceridaemia.

As genetic variants of the ApoE gene are common and are known to affect lipoprotein metabolism, it is a suitable candidate gene for studying the pathophysiology of diabetes mellitus.

1.4.3 Angiotensin-converting (ACE) gene polymorphism

The ACE gene spans 21 KB on chromosome 17 (17q23) and includes 26 exons and the mature polypeptide has a MW of 80 073 [139]. Angiotensin-converting enzyme or kininase II is a dipeptidyl carboxypeptidase that plays a pivotal role in regulating blood pressure and electrolyte balance by hydrolysing angiotensinogen I to angiotensinogen II, a potent vasopressor and aldosterone-stimulating peptide. This enzyme also is able to inactivate bradykinin, a potent vasodilator. ACE catalyses cleavage of angiotensin I to the octapeptide angiotensin II, an active component of the RAS which affects vasoconstriction and sodium retention and as a result alters the haemodynamics of the kidney and elevates systemic blood pressure [140, 141]. The ACE gene encodes 2 isoenzymes, the somatic enzyme is expressed in many tissues, such as the vascular endothelial cells, renin epithelial cells and testicular epithelial cells, whereas the testicular or germinal isoenyme is expressed only in sperm.

The insertion/deletion (I/D) polymorphism in intron 16 of the human ACE gene is a major determinant (up to 50%) of circulating ACE levels), with the deletion type associated with higher plasma ACE levels, a situation that may activate the intra-renal renin-angiotensin system [142-144]. The RAS is physiologically implicated in the regulation of blood pressure. Although there is strong evidence that supports the idea of the involvement of RAS in the pathogenesis of essential hypertension, the association of ACE gene insertion/deletion polymorphism with blood pressure is still contradictory, and this could be due to environmental, gender and racial differences [145-147].

The association of the deletional allele of the ACE gene ID polymorphism with CAD and diabetic nephropathy has remained a controversial issue for some time [148-152]. In Caucasian subjects, the 287-bp the insertion/deletion (I/D) polymorphism of the ACE gene is characterised by the presence (I) or the absence (D) of a 287 –bp alu repeat sequence within the intron 16 of the ACE gene. Polymorphism in the renin-angiotensin system such as the insertion/deletion polymorphism in the angiotensin I converting enzyme may predispose to hypertension and related disorders because of the advantage they confer to thermoregulation (note hypertension is a polygenic disease). The I/D polymorphism is a candidate genetic locus in the development of CVD in T2DM patients [153] and renal disease [154]. The ACE gene DD genotype is closely associated with the presence of diabetic nephropathy in diabetic patients [155] and the I allele is associated with higher plasma insulin levels [156]. A study of African-Americans concluded that the I allele is associated with insulin resistance in glucose-tolerant and normotensive subjects [157].

ACE gene polymorphism has been shown to have a significant impact on the progression of diabetic nephropathy. Diabetic nephropathy itself is a major complication in patients with uncontrolled diabetes and is a glomerular disease with genetic factors. Diabetic nephropathy is the major determinant of premature morbidity and mortality in T1DM [158]. Variants of the ACE gene I/D polymorphism may result in elevated production of angiotensin II, which in turn increases intraglomerular pressure and glomerular filtration rate, leading to renal damage. Strong evidence of the association of ACE gene I/D polymorphism with diabetic nephropathy comes from several studies in T1DM patients. Severity of

renal involvement was associated with ACE I/D polymorphism in uncontrolled Type I French and Belgian subjects [159]. The DD genotype was more prevalent in Type 1 Austrian diabetic patients with renal disease, and nephropathy was more frequent in DD genotypes with more than 20 years of diabetes [160].

A recent study has associated the ACE gene polymorphism with features of the insulin resistance syndrome (IRS) [161]. Normoglycaemic probands with MI and the ACE polymorphism DD genotype had characteristics of the IRS and had significant higher levels of blood glucose and FFAs levels after an oral glucose test. They also had significantly higher levels of total and VLDL triglycerides than the probands with the ID or the II genotypes. Thus dyslipidaemia may have a role to play in diabetic nephropathy.

Since subjects with diabetes mellitus have an increased risk of developing diabetic nephropathy and cardiovascular diseases, investigating the polymorphisms of the ACE gene may assist in understanding the genetic aberrations that influence the intra-renal renin-angiotensin system which is implicated in regulation of blood pressure.

1.4.4 Tumour necrosis factor–alpha (TNF-alpha) gene polymorphism

The tumour necrosis factor alpha (TNF-alpha) gene which is located on chromosome 6 (6p21.3), spans about 3 kb and contains 3 introns [162]. Four exons code for a mature product of 157 amino acids and the last exon codes for more than 80% of the secreted protein. TNF alpha itself is a multifunctional cytokine constituitively produced by adipose tissue, that has an effect on lipid

metabolism, on endothelial function as well as on coagulation and may mediate insulin resistance. A study in Caucasian subjects has suggested that the 308G丿A polymorphism in the 5! region of the TNF-alpha gene may be associated with insulin resistance and obesity [163], factors that underlie the clustering of T2DM, hypertension, and dyslipidaemia found in the metabolic syndrome. Thus TNF-alpha might play an important role in insulin resistance, a frequent correlate of obesity, likely augmenting some obesity-associated complications.

It has been shown that the adipocyte expression of TNF-alpha increases with increasing adipocyte mass [164] and this increase may contribute to insulin resistance in obesity and in T2DM. The adipocytes are highly sensitive to insulin and TNF-alpha has been shown to inhibit insulin-stimulated tyrosine phosphorylation of the insulin receptor (IR) as well as insulin receptor substrate-1, and to downregulate the insulin-sensitive glucose transporter, GLUT4, in adipocytes [164, 165]. Thus it is postulated that the increased expression of TNF-alpha through the activation of the cytokine system in obese individuals may play a role in the insulin resistant state associated with T2DM.

By acting on phosphorylation of insulin receptor substrate-1 (IRS-1) and phosphatidylinositol-3-kinase (PI-3 kinase), TNF-alpha could promote significantly to insulin resistance and cause deterioration of diabetes. It is known that when the hormone insulin binds to its receptor, the next step involves the instigation of the phosphorylation of its substrates, (IRS-1) and insulin receptor substrate-2 (IRS-2). This step consists of receptor substrate autophosphorylation, then binding to the 85kDa subunit, PI-3 kinase. This interaction with the p85

alpha subunit of PI3K stimulates the tyrosine kinase activity, a step required in insulin-stimulated glucose transport into muscle.

Central obesity is much more strongly associated with insulin resistance than overall obesity [166], and it is possible that the increase of both the influx of free fatty acid to liver and the production of TNF-alpha by the adipose tissue may play a vital role in mechanism of insulin resistance associated with central obesity. Therefore, possible mechanisms of the metabolic consequences of TNF-alpha could include (a) downregulation of genes that are necessary for normal insulin action, (b) direct effects on insulin signalling and (c) induction of elevated free fatty acids via stimulation of lipolysis. Finding genes that are specifically expressed in visceral fat as well as relating them to adipocytokines should facilitate clarification of the mechanism for the development and complications such as insulin resistance and obesity, conditions associated with visceral fat accumulation and conditions that are potential prediabetic states.

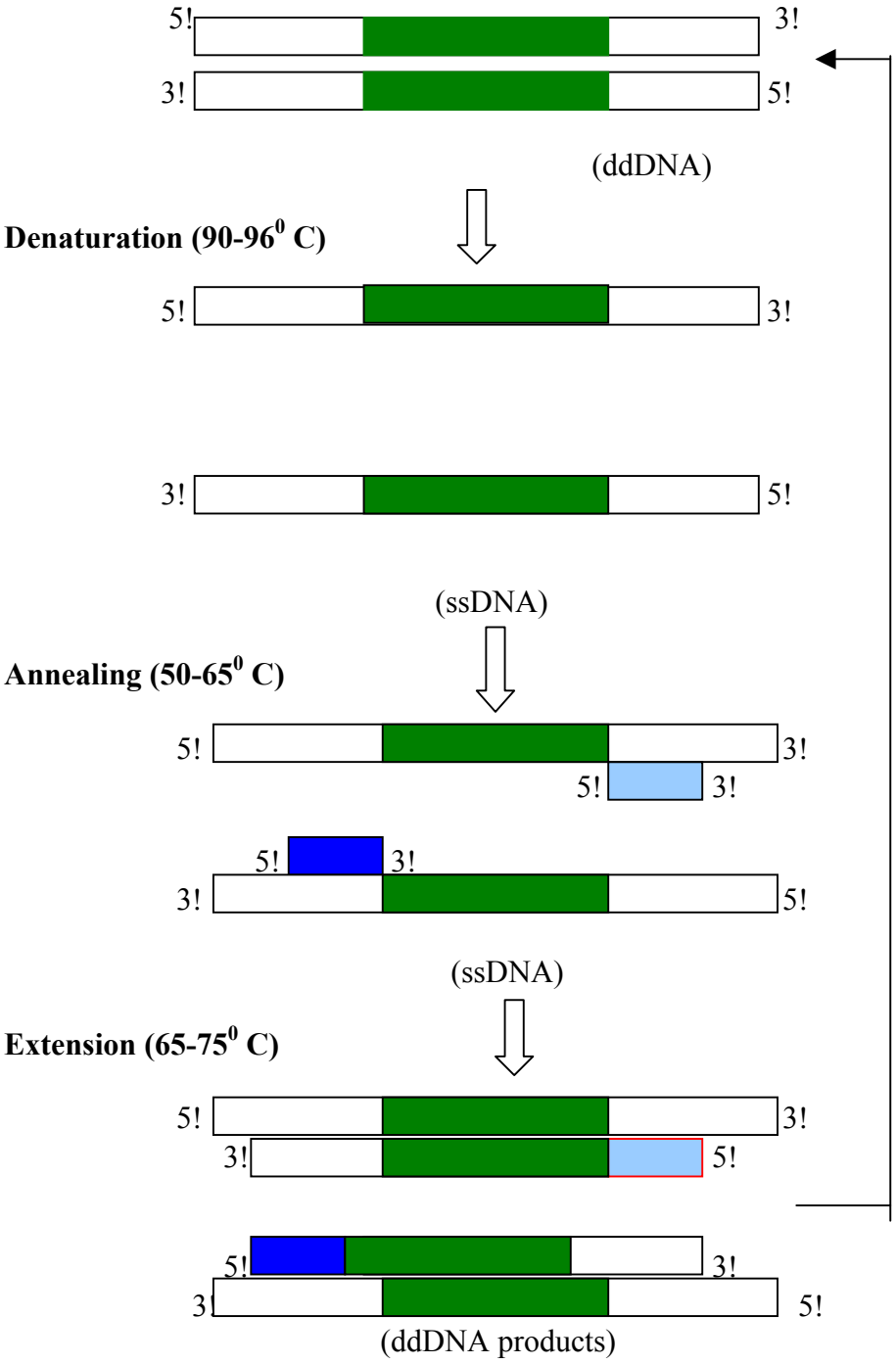
Obesity has become the most common metabolic disorder in the developed world and is strongly associated with insulin resistance and T2DM diabetes mellitus [69], but the molecular mechanisms underlying these disorders are poorly understood. Thus the hypothesis is that the TNF-alpha -308 polymorphism, with substitution of guanine for adenine in the promoter region, may strongly influence genetic susceptibility to overexpression of TNF-alpha in adipocytes. In this way it may act as a candidate mediator of insulin resistance associated with obesity, and insulin resistance itself is a major risk factor for the development of T2DM.

1.5 POLYMERASE CHAIN REACTION (PCR) TECHNIQUE

The PCR technique is conceptually a very simple method for amplifying nucleic acids and somewhat mimics the natural DNA replication process [167-169]. The process of the PCR involves amplifying DNA up to a million-fold *in vitro*, and the principle is based on the fact that when DNA is heated above a certain temperature, the two component strands separate (melt) and that when it is cooled down again, the strands come together (anneal). The PCR method relies on the repetitive cycling of 3 basic reactions, denaturation, primer annealing and primer-directed extension (Figure 1.9.). The most reliable source of template for the PCR is 20 ng to 1 ③g of genomic blood.

Thus the PCR is based on the repetitive cycling of three reactions, denaturation, primer annealing and primer-directed extension in microtubes containing a solution with target DNA, oligonucleotide primers, buffer, dNTPs and heat-stable *Taq* DNA polymerase (enzyme catalysing synthesis of DNA). The initial stage of the reaction (denaturation) is carried out for approximately 5 minutes at temperatures between 90 to 96^{o} C and this results in separation of the target sequence into 2 DNA strands. Primer (oligonucleotide) annealing, the second stage, is a process that involves subsequent cooling of the microtubes to temperatures between 50 and 65^{o} C, to allow the two short DNA primers to bind to their complimentary sequences in the target DNA which flank the target sequences.

Figure 1.9. Schematic diagram of the PCR method which relies on the repetitive cycling of 3 basic reactions, denaturation, primer annealing, and primer-directed extension (by Taq polymerase).



The smallest light rectangle is primer 1, the smallest dark rectangle is primer 2 and the continuous arrow indicates the repeat cycle.

The primers themselves define the 2-ends of the DNA that are to be amplified. In the final stage (primer-directed extension) of the reaction, the microtubes are brought to temperatures between 65 and 75^{o} C which are around the optimal temperature of the thermostable *Taq* DNA polymerase, the primer extension enzyme. This process results in the synthesis of a complimentary strand of new DNA by enzyme-mediated addition of nucleoside triphosphates onto the 3! ends of each primer.

After one complete cycle of the PCR, the amount of DNA between the primers has doubled and the cycle of denaturation, primer annealing and primer-directed extension is repeated so that after n cycles the theoretical amplification is 2^{n} . It must be noted that in actual practise the PCR is approximately 85% efficient, so that the net amplification is 1.85^{n} . Therefore this method is rapid (as it is automated), specific (through selection of a starting block for the primers, i.e. single stranded DNA) sensitive (uses minute quantities of DNA as starting material) and is rapidly becoming the standard method for analysis of DNA.

PCR is a revolutionary research method and an indispensable tool in medical diagnostics and human genetics. The scope for using this technology is unlimited, as it can be used to decode, compare and alter genetic material in basic biological and medical research. The recently completed work in decoding the entire human genome, through mapping and sequencing DNA by the Human Genome Project [170] is a classic example of the utility of the PCR technique. PCR can also be used to introduce point mutations by altering the nucleotide sequence of a known gene by synthesising primers containing the desired changes in the sequence of

bases. The primers attach themselves to related individual strands, which are not necessarily complimentary, and as soon as the starting blocks have been generated, amplification takes place and each double strand of DNA produced will contain the new sequence of bases. In basic biological research, this altered gene can be used to investigate gene structure and the biological function of the "new" protein product.

The PCR can identify inherited disorders by comparing specific sequences of bases in healthy genes and in genes from individuals suspected of carrying defective genetic material. The only prerequisite is that we should know the precise location of the genetic alteration as well as the type of genetic alteration to enable us to synthesise the appropriate primers. The advantage of PCR method is that it can easily detect inherited diseases caused by the absence, the exchange or the repetition of individual base pairs, compared to methods that can only identify genetic diseases in which long DNA sequences are missing or are repeated many times.

The PCR technique is useful in forensic medicine, as tiny amounts of blood, semen, hair or other tissues left at the scene of crime can be amplified and the product can be compared with DNA sequences from the suspect. As HLA gene sequences are inherited, paternity suits and consanguinity cases can be resolved by comparisons of DNA sequences of the affected individuals against the "suspects". In organ transplants, specific HLA sequences from the donor and the recipient can speedily and easily be compared after PCR amplification to check for compatibility. In future PCR may be useful in the prediagnosis of cancer by

identifying changes in the genetic material that normally occur before malignancy and metastasis in susceptibly individuals. Fragments of DNA from archaeological finds and from museum samples can be amplified by PCR, and comparisons made with DNA from living plants as well as animals, to unravel the evolution of species.

The PCR method can also be used in the diagnosis of infectious diseases caused by bacterial, viral, fungal and parasitic pathogens [171-174]. This is possible because the genetic information of the pathogens residing in the host (foreign DNA) can be determined by the amplification of the specific DNA sequence of the pathogen by the extremely sensitive PCR technique. Nowadays, diagnosis of bacterial diseases such as *mycobacterium tuberculosis*, *chlamydia trachomatis*, viral diseases such as HIV, hepatitis, fungal infections such as *cryptococcus neoformans*, *candida albicans* and parasitic diseases such as *pneumocystis carinii* and *toxoplasma gondii* can be achieved by the PCR method.

1.6 PCR AND DETECTION OF DNA POLYMORPHISMS

A change (mutation) in a single base-pair in genomic DNA in a complimentary sequence prevents hybridisation and can thus be detected with suitable oligonucleotide probes. In order to demonstrate that a mutation has taken place in the study of an inherited disease, the gene is amplified by the PCR method. The essential requirement is to know the exact location and type of genetic changes that have taken place in order to synthesise suitable primers. Purified enzymes (restriction endonucleases), isolated from bacteria, are used to cut DNA into manageable fragments that can be used to study genes [97, 175, 176].

The endonucleases work by recognising and cleaving specific nucleotide sequences of double-stranded DNA. Therefore an amplified DNA sequence, after digestion by a restriction endonuclease, recognises an allele which is linked to or causes an inherited disorder. The DNA amplified a million-fold by the PCR technique, can be directly visualised on a gel without further techniques or probes (for instance without cloning or without need for Southern or Northern blotting). The standard medium used for the separation and visualisation of restriction fragments obtained by endonuclease digestion of amplified genomic DNA is agarose gel.

1.7 JUSTIFICATION, HYPOTHESIS AND OBJECTIVES OF THE STUDY

T2DM and its complications have a multifactorial aetiology that include both environmental and genetic factors, and it is rather difficult to isolate susceptibility genes because of the contribution of multiple genes, the wide variety of environmental factors that are involved, the lack of a clear mode of inheritance and the presence of genetic heterogeneity. Genes underlie numerous human diseases and traits and the mission of identifying disease-susceptible genes in complex disorders such as diabetes mellitus is challenging. However, it is still imperative to search for T2DM genetic lesions, as the ultimate aim of modern medical and human genetics, should be to comprehend at a molecular level, the basis for mutations that lead to a genetic disease, in order to improve methods of diagnosis as well as treatment.

The risk factors for multifactorial diseases such as dyslipidaemia and diabetes mellitus generally have a continuous distribution in a population as a result of interaction between the genetic loci variation, and the gene-environment interaction. Thus, investigating the genetics of intermediate traits such as lipids, obesity, hypertension, microalbuminuria, hyperinsulinaemia and insulin resistance, all components of the metabolic syndrome, may lead to useful information about gene-gene interactions and the major candidate genes associated with the heterogeneous disorder of diabetes mellitus. Also metabolic studies may provide the necessary aetiological link between genetic and clinical manifestation of T2DM.

It is debatable, whether the aetiology of T2DM is similar in different populations with geographical differences, but what is certain is that the disease is heterogenous with strong genetic and familial components. As dramatically high rates of T2DM have been observed in historically undernourished recently urbanized populations, as a result of rapid changes in their environment and lifestyle, it is possible that alterations in diet and behaviour could transform genes that once conferred selective biologic advantage into health hazards. In this way, T2DM can be regarded as a disease composed of subtypes that are strongly associated with environmental factors at one end of the spectrum and highly genetic forms at the other end.

The hypothesis in this study is that:

(i) certain genetic polymorphisms have a substantial influence in metabolic programming that increases susceptibility to T2DM

and (ii) the study of human genetic variations, particularly the distribution of single nucleotide polymorphisms (SNP), between affected and non-affected individuals could provide crucial information about the genetic contributions to complex disorders such as T2DM.

