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Exploring the role of genetic variation at the leptin and leptin receptor genes (LEP and LEPR) in obesity and hypertension in a black South African cohort

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Exploring the role of genetic variation at the leptin and leptin receptor genes (*LEP* and *LEPR*) in obesity and hypertension in a black South African cohort

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A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of Master of Science (Medicine) in Human Genetics

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Declaration

I, Thandiswa Ngcungcu, declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science (Medicine) in Human Genetics to the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

Thandiswa Ngcungcu

Date

To my friends and family.

Presentations arising from this study

1. **Chronic Non-communicable Diseases and Disorders Research Training Networking Meeting (Bethesda, 21-23 March 2012).** Presented a poster entitled "Exploring the role of genetic variation at the leptin and leptin receptor genes (*LEP* and *LEPR*) in obesity and hypertension in a black South African cohort".

2. **Wits Health Sciences Research Day & Postgraduate Expo 2012 (Johannesburg, 19 September 2012).** Presented a poster entitled "Exploring the role of genetic variation at the leptin and leptin receptor genes (*LEP* and *LEPR*) in obesity and hypertension in a black South African cohort".

3. **5th Cross Faculty Graduate Symposium (Johannesburg, 1-2 August 2013).** Oral presentation entitled "Exploring the role of genetic variation at the leptin and leptin receptor genes (*LEP* and *LEPR*) in obesity and hypertension in a black South African cohort".

Abstract

Obesity and hypertension often occur together and are risk factors for cardio-metabolic disorders. Single nucleotide polymorphisms (SNPs) in the leptin (*LEP*) and leptin receptor (*LEPR*) genes have been shown to be associated with obesity and hypertension, but have not been well explored in African populations. The aims of this study were to determine the heritability estimates of anthropometric and blood pressure (BP) measures and leptin levels; to identify additional informative SNPs in and around the *LEP* and *LEPR* genes; and to examine the potential relationships between these SNPs and measures of obesity, hypertension and leptin levels in a black South African cohort. Participants from the African Programme on Genes in Hypertension (APOGH) with various anthropometric and BP measurements were genotyped for *LEP* and *LEPR* SNPs using the BeadXpress platform. Heritability estimates were determined using Statistical Analysis for Genetic Epidemiology (S.A.G.E.) software and relationships between *LEP* or *LEPR* SNPs and obesity, leptin levels and hypertension were assessed using SAS 9.3 and gPLINK vs2.050, taking into account family relationships, various confounders and correcting for multiple testing. The Bonferroni method was used to correct for multiple testing and $P \leq 0.00076$ was considered as statistically significant for SNP association tests. Seven-hundred-and-thirteen individuals were successfully genotyped and there were more women (66%) than men. The prevalence of obesity (42%) and hypertension (46%) were high in the sample. Significant heritability (h^2 %, $P<0.05$) was noted for body weight (38%), body mass index (26%), waist (35%) and hip circumference (42%), waist-to-hip ratio (46%), skinfold thickness (44%), systolic (34%), diastolic (27%) and central systolic (33%) BP; but leptin levels were not significantly heritable (h^2 %=15%, P=0.228). *LEP* rs17151914 (P=0.0002) and *LEPR* rs6690661 (P=0.0007) were significantly associated with leptin levels and diastolic BP, respectively, in women. The *LEP* rs17151913T-rs6956510G haplotype was associated with an increase in central systolic BP in

women (P=0.012 with Bonferroni correction) whereas the *LEPR* rs2154381C-rs1171261T haplotype was associated with lower systolic BP in men (P=0.0359 with Bonferroni correction). *LEP* gene variants were significantly correlated with effects on leptin levels in women and the *LEPR* gene variants were significantly correlated with effects on diastolic BP also in women. These results indicate that further exploration of the role of genetic variation in the *LEP* and *LEPR* genes in obesity and hypertension in individuals of African ancestry is warranted.

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Abbreviations

°C	Degrees Celsius
µg	Microgram
µl	Micro litre
µM	Micro molar
A	Adenine
AA	African American
ACE	Angiotensin converting enzyme
ADT	Assay design tool
AFR	Africa
AGT	Angiotensinogen
AIM	Ancestry informative marker
AMR	Americas
ANS	Automatic nervous system
APOGH	African Programme on Genes in Hypertension
ARC	Acuate nucleus
ASO	Allelic specific oligonucleotides
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BDNF	Brain-derived neurotropic factor
BMI	Body mass index
bp	Base pair
BP	Blood pressure

bpm	Beats per minute
BW	Body weight
C	Cytosine
CEU	CEPH (Utah residents with ancestry from northern and western Europe)
CHB	Han Chinese in Beijing, China
cm	Centimetre
CNS	Central nervous system
CVD	Cardiovascular disease
ddH2O	Double distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
eg.	Example
eQTL	Expression quantitative trait loci
EtBr	Ethedium bromide
FTO	Fat Mass and Obesity Associated
g	Gram
GWAS	Genome wide association studies
h^2	Heritability coefficient
Hg	Mercury
hg	Human genome build
HREC	Human Research Ethics Committee

HWE	Hardy-Weinberg equilibrium
IDF	International Diabetes Federation
IGF-BP3	Insulin-like growth factor binding protein-3
JAK	Janus kinase
JPT	Japanese in Tokyo, Japan
K^+	Potassium
kb	Kilo base
kg	Kilogram
KOH	Potassium hydroxide
KZN	KwaZulu Natal
LD	Linkage disequilibrium
LEP	Leptin gene
LEPR	Leptin receptor gene
LSO	Locus specific oligonucleotide
LWK	Luhya in Webuye, Kenya
m^2	Metres squared
MAF	Minor allele frequency
MAPK	Mitogen-activated protein kinase
MC4R	Melanocortin receptor 4 gene
MCH	melanin-concentrating hormone
MGB	Minor groove binder
MgCl2	Magnesium chloride
ml	Milli litre

mM	Milli molar
MSS	Maasai in Kinyawa, Kenya
MUAC	Mid-upper arm circumference
n	Number
NA^+	Sodium
NaOH	Sodium hydroxide
NCEP	National Cholesterol Education Program
NEGR1	*Neuronal growth regulator 1* gene
NPY	neuropeptide Y
ng	Nano gram
ns	Non-synonymous
PCR	Polymerase chain reaction
PCSKKI1	*Proprotein convertase subtilisin/kexin type 1* gene
PFM	Percentage fat mass
POMC	proopiomelanocortin
rpm	Revolutions per minute
r^2	Correlation coefficient
RAS	Renin-angiotensin system
RNA	Ribonucleic acid
rs	Reference sequence
RSA	South Africa
s	Synonymous
S.A.G.E.	Statistical Analysis for Genetic Epidemiology

SD	Standard deviation
SDS	Sequence Detection System
SFT	Skinfold thickness
SH2B1	Src homology 2 B1 gene
SNP	Single nucleotide polymorphism
SNS	Sympathetic nervous system
SPSmart	SNPs-for-population-Studies-mart
STAT	Signal transducer and activator of transcription
T	Thymine
T2D	Type II diabetes
Taq	Thermus aquaticus polymerase
TDT	Transmission disequilibrium test
TE	Tris/EDTA buffer
TF	Transcription factor
U	Uracil
UCSC	University of California, Santa Cruz
UK	United Kingdom
USA	United States of America
UTR	Untranslated region
V	Volts
VEP	Variant Effect Predictor
vs.	Versus
WC	Waist circumference

WHO	World Health Organisation
WHR	Waist-to-hip ratio
YRI	Yoruba in Ibadan, Nigeria

1. Introduction

Obesity and hypertension are common, preventable disorders that are associated with increased morbidity and mortality worldwide (Frayling et al., 2007, Willer et al., 2009, Minor et al., 2008). They have already reached epidemic levels in many industrialised countries and are fast becoming a major problem in the developing world (Scuteri et al., 2007, Prentice, 2006, Ma et al., 2009). A large number of South African individuals are affected by obesity and hypertension, predisposing them to other non-communicable disorders like diabetes and stroke. The number of affected individuals is rapidly increasing due to lifestyle changes, but contributing factors that lead to obesity and hypertension, particularly the role of genetics in disease development and progression are not well understood. This study aims to build on current knowledge of the genetic predisposition to obesity and hypertension, particularly in individuals of African descent.

In the introduction chapter, I will provide a detailed background of obesity and hypertension. This will include definitions, associated complications of each disorder and causes, including the role of genetics in disease pathogenesis. The interaction between obesity and hypertension, pathways involved in energy and blood pressure regulation and the role of genetic variation in the development and pathogenesis of both disorders will also be discussed.

1.1. Obesity

Obesity is the most common non-communicable human disorder and is the leading cause of preventable disease and death internationally (Han et al., 2010, Tung et al., 2010, Thorleifsson et al., 2009, Willer et al., 2009). Obesity is a medically important disorder defined as having excess body fat (white adipose tissue) (Friedman, 2009, Zhang et al., 2012). Obesity is caused by an energy imbalance

that results from excess energy intake relative to what is required by the body, coupled with insufficient physical activity (World Health Organisation, 2010).

1.1.1. Adipose tissue

Adipose tissue is an endocrine organ (Ailhaud, 2006) which is distributed among different regions of the body including the internal (eg. around organs or abdominal/visceral fat) and subcutaneous (under the skin) regions (Palou et al., 2008). Based on the location, adipocyte tissue carries out different metabolic activities and different functions (Casteilla and Dani, 2006, Palou et al., 2008). In essence, although adipose tissue is essential in protecting internal organs and in body insulation, its main function is to maintain and regulate energy homeostasis (Paracchini et al., 2005, Zhang et al., 2012).

Adipose tissue is present in two forms, brown adipose tissue (BAT) and white adipose tissue (WAT). WAT has a role in energy storage and provides energy in the form of lipid substrates to different tissues whereas BAT uses fatty acids in heat production (Pénicaud, 2010, Zhang et al., 2012). WAT acts as an important site in energy or fuel storage in the form of fats (Zhang et al., 2012). Excess energy is converted into lipids in the liver, hydrolysed into fatty acids and then taken up by adipocytes, where they are esterified with glycerol and stored as triacylglycerides (Zhang et al., 2012). During fasting states and periods of high energy demand, stored triacylglycerides undergo lipolysis, a process which breaks down lipids, to release usable, non-esterified fatty acids that can be used as an energy source in other tissue such as muscle and liver tissue (Palou et al., 2008, Zhang et al., 2012). As WAT primarily stores energy in the form of fat, it is therefore very important in obesity.

Adipose tissue is made of several cell types including mesenchymal stem cells, preadipocytes, adipocytes, fibroblasts and macrophages (Guerre-Millo, 2004). Adipocytes or fat cells are the most abundant cell type in adipose tissue and are formed in a process called adipogenesis (Figure 1.1) (Ailhaud, 2006, Casteilla and Dani, 2006, Romao et al., 2011, Pénicaud, 2010, Zhang et al., 2012). During adipogenesis, mesenchymal stem cells found in adipose tissue commit to the adipose lineage and in response to signals, differentiates into preadipocytes. These preadipocytes have self-renewing properties that allow them to expand continuously (Ailhaud, 2006). Preadipocytes then differentiate to form mature adipocytes which are characterised by a large fat droplet that occupies the majority of the cell (Romao et al., 2011).

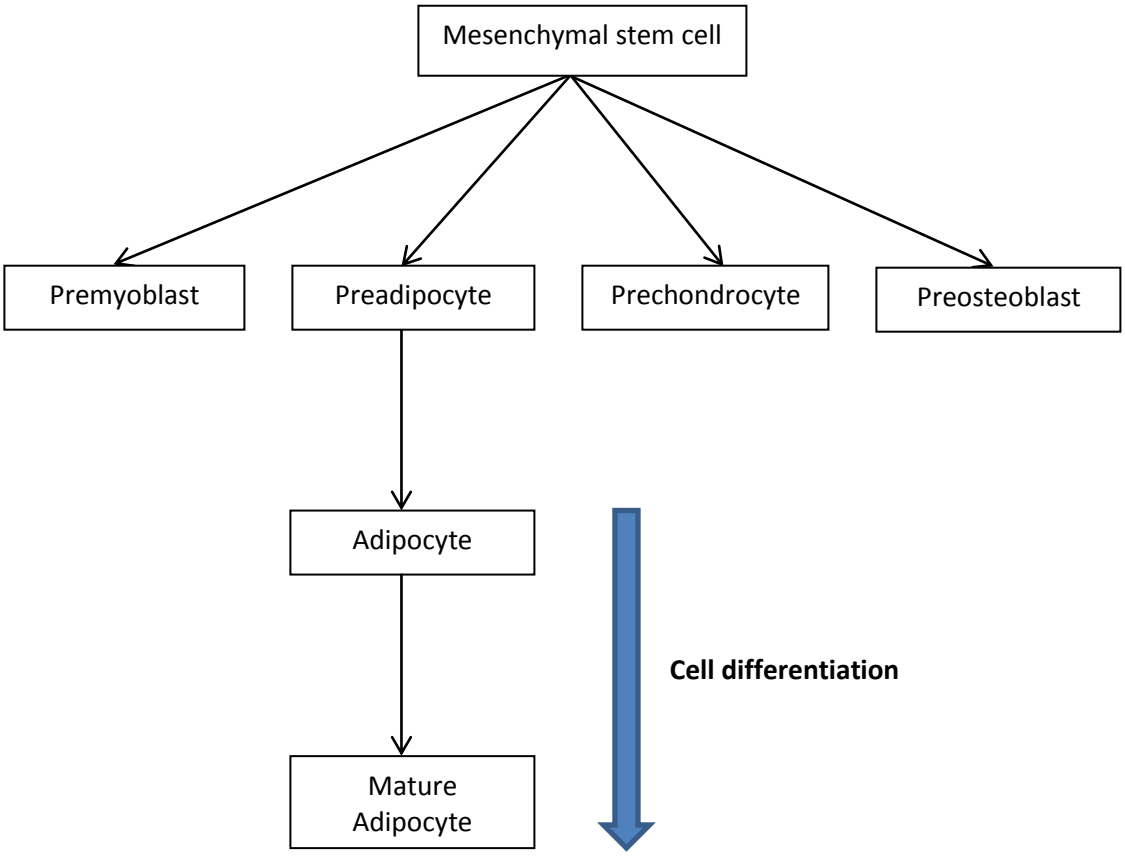


Figure 1.1: Formation of mature adipocytes. Mesenchymal stem cells are capable of differentiating into different cells that give rise to other cells types such as osteocytes which give rise to bone cells and preadipocytes which through the process of adipogenesis to give rise to mature adipocytes (Adapted from Romao et al., 2011).

Adipocytes and the other cells present in adipose tissue secrete adipokines which enable adipose tissue to relay signals to various organs in the body (Ailhaud, 2006). Examples of adipokines secreted by adipocytes include leptin which is involved in energy balance and appetite regulation; angiotensinogen which has a role in BP regulation and adiponectin which has a role in fatty acid oxidation and insulin sensitivity (Ailhaud, 2006, Awazawa et al., 2011). An increase in the size and number of adipocytes (such as in obesity) results in increased secretion of adipokines. Consequently, signalling pathways are impaired resulting in a range of metabolic disorders including dyslipidaemia and insulin resistance (precursor for type II diabetes mellitus) (Ailhaud, 2006, Palou et al., 2008).

1.1.2. Anthropometric measures of obesity

Obesity is defined by a number of quantitative traits such as increased waist circumference, ratio of waist to hip circumference (WHR), body fat percentage, weight and body mass index (BMI). BMI is an indirect, approximate measure of adiposity, whereas percentage fat mass (PFM) is a direct measure for adiposity (Willer et al., 2009) and both measures are indicators for general obesity. BMI is a more commonly used obesity index because determining BMI is less complicated compared to measuring fat mass (Hofker and Wijmenga, 2009, Scuteri et al., 2007, Willer et al., 2009). BMI is calculated by dividing the body mass (kg) by the square of the individual's height (m^2) (Frayling et al., 2007, James et al., 2001). Although body composition is also influenced by other factors such as changes in lean mass, fat mass and bone mass, it is mainly influenced by changes in adiposity (Frayling et al., 2007). Children and adult BMIs have different cut off values for obesity but are calculated the same way. Adult BMI values and their interpretations are shown in Table 1.1.

Table 1.1: Adult body mass index values and their interpretation[1]

Body mass index (kg/m^2)	Weight range/Status
< 18.5	Underweight
18.5 - 24.9	Normal
25 - 29.9	Overweight
≥ 30	Obese
≥ 40	Morbidly/severely obese

[1](James et al., 2001, Scuteri et al., 2007, Willer et al., 2009, Frayling et al., 2007)

Percentage fat mass (PFM) is the most accurate measure of obesity and can be measured by underwater weighing, dual-energy X-ray absorptiometry [DXA], total body water assessment, body electrical resistance and skinfold thickness (Fogelholm and van Marken Lichtenbelt, 1997). These methods require expensive facilities and equipment and high levels of expertise (Aboul-Seoud and Aboul-Seoud, 2001). Of these methods, skinfold thickness measurements are relatively easy to determine and far less expensive than the other methods. Measurements are usually taken from biceps, triceps, subscapular and supra-iliac areas of the body using a calliper (Aboul-Seoud and Aboul-Seoud, 2001).

Waist circumference (WC) and waist-to-hip ratio (WHR) are indicators for abdominal obesity also known as central or visceral obesity and both measures are correlated with BMI (Tanyolac et al., 2007, World Health Organisation, 2011b). WC is also used, along with other measures, to diagnose metabolic syndrome which groups abdominal obesity together with other CVD risk factors (Alberti et al., 2009). Like other measures of obesity, WC and WHR values differ between ethnic groups and between males and females (World Health Organisation, 2011b). The current WC cut-off values used in the diagnosis of abdominal obesity in Sub-Saharan Africa, as recommended by the International Diabetes Federation (IDF), are ≥94 cm for men and ≥80 cm in women (Alberti et al., 2009). Crowther

and Norris (2012) reported that the IDF recommended cut-off values for diagnosing metabolic syndrome were inappropriate for diagnosis in South African women (an urban (Soweto) sample was used) and recommended that the cut-off value should be increased from 80 cm to 91.5 cm (Crowther and Norris, 2012). Similar recommendations were made by Motala et al. (2011) for rural KwaZulu Natal women (92 cm) using the joint interim statement. A cut-off value of 86 cm was recommended for black South African men (rural) to predict at least two other components of the metabolic syndrome using the JIS statement (Alberti et al., 2009, Motala et al., 2011b). In this study, the IDF cut-off values for WC were used.

1.1.3. Prevalence of obesity

Obesity has increased over the past few decades in many countries, in both sexes, all ages and across all ethnic groups (Thorleifsson et al., 2009). According to the World Health Organisation (WHO), at least 500 million adults across the world were obese in 2008 and it is predicted that by 2015, 700 million adults will be obese (World Health Organisation, 2010). Obesity is common in both developing and developed countries and the overall levels of obesity are comparable but obesity statistics are often different between developed and developing countries. Many developed countries such as the United States of America (USA) and the United Kingdom (UK) have alarmingly high incidences of overweight and obese individuals (Table 1.2). In these countries, obesity is common in children and adults and the prevalence is relatively similar in both sexes (Troiano & Flegal, 1998 as cited by Grant et al., 2008, Prentice, 2006).

