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Screening for the presence of Single Nucleotide Polymorphisms associated with Type 2 Diabetes in a black South African population

By

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In the Department of Genetics

Faculty of Natural and Agricultural Sciences

University of the Free State

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South Africa

DECLARATION

I Lerato Gloria Diseko, declare that the Master's Degree research dissertation that I herewith submit for the Master's degree qualification M.Sc Human Molecular Genetics at the University of the Free State is my independent work, and that I have not previously submitted it for a qualification at another institution of higher education.

LG Diseko

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“Therefore I say to you, whatever things you ask when you pray, believe that you receive them, and you will have them.” Mark 11:24

ABSTRACT

Introduction: The number of people suffering from type 2 diabetes (T2D) is expected to rise to 642 million by 2040. It is estimated that the highest proportion of undiagnosed individuals are African. The increasing prevalence of T2D has become a leading public health challenge throughout the world and has led to an intense search for genetic risk factors to this disease. The large increase in the prevalence of T2D coincides with a higher prevalence of obesity and insulin resistance. The aetiology of T2D is not fully understood, as a result, impairing the development of curative interventions to relieve the burden of T2D.

Objective: The present study was conducted to screen for the association of SNPs in candidate genes identified through genome wide association studies, with T2D in a black South African population. The identification of T2D risk associated alleles could aid in preventing the clinical onset of the diabetes by intervention of a modified lifestyle.

Methods: A descriptive case control study was performed on a cohort of 188 South African participants (T2D patients: n=96 and non T2D controls: n=92) of mostly Sotho descent, living in the central Free State area of Mangaung. The two groups were individually matched according to gender, age (20-60 years) and Body Mass Index (BMI) conferring to WHO categories. HbA1c levels were recorded for both groups. Non-T2D controls were included only if their HbA1c<6.5%.

Genotyping was determined using Real time PCR on a Quant Studio 5 qPCR system (Applied Biosystems) using hydrolysis probe technology. Genotype of the following six SNPs were determined: *TCF7L2* (rs7903146, rs12255372), *IRS1* (rs2943641), *CDKAL1* (rs7754840), *KCNJ11* (rs5219) and *RND3-RBM43* (rs7560163). Each qPCR run was performed with a technical homozygous control for each genotype as well as a non-template control. All the reactions were set up in duplicate. Control samples were sequenced using Sanger sequencing to confirm genotype, and for the rare allele control, synthetic oligomers (gBlocks® Gene Fragments; IDT) were purchased and applied.

Differences in allele and genotype frequencies between patients and controls were calculated with Chi-squared and 2x2 contingency tables (VassarStats). Odds ratios and 95% confidence intervals were determined. A $p<0.05$ indicated statistical significance.

Results: *TCF7L2* rs12255372 showed a significantly higher allele frequency in the T2D patient group than in non-T2D control group with value of p= 0.0000708. Increased homozygosity for the mutant TT genotype at *TCF7L2* rs12255372 was observed in 23% of T2D patients vs 3% in non-T2D controls (odds ratio 8.82, 95% confidence interval: 2.54-30.63 p= 0.0000599). The same trend was observed for the rare C allele of *IRS1* rs2943641, but without significance (p= 0.559). An increase in heterozygosity was observed for the *CDKAL1* rs7755840 in T2D patients, also without statistical significance p=1.89. No mutant homozygyotes for *KCNJ11* rs5219 were found in both cohorts. The rare/mutant allele of both *IRS1* and *RND3-RBM43* were higher in both patients and controls than the ancestral allele.

Conclusion: The *TCF7L2* rs12255372 is the only SNP that is significantly associated with T2D. Differences in ethnic background or environmental factors can possibly be attributable to differences in the results found in this study of black South African population compared to other ethnic groups. Results from this study emphasizes the need to investigate genetic variants associated with complex diseases such as T2D in the black South African population.

Keywords: T2D, SNP, genetics*, TCF7L2, KCNJ11, IRS1, CDKAL1, RND3-RBM43*, GWAS, South African population

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List of Abbreviations and acronyms

°C	Degree Celsius
%	Percentage
µl	Microliter
2-h PG	Two-hour plasma glucose
3’	3 prime
5’	5 prime
B-cat/TCF	Beta-catenin/ T cell transcription factor
β-cells	Beta cells
A	Adenine
A260/A280	Absorbance ratio at 260nm and 280nm
ABCC8	ATP-binding cassette sub-family C member 8
ABI	Applied Biosystems
ADA	American Diabetes Association
ADAMTS9	ADAM metallopeptidase with thrombospondin type 1 motif
ATP	Adenosine triphosphate
BMI	Body Mass Index
bp	base pairs
C	Cytosine
CI	Confidence Interval
CDK5	Cyclin dependent kinase 5

CDKAL1	CDK5 regulatory subunit associated protein 1-like 1
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CDKN2B	Cyclin-dependent kinase inhibitor 2B
CEO	Chief Executive Officer
CI	Confidence interval
COOH-	Carboxylic acid
Ct	Cycle threshold
CTLA	Cytotoxic T-lymphocyte antigen-4
CTNNB	beta-catenin
DBD	DNA Binding Domain
DCCT	Diabetes Control and Complications Trial
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
ECUFS	Ethics Committee of the Faculty of Health Sciences of the Free State
EDTA	Ethylenediamine tetra acetic acid
Et al.	*et alia* (and others)
Exo-SAP-IT	Exonuclease 1 and Shrimp Alkaline Phosphatase
FAM	Fluorescein amidite
FAF1	Fas Associated Factor 1
FoXO	Forkhead box class O
FPG	Fasting plasma glucose

FTO	Fat mass and obesity-associated
g	Gram
g/L	Gram per litre
G	Guanine
GAPs	GTPase-activating proteins
GCK	Glucokinase
GDM	Gestational Diabetes Mellitus
GEFs	Guanine nucleotide exchange factors
GGTases	Geranyl-geranyltransferases
GIP	Glucose dependent insulinotropic peptide
GLP	Glucagon-like peptide
GLP-1	Glucose-like peptide 1
GLP-2	Glucose-like peptide 2
GLUT	Glucose transporters
GMOs	Genetically Modified Organisms
GRB	Growth Factor Receptor Bound Protein
GTP	Guanosine triphosphate
GTPases	Guanosine triphosphatases
GSIS	Glucose-Stimulated Insulin Secretion
GWAS	Genome-Wide Association Studies
HAART	Highly Active Antiretroviral Therapy

HbA1c	Haemoglobin A1c
HDL	High density lipoprotein
HEX	Hexachlorofluorescein
HIV/AIDS	Human immunodeficiency virus/ Acquired immunodeficiency virus
HLA	Human Leukocyte Antigen A
HMG	High mobility group
HNF4α	Hepatocyte Nuclear Factor 4 alpha
HRM	High Resolution Melting
HRSEC	Health Sciences Research Ethics Committee
HWE	Hardy-Weinberg equilibrium
IDDM	Insulin-dependent diabetes mellitus
IDDM2	Insulin-dependent diabetes mellitus 2
IDDM12	Insulin-dependent diabetes mellitus 12
IDF	International Diabetes Federation
IDT	Integrated DNA Technologies
IFG	Impaired fasting glucose
IGF2BP2	Insulin-like growth factor 2 mRNA binding protein 2
IGT	Impaired glucose tolerance
IR	Insulin resistance
IRS1	Insulin receptor substrate 1
KATP	ATP-sensitive potassium channel

Kbp	Kilo base pairs
KCNJ11	Potassium inwardly-rectifying channel, subfamily J, member 11
Kg	Kilogram
LBD	Ligand-binding domain
LD	Linkage disequilibrium
LDL	Low-density lipoproteins
Lys23Gln	Lysine23Glutamine
M	Molar
MC4R	Melanocortin 4 receptor
Min	minutes
ml	Millitre
mmol/L	Millimoles per litre
mM	Millimolar
MODY	Maturity-Onset-Diabetes of the Young
MTNR1B	Melatonin receptor 1B
n	Number
NaN	No real number
nm	newton meter
NTC	No template control
OGTT	Oral glucose tolerance test
OR	Odds ratio

PCR	Polymerase chain reaction
PH	Pleckstrin homology
PI3K	Phosphoinositide 3-kinase
pmol	Picomoles
PPARG	Peroxisome proliferator-activated receptor gamma
PTB	Phosphotyrosine binding
qPCR	Quantitative PCR
RBM43	RNA binding motif protein 43
Rho GTPase	Ras homolog gene family member E
RND3	Rho family GTPase 3
ROX	6- Carboxyl-X-Rhodamine dye
RPG	Random plasma glucose
Rs	Reference single nucleotide polymorphism number
SAP	Shrimp Alkaline Phosphatase
Sec	Seconds
SHP2	Src homology 2-containing phosphotyrosine phosphatase
SLC30A8	Solute carrier family 30 (zinc transporter), member 8
SLC16A11	Solute Carrier Family 16 Member 11
SNPs	Single nucleotide polymorphisms
SUR1	Sulfonylurea receptor 1
SYBR green	cyanine dye used as nucleic acid stain

T	Thymine
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TAE	Tris-acetate-EDTA
Taq	Thermus aquaticus
TCF7L2	Transcription factor 7-like 2
TE	Tris-EDTA
Tm	Melting temperature
TNFα	Tumor necrosis factor alpha
Tris	Tris hydromethyl aminimethane
tRNA	Transfer Ribonucleic acid
tRNAlys	Lysine transfer ribonucleic acid
UUU	Urasil codon
VIC	Fluorescent dye is proprietary to ABI (now Life Technologies) and its chemical structure is currently not publically available. Wavelength 551nM
VNTR	Variable number of tandem repeats
v/v	Volume per volume
w/v	Weight per volume
WHO	World Health Organization
www	World wide web

X^2	Chi-Square

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CHAPTER 1: INTRODUCTION

The increasing prevalence of T2D has become a leading major public health challenge throughout the world, in both rural and urban areas. In 2015, data from the International Diabetes Federation (IDF) estimated 415 million people between the ages of 20-79 to be diabetic, this number was estimated to rise to 642 million by 2040. The highest proportion of undiagnosed individuals (66.7%) was estimated to be African (Orgurtsova *et al.* 2017). However, undiagnosed T2D is common worldwide with an estimated lag of 5-7 years (Kronenberg *et al.* 2008). Based on the most recent 2015 IDF estimates for South Africa, the prevalence of undiagnosed T2D adults between the ages of 20-79 was 7% with a comparative prevalence of 7.6% (Amod *et al.* 2017).

Diabetes has become a major burden on health systems and even greater burden on the economy throughout the world. The total global health expenditure due to diabetes was estimated at 673 billion US dollars in the year 2015 (Orgutsova *et al.* 2017). According to American Diabetes Association (2004), chronic hyperglycaemia of diabetes is associated with long term damage and a failure of various organs specifically the eyes, the kidneys, nerves, heart and blood vessels accounting for a high mortality rate. The large increase in the prevalence of T2D coincides with a higher prevalence of obesity and obesity itself causes some degree of insulin resistance (Kahn *et al.* 2006). The burden of disease on patients has created an urgency for innovative research on diabetes to lessen its burden on both people with the disease and those affected by the disease by cutting down costs of care.

Research has proven that T2D is a complex multifactorial metabolic disorder with genes and environmental factors playing a role in its onset. T2D is postulated to have a strong genetic aetiology, though its molecular mechanisms and the underlying genetic architecture still needs to be elucidated (Kirchhoff *et al.* 2008). Nutrition therapy and lifestyle interventions including weight reduction and physical activity are powerful tools for primary prevention and minimizing associated risk (Psaltopoulou *et al.* 2010). Modern medical care uses a huge array of pharmaceutical interventions aimed at preventing and

controlling high blood glucose (Fowler 2008). Efforts to understand specific features of T2D may help in containing the escalating epidemic.

The strong genetic component of T2D has been indicated by high concordance rates in twin studies, but the aetiology is not well understood. As a polygenic disorder, T2D has many different combinations of gene defects that exist among diabetic patients. Although these genes may contribute susceptibility to T2D, environmental factors interacting with these genetic aberrations may trigger the clinical disease. The majority of Genome Wide Association Studies (GWAS) are aimed at identifying genetic factors as predictive disease markers with a total of 83 susceptibility loci identified (Wang *et al.* 2015). Early identification of individuals at high T2D risk enables delay or prevention of T2D onset through effective lifestyle and/or pharmacological interventions and in turn reduce the costs of care (Ashraf *et al.* 2013).

1.1 Problem statement

There is limited information on common variants performed by the GWAS associated with T2D in the black South African population. The increasing prevalence of T2D has become a leading major burden on health systems and the economy of the country. Urbanization and adoption of Western dietary and lifestyle habits are the major environmental risk factors associated with the increase in prevalence of diabetes in South Africa. Relieving the burden of this disease should be a number one priority as the mortality rate attributable to this disease exceeds that of HIV/AIDS, tuberculosis and malaria combined in the year 2015 (Amod *et al.* 2017).

Only a handful of studies have used the genome-wide approach to identify genomic regions linked to or associated with T2D in African populations. T2DM associated SNPs have therefore not been thoroughly investigated for genetic prediction in African populations. There is therefore a high probability that genetic variants associated with T2D may be overlooked in the understudied African genome with the highest number of undiagnosed individuals (Nienaber 2016). More T2D genetics studies are required in the African setting since the genetic make-up poses different effects on different ethnic

groups (Chikowore 2015). This research will contribute to the genetic prediction of T2D and add to the future health care interventions for disease prevention.

1.2 Aim

To screen for single nucleotide polymorphisms associated with T2D in a black South African population that live in Bloemfontein and the central Free State area.

1.3 Objectives

To perform Real Time PCR genotype screening to determine the presence of diabetes associated SNPs in patients suffering from T2D and non-Diabetic participants. The genotypes of the black central South African population have not been documented for genes associated with T2D in the published literature.

1.4 Structure of the dissertation

Chapter 1 is an explanation of the burden of T2D globally, the prevalence of T2D, possible risk factors associated with T2D and the importance of knowledge of the genetic basis of this disease. The problem statement, aim and objective is also stated in Chapter 1. Chapter 2 is a detailed review of the literature concerning T2D, the genes associated with and how this study could potentially add to the knowledge about genetic variants associated with T2D in black South Africans of Sotho descent. Chapter 3 describes the methods used to screen for SNPs associated with T2D the study design, sample size, inclusion criteria and exclusion criteria of the population as well as data analysis methodology. Chapter 4 focuses on the results from this study and their implications. Chapter 5, the final chapter of the dissertation that concludes the study and mentions the limitations and the potential impact of the study in future.

CHAPTER 2: LITERATURE REVIEW

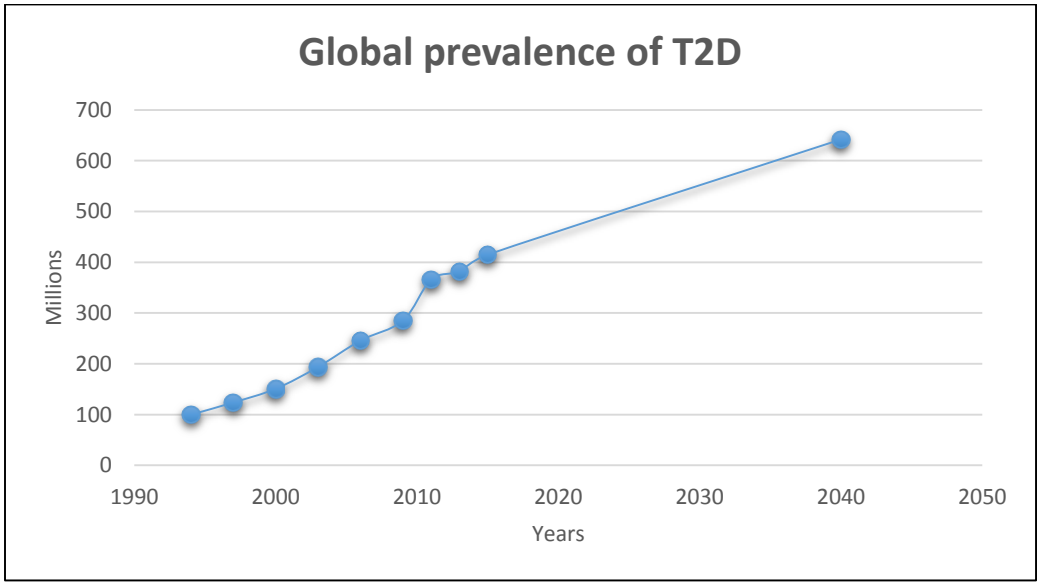
2.1 Diabetes mellitus, symptoms and complications

Diabetes mellitus (DM) is a group of metabolic disorders of multiple aetiologies characterized by increased levels of glucose caused by either insufficient insulin production by the pancreas, improper response of the body cells to insulin or both (American Diabetes Association 2004). Diabetic individuals have abnormalities in the metabolism of carbohydrates, fat and protein as a result of the inadequate action of insulin on target tissues (American Diabetes Association 2011). Patients with uncontrolled diabetes mellitus may present with acute, life threatening outcomes such as hyperglycaemia with ketoacidosis, a metabolic state associated with high concentrations of ketone bodies or the non-ketotic hyperosmolar syndrome (American Diabetes Association 2014). This disease has early and late stage complications with hyperglycaemia, polyphagia, polyuria, polydipsia and blurred vision being part of the early stage complications leading to late stage complications such as vascular disease, heart disease, stroke, peripheral nephropathy with risk foot ulcers; Charcot joints and amputations, neuropathy and predisposition to infection (Haghvirdizadeh *et al.* 2014; Nathan 1993). Abnormalities of lipoprotein metabolism and hypertension may also be present in people suffering from diabetes (Papatheodorou *et al.* 2015)

Because of the silent and progressive nature of diabetes and its complications, diabetes may progress undetected for a long time. Thus, many individuals are often undiagnosed until noticeable signs and symptoms appear (Yin *et al.* 2014). It is estimated that 30-80% of diabetes cases are undiagnosed and among those diagnosed, 25% would have developed microvascular complications by the time of diagnosis (Chikowore 2015). There is a major need for improved screening for diabetes, this disease, if untreated could lead to death (Amod *et al.* 2017). Early intervention treatment and a healthier lifestyle could reduce the risk of long term complications.

2.2 Prevalence and burden of Diabetes Mellitus

The number of people suffering from diabetes has increased in nearly all countries and continues to rise, as economic development and urbanization lead to evolving lifestyles characterized by reduced physical activity and increased obesity (Whiting *et al.* 2011). The earliest global estimates were from the year 1994 to the year 1997 and in those years, 100-124 million people were estimated to have diabetes (Jaacks *et al*. 2016). The International Diabetes Federation (IDF) estimated the prevalence of diabetes to be 151 million in 2000, 194 million in 2003, 246 million in 2006, 285 million in 2009, 366 million in 2011 and 382 million in 2013 globally. In 2015, people between the ages of 20-79, 415 million people (8.8% of the population) were estimated to be diabetic and an estimated 193 million people to have undiagnosed diabetes globally (Figure 2.1). This number was predicted to increase to 642 million by 2040 (Orgurtsova *et al*. 2017).



(Orgurtsova *et al.* 2017 and Jaacks *et al.* 2016)

Figure 2.1 Global prevalence of Diabetes

Disturbingly, the number of children with newly diagnosed diabetes has increased from 1-2% to 8-45%, the majority of these children are usually overweight/obese when

diagnosed (American Diabetes Association 2000). Globally, diabetes is also an increasing cause of premature death (Bradshaw *et al.* 2007). An estimation of 5 million people between the ages of 20-79 years died from diabetes. This number is higher than that of deaths due to HIV/AIDS (1.5 million), tuberculosis (1.5 million) and malaria (0.6-million) combined in the year 2013 (Amod *et al.* 2017).

The regional prevalence of diabetes on the African continent in 2015, was 3.2% and is expected to rise to 3.7 % by 2040 (Amod *et al.* 2017). An estimated 46.5% of adults between the ages of 20-79 years are undiagnosed and the highest percentage (66.7%) of undiagnosed individuals are in Africa (Orgustova *et al.* 2017). In the Africa region, 321-100 deaths were due to diabetes in 2015 of which 1.7 times more in women than men. The total health expenditure on diabetes was estimated to be 3.4 billion in Africa. South Africa is the country with the highest amount of diabetic individuals in Africa (2.3 million) due to urbanization (Amod *et al.* 2017). In South Africa alone, 57 319 deaths attributable to diabetes occurred in 2015 and with that said, 60-80% of people with diabetes die before the age of 60, all falling within the working class (Amod *et al.*2017). In 2015, the total global expenditure due to diabetes was estimated at 673 billion US dollars, which is approximately 9.24 trillion South African Rands (Orgutsova *et al.* 2017). The cost per person, per annum of individuals with diabetes was approximately 26 743.69 South African Rands (International Diabetes Federation 2015). Changes associated with urbanization, globalization and development are dramatically adding to the burden of diabetes in all countries, mostly in low and middle-income countries where resources for dealing with the associated clinical problems are most scarce (Whiting *et al.* 2011).

Estimates of the current and future burden of diabetes are important to allow rational planning and allocation of resources and emphasize the role of lifestyle and encourage measures to counteract trends for increasing prevalence (Whiting *et al.* 2011; Wild *et al.* 2004). The latest evidence shows that diabetes continues to be a huge and increasing global health burden and likely to continue to escalate significantly in the next decades if current trends continue. Combined with comparatively slow development of health systems, this disease and its complications will continue to be elevated, especially in low and middle income countries (Guariguata *et al.* 2014).

2.3. Classification of Diabetes Mellitus

Several guidelines for the classification and diagnosis of diabetes have been published since 1965, globally (Amod *et al.* 2017). The classification encompasses both the clinical stages and aetiological types of diabetes, and other categories of hyperglycaemia (Amod *et al*. 2017). In T1D, the individual needs insulin for survival whereas in T2D, other specific types and gestational diabetes only need insulin to control the hyperglycaemia (Amod *et al*. 2017; Alberti *et al*. 1998). The aetiological classification reflects the fact that the defect or process which may lead to diabetes may be detectable at any stage in the development of diabetes even at the stage of normoglycaemia (Alberti *et al*. 1998). Diabetes is classified according to aetological types and clinical stages indicated in Table 2.1 (Amod *et al*. 2017).

The three most common types of Diabetes are discussed in more detail to follow, however, T2D is by far the most common type of diabetes and is also the focus of this research study.

Table 2.1 Aetiological classification of diabetes mellitus

I.	**T1D (β cell destruction, usually leading to absolute insulin deficiency)** A. Immune mediated B. Idiopathic
II.	**T2D** May range from predominantly insulin resistance with relative insulin deficiency, to a predominantly secretory defect with insulin resistance. Also includes a subset who have ketosis-prone diabetes
III.	**Other specific types** A. Genetic defects of β cell function Maturity onset diabetes of the young (MODY) –neonatal diabetes mellitus, mitochondrial DNAs

	B. Genetic defects in insulin action Type A Insulin resistance, Donahue syndrome (Leprechaunism), Rabson-Mendehall syndrome, lipoatrophic diabetes and a few extremely rare syndromes C. Diseases of the exocrine pancreas Including Pancreatitis, trauma/pancreatectomy, neoplasia, cystic fibrosis, haemochromatosis, fibrocalculous pancreatopathy, and others D. Endocrinopathies Acromegaly, Cushing's syndrome, glucagonoma, phaeochromocytoma, hyperthyroidism and others E. Drug or chemical induced Glucocorticoids, nicotinic acid, thyroid hormone, β-adrenergic agonists, thiazides, phenytoin, Interferon, pentamidine, diazoxide, atypical antipsychotics, highly active antiretroviral therapy (HAART) F. Infections Congenital rubella, cytomegalovirus and others G. Uncommon forms of immune-mediated diabetes "Stiff-man" syndrome, anti-insulin receptor antibodies, others H. Other genetic syndromes sometimes associated with diabetes Down syndrome, Klinefelter syndrome, Turner syndrome, Wolfram syndrome, Friedreich ataxia, Hintington chorea, Laurence-Moon-Bledi syndrome, myotonic dystrophy, porphyria, Prader-Will syndrome, others
IV.	**Hyperglycaemia first detected in pregnancy** A. Gestational diabetes B. Diabetes mellitus in pregnancy

Amod *et al.* (2017)

2.3.1 Type 2 Diabetes Mellitus

T2D mellitus is the most common type of diabetes and constitutes about 90-95% of diabetes cases globally (Bao *et al*. 2013; Kronenberg *et al*. 2008). T2D mellitus, also

known as non-insulin-dependent diabetes mellitus is a complex multifactorial metabolic disorder characterized by hyperglycaemia, with a varying degree of insulin resistance, impaired insulin secretion and increased hepatic glucose production (Dayeh 2015; Alejandro *et al*. 2015). The earliest predictor of the development of T2D is low insulin sensitivity in skeletal muscle (Cree-Green *et al.* 2013). However, often, symptoms may be absent or not severe, as a result diagnosis of the disease is prolonged. With a percentage of 30-80 cases undiagnosed, there is a major need for improved screening for diabetes (Amod *et al*. 2017).

The increasing prevalence of T2D has become a leading major public health challenge throughout the world and has led to an intense search for genetic risk factors to this disease (Wang *et al*. 2015). The rapid and continuous increase in T2D globally and specifically in low income countries such as South Africa, warrants the need for scientists to come up with innovative research on diabetes to diminish the burden of the disease and also to reduce costs of care.

The aetiology of T2D is not fully understood, but it is currently thought to occur in genetically predisposed individuals exposed to a series of environmental influences that precipitate the onset of clinical disease (Hertel *et al.* 2013; Kronenberg *et al*. 2008). Epidemiologic determinants and risk factors of T2D can be divided into genetic factors (genetic markers, family history), demographic characteristics (sex, age and ethnicity), behavioral and lifestyle-related risk factors (obesity, physical inactivity, diet, stress) and metabolic determinants (impaired glucose intolerance, insulin resistance) (Zimmet *et al.* 2001) (Table 2.2). Other major risk factors for T2D include obesity, Impaired Fasting Glucose (IFG) or Impaired Glucose Tolerance (IGT), hypertension and high density lipoprotein (HDL) cholesterol of greater than 0.9 mmol/L or a triglyceride level of greater or equal to 2.8 mmol/L (American Diabetes Association 2006).

Treatment of T2D involves lifestyle changes and oral anti-diabetic drugs that lead to an increase in insulin secretion from the pancreas or increased insulin sensitivity in the tissues (Gough *et al*. 2010). The use of insulin is needed for survival in severe cases (Amod *et al.* 2017). Blood glucose control is usually monitored in patients by determination of HbA1C, a measure of glucose-bound (glycated) haemoglobin (Gough *et*

al. 2010). The normal range for the HbA1c test is between 4% and 5.6% for people without diabetes, levels between 5.7% and 6.4% indicate increased risk of diabetes while levels of 6.5% or higher indicate diabetes (Dansinger 2015). People with diabetes should have the glycated haemoglobin test every 3 months, since this is the lifespan of red blood cells, to determine whether their blood sugar level have reached the control target level (Dansinger 2015; Gough *et al.* 2010).

2.3.2 Type 1 Diabetes Mellitus

Type 1 diabetes (T1D) accounts for about 5-10% of all cases of diabetes. However, its incidence continues to increase worldwide and it is known to have serious short-term and long-term implications (Danerman 2006). The incidence of type 1 disease is rapidly increasing in specific regions such as Africa and Asia and shows a trend towards earlier onset (Atkinson and Eisenbarth 2001). T1D is a chronic disorder characterized by hyperglycaemia, primarily caused by autoimmune pancreatic beta cell destruction and by absolute insulin deficiency (Yagnik 2015). β-cell mass is destroyed gradually over time in genetically susceptible individuals after exposure to environmental triggers that induce T-cell mediated β-cell injury and the production of humoral antibodies. T1D individuals require insulin for survival (Kaufman 2003; Alberti *et al*. 1998).The beta cell destruction is variable, usually rapid in infants and children and usually slower in adults (American Diabetes Association 2004).

There are two forms of T1D identified namely type 1A diabetes, a cell-mediated autoimmune attack on beta cells and type 1B which is less frequent, has no known cause and occurs mostly in individuals of African and Asian descent with varying degrees of insulin deficiency between sporadic episodes of ketoacidosis (Danerman 2006). T1D can occur when approximately two/thirds of the islets are devoid of insulin-producing cells. Among individuals who have had T1D for more than 5 years, most of the remaining islets are insulin deficient, however still contain a normal complement of other hormone secreting cells (Atkinson *et al*. 2014). T1D is known to be a polygenic disease with almost 40 loci known to affect its susceptibility, the HLA region on chromosome 6 provides almost half of the genetic susceptibility that leads to T2D risk (Atkinson *et al*. 2014).

T1D has traditionally been diagnosed based on clinical catabolic symptoms suggestive of insulin deficiency: polyuria, polydipsia, weight loss and marked hyperglycaemia that is non-responsive to oral agents (Chiang *et al.* 2014). Currently diagnosis is based on the level of sugar in blood, done by either an HbA1c test, OGTT, RPG or an FPG (American Diabetes Association 2018). Patients with T1D are prone to other autoimmune disorders such as Graves disease, Hashimoto's thyroiditis, Addison's disease, vitiligo, autoimmune hepatitis, myasthenia gravis and pernicious anaemia (American Diabetes Association 2004).

2.3.1 Gestational diabetes

Pregnancy is associated with insulin resistance (IR) and hyperinsulinemia that may predispose some women to develop diabetes (Alfadhli 2015). Gestational diabetes mellitus (GDM) is defined as glucose intolerance of various degrees that is first detected during pregnancy (Buchanan and Xiang 2005). The prevalence of GDM fluctuates from 1-20%, and is rising worldwide, parallel to the increase in the prevalence of obesity and T2D mellitus (Alfadhli 2015). In 2013, the global prevalence of hyperglycaemia in pregnancy was estimated to be 16.9% (Guariguata *et al.* 2014). GDM is found to be higher in African, Hispanic, Indian and Asian women than for Caucasian women (Alfadhli 2015). GDM women usually have an increased positive family history of T2D, with significantly greater parental history and their offspring are at higher risk of getting T2D (Kwak *et al.* 2012), indicating that gestational diabetes is assumed to have a strong heritability. Furthermore, women with gestational diabetes history have a high risk (20-60 %) of developing T2D 5-10 years post pregnancy (Amod *et al.* 2017).

Pregnancy can be associated with many metabolic, biochemical, physiological, haematological and immunological changes. Healthy pregnancies can be associated with resistance to the action of insulin and utilization since the placenta produces hormones such as oestrogen, cortisol, progesterone and human placental lactogen that have insulin-desensitizing effects as well as increased maternal adiposity particularly in the third trimester (Buchanan and Xiang 2005). The pancreatic beta cells normally increase their insulin secretion to compensate for the insulin resistance of pregnancy, resulting in changes in circulating glucose levels (Buchanan and Xiang 2005). Women at high risk for

gestational diabetes include older women, those with previous history of glucose intolerance, those with babies born at 4 kg and above, women from high risk ethnic groups, history of recurrent abortions, history of unexplained stillbirths, history of hypertension and woman who has elevated fasting blood glucose levels during pregnancy (Alberti *et al*. 1998 and Alfadhli 2015). Several clinical studies suggested that GDM women have limited insulin secretion capacity which cannot compensate for the increased insulin resistance (Kwak *et al*. 2012). Glycaemic control is especially in important in women with GDM. Numerous other symptoms also include diabetes symptoms, however the focus of this study is on T2D.

2.4 Age, Ethnicity and Family history of T2D

Genetic, environmental and demographic factors together with their interaction, determine an individual's risk for T2D; its heritability has been estimated as approximately 25% (Kato 2013). In individuals between the ages of 40-65, diabetes has reached epidemic numbers and is expected to continue to rise even higher (Psaltopulou et al. 2010). Diabetes mellitus is highly prevalent and increasing in persons aged 40 and older, particularly among racial and ethnic minorities (California Healthcare Foundation 2003). Older persons with T2D have higher rates of premature death, functional disability, geriatric syndromes and coexisting illnesses such as hypertension, coronary heart disease and stroke (California Healthcare Foundation 2003).

African Americans have a disproportionately high risk for developing T2D with an estimated prevalence, twice that observed for their European-American counterparts (Palmer *et al*. 2012). Overall, Africans are affected earlier by T2D and with more severe complications than their Caucasian counterparts, causes being delayed diagnosis and poor management due to a low socio-economic status (Danquah *et al*. 2013). Cumulative evidence suggests that Asians, Egyptians, Iranian and African Americans maybe more susceptible to insulin resistance compared with European ancestry (Shu *et al*. 2010; Ashraf *et al*. 2013; Sale *et al*. 2007; Keshavarz *et al*. 2014).

T2D is postulated to have a strong genetic aetiology. A family history confers an up to 3-fold increased risk for first degree relatives to develop the disease, concordance for diabetes is approximately 70% higher in monozygotic twins compared to, up to 20% in dizygotic twins (Schafer *et al*. 2011). A study by Köbberling (1982) reported 40% of first-degree relatives of T2D patients to have developed T2D, compared to a baseline population of 6% confirming the strong genetic basis of T2D.

2.5 T2D diagnosis

The clinical diagnosis of T2D is often prompted by signs and symptoms such as increased thirst and frequent urination, recurrent infections, unexplained weight loss and in severe cases, drowsiness and coma (Alberti *et al.* 1998). The diagnosis of diabetes is based on the measurement of plasma glucose levels (Kronenberg *et al*. 2008). A random blood sugar test is taken at any time regardless of if an individual had something to eat. A random blood sugar level of 11.1 mmol/L or higher suggests diabetes (Amod *et al.* 2017). The fasting plasma glucose test checks an individual's fasting glucose levels. This test is usually done in the morning before and after 2 hours that an individual eats anything. Diabetes is diagnosed at fasting blood glucose greater than 7 mmol/L. The oral glucose tolerance test is a two hour test that checks an individual's blood glucose levels before and after 2 hours an individual drinks a special sweet drink. A 2-hour blood glucose of greater than 11.1 mmol/L or higher indicates diabetes while that of 7.8 mmol/L to 11.1 mmol/L indicates pre-diabetes (American Diabetes Association 2015).

