Evaluation of specific genotypes in the context of the type 2 diabetes risk phenotype in the black South African population.

BY

DR. H.J. KOTZé, MBChB

Thesis submitted for the degree Philosophiae Doctor (Ph.D.)
in Biochemistry at the North-West University

SUPERVISOR: Professor Antonel Olckers
Centre for Genome Research, North-West University (Potchefstroom Campus)

CO-SUPERVISOR: Doctor Wayne Towers
Centre of Excellence for Nutrition, North-West University (Potchefstroom Campus)

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Evaluasie van spesifieke genotipes in konteks met die tipe 2 diabetes risiko fenotipe in die swart Suid-Afrikaanse populasie

DEUR

DR. H.J. KOTZé, MBChB

Proefskrif voorgelê vir die graad Philosophiae Doctor (Ph.D.)
in Biochemie aan die Noordwes-Universiteit

Studieleier: Professor Antonel Olckers
Sentrum vir Genomiese Navorsing, Noordwes-Universiteit (Potchefstroom Kampus)

Medestudieleier: Dokter Wayne Towers
Sentrum vir Uitnemendheid vir Voeding, Noordwes-Universiteit (Potchefstroom Kampus)

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To my Parents
and those
who inspire me
to be
the best I can be

ABSTRACT

Type 2 diabetes (T2D) is a complex disease that affects 4% of the general population and is expected to increase to 5.4% by the year 2025. A clear understanding of the aetiology of T2D susceptibility and pathogenesis will thus have a noticeable impact on global health. The black South African population is currently under increased risk for developing T2D due to the impact of urbanisation. Since the mechanisms of disease risk in this population differ to that of the so-called developed countries, it is necessary that the exact pathogenesis of this disease be elucidated in order to define suitable screening and therapeutic strategies for the black South African population. The purpose of this study was to initiate this process. Four genotypes were investigated, including alterations in the IRS-1, IRS-2, PPARγ2 and calpain 10 genes. This study was therefore the first to evaluate these specific genotypes in the context of the T2D risk phenotype in the black South African population, aiming towards a novel and population specific contribution towards current T2D research.

The results of this study indicated that none of the screened genotypes were significant predictors of impaired glucose in the black South African population. A biphasic glucose curve shape (GCS) was associated with female gender, whereas a monophasic GCS, a high BMI, female gender as well as a high HbA1c level were linked to glucose intolerance. A high HbA1c level proved to be a significant predictor for glucose intolerance, although the four screened loci were not good predictors of the HbA1c level. The study also illustrated that it is not possible to simply adopt T2D screening strategies from those developed in other ethnic groups and that different genetic and environmental risk factors that play a role in the pathophysiology of T2D should be taken into account. The need for optimised and population specific T2D screening strategies is therefore emphasised.

By further elucidating the complexities of T2D, a step towards providing more accurate screening strategies to the immediate population will be achieved. This will directly result in a significant decrease in the national burden of care, morbidity and mortality, paving the way to optimal health care strategies for this developing country.

OPSOMMING

Tipe 2 diabetes (T2D) is ‘n komplekse siektetoestand wat 4% van die wêreld bevolking affekteer en daar word verwag dat dit teen die jaar 2025 tot 5.4% sal verhoog. ‘n Goeie begrip van die etiologie van T2D vatbaarheid en patogenese sal dus ‘n sterk impak hê op globale gesondheid. As gevolg van verstedeliking het die swart Suid-Afrika populasie ‘n verhoogde risiko vir die ontwikkeling van T2D. Aangesien die meganismes wat betrokke is by die risiko vir T2D in hierdie bevolking, verskil van die in sogenaamde ontwikkelde lande, is dit nodig dat die presiese patogenese vasgestel word om sodoende gepaste evaluasie- en behandelings- strategieë vir die swart Suid-Afrika bevolking te bepaal. Die doel van hierdie studie was om die proses te inisieer. Vier genotipes is ondersoek, insluitende alterasies in die IRS-1, IRS-2, PPARγ2 en calpain 10 gene. Hierdie studie was dus die eerste om die spesifieke genotipes in die konteks van die T2D risiko fenotipe in die swart Suid-Afrikaanse populasie te evalueer, ten einde ‘n nuwe en populasie spesifieke bydrae te lewer tot die huidige T2D navorsing.

Die resultate van hierdie studie het aangetoon dat nie een van die genotipes wat ondersoek is, beduidende aanwysers van ingekorte glukose in die swart Suid-Afrika populasie is nie. ‘n Bifasiese glucose kurwe vorm (GKV) is geassosieer met vroulike geslag en ‘n monofasies GKV, verhoogde liggaamsmassa-indeks, vroulike geslag sowel as ‘n verhoogde HbA1c vlak is geassosieer met glukose intoleransie. ‘n Hoë HbA1c vlak is bewys as beduidende aanwyser van glukose intoleransie, alhoewel die vier geen alterasies wat ondersoek is, nie goeie voorspellers is van die HbA1c vlak nie. Hierdie studie het ook aangedui dat dit nie moontlik is om T2D evaluasie-strategieë wat ontwikkel is vir ander etniese groepe, net aan te pas nie en dat verskillende genetiese- en omgewings-risiko faktore wat ‘n rol speel in die patofisiologie van T2D, in oorweging gebring moet word. Die noodsaaklikheid vir geoptimaliseerde en populasie spesifieke T2D evaluasie-strategieë is dus beklemtoon.

Meer akkurate evaluasie-strategieë vir die onmiddellike populasie sal slegs bereikbaar wees met ‘n beter begrip van die kompleksiteite van T2D. Die direkte gevolg sal ‘n beduidende verlaging in die nasionale las van sorg, morbiditeit en mortaliteit wees wat die weg sal baan na optimale gesondheidsorg-strategieë vir hierdie ontwikkelende land.

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LIST OF ABBREVIATIONS AND SYMBOLS

Symbols and abbreviations are listed in alphanumerical order.

LIST OF SYMBOLS

α	alpha
β	beta
$\chi 2$	Chi square
°C	degrees centigrade
=	equal to
γ	gamma
↑	increase
<	less than
$\bar{x}$	mean statistic
‘	minute
μ	mu, denoting micro: 10^{-6}
>	more than
%	percentage
®	registered trademark
I	Roman numeral one
II	Roman numeral two
III	Roman numeral three
IV	Roman numeral four
VI	Roman numeral six
√	square root
™	trademark

LIST OF ABBREVIATIONS

A or a	adenine
A_{260}/A_{280}	ratio of absorbency measured at 260 nm and 280 nm
ACRS	amplification created restriction site
ADA	American Diabetes Association
AIDS	acquired immune deficiency syndrome
Ala	alanine
AMPK	adenosine monophosphate-activated protein kinase
ANOVA	analysis of variance
Arg	arginine
Asp	aspartic acid
AUC	area under the curve
bi	biphasic in terms of glucose curve shape
BLAST	Basic Local Alignment Search Tool
bp	base pair
BMI	body mass index
BSA	bovine serum albumin
Bst UI	restriction endonuclease *Bst* UI
Bst NI	restriction endonuclease *Mva* I (*Bst* NI)
C or c	cytosine (in DNA sequence)
CAPN10	calpain 10
CI	confidence interval

cm	centimetre: 10^{-2} metre
d	biological significance
ddH_2O	double distilled water
ddNTP	2',3'-dideoxynucleotide-5'-triphosphate
DKA	diabetic ketoacidosis
DM	diabetes mellitus
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
E	expected number
EDTA	ethylenediamine tetraacetic acid: $C_{10}H_{16}N_2O_8$
e.g.	*exempli gratia* (for example)
et al.	*et alii* (and others)
EtBr	ethidium bromide: $C_{21}H_{20}BrN_3$
EtOH	ethanol: CH_3CH_2OH
F	female
FFA	free fatty acids
g	gram
G or g	guanine
GCS	glucose curve shape
gDNA	genomic DNA
Genbank	Genbank®: United States repository of DNA sequence information
GLUT	glucose transporter
GLUT-1	glucose transporter 1
GLUT-2	glucose transporter 2
GLUT-4	glucose transporter 4
Gly	glycine
h	hour
HbA1c	glycosylated haemoglobin
H_2O	water
HCl	hydrochloric acid
HDL	high density lipoprotein
Hha I	restriction endonuclease *Cfo* I
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
H-W	Hardy-Weinberg
IDDM	insulin dependent diabetes mellitus
IDT	integrated DNA technology
i.e.	*id est* (that is)
IFG	impaired fasting glucose
IGR	impaired glucose regulation
IGT	impaired glucose tolerance
Ile	isoleucine
IQR	inter-quartile range
IRS-1	insulin receptor substrate-1 gene
IRS-2	insulin receptor substrate-2 gene
IV	intravenous
$kg.m^{-2}$	kilogram per metre squared: unit of body mass index
K-W	Kruskal-Wallis
L	litre
Leu	leucine
LIPC	hepatic lipase
Ltd.	Limited
μg	microgram
$\mu g.mL^{-1}$	microgram per millilitre
μL	microlitre
μM	micromolar
m	metre
M	male
Max	maximum

$MgCl_2$	magnesium chloride
$mg.dL^{-1}$	milligram per decilitre
min	minute
Min	minimum
mL	millilitres
mM	millimolar concentration
$mmol.L^{-1}$	millimole per litre
MODY	maturity onset diabetes of the young
MODY1	locus associated with maturity onset diabetes of the young 1
MODY2	locus associated with maturity onset diabetes of the young 2
MODY3	locus associated with maturity onset diabetes of the young 3
mol	mole: unit describing the amount of a particular chemical species; the amount being equal to one Avogadro's number (6.02×10^{23}) of atoms, ions, molecules, or electrons
mono	monophasic in terms of glucose curve shape
MS	metabolic syndrome
M-W	Mann-Whitney
n	number
n	nano: 10^{-9}, when referring to the metric scale
Na^+	sodium ion
Na_2EDTA	di-sodium ethylenediamine tetra-acetic acid: $C_{10}H_{14}N_2Na_2O_8.2H_2O$
neg	negative
NFG	normal fasting glucose
ng	nanogram
$ng.\mu L^{-1}$	nanogram per microlitre
NIDDM	non insulin dependent diabetes mellitus
NIH	national institute of health
NGT	normal glucose tolerance
NIDDM	non-insulin dependent diabetes mellitus
NIH	National Institute of Health, USA
NF4α	nuclear transcription factor 4 alpha
NKHHC	non-ketotic hyperglycaemic-hyperosmolar coma
nm	nanometre
nt	nucleotide
O	observed number
ob	obesity
OGTT	oral glucose tolerance test
OR	odds ratio
p	p-value: in a statistical context
p	pico: 10^{-12}, when referring to volume
p	short arm of a chromosome: in a genetic context
PAI-1	plasminogen activator inhibitor type-1
PCR	polymerase chain reaction
pH	indicates acidity: numerically equal to the negative logarithm of H^+ concentration expressed in molarity
pmol	picomole
pos	positive
PPARγ2	peroxisome proliferator-activated receptor gamma 2 gene
PRIMER	Profiles of Resistance to Insulin in Multiple Ethnicities and Regions
Pro	proline
Pty	propriety
PURE	Prospective Urban and Rural Epidemiology
q	long arm of chromosome: in a genetic context
rad	ras associated with diabetes
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	revolutions per minute
SA	South Africa
SD	standard deviation
SD_{max}	maximum standard deviation between two means
Ser	serine

Sma I	restriction endonuclease *Sma* I
S_{max}	maximum standard deviation
SNP	single nucleotide polymorphism
S-W	Shapiro-Wilk
t	time
T or t	thymine
T_a	annealing temperature
$T_{a(calc)}$	calculated annealing temperature
$T_{a(opt)}$	optimised annealing temperature
Taq	*Thermus aquaticus*
TBE	Tris® borate-EDTA buffer
T1D	type 1 diabetes
T2D	type 2 diabetes
T2DM	type 2 diabetes mellitus
Thr	threonine
T_m	melting temperature
TNFα	tumour necrosis factor alpha
Triton X-100®	Triton X-100®: octylphenolpoly(ethylene-glycolether)$_n$: $C_{34}H_{62}O_{11}$, for n = 10
Tris	Tris: tris(hydroxymethyl)-amino-methane: 2-amino-2-(hydroxymethyl)-1,3-propanediol: $C_4H_{11}NO_3$
Tris-HCl	2-amino-2(hydroxymethyl)-1,3-propanediol hydrochloride: $C_4H_{11}NO_3.H_2O$
$tRNA^{Leu}$	transfer ribonucleic acid specific for leucine
Trp	tryptophan
Tyr	tyrosine
unclas	unclassified in terms of glucose curve shape
UCSNP	University of Chicago single nucleotide polymorphism
UCSNP19	insertion deletion alteration within intron 6 of the calpain 10 gene
UCSNP43	alteration of a guanine to an adenine within intron 3 of the calpain 10 gene
UCSNP44	alteration of a thymine to a cytosine within intron 3 of the calpain 10 gene
UCSNP56	alteration of a guanine to an adenine within intron 6 of the calpain 10 gene
UCSNP63	alteration of a cytosine to a thymine within intron 13 of the calpain 10 gene
USA	United States of America
UV	ultraviolet
V	volt
WHR	waist-to-hip ratio
WHO	World Health Organisation
WT	wild type
x g	gravitational force or gravitational acceleration

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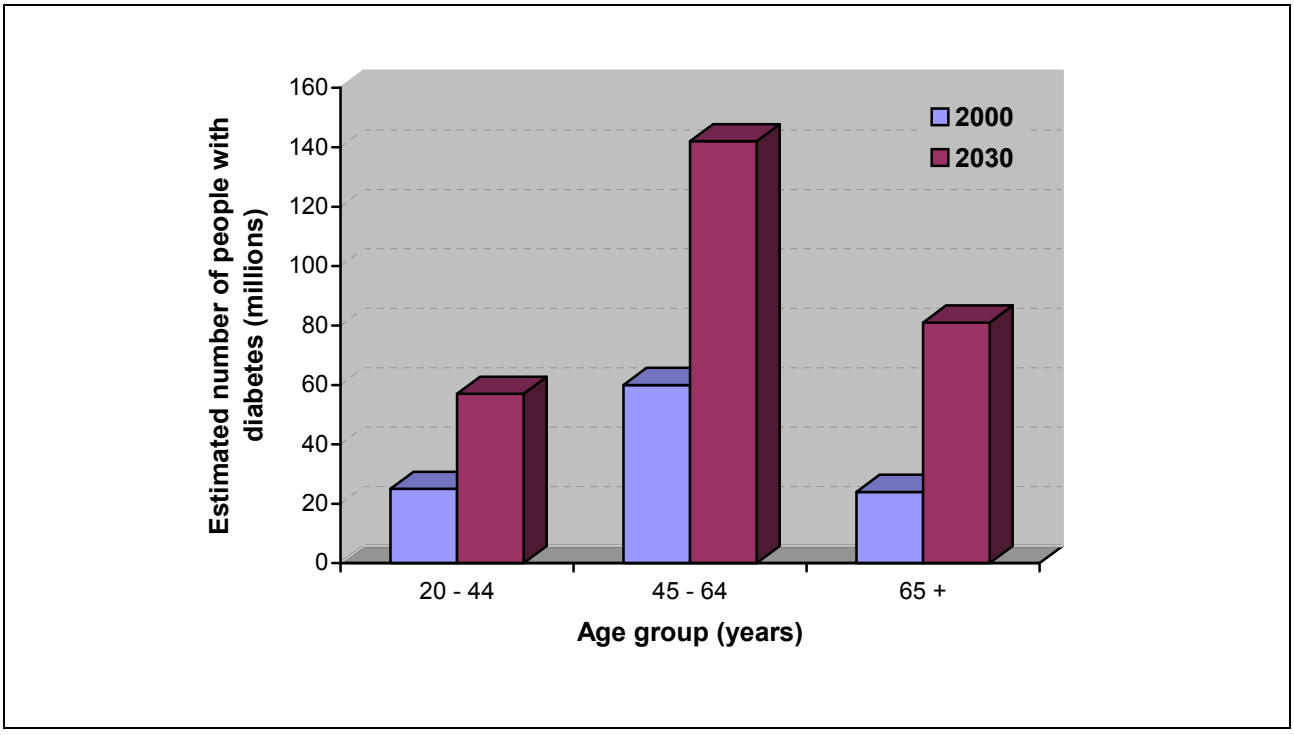
CHAPTER ONE

INTRODUCTION

Hyperglycaemia, as a final biochemical outcome, unites a diverse collection of metabolic disorders classified as diabetes mellitus (DM). One of these disorders, namely type 2 diabetes (T2D), is a multifactorial disease with a high global prevalence and devastating complications when left untreated. It is estimated that the incidence of T2D will increase to 5.4% by the year 2025. The third largest incidence increase of 185% will be observed in the developing region of sub-Saharan Africa (King *et al.,* 1998). The estimated increase in the number of people with diabetes in developing countries, including South Africa, is depicted in Figure 1.1. The best argued causes of this massive rise in diabetes prevalence are an increase in obesity prevalence as well as urbanisation (Wild *et al.*, 2004). The prognosis of this devastating disease is determined by both the time of diagnosis and effective treatment initiation.

Figure 1.1: Estimated number of adults with diabetes in 2000 and 2030 in developing countries



Adapted from Wild *et al.* (2004).

Genetic background plays an important role in the susceptibility of an individual to T2D, but this is only one component in a multifaceted network of determinants that bring about

increased diabetes risk. This risk is further compounded by various environmental, biochemical, including glucose curve shape (GCS), as well as anthropometrical factors. Research on this complex disease is extensive. However, there is a desperate need for the development of population specific screening, prevention and treatment strategies due to the strain that DM will place on healthcare systems.

Screening for T2D is imperative and allows for identifying asymptomatic individuals with the risk of developing T2D. This will permit timeous commencement of treatment and preventive management of this disease. Successful prevention and individualised treatment strategies are the gold standard to which national health services should strive in order to optimise use of both time and money. Previous reports highlight the fact that a specific screening strategy does not implicitly apply to different populations with varying environment, socio-economic and clinical characteristics (Engelgau *et al.*, 2009). When optimising such a strategy within a specific population, the availability of risk parameter data, the best possible cut-off point to identify an affirmative test, cost effectiveness as well as the simplicity and frequency of the strategy are factors that need to be assessed in order to ensure the usefulness of a screening model. This definitive strategy is hypothesised to be feasible only with a complete understanding of all factors involved in T2D susceptibility within a specific population.

The purpose of this study was to initiate the process of developing a population specific T2D susceptibility screening strategy for black South Africans, as suggested to be essential in the editorial by Herman (2009). Blood samples were collected from 443 black South African individuals during a two-hour oral glucose tolerance test (OGTT) at the time of the PRIMER (Profiles of Insulin Resistance in Multiple Ethnicities and Regions) study. The investigated genotypes included alterations in the insulin receptor substrate-1 (IRS-1), insulin receptor substrate-2 (IRS-2), peroxisome proliferator-activated receptor gamma 2 (PPARγ2) and calpain 10 (CAPN10) genes. The cohort was stratified into subgroups according to GCS (biphasic, monophasic and unclassified), physical environment (urban, rural), genotype (IRS-1, IRS-2, PPARγ2 and CAPN10 genes), HbA1c levels (normal, high), glucose tolerance (normal, impaired), HIV status (positive, negative), as well as phenotype. Phenotypic differences were evaluated with regard to anthropometrical measures including body mass index (BMI), age and gender, as well as environmental factors encompassing diet and physical activity. This study was therefore the first to evaluate specific genotypes in the context of the T2D risk phenotype in the black South

African population, aiming towards a novel and population specific contribution towards current T2D research.

Clinical, phenotypic and genetic aspects of diabetes are discussed in Chapters Two to Four, whilst the study design and the various screening strategies utilised to fulfil the aims of this study are described in Chapter Five. The results obtained during the study and the conclusions reached, subsequent to data and statistical analyses, are discussed in Chapters Six and Seven, respectively.

CHAPTER TWO

CLINICAL ASPECTS OF DIABETES

Diabetes is a common disorder with the classic clinical presentation of hyperglycaemia related symptoms, signs and complications (Berkow *et al.*, 1992). A variety of diagnostic and treatment modalities are currently used and will be discussed.

2.1 CLINICAL PRESENTATION OF DIABETES MELLITUS

The clinical presentation of the various diabetes types, differ substantially. A discussion of the presenting features of type one and type two diabetes follows. Other observed forms of diabetes are also discussed briefly.

2.1.1 Type 1 diabetes mellitus

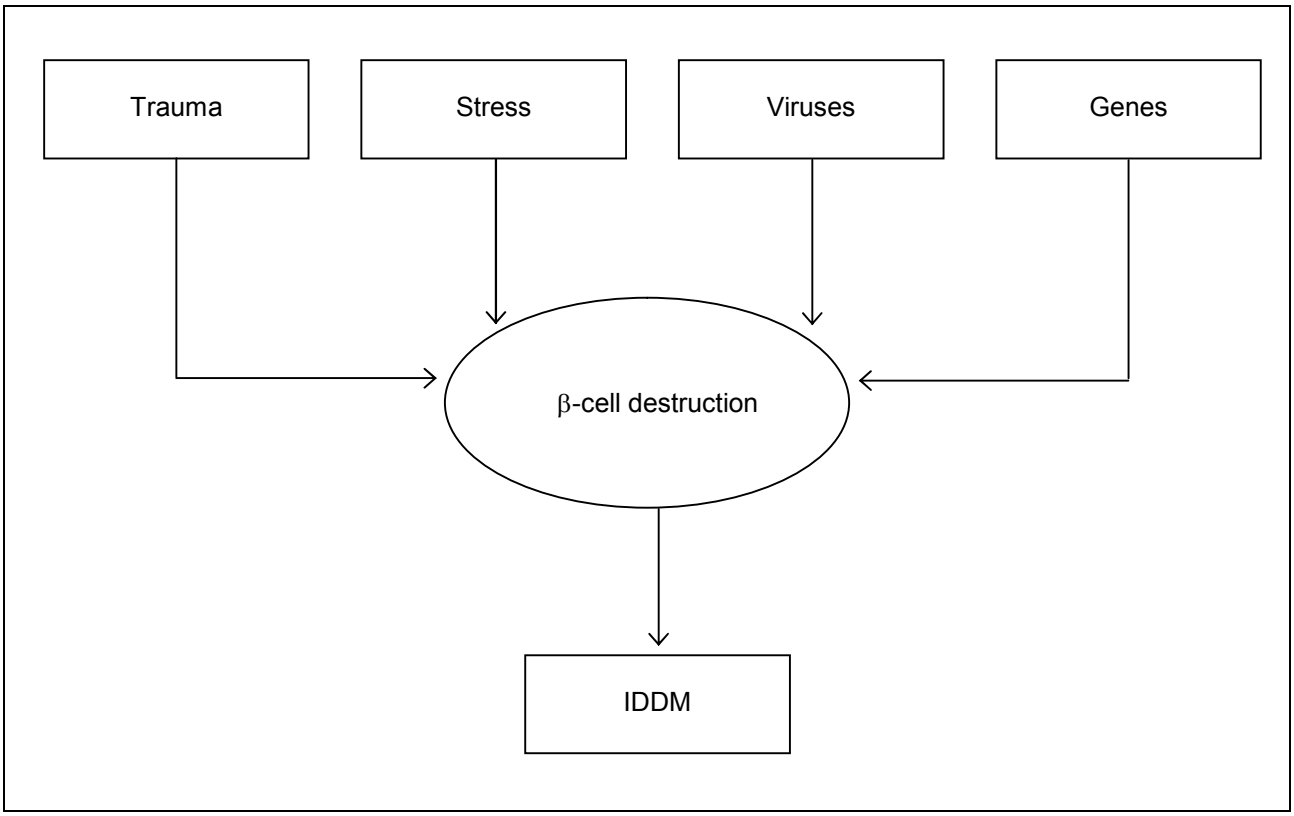
Hyperglycaemia and a predisposition to DKA, as well as loss of body weight, due to the degradation of the insulin producing pancreatic β-cells, are the main clinical features of type 1 diabetes (T1D) or insulin dependent diabetes mellitus (IDDM). This disorder is the result of autoantibody degradation of the insulin-producing β-cells present in the pancreas. The decreased insulin secretion, which results directly from the β-cell destruction, is the end result of this autoimmune process. It proceeds for several years and is influenced by various genetic and environmental factors (Olefsky, 2001), as depicted in Figure 2.1.

Detectable islet cell cytoplasmic and/or surface antibodies are associated with specific human leukocyte antigen (HLA) phenotypes. More than 90% of the β-cells in patients with this condition are damaged due to both genetic and immune factors. Pancreatic islet cells, excluding the α-cells (glucagon secreting), are infiltrated by T- and B-lymphocytes as well as macrophages, causing destruction, as observed as an insulitis during autopsy (Berkow *et al.*, 1992).

Only 10% to 12% of newly diagnosed children with IDDM have a first-degree relative with the disease. There is less than 50% concordance rate for IDDM in monozygotic twins and some environmental factor like a virus, may be responsible for initiating the process of autoimmune β-cell destruction in individuals with a genetic susceptibility, which can thus

result in IDDM (Berkow *et al.,* 1992). This is the predominant type of DM diagnosed in juveniles, even though it may occur at any age. Ten to fifteen percent of the global DM incidence is accounted for by IDDM (Jun *et al.,* 1999). Individuals diagnosed with IDDM have little or no ability to produce insulin and are entirely dependent on insulin injections for survival. Diet must also be carefully controlled, including adequate carbohydrates to provide for the constant need of the body.

Figure 2.1 Schematic representation of the pathogenesis of T1D



Adapted from Elbein (1997). IDDM = non-insulin dependent diabetes mellitus; β = beta. The diagram presents the interaction of multiple susceptibility loci and environmental factors influencing the disease of which a β-cell defect is presumed as the final stage.

Environmental factors observed to have an effect, are congenital rubella, mumps and coxsackie B viruses. Stress and trauma were however also confirmed to have a causative effect (Berkow *et al.,* 1992). Data from an investigation in Cuba has revealed that the echovirus 16 infection can possibly initiate the autoimmune destruction of the pancreatic β-cells (Cabrera-Rode *et al.,* 2003). This diabetes, which starts in childhood or adolescence, is usually more severe than type 2 diabetes mellitus (T2DM).

2.1.2 Type 2 diabetes mellitus

The most important clinical feature of T2D or non-insulin dependent diabetes mellitus (NIDDM) is also hyperglycaemia, but unlike individuals with IDDM, these patients are

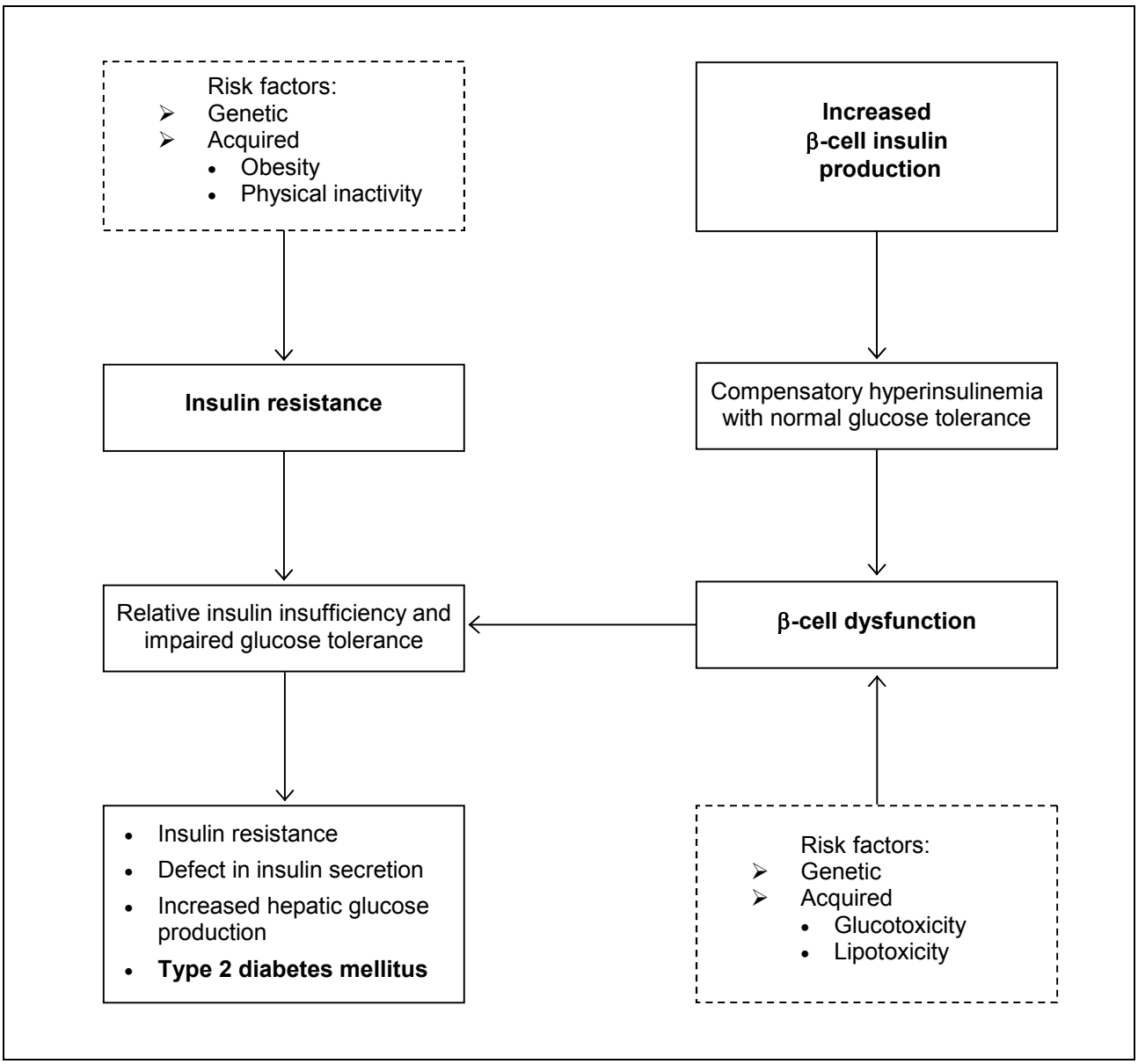
repeatedly observed to be obese and older than 30 years without a tendency for diabetic DKA. T2D is generally observed in adults and includes 85% of diabetic individuals globally. Five percent of the general population is affected by this disorder (Jun *et al.,* 1999) and although the pancreas of these individuals retains some ability to produce insulin, it is inadequate for the body's needs.

Genetic aspects play a significant role in the development of this disorder and the concordance rate for NIDDM in monozygotic twins is > 90% (Berkow *et al.,* 1992). This high concordance rate for the disease, together with familial aggregation of the disorder suggests that genetic factors play an important role in the development of NIDDM. Individuals have different genetic and environmental factors contributing to NIDDM, although tissue resistance to insulin and a decreased secretion of glucose-stimulated insulin are the common defects resulting in T2D (Jun *et al.,* 1999).

In T2D, no association has been demonstrated between the disorder and specific HLA phenotypes. Whereas degradation of pancreatic β-cells is observed in IDDM, the β-cells are mostly conserved in NIDDM. The elevated plasma glucose levels observed in NIDDM individuals is not only due to the inefficient insulin response to exogenous glucose, despite the occasionally significant secretion of insulin by the pancreas, but is also due to the resistance of tissue to insulin (Berkow *et al.,* 1992).

As NIDDM patients are frequently observed to be obese, appropriate drug and/or persistent diet therapy often improves the secretion of insulin. The β-cell dysfunction combined with a genetic or environmental aspect like obesity is required for the development of NIDDM. The normal regulation of glucose entails glucose production by the liver and the utilisation of glucose by skeletal muscle, which are both reliant on normal insulin action. The decreased efficacy of insulin present in T2D individuals also exists in obesity. The complex aetiology of T2D is presented in Figure 2.2. The criteria required for initiating insulin treatment include ketonuria, a blood glucose level higher than 25 $mmol.L^{-1}$, the sudden onset of hyperglycaemia or weight loss and dehydration. A valid statement formulated by Berkow *et al.* (1992) states that T1D always exists in the presence of a ketoacidosis.

Figure 2.2 Schematic representation of the aetiology of type 2 diabetes mellitus



Adapted from Olefsky (2001). β = beta.

2.1.3 Maturity onset diabetes of the young (MODY)

Maturity onset diabetes of the young (MODY) is defined as a form of NIDDM, as it is observed mostly in normal weight, young adolescents without symptoms of DM. This disorder has an autosomal dominant inheritance pattern (Berkow *et al.,* 1992). The MODY1 (locus associated with maturity onset diabetes of the young 1) gene was localised to chromosome 20. NIDDM and its complications have been associated with a rare nonsense mutation (Glu268X) in the hepatocyte nuclear transcription factor 4α (NF4α) as observed by Yamagata *et al.* (1996). A candidate gene, glucokinase, was studied via linkage analyses to identify MODY2 (locus associated with maturity onset diabetes of the young 2). The mutations within the glucokinase gene were observed in 50% of MODY cases observed in France. The most important solitary gene causing NIDDM may be

MODY3 (locus associated with maturity onset diabetes of the young 3), which is associated with 25% of MODY in French families (Elbein, 1997).

2.1.4 Other types of diabetes

Diabetes is also associated with other endocrine diseases like Cushing's syndrome, pheochromocytoma, acromegaly, primary aldosteronism, glucagonoma or somatostatinoma. In these disorders insulin effectiveness and/or secretion is influenced by a major endocrine defect. IDDM generally occurs in patients suffering from specific autoimmune endocrine disorders, for example Hashimoto's, Graves' disease and idiopathic Addison's disease (Berkow *et al.,* 1992).

The heterozygous inheritance of an abnormal gene leading to an increased incidence of insulin defectively binding to the insulin receptor, resulting in DM, can lead to individuals presenting with symptoms of NIDDM. Despite the glucose responding to the exogenous insulin as expected, immunoreactive insulin levels are considerably elevated (Berkow *et al.,* 1992). Pancreatic disease also often manifests as diabetes *e.g.* alcohol induced chronic pancreatitis, which is associated with DM (Berkow *et al.,* 1992).

Insulin resistance at the insulin receptor level is evident in disorders associated with acanthosis nigricans. Two different types are distinguishable, namely type A, which is due to alterations in the insulin receptor on a genetic level and type B, which is due to the insulin receptor being targeted by circulating antibodies (Berkow *et al.,* 1992).

Lipoatrophic diabetes is another type of hyperglycaemia that is occasionally observed. Subcutaneous adipose tissue partially ceases to exist in insulin resistant DM. This has been linked to genetic alterations in the insulin receptor (Berkow *et al.,* 1992). Diabetes can at times also be induced by β-cell toxins such as streptozocin that is utilised for the treatment of pancreatic islet carcinomas. This toxin has been determined to induce diabetes in experiments on rats (Berkow *et al.,* 1992).

2.2 SYMPTOMS AND SIGNS OF DIABETES MELLITUS

Common clinical signs of T2D include hyperglycaemia and glucosuria, which in turn results in the classic clinical DM symptoms including polyuria, polydipsia and polyphagia associated with weight loss, blurred vision, recurrent candidal vaginitis, soft tissue

infections or dehydration. All populations and age groups are affected by diabetes, but the disorder is more frequently observed in older individuals as well as in black Africans, Hispanics, Native Americans and Asians. Life expectancy is reduced with up to 15 years in individuals with DM (Olefsky *et al.,* 2001).

Complications occurring at a later stage of the disease include retinopathy (due to thickening of the arteries), nephropathy, atherosclerotic coronary and peripheral arterial disease as well as peripheral and autonomic neuropathies. A general examination or observation of hyperglycaemic symptoms mostly results in the diagnoses of T2D. A diagnosis is however often only made at times of presentation with NKHHC or another diabetic complication (Berkow *et al.,* 1992).

2.3 DIAGNOSIS OF DIABETES MELLITUS

The classic symptoms of DM *i.e.* polyuria, polydipsia and polyphagia associated with weight loss, are used to raise the question of a possible DM diagnosis. Many cases will however be asymptomatic and only noticed upon routine blood glucose screening. Complications such as infections, neuropathy, retinopathy and arterial disease may be the presenting feature. A simple, finger prick test to determine a random plasma glucose level, can give an indication of a possible DM diagnosis (Berkow *et al.,* 1992). A random glucose level above 5.6 $mmol.L^{-1}$ should according to the World Health Organisation (WHO) criteria raise suspicion of existing diabetes or at least impaired glucose tolerance, warranting further investigation.

In cases of suspected disease, further laboratory tests should be performed. The essence of diagnosing DM lies in identifying patients at risk for symptomatic hyperglycaemia, for DKA or NKHHC, and for late clinical complications. The WHO has set up diagnostic criteria, as listed in Table 2.1, for both random and fasting glucose levels, which have to be met in order to make the diagnosis of DM. If these criteria are met, performing an OGTT is not required. Glucosuria always requires further investigation, even when symptomless, due to the 32% sensitivity and 99% specificity of this straightforward test (Hope *et al.,* 1998).

Impaired fasting glucose (IFG) implies a fasting glucose between 6 and 7.8 $mmol.L^{-1}$ and a 2-hour glucose value between 7.8 and 11.1 $mmol.L^{-1}$ is required to diagnose impaired glucose tolerance (IGT). IGT increases the risk of death from myocardial infarction (MI)

by two fold. Patients observed with either an impaired fasting glucose or impaired glucose tolerance is referred to as having pre-diabetes or impaired glucose regulation (IGR). One to five percent of these patients with IGR will within a year progress to DM (Chen *et al.,* 2004).

Table 2.1 World Health Organisation diagnostic criteria for DM

Random plasma glucose	Fasting plasma glucose	Diagnosis
< 5.6 mmol.L^{-1}	< 6.0 mmol.L^{-1}	Diabetes mellitus excluded
5.6 - 6.1 mmol.L^{-1}	6.0 – 7.8 mmol.L^{-1}	Impaired fasting glucose
> 6.1 mmol.L^{-1}	> 7.8 mmol.L^{-1}	Diabetes mellitus

Adapted from Hope *et al.* (1998); DM = diabetes mellitus; mmol.L^{-1} = millimole per litre.

A screening test for DM should be undertaken in all patients at age 45 and older and repeated every three years. More frequent testing as well as initial testing before age 45 should be considered for individuals that have one or more of the following risk factors: Obesity with a BMI > 27 kg.m^{-2}, member of a high-risk group (African-American, Asian, Native-American or Hispanic), first degree relative with DM, hypertensive, high density lipoprotein (HDL) < 35 mg.dL^{-1}, triglyceride level > 250 mg.dL^{-1}, or a history of IGR (impaired fasting glucose or impaired glucose tolerance) on former testing (Chen *et al.,* 2004).

In more complicated cases, T2D is often suspected even though fasting or symptomatic hyperglycaemia is absent. In these patients, OGTTs are performed to support the diagnosis of T2D. Plasma glucose levels and the values according to which clinical diagnosis is determined are presented in Table 2.2. Prior to the ingestion of a 75 g glucose load and glucose plasma level testing, the patient has to be fasting for ten to sixteen hours with no evidence of metabolic stress (systemic infection). Plasma glucose is measured initially and again at 30, 60, 90 and 120 minutes post prandially. Various drugs (thiazides, glucocorticoids, indomethacin) and other conditions (renal, central nervous system, cardiovascular or endocrine disease) can cause abnormalities in the OGTT results (Berkow *et al.,* 1992).

Table 2.2 OGTT results for diagnosing DM

Initial plasma glucose	2 hour plasma glucose	Diagnosis
6.0 – 7.8 mmol.L^{-1}	7.8 – 11.0 mmol.L^{-1}	Impaired glucose tolerance
> 7.8 mmol.L^{-1}	> 11.1 mmol.L^{-1}	Diabetes mellitus

Adapted from Berkow *et al.* (1992); DM = diabetes mellitus; OGTT = oral glucose tolerance test; mmol.L^{-1} = millimole per litre.

The OGTT was described for the first time in 1922 by John (McCance *et al.,* 1997) and the National Diabetes Data Group (1979) described the procedure as it is used at present as a diagnostic tool. Clinical use of the test is however on the decrease and the validity of performing an OGTT routinely is widely debated (McCance *et al.,* 1997). The value of using OGTT results for the screening of T2D is however currently still evident (Barr *et al.,* 2002). Zhou *et al.* (2006) reported cut-off values for the various OGTT intervals that can be used for the diagnoses of IGR and T2D, as presented in Table 2.3.

Table 2.3 OGTT cut-off values for diagnosing IGR and DM

OGTT interval	Cut-off value for IGR	Cut-off value for DM
0 min	5.6 mmol.L^{-1}	6.8 mmol.L^{-1}
30 min	9.7 mmol.L^{-1}	11.2 mmol.L^{-1}
60 min	10.1 mmol.L^{-1}	13.0 mmol.L^{-1}
120 min	7.8 mmol.L^{-1}	11.1 mmol.L^{-1}

Adapted from Zhou *et al.* (2006); DM = diabetes mellitus; OGTT = oral glucose tolerance test; IGR = impaired glucose regulation; min = minutes; mmol.L^{-1} = millimole per litre.

The plasma glucose level over the past couple of months is well indicated by the HbA1c level. It is argued that this level cannot be utilised as a reliable tool for DM screening due to the fact that it can be unaffected in patients with impaired glucose tolerance. However, with the specificity of the HbA1c measurement for the diagnosing of T2D, being more than 98% when more than one percent above normal (6.5%), it is evidently a useful tool in screening for T2D, and also in the decision of initiating insulin therapy (Chen *et al.,* 2004).

According to clinical findings, it is generally possible for a clinician to distinguish between T1D and T2D. This may however be more problematic and the c-peptide levels may be used to assist. The c-peptide is a product of the cleavage of pro-insulin to insulin and is present in T2D, but insignificant or lacking in T1D. If any uncertainty exists, it is useful to repeat the c-peptide after a glucose load, where it will be significantly increased in T2D, but unchanged in T1D (Chen *et al.,* 2004).

2.4 TREATMENT OF DIABETES MELLITUS

The high rate of morbidity and mortality associated with this disorder is due to the common complications of DM resulting from the constant hyperglycaemia. Mostly in IDDM patients, recurrent incidents of significant hypoglycaemia result from the inadequate attempt to maintain normal plasma glucose. Developing the complications associated with this disease is not prevented by the therapy initiated to control fluctuating glucose

levels (Berkow *et al.,* 1992). Equally important to reducing plasma glucose levels is patient education, which is essential in ensuring the effectiveness of the therapy. Treatment consists of diet, physical activity and various pharmaceutical treatment regimens, as discussed in the sections to follow.

2.4.1 Diet and increased physical activity

As depicted in Figure 2.2, environmental factors such as obesity and physical inactivity form an integral part of the pathogenesis of T2D. These easily modifiable factors could readily assist in reducing the fasting plasma glucose and increasing oxygen uptake, resulting in an enhanced regulation of glucose (Eriksson and Lindgärde, 1991).

The deterioration from impaired glucose regulation to T2D is always a possibility, but can be largely prevented by eliminating the risk factors. The importance of this elimination process should not be ignored, even though drug therapy sometimes needs to be included to reach optimal therapy goals. Not only does weight loss assist in this prevention strategy, but it also plays a role in decreasing triglyceride levels and therefore in lowering the associated risk for hypercholesterolaemia and hypertension.

T1D patients should follow inflexible diet plans in order to avoid a variable level of blood glucose. T2D patients must follow a similar restrictive diet, although their hyperglycaemia is often overcome with weight loss alone. If meals are delayed, although this should ideally be avoided, 10 g of carbohydrate should be ingested per half hour. Caloric need for an adult with average activity is 40 kcal.kg.24 hours^{-1}. With added exercise, energy expenditure will be increased and therefore energy consumption may be increased by adding 10 g of extra carbohydrate per hour with modest exercise and 20 to 30 g.hr^{-1} of carbohydrate with vigorous exercise (Chen *et al.,* 2004).

A complete nutrition assessment has to include an overview of the specific individual's previous eating plan, lifestyle, fitness level, anthropometric and biochemical data as well as a physical examination. Important history includes chronic disease and concomitant medication, family health history, eating disorders and physical activity.

Different food sources are not necessarily good or bad; however a diet could either be good or bad for a specific individual, where the aim should be for a normal BMI

(weight/height²) of 20 - 25. For every additional unit of BMI above 22 $kg.m^{-2}$, the risk of diabetes increases by 25% (Bethesda, 1998).

A good diet consists of starchy foods (bread, rice, pasta, potatoes) as the most important energy supply, as presented in Table 2.4. A target daily fluid intake of two litres should be reached. Five portions of fruit and vegetables should be included in the daily food intake. Small portions of lean meat, cooked without additional fat can be eaten, although lower fat alternatives such as white meat, white fish, pulses and soya should rather be encouraged. Dairy products should be taken in moderation and low fat products preferred to full fat products, *e.g.* skimmed milk, low fat yoghurts and cottage cheese. Extra fat for cooking should be avoided and fatty spreads and snack foods such as sweets, biscuits, crisps or cake should be kept to a minimum. Alcohol intake should be moderate, *i.e.* female < 15 units per week, male < 20 units per week (Hope *et al.,* 1998). As confirmed in men with impaired glucose tolerance in a study by Kosaka *et al.* (2005), achieving ideal body weight is a very simple and effective means of lifestyle intervention in order to prevent the onset of T2D.

Table 2.4 Recommended daily dietary intake

Food source	Percentage daily intake
Carbohydrates	60 – 65%
Fats	25 – 35%
Protein	10 – 20%

Adapted from University of Iowa, Family Practice Handbook (2004). % = percentage.

2.4.2 Insulin therapy

Insulin is utilised as a treatment modality in T1D, as well as severe T2D where the ability to produce insulin is lacking. The exogenous insulin does not affect the β-cells and further depletion is therefore prevented (Scarlett *et al.,* 1982). The entry of exogenous insulin into the body decreases the plasma glucose levels. The target plasma glucose level differs between individuals, but IDDM always requires a chronic insulin regimen.

2.4.3 Oral hypoglycaemic drugs

Due to the fact that so many obese T2D individuals are asymptomatic, a relevant reduction in weight is initially preferred. This avoids the risks associated with drug therapy. However, if the weight reduction on its own does not improve the

hyperglycaemia, treatment with oral hypoglycaemic agents or insulin are vital. Due to the risks involved in drug therapy, the decision to start these drug regimens, especially in cases of mild hyperglycaemia, is often a difficult one. Possible assistance in this task could be gained by the determination of the HbA1c level (Berkow *et al.,* 1992). Due to the fact that these oral hypoglycaemic drugs have no preventative effect on symptomatic hyperglycaemia or DKA, it is never considered as a treatment option for T1D patients.

There are two major drug groups to choose from at times of initiating oral drug therapy for T2D. Firstly, the fast acting sulphonylureas are used in non-obese diabetic individuals. This group of drugs stimulates the secretion of insulin and in addition intensifies the effects of insulin in some target tissues and inhibits the synthesis of glucose by the liver (Hope *et al.,* 1998).

In obese individuals, the second group of drugs are used, namely the biguanides. A biguanide is the drug of choice not only because it can be used in obese individuals without causing weight gain, but also due to its improvement of plasma lipid and fibrinolytic profiles associated with T2D. This class of drugs improves the insulin sensitivity of the liver, as well as peripheral tissues and thereby decreases the plasma glucose levels. Insulin secretion as well as glucose production by the liver remains unaffected whilst glucose uptake is increased (Hope *et al.,* 1998).

2.4.4 Prevention

Curing T1D lies in the possibility of replacing lost β-cell function through islet cell transplantation, regeneration of β-cells or the development of immortalised insulin secreting cell lines (Olefsky *et al.,* 2001). Lifestyle changes can have a remarkable effect on T2D incidence, due to the evident impact that environmental factors (see Chapter Three), including diet and weight reduction, have on glucose intolerance as well as on diabetic complications (Cheng, 2005). Establishing prevention strategies for both T1D and T2D is of very high importance in decreasing morbidity and mortality worldwide. These prevention strategies firstly require the identification of all the genes possibly involved in the predisposition to DM, as well as all the interacting environmental factors. Subsequent to identification, population specific prevention strategies could be developed and introduced to ensure the prevention of this devastating disease.

CHAPTER THREE

PHENOTYPIC ASPECTS OF DIABETES

In addition to genetic background and ethnicity, the susceptibility to T2D is associated with various biochemical, environmental as well as anthropometrical factors. Specific ranges of these factors are widely utilised in the assessment of disease risk as well as disease progression, treatment and prognosis. The necessity of an individualised risk assessment is thus evident and needs to include the most important biochemical factors associated with diabetes risk, such as the HbA1c, OGTT, insulin, leptin and adiponectin as well as anthropometrical factors including age, gender, body weight, waist-to-hip ratio (WHR) and waist circumference. It is proposed that the individualised approach also includes the relevant environmental factors linked to diabetes risk, encompassing physical environment, diet, physical activity and lifestyle.

3.1 BIOCHEMICAL FACTORS

The biochemical factors discussed are all implicated in T2D susceptibility. These factors are widely utilised in diverse approaches to diagnose disease as well as to determine disease risk and treatment success.

3.1.1 Glycosylated haemoglobin

Glucose glycosylates the β-chain of haemoglobin without the help of an enzyme, producing glycosylated haemoglobin (HbA1c) that increases as the plasma glucose levels increase (Berkow *et al.,* 1992). The use of an HbA1c cut-off level of 6.1% for diagnosing T2D was reported to be significantly more specific than the fasting plasma glucose on its own (Perry *et al.*, 2001). The individuals diagnosed with T2D according to their OGTT results also had high HbA1c levels. However, 19% of these DM diagnosed individuals had fasting glucose levels of less than 7.0 $mmol.L^{-1}$. It is therefore evident that evaluating the HbA1c value in addition to the glucose levels is useful in increasing the sensitivity of the T2D screening tests in individuals at high risk for the disease.

The HbA1c measurement is a reliable tool in the screening for T2D, but values can however be influenced by various factors. Most commonly, these fluctuations will be

observed when taking drugs like sulphonamides, as well as in the presence of diseases like haemolytic anaemia, which results in cell breakage (Conrad and Gitelman, 2006).

3.1.2 Oral glucose tolerance test

Results from an OGTT do not only include the glucose values, but also the shape of the glucose curve over a two-hour period. As stated previously, the 120 minute (min), glucose value has been described as the best determinant of glucose tolerance (Barr *et al.,* 2002). However, the use of the GCS may elucidate further mechanistic data in the pathogenesis of insulin resistance. Limited research has been done on the use of the curve shape as predictor of glucose tolerance. Population specific curve shape classification will have to be the first step in this investigation, followed by the development of standardised criteria for classification into the different GCS groups. The associations ascertained between various traits involved in T2D susceptibility, including genetic, biochemical, anthropometrical and environmental factors, and these GCS groups, will be indicative of the efficacy of a GCS classification for use in the screening and diagnosis of diabetes. This was therefore evaluated within the studied black South African cohort.

3.1.2.1 Glucose regulation

Impaired glucose regulation is a pre-diabetic state that is determined by either an impaired fasting glucose or impaired glucose tolerance (see Table 2.3), as suggested by the 120 min OGTT glucose level (Zhou *et al.,* 2006). The diagnosis of T2D currently relies on the level of the fasting glucose, despite research indicating the high frequency of misdiagnosis when evaluating this biochemical factor on its own (Perry *et al.,* 2001). Although the two-hour OGTT is not used in practise as often as it used to be, the value of the 120 min glucose level as an indicator of glucose tolerance remains the absolute diagnostic tool for diabetes (Tschritter *et al.,* 2003). The fasting glucose level is the WHO recommended assay for diagnosing T2D (WHO, 2006), in light of the argument that performing an OGTT is time consuming, laborious and inconvenient to the patient. The sensitivity and efficacy of the OGTT can however not be disregarded.

3.1.2.2 Glucose curve shape

Research on the shape of the glucose curve is limited. A Japanese article published in the *Japanese Journal of Geriatrics* (*Nippon Ronen Igakkai Zasshi*) by Fuchigami *et al.* (1994) and a study on a Caucasian cohort by Tschritter *et al.* (2003) is of the few

publications. Fuchigami *et al.* (1994) suggested that the shape of the glucose curve is primarily a result of the early insulin response. They concluded that in individuals with T2D an "upward" and "domed" shape was more prevalent than a "biphasic" shape. A need for adequate description of shape, within different populations, therefore exists.

Tschritter *et al.* (2003) demonstrated in their assessment of the GCS in non-diabetic Caucasian individuals that a correlation existed between the unadjusted shape index and BMI, waist-to-hip ratio (WHR), HbA1c, age, and various insulin secretion parameters. Furthermore, a strong relationship between the shape index (difference between 90 and 120 min glucose) and the plasma glucose AUC (area under the glucose curve, indicative of the absolute glucose level) was also reported. A significant correlation was observed between the biphasic shape and female gender, which was to some extent explained by the low WHR, high early insulin secretion and low fasting glucagon levels observed within the female cohort. Following the subsequent adjustment for these covariates, the correlation however remained significant to some extent ($p = 0.04$) and was suggested to be a result of hormone regulated metabolic variation (Tschritter *et al.,* 2003).

Tschritter *et al. (*2003) screened this same cohort for polymorphisms associated with T2D, including alterations in the IRS-1, IRS-2, CAPN10, LIPC (hepatic lipase) and PPARγ2 genes. Subsequent to adjustment for plasma glucose AUC and gender, the only significant association observed was between the monophasic shape and individuals homozygous for the UCSNP44 T-allele in the CAPN10 gene. Prior to adjustment however, the Asp allele in the IRS-2 gene was also significantly associated with the biphasic curve shape.

The unadjusted correlation between the parameters and the GCS illustrated the biphasic shape in association with normal glucose tolerance (NGT) as recognised by a lower BMI, WHR, fasting glucose, HbA1c, plasma glucose AUC, insulin concentration, age as well as higher insulin sensitivity and insulin secretion. As mentioned above, the correlation was however not significant after adjusting for plasma glucose AUC.

3.1.3 Insulin

The only source of the peptide hormone insulin is the β-cells of the pancreatic islets of Langerhans. Glycogen, fats and proteins are synthesised and the uptake of glucose

stimulated upon insulin stimulation of muscle cells, hepatic cells and adipocytes, excluding brain and liver cells (DeFronzo, 1992).

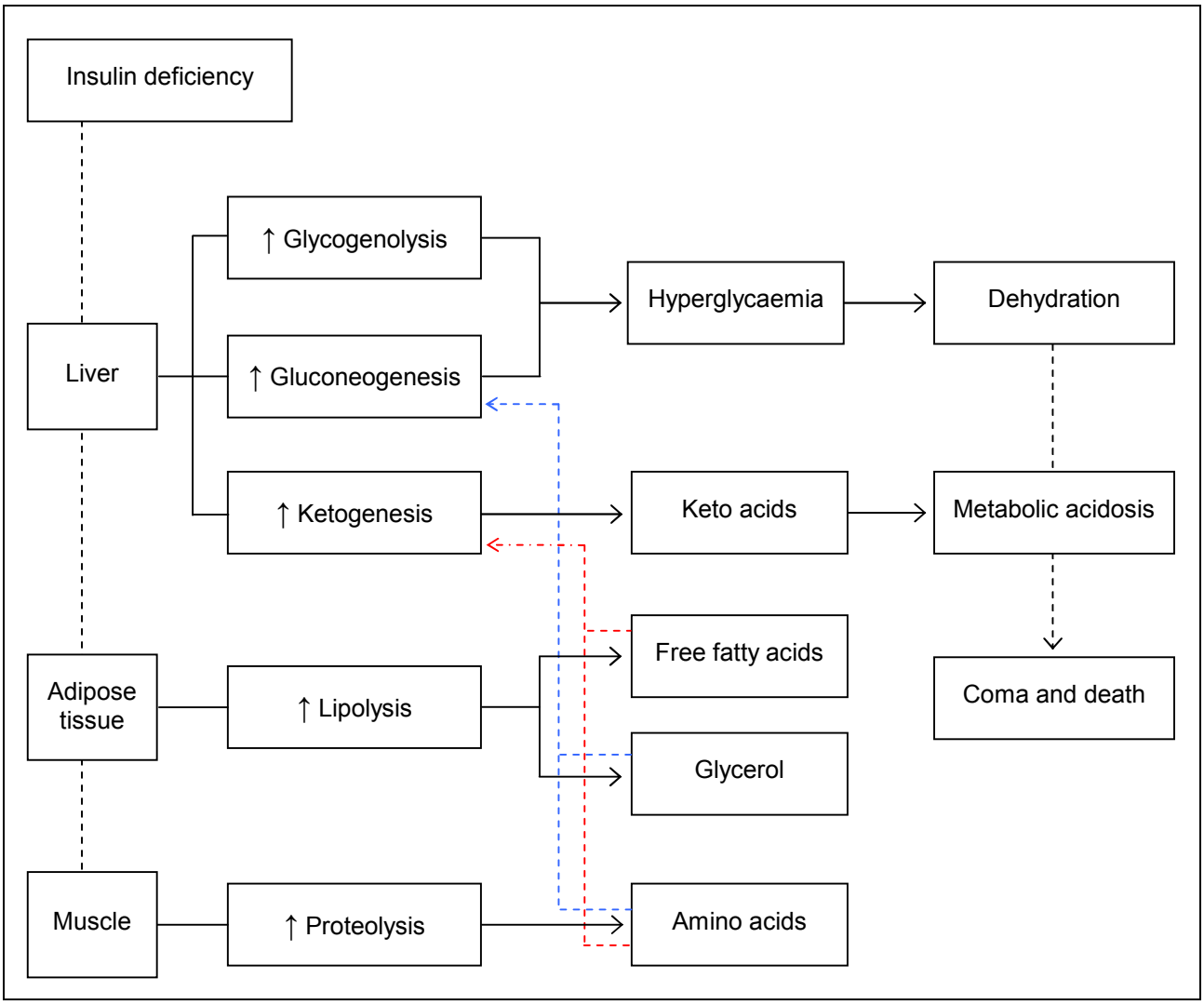
It is hypothesised that with overeating, the relevant increase in the production of insulin will result in the restraint of the glycoprotein synthesis of the insulin receptor, explaining the fact that diet can be sufficient on its own to control T2D. Insulin release is a secretion process observed in two different phases. The initial rapid release of insulin within 10 minutes of the glucose stimulus is followed by a steady increase in insulin secretion, provided that the stimulus is continuous. This initial rapid release phase is absent in T2D susceptible individuals (DeFronzo, 1992).

The pulsatile fashion in which insulin is secreted results in more effective glucose disposal. Studies proved that in DM, it was the amplitude of the pulses that were less and their relationship to meals that were distorted. During a 24-hour period however, the number of pulses did not differ between T2D and non-T2D individuals (Polonsky, 1988).

The insulin in skeletal, myocardial and adipose cells is responsible for the augmentation of glucose uptake. Glucose transport in the liver is however not affected by insulin. A similar resistance to insulin is observed in obesity, possibly influenced by an increase in the free fatty acid levels. Typical insulin action is initiated with the binding of insulin to its cell membrane receptor in muscle cells and adipocytes, followed by the subsequent mobilisation of the major glucose transporter protein, GLUT-4, to the cell surface for the uptake of glucose (Voet and Voet, 1991).

Insulin is produced by the pancreatic β-cells as a pre-prohormone. This pre-proinsulin consists of three major subunits: A-, B- and C-peptides as well as a signal peptide for targeting towards the endoplasmic reticulum. Subsequent to the cleavage of the signal peptide with insertion into the membrane, the production of proinsulin follows. Active insulin is produced from cleavage of the C-peptide, whilst the A- and B-peptides remain attached through the disulphide bonds (Narang *et al.,* 1984). The biochemical consequences of insulin deficiency are presented in Figure 3.1.