South Africa is a developing country and, unlike the situation in the USA, many parts of the country are poverty stricken, yet the prevalence of overweight and obesity is amongst the highest in the world (Puoane et al., 2002). In South Africa and many of the other African developing countries, adult

obesity is influenced by sex, with women tending to be heavier than men. As seen in Table 1.2, higher BMIs are more common in women. Puoane et al. (2002), showed that the move of women from rural areas to urban areas, where high calorie food is readily available and less physical activity is required, has led to South African women living in urban areas being more prone to obesity compared with their counterparts living in rural areas.

Table 1.2: Prevalence (%) of overweight and obesity in 15-100 year old individuals resident in developed and developing countries[1]

Status	Sex	Developed countries			Developing countries		
		New Zealand	United Kingdom	United States	Kenya	Mexico	South Africa
Overweight	Males	45	43.1	36.3	7.6	7.3	33.7
	Females	34.3	37.5	28.4	21.3	33	31.7
Obese	Males	28.9	23.7	44.2	0.1	30.1	7.6
	Females	39.9	26.3	48.3	2.2	41.0	36.8

[1] (https://apps.who.int/infobase/Comparisons.aspx)

1.1.4. Complications associated with obesity

Obesity is associated with increased morbidity and mortality worldwide (Frayling et al., 2007, Willer et al., 2009) and has been shown to reduce life expectancy (Do et al., 2008). Being overweight or obese predisposes individuals to an increased risk for a range of medical disorders such as hypertension, stroke, some forms of cancer, heart disease and insulin resistance which leads to type II diabetes mellitus (Frayling et al., 2007, Grant et al., 2008, Scuteri et al., 2007). Due to health problems associated with obesity, obese individuals also contribute to reduced productivity in the work place causing economic losses (Scuteri et al., 2007) and inflicting severe economic burdens on health systems (Flegal et al., 2007 as cited by Willer et al., 2009).

1.1.5. Causes of Obesity

Obesity is a multifactorial trait, with the major contributors to obesity being inappropriate nutrition, behaviour, environmental factors, social pressures and genetic factors (Scuteri et al., 2007, Puoane et al., 2002). Reduced physical activity, increased urbanisation and a change in mode of transport along with a calorie and sugar rich diet and employment that does not require a lot of physical activity are all major contributors to obesity in the USA and the world at large (Prentice, 2006).

People in different parts of the world have different views on obesity. In many South African cultures, obesity is not regarded as a health problem, but is rather seen as a sign of beauty and fertility in women and is also regarded as a sign of health and wealth (Kruger et al., 2001). South Africa has the highest rate of HIV infection in the world and leanness is often associated with HIV infection, so most people see being overweight as an indicator of health or being HIV negative. Environmental and social determinants contribute greatly to the development and persistence of obesity (Prentice, 2006), however genetic factors also play a role.

Genetic factors play an important role in determining the development of obesity and the distribution of body fat but it is not well understood how genetic variations influence adiposity (genetic factors influencing obesity are discussed in section 1.4). Genetic factors strongly influence how environmental factors affect a person and play a big role in determining if the person will become obese or not (Willer et al., 2009). The interaction between genes and the environment is very important in obesity and other multifactorial disorders. A genetically susceptible individual with an unhealthy diet and lack of sufficient physical activity or exercise is more likely to become overweight and eventually obese than a non-susceptible person with similar eating and lifestyle habits.

1.2. Hypertension

Hypertension, also known as high blood pressure (BP), is a common disorder (Chobanian et al., 2003, Minor et al., 2008) that is often asymptomatic and is therefore sometimes referred to as the "silent epidemic" (Chobanian et al., 2003, Minor et al., 2008). BP can be increased during normal physiological processes such as in response to physical activity, but it is a problem when BP is elevated in a persistent state even when the person is at rest (Nakayama and Yamamoto, 2009).

1.2.1. Definition of hypertension

BP is determined by measuring the pressure on the artery walls when the heart muscle is at rest (diastolic BP) and when muscles are contracted (systolic BP). Hypertension is defined as a systolic BP of ≥140 mm Hg or diastolic BP of ≥90 mm Hg (average two to three measurements of systolic BP and diastolic BP measured at two conservative clinical check-ups) or taking antihypertensive drugs (Chobanian et al., 2003, Minor et al., 2008). Table 1.3 shows the different BP ranges and categories of hypertension. Hypertension is diagnosed by taking brachial (on the arm) BP measurements using a mercury sphygmomanometer with the patient is usually sitting down.

Table 1.3: Classification of blood pressure levels[1]

Disease state	Category	Systolic BP (mm Hg)		Diastolic BP (mm Hg)
Normal	Optimal	<120	and	<80
Prehypertension	Normal	<130	and	<85
	High normal	130-139	or	85-89
Hypertension	Stage 1	140-159	or	90-99
	Stage 2	160-179	or	100-109
	Stage 3	≥180	or	≥110
	Isolated Systolic hypertension	≥140	or	<90

[1](Adapted from Carretero & Oparil, 2000)

1.2.1.2. Primary hypertension vs. secondary hypertension

Two forms of hypertension exist in humans, essential (primary) hypertension and secondary hypertension. Secondary hypertension is a result of other underlying conditions such as renal disease aldosteronism or due to pregnancy whereas the cause of essential hypertension is unknown (Adeyemo et al., 2009, Wolff and Miller, 2007). Glucocorticoid remedial aldosteronism, Liddles Syndrome, Apparent Mineralocorticoid Excess and Autosomal Dominant Hypertension with Brachydactyly are well characterised monogenic forms of secondary hypertension caused by congenital defects (Carretero and Oparil, 2000). Essential hypertension is a heterogeneous disorder that accounts for 90-95% of hypertension cases (Carretero and Oparil, 2000, Caulfield et al., 1994, Jeunemaitre et al., 1992) and it occurs at all ages but is more common in adults (Adeyemo et al., 2009, Wolff and Miller, 2007).

1.2.2. Prevalence

Hypertension has an alarmingly high prevalence throughout the world. According to the WHO, 12.8% (approximately 7.5 million) of all deaths are attributable to hypertension (World Health Organisation, 2012). In 2008, the global prevalence of hypertension was estimated to be 40% with the lowest prevalence in high income countries (World Health Organisation, 2012). Among the WHO regions, the Americas region had the lowest prevalence of hypertension (35%) (Figure 1.2). In the USA (high income country part of the region of the Americas), one third (33.5%) of the adult population is hypertensive and hypertension is particularly high in African-Americans (black) where 43% of men and 45.7% women were hypertensive (Roger et al., 2012).

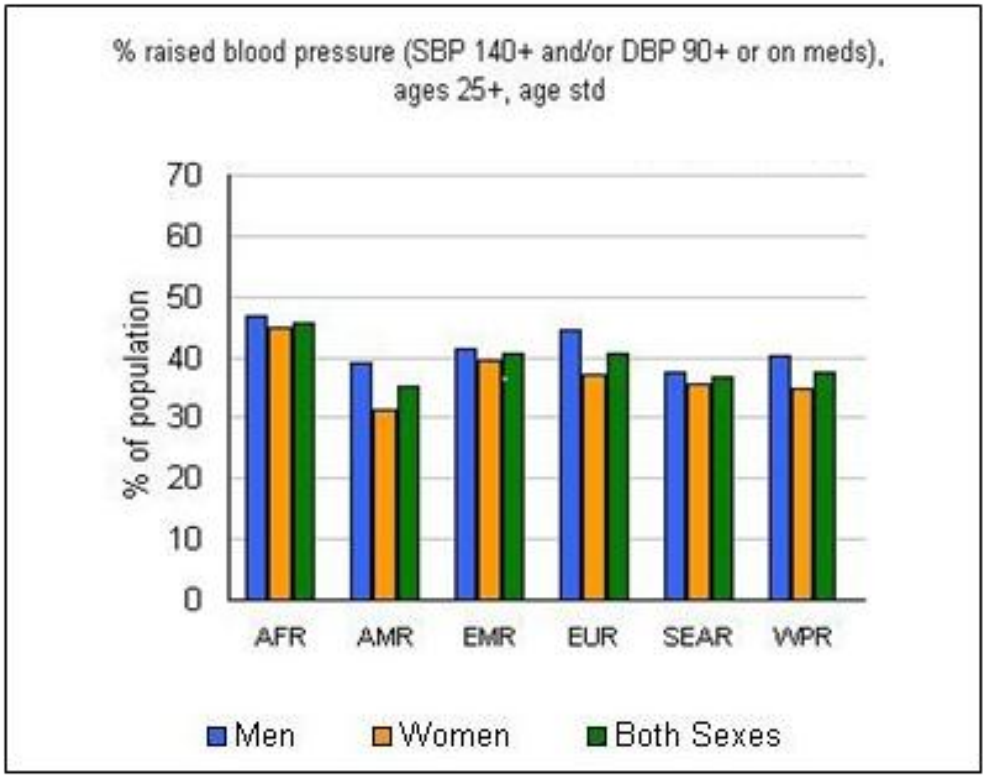


Figure 1.2: Prevalence of hypertension across WHO regions. Figure shows that hypertension is common in all regions, affecting at least 30% of the population in all regions and is more prevalent in the African region (AFR) and lowest in the region of the Americas (AMR). In all regions, hypertension is more common in men (World Health Organisation, 2012). EMR: Eastern-Mediterranean Region, EUR: European region, SEAR: South East Asia Region, WPR: Western Pacific Region.

According to the WHO, hypertension is more prevalent in Africa than in any other region (World Health Organisation, 2012) with a prevalence of 46% (Figure 1.2) (Adeyemo et al., 2009, Wolff and Miller, 2007). In contrast to African Americans (43% in men and 45.7% in women (Roger et al., 2012)), hypertension is slightly higher in men than that in women residing in Africa (majority are of African descent) (Figure 1.2). In South Africa, an estimated 42% of adults are hypertensive (Steyn et al., 2008). Like in many other countries, men have a higher prevalence of hypertension than women with 43.1% and 41.14% of adult men and women respectively, being hypertensive (Steyn et al., 2008). According to numerous studies conducted in different regions of the country, the prevalence of hypertension is highest in black South Africans (20-25%) and lowest in Indians (14.2-19%) (Connor et al., 2005, Seedat, 1999). It is still unclear why hypertension is more common in people of African descent. It has been suggested that the ethnic difference in the prevalence of hypertension are due to both genetic and environmental factors (Cooper et al., 1997).

1.2.3. Blood pressure regulation

The term blood pressure usually refers to the mean arterial BP, which is the product of the cardiac output and total peripheral resistance (Carretero and Oparil, 2000). BP regulation is quite complex and requires many local, humoral and neural factors to regulate cardiac output and total peripheral resistance (Figure 1.3) (Monahan, 2007, Sherwood, 2012). Changing any of the factors that determine the cardiac output or total peripheral resistance will change BP. It is important to regulate arterial BP because when the BP is too high, the heart is overworked and the risk for rupturing small blood vessels and vascular damage is increased (Sherwood, 2012) which results in CVD. Many of the determinants are regulated by other systems such as the baroreceptor reflex system and the angiotensin system.

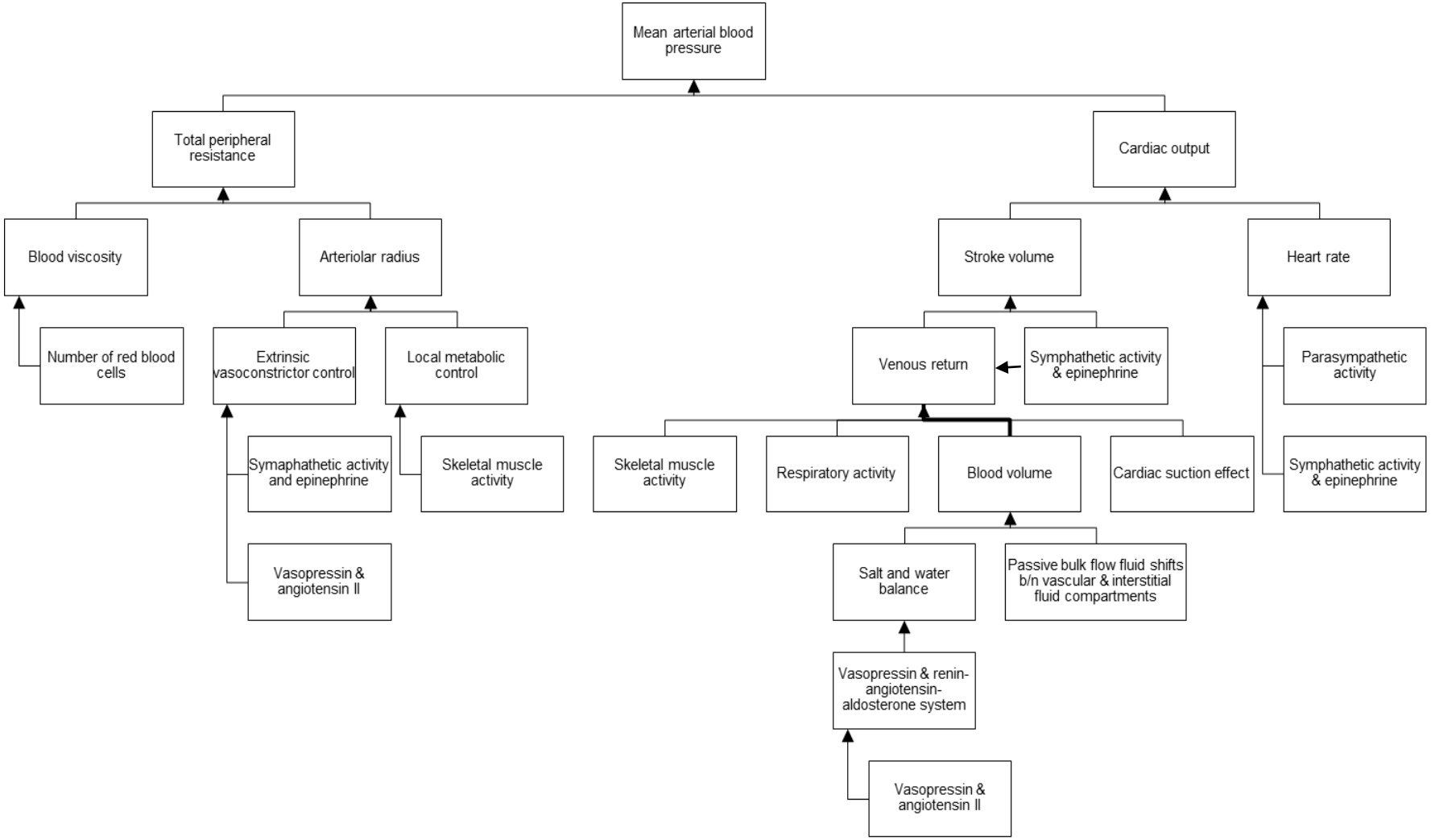


Figure 1.3: Determinants of mean arterial blood pressure in humans. Total peripheral resistance is determined by blood viscosity and arteriolar radius whereas cardiac output is determined by stroke volume and heart rate. These determinants are also controlled by other determinants such as vasopressin and angiotensin which regulates determinants of cardiac output and total peripheral resistance (Adapted from Sherwood, 2012).

1.2.3.1. The baroreceptor reflex

The baroreflex regulates short and long term BP through the autonomic nervous system (ANS) (involuntary system that controls heartbeat, digestion, respiration and other systems) which operates in a negative feedback manner to maintain circulatory homeostasis (Monahan, 2007). The ANS regulates BP by means of baroreceptors which are nerve endings that are located in many parts of the cardiovascular system such as the aorta and carotid arteries (Monahan, 2007, Stauss, 2002).

In short term BP regulation, the baroreceptors receive a stimulus in response to increased BP and they transmit a signal to the central nervous system (CNS) where the signals are integrated and sent to the ANS (parasympathetic and sympathetic nervous systems) (Monahan, 2007). The stimulation of the sympathetic nervous system (SNS) results in increased BP as shown in Figure 1.4 (Sherwood, 2012) hence its activation is decreased when the BP is too high. The activation of the parasympathetic system which results in BP reduction is increased in an effort to lower systemic BP (Monahan, 2007).

SNS activity can also be stimulated by a number of humoral factors such as angiotensin II and leptin, it is therefore essential to regulate these hormones to ensure the regulation of SNS activity (Albaghdadi, 2007, Yang and Barouch, 2007). Over stimulation of SNS activity can result in increased arterial BP which will lead to CVDs.

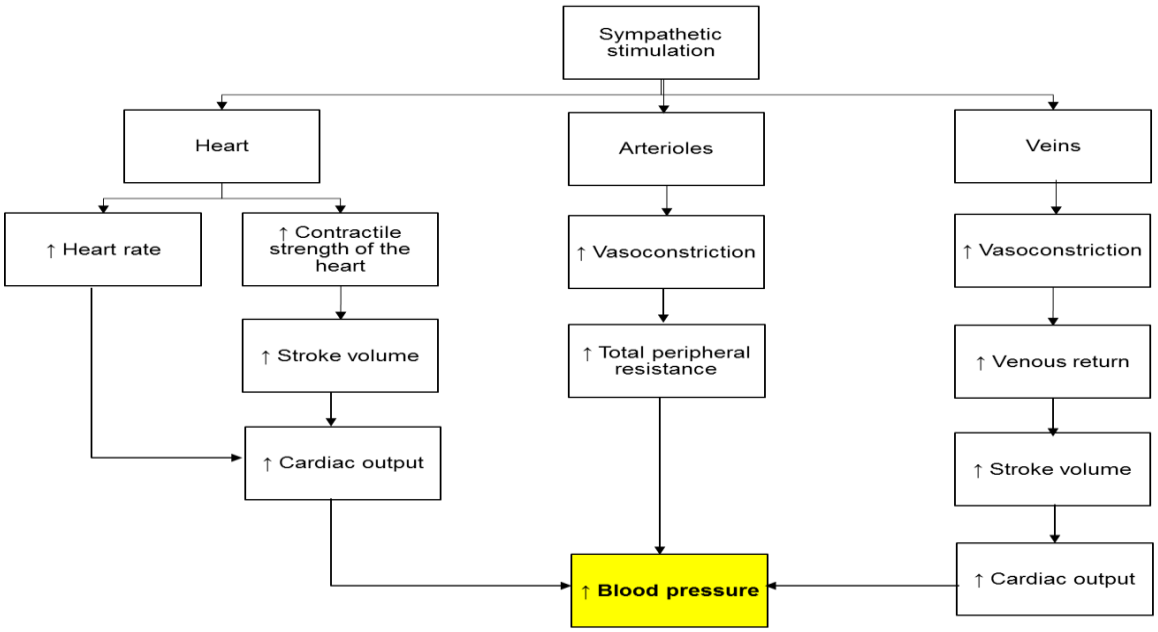


Figure 1.4: Blood pressure regulation through the sympathetic nervous system. Sympathetic stimulation affects the heart and veins and increases various blood pressure determinants to increase cardiac output. The arterioles are also affected as vasoconstriction results in increased peripheral resistance. An increase in cardiac output and total peripheral resistance results in increase blood pressure (Adapted from Sherwood, 2012).