A strong correlation between the concentration of glycated haemoglobin and the mean level of blood glucose over 3 months was indicated by clinical studies. The glycated haemoglobin (HbA1c) test indicates the average blood sugar level for the past two to three months because the red blood cells lifespan is four months. It measures the percentage of the glycated hemoglobin, the oxygen- carrying protein in red blood cells (Gough *et al.* 2010). The glucose binds to the haemoglobin of erythrocytes hence the term glycated haemoglobin. The higher the blood glucose levels, the more the glycated hemoglobin. An HbA1c below 5.7% is considered normal, between 5.7 and 6.4%, prediabetic and an HbA1c level of 6.5% and higher indicates diabetes (American

Diabetes Association 2018). The complete recommendations for the diagnosis for diabetes, according to the 2017 SEMDSA guidelines (Amod *et al.* 2017) can be viewed in Table 2.2

Table 2.2. Diagnosis of diabetes

SEMDSA 2017 Recommendations
The diagnosis of diabetes is confirmed: a. In patients with symptoms of hyperglycaemia (polyuria, polydipsia, blurred vision, weight loss) or metabolic decompensation (diabetic ketoacidosis or hyperosmolar non-ketotic state), when any one single test confirms that the: • Random plasma glucose is ≥ 11.1 mmol/L • Fasting plasma glucose is ≥ 7.0 mmol/L • HbA1c is ≥ 6.5% • 2-hour post-load glucose is ≥ 11.1 mmol/L. However, a GTT is rarely needed in this category of patient. b. In an asymptomatic individual, when any one of the following tests, repeated on separate days within a 2 week period confirms that the: • Fasting plasma glucose is ≥ 7.0 mmol/L • 2 hr-post load glucose (OGTT) is ≥ 11.1 mmol/L • HbA1c is ≥ 6.5% If the diagnosis of diabetes is not confirmed with the repeated test, institute lifestyle modification and retest in 3 to 6 months.
HbA1c alone can be used as a diagnostic test for diabetes providing that stringent quality assurance tests are in place and assays are standardised to criteria aligned to the international reference values, and there are no conditions present which preclude its accurate measurement.

Bedside or point-of-care devices (for glucose or HbA1c) must not be used to diagnose diabetes.
HbA1c of 6.5% is recommended as the cut-point for diagnosing diabetes. A value of less than 6.5% does not exclude diabetes diagnosed using glucose tests. A glucose based measurement is desirable in individuals with HbA1c values close to the diagnostic cut-point (e.g. 6.0 to 6.4%).
The diagnosis of type 2 diabetes is confirmed when all other causes of diabetes are reasonably excluded
Impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) are categories of intermediate hyperglycaemia that identify individuals at risk for future diabetes and cardiovascular disease. IFG and IGT are modifiable risk factors. Refer to Chapter 25 for management of these risk factors.
Impaired fasting glucose is present when 2 consecutive tests performed on different days confirm that the fasting plasma glucose is 6.1 to 6.9 mmol/L, in the absence of diabetes and impaired glucose tolerance by other tests.
Impaired glucose tolerance is present when 2 consecutive tests performed on different days confirm that the 2-hour post-load plasma glucose is 7.8 to 11.0 mmol/L, in the absence of diabetes by any other test.
Screening for type 2 diabetes: Screen all overweight adults at any age if they have at least one other risk factor for diabetes. For all other adults, start screening for diabetes at age 45. The frequency of rescreening for diabetes depends on individual risk and can range from 3 months (e.g. the obese individual with IGT and multiple other risk factors for diabetes) to 3 years (e.g. the normal-weight individual with no risk factors for diabetes). The preferred screening test for high-risk individuals is the OGTT as it is more sensitive and is the only method for detecting impaired glucose tolerance.

Adapted from SEMDSA guidelines 2017- (Amod *et al.* 2017).

2.6. Genetics of Diabetes

2.6.1 Monogenic Diabetes types

Several types of diabetes are associated with monogenic defects in beta cell function. The most prevalent monogenic diabetes is referred to as maturity onset diabetes of the young (MODY). MODY is characterized by an onset of high blood sugar level at an early age usually before the age of 25 years. Individuals with this type of diabetes have impaired insulin secretion with minimal or no defects in insulin action (American Diabetes Association 2004). MODY patients display a familial form of noninsulin-dependent diabetes with an autosomal dominant pattern of inheritance. To date, mutations in nine different genes were identified causative to the MODY phenotype which accounts for approximately 1-2% of patients diagnosed with diabetes (Gardner and Tai 2012). Mutations on chromosome 12 in a hepatic nuclear transcription factor have been reported to be associated with the most common form of MODY (Alberti and Zimmet 1998).

2.6.2 T1D

T1D is known to result from an immunological destruction of insulin producing islet pancreatic beta cells. An interaction of genetic susceptibility and environmental factors are thought to provide the fundamentals for disease development (Atkinson and Eisenbarth 2001). The major histocompatibility (HLA) gene region is reported to have multiple genetic loci that predispose the development of T1D (Kaufman 2003). To date, 40 loci have been identified to affect the susceptibility of the disease. The HLA region on chromosome 6 has half of the genetic susceptibility loci that leads to risk of T1D. HLA class II has been reported to show the strongest association with T1D (Atkinson *et al.* 2014). Other genes known to be associated with T1D are the *insulin-VNTR (IDDM2)* and *CTLA* (*IDDM12*) which account for roughly 15% of the susceptibility loci associated with T1D, with minor contributions from the other *IDDM* genes. The important regulators of the immune response are thought to be these susceptibility genes (Danerman 2006).

2.6.3 T2D

To date, genome wide association studies (GWAS) have identified a total of 83 T2D susceptibility loci in over 65 genes. The strong genetic contribution of diabetes is supported by a concordance rate of 26-73% in twin studies and 76% in monozygotic twins (Wang *et al.* 2015). Different ethnic groups and different environmental factors also play a role in the variation of T2D prevalence (Kato 2013). The majority of SNPs were discovered in the European population, secondly in the East and South Asian populations, with very few identified in African, African-American and Hispanic populations (Wang *et al*. 2015). Extensively studied genes include: peroxisomal proliferator activated-receptor gamma (*PPARG*) potassium channel, inwardly rectifying subfamily J, member 11 (*KCNJ11*), transcription factor 7-like 2 (T-cell specific, HMG-box) (*TCF7L2*), solute carrier family 30 (zinc transporter) member 8 (*SLC30A8*), ADAM metallopeptidase with thrombospondin type 1 motif (*ADAMTS9*), insulin receptor substrate 1 (*IRS1*), CDK5 regulatory subunit associated protein 1-like 1(*CDKAL1*), cyclin-dependent kinase inhibitor 2A (*CDKN2A/B*), insulin-like growth factor 2 mRNA binding protein 2 (I*GF2BP2*), fat mass and obesity associated (*FTO*) and melatonin receptor 1B (*MTNR1B*) (Saxena *et al.* 2007; Scott *et al.* 2007; Zeggini *et al*. 2008; Rung *et al*. 2009; Dupuis *et al*. 2010).

Since T2D is a complex disease that is thought to occur in genetically predisposed individuals, a single gene mutation will probably not impose any effect unless an individual is exposed to a series of environmental influences that precipitate the onset of the clinical disease (Kronenberg *et al.* 2008). Also the effect imposed by the gene mutation or polymorphism might not be the same amongst individuals within a population or different populations. This variation is directly or indirectly affected by the genetic background, family or population level of the individual and can be further complicated by interaction with environmental factors (Kharroubi and Darwish 2015). Despite the knowledge of genetic polymorphisms associated with T2D, the pathogenesis of their influence is still unclear. Most studies on T2D have now been conducted amongst the African American, a meta-analysis by Ng *et al.* (2014) in which association results for approximately 2.6 million SNPs were subsequently examined. The sub-Saharan Africa has a high rate of

T2D yet remains largely understudied. A study by Ademeyemo *et al.* (2015) evaluated 106 reported T2D GWAS loci in continental Africans (Nigeria, Ghana and Kenya) and found the TCF7L2 rs7903146 to be the most significant locus.

Only a few studies have screened for the presence of polymorphisms associated with T2D in South Africa. A study by Nienaber (2016) screened for the presence of SNPs associated with T2D in the *PPARG* gene and found no association in South African Sotho population, in contrast to data on the *PPARG* gene from the European region (Wang *et al.* 2016). A study by Pirie *et al.* (2010) screened for the presence of SNPs in the *PPARG, KCNJ11, TCF7L2, FTO* and *HHEX* genes in South Africa (Zulu descent) and found only the rs7903146 in the *TCF7L2* to be associated with T2D. A study by Chikowore (2015) evaluated the association of genetic variants; with impaired glucose tolerance in South Africa (Tswana descent) and found no association. A study by Olckers *et al.* (2007) investigated the C-11377G and G-11391A, within the promoter of the adiponectin gene. The variant allele at G-11391A as well as the 12 haplotypes were found to be significantly associated with a protective factor with regard to T2D susceptibility (Olckers *et al.* 2007). Schwarz *et al.* (2009) did a meta-analysis study of the C-11377G locus within the adiponectin gene in a black South African, a Cuban Hispanic and a German Caucasian cohort. No significant difference was found between the black South African control and diabetic cohorts and thus C-11377G is not a significant factor within the South African population. The homozygous genotype for the risk factor variant and increased risk towards T2D indicates the possible role of this alteration within the Cuban cohort (Shwarz *et al.* 2009). A study by Vergotine *et al.* (2015) investigated the presence of PPARG Pro12Ala (rs1801282, G>C), Pro115Gln (rs1800571, G>T), Val290Met (rs72551362, G>A), Pheu388Leu (rs72551363, T>A), Arg397Cys (rs72551364, C>T), His449His (rs3856806, C>T) and IRS1 Gly972Arg (rs 1801278, G>A) and their association with T2D and obesity in South Africans from Cape Town. In their study they found the PPARG Pro12 to be associated with insulin resistance and showed a gene-gene interaction with the unfavourable polymorphism IRS1 Gly972Arg leading to increased risk of T2D, while the PPARG His449His T allele showed a protective effect against the risk of developing diabetes (Vergotine *et al.* 2015). Some of the most studied genes published by GWAS have not been identified in central South African populations include *TCF7L2, IRS1,*

KCNJ11, CDKAL1 and *RBM43-RND3*. Gene polymorphisms from these genes are discussed below in more detail, and will be the focus of the study. The time and budget limitation of an MSc study required the selection of only a few gene polymorphisms to study. The *TCF7L2, IRS1, KCNJ11, CDKAL1* and *RBM43-RND3* gene polymorphisms were selected based on the presence of these genes being studied repeatedly in Caucasian populations and also in populations from African descent.

TCF7L2

TCF7L2 is located on the long arm of chromosome 10, consists of 17 exons and is expressed in the pancreatic beta cell (Saxena *et al.* 2012*;* Hansson *et al.* 2010; Pilgaard *et al.* 2009). It encodes a high mobility group (HMG) box-containing transcription factor that plays a key role in the Wnt signalling pathway (Frietze *et al.* 2012). Wnts are secreted glycoproteins that bind to frizzled seven-transmembrane-span receptors, which may be coupled to heterotrimeric G proteins (Huelsken and Behrens 2002). The Wnt pathway is involved in lipid metabolism and glucose homeostasis (Jin 2008). The TCF7L2 protein mediates the downstream effects of Wnt signalling via its interaction with CTNNBI (beta-catenin) and it can function as a repressor or an activator, depending on the availability of CTNNB1 in the nucleus (Figure 2.2)(Frietze *et al.* 2012).

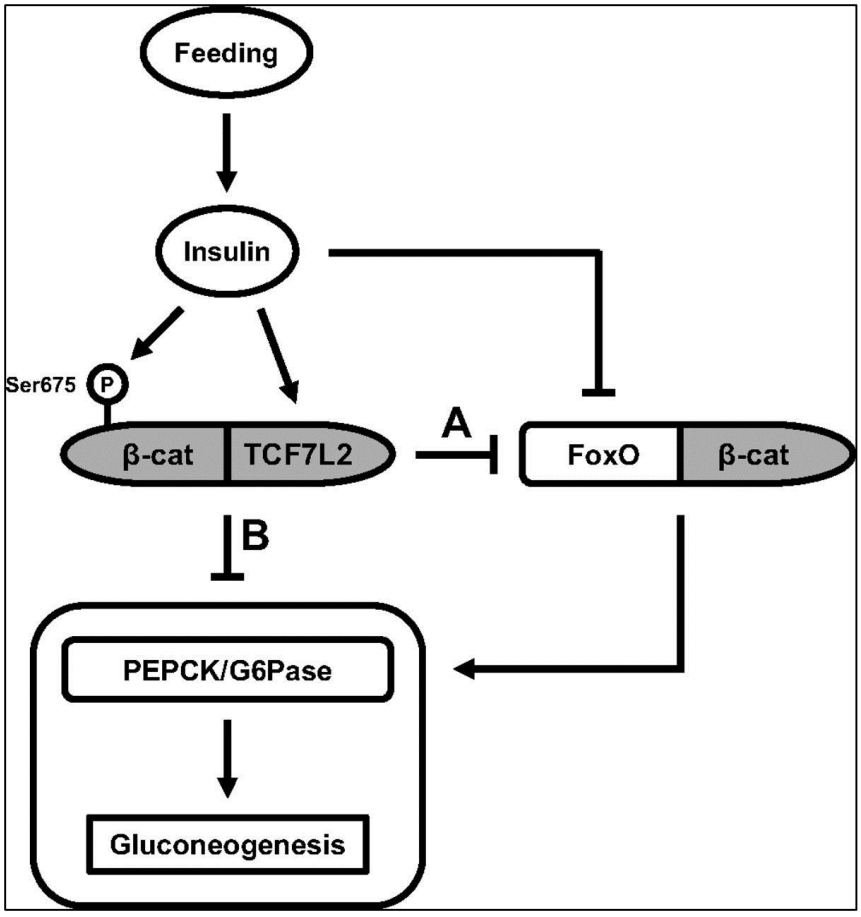


Figure 2.2 A schematic presentation of *TCF7L2* and Wnt Signalling in hepatic gluconeogenesis. The key effector of the Wnt signalling pathway is the bipartite transcription factor β-cat/TCF. Feeding upregulates plasma insulin levels, leading to β-cat Ser675 phosphorylation and *TCF7L2* expression. A: Increased levels of *TCF7L2* may possess an intrinsic repressive effect on gluconeogenesis (in B) by competing with FoxO for β-cat (Ip *et al.* 2012).

The *TCF7L2* gene plays an important role in T2D by regulating adipogenesis, myogenesis and pancreatic islands. It has an effect on the function of beta cells and granules responsible for insulin secretion as well as regulating expression of protein involved in exocytosis of insulin granules (Demirsoy *et al.* 2016). *TCF7L2* encodes a transcription factor that binds to the promoter of the proglucagon gene, which in turn encodes glucagon, glucagon-like peptide (GLP)-1 and GLP-2 (Pilgaard *et al.* 2009). GLP-1 is

secreted in response to nutrient intake and plays an essential role in postprandial glucose regulation by potentiating glucose-stimulated insulin secretion (Kuhre *et al.* 2015). GLP-1 secreted from the gut enhances glucose-stimulated insulin secretion (GSIS), and suppresses glucagon secretion (Dayeh 2015). GLP-2 is a proglucagon-derived peptide that functions as an intestinal epithelial growth and is involved in diabetes associated bowel growth (Dayeh 2015). *TCF7L2* is an important gene for determining susceptibility to T2D mellitus and it transgresses the boundaries of ethnicity (Chandak *et al.* 2007).

It has been reported that individuals who carries risk alleles for SNPs within *TCF7L2* showed impaired insulin secretion, increase in gastric inhibitor and impairment in glucose metabolism. The molecular mechanism of impaired insulin secretion due to *TCF7L2*, however is still unknown (Dermisoy *et al.* 2016). *TCF7L2* variants have been consistently associated with type 2 in populations of different ethnic descent (Cauchi *et al.* 2007). There are at least four well-studied polymorphic markers in the human *TCF7L2* gene, which are associated with T2D, rs7903146, rs7901695, rs12255375 and rs1196205, the majority of published epidemiological studies have placed emphasis on rs7903146 (C/T) and rs12255372 (G/T) variants (Alami *et al.* 2012). The single nucleotide polymorphism (SNP) rs7903146 located within intron 4 in the Transcription factor 7-like 2 (*TCF7L2*) gene has shown the strongest association with T2D and has been investigated in several studies (Table 2.3) (Zhou *et al*. 2016; Hansson *et al*. 2010). Individuals with the risk TT and CT genotypes were found to have a predisposition to T2D more so than individuals with the CC genotype (Hansson *et al*. 2010; Wang *et al*. 2015). In studies of African American, a study by Palmer *et al.* (2010) found rs7903146 to be significantly associated with T2D and a study by McComack *et al.* (2013) found an association with T2D (Table 2.3).

The risk T allele is associated with impaired insulin secretion and it was suggested that individuals with this allele exhibit elevated hepatic glucose production (Hansson *et al*. 2010). The association of the *TCF7L2* rs12255372 SNP with T2D has also been widely studied (Table 2.4). A study by Chandak *et al.* (2007) found non-coding variants of the *TCF7L2*, rs12255372 and rs7903146 to be strongly associated with increased risk of T2D, in a South Asian Indian population. Tabara *et al.* (2009) found the T allele of the *TCF7L2*

rs12255372 significantly associated with T2D in a Japanese population. A study by Alami *et al.* (2012) also found the minor T allele of *TCF7L2* rs12255372 significantly increased T2D risk in an Iranian population. Although recent studies indicate TCF7L2 SNPs may directly affect insulin expression and/or insulin secretion, there is no clear method of how these SNPs affect the function of pancreatic beta cells (Jin 2008). A study by Dermisoy *et al.* (2016) found the T allele of the rs7903146 to be associated with T2D but not the T allele of the rs12255372. Further studies in other European populations, Mexican Americans and Asian Indians also confirmed the strong associations with an estimated population attributable risk of 17-28% (Chang *et al.* 2007).

Table 2.3 Summary data from studies conducted to investigate the association of *TCF7L2* rs7903146 polymorphism with T2D mellitus.

Population	Sample size	Association with T2D	Study reference
African-American	577 patients; 596 controls	Yes	Sale *et al*. 2007
African American	3132 patients;3317 controls	Yes	Palmer *et al.* 2012
Ashkenazi	1131 patients; 1147 controls	Yes	Bronstein et al. 2008
Austrian	486 patients; 1075 controls	No	Cauchi *et al.* 2007
Brazil	110 patients; 110 controls	No	Barros *et al.* 2014
Brazillian	953 patients; 535 controls	Yes	Assmann *et al.* 2014
Cameroonian	37 patients; 37 controls	Yes	Guewo-Fokeng *et al.* 2015
Chinese Han	760 patients; 760 controls	No	Chang *et al.* 2007
European	14,073 patients; 57,489 controls	Yes	Saxena *et al.* 2012
Finnish	1161 patients; 1174 controls	Yes	Scott *et al.* 2007
Ghanaian	675 patients; 377 controls	Yes	Danquah *et al.* 2013
Hungarian	1297 patients; 1497 controls	Yes	Lukacs *et al.* 2011
India	758 patients; 621 controls	Yes	Jyothi and Reddy 2015
Iranian	258 patients; 168 controls	Yes	Amoli *et al.* 2010
Malaysian	105 patients; 60 controls	No	Vasudevan *et al.* 2009
Morrocan	504 patients; 406 controls	No	Cauchi *et al.* 2007
Norwegian	869 patients; 2080 controls	Yes	Thorsby *et al.* 2009
South African Zulus	178 patients; 200 controls	Yes	Pirie *et al.* 2010
Turkish	100 patients; 100 controls	Yes	Dermisoy *et al.* 2016

Table 2.4 Summary data from studies conducted to investigate the association of *TCF7L2* rs12255372 polymorphism with T2D mellitus.

Population	Sample size	Association with T2D	Study reference
African-American	577 patients; 596 controls	Yes	Sale *et al.* 2007
African American	3132 patients; 3317 controls	Yes	Palmer *et al.* 2012
Brazillian	110 patients; 110 controls	No	Barros *et al.* 2014
Chinese Han	760 patients; 760 controls	No	Chang *et al.* 2007
European	14,073 patients; 57,489 controls	Yes	Saxena *et al.* 2012
Finnish	1161 patients; 1174 controls	Yes	Scott *et al.* 2007
India	758 patients; 621 controls	Yes	Jyothi and Reddy 2015
Japanese	506 patients; 402 controls	Yes	Tabara *et al.* 2009
Norwegian	869 patients; 2080 controls	Yes	Thorsby *et al.* 2009
South African Zulus	178 patients; 200 controls	Yes	Pirie *et al.* 2010
Turkish	100 patients; 100 controls	No	Dermisoy *et al.* 2016

CDKAL1

CDKAL1 is located on chromosome 6p22.3, spans 37 kbp and encodes 579 amino acids (Li *et al.* 2013). It encodes a tRNA enzyme methylthiotransferase that catalyses methylthiolation of tRNALys(UUU) by adding a lysine residue during protein synthesis and plays an important role in fine tuning of translation fidelity (Figure 2.3) (Locke *et al.* 2015; Wanatabe *et al*. 2013; Kaufman 2011; Wei *et al.* 2011). This protein was speculated to share protein domain similarity with a protein that has a role in the decrease of insulin gene expression by inhibiting the activation of CDK5. *CDKAL1* was hypothesized to also

have an effect on insulin secretion, reduced expression of *CDKAL1* would result in enhanced activity of CDK5 in beta cells, eventually leading to decreased insulin secretion (Dehwah *et al.* 2010).

Single nucleotide polymorphisms in the intronic region of *CDKAL1* were found to be associated with decreased insulin secretion and the development of T2D, however, the physiological functions of *CDKAL1* are unclear (Watanabe *et al.* 2013). *CDKAL1* risk allele carriers display an insulin secretory defect that is concomitant with higher levels of proinsulin. It has been found that beta cell-specific deletion of *CDKAL1* in mice results in glucose intolerance due to reduced insulin secretion and impaired proinsulin conversion (Locke *et al.* 2015). Five SNPs have been identified with GWAS to be associated with T2D in the *CDKAL1,* they include: rs4712523, rs10946398, rs7754840, rs7756992 and rs9465871 all located in intron 5 (Wanatabe *et al.* 2013).

SNP rs7754840 was found to have the strongest association with T2D. The risk C allele of rs7754840 was associated with reduced insulin secretion in non-diabetic subjects (Stancakova *et al.* 2008). Numerous studies have been conducted to determine the risk association of the *CDKAL1* rs7754840 SNP with T2D, they are listed in Table 2.5. The biological role these non-coding SNPs play is still unclear however it is speculated that they are involved in the regulation of *CDKAL1* expression and the formation of splicing variants (Wei and Tomizawa 2011). A study by Chistiakov *et al.* (2011) found a minor allele of rs10946398, rs7754840 and rs7756992 of *CDKAL1* to have an association with higher risk of T2D in a Russian population. The only study results published on an African population was by Ashraf *et al*. (2013), on an Egyptian study group consisting of 49 patients and 22 controls. The study concluded the rs7754840 to be significantly associated with T2D in this cohort.

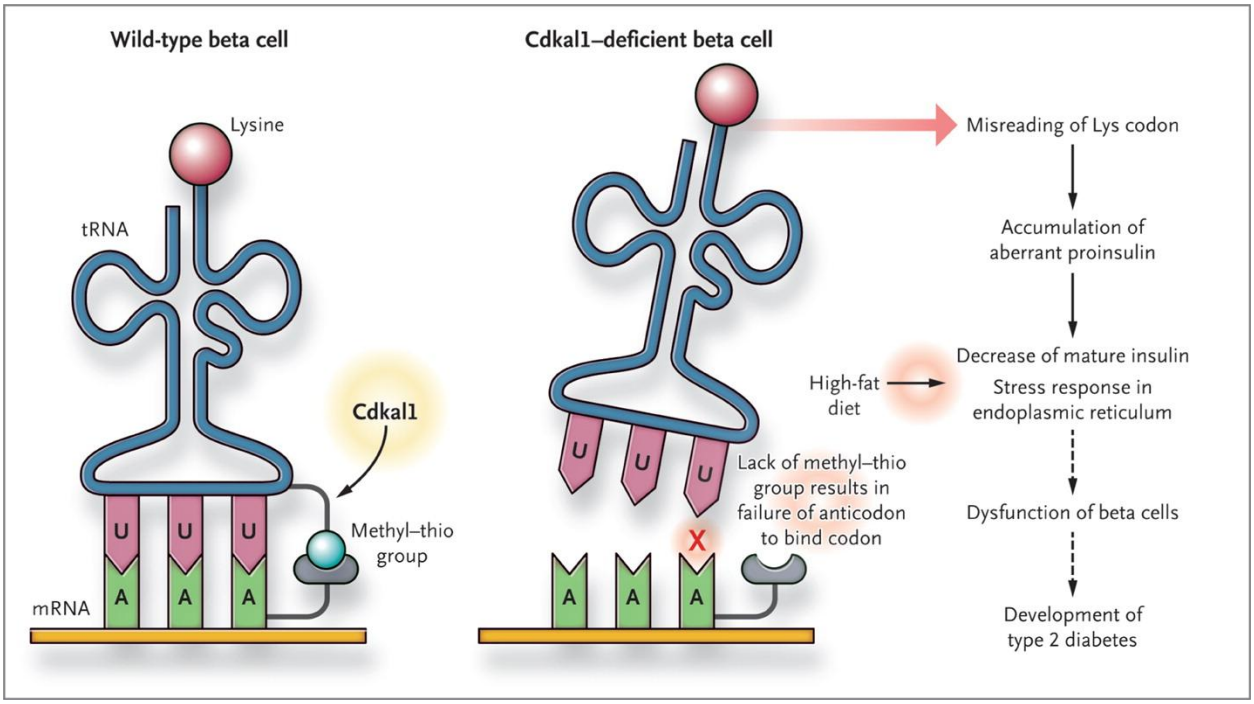


Figure 2.3 Schematic illustration of tampering Transfer RNA. T2D is associated with variant *CDKAL1.* Wei *et al*. (2011) recently ascribed a function to this gene: it critically modifies a specific transfer RNA (tRNA) by catalyzing the addition of a methyl–thio moiety (ms2t6) to a residue adjacent to the anticodon (pink) (Copied from Kaufman 2011).

Table 2.5 Summary data from studies conducted to investigate the association of *CDKAL1* rs7754840 polymorphism with T2D mellitus.

Population	Sample size	Associated with T2D	Study reference
Ashkenazi Jewish	1131 patients; 1147 controls	Yes	Bronstein *et al.* 2008
Egyptian	49 patients; 22 controls	No	Ashraf *et al*. 2013
European	993 patients; 1054 controls	No	Lewis *et al.* 2008
Finnish	533 patients; 3367 controls	Yes	Stancáková *et al.* 2008
Indian	758 patients; 621 controls	Yes	Jyothi and Reddy 2015
Iranian	140 patients; 140 controls	Yes	Mansoori *et al.* 2015
Japanese	506 patients; 402 controls	Yes	Tabara *et al.* 2009
Korean	908 patients; 502 controls	Yes	Lee *et al.* 2008
Lebanese Arab	630 patients; 792 controls	Yes	Nemr *et al.* 2012
Mexican Mestizo	1027 patients; 990 controls	Yes	Gamboa-Meléndez *et al.* 2012
Norwegian	1638 patients;1858 controls	Yes	Hertel *et al.* 2008
Uyghur	51 patients; 51 controls	Yes	Song *et al.* 2015

KCNJ11

The *KCNJ11* gene is a member of the potassium channel gene family, is located on the short arm of chromosome 11 and encodes the islet ATP-sensitive potassium (KATP) channel Kir6.2 protein (Florez *et al.*2007; Zhou *et al.* 2009). The Kir6.2 protein, together with the high-affinity sulfonylurea receptor 1 (SUR1), forms the KATP channel. The KATP channel function and downstream molecules within the cells as illustrated in Figure 2.4. In Pancreatic beta-cells, the ATP-sensitive K+ (KATP) channel is a key component (Yang

et al. 2011). Potassium channels mediate the glucose-induced insulin secretion from pancreatic β-cell by coupling cell metabolism to electrical activity of the plasma membrane. Elevation of the blood glucose concentration increases glucose uptake and metabolism by the beta cell, producing changes in cytosolic nucleotide concentrations that cause KATP channel closure (Hattersley and Ashcroft 2005). The importance of *KCNJ11* for insulin secretion in humans was supported by the demonstration of its role in familial persistent hyperinsulinaemic hypoglycaemia of infancy and in permanent neonatal diabetes (Yang *et al.* 2011).

Approximately 219 SNPs have been identified within the *KCNJ11* gene, 6 of the most common that have been shown to be involved in diabetes include rs5219, rs5215, rs5210, rs5218, rs886288, and rs2285676. Table 2.6 is a summary of the data from studies conducted to investigate the association of *KCNJ11* rs5219 polymorphism with T2D mellitus. SNP rs5219 is located in exon 1 of the *KCNJ11* gene and the polymorphism is a substitution of A to C (AAG—CAG) and changes the amino acid from lysine to glutamine (Lys23Gln) at the NH2-terminal tail of Kir6.2 (Haghvirdizadeh *et al.* 2014). The A allele of this locus impairs this pathway by reducing ATP sensitivity of the KATP channel resulting in over activity of the channel and subsequent suppression of insulin secretion (Haghvirdizadeh *et al.* 2014). A study by Zhou *et al.* (2009) found the rs5219 SNP in *KCNJ11* to be associated with T2D in the East Asian population. The presence rs5219 SNP is involved in the transduction of glucose signalling in the β cell, however this SNP was found to be highly correlated with another missense SNP in the ABCC8 gene, both genes are next to each other on the gene cassette and both encode a separate subunit of the islet ATP-sensitive potassium channels (figure 2.4). Carriers of rs5219 SNP risk allele have been found to usually also carry the risk A allele at *ABCC8* A1369S in most ethnic groups (Table 2.6) (Willard and Ginsburg 2008).

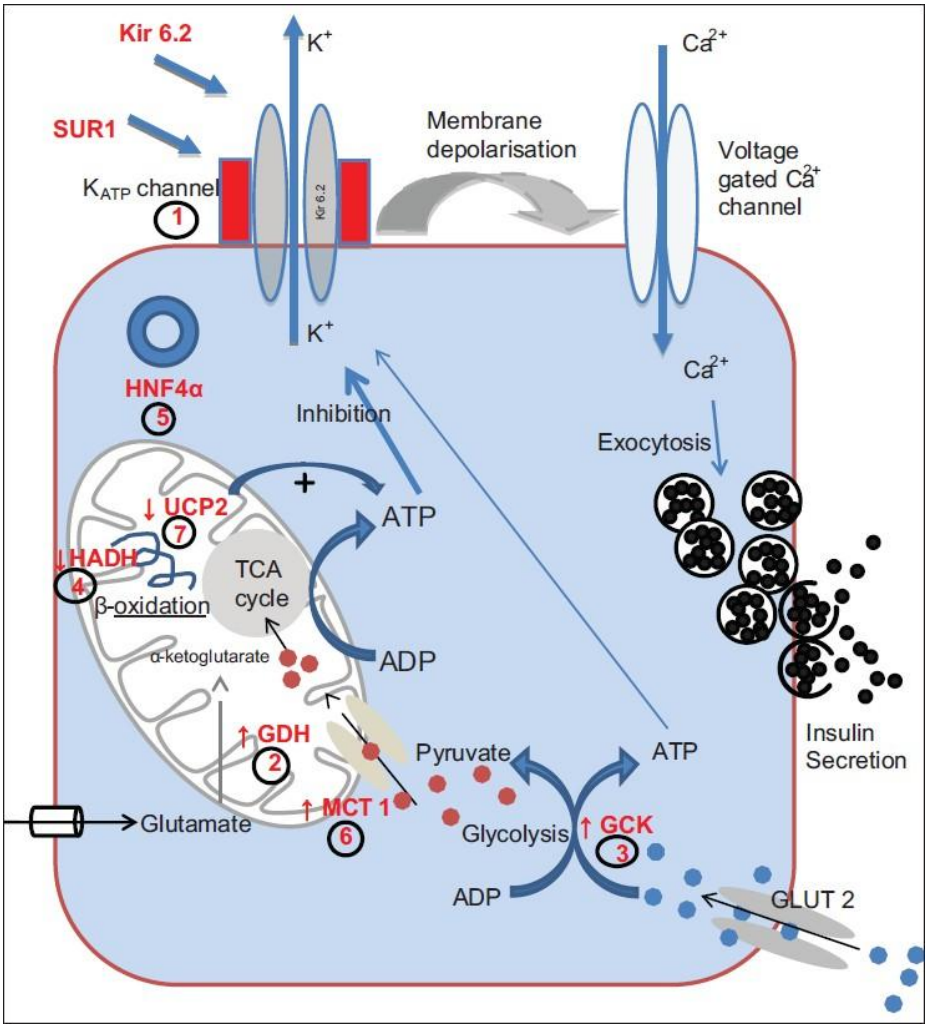


Figure 2.4 Schematic illustration of the of the KATP channel function:

The ATP gated K+ channel (KATP) is encoded by the *ABCC8* and *KCNJ11*. The inactivating mutations in *ABCC8/KCNJ11* reduce or completely abolish the activity of the KATP channel, leading to unregulated insulin release despite severe hypoglycemia

(1) ATP gated K+ channel (KATP) encoded by *ABCC8* and *KCNJ11*; (2) Glutamate Dehydrogenase (GDH) encoded by GLUD1; (3) Glucokinase (GCK) encoded by GCK gene; (4) L-3-hyroxyacyl-coenzyme A dehydrogenase (HADH) encoded by HADH; (5) Hepatocyte Nuclear Factor 4α (HNF4α) encoded by HNF4A gene; (6) The moncarboxylate transporter (MCT1) encoded by SLC16A1;(7) Uncoupling Protein 2 (UCP2) (Copied from Senniappan *et al.* 2013).