Figure 3.1 Diagrammatic representation of the biochemical consequences of insulin deficiency



↑ = increased.

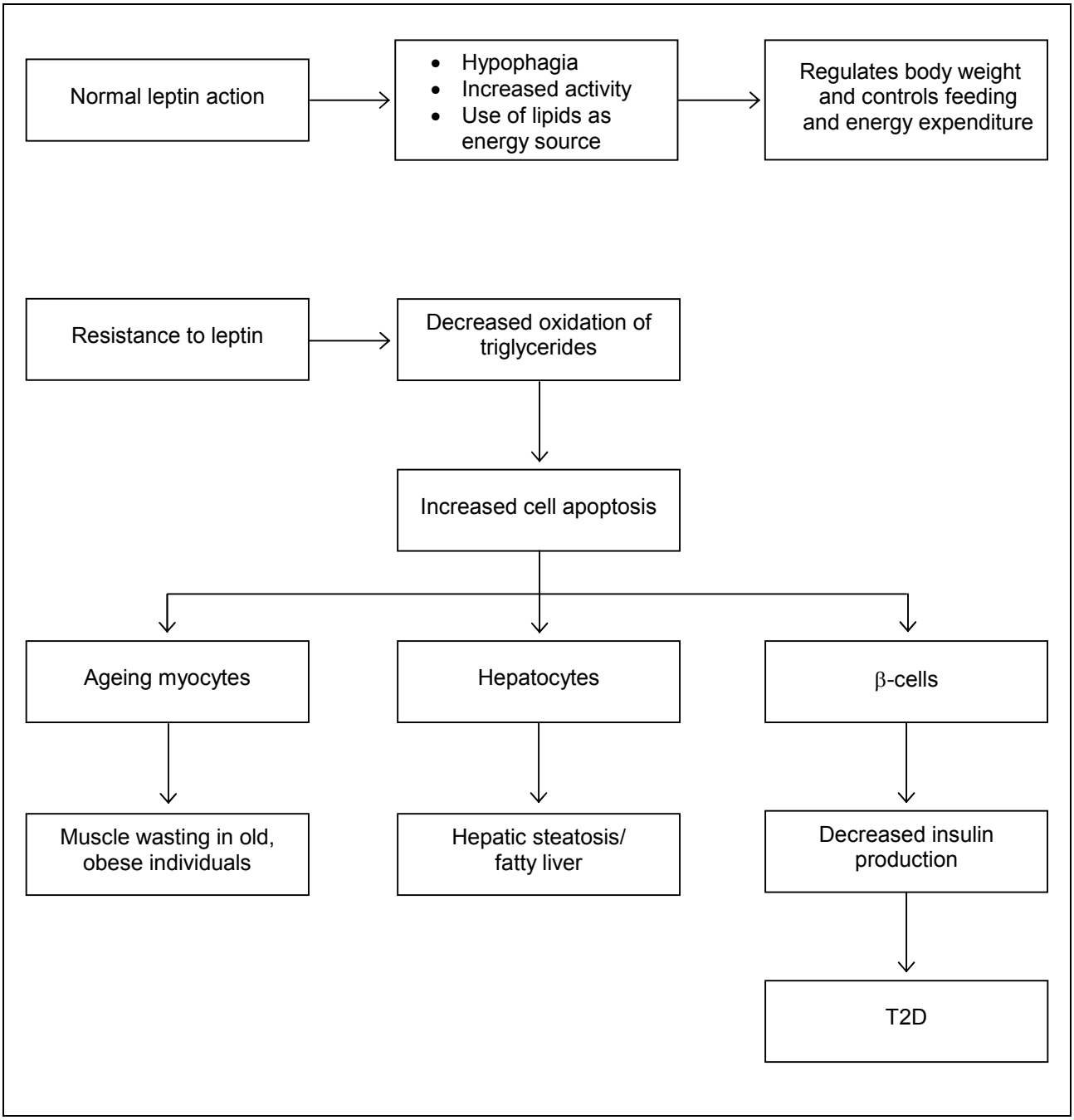
As illustrated in Figure 3.1, an insulin deficiency in the body stimulates various processes in the liver, fat and muscle cells. The breakdown of glycogen, lipid and protein as well as the production of glucose and ketones results in dehydration and metabolic acidosis which finally ends in coma and death.

3.1.4 Leptin

Adipocytes produce leptin hormone according to the amount of fat stored in these cells. An increase in stored fat correlates with an increase in leptin release into the blood, as well as increased messages to the brain indicating that sufficient food has been ingested (NIH publication, 1998). Leptin levels were determined to be high in obese individuals and are associated with BMI and body fat in non obese, obese and T2D individuals. A strong association between leptin and insulin concentrations also exists. It has been proven that

leptin improves the insulin sensitivity. Leptin was also observed to correlate with insulin and proinsulin levels (Abdelgadir *et al.,* 2002). Higher serum leptin levels of up to 40% were present in females, compared to males with similar body mass index and fat mass percentage (Saad *et al.,* 1997). The normal action of leptin as well as leptin resistance due to obesity that leads to T2D is depicted in Figure 3.2.

Figure 3.2 Diagram of normal leptin action as well as leptin resistance in obesity leading to T2D



T2D = type 2 diabetes; β = beta.

Leptin receptor resistance can cause obesity as well as DM and liver disease. Studies also revealed that leptin levels in newborns were higher in females and that a direct

correlation existed with their birth weight (Matsuda *et al.,* 1997). The normal action of the leptin hormone ensures appropriate regulation of body weight as well as controlled feeding and energy expenditure, as illustrated in Figure 3.2. Leptin resistance in obesity however, decreases the oxidation of triglycerides that causes muscle wasting in obese individuals, liver steatosis as well a decrease in insulin production due to β-cell apoptosis. These effects, as a result of the increased cell death, bring about T2D.

3.1.5 Adiponectin

Adiponectin is produced and secreted only from adipose tissue. The plasma adiponectin level decreases with a rise in insulin resistance. When visceral fat accumulates, adiponectin concentration decreases (Kondo *et al.,* 2002). According to a study by Funahashi *et al.* (2004), adiponectin is protective against the development of diabetes in Pima Indians and is one of the first adipocytokines proven to have this characteristic.

Low levels of adiponectin are observed in individuals with obesity and T2D while elevated levels, as observed in non-obese patients, are associated with an improvement in insulin action. Adiponectin levels were also reported to be lower in Pima Indians with impaired glucose tolerance and T2D. Insulin resistance seems to be the main reason for these decreased adiponectin levels, due to the fact that insulin sensitivity seems to relate to the adiponectin level being decreased in obese and NIDDM patients, more than by the variation in body fat (LeRoith, 2002).

Adiponectin has, in addition, effects on several different tissues. The plasma protein causes a decrease in the triglyceride stores of muscle tissue by the induction of gene expression of molecules involved in fatty acid uptake and oxidation. Adiponectin also positively affects insulin sensitivity in the liver by increasing fatty acid oxidation, and lowers triglyceride levels whilst suppressing the production of glucose (LeRoith, 2002).

Studies have revealed that there is an increase in leptin levels as a result of the decrease in adiponectin (Unger *et al.,* 1999; Yamauchi *et al.,* 2002). Therefore, when the leptin receptors are not functional and the adiponectin levels decrease, triglycerides will accumulate in non-adipocyte tissues. Further investigation into this statement will be necessary. Yamauchi *et al.* (2002) observed in a study on rats that adiponectin controls the metabolism of glucose as well as the tissue sensitivity of insulin, by triggering 5'-AMP-activated protein kinase (AMPK).

3.2 ANTHROPOMETRICAL FACTORS

Anthropometrical parameters play an integral part in the risk for developing T2D. Susceptibility is observed to be higher in individuals grouped together according to age, gender and body weight (Wild *et al.*, 2004). Evidence illustrates that the increased risk for the disease in terms of body weight, is best described by calculated ratios like the BMI and WHR (Osman *et al.,* 1994).

3.2.1 Age and gender

An increase in age is associated with an increase in the resistance to insulin. This association can be explained by the decline in insulin sensitivity with increasing age. This hypothesis of age being linked to insulin resistance can, in addition, be supported by the higher incidence of T2D in individuals older than 45 years (DeFronzo *et al.,* 1991). A biphasic GCS has been associated with female gender and adequate glucose tolerance in a Caucasian population by Tschritter *et al.* (2003).

Age was reported to be an important susceptibility factor for T2D and the number of people older than 64 years and diagnosed with diabetes in developing countries was predicted to be more than 82 million in the year 2030. Furthermore, T2D was reported in a Caucasian population to be more prevalent in males when compared to females (Wild *et al.*, 2004).

Gale and Gillespie (2001) reported an increase in T2D prevalence in both African American males and females. The rapid increase observed in males was explained by their inactive Westernised lifestyles resulting in obesity whereas decreasing parity elucidated the increased prevalence in females.

3.2.2 Body weight

A well-researched topic is the link between obesity and T2D (Olefsky, 2001). Therefore, by elucidating the genetic and environmental factors contributing to obesity, a better understanding of DM aetiology will become evident. According to studies by Elbein (1997), the age of onset of NIDDM may be decreased in obese individuals. It was also stated that the expression of the T2D susceptibility loci seems to be regulated by the obesity loci as well as the environmental factors influencing obesity.

An understanding of the mechanism of insulin action in obese individuals is needed to clarify the proposed link between obesity and T2D. Obese individuals have a higher level of insulin and glucose in their plasma, than normal weight individuals, explaining the small increase in insulin release after a meal. A similar response is observed in T2D individuals presenting with an insignificant increase in insulin after a meal, regardless of the glucose level already being high due to insulin resistance. The degree of fasting hyperglycaemia is in direct correlation with the peripheral insulin response to glucose ingestion, both in obese and non-obese T2D patients (Berkow *et al.,* 1992). Obesity is proposed to result in an increase in the release of non-esterified fatty acids, causing insulin resistance, which in turn results in post-receptor defects in insulin action (Hope *et al.,* 1998). It was observed in obese individuals that a significant reduction in body weight, due to a healthy diet and increased physical activity, increased the binding as well as post binding of insulin to its receptor and therefore improved glucose tolerance (Osman *et al.,* 1994).

The BMI is utilised as an indicator of diabetes susceptibility according to body weight in relation to individual height. A normal BMI (weight/height^2) ranges between 20 and 25 kg.m^{-2}. For every additional unit above 22 kg.m^{-2}, the risk of diabetes increases significantly (Bethesda, 1998). The BMI is an effortlessly calculated ratio from readily available or obtainable data and has proved to be an indispensable tool in the determination of disease risk, including diabetes and cardiovascular disease (Chen *et al.,* 2004). Whilst a strong correlation was observed between high BMI and T2D incidence in females, the risk for the disease was not influenced by body weight in males (Lasky *et al.,* 2002).

3.2.3 Waist-to-hip ratio and waist circumference

Both WHR and waist circumference are anthropometric indexes which can be utilised in the measurement of regional fat distribution, and therefore, aid in determining T2D risk. Due to the elevated sensitivity of abdominal fat to lipolytic stimuli, compared to other adipose cells, and measurement of this anatomic region being included in the WHR, the value of this parameter as indicator of T2D risk is evident (Osman *et al.,* 1994).

3.3 ENVIRONMENTAL FACTORS

Daily exposure to various environmental factors has a significant effect on the risk of developing diabetes (Sobngwi *et al.,* 2001). The majority of these factors are readily

modified and therefore vital in the initial assessment of disease risk as well as at times of prospective treatment evaluation.

3.3.1 Diet, physical activity and lifestyle

The significance of diet and physical activity in the risk for developing diabetes has previously been confirmed (Young *et al.,* 1990). Subsequent to the diagnosis of T2D, modification of these factors should be the initiated as first line treatment, as discussed in Chapter Two.

3.3.2 Physical environment

Westernisation, as observed due to dietary changes, encompassing an increase in saturated fat and refined sugar intake, combined with decreased physical activity, results in the higher prevalence of diabetes worldwide. The rapid increase in urbanisation in Africa explains the expected increase in diabetes incidence (Sobngwi *et al.,* 2001). Research elucidating the pathogenesis of this complex disease in the black South African population is therefore vital and validates the aims of this study.

A study by Osman *et al*. (1994) in Malaysia observed less urban Malays with IGT, compared to a rural cohort, hypothesising that negative environmental factors increase the risk for diabetes in individuals with an existing genetic susceptibility. The study indicated the significant effect of environmental factors on T2D prevalence by comparing 120 min glucose values following an OGTT, as well as HbA1c values, between different communities.

3.4 AIMS OF THE STUDY

The aims of the study incorporated assessment of the five point OGTT data collected during the PRIMER study and the classification of the GCSs as biphasic, monophasic or unclassified, in accordance with the criteria described by Tschritter *et al.* (2003). The cohort was stratified into subgroups according to GCS (biphasic, monophasic and unclassified), physical environment (urban, rural), genotype (IRS-1, IRS-2, PPARγ2 and CAPN10 genes), HbA1c (normal, high), glucose tolerance (NGT, IGT), HIV status (positive, negative), as well as phenotype. Phenotypic differences were evaluated with

regard to anthropometrical measures including BMI, age and gender, as well as environmental factors encompassing diet and physical activity.

Genomic deoxyribonucleic acid (DNA) was isolated and amplified via a polymerase chain reaction (PCR) strategy. Analyses of the four T2D susceptibility gene alterations entailed PCR and restriction fragment length polymorphism (RFLP) methods. The main objective of this study was to assess the GCS during a two-hour OGTT and evaluate specific genotypes in the context of the T2D risk phenotype in the black South African population. Fulfilment of this aim resulted in a novel and population specific contribution towards current T2D research.

CHAPTER FOUR

GENETIC ASPECTS OF DIABETES

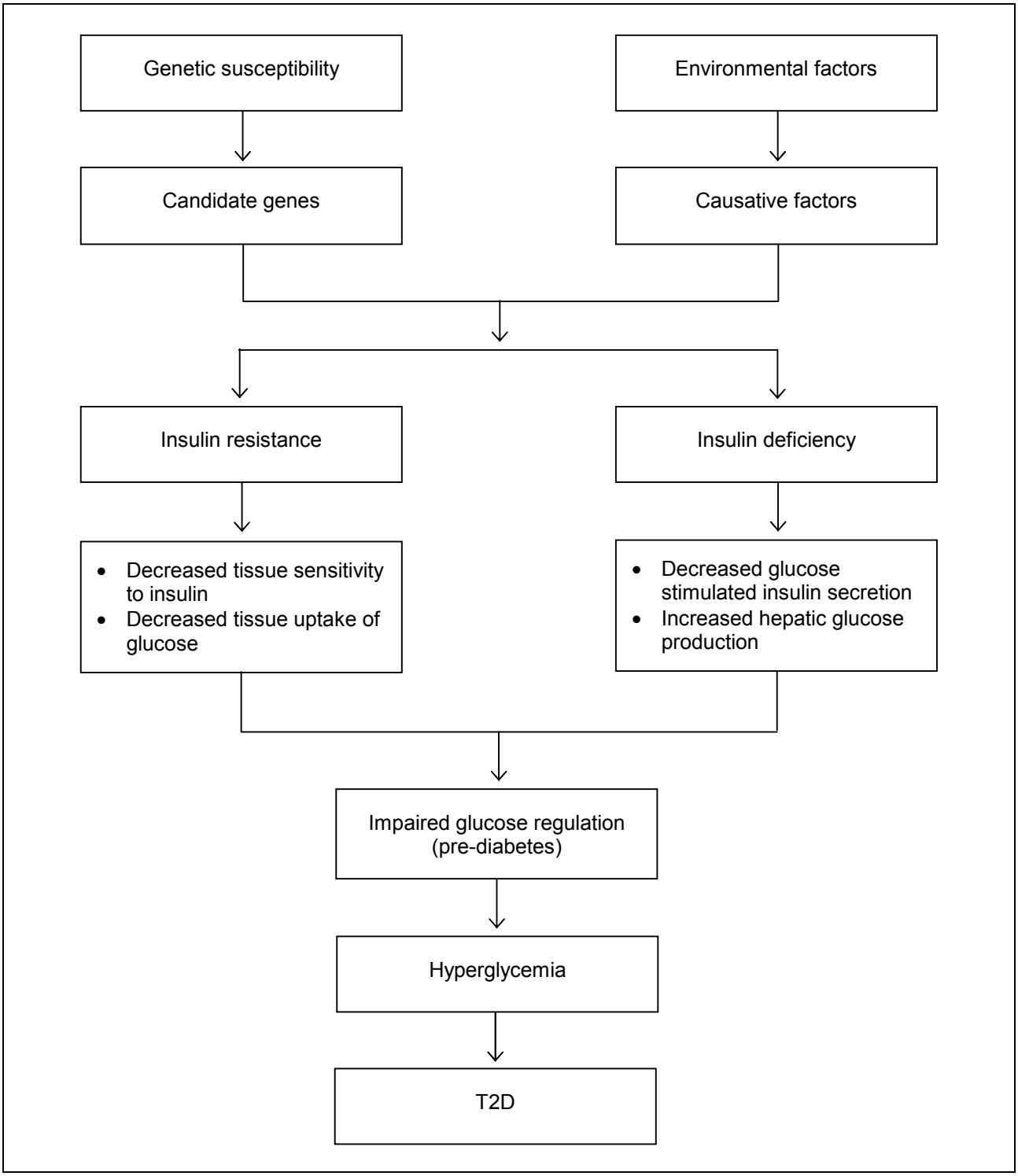
The pathogenesis of DM comprises not only of pathogenic and environmental factors as discussed in Chapter Two, but also various genetic aspects. T2D that develops due to insulin resistance and/or insulin deficiency is linked both to gene mutations and metabolism-influencing factors, of which obesity is the most important. The role of these environmental factors, such as body weight and physical activity, as well as diet cannot be ignored, in light of the significant difference that simple lifestyle modifications can have on diabetes susceptibility. Comprehension of the interaction between the genetic and environmental factors and its role in the pathogenesis of DM is necessary in order to formulate possible therapeutic and preventative strategies for this complex disease (Jun *et al.,* 1999).

As South Africa is so heavily affected by the infection and consequences of the human immunodeficiency virus (HIV), it is of great importance that prevention strategies are established and implemented for diseases, such as T2D, which can be detected early or even prevented by the mentioned lifestyle modifications. To make these screening tests worthwhile, it is necessary to understand which genes play a role in the susceptibility to T2D. By establishing a means to prevent diseases like T2D, and thus saving millions of Rands on management of DM and its complications, our country will be in a position to rather treat people with diseases like the acquired immune deficiency syndrome (AIDS), which are preventable in some circumstances, but unfortunately not yet curable or even efficiently treatable. In addition, antiretroviral treatment can cause T2D, impaired glucose tolerance, hyperlipidaemia and/or lipodystrophy (Walli *et al.,* 1998) as occurring side effects, especially in the case of treatment with protease inhibitors. It is hypothesised that being able to determine the genetic risk for T2D would therefore not only improve the diabetes care, but could in future also assist in individualising and optimising other treatments such as that used for HIV.

In T2D, there are a variety of genes involved in the pathogenesis of the disease and multiple genes may together ascertain an individual's predisposition to diabetes. As discussed in Chapter Two, one of the major environmental factors contributing to T2D is body weight, which is in turn affected by numerous other genetic, hormonal and

environmental factors. The evidence generated from this investigation in South African individuals could support the hypothesis that various populations have different genetic risk factors for T2D and that this should be kept in mind during the future implementation of prevention strategies in certain populations. Figure 4.1 depicts a schematic diagram of the progressive pathogenesis of T2D, including all the various factors involved.

Figure 4.1 Schematic diagram of the progressive pathogenesis of T2D



Adapted from Jun *et al.* (1999). T2D = type 2 diabetes.

The genetic component plays a strong etiological role in the development of T2D, as suggested by the high concordance rate in monozygotic twins (Newman *et al.,* 1980), familial aggregation, the high prevalence of T2D in certain ethnic groups (Knowler *et al.,* 1990) and familial transmission patterns. The inheritance mode and number of genes involved however still remain undefined.

4.1 ETHNICITY

Ethnicity is one of the risk factors involved in developing T2D that cannot be modified. Sobngwi *et al.* (2001) illustrated the difference in diabetes prevalence between various ethnic groups, with the Indian population being most affected, followed by Black and Caucasian populations. This emphasizes the importance of developing population specific T2D screening strategies and hence the investigation of black South African individuals in this study.

4.2 GENES ASSOCIATED WITH INSULIN RESISTANCE

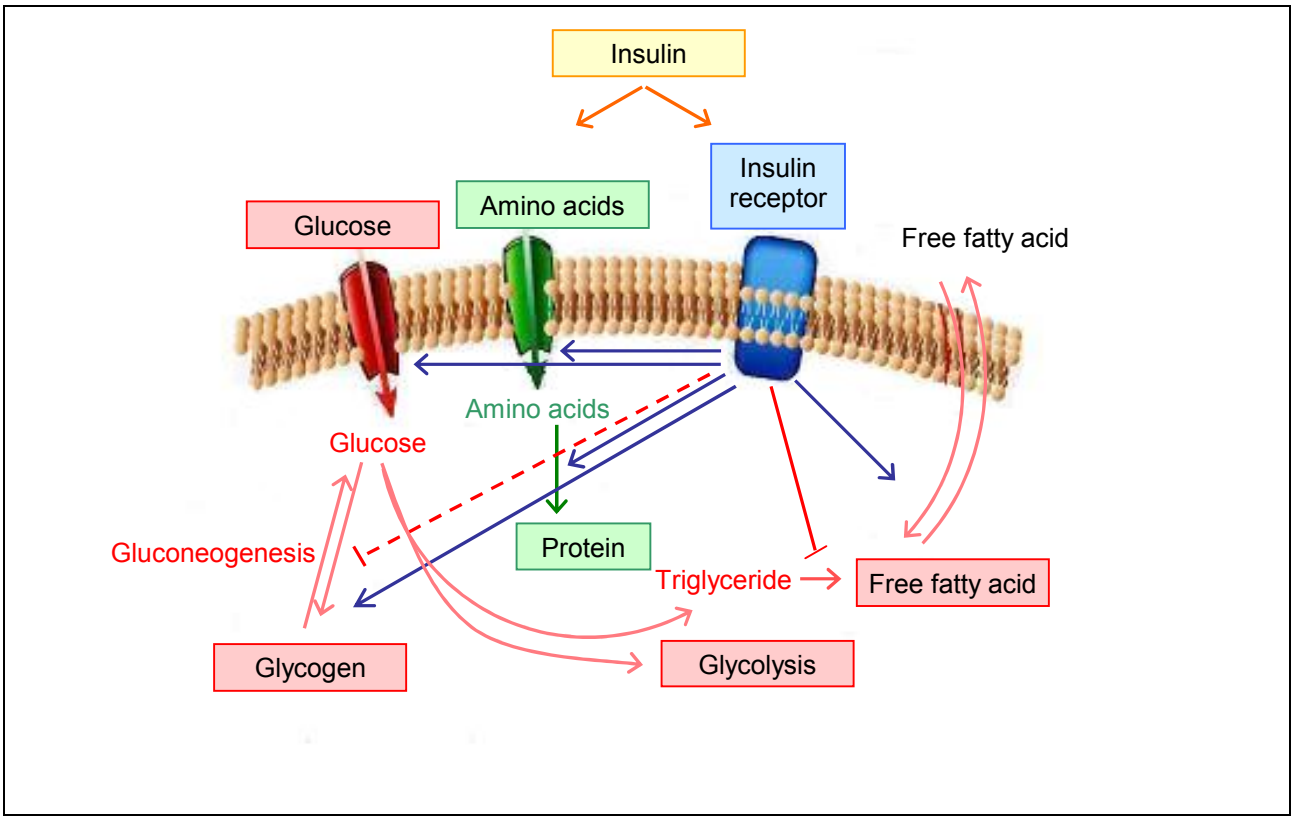
The best-described genes involved in the genetics of diabetes are those associated with insulin resistance. These genes include the insulin receptor, IRS-1, IRS-2, PPARγ2, CAPN10, prohormone convertase-2, islet amyloid polypeptide, calcitonin gene related peptide, both the glucose transporter-1 and -4 (GLUT-1 and -4), hexokinase II, glycogen synthase, beta-3-adrenergic receptor, ras associated with diabetes (rad) as well as various obesity related genes.

Gene alterations that have previously been investigated for possible association with the glucose curve shape during a two hour OGTT include polymorphisms in the CAPN10, IRS-1, IRS-2 and PPARγ2 genes. It is hypothesised that a positive correlation between susceptibility genes and the GCS could assist in the establishment and implementation of effective screening tools utilised for the risk analyses of developing T2D. It is speculated that these screening strategies would have to be based on clinical, biochemical as well genetic profiles. Tschritter *et al.* (2003) investigated Caucasian individuals for association between specific genotypes and the shape of the glucose curve. The black South African population has however not been screened previously and this is undertaken during this investigation

4.3 INSULIN RECEPTOR GENE

Insulin resistance in T2D has been observed to be due to both receptor defects and post receptor defects, as depicted in Figure 4.2. In previous studies, it has been revealed that T2D results in a reduction in insulin receptor binding in both monocytes and adipocytes whilst the affinity to the insulin receptor is still unaffected. This is due not only to defective receptors but also defective receptor processing (Olefsky *et al.,* 1981). Insulin regulates metabolism as a hormone that not only controls the production and storage of carbohydrates, lipids and proteins, but also inhibits their breakdown and release. As illustrated in Figure 4.2, the transfer of glucose, amino acids and fatty cells into the cell is stimulated by insulin. Furthermore, insulin enhances enzyme activity involved in glycogen breakdown as well as lipid and protein production, as illustrated by the arrows in Figure 4.2.

Figure 4.2 A graphic representation of insulin action



Adapted from Saltiel and Kahn (2001).

Abnormalities were however also detected subsequent to the binding of insulin to its receptor which was evident in the decreased production of the second messenger of insulin, decreased transport of glucose into the cells, as well as a transport abnormality during critical enzymatic steps involved in the use of glucose (DeFronzo *et al.,* 1992). Currently more than thirty mutations resulting in insulin resistance due to defects at the

insulin receptor have been identified in genetic syndromes associated with severe insulin resistance (Jun *et al.,* 1999).

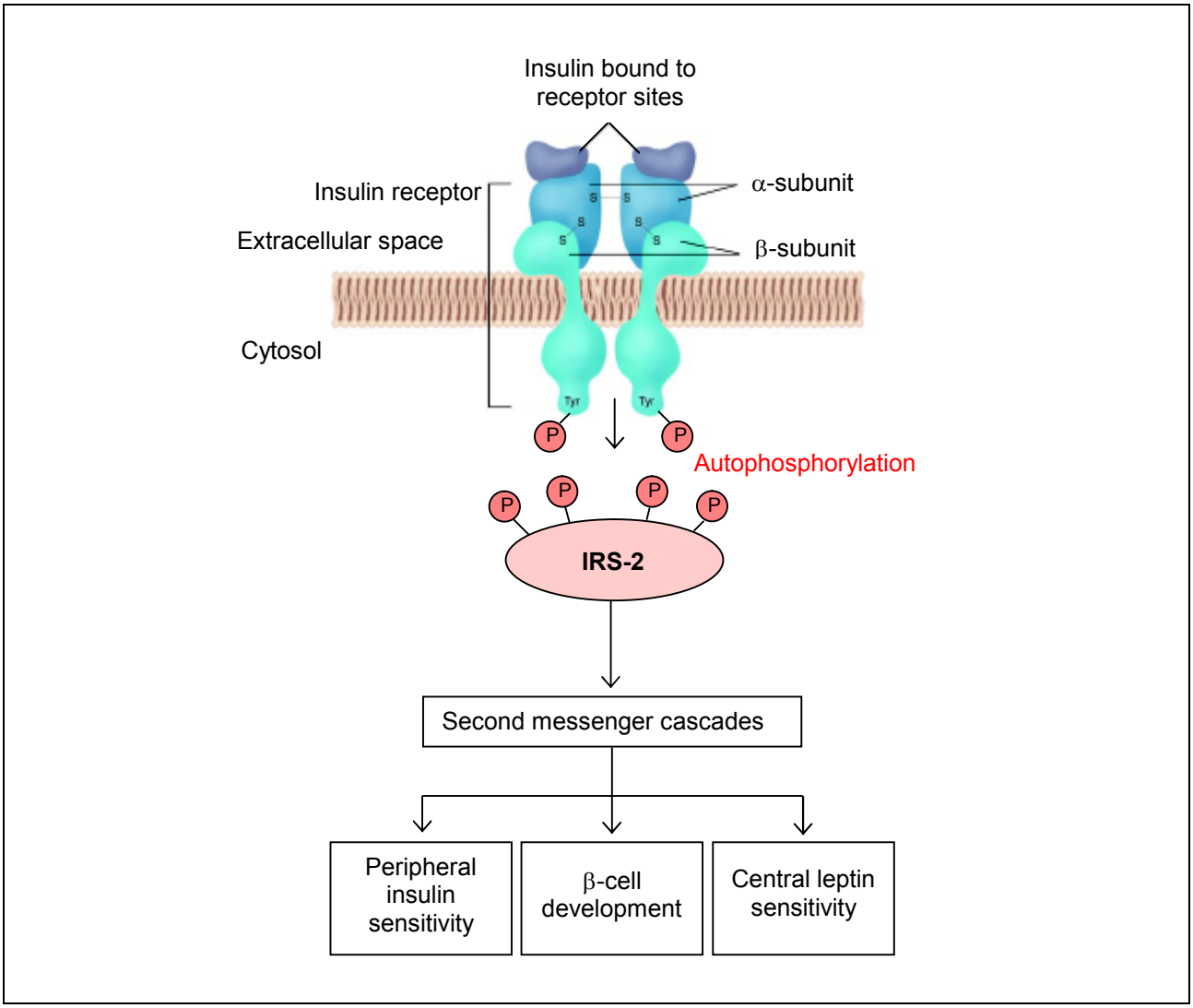
4.4 INSULIN RECEPTOR SUBSTRATE-1 GENE

The insulin receptor substrate is a substrate for the tyrosine kinase action of the insulin receptor. The IRS-1 gene is involved in the insulin-signalling pathway and is localised to chromosome 2q36 (Stoffel *et al.,* 1996). Possible T2D association is considered for this gene via β-cell dysfunction. In Danish Caucasian T2D patients, two amino acid mutations (Gly972Arg and Ala513Pro) in IRS-1 were observed three times more often than in control subjects (Almind, 1993). The association of the Gly972Arg alteration with increased disease risk has been suggested to be due to the association of the Arg allele with obesity (Jellema *et al.,* 2003). This meta-analysis of 27 studies estimated an odds ratio of 1.25 for diagnosing T2D in individuals harbouring the 972Arg variant. Abate *et al.* (2003) reported a prevalence of 5.8% in normal and 10.7% in T2D Caucasian individuals. The results on the association between the Gly972Arg alteration and the prevalence of T2D are controversial, and the findings from previous studies could not support this association (Florez *et al.,* 2004.) Evidence of association between the polymorphism and the two-hour postprandial glucose curve was proposed, but not confirmed by Tschritter *et al.* (2003) in non-Africans. This polymorphism was thus selected to be evaluated within a black South African cohort.

4.5 INSULIN RECEPTOR SUBSTRATE-2 GENE

The IRS-2 gene plays an integral role in the action of insulin within various cells (Brady, 2004). Altered expression or function of the gene may therefore result in β-cell failure or insulin resistance, as presented in Figure 4.3. Although analyses have suggested the IRS-2 gene to be causally involved in T2D (‘T Hart *et al.,* 2002), a study by Bernal *et al.* (1998) demonstrated that this gene was not associated with the disease in a Caucasian population. ‘T Hart *et al.* (2002) also demonstrated that the Gly1057Asp polymorphism in IRS-2 was not associated with the secretion of insulin following a glucose load in two populations from the Netherlands.

Figure 4.3 The role of IRS-2 in the signalling pathway of insulin



Adapted from Brady, 2004. α = alpha; β = beta; IRS-2 = insulin receptor substrate-2. The tyrosine kinase in the β-subunit of the insulin receptor is activated by the binding of insulin, resulting in autophosphorylation (P) on tyrosine (Tyr) residues. The activation of IRS-2 initiates second messenger cascades that play a role in peripheral insulin sensitivity, central leptin sensitivity as well as β-cell development. S-S = disulphide bonds.

The IRS-2 Gly1057Asp polymorphism has however been determined to have a significant augmenting effect on the insulin secretion in a German population (Stumvoll *et al.,* 2001a). Tschritter *et al.* (2003) attempted, but failed to confirm an association between this genotype and the GCS in non-Africans.

4.6 PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA 2 GENE

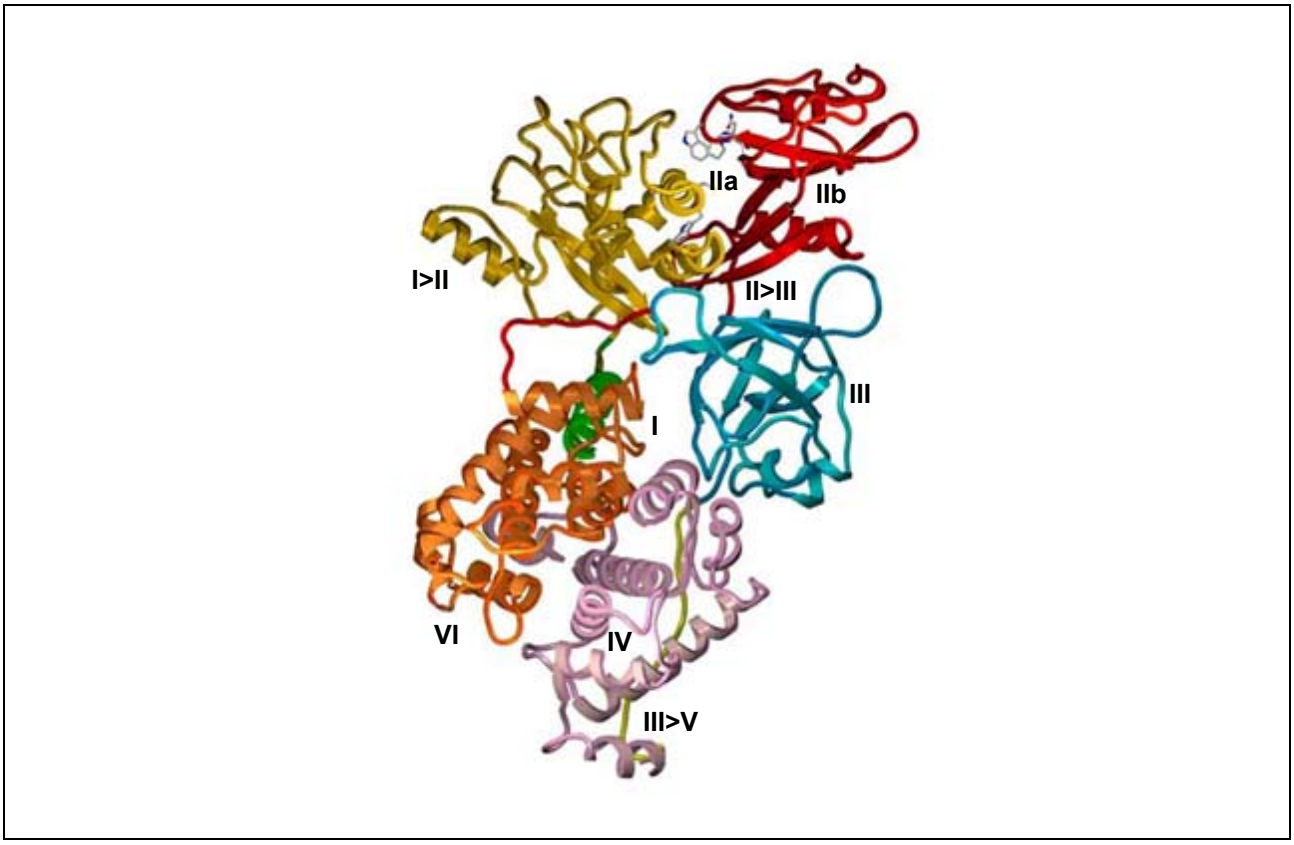
PPARγ is a ligand activated transcription factor mainly expressed in adipose tissue and the Pro12Ala polymorphism has been associated with increased antilipolytic insulin sensitivity by Stumvoll *et al.* (2001b). A frequency of 15% was observed for the Pro12Ala alteration in Caucasians in a study by Rhee *et al.* (2006), who hypothesised that glucose stimulated insulin secretion was increased by inhibition of lipolysis in individuals

harbouring the Ala allele. Various associations with the Pro12Ala variant have been evaluated, including T2D, the Metabolic Syndrome (MS) and hypertension, with different causative and protective results being reported in various populations (Rhee *et al.,* 2006). The Pro12Ala polymorphism was associated with protection against T2D in Caucasians (Desai *et al.,* 2009). A significant association between the PPARγ2 gene and the shape of the glucose curve following a five point OGTT are yet to be established and this was attempted within the investigated black South African cohort.

4.7 CALPAIN 10 GENE

Calpain 10, a non-lysosomal cysteine protease encoded by the CAPN10 gene, is expressed in skeletal muscle, liver and pancreatic tissue (Baier *et al.,* 2000). Calpains are calcium dependent intracellular proteases that cleave proteins at a minimum number of sites, thus modifying the protein (Horikawa *et al.,* 2000). Calpains consist of various domains and the ribbon structure of human m-calpain is depicted in Figure 4.4.

Figure 4.4 The ribbon structure of human m-calpain



Adapted from Strobl *et al.* (2000). I = autolytic activation domain; II = cysteine catalytic site; III = electrostatic switch domain; IV = right-side calmodulin-like calcium binding site and VI = left-side calmodulin domain.

In the CAPN10 gene, various polymorphisms have been associated with T2D (Horikawa *et al.,* 2000). These alterations were initially characterised by research undertaken at the University of Chicago and therefore the single nucleotide polymorphisms are named UCSNP. UCSNP43, UCSNP19 and UCSNP63 in various intronic areas of the CAPN10 gene were previously associated with T2D (Horikawa *et al.,* 2000). UCSNP19 is present in intron six and is an insertion/deletion mutation of 32 base pairs at nucleotide 7920. Three 32 bp repeats are observed in the "risk" allele and this has been associated within the Caucasoid population with a decrease in insulin sensitivity due to a higher fasting insulin level (Elbein *et al.,* 1997). It has previously been hypothesised that the CAPN10 gene only induces diabetes susceptibility in specific populations (Tsai *et al.,* 2001), which has been confirmed by the observed protection towards T2D within black South African individuals that are homozygous for the wild type allele at the UCSNP56 locus (Towers, 2005).

UCSNP44 is a thymine to cytosine alteration in intron three of the CAPN10 gene and an increase in plasma glucose was observed for individuals included in the non T/T CAPN10 genotype group reported by Wang *et al.* (2002). An association was also reported between a monophasic glucose curve shape and the UCSNP44 homozygous T allele genotype in non-Africans (Tschritter *et al.,* 2003). This gene was therefore evaluated in terms of an association with the shape of the glucose curve in the investigated black South African cohort.

4.8 OTHER GENES

Prohormone convertase-2 and -3 and carboxypeptidase are required for the conversion of proinsulin to insulin. T2D individuals demonstrate a high level of biologically inactive proinsulin and cleaved proinsulin products (Yoshioka *et al.,* 1988). In patients without diabetes the amount of proinsulin is small compared to insulin (DeFronzo *et al.,* 1992). Amyloid deposits in the islets are often observed as a pathological characteristic of T2D and could possibly explain the association of the islet amyloid polypeptide gene with T2D. This explanation involves amilyn which is a 37 amino acid peptide secreted together with insulin, by the pancreatic β-cells (DeFronzo *et al.,* 1992). Resistance to insulin is caused via this peptide, which negatively affects the synthesis of glycogen, similar to the defect in insulin action as reported with the calcitonin gene related peptide. Most recent studies have proven though that the role of these polypeptides in insulin resistance in T2D is only observed in isolated cases (Koopmans *et al.,* 1991).

The insulin sensitive glucose transporter-4, as observed in muscle and adipose tissue, has been associated with T2D where the transport of glucose was not only affected in adipocytes but also in muscle cells. Each specific tissue has its own glucose transporter and associated hexokinase, which are responsible for normal glucose metabolism. Therefore, it would be impossible to differentiate between an abnormality in hexokinase action and a defect in a glucose transporter (DeFronzo *et al.,* 1992). Following a study in rats (Garvey *et al.,* 1989), it has been stated that a reduction in the expression of the glucose transporter-4 could be responsible for subsequent resistance to insulin.

Muscle and liver tissue requires glycogen synthase as an essential enzyme in non-oxidative glucose metabolism. The activity of glycogen synthase is enhanced by insulin in two ways. Firstly, it may be increased by direct dephosphorylation of glycogen synthase (Dent *et al.,* 1990) or secondly, through the inactivation of glycogen synthase kinase-3 (Embi *et al.,* 1980). These enzyme defects are evident in the initial phases of T2D (DeFronzo *et al.,* 1992).

The beta-3-adrenergic receptor is located in adipocytes and regulates both the breakdown of lipids and the resting metabolic rate. In Pima Indians, Mexican Americans and African Americans with T2D and obesity, the Trp64Arg mutation was identified and association with an earlier onset of T2D (Widen *et al.,* 1995) was determined. Thus, susceptibility to both obesity and insulin resistance appear to be increased by alterations in this gene.

The ras associated with diabetes gene encodes a product which inhibits the uptake of insulin-stimulated glucose, and is located on chromosome 16q22 (Thoren *et al.,* 1982). The report also stated that the expression of this gene was enhanced in individuals with IDDM.

4.9 GENES ASSOCIATED WITH OBESITY

Adipose tissue acts as an endocrine organ and produces hormones and cytokines that may link free fatty acids (FFAs) with insulin action and β-cell function. Proteins involved are leptin, tumour necrosis factor α (TNFα), adiponectin, resistin, plasminogen activator inhibitor type-1 (PAI-1) and interleukin (LeRoith, 2002).

In obese individuals, the mRNA expression of the obesity gene (ob gene) is enhanced. It is therefore suggested that the ob gene could be involved in T2D that is associated with

increased bodyweight (Maffei *et al.,* 1995). Further investigation is however required to determine the association of this gene with T2D susceptibility.

TNFα influences weight control via adipocyte expression, which is excessive in obese individuals. The TNFα gene is located on chromosome 6p21.3 from where it inhibits the insulin receptor kinase enzyme and is therefore hypothesised to cause insulin resistance due to the serine phosphorylation of IRS-1, which in turn decreases the tyrosine kinase activity of the insulin receptor (Hotamisligil and Spiegelman, 1995).

The adiponectin gene is located on chromosome 3q27, and is linked to susceptibility towards the MS and T2D. It has been hypothesised that the MS was associated with an Ile164Thr mutation of the adiponectin gene in individuals with low levels of adiponectin (Kondo *et al.,* 2002). Insulin resistance could partly be explained by the genetic polymorphisms of the adiponectin gene, which result in a decrease not only in adiponectin production and/or secretion, but also an altered production of TNFα, leptin, angiotensinogen, PAI-1 and resistin. In a study by Kondo *et al.* (2002) it was revealed that the development of T2D was directly related to a mutation (Ile164Thr) in the coding region of the adiponectin gene.

4.10 GENES ASSOCIATED WITH DEFECTS IN INSULIN SECRETION

Various genes are associated with defects in insulin secretion. These include the insulin gene, glucose transporter-2 (GLUT-2) gene, glucokinase (MODY2) gene, MODY1 and MODY3 genes, mitochondrial genes, glucagon-like peptide-1 receptor gene as well as the glucagon receptor gene

The two diverse mutations in the insulin gene, reported to be linked to T2D, include a mutation in the main sequence of the A and B chains, which increases the quantity of circulating bioactive abnormal insulin molecules via defects in receptor binding. The second mutation type is implicated in the augmented processing of proinsulin. The mentioned alterations have seldom been observed to date and are therefore rare in the susceptibility to T2D (Jun *et al.,* 1999).

The GLUT-2 genes are expressed in hepatocytes and in pancreatic β-cells. Association with T2D has not been established by Jun *et al.,* 1999 but in a Finnish Diabetes Prevention study (Laukkanen *et al.,* 2005) four SNPs of a GLUT-2 encoding gene

predicted the conversion from IGT to T2D. Glucokinase, similar in structure to hexokinase, is an enzyme involved in the initiation of glucose metabolism. It is expressed in the pancreatic islets and the liver. In families with MODY, linkage was observed (Hattersley *et al.,* 1992) and mutations in the glucokinase gene have also been linked to French MODY families (Froguel *et al.,* 1993).

The MODY1 locus on chromosome 20q is closely associated with MODY in a single large American family (Rothschild *et al.,* 1993). The MODY3 locus on chromosome 12q was observed to be responsible for up to 25% of French MODY cases (Vaxillaire *et al.,* 1995).

Mitochondrial oxidative phosphorylation is involved in energy production and in glucose-stimulated insulin secretion in pancreatic β-cells (Gerbitz *et al.,* 1992). One of the mitochondrial gene defects that could possibly play a role in causing T2D is the point mutation at nucleotide position 3243 of the transfer ribonucleic acid specific for leucine ($tRNA^{Leu}$) gene, which causes reduced insulin secretion and was observed in patients with diabetes and deafness (Schulz *et al.,* 1993). The A3243G mutation is suggested to be associated with a premature aging of the β-cells, resulting in reduced glucose stimulated insulin secretion (Maassen *et al.,* 2004).

Age related β-cell dysfunction, similar to that seen in individuals harbouring the mitochondrial DNA A3243G mutation (Maasen *et al.,* 2004), suggests the possible role of the mitochondria in the susceptibility to T2D. This is further supported by the fact that mitochondrial oxidative phosphorylation is involved in energy production and in glucose-stimulated insulin secretion in pancreatic β-cells (Gerbitz *et al.,* 1992).

The glucagon-like peptide-1 receptor gene is located on chromosome 6p21 and glucagon-like peptide is produced in the small intestine. It increases insulin secretion by binding to a receptor on the β-cells. The gene was however observed at similar frequencies in diabetic and non-diabetic African American individuals (Jun *et al.,* 1999).

The hormone glucagon takes part in insulin secretion, and the glucagon receptor gene located on chromosome 17q25. In a number of T2D patients, high levels of circulating glucagon were observed, predicting the possible link to T2D susceptibility. Despite the detection of the Gly40Ser mutation in the glucagon receptor gene of French Caucasian T2D individuals, the association with NIDDM is yet to be completely described (Jun *et al.,* 1999).

CHAPTER FIVE

MATERIALS AND METHODS

All reagents used during this investigation were analar grade, unless otherwise stated, and supplied by Promega® [1], Sigma® [2] or Roche® [1]. Protocols provided with the various kits were used and any deviations from these standard protocols reported. Twenty millilitres (mL) of whole blood was collected in ethylenediamine tetra-acetic acid (EDTA) tubes during the PRIMER study for DNA analysis. DNA was isolated according to standard protocols. UCSNP44 in the CAPN10 gene, the G3494A alteration in the IRS-1 gene, the G3684A alteration in the IRS-2 gene as well as the G8492C alteration in the PPARγ2 gene were all screened via a PCR and RFLP procedure. Automated cycle sequencing was used to confirm that the correct regions were amplified. The genotype data as well as the OGTT, questionnaire and measured anthropometrical data were evaluated and compared within different subgroups, as discussed in Section 5.4.2. The statistical methods that were used are discussed in Section 5.5.

5.1 ETHICAL APPROVAL

The research study was approved by the Ethics Committee of the North-West University (Potchefstroom Campus) under the titles of “Molecular analysis of non-insulin dependent diabetes mellitus in the South African population” with approval number 02M08 and subsequently under the title “Molecular analysis of type 2 diabetes (T2D)” with approval number NWU-00039-07-S9. Details of the study procedures, including sample collection, related risks and benefits were explained to each participating individual as part of obtaining informed consent. Participant confidentiality was maintained by assigning an anonymous sample number to each individual and the use of limited access and password protected databases.

[1] Promega® is the registered trademark of the Promega Corporation, Madison, WI, USA.
[2] Sigma® is a registered trademark of Sigma-Aldrich Corporation, St. Louis, MO, USA.

5.2 PATIENT POPULATION

The PRIMER study was an investigation of a cohort including 443 black South African individuals. The cohort predominantly included Tswana-speaking (76%) individuals. The remaining 24% of the cohort consisted of individuals from various ethnic backgrounds including 28 Xhosa, 28 Sotho, 4 Northern and 19 Southern Sotho, 10 Zulu, 8 Coloured, 3 Ndebele, 1 Pedi, 1 Swazi and 1 Tsonga individual. The ethnic origins of three individuals included in the cohort were not reported. Exclusion criteria included pregnancy, lactation, any identified acute infection or history of chronic disease or chronic treatment. In addition, individuals were only included when 'apparently healthy' and between the ages of 35 and 60. Although data collected from a cross-sectional study such as this only provides an overview of the investigated characteristics at a specific point in time and cannot always be used to differentiate cause and effect from simple association, it is the most effective approach to determine prevalence and identify important associations that can then be further investigated by means of a cohort or randomised controlled study.

5.3 METHODS

The different methods used for DNA extraction, amplification and screening of the four genetic alterations are discussed. These include DNA isolation, PCR and primer design, RFLP, agarose gel electrophoresis as well as automated cycle sequencing.

5.3.1 Genomic DNA isolation

DNA was isolated from the whole blood samples of 443 PRIMER individuals. The extraction of DNA was achieved via the use of a modified protocol of the FlexiGene™ [2] DNA Isolation Kit (QIAGEN®). In a 15 mL centrifuge tube, 2.5 mL of buffer FG1 Cell Lysis Solution was mixed with one mL of blood. The tube was gently inverted until thoroughly mixed, followed by centrifugation at 2,000 x g (gravitational force) for five minutes, at 4 degrees centigrade (°C). As much supernatant as possible was removed, without disturbing the visible white pellet, after which the tube was left inverted on a clean piece of absorbent paper for at least two minutes. A solution of 0.5 mL of buffer FG2 and 5 microlitre (µL) QIAGEN protease, which was mixed prior to DNA isolation, was added to

[1] Roche® is a registered trademark of Hoffmann-La Roche Ltd., Basel, Switzerland.
[2] FlexiGene® DNA kit is a registered trademark of QIAGEN, Clifton Hill, Victoria, Australia.

the tube containing the pellet. The tube was vigorously vortexed until the white blood cells were resuspended (10 – 15 seconds). On inspection of complete homogenisation, the tube was inverted three times, after which it was incubated at 65°C for 10 min. The incubation step allowed for protein digestion, as indicated by the solution turning olive green.

Subsequent to incubation, 0.5 mL of isopropanol was added to the tube and mixed by inversion until visualisation of the DNA precipitate as a clump. The sample was centrifuged at 2,000 x g for three minutes. The DNA was visible as a small white pellet.
The supernatant was decanted and 0.5 mL of a 70% ethanol (EtOH) solution at room temperature was added to the DNA. The tube was gently inverted several times to wash the DNA pellet and the sides of the centrifuge tube. Centrifugation was repeated as indicated in the previous step. The ethanol was carefully aspirated. The tube was left inverted on a clean piece of absorbent paper for five minutes, followed by air-drying for another five minutes. Five seconds of low speed vortexing was performed subsequent to adding 200 µL of the DNA Rehydration Solution. The DNA was incubated at 65°C for one hour. Rehydration of DNA was obtained by incubating overnight at room temperature. A 50 nanograms per microlitre ($ng.\mu L^{-1}$) final DNA working dilution was prepared with double distilled water (ddH_2O) and stored at 2 to 8°C. The stock DNA was stored at minus 20°C and thawed at room temperature prior to use.

5.3.2 Polymerase chain reaction and restriction fragment length polymorphism analysis

The polymerase chain reaction involves denaturation of the DNA at 94°C, annealing of the primers to the template at a specific optimised temperature and template elongation. The annealing temperatures of the different primer sets were optimised and are discussed in Chapter Six. Primers were either synthesised from previously described sequences or specifically designed according to the protocol discussed in Section 5.3.2.1. Optimisation of the PCR included evaluation of amplification by visualising the PCR product on an agarose gel, prepared according to the protocol discussed in Section 5.3.4. The annealing temperature (Ta) was subsequently adjusted according to the appearance of the observed fragment and the PCR repeated. Successfully amplified DNA fragments fluoresced intensely on the agarose gel and presented with no secondary amplification products. The first T_a was 5°C below the calculated annealing temperature ($T_{a(calc)}$) followed by a temperature increase in increments of 2°C, until the optimised T_a ($T_{a(opt)}$)

was reached, as judged by the PCR product observed on the agarose gel. The formula described by Thein and Wallace (1986) was used to calculate the $T_{a(calc)}$ and is presented in Equation 5.1.

Equation 5.1 Equation for the calculation of annealing temperature

$$T_{a(calc)} = 2 (A + T) + 4 (G + C)$$

$T_{a(calc)}$ = calculated annealing temperature; A + T = adenine and thymine content of the primer sequence; G + C = guanine and cytosine content of the primer sequence (Thein and Wallace, 1986).

The following reaction mixture was used for amplification of fragments: 1 X PCR buffer [50 mM potassium chloride; 10 mM Tris®-HCl[1] (pH 9.0); 0.1% Triton® [2] X-100]; 0.5 to 2.0 mM $MgCl_2$; 200 µM 2'- deoxynucleotide triphosphate (dNTP) mixture; 5.0 picomole (pmol) of both the forward and reverse primers, 100 ng of gDNA, 0.5 units of Go *Thermus aquaticus* (*Taq*)® [3] Flexi (Promega) DNA polymerase, or FastStart *Taq*® [4] (Roche) DNA polymerase with GC-RICH® [5] solution (Roche). The final volume of the PCR reaction was 12.5 µl, which was overlaid with 12.5 µL mineral oil to prevent evaporation. Amplification of all fragments was performed in a Thermo Hybaid MultiBlock System 0.5 Satellite (MBS 0.5S) thermal cycler, using the thermal cycling parameters presented in Table 5.1.

Table 5.1 Thermal cycling conditions used for the PCR reaction

Cycles	PCR Step	Duration	Temperature
1 cycle	denaturation	10 minutes	94°C
30 cycles	denaturation	30 seconds	94°C
	annealing	30 seconds	$T_{a(opt)}$
	extension	60 seconds	72°C
1 cycle	final elongation	7 minutes	72°C
1 cycle	hold	indefinitely	4°C

$T_{a(opt)}$ = optimised annealing temperature; °C = degrees centrigrade.

A RFLP strategy with the appropriate restriction endonuclease was used for the digestion of the specific amplified region harbouring the alteration in each of the four genes investigated. The resultant fragments were separated via agarose gel electrophoresis,

[1] Tris® is a registered trademark of Rohm & Haas Company, Philadelphia, PA, USA.
[2] Triton X-100® is a registered trademark of Union Carbide Chemicals and Plastics Technology Co., Inc., Swanpscott, MA, USA.
[3] Go *Taq*® Flexi DNA polymerase is a registered trademark of the Promega Corporation, Madison, WI, USA.
[4] FastStart *Taq*® DNA polymerase is a registered trademark of Roche Diagnostics GmbH, Mannheim, Germany.
[5] GC-RICH solution® is a registered trademark of Roche Diagnostics GmbH, Mannheim, Germany.

which was prepared and loaded according to the protocol discussed in Section 5.3.3. Incubation temperatures and times used were according to the manufacturer's protocol. Incomplete digestion was corrected for by increasing the incubation time. Separating the RFLP products via agarose gel electrophoresis and comparing the sizes of the observed fragments in relation to the molecular marker enabled evaluation of successful digestion. Digestion was complete when no fragment with the same size as the original PCR product was observed.

The sequence of the regions harbouring the alteration in both the PPARγ2 and CAPN10 genes were not recognised by a commercially available restriction endonuclease and a restriction site was therefore created during amplification (amplification created restriction site, ACRS).

5.3.2.1 Primer design

The forward primers for both the CAPN10 and IRS-1 genes were specifically designed via PrimerQuest^{SM}, a software programme provided by Integrated DNA Technologies (IDT). The DNA sequence of regions opposing the alteration was entered and recommended primer sets subsequently provided by PrimerQuest^{SM}. The primer sets were analysed via the IDT OligoAnalyser and the most appropriate set selected based on the melting temperature (T_m), self-dimer and hairpin loop formations of that specific primer set. Guidelines used for designing the primers included a primer length between 18 and 25 bases, less than a 5°C difference in T_m between the primer pairs, a GC content of 45% to 60% and no formation of hairpins or self or cross–dimers, which will adversely affect the amplification reaction (Sambrook and Russell, 2001). The Basic Local Alignment Search Tool (BLAST) programme version 2.2.9 (Altschul *et al*., 1997) was used to determine cross homology with any other sequence in the human genome. Primer sets annealing only to the specific regions of interest were used for amplification.

5.3.2.2 Insulin receptor substrate-1 gene

The G3494A alteration in the IRS-1 gene was analysed via PCR and RFLP analysis using the Promega Go *Taq*® Flexi DNA polymerase and *Bst* NI restriction endonuclease, respectively. The 30 µL RFLP reaction mixture contained 10 µL of PCR product, 1 x restriction enzyme buffer 2, two units *Bst* NI restriction enzyme and ddH_2O. The reaction was supplemented with 0.3 µL of 100 $\mu g.mL^{-1}$ bovine serum albumin (BSA).

Incubation was performed at 60°C for an hour, which was the optimised time period ensuring complete digestion. The primers used for the amplification of the region containing the G3494A alteration in the IRS-1 gene, as well as the calculated annealing temperatures for the primer set, are indicated in Table 5.2. The reverse primer was synthesised according to the sequence described by Federici *et al.* (2003) and the forward primer design consistent with the procedure described in Section 5.3.2.1.

Table 5.2 Primers used for amplification of the region containing the G3494A alteration in the IRS-1 gene

Genotype	Primer sequence	$T_{a(calc)}$
G3494A	**F:** 5'- tcc aga gcc caa gag ccc–3'	59.9°C
	R: 5'-tgg cga ggt gtc cac gta gc–3'	58.0°C

IRS-1 = insulin receptor substrate-1 gene; G = guanine; A = adenine; F and R indicate forward and reverse primers respectively; $T_{a(calc)}$ = calculated annealing temperature for each primer.

The published sequence of the IRS-1 gene harbouring the alteration of interest is presented in Table 5.3. The positions of the forward and reverse primers as well as the position of the alteration are also indicated.

Table 5.3 Partial sequence of the IRS-1 gene, encompassing the region between nucleotides 3241 and 3601

Nucleotide number	DNA Sequence: IRS-1
3241	ccctccagag cccaagagcc cgggggaata tgtcaatatt gaatttggga gtgatcagtc
3301	tggctacttg tctggcccgg tggctttcca cagctcacct tctgtcaggt gtccatccca
3361	gctccagcca gctcccagag aggaagagac tggcactgag gagtacatga agatggacct
3421	ggggccgggc cggagggcag cctggcagga gagcactggg gtcgagatgg gcagactggg
3481	ccctgcacct cccgggctg ctagcatttg caggcctacc cgggcagtgc ccagcagccg
3541	gggtgactac atgaccatgc agatgagttg tccccgtcag agctacgtgg acacctcgcc
3601	agctgccccct gtaagctatg ctgacatgcg aacaggcatt gctgcagagg aggtgagcct

IRS-1 = insulin receptor substrate-1 gene. The forward primer sequence is indicated by the red text and the position of the reverse primer is indicated by the blue text. The text highlighted in turquoise represents the codon sequence wherein the investigated IRS-1 gene mutation occurs. The sequence has been retrieved from Genbank accession number S 85963.