1.2.3.2. The renin-angiotensin system

The renin-angiotensin system (Figure 1.5) is essential in the long term regulation of BP as it regulates arterial BP and electrolyte balance through the renal, cardiovascular and adrenal systems (Carey and Siragy, 2003). Angiotensinogen, which is coded for by the angiotensinogen *(AGT*) gene is essential in the cascade as it is the only known precursor for downstream angiotensin I. Mutations in the *AGT* gene have been shown to cause BP irregularities (Jeunemaitre et al., 1992). Angiotensinogen is mainly produced by the liver but it is also secreted by other organs such as the heart and adipose tissue (Carey and Siragy, 2003). Angiotensinogen is cleaved by renin (produced in the renal afferent arterioles in the kidney) to produce inactive angiotensin I, which is then hydrolysed by the angiotensin converting enzyme (ACE) to form active angiotensin II (Carey and Siragy, 2003, Fyhrquist and Saijonmaa, 2008).

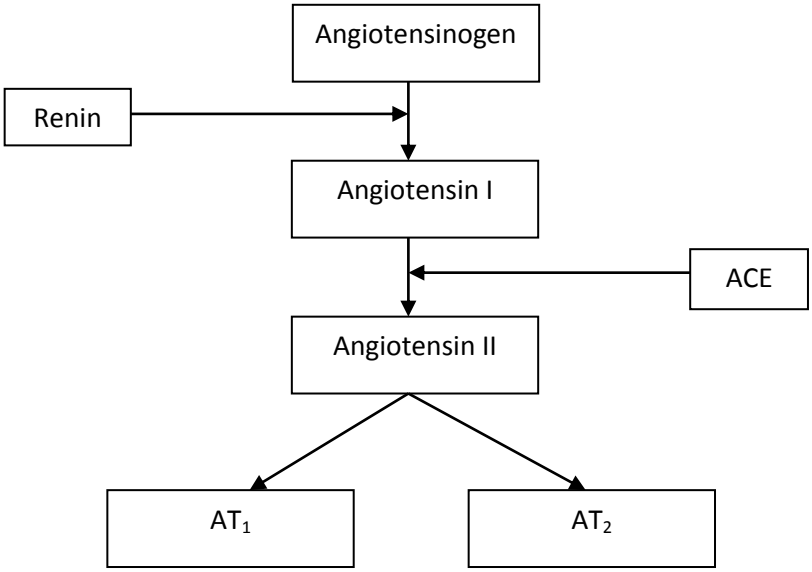


Figure 1.5: The renin-angiotensin system. Angiotensinogen is cleaved by rennin to produce angiotensin I. Angiotensin I is hydrolysed by ACE to form angiotensin II which has a high affinity for AT_1 and AT_2 receptors (Adapted from Carey and Siragy, 2003).

Angiotensin II carries out its function using angiotensin type 1 receptors (AT_1) that are located in renal, adrenal and cardiovascular tissue (Carey and Siragy, 2003). Angiotensin II, using the AT_1 receptors facilitates smooth muscle contraction, vasoconstriction, cell growth and dedifferentiation, tachycardia responses and inhibition of renin secretion (Carey and Siragy, 2003). Angiotensin also plays an important role in regulating intravascular potassium, sodium and water balance in the vascular system by regulating aldosterone, a steroid which promotes sodium and water re-absorption and potassium excretion (Maron and Leopold, 2010). Through the use of a second angiotensin type receptor 2 (AT_2), angiotensin also acts as a vasodilator, growth inhibitor and aids in the excretion of sodium (Carey and Siragy, 2003), essentially performing the opposite function to the AT_1 receptor. Since the final products of the renin angiotensin system are important in vasoconstriction and vasodilatation, it is critical that this pathway works optimally to ensure BP regulation.

1.2.4. Complications of hypertension

Hypertension is associated with increased risk for CVDs (for example, cerebrovascular ischemic heart disease, stroke and myocardial infarction), chronic renal disease and blindness (Chobanian et al., 2003, Minor et al., 2008). If not properly treated, hypertension can result in target organ damage and eventually, death (Nakayama and Yamamoto, 2009, Steyn, 2006). Understanding hypertension and its role in CVDs is critical because of the high mortality attributable to CVDs throughout the world.

The risk for a cardiovascular event increases with the number of risk factors and these can be gender or age specific and family history and life style preferences are important. These risk factors include tobacco use, age and gender (men older than 55 and women older than 65), high cholesterol levels, obesity, physical inactivity and history of a cardiovascular disease in a first degree relative before the age of 50 (Kaplan et al., 2003). The co-existence of hypertension with these other risk factors increases the risk for CVDs and the increased risk is important in deciding how to treat hypertension (Kaplan et al., 2003). Table 1.4 shows the risk of cardiac event in 10 years, based on hypertension co-existing with other risk factors. Many CVD risk factors such as obesity and hypertension are modifiable. It is important to treat BP and keep levels below the pathogenic levels because BP increases the risk of CVD which results in increased morbidity and mortality.

Table 1.4: Risk of a major cardiac event due to hypertension and other risk factors in 10 years

Category	Systolic BP (mm Hg)	Diastolic BP (mm Hg)	CVD risk	Risk factors*
Stage 1	140-159	90-99	Low (<15%)	0
Stage 2	160-179	100-109	Medium (15-20%)	0-2
Stage 3	≥180	≥110	High (>20%)	≥3

*Risk factors; target organ damage or associated conditions (Adapted from Kaplan et al., 2003).

1.2.5. Causes of hypertension

Hypertension often co-exists with other NCDs such as diabetes mellitus and obesity (Steyn, 2006) and is usually a complication of another NCD hence the co-occurrence. Like obesity, hypertension is a complex and heterogeneous disease that is contributed to by age (Carretero and Oparil, 2000), environmental and genetic factors.

High alcohol intake, tobacco usage, poor diet, stress and lack of physical activity are among the leading lifestyle and environmental factors that increase BP and each of these factors has an additive effect on the increase in BP (Carretero and Oparil, 2000). Diet plays a crucial role in BP regulation as many nutrients such as potassium and sodium need to be kept at optimal levels. Salt sensitivity which has been defined as BP that varies directly with salt intake, is more common in individuals of African descent (Fujita et al., 1980, Morris et al., 1999). Sodium which is one of two elements that make salt is a major dietary risk factor for hypertension and is the key factor in salt sensitivity. Sodium has an important role in the regulation of extracellular fluid volumes which can influence BP fluctuations (Mohan and Campbell, 2009). Elevated sodium levels result in elevated extracellular fluid which results in plasma volume increases leading to raised BP (Mohan and Campbell, 2009). Although sodium levels are important in salt sensitivity, potassium levels also play an important role because salt sensitivity is observed in conjunction with reduced potassium levels or potassium deficiency (Morris et al., 1999). Low levels of potassium and high levels of sodium result in impaired BP regulation causing hypertension.

Migration from the rural areas to urban areas is associated with a change in diet and reduced physical activity (Puoane et al., 2002). A study in Kenyans by Poulter et al. (1990) showed that individuals who migrated from the rural areas to urban areas (Nairobi) had higher pulse rates, BP, BMI and urinary sodium:potassium ratios compared to those in the rural areas (Poulter et al.,

1990 as cited by Steyn, 2006). A move from the rural to urban areas often leads to a change in diet from one that is high in fruit and vegetables therefore high in potassium and calcium, low in salt (low sodium) and low in calories. In urban areas, the new diet consists of little or no fruit and vegetables, low potassium intake, increased salt/sodium intake and high in calories coupled with low physical activity, hence an increase in BMI (Poulter et al., 1990 as cited by Steyn, 2006). All these factors lead to impaired BP regulation, resulting in hypertension.

Molecular and cellular pathways are very important in regulating BP. Defects in salt regulation by the kidneys, abnormalities in the activity of vasoactive chemicals (for example, endothelin and nitric oxide), gene mutations (eg. angiotensinogen mutations) and defects in plasma membrane pumps such as the sodium-potassium (Na^+-K^+) pumps can all lead to hypertension (Sherwood, 2012). People with hypertension tend to be overweight/obese and diabetic. Therefore understanding pathways involved in obesity and diabetes will aid in understanding of the pathophysiology of hypertension. Genetics are also thought to have an important role in hypertension and although pathways involved in BP regulation are well defined, the genetic contribution to hypertension is not well understood.

1.3. Relationship between obesity and hypertension

Adipose tissue has been implicated in both obesity and hypertension. Excess adipose tissue is directly associated with obesity and secretes components important in appetite regulation such as leptin. In relation to hypertension, adipocytes also secrete components required in BP regulation such as angiotensinogen (AGT). In obesity, fat mass is increased and this may result in increased production of AGT which may lead to elevated BP (Ailhaud, 2006).

Obesity and hypertension are multifactorial traits, with the major contributors to disease development being environmental factors and genetic factors (Onions et al., 1998, Puoane et al., 2002). Hypertension is twice as common in obese individuals compared to non-obese individuals (Kotchen, 2008). Twin studies have shown a 15-31% concordance for joint occurrence of obesity and hypertension (Carmelli et al., 1994). Since these traits are related, this suggests that there may be common environmental and genetic susceptibility factors (Onions et al., 1998). Some genetic factors that play an important role in determining BMI and BP have been identified, but it is not well understood how genetics influences these traits concurrently (Shintani et al., 2002).

1.4. The Genetics of obesity and hypertension

Obesity and hypertension have been shown to be heritable, supporting the idea that the pathogenicity of obesity and hypertension is not only due to lifestyle or environmental factors, but is also attributable to genetics. Familial occurrence of obesity and the concordance amongst twins has shown that obesity has a heritable component thus implying that there are genetic factors driving obesity. Twin studies show that monozygotic twins have 70-90% concordance for obesity compared to 35-45% in dizygotic twins (Stunkard et al., 1986 as cited by Grant et al., 2008).

BMI has 50-84% heritability (Frayling et al., 2007, Stunkard et al., 1986 as cited by Grant et al., 2008) and an estimated 60-81% in variation in total body weight, not related to height, is due to heritability or a genetic component (Allison et al., 1994, Stunkard et al., 1986 as cited by Grant et al., 2008). WC has a heritability of 41-72% (Fox et al., 2004, Rose et al., 1998) and WHR has 48% heritability in white women (Rose et al., 1998). Systolic BP has a heritability of 45% in Nigerians, 68% in African-Americans and 43% in Caucasian Americans (Gu et al., 1998, Rotimi et al., 1999).

Diastolic BP has a heritability of 43% in Nigerians and 56% in African-Americans and only 24% in Caucasian Americans (Gu et al., 1998, Rotimi et al., 1999).

1.4.1. Genes associated with obesity and hypertension

Multiple genes identified in genome wide association studies (GWAS) are associated with obesity, hypertension and both disorders concurrently. Most of these studies have been done in populations of European descent and very little is known about which genes are associated with obesity and hypertension in people of African descent. To date, many genes associated with either obesity or hypertension have been identified using association studies. These association studies provide insight into which genes and pathways may be involved in disease pathogenesis and provide candidate genes that can be interrogated in detail.

The *AGT* and *ACE* genes which code for the angiotensin and angiotensin converting enzymes, respectively (Carey and Siragy, 2003, Fyhrquist and Saijonmaa, 2008) are involved in the renin angiotensin system which is important in BP regulation. The *AGT* gene was among the first genes to be associated with hypertension (Jeunemaitre et al., 1992).

As expected, an overlap occurs between genes associated with obesity and genes associated with hypertension. The *Fat Mass and Obesity associated* (*FTO*) and *Melanocortin receptor 4* (*MC4R*) genes were amongst the first genes to be associated with human obesity. *FTO*, like many of the other obesity associated genes is strongly associated with weight and fat mass, rather than height (Willer et al., 2009). Both the *FTO* and *MC4R* genes have also been associated with hypertension (Greenfield, 2011, Melka et al., 2012).

Many genes that have been associated with obesity or hypertension are involved in multiple pathways and therefore affect multiple phenotypes (pleiotropy; mutation or gene that influences multiple phenotypes or traits (de Visser et al., 2011, Tyler et al., 2009)). Many of the genes associated with obesity are genes that are involved in neural development, such as the *BDNF* gene which is involved in neural development and has also been associated with some psychiatric disorders (Thorleifsson et al., 2009). The leptin receptor encoded by the *LEPR* gene is primarily expressed in the hypothalamus and receives signals from leptin (coded for by the *LEP* gene) to signal satiety (Ailhaud, 2006). Both genes have been implicated in obesity and BP (Ben Ali et al., 2008, Ma et al., 2009, Shintani et al., 2002). Genes such as *SH2B1* are involved in the leptin signalling pathway (Willer et al., 2009) and can therefore also be considered to have an indirect role in obesity and hypertension.

1.4.2. Leptin and its receptor as candidate genes for obesity and hypertension

1.4.2.1. Leptin

The word leptin is derived from the Greek word, "leptos" which means thin (Hegyi et al., 2004). The hormone was named based on its anti-obesity properties (Hegyi et al., 2004) attained by acting as a satiety signal in the CNS (Iglesias et al., 2006). Leptin is a non-glycosylated, 167 amino acid cytokine that has 4 helices (characteristic of cytokines) (Ailhaud, 2006, Hegyi et al., 2004, Zhang et al., 1994) . It circulates in the blood stream and is mainly produced by adipocytes (Hegyi et al., 2004, Paracchini et al., 2005). Leptin is encoded by the leptin gene (*LEP*), formerly known as the *OB* gene. The *LEP* gene was first identified in humans and animals by Zhang et al. (1994) using positional cloning. *LEP* is a three exon gene that is located at chromosome 7q31.3 (http://ghr.nlm.nih.gov/gene). The mouse *Lep* gene encodes the Lep protein which is predicted to have 84% homology with the human *LEP* amino acid sequence (Zhang et al., 1994).

1.4.2.2. The leptin receptor

Leptin acts through the leptin receptor by binding to the leptin receptors to send an intracellular signal (Hegyi et al., 2004). The *LEPR* or *OB-R* gene is a 20 exon gene located on chromosome 1p31 and shares a promoter and the first two exons with an overlapping gene, *LEPROT* (*Leptin receptor overlapping transcript*) (Jeon et al., 2010). *LEPR* is alternatively spliced and codes for eight transcripts, of which six are protein coding transcripts (http://www.ensembl.org). The leptin receptor is expressed in different organs including the liver, kidney, heart, adrenal gland and skeletal muscle, with the longest isoform being expressed mainly in the hypothalamus (Paracchini et al., 2005). Leptin receptors have also been located in the part of the hypothalamus that is involved in cardiovascular control, suggesting that leptin could affect BP regulation through the CNS (Stenvinkel, 2000).

1.4.2.3. Function and action of leptin and its receptor

Leptin, along with many other hormones and neurotransmitters, is involved in energy balance (intake and expenditure) (Ailhaud, 2006, Hegyi et al., 2004). It does this by sending a signal from the peripheral system to the central nervous system (CNS). Leptin is transported from the adipose tissue to the hypothalamus through the bloodstream (Paracchini et al., 2005). In the CNS (hypothalamus), leptin inhibits appetite by suppressing orexigenic neuropeptides such as the melanin-concentrating hormone (MCH) and neuropeptide Y (NPY) while up-regulating anorexigenic neuropeptides such as the melanocortin-4 receptors (MC4R) and proopiomelanocortin (POMC) (Paracchini et al., 2005). Although leptin is the central hormone in the adiposity sensing pathway (Gaukrodger et al., 2005) and its main function is to regulate energy balance, due to the diversity and multiple expression sites of the leptin receptor, leptin is pleiotropic. Based on the location of a leptin receptor, leptin can affect multiple pathways and

phenotypes including insulin and glucose metabolism, BP, adiposity, fertility and reproduction and immune response (Hegyi et al., 2004, Paracchini et al., 2005, Iglesias et al., 2006).

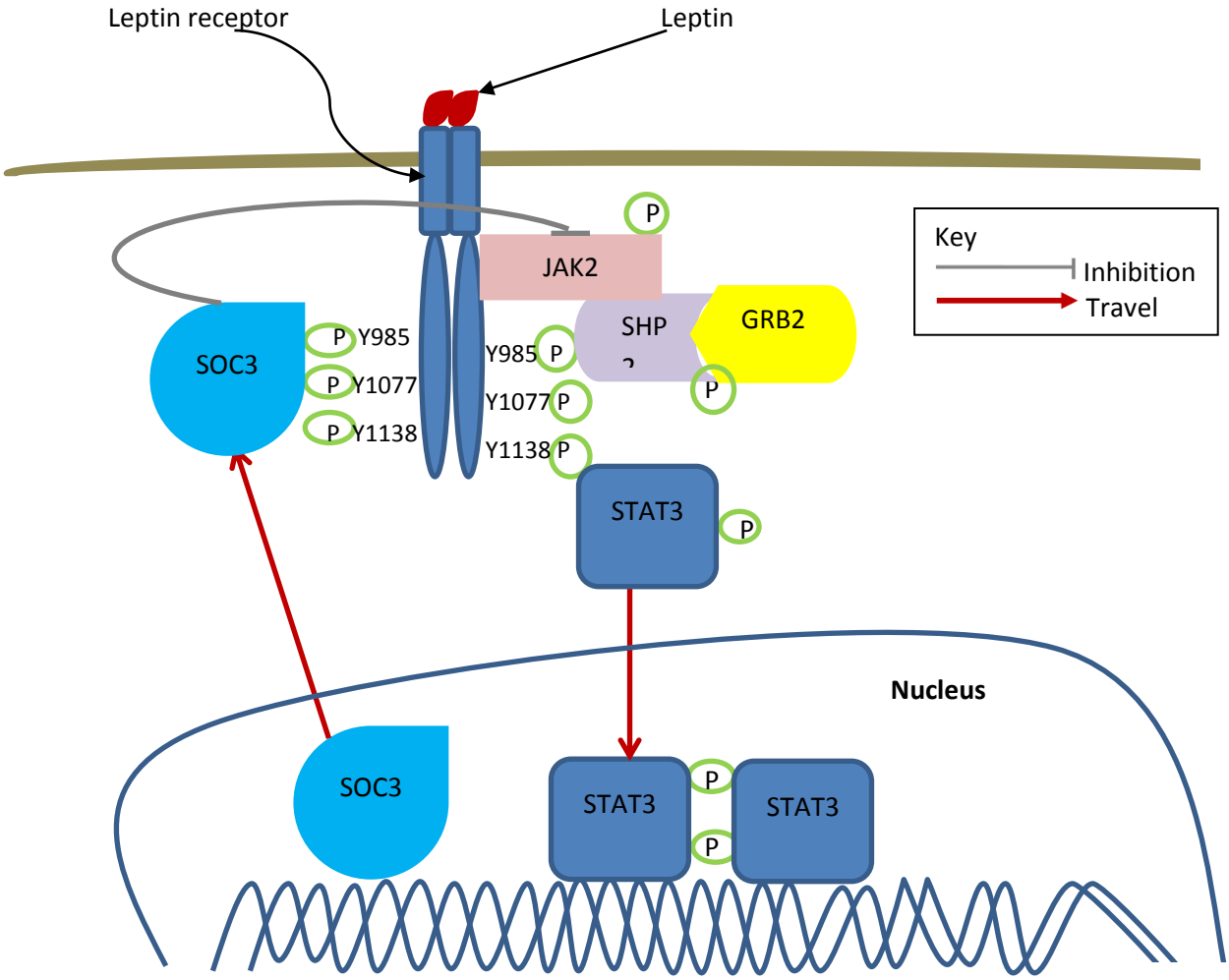


Figure 1.6: Leptin signalling using the JAK/STAT pathway. When leptin binds to the leptin receptor, the receptor recruits JAK to form a phosphorylated leptin receptor/JAK complex. The complex then recruits STAT3 and phosphorylates it. Phosphorylated STAT3 migrates to the nucleus to activate transcription (Adapted from Yang and Barouch, 2007).