Table 2.6 Summary data from studies conducted to investigate the association of *KCNJ11* rs5219 polymorphism with T2D mellitus.

Population	Sample size	Associated with T2D	Study reference
African-Americans	577 patients; 596 controls	Yes	Sale *et al.* 2007
Caucasians	854 patients; 1182 controls	Yes	Gloyn *et al.* 2003
Chinese Han	1848 patients; 1910 controls	Yes	Zhou *et al.* 2009
Chinese She	2210 patients; 1119 controls	Yes	Chen *et al.* 2013
Ghanaian	675 patients; 377 controls	No	Danquah *et al*.2013
Iranian	400 patients; 420 controls	No	Kershavarz *et al.* 2014
Japanese	1630 patients; 1064 controls	Yes	Omori *et al.* 2008
Korean	761 patients; 630 controls	Yes	Yang *et al.* 2011
Mauritanian	135 patients; 135 controls	Yes	Abdelhamid *et al.* 2014
Mexican Mestizo	990 patients; 1027 controls	No	Gamboa-Meléndez *et al.* 2012
Mongolian	177 patients; 216 controls	No	Odgerel *et al.* 2012
Netherlands	725 patients; 742 controls	Yes	Van Dam *et al.* 2004
Norwegian	869 patients; 2080 controls	No	Thorsby *et al.*2009
Russian	127 patients; 117 controls	Yes	Chistiakov *et al.* 2009
Saudi Arabians	550 patients; 335 controls	Yes	Alsmadi *et al.* 2008
South Asian	3831 patients; 3543 control	No	Phani *et al.* 2014
South Indian	400 patients; 400 controls	Yes	Phani *et al.* 2014
Tunisian	250 patients; 267 controls	Yes	Lasram *et al.* 2014
Turkish	169 patients; 119 controls	Yes	Gonen *et al.* 2012

IRS1

Insulin receptor substrate 1 (IRS1) protein is a substrate of the insulin receptor tyrosine kinase and appears to have a central role in the insulin-stimulated signal transduction pathway (Kovacs *et al.* 2003). Insulin signalling is initiated by insulin binding to its receptor to activate tyrosine kinase, an enzyme that phosphorylates selected tyrosine residues of the IRS1 protein to activate the downstream phosphatidylinositol 3-kinase (PI3K) pathway, leading to glucose uptake and glycogen synthesis (Zheng *et al*. 2013). IRS proteins have a similar structure consisting of an NH2-terminal pleckstrin homology (PH) domain adjacent to a phosphotyrosine binding (PTB) domain followed by a variable-length COOH-terminal tail (Fig 2.5). The PH domain is essential for effective IR–IRS1 interactions (Arikoglu *et al.* 2014). Tissue knockout experiments in mice show that IRS1 is an important component of insulin action in adipose tissue, skeletal muscle and pancreatic beta cells and its gene *IRS1* has been identified to have a pathogenic role in the development of T2D (Li *et al* 2015).

The rs2943641 SNP is a genetic variant about (C/T) situated approximately 570 kbp downstream of the transcription start codon of the *IRS1* gene and, was found to be associated with T2D, insulin resistance, hyperinsulinemia and adiposity in GWAS (Li *et al*. 2015 and Feng *et al*. 2013) (Table 2.7). This particular genetic variant was identified by the GWAS to be associated with T2D in the European population. Additionally the gene has been shown to have interaction with dietary carbohydrates and fat resulting in insulin resistance in an intervention study (Zheng *et al.* 2013). Even though the genetic variant rs2943641 was associated with T2D based on 14,358 Europeans, the association of this variant and T2D or any related traits did not reach significance is a study on Hispanics particularly from Boston Puerto Rican (Feng *et al.* 2013). Following the same trend, the association between T2D and rs2943641 was significant in a codominant model in a Caucasian population (Li *et al.* 2015), however a study by Tang *et al.* (2012) could not demonstrate an association between rs2943641 and T2D in a Chinese Han population. A significant association of T2D and rs2943641 was identified in the Japanese population also in the Saudi population (Alharbi *et al.* 2014; Ohshige *et al.* 2011).

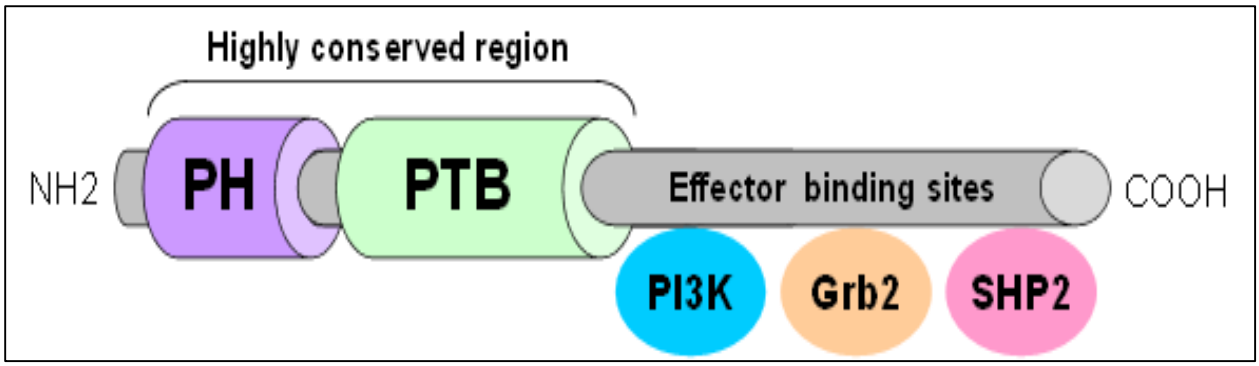


Figure 2.5 Schematic structure of *IRS*. Interaction domain: pleckstrin homology (PH) domain, phosphotyrosine binding (PTB) domain and the effector binding sites including the Phosphoinositide 3-kinase (PI3K), Growth factor receptor-bound protein 2 (Grb2) and Src homology 2-containing phosphotyrosine phosphatase (SHP2) (Machado-Neto and Traina 2013).

Table 2.7 Summary data from studies conducted to investigate the association of *IRS1* rs2943641 polymorphism with T2D mellitus.

Population	Sample size	Associated with T2D	Study reference
Bosnia and Herazegovina	437 patients; 252 controls	No	Halilovic *et al.* 2016
Boston Puerto Rican	444 patients; 688 controls	Yes	Feng *et al.* 2013
Caucasians	11701 patients; 29015 controls	Yes	Li *et al.* 2015
Chinese Han	1177 patients; 1113 controls	No	Tang *et al.* 2012
Japanese	2839 patients; 2125 controls	Yes	Ohshige *et al.* 2011
European	14, 073 patients; 57,489 controls	Yes	Saxena *et al.* 2012
Pakistani	1678 patients;1584 controls	Yes	Rees *et al.* 2011
Saudi	376 patients; 380 controls	Yes	Alharbi *et al.* 2014

RND3-RBM43

The *RND3-RBM43* gene is located on the long arm of chromosome 2 (Wang *et al.* 2015). Although the SNP associations observed reside between genes, the nearest annotated gene to SNP rs7560163 is *RND3* (Palmer *et al.* 2012). *RND3*, also known as RhoE, encodes small guanosine triphosphatase (GTPase) proteins that belongs to the Rho family of the Ras GTPases superfamily. The expression levels of *RND3* have been suggested to be associated with human diseases, indicating its important role in the pathogenesis of human diseases (Jie *et al.* 2016).

A recent study showed that *RND3* could regulate a diverse set of biological activities including actin cytoskeleton organization in response to extracellular growth factors, cell motility, cell-cycle progression, apoptosis and development (Hongwei *et al.* 2013; Palmer *et al.* 2012 and Jie *et al.* 2016) (Figure 2.6). Small GTPases are a family of hydrolase enzymes that can bind and hydrolyze guanosine triphosphate (GTP) and they are a certain type of G-protein that are homologous to the alpha subunit of heterotrimeric G-proteins (Jie *et al.* 2016).

The rs7560163 (C/G) SNP is located between two different genes, the (Ras homolog gene family, member E) Rho family GTPase 3 (*RND3*) and the RNA binding motif protein 43 (RBM43). There is limited data regarding this particular SNP, and it seems to be conducted only on African American individuals. The first T2D associated GWAS in African Americans was reported by Palmer and team in 2012 and identified a novel locus near the *RND3* and *RBM43* genes from a sample consisting of 1994 discovery and 4455 validation samples (Ng 2015). This SNP was later found to be association with T2D in the African American population in a GWAS study conducted on 6449 individuals (Mohlke and Boehnke 2015; Palmer *et al.* 2012). These studies concluded that there is a strong significantly association with this particular SNP amongst the African American individuals.

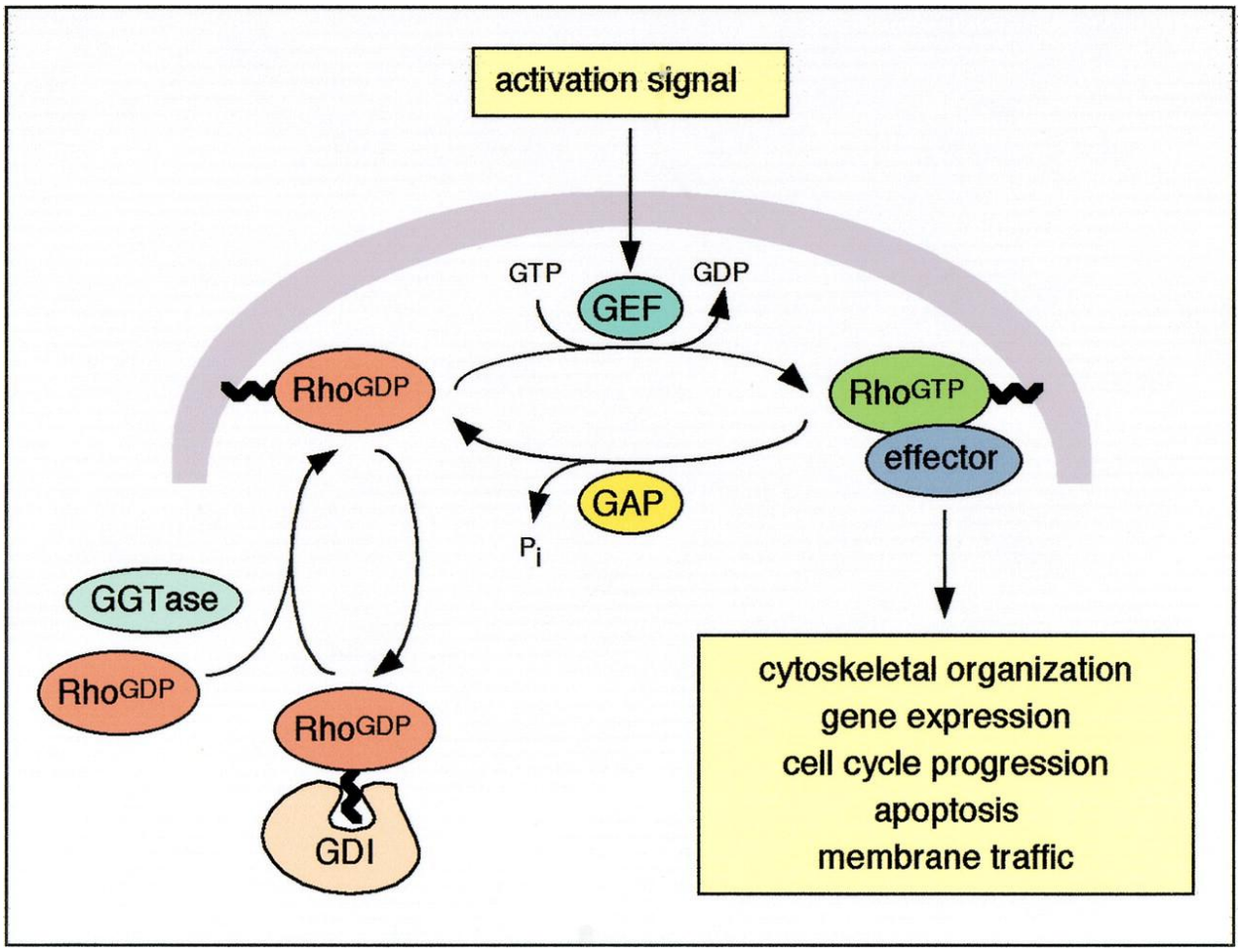


Figure 2.6 Schematic illustration of the Rho GTPase switch. Phosphorylation of Rho GTPases can signal for various regulatory mechanisms. Rho GTPases are targeted to the membrane by posttranslational attachment of prenyl groups by geranyl-geranyltransferases (GGTases). Cycling between the inactive (GDP-bound) and active (GTP-bound) forms is regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). Active, GTP-bound GTPases interact with effector molecules to mediate various cellular responses. (Hodge and Ridley 2016; Schmidt and Hall 2002).

2.7 Genotyping in T2D research

Recent advances in molecular biology has brought about numerous techniques to determine the genotype or nucleotide sequence of an individual. A few of the most

common techniques include: micro arrays, next generation sequencing and various applications of PCR, one of which is Real-Time PCR also called Quantitative Real-Time Polymerase Chain Reaction (qPCR). All of these techniques have advantages and disadvantages, depending on the resources, cost and time-frame available to a specific study. Consequently, the most suitable technique should be applied according to study conditions. Genotyping can thus be accurately performed with several different techniques.

Genotyping results in GWAS studies need to be produced for thousands of individuals and usually for multiple regions of the genome if not for the complete genome. For this reason, high-throughput techniques, such as micro array chip techniques or next generation sequencing are applied. For example, a study by Chikowore (2015) used Illumina Veracode technology on BeadXpress platform to genotype variants associated with T2D in South Africans of Tswana descent. A GWAS on 24 409 participants, genotyped type 2 diabetic patients and their controls in Asian population using the Affymetrix Genome-Wide Human SNP Array 6.0 Extensive quality control procedure (Shu *et al.* 2010). In a study with fewer study participants by Chandak *et al.* (2007) 1354 individuals were genotyped using direct sequencing

Population based studies determining association with single nucleotide polymorphisms and a trait or disease state, often utilizes PCR based genotyping techniques such as qPCR, electrophoresis based PCR, reverse transcriptase PCR or combination of these. A study by Lu *et al.* (2012), performed genotyping on 900 individuals by high resolution melting (HRM) of small amplicons to determine the association with T2D in a Chinese population. A study by Pirie *et al.* (2010), performed genotyping on 378 individuals using qPCR to determine association with T2D on a South African cohort of Zulu descent. Real time PCR was also used to genotype 908 individuals by Tabara *et al.* (2009) on a Japanese population. According to literature, the use of qPCR for genotyping would be suitable for this study and consequently this technique is discussed in more detail.

2.8 Quantitative Real-Time Polymerase Chain Reaction

Real-time quantitative PCR (qPCR) allows the reliable detection and measurement of products generated during each cycle of the PCR process, directly proportional to the amount of template prior to the start of the PCR process (Arya *et al.* 2005). Real-time PCR relies on the detection and quantification of a fluorescent reporter molecule, which can be in the form of an intercalating fluorescent dye or attached to a DNA probe specifically designed to hybridise to the template DNA, which may or may not be hydrolysed after each PCR cycle (Heid *et al.* 1996). Usually, one of two types of chemistries is thus applied in qPCR, either SYBR green (intercalating dye) or hydrolysis probe (fluorescent probe) chemistry, where the fluorescence of all dye molecules are measured during each cycle. Real-time PCR is highly suited for a wide range of applications, such as gene expression analysis, determination of viral load and other pathogen-detection applications, detection of genetically modified organisms (GMOs), SNP genotyping and allelic discrimination.

2.8.1 Fluorescently labeled sequence-specific probes

Fluorescently labeled sequence-specific probes provide a highly sensitive and specific method of detection, as they only detect the template target region complementary to the probe. The most widely applied is the hydrolysis probe, or commonly referred to as TaqMan probes, which carries a fluorophore and a quencher moiety (Bustin *et al*. 2009). The fluorophore is always attached at the 5’ end of the probe while the quencher is attached at the 3’ end. When the probe is intact, the quencher dye is in close proximity to the reporter dye and suppresses the reporter fluorescence signal. The probe is cleaved by the 5’-3’ exonuclease activity of Taq DNA polymerase during the combined annealing/extension phase of PCR (Figure 2.7). The result of this is a detectable fluorescence signal that is directly proportional to the amount of accumulated PCR product.

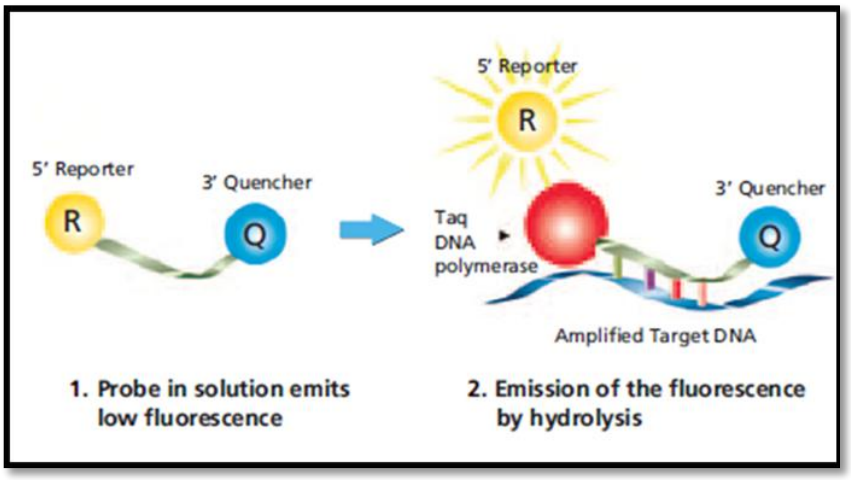


Figure 2.7 Schematic presentation of Mechanism of Dual-labelled Probes. Taq DNA polymerase extends the primer situated on the same strand as the probe until it reaches the probe position. The inherent exonuclease activity hydrolyzes the probe from 5' to 3', which releases the reporter dye into solution and thereby causes an increase in fluorescence. The measured fluorescence signal is directly proportional to the amount of target DNA (Sigma-Aldrich 2018).

2.8.2 Intercalating dye chemistry based qPCR

The most abundantly used intercalating dye is SYBR Green which binds to all double stranded DNA molecules, emitting a fluorescent signal of a defined wavelength when bound. Detection takes place in the extension step of real-time PCR. This type of detection enables detection without the use of target-specific labelled probes. One of the few disadvantages of this chemistry is that nonspecific PCR products and primer-dimers also contribute to the fluorescent signal. Additionally, SYBR green is not as sensitive as other intercalating dyes, such as the ones used in high resolution melt analysis, due to the lower saturation of SYBR green to the double stranded DNA molecule. However, depending on the design and application of the chemistry, it may be the most suitable method, such as gene expression studies or genotyping of large gene segments.

2.8.3 Advantages of using qPCR

The application of qPCR is much faster than end-point methods because the time consuming separate analysis step of gel electrophoresis is eliminated. Furthermore, it allows for the detection of amplifications during the early phases of the reaction and it collects data at the exponential growth phase of PCR. Real-time PCR produces very reliable results because of the sophisticated detection chemistry and instrument being far more sensitive than electrophoresis DNA detection dyes such as ethidium bromide staining. It is rapid and possible to analyse several transcripts simultaneously. Real-time PCR is less labour intensive compared to conventional PCR playing a big role in making it more cost effective. Real-time PCR minimizes the chance of contamination since both amplification and detection take place in a single closed tube system (Mackay *et al.* 2002). Sample throughput increases dramatically because there is no post-PCR processing time (Heid *et al.* 1996). The MIQE guidelines have been published to serve as a guide in the execution of qPCR experiments ensuring the accuracy, repeatability and correct interpretation of data (Bustin *et al*. 2009). The advantages of real time PCR makes it easy to genotype large amount of samples in research projects as well as industry driven genotyping. Therefore making it the method of choice for genotyping applied in this study.

2.9 Conclusion

T2D is a chronic disease that is recognized as one of the leading causes of disability and death worldwide. It places a huge burden on health systems, global economy and ultimately on patient's daily existence. The number of deaths globally attributable to diabetes exceeded the combined mortality from HIV/AIDS, tuberculosis and malaria in 2015 (Amod *et al.* 2017). The highest proportion of undiagnosed individuals was found to be in Africa with a percentage of 66.7 (Ogurtsova *et al.* 2017). Based on the most recent 2015 IDF estimates for South Africa, the prevalence of adults between the ages of 20-79 was 7% with a comparative prevalence of 7.6% (Amod *et al.* 2017).

Urbanisation and adoption of Western dietary patterns are the major environmental risk factors associated with the increase in prevalence of diabetes in South Africa. Relieving

the burden of the disease should be the priority and researchers worldwide has approached this by making use of the GWAS. More than 60 SNPs were found to be associated with T2D reaffirming the polygenic and complex nature of T2D. Knowledge on the genetic bases of a disease will allow medical professions to predict and diagnose before the onset of its complications.

The strong genetic component of T2D has been indicated by high concordance rates in twin studies, however the development of it is not well understood. As a polygenic disorder, T2D has many different combinations of gene defects that exist among diabetic patients. Although these genes may contribute susceptibility to T2D, environmental factors interacting with these genetic aberrations may trigger the clinical disease. The majority of GWAS are aimed at identifying genetic factors as predictive disease markers with a total of 83 susceptibility loci identified (Wang *et al.* 2015). Early identification of individuals at high T2D risk enables delay or prevention of T2D onset through effective lifestyle and/or pharmacological interventions and in turn reduce the costs of care (Ashraf *et al.* 2013).

CHAPTER 3: METHODOLOGY

INTRODUCTION

This chapter describes the methodology applied to investigate the study consisting of the study design, population group, sample selection, materials, methods and techniques used in the study.

3.1 Study design

A case control study was performed on 188 participants, 96 patients diagnosed with T2D and 92 control subjects. The cohorts were matched for gender, age and body mass index (BMI).

3.2 Summary of study procedure

Figure 3.1 is a schematic representation summarizing the flow of this study. Ethical approval to conduct this study was obtained from the Health Sciences Research Ethics Committee at the University of the Free State (HRSEC 80/2016). Pre-existing participant DNA was obtained from blood collected under Ethics number ECUFS 162/2012, with informed consents allowing the DNA to be used also for future research on T2D. Additional participants were recruited from public areas and a private practice. Firstly, informed consent was obtained from every participant thereafter measurements were taken and blood collected. HbA1c levels were tested at an accredited private laboratory and DNA was isolated from peripheral blood samples.

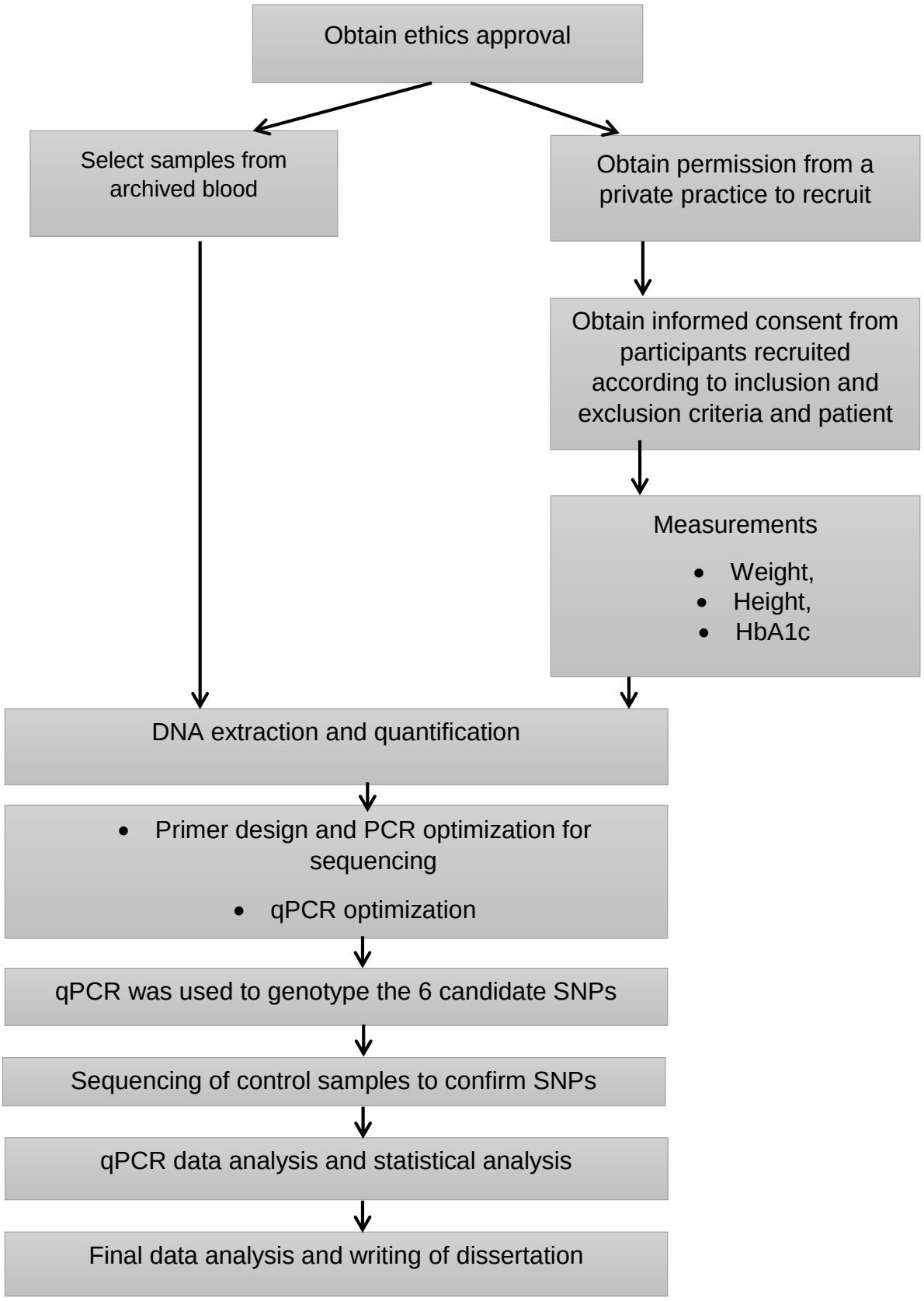


Figure 3.1 Flow chart illustrating the different steps of the procedure

Six qPCR assays were designed and optimized to enable screening of 6 SNPs that were chosen for the study based on literature, in both the patient and control cohorts. Genetic testing and genotype determination was conducted at the University of the Free State, in the department of Genetics in the Faculty of Natural and Agricultural Science. The genetic results were used to determine the association of the single nucleotide polymorphisms with T2D in the study population.

The study population consisted of black participants living in and around Manguang in the Free State, South Africa. The languages spoken are Sesotho, Afrikaans, English, Setswana and isiXhosa. The Mangauang population has approximately 747 431 people and an area that covers more than 6,284 km^2 (Municipality: Managaung Metropolitan Municipality 2014).

A sample of convenience approach was taken to recruit T2D patients from the diabetes clinics of the Pelonomi Regional Hospital and Universitas Academic complex in Mangaung, both referral hospitals representing the Mangaung Metropolitan black population. Archived samples were collected under ECUFS 162/2012, according to the inclusion criteria mentioned in section 3.3.3. Control participants were recruited to match criteria ranges for age and body mass index with that of the recruited T2D patients. Participants recruited with ethics approval number HRSEC 80/2016 were recruited as a convenience sample from public places and were remunerated for transport to the participating private practice. Informed consent were obtained from individuals before participation.

3.3 Sampling

3.3.1 T2D patient sampling

T2D patients were recruited from a private practice of general practitioners, physicians and endocrinologists in the Mangaung region. A letter was written to the doctors to ask for permission to recruit patients from their practices (Appendix A). The investigator visited the private practices of doctors that gave permission, on a weekly basis. Patients were only asked to take part in the study after their appointment with the doctor according

to best ethical practice. The study was explained to the patients and voluntary signed consent was obtained from each patient before measurements or blood was taken. Refusals to take part in the study were not documented, and upon study refusal, another person was approached. A small private area was set up in the nurse's suite of the private practice for measurements to be taken and blood to be drawn.

3.3.2 Control participant sampling

Healthy volunteers were recruited for the control cohort from public areas such as bus stops and public shopping centres in the Mangaung area. The study was explained to the participants and voluntary signed consent was obtained from each person before inclusion in the study. Refusals to take part in the study were not documented, and upon study refusal, another person was approached. Voluntary participants were asked to use public transport to the participating private practice. A finger prick point of care blood glucose test was performed on all volunteers. Volunteers with a random blood glucose test of <11mmol/L continued with the study as control participants. Volunteers with a random blood test >11mmol/L were referred to a local clinic for a medical examination. The healthy volunteers' data were included in the study if; HbA1c was less than 6.5%, aged between 20 and 60 years and a body mass index (WHO category, Table 3.1) in the same range as T2D patients.

The following inclusion and exclusion criteria were used to select archived samples as well as participant samples recruited during this study:

Inclusion criteria for T2D patients

The sample consisted of black male and female patients

- Diagnosed with T2D by a medical doctor;
- Glycated haemoglobin (HbA1c) ≥ 6.5%;
- Between 20-60 years old;
- That were able to give consent

Exclusion criteria for T2DM patients

Patients were excluded from the study if they:

- Were diagnosed with any other form of Diabetes Mellitus;
- Were pregnant;
- Were younger than 20 years or older than 60 years old.
- Were unable to give consent:

Table 3.1 BMI Classification according to the World Health Organization (WHO 2015)

BMI (Kg/m^2)	Classification
<18.5	Underweight
18.5-24.9	Normal
25-30	Overweight
>30	Obese

3.4 Measurements and Techniques

Anthropometry was included to obtain physical measurements of an individual. Anthrometrical measurements that were measured in this study included weight, height and BMI (body mass index). The BMI was calculated with the following formula: (weight in Kg/ height in m^2).

3.4.1 Weight

Actual body weight is the body weight measurement obtained at the time of examination and can be influenced by changes in fluid status (Hammond and Litchford 2012). Weight

was measured with a periodically calibrated scale for accuracy. The subjects were weighed in light clothing without shoes. The weight was recorded to the nearest 0.1 kg.

3.4.2 Height

To determine their height participants were asked to stand flat (Hammond and Litchford 2012). Height was measured without shoes; the subject's feet were together, with heels against the measuring board. During the measurement, the participant stood up right, looked straight ahead without tipping the head up or down. The top of the ear and outer corner of the eye was in a line parallel to the floor. A ruler was placed on top of the head at a 90 degree angle to the wall to note the correct height measurement. The height was read to the nearest 0.1 centimetre (De Bruyne *et al.* 2012).

3.4.3 HbA1c measurements

The HbA1c percentage levels of all participants was analysed by an accredited private laboratory. HbA1c is a reflection of the average blood glucose levels for the preceding two to three months and does not reflect recent changes in glucose levels. HbA1c is expressed as the percentage of total haemoglobin. An HbA1c level above 6.5% is an indication of diabetes (Amod *et al.* 2017).

3.5 Materials and methods

3.5.1 DNA isolation

Peripheral blood was collected in Ethylene diamine tetra acetic acid (EDTA) tubes (BD Vacutainer, Reference no: 368861, Becton Dickinson, South Africa). Genomic DNA was extracted using the salting out method also known as anti-solvent crystallization or precipitation crystallization, with alterations (Salazar *et al.* 1998). Initially, erythrocytes were lysed by incubating 1 ml of peripheral blood with Tris buffer 1 (0.01 M Tris-HCl, pH 8.0, 0.01 M KCl, 0.01 M MgCl and 0.002 M EDTA, 0.4 M NaCl and 10 g/L SDS) and Triton X (1M Triton-X-100). The sample was mixed well by inversion of tube several times and centrifuged at 2444 xg for 10 min at room temperature. The supernatant was discarded and the pellet saved and washed twice with 1 ml of Tris Buffer 1 and Triton-X followed by

centrifugation at 2444 xg for 10 min at room temperature. The supernatant was discarded and the pellet was disrupted before incubation in 160 µl of Tris Buffer 2 (0.01 M Tris-HCl, 0.01 M KCl, 0.02 M EDTA, 0.4 M NaCl and 10 g/L SDS) at 55°C for 30 min. Sixty µl of 5-M NaCl were added and vortexed and transferred to a new tube. The sample was centrifuged at 24 900 xg for 5 min and the supernatant was transferred to a new tube. The DNA was precipitated by adding 250 µl of 100% isopropanol at room temperature to the supernatant by inversion. The sample was centrifuged at 24 900 xg for 5 min and the supernatant was discarded. The DNA pellet was washed twice with 500 µl cold 70% (v/v) ethanol and was then centrifuged at 18 675 xg for 5 min at room temperature. The DNA pellet was dried in a heating block and resuspended in 100 µl of Tris-EDTA buffer (0.01 M Tris-HCl and 0.001 M EDTA) at 65°C for an hour. The quantity and quality of the DNA was assessed using a Thermo Scientific™ NanoDrop 2000 Spectrophotometer.

3.5.2 DNA quantity and integrity determination

Initially, a blanking step was performed on the NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA), in which 1.5 µl of 0.1X TE Buffer was pipetted on the measurement pedestal. The concentrations of the DNA were measured on the NanoDrop 2000 Spectrophotometer by pipetting 1.5 µl of the DNA isolated on the measurement pedestal. The measurement was performed for all samples in duplicate and an average used for downstream calculations. The A260/A280 ratio was used to analyze the purity of the DNA and the DNA was considered pure with a ratio of above 1.8. Samples were stored at -20 °C.