Therefore the main objective of this study is to study genetic polymorphisms of metabolic traits in Black Zimbabweans with T2DM. This will be achieved by investigating potential genes involved in insulin action and or secretion, lipid metabolism and blood pressure regulation:

(i) ApoE gene mainly for dyslipidaemia, obesity and nephropathy. Surveys of ApoE polymorphism in different populations reflect an allelic variation in serum triglycerides, total cholesterol and HDL-C and this variation makes the ApoE gene a key candidate gene in insulin resistance and CAD.

(ii) ACE gene mainly for hypertension and nephropathy. Hypertension and diabetic nephropathy clusters in T2DM families, and this suggests genetic susceptibility plays an important role in these two disorders in diabetic subjects. As Afro-Americans and Afro-Caribbeans have a higher prevalence of hypertension and renal disease than their Caucasian counterparts, it is imperative to study genes associated with the renin-angiotensin system in Black Africans, and the ACE gene is one such candidate

(iii) TNF-alpha gene mainly for obesity and insulin resistance. Obesity has become the most common metabolic disorder in the developed world and is strongly associated with insulin resistance and T2DM diabetes mellitus. It is

likely that it will also become a problem in developing nations because of their rapidly changing environments. Obesity and insulin resistance are risk factors for T2DM as subjects with a family history of T2DM have a greater WHR and are more insulin resistant than subjects without a family history of T2DM. Since TNF-alpha, a product of the TNF-alpha gene is produced by the adipose tissue, that in turn has an effect on lipid metabolism, it is tempting to speculate that the gene has an important role to play in obesity, and that it may mediate insulin resistance.

Thus the major rationale for choosing the 3 genes, ApoE, ACE and TNF-alpha is that they have been found to be consistently associated with the major components of the metabolic syndrome, and the 3 genes have been extensively studied in several populations. The components of the metabolic syndrome are the key elements in the pathophysiology of T2DM. Also, the association of these genes with the metabolic syndrome makes them firm candidates for thrifty genes. It is important to note that the acquisition of thrifty genes, genes that predispose to the accumulation of abdominal fat, energy preservation and insulin resistance, may be responsible for the aetiology of T2DM in developing countries. The 3 genes, ApoE, ACE and TNF-alpha are classic “metabolic genes” that may fulfil the criteria of thrifty genes.

CHAPTER 2. MATERIALS AND METHODS

2.1 ETHICAL ISSUES

This study was approved by the Medical Research Council of Zimbabwe and by the Ethics Committees of the 3 main referral Hospitals, namely Chitungwiza, Harare and Parirenyatwa. It was emphasised that the patients were invited to participate on a voluntary basis, that they had a right to refuse to join the study, that they were free to withdraw from the study any time and that refusal to participate in the study would not affect their care by the medical staff. Only those patients who gave written and/or verbal informed consent were allowed to participate in the study. The patients were assured that the information they volunteered, and all the laboratory results would remain confidential. The interviews were carried out in a private consultation room.

2.2 SUBJECTS

The Epi Info version 6 statistical package was used to calculate the sample size. Assuming the size of the population to be greater than 100 000, the desired precision to be 6% and the expected prevalence of the metabolic syndrome to be 70% in diabetic subjects and 10% in healthy youths, a sample size of at least 224 diabetic and 96 non-diabetic subjects at 95% confidence level was computed. Unrelated and consecutive Zimbabwean blacks with an onset of T2DM at least 40 years after birth were recruited to investigate the metabolic aberrations of diabetes mellitus with certain genetic divergences. Diagnosis of T2DM was made by medical practitioners in the 3 referral hospitals, using a combination of features including age at diagnosis, no absolute requirement for insulin and

absence of a history of diabetic ketoacidosis, according to the World Health Organization criteria [44]. All consenting subjects who were on a combination of diet and/or hypoglycaemic treatment for T2DM were eligible to participate in the study. In addition, a subset of consecutive, young healthy Zimbabwean blacks was recruited for similar investigations. The spin-off of this selection is that useful and novel information about the relation of genetic abnormalities with metabolic phenotypes (intermediate traits) of young Zimbabweans, because of their rapidly changing environment, such as urbanisation, a rise in socio-economic status, a change in dietary habits and a decline in physical activity, all attributes of the modern Western lifestyle could be obtained. This lifestyle is potentially damaging as it can result in metabolic disorders, which can precipitate diabetes mellitus.

2.3 BLOOD PRESSURE MEASUREMENTS

Blood pressure (systolic and diastolic) was measured in the sitting position after a 15-minute rest from the antecubital fossa of the right arm. The auscultation method was used, that is, a conventional mercury column sphygmomanometer with an appropriately sized cuff and the arm supported at heart level. Disappearance of the Korotkoff sounds (phase V) was taken as the diastolic pressure. Two readings were obtained, with at least 1 minute between the measurements. However if there was a difference of > 5mmHg between the first 2 readings, a third reading was obtained and the mean of the 2 closest readings was used to obtain the average blood pressure reading. Blood pressure measurements were carried out by a qualified (state registered nurse) research midwife, using a standard protocol.

2.4 BLOOD AND URINE SPECIMEN COLLECTION

Blood and urine samples were collected from patients who had been on an overnight fast for at least 12 hours. Factors such as posture of the patient, the selection of a suitable vein, choice of anticoagulant, choice of skin cleansing agent, the extent of venous stasis, and precautions to avoid haemolysis were taken into consideration. Blood was collected in a sitting position, by venipuncture into sterile plain, Na-EDTA and fluoride/oxalate tubes and when an anticoagulant was used, the blood was mixed with the anticoagulant by repeated gentle inversion. Early morning mid-stream urine specimens were collected in privacy, into sterile containers.

2.5 DATA COLLECTION

Patients attending the 3 main referral diabetic outpatients-polyclinics, namely, Chitungwiza, Harare and Parirenyatwa between 6.30 am and 9.30 am from Monday to Friday were randomly selected. Demographic data such as age, sex, educational background, residence was collected from each patient and from the patients' medical cards and notes using a standardised interviewer –administered questionnaire. This questionnaire was also designed to obtain information on the history of diabetes and hypertension, previous and current symptoms related to diabetes mellitus, medication, risk factors of diabetes as well as other symptoms.

2.6 ANTHROPOMETRIC MEASUREMENTS

Height was measured to the nearest 0.1 cm using a stadiometer and weight measurements up to the nearest 0.1 Kg, on a calibrated scale, with patients bare footed and wearing minimal clothing. Body mass index (BMI) was calculated

from height and weight measurements (weight in kilograms divided by height in metres, Kg/m^2). Fat distribution was assessed through waist and hip measurements by using a flexible tape measure (in centimetres) on a subject who was standing and breathing shallowly. For the waist circumference, the measurement was taken at a level halfway between the lower rib margin and the iliac crest, and for the hip circumference, the measurement was taken over the widest hip circumference. The hip circumference over the waist circumference was expressed as the waist to hip ratio (WHR). A qualified research (state registered nurse) midwife, using a standard protocol, carried out these anthropometric measurements.

2.7 SPECIMEN PREPARATION AND STORAGE FOR BIOCHEMICAL ASSAYS AND DNA STUDIES

Plasma or serum for biochemical assays such as glucose, insulin and lipids was separated from blood within 30 minutes of blood collection by centrifugation at 3000 g for 5 minutes. The separated plasma for insulin tests was stored frozen at -80^{o} C, and insulin assays were performed within one month of specimen collection. These frozen samples were thawed overnight at 2-8^{o} C and mixed thoroughly before analysis, to ensure consistency of results. Repeated thawing and freezing were totally avoided. The rest of the biochemical assays were carried out within two hours of serum or plasma separation. The urine samples were stored at 2-8^{o} C in a container with 0.02% sodium azide, an anti-microbial agent, and microalbumin assays were done within 2 weeks of specimen collection. The Na-EDTA whole blood samples for genomic DNA extraction were stored frozen at -80^{o} C.

2.8 GENOMIC DNA EXTRACTION AND QUANTITATION

High molecular weight genomic DNA was prepared from peripheral white blood cells [177]. Stored, frozen Na-EDTA whole blood samples were left to thaw overnight at ordinary refridgerator tempaeratures (4-8^{o} C. Then the procedures below were carried out in the following order on each sample.

1. 1,5 volume of sterile LYSIS BUFFER (refer to section 2.10) was added to each thawed sample in sterile 50 ml polypropylene tubes (e.g. for 5 ml of patients blood sample, 7.5 ml of lysis buffer would be added) using sterile glass pipettes. The contents were then thoroughly mixed by gently inversion several times. The tubes were then placed in an ice bath for 30 minutes (or for one hour if the red cells were clumped). For the duration of the lysis period, the tube were periodically inverted several times to promote effective cell lysis.

2. The lysed samples were then centrifuged for 30 minutes at 3000 rpm at 4^{o} C in a MSE Mistral 600 centrifuge to pellet white cell nuclei.

3. The samples were immediately placed in an ice bath. After discarding the supernatant from the samples, 1.5 ml of sterile EXTRACTION SOLUTION (refer to section 2.10) was added to the pellet, which contains nuclei with DNA-containing white cells. The extraction solution lyses the nuclei. The contents were mixed gently to loosen the pellet from the base of the tube. Then the pellet was transferred to a 10 ml polypropylene tube containing 200 ③L of 20% sterile SDS detergent to dissociate DNA-protein complexes.

4. The tubes with the pellet and SDS were then mixed gently several times and then incubated at 65^{o} C for 10 minutes to solubilize the pellet.

5. Then 10 ③L of 20mg/ml defrosted and sterile PROTEINASE K, equivalent to 200 ③g, was added to each tube using a sterile micropipette with sterile tips. The

contents were then mixed gently and the tubes were incubated at 37 ^{o}C overnight for maximum digestion of the pellet to release DNA and remove contaminating protein.

6. The tubes were removed from the water bath and their contents allowed to reach room temperature. To each tube, 4 ml of sterile TE (Tris/EDTA buffer) was added through use of sterile glass pipettes, and the contents thoroughly mixed.

7. Then 2ml of sterile and equilibrated PHENOL was added to each digest to extract DNA and remove the bulk of residual protein and membrane contamination, through sterile graduated pasteur pipettes, and the tubes were gently mixed in a rotor (Heto Rotamix RS) for 10 minutes.

8. The phenol tubes were then spun at 3000 rpm for 15 minutes in a centrifuge at room temperature and the viscous upper layer of each tube was carefully removed by sterile pasteur pipettes, avoiding the contaminants of the lower layer, and this was transferred to a new set of polypropylene tubes.

9. Then 2 ml of sterile PHENOL:CHLOROFORM/ISOAMYL ALCOHOL, in the ratio 1:1 (ratio of chloroform/isoamyl alcohol is 24:1) mixture was added to each tube to extract and further purify the DNA, using sterile graduated pasteur pipettes, and the tubes were mixed gently in a Heto rotor for 10 minutes. The tubes were then centrifuged for 15 minutes at 3000 rpm at room temperature.

10.A mixture of 4 ml sterile CHLOROFORM/ISOAMYLALCOHOL in the ratio 24:1 was then added to each tube to extract DNA, using sterile graduated pasteur pipettes, and the contents gently mixed in a Heto rotor for 10 minutes. The tubes were then spun at 3000 rpm at room temperature for 15 minutes.

11.The upper layer in each tube was then carefully removed by sterile pasteur pipettes, and transferred to sterile 15 ml polypropylene tubes. Then 8 ml of

chilled 95% analytical grade ETHANOL (stored at –20^{o} C) was gently added to each tube by use of sterile graduated pasteur pipettes. After addition of ethanol in each tube, the tube was slightly tilted, keeping it fairly horizontal to allow the DNA to precipitate out as a small cloud. The racked tubes were left in a freezer at –20^{o} C overnight.

12. The DNA was then spooled out using a sterile glass pasteur pipette that had been previously hooked by a flame from a burner and rinsed in 1 ml of 70% analytical grade ethanol in a 1.5 ml sterile microtube.

13. Then as much ethanol was squeezed out of the DNA strand and the DNA was left to dry on the glass hook for a couple of minutes at room temperature.

14. The DNA was then resuspended in 400 ③L of TE (refer to section 2.10) in a 2 ml sterile microtube and left to dissolve overnight at room temperature.

15. The samples were then stored at 4^{o} C for 7 days to thoroughly dissolve the extracted DNA, before quantitation of DNA concentration in each sample.

16. The concentration of DNA in ③g/ml of each sample was determined by the GeneQuant RNA/DNA calculator (Pharmacia LKB Biochrom Ltd). The instrument measures DNA and RNA samples in uv cells at 230 nm (as a guide for protein determination), 260 and 280 nm (for DNA quantitation and purity checking, i.e. protein contamination) as well as 320 nm (for background compensation) simultaneously. Appropriate dilutions were made and the working DNA stored between 2-8^{o} C, but bulk DNA was stored at –20^{o} C.

2.9 MOLECULAR BIOLOGY GRADE REAGENTS FOR DNA EXTRACTION

1. Chloroform – SIGMA
2. EDTA disodium, 0.5M – SIGMA
3. Isoamyl alcohol – SIGMA
4. MgCl hexahydrate – SIGMA
5. Phenol equilibrated – SIGMA
6. Proteinase K (20mg/ml) - SIGMA
7. Sucrose – SIGMA
8. SDS (lauryl sulphate) - SIGMA
9. TRIZMA Base – SIGMA
10. Triton X-100 – SIGMA

2.10 SOLUTIONS FOR DNA EXTRACTION AND ELECTROPHORESIS

1. **EXTRACTION SOLUTION:** 1 ml TE was added to 100 ③l EDTA. The mixture was then autoclaved for sterilisation.

2. **LYSIS BUFFER:** 218 g of sucrose was added to 20 ml 1M TRIS (pH 7.5), 20 ml I M $MgCl_2$ (hydrous) and 20 ml Triton X-100. This mixture was then made up to 1000 ml with distilled water. The solution was filtered to sterilise it and stored at 4^0 C.

3. **TE:** 1 ml 0.5M EDTA was added to 5 ml of 1M TRIS (pH 8). The mixture was made up to 500 ml with distilled water. It was then filtered to sterilise it.

4. **TBE (10x):** 108 g of Tris was added to 55 g of boric acid and 9.8 g of EDTA in 900ml of sterile water. The mixture was thoroughly mixed with a magnetic stirrer and finally brought to a volume of 1000 ml with sterile water.

2.11 BIOCHEMICAL METHODS

All biochemical tests, except where indicated, were performed on the Synchron CX5 Beckman autoanalyzer (Beckman Instruments, INC, Brea, CA, USA), using Randox (Randox Laboratories LTD, Crumlin, Antrim Co., UK) or Beckman diagnostic reagent kits.

2.11.1 Plasma glucose

The Randox Glucose (GOD/PAP) reagent was used to determine fasting blood plasma glucose concentration. This is a colorimetric glucose oxidase method, which also incorporates phenol, 4-aminophenazone and peroxidase reagents. The resultant red-violet quinoneimine dye was measured at 600 nm [178].

2.11.2 Serum creatinine

Serum creatinine was measured by the automated Jaffe kinetic method, using alkaline picric acid to measure the red colour complex formation at 520 nm [179, 180]. The Beckman Reagent Diagnostic Kit (Beckman Instruments, INC, Galway, Ireland) was used for the assays.

2.11.3 Serum urea

The assay was performed using the Randox urea enzymatic kinetic method, which incorporates urease, 2 α-oxoglutarate, glutamate dehydrogenase and

NADH [181, 182]. The rate of the disappearance of NADH was followed at 340nm and was related to the concentration of urea in the patient's sample.

2.11.4 Serum urate

The Beckman Syncron CX systems uric acid reagent was used to measure serum uric acid in a timed-endpoint and enzymatic method. The reagents include, uricase, 4-aminoantipyrine, 3,5-dichloro-2-hydroxybenzene sulphonate and peroxidase and the change in absorbance at 520 nm due to the quinoneimine dye was related to the concentration of uric acid [183].

2.11.5 Serum lipids

Fasting serum total cholesterol concentration was measured by a timed end point enzymatic method utilising a Randox kit. This method contains the following reagents, cholesterol esterase, cholesterol oxidase, phenol, 4-aminoantipyrine and peroxidase [184-186]. The end product, o-quinoneimine dye, was measured photometrically at 520nm.

The Randox direct HDL-cholesterol technique incorporating the above cholesterol method was used to determine fasting serum HDL-cholesterol levels. This requires preincubation of a patient's sample with sulphated cyclohexidine buffer to form water-soluble LDL, VLDL and chylomicron complexes, which are, resistant to polyethylene glycol modified enzymes which act on HDL [187, 188]. The quinoneimine dye so formed was measured at 600 nm.

A Randox enzymatic colorimetric method was used to measure fasting serum triglyceride levels. The method uses lipase, ATP, glycerol kinase, glycerophosphate oxidase aminophenazone3-5-di-2-hydroxybenzenesulphonic acid and peroxidase as reagents [189, 190]. The stable quinoneimine dye formed was read at 520 nm.

2.11.6 Plasma insulin

Fasting levels of plasma insulin were carried out in an Abbott Laboratories IMx analyser using an Abbott insulin kit, by the microparticle enzyme immunoassay (MEIA) technique. This method exhibits no cross-reactivity with proinsulin and an optical assembly that reads fluorescence measures the final product. The reagents include anti-insulin mouse monoclonal coated microparticles (to form the antibody-antigen complex), alkaline phosphatase conjugate (which binds to the antibody-antigen complex) and 4-methylumberlliferyl phosphate (to produce the fluorescent product)

2.11.7 Urinary albumin

The Randox immunoturbidimetric assay was utililized to measure microalbumin in mid-stream urine samples of patients on the Synchron CX5 Beckman autoanalyzer. The urine sample was added to a buffer containing an antibody specific for human serum albumin which spontenously precipitated immunocomplexes and the turbidity of these precipitants was measured at 340 nm. Any urinary specimen exhibiting cloudness was centrifuged at 3000 g for 5 minutes (to obtain a clear supernatant), before carrying out the assay [191-193].

2.11.8 Insulin resistance

Relative insulin resistance was calculated using fasting glucose and insulin levels by the Homeostasis Assessment Model (HOMA), a computer generated model: HOMA = {insulin (③U/ml) x glucose (mmol/L)}/22.5, [194].

2.12 CLASSIFICATION CRITERIA OF PARTICIPANTS FROM ANTHROPOMETRIC, BLOOD PRESSURE AND BIOCHEMICAL RESULTS

The participants were classified as (1) overweight if their BMI was greater than 25 Kg/m^2, (2) obese if their BMI was equal to or > 30 Kg/m^2 or a waist to hip ratio > 0.85 in females and > 0.90 in males, (3) hypertensive if the SBP was > 140 mmHg and/or a DBP > 90 mmHg or if they were on antihypertensive drug therapy (4) hyperinsulinaemic if plasma insulin levels were > 9.2 ③U/ml, (5) dyslipidaemic if the fasting serum triglycerides levels were >1.7 mmol/L or fasting serum HDL-C was < 1.0 mmol/L in women and < 0.9 mmol/L in men or if the patient had both hypertriglyceridaemia and hypoHDL-C, (6) hypercholesterolaemic if fasting total cholesterol levels were > 5.2 mmol/L, (7) hyperuricaemic if serum uric acid levels were > 365 ③mol/L in females and > 415 ③mol/L in males (8) microalbuminuric if a random morning urine albumin/creatinine ratio was > 2.5 mg/mmol, and (9) insulin resistant if HOMA-IR was >3.