Leptin carries out its function by binding to the leptin receptor and activating various pathways such as the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) or JAK/STAT pathway to signal satiety and decrease food intake while increasing energy expenditure (Hegyi et al., 2004, Paracchini et al., 2005). Leptin also utilises the Mitogen-activated protein kinase (MAPK) pathway but leptin uses the JAK/STAT pathway as a primary signal transduction pathway (Hegyi et

al., 2004). Leptin sends a signal by binding to leptin receptors (*LEPR*) extracellularly (Figure 1.6). The binding of leptin results in the initiation of *LEPR* homo-oligomer biosynthesis to create oligomers, which are composed of a small number of identical monomers which then binds to JAK to form a LEPR/JAK complex. Once the homo-oligomerised leptin receptor binds to JAK (primarily JAK2), JAK is auto-phosphorylated and the Tyr985, Tyr1077 and Tyr1138 amino acids of the leptin receptor are also phosphorylated (Yang and Barouch, 2007). The phosphorylated Try1138 recruits STAT3 (only STAT stimulated by leptin in the hypothalamus) (Hegyi et al., 2004). The phosphorylated STAT3 dimerises and moves to the nucleus to activate transcription of genes involved in energy and appetite regulation and those that inhibit the leptin signalling pathway and leads to negative feedback on leptin signalling, such as the *SOC3* gene (Yang and Barouch, 2007).

1.4.2.4. Leptin, the leptin receptor and their association with obesity and hypertension

1.4.2.4.1. Leptin levels and leptin resistance

Elevated leptin levels are associated with elevated fat mass, WC and BP (Ailhaud, 2006, Barba et al., 2003). Obese and hypertensive individuals have higher leptin levels (hyperleptinemina) compared to normotensive or normal weight individuals (Ailhaud, 2006, Malmqvist et al., 2002, Martin et al., 2008). Since leptin's role is to regulate energy homeostasis by reducing energy intake and increasing expenditure, one would expect higher leptin levels to result in less fat mass. As observed in many obese individuals, higher leptin levels do not protect against weight gain. This is due to leptin resistance. Leptin resistance may result from defects in the leptin receptor, leptin regulating proteins and leptin signalling pathway. In addition, elevated leptin levels result in some parts of the body being desensitised to leptin (Martin et al., 2008).

Selective resistance has also been observed, where even with elevated leptin levels, leptin is still able to perform some of its functions in a spatial specific manner. When leptin is continuously over expressed, it is unable to perform its functions in the hypothalamus and therefore cannot regulate appetite, but it is still able to activate the sympathetic nervous system (SNS) (Yang and Barouch, 2007). When the SNS is activated long term or persistently, BP will also be elevated persistently, therefore increased levels of leptin will result in more activation of the SNS thereby resulting in hypertension (Kotsis et al., 2010).

In murine studies, leptin was shown to have a direct effect on BP. The direct effect of leptin in hypertension pathogenesis was demonstrated in a study by Aizawa-Abe and colleagues (2000) where transgenic lean mice that were overexpressing leptin had elevated BP, demonstrating high BP which was independent of obesity (Aizawa-Abe et al., 2000). In another study by Shek et al. (1998), rats that had chronically elevated leptin levels due to chronic leptin infusions showed a sustained elevation in BP (Shek et al., 1998). All this evidence from murine and human studies suggests that leptin levels are associated with disease pathogenesis in both obesity and hypertension.

1.4.2.4.2. Leptin and leptin receptor deficiency

Leptin deficiency is a rare monogenic, autosomal recessive disorder that is caused by mutations on both copies of the leptin gene, which has been shown to result in early onset morbid obesity and hyperphagia in humans (Gibson et al., 2004) and mice. Leptin deficiency was first characterised in ob/ob mice that were characterised by morbid obesity and type II diabetes (Ingalis and Snell, 1950 as cited by Zhang et al., 1994). Leptin deficiency in humans was first reported in two children (cousins) of Pakistani origin from a consanguineous family (Montague et al., 1997). These children were found to have very low serum leptin levels and both were

homozygous for a frameshift mutation caused by a deletion of a guanine at codon 133 of the *LEP* gene (Montague et al., 1997). Leptin replacement therapy has been shown to be an effective treatment for early onset obesity in leptin deficient individuals (Gibson et al., 2004).

Leptin can only perform its function if functional leptin receptors are present. Mutations in the *LEPR* gene show epistasis (the interaction between genes or variants that results in one gene masking the effects of another gene to affect phenotype (Cordell, 2002) to *LEP* mutations. *LEPR* deficiency shows similar phenotypes to *LEP* deficiency. In murine studies, Kowalski and colleagues (2001) found that the absence of the long isoform of the leptin receptor (expressed in the hypothalamus) resulted in obesity and diabetes (Kowalski et al., 2001). Leptin receptor deficiency has also been described in humans. In an individual from a family with a family history of morbid obesity, a homozygous mutation (splice donor site G, substituted by A) in exon 16 of *LEPR*, which causes exon 16 to be spliced out, was identified (Clément et al., 1998). The substitution results in a shorter receptor that lacks the transmembrane and intracellular domains and is therefore unable to perform its function (Clément et al., 1998). Individuals with this mutation tend to over eat, have early onset morbid obesity, absence of pubertal stage and short stature because they secrete low levels of IGF-I and IGF-binding protein 3 (IGF-BP3) (Clément et al., 1998).

1.4.2.4.3. Variation in the *LEP* and *LEPR* genes

Leptin and its receptor have been implicated in monogenic forms of morbid obesity and have also been shown to have a role in multifactorial obesity and hypertension. Several studies in different populations have shown that *LEP* and *LEPR* variants are associated with multifactorial forms of obesity and/or hypertension whereas some studies have shown that variants in these genes are not associated with either phenotype. Ethnic and gender specific differences have been noted for single nucleotide polymorphisms (SNPs) within the *LEP* and *LEPR* genes.

The *LEP* G2548A (rs7799039) SNP was associated with obesity in Taiwanese Aborigines (Wang et al., 2006), but not in Romanians (Constantin et al., 2010). rs7799039 was also found to be associated with plasma leptin levels in obese, Tunisian patients (Ben Ali et al., 2009b). The same SNP was associated with obesity or hypertension in a gender specific manner in some populations. rs7799039 was associated with hypertension in male and female Tunisians (Ben Ali et al., 2008) whereas an association between rs7799039 and hypertension was only found in American women but not in men (Ma et al., 2009). In a study done by Gaukrodge et al. (2005), they found that rs7799039, along with rs2060713 and other SNPs were not associated with measures of hypertension. In the same study, they found that for *LEP* C538T, which is located in the 3'untranslated region (3'UTR), the minor allele, T, was protective against hypertension and was associated with low pulse pressure and systolic BP (Gaukrodge et al., 2005). This SNP is very rare in the general Caucasian population (5%) and has a large effect in people who carry the T allele (20% lower systolic BP per allele) (Gaukrodge et al., 2005).

Several studies have also been done in South Africa to determine the role of leptin in obesity and hypertension. In a South African study, *LEP* SNPs rs10954174 and rs6966536 were found to be associated with BMI in black adolescents living in Soweto (Lombard et al., 2012). A haplotype constructed from *LEP* rs2167270, rs111650308 and rs17151919 was associated with hip circumference, BMI and mid-upper arm circumference (MUAC) in a small cohort of black and mixed ancestry adolescents living in the Western Cape (Yako et al., 2011).

LEPR rs1137101 was associated with obesity in Pacific Islanders (Furusawa et al., 2010) but not in Romanians (Constantin et al., 2010). The minor allele of the same SNP is associated with reduced leptin levels and low BMI in Tunisian women (Ben Ali et al., 2009). rs1137101, along with rs1137100, rs1805096 and 1805134 were associated with serum leptin levels and increased risk

for cardiovascular disease in obesity in Japanese children (Okada et al., 2010). *LEPR* rs1137101 and rs1137100 were not associated with BMI in South African black adolescents living in Johannesburg (Lombard et al., 2012), but rs1137100 was associated with BMI, WC and MUAC in black and mixed ancestry adolescents living in the Western Cape (Yako et al., 2011).

1.5. Problem statement

The role of leptin and its receptor in appetite regulation, the relationship between leptin levels and BP and the fact that SNPs within these genes have been shown to be associated with obesity and hypertension in other populations make the *LEP* and *LEPR* genes compelling candidate genes for obesity and hypertension. Recent evidence that *LEP* SNPs are associated with BMI and WC in South Africans (Lombard et al., 2012, Yako et al., 2011), merit the further exploration the role of *LEP* and *LEPR* SNPs in black South Africans.

1.6. Aims and Objectives

The aims of this study were to determine the heritability estimates of anthropometric and blood pressure (BP) measures and leptin levels; to identify additional informative SNPs in and around the *LEP* and *LEPR* genes; and to examine the potential relationships between these SNPs and measures of obesity, hypertension and leptin levels in a black South African cohort.

Objectives

- Select participants from the African Programme on Genes in Hypertension (APOGH) cohort with relevant anthropometric and BP data
- Determine heritability estimates for anthropometric measures, BP and leptin levels
- Select tag SNPs that capture the *LEP* and *LEPR* genes using 1000 Genomes data, HapMap data and published data

- Genotype DNA from participants for the selected SNPs using the BeadXpress platform
- Determine whether *LEP* and *LEPR* SNPs are associated with anthropometric measures, BP and leptin levels

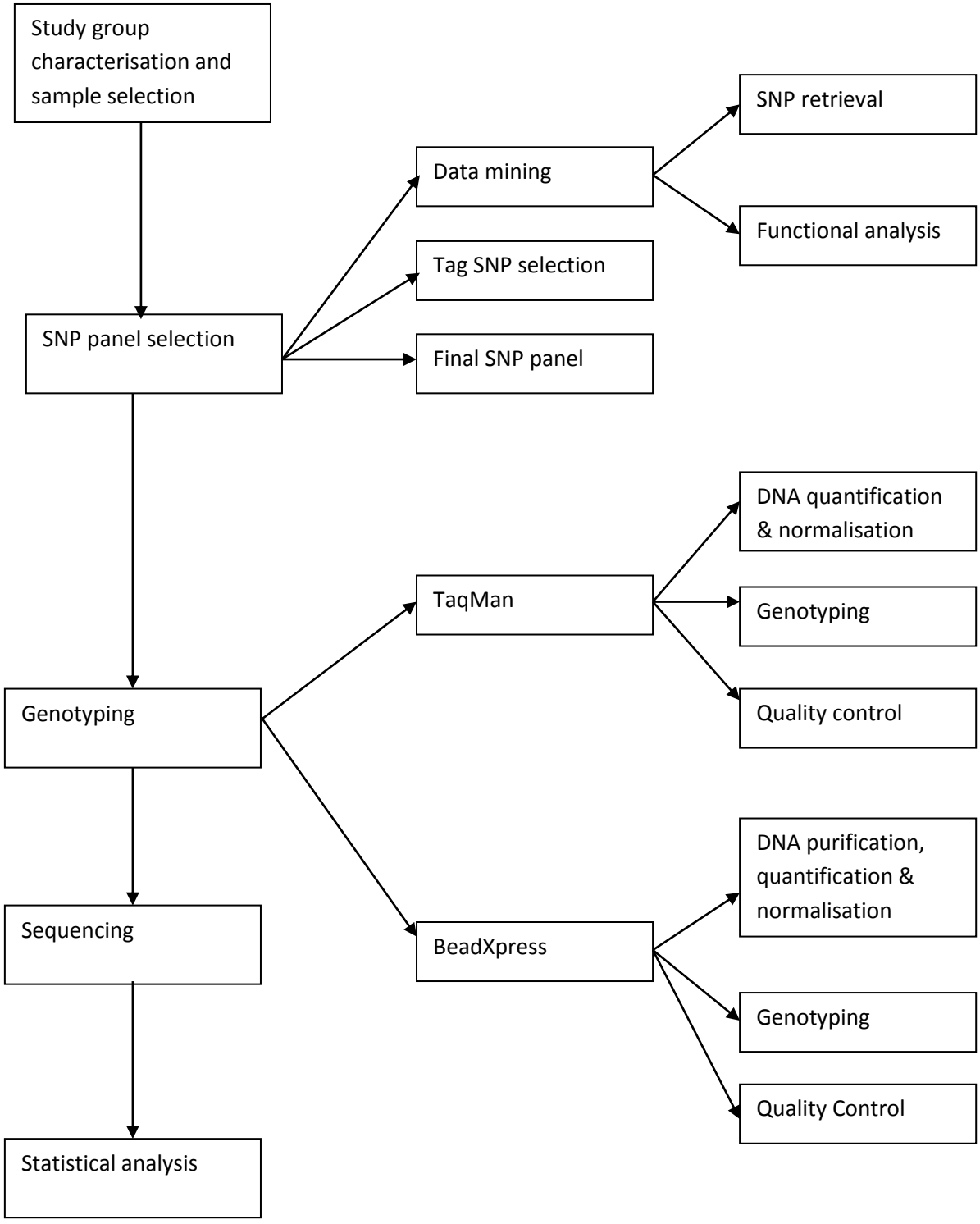


Figure 2.1: Schematic outline of methods carried out in the project.

2.1. Subjects

DNA samples from participants of the African Programme on Genes in Hypertension (APOGH) cohort were used for this study (Majane et al., 2007, Shiburi et al., 2006). The APOGH study was approved by the Human Research Ethics Committee of the University of the Witwatersrand (HREC) (approval numbers: M02-04-72 renewed as M07-04-69 and M12-04-108, Profs Norton and Woodiwiss as principal investigators). Permission was granted by the HREC to use APOGH DNA samples and data in this study (approval numbers: M110464, Appendix A).

The APOGH cohort comprises a random sample of nuclear families of black South African ancestry, from the Nguni and Sotho chiefdoms. All participants were residing in the metropolitan area of Johannesburg at the time of data and sample collection. The nuclear families consist of one or two offspring that were at least 16 years old and one or both parents. APOGH records were examined and 1143 participants for whom DNA samples and the relevant clinical data for this study were available (discussed in section 2.2.1) were identified. Of the 1143 samples, only 927 were genotyped and 481 of these 927 had leptin levels.

2.2. Methods

2.2.1. DNA and phenotypic data collection

All data and samples were available at the time the study started and all collections were conducted prior to the study as a part of the APOGH program by the School of Physiology at the University of the Witwatersrand (Wits) Medical School. All participants filled in a standardised questionnaire that required demographic data, medical history, names of medication taken and smoking and drinking habits. Blood samples and phenotype data were collected by a qualified nurse. DNA from these blood samples was extracted using the Rapid Method (Higuchi, 1989) (Appendix B) and stored at -20°C. Leptin levels were determined from blood plasma by ELISA.

2.2.1.1. Clinical, demographic, anthropometric and blood pressure data collection

Height, weight, waist circumference (WC), hip circumference (HC), waist-to-hip ratio (WHR) and skinfold thickness were used as anthropometric measurements to determine the presence/absence of obesity. Body mass index (BMI) was calculated by measuring the weight and height of the participants then dividing the weight (kg) by the height (m^2). Height and weight were measured in standing position, with no shoes, in light dress (Majane et al., 2007, Shiburi et al., 2006) using a stadiometer and an electronic scale. Skinfold thickness was determined by measuring triceps and subscapular skinfold thickness using a skinfold calliper. The means of the triceps and subscapular skinfold thickness values were reported as skinfold thickness (Majane et al., 2007). WC and HC were measured at standing position, to the nearest 0.5 cm using a soft measuring tape. WC was measured at the smallest girth above the umbilicus and HC was measured at the widest part of the gluteal region (Crowther and Norris, 2012). WHR was then calculated using waist and hip circumference values.

To determine the hypertensive state of the participants, brachial artery and aortic (central) BP measurements were collected. Brachial systolic and diastolic BP were measured using auscultation five times (average of five measurements taken as final value), from the left arm, in the sitting position, to the nearest 2 mm Hg (Majane et al., 2007; Shiburi et al., 2006). Standard cuffs with an inflatable bladder (22 cm length by 12 cm width or 31 by 15 cm for larger cuffs) were used to determine systolic and diastolic BP. Aortic BP was derived from radial BP. The radial waveform was measured using an applanation tonometry and a SPC micromanometer. SphygmoCor software was used to calculate aortic BP from the radial waveform signal (Shiburi et al., 2006).

2.2.2. SNP panel selection

Genotype data from the International HapMap Project (The International HapMap Consortium, 2003) and 1000 Genomes Project (The 1000 Genomes Project Consortium, 2012) were used for SNP selection. Two African populations from HapMap project, the Luhya in Webuye of Kenya (LWK) and the Yoruba in Ibadan of Nigeria (YRI) were genotyped by both the International HapMap Project and the 1000 Genomes Project. These two populations were used as proxies for the South African, black population. YRI is often used as a proxy for African populations, but the LWK was also included as a proxy population for South Africans because the LWK has the highest correlation of allele frequencies with YRI (Joubert et al., 2010). The Maasai in Kinyawa, of Kenya (MSS), the third African population genotyped by the International HapMap Project was excluded as a proxy population because it was not included in the 1000 Genomes Project.

The genomic locations of the *LEP* and *LEPR* genes were retrieved for the human genome build 19 (hg19)/GRCh37) using Ensembl version 62 (http://www.ensembl.org/index.html). The coordinates from the hg18/GCRh36 build were retrieved from HapMap using the HapMap Genome Browser release #27 (Phase 1, 2 & 3 - merged genotypes & frequencies). The NM_002303 processed_transcipt: UCSC_1 (NM_002303) leptin receptor isoform 1 [*LEPR*] was used to retrieve hg18 coordinates for *LEPR* as this was the largest isoform (http://hapmap.ncbi.nlm.nih.gov/cgi-perl/gbrowse/hapmap27_B36/). Coordinates for both builds are shown in Table 2.1.

Table 2.1: Genome coordinates for *LEP* and *LEPR* in the two most recent genome assemblies[1]

Gene	Chromosome	Hg19 (GCRh37)		Hg18 (GCRh36)	
		Start	End	Start	End
LEP	7	127881337	127897681	127668567	127684917
LEPR	1	65886248	66107242	65658906	65875408

[1]The two most recent genome builds are Hg18 (GCRh36) and Hg19 (GCRh37).

