Identifying relevant novel markers of cardiometabolic risk in the Cardiovascular Risk in Black South Africans (CRIBSA) Study

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Declaration

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Abstract

Non-communicable diseases are the second leading cause of death in South Africa. In South African Black individuals, risk factors for cardiovascular disease, such as hypertension, obesity and type 2 diabetes mellitus, are common. These individual risk factors for cardiovascular disease (dyslipidaemia, hypertension, obesity and type 2 diabetes) are the focus of the present study in the Black isiXhosa-speaking population of Cape Town.

Previous studies, in European and North American populations, have identified single nucleotide polymorphisms (SNPs) in various genes to be associated with non-communicable diseases that are risk factors for cardiovascular disease. However, few studies have been conducted in sub-Saharan Africa and even fewer have been conducted in South Africa.

Identifying genes that contribute to the development of cardiovascular disease may help to understand its pathophysiology, identify individuals at higher risk and novel targets may aid preventative and treatment strategies.

The aim of this study was to determine if selected genetic markers in genes encoding the angiotensin-converting enzyme (*ACE*), angiotensinogen (*AGT*), angiotensin II type I receptor (*AT1R*), transcription factor 7-like 2 (*TCF7L2*), fat-mass and obesity associated (*FTO*), melanocortin 4 receptor (*MC4R*) and tumour necrosis factor-alpha (*TNFα*) are associated with cardiovascular disease risk in South African Black individuals.

Of the 1 116 samples available for this study, DNA was extracted from 936 samples. SNPs in each of these genes were selected based on previous findings of association with disease in other African populations. Genotypes were analysed under additive, dominant and recessive association models using the R Statistical Package, *snpassoc.*

The I/I genotype of rs4646994 of *ACE* was associated with blood pressure (p=0.014) and LDL-C (p=0.038) under a recessive inheritance model, while the D/D genotypes was associated with obesity and waist circumference under additive (p=0.047 and p=0.044, respectively) and dominant (p=0.04351 and p=0.04437, respectively) inheritance models. rs17782313 of *MC4R* was nominally associated with type 2 diabetes mellitus under dominant (T/C and C/C genotypes) (p=0.054) and recessive (T/T genotype) (p=0.075) inheritance models; and rs229616 (A/A genotype) was nominally associated with HDL-C (p=0.059) and rs1297034 was nominally associated with type 2 diabetes mellitus (p=0.075) under recessive inheritance models. Suggestive evidence of association with disease was observed for many of the genes, but further studies are needed to confirm this.

Genetic associations with obesity and type 2 diabetes mellitus, risk factors for cardiovascular disease, observed in other African, as well as European and American populations, were replicated in this study. Novel associations with disease in South Africa and sub-Saharan Africa are reported and cross-phenotype associations were observed. This study suggests that these genes are

potentially causal in disease predisposition and progression in the South African Black population, where the prevalence of these diseases is high. This study suggests that this is an important population to study and further studies are warranted.

Opsomming

Nie oordraagbare siekte is die tweede hoofoorsaak van dood in Suid Afrika. In Suid Afrikanse Swart mense, risiko faktore vir hart siekte, soos hoë bloeddruk, vetsug en suiker siekte, is algemeen. Metaboliese sindroom beskryf die groepering van hierdie risiko faktore, wat 'n individu plaas met hoër risiko vir hart siekte.

Voorige studies, in Europa en Amerika, het enkel nukleotied polimorfismes (ENP) in verskeie gene geidentifiseer wat verband hou met nie oordraagbare siektes, wat risiko faktore is vir hart siekte. Maar min studies is in sub-Sahariese Afrika gedoen, en nog minder in Suid Afrika.

Die identifikasie van gene wat bydra tot die ontwikeling van hart siekte mag dalk help om die patofisiologie te verstaan, en om hoë risiko individu te identifiseer. Nuwe genetiese teikens mag ook dalk voorkomende en behandelings strategieë help.

Die doel van hierdie study was dus om te bepaal of spesfieke genetiese teikens (die angiotensienomskekling ensiem (*ACE*), angiotensinogen (*AGT*), angiotnsien II tipe I reseptor (*AT1R*), transkripsiefator 7, 2-agtige (*TCF7L2*), vetmassa en vetsugverwante (*FTO*), melanocortine 4 reseptor (*MC4R*) en tumor nekrose factor-alfa (*TNFα*) gene) is verband met hart siekte risiko in Suid Afrikanse Swart mense.

Uit 1 116 monsters beskikbaar vir die study, DNS van 936 monsters was onttrek. ENP gekies in elk van die gene was gebasseer op voorige vindings van verbanding met siekte in Afrika lande. Genotipes was ontleed onder toevoeging, dominante en ressesiewe assosiasie modelle met die R statistike paket, *snpassoc.*

Die I/I genotype van rs4646994 van *ACE* was met hoë bloeddruk (p=0.014) en LDL-C (p=0.038) geassosieer onder 'n resessiewe model, terwyl die D/D genotype met vetsug en middellyf omtrek onder toevoeging (p=0.047 en p=0.044, onderskeidelik) en dominante (p=0.044 en p=0.044, onderskeidelik) modelle geassosieer was. Rs17782313 van *MC4R* was nominal geassosieer met suiker siekte geassosieer onder dominante (T/C en C/C genotype) (p=0.054) en resessiewe (T/T genotype) (p=0.075) modelle; rs229616 (A/A genotype) was nominaal geassosieer met HDL-C (p=0.059) en rs1297034 was nominaal geassosieer met suiker siekte (p=0.075) onder toevoeging modelle. Aanduidende bewyse vie assosiasie met hart siekte risiko faktore was waargeneem vir baie van die gene, maar meer studies is nodig om dit te bevestig.

Genetiese assosiasies met vetsug en suiker siekte, risiko faktore vir hart siekte, waargeneem in ander Afrika, sowel as Europese en Noord Amerikanse mense, was herhaal in dié study. Nuwe assosiasies met siekte in Suid Afrika is berig en kruis-fenotipe assosiasies waargeneem. Dié study dui daarop dat hierdie gene moontlik oorsaaklik in die vatbaarheid en vordering van siekte is in Suid Afrikanse Swart mense, waar die voorkoms van hierdie siektes hoog is. Dié study dui ook daarop dat die Swart mense van Suid Afrika belangrik is om te studeer en meer studies geregverdig is.

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Abbreviations

> - Greater than

≥ - Greater than or equal to

< - Less than

≤ - Less than or equal to

± - Plus/minus

x *g* - Times gravity (9.8m/s^2)

% - Percent/Percentage

%E – Dietary fat intake

°C – Degrees Celsius

95% CI – 95% confidence interval

AACE – American Association of Clinical Endocrinology

ACE – Angiotensin converting enzyme gene

ACE2 – Angiotensin converting enzyme 2

AGT – Angiotensinogen gene

AngI – Angiotensin I

AngII – Angiotensin II

APOB – Apolipoprotein B

AT1R – Angiotensin II type I receptor gene

BMI – Body mass index

BP – Blood pressure

CAD – Coronary artery disease

CETP – Cholesteryl ester transfer protein

CHD – Coronary heart disease

Chr - Chromosome

cm – Centimetre

CRIBSA – Cardiovascular Risk in Black South Africans

CVD – Cardiovascular disease

DBP – Diastolic blood pressure

DNA – Deoxyribonucleic acid

EDTA – Athylene diamine triacetic acid

EGIR – European Group for study on Insulin Resistance

EtBr – Ethidium bromide

FRET – Fluorescence resonance energy transfer

FTO – Fat-mass and obesity associated gene

g – Grams

GLM – General linear modelling

GWAS – Genome-wide association study

H^+ - Hydrogen ions

H_2O - Water

HDL-C – High-density lipoprotein cholesterol

HIV – Human immunodeficiency virus

HWE – Hardy-Weinberg equilibrium

I/D – Insertion/deletion

IDF – International Diabetes Federation

IGT – Impaired glucose tolerance

IHD – Ischaemic heart disease

IQRs – Interquartile ranges

IR – Insulin resistance

JIS – Joint Interim Statement

K^+ - Potassium ions

KASP – Kompetitive allele-specific PCR

kb - Kilobases

kg/m^2 – Kilogram per metre squared

LD – Linkage disequilibrium

LDL-C – Low-density lipoprotein cholesterol

LDLR – Low-density lipoprotein receptor

LEP – Leptin

LEPR – Leptin receptor

MAF – Minor allele frequency

MC4R – Melanocortin 4 receptor gene

MetS – Metabolic syndrome

MI – Myocardial infarction

min – Minute/s

ml - Millilitre

mmol/l – Millimole per litre

MODY – Maturity onset diabetes of the young

MTHFR – Methylene tetrahydrofolate reductase

NaCl – Sodium chloride

NCD – Non-communicable disease

NCEP ATPIII – National Cholesterol Education Program Adult Treatment Panel III

NDoH – National Department of Health

ng/µl – Nanogram per microlitre

OR – Odds ratio

p – P-value (significance, <0.05)

PCR – Polymerase chain reaction

PCSK9 – proprotein convertase subtilisin/kexin type 9

PE – Preeclampsia

QKI – Quaking Homolog

RAAS – Renin-angiotensin-aldosterone system

RBC – Red blood cell

RT – Room temperature

SA – South Africa/South African

SADHS – South African Demographic and Health Survey

SANHANES-1 – South African National Health and Nutrition Examination Survey

SB – Sodium borate

SBP – Systolic blood pressure

SD – Standard deviation

SINEs – Short interspersed nucleotide elements

SNP – Single nucleotide polymorphism

SSA – Sub-Saharan African

STD – Sexually transmitted disease

T2DM – Type 2 diabetes mellitus

TB – Tuberculosis

TC – Total cholesterol

TCF7L2 – Transcription factor 7-like 2 gene

TNFα – Tumour necrosis factor alpha

WC – Waist circumference

WHO – World Health Organization

Chapter 1

Introduction

1. Introduction

Non-communicable diseases (NCDs) are non-transmittable diseases that occur mainly due to lifestyle choices. For the past 30 years, NCDs have been recognised as a major cause of death and disability (Nojilana *et al.,* 2016). The four most prevalent NCDs are cardiovascular disease (CVD), type 2 diabetes mellitus (T2DM), cancer and chronic respiratory disease (WHO, 2008). At the 2011 United Nations high-level meeting on NCD disease burden, NCDs were recognised as a growing threat to human health (Parry *et al.,* 2011), reiterating the emphasis the World Health Organization (WHO) put on NCDs as a neglected global health issue (WHO, 2005).

Some NCDs, such as hypertension, T2DM and stroke, are risk factors for CVD, and in the Black population of South Africa (SA), these CVD risk factors are the most frequently found morbidities and mortalities associated with chronic diseases, such as hypothyroidism, cancer and renal disease (Dalal *et al.,* 2011; Tibazarwa *et al.,* 2009; Alberts *et al.,* 2005; Akinboboye e*t al.,* 2003).

Risk factors for CVD and its associated diseases include unmodifiable risk factors, modifiable/lifestyle risk factors, environment risk factors and physiological intermediate risk factors (Figure 1.1) (Tekola-Ayele e*t al.,* 2013; Mayosi *et al.,* 2009; Mollentze, 2003).

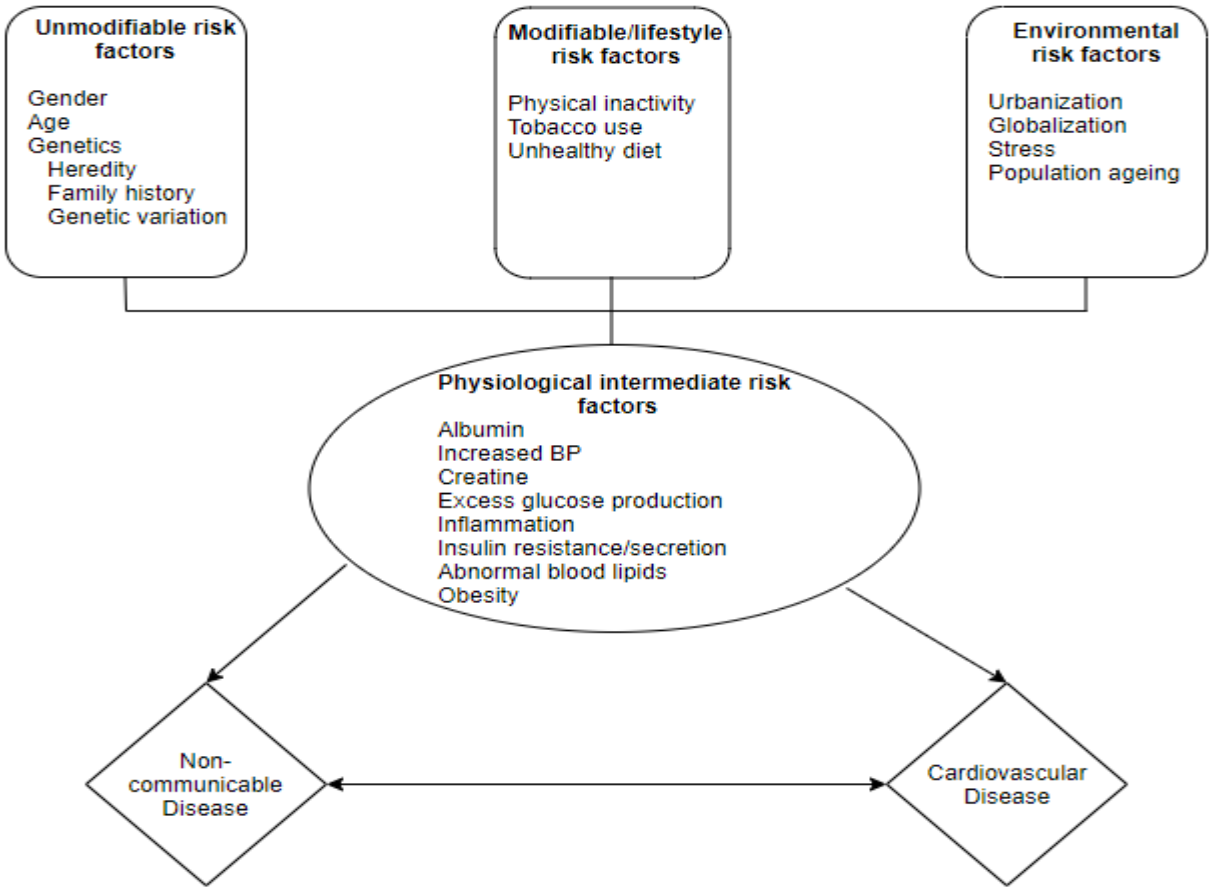

Figure 1.1 Interactions between genes and environment in the pathophysiology of CVD (Adapted from Tekola-Ayele et al., 2013).

By 2030, death due to NCDs is estimated to increase from 28.0% in 2004 to 46.0% in sub-Saharan Africa (SSA), with the highest death rates observed in the Democratic Republic of Congo, Ethiopia, Nigeria and SA (Dalal *et al.,* 2011). The rising trend of NCDs in SA is evident. In 2003, infectious diseases accounted for 28.0% of life years lost in SA, while death due to NCDs accounted for 25.0% (Steyn *et al.,* 2003). By 2004, death due to NCDs in SA had increased to 28.0% (Mayosi e*t al.,* 2009, WHO, 2008), of which 12.0% was due to CVD, cancers, respiratory disease and T2DM, and 6.0% was due to neuropsychiatric disorders including bipolar depression, dementia, epilepsy and schizophrenia (WHO, 2008). In 2010, 594 710 deaths, a 3.85% increase from 2009, were reported in SA. Of those, NCDs accounted for 38.9% (Nojilana *et al.,* 2016). Forty-four percent of the latter was due to CVD (of which 17.5% was due to stroke), 18.0% due to cancers, 9.3% due to chronic respiratory diseases and 8.0% due to T2DM (Nojilana *et al.,* 2016). The age-standardised death rates in SA, per 100 000 population, has been reported as 287, 114, 58 and 52 for CVD, cancers, chronic respiratory disease and T2DM, respectively (Nojilana *et al.,* 2016). The SA Coloured as well as the Indian populations, and Black populations have been found to have NCD mortality rates at 1.4-fold and 1.3-fold higher than in the SA Caucasian population (Nojilana *et al.,* 2016).

Globally, low- and middle-income countries have the highest proportion of NCD burden (Nojilana *et al.,* 2016). In the low-income countries of SSA, particularly in the Democratic Republic of Congo, Ethiopia, Nigeria and SA, death due to NCDs far exceeds that of higher income countries (Dalal *et al.,* 2011; Mayosi *et al.,* 2009; Lopez *et al.,* 2006). Specifically, SA's NCD burden was 2-3 times higher than in developed countries (WHO, 2008; WHO, 2005). However, by 2017, death due to NCDs in SA was 2-3 times higher than in developing countries, while that of developed countries surpassed SA (WHO, 2017). In the 1990s, population-based surveys in the Black population of SA revealed a high prevalence of hypertension (14.0-33.0%) and T2DM (4.8-6.0%), as well as smoking (13.0-33.0%), which is a known risk factor for many NCDs, but other risk factors were not examined in these surveys (Mollentze *et al.,* 1995; Steyn *et al.,* 1991). Surveys of the same population, conducted in the early 2000s, confirmed the high prevalence of hypertension and T2DM observed in the 1990s, and also reported a high prevalence of overweight and obesity, especially in females, with more than 50.0% of the female population being overweight or obese (Thorogood e*t al.,* 2007; Alberts *et al.,* 2005). A later study of age-standardised mortality rates in Khayelitsha, Cape Town, found that 856.4 deaths per 100 000 were due to NCDs, compared to 450-500 deaths per 100 000 in wealthier districts of Cape Town (Groenewald *et al.,* 2008).

Since the early 2000s, marked declines in the age-standardised death rates due to NCDs in SA Coloured (de Wit *et al.,* 2010) and Indian individuals have been observed; however, only slight declines were observed in SA Black and Caucasian individuals (Nojilana *et al.,* 2016). Age-standardised death rates, due to CVD and T2DM, in particular, were reduced in SA Coloured and Indian individuals, while there was an increase of death rates due to CVD and T2DM in SA Black individuals (Nojilana *et al.,* 2016). High mortality rates of cardiomyopathy, hypertensive heart

disease, stroke and T2DM have been reported in SA Black individuals, who are currently undergoing a CVD epidemic. These increased rates may lead to high rates of ischaemic heart disease (IHD) and renal disease, as is observed in SA individuals of Indian descent (Nojilana *et al.,* 2016).

As previously mentioned, death due to NCDs in the adult SA population is second only to infectious diseases (human immunodeficiency virus (HIV), malaria and tuberculosis (TB)) (Mayosi *et al.,* 2009). However, in SA, it is predicted that, by 2020, death due to NCDs will exceed that of infectious diseases (Murray & Lopez, 1997), and it will continue to increase if measures are not put in place to overcome the burden of NCDs (Mayosi *et al.,* 2009; Abegunde *et al.,* 2007). In 2013, because NCD burden in SA is predicted to rise, the SA National Department of Health (NDoH) set out national goals and targets for the prevention and control of NCDs (NDoH, 2013). Table 1.1 compares the SA NDoH goals and targets to that of the WHO's global target for 2025.

Table 1.1 Goals and targets for the prevention and control of NCDs in South Africa, by 2020, as set out by the NDoH and globally, by 2025, as set out by WHO.

NDoH NCD prevention and control: 2020 SA goals and targets	**WHO NCD prevention and control: 2025 global goals and targets (66TH World Health Assembly)**
Relative premature mortality (<60 years): reduce by 25%	NCD premature mortality reduce by 25% (all 4 major NCDs)
Tobacco use: reduce by 20%	Behavioural risk factors Tobacco use: reduce by 30% (aged >15years) Alcohol use: reduce by 10% Salt/sodium intake: reduce by 30%
Alcohol consumption (per capita): reduce by 20%	
Salt intake (mean population): <5g per day	
Percentage overweight/obese: reduce by 10%	Biological risk factors Prevent rise in diabetes and obesity Prevalence of raised BP: reduce by 25%
Prevalence of raised BP: reduce by 20% (via lifestyle and medication)	
Physical activity: increase by 10% (150min moderate-intensity per week)	Behavioural risk factors Physical activity: increase by 10%
Females with STDs: screened for cervical cancer every 5years. Healthy females: 3 screens in a lifetime (and as per policy for females who are HIV-positive)	National systems response Drug therapy and counselling to prevent heart attack and stroke: 50% eligible candidates receive care (eligibility: >40years with 10year CVD risk <30%). Treatment of major NCDs: 80% availability of affordable basic technologies and essential medicines in public and private facilities
Asthma, diabetes and hypertension control: increase by 30% in sentinel sites	
Mental disorders (screening and treatment): increase by 30% (by 2030)	

NDoH – National Department of Health; WHO – World Health Organization; SA – South Africa; NCD – Non-communicable disease; g – grams; BP – Blood pressure; STD – Sexually transmitted disease; HIV – Human immunodeficiency virus

This rapid, predicted increase in NCD burden is thought to be largely due to the rapid urbanization and accompanying demographic and epidemiological transitions (Motala *et al.,* 2011). Such transitions are characterised by a quadruple burden of disease: communicable (transmittable); non-communicable (non-transmittable); perinatal and maternal; and injury related diseases/disorders (Mayosi *et al.,* 2009).

1.1 Metabolic syndrome (MetS)

In 1988 the term “Syndrome X” was coined by Reaven (1988) to describe a clustering of metabolic abnormalities, with insulin action as the underlying cause. Syndrome X was later termed metabolic syndrome (MetS) (Alberti *et al.,* 2006). Metabolic syndrome is often related to the four major NCDs (cardiovascular disease, cancer, chronic respiratory disease and T2DM), as MetS is a cluster of risk factors found together more often than by chance alone that place an individual at higher risk for the development of CVD and T2DM (O’Neill & O’Driscoll, 2015; Emmanuela *et al.,* 2012; Motala *et al.,* 2011; Sookoian & Pirola, 2011; Gallagher *et al.,* 2010; Ntyintyane *et al.,* 2009; Ntyintyane *et al.,* 2006; Aizawa *et al.,* 2006; Eckel *et al.,* 2005).

Metabolic syndrome is related to increased risk of morbidity and mortality (Ntyintyane *et al.,* 2006), with insulin resistance (IR), a common thread among all risk factors for CVD, proposed as a linking factor to disease (Motala *et al.,* 2011; Gallagher *et al.,* 2010; Boura-Halfon & Zick, 2009; Eckel *et al.,* 2005; Grundy *et al.,* 2005; Smith & LeRoith, 2004; Hu *et al.,* 2004; Stephens *et al.,* 1997; Hotamisligil *et al.,* 1994; Reaven, 1988).

Metabolic syndrome is accompanied by sustained inflammation (Ntyintyane *et al.,* 2009; Boura-Halfon & Zick, 2009; Grundy *et al.,* 2005; Hu *et al.,* 2004; Stephens *et al.,* 1997; Hotamisligil *et al.,* 1994); the fundamental, unifying pathogenic mediator of CVD, T2DM and obesity (Lontchi-Yimagou *et al.,* 2013). The low-grade, persistent presence of inflammation, due to obesity, may be correlated with the development of CVD and T2DM (Medzhitov, 2008; Hotamisligil, 2006). Risk factors for, and the diagnosis of MetS, as defined by the International Diabetes Federation’s (IDF) Joint Interim Statement (JIS), and subsequently CVD and its associated risk factors include abdominal obesity (presenting as an increased waist circumference (WC)); raised triglycerides (>1.7mmol/l (150mg/dl)) and reduced high-density lipoprotein cholesterol (HDL-C) (males: <1.03mmol/l (40mg/dl); females: <1.29mmol/l (50mg/dl)), referred to as dyslipidaemia; hypertension (systolic BP: >130mmHg; diastolic BP: >85mmHg), often present with obesity and/or IR; and elevated fasting glucose (>5.6mmol/l (100mg/dl)) (Alberti *et al.,* 2009; Kooner *et al.,* 2008; Ntyintyane *et al.,* 2006; Vague, 1956). To be diagnosed with MetS, three of the above mentioned five risk factors need to be present in an individual (Alberti *et al.,* 2009).

Various definitions exist for MetS (Table 1.2), but for the purpose of this study, the JIS criteria will be used for diagnosis.

Table 1.2 Various definitions of the metabolic syndrome.

	NCEP ATPiii	WHO	IDF	EGIR	AACE	JIS
Obesity	**Increased WC** Males: >102cm Females: >88cm	**Increased waist-to-hip ratio** Males: >0.09 Females: >0.82 or BMI >30kg/m2	**Increased WC** Males: >90cm Females: >80cm	**Increased WC** Males: >94cm Females: >80cm		**Increased WC** Males: >94cm Females: >80cm
Triglycerides	**Elevated plasma triglycerides** >1.69mmol/l	**Elevated plasma triglycerides** >2mmol/l	**Elevated plasma triglycerides** >1.69mmol/l	**Elevated plasma triglycerides** >1.7mmol/l Treatment	**Elevated plasma triglycerides** >1.7mmol/l	**Elevated plasma triglycerides** >1.7mmol/l
Glucose	**Impaired fasting glucose** >6.1mmol/l	Glucose intolerance, IGT, T2DM	**Impaired fasting glucose** >5.6mmol/l	**Impaired fasting glucose** >6.1mmol/l	**Impaired fasting glucose** >6.1-6.9mmol/l **2h glucose tolerance** 7.8-11.mmol/l	**Impaired fasting glucose** >5.6mmol/l
Hypertension	**Elevated BP** >130/85mmHg antihypertensive medication	**Elevated BP** >140/90mmHg	**Elevated BP** >130/85mmHg antihypertensive medication	**Elevated BP** >140/90mmHg antihypertensive medication	**Elevated BP** >130/85mmHg	**Elevated BP** >130/85mmHg
HDL-C	**Low plasma HDL-C** Males: <1.04mmol/l Females: <1.29mmol/l	**Low plasma HDL-C** Males: <0.9mmol/l Females: <1.0mmol/l	**Low plasma HDL-C** Males: <1.04mmol/l Females: <1.29mmol/l	**Low plasma HDL-C** <1.0 mmol/l Treatment	**Low plasma HDL-C** Males: <1.04mmol/l Females: <1.29mmol/l	**Low plasma HDL-C** Males: <1.0mmol/l Females: <1.3mmol/l

AACE – American Association of Clinical Endocrinology; EGIR – European Group for study on Insulin Resistance; IDF – International Diabetes Federation; JIS – Joint Interim Statement; NCEP ATPiii – National Cholesterol Education Program Adult Treatment Panel III; WHO – World Health Organization

1.2 Cardiovascular disease (CVD)

Cardiovascular disease (CVD) refers to disorders of the heart and its blood vessels. Cardiovascular diseases are divided into 2 groups: acute CVDs, such as heart attack and stroke, and chronic CVDs such as cerebrovascular disease, congenital heart disease, coronary heart disease (CHD), deep vein thrombosis and pulmonary embolism, peripheral arterial disease and rheumatic heart disease (WHO, 2014; Motawi *et al.,* 2011). Heart disease/failure due to non-ischaemic causes of hypertension, idiopathic cardiomyopathy (enlarged chambers or decreased muscle contractions) and rheumatic heart disease (damage to the valves) are the most common reasons for admission to hospital in the SA Black population, while ischaemic heart diseases account for 10% of hospitalization due to heart diseases/failure (Mayosi *et al.,* 2009)

In general, CVD patients report a higher frequency of hypertension, family history of CVD, smoking habits, T2DM and increased waist-to-hip ratios (Abd El-Aziz *et al.,* 2012; Mayosi *et al.,* 2009). These patients also have increased levels of low-density lipoprotein cholesterol (LDL-C), total cholesterol (TC) and triglycerides, while their HDL-C levels are decreased (Abd El-Aziz *et al.,* 2012). All these factors are independently associated with CVD (Abd El-Aziz *et al.,* 2012). In SSA, heart failure has been found to occur at a younger age and results in a higher rate of hospitalised mortality than in American and European countries (Carlson *et al.,* 2017). The impact on CVD of its risk factors, such as BP and T2DM, differs dramatically between the sexes (Um *et al.,* 2003).

1.2.1 Epidemiology of cardiovascular disease in Africa

Three quarters of the world's annual deaths due to CVD occurs in low- and middle-income countries (WHO, 2014). After HIV/AIDS, CVD is the leading cause of death in SA (Msemburi *et al.,* 2014), responsible for 1 in 6 deaths (Stats SA, 2015). This figure is greater than the deaths for all forms of

cancer combined (Msemburi *et al.,* 2014). Per hour in SA, 5 people have a heart attack, 10 people suffer a stroke (HSFSA, 2016), and 215 people die per day from CVD (Stats SA, 2015). In 2013, nearly 1 million deaths in SSA (512 269 females and 445 445 males), an 81% increase from 1990 (Mensah e*t al.,* 2015), was due to CVD, equating to 11.3% of all deaths and 38% of NCD-related deaths reported in SSA (Keates e*t al.,* 2017; Mensah e*t al.,* 2015).

Cardiovascular disease is a polygenic disease that is influenced by many risk factors (Figure 1.1) (Abd El-Aziz *et al.,* 2012; Motawi *et al.,* 2011; Shaker *et al.,* 2009; Abbate *et al.,* 2008). Until the early 2000s (Motala *et al.,* 2011; Mensah, 2008; Sliwa *et al.,* 2008; Ntyintyane *et al.,* 2006; Joubert *et al.,* 2000; Walker & Sareli, 1997; Seedat *et al.,* 1977; Mollentze *et al.,* 1995), coronary artery disease (CAD) was believed to be rare in Black Africans (Mayosi *et al.,* 2009; Ntyintyane *et al.,* 2006; Steyn *et al.,* 2005; Akinboboye *et al.,* 2003; Ntyintyane *et al.,* 2006; Muna, 1993). However, evidence now suggests that CAD is increasing in this population (Ntyintyane *et al.,* 2009).

1.2.1.1 CVD in South Africa

In the 1990s, nearly 70 black patients were hospitalised at the Chris Hani Baragwanath Hospital in Soweto for CVD, and this number increased to 85 in 2002 and 150 in 2006 (Ntyintyane *et al.,* 2006). In 1940, only a single death of a total of 352 autopsies that were performed on Black African adults was as a result of myocardial infarction and three decades later, CHD was still rare among Black individuals living in Durban and Johannesburg (Mensah, 2008). In 1990, CHD was the most common form of death due to diseases of the circulatory system in SA Caucasian and Asian individuals (165.3 and 101.2 per 100 000, respectively), but was rare in SA Coloured (55.1 per 100 000) and Black (5.3 per 100 000) individuals (Central Stats Unit, 1990). Until 2010, CHD was still rare in SA Black individuals, accounting for only 10.0% of all heart disease patients that present at hospital (Mayosi *et al.,* 2009; Sliwa *et al.,* 2008; Mayosi *et al.,* 2009) and is still rare in Black individuals from Nigeria and Uganda (Nkoke & Luchuo, 2016; Mensah, 2008). Ischaemic heart disease was estimated to be found in only 10.0% of SA Black patients diagnosed with heart disease in 2009 (Mayosi e*t al.,* 2009), and remains uncommon in the SA Black population (Churchill, 2013), representing the lowest IHD death rates worldwide (Mensah e*t al.,* 2015).

In Soweto, the annual number of heart failure diagnosis is fast exceeding that of previously diagnosed cases (Sliwa *et al.,* 2008), and more than 78.0% of the population within Soweto has at least one risk factor for CVD (Tibazarwa *et al.,* 2009), as is observed in Black individuals all over SA (Motawi *et al.,* 2011). A study found that in SA Black individuals living in Soweto, <1.0% of deaths was attributable to CAD, whereas in SA Caucasian individuals it was responsible for 5% of deaths (Joubert *et al.,* 2000). This low trend was also observed in SA black stroke patients (Joubert *et al.,* 2000), where a meta-analysis of NCD studies in SSA found that the prevalence of stroke ranged between 0.07% and 0.3% (Dalal *et al.,* 2011). In 1995 the incidence of stroke was reported to be 1.01 per 1 000 in the urban Black population of Mangaung (Mollentze *et al.,* 1995). However, reports are emerging that Africans are disproportionately affected by stroke at a younger age, with attacks

more severe and leading to accelerated end-organ damage (Moran *et al.,* 2013; Dalal *et al.,* 2011; Tibazarwa *et al.,* 2009; Vorster, 2002), possibly due to hyperfibrinogenaemia, increased hypertension, obesity and tobacco use (Vorster, 2002).

A study of 4 162 individuals, of which 1 359 were Black Africans, found that Black individuals were more frequently diagnosed with heart failure and less frequently diagnosed with CAD than other SA populations (Sliwa *et al.,* 2008). This was also observed in an earlier study of Black individuals in the Cape Peninsula (Steyn *et al.,* 1991). A survey of 10 000 individuals in 200 hospital across SA found that 76.0% had one or more risk factors for stroke, an acute form of CVD, and 40.0% had two or more risk factors (Connor *et al.,* 2005). In 2008 and 2009 all forms of CVD were responsible for 13.7% and 14.0%, respectively, of deaths in SA (Raal e*t al.,* 2013).

1.2.1.2 CVD in sub-Saharan Africa

From 1997 to 2009, deaths as a result of CAD in older individuals increased from 70 deaths per 100 000 population to 87 deaths per 100 000 population in Tunisian males (>55 years), and from 28 deaths per 100 000 population to 41 deaths per 100 000 population in Tunisian females (>65 years) (Keates *et al.,* 2017). This is in stark contrast to Sudan, where by 2002, CAD-associated deaths were already reported at 205 deaths per 100 000 population (Keates *et al.,* 2017). In Kenya between 2005 and 2009, CVD was responsible for 13% of all deaths, while a retrospective analysis of hospital admissions in Ethiopia reported that CVD was responsible for 32% of all deaths in the years 1981-1982, 1991-1992, 2001-2002 and 2011-2012 (Keates *et al.,* 2017).

Ischaemic heart disease is the eighth leading cause of death in SSA (Ebireri *et al.,* 2016; Mensah, 2008). The southern region of SSA (Botswana, Namibia, SA) has the highest CVD burden, while the Western region (including, but in no way limited, to Ghana, Mali, Nigeria and Togo) has the lowest (Moran *et al.,* 2013). In 2005, 361 000 deaths as a result of IHD were reported in Africa, and this number is estimated to double by 2030 (Mensah, 2008). Surprisingly, fewer deaths as a result of IHD were reported for SSA in 2013 than in 2005 (258 939 vs. 361 000, respectively), but this was still an 87.0% increase since 1990 (Mensah *et al.,* 2015). In 2010, stroke was the leading cause of death and disability in SSA (Moran *et al.,* 2013). In 2013, 409 840 deaths, almost double that reported in 1990, as a result of stroke were reported in SSA, but overall stroke mortality has increased by 1.0% (Mensah e*t al.,* 2015).

IHD is rare in the SA Black population (10% of CVD patients in 2009) (Churchill, 2013; Mayosi *et al.,* 2009) and is thought to be due to favourable serum lipid profiles (decreased serum cholesterol, but stable HDL-C) and low homocysteine values, which have been suggested to protect SA Black individuals against IHD (Mayosi *et al.,* 2009; Lemogoum *et al.,* 2003; Vorster, 2002). However, SA Black individuals may soon transition to IHD, as the presence of risk factors associated with CVD is widespread (Table 1.3), and this ethnic population is undergoing a CVD epidemic, with high mortality rates for cardiomyopathy, hypertensive heart disease, stroke and T2DM (Nojilana *et al.,* 2016).

A low frequency of the methylene tetrahydrofolate reductase (*MTHFR*) 677C-T mutation, which impairs remethylation of homocysteine (20.0% in SA Black individuals versus 56.0% in SA Caucasian individuals), thus contributing to the low homocysteine values observed in the SA Black population group, was proposed as a protective mechanism against IHD in SA Black individuals in the THUSA study (Loktionov *et al.,* 1999). Increased levels of homocysteine contribute to CVD by promoting atherosclerosis and thrombosis (Sengwayo *et al.,* 2013). Atherosclerosis is achieved by damaging the inner lining of arteries while thrombosis is achieved through sustained collagen activation, endothelial dysfunction, impaired thrombolysis, and oxidative stress (increased production of hydrogen peroxide and oxidation of low-density lipoproteins) (Sengwayo e*t al.,* 2013). Homocysteine plays a role in the aetiology of T2DM by regulating glucose metabolism and insulin absorption, and is also thought to contribute to the development of essential hypertension by inducing arteriolar constriction and increasing sodium reabsorption thereby enhancing arterial stiffness (Sengwayo *et al.,* 2013). Homocysteine also increases oxidative stress, a common abnormal physiological process between hypertension, obesity and T2DM (Sengwayo e*t al.,* 2013).

Table 1.3 Population groups in South Africa: Prevalence and level of selected CVD risk factors (Adapted from Vorster *et al*., 2002)

Population group in South Africa	Gender	Prevalence (%)		Level
		Hypertension (BP ≥160/95 mmHg)	Obesity (BMI > 30kg/m)	Dyslipidaemia (45-54 years) (mmol/l)
Black	Female	13.0	30.5	4.70
	Male	10.3	7.7	4.20
Coloured	Female	17.1	28.3	6.30
	Male	12.4	9.1	6.09
Indian	Female	9.3	20.2	5.86
	Male	9.9	8.7	6.28
Caucasian	Female	12.0	24.3	6.62
	Male	15.2	19.8	6.39
All	Female	13.2	29.4	
	Male	11.0	9.1	

1.2.2 Genetics of cardiovascular disease

The heritability of CVD and its associated risk factors has strongly and consistently been supported by genome-wide association studies (GWAS) and twin and/or family studies (Dehghan e*t al.,* 2016). Overall, only about 10.0% of the predicted heritable risk for CVD, specifically CHD, has been explained by GWAS (McPherson, 2014; Zeller *et al.,* 2012).

A Swedish study that followed 10 500 twin pairs for 35 years estimated the heritability of fatal CAD at 57.0% and 38.0% for males and females, respectively (Zdrakovic *et al.,* 2002). However, a study in an American cohort, over a 9 to 26-year follow-up period, showed that single nucleotide polymorphisms (SNPs) associated with incident CAD in Caucasian Americans was not associated with CAD in their Black counterparts (Franceschini *et al.,* 2011). This was also observed in another study which found that SNPs associated with 28 different disease phenotypes in European populations had low replicative results in African-ancestry populations (Marigorta & Navarro, 2013). These studies suggest that even though there is evidence for the heritability of CAD, it is variable among different ethnic population groups.

In 2007, three GWAS reported a locus on chromosome 9p21.3 to be associated with CAD and myocardial infarction (MI) risk (Helgadottir *et al.,* 2007; McPherson *et al.,* 2007; Samani *et al.,* 2007). This finding was later replicated in a GWAS of 8 090 African Americans (AfAms) from 5 population-based cohorts, which also replicated 16 other SNPs associated with CVD and its associated risk factors in Europeans (Lettre *et al.,* 2011). Homozygosity of these SNPs in the region of 9p21 has been associated with a 30.0-40.0% increased risk of CAD and a 15.0-20.0% increased risk in heterozygotes (Cambien & Tiret, 2007). Since the first associations of the 9p21 region with CAD, it has also been hypothesized to be associated with other disease, such as aggressive periodontitis (Schaefer *et al.,* 2009), aortic aneurysm (Helgadottir *et al.,* 2008), glioma (Shete *et al.,* 2009), ischaemic stroke (Gschwendtner *et al.,* 2009), malignant melanoma (Bishop *et al.,* 2009) and T2DM (Zeggini *et al.,* 2007).

