Cape Peninsula University of Technology

The role of *FTO*, *ENPP1* and *TCF7L2* in the pathogenesis of diabetes in an adult population from Bellville South, Cape Town, South Africa

By

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DECLARATION

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

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ABSTRACT

Background: The Mixed Ancestry population of South Africa has recently been reported to have a higher prevalence of type 2 diabetes (T2DM). However, the genetic risk factors that may contribute to the development of T2DM are currently unknown. We investigated the association of fat mass and obesity-associated gene (FTO), ectonucleotide pyrophosphatase/phosphodiesterase gene (ENPP1) and transcription factor 7-like 2 gene (TCF7L2) with T2DM risk in a community residing in Bellville South, Cape Town.

Methods: Five hundred and sixty six participants (11.7% males) who consented to genetic analyses were genotyped for six single nucleotide polymorphisms (SNPs): *ENPP1*-rs997509 and -rs1044498, *FTO*-9941349 and -rs3751812, *TCF7L2*-rs12255372 and -rs7903146. The SNPs were genotyped using their corresponding Taqman genotyping assays, and validated by automated sequencing. Allele and genotype frequencies were determined and regression analyses was conducted to assess the association of the polymorphisms with T2DM and its related,traits.

Results: Overall and in subgroups defined by diabetes and obesity statuses, there were present no significant differences in the distribution of alleles and genotypes, except for the polymorphisms observed in the FTO and ENPP1 genes. In logistic regression models adjusted for age, sex, body mass index (BMI) and insulin resistance, minor alleles of *ENPP1*-rs997509 and *ENPP1*-rs1044498 were associated with risk for T2DM respectively, 4.55 (1.06-19.49) (p=0.041) and 1.81 (1.09-2.98) (p=0.021) assuming a recessive genetic model. Furthermore, the *FTO* rs9941349 minor allele was associated with the prevalent T2DM under the log-additive model: 1.40 (1.00 to 1.96) (0.049). The *TCF7L2* polymorphisms showed no evidence of association with T2DM and/or insulin sensitivity/resistance indicators.

Conclusion: Our results demonstrate that *ENPP1* and *FTO* polymorphisms may contribute to T2DM susceptibility in this population, confirming previous findings that insulin resistance may mediate the development of the disease in the Mixed Ancestry population group of South Africa.

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ABBREVIATIONS

β	Beta
%	Percentage
®	Registered Trademark
™	Trademark
ADA	American Diabetes Association
Akt	Atypical Protein Kinase B
aPKC ζ\ λ	Atypical Protein Kinase C Isoforms ζ and λ
AS160	Akt Substrate 160
β-cat	βeta-catenin
BMI	Body Mass Index
CAP	C-Cbl Associated Protein Catabolite Activator Protein
Cbl	Cellular Homologue of Cas NS-1 Oncogene
CBP	Creb Binding Protein
CDKAL1	Cyclin-Dependant Kinase-5 Subunit Associated Protein 1Like 1
CDKN2A	Cyclin-Depedent Kinase Inhibitor 2A
CDKN2B	Cyclin-Depedent Kinase Inhibitor 2B
CI	Confidence Interval
Crk	CT10 Regulator Kinase
CRP	C-Reactive Protein
Csk	C-Terminal Src Kinase
CT	Computed Tomography
CtBP-1	C-Terminal Binding Protein
C3G	Cyanidin 3-O-β-D-Glucoside
CV	Confidence Interval
CVD	Cardiovascular Disease
DM	Diabetes Mellitus
dNTPs	Deoxyribonucleotides
ds-DNA	Double-Stranded DNA
EDTA	Ethylenediamine Tetra-Acetic Acid
eGFR	Estimated Glomerular Filtration Rate
ENPP1	Ectonucleotide Pyrophosphatase/Phosphodiesterase 1
EtOH	Ethanol
ETX2	Exostosin-2 gene
ExoSAP	Exonuclease I and Shrimp Alkaline Phosphatase
FFA	Free Fatty Acids
FIRI	Fasting Insulin Resistance Index
FOXO-1	Forkhead box protein 01
Ft	Fused Toes
FTO	Fat Mass and Obesity Associated-gene
Fyn	Src Family Tyrosine-Protein Kinase
Gab-1	Grb-2 Associated Binder-1
GCK	Glucokinase
GDM	Gestational Diabetes Mellitus
GGT	γ-Glutamyltransferase
GLP-1	Glucagon-Like Peptide-1
GLUTs	Glucose Transporters
GLUT 2	Glucose Transporter 2

GLUT 4	Glucose Transporter 4
Grb-2	Growth Factor Receptor-Binding Protein -2
GSK-3	Glycogen Synthase Kinase
GTP	Glucose Triphosphate
GWA	Genome-Wide Approach
G6P	Glucose-6-Phosphate
G6P-ase	Glucose-6-phosphatase
HbA1c	Glycated Hemoglobin A1c
HBP1	HMG-Box Transcription Factor-1
HDL	High Density Lipoproteins
HDL-C	High Density Lipoprotein-Cholesterol
HHEX	Human Homeobox Gene
HMG	High Mobility Group
HNF-1α,	Hepatocyte Nuclear Factor -1α
HNF-4α	Hepatocyte Nuclear Factor- 4α
HNF-1β	Hepatocyte Nuclear Factor -1β
HOMA-B%	Functional β-Cells
HOMA-IR	Homeostatic Model Assessment of Insulin Resistance
HWE	Hardy-Weinberg Equilibrium
IDF	International Diabetes Federation
IFG	Impaired Fasting Glycaemia
IGF2BP2	Insulin-Like Growth Factor mRNA-Binding Protein 2
IGT	Impaired Glucose Tolerance
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IPF-1	Insulin Promoter Factor-1
IR	Insulin Receptor
IRS	Insulin Substrate
IRS-1	Insulin Receptor Substrate 1
IRS-2	Insulin Receptor Substrate 2
JNK	Jun n-Terminal Kinase
KCNJ11	Potassium Inwardly-Rectifying Chanel, Subfamily J, Member
LD	Linkage Disequilibrium
LDL	Low Density Lipoproteins
LEF	Lymphoid Enhancer Factor
LY-294002	2-(4-Morpholinyl)-8-Phenylchromone
MCP-1	Monocyte Chemoattractant Protein -1
MODY	Maturity Onset Diabetes of the Young
MS	Metabolic Syndrome
MUAC	Mid-Upper-Arm Circumference
NaCl	Sodium Chloride
Nck	Nck Adapter Protein
NGSP	Glycohemoglobin Standardization Program
OGTT	Oral Glucose Tolerance Test
p85	Regulatory Subunit of Phosphoinositide-3-Kinase
p110	Catalytic Subunit of Phosphoinositide-3-Kinase
p300/CBP	Cofactor Family, Creb Binding Protein.
PBS	Phosphate Buffered Saline
PC-1	Plasma cell-1
PCR	Polymerase Chain Reaction
PDK1	Phosphatidylinositol-Dependent Kinase-1
PEPCK	Phosphoenolpyruvate carboxykinase
PIP2	Phosphatidylinositol 4,5-Bisphosphate
PIP3	Phosphatidylinositol 3,4,5-Trisphosphate
PI3K	Phosphoinoside-3 Kinase
PKB	Protein Kinase B
PKC ζ\ λ	Protein Kinase C Isoforms ζ and λ

PO_4	Phosphate
POMC	Proopiomela-Nocortin
PPARG	Peroxisome Proliferator Activated Receptor Gamma
PTPases	Protein-Tyrosine Phosphatases
QUICKI	the Quantitative Insulin-Sensitivity Check Index
RIA	Radioimmunoassay
RT-PCR	Real-Time Polymerase Chain Reaction
Rpm	Revolutions per Minute
SB	Sodium Boric
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
Shc	Adapter Protein
SH2	Src-Homology-Phosphatase 2
SNP	Single Nucleotide Polymorphism
Src	Pro-Oncogene Tyrosine-Protein Kinase
SR160	Systemin Binding Receptor 160
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
TE	Tris EDTA
TC10	Member of the GTP-Binding Protein
TCF7L2	Transcription Factor 7-Like 2
TNF-α	Tumor Necrosis Factor -α
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
UV	Ultraviolet Light
V	Volts
WC	Waist Circumference
WHO	World Health Organization
WHR	Waist Height Ratio
WHR	Waist Hip Ratio

CHAPTER 1
LITERATURE REVIEW

1.1 Study outline

The present study is of cross-sectional design and aimed to investigate the role of *FTO*-rs3751812 and -rs9941349, *ENPP1*-rs997509 -rs1044498 and *TCF7L2*-rs7903146 rs12255372 polymorphisms in the development of type 2 diabetes in a Mixed Ancestry population group that resides in Bellville South, Western Cape South Africa. Single nucleotide polymorphisms (SNPs) are sequence variations in the genome of biological species which can sometimes cause a difference in gene expression by altering splicing, transcription or translation and thereby leading to a malfunctioning polypeptide. Thus SNPs are used as biological markers of diseases. In the present study six SNPs that are associated with the pathogenesis of the type 2 diabetes were investigated. Chapter 1 provides brief background information on type 2 diabetes, its prevalence and the pathophysiology of the disease. Both genetic and environmental factors such as physical inactivity, poor diet, socioeconomic status and environmental pollutants are discussed. Thereafter, the genes investigated in this study are introduced, followed by the aims and objectives of the study. Chapter 2 describes the methods used in the thesis. Chapter 3 describes the findings of the study and their relevance (results and discussion) in the Mixed Ancestry population of South Africa, with concluding remarks presented at the end of the chapter. Chapter 6 is the references to all the papers and journals that are used in all the chapters. The appendix is indicated at the end of the thesis.

1.2. Definition and classification of diabetes mellitus

Diabetes Mellitus (DM) is a chronic condition that occurs when the body fails to effectively use the insulin it produces or when the pancreas does not produce enough insulin resulting in hyperglycemia (Gardner and Shoback, 2011). Several forms of diabetes exist and these are classified based on their underlying cause. These include type 1 diabetes (T1DM), type 2 diabetes (T2DM), gestational diabetes mellitus (GDM) and maturity onset diabetes of the young (MODY). T1DM and T2DM are the two principal forms of diabetes, which vary according to the underlying cause and pathogenesis (Tilburg *et al*., 2001). T1DM accounts for 5% to 10% of all diabetes cases, and is thought to be caused by an auto-immune reaction whereby the body's defense system attacks the beta-cells of the islets of Langerhans in the pancreas, thus resulting in the body being unable to produce enough insulin required to regulate glucose levels. People with T1DM require insulin to regulate their glucose levels and to prevent progression of the disease (Tilburg *et al*., 2001; IDF Diabetes Atlas, 2012). On the other hand, Type 2 diabetes mellitus (T2DM) is the most common form of diabetes accounting for approximately 90% of all diagnosed cases. It was initially referred to as adult-onset diabetes as it commonly affected adults. However, it is now recognized as the disease

of people of all ages. T2DM is characterized by reduced responsiveness of cells to insulin (known as insulin resistance) and relative insulin deficiency (insulin produced but not sufficient to prevent hyperglycemia) thus leading to increased levels of glucose in the blood. As the disease progresses, secretion of insulin by the pancreatic beta cells is impaired (Larsen, 2000; Kahn and Flier, 2000). Many affected individuals do not require exogenous insulin but may require it to control glucose levels if this is not achieved by recommended diet changes or hypoglycaemic drugs (Tilburg *et al*., 2001; Thevenod *et al*., 2008). T2DM can remain undiagnosed for many years with symptoms only presenting at a later stage or adulthood (IDF Diabetes Atlas, 2012).

Gestational diabetes mellitus affects women during pregnancy, who were not previously diagnosed with diabetes and accounts for about 2% - 10% of pregnant women. It usually occurs later in pregnancy and most women revert to normoglycemia after birth (Tilburg *et al*., 2001; IDF Diabetes Atlas, 2012).

Maturity onset diabetes of the young is a clinical heterogeneous group of disorders that is characterized by familial hyperglycemia with an autosomal dominant mode of inheritance. This mode of inheritance therefore implies half the subjects of both sexes will be affected in each generation of MODY family (i.e. it has a pedigree of multigenerational) (Velho and Robert, 2002; Fajans and Bell, 2011). Hyperglycemia in MODY patients is as the result of beta-cell dysfunction. This form of diabetes usually occurs before the age of 25 years (frequently seen in childhood or adolescence). Most members of MODY families are non-obese, in contrast to T2DM (Fajans and Bell, 2011). Studies have revealed that MODY is a polygenic entity characterised by metabolic as well as clinical heterogeneity (Velho and Robert, 2002). Mutations in *GCK, HNF-1α, HNF-4α, HNF-1β a*nd *IPF*-1 have been described as causes of the disease (Fajans and Bell, 2011). Treatment of MODY depends primarily on the cause and the severity of Hyperglycemia (Velho and Robert, 2002).

If remained untreated, DM progresses to a number of serious health problems such as macro- and microvascular complications, regress, or stays the same and all these complications may lead to a reduced quality of life and life expectancy (Thevenod *et al*., 2008). Microvascular complications include retinopathy, neuropathy, nephropathy which may lead to blindness, lower limb amputation and renal failure in adults. Macrovascular complications include cardiovascular disease (CVD) and stroke. Diabetes and its complications cause a burden on public health services due to their high management costs, and therefore strategies should be developed for disease management (Tilburg *et al*., 2001; Diabetes Report Card, 2012).

1.3. Blood glucose homeostasis

Normal glucose homeostasis is controlled by a balance between the production of glucose by the liver and kidneys, and the uptake by insulin-responsive tissues such as the brain, kidneys, adipose and muscle tissue (Tilburg *et al*., 2001). Glucose in the blood is obtained through consumption of carbohydrate-containing food, or via its synthesis from non-carbohydrate sources such as lactate, glycerol and glucogenic amino acids; a metabolic process called gluconeogenesis. Gluconeogenesis occurs during periods of fasting, starvation, low-carbohydrate diets or intense exercise. Due to its importance in regulating blood glucose levels, gluconeogenesis is a target of therapy for type 2 diabetes. Blood glucose levels can also be regulated through degradation of glycogen (glycogenolysis). While glucose production is stimulated by glucagon through stimulation of gluconeogenesis and glycogenolysis, insulin acts in the opposite direction by decreasing blood glucose concentration (Barnard and Youngren, 1992; Tilburg *et al*., 2001). Insulin and glucagon are secreted by pancreatic beta cells in response to blood glucose levels. Insulin regulates glucose homeostasis at several sites by increasing the rate of glucose uptake in insulin-responsive tissues and enhances its conversion to glycogen and triglycerides. Normally, blood glucose levels are kept within a small range of 4.4 to 6.1 mmol/L through several mechanisms. For example, after a meal blood glucose levels increase resulting in an enhanced secretion of insulin by the pancreatic β-cells, thereby reducing the production of glucose by the liver and enhancing the uptake of glucose by muscle and adipose tissue. Glucose counter-regulatory hormones such as glucagon, cortisol, epinephrine and norepinephrine increase the levels of blood glucose and thus counteracts the hypoglycaemia state. Balance between insulin action and the effects of the counter-regulatory hormones ensure normal glucose homeostasis (Tilburg *et al*., 2001).

In response to the increased levels of insulin, the insulin-responsive tissues express membrane proteins called glucose transporters (GLUTs) that are known to facilitate the transportation of glucose molecules from the cytosol to the plasma membrane. A number of GLUTs (GLUT1-14) have been identified and these are expressed in different tissues and have a different affinity for glucose. For example, GLUT4 which has been shown to play a pivotal role in glucose homeostasis is mostly expressed in muscle and fat cells. GLUT2 is prominent in pancreatic β-cells and in the liver (Sesti, 2006). These GLUTs are localized intracellularly and in response to the increased levels of insulin are translocated from the cytosolk to the plasma membrane of myocytes and adipocytes (Barnard and Youngren,

1992; Inoue *et al.*, 2006). In unstimulated cells, GLUTs are stored in endosomes, the intracellular reservoir compartments and can only be translocated from the plasma membrane when the insulin receptor is activated (Inoue *et al.*, 2006).

Insulin Signalling: Insulin responsive cells such as adipocytes, myocytes and liver possess insulin-binding receptors (IRs) at their cell surfaces. Insulin receptor is a heterotetramic glycoprotein comprising of two α-subunits which are entirely extracellular with insulin binding sites and two β-subunits which is composed of extracellular domain, a transmembrane domain and an intracellular domain. These two subunits are linked together by disulphide bonds. Insulin binds to the IR extracellular α-subunits and stimulates autophosphorylation on multiple tyrosine residues of the cytoplasmic portion of the transmembrane β-subunit (Sesti, 2006; Saina, 2010). This results in the activation of the its intrinsic tyrosine kinase which catalyses the phosphorylation of cellular proteins including the members of the insulin receptors substrate family (IRS 1-4), Shc adapter protein, Grb-2 associated binder-1 (Gab-1) and casitas B-lineage lymphoma 1 (Cbl) (Sesti, 2006; Saina, 2010; Siddle, 2011). When IRS is phosphorylated, it binds and activates several signalling molecules containing Src homology 2 (SH2) domain proteins such as the p85 regulatory subunit of phosphoinoside-3 kinase (PI3K), tyrosine kinases (e.g. src family tyrosine-protein and c-terminal src kinases), tyrosine protein phosphatase (e.g. SHP-2), growth factor receptor-binding protein (Grb-2), CT10 sarcoma oncogene cellular homolog (Crk) and Nck adapter protein (Sesti, 2006; Saina, 2010; Siddle, 2011). Phosphoinoside-3 kinase has been widely investigated because of its important role in insulin-stimulated intracellular translocation of GLUT4 (Sesti, 2006). The phosphorylated catalytic subunit of PI3K, p110, transforms phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PIP3). In turn, the increased level of PIP3 activates downstream effectors such as phosphatidylinositol-dependent kinase-1 (PDK1), thus phosphorylating and activating serine\threonine kinase B (also known as AKT) and atypical protein kinase C isoforms ζ and λ (PKC ζ/λ) (Standaerte *et al.*, 2002; Sesti, 2006; Saina, 2010; Siddle, 2011). These effectors act in conjunction to phosphorylate protein kinase B's (PKB's) Thr 308 and Ser 473 residues, and ultimately regulate insulin signalling pathway (Figure 1.1) (Kahn and Flier, 2000).

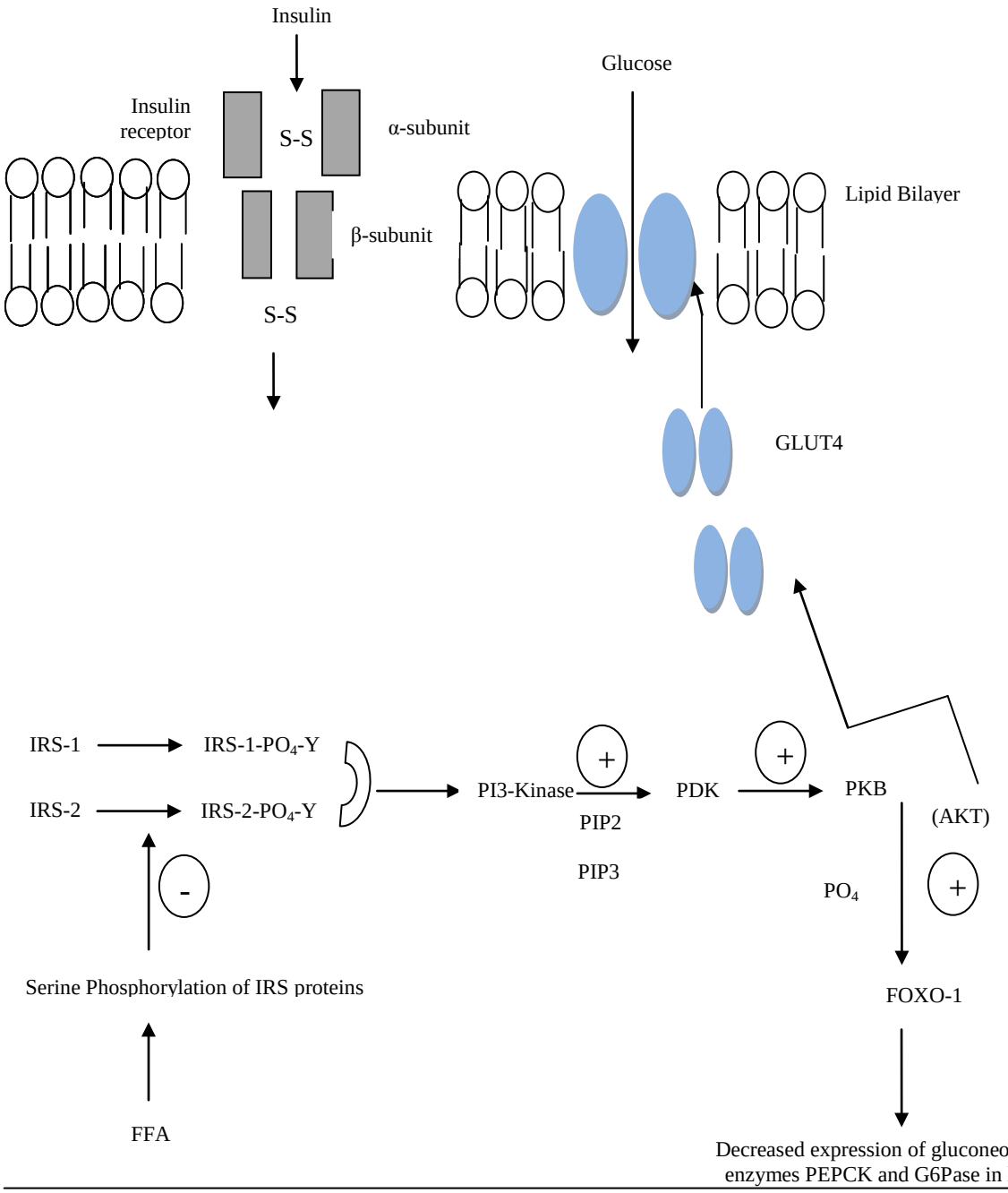


Figure 1.1. A diagrammatic summary of the insulin signalling pathway. Binding of the insulin to its receptor leads to the activation of GLUT4 which then imports glucose to the cell. The activated IR activates PI3-Kinase which produces PI3,4,5P2 and 5P3 which then serve as docking sites for PDK1.PDK1 mediates activation of PKB that is responsible for regulating transcription of target genes-PEPCK and G6Pase via Foxo-1. The increased levels of FFA may cause serine phosphorylation of IRS proteins which in turn decreases IRS-tyrosine phosphorylation, impairing downstream effectors (*Adapted from Saina, 2010*).
Abbreviations: GLUT4, glucose transporter-4; IRS-1, insulin receptor substrate-1; IRS-2, insulin receptor substrate-2, IRS-1-PO_4-Y; IRS-2-PO_4-Y; PI3 Kinase, phosphatidylinositol 3-Kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PDK, phosphatidylinositol-dependent kinase; PKB, protein kinase B; AKT, Atypical Protein Kinase B; PO4,phophate; FOXO-1, fork head box protein 01; PEPCK phosphoenolpyruvate carboxykinase; G6Pase, glucose-6-phosphatase; FFA, free fatty acids.

Other insulin-regulating mechanisms such as the CAP/Cbl/TC10 pathway have been described (Baumann *et al*, 2000). The Cbl-CAP complex is known to play a role in recruitment of GLUT4 to the plasma membrane in a PI3K-independent pathway (Chiang *et al*, 2001; Siddle, 2013). Its activation through IRS phosphorylation results in the interaction of the complex with adapter protein Crk. The adapter protein Crk is always associated with cyanidin 3-O-β-d-glucoside (C3G), a Rho-family guanine nucleotide exchange factor. In turn C3G activates TC10, a member of the GTP-binding proteins, which then interacts with GLUT4 and promotes its translocation to the plasma membrane (Bjornholm and Zierath; 2005; Sesti, 2006). The activated TC10 functions parallel to PI3 kinase in promoting insulin-stimulated GLUT4 translocation. It is speculated that TC10 might stimulate GLUT4 translocation and glucose uptake by either directly producing a cytoskeleton rearrangement (actin filament to be specific) to facilitate the exocytosis of GLUT4 or through docking and fusing with the plasma membrane (Chiang *et al*, 2001). Experimental evidence has shown that disruption of this pathway results in the attenuation of insulin-stimulated glucose transport in 3T3-L1 adipocytes (Baumann *et al*, 2000; Chiang *et al*, 2001). For example, blocking the phosphorylation step completely inhibits insulin-stimulated GLUT4 translocation. However, the pathway does not appear to have a role in skeletal muscle (JeBailey *et al*., 2000; Bjornholm and Zierath; 2005).

Insulin signalling pathway is a very complex process with many steps at which defects can potentially occur thus causing reduction of insulin-mediated glucose transport. One of the potential mechanisms includes reduction of receptor density and defects in phosphorylation of intracellular enzymes (Sesti, 2006). For example, protein-tyrosine phosphatases (PTPases) (e.g. PTPase 1B) function as negative regulators of the signalling cascade by degrading PIP3s to form PIP2s and thus terminating insulin signalling (Sesti, 2006). Internalization of the insulin-insulin receptor complex into endosomes is known to result in the termination of insulin signalling. It has also been observed that mutagenesis in any of the three major phosphorylation sites of IR (residues 1158, 1162 and 1163) results in impairment of IR phosphrylation activity thus reducing insulin-stimulated glucose disposal (Abdul-Ghani and DeFronzo, 2008). Futhermore, insulin degrading enzymes may also cause reduction of insulin-mediated glucose transport. Moreover, inhibition of PI3K by inhibitors such as Wortmannin or 2-(4-Morpholinyl)-8-Phenylchromone (LY-294002) has been shown to block the formation of these lipid products, thus inhibiting several intracellular events including GLUT4 translocation in the skeletal muscle (Bjornholm and Zierath; 2005), reduction of receptor density and defects in phosphorylation of IRS of intracellular enzymes (Sesti, 2006).

1.4 Diagnosis of Diabetes Mellitus

The most commonly used methods to diagnose DM are those of the World Health Organization (WHO), the International Diabetes Federation (IDF) and the American Diabetes Association (ADA) (Table 1.1). All these criteria are based on plasma glucose levels, however recently some of these criteria have also included A1c. The A1c test is a blood test that provides information about a person's average levels of blood glucose over the past three months. It is sometimes referred to as glycosylated hemoglobin A1c (HbA1c). A1c is formed by the attachment of glucose to various amino groups of haemoglobin and is used to help diagnose diabetes and prediabetes. In contrast to other methods of diagnosing diabetes, A1c can be performed at anytime of the day as it does not require any special preparation such as fasting (WHO, 2011). A1c results are reported as a percentage, the higher the percentage, the higher a person's blood glucose levels have been (The International Experts Committee, 2009). WHO recommends a cut-off point of 6.5% for diagnosing diabetes but values less than 6.5% does not exclude diabetes (WHO, 2009). ADA has recommended the lower cut-off point of 5.7 – 6.4% which can be used to indicate intermediate hyperglycemia (ADA, 2003). Our research group has found a cut-off point of 6.1% to be optimum for the Bellville South community of South Africa thus emphasizing the need for evidenced based values to be established for all population groups (Zemlin *et al*., 2011).

Table 1.1. Values for the diagnosis of DM and its related traits as classified by ADA, WHO and IDF. (*ADA report, 2003*)

CONDITION	**VENOUS PLASMA GLUCOSE (MMOL/L)**		
	ADA	**WHO**	**IDF**
Diabetes mellitus:			
A1C levels	≥ 6.5%	≥ 6.5%	6.5%
Fasting or	≥7.0	≥7.0	≥7.0
2-h post glucose load	≥11.1	≥11.1	≥11.1
Impaired glucose tolerance (IGT):			
Fasting concentration (if measured)	≥6.1 and <7.0	<7.0	≥6.1
2-h glucose load	<7.8	≥7.8 and <11.1	<7.8
Impaired glycaemia (IFG) fasting			
Fasting	≥6.1 and <7.0	6.1-6.9	≥6.1 and <7.0
2-h (if measured)	<7.8	<7.8	<7.8

Abbreviation: ADA, American Diabetes Association; International Diabetes Federation; IDF, WHO, World Health Organization; A1C, IGT, impaired glucose tolerance; IFG, impaired glycaemia.

1.5 Prevalence of Diabetes

Diabetes Mellitus is now recognized as one of the main public health challenges of the 21st century. In the past two decades, the prevalence of diabetes has increased at an alarming rate in both developed and developing countries (Thevenod *et al,* 2008). The prevalence and incidence rates are much higher than predicted some 20 years ago (Uusitipa, 2010). It was stipulated that there will be about 300 million people with diabetes by 2025 (Tilburg *et al*, 2001) and this number has already been exceeded (IDF Diabetes Atlas, 6th Edition, 2013). Statistically, there are about 382 million people living with diabetes globally and this number is approaching that (592 million) estimated for 2035. The highest number of people with diabetes is between 40 and 59 years of age. Statistics show that about 5.1 million deaths due to diabetes were reported in 2013, meaning that every six seconds a person dies from diabetes (IDF Diabetes Atlas, 6th Edition, 2013). This epidemic relates particularly to T2DM which accounts for the most diagnosed cases of DM. According to epidemiological data, the prevalence of diabetes is increasing in every country including those on the African continent. It is reported that Africa has the highest proportion of undiagnosed cases (at least 63%). In 2013 alone, it was estimated that about 522,600 deaths that are diabetic-related deaths were reported in Africa, representing a total of 8.6% of deaths from all causes in adults. These deaths occur in people under the age of 60 years and more deaths occur in women compared to men (IDF, Diabetes Atlas, 2013). In 2013 in Africa about 19.8 million individuals were estimated to have diabetes and this number is expected to increase to about 41.5 million by 2035. A highest prevalence in Africa is seen in Reunion island (15.4%), followed Seychelles (12, 1%), Gabon (10.7%) and Zimbabwe (9.7%). High prevalence is also seen in the more populated countries such as South Africa (6.5 million), followed by Nigeria (3.9 million), Ethiopia (1.9 million) and the United Republic of Tanzania (1.7 million) (IDF, Diabetes Atlas, 2013).

This rapid epidemiological increase of diabetes in Africa is linked to the rapid transformation in lifestyles and urbanization where the traditional lifestyle is replaced by western lifestyles where there is cheap availability of high-fat and high-energy food (Bos and Agyemang, 2013). These two factors combined lead to obesity which is a strong risk factor of diabetes. In South Africa there are about 6.5 million individuals that are diabetic but only a low percentage of them are registered with the Diabetes Society of South Africa (IDF, Diabetes Atlas, 2013; SAhealthBlog.com). This clearly shows a concern and the lack of therapeutic education covering the aspect of management of the disease. Therapeutic education is a responsibility that should not be underestimated as it is the linker between therapy compliance and physician recommendation (Boudiba, 2013).

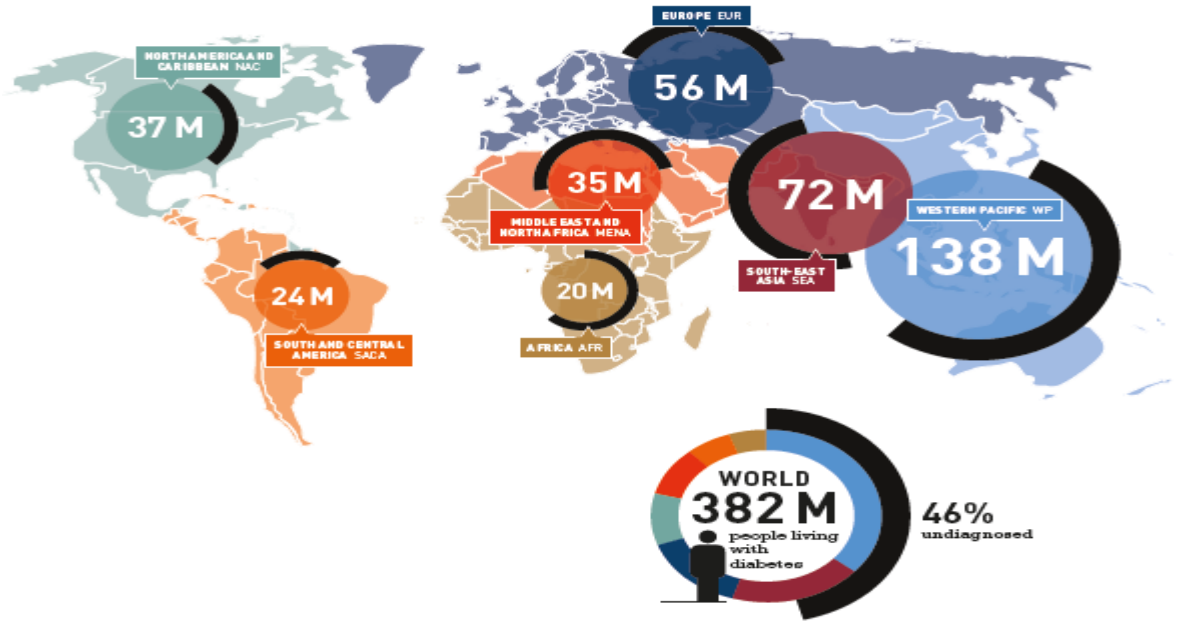


Figure 1.2. Regional estimates of people with diabetes mellitus (in millions) (*Adapted from IDF Diabetes Atlas, 6th Edition, 2013*).

Studies have shown the prevalence of T2DM varies widely across populations with rates of 5% or less in white and Asian to 50% or more in Pima Indians. This is likely influenced by genetic variation among ethnic groups (Huang *et al*., 2006). In South Africa, studies have shown that the prevalence of T2DM is high in Indian communities, followed by Mixed Ancestry, Caucasians and Black African ethnic groups (Molleutze and Levitt, 2005; Rheeder, 2006). As illustrated in Table 1.2 women are more affected than men in most ethnic groups.

Table 1.2. Self-reported prevalence of diabetes (%) in South African men and women aged ≥15 years (*Molleutze and Levitt, 2005*).

PROVINCE	**WOMEN**	**MEN**
Western Cape	4.9	3.2
Eastern Cape	3.5	2.7
Northern Cape	2.9	2.1
Free State	2.3	1.3
KwaZulu-Natal	5.9	3.1
North West	1.1	0.9
Gauteng	4.3	3.3
Limpopo	1.2	0.9
POPULATION GROUP		
Black African	3.0	1.6
Urban	3.7	1.6
Rural	2.2	1.5
Mixed Ancestry	5.8	3.1
Caucasians	4.8	6.0

1.6. Pathophysiology of T2DM

Diabetes mellitus is a complex multifactorial disease that results from the interaction of multiple defective genes and several environmental factors. With the increasing prevalence of obesity throughout the world, diabetes has now reached epidemic proportions. According to the World Health Organisation (WHO), the escalating prevalence of diabetes and obesity is a result of Westernized diet and lack of exercise (Figure 1.3) (Wild *et al*, 2004; Clement, 2006).

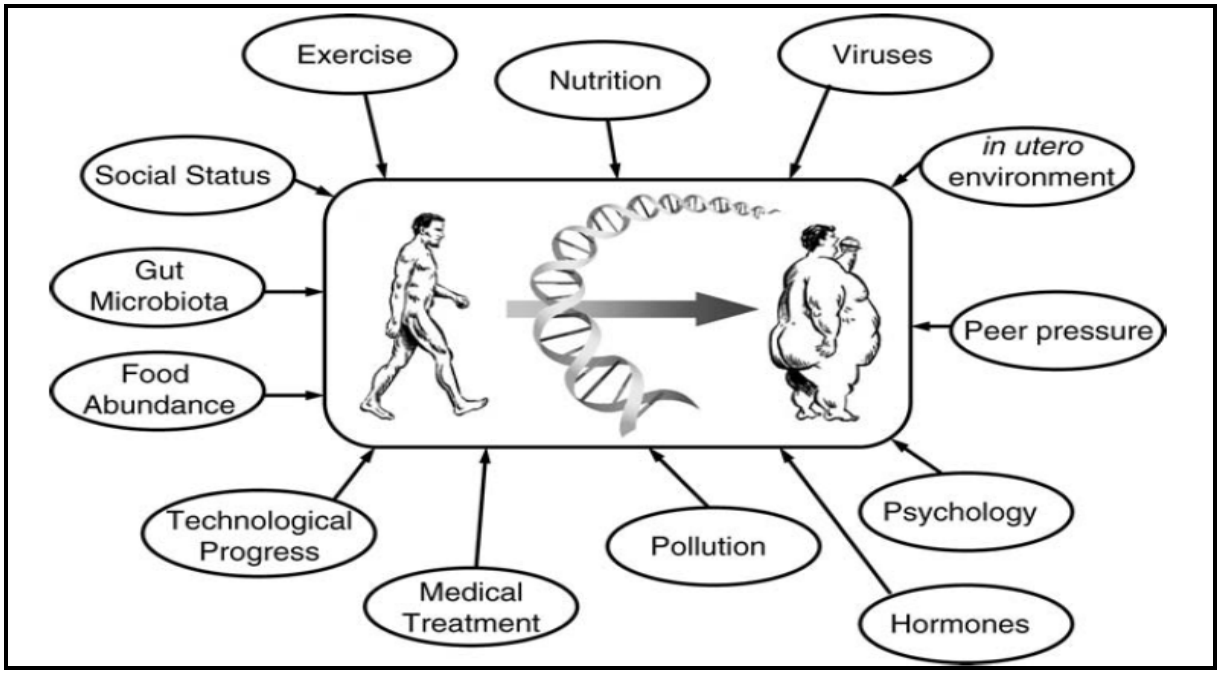


Figure 1.3. Genetic and environment risk factors contributing to the pathogenesis of obesity and diabetes (*Adapted from Mutch and Clement, 2006*).

1.6.1. Obesity as a risk factor for T2DM

Obesity is the primary risk factor for T2DM, with approximately 80% of T2DM patients having a body mass index (BMI) > 29.99 kg/m^2 (Thevenod *et al,* 2008). Obesity is measured by the BMI, which is defined as an individual's weight in kilograms divided by the square of the height in meters (James *et al*., 2001). Several studies have demonstrated that the risk of developing T2DM increases proportionally with BMI or obesity (Table 1.3) (James *et al*., 2001, Johnstone *et al*, 2001). However, the use of BMI as a biomarker for metabolic diseases is controversial due to its heterogeneous nature. It has been clearly demonstrated that it is influenced by many factors such as height, bone mass and lean mass (Franks *et al*., 2008).

Table 1.3. Classification of weight by BMI (*WHO report, 1998*).

Classification	BMI (kg/m^2)	Risk of co-morbidities
Underweight	<18.5	Low (but risk of other clinical problems increased)
Normal range	18.5 to 24.9	Average
Overweight	≥ 25	
Pre-obese	25.0 to 29.9	Increased
Obese class 1	30.0 to 34.9	Moderate
Obese class 2	35.0 to 39.9	Severe
Obese class 3	≥40.0	Very severe

Studies have revealed that the development of metabolic syndrome, T2DM and cardiovascular disease (CVD) are more likely influenced by the percentage and distribution of body fat (Meisinger *et al*., 2006; Jennings *et al*., 2009; Luo *et al*., 2013; Borné *et al*., 2014). It is therefore suggested that waist circumference (WC) be measured in addition to BMI to assess the risk of type 2 diabetes in both men and women. As illustrated in the table 1.4, waist circumference cut-off values as recommended by the IDF differ according to gender and ethnicity, and are not related to the BMI values. When matched for BMI, women have more body fat than men due to greater subcutaneous adipose tissue in females. It has also been observed that females at menopause have a selective deposition of visceral fat, and as a result this poses challenges with the use of waist circumference as a predictor of metabolic diseases. Whether or not gender differences in waist circumference values are translated into the clinical metabolic outcome is debatable as studies have reported different findings, some reporting a higher incidence of metabolic diseases among men in each category of BMI, WC, and waist height ratio (WHR) (Meisinger *et al*., 2006; Regitz-Zagrosek *et al.,* 2006; Fezeu *et al*., 2007); while other studies have found a higher risk in women (Beigh and Jain, 2012). These inconsistent findings may be due to other related factors such as genetic background, aging, physical inactivity, drugs and hormones, and ethnicity.

Ethnic variations in fat distribution have also been observed in both men and women. Several studies have highlighted variation in fat distribution among different populations. For example, it has been observed that Asians have higher visceral body fat (Deurenberg-Yup *et al*, 2000; Chang *et al.,* 2003; Sandeep *et al*., 2010; Zhu *et al.,* 2011; He *et al*., 2013) whereas Caucasian women were found to accumulate more fat centrally compared to their black African counterparts (Lovejoy *et al*. 1996; Punyadeera *et al*. 2001). More interesting is the differential distribution of metabolic-related traits among Caucasian and black African women: obese black Southern African women have a higher prevalence of diabetes (7.0% vs. 3.6%) and hypertension (30% vs. 15%) compared to obese Caucasian women who

commonly present with coronary heart disease and hypercholesterolaemia (Seedat, 1983; Schutte *et al.* 2008). It is speculated that the preferential accumulation of more gluteofemoral and subcutaneous adipose tissue (SAT) in black African women may be strongly correlated with the development of insulin resistance.

In addition to waist circumference, the waist-to-hip circumference ratio (WHR) (Hughes *et al.*, 2004), or to a lesser extent sagittal abdominal diameter (SAD) (Harris *et al.,* 2000) have been used to determine abdominal obesity. Other non-anthropometric methods such as underwater weighing, dilution techniques and dual-energy X-ray absorptiometry (DXA) are also available and can be used reliably to obtain accurate measures of total body fat (Jebb *et al*., 1993; Snijder *et al.,* 2002; Mattsson and Thomas, 2006). Furthermore, techniques such as magnetic resonance imaging (MRI) and computed tomography (CT) have been found useful in differentiating fat depots at the waist level. However, due to their costs in terms of time and money, these methods are not practical for use in large epidemiological studies and clinical settings.

Table 1. 4. Ethnic-specific values for waist circumference according to International Diabetes Federation (*IDF, 2011*).

Country/ethnic group	**Waist circumference value**	
	Male	**Female**
Europids*	≥94 cm	≥80 cm
South Asians‡	≥90 cm	≥80 cm
Chinese	≥90 cm	≥80 cm
Japanese	≥85 cm	≥90 cm
Ethnic South and Central Americans	Use South Asian recommendations until more specific data are available	
Sub-Saharan Africans	Use European data until more specific data are available	
Arabs	Use European data until more specific data are available	

*In the USA, the ATP III values (> 102 cm male; > 88 cm female) are likely to continue to be used for clinical purposes, ‡Based on Chinese, Malay and Asian-Indians populations.

The mechanism by which visceral fat causes diabetes is not fully understood but it is hypothesized that visceral fat adipocytes are more lipolytically active and thus increase circulating levels of free fatty acids (FFAs) (Kahn *et al.*, 2000). The increased levels of FFAs are known to inhibit insulin clearance leading to hyperinsulinemia, as well as competiung with glucose thus leading to insulin resistance (Kahn *et al.*, 2000). There is also evidence that suggest that visceral fat could also induce secretion of inflammatory adipokines such as tumor necrosis factor α (TNF-α), interleukin-6- (IL-6), monocyte chemoattractant-1 protein (MCP-1), and resistin which all leads to insulin resistance (Fantana *et al.,* 2007).

It has also been speculated that insulin resistance is a link between obesity and T2DM. However, not all individuals with insulin resistance progress to T2DM as only about 20% of obese individuals develop the disease, while most of them are able to maintain euglycemia throughout their life span in spite of insulin resistance (Lan *et al.*, 2002). Insulin resistance precedes the development of T2DM. This has been supported by the consistent presence of insulin resistance in patients with T2DM (Savage *et al.*, 2005). However, not all people with insulin resistance develop T2DM, the disease develops only if their β-cells produce insufficient insulin. Beta-cell dysfunction is thought to be the determining factor for progression of insulin resistance to T2DM. Several studies have documented that individuals at increased risk of subsequently developing diabetes exhibit β-cell dysfunction well before they would be considered to have reduced glucose tolerance (Fonseca, 2009). Such people include women with a history of gestational diabetes or polycystic ovarian syndrome, aging individuals who frequently develop Hyperglycemia as they continue to grow older, and those with impaired glucose tolerance. In individuals with β-cell dysfunction and a resultant inadequate insulin secretion, blood glucose levels continue to increase due to the impaired inhibition of hepatic glucose production and its uptake by the liver and muscle. This was also evident in first-degree relatives of people with T2DM from different ethnic backgrounds who had impaired β-cell function in common in addition to being genetically at risk of developing the disease as reported by Jensen and co-workers (2002). The highly elevated blood glucose concentration may further contribute to the glucotoxic and harmful effect on the β-cells and insulin sensitivity. In contrast, increasing blood glucose levels in healthy individuals for 20 hours has been reported to have an opposite effect, increasing insulin sensitivity and enhancing β-cell function (Kahn *et al*, 1992). In these individuals insulin resistance and impaired β-cell function seemed to be the common mechanisms underlying the development of T2DM.

The relationship between fat accumulation in adipose tissue and myocytes, and insulin resistance remain elusive, but is speculated to involve enhanced systemic chronic inflammation that is characterized by altered cytokine production and increased acute-phase reactants, which ultimately result in impaired insulin signalling pathway (Figure 1.4) (Wellen and Hotamisligil, 2003). Increased levels of inflammatory intermediates can be used as biomarkers for diabetes and other related diseases including insulin resistance (Qatanani and Lazar, 2007; Qi *et al.*, 2009).

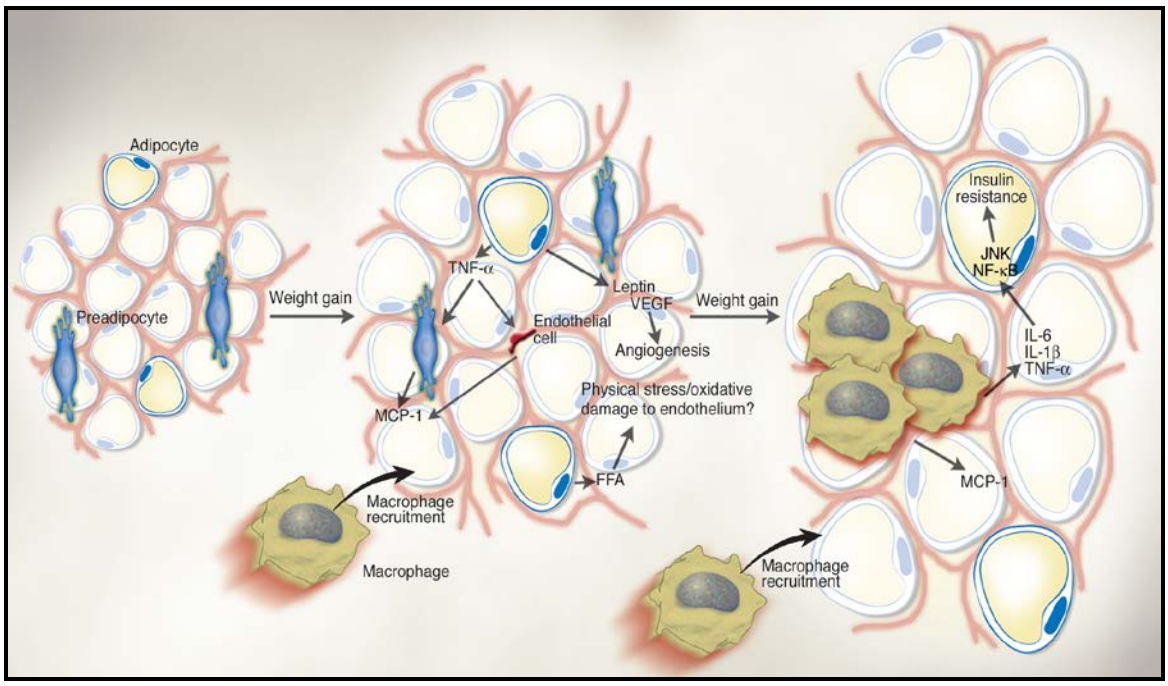


Figure 1.4. Changes in adipocytes as adiposity increases from the lean to the obese state. The adipose tissue is characterized by inflammation and infiltration of macrophages which may induce insulin resistance (*Adapted from Wellen and Hotamisligil, 2003*).
Abbreviations: TNF-α, tumor necrosis factor- α; MCP-1, mnjonocyte chemoattractant protein -1; FFA, Free fatty acids; EGF; IL-6, interleukin-6; IL-1β, interleukin-1β; JNK, Jun N-terminal Kinase; NF-κβ, nuclear factor kinase-β.

1.6.2. Environmental factors predisposing to T2DM

Physical inactivity: The past several decades has been characterised by globalisation and urbanisation, which are considered the main driving forces of the increase in prevalence of T2DM. Supporting this is the observation of the effect of demographic changes in the prevalence of T2DM as people migrate from rural to urban areas societies (Pouane *et al*., 2005). As a result of this migration, traditional manual labour and walking have been replaced with sedentary lifestyle which is characterized by lack of physical activity that is accompanied by the consumption of calorie-dense processed food (Bianchini *et al.,* 2002). The combination of sedentary lifestyles and diets rich in both animal fat and soluble carbohydrates play significant roles in the development of obesity and T2DM (Mollentze *et al*., 1993; Pouane *et al*., 2005). This has been supported by studies which have shown that lifestyle changes especially exercise and low-fat diets can reduce the conversion of impaired glucose tolerance to diabetes by up to 58%, whereas oral hypoglycemic medication only reduce risk of progression to T2DM by 30% (Tuomilehto *et al*., 2001; Vinik, 2007). Exercise is known to improve glucose and lipid metabolism and thus decreasing the risks of developing.

Poor diet: Diets high in fat, calories and cholesterol increase the risk of developing T2DM as these lead to obesity, a strong risk factor for the disease (Bianchini *et al.,* 2002). In addition to quality of food, quantity of food is also important. According to Ahlqvist *et al* (2011)

affluence is not a factor in the increased prevalence of T2DM but the problem is that seemingly humans are programmed to overeat and this is linked to the genes that select and favour energy-preserving genotypes which maximize the storage of energy and thus maximizing the probability of survival are programmed (Ahlqvist *et al*., 2011).

Socioeconomic status: There is increasing evidence that suggest that socioeconomically disadvantaged groups experience a high risk of T2DM (Stringhini *et al*, 2013). Supporting this is the evidence that show that the prevalence of T2DM varies with social economic class within populations (Robbins *et al.* 2001). The high prevalence of T2DM in low socioeconomic class is pronounced in the ages between 40 – 69 years (Connolly *et al*, 2000). The mechanism by which low socioeconomic status causes T2DM is not fully understood. However, it is known that people in low social class are less likely to go for routine health checks thus impacting on people's wellness by remaining undiagnosed until such time when the disease is complicated with associated comorbidities. Further, low economic status is hypothesized to partly mediate T2DM through the elevated inflammatory state that results from the altered gene expression and unhealthy lifestyles. Low social economic is now used as a risk factor for inflammation-related chronic conditions such as cardiovascular disease (CVD) and T2DM (Stringhini *et al*, 2013). As discussed above, inflammation affects insulin signalling and increases beta-cell death through elevated biomarkers such as TNF-α, IL-6 and C-reactive protein. Socioeconomic status is a complex construct that is often measured by different variables such as education, income and occupation (Robbins *et al.* 2001). The increasing evidence suggests that early life factors such as low birth weight and early life social adversities such as poverty may lead to programming a vulnerable phenotype through elevated inflammatory responses in adulthood (Stringhini *et al*, 2013).

Environmental pollutants: Several studies have revealed that the environmental toxins such as fire, car exhaust (nitrogen dioxide, carbon monoxide) and particulate matter (PM) are contributing factors for non-communicable diseases such as hypertension, cardiovascular disease and diabetes (Coogan *et al*, 2012). An example is the A U.S study that has revealed that diabetes prevalence among adults is higher in areas with higher PM2.5 concentration (Pearson *et al.* 2010). It has been observed that longer-term exposure over years does more harm than shorter-term exposure (Coogan *et al*, 2012). The mechanism by which these air pollutants confer diabetes is still to be elucidated but evidence suggests that air pollution promotes insulin resistance, fat formation, inflammation, oxidative stress and endothelial dysfunction (Coogan *et al*, 2012; Kelishadi *et al.* 2009). Xu and coworkers demonstrated that mice exposed to high levels of PM in their early life developed insulin resistance, and decreased glucose tolerance in adulthood (Xu *et al*. 2011). PM carries contaminants that are

able to trigger the production of free radicals and immune system cells known as cytokines that are involved in inflammation. The production of cytokines and free radicals may then affect other organs that are sensitive to oxidative stress (MohanKumar *et al*. 2008). For example, it is known that beta cells are sensitive to oxidative stress and that free radicals produced may cause beta cell destruction (Lenzen 2008a).

1.6.3. Genetics of T2DM

The contribution of genetic variation to the development and differences in the prevalenckkke of T2DM observed in various populations around the world had long been established (Tilburg *et al*., 2001). For example, it has been demonstrated that the sibling relative risk (λ_s), which is obtained by comparing the risk of developing T2DM between relatives of affected patients and the background population, is approximately 3 in most populations (Tilburg *et al*., 2001; Lyssenko *et al.,* 2005). However, for the disease to manifest the susceptible individuals need to be exposed to a risk environment. Studies have also shown that the lifetime risk of developing T2DM is 40% for individuals with only one parent affected with the disease (might be even higher if the mother is affected), but it can go up to 70% if both parents are affected (Ahlqvist *et al*., 2011). Further supporting evidence was obtained from twin studies which showed that the concordance rate of T2DM in monozygotic twins is about 70% compared to 20%–30% observed in dizygotic twins (Newman *et al*., 1987; Kaprio *et al*,. 1992). The completion of the Human Genome Project brought about new supporting evidence, enabling identification of risk alleles of T2DM in several genes. Since then several efforts have been made to identify the polymorphisms that may contribute to the development of T2DM in different populations, in order to better understand the pathogenesis of this disease, to find new targets for clinical therapy, and allow prediction of disease (Ahlqvist *et al*., 2011). However, these studies have been hindered by the complex nature of T2DM, which seems to be characterized by genetic heterogeneity and low penetrance of some of the reported risk alleles. To date, more than 50 candidate genes have been identified in various populations throughout the world (Table 1.5) (Ahlqvist *et al*., 2011). These polymorphisms were identified in genes that play important roles in insulin signalling and glucose metabolism (Kim and Misra; 2007).

Table 1.5. Few examples of genetic variants associated with T2DM. (*Ahlqvist et al., 2011*).

Variants	Nearest gene	Full gene name	References
rs4607103 rs6795735	*ADAMTS9*	ADAM metallopeptidase with thrombospondin type 1 motif 9	Voight *et* al. Zeggini *et* al
rs15552224	*ARAP1(CENTD2)*	ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 1	Voight *et* al
rs243021	*BCL11A*	B-cell CLL/lymphoma 11A (zinc finger protein)	Voight *et* al
rs2975760 rs3792267	*CAPN10*	Calpain 10	Weedon *et* al
rs127797m90	*CDC123/CAMK1D*	Cell division cycle 123 homologue- Calcium/calmodulin-depended protein Kinase ID	Voight *et* al. Zeggini *et* al
rs7754840	*CDKAL1*	CDKS regulatory subunit associated Protein 1-like 1	Saxena *et* al. Scott *et* al. Voight *et* al. Steinthorsdottie *et* al.
rs13292136	*CHCHD9/TLE4*	Coiled-coil helix-coil-coiled-helix domain containing 9/transducin-like enhancer of split 4	Voight *et* al
rs10811661	*CDKN2A/2B*	Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)-cyclin- dependent kinase inhibitor 2B (p15, inhibits CDK4)	Saxena *et* al. Scott *et* al. Voight *et* al
rs5945326 rs1111875 rs5015480	*DUSP9* *HHEX*	Dual specificity phosphate 9 Hematopoietically expressed homeobox	Voight *et* al Saxena *et* al Voight *et* al Sladek *et* al
rs1531343	*HMGA2*	High mobility group AT-hook 2	Voight *et* al
rs7957197	*HNF1A*	HNF1 homeobox A	Voight *et* al
rs1920792 rs7501939 rs757210 rs4430796	*HNF1B*	HNF1 homeobox B	Winckler et al Voight *et* al. Gudmundsson *et* al.
rs2334499	*HCCA2*	YY1 associated protein	Kong *et* al.
rs4402960	*IGF2BP2*	Insulin-like growth factor 2 mRNA binding protein 2	Saxena *et* al Scott *et* al. Voight *et* al
rs2943641 rs7578326	*IRS1*	Insulin receptor substrate 1	Almind *et* al Voight *et* al
rs864745 rs849134	*JAZF1*	JAZF1 zinc finger 1	Voight et al Zeggini *et* al

Table 1.5. *Continues*

Variants	Nearest gene	Full gene name	References
rs5219	*KCNJ11*	Potassium voltage-gated channel, KQT-like subfamily, member 11	Hani *et* al. Saxena *et* al Voight *et* al
rs2237892 rs163184 rs231362	*KCNQ1*	Potassium voltage-gated channel, KQT-like subfamily, member 1	Voight *et* al Unoki *et* al. Yasuda *et* al.
rs972283	*KLF14*	Kruppel-like factor 14	Voight *et* al
rs10923931	*NOTCH2/ADAM30*	Notch 2-ADAM metallopeptidase domain 30	Voight *et* al Zeggini *et* al
rs1801282 rs13081389	*PPARG*	Peroxisome proliferator-activated receptor gamma	Deeb *et* al Saxena *et* al Voight *et* al

Although a number of loci associated with diabetes have been identified through genome-wide association studies (GWAS), these have not been replicated in other studies involving other populations. A large amount of genetic data on T2DM risk polymorphisms is now available, however; we are still far from elucidating the mechanism underlying the pathogenesis of T2DM due to inconsistent findings from different genetic association studies (Torres *et al*., 2013). The inconsistencies could be attributed to differences in study design, populations analyzed, and sample sizes across studies.

1.6.3.1. Strategies used for identification of T2DM risk polymorphisms

The section below briefly describes the three strategies that have been used in many studies to elucidate the role of polymorphisms in common polygenic diseases, with specific reference to T2DM.

***Candidate gene approach method*:** This is based on the knowledge of the gene or protein function in relation to the disease in question, and determines the association between certain polymorphisms and the disease-related trait(s). Identification of polymorphisms and association testing using the candidate gene approach can also be conducted in populations rather than in families only as is done in linkage analyses. The short linkage disequilibrium (LD) regions in unrelated individuals require that a large number of markers be tested at a genome-wide level (Ahlqvist *et al.*, 2011). In this approach, a random sample of unrelated T2DM patients is compared with a matched controls, thus determining a polymorphic allele that occurs at a higher frequency in the patient group. The method allows testing a large number of samples for a modest number of targeted SNPs at low cost. In this method a

priori hypothesis about the biological function of the gene is required and this is a challenge because the role of many genes is not yet clear and can thus lead to bias positive findings that are difficult to replicate (Wilkening *et al*., 2008; Edenberg and Liu; 2009). A large number of studies have reported several functional candidate genes, however association could be replicated for only 6 genes: peroxisome proliferator-activated receptor gamma (*PPARG*), insulin receptor substrate 1 (*IRS1*), potassium inwardly-rectifying channel, subfamily J, member 11 (*KCNJ11*), Wolfram syndrome 1 (wolframin) (*WFS1*), HNF1 homeobox A (*HNF1A*), and HNF1 homeobox B (*HNF1B*). Genes such as *KCNJ11*, *WFS1*, *HNF1A*, and *HNF1B* that were also investigated due to their role in monogenic diabetes disorders were found to increase the risk of T2DM (Ahlqvist *et al.*, 2011).

***Linkage analysis method*:** This is a traditional method of identifying disease-causing genes which employs long LD genomic regions in affected families (Elbers *et al*., 2006). This strategy is based on the principle that affected family members share a certain marker that is identical by descent. This means that if the marker was inherited from the same affected parent, more often than expected by chance, a disease-causing variant is in LD with the genotyped marker (Gulcher *et al*., 2001). Using this method, one can screen the entire gene with a framework set of polymorphic markers in families and sibling pairs, and calculate the degree of linkage marker to the disease trait. This can be done to determine their occurrence or frequency in diseased compared to healthy individuals, and thus identifying known and unknown genes predisposing to T2DM or any phenotypic characteristic being studied. Although linkage analysis has been a method of choice for monogenic disorders, it has been less successful in identifying susceptibility alleles of complex diseases such as T2DM (Ahlqvist *et al*., 2011). According to a review by Elbers and co-authors (2006), more than 18 susceptibility loci were reported for T2DM by several studies, but only 2 genes have been consistently associated with T2DM: calpain 10 (*CAPN10*) and transcription factor 7-like 2 (T-cell specific, HMGbox) (*TCF7L2*) (Elbers *et al*., 2006; Ahlqvist *et al*, 2011). The figure below illustrates the chromosomal regions (q32,6q22-6q24,11q24,12q24,and 20q12 – 20q13) which encompasses about 612 genes which were found to be linked to both T2DM and obesity susceptibility (Elbers *et al*., 2006). Linkage analysis is known to be ineffective in finding rare polymorphisms with minor effects (Gulcher *et al*., 2001).

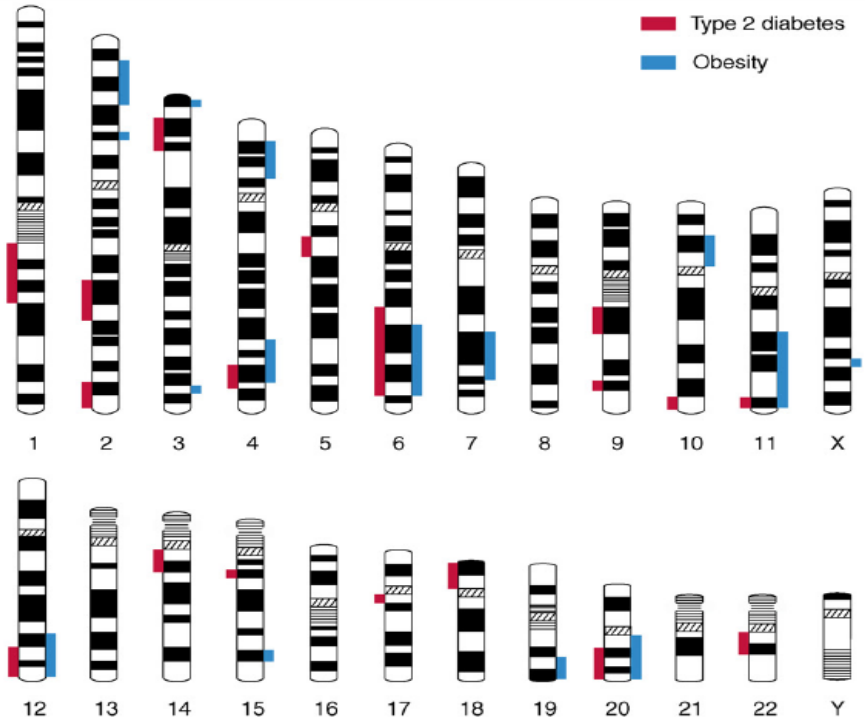


Figure 1.5. Genetic linkage map for T2DM and obesity. The red bars indicate the susceptible loci for T2DM and the blue bars for obesity (*Adapted from Elbers et al., 2006*).

***Genome-wide association*:** Genome-wide association studies (GWAS) is a high-through put technologies that is comprehensive and unbiased allowing simultaneous genotyping of thousands of SNPs. In contrast to candidate gene and linkage studies, genome wide association allows analyses of SNPs in the entire genome regardless of their functionality, and statistically tests their association with quantitative phenotypes (Huang *et al*., 2006). The goal of this approach is to uncover the genetic basis of a given disease by genotyping SNPs in cases and controls of a large population. In this approach only a subset of SNPs are collected and these are used as proxies for the uncollected SNPs thus taking advantage of linkage disequilibrium between SNPs. The disadvantage of this method is that it requires large numbers of individuals to reach a high statistical power to detect risk alleles with modest effects (Huang *et al*., 2006). Of the polymorphisms identified through GWA in genes such as at *PPARG*, *IRS1*, *KCNJ11*, *WFS1*, *HNF1A* and *HNF1B*; only those in *PPARG* and *KCNJ11* have shown replication across multiple studies (Torres *et al*, 2013). In addition to identifying more replicable association, GWAS have unravelled novel genes that were missed by the candidate approach and linkage analyses, and these include *SLC30A8 encoding* a zinc transporter (ZnT8).

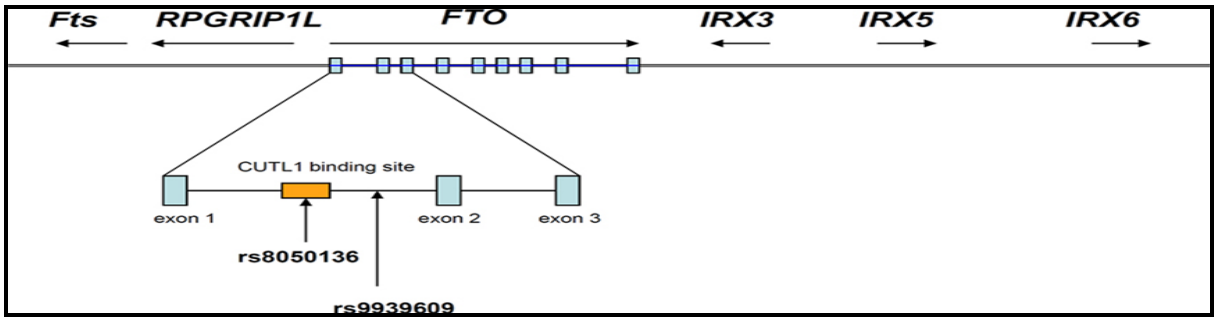
1.6.3.2. Genes selected for the present study

Although a large number of polymorphisms have been reported as genetic risk factors for T2DM, only six variants were chosen to be investigated in the present study, and these are discussed in the section below.

i. Fat Mass and Obesity-associated transcript (FTO) Gene

Structure: Fat mass and obesity- associated transcript gene (*FTO*) was identified as one of six genes (*Irx3*, Irx5, *Irx6*, *Fts* and *Rpgrip1*) located in a 1.6-Mb deleted region of chromosome 8 in a mutant mouse model (Frayling *et al*., 2007; Cheung and Yeo, 2011). It is a large gene with 9-exons. It spans more than 400 kb on chromosome 16q12.2 in humans and is well conserved in vertebrates and algae (Cheung and Yeo, 2011). The FTO gene encodes a 2-oxoglutarate-dependent dioxygenase, a member of the non-heme (Fe[II]) dioxygenase superfamily localized in the nucleus (Loos & Bouchard, 2008). The *FTO*-encoded protein consists of an N-terminal domain carrying a catalytic core and a C-terminal domain of unknown structural homology (Han *et al*., 2010) (Figure 1.6). According to Han and co-workers (2010), *FTO* has a substrate preference for single-stranded nucleic acids as evident in its crystal structure, which shows a Loop 1 extra region that hinders the unmethylated strand of dsDNA/RNA from gaining access to the substrate-binding site. The Loop 1 region is highly conserved among *FTO*s of different species, but is absent from AlkB and ABH proteins. The study also identified residues in the catalytic domain that are conserved in all members of the *FTO* superfamily: histidine and aspartic acid required for binding Fe(II); and three residues, histidine and two arginines separated by six amino acids required for 2-OG binding.

(A)



(B)

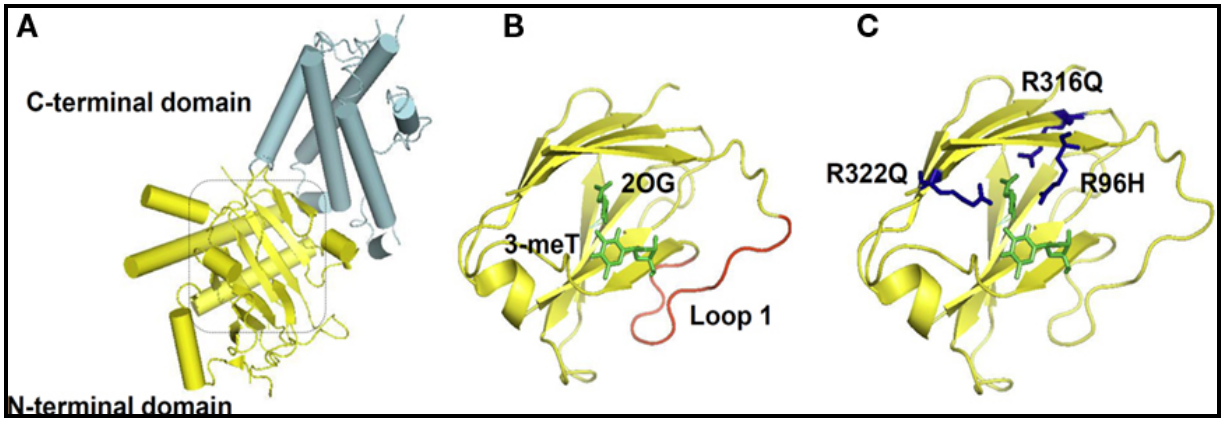


Figure 1.6. Genomic (A) and protein (B) structure of FTO gene. **A**: GenomicÑ structure of *FTO* and its neighboring genes (Cheung and Yeo; 2011). **B**: Protein structure of *FTO* illustrating the C-terminal (colored in cyan) with unknown structural homology and the N-terminal domain with a catalytic core (colored in yellow). Bound to the catalytic core is a substrate, 3-met and 2-oxoglutarate (colored in green). Residues R316 and R322 are known to cause a complete loss of function of FTO gene and these are required for binding of substrate (2-OG) to the gene (*Adapted from Cheung and Yeo, 2011*).

***Physiological function and association of FTO with T2DM*:** The FTO gene is expressed in numerous tissues (including adipose tissue, pancreatic islets, skeletal muscle and hypothalamus) in both fetus and adults (Stratigopoulos *et al*., 2008; Boissel *et al*., 2009), but predominantly in the hypothalamus suggesting a role in regulation of energy homeostasis (Fischer *et al.,* 2009). Evidence of its role in controlling energy expenditure was demonstrated in animal studies in which *FTO*-deficient mice developed increased energy expenditure and had an activated sympathetic nervous system regardless of reduced spontaneous locomotor activity and relative hyperphagia (Fischer *et al.,* 2009). Fat Mass and Obesity-associated transcripts has also been reported to play a role in DNA methylation (Attaoua *et al*., 2009; Wu *et al*., 2010). The *FTO* mutations detected in humans have been linked with polymalformation syndrome, which is characterised by postnatal growth retardation, microcephaly, severe psychomotor delay, functional brain deficits and facial dysmorphic features (Boissel *et al*., 2009). The effect of these mutations is suggestive of the role of *FTO* in the normal development of the central nervous system and cardiovascular systems. As a risk factor, *FTO* has been associated with obesity (Frayling *et al*., 2007; Cheung and Yeo, 2011). The first GWAS study to establish the association of *FTO*

polymorphisms with obesity was conducted in 1,924 T2DM-affected individuals and 2,938 controls, findings that were replicated in 13 cohorts consisting of 38,759 individuals (Frayling *et al*., 2007). Scott and co-workers (2007) reported an association of the *FTO* rs9939609 SNP with T2DM, but the relationship was weakened after adjusting the analysis for BMI. The association was replicated in a large study (Wellcome Trust Case Control Consortium, 2007), which reported odds ratio of heterozygotes is 1.34 for heterozygotes and 1.55 for homozygotes; as well as an isolated Norwegian population (Hertel *et al*., 2008). In an American Indian population, the rs9939609 SNP was significantly associated with T2DM after adjusting the analysis for age, gender, and BMI (Sanghera *et al*., 2008). The rs3751812 and rs9941349 SNPs, which is the focus of the present study, showed strong association with BMI (P=2.58 x10 10 6 and 3.61 x 10^6 respectively) in a meta-analysis of 9881 African-derived population. Based on the results observed, these two SNPs influenced BMI at a similar level of association and thus speculated that one of them could be the causal polymorphism at this locus (Hassanein *et al*., 2010). The mechanism underlying the association of *FTO* polymorphisms with obesity and T2DM is yet to be elucidated. Although it is speculated that intronic polymorphisms may affect the expression level of their corresponding genes, in their study Grunnet and co-workers (2009) reported that the expression levels of *FTO* in human skeletal muscles and adipose tissue was not influenced by the presence of the rs9939609 polymorphism which is located in intron 1. An age-related reduction of the *FTO* expression levels has been suggested to affect glucose and lipid metabolism (Wåhlén *et al*., 2008).

ii. Ectonucleotide pyrophosphatase/phosphodiesterase1 gene (*ENPP1*)

Structure: Ectonucleotide pyrophosphatase/phosphodiesterase1 (also known as plasma cell-1, *PC-1*) is a 25-exon long gene located on chromosome 6q22-23 (Buckley *et al*., 1990; Lee *at al.,* 2010). It is a member of a family of five enzymes (ENPP1-5) that are known to regulate nucleotide metabolism. Ectonucleotide pyrophosphatase/phosphodiesterase1 is a homodimer consisting of a short intracellular N-terminal domain (with 10–80 residues), a single transmembrane domain (with approximately 20 residues), and a large extracellular part (with approximately 800 residues) comprising of several domains such as two consecutive somatomedin B-like domains responsible for *ENPP1* dimerization (Figure 1.7) (Goldfine *et al.,* 2008; Dimatteo *et al*., 2013).

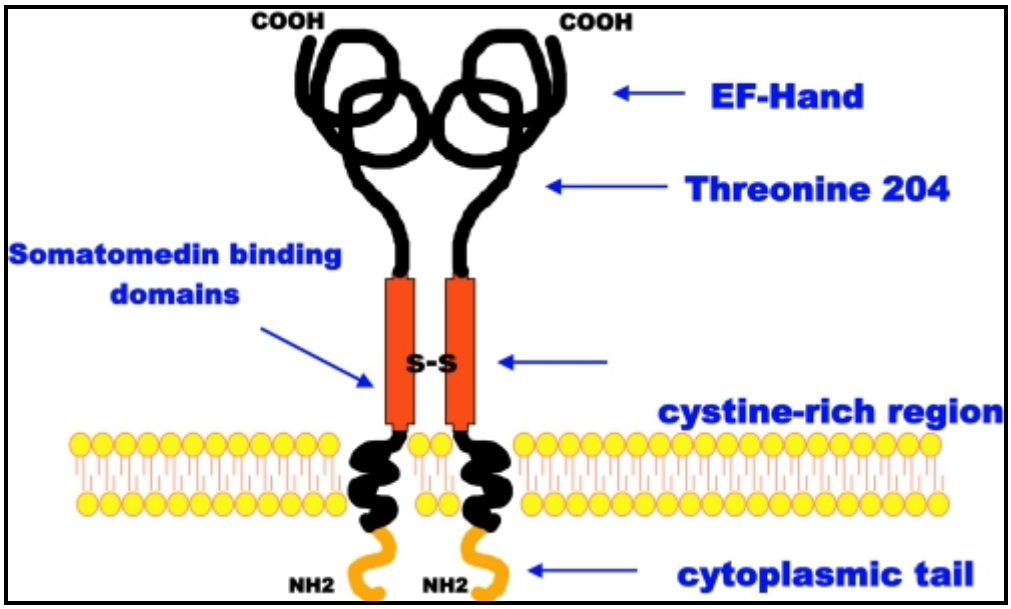


Figure 1.7. Structure of *ENPP1*. Major domains include EF-Hand, Ca^{2+} binding domain that maintains the structure, Threonine 204, phosphodiesterase/pyrophosphatase site and somatomedin-binding domans (*Adapted from Goldfine et al., 2008*).

***Physiological function and association of ENPP1 with T2DM*:** The physiological function of *ENPP1* is not fully understood, but there is evidence supporting its role in bone and cartilage metabolism (Johnson *et al*., 1999; Rutsch *et al.,* 2001). Ectonucleotide pyrophosphatase/phosphodiesterase1 is ubiquitously expressed, most importantly in insulin-sensitive tissues such as liver, skeletal muscle and adipose tissue. The role of *ENPP1* in relation to insulin resistance and T2DM has been widely investigated. Several studies have reported that the genomic region where *ENPP1* was mapped is linked to insulin resistance, highlighting its disease risk-associated effect (Goldfine *et al*., 2008). Ectonucleotide pyrophosphatase/phosphodiesterase1 has been reported to play a role in the pathogenesis of insulin resistance through its effect on insulin receptor function (Goldfine *et al.*, 2008). It is believed that the *ENPP1* binds to the domain of the insulin receptor α-subunit that is located at residues 485 – 599, thus inhibiting the insulin-induced autophosphorylation activity of tyrosinase kinase (Bjornholm and Zierath; 2005; Lyon *et al*., 2006; Goldfine *et al.,* 2008; Lee *et al*., 2010). Other lines of evidence exist, for example, *in vitro* studies demonstrated that culture cells overexpressing *ENPP1* were less responsive to insulin (Maddux *et al*., 1995). Moreover, insulin resistance and diabetes were observed in transgenic animal models overexpressing *ENPP1* (Maddux *et al*., 2006). It has also been observed that overexpressed *ENPP1* correlates with increased BMI and decreased insulin stimulation of muscle glucose transport (Mackenzie *et al*, 2012), however, the mechanism is not fully understood. Although several *ENPP1* polymorphisms have been reported to contribute to the development of

insulin resistance, only the K121Q (Lys121Glu, rs1044498) polymorphism has been widely studied. This polymorphism has been associated with insulin resistance and its related comorbidities including T2DM by many but not all studies (Lyon *et al*., 2006; Stolerman *et al.,* 2008; Müssig *et al*., 2010). According to the studies, the K121Q polymorphism contributes to insulin resistance through inhibition of the insulin receptor, and enhanced binding affinity to the cell membrane (Goldfine *et al.,* 2008). Other polymorphisms such as rs997509 and rs1044498 have received little attention. The rs997509 polymorphism is an intronic SNP which has been associated with T2DM (Bochenski *et al*., 2006), metabolic syndrome and impaired glucose tolerance (Santoro *et al*., 2009) in the presence of obesity.

iii. Transcription factor 7-like 2 gene (*TCF7L2*)

Structure: Transcription factor 7-like 2 (formerly known as TCF4) is a 17-exon gene mapped on chromosome 10q25.3. The TCF7L2 gene encodes the transcription factor 4 (TCF4), which consists of two major domains: a catenin-binding domain (exon 1) and a central DNA-binding high mobility group (HMG) domain (exon 10 and 11) (Yu-ting *et al.,* 2012). At least five of the *TCF7L2* exons are alternatively spliced (white boxes in Figure 1.8A) to form numerous transcripts which give rise to a number of isoforms (Hansson *et al.,* 2010; Pang *et al*., 2012; Yu-ting *et al.,* 2012).

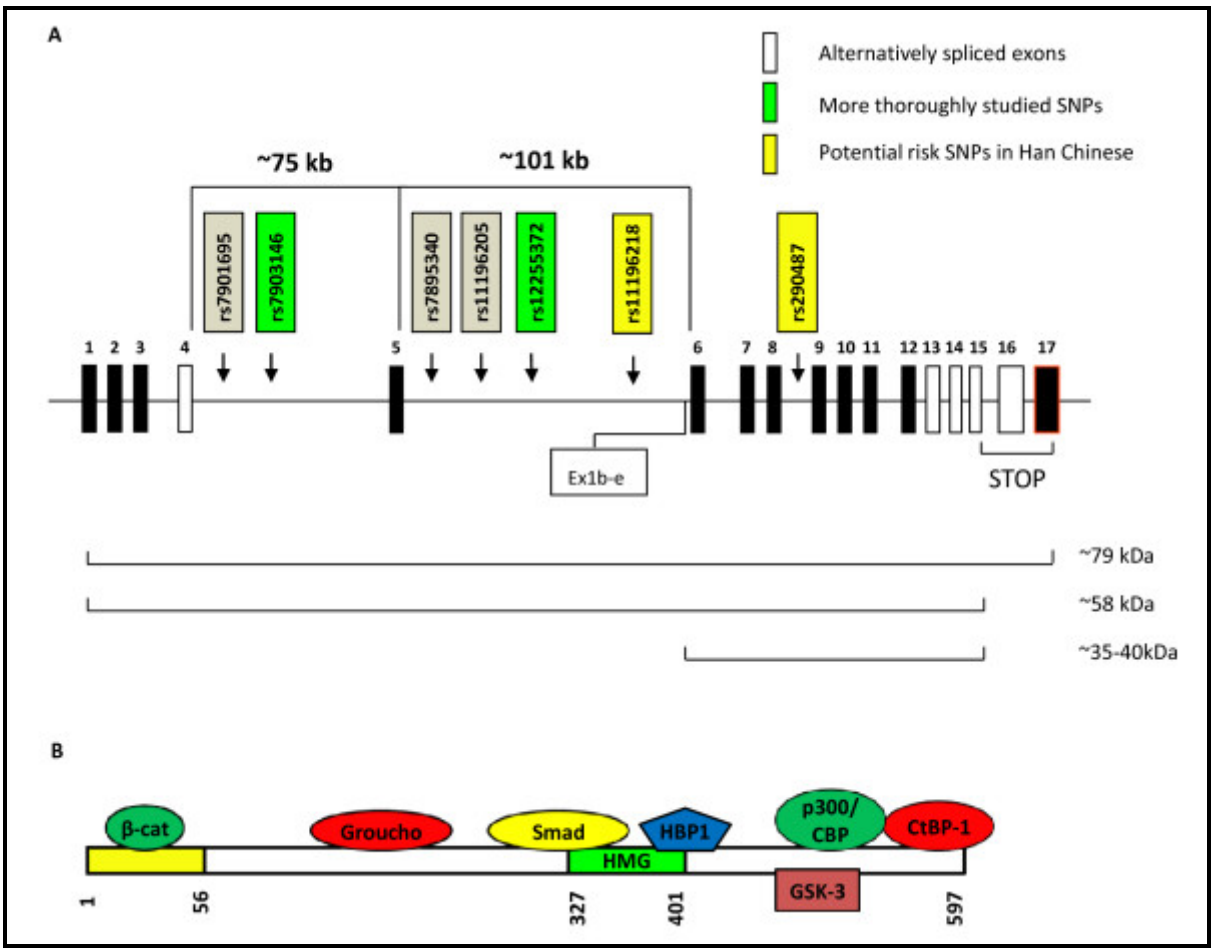


Figure 1.8. Genetic (A) and protein (B) structure of *TCF7L2.* Genetic structure illustrates T2DM risk SNPs located on chromosome 10q25.3. Protein structure illustrates the two major domains, the β-cat binding at the N-terminal and the HMG-box for binding to DNA (*Adapted from Ip et al., 2012*). *Abbreviation*: β-cat, βeta-catenin; HMG, high mobility group; GSK-3, glycogen synthase kinase-3, CtBP-1, C-terminal binding protein, HBP1, HMG-box transcription factor-1; p300/CBP, cofactor family, Creb binding protein.

***Physiological function and association of TCF7L2 with T2DM*:** *TCF7L2* has a wide tissue distribution, but is expressed predominantly in the pancreatic islet and adipose tissue. TCF4 is a member of the T-cell specific high mobility group (HMG) box, a family of transcription factors which play an important role in downstream signals of the canonical morphogenic wingless type (MMTV) integration site family Wnt pathway (Yang *et al*., 2012). The Wnt signalling pathway is involved in a wide range of cellular processes such as embryonic development, adipogenesis, pancreatic islet development, stem cell maintenance, cell fate, cell proliferation, cell migration, tumor suppression, and oncogenesis (Chien *et al*., 2009). The activated Wnt signalling results in the accumulation of e β-catenin in the nucleus which then interacts with the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors thereby regulating the expression of Wnt target genes involved in the regulation of beta-cell proliferation, differentiation and insulin secretion (Chien *et al*., 2009; Buraczynska *et al*., 2011; Yang *et al*. 2012). Transcription factor 7-like 2 plays a vital role in the development of the pancreas. It is required for SDF-1/CXCR4-induced cytoprotection of

β-cells which helps to increase the number of β-cells (Hansson *et al.,* 2010). There is also evidence suggesting that TCF4 interacts with glucagon-like peptide 1 (GLP-1) to regulate pancreatic islet function (Yu-ting *et al.,* 2012). The role of GLP-1 is to maintain the nondiabetic state by promoting the satiety signal in the hypothalamus and slowing down gastric emptying (Hansson *et al*., 2010). In 2006 GWAS identified *TCF7L2* as a strong T2DM susceptible gene and this has subsequently been confirmed by many studies (Franklin *et al*., 2010). Decreased beta-cell mass, impaired insulin processing or release, impaired glucagon-like peptide-1-(amide) signalling in beta-cells, and liver insulin resistance have been suggested as the underlying effects of *TCF7L2* polymorphisms (Pearson, 2009). The association of *TCF7L2* with T2DM was first reported in Mexican-American populations *(*Duggirala *et al*., 1999)*,* however at that time the specific gene was unknown until it was reported in 2006 (Grant *et al*., 2006*)*. Since then the association of *TCF7L2* SNPs with T2DM has been reported in several studies in different ethnic population groups (Ip *et al*., 2012). Two of *TCF7L2* SNPs, the rs7903146 and rs12255372 (green bars in Figure 1.7A) has shown the most strong association with T2DM, with rs7903146 having the greatest effect in Caucasian populations (Ip *et al*., 2012). The mechanisms by which *TCF7L2* affects diabetes susceptibility are yet to be elucidated.

1.7 Problem statement and motivation

Type 2 diabetes mellitus is a result of genetic predisposition of certain individuals with the disease precipitating when carriers of risk alleles are exposed to a risk environment such as sedentary lifestyle and or/overconsumption high-fat diet. Patients with diabetes mellitus have increased risk of cardiovascular disease, blindness, renal failure and many other diabetes associated-traits. Type 2 diabetes mellitus has become an increasing public health problem globally, and it is estimated that by 2035 about 592 million people worldwide will be affected. This poses a financial burden on the government due to the costs (estimated to be between 213 and 296 billion worldwide in international dollar currencies) associated with the treatment of diabetes patients. There is limited data on the epidemiology of T2DM in Africa and as a result its impact on people's wellness remains underestimated. Type 2 diabetes was initially thought to present late in life, but recent data provide evidence for its existence in children and young adults. In those countries with limited or old prevalence reports many people remain undiagnosed until such time when the disease is complicated with associated comorbidities. In the 1960s and 1980s the prevalence of type 2 diabetes in South Africa ranged from 0.6 to 3.6% (McLarty *et al*., 1990). However this number has increased drastically and is expected to increase even further as projected by the IDF (IDF, 2013). Therefore, understanding the mechanisms underlying the development of T2DM and the

discovery of risk factors, be it environmental or genetic, which may contribute to its pathogenesis is crucial for early detection of the disease. Recently, our group reported a high prevalence of T2DM (26%) in a Bellville South community in Cape Town (Erasmus *et al*., 2012). In addition, this community has a high prevalence of obesity, but the risk factors associated with the disease and T2DM are currently unknown. There are few studies in African populations on genetic variants; in fact most data on genetic variants is derived from African Americans population which predominantly originates from West Africa. African American somehow exhibit varying degrees of genetic admixture from that of subjects in sub-Saharan Africa. It will be interesting to see whether or not genetic loci of T2DM identified in African-Americans are similar to those that may predispose indigenous Africans to T2DM. This therefore suggests that more work still needs to be done to understand the diabetogenic effect of genes in subjects from sub-Saharan African countries. In European populations several risk polymorphism have been consistently shown to be associated with T2DM and these include *PPARG*, *KCNJ11*, *PKN2*, *IGF2BP2*, *CDKAL1*, *SLC30A8*, *CDKN2A*/ *CDKN2B*, *EXT*/*ALX4*, *FTO*, *TCF7L2* and *HHEX.* The association of some loci (*PPARG*, *KCNJ11*, *FTO*, *TCF7L2* and *HHEX*) with T2DM could not be replicated in South African Zulu subjects (Pirie *et al.,* 2010). The adiponectin gene (*ACDC*), which was demonstrated to play a major role in T2DM susceptibility in a French Caucasian population (Vasseur *et al*., 2002) was not a risk factor in black South African populations (Olckers, *et al*, 2007). This therefore suggests that SNPs have differential effects on different populations. Our research group has recently shown that *PPARG* Pro12 is significantly associated with type 2 diabetes and it conferred about 64% risk of type diabetes (Vergotine *et al*, 2014). The community of Bellville South has been identified as a high risk population group for type 2 diabetes, but the genetics that predispose this ethnic group to the disease is still poorly understood. In this study the *FTO, TCF7L2* and *ENPP1* genes which are known risk factors of either T2DM or obesity were investigated for their association with T2DM in this community of Bellville South, Cape Town.

Study aims and Objectives

- To determine the allele and the genotype distribution of *FTO* rs3751812 and rs9941349, *TCF7L2* rs7903146 and rs12255372, and *ENPP1* rs997509 rs1044498 SNPs in Mixed Ancestry ethnic group that resides in Bellville South, Cape Town.

- To examine the association between the selected SNPs and T2DM-related phenotypes such as anthropometric measurements, blood pressure (BMI, weight, height, waist circumference) and metabolic traits (oral glucose tolerance test (OGTT), impaired fasting glycaemia (IFG), hemoglobin a1c (HbA1c),homeostasis model

assessment (HOMA), high density lipoprotein-cholesterol (HDL-C), total cholesterol, and insulin sensitivity) in the study population.

CHAPTER 2
MATERIALS & METHODS

2.1 Ethical considerations, confidentiality and study design

The present study was an extension of a research project conducted in a Bellville South community that aimed at establishing a cohort that could be followed up for insulin resistance and other metabolic syndrome risk factors. The parent study was approved by the Faculty of Health and Wellness Sciences Ethics Committee of the Cape Peninsula University of Technology (CPUT) (reference number: CPUT/HW-REC 2008/002), and was conducted according to the code of ethics of the World Medical Association (Declaration of Helsinki). For the current study, additional ethics was obtained. Permission to conduct the study in the Bellville South community was granted by relevant authorities such as the city municipality and community leaders. All recruited participants had the right to decide voluntarily whether to participate in the study or to withdraw without the risk of incurring penalties or prejudicial treatment. They also had the right to refuse to give information or to ask clarity about the objectives of the study or specific procedures. The data was collected from mid-January 2008 to March 2009. All participants who were recruited for the study consented for their blood sample to be stored and used for future studies, including genetic analysis after the procedures had been fully explained in the language of their choice.

The detailed setting of the parent research study is described in Matsha *et al.,* 2012. Briefly, Bellville South is traditionally a mixed-ancestry township formed in the late 1950s and according to 2001 population census, the community has approximately 26 758 people, with 80.48% of mixed ancestry. Using a map of Bellville South, 16 short streets, representing approximately 190 houses, 15 medium streets representing approximately 410 houses and 12 long streets representing approximately 400 houses were randomly selected across the different strata. The household members from the selected streets who were eligible to participate in the study were approached and invited to participate in the study.

2.2. Study population

The study population was selected from 946 (722 females and 224 males) individuals of Mixed Ancestry, of whom 642 of them were randomly selected subjects (age 35-65 years) and 304 volunteered to participate (age between 16-95 years). The participants were then subdivided into diabetic (cases) and non-diabetic (controls) groups based on their diabetes status, which was defined as FPG ≥ 7 mmol/l or ≥ 11.1mmol/L and a HbA1c of greater than or equal to 6.1%. The selection criteria were as follows:

2.2.1 Inclusion criteria:

- All participants who consented to genetic analyses and who had blood samples collected were included.
- Unrelated Mixed Ancestry individuals, both females and males aged 35–65 years.
- The case study group consisted of individuals with known T2DM according to their medical records (disease diagnosed after the age of 25 years), and those who were diagnosed during the study period using the 75-g oral glucose tolerance test (OGTT) according to the World Health Organisation (WHO Consultation, 1999) definition, and the American Diabetes Association (ADA) criteria using fasting blood glucose.
- Unrelated individuals without a known diagnosis of diabetes or renal disease during the time of the study were included as controls. This group was age-, gender- and ethnic-matched to the cases.
- Participants who had all the required measurements and biochemical analysis data.

2.2.2 Exclusion criteria

- Participants who chose not to give blood for DNA analysis because of various personal reasons such as low blood pressure, anxiety problems related to the sight of blood and fear of surgical needles, and those who did not consent to participate in the study.
- Participants with diabetes-independent nephropathy and other forms of diabetes according to their medical records they provided and age of diagnosis (below the age of 25 years) were also excluded.

2.3. Data collection

Biochemical analysis and anthropometric measures were performed in order to obtain relevant information about biological or physiological parameters, which may be correlated with the genetic data that was obtained from the proposed study. All measurements and biochemical analysis were conducted by professional medical nurses and qualified technologists with strict adherence to quality control. In addition, questionnaires were designed to obtain information about lifestyle behavior, family history of T2DM and other chronic diseases. These parameters obtained are as follows:

a) Questionnaire design

All questionnaires were designed to contain adequate indicators of the family history of diabetes, lifestyle behaviour, dietary patterns, physical activity and leisure activities of all the participants. These questionnaires were developed in English and translated into Afrikaans,

and pre-tested in a pilot study with respect to the ability of respondents to understand and respond to questions well. Questionnaires were pre-tested in a random small sub-sample of individuals with similar characteristics as the study population (Somers, 2004). Reliability of the answers received was ensured by re-administering these questionnaires to the same group of people a week after the initial administration in a sub-group.

b) Measurement of anthropometric parameters

Anthropometry is the measurement of the human body in terms of dimensions of bone, muscle and adipose tissue. Example of anthropometric measurement include human body measurements such as weight, stature (standing height), recumbent length, skinfold thickness, circumference (head, waist, limb etc), limb length and breadth (e.g. shoulder, wrist etc) and many others (Ulasjaszek, 1994). Anthropometric measurements such as BMI, waist circumference, waist-hip-ratio, and skinfold thickness have been widely used as measures of obesity. To ensure that high quality body measurement data was obtained, professional nurses and field workers were trained to follow the prescribed and standardized techniques using calibrated equipments. All instruments were assessed prior to use for validity and reliability. Firstly, the literature evidence of content validation studies and the feedback regarding the instruments were reviewed. The instruments were then pretested and calibrated to minimize measurement errors. To check for reliability and stability of instruments, same standards were used at different times to check for the internal consistency of the instrument. The scale was calibrated and standardised using a weight of known mass to test the stability and internal consistency of the instrument. Weight was measured to the nearest 0.1 kg using a balanced Sun-beam® EB710 digital bathroom electronic scale (Fuzhou Sunny Electronic Co., Ltd, China). Height without shoes was measured to the nearest 0.1 cm using a Stadiometer. Using this data, the body mass index (BMI) (kg/m^2) was calculated as body weight/height^2. Waist and hip circumferences were measured using an anthropometric fiberglass tape measure to the nearest 0.2 cm on the left arm mid-way between the acromion and radial points, with both arms loosely and comfortably at the sides.

c) Biochemical analysis

All participants underwent a standard 75-g OGTT and had their blood taken to test for fasting glucose. Type 2 diabetes mellitus was diagnosed according to the ADA and WHO criteria by measuring blood glucose in a fasted state or 2 hours after a 75-g glucose intake (OGTT). In both these methods, the enzymatic hexokinase method was used to test for blood glucose level (Cobas 6000, Roche Diagnostic). Glycosylated haemoglobin (HbA1c) was tested using a turbidimetric inhibition immunoassay which has a within-run confidence interval (CV) of 1.4% and between-run CV of 2.8% (a method certified by National Glycohemoglobin

Standardization Program (NGSP). Insulin was determined by a microparticle enzyme immunoassay (Axsym, Abbot). High-density lipoprotein cholesterol (HDL-C) and triglycerides (TG) were estimated by enzymatic colorimetric methods (Cobas 6000, Roche Diagnostics). Low-density lipoprotein cholesterol (LDL-C) was calculated using Friedwald's formula.

d) Definitions and calculations

Individuals were considered to have T2DM if blood glucose concentration is ≥ 7 mmol/L (or 126 mg/dL) or ≥ 11.1mmol/L (or 200mg/dL). An HbA1c of greater than or equal to 6.1% was considered optimal for diagnosis of T2DM in the Bellville South community (Zemlin *et al*., 2011). Insulin resistance was determined by calculating the homeostatic model assessment of insulin resistance (HOMA-IR) according to the formula: HOMA-IR= [fasting insulin concentration (mIU⁄ L) x fasting plasma glucose (mmol⁄ L]/ 22.5. The functional β-cells (HOMA-B%) were estimated using the formula: 20 × fasting insulin (μIU/ml)/fasting glucose (mmol/ml) − 3.5.

e) Statistical analysis of data

Each participant who agreed to participate in the study was assigned a unique code, which was then used for all data collection instruments, as well as biological specimen containers. All data obtained from the questionnaires and laboratory tests were recorded on Microsoft Excel data-capturing sheet in order to generate a STATISTICA data file that was analysed by STATISTICA (STATISTICA 7, StatSoft, 1984-2004). All statistical analyses were carried out by a qualified statistician.

2.4 Genetic Analysis

2.4.1 Genomic DNA isolation

Whole blood obtained from participants was collected in vacutainer ethylenediamine tetra-acetic acid (EDTA) tubes and was kept at -20°C until use. Genomic DNA was isolated from whole blood using a salting-out method as described by Miller *et al*., 1988. A volume of approximately 2 ml of whole blood was transferred from an EDTA tube and placed in a 50-ml Falcone tube. About 10 ml of cold lysis buffer (Appendix) was then added. The mixture was placed on ice for 15 minutes and mixed by inverting the tube every five minutes. This mixture was then centrifuged for 10 minutes at 15000 rpm (400 x g) at 4°C (J-6M/E centrifuge, Beckman, United Kingdom). The supernatant was discarded and the pellet (white blood cells) rinsed with 10 ml of 0.9% phosphate buffered saline (PBS) (Appendix). The pellet was resuspended in 10 ml of PBS and centrifuged for 10 minutes at 1500 rpm at 4°C

after which the supernatant was discarded. The pellet was then dissolved in a mixture of 1.5 ml of nuclear lysis buffer (Appendix), 20 µl proteinase K (10 mg/ml) and 150 µl 10% sodium dodecyl sulphate (SDS) (Appendix). The mixture was thoroughly mixed and then incubated overnight at 55°C. Thereafter, about 700 µl of 6 M sodium chloride (NaCl) was added to the solution and vortexed vigorously for 1 minute. This mixture was then centrifuged at 2500 rpm (1500 x g) for 30 minutes. The supernatant was transferred into a clean Falcon tube, vortexed for 15 seconds and then centrifuged at 2500 rpm (1500 x g) for 15 minutes. The supernatant was carefully transferred to a new Falcon tube and two volumes of 99.9% (v/v) ethanol (EtOH) carefully added to precipitate the DNA. The DNA was pulled out using a sterile pipette tip and placed in 750 µl of cold 70% (v/v) ethanol in a clean 1.5 ml eppendorf tube. The tubes were centrifuged using a benchtop microcentrifuge (Microcentrifuge® Lite, Beckman Coulter™) at 8000 rpm (6000 x g) for 2 minutes. The ethanol was discarded and the tubes were left to dry at room temerature. The DNA pellet was dissolved in approximately 150 µl of 1 x tris ethylediamine tetra acetic acid (TE) buffer.

DNA quantification: DNA was quantified using Nanodrop UV/VIS spectrophotometer (NanoDrop Technologies Inc, DE and USA). The 260/280 nm ratio was used to assess the purity of DNA and values between 1.8-2.0 considered for high quality.

2.4.2 Molecular Analysis of Single Nucleotide Polymorphisms (SNPs) in the selected genes of interest (*FTO*, *ENPP1* and *TCF7L2*)

The study population was genotyped using real-time polymerase chain reaction (RT-PCR). Firstly, conventional PCR and automated sequencing were used to identify positive controls (samples with known genotypes) that can be incorporated in each RT-PCR run. Convention PCR and automated sequencing were also used for validating genotypes that could not be determined by RT-PCR. Below is a brief description of each method used.

Oligonucleotides Primers: The success of PCR is highly depended on the quality of primer sets, and the following parameters are used as a guideline for designing primers:

- melting temperature (T_m): in the range of 52 -58°C is desirable. The two primers of a pair should have closely matched temperatures (difference of no more than 5°C) for maximum amplicon yield. Primers with a melting temperature above 65°C tend to anneal non-specifically to other regions. The melting temperatures of primers are used to calculate the annealing temperature. High annealing temperature produces insufficient primer-template hybridization and subsequently low amplicon yield. Lower annealing temperatures result in non-specific binding through mismatches.
- primer length: preferable optimal length is 18 – 22bp.

- GC content: the number of G's and C's in the primer should be between 40–60%
- hairpin loops and secondary structures: These are produced by intermolecular interaction and can result in poor or no amplicon yield.
- GC clamp: the presence of G or C within the last five nucleotides at the 3' end of the primer sequence helps promote specific binding. However, more than 3 G's or C's should be avoided in the last 5 nucleotides at the 3' end.
- complementarity of primers: these can exist as either self or cross dimers. Self-dimers are formed when a primer is homologous to itself. When primers self-dimerise more readily than hybridising with a target region, the PCR product yield is reduced. Cross-dimers are formed by intermolecular interaction between sense and antisense primers and also reduces amplicon yield. Optimally, a 3' end self-dimer and cross-dimer with a ΔG of -5 kcal/mol and an internal cross-dimer with ΔG of -6 kcal/mol are tolerable.

Primers were designed from the reference nucleotide sequence flanking the polymorphisms of interest using freely available primer design programs such as Primer3plus (http://www.primer3plus.com) and Integrated DNA Technologies (http://eu.idtnda.com). Primers (Table 2.1) were synthesized at Integrated DNA Technologies and supplied lyophilised by Whitehead Scientific (Cape Town, South Africa).

Table 2.1. Oligonucleotide primers used for the synthesis of the SNPs of interest.

Primer name	Sequence (5'-3')	Length	Ta (°C)	Product Size (bp)
FTO gene				
rs3751812 F	TCATGTGGGTTAGCTCTTCAACC	23	56	535
rs3751812 R	CCACCTTGGCAGAGGTAAATG	21		
rs9941349 F	TGGTTGTAGGTGGTTGTAGGC	21	54	354
rs9941349 R	TTGAAACGTTGCATAAAACTCTG	23		
TCF7L2 gene				
rs7903146 F	TTCTTGCTTAGTCACTTTCTGTTTG	25	57	600
rs7903146 R	GTGAAGTGCCCAAGCTTCTC	20		
rs12255372 F	TTGTCCCTTGAGGTGTACTGG	21	56	300
rs12255372 R	TGCACTAAAGACGTGGATTCTG	22		
ENPP1 gene				
rs1044498 F	TGGTAGTGGCAGATTCTGTGAG	22	55	378
rs1044498 R	GGCCAATAGCCATGACTCC	19		
rs997509 F	ACTTTGCAGGCGGTTTGTAGGGC	23	55	535
rs997509 R	TGGGAACGAGCCCTGAAGAGG	21		

Conventional Polymerase Chain Reaction (PCR): It is one of the techniques used for amplifying DNA. Three major stages of conventional PCR include denaturation of DNA to separate the two strands; annealing of primers to the denatured DNA template at a temperature calculated from the melting temperature of primers; and extension of the primers by incorporating dNTPs complementary to the DNA template in a 5'-3' direction. Extension of the annealed primers is catalysed by a DNA polymerase. These steps are then repeated for 25-45 cycles to make million copies of the DNA template (Burpo, 2001). The PCR protocols for amplifying the regions flanking the *FTO* rs3751812 and rs9941349, *TCF7L2* rs7903146 and rs12255372, and *ENPP1* rs997509 and rs1044498 were prepared as follows: 25 microlitre (µl) PCR reaction was prepared for each DNA sample per gene of interest and contained 1.5 millimolar (mM) magnesium chloride ($MgCl_2$), 0.2 mM dNTPs mix, 0.24 µM of each forward and reverse primers, 1.25 units of GoTaq Polymerase, 1 x PCR buffer, and nuclease-free water added to a final volume of 25 µl. In each PCR tube, DNA template (200

ng/µl) of each individual was added. The reaction was carried out in a Perkin Elmer GeneAmp 2720 thermal cycler (Applied Biosystems, CA, U.S.A) as follows: initial denaturation step at 95°C for 2 minutes, 35 cycles of; denaturation at 95°C for 30 seconds, annealing temperature (differed for each primer set, 54°C – 56°C as illustrated in table 2.1) for 30 seconds, extension at 72°C for 30 seconds, and final extension at 72°C for 5 minutes.

Agarose Gel Electrophoresis: The success of the PCR reactions was confirmed by running PCR products or amplicons on 2% agarose gels and visualised under ultraviolet (UV) light. The gel was prepared by weighing 2 g of agarose powder and dissolving it in 100 ml of 1 X Sodium Boric (SB) buffer (Appendix). The solution was heated in a microwave oven until the powder was completely dissolved. The solution was then allowed to cool down without solidifying before adding 5 µl of ethidium bromide (10 mg/ml). This was then poured in a casting tray (7 x 15 cm), combs inserted to create wells, and allowed to set at room temperature before loading the samples. The SB buffer was the buffer of choice since it has a lower conductivity and thus can be used to run gels at higher voltage and speed, thereby separating DNA fragments faster than those gels prepared from tris borate-EDTA (TBE) and tris acetate-EDTA (TAE) buffers. Amplicons (8 µl each) were mixed with 5 µl of the 6 x loading buffer (Fermentas, Thermo Scientific, Denmark), and loaded in the gel. The amplicons were run in a gel along with a 100-base pair DNA molecular marker so as to determine the amplicon sizes. The gels were run at 140 volts for 30 min and viewed under the GelDoc XR System (Bio-Rad Laboratories, Inc., CA, U.S.A).

PCR Product Purification: The DNA purity and amplicon concentration are important parameters that determine the quality of sequence chromatograms. Various methods of purifying amplicons are available but in the present study *Exonuclease I* (*Exo* 1) and Shrimp Alkaline Phosphatase (SAP) (20 U/µL) (New England Biolabs) were used to purify and prepare the amplicons prior to sequencing. Exonculease I is known to degrade the residual single-stranded primers and the unused single-stranded DNA whereas the SAP hydrolyses and removes dNTPs. Equal volumes of *Exo I* and SAP were mixed together, and 1 µl of the solution was added to 5 µl of the amplicon. The mixture was then incubated at 37°C for 15 minutes and then at 80°C for another 15 minutes to inactivate the enzymes.

Automated Sequencing: DNA sequencing is the method of determining the precise order of nucleotides (adenine, guanine, cytosine, and thymine) within a DNA molecule. Two type of sequencing methods were developed, Maxam-Gilbert and chain-termination (dideoxy or Sanger), with the latter being the method of choice due to its relative ease, safety, and reliability (Franca *et al.,* 2002). Initially, Sanger sequencing used fewer toxic and radioactive reagents, and due to its comparative ease of use was automated (Franca *et al*., 2002). The

method has advanced over the years, with fluorescent labeling and capillary electrophoresis currently being used (Franca *et al.,* 2002). This is the method used to generate the first human genome 2001. The Sanger sequencing method uses fluorescently-labeled dideoxynucleotides (ddNTP) in addition to normal dNTPs (Russell, 2002). Similar to PCR, the reaction involves denaturation of double stranded DNA, annealing of primers, and extension that is terminated when the ddNTP is incorporated at a certain position instead of the dNTP (Franca *et al*., 2002). The presence of a hydrogen group on the 3' carbon instead of a hydroxyl group in the ddNTP prevents addition of the incoming dNTP.

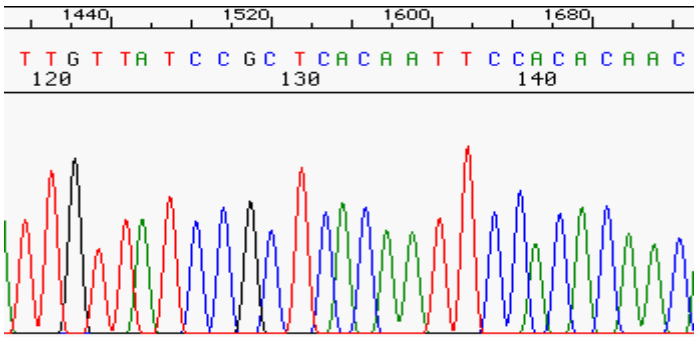


Figure 2.1. A typical automated sequence shown as a chromatogram. The coloured peaks represent four nucleotides as follows: blue is cytosine (C), green is adenine (A), black is guanine (G) and red is thymine (T).

In the present study. amplicons were sequenced using a BigDye terminator version 3.1 cycle sequencing kit (Applied Biosystems, CA, U.S.A) according to the manufacturer's instructions. The sequence reaction was prepared as follows: 3 µl of the purified amplicon was added to an equal volume of the 1.3 µM primer (one primer per reaction) and 4 µl of the sequencing solution. The sequence reaction was carried out as follows: one cycle of initial denaturation at 96 °C for 1 minutes, 25 cycles of denaturation at 96°C for 30 seconds, annealing at 55°C for 15 seconds, and extension at 60°C for 4 minutes. The samples were electrophoresed at the Central Analytical Facility at Stellenbosch University.

Real-time Polymerase reaction (RT-PCR): It is well known for its good sensitivity, accuracy and precision. This method of analysis is used for primarily validation of gene expression, bacterial species identification, and single nucleotide polymorphism genotyping (Dvorak *et al*, 2003). RT-PCR allows one to view and monitor the increase in the amount of DNA as it is amplified. It uses various detective methods including DNA binding dye, hydrolysis probes, hairpin probes and simple hybridization dye. The signal of the dye is depended on the amount bound to the ds-DNA, thus increasing proportionally with the generated amount of amplicon. The fluorescence increases linearly until a plateau is reached (Dvorak *et al*, 2003).

Selected individuals were genotyped for polymorphisms by real-time polymerase chain reaction (RT-PCR) using SNP-specific Taqman genotyping assays ((Applied Biosystems, CA, U.S.A). Taqman genotyping combines hybridization and 5' nuclease activity of polymerase coupled with fluorescence detection. This method utilizes four oligonucleotides: two allele-specific probes and a pair of PCR primer flanking the region containing the polymorphism of interest. The allele-specific probes carry a fluorescence reporter dye at one end and a non-fluorescence quencher at the other end. During the extension stage of PCR, the polymerase enzyme only cleaves on the hybridized probe that is perfectly matched and thus freeing the reporter dye from the quencher. This generates the florescence signal in the absence of the quencher. On the other hand, the mismatched probe remains intact and shows no florescence. This allows detection of both alleles during the reaction based on hybridization of respective probes (Kim and Misra, 2007). The following polymorphisms were selected to be investigated in the present study: *ENPP1* rs1044498 and *ENPP1* rs997509, *FTO* rs3751812, *FTO* rs9941349, *TCF7L2* rs7903146, and *TCF7L2* rs12255372. Genotyping of subjects was conducted on the BioRad MiniOpticon Real time PCR system (Biorad), and the data was analysed using corresponding software (CFX Manager software).

2.5 Statistical analysis

All statistical analysis was carried out by a qualified Genetic Statistician using various programs including freely available programming language R (www.r-project.org) and specific packages such as LD, Hardy-Weinberg, genotype and allelic association and haplotype analysis.

A. Statistical analysis of clinical data

General characteristics of the study group are summarized as count and percentage for dichotomous traits, mean and standard deviation (SD) or median and 25^{th}-75^{th} percentiles for quantitative traits. Traits were log-transformed to approximate normality, where necessary, prior to analysis. These main variables were compared between obese-overweight, normal, diabetic, non-diabetic and genders.

B. Statistical analysis of genetic data

The allele and genotype distribution of the selected genetic polymorphisms were determined and compared between case and control groups of the study population. Single nucleotide polymorphisms were tested for departure from Hardy-Weindberg Equilibrium (HWE) expectation via a chi square goodness of fit test. Hardy- Weinberg principle is used to predict how gene frequencies are inherited from generation to generation given a set of assumptions. According to this principle, in a large randomly breeding population the allelic frequency will remain the same from generation to generation provided that there is no

mutation, gene migration, natural selection or genetic drift. Mathematically the principle is illustrated as $p^2+2pq+q^2=1$, where "p" and "q" represent the frequency of alleles. However, when populations do not always meet the Hardy-Weinberg law, the evolutionary forces within the population causes a violation of the assumptions.

Linkage disequilibrium (LD) was estimated using the D' statistic. Linkage disequilibrium (LD) is the non-random association of alleles at two loci, not necessarily on the same chromosome. For an example, the loci are said to be in disequilibrium when a particular allele at one locus is found together with another allele at a second locus more often than expected and segregate independently in a population. The term linkage disequilibrium can be misleading sometimes for two reasons. Firstly, non-random association of alleles at two loci can occur even if the two genes are unlinked. Secondly, genes can be linked but may not be in linkage disequilibrium. Linkage disequilibrium in a population is a result of chromosome rearrangement, natural selection, random drift, genetic hitchhiking, gene flow and epistatic. A wide variety of statistics methods have been proposed to measure the extent of LD. LD is measured by comparing the observed and expected frequency of one haplotype and the differences between the two values is considered the deviation or D. However, the numerical value measured is of little use in measuring strength and comparing levels of LD due to the dependence of D on allele frequencies. The most common measures that are used to quantify LD are the absolute value of D' and r^2. D' value is determined by dividing D by its maximum possible value, given the allele frequency at the two loci. Two loci are said to be in complete LD if D' = 1, a value of D'>1 indicates that the complete ancestral LD has been disrupted whilst a value of D'<1 has no clear interpretation. The r^2 value is complementary to D' and it is equal to D^2 divided by the product of allele frequencies at two loci.

Association between alleles, genotypes with their constructed haplotypes and quantitative traits (including the presence of type 2 diabetes) was assessed using regression models (Umbach and Weinberg, 1997). Logistic regression models were used to test the association between SNPs and traits, assuming both recessive and log-additive models. We investigated the association of each SNP with each trait, overall and tested for heterogeneity by major subgroups by adding the interaction term of major grouping variables and each SNP to a model that contained the main effects of grouping variable and the relevant SNP. Results corresponding to p-values below 5% are described as significant. Adjust for multiple testing was conducted via Bonferroni methods. All analyses used the statistical software R (version 3.0.0 [2013-04-03], The R Foundation for statistical computing, Vienna, Austria). SNPs analyses used the packages '*genetic*', '*gap*', '*SNPassoc*' and '*hapassoc*'.

CHAPTER 3:
RESULTS

3.1 Clinical Characteristics of Participants

Of the 946 participants who took part in the survey, 566 consented for genetic studies. Among the latter, 72 were excluded for missing data on the genotypes and/or trait. Therefore, 494 had valid data for the current analyses. The clinical characteristics of the participants are presented in Table 3.1. Clinical traits of participants were compared between obese, overweight and normal, diabetic and non-diabetic and are summarized as mean ± SD unless indicated otherwise. The data show significant differences in the gender, age and weight in the studied population. The participants were mainly female, middle-aged and over-weight.

An age-adjusted T2DM prevalence of 26.3% (95% confidence interval 22.0–30.3) was observed in this population. Expectedly, T2DM-related traits differed significantly according to diabetes status. For example, significant differences between diabetic and non-diabetic individuals were observed for all insulin resistance/sensitivity indicators ($p < 0.0001$; glucose/insulin ratio $p = 0.009$). When compared to the non-diabetic group, participants with diabetes were older ($p < 0.0001$), had higher body mass index ($p = 0.023$), higher waist circumference and waist-hip-ratio (both $p < 0.0001$), increased systolic blood pressure (129 vs 121 mmHg, $p < 0.0001$), but few of them were hypertensive. Higher triglycerides ($p < 0.001$) and GGT ($p = 0.0002$) were observed in diabetic individuals.

Table 3.1. Baseline Profile of the Study Population (Distribution of variables comparing them between Diabetic and Normal).

Characteristics	No diabetes	Diabetes	p-value	Normal weight	overweight	obese	p-value	Overall
N	336	158		125	129	240		494
Gender, male n (%)	19	39	<0.0001	21	17	20	0.049	58 (11.7)
Mean Age, year (SD)	53.3 (13.6)	60.3 (11.8)	<0.0001	54.6 (14.9)	56.2 (12.8)	55.6 (13.0)	0.631	55.2 (13.4)
Mean Systolic blood pressure, mmHg (SD)	121 (18)	129 (22)	<0.0001	119 (21)	122 (17)	127 (20)	0.0006	124 (20)
Mean Diastolic blood pressure, mmHg (SD)	74 (12)	77 (16)	0.113	73 (17)	74 (11)	77 (12)	0.016	75 (13)
Hypertension, n (%)	225	123	0.013	69	92	127	<0.0001	348 (70.4)
Mean Body mass index, kg/m^2 (SD)	30.1 (7.5)	31.7 (7.2)	0.023	22.0 (2.2)	27.7 (1.4)	36.5 (5.7)	<0.0001	30.6 (7.4)
Mean Waist circumference, cm (SD)	96 (15)	103 (14)	<0.0001	82 (9)	93 (7)	109 (11)	<0.0001	98 (15)
Mean Hip circumference, cm (SD)	111 (15)	112 (15)	0.776	96 (9)	105 (6)	122 (13)	<0.0001	111 (15)
Mean Waist/hip ratio, (SD)	0.86 (0.08)	0.92 (0.08)	<0.0001	0.85 (0.07)	0.89 (0.08)	0.89 (0.09)	<0.0001	0.88 (0.08)
Mean HbA1c, % (SD)	5.7 (0.4)	7.7 (1.9)	<0.0001	5.8 (0.9)	6.6 (1.8)	6.5 (1.4)	<0.0001	6.3 (1.4)
Mean Fasting blood glucose, mmol/l (SD)	5.3 (0.7)	9.6 (3.8)	<0.0001	6.0 (2.3)	7.1 (3.7)	6.8 (2.9)	0.016	6.7 (3.0)
Mean 2h glucose, mmol/l (SD)	6.9 (1.6)	13.4 (4.8)	<0.0001	7.3 (2.6)	7.8 (3.6)	8.7 (4.0)	0.002	8.1 (3.6)
Mean eGFR, ml/min (SD)	83.9 (21.5)	82.0 (24.2)	0.387	85.9 (23.7)	82.7 (21.0)	82.2 (22.4)	0.317	83.3 (22.4)
Mean Triglycerides, mmol/l (SD)	1.4 (0.9)	1.8 (0.9)	<0.0001	1.3 (1.0)	1.6 (0.9)	1.6 (0.8)	0.002	1.5 (0.9)
Mean HDL cholesterol, mmol/l (SD)	1.3 (0.3)	1.2 (0.3)	0.0007	1.4 (0.4)	1.3 (0.3)	1.2 (0.3)	0.0002	1.3 (0.3)
Mean LDL cholesterol, mmol/l (SD)	3.7 (1.0)	3.8 (1.1)	0.824	3.5 (1.0)	3.8 (1.0)	3.9 (1.0)	0.023	3.8 (1.0)
Mean Total cholesterol, mmol/l (SD)	5.7 (1.2)	5.8 (1.2)	0.341	5.5 (1.2)	5.8 (1.2)	5.8 (1.2)	0.032	5.7 (1.2)
Median GGT (25^{th}-75^{th} percentiles)	25 [17-38]	31 [23-50.5]	0.0002	26 [18-42]	27 [16-39]	28 [20-45]	0.274	27 [19-41]
Median Insulin mmol/l(25^{th}-75^{th} percentiles)	6.7 [2.7-11.9]	8.3 [2.3-14.7]	0.088	3.4 [1.2-7.7]	7.0 [3.4-11.1]	8.9 [4.2-14.7]	<0.0001	7.2 [2.7-12.9]
Median 2h insulin mmol/l(25^{th}-75^{th} percentiles)	39.1 [25.2-68.1]	56.6 [20.6-115.6]	0.025	28.4 [17.0-45.1]	36.7 [22.7-65.3]	57.5 [30.4-110.6]	<0.0001	40.4 [21.2-76.2]
Median Glucose/insulin (25^{th}-75^{th} percentiles)	0.77 [0.45-1.81]	1.10 [0.56-3.71]	0.009	1.50 [0.71-5.00]	0.53 [0.12-1.91]	0.68 [0.40-1.54]	<0.0001	0.82 [0.49-2.14]
Median HOMA-IR (25^{th}-75^{th} percentiles)	1.54 [0.57-2.89]	2.92 [1.56-5.82]	<0.0001	0.86 [0.32-2.06]	1.95 [0.82-3.35]	2.49 [1.07-4.36]	<0.0001	1.83 [0.68-3.64]
Median HOMA-B% (25^{th}-75^{th} percentiles)	73.1 [31.8-141.7]	30.4 [7.2-66.2]	<0.0001	38.6 [10.7-90.6]	55.8 [15.4-95.2]	73.9 [26.5-140.9]	0.0003	58.5 [18.7-115.0]
Median QUICKI (25^{th}-75^{th} percentiles)	0.15 [0.14-0.18]	0.14 [0.13-0.16]	<0.0001	0.17 [0.15-0.21]	0.15 [0.14-0.17]	0.14 [0.13-0.16]	<0.0001	0.15 [0.14-0.18]
Median FIRI (25^{th}-75^{th} percentiles)	1.38 [0.51-2.60]	2.63 [1.04-5.24]	<0.0001	0.77 [0.29-1.85]	1.75 [0.74-3.01]	2.24 [0.96-3.92]	<0.0001	1.67 [0.61-3.28]
Median 1/HOMA-IR (25^{th}-75^{th} percentiles)	0.65 [0.35-1.76]	0.34 [0.17-0.86]	<0.0001	1.16 [0.48-3.15]	0.51 [0.30-1.22]	0.40 [0.23-0.93]	<0.0001	0.54 [0.27-1.48]

Abbreviations: **HbA1c**, glycated haemoglobin ; **eGFR**, estimated glomerular filtration rate; **HDL** ,High Density Lipoproteins; **LDL**, Low Density Lipoproteins; **GGT,** γ-glutamyltransferase , **CRP,** C-reactive protein; **HOMA-IR,** homeostatic model assessment of insulin resistance; **HOMA-B%,** functional β-cells ;**QUICKI,** the quantitative *insulin*-sensitivity check index; **FIRI,** fasting *insulin* resistance index; **SD,** standard deviation.

3.2. Genotyping of the study population

3.2.1 *FTO* Variants

FTO variants (rs9941349 and rs3751812) were successfully amplified by PCR and separated on a 2% agarose gel (Figure 3.2.1.1 A & B) and had sequence variant analysis determined by semi-automated DNA sequence analysis (see chromatograms in Figure 3.2.1.2 A & B). These chromatograms illustrate the polymorphisms: *FTO* (Homozygote (wild type) C>C: rs9941349 and Homozygote (wild type) G/G: rs3751812) and *TCF7L2* (Heterozygote G>T: rs12255372 and Heterozygote C>T: rs7903146).

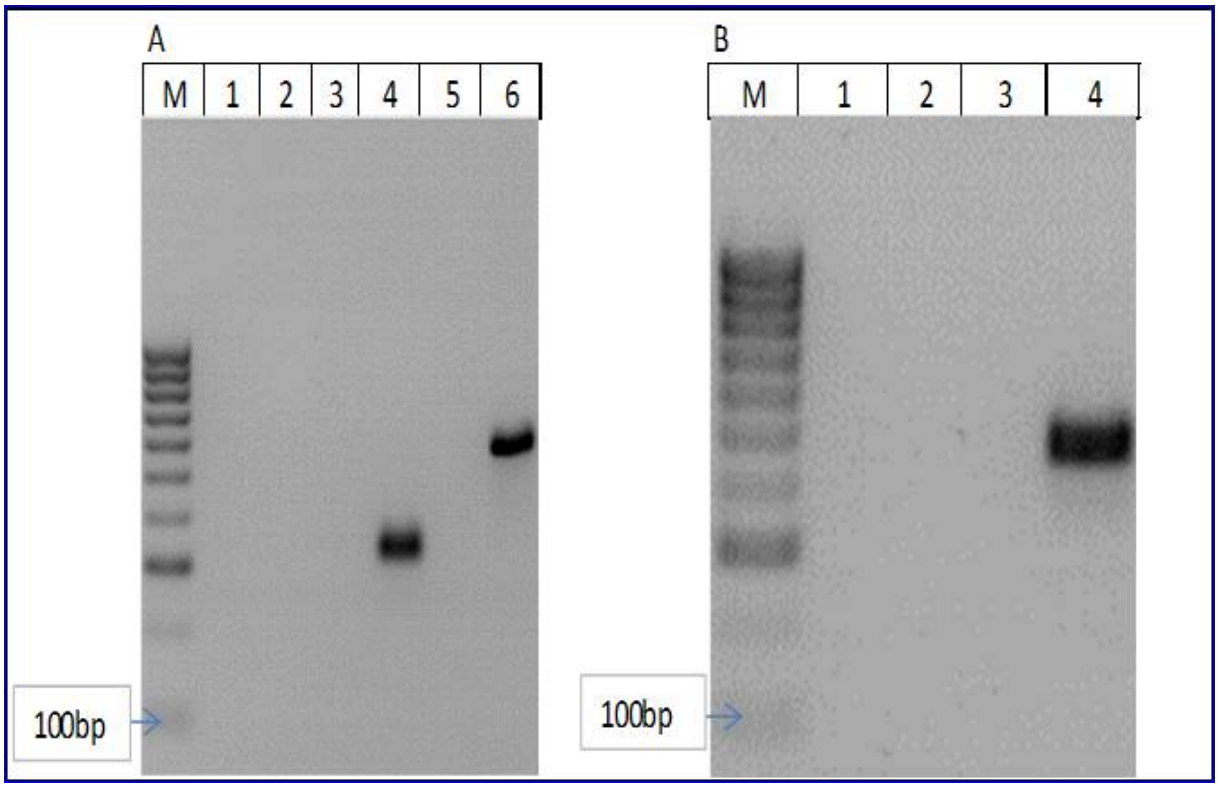


Figure 3.2.1.1: 2% Agarose gel showing amplicons of the nucleotide region flanking the *FTO* rs3751812 (A) and rs9941349 (B) polymorphisms obtained by conventional polymerase chain reaction. (A): Lane M, 100-base pair DNA molecular marker; lane 2, non-template control; lane 4, positive control; lane 6, amplicon (535 bp). (B): Lane M, 100-base pair DNA molecular marker, lane 2, non-template control; lane 4, amplicon (354bp).

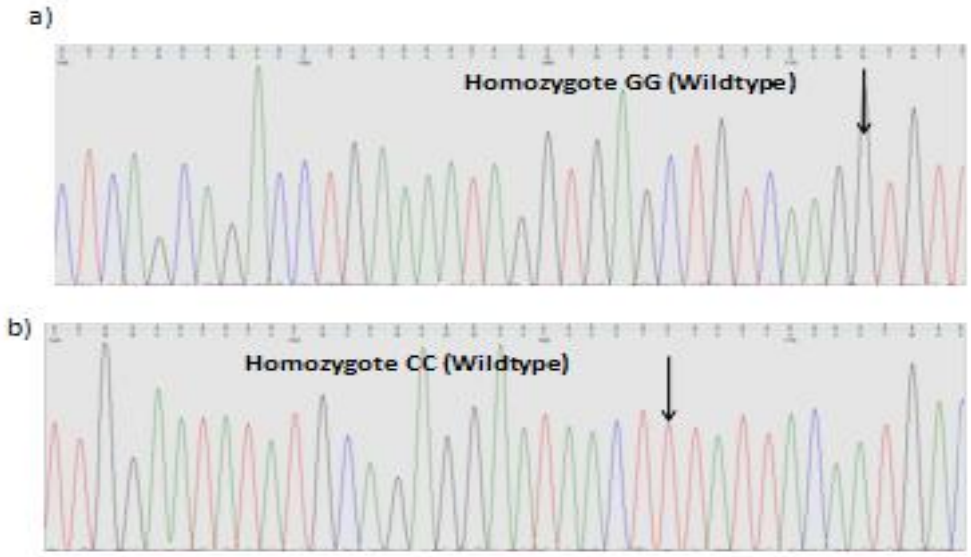


Figure 3.2.1.2: Sequence chromatographs illustrating *FTO* a) rs3751812 SNP and b) rs997509

3.2.2 *ENPP1* Variants

Selected regions of *ENPP1* (rs997509 and rs1044498), were also successfully amplified by PCR and separated on a 2% agarose gel (Figure 3.2.2.1 A & B). Sequence variant analysis was determined by semi-automated DNA sequence analysis and Figure 3.2.2.2 A & B are the chromatograms illustrating the polymorphisms. *ENPP1* rs997509 lies at the 3' end of intron 1 and causes a cytosine (C) to thymine (T) transition at the nucleotide position (c.258-939C>T). *ENPP1* rs1044498 is located on exon 4 causing an adenine (A) to cytosine (C) transversion at nucleotide number (c.534A>C).

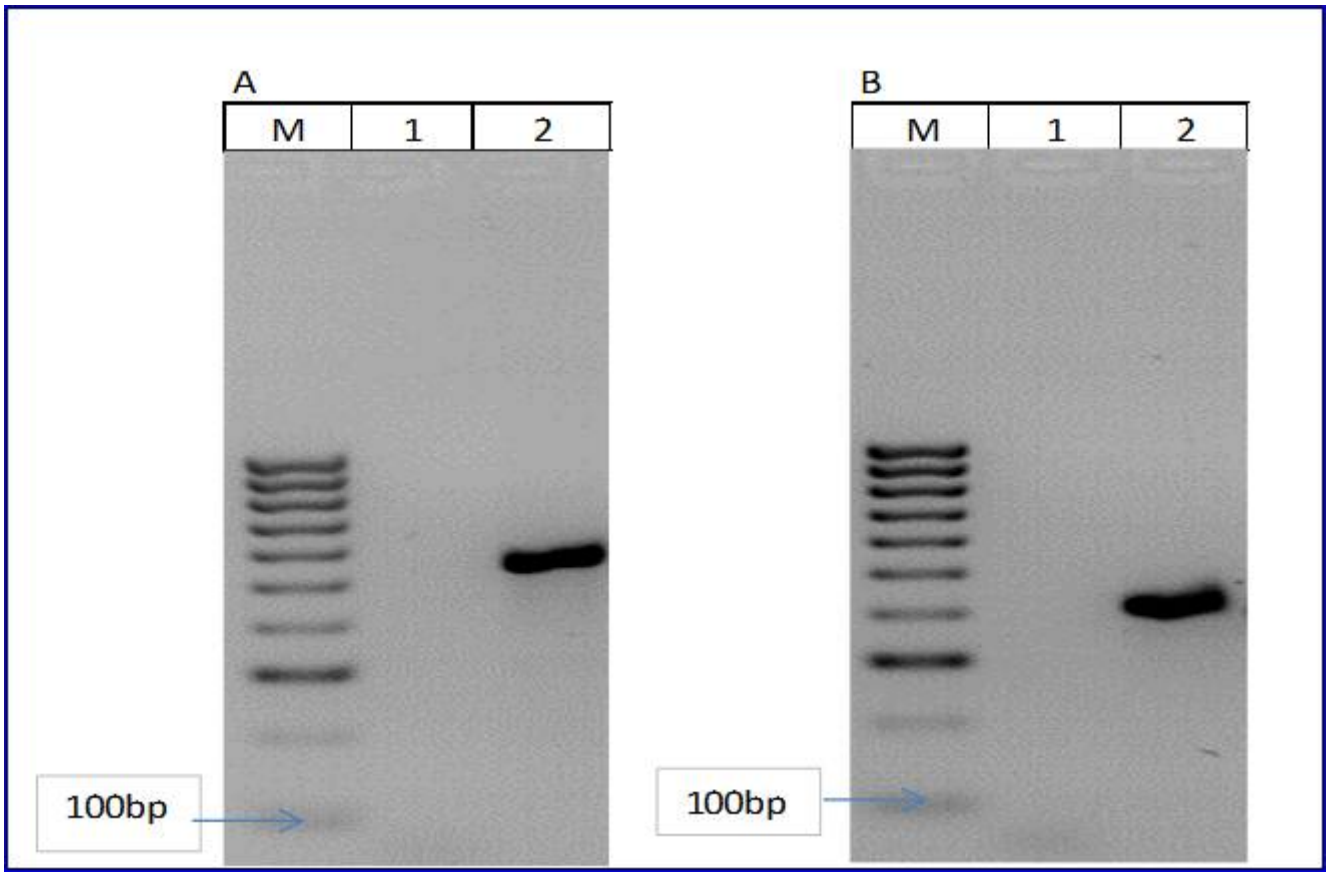
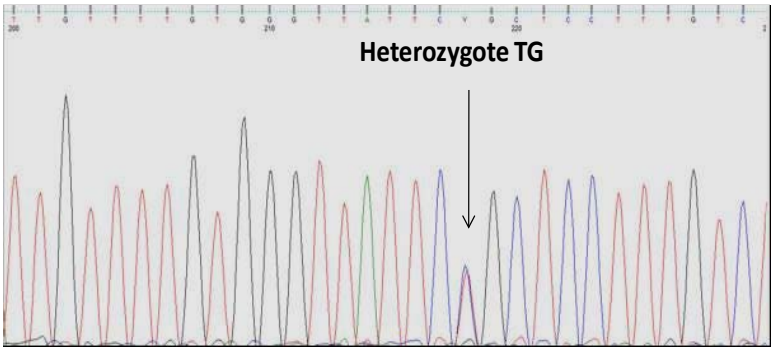


Figure 3.2.2.1: 2% Agarose gel showing amplicons of the nucleotide region flanking the *ENPP1* rs997509 (A) and rs1044498 (B) polymorphisms obtained by conventional polymerase chain reaction. Lane 1, 100-base pair DNA molecular marker; lane 2, non-template control; lanes 3-6, amplicons (535 bp and 378 bp respectively).

a)



b)

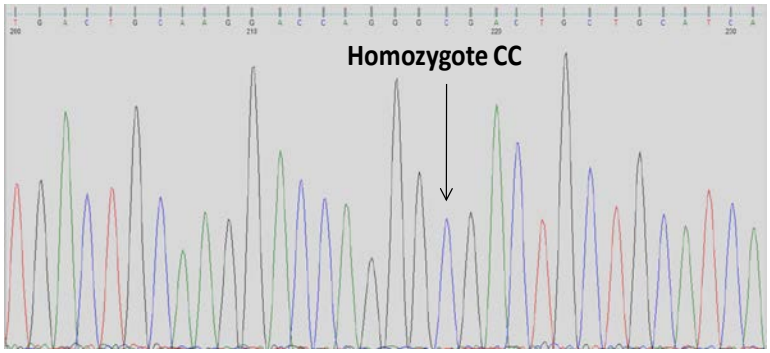


Figure 3.2.2.2: Sequence chromatographs illustrating *ENPP1* a) rs997509 SNP C>T b) rs1044498.

3.2.3 *TCF7L2* Variants

Lastly, *TCF7L2* variants (rs9941349 and rs3751812) were likewise successfully amplified by PCR and separated on a 2% agarose gel (Figure 3.2.3.1 A & B) and had sequence variant analysis determined by semi-automated DNA sequence analysis (chromatograms in Figure 3.2.3.2 A & B). These chromatograms illustrate the polymorphisms: *TCF7L2* (Heterozygote G>T: rs12255372 and Heterozygote C>T: rs7903146).

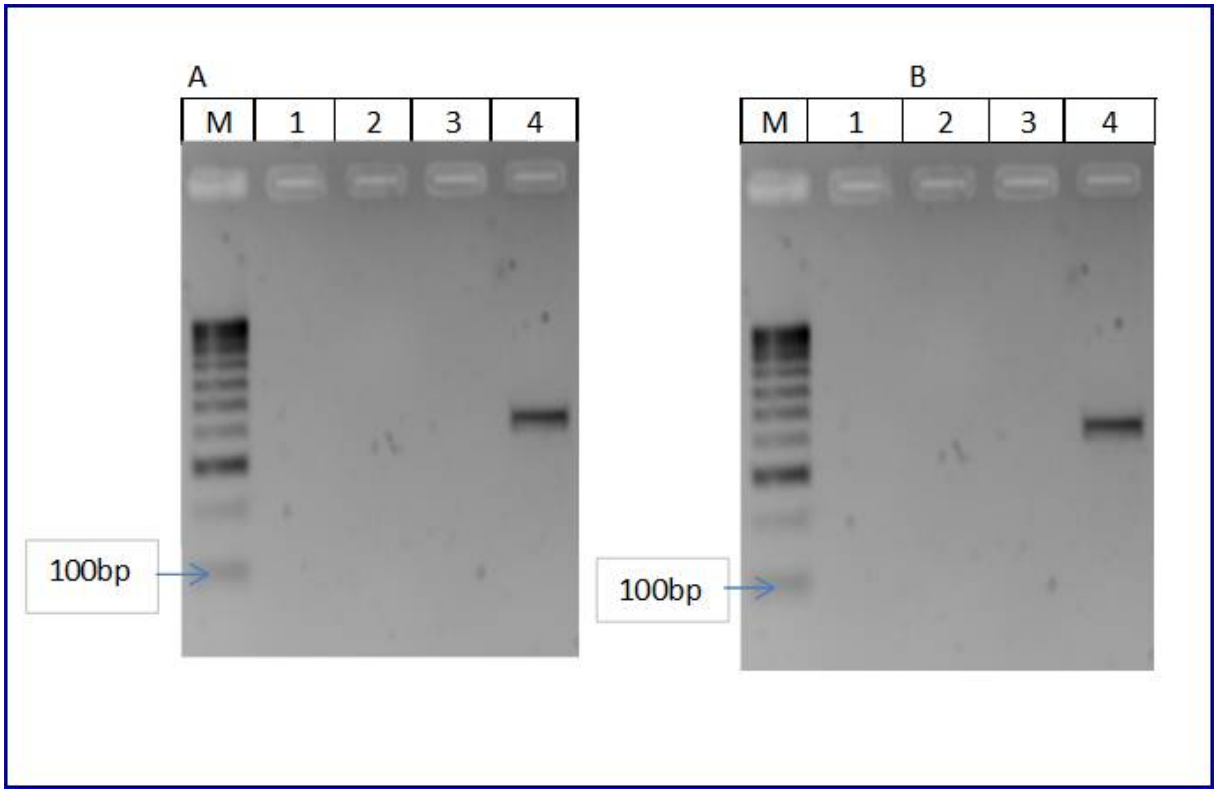


Figure 3.2.3.1: 2% Agarose gel showing amplicons of the nucleotide region flanking the *TCF7L2* rs7903146 (A) and rs12255372 (B) polymoprhisms obtained by conventional polymerase chain reaction. Lane M, 100-base pair DNA molecular marker; lane 3, non-template control; lanes 4, amplicons (600 bp and 300 bp respectively).

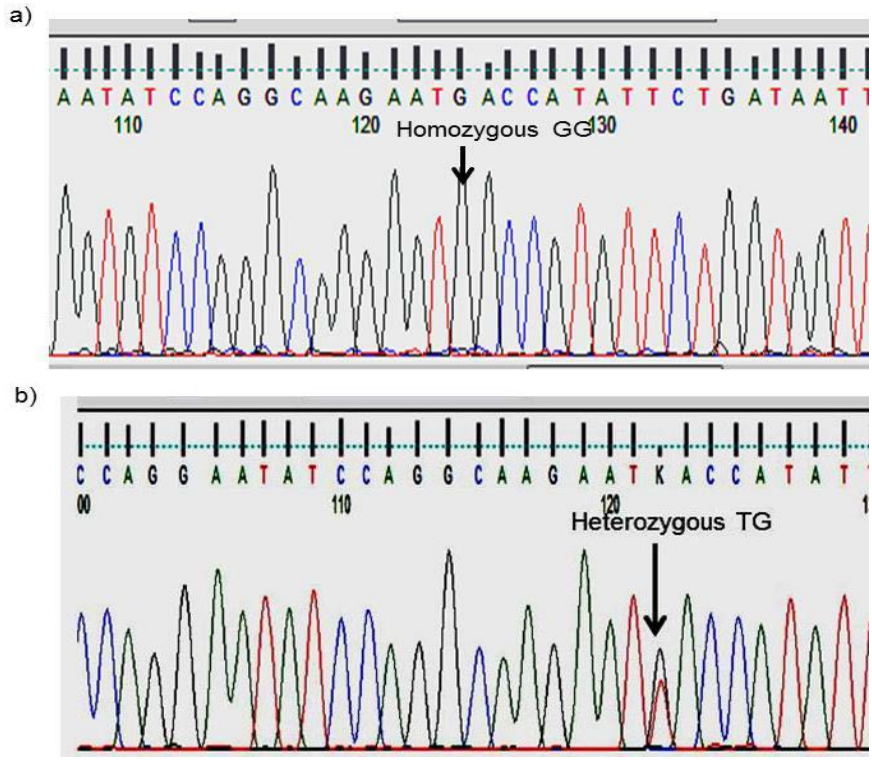


Figure3.2.3.2: TCF7L2 SNP Sequence chromatographs illustrating a) rs12255372 homozygous GG, and b) rs12255372 heterozygous GT, c) rs7903146 homozygous XX and d) rs7903146 heterozygous XY.

3.3 Allele and genotype distribution

Table 3.2 shows the distribution of alleles and genotypes of the selected SNPs in the study population. The comparison was done between diabetic and normoglycemic; obese, overweight and normal weight subgroups. Except for *TCF7L2* rs12255372 and *FTO* 3751812 all the SNPs investigated were in Hardy-Weinberg equilibrium (p>0.05). The SNP were in linkage disequilibrium in all sub-groups of the study population: *ENPP1*-rs997509 and –rs1044498; overall D'=0.969, in participants without diabetes D'=0.949, in those with diabetes D'=0.999, in normal weight (D'=0.998), overweight (D'=0.999) and obese participants (D'=0.944). *FTO*-9941349 and -rs3751812; overall D'=0.632, in participants without diabetes D'=0.623, in those with diabetes D'=0.646, in normal weight D'=0.581, overweight D'=0.667 and obese participants D'=0.635. *TCF7L2*-rs12255372 and -rs7903146; overall D'=0.491, in participants without diabetes D'=0.450, in those with diabetes D'=0.568, in normal weight D'=0.437, overweight D'=0.466, and obese participants D'=0.555. Except for *TCF7L2*- rs7903146 (diabetic vs non-diabetic, p = 0.046), there was no significant difference in the distribution of alleles and genotypes of other SNPs, overall and between subgroups.

Table 3.2. Genotype distributions, minor allele frequencies, and unadjusted p-values for comparing genotype distributions according to diabetes and BMI status.

	No diabetes	Diabetes	p-value	Normal weight	Overweight	Obese	p-value	Overall
N	336	158		125	129	240		494
***ENPP1* rs997509**								
C/C, n (%)	257 (72.5)	126 (79.7)	0.144	101 (80.8)	96 (74.4)	186 (77.5)	0.688	383 (77.5)
C/T, n (%)	75 (22.3)	27 (17.1)		21 (16.8)	31 (24.0)	50 (20.8)		102 (20.6)
T/T, n (%)	4 (1.2)	5 (3.2)		3 (2.4)	2 (1.6)	4 (1.7)		9 (1.8)
T, n (%)	83 (12.4)	37 (11.7)	0.777	27 (10.8)	35 (13.6)	58 (12.1)	0.634	120 (12.1)
HWE (p-value)	0.800	0.041		0.150	>0.999	0.759		0.524
***ENPP1* rs1044498**								
C/C, n (%)	85 (25.3)	42 (26.6)	0.147	37 (29.6)	33 (25.6)	57 (23.8)	0.746	127 (25.7)
C/A, n (%)	183 (54.5)	73 (46.2)		64 (51.1)	66 (51.2)	126 (52.5)		256 (51.8)
A/A, n (%)	68 (20.2)	43 (27.2)		24 (19.2)	30 (23.3)	57 (23.8)		111 (22.5)
A, n (%)	319 (47.5)	159 (50.3)	0.403	112 (44.8)	106 (41.1)	240 (50.0)	0.080	478 (48.4)
HWE (p-value)	0.102	0.342		0.856	0.861	0.518		0.419
***FTO* 9941349**								
C/C, n (%)	193 (57.4)	76 (48.1)	0.151	66 (52.8)	65 (50.4)	138 (57.5)	0.589	269 (54.5)
C/T, n (%)	119 (35.4)	68 (43.0)		47 (37.6)	55 (42.6)	85 (35.4)		187 (37.9)
T/T, n (%)	24 (7.1)	14 (8.9)		12 (9.6)	9 (7.0)	17 (7.1)		38 (7.7)
T, n (%)	167 (24.9)	96 (30.4)	0.067	71 (28.4)	73 (28.3)	119 (24.8)	0.449	263 (26.6)
HWE (p-value)	0.380	>0.999		0.385	0.667	0.488		0.490
***FTO* rs3751812**								
G/G, n (%)	148 (44.0)	59 (37.3)	0.320	41 (32.8)	53 (41.1)	113 (47.1)	0.130	207 (41.9)
G/T, n (%)	181 (53.9)	94 (59.5)		81 (64.8)	73 (56.6)	121 (50.4)		275 (55.7)
T/T, n (%)	7 (2.1)	5 (3.2)		3 (2.4)	2 (1.6)	6 (2.5)		12 (2.4)
T, n (%)	195 (29.0)	104 (32.9)	0.215	87 (34.8)	77 (29.8)	133 (27.7)	0.139	299 (30.3)
HWE (p-value)	<0.0001	<0.0001		<0.0001	0.0001	<0.0001		<0.0001 *
***TCF7L2* rs12255372**								
G/G, n (%)	40 (11.9)	15 (9.5)	0.633	16 (12.8)	17 (13.2)	22 (9.2)	0.407	55 (11.1)
415G/T, n (%)	281 (83.6)	134 (84.8)		100 (80.0)	105 (81.4)	208 (86.7)		415 (84.0)
T/T, n (%)	15 (4.5)	9 (5.7)		9 (7.2)	7 (5.4)	10 (4.2)		24 (4.9)
T, n (%)	311 (46.3)	152 (48.1)	0.590	118 (47.2)	119 (46.1)	228 (47.5)	0.937	463 (46.9)
HWE (p-value)	<0.0001	<0.0001		<0.0001	<0.0001	<0.0001		<0.0001 *

***TCF7L2* rs7903146**								
C/C, n (%)	187 (55.7)	70 (44.3)	0.046	62 (49.6)	67 (51.9)	128 (53.3)	0.328	257 (52.0)
C/T, n (%)	127 (37.8)	78 (49.4)		50 (40.0)	54 (41.9)	101 (42.1)		205 (41.5)
T/T, n (%)	22 (6.5)	10 (6.3)		13 (10.4)	8 (6.2)	11 (4.6)		32 (6.5)
T, n (%)	171 (25.4)	98 (31.0)	0.067	76 (30.4)	70 (27.1)	123 (25.6)	0.389	269 (27.2)
HWE (p-value)	>0.999	0.064		0.530	0.656	0.129		0.363

*HWE p-values are from exact tests. *Abbreviations*: **HWE**, Hardy-Weinberg Equilibrium ; ***ENPP1*,** Ectonucleotide pyrophosphate/phosphodiiesterase-1; ***FTO***, Fat mass and obesity- associated gene; ***TCF7L2***, Transcription factor 7-like 2.

3.4 Genetic association analyses

The association analyses between the SNPs and T2DM were conducted under dominant, additive and recessive models. In a generalized linear regression analysis adjusted for age, gender, BMI and HOMA IR, minor alleles of *ENPP1* SNPs and *FTO*-rs9941349 were associated with a higher risk of T2DM, respectively, under recessive and additive models but with no evidence of significant statistical interaction with BMI categories (Table 3.3). In the recessive model, the minor alleles of *ENPP1* rs997509 and rs1044498 conferred 4.55 (95% confidence interval; 1.06 to 19.49; p = 0.041) and 1.81 (1.09 to 2.98; p = 0.021) respectively. Only the *FTO* rs9941349 showed significant association with T2DM under log additive model [p= 0.049; 1.40 (1.00 to 1.96)]. Although there was a statistically significant difference in the distribution of the *TCF7L2*-rs7903146 polymorphisms, no association was observed with T2DM or any insulin resistance/sensitivity indicators. Inferred haplotypes of the SNPs also showed no statistical significant association with T2DM and its related traits.

Table 3.3. Logistic regression models showing the effects of genes on prevalent diabetes risk (recessive model).

		Overall		BMI categories*SNP interaction
SNP	**Allele**	**Effects size (95%CI)**	**P**	
Recessive model				
ENPP1 rs997509	T/T	4.55 (1.06 to 19.49)	0.041	0.210
ENPP1 rs1044498	A/A	1.81 (1.09 to 2.98)	0.021	0.300
FTO 9941349	T/T	1.04 (0.47 to 2.31)	0.925	0.438
FTO rs3751812	T/T	0.95 (0.24 to 3.73)	0.937	0.709
TCF7L2 rs12255372	T/T	1.30 (0.49 to 3.46)	0.604	0.231
TCF7L2 rs7903146	T/T	0.81 (0.34 to 1.95)	0.637	0.934
Log additive model				
ENPP1 rs997509	T	0.93 (0.59 to 1.46)	0.757	
ENPP1 rs1044498	A	1.10 (0.80 to 1.51)	0.541	
FTO 9941349	T	1.40 (1.00 to 1.96)	0.049	
FTO rs3751812	T	1.14 (0.76 to 1.71)	0.526	
TCF7L2 rs12255372	T	1.14 (0.64 to 2.01)	0.658	
TCF7L2 rs7903146	T	1.20 (0.85 to 1.70)	0.296	

Models are adjusted for age, gender, BMI and HOMA IR. Effect estimates are odd ratio and 95% confidence intervals. *Abbreviations*: **CI**, confidence interval; ***ENPP1*,** Ectonucleotide pyrophosphate/phosphodiiesterase-1; ***FTO***, Fat mass and obesity- associated transcript gene; ***TCF7L2***, Transcription factor 7-like 2.

Table 3.4. Logistic regression models showing the haplotype effects of genes on prevalent diabetes risk

				Overall	
SNP 1	**SNP 2**	**Haplotype**	**Estimated frequency (%)**	**Effects size (95%CI)**	**p**
ENPP1 rs997509	ENPP1 rs1044498	CA	48.0	1.14 (0.88 to 1.88)	0.593
		CC	39.6	1.01 (0.99 to 1.04)	0.954
		TA	0.2	-	-
		TC	12.1	-	-
	Global effects				0.753
FTO 9941349	FTO rs3751812	CG	63.6	1 (reference)	
		CT	10.3	0.78 (0.47 to 1.27)	0.424
		TG	6.4	1.25 (0.80 to 1.95)	0.491
		TT	19.7	1.34 (0.75 to 2.40)	0.182
	Global effects				0.206
TCF7L2 rs12255372	TCF7L2 rs7903146	GC	45.6	1 (reference)	
		GT	7.4	0.76 (0.43 to 0.44)	0.548
		TC	27.2	0.81 (0.54 to 1.22)	0.591
		TT	19.8	1.24 (0.81 to 1.91)	0.778
	Global effects				0.514

Models are adjusted for age, gender, BMI and HOMA-IR. Effect estimates are odd ratio and 95% confidence intervals for qualitative traits. For the *ENPP1* SNPs, estimations were not possible for the haplotypes TA and TC. *Abbreviations*: **CI**, confidence interval; ***ENPP1*****,** Ectonucleotide pyrophosphate/phosphodiiesterase-1; ***FTO***, Fat mass and obesity- associated gene; ***TCF7L2***, Transcription factor 7-like 2.

CHAPTER 4:
DISCUSSION AND CONCLUSION

DISCUSSION

The number of confirvmed loci implicated in the development of T2DM has increased from 3 in 2006 to over 20 to date (Singh, 2011). The susceptibility of different ethnic groups differ and this is due to genetic and environmental factors. Most of these variants have modest effects on disease risk (odds ratio ~ 1.2) and their role in the development of T2DM is still poorly understood. In this present case-control study, we wanted to investigate the involvement of the *ENPP1*, *FTO* and *TCF7L2* genes in development of T2DM so as to unravel the genetics of T2DM in the Mixed Ancestry population of South Africa. According to the peri-urban studies conducted about 20 years ago the Mixed Ancestry population has the second-highest prevalence of T2DM in South Africa (Levitt *et al*, 1999). However, since then South Africa has undergone significant political and economic changes which has resulted in the rapid urbanisation particularly of the previously disadvantaged populations such as Mixed-ancestry and Black ethnic groups. Urbanisation is accompanied by nutritional transitions where traditional foods rich in fruits and vegetables are replaced by a western diet that is high in animal fat and low in complex carbohydrates. Our research group has found a prevalence of diabetes of about 26% in the Mixed-Ancestry population residing in Bellville South, Cape Town (Matsha *et al*, 2002); a rapid increase from what was published by Levitt and co-workers in 1999 (Levitt *et al*, 1999).

In this study, the data has shown that most participants that had T2DM were mainly overweight, middle-aged and they were female. In this investigation we found that BMI, WC, and WHR ranged from moderate to significantly higher in participants with diabetes when compared to the healthy control group. For example, the average BMI was 31.7 (7.2) for the diabetic group compared to the 30.1 (7.5) of non-diabetic controls, $p=0.023$. On the other hand, the WC average was 103 (14) in the diabetic group and 96 (15) in the non-diabetic controls, $p<0.0001$. The WHR was also significantly higher ($p<0.0001$) in the diabetic group compared to the controls.

The association between obesity and T2DM has long been established and has been recognized across all ethnic groups. In South Africa the high obesity rates were linked to cultural beliefs. Generally, in the South African black communities large body size is perceived to reflect wealth and happiness (Puone *et al*, 2005) whilst being thin is equated with HIV/AIDS (Mvo *et al*, 1999). Thus, there is a need to educate people with the increased risks of metabolic diseases associated with large body size to help reduce the increasing rate of obesity and its related traits. In morbid obesity, the expression of various insulin signalling molecules is reduced and mechanisms such as translocation, docking and fusion of GLUT-4 containing vesicles with the plasma membrane are impaired, thus resulting in

insulin resistance, a major risk factor for T2DM (Kahn *et al*, 1992; Goodyear *et al*., 1995). Insulin resistance is a fundamental aspect of the etiology of T2DM and many other metabolic traits. However, it has been shown that not all individuals with insulin resistance progress to T2DM, only about 20% of obese individuals develop the disease while most of them are able to maintain euglycemia state (Lan *et al.*, 2002). Indeed, in our study population obese individuals were normoglycemic. Aging has also been identified as an important risk factor for many metabolic disorders including T2DM. In the current study, we observed a significant age difference between the diabetic [60.3 (11.8)] and control groups [53.3(13.6)], $p<0.0001$, indicating that age was one of the contributing factors to the high prevalence of T2DM noted in this population. Szoke and coworkers (2008) demonstrated that insulin secretion decreases at a rate of approximately 0.7 % per year with aging and the decrease is accelerated up to two-fold in people with impaired glucose tolerance. It is not clear yet if the increased rate of deterioration is linked to glucotoxicity or if there are any underlying genetic defects. In addition to this, it was also observed that the activity of β-cells was low in a diabetic group when compared to the healthy control group ($p<0.0001$) as measured by HOMA-B, a confirmation of what has been reported that β-cell mass is reduced by up to 50% in people with impaired glucose tolerance (Butler *et al*, 2003).

In the present case-control study, we evaluated the effects of *ENPP1*, *FTO* and *TCF7L2* variants on T2DM in a Mixed Ancestry population of Bellville South in South Africa. Our results demonstrate that *ENPP1* and *FTO* rs9941349 genetic variants may contribute to the development of T2DM in the studied population. The presence of *ENPP1*-rs997509 T allele conferred a 4 times higher risk of developing T2DM ($p = 0.041$), while the *ENPP1*-rs1044498 A allele had a lower susceptibility effect, 1.81 (1.09- 2.98) ($p = 0.021$). The association of *ENPP1* rs997509 with T2DM was first described by Bochenski et al in 2006, where it was found to confer a high risk of T2DM in Polish individuals. The role of *ENPP1*-rs997509 polymorphism in the development of T2DM and insulin resistance has also been reported elsewhere, in obese Caucasians (Santoro *et al*., 2009), while in our study the association was adjusted for BMI. The *ENPP1* rs997509 polymorphism is a cytosine (C) with a thymine (T) substitution located at the 3' end of intron 1, in a region that may contain a regulatory element and may have a functional role although at present it is unclear. It is possible that the association we observed is through the linkage of rs997509 with rs1044498 ($D' > 0.969$). The association of *ENPP1*-rs1044498 with T2DM risk has been reported in different ethnic population groups (but not all) worldwide. A meta-analysis (including all studies published until 2008) conducted by McAteer and co-authors (2008) reported a 38% increased risk of T2DM in European carriers of the 121QQ genotype. An association between *ENPP1*-rs1044498 polymorphism and T2D that followed model of inheritance was reported in a

Chinese population (Li *et al*., 2012). Dane carriers of the 121Q allele also exhibited increased risk of T2DM, according to a meta-analysis conducted by Grarup and co-authors (2006). A study conducted in a Moroccan population provided further evidence for the role of the *ENPP1*-rs1044498 polymorphism in the development of T2DM (El Achhab *et al*., 2009). However, this was not supported by findings from other population groups such as the British (Weedon *et al*., 2006), African- and European-Americans, Polish (Lyon *et al*., 2006), Indians (Bhatti *et al*., 2010), and Tunisian Arabs (Ezzidi *et al.,* 2009).

ENPP1 encodes a transmembrane glycoprotein, which interacts with and autophosphorylates the insulin receptor beta-subunit, thus causing insulin resistance. However, in the present study we did not find any significant association between *ENPP1* SNPs and indicators of insulin resistance/sensitivity. Instead, in a previous study, we demonstrated that the *PPARG*-Pro12 allele was associated with insulin resistance in the Mixed-ancestry cohort. Like *ENPP1*, *PPARG* plays a role in insulin sensitivity. Although the role of *ENPP1* SNPs in insulin resistance was not evident in the present study, combining the findings of our two studies and based on the functions of the two proteins suggest that insulin resistance rather than a defect in insulin secretion is a primary defect that may lead to the development of T2DM in this population group. Further supporting this hypothesis is the lack of association between *TCF7L2* SNPs that were investigated in the present study and T2DM and/or the measure of β-cell function (HOMA-B%). While SNPs within *ENPP1* and *PPARG* have been reported to contribute to altered insulin sensitivity, polymorphisms within the TCF7L2 *gene* impair β-cell function and deficit in insulin secretion (Grant *et al.,* 2006; Zhang *et al.,* 2006; Groves *et al*., 2006; Florez *et al.,* 2006). First identified in 2006 by Grant *et al*, *TCF7L2* has been the most significantly and consistently associated genetic risk of T2DM in many ethnic population groups. It has been shown to increase the risk of T2DM by approximately 1.45 in heterozygotes and 2.41 in homozygotes (Grant *et al*, 2006). Even though *TCF7L2* SNP has been widely investigated and has been observed to have a strong association with T2DM in multiple populations (mostly in Europe and North America), there are few publications that have examined its effect on T2DM in African-native populations. African-native populations have unique characteristics for example, the occurrence of the ketosis-prone T2DM (Umpierrez *et al*, 2006), thus limiting the comparison of genetic risk for T2DM between them and other populations. Thus, the fine mapping of candidates genes are needed to identify the genes affecting the African-native populations. Pirie et al (2010) observed association between the heterozygote genotype of rs7903146 (TCF7L2) and T2DM in the Zulu population of South Africa. However, rs12255372 of TCF7L2 did not have association with T2DM in the same population (Pirie *et al*, 2010). In the current study we

could not replicate the results of several studies which have showed the link between *TCF7L2* SNPs and T2DM.

In some cases insulin resistance is secondary to obesity, and several studies have demonstrated that the association of *ENPP1* and *FTO* polymorphisms with T2DM is mediated by obesity (Bochenski *et al*., 2006; Cauchi *et al.,* 2008; Meyre *et al.,* 2007; Frayling *et al*., 2007; Stolerman *et al*., 2008; McAteer *et al*., 2008; Robiou-du-Pont *et al*., 2013). However, our study failed to show any association of *ENPP1* and *FTO* with obesity. In the present study, the *FTO*-rs9941349 T allele under an additive model was associated with an increased risk of T2DM, while in African-Americans this polymorphism conferred a higher risk of obesity (Hassanein *et al*, 2010). As observed in other studies for other *FTO* variants (Li *et al.*, 2012; Binh *et al*., 2013; Ali *et al*., 2013), the effect of this polymorphism on T2DM was not mediated by obesity in our study population. The *FTO* polymorphisms have been widely associated with BMI or increased obesity risk mostly in European populations (Frayling *et al*., 2007; Dina *et al*., 2007; Thorleifsson *et al*., 2009; Burgdörfer *et al*., 2013; Qian *et al*., 2013), but showed inconsistent effects on African-ancestry ethnic groups (Scuteri *et al*., 2007; Grant *et al*., 2008; Song *et al.,* 2008; Hennig *et al*., 2009; Hassanein *et al*., 2010). It is possible that the effect of *FTO* polymorphisms on T2DM and obesity is dependent on ancestry. For example, the T2DM susceptibility effect of the rs9939609 is mediated by obesity in Caucasians while the obesity-risk allele of the rs1421085 may be protecting African-Americans against T2DM (Bressler *et al*., 2010). In East and South Asians, the rs9939609 minor allele increased the risk of T2DM (Li *et al*., 2012).

CONCLUSION

The present study is of cross-sectional design that aimed at investigating the role of *FTO*-rs3751812 and -rs9941349, *TCF7L2*-rs7903146 and -rs12255372, and *ENPP1*-rs997509 -rs1044498 polymorphisms in the development of type 2 diabetes in a Mixed Ancestry ethnic population group that resides in Bellville South, Western Cape, South African. This community is characterised with a higher prevalence of T2DM, 26%, as reported previously (Erasmus *et al*., 2012). Genetic risk factors predisposing this ethnic group to T2DM are still poorly understood. Our research group recently demonstrated that in this ethnic group the peroxisome proliferator-activated receptor gamma gene (*PPARG*), Pro12Ala polymorphisms was associated with higher risk of T2DM, an effect that may be mediated by insulin resistance as carriers of the Pro12 allele had increased 2-hour post-load insulin. The present study further suggests that genes playing a role in the regulation of insulin sensitivity may contribute to the development of T2DM in the South African Mixed Ancestry ethnic population group. We demonstrated that the minor alleles of the *ENPP1*-rs997509 and -rs1044498 polymorphisms were associated with a higher risk of T2DM in a recessive model (T allele, $p = 0.041$ and A allele, $p = 0.021$), but without any effect on insulin sensitivity/resistance indicators. The study also showed that the *FTO*-rs9941349 T allele under an additive model was associated with an increased risk of T2DM, but not in the presence of obesity as observed in other population groups. Further studies are required to confirm these findings in a larger study population, stratified according to obesity status and accounting for other confounding factors such as population stratification and environmental factors.

Association of *ENPP1* and *FTO* may have been confounded by lifestyle effects as reported elsewhere. For example, Moore and co-workers (2009) showed that the significant association between *ENPP1* and increased incidence of T2DM was abolished when lifestyle or metformin intervention was included in the analysis. Ortega-Azorin and co-workers (2012) also found an interaction between *FTO* and the Mediterranean diet in determining T2DM, with carriers of the *FTO*-rs9939609 minor allele on a low Mediterranean diet having a higher risk of prevalent T2DM than individuals homozygous for the major allele. Due to a small sample size, we did not match cases of T2DM and controls by BMI to investigate whether or not obesity modulates the effect of polymorphisms on T2DM. Only two SNPs in the genes of interest were studied, and therefore we cannot rule out the possible role of other polymorphisms that may be in linkage disequilibrium with these loci. At this point the observed association of polymorphisms with T2DM in the present study has no clinical implications. Replication of these findings is critical and should be based on multiple

independent samples of substantial sizes. Once replicated, clinical trials will be required to assess the effectiveness of genotype-based intervention programs against T2DM.

Due to the genetic nature of the Mixed-Ancestry ethnic population group, it is possible that population admixture may have interfered with the association analysis. Ancestry-informative markers were not used to account for population admixture. That these findings may be false-positive results cannot be ruled-out. If not accounted for, potential population stratification in unrelated samples may cause spurious positive or negative associations in population-based association studies (Deng, 2001). However, when tested for HWE, only the *TCF7L2* rs12255372 and *FTO* 3751812 deviated and did not show any significant association with T2DM. Structure analysis conducted in the South African Mixed-Ancestry population revealed that its origin is predominantly Khoisan (32-43%), Bantu-speaking African (20-36%), European (21-28%), and a small proportion Asian (9-11%) (de Wit *et al*., 2010). However, this analysis was based on Affymetrix 500k SNP chip containing markers primarily designed for use in Europeans, and may not be accurate. Several statistical methods have been proposed to reduce the effect of population stratification on population-based association analyses, and these include structured association (SA) (Pritchard *et al.,* 2000), genomic control (GC) (Devlin *et al*., 1999), and principal components analysis (PCA) (Price *et al.,* 2006). We recommend that either SA (when appropriate markers are available) or PCA be conducted to correct for population stratification if further studies are to be conducted to confirm the association detected in the present study.

CHAPTER 6
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APPENDIX

APPENDIX

Phosphate Buffered Saline (PBS) pH7.4 (1L)

8.0 g NaCl

1.44 g Na_2HPO_4

0.24 g KH_2PO_4

0.20 g KCl

Add components one at a time to 900 mL dH_2O, dissolve and adjust pH then fill with dH_2O to 1L

Nuclear Lysis Buffer pH 8.2 (500 mL)

11.5 g Nacl

10 ml Tris (1M stock)

10 ml EDTA (10 mM stock)

Preparation of lysis buffer pH 7.4

31 ml from NH_4Cl (1M stock)

1 ml of $KHCO_3$ (1M stock)

100 µl of EDTA (100 mM stock)

For all solutions, use HCL / NaOH to adjust pH

6M Sodium Chloride (NaCL)

350.64 g of NaCl dissolved in 800 ml of dH_2O and then adjust to 1L with dH_2O.

1x Tris EDTA (TE) Buffer

10 mM Tris (10 ml 1 mM stock)

1 mM EDTA (2 ml of 0.5 M Stock)

Adjust pH and then fill up 1L with dH_2O

10% (w/v) Sodium Dodecyl Sulphate (pH 7. 2)

Dissolve 10 g of electrophoresis-grade SDS in 100 ml dH_2O.

Heat and stir the solution with a magnetic stirrer to assist dissolution.

20x SB buffer (1L)

8 g NaOH

45 g Boric Acid

Dissolve in 800 ml of dH_2O and then adjust pH accordingly before filling up with dH_2O to 1L

1x SB buffer (1L)

Dilute 100 ml of 20x SB buffer in 1900 ml of dH_2O

Ethidium Bromide (EtBr) Stain (10ng/ml)

1 g EtBr powder in 100 ml dH_2O.

Store in a dark bottle