3.5.3 Genotyping

Genotyping was performed on six SNPs from five genes (Table 3.2) using qPCR with Taqman® chemistry (Thermo Fisher Scientific, USA) on the Quant studio 5 (Applied Biosysterms by Thermo Fisher Scientific) qPCR instrument. The assay was developed to distinguish between different alleles of genes mentioned in Table 3.2

Primers and probes were custom designed and manufactured to bind to the target SNP by Thermo Fisher Scientific. Sequences and fragment sizes of primers and probes from the Taqman® Genotyping SNP assays (Thermo Fisher scientific, Taqman® Genotyping

SNP assays, Human, ordering part number: 4351379) are not made publically available. These assays use TaqMan® minor groove-binding (MGB) probes for superior allelic discrimination, improved signal-to-noise ratios, and design flexibility. According to the manufacturer, all human SNP genotyping assays are functionally tested on 20 unique DNA samples to ensure assay amplification and sample clustering. These custom assays are designed, synthesized, formulated, optimized, and quality control tested by the manufacturer.

The context sequences for each of the SNP's can be viewed in Table 3.3, which indicates the SNP alleles. The probe complimentary to allele 1 was labelled with VIC fluorescent dye and the probe complimentary to allele 2 was labelled with FAM fluorescent dye. In Table 3.3, Allele 1 is shown first in the block brackets and Allele 2 second. It is important to note that the design, of the Taqman® Genotyping SNP assays, does not as a rule indicate allele 1 as wild type and allele 2 as mutant alleles. Table 3.2 shows the wild type of common allele and mutant allele according to the Genome Reference Consortium Human Build 38 (https://www.ncbi.nlm.nih.gov).

Table 3.2 Candidate genes with annotation numbers, alleles and genome position

Gene	SNP	Chromosome	Position	Alleles(+/-)
TCF7L2	rs7903146	10	112 998 590	T/C
TCF7L2	rs12255372	10	113 049 143	T/G
CDKAL1	rs7754840	6	20 661 019	C/G
KCNJ11	rs5219	11	17 388 025	T/C
IRS1	rs2943641	2	266 801 989	C/T
RND3-RBM43	rs7560163	2	151 346 182	C/G

(+) indicates the mutant allele and (–) the wild type allele

Table 3.3 The SNPs and the corresponding context sequences

Gene	SNP	Context sequence [VIC/FAM]
TCF7L2	rs7903146	TAGAGAGCTAAGCACTTTTTAGATA**[C/T]**TATATAATTTAATTGCCGTATGAGG
TCF7L2	rs12255372	TGCCCAGGAATATCCAGGCAAGAAT**[G/T]**ACCATATTCTGATAATTACTCAGGC
CDKAL1	rs7754840	GGGGAAGAAGTAGTAATGTTGGAAA**[C/G]**GTTGACTTGATAGAGGATTTTGTAA
KCNJ11	rs5219	CGCTGGCGGGCACGGTACCTGGGCT**[C/T]**GGCAGGGTCCTCTGCCAGGCGTGTC
IRS1	rs2943641	CAACATAGTTGGAAATGAGAGGAAC**[C/T]**CTTCTAACTATTAGCCCTGATATTC
RND3-RMB43	rs7560163	AAAACTTCAACCCTGCTTCAACTCA**[C/G]**AGTTGCCTGGTCTTCAGGTTTTCCC

3.5.4 Real-time PCR optimization

Several reactions were performed with the TaqMan® Genotyping Assay kit reagents (Thermo Fisher Scientific, Taqman® Genotyping SNP assays, Human, ordering part number: 4351379), as described by the manufacturer with the addition of template DNA concentrations of 12.5 ng per 20 µl reaction. The different annealing temperatures during the PCR cycle were 61°C, 63°C and 65°C for a minute, performed on the SimpliAmp Thermal cycler (Applied Biosystems by Life Technologies, USA) to determine the optimal annealing temperature for the assays. The annealing temperature of 60°C was optimal for every assay.

3.5.5 Gel electrophoresis

Gel electrophoresis was performed to determine the amplicon size and integrity of the amplicon generated by the qPCR genotyping SNP assay optimization reactions as well as for the pre sequencing reactions. A 1% (w/v) agarose gel using 1XTAE buffer (40 mM Tris-acetate and 1 mM EDTA) was used to make up the gel and also to separate the

amplicons for 30 min at 120V. The gel was stained with gel red (Thermo Fisher Scientific, USA). The gel was visualized under UV light to determine the fragment sizes according to the known DNA size marker (Hyperladder II, Invitrogen USA) and loaded with the samples on the gel. The gel was run to ensure that there was amplification and to have an idea of the amplicon fragment size, however no measurements were taken from the gel.

3.5.6 Real time PCR

Real-time PCR was performed on the QuantStudio 5 qPCR instrument (Applied Biosysterms by Thermo Fisher Scientific), calibrated with several fluorescent dyes inducing VIC, FAM and ROX. The reaction mixture contained 10 µl of Taqman® Genotyping SNP assay kit Master mix, 1 µl Taqman® Genotyping SNP assay kit Working solution (Thermo Fisher scientific, Taqman® Genotyping SNP assays, Human, ordering part number: 4351379) which contain primers and probes, 8 µl nuclease free water and isolated sample DNA of 12.5 ng in a 20 µl reaction. The cycling conditions were; 95°C for 5 min followed by 40 repeats of a two-step cycling between 95°C for 10 sec and an annealing temperature of either 65°C (*TCF7L2, RND3-RBM43* and *CDKAL1* gene SNPs) or 63°C (*KCNJ11* and *IRS1* SNPs) for 1 min.

For quality control purposes, six control reactions were included per plate. Two non-template controls (NTC), two homozygote wild type reactions and two homozygote mutant reactions. Table 3.4 indicates the origin of the genotype confirmed run controls, the genotype confirmation was either performed by sequencing of a study participant sample or by purchasing synthetic oligomers (gBlocks® Gene Fragments, IDT). The sequence confirmed wild type participant sample electropherograms can be viewed in Appendix D1. Manufacturing sheets confirming the genotypes of the gBlocks® Gene Fragment oligomers are included in Appendix C. Heterozygote controls were not included in each qPCR run, since mixing of isolated DNA and manufactured oligomers were problematic. However, all heterozygote genotyped samples were sequenced afterwards to confirm heterozygosity. Additionally, all samples showing an ‘undetermined’ result in the allelic discrimination plot were repeated.

Table 3.4 Genotype confirmation of qPCR run controls.

Gene	SNP	Allele	Assignment	Genotyped
TCF7L2	rs7903146	T C	Mutant / Allele 2 Wild type / Allele 1	Synthetic oligo (gBlocks®) Sequencing
TCF7L2	rs12255372	T G	Mutant / Allele 2 Wild type / Allele 1	Synthetic oligo (gBlocks®) Sequencing
CDKAL1	rs7754840	C G	Mutant / Allele 1 Wild type / Allele 2	Synthetic oligo (gBlocks®) Sequencing
KCNJ11	rs5219	T C	Mutant / Allele 2 Wild type / Allele 1	Not determined Synthetic oligo (gBlocks®)
IRS1	rs2943641	C T	Mutant / Allele 1 Wild type / Allele 2	Synthetic oligo (gBlocks®) Sequencing
RND3-RBM43	rs7560163	C G	Mutant / Allele 1 Wild type / Allele 2	Synthetic oligo (gBlocks®) Sequencing

3.5.7 Sequencing

Sequencing was performed in this study for two purposes, implementing the same procedures. Firstly, to determine the genotype of qPCR positive run controls for wild type or common alleles and secondly to confirm heterozygote and homozygote mutant or rare allele genotypes. Synthetic oligomers were not sequenced all their sequences were already known. All the information of the gBlocks® Gene Fragments are presented in Appendix C.

3.5.7.1 Primer design

Primers were designed to include the SNPs of interest. The primers were designed using an online oligo design tool (IDT, USA). The IDT Oligo Analyzer online tool was used to calculate oligonucleotide parameters such as melting temperatures, hairpin loop formation and self-dimerization (Integrated DNA Technology 2017). The primers' length

ranged from 18bp to 23bp, annealing temperature between 53°C and 55°C and GC content ranged from 43.5% to 50% with the smallest to no possibility of formation of primer dimers or hairpin loops (Table 3.5).

3.5.7.2 PCR clean up

The PCR amplicon was cleaned up using ExoSAP-IT® Express PCR product Clean up (Affymetrix USB, USA). The above mentioned reagent consists of a patented combination of Exonuclease 1 and Shrimp Alkaline Phosphatase (SAP) enzymes that remove unwanted primers and nucleotides form PCR amplification product. Two microliter of ExoSAP-IT reagent was added to 5 µl of PCR amplicon and incubated at 37°C for 4 min and further incubated at 80°C for 1 min to inactivate the enzymes.

Table 3.5 Characteristics of the PCR primers for DNA sequencing

Primer name	Sequence 5'-3'	Length (bp)	Tm (°C)	GC%
***TCF7L2* rs7903146_F**	GCCGGACCAAAGAGAAGATT	20	55.0	50.0
***TCF7L2* rs7903146_R**	TGCCAGTCAGCAAACACA	18	55.0	50.0
***CDKAL1*_F**	GTGTTTGGCCTTGAGTTTGG	20	54.8	50.0
***CDKAL1*_R**	ACAGAGACATCACTGTCCTT	20	53.0	45.0
***IRS1*_F**	TCTGACCAAGGGACAGTCTTA	21	54.9	47.6
***IRS1*_R**	GCTAGTGGCTACCATGTTGAA	21	54.8	47.6
***RND3-RBM43*_F**	AAGATGAAAGCCACTACCTCAG	22	54.4	45.5
***RN3-RBM43*_R**	CATGGTGAAATCCCGTCTCTAC	22	55.1	50.0
***KCNJ11*_F**	GACATGGTGAAGATGAGCAATG	22	54.1	45.5
***KCNJ11*_R**	AGGTGGAGGTAAGGAAGAGT	20	54.7	50.0
***TCF7L2* rs1225537_F**	CCTTGAGGTGTACTGGAAACTAA	23	54.2	43.5
***TCF7L2* rs1225537_R**	GTCGATGTTGTTGAGCTTTACTG	23	54.2	43.5

Bp- base pair; T-thymine; A-adenine; G-guanine; C-cytosine; F-forward; R-reverse °C-degree Celsius; Tm-annealing temperature

3.5.7.3 Sequencing PCR

The Big Dye Terminator V3.1 cycle sequencing kit (Applied Biosystems by Life Technologies, USA) was used to prepare sequence reactions. Two microliters of cleaned PCR product was mixed with 2 µl of 5x sequencing buffer, 1 µl of premix reagent, 4 µl nuclease free water, and this was done in duplicate in which the other tube had 1 µl of the forward primer (3.2 pmol) and the other tube 1 µl of the reverse primer (3.2 pmol) in a 10 µl reaction. The cycling conditions were as followed: 96°C for 1 min followed by 25 cycles of 96°C for 10 sec, 65°C for 5 sec, 60°C for 4 min, lastly a rapid thermal ramp and hold at 4°C. The contents of the tubes were spun down in a micro centrifuge and 10 µl of nuclease free water was added for a total volume of 20 µl.

3.5.7.4 Purification of the sequencing product

The sequencing PCR product was transferred to a new micro centrifuge tube with the addition of 5 µl of 125 mM EDTA. Sixty microliter of ice cold 100% ethanol (-20°C) was added, tubes were incubated at room temperature for 15 min in a dark room and then centrifuged for 30 min at a speed of 21 200 xg. The supernatant was removed by vacuum aspiration and 200 µl of 70% (v/v) ethanol was added to the tubes and mixed by vortexing, then centrifuged at room temperature for 30 min at a speed of 21 200 xg. The supernatant was removed by vacuum aspiration and the pellet was air dried. Hi Dye injection buffer of 30 µl was added, mixed by pipetting and then briefly centrifuged. The sample was denatured for 5 min at 94°C and then cooled for 5 min in ice. The sample was mixed by pipetting and loaded onto the 3500 ABI Genetic Analyzer (Applied Biosystems by Thermo Fisher Scientific) for sequence analysis.

3.6 Ethical aspects and informed consent

Ethical approval for the study was obtained from the Ethics Committee of the Faculty of Health Sciences, University of the Free State before onset of the study (HRSEC 80/2016).

Written informed consent forms for participants were translated in Sesotho, English and Afrikaans with the risks associated with drawing of blood, the use of genetic material and travel remuneration explained to participants. Only individuals that voluntarily gave written informed consent were included in the study.

3.7 Statistical analysis

Statistical analysis of data in this study was done by calculating odds ratios, confidence intervals and p values of allelic frequencies and gene frequencies using the 2x2 contingency table using online statistical tools (vassarstats) as well as statistical calculation tools in Microsoft Excel. Statistical significance was set at p-values less than 0.05 % confidence interval with no Bonferroni corrections utilized.

The Hardy-Weinberg equilibrium (HWE) was piloted to determine if the genotypic frequencies of selected SNPs adhered to the assumptions of the HWE model. The HWE entails the following assumptions: infinite population; discrete generations; no selection; random mating; no new mutations; no migration in and out of the population and equal initial genotype frequencies in the two sexes. The observed genotypic frequencies were compared with the expected genotypic frequencies using the chi-square statistical method. A statistically significant difference between the observed and expected genotype frequencies was indicated by a p-value of less than 0.5 and used to indicate if the SNP adhered to the HWE model. HWE calculations are as follows: $(\text{Observed} - \text{Expected})^2 / (\text{Expected})$ (Court 2005-2008).

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Study population

The study population was recruited and samples were collected as described in Chapter 3: Methodology. According to the study methodology followed, the T2D patients were recruited first as a convenience sample. Consequently, healthy volunteers subsiding within the broad age group and BMI were collected to match the T2D patient group. Tables containing participant number, age, weight, height, BMI and HbA1c information are provided in Appendix B. Since very few male T2D patients could be recruited, it was decided to only include female participants in the study. Originally, 103 T2D patients were recruited, however only 96 were included in the results, similarly 93 healthy volunteer controls were recruited and data from 92 were included due to incomplete data.

4.1.1 Participant phenotypes

The control group was selected to match the patient group for sex, age and BMI. Figure 4.1 shows an almost equal distribution of age groups for both cohorts. Both the patient and control groups display a similar average age. The age of the participants ranged between 20-60 years with the largest grouping of T2D patients between the ages of 50-59 and that of controls between the ages of 40-49 (Table 4.1). These results are similar to studies indicating the average age of T2D development between 50 and 60 years (Orgurtsova *et al.* 2017). A slight increase of people between the ages of 30-39 in controls than in T2D patients was observed in Figure 4.1, however with no statistical significant difference (p=0.11).

The BMI of the participants ranged from normal to obese with no study participants in the underweight category (Figure 4.2)(Table 4.1). The largest group of participants were in the obese group. Only a slight difference was seen between the BMI categories of the two cohorts, with a p value of 0.011. The highest difference is seen in the obese category

with only 9.6% difference. This can be explained by convenience sampling of recruiting the control group to coincide with the age and BMI range of the T2D patient group.

The HbA1c percentage level of participants ranged from 4.3% to 15.8% (Figure 4.3) (Table 4.1). Only 4.2% of T2D patients had normal glycated haemoglobin levels of below 6.5%. The HbA1c results of the control cohort healthy volunteer participants were below 6.5%. However, within this group, 11.5% of healthy volunteers had an HbA1c percentage above 5.7%, indicating that they could be at risk of developing T2D.

The largest group of T2D patients had HbA1c levels above 6.5%. Optimal glycemic control for T2D patients has been indicated at 7.5% (Vaaler 2000). Figure 4.3 indicates that 68.7% of T2D patients have an HbA1c level above 7.5%. This sadly point out that either patients are not going for regular check-ups or may not be following prescribed treatment procedures to achieve optimal glycaemic control.

In the UK Prospective Diabetes Study, failure to attain an HbA1c target of less than 7% has been associated with a two to four fold increased risk of hospitalization and duration of hospital stay in an international prospective survey of real-world practice. Patients with HbA1c ≥10% had three-fold increased risk of complications than those with HbA1c <6% in the epidemiological analysis of the UK Prospective Diabetes Study (Yin *et al*. 2014).

The percentages of age distribution, body mass index (BMI) and HbA1c for both the patient and control participants are illustrated in Figure 4.1, 4.2 and Figure 4.3 with data distribution and p-values in Table 4.1. Phenotypic data (age, weight, height, BMI and HbA1c) of each participant can be viewed in Appendix B of each participant in the T2D patient and control groups. .

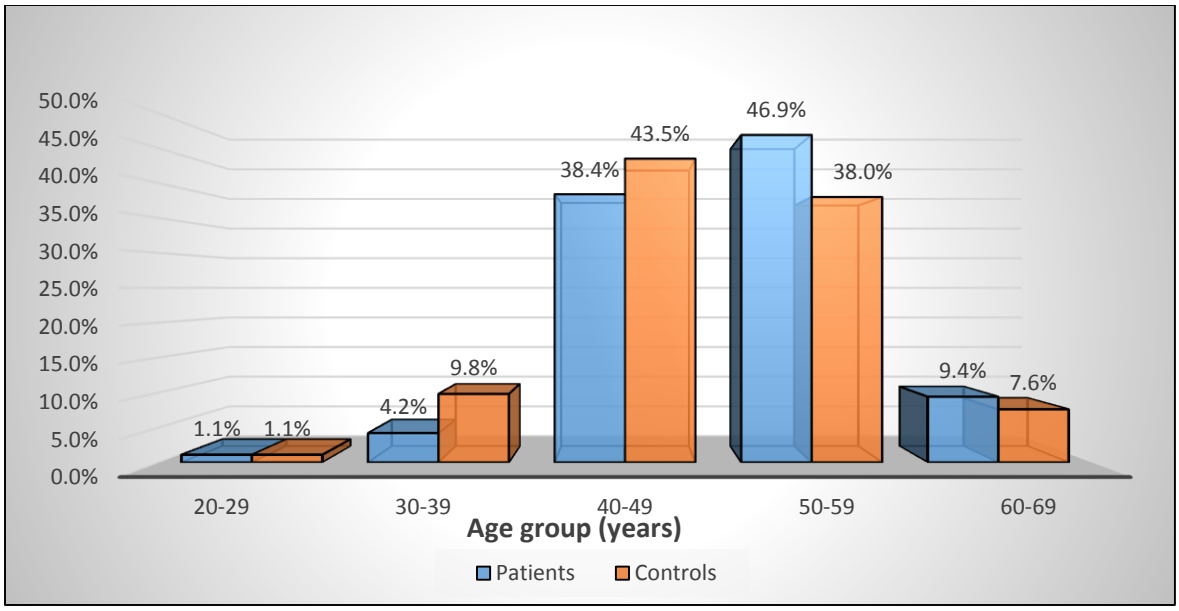


Figure 4.1 Age distribution of the study population. T2D patients (n=96), controls (n=92)

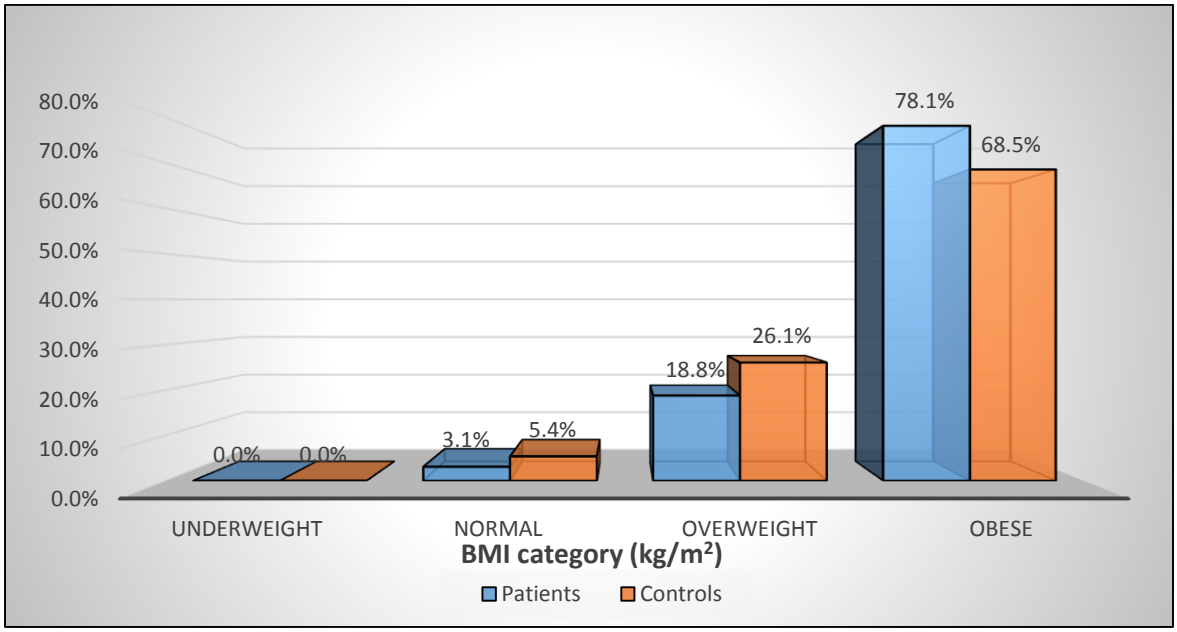


Figure 4.2 BMI categories of T2D patient and control participants. The BMI ranges (kg/m^2) for each category: Underweight (<18.5), Normal (18.5-24.9), Overweight (25-29.9) and Obese (≥30).

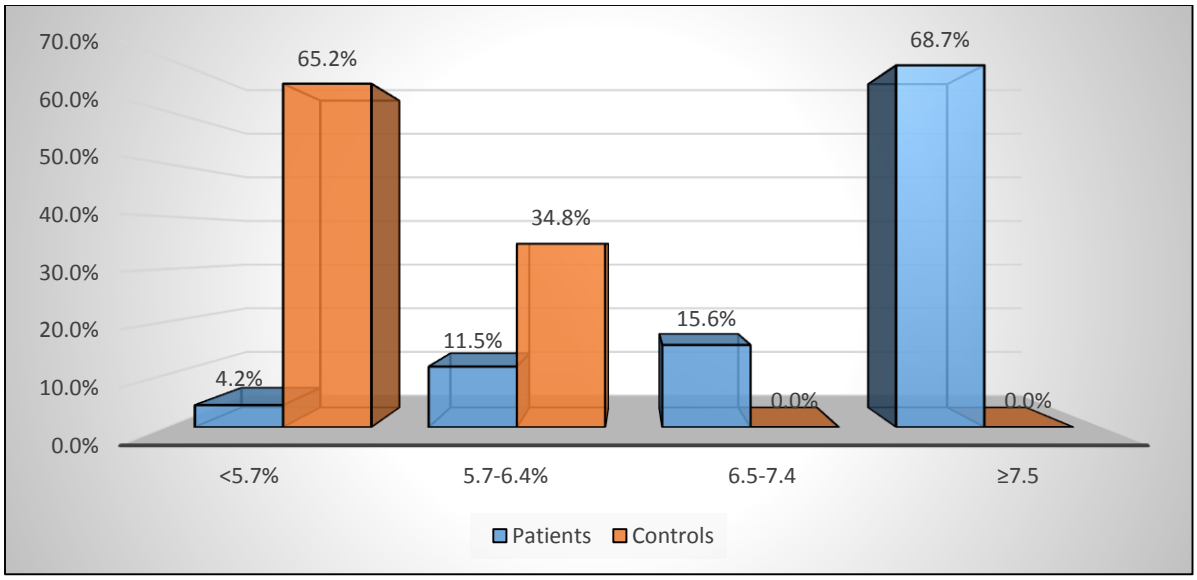


Figure 4.3 HbA1c percentage levels of patient and control participants. The HbA1c classes: <5.7%; 5.7-6.4%; 6.4-7.4% and ≥7.5%.

Table 4.1 Summary of data distribution and p-values of patients with T2D (n=96) and control participants (n=92).

	Patients				Controls				
Variable	Min	Max	Median	Mean (SD)	Min	Max	Median	Mean (SD)	p-value
Age	35	61	51	50.40 (6.51)	36	62	49	48.63 (7.45)	0.110
Weight (kg)	52.3	130.6	84.7	89.26 (20.33)	52	120.2	82.2	83.49 (16.14)	0.033
Height (m)	1.415	1.72	1.57	1.571 (5.88)	1.36	1.744	1.57	1.578 (6.49)	0.437
BMI (kg/m^2)	21.5	52.2	34.4	36.08 (7.55)	20.98	47.3	32.3	33.51 (6.19)	0.011
HbA1c (%)	5.3	15.8	8.3	8.73 (2.33)	4.3	6.4	5.5	5.51 (0.41)	<0.001

SD= Standard deviation

4.2 Participant genotypes

4.2.1 DNA isolation

The mean yield of the DNA after extraction was 169.9 ng/µl. The concentration and the purity of the DNA was evaluated using the NanoDrop® spectrophotometer as described in section 3.5.2. An absorbance ratio at A260/280 of above 1.8 was considered good and samples for which this was not acquired was re-isolated until the desired absorbance value was obtained. The DNA samples were further diluted to a final working solution of 12.5 ng/µl required for genotyping with qPCR.

4.2.2 Sequencing of positive run controls to be used for quality control in qPCR

Sanger sequencing was performed on control group participants DNA samples for the regions containing the SNPs to serve as a positive wild type allele control in each qPCR run (Appendix D1). This was performed for the regions that contain the following six polymorphisms; rs12255372, rs7903146, rs5219, rs2943641, rs7754840 and rs7560163. Initial pre-sequencing electrophoresis based PCR amplification using the six different primer sets, yielded amplicon sizes for all SNPs at approximately 320bp (Figure 4.4). The optimized cycling conditions were as follows: 95°C for 10 minutes, followed by 35 repeats of three-step cycling between 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 1 minute. These conditions were used for all assays. Figure 4.5 is an example of a sequence electropherograms of wild type allelic control samples. The non-template controls were run to ensure that there was no contamination of any of the PCR reagents.

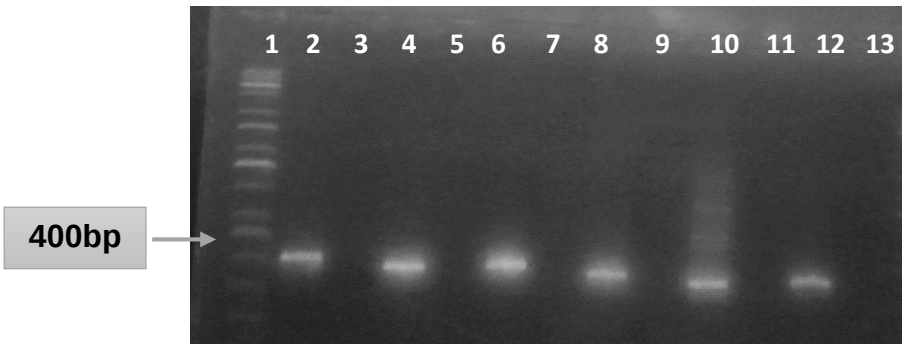


Figure 4.4. Electrophoresis gel image of PCR amplification products prior to Sanger sequencing of the wild type allelic samples of the six SNPs. A 1% (v/w) TAE agarose gel was electrophoresed and visualized by gel red stain. Lane 1: 100bp DNA marker (Hyperladder II Invitrogen, USA); lane 2: *TCF7L2* rs7903146; lane 4: *TCF7L2* rs1225537: lane 6: *RND3-RBM43* rs7560163; lane 8: *KCNJ11* rs5219; lane 10: *CDKAL1* rs7754840 and lane 12: *IRS1* (rs2943641) with non-template PCR controls in Lanes 3, 5, 7, 9, 11, 13 respectively.

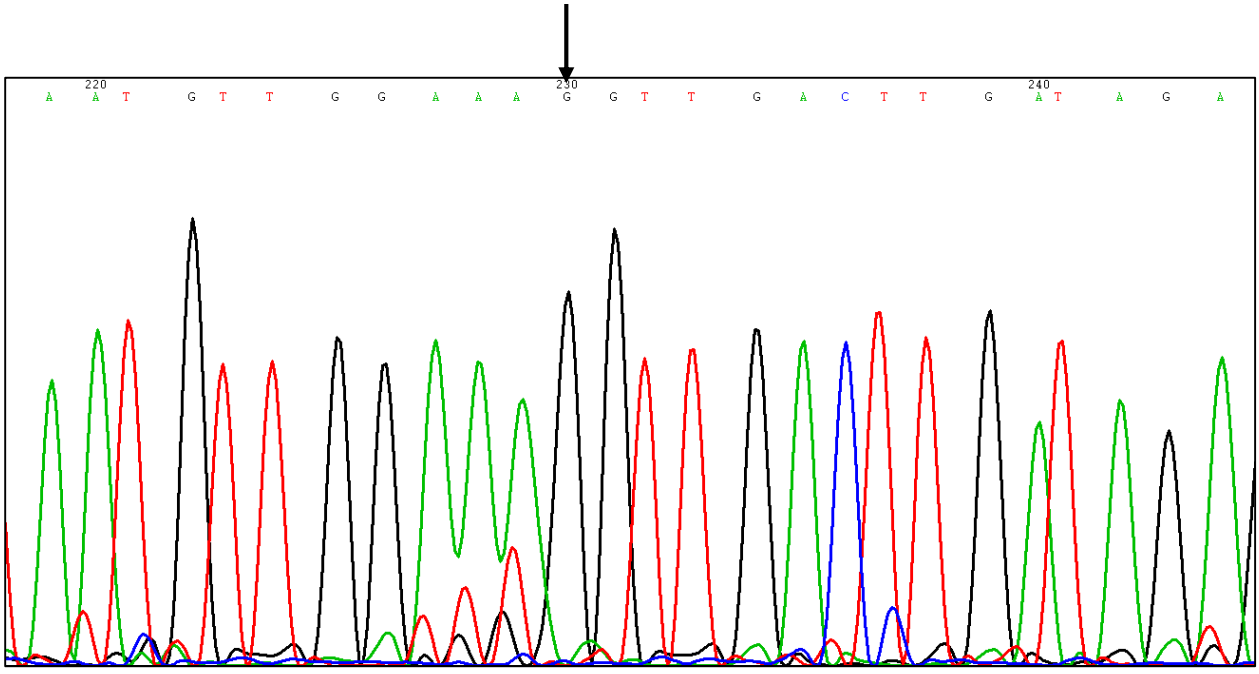


Figure 4.5 Example of sequencing electropherogram of an individual (Control C7) with a mutant allele (GG) on position 230 in the *CDKAL1* gene. The black arrow indicates the SNP position.

4.2.3 Quantitative PCR optimization results

To attain optimal genotyping results, gel electrophoresis based PCR was performed with three annealing temperature differences in the cycling conditions on a SimpliAmp Thermal cycler (Applied Biosystems by Life Technologies, USA). The annealing temperatures tested were; 60°C, 63°C and 65°C on sample C7 for all six primer pairs. An example of the qPCR amplification plot for SNP *IRS1* rs2943641 (heterozygous genotype) is shown in figure 4.6. Amplification curves indicated with black arrows had annealing temperatures of 60°C, and produced the best amplification curves compared to the runs programmed at 63°C and 65°C (Figure 4.6). Both the VIC (Allele 1) and FAM (Allele 2) produced superior amplification curves with earlier Ct values, compared to the reactions with annealing temperatures at 63°C (green arrow indication) and 65°C (blue arrow indication) (Figure 4.6). Figure 4.7 is a gel electrophoresis photograph taken of reactions performed at the optimized cycling conditions to confirm amplicon size. The optimized cycling conditions were as follows: 95°C for 10 min and then 40 repeats of a two-step cycling between 95°C for 15 seconds and 60°C for 1 minute. These conditions were used for all patient and control samples for all six the SNPs.

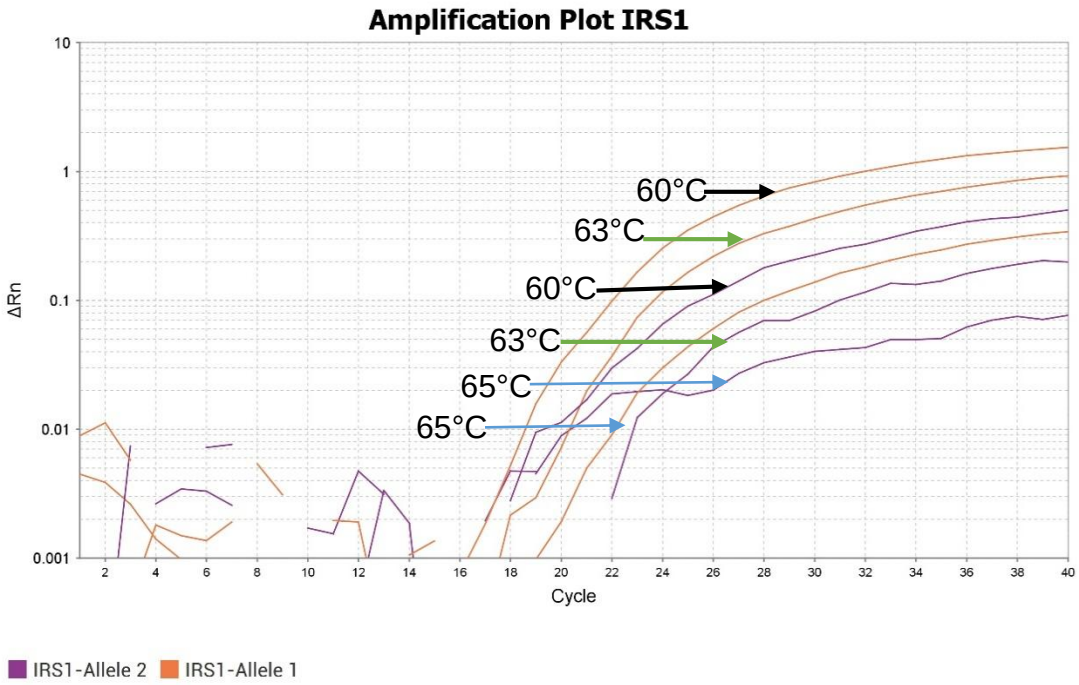


Figure 4.6. Example of an amplification plot showing three different annealing temperatures of 60°C, 63°C and 65°C for Gene SNP *IRS1* rs2943641. Annealing temperature of 60°C is indicated with black arrows, 63°C with green arrows and 65°C with blue arrows, for the probes labelled with VIC (Allele 1) and FAM (Allele 2).