A GWAS of 64 297 European individuals identified 3 loci significantly associated with CHD, of which rs6941513, close to the Quaking homolog (*QKI*) gene, was the strongest hit of all the SNPs reported. However, this association was not replicated in 8 201 AfAms (Dehghan *et al.,* 2016).

No studies have thus far been conducted to determine the genetics of CVD in Africa. However, the renin-angiotensin-aldosterone system (RAAS) has been proposed as one of the major players in CVD progression and diagnosis, as well as in the risk phenotypes associated with CVD. And thus, the genes involved in the RAAS could be implicated in disease.

1.2.2.1 The renin-angiotensin-aldosterone system

A key mechanism of CVD initiation and progression is inflammation (Farrario & Strawn, 2006). The main effector in the renin-angiotensin-aldosterone system (RAAS) (Figure 1.2), angiotensin II (Ang II) (Munóz-Duranga *et al.,* 2016; Farrario & Strawn, 2006), plays an important role in inflammatory diseases, especially atherogenesis and renal disease (Farrario & Strawn, 2006).

The RAAS (Figure 1.2) has been proposed to be involved in atherosclerosis pathogenesis, the leading cause of death worldwide (Shaker *et al.,* 2009), and CAD prognosis (Abd-El Aziz *et al.,* 2012). It is involved in many of the diseases that are risk factors for CVD (Munóz-Duranga *et al.,* 2016; Farrario & Strawn, 2006; Lovati *et al.,* 2001). The RAAS is a crucial hormonal pathway that

controls haemodynamic stability by regulating blood pressure, cardiac and vascular trophic effects, extracellular fluid volume and sodium-potassium balance (Munóz-Duranga *et al.,* 2016; Farrario & Strawn, 2006).

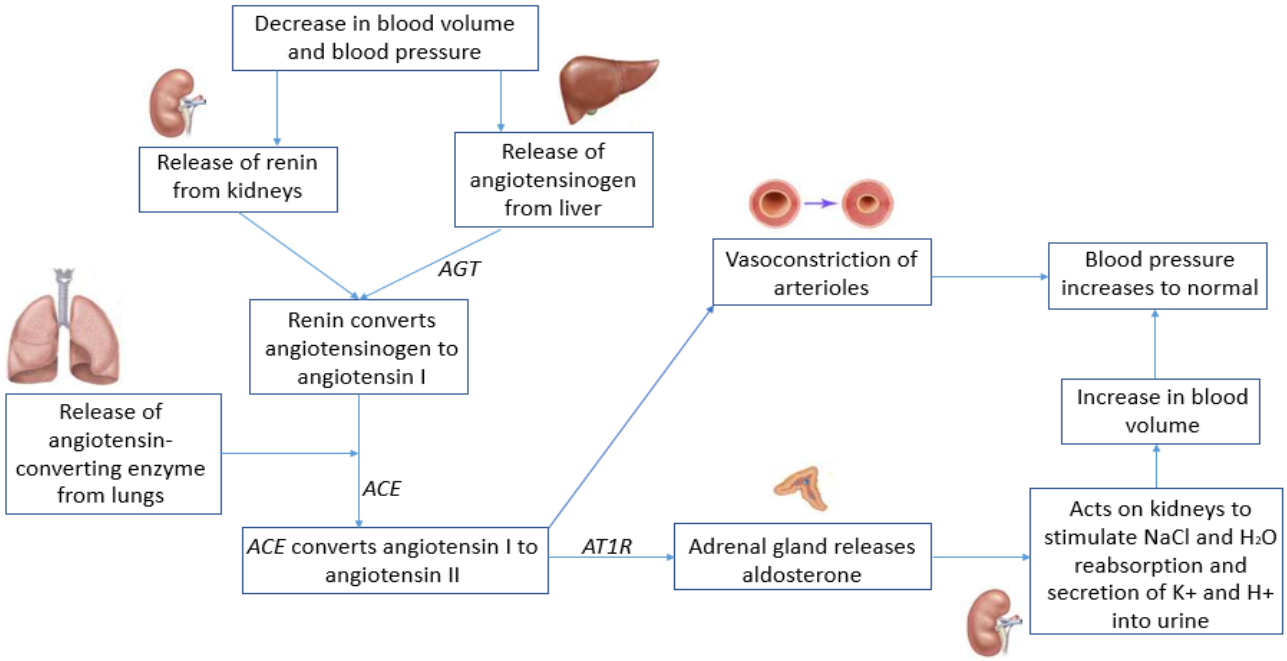

Figure 1.2 The renin-angiotensin-aldosterone system (RAAS). Adapted from Encyclopaedia Brittanica.

ACE – Angiotensin-converting enzyme; *AGT* – Angiotensinogen; *AT1R* – Angiotensin II type I receptor; NaCl – Sodium chloride; H_2O – Water; K^+ - Potassium; H^+ - Hydrogen

In response to dehydration, haemorrhage, low blood pressure in renal glomerulus arterioles or sodium deficiency, an inactive form of renin is released from the kidneys. Inactive renin is activated in the bloodstream by either proteolytic or non-proteolytic mechanisms. Angiotensinogen (AGT), released by the liver at the same time as renin being released from the kidneys, is cleaved by the active renin to form angiotensin I (Ang I), which is further cleaved by the angiotensin-converting enzyme (ACE), released from the lungs, into angiotensin II (Ang II) (Munóz-Duranga *et al.,* 2016; Farrario & Strawn, 2006). The presence of AngII results in the increase of blood pressure until it returns to normal (120/80 mmHg) through four mechanisms: (a) by inducing arteriole vasoconstriction; (b) stimulation of the sympathetic nervous system; (c) renal action or (d) by stimulating the adrenal cortex, through the angiotensin II type I receptor (AT1R), to release aldosterone, another major effector of the RAAS. The latter stimulates the kidneys to increase salt (NaCl) and water (H_2O) reabsorption as well as increasing the secretion of potassium (K^+) and hydrogen (H^+) into the urine (Munóz-Duranga *et al.,* 2016) (Figure 1.2).

Previously, genes encoding parts of the RAAS have been associated with CAD (Zitouni *et al.,* 2018; Abd El-Aziz *et al.,* 2012; Shaker *et al.,* 2009). A study of Egyptian CAD patients and unaffected

controls found that the patient group (cases) showed a higher frequency of diabetes, family history of CAD, hypertension, increased waist-to-hip ratio and smoking, as well as homozygosity of the D-, T- and C-alleles of the angiotensin-converting enzyme (*ACE*), angiotensinogen (*AGT*) and angiotensin II type I receptor (*AT1R*) genes respectively, and the presence of these factors were shown to be associated with CAD in the patient group (cases) . The risk observed for CAD was increased by the presence of other risk factors such as diabetes, dyslipidaemia, hypertension, obesity and smoking (Abd El-Aziz *et al.,* 2012). Renin contributes to BP elevation, through the action of AngII (Gafane e*t al.,* 2016). Angiotensin II promotes vasoconstriction and promotes increased renin synthesis. Angiotensin II also has damaging effects on the vasculature by activating profibrotic and proinflammatory pathways (Gafane e*t al.,* 2016), as well as contributing to cardiac remodelling, plaque rupture and thrombosis (Burrel *et al.,* 2013). Increased levels of AngII also affects cell growth, immune response, inflammation, neuromodulation and proliferation (Zarebska *et al.,* 2013). The vasoconstrictive action and damaging effects of AngII can be overcome by the action of the angiotensin-converting enzyme 2 (*ACE2*) protein, a 805 amino acid long protein located on chromosome Xp22, that degrades AngII (Burrel *et al.,* 2013). In failing hearts and atherosclerotic vessels, increased levels of ACE2 have been found, associating ACE2 with CHD and heart failure (Burrel *et al.,* 2013).

Genes involved in the RAAS (Figure 1.2), such as *AGT*, *ACE* and *AT1R* have been found to be involved in CVD in Africa and are discussed below (Table 1.4).

Angiotensinogen (*AGT*)

The gene encoding angiotensinogen (AGT), spans 12 kilobases on chromosome 1 (1q42-q43) and consists of 5 exons. It is a member of the serpin gene superfamily, which is important for cardiovascular remodelling, and regulating blood pressure (BP) and the body's fluid and salt balance (Shaker *et al.,* 2009) (Figure 1.2).

The T allele of the rs699 SNP, located in exon 2 of *AGT*, results in a missense amino acid substitution (methionine to threonine) at residue 235 (M235T); the T allele of the rs4762 SNP, also located in exon 2 of *AGT* results in a missense amino acid substitution (threonine to methionine) at residue 174 (T174M) (Zarebska *et al.,* 2013).

A study of Egyptian CAD patients found the T allele of rs699 to be significantly associated with CAD and positively correlated with BP (Shaker *et al.* 2009). Higher frequencies of the rs699 T allele and rs4762 T allele were observed in Tunisian CAD patients when compared to controls (Abboud *et al.,* 2010), this was also observed by both Abd El-Aziz *et al.* (2012) and Motawi e*t al.* (2011) in Egyptian CAD patients. In association with dyslipidaemia, hypertension, smoking and T2DM, T homozygosity of these SNPs resulted in a 2.7-fold increased risk of CAD development (Abd El-Aziz *et al.,* 2012) (Table 1.4).

Angiotensin-converting enzyme (*ACE*)

The gene encoding angiotensin-converting enzyme (*ACE*), is located on chromosome 17 (17q23.3) and is 21kb long with 26 exons (Baroudi *et al.,* 2009). *ACE* is a member of the metallopeptidase protein family, responsible for cleaving the decapeptide angiotensin I (Ang I) to the octapeptide angiotensin II (Ang II) (Figure 1.2) (Baroudi *et al.,* 2009).

Alu repeats are short, interspersed elements (SINEs) that have recently amplified within the human genome and are believed to have an African origin (Batzer & Deininger, 2002). The deletion (D) of a 287bp Alu repeat at intron 16 of the *ACE* gene has been found to affect the activity of ACE intracellularly and in cardiovascular tissues (Abd El-Aziz *et al.,* 2012), and has been associated with an increased risk of CAD associated with this allele (Abd El-Aziz *et al.,* 2012). The D allele was also found to be more frequent in CAD patients than in controls in this population (Abd El-Aziz *et al.,* 2012). However, in Tunisian CAD patients, a higher frequency of the insertion (I) allele was observed in CAD patients and I homozygosity placed an individual at higher risk for CAD (Abboud *et al.,* 2010). In association with dyslipidaemia, hypertension, obesity, smoking and T2DM, D homozygosity of this SNP resulted in a 2.8-fold increased risk of CAD development (Abd El-Aziz *et al.,* 2012) (Table 1.4).

Table 1.4 A summary of genes that have been found to be associated with CVD in Africa.

Gene name	Gene function	Chr. Location	Chr coordinates (GRCh 38) (From - To)	SNP	Risk allele	Population studied	No. samples		Results	Reference
							Cases	Controls		
Angiotensinogen (*AGT*)	Cardiovascular remodelling; blood pressure control	1q42-q43	230702523 - 230714590	rs699	T	Egyptians	70	60	Associated with CAD	Shaker *et al.,* 2009
						Egyptians	230	119	Increased risk of CAD, more frequent in patients	Abd El-Aziz *et al.,* 2012
						Egyptians	100	50	More frequent in CAD patients	Motawi *et al*., 2011
						Tunisians	341	316	Associated with CAD (p=0.001)	Abboud *et al.,* 2010
				rs4762	T	Tunisians	341	316	Associated with CAD (p=0.026)	Abboud et al., 2010
Angiotensin II type I receptor (*AT1R*)	Vasoconstriction through AngII; mediates major cardiovascular effects of AngII	3q21-q25	148697871 - 148743003	rs5186	C	Egyptians	70	60	Associated with CAD	Shaker *et al.,* 2009
						Egyptians	230	119	Increased risk of CAD, more frequent in patients	Abd El-Aziz et al., 2012
						Tunisians	341	316	No difference in frequency between cases and controls	Abboud *et al*., 2010
Angiotensin converting enzyme (*ACE*)	Catalyzes conversion of AngI to the physiologically active AngII	17q23	63477061 - 63498380	rs4646994 (I/D)	D	Egyptians	230	119	Increased risk of CAD, more frequent in patients	Abd El-Aziz et al., 2012
					I	Tunisians	341	316	I homozygosity as risk for CAD (p=0.02)	Abboud et al., 2010

ACE – Angiotensin converting enzyme; *AGT* – Angiotensinogen; *AT1R* – Angiotensin II type I receptor; AngI – Angiotensin I; AngII – Angiotensin II; CAD - Coronary artery disease; CHD – Coronary heart disease; Chr – Chromosome; I/D – Insertion/deletion; MI – Myocardial infarction; SSA – sub-Saharan Africa

Angiotensin II type I receptor (*AT1R*)

The angiotensin II type I receptor (*AT1R*) gene, a 60 kilobase gene located on chromosome 3 (3q24) and consisting of 5 exons and 4 introns, is a member of the G-protein coupled receptor superfamily. It is responsible for vasoconstriction through its effector molecule, angiotensin II (AngII) (Figure 1.2), as well as cardiac and vessel hypertrophy (Kooffreh *et al.,* 2013).

The rs5186 SNP is an A to C substitution in *AT1R* that occurs at position 1166 (A1166C) in the 3' untranslated region of the *AT1R* gene (Ghogomu *et al.,* 2016; Mehri *et al.,* 2011). The SNP has been found to be associated with CAD in Egyptians (Shaker *et al.,* 2009) and this association was replicated by Abd El-Aziz *et al.* (2012) in a different sample from the same population. This SNP has been associated with an increased risk of diabetic nephropathy, heart disease and hypertension (Aung *et al.,* 2017), and through an epistatic interaction with the *ACE* insertion/deletion (I/D) polymorphism (rs46464994), at intron 16, *AT1R* is correlated with CHD (Aung *et al.,* 2017; Wang & Staessen, 2000). In Tunisians, there was no allele frequency difference observed between CAD cases and controls for the rs5186 C risk allele (Abboud *et al.,* 2010), suggesting that in this population the variant may not be involved in CAD pathogenesis.. In association with dyslipidaemia, hypertension, smoking and T2DM, C homozygosity of this SNP results in a 2.8-fold increased risk of CAD development (Table 1.4) (Abd El-Aziz *et al.,* 2012).

1.2.3 Concluding remarks - CVD

Cardiovascular disease is the second leading cause of death in SA with the main contributor being stroke, which is disproportionately high in the SA Black population. Black CVD patients have high rates of diseases that are risk factors for CVD, such as T2DM, obesity and hypertension. Genes encoded by the RAAS, involved in controlling BP and sodium reabsorption, have been associated with CVD and its associated diseases. However, very few studies have associated genes of the RAAS with CVD in Africa, suggesting that the causal variant has not been found yet or that these genes, which play a very small role in CVD risk, may act together to increase risk of disease.

1.3 Type 2 diabetes mellitus (T2DM)

Various forms of diabetes mellitus exist. Type 1 diabetes mellitus is an inherited form that exists as either type 1A (autoimmune) or type 1B (ketosis-prone type 2 diabetes), while T2DM is considered a disease of lifestyle, to which there is also a genetic component which may contribute to susceptibility, and accounts for 90.0% of all diabetes cases worldwide (Levitt, 2008; Kengne *et al.,* 2005). Gestational diabetes is a pregnancy specific form of diabetes that occurs in 2.0-10.0% of all pregnant females (Madubedube, 2015). Maturity onset diabetes of the young (MODY), a heterogeneous group of disorders, is inherited in an autosomal dominant mode and is characterised by familial hyperglycaemia (Madubedube, 2015).

Type 2 diabetes mellitus is a multifactorial, multiorgan metabolic disease, which occurs as a result of an interaction between environmental and genetic factors, with acute and chronic complications

(Mato *et al.,* 2016; Guewo-Fokeng *et al.,* 2015; Ouedemi *et al.,* 2009), and which is characterised by IR (Baroudi *et al.,* 2009). The disease occurs due to hyperglycaemia, when insufficient insulin is produced by the pancreas or when the body is unable to successfully utilise the insulin produced. It may also be as a result of reduced insulin secretion, increased peripheral IR or increased hepatic glucose output (Mato *et al.,* 2016; Madubedube, 2015; Guewo-Fokeng *et al.,* 2015; Nanfa *et al.,* 2015; Turki *et al.,* 2013a; Turki *et al.,* 2012; Ouedemi *et al.,* 2009), leading to impaired glucose tolerance (IGT) (Christensen e*t al.,* 2009). Disease occurrence is also modulated by age, dietary habits, lifestyle, obesity and physical activity (Ouedemi *et al.,* 2009).

The complex, multifactorial nature of T2DM and the interplay between diseases is evidenced by a systematic review which stated that 2 out of 3 diabetic patients will die as a result of cardiovascular complications, while approximately 30.0% of CVD treated individuals are diabetic (Kengne *et al.,* 2005). The authors also reported that 5-8% of T2DM patients may be affected by CHD, 15% of patients who previously had a stroke are diabetic and 4-28% of such patients have peripheral vascular disease (Kengne et *al.,* 2005). In Black African T2DM individuals, 44.4% had coexisting hypertension and dyslipidaemia, 42.5% had coexisting obesity and dyslipidaemia, and 33.1% had coexisting hypertension and obesity (Isezou & Ezunu, 2005).

The rising rates of T2DM worldwide are attributable to the increased presence of obesity, physical inactivity and urbanization (Ganu *et al.,* 2016; Levitt, 2008). Obesity, the most modifiable risk factors of T2DM, has been found to be a major risk factor for T2DM onset (Adeniyi *et al.,* 2015; Lontchi-Yimagou *et al.,* 2013; Bouhaha *et al.,* 2010a), as the development of T2DM and CVD risk factors is likely linked to body fat percentage and distribution (Borné *et al.,* 2014; Luo *et al.,* 2013; Jennings *et al.,* 2009; Meisinger *et al.,* 2006). Approximately 80.0% of all T2DM patients worldwide are obese (Thevenod *et al.,* 2008), while in SA, 87% of all T2DM in males and females is attributed to excess body fat (Goedecke e*t al.,* 2009).

The development of T2DM is preceded by IR (Grundy *et al.,* 2005). This notion is supported by the persistent presence of IR in T2DM patients (Savage *et al.,* 2005). However, T2DM only develops if insufficient insulin is produced by the patients' β-cells. Not all IR patients develop T2DM, but all T2DM patients are IR (Savage e*t al.,* 2005). The IR observed in T2DM patients is as a result of increased activation of the inflammatory pathways, which is intensified by hyperglycaemia, another characteristic of T2DM, promoting the chronic complications of disease (Lontchi-Yimagou *et al.,* 2013). Black females in SA have been found to be more IR than their Caucasian counterparts (Goedecke *et al.,* 2009). Disease progression of T2DM may lead to macrovascular (CVD and stroke) and microvascular (lower limb amputation, nephropathy leading to blindness, neuropathy, renal failure and retinopathy) complications (Thevenod *et al.,* 2008; van Tilburg *et al.,* 2001). Indeed, β-cell dysfunction has been observed mostly in African T2DM patients (Oli *et al.,* 2009; Kahn *et al.,* 2003; Jensen *et al.,* 2002; Bell *et al.,* 1996; Joffe *et al.,* 1992).

1.3.1 Epidemiology of type 2 diabetes mellitus in Africa

In 2013, 8.6% (522 600) of all deaths reported in Africa were diabetes-related (IDF, Diabetes Atlas, 2013). The burden of T2DM is high in SSA and continues to rise, surpassing that of developed countries. By 2006, 10.8 million people in SSA were estimated to have diabetes by the IDF Atlas (Levitt, 2008) and by 2010 T2DM accounted for 6% of the total deaths that year (Mbanya *et al.,* 2010).

The prevalence of T2DM in SSA is estimated to increase by 109.6% to 41.5 million people in 2035, from 19.8 million in 2013 (Guewo-Fokeng *et al.,* 2015), exceeding the 50% predicted worldwide increase (Roglic e*t al.,* 2005). In addition, the prevalence of IGT is expected to increase by 75.8% to 47.3 million in 2030, from 26.9 million in 2010 (Mbanya *et al.,* 2010). Early on in disease progression, levels of insulin are increased to compensate for the increased glucose levels. However, this increased level of insulin secretion is not able to manage the increased glucose levels accompanying disease progression for extended periods of time and thus a state of IR is reached (Wiegend e*t al.,* 2005). The progression from normal glucose tolerance to IGT, and thus T2DM, is modulated by IR (Wiegend *et al.,* 2005). Impaired glucose tolrance precedes T2DM and may thus be an indication of T2DM progression in high-risk individuals (Wiegend *et al.,* 2005; Motala *et al.,* 2005). It has also been found that individuals with IGT progress to T2DM 2-10-fold faster than individuals with normal glucose tolerance (Ferrannini *et al.,* 2004).

In 2014, the prevalence of T2DM in Africa (7.1%) was similar to that of the Americas (8.3%) and European countries (7.3%). However, death per 100 000 population was much higher in the African compared to the America and European countries (111.3 versus 72.6 and 55.7, respectively) due to poor disease management (WHO, 2016).

No clear gender distribution pattern of T2DM has been observed in any population (Tuei *et al.,* 2010). In SA (Cape Town), Sudan and Tanzania equal rates of T2DM have been reported in males and females. However, in urban Cameroon, Sudan and Tanzania T2DM is more common in males; while in rural Cameroon, Mali, Mauritania, SA (Durban) and Sudan, T2DM was more common in females (Tuei *et al.,* 2010).

1.3.1.1 T2DM in South Africa

In the year 2000, nearly 1.5 million people were estimated to be diabetic in SA and T2DM accounted for 2.6% of the total deaths in that year, making it the tenth leading cause of death in SA (Bradshaw *et al.,* 2007). There has been a steady increase in the prevalence of T2DM and IGT in SA (Table 1.5). From 1990 to 1993 the prevalence of T2DM in SA increased by 2-3%, and continued to rise (Mayosi *et al.,* 2009; Walker e*t al.,* 1997; Levitt e*t al.,* 1993) (Table 1.5). Between 1999 and 2006, T2DM prevalence increased by 38% in SA (Mayosi *et al.,* 2009) and remains the second leading cause of death in the SA adult population (WHO, 2016; Mayosi *et al.,* 2009) . 5In SA, Black individuals have the lowest prevalence of T2DM (Rheeder, 2006; Molleutze & Levitt, 2005) (Table

1.5). In 2003, the prevalence of T2DM in SA Xhosa- and Zulu-speaking individuals was reported at 8% and 5.3%, respectively (Motala *et al.,* 2003), and by 2006, the prevalence of T2DM in SA Blacks, as a whole, was reported at 6%, compard to Indians (13%), Coloureds (10.8%) and Caucasians (8.5%) (Molleutze & Levitt, 2005; Rheeder, 2006). However, with difficulty managing disease observed in Black SA T2DM patients (Gill *et al.,* 2009; Westaway *et al.,* 2005), this population is at increased risk of death due to T2DM (Gill *et al.,* 2009).

The high prevalence of IGT is indicative of a population at risk for the development of T2DM as nearly 70.0% of all individuals with IGT may progress to T2DM (Christensen *et al.,* 2009; Motala, 2002). A study in Cape Town found that IGT prevalence was 7.0% in 1969 and had increased to 12.2% in 1993 (Table 1.5) (Motala, 2002), compared to the overall prevalence of IGT reported for SA in the same year (7%) (Levitt *et al.,* 1993). However, it has been noted that in areas of high IGT prevalence there is usually a low prevalence of T2DM and vice versa (Motala *et al.,* 2003). Impaired glucose tolerance has also been associated with having an overweight or obese body mass index (BMI) and an obese WC (Christensen *et al.,* 2009).

Table 1.5 The epidemiology and rising trends of T2DM in South Africa.

Prevalence	Area/Town/Province	Year	Comments	Reference
		T2DM		
0.6 – 3.6	SA	1960 - 1980		McLarty *et al.,* 1990
2.9	Mamelodi, Pretoria	1973		Levitt *et al.,* 1993
3.6	Gugulethu, Cape Town			
4.8 – 8	SA	>1984		Motala *et al.,* 2003
2.2 – 3.6	SA	<1985		Christensen *et al.,* 2009; Levitt, 2008
4.8 – 6	SA	1990	Population based survey	Mayosi *et al.,* 2009
5.3	SA	1990s	Blacks	Walker *et al.,* 1997
8	SA	1993		Christensen *et al.,* 2009; Levitt e*t al.,* 1993
4.2	Durban	1993	More females (5,2%) than males (2,3%)	Omar *et al.,* 1993
4.8	QwaQwa	1995		Mollentze *et al.,* 1995
6	Mangaung			
2.45	Transkei	2001	Factory workers	Erasmus *et al.,* 2001a
4.8 / 6	Orange Free State	2003	Semi-urban/Urban	Motala *et al.,* 2003
5.3	Durban		Zulu	
8	Cape Town		Xhosa	
13	SA	2005 - 2006	Indians	Molleutze & Levitt, 2005; Rheeder, 2006
10.8			Coloureds	
8.5			Caucasians	
6			Blacks	
4.5	SA	2008	Individuals >15 years	WHO, 2008
3.9	Ubombo	2008		Motala *et al.,* 2008
26	Bellville South, Cape Town	2012	Coloureds; 28,7% in individuals <60 years	Erasmus *et al.,* 2012a
		IGT		
7	SA	1993		Levitt *et al.,* 1993
12.2	Cape Town	1969 -1993	Increase from 7,0 in 1963	Motala *et al.,* 2012
6.9	Durban	1993	More males (11,5%) than females (5,5%)	Omar *et al.,* 1993
2.7	Transkei	2001	Factory workers	Erasmus *et al.*, 2001a
4.8	KwaZulu Natal	2008	Similar between males and females	Motala *et al.,* 2008
15.3	Bellville South, Cape Town	2012	Coloureds	Erasmus *et al.,* 2012b

1.3.1.2 T2DM in sub-Saharan Africa

The earliest study to determine the prevalence of T2DM in SSA countries was conducted in Ghana in 1958 (Christensen *et al.,* 2009) and approximately 20 years later, in 1980, the prevalence of T2DM was still below 1% (Table 1.6) (McLarty *et al.,* 1990). By 1985 the prevalence of T2DM for most countries in SSA was still below 1%, with the exception of Côte D'Ivoire, Egypt and Northern Sudan (Table 1.6) and a clear rural-urban difference was starting to emerge. From the 1990s the prevalence of T2DM started increasing rapidly and will continue to do so, as suggested by the studies presented in Table 1.6. In 2011, the overall prevalence of T2DM in SSA was reported to range from 0-16% (Dalal *et al.,* 2011). The estimated prevalence of T2DM in African populations increased from 3 million individuals in 1994 to 7.1 million individuals in 2000 (Tuei *et al.,* 2010). Particular African countries have also reported high numbers of T2DM patients (SA, 6.5 million; Nigeria, 3.9 million; Ethiopia, 1.9 million and Tanzania, 1.7 million) (IDF, 2013). The prevalence of IGT is also increasing with an overall estimate of 9.1% for Africa in 2013 (Table 1.6) (IDF, 2013).

Table 1.6 The epidemiology and rising trends of T2DM in sub-Saharan Africa.

Prevalence	Country	Year	Comments	Reference
T2DM				
0.4	Ghana	1958	Urban	Christensen *et al.,* 2009
<1	SSA	1960 – 1980		McLarty *et al.,* 1990
5.7	Côte D'Ivoire			
0.5 / 2.5	Tanzania	1984	Rural, Sukuma/Haya tribe	Ahren *et al.,* 1984
1.9			Urban, Mwanza Town	
<1	SSA	<1985	Rural (Ethiopia, Ghana, Lesotho, Malawi, Uganda)	Ahren *et al.,* 1984; Levitt, 2008
0.6 – 1.2			Urban Africa	
5.7	Ivory Coast	<1985		Levitt, 2008
0	Togo	>1984		Motala *et al.,* 2003
9.3	Egypt			
10.4	Northern Sudan			
0.3	Gambia	1990s		Christensen *et al.,* 2009
2	Nigeria	1990s		Cooper *et al.,*1997
3.4 – 8.3	Northern Sudan	1990s		Elgabir *et al.,* 1996 & 1998
6.8	Tanzania	1996	Males, Increase from 3,3 in 1980	Motala, 2002
4.8			Females, Increase from 0,9 in 1980	
6.3	Ghana	2002		Amoah *et al.,* 2002
6.1	Guinea	2008		Balde *et al.,* 1997
4	West Africa	2008	Urban	Abubakari *et al.,* 2008
2.6			Rural	
4.2	Kenya	2009		Christensen *et al.,* 2009
10	Tunisia	2009		Baroudi *et al.,* 2009
14.3	Africa	2012		Crowther & Norris, 2012
3.6 / 5.5	Ghana	2016	Rural males/females	Agyemang *et al.,* 2016
10.3 / 9.2			Urban males/females	
0 – 16	SSA	<2011	Meta-analysis	Dalal *et al.,* 2011
15.4	Reunion Island	2013		IDF, 2013
12.1	Seychelles			
10.7	Gabon			
9.7	Zimbabwe			
IGT				
2.9 – 7.9	Northern Sudan	1990s		Elgabir *et al.,* 1996 & 1998
1.8	Cameroon	2003	Urban	Motala *et al.,* 2003
13.1	Egypt		Rural	
13.4	Ghana	2008		Balde *et al.,* 1997
12	Kenya	2009		Christensen *et al.,* 2009
9.1	Africa	2013		IDF, 2013

1.3.2 Genetics of type 2 diabetes mellitus

Although the aetiology of T2DM comprises a genetic component, genetic studies have thus far only explained ~15% of the heritability of T2DM (Groop & Pociot, 2014). The disease is believed to arise from the inheritance of a variety of susceptibility genes that exert a partial effect on T2DM development, and only when combined with specific risk factors is the disease expressed (Ouedemi *et al.,* 2009; Freeman & Cox, 2006). Genes that confer risk of T2DM are called "diabetogenes" and have been found to encode proteins responsible for glucose metabolism, insulin activity and/or signal transduction for insulin action (Ouedemi *et al.,* 2009).

The risk of developing T2DM is greater on the offspring if the mother has T2DM (Groop & Pociot, 2014), suggesting that DNA methylation and imprinting contribute to unique risk allele parent-of-origin transmission (Kong *et al.,* 2009). Offspring with either parent affected by T2DM have a 40% risk of disease development, whereas having two T2DM affected parents pushes the lifetime risk up to 70% (Ahlqvist *et al.,* 2011; Poulson *et al.,* 1999). Offspring of diabetic parents are also more likely to suffer from β-cell dysfunction and glucose intolerance (Sobngwi *et al.,* 2002; Mbanya *et al.,* 2000). In a study of Black SA T2DM individuals, family history, and specifically maternal T2DM, was found to play an important role in the heritability of T2DM, with 27.3% reporting a family history (Erasmus *et al.,* 2001b). Of the 27.3% that reported a positive family history, 82.6% had one affected family member and 17.4% had two or more affected family members, 87.8% of which was a first-degree family member. Maternal diabetes was reported for 64.7% of the affected individuals, while paternal diabetes was reported for only 27% of the affected individuals (Erasmus *et al.,* 2001b). Both maternal and paternal T2DM was reported for 6.6% of the affected individuals (Erasmus *et al.,* 2001b).

Rare monogenic forms of diabetes observed in families has made it evident that there is a genetic component to T2DM (Gottlied, 1980). Genetic predisposition to T2DM and abnormal glucose tolerance has been shown in twin studies, with heritability ranging from 26-77% (Poulsen *et al.,* 1999; Carlsson *et al.,* 2013) and 30-61%, respectively (Poulsen *et al.,* 1999). This notion is strengthened by discordant rates of T2DM between mono- and dizygotic twins (Newman *et al.,* 1987). Monozygotic twins have a higher concordance than dizygotic twins for both T2DM and abnormal glucose (50% vs. 37% and 43% vs. 37%, respectively) (Poulsen *et al.,* 1999). It has also been shown that 18% of the genetic predisposition to T2DM is shared with the genetic predisposition to BMI (Carlsson *et al.,* 2013). The high prevalence of T2DM in certain ethnic groups and admixture modifications (Stern & O'Connel, 1999; Knowler *et al.,* 1990; Zimmet *et al.,* 1983) of individuals in the same areas allows us to investigate gene-environment interactions that may influence susceptibility to T2DM (Zimmet *et al.,* 1983). Susceptibility loci identified in recently admixed populations give an indication of genes that may be present in the ancestry population.

A meta-analysis of 13 Tunisian studies (4608 controls and 5080 cases) found that SNPs in the genes transcription factor 7-like 2 (*TCF7L2*), tumour necrosis factor α (*TNF*α) and *ACE* amongst others were associated with T2DM in Tunisians with OR ranging from 1.43 – 6.72 (Berhouma *et al.,* 2012).

A meta-analysis of 60 African studies (14 302 controls and 20 464 cases) found associations between *ACE*, melanocortin 4 receptor (*MC4R*), *TCF7L2* and *TNFα* amongst others and T2DM (Yako *et al.,* 2016). A summary of all the genes discussed below that have been found to be involved in T2DM in Africa are presented in Table 1.7.

1.3.2.1 Transcription factor 7-like 2 (*TCF7L2*)

The transcription factor 7-like 2 (*TCF7L2*) gene is located on chromosome 10 (10q25.3) and consists of 19 exons that span across a 200kb-long DNA region. The protein encoded by *TCF7L2* is a member of the T-cell specific family and encodes a high-mobility group box-containing transcription factor which is an effector in the Wnt signalling pathway. The Wnt signalling pathway has been shown to downregulate adipogenesis in adipocytes via TCF7L2 (Nguimmo-Metsadjio *et al.,* 2017).

The rs7903146 SNP of *TCF7L2* is the most consistently replicated locus associated with T2DM in SSA populations (Table 1.7) (Adeyemo *et al.,* 2015; Turki *et al.,* 2013a; Berhouma *et al.,* 2012), as well as in AfAms, Hispanics, Asians, American Indians (Florez *et al.,* 2006), Danes and Icelandics (Helgason *et al.,* 2007). 777The T allele of the rs7903146 SNP has consistently been described as the risk allele for T2DM in the Sudanese (Ibrahim *et al.,* 2016) and Ghanaian (Danquah e*t al.,* 2013) populations, with individuals harbouring at least one T allele being at a 39% increased risk of developing T2DM (Table 1.7) (Danquah *et al.,* 2013). These fidnings were supported by a meta-analysis of 60 African studies (14 302 controls and 20 464 cases), which found that cases harbouring the TT genotype of the rs7903146 SNP in *TCF7L2* had 40-45% greater odds of developing T2DM than those that were C homozygotes (Yako *et al.,* 2016). A Tunisian study, comprising 884 cases and 513 controls, found that the T allele of rs7903146 increased T2DM susceptibility (OR=1.25, 95%CI=1.06-1.47, p=0.006), with TT carriers having a 56% risk of T2DM susceptibility compared to CC carriers (Table 1.7) (Ezzidi *et al.,* 2009). However, individual studies have reported contradictory findings. For example, in the Cameroonian population, the C allele (OR=16.56), and particularly the CT genotype, has been associated with increased risk of T2DM, while the TT genotype has been found to be protective against T2DM (OR=0.054) (Table 1.7) (Guewo-Fokeng *et al.,* 2015; Ngwa *et al.,* 2015). As observed in Cameroonians (Guewo-Fokeng *et al.,* 2015; Ngwa *et al.,* 2015), the C allele and CT genotype was also associated with T2DM in a SA Zulu population (Table 1.7) (Pirie *et al.,* 2010), suggesting that the C allele is heterozygous dominant and is associated with disease in this population.

In the SA Coloured population, the rs7903146 T allele (p=0.011) was more frequent in T2DM patients and the CT or TT genotypes (p=0.025) were significantly associated with T2DM (Yako *et al.,* 2015a). Under an additive model, the rs7903146 SNP was nominally associated with an increased risk of T2DM (OR=1.43, 95%CI=1.00-2.04, p=0.053) (Table 1.7) (Yako *et al.,* 2015a). A study in Lebanese and Tunisian Arabs found that *TCF7L2* rs7903146 TT genotype was significantly associated with T2DM in both populations (OR 1.38, 95% CI 1.2-1.59, p<0001 and OR 1.36, 95%CI 1.18-1.86, p<0.001, respectively) 7 (Mtiraoui *et al.,* 2012a). However, in another study of 331 Tunisian cases

and 403 Tunisian controls, rs7903146 was found to have no significant differences between the two groups (Kifagi *et al.,* 2011). In Tunisians the TT genotype was associated with T2DM, but not obesity, which modulated the effect of this genotype (TT) on T2DM risk 7 (Bouhaha *et al.,* 2010a). Thus, TT was only associated with T2DM in nonobese individuals. Under a dominant (OR=1.68, 95%CI=1.39-2.03, p=9.57x10^{-8}), recessive (OR=1.65, 95%CI=1.31-2.08, p=1.98x10^{-5}) and log-additive (OR=1.47, 95%CI=1.29-1.66, p=4.06x10^{-9}) model, rs7903146 was significantly associated with T2DM in Moroccans (1 193 cases and 1 055 controls) (Table 1.7) (Cauchi *et al.,* 2012). It was also associated with T2DM in Tunisians (1 446 cases and 943 controls) under a dominant (OR=1.32, 95%CI=1.06-1.63, p=0.01) and log-additive (OR=1.39, 95%CI=1.04-1.37, p=0.01) model (Table 1.7) (Cauchi *et al.,* 2012). The rs7903146 SNP is located in intron 4 of the *TCF7L2* gene and although the functionality is still unclear, it is believed to affect insulin secretion (Danquah *et al.,* 2016).

Another study in Tunisian Arabs (900 cases and 875 controls) found several SNPs in intron 4 of the *TCF7L2* gene, with unclear functional roles, to be associated with T2DM. The minor allele frequencies (MAF) of these SNPs were significantly higher in cases than in controls. These SNPs included rs4506565 (p=2.4x10^{-8}), rs7903146 (p=1.2x10^{-6}), rs12243326 (p=8.4x10^{-8}) and rs12255372 (p=1.1x10^{-5}) 7 (Turki *et al.,* 2013a). In a study of 566 SA Coloured individuals, neither rs7903146 or rs12255372 were associated with T2DM (Table 1.7) (Madubedube, 2015). The association of the *TCF7L2* rs12255372 TT genotype with T2DM in Arab Africans replicated a case-control study in the SA Zulu population (178 cases and 200 controls) (OR 1.84, 95%CI 1.19-2.83, p=0.0035) (Pirie *et al.,* 2010) and a replication study (621 cases and 448 controls) and meta-analysis in West African populations (Tong *et al.,* 2009; Helgason *et al.,* 2007). The association of rs12255372 was later replicated in two case-controls studies (60 cases and 60 controls each) in a Cameroonian population (OR 3.92, 95%CI 2.04-7.67, p<0.0001) (Nanfa *et al.,* 2015; Ngwa *et al.,* 2015) (Table 1.7). A study of 240 cases and 128 controls in Sudan found that the rs7903146 TT genotype (OR 1.69, 95%CI 1.21-2.38, p=0.002) and rs12255372 TT genotype (OR 1.72, 95% CI 1.2-2.41, p=0.003) of *TCF7L2* were associated with T2DM (Ibrahim *et al.,* 2016).