Expected fragment sizes for the presence or absence of the G3494A alteration in the IRS 1 gene is presented in Table 5.4. As indicated, the 358 bp amplification product is digested in either two or three fragments, depending on the genotype.

Table 5.4 Position, alteration and expected fragment sizes for the IRS-1 gene alteration

IRS-1 gene	Alteration	Genotype	Expected size (bp)
G3494A	G	wild type	199, 159
	A	mutant	199, 108, 51

IRS-1 = insulin receptor substrate-1 gene; bp = base pair; G = guanine; A = adenine. Expected fragment sizes after restriction enzyme digest with *Bst* NI.

5.3.2.3 Insulin receptor substrate-2 gene

The region in the IRS-2 gene containing the G3684A alteration was analysed via PCR and RFLP methods, as discussed in Section 5.3.2, with addition of the FastStart *Taq*® DNA polymerase and GC-RICH® solution to improve the efficiency of the PCR. Primers described by Fritsche *et al.* (2001) were synthesised and used for amplification and the sequences of the primers as well as the calculated annealing temperatures are presented in Table 5.5. The T_a for the primer set was optimised prior to analysis and discussed in Chapter Six.

Table 5.5 Primers used for amplification of the region containing the G3684A alteration in the IRS-2 gene

Genotype	Primer sequence	$T_{a(calc)}$
G3684A	**F:** 5'- ccg act aca tga acc tcg ac-3'	54.9°C
	R: 5'-gag gag aag gtc tcg gaa ct-3'	55.7°C

IRS-2 = insulin receptor substrate-2 gene; G = guanine; A = adenine; bp = base pair; F and R indicate forward and reverse primers respectively; $T_{a(calc)}$ = calculated annealing temperature for each primer.

The 30 µL RFLP reaction mixture consisted of 10 µL PCR product, 1 x restriction enzyme buffer 4, two units *Sma* I restriction enzyme and ddH_2O. Incubation was performed at 25°C for an hour, ensuring complete digestion, as confirmed visually on the agarose gel. The 535 bp amplification product was digested into three different sized products, as indicated in Table 5.6, when the coding sequence for Gly was observed at the point of the alteration. Only two fragments were observed when the sequence coding for Asp was present.

Table 5.6 Position, alteration and expected fragment sizes generated via RFLP for the IRS-2 gene

IRS-2 gene	Alteration	Genotype	Expected size (bp)
G3684A	G	wild type	294, 187, 54
	A	mutant	348, 187

IRS-2 = insulin receptor substrate-2 gene; bp = base pair; G = guanine; A = adenine. Expected fragment sizes after restriction enzyme digest with *Sma* I.

The published sequence of the IRS-2 gene harbouring the G3684A alteration is presented in Table 5.7. The table also indicates the positions of the codon wherein the mutation arises, as well as the forward and reverse amplification primers.

Table 5.7 Partial sequence of the IRS-2 gene, encompassing the region between nucleotides 3421 and 3961

Nucleotide number	DNA Sequence: IRS-2
3421	ccggcagcgg tctccgctct ccgactacat gaacctcgac ttcagctccc ccaagtctcc
3481	taagccgggc gccccgagcg gccaccccgt gggctccttg gacggcctcc tgtcccccga
3541	ggcctcctcc ccgtatccgc cgttgccccc gcgtccgtcc gcgtccccgt cgtcgtctct
3601	gcagccgccg ccaccgccgc cggccccggg ggagctgtac cgcctgcccc ccgcctcggc
3661	cgttgccacc gcccagggcc cgggccgccgc ctcatcgttg tcctcggaca ccggggacaa
3721	tggtgactac accgagatgg cttttggtgt ggccgccacc ccgccgcaac ctatcgcggc
3781	ccccccgaag ccagaagctg cccgcgtggc cagcccgacg tcgggcgtga agaggctgag
3841	cctcatggag caggtgtcgg gagtcgaggc cttcctgcag gccagccagc ccccggaccc
3901	ccaccgcggc gccaaggtca tccgcgcaga cccgcagggg ggccgccgcc gccacagttc
3961	cgagaccttc tcctccacca cgacggtcac ccccgtgtcc ccgtccttcg cccacaaccc

IRS-2 = insulin receptor substrate-2 gene. The forward primer sequence is indicated by the red text and the position of the reverse primer is indicated by the blue text. The text highlighted in turquoise represents the codon sequence wherein the investigated IRS-2 gene mutation occurs. The sequence has been retrieved from Genbank accession number NM 003749.

5.3.2.4 UCSNP44 in the calpain 10 gene

UCSNP44 is a thymine to a cytosine alteration in intron three of the CAPN10 gene. The region containing the alteration was amplified according to the PCR protocol in Section 5.3.2, using the standard protocol with the Promega Go *Taq*® Flexi DNA polymerase. The sequences of the amplification primers as well as the calculated annealing temperatures are presented in Table 5.8. The reverse primer was synthesised according to the sequence described by Towers (2004) and the forward primer design was consistent with the procedures discussed in Section 5.3.2.1.

Table 5.8 Primers used for amplification of the UCSNP44 region in the CAPN10 gene

Genotype	Primer sequence	$T_{a(calc)}$
T22751C	**F: 5'-**cag ggc gct cac gct tgc cg**-3'**	66.8°C
	R: 5'-ggt ctg tag cac ccc aaa tcg**-3'**	58.0°C

CAPN10 = calpain 10 gene; UCSNP44 = University of Chicago single nucleotide polymorphism; T = thymine; C = cytosine; F and R indicate forward and reverse primers respectively; $T_{a(calc)}$ = calculated annealing temperature for each primer; bp = base pair.

The RFLP reaction, with a total volume of 30 µL, contained a mixture of 10 µL PCR product, 1 x restriction enzyme buffer 2, two units of *Bst* UI and ddH_2O. Incubation was performed at 60°C for an hour. The WT genotype harbours a thymine nucleotide at position 22751, preventing digestion by the restriction enzyme. A cytosine at the same position resulted in the amplification product of 140 bp, being digested into two fragments. The expected fragment sizes generated via RFLP for the CAPN10 gene are presented in Table 5.9.

Table 5.9 Position, alteration and expected fragment sizes generated via RFLP for the CAPN10 gene

CAPN10 gene	Alteration	Genotype	Expected size (bp)
T22751C	T	wild type	140
	C	mutant	120, 20

T = thymine; C = cytosine; bp = base pair; nt = nucleotide; CAPN10 = calpain 10 gene, UCSNP44 = University of Chicago single nucleotide polymorphism 44. Expected fragment sizes after restriction enzyme digest with the *Bst* UI restriction endonuclease.

The partial nucleotide sequence of intron three in the CAPN10 gene, which contains the region of the UCSNP44, is presented in Table 5.10. The annealing sites of the primers and the ACRS site are also indicated.

Table 5.10 Partial sequence of the CAPN10 gene, encompassing the region between nucleotides 22621 and 22921

Nucleotide number	DNA Sequence: UCSNP44
22681	ctgtgcccac accggatgcc agagagtttc tgtgtgtggg cagaggactg cagggcgctc
22741	acgcttgctg tgaagtaagg cgtttgaagg tgaggctaag ccttgacttg gtgaggatga
22801	ggaagaaggc agaggggagt aaagaggtgg gattgaggca gcggttggac gatttggggt
22861	gctacagacc atgggaatca gagaggggcc catgctcaat gccagaggct cactcccatg

The forward primer sequence is indicated by the red text, the position of the ACRS site is highlighted in yellow and the position of the reverse primer is indicated by the blue text. The position of UCSNP44 is highlighted in turquoise. Nucleotide numbering is according to Horikawa *et al* (2000). The sequence has been retrieved from Genbank accession number AF 158748.

5.3.2.5 Peroxisome proliferator-activated receptor gamma 2

The PCR and RFLP protocols discussed in Section 5.3.2 were used for the screening of the PPARγ2 gene alteration. The Promega Go *Taq*® Flexi DNA polymerase was used in the amplification reaction. A restriction site was created during amplification in order to allow for enzyme digestion with *Hha* I. A 30 µL total RFLP reaction volume was used, which contained 10 µL PCR product, 1 x restriction enzyme buffer 4, two units *Hha* I restriction enzyme, and ddH_2O supplemented with 0.3 µL of 100 $\mu g.mL^{-1}$ BSA. Incubation was optimised at 37°C for an hour.

Primers used for the amplification of the region harbouring the C8492G alteration in the PPARγ2 gene are presented in Table 5.11. Kim *et al.* (2004) described the sequences that were used for the synthesis of both the amplification primers. The calculated annealing temperatures for the primers are also included.

Table 5.11 Primers used for amplification of the region containing the G8492C alteration in the PPARγ2 gene

PPARγ2 gene	Primer sequence	$T_{a(calc)}$
C8492G	**F:** 5'-tct ggg aga ttc tcc tat tgg-3'	53.2°C
	R: 5'-ctg gaa gac aac tac aag ag-3'	50.0°C

PPARγ2 = peroxisome proliferator-activated gamma 2 gene; G = guanine; C = cytosine; F and R indicate forward and reverse primers respectively; $T_{a(calc)}$ = calculated annealing temperature for the primers. bp = base pair.

The alteration in the PPARγ2 gene, and the expected fragment sizes as separated via agarose gel electrophoresis are presented in Table 5.12. The wild type genotype sequence produced a 154 bp amplification product that remained undigested. The presence of a guanine nucleotide at position 8492 resulted in an ACRS that allowed for digestion of the amplified product into two fragments.

Table 5.12 Position, alteration and expected fragment sizes generated via RFLP for the PPARγ2 gene

PPARγ2 gene	Alteration	Genotype	Expected size (bp)
C8492G	C	wild type	154
	G	mutant	131, 23

PPARγ2 = peroxisome proliferator-activated gamma 2 gene; bp = base pair; G = guanine; C = cytosine. Expected fragment sizes after restriction enzyme digest with *Hha* I.

The published sequence retrieved from Genbank, harbouring the C8492G alteration as well as the position of the forward and reverse amplification primers are presented in Table 5.13. The position of the ACRS is also highlighted.

5.3.3 Agarose gel electrophoresis

Prior to using the RFLP strategies, the PCR reactions were optimised and the amplified products separated via agarose gel electrophoresis. Genotypes were determined by evaluating the separated RFLP fragments on an agarose gel and comparing the sizes of the observed fragments to the expected sizes, as discussed in Section 5.3.2.

Table 5.13 Partial sequence of the PPARγ2 gene, encompassing the region between nucleotides 841 and 1021

Nucleotide number	DNA Sequence: PPARγ2
841	ccctattcc atgctgttat gggtgaaact ctgggagatt ctcctattga ccccagaaagc
901	gattccttca ctgatacact gtctgcaaac atatcacaag gtaaagttcc ttccagatac
961	ggctattggg gacgtggggg catttatgta agggtaaaat tgctcttgta gtttgtcttc
1021	caggttgtgt ttgttttaat actatcatgt gtacactcca gtattttaat gcttagctcg

PPARγ2 = peroxisome proliferator-activated gamma 2 gene. The forward primer sequence is indicated by the red text, the position of the ACRS is highlighted in yellow and the position of the reverse primer is indicated by the blue text. The text highlighted in turquoise represents the codon sequence wherein the investigated PPARγ2 gene mutation occurs. The sequence has been retrieved from Genbank accession number AB 005520.

Midi gels, with a total gel volume of 100 mL, were prepared to visualise the products. The gels consisted of either 2% or 3% molecular grade agarose (Promega®), 1 X TBE buffer [89.15 mM tris(hydroxymethyl)aminomethan:2-amino-2-(hydroxymethyl)-1,3-pro-panediol or Tris (pH 8.1), 88.95 mM boric acid, 2.5 mM disodium ethylenediamine tetra-acetic acid or Na_2EDTA] and 0.5 $\mu g.mL^{-1}$ ethidium bromide (EtBr) from Sigma®. A volume of 2.5 µL of 1 X loading buffer [15% Ficoll and 0.04% orange G (Sigma® [1])] was used to load 2.5 µL of the PCR products along with 250 ng of a 100 bp DNA ladder (Promega®). The gel was electrophoresed at 10 Volts (V) per cm for 30 minutes in 1 X TBE buffer. For the various RFLP analyses, a volume of 8 µL of the RFLP product was loaded with 3 µL of 1 X loading buffer, along with 125 ng of a 50 bp DNA ladder (Promega®) followed by electrophoresis at 10 V per cm for 50 minutes. Electronic copies of the images were captured via a video documentation system following illumination of the DNA with an ultraviolet (UV) light.

5.3.4 Automated cycle sequence analysis

Control samples for each of the four genes were sequenced in order to confirm that the correct regions were amplified. For each of the investigated genes, these included samples from individuals that harboured a wild type homozygous, heterozygous or homozygous mutant DNA sequence. The genotypes were determined via RFLP and subsequently confirmed by automated cycle sequence analysis, following purification of the PCR product.

5.3.4.1 PCR purification

The Zymo Research DNA Clean & Concentrator-5™ [2] PCR purification kit was used for the direct purification of the PCR product from the reaction mixture. The manufacturer's protocol was followed. To bind the PCR product, two volumes of DNA binding buffer was added to one volume of PCR sample and vortexed. The mixture was applied to the Zymo-Spin™ column and centrifuged for one min at 13,000 rpm. The sample was washed by adding 200 µL of the wash buffer and centrifugation at 13,000 rpm for one min. Centrifugation was repeated at the same conditions after discarding the filtrate. Subsequent to placing the spin column in a clean 1.5 mL microcentrifuge tube and elution of the DNA by adding 30 µl of ddH_2O, centrifugation was repeated for one min at 13,000 rpm. A spectrophotometer was used to determine the DNA concentration in order to optimise the amount of DNA template required in the sequencing reaction. DNA concentrations were calculated according to the equation listed in Equation 5.2.

Equation 5.2 Formula for determining DNA concentration

$$\text{Unknown } (\mu g.mL^{-1}) / A_{260\ nm} = 50\ \mu g.mL^{-1} / 1.0\ A_{260\ nm}$$

A = absorbency; $\mu g.mL^{-1}$ = microgram per millilitre; nm = nanometre.

5.3.4.2 Chain termination sequencing

Automated sequencing was performed in order to confirm the nucleotide sequence of the control DNA fragments amplified and digested via the PCR-RFLP technique. The determination of a nucleotide sequence involves a method developed by Sanger *et al.* (1977), which entails dye terminators in the form of fluorescently labelled 2',3'-dideoxynucleotide triphosphates (ddNTPs).

Sequencing of samples was performed using the ABI Prism® [3] BigDye™ [4] Terminator version 3.0 Ready Reaction Cycle Sequencing Kit, which contains a Ready Reaction Mix consisting of dNTPs, dye terminators, Ampli*Taq*® [1] DNA polymerase, $MgCl_2$ and buffer. A final reaction volume of 10 µl was prepared, consisting of 10 ng purified PCR product, 2 µL Ready Reaction Premix, 2 µL of 5 X sequencing buffer ($MgCl_2$ and Tris-HCL,

[1] Sigma® is a registered trademark of Sigma Chemical Company, St. Louis, MO, USA.
[2] Zymo Research DNA Clean & Concentrator-5™ is a registered trademark of the Zymo Research Corporation, Orange County, CA, USA.
[3] ABI Prism® is a registered trademark of Applera Corporation, Foster City, CA, USA.
[4] BigDye™ is a trademark of Applera Corporation, Foster City, CA, USA.

pH 9.0), 3.2 pmol forward amplification primer and deionised water. The Hybaid® [2] MultiBlock System was used for sequencing according to the protocol presented in Table 5.14.

Table 5.14 Cycle sequencing protocol

Cycles	PCR Step	Duration	Temperature
25 cycles	denaturation	10 seconds	96°C
	annealing	5 seconds	50°C
	elongation	4 minutes	60°C
	hold	indefinitely	4°C

5.3.4.3 Sequence product precipitation

Precipitation and electrophoresis of the sequencing product was performed on contract. The protocol used for nucleic acid precipitation involved the Centri-sep™ [3] 96 well clean-up kit. Excess dye terminators were removed from the completed sequencing reaction via the Centri-sep™ 96 well filter plate. The sequences were analysed on an Applied Biosystems® [4] 3130xl genetic analyser. Resultant sequences were aligned to the reference sequence retrieved from Genbank. Sequence alignments were performed via the BioEdit Sequence Alignment Editor Version 5.0.9 programme.

5.4 DATA ANALYSES

During the PRIMER study, data was collected from 443 “apparently healthy”, fasting black South African individuals. The data was gathered via questionnaires, glucose and anthropometrical measurements and specific biochemical assays. Individuals were only included in the phenotypic and genotypic analyses when complete OGTT, biochemical and anthropometrical data were available.

5.4.1 Glucose measurements

Blood samples were collected from all participants prior to initiating the OGTT, including a capillary sample after a finger prick and a plasma sample following intravenous (IV)

[1] AmpliT*aq*® is a registered trademark of Roche Molecular Systems Inc., Pleasanton, CA, USA.
[2] Hybaid® is a registered trademark of Hybaid Limited, Ashford, Middlesex, UK.
[3] Centri-sep™ 96 is a trademark of Princeton Separations, Adelphia, NJ, USA.
[4] Applied Biosystems® 3130xl genetic analyser is a registered trademark of Applera Corporation, Foster City, CA, USA.

catheterisation. Plasma samples were also collected post prandially at 30, 60, 90 and 120 min for glucose level measurements. Both capillary and plasma blood glucose levels were measured via a handheld MediSens® Optium™ [1] Sensor glucose machine, together with MediSens® Optium™ *Plus* blood glucose test strips [2]. This glucose machine measures blood glucose when a sample is applied to the electrode and the blood glucose reacts with the chemicals on the electrode. The reaction produces a small electrical current that is equivalent in size to the amount of glucose in the blood sample. The machine measures the generated current and derives the blood glucose concentration in the sample, subsequently displaying the measurement within 20 seconds of application.

5.4.2 Oral glucose tolerance test

Individuals arriving for the OGTT were only included for testing if they were fasting for at least ten hours and not clinically stressed, as defined by harbouring an acute infection or suffering from chronic disease. Following a finger prick, a fasting glucose measurement was determined to ensure normal glucose tolerance, followed by IV catheterisation and the subsequent ingestion of 75 grams (g) of glucose dissolved in 300 mL water. Plasma samples were collected prior to glucose consumption (at 0 min) and thereafter at 30, 60, 90 and 120 min post prandially.

5.4.3 Questionnaires

Members of the PRIMER team questioned all study participants in their home language and thereafter transcribed the questionnaire data in English. The data recorded for each individual included age, gender, residing location, ethnicity, education, medical history, diabetes risk score criteria (diet and physical activity), habits in terms of smoking and alcohol consumption as well as diabetic complications. All questionnaire data was captured electronically using the Microsoft® [3] EXCEL® [4] spreadsheet programme and exported to the STATISTICA7™ [5] programme, version 7 [Statsoft (2004)] for statistical analyses. Data on the daily intake of vegetables, fruits or berries as well as physical activity levels were used to assess diabetes risk according to methods described by Lindström and Tuomilehto (2003).

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[1] MediSens® Optium™ is a registered trademark of Abbott Diabetes Care, Doncaster, Australia.
[2] Glucose measurements were performed by members of the PRIMER team.
[3] Microsoft® is a registered trademark of Microsoft Corporation, Seattle, WA, USA.
[4] Excel® is a registered trademark of Microsoft Corporation in the United States and/or other countries.
[5] STATISTICA7™ is a registered trademark of StatSoft Inc., Tulsa, OK, USA.

5.4.4 Anthropometrical measures

All anthropometrical measures were determined by members of the PURE team under the supervision of Prof Hans de Ridder and Dr Hanlie Moss. Height and weight measurements [1] were obtained in duplicate from all study participants according to the guidelines adopted from the National Institutes of Health sponsored Arlie Conference (Lohman *et al*., 1988). Weight was measured in kilograms (kg) using a portable electronic scale while participants only wore undergarments (Precision Health Scale, A&D Company, Tokyo, Japan). Height measurements were recorded in centimetres (cm) following assessment using a Stadiometer (IP 1465, Invicta, London, UK) with all participants standing upright, wearing no shoes and their heads in the Frankfort plane. Once captured, BMI data was generated according to Equation 5.3.

Equation 5.3 Calculation of BMI

$$\text{BMI} = \frac{\text{weight (kg)}}{\text{height (m)}^2}$$

BMI = body mass index; kg = kilograms; m = metre.

5.4.5 Biochemical assays

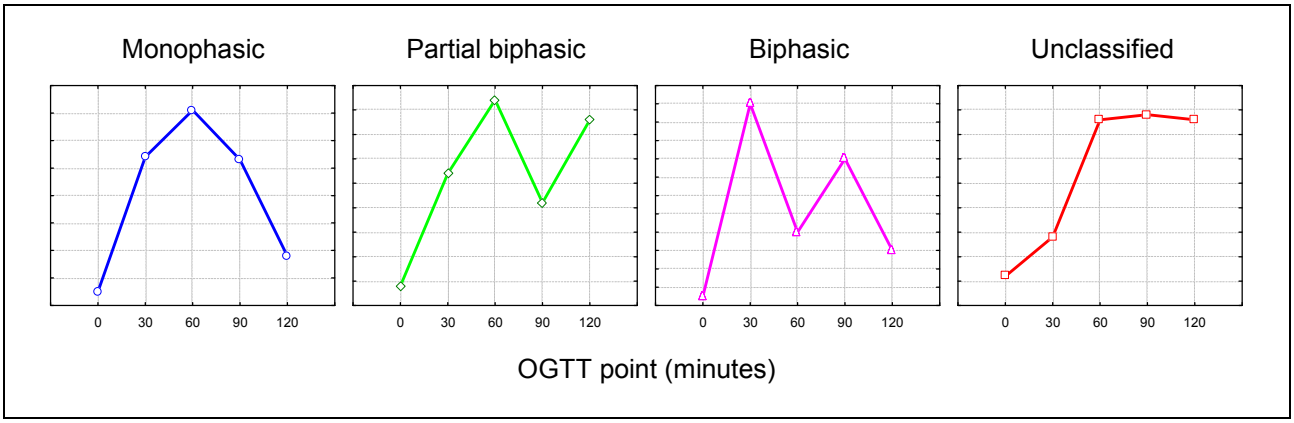
Members of the PRIMER team measured HbA1c levels from whole blood EDTA samples by means of the Bio-Rad D-10™ [2] HbA1c kit [3], which operates via cation exchange high performance liquid chromatography. The HIV testing was performed for the PURE-SA study, with results being presented in this thesis with permission from Prof A Kruger, leader of the South African leg of the PURE study. The HIV status [4] was determined by using the First Response® HIV Card Test 1-2.O™ [5]. All positive HIV test results were confirmed with the SD HIV-1/2 3.0™ [6] rapid confirmation test. The two types of HIV tests used in this study have sensitivities of 100% and specificities of greater than 99%. The tests are based on immunochromatographic technology that differentially and qualitatively detect all antibodies specific to HIV-1 and HIV-2 simultaneously in human samples.

[1] Height and weight measurements were obtained by members of the PURE team and are presented in the thesis with permission from Prof A Kruger, leader of the South African leg of the PURE study.
[2] Bio-Rad D-10™ is a registered trademark of Bio-Rad Laboratories, Inc., Hercules, France.
[3] The HbA1c level measurements were performed by members of the PRIMER team.
[4] HIV testing was performed by members of the PURE team and is used with permission.
[5] First Response® HIV Card Test 1-2.O™ is a registered trademark of PMC Medical Pvt., Ltd., Daman, India.
[6] SD HIV-1/2 3.0™ is a registered trademark of Standard Diagnostics, Inc., Kyonggi-do, Korea.

5.4.6 Glucose curve shape classification

The shape of the glucose curves were graphically plotted and classified according to the criteria defined by Tschritter *et al.* (2003). The different glucose curve shapes observed within the investigated cohort, following the two hour OGTT, were classified into three groups namely a biphasic, a monophasic and an unclassified group. An example of each of the GCSs is presented in Figure 5.1.

Figure 5.1 GCS classification examples



GCS = glucose curve shape; OGTT = oral glucose tolerance test.

The characteristics of a monophasic curve entailed a maximum increase in glucose within 30 to 90 minutes after the glucose load. This is followed by a decrease until the end of the two hours, with a final decline of at least 0.25 mmol.L^{-1} in the last 30 minutes. The biphasic curves first increased and then decreased in cycles of 30 minutes over the two-hour period. The curve of the partial biphasic shape increased at first, and then reached a low point followed by a second increase of more than 0.25 mmol.L^{-1}, until 120 minutes. Both the complete and partial biphasic shapes were included in the biphasic GCS group. Curves included in the unclassified group did not reach the 0.25 mmol.L^{-1} cut-off defined for the increases and decreases of the monophasic or biphasic GCS. This empirically selected 0.25 mmol.L^{-1} level was defined by Tschritter *et al.* (2003) to avoid incorrect classification. The glucose values measured over the two hour OGTT period were entered into the STATISTICA7^{TM} [1] programme, version 7 [Statsoft (2004)]. The programme was used to plot the graphs, followed by individual assessment and classification of the curves by the author.

[1] STATISTICA7^{TM} is a registered trademark of StatSoft Inc., Tulsa, OK, USA.

5.4.7 Cohort subgroups

The investigated cohort included 443 individuals recruited from the black South African population. This cohort was further divided into subgroups, stratified according to fasting glucose, glucose tolerance, HbA1c, BMI, age, gender, physical environment, diet, exercise, HIV and genotype, as discussed in Chapter Six.

5.5 STATISTICAL ANALYSES

Various statistical analyses were performed during this investigation via the use of the STATISTICA7$^{TM\ 1}$ programme [Statsoft (2004)] and with the assistance of Dr Wayne Towers. Allelic and genotypic frequencies were analysed to determine whether they were in Hardy-Weinberg equilibrium, as described in Section 5.5.1. All continuous data distributions were tested for normality, as discussed in Section 5.5.2, and subsequently compared between the various genotypic subgroups for the determination of statistically significant differences. Statistically significant findings were further evaluated for practical significance, as discussed in Section 5.5.4.

5.5.1 Hardy-Weinberg equilibrium

The Hardy-Weinberg (H-W) method was defined by G.H. Hardy and W. Weinberg in the early 1900s and is used to determine the expected genotype frequencies from the allele frequencies, as presented in Equation 5.4. Assumptions of this model include random mating, no genetic drift or mutation, no migration and no affect on the alleles due to natural selection. With the genotypes in H-W equilibrium it can be hypothesised that the observed differences are most likely brought about by association of a specific genotype to disease susceptibility and not by the above-mentioned mechanisms of evolution.

Equation 5.4 Calculation of allele frequencies

$$p = \frac{[\text{number of homozygotes} + 0.5(\text{number of heterozygotes})]}{\text{total number of individuals}}$$

$$q = 1 - p$$

p = frequency of allele 1; q = frequency of allele 2.

The chi square (χ^2) test (Motulsky, 1995) was used to test the null hypothesis stating that the genotypes are in H-W equilibrium. A high chi square value and resultant small p-value

(< 0.05) rejected the null hypothesis, suggesting that the genotypes were not in H-W equilibrium. The chi square value was determined by the test equation presented in Equation 5.5, which is discussed further in Chapter Six.

Equation 5.5 The chi square test

$$\chi^2 = \frac{(O-E)^2}{E}$$

χ^2 = chi square; O = observed number; E = expected number.

5.5.2 Normal distribution

Continuous data in all comparison subgroups were entered into the STATISTICA7™ [1] programme, version 7 [Statsoft (2004)]. Due to the advanced power properties of the Shapiro-Wilk's (SW) test (Royston, 1982), it was used to evaluate the normality of the data. Data within the subgroups were compared via parametric or non-parametric tests, depending on the distribution, as determined by the SW p-value.

A significant p-value (< 0.05) as determined via a SW test did not support the hypothesis of normality and all subsequent comparisons were performed via non-parametric tests. The Mann-Whitney (M-W) test (Motulsky, 1995) was used to compare non-parametric parameters between two unpaired subgroups. The data compared via a M-W test had to include randomly selected samples, where the two subgroups as well as the parameters within each subgroup were obtained independently and the data not distributed normally. More than two subgroups were compared via a Kruskal-Wallis (K-W) analysis of variance (ANOVA) test (Motulsky, 1995), followed by multiple comparison analysis on significant findings. The data compared via the K-W ANOVA had to follow a non-normal distribution and included randomly selected samples with independently obtained parameters within the subgroups.

A non-significant p-value (> 0.05) generated via the Shapiro-Wilk's test supported a normal distribution of data within the compared subgroups. Two subgroups were compared via a student t-test (Motulsky, 1995). The assumptions of the t-test include randomly selected samples, with parameters within the two subgroups obtained independently and following a normal distribution. A one-way ANOVA (Motulsky, 1995)

strategy was used to compare more than two subgroups. Assumptions of the ANOVA test are the same as for the K-W ANOVA as discussed above, with the exception of a normal distribution assumed for the parametric ANOVA.

5.5.3 Contingency table, odds ratio and 95% confidence interval analysis

Contingency tables with different determining factors were used to evaluate the association between various categorical variables. An example of a contingency table that was used is presented in Table 5.15. All numbers presented in the rows and columns were mutually exclusive and indicated the number of individuals within a certain category, with or without the presence of a specific variable (Motulsky, 1995).

Table 5.15 An example of the contingency table used

Genotype	Abnormal variable	Normal variable	Total	Odds ratio
At-risk	a	b	a + b	$\frac{ad}{bc}$
Wild type	c	d	c + d	
Total	a + c	b + d	a + b + c + d	
95% CI	95% CI of ln(OR) = ln(OR) ± 1.96 $\sqrt{\frac{1}{a}+\frac{1}{b}+\frac{1}{c}+\frac{1}{d}}$			

a = The number of individuals harbouring the 'risk' allele and falling into the abnormal variable group; d = the number of individuals harbouring the wild type genotype in the normal variable group; b = the number of individuals with the 'risk' allele and in the normal variable group; c = the number of individuals included in the abnormal variable group harbouring the wild type genotype. OR = odds ratio; CI = confidence interval.

The ratio of the odds (OR) for having the variable in one group to the odds of having it in another group was determined by the equation presented in Table 5.15. The OR will always be greater than or equal to zero. When the OR is equal to one, the investigated condition is equally possible in both groups. An OR lesser or greater than one, indicates that the condition is more probable in either group specifically. The 95% confidence intervals (CI) were calculated subsequent to the determination of a two-tailed p-value. The natural logarithm of the OR and subsequently the symmetrical 95% CI thereof, was calculated by using Woolf's method, as presented in Table 5.15. The disease risk within the population is directly assessed with this method, by using the actual incidence rates (Woolf, 1955).

[1] STATISTICA7™ is a registered trademark of StatSoft Inc., Tulsa, OK, USA.

5.5.4 Practical significance

Statistically significant findings were further evaluated for their practical or biological significance, in order to evaluate the importance of the results in practice. The biological significance of statistically significant results supports the clinical importance of the observed relationships between the samples. The validity of statistically significant results can however not be disregarded where effects were too small to demonstrate biological significance. The calculation that was used to determine the biological significance, as defined by Ellis and Steyn (2003), is presented in Equation 5.6.

Equation 5.6 Calculation of biological significance

$$d = \frac{|\bar{x}_1 - \bar{x}_2|}{S_{max}}$$

d = biological significance; $\bar{x}$ = means; S_{max} = maximum standard deviation.

The calculation in Equation 5.6 is not affected by sample size. The p-value determined via statistical significance tests however, are likely to be lower when evaluating data from larger sample sizes. A d-value of more than 0.8 indicates biological significance (Ellis and Steyn, 2003), as calculated and discussed in Chapter Six.

CHAPTER SIX

RESULTS AND DISCUSSION

Research on the glucose curve shape (GCS) as a screening tool for T2D is absent in the black South African population. This study was undertaken to evaluate the association between glucose curve shapes, various phenotypes, OGTT results and specific genotypes in this population in order to contribute to the current understanding of T2D. This chapter describes method optimisation for the collection and analysis of samples in this investigation as well as the generation of the phenotypic and genotypic data. Comparison analyses of the different subgroups as well as a discussion thereof follow.

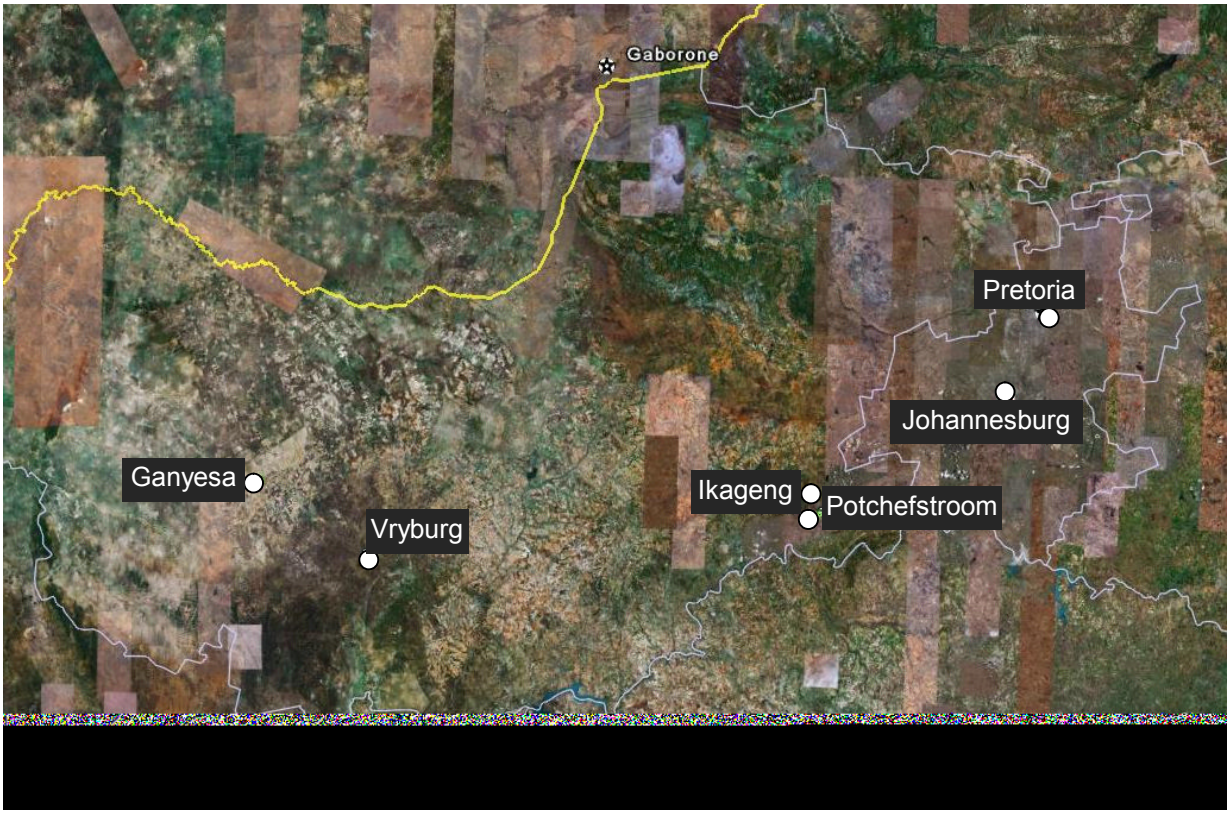
6.1 STUDY DESIGN AND METHOD OPTIMISATION

A description of the participants, their residing environment, the inclusion criteria of the study as well as the sample collection and analysis methods are discussed. The methods used for screening the IRS-1, IRS-2, CAPN10 and PPARγ2 gene alterations are discussed in Chapter Five and the optimisation results are discussed in the sections that follow.

6.1.1 Participants and location

During the PRIMER study, samples and data were collected from 443 “apparently healthy”, fasting black South African individuals. The participants reside in either the rural Ganyesa (250 individuals) or the urban Ikageng (193 individuals) areas. In both of these regions, individuals from two different communities were sampled, which included Ganyesa and Tklagameng in the Ganyesa district and Ikageng and Sonderwater in Potchefstroom. The participants were predominantly Tswana-speaking (76%), as discussed in Section 5.2, and between the ages of 35 and 60 years. Ethical approval was obtained from the Ethics Committee of the North-West University (Potchefstroom Campus), as discussed in Section 5.1. The North-West Province location of the two communities is indicated on the map represented in Figure 6.1. Individuals arriving for the OGTT were excluded from further testing if they were not fasting for at least ten hours or identified to be clinically stressed, defined as harbouring an acute infection or having a chronic disease.

Figure 6.1 Map location of the urban Ikageng and rural Ganyesa communities



The urban Ikageng and rural Ganyesa communities, both situated in the North-West province of South Africa, are indicated on the map. Adapted from Google™ [1] Earth.

6.1.2 DNA extraction

DNA was isolated from 443 participants via the protocol discussed in Section 5.3.1. The average DNA yield was 179 ng.μL^{-1} (50 – 676 ng.μL^{-1}) and the average A260/A280 ratio was 1.73 (1.16 – 1.87), which indicated a sufficient amount of purified DNA, to be used in the subsequent amplification and analysis techniques.

6.1.3 Polymerase chain reaction and restriction fragment length polymorphism

Primers were synthesised from either previously reported or newly designed sequences, as discussed in Section 5.3.2.1. The alterations in the IRS-1, IRS-2, CAPN10 and PPARγ2 genes were screened via PCR and RFLP methods, as discussed in Section 5.3.2. Secondary amplification fragments were however observed when using the standard PCR protocol for the PCR of IRS-2 gene region. The PCR was subsequently optimised by using a FastStart *Taq*® DNA polymerase, which remains inactive at room

[1] Google™ Earth is a registered trademark of Google, Mountain View, CA, USA.

temperature during the set up of the PCR reaction. The activity of this DNA polymerase is only initiated at temperatures above 95°C, resulting in more specific amplification and preventing the formation of secondary structures and primer-dimers (Roche, 2007). A GC-RICH® solution (Roche) was also added to the PCR to increase the sensitivity of amplification. This additive enhanced amplification by altering the melting characteristics of the template but it did not affect the activity of the FastStart *Taq*® DNA polymerase, unlike the reduction in activity observed when using other *Taq* DNA polymerase (Hermann and Foernzler, 2002) with the solution. The FastStart *Taq*® DNA polymerase and GC-RICH® solution therefore improved the efficiency of the PCR of the IRS-2 gene region. The optimised amplification conditions for the four genes that were screened are listed in Table 6.1.

Table 6.1 Optimised amplification conditions for the screening of the IRS-1, IRS-2, CAPN10 and PPARγ2 gene alterations

Gene	Alteration	Primer set	$T_{a(opt)}$
IRS-1	G3494A	F and R	70°
IRS-2	G3684A	F and R	54°
CAPN10	T22751C	F and R	62°
PPARγ2	C8492G	F and R	58°

IRS-1 = insulin receptor substrate-1; IRS-2 = insulin receptor substrate-2; PPARγ2 = peroxisome proliferator-activated gamma 2; CAPN10 = calpain 10; G = guanine; A = adenine; T = thymine; C = cytosine; F = forward primer; R = reverse primer; $T_{a(opt)}$ = optimised annealing temperature for each primer set.

An RFLP technique with the appropriate restriction endonuclease was used for the digestion of the specific amplified regions harbouring the alterations of interest in the CAPN10, IRS-1, IRS-2 and PPARγ2 genes respectively. The sequence of the regions harbouring the alteration in both the PPARγ2 and CAPN10 genes were not recognised by commercially available restriction endonucleases and a restriction site was therefore created during amplification via ACRS, as discussed in Section 5.3.2. The digested products were evaluated via agarose gel electrophoresis (see Section 5.3.2) and the respective optimised RFLP conditions are listed in Table 6.2, including the optimised RFLP temperatures and digestion times.

Table 6.2 Optimised RFLP conditions as used for the screening of the IRS-1, IRS-2, CAPN10 and PPARγ2 gene alterations

Gene	Alteration	Digest enzyme	RFLP temp	RFLP time°
IRS-1	G3494A	*Bst* NI	60°	1 hour
IRS-2	G3684A	*Sma* I	25°	1 hour
CAPN10	T22751C	*Bst* UI	60°	1 hour
PPARγ2	C8492G	*Hha* I	37°	1 hour

IRS-1 = insulin receptor substrate-1; IRS-2 = insulin receptor substrate-2; PPARγ2 = peroxisome proliferator-activated gamma 2; CAPN10 = calpain 10; RFLP = restriction fragment length polymorphism; G = guanine; A = adenine; T = thymine; C = cytosine; temp = temperature.

The expected fragment sizes of the amplified products subsequent to performing the RFLP procedure, as discussed in Section 5.3.2, are listed in Tables 5.3, 5.6, 5.9 and 5.12 for the IRS-1, IRS-2, CAPN10 and PPARγ2 genes respectively. The resultant RFLP fragments were separated via agarose gel electrophoresis as discussed in Section 5.3.3. The sizes of the observed fragments were compared to the expected fragment sizes and molecular weight markers as indicated in Section 5.3.2.

6.1.4 Agarose gel electrophoresis

Successful amplification was determined by separation of the amplicons via agarose gel electrophoresis, as discussed in Section 5.3.4, and visualisation using an EtBr-based detection system. Genotypes were determined by evaluating the separated RFLP fragments on an agarose gel and comparing the sizes of the observed fragments to that of expected sizes, as discussed in Section 5.3.2.

6.1.4.1 Artefacts observed in agarose gels

A smear, as indicated by the single asterisk (*) in Figure 6.2, Figure 6.4, Figure 6.7 and Figure 6.9 was sometimes observed on the gels. The smears were due to background amplification (Sambrook and Russell, 2001) that can be avoided by further optimisation of the PCR. Varied intensities of the smears were observed for different samples. This was due to the fact that the PCR and RFLP products originated from different individuals and that these samples had different PCR efficiencies. Even though DNA was purified and diluted to equal concentrations, variations in the purified product will inevitably be expected. The smears did however not affect visualisation of the products of interest when analysing the PCR and RFLP products.

Varying degrees of primer-dimer formation, as indicated by the two asterisks (**) in Figure 6.2, Figure 6.7 and Figure 6.9 were observed for the different samples loaded on the gels. The variation in primer-dimer formation as well as in fragment intensity observed on these gels was due to the variation in PCR efficiency (Sambrook and Russell, 2001) between the samples. Further optimisation of the PCR could have been achieved by decreasing the primer concentrations. However, as was the case with the smear artefacts, these formations did not interfere with the visualisation of the PCR products of interest.

The three asterisks (***) indicated in Figure 6.2, Figure 6.4, Figure 6.7 and Figure 6.9 designate artefacts observed as white spots, which were present on the gels due to lint from the paper towels used to clean the gel casting trays. These white spot artefacts were set in the gel itself and were not a result of the amplification or RFLP techniques. In future, it would be recommended that lint-free towels be used to clean the casting tray. None of the PCR and RFLP products observed in this study were interfered with by any of these artefacts.

The faint 535 bp fragment observed in lane 3 of Figure 6.4 is present due to incomplete digestion of the PCR product, which resulted in the more intense 348 bp fragment when compared to the expected equal intensity 294 bp fragments on this gel. Increasing the time at the optimised temperature for restriction enzyme digestion would ensure complete digestion and avoid the presence of this fragment. Nonetheless, this did not affect interpretation of the resultant IRS-2 RFLP products, since the restriction enzyme consistently digested the PCR product into distinguishable fragment sizes irrespective of whether the polymorphism was present or not.

Overloading of the molecular weight marker (lane M) resulted in uneven fragment shapes in lane M of the gel observed in Figure 6.4. Although the different fragment sizes could still be observed, loading a lesser amount of the marker would result in more clearly defined fragments.

6.1.5 Automated cycle sequence analysis

Successful amplification, as discussed in Section 5.3.2, was determined by separation of the amplicons via agarose gel electrophoresis prior to automated cycle sequence analysis. Accurate amplification as well as RFLP results of control individuals was subsequently

confirmed via automated cycle sequence analysis for each of the four genes, according to the protocol discussed in Section 5.3.4. Confirmation sequencing was used to verify the status of each of the respective homozygous WT, heterozygous and homozygous mutant genotypes, as determined by the RFLP procedures. Following purification of the PCR product, as discussed in Section 5.3.4, the concentration of the purified products was determined via spectrophotometry. The average concentration for all the purified PCR products was approximately 9 $ng.\mu L^{-1}$. Automated cycle sequence analysis was subsequently performed according to the protocol described in Section 5.3.4. Sequences were aligned to the respective reference sequences that were retrieved from Genbank, as listed in Section 5.3. Sequence alignments were performed via the BioEdit Sequence Alignment Editor Version 5.0.9 programme, as discussed in Section 5.3.4.

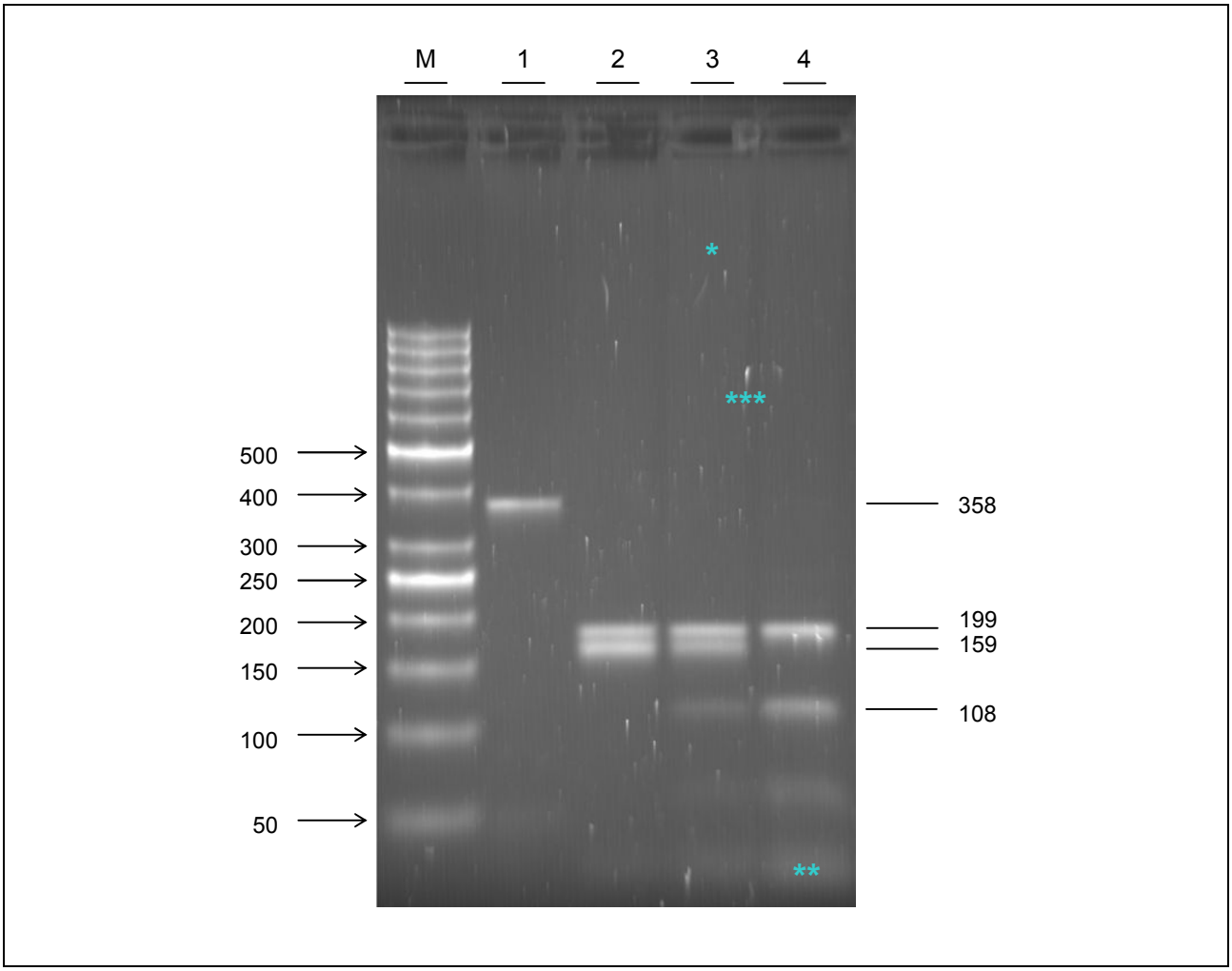
6.1.6 Mutation analyses

The four alterations were screened via PCR and RFLP methods as discussed in Section 5.3.2 and the resultant RFLP fragments separated via agarose gel electrophoresis. Sequences were confirmed via automated cycle sequence analysis, as discussed in Section 5.3.4. Photographic representations of the agarose gels as well as the electropherograms of the control DNA sequences are presented and discussed in the sections that follow.

6.1.6.1 Insulin receptor substrate-1 gene

Amplification of the region containing the G3494A alteration in the IRS-1 gene was performed via the PCR protocol discussed in Section 5.3.2. The primer set that was used for amplification is presented in Table 5.5. The $T_{a(opt)}$ for this reaction was optimised at 70°C. The expected fragment sizes subsequent to the RFLP procedure discussed in Section 5.3.2 are presented in Table 5.6. The digestion reaction with *Bst* NI was optimised at 60°C for a period of an hour. A PCR product as well as the resulting RFLP products from individuals harbouring a homozygous WT, heterozygous or homozygous mutant genotype, is presented in Figure 6.2. As anticipated, the small 51 bp fragment expected for individuals harbouring the heterozygous or homozygous mutant genotype could not be visualised on the agarose gel. This fragment was assumed to be present based on the visualisation of the 108 bp fragment, indicating the presence of the A allele at position 3494 in the DNA sequence.

Figure 6.2 Photographic representation of the amplification and RFLP products of the G3494A alteration within the IRS-1 gene

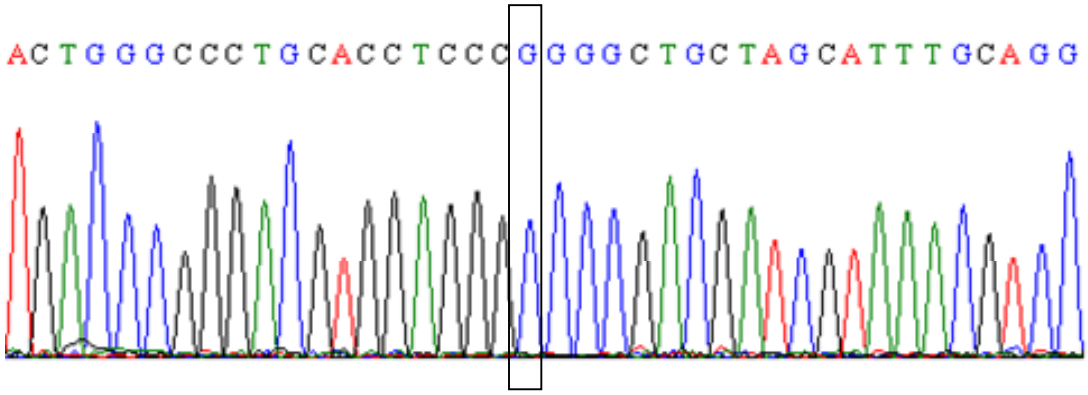


IRS-1 = Insulin receptor substrate-1 gene; RFLP = restriction fragment length polymorphism; M = 50 base pair molecular weight marker; 1 = sample of individual # 3084, undigested amplification product; 2 = sample individual # 3010, homozygote wild type genotype; 3 = sample of individual # 3023, heterozygote genotype; 4 = sample of individual # 3006, homozygote mutant genotype; * = grey smear artefact; ** = primer-dimer formation; *** = white spot artefact. All fragment sizes are indicated in base pairs.

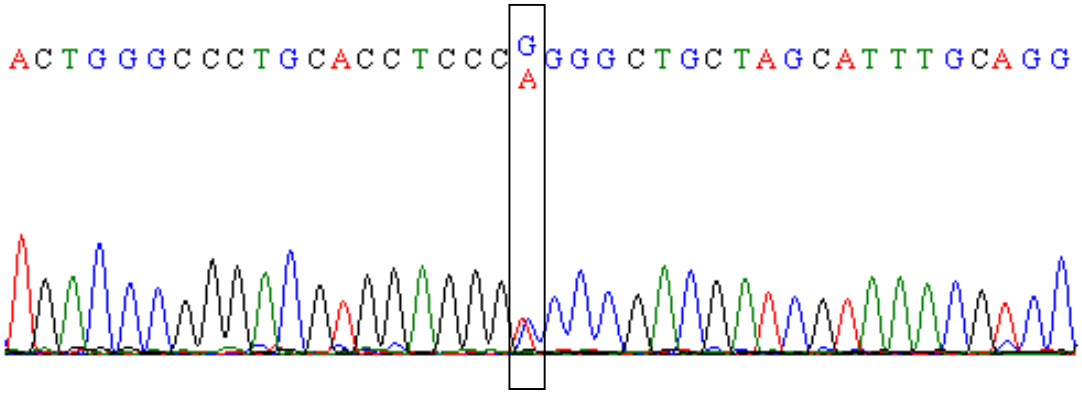
As confirmation of the RFLP analyses, DNA sequences were determined via automated cycle sequence analysis from three individuals that were either homozygous for the WT allele, heterozygous or homozygous for the variant allele. Examples of the sequences generated in the study for the region containing the G3494A alteration in the IRS-1 gene are represented in Figure 6.3.

Figure 6.3 Representative electropherograms of the gDNA sequence encompassing the G3494A alteration in the IRS-1 gene

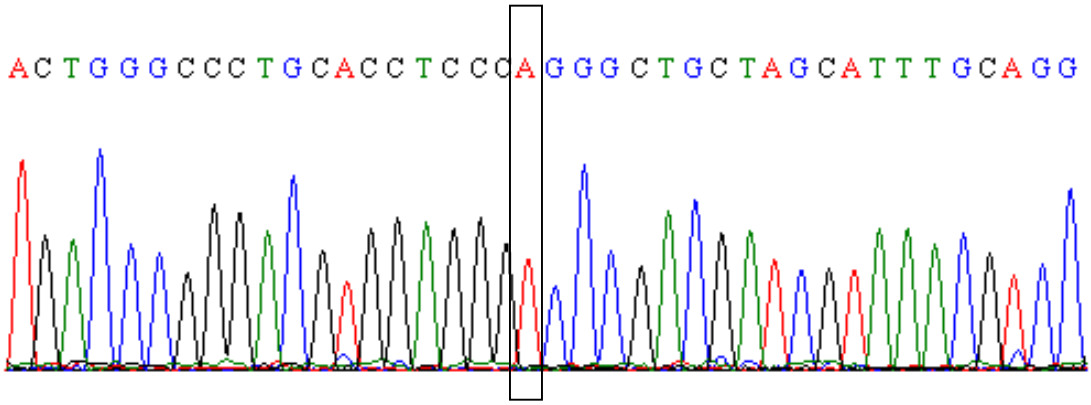
A. Sample of individual # 5008, homozygous WT genotype



B. Sample of individual # 2021, heterozygous genotype



C. Sample of individual # 2103, homozygous mutant genotype



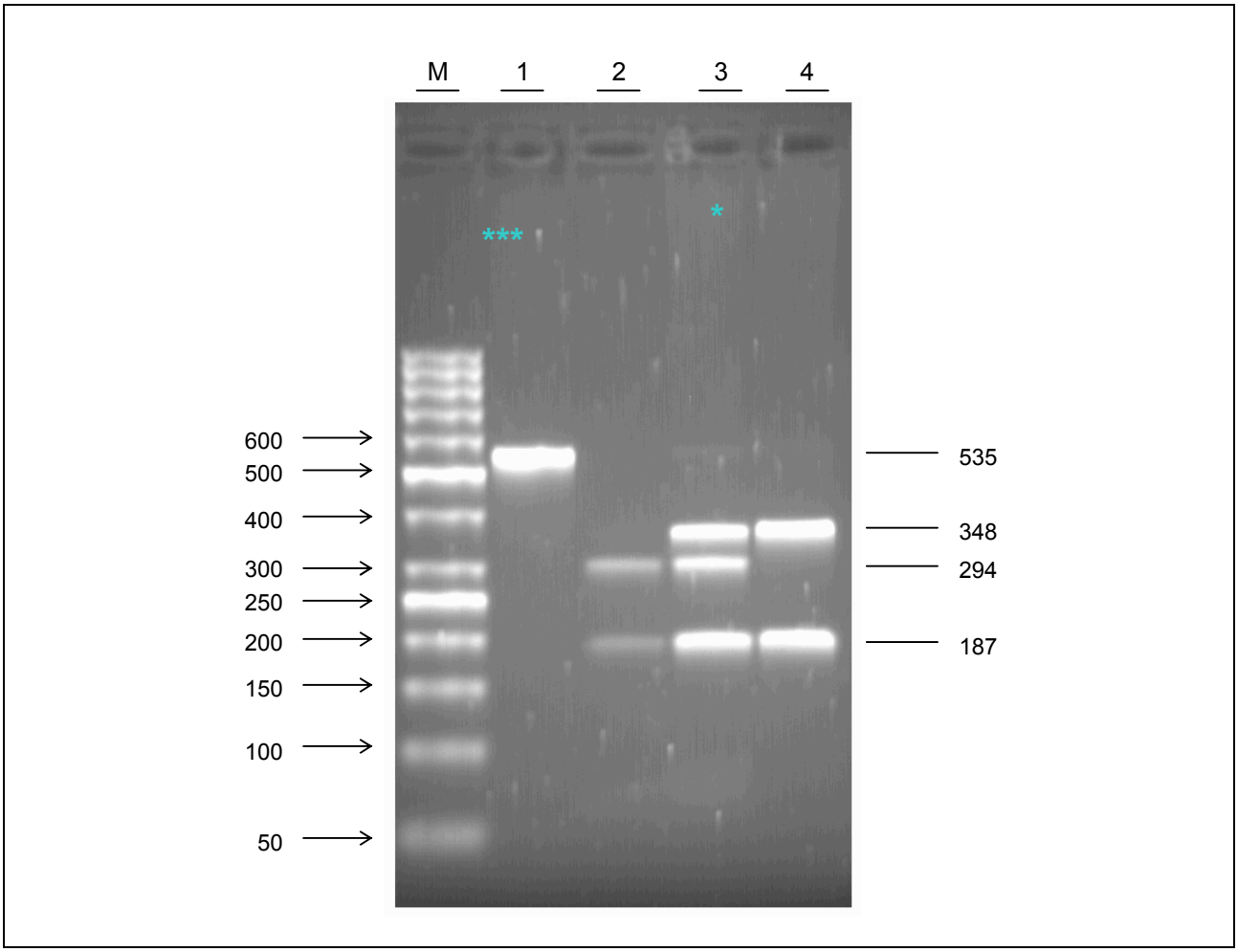
WT = wild type; A = adenine; C = cytosine; G = guanine, T = thymine. The text inside the textbox (▯) indicates the alteration.

The electropherograms in Figure 6.3 indicate DNA sequences of the regions encompassing the investigated alteration in the IRS1 gene, including samples from individuals harbouring the homozygous WT, heterozygous or homozygous mutant genotypes, respectively. The position of the G3494A alteration in the DNA sequence is indicated via the textbox.