2.13 GENETIC METHODS

2.13.1 ApoE gene amplification and RFLP analysis

The amino acid substitutions of cysteine and arginine at codons 112 and 158 of the 3 common alleles, epsilon 2 (E2), epsilon 3 (E3) and epsilon 4 (E4) in the ApoE gene were investigated by PCR method of DNA amplification by using the Hixson and Vernier method [195]. Bulk PCR mix was prepared in an ice bath, by adding the components listed (refer to i), except DNA and care was taken on all occasions avoiding foaming which can denature proteins.

i. PCR MASTER MIX

Component	**Volume (③L)**
1. Sterile water	2.35
2. Taq buffer (10x)	1.50 (final concentration, 1x)
3. DMSO (10%)	1.50
4. dNTPs (2 mM)	1.50 (final concentration, 0.2 mM)
5. Primer 4 (10 ③M)	1.50 (final concentration, 1.0 ③M)
6. Primer 6 (10 ③M)	1.50 (final concentration, 1.0 ③M)
7. DyNazyme (2 U/③L)	0.15 (final concentration, 0.02 U/③L)
8. DNA (100 ng/ ③L)	5.00
Total volume	15.00

The sequence of sense primer (Primer 4), consisted of 26 bases in the following order, (5!-ACA GAA TTC GCC CCG GCC TGG TAC AC-3!), and the antinsense primer (primer 6) consisted of 25 bases (5!-TAA GCT TGG CAC

GGC TGT CCA AGG A-3!. Then the mixture was dispensed into microtubes (in an ice bath) and the DNA from each participant was then added. The microtubes were overlaid with a few drops of mineral oil to seal the reaction and arrest evaporation. Amplification of DNA was then performed by a standard PCR amplification protocol for 35 cycles, which included denaturation at 95 ^{o}C (1 minute), primer annealing at 60^{o} C (1 minute), primer extension at 70^{o} C (2 minutes) and final extension at 72^{o} C (5 minutes) on the Perkin Elmer programmable thermal cycler 480. Finally, chilling at 4^{o} C stopped the reaction.

The digestion mix (refer to ii) was dispensed into the PCR product of each participant in an ice bath. Digestion of the PCR product by the restriction enzyme *Hha* I (from an *E Coli* strain carrying the cloned *Hha* I gene from *Haemophilus haemolyticus*) was carried out at 37 ^{o}C overnight. The digestion by restriction endonucleases is to cut DNA into fragments starting from the original product of the PCR. In this way, it is possible from the size of the obtained fragments to deduce the alleles of the gene under study.

ii. DIGESTION COMPONENTS

Component	**Volume (③L)**
1. Sterile water	2.6
2. Taq buffer (10x)	2.0 (final concentration, 1x)
3. *Hha* I (20 U/③L)	0.4 (final concentration, 0.4 U/③L)
4. PCR product	15.0
Total volume	20.0

The digest product together with a specific molecular weight marker (SIGMA pBR322 Hae III digest, D-9655) that had been incubated in a thermo dry bath at 65^{o} C for 10 minutes were then loaded onto specific wells on 8% polyacrylamide gel (iii), after addition of a gel loading solution, SIGMA G 2526, (which serves as a tracking dye and also allows the amplified DNA to stay in the wells) using microcapillary tips. PCR-amplified DNA fragments in the size range of <100 up to 2000 bp are amenable to high resolution polyacrylamide gel electrophoresis on ultrathin-layer gels.

iii. 8% POLYACRYLAMIDE ELECTROPHORESIS WORKING GEL (for 20cm x 20 cm electrophoresis slabs and 8 mm line spacers).

Component	**Volume**
1. Stock (40% acrylamide + bis acrylamide 29:1)	7 ml
2. Sterile water	24.45 ml
3. TBE (10x)	3.5 ml
4. Ammonium persulphate (10%)	250 ③L
5. TEMED	25 ③L
Total volume	35 ml

Electrophoresis to resolve the digest product was carried out at 35 mA after an initial 30 minute prerun at a constant current of 35 mA and at room temperature for 1.5 hours. At completion of electrophoresis, the gel was gently removed from the slabs and carefully transferred to a tray containing ethidium bromide, a fluorescent dye that avidly binds to DNA. The gel was left in this ethidium bromide (40③g/ml) for 30 minutes in a fume cupboard.

Ethidium bromide binds by intercalating between the base pairs, thereby causing the DNA helix to unwind and allows the visualisation of the different fragments (alleles) in uv light. The gel was then photographed on a dark background with 30 seconds exposure and 30 seconds development, using a Polaroid black and white print, film 667.

2.13.2 ACE gene amplification and RFLP analysis

The 287-bp insertion/deletion (I/D) polymorphism in intron 16 of the ACE gene was determined by the polymerase chain reaction [196]. Preparation of the PCR master mix (refer to i) was similar to that for ApoE above. The sequence of the sense primer (24 bases) was 5!-CTG GAG ACC ACT CCC ATC CTT TCT-3! with a MW of 7199.763, and that of the antinsense primer (25 bases) was 5!-GAT GTG GCC ATC ACA TTC GTC AGA T-3! with a MW of 7657.073.

i. PCR MASTER MIX

Component	**Volume (③L)**
1. Sterile water	5.35
2. Taq buffer (10x)	1.50 (final concentration, 1x)
3. dNTPs (2mM)	1.50 (final concentration, 0.2 mM)
4. Primer 1 (10 ③M)	0.75 (final concentration, 0.5 ③M)
5. Primer 2 (10 ③M)	0.75 (final concentration, 0.5 ③M)
6. DyNazyme (2 U/③L)	0.15 (final concentration, 0.02 U/③L)
7. DNA (100 ng/ ③L)	5.00
Total volume	15.00

The DNA polymerase enzyme used was DyNAzyme II, isolated and cloned from an E coli strain that carries a plasmid with the cloned DyNAzyme DNA polymerase gene from *Thermus brockianus* (supplied by FINNZYMES OY, Finland). Amplification was carried out for 3 hours by a standard PCR amplification protocol for 30 cycles, which included denaturation at 94 ^{o}C (1 minute), primer annealing at 54^{o} C (1 minute), primer extension at 72^{o} C (1 minute) and final extension at 72^{o} C (5 minutes) using the Epperndorf Master Cycler 5330 programmable thermocycler. As the allelic differences in the ACE gene were either insertion (I) or deletion (D) in the intron of the gene, no enzymatic digestion was performed and the PCR product was directly resolved in an agarose gel.

ii. AGAROSE GEL PREPARATION AND ELECTROPHORESIS

Agarose gel was used for separating the PCR ACE gene products. At a pH of 7, DNA is negatively charged and migrates from cathode to anode with mobility that is related to molecular weight, that is, fragment size. Agarose is one of the standard methods used for separation of restriction fragments obtained by endonuclease digestion of genomic DNA. The gel was prepared by dissolving 2.5 g of agarose in 100 ml of TBE buffer 1x (see section 2.10) in a microwave oven. The agarose mixture was removed from the oven to a fume cupboard, and 5 ③L of ethidium bromide (40③g/ml) was added. The flask was then shaken gently to avoid air bubbles and the final concentration of ethidium bromide in the gel was 0.5 ③g/ml. This mixture was then poured into a gel-casting tray with combs for wells and this was left at room temperature for at least 30 minutes before use. The PCR amplification product together with a specific marker (SIGMA pBR322 *Hae*

III digest, D-9655) that had been incubated in a thermo dry bath at 65^{o} C for 10 minutes were then loaded with the loading solution, SIGMA G2526, onto different wells on the agarose gel using microcapillary tips and the mixture was fractionated by electrophoresis. Electrophoresis was carried out at constant voltage of 120 V using TBE buffer (1x) at room temperature for 1 hour. At completion of electrophoresis, the gel was gently removed from the gel-casting trays and was visualised in uv light for allele identification (fluorescent bands). The gel was photographed by a Polaroid camera, on a dark background with 10 seconds exposure and 30 seconds development, using a Polaroid black and white print, film 667.

2.13.3 TNF -alpha gene amplification and RFLP analysis

The promoter region of the TNF-alpha gene was amplified by the PCR using the Day *et al* method [197]. The presence or absence of the 308 polymorphism (308G⌋A) in the 5! region of the gene was determined by electrophoretic resolution of the digested PCR product. The PCR master mix (refer to i) was prepared in a similar manner to ApoE above and amplification was carried out for 3 hours 15 minutes by a standard PCR amplification protocol for 35 cycles, which included denaturation at 94 ^{o}C (1 minute), primer annealing at 60^{o} C (1 minute), primer extension at 72^{o} C (1 minute) and final extension at 72^{o} C (5 minutes) using the Epperndorf Master Cycler 5330 programmable thermocycler. The sequence of sense primer, was made up of 23 bases (5!-AGG CAA TAG GTT TTG AGG GCC AT-3!) with a molecular weight of 7143.737, and the antinsense

primer consisted of 20 bases in the following sequence 5!-TCC TCC CTG CTC CGA TTC CG-3! with a molecular weight of 5955.931.

i. PCR MASTER MIX

Component	**Volume (③L)**
1. Sterile water	2.35
2. Taq buffer Taq (10x)	1.50 (final concentration, 1x)
3. dNTPs (2mM)	1.50 (final concentration, 0.2 mM)
4. Primer TNF1 (10 ③M)	0.75 (final concentration, 0.5 ③M)
5. Primer TNF2 (10 ③M)	0.75 (final concentration, 0.5 ③M)
6. DyNazyme (2 U/③L)	0.15 (final concentration, 0.02 U/③L)
7. DNA (100 ng/ ③L)	8.00
Total volume	15.00

Digestion (refer to ii) of the PCR amplification product by *Nco* I restriction endonuclease from an *E Coli* strain carrying the cloned *Nco* I gene from *Nocardia corallina* was performed at 37^{o} C overnight. The digest fragments were then subjected to electrophoresis in 2.5% agarose gel (2.5 grammes per 100ml TBE buffer). The *Nco* I RFLP was detected by ethidium bromide staining under ultraviolet light. To avoid false positives, digestion and electrophoresis were repeated on homozygous A carriers to ensure the original digestion had not failed. The components of the PCR master mix and digestion were as shown below.

ii. DIGESTION COMPONENTS

Component	Volume (③L)
1. Sterile water	3.4
2. Taq buffer (10X)	1.5 (final concentration, 0.75x)
3. *Nco* I (50 U/③L)	0.1 (final concentration, 0.25 U/③L)
4. PCR product	15.0
Total volume	20.0

2.14 CRITERIA FOR ELECTROPHORETIC IDENTIFICATION AND INTERPRETATION OF THE VARIOUS PCR AMPLIFICATION PRODUCTS

Figures 2.1, 2.2 and 2.3 are schematic diagrams, showing how ApoE, ACE and TNF-alpha gene mutations can be detected after electrophoretic separation of the respective PCR products.

2.14.1 ApoE gene

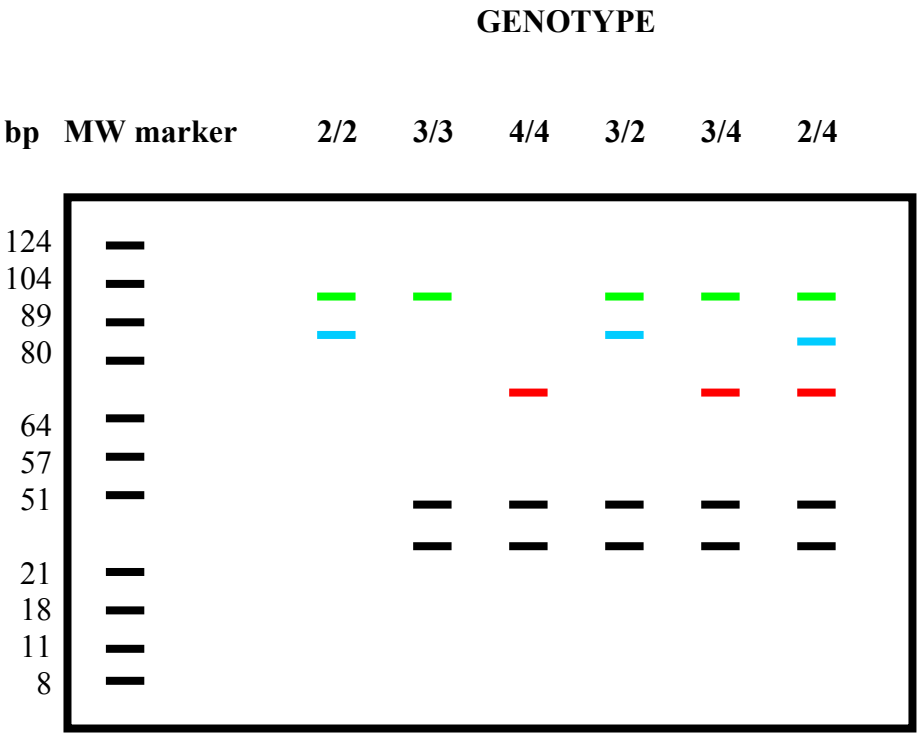


Figure 2.1 Schematic diagram showing the comparison of amino acid substitutions of cysteine and arginine at codons 112 and 158 of the ApoE gene by electrophoretic resolution on 8% polyacrylamide gel, after PCR amplification and *Hha* I endonuclease digestion. The fragments characterising the genotypes are 91 and 83 bp for 2/2, 91, 48 and 35 bp for 3/3, 72, 48 and 35 bp for 4/4, 91, 83, 48 and 35 bp for 3/2, 91, 72, 48 and 35 bp for 3/4 and 91, 83, 72, 48 and 35 bp for 2/4. The 2/2 genotype reflects the absence of sites at 112cys and 158cys, the 3/3 genotype contains the 91 bp fragment (112 cys) as well as the 48 and 35 bp fragments from cleavage at 158arg, in addition to the 48 and 35 bp fragments, the 4/4 genotype also contains the 72 bp fragment from the cleavage at 112arg.

2.14.2 ACE gene

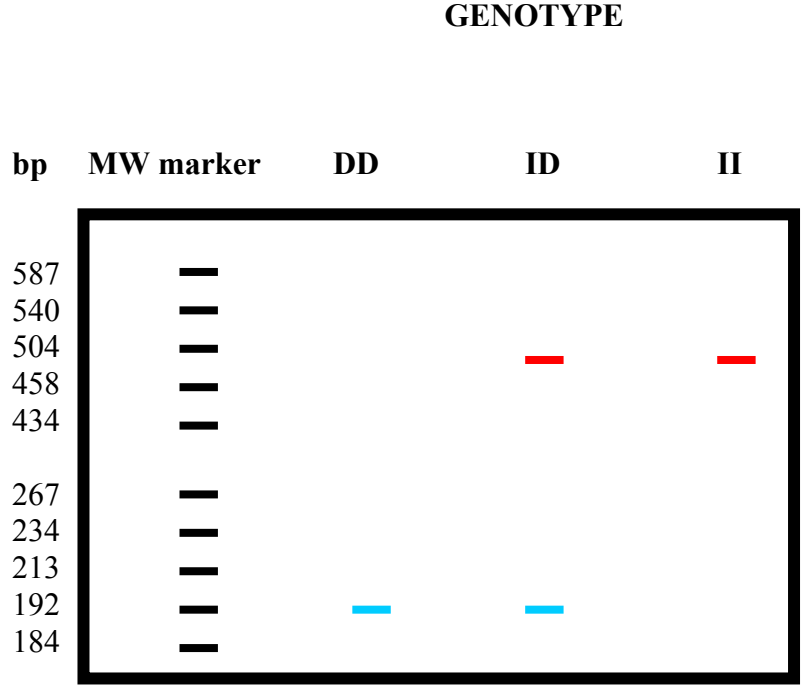


Figure 2.2 Schematic representation showing the electrophoretic detection of ACE 287-bp insertion/deletion (I/D) polymorphism on a 2.5% agarose gel after PCR amplification. The fragments characterising the genotypes are 190 bp for DD, 490 and 190 bp for ID and 490 bp for II.

2.14.3 TNF-alpha gene

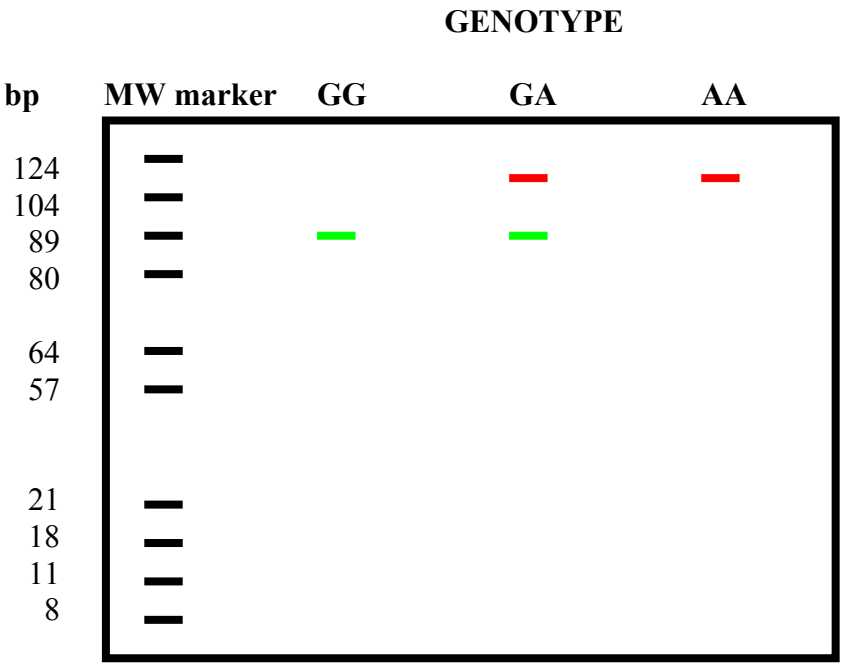


Figure 2.3. Schematic diagram of the electrophoretic analysis of the 308G⌡A mutation in the 5! region of the TNF-alpha gene on a 2.5% agarose gel after PCR amplification and *Nco* I endonuclease digestion. The fragment 87 bp corresponds to the G allele (restriction site present), the fragment 107 bp corresponds to the A allele (restriction site absent).

2.15 QUALITY ASSURANCE MEASURES

Expiry dates of all reagents indicated on the labels were observed. Also storage temperatures and periods including the stability of reagents, calibrators and controls were noted and adhered to. Reconstitution times were religiously followed for freeze-dried (lyophilised) material to ensure complete dissolution. All blood and urine samples from diabetic patients and young healthy participants were handled and disposed of using precautions designed for potentially pathogenic materials. This was also true for primary and secondary control material.

Safety precautions required for handling and/or disposing of laboratory reagents were exercised. Participants were given progressive, unique and reproducible identification numbers and these were constantly checked to ensure unequivocal identification as well as to avoid mixing of samples. The results of the participants for biochemical and DNA assays were accepted only if control material values fell within manufacturer's and/or laboratory's established limits. Where implicated, haemolyzed, lipaemic, icteric and turbid samples from participants were avoided. For biochemistry tests, any turbid sample or any sample suspected to be turbid, was centrifuged at 3000 rpm for 5 minutes before any biochemical analysis was carried out.

A separate and isolated room was used for DNA extraction and all equipment, glassware, gloves and plasticware were sterilised before use. All the protocols for extraction and processing of DNA were performed with techniques that avoided possible carry over from previous amplification sequences, or from laboratory

equipment or from other supplies and also from cross-contamination between samples. Most of the bulk reagents and reaction buffers for PCR and digestion of PCR products were divided into small aliquots and stored at –20^{o} C. Each aliquot was used only once so as to avoid repeated thawing and freezing. The reagents for DNA extraction and analysis were of molecular biology grade. Positive-displacement pipettes were used to avoid cross-contamination of samples and precautions were taken to prevent aerosols, for example, by carefully uncapping and closing tubes. Strict observance of routine maintenance and calibration procedures of all equipment including the autoanalyzers, the PCR thermocyclers and the electrophoresis units were followed.

2.16 STATISTICAL ANALYSIS

The results for continuous data are given as mean ! SD except were indicated. The student's two tailed t-test and ANOVA was used for continuous variables and Chi-squared test for categorical variables. Skewed data were logarithmically transformed and expressed as geometric means with 95% confidence limits. The allele and genotype frequency was worked out from direct counts and the X^2 test was used for comparisons, assuming Hardy-Weinberg equilibrium. For metabolic disorders and other variables, binary logistic regression was utilised and relative risk was then determined by the odds ratios. The statistical package was the WHO Epi-info version 6 and significance was set at 5%.