2.2.2.1. SNP mining

A subset of SNPs that was interrogated in this study were selected from published data and have been shown to be associated with either obesity or hypertension or related phenotypes/disorders. These SNPs will be referred to as literature SNPs. Functional analysis was done to identify synonymous and non-synonymous SNPs and the possible deleterious effects of non-synonymous SNPs within the list of SNPs identified from the VarioWatch search (discussed below). These SNPs were subjected to SIFT (http://sift.jcvi.org/) and PolyPhen (http://genetics.bwh.harvard.edu/pph/) analysis to assess potential functional impact. In the SIFT query, the "SIFT dbSNP rs IDs" option was used to input the list of SNPs from the Engines query. In the PolyPhen query, the SNPs were queried using the "Batch query" option, using the HumVar classifier model, the GRCh37/hg19 genome assembly and all transcripts and annotations options. Literature SNPs and potentially functional SNPs are listed in Appendix C.

VarioWatch and SNPs-for-population-Studies-mart (SPSmart) interface Engines tool were used to identify known SNPs that have been identified in the *LEP* and *LEPR* genes. VarioWatch (http://genepipe.ncgm.sinica.edu.tw/variowatch/main.do) is a tool that can be used to retrieve all known genomic variants with a detailed description of each variant. Variant descriptions include the gene and transcript name, transcript start and end sites, SNP ID and position, variant risk type and risk level, cDNA change and protein change and variation type (SNP or mutation). The

physical positions (hg19) of each gene, with 1000 bp upstream and downstream regions flanking each gene were used to query VarioWatch for all known variants in and around each gene.

The SPSmart Engines tool (Amigo et al., 2011) is a pre-processing engine that summarises information from genotype databases into different population genetics indices such as allele frequencies. The SPSmart Engines tool was queried using *LEP* and *LEPR* SNPs (only variants with reference sequence (rs) numbers) that resulted from the VarioWatch query by pasting all SNPs in the "Search by SNPs" option. The LWK and YRI populations were used as proxies for the South African population. HapMap Release #28 and 1000 Genomes Phase I data were used to compare population groups and determine minor allele frequencies (MAF) for each SNP in the proxy populations (LWK and YRI). The SNP list was then filtered by only selecting SNPs that were present in both the LWK and YRI populations. SNPs that had a MAF of 0.05 or more in both proxy populations (LWK and YRI) were kept and a minimum MAF of 0.02 in either the LWK or YRI population was used as a cut-off for literature SNPs and potentially functional SNPs.

2.2.2.2. Tag SNP Selection

Genotype data for SNPs compatible with the GoldenGate® Genotyping assay were retrieved using 1000 Genomes and HapMap data for LWK and YRI. 1000 Genomes family and genotype data were retrieved by a bioinformatician. HapMap family data were retrieved from the HapMap website using "Bulk data download" and genotype data were retrieved from HapMap Genome Browser release #27 (Phase 1, 2 & 3 merged genotypes & frequencies) using the genotype frequencies option. 1000 Genomes data were merged with HapMap data using Microsoft Excel 2010.

Tag SNPs are SNPs that are able to act as tags for other SNPs in a specific region (Montpetit et al., 2006). The Tagger algorithm under "Linkage format" on HaploView 4.2 was used to select tag SNPs that represent the *LEP* and *LEPR* genes (Barrett et al., 2005, de Bakker et al., 2005). Pairwise tagger, r^2 threshold of 0.8 and LOD threshold of 0 were used for tag SNP selection.

Force exclude and force include files were created for both genes to prioritise or exclude SNPs as tag SNPs (Appendix C). Force include files included SNPs that Tagger algorithm forces to be chosen as tag SNPs whereas force exclude SNPs are SNPs that are prohibited by the Tagger algorithm from being chosen as tag SNPs. All potentially functional and literature SNPs from the *LEP* and *LEPR* genes were included in the force include file for the respective gene, given that they had a MAF of 0.02 or more in at least one proxy population.

Selected tag SNPs were analysed using Illumina's Assay Design Tool (ADT) to determine if selected SNPs were compatible with the GoldenGate® Genotyping assay. SNPs that failed the ADT assessment were excluded and made part of the force exclude files based on the criteria below. SNPs that had low design scores (≥0.5) and 360 failure codes (low score warning, critical design failure causing the SNP to be un-designable for the GoldenGate® Genotyping assay) were flagged for exclusion. Some SNPs had an ADT failure code 340 (another polymorphism in SNP list is equal to or less than 60 nucleotides away); in this case, if both SNPs were selected as tag SNPs, one of these SNPs had to be excluded. Exclusion related to the 340 failure code was based on priority; literature and potentially functional SNPs were prioritised. If both SNPs were potentially functional, non-synonymous (ns) SNPs were prioritised over synonymous (s) SNPs. Where both SNPs were not priority SNPs or both were synonymous or both non-synonymous, the SNPs with the higher final score and design score were selected for genotyping.

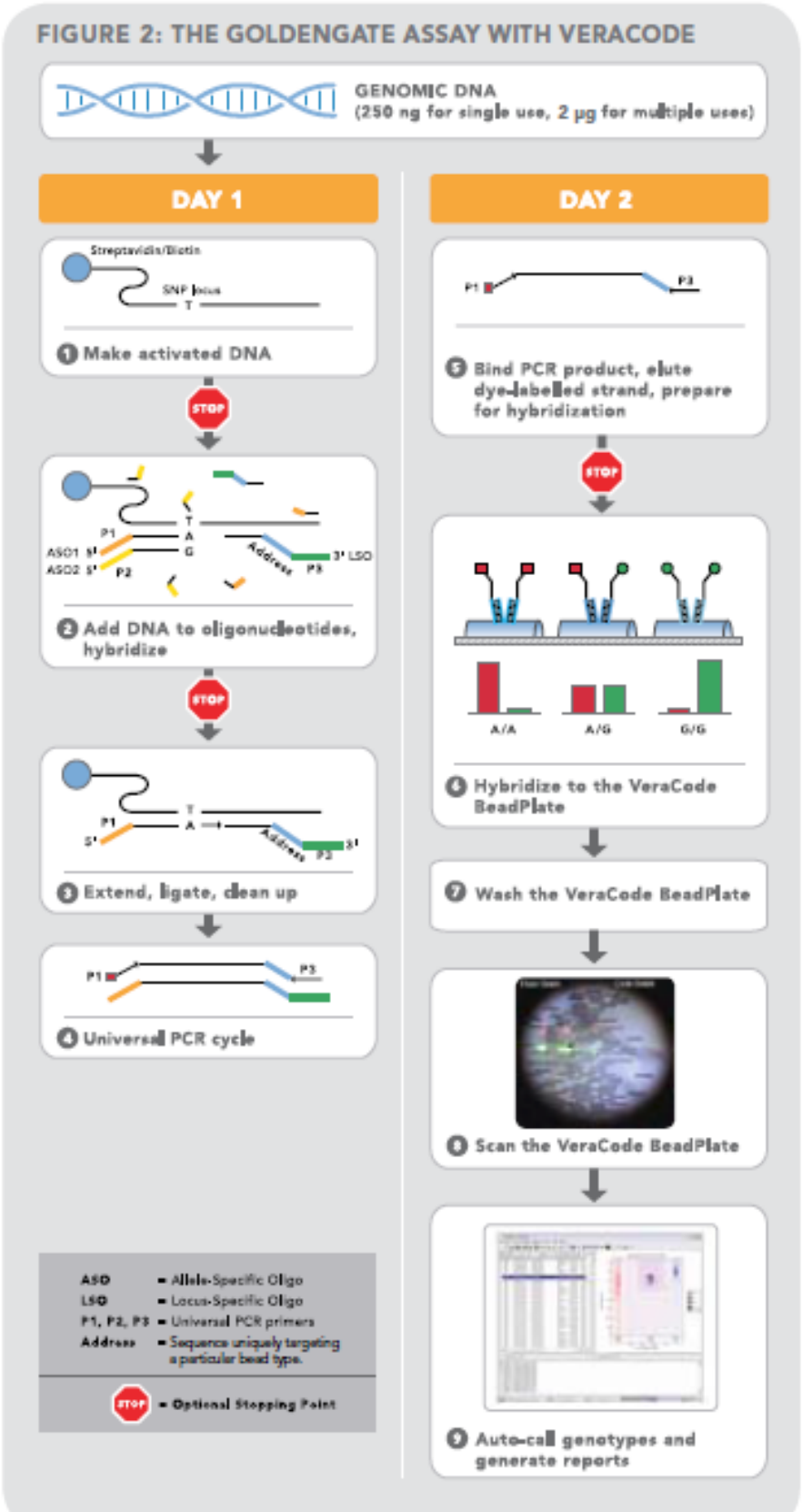
In the final list of SNPs, only SNPs compatible with the GoldenGate® Genotyping assay remained. *LEP* rs7799039, an important literature SNP which was not compatible with the assay was genotyped using the TaqMan® assay. For *LEP*, there were 23 tag SNPs and for *LEPR*, there were 118 tag SNPs. All potentially functional tag SNPs, tag SNPs that tagged potentially functional/literature SNPs and literature tags SNPs were kept for genotyping. For the remainder of the tag SNPs, tag SNPs were prioritised based on location within the intron and the number of SNPs that they tagged. SNPs that were closer to intron borders and tagged at least one other SNP were selected. For tag SNPs that were deeper in the intron, particularly tag SNPs in *LEPR* intron 2-3, tag SNPs were selected based on the number of SNPs they tag. Overall, there were 80 SNPs from the *LEP* and *LEPR* genes selected for genotyping with the GoldenGate® Genotyping assay. Of the 80 tag SNPs, 17 were *LEP* tag SNPs and 63 were *LEPR* tag SNPs (Appendix C).

2.2.3. Genotyping

To determine whether variation in the *LEP* and *LEPR* genes is associated with hypertension and obesity in the APOGH cohort, tag SNPs spanning the *LEP* and *LEPR* gene regions were genotyped using the GoldenGate® Genotyping Assay with VeraCode® Technology (will be referred to as the GoldenGate assay). Eighty *LEP* and *LEPR* SNPs, along with 18 ancestry informative markers (Appendix C) were genotyped using the GoldenGate assay on the BeadXpress platform. AIMs are be used to determine individual ancestry and detect population stratification ((Nassir et al., 2009, Parra et al., 2004). The 18 AIMs were selected using 10 SNPs that are informative for geographic population structure and the other ten SNPs were informative for the southern African populations (Lao et al., 2006, Schlebusch, 2011). *LEP* rs7799039, an important literature SNP, failed the ADT designability test and was genotyped using the TaqMan® SNP Genotyping Assay on the Applied Biosystems 7900HT Fast Real-Time PCR System.

2.2.3.1. Genotyping using the GoldenGate® Genotyping Assay with VeraCode™ Technology

The GoldenGate assay is a medium-to-high-throughput SNP genotyping assay that is flexible, has a fast turn-around time and is relatively affordable as described by Lin and colleagues (2009) (Figure 2.2). The assay runs over two days and starts with biotinylation of DNA. The biotinlyated DNA is then attached to streptavidin coated, paramagnetic beads while simultaneous hybridisation of two allelic specific oligonucleotides (ASO, each specific to an allele of the target SNP) and hybridisation of a locus specific oligonucleotide (LSO, which has a unique address sequence to identify the SNP) to genomic DNA occurs. Hybridization is followed by allele specific extension and ligation of hybridized ASOs and LSOs to form a synthetic template that can be amplified by PCR. During PCR, the template is amplified by a biotinylated universal primer and one of two fluorophore-labeled universal primers, depending on the hybridized ASO. The non-labeled, biotin attached strand is discarded leaving behind single stranded, fluorophore-labeled strands that can hybridize to SNP specific VeraCode™ beads using LSOs. The VeraCode™ beads were scanned on the BeadXpress Reader to call genotypes (Lin et al., 2009).



http://www.uthscsa.edu/csb/CSBPDFfiles/genomics-GoldenGateGenotypingOverview.pdf

Figure 2.2: GoldenGate® Genotyping with VeraCode® Technology.

2.2.3.1.1. Sample purification

The first BeadXpress plate was run without any sample purification or heat treatment. Due to the low quality of results from the first plate, all subsequent samples were heat treated and the DNA was further purified to remove contaminating reagents. Samples were diluted in a 1:1 ratio of ddH_2O to stock DNA. Diluted samples were heat-treated using a desktop incubator by heating the samples to 95°C for 15 minutes and allowing them to cool to room temperature (approximately 22-27°C). Heat treated samples were then quantified to determine DNA concentration and the A260/A280 ratio for purity of the DNA samples. The PCR buffer used as an elution buffer during DNA extraction was used as a blank during quantification. Samples that had very low concentrations (10 ng/µl or less) were excluded and DNA for those samples was recollected and quantified.

Samples were then purified using the QIAEX II Gel Extraction Kit. The Desalting and Concentrating DNA Solutions protocol was used for purification (Appendix D). After PE buffer wash up steps, the buffer was evaporated by placing the tubes in a 25°C incubator until the ethanol in the sample was completely evaporated. DNA was eluted in 1X 10 mM Tris-HCl 1 mM EDTA (TE) buffer and the elution step was done twice using 20 µl TE buffer. Purified samples were then quantified using the NanoDrop. The GoldenGate Genotyping Assay for VeraCode™ manual recommends that the DNA concentration should be 50 ng/µl, but due to the DNA purification process, most of the samples had concentrations lower than 50 ng/µl. Samples that had concentrations below 5 ng/µl were excluded. The 260:280 and 230:280 ratios were not used as a sample exclusion criterion because the ratios were still below 1.8 for most of the samples. Purified samples were stored at 4°C until they were used.

2.2.3.1.2. Genotyping

The GoldenGate assay was used to genotype 98 *LEP* and *LEPR* SNPs and AIMs, using a 192 plex genotyping assay (the other SNPs were for a different study). Prior to each run, the fluidics of the BeadXpress were initialised and primed. The bead cell was reconditioned by replacing the ethanol bottle with a bottle of 10% KOH. DNA plates with 20 µl of purified DNA were prepared in a 96 well PCR plate within 24 hours prior to starting the assay and stored at 4°C. Samples that had concentrations higher than 50 ng/µl were normalised to 50 ng/µl using 1X TE buffer to a final volume of 20 µl. The GoldenGate assay was done as per Illumina GoldenGate Genotyping Assay for VeraCode™ manual and the prepared plate was run on the Illumina BeadXpress Reader.

2.2.3.1.3. Quality control

Plates were grouped for separate analysis based on major changes that may have occurred during the project. Previous experience with the BeadXpress Reader has shown that a major service on the BeadXpress may alter genotype calling and if results from before and after a major service are combined, the results become unreliable. As a result, plates were analysed based on whether they were run before or after a major service. Scan results for each plate were evaluated to determine the red and green fluor intensities, VeraCode™ bead counts and bed types using the BeadXpress Scan Results.cvs file generated as part of the run results to determine plate performance. Illumina's BeadStudio 3.1.3.0, Genotyping Module was used to visualise BeadXpress run results. The contamination dashboards were analysed to determine if any of the plates were contaminated.

2.2.3.2.3.1. Sample and SNP exclusion

The GoldenGate assay has five built-in controls that allow assessment of samples, reagents, BeadChips and equipment. The controls are allele specific extension, PCR uniformity, extension gap, first hybridisation, second hybridization and gender controls (not used as a control in this study). These controls assess performance at different stages of the assay. Samples that failed one or more of these controls were excluded. From the excluded samples, 33 samples that had a call frequency of 0.9, but had failed one control, were selected to be repeated in the final BeadXpress plate.

SNPs were excluded based on exclusion criteria provided by the Illumina's product specific technoteshttp://www.illumina.com/documents/products/datasheets/datasheet_GGGT_DataAnalysis_TN.pdf and http://www.illumina.com/Documents/products/technotes/technote_infinium_genotyping_data_analysis.pdf). SNPs that had a call rate of less than 0.85 and 10% GC below 0.5 were excluded (Figure 2.2). SNPs were also excluded based on the quality of SNP clusters (Figure 2.3), low intensities and lack of Hardy Weinberg equilibrium (P<0.05).

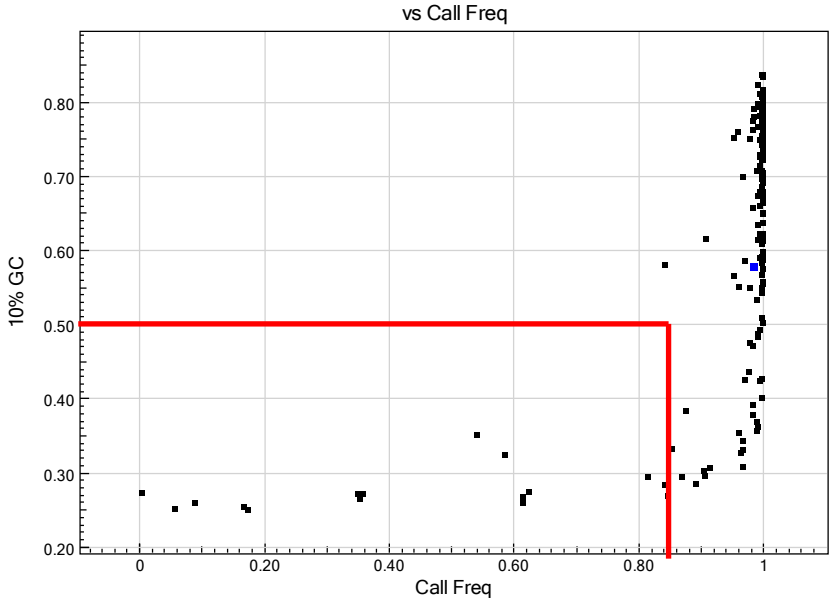
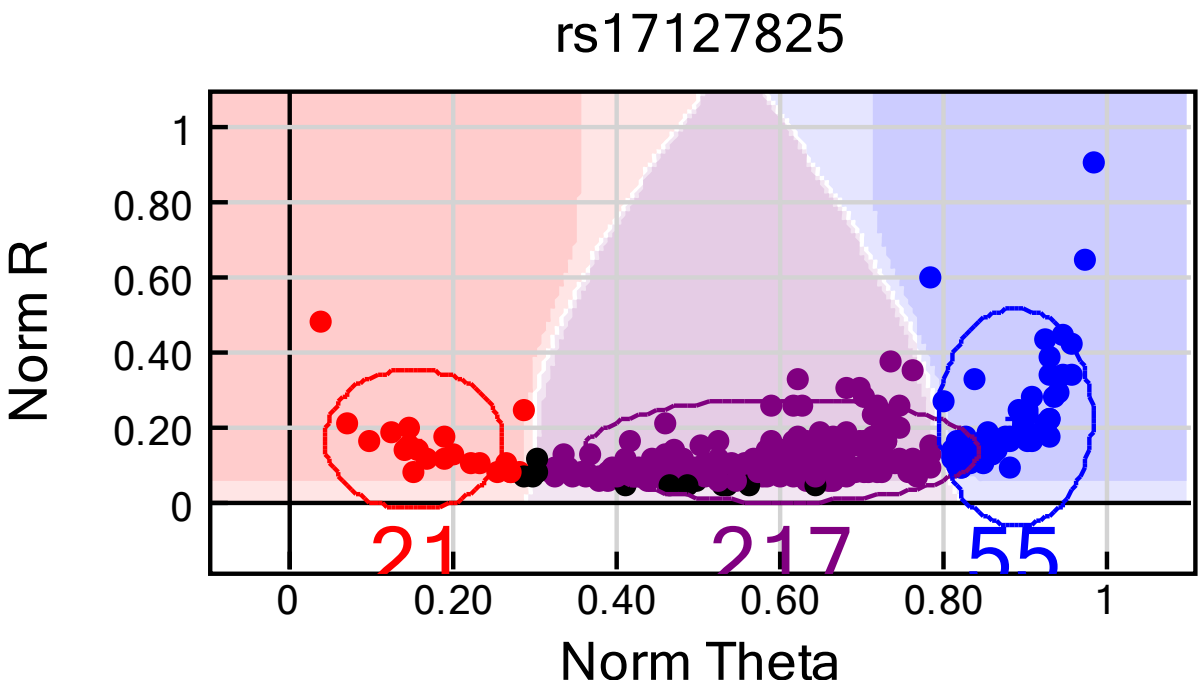