6.1.6.2 Insulin receptor substrate-2 gene

The G3684A alteration in the IRS-2 gene was detected via the PCR and RFLP procedures described in Section 5.3.2. The $T_{a(opt)}$ of the PCR was 54°C and the primer set used for amplification is presented in Table 5.8. The fragment sizes that were expected subsequent to the digestion reaction are presented in Table 5.9. The RFLP was performed with *Sma* I and digestion was optimised at 25°C for an hour. The resulting RFLP products of individuals harbouring an IRS-2 homozygous WT, heterozygous and homozygous mutant genotype are represented in Figure 6.4. Included in the figure, is the amplification product of a sample from an individual harbouring the IRS-2 G3684A alteration.

Figure 6.4 Photographic representation of the amplification and RFLP products of the G3684A alteration within the IRS-2 gene



IRS-2 = Insulin receptor substrate-2 gene; RFLP = restriction fragment length polymorphism; M = 50 base pair molecular weight marker; 1 = sample of individual # 2096, undigested amplification product; 2 = sample of individual # 5119, homozygote wild type genotype; 3 = sample of individual # 4075, heterozygote genotype; 4 = sample of control individual, homozygote mutant genotype; * = grey smear artefact; *** = white spot artefact. All fragment sizes are indicated in base pairs.

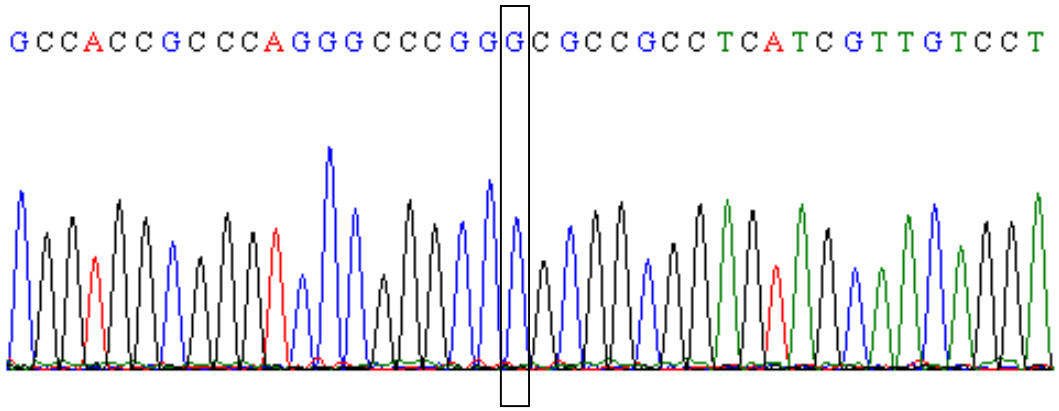
The 54 bp fragment that was expected for individuals with a heterozygous or homozygous wild type genotype was assumed to be present, although it was not detected on the

agarose gel due to its small size. The assumption was based on the clear visualisation of the 294 bp and 187 bp fragments, as would be expected to be present with the 54 bp fragment on accurate digestion.

The presence of the G3684A alteration in the IRS-2 gene was determined via RFLP. Positive results were confirmed via automated cycle sequence analysis. Electropherogram excerpts of the interested IRS-2 gene regions are presented in Figure 6.5 and Figure 6.6.

Figure 6.5 Representative electropherograms of the gDNA sequence encompassing the G3684A alteration in the IRS-2 gene

A. Sample of control individual, homozygous WT genotype



B. Sample of control individual, heterozygous genotype

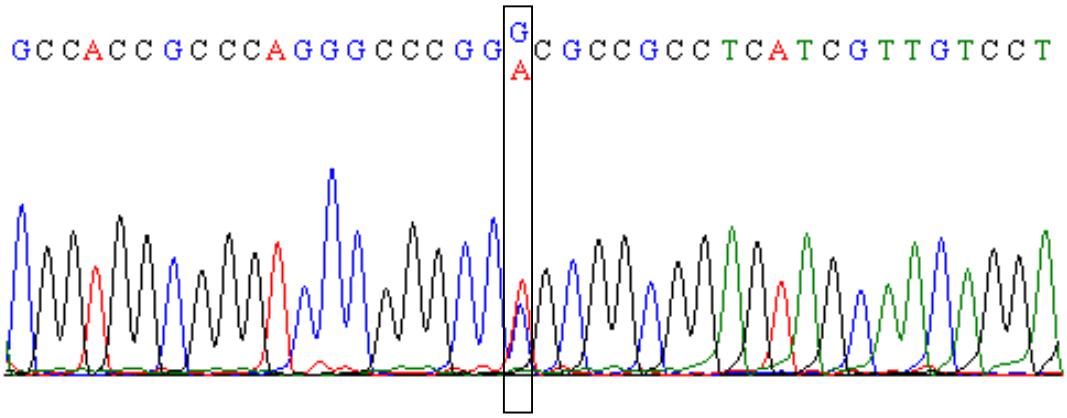
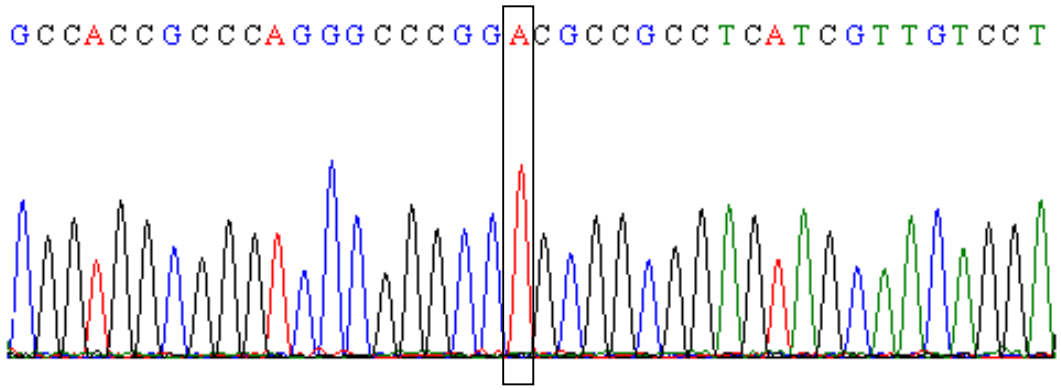


Figure 6.6 Representative electropherograms of the gDNA sequence encompassing the G3684A alteration in the IRS-2 gene

C. Sample of control individual, homozygous mutant genotype



WT = wild type; A = adenine; C = cytosine; G = guanine, T = thymine. The text inside the textbox (□) indicates the alteration.

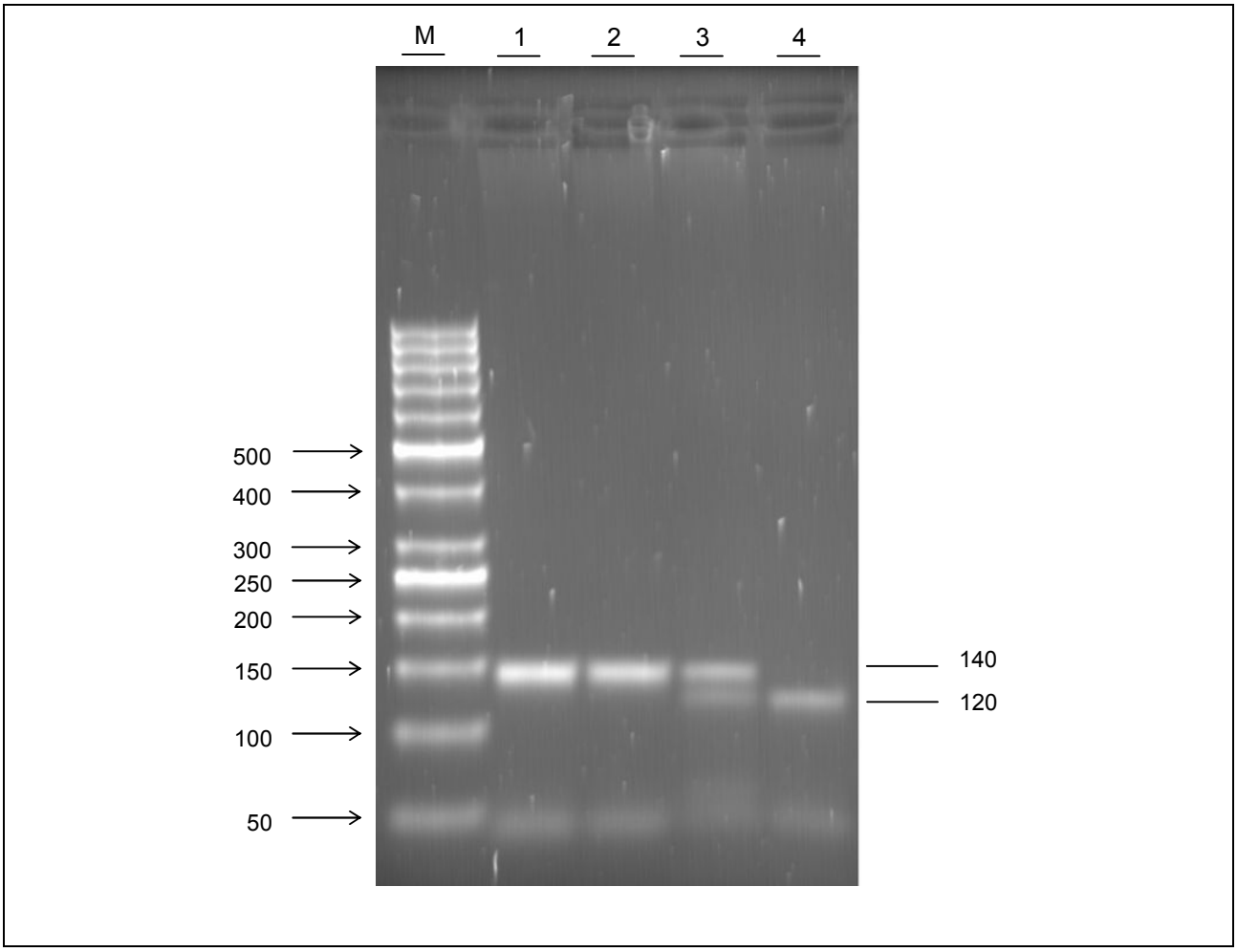
Figure 6.5 indicates examples of the three possible IRS-2 genotypes, including the homozygous WT, heterozygous and homozygous mutant genotypes. The textbox in the figure points out the investigated G3684A alteration in the IRS-2 gene.

6.1.6.3 Calpain 10 gene

Amplification of the region containing UCSNP44 within the CAPN10 gene was performed via the PCR and RFLP procedures that are described in Section 5.3.2. The $T_{a(opt)}$ for the amplification reaction was 62°C and the primer set that was used is indicated in Table 5.2. The fragment sizes that are expected subsequent to the RFLP procedure are presented in Table 5.3. The RFLP was performed with *Bst* UI enzyme, allowing digestion for one hour at 60°C. The RFLP products of samples from individuals harbouring a CAPN10 homozygous WT, heterozygous or homozygous mutant genotype are represented in Figure 6.7. In addition, an undigested amplification product of an individual harbouring the CAPN10 gene alteration is represented in lane 1 of this figure.

The small 20 bp fragment that was expected for individuals with a heterozygous or homozygous mutant genotype could not be detected on the agarose gel due to the small fragment size as well as the one hour run time of the gel required for the appropriate separation of the 140 bp and 120 bp fragments. The presence of this fragment was however assumed in accordance with the sizes of the larger fragments that were visualised on the gel.

Figure 6.7 Photographic representation of the amplification and RFLP products of UCSNP44 within the CAPN10 gene

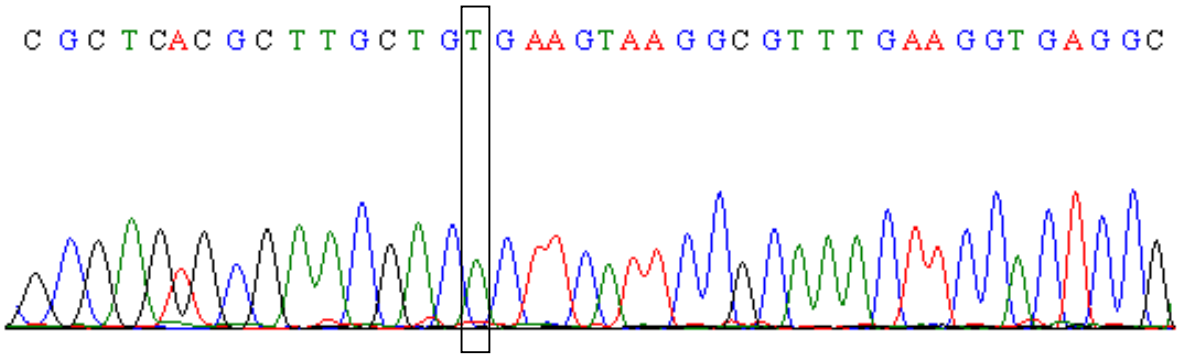


CAPN10 = calpain-10 gene; RFLP = restriction fragment length polymorphism; M = 50 base pair molecular weight marker; 1 = sample of individual # 5110, undigested amplification product; 2 = sample of individual # 5116, homozygote wild type genotype; 3 = sample of individual # 4053, heterozygote genotype; 4 = sample of individual # 5001, homozygote mutant genotype; * = grey smear artefact; ** = primer-dimer formation; *** = white spot artefact. All fragment sizes are indicated in base pairs.

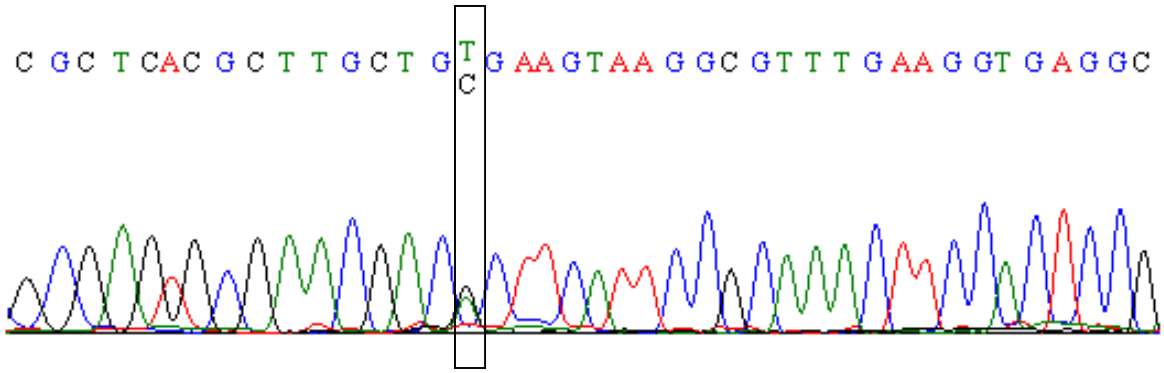
Representative electropherograms for the DNA sequence encompassing UCSNP44 of samples from individuals with homozygous WT, heterozygous or homozygous mutant genotypes are represented in Figure 6.8. Presence of the SNP was determined via the RFLP procedure discussed in Section 5.3.2 and confirmed by automated cycle sequence analysis, as discussed in Section 5.3.4.

Figure 6.8 Representative electropherograms of the gDNA sequence encompassing UCSNP44 in the CAPN10 gene

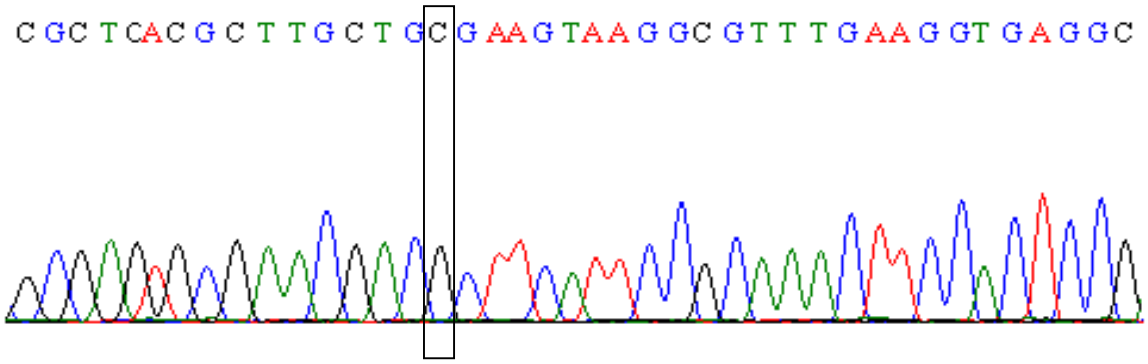
A. Sample of individual # 5008, homozygous WT genotype



B. Sample of individual # 5014, heterozygous genotype



C. Sample of control individual, homozygous mutant genotype



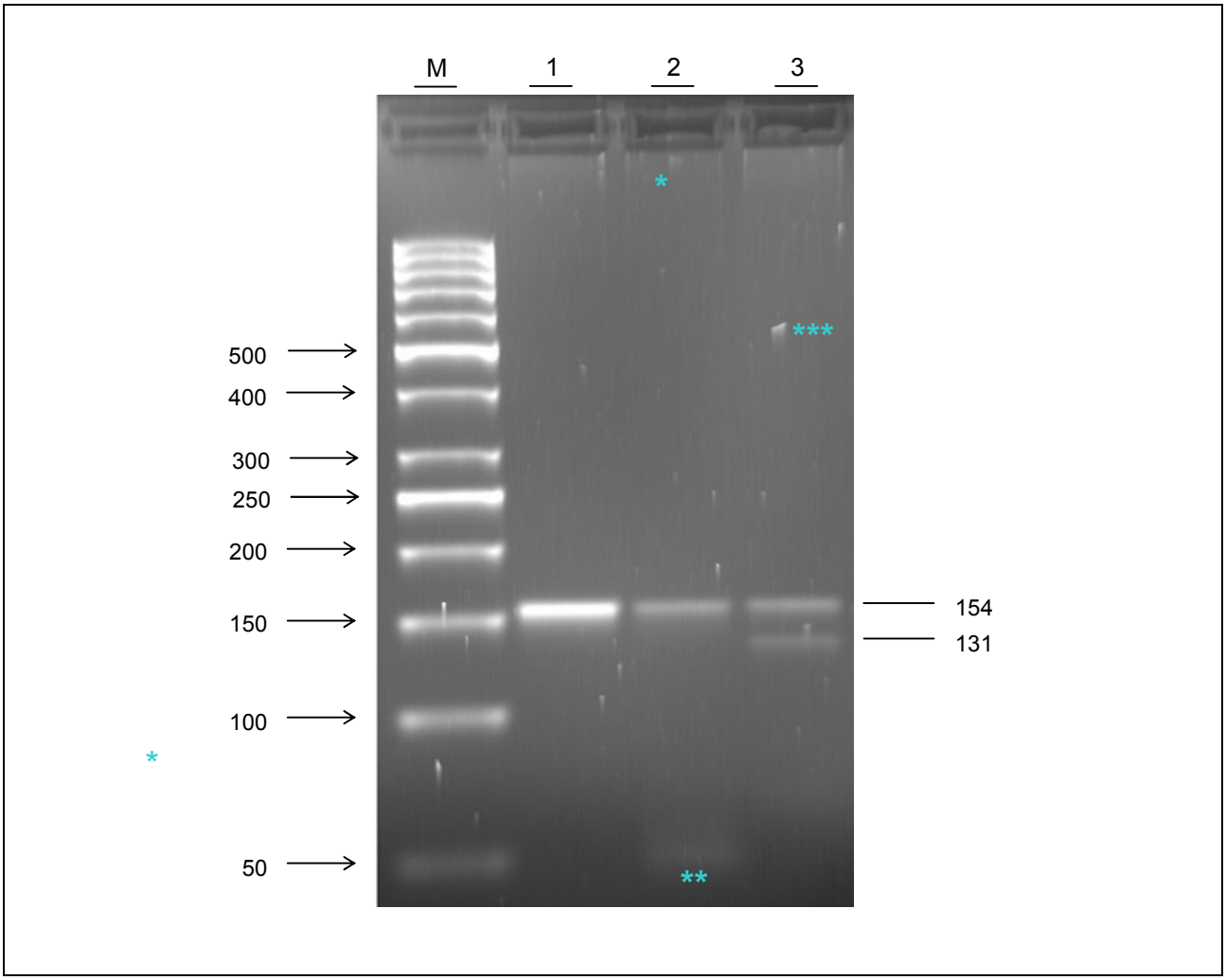
WT = wild type; UCSNP44 = University of Chicago single nucleotide polymorphism 44; A = adenine; C = cytosine; G = guanine, T = thymine. The text inside the textbox (▯) indicates the alteration.

The textboxes in Figure 6.8 indicate the position of UCSNP44 in the investigated CAPN10 gene. The electropherogram examples include DNA sequences from participants harbouring either the homozygous WT or heterozygous genotype as well as the DNA sequence from a control individual harbouring the homozygous mutant genotype.

6.1.6.4 Peroxisome proliferator-activated gamma 2 gene

Screening for the C8492G alteration in the PPARγ2 gene was performed via the PCR and RFLP strategies that are discussed in Section 5.3.2.5. The primer set that is presented in Table 5.10 was used for the amplification of this region and the $T_{a(opt)}$ for the reaction was determined at 58°C. The expected fragment sizes following RFLP digestion with *Hha* I at 37°C for one hour are presented in Table 5.12. Figure 6.9 presents a gel image of the resultant RFLP products of individuals harbouring either a PPARγ2 homozygous WT, heterozygous or homozygous mutant genotype. Included in the figure is an undigested amplification product of the PPARγ2 C8492G region of interest.

Figure 6.9 Photographic representation of the amplification and RFLP products of the C8492G alteration within the PPARγ2 region



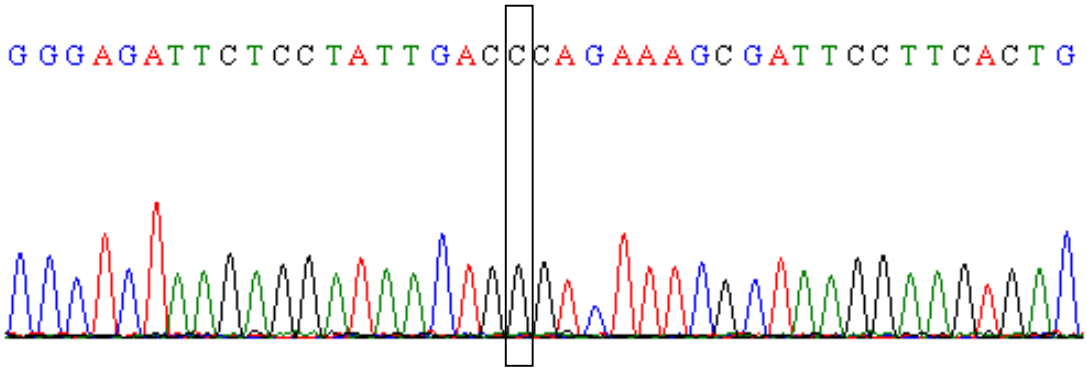
PPARγ2 = Peroxisome proliferator-activated receptor gamma 2; RFLP = restriction fragment length polymorphism; M = 50 base pair molecular weight marker; 1 = sample of individual # 2020, undigested amplification product; 2 = sample of individual # 2020, homozygote wild type genotype; 3 = sample of individual # 2045, heterozygote genotype; * = grey smear artefact; ** = primer-dimer formation; *** = white spot artefact. All fragment sizes are indicated in base pairs.

The DNA sequence of the region encompassing the C8492G alteration in the PPARγ2 gene, as determined via the RFLP procedure, was confirmed by using automated cycle

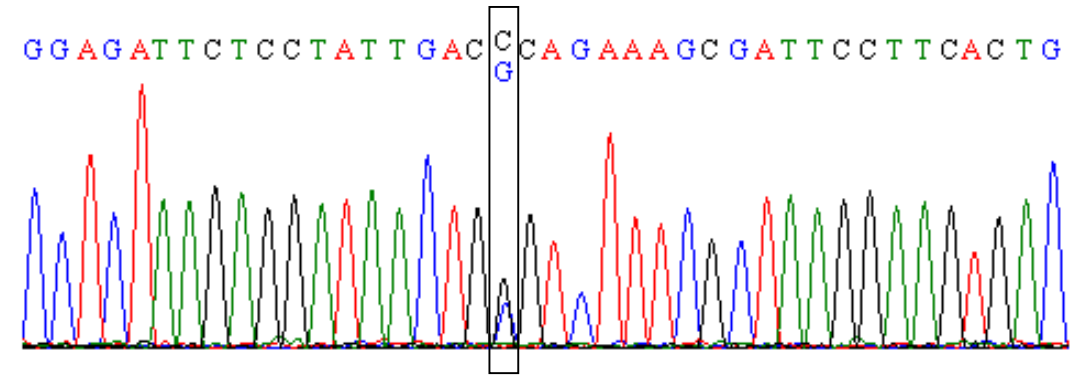
sequence analysis. The representative electropherograms of individuals with the C8492G alteration in the PPARγ2 gene are depicted in Figure 6.10.

Figure 6.10 Representative electropherograms of the gDNA sequence encompassing the C8492G alteration in the PPARγ2 gene

A. Sample of individual # 2020, homozygous WT genotype



B. Sample of individual # 2045, heterozygous genotype



WT = wild type; A = adenine; C = cytosine; G = guanine, T = thymine. The text inside the textbox (□) indicates the alteration.

Figure 6.10 represents electropherograms of two individuals harbouring either the homozygous WT or heterozygous genotype. The C8492 alteration in the PPARγ2 gene sequences is indicated via the textbox.

6.2 DATA AND STATISTICAL ANALYSES

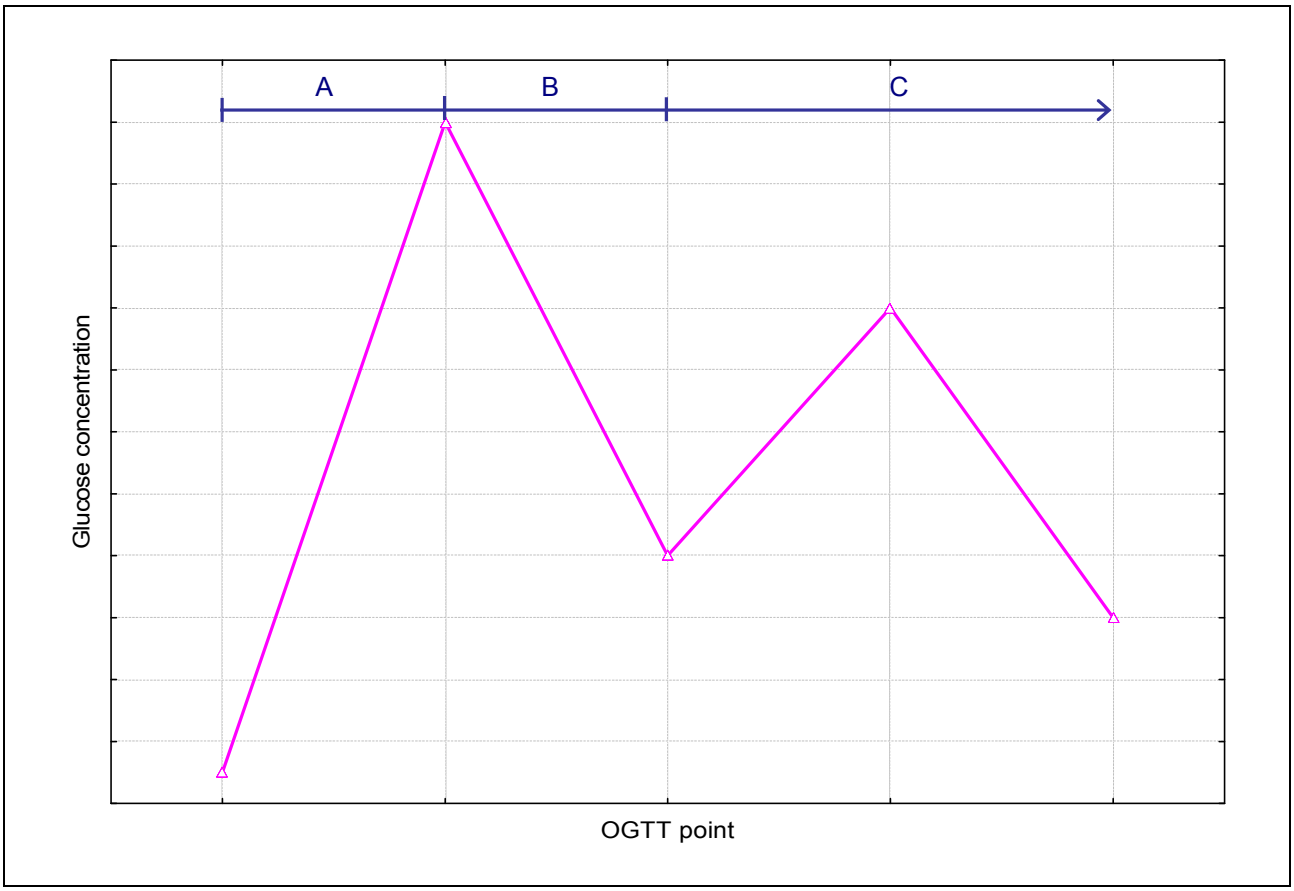
Descriptive statistical analysis was performed on all continuous biochemical and clinical data. The Shapiro-Wilk's test, as discussed in Section 5.5.2, was performed to determine if the continuous data adhered to a normal distribution. A significant SW p-value indicates that the data is not normally distributed and should hence be analysed via non-parametric methods as discussed in Section 5.5.2. Conversely, a non-significant SW p-value indicates a normal distribution of data to be analysed by parametric methods, as

discussed in Section 5.5.2. Biological significance of all statistically significant results was determined via Equation 5.5.

6.2.1 Glucose curve shape

The study evaluated the classification of the GCS and its potential use in screening for T2D in the black South African population. Research on the GCS, as determined by a five point OGTT has not been reported to date in this population. Tschritter *et al*. (2003) observed a significant correlation in a non-T2D Caucasian cohort between the GCS, female gender and specific genotypes, as discussed in Section 3.1.2.2. The frequencies of the GCSs that were observed within the investigated T2D susceptibility gene subgroups are presented and discussed in Section 6.2.4. The GCS, as derived from the glucose values during a two hour OGTT, measures the body's physiological response to a glucose load. Preceding an OGTT, an exogenous glucose load is ingested, explaining the initial rise in plasma glucose concentration, as indicated by timeline A in Figure 6.11.

Figure 6.11 Graphic description of the glucose curve shape during a two hour OGTT



OGTT = oral glucose tolerance test.

Timeline B, as indicated in Figure 6.11, depicts the period between reaching the maximum glucose level after the oral glucose load and the resulting hypoglycaemia due to normal insulin action (Trujillo-Arriaga and Román-Romas, 2008).

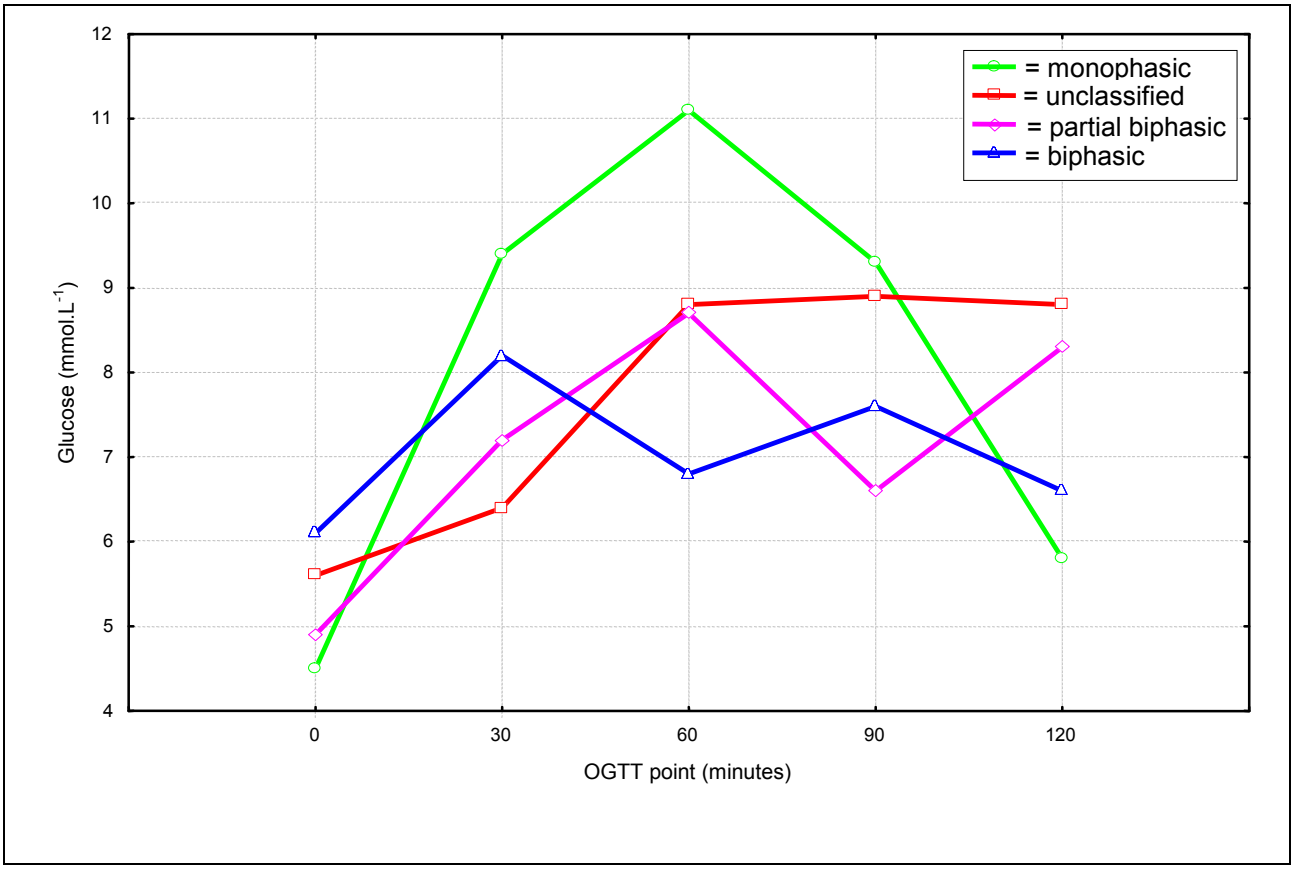
The insulin action is however affected by various factors such as gender differences in terms of body composition (fat versus muscle) as well as hormonal status (Fuchigami *et al.,* 1994). The rest of the curve, indicated by timeline C, is a result of a combination of endogenous stimuli that include the gastric emptying rate and intestinal motility that determine the rate of glucose absorption, elimination as well as the cessation of liver gluconeogenesis (Dedik *et al*., 2007). When a solid meal reaches the stomach of a non-diabetic individual, no gastric emptying is observed within the first 30 minutes, after which there is a linear increase in emptying (Schirra *et al.,* 1996). The studied individuals however, were fasting for ten hours prior to the consumption of water-dissolved glucose. Stomach emptying depends on both volume and composition of ingested matter and therefore, with the entering of only liquid into the stomachs of the participants, the solid-food-stimulus was absent and gastric emptying delayed. Emptying of the stomach would however increase at an exponential rate with an increase in volume size, subsequent to the initial delay period. The intake of high concentrations of glucose would further slow down emptying of the stomach (Schirra *et al.,* 1996). The fact that the individuals were in a supine position during the test, would cause the ingested glucose drink to redistribute into the proximal stomach, stimulating chemoreceptors and thus resulting in an auxiliary delayed stomach emptying (Feinle *et al.,* 1999). These factors will be involved in the GCS of all individuals undertaking an OGTT under these same conditions.

Jones *et al.* (2001) observed a slower stomach emptying rate in non-diabetic females ($p < 0.001$) compared to males, following both solid and liquid meals. No correlation was observed between the stomach emptying rate and BMI, age or plasma glucose. Significant reduction in the velocity of gastric emptying was observed when taking stomach bloating, female gender and plasma glucose collectively into consideration. These results were however in contrast to the findings by Tschritter *et al.* (2003), where an association of the biphasic curve shape with non-T2D females was demonstrated. However, as the study cohort ($n = 443$) consisted mostly of females (63%), further investigation into gastric emptying is suggested in order to evaluate the association between a slow stomach emptying rate, female gender and a monophasic GCS. Fuchigami *et al.* (1994) suggested that the GCS is primarily a result of early insulin

response and concluded that an “upward” and “domed” (monophasic) shape in T2D Caucasian individuals was more prevalent than a “biphasic” shape (Section 3.1.2.2).

The GCS following an OGTT thus depends on various processes. Upon plotting the OGTT data of the studied cohort, various GCS were observed. Unambiguous classification of the GCSs was not possible without stringent criteria and was therefore classified according to that described by Tschritter *et al.* (2003), as discussed in Section 5.4.6. An example of each of the GCSs is presented in Figure 6.12. The decision to use these criteria was to enable accurate comparison between the results of this study, including black South Africans, and the results reported in non-Africans by Tschritter *et al.* (2003).

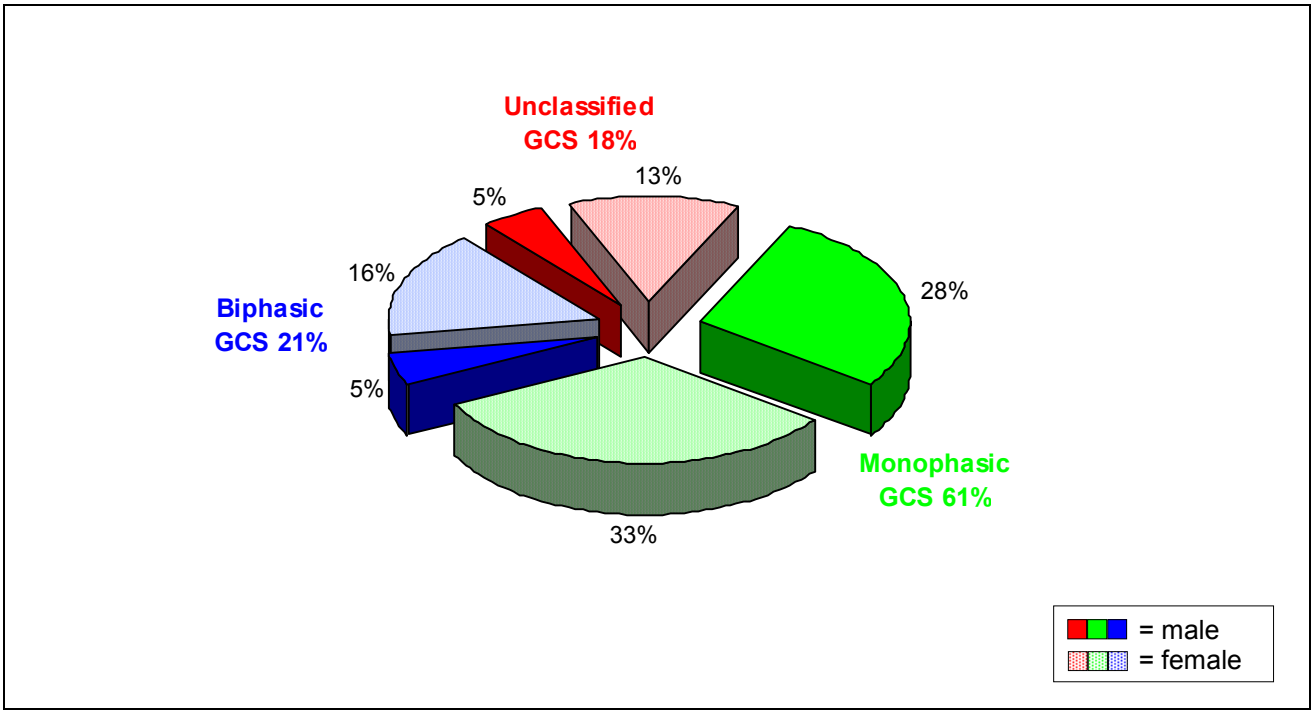
Figure 6.12 Examples of the glucose curve shapes as observed within the investigated cohort



OGTT = oral glucose tolerance test; mmol.L^{-1} = millimole per litre.

The number of GCSs according to category and gender subgroup is depicted in Figure 6.13. A biphasic GCS was observed in 21% of the cohort, of which 21 were male and 71 were female participants. The monophasic curve shape subgroup included 123 male and 149 female individuals, constituting 61% of the cohort. The residual 18% were unclassified curve shapes and comprised of 21 males and 58 females.

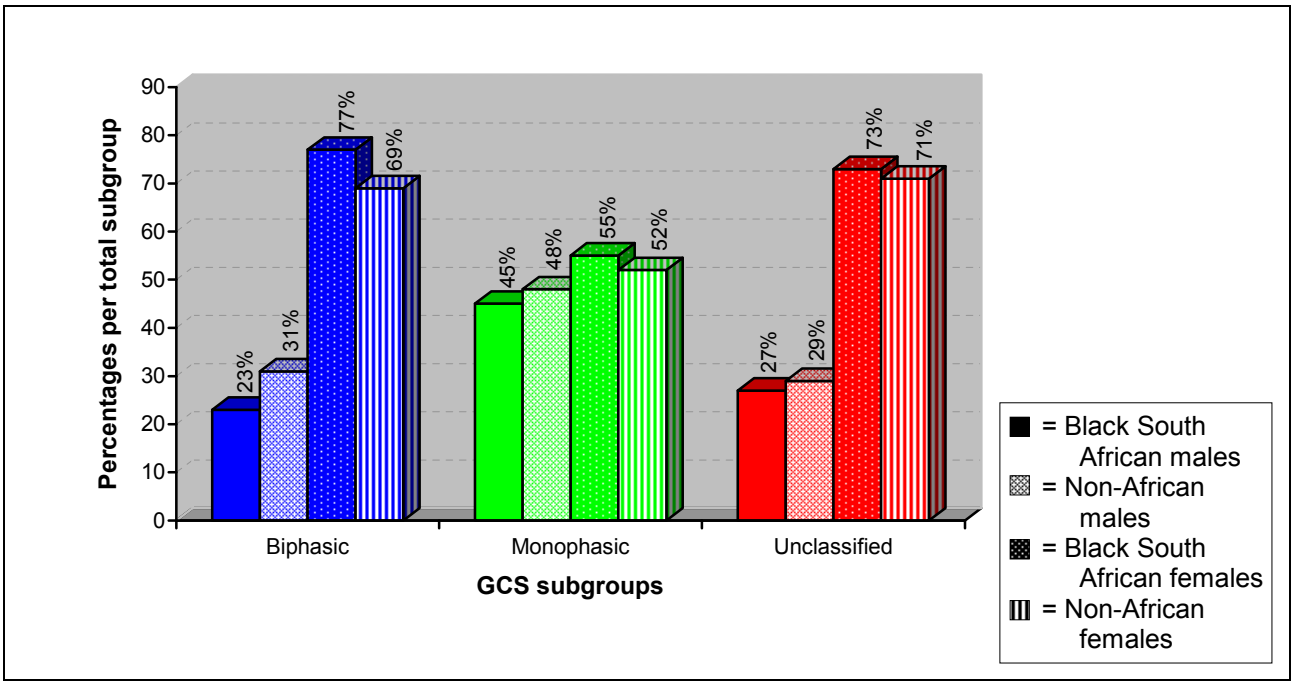
Figure 6.13 Percentages observed in each of the GCS subgroups



Percentages calculated from the total study cohort number (443). GCS = glucose curve shape; % = percentage.

The gender percentage distributions in the three investigated black South African GCS subgroups were similar when compared to those reported by Tschritter *et al.* (2003) in non-Africans. Both the investigated and reported percentages are graphically presented in Figure 6.14.

Figure 6.14 Percentages observed in each of the GCS subgroups in both the investigated black South African and reported non-African cohorts



Black South African percentages as observed within the studied cohort; Non-African percentages as reported by Tschritter *et al.* (2003). GCS = glucose curve shape; % = percentage.

As depicted in Figure 6.14, 77% of the South African individuals included in the biphasic GCS subgroup were female and 23% male. This finding is in accordance with the reported non-African gender percentage distribution for individuals with a biphasic GCS (Tschritter *et al.*, 1993). A significant association was also reported between the non-African female gender and a biphasic GCS and this was evaluated in the investigated cohort. The contingency table results are listed in Table 6.3.

Table 6.3 Contingency table summary for the association between GCS and gender

Observed numbers	Female	Male	Total	p-value
Biphasic	71	21	92	0.000
Monophasic	149	123	272	
Total	**220**	**144**	**364**	
OR (95% CI)	2.79 (1.62 – 4.80)			

A contingency table with two-tailed Fisher exact p-value as well as odds ratio and 95% confidence interval were determined. IGT = impaired glucose tolerance; NGT = normal glucose tolerance; OR = odds ratio; CI = confidence interval; CGS = glucose curve shape. The p-values indicated in red text are significant at a significance level < 0.05.

The OR and significant Fisher exact p-value indicate that there was an almost three fold increased chance for being female when harbouring a biphasic GCS. This result was also statistically significant ($p < 0.001$) and therefore supports the reported association between female gender and a monophasic GCS as determined in non-Africans (Tschritter *et al.*, 1993).

Descriptive analyses were performed on all phenotypic and OGTT data and the clinical parameters compared between the biphasic, monophasic and unclassified shape subgroups via a K-W test, as discussed in Section 5.5.2. The results are presented in Table 6.4. A statistically significant increase in the 30, 60, and 90 min glucose levels was observed when comparing the monophasic GCS subgroup to the other two GCS subgroups. This is in support of the findings reported by Tschritter *et al.* (1993) in non-Africans, where the monophasic GCS was associated with impaired glucose regulation. In the latter publication, the monophasic GCS was also demonstrated to in association with the homozygous UCSNP44 T allele in the CAPN10 gene. This genotype and GCS association in the black South African population is discussed further in Section 6.2.4.3.

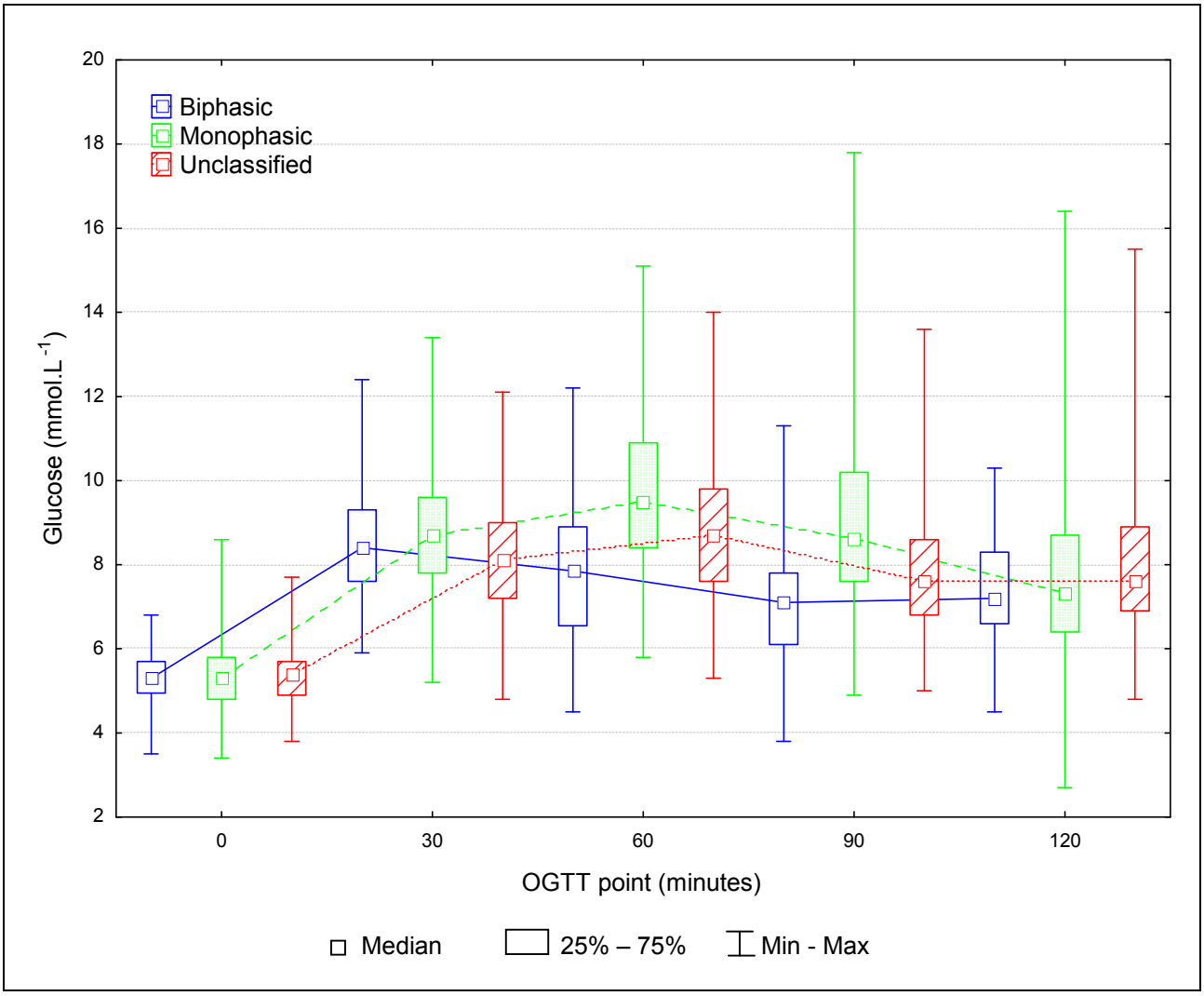
Table 6.4 Clinical parameters stratified according to glucose curve shape

Parameter	Biphasic GCS		Monophasic GCS		Unclassified GCS		K-W p-value
	SW p-value	Median (IQR)	SW p-value	Median (IQR)	SW p-value	Median (IQR)	
Number (M/F)	--	92 (21 / 71)	--	272 (123 / 149)	--	79 (21 / 58)	--
Age (years)	0.097	41.0 (39.0 – 45.0)	0.000	42.0 (38.0 – 47.0)	0.002	42.0 (38.0 – 46.0)	0.649
Weight (kg)	0.001	59.0 (50.0 – 73.0)	0.000	58.0 (52.0 – 67.0)	0.000	63.0 (52.0 – 76.0)	0.298
Height (cm)	0.128	159.5 (155.0 – 165.0)	0.001	162.0 (157.0 – 169.0)	0.142	161.0 (154.0 – 166.0)	0.005
BMI ($kg.m^{-2}$)	0.000	22.8 (18.8 – 29.4)	0.000	21.5 (19.0 – 26.5)	0.000	24.1 (19.8 – 29.1)	0.039
0' Glucose ($mmol.L^{-1}$)	0.150	*5.4 ± 0.6	0.331	*5.3 ± 0.8	0.587	*5.4 ± 0.6	**0.587
30' Glucose ($mmol.L^{-1}$)	0.072	8.4 (7.6 – 9.3)	0.025	8.7 (7.8 - 9.7)	0.757	8.1 (7.2 – 9.0)	0.001
60' Glucose ($mmol.L^{-1}$)	0.134	7.9 (6.6 – 8.9)	0.010	9.5 (8.4 - 10.9)	0.017	8.7 (7.6 – 9.8)	0.000
90' Glucose ($mmol.L^{-1}$)	0.454	7.1 (6.1 – 7.8)	0.000	8.6 (7.6 - 10.2)	0.000	7.6 (6.8 – 8.6)	0.000
120' Glucose ($mmol.L^{-1}$)	0.044	7.3 (6.6 – 8.3)	0.000	7.3 (6.4 - 8.7)	0.000	7.6 (6.9 – 8.9)	0.045
HbA1c (%)	0.001	5.5 (5.2 – 5.8)	0.800	5.5 (5.2 - 5.7)	0.040	5.5 (5.3 – 5.7)	0.746
HIV number (pos/neg)	--	92 (22 / 70)	--	272 (66 / 206)	--	79 (22 / 57)	--

SW = Shapiro Wilk's test. K-W = Kruskal-Wallis test unless the p-value is marked with asterisks (**) = ANOVA p-value. Values are indicated as median (inter-quartile range) unless marked with an asterisk (*) = means ± standard deviation. GCS = glucose curve shape; M = male; F = female; BMI = body mass index; IQR = inter-quartile range; pos = positive; neg = negative; WT = wild type; kg = kilogram; cm = centimetre, $kg.m^{-2}$ = kilogram per metre squared; $mmol.L^{-1}$ = millimole per litre; HbA1c = glycosolated haemoglobin; HIV = human immunodeficiency virus. The p-values indicated in red text are significant at a significance level < 0.05.

Despite the fact that the median 120 min glucose value in the monophasic GCS subgroup indicates normal glucose tolerance, the median 30, 60 and 90 min glucose values were higher than that observed in the other two GCS subgroups. However, none of the glucose values exceeded the IGR cut-off values (Zhou *et al*., 2006) at these time intervals. An argument therefore exists for the use of these specified IGR cut-off values within the black South African population. Possible future strategies will include the optimisation of the IGR cut-off values in order to develop population specific T2D prevention and diagnostic approaches. The OGTT values observed within the three GCS subgroups, as indicated in Table 6.4, were plotted graphically and presented in Figure 6.15.

Figure 6.15 Graphic representation of the OGTT values stratified according to GCS



OGTT = oral glucose tolerance test; mmol.L^{-1} = millimole per litre; Min = minimum; Max = maximum ; % = percentage.

Multiple comparison test results were generated for statistically significant K-W p-values as discussed in Section 5.5.2. Biological significance, as discussed in Section 5.5.4, was calculated on all statistically significant multiple comparison data and the results presented in Table 6.5.

Although the difference in body height between the individuals included in the monophasic and biphasic GCS subgroups is statistically significant, the practical irrelevance of a height difference of 2.5 centimetres (see Table 6.4) is supported by the non-significant biological d-value of 0.33. The statistically significant differences observed between the unclassified and monophasic GCS subgroups, when comparing BMI, 30, 60, 90 and 120 min glucose as well as between the unclassified and biphasic GCS subgroups, in terms of the 60 and 90 min glucose values, were determined to not be of biological significance. This finding is expected since all the glucose levels are distributed within the normal glucose range. A

GCS was regarded to be unclassified, when criteria for the biphasic or monophasic GCS subgroup were not met. This lack of classification makes it not possible to use the data within the unclassified group for clinical inference. Further investigation into the physiological explanation for the existence of the unclassified GCS is thus warranted.

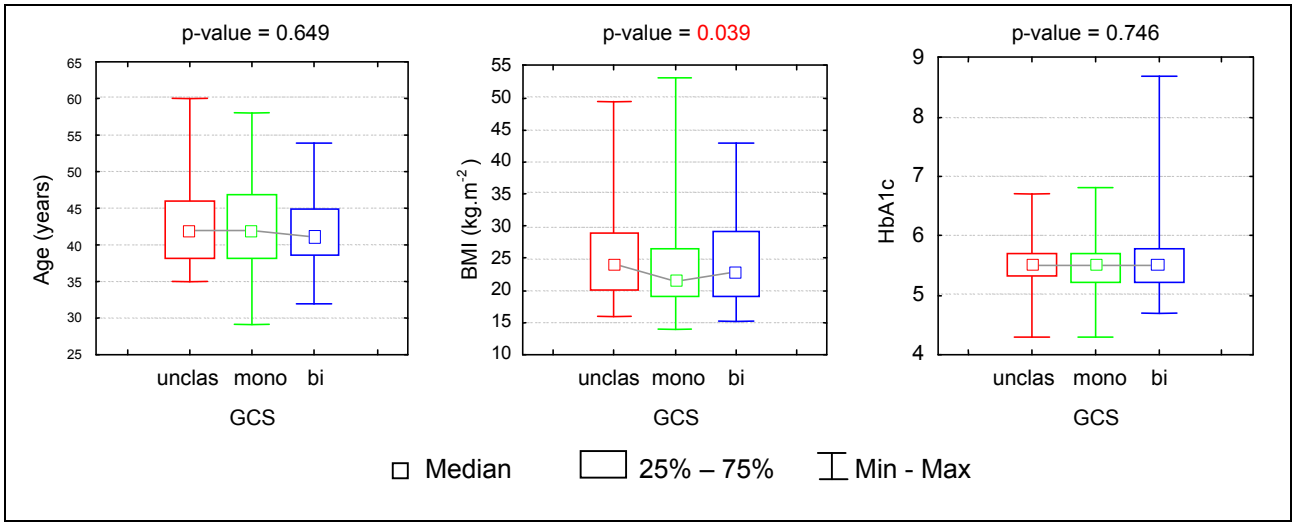
Table 6.5 Multiple comparison and biological significance results observed within the GCS subgroups

Parameter		K-W p-value	d-value	Parameter		K-W p-value	d-value
Height				**BMI**			
Unclassified	Monophasic	0.106	--	Unclassified	Monophasic	0.045	0.28
Unclassified	Biphasic	1.000	--	Unclassified	Biphasic	0.988	--
Monophasic	Biphasic	0.010	0.33	Monophasic	Biphasic	0.546	--
30 min Glucose				**60 min Glucose**			
Unclassified	Monophasic	0.001	0.48	Unclassified	Monophasic	0.001	0.43
Unclassified	Biphasic	0.353	--	Unclassified	Biphasic	0.003	0.55
Monophasic	Biphasic	0.187	--	Monophasic	Biphasic	0.000	1.02
90 min Glucose				**120 min Glucose**			
Unclassified	Monophasic	0.000	0.50	Unclassified	Monophasic	0.050	0.30
Unclassified	Biphasic	0.004	0.56	Unclassified	Biphasic	0.117	--
Monophasic	Biphasic	0.000	0.97	Monophasic	Biphasic	1.000	--

K-W = Kruskal-Wallis multiple comparison p-value. d-value = biological significance; GCS = glucose curve shape. The d-values indicated in red text are significant at a significance level > 0.8.

Based on the multiple comparison data presented in Table 6.5, there is a statistically significant difference between the biphasic and monophasic GCS subgroups when comparing the 60 and 90 min glucose levels. In addition, the biological significance is expected, given that the 60 and 90 min OGTT points are the criteria that differentiate the monophasic and biphasic GCS from each other. There was no significant difference between the three GCS subgroups when comparing either age or HbA1c values. The observed values for the BMI, age and HbA1c within the three subgroups are graphically represented in Figure 6.16.

Figure 6.16 Graphic representation of age, BMI and HbA1c values stratified according to GCS



BMI = body mass index; $kg.m^{-2}$ = kilogram per metre squared; HbA1c = glycosylated haemoglobin; unclas = unclassified; mono = monophasic; bi = biphasic; min = minimum; max = maximum; % = percentage; p-value = Kruskal-Wallis p-value, see Table 6.4. The p-values indicated in red text are significant at a significance level < 0.05.

It is important to note from the multiple comparison data presented in Table 6.5 that there is no significant difference between the monophasic and biphasic GCS subgroups when comparing the 120 min glucose value. Due to the available data in non-Africans indicating an association between the monophasic GCS and glucose intolerance (Tschritter *et al.,* 1993), this association was evaluated within the studied cohort via a contingency table and the results listed in Table 6.6. The table presents numbers of monophasic and biphasic GCSs in relation to glucose tolerance as defined by the 120 min glucose level.

Table 6.6 Contingency table summary for the association between CGS and glucose tolerance

Observed numbers	IGT	NGT	Total	p-value
Monophasic	69	203	272	0.045
Biphasic	14	78	92	
Total	**83**	**281**	**364**	
OR (95% CI)	1.89 (1.00 - 3.55)			

A contingency table with two-tailed Fisher exact p-value as well as odds ratio and 95% confidence interval were determined. IGT = impaired glucose tolerance; NGT = normal glucose tolerance; OR = odds ratio; CI = confidence interval; CGS = glucose curve shape. The p-values indicated in red text are significant at a significance level < 0.05.