CHAPTER 3. RESULTS AND DISCUSSION - METABOLIC COMPONENTS

3.1 DEMOGRAPHIC AND METABOLIC ANALYSES

Two hundred and thirty six unrelated adult diabetic patients comprising of 164 females (69.5%) and 72 males (30.5%) were recruited (Table 3.1). In addition, 115 young, unrelated, non-diabetic and healthy adults, made up of 41 females (36%) and 74 males (64%) were recruited. The average age of the diabetic subjects was 57 years and of the youth was 21 years. Independent analysis of the means of age in the 2 groups according to gender did not show any statistically significant differences. The duration of diabetes in the subjects varied from 1 to 40 years, with a mean of 6 years in females and 7 years in males and the difference between the 2 means was not significant. Since the adopted selection criteria used to recruit participants in the study were different, no attempt was made to compare the two divergent groups in all aspects of the statistical analysis.

The means of the glycaemic indices, insulin and the homeostasis model assessment were statistically significant between the genders in the youth, $p = 0.0224$ and 0.0276 respectively. Males tended to have elevated mean plasma sugar levels and females elevated mean plasma insulin and HOMA-IR levels, reflecting a higher degree of hyperinsulinaemia and insulin resistance in the female diabetic and healthy subjects.

Table 3.1. Baseline means !SD of age, gender, and glycaemic indices in adult 236 diabetic patients and 115 young participants.

Adult diabetic patients		**Young adults**	
CHARACTERISTIC	**MEAN!SD**	**CHARACTERISTIC**	**MEAN!SD**
AGE		**AGE**	
Combined	57!12	Combined	21!1
By gender	F:58!12	By gender	F:21!1
	M:57!12[a]		M:22!1[b]
GENDER*		**GENDER***	
	F:69.5%		F:36%
	M:30.5%		M:64%
DURATION OF DIABETES		**DURATION OF DIABETES**	
Combined	6!7	Combined	-
By gender	F:6!7	By gender	-
	M:7!8		-
GLYCAEMIC INDICES		**GLYCAEMIC INDICES**	
Glucose (A)	11.4!5.5	Glucose (A)	4.4!0.6
Glucose (≅)	F:11.2!5.4	Glucose (≅)	F:4.3!0.5
	M:11.7!6.0[a]		M:4.4!0.7[b]
Insulin (A)	12.2!16.6	Insulin (A)	7.4!6.3
Insulin (≅)	F:12.8!18.8	Insulin (≅)	F:9.6!9.7
	M:11.0!9.9[a]		M:6.3!3.3[c]
HOMA-IR (A)	5.5!8.3	HOMA-IR (A)	1.4!1.1
HOMA-IR (≅)	F:5.5!9.3	HOMA-IR (≅)	F:1.7!0.7
	M:5.3!5.4[a]		M:1.2!0.7[c]

A, combined; ≅, by gender; *, gender frequencies. The units for glucose are mmol/L, ③U/ml for insulin, years for age and duration of diabetes. The superscripts a and b denote a p value that is not statistically significant among diabetic patients and young participants respectively and c a p value of < 0.05. No comparisons were made between the two groups because of the adopted selection criteria.

The anthropometric, blood pressure, uric acid and renal indices of all participants are described in Table 3.2 below. Females had higher mean values of BMI (10% in diabetes and 5% in the youth) and the means were statistically significant only between the sexes ($p < 0.0001$) in the diabetic population. However, males had higher means of WHR than females in both groups 8% in diabetics and 6% in the youth. These means were statistically significant in both diabetic adults and young healthy adults $p < 0.0001$ and 0.0003 respectively according to gender. No statistically significant gender differences were found in the means of systolic blood pressure and diastolic blood pressure in the diabetic population.

Blood uric acid as expected, showed some statistically significant differences between the genders, $p = 0064$ in diabetics, and $p < 0.0001$ in young adults. Males had higher levels of serum creatinine and urea levels in both groups of participants, and in the diabetic population the difference between the creatinine means was statistically significant ($p < 0.0001$). The urinary microalbumin index in diabetic patients was elevated by 24% in females, although this was not statistically significant.

Table 3.2. Baseline means !SD of anthropometric, blood pressure and uric acid indices in 236 adult diabetic patients and 115 young participants.

Adult diabetic patients		Young adults	
CHARACTERISTIC	**MEAN!SD**	**CHARACTERISTIC**	**MEAN!SD**
ANTHROPOMETRIC INDICES		**ANTHROPOMETRIC INDICES**	
BMI (A)	28!6	BMI (A)	22!3
BMI (≅)	F:29!6	BMI (≅)	F: 22!4
	M:26!5[a]		M: 21!2[e]
WHR (A)	0.89!0.09	WHR (A)	0.77!0.06
WHR (≅)	F:0.87!0.08	WHR (≅)	F: 0.74!0.06
	M:0.94!0.08[a]		M: 0.79!0.05[f]
BLOOD PRESSURE INDICES		**BLOOD PRESSURE INDICES**	
SBP (A)	144!21	SBP (A)	-
SBP (≅)	F:144!21	SBP (≅)	-
	M:144!24[b]		-
DBP (A)	90!13	DBP (A)	-
DBP (≅)	F:91!13	DBP (≅)	-
	M:89!13[b]		-
URIC ACID		**URIC ACID**	
Uric acid (A)	328!98	Uric acid (A)	323!59
Uric acid (≅)	F:314!86	Uric acid (≅)	F:288!55
	M:356!111[c]		M:343!51[f]
RENAL INDICES		**RENAL INDICES**	
Creatinine (A)	98!28	Creatinine (A)	83!19
Creatinine (≅)	F:92!24	Creatinine (≅)	F:79!17
	M:111!32[d]		M:85!20[e]
Urea (A)	4.0!1.3	Urea (A)	3.7!0.9
Urea (≅)	F:3.9!1.3	Urea (≅)	F:3.5!0.9
	M:4.1!1.4[b]		M:3.7!0.9[e]
A/C ratio (A)	7.6!12.1		
A/C ratio (≅)	F:8.2!12.8		
	M:6.2!10.3[b]		

A, combined; ≅, by gender. The units for BMI are, Kg/m^2, mmHg for blood pressure, ③mol/L for uric acid and creatinine, and mol/L for urea. The superscripts (a) (d) and (f) denote a p value < 0.0001, (b) and (e) a p value that is

not statistically significant, respectively, (c) by a value < 0.01. No comparisons were made between the two groups because of the adopted selection criteria

Table 3.3 below represents the lipid and metabolic derangements indices in both diabetic and healthy adults. When the means of serum lipids, total cholesterol and LDL-cholesterol, HDL-cholesterol and triglyceride in males and females were compared, no statistically significant differences were detected in diabetic subjects. Comparison of means of total cholesterol and LDL-C between genders in young non-diabetic participants yielded no statistically significant differences. However, there were statistically significant gender differences in the means of HDL-C ($p = 0.0004$) and triglycerides ($p = 0.0012$), females exhibiting higher HDL-C levels (18%) and males higher triglycerides levels (22%). Over fifty percent of diabetic patients were hypertensive (69%), overweight (64%), microalbuminuric (62%) and insulin resistant (57%). Dyslipidaemia and hyperinsulinaemia was found in more than 40% of the diabetic patients. The prevalence of obesity was 31%, that of hyperuricaemia was 30%, and 36% of the diabetic subjects were afflicted by the metabolic syndrome.

Comparisons of the means of glycaemic, uric acid, anthropometric and blood pressure indices with metabolic disorders in the diabetic population are shown in Tables 3.4 and 3.5 and some of their odds ratios together with confidence limits and p values are represented in Table 3.6. Obese and overweight subjects had statistically significant lower blood glucose levels than their non-obese and non-overweight counterparts, 10.3!4.9 vs 11.9! 5.7 mmol/L and 10.7!5.2 vs 11.3!5.5 mmol/L respectively. Also those subjects who were hyperuricaemic, had lower

blood glucose levels that were statistically significant, compared to those that had normal serum uric acid levels, 9.4!4.1 vs 12.2!5.9 mmol/L.

Table 3.3. Baseline means or frequencies of lipid and metabolic indices in 236 adult diabetic patients as well as 115 young participants.

Adult diabetic patients		**Young adults**	
CHARACTERISTIC	**MEAN!SD**	**CHARACTERISTIC**	**MEAN!SD**
LIPID INDICES		**LIPID INDICES**	
Total cholesterol (A)	4.5!1.1	Total cholesterol (A)	3.6!0.8
Total cholesterol (≅)	F:4.5!1.1	Total cholesterol (≅)	F:3.7!0.7
	M:4.5!1.0[a]		M:3.5!0.8[b]
LDL-C (A)	2.8!1.1	LDL-C (A)	1.9!0.8
LDL-C (≅)	F:2.8!1.0	LDL-C (≅)	F:1.9!0.9
	M:2.9!1.0[a]		M:2.0!0.7[b]
HDL-C (A)	1.3!0.43	HDL-C (A)	1.47!0.41
HDL-C (≅)	F:1.32!0.44	HDL-C (≅)	F:1.66!0.41
	M:1.25!0.43[a]		M:1.37!0.37[c]
Triglycerides (A)	1.9!1.1	Triglycerides (A)	0.75!0.30
Triglycerides (≅)	F:1.8!1.2	Triglycerides (≅)	F:0.63!0.22
	M:2.0!1.1[a]		M:0.81!0.32[d]
METABOLIC INDICES#		**METABOLIC INDICES#**	
Obesity*	31%	Obesity	1%
Overweight*	64%	Overweight	10%
Hypertension	69%	Hypertension	4%
Dyslipidaemia	47%	Dyslipidaemia	5%
Hyperinsulinaemia	44%	Hyperinsulinaemia	18%
Insulin resistance**	57%	Insulin resistance**	4%
Hyperuricaemia	30%	Hyperuricaemia	11%
Microalbuminuria***	62%		
Metabolic syndrome****	36%		

A, combined; ≅, by gender; * , according to BMI; **, defined by HOMA-IR; ***, defined by albumin:creatinine ratio; **** defined by a combination of 2 of the following, dyslipidaemia, hypertension and obesity in insulin resistant patients. The units for all biochemical parameters are in mmol/L and the superscripts *a* and *b* denote a p value that is not statistically significant, *c* a value < 0.001, *d* a value < 0.01 and *e* a value <0.0001. No comparisons were made between the two

groups because of the adopted selection criteria. # = frequencies of metabolic indices.

Obese and overweight diabetic subjects showed marked fasting hyperinsulinaemia compared to their counterparts with normal BMI, 19.2!26.0 vs 9.6!9.6 ③U/ml and 15.5!19.8 vs 7.1!6.9 ③U/ml respectively, and this was statistically significant. Similarly, statistically significant results were evident in those subjects who had high values of HOMA-IR. Hyperinsulinaemic individuals were about four times more likely to be obese or overweight and subjects with elevated HOMA-IR were four times more likely to be obese and three times more likely to be overweight.

There was a statistically significant association between hyperuricaemia and the following metabolic disorders, obesity, hypertension, dyslipidaemia and microalbuminuria. Hyperuricaemic patients were twice likely to be obese, hypertensive or dyslipidaemic, respectively, and almost three times likely to be microalbuminuric.

Compared with the non-obese and non-overweight individuals, the means of systolic and diastolic blood pressure readings were higher in those that were obese and overweight, 149!20 vs 142!20, 146!20 vs 139!23 and 93!13 vs 89!13, 92!12 vs 87!14 mmHg, respectively, and these were statistically significant (Table 3.5). Hypertensive subjects were almost 3 times more likely to be obese or overweight compared to their normotensive counterparts.

There was a weak association between hypertension and hyperuricaemia with hyperinsulinaemia. Hyperinsulinaemic patients were about 2 times more likely to be hypertensive (p = 0.0530, 95% confidence limits 0.9-3.2) or 2 times more likely to be hyperuricaemic (p = 0.083, 95% confidence limits 0.8-3.0).

Table 3.4. P values after comparisons of means of glycaemic indices with metabolic derangements in diabetic patients.

Metabolic disorder	Glucose	Insulin	HOMA-IR
Obesity*	0.0387	< 0.0001	0.0010
Overweight*	0.0137	< 0.0001	< 0.0001
Hypertension	0.6220	0.0857	0.2510
Dyslipidaemia	0.1394	0.4328	0.3554
Hyperinsulinaemia	-	-	-.
Insulin resistance	-	-	-
Hyperuricaemia	0.0001	0.0873	0.5866
Microalbuminuria	0.7809	0.7870	0.8074

Table 3.5. P values after comparisons of means of anthropometric as well as blood pressure indices with metabolic derangements in diabetic patients.

Metabolic disorder	BMI	SBP	DBP
Obesity*	-	0.0240	0.0213
Overweight*	-	0.0281	0.0060
Hypertension	0.<0.0001	-	-
Dyslipidaemia	0.6593	0.5642	0.5433
Hyperinsulinaemia	< 0.0000	0.1515	0.3305
Insulin resistance	0.0001	0.7389	0.5751
Hyperuricaemia	0.0053	0.0060	0.0007
Microalbuminuria	0.1401	0.2135	0.1087

NB. For Table 3.4 and 3.5, refer to section 2.12, page 68 for the definition of the metabolic derangements. * , according to BMI.

Table 3.6. Odds ratio (OR), 95% confidence limits (CL) and respective significance values of various metabolic derangements in diabetic patients according to the classification criteria of 2.12 (page 68).

Characteristic	OR	CL	p value
HYPERINSULINAEMIA			
Vs obesity*	3.7	1.8-7.3	< 0.0001
Vs overweight*	4.4	2.2-8.6	< 0.0001
INSULIN RESISTANCE			
Vs obesity	3.6	1.7-7.4	0.0001
Vs overweight	3.3	1.8-6.3	< 0.0001
HYPERURICAEMIA			
Vs obesity	2.2	1.1-4.1	0.0084
Vs hypertension	2.2	1.1-4.6	0.0114
Vs dyslipidaemia	1.8	1.0-3.4	0.0248
Vs microalbuminuria	2.9	1.2-7.5	0.0091
OBESITY			
Vs hypertension	2.6	1.3-5.5	0.0032
HYPERTENSION			
Vs overweight	2.6	1.4-4.8	0.0012

* , according to BMI. All other possible combinations were not statistically significant.

3.2. DISCUSSION

3.2.1 METABOLIC SYNDROME FACTORS

The data in this study clearly demonstrates a rather high prevalence of the metabolic syndrome, characterised by hypertension, microalbuminuria, insulin resistance, dyslipidaemia and obesity in the diabetic patients. This is worrying, as in the last few years it has been demonstrated that these metabolic disorders are aetiological factors for cardiovascular and cerebrovascular disease [48]. Cardiovascular and cerebrovascular disease are two of the most important causes of morbidity and mortality in the developed world [48]. The association of obesity, insulin resistance, hyperinsulinaemia and nephropathy may aggravate the atherogenic lipid profile in most of these subjects. It is therefore important to modify these risk factors where possible in this population, so as to reduce the risk of complications of macrovascular disease.

It is important to note that some indices, for example, glycaemic, blood pressure, anthropometric, as well as uric acid levels could have been influenced by drug therapy and/or weight management regimes, resulting in potential limitations to the interpretation of the data in this study. However, none of the patients were on drugs used in the treatment of gout or were on lipid-lowering therapy.

3.2.2 Hypertension

Hypertension was the most prevalent disorder as it was found in 69% of the patients and this makes them prime targets for the development of diabetic nephropathy. Despite the fact that a strong association between insulin resistance and hypertension in lean rather than obese subjects has been reported [198, 199], this relationship remains the most controversial part of the metabolic syndrome [200-202]. This study demonstrated a weak association between hyperinsulinaemia and hypertension, but as 64% of the study population was overweight, this could explain the weaker association between blood pressure and plasma insulin levels. It is also possible that the weak relationship could be derived from ethnic differences. Although the pathogenesis of the association between hyperinsulinaemia and hypertension is poorly understood, it is possible that high blood insulin levels may contribute to the development of hypertension by affecting the following, renal sodium reabsorption, the sympathetic nervous system, the transmembranous cation transport, the renin-angiotensin system, cardiovascular reactivity, and the atrial natriuretic peptide. It is also feasible to postulate that in some populations polygenic hypertension (a complex and multifactorial trait) per se is not associated with hyperinsulinaemia, but monogenic hypertension may be, for example glucocorticoid-remediable aldosteronism and Liddle's syndrome (rare monogenic forms of hypertension).

In this study we demonstrated a positive association between blood pressure and obese T2DM subjects. The evolution from obesity to T2DM may represent a continuum, that progresses through different phases, in which defects in both insulin action and insulin secretion play a critical interaction, and these must be

looked at in concert. Defective insulin action and/or secretion may result in insulin resistance, which in turn may precipitate hyperinsulinaemia. The resultant hyperinsilunaemia may then contribute to the development of hypertension. Thus hypertension can be viewed as one of the co-morbidities of obesity.

It has been reported previously that in hypertensive patients, there is a cluster of elevated serum uric acid with insulin resistance, dyslipidaemia and obesity [203-205]. The significant association between hypertension and hyperuricaemia in this study could be attributed to some renal involvement, which is probably driven by obesity, dyslipidaemia, and hyperinsulinaemia, resulting in renal impairment and accelerated microalbuminuria.

3.2.3 Hyperuricaemia and microalbuminuria

Literature reports demonstrate a cluster of elevated blood uric acid with insulin resistance, dyslipidaemia and obesity [206-208]. In this study, we found statistically significant associations of elevated serum uric acid with dyslipidaemia, hypertension, microalbuminuria and obesity, a weak relationship with hyperinsulinaemia and a non statistically significant relationship with insulin resistance. It is possible that the elevated levels of serum uric acid in T2DM is due to insulin resistance and hyperinsulinaemia, leading to decreased renal clearance of this metabolite [209, 210]

Obesity is an important factor as well as a consequence of abnormal renal function and although the mechanisms of the abnormal renal function are complex [211, 212] altered function of the renin-angiotensin aldosterone system, altered function of the sympathetic nervous system and hyperinsulinaemia may be

involved. The kidney plays a crucial role in the excretion of uric acid, and as uric acid is less soluble in acidic solutions such as kidney tubular fluids and in hyperuricaemia, tubulointerstitial nephropathy may ensue. Obesity may incite changes in uric acid metabolism in a hyperinsulinaemia-driven process by decreasing its renal clearance, thus giving rise to hyperuricaemia.

It has been reported that in hypertensive patients there is a cluster of elevated serum uric acid with insulin resistance, dyslipidaemia and obesity [213-215]. We found a statistically significant association between hyperuricaemia and hypertension and this could be due to some renal involvement as a result of hyperinsulinaemia. The hyperinsulinaemia may increase tubular reabsorption of sodium, which is known to increase renal reabsorption of uric acid [216].

In the current study the statistically significant association between hyperuricaemia and dyslipidaemia was due to elevated serum triglycerides levels. The mechanism for this association may be related to obesity, hypertension or hyperinsulinaemia. Elevated blood triglycerides may cause glomerular cell proliferation and matrix accumulation that may lead to loss of nephron function and eventually to decreased renal excretion of uric acid and therefore hyperuricaemia. This process could involve alterations in insulin action on the synthesis of apolipoproteins, action of insulin on adipocytes, action of CETP and modification of blood lipoproteins by hyperglycaemia to cause endothelial dysfunction. The association of hyperuricaemia with triglycerides has been reported in several studies [217-220].

Insulin has been shown to decrease the urinary excretion of sodium and potassium [221, 222], therefore impaired membrane transport of these ions may be the reason for the impaired urinary excretion of uric acid (elevated uric acid absorption) especially in hypertensive subjects. Since the relationship between HOMA-IR and serum levels of serum uric acid were not statistically significant, the data in this study suggests that it may be plasma insulin levels per se, rather than insulin resistance that influence hyperuricaemia in this T2DM population, and this is supported by a recent study [223].