Figure 2.2: Call frequency vs. 10% GC graph used to exclude poorly performing SNPs. SNPs that had a call rate of 0.85 and a 10% GC value lower than 0.5 were excluded.

a. SNP with low signal intensity and undefined genotypes clusters



b. SNP with high signal intensity and well defined genotypes

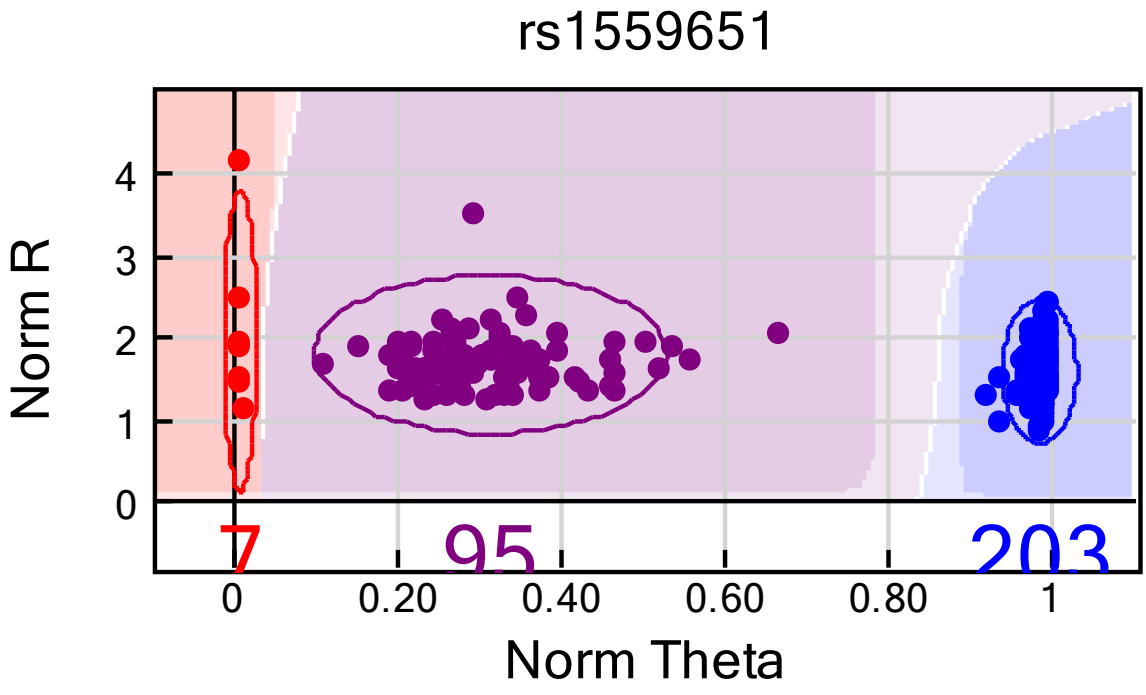
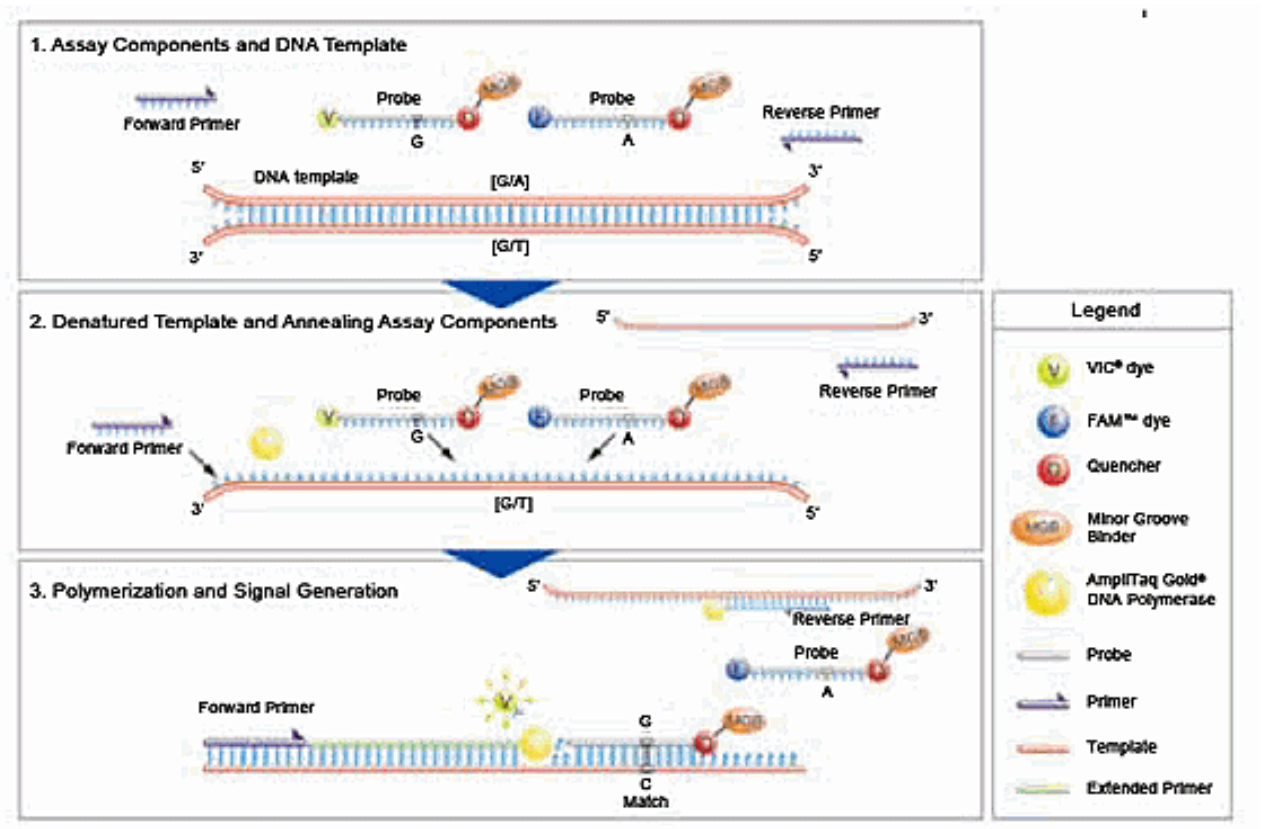


Figure 2.3: BeadStudio SNP graph. The dots represent samples and the different colours represent genotypes with the purple dots representing heterozygous genotypes. Samples indicated in black have not been called as the genotype is uncertain and is therefore excluded. Graphs are plotted based on signal intensity (Norm R) and observed genotypes (Norm Theta). For rs17127825, the signal intensity is low (<1) and the genotype clusters are not well defined, therefore this SNP would be excluded. On the other hand, the signal intensity for rs1559651 is high and the genotype clusters are well defined.

2.2.3.2. Genotyping using the TaqMan® SNP Genotyping Assay

The TaqMan® SNP Genotyping Assay has been previously described (McGuigan and Ralston, 2002) (Figure 2.4). In summary, the TaqMan® SNP Genotyping Assay is an application of real-time PCR. Forward and reverse primers along with two probes, each specific to an allele of the SNP, are labeled with different reporter fluorophores and quencher molecules required for PCR and genotype calling. During PCR, as the forward primer extends the template, the probe bound to the genomic strand is degraded in a 5'-3' direction by the DNA *Taq* polymerase allowing the allele specific fluoropore to be released. With successive amplifications, the fluoropore intensities increase based on the alleles that were present and these intensities are recorded. Once the PCR is complete, fluoropore intensities corresponding to each probe (allele) are compiled to produce a scatter plot from which genotypes can to identified (McGuigan and Ralston, 2002).



http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/PCR/real-time-pcr/qpcr-education/what-can-you-do-with-qpcr/genotyping.html

Figure 2.4: Allelic discrimination using the TaqMan® SNP Genotyping Assay

2.2.3.2.1. DNA normalisation and quantification

Samples were diluted with deionised distilled water (ddH_2O) by diluting 5 µl of DNA in 15 µl of ddH_2O. Diluted samples were then heat-treated in a thermal cycler by heating the samples to 95°C for 5 minutes and then allowed to cool at 27°C for 3 minutes. DNA was quantified on the Applied Biosystems 7900HT Fast Real-Time PCR System with SDS Software v2.0, using the Quantifiler™ Human DNA Quantification Kit. Fifty percent of the recommended reaction volumes were used instead of full reactions (Table 2.2). For each quantification run, 8 DNA standards (in duplicate) were prepared to draw the standard curve required to calculate the concentration in each sample (Table 2.3).

To quantify samples, 2 µl of heat treated DNA or 2 µl of DNA standard was mixed with 11.5 µl of the Quantifiler™ master mix shown in Table 2.2. Plates were sealed with MicroAmp adhesive seals, vortexed and centrifuged with a table top plate centrifuge. Samples were then quantified using the 7900HT Fast Real-Time PCR System. Based on the quantification results, heat treated DNA was normalised to 4 ng/µl using ddH_2O to a final volume of 20 µl in a 96 well plate. Samples were than stored at -20°C until they were genotyped.

Table 2.2: Quantifiler™ reagent volumes used per sample

Component	Volume (µl) per full reaction	Volume (µl) for 50% reaction
Quantifiler PCR Reaction Mix	12.5	6.25
Quantifiler Human Primer Mix	10.5	5.25
Total master mix	23	11.5

Table 2.3: Preparing the DNA Quantification Standards and final standard final concentration

Standard	Volume of standard	Volume of TE buffer	Standard final Concentration (ng/µl)
1	10 µl [Quantifiler Human DNA Standard] [1]	30 µl	50
2	10 µl [Standard 1]	20 µl	16.7
3	10 µl [Standard 2]	20 µl	5.56
4	10 µl [Standard 3]	20 µl	1.85
5	10 µl [Standard 4]	20 µl	0.62
6	10 µl [Standard 5]	20 µl	0.21
7	10 µl [Standard 6]	20 µl	0.068
8	10 µl [Standard 7]	20 µl	0.023

[1] 200 ng/µl purified DNA standard (stock)

2.2.3.2.2. Genotyping

Normalised DNA was genotyped using the dried down method, where 2 µl of DNA was dried overnight in a MicroAmp Optical 384-well reaction plate. The TaqMan® Pre-designed SNP Genotyping Assay, for rs7799039 (small scale, human) and the TaqMan® SNP Genotyping Master Mix were used to make the master mix for genotyping as shown in Table 2.4 which was added to the dried down DNA. All genotyping runs had 16 negative controls (no DNA) and four positive controls (2 GG and 2 AG), with the exception of the first run which had 8 negative controls and no positive controls. Genotypes of positive controls were confirmed using Sanger sequencing that will be discussed in section 2.2.4.

Table 2.4: TaqMan® SNP Genotyping Assay master mix preparation

Component	Volume per well/reaction (µl)
TaqMan® Universal PCR Master Mix	2.5
20x working stock of SNP genotyping assay	0.25
ddH_2O	2.25
Total Volume	5

2.2.3.2.2. Quality control

Genotyping results were exported from the SDS software to Excel and genotype clusters were viewed and modified on the SDS program. Samples that were called as unknown or had low intensities (clustered near the negative controls) or were too far away from the designated (called) cluster were repeated. Repeated samples that still failed to be accurately called were excluded from analysis.

2.2.4. Sequencing

Sanger Sequencing was used to confirm rs7799039 genotypes of 8 samples that were genotyped using the TaqMan® SNP Genotyping Assay. Of the samples, 4 were called as homozygous for the common allele (GG) and the other 4 samples were called heterozygotes (AG). Polymerase chain reaction (PCR) reverse and forward primers were designed using Primer 3 (version 0.4.0) software. The Primer 3 input sequence, which was the flanking sequence around the SNP, was retrieved from Ensembl release 62 (Appendix E) (http://www.ensembl.org). PCR product sequence and size were determined using UCSC *In-Silico* PCR (Appendix E) (Kent et al., 2002). The primer sets and PCR product size for rs7799039 are shown in Table 2.5.

Table 2.5: sets and PCR product sizes for rs7799039

SNP	Direction	Primer sequence	PCR product size (kb)
rs7799039	Forward	5’ AGCCAAGGCAAAATTGAGG 3’	250
	Reverse	5’ TCCAGCCGATCTCTCTGTTC 3’	

2.2.4.1. Polymerase chain reaction

Polymerase chain reaction (PCR) was used to amplify DNA fragments that encompass rs7799039. Gradient PCR using Cycler Gradient Eppendorf was done to determine the optimal annealing temperature for rs7799039. Gradient PCR annealing temperatures were 55°C and 60°C, with 60°C

showing the best results. All PCR reactions done after gradient PCR were done using the Applied Biosystems, 2720 Thermal Cycler. PCR reaction mixtures are shown in Table 2.6 and PCR conditions are shown in Table 2.7.

Table 2.6: Concentrations and volumes of reagents for PCR reactions

Reagent	Stock concentration	Final concentration	Volume per tube (µl)
ddH_2O	-	-	14.3
10X buffer, no MgCl	10X	1X	2.5
$MgCl_2$	25 mM	2.5 mM	2.5
dNTPs	10 mM	0.125 mM	2.5
Forward primer	100 µM	0.4 µM	1
Reverse primer	100 µM	0.4 µM	1
Amplitaq gold	5U	1U	0.2
DNA	100 ng/µl	100 ng/µl	1
Total volume	-	-	25

Table 2.7: PCR run conditions

Step	Temperature (°C)	Time	
Initialisation	95	5 min	
Denaturation	95	15 sec	45 cycles
Annealing	60	30 sec	
Extension	72	15 sec	
Final extension	72	5 min	
Hold	15	∞	

Gel electrophoresis on a 3% agarose gel was used to determine if the PCR reaction was successful, check if the correct DNA fragment (according to size) was amplified and to check for contamination (Appendix F). PCR products were loaded to the solidified 3% agarose gels along with Fermentas GeneRuler™ 50 bp DNA ladder by mixing 3 µl Ficoll loading dye with 2 µl of the

PCR product. The gels were run at 5V/cm for 30 min on Sigma-Aldrich gel tanks and analysed using Syngene's G-Box and GeneSnap (version 6.08) gel documentation system.

The PCR products were cleaned using the Nucleospin Kit (Appendix G). Cycle sequencing, cycle sequencing product clean-up (using Qiagen DyeEX) and sequencing preparation are outlined in Appendix G. Sequencing run was done on the Applied Biosystems/Hitachi Genetic Analyzer and results were viewed on Foundation Data Collection version 3.0. All reagent and machine company names are listed in Appendix H.

2.5. Statistical analysis

2.5.1. Descriptive statistics

SAS Enterprise Guide version 5.1 and SAS 9.3 were used to describe demographic and phenotype data in the sample. The Chi-square test for goodness-of-fit was used to determine the normality of the distribution for all phenotypes and demographic data. All measures were normally distributed except leptin levels (log transformed for all association tests). For quantitative traits, the two-sample t Test (SAS Enterprise Guide 5.1) was used to determine variable mean values and to assess inter-gender differences. For the t Test, where equality of variance was significant, the Satterthwaite method was adopted and for non-significant equality variance, the pooled method was adopted. The Pearsons Chi-Square test (SAS 9.3) was used to assess inter-gender differences for dichotomous variables.

2.5.2. Heritability and Familial aggregation

Heritability (h^2) was estimated using Statistical Analysis for Genetic Epidemiology (S.A.G.E) version 6.0.1 using the Marker-Trait Associations in Pedigree data (ASSOC) program which calculates the amount of additive genetic factors divided by total trait variance (Martin et al., 2008). The ASSOC

program estimates and tests the association between a trait (binary and/or continuous (normally distributed) and covariates from pedigree data in the presence of familial correlations (Elston and Gray-McGuire, 2004). Unadjusted and multivariate adjusted heritability estimates were determined for each trait and the multivariate adjusted estimates are reported in the results. Confounding factors that were adjusted for in the ASSOC test are listed in Table 2.8. The number of parent pairs, mother and child pair and sibling pairs used for estimating heritability and familial aggregation correlations are shown in Table 2.9.

Intra-familial aggregation correlation coefficients were determined using the PROC GENMOD procedure on SAS 9.3. This procedure determines concordance between family members, ie. father-mother pairs, parent-child pairs and sibling pairs. Because not all the individuals had all the phenotype data, sample sizes varied for the different phenotypes (Table 2.9). Unadjusted and multivariate adjusted correlation coefficients were determined in different pairs. Inter-pair differences between the pairs were determined.

Table 2.8: Confounding factors adjusted for in heritability estimates and association tests

Variable	**Confounding factors**
Anthropometric measures	Age, sex, smoking and drinking
Leptin levels	Age, sex, smoking, drinking and WC
Systolic and diastolic BP	Age, sex, smoking, drinking, poor glucose control or HbA1C>6.1%, hypertension treatment and WC
Central systolic BP	Age, sex, smoking, drinking, poor glucose control or HbA1C>6.1%, hypertension treatment, WC, mean arterial pressure and heart rate

Table 2.9: Number of pairs used for familial aggregation and heritability estimates

Pairs	**n**
Height, Weight and BMI	
Father-mother	29
Parent-child	184
Sibling-sibling	82
Skinfold thickness	
Father-mother	27
Parent-child	170
Sibling-sibling	70
Waist and hip circumference, waist-hip-ratio	
Father-mother	27
Parent-child	176
Sibling-sibling	81
Leptin levels	
Father-mother	18
Parent-child	107
Sibling-sibling	43
Systolic and diastolic BP	
Father-mother	29
Parent-child	184
Sibling-sibling	82
Central systolic BP	
Father-mother	27
Parent-child	173
Sibling-sibling	77

2.5.3. SNP association

gPLINK version 2.050 (Purcell et al., 2007) was used to determine minor allele frequencies (MAF), Hardy-Weinberg equilibrium (HWE) and SNP Mendelian errors. SNPs that had MAF<0.01 or HWE P-value<0.05 or Mendelian errors >0.05 were excluded from further analysis. The power of the study was calculated for each tested trait using the additive model on Quanto version 1.2.4 (Gauderman and Morrison, 2006) using the number of individuals available for each specific trait (Table 2.10).