1.3.2.2 Tumour necrosis factor-alpha (*TNFα*)

The *TNFα* gene, a 3 kilobase gene located on chromosome 6 (6p21.33) and consisting of 4 exons, is a member of the tumour necrosis family type II transmembrane protein *TNFα* is a proinflammatory cytokine that is involved in the acute phase reaction in systemic inflammation (Berhouma *et al.,* 2012).

The rs1800629 SNP in the *TNFα* gene is a functional G to A substitution at position 308 upstream from the transcription initiation site in the promoter (Bouhaha *et al.,* 2010b). The AG or AA genotype of rs1800629 leads to the overexpression of TNFα, which plays a role in the transition of IR to T2DM and has also been reported in obese subjects (Bouhaha *et al.,* 2010b). Increased levels of *TNF*α directly correlates with obesity grade by contributing to IR and thus potentially playing a role in T2DM etiology (Bouhaha *et al.,* 2010b). Two separate meta-analyses of Tunisian studies, which included

20 464 and 5 080 cases, and 14 302 and 5 608 controls, respectively, associated SNP rs1800630 in the *TNFα* gene with T2DM (Table 1.7) (Yako *et al.,* 2016; Berhouma *et al.,* 2012). However, this association was not seen in an earlier study in Tunisians 7 (Bouhaha *et al.,* 2010b).

The rs1800630 is a C to A substitution at position 386 in the promoter region of *TNFα* (Kallel *et al.,* 2013). The CA and AA genotypes were more frequent in the cases (35.5%) than controls (22.3%) (OR=1.91, 95%CI=1.31-2.80, p=0.001) 7 (Kallel *et al.,* 2013), however no association analysis results have been reported.

1.3.2.3 Angiotensin-converting enzyme (*ACE*)

As discussed in section 1.2.2.3, the angiotensin-converting enzyme (*ACE*) is a metallopeptidase protein responsible for cleaving angiotensin I (Ang I) to angiotensin II (Ang II).

In a meta-analysis of 13 Tunisian studies (5 080 cases and 4 608 controls), the deletion (D) of a 287bp Alu repeat was associated with T2DM (Berhouma e*t al.,* 2012), in support of findings reported by other studies. In a study of 272 (172 cases and 100 controls) Arabs and Berbers from Tunisia, the DD genotype was associated with T2DM susceptibility (Baroudi *et al.,* 2009). This finding was replicated in two other subsets of the same population, consisting of 115 cases and 116 controls (Mehri e*t al.,* 2010a), and 114 cases and 175 controls (Mehri e*t al.,* 2010b): D allele: OR=3.08, 95%CI=2.09-4.51, p<0.0001 and OR=3.10, 95%CI=2.1-4.4, p<0.001, respectively; DD genotype: OR=4.93, 95%CI=2.71-8.97, p<0.0001 and OR=7.2, 95%CI==3.5-14.8, p<0.001, respectively 7, and is thought to act synergistically with the *MTHFR* C677T SNP (Mehri *et al.,* 2010a). The *ACE* DD genotype, in conjunction with hypertension, was found to be the most significant T2DM contributor (Mehri *et al.,* 2010b).

However, in a study of 203 Egyptian cases and 311 Egyptian controls, the I allele was more frequent in cases than in controls (68.8% vs 52.5%) and the ID and II genotypes were significantly associated with increased T2DM susceptibility (OR=2.0, p=0.0007) 7 (Settin *et al.,* 2015).

1.3.2.4 Fat-mass and obesity associated (*FTO*) gene

The *FTO* gene, a 457kb gene located on chromosome 16 (16q12.2) and consisting of 9 exons, is a member of the alphaketoglutarate-dependent hydrolase superfamily, is involved in DNA demethylation (Yang *et al.,* 2017).

The rs9939609 SNP results in a functional T to A substitution, controlling food intake and preference, in intron 1 of the *FTO* gene (Leońiska-Duniec *et al.,* 2018). Individuals without this SNP manage to limit their food intake while those with this SNP have a greater appetite (Leońiska-Duniec *et al.,* 2018). In a study of participants of Zulu ethnicity (178 cases and 200 ethnically matched controls), the TT genotype of SNP rs9939609 had no association with T2DM (Table 1.7) (Pirie e*t al.,* 2010). The rs17817499 SNP was not associated with T2DM in North Africans, even after adjustment for BMI in a meta-analysis (Table 1.7) (Yang e*t al.,* 2017).

However, in SA Coloured individuals, under a log additive model, the minor allele (T) of the rs9941349 SNP of the *FTO* gene was associated with T2DM (OR=1.40, 95%CI=1.00-1.47, p=0.049) (Table 1.7) (Madubedube, 2015). Similar values were observed in the same population by Yako *et al.* (2015a) (Table 1.7).

A study conducted in Moroccans (1 193 cases and 1 055 controls) found that the rs1421085 SNP of the *FTO* gene is significantly associated with T2DM in Moroccans under a dominant (OR=1.34, 95%CI=1.03-1.49, p=0.02) and log-additive (OR=1.18, 95%CI=1.04-1.35, p=0.01) model (Table 1.7), while under a recessive model it was only nominally associated with T2DM (p=0.07) (Cauchi *et al.,* 2012).

1.3.2.5 Melanocortin 4 receptor (*MC4R*)

The *MC4R* gene, a 2 kilobase gene located on chromosome 18 (18q21.32) and consisting of 1 exon, is G-protein coupled receptor of the melanocortin receptor family, that regulates energy homeostasis and food intake (Logan e*t al.,* 2016).

The rs17782313 SNP, a C to T substitution near *MC4R* with unknown function*,* was significantly associated with T2DM in Moroccans (1 193 cases and 1 055 controls) under a log-additive model (OR=1.18, 95%CI=1.00-1.39, p=0.05) (Table 1.7), while under a dominant and recessive model it was only nominally associated (p=0.10 and p=0.09, respectively) (Cauchi *et al.,* 2012). In Tunisians (1 446 cases and 943 controls), the C genotype of this SNP was only nominally associated with T2DM under a dominant model (p=0.08) (Cauchi *et al.,* 2012).

A study 900 Tunisian cases and 748 Tunisian controls found that the T allele of the rs1942872 SNP 3' of *MC4R* was more frequent in cases than in controls and that it was significantly associated with T2DM 7 (Turki *et al.,* 2013b).

Table 1.7 A summary of genes found to be associated with T2DM in Africa.

Gene name	Gene function	Chr. Location	Chr coordinates (GRcH 38) (From - To)	SNP	Risk allele	Population studied	No. samples		Results	Reference
							Cases	Controls		
Transcrption factor 7-like 2 (*TCF7L2*)	Encodes effector for Wnt signalling pathway	10q25.3	112950220 - 113167678	rs7903146	T	Africans	20 464	14 302	TT genotype 40-45% higher odds of developing T2DM	Yako *et al.*, 2016
						Sudanese	240	128	Associated (OR 1.69, 95% CI 1.21-2.38, p=0.002)	Ibrahim *et al.*, 2016
						Tunisians	900	883	Associated	Turki *et al.*, 2014
						Tunisians	Meta-analysis		Associated	Berhouma *et al.*, 2012
						Moroccans	1 193	1 055	Signicantly associated under a dominant, recessive and log-additive model	Cauchi *et al.*, 2012
						Tunisians	1 446	943	Significantly associated under a dominant and log-additive model	
						Africans	1 035	740	Most significantly associated locus	Adeyemo *et al.*, 2015
						SA Coloureds	566		No association with T2DM or IR	Madubedube, 2015
						SA Coloureds	566		T allele more prevalent in cases than controls (32.2% vs 24.2%). Nominal under additive model: OR 1.43, 95% CI 1.00 - 2.04, p=0.053	Yako *et al., 2015b*
					C	SA Zulu	178	200	CT risk genotype	Pirie *et al.*, 2010
						Cameroonians	37 / 60	37 / 60	C risk allele (OR 16.56), CT risk genotype (OR18.56), T allele protective	Guewo-Fokeng *et al.*, 2015; Ngwa *et al.*, 2015
				rs12243326	C	Tunisians	900	883	Gender-independent association	Turki et al., 2014
				rs12255372	T	Sudanese	240	128	Associated (OR 1.7, 95% CI 1.2-2.41, p=0.003)	Ibrahim et al., 2016
						West Africans	621	448	TT genotype associated	Tong *et al.*, 2009
						Cameroonians	60 / 60	60 / 60	Associated (OR 3.92, 95% CI 2.04 - 7.67)	Nanfa *et al.*, 2015; Ngwa *et al.*, 2015
						SA Zulu	178	200	Associated (OR 1.84, 95% CI 1.19 - 2.83, p=0.0035)	Pirie et al., 2010
						Tunisians	900	883	Gender-independent association	Turki et al., 2014
						SA Coloureds	566		No association with T2DM or IR	Madubedube, 2015
Tumor necrosis fator alpha (*TNFa*)	Proinflammatory cytokine	6p21.3	31575567 - 31578336	rs1800629	A	Tunisians	Meta-analysis		Associated	Yako *et al.*, 2016
						Tunisians	Meta-analysis		Associated	Berhouma *et al.*, 2012
Angiotensin-converting enzyme (*ACE*)	Cleaves AngI to AngII	7q23.3	63477061 - 63498380	rs4646994	D	Tunisians	Meta-analysis		Associated	Berhouma et al., 2012

Fat-mass and obesity associated (*FTO*)	Energy homeostasis and food intake	18q21.32	53701692 - 54158512	rs9939609	T	SA Zulu	178	200	No significant association	Pirie et al., 2010
				rs17817499	A	North Africans	Meta-analysis		No association, even after adjustment for BMI	Yang *et al.*, 2017
				rs9941349	T	SA Coloureds	566		Associated under a log-additive model (OR 1.4, 95% CI 1 - 1.96, p=0.049)	Madubedube, 2015
						SA Coloureds	566		Nominal under log-additive model (OR 1.43, 95% CI 1 - 1.96, p=0.052)	Yako *et al.*, 2015b
				rs1421085	C	Moroccans	1 193	1 055	Significantly associated under dominant and log-additive models	Cauchi et al., 2012
Melanocortin 4 receptor (*MC4R*)	Energy homeostasis and food intake	18q21.32	60371110 - 60372775	rs17782313	C	Moroccans	1 193	1 055	Significantly associated under a log-additive model; nominal under dominant and recessive models	Cauchi et al., 2012

Unless specified, ethnicity of SA Blacks was not stated in the article.

ACE – Angiotensin converting enzyme; AngI – Angiotensin I; AngII – Angiotensin II; Chr - Chromosome; *FTO* – Fat-mass and obesity associated; I/D – Insertion/deletion; OR – Odds ratio; SA – South Africa; SNP – Single nucleotide polymorphism; T2DM – Type 2 diabetes mellitus; *TCF7L2* -Transcription factor 7-like 2; *TNFα* – Tumour necrosis factor alpha; 95% CI – 95% Confidence interval

1.3.3 Concluding remarks – T2DM

Type 2 diabetes mellitus is the most common form of diabetes worldwide and can be in part due to obesity, a major contributor, and the accompanying IR and inflammation. Type 2 diabetes mellitus is often preceded by and/or accompanied by obesity, hypertension and dyslipidaemia. In SSA, *TCF7L2* is the most consistently replicated gene associated with T2DM, more specifically the rs7903146 SNP. Based on studies reviewed here, *TCF7L2* is a promising target for genetic studies in our populations. *TCF7L2* is possibly the gene with the biggest effect on T2DM risk and other variants in other genes may have a very small influence on T2DM risk. Although *FTO* was not associated with T2DM in SA Zulus, association has been seen in SA Coloured individuals and it may be important in SA Xhosas. *FTO* has also been associated with obesity, the most modifiable risk factor of T2DM.

1.4 Obesity

Obesity is a chronic state of inflammation that results from a continuing imbalance between energy intake and expenditure (Mato *et al.,* 2016; Ngwa *et al.,* 2015; Joffe *et al.,* 2010), characterised by increased body fat stores (Tuei *et al.,* 2010). This energy imbalance affects feeding behaviours and time spent active (Mato *et al.,* 2016; Ngwa *et al.,* 2015), and often precedes other risk factors for CVD (Deedwania & Gupta, 2006; Despres & Lemieux, 2006). Other factors associated with obesity include aging, economic transition, genetics, globalization, industrialization, physical inactivity and urbanization (Adeyemo *et al.,* 2015), as well as educational attainment, female gender and tobacco use (Adeniyi *et al.,* 2015). Currently, there are three measures of obesity used to assess health risk, namely BMI, WC and waist-to-hip ratio (Matsha *et al.,* 2013). The gold standard of obesity measurement is BMI, where weight (in kg) is divided by the square root of the height (Adab *et al.,* 2018). However, BMI measurement has a low sensitivity, is unable to differentiate between lean and fat mass, provides no details of fat distribution or no clear insight into the correlation between total body fat and obesiy risk (Adab *et al.,* 2018). Determining body fat distribution is essential as the distribution of fat around the abdomen/central body region is a major predictor of disease risk (Adab *et al.,* 2018). Both WC and waist-to-hip ratios are measures of central obesity and are better at predicting cardiometabolic disease, mortality and visceral adiposity than BMI (Adab *et al.,* 2018; Matsha *et al.,* 2013); however, population-specific cut-points are lacking and generalized cut-points are not good predictors in African populations (Matsha *et al.,* 2013; Crowther & Norris, 2012).

Abdominal/visceral obesity is the distribution of fat around the central (stomach) region, while peripheral obesity is the distribution of fat in the lower body (Keswell *et al.,* 2016). Visceral/abdominal obesity, measured by waist circumference, rather than generalised obesity which is measured by body mass index (BMI), is a risk factor for CVD and T2DM (Alberti *et al.,* 2009; Yusuf *et al.,* 2005; Ohlson *et al.,* 1985). The distribution of body fat, rather than percent body fat, is also a risk factor for CVD and T2DM (Borné *et al.,* 2014; Luo *et al.,* 2013; Jennings *et al.,* 2009; Meissinger *et al.,* 2006), as the distribution of body fat is also a greater predictor of dyslipidaemia and IR than percent body

fat (Keswell *et al.,* 2016). Abdominal obesity is more common in males and is associated with a higher risk of IHD, while peripheral obesity is more common in females and carries a lower risk of CVD (Sengwayo *et al.,* 2013).

Increase in body fat promotes the expression and secretion of inflammatory cytokines such as TNFα. Increased levels of TNFα during inflammation promote proinflammatory cytokine secretion (Gallagher *et al.,* 2010; Wang & Trayhurn, 2006) and adipocyte apoptosis, which modifies adipose tissue mass in an effort to limit the development or extent of obesity (Joffe *et al.,* 2010; Prins *et al.,* 1997). Tumour necrosis factor-α has also been found to promote IR (Hotamisligil *et al.,* 1996), by inhibiting the insulin receptor substrate I pathway (Boura-Halfon & Zick, 2009; Stephens *et al.,* 1997; Hotamisligil *et al.,* 1996; Hotamisligil *et al.,* 1994). Insulin resistance is important in the pathophysiology and control of T2DM and is associated with obesity, a risk factor for T2DM (Joffe *et al.,* 2010; Ntyintyane *et al.,* 2006). Insulin resistance and vascular resistance during obesity are mediated by dysfunctional endothelial and microvascular systems, present early on in obese subjects (Gallagher *et al.,* 2010; Villela *et al.,* 2009; Faloia *et al.,* 2000). Reduced insulin sensitivity and higher levels of blood insulin concentrations, associated with increased weight, have been associated with obesity (Makuyana *et al.,* 2004). Raised serum triacylglycerol and decreased levels of HDL-C have also been associated with increased levels of TNFα (Joffe *et al.,* 2010).

1.4.1 Epidemiology of obesity in Africa

1.4.1.1 Obesity in South Africa

In SA it has been found that obesity is extremely common in females (Keswell *et al.,* 2016). Black females have been found to be more obese than their Caucasian counterparts, with more peripheral fat seen in Black females compared to central fat in Caucasian females (Keswell *et al.,* 2016). Over a period of 15 years, from 1998 to 2013, the prevalence of overweight and obesity in SA males increased by 1.5% (from 29.0% in 1998 to 30.7% in 2013) and by 8% in SA females (from 56.0% in 1998 to 64% in 2013) (Table 1.8) (Puoane *et al.,* 2002; Shisana *et al.,* 2013). However, no clear gender pattern is observed. In the Black, Coloured and Indian populations of SA, females were found to be more obese than males, while in the Caucasian population of SA, males have been found to be more obese than females (Table 1.8) (Shisana *et al.,* 2013; Sengwayo *et al.,* 2013). In the Heart of Soweto Study, obesity was found to be the most prevalent CVD risk factor, with more females than males affected (Adeniyi *et al.,* 2015; Tibazarwa *et al.,* 2009; Voster, 2002). In 2008, physical inactivity, a known factor in obesity onset and diagnosis, was reported in 46-56% of the SA population (NDoH, 2013), with overweight and obesity, partly as a result of physical inactivity, at 58.5% and 71.8%, respectively (NDoH, 2013; Bradshaw *et al.,* 2007). The 2008 WHO estimate of obesity was much lower than that of the NDoH (31.3% by WHO compared to 71.8% by the NDoH) (Table 1.8) (NDoH, 2013; WHO, 2008).

The prevalence of T2DM and hypertension in Black obese SA females has been found to be higher than in their Caucasian counterparts (7% vs 3.6% and 30% vs 15%, respectively), who are prone to CHD and hypercholesterolaemia (Schutte *et al.,* 2008; Seedat, 1983). However, studies have suggested that within the obese population of SA, up to 40% may not be at a greater risk of developing CVD and T2DM, as they are metabolically normal (Stefan *et al.,* 2008; Brochu *et al.,* 2001).

Table 1.8 The epidemiology and rising trends of obesity in South Africa.

Prevalence	Area/Town /Province	Year	Comments	Reference
22.2	Transkei	2001	Factory workers	Erasmus *et al.,* 2001b
42.5	SA	2005	Blacks	Isezou & Ezunu, 2005
58.5	SA	2008	Overweight	NDoH, 2013; Bradshaw *et al.,* 2007
71.8			Obese	
31.3	SA	2008	Overall	WHO, 2008
42	SA	2013	Highest prevalence in SSA females	Ajayi *et al.,* 2016
43	Soweto	2013	More prevalent in Black females (55.0%) than males (23.0%)	Adeniyi *et al.,* 2015; Tibazarwa *et al.,* 2009; Vorster, 2002
40 – 70	Soweto	2013	Obese BMI	Logan *et al.,* 2016
30.7	SA	2013	Overweight/obese males, Increase from 29,0% in 1998	Puaone *et al.,* 2012; Shisana *et al.,* 2013
64			Overweight/obese females; Increase from 56,0 in 1998	
9.4 / 15.1 / 7.6 / 26.5	SA	2013	Males; Black/Coloured/Indian/Caucasian	Shisana *et al.,* 2013
39.9 / 34.9 / 32.4 / 22.7			Females; Black/Coloured/Indian/Caucasian	
25.9 – 54.3	SA	2013	Females	Sengwayo *et al.,* 2013
3 – 20.4			Males	
60.2	Mthatha	2015		Adeniyi *et al.,* 2015
85 / 54	SA	2016	Overweight + Obese/Obese only	Ajayi *et al.,* 2016
27	SA	2017	Overall	Keates *et al.,* 2017

1.4.1.2 Obesity in sub-Saharan Africa

A meta-analysis of NCDs in SSA reported an overall prevalence of obesity ranging from 0.4-43% between all countries (Dalal *et al.,* 2005) and in 2014, WHO estimated that in most African countries, less than 15.0% of the adult population is obese (Table 1.9) (Keates *et al.,* 2017). As shown in Table 1.9, obesity is rising in SSA and there is a clear rural-urban difference in the prevalence of obesity.

Contrary to what is seen in other countries, in SSA countries, obesity is more prevalent in females (10-15%) than in males (4-5%) (Keates e*t al.,* 2017). In 1995, in 18 SSA countries, 1-7.1% of females were found to have an obese BMI, but this figure was greatly inflated in SA females (31%) (table 1.9) (Levitt, 2008). Overall for Africa, obesity prevalence was highest in females, ranging from 10.8% in Ethiopia to 34.7% in Ghana, compared to males (2.0% in Ethiopia, 12.2% in the Democratic Republic of Congo and 13.8% in Cameroon) (Table 18) (Peer *et al.,* 2014a). In 2013, females in SA had the highest prevalence of obesity (42.0%) while their counterparts in Ethiopia had the lowest (1.8%) (Table 1.9) (Ajayi *et al.,* 2016). In males the highest prevalence was reported in Equatorial Guinea (25.0%) and the lowest in Uganda (1.8%) (Table 1.9) (Ajayi *et al.,* 2016).

Interestingly, the prevalence of obesity in Cameroon follows that seen in non-African countries. From 1994 to 2003 the prevalence of an overweight BMI in rural Cameroon increased by 54% in females and 82% in males and that of abdominal obesity in urban Cameroon increased by 32% in females and 190.0% in males (Table 1.9) (Fezeu *et al.,* 2008). By 2010, the prevalence of abdominal obesity in Cameroon was 67% in females and 18.0% in males (Table 1.9) (Tuei *et al.,* 2010). Although the observed increase of obesity is greater in males, the overall prevalence of obesity in males remains lower than that observed in females. This could possibly be explained by the fact that males generally do more physical work, but with the adoption of westernised diets and lifestyles, males are more at risk to gain weight than females, which have generally been more overweight than males due to traditional views of a bigger woman being healthy and more fertile.

In 2012 the prevalence of obesity in Africa was 50.1% (Table 1.9) (Crowther & Norris, 2012). In Nigeria, SA, Tanzania, rural Uganda and urban Uganda the prevalence of overweight and obesity combined was 68%, 85%, 75%, 46% and 48%, while the prevalence of obesity alone was 31%, 54%, 40%, 10% and 14%, respectively (Table 1.9) (Ajayi e*t al.,* 2016).

In Africa it is estimated that 20-50% of the urban population is overweight or obese (Ajayi e*t al.,* 2016), and the high prevalence of obesity seen in Egypt (29%) and SA (27%) is comparable to that of high-income countries (Keates e*t al.,* 2017).

Table 1.9 The epidemiology and rising trends of obesity in sub-Saharan Africa.

Prevalence	Country	Year	Comments	Reference
3.7	Tanzania	1984	9,1% of diabetic population obese	Ahren *et al.,* 1984
1 – 7.1	SSA	1995	18 Countries; Females; Obese BMI; 31% in SA	Levitt, 2008
30 / 33	Cameroon	1994 – 2003	Overweight BMI; Males/Females	Fezeu *et al.,* 2008
7 / 22			Obesity; Males/Females	
18 / 67			Obese BMI; Males/Females	
0.4 – 43	SSA	2005	Meta-analysis	Dalal *et al.,* 2005
5.1	Guinea	2007		Balde *et al.,* 2007
39.8 / 15.8	Kenya	2008	Overweight; Urban/Rural	Christensen *et al.,* 2009
15.5 / 5.1			Obese; Urban/Rural	
28.2 / 52.2	Benin	2009	Abdominal obesity; Rural/Urban	Ntandou *et al.,* 2009
18 / 32	Cotonou, Benin		Obese/Abdominal obesity	
10.6 / 23.8	Ouidah, Benin		Obese/Abdominal obesity	
67 / 18	Cameroon	2010	Abdominal obesity; Females/Males	Tuei *et al.,* 2010
50.1	Africa	2012		Crowther & Norris, 2012
1.8	Ethiopia	2013	Lowest prevalence in females	Ajayi *et al.,* 2016
25	Equatorial Guinea		Highest prevalence in males	
1.8	Uganda		Lowest prevalence in males	
<15	SSA	2014	Overall	Keates *et al.,* 2017
10.8	Ethiopia	2014	Females	Peer *et al.,* 2014a
34.7	Ghana			
2	Ethiopia		Males	
12.2	Democratic Republic of Congo			
13.8	Cameroon			
68 / 31	Nigeria	2016	Overweight + Obese / Obese only	Ajayi *et al.,* 2016
75 / 40	Tanzania		Overweight + Obese / Obese only	
46 / 10	Uganda		Rural; Overweight + Obese / Obese only	
48 / 14			Urban; Overweight + Obese / Obese only	
1.3 / 6.9	Ghana	2016	Males; Rural/Urban	Agyemang *et al.,* 2016
8.3 / 33.9			Females; Rural/Urban	
20 / 50	Africa	2016	Urban; Overweight/Obese	Ajayi *et al.,* 2016
29	Egypt	2017		Keates *et al.,* 2017

1.4.2 Genetics of obesity

Twin studies have noted a strong genetic component linked to BMI, with heritability estimates ranging from 55.0-87.0% (Min *et al.,* 2013; Tarnoki *et al.,* 2014a). In Nigeria, the heritability estimate is reported at only 36.0% (Kramer *et al.,* 2005). However, the effects of genes predisposing an individual to obesity have been shown to be modified by physical inactivity, with dietary protein intake having no such effects (Silventoinen *et al.,* 2009). Monogenic obesity is an autosomal form of obesity that is characterised by severe obesity without developmental delays (O'Rahilly, 2009; Coll *et al.,* 2004). It occurs as a result of increased appetite due to weakened satiety (Coll *et al.,* 2004). The fat mass and obesity-associated gene (*FTO*) was the first locus identified by GWAS to harbour common variants associated with obesity predisposition (Ndiaye e*t al.,* 2011).

A systematic review of 43 SSA studies, ranging between 85 and 2 332 participants, identified 300 polymorphisms in 42 genes that were associated with obesity in Africa (Yako e*t al.,* 2015b). This included some of the genes in the current study such as *ACE*, *FTO*, and *MC4R.* Of the genes identified by Yako *et al.* (2015b), only SNPs in *FTO* and *MC4R* were previously validated by a genome-wide association study (GWAS) in Ghanaian, Nigerian and SA Black individuals (Yako *et al.,* 2015b). A summary of all the genes discussed below that have been found to be involved in obesity and other factors of body mass in Africa are presented in Table 1.10.

1.4.2.1 Fat-mass and obesity associated (*FTO*) gene

Variants in the *FTO* gene (section 1.3.3.4) are the most consistently replicated variants for obesity in many populations (Lombard e*t al.,* 2012; Adeyemo *et al.,* 2010; Hennig *et al.,* 2009), with an effect size of 0.35 kg/m^2 per susceptibility allele (Hennig *et al.,* 2009); however, data is scarce in African populations (Adeyemo *et al.,* 2010).

The rs1121980 and rs7204609 SNPs, located in intron 1 of the *FTO* gene, were associated with obesity in 517 West Africans (Table 1.10) (Adeyemo *et al.,* 2010). The rs7204609 SNP, as well as the SNPs rs17817288 and rs12447107 in intron 8 of the *FTO* gene, were also found to be associated with percentage body fat in West Africans, while rs16952624 showed no association (Table 1.10) (Adeyemo *et al.,* 2010). A study of 990 SA Black adolescents associated the minor allele (G) of rs17817449 with a 2.5% increased risk of BMI per susceptibility allele (Lombard *et al.,* 2012) while it has been reported much lower in Europeans and Asians (1.53 and 1.32, respectively) (Jacobsson *et al.,* 2012).

Some of these SNPs, plus others of the *FTO* gene (rs17817288, rs17817449, rs7204609, rs3571812, rs9931494, rs1121980 and rs8050136) have been found to be significantly associated with obesity in Ghanaian, Nigerian and SA black individuals by GWAS (Yako *et al.,* 2015a). An earlier study of 2 208 Gambian males and females, the first of its kind in Africa, observed no association between any SNPs in the *FTO* gene and obesity (Table 1.10) (Hennig *et al.,* 2009).

1.4.2.2 Melanocortin 4 receptor (*MC4R*)

So far 20 genetic variations, positioned in the leptin/melanocortin pathway, involved in the regulation of inflammation and whole-body energy homeostasis (Coll *et al.,* 2004), have been identified to play a role in monogenic obesity (Lombard *et al.,* 2012; O'Rahilly, 2009; Coll *et al.,* 2004). The *MC4R* gene (section 1.2.3.5), is a G-protein coupled receptor of the melanocortin receptor family, that regulates energy homeostasis and food intake (Logan e*t al.,* 2016).

A deficiency in MC4R, resulting in decreased cell surface receptor expression, decreased or absent ligand binding, incorrect protein folding and reduced signal transduction, is the most common form of monogenic obesity (Logan *et al.,* 2016). The *MC4R* genetic variants responsible for monogenic obesity are found in only 0.05% of the population, with a prevalence between 0.5-1.0% in obese patients and accounting for 6.0% of severe cases of obesity that manifest during childhood (Logan *et al.,* 2016).

The rs17782313 SNP of *MC4R*, a C to T substitution with unknown function, was found to be associated with BMI in Black adolescents, particularly the T allele conferred a2.5% increased risk of an obese BMI (Table 1.10) (Lombard *et al.,* 2012). In a study of 297 SA non-monogenic, obese subjects (63% Black Africans), 42 Black participants had 8 *MC4R* SNPs previously identified in a South African cohort to play a role in monogenic obesity. The most prevalent SNPs were rs229616 (4%), rs121913560 (1.5%) and rs61741819 (1.2%) (Table 1.10). The remaining 5 *MC4R* variants were found in 1.18% of the total study population (Logan *et al.,* 2016). This study also found that carrying any one of the 8 *MC4R* variants identified, leads to a 4.5-fold increased risk of developing monogenic obesity (Logan *et al.,* 2016). The presence of any of these *MC4R* variants also resulted in an elevated prevalence of MetS amongst obese individuals (Logan *et al.,* 2016).

1.4.2.3 Angiotensinogen (*AGT*)

The missense rs699 (threonine to methione) and rs4762 (methionine to threonine) SNPs of the angiotensinogen gene (section 1.2.2.2) was associated with increased BMI in 550 preeclamptic (PE) pregnant Tunisian females (Table 1.10) (Zitouni *et al.,* 2018). In Nigerians and Zimbabweans, average BMI has been associated with circulating AGT levels, however no genetic association analyses were conducted to determine whether or not the AGT levels were influenced by the presence of DNA sequence variants (Cooper *et al.,* 1998).

1.4.2.4 Transcription factor 7-like 2 (*TCF7L2*)

The TCF4 protein, encoded by *TCF7L2,* was shown to downregulate adipogenesis in adipocytes (section 1.2.3.2). No association with obesity was observed for the *TCF7L2* rs12255372 (Ngwa *et al.,* 2015) and rs7903146 (Nguimmo-Metsadjio *et al.,* 2017) SNPs in Cameroon (Table 1.10). No other studies investigating the genetic association ofthese or any other SNPs in this gene with T2DM in African populations was found.

1.4.2.5 Angiotensin-converting enzyme (*ACE*)

The rs4646994 I/D polymorphism of the *ACE* gene (section 1.2.2.3) has been found to be associated with the risk of obesity, with the differences in risk observed attributable to sex and racial influences (Kramer *et al.,* 2005). Although a study conducted in 1 158 Black individuals from Ibadan, Nigeria and 1 080 AfAms, found no association between obesity and the rs4646994 I/D polymorphism, they did find that the *ACE1-ACE5* (TACAT) haplotype in the promoter region of the gene was over-transmitted from parents to their obese offspring (Table 1.10) (Kramer *et al.,* 2005). A meta-analysis of 14 studies (3 371 cases and 4 490 controls) found that the D allele (p=0.026) and DD genotype (p=0.010) was significantly associated with the risk of overweight or obesity (Table 1.10) (Mao & Huang, 2015). In Ethiopian females, the DD genotype was associated with IR and high BMI, IR and WC, and IR and waist-to-height ratio (Table 1.10) (Motawi *et al.,* 2016).

Table 1.10 A summary of genes found to be associated with obesity and other measures of body fat distribution in Africa.

Gene name	Gene function	Chr. Location	Chr coordinates (GRCh 38) (From - To)	SNP	Risk allele	Population studied	No. samples		Results	Reference
							Cases	Controls		
Fat mass and obesity associated (*FTO*)	DNA demethylase	16q12.2	53701692 - 54158512	rs17817288	G	Ghanaians; Nigerians, SA blacks	GWAS		Significantly associated	Yako *et al.,* 2015
						West Africans	517		Associated with percent body fat	Adeyemo et al., 2010
				rs17817449	G	Ghanaians; Nigerians, SA blacks	GWAS		Significantly associated	Yako et al., 2015
						SA Blacks	990		Assoicated with BMI, 2.5% increased risk per allele	Lombard *et al.,* 2012
				rs7204690	T	Ghanaians; Nigerians, SA blacks	GWAS		Significantly associated	Yako *et al.,* 2015
						West Africans	517		Associated with obesity and percent body fat	Adeyemo *et al.,* 2010
				rs3571812	T	Ghanaians; Nigerians, SA blacks	GWAS		Significantly associated	Yako *et al.,* 2015
				rs9931494	G	Ghanaians; Nigerians, SA blacks	GWAS		Significantly associated	Yako *et al.,* 2015
				rs1121980	A	Ghanaians; Nigerians, SA blacks	GWAS		Significantly associated	Yako *et al*., 2015
						West Africans	517		Associated	Adeyemo *et al.,* 2010
				rs8050136	A	Ghanaians; Nigerians, SA blacks	GWAS		Significantly associated	Yako *et al.,* 2015
				rs12447107	G	West Africans	517		Associated with percent body fat	Adeyemo et al., 2010
				rs16952624	T	West Africans	517		No association with obesity or percent body fat	Adeyemo et al., 2010
				All SNPs above		Gambians	2 208		Does not influence measures of body mass	Hennig *et al.,* 2009

Table 1.10 Continued

Melanocortin 4 receptor (*MC4R*)	Regulates energy homeostasis and food intake	18q21.32	60371110 - 60372775	rs229616	G	SA blacks	198	99	Highly prevalent in obese individuals	Logan *et al.,* 2016
				rs121913560	G	SA blacks	198	99	Highly prevalent in obese individuals	Logan et al., 2016
				rs61741819	G	SA blacks	198	99	Highly prevalent in obese individuals	Logan et al., 2016
				rs17782313	T	SA Blacks	990		Assoicated with BMI, 2.5% increased risk per allele	Lombard et al., 2012
Angiotensinogen (*AGT*)	Cardiovascular remodelling; blood pressure control	1q42-q43	230702523 - 230714590	rs699	T	Tunisians	550	278	Associated with increased BMI in pregnant women	Zitouni *et al.,* 2018
				rs4760	T	Tunisians	500	278	Associated with increased BMI in pregnant women	Zitouni et al., 2018
Transcription factor 7-like 2 (*TCF7L2*)	Encodes effector for Wnt signalling pathway	10q25.3	112950220 - 113167678	rs12255372	T	Cameroonians	35	30	No association with obesity	Ngwa *et al.,* 2015
				rs7903146	T	Cameroonians	35	30	No association with obesity	Nguimmo-Metsadjio *et al.,* 2017
Angiotensin-converting enzyme (*ACE*)	Cleaves AngI to AngII	17q23.3	63477061 - 63498380	rs4646994	D	Nigerians, AfAms	1 158/1 080		TACAT (ACE1-ACE5) haplotype over-transmitted from parents to obese offspring	Kramer *et al.,* 2005
						Africans	3 371	4 490	D (p=0.026) and DD (p=0.010) associated with risk of overweight and obesity	Mao & Huang, 2015
						Ethiopians	80		DD carriers: siginificant associations between IR and high BMI (OR=8.89, 95%CI=1.94-40.71, p=0.004); IR and WC (OR=9.63, 95%CI=2.14-43.36, p=0.003); IR and waist:height (OR=6.86, 95%CI=1.25-37.61, p=0.034)	Motawi *et al.,* 2016

Unless specified, ethinicity of SA Blacks was not stated in the articles.

AGT – Angiotensinogen; BMI – Body mass index; Chr – Chromosome; DNA – Deoxyribonucleic acid; *FTO* – Fat-mass and obesity associated; GWAS – Genome-wide association study; *MC4R* – Melanocortin 4 receptor; SA – South Africa; TCF7L2 – Transcription factor 7-like 2; WC – waist circumference

1.4.3 Concluding remarks – obesity

Obesity often precedes other diseases associated with CVD and is associated with IR and inflammation. Obesity, specifically peripheral obesity, is more common in Black females than in their Caucasian counterparts or in males, contrary to what is seen in other countries. The *FTO* gene is the most consistently replicated gene associated with obesity, although no specific SNP stands out in Africans as the causal variant. In other countries, the rs9939609 SNP stands out. Another top gene includes *MC4R.* The various SNPs within genes and between genes could potentially interact to increase obesity risk.

1.5 Hypertension

Hypertension is characterised by stiff arterial walls that increases resistance to blood flow, requiring the heart to beat with more force, increasing the pressure of the blood being pumped out of the heart (Sengwayo *et al.,* 2013). Mechanical damage to the heart, kidneys and vascular system occurs as a result of the high pressure observed in hypertension (Munóz-Duranga e*t al.,* 2016) due to low plasma renin concentrations and increased cardiovascular response to stress (Gafane *et al.,* 2016). Hypertension occurs as a result of sustained elevated BP (Reiter *et al.,* 2016) and can present in different forms. Primary/essential hypertension is diagnosed in an individual when the cause of arterial hypertension is unknown, while secondary hypertension is preceded by abnormal physiological conditions that affect kidneys, heart or the endocrine system (Munóz-Duranga *et al.,* 2016). Additionally, hypertension can occur in preeclampsia, a multisystem pregnancy disorder (Zitouni *et al.,* 2018; Aung *et al.,* 2017). All forms of hypertension predispose an individual to many serious medical problems such as blindness, heart failure and stroke, renal failure, and ventricular arrhythmias (Rayner & Spence, 2017), and contributes to 75.0% of all heart attacks and strokes (Sengwayo *et al.,* 2013). Hypertension is a widespread problem due to its severe associated complications and frequent underdiagnosis (Schutte e*t al.,* 2008).

Essential hypertension and the associated salt-sensitive phenotype (Figure 1.3) appears polygenic in nature (Poch *et al.,* 2001). A high dietary salt intake mediates BP response to the environment (Poch *et al.,* 2001). This salt-sensitive phenotype is said to be heterogenous in nature as it is found in 50.0% of hypertensive patients (Poch *et al.,* 2001). A genetic component to salt-sensitivity (Figure 1.3) has also been established, as is exhibited by the familial history of hypertension, and familial resemblance of BP response to salt, found in normotensive and hypertensive individuals when compared to salt-resistant individuals (Poch *et al.,* 2001). In addition, the genetic nature of essential hypertension was supported by the sodium retention hypothesis which was proposed to explain the higher prevalence of hypertension in Africans, as hypertensive indigenous Africans appear to be more salt-sensitive (Figure 1.3), with reduced plasma renin levels (Rayner & Spence, 2017; Edina-Melenge *et al.,* 2017; Reiter *et al.,* 2016; Weissberg *et al.,* 1987), than their normotensive counterparts (Rayner & Spence, 2017; Weissberg *et al.,* 1987). It is thought that the retention of sodium and water, especially in West Africans, was a survival adaptation due to limited access to

fluid and periods of drought in rural areas but may now predispose the more urban individuals to hypertension (Reiter *et al.,* 2016).