Microalbuminuria is considered as an indicator of endothelial dysfunction and diabetic nephropathy manifests initially by microalbuminuria, then by clinical proteinuria leading to progressive chronic renal failure. This study found an association between microalbuminuria (urinary albumin/creatinine ratio) and hyperuricaemia. It is likely that the mechanisms that govern this association are multifaceted and the process could operate in the following sequence: **obesity** → insulin resistance → hyperinsulinaemia → dyslipidaemia and hypertension → renal impairment → microalbuminuria and hyperuricaemia. Thus in T2DM, microalbuminuria and hyperuricaemia can be seen in close association with obesity, insulin resistance, lipid changes and hypertension within the framework of the metabolic syndrome.

Hypertriglyceridaemia, because of its effects on CETP, leads to the formation of a small dense LDL, which are susceptible to oxidation and these may damage the endothelium. The damaged endothelium leads to nephropathy, hence elevated urinary albumin excretion (microalbuminuria) and hyperuricaemia. Some studies

have suggested that endothelial dysfunction may be a consequence of diabetic dyslipidaemia and that the damaged endothelium is a key accessory to microangiapathy [224].

3.2.4 Obesity

In this study, obesity was statistically significant associations with hypertension, hyperglycaemia, insulin resistance, hyperinsulinaemia and hyperuricaemia were obtained, suggesting that obesity is a heterogeneous condition and a very important component of T2DM. Hyperinsulinaemia had a consistent positive relation with all the weight indices, that is, overweight and obesity and BMI was a strong predictor of fasting hyperinsulinaemia. The hyperinsulinaemia in these patients could be explained by an increase in android fat. Being overweight or obese had significant adverse effects on blood pressure levels. Thus anthropometric measures can be used as indicators of the probability of hyperuricaemia, high blood pressure and hyperinsulinaemia in this T2DM population.

Obesity can lead to an insulin resistant state and hyperinsulinaemia in T2DM with the metabolic syndrome. Hyperinsulinaemia is known to stimulate the sympathetic nervous system, to promote sodium retention, to inhibit the sodium pump and to disturb membrane ion transport. The above processes have effects on a number of vascular and cardiac parameters leading to a subset of hypertension, and therefore offer a possible explanation for the association of hypertension and the anthropometric indices in this study. Thus in T2DM, a subset of hypertension can be seen in close association with obesity, insulin resistance, and hyperglycaemia within the framework of the metabolic syndrome.

Overweight and obesity were strong predictors of fasting hyperinsulinaemia and the hyperinsulinaemia in these patients could be explained by an increase in android fat. Obesity has the potential to cause defective insulin action and/or secretion, which may result in insulin resistance and hyperinsulinaemia. This appears to be the case in our T2DM subjects as insulin resistance and hyperinsulinaemia were the two parameters that had the strongest association with obesity and overweight. Being obese and overweight had significant adverse effects on blood pressure and blood glucose levels. The constellation of the metabolic disorders such as hypertension, prolonged hyperglycaemia, insulin resistance, hyperinsulinaemia and hyperuricaemia may expose the T2DM subjects to coronary artery disease as well as to the severe microvascular complications of diabetes.

Although no statistically significant difference was found between obesity and microalbuminuria, the majority of the T2DM subjects were obese and insulin resistant and this may lead to endothelium dysfunction, resulting in renal impairment [225].

3.2.5 Dyslipidaemia

It is generally accepted that alterations in insulin action and hyperglycaemia play a key role in the modification of blood lipoproteins, but obesity and insulin resistance in the absence of hyperglycaemia are also known to contribute to dyslipidaemia. Diabetic dyslipidaemia could be influenced by insulin action on the synthesis of hepatic apolipoproteins as well as insulin action on fat cells and

muscles, action of CETP and regulation of LpL as previously discussed. The Zimbabwean Black T2DM phenotype was characterised by a high prevalence of dyslipidaemia (47%) and forty two percent of the subjects had hypertriglyceridaemia.

The high fasting blood glucose levels in the diabetic group implies poor glycaemic control and this may exacerbate the lipid and lipoprotein abnormalities as total cholesterol and LDL-C levels are often elevated in poor glycaemic control. The lipid abnormalities in Zimbabwean T2DM subjects may be a secondary consequence of insulin resistance, which may arise from obesity-related causes and may expose the afflicted subjects to increased vascular risk through both quantitative and qualitative abnormalities that impair normal lipid metabolism. The major quantitative abnormalities include elevated blood triglyceride levels, increased VLDL and IDL and decreased HDL-C levels as a result of decreases in the HDL2 subfraction. The qualitative abnormalities include alteration in the lipoprotein size, that is, large VLDL and small LDL, elevated triglyceride content of LDL and HDL, glycation of apolipoproteins, and increased susceptibility of LDL to oxidation. The compositional changes in VLDL and the conversion of LDL particles lead to smaller dense LDLs, which are potentially atherogenic. Subjects with a combination of hypertriglyceridaemia and hypoHDL-C may be at a higher risk of atherogenesis and subsequent manifestation of cardiovascular disease [226].

Although the physiopathology of lipid disorders in diabetes mellitus is multifactorial and still imperfectly known, factors such as hyperglycaemia and insulin resistance are likely to play a pivotal role.

3.2.6 Insulin resistance and hyperinsulinaemia

As the data in the current study demonstrated an association of hyperinsulinaemia with obesity, dyslipidaemia and hypertension in T2DM subjects, there is a temptation to conclude that hyperinsulinaemia is a common physiopathological feature of these metabolic derangements. Since obesity correlates strongly with insulin resistance and hyperinsulinaemia, this association may be worsened in hypertensive and/or dyslipidaemic patients by an additive effect.

It is possible that the insulin resistance in adipocytes may precipitate dyslipidaemia by increased release of FFAs into plasma, resulting in secretion of VLDL, hypertriglyceridaemia and exchange of cholesteryl esters from HDL and LDL for VLDL-TG. ApoA-1 may then dissociate from TG-rich HDL and rapidly cleared from plasma by the kidney, thus reducing the availability of HDL for cholesterol transport. TG-rich LDL through lipolysis could be altered to small dense LDL, which is more atherogenic. Thus hypo HDL-C and small dense LDL could present as independent risk factors for CVD in the T2DM subjects.

There is still a paucity of epidemiological data to support a role for hyperinsulinaemia in the etiology of hypertension. From the available literature it appears that blood insulin expresses itself differently in diverse ethnic groups and this could indicate genetic as well as environmental variation [227, 228]. For instance, a substantial number of Pima Indians have hyperinsulinaemia, but

exhibit low rates of hypertension. Blacks show a weak relationship between hyperinsulinaemia and hypertension, whereas in some lean Caucasians hyperinsulinaemia is implicated in the development of hypertension [229, 198, 199]. Our study demonstrated a weak association between hyperinsulinaemia and hypertension in Black T2DM subjects. Since there was a high prevalence of overweight in the study population, this could explain the weak association. Alternatively, the weak relationship could be explained by ethnic variations. The contribution of hyperinsulinaemia in the development of hypertension remains a controversial area, and further studies on this relationship are needed to clarify the mechanisms involved. It is worth postulating that in T2DM-related hypertension, this is a multifaceted process, where insulin resistance, the resultant hyperinsulinaemia as well as dyslipidaemia are spokes of a wheel and obesity the hub of the wheel.

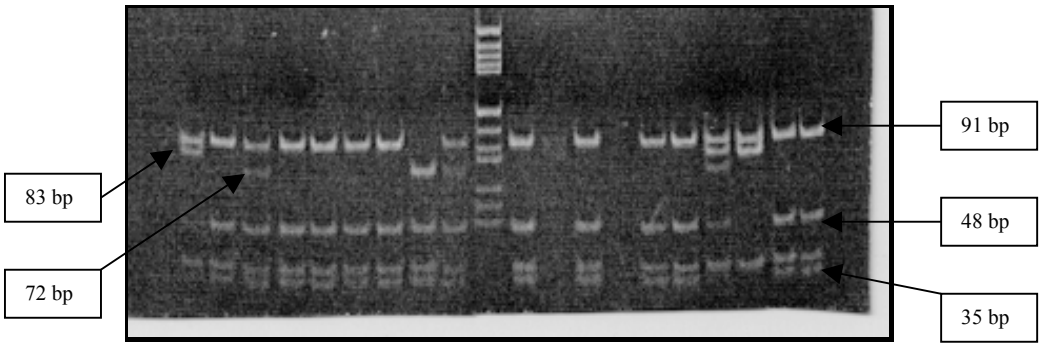
Data from the current study demonstrates that hyperinsulinaemia is strongly related to most of the components of the metabolic syndrome, thus the T2DM subjects are at a high risk of developing cardiovascular disorders. As fasting hyperinsulinaemia, not insulin resistance was strongly associated with a clustering of metabolic derangements in this study, it appears that hyperinsulinaemia is the one that is strongly related to the plurimetabolic syndrome than insulin resistance per se in Zimbabwean T2DM subjects. This is supported by a recent study on insulin sensitivity and clustering of cardiovascular disease risk factors [71]. Since obesity has similar effects as hyperinsulinaemia, it is tempting to speculate that obesity may be the main driving force of hyperinsulinaemia in a subset of these diabetic subjects.

CHAPTER 4. RESULTS AND DISCUSSION - APOLIPOPROTEIN E GENE POLYMORPHISM

4.1 Genotype and allelic frequencies

All the six ApoE genotypes, E2/2, E2/3, E2/4, E3/3, E3/4 and E4/4 of the amino acid substitutions of cysteine and arginine at codons 112 and 158 were detected after PCR amplification, *Hha* I endonuclease digestion of the PCR product and electrophoretic resolution on 8% polyacrylamide gels (Figure 4.1). The fragments characterizing the genotypes were 91 and 83 bp for 2/2, 91, 48 and 35 bp for 3/3, 72, 48 and 35 bp for 4/4, 91, 83, 48 and 35 bp for 3/2, 91, 72, 48 and 35 bp for 3/4 and 91, 83, 72, 48 and 35 bp for 2/4, respectively. The 3/3 genotype was the most prevalent, 36% in the diabetic population followed by the 3/4 genotype, 29% (Table 4.1). The frequency of the 4/4 genotype was 8% and the 2/2 genotype was the least prevalent, 1%. The corresponding allele frequency of ε2, ε3 and ε4 was 11%, 60% and 29%, respectively. The same distribution of the genotype and allele frequency was evident in the young non-diabetic participants and the allele frequency in the two groups followed the Hardy-Weinberg equilibrium.

Figure 4.1. The amino acid substitutions of cysteine and arginine, at codons 112 and 158 of the ApoE gene, after PCR amplification, *Hha* I endonuclease digestion and electrophoretic resolution on an 8% polyacrylamide gel.



1 20

The fragments characterizing the genotypes were 91 and 83 bp for 2/2, 91, 48 and 35 bp for 3/3, 72, 48 and 35 bp for 4/4, 91, 83, 48 and 35 bp for 3/2, 91, 72, 48 and 35 bp for 3/4 and 91, 83, 72, 48 and 35 bp for 2/4 respectively. For example, lane 10 is the MW marker, lane 18 represents the 2/2 genotype, lane 17 represent the 2/4 genotype, lane 1 represents the 3/2 genotype, lane 2 represents the 3/3 genotype, lane 3 represents the 3/4 genotype, lane 8 represents the 4/4 genotype, lanes 12 and 14 represent non-detectable DNA products or low yields of the desired DNA products.

Table 4.1. Frequencies of ApoE genotypes and alleles in 236 adult diabetic patients and 115 young healthy participants.

Genotype	Genotype frequency		Allele frequency (%)					
	PATIENTS	**YOUTH**	**PATIENTS**			**YOUTH**		
APOE GENE			**ε2**	**ε3**	**ε4**	**ε2**	**ε3**	**ε4**
2/2	1.3%	1.8%						
2/3	16.5%	21.4%	11	60	29	13	61	27
2/4	8.9%	4.5%						
3/3	36.4%	37.5%						
3/4	28.8%	27.7%						
4/4	8.1%	7.1%						

4.2 Biochemical assays, anthropometric and blood pressure features according to apolipoprotein E genotypes

Baseline glycaemic, anthropometric, blood pressure, lipid, uric acid and renal indices according to the ApoE genotypes in diabetic patients are shown in Table 4.2. Although comparisons of the means of the fasting plasma glucose levels among the ApoE genotypes were not statistically significant, the E2/2 genotype exhibited the lowest value, 6.3 mmol/L compared to 12.3 mmol/L in the E2/3 genotype which had the highest mean. Similarly, the E3/4 genotype had the least fasting plasma insulin mean (9.6 ③U/ml compared to the E2/4 genotype (21.5 ③U/ml), although the differences in the means of all genotypes were not statistically significant. The E2/4 genotype displayed the worst insulin resistant state, with a mean HOMA-IR of 9.8 compared to the E2/2, the least resistant genotype, with a mean HOMA-IR of 3.4, but this did not reach statistical significance.

Comparison of the means of BMI, showed that the E2/2 diabetic genotype had the highest value (31 Kg/m^2) and the E2/4 together with the E4/4 genotype had the lowest value of 27 Kg/m^2). Although not statistically significant, the E2/2 genotype was approximately 12% more obese than the E2/4 and E4/4 genotypes. The E2/2 genotype showed the lowest systolic and diastolic blood pressure means, 130 and 83 mmHg, and the E3/3 genotype the highest means, 146 and 92 mmHg. However, the differences in the means of all blood pressure indices among the 6 genotypes were not statistically significant.

Table 4.2. Baseline means !SD of biochemical, anthropometric, and blood pressure indices in 236 adult diabetic patients according to ApoE genotypes.

CHARACTERISTIC	2/2	2/3	2/4	3/3	3/4	4/4
GLYCAEMIC INDICES						
Glucose	6.4!4.2	12.2!4.9	11.2!6.6	11.90!5.6	10.7!5.4	11.4!5.7[a]
Insulin	11.2!2.4	11.6!12.3	21.5!41.8	11.6!11.2	9.6!8.1	15.9!20.4[a]
HOMA-IR	3.4.!2.6	5.3!4.6	9.8!22.0	5.7!6.3	4.0!3.1	5.8!7.6[a]
ANTHROPO* INDICES						
BMI	31!6	27!6	28!6	28!6	28!6	27!5[a]
WHR	0.82!0.04	0.90!0.08	0.90!0.08	0.89!0.09	0.90!0.09	0.90!0.10[a]
BP INDICES						
SBP	130!10	142!20	136!15	147!23	145!22	140!20[a]
DBP	83!12	88!12	89!10	92!14	91!14	85!14[a]
LIPID INDICES						
Total-cholesterol	3.3.!1.1	4.2!1.0	4.4!0.7	4.6!1.1	4.7!1.1	4.5!0.9[b]
LDL-cholesterol	1.4.!1.2	2.5!0.9	2.6!0.8	2.9!1.2	3.0!1.0	2.9!0.8[b]
HDL-cholesterol	1.6.!0.2	1.3!0.4	1.3!0.4	1.4!0.5	1.2!0.4	1.2!0.5[a]
Triglycerides	1.33.!0.45	1.90!1.23	2.01!1.30	1.71!0.91	2.00!1.35	1.75!0.82[a]
URIC ACID						
Uric acid	308!53	346!84	294!52	330!104	334!108	300!64[a]
RENAL INDICES						
Creatinine						
Urea	102!5	107!36	94!19	95!28	99!26	97!23[a]
A/C ratio	3.2.!0.3	4.2!1.3	3.5!0.9	4.0!1.4	3.9!1.3	4.0!1.4[a]
	4.7.!4.5	12.3!22.0	5.1!11.2	7.0!8.5	7.1!9.0	5.9!5.9[a]

*, anthropo = anthropometric. The units for glucose, cholesterol, triglycerides, and urea are mmol/L, Kg/m^2 for BMI, mmHg for blood pressure, ③mol/L for creatinine and uric acid. The superscript (a) denotes a p value that is not statistically significant and (b) a value < 0.05.

Statistically significant differences in the means of fasting serum total cholesterol and LDL-C were detected among the 6 ApoE genotypes, p = 0.0465 and 0.0188 respectively. ApoE2/2 genotype had the lowest cholesterol mean, 3.1 mmol/L and the E3/4 the highest mean, 4.7 mmol/L and the difference between these means was almost 30%. The differences in the means of cholesterol among the genotypes gave a p value of 0.0049 between E2/2 and E3/3 and a p value of 0.0097 between E2/2 and E4/4. There was a 53% difference in the LDL-C means of the E2/2 genotype and the E3/4 genotype. The corresponding mean differences yielded a p value of 0.0144 between the E2/2 and E3/3 genotypes and a p value of 0.0024 between the E2/2 and E4/4 genotypes.

Although not statistically significant, there was a 26% difference in the HDL-C means of the E2/2 genotype (1.9 mmol/L) compared to the E3/3 and E4/4 genotypes (1.4 mmol/L) in diabetic patients. The E2/2 genotype exhibited the lowest triglycerides mean (0.70 mmol/L), whereas the E4/4 genotype had the highest mean (0.84mmol/L), but this did not attain statistical significance. There were no statistically significant differences in the uric acid means among the 6 genotypes, but the e3 allele was associated with higher levels of this analyte. A similar picture was observed in the 3 renal indices, creatinine, urea and albumin/creatinine ratio.

Baseline glycaemic, anthropometric, lipid, uric acid and renal indices according to the ApoE genotypes in young adults are shown in Table 4.3. Although not statistically significant, the E2/2 genotype displayed the highest mean in plasma glucose, but the lowest means in the anthropometric indices (BMI and WHR) and serum uric acid. In addition the E2/2 genotype had the lowest serum urea mean compared to other genotypes and this was statistically significant.

Lipid indices in the young non-diabetics mirrored the pattern that was exhibited by the diabetic patients. The differences in the means of fasting serum cholesterol among the 6 genotypes were statistically significant, $p = 0.0003$ and the differences in the means of LDL-C were statistically significant, with a p value < 0.0001. Corresponding mean differences between genotypes E2//2 and E3/3 gave a p value of 0.0007, whereas E2/2 and E4/4 gave a p value of 0.0002 for serum cholesterol. For LDL-C, the differences of the means of the E2/2 genotype versus either the E3/3 or the E4/4 genotype were statistically significant and yielded p values < 0.0001.

Table 4.3. Baseline means !SD of biochemical, anthropometric, blood pressure and lipid indices in 115 young adults according to ApoE genotypes.

CHARACTERISTIC	2/2	2/3	2/4	3/3	3/4	4/4
GLYCAEMIC INDICES						
Glucose	4.9!0.2	4.4!0.6	4.1!0.6	4.2!0.6	4.5!0.7	4.6!0.5[a]
Insulin	7.4!1.6	8.8!10.0	6.0!3.3	5.8!3.3	8.0!3.5	10.1!11.0[a]
HOMA	1.6.!0.3	1.7!1.9	1.4!0.7	1.1!0.6	1.6!0.9	1.3!0.5[a]
ANTHROPO* INDICES						
BMI	21!3	22!3	20!3	21!2	21!3	23!6[a]
WHR	0.76!00.13	0.76!0.06	0.78!0.04	0.78!0.05	0.78!0.08	0.78!0.02[a]
LIPID INDICES						
Total cholesterol	2.7.!0.1	3.1!0.7	3.0!0.8	3.7!0.7	3.9!0.7	4.1!1.0[b]
LDL-cholesterol	0.62.!0.25	1.38!0.47	1.30!0.56	2.07!0.66	2.24!0.66	2.54!0.94[c]
HDL-cholesterol	1.9.!0.2	1.5!0.4	1.6!0.9	1.4!0.4	1.5!0.4	1.3!0.4[a]
Triglycerides	0.80.!0.00	0.75!0.31	0.72!0.19	0.72!0.34	0.73!0.27	0.85!0.29[a]
URIC ACID						
Uric acid	312!59	316!63	341!63	331!55	315!60	347!35[a]
RENAL INDICES						
Creatinine	92!29	84!21	89!37	81!12	83!22	79!16[a]
Urea	3.2.!0.5	3.4!0.9	3.6!0.8	4.1!1.0	3.5!0.7	3.6!0.9[d]

*, anthropo = anthropometric. The units for glucose, cholesterol, triglycerides creatinine and urea are mmol/L, Kg/m^2 for BMI, and ③mol/L for uric acid. The superscript (a) denotes a p value that is not statistically significant, (b) a value < 0.001, (c) a value <0.0001 and (d) a value <0.05.