Table 2.10: Sample sizes needed to detect nominal effects on phenotype at 5% allele frequencies

Trait	Actual APOGH sample size	Effect*	Required sample size†
Body weight	713	7 kg	624
BMI	713	3 kg/m^2	569
Skinfold thickness	694	0.4 cm	512
Waist circumference	678	6 cm	667
Hip circumference	676	6 cm	526
WHR	676	0.04	512
Leptin levels	339	13 ng/ml	332
Systolic BP	713	8 mm Hg	679
Diastolic BP	713	5 mm Hg	555
Central systolic BP	704	8 mm Hg	679

***Lowest** effect size that can be detected at a minor allele frequency of 5% given the available APOGH sample size. † Minimum sample size required to detect effect size in the "Effect" column.

SNP analysis was performed on up to 713 participants and SNP associations with leptin levels were performed on 339 individuals with leptin level data. All association tests were performed in the entire sample and in men and women separately. SNP association tests were done on SAS 9.3 using the PROC MIXED test which uses the genotypic model and takes into account the family

relationships therefore adjusting for relatedness. Multivariate analyses were done adjusting for confounding factors listed in Table 2.8. The Bonferroni correction was used to correct for multiple testing. A significance level threshold of P= 0.00076 (ie. 0.05/66 successfully genotyped SNPs) was adopted for all tests. Because the Bonferroni correction is very stringent, significance levels 0.05>P>0.00076 were reported and considered as a nominally significant. P-values were rounded-off to four decimal places.

The Ensembl Variant Effect Predictor (VEP) tool (http://www.ensembl.org/tools.html) was used to determine whether SNPs were within exons, introns and intergenic regions or within regulatory regions. RegulomeDB (Boyle et al., 2012) was used to determine if SNPs overlapped with potentially regulatory regions such as DNAse hypersensitivity sites, transcription factor binding sites and promoter regions. LocusZoom (Pruim et al., 2010) was used to visualise significantly associated SNPs within their respective genes by plotting the logged P-value (-log10) for each SNP and showing degree of linkage disequilibrium (LD) between all SNPs in that gene with the most significantly associated SNP. The LD values in the plots are based on data from African populations genotyped in the 1000 Genomes Project (March 2012 release which uses hg19). The SPSmart Engines tool (Amigo et al., 2011) was used to retrieve allele frequencies in HapMap population and allele frequency pie chart were drawn on Excel 2010.

2.5.4. Linkage disequilibrium and haplotype analysis

Haplotype blocks were constructed using Haploview version 4.2 (Barrett et al., 2005) using 228 singletons (not related to anyone else in the sample) and 35 trios. Individuals with only one parent were automatically excluded. Haplotype blocks were constructed using confidence intervals (Gabriel et al., 2002) which by default only use SNPs with MAF≥0.05.

gPLINK was used to perform haplotype association tests on 353 unrelated individuals because relatedness could not be corrected for while running multivariate analysis. Haplotype blocks constructed on Haploview were used for haplotype association tests and association tests were performed in the entire sample and in men and women separately. Multivariate haplotype analyses were done using the "hap-linear" function on gPLINK, adjusting for confounding factors listed in Table 2.8. The Bonferroni correction was used to correct for multiple testing using the "adjust" function which based correction on the number of haplotypes observed in the sample. A significance level threshold of $\alpha = 0.05$ after correcting for multiple testing was adopted for all tests.

3. Results

Section 3.1 summarises and describes the data used in sections 3.2 to 3.5. In section 3.1, demographic and phenotype data are described and the heritability of all tested traits is reported in section 3.2. Genotyping results are outlined in section 3.3 and Parent Transmission disequilibrium test (TDT) results are reported in section 3.4. SNP and haplotype association results for anthropometric measures, leptin levels and BP are outlined in section 3.5 and 3.6, respectively.

3.1. Summary and descriptive statistics

A total of 713 APOGH participants were successfully genotyped and analysed in association tests whereas a subset of participants were analysed in heritability and familial aggregation tests, because not all individuals in all pedigrees were genotyped. For haplotype analysis, 353 unrelated individuals were used. The descriptive statistics of the entire sample are shown in Table 3.1 and the descriptive statistics of the unrelated individuals are shown in Table 3.2. For both the mixed (related individuals and singletons) sample and unrelated individuals, the number of women was significantly different to the number of men. Women were significantly shorter in height and had higher anthropometric measures, diabetes prevalence and leptin levels compared to men. Overweight rather than obesity was more common in men whereas obesity was more common in women compared to overweight. There were no significant differences in BP and the prevalence of hypertension between men and women but heart rate was significantly higher in women and the prevalence of smoking and drinking were significantly higher in men. The number of individuals with both overweight/obesity and hypertension was high and only 8% of the individuals had hypertension that was independent of overweight/obesity (BMI>25 kg/m^2) (Figure 3.1).

Table 3.1: Demographic, anthropometric, haemodynamic and blood pressure characteristics of successfully genotyped individuals, with inter-gender comparisons

	All (n=713)	Male (n=240)	Female (n=473)	P
Sex (%)		33.7	66.3	<.0001
Age (years)	43.3±18	43±18.9	43.4±17.6	0.7554
Body height (cm)	161±8.6	169±7.0	157±6.2	<.0001
Body mass index (kg/m^2)	29.3±7.9	25.2±5.3	31.4±8.1	<.0001
Body weight (kg)	75.5±19.3	72±16.2	77.3±20.5	0.0002
Skinfold thickness (cm)	2.1±1.0	1.5±0.9	2.4±1.0	<.0001
Waist circumference	90.1±17.1	85.8±140	92.3±18.1	<.0001
Hip circumference (cm)	107.6±15.2	99.4±11.6	111.7±15.2	<.0001
Waist to hip ratio (cm)	0.8±0.1	0.9±0.1	0.8±0.1	<.0001
Elevated waist circumference(%) ≥94/80 *	65.3	55.2	70.4	<.0001
Overweight (%)[†]	24.4	31.7	20.7	0.0013
Obese (%)[‡]	42.4	18.8	54.3	<.0001
Poor glucose control or HbA1C>6.1% (%)	26.7	21.3	29.31	0.0202
Leptin levels (ng/ml)	26.2±26.2	7.5±8.3	36.3±27.0	<.0001
Systolic blood pressure (mm Hg)	129±23	85±12	83±14	0.0686
Diastolic blood pressure (mm Hg)	84±13	131±22	128±23	0.0681
Central systolic blood pressure (mm Hg)	120±23	122±23	120±24	0.2595
Heart rate (bpm)	65±12	61±12	68±12	<.0001
Mean arterial blood pressure (mm Hg)	100±17	101±15	100±17	0.2678
Treated hypertension (%)	23.4	17.92	26.2	0.0134
Hypertensive (%)[◊]	45.6	47.9	44.4	0.3279
Regular alcohol intake (%)	22.1	38.8	13.7	<.0001
Regular tobacco intake (%)	14.7	32.9	5.5	<.0001

All values are mean values ±SD values unless otherwise stated, *Waist circumference ≥80 in women or ≥94 in men, [†]25≤BMI<30 kg/m^2, [‡] BMI≥30 kg/m^2, HbA1C: Glycated haemoglobin, bpm: Beats per minute, [◊]Systolic blood pressure ≥140 or diastolic blood pressure ≥90mm Hg or taking antihypertensive medication.

Table 3.2: Demographic, anthropometric, haemodynamic and blood pressure characteristics of successfully genotyped, unrelated individuals, with inter-gender comparisons

	All (n=353)	Male (n=119)	Female (n=234)	P
Sex (%)		33.7	66.3	<.0001
Age (years)	56.1±14.0	56.6±15.0	55.9±135.5	0.6536
Body height (cm)	160.2±8.1	167.8±6.2	156.3±5.9	<.0001
Body mass index (kg/m^2)	32.4±7.8	27.2±5.4	35.1±7.6	<.0001
Body weight (kg)	82.8±18.9	76.8±16.4	85.8±19.4	<.0001
Skinfold thickness (cm)	2.3±1.0	1.7±0.9	2.6±0.9	<.0001
Waist circumference	98.2±15.9	92.5±13.4	101.2±16.2	<.0001
Hip circumference (cm)	112.0±15.2	102.3±12.6	116.9±14.1	<.0001
Waist to hip ratio (cm)	0.88±0.10	0.90±0.07	0.86±0.11	0.0009
Elevated waist circumference(%) ≥94/80 *	75.4	46.2	91	<.0001
Overweight (%)[†]	52.6	41.2	17.9	<.0001
Obese (%)[‡]	58.9	28.6	74.4	<.0001
Poor glucose control or HbA1C>6.1% (%)	40.5	30.25	45.7	0.0051
Leptin levels (ng/ml)	30.9±28.5	9.3±7.5	43.3±28.9	<.0001
Diastolic blood pressure (mm Hg)	88±13	87±11	88±14	0.9161
Systolic blood pressure (mm Hg)	139±24	140±23	138±24	0.4322
Central systolic blood pressure (mm Hg)	130.±24	131±22	129±24	0.5721
Heart rate (bpm)	66±13	63±13	68±12	0.0025
Mean arterial blood pressure (mm Hg)	106±16	106±14.9	106±17	0.9622
Treated hypertension (%)	41.36	35.3	44.4	0.0989
Hypertensive (%) [◊]	69.12	70.59	68.4	0.6706
Regular alcohol intake (%)	21.0	37.8	12.4	<.0001
Regular tobacco intake (%)	12.8	28.6	4.7	<.0001

All values are mean ±SD values unless otherwise stated, *waist circumference ≥80 in women or ≥94 in men, [†] BMI 25≤BMI<30 kg/m^2, [‡] BMI≥30 kg/m^2, bpm: Beats per minute, HbA1C: Glycated haemoglobin, [◊]Systolic blood pressure ≥140 or diastolic blood pressure ≥90mm Hg or taking antihypertensive medication.

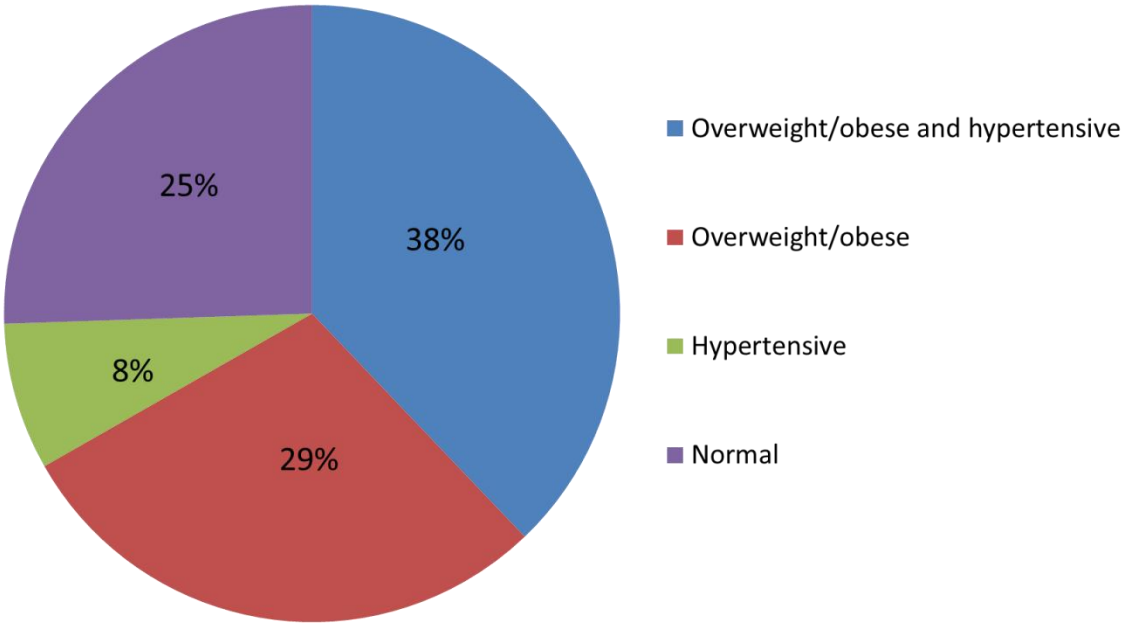


Figure 3.1: Prevalence of overweight/obesity and hypertension in the APOGH sample. More than a third (38%) of the 713 individuals from the APOGH sample are both overweight/obese and hypertensive (Blue). Only a quarter of the sample has BMIs within the normal range and has normal blood pressure (Purple). A small number (8%) of individuals have high blood pressure in the absence of overweight/obesity and 29% are either overweight or obese but do not have hypertension (Red).

Familial aggregation and heritability tests were performed in up to 122 families which comprised 29 parent pairs and 255 offspring of which 164 were siblings. For parent-child pairs, mother-child and father-child pairs were tested with some parents and children being tested more than once in pedigrees with more than one child. Also, in large families with at least three generations, parents were also offspring if individuals from at least three generations were available. Because some individuals had missing data for some of the variables (eg. WC, leptin levels, central systolic BP), the number of pairs differed for different tests. Table 3.3 shows the descriptive statistics of the individuals used in the familial aggregation correlations. There was a borderline significant difference in the number of men compared to women between the parents and offspring. No significant differences were noted in height between parents and offspring. Parents had significantly higher anthropometric measures, diabetes prevalence, leptin levels, BP and the

prevalence of hypertension compared to their offspring. There were no significant differences in heart rate and the number of smokers and drinkers between parents and offspring.

Table 3.3: Demographic, anthropometric and haemodynamic characteristics of parents and offspring of the study sample used in intra-familial correlation

	Parents (n=58)	Offspring (n=255)	P
Sex (% Females)	50	64	0.0494
Age (years)	57.1±9.6	30.7±10.6	<0.0001
Body height (cm)	161±7.8	162±9.2	0.3155
Body mass index (kg/m^2)	31.3±7	26.3±6.6	<0.0001
Body weight (kg)	80.7±17.2	69.1±17.0	<0.0001
Skinfold thickness (cm)	2.4±0.9	1.8±1.0	<0.0001
Waist circumference (cm)	98.8±11.8	82.0±13.5	<0.0001
Hip circumference (cm)	110.1±15.0	103.1±14.0	0.0013
Waist to hip ratio	0.9±0.1	0.8±0.1	<0.0001
Overweight (%)†	34.5	20.8	0.0260
Obese (%)‡	51.7	29.4	0.0012
Poor glucose control or HbA1C>6.1% (%)	31.0	1.8	0.0002
Leptin levels (ng/ml)	29.0±24.7	17.8±17.0	0.0054
Diastolic blood pressure (mm Hg)	88±9	82±13	<0.0001
Systolic blood pressure (mm Hg)	139±18	121±18	<0.0001
Central systolic blood pressure (mm Hg)	132±17	113±20	<0.0001
Mean arterial pressure (mm Hg)	107±13	97±16	<0.0001
Heart rate (bpm)	64±11	64±11	0.8199
Hypertensive (%) $^{\lozenge}$	69	22	<.0001
Treated hypertension (%)	36	4.3	<.0001
Regular alcohol intake (%)	27.6	26.7	0.8866
Regular tobacco intake (%)	12.1	16.5	0.4050

All values are mean±SD values unless otherwise stated, †25≤ BMI<30 kg/m^2, ‡ BMI≥30 kg/m^2, HbA1C: Glycated haemoglobin, bpm: Beats per minute, $^{\lozenge}$Systolic blood pressure ≥140 or diastolic blood pressure ≥90mm Hg or taking antihypertensive medication.

3.2. Heritability

With appropriate adjustments for potential confounding factors (age and sex), height was significantly heritable and showed the strongest heritability (h^2=0.764) among all the tested measures. With adjustments for age, sex, smoking and drinking, significant heritability was noted for body weight, BMI, waist and hip circumference, WHR and skinfold thickness. With adjustments for covariates (for age, sex, smoking and drinking and WC), leptin levels were not significantly heritable (P=0.228). After adjusting for appropriate confounders (age, sex, smoking, drinking, treatment for hypertension, type II diabetes and WC for all BP with additional mean arterial BP and heart rate for central systolic BP), systolic, diastolic and central systolic BP were all significantly heritable. Familial aggregation results are outlined in Appendix I.

Table 3.4: Multivariate adjusted heritability estimates for anthropometric measures, leptin levels and blood pressure

Trait	h^2±S.E	P-value
Anthropometry and leptin levels		
Body height (cm)	0.764±0.078	<0.0001
Body weight (kg)	0.383±0.098	<0.0001
Body mass index (kg/m^2)	0.257±0.103	0.0060
Waist circumference (cm)	0.351±0.104	0.0004
Hip circumference (cm)	0.418±0.098	<0.0001
Waist to hip ratio	0.461±0.098	<0.0001
Skinfold thickness (cm)	0.444±0.089	<0.0001
Leptin levels (ng/ml)	0.148±0.199	0.2280
Blood pressure		
Systolic blood pressure (mm Hg)	0.342±0.115	0.0014
Diastolic blood pressure (mm Hg)	0.269±0.103	0.0045
Central systolic blood pressure (mm Hg)	0.332±0.132	0.0060

3.3. SNP Genotyping

LEP rs7799039 was genotyped using the TaqMan® assay because it was not compatible with the GoldenGate assay. Seven-hundred and eighty-six individuals were successfully genotyped for rs7799039. rs7799039 was non-polymorphic in unrelated individuals and no further tests were run for this SNP (Table 3.4). The rest of the SNPs were genotyped using the GoldenGate SNP genotyping assay on the BeadXpress platform (results are outlined in section 3.3.1 and 3.31.)

3.3.1. BeadXpress Plate performance

Plates were clustered for separate analysis based changes that may have occurred during the project. Plate 1 was analysed separately because the samples used in this plate were not purified like all samples in subsequent plates. Plates 2-5 were analysed separately from plates 6-9 due to a major service of the BeadReader after plate 5 was run. Results from plate 10 were analysed separately because according to the red and green laser intensity fluor means, many samples in the plate failed (some samples were samples that had failed in previous runs).

3.3.2. Successfully genotyped SNPs using the GoldenGate Assay

Of the 927 samples that were genotyped, 713 samples were successfully genotyped without any errors (discussed in section 2.2.3.2.3.1). Of the 98 SNPs that were genotyped with the GoldenGate assay, 24 SNPs were excluded because they failed to meet the quality control thresholds discussed in Chapter 2. From the remaining 74 SNPs (including rs7799039), six SNPs were excluded because they were not in Hardy-Weinberg Equilibrium ($P<0.05$) or they were non-polymorphic (minor allele frequency (MAF) <0.01) (Table 3.5). A total of 66 SNPs were analysed for association with anthropometry, leptin levels and BP data.