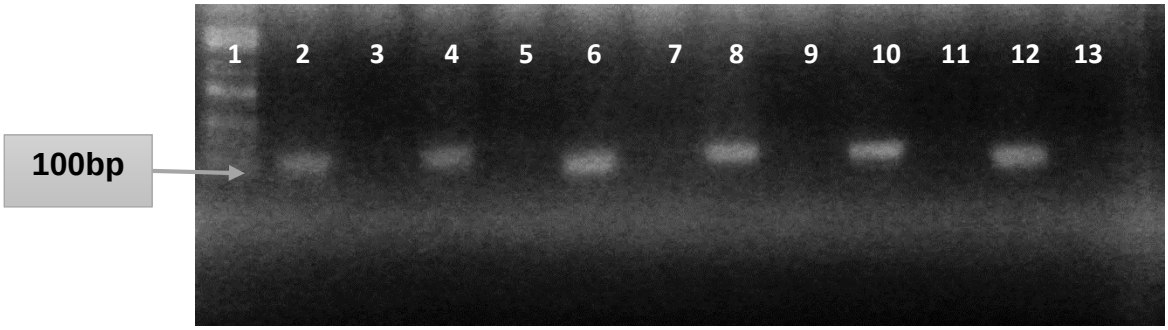


Figure 4.7. Electrophoresis gel image of PCR amplification depicting the PCR product of the wildtype allele of different participants used as positive controls for quality control for qPCR for all six primer pairs products. A 1% TAE buffer based agarose gel was used to separate the PCR products. Lane 1: 100bp DNA marker (Hyperladder II Invitrogen, USA). Lanes 2-7: PCR products of all 6 assays as follows: lane 2 (*TCF7L2* rs7903146), lane 3 (*TCF7L2* rs12255372), 4 lane (*RND3-RBM43* rs7560163), lane 5 (*KCNJ11* rs5219), lane 6 (*CDKAL1* rs7754840) and lane 7 (*IRS1* rs2943641).

4.2.4 qPCR Genotyping results

The patient and control cohorts (n=188) with 96 T2D patients and 92 controls were screened for all six SNPs known to be associated with the development of T2D among the European, the Caucasian and the African American descent. Figure 4.8, is an example of an allelic discrimination plot of the rs7754840 SNP in *CDKAL1* of 14 participants generated by the Quant studio 5 (Applied Biosystems by Thermo Fisher Scientific) qPCR instrument. In this allelic discrimination plot, three distinct clusters represent the three genotypes. These clusters are representative of samples genotyped grouping together in their respective genotypes. Samples represented by red dots indicate a homozygous genotype for allele 1 this represents the C/C genotype which is the mutant according to the Genome Reference Consortium Human Build 38 (https://www.ncbi.nlm.nih.gov). These dots are positioned on the graph where the fluorescence measured on the x-axis (measuring allele 1 – probe labelled with VIC) is the highest and the measurement on the y-axis is the lowest. The run control for allele 1 used

was a technical gBlocks® synthesized control, indicated by a circle around the duplicate repeats.

The blue dots represent the homozygous genotype for allele 2, this represents the G/G genotype which is the wild type genotype according to the Genome Reference Consortium Human Build 38 (https://www.ncbi.nlm.nih.gov). These dots are positioned on the graph where the fluorescence measured on the y-axis (measuring allele 2 – probe labelled with FAM) is the highest and the measurement on the x-axis is the lowest. In this case the run control for the allele 2 homozygous genotype was a sequence confirmed genotype (Figure 4.5), the two repeats are again circled. The green dots represent samples with a heterozygous genotype (C/G). These dots are positioned on the graph with almost equal fluorescence from both the x- and y-axis indicate equal fluorescence from both FAM and VIC dyes. Indicating that one allele is a C (VIC) and the other is a G (FAM). The black squares represented non-template controls, these reactions are set up from the same master mix reagent as the samples and run controls, with the exclusion of template DNA. Instead of template DNA, the 20 µl reaction is filled up with nuclease free water. The black squares are positioned on the graph with the lowest levels of fluorescence from both dyes. Additional allelic discrimination plots have been included in Appendix E.

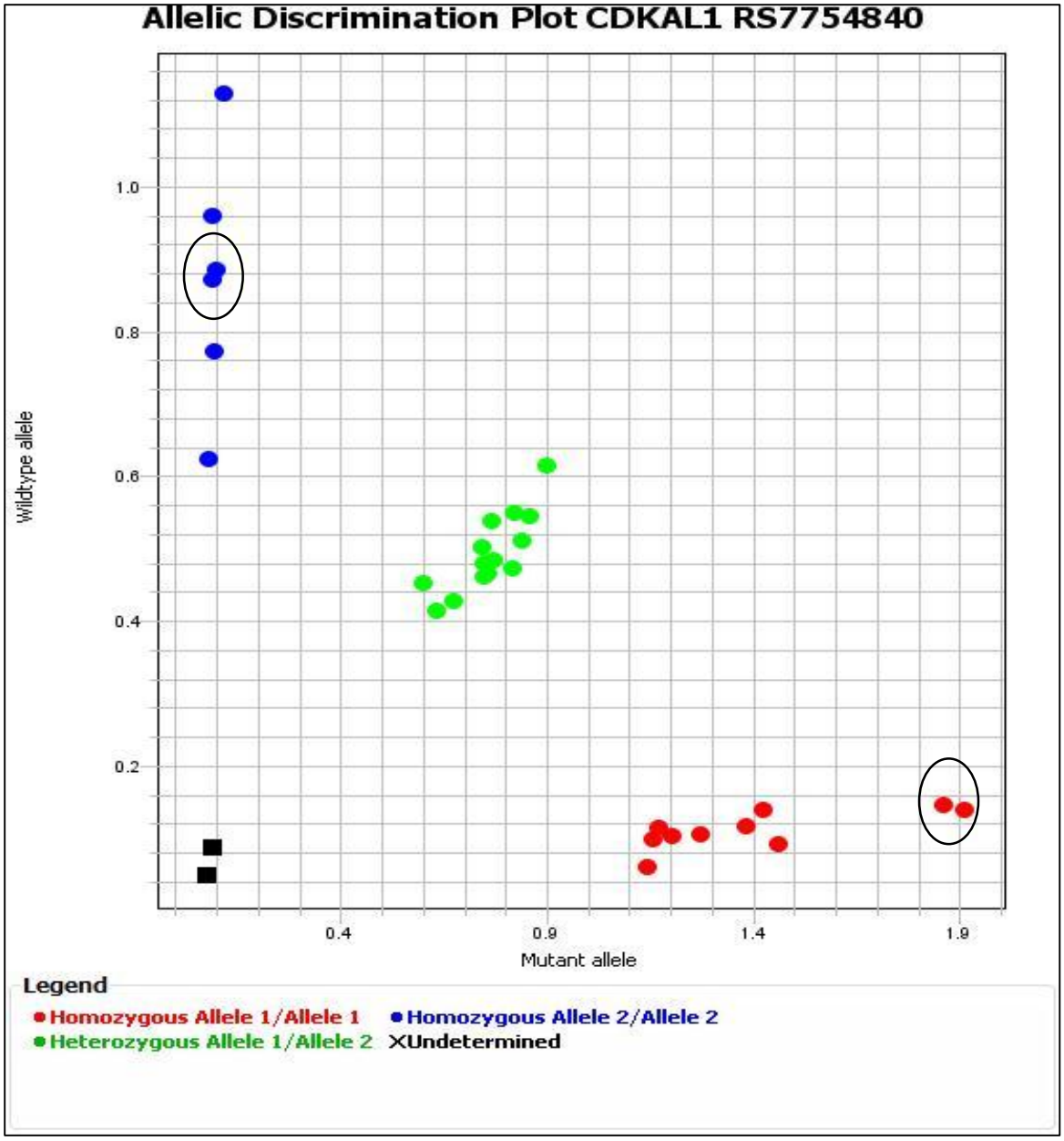


Figure 4.8 Example of an Allelic discrimination plot of the rs7754840 SNP in *CDKAL1*. Genotypes of 14 participants are represented as follows: Blue colour = (GG) homozygous wild type / common allele genotype; the green colour = (GC) heterozygous genotype; the red colour = (CC) homozygous mutant / rare allele genotype; and the black colour = Non template control.

The allelic discrimination plots are based on the fluorescence emitted by the fluorophore attached to the specific probe, once it is cleaved from its quenching molecule, during the

PCR amplification. The fluorescence produced during PCR are depicted by the qPCR instrument in amplification plots, showing the variation in PCR cycle number that amplification curves cross the baseline value. The threshold cycle or Ct value, is the intersection between an amplification curve and a threshold line. It is a relative measure of the concentration of target in a PCR reaction. Figure 4.9 is the amplification curve produced by the qPCR instrument of one of the synthetic manufactured oligo reactions serving as the homozygous wild type (CC) run control of the allelic discrimination plot as seen in figure 4.8. Figure 4.10 is the amplification curve produced by the qPCR instrument of one of the sequence genotype confirmed reactions serving as the homozygous mutant (GG) run control and Figure 4.11 shows the amplification plot of a heterozygous sample.

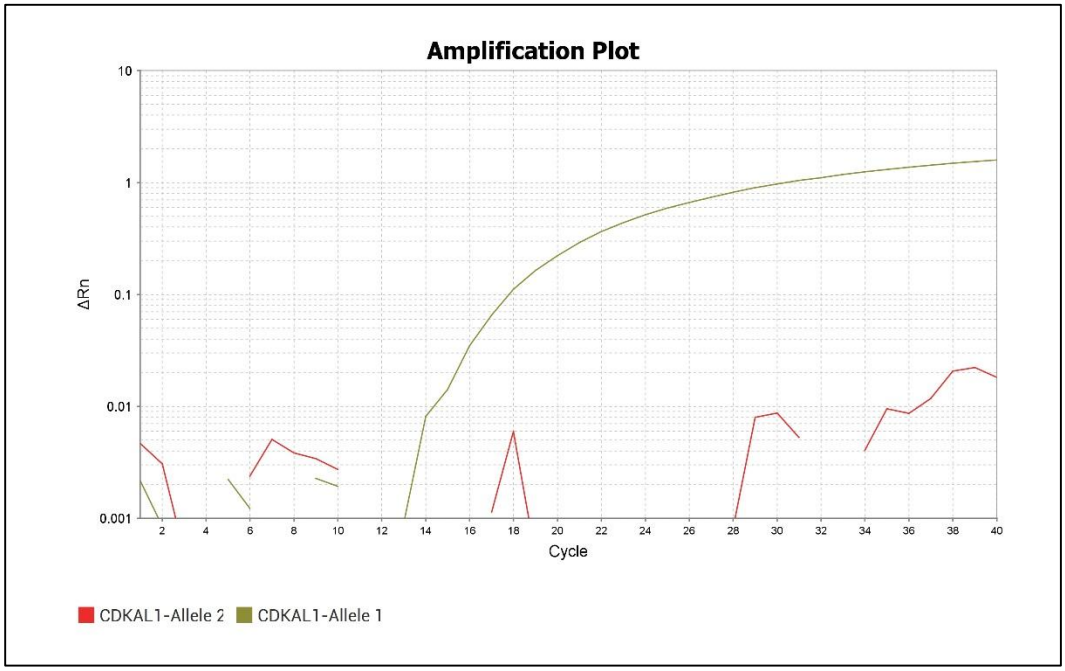


Figure 4.9 Amplification curve of an Allele 1 run control synthetic manufactured oligo reaction for the rs7754840 SNP in *CDKAL1*.

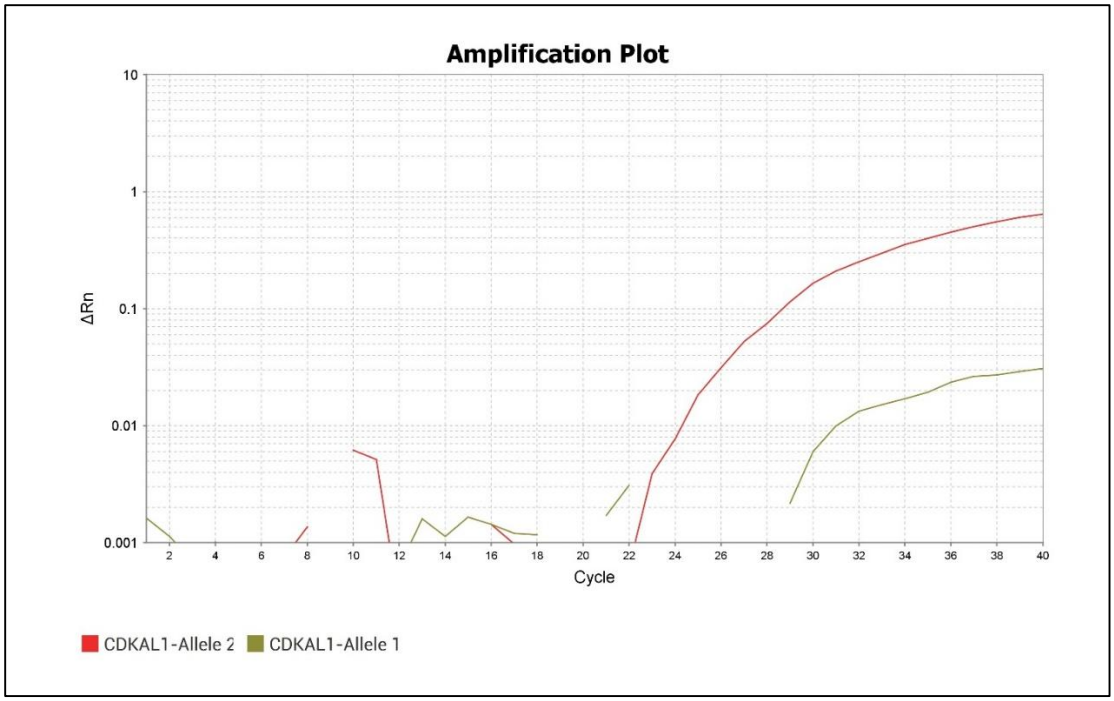


Figure 4.10 Amplification curve of an Allele 2 run control sequence confirmed reaction for the rs7754840 SNP in *CDKAL1*.

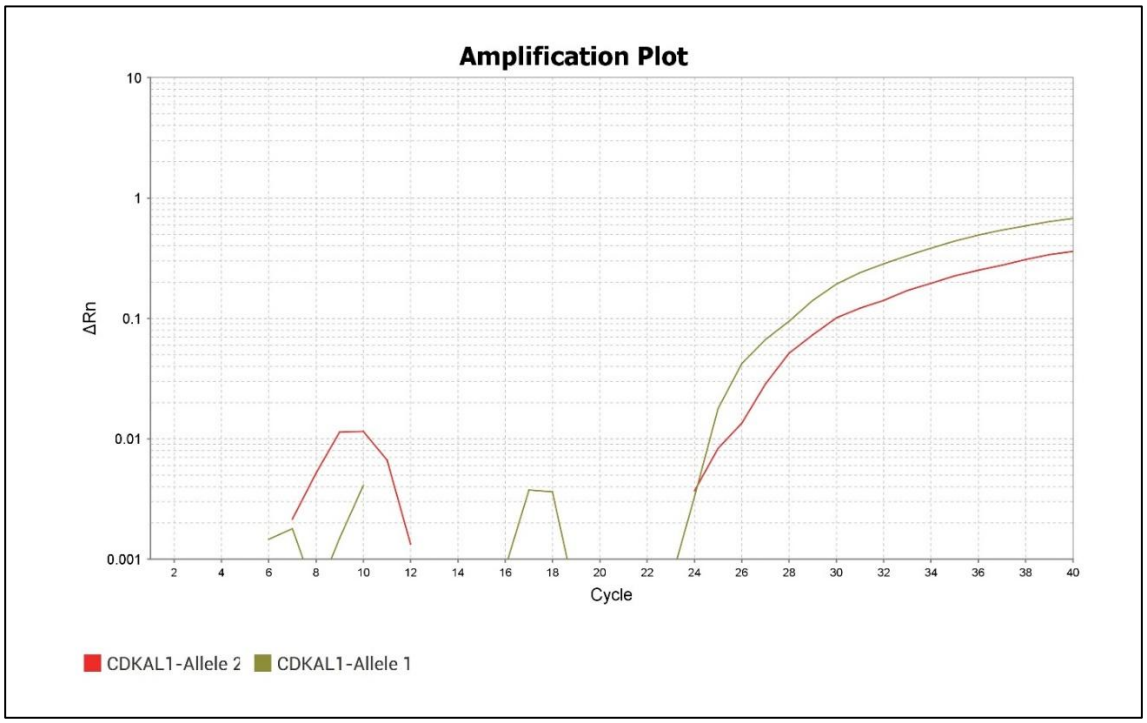


Figure 4.11 Amplification curve of a heterozygous reaction for the rs7754840 SNP in *CDKAL1*.

The allele frequencies and genotype frequencies of the six candidate SNPs observed during the screening are shown in Table 4.2 and 4.3. The percentage distribution of alleles and genotypes in both cohorts have been plotted in Figure 4.12 and Figure 4.13. Increased homozygosity for the mutant T allele at *TCF7L2* rs12255372 was observed in T2D patients of 23% vs the 3% in non-T2D controls (Table 4.3 and Figure 4.13) (odds ratio 8.82, 95% confidence interval: 2.54-30.63 p value= 0.0000599). The rare C allele of IRS1 rs2943641 was also present at a high percentage in both the cohorts, without statistical significance (Table 4.3 and Figure 4.13).

Table 4.2 Allele frequencies of participants for six candidate SNPs screened

Gene	SNP	Allele	T2D N=96	Controls N=92	Odds ratio	95% CI	p-value
TCF7L2	rs7903146	C T	0.63 0.37	0.62 0.38	0.98 1.02	0.64-1.48 0.67-1.55	0.92
TCF7L2	rs12255372	G T	0.56 0.44	0.76 0.24	2.47 0.40	1.59-3.85 0.26-0.63	0.0000543
IRS1	rs2943641	T C	0.24 0.76	0.30 0.70	1.39 0.72	0.88-2.19 0.46-1.14	0.166
RND3	rs7560163	G C	0.30 0.70	0.25 0.75	0.75 1.34	0.47-1.18 0.85-2.11	0.248
CDKAL1	rs7754840	G C	0.48 0.52	0.48 0.52	1 1	0.67-1.49 0.67-1.50	1
KCNJ11	rs5219	C T	1 0	1 0	0 Infinity	0-NaN NaN-Infinity	0.489

NaN- No real number

Table 4.3 Genotype frequencies of participants for all six candidate SNPs screened.

Gene	Genotypes	Patients (n=96)	Controls (n= 92)	Odds ratio	95% CI	P-Value
TCF7L2 **rs7903146**	CC CT TT	0.37 0.52 0.11	0.39 0.46 0.15	0.89 1.29 3.37	0.50-1.61 0.73-2.30 0.91-12.51	0.746 0.386 0.0910
TCF7L2 **rs12255372**	GG GT TT	0.35 0.42 0.23	0.56 0.41 0.03	0.44 1.02 8.82	0.25-0.79 0.57-1.81 2.54-30.63	0.00818 1 0.00005 99
IRS1 **rs2943641**	TT CT CC	0.03 0.42 0.55	0.11 0.39 0.50	0.27 1.11 1.23	0.07-0.99 0.62-1.99 0.70-2.19	0.045 0.767 0.559
CDKAL1 **rs7754840**	GG GC CC	0.21 0.55 0.24	0.26 0.45 0.29	0.76 1.533 0.76	0.38-1.47 0.86-2.73 0.40-1.45	0.491 1.89 0.415
RND3-RBM43 **rs7560163**	GG GC CC	0.10 0.40 0.50	0.07 0.36 0.57	1.67 1.17 0.74	0.58-4.79 0.65-2.12 0.41-1.31	0.436 0.653 0.310
KCNJ11 **rs2943641**	CC CT TT	1 0 0	0.99 0.01 0	Infinity 0 Nan	NaN-Infinity 0-NaN	0.489 0.489 1

NaN – No real number

The T allele of *TCF7L2* rs12255372 showed a higher allele frequency in the T2D patient group than in the non-T2D control group, with a statistical significance value of p= 0.0000543 (Table 4.2). The risk T allele of the *TCF7L2* rs12255372 was found in literature to be associated in the Japanese population (OR: 2.082, 95% CI: 1.112-3.898, P value: 0.022) (Tabara *et al.* 2009). This strong association of the T allele of the *TCF7L2* rs12255372 was also documented in the Indian population with a statistical significance value of p= 0.000004. In the study on the Indian population, Chandak *et al.* (2007) observed that the non-diabetic group that possessed the mutant T allele of the rs12255372 had higher fasting and 2-h plasma glucose concentrations, suggesting both a defect in insulin secretion from the beta cells and an increase in insulin resistance. Although it is not yet clear how *TCF7L2* contributes to the pathogenesis of T2D, a study

by Jin (2008) suggested that the *TCF7L2* SNPs may modify disease susceptibility by affecting intestinal glucagon expression and plasma levels of GLP-1. This particular SNP was found to be in be in strong linkage disequilibrium with a microsatellite DG10S478 located within intron 3 of the *TCF7L2* gene, which was also found to be associated with T2D (Jin 2008). The statistical significant association resulting from the *TCF7L2* rs12255327 SNP with the T2D patient group, in the small study population of this study, warrants further investigation.

No statistically significant difference was observed between the T2D patient and control group allele frequencies for *TCF7L2* rs7903146, *CDKAL1* rs7755840, *IRS1* rs2943641, *RND3-RBM43* rs7560163 and *KCNJ11* rs5219 (Table 4.2). In contrast, to the results observed in this study, Pirie *et al.* (2010) found an association with *TCF7L2* rs7903146 rather than the rs12255372 in the black South African Zulu population. Tabara *et al.* (2009) found both these SNPs, the *TCF7L2* gene rs12255372 and rs7903146 to be associated with T2D in the Japanese population. A case control study by Palmer *et al.* (2010) found rs7903146 to be the most significantly associated with T2D more than other variants in the *TCF7L2,* while a study by McCormack (2013) found an association with T2D in African Americans. Data from 1000 genomes shows percentages of the T alleles of the rs7903146 and rs122553372 in Africans to be 26% and 39% respectively (Table 4.4).

The rare or mutant allele of the *IRS1* and *RND3-RBM43* gene SNPs were higher in both patients and controls than the ancestral allele. This result is in accordance with the 1000 genomes allele frequencies as observed in African populations from ENSEMBL (Table 4.4). Palmer *et al.* (2012) found an association of the mutant C allele of *RND3-RBM43* to be associated with T2D in African Americans (OR: 0.75, 95% CI: 0.67-0.84 and $p=7.0x10^{-9}$). A study by Tang *et al.* (2012) found an even higher association of the C mutant allele with T2D in the Chinese Han population. *IRS1* is known to play an important role in insulin signalling and the rs2943641 SNP has been identified as a susceptibility locus for T2D in Caucasian patients (Tang *et al.* 2012). Association of the *IRS1* rs2943641 with T2D was identified in the Saudi population by a study by Alharbi *et al.* (2014) (OR=1.482, 95% CI

1.002-3.121 and P=0.04). However, no trend or association with T2D resulted from the study population investigated.

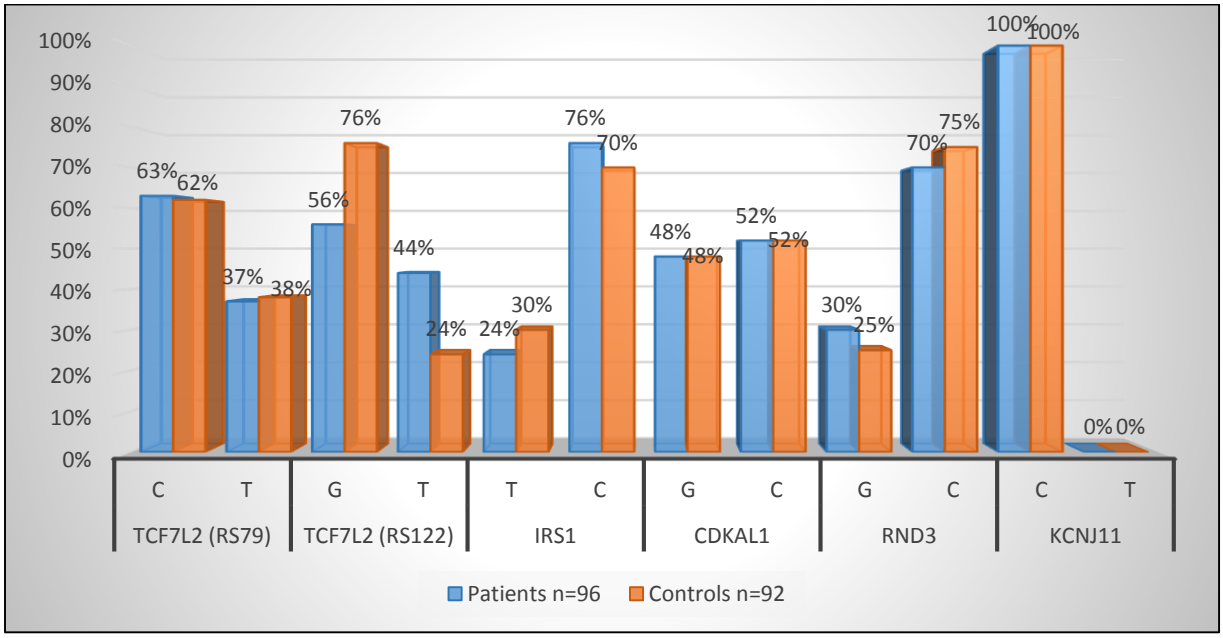
An increase in heterozygosity was observed for the *CDKAL1* rs7755840 SNP in T2D patients, also without statistical significance p=1.89, yet the allele frequencies of the wildtype and the mutant alleles was exactly the same in both the patient and control cohorts. In literature, the rs7754840 in the *CDKAL1* gene presented a significantly stronger effect in the Ashkenazi Jewish population as compared to the general Caucasian population (OR=1.29, 95% CI: 1.14-1.45 and p=0.00003) (Bronstein *et al.* 2008). A study by Watanabe *et al.* (2013) found homozygous carriers of risk allele of the *CDKAL1* rs7754840 to have a 22% or 24% lower insulin secretion than non-carriers in non-T2D individuals in the Japanese population, however the physiological functions of *CDKAL1* gene are unclear.

Only one heterozygote genotype was observed for the *KCNJ11* rs5219 and no mutant homozygote was detected, again this is in accordance with the 1000 genome allele frequencies in Africa in which only 2% of the population had the mutant T allele (Table 4.4). In a study by Haghvirdizadeh *et al.* (2014), the mutant T allele of the *KCNJ11* rs5219 was shown to suppress insulin secretion by over activity of the channel, resulting from the impaired pathway by reduced ATP sensitivity of the K ATP channel. In the Caucasian population and in some Asian populations, the rs5219 polymorphism was found to be a risk factor for developing T2D (Haghvirdizadeh *et al.* 2014). Also a study by Lasram *et al.* (2014) reported a significant association of the mutant T allele of the *KCNJ11* rs5219 with T2D in the Tunisian population (OR=1.6, 95% CI: 1,14-2.27 and p=0.007). A potential limitation to this study is the relatively small number of patients and control participants, thus estimates of the association have relatively large confidence risks especially for homozygous participants

Table 4.4 Data from the ENSEMBL 1000 genomes database of allele percentages as well as allele frequencies in the African region.

Gene	Percentages	Frequencies
***TCF7L2* rs7903146**	C:74% T: 26%	C:0.74(978) T:0.26(344)
***TCF7L2* rs12255327**	G:61% T:39%	G:0.61(806) T:0.39(516)
***RND3* rs7560163**	C:88% G:12%	C:0.884(1169) G:0.116(153)
***IRS1* rs2943641**	T:31% C:69%	T:0.315(416) C:0.685(906)
***CDKAL1* rs7754840**	C:63% G:37%	G:0.368(487) C:0.632(835)
KCNJ11 rs5219	T:2% C:98%	T:0.023(31) C:0.977(1291)

Numbers in brackets indicate the number of people included in the statistical calculations



rs79= rs7903146 and rs122= rs12255372

Figure 4.12 The graph indicates the percentage distribution of allele variants in T2D patient and control participants.

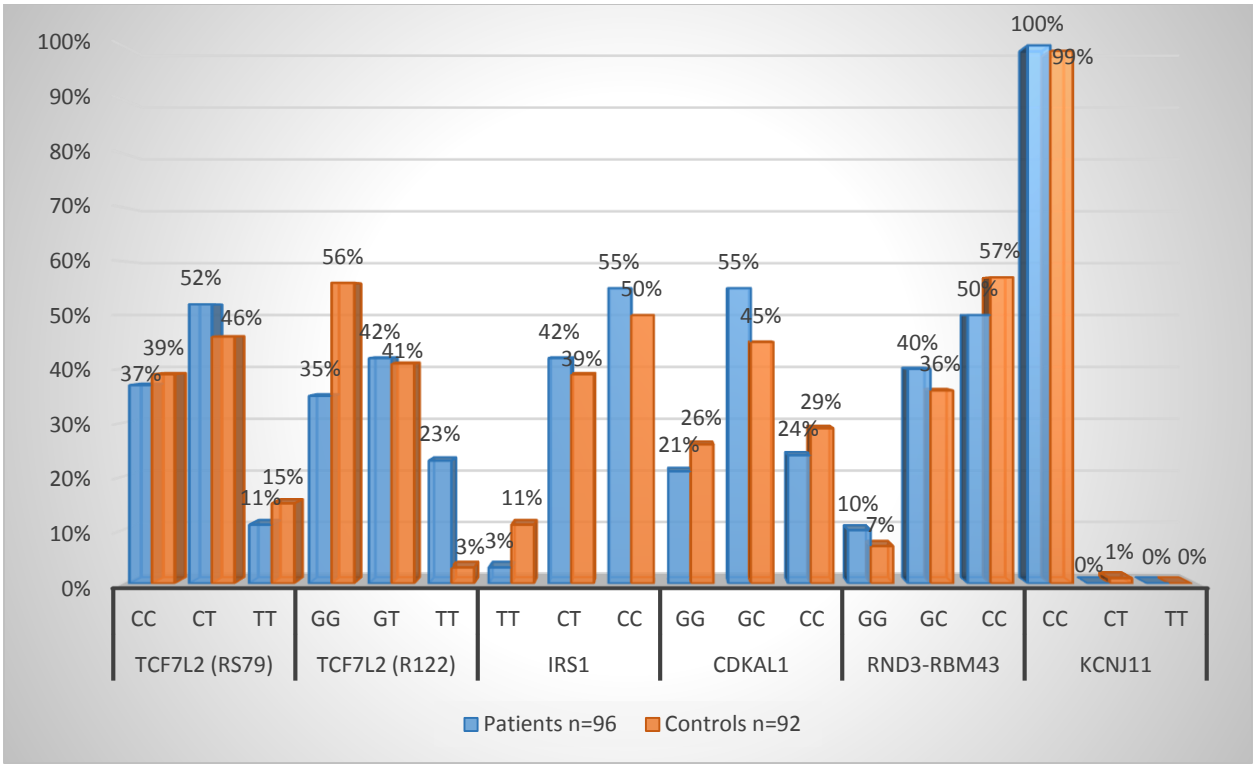


Figure 4.13 The graph indicates the percentage distribution of genotype variants in T2D patient and control participants

4.3 Sequencing results to confirm genotyping

To confirm the SNPs observed from the qPCR genotyping, sequencing was performed on samples with the heterozygote and the mutant homozygote genotypes for each SNP assay. An example of a homozygote mutant allele sequence can be viewed in Figure 4.14 and a heterozygote allele sequence in Figure 4.15. All sequencing results have been captured in Appendix D2.

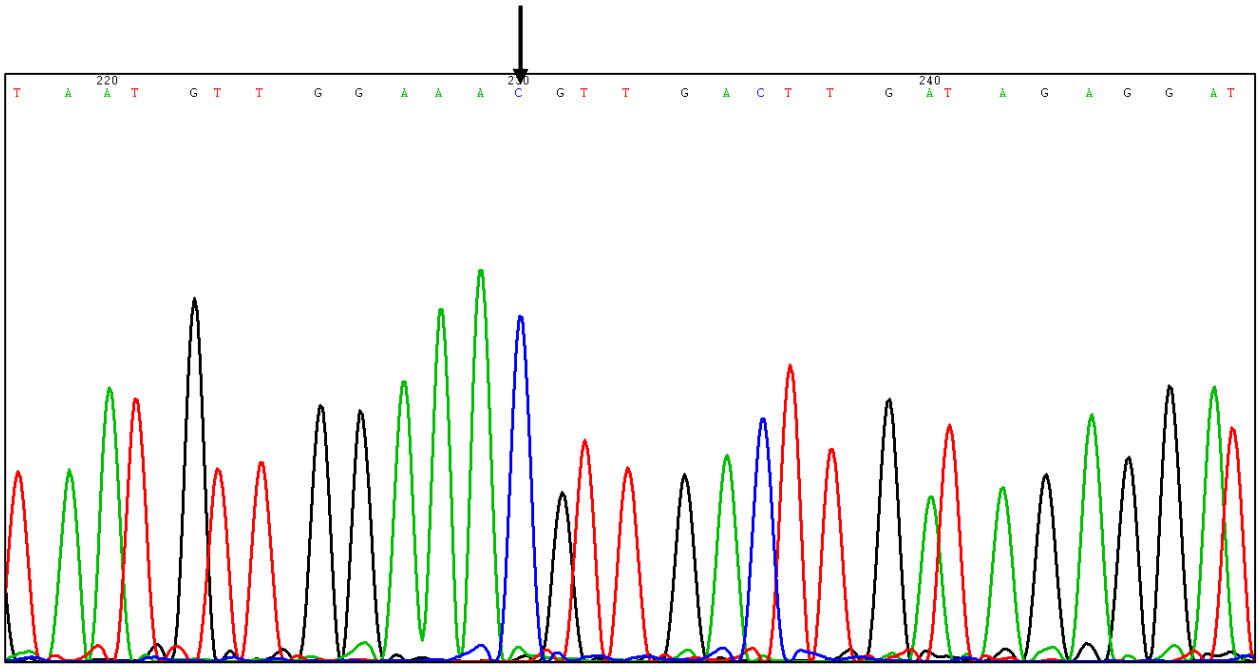


Figure 4.14 Example of a sequencing electropherogram result of individual C2 with a homozygote (mutant) allele (CC) on position 230 in the *CDKAL1* gene. The black arrow indicates the SNP position.