4.3 DISCUSSION

4.3.1 General Introduction

Diseases with a marked inherited component are likely to show the strongest associations with DNA variants. That T2DM has a major genetic component is unquestionable, but neither the exact mode of transmission, nor the chromosomal location of any genetic defect is known. Efforts to identify those genes that contribute to the aetiology of T2DM will always be handicapped by the complex nature of this disorder as the T2DM phenotype is characterised by multiple defects as well as other secondary contributing factors. Therefore, a new approach of investigating the pathogenesis of diabetes mellitus, a heterogenous disorder, should make a calculated guess, that particular genes of metabolic components, such as insulin resistance, dyslipidaemia, obesity, hypertension and microalbuminuria, play an important role in influencing susceptibility to the disease.

4.3.2 Apolipoprotein E gene polymorphism

Data from the current study shows substantial differences in relative frequencies of the ApoE alleles in Zimbabwean blacks when compared to numerous reports from Caucasian studies. The data also demonstrate that serum lipids and

lipoproteins are influenced by ApoE polymorphism and the effects are already evident in young healthy adults.

The frequency of the ε4 allele in Zimbabweans is much higher than in Caucasians and is comparable to data from the few studies carried out in Afro-Americans and Black Africans (Table 4.4). Studies on ApoE show heterogeneity of ApoE phenotype frequency in diverse ethnic populations, North European populations tend to have significantly elevated frequencies of the ε4 allele [117], whereas some Asian populations have lower ε2 and ε3 alleles [118]. The Yanomami Indians of northwestern Brazil exhibit only two alleles, ε3 and ε4 (the most common alleles in Caucasians), and lack the ε2 allele [119]. Zimbabweans have a relatively low frequency of the ε3 allele and this has a net effect of increasing the ε2 and ε4 allele frequencies. The frequencies of ε3 (60%) and ε4 (29%) found in Zimbabweans are comparable to those of Nigerian Blacks, 67% and 30% respectively, but Zimbabwean Blacks have a higher frequency of the ε2 allele (11%) compared to their Nigerian counterparts (3%) [230]. Generally the data from the current study is in agreement with information obtained from recent studies investigating ApoE polymorphism in Black populations in which ε4 allele frequency is very high. The reported frequencies for Pygmies are ε2 allele 5.7 %, ε3 allele 56.3 % and ε4 allele 40.7%, for the South African Zulu are ε2 allele 19%, ε3 allele 56% and ε4 allele 25% [231-233], and for the Black Americans the ε4 allele frequency is 20% [234].

Table 4.4. ApoE allelic frequencies (%) in various ethnic groups.

ETHNIC GROUP	FREQUENCY OF ALLELES		
	ε2	ε3	ε4
Zimbwabwean blacks (current study)	11	60	29
Nigerian blacks [230]	3	67	30
Pygmies [123, 231]	6	56	41
South African Zulu [123, 232, 233]	19	56	25
Sudanese [235]	8	62	29
Italians (Sardinians) [236]	5	90	5
Swedes [235]	8	72	20
Germans [237]	12	76	12
Afro-Americans [234, 238]	12	68	20
United States whites [234, 238]	7	80	13
Chinese [239]	8	85	6
Japanese [235]	8	85	7
Indians [240]	6	86	8
American Indians[241]	2	85	13

It is worth noting that Black Americans are more prone to diabetes, hypertension and cardiovascular disease than their white counterparts and have a high frequency of the ε4 allele. The sustenance of a high frequency of the ε4 allele in Black Africans could have arisen due to sporadic and scarce food availability, thus making this a thrifty allele in that environment. Since the "western lifestyle" characterised by more sedentary occupations, high energy fuel resources, as well as overabundant food sources is spreading, the high prevalence of this allele is likely to be a heavy genetic burden as it has been shown to enhance susceptibility to coronary artery disease and Alzheimer disease in several populations [242-244]. The current study yields one of the highest ε2 allele frequencies reported in a Black population to date or in any Caucasian population. It appears that the ε3 allele is very high in populations with long established agricultural economy but the ε4 allele may be considered as the ancestral allele and is still high in some ethnic groups such as Nigerians, Pygmies, the Zulus and Khoi San of Africa, aborigines of Australia and Malaysia, Native Americans, Lapps and Papuans.

Apo E4 allele is known to confer a high capacity of intestinal cholesterol absorption as well as reduced elimination of LDL [245] and for this reason one can speculate that the sustenance of a high ε4 allele frequency in a population like this (Zimbabweans) was due to a selection for a thrifty allele as a result of

sporadic and scarce food availability. Since the polymorphism of ApoE is a major source of genetic differences in lipid metabolism, this study compared plasma lipids in the different ApoE classes. Significant differences among ApoE phenotypes were noted for total cholesterol and LDL-C in Zimbabwean diabetic subjects as well as healthy subjects. The ε2 allele was associated with lower total and LDL-C, whereas the ε4 allele was associated with elevated concentrations of both. A trend of gradual increase in LDL-C levels from ε2 to ε4 was observed in both diabetic and healthy Zimbabwean Blacks. In diabetic subjects there was an increase in LDL-C from the ε2 allele to the ε3, with a plateau between the ε3 and the ε4. The full genetic effects of the different ApoE alleles, characterised by a progressive increase in LDL-C from the ε2 allele to the ε3 right up to the ε4 is evident in young, healthy Zimbabweans, who are not exposed to a high glucose environment. The information from the current study is in agreement with most reports on the quantitative effects of the ε2 allele and the ε4 allele on lipid and lipoprotein levels [246, 247].

However, data from the current study contrasts that of Kamboh et al, as in the Mayans of the Yucatan Peninsula in Mexico, who lack the ε2 allele and have a relatively low incidence of the ε4 allele, the ε4 allele is associated with approximately 4% decrease in mean serum cholesterol [120]. Similarly, the well-established association between ε4 allele and LDL-C is absent in the Evenki herders of Siberia [121]. The Japanese are yet another population that does not exhibit the association between ApoE polymorphism and cholesterol as their diet is low in energy, fat, and cholesterol and has a high ratio of polyunsaturated fat to saturated fat. This diet has the potential to reduce elevations in blood LDL

concentration, thus masking the effects of ApoE polymorphism [248]. Lipid responses to varied diets are highly variable in individuals, and part of this variation may be accounted for by differences in lipid-regulating genes among individuals. When these regulatory genes interact with diet they induce some changes in lipoprotein metabolism, and a recent report on Apo E4 and lipid responses to a low-fat, low-cholesterol diet in obese, postmenopausal women confirms this [249].

There was a trend associated with higher HDL-C and lower triglycerides in those few Zimbabweans with the E2/2 genotype. This data mirrors that obtained from indigenous Australian subjects, where the ε4 allele had higher triglyceride concentrations and had statistically significant HDL-cholesterol concentrations that were lower than those in the apoE3/3 and 3/2 genotypes [250]. This trend has been variably reported in the literature, maybe due to the small numbers of E2/2 analysed and characterised [137, 241 251]. The intriguing relationship between ApoE polymorphism and HDL-C concentrations has not been fully elucidated, but could be attributed to several factors, such as modulation of lipoprotein lipase activity [252], regulation of cholesterol ester transfer protein activity [253] and extra cellular efflux of cholesterol [254].

The current study demonstrates that the polymorphism of ApoE is an important source of phenotypic differences in lipid metabolism. The 3 different alleles code for proteins that differ in lipoprotein receptor binding activity, but it's also possible that the effects may be due to allelic-rate differences of lipoprotein catabolism and that dietary (environmental) factors modulate these processes.

Since the ε2 allele has the least receptor-binding affinity, it exhibits low serum cholesterol levels, and the ε4 allele has the greatest receptor-binding affinity, and is prone to high levels of LDL-C, it is possible to speculate that the ε4 allele might represent a thrifty allele [123].

The present data reveals yet another fundamental finding, the association of ApoE polymorphism with serum lipoproteins is already manifest in early adulthood in absence of diabetes, where the ε4 allele, is associated with higher concentrations of serum total cholesterol, LDL-C and total and lower serum HDL-C, a well known highly atherogenic condition. It is important to determine the prevalence of the different ApoE alleles and to explore their significance in coronary artery disease in developing African Black populations. It appears that the role of the ApoE polymorphism in determining serum total cholesterol seems to be homogenous in most ethnic populations, but it is worth pursuing investigations on gene-diet interactions in the different ApoE genotypes and their influence on serum HDL-C and triglycerides concentrations. It is now unquestionable that the cholesterol-increasing effect of the ε4 allele is genetically determined, but data from this study and others suggest that the process is also environmentally mediated. This may create problems for Zimbabweans who are in the process of "modernisation". Zimbabwean Blacks with their high frequency of the ε4 allele, potentially carry a heavy genetic burden for coronary heart disorders and Alzheimer disease if exposed to a Western lifestyle.

The current study did not find any statistically significant differences between ApoE genotypes and insulin resistance (HOMA-IR) in T2DM subjects with

hypertension, obesity, dyslipidaemia and microalbuminuria. This finding is similar to a recent American study investigating the relationship between insulin resistance and ApoE polymorphism among participants with impaired glucose tolerance, hypertension, obesity, and dyslipidaemia [255]. These investigators found no significant differences in proportions of ApoE isoforms or alleles across increasing quantiles of HOMA-IR, although there were some increases in proportions with elevated triglycerides associated with increasing HOMA-IR among E2/2 and E2/3 genotypes compared with the other genotypes. Their results suggest that there is no association between Apo E polymorphisms and features of the insulin resistance syndrome.

A study in Japan found that the ε2 allele frequency was higher in diabetic patients with nephropathy and with renal failure than in diabetic patients without nephropathy and in the general Japanese population, and this was statistically significant [256]. This was confirmed by Kimura et al who reported that in T2DM subjects, ApoE polymorphism is associated with the progression of diabetic nephropathy and that the presence of the ε4 allele is a protective factor, and other alleles are risk factors [134]. Although data from the current study does not show statistically significant, but only marginal effects between the ApoE alleles and microalbuminuria (albumin/creatinine ratios) in the diabetic patients, the rather high frequency of the ε2 allele in Zimbabweans may contribute as a risk factor in the development of renal insufficiency, and nephropathy [257]. It is also worth noting that the E2/2 genotype had a mean BMI level of > 30 Kg/m^2 and this could generate obesity-related hyperinsulinaemia, leading to renal impairment.

CHAPTER 5. RESULTS AND DISCUSSION - ANGIOTENSIN CONVERTING ENZYME GENE POLYMORPHISM

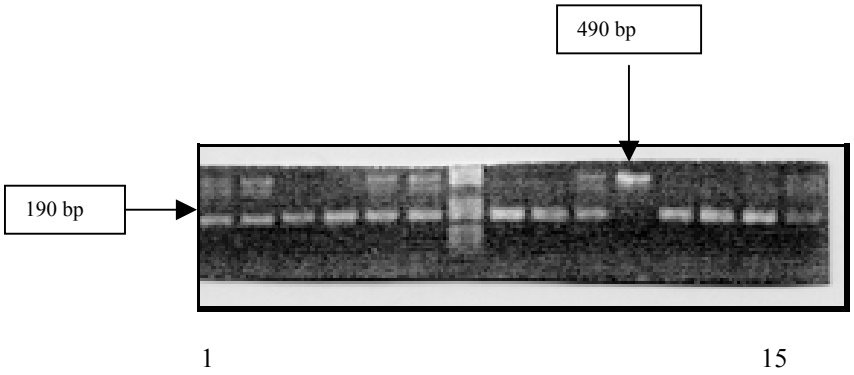
5.1 Genotype and allelic frequencies

The PCR product of the ACE gene subjected to agarose electrophoresis revealed a 287-bp insertion/deletion (I/D) polymorphism. The fragments characterizing the genotypes were 190 bp for DD, 490 and 190 bp for ID and 490 bp for II (Figure 5.1). The genotype frequency in the diabetic population was 42% for DD. 45% for ID and 13% II, and in the non-diabetic youth, it was 48% for DD, 37% for ID and 15% for II (Table 5.1). The frequency was 64% for the deletion allele, 36% for the insertion allele in diabetics, and 63% for the deletion allele, 37% for the insertion allele in non-diabetic participants. All observed genotype frequencies were in Hardy-Weinberg equilibrium.

Table 5.1. Frequencies of ACE genotypes and alleles in 236 adult diabetic patients and 115 young healthy participants.

Genotype	Genotype frequency		Allele frequency (%)			
	PATIENTS	**YOUTH**	**PATIENTS**		**YOUTH**	
ACE GENE			**D**	**I**	**D**	**I**
DD	41.5%	40.0%	64	36	63	37
ID	45.3%	46.1%				
II	13.1%	13.9%				

Figure 5.1. Electrophoretic detection of ACE 287-bp insertion/deletion (I/D) polymorphism on a 2.5% agarose gel after PCR amplification.



The fragments characterising the genotypes are 190 bp for DD (deletion product), 490 and 190 bp for ID and 490 bp for II (insertion product). For example, lane 7 is the MW marker, lanes 1 and 2 represent the ID genotype, lane 11 represents the II genotype, lanes 3 and 4 represent the DD genotype.

5.2 Biochemical assays, anthropometric and blood pressure features according to ACE genotypes

Although the means of the baseline, glycaemic, anthropometric, blood pressure, lipid, uric acid and renal indices did not show any statistically significant differences among the ACE genotypes, several patterns were derived (Table 5.2). The II genotype exhibited the highest insulin, systolic blood pressure, diastolic blood pressure, as well as the highest serum uric acid levels in the diabetic population. Generally, the diabetic ID genotype had the lowest levels of the glycaemic, blood pressure and lipid indices, but the highest levels in serum creatinine and uric acid levels. The diabetic DD genotype displayed the highest HOMA-IR score among the 3 genotypes. The ACE 287-bp insertion/deletion (I/D) polymorphism did not have any apparent impact on the anthropometric indices.

In the non-diabetic youth, the lowest means in glycaemic, lipid (except for serum triglcerides), and renal indices were evident in the II genotype. Generally, the glycaemic, lipid and renal indices exhibited intermediate levels in the non-diabetic ID genotype, compared to the DD and ID genotypes. The ID genotype had the lowest triglyceride level (0.61 mmol/L) compared to the II genotype, which had the highest level (0.80 mmol/L), a 24% difference. On the whole, the DD genotype had the highest levels of analytes associated with glycaemia, anthropometry, lipids and the kidney.

Table 5.2. Baseline means !SD of biochemical, anthropometric, and blood pressure indices in 236 adult diabetic patients and 115 young adults according to ACE genotypes.

CHARACTERISTIC	ACE GENOTYPES					
	DIABETIC PATIENTS			YOUNG ADULTS		
	DD	ID	II	DD	ID	II
GLYCAEMIC INDICES						
Glucose	12.0!6.24	11.2!5.1	10.1!4.2	4.4!0.6	4.4!0.7	4.3!0.5
Insulin	12.1!12.3	11.4!19.5	15.8!17.3	7.5!3.5	6.4!8.6	5.4!2.4
HOMA	6.0.!6.7	5.2!10.1	4.5!5.0	1.4!0.6	1.4!1.5	1.0!0.5
ANTHROPO* INDICES						
BMI	28!6	28!6	29!5	22!3	22!2	22!3
WHR	0.90!0.09	0.90!0.09	0.87!0.08	0.78!0.06	077!0.06	0.77!0.06
BP INDICES						
SBP	144!19	142!24	150!19	-	-	-
DBP	90!13	89!14	94!10	-	-	-
LIPID INDICES						
Total cholesterol	4.5.!1.0	4.5!1.0	4.6!1.3	3.7!0.7	3.5!0.8	3.3!0.9
LDL-cholesterol	2.9.!1.0	2.8!1.0	2.9!1.3	2.0!0.7	1.9!0.8	1.9!0.8
HDL-cholesterol	1.3.!0.4	1.3!0.5	1.3!0.5	1.5!0.4	1.5!0.4	1.3!0.3
Triglycerides	1.92.!0.95	1.77!1.27	1.91!1.18	0.81!0.35	0.67!0.24	0.80!0.29
URIC ACID						
Uric acid	316!84	332!105	348!97	332!67	315!48	323!56
RENAL INDICES						
Creatinine	99!32	101!29	97!28	84!20	82!16	80!25
Urea	4.0.!1.4	4.2!1.4	4.0!1.4	3.7!1.0	3.7!1.0	3.5!0.7
A/C ratio	7.6.!13.3	7.5!12.4	7.4!5.6	-	-	-

*, anthropo = anthropometric. The units for glucose, cholesterol, triglycerides creatinine and urea are mmol/L, Kg/m^2 for BMI, mmHg for blood pressure, and ③mol/L for uric acid. No statistically significant values were obtained in independent analysis of diabetic and young adults respectively.

5.3 DISCUSSION

Hypertension is known to contribute to the progression to renal failure and it is possible that a genetic susceptibility to hypertension may predispose to the development of ESRD and promote a faster progression to ESRD in individuals with renal diseases. Therefore it is necessary to seek candidate genes for abnormal blood pressure regulation. Evaluating the role of polymorphisms in the genes encoding for components of the renin-angiotensin system (RAS) in the development and/or progression of renal diseases is a viable starting point.

The genotype frequencies in Black Zimbabweans were similar to British Blacks of African descent and to Caucasian subjects but considerably different from Asians (Table 5.3). Black and Caucasians exhibit a much higher frequency of the DD genotype and the lowest frequency of the II genotype. The opposite is true for the Asian communities, who have the highest I allele frequency and the lowest D allele frequency in the world. The information derived from Table 5.3 shows that the distribution of the ACE gene insertion/deletion polymorphism widely differs in various populations and depends on ethnic background.

Table 5.3. ACE genotype frequencies (%) in various ethnic groups.

ETHNIC GROUP	GENOTYPE FREQUENCY		
	DD	**ID**	**II**
Zimbabweans blacks (current study)	42	45	13
Afro-British (blacks) [258]	31	51	18
British (whites) [258]	32	50	18
Afro-Americans [259]	33	46	21
Italians [260]	43	44	14
Germans [261]	42	37	21
Swedes [262]	30	49	21
South Koreans [263]	17	41	49
Thais [264]	10	39	51
Chinese [265]	8	38	54
Japanese general population [266]	14	43	43
British of South Asian origin [258]	18	42	40
Pima Indians [267]	7	39	55

The frequent association of nephropathy with hypertension must lead to research on genes related to hypertension and ACE is a viable candidate gene. Although the ACE gene I/D polymorphism in the current study detected statistically insignificant differences in systolic blood pressure and diastolic blood pressure, the II subjects had the highest values than the DD subjects. This is difficult to explain as the deletion allele is associated with higher plasma ACE levels, a situation that would activate the intra-renal renin-angiotensin system. The II genotype is assumed to produce low plasma ACE concentration, to reduce renal angiotensin II production and to inactivate kinin, thus protecting against diabetic nephropathy. Elevated fasting plasma insulin as well as an elevated BMI in the diabetic subjects carrying the II genotype could explain this discrepancy. Interestingly, the I allele has been shown to be associated with hypertension, insulin resistance and metabolic syndrome in a few studies [268-271]. Another plausible explanation for the association of the I allele with hypertension, insulin resistance and metabolic syndrome is that there may be a synergistic effect arising from gene-gene interactions.