Table 3.5: Allele frequencies, Hardy-Weinberg equilibrium P-values and genomic location of all successfully genotyped SNPs

SNP	Chr	Gene	Position	MA[#]	A2	HWE[†]	MAF
rs10249476	7	*LEP*	127877026	T	G	0.0755	0.2657
rs11761556	7	*LEP*	127897069	A	C	0.6048	0.1179
rs11763517	7	*LEP*	127890062	C	T	0.8357	0.1506
rs12706832	7	*LEP*	127887139	G	A	0.3375	0.0984
rs17151913	7	*LEP*	127894018	A	T	1	0.0595
rs17151914	7	*LEP*	127894407	T	C	0.5344	0.1537
rs17151919	7	*LEP*	127894592	A	G	1	0.1182
rs28959478	7	*LEP*	127897989	A	C	0.2865	0.0312
rs4236625	7	*LEP*	127883695	T	A	0.5517	0.1618
rs6956510	7	*LEP*	127894240	A	G	0.8453	0.1662
rs7795794	7	*LEP*	127890151	A	G	1	0.0994
rs7799039	7	*LEP*	127878783	A	G	1	0.0071
rs10493379	1	*LEPR*	66045927	A	G	0.0498	0.2368
rs10749754	1	*LEPR*	66054640	G	A	1	0.4871
rs10889556	1	*LEPR*	65969552	G	A	0.509	0.2847
rs10889557	1	*LEPR*	65970704	A	G	1	0.2837
rs11208656	1	*LEPR*	65970787	G	A	0.8063	0.3255
rs1137100	1	*LEPR*	66036441	G	A	1	0.0598
rs1137101	1	*LEPR*	66058513	A	G	1	0.4602
rs1171261	1	*LEPR*	66001402	T	C	0.4537	0.1239
rs1171273	1	*LEPR*	65980215	C	G	0.8544	0.1805
rs11801408	1	*LEPR*	66075136	C	T	0.9145	0.4658
rs11808888	1	*LEPR*	65952781	G	A	0.0996	0.4346
rs12067730	1	*LEPR*	66049022	C	T	0.3476	0.1311
rs12145690	1	*LEPR*	65887013	C	A	0.1245	0.3563
rs12239395	1	*LEPR*	66046013	C	T	0.0126	0.2659
rs12403114	1	*LEPR*	66005603	G	C	0.6082	0.0724
rs1475397	1	*LEPR*	65983158	C	T	0.3226	0.2049
rs1571151	1	*LEPR*	65988706	G	A	0.2133	0.2593
rs1627238	1	*LEPR*	65986079	C	T	0.7336	0.4587

SNP	Chr	Gene	Position	MA[#]	A2	HWE[†]	MAF
rs17127828	1	*LEPR*	66095526	G	A	0.4465	0.12
rs1751484	1	*LEPR*	66019891	C	T	0.0052	0.1257
rs1751492	1	*LEPR*	65992625	C	T	0.4331	0.2195
rs1782763	1	*LEPR*	66007900	C	T	0.0301	0.2435
rs1805095	1	*LEPR*	66102158	T	C	1	0.0187
rs1805096	1	*LEPR*	66102257	T	C	0.8302	0.4957
rs1805134	1	*LEPR*	66067109	C	T	1	0.3136
rs1887285	1	*LEPR*	65897747	C	T	0.0967	0.1782
rs1892534	1	*LEPR*	66105944	G	A	0.523	0.483
rs1938484	1	*LEPR*	66081282	A	C	0.0629	0.0584
rs2154381	1	*LEPR*	65995701	C	T	0.7779	0.2536
rs34499590	1	*LEPR*	66081791	A	G	1	0.0244
rs3790425	1	*LEPR*	66043112	C	T	<0.0001	0.2017
rs3828039	1	*LEPR*	66053556	C	T	0.078	0.2771
rs4655557	1	*LEPR*	66080787	T	C	0.1822	0.409
rs4655564	1	*LEPR*	66083537	A	G	0.1766	0.0657
rs4655811	1	*LEPR*	65923157	C	G	1	0.0968
rs61090190	1	*LEPR*	65939974	A	G	0.3485	0.2759
rs6673324	1	*LEPR*	66031063	A	G	0.4383	0.4066
rs6690661	1	*LEPR*	66086629	C	G	0.3444	0.2376
rs6694528	1	*LEPR*	65963016	T	C	0.8222	0.4417
rs6700896	1	*LEPR*	66089782	T	C	0.5916	0.4756
rs7518849	1	*LEPR*	65948791	C	T	0.3342	0.2098
rs7535099	1	*LEPR*	66050997	G	A	0.3891	0.1939
rs8179183	1	*LEPR*	66075952	C	G	1	0.1835
rs9436298	1	*LEPR*	65891183	A	T	0.0269	0.1637
rs9436739	1	*LEPR*	65890699	A	T	0.145	0.2104
rs970468	1	*LEPR*	65906490	T	G	1	0.4914

Ancestry informative markers							
SNP	**Chr**	**Gene**	**Position**	**MA[#]**	**A2**	**HWE[†]**	**MAF**
rs1363448	5	AIM	140783596	C	T	0.107	0.0737
rs1344870	3	AIM	21307401	C	A	0.5208	0.0453
rs1823718	15	AIM	74147244	C	T	0.3778	0.1871
rs1858465	17	AIM	51142920	T	A	0.0591	0.1304
rs1876482	2	AIM	17362568	T	C	1	0.0057
rs2089740	15	AIM	36310531	T	G	0.3401	0.3329
rs2112527	19	AIM	9603751	G	A	<0.0001	0.3319
rs217538	6	AIM	108483470	G	C	1	0.0156
rs65264	7	AIM	28545611	C	T	0.3593	0.1761
rs679047	9	AIM	12883664	A	T	0.2926	0.2847
rs714857	11	AIM	15974389	C	T	1	0.217
rs722869	14	AIM	97277005	G	C	0.5885	0.1097
rs723854	1	AIM	192511012	G	C	0.1575	0.1847
rs735612	15	AIM	34076642	T	G	0.4462	0.4618
rs952718	2	AIM	215888624	C	A	0.1152	0.2865
rs953386	13	AIM	110943692	A	G	0.5191	0.4713

SNPs with red font are the SNPs that were excluded. AIM: Ancestry informative marker, Chr: Chromosome, [†]P-value for Hardy-Weinberg equilibrium, [‡] MAF; Minor allele frequency [#]MA: Minor allele, A2; Major allele.

3.4. Over transmitted SNPs and haplotypes

The Parent Transmission disequilibrium test (TDT) was performed on Haploview to determine if any SNPs were over transmitted from parents with increased WC (≥80 cm in women and ≥94 cm in men) or overweight/obese (BMI≥25 kg/m^2) or hypertensive (diastolic BP≥90 mm Hg or systolic BP≥140 mm Hg or treated for hypertension) to affected offspring. In this analysis, only parent-child pairs were used and no SNPs or haplotypes were over transmitted for all three phenotypes.

3.5. SNP Association Results

There were 66 SNPs that met HWE and the MAF threshold, therefore the P-value cut-off for multiple testing using the Bonferroni method, was 0.00076. P-values that were $P<0.0076$ were considered as statistically significant for associations whereas $0.05 \geq P > 0.00076$ were considered to be nominally associated. Because of the significant differences noted between men and women for anthropometric measures and leptin levels, all association tests, including those for BP, were done in the entire sample and in men and women separately.

All associated (nominally and significantly) SNPs were scored using RegulomeDB to determine if the SNPs overlapped with potentially regulatory regions such as DNAase hypersensitivity sites, transcription factor binding sites and promoter regions. Each SNP was scored based on the regulatory regions that it overlaps with as shown in Table 3.6. SNPs that had no known/predicted regulatory function are reported to have "No data" on RegulomeDB. SNP locations with respect to gene were retrieved to determine if SNPs were exonic, intronic, regulatory or intergenic. For exonic SNPs, synonymous and non-synonymous SNPs were coded as Coding_S and Coding_NS, respectively. Regulatory SNPs were SNPs within the 5' and 3' untranslated regions (UTRs) or upstream SNPs that were generally in promoter regions.

Where a significant association was observed, the gene with the significantly associated SNP was visualised in a LocusZoom plot to illustrate the degree of significance of that SNP and to visually compare the logged P values for each SNP with in a gene of interest. These plots also illustrate the level of LD between the most significant SNP with the rest of the genotyped SNPs in that gene.

Table 3.6: RegulomeDB scoring scheme[1]

Score	Supporting data
1a	eQTL + TF binding + matched TF motif + matched DNase Footprint + DNase peak
1b	eQTL + TF binding + any motif + DNase Footprint + DNase peak
1c	eQTL + TF binding + matched TF motif + DNase peak
1d	eQTL + TF binding + any motif + DNase peak
1e	eQTL + TF binding + matched TF motif
1f	eQTL + TF binding / DNase peak
2a	TF binding + matched TF motif + matched DNase Footprint + DNase peak
2b	TF binding + any motif + DNase Footprint + DNase peak
2c	TF binding + matched TF motif + DNase peak
3a	TF binding + any motif + DNase peak
3b	TF binding + matched TF motif
4	TF binding + DNase peak
5	TF binding or DNase peak
6	other

[1] http://regulome.stanford.edu/help

eQTL: Expression quantitative trait loci, TF: Transcription factor binding site

3.5.1. SNP association tests for anthropometric measures

In the cohort and in women, no *LEP* SNPs showed any association with anthropometric measures for general obesity (Table 3.7). Five *LEPR* SNPs were nominally associated with body weight in men and rs6690661 showed the strongest association with general obesity in men (Table 3.7 and Figure 3.2); however, this association did not remain after correcting for multiple testing. Interestingly, rs6690661 was significantly associated with diastolic BP in women, but not in men (Table 3.10). *LEP* and *LEPR* SNPs were nominally associated with measures of abdominal obesity (Table 3.8) with most of the SNPs being nominally associated with WHR, in a gender specific manner. Interestingly, rs1823718, an ancestry informative marker (AIM) located on chromosome 15 showed a nominal association with skinfold thickness in the entire sample and with body weight in women, but was not associated with any measures of abdominal obesity (Table 3.8).

Table 3.7: SNPs associated with different anthropometric measures for general obesity prior to correction for multiple testing

SNP	Chr*	Gene	Function†	RegulomeDB score‡	P-value		
					Body weight	Body mass index	Skinfold thickness
All							
rs1823718	15	AIM	Intergenic	6	0.0603	0.1405	0.0417
rs9436298	1	*LEPR*		No data	0.021	0.0437	0.4097
Female							
rs1823718	15	AIM	Intergenic	6	0.0186	0.0573	0.0775
Male							
rs11208656	1	*LEPR*	Intronic	6	0.0318	0.0882	0.9103
rs1805096	1	*LEPR*	Coding_S	No data	0.0214	0.0433	0.1741
rs6690661	1	*LEPR*	Intronic	No data	0.0033	0.0111	0.0215
rs6700896	1	*LEPR*	Intronic	3a	0.0323	0.0736	0.1698
rs9436298	1	*LEPR*	Intronic	No data	0.0202	0.0151	0.2302

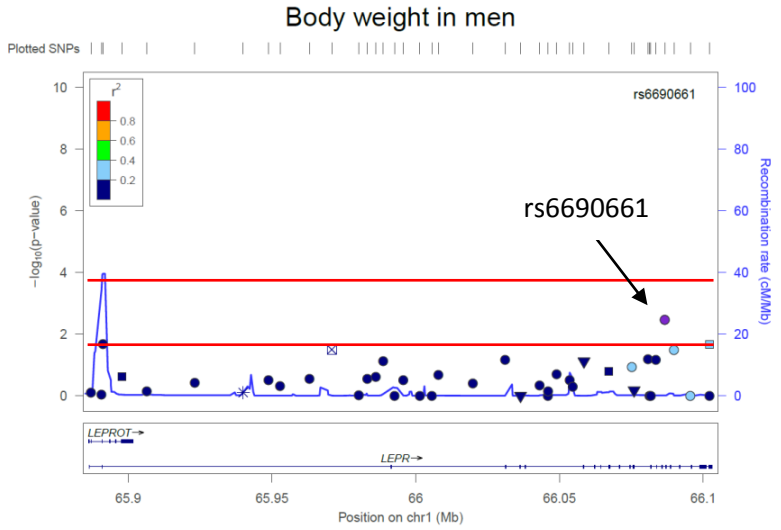
*Chr; Chromosome, †Coding_S; synonymous SNP within coding region and Coding_NS; Non-synonymous SNP within coding region, ‡RegulomeDB score means that a SNP overlaps with: 3a; a Transcription factor (TF) binding site and any motif and a DNase peak, 6; has other regulatory role.

Table 3.8: SNPs associated with different anthropometric measures for abdominal obesity prior to correction for multiple testing

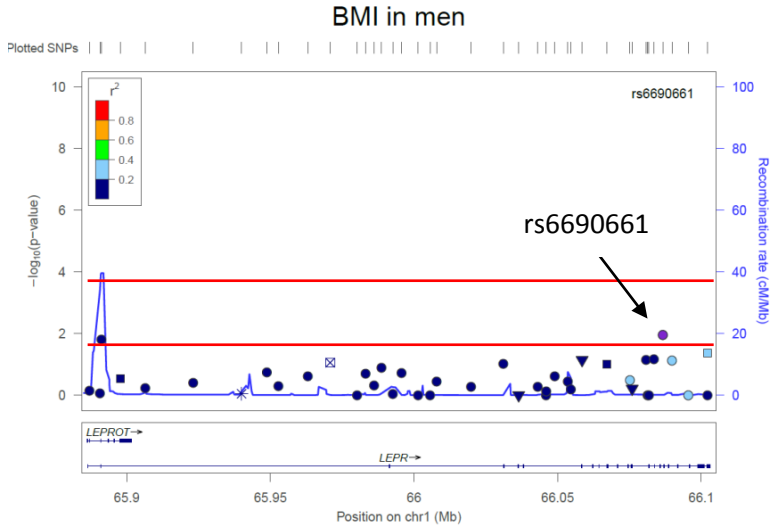
SNP	Chr*	Gene	Function†	RegulomeDB score‡	P-value		
					Waist circumference	Hip circumference	Waist-to-hip ratio
All							
rs1137101	1	*LEPR*	Coding_NS	6	0.03	0.0443	0.5262
rs11801408	1	*LEPR*	Intronic	No data	0.3936	0.032	0.6592
Women							
rs6956510	7	*LEP*	Intronic	6	0.7459	0.1527	0.0471
rs12067730	1	*LEPR*	Intronic	5	0.2174	0.7312	0.035
rs10889556	1	*LEPR*	Intronic	No data	0.4749	0.0423	0.1562
rs7535099	1	*LEPR*	Intronic	6	0.7496	0.2202	0.0412
Men							
rs28959478	7	*LEP*	Regulatory	5	0.0211	0.033	0.3991
rs10493379	1	*LEPR*	Intronic	No data	0.5985	0.4586	0.0427
rs10889556	1	*LEPR*	Intronic	No data	0.3365	0.4005	0.0055
rs10889557	1	*LEPR*	Intronic	No data	0.23	0.8993	0.0227
rs1805096	1	*LEPR*	Coding_S	No data	0.0321	0.2506	0.0174
rs34499590	1	*LEPR*	Coding_S	2b	0.0792	0.5776	0.0257
rs6700896	1	*LEPR*	Intronic	3a	0.0475	0.2828	0.0315
rs8179183	1	*LEPR*	Coding_S	No data	0.0264	0.2622	0.4138
rs735612	15	AIM	Intronic	No data	0.4694	0.0403	0.1034

*Chr; Chromosome, †Coding_S; synonymous SNP within coding region and Coding_NS; Non-synonymous SNP within coding region, ‡RegulomeDB score means that a SNP overlaps with: 2b; a Transcription factor (TF) binding site and matched TF motif and matched DNase Footprint and a DNase peak; 3a; a TF binding site and any motif and a DNase peak, 5; a TF binding site or DNase peak , 6; has other regulatory role.

a.



b.



c.

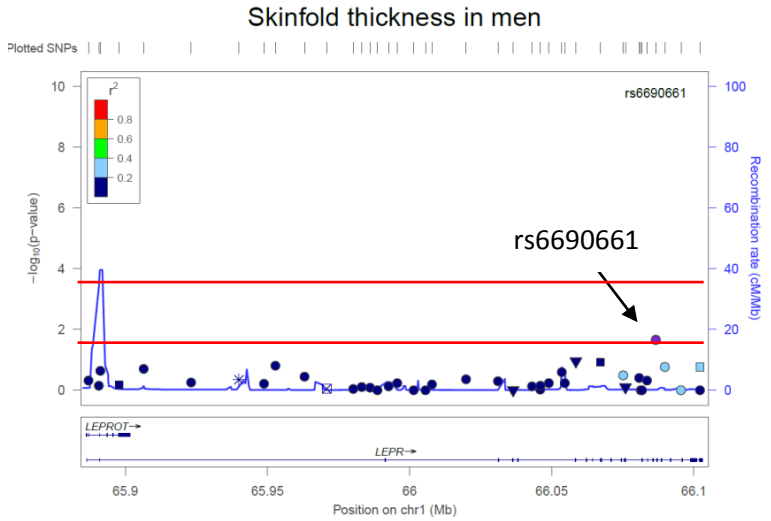


Figure 3.2: LocusZoom plots for *LEPR* in body weight, BMI and skinfold thickness in men. rs6690661 (purple) showed the strongest association with body weight (a), BMI (b) and skinfold thickness (c) in men. The lower red line indicates P≤0.05 and the upper red line indicates P≤0.00076. In the APOGH sample, rs6690661 was not in LD with any markers but appears to be in LD with selected genotyped markers (blue; r^2 between 0.2 and 0.4) in the general African population.

3.5.2. SNP association tests for leptin levels

SNPs in both the *LEP* and *LEPR* genes showed nominal association with leptin levels and only rs17151914 showed a significant association with leptin levels (Table 3.9 and Figure 3.3). *LEP* rs17151914 was significantly associated with leptin levels in the entire sample (P<0.0001) and in women (P=0.0002) but not in men (P=0.064). Although rs17151914 was not associated with leptin levels in men, this SNP showed the strongest association with leptin levels among the *LEP* SNPs (Figure 3.3). The rs17151914 TT genotype was rare in the APOGH sample (TT frequency =1.7% (12/705) in the entire sample, observed in one man) and was not observed in men with leptin level data (Figure 3.4). Although the TT genotype appears to have the highest mean leptin levels, the TT (n=3) sample is very small and there is no clear additive effect of the alleles. rs17151914 has no known regulatory function and is polymorphic in the APOGH sample with similar allele frequencies in HapMap African populations but it is non-polymorphic in non-African populations (Figure 3.5).

LEPR rs1805096, rs1892534 and rs6700896, which are all in LD in the APOGH sample (Figure 3.10), were nominally associated with leptin levels in men but not in women. rs1823718, an ancestry informative marker which showed nominal association with skinfold thickness in the entire sample and with body weight in women, was also nominally associated with leptin levels in men.

Table 3.9: SNPs associated with leptin levels prior to correction for multiple testing

SNP	Chr*	Gene	Function	RegulomDB score†	P-value
All					
rs17151914	7	*LEP*	Intronic	No data	<0.0001
rs1892534	1	*LEPR*	3' UTR	No data	0.0454
rs8179183	1	*LEPR*	Coding_NS	No data	0.0499
rs9436298	1	*LEPR*		No data	0.0181
rs17151914	7	*LEP*	