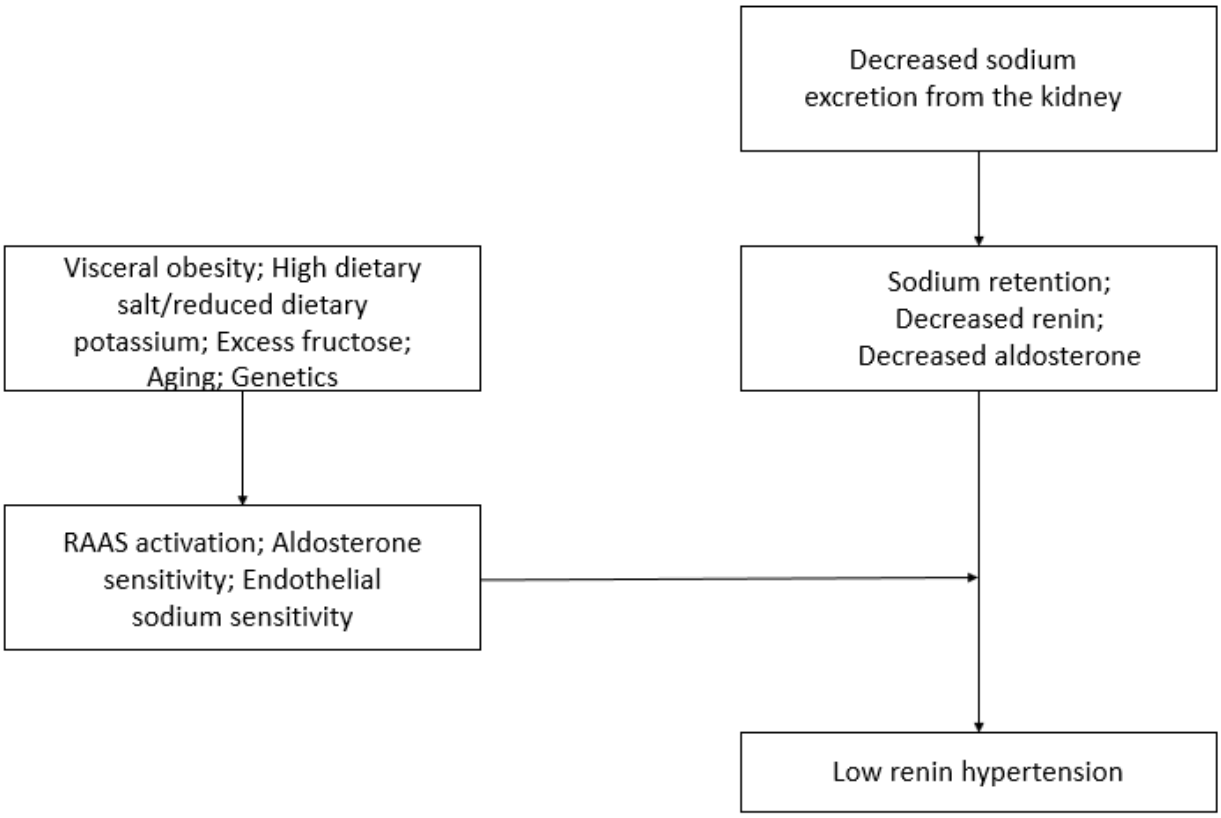

Figure 1.3 The mechanism of salt-sensitive hypertension (Adapted from Rayner & Spence, 2017).

An individual is predisposed to essential hypertension by factors such as age, cigarette smoking, diet, ethnicity, hormones, physical inactivity, sex and stress. This mosaic basis of hypertension was proposed by Paige in 1960 (Frolich *et al.,* 1991). Essential hypertension, as a result of environmental and genetic factors, is the most common disorder of aging and is the leading cause of CVD (Rayner & Spence, 2017; Poch *et al.,* 2001), that results in a continual elevation in BP (Schutte *et al.,* 2008). It is linked to IR and oxidative stress, promoting inflammatory pathways (Ceriello & Motz, 2004; Saad e*t al.,* 2004; Sowers & Frolick, 2004; Facchini *et al.,* 2001; Griendling & Alexander, 1997), which are in turn associated with obesity and T2DM (Ceriello & Motz, 2004; Dandona *et al.,* 2004; Sattar *et al.,* 2003). IR, oxidative stress and inflammation are known risk factors for CVD (Mayosi e*t al.,* 2009; Medzhitov, 2008; Hotamisligil, 2006; Farrario & Strawn, 2006; Mollentze, 2003), T2DM (Medzhitov, 2008; Hotamisligil, 2006) and obesity (Stenlöf *et al.,* 2006; Coll *et al.,* 2004). The oxidative stress and inflammation induced by hypertension is responsible for end-organ damage, mainly as a result of fibrosis (Munóz-Duranga *et al.,* 2016). Low renin hypertension is common in Black individuals, as they have been found to have suppressed RAAS (Figure 1.2) activity and a resultant low renin status, associated with a sustained increase in BP and end-organ damage (Gafane *et al.,* 2016).

Hypertension is the most prevalent (Gafane *et al.,* 2016; Munóz-Duranga *et al.,* 2016) and most modifiable (Edinga-Melenge *et al.,* 2017) risk factor for CVD and stroke (Munóz-Duranga *et al.,* 2016), but it is the leading cause of death due to CVD worldwide (Edinga-Melenge *et al.,* 2017). In 40.0–60.0% of T2DM patients, hypertension and T2DM coexist (Edinga-Melenge *et al.,* 2017). Hypertension is 1.5–3 times more prevalent in T2DM patients compared to nondiabetic individuals (Edinga-Melenge *et al.,* 2017). In Black children BP increases faster over time than in their Caucasian counterparts, resulting in increased prevalence of hypertension in Black adults (Bloem *et al.,* 1995).

The progression of renal failure to end-stage renal disease (ESRD) is driven by hypertension (Edinga-Melenge *et al.,* 2017; Lovati *et al.,* 2001). Both hypertension and ESRD are as a result of an excess production of AngII (Figure 1.2), which mediates vasoconstriction, and thereby hypertension, and the progression to renal disease (Lovati *et al.,* 2001).

1.5.1 Epidemiology of hypertension in Africa

Insulin resistance and obesity are the leading causes of hypertension (Duvnjak *et al.,* 2008; Sowers, 2004). Hypertension contributes to 75% of all heart attacks and strokes (Sengwayo *et al.,* 2013) and 85% of MetS patients are hypertensive (Duvnjak *et al.,* 2008; Sowers, 2004).

1.5.1.1 Hypertension in South Africa

Since 1998, the prevalence of hypertension in SA has increased (Table 1.11), being present in more than 40.0% of the population over 25 years (Yako *et al.,* 2018; Reiter *et al.,* 2016; Day *et al.,* 2014). In the Black population of SA, hypertension has been found to be common, with prevalence estimates ranging between 9%-70% (Table 1.11) (Schutte *et al.,* 2008; Cappucio *et al.,* 2004; Vorster, 2002; Mollentze *et al.,* 1995; Akinkugbe *et al.,* 1985), with a high stroke mortality rate 2-fold higher in hypertensives than in their Caucasian counterparts (Schutte *et al.,* 2008; Opie *et al.,* 2005). Black individuals are also at increased risk of heart failure, peripheral vascular disease and renal failure due to hypertension (Seedat, 1999). This is believed to be as a result of the increased fibrinogen levels observed in Africans (Schutte *et al.,* 2008). A study of Black SA females found that they had higher BP when compared to their Caucasian counterparts, with increased peripheral vascular disease with a link to IR (Schutte *et al.,* 2008).

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Table 1.11 The epidemiology and rising trends of hypertension in South Africa.

Prevalence	Area/Town/Province	Year	Comments	Reference
9.4	SA	1982	Zulu	Seedat, 1982
25	Durban	1983	Urban Zulu	Seedat, 1983
17.2			Caucasians	
14.2			Ethnic Indians	
29	QwaQwa	1995		Mollentze *et al.,* 1995
30.3	Mangaung			
69.8	QwaQwa & Mangaung		Stroke patients	
40	SA	1998 - 2018	Individuals >25 years	Yako *et al.,* 2018; Reiter *et al.,* 2016; Day *et al.,* 2014
55	SA	2005	Overall	Connor *et al.,* 2003
59			Blacks	
43 / 41	SA	2008	Males / Females	NDoH, 2013
42.2	SA	2008	Overall	WHO, 2008
33	Soweto	2009		Tibazarwa *et al.,* 2009

1.5.1.2 Hypertension in sub-Saharan Africa

In 6 low- and middle-income countries (China, Ghana, India, Mexico, Russian Federation and SA), the highest prevalence of hypertension (77.9%) was reported in people over the age of 50 years (Lloyd-Sherlock *et al.,* 2014). By 2025, the prevalence of hypertension in SSA is estimated to increase from 80 million in 2000 to 150 million (Ghogomu *et al.,* 2016). In the year 2000, hypertension in SSA was reported in approximately 12% of the SSA population (Table 1.12) and this number is projected to double by 2025 (Yako *et al.,* 2018).

Hypertension prevalence varies between rural and urban populations (Table 1.12). In West Africa the prevalence of hypertension in rural and urban populations was 16% and 20% (Table 1.12), respectively, and is believed to be due to the presence of obesity in these communities (Cooper & Rotimi, 1997; Seedat *et al.,* 1982).

In 1985 the hypertension prevalence in SSA was below that of either the West Indies or the United States of America (Akinkugbe, 1985), but an ever-rising increase of hypertension in SSA will result in the prevalence exceeding that of either country.

Table 1.12 The epidemiology and rising trends of hypertension in sub-Saharan Africa.

Prevalence	Country	Year	Comments	Reference
7	Lesotho	1976		Mokhobo *et al.,* 1976
4.1 – 11	Ghana	1977		Pobee *et al.,* 1977
5.9	Nigeria	1978		Oviasu *et al.,* 1978
16 / 20	West Africa	1982 - 1997	Rural/Urban	Cooper & Rotimi, 1997; Seedat, 1982
11	Cameroon	1998		Mbanya *et al.,* 1998
12	SSA	2000	Overall	Yako *et al.,* 2018
54.3	Africa	2005	T2DM patients	Isezou & Ezunu, 2005
24.1 / 26.5	Benin	2009	Rural/Urban	Ntandou *et al.,* 2009
23	Cotonou, Benin			
14.7 – 69.9	SSA	2014	Overall	Ataklte *et al.,* 2014

1.5.2 Genetics of hypertension

Many genes, with mild effects on BP, have been associated with hypertension (Butler, 2010), However, the underlying genetics are poorly understood (Edinga-Melenge *et al.,* 2017; Tiffin e*t al.,* 2010). The heritability of hypertension ranges between 30.0-60.0% (Tarnoki *et al.,* 2014b), with inconsistent clinical presentations and drug response. However, approximately only 2.0% of the total variation in BP has been explained by the discovery of associated genetic loci, which have been broadly replicated with modest odd's ratios (Levy *et al.,* 2009; Newton-Cheh *et al.,* 2009, Fox *et al.,* 2011; Johnson *et al.,* 2011; Zeller *et al.,* 2012).

A genetic component is responsible for 50.0% of all hypertensive cases (Ghogomu *et al.,* 2016; Butler, 2010; Jeanemaitre *et al.,* 2007). Disease expression is believed to be modified by genetic factors as seen by the female predominance, reduced penetrance and variable expressivity of pulmonary arterial hypertension (Austin & Loyd, 2014). The RAAS (Figure 1.2) has been demonstrated to play a central role in BP response to a high-salt diet (Figure 1.3) (Poch *et al.,* 2001; Svetkey *et al.,* 2001), as well as in the genesis of arterial hypertension (Munóz-Duranga *et al.,* 2016). A summary of all the genes discussed below that have been found to be involved in hypertension and other measures of BP in Africa are presented in Table 6.

1.5.2.1 Angiotensinogen (*AGT*)

Angiotensinogen, produced in the liver, is converted to AngI, the prohormone of AngII, by interaction with renin (Figure 1.2) (Bloem *et al.,* 1995). Sodium retention and vascular resistance is increased by AngII, making it important for BP regulation (Bloem *et al.,* 1995).

Elevated levels of AGT in plasma, due to the rs699 (TT) SNP in this gene (section 1.2.2.2), leads to increases in both systolic and diastolic BP (Table 1.13) (Zitouni *et al.,* 2018; Bloem *et al.,* 1995). In Nigerians and Zimbabweans, hypertensive individuals were found to possess higher levels of

circulating AGT than normotensives (Cooper *et al.,* 1998), but the genetic changes that may contribute to the increased levels of this protein were not investigated in this study. The T allele of rs699 has been reported to be the predominant allele in African American Black individuals (Bloem *et al.,* 1995) and may thus contribute to the higher BP observed in SA Black individuals.

The T-allele of the *AGT* rs699 variant has a frequency ranging from 40% in Caucasian individuals to 90% in Nigerians (Barlassina *et al.,* 2000; Wang & Staessen, 2000). The association of rs699 with hypertension is the most replicated association for hypertension in many populations (Barlassina *et al.,* 2000; Wang & Staessen, 2000). This was also observed in a meta-analysis of individuals of West African descent, where the frequency of the T allele was 81% in individuals of West African descent and 42% in their Caucasian African counterparts (Reiter *et al.,* 2016). Another meta-analysis of individuals of West African descent showed a strong tendency to increased risk for hypertension among individuals with the TT genotype, but this association was not observed in an earlier study of 1 308 Nigerians (Table 1.13) (Kooffreh *et al.,* 2013a). In Egyptian CAD patients, the TT genotype of rs699 was positively correlated with BP (Table 1.13) (Shaker *et al.,* 2009), and may thus modulate the association with CVD (section 1.2.2.2). In one SA study, the T allele of rs699 was found to be more common in Black Xhosa or Zulu soeaking PE and late-onset PE patients, and was associated with a 2-fold higher risk of PE development in hypertensive females (Table 1.13) (Aung *et al.,* 2017). In a Tunisian Black cohort, the T allele was significantly higher in PE patients and associated with increased risk and severity of PE (Table 1.13) (Zitouni *et al.,* 2018). The high prevalence of the rs699 T allele in Black individuals, found to be associated with hypertension, may indicate a genetic cause for the high prevalence of hypertension observed in SA Black individuals (Tibazarwa e*t al.,* 2009; Ataklte *et al.,* 2014) and could be a valuable gene variant to look at (Yako *et al.,* 2018; Reiter *et al.,* 2016; Day *et al.,* 2014; Schutte *et al.,* 2008).

The rs4762 SNP found in exon 2 of the AGT has been reported in a Ghanaian population, and associated with both higher SBP and higher DBP and may play a role in the high prevalence of hypertension observed in this Black population (Robinson & Williams, 2004), but this association was not replicated in an Algerian population (Table 1.13) (Amrani *et al.,* 2015). Neither rs699 and rs4762 was found to be associated with hypertension in a Nigerian cohort (Table 1.13) (Rotimi *et al.,* 1997).

33In a Tunisian cohort, the T allele of the rs4762 SNP was associated with an increased risk of developing PE (Table 1.13) (Zitouni *et al.,* 2018). These findings were not observed in an earlier study of an SA Black, Zulu speaking cohort (Roberts *et al.,* 2004). In a recent study, having either the rs699 and rs4762 risk alleles was also associated with increased BMI (Zitouni *et al.,* 2018).

1.5.2.2 Angiotensin-converting enzyme (*ACE*)

The ACE protein, encoded by the *ACE* gene on chromosome 17 (section 1.2.2.3), cleaves Ang I to Ang II. Angiotensin II is involved in BP regulation, and variations in ACE levels may thus affect BP

(Lombard *et al.,* 2012). The rs4343 (A262T) and rs4291 (A11860G) variants, located in exon 16 and the promoter region of rs4646994, have the strongest effect on circulating ACE levels, and were associated with systolic BP in a combined linkage and association study of 1343 individuals from 332 Nigerian families (Zhu *et al.* 2001). The CAD haplotype, thought to be involved in regulating circulating ACE levels and covering approximately 9kb of the *ACE* gene, consisting of the variants rs4309 (C5467T), rs4331 (A9596G) and rs4646994 (I/D), was significantly associated with hypertension in a Nigerian cohort (Table 1.13) (Bouzekri *et al.,* 2004).

Although ACE levels are similar in Black and Caucasian individuals, ACE levels was inversely associated with diastolic BP in Black individuals, but positively associated with diastolic BP in Caucasian individuals, which may be indicative of possible underlying ethnic differences in BP regulation (He *et al.,* 1999). No association was observed between the *ACE* rs4646994 I/D polymorphism and hypertension in Tunisians (388 cases and 425 controls) (Table 1.13) (Kabadou *et al.,* 2013).

1.5.2.3 Angiotensin II type I receptor (*AT1R*)

The angiotensin II type I receptor (AT1R) (section 1.2.2.4) is involved in mediating the salt-conserving and vasoconstrictive actions of the RAAS (Figure 1.2) (Ghogomu *et al.,* 2016; Kooffreh *et al.,* 2013b). A computational analyses of candidate genes of hypertension in SA Black individuals ranked the rs5186 SNP of the *AT1R* gene (section 1.1.3.4), implicated in BP control and blood volume in the cardiovascular system, as the most plausible candidate gene for disease (Table 1.13) (Tiffin *et al.,* 2010). However, in the same population, this SNP was found to have similar distributions in PE patients and normotensive pregnant controls (Table 1.13) (Aung *et al.,* 2017), suggesting that this SNP may not be associated with increased BP in pregnant Black females from SA. No association was observed between the rs5186 SNP and hypertension in the Calabar and Uyo cities of Nigeria (Kooffreh *et al.,* 2013b) and in a Cameroonian population from the South West region (Ghogomu *et al.,* 2016), with similar genotype distributions between normotensive and hypertensive individuals (Table 1.13) (Ghogomu *et al.,* 2016; Kooffreh *et al.,* 2013b).

Table 1.13 A summary of genes associated with hypertension and other features of blood pressure in Africa.

Gene name	Gene function	Chr. Location	Chr coordinates (GRCh 38) (From - To)	SNP	Risk allele	Population studied	No. samples		Results	Reference
							Cases	Controls		
Angiotensinogen (*AGT*)	Cardiovascular remodelling; blood pressure control	1q42-q43	230702523 - 230714590	rs699	T	SA black	357	246	Significantly higher in PE and late-onset PE than normotensives	Aung *et al.,* 2017
						Tunisians	544	278	Associated with increased PE risk and severity; T homozygosity positively associated with PE	Zitouni *et al.,* 2018
						Nigerians	138	116	No association with hypertension; possitively associated with concentration in males	Rotimi *et al.,* 1997
						West Africans; Nigerians	Meta-analysis		TT associated with increased risk in West Africans, but not in Nigerians	Kooffreh *et al.,* 2013a
						Egyptians	70	60	TT positively correlated with BP in CAD patients	Shaker *et al.,* 2009
						Algeria	82	72	No association with BP	Amrani *et al.,* 2015
				rs4762	T	SA black	544	278	Associated with increased PE risk	Zitouni *et al.,* 2018
						Ghanaians; Algerian	82	72	Higher SBP and DBP in Ghanaians, not in Algerians	Robinsson & Williams, 2004; Amrani *et al.,* 2015
						Nigerians	138	116	No association	Rotimi *et al.,* 1997
				H1 haplotype (-1074T/G, -532C/T, 384A/G, 1164A/G, 2186T/G),	TTAAT	Nigerians	595 people from 184 families		Associated with increased serum angiotensinogen (p=0.042)	Fejerman *et al.,* 2006
				-255G/A; -44G/A	A	Mali (Dogon)	199		No association between BMI and systolic or diastolic BP	Taylor et al., 2013
Angiotensin converting enzyme (*ACE*)	Catalyzes conversion of AngI to the physiologically active AngII	17q23	63477061 - 63498380	C5467T, A9596G, I/D	CAD	Nigerians	1158		Haplotype associated with hypertension (p=0.007)	Bouzekri et al., 2004
				A-262T; A11860G		Nigerians	1343 people from 332 families		Associated with systolic BP	Zhu *et al.,* 2001
				rs4646994	D	Tunisians	388	425	No association	Kabadou *et al.,* 2013
Angiotensin II type I receptor (*AT1R*)	Vasoconstriction through AngII; mediates major cardiovascular effects of AngII	3q21-q25	148697871 - 148743003	rs5186	C	SA black	Computational analysis		Top ranked candidate gene for hypertension	Tiffin *et al.,* 2010
						Nigerians; Cameroonians	612/32	612/50	No association with hypertension	Kooffreh *et al.,* 2013b; Ghogomu *et al.,* 2016
						SA black	357	246	Similar distribution in PE and normotensives	Aung *et al.,* 2010

ACE – Angiotensin converting enzyme; *AGT* – Angiotensinogen; AngI – Angiotensin I; AngII – Angiotensin II; *AT1R* – Angiotensin II type I receptor; BMI – Body mass index; BP – Blood pressure; Chr – Chromosome; DBP – Diastolic blood pressure; PE – Pre-eclampsia; SA – South Africa; SBP – Systolic blood pressur

1.5.3 Concluding remarks - hypertension

Hypertension is the most common disorder of aging and is the leading cause of CVD. It is linked to obesity and T2DM by its accompanying IR and inflammation and is thus the most modifiable risk factor of CVD. Hypertension is very common in Black Africans. The RAAS is responsible for BP regulation, and *AGT*, the main regulator of the system has repeatedly been associated with hypertension and BP control in Black Africans and is a promising target for genetic studies of BP. Other genes of the RAAS have also been associated with hypertension and may interact with *AGT* to exert their effects.

1.6 Dyslipidaemia

Dyslipidaemia is characterised by increased TC, triglycerides and LDL-C, and decreased HDL-C levels (Ellman *et al.,* 2015; Bentley *et al.,* 2012; Brown *et al.,* 2006). Dyslipidaemia is associated with increased CVD risk (Bentley & Rotimi, 2012; Kathiresan *et al.,* 2008; Brown *et al.,* 2006; Kotowski *et al.,* 2006; Chien *et al.,* 2005; Knoblauch *et al.,* 2004; Lai *et al.,* 2004). Dyslipidaemia is common in T2DM patients, predating glucose intolerance (Adeyemo *et al.,* 2005) and is associated with obesity (Ellman *et al.,* 2015). Higher cholesterol has been associated with increased atherogenesis (Karaye & Habib, 2014). Atherogenic dyslipidaemia is a combination of increased triglycerides and decreased HDL-C concentrations (Ntyintyane *et al.,* 2006).

Hypercholesterolaemia is diagnosed when TC is greater than 5 mmol/l (Karaye & Habib, 2014). Familial hypercholesterolaemia is an autosomal dominant disease that contributes to CHD mortality (Kotze & Theart, 2003), and in the 1970s, familial hypercholesterolaemia was found to have a high prevalence in SA (Seftel, 2003). In 2016 familial hypercholesterolaemia was still common (1 in 200 to 1 in 500) and is said to be the most significant monogenic disorder of lipoprotein metabolism (Khine & Marais, 2016). If untreated, homozygous familial hypercholesterolaemia is associated with a 20-fold increased risk of CHD; and untreated heterozygous familial hypercholesterolaemia in males results in a 2-fold increased risk of a coronary event by 50 years, and a 0.3-fold increase in females (Khine & Marais, 2016).

Postprandial lipaemia, a rise in triglycerides and triglyceride-rich lipoproteins after eating, is common in SA Black CAD patients (Ntyintyane *et al.,* 2008). In addition to the increased triglyceride concentrations, a prolonged postprandial response is also observed in CAD patients (Ntyintyane *et al.,* 2008). Postprandial lipaemia results in hypertriglyceridaemia with decreased HDL-C and increased LDL-C (Ntyintyane *et al.,* 2008). Increased triglyceride concentrations after eating has also been associated with hypertension, MetS, obesity and T2DM (Ntyintyane *et al.,* 2008).

In urban Black populations in SA, mean serum cholesterol is twice as high as that found in traditional, rural populations (Walker *et al.,* 1997). Total cholesterol, triglycerides and LDL-C serum levels have been positively associated with CHD, while HDL-C serum levels is negatively associated with CHD (Adeyemo *et al.,* 2005). In T2DM patients, increased triglycerides and decreased HDL-C are the

most common lipid abnormalities observed (Makuyana *et al.,* 2004). The degree of insulin production and IR, and thus progression to T2DM, has been found to be determined by the level of tissue triglycerides (Makuyana e*t al.,* 2004). The prevalence of dyslipidaemia in SA follows the trend seen for CVD in SA with SA Indian and Caucasian individuals having the highest prevalence, followed by Coloured individuals and generally uncommon in Black individuals (Mayosi *et al.,* 2009; Seftel, 2003). This is supported by the finding that in the absence of dyslipidaemia, CHD is rare in Black individuals, even though the prevalence of T2DM, hypertension and smoking is high (Seftel, 2003).

1.6.1 Epidemiology of dyslipidaemia in Africa

1.6.1.1 Dyslipidaemia in South Africa

In Xhosa speaking Black females, it has been found that they have lower levels of TC, triglycerides, HDL-C and LDL-C compared to their Caucasian counterparts, and this could explain the lower rates of lipid related mortalities in the Black population (47 vs. 152 per 100 000, respectively) (Ellman *et al.,* 2015).

As shown in Table 1.14, the prevalence of dyslipidaemia is the lowest in the Black population of SA, when compared to Caucasian, Indian and Coloured individuals in SA. A survey conducted in 2005 involving 10 000 individuals from 200 hospital across SA reported a dyslipidaemia prevalence of 37.0% in Caucasian individuals, but only 5.0% in Black individuals (Connor *et al.,* 2005). In the same year, a study in indigenous Nigerians found the prevalence to be 72.4% (Table 1.14) (Isezou & Ezunu, 2005). This vast difference in the prevalence of dyslipidaemia could be attributed to varying nutritional habits across these geographical locations. The overall prevalence of hypercholesterolaemia was reported at 47.6% in a subset of the SA adult population (Table 1.14) (Karaye & Habib, 2014) with the prevalence of hypercholesterolaemia lowest amongst adult SA Black individuals at 27.6%, followed by Coloured (80.7%), Asian and Indian (81.7%) and highest amongst Caucasian (89.2%) individuals (Table 1.14) (Karaye & Habib, 2014). This huge difference in the prevalence of hypercholesterolaemia across different populations in SA may be as a result of the adoption of a more westernised diet containing more fats in Caucasian, Coloured and Indian populations, whereas the Black population may still be following a traditional diet that contains more fibre. The lower prevalence of hypercholesterolaemia in SA Black individuals may be as a result of their favourable serum lipid profiles (increased serum cholesterol, but stable HDL-C) and low homocysteine, which has also been proposed to protect SA black individuals from IHD (Mayosi *et al.,* 2009; Lemogoum *et al.,* 2003; Vorster, 2002).

Table 1.14 The epidemiology and rising trends of dyslipidaemia in South Africa.

Prevalence	Area/Town/Province	Year	Comments	Reference
16.5 / 25.8	Cape Peninsula	1991	High-risk TC; Black males/Females	Steyn *et al.,* 1991
12.5	QwaQwa	1995	High-risk hypercholesterolaemia	Mollentze *et al.,* 1995
6	Mangaung			
34	QwaQwa		Moderate-risk hypercholesterolaemia'	
44.8	Mangaung			
37	SA	2005	Caucasians	Connor *et al.,* 2005
5			Blacks	
72.4	SA	2005	Blacks	Isezou & Ezunu, 2005
31 / 37	SA	2008	Raised blood cholesterol; Males/Females	Nojilana *et al.,* 2016
13	Soweto	2009	Total cholesterol	Tibazarwa *et al.,* 2009
47.6	SA	2014	Hypercholesterolaemia; Overall	Karaye & Habib, 2014
27.6			Blacks	
80.7			Coloureds	
81.7			Asians & Indians	
89.2			Caucasians	

1.6.1.2 Dyslipidaemia in sub-Saharan Africa

A meta-analysis of 16 studies of dyslipidaemia in SSA (Kenya, Nigeria, South Africa, Sudan), estimated a prevalence of 38.4% across all studies (Table 1.15) (Karaye & Habib, 2014). Dyslipidaemia prevalence was highest amongst IHD patients (49.6%), followed by stroke patients (26.5%) and heart failure patients (11.4%), with high cholesterol responsible for a big part of the burden (Karaye & Habib, 2014).

Another meta-analysis in SSA reported a fairly low prevalence of hypercholesterolaemia in West African countries (Nigeria and Sierra Leone), while in North African countries (Egypt and Tunisia) it was much higher (Table 1.15) (Keates *et al.,* 2017), similar to that reported for SA (47.6%) (Karaye & Habib, 2014). In all studies included in this meta-analysis, females had significantly higher values for total cholesterol, triglycerides, HDL-C, LDL-C and HDL-C/TC than males. This is thought to be due to a diet higher in fat and a less physically active lifestyle (Keates *et al.,* 2017). Interestingly, in other studies, it has been found that females consistently have higher hypercholesterolaemia values, while low HDL-C and relatively high LDL-C and triglycerides levels are consistently reported in both males and females (Keates *et al.,* 2017).

Table 1.15 The epidemiology and rising trends of dyslipidaemia in sub-Saharan Africa.

Prevalence	Country	Year	Comments	Reference
25.3 / 37.5	Benin	2009	Reduced HDL-C; Rural/Urban	Ntandou *et al.,* 2009
38.4	SSA	2014	Meta-analysis	Karay & Habib, 2014
48	North Africa	2017	Egypt & Tunisia	Keates *et al.,* 2017
20 - 30	East & Central Africa			

1.6.2 Genetics of dyslipidaemia

A twin study investigating lipid profiles was the first of its kind to include AfAms and reported a heritability range of 69-92% (Iliadou *et al.,* 2005), but genes involved in the pathogenesis has not been fully elucidated (Frikke-Schmidt *et al.,* 2004). For all components of abnormal lipids, a combined heritability of 30-80% has been reported in Mexican-Americans (Adeyemo *et al.,* 2005). However, the heritability of the individual measures of abnormal lipids differs. The heritability of total cholesterol, HDL-C and triglycerides has been reported at 8-72%, 21-79% and 19-72%, respectively (Elder *et al.,* 2009).

Of all the SNPs associated with dyslipidaemia by GWAS, two thirds have been successfully replicated in an array of populations. (Ndiaye *et al.,* 2011). Most notably is 3 SNPs in the vicinity of the cholesteryl ester transfer protein (*CETP*) gene, which have been associated with high values of LDL-C, total cholesterol and triglycerides (Teslovich *et al.,* 2010). Genes identified by GWAS involved in familial hypercholesterolaemia include the low-density lipoprotein receptor (*LDLR*), apolipoprotein B (*APOB*) and proprotein convertase subtilisin/kexin type 9 (*PCSK9*) (Zeller *et al.,* 2012) and many of the genes associated with blood lipid concentrations have also been associated with CAD risk (Teslovich *et al.,* 2010).

It is thought that the aggregation of numerous genetic loci, that on their own exert a small influence on lipoprotein levels, exert a significant effect on CVD risk (Kotze & Thiart, 2003). It is also thought that due to additive effects, low-penetrance CVD-associated genetic loci are pronounced in familial hypercholesterolaemia (Kotze & Thiart, 2003).

Dyslipidaemia has not been studied to any great extent in African populations. However, studies of recent African-ancestry populations, i.e. AfAms, have identified genes that are likely to also be present in populations from the African continent. The apolipoprotein gene cluster (apolipoprotein A1 (*APOA1)/*apolipoprotein C3 (*APOAC3)/*apolipoprotein A4 (*APOA44)/*apolipoprotein A5 (*APOA5*)), leptin (*LEP*) and leptin receptor (*LEPR*) have repeatedly been associated with dyslipidaemia (Chien *et al.,* 2005; Frikke-Schmidt *et al.,* 2004). Although genes involved in familial hypercholesterolaemia have been identified in other populations, this association has not been confirmed in African populations. Instead, a study conducted in a SA population reported an association of the *TNFα* with dyslipidaemia. (Joffe *et al.,* 2011; Joffe *et al.,* 2010). Evidence from this

study suggests that this may be an important gene to study in the pathogenesis of dyslipidaemia, and it has also been associated with T2DM.

1.6.2.1 Tumour necrosis factor-alpha (*TNFα*)

Tumour necrosis factor-alpha concentrations have been associated with obesity and increased IR (section 1.3). Whole-body lipid metabolism is influenced by TNFα and raised concentrations thereof has been associated with increased levels of triglycerides and low levels of HDL-C (Joffe *et al.,* 2011).

The rs1800629 SNP in the *TNFα* gene (section 1.3.3.2) is a functional G to A substitution at position 308 upstream from the transcription initiation site in the promoter (Bouhaha *et al.,* 2010b). The A allele has been associated with increased TNFα production. In SA Black females, the relationship between dietary fat intake (%E), risk of obesity and lipid concentrations was found to be modified by SNP rs1800629 (Joffe *et al.,* 2010). In SA Caucasian females, total cholesterol was found to be acted upon by the interaction of the rs1800629 genotype and dietary fat intake (%E). In GG, carriers decreased TC levels were observed with increased %E; while in AG and AA carriers, increased %E was associated with increased TC (Joffe *et al.,* 2011).

1.6.3 Concluding remarks - dyslipidaemia

Dyslipidaemia is found in T2DM patients, predating IGT, and is associated with obesity. However, overall dyslipidaemia in Black Africans is rare, with prevalence higher in females than in males. The *LEP* and *LEPR* genes have consistently been associated with dyslipidaemia in other countries, but no such studies have been performed in African populations. Thus, more studies regarding the genes involved in dyslipidaemia in African populations are needed.

1.7 Study rationale

Identifying genes that contribute to the development of CVD and its associated risk factors, especially diabetes and hypertension, particularly in the isiXhosa-speaking Black population of South Africa may help to provide a better understanding of the pathways involved in their development. Identifying genes associated with disease may enable the early identification of individuals at high-risk of CVD, as well as identify novel targets for pharmaceutical and preventative strategies. New therapeutic strategies may also be developed by the discovery of functional mutations that reduce disease risk.

The focus of this study was on the genetic risk factors associated with CVD and its associated risk factors, particularly T2DM, hypertension, obesity and dyslipidaemia, in the isiXhosa-speaking Black population of Cape Town. Of particular interest are the single nucleotide polymorphisms (SNPs) found in the transcription factor 7-like 2 (*TCF7L2*); melanocortin 4 receptor (*MC4R*); angiotensinogen (*AGT*); angiotensin II type I receptor (*AT1R*); fat-mass and obesity associated (*FTO*) and tumour necrosis factor alpha (*TNFα*) genes, as they have previously been associated with the diseases that this study will focus on, both globally and in SSA.

In particular, the SA Black population was chosen as very few studies investigating the genetic risk of CVD and its associated risk factors have been conducted in this population (Yako *et al.,* 2016; Logan *et al.,* 2016; Pillay *et al.,*2015; Tekola-Ayele *et al.,* 2013; Pirie *et al.,* 2010; Barlassina *et al.,* 2000). However, studies in other African countries, particularly Nigeria (Yako *et al.,* 2016; Barlassina *et al.,* 2000), Cameroon, Ghana (Yako e*t al.,* 2016; Jeck *et al.,* 2004), and the predominantly Arab North African countries of Egypt (Abd El-Aziz *et al.,* 2012; Shaker *et al.,* 2009), Lebanon (Mtiraoui *et al.,* 2012a) and Tunisia (Yako *et al.,* 2016; Turki *et al.,* 2014; Turki *et al.,* 2013a; Turki *et al.,* 2012; Berhouma *et al.,* 2012; Mtiraoui *et al.,* 2012a), have replicated some of the genetic findings of other countries, mainly the United States of America and Europe. Thus, not much is known about the genetic associations with CVD related diseases in the SA Black population.

1.8 Aim and objectives

1.8.1 Aim

To determine if genetic variants selected from previous studies performed in sub-Saharan African populations are associated with CVD and its associated risk factors in Black South Africans participating in the Cardiovascular Risk in Black South Africans (CRIBSA) Study.

1.8.2 Objectives

1. To select genetic markers associated with disease in studies previously performed in African populations that could potentially be associated with cardiovascular disease, such as type 2 diabetes mellitus, obesity, hypertension, dyslipidaemia and MetS , in South African Black individuals.

2. To optimise the DNA extraction protocol using the Gentra PureGene blood kit and extract DNA from buffy coat samples.

3. To optimise the PCR amplification protocol and genotype characterise samples by gel electrophoresis, and to test variants for association with CVD and its associated disease risk factors.

4. To test variants for association with CVD and its associated disease risk factors (as mentioned in Objective 1) using relevant bioinformatic tools after KASP genotyping.

Articles cited in this thesis were found using the following searches: "*cardiovascular diseases in South Africa"; "metabolic syndrome in South Africa"; "hypertension in South Africa"; "obesity in South Africa"; "type 2 diabetes mellitus in South Africa"; "dyslipidaemia in South Africa"; "cardiovascular diseases in sub-Saharan Africa"; "metabolic syndrome in sub-Saharan Africa"; "hypertension in sub-Saharan Africa"; "obesity in sub-Saharan Africa"; "type 2 diabetes mellitus in sub-Saharan Africa"; "dyslipidaemia in sub-Saharan Africa"; "cardiovascular diseases in Africa"; "metabolic syndrome in Africa"; "hypertension in Africa"; "obesity in Africa"; "type 2 diabetes mellitus in Africa"; "dyslipidaemia in Africa"; "genes involved in cardiovascular diseases"; "genes involved in metabolic syndrome"; "genes involved in hypertension"; "genes involved in obesity"; "genes involved in type 2 diabetes mellitus"; "genes involved in dyslipidaemia"; "genes involved in cardiovascular diseases in South Africa"; "genes involved in metabolic syndrome in South Africa"; "genes involved in hypertension in South Africa"; "genes involved in obesity in South Africa"; "genes involved in type 2 diabetes mellitus in South Africa"; "genes involved in dyslipidaemia in South Africa"; "genes involved in cardiovascular diseases in sub-Saharan Africa"; "genes involved in metabolic syndrome in sub-Saharan Africa"; "genes involved in hypertension in sub-Saharan Africa"; "genes involved in obesity in sub-Saharan Africa"; "genes involved in type 2 diabetes mellitus in sub-Saharan Africa"; "genes involved in dyslipidaemia in sub-Saharan Africa"; "genes involved in cardiovascular diseases in Africa"; "genes involved in metabolic syndrome in Africa"; "genes involved in hypertension in Africa"; "genes involved in obesity in Africa"; "genes involved in type 2 diabetes mellitus in Africa"; "genes involved in dyslipidaemia in Africa"; "genetics of cardiovascular diseases in South Africa"; "genetics of metabolic syndrome in South Africa"; "genetics of hypertension in South Africa"; "genetics of obesity in South Africa"; "genetics of type 2 diabetes mellitus in South Africa"; "genetics of dyslipidaemia in South Africa"; "genetics of cardiovascular diseases in sub-Saharan Africa"; "genetics of metabolic syndrome in sub-Saharan Africa"; "genetics of hypertension in sub-Saharan Africa"; "genetics of obesity in sub-Saharan Africa"; "genetics of type 2 diabetes mellitus in sub-Saharan Africa"; "genetics of dyslipidaemia in sub-Saharan Africa"; "genetics of cardiovascular diseases in Africa"; "genetics of metabolic syndrome in Africa"; "genetics of hypertension in Africa"; "genetics of obesity in Africa"; "genetics of type 2 diabetes mellitus in Africa"; "genetics of dyslipidaemia in Africa"*

Chapter 2

Methods and materials

2. Methods and materials

Sections 2.1 – 2.3 were completed before the commencement of this MSc project as part of the Cardiovascular Risk in Black South Africans (CRIBSA) Study (PI: Dr Nasheeta Peer). Section 2.4 onwards were completed for this MSc thesis.