Data from the current study contrasts that from a Japanese study that demonstrated statistically significant association between systolic blood pressure as well as diastolic blood pressure, with the highest values exhibited by the DD subjects [153]. Also Bengtsson et al found out that the D-allele of the ACE gene ID polymorphism increases susceptibility to hypertension in Swedes, and that the strongest association was a combination of T2DM and hypertension [272]. In a study to investigate an association between ACE-D/D polymorphism and hypertension in T2DM subjects, Pujia et al concluded that the ACE-I/D

polymorphism seems to play a role in the development of hypertension, at least in diabetic subjects [145].

However, data of the current study is in agreement with a Turkish study that reported no difference in genotypic and allelic frequencies of the ACE I/D polymorphism between the hypertensive and normotensive diabetic patients and no statistically significant differences in systolic and diastolic blood pressure among the three different genotypes [273]. In Chinese populations where hypertension is a common occurrence, no statistically significant relationship was identified between ACE polymorphism and blood pressure and the authors concluded that this polymorphism was unlikely to be involved in the pathogenesis of hypertension [270]. In a British population-based study of three ethnic groups, there were no statistically significant associations between the I/D polymorphism and hypertension in whites and in those of South Asian origin, but a highly significant association between the D allele and hypertension in women of African descent [258].

Thus reports about the ACE insertion/deletion polymorphism and its role in the pathogenesis of hypertension are conflicting. Taking into consideration findings from other researchers, this polymorphism has a potential role to play in the development of hypertension, at least in diabetic subjects as well as in some selected ethnic groups. It is possible that there are gender-dependent interactions between genetic background and expression of the hypertensive phenotype. Data from the current study is not totally incompatible with the proposition that this polymorphism has some influence on blood-pressure variability, and in future

studies, blood ACE levels should be determined and compared to the 3 ACE genotypes in diabetic subjects as well as in non-diabetic subjects with or without hypertension.

Apart from metabolic control and duration of diabetes, genetic predisposition plays an important role in genesis of diabetic nephropathy. Good evidence that genetic predisposition plays a major role in development of diabetic nephropathy is based on the observation that diabetic nephropathy clusters within families as well as in T2DM. Nephropathy is a major cause of premature morbidity and mortality in patients with T2DM and approximately 30% of patients with T2DM develop diabetic nephropathy. No genes responsible for predisposition to diabetic nephropathy have been identified, but those of the RAS are plausible candidates and it has been found that the I/D polymorphism of ACE is a genetic determinant of plasma ACE levels. The involvement of deletion polymorphism of the ACE gene in the pathogenesis of nephropathy has been described predominantly in Caucasian populations.

Data presented in this study failed to demonstrate any statistically significant association between the ACE insertion/deletion polymorphism and microalbuminuria as defined by the albumin/creatinine ratio in the 3 ACE genotypes, DD, ID and II. As there was also no difference in the frequency of DD, ID and II genotypes, or of I and D alleles among T2DM patients with or without microalbuminuria, the current study suggests that this polymorphism may not play a key role in the development of nephropathy in Black Zimbabweans. The results are consistent with a Finnish 9-year follow-up study to investigate the

relationship between ACE gene I/D polymorphism and diabetic albuminuria in T2DM subjects, which found no statistically significant differences in the ACE genotype distribution and allele frequencies between patients with or without albuminuria [274]. Also a study in a Caucasian Mediterranean population found no differences in genotypic or allelic distribution between T2DM patients for either the presence or the absence of nephropathy [275].

However data from this study, on ACE I/D polymorphism and diabetic nephropathy, differs from the majority of studies carried out so far on T2DM subjects. A ten-year follow-up study in Japanese T2DM patients to assess the risk factors for the progression of microalbuminuria demonstrated that glycaemic control and age are important risk factors, and that the DD genotype of the ACE gene acts as a risk factor for the progression of microalbuminuria [276]. Their findings agree yet with another Japanese study conducted by Kuramoto et al on T2DM subjects with insulin resistance [277]. A UK study demonstrated that the ACE DD genotype was associated with higher urinary albumin levels in T2DM subjects [278]. Using urinary albumin index US investigators found that the ACE DD genotype was independently associated with the presence of diabetic nephropathy in non-Hispanic white T2DM patients [149]. Their results imply that the DD genotype may be potentially used as a marker for T2DM patients at risk for developing diabetic nephropathy.

It is well known that diabetes is one of the major causes of end-stage renal failure. The fact that the frequency of ACE DD genotype is higher and statistically significant in T2DM subjects compared with normal subjects, as well as in

patients with diabetic nephropathy, than in patients without nephropathy in certain populations, implies that the ACE DD genotype is associated with T2DM and diabetic nephropathy. Zimbabwean Blacks have a high frequency of the DD genotype and thus, potentially, carry a heavy genetic risk of developing T2DM and diabetic nephropathy.

The results in the current study show a trend toward reduced fasting blood glucose and elevated insulin, and uric acid levels among T2DM subjects with the II genotype. In addition, homozygotes for this rare allele (I) had a tendency toward higher mean values of BP and BMI compared with the other genotypes. It is interesting to note that the I allele in African-Americans was found to be associated with insulin resistance in glucose-tolerant and normotensive subjects [157]. In Hong Kong Chinese subjects with T2DM, the ACE insertion allele was more frequent in subjects with the metabolic syndrome and statistically significant, and the insertion allele was associated with higher fasting plasma glucose levels [270]. Also, American women who were homozygous for the I allele of the ACE gene had greater insulin resistance and a potential risk for T2DM [156].

The data in the current study is somewhat divergent from a Finnish study, as the DD genotype in T2DM patients displayed high blood glucose levels that were statistically significant (same trend in our results), but no statistically significant differences were found between the ACE genotype and serum insulin, body mass index, blood pressure, or serum lipids between the three genotypes [279]. Similar results were obtained from Pima Indians, as the ACE genotype did not correlate

with blood lipids, plasma glucose, and blood pressure [267]. Also a Japanese investigation failed to detect any statistically significant association between ACE polymorphism with obesity, dyslipidaemia, hypertension, insulin resistance, hyperinsulinaemia and microalbuminuria, although they found that there was a trend that all four disorders were more frequent in ID and DD subjects than in II subjects, and this was significant [153].

The majority of studies carried out so far indicate that the insertion/deletion polymorphism of the ACE gene may be a candidate for further understanding the interindividual and interethnic differences in influencing the degree of hypertension and predisposition of onset of diabetic nephropathy in T2DM. It is imperative to find out the consequences of the different distribution of ACE genotypes in various ethnic populations, and the magnitude of the effect of the polymorphism within subsets of subjects with specific metabolic derangements of T2DM must be evaluated further.

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Although the data in the current study seems to suggest that the ACE gene I/D polymorphism is unlikely to serve as a clinically useful predictor of either nephropathy or hypertension in Black Zimbabwean T2DM subjects, the pathogenesis of diabetic nephropathy is not yet clearly defined and thus is not yet elucidated. It is apparent that glycaemic control and blood pressure regulation are involved in the initiation and progression of renal disease in diabetes. Thus, it is important to further explore the complex genetics of diabetes and diabetic nephropathy, especially relating them to individual metabolic derangements. There is no doubt that the RAS system, in particular the ACE gene is a useful

candidate gene that plays some role in this complex issue, and in Zimbabweans, the I allele could play an important role in metabolic derangements. The fragmentary results in the study of ACE gene I/D polymorphism from various studies may emanate from differences in definition, sample size, age, duration of diabetes, gender ratio and ethnic background of the sample. Clearly, more rigorous methodology for investigating the ACE gene I/D polymorphism needs to be applied in future studies.

CHAPTER 6. RESULTS AND DISCUSSION - TUMOUR NECROSIS FACTOR ALPHA GENE POLYMORPHISM

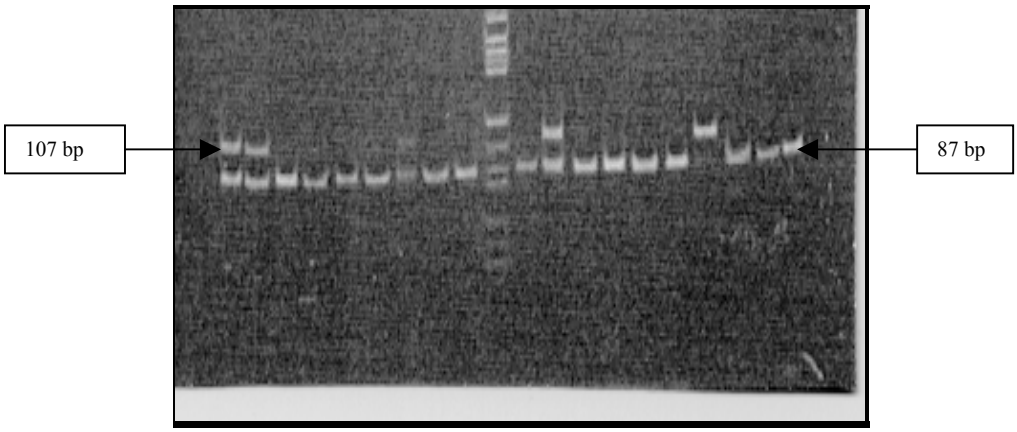
6.1 Genotype and allelic frequencies

Electrophoretic analysis of the -308G⟩A mutation in the 5' region of the TNF-alpha gene on 2.5% agarose gel after PCR amplification and *Nco* I endonuclease digestion revealed the fragments characterising the GG genotype (87 and 20 bp), the AG genotype (107 and 87 bp) and the AA genotype (107 bp), Figure 6.1. The genotype frequency was 74% for GG, 24% for AG, 2% for AA in diabetic adults and 72% for GG, 25% for AG and 3% for AA in young adults (Table 6.1). The TNF-alpha gene allele frequency followed the Hardy-Weinberg equilibrium, being 86% for G, 15% for A in diabetic patients and 85% for G, 16% for A in the youth.

Table 6.1. Frequencies of TNF-alpha genotypes and alleles in 236 adult diabetic patients and 115 young healthy participants.

Genotype	Genotype frequency		Allele frequency (%)			
	PATIENTS	**YOUTH**	**PATIENTS**		**YOUTH**	
TNF-α GENE			**G**	**A**	**G**	**A**
GG	74.1%	72.1%	86	15	85	16
AG	23.7%	25.2%				
AA	2.2%	2.7%				

Figure 6.1. Electrophoretic analysis of the –308G→A mutation in the 5' region of the TNF-alpha gene on a 2.5% agarose gel after PCR amplification and *Nco* I endonuclease digestion.



Fragment 87 bp corresponds to the G allele (restriction site present) and fragment 107 bp corresponds to the A allele (restriction site absent). For example, lane 10 is the MW marker, lanes 1 and 2 represent the AG genotype, lanes 3 and 20 represent the GG genotype, lane 17 represents the AA genotype.

6.2 Biochemical assays, anthropometric and blood pressure features according to TNF-alpha genotypes.

Comparative analyses of the means of glycaemic, anthropometric, blood pressure, lipid, uric acid and renal indices of the -308G⌋A mutation in the 5' region of the TNF-alpha gene mostly yielded differences in means that were not statistically significant (Table 6.2). However, the means of total cholesterol and LDL-C in non-diabetic youth, BMI, WHR, DBP and serum uric acid levels in diabetic adults were different among the three genotypes, GG, AG and AA and statistically different.

The mean of BMI among the three genotypes gave a p value of 0.0080, the AA genotype having the highest mean. Similarly the p value for DBP was 0.0174, and AA had the highest value. Differences in the means of WHR in the three genotypes gave a p value of 0.0344, the AG genotype having the highest mean and the AA genotype the lowest mean. The AA genotype had the highest uric acid mean (374 ③mol/L) and the GG genotype the lowest mean (319 ③mol/L) and the p value among the three TNF-alpha genotypes was 0.0215. Thus in adult diabetic and young subjects the A allele conferred the highest levels of anthropometric, blood pressure and uric acid indices. In addition, the A allele exhibited elevated total cholesterol and LDL-C blood levels compared to the G allele.

Table 6.2. Baseline means !SD of biochemical, anthropometric, and blood pressure indices in 236 adult diabetic patients and 115 young adults according to TNF-alpha genotypes.

CHARACTERISTIC	TNF-alpha GENOTYPES					
	DIABETIC PATIENTS			YOUNG ADULTS		
	GG	AG	AA	GG	AG	AA
GLYCAEMIC INDICES						
Glucose	11.6!5.7	10.4!3.5	10.1!3.5	4.4!0.7	4.3!0.5	4.5!0.3
Insulin	13.3!18.9	9.8!6.9	11.4!4.1	6.9!3.5	7.8!10.0	16.4!17.3
HOMA	5.9.!9.4	4.1!3.6	4.8.6!2.6	1.4!0.7	1.5!2.0	1.3!0.4
ANTHROPO* INDICES						
BMI	28!6	28!6	36!4[a]	21!2	22!3	27!8
WHR	0.89!0.08	0.92!0.11	0.85!0.06[b]	0.77!0.06	077!0.06	0.77!0.01
BP INDICES						
SBP	143!22	146!20	163!16[c]	-	-	-
DBP	90!13	89!12	106!11[b]	-	-	-
LIPID INDICES						
Total cholesterol	4.5.!1.1	4.5!0.93	4.4!0.82	3.5!0.7	3.7!0.9	4.7!1.4[b]
LDL-cholesterol	2.8.!1.1	2.9!1.0	2.9!0.5	1.9!0.7	2.0!0.9	3.1!1.2[b]
HDL-cholesterol	1.3.!0.5	1.3!0.4	1.2!0.3	1.5!0.4	1.5!0.4	1.4!0.4
Triglycerides	1.81!1.18	2.00!0.99	2.00!1.1	0.74!0.31	0.78!0.31	0.83!0.12
URIC ACID						
Uric acid	319!94	356!101	377!79[b]	320!55	334!61	366!40
RENAL INDICES						
Creatinine	98!28	100!28	94!35	83!20	83!16	78!18
Urea	4.0.!1.3	3.8!1.4	3.7!1.3	3.7!0.9	3.8!0.9	3.4!0.2
A/C ratio	7.2.!10.8	8.5!15.5	5.1!3.6	-	-	-

*, anthropo = anthropometric. The units for glucose, cholesterol, triglycerides creatinine and urea are mmol/L, Kg/m^2 for BMI, mmHg for blood pressure, and ③mol/L for uric acid. The superscript (a) denotes a p value < 0.01 in diabetic subjects, (b) denotes a p value <0.05 in diabetic as well as healthy subjects and (c) a p value =0.0883. No statistically significant values were obtained in independent analysis of diabetic and young adults for the rest of the parameters.

6.3 DISCUSSION

Zimbabwean Blacks are genetically close in genotype frequencies to most of the European Caucasians (except the Italians), but differ considerably from the Japanese (Table 6.3).

Recent studies in Caucasian subjects have suggested that the –308G⌋A transition in the 5' region of the TNF-alpha gene may be associated with insulin resistance and obesity. In this study, the AA genotype of the TNF-alpha gene *Nco* I polymorphism had the highest fasting blood levels of uric acid in T2DM subjects, and elevated fasting total cholesterol, LDL-C and triglycerides and uric acid in healthy youths compared to the GG genotype. In addition, the AA genotype displayed the highest BMI, SBP and DBP values, with statistically significant differences in BMI and DBP mean values in T2DM subjects.

The data in the current study is in agreement with that of Rosmond et al as they reported that the homozygotes for the rare allele (AA) of TNF-alpha gene tended to have higher mean values of body mass index, waist-to-hip ratio, and abdominal sagittal diameter compared with the other genotype groups [280]. These investigators also found a weak trend toward elevated insulin and glucose levels among men with the AA genotype. In a Swedish study, the investigators found that the *Nco* I-sensitive polymorphism (–308G⌋A) in the promoter region of the TNF alpha gene was related to body weight [290]. Females carrying the AA genotype were markedly more obese than both the AG and GG (in terms of BMI)

and the body fat content of female AA carriers was increased by one-third compared with AG/GG carriers.

Table 6.3. TNF-alpha genotype frequencies (%) in various ethnic groups.

ETHNIC GROUP	GENOTYPE FREQUENCY		
	GG	**AG**	**AA**
Zimbabwean blacks (current study)	75	22	3
Swedes [280]	71	27	2
Italians [281]	84	14	2
British [282]	73	25	2
Germans [283]	73	24	3
Swedes [284]	67	29	4
European whites [285]	75	23	2
Afro-Americans [286]	77	21	2
Chinese [287]	74	21	5
Japanese [288]	96.7	3.1	0.2
Taiwanese [289]	76	22	2

No statistically significant differences were found between genotypes with respect to waist-to-hip ratio, blood pressure or metabolic variables. Thus the AA-variant at position -308 in the promoter region of the TNF alpha gene could be an important genetic factor behind excessive fat accumulation in women. Recently, Dalziel et al found out that the –308G⌋A TNF-alpha gene variant is associated with insulin resistance, anthropometric measures and systolic blood pressure in obese Australians [291]. Also Brand et al reported that in Caucasian Germans, the –308G⌋A polymophism of the TNF-alpha gene is associated with BMI and concluded that this polymorphism may represent a genetic marker for increased susceptibility for obesity in Caucasians [292].

Gender-pooled quantitative sib-pair analysis in Franks of Canadian origin, found a statistically significant association between the TNF-alpha gene locus and both obesity and obesity-associated hypertension especially in individuals with non-morbid obesity [293]. The investigators reported that the impact of the locus on obesity is most significant in the abdominal region in males and in the thigh region in females. This linkage and association analyses suggest that in hypertensive pedigrees of French-Canadian origin, the TNF-alpha gene locus contributes to the determination of obesity and obesity-associated hypertension and that gender has modifying effects on the regional distribution of body fat.

However, data from the current study is at variance with that from a Japanese investigation made up of healthy young men, healthy old men and T2DM males

[294]. The investigators found no statistically significant differences between the GG and the AA genotypes in any measured anthropometric or metabolic parameter. In the older group fasting plasma glucose was higher in the AA genotype and this was statistically significant. In the 2 healthy groups, plasma triglycerides and the insulin resistance index tended to be higher and HDL-C tended to be lower in the AA genotype, although these differences were not statistically significant. In T2DM subjects, there were no statistically significant differences between the two genotypes in any parameter and HDL cholesterol tended to be lower, although it was not statistically significant. From this data it is apparent that TNF-alpha –308G⁄A polymorphism does not have major effects on obesity, blood pressure, lipids, or glucose in young, older, or diabetic Japanese men.

Healthy and overlapping groups of Hong Kong Chinese with T2DM, hypertension, dyslipidemia, or obesity were investigated for the TNF-alpha gene –308G⁄A polymorphism and the components of the metabolic syndrome. The mutation was not associated with any component of the metabolic syndrome [295]. Data from the current study also conflicts with that of Rasmussen et al, who found no relation between the genetic variants of TNF-alpha and altered insulin sensitivity index or other features of the insulin resistance syndrome, such as body mass index, waist to hip ratio, fat mass, fasting serum lipids or fasting serum insulin or C-peptide in Caucasian Danes. Thus in this Danish population the -308 substitution of the TNF-alpha gene does not seem to play a major role in the pathogenesis of insulin resistance. [296].

Mishima et al found that high levels of serum TNF-alpha in BMI matched Japanese T2DM patients with insulin resistance are related to high levels of fasting insulin and HOMA-IR. Thus TNF-alpha may have a role to play in the pathogenesis of T2DM in obese Japanese men [297]. This mirrors the data from Zinman et al, who investigated the relationship between TNF-alpha and anthropometric and physiologic variables associated with insulin resistance and diabetes in an isolated native Canadian population with very high rates of T2DM [298]. They found moderate, but statistically significant correlations between TNF-alpha and fasting insulin, HOMA-IR, waist circumference, fasting triglycerides, and systolic blood pressure and these relationships were stronger in females than in males. Thus data from this homogeneous Canadian population suggests a possible role for circulating TNF-alpha in the pathophysiology of insulin resistance.