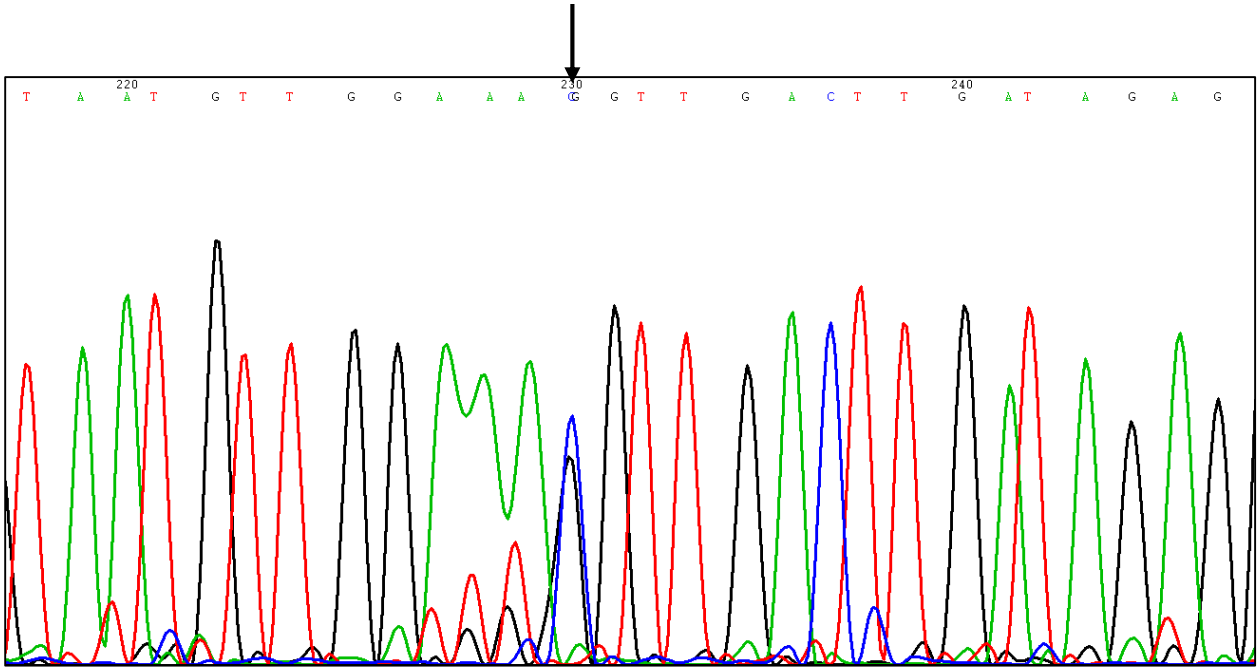


Figure 4.15 Example of sequencing electropherogram of individual C9 with a heterozygous (GC) on position 230 in the *CDKAL1* gene. The black arrow indicates the SNP position.

4.4 Limitations with sequencing results

In a few cases sequence electropherograms showed background peaks, even after several repeat attempts to obtain a desired result. One in particular is the heterozygote *KCNJ11* rs5219 SNP determined by the qPCR optimized kit. Since this heterozygote genotype could not be confirmed the sample and participant was removed from the data set. The homozygote wild type control sequences (Appendix D1) for the following gene SNP's showed a degree of background, however the genotype of the SNP is clearly identifiable; *TCF7L2* rs12255327; *RND3-RBM43* rs7560163. In addition the optimized qPCR kit reactions also called the genotypes as homozygous for allele 1 (wild type). Two other wild type control sequence reactions *IRS1* rs2943641 and the *TCF7L2* rs7903146 was repeated to obtain better quality sequences, unfortunately the peaks of the adjacent nucleotides are in close proximity to that of the SNP nucleotide, but not superimposed as to suggest heterozygosity and the genotype can be confirmed. The sequence fasta file, also did not indicate heterozygosity. Both the sequences confirming heterozygosity of the *TCF7L2* rs12255327 and *TCF7L2* rs7903146 were of good quality initially, the best possible sequence after repeating PCR and sequencing reactions were captured in Appendix D2. The sequence of *TCF7L2* rs7903146 still contain some background peaks, however, the heterozygous genotype can clearly be seen. In the case of *TCF7L2* rs12255327 repeat sequence attempts produced a heterozygous genotype called by the sequence software, however upon close inspection the peaks are not fully superimposed.

The possible reasons encountered during sequencing could have been because of the salt residue that could have inhibited the sequencing reaction, since the salting out method was used for DNA isolation. Another option is that the concentration of the DNA could have been too high. PCR artefacts under each peak are normal and follows a specific pattern in that red (A) always pairs-up with black (C) and blue (T) always pairs-up with green (G), however these could not be reduced to confirm the above genotype. One other reason for inconclusive sequences results could have been a low signal which causes reduced sequence reliability. Nevertheless, the optimized and quality control tested Taqman® genotyping assay (Thermo Scientific) has been tested and designed to

accurately determine the genotype of the designed SNP and thus we conclude that only the sequencing is not accurate and the qPCR result is reliable.

4.5 Hardy–Weinberg equilibrium (HWE) results

Results from the HWE has been captured in Table 4.5. The HWE distribution pattern of the SNPs were performed using the Michael H Court (2005-2008) method. All the SNPs adhered to the HWE model with the exception of the *KCNJ11* rs5219 SNP in the T2D patient group for which no p value could be calculated. This result can mainly be ascribed to the small number of participants in the study population. If the study population had a higher statistical strength with more individuals, a homozygote for the minor allele might have been captured or the minor allele frequency could have been increased. Nevertheless, the minor allele T of the rs5219 SNP is very rare in the studied population, contributing to the T2D patient group not meeting Hardy–Weinberg equilibrium. An increase in numbers of the study population should be able to rectify this, and has been planned for future research.

Table 4.5 Hardy Weinberg Equilibrium model of selected SNPs

SNP	Major allele	Minor allele	MAF	Genotype Freq T2D Patients AA/AB/BB	Genotype Freq Controls AA/AB/BB	T2D Patients HWE (p-value)	Controls HWE (p-value)
rs7903146	**C**	**T**	**0.38**	**11/50/35**	**14/42/36**	**0.26**	**0.76**
rs12255372	**G**	**T**	**0.34**	**22/40/34**	**3/38/51**	**0.13**	**0.20**
rs7754840	**C**	**G**	**0.48**	**20/53/23**	**24/41/27**	**0.16**	**0.14**
rs5219	**C**	**T**	**0.01**	**0/0/96**	**0/1/91**	**No Value**	**0.91**
rs2943641	**C**	**T**	**0.27**	**3/40/53**	**10/36/46**	**0.16**	**0.46**
rs7560163	**C**	**G**	**0.27**	**10/38/48**	**6/33/53**	**0.54**	**0.75**

A-adenine; C-cytosine; G-guanine; T-thymine; MAF- Minor allele frequency; HWE- Hardy Weinberg Equilibrium; MAF- Minor allele frequency; Freq- frequency; A- minor allele; B-major allele; No Value could be calculated since the group is not within HWE.

4.6 Conclusion

Recent studies have suggested that the high prevalence of T2D in South Africans could be partially due to obesity and altered distribution of fat and muscle mass (Amod *et al.* 2017). The high percentage individuals in the control group with an HbA1c between 5.7 and 6.4%, that regard themselves as being healthy, is an indication that some black South Africans know little or nothing about the dangers of persistent high blood glucose levels and on how to prevent or control it. Alarmingly, only 30 of T2D patients could succeed in obtaining optimal glycaemic control. Health education about chronic diseases such as diabetes is very important to the general population. Even though diabetes education is a primary goal in health care systems, individuals in the larger populations are not reached. In order to aid in combating the increase of diabetes in South Africa more should be done to increase knowledge about diabetes to reduce costs of care and the burden of the disease on patients and healthcare systems.

The *TCF7L2* rs12255372 is the only SNP showing statistical significance to T2D, no association was found between T2D and the other polymorphisms identified from GWAS in the population group studied. The association of *TCF7L2* rs12255372 to T2D has also been identified in the Iranian population. Black South Africans might not share the common genetic variants associated with T2D as observed in other ethnic groups such as the European population. In this study The *TCF7L2* rs7903146 SNP did not prove to be significantly associated with T2D (p=0.92). However, in two other studies on individuals from African descent the *TCF7L2* rs7903146 SNP was significantly associated with T2D (Pirie *et al*. 2010 and Adeyemo *et al*. 2015). The study by Adeyemo *et al*. (2015) was performed on 1035 T2D patients and 740 control volunteers on persons residing in north to central Africa (Nigeria, Ghana and Kenya). In the study by Pirie *et al*. (2010) 178 T2D patients and 200 control individuals, from Zulu descent were genotyped. The difference in results from this study can be attributed to lack of statistical power in numbers from the current study. Another hypothesis, could be that the Zulu population are genetically closer related to central African populations than the Sotho, Tswana or Xhosa speaking individuals residing in central South Africa.

Some SNPs however, which are known to be mutant seem to be more common in this population as seen with the *RND3-RBM43* rs7560163 and *IRS1* rs2943641 and merits further investigation. This study further confirms the *TCF7L2* gene as having an enhancing susceptibility to the development of T2D. However, it is important to note that from a statistical point of view, this study was performed on a very small group of individuals. Therefore, further studies are required to confirm and clarify the exact mechanism of its effect.

All the SNPs adhered to the HWE model except the *KCNJ11* SNP in the T2D patient cohort, which could not be determined, since no mutant allele was observed. The HWE results is an indication that the genotyping results are correct and that migration, inbreeding or other factors are not at play in the study population. Results from this study put an emphasis on the need to investigate genetic variants associated with complex diseases such as T2D in the black South African population.

CHAPTER 5: CONCLUSION

T2D has become a leading public health challenge globally and a great burden to the economy and health systems (Haghvirdizadeh *et al.* 2014). Africa has the greatest number of diabetic people but the genetic basis of T2D in Africa is poorly understood. To date a total of 83 susceptibility loci for T2D have been identified by the GWAS to be associated with T2D in various ethnic groups reaffirming the polygenic and complex nature of T2D (Wang *et al.* 2015). Only a handful of studies have used the genome wide approach to identify genomic regions linked to or associated with T2D in the African population. T2D associated SNPs have therefore not been thoroughly studied for genetic prediction in African populations. In this study black participants living in the central South African region of Mangaung were included, speaking a mixture of African languages as well as English. The specific ethnicity of each participant was not documented, since it would be very difficult to accurately determine this. Even though, the ethnic groups differ (Sotho, Xhosa, Zulu and Tswana), to our knowledge they consist of Southern African black ancestry, which differs from the genetic ancestry of Caucasian populations. The aim of the study was to determine if gene polymorphisms associated with T2D are also present in the population of individuals with African ancestry, residing in central South Africa.

Diabetes is fast becoming a major burden on health systems and an inhibitor of financial growth to struggling economies in developing countries such as South Africa. Urbanisation and adoption of Western dietary patterns are the major environmental risk factors associated with the increase in prevalence of diabetes in South Africa (Amod *et al.* 2012). Because of the silent and progressive nature of diabetes and its complications, quality improvement strategies should be recommended for effective improvement of glycaemic control as well as periodic assessments of patients (Yin *et al.* 2014). Early identification of individuals at high T2D risk enables delay or prevention of T2D onset through effective lifestyle and/or pharmacological interventions and in turn reduce the costs of care (Ashraf *et al.* 2013).

Twin studies indicate a strong genetic aetiology in T2D with a high concordance rate of 70% in monozygotic twins and a concordance rate of 20% in dizygotic twins (Schafer *et al.* 2011). The aim of this study was to screen for the association of SNPs with T2D susceptibility in black South African Free State population. The identification of T2D risk associated alleles could aid in preventing the clinical onset of the diabetes by intervention of a modified life style.

The percentage of individuals that have a HbA1c between 5.7-6.4% in the control group was as high as 34.8%. This indicates that some black South Africans do not know much about diabetes, the dangers associated with T2D and how to prevent it or control it. It also means some black South Africans do not test for it. Distressingly, only 31.3% of T2D patients succeeded in obtaining optimal glycaemic control. Health education about diseases in general is an essential need in every population to aid in combating these types of diseases in this age. Even though diabetes education is a primary goal in health care systems, individuals in the larger populations are not reached. More should be done to increase the knowledge about diabetes in South Africa to reduce costs of care and the burden of the disease on patients and health systems

One of the biggest limitations of this study is the small sample size bringing about a lack of statistical power. This is of particular importance when dealing with gene associations. Therefore the results from this study should be viewed from this point of view. Results from this study indicate that only the *TCF7L2* rs12255372 SNP is associated with T2D in the studied black South African population. This association has also been found in a number of populations including the Japanese, the Iranian, Indian and African American population but not in any true African group. The *KCNJ11* rs5219 SNP homozygous rare or mutant allele, that has been associated with decreased insulin secretion in the Japanese and the Caucasian ethnic groups was absent in this cohort, only one heterozygote was detected in the control group.

Some SNPs known to be associated with T2D, particularly the *IRS1* and the RND3-RBM43 in the European and the African American respectively, had no association with T2D in this cohort as there was a high frequency of the mutant alleles in both the patient and control cohorts. Mutant and wildtype allele frequencies were exactly the same in both

the patient and control groups, however there was a slight increase in the heterozygotes in the patient cohort than the control group. This study further confirms the *TCF7L2* gene as having an enhancing susceptibility to the development of T2D. The relatively small sample size of the study has a weakening effect on statistical power and is a limitation of the study. Further studies are required to clarify the exact mechanism of its effect. Results from this study put an emphasis on the need to investigate genetic variants associated with complex diseases such as T2D in the black South African population

Genetic prediction models based only on GWAS data have not proven to be useful for all populations regarding T2D. Future investigations could use Next Generation Sequencing to fine map both intronic and exon regions to identify loss of function of markers with T2D predictive value to African ancestry. Furthermore, different genetic influences may be found specifically in people of African descent to reduce the future incidence of T2DM in Africa or even provide new insights into potential therapeutic targets. Innovative approaches are needed to limit the increase and relieve the current burden of the disease. Knowledge on the genetic basis of a disease will allow medical professionals to predict and diagnose diabetes with its complications before disease onset.

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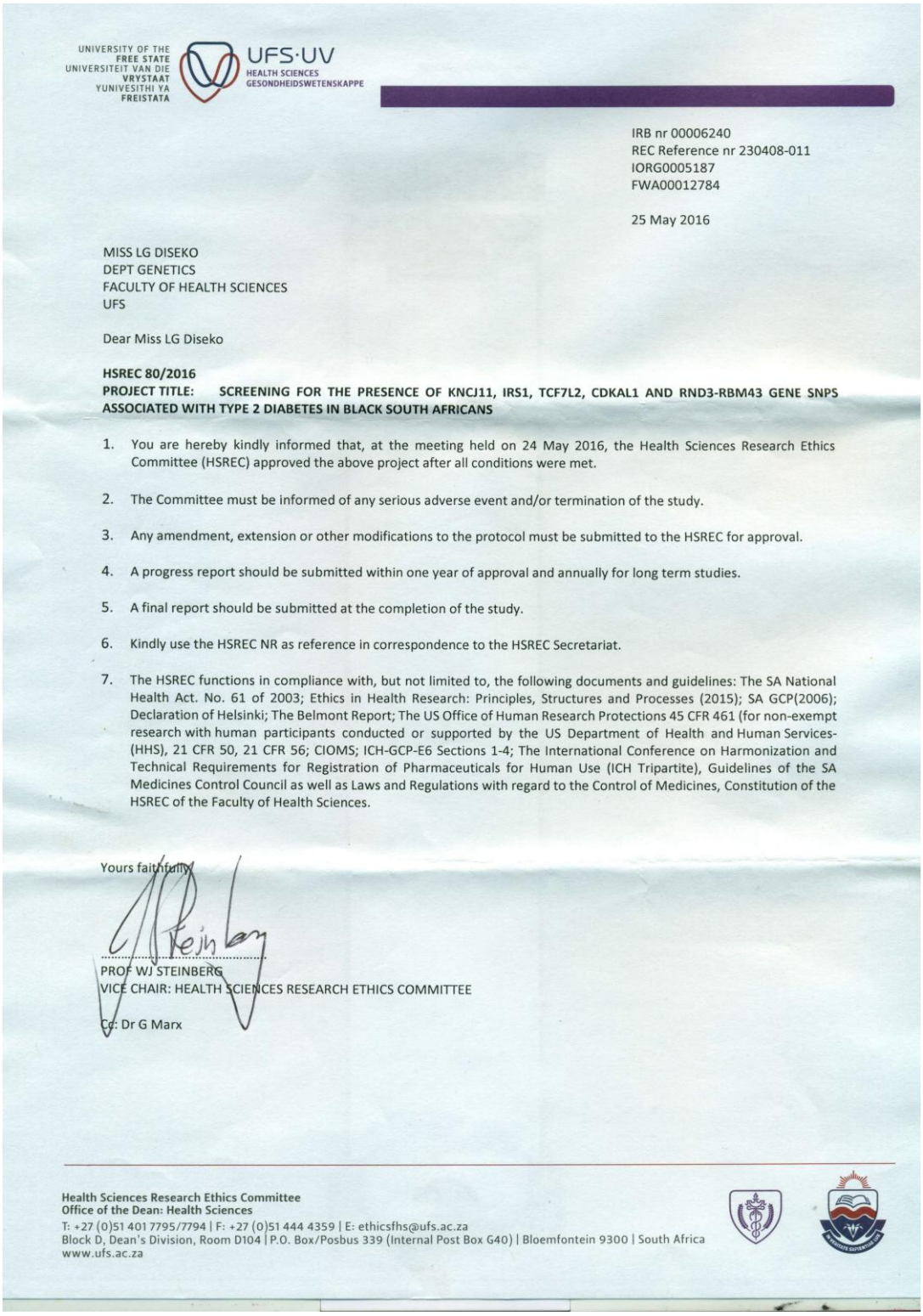
APPENDICES

APPENDIX A: Ethical approval letters for study HRSEC 80/2016 from the Health Sciences Research Ethics Committee of the Free State



UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
HEALTH SCIENCES
GESONDHEIDSWETENSKAPPE

IRB nr 00006240
REC Reference nr 230408-011
IORG0005187
FWA00012784

25 May 2016

MISS LG DISEKO
DEPT GENETICS
FACULTY OF HEALTH SCIENCES
UFS

Dear Miss LG Diseko

HSREC 80/2016
PROJECT TITLE: SCREENING FOR THE PRESENCE OF KNCJ11, IRS1, TCF7L2, CDKAL1 AND RND3-RBM43 GENE SNPS ASSOCIATED WITH TYPE 2 DIABETES IN BLACK SOUTH AFRICANS

1. You are hereby kindly informed that, at the meeting held on 24 May 2016, the Health Sciences Research Ethics Committee (HSREC) approved the above project after all conditions were met.
2. The Committee must be informed of any serious adverse event and/or termination of the study.
3. Any amendment, extension or other modifications to the protocol must be submitted to the HSREC for approval.
4. A progress report should be submitted within one year of approval and annually for long term studies.
5. A final report should be submitted at the completion of the study.
6. Kindly use the HSREC NR as reference in correspondence to the HSREC Secretariat.
7. The HSREC functions in compliance with, but not limited to, the following documents and guidelines: The SA National Health Act. No. 61 of 2003; Ethics in Health Research: Principles, Structures and Processes (2015); SA GCP(2006); Declaration of Helsinki; The Belmont Report; The US Office of Human Research Protections 45 CFR 461 (for non-exempt research with human participants conducted or supported by the US Department of Health and Human Services-(HHS), 21 CFR 50, 21 CFR 56; CIOMS; ICH-GCP-E6 Sections 1-4; The International Conference on Harmonization and Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH Tripartite), Guidelines of the SA Medicines Control Council as well as Laws and Regulations with regard to the Control of Medicines, Constitution of the HSREC of the Faculty of Health Sciences.

Yours faithfully

PROF WJ STEINBERG
VICE CHAIR: HEALTH SCIENCES RESEARCH ETHICS COMMITTEE

Cc: Dr G Marx

Health Sciences Research Ethics Committee
Office of the Dean: Health Sciences
T: +27 (0)51 401 7795/7794 | F: +27 (0)51 444 4359 | E: ethicsfhs@ufs.ac.za
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UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
HEALTH SCIENCES
GESONDHEIDSWETENSKAPPE

IRB nr 00006240
REC Reference nr 230408-011
IORG0005187
FWA00012784

01 March 2017

MISS LG DISEKO
DEPT OF GENETICS
FACULTY OF HEALTH SCIENCES
UFS

Dear Miss LG Diseko

~~HSREC 80/2016~~
PROJECT TITLE: SCREENING FOR THE PRESENCE OF SINGLE NUCLEOTIDE POLYMORPHISMS ASSOCIATED WITH TYPE2 DIABETES IN A BLACK SOUTH AFRICAN POPULATION

1. You are hereby kindly informed that the Health Sciences Research Ethics Committee (HSREC) approved the following at the meeting held on 28 February 2017:

   - *Change title from "Screening for the presence of KNCJ11, IRS1, TCF7L2, CDKAL1 and RND3-RBM43 gene SNPs associated with Type 2 Diabetes in black South Africans " to "Screening for the presence of single nucleotide polymorphisms associated with type2 diabetes in a black South African population"*
   - *Give travel remuneration of R50 to participants*

2. Kindly use the **HSREC NR** as reference in correspondence to HSREC Administration.

3. The HSREC functions in compliance with, but not limited to, the following documents and guidelines: The SA National Health Act. No. 61 of 2003; Ethics in Health Research: Principles, Structures and Processes (2015); SA GCP(2006); Declaration of Helsinki; The Belmont Report; The US Office of Human Research Protections 45 CFR 461 (for non-exempt research with human participants conducted or supported by the US Department of Health and Human Services- (HHS), 21 CFR 50, 21 CFR 56; CIOMS; ICH-GCP-E6 Sections 1-4; The International Conference on Harmonization and Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH Tripartite), Guidelines of the SA Medicines Control Council as well as Laws and Regulations with regard to the Control of Medicines, Constitution of the Ethics Committee of the Faculty of Health Sciences.

Yours faithfully

.....
DR SM LE GRANGE
CHAIR: HEALTH SCIENCES RESEARCH ETHICS COMMITTEE

Health Sciences Research Ethics Committee
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APPENDIX B

Table 7: Table showing all T2D patient samples collected. Included is the participant number, age, weight, height, BMI, HbA1c level and genotypes obtained from the Real Time PCR results.

Participant No	Age (Years)	Weight (kg)	Height (m)	BMI (kg/m^2)	HbA1c (%)	TCF7L2 RS79	TCF7L2 RS122	IRS1	CDKAL1	RND3	KCNJ11
P1	43	75.45	1.49	34	10	CC	GG	CT	CG	CG	CC
P2	53	72.85	1.7	25.2	9.6	TT	GT	CC	CG	CC	CC
P3	49	100.15	1.66	36.6	10.3	CC	GG	CT	CG	CG	CC
P4	41	69.55	1.64	25.86	8.2	CT	GT	CC	GG	GG	CC
P5	59	125.3	1.62	47.74	9.2	CC	GG	CT	CG	CC	CC
P6	59	74.85	1.52	32.61	8	CT	GG	CT	CC	CG	CC
P7	47	89	1.55	37.04	7.1	CC	GG	CT	CG	GG	CC
P8	51	82.9	1.49	37.3	8.9	CT	GT	CT	CG	CG	CC
P9	45	116.1	1.645	42.9	7.5	CT	GG	CT	CG	CC	CC
P10	53	117.4	1.568	47.8	7.7	CT	GG	CC	GG	CC	CC
P11	43	120.6	1.65	44.3	7.6	CC	GG	CC	GG	CC	CC
P12	55	86.6	1.595	34	6.7	CC	GG	TT	CC	CC	CC
P13	43	82	1.622	31.2	5.8	TT	GG	CT	CG	CC	CC
P14	57	104	1.52	45	7.2	CC	GG	CT	CG	CC	CC
P15	55	94.4	1.612	36.3	11.1	TT	GT	CC	CG	CC	CC
P16	40	53.1	1.529	22.7	6.5	CT	TT	CC	CG	CC	CC
P17	41	106.4	1.676	37.9	12.8	CT	GT	CT	CC	CG	CC
P18	52	114.95	156.7	46.8	8.5	CT	GG	CC	CG	CC	CC

Participant No	Age (Years)	Weight (kg)	Height (m)	BMI (kg/m^2)	HbA1c (%)	TCF7L2 RS79	TCF7L2 RS1222	IRS1	CDKAL1	RND3	KCNJ11
P19	49	84.3	1.59	33.3	8.1	CC	GT	CT	CG	CG	CC
P20	41	70.45	1.531	30.1	6.5	TT	GT	CC	CG	CG	CC
P21	48	105.6	1.55	44	8.4	CC	GT	TT	CG	CG	CC
P22	50	60.9	1.523	26.3	13.8	CT	TT	CC	CG	CG	CC
P23	46	52.3	1.559	21.5	12.3	TT	GG	CC	CG	CG	CC
P24	60	109.05	1.495	48.8	10.7	CT	TT	CT	CG	CG	CC
P25	54	109.9	1.539	46.4	6.2	CT	GG	CC	CG	CG	CC
P26	56	128.15	1.66	46.5	8.5	CC	GG	CC	CC	CG	CC
P27	41	73.5	1.52	31.8	5.5	CC	GG	CC	CC	CG	CC
P28	52	83	1.58	33.2	8.3	CT	GG	CC	CG	GG	CC
P29	60	101.8	1.57	41.3	6.8	CC	GG	CC	CG	GG	CC
P30	60	121.4	1.62	46.3	8.3	CT	GT	CC	CG	CG	CC
P31	56	63	1.46	29.6	7.1	CT	GG	CC	GG	GG	CC
P32	51	96.8	1.657	35.3	12.6	CT	GT	CC	CG	GG	CC
P33	46	87.6	1.605	34	6.5	CC	GT	CT	CG	CG	CC
P34	52	79.4	1.572	32.1	11.7	CT	GT	CC	CG	CG	CC
P35	47	100.4	1.56	41.3	10	CT	GT	CT	CC	GG	CC
P36	59	90.3	1.56	37.1	6.1	CT	GG	CT	CG	CG	CC
P37	54	80.65	1.532	34.4	10.6	CT	GG	CC	CC	CG	CC
P38	48	67	1.61	25.8	10.6	TT	GT	CC	CG	CG	CC
P39	59	93.25	1.52	40.4	8.3	CC	GT	CC	GG	GG	CC

Participant No	Age (Years)	Weight (kg)	Height (m)	BMI (kg/m^2)	HbA1c (%)	TCF7L2 RS79	TCF7L2 RS1222	IRS1	CDKAL1	RND3	KCNJ11
P40	53	78.3	1.545	32.8	6.4	TT	GT	CT	GG	CG	CC
P41	54	84.7	1.611	32.6	8.7	CC	TT	CC	GG	GG	CC
P42	57	122.95	1.586	48.9	6.5	CC	GG	CC	CG	CC	CC
P43	41	117.5	1.63	44.2	12.6	CT	GT	CC	CG	CC	CC
P44	61	97.45	1.58	39	8.5	CT	GG	CT	CG	CG	CC
P45	59	87	155.5	36	6.1	CT	GT	CT	CC	CG	CC
P46	60	82.45	1.572	33.4	7.7	CT	GT	CT	CC	GG	CC
P47	59	73.5	1.513	32.1	8	CT	GT	CT	CG	CG	CC
P48	56	84.8	1.72	28.7	5.6	CT	GT	CC	GG	CG	CC
P49	55	108.2	1.62	41.2	9.9	CT	TT	CT	CC	CC	CC
P50	57	125.5	1.566	51.2	7.6	CC	GT	CT	CG	CC	CC
P51	52	53.35	1.415	26.6	6	CC	TT	CC	CC	CC	CC
P52	48	82.3	1.581	32.9	8.4	CT	TT	CC	GG	CC	CC
P53	57	94.15	1.525	40.5	9.3	CT	GT	CC	CG	CC	CC
P54	60	121.2	1.567	49.4	8.6	CC	GT	CC	CG	CC	CC
P55	44	79.2	1.518	34.4	7.7	CT	GT	CC	CG	CG	CC
P56	51	101.4	1.511	44.4	6.8	TT	GT	CC	CG	CC	CC
P57	57	70.8	1.5	31.5	8.9	CT	TT	CC	CC	CG	CC
P58	38	90.6	1.588	35.9	11.7	CC	TT	CC	CC	CC	CC
P59	44	52.35	1.48	23.9	5.8	TT	TT	CC	GG	CC	CC
P60	60	121.2	1.567	49.4	8.6	CC	GT	CC	CG	CC	CC

Participant No	Age (Years)	Weight (kg)	Height (m)	BMI (kg/m^2)	HbA1c (%)	TCF7L2 RS79	TCF7L2 RS1222	IRS1	CDKAL1	RND3	KCNJ11
P61	55	74.65	1.635	27.9	12.5	CC	GT	CC	CG	CC	CC
P62	49	96.2	1.577	38.7	10.9	CC	TT	CC	CG	CG	CC
P63	34	72.7	1.604	28.3	5.3	CT	TT	CC	GG	CG	CC
P64	48	82.3	1.581	32.9	8.4	CT	TT	CC	GG	CC	CC
P65	45	83.8	1.63	31.5	10.9	CT	TT	CC	CG	CC	CC
P66	39	66.7	1.531	28.5	12.3	CT	GT	CC	CG	CC	CC
P67	56	67.7	1.45	32.2	5.7	CC	GT	CC	CC	CG	CC
P68	40	127.15	1.561	52.2	7.8	CT	GT	CC	CC	CC	CC
P69	57	81.55	1.53	34.8	13.2	CT	GT	CT	GG	CG	CC
P70	46	86.1	1.623	32.7	9.5	CT	GT	CT	CG	CG	CC
P71	57	58.5	1.515	25.5	7.8	CT	GT	CC	CG	CC	CC
P72	47	73.3	1.64	27.3	6.8	CC	GT	TT	GG	CG	CC
P73	53	101.9	1.6	39.8	9.6	CT	GT	CC	CG	CC	CC
P74	60	80.05	1.495	35.8	7.4	CC	GT	CT	CG	CC	CC
P75	35	97.25	1.59	38.5	10	CT	TT	CT	CG	CC	CC
P76	51	93.9	1.47	43.5	8.7	CT	TT	CC	CG	CC	CC
P77	49	105.95	1.596	41.6	5.3	CT	GT	CT	CC	CC	CC
P78	50	130.55	1.612	50.2	8.2	CC	GT	CC	CG	CC	CC
P79	54	121.55	1.69	42.6	6.7	CC	GG	CT	GG	CG	CC
P80	25	72.95	1.526	31.3	6.5	CT	TT	CT	CG	CC	CC
P81	53	64.2	1.535	27.2	6	CT	GG	CT	CC	CC	CC

Participant No	Age (Years)	Weight (kg)	Height (m)	BMI (kg/m^2)	HbA1c (%)	TCF7L2 RS79	TCF7L2 RS1222	IRS1	CDKAL1	RND3	KCNJ11
P82	49	60.55	1.52	26.2	15.8	CT	GG	CT	CG	CC	CC
P83	49	74.55	1.57	30.2	13.3	CC	TT	CT	GG	CC	CC
P84	51	79.9	1.54	33.7	5.9	CT	TT	CT	CC	CC	CC
P85	47	100.9	1.67	36.2	13.1	CT	GG	CC	CG	CC	CC
P86	50	84.35	1.57	34.2	9.8	TT	TT	CC	CC	CG	CC
P87	60	82	1.45	39	9.4	CT	TT	CT	CC	CC	CC
P88	41	60	1.53	25.6	11.3	CC	GT	CT	CG	CC	CC
P89	59	125.3	1.62	47.7	9.2	CT	GG	CC	CC	CG	CC
P90	46	69.8	1.56	28.7	12.6	TT	TT	CT	GG	CC	CC
P91	44	98	1.6	38.3	6	CC	GG	CT	GG	CC	CC
P92	59	89.15	1.625	33.8	8.4	CC	GG	CC	CC	CG	CC
P93	42	69.8	1.6	27.3	7.1	CC	GG	CT	GG	CC	CC
P94	42	123.6	1.59	48.9	7.9	CC	GG	CT	GG	CG	CC
P95	41	79.25	1.57	32.2	12.6	CT	GG	CC	CC	CC	CC
P96	57	59.95	1.55	25	7.3	CC	GG	CT	CG	CC	CC

Table 7: Table showing all control samples collected. Included is the participant number, age, weight, height, BMI, HbA1c level and genotypes obtained from the Real Time PCR results.