2.1 Sample cohort and sampling procedure

The CRIBSA sample cohort consists of 1 116 isiXhosa-speaking Black participants (n=1 116), between the ages of 25 and 74 years. Participants were recruited in 2008/09 from predominantly Black areas of Cape Town (Khayelitsha, Gugulethu, Crossroads, Nyanga and Langa). A three-stage cluster sampling, stratified by area and housing type, was included in the sampling procedure which has been described previously (Peer *et al.,* 2014b; Peer *et al.,* 2012). Participants were excluded if they were unable to give consent, on tuberculosis treatment or anti-retroviral therapy, bedridden, cancer patients who had received treatment within the last year prior to sampling, pregnant or lactating mothers, or were resident in Cape Town for less than 3 months. The 2001 census was used to calculate prespecified age and gender quotas, ensuring at least 50 participants in each gender category.

2.2 Data collection

Questionnaires regarding socio-demographic status such as gender, age, level of education, employment status, household type and number of occuoants, acces to electricity and water, household appliances and mode of transport and relevant medical history, including form of treatment (traditional/herbal/western), family history of disease, smoking and alcohol consumption status, early deaths in family and personal history of disease, were administered by trained fieldworkers. Three BP measurements, using an Omron BP monitor with an appropriately sized cuff, were taken at 2-minute intervals after the participant was seated for 5 minutes. The BP measurement used in the analysis was the average of the second and third BP measurement.

Anthropometric measurements were obtained by three trained staff members, of which two were nurses. Weight to the nearest 0.5kg was determined using a calibrated digital scale with participants barefoot and wearing light clothing. Height to the nearest 0.1cm was measured with a stadiometer. Waist and hip circumference to the nearest 0.1cm was measured using a flexible, non-elastic tape measure. The smallest circumference between the lower sternum and the umbilicus during exhalation while standing was taken as the waist measurement; and the measurement of the maximum posterior perturbance of the buttocks was taken as the hip measurement (Alberti e*t al.,* 2006).

Blood samples were drawn for glucose and lipid estimations following a 10-hour overnight fast. An oral glucose tolerance test was then administered, and blood samples were again drawn 2 hours later (WHO, 1999).

2.3 Definitions used for the diagnosis of diseases in this study

Cardiovascular disease can occur secondary to other diseases. Having one of these diseases places an individual at higher risk of CVD, and each consecutive disease increases the risk of CVD. The disease components that increase the risk of CVD, and used in this study, are type 2 diabetes mellitus, obesity, hypertension, dyslipidaemia and MetS.

The definitions of each risk phenotype used in this study to diagnose disease, reported elsewhere (Peer *et al.,* 2012), are as follow: *Diabetes* was defined using the 1998 WHO criteria of fasting glucose >7mmol/l or glucose tolerance >11mmol/l; use of hypoglycaemic medication or diagnosis by a doctor or nurse (Table 2.1). *Hypertension* was defined as an average blood pressure (BP) measurement ≥140/90mmHg or the use of antihypertensive agents (Table 2.1). *Obesity* was defined by body mass index (BMI) ≥30kg/m^2; waist circumference (WC) ≥94cm for males, ≥80cm for females and waist-to-hip ratio >1.0 for males and >0.85 for females (Table 2.1). *Dyslipidaemia* was defined as total cholesterol >5mmol/l; triglycerides >1.5mmol/l, HDL-C <1.2mmol/l; LDL-C >3.0mmol/l and HDL-C/total cholesterol <20% (Table 2.1).

According to WHO (1999) and the IDF's Joint Interim Statemenst (JIS) (Alberti *et al.,* 2009), metabolic syndrome (MetS) is defined as a collection of risk factors of CVD that occur together more often than by chance alone. To be diagnosed with MetS, using the JIS criteria, 3 out of the 5 abnormal findings are required: WC: ≥94cm for males and ≥80cm for females; Triglycerides: >1.7mmol/l; HDL-C: <1mmol/l for males and <1.3mmol/l for females; BP: ≥130/85mmHg; and Fasting glucose: ≥5.6mmol/l (Table 2.1).

Table 2.1 Definitions of risk phenotypes used for the diagnosis of disease in this study (Peer *et al.,* 2012).

Diabetes	
Fasting glucose	>7mmol/l
Glucose tolerance	>11mmol/l
Obesity	
Body mass index	Overweight: 25-29,99 kg/m2
	Obese: >30kg/m2
Waist circumference	Males: >94cm
	Females: >80cm
Waist-to-hip ratio	Males: >1.0
	Females: >0.85
Hypertension	
Blood pressure	>140/90mmHg
Dyslipidaemia	
Total cholesterol	>5mmol/l
Triglycerides	Males: <1mmol/l
	Females: <1.3mmol/l
HDL-C	<1.2mmol/l
LDL-C	>3.0mmol/l
HDL-C/total cholesterol	<20%
Metabolic syndrome	
Waist circumference	Males: >94cm
	Females: >80cm
Triglycerides	>1.7mmol/l
HDL-C	Males: <1mmol/l
	Females: <1.3mmol/l
Blood pressure	>130/85mmHg
Fasting glucose	>5.6mmol/l

2.4 DNA extraction

Whole blood was collected from 1 116 participants in ethylene diamine triacetic acid (EDTA) blood collection tubes and separated into individual components (plasma, buffy coat and red blood cells) by gradient centrifugation. The buffy coat, containing white blood cells and platelets, was stored at -80°C for deoxyribonucleic acid (DNA) extraction. In order to obtain a high quantity and quality of DNA, the DNA extraction protocol was optimised. This was necessary because the samples were stored for many years (~10 years) and underwent many freeze-thaw cycles. There was also a definite period of time, over a long weekend in summer, where the freezers were off due to an electric outage with no back-up to maintain the temperature of the samples. These are all factors that contribute to the degradation of DNA and lead to a low quantity and quality of DNA.

Deoxyribonucleic acid (DNA) was extracted using the Gentra Puregene blood kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Briefly, 1.5ml red blood cell (RBC) lysis solution was added to 500 µl buffy coat, and this was incubated for 10 minutes (min) at room temperature

(RT). Samples were then centrifuged for 5 min at 2 000 x *g* and the supernatant was subsequently discarded. The pellet was resuspended in the residual liquid by vortex-mixing and 3 ml lysis solution was added to the re-suspended pellet. After adding 15 µl RNase A to the re-suspended pellet, and mixing by inversion, samples were incubated for 10 min at 37 °C and then for 3 min on ice. Following the addition of 1 ml protein precipitation solution, samples were centrifuged for 5 min at 2 000 x *g*. In a clean 15 ml centrifuge tube, 3 ml isopropanol was added along with the supernatant from the centrifugation step after the addition of protein precipitation solution and mixed by inversion. Samples were then centrifuged at 2 000 x *g* for 3 min, the supernatant discarded, and the pellet drained. The pellet was then washed with 3 ml 70% ethanol (EtOh) and centrifuged for 1 min at 2 000 x *g.* The pellet was then allowed to air dry at RT. The samples were then rehydrated with 150 µl DNA hydration solution and incubated at 65 °C for one hour followed by an overnight incubation at RT with shaking. DNA quality was assessed using the NanoDrop 2000c spectrophotometer (Thermo Scientific, Delaware, USA) using the NanoDrop 2000/2000c software (version 1.5). Values between 1.8-2.0 nm for the 260/280 ratio and 2.0-2.2 nm for the 260/230 ratio were deemed suitable.

2.4.1 Optimization of the DNA extraction protocol

After attempting the DNA extraction following the manufacturer's instructions and obtaining a low DNA yield and poor 260/280 ratios, the extraction was attempted using twice the amount of buffy coat (500 µl) as specified by the manufacturer's instructions. The extraction of another batch of samples was attempted using twice the volume of reagents (3 ml RBC lysis solution, 6 ml cell lysis solution, 30 µl RNase A, 2 ml protein precipitation solution, 6ml isopropanol, 6ml 70% EtOH) to determine if increasing the amount of buffy coat required more reagents, but did not change the volume of DNA hydration solution (150 µl) and compared the results to when following the manufacturer's instructions. A low DNA yield was still obtained, and the DNA extraction was then attempted using 500 µl buffy coat and half the stipulated volume of reagents (750 µl RBC lysis solution, 1.5 ml cell lysis solution, 7.5 µl RNase A, 500 µl protein precipitation solution, 1.5 ml isopropanol, 1.5 ml 70% ethanol and 100 µl DNA hydration solution), optimising the extraction protocol.

The optimised protocol thus used 500 µl of buffy coat with 750 µl RBC lysis solution, 1.5 ml cell lysis solution, 7.5 µl RNase A, 500 µl protein precipitation solution, 1.5 ml isopropanol, 1.5 ml 70% ethanol and 100 µl DNA hydration solution. Following optimization of the protocol, DNA was extracted using half the amounts of reagent as described above and DNA quality was assessed using the NanoDrop 2000c spectrophotometer (Thermo Scientific, Delaware, USA) using the NanoDrop 2000/2000c software (version 1.5). Values between 1.8-2.0 for the 260/280 ratio and 2.0-2.2 for the 260/230 ratio were deemed suitable for DNA purity.

2.4.2 DNA dilution

DNA samples with a concentration greater than 100 ng/µl were diluted to 50 ng/µl in dH_2O for downstream analyses. DNA quality of the dilutions was assessed using the Nanodrop 2000c spectrophotometer (Thermo Scientific, Delaware, USA) using the Nanodrop 2000/2000c software (version 1.5). Values between 1.8-2.0 for the 260/280 ratio and 2.0-2.2 for the 260/230 ratio were deemed suitable.

2.4.3 Gel electrophoresis

All DNA samples were electrophoresed on an agarose gel to assess DNA quality. Prior to gel electrophoresis, ~5 µl DNA samples were mixed with cresol and together with a 50 bp Kapa Universal DNA Ladder (Kapa Biosystems, Western Cape, South Africa) were electrophoresed on a 1% agarose gel (Addendum A) at 160 V, for approximately 1 hour in 1x sodium borate (SB) buffer (Addendum A). DNA visualization was aided by ethidium bromide (EtBr) using the G box (Syngene, Maryland, USA) with GeneSnap Syngene software (version 7.12.06).

2.5 PCR genotyping of *ACE*

The *ACE* insertion/deletion (I/D) polymorphism (rs4646994) was amplified using the primers used by Youssof *et al.* (2009) (Table 2.2) (Integrated DNA technologies, Iowa, USA). The region of interest was amplified using the polymerase chain reaction (PCR) in a final volume of 25 µl (1 x dyed Kapa Taq Ready Mix PCR Kit, 10 µM of each primer and 50 ng/µl genomic DNA). The PCR was performed in a 2720 Thermal Cycler PCR machine (Applied Biosystems, California). The optimised conditions of the PCR were an initial denaturation step, 35 cycles of denaturation, annealing and extension, followed by a final extension and cooling (Table 2.2). Optimization of the annealing step was required as non-specific binding was observed.

Table 2.2 Primer sequence and PCR cycling conditions used for the genotyping of the *ACE* rs4646994 I/D polymorphism (Youssof *et al.,* 2009).

***ACE* primers**			
	Primer sequence	**Tm (°C)**	**GC content (%)**
Forward primer	5' - CTG GAG ACC ACT CCC ATC CTT TCT - 3'	60.0	54.2
Reverse primer	5; - GAT GTG GCC ATC ACA TTC GTC AGA T - 3'	59.2	48.0
PCR cycling conditions			
Step	**Temp (°C)**	**Time (min)**	**Cycles**
Initial denaturation	95	5	
Denaturation	95	1	35
Annealing	60	1	
Extension	72	2	
Final extension	72	10	
Hold	4	∞	

2.5.1 Gel electrophoresis of *ACE*

All PCR products and a 50 bp Kapa Universal DNA Ladder (Kapa Biosystems, Western Cape, South Africa) were electrophoresed on a 1% agarose gel (Addendum A) at 160V for approximately 1 hour in 1 x sodium borate (SB) buffer (Addendum A). PCR product visualization was aided by ethidium bromide (EtBr) using the G box (Syngene, Maryland, USA) with GeneSnap Syngene software (version 7.12.06).

2.6 KASP genotyping

Samples with a concentration ≥20 ng/µl were diluted to 5-20 ng/µl for genotyping by Kompetitive Allele-Specific PCR (KASP) genotyping at LGC Genomics (Hertfordshire, United Kingdom). The DNA sequences (Table 2.3) flanking the SNPs of interest were sent to LGC Genomics and the primers were designed in-house.

Table 2.3 DNA sequences flanking the SNPs of interest for KASP genotyping.

Gene	SNP	SNP sequence
ACE	I/D	CCCATCCTTTCTCCCATTTCTCTAGACCTGCTGCCTATACAGTCACTTTT[TT TTTTTTTTTGAGACGGAGTCTCGCTCTGTCGCCCAGGCTGGAGTGCAG/-]TTTTATGTGGTTTCGCCAATTTTATTCCAG CTCTGAAATTCTCTGAGCTC
AGT	rs699	TGACAGGTTCATGCAGGCTGTGACAGGATGGAAGACTGGCTGCTCCCTGA C/TGGGAGCCAGTGTGGACAGCACCCTGGCTTTCAACACCTACGTCCACTT
AT1R	rs5186	AAGAAGGAGCAAGAGAACATTCCTCTGCAGCACTTCACTACCAAATGAGCA/ CTTAGCTACTTTTCAGAATTGAAGGAGAAAATGCATTATGTGGACTGAACC
MC4R	rs1782313	CTTTAATGACTACAACATTATAGAAGTTTAAAGCAGGAGAGATTGTATCCC/T GATGGAAATGACAAGAAAAGCTTCAGGGGGAAGGTGACATTTAAGTTGG
	rs12970134	CAGACATTTTTTCGTTAATAATTCATCCTTTCGTACTCATTAATCCTTACA/GT ATATCTTACATAATTTCAGTGTTTCTGGGTTGCCTATTCTGTTCTATGC
	rs2229616	GCAGCTCTAAATGCACAGTCCAGAGTCATCCTTACCTGCCTTCTGCCACCA/ GCCGACCCCAGGCTATATTTTGAGTAGGATGGGACCTGAGAAGGCTTGCC
FTO	rs17817499	CCACTACTTTACAAATATTACTCAATAAATATCAGTTTAATTAAGTTGGGA/TT TTTTCTTATTATTTTAGTAACTTTGGATTCTAAATGTGCTTCTGGGTAT
TCF7L2	rs7903146	ACCTAGCACAGCTGTTATTTACTGAACAATTAGAGAGCTAAGCACTTTTTAG ATAC/TTATATAATTTAATTGCCGTATGAGGCACCCTTAGTTTTCAGACGAGA
TNFα	rs1800629	CAGAGAAGGGAAGCAGTTTGAGAAAAAAAATGGGAATCCAAAGTACAAGAA/ GGGGGCCCTGTTACAGTGGCCAGGATAGAGGGAATGTCTCTTCCAGAA

DNA sequences in red represent the base change of the SNP. rs180692 and rs4646994 was excluded from the analysis as reproducible results were not obtained. *ACE* – Angiotensin-converting enzyme; *AGT* – Angiotensinogen; *AT1R* – Angiotensin II type I receptor; *MC4R* – Melanocortin 4 receptor; *FTO* – fat-mass and obesity associated; *TCF7L2* – Transcription factor 7-like 2; *TNFα* – Tumour necrosis factor-alpha.

KASP is a homogenous, fluorescence-based assay which enables the bi-allelic discrimination of known DNA sequence variants by making use of allele-specific fluorescence labelled primers. The KASP assay mix contains two allele-specific forward primers and one common reverse primer. Each allele-specific primer contains either a FAM dye labelled tail sequence, or a HEX dye labelled tail

sequence, which corresponds to a universal fluorescence resonance energy transfer (FRET) cassette. During the PCR amplification, the relevant allele-specific primer binds the template DNA and elongates adding the dye labelled tail sequence to the newly synthesized strand. As the compliment of this dye labelled tail sequence is generated, the FRET cassette is then able to bind to the DNA, enabling the fluorescence emission. One fluorescent signal indicates homozygosity for a specific SNP while heterozygosity generates a mixed fluorescence signal (Figure 2.1).

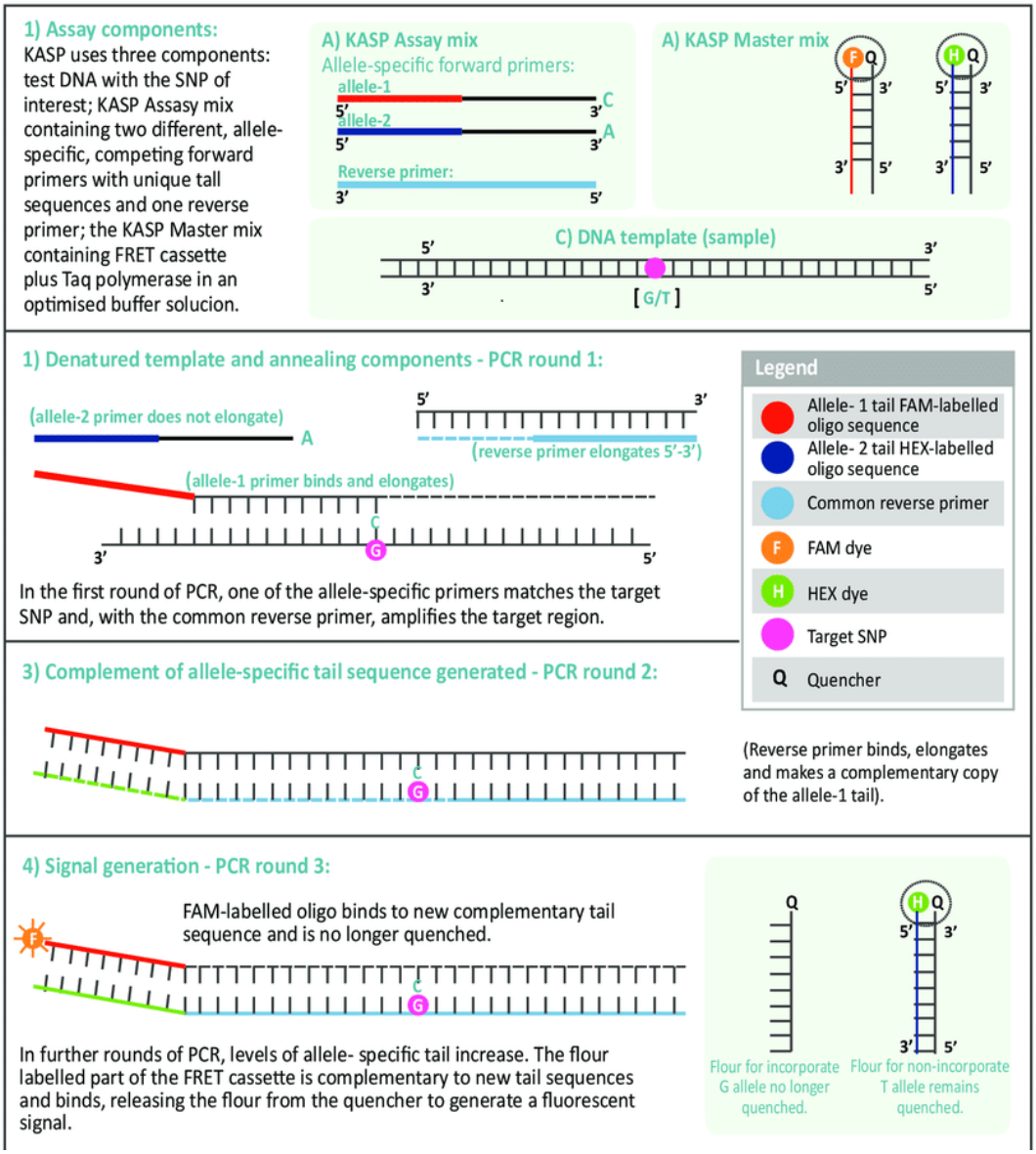

Figure 2.1 KASP genotyping is based on the competitive binding of allele-specific primers and FRET to discriminate between known SNPs (He *et al.,* 2014).

2.7 Statistical analysis

Various definitions are used for the diagnosis of disease as there is no “gold standard” for diagnosis, and as such, this study looked at the most common definitions used.

Data analysis was performed using R statistical software (v3.5.1). Demographic and clinical characteristics were summarised as mean and standard deviation if approximately normally distributed, and as medians and interquartile ranges (IQRs) if unevenly distributed. Genotype counts (%) and Hardy-Weinberg equilibrium (HWE) p-values were summarised separately for each of the CVD risk phenotypes (Type 2 diabetes mellitus, obesity, hypertension, dyslipidaemia and metabolic syndrome) under investigation (Table 2.1).

General linear modelling (GLM) was used to express the risk phenotype as a function of genotype, under the log-additive allelic model, adjusting for possible confounders. For association testing, the confounders were age and gender, and these were included in the GLM. Alleles were coded as the number of minor alleles (0, 1, 2) and each haplotype was similarly coded as number present. Haplotypes were only inferred in the blocks constructed using the solid spine of linkage disequilibrium (LD) method, implemented in Haploview software (v4.2) and were investigated under log-additive models only.

We used multiplicative (log-additive) genetic inheritance as an initial “screening” mechanism to identify signals that were worth pursuing in other genetic models (namely the dominant and recessive inheritance models). This is because the multiplicative model uses chromosomes (alleles) as the unit of analysis, and thus increases the power for detecting significant association that may be missed in other genetic modelling. Here, genotypes were coded as 3 categories (2 degrees of freedom).

Linear regression analysis was used to express the risk phenotype as a function of its measurements to determine continuous risk, adjusting for the possible confounding effects of gender and age. Alleles were coded as the number of minor alleles (0, 1, 2).

All modelling was done separately for the various CVD risk phenotypes described. When significant effects were detected using a multiplicative (log-additive) model, (<0.05), further investigation in other genetic models was performed. A trend towards significance (p=0.05-0.075) was used in the initial screening (log-additive model) of association.

Effects corresponding to p-values below 0.05 were described as significant, except for the HWE tests, where 0.01 was the critical p-value. All analyses were done using R and functions from R packages, *SNPassoc* and *haplo.stats*.

Chapter 3

Results

3. Results

3.1 Protocol optimization

3.1.1 DNA extraction protocol

Following the manufacturer's protocol for the extraction of DNA using the Gentra Puregene blood kit, low yields and quality of DNA was obtained. The extraction was then attempted using the double the amount of buffy coat and reagents, but low yields and quality was still obtained.

The protocol was thus optimised using double the amount of buffy coat (500 µl) with half the amount of reagents stipulated (750 µl RBC lysis solution, 1.5 ml cell lysis solution, 7.5 µl RNase A, 500 µl protein precipitation solution, 1.5 ml isopropanol, 1.5 ml 70% ethanol and 100 µl DNA hydration solution). DNA quantity and quality were assessed using the NanoDrop 2000 spectrophotometer. Although not degraded (Figure 3.1), many of the DNA samples were not pure as they did not meet the values of 1.8-2.0 for the 260/280 ratio or 2.0-2.2 for the 260/263 ratio indicating protein and RNA contamination.

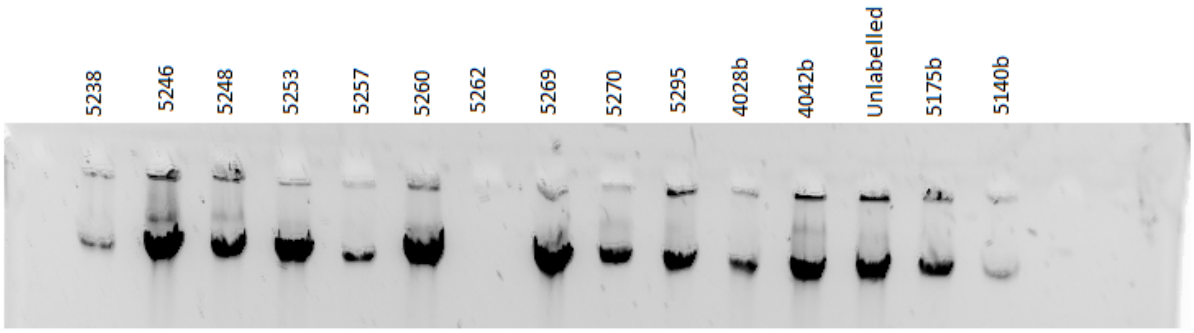

Figure 3.1 DNA quality was assessed using agerose gel electrophoresis to determine if sample degradation had occurred.

3.1.2 PCR amplification protocol

The *ACE* rs4646994 I/D polymorphism was PCR amplified using the protocol and primers used by Youssof *et al.* (2009) (Table 2.2). The PCR amplification was first attempted with an annealing temperature of 58°C, but non-specific binding was observed (Figure 3.2 A). The annealing temperature was thus increased to 60°C, overcoming the non-specific binding (Figure 3.2 B), and this temperature was used throughout.

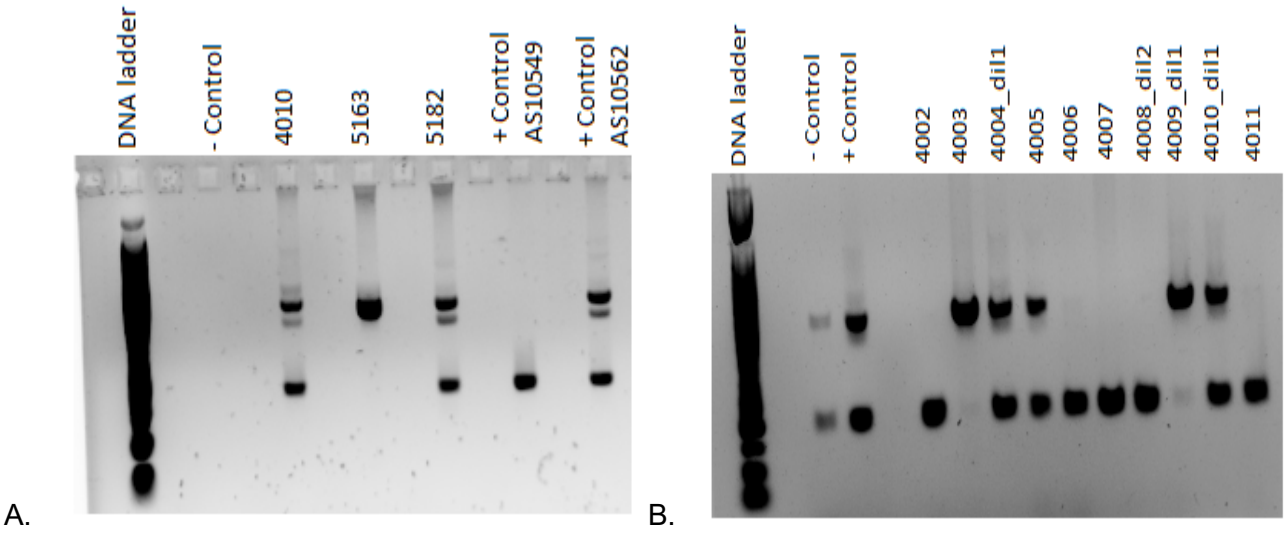

Figure 3.2 Optimization of the PCR protocol annealing temperature to overcome the observed non-specific binding. (A) annealing at 58°C with non-specific binding observed (lanes 4010, 5182 and the positive control); (B) annealing at 60°C where the non-specific binding has been overcome (lane 4010_dil1).

3.2 Genotyping cohort

Five hundred and eighty-five DNA samples were available for KASP genotyping (n=194 (33.2%) male; n=391 (66.8%) female). The median age was 43 years (IQR: 23 years – 53 years) (Figure 3.3).

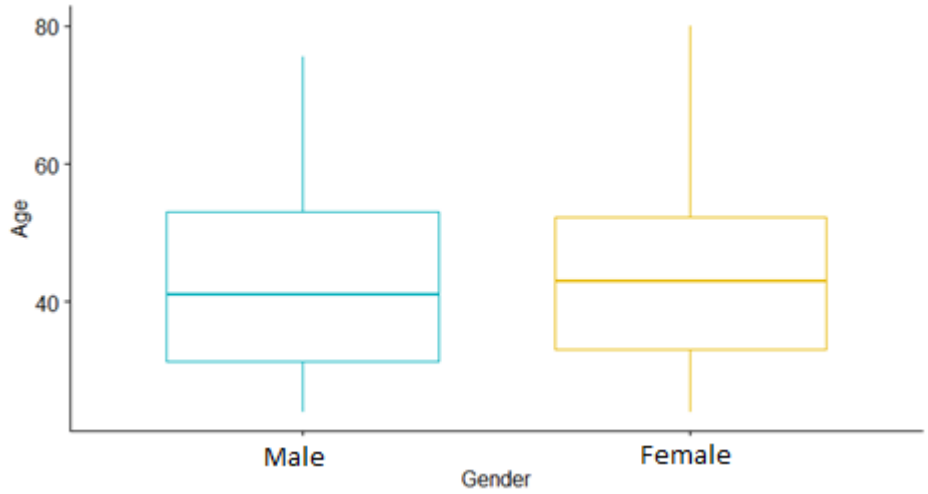

Figure 3.3 Median age (IQR) of males and females in the KASP-genotyped cohort.

3.3 Sample characteristics

3.3.1 CVD risk phenotypes according to age

Older individuals were found to exhibit significantly more risk phenotypes associated with CVD than younger individuals (Table 3.1). This was true for all diseases, except for the presence of dyslipidaemia which was not statistically different between younger and older individuals.

Table 3.1 The association of age with risk phenotypes.

Disease definition	<25	25-34	35-44	45-54	55-64	>65	p-value
Overall T2DM	0	7	31	16	11	0	**$3.038x10^{-10}$**
T2DM by fasting glucose	0	6	27	13	8	0	**$6.432x10^{-12}$**
T2DM by glucose tolerance	0	5	25	11	8	0	**$5.646x10^{-7}$**
Overall obesity	1	108	196	60	31	1	**$1.709x10^{-6}$**
Obesity by BMI	1	77	145	37	21	1	**0.00077**
Obesity by waist circumference	1	107	193	57	29	2	**$8.665x10^{-5}$**
Obesity by waist-to-hip ratio	0	44	106	36	17	1	**$1.157x10^{-7}$**
Hypertension	0	6	55	32	12	1	**$2.224x10^{-15}$**
Overall dyslipidaemia	3	176	227	80	35	3	0.508
Dyslipidaemia by total cholesterol	1	29	73	40	16	1	**$2.927x10^{-10}$**
Dyslipidaemia by triglycerides	0	15	44	15	13	0	**$2.470x10^{-7}$**
Dyslipidaemia by HDL-C	2	144	174	52	20	2	**0.0019**
Dyslipidaemia by LDL-C	1	75	125	57	22	2	**$2.698x10^{-7}$**
Dyslipidaemia by HDL-C/total cholesterol	0	47	55	24	8	0	**0.038**
Metabolic syndrome	0	14	91	33	15	0	**$<2.2x10^{-16}$**

3.3.2 CVD risk phenotypes according to gender

Females were found to be more obese than males, with higher BMI ($p<2.2x10^{-16}$), WC ($p=2.2x10^{-16}$) and waist-to-hip ratio ($p=9.11x10^{-6}$) (Table 3.2). The overall prevalence of obesity was 68.72%, with 23.71% of all males and 91.05% of all females classified as being obese ($p<2.2x10^{-16}$) according to BMI, WC and/or waist-to-hip ratio (Figure 3.4 and Table 3.3). The prevalence of obesity in males and females by BMI, WC and waist-to-hip ratio, respectively, was 10.82% and 66.75% ($p<2.2x10^{-16}$); 20.62% and 89.26% ($p<2.2x10^{-16}$); and 8.25% and 48.08% ($p<2.2x10^{-16}$) (Table 3.3).

The overall prevalence of T2DM was 11.79% (n=69), with 8.25% male (n=16) and 13.55% female (n=53) (p=0.082) according to fasting glucose (>7 mmol/l) and glucose tolerance (<11 mmol/l) values

(Figure 3.4 and Table 3.3). The prevalence of T2DM by fasting glucose and 2h glucose tolerance respectively was 6.19% in males (n=12) and 9.72% in females (n=38) (p=0.331), and 4.12% in males (n=8) and 10.23% in females (n=40) (p=0.015), respectively (Table 3.3). In total, 49.28% (n=34) of the participants (25% of males (n=4) and 56.60% of females (n=30)) with T2DM by fasting glucose (<7 mmol/l) and/or glucose tolerance (<11 mmol/l) were on insulin treatment. Fasting glucose levels and glucose tolerance were similar in males and females (p=0.651 and p=0.053, respectively) (Table 3.2).

Table 3.2 The median and IQRs of CVD risk factors by gender in the total sample genotyped (n=585).

	Males (n=194)	Females (n=391)	Total (n=585)	p-value
Anthropometry				
BMI	22.17 (19.92-38.58)	33.04 (27.92-38.58)	29.52 (23.21-35.57)	**<2.20X10^{-16}**
Waist circumference	80.5 (74.63-91.5)	97.65 (88.13-108.23)	92.5 (80.93-105.0)	**<2.20X10^{-16}**
Waist-to-hip ratio	0.87 (0.83-0.94)	0.85 (0.8-0.9)	0.86 (0.81-0.91)	**9.108X10^{-6}**
Blood pressure				
Systolic	127 (115-140)	118 (107-132)	121 (110-136)	**1.722x10^{-6}**
Diastolic	80 (73-90)	80 (72-89)	80 (72-89)	0.651
Glucose				
Fasting glucose	4.9 (4.5-5.3)	4.8 (4.3-5.4)	4.9 (4.4-5.4)	0.651
Glucose tolerance	5.9 (4.6-7.3)	6.0 (4.9-7.85)	5.9 (4.8-7.5)	0.053
Lipid profile				
Total cholesterol	4.28 (3.52-5.02)	4.37 (3.69-5.12)	4.34 (3.64-5.04)	0.214
Triglycerides	0.96 (0.71-1.3)	0.98 (0.72-1.29)	0.97 (0.71-1.29)	0.930
HDL-C	1.45 (0.93-1.41)	1.04 (0.87-1.27)	1.06 (0.89-1.32)	**0.0018**
LDL-C	2.81 (2.2-3.43)	3.06 (2.47-3.74)	2.97 (2.38-3.66)	**0.0011**
HDL-C/Total cholesterol ratio	26.89 (21.98-34.99)	24.26 (19.98-29.14)	25.1 (20.69-30.42)	**1.208X10^{-5}**

BMI – Body mass index; HDL-C – High-density lipoprotein cholesterol; LDL-C – Low-density lipoprotein cholesterol.

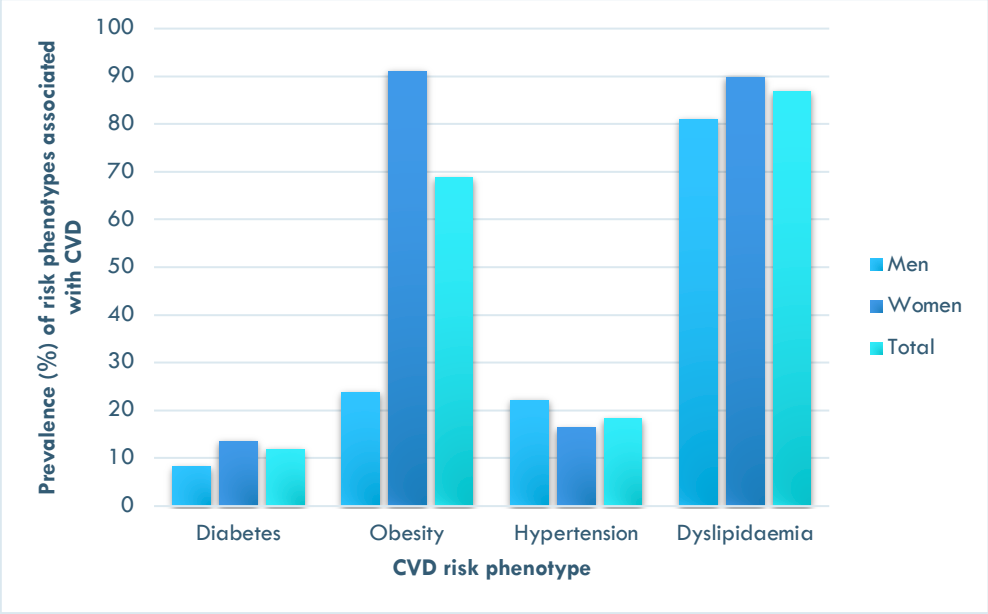

Figure 3.4 Prevalence of CVD risk phenotypes according to gender in the KASP-genotyped cohort.

Percentage refers to number of affected individuals per total sample (n=585), per total number of males (n=194) and per total number of females (n=391) in the KASP genotyped samples.

Diabetes: fasting glucose >7mmol/l, glucose tolerance >11mmol/l; Hypertension: BP ≥140/90mmHg; Dyslipidaemia: total cholesterol >5mmol/l, triglycerides >1.5mmol/l, HDL-C <1.2mmol/l, LDL-C >3.0mmol/l, HDL-C/total cholesterol ratio <20%; Obesity: BMI ≥30kg/m2, waist circumference males ≥94cm, females ≥80cm, waist-to-hip ratio males >1, females >0.85.

Systolic BP was higher in males ($p=1.72x10^{-6}$), but diastolic BP was similar in males and females (p=0.651) (Table 3.2). The overall prevalence of hypertension was 18.29% (n=107), with 22.17% of those being male (n=43) and 16.37% female (n=64) (0.111) (Figure 3.4 and Table 3.3). Of those with raised BP, 13.95% of males (n=6) and 46.88% of females (n=30) were on antihypertensive medication.

Total cholesterol and triglyceride levels (p=0.214 and p= 0.930, respectively) were similar in males and females (Table 3.2). HDL-C, LDL-C and HDL-C/total cholesterol levels were found to be higher in males than females (p=0.0018, p=0.0011, and $p=1.21x10^{-5}$, respectively) (Table 3.2). The overall prevalence of dyslipidaemia was 86.84% (n=508), with 80.93% of all males (n=157) and 89.77% of all females (n=351) having lipid abnormalities (p=0.0044) (Figure 3.4 and Table 3.3). The prevalence of dyslipidaemia by raised TC (>5 mmol/l) was 24.74% (n=48) in males and 27.62% (n=108) in females (p=0.487) (Table 3.3). The prevalence of raised triglycerides (>1.5 mmol/l) was 17.53% (n=34) in males and 14.32% (n=56) in females (p=0.395) (Table 3.3). The prevalence of decreased HDL-C levels (<1.2 mmol/l) in males was 59.28% (n=115) and 68.29% (n=267) in females (p=0.028) (Table 3.3). The prevalence of raised LDL-C levels (<3 mmol/l) was 41.75% (n=81) in males and 51.15% (n=200) in females (p=0.027) (Table 3.3). The prevalence of decreased HDL-C/total cholesterol (<20%) was 14.95% (n=29) in males and 24.81% (n=97) in females (0.0076) (Table 3.3). Overall, the majority of females were obese, type 2 diabetic and had abnormal lipid profiles. All

medians and IQRs of CVD risk factors (Table 3.2) is graphically represented in Addendum B (Figure B1-12).