Adipose tissue is not only a passive storage organ for excess energy intake, but is able to act as an endocrine organ that produces and secretes some bioactive molecules, the adipocytokines, which can regulate cell growth and/or metabolic pathways. The cytokine TNF-alpha, implicated in the development of central obesity and peripheral insulin resistance probably mediates the insulin-resistance of obesity through its overexpression in the adipose tissue, thus influencing fat mass. The fact that there is an association between high plasma levels of TNF-alpha and several metabolic aberrations characteristic for the insulin resistance syndrome suggests that TNF-alpha gene mutations may be involved in the pathogenesis of T2DM.

Insulin resistance is linked to numerous common disorders such as diabetes mellitus, dyslipidaemia and hypertension, and has a substantial role in determining their clinical courses. Obesity, a multifactorial syndrome, characterized by storage of excessive adipose tissue, is frequently associated with acquired insulin resistance and abnormal glucose homeostasis. As the molecular mechanism of insulin resistance has not been elucidated, it is conceivable that insulin receptor dysfunction can be responsible for insulin resistance in certain pathological states and obesity is known to be one of the key factors that induce insulin receptor dysfunction. In insulin resistance-related obesity, an elevated expression of TNF-alpha could mediate skeletal muscle insulin resistance, which is characteristic of T2DM. Thus TNF-alpha may be a key component in obesity-linked insulin resistance by inhibiting insulin receptor signaling and glucose transport in insulin-sensitive tissues. However other factors such as persistent hyperglycemia, hypertension and dyslipidemia must be considered in the induction of impaired insulin receptor function.

The majority of population studies show that the TNF-alpha gene –308G⌋A polymorphism is likely to play a key role in the development of metabolic syndrome disorders [295]. Data from the current study suggests that the 308G⌋A mutation in the 5! region of the TNF-alpha gene may have a complex relationship with T2DM, and that it may be a useful tool in determining subjects at high risk of developing the metabolic syndrome.

CHAPTER 7. CONCLUSION

Despite intense ongoing research to identify the putative diabetic genes, no major diabetogene has been identified and the genetics of T2DM still remain an area of speculation and controversy. The imperfect correlation between genotype and phenotype makes the task of finding the diabetogenes a formidable one. It is unlikely that T2DM is a single gene defect, because of the complexity of the pathophysiology that includes contributions from impaired beta-cell function, insulin resistance and obesity, but it is likely to be a polygenic disorder with variable penetrance. Genetic polymorphisms, in particular RFLPs, may not be useful only as markers of human disease, but as valuable tools for constructing a genetic linkage map of the human genome, as has been demonstrated by the Human Genome Program. This affords us an opportunity to follow human disease genes in families afflicted with a heterogenous disorder such as T2DM.

The major findings in this project are:

1. The metabolic syndrome in Zimbabwean diabetic patients is very high and is characterised by high levels of hypertension, microalbuminuria, dyslipidaemia and obesity in that order.

2. The nucleotide substitutions in codons 112 and 158 of the ApoE gene reveal that Zimbabweans have an unusually high frequency of ε4 and ε2 alleles, compared to Caucasian and Asian populations.

3. ApoE polymorphism has a significant influence on blood total cholesterol, and LDL-C in this Black population and a marked influence on triglycerides and HDL-C.

4. The ACE 287-bp insertion/deletion (I/D) polymorphism does not seem to play a major role in influencing blood pressure or nephropathy, despite the fact that Black Zimbabweans have a high D allele frequency. The relation of the I allele with hypertension, insulin and BMI suggests that its conferred risk is much higher in the Zimbabwean Black population.

5. The *Nco* I-sensitive polymorphism (-308G⌋A) in the promoter region of the TNF-alpha gene is related to BMI, blood pressure, uric acid and some lipid indices in this populace. These results suggest a role for TNF-alpha in obesity in Black Zimbabweans.

6. The association of ApoE polymorphism with serum lipoproteins and of TNF-alpha polymorphism with BMI, and uric acid is already manifest in early adulthood in the absence of diabetes.

From extensive literature review, the current study provides the only published data on combined polymorphisms of, ApoE, ACE, and TNF alpha genes in an African diabetic as well as in a healthy population. The data contributes to the knowledge of the genetic architecture of the Zimbawean population, which is largely untapped. In addition, the data brings to the fore the fundamental metabolic profiles of the T2DM phenotypes in Black Zimbabweans. It is important to note that in a complex disorder like T2DM, different populations may have different genetic influences operating, and this may allow a metabolic trait to become expressed clinically, resulting in a diseased state.

As the frequency of the metabolic syndrome is high in this population, this is disturbing, since in the last few years it has been demonstrated that these metabolic disorders are aetiological factors for cardiovascular and cerebrovascular disease. The association of obesity, insulin resistance, hyperinsulinaemia and nephropathy may aggravate the atherogenic lipid profile in most of the Zimbabwean diabetic subjects. It is critical to modify these risk factors in this population through therapeutic and nutritional interventions where possible, so as to reduce the risk of complications of macrovascular disease.

This study has established that ApoE is an important genetic determinant of serum lipoprotein concentrations in this Black population. As ApoE4 may promote atherosclerosis by its low-density lipoprotein raising effect and its compositional changes of LDL (an atherogenic state), the establishment of apolipoprotein E isoforms may be important for patients with diabetes mellitus and those who are at a high risk of developing diabetes mellitus.

A striking feature of this work is the finding that the association of ApoE polymorphism with serum lipoproteins is already manifest in early adulthood in absence of diabetes, where the ε4 allele, is associated with higher concentrations of serum total cholesterol, LDL-C and lower serum HDL-C, a well known highly atherogenic condition. Zimbabwean Black youths with their high frequency of the ε4 allele, potentially carry a heavy genetic burden for coronary heart disorders and Alzheimer disease, as they are now exposed to a Western lifestyle that also has a potential to accelerate obesity, dyslipidaemia, hypertension and diabetes

mellitus. Further studies are required to investigate gene-diet interactions in the different ApoE genotypes and their influence on serum HDL-C and triglycerides concentrations in the general population as their influence can be significant on the efficacy of nutritional or therapeutic interventions.

The D allele of ACE 287-bp insertion/deletion (I/D) polymorphism appears not to have a statistically significant impact on hypertension and diabetic nephropathy in Black Zimbabweans. This may have therapeutic implications, as the majority of this population may be resistant to ACE inhibitors. Although the ACE gene polymorphism has the potential link to diabetic nephropathy as most definitive studies show, large epidemiologic studies must be conducted to determine the implications this polymorphism holds for the best treatment strategies in the care of hypertensive subjects in this population. It is interesting to note that a relationship between circulating ACE and IRS-1 has been shown, since angiotensin II utilizes IRS-1 to relay its signals. The intriguing relation of the I allele with hypertension, insulin and BMI deserves further investigation, as there is a possibility that its conferred risk is much higher in the Zimbabwean Black population.

TNF-alpha gene *Nco* I polymorphism in the promoter region of the TNF alpha gene seems to be an essential determinant in the interindividual variations of body mass indices in healthy subjects as well as diabetic subjects in Zimbabwean Blacks. It is therefore possible that this polymorphism may contribute to obesity-related metabolic disorders, as visceral fat accumulation often accompanies various complications such as insulin resistance, hypertension, dyslipidaemia and

atherosclerosis. T2DM may present as a late phenomenon in obesity after many years characterised by phases of lipotoxicity and glucotoxicity, resulting in impaired glucose tolerance and metabolic deterioration.

Lipotoxicity and glucotoxicity are responsible for causing defects in insulin action and insulin secretion and may lead to an insulin resistant state, and eventually to T2DM. Interestingly, all the elements in the renin-angiotensin system are produced in adipose tissue, and in this study there was an association of TNF-alpha gene *Nco* I polymorphism with blood pressure. Finding the genes specifically expressed in visceral fat and new adipocytokines, should facilitate clarification of the mechanism for the development of visceral fat accumulation, and the associated complications such as insulin resistance, hypertension and dyslipidemia.

The data in the current study categorically shows that the investigated genetic polymorphisms contribute to biological variation in various target tissues, and one can deduce that they are capable of influencing disease susceptibility and progression. Taken together, the findings in this study suggest that genetic polymorphisms could be used as potential markers in T2DM subjects at risk of developing certain metabolic complications and may be useful in the determination of severity of these disorders. The multiple molecular mechanism whereby these polymorphisms induce the metabolic disorders is not well understood and requires further investigation. The association of certain genetic markers with some metabolic derangements, suggest that it may be possible to generate an individual risk profile based on genetic markers (genetic

susceptibility), and theoretically, to design direct therapeutic strategies using such genetic markers. This may also enable us to predict the clinical course of disease in a given patient with a high degree of reliability, even when no clinical symptoms are yet evident. However, it is important to stress that any single mutation in a single gene is only likely to play a small role in determining the total risk of developing any one metabolic trait in a polygenic disorder such as T2DM. Therefore, an intense effort is required to find related genes (gene-gene interactions) that combine to generate most, if not the overall risk.

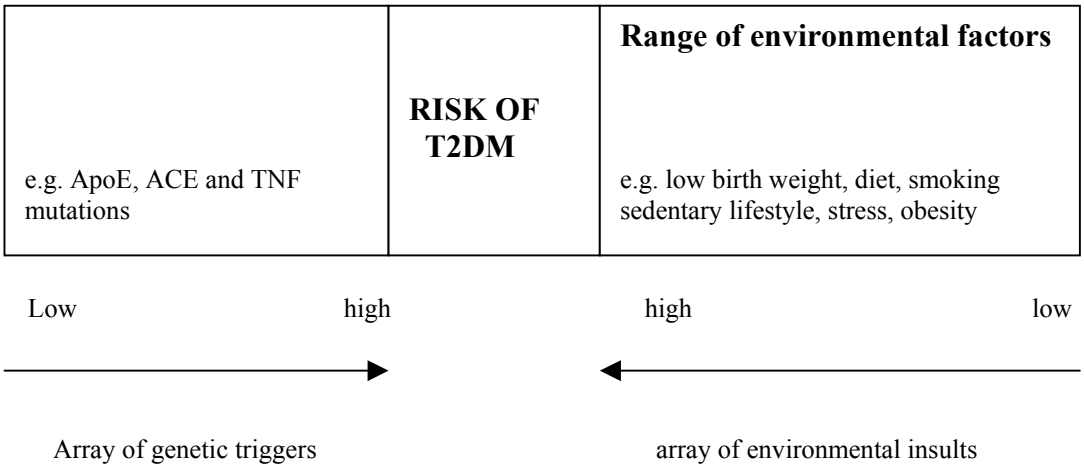
Although the role for newer drugs and targeting high risk groups using genetic markers is still uncertain, it is interesting to note that recently, Katsuki et al reported that troglitazone significantly reduces plasma glucose, C-peptide, HbA_{1c}, and TNF-alpha levels in obese Japanese, T2DM patients [299]. Troglitazone may be a useful drug in the subset of obese diabetic patients homozygous for the defective TNF-alpha A allele, in Zimbabwe. However it is necessary at this stage to establish the functional significance of the associations of the various gene polymorphisms with a cluster of metabolic abnormalities. Thus, in the future, a person's genetic polymorphic architecture could be used by the pharmaceutical industry to propagate new therapeutic approaches for T2DM patients.

It is possible that some genes may promote obesity, hypertension, dyslipidaemia, insulin resistance, microalbuminuria by gene-gene interactions or by some gene-environment interactions or by gene-gender interactions. Conceptually, complex disorders such as T2DM pose a challenge of determining the ways in which the interactive or the additive effects of multiple genes generate the predisposition to

diseases, which will in turn manifest themselves only in the presence of appropriate environmental insults (Figure 7.1). It is imperative to search for these environmental triggers on those individuals who are at most genetic risk of developing multifactorial disorders such as T2DM. A useful future strategy in studying the genetics of T2DM should use a simpler approach, for instance, the investigation of individuals at greatest risk for T2DM, such as siblings, first-degree relatives and offspring of patients with T2DM. Here the assumption is that genetic factors, together with metabolic abnormalties that predispose individuals to T2DM cluster and co-segregate together in selected individuals, as well as in specific environments. This approach may be the key to the discovery of genetic mutations in individuals with a propensity to develop T2DM.

Now that the Human Genome Program is complete, it is important to intensify our efforts of locating new genes that may define susceptibility to T2DM. This will assist in the determination of the function of these genes, definition of the disease associations and elucidation of the correlation between the genotype and the phenotype. And this will lead to investigations of molecular mechanisms that explain the relationship between environmental and inheritable factors directing the metabolic programming responsible for T2DM. Thus, unravelling the role functional gene polymorphisms play in determining risk of T2DM and intermediate phenotypes in assorted populations, may be the crucial key in comprehending the major metabolic pathways and physiology in T2DM, as well as in health.

Figure 7.1. A model depicting how gene-gene and gene-environment interactions conspire in T2DM. The arrows indicate an increased array of genetic and environmental factors that augument the risk of developing diabetes mellitus.



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ABBREVIATIONS

ATP	adenosine triphosphate	AER	albumin excretion rate
ANOVA	one-way analysis of variance		
ACE	angiotensin-converting enzyme	Apo	apolipoprotein
arg	arginine	bp	base pair
BMI	body mass index	CVD	cardiovascular disease
C	centigrade	CE	cholesterol ester
CETP	cholesterol ester transfer protein		
C-peptide	connecting peptide	CAD	coronary artery disease
CHD	coronary heart disease	cys	cysteine
dl	decilitre	DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphates	DM	diabetes mellitus
DBP	diastolic blood pressure	dd	doublestranded
DMSO	dimethyl sulphoxide	ESRD	end stage renal failure
EDTA	ethylene diamine tetra-acetic acid	FFA	free fatty acid
G6PD	glucose 6 phosphate dehydrogenase	gly	glycine
Hb	haemoglobin	HL	hepatic lipase
HDL-C	high density lipoprotein cholesterol	HT	hypertension
HOMA	homeostasis model assessment		
HIV	human immunodeficiency virus	HLA	human leukocyte antigen
IGT	impaired glucose tolerance		
IRS-1	insulin receptor substrate-1		
IDDM	insulin-dependent diabetes mellitus	IR	insulin receptor
IRS	insulin resistance syndrome		

IDL	intermediate density lipoprotein		
KB	kilobase	Kda	kilodalton
Kg	kilogram	Lpl	lipoprotein lipase
L	litres	lys	lysine
LDL-C	low density lipoprotein cholesterol	m	metres
MA	microalbuminuria	mA	milliamp
Mg	milligrams	ml	millilitre
mmol	millimoles	min	minute
MW	molecular weight	ε	epsilon
③g	microgram	③l	microlitre
③M	micromole	③U	microunit
NADH	reduced nicotineamide adenine dinucleotide		
ng	nanogram	nm	nanometre
NIDDM	non-insulin-dependent diabetes mellitus		
PCR	polymerase chain reaction	RAS	renin-angiotensin system
rpm	revolutions per minute		
RFLP	restriction fragment length polymorphism		
ser	serine	ss	single stranded
SDS	sodium dodecyl sulphate	SD	standard deviation
SBP	systolic blood pressure		
TEMED	tetramethyl ethylene diamine	Taq	thermus aquaticus
TG	triglyceride		
TRLs	triglyceride-rich lipoproteins	TE	TRIS/EDTA
TBE	TRIS/Boric acid/EDTA buffer		
TNF-alpha	tumour necrosis factor alpha		

T1DM	type 1 diabetes mellitus	T2DM	type 2 diabetes mellitus
uv	ultra violet		
VLDL	very low density lipoprotein	V	volts
WHR	waist to hip ratio		
WHO	World Health Organization		

ABSTRACT

Objectives: The main objectives of the study were (i) to determine the prevalence of the metabolic syndrome disorders, (ii) to determine the frequency, of ApoE gene polymorphism at codons 112 and 158, of the ACE gene 287-bp insertion/deletion polymorphism, of the -308G\A mutation in the 5! region of the TNF-alpha gene, and (iii) to investigate the effects of these polymorphisms on the components of the metabolic syndrome in type 2 diabetic patients.

Methods: Dyslipidaemia, obesity, hypertension, microalbuminuria, hyperinsulinaemia and insulin resistance, all intermediate traits and metabolic components found in type 2 diabetes mellitus, were determined in diabetic patients as well as non-diabetic participants. Apolipoprotein E (ApoE) gene polymorphism of the amino acid substitutions of cysteine and arginine at codons 112 and 158, angiotensin converting enzyme (ACE) gene 287-bp insertion/deletion polymorphism and the -308G\A mutation in the 5! region of the tumour necrosis factor alpha (TNF-alpha) gene were determined by the polymerase chain reaction (PCR). Polyacrylamide and agarose gels were used to electrophoretically identify the genotypes from the PCR DNA products. The genotypes from the 3 different polymorphisms were related to lipid metabolism, anthropometric measurements, microalbuminuria, hypertension, insulin resistance or hyperinsulinaemia.

Results: The data in the current study clearly demonstrates a rather high prevalence of the metabolic syndrome (36%) in Black Zimbabweans with type 2 diabetes mellitus, characterised by hypertension (69%), microalbuminuria (62%), insulin resistance (57%), dyslipidaemia (47%) and obesity (31%). The nucleotide substitutions in codons 112 and 158 of the ApoE gene reveal that Zimbabweans have an unusually high frequency of ε4 (29%) and ε2 (11%) alleles, compared to general Caucasian and Asian populations. The E4/4 genotype has a statistically significant influence on blood total cholesterol ($p < 0.05$), and low density lipoprotein cholesterol ($p < 0.05$) in this Black population compared to other genotypes. The ApoE polymorphism has a marked influence on triglycerides and high density lipoprotein cholesterol even if this is not statistically significant. Although Black Zimbabweans have a high D (deletion) allele frequency (64%), the ACE 287-bp insertion/deletion (I/D) polymorphism, blood pressure and microalbumin levels were not statistically significant in hypertensive versus non-hypertensive and microalbuminuric versus non-albuminuric patients, respectively. The I allele had a relationship with hypertension, insulin and BMI, although this was not statistically significant. The AA genotype compared to the AG and GG genotypes of the *Nco* I-sensitive polymorphism (-308 G\A) in the promoter region of the TNF-alpha gene is significantly associated with BMI ($p < 0.01$), diastolic blood pressure ($p < 0.05$), and uric acid ($p < 0.05$) in this populace.

Discussion: In the last few years it has been demonstrated that metabolic disorders investigated in this study are aetiological factors for cardiovascular and cerebrovascular disease. Obesity, insulin resistance, hyperinsulinaemia and nephropathy may aggravate the atherogenic lipid profile in most of these patients. Therefore, it may be critical to modify these risk factors in this population through therapeutic and nutritional interventions where possible, so as to reduce the risk of complications of macrovascular disease. The high prevalence of the ε2 and ε4 alleles is likely to be a heavy genetic burden as it has been shown to

enhance susceptibility to diabetic nephropathy, coronary artery disease and Alzheimer disease in several populations. The ACE 287-bp insertion/deletion (I/D) polymorphism does not seem to play a major role in influencing blood pressure or diabetic nephropathy, despite the fact that Black Zimbabweans have a high D allele frequency. However the relation of the I allele with hypertension, insulin and BMI, although not statistically significant, suggests that its conferred risk may be much higher in the Zimbabwean Black population. Data from the current study suggests that TNF-alpha gene polymorphism may have a complex relationship with T2DM and that it may be a useful tool in determining subjects at high risk of developing the metabolic syndrome.

Conclusion: The data in the current study categorically shows that genetic polymorphisms contribute to biological variation in various target tissues, and one can deduce that they are capable of influencing disease susceptibility and progression. Taken together, the findings in this study suggest that these polymorphisms could be used as potential markers in T2DM subjects at risk of developing certain metabolic complications and that they may be useful in the determination of severity of these disorders. The multiple molecular mechanism whereby these polymorphisms induce the metabolic disorders is not well understood and requires further investigation.

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