Participant No	Age (Years)	Weight (kg)	Height (m)	BMI (kg/m2)	HbA1c (%)	TCF7L2 RS79	TCF7L2 RS122	IRS1	CDKAL1	RND3	KCNJ11
C1	45	77.3	1.57	31.36	5.8	TT	GG	CT	GG	CC	CC
C2	40	53.7	1.6	20.98	5.6	CC	GT	CT	CC	CG	CC
C3	49	71	1.51	31.14	4.8	CC	GT	CT	CC	CC	CC
C4	43	107.1	1.53	45.75	5.4	CC	GT	CC	CG	CC	CC
C5	53	85.9	1.51	37.67	5.7	CC	GG	CC	CG	CC	CC
C6	49	113	1.63	42.53	5.4	CT	GT	CT	CC	GG	CC
C7	43	66.9	1.6	26.13	7.2	TT	GT	CT	GG	CG	CC
C8	61	77.1	1.55	32.09	5.8	CC	GG	CT	CG	CG	CC
C9	51	64	1.48	29.22	5.4	CT	GG	CC	CG	CG	CC
C10	57	101.3	1.64	37.65	6.3	CC	GG	CT	CC	CC	CC
C11	46	96.6	1.56	39.7	5.5	CT	GG	CC	CC	CG	CC
C12	49	69.5	1.54	29.3	4.3	TT	GG	CC	GG	CC	CC
C13	57	76.4	1.53	32.6	5.9	CC	GG	CC	GG	CG	CC
C14	59	109.6	1.64	40.7	5.6	CT	GT	CC	CG	CC	CC
C15	58	75.2	1.69	26.3	5.5	CT	GT	CT	CG	CC	CC
C16	54	95.8	1.63	36.1	4.9	CC	GG	CC	CG	CC	CC
C17	54	64.1	1.62	24.4	4.5	CT	GG	CT	CC	CC	CC

Participant No	Age (Years)	Weight (kg)	Height (m)	BMI (kg/m^2)	HbA1c (%)	TCF7L2 RS79	TCF7L2 RS122	IRS1	CDKAL1	RND3	KCNJ11
C18	59	99.5	1.51	43.6	5.5	TT	GG	CC	GG	CC	CC
C19	42	91.1	1.54	38.41	5.9	CT	GG	CC	CG	GG	CC
C20	52	81.2	1.68	28.77	5.6	CT	GT	TT	GG	CG	CC
C21	59	52	1.46	24.39	5.6	CC	GT	CC	CG	GG	CC
C22	52	67.8	1.46	31.81	5.7	CC	GT	CC	CC	CG	CC
C23	43	83.4	1.6	32.58	5.3	CC	GG	CC	GG	CC	CC
C24	52	86.9	1.53	37.12	5.2	CT	GG	CT	CG	CC	CC
C25	41	105	1.49	47.3	5.8	CT	TT	CT	GG	CG	CC
C26	52	100	1.56	41.09	6.1	CC	GT	TT	CG	CC	CC
C27	54	89	1.65	32.69	5.8	CC	GG	CC	GG	CG	CC
C28	46	81.7	1.49	36.8	5.8	TT	GG	CT	CC	CC	CC
C29	58	79	1.58	31.65	5.5	CC	GG	CC	GG	CG	CC
C30	53	79.7	1.57	32.33	5.9	CC	GT	CT	CG	CC	CC
C31	58	82.9	1.68	29.37	6.1	CT	GT	CT	GG	CG	CC
C32	62	82.2	1.64	30.56	5.6	CT	TT	CT	CG	CC	CC
C33	57	83.9	1.49	37.79	5.8	CC	GG	CT	CG	CC	CC
C34	60	99.3	1.545	41	5.6	CT	GG	TT	CC	CC	CC
C35	56	110	1.564	44	5.3	CT	GG	CT	GG	CG	CC
C36	37	91	1.672	32	5.4	CT	GT	TT	CC	CC	CC
C37	37	89.4	1.54	37	5.3	CT	GG	TT	CC	CG	CC
C38	46	105	1.588	42	5.2	CT	GG	CT	CG	CG	CC

Participant No	Age (Years)	Weight (kg)	Height (m)	BMI (kg/m^2)	HbA1c (%)	TCF7L2 RS79	TCF7L2 RS122	IRS1	CDKAL1	RND3	KCNJ11
C39	48	89.9	1.605	35	5.5	CC	GG	CT	GG	CG	CC
C40	58	71.9	1.59	28	6.1	CT	TT	CT	GG	GG	CC
C41	40	116.9	1.585	41	5.6	CC	GT	CC	GG	CC	CC
C42	37	101	1.503	45	4.9	CT	GG	CC	CG	GG	CC
C43	40	85.2	1.647	32	5.1	CC	GG	TT	GG	CC	CC
C44	53	62.8	1.5	28	5.2	CT	GG	CC	GG	CC	CC
C45	52	58.3	1.468	26	5.6	CT	GG	CC	CG	CC	CC
C46	62	59.2	1.452	28	5.4	CT	GT	CT	CC	CC	CC
C47	55	62.5	1.57	25	5.8	TT	GG	CT	CG	CC	CC
C48	62	70.8	1.622	28	4.9	CT	GG	TT	CG	CG	CC
C49	47	85.2	1.553	36	5.6	CC	GT	CC	CG	CG	CC
C50	52	83	1.54	34	5.5	CC	GT	CC	CG	CG	CC
C51	44	105.9	1.744	33	6.4	CT	GT	TT	CG	GG	CC
C52	42	87.4	1.59	34.57	5.4	CT	GT	TT	CC	CC	CC
C53	61	65	1.515	29	5.6	CC	GG	CT	CG	CC	CC
C54	55	71.8	1.548	29	5	CC	GG	CT	CC	CC	CC
C55	64	83.8	1.84	34	5.9	TT	GG	CC	CG	CC	CC
C56	51	78.65	1.57	31	5.4	CT	GG	CC	CC	CG	CC
C57	48	63.1	1.64	23.46	5.6	TT	GT	CC	CG	CC	CC
C58	46	75.8	1.66	27.5	4.8	TT	GT	CC	CG	CC	CC
C59	53	93.65	1.52	40.53	5.4	CC	GT	CT	CG	CG	CC

Participant No	Age (Years)	Weight (kg)	Height (m)	BMI (kg/m^2)	HbA1c (%)	TCF7L2 RS79	TCF7L2 RS122	IRS1	CDKAL1	RND3	KCNJ11
C60	59	102.3	1.69	35.82	5	CC	GT	CC	CC	CG	CC
C61	40	79	1.63	29.73	5.9	CC	GT	CC	CC	CC	CC
C62	39	67.4	1.48	30.8	5.2	CC	GT	CC	CG	CC	CC
C63	57	111.9	1.59	44.26	5.4	CT	GG	CT	CC	CC	CC
C64	53	95.3	1.66	34.58	5.3	CT	GG	CT	CG	CG	CC
C65	46	96	1.52	41.55	5.8	CC	GG	CC	CC	CC	CC
C66	41	59.7	1.49	26.9	5	CT	GT	CT	GG	CG	CC
C67	46	75.8	1.66	27.5	4.8	TT	GT	CC	CG	CC	CC
C68	41	114	1.62	43.44	5.8	CC	GG	CT	GG	CG	CC
C69	41	81.3	1.61	31.36	5	TT	GT	CC	GG	CG	CC
C70	40	65.8	1.57	26.69	5.4	CC	GT	CC	CC	CC	CC
C71	48	85.5	1.57	34.69	4.9	CT	GG	CT	CG	CG	CC
C72	50	78.1	1.58	31.29	5.7	CC	GG	CC	GG	CG	CC
C73	45	87.7	1.57	35.58	6.1	CT	GG	CC	GG	CC	CC
C74	45	74.3	1.58	29.76	6.1	CT	GT	CC	CG	CG	CC
C75	27	94.7	1.59	37.46	5.2	CT	GT	CC	CG	CC	CC
C76	49	61.8	1.36	33.41	5	TT	GT	CT	CG	CC	CC
C77	53	70.3	1.59	27.8	6	CT	GG	CC	CG	CC	CC
C78	38	97	1.68	34.3	5.2	CT	GT	CC	CC	CC	CC
C79	53	86.5	1.6	33.8	5.3	CC	GG	CC	CC	CC	CC
C80	41	68.7	1.58	27.5	6.3	CT	GG	CC	GG	CG	CC

Participant No	Age (Years)	Weight (kg)	Height (m)	BMI (kg/m^2)	HbA1c (%)	TCF7L2 RS79	TCF7L2 RS122	IRS1	CDKAL1	RND3	KCNJ11
C81	44	64.5	1.52	27	5.4	CT	GG	CT	GG	CG	CC
C82	38	55.3	1.57	22.4	5.4	CC	GG	CT	CC	CG	CC
C83	36	106	1.6	41.4	5.8	CC	GT	CT	CC	CC	CC
C84	49	72	1.51	31.6	5.8	CT	GT	CC	CG	CC	CC
C85	36	82.7	1.54	34.9	5.4	CC	GG	CC	CG	CC	CC
C86	40	65	1.52	28.1	4.8	CT	GT	CT	CG	CC	CC
C87	49	120.1	1.64	44.7	5.6	CT	GG	CC	CC	CC	CC
C88	52	81	1.76	26.5	5.2	CT	GT	CT	CG	CC	CC
C89	41	81	1.57	32.9	5.9	CT	GG	CC	CC	CC	CC
C90	38	77.1	1.6	30.1	5.9	TT	GG	CC	CC	CG	CC
C91	41	103	1.63	38.8	5.1	CC	GG	TT	CG	CC	ND
C92	44	103.9	1.57	42.2	5.7	TT	GG	CC	CG	CC	CC

ND=not determined

APPENDIX C: Gene fragment manufacturing sheet of qPCR mutant controls-gBlocks®

Gene: KCNJ11 rs5219

INTEGRATED DNA TECHNOLOGIES

SPECIFICATION SHEET www.idtdna.com

12-Dec-2016

Order No. 2638053
Ref. No. 72579193

Name - KCNJ11 rs5219 Mut PC

gBlocks® Gene Fragments 420 base pairs

```
5' - CGT GGG CGA AGG CGA TGA GCC ACC AGG CCA TGG CGA AGA GCA GCC AGC TGC ACA GGA AGG ACA TGG
TGA AGA TGA GCA ATG TGT GTG GCC ACT TGA GGT CCA CCA GCG TGG TGA ACA CGT CCT GCA GGA AGC GGC
CCT GCT CCC GGA TGT TCT TGT GGG CCA CGT TGC AGT TGC CTT TCT TGG ACA CAA AGC GGG CCC TCC GCT
GGC GGG CAC GGT ACC TGG GCT CGG CAG GGT CCT CTG CCA GGC GTG TCA GCA CGT ATT CCT CGG GGA TGA
TGC CCT TGC GGG ACA GCA TGG CTC CGG TGA CCC CCA GGG AGG GGC TTC CCC CAT CGG AGG CAC CCC TCG
GAC GTG GCC TAG GGC CTC ACT GCA GAG TCC TCT CGG TGG GCA CCT TCT . . . - 3'
```

Note 1: The sequence information of the sense strand displayed above is intentionally truncated to the first 400 bases. The complete sequence can be verified in FASTA format in your order history.

Note 2: gBlocks® Gene Fragments are delivered as double-stranded DNA. Conformance to quality standards is established in multiple ways, including size verification by capillary electrophoresis, sequence identification by mass spectrometry and consensus sequence verification.

Properties

Length:	420
Amount Delivered:	500ng
GC Content:	65.24%
Molecular Weight:	259456.6
fmoles/ng:	3.85
µg/OD_{260}:	50

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Instructions

Dried contents may appear as either a translucent film or a white powder. This variance does not affect the quality of the gBlocks Gene Fragment.

Resuspending your gBlocks Gene Fragment

1. Centrifuge the tube for 3-5 sec at a minimum of 3000 x g to ensure the material is in the bottom of the tube.
2. Add TE to reach a final concentration of 10ng\µL.
3. Vortex briefly.
4. Incubate at 50°C for 20 minutes.
5. Briefly vortex and centrifuge.

Amplifying your gBlocks Gene Fragment

- For gBlocks Gene Fragments ≤1kb, amplification can be performed using a high fidelity polymerase. To avoid sequence mutations due to amplification errors, limit cycles to 12 or fewer.
- For gBlocks Gene Fragments >1kb, we do not recommend amplification.

For additional information please see www.idtdna.com/gblockssupport or contact genes@idtdna.com.

Gene: RND3-RBM43 rs7560163

INTEGRATED DNA TECHNOLOGIES

SPECIFICATION SHEET www.idtdna.com

12-Dec-2016

Order No. 2638053
Ref. No. 72579196

Name - RND3-RBM43 rs7560163 Mut PC gBlocks® Gene Fragments 420 base pairs

```
5' - GTA CTA CTC TGA CAG CTA TCC AAT GGG ATT ACA TCA ACA ATC CTG AGC CGG AAG CAA GAA AAC CAG
AAC GTC GGT AAA TTT CAC AGC AAT TAA AGA TGA AAG CCA CTA CCT CAG CCA AAC CAC AAA AAT ACA TAC
TCC CCT CTT CTC CTT ACC CTG TAA AAC TTC AAC CCT GCT TCA ACT CAC AGT TGC CTG GTC TTC AGG TTT
TCC CTG GTG TGT ATC AGC TGA ATG AAG TCT TTA AGT TCT ATT TGA GTT TGT TTG TTT GTT TGT TGA GAT
GGA GTC TCG CTC TGT CGC CCA GGC CGG AGT GCA GTG GCG CGA TCT CTG CTC ACT GCA ACC TCT ACC TCC
CAG GTT CAA GCA ATT CTC CTG CAT CAG CCT CCT GAG TAG CTG GGA TTA . . . - 3'
```

Note 1: The sequence information of the sense strand displayed above is intentionally truncated to the first 400 bases. The complete sequence can be verified in FASTA format in your order history.

Note 2: gBlocks® Gene Fragments are delivered as double-stranded DNA. Conformance to quality standards is established in multiple ways, including size verification by capillary electrophoresis, sequence identification by mass spectrometry and consensus sequence verification.

Properties

Length:	420
Amount Delivered:	500ng
GC Content:	47.62%
Molecular Weight:	259383.6
fmoles/ng:	3.86
µg/OD_{260}:	50

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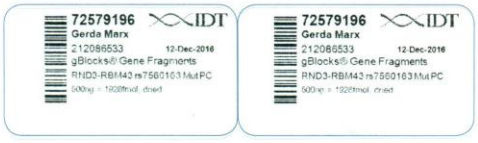

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Instructions

Dried contents may appear as either a translucent film or a white powder. This variance does not affect the quality of the gBlocks Gene Fragment.

Resuspending your gBlocks Gene Fragment

1. Centrifuge the tube for 3-5 sec at a minimum of 3000 x g to ensure the material is in the bottom of the tube.
2. Add TE to reach a final concentration of 10ng/µL.
3. Vortex briefly.
4. Incubate at 50°C for 20 minutes.
5. Briefly vortex and centrifuge.

Amplifying your gBlocks Gene Fragment

- For gBlocks Gene Fragments ≤1kb, amplification can be performed using a high fidelity polymerase. To avoid sequence mutations due to amplification errors, limit cycles to 12 or fewer.
- For gBlocks Gene Fragments >1kb, we do not recommend amplification.

For additional information please see www.idtdna.com/gblockssupport or contact genes@idtdna.com.

Gene: TCF7L2 rs12255372

INTEGRATED DNA TECHNOLOGIES

SPECIFICATION SHEET www.idtdna.com

12-Dec-2016

Order No. **2638053**
Ref. No. **72579197**

Name - TCF7L2 rs12255372 Mut PC gBlocks® Gene Fragments 420 base pairs

```
5' - GAT TTT CAT CTT TTT AAA TTA ATT ATC ATA GAA GGA GAA AAC AAC TGG ATT TCA GAA TTG TCC CTT
GAG GTG TAC TGG AAA CTA AGG CGT GAG GGA CTC ATA GGG GTC TGG CTT GGA AAG TGT ATT GCT ATG TCC
AGT TTA CAC ATA AGG ATG TGC AAA TCC AGC AGG TTA GCT GAG CTG CCC AGG AAT ATC CAG GCA AGA ATT
ACC ATA TTC TGA TAA TTA CTC AGG CCT CTG CCT CAT CTC CGC TGC CCC CCC GCC CCC TGA CTC TCT TCT
GAG TGC CAG ATT CAG CCT CCA TTT GAA TGC CAA ATA GAC AGG AAA TTA GCA TGC CCA GAA TCC ACG TCT
TTA GTG CAC TCT CTC CCC AGC TCC AAA CCT GTT ACT GCT TGT GTT CAA  . . .  - 3'
```

Note 1: The sequence information of the sense strand displayed above is intentionally truncated to the first 400 bases. The complete sequence can be verified in FASTA format in your order history.

Note 2: gBlocks® Gene Fragments are delivered as double-stranded DNA. Conformance to quality standards is established in multiple ways, including size verification by capillary electrophoresis, sequence identification by mass spectrometry and consensus sequence verification.

Properties

Length:	420
Amount Delivered:	500ng
GC Content:	45.71%
Molecular Weight:	259375.7
fmoles/ng:	3.86
µg/OD_{260}:	50

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Instructions

Dried contents may appear as either a translucent film or a white powder. This variance does not affect the quality of the gBlocks Gene Fragment.

Resuspending your gBlocks Gene Fragment

1. Centrifuge the tube for 3-5 sec at a minimum of 3000 x g to ensure the material is in the bottom of the tube.
2. Add TE to reach a final concentration of 10ng\µL.
3. Vortex briefly.
4. Incubate at 50°C for 20 minutes.
5. Briefly vortex and centrifuge.

Amplifying your gBlocks Gene Fragment

- For gBlocks Gene Fragments ≤1kb, amplification can be performed using a high fidelity polymerase. To avoid sequence mutations due to amplification errors, limit cycles to 12 or fewer.
- For gBlocks Gene Fragments >1kb, we do not recommend amplification.

For additional information please see www.idtdna.com/gblockssupport or contact genes@idtdna.com.

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Gene: TCF7L2 rs7903146

INTEGRATED DNA TECHNOLOGIES

SPECIFICATION SHEET www.idtdna.com

12-Dec-2016

Order No. 2638053
Ref. No. 72579194

Name - TCF7L2 rs7903146 Mut PC gBlocks® Gene Fragments 420 base pairs

```
5' - TTG GAG GGT TGC ACA TGT GAA AGA AAA AGG GAG AAA GCA GGA TTG AGC AGG GGG AGC CGT CAG ATG
GTA ATG CAG ATG TGA TGA GAT CTC TGC CGG ACC AAA GAG AAG ATT CCT TTT TAA ATG GTG ACA AAT TCA
TGG GCT TTC TCT GCC TCA AAA CCT AGC ACA GCT GTT ATT TAC TGA ACA ATT AGA GAG CTA AGC ACT TTT
TAG ATA TTA TAT AAT TTA ATT GCC GTA TGA GGC ACC CTT AGT TTT CAG ACG AGA AAC CAC AGT TAC AGG
GAA GGC AAG TAA CTT AGT CAA TGT CAG ATA ACT AGG AAA AGG TTA GAG GGG CCC TGG ACA CAG GCC TGT
GTG ACT GAG AAG CTT GGG CAC TTC ACT GCT ACA TTT CAT CTC TTC GCT  . . .  - 3'
```

Note 1: The sequence information of the sense strand displayed above is intentionally truncated to the first 400 bases. The complete sequence can be verified in FASTA format in your order history.

Note 2: gBlocks® Gene Fragments are delivered as double-stranded DNA. Conformance to quality standards is established in multiple ways, including size verification by capillary electrophoresis, sequence identification by mass spectrometry and consensus sequence verification.

Properties

Length:	420
Amount Delivered:	500ng
GC Content:	42.38%
Molecular Weight:	259361.9
fmoles/ng:	3.86
µg/OD_{260}:	50

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Instructions

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Resuspending your gBlocks Gene Fragment

1. Centrifuge the tube for 3-5 sec at a minimum of 3000 x g to ensure the material is in the bottom of the tube.
2. Add TE to reach a final concentration of 10ng/µL.
3. Vortex briefly.
4. Incubate at 50°C for 20 minutes.
5. Briefly vortex and centrifuge.

Amplifying your gBlocks Gene Fragment

- For gBlocks Gene Fragments ≤1kb, amplification can be performed using a high fidelity polymerase. To avoid sequence mutations due to amplification errors, limit cycles to 12 or fewer.
- For gBlocks Gene Fragments >1kb, we do not recommend amplification.

For additional information please see www.idtdna.com/gblockssupport or contact genes@idtdna.com.

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Gene: IRS1 rs2943641

INTEGRATED DNA TECHNOLOGIES

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12-Dec-2016

Order No. **2638053**
Ref. No. **72579195**

Name - IRS1 rs2943641 Mut PC gBlocks® Gene Fragments 420 base pairs

```
5' - TGG TCT TCC AAT GTT TCC TAT CAC TCT GAA GCA GTA GTA TGA TAG AAT AGT GCA ATA GCT TTT AGA
AAA ATC AGT TAT GGC ACC AAT TGG GTA AGA ACA CCT TAT GAG GCT GAG ATA ATG TCC TCC AGG ATG CAG
TAT ATG CTT TAA CTC AGC AGC TAT ACA TAG TGC TGT TTC CCC ATA TCC AAA ATT CAT GGG TCC AAG GAT
CAA CAT AGT TGG AAA TGA GAG GAA CCC TTC TAA CTA TTA GCC CTG ATA TTC CCC TAG TTA AAA TGT TGT
TTG TTA ACA AAA CTT TGA ATT CTG GTG GTC TCT AGA CCA GAT AAC ACT GTT CCA TAT TTG TGT AAT TCA
ACA TGG TAG CCA CTA GCT ACA TGT GGC TAT TGA ACT CTT GAA AGA TGG  . . .  - 3'
```

Note 1: The sequence information of the sense strand displayed above is intentionally truncated to the first 400 bases. The complete sequence can be verified in FASTA format in your order history.

Note 2: gBlocks® Gene Fragments are delivered as double-stranded DNA. Conformance to quality standards is established in multiple ways, including size verification by capillary electrophoresis, sequence identification by mass spectrometry and consensus sequence verification.

Properties

Length:	420
Amount Delivered:	500ng
GC Content:	37.38%
Molecular Weight:	259341.1
fmoles/ng:	3.86
µg/OD_{260}:	50

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Instructions

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Resuspending your gBlocks Gene Fragment

1. Centrifuge the tube for 3-5 sec at a minimum of 3000 x g to ensure the material is in the bottom of the tube.
2. Add TE to reach a final concentration of 10ng\µL.
3. Vortex briefly.
4. Incubate at 50°C for 20 minutes.
5. Briefly vortex and centrifuge.

Amplifying your gBlocks Gene Fragment

- For gBlocks Gene Fragments ≤1kb, amplification can be performed using a high fidelity polymerase. To avoid sequence mutations due to amplification errors, limit cycles to 12 or fewer.
- For gBlocks Gene Fragments >1kb, we do not recommend amplification.

For additional information please see www.idtdna.com/gblockssupport or contact genes@idtdna.com.

Gene: CDKAL1 rs7754840

INTEGRATED DNA TECHNOLOGIES

SPECIFICATION SHEET www.idtdna.com

12-Dec-2016

Order No. **2638053**
Ref. No. **72579192**

Name - CDKAL1 rs7754840 Mut PC gBlocks® Gene Fragments 300 base pairs

```
5' - GCA TTA ATA TCA GAA CAC TTG TCT TAC ATT TGA GTT TCA AAT TGT CCA GAT TTG AGA GTG AGC AAA
ATA CAC CAG ATA TAC CAC CAA AAT TGA AAA AAA AAT CAA CTG CTT GCT GTT GGG GAA GAA GTA GTA ATG
TTG GAA ACG TTG ACT TGA TAG AGG ATT TTG TAA GAT GAG TGA AAA AGA TCT AAA AGG ACA GTG ATG TCT
CTG TTA TTG ACT GAG GTA TCC TTG GTC TCT AGA ATA GTG CCT GAT GAA CAG CAT TGA GTT ACT TAT TCA
CTG AAT TGA ATT GAA AGA TTT GAT GAA - 3'
```

Note 1: The sequence information of the sense strand displayed above is intentionally truncated to the first 400 bases. The complete sequence can be verified in FASTA format in your order history.

Note 2: gBlocks® Gene Fragments are delivered as double-stranded DNA. Conformance to quality standards is established in multiple ways, including size verification by capillary electrophoresis, sequence identification by mass spectrometry and consensus sequence verification.

Properties

Length:	300
Amount Delivered:	500ng
GC Content:	34.67%
Molecular Weight:	185200.2
fmoles/ng:	5.40
$\mu g/OD_{260}$:	50

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Instructions

Dried contents may appear as either a translucent film or a white powder. This variance does not affect the quality of the gBlocks Gene Fragment.

Resuspending your gBlocks Gene Fragment

1. Centrifuge the tube for 3-5 sec at a minimum of 3000 x g to ensure the material is in the bottom of the tube.
2. Add TE to reach a final concentration of 10ng/µL.
3. Vortex briefly.
4. Incubate at 50°C for 20 minutes.
5. Briefly vortex and centrifuge.

Amplifying your gBlocks Gene Fragment

- For gBlocks Gene Fragments ≤1kb, amplification can be performed using a high fidelity polymerase. To avoid sequence mutations due to amplification errors, limit cycles to 12 or fewer.
- For gBlocks Gene Fragments >1kb, we do not recommend amplification.

For additional information please see www.idtdna.com/gblockssupport or contact genes@idtdna.com.

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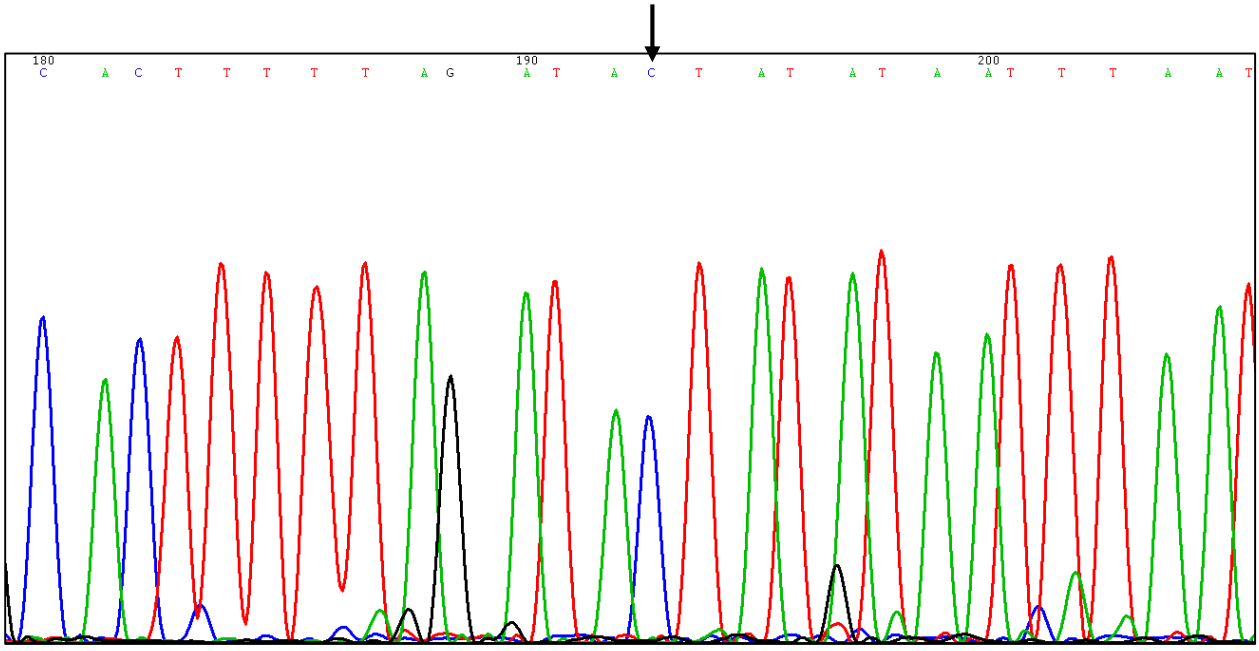
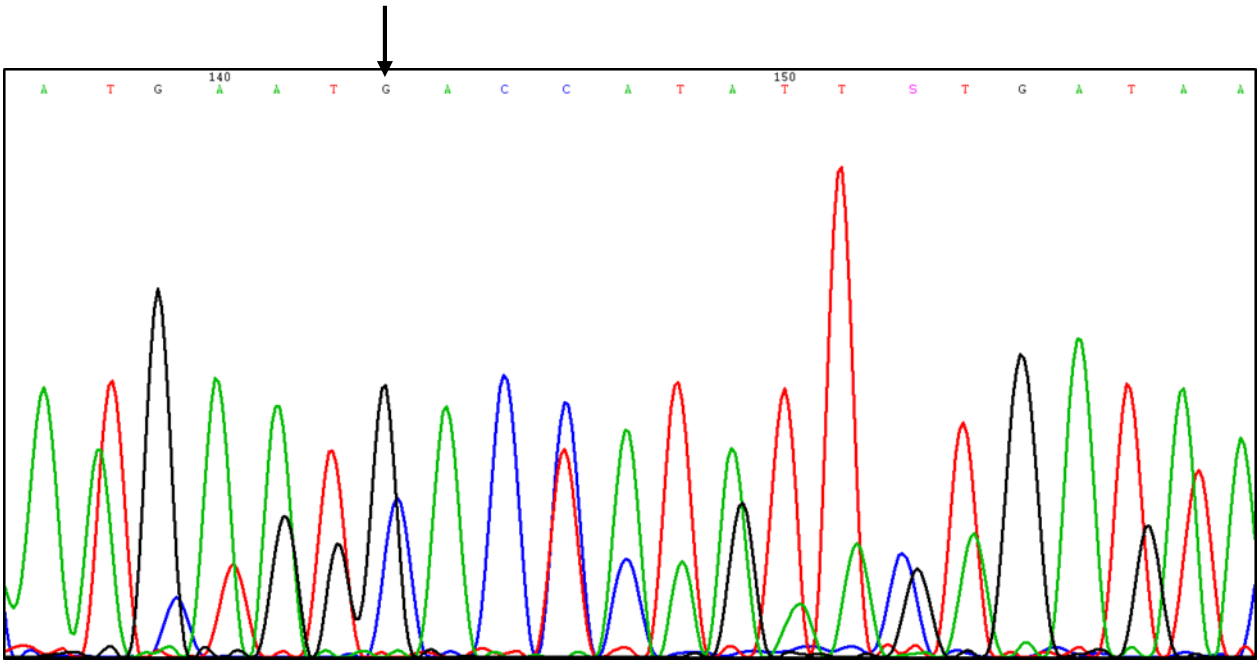
APPENDIX D: Sequence electropherograms

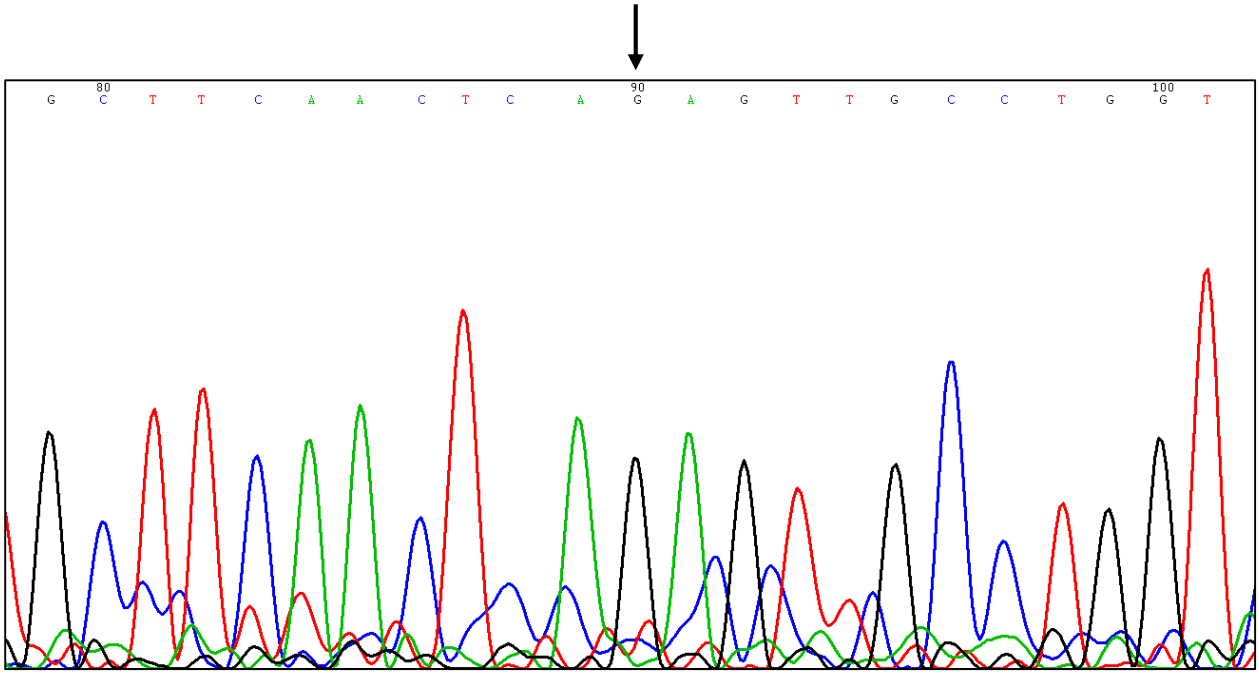
APPENDIX D1: Sequencing electropherograms confirming homozygous wild type genotypes to serve as homozygous wild type qPCR run controls.



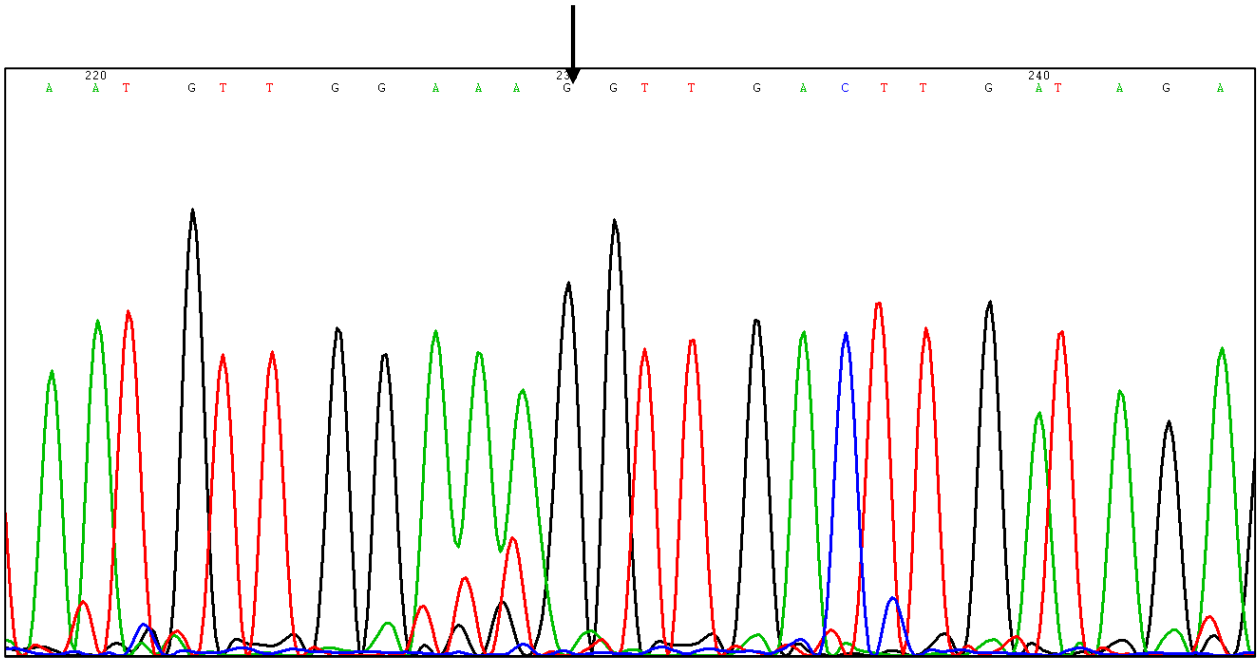
Sequencing electropherogram result of individual P12 with a homozygote (wildtype) allele (CC) on position 193 in the *TCF7L2* (rs7903146) gene.



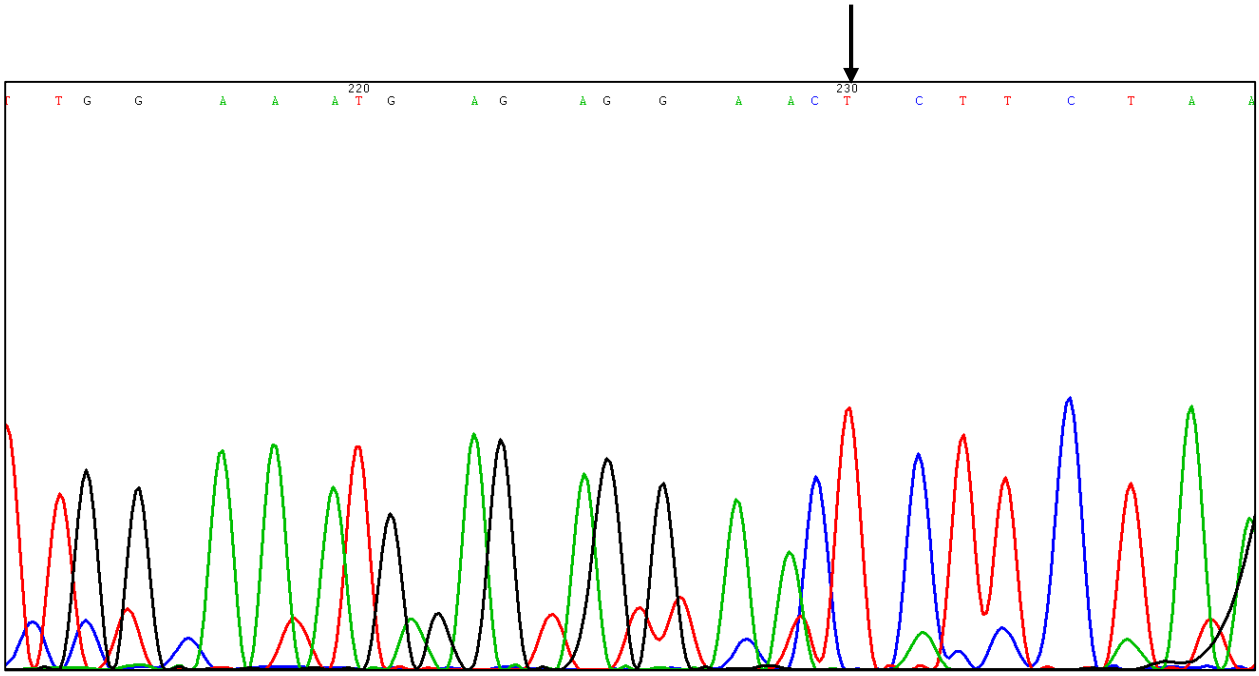
Sequencing electropherogram result of individual P12 with a homozygote (wildtype) allele (GG) on position 144 in the TCF7L2 (rs122553372) gene.



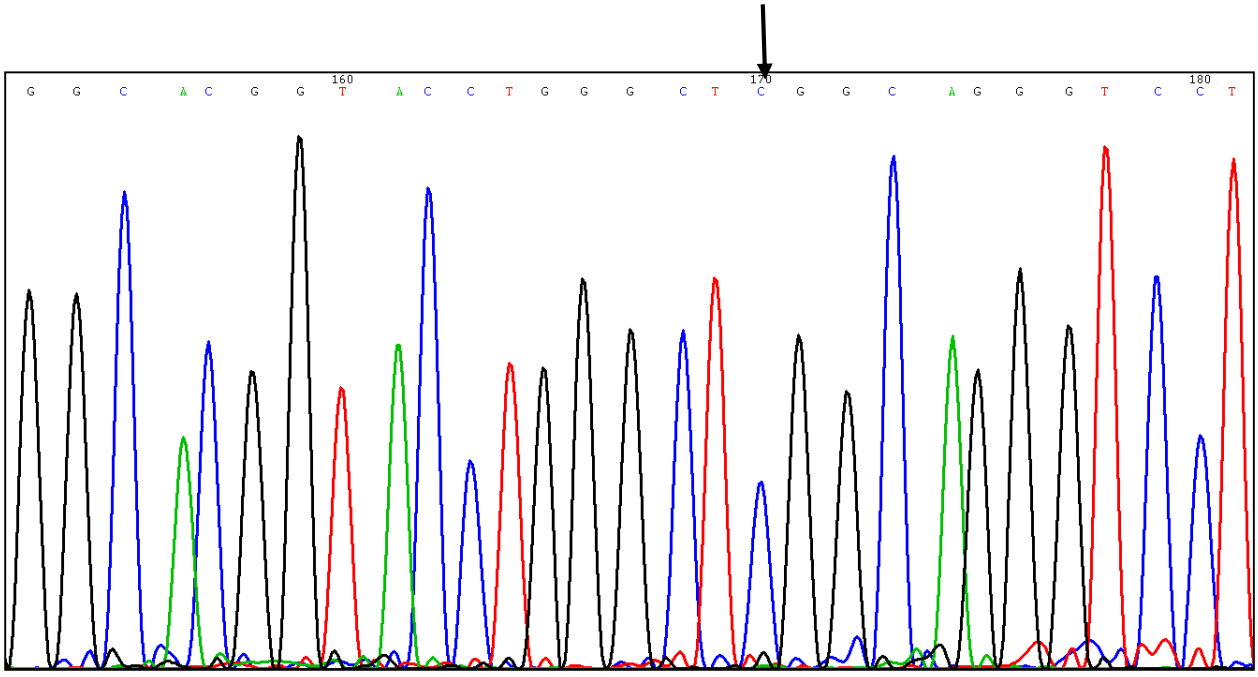
Sequencing electropherogram result of individual P65 with a homozygote (wildtype) allele (GG) on position 90 in the *RND3-RBM43* (rs7560163) gene



Sequencing electropherogram of an individual (Control C7) with a wildtype allele (GG) on position 230 in the *CDKAL1* gene

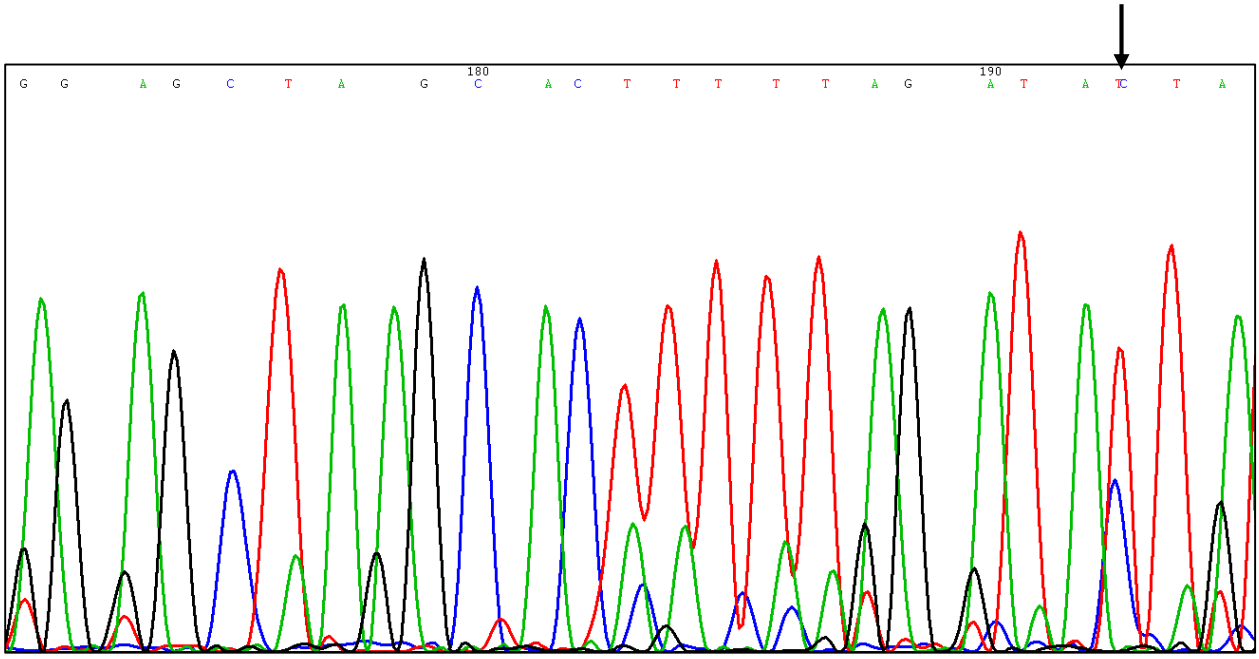


Sequencing electropherogram of individual P12 with a homozygyous wildtype (TT) on position 230 in the *IRS1* gene

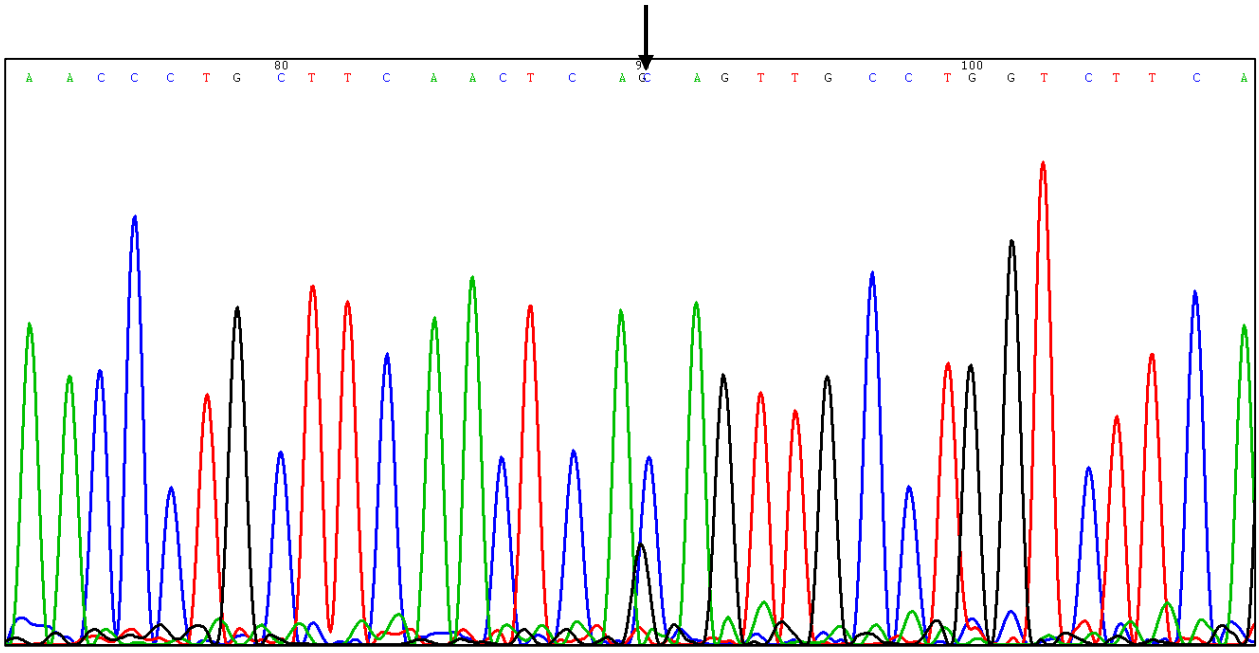


Sequencing electropherogram result of individual C2 with a homozygote (wildtype) allele (CC) on position 170 in the *KCNJ11* (rs5219) gene

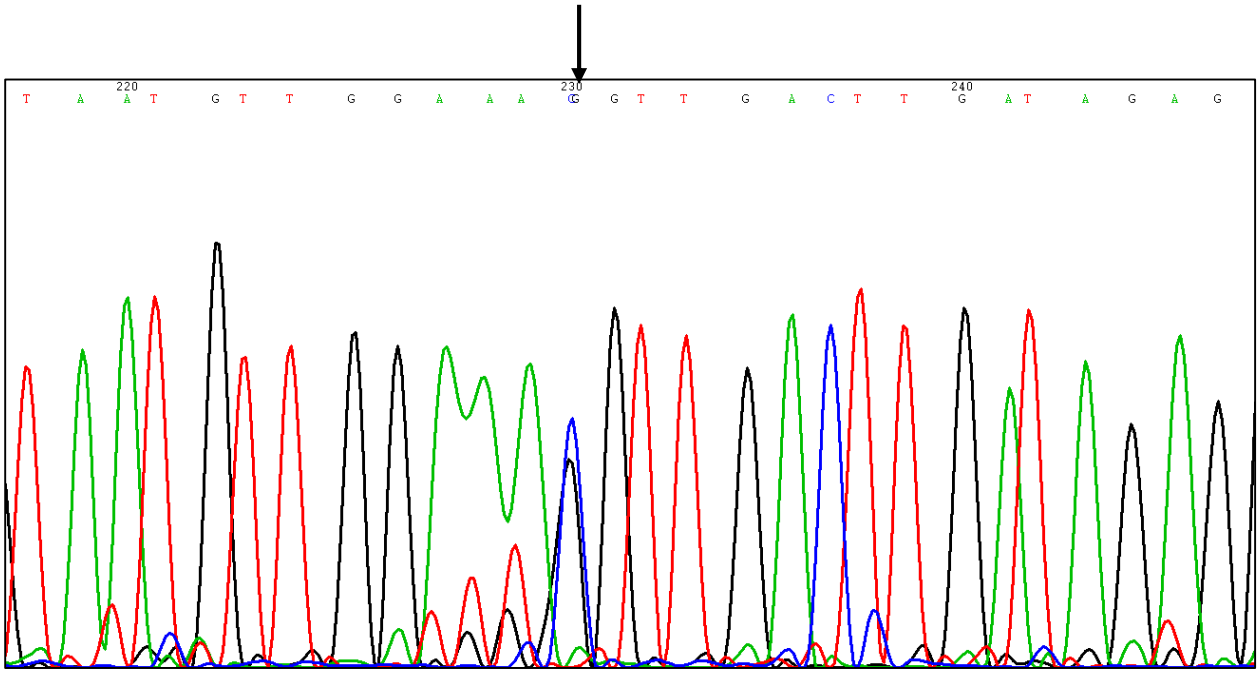
APPENDIX D2: Sequence electropherograms confirming heterozygosity



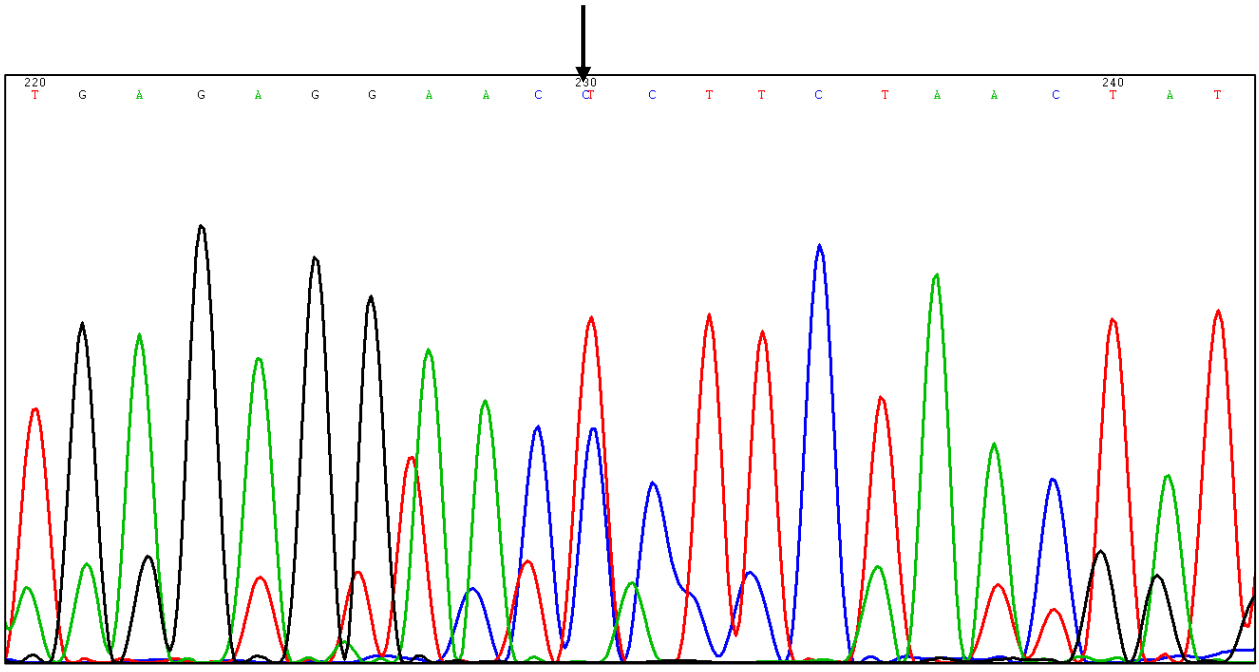
Sequencing electropherogram result of individual P8 with a heterozygote allele (CT) on position 193 in the *TCF7L2* (rs7903146) gene.



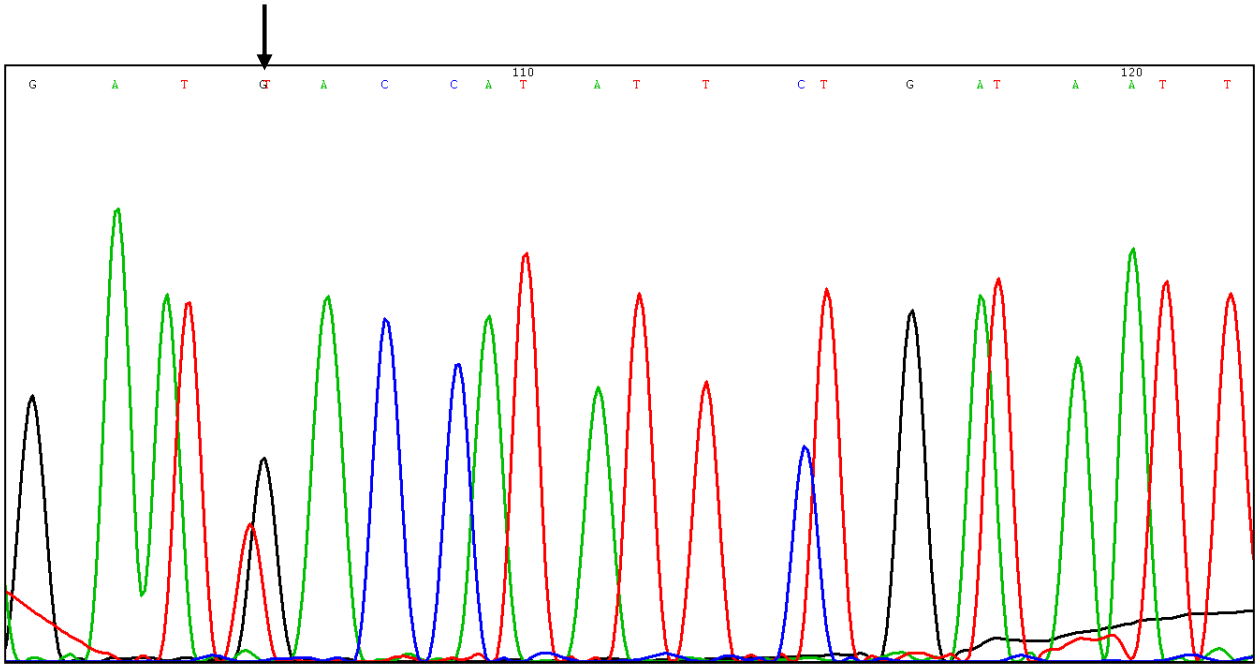
Sequencing electropherogram result of individual P65 with a heterozygote allele (GC) on position 90 in the *RND3-RBM43* (rs7560163) gene



Sequencing electropherogram of individual C9 with a heterozygyous (GC) on position 230 in the *CDKAL1* gene

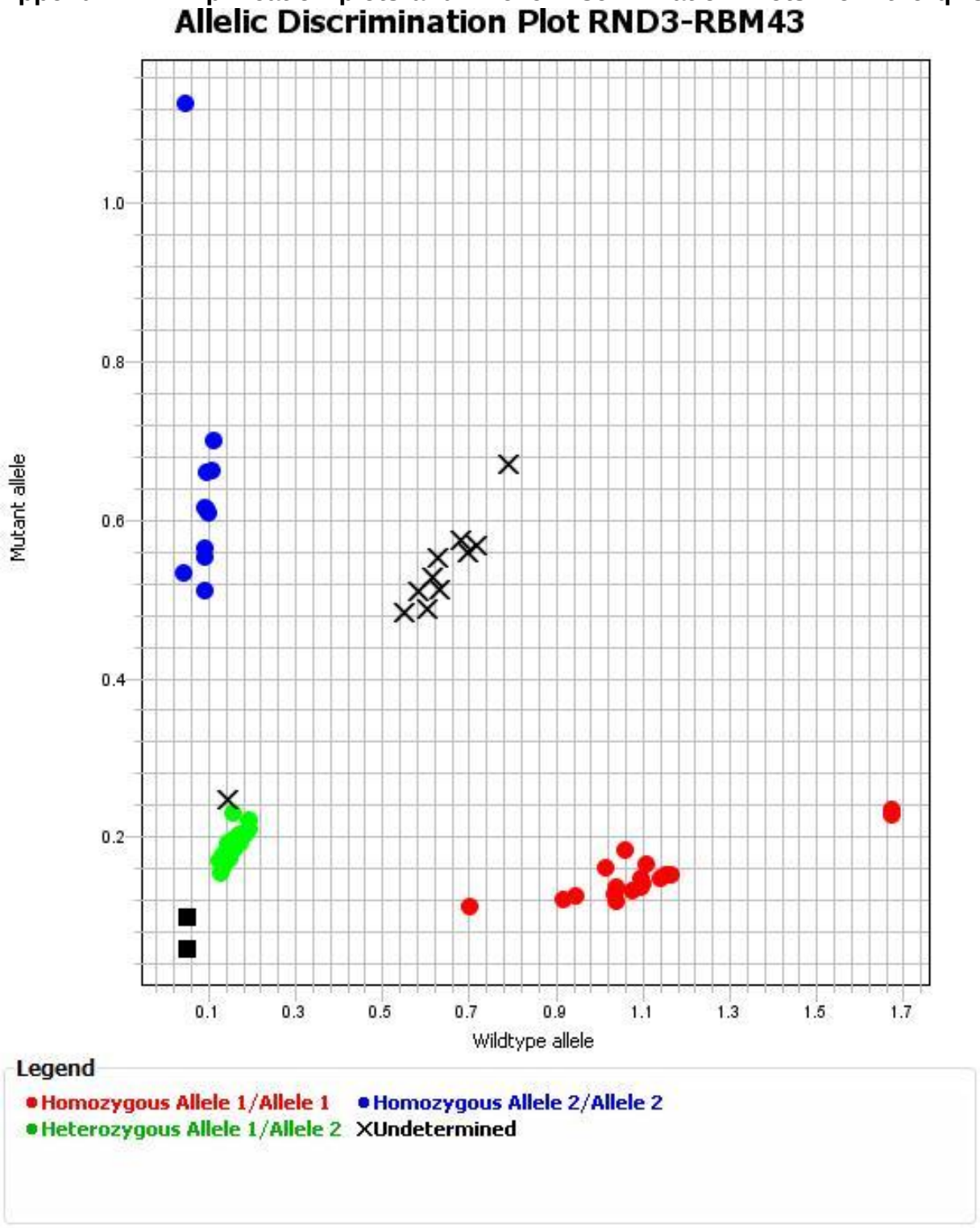


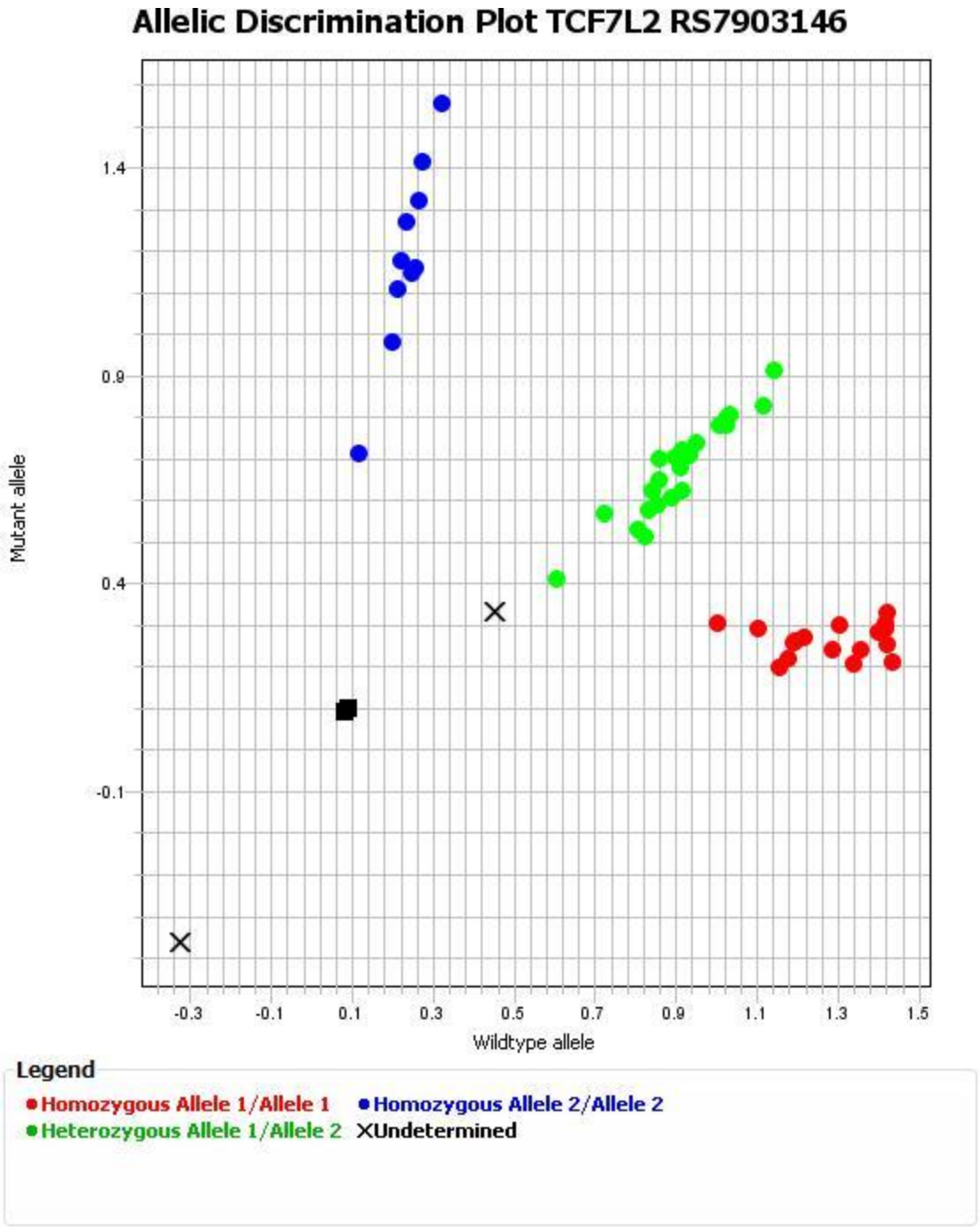
Sequencing electropherogram result of individual P8 with a heterozygote allele (GC) on position 230 in the *IRS1* gene.

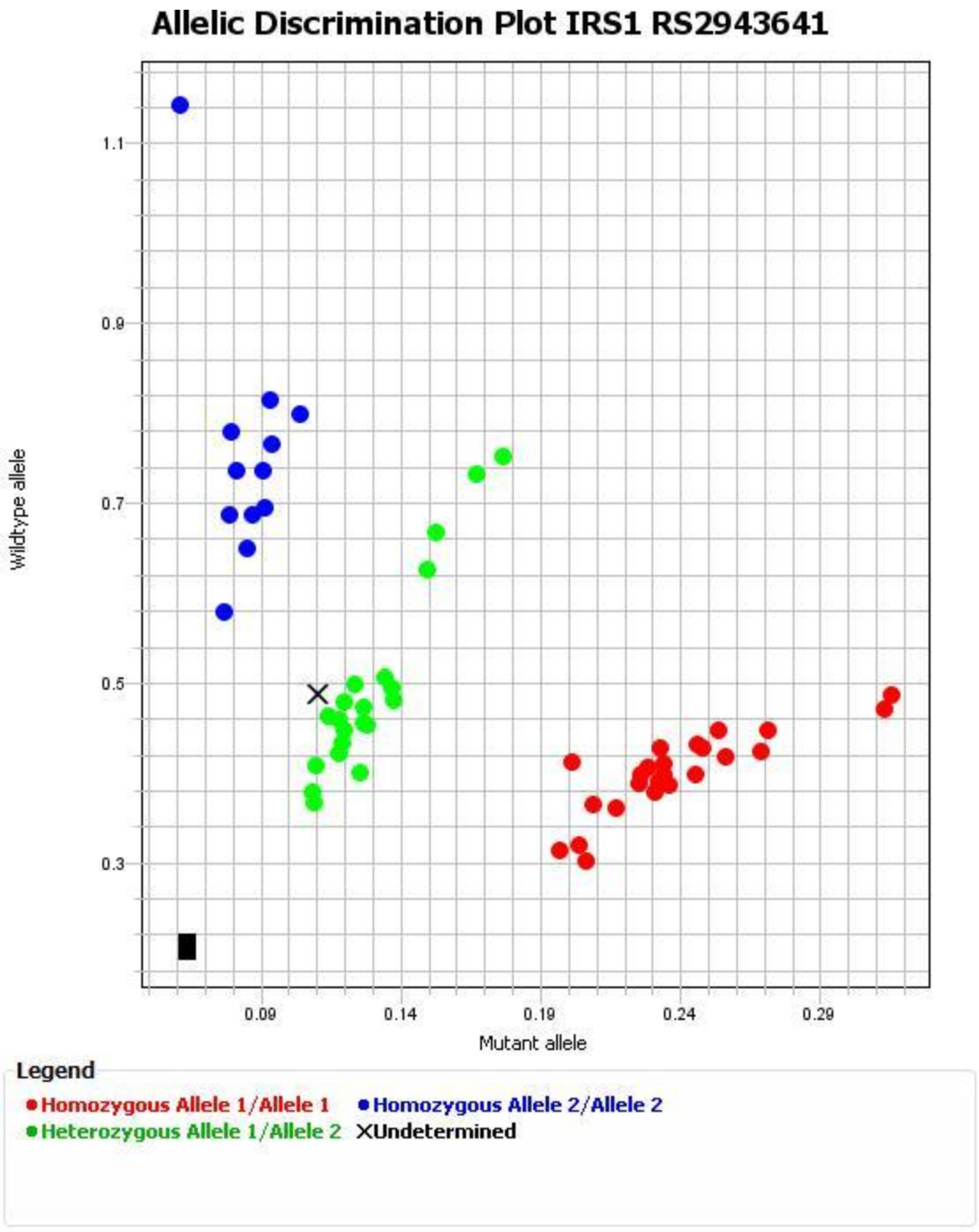


Sequencing electropherogram result of individual P2 with a heterozygote allele (GT) on position 106 in the *TCF7L2* (rs12255372) gene

Appendix E: Amplification plots and Allelic Discrimination Plots from the qPCR




Allelic Discrimination Plot TCF7L2 RS7903146
Mutant allele
1.4
0.9
0.4
-0.1
-0.3
-0.1
0.1
0.3
0.5
0.7
0.9
1.1
1.3
1.5
Wildtype allele
Legend
Homozygous Allele 1/Allele 1
Homozygous Allele 2/Allele 2
Heterozygous Allele 1/Allele 2
XUndetermined


Allelic Discrimination Plot IRS1 RS2943641
Wildtype allele
1.1
0.9
0.7
0.5
0.3
0.09
0.14
0.19
0.24
0.29
Mutant allele
Legend
Homozygous Allele 1/Allele 1
Homozygous Allele 2/Allele 2
Heterozygous Allele 1/Allele 2
XUndetermined