Table 3.3 Counts and percentage of affected males and females for the measures of CVD risk factors (n=585).

	Males (n=194) n (%)	Females (n=391) n (%)	Total sample (n=585) n (%)	p-value
Obesity				
Obese	46 (23.71)	356 (91.05)	402 (68.72)	**$<2.20x10^{-16}$**
BMI	21 (10.82)	261 (66.75)	282 (48.21)	**$<2.20x10^{-16}$**
Waist circumference	40 (20.62)	349 (89.26)	389 (66.5)	**$<2.20x10^{-16}$**
Waist-to-Hip ratio	16 (8.25)	188 (48.08)	204 (34.87)	**$<2.20x10^{-16}$**
Type 2 diabetes mellitus				
Diabetic	16 (8.25)	53 (13.55)	69 (11.79)	0.082
Fasting glucose	12 (6.19)	38 (9.72)	50 (8.55)	0.331
Glucose tolerance	8 (4.12)	40 (10.23)	48 (8.21)	**0.015**
Hypertension				
Blood pressure	43 (22.17)	64 (16.37)	107 (18.29)	0.111
Dyslipidaemia				
Dyslipidaemic	157 (80.93)	351 (89.77)	508 (86.84)	**0.0044**
Total cholesterol	48 (24.74)	108 (27.62)	156 (26.67)	0.487
Triglycerides	34 (17.53)	56 (14.32)	90 (15.38)	0.395
HDL-C	115 (59.28)	267 (68.29)	382 (65.3)	**0.028**
LDL-C	81 (41.74)	200 (51.15)	281 (48.03)	**0.028**
HDL-C/Total cholesterol ratio	29 (14.95)	97 (24.81)	126 (21.54)	**0.0076**

BMI – Body mass index; HDL-C – High-density lipoprotein cholesterol; LDL-C – Low-density lipoprotein cholesterol. Percentage refers to number of affected individuals per total sample (n=585), per total number of males (n=194) and per total number of females (n=391) in the KASP genotyped samples.

Diabetes: fasting glucose >7mmol/l, glucose tolerance >11mmol/l; Dyslipidaemia: total cholesterol >5mmol/l, triglycerides >1.5mmol/l, HDL-C <1.2mmol/l, LDL-C >3mmol/l, HDL-C/total cholesterol ratio <20%; ; Hypertension: BP ≥140/90mmHg; Obesity: BMI ≥30kg/m^2, waist circumference males ≥94cm, females ≥80cm, waist-to-hip ratio males >1, females >0.85.

One hundred and fifty-four were diagnosed as having MetS by the JIS criteria (Alberti *et al.,* 2009). The overall prevalence of MetS was 26.32% (n=154), with 14.95% of males (n=29) and 31.71% of females (n=125) being diagnosed ($p=1.698x10^{-5}$) (Figure 3.5 and Table 3.4).

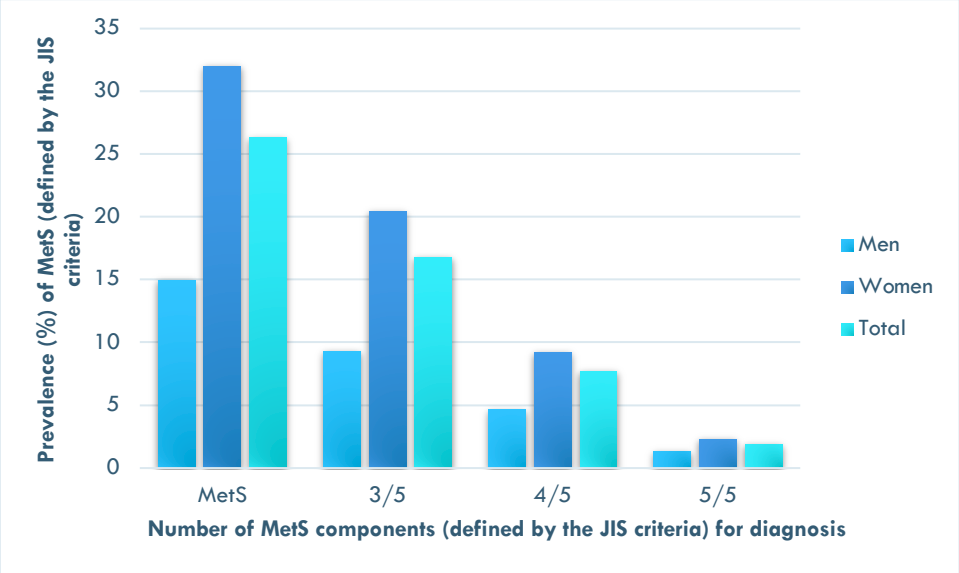

Figure 3.5 Prevalence of MetS, as defined by the JIS, by number of components in affected males and females.

MetS calculated per total sample (n=585), number of componets calculated per number of affected individuals in total (n=154), number of affected males (n=29) and affected females (n=125)

Blood pressure (BP): ≥130/85mmHg; Fasting glucose: ≥5.6mmol/l; High-density lipoprotein cholesterol (HDL-C): males <1.0mmol/l, females <1.3mmol/l; Triglycerides: >1.7mmol/l; Waist circumference (WC): males >94cm, females >80cm.

The prevalence of raised BP (82.76% (n=24) males vs. 63.20% (n=79) females, p=0.0891) and triglyceride levels (65.52% (n=19) males vs. 25.60% (n=32) females, p= $4.79x10^{-5}$) was highest among affected males than affected females (Figure 3.6 and Table 3.4). The prevalence of increased fasting glucose (62.40% (n=78) females vs. 55.17% (n=16) males, p=0.5152), WC (100.00% (n=125) females vs. 72.41% (n=21) males, p=$1.767x10^{-7}$) and HDL-C levels (91.20% (n=114) females vs. 68.97% (n=20) males, p=0.0027) was higher in affected females than in affected males (Figure 3.6 and Table 3.4).

Table 3.4 Counts and percentage of affected males and females for the measures of MetS in the KASP genotyped samples.

	Males	Females	Total sample	p-value
MetS	29 (14.95)	125 (31.71)	154 (26.32)	**$1.698x10^{-5}$**
Waist circumference	21 (72.41)	125 (100.00)	146 (94.81)	**$1.767x10^{-7}$**
Fasting glucose	16 (55.17)	78 (62.40)	94 (61.04)	0.5152
Blood pressure	24 (82.76)	79 (63.20)	103 (66.88)	0.0891
Triglycerides	19 (65.52)	32 (25.60)	51 (33.12)	**$4.79x10^{-5}$**
HDL-C	20 (68.97)	114 (91.20)	134 (87.01)	**0.0027**

BP: ≥130/85mmHg; Fasting glucose: ≥5.6mmol/l; HDL-C: males <1mmol/l, females <1.3mmol/l; MetS: Diagnosis by JIS criteria; Triglycerides: >1.7mmol/l; Waist circumference: males ≥94cm, females ≥80cm.

Overall, an obese WC (males ≥94 cm; females ≥80 cm) (94.81%, n=146) was the component most prevalent among individuals with MetS, with all affected females meeting this criterion. However, in affected males, BP (≥130/85 mmHg) (82.76%, n=24) was the most prevalent component (Figure 3.6

and Table 3.4). Decreased HDL-C (males <1 mmol/l; females <1.3 mmol/l) was the second most common criterion met by all individuals with MetS (87.01%, n=134) and by affected females (91.20%, n=114), while in affected males the second most common criterion met was an obese WC (72.43%, n=21) (Figure 3.6 and Table 3.4). Raised triglycerides (>1.7 mmol/l) was the least common among all affected individuals (33.12%, n=51) and affected females (25.60%, n=32), while in affected males fasting glucose (≥5.6 mmol/l) was the least common criterion met (55.17%, n=16) (Figure 3.6 and Table 3.4).

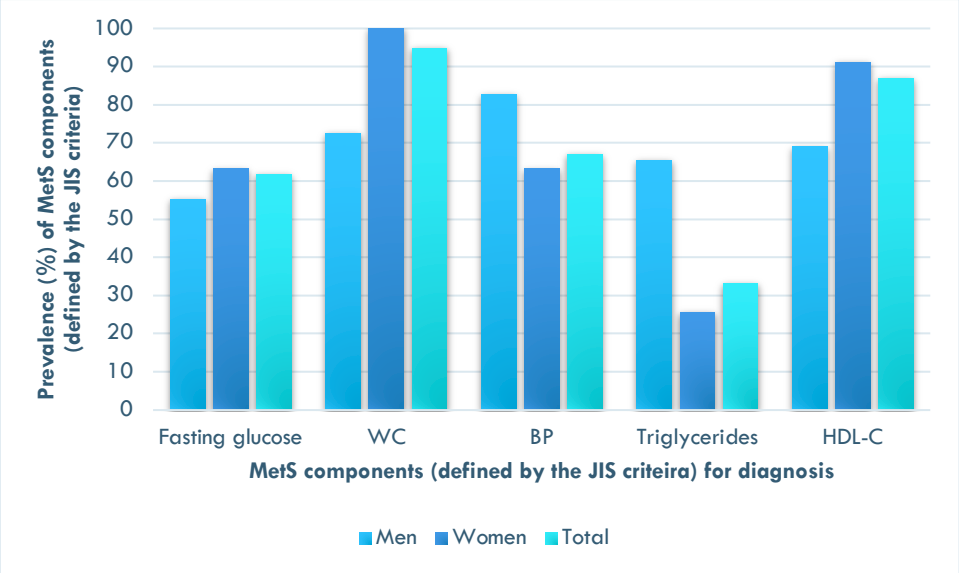

Figure 3.6 Prevalence of MetS components by gender among affected individuals.

Blood pressure (BP): ≥130/85mmHg; Fasting glucose: ≥5.6mmol/l; High-density lipoprotein cholesterol (HDL-C): males <1.0mmol/l, females <1.3mmol/l; Triglycerides: >1.7mmol/l; Waist circumference (WC): males >94cm, females >80cm.

3.4 Genotype distributions

3.4.1 PCR genotyping

Three hundred and thirty-four samples were genotyped for the *ACE* I/D variation using PCR amplification (Figure 3.7). One hundred and fifty-six (156) (46.71%) were homozygous for the D allele (DD genotype) (represented by lanes 1089 and 1097 at 190bp in Figure 3.7), 31 (9.28%) were homozygous for the I allele (II genotype) (represented by lanes 1094 and 1100 at 490bp in Figure 3.5) and 147 (44.01%) were heterozygous (ID genotype) (represented by lanes 1088, 1090, 1092, 1101, 1102 and 1105 in Figure 3.7).

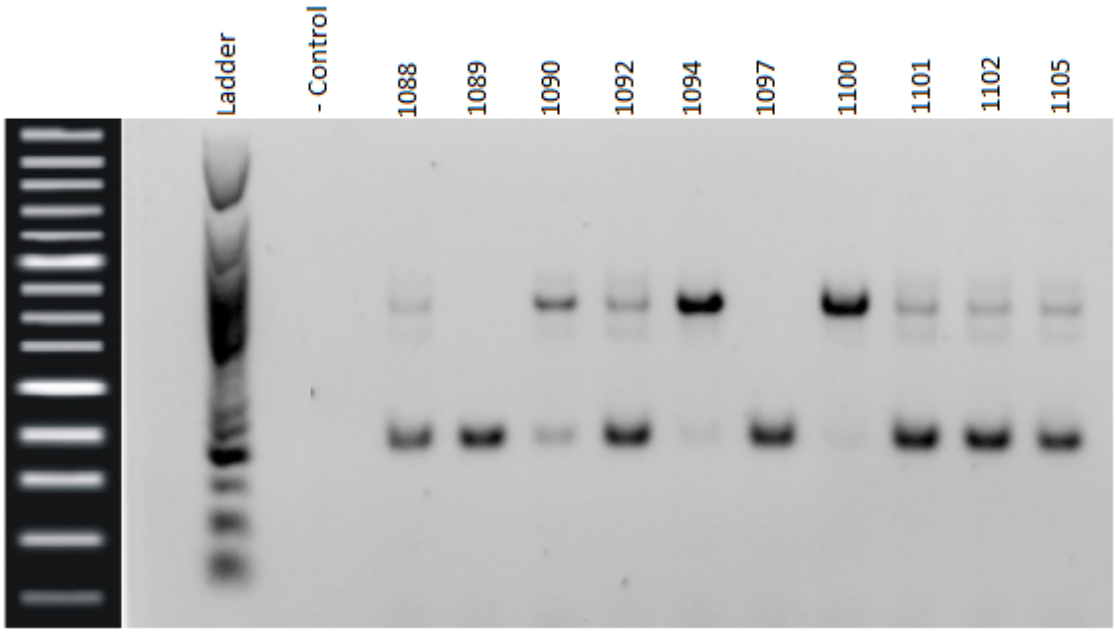

Figure 3.7 A representative image of the gel electrophoresis following PCR amplification of the *ACE* rs4646994 I/D polymorphism.

3.4.2 KASP genotyping

3.4.2.1 Hardy-Weinberg equilibrium

All SNPs were in Hardy-Weinberg equilibrium for cases and controls ($p>0.05$), except rs17817499 and it was thus excluded from further analysis.

3.4.2.2 Genotype frequencies

The following genes were genotyped using the KASP method:

Fat-mass and obesity associated (*FTO*) gene

Transcription factor 7-like 2 (*TCF7L2*)

Melanocortin 4 receptor (*MC4R*)

Angiotensinogen (*AGT*)

Angiotensin II type I receptor (*AT1R*).

Genotype frequencies of each SNP can be found in the table below (Table 3.5). The genotype distributions are also graphically represented in Addendum C (Figure C1-7).

Table 3.5 Genotype distributions of samples genotyped using KASP technology.

TCF7L2		
rs7903146	T/C	260 (45.90%)
	C/C	243 (42.19%)
	T/T	64 (11.30%)
MC4R		
rs229616	G/G	326 (56.79%)
	G/A	209 (36.41%)
	A/A	39 (6.79%)
rs17782313	T/T	304 (52.69%)
	T/C	232 (40.21%)
	C/C	41 (7.11%)
rs1297034	G/G	463 (80.94%)
	G/A	102 (17.83%)
	A/A	7 (1.22%)
AGT		
rs699	C/C	496 (86.26%)
	C/T	76 (13.22%)
	T/T	3 (0.52%)
AT1R		
rs5186	A/A	562 (99.47%)
	C/A	3 (0.3%)
	C/C	0

AGT – Angiotensinogen; *AT1R* – Angiotensin II type I receptor; *FTO* – Fat-mass and obesity associated; *MC4R* – Melanocortin 4 receptor; *TCF7L2* – Transcription factor 7-like 2.

3.4.3 Genotype distributions by disease under a log-additive model

An additive model was used to determine if any of the SNPs were associated with any of the CVD risk variables in this study.

3.4.3.1 Type 2 diabetes mellitus

No SNPs were associated with overall T2DM diagnosis by fasting glucose (>7 mmol/l) and/or glucose tolerance (>11 mmol/l) under an additive model adjusted for gender and age (Table 3.6).

Table 3.6 Genotype distributions and frequencies of all SNPs in controls and T2DM cases by fasting glucose and/or glucose tolerance under a log-additive inheritance model, adjusted for gender and age.

Gene	SNP	Genotype	Controls	Cases	OR	95% CI	p-value
ACE	rs4646994	D/D	136 (45.6)	20 (55.6)	0.87	0.49-1.54	0.633
		I/D	135 (45.3)	12 (33.3)			
		I/I	27 (9.1)	4 (11.1)			
TCF7L2	rs7903146	T/T	209 (41.7)	34 (51.5)	0.69	0.45-1.07	0.092
		T/C	232 (46.3)	28 (42.4)			
		C/C	60 (12.0)	4 (6.1)			
MC4R	rs229616	G/G	282 (55.7)	44 (64.7)	0.80	0.51-1.26	0.331
		G/A	189 (37.4)	20 (29.4)			
		A/A	35 (6.9)	4 (5.9)			
	rs17782313	T/T	273 (53.7)	31 (44.9)	1.29	0.85-1.95	0.238
		T/C	200 (39.4)	32 (46.4)			
		C/C	35 (6.9)	6 (8.7)			
	rs1297034	G/G	407 (80.9)	56 (81.2)	0.92	0.50-1.72	0.8
		G/A	89 (17.7)	13 (18.8)			
		A/A	7 (1.4)	0			
AGT	rs699	C/C	437 (86.0)	59 (88.1)	0.81	0.38-1.73	0.577
		C/T	68 (13.4)	8 (11.9)			
		T/T	3 (0.6)	0			
AT1R	rs5186	A/A	496 (99.4)	66 (100.0)	-	-	-
		C/A	3 (0.6)	0			
		C/C	0	0			

T2DM diagnosis by fasting glucose (>7mmol/l) and/or glucose tolerance (>11 mmol/l).

ACE – Angiotensin-converting enzyme; *AGT* – Angiotensinogen; *AT1R* – Angiotensin II type I receptor; *MC4R* – Melanocortin 4 receptor; *TCF7L2* – Transcription factor 7-like 2.

High-risk and diabetic individuals were first separated and then combined as cases for genetic analysis to determine if any SNP is involved in disease predisposition and/or on disease diagnosis.

Under a log-additive genetic model, adjusted for gender and age, rs17782313 (C/C genotype) of *MC4R* was significantly associated with T2DM (assessed using fasting glucose levels) when controls, high-risk (5.6-7 mmol/l) and diabetic (>7 mmol/l) individuals were investigated (p=0.043) (Table 3.7).

Table 3.7 Genotype distributions and frequencies of all SNPs in controls, high-risk and T2DM cases by fasting glucose levels under a log-additive inheritance model, adjusted for gender and age.

Gene	SNP	Genotype	Controls	High-risk	Cases	Diff	95% CI	p-value
ACE	rs4646994	D/D	119 (44.4)	24 (63.2)	13 (46.4)	-0.023	-0.121-0.075	0.649
		I/D	124 (46.3)	31 (31.6)	11 (39.1)			
		I/I	25 (9.3)	2 (5.6)	4 (14.3)			
TCF7L2	rs7903146	T/T	191 (42.9)	25 (35.7)	27 (56.2)	-0.036	-0.11-0.037	0.335
		T/C	203 (45.6)	38 (54.3)	18 (37.5)			
		C/C	51 (11.5)	7 (10.0)	3 (6.2)			
MC4R	rs229616	G/G	249 (55.3)	42 (60.0)	32 (65.3)	-0.197	-0.099-0.058	0.61
		G/A	173 (38.4)	21 (30.0)	13 (26.5)			
		A/A	26 (6.2)	7 (10.0)	4 (8.2)			
	rs17782313	T/T	250 (55.3)	30 (42.9)	23 (46.0)	0.081	0.003-0.16	**0.043**
		T/C	175 (38.7)	33 (47.1)	22 (44.0)			
		C/C	27 (6.0)	7 (10.0)	5 (10.0)			
	rs1297034	G/G	357 (79.9)	61 (87.1)	42 (84.0)	-0.089	-0.203-0.025	0.126
		G/A	83 (18.6)	9 (12.9)	8 (16)			
		A/A	7 (1.6)	0	0			
AGT	rs699	C/C	388 (85.8)	61 (88.4)	42 (85.7)	-0.029	-0.162-0.103	0.666
		C/T	61 (13.5)	8 (11.6)	7 (14.3)			
		T/T	3 (0.7)	0	0			
AT1R	rs5186	A/A	441 (99.5)	68 (98.6)	48 (100.0)	-0.057	-0.729-0.614	-
		C/A	2 (0.5)	1 (1.4)	0			
		C/C	0	0	0			

The difference instead of an OR is reported as the analysis was performed with quantitative variables (3 categories) and not dichotomous variables.

T2DM diagnosis by fasting glucose (>7mmol/l; High-risk: 5.6-7 mmol/l).

ACE – Angiotensin-converting enzyme; *AGT* – Angiotensinogen; *AT1R* – Angiotensin II type I receptor; *MC4R* – Melanocortin 4 receptor; *TCF7L2* – Transcription factor 7-like 2

Under a log-additive inheritance model, adjusted for gender and age, rs17782313 (CC genotype) of *MC4R* was significantly associated with T2DM by fasting glucose when high-risk (5.6-7 mmol/l) and diabetic (>7 mmol/l) individuals were combined to represent cases (OR=1.48, 95%CI=1.06-2.06, p=0.022) (Table 3.8). The rs1297034 SNP of *MC4R* was also nominally associated with T2DM by fasting glucose when high-risk (5.6-7 mmol/l) and diabetic (>7 mmol/l) individuals were combined (OR=0.62, 95%CI=0.35-1.07, p=0.072) (Table 3.8).

Table 3.8 Genotype distributions and frequencies of all SNPs in controls and T2DM cases by fasting glucose levels when high-risk and diabetic and individuals are combined (cases) under a log-additive inheritance model, adjusted for gender and age.

Gene	SNP	Genotype	Controls	Cases	OR	95% CI	p-value
ACE	rs4646994	D/D	119 (44.4)	37 (56.1)	0.78	0.50-1.22	0.269
		I/D	124 (46.3)	23 (34.9)			
		I/I	25 (9.3)	6 (9.1)			
TCF7L2	rs7903146	T/T	191 (42.9)	52 (44.1)	0.95	0.68-1.32	0.766
		T/C	203 (45.6)	56 (47.5)			
		C/C	51 (11.5)	10 (8.5)			
MC4R	rs229616	G/G	249 (55.3)	74 (62.2)	0.96	0.68-1.5	0.8
		G/A	173 (38.4)	34 (28.6)			
		A/A	28 (6.2)	11 (9.2)			
	rs17782313	T/T	250 (55.3)	53 (44.2)	1.48	1.06-2.06	**0.022**
		T/C	175 (38.7)	55 (45.8)			
		C/C	27 (6.0)	12 (10.0)			
	rs1297034	G/G	357 (79.9)	103 (85.8)	0.62	0.35-1.07	0.072
		G/A	83 (18.6)	17 (14.2)			
		A/A	7 (1.6)	0			
AGT	rs699	C/C	388 (858.8)	103 (87.3)	0.82	0.45-1.49	0.505
		C/T	61 (13.5)	15 (12.7)			
		T/T	3 (0.7)	0			
AT1R	rs5186	A/A	441 (99.5)	116 (99.1)	1.48	0.13-16.59	-
		C/A	2 (0.5)	1 (0.9)			
		C/C	0	0			

T2DM diagnosis by fasting glucose (>5.6 mmol/l).

ACE – Angiotensin-converting enzyme; *AGT* – Angiotensinogen; *AT1R* – Angiotensin II type I receptor; *MC4R* – Melanocortin 4 receptor; *TCF7L2* – Transcription factor 7-like 2

No SNPs were associated when T2DM was diagnosed according to glucose tolerance under a log-additive inheritance model, adjusted for gender and age (Table 3.9).

Table 3.9 Genotype distributions and frequencies of all SNPs in controls and T2DM cases by impaired glucose tolerance under a log-additive inheritance model, adjusted for gender and age.

Gene	SNP	Genotype	Controls	Cases	OR	95% CI	p-value
ACE	rs4646994	D/D	437 (86.0)	42 (89.4)	0.89	0.49-1.62	0.695
		I/D	68 (13.4)	5 (10.6)			
		I/I	3 (0.6)	0			
TCF7L2	rs7903146	T/T	211 (42.0)	19 (42.2)	0.91	0.55-1.50	0.712
		T/C	235 (46.8)	23 (51.1)			
		C/C	56 (11.2)	3 (6.7)			
MC4R	rs229616	G/G	279 (55.1)	32 (68.1)	0.67	0.39-1.18	0.154
		G/A	193 (38.1)	13 (27.7)			
		A/A	34 (6.7)	2 (4.3)			
	rs17782313	T/T	270 (53.0)	21 (43.8)	1.26	0.77-2.04	0.358
		T/C	204 (40.1)	23 (47.9)			
		C/C	35 (6.9)	4 (8.3)			
	rs1297034	G/G	408 (81.0)	40 (83.3)	0.80	0.38-1.71	0.554
		G/A	89 (17.7)	8 (16.7)			
		A/A	7 (1.4)	0			
AGT	rs699	C/C	437 (86.2)	42 (89.4)	0.76	0.30-1.92	0.5451
		C/T	67 (13.2)	5 (10.6)			
		T/T	3 (0.6)	0			
AT1R	rs5186	A/A	497 (99.4)	45 (100.0)	-	-	-
		C/A	3 (0.6)	0			
		C/C	0	0			

T2DM diagnosis by glucose tolerance (>11 mmol/l).

ACE – Angiotensin-converting enzyme; *AGT* – Angiotensinogen; *AT1R* – Angiotensin II type I receptor; *MC4R* – Melanocortin 4 receptor; *TCF7L2* – Transcription factor 7-like 2

3.4.3.2 Obesity

Under a log-additive genetic model, adjusted for gender and age, rs4646994 (D/D genotype) of *ACE* was significantly associated with overall obesity diagnosis by BMI (≥30kg/m^2), WC (males >94cm, females >80cm) and/or waist-to-hip ratio (males >1, females >0.85) (OR=0.55, 95%CI=0.32-0.96, p=0.034) (Table 3.10).

Table 3.10 Genotype distributions and frequencies of all SNPs in controls and obese cases by BMI, waist circumference and/or waist-to-hip ratio under a log-additive inheritance model, adjusted for gender and age.

Gene	SNP	Genotype	Controls	Cases	OR	95% CI	p-value
ACE	rs4646994	D/D	39 (43.3)	117 (48.0)	0.55	0.32-0.96	**0.034**
		I/D	41 (45.6)	106 (43.4)			
		I/I	10 (11.1)	21 (8.6)			
TCF7L2	rs7903146	T/T	75 (42.1)	168 (43.2)	0.99	0.68-1.44	0.958
		T/C	81 (45.5)	179 (46.0)			
		C/C	22 (12.5)	42 (10.8)			
MC4R	rs229616	G/G	99 (55.0)	227 (57.6)	0.72	0.48-1.07	0.107
		G/A	64 (35.6)	145 (36.8)			
		A/A	17 (9.4)	22 (5.6)			
	rs17782313	T/T	98 (54.4)	206 (51.9)	0.97	0.65-1.45	0.893
		T/C	67 (37.2)	165 (41.6)			
		C/C	15 (8.3)	26 (6.5)			
	rs1297034	G/G	147 (81.7)	316 (80.6)	0.87	0.49-1.55	0.642
		G/A	32 (17.8)	70 (17.9)			
		A/A	1 (0.6)	6 (1.5)			
AGT	rs699	C/C	149 (83.7)	347 (87.4)	0.88	0.44-1.72	0.701
		C/T	27 (15.2)	49 (12.3)			
		T/T	2 (1.1)	1 (0.3)			
AT1R	rs5186	A/A	175 (100.0)	387 (99.2)	-	-	-
		C/A	0	3 (0.8)			
		C/C	0	0			

Overall obesity diagnosis by BMI (≥30kg/m$^{2)}$, waist circumference (males ≥94cm; females ≥80cm) and/or waist-to-hip ratio (males >1, females >0.85).

ACE – Angiotensin-converting enzyme; *AGT* – Angiotensinogen; *AT1R* – Angiotensin II type I receptor; *MC4R* – Melanocortin 4 receptor; *TCF7L2* – Transcription factor 7-like 2

Overweight and obese individuals were first separated and then combined as cases for genetic analysis to determine if any SNP is involved in disease predisposition and/or on disease diagnosis.

No association with obesity by BMI when overweight (25-29.99kg/m^2) and obese (≥30kg/m^2) individuals were separated was observed under a log-additive model of inheritance adjusted for gender and age (Table 3.11).

Table 3.11 Genotype distributions and frequencies in controls, overweight and obese cases by BMI under a log-additive inheritance model, adjusted for gender and age.

Gene	SNP	Genotype	Controls	Overweight	Cases	Diff	95% CI	p-value
ACE	rs4646994	D/D	42 (43.3)	31 (47.7)	83 (47.7)	-0.095	-0.207-0.017	0.097
		I/D	45 (46.4)	24 (38.1)	78 (44.8)			
		I/I	10 (10.3)	8 (12.7)	13 (7.5)			
TCF7L2	rs7903146	T/T	80 (45.1)	49 (45.4)	112 (40.4)	0.057	-0.031-0.144	0.205
		T/C	78 (42.9)	49 (45.4)	133 (48.0)			
		C/C	22 (12.1)	10 (9.3)	32 (11.6)			
MC4R	rs229616	G/G	100 (54.1)	67 (59.8)	159 (57.4)	-0.06	-0.154-0.033	0.204
		G/A	67 (36.2)	42 (37.5)	100 (36.1)			
		A/A	18 (9.7)	3 (2.7)	18 (6.5)			
	rs17782313	T/T	108 (58.1)	50 (45.0)	146 (52.1)	0.006	-0.087-0.098	0.907
		T/C	62 (33.3)	53 (47.7)	117 (41.8)			
		C/C	16 (8.6)	8 (7.2)	17 (6.1)			
	rs1297034	G/G	151 (82.1)	88 (80.0)	224 (80.6)	0.001	-0.134-0.136	0.989
		G/A	33 (17.9)	19 (17.3)	50 (18.0)			
		A/A	0	3 (2.7)	4 (1.4)			
AGT	rs699	C/C	154 (84.2)	102 (91.1)	240 (85.7)	0.034	-0.124-0.193	0.67
		C/T	27 (14.8)	10 (8.9)	39 (13.9)			
		T/T	2 (1.1)	0	1 (0.4)			
AT1R	rs5186	A/A	182 (100.0)	108 (100.0)	272 (98.9)	0.792	-0.013-1.597	-
		C/A	0	0	3 (1.1)			
		C/C	0	0	0			

Obesity diagnosis by BMI (≥30kg/m^2; high-risk: 25-29.99kg/m^2).

The difference instead of an OR is reported as the analysis was performed with quantitative variables (3 categories) and not dichotomous variables.

ACE – Angiotensin-converting enzyme; *AGT* – Angiotensinogen; *AT1R* – Angiotensin II type I receptor; *MC4R* – Melanocortin 4 receptor; *TCF7L2* – Transcription factor 7-like 2

No association with obesity by BMI when overweight (25-29.99kg/m^2) and obese (≥30kg/m^2) individuals were combined as cases was observed under a log-additive genetic model adjusted for gender and age (Table 3.12). However, a nominal association for rs229616 (G/G genotype) of *MC4R* was observed (OR=0.72, 95%CI=0.51-1.01, p=0.059) (Table 3.12).

Table 3.12 Genotype distributions and frequencies of all SNPs in controls and obese cases by BMI when overweight and obese individuals are combined (cases) under a log-additive inheritance model, adjusted for gender and age.

Gene	SNP	Genotype	Controls	Cases	OR	95% CI	p-value
ACE	rs4646994	D/D	42 (43.3)	114 (48.3)	0.69	0.44-1.10	0.12
		I/D	45 (46.4)	102 (43.2)			
		I/I	10 (10.3)	21 (8.9)			
TCF7L2	rs7903146	T/T	82 (45.1)	161 (41.8)	1.15	0.83-1.59	0.402
		T/C	78 (42.9)	182 (47.3)			
		C/C	22 (12.1)	42 (10.9)			
MC4R	rs229616	G/G	100 (54.1)	226 (58.1)	0.72	0.51-1.01	0.059
		G/A	67 (36.2)	142 (36.5)			
		A/A	18 (9.7)	21 (5.4)			
	rs17782313	T/T	108 (58.1)	196 (50.1)	1.17	0.83-1.64	0.368
		T/C	62 (33.3)	170 (43.5)			
		C/C	16 (8.6)	25 (6.4)			
	rs1297034	G/G	151 (82.1)	312 (80.4)	1.09	0.65-1.81	0.744
		G/A	33 (17.9)	69 (17.8)			
		A/A	0	7 (1.8)			
AGT	rs699	C/C	154 (84.2)	342 (87.2)	0.86	0.48-1.52	0.599
		C/T	27 (14.8)	49 (12.5)			
		T/T	2 (1.1)	1 (0.3)			
AT1R	rs5186	A/A	182 (100.0)	380 (99.2)	-	-	-
		C/A	0	3 (0.8)			
		C/C	0	0			

Obesity diagnosis by BMI when high-risk (≥25kg/m^2) and obese (≥30kg/m^2) individuals are combined. *ACE* – Angiotensin-converting enzyme; *AGT* – Angiotensinogen; *AT1R* – Angiotensin II type I receptor; *MC4R* – Melanocortin 4 receptor; *TCF7L2* – Transcription factor 7-like 2

Under a log-additive genetic model adjusted for gender and age, rs4646994 (DD genotype) of *ACE* was significantly associated with obesity by WC (males ≥94cm, females ≥80cm) (OR=0.58, 95%CI=0.34-0.99, p=0.044) (Table 3.13).

Table 3.13 Genotype distributions and frequencies of all SNPs in controls and obese cases by waist circumference under a log-additive inheritance model, adjusted for gender and age.

Gene	SNP	Genotype	Controls	Cases	OR	95% CI	p-value
ACE	rs4646994	D/D	40 (43.0)	116 (48.1)	0.58	0.34-0.99	**0.044**
		I/D	43 (46.2)	104 (43.2)			
		I/I	10 (10.8)	21 (8.7)			
TCF7L2	rs7903146	T/T	79 (41.8)	164 (43.4)	0.92	0.64-1.33	0.661
		T/C	86 (45.5)	174 (46.0)			
		C/C	24 (12.7)	40 (10.6)			
MC4R	rs229616	G/G	106 (55.5)	220 (57.4)	0.75	0.51-1.10	0.139
		G/A	67 (35.1)	142 (37.1)			
		A/A	18 (9.4)	21 (5.5)			
	rs17782313	T/T	105 (54.7)	199 (51.7)	1.02	0.69-1.51	0.904
		T/C	72 (37.5)	160 (41.6)			
		C/C	15 (7.8)	26 (6.8)			
	rs1297034	G/G	158 (82.3)	305 (80.3)	1.00	0.56-1.76	0.986
		G/A	33 (17.2)	69 (18.2)			
		A/A	1 (0.5)	6 (1.6)			
AGT	rs699	C/C	157 (83.1)	38 (87.8)	0.77	0.40-1.48	0.434
		C/T	30 (15.9)	46 (11.9)			
		T/T	2 (1.1)	1 (0.3)			
AT1R	rs5186	A/A	186 (100.0)	376 (99.2)	-	-	-
		C/A	0	3 (0.8)			
		C/C	0	0			

Obesity diagnosis by waist circumference (males ≥94cm; females ≥80cm).

ACE – Angiotensin-converting enzyme; *AGT* – Angiotensinogen; *AT1R* – Angiotensin II type I receptor; *FTO* – Fat-mass and obesity associated gene; *MC4R* – Melanocortin 4 receptor; *TCF7L2* – Transcription factor 7-like 2

No SNPs were associated with obesity by waist-to-hip ratios (males >1, females >0.85) under a log-additive genetic model adjusted for gender and age (Table 3.14).

Table 3.14 Genotype distributions and frequencies of all SNPs in controls and obese cases by waist-to-hip ratio under a log-additive inheritance model, adjusted for gender and age.

Gene	SNP	Genotype	Controls	Cases	OR	95% CI	p-value
ACE	rs4646994	D/D	97 (44.9)	59 (50.0)	0.77	0.53-1.13	0.174
		I/D	96 (44.4)	51 (43.2)			
		I/I	23 (10.7)	8 (6.8)			
TCF7L2	rs7903146	T/T	153 (41.5)	90 (45.5)	0.86	0.64-1.15	0.314
		T/C	171 (46.3)	89 (44.9)			
		C/C	45 (12.2)	19 (9.6)			
MC4R	rs229616	G/G	210 (56.0)	116 (58.3)	0.99	0.73-1.36	0.957
		G/A	141 (37.6)	68 (34.2)			
		A/A	24 (6.4)	15 (7.5)			
	rs17782313	T/T	203 (54.1)	101 (50.0)	1.06	0.78-1.45	0.698
		T/C	145 (38.7)	87 (43.1)			
		C/C	27 (7.2)	14 (6.9)			
	rs1297034	G/G	302 (81.4)	161 (80.1)	1.08	0.70-1.66	0.731
		G/A	66 (17.8)	36 (17.9)			
		A/A	3 (0.8)	4 (2.0)			
AGT	rs699	C/C	326 (87.2)	169 (84.5)	1.52	0.88-2.60	0.134
		C/T	46 (12.3)	30 (15.0)			
		T/T	2 (0.5)	1 (0.5)			
AT1R	rs5186	A/A	366 (99.7)	196 (99.0)	4.48	0.25-81.62	
		C/A	1 (0.3)	2 (1.0)			
		C/C	0	0			

Obesity diagnosis by waist-to-hip circumference (males >1; females >0.85).

ACE – Angiotensin-converting enzyme; *AGT* – Angiotensinogen; *AT1R* – Angiotensin II type I receptor; *MC4R* – Melanocortin 4 receptor; *TCF7L2* – Transcription factor 7-like 2

3.4.3.3 Hypertension

No SNPs were associated with BP (≥140/90 mmHg) under a log-additive genetic model adjusted for gender and age (Table 3.15).

Table 3.15 Genotype distributions and frequencies of all SNPs in controls and hypertensive cases by blood pressure under a log-additive inheritance model, adjusted for gender and age.

Gene	SNP	Genotype	Controls	Cases	OR	95% CI	p-value
ACE	rs4646994	D/D	129 (47.3)	27 (44.3)	1.51	0.96-2.39	0.073
		I/D	123 (45.1)	24 (39.3)			
		I/I	21 (7.7)	10 (16.4)			
TCF7L2	rs7903146	T/T	198 (43.0)	45 (42.5)	0.94	0.67-1.33	0.74
		T/C	206 (44.7)	54 (50.9)			
		C/C	57 (12.4)	7 (6.6)			
MC4R	rs229616	G/G	262 (56.0)	64 (60.4)	0.98	0.68-1.41	0.903
		G/A	175 (37.4)	34 (32.1)			
		A/A	31 (6.6)	8 (7.5)			
	rs17782313	T/T	252 (53.5)	52 (49.1)	1.18	0.82-1.68	0.372
		T/C	187 (39.7)	45 (42.5)			
		C/C	32 (6.8)	9 (8.5)			
	rs1297034	G/G	376 (80.9)	87 (81.3)	1.06	0.63-1.79	0.815
		G/A	84 (18.1)	18 (16.8)			
		A/A	5 (1.1)	2 (1.9)			
AGT	rs699	C/C	410 (87.2)	86 (81.9)	1.47	0.83-2.60	0.196
		C/T	58 (12.3)	18 (17.1)			
		T/T	2 (0.4)	1 (1.0)			
AT1R	rs5186	A/A	458 (99.6)	104 (99.0)	1.72	0.15-19.3	-
		C/A	2 (0.4)	1 (1.0)			
		C/C	0	0			

Hypertension diagnosis by blood pressure (≥140/90 mmHg).