The results in Table 6.6 indicate that there was an almost two fold increased risk for being glucose intolerant when harbouring a monophasic GCS. This result was also statistically significant and although there were three times as many individuals with monophasic GCSs than those with biphasic GCSs, there is certainly value in determining the GCS in addition to assessing the 120 min glucose value to evaluate glucose tolerance. It is

therefore suggested that a review of the CGS could be of value when included in the assessment of glucose tolerance.

6.2.2 Oral glucose tolerance test results

Fasting glucose was determined on two occasions, the first prior to the OGTT, measured from capillary blood after a finger prick, and the second, a plasma glucose measurement following IV catheterisation, prior to initiating the glucose tolerance test. The median glucose and IQR measurements recorded at the various OGTT points are represented in Table 6.7.

Table 6.7 OGTT results observed in the cohort

Glucose (mmol.L^{-1})	Median (IQR)	Glucose (mmol.L^{-1})	Median (IQR)
Finger prick	5.0 (4.6 – 5.3)	60 minutes	9.1 (7.8 – 10.4)
0 minutes	5.3 (4.9 – 5.7)	90 minutes	8.1 (7.0 – 9.6)
30 minutes	8.6 (7.6 – 9.5)	120 minutes	7.3 (6.5 – 8.6)

OGTT = oral glucose tolerance test; IQR = inter-quartile range; mmol.L^{-1} = millimole per litre.

Plasma blood samples were thus collected at 0 min, prior to ingesting the glucose load, and at 30, 60, 90 and 120 min post prandially. Both the capillary and plasma blood glucose levels were measured via a handheld MediSens® Optium™ [1] Sensor glucose machine together with MediSens® Optium™ *Plus* blood glucose test strips [2].

6.2.2.1 Fasting glucose

Despite the accuracy of a T2D diagnosis based upon OGTT results (Tschritter *et al.,* 2003), this test is not the recommended screening test of the ADA. The test of choice remains the fasting glucose level. The reasoning for this is that a fasting glucose test is easier, cheaper, more precise and consistent (McCance *et al.,* 1997) when compared to an OGTT.

The subgroups were divided according to the WHO values for normal fasting glucose (NFG), grouping together the impaired and diabetic fasting glucose groups. The Mann-Whitney statistical method, as discussed in Section 5.5.2, was used to compare values between the two fasting glucose subgroups. The different clinical parameters for

[1] MediSens® Optium™ is a registered trademark of Abbott Diabetes Care, Doncaster, Australia.
[2] Glucose measurements were performed by members of the PRIMER team.

these two fasting glucose subgroups are presented in Table 6.8, with the fasting glucose values included as informative data. This parameter was not compared, due to the fact that the two subgroups were stratified according to this value.

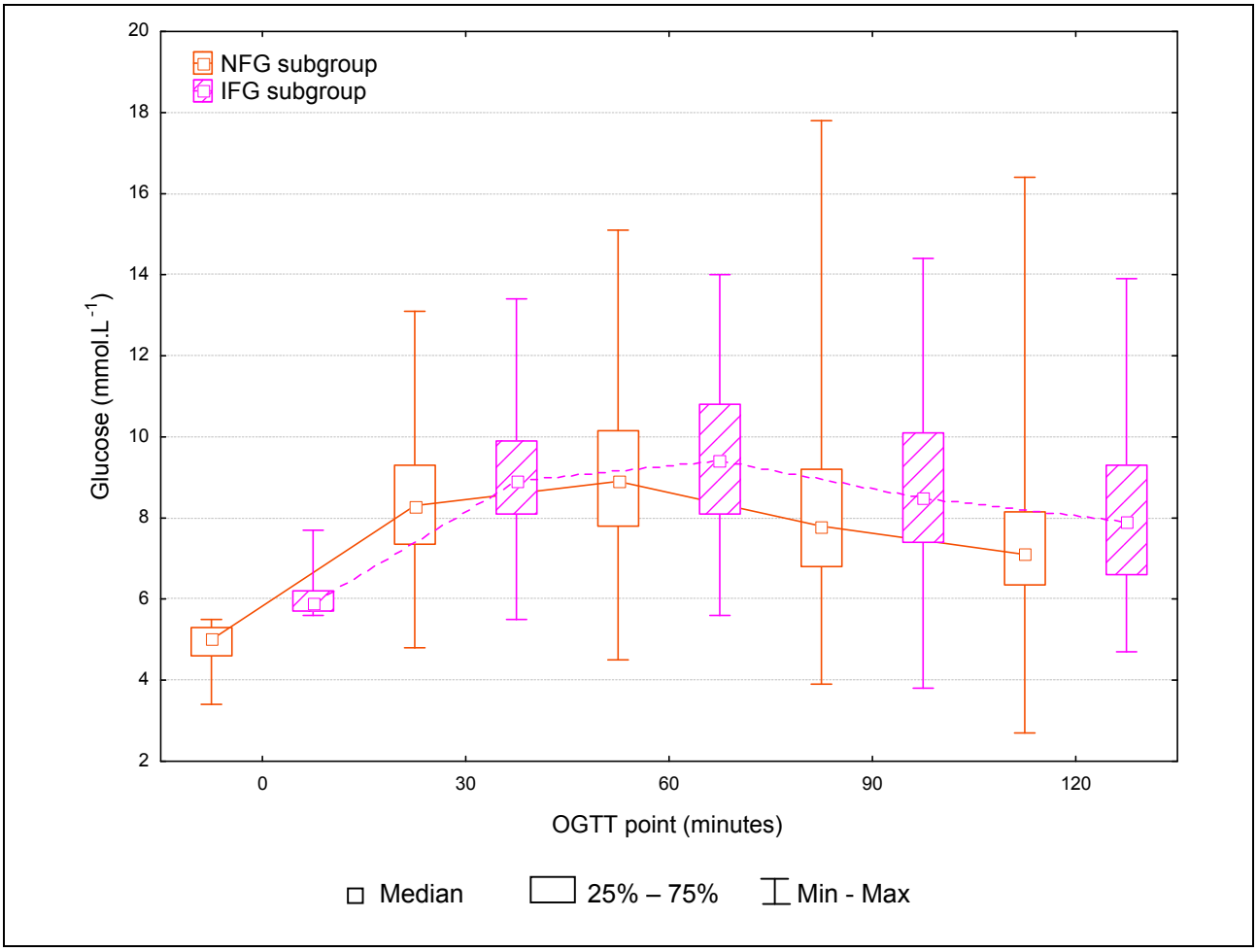
Table 6.8 Clinical parameters stratified according to fasting glucose

Parameter	NFG subgroup		IFG subgroup		M-W p-value	d-value
	SW p-value	Median (IQR)	SW p-value	Median (IQR)		
Number (M/F)	--	280 (123 / 157)	--	163 (42 / 121)	--	--
Age (years)	0.000	42.0 (38.0 – 46.0)	0.003	43.0 (39.0 – 47.0)	0.086	--
Weight (kg)	0.000	58.0 (51.0 – 67.0)	0.000	62.0 (53.0 – 77.0)	0.000	0.29
Height (cm)	0.003	162.0 (157.0 – 169.0)	0.004	159.0 (154.0 – 164.0)	0.002	0.35
BMI ($kg.m^{-2}$)	0.000	21.2 (18.7 – 25.5)	0.000	23.8 (20.2 – 31.2)	0.000	0.39
0' Glucose ($mmol.L^{-1}$)	0.000	5.0 (4.6 – 5.3)	0.000	5.9 (5.7 – 6.2)	--	--
30' Glucose ($mmol.L^{-1}$)	0.005	8.3 (7.4 – 9.3)	0.192	8.9 (8.1 – 9.9)	0.000	0.41
60' Glucose ($mmol.L^{-1}$)	0.001	8.9 (7.8 – 10.2)	0.333	9.4 (8.1 – 10.8)	0.015	0.20
90' Glucose ($mmol.L^{-1}$)	0.000	7.8 (6.8 – 9.2)	0.226	8.5 (7.4 – 10.1)	0.000	0.26
120' Glucose ($mmol.L^{-1}$)	0.000	7.1 (6.4 – 8.2)	0.002	7.9 (6.6 – 9.3)	0.000	0.39
HbA1c (%)	0.000	5.4 (5.2 – 5.7)	0.072	5.7 (5.3 – 5.9)	0.701	--

SW = Shapiro Wilk's test p-value; M-W = Mann-Whitney test p-value; d-value = biological significance. Values are indicated as median (inter-quartile range). NFG = normal fasting glucose; IFG = impaired fasting glucose; M = male; F = female; BMI = body mass index; SD = standard deviation; pos = positive; neg = negative; kg = kilogram; cm = centimetre, $kg.m^{-2}$ = kilogram per metre squared; $mmol.L^{-1}$ = millimole per litre; HbA1c = glycosylated haemoglobin; HIV = human immunodeficiency virus. The p-values and d-values indicated in red text are significant at a significance level < 0.05 and > 0.8 respectively.

An anticipated trend is observed in the increased OGTT glucose values within the IFG subgroup. This subgroup had significantly higher glucose values throughout the two hours when compared to the normal fasting glucose subgroup, despite the non-significant biological values. The cut-off values for IGR (Zhou *et al*., 2006) were reached at 0 min (> 5.6 $mmol.L^{-1}$) and 120 min (> 7.8 $mmol.L^{-1}$) in the IFG subgroup. Using the proposed (Zhou *et al*., 2006) 60 min cut-off value of > 10.1 $mmol.L^{-1}$ to predict pre-diabetes would be insufficient in this cohort (median 60 min glucose = 9.4 $mmol.L^{-1}$), which therefore calls into question the relevance of this predictor in different ethnic populations. Follow-up of the individuals included in the IFG subgroup in order to determine their future development of T2D is necessary to determine the accuracy of the 60 min cut-off value proposed in Chinese subjects (Zhou *et al*., 2006). The statistical differences between these glucose values were however not large enough to be of biological significance. The OGTT values are graphically represented in Figure 6.17, indicating the significantly increased 30, 60 and 90 min glucose values in the impaired glucose tolerance subgroup. The clinical significance of this GCS is further discussed in Chapter Seven.

Figure 6.17 Graphic representation of the OGTT values stratified according to fasting glucose

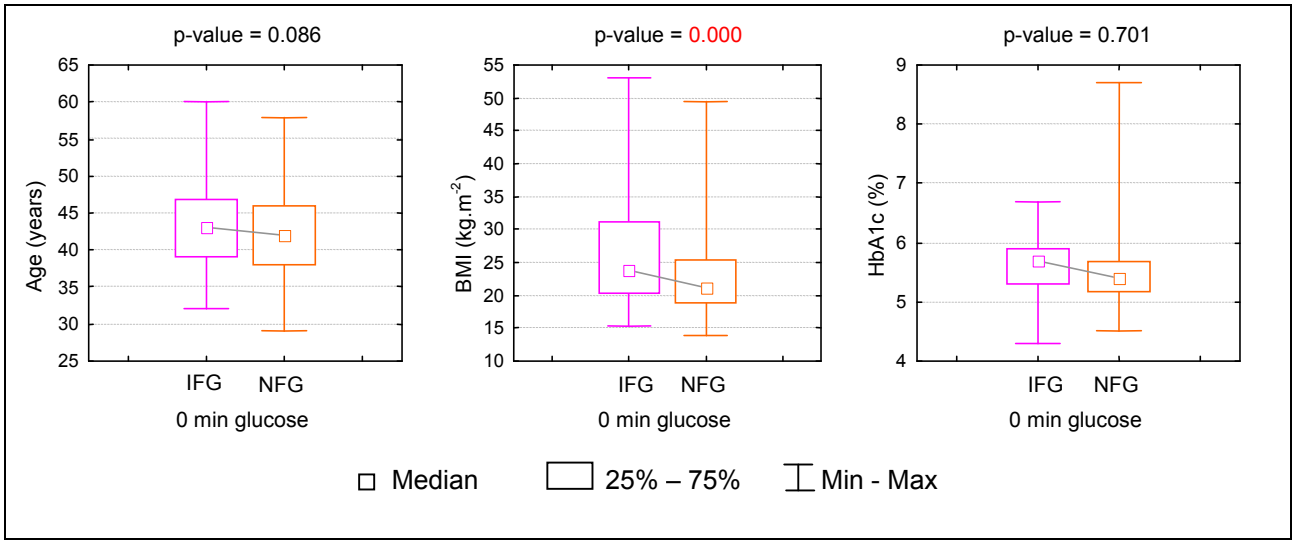


OGTT = oral glucose tolerance test; mmol.L^{-1} = millimole per litre; Min = minimum; Max = maximum.

The age, BMI and HbA1c values within the fasting glucose subgroups, as listed in Table 6.8, are represented in Figure 6.18. The median BMI value within the IFG subgroup does not indicate obesity, excluding this parameter as the possible cause of the impaired glucose tolerance (Olefsky, 2001). The trend towards a higher BMI within this subgroup is however similar due to the reported association between excess body weight and an impaired fasting glucose level (DeFronzo *et al.*, 1991).

Future variations in the BMI as well as glucose tolerance of these individuals requires follow-up in the subsequent assessments. Although the HbA1c level is increased in the impaired glucose tolerance subgroup, the difference is not statistically significant. The higher HbA1c value does not reach the greater than 6.5% cut-off level for diagnosing diabetes in other populations (Chen *et al.,* 2004) and is therefore not of clinical importance, although it could be indicative of a future glucose metabolism irregulation.

Figure 6.18 Graphic representation of the age, BMI and HbA1c values stratified according to fasting glucose



BMI = body mass index; $kg.m^{-2}$ = kilogram per metre squared; HbA1c = glycosylated haemoglobin; imp = impaired; Min = minimum; Max = maximum. p-value = Mann-Whitney test p-value, see Table 6.8. The p-values indicated in red text are significant at a significance level < 0.05.

The fact that the two-to-three month average blood glucose concentration, as represented by the HbA1c level, which is expected to be impaired when diabetic, was not observed within this cohort, questions the accuracy of the 6.5% cut-off value within the black South African population. However, due to the young median age of 42 years and apparently healthy state of the individuals included in the study, future assessment of the glucose metabolism is recommended in order to predict the clinical relevance of the currently observed higher HbA1c level as well as the relevant diagnostic cut-off value for this specific population.

6.2.2.2 Glucose tolerance

A definitive diagnosis of impaired glucose tolerance can only be determined following an OGTT, since the fasting glucose value is often observed to be within the normal range whilst the 120 min glucose value confirms glucose intolerance. In view of the fact that the risk for developing T2D is increased when an individual is glucose intolerant, this diagnosis is essential. Glucose intolerance is expected to be prevalent in the studied population due to its ongoing urbanisation, as discussed in Section 6.2.3.2.

The clinical parameters observed within the two glucose tolerance subgroups, as determined by the 120 min glucose value, are listed in Table 6.9. The listed values were compared between the two subgroups and the biological significance determined via the statistical methods described in Section 5.5. A comparison of 120 min glucose was not

performed due to the fact that the two subgroups were stratified according to glucose tolerance.

Table 6.9 Clinical parameters stratified according to glucose tolerance

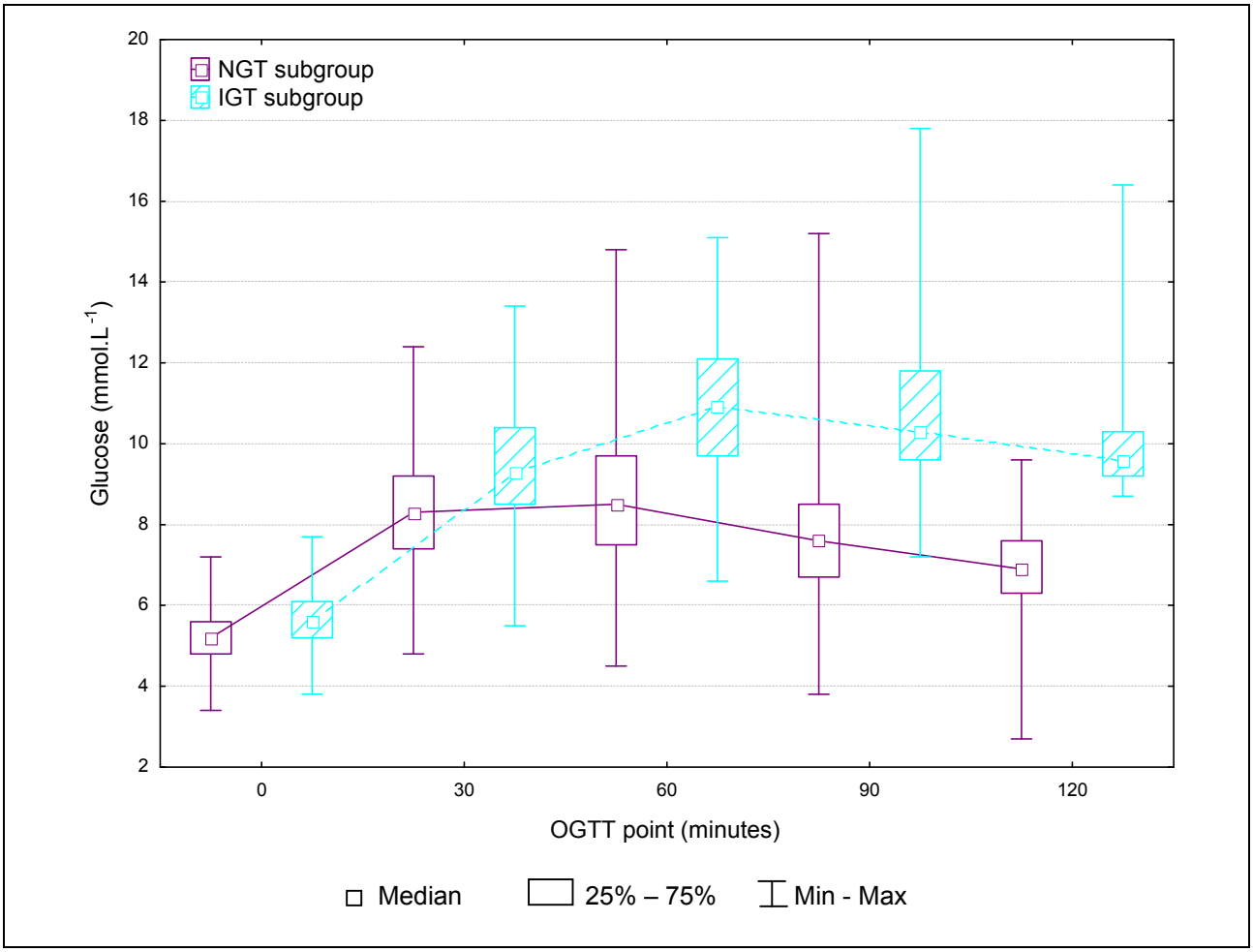
Parameter	NGT subgroup		IGT subgroup		M-W p-value	d-value
	SW p-value	Median (IQR)	SW p-value	Median (IQR))		
Number (M/F)	--	338 (138 / 200)	--	105 (27 / 78)	--	--
Age (years)	0.000	41.0 (38.0 – 46.0)	0.105	43.0 (40.0 – 47.0)	0.002	0.34
Weight (kg)	0.000	58.0 (52.0 – 67.0)	0.001	63.0 (54.0 – 77.0)	0.008	0.28
Height (cm)	0.001	162.0 (156.0 – 168.0)	0.004	160.0 (155.0 – 164.0)	0.037	0.19
BMI ($kg.m^{-2}$)	0.000	21.6 (19.0 – 26.6)	0.000	24.4 (20.0 – 31.6)	0.003	0.33
0' Glucose ($mmol.L^{-1}$)	0.004	5.2 (4.8 – 5.6)	0.400	5.6 (5.2 – 6.1)	0.000	0.44
30' Glucose ($mmol.L^{-1}$)	0.013	8.3 (7.4 – 9.2)	0.844	9.3 (8.5 – 10.4)	0.000	0.70
60' Glucose ($mmol.L^{-1}$)	0.002	8.5 (7.5 – 9.7)	0.589	10.9 (9.7 – 12.1)	0.000	1.15
90' Glucose ($mmol.L^{-1}$)	0.000	7.6 (6.7 – 8.5)	0.003	10.3 (9.6 – 11.8)	0.000	1.67
120' Glucose ($mmol.L^{-1}$)	0.000	6.9 (6.3 – 7.6)	0.000	9.6 (9.2 – 10.3)	--	--
HbA1c (%)	0.000	5.5 (5.2 – 5.7)	0.092	5.6 (5.2 – 5.8)	0.011	0.25

NGT = normal glucose tolerance; IGT = impaired glucose tolerance; SW = Shapiro Wilk's test p-value; M-W = Mann-Whitney test p-value; d-value = biological significance. M = male; F = female; BMI = body mass index; SD = standard deviation; pos = positive; neg = negative; kg = kilogram; cm = centimetre, $kg.m^{-2}$ = kilogram per metre squared; $mmol.L^{-1}$ = millimole per litre; HbA1c = glycosylated haemoglobin; HIV = human immunodeficiency virus. The p-values and d-values indicated in red text are significant at a significance level < 0.05 and > 0.8 respectively.

The OGTT results observed within the two glucose tolerance subgroups, as indicated in the Table 6.9, are graphically represented in Figure 6.19. The significantly increased OGTT values in the IGT subgroup are anticipated, given that the subgroups are stratified according to the 120 min glucose value. The IGR cut-off values to predict pre-diabetes were reached at the 0, 60 and 120 min intervals. Assessing the 60 min glucose to predict glucose metabolism irregularities and therefore possible future diabetes according to Zhou *et al*. (2006), would prove sensible within this subgroup with a median 60 min glucose value of 10.9 $mmol.L^{-1}$ (*i.e.* > 10.1 $mmol.L^{-1}$). Pre-diabetes, as defined by Zhou *et al*. (2006), included individuals with IFG and/or IGT. Within the studied black South African population, the diagnoses of pre-diabetes according to this reported 60 min glucose value would be inaccurate due to the fact that even though some of the individuals included in the IGT subgroup (Table 6.9) would have been correctly identified (median 60 min glucose, 10.9 and IQR, 9.7 – 12.1), the majority of the individuals included in the impaired fasting glucose subgroup (Table 6.8) would have been excluded (9.4 $mmol.L^{-1}$ median 60 min glucose, and 8.1 – 10.8 $mmol.L^{-1}$ IQR). This finding emphasises the importance of

population specific disease cut-off values in order to ensure accurate evaluation of glucose regulation.

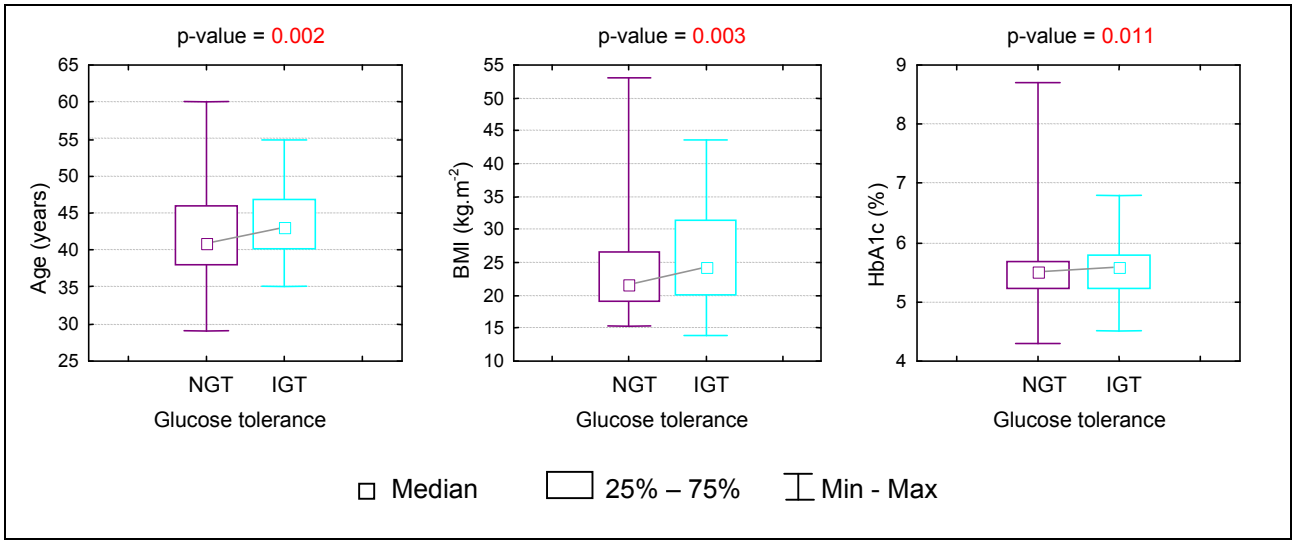
Figure 6.19 Graphic representation of the OGTT values stratified according to glucose tolerance



NGT = normal glucose tolerance; IGT = impaired glucose tolerance; OGTT = oral glucose tolerance test; mmol.L^{-1} = millimole per litre; Min = minimum; Max = maximum.

The age, BMI and HbA1c values observed within the glucose tolerance subgroups are presented graphically in Figure 6.20. Despite the statistically significant age difference between the two glucose tolerance subgroups, the biological significance value of 0.34 (Table 6.9) indicates the clinical irrelevance of the two-year difference in age. The BMI, as determined by weight and height, was significantly increased in the IGT subgroup. This finding supports the reported association between increased body weight and glucose intolerance in non-Africans (Olefsky, 2001).

Figure 6.20 Graphic representation of the age, BMI and HbA1c values stratified according to glucose tolerance



BMI = body mass index; kg.m^{-2} = kilogram per metre squared; HbA1c = glycosylated haemoglobin; NGT = normal glucose tolerance; IGT = impaired glucose tolerance; Min = minimum; Max = maximum. p-value = Mann-Whitney test p-value, see Table 6.9. The p-values indicated in red text are significant at a significance level < 0.05

Despite the non-significant biological significance value (see Table 6.9), the high BMI (> 25kg.m^{-2}) observed for the majority of the individuals included in the IGT subgroup is of clinical importance and indicates an increased T2D disease risk. The increased HbA1c value in the IGT subgroup supports the reported association of a high HbA1c level with glucose intolerance observed in non-Africans (Perry *et al.*, 2001). The higher HbA1c value within the IGT subgroup is however not high enough to reach the cut-off (> 6.5%) level indicative of T2D in non-Africans. Further investigation into this cut-off level for HbA1c within the black South African population is suggested as studies performed in various ethnic groups have reported dissimilar HbA1c cut-off points. In addition to ethnicity, age, gender and population prevalence of T2D were suggested to be important factors to consider when optimising an HbA1c level for use in diagnosing T2D (Bae *et al.*, 2009).

6.2.3 Phenotype

Descriptive statistics were generated for the continuous clinical data that was collected in the investigated cohort. The phenotype, with respect to environmental factors, including diet, physical activity and physical environment, as well as anthropometrical measures including age, gender and BMI, and biochemical assays such as HbA1c and HIV status was analysed. The observed phenotypic data within the investigated cohort are represented in Table 6.10.

Table 6.10 Observed phenotype within the investigated cohort

Parameter	Median (IQR)	Parameter	Median (IQR)
Total number	443	Height (cm)	161.0 (156.0 – 167.0)
Gender (M/F)	165 / 278	BMI ($kg.m^{-2}$)	21.9 (19.0 – 27.8)
Age (years)	42.0 (30.8 – 46.0)	HbA1c	5.5 (5.2 – 5.7)
Weight (kg)	59.0 (51.0 – 71.0)	HIV (pos/neg)	110 / 333

M = male; F = female; BMI = body mass index; IQR = inter-quartile range; HbA1c = glycosylated haemoglobin; HIV = human immunodeficiency virus; pos = positive; neg = negative; kg = kilogram; cm = centimetre, $kg.m^{-2}$ = kilogram per metre squared; $mmol.L^{-1}$ = millimole per litre.

The statistical methods described in Section 5.5.3 were used to evaluate the association between the physical environment and various parameters including physical activity, diet, glucose tolerance and GCS. In addition, an association was evaluated between glucose tolerance and both physical activity and diet.

6.2.3.1 Environmental factors

Questionnaire data was collected from every participant [1]. The data recorded for each individual included age, gender, residing location, ethnicity, education, medical history, diabetes risk score criteria (diet and physical activity), habits in terms of smoking and alcohol consumption and diabetic complications. T2D and obesity are increasingly prevalent world-wide, mainly due to urbanisation which results in an increased intake of animal fat and sugar, a decreased vegetable and fruit intake, as well as a lack of physical activity (Osman *et al.,* 1994). The value of therapeutic diet restriction and exercise in the management and prevention of T2D are therefore evident, although often underestimated.

6.2.3.1.1 Diet and physical activity

Daily intake of vegetables, fruits or berries was determined via the questionnaire to assess diabetes risk regarding diet, as described by Lindström and Tuomilehto (2003). Individuals were classified according to physical activity and the observed numbers within the cohort as well the physical environment subgroups are listed in Table 6.11.

[1] Questionnaire data was obtained by members of the PRIMER team.

Table 6.11 Number of individuals stratified according to physical activity

Number	Cohort	Rural subgroup	Urban subgroup
Number (M/F)	441 (163 / 278)	248 (88 / 160)	193 (75 / 118)
Physically active (M/F)	148 (62 / 86)	98 (38 / 60)	50 (24 / 26)
Physically inactive (M/F)	293 (101 / 192)	150 (50 / 100)	143 (51 / 92)

The physically active subgroup does physical activity for a minimum of 30 minutes, at least 4 days a week; the physically inactive subgroup does physical activity for less than 30 minutes or less than 4 days a week. M = male; F = female.

In the investigated cohort, 66% of the individuals were physically active for less than 30 min at a time or less than 4 days a week, whilst the other 34% exercised on a regular basis. Less than half of the participating males (38%) as well as females (31%) were physically active. Physical activity is an important environmental factor playing a role in T2D susceptibility. The effect of exercise on energy expenditure, appetite, serum lipoproteins, blood pressure, insulin response and glucose regulation, as well as coronary heart disease, emphasises this importance (Osman *et al.,* 1994). Individuals included in the total cohort, as well as the physical environment subgroups, were stratified according to diet and the observed numbers listed in Table 6.12.

Table 6.12 Number of individuals stratified according to diet

	Cohort	Rural subgroup	Urban subgroup
Number (M/F)	443 (165 / 278)	248 (88 / 160)	193 (75 / 118)
Daily consumption (M/F)	389 (142 / 247)	234 (85 / 149)	155 (57 / 98)
Non-daily consumption (M/F)	54 (23 / 31)	16 (5 / 11)	38 (18 / 20)

The daily consumption subgroup eats vegetables, fruits or berries daily and the non-daily subgroup eats vegetables, fruits or berries less often than daily. M = male; F = female.

As derived from the numbers listed in Table 6.12, more than 87% of the cohort, including 89% of the women and 86% of the men, did consume vegetables, fruits or berries on a daily basis according to the questionnaire data. The rural community had a higher percentage of individuals being physically active (40%) compared to the urban subgroup (26%), as indicated in Table 6.11. The rural subgroup also had a higher daily fruit and vegetable intake (94%) when compared to the urban subgroup (80%). These findings correlate with the reported effects of urbanisation on diet and physical activity (Osman *et al.,* 1994) and are further supported by the association between the physical environment and physical activity as well as diet, as presented in Table 6.13 and Table 6.14.

Table 6.13 Contingency table for the association between environment and physical activity

Observed numbers	Not physically active	Physically active	Total	p-value
Urban subgroup	143	50	193	0.002
Rural subgroup	149	99	248	
Total	**292**	**149**	**441**	
OR (95% CI)	1.90 (1.26 - 2.86)			

A contingency table with two-tailed Fisher exact p-value as well as odds ratio and 95% confidence interval were determined. The physically active subgroup does physical activity for a minimum of 30 minutes, at least 4 days a week; the physically inactive subgroup does physical activity for less than 30 minutes or less than 4 days a week. OR = odds ratio; CI = confidence interval. The p-values indicated in red text are significant at a significance level < 0.05.

The predictive value for the association between physical environment and physical activity was determined and presented in Table 6.13. The statistically significant OR and 95% CI indicate an almost two fold increased risk of being physically inactive when residing in an urban community. Similarly the urban residents presented with a statistically significant four fold increased risk of not including fruit, vegetables and berries into their diet, as represented in Table 6.14. These findings within the studied urban community are expected and correlate with the well-described association between urbanisation, diet and exercise levels in non-Africans (Osman *et al.,* 1994).

Table 6.14 Contingency table for the association between environment and diet

Observed numbers	Non-daily consumption	Daily consumption	Total	p-value
Urban subgroup	39	154	193	0.000
Rural subgroup	15	235	250	
Total	**54**	**389**	**443**	
OR (95% CI)	3.96 (2.11 - 7.44)			

A contingency table with two-tailed Fisher exact p-value as well as odds ratio and 95% confidence interval were determined. The daily consumption subgroup eats vegetables, fruits or berries daily and the non-daily consumption subgroup eats vegetables, fruits or berries less often than daily. OR = odds ratio; CI = confidence interval. The p-values indicated in red text are significant at a significance level < 0.05.

The association between physical activity, diet and glucose tolerance was determined and is presented in Table 6.15 and Table 6.16. An OR of 1.19 confirms the positive association between regular physical activity and glucose tolerance, although this association was determined not to be statistically significant. The wide 95% CI however indicates that further investigation is required in order to confirm this finding in the greater black South African population.

Table 6.15 Contingency table for the association between physical activity and glucose tolerance

Observed numbers	IGT	NGT	Total	p-value
Not physically active	73	222	295	0.480
Physically active	32	116	148	
Total	**105**	**338**	**443**	
OR (95% CI)	1.19 (0.74 - 1.91)			

A contingency table with two-tailed Fisher exact p-value as well as odds ratio and 95% confidence interval were determined. The physically active subgroup does physical activity for a minimum of 30 minutes, at least 4 days a week; the physically inactive subgroup does physical activity for less than 30 minutes or less than 4 days a week. OR = odds ratio; CI = confidence interval.

Based on the data generated in this study, an unhealthy diet was not a good predictor of glucose intolerance in the investigated cohort. The OR and 95% CI, as presented in Table 6.16, indicates that the daily consumption of fruits, berries or vegetables is associated with impaired glucose tolerance. This finding is not expected and not consistent with the importance of a healthy diet on T2D susceptibility (Osman *et al.,* 1994). These findings were however based on the daily environment and diet of non-Africans. The number of individuals (n = 5) without daily consumption of fruits, berries or vegetables, included in the IGT subgroup is very small. These five individuals were all residing in an urban environment and followed a Westernised diet without consuming fruits, berries or vegetables daily. All five of the individuals included in the IGT subgroup were also obese and physically inactive. Further investigation into the effect of diet on disease risk is therefore required within the black South African population. The fact that 75% of the individuals that included fruits, berries or vegetables in their diet were NGT, excludes the possibility of an unhealthy diet being protective against glucose intolerance, as indicated by the contingency table results listed in Table 6.16. Several different approaches however exist for measuring individual dietary intake, each with its own advantages and limitations. During this study a questionnaire on daily intake was completed, however more accurate approaches would include a 24 hour dietary recall or food record or a food frequency questionnaire that minimizes day-to-day variation, as suggested by the WHO (Bazzano, 2005).

Table 6.16 Contingency table for the association between diet and glucose tolerance

Observed numbers	IGT	NGT	Total	p-value
Non-daily consumption*	5	49	54	0.006
Daily consumption**	100	289	389	
Total	**105**	**338**	**443**	
OR (95% CI)	0.29 (0.11 - 0.76)			

A contingency table with two-tailed Fisher exact p-value as well as odds ratio and 95% confidence interval were determined. The daily consumption** subgroup eats vegetables, fruits or berries daily and the non-daily consumption* subgroup eats vegetables, fruits or berries less often than daily. NGT = normal glucose tolerance; IGT = impaired glucose tolerance; OR = odds ratio; CI = confidence interval. The p-values indicated in red text are significant at a significance level < 0.05.

The five IGT individuals with an unhealthy diet were obese and glucose intolerance may be as a result of a combination of factors including physical environment, obesity, diet and exercise levels. The tolerance may therefore not be determined by the daily consumption of fruits, berries or vegetables only. The possibility of untruthful questionnaire responses also needs to be considered as an explanation for the observed negative association between a healthy diet and glucose tolerance. Therefore, it is evident that there are several genetic and environmental confounding factors that need to be taken into consideration when determining IGT risk.

6.2.3.1.2 Physical environment

The effect of physical environment on T2D risk has previously been described in non-Africans (Osman *et al.,* 1994). Westernisation, as observed with dietary changes, encompassing an increase in saturated fat and refined sugar intake, combined with decreased physical activity, results in the world-wide higher prevalence of T2D. The rapid increase in urbanisation in Africa explains the increase in T2D incidence (Sobngwi *et al.,* 2001). This preliminary study evaluated the association between urbanisation and disease risk, which will be assessed further over the next 12 years of the study. Clinical parameters were compared between the rural and urban environment subgroups via a Mann-Whitney statistical method, unless the data was distributed normally, in which case the Student t-test was used, as discussed in Section 5.5.2. The biological significance was determined when statistically significant M-W results were observed, as discussed in Section 5.5.4. Results of the comparison analysis between the two physical environment subgroups are represented in Table 6.17.

Table 6.17 Clinical parameters stratified according to physical environment

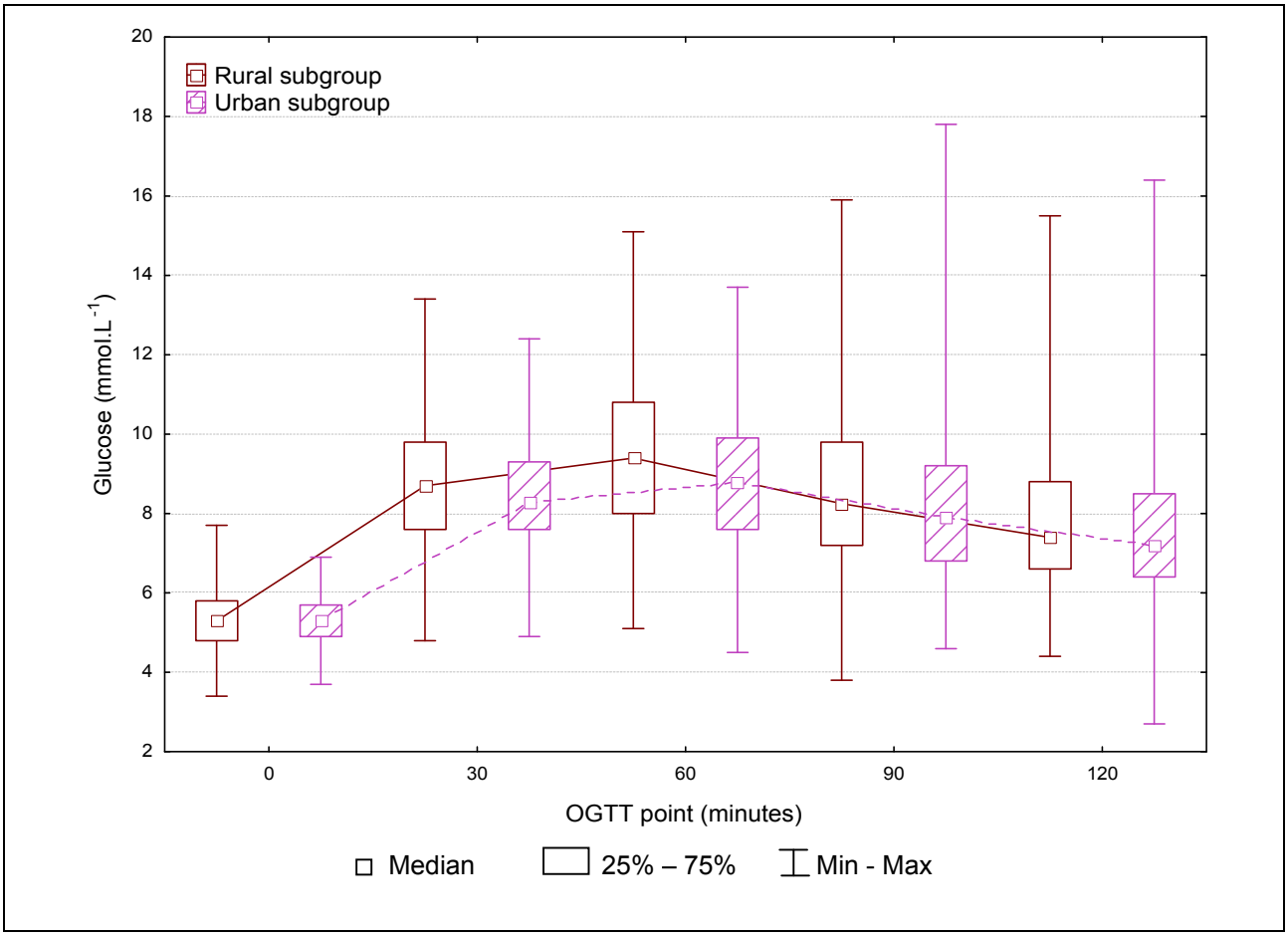
Parameter	Rural subgroup		Urban subgroup		M-W p-value	d-value
	SW p-value	Median (IQR)	SW p-value	Median (IQR)		
Number (M/F)	--	250 (90 / 160)	--	193 (75 / 118)	--	--
Age (years)	0.000	42.0 (38.0 – 47.0)	0.048	42.0 (39.0 – 46.0)	0.513	--
Weight (kg)	0.000	58.0 (50 – 67.0)	0.000	60.0(53.0 – 73.0)	0.039	0.01
Height (cm)	0.001	161.0 (156.0 – 167.0)	0.038	161.0 (155.0 – 167.0)	0.958	--
BMI ($kg.m^{-2}$)	0.000	21.5 (19.0 – 26.8)	0.000	23.0 (19.3 – 28.2)	0.061	--
0' Glucose ($mmol.L^{-1}$)	0.403	*5.3 ± 0.8	0.113	*5.3 ± 0.6	**0.715	--
30' Glucose ($mmol.L^{-1}$)	0.052	*8.76 ± 1.5	0.236	*8.4 ± 1.3	**0.014	0.22
60' Glucose ($mmol.L^{-1}$)	0.044	9.4 (8.0 – 10.8)	0.068	8.8 (7.6 – 9.9)	0.006	0.27
90' Glucose ($mmol.L^{-1}$)	0.000	8.3 (7.2 – 9.8)	0.000	7.9 (6.8 – 9.2)	0.048	0.18
120' Glucose ($mmol.L^{-1}$)	0.000	7.4 (6.6 – 8.8)	0.000	7.2 (6.4 – 8.5)	0.035	0.21
HbA1c (%)	0.000	5.5 (5.2 – 5.8)	0.111	5.5 (5.2 – 5.7)	0.912	--
HIV (pos/neg)	--	61 / 189	--	49 / 144	--	--

SW = Shapiro Wilk's test p-value; M-W = Mann-Whitney test p-value unless the p-value is marked with asterisks (**) = student t-test p-value; d-value = biological significance. Values are indicated as median (inter-quartile range) unless marked with an asterisk (*) = means ± standard deviation. M = male; F = female; BP = blood pressure; BMI = body mass index; IQR = inter-quartile range; pos = positive; neg = negative; kg = kilogram; cm = centimetre, $kg.m^{-2}$ = kilogram per metre squared; $mmol.L^{-1}$ = millimole per litre; HbA1c = glycosylated haemoglobin; HIV = human immunodeficiency virus. The p-values and d-values indicated in red text are significant at a significance level < 0.05 and > 0.8 respectively.

The differences observed in the OGTT results between the rural and urban subgroups are further represented graphically in Figure 6.21. The statistically significantly increase in the 30, 60, 90 and 120 min glucose values within the rural subgroup, when compared to the urban subgroup, can partly be explained by the rapid urbanisation currently observed within the rural communities of South Africa (Sobngwi *et al.,* 2001).

Individuals from the rural subgroup are expected to not be accustomed to high fat and sugar content diets and it can therefore be hypothesised that their glucose levels would take a longer time to stabilise subsequent to the glucose load. The observed glucose intolerance could also be explained by their limited access to healthcare, resulting in fewer check-ups and a greater proportion of undiagnosed diabetics. It is hence suggested that negative environmental factors, such as the physical environment, diet and exercise levels may increase the risk for diabetes in individuals with an existing genetic susceptibility to the disease (Sobngwi *et al.,* 2001), similar to that observed in the study by Osman *et al.* (1994) in non-Africans.

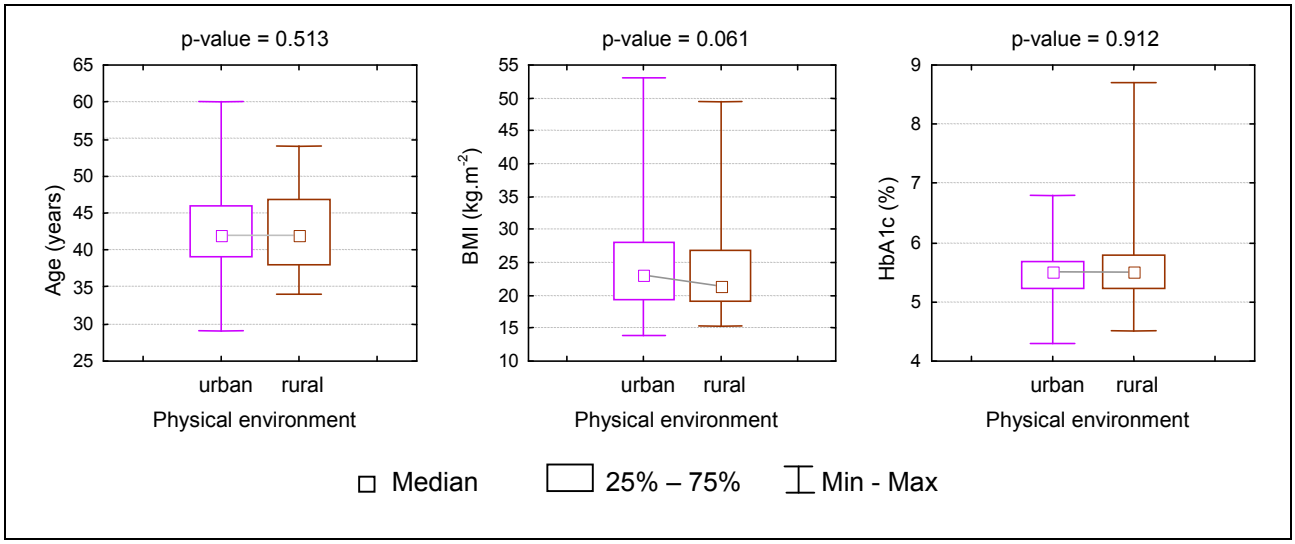
Figure 6.21 Graphic representation of the OGTT values stratified according to physical environment



OGTT = oral glucose tolerance test; mmol.L^{-1} = millimole per litre; Min = minimum; Max = maximum.

Both age and HbA1c values were determined to be similar within the two physical environment subgroups. The statistically significant difference in weight between the two subgroups did not result in a clinically significant difference in BMI, as indicated in Table 6.17. The age, BMI and HbA1c values are represented in Figure 6.22.

Figure 6.22 Graphic representation of the age, BMI and HbA1c values stratified according to physical environment



BMI = body mass index; $kg.m^{-2}$ = kilogram per metre squared; HbA1c = glycosylated haemoglobin; Min = minimum; Max = maximum. p-value = Mann-Whitney test p-value, see Table 6.17.

The OR of 1.41, as presented in Table 6.18, indicates that physical environment is not a good predictor of disease risk in the studied cohort, despite the statistically significant difference observed within the rural and urban subgroups when comparing the 120 min glucose values (Table 6.17). The widely distributed 95% CI also warrants further investigation to confirm this finding within the greater black South African population.

Table 6.18 Contingency table for the affect of physical environment on glucose tolerance

Observed numbers	IGT	NGT	Total	p-value
Rural subgroup	66	184	250	0.144
Urban subgroup	39	154	193	
Total	**105**	**338**	**443**	
OR (95% CI)	1.41 (0.90 - 2.22)			

A contingency table with two-tailed Fisher exact p-value as well as odds ratio and 95% confidence interval were determined. NGT = normal glucose tolerance; IGT = impaired glucose tolerance; OR = odds ratio; CI = confidence interval.

The number of different GCSs observed within the rural and urban subgroups is listed in Table 6.19. The GCS distribution within the two environmental subgroups was observed to be very similar.

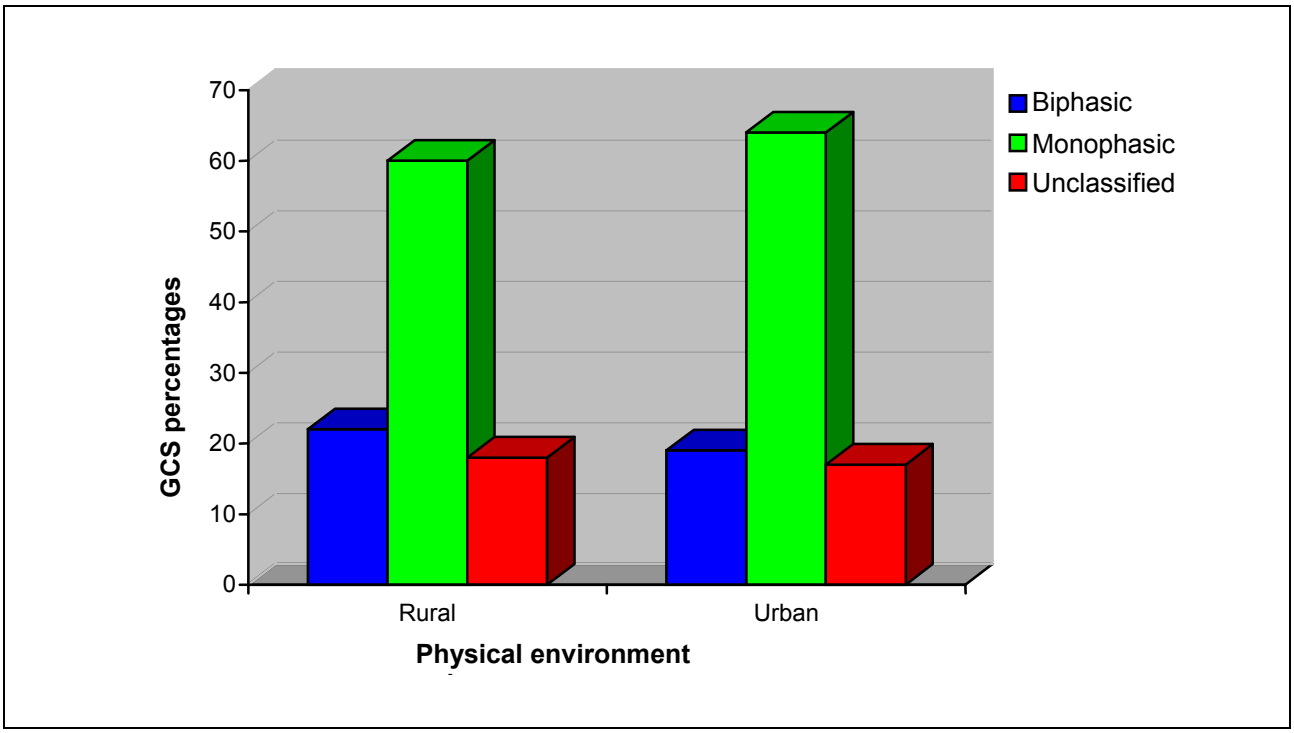
Table 6.19 GCS numbers observed within the physical environment subgroups

GCS	Rural subgroup	Urban subgroup
Biphasic	56	36
Monophasic	149	123
Unclassified	45	34
Total	250	193

GCS = glucose curve shape.

A biphasic GCS was observed in 22% and 19% of the individuals included in the rural and urban subgroups respectively. Sixty percent of individuals living in a rural environment presented with a monophasic GCS, with the remaining 18% being unclassified. A monophasic GCS was observed in 64% of individuals in the urban subgroup, whilst 17% of this subgroup had an unclassified GCS. The observed GCS percentages within the two physical environment subgroups are presented in Figure 6.23.

Figure 6.23 GCS percentages observed within the physical environment subgroups



Percentages calculated from the total rural (250) and urban (193) subgroups. GCS = Glucose curve shape.

The association between physical environment and GCS was determined by means of a contingency table, but did not yield a statistically significant result. The contingency table results are listed in Table 6.20.

Table 6.20 Contingency table for the association between physical environment and GCS

Observed numbers	Biphasic	Monophasic	Total	p-value
Rural subgroup	56	149	205	0.332
Urban subgroup	36	123	159	
Total	**92**	**272**	**364**	
OR (95% CI)	1.28 (0.79 - 2.07)			

A contingency table with two-tailed Fisher exact p-value as well as odds ratio and 95% confidence interval were determined. OR = odds ratio; CI = confidence interval.

According to the results listed in Table 6.20, the physical environment cannot be used to accurately predict GCS. However, further investigation within a larger cohort is required to confirm this finding in the greater black South African population, as indicated by the widely distributed 95% CI.

6.2.3.2 Anthropometrical measures

Anthropometrical measures such as age, gender and BMI play an important role in the susceptibility to T2D, as discussed in Section 3.2. The study cohort was stratified according to the reported T2D risk levels of anthropometrical measures (Chen *et al.,* 2004) and the clinical parameters compared between the various subgroups via the statistical procedures discussed in Section 5.5.

6.2.3.2.1 Age and gender

De Fronzo *et al.* (1991) observed a higher incidence of T2D in Caucasian individuals older than 45 years of age. This observation was further supported by the findings of Wild *et al.* (2004), which validated advanced age as an important susceptibility factor for T2D. The two age subgroups therefore included individuals less than 45 years of age and those over 45 years of age. The cohort was also divided into subgroups of gender and the observed parameters presented in Table 6.17. The parameters within the gender subgroups were compared via the statistical methods discussed in Section 5.5.2 and the results listed in Table 6.21.

Table 6.21 Clinical parameters stratified according to gender

Parameter	Male		Female		M-W p-value	d-value
	SW p-value	Median (IQR)	SW p-value	Median (IQR)		
Number	--	165	--	278	--	--
Age (years)	0.000	42.0 (38.0 – 46.0)	0.001	42.0 (38.0 – 46.0)	0.445	--
Weight (kg)	0.000	55.0 (50.0 – 60.0)	0.000	63.5 (54.0 – 78.0)	0.000	0.59
Height (cm)	0.171	168.0 (163.0 – 173.0)	0.000	158.0 (154.0 – 162.0)	0.000	1.52
BMI ($kg.m^{-2}$)	0.000	19.4 (18.0 – 21.2)	0.000	25.1 (21.2 – 31.4)	0.000	0.98
0' Glucose ($mmol.L^{-1}$)	0.051	*5.1 ± 0.44	0.155	*5.5 ± 0.67	**0.000	0.58
30' Glucose ($mmol.L^{-1}$)	0.268	8.6 (7.8 – 9.6)	0.004	8.5 (7.5 – 9.4)	0.293	--
60' Glucose ($mmol.L^{-1}$)	0.005	9.1 (7.9 – 10.7)	0.049	9.1 (7.8 – 10.3)	0.586	--
90' Glucose ($mmol.L^{-1}$)	0.000	7.8 (6.6 – 9.2)	0.000	8.3 (7.2 – 9.7)	0.011	0.14
120' Glucose ($mmol.L^{-1}$)	0.000	6.8 (6.2 – 7.6)	0.000	7.7 (6.8 – 8.9)	0.000	0.42
HbA1c (%)	0.000	5.5 (5.1 – 5.7)	0.102	5.6 (5.3 – 5.8)	0.005	0.19
HIV number (pos/neg)	--	43 / 122	--	67 / 211	--	--

SW = Shapiro Wilk's test p-value; M-W = Mann-Whitney test p-value unless the p-value is marked with asterisks (**) = student t-test p-value; d-value = biological significance. Values are indicated as median (inter-quartile range) unless marked with an asterisk (*) = means ± standard deviation. M = male; F = female; BMI = body mass index; IQR = inter-quartile range; pos = positive; neg = negative; kg = kilogram; cm = centimetre, $kg.m^{-2}$ = kilogram per metre squared; $mmol.L^{-1}$ = millimole per litre; HbA1c = glycosylated haemoglobin; HIV = human immunodeficiency virus. The p-values and d-values indicated in red text are significant at a significance level < 0.05 and > 0.8 respectively.

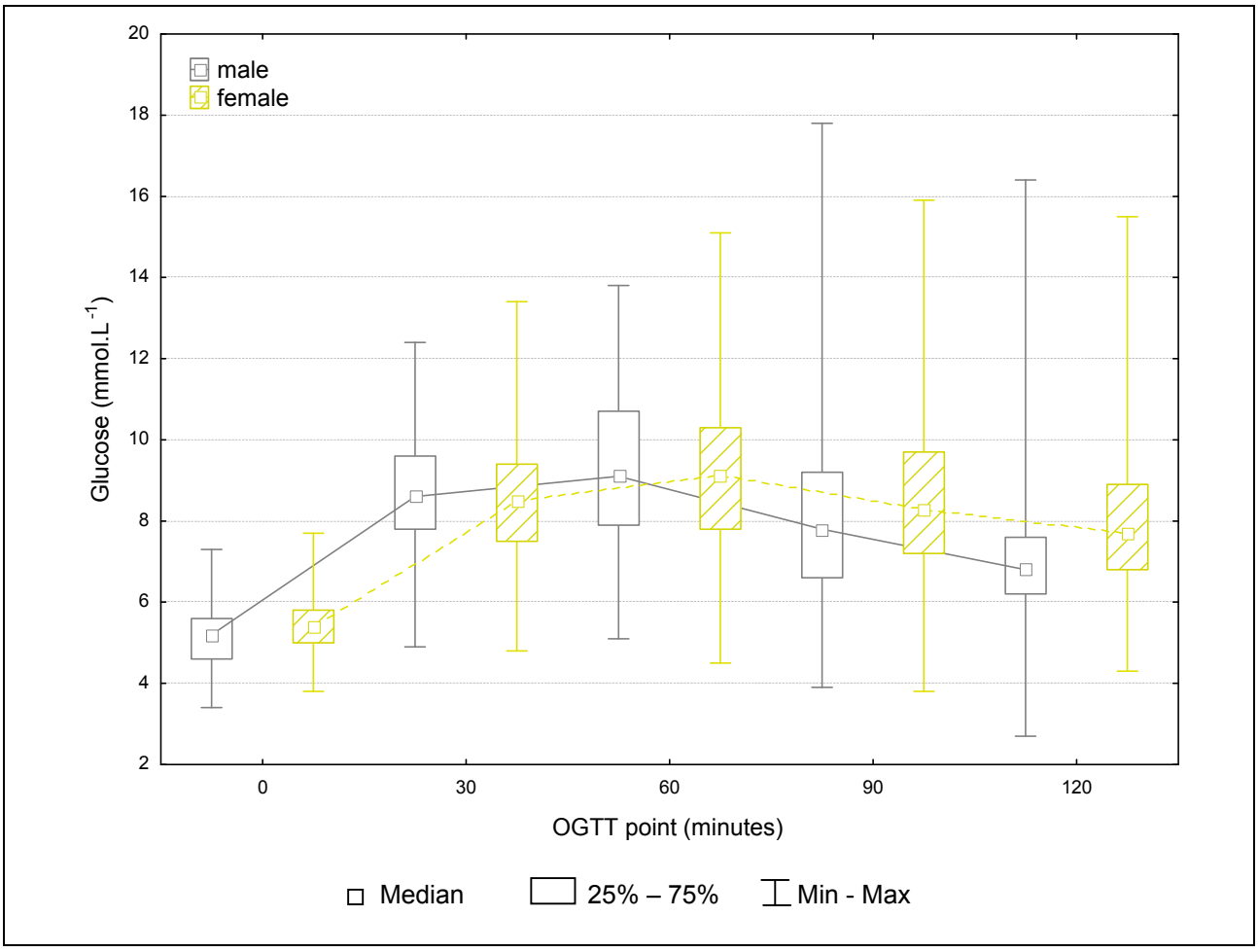
There was no significant statistical difference between the two gender subgroups when comparing age. The M-W p-values listed in Table 6.17 indicate a statistically significant difference when comparing the anthropometrical parameters, namely weight, height and BMI between the two gender subgroups. Not only did BMI differ statistically and biologically significantly between the two gender subgroups, but the median female BMI was above 25 $kg.m^{-2}$, indicating that the female population was on average obese (Chen *et al.,* 2004). Consequently, the females included in this study were at greater risk for developing T2D, similar to that reported by Osman *et al.* (1994) in Malaysian individuals.

The fasting glucose levels differed statistically significantly between the two gender subgroups, which can also be observed graphically in Figure 6.24. This glucose level does, however neither reach the cut-off value for impaired fasting glucose (> 6 $mmol.L^{-1}$) according to Berkow *et al.* (1992), nor the IGR cut-off value of greater than 5.6 $mmol.L^{-1}$ reported by Zhou *et al.* (2006).

The 120 min glucose value was significantly higher in the female subgroup when compared to the male subgroup. However, the median 120 min glucose levels for both subgroups were below the glucose intolerance level of 7.8 $mmol.L^{-1}$. On the other hand,

the 120 min glucose level for the majority of females was within the impaired glucose tolerance range between 7.8 and 11.1 mmol.L^{-1}, as graphically represented in Figure 6.24. Although the glucose curves suggest a trend towards poorer glucose regulation in the female subgroup compared to the male subgroup, none of the OGTT points reached the IGR cut-off values (Zhou *et al.,* 2006). The association between gender and the risk for glucose intolerance was therefore questioned and evaluated via a contingency table, as presented in Table 6.22.

Figure 6.24 Graphic representation of the OGTT values observed within the gender subgroups

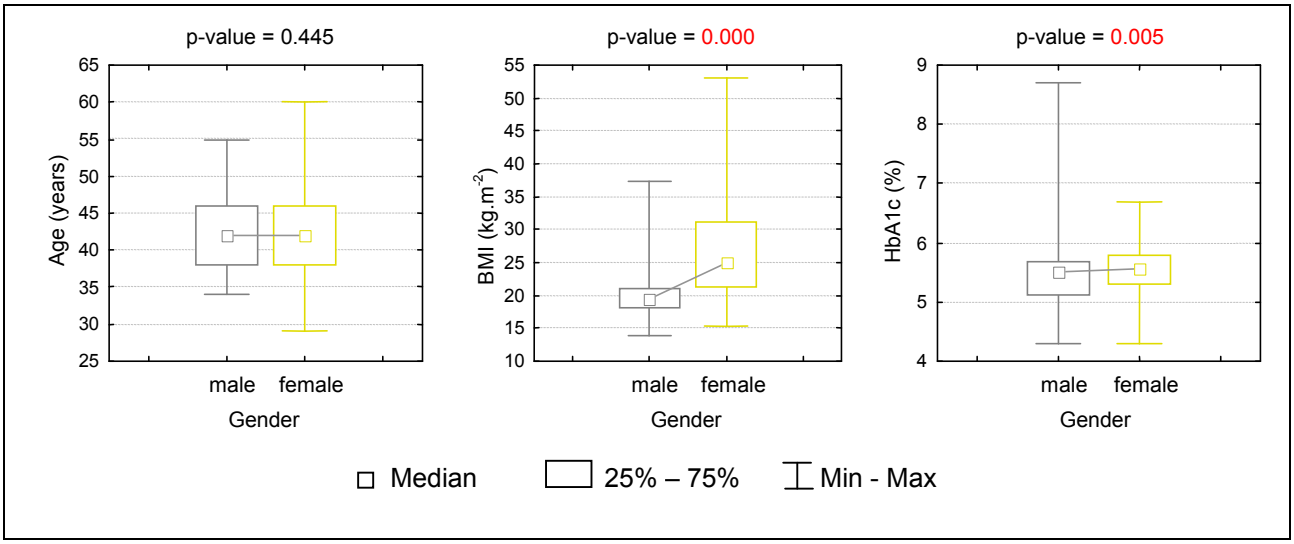


OGTT = oral glucose tolerance test; mmol.L^{-1} = millimole per litre; Min = minimum; Max = maximum; % = percentage.

The difference in the HbA1c levels between the two gender subgroups was determined to be statistically significant ($p = 0.005$), as listed in Table 6.21. A difference of 0.1% is however not of clinical importance, as indicated by the biological significance value of 0.19. The fact that the HbA1c levels for both subgroups were within the normal range (> 6.5%) supports the lack of biological significance. The accuracy of the high HbA1c level cut-off (> 6.5%) that is used in other non-African populations (Perry *et al.,* 2001) is also therefore questionable within the context of a black South African population, as

further discussed in Section 6.2.3.3. The differences observed within the two gender subgroups in terms of age, BMI and HbA1c are graphically represented in Figure 6.25.

Figure 6.25 Graphic representation of the age, BMI and HbA1c values stratified according to gender



BMI = body mass index; $kg.m^{-2}$ = kilogram per metre squared; HbA1c = glycosylated haemoglobin; Min = minimum; Max = maximum; % = percentage. p-value = Mann-Whitney test p-value, see Table 6.21. The p-values indicated in red text are significant at a significance level < 0.05.

The reported higher prevalence of T2D in non-African males (Wild *et al.,* 2004) compared to non-African females was not observed within the studied black South African cohort. There was however a statistically significant two fold increased risk for being glucose intolerant when female, as indicated by the OR and 95% CI represented in Table 6.22. The observed obesity amongst females (see Table 6.21) may explain the higher risk for being glucose intolerant when compared to males (Elbein, 1997).

Table 6.22 Contingency table for the association between gender and glucose tolerance

Observed numbers	IGT	NGT	Total	p-value
Female	78	200	154	0.006
Male	27	138	289	
Total	**105**	**338**	**443**	
OR (95% CI)	1.99 (1.22 - 3.24)			

A contingency table with two-tailed Fisher exact p-value as well as odds ratio and 95% confidence interval were determined. OR = odds ratio; CI = confidence interval; IGT = impaired glucose tolerance; NGT = normal glucose tolerance. The p-values indicated in red text are significant at a significance level < 0.05.

The finding of female gender as a good predictor of glucose intolerance within the studied cohort once more supports the necessity of population specific T2D screening tools. An evaluation of the association between age and glucose tolerance in the studied cohort

revealed a non-significant Fisher exact p-value, as indicated by the contingency results in Table 6.23.

Table 6.23 Contingency table for the association between age and glucose tolerance

Observed numbers	IGT	NGT	Total	p-value
Subgroup > 45 years	43	111	154	0.130
Subgroup < 45 years	62	227	289	
Total	**105**	**338**	**443**	
OR (95% CI)	1.41 (0.90 - 2.22)			

A contingency table with two-tailed Fisher exact p-value as well as odds ratio and 95% confidence interval were determined. OR = odds ratio; CI = confidence interval; IGT = impaired glucose tolerance; NGT = normal glucose intolerance.

According to the OR and 95% CI listed in Table 6.23, age was not a good predictor of glucose tolerance at the WHO recommended T2D risk cut-off age (Chen *et al.,* 2004) of 45 years within the study cohort. It is important to note that these results do not necessarily indicate that age is not important in increased T2D disease risk, but rather that the cut-off value for age should possibly be re-evaluated in the black South African population.

6.2.3.2.2 Body mass index

The BMI values were calculated, as discussed in Section 5.4.4, from the height and weight measurements [1] obtained during the study. Osman *et al.* (1994) observed in a Malaysian population that the risk for T2D was significantly higher when an individual had a BMI greater than 25 $kg.m^{-2}$. Malaysian individuals who were 40% overweight had a seven fold increased risk for developing T2D, similar to the findings by Young *et al.* (1990). Correspondingly, significant weight loss was reported to decrease the risk of developing T2D (Osman *et al.,* 1994). The investigated cohort was stratified according to reported T2D risk in terms of the BMI level (Chen *et al.,* 2004) and the determined clinical parameters were compared between the normal (< 25 $kg.m^{-2}$) and high (> 25 $kg.m^{-2}$) BMI subgroups. Comparisons were performed via the statistical methods discussed in Section 5.5.2 and the results presented in Table 6.24.

[1] Height and weight measurements were performed by the PURE-SA study and are used in this thesis with permission from Prof A Kruger, leader of the South African leg of the PURE study.

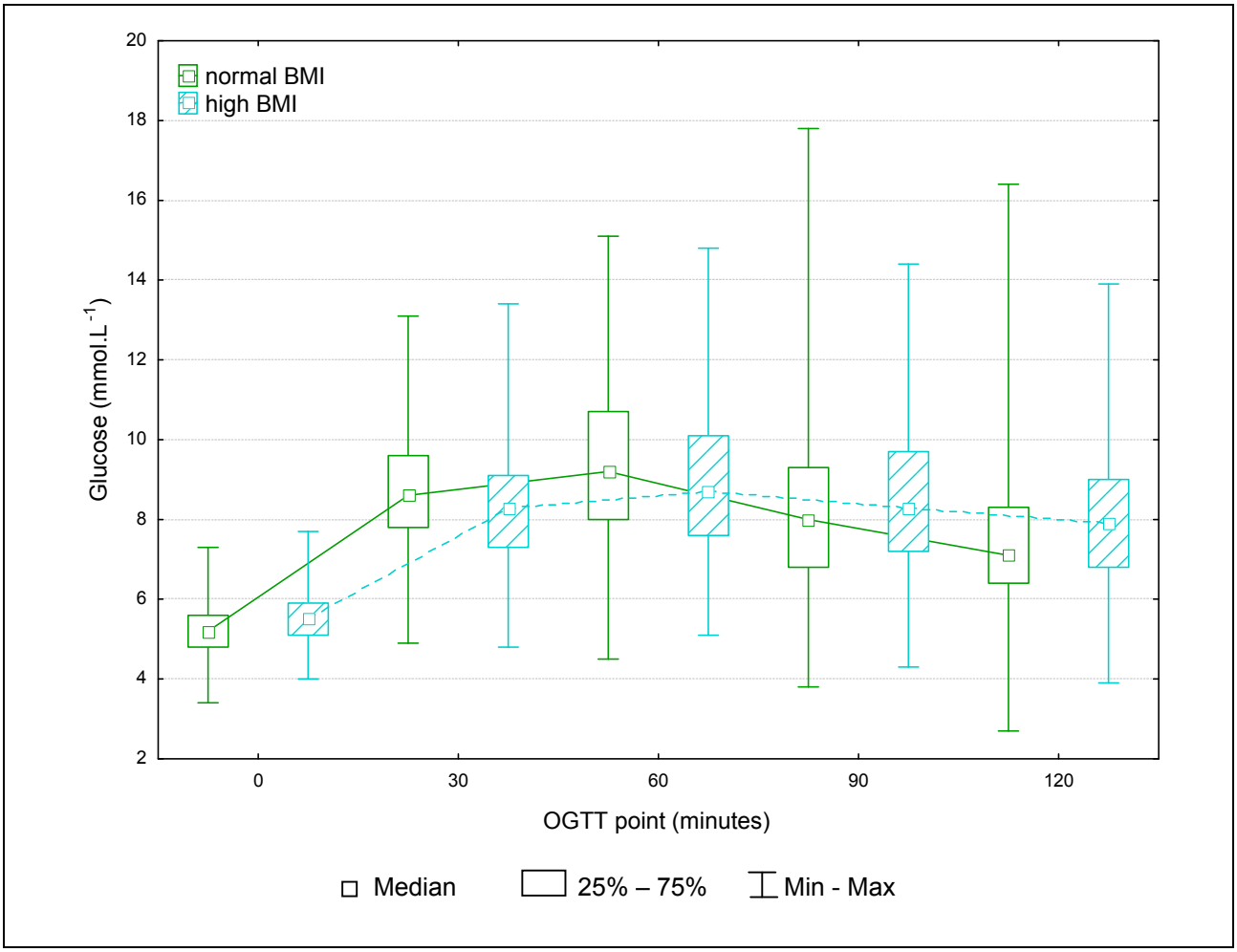
Table 6.24 Clinical parameters stratified according to BMI

Parameter	Normal BMI		High BMI		M-W p-value	d-value
	SW p-value	Median (IQR)	SW p-value	Median (IQR)		
Number (M/F)	--	281 (156 / 125)	--	162(9 / 153)	--	--
Age (years)	0.000	42.0 (38.0 – 46.0)	0.002	42.0 (38.0 – 46.0)	0.490	--
Weight (kg)	0.324	53.0 (48.0 – 58.0)	0.000	76.0 (67.0 – 87.0)	0.000	1.75
Height (cm)	0.001	163.0 (158.0 – 170.0)	0.118	158.0 (154.0 – 162.0)	0.000	0.67
BMI ($kg.m^{-2}$)	0.001	19.7 (18.1 – 21.6)	0.000	30.2 (26.9 – 34.4)	--	--
0' Glucose ($mmol.L^{-1}$)	0.080	*5.2 ± 0.70	0.184	*5.5 ± 0.67	**0.000	0.44
30' Glucose ($mmol.L^{-1}$)	0.005	8.6 (7.8 – 9.6)	0.158	8.3 (7.3 – 9.1)	0.004	0.31
60' Glucose ($mmol.L^{-1}$)	0.012	9.2 (8.0 - 10.7)	0.177	8.7 (7.6 – 10.1)	0.025	--
90' Glucose ($mmol.L^{-1}$)	0.000	8.0 (6.8 – 9.3)	0.134	8.3 (7.2 – 9.7)	0.225	--
120' Glucose ($mmol.L^{-1}$)	0.000	7.0 (6.4 – 8.3)	0.009	7.9 (6.8 – 9.0)	0.000	0.29
HbA1c (%)	0.000	5.4 (5.2 – 5.7)	0.173	5.6 (5.3 – 5.8)	0.000	0.37
HIV number (pos/neg)	--	83 / 198	--	27 / 135	--	--

SW = Shapiro Wilk's test p-value; M-W = Mann-Whitney test p-value unless the p-value is marked with asterisks (**) = student t-test p-value; d-value = biological significance. Values are indicated as median (inter-quartile range) unless marked with an asterisk (*) = means ± standard deviation. M = male; F = female; BMI = body mass index; IQR = inter-quartile range; pos = positive; neg = negative; kg = kilogram; cm = centimetre, $kg.m^{-2}$ = kilogram per metre squared; $mmol.L^{-1}$ = millimole per litre; HbA1c = glycosylated haemoglobin; HIV = human immunodeficiency virus. The p-values and d-values indicated in red text are significant at a significance level < 0.05 and > 0.8 respectively.

The 0 and 30 min glucose levels were significantly higher within the high BMI subgroup, although not reaching the IGR cut-off levels (Zhou *et al.,* 2006). The significantly higher 120 min glucose level observed within the high BMI subgroup was above 7.8 $mmol.L^{-1}$ and was therefore indicative of glucose intolerance. The increased risk for T2D reported in overweight (BMI > 25 $kg.m^{-2}$) non-African individuals (Osman *et al.,* 1994) was also observed within the studied cohort. The individuals with a 120 min glucose value below 7.8 $mmol.L^{-1}$, who are included in the high BMI subgroup, are expected to develop glucose intolerance in the near future, unless significant weight reduction and diet restrictions are initiated. The distribution of the OGTT values within the two BMI subgroups is represented in Figure 6.26.

Figure 6.26 Graphic representation of the OGTT values stratified according to BMI



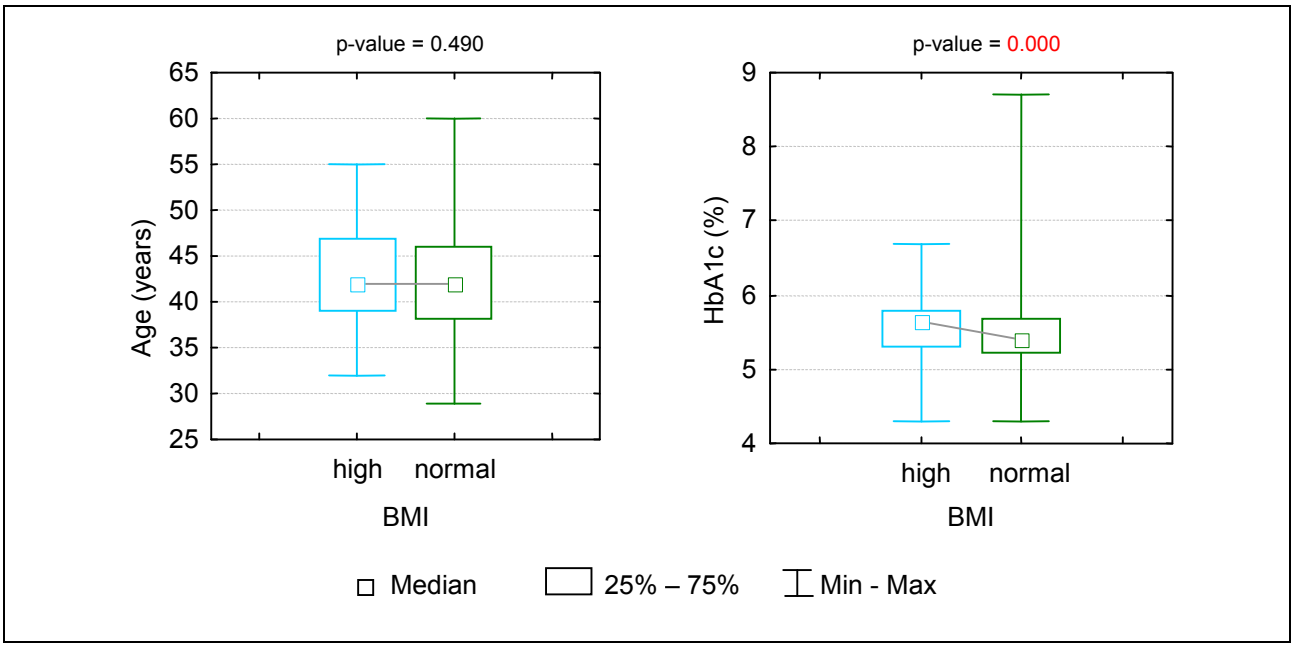
OGTT = oral glucose tolerance test; Min = minimum; Max = maximum; mmol.L^{-1} = millimole per litre; % = percentage.

The two BMI subgroups were significantly different in terms of height and weight (Table 6.24) as expected due to the fact that the BMI is a ratio derived from these two measurements. The difference in BMI between the two subgroups could be accounted for by the biologically significant increased weight of the individuals included in the high BMI subgroup. The statistically significant ($p = 0.000$) higher HbA1c value observed within the high BMI subgroup, compared to the HbA1c level of the normal BMI subgroup, is depicted in Figure 6.27. This difference is anticipated, when keeping in mind that a link was reported between obesity and increased T2D susceptibility (Osman *et al.,* 1994).

Although the HbA1c value is still within the normal limits (< 6.5%), a trend was observed towards poor glucose regulation in the high BMI subgroup, as indicated by the minimal glucose decrease in the last hour of the glucose curve as well as the high final 120 min glucose value, as represented in Figure 6.26. This necessitates future follow-up of the glucose tolerance and HbA1c levels of the individuals included in the study cohort. An evaluation of the future variation in HbA1c levels, according to the change in glucose

tolerance and body weight, will define the value of the HbA1c level as a screening tool for T2D risk in the black South African population. The similar distribution of the age and HbA1c levels within the normal and high BMI subgroups are graphically depicted in Figure 6.27.

Figure 6.27 Graphic representation of the age and HbA1c values stratified according to BMI



BMI = body mass index; HbA1c = glycosylated haemoglobin; Min = minimum; Max = maximum; % = percentage. p-value = Mann-Whitney test p-value, see Table 6.24. The p-values indicated in red text are significant at a significance level < 0.05.

The OR and 95% CI were calculated in order to determine the association between BMI and glucose tolerance, as presented in Table 6.25. The results indicate that there was an almost two fold increased risk within the studied cohort for being glucose intolerant in the presence of a high BMI. This statistically significant result supports the finding by Young *et al.* (1990) in Caucasian individuals, signifying a higher risk for T2D when overweight.

Table 6.25 Contingency table for the association between BMI and glucose tolerance

Observed numbers	IGT	NGT	Total	p-value
BMI > 25 kg.m^{-2}	51	111	162	0.005
BMI < 25 kg.m^{-2}	54	227	281	
Total	**105**	**338**	**443**	
OR (95% CI)	1.93 (1.23 - 3.01)			

A contingency table with two-tailed Fisher exact p-value as well as odds ratio and 95% confidence interval were determined. OR = odds ratio; CI = confidence interval; IGT = impaired glucose tolerance; NGT = normal glucose intolerance; BMI = body mass index. The p-values indicated in red text are significant at a significance level < 0.05.

6.2.3.3 Biochemical assays

HbA1c levels as well as HIV status [1] was evaluated in the individuals included in the investigated cohort. Subgroups were stratified according to the presence or absence of HIV infection as well as according to the HbA1c level under or above the reported T2D risk level of 6.5% (Chen *et al.,* 2004). The clinical parameters were compared within the normal and high HbA1c subgroups according to the statistical methods discussed in Section 5.5.2. The relevance of using the HbA1c value as a predictor of T2D risk was evaluated and a discussion thereof follows. The HIV status of the cohort as well as the collected clinical data within the HIV positive and negative subgroups respectively is discussed. Biological significance was determined for all statistically significant results via the protocol discussed in Section 5.5.4.

6.2.3.3.1 Glycosylated haemoglobin

The HbA1c levels were measured daily from the collected whole blood samples by using the method discussed in Section 5.4.5. Previous reports on samples from non-African individuals stated that the HbA1c level was a good indicator of T2D, even more so than the fasting glucose level on its own (Perry *et al.,* 2001). The HbA1c value is therefore useful in increasing the sensitivity of T2D screening tests in individuals at risk for the disease. The HbA1c values can however be influenced by various factors including haemolysis, drugs (*e.g*. sulphonamides) and diseases which cause cell breakage like haemolytic anaemia (Conrad and Gitelman, 2006).

The ADA does not include the HbA1c value in the screening method for T2D due to the various measuring methods available as well as the different proposed 'normal' ranges of the assay (Berkow *et al.,* 1992). The advantages of this test however include the fact that it can be determined during any health investigation and that it is not dependent on fasting status. An elevated HbA1c level is indicative of glucose intolerance whereas a normal value can either signify normal tolerance or well-controlled T2D (Berkow *et al.,* 1992). The observed parameters as well as the comparison results of the normal (< 6.5%) and high (> 6.5%) HbA1c subgroups are represented in Table 6.26.

[1] HIV testing was performed by the PURE-SA study and is used in this thesis with permission from Prof A Kruger, leader of the South African leg of the PURE study.

Table 6.26 Clinical parameters stratified according to HbA1c

Parameter	Normal HbA1c		High HbA1c		M-W p-value	d-value
	SW p-value	Median (IQR)	SW p-value	Median (IQR)		
Number (M/F)	--	437 (161 / 276)	--	6 (4 / 2)	--	--
Age (years)	0.000	42.0 (38.0 – 46.0)	0.444	44.0 (41.0 – 50.0)	0.157	--
Weight (kg)	0.000	59.0 (51.0 – 71.0)	0.639	60.5 (44.0 – 74.0)	0.912	--
Height (cm)	0.001	161.0 (156.0 – 167.0)	0.518	168.0 (163.0 – 173.0)	0.075	--
BMI ($kg.m^{-2}$)	0.000	21.9 (19.1 – 27.6)	0.244	20.1 (16.6 – 30.0)	0.391	--
0' Glucose ($mmol.L^{-1}$)	0.031	5.3 (4.9 – 5.7)	0.399	5.3 (4.6 – 6.2)	0.916	--
30' Glucose ($mmol.L^{-1}$)	0.001	8.5 (7.6 – 9.4)	0.242	10.0 (8.9 – 10.9)	0.012	0.95
60' Glucose ($mmol.L^{-1}$)	0.001	9.1 (7.8 – 10.3)	0.835	11.7 (11.1 – 12.7)	0.004	1.29
90' Glucose ($mmol.L^{-1}$)	0.000	8.1 (7.0 – 9.6)	0.305	11.0 (10.6 – 11.8)	0.016	0.83
120' Glucose ($mmol.L^{-1}$)	0.000	7.3 (6.5 – 8.5)	0.550	10.0 (9.3 – 12.1)	0.003	1.01
HbA1c	--	5.5 (5.2 – 5.7)	--	6.8 (6.7 – 6.8)	--	--
HIV number (pos/neg)	--	109 / 328	--	1 / 5	--	--

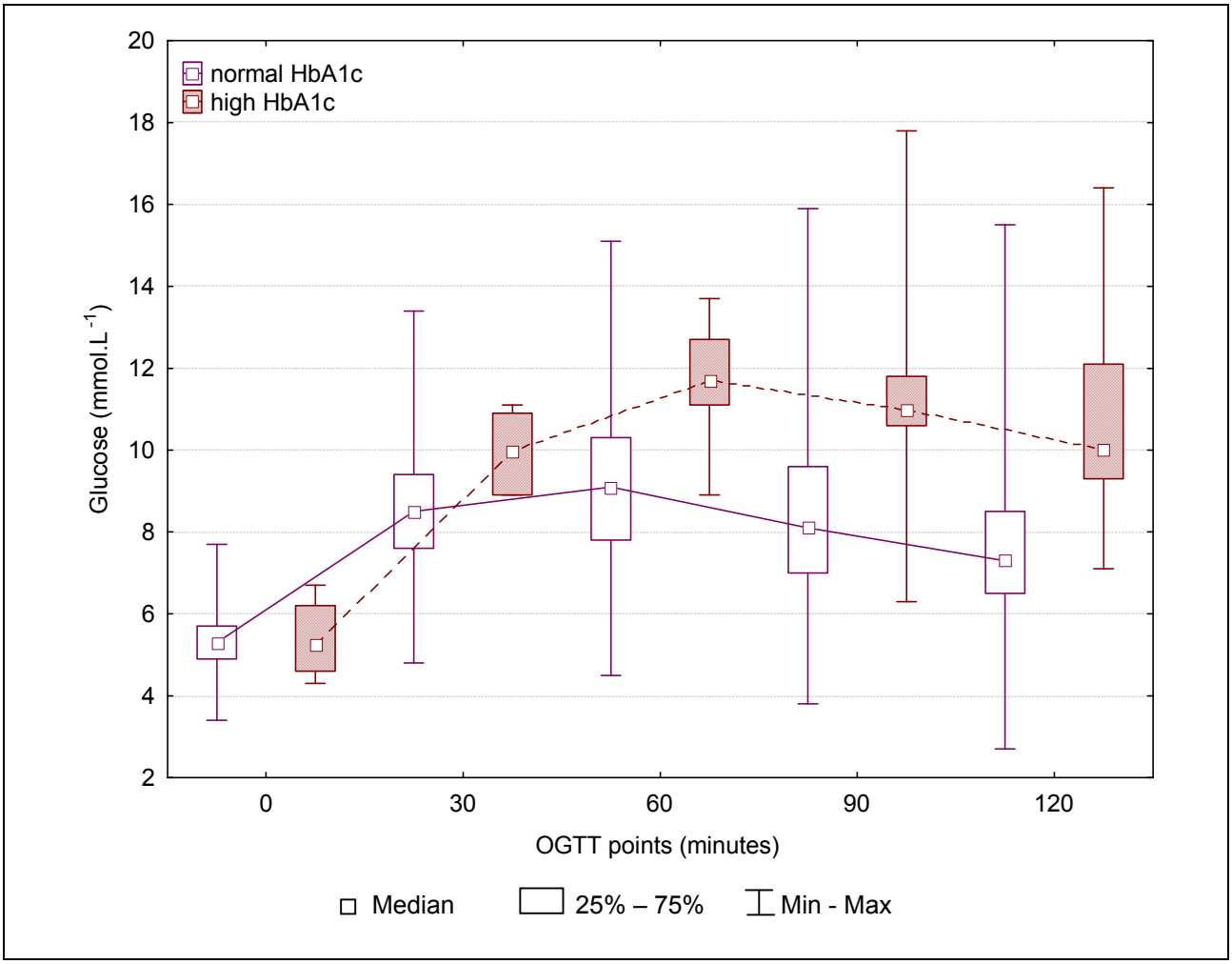
SW = Shapiro Wilk's test p-value; M-W = Mann-Whitney test p-value; d-value = biological significance; M = male; F = female; BMI = body mass index; IQR = inter-quartile range; pos = positive; neg = negative; kg = kilogram; cm = centimetre, $kg.m^{-2}$ = kilogram per metre squared; $mmol.L^{-1}$ = millimole per litre; HbA1c = glycosylated haemoglobin; HIV = human immunodeficiency virus. The p-values and d-values indicated in red text are significant at a significance level < 0.05 and > 0.8 respectively.

The number of individuals (n = 6) included in the high HbA1c subgroup is very small and therefore all comparison results for this part of the study require future confirmation within a bigger cohort. No statistically significant difference (p = 0.916) was observed on comparison of the 0 min glucose values between the two HbA1c subgroups, suggesting inaccurate assessment of diabetes risk when judged on fasting glucose only. In addition, it substantiates the efficacy of evaluating both the HbA1c and fasting glucose levels, which provides a better reflection of glucose use. The HbA1c level in both the impaired fasting glucose (Table 6.8) and the glucose intolerant (Table 6.9) subgroups were higher than in the normal glucose tolerance subgroup, but did not reach the widely used cut-off value of 6.5% (Chen *et al.,* 2004). It is therefore argued that this reported cut-off HbA1c value may require population specific evaluation prior to accurately assessing the value of this parameter as a T2D screening tool in the black South African population.

A trend towards higher glucose values at the other four OGTT points, as listed in Table 6.26, was observed within the high HbA1c subgroup, despite the small number of individuals included in this subgroup. This preliminary study will have to be followed up in order to confirm these results within a larger cohort. The 120 min glucose level was statistically and biologically significantly higher within the high HbA1c subgroup, ranging between 7.8 and 11.1 $mmol.L^{-1}$ and confirming glucose intolerance, as expected with a

high HbA1c level. This again reflects the appropriateness of including the HbA1c level in screening strategies for T2D risk within the black South African population. The entire GCS is however also indicative of IGR, as suggested by Zhou *et al.* (2006) and the 30, 60 and 120 min glucose values were above the described cut-off values for IGR (Table 2.3). Evaluating the other OGTT time intervals, instead of only the 0 min glucose value, would therefore prevent missing an imperative glucose intolerance diagnosis. The OGTT results observed within the investigated normal and high HbA1c subgroups are graphically represented in Figure 6.28.

Figure 6.28 Graphic representation of the OGTT values stratified according to HbA1c

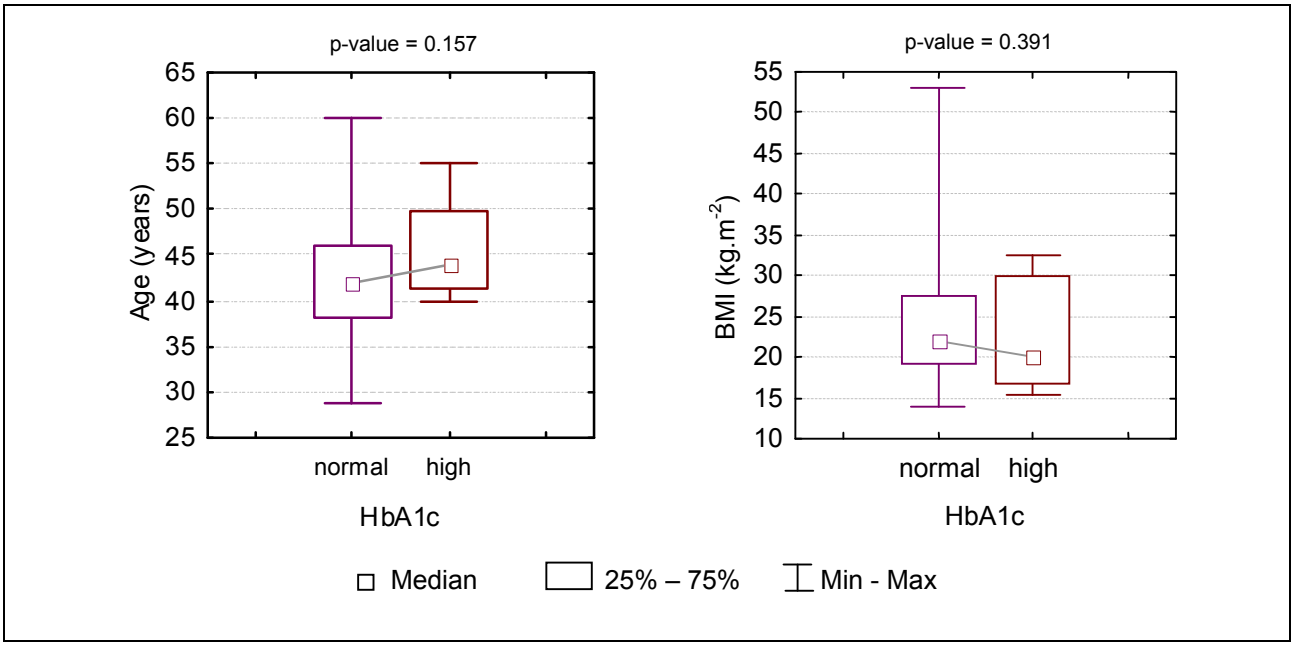


OGTT = oral glucose tolerance test; HbA1c = glycosylated haemoglobin; Min = minimum; Max = maximum; % = percentage; $mmol.L^{-1}$ = millimole per litre.

No significant difference was observed between the two studied HbA1c subgroups in terms of age or BMI with the resultant p-values being 0.157 and 0.391 respectively, as listed in Table 6.26. Any other differences observed between the two HbA1c subgroups, was therefore not influenced by age or BMI of the individuals included in the HbA1c

subgroups. The similar distribution of the BMI and HbA1c data is graphically depicted in Figure 6.29.

Figure 6.29 Graphic representation of the age and BMI values stratified according to HbA1c



BMI = body mass index; $kg.m^{-2}$ = kilogram per metre squared; HbA1c = glycosylated haemoglobin; Min = minimum; Max = maximum; % = percentage. p-value = Mann-Whitney test p-value, see Table 6.26.

The association between a high HbA1c level and glucose intolerance was determined in the investigated cohort and the contingency table results listed in Table 6.27. The OR and significant Fisher exact p-value indicated an almost 17 fold increased risk for glucose intolerance with the HbA1c level above 6.5% within the studied cohort similar to the results reported in other populations (Perry *et al.,* 2001). However, the small number of individuals included in the high HbA1c subgroup should be kept in mind, as it may be a factor exaggerating the level of statistical significance.

Table 6.27 Contingency table for the association between the HbA1c and glucose tolerance

Observed numbers	IGT	NGT	Total	p-value
HbA1c > 6.5%	5	1	6	0.003
HbA1c < 6.5%	100	337	437	
Total	**105**	**338**	**443**	
OR (95% CI)	16.8 (1.94 – 145)			

A contingency table with two-tailed Fisher exact p-value as well as odds ratio and 95% confidence interval were determined. OR = odds ratio; CI = confidence interval; IGT = impaired glucose tolerance; NGT = normal glucose intolerance. The p-values indicated in red text are significant at a significance level < 0.05.

Despite the widely distributed 95% CI, the lower cut-off value already indicates a significant 1.94 fold increase in IGT risk, which excludes random variation as a cause for the observed findings. The OR of 16.8 supports the clinical importance of the HbA1c level being a predictor of glucose tolerance and therefore T2D susceptibility in the black South African population. These findings are however based on subgroups that included only a few individuals, hence requiring confirmation within a larger cohort.

6.2.3.3.2 HIV status

HIV testing was performed by the PURE-SA study, and is used in this thesis with permission from Prof A Kruger, leader of the South African leg of the PURE study. The HIV status was determined and confirmed via the method discussed in Section 5.4.5. The individuals included in the studied cohort were all without evident clinical infection despite the fact that 110 were HIV positive. HIV infection, especially when in conjunction with lipodystrophy is linked to glucose intolerance and hyperglycaemia (Hadigan *et al.,* 2001) in non-African individuals. Since individuals included in the HIV positive subgroup had a normal median BMI value, this previously reported finding could however not be evaluated within the studied cohort. The parameters observed within the HIV subgroups are represented in Table 6.28 and was compared by means of a M-W statistical method, as discussed in Section 5.5.2.

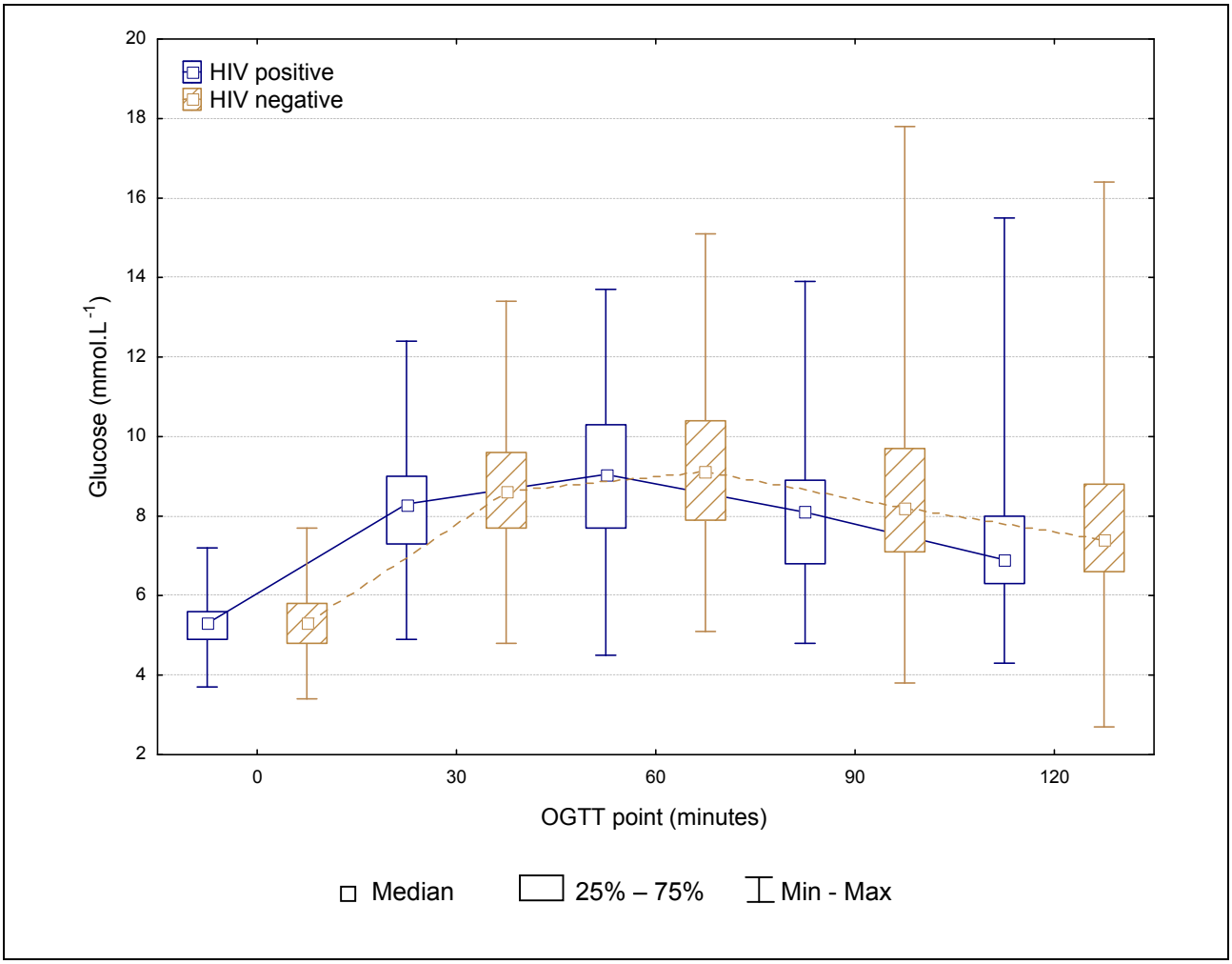
Table 6.28 Clinical parameters stratified according to HIV status

Parameter	HIV Positive		HIV Negative		M-W p-value	d-value
	SW p-value	Median (IQR)	SW p-value	Median (IQR)		
Number (M/F)	--	110 (43 / 67)	--	333 (122 / 211)	--	--
Age (years)	0.041	40.5 (36.0 – 44.0)	0.000	42.0 (39.0 – 47.0)	0.000	2.39
Weight (kg)	0.000	56.5 (49.0 – 64.0)	0.000	60.0 (52.0 – 73.0)	0.002	0.37
Height (cm)	0.818	162.0 (157.0 – 167.0)	0.000	161.0 (155.0 – 167.0)	0.469	--
BMI ($kg.m^{-2}$)	0.000	20.6 (18.7 – 24.5)	0.000	22.7 (19.3 – 28.7)	0.002	0.37
0' Glucose ($mmol.L^{-1}$)	0.045	5.3 (4.9 – 5.6)	0.299	5.3 (4.8 – 5.8)	0.312	--
30' Glucose ($mmol.L^{-1}$)	0.146	8.3 (7.3 – 9.0)	0.012	8.6 (7.7 – 9.6)	0.007	0.31
60' Glucose ($mmol.L^{-1}$)	0.574	9.1 (7.7 – 10.3)	0.001	9.1 (7.9 – 10.4)	0.497	--
90' Glucose ($mmol.L^{-1}$)	0.000	8.1 (6.8 – 8.9)	0.000	8.2 (7.1 – 9.7)	0.160	--
120' Glucose ($mmol.L^{-1}$)	0.000	6.9 (6.3 – 8.0)	0.000	7.4 (6.6 – 8.8)	0.025	--
HbA1c (%)	0.526	5.5 (5.2 – 5.8)	0.000	5.5 (5.2 – 5.7)	0.408	--

SW = Shapiro Wilk's test p-value; M-W = Mann-Whitney test p-value; d-value = biological significance. Values are indicated as median (inter-quartile range). M = male; F = female; BMI = body mass index; IQR = inter-quartile range; pos = positive; neg = negative; kg = kilogram; cm = centimetre, $kg.m^{-2}$ = kilogram per metre squared; $mmol.L^{-1}$ = millimole per litre; HbA1c = glycosylated haemoglobin; HIV = human immunodeficiency virus. The p-values and d-values indicated in red text are significant at a significance level < 0.05 and > 0.8 respectively.

The only statistically significant difference amongst the OGTT results was the higher 30 min glucose value ($p = 0.007$) in the HIV negative subgroup when compared to that of the HIV positive subgroup. However, this 30 min glucose value did not reach the IGR cut-off (> 9.7 mmol.L^{-1}), as supported by the biological significance value of 0.31. The OGTT results observed within the HIV subgroups are graphically depicted in Figure 6.30.

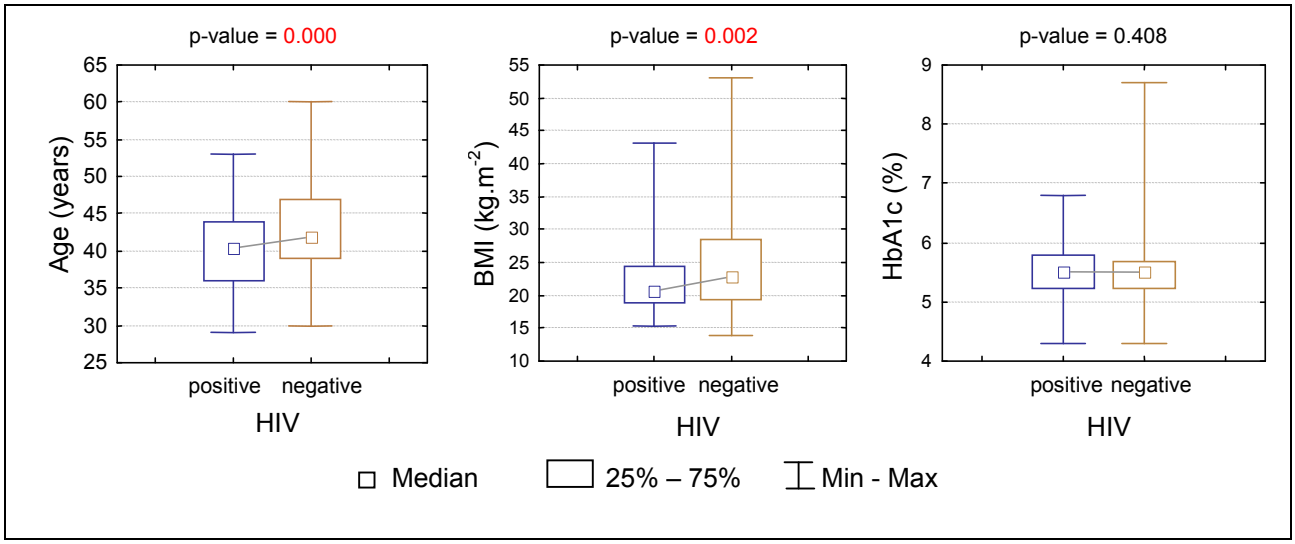
Figure 6.30 Graphic representation of the OGTT values stratified according to HIV status



HIV = human immunodeficiency virus; OGTT = oral glucose tolerance test; Min = minimum; Max = maximum; % = percentage; mmol.L^{-1} = millimole per litre.

According to the data listed in Table 6.28, HIV infection was confirmed in 26% and 24% of the male and female subgroups respectively. The average prevalence of HIV within this random black South African cohort was therefore 25%. The age, as well as the similarly distributed BMI and HbA1c values within the two subgroups is graphically represented in Figure 6.31.

Figure 6.31 Graphic representation of the age, BMI and HbA1c values stratified according to HIV status



HIV = human immunodeficiency virus; BMI = body mass index; $kg.m^{-2}$ = kilogram per metre squared; HbA1c = glycosylated haemoglobin; Min = minimum; Max = maximum; % = percentage. p-value = Mann-Whitney test p-value, see Table 6.28. The p-values indicated in red text are significant at a significance level < 0.05.

Statistically, a significantly (p = 0.000) higher age was observed within the HIV negative subgroup when compared to the median age of the HIV positive subgroup, as listed in Table 6.28. Neither one of the subgroups, however, exceeded the T2D risk age of 45 years, similar to that reported by DeFronzo *et al.* (1991). The statistically significant weight difference between the two subgroups, represented in Table 6.28, resulted in a significantly increased BMI within the HIV negative subgroup (p = 0.002). As supported by the biological significance value, the BMI in both subgroups was still below the obesity cut-off level of 25 $kg.m^{-2}$ reported in non-Africans (Berkow *et al.,* 1992). The HIV positive subgroup had a median BMI of 20 $kg.m^{-2}$ that is at the lower end of the normal BMI range (Berkow *et al.,* 1992).

6.2.4 Genotype

T2D susceptibility is the collective result of both environmental factors (Jun *et al.,* 1999; Sobngwi *et al.,* 2001), and the genetic background of an individual. These factors do not only determine disease risk on their own, but also function in combination with each other (Jun *et al.,* 1999). The aim of this study was to screen the black South African population for T2D susceptibility loci that have previously been reported in non-African populations. Furthermore, these genotypes were evaluated in context of the T2D risk phenotype. The screened T2D susceptibility loci investigated in this cohort are located in IRS-1, IRS-2, PPARγ2 and CAPN10 genes in the investigated cohort.

Jellema *et al.* (2003) reported that individuals with T2D had a 1.25 fold increased probability of harbouring the A allele at the IRS-1 gene locus. In addition, Stumvoll *et al.* (2001a) suggested an association between normal glucose tolerant individuals harbouring the IRS-1 A allele and β-cell dysfunction. Inheritance was therefore assumed to be dominant and participants with either a heterozygous or homozygous mutant genotype (X/A) were combined for analysis within the studied cohort. The clinical parameters within the IRS-1 X/A genotype subgroup were subsequently compared to that of the homozygous WT (G/G) subgroup.

The involvement of the A allele at the IRS-2 locus in T2D susceptibility is controversial (Stumvoll *et al.,* 2005). Therefore, the association between the IRS-2 A allele and T2D risk was evaluated within the studied cohort. As with IRS-1, the combined clinical parameters of the heterozygous and homozygous mutant genotypes (X/A) were compared with that of the homozygous WT (G/G) subgroup.

Various polymorphisms in the CAPN10 gene were previously associated with T2D (Horikawa *et al.,* 2000). The association between T2D risk and one of these polymorphisms, namely UCSNP44, was evaluated within the studied cohort. An increase in plasma glucose was observed in individuals included in the non-TT CAPN10 genotype group i.e. thus individuals harbouring either the CC or TC genotypes, as reported by Wang *et al.* (2002) and an association was confirmed between the homozygous T allele genotype and a monophasic GCS (Tschritter *et al.,* 2003). Therefore, study participants were divided into two genotypic subgroups depending on whether they harboured the UCSNP44 C allele or not. Individuals with a heterozygous or homozygous mutant genotype were included in the X/C subgroup and those harbouring the homozygous wild type genotype were included within the T/T subgroup.

Increased insulin sensitivity has been reported in individuals harbouring a G allele at the PPARγ2 locus (Stumvoll *et al.,* 2001b). In the investigated cohort, clinical data was compared between a subgroup including participants without the G allele, therefore harbouring the homozygous wild type genotype (C/C), and a subgroup of participants harbouring the G allele, including both the heterozygous and homozygous mutant genotypes (X/G). The number of participants harbouring the abovementioned genotypes for the various susceptibility loci investigated in this study are listed in Table 6.29.

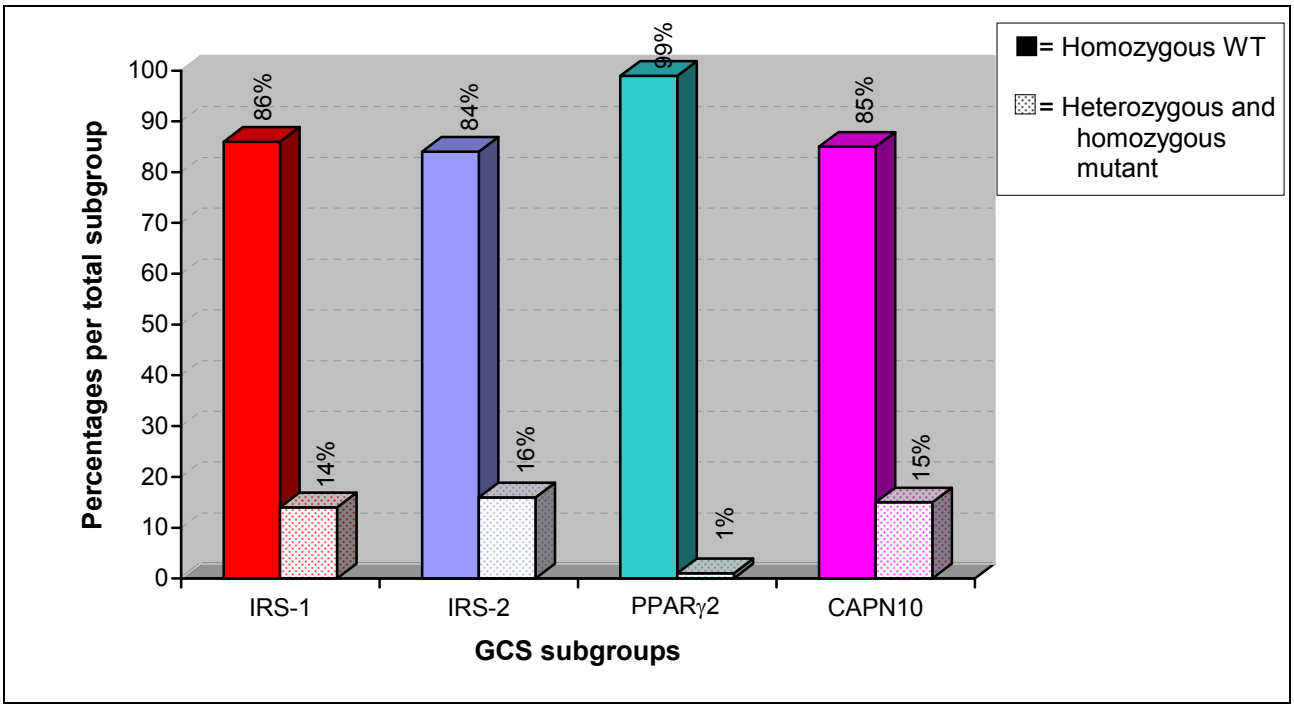
Table 6.29 Number of individuals observed in the genotypic subgroups

Gene	Homozygous WT		Heterozygous		Homozygous mutant	
IRS-1	G/G	383 (143 / 240)	G/A	57 (20 / 37)	A/A	3 (2 / 1)
IRS-2	G/G	372 (136 / 236)	G/A	71 (29 / 42)	A/A	0
PPARγ2	C/C	437 (163 / 274)	C/G	5 (2 / 3)	G/G	1 (0 / 1)
CAPN10	T/T	379 (146 / 233)	T/C	62 (19 / 43)	C/C	2 (0 / 2)

Numbers are presented as total (male/female). Total number of individuals included in the genotype analysis is 443. G = guanine; C = cytosine; T = thymine; A = adenine; IRS-1 = insulin receptor substrate-1; IRS-2 = insulin receptor substrate-2; PPARγ2 = peroxisome proliferator-activated receptor gamma 2, CAPN10 = calpain 10, WT = wild type.

Of the total cohort of 433, 14% of the participants harboured at least one A allele at the IRS-1 locus whereas 16% were heterozygous at the IRS-2 locus with none harbouring the homozygous mutant genotype. Only 1% of the total number of participants harboured the G allele at the PPARγ2 locus while 85% were homozygous wild type at the CAPN10 locus. These observed percentages in each of the genotype subgroups are graphically depicted in Figure 6.32.

Figure 6.32 Percentages observed in each of the genotype subgroups



Total number of individuals included in the genotype analysis is 443. IRS-1 = insulin receptor substrate-1; IRS-2 = insulin receptor substrate-2; PPARγ2 = peroxisome proliferator-activated receptor gamma 2, CAPN10 = calpain 10, WT = wild type; GCS = glucose curve shape.

6.2.4.1 Hardy-Weinberg equilibrium

Genotypic frequencies were analysed via the chi square test and used to determine whether the investigated genotypes were in H-W equilibrium. The results are listed in Table 6.30. If the cohort was in H-W equilibrium, it could be hypothesised that the differences in genotypic frequencies were most likely brought about by association of a

specific genotype to disease susceptibility rather than by the parameters influencing H-W equilibrium, as discussed in Section 5.5.1. The chi square test results for goodness-of-fit to the H-W proportions of the study population for the IRS-1, IRS-2, PPARγ2 and CAPN10 genes are listed in Table 6.30.

Table 6.30 Chi square test for goodness-of-fit to the H-W proportions of the study population for the IRS-1, IRS-2, PPARγ2 and CAPN10 genes

Genotypes	Observed (O)	Frequency	Expected (E)	$\chi^2 = (O-E)^2/E$	p-value
IRS-1 gene					
G/G	383	0.86	382.24	0.00	0.861
G/A	57	0.13	58.52	0.04	
A/A	3	0.01	2.24	0.26	
Total	443	1.00	443.00	0.30	
IRS-2 gene					
G/G	372	0.84	374.84	0.02	0.186
G/A	71	0.16	65.31	0.50	
A/A	0	0.00	2.84	2.84	
Total	443	1.00	443.00	3.36	
PPARγ2 gene					
C/C	437	0.99	436.03	0.00	0.000
C/G	5	0.01	6.94	0.54	
G/G	1	0.00	0.03	34.19	
Total	443	1.00	443.00	34.74	
CAPN10 gene					
T/T	379	0.86	379.46	0.00	0.951
T/C	62	0.14	61.08	0.01	
C/C	2	0.00	2.46	0.09	
Total	443	1.00	443.00	0.10	

χ^2 = Chi square value; O = observed numbers; E = expected numbers; p = frequency of wild type allele; q = frequency of mutant allele; H-W = Hardy-Weinberg; CAPN10 = calpain 10; IRS-1 = insulin receptor substrate-1; IRS-2 = insulin receptor substrate-2; PPARγ2 = peroxisome proliferator-activated receptor gamma 2; G = guanine; A = adenine; C = cytosine; T = thymine. The p-values indicated in red text are significant at a significance level < 0.05.

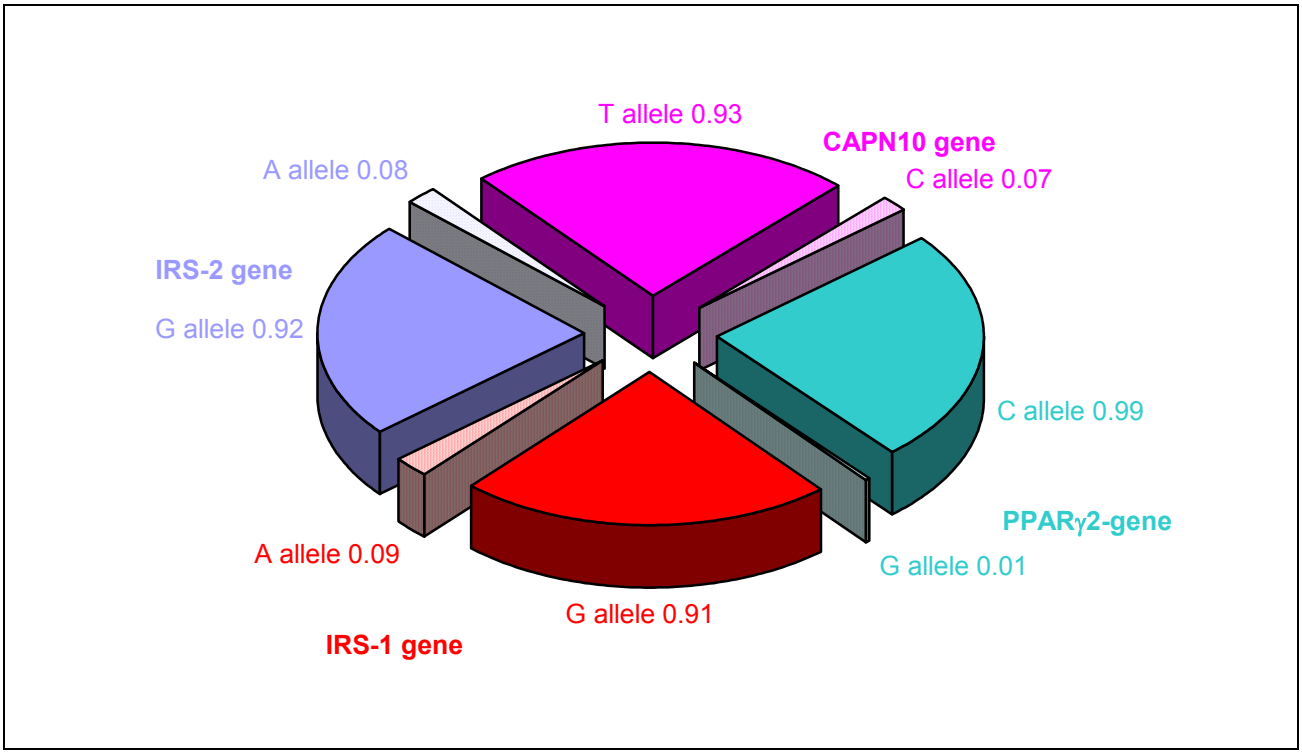
The G3494A alteration in the IRS-1 gene was observed three times more often in Danish Caucasian diabetic patients, compared to non-diabetic controls (Almind, 1993). In the studied non–diabetic cohort, the allelic frequencies for the screened IRS-1 gene alteration were 0.91 for the G allele and 0.09 for the A allele (Equation 5.3). Since the risk A allele is more frequent in T2D individuals (Almind, 1993), the observed low frequency in the non-diabetic cohort is expected. The p-value of 0.861 in Table 6.30 indicates that the IRS-1 gene is in H-W equilibrium.

Various levels of involvement of the IRS-2 gene in T2D susceptibility have been reported in a number of different populations (Bernal *et al.,* 1998 and Stumvoll *et al.,* 2001a). Allele frequencies of 0.92 and 0.08 for the G allele and A allele of IRS-2 were calculated respectively. As listed in Table 6.30, the non-significant Chi square test p-value of 0.186 is indicative of the data being in H-W equilibrium. Reports to date have demonstrated that this alteration is not associated with T2D in Caucasian individuals. The low frequency of the IRS-2 A allele in the studied black South African cohort also indicated the rarity of the risk allele in this non-diabetic cohort. Further study to evaluate the presence of both the IRS-1 and IRS-2 A alleles in a diabetic cohort would elucidate the actual involvement of these polymorphisms in South Africans.

Equation 5.3 was used to calculate the allelic frequencies of the CAPN10 gene UCSNP44 alteration as presented in Figure 6.33. The frequencies were 0.93 for the common T allele and 0.07 for the risk C allele in the studied cohort. Due to the inclusion of only non-diabetic individuals, the low risk C allele frequency was expected and in line with previous reports associating the C allele with increased glucose levels (Wang *et al.,* 2002). The non-significant p-value of 0.951 determined by Chi square testing (Table 6.30) for the CAPN10 genotypes again indicated that the data adhered to the assumptions of H-W equilibrium. Thus, the factors involved in H-W equilibrium, as discussed in Section 5.5.1, therefore had no influence on the IRS-1, IRS-2 and CAPN10 genotype frequencies in the investigated cohort.

For the C8492G alteration in the PPARγ2 gene, the calculated allelic frequencies (Equation 5.3) were 0.99 for the C allele and 0.01 for the G allele, as presented in Figure 6.33. The PPARγ2 G allele in the investigated cohort was therefore rare, similar to the low frequency of less than 0.05 reported in Korean and Asian populations (Rhee *et al*., 2006). Li *et al*. (2003) also reported a low G allele frequency of less than 0.02 in a screened black population.

Figure 6.33 Allele frequencies observed in each of the four genotype subgroups



CAPN10 = calpain 10; IRS-1 = insulin receptor substrate-1; IRS-2 = insulin receptor substrate-2; PPARγ2 = peroxisome proliferator-activated receptor gamma 2, A = adenine; G = guanine; C = cytosine; T = thymine. Each quarter of the pie represents the allele distribution of the specific variant in the 443 individuals investigated.

The chi square test result for the PPARγ2 alteration, as presented in Table 6.30, indicates that the genotypes were not in H-W equilibrium ($p < 0.05$). The resultant chi square statistic of 34.19 is most likely due to the fact that the G allele was present at such a low frequency (0.01) in the studied cohort. It is unlikely that any of the other factors affecting H-W equilibrium, namely non-random mating, genetic drift, mutation and natural selection, played a role in the reported lack of equilibrium within this population.

6.2.5 Phenotype and genotype results observed in various subgroups

The clinical parameters were evaluated within the various genotypic subgroups, as outlined in Section 5.4, and will be discussed in the sections that follow. Individuals were categorised according to GCS, glucose tolerance, HbA1c level as well as physical environment and the association with the respective genotypes determined.

6.2.5.1 Genotype subgroups

The collected phenotypic data including the OGTT results were evaluated in the context of the genotypic data from the four previously reported T2D susceptibility genes, *i.e.* IRS-1, IRS-2, PPARγ2 and CAPN10. For each of the four genes, the individuals were divided

into two subgroups according to genotype, as discussed in Section 6.2.4. The first genotypic subgroup contained individuals harbouring the homozygous WT genotype, while the second subgroup consisted of individuals with both the heterozygous and the homozygous mutant genotype. Prior to analysing the continuous clinical data, the Shapiro Wilk's test was used to determine the distribution of the data, as discussed in Section 5.5.2. A significant p-value ($p < 0.05$) did not support the hypothesis of normality, in which case the subgroups were analysed via a non-parametric Mann-Whitney test. The non-parametric statistical methods were used when either one or both of the data sets being compared were not distributed normally. Furthermore, non-parametric descriptive statistics, *i.e.* median and IQR, were used. In cases where the data in both subgroups were normally distributed, a parametric Student t-test, as discussed in Section 5.5.2, was used for comparisons. Descriptive statistics for these datasets were therefore indicated as means ± SD and are designated with an asterisk (*) and the resulting p-values for the student t-test indicated by two asterisks (**) in the sections to follow.

6.2.5.1.1 Insulin receptor substrate-1 gene

The observed clinical parameters within the IRS-1 genotypic subgroups are presented in Table 6.31, indicating that neither the phenotypic parameters nor the observed OGTT results differed significantly between the two IRS-1 genotypic subgroups. Clinical parameters that increase the risk for T2D, such as glucose intolerance, a high BMI, female gender and old age, were not significantly associated with the previously reported (Jellema *et al.,* 2003) risk X/A genotypic subgroup. The studied South African cohort thus differs from reported non-African individuals in terms of the association of the IRS-1 genotype with reported T2D risk factors.

The OGTT results observed within the IRS-1 X/A genotype subgroup do not illustrate any trend towards glucose intolerance according to either the diagnostic T2D criteria suggested by the WHO (see Table 2.2) or the IGR cut-off values reported by Zhou *et al.* (2006). Changes in glucose tolerance status as well as the possible progression to diabetes will have to be evaluated in a follow-up study, taking into account the reported glucose tolerance results in non-Africans (Jellema *et al.,* 2003), that suggested a 25% increased risk for developing T2D when harbouring the X/A genotype. This increased risk for T2D in individuals harbouring the A/A genotype, was in addition reported (Jellema *et al.,* 2003) to be associated with an increased BMI. In the investigated South African

cohort however, no significant difference was observed when comparing body weight within the two IRS-1 genotypic subgroups.

Table 6.31 Clinical parameters stratified according to the IRS-1 genotypes

Parameter	G/G genotype		X/A genotype		M-W p-value
	SW p-value	Median (IQR)	SW p-value	Median (IQR)	
Number (M/F)	--	383 (143 / 240)	--	60 (22 / 38)	--
Age (years)	0.000	42.0 (38.0-46.0)	0.144	42.5 (38.0-47.0)	0.795
Weight (kg)	0.000	59.0 (51.0-71.0)	0.192	61.0 (50.5-69.5)	0.995
Height (cm)	0.002	161.0 (156.0-167.0)	0.011	160.5 (157.0-169.0)	0.694
BMI ($kg.m^{-2}$)	0.000	21.9 (19.2-27.9)	0.006	22.2 (18.6-27.2)	0.688
0' Glucose ($mmol.L^{-1}$)	0.130	*5.3 ± 0.7	0.121	*5.4 ± 0.6	**0.234
30' Glucose ($mmol.L^{-1}$)	0.006	8.5 (7.6-9.4)	0.287	8.6 (7.9-9.7)	0.248
60' Glucose ($mmol.L^{-1}$)	0.011	9.1 (7.8-10.4)	0.014	8.9 (8.2-10.1)	0.882
90' Glucose ($mmol.L^{-1}$)	0.000	8.1 (6.9-9.6)	0.001	8.2 (7.3-9.5)	0.466
120' Glucose ($mmol.L^{-1}$)	0.000	7.3 (6.5-8.6)	0.003	7.2 (6.6-8.2)	0.466
HbA1c	0.000	5.5 (5.2-5.7)	0.464	5.6 (5.3-5.9)	0.218
HIV (pos/neg)	--	383 (100/283)	--	60 (10/50)	--

IRS-1 = insulin receptor substrate-1; SW = Shapiro Wilk's test p-value; M-W = Mann-Whitney test p-value, unless marked with a double asterisk (**) = t-test p-value; d = biological significance. Values are indicated as median (inter-quartile range) unless marked with an asterisk (*) = means ± standard deviation. G/G = homozygous wild type; X/A = heterozygous plus homozygous mutant; M = male; F = female; BMI = body mass index; IQR = inter-quartile range; pos = positive; neg = negative; kg = kilogram; cm = centimetre, $kg.m^{-2}$ = kilogram per metre squared; $mmol.L\text{-}1^{-1}$ = millimole per litre; HbA1c = glycosylated haemoglobin; HIV = human immunodeficiency virus. The p-values indicated in red text are significant at a significance level < 0.05.

Contingency table analyses as well as odds ratios and 95% CIs, were calculated via statistical methods discussed in Section 5.5.3. The results that were used to determine the association between the IRS-1 genotype and glucose tolerance, are listed in Table 6.32. A non-significant Fisher exact p-value (0.518) was generated on evaluating this association. The IRS-1 genotype was therefore not a good predictor of impaired glucose in the investigated black South African cohort.

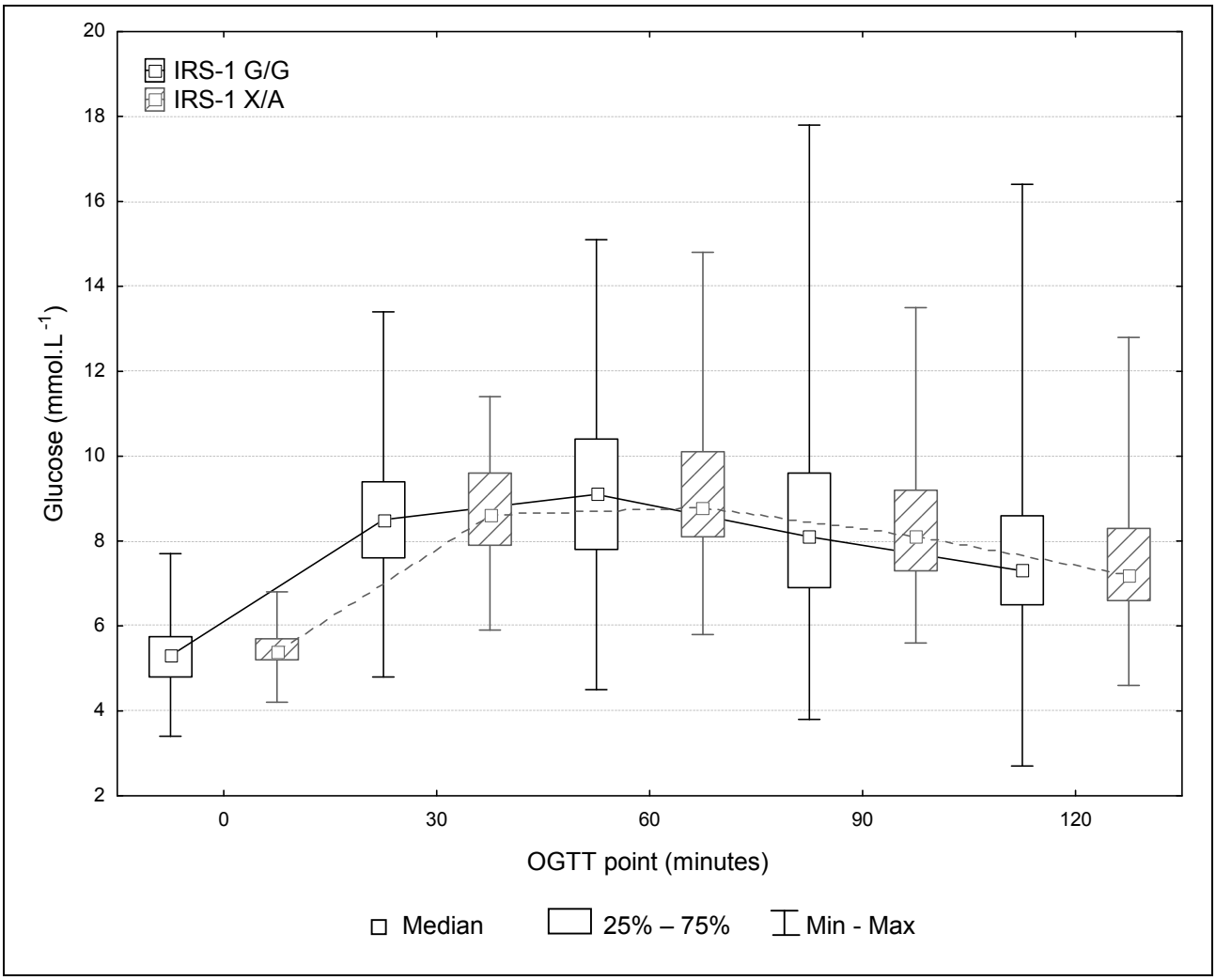
Table 6.32 Contingency table summary for the association between the IRS-1 genotype and glucose tolerance

Genotype	IGT	NGT	Total	Fisher exact p-value	OR (95% CI)
IRS-1 gene					
X/A	12	48	60	0.518	0.77 (0.39 - 1.53)
G/G	93	290	383		
Total	**105**	**338**	**443**		

A contingency table including the two-tailed Fisher exact p-value as well as the odds ratio and 95% confidence interval was determined. IGT = impaired glucose tolerance; NGT = normal glucose tolerance; OR = odds ratio; CI = confidence interval; IRS -1 = insulin receptor substrate-1; A = adenine; G = guanine. The X/A subgroup includes both heterozygous and homozygous mutant genotypes.

The IRS-1 X/A genotype was thus not associated with any of the T2D risk factors, previously reported in other populations by Jellema *et al.* (2003). The OGTT values of the two IRS-1 genotypic subgroups were not significantly different, as indicated in Table 6.31 and are graphically represented in Figure 6.34.

Figure 6.34 Graphic representation of the OGTT values stratified according to the IRS-1 genotypes



IRS-1 = insulin receptor substrate-1; OGTT = oral glucose tolerance test; G = guanine; A = adenine; mmol.L^{-1} = millimole per litre; Min = minimum; Max = maximum; % = percentage; G/G = homozygous wild type genotype; X/A = heterozygous plus homozygous mutant genotypes.

The association of the IRS-1 genotypes with the HbA1c level was determined according to the methods described in Section 5.5.3, with the results listed in Table 6.33. The Fisher exact p-value was not significant (p = 0.585) and the wide 95% CIs indicate that the IRS-1 genotype was not a good predictor of a high HbA1c level within the studied cohort.

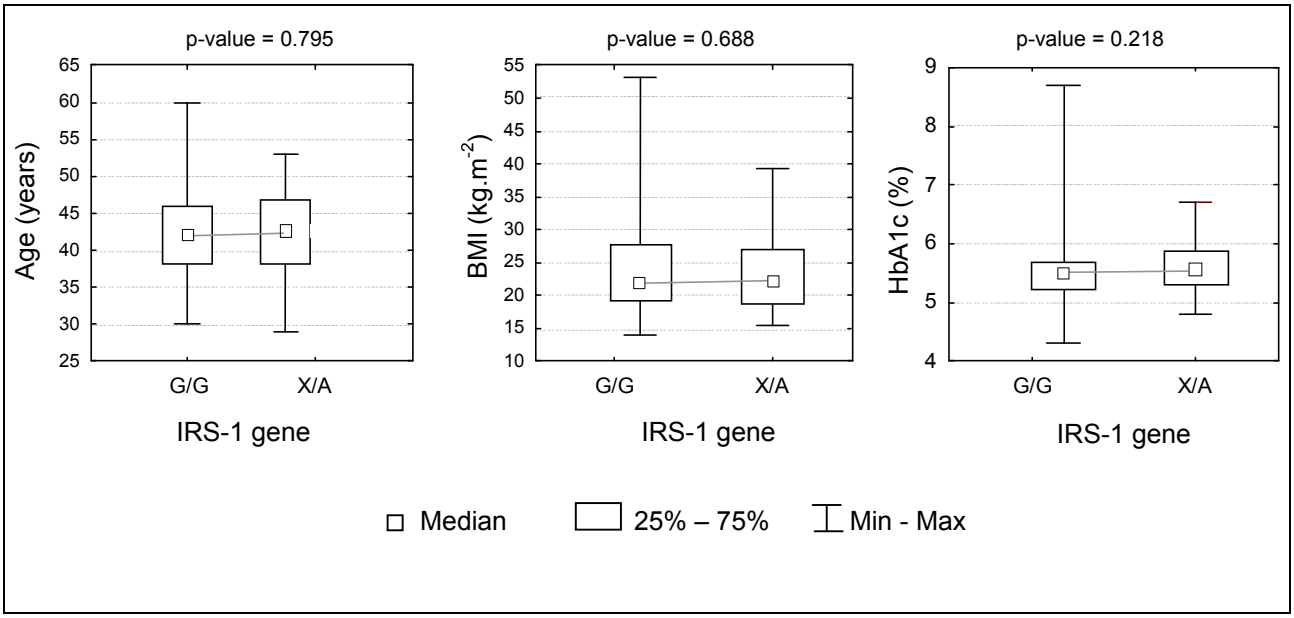
Table 6.33 Contingency table summary for the association between the IRS-1 genotype and HbA1c

Genotype	High HbA1c	Normal HbA1c	Total	Fisher exact p-value	OR (95% CI)
IRS-1 gene					
X/A	1	59	60	0.585	1.28 (0.14 - 11.1)
G/G	5	378	383		
Total	**6**	**437**	**443**		

A contingency table with two-tailed Fisher exact p-value as well as odds ratio and 95% confidence interval were determined. HbA1c = glycosylated haemoglobin; OR = odds ratio; CI = confidence interval; IRS-1 = insulin receptor substrate-1; A = adenine; G = guanine. The X/A subgroup includes both heterozygous and homozygous mutant genotypes.

The age, BMI and HbA1c values were compared between the two IRS-1 genotype subgroups and are graphically represented in Figure 6.35. A similarity in distribution of this data within the two IRS-1 genotypic subgroups is observed.

Figure 6.35 Graphic representation of the age, BMI and HbA1c values stratified according to the IRS-1 genotypes



IRS-1 = insulin receptor substrate-1; G = guanine; A = adenine; BMI = body mass index; $kg.m^{-2}$ = kilogram per metre squared; HbA1c = glycosylated haemoglobin; Min = minimum; Max = maximum; % = percentage. p-value = Mann-Whitney test p-value, see Table 6.31.

The differences observed between the two IRS-1 genotypic subgroups in terms of age, BMI or HbA1c were not statistically significant. Despite the much higher maximum BMI and HbA1c levels in the G/G subgroup, compared to the highest BMI and HbA1c levels in the X/A subgroup, the median and IQRs were similar for both the IRS-1 genotypic subgroups. As discussed in Section 6.2.4.1, the allele frequency of the risk A allele was only 0.09 (Figure 6.33), as was expected within the non-diabetic cohort when keeping in mind the similar low frequency observed in Caucasian non-diabetic individuals (Almind, 1993). Furthermore, the reported T2D risk associated with the A allele was only reported

in obese individuals (Jellema *et al.,* 2003). This compounded risk determined by Jellema *et al.* could however not be validated within this black South African cohort since the BMI (Table 6.31) of the individuals with a X/A genotype were within the normal limits. The association between the IRS-1 polymorphism and the prevalence of T2D therefore remains controversial as reflected by the Florez *et al*. (2004) report.

6.2.5.1.2 Insulin receptor substrate-2 gene

The clinical parameters of the IRS-2 genotype subgroups were compared via the statistical procedures described in Section 5.5.2. The results are presented in Table 6.34.

Table 6.34 Clinical parameters stratified according to the IRS-2 genotypes

Parameter	G/G genotype		X/A genotype		M-W p-value
	SW p-value	Median (IQR)	SW p-value	Median (IQR)	
Number (M/F)	--	372 (136 / 236)	--	71 (29 / 42)	--
Age (years)	0.000	42.0 (38.0 – 46.0)	0.058	42.0 (40.0 – 48.0)	0.219
Weight (kg)	0.000	59.0 (51.0 – 71.0)	0.000	60.0 (53.0 – 71.0)	0.247
Height (cm)	0.003	161.0 (155.0 – 167.0)	0.023	162.0 (158.0 – 170.0)	0.108
BMI ($kg.m^{-2}$)	0.000	22.0 (19.1 – 27.5)	0.000	21.8 (19.0 – 28.6)	0.702
0' Glucose ($mmol.L^{-1}$)	0.063	5.3 (4.8 – 5.8)	0.014	5.3 (4.9 – 5.7)	0.940
30' Glucose ($mmol.L^{-1}$)	0.007	8.6 (7.6 – 9.6)	0.301	8.5 (7.4 – 9.4)	0.471
60' Glucose ($mmol.L^{-1}$)	0.003	9.1 (7.9 – 10.4)	0.496	9.1 (7.7 – 10.2)	0.584
90' Glucose ($mmol.L^{-1}$)	0.000	8.1 (7.0 – 9.6)	0.001	8.1 (6.9 – 9.8)	0.809
120' Glucose ($mmol.L^{-1}$)	0.000	7.3 (6.5 – 8.5)	0.000	7.3 (6.3 – 8.9)	0.789
HbA1c (%)	0.000	5.5 (5.2 - 5.8)	0.059	5.5 (5.2 – 5.7)	0.394
HIV number (pos/neg)	--	372 (96 / 276)	--	71 (14 / 57)	--

IRS-2 = insulin receptor substrate-2; SW = Shapiro Wilk's test p-value; M-W = Mann-Whitney test p-value; d = biological significance. Values are indicated as median (inter-quartile range). G = guanine; A = adenine; G/G = homozygous wild type genotype; X/A = heterozygous plus homozygous mutant genotypes; M = male; F = female; BMI = body mass index; IQR = inter-quartile range; pos = positive; neg = negative; kg = kilogram; cm = centimetre, $kg.m^{-2}$ = kilogram per metre squared; $mmol.L^{-1}$ = millimole per litre; HbA1c = glycosylated haemoglobin; HIV = human immunodeficiency virus. The p-values indicated in red text are significant at a significance level < 0.05.

Similar to the IRS-1 results, there was no significant difference upon comparison of the clinical data between the two IRS-2 genotypic subgroups. The glucose levels of all the OGTT intervals in both genotype subgroups were below the reported cut-off levels for IGR (Zhou *et al.*, 2006). The controversial association between the IRS-2 genotype and glucose tolerance in previous reports (Bernal *et al.,* 1989; 'T Hart *et al.,* 2002) was evaluated within the studied cohort via statistical methods discussed in Section 5.5.3 and the contingency table results listed in Table 6.35.

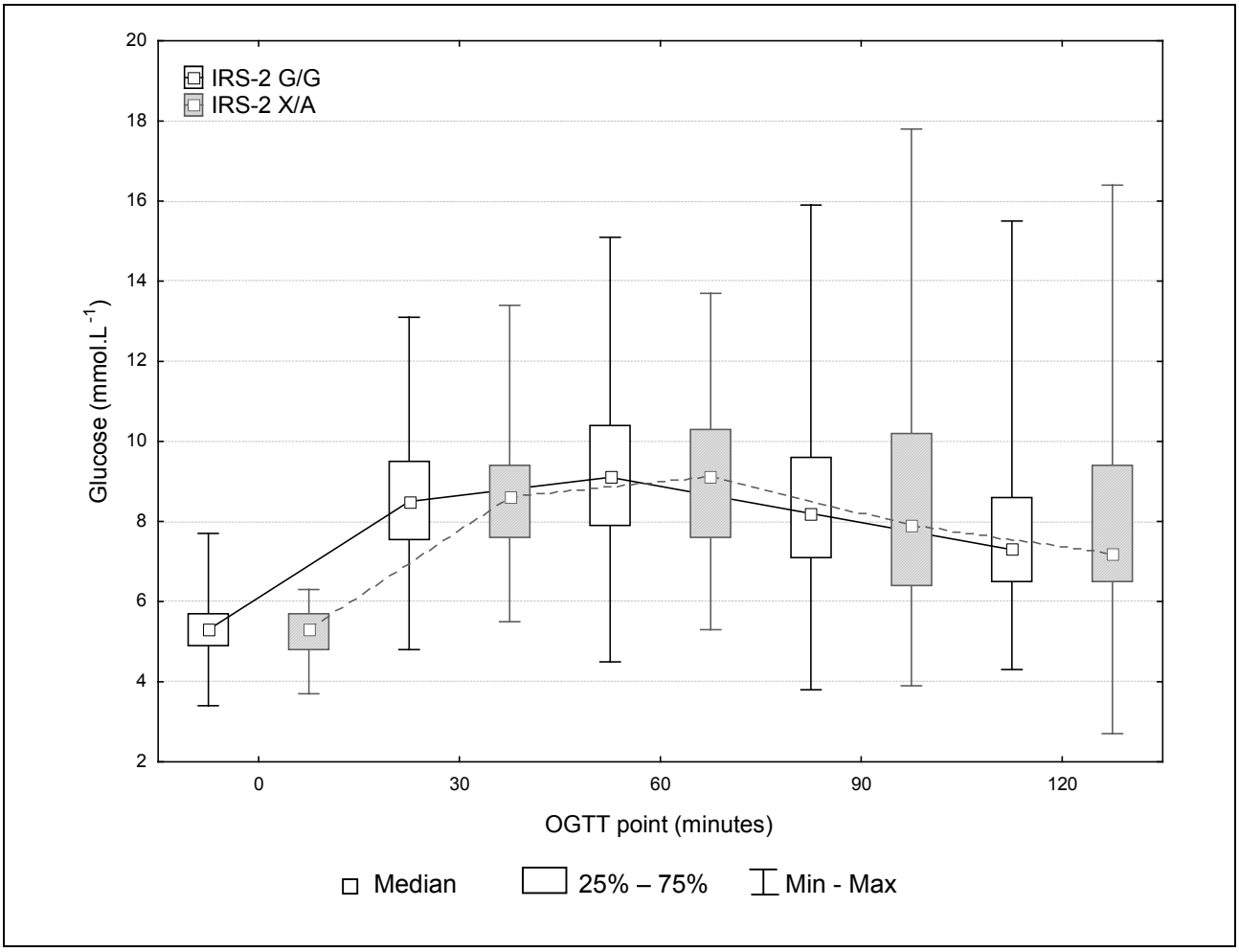
Table 6.35 Contingency table summary for the association between the IRS-2 genotype and glucose tolerance

Genotype	IGT	NGT	Total	Fisher exact p-value	OR (95% CI)
IRS-2 gene					
X/A	20	51	71	0.361	1.32 (0.74 - 2.34)
G/G	85	287	372		
Total	**105**	**338**	**443**		

A contingency table with two-tailed Fisher exact p-value as well as odds ratio and 95% confidence interval were determined. IGT = impaired glucose tolerance; NGT = normal glucose tolerance; OR = odds ratio; CI = confidence interval; IRS-2 = insulin receptor substrate-2; A = adenine; G = guanine. The X/A subgroup includes both heterozygous and homozygous mutant genotypes.

Figure 6.36 depicts the OGTT values stratified according to the IRS-2 genotypes. As observed from this graphic representation the distribution of OGTT data between the two IRS-2 genotypic subgroups are consistently similar at the various time intervals.

Figure 6.36 Graphic representation of the OGTT values stratified according to the IRS-2 genotypes



IRS-2 = insulin receptor substrate-2; OGTT = oral glucose tolerance test; G = guanine; A = adenine; mmol.L^{-1} = millimole per litre; Min = minimum; Max = maximum; % = percentage; G/G = homozygous wild type genotype; X/A = heterozygous plus homozygous mutant genotypes.

The non-significant Fisher exact p-value (0.361) proved the IRS-2 A allele to not be a good predictor of impaired glucose tolerance in the black South African population. Nonetheless, the odds ratios indicated a more than 1.3 fold increase in risk for glucose intolerance when harbouring the IRS-2 A allele, although the 95% CI was too widely distributed for these genotypes to be confirmed as good indicators of glucose tolerance (p = 0.361).

The association of the IRS-2 genotypes with the HbA1c level was determined according to the methods described in Section 5.5.3 and the results listed in Table 6.36. Similar to the results observed for the IRS-1 gene, the Fisher exact p-value were not significant (p = 1.000) and the 95% CIs widely distributed, indicating that the IRS-2 genotype was also not a good predictor of a high HbA1c level within the studied cohort. The small number (n = 6) of individuals included within the high HbA1c subgroup may again explain the wide 95% CIs.

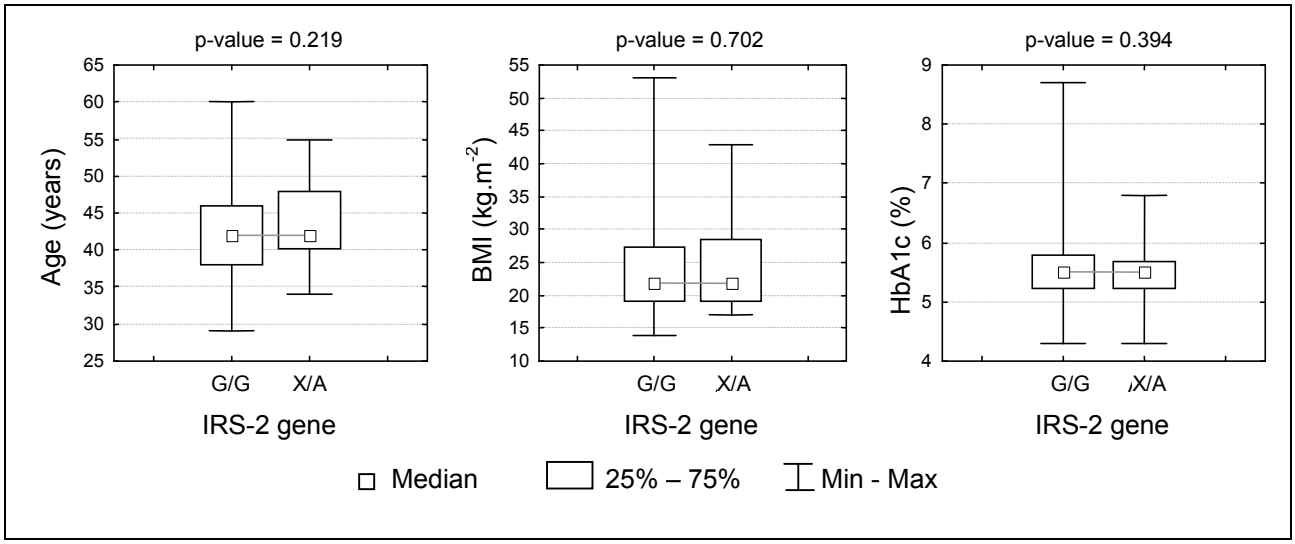
Table 6.36 Contingency table summary for the association between the IRS-2 genotype and HbA1c

Genotype	High HbA1c	Normal HbA1c	Total	Fisher exact p-value	OR (95% CI)
IRS-2 gene					
X/A	1	70	71	1.00	1.0 (0.12 - 9.11)
G/G	5	367	372		
Total	**6**	**437**	**443**		

A contingency table with two-tailed Fisher exact p-value as well as odds ratio and 95% confidence interval were determined. HbA1c = glycosylated haemoglobin; OR = odds ratio; CI = confidence interval; IRS-2 = insulin receptor substrate-2 gene; A = adenine; G = guanine. The X/A subgroup includes both heterozygous and homozygous mutant genotypes.

The age, BMI and HbA1c data within the two IRS-2 genotype subgroups did not differ significantly. The similarity in the distribution of this data between the two genotypic subgroups is evident in the graphs displayed in Figure 6.37. Previously reported findings of an association between the X/A genotype and an increase in body weight in non-Africans (Tschritter *et al.,* 2003) were not observed within the studied cohort, as indicated by the non-significant p-value of 0.702 in Table 6.34 when comparing the BMI between the two genotype subgroups.

Figure 6.37 Graphic representation of the age, BMI and HbA1c values stratified according to the IRS-2 genotypes



IRS-2 = insulin receptor substrate-2; G = guanine; A = adenine; G/G = homozygous wild type genotype; X/A = heterozygous plus homozygous mutant genotypes; BMI = body mass index; $kg.m^{-2}$ = kilogram per metre squared; HbA1c = glycosylated haemoglobin; Min = minimum; Max = maximum; % = percentage. p-value = Mann-Whitney test p-value, see Table 6.31.

No association could therefore be confirmed in the studied cohort between either the IRS-1 or the IRS-2 genotypes and previously reported T2D risk factors, as discussed in Chapter Three. However, the findings of this study are only preliminary and further testing within bigger sample sets are required in order to draw conclusions on the T2D risk prediction value of the IRS-1 and IRS-2 genotypes in the black South African population.

6.2.5.1.3 Peroxisome proliferator-activated gamma 2 gene

The recorded parameters observed within the PPARγ2 genotypic subgroups are presented in Table 6.37. No significant differences were observed on comparison of the clinical data. Comparison analysis was performed using the statistical methods discussed in Section 5.5.2.

According to WHO criteria (Table 2.2), the median 120 min glucose value (Table 6.37) of the X/G genotype subgroup falls within the glucose intolerant category (> 7.8 $mmol.L^{-1}$) despite the fasting 0 min glucose level being within the normal range (< 6.0 $mmol.L^{-1}$). The graphs represented in Figure 6.38 indicate that the majority of individuals that are included in the X/G genotype subgroup had a 120 min glucose value greater than 7.8 $mmol.L^{-1}$.

Table 6.37 Clinical parameters stratified according to the PPARγ2 genotypes

Parameter	C/C genotype		X/G genotype		M-W p-value
	SW p-value	Median (IQR)	SW p-value	Median (IQR)	
Number (M/F)	--	437 (163 / 274)	--	6 (2 / 4)	--
Age (years)	0.000	42.0 (38.0 – 46.0)	0.746	44.5 (42.0 – 47.0)	0.348
Weight (kg)	0.000	59.0 (51.0 – 71.0)	0.134	60.0 (53.0 – 65.0)	0.694
Height (cm)	0.000	161.0 (156.0 – 167.0)	0.281	163.0 (161.0 – 166.0)	0.384
BMI ($kg.m^{-2}$)	0.000	21.9 (19.1 – 27.9)	0.218	22.3 (19.7 – 25.1)	0.666
0' Glucose ($mmol.L^{-1}$)	0.060	5.3 (4.8 – 5.7)	0.006	5.1 (5.1 – 5.6)	0.837
30' Glucose ($mmol.L^{-1}$)	0.003	8.6 (7.6 – 9.4)	0.848	9.2 (7.5 – 10.7)	0.394
60' Glucose ($mmol.L^{-1}$)	0.002	9.1 (7.8 – 10.3)	0.137	8.8 (7.8 – 12.6)	0.821
90' Glucose ($mmol.L^{-1}$)	0.000	8.1 (7.0 – 9.6)	0.306	8.8 (7.9 – 9.9)	0.302
120' Glucose ($mmol.L^{-1}$)	0.000	7.3 (6.5 – 8.6)	0.389	8.1 (7.7 – 9.6)	0.117
HbA1c (%)	0.000	5.5 (5.2 – 5.8)	0.168	5.5 (5.1 – 5.7)	0.558
HIV (pos/neg)	--	437 (109 / 328)	--	6 (1 / 5)	--

Shapiro Wilk's (SW) test is performed for normality testing of all comparison subgroups and p-values are indicated. The p-value comparing parameters between individuals within the wild type (Pro/Pro) and heterozygous plus homozygous mutant (X/Ala) genotype subgroups in the PPARγ2 gene are generated via a Mann-Whitney test. C = cytosine; G = guanine; C/C = homozygous wild type genotype; X/G = heterozygous plus homozygous mutant genotypes; M = male; F = female; BP = blood pressure; BMI = body mass index; SD = standard deviation; pos = positive; neg = negative; WT = wild type; kg = kilogram; cm = centimetre, $kg.m^{-2}$ = kilogram per metre squared; $mmol.L^{-1}$ = millimole per litre; HbA1c = glycosylated haemoglobin; HIV = human immunodeficiency virus. The p-values indicated in red text are significant at a significance level < 0.05.

A wide interquartile distribution at the 30, 60 and 90 min time intervals is however observed between the six individuals included in the X/G genotype subgroup, although the median glucose levels do not reach the cut-off levels for IGR (Zhou *et al.*, 2006). This finding questions the accuracy of these cut-off values within the black South African population, since the 120 min glucose level definitively indicates glucose intolerance in the X/G genotype subgroup. Due to the fact that the observed glucose intolerance within the small number (n = 6) PPARγ2 X/G genotype subgroup is not in concordance with the reported association between the G allele and protection against T2D in non-Africans (Stumvoll *et al.*, 2001b), further investigation within a larger cohort is required in order to confirm or reject the observed trend within the black South African population. The association between the PPARγ2 X/G genotype and glucose intolerance were however further evaluated according to the statistical methods discussed in Section 5.5.3 and the contingency table results listed in Table 6.38.

Table 6.38 Contingency table summary for the association between the PPARγ2 genotype and glucose tolerance

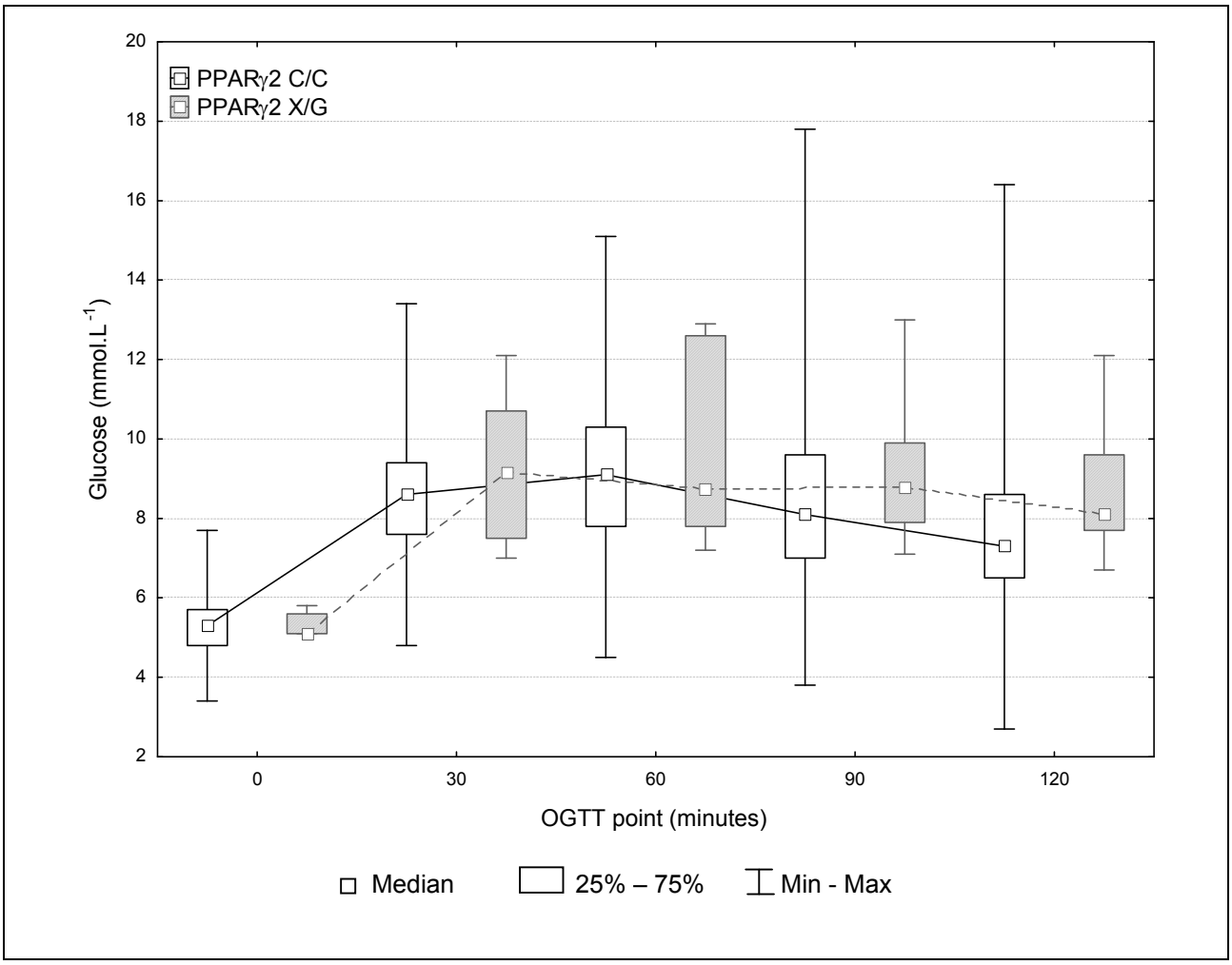
Genotype	IGT	NGT	Total	Fisher exact p-value	OR (95% CI)
PPARγ2 gene					
X/G	2	3	6	0.340	2.16 (0.35 – 13.1)
C/C	103	335	437		
Total	**105**	**338**	**443**		

A contingency table with two-tailed Fisher exact p-value as well as odds ratio and 95% confidence interval were determined. IGT = impaired glucose tolerance; NGT = normal glucose tolerance; OR = odds ratio; CI = confidence interval; PPARγ2 = peroxisome proliferator-activated gamma 2 gene; G = guanine; C = cytosine. The X/G subgroup includes both heterozygous and homozygous mutant genotypes.

The Fisher exact p-value (0.340) generated by evaluating the risk of the PPARγ2 G allele imparted on glucose tolerance was not significant. The small number (n = 2) of individuals included in the IGT subgroup may explain the wide 95% CI. The odds ratio of 2.16 as listed in Table 6.38 does however suggest a risk for glucose intolerance when harbouring the G allele, which is in direct contrast with findings in other populations that associated this allele with increased insulin sensitivity and a protective effect against T2D (Stumvoll *et al.,* 2001b). The OGTT values within the two PPARγ2 genotypic subgroups are graphically depicted in Figure 6.38.

Although not statistically significant, the body weight of the majority of individuals that were included in the PPARγ2 C/C genotype subgroup indicated a trend of being higher than the body weight observed within the small (n = 6) X/G genotypic subgroup. Further investigation will however be necessary in larger cohorts in order to evaluate the effect of the G allele in suppressing lipolysis, as reported in non-Africans (Stumvoll *et al*., 2001b). This is in direct contrast with the increased weight findings in individuals harbouring the X/G genotype, as reported by Rhee *et al*. (2006).

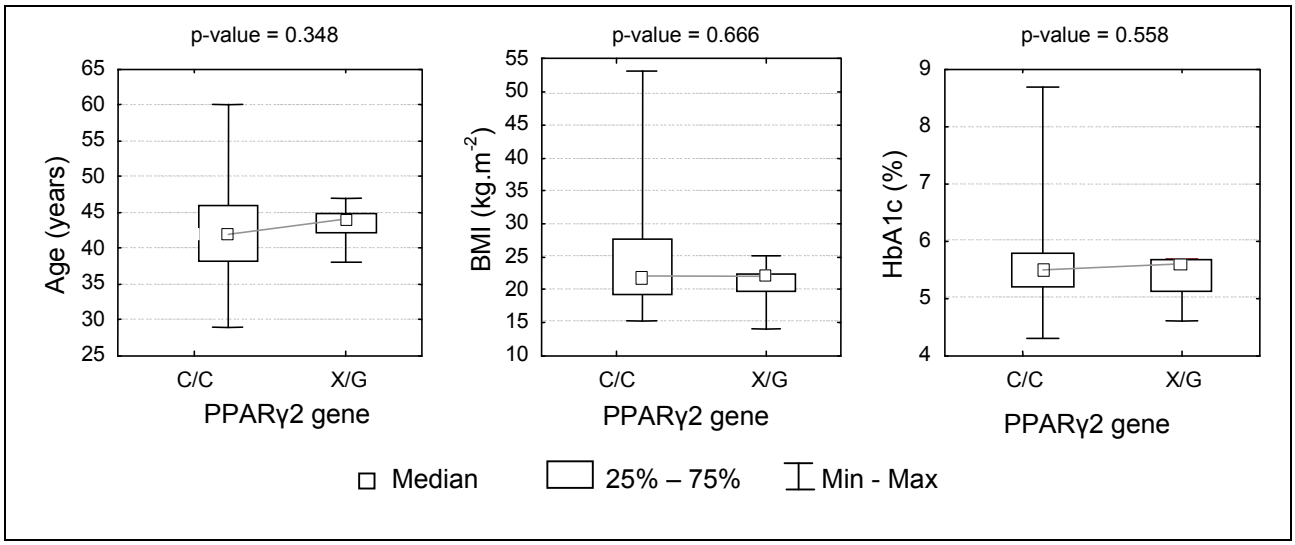
Figure 6.38 Graphic representation of the OGTT values stratified according to the PPARγ2 genotypes



PPARγ2 = peroxisome proliferator-activated gamma 2; OGTT = oral glucose tolerance test; C = cytosine; G = guanine; mmol.L^{-1} = millimole per litre; Min = minimum; Max = maximum; % = percentage; C/C = homozygous wild type genotype; X/G = heterozygous plus homozygous mutant genotypes.

The age, BMI and HbA1c values within the two genotypic subgroups were not significantly different. These parameters as stratified according to the PPARγ2 genotypes are represented in Figure 6.39.

Figure 6.39 Graphic representation of the age, BMI and HbA1c values stratified according to the PPARγ2 genotypes



PPARγ2 = peroxisome proliferator-activated gamma 2; C = cytosine; G = guanine; C/C = homozygous wild type genotype; X/G = heterozygous plus homozygous mutant genotypes; BMI = body mass index; $kg.m^{-2}$ = kilogram per metre squared; HbA1c = glycosylated haemoglobin; Min = minimum; Max = maximum; % = percentage. p-value = Mann-Whitney test p-value, see Table 6.37.

An association between the PPARγ2 genotype and the HbA1c level could not be evaluated due to the fact that none of the six individuals with high HbA1c levels (> 6.5%) harboured a X/G genotype. Further investigation into the role of the PPARγ2 gene in T2D susceptibility in black South Africans is therefore suggested.

6.2.5.1.4 Calpain 10 gene

The clinical parameters were compared between the two CAPN10 genotypic subgroups via the statistical methods discussed in Section 5.5.2. Biological significance was determined on all statistically significant results according to the procedure describe in Section 5.5.4. The results are presented in Table 6.39.

Table 6.39 Clinical parameters stratified according to the CAPN10 genotypes

Parameter	T/T genotype		X/C genotype		M-W p-value	d-value
	SW p-value	Median (IQR)	SW p-value	Median (IQR)		
Number (M/F)	--	379 (146 / 233)	--	64 (19 / 45)	--	--
Age (years)	0.000	42.0 (38.0 – 46.0)	0.064	44.0 (40.0 – 48.0)	0.016	0.35
Weight (kg)	0.000	59.0 (51.0 – 71.0)	0.000	60.5 (51.0 – 67.0)	0.865	--
Height (cm)	0.001	161.0 (156.0 – 167.0)	0.185	162.0 (156.0 – 166.0)	0.865	--
BMI ($kg.m^{-2}$)	0.000	21.9 (19.1 – 28.0)	0.000	22.2 (18.9 – 27.2)	0.968	--
0' Glucose ($mmol.L^{-1}$)	0.099	*5.3 ± 0.7	0.142	*5.3 ± 0.8	**0.750	--
30' Glucose ($mmol.L^{-1}$)	0.001	8.4 (7.4 – 9.4)	0.432	8.8 (8.1 – 9.8)	0.013	0.29
60' Glucose ($mmol.L^{-1}$)	0.003	9.0 (7.8 – 10.3)	0.125	9.5 (8.3 - 11)	0.026	0.32
90' Glucose ($mmol.L^{-1}$)	0.000	8.0 (6.9 – 9.6)	0.051	8.6 (7.8 – 10.4)	0.004	0.35
120' Glucose ($mmol.L^{-1}$)	0.000	7.3 (6.4 – 8.5)	0.928	7.8 (6.7 – 9.2)	0.040	0.20
HbA1c (%)	0.000	5.5 (5.2 – 5.8)	0.718	5.5 (5.2 – 5.7)	0.444	--
HIV (pos/neg)	--	379 (93 / 286)	--	64 (17 / 47)	--	--

SW = Shapiro Wilk's test p-value; M-W = Mann-Whitney test p-value; d-value = biological significance. Values are indicated as median (inter-quartile range) unless marked with an asterisk (*) = means ± standard deviation. CAPN10 = calpain 10; C = cytosine; T = thymine; T/T = homozygous wild type genotype; X/C = heterozygous plus homozygous mutant genotypes; M = male; F = female; BMI = body mass index; IQR = inter-quartile range; pos = positive; neg = negative; kg = kilogram; cm = centimetre, $kg.m^{-2}$ = kilogram per metre squared; $mmol.L^{-1}$ = millimole per litre; HbA1c = glycosylated haemoglobin; HIV = human immunodeficiency virus. The p-values and d-values indicated in red text are significant at a significance level < 0.05 and > 0.8 respectively.

The two CAPN10 genotypic subgroups differed significantly when comparing the 30, 60, 90 and 120 min glucose values. However, it should be noted the 30 and 60 min glucose levels did not reach the IGR cut-off levels as defined by Zhou *et al.* (2006). The median glucose values of the X/C subgroup follows a glucose curve with values all above 7.8 $mmol.L^{-1}$ throughout the whole two hour OGTT, similar to the curve shape observed in non-diabetic individuals anticipated to develop glucose intolerance as reported by Trijillo-Arriaga and Roman-Ramos (2007).

The median 120 min glucose level of the X/C genotype subgroup was on the lower limit of indicating glucose intolerance (7.8 $mmol.L^{-1}$) according to the WHO criteria (Table 2.2) for the diagnosis of T2D. This finding is similar to that reported by Wang *et al.* (2002) for individuals included in a non-T/T genotype group and therefore supports the association between this genotype and an increase in plasma glucose. The non-significant d-value of 0.2 indicates that the finding is practically not relevant which may be explained by the fact that the 120 min glucose inter-quartile range indicates that only certain individuals included in the X/C genotype subgroup were in fact glucose intolerant (> 7.8 $mmol.L^{-1}$), whereas the rest had normal glucose tolerance as observed in Figure 6.40. The association between the X/C genotype and glucose tolerance was evaluated via statistical

methods as discussed in Section 5.5.3 and the contingency table results listed in Table 6.40.

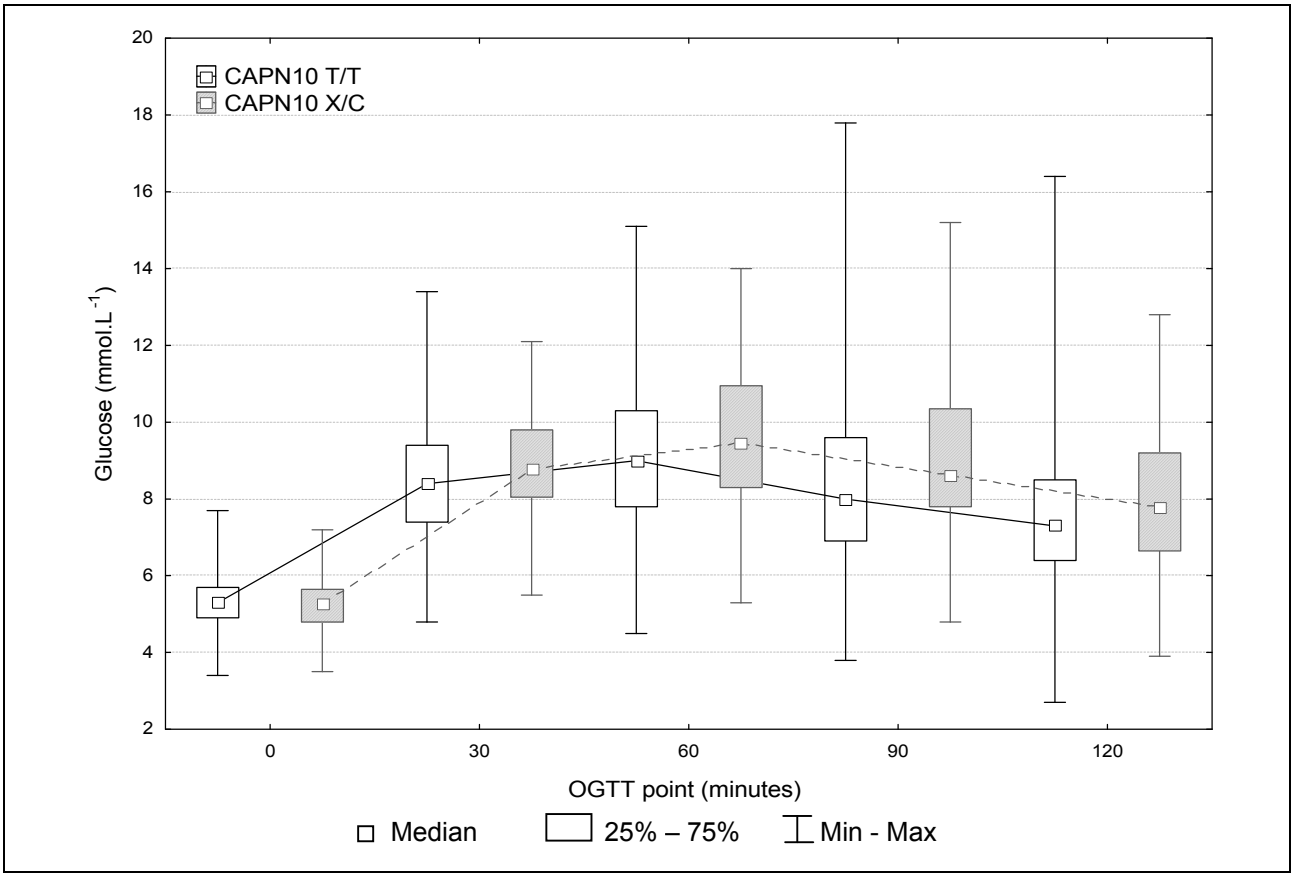
Table 6.40 Contingency table summary for the association between the CAPN10 genotype and glucose tolerance

Genotype	IGT	NGT	Total	Fisher exact p-value	OR (95% CI)
CAPN10 gene					
X/C	18	46	64	0.427	1.31 (0.72 - 2.38)
T/T	87	292	379		
Total	**105**	**338**	**443**		

A contingency table with two-tailed Fisher exact p-value as well as odds ratio and 95% confidence interval were determined. IGT = impaired glucose tolerance; NGT = normal glucose tolerance; OR = odds ratio; CI = confidence interval; CAPN10 = calpain 10; C = cytosine; T = thymine. The X/C subgroup includes both heterozygous and homozygous mutant genotypes.

According to the contingency table results listed in Table 6.40, the CAPN10 genotypes were not good predictors of impaired glucose tolerance in the black South African population ($p = 0.427$). The odds ratios for the investigated cohort indicated a more than 1.3 fold increase in risk for glucose intolerance when harbouring the CAPN10 T allele, although the 95% CI was too widely distributed for these genotypes to be confirmed as good indicators of glucose tolerance. The differences observed between the OGTT glucose values within the two CAPN10 genotypic subgroups are represented in Figure 6.40.

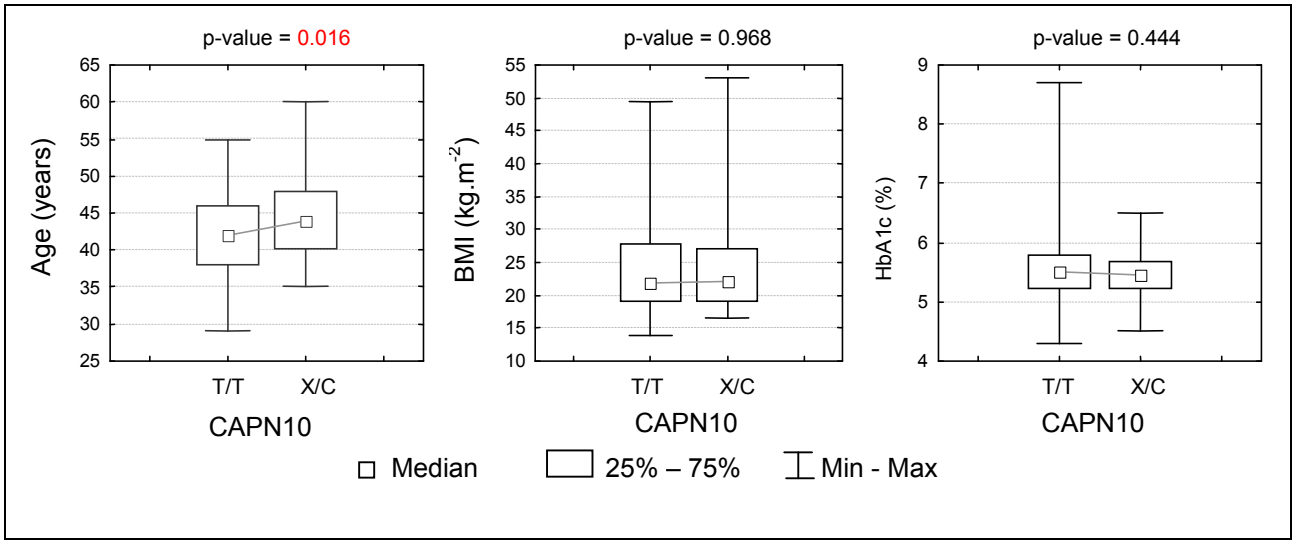
Figure 6.40 Graphic representation of the OGTT values stratified according to the CAPN10 genotypes



CAPN10 = calpain 10; OGTT = oral glucose tolerance test; C = cytosine; T = thymine; mmol.L^{-1} = millimole per litre; Min = minimum; Max = maximum; % = percentage; T/T = homozygous wild type genotype; X/C = heterozygous plus homozygous mutant genotypes.

There was no significant difference between the two genotypic subgroups when comparing BMI and HbA1c values, despite the highest HbA1c level being above 8% within the T/T subgroup, compared to the highest HbA1c level in X/C subgroup being well under 7%. Similar to the numbers observed in the PPARγ2 genotype subgroup, none of the six individuals with high HbA1c levels (> 6.5%) harboured an X/C genotype and therefore the association between the CAPN10 genotype and the HbA1c level could not be evaluated. The similar distribution of the IQRs for BMI and HbA1c in both the CAPN10 genotype subgroups is graphically depicted in Figure 6.41.

Figure 6.41 Graphic representation of the age, BMI and HbA1c values stratified according to the CAPN10 genotypes



CAPN10 = calpain 10; C = cytosine; T = thymine; T/T = homozygous wild type genotype; X/C = heterozygous plus homozygous mutant genotypes; BMI = body mass index; $kg.m^{-2}$ = kilogram per metre squared; HbA1c = glycosylated haemoglobin; Min = minimum; Max = maximum; % = percentage. p-value = Mann-Whitney test p-value, see Table 6.39. The p-values indicated in red text are significant at a significance level < 0.05.

Despite the statistically significant age difference between the two CAPN10 genotypic subgroups, the non-significant d-value of 0.35 supports the fact that this two-year age difference is clinically not important, especially since the median age in both subgroups is below the T2D risk age of 45 years. This similarity in age between the two CAPN10 genotypic subgroups can be viewed in Figure 6.41.

6.2.5.2 Glucose curve shape

Individuals were divided into the three GCS subgroups and stratified according to the various investigated genotypes. This stratification is presented in Table 6.41.

None of the individuals with a biphasic postprandial GCS harboured either the IRS-1 A/A or the IRS-2 A/A genotypes. The three individuals harbouring the IRS-1 A/A genotype had a monophasic GCS, whereas none of the investigated individuals with a monophasic GCS harboured the IRS-2 A/A genotype.

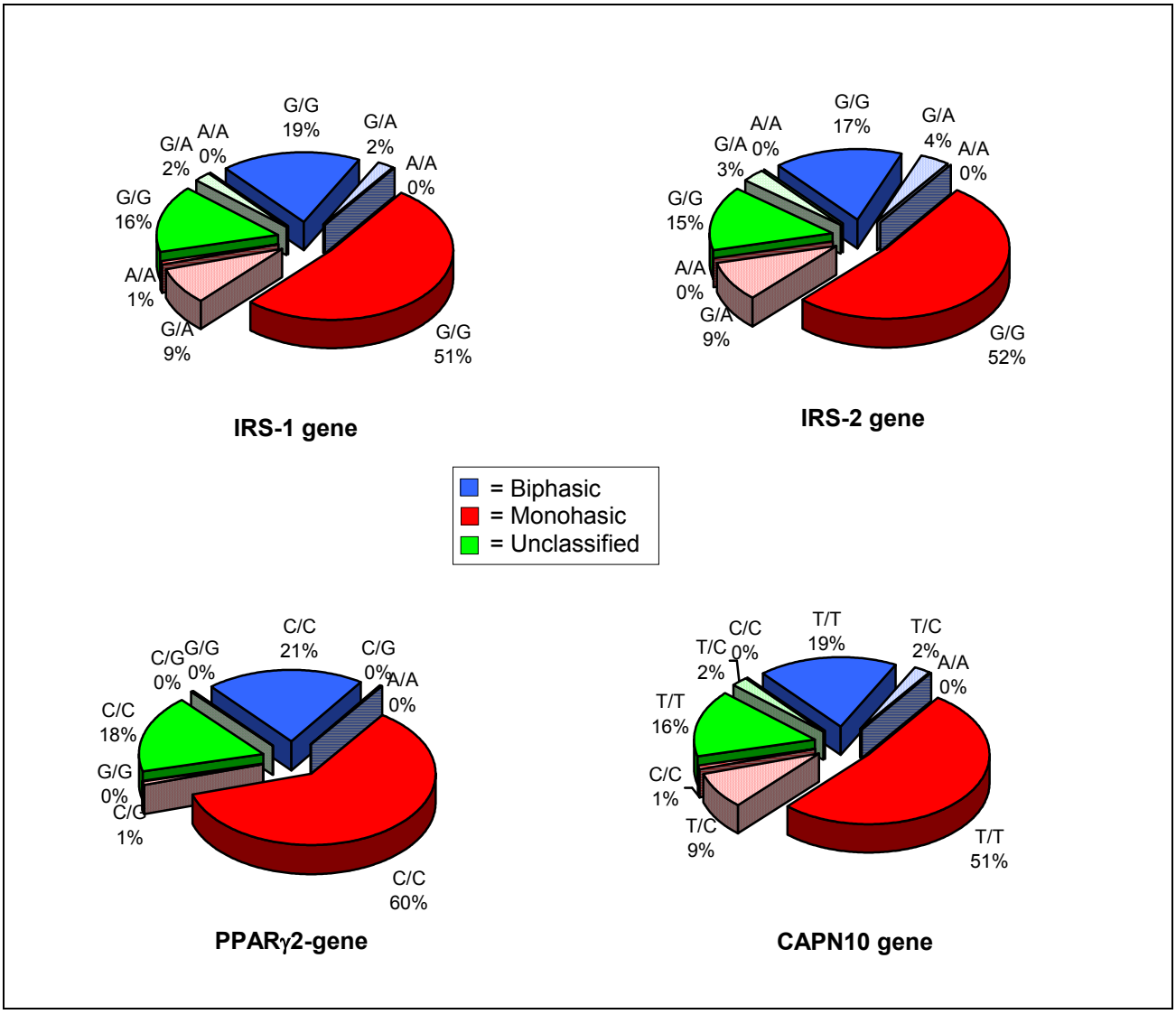
Table 6.41 Observed genotypic subgroup numbers stratified according to glucose curve shape

Genotype	Biphasic	Monophasic	Unclassified
IRS-1 gene			
G/G	83	231	69
G/A	9	38	10
A/A	0	3	0
Total	**92**	**272**	**79**
IRS-2 gene			
G/G	75	232	65
G/A	17	40	14
A/A	0	0	0
Total	**92**	**272**	**79**
PPARγ2 gene			
C/C	91	269	78
C/G	1	3	1
G/G	0	0	0
Total	**92**	**272**	**79**
CAPN10 gene			
T/T	80	227	72
T/C	12	43	7
C/C	0	2	0
Total	**92**	**272**	**79**

GCS = glucose curve shape; IRS-1 = insulin receptor substrate-1; IRS-2 = insulin receptor substrate-2; PPARγ2 = peroxisome proliferator-activated gamma 2; CAPN10 = calpain 10; A = adenine; G = guanine; C = cytosine; T = thymine.

The low frequencies of both the IRS-1 and IRS-2 A alleles in all three the GCS subgroups indicate the rarity of these alleles in the investigated cohort, as graphically presented in Figure 6.33. The association between the IRS-1 and IRS-2 polymorphisms and a specific GCS was evaluated in the investigated cohort according to the methods described in Section 5.5.3.

Figure 6.42 Percentage of genotypes according to glucose curve shape



GCS = glucose curve shape; CAPN10 = calpain 10; IRS-1 = insulin receptor substrate-1; IRS-2 = insulin receptor substrate-2; PPARγ2 = peroxisome proliferator-activated receptor gamma 2, A = adenine; G = guanine; C = cytosine; T = thymine.

A summary of the contingency tables, Fisher exact p-values as well as the odds ratios for both IRS-1 and IRS-2 are presented in Table 6.42. Due to the fact that the unclassified group could by definition not be categorised and would therefore not be useful as an indicator of T2D, it was excluded from the association testing.

Tschritter *et al.* (2003) could not find evidence to demonstrate an association between the IRS-1 or IRS-2 polymorphisms and a specific GCS in non-Africans. The association of the WT and the X/A genotypes with GCS was evaluated for the IRS-1 and IRS-2 genes. The non-significant p-values and wide 95% CIs, as listed in Table 6.42, indicate that neither one of the two IRS genes were however good predictors of GCS.

Table 6.42 Contingency table summary for the association between the IRS-1 and IRS-2 genotypes and GCS

Genotype	Monophasic	Biphasic	Total	Fisher exact p-value	X/N OR (95% CI)	WT OR (95% CI)
IRS-1 gene						
X/A	41	9	50	0.225	1.63 (0.76 - 3.51)	0.61 (0.28 – 1.31)
G/G	231	83	314			
Total	**272**	**92**	**364**			
IRS-2 gene						
X/A	40	17	57	0.408	0.76 (0.40 - 1.42)	1.31 (0.70 – 2.45)
G/G	232	75	307			
Total	**272**	**92**	**364**			

A contingency table with two-tailed Fisher exact p-value as well as odds ratio and 95% confidence interval were determined. OR = odds ratio; CI = confidence interval; WT = wild type; IRS-1 = insulin receptor substrate-1; IRS-2 = insulin receptor substrate-2; GCS = glucose curve shape; A = adenine; G = guanine; C = cytosine; T = thymine. The X/nucleotide subgroup includes both heterozygous and homozygous mutant genotypes.

Due to the fact that the study included only non-diabetic individuals, a higher frequency of the reported T2D protective PPARγ2 G allele would be expected if this trait also held true in black South Africans as reported in non-Africans (Stumvoll *et al*., 2001b). The non-diabetic individuals included could however still progress to glucose intolerance in the future and that would clarify the low protective G allele frequency observed at present. The observed glucose intolerance (120 min glucose = 8.1 mmol.L^{-1}) in the PPARγ2 X/G genotype subgroup, as indicated in Table 6.37, therefore defies the G allele protection against T2D as reported within other populations (Stumvoll *et al*., 2001b). Further evaluation is however required into a larger PPARγ2 X/A genotype population.

The association between the screened PPARγ2 genotype and GCS was determined according to the methods described in Section 5.5.3. The results of the contingency tables, Fisher exact p-values as well as the odds ratios and 95% CIs are listed in Table 6.43.

Table 6.43 Contingency table for the association between the PPARγ2 genotype and GCS

Genotype	Monophasic	Biphasic	Total	Fisher exact p-value	X/N OR (95% CI)	WT OR (95% CI)
PPARγ2 gene						
X/G	3	1	4	1.00	1.01 (0.10 - 9.87)	0.98 (0.10 - 9.59)
C/C	269	91	360			
Total	**272**	**92**	**364**			

A contingency table with two-tailed Fisher exact p-value as well as odds ratio and 95% confidence interval were determined. OR = odds ratio; CI = confidence interval; WT = wild type; PPARγ2 = peroxisome proliferator-activated gamma 2 gene; GCS = glucose curve shape; A = adenine; G = guanine; C = cytosine; T = thymine. The X/nucleotide subgroup includes both heterozygous and homozygous mutant genotypes.

No association between the PPARγ2 genotypes and a specific GCS has been described to date. According to the non-significant p-values and wide 95% CIs, as listed in Table 6.43, it is evident that this gene is also not a good predictor of GCS within the studied cohort.

The frequencies of the CAPN10 T allele were 0.91 within the monophasic subgroup, 0.93 in the biphasic subgroup and 0.96 in the unclassified subgroup, as calculated with Equation 5.3 from the observed numbers presented in Table 6.41. The majority of the individuals with either the X/C (70%) or the T/T (60%) genotype had a monophasic GCS, with a biphasic GCS being observed in 19% and 21% of the two subgroups, respectively. The reported association in Caucasians between a monophasic GCS and the UCSNP44 T allele (Tschritter *et al.,* 2003) is therefore also proposed within the black South African population and was tested along with the possible association of the WT allele and GCS via the methods described in Section 5.5.3. Results of this testing are listed in Table 6.44.

Table 6.44 Contingency table for the association between the CAPN10 genotype and GCS

Genotype	Monophasic	Biphasic	Total	Fisher exact p-value	X/N OR (95% CI)	WT OR (95% CI)
CAPN10 gene						
X/C	45	12	57	0.508	1.32 (0.66 - 2.62)	0.75 (0.38 – 1.50)
T/T	227	80	307			
Total	**272**	**92**	**364**			

A contingency table with two-tailed Fisher exact p-value as well as odds ratio and 95% confidence interval were determined. OR = odds ratio; CI = confidence interval; WT = wild type; IRS-1 = insulin receptor substrate-1; IRS-2 = insulin receptor substrate-2; PPARγ2 = peroxisome proliferator-activated gamma 2 gene; CAPN10 = calpain 10; GCS = glucose curve shape; A = adenine; G = guanine; C = cytosine; T = thymine. The X/nucleotide subgroup includes both heterozygous and homozygous mutant genotypes.

In the studied black South African cohort though, the association between the CAPN10 genotypes and the GCS was not significant, as reflected by the non-significant Fisher exact p-value of 0.508 in Table 6.44. None of the four investigated genes were therefore good predictors of GCS within the studied black South African cohort.

6.2.5.3 Physical environment

Individuals included in the studied cohort resided in either a rural or an urban environment, as discussed in Section 5.2. The numbers of individuals harbouring the specific genotypes according to physical environment are presented in Table 6.45.

Table 6.45 Genotype numbers observed within the physical environment subgroups

Genotype	Urban subgroup	Rural subgroup
IRS-1 gene		
G/G	164	219
G/A	27	30
A/A	2	1
Total	**193**	**250**
IRS-2 gene		
G/G	160	212
G/A	33	38
A/A	0	0
Total	**193**	**250**
PPARγ2 gene		
C/C	190	248
C/G	3	2
G/G	0	0
Total	**193**	**250**
CAPN10 gene		
T/T	167	212
T/C	25	37
C/C	1	1
Total	**193**	**250**

IRS-1 = insulin receptor substrate-1; IRS-2 = insulin receptor substrate-2; PPARγ2 = peroxisome proliferator-activated gamma 2; CAPN10 = calpain 10; A = adenine; G = guanine; C = cytosine; T = thymine.

Based on the information observed in Table 6.45, it can be deduced by viewing the genotype numbers that the mutant alleles, when present, were consistently in the minority

and present in both the rural and urban subgroups. The association between the physical environment and the screened genotypes was measured and the results are listed in Table 6.46.

Table 6.46 Contingency table summary for the association between physical environment and the genotype

IRS-1 gene					
Environment	**X/A**	**G/G**	**Total**	**Fisher exact p-value**	**OR (95% CI)**
Rural	31	219	250	0.484	0.80 (0.46 - 1.38)
Urban	29	164	193		
Total	**60**	**383**	**443**		
IRS-2 gene					
Environment	**X/A**	**G/G**	**Total**	**Fisher exact p-value**	**OR (95% CI)**
Rural	38	212	250	0.604	0.86 (0.52 - 1.44)
Urban	33	160	193		
Total	**71**	**372**	**443**		
PPARγ2 gene					
Environment	**X/G**	**C/C**	**Total**	**Fisher exact p-value**	**OR (95% CI)**
Rural	2	248	250	0.657	0.51 (0.08 - 3.08)
Urban	3	190	193		
Total	**6**	**437**	**443**		
UCSNP44 gene					
Environment	**X/C**	**T/T**	**Total**	**Fisher exact p-value**	**OR (95% CI)**
Rural	38	212	250	0.683	1.15 (0.67 - 1.97)
Urban	26	167	193		
Total	**64**	**379**	**443**		

A contingency table with two-tailed Fisher exact p-value as well as odds ratio and 95% confidence interval were determined. OR = odds ratio; CI = confidence interval; IRS-1 = insulin receptor substrate-1; IRS-2 = insulin receptor substrate-2; PPARγ2 = peroxisome proliferator-activated gamma 2 gene; CAPN10 = calpain 10; A = adenine; G = guanine; C = cytosine; T = thymine. The X/nucleotide subgroup includes both heterozygous and homozygous mutant genotypes.

No association was previously reported for genotype and a specific environment. The observed p-values in Table 6.46 were all non-significant and therefore indicated that the physical environment was not associated with any of the investigated genotypes within the studied cohort.

6.3 SUMMARY OF PHENOTYPE AND GENOTYPE RESULTS

Four reported genes involved in T2D susceptibility in other populations were identified and screened in the black South African population via the procedures discussed in Chapter Five. The genotypic data was analysed in relation to various phenotypic measures and a discussion of the clinically relevant results is outlined below.

The risk allele frequencies for all four the investigated genes were low, as indicated in Figure 6.33. These findings may have been expected since all the individuals included in the studied cohort were non-diabetic. The GCSs of the 443 individuals were classified as discussed in Section 6.2.1 and the majority (61%) were included in the monophasic GCS subgroup, whilst 21% were biphasic and 18% grouped as unclassified. A summary of the findings that were of statistical and biological significance is presented in Table 6.47 below.

Upon stratifying the data of the studied cohort into various subgroups, glucose intolerance (120 min glucose level > 7.8 $mmol.L^{-1}$) was observed in individuals with high HbA1c levels (> 6.5%), those with impaired fasting glucose, those coming from a rural physical environment, those who were female, those with a high BMI (> 25 $kg.m^{-2}$) as well as the subgroup harbouring the UCSNP44 X/C genotype. A statistically significant association was however only confirmed between glucose intolerance and high HbA1c levels (> 6.5%), female gender, high BMI and monophasic GCS subgroups.

None of the screened genotypes had a significant association with GCS, as discussed in Section 6.2.4, nor was there a significant difference between the three GCS subgroups when compared with either age or HbA1c values. There was a statistically significant difference when comparing the anthropometrical parameters, namely weight, height and BMI between the two gender subgroups as would be expected. Not only did BMI differ statistically and biologically significantly between the two gender subgroups, but the median female BMI was above 25 $kg.m^{-2}$, indicating that the female population was on average overweight. Consequently, the females included in this study were at greater risk for developing T2D, similar to that reported by Osman *et al.* (1994) in Malaysian individuals.

Table 6.47 Summary of statistically and biologically significant results

Parameter	Comparison subgroups	Parameter	p-value
Glucose curve shape	Monophasic vs biphasic	Height	0.010
		60 min glucose	0.000
		90 min glucose	0.000
Fasting glucose	Normal (< 6.0 mmol.L^{-1}) vs impaired (6.0 – 7.8 mmol.L^{-1})	Weight	0.000
		Height	0.002
		BMI	0.000
		30 min glucose	0.000
		60 min glucose	0.015
		90 min glucose	0.000
		120 min glucose	0.000
Glucose tolerance	Normal (< 7.8 mmol.L^{-1}) vs impaired (> 7.8 mmol.L^{-1})	Age	0.002
		Weight	0.008
		Height	0.037
		BMI	0.003
		0 min glucose	0.000
		30 min glucose	0.000
		60 min glucose	0.000
		90 min glucose	0.000
Environment	Rural vs urban	Weight	0.039
		30 min glucose	*0.014
		60 min glucose	0.006
		90 min glucose	0.048
		120 min glucose	0.035
Gender	Male vs female	Weight	0.000
		Height	0.000
		BMI	0.000
		0 min glucose	*0.000
		90 min glucose	0.011
		120 min glucose	0.000
		HbA1c	0.005
BMI	Normal(< 25 kg.m^{-2}) vs high (> 25 kg.m^{-2})	Weight	0.000
		Height	0.000
		0 min glucose	*0.000
		30 min glucose	0.004
		120 min glucose	0.000
		HbA1c	0.000
HbA1c	Normal (< 6.5%) vs high (> 6.5%)	30 min glucose	0.012
		60 min glucose	0.004
		90 min glucose	0.016
		120 min glucose	0.003
CAPN10 gene	T/T vs X/C genotypes	Age	0.016
		30 min glucose	0.013
		60 min glucose	0.026
		90 min glucose	0.004
		120 min glucose	0.040

p-value = Mann-Whitney test p-value, unless marked with an asterisk (*) = t-test p-value. The statistically significant findings that were also of biological significance were indicated by the grey highlight. CAPN10 = calpain 10; T/T = homozygous wild type genotype; X/C = heterozygous plus homozygous mutant genotypes; C = cytosine; T = thymine; min = minute; BMI = body mass index; HbA1c = glycosylated haemoglobin; vs = versus. The p-values indicated in red text are significant at a significance level < 0.05.

A summary of the statistically significant association analyses results is listed in Table 6.48. There was an almost three fold increased chance for harbouring a biphasic GCS when a participant was female and an almost two fold increased risk for being

glucose intolerant when harbouring a monophasic GCS. Similarly, individuals with either a high BMI or HbA1c level had an increased risk for glucose intolerance, whereas females were twice as likely to be glucose intolerant as compared to males. Individuals from the urban environment had double the chance of being physically inactive as compared to those from the rural subgroup and were four times more likely to have an unhealthy diet.

Table 6.48 Summary of statistically significant association analyses results

Parameter	Risk factor	Contingency table results	
		OR (95% CI)	p-value
IGT	Monophasic GCS	1.89 (1.00 - 3.55)	0.045
	High BMI	1.93 (1.23 - 3.01)	0.005
	High HbA1c	16.8 (1.94 – 145)	0.003
	Female gender	1.99 (1.22 - 3.24)	0.006
Urban environment	Physical inactivity	1.90 (1.26 - 2.86)	0.002
Female gender	Biphasic GCS	2.79 (1.62 – 4.80)	0.000
Unhealthy diet	Urban environment	3.96 (2.11 - 7.44)	0.000

A contingency table with two-tailed Fisher exact p-value as well as odds ratio and 95% confidence interval were determined. OR = odds ratio; CI = confidence interval; IGT = impaired glucose tolerance; GCS = glucose curve shape; BMI = body mass index; HbA1c = glycosylated haemoglobin. The p-values indicated in red text are significant at a significance level < 0.05.

None of the four investigated genes were good predictors of GCS within the studied black South African cohort. However, since the numbers of individuals included in some of the genotypic subgroups were small, investigation within larger cohorts will be required to further evaluate this association. As discussed in Section 6.2.3.1, a rural physical environment was not a good predictor of glucose intolerance (OR = 1.41). In addition, the physical environment was not associated with any of the four investigated genotypes within the studied cohort.

Similar to the lack of association of the genotypes with GCS, none of the screened genotypes were good predictors of impaired glucose in the black South African population. No significant differences were observed when comparing the various genotype subgroups in terms of the T2D related quantitative traits, such as BMI, glycaemia and the female gender. According to these findings, none of the screened gene alterations, with exception of the CAPN10 alteration, were good predictors of T2D risk in the black South African population.

A high HbA1c level (> 6.5%) proved to be a significant predictor for glucose intolerance although the four screened loci were not good predictors of the HbA1c level. However,

only six individuals were included in the high HbA1c subgroup (> 6.5%). These findings are preliminary and further testing within bigger sample sets are suggested in order to draw conclusions on the actual T2D risk prediction value of the investigated genes in the extended black South African population.

CHAPTER SEVEN

CONCLUSIONS

T2D is a multifactorial disease with a high global prevalence and devastating complications when left untreated. Prognosis is determined by the time of both diagnosis and the effective initiation of treatment. Susceptibility to T2D depends on the genetic background of an individual, as discussed in Chapter Four, but this is only one component in a multifaceted network of determinants that bring about increased diabetes risk. This risk is further compounded by various environmental, biochemical and anthropometrical factors, as discussed in Chapter Three.

Research on this complex disease is extensive. However, there is a desperate need for population specific screening, prevention and treatment strategies. The main objective of this study was to assess the glucose curve shape during a two-hour OGTT and evaluate specific genotypes in the context of the T2D risk phenotype in the black South African population. The GCS, specific genotype and phenotype parameters of 443 black South African individuals were therefore evaluated in order to determine the value of using them in the screening for T2D risk in this population. These specific parameters were selected based on a reported study in a non-African cohort by Tschritter *et al.* (2003).

7.1 POPULATION AND ENVIRONMENT SPECIFIC T2D SUSCEPTIBILITY

The evidence generated from this investigation supports the hypothesis that various populations have different but specific risk factors for T2D and that these should be taken into consideration during the future implementation of screening, prevention and treatment strategies. The increase in diabetes prevalence in Africa (Sobngwi *et al.,* 2001), as explained by rapid urbanisation, emphasises the importance of early diagnosis and effective treatment strategies. The final effect of urbanisation on disease risk will be effectively determined over the 12 years of the PURE study, although this initial investigation has already indicated that glucose regulation is a direct result of daily lifestyle as discussed in Chapter Six. The negative effect of urbanisation was confirmed by the high percentage of individuals being physically inactive (74%) when living in an urban environment, as discussed in Section 6.2.3. The statistically significant association

between physical inactivity and an urban physical environment (OR = 0.29; p-value = 0.006) further substantiated this finding.

This study confirms that T2D is not only a heterogenic disease, involving different genetic as well as environmental factors, but that it is also population specific, given that findings on T2D susceptibility in other populations were not similar when compared within the investigated cohort. It is argued that current T2D screening strategies can therefore not simply be adopted in all populations, especially since these have generally been optimised in populations of specific origin, namely the Europeans and Americans. A need therefore exists to further characterise the black South African population both genotypically and phenotypically, specifically in terms of environmental factors, anthropometrical measures and biochemical assays, in order to optimise T2D screening as well as diagnostic, prognostic and treatment strategies. Even within a single population, lifestyle and environmental factors should be evaluated and diagnoses and treatment adjusted accordingly. Physical environment i.e. rural versus urban, did not have a statistically significant association with the GCS (p = 0.332) in the studied cohort, however the widely distributed 95% CI may indicate that this association cannot be definitively excluded within the greater black South African population and that further evaluation is therefore suggested within a larger cohort.

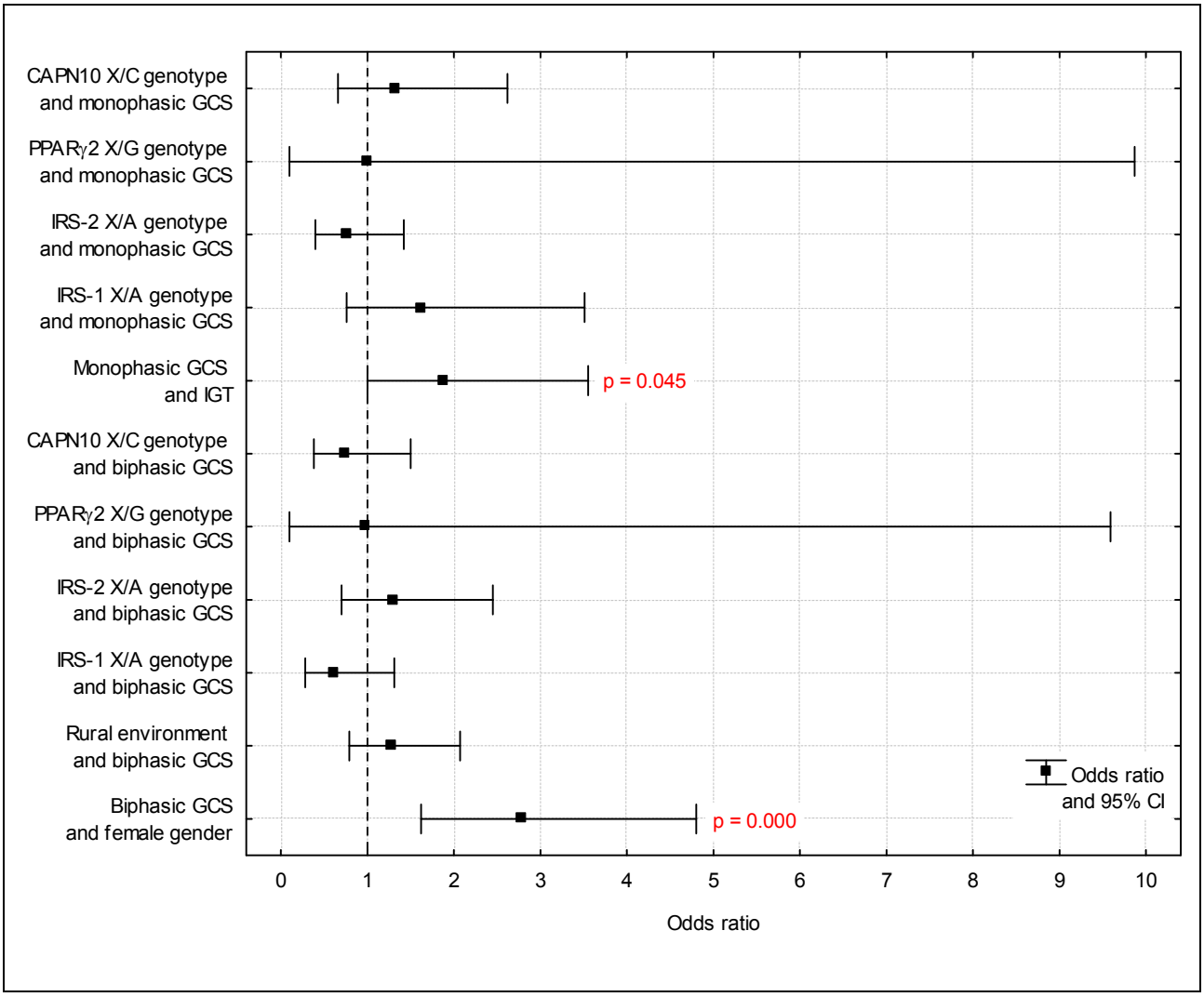
Similar allelic and genotypic frequencies were observed within the rural and urban subgroups (Figure 6.2) when the alterations of interest in the IRS-1, IRS-2, PPARγ2 and CAPN10 genes were investigated. Furthermore, there was no statistically significant association between physical environment and any of the investigated genotypes (Table 6.38).

7.2 EVIDENCE GENERATED FROM GLUCOSE CURVE SHAPE ANALYSES

The inability to classify all individuals included in this study into either a biphasic or monophasic GCS, based on the criteria defined by Tschritter *et al.* (2003), emphasises the necessity for developing criteria that are population and environment specific. It is argued that these stringent reported criteria do not allow for inter-sample variation, resulting in the classification of a large number of samples into the unclassified GCS subgroup. As a consequence, a large percentage of individuals (18%) were not included in further GCS related analyses. A more precise GCS classification would contribute considerably to the screening of T2D when including GCS as risk parameter. Figure 7.1 is a graphic

representation of all the results of the association analyses performed with GCS. It is presented in the form of a Forest plot indicating odds ratios, 95% CIs and statistically significant p-values.

Figure 7.1 Summary of association analyses performed with glucose curve shape



Odds ratios and 95% confidence intervals determined via contingency table analysis are presented with statistically significant two-tailed Fisher exact p-values (< 0.05). IGT = impaired glucose tolerance; UCSNP44 = University of Chicago single nucleotide polymorphism; PPARγ2 = peroxisome proliferator-activated gamma 2; IRS-1 = insulin receptor substrate-1; IRS-2 = insulin receptor substrate-2; GCS = glucose curve shape; OR = odds ratio; CI = confidence interval. The X/nucleotide subgroup includes both heterozygous and homozygous mutant genotypes.

The finding that most of the individuals in the investigated cohort presented with a monophasic GCS (61%) could be explained, to some extent, by the BMI and the gender of participants. This is due to the fact that both a monophasic GCS ($p = 0.045$) and BMI ($p = 0.005$), as presented in Figure 7.2, was significantly associated with glucose intolerance. Furthermore, 63% of the individuals in the monophasic GCS were females with a high median BMI of 25.1 $kg.m^{-2}$. This effect can be due to the decreased leptin levels associated with obesity (Abate *et al.,* 1995), resulting in decreased insulin sensitivity

and therefore delayed stomach emptying and decreased glucose tolerance but further investigation will be needed to confirm this possibility. The effect may however also be explained by the observed difference in dietary intake and exercise levels. Similar to the findings reported by Tschritter *et al.* (2003), a two fold increased risk was reported for being glucose intolerant when having a monophasic GCS ($p = 0.045$). The monophasic GCS is therefore confirmed to be associated with glucose intolerance in the black South African population.

As presented in Figure 7.2, the females included in this study had a two fold increased risk of being glucose intolerant [OR = 1.99 (1.22 - 3.24); $p = 0.006$]. This finding was expected due to the high median BMI of 25.1 $kg.m^{-2}$ observed within the female gender subgroup, keeping in mind the reported association between a high BMI and glucose intolerance (Osman *et al.,* 1994). The female gender group was also statistically significantly associated with a biphasic GCS ($p = 0.000$), similar to findings reported in non-Africans (Tschritter *et al.,* 2003). However, since glucose intolerance was also determined to be associated with the monophasic GCS ($p = 0.045$), it could be argued that the female gender should also be associated with the monophasic GCS and not the biphasic GCS. However, there are several confounding factors influencing the latter association that should be taken into account, such as the fact that both males and females were included in the association analysis and that the BMI levels were widely distributed, particularly amongst the investigated females. Furthermore, the impact of variable glucose metabolism cannot be ignored.

Jellema *et al.* (2003) reported an association of the IRS-1 X/A genotype with T2D in a non-African population. However, within this study, in the biphasic GCS subgroup, only 2% of individuals harboured the IRS-1 X/A genotype, whereas it was observed in 10% of the individuals presenting with a monophasic GCS. Neither of these GCSs were however statistically significantly associated with the risk X/A genotype ($p = 0.225$), as indicated in Figure 7.1. It is however recommended that a larger cohort be investigated prior to definitively excluding this genotype as a predictor of T2D risk in the black South African population.

The IRS-2 gene A allele was observed at a low frequency of 0.08 in the studied cohort, as discussed in Section 6.2.5.2. The X/A genotype did not have a statistically significant association with either the monophasic ($p = 0.408$) or biphasic ($p = 0.408$) GCS, as presented in Figure 7.1. Furthermore, the previously reported finding of an association

between the X/A genotype and an increase in body weight in non-Africans (Tschritter *et al.,* 2003) was not observed within the studied cohort, as indicated by the non-significant p-value of 0.702 in Table 6.34 of Chapter Six.
Although Tschritter *et al.* (2003) reported an association between this IRS-2 alteration and T2D risk in an overweight (mean BMI = 27.8 $kg.m^{-2}$) Caucasian population, this was not the case in this black South African cohort including individuals with a lower median BMI (median male BMI = 19.4 $kg.m^{-2}$; median female BMI = 25.1 $kg.m^{-2}$).

The number of individuals harbouring the PPARγ2 X/G genotype, which is in association with improved glucose tolerance in other populations (Stumvoll *et al*., 2001b), was very small (n = 6) and this genotype could not be associated with either the monophasic or biphasic GCS in the investigated cohort. In addition, it appears that the protective effect of the G allele is not the case in the black South African population, due to the glucose intolerance (median 120 min glucose = 8.1 $mmol.L^{-1}$) observed in this genotype subgroup.

Despite the observed high frequency of the CAPN10 gene UCSNP44 T allele (0.93), the finding by Tschritter *et al.* (2003) associating the monophasic GCS with this allele, could not be reported within the studied cohort. Furthermore, as presented in Figure 7.1, the CAPN10 genotypes had no statistically significant association with either of the analysed GCSs.

The fact that the investigated variants in the IRS-1, IRS-2 and PPARγ2 genes were observed at relatively low frequencies and not associated with glucose intolerance, reduces the likelihood of including these variants in a T2D screening strategy that is specific for the black South African population. There is therefore an urgent need to evaluate all possible susceptibility loci within this population in order to determine the more common susceptibility variants to include in an effective screening strategy. Furthermore, the lack of association between the UCSNP-44 alteration and glucose intolerance supports the argument that genetic susceptibility varies between populations.

7.3 EVIDENCE GENERATED FROM GLUCOSE LEVEL ANALYSES

The importance of always including the fasting glucose level in screening strategies for T2D was supported in the studied cohort by the statistically significant increase in the OGTT values at 30, 60, 90 and 120 min observed in the impaired fasting glucose subgroup. These values were consistently higher when compared to the OGTT values in

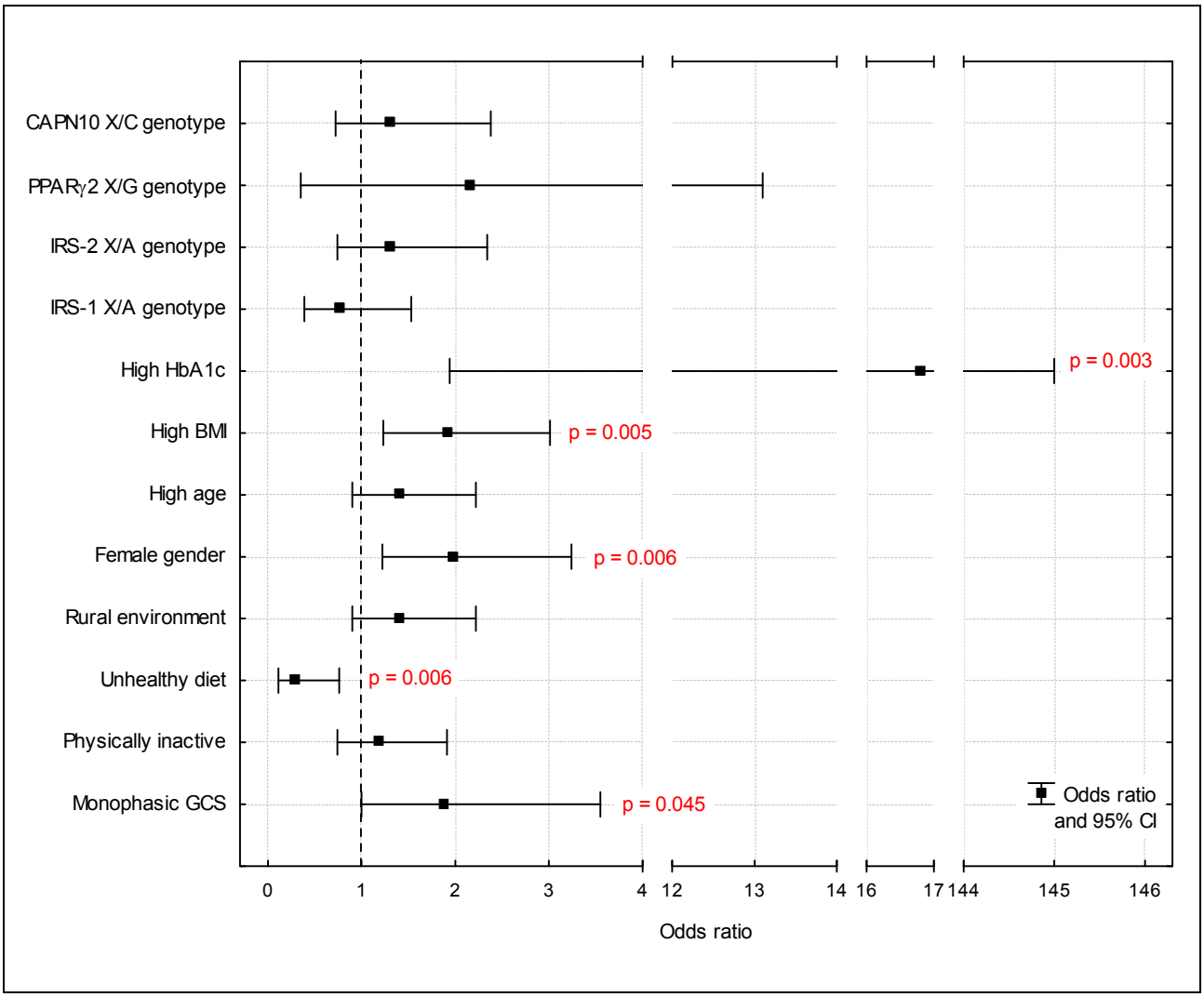
the normal fasting glucose subgroup (Table 6.5). The fasting glucose was however not increased in the high HbA1c subgroup (median 0 min glucose = 5.3 mmol.L^{-1}), despite the observed glucose intolerance (median 120 min glucose = 10 mmol.L^{-1}), emphasising the appropriateness of including the HbA1c as a screening tool. The screening of T2D risk parameters within these individuals with a high HbA1c (> 6.5%) would therefore result in omitting the diagnoses of glucose intolerance if judged on fasting glucose alone. It is therefore recommended that fasting glucose should always be included with the 120 min glucose and HbA1c levels, when screening for T2D.

The subgroups with glucose intolerance included those with a high HbA1c level, impaired fasting glucose, female gender, high BMI as well as individuals with the UCSNP44 X/C genotype. As expected, the glucose levels at 0, 30, 60 and 90 min, as well as the HbA1c level were statistically significantly increased when comparing the IGT and NGT subgroups, as listed in Table 6.9. An association between these quantitative traits and T2D risk is therefore suggested within the investigated cohort. Not only was there statistical significance reported when comparing the 120 min glucose values in these subgroups, but the value was high enough to indicate glucose intolerance (> 7.8 mmol.L^{-1}) and can therefore be directly linked to T2D susceptibility.

Odds ratios with 95% CIs and statistically significant p-values, as generated via the contingency table analyses, are represented in Figure 7.2 in the form of a Forest plot. The results listed are for the T2D susceptibility factors, including the IRS-1, IRS-2, PPARγ2 and CAPN10 genotype subgroups, physical environment, physical activity, diet, gender, age, BMI, HbA1c and monophasic GCS subgroups.

A statistically, as well as biologically, significant increase in the 120 min glucose value ($p = 0.003$; $d = 2.39$) was observed within the high HbA1c subgroup when compared to the normal HbA1c subgroup. This finding, in addition to the statistically significant association observed between the HbA1c level and glucose intolerance (OR = 16.8; $p = 0.0003$), confirms the importance of including the HbA1c value in the screening strategy for T2D risk in black South African individuals. However, since only a small number ($n = 6$) of individuals were included in the high HbA1c (> 6.5%) subgroup, this finding should be evaluated within a larger cohort.

Figure 7.2 Summary of associations of T2D susceptibility factors with glucose intolerance



Odds ratios and 95% confidence intervals determined via contingency table analysis are presented with statistically significant two-tailed Fisher exact p-values (< 0.05). HbA1c = glycosylated haemoglobin; BMI = body mass index; UCSNP44 = University of Chicago single nucleotide polymorphism; PPARγ2 = peroxisome proliferator-activated gamma 2; IRS-1 = insulin receptor substrate-1; IRS-2 = insulin receptor substrate-2; OR = odds ratio; CI = confidence interval. Listed subgroups include the reported risk genotype subgroups for the IRS-1, IRS-2, PPARγ2 and CAPN10 genes, high HbA1c > 6.5%; high BMI > 25 $kg.m^{-2}$; high age > 45 years; unhealthy diet subgroup consuming vegetables, fruits or berries less often than daily; physically inactive subgroup doing physical activity for less than 30 minutes or less than 4 days a week.

The elevated 120 min glucose and HbA1c values in the high BMI subgroup (Table 6.19) were statistically significantly higher as compared to the subgroup with a normal BMI. The statistically significant association and almost two fold increased risk (OR = 1.93; p = 0.005) of glucose intolerance when obese, substantiates the well-defined association between these two traits (Osman *et al.,* 1994) and therefore T2D susceptibility.

There is an almost two fold increased risk of being glucose intolerant when female (OR = 1.99; p = 0.006). This finding is further supported by the statistically as well as biologically significant increase in BMI (p = 0.000; d = 0.98), and the statistically significant

increase in fasting glucose (p = 0.000) and 120 min glucose (p = 0.000) levels in the female subgroup when compared to the male subgroup.

Physical activity is an important environmental factor playing a role in T2D susceptibility. The effect of exercise on energy expenditure, appetite, serum lipoproteins, blood pressure, insulin response and glucose regulation, as well as coronary heart disease, emphasises this importance (Osman *et al.,* 1994). An odds ratio of 1.19 suggests an association of regular physical activity with glucose tolerance. Even though this association was not of statistical significance, it is evident from the 95% CI plotted in Figure 7.2 that the majority of individuals that will be investigated within this population will present with an association between these two traits.

The statistically significantly increased OGTT values at 30, 60, 90 and 120 min (Table 6.13) within the rural subgroup as compared to the urban subgroup can be argued to be the result of delayed urbanisation and limited healthcare. Although the association of a rural physical environment with glucose tolerance could not be confirmed (OR = 1.41; p-value = 0.144), it does suggest a trend towards glucose intolerance in association with physical environment, but requires confirmation within a larger cohort. Individuals residing in an urban environment did not only have a statistically significant association with physical inactivity (OR = 0.29; p = 0.006), but also with an unhealthy diet (OR = 3.96; p = 0.000), as graphically depicted in Figure 7.2. It may therefore be expected that individuals residing in an urban environment will be more susceptible to developing T2D.

7.4 EVIDENCE GENERATED FROM ANTHROPOMETRICAL MEASURE ANALYSES

The female participants in this study had a statistically significant increase in their risk for being glucose intolerant (OR = 1.99; p = 0.006), as depicted in Figure 7.2. This finding was expected due to the high median BMI of 25.1 $kg.m^{-2}$ observed within the female gender subgroup and is further supported by both the statistically significant increase in median height (158 cm) and weight (63.5 kg) values when compared to that of the male individuals.

Individuals included in the high BMI subgroup had an almost two fold increased risk for being glucose intolerant (OR = 1.93; p = 0.005), as supported by the 120 min glucose

level of 7.9 mmol.L^{-1} reported in this subgroup. This finding is in accordance with that reported for non-Africans (Osman *et al.,* 1994).

The importance of including both gender and body mass into an effective T2D risk screening strategy is therefore evident. This is in accordance with other widely used risk scores such the Indian (Mohan *et al*., 2005) and German (Schulze et al., 2007) Diabetes Risk Scores, as well as with the revised classification and diagnostic criteria published by the WHO (Hope *et al.,* 1998).

7.5 EVIDENCE GENERATED FROM HbA1c VALUE ANALYSES

When comparing the 120 min glucose values, statistically and biologically significantly higher values were reported within the high HbA1c subgroup ($p = 0.003$; $d = 1.01$) when compared to the normal HbA1c subgroup, which was expected since a high HbA1c level has previously been linked to glucose intolerance (Berkow *et al.,* 1992). This glucose value is however not only higher than that of the normal HbA1c subgroup, but also falls within the range of the glucose intolerance category according to the WHO guidelines for the two hour post prandial glucose level (between 7.8 and 11.1 mmol.L^{-1}). Furthermore, there was a statistically significant, almost 17 fold increased risk (OR = 16.8; $p = 0.003$) for glucose intolerance when having an HbA1c level > 6.5%. Despite the widely distributed 95% CI (1.94 - 145), as depicted in Figure 7.2, which is argued to be a result of the small number of individuals ($n = 6$) included in this subgroup, the lower limit still indicated an almost two fold increased risk. The aptness of including the HbA1c level in screening for T2D risk in the black South African population is therefore substantiated.

The subgroups that were statistically significantly associated with an increased risk for glucose intolerance, and therefore T2D, included the female gender (OR = 1.99; $p = 0.006$) and high BMI (OR = 1.93; $p = 0.005$) subgroups. These subgroups also had a statistically significant increase in HbA1c level with p-values of 0.005 and 0.000, respectively. The HbA1c value did however not reach the cut-off (> 6.5%) for the diagnosis of glucose intolerance and a re-evaluation of this cut-off value within the black South African population is suggested, keeping in mind the population specific best possible cut-off points proposed by Engelgau *et al.* (2009). The statistically significant association of a high HbA1c level with glucose intolerance (OR = 16.8; $p = 0.003$) further supports this suggestion. The HbA1c level is thus determined to be a good predictor of glucose intolerance and it is recommended that the change in the HbA1c levels be

evaluated in this study cohort over the total study period. The association analyses determined that none of the four screened loci were good predictors of HbA1c (Table 6.36). However, due to the nature of this study, including only 'apparently healthy' individuals, the number of participants included in the high HbA1c subgroup (n = 6) was relatively small, as would be expected.

7.6 EVIDENCE GENERATED FROM GENOTYPE ANALYSES

Genetic variants reported to be involved in T2D susceptibility in various other non-African populations were screened within black South African individuals and their value in predicting glucose intolerance evaluated. Due to the small sample size observed for some of the genotype subgroups (Table 6.1), the findings within this cohort are only a preliminary suggestion of risk indication according to genotype, and further testing will be required within a larger black South African cohort.

The allele frequencies of both the IRS-1 and IRS-2 A alleles, previously linked to T2D susceptibility in other populations (Jellema *et al.,* 2003 and Bernal *et al.,* 1989), were 0.09 and 0.08 respectively in the investigated cohort. The reported high BMI observed in individuals harbouring the IRS-1 X/A genotype in non-Africans (Jellema *et al.,* 2003) was not evident within the studied cohort (median BMI = 22.2 $kg.m^{-2}$). During the follow up of this preliminary study, future glucose tolerance status as well as progression to diabetes should be evaluated to determine the association of these genotypes with the increased risk for T2D.

Despite the small number of individuals (n = 6) included in the PPARγ2 X/G genotype subgroup, the 120 min glucose (median = 8.1 $mmol.L^{-1}$) indicated glucose intolerance, although this level did not differ statistically significantly to that of the WT subgroup (median = 7.3 $mmol.L^{-1}$). This is in direct contrast with the findings in other populations (Stumvoll *et al*., 2001b) that linked the G allele to protection against glucose intolerance. The association analyses results indicated however that there was no statistically significant association of the PPARγ2 X/G genotype with glucose intolerance (p = 0.340). The involvement of this alteration in T2D risk requires follow-up and the expansion to a larger cohort in an attempt to fully resolve the association of this genotype in the black South African population.

The WT and X/C CAPN10 genotypic subgroups differed significantly when comparing the 30, 60, 90 and 120 min glucose values (Table 6.11). However, the 30 and 60 min glucose levels observed in the X/C genotype subgroup did not reach the IGR cut-off levels as defined by Zhou *et al.* (2006). However, it should be noted that even though these cut-off levels were not reached, they may become more pronounced should these individuals develop T2D. Glucose intolerance was nevertheless observed in this subgroup, according to the 120 min glucose level of 7.8 mmol.L^{-1}. This finding is similar to that reported by Wang *et al.* (2002) in non-Africans. The evaluation of the association between the CAPN10 genotypes and glucose tolerance were however determined to not be statistically significant, as depicted in Figure 7.2. The X/C genotype, to some extent, is therefore suggested to be involved in T2D risk within the black South African population, but further investigation is required to fully elucidate this association.

The lack of significant associations between the investigated genotypes and T2D are contradictory to previously reported studies. This could be explained by the complexity of the biological mechanisms stemming from these genetic variants as well as their practical significance and physiological functions in glucose metabolism, which is yet to be comprehensively defined. These complexities are further compounded by the evident variation observed when comparing populations with different ethnic backgrounds.

In addition, none of the four investigated gene variants were determined to be good predictors of GCS (Table 6.13) within the studied black South African cohort. Since the numbers of individuals included in some of the genotypic subgroups were small, follow-up of the studied cohort is required to further evaluate the possible association of these genotypes with GCS.

The screened susceptibility loci within the IRS-1, IRS-2, PPARγ2 and CAPN10 genes were thus not determined to be accurate genetic markers in identifying black South African individuals at risk for T2D. The glucose intolerance observed within the various investigated subgroups however warrants further investigation into the genotypes associated with T2D risk, specifically in this population. The findings of this preliminary study suggest that the reported association of the investigated genetic variants in other populations does not hold true in the investigated population. Therefore, it is recommended that additional susceptibility loci and an expanded cohort be evaluated in the black South African population in order to definitively determine the genetic factors associated significantly with T2D risk.

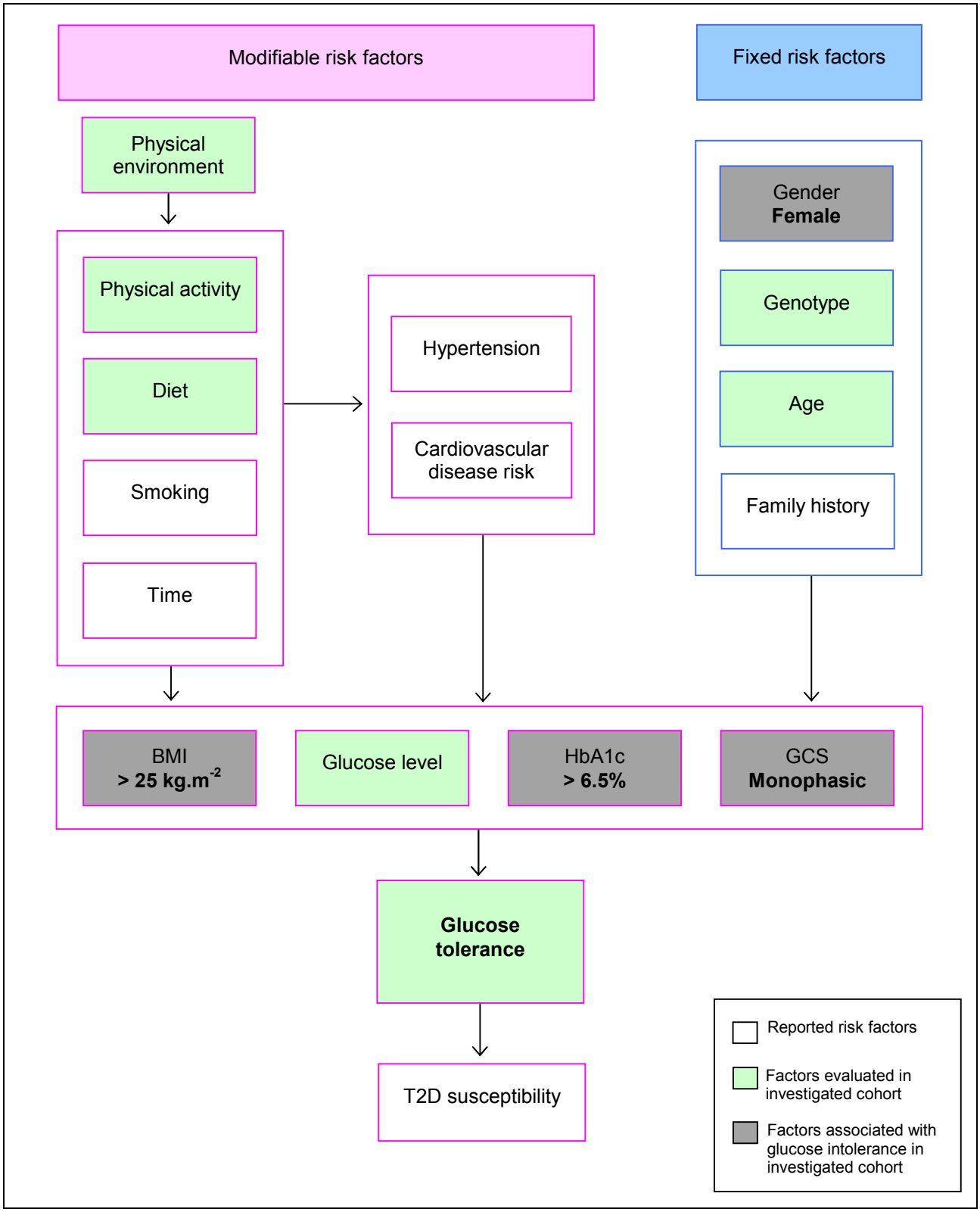
7.7 CLINICAL IMPORTANCE AND FUTURE APPLICATIONS

The association of various factors with the 120 min glucose value, as predictors of glucose tolerance, were evaluated to determine the most informative parameters required to effectively screen for T2D in the black South African population. The most common T2D screening tests include the WHO criteria of BMI, age, gender, diet and physical activity (Balkau *et al*., 2008). These parameters, as well as the HbA1c level and certain susceptibility loci, were evaluated within the black South African population. Figure 7.3 is a diagrammatic representation of the reported factors influencing T2D risk, including those investigated in the studied cohort. The factors that had a significant statistical association (Figure 7.1) with glucose intolerance are indicated in grey shading. As reported previously (Osman *et al.,* 1994), and as observed in this study, the risk of developing T2D depends on both modifiable and fixed factors. Physical environment, as well as easily adjustable lifestyle factors, form an integral part of the modifiable risk factor group. At present, factors proposed to be included in the fixed factor group are gender, genotype, age, family history and time.

Time, as a fixed risk factor, negatively affects disease susceptibility in the sense that older age on its own increases the risk for developing T2D (DeFronzo *et al.,* 1991). Time can however be argued, to some extent, to already be modifiable in two ways. Firstly, by improving diagnostic strategies in order to provide early diagnosis of glucose intolerance it is possible to implement appropriate lifestyle interventions to prevent disease onset. Secondly, time can be modified via early initiation of effective treatment strategies in order to delay onset of disease complications.

Another risk factor to take into account is that of time, and more specifically not only the time to diagnosis, but also the time to treatment. The time to diagnosis is of particular importance in the South African context, with not all communities having the necessary access to appropriate healthcare facilities. This is compounded by the fact that there is a need for improving diagnostic strategies in order to firstly, identify individuals at risk of developing T2D, and secondly to provide early diagnosis of glucose intolerance. These improvements, in addition to considering lifestyle intervention strategies, will contribute immensely to the prevention of the disease. The time to treatment plays an important role in decreasing the burden of care in our healthcare systems. The early initiation of effective treatment strategies will make a contribution in preventing or at least delaying the onset of disease complications.

Figure 7.3 Risk factors associated with T2D susceptibility



T2D = type 2 diabetes; BMI = body mass index; GCS = glucose curve shape; HbA1c = glycosylated haemoglobin. The parameters shaded in green and grey were evaluated and those in grey were statistically significantly associated with glucose intolerance (p-value < 0.05) within the investigated cohort, with the associated risk subgroup indicated in **bold** text.

As indicated in Figure 7.3, glucose tolerance is affected by various reported factors of which some entail a combination of other factors. Cardiovascular disease risk for

instance, is affected by, amongst others, hypertension, dyslipidaemia, smoking status and left ventricular hypertrophy. Diet, as risk factor, does not only include the investigated daily vegetable, fruit or berry intake but also the consumption of meat, coffee, fibre and alcohol. Although there are many susceptibility loci reported for T2D, only four common alterations were screened in the investigated cohort. It could therefore be argued that the many additional factors that were not investigated may have had an influence on the results of the study. This emphasises the importance of extensive follow-up of the individuals included in the studied cohort in terms of both the parameters included in this study as well as other identified risk factors.

Screening for this multifactorial disease is therefore imperative and allows for identifying asymptomatic individuals with a risk of developing T2D, in order to permit timeous commencement of preventive management. Successful prevention and individualised treatment strategies are the gold standard to which national health services should strive in order to optimise use of both time and money. Previous reports highlight the fact that a specific screening strategy does not necessarily apply to different populations with varying environment, socio-economic and clinical characteristics (Engelgau *et al.*, 2009). When optimising such a strategy within a specific population, the availability of risk parameter data, the best possible cut-off point to identify an affirmative test, cost effectiveness as well as the simplicity and frequency of the strategy are factors that need to be assessed in order to ensure the usefulness of the screening model. This ambitious strategy is hypothesised to be feasible only with a complete understanding of all factors involved in T2D susceptibility within a specific population. The purpose of this study was therefore to initiate the process of developing a population specific T2D susceptibility screening strategy for black South Africans, as suggested to be essential in the editorial by Herman, (2009).

The genotypes investigated in this study appeared to have minimal effects in altering the assessment of T2D risk in this cohort, similar to the analyses of the SNPs involved in T2D risk in non-Africans (Balkau *et al*., 2008). The importance of assessing simple parameters such as glucose level and BMI when determining T2D risk, as reported by Balkau *et al*. (2008), were however also confirmed in the studied cohort. Three of the parameters that are included in the Indian Diabetes Risk Score (Mohan *et al*., 2005), which was optimised for a developing country, namely age, obesity and physical activity were evaluated and the relevance substantiated in terms of T2D risk in the black South African cohort as depicted in Figure 7.3. The relevance of these findings were further supported

by the widely used German Diabetes Risk Score (Schulze et al., 2007) that also includes anthropometrical, dietary and lifestyle parameters that primarily represent modifiable risk factors for T2D, to accurately predict the 5-year probability of developing T2D in Caucasians. The assessment of GCS indicated the significance of harbouring a monophasic GCS when being glucose intolerant, as reported by Tschritter *et al*. (2003). This signifies the importance of including this parameter in the T2D risk screening strategy within the investigated population.

The results of this study emphasise the fact that a T2D screening strategy needs to be optimised within a specific population. A strategy cannot simply be adopted from those developed in other ethnic groups, and should take into account the different genetic as well as environmental risk factors that play a role in the pathophysiology of T2D. In order to develop a comprehensive understanding of T2D susceptibility and therefore optimise a population specific T2D screening strategy, a genome wide scan for susceptibility variants is essential in black South Africans, followed by powerful fine mapping and comprehensive case-control studies. By fully elucidating the complexities of T2D on the individual level, a step towards providing more accurate screening strategies to the immediate population will be achieved.

The generation of an optimised T2D screening strategy that is simple, effective, reliable, easy to use and cost effective for black South Africans will therefore ensure effective screening, early prevention and diagnoses of T2D. This will directly result in a significant decrease in the national burden of care, morbidity and mortality, paving the way to optimal health care strategies for this developing country.

CHAPTER EIGHT

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8.1 GENERAL REFERENCES

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