ACE – Angiotensin-converting enzyme; *AGT* – Angiotensinogen; *AT1R* – Angiotensin II type I receptor; *MC4R* – Melanocortin 4 receptor; *TCF7L2* – Transcription factor 7-like 2

3.4.3.4 Dyslipidaemia

No SNPs were associated with overall dyslipidaemia diagnosis by total cholesterol (>5 mmol/l), triglycerides (>1.5 mmol/l), HDL-C (<1.2 mmol/l), LDL-C (>3 mmol/l) and/or HDL-C/total cholesterol (<20%) under a log-additive genetic model adjusted for gender and age (Table 3.16).

Table 3.16 Genotype distributions and frequencies of all SNPs in controls and dyslipidaemia cases total cholesterol, triglycerides, HDL-C, LDL-C and/or HDL-C/total cholesterol measurements under a log-additive inheritance model, adjusted for gender and age.

Gene	SNP	Genotype	Controls	Cases	OR	95% CI	p-value
ACE	rs4646994	D/D	25 (54.4)	131 (45.5)	1.34	0.80-2.22	0.257
		I/D	18 (39.1)	129 (49.8)			
		I/I	3 (6.5)	28 (9.7)			
TCF7L2	rs7903146	T/T	32 (43.2)	211 (42.8)	0.95	0.66-1.37	0.779
		T/C	31 (41.9)	229 (46.5)			
		C/C	11 (14.9)	53 (10.8)			
MC4R	rs229616	G/G	44 (59.5)	282 (56.4)	1.02	0.69-1.51	0.912
		G/A	24 (32.4)	185 (37.0)			
		A/A	6 (8.1)	33 (6.6)			
	rs17782313	T/T	39 (52.7)	265 (52.7)	0.85	0.58-1.24	0.389
		T/C	26 (35.1)	206 (41.0)			
		C/C	9 (12.2)	32 (6.4)			
	rs1297034	G/G	62 (83.8)	401 (80.5)	1.17	0.64-2.15	0.603
		G/A	11 (14.9)	91 (18.3)			
		A/A	1 (1.4)	6 (1.2)			
AGT	rs699	C/C	62 (84.9)	434 (86.5)	0.92	0.48-1.74	0.789
		C/T	11 (15.1)	65 (12.9)			
		T/T	0	3 (0.6)			
AT1R	rs5186	C/C	74 (100.0)	488 (99.4)	-	-	-
		C/A	0	3 (0.6)			
		A/A	0	0			

Overall dyslipidaemia diagnosis by total cholesterol (>5 mmol/l), triglycerides (>1.5 mmol/l), HDL-C (<1.2 mmol/l), LDL-C (>3 mmol/l) and/or HDL-C/total cholesterol (<20%).

ACE – Angiotensin-converting enzyme; *AGT* – Angiotensinogen; *AT1R* – Angiotensin II type I receptor; *MC4R* – Melanocortin 4 receptor; *TCF7L2* – Transcription factor 7-like 2.

No SNPs were associated with dyslipidaemia by TC (>5 mmol/l) under a log-additive genetic model adjusted for gender and age (Table 3.17).

Table 3.17 Genotype distributions and frequencies of all SNPs in controls and dyslipidaemia cases by levels of total cholesterol under a log-additive inheritance model, adjusted for gender and age.

Gene	SNP	Genotype	Controls	Cases	OR	95% CI	p-value
ACE	rs4646994	D/D	111 (47.4)	45 (45.0)	1.30	0.88-1.92	0.184
		I/D	104 (44.4)	43 (43.0)			
		I/I	19 (8.1)	12 (12.0)			
TCF7L2	rs7903146	T/T	170 (41.2)	72 (47.4)	0.98	0.73-1.32	0.908
		T/C	201 (48.7)	59 (38.8)			
		C/C	42 (10.2)	21 (13.8)			
MC4R	rs229616	G/G	241 (57.4)	83 (54.6)	1.16	0.85-1.57	0.352
		G/A	151 (36.0)	58 (38.2)			
		A/A	28 (6.7)	11 (7.2)			
	rs17782313	T/T	222 (52.6)	81 (52.9)	0.95	0.70-1.30	0.757
		T/C	170 (40.3)	62 (40.5)			
		C/C	30 (7.1)	10 (6.5)			
	rs1297034	G/G	336 (80.6)	126 (82.4)	0.86	0.54-1.36	0.516
		G/A	75 (18.0)	26 (17.0)			
		A/A	6 (1.4)	1 (0.7)			
AGT	rs699	C/C	365 (87.1)	130 (84.4)	1.24	0.74-2.06	0.417
		C/T	52 (12.4)	23 (14.9)			
		T/T	2 (0.5)	1 (0.6)			
AT1R	rs5186	A/A	410 (99.5)	150 (99.3)	1.07	0.1-11.96	-
		C/A	2 (0.5)	1 (0.7)			
		C/C	0	0			

Dyslipidaemia diagnosis by total cholesterol (>5 mmol/l).

ACE – Angiotensin-converting enzyme; *AGT* – Angiotensinogen; *AT1R* – Angiotensin II type I receptor; *MC4R* – Melanocortin 4 receptor; *TCF7L2* – Transcription factor 7-like 2

No SNPs were associated with dyslipidaemia by triglycerides (>1.5 mmol/l) under a log-additive genetic model adjusted for gender and age (Table 3.18).

Table 3.18 Genotype distributions and frequencies of all SNPs in controls and dyslipidaemia cases by levels of triglycerides under a log-additive inheritance model, adjusted for gender and age.

Gene	SNP	Genotype	Controls	Cases	OR	95% CI	p-value
ACE	rs4646994	D/D	126 (45.0)	30 (55.6)	0.76	0.47-1.24	0.271
		I/D	127 (45.4)	20 (37.0)			
		I/I	27 (9.6)	4 (7.4)			
TCF7L2	rs7903146	T/T	203 (42.4)	39 (45.3)	0.86	0.60-1.25	0.432
		T/C	219 (45.7)	41 (47.7)			
		C/C	57 (11.9)	6 (7.0)			
MC4R	rs229616	G/G	269 (55.7)	55 (61.8)	0.92	0.63-1.35	0.676
		G/A	182 (37.7)	27 (30.3)			
		A/A	32 (6.6)	7 (7.9)			
	rs17782313	T/T	253 (52.2)	50 (55.6)	0.92	0.63-1.35	0.679
		T/C	199 (41.0)	33 (36.7)			
		C/C	33 (6.8)	7 (7.8)			
	rs1297034	G/G	388 (80.5)	74 (84.1)	0.75	0.41-1.35	0.32
		G/A	87 (18.0)	14 (15.9)			
		A/A	7 (1.5)	0			
AGT	rs699	C/C	421 (86.8)	74 (84.1)	1.11	0.60-2.05	0.739
		C/T	61 (12.6)	14 (15.9)			
		T/T	3 (0.6)	0			
AT1R	rs5186	A/A	474 (99.8)	86 (97.7)	9.04	0.81-101	-
		C/A	1 (0.2)	2 (2.3)			
		C/C	0	0			

Dyslipidaemia diagnosis by triglycerides (>1.5 mmol/l).

ACE – Angiotensin-converting enzyme; *AGT* – Angiotensinogen; *AT1R* – Angiotensin II type I receptor; *MC4R* – Melanocortin 4 receptor; *TCF7L2* – Transcription factor 7-like 2

No SNPs were associated with dyslipidaemia by HDL-C (<1.2 mmol/l) under a log-additive genetic model adjusted for gender and age (Table 3.19).

Table 3.19 Genotype distributions and frequencies of all SNPs in controls and dyslipidaemia cases by levels of HDL-C under a log-additive inheritance model, adjusted for gender and age.

Gene	SNP	Genotype	Controls	Cases	OR	95% CI	p-value
ACE	rs4646994	D/D	55 (45.1)	101 (47.6)	0.93	0.65-1.32	0.68
		I/D	57 (46.7)	90 (42.5)			
		I/I	10 (8.2)	21 (9.9)			
TCF7L2	rs7903146	T/T	82 (42.1)	160 (43.2)	0.87	0.66-1.13	0.285
		T/C	86 (44.1)	174 (47.0)			
		C/C	27 (13.8)	36 (9.7)			
MC4R	rs229616	G/G	104 (53.6)	220 (58.2)	0.85	0.64-1.13	0.262
		G/A	76 (39.2)	133 (35.2)			
		A/A	14 (7.2)	25 (6.6)			
	rs17782313	T/T	107 (54.0)	196 (52.0)	1.03	0.78-1.37	0.819
		T/C	76 (38.4)	156 (41.4)			
		C/C	15 (7.6)	25 (6.6)			
	rs1297034	G/G	161 (82.6)	301 (80.3)	1.14	0.75-1.72	0.547
		G/A	32 (16.4)	69 (18.4)			
		A/A	2 (1.0)	5 (1.3)			
AGT	rs699	C/C	162 (83.5)	333 (87.9)	0.76	0.47-1.21	0.244
		C/T	31 (16.0)	44 (11.6)			
		T/T	1 (0.5)	2 (0.5)			
AT1R	rs5186	A/A	195 (100.0)	365 (99.5)	-	-	-
		C/A	0	3 (0.8)			
		C/C	0	0			

Dyslipidaemia diagnosis by HDL-C (<1.2 mmol/l).

ACE – Angiotensin-converting enzyme; *AGT* – Angiotensinogen; *AT1R* – Angiotensin II type I receptor; *MC4R* – Melanocortin 4 receptor; *TCF7L2* – Transcription factor 7-like 2

No SNPs were associated with dyslipidaemia by LDL-C (>3 mmol/l) under a log-additive genetic model adjusted for gender and age (Table 3.20).

Table 3.20 Genotype distributions and frequencies of all SNPs in controls and dyslipidaemia cases by levels of LDL-C under a log-additive inheritance model, adjusted for gender and age.

Gene	SNP	Genotype	Controls	Cases	OR	95% CI	p-value
ACE	rs4646994	D/D	76 (48.4)	80 (45.2)	1.32	0.93-1.87	0.116
		I/D	71 (45.2)	76 (42.9)			
		I/I	10 (3.4)	21 (11.9)			
TCF7L2	rs7903146	T/T	119 (41.5)	120 (44.0)	0.99	0.77-1.28	0.0967
		T/C	137 (47.7)	121 (44.3)			
		C/C	31 (10.8)	32 (11.7)			
MC4R	rs229616	G/G	168 (57.5)	152 (55.3)	1.09	0.83-1.44	0.516
		G/A	104 (35.6)	104 (37.8)			
		A/A	20 (6.8)	19 (6.9)			
	rs17782313	T/T	154 (52.6)	146 (52.7)	0.99	0.75-1.30	0.932
		T/C	119 (40.6)	111 (40.1)			
		C/C	20 (6.8)	20 (7.2)			
	rs1297034	G/G	240 (82.5)	218 (79.6)	1.14	0.77-1.69	0.514
		G/A	47 (16.2)	53 (19.3)			
		A/A	4 (1.4)	3 (1.1)			
AGT	rs699	C/C	250 (85.6)	241 (87.3)	0.92	0.57-1.47	0.715
		C/T	41 (14.0)	33 (12.0)			
		T/T	1 (0.3)	2 (0.7)			
AT1R	rs5186	A/A	284 (99.3)	271 (99.6)	0.41	0.04-4.63	-
		C/A	2 (0.7)	1 (0.4)			
		C/C	0	0			

Dyslipidaemia by LDL-C (>3 mmol/l).

ACE – Angiotensin-converting enzyme; *AGT* – Angiotensinogen; *AT1R* – Angiotensin II type I receptor; *MC4R* – Melanocortin 4 receptor; *TCF7L2* – Transcription factor 7-like 2

Under an additive model adjusted for gender and age, rs229616 (GG genotype) of *MC4R* was significantly associated with dyslipidaemia by HDL-C/total cholesterol (<20%) (OR=1.39, 95%CI=1.01-1.90, p=0.043) (Table 3.2) under a log-additive genetic model adjusted for gender and age.

Table 3.21 Genotype distributions and frequencies of all SNPs in controls and dyslipidaemia cases by HDL-C-to-total cholesterol ratios under a log-additive inheritance model, adjusted for gender and age.

Gene	SNP	Genotype	Controls	Cases	OR	95% CI	p-value
ACE	rs4646994	D/D	120 (46.5)	36 (47.4)	1.02	0.68-1.53	0.917
		I/D	115 (44.6)	32 (42.1)			
		I/I	23 (8.9)	8 (10.5)			
TCF7L2	rs7903146	T/T	183 (41.3)	59 (48.4)	0.82	0.60-1.13	0.226
		T/C	209 (47.2)	51 (41.8)			
		C/C	51 (11.5)	12 (9.8)			
MC4R	rs229616	G/G	261 (58.3)	63 (50.8)	1.39	1.01-1.90	**0.043**
		G/A	161 (35.9)	48 (38.7)			
		A/A	26 (5.8)	13 (10.5)			
	rs17782313	T/T	236 (52.2)	67 (54.5)	0.89	0.64-1.23	0.47
		T/C	183 (40.5)	49 (39.8)			
		C/C	33 (7.3)	7 (5.7)			
	rs1297034	G/G	362 (81.0)	100 (81.3)	0.99	0.62-1.57	0.955
		G/A	80 (17.9)	21 (17.1)			
		A/A	5 (1.1)	2 (1.6)			
AGT	rs699	C/C	382 (85.3)	113 (90.4)	0.63	0.33-1.18	0.131
		C/T	63 (14.1)	12 (9.6)			
		T/T	3 (0.7)	0			
AT1R	rs5186	A/A	438 (99.5)	122 (99.2)	1.64	0.14-18.67	-
		C/A	2 (0.5)	1 (0.8)			
		C/C	0	0			

Dyslipidaemia diagnosis by HDL-C/total cholesterol (<20%).

ACE – Angiotensin-converting enzyme; *AGT* – Angiotensinogen; *AT1R* – Angiotensin II type I receptor; *MC4R* – Melanocortin 4 receptor; *TCF7L2* – Transcription factor 7-like 2

3.4.3.5 Metabolic syndrome

No SNPs were associated with MetS, as defined by the JIS criteria (waist circumference: males ≥94cm, females ≥80cm; triglycerides: >1.7 mmol/l; HDL-C: males <1 mmol/l, females <0.85 mmol/l; blood pressure: ≥130/85 mmHg; fasting glucose: ≥5.6 mmol/l), under a log-additive genetic model adjusted for gender and age (Table 3.22).

Table 3.22 Genotype distributions and frequencies of all SNPs in controls and metabolic syndrome cases by the JIS criteria under a log-additive inheritance model, adjusted for gender and age.

Gene	SNP	Genotype	Controls	Cases	OR	95% CI	p-value
ACE	rs4646994	D/D	106 (44.4)	50 (52.6)	0.87	0.58-1.31	0.506
		I/D	111 (46.4)	36 (37.9)			
		I/I	22 (9.2)	9 (9.5)			
TCF7L2	rs7903146	T/T	177 (42.4)	66 (44.0)	0.88	0.64-1.20	0.408
		T/C	187 (44.8)	73 (48.7)			
		C/C	53 (12.7)	11 (7.3)			
MC4R	rs229616	G/G	231 (54.7)	95 (62.5)	0.83	0.60-1.16	0.274
		G/A	162 (38.4)	47 (30.9)			
		A/A	29 (6.9)	10 (6.6)			
	rs17782313	T/T	230 (54.2)	74 (48.4)	1.14	0.83-1.57	0.435
		T/C	164 (38.7)	68 (44.4)			
		C/C	30 (7.1)	11 (7.)			
	rs1297034	G/G	340 (81.0)	123 (80.9)	0.99	0.63-1.56	0.96
		G/A	75 (17.9)	27 (17.8)			
		A/A	5 (1.2)	2 (1.3)			
AGT	rs699	C/C	365 (86.3)	131 (86.2)	0.98	0.57-1.71	0.952
		C/T	55 (13.0)	21 (13.8)			
		T/T	3 (0.7)	0			
AT1R	rs5186	A/A	413 (99.5)	149 (99.3)	1.02	0.08-12.19	-
		C/A	2 (0.5)	1 (0.7)			
		C/C	0	0			

Metabolic syndrome diagnosis by the JIS criteria (waist circumference: males ≥94cm, females ≥80cm; triglycerides: >1.7 mmol/l; HDL-C: males <1 mmol/l, females <1.3 mmol/l; blood pressure: ≥130/85 mmHg; fasting glucose: >5.6 mmol/l).

ACE – Angiotensin-converting enzyme; *AGT* – Angiotensinogen; *AT1R* – Angiotensin II type I receptor; *MC4R* – Melanocortin 4 receptor; *TCF7L2* – Transcription factor 7-like 2

3.4.4 Post-hoc investigation of significant findings

SNPs that were significantly and nominally associated with disease under a log-additive genetic model were further investigated under dominant and recessive genetic models. These include the significant associations of rs17782313 with T2DM by fasting glucose when high-risk and diabetic individuals were separated (p=0.043) and combined as cases (p=0.022); rs4646994 with overall obesity (p=0.034) and obesity by WC (p=0.044); and rs229616 with dyslipidaemia by HDL-C/total cholesterol (p=0.043). As well as the nominal associations of rs1297034 with T2DM when high-risk and diabetic individuals are combined as cases (p=0.072) and rs229616 with obesity by BMI when overweight and obese individuals are combined as cases (p=0.059).

3.4.4.1 rs4646994 of *ACE* is associated with obesity and WC under a dominant model

3.4.4.1.1 Angiotensin-converting enzyme (*ACE*)

Under a dominant model adjusted for gender and age, rs4646994 (D/D genotype) was significantly associated with obesity by BMI (≥30kg/m^2), WC (males ≥94cm, females ≥80cm) and/or waist-to-hip ratio (males >1, females >0.85), while the I/D and I/I genotypes were protective (OR=0.46, 95%CI=0.21-1.01, P=0.047) (Table 3.23). This SNP was also associated with obesity by WC (males ≥94cm, females ≥80cm) (OR=0.48, 95%CI=0.23-1.00, p=0.044) (Table 3.23).

The nominal associations with obesity by BMI when overweight (25-29.9kg/m^2) and obese (≥30kg/m^2) individuals were separated (Table 3.11) and BP (≥140/90 mmHg) (Table 3.15) observed under a log-additive inheritance model adjusted for gender and age was not observed under a dominant model of inheritance adjusted for gender and age (Table 3.23).

3.4.4.1.2 Transcription factor 7-like 2 (*TCF7L2*)

The nominal association of rs7903146 (T/C and T/T genotypes) with T2DM by fasting glucose (>7 mmol/l) and/or glucose tolerance (>1 mmol/l) observed under an additive model adjusted for gender and age (Table 3.6) was not observed under a dominant model adjusted for gender and age (Table 3.23).

3.4.4.1.3 Melanocortin 4 receptor (*MC4R*)

Under a dominant model adjusted for gender and age, rs17782313 (T/C and C/C genotypes) was nominally associated with T2DM by fasting glucose when high-risk (5.6-7 mmol/l) and diabetic (>7 mmol/l) individuals when they were combined as cases, while the T/T genotype was protective (OR=1.51, 95%CI=0.99-2.13, p=0.054) (Table 3.23).

The nominal associations observed between rs229616 (GA and AA genotypes) and dyslipidaemia by HDL-C/total cholesterol or between rs1297034 (GA and AA genotypes) and T2DM by fasting glucose when high-risk (5.6-7 mmol/l) and diabetic (>7 mmol/l) individuals combined under log-

additive inheritance models, adjusted for gender and age (Table 3.21 and Table 3.8, respectively), was not observed under a dominant model adjusted for gender and age (Table 3.23).

Table 3.23 SNPs associated with CVD risk phenotypes under a dominant inheritance model, adjusted for gender and age.

Gene	SNP	Associated genotype	Disease variable	Adjusted		
				OR	95% CI	p-value
ACE	rs4646994	D/D	Ob	0.46	0.21-1.01	**0.047**
		D/D	ob_wc	0.48	0.23-1.00	**0.044**
		I/I	Bp	1.29	0.71-2.33	0.407
TCF7L2	rs7903146	C/C	Db	1.00	0.51-1.95	0.995
MC4R	rs17782313	C/C or T/C	db_2h_hr	1.51	0.99-2.31	0.054
	rs229616	G/G	dl_hdlctc	1.38	0.92-2.06	0.121
	rs1297034	A/A	db_2h_hr	0.64	0.36-1.14	0.117
Gene	SNP	**Associated genotype**	**Disease variable**	**Adjusted**		
				Diff	**95% CI**	**p-value**
ACE	rs4646994	D/D	ob_bmi	-0.10097	-0.2463-0.04437	0.1743
MC4R	rs17782313	T/T	db_2h	0.08352	-0.0141-0.1812	0.09422

95%CI – 95% Confidence interval; BMI – Body mass index; bp – Blood pressure; db_2h – Diabetes by fasting glucose when high-risk and diabetic individuals are separated; db_2h_hr – Diabetes by fasting glucose when high-risk and diabetic individuals combined; Diff – Difference; dl_hdlctc – Dyslipidaemia by HDL-C/TC; dl_ldlc – Dyslipidaemia by LDL-C; HDL-C – High-density lipoprotein cholesterol; HDL-C/TC – HDL-C to total cholesterol ratio; IGT – Impaired glucose tolerance; LDL-C – Low-density lipoprotein cholesterol; ob – obesity by any definition; ob_bmi – Obesity by BMI when overweight and obese individuals are separated; db_wc – Obesity by waist circumference; OR – Odds ratio; SNP – Single nucleotide polymorphism; TC – Total cholesterol.

3.4.4.2 rs4646994 of *ACE* is associated with BP and LDL-C under a recessive model

3.4.4.2.1 Angiotensin-converting enzyme (ACE)

Under a recessive model adjusted for gender and age, rs4646994 (I/I genotype) was significantly associated with hypertension by BP (≥140/90 mmHg) while the I/D and D/D genotypes were protective (OR=3.25, 95%CI=1.32-7.99, p=0.014) (Table 3.24). It was also significantly associated with dyslipidaemia by LDL-C (>3 mmol/l) (OR=2.31, 95%CI=1.02-5.23, p=0.038) (Table 3.24).

The significant associations observed under a log-additive inheritance model, adjusted for gender and age, with obesity by BMI (≥30kg/m^2), WC (males ≥94cm, females ≥80cm) and/or waist-to-hip ratio (males >1, females >0.85) (Table 3.10) and obesity by WC (males ≥94cm, females ≥80cm) (Table 3.13); and the nominal association with obesity by BMI when overweight (25-29.9kg/m^2) and

obese (≥30kg/m^2) individuals were separated (Table 3.11) was not observed under a recessive model adjusted for gender and age (Table 3.24).

3.4.4.2.2 Transcription factor 7-like 2 (*TCF7L2*)

Under a recessive model adjusted for gender and age, rs7903146 (T/T genotype) was nominally associated with dyslipidaemia by total cholesterol (>5 mmol/l), while the T/C and C/C genotypes were protective (OR=1.72, 95%CI=0.96-3.08, p=0.075) (Table3.24).

The nominal association observed with T2DM by fasting glucose (>7 mmol/l) and/or glucose tolerance (>11 mmol/l) observed under a log-additive inheriatnce model, adjusted for gender and age (Table 3.6), was not observed under a recessive inheritace model adjusted for gender and age (Table 3.24).

3.4.4.2.3 Melanocortin 4 receptor (*MC4R*)

Under a recessive inheritance model, adjusted for gender and age, rs1297034 was nominally associated with T2DM by fasting glucose when high-risk (5.6-7 mmol/l) and diabetic (>7 mmol/l) individuals were combined (p=0.075); however, no conclusion can be drawn regarding the associated genotype as the OR was 0, which could be an artefact of the cohort, as there was no cases with the A/A genotype (Table 3.24).

The rs17782313 (C/C genotype) was nominally associated with T2DM by fasting glucose when high-risk (5.6-7 mmol/l) and diabetic (<7 mmol/l) individuals were combined as cases, while the T/C and T/T genotypes were protective (OR=2.02, 95%CI=0.96-4.26, p=0.075) (Table 3.24).

The rs229616 (A/A genotype) was nominally associated with dyslipidaemia by HDL-C/total cholesterol (<20%), while the T/A and T/T genotypes were protective (OR=2.03, 95%CI=1.00-4.13, p=0.059) (Table 3.24).

3.4.4.2.4 Angiotensinogen (*AGT*)

Under a recessive inheritance ,model adjusted for gender and age, rs699 was nominally associated with MetS as defined by the JIS criteria (WC: males ≥94cm, females ≥80cm, triglycerides: >1.7 mmol/l; HDL-C: males <1 mmol/, females <1.3 mmol/l; BP: ≥130/85 mmHg; fasting glucose: >5.6 mmol/l) (p=0.076) however, no conclusion can be drawn regarding the associated genotype as the OR was 0, which could be an artefact of the cohort, as there was no cases with the T/T genotype (Table 3.24).

Table 3.24 SNPs associated with CVD risk phenotypes under a recessive inheritance model, adjusted for gender and age.

Gene	SNP	Associated genotype	Disease variable	Adjusted		
				OR	95% CI	p-value
ACE	rs4646994	I/I	Bp	3.25	1.32-7.99	**0.014**
		I/I	dl_ldlc	2.31	1.02-5.23	**0.038**
		D/D	Ob	0.47	0.16-1.38	0.179
		D/D	ob_wc	0.55	0.19-1.56	0.268
TCF7L2	rs7903146	T/T	dl_tc	1.72	0.93-3.08	0.075
		C/C	Db	0.59	0.20-1.73	0.306
MC4R	rs1297034	-	db_2h_hr	0	0	0.075
	rs17782313	T/T	db_2h_hr	2.02	0.96-4.26	0.075
	rs229616	A/A	dl_hdlctc	2.03	1.00-4.13	0.059
AGT	rs699	-	Ms	0	0	0.075
Gene	SNP	**Associated genotype**	**Disease variable**	**Adjusted**		
				Diff	**95% CI**	**p-value**
ACE	rs4646994	D/D	ob_bmi	-0.17360	-0.4233-0.07613	0.174
MC4R	rs17782313	T/T	db_2h	0.16583	-0.02756-0.3592	0.093

95%CI – 95% Confidence interval; BMI – Body mass index; bp – Blood pressure; db – Diabetes by any definition; db_2h – Diabetes by fasting glucose when high-risk and diabetic individuals are separated; db_2h_hr – Diabetes by fasting glucose when high-risk and diabetic individuals combined; Diff – Difference; dl_hdlctc – Dyslipidaemia by HDL-C/TC; dl_ldlc – Dyslipidaemia by LDL-C; HDL-C – High-density lipoprotein cholesterol; HDL-C/TC – HDL-C to total cholesterol ratio; IGT – Impaired glucose tolerance; LDL-C – Low-density lipoprotein cholesterol; ms – Metabolic syndrome by JIS criteria; ob – obesity by any definition; ob_bmi – Obesity by BMI when overweight and obese individuals are separated; ob_wc – Obesity by waist circumference; OR – Odds ratio; SNP – Single nucleotide polymorphism; TC – Total cholesterol.

3.4.4.3 Investigating the continuous measurements of glucose and blood pressure

As the risk of T2DM and hypertension is continuous and the cut-offs for diagnosis are constantly changing (Matsha e*t al.,* 2013), linear regression analysis was performed to determine the association of each SNP with continuous measurements of glucose and blood pressure adjusted for gender and age as covariates (Table 3.25). Individuals on glucose management or blood pressure lowering medication were excluded from this analysis as although they are diseased, treatment will lower their measurements and classify them as “healthy” affecting the association outcome.

Table 3.25 The association of each SNP to the variation in disease diagnosis measures.

Disease measure	*AGT* rs699	*MC4R* rs229616	*MC4R* rs1297034	*MC4R* rs17782313	*TCF7L2* rs7903146	*AT1R* rs5186
Glucose measurements						
Fasting glucose	R^2=0,059 p=0,239	R^2=0,052 p=0,723	$R^2$0,05 p=0,862	R^2=0,051 p=0,255	R^2=0,054 p=0,923	R^2=0,051 p=0,8646
Glucose tolerance	R^2=0,101 p=0,160	R^2=0,098 p=0,9933	R^2=0,10 p=0,1983	R^2=0,099 p=0,319	R^2=0,103 p=0,8703	R^2=0,103 p=0,4534
Blood pressure measurements						
Systolic BP	R^2=0,193 p=0,798	R^2=0,199 p=0,0987	R^2=0,195 p=0,681	R^2=0,198 p=0,303	R^2=0,194 p=0,61	R^2=0,196 p=0,676
Diastolic BP	R^2=0,042 p=0,063	R^2=0,039 p=0,146	R^2=0,037 p=0,337	R^2=0,035 p=0,649	R^2=0,038 p=0,229	R^2=0,035 p=0,845

AGT – Angiotensin; *AT1R* – Angiotensin II type I receptor; *MC4R* – Melanocortin 4 receptor; R^2 – Multiple R-squared; *TCF7L2* – Transcription factor 7-like 2

No SNPs in any of the genes were associated with continual risk of T2DM or hypertension. However, a nominal association was observed between *AGT* rs699 and diastolic BP measurements, associated with a 4.2% increased risk of hypertension (R^2=0.042). R^2 explains the percentage of data variability around the mean.

3.5 Haplotype analysis of *MC4R*

Haplotype analysis of the *MC4R* SNPs was not performed, as the SNPs were not in LD (Table 3.26).

Table 3.26 Linkage disequilibrium analysis of the MC4R SNPs rs17782313, rs229616 and rs1297034.

L1	L2	D'	R^2
rs17782313	rs1297034	0.106	0
rs17782313	rs229616	0.017	0
rs1297034	rs229616	0.025	0

D^2 and r^2 values obtained using the "solid spine of LD" setting in Haploview v4.2.

Chapter 4

Discussion

4. Discussion

To our knowledge, the present study represents one of the first to investigate candidate genes involved in risk phenotypes associated with CVD in isiXhosa-speaking SA Black individuals. Many genetic studies (both candidate and GWAS-based) have previously been performed in European populations and have identified candidate genes involved in risk phenotypes associated with CVD, however very few genetic investigations have been done on African populations, where the risk phenotypes associated with CVD have been found to be highly prevalent and continue to increase (IDF, 2013; Shisana *et al.,* 2013; Sengwayo *et al.,* 2013; Schutte *et al.,* 2008). However, the results in this study differed to that of a study conducted in SA Blacks of Zulu descent (Pirie e*t al.,* 2010). In the Pirie *et al.* (2010) study, an association was observed between rs7903146 of *TCF7L2* and T2DM, which was not observed in the present study of isiXhosa-speaking Black individuals. The present study also did not investigate the same genes as chosen by Pirie *et al.* (2010). This difference highlights the need to study SA Black individuals with different ethnicities, as the allele frequencies may differ between them. Individuals of different ethnicities also follow different ways of life, and thus, as has been shown previously (Ouedemi *et al.,* 2009), their environment, diet and lifestyles may also predispose them to disease.

Candidate genes in the present study were selected based on a thorough review of the literature, as discussed in the introduction, and we have replicated the associations of SNPs present in *ACE* (rs4646994) (Zitouni *et al.,* 2018; Aung *et al.,* 2017; Motawi *et al.,* 2016; Amrani *et al.,* 2015; Mao & Huang, 2015; Kooffreh *et al.,* 2013a; Shaker *et al.,* 2009; Kramer e*t al.,* 2005; Rotimi *et al.,* 1997) and *MC4R* (Cauchi e*t al.,* 2012) (rs17782313) and risk phenotypes associated with CVD in various ethnic African populations. The sections below provide detailed discussion on these findings.

4.1 Angiotensin-converting enzyme (*ACE*)

The D/D genotype of *ACE* rs4646994 was found to be associated with overall obesity (BMI (≥30kg/m^2), waist circumference (males≥94cm, females ≥80cm) and/or waist-to-hip ratio (males >1, females >0.85)) (p=0.047, OR=0.46) and WC (p=0.044, OR=0.48) under a dominant model of inheritance, after correcting for gender and age (Table 3.23). Here, it was found that individuals who carried at least one I allele were at significantly reduced risk of obesity, compared to individuals homozygous for the D-allele. This is in accordance with what was seen in a sample of Ethiopian females with a mean age of 48.8 years, where rs4646994 (D/D genotype) was associated with obesity overall as measured by BMI, WC and waist-to-hip ratios in the presence of IR (Motawi *et al.,* 2016) and with increased risk of obesity in a meta-analysis of African studies, that included 3 371 cases and 4 490 controls (Mao & Huang, 2015).

Under a recessive model of inheritance, rs4646994 (I/I genotype) was significantly associated with BP after adjustment for gender and age (p=0.014) (Table 3.24). Individuals with the I/I genotype were at significantly higher risk of having increased BP, compared to those who carried at least one

D-allele (p=0.014, OR=3.25). This contradicts a previous finding in 388 hypertensive Tunisians, where no association of the rs4646994 D-allele with BP was observed (Kabadou *et al.,* 2013). Although this difference could be as a result of a difference in the frequency of the I/I genotype (10.1% (n=39) in Kabadou *et al.* (2013) study vs. 16.4% (n=10) in the present study), it appears to be instead as a result of varying genetic architecture between the populations – the I-allele may be in linkage disequilibrium (LD) with a causal variant that affects BP in our South Afrcan population, but this causal variant may not be in LD with the causal variant in the Tunisian population.

rs4646994 was not associated with T2DM in the present study, contrary to findings of association of rs4646994 D/D genotype in Tunisians (Berhouma *et al.,* 2012; Mehri *et al.,* 2010a; Mehri *et al.,* 2010b; Baroudi *et al.,* 2009) and rs4646994 I/I genotype in Egyptians (Settin *et al.,* 2015) being associated with T2DM. This could be due to varying genetic architecture in different populations and geographical locations.

After adjustment for gender and age, rs4646994 (I/I genotype) was significantly associated with dyslipidaemia by increased LDL-C levels (p=0.038) under a recessive model of inheritance (Table 3.24), Here, individuals homozygous for the I-allele were found to possess increased risk for increased LDL-C levels, compared to those individuals who possessed at least one D-allele (OR=2.31). This is an association not previously reported in Black individuals as the association of the *ACE* gene with dyslipidaemia has not been studied in the African Black population.

The angiotensin converting enzyme (*ACE*) is important for the RAAS, involved in BP control (Figure 1.2), and has previously been associated with T2DM (Settin *et al.,* 2015; Berhouma *et al.,* 2012; Mehri *et al.,* 2010a; Mehri *et al.,* 2010b; Baroudi *et al.,* 2009), hypertension (Motawi *et al.,* 2016; Mao & Huang, 2015; Kramer *et al.,* 2005) and obesity (Kabadou *et al.,* 2013; Baroudi *et al.,* 2004; Zhu *et al.,* 2001) in different populations. The angiotensin-converting enzyme is required for the cleavage of Ang I to Ang II for the optimal functioning of the RAAS to control BP. The D allele has been found to affect the activity of ACE, reducing the functioning of the RAAS in BP control (Gard, 2010). Interestingly, in the present study, both the I and D alleles conferred increased risk for various CVD risk phenotypes. The I/I genotype was significantly associated with raised BP and raised LDL-C associated with dyslipidaemia, while the D/D genotype was associated with overall obesity and an obese WC. LDL-C is associated with cholesterol deposits in arteries which may result in restricted blood flow, which in turn results in a rise in BP. Blood pressure is also proportional to pulse rate which is affected by the deposits in arteries. Thus, the association of the I/I genotype of rs4646994 with both BP and LDL-C suggests that these two risk phenotypes are in fact related. Although one would expect obesity to be associated with BP and dyslipidaemia, they are not necessarily causal factors, so while expecting the same genotypes to be responsible for the observed phenotypes, many factors such as dietary composition and physical exercise, which affects both fat stores as well as cholesterol deposits, and in turn BP, were not taken into account and may be why in the present

study the D/D genotype is associated with obesity. These observations suggest a cross-phenotype association and increased risk of CVD associated with this SNP.

4.2 Angiotensinogen (*AGT*)

Angiotensinogen (*AGT*) is involved in regulating the BP and fluid and salt balance (Figure 1.2). Previous studies have found the T allele of the rs699 SNP to be associated with BMI in Tunisian pregnant females (Zitouni *et al.,* 2018) and the T/T genotype associated with hypertension (pre-eclampsia) in Tunisian and SA Black Zulu-speaking pregnant females (Zitouni *et al.,* 2018; Aung *et al.,* 2017), but not with hypertension in non-pregnant Algerians (Amrani *et al.,* 2015) and Nigerians (Rotimi e*t al.,* 1997). No association of the *AGT* rs699 variant with disease was observed in this study, potentially due to the fact that the study population for the present study were not pregnant, and rs699 appears to only have an effect in pregnancy-specific (pre-eclampsia) hypertension and thus rs699's harmful affects are context-dependent.

4.3 Angiotensin II type I receptor (*AT1R*)

The angiotensin II type I receptor (*AT1R*) is responsible for vasoconstriction through AngII and cardiac and vessel hypertrophy. A computational analysis ranked rs5186, an A to C substitution in the 3' untranslated region, as the most plausible genotype to be associated with hypertension in Xhosa and Zulu-speaking SA Black individuals (Tiffin *et al.,* 2010), but as seen in Nigerians and Cameroonians, no association with hypertension was found in the present study (Table 3.15). Previous studies have also associated this SNP with CAD in Egyptians (Abd El-Aziz *et al.,* 2012; Motawi *et al.,* 2011; Shaker *et al.,* 2009) and Tunisians (Abboud e*t al.,* 2010), but as CAD was not looked at as a phenotype in the present study, no conclusion can be made regarding the association of rs5186 with CAD in this population. However, no association was observed between rs5186 and any of the CVD risk phenotypes investigated in the current study. Although selection of candidate SNPs was made based on literature, the absence of association in the present study suggests that rs5186 may indeed not be associated with hypertension as suggested by a computational analysis (Tiffin *et al.,* 2010), potentially related to the ethnic differences within Black individuals and the variability between computational and biological tests.

4.4 Melanocortin 4 receptor (*MC4R*)

The melanocortin 4 receptor (*MC4R*) is involved in regulating energy homeostasis and food intake (Logan *et al.,* 2016). In Moroccans, the C/C genotype of the rs17782313 SNP of *MC4R* was associated with T2DM under a log-additive model of inheritance (Cauchi *et al.,* 2012). In the same study, under a dominant model of inheritance in Moroccans and Tunisians (T/C and C/C genotype), and a recessive model of inheritance in Moroccans (T/T genotype), rs17782313 was nominally associated with increased T2DM risk (Cauchi *et al.,* 2012). In line with these resuts, we found that the rs17782313 T/C and C/C genotypes were nominally associated with increased T2DM risk under a dominant model of inheritance, when high-risk (fasting glucose 5.6-7.0 mmol/l) and diabetic (fasting

glucose >7mmol/l) individuals were combined as cases, after adjustment for gender and age (p=0.054) (Table 3.26). High-risk and diabetic individuals were first separated and then combined as cases for genetic analysis to determine if any SNP is involved in disease predisposition and/or on disease diagnosis. Those individuals who carried at least one C-allele were found to be at significantly increased risk (OR=1.51) of developing T2DM, according to fasting glucose and glucose tolerance, compared to T/T homozygotes.

In SA Black individuals from Soweto, the rs17782313 SNP (C/C genotype) was found to be associated with BMI and a 2.5% increased risk of BMI (Lombard *et al.,* 2012). However, in this study, no association between rs17782313 and BMI or obesity was observed. These contradictory results could potentially be because the study by Lombard *et al.* (2012) investigated the predisposition to obesity in early life in adolescents, whereas the population in the current study are mostly between the ages of 25-44 years. Possibly indicating an age specific association between rs17782313 and obesity. The nominal association of increased risk observed between rs17782313 (T/C and C/C genotypes) and T2DM in our population may, however, indicate a possible connection to BMI, not observed in the present study, as T2DM is modulated by obesity and the accompanying IR (Ouedemi *et al.,* 2009), an underlying cause of T2DM. rs17782313 is located downstream near the *MC4R* gene, and although its function is unknown, its proximity to *MC4R* suggests that it may play a role in food intake and energy homeostasis. rs17782313 has also been found to be in LD with rs17700144, rs4450508 and rs12970134 in Europeans and East Asians, previously found to be associated with obesity (Xi *et al.,* 2012). However, it should be noted that rs17700144 and rs4450508 were not investigated in the present study, so the LD with rs17782313 is not know in our sample.

The melanocortin 4 receptor is involved in energy homeostasis and food intake, and given that T2DM, in addition to genetic predisposition, is a disease of lifestyle, *MC4R* represents a valuable genetic target to investigate for treatment of T2DM. However, the absence of association with obesity observed in the present study, contrary to what was seen in Black individuals from Soweto (Lombard *et al.,* 2012), may suggest a more profound environmental effect in the isiXhosa-speaking Black population from Cape Town.

Although rs17782313 of *MC4R* was associated with T2DM diagnosis by fasting glucose, no SNPs were associated with T2DM diagnosis by glucose tolerance in the present study, or others. One would expect that rs17782313 would also be associated with glucose tolerance, as both fasting glucose and glucose tolerance represent the same end-point disease, T2DM. This may however be attributable to the fact that these are in themselves separate phenotypes that we are able to measure in order to evaluate the physiological mechanisms of glucose utility and the affect these phenotypes have on disease progression may thus differ (Karakas *et al.,* 2010) or that rs17782313 may be associated only with specific T2DM endophenotypes (in this case, fasting glucose levels).

The G allele of *MC4R* rs229616 has previously been found to be highly prevalent in obese SA Black individuals compared to normal weight SA Black individuals and obese SA Caucasian individuals (Logan *et al.,* 2016). Caucasian. However, in the current study, a nominal association (p=0.059) was however observed between rs229616 (G/G genotype) and obesity by BMI when overweight and obese individuals were combined as cases under a log-additive model of inheritance. This difference in association could be due to the difference in sample size of the study populations used in both studies, as a larger sample sze would increase the power to detect an association. No SNPs were associated with obesity diagnosis by waist-to-hip ratio under any of the models of inheritance. This could potentially be due to the inefficiency of the waist-to-hip ratio cut-points to diagnose obesity in the Black population of SA (Motala *et al.,* 2011).

After adjustment for gender and age, rs229616 (GG genotype) was associated with increased HDL-C/TC ratios under a log-additive inheritance model (p=0.043) (Table 3.21), and suggestive evidence was observed after adjustment for gender and age under a recessive model (p=0.059) (Table 3.24). Individuals who carried at least one A-allele were at increased risk of increased HDL-C/TC (OR=2.03), compared to G/G homozygotes.

Although rs229616 has not yet been found to be functional, based on the association with HDL-C/TC in the present study, and its location within the *MC4R* gene, it could be involved in metabolism and energy imbalance, affecting the levels of HDL-C and TC. The present study demonstrates that rs229616 may be involved in dyslipidaemia, but since dyslipidaemia is understudied in Africa, no definite conclusions can be drawn until further studies are undertaken. No studies have previously reported this association between rs229616 and dyslipidaemia and HDL-C/TC as a measure thereof in African or SA populations. The difference in genetics observed in the present study with regards to the various measures of diagnosing dyslipidaemia may be attributable to the fact that these are in themselves separate physiological parameters of lipid profile, and may therefore play different rolesin the mechanisms underlyin dyslipidaemia (Bitzur *et al.,* 2009); highlighting an inconsistency that will need to be further investigated. There may also be underlying genetic causes not investigated in the present study.

No significant association was observed between rs1297034 and any CVD risk phenotpye in this study, but suggestive evidence was observed for T2DM by fasting glucose when high-risk and diabetic individuals were combined as “cases”, after adjustment for gender and age under log-additive (p=0.072) (Table 3.8) and recessive (p=0.075) (Table 3.24) models of inheritance. However, no conclusion can be drawn with regards to the associated genotype as the OR could not be calculated and was 0, as there were no cases with the A/A genotype. Although haplotype analysis was not performed in the present study due to the selected SNPs not being in LD, the rs1297034 SNP may be in LD with other causal variants, not investigated in the present study, that may be implicated in disease.

Overall, the present study demonstrates that the *MC4R* gene may be involved in T2DM in the isiXhosa-speaking Black population of Cape Town, as seen in Moroccans and Tunisians. The *MC4R* gene regulates energy homeostasis and food intake, contributing to obesity. However, in the present study, *MC4R* was not significantly associated with obesity and we were thus unable to replicate the findings of Lombard *et al.* (2012) which reported an association with increased BMI in Black individuals from Soweto. This difference in association could be due to the difference in sample size of the study populations used in both studies, , as a larger sample sze would increase the power to detect an association.

4.5 Transcription factor 7-like 2 (*TCF7L2*)

Transcription factor 7-like 2 (*TCF7L2*) is involved in the downregulation of adipogenesis via the Wnt signalling pathway and is the most replicated gene for T2DM (Nguimmo-Metsadjio *et al.,* 2017). The T/T genotype of *TCF7L2* rs7903146 has been found to be associated with T2DM in Lebanese (Mtiraoui *et al.,* 2012), Tunisian (Berhouma *et al.,* 2014; Turki e*t al.,* 2014; Cauchi *et al.,* 2012; Mtiraoui *et al.,* 2012a; Bouhaha *et al.,* 2010a), Sudanese (Ibrahim *et al.,* 2016) and Moroccan (Cauchi e*t al.,* 2012) populations. However, as observed in SA Coloured individuals (Madubedube *et al.,* 2015), rs7903146 was not associated with T2DM in this study. It may be that in populations where significant associations have been observed, rs7903146 was in LD with a causal variant, whereas in the isiXhosa-speaking Black population of SA, rs7903146 is not in LD with a causal variant. Moreover, no association was observed between rs7903146 and obesity in this study, in line with results from Cameroonians (Nguimmo-Metsadjio *et al.,* 2017).

4.6 Limitations

The present study was conducted with some limitations, which are important to acknowledge and discuss. First, the samples for this study were collected during working hours, which could explain the over-representation of females during sampling, and may be biased to unemployed individuals who are usually less active than employed individuals, as individuals living in these communities are usually labourers (Peer *et al.,* 2014). In addition, the number of samples collected in Langa was much more than for any other area as this area was sampled for a secondary study (Peer *et al.,* 2012).

Second, CVD is a multifactorial disease, with numerous genes contributing to its aetiology. This complexity is further amplified by gene-gene and gene-environment interactions. Therefore, it would be pertinent to investigate these genetic variants using larger sample sizes (imparting increased power), in which epistatic and gene-environment interactions can be investigated.

Third, based on home language reporting, all participants in the present study were assumed to be of isiXhosa ethnicity. However, as this is done by means of self-report, it is only a proxy for ethnicity. We also did not take into account the genetic make-up of the isiXhosa population and assumed that it is homogenous (Shim *et al.,* 2014; Magazi *et al.,* 2008), as we did not employ any population

stratification methodologies, such as genomic control (Devlin & Roeder, 1999), structured analysis (Pritchard e*t al.,* 2000), mixed models (Yu *et al.,* 2006) and principal component analysis (Price e*t al.,* 2006), in the present study.

Fourth, the DNA quality of the samples have been compromised as the samples for this study were collected 10 years ago and have undergone multiple freeze-thaw cycles (Shao *et al.,* 2012). Of the 1 116 samples available for this study, DNA from 963 samples was extracted. Of the 963 samples, only 585 were eligible for KASP genotyping of the selected SNPs. However, two SNPs, rs1800629 in *TNFα* and rs4646994 in *ACE*, were not genotyped as the results could not be validated on an in-house system. The rs4646994 SNP was genotyped by PCR, but only 344 samples were completed, and, despite numerous attempts to optimise the genotyping procedure, negative control contamination, and possibly sample contamination, persisted throughout the genotyping of this variant. Results pertaining to this variant should thus be interpreted with caution, and require replication using a larger sample size.

Fifth, multiple testing was not corrected for, and if it had been, none of the significant associations would have been retianed. In total, six genes were investigated under three inheritance models, but multiple testing correction was not performed due to the exploratory nature of the present study. Increasing the sample size would increase the level of confidence and the power to detect differences, while decreasing the margin of error, and effect size.

Sixth, validation of both genotyping methods, PCR and KASP genotyping, was not done. To show reliability of these genotyping methods, samples could be sent for Sanger sequencing and blindly compared to the results obtained for PCR and KASP genotyping.

Finally, besides the SNPs in *MC4R*, only one SNP in the other genes were investigated, and thus the investigation of haplotypes could not be performed. Therefore, investigating multiple SNPs in one gene may help to detect SNPs that may otherwise not have enough penetrance within a given population, as many complex diseases are as a result of the combined effect of multiple variants and/or genes and this joint effect may be helpful for investigating causal genes and/or variants.

4.7 Future studies

Replicating the significant associations observed between *ACE* rs4646994 and overall obesity, WC, BP and LDL-C, and the nominal association between *MC4R* rs17782313 is crucial to understand the genetic risk factors that predispose SA Black individuals to CVD and diseases associated therewith, given that these risk phenotypes are common in Black population of SA, but the underlying genetics remains under-studied in this Black population. The results can be further validated by extending the sampling to predominantly Black communities in other provinces within South Africa, as environmental and dietary effects may further predispose an individual to disease (Tekola-Ayele *et al.,* 2013). The effect of disease coexistence could also be analysed, as studies have shown that

T2DM is modulated by obesity, which are both modulated by IR and often times are end-points of complex physiological processes that involve gene-environment interactions.

Knowledge of family history of disease and identifying shared genes between members of affected families could also add valuable insights into genes potentially predisposing an individual to increased risk of disease or genes that are the underlying cause of disease.

In addition, the present study investigated only a handful of the many genes that have been associated with CVD risk phenotypes in Africa and invesitgating other genes will further help to understand the pathophysiology of the studied disease in Africa. Investigating more SNPs in each gene would allow for haplotype analysis which may further elucidate genes associated with disease as single SNPs may have small effect sizes that could potentially aggregate in a haplotype to cause disease. Haplotype analysis has also been shown to explain more of the observed phenotypic variance when compared to a single SNP that has previously been found to be the best candidate (Barendse, 2011).

4.8 Conclusion

Non-communicable diseases are a major public health problem in SA. Insulin resistance, inflammation and oxidative stress are linking factors among various NCDs (Lontchi-Yimagou *et al.,* 2013; Motala *et al.,* 2011; Gallagher *et al.,* 2010; Boura-Halfon & Zick, 2009; Eckel e*t al.,* 2005; Grundy *et al.,* 2005; Hu *et al.,* 2004; Smith & LeRoith, 2004; Stephens *et al.,* 1997; Hotamisligil *et al.,* 1994; Reaven, 1988). The prevalence of CVD in the Black population of SA is low (Mensah e*t al.,* 2015; Mayosi *et al.,* 2009; Sliwa *et al.,* 2008), but that of NCDs such T2DM, obesity, hypertension and dyslipidaemia, risk factors for CVD, is high (Nojilana *et al.,* 2016; Abd El-Aziz e*t al.,* 2012; Mayosi *et al.,* 2009; Tibawarza e*t al.,* 2009; Alberts *et al.,* 2005; Akinboboye *et al.,* 2003). This could partly be as a result of westernisation and the economic and social transitions SA is undergoing, in addition to underlying genetic causes. To date only about 10.0-20.0% of the heritable risk of CVD and the associated disease risk factors have been explained. Heritability estimates of 35.0-57.0% for CVD, 26.0-77.0% for measures of diabetes, 50.0-89.0% for measures of obesity, 30.0-60.0% for hypertension and 8.0-72.0% for measures of dyslipidaemia have been reported.

In the present study we investigated the role of the transcription factor 7-like 2 (*TCF7L2*), melanocortin 4 receptor (*MC4R*), angiotensinogen (*AGT*), angiotensin II type I receptor (*AT1R*), angiotensin converting enzyme (*ACE*), fat-mass and obesity associated (*FTO*) and tumour necrosis factor-alpha (*TNFα*) genes in the development of CVD, T2DM, obesity, hypertension and dyslipidaemia in the SA Black population, as few genetic studies of CVD risk has been conducted in SA.

In summary, two SNPs (rs4646994 and rs17782313) in two genes (*ACE* and *MC4R*, respectively) were associated with risk phenotypes associated with CVD. These risk phenotypes include T2DM and fasting glucose and IGT as measures thereof; obesity and BMI, WC and waist-to-hip ratios as

measures thereof; and dyslipidaemia and TC, triglycerides, HDL-C, LDL-C and HDL-C/TC as measures thereof. Two SNPs in *MC4R* (rs1297034 and rs229616) were also nominally associated with T2DM and HDL-C/TC, respectively. Some of these genes have not previously been associated with these diseases in SA and/or SSA and cross-phenotype associations were observed, providing valuable insight to understand the role genetic risk factors have on predisposing an individual to disease and the contribution of one disease to the risk of another disease. However, no genes were associated with the measurements of glucose and/or blood pressure.

Overall, genetic associations with CVD risk phenotypes were replicated in the isiXhosa-speaking SA Black population and novel asssociations were reported. The results of the present study indicate that the rs4646994 SNP in *ACE* and rs17782313 in *MC4R* need to be further studied in this population to validate these findings, as they could essentially be the predisposing genetic factors for disease, offering novel targets for treatment and prevention. However, further studies in this population is required to identify genes associated with CVD risk phenotypes, especially T2DM and hypertension.

The prevalence of CVD risk factors is high in this population of isiXhosa-speaking SA Black individuals (Peer *et al.,* 2014b; Peer *et al.,* 2012), and very few studies to determine the genetic predisposition of these individuals to these diseases have been conducted. The results of this study suggest that this is an important population to study as there appears to be a genetic predisposition to CVD risk phenotypes in this population and genetic differences due to ethnicity need to be taken into account. The results of the present study lay the groundwork to understand the pathways/mechanisms leading to the development of these risk phenotypes in this population. The genes investigated and associated with CVD risk phenotypes in this study represent potential targets for treatment and preventative strategies as well as enabling the early identification of at-risk individuals.

5. References

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Chapter 6

Addenda

Addendum A: Agarose gel electrophoresis

A.1. 1x Sodium borate (SB) buffer

Dissolve 1.907g di-sodium tetraborate decahydrate in 1l distilled water.

A.2. 1% Agarose gel

Add 0.5g agarose powder in 50ml SB buffer.

Microwave on high until the agarose powder is dissolved.

Allow agarose to cool slightly and add 2µl ethidium bromide.

Pour agarose mixture into a gel mould and insert well comb.

Allow agarose gel to set.

A.3. Gel electrophoresis

Insert the set gel into a gel dock filled with SB buffer and remove the comb.

Add 1µl 50bp Kapa Universal DNA Ladder (Kapa Biosystems, Western Cape, South Africa) to the first well of the gel.

Add 10µl of the sample to the consecutive wells.

Electrophorese at 160V for 1 hour.

Visualise using the G box (Syngene, Maryland, USA) with GeneSnap Syngene software (version 7.12.06).

Addendum B: Medians and IQR of CVD risk factors

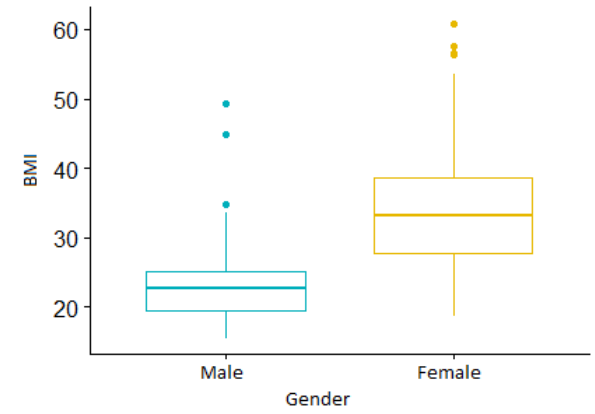

Figure B1 Median and IQR for BMI as a CVD risk factor by gender

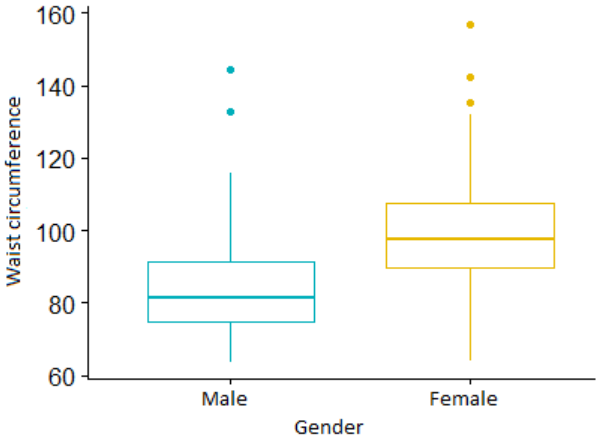

Figure B2 Median and IQR for WC as a CVD risk factor by gender

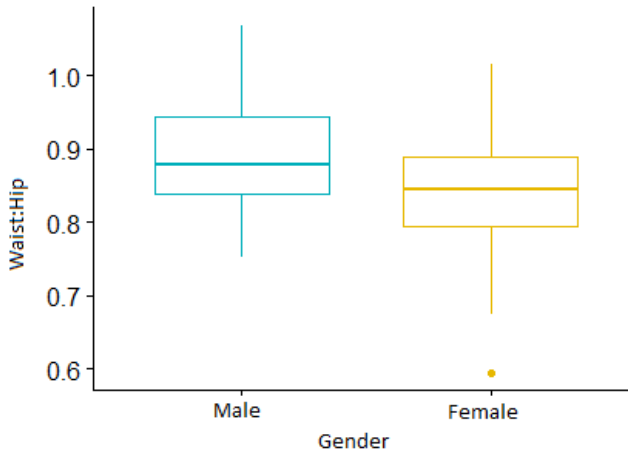

Figure B3 Median and IQR for waist-to-hip ratio as a CVD risk factor by gender

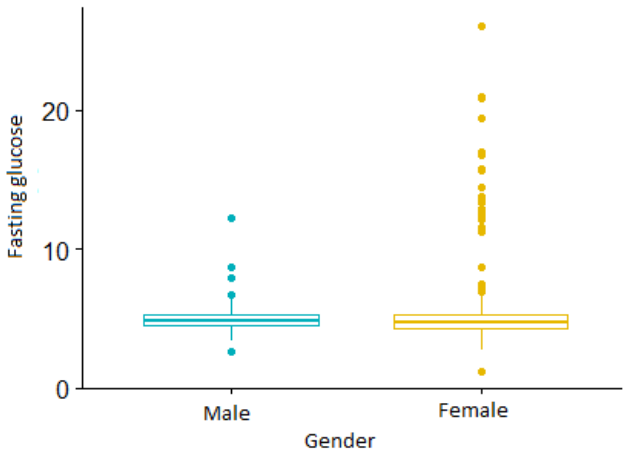

Figure B4 Median and IQR for fasting glucose as a CVD risk factor by gender

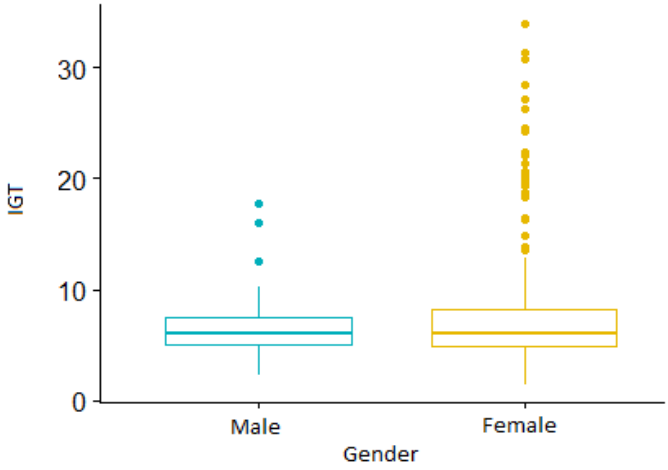

Figure B5 Median and IQR for impaired glucose tolerance as a CVD risk factor by gender

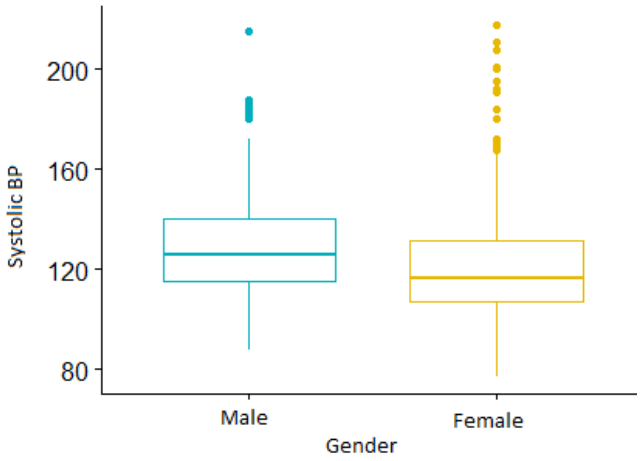

Figure B6 Median and IQR for systolic BP as a CVD risk factor by gender

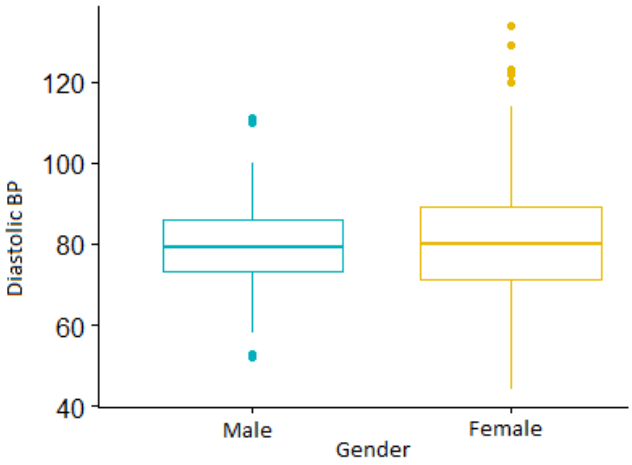

Figure B7 Median and IQR for diastolic BP as a CVD risk factor by gender

Figure B8 Median and IQR for total cholesterol as a CVD risk factor by gender

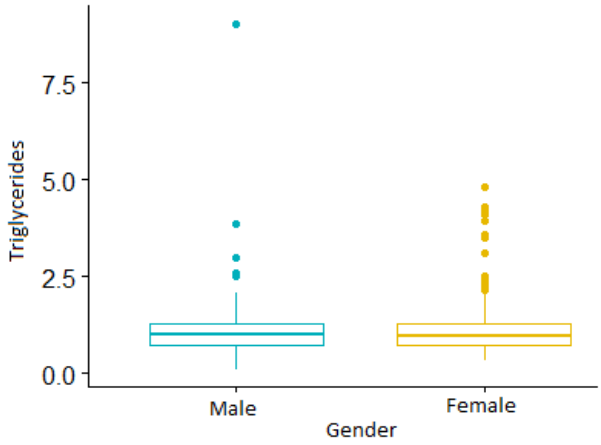

Figure B9 Median and IQR for triglycerides as a CVD risk factor by gender

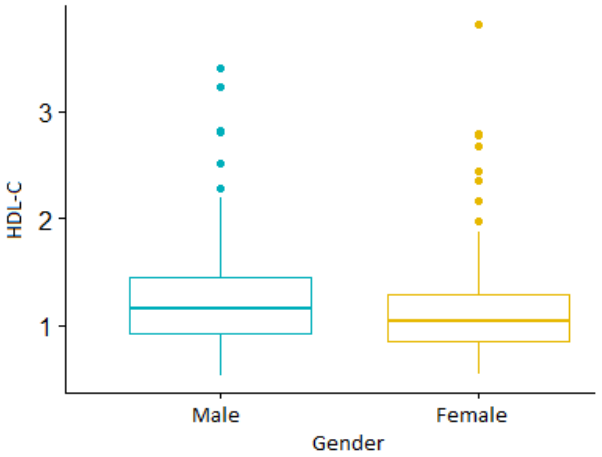

Figure B10 Median and IQR for HDL-C as a CVD risk factor by gender

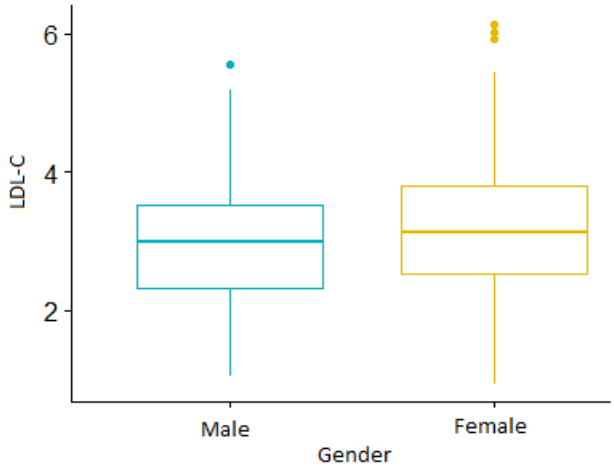

Figure B11 Median and IQR for LDL-C as a CVD risk factor by gender

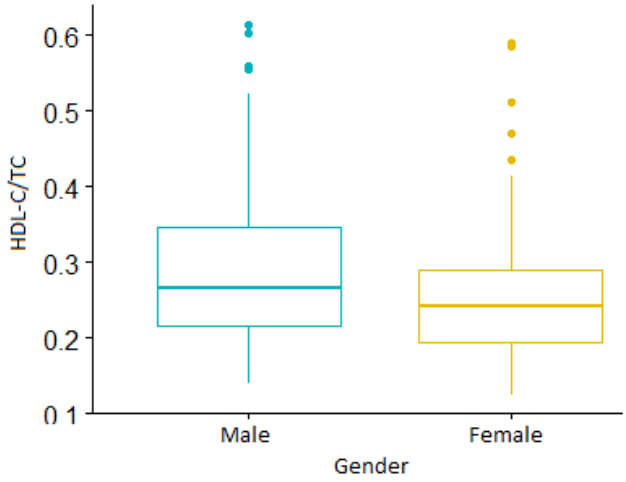

Figure B12 Median and IQR for HDL-C/TC as a CVD risk factor by gender

Addendum C: KASP genotype distributions

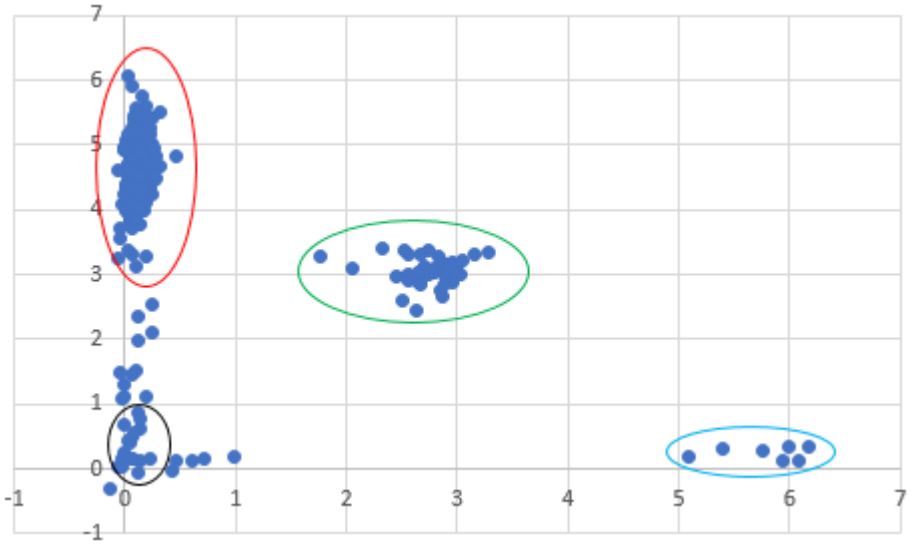

Figure C1 Genotype distribution of *FTO* rs17817499 Points circled in red: TT (87.52%); green: TA (6.32%): blue: AA (1.20%); black: no template control (NTC); not circled: no genotype results (4.96%).

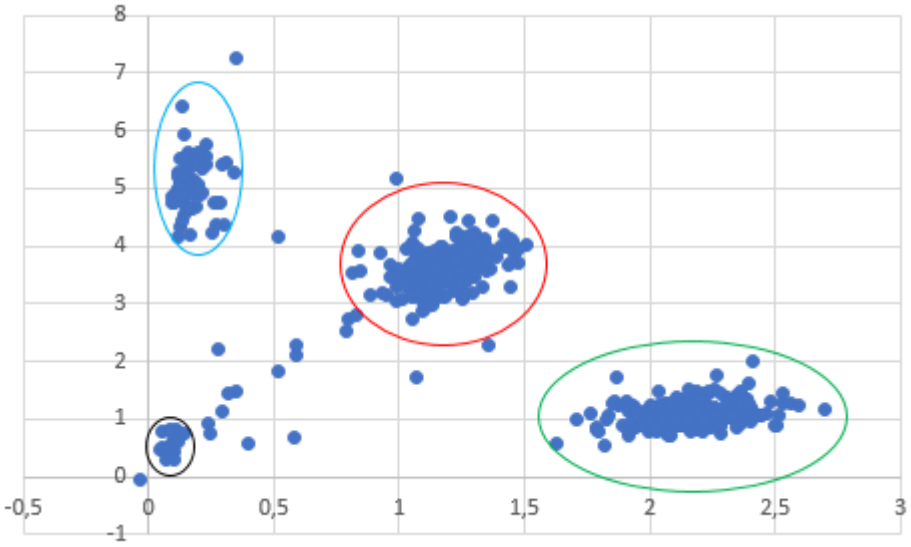

Figure C2 Genotype distribution of *TCF7L2* rs7903146 Points circled in red: TC (44.07%); green: CC (41.71%); blue: TT (10.43%); black: NTC; not circled: no genotype results (3.59%).

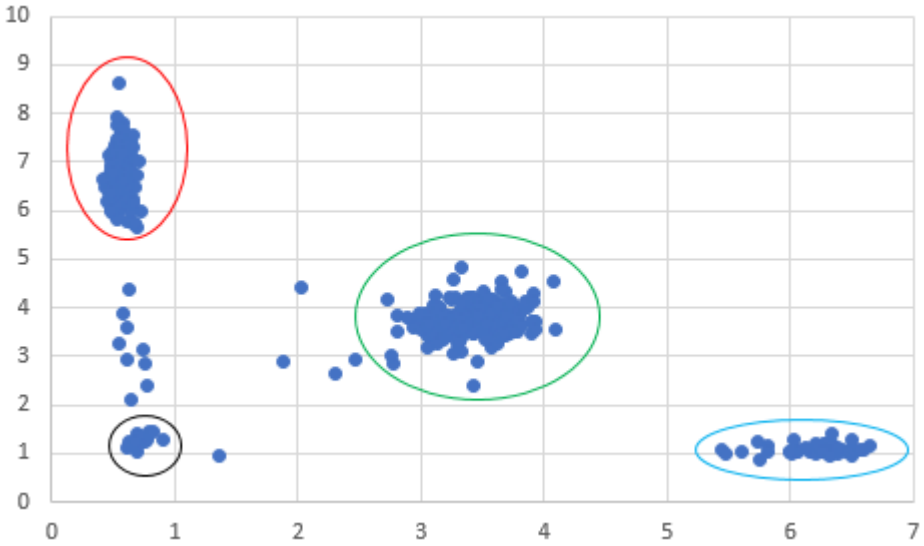

Figure C3 Genotype distribution of *MC4R* rs229616 Points circled in red: GG (55.21%); green: GA (35.56%); blue: AA (6.67%); black: NTC; not circled: no genotype results (2.56%).

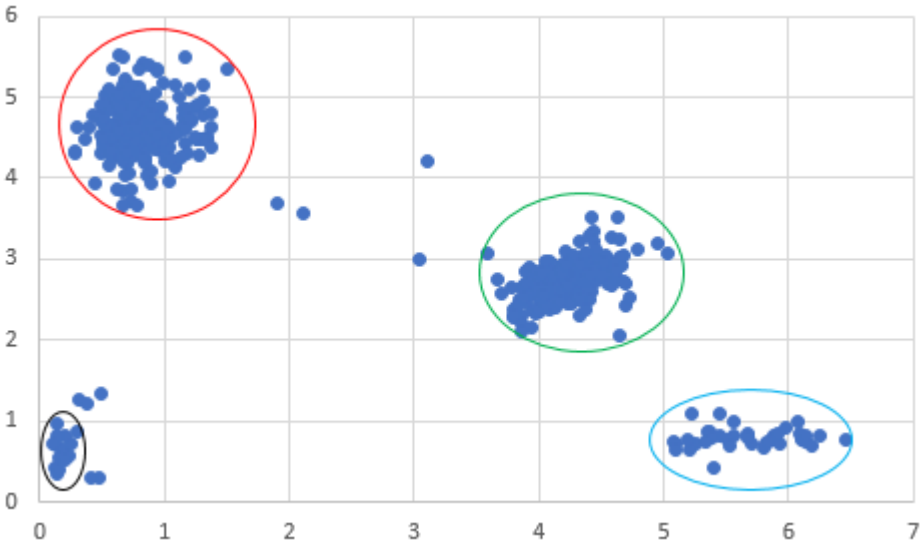

Figure C4 Genotype distribution of *MC4R* rs17782313 Points circled in red: TT (51.79%); green: TC (39.49%); blue: CC (6.67%); black: NTC; not circled: no genotype results (2.05%).

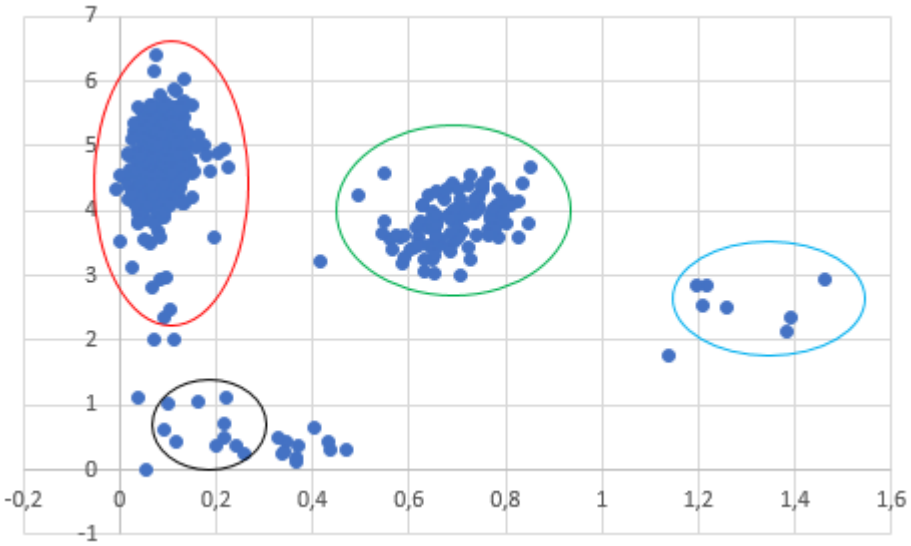

Figure C5 Genotype distribution of *MC4R* rs1297034 Points circled in red: GG (78.80%); green: GA (17.09%); blue: AA (1.20%); black: NTC; not circled: no genotype results (2.91%).

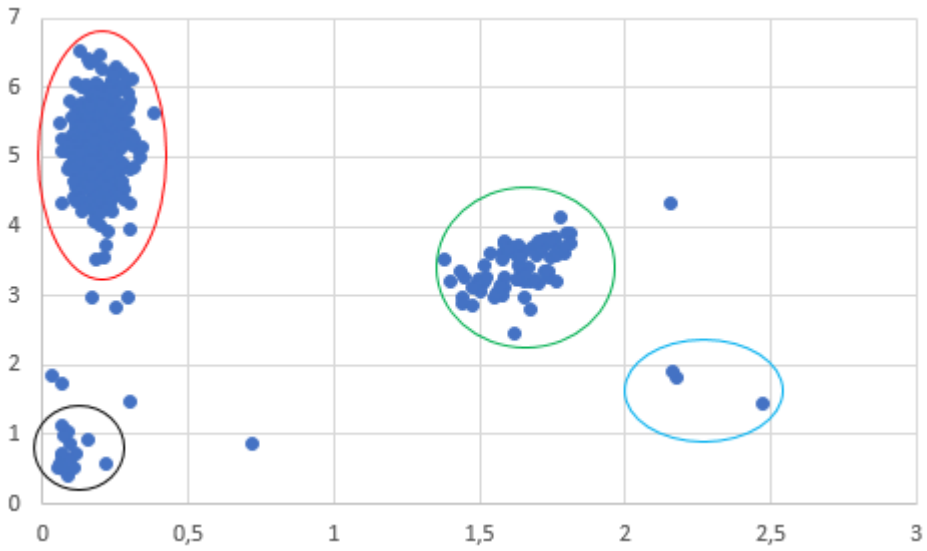

Figure C6 Genotype distribution of *AGT* rs699 Points circled in red: CC (83.93%); green: CT (13.16%); blue: TT (0.51%); black: NTC; not circled: no genotype results (2.39%).

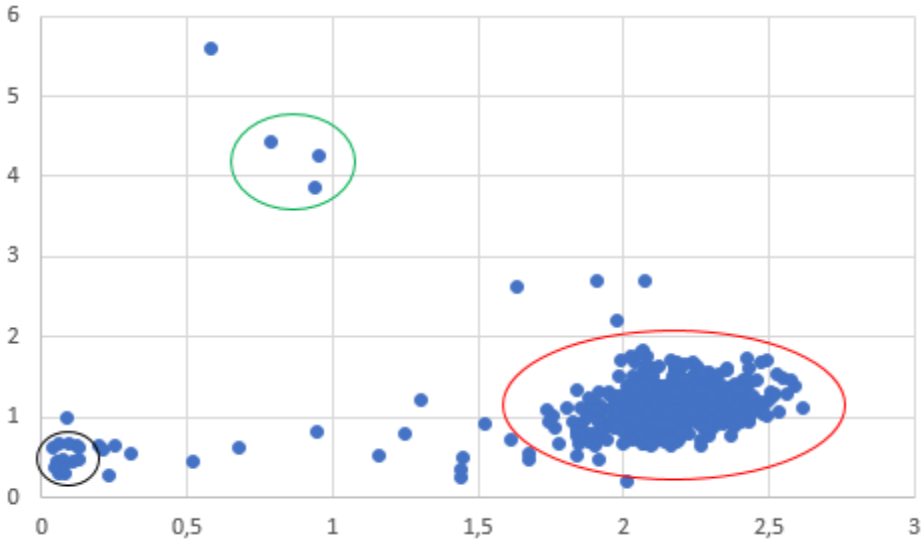

Figure C7 Genotype distribution of *AT1R* rs5186. Points circled in red: AA (95.39%); green: CA (0.51%); blue: CC (0%); black: NTC; not circled: no genotype results (5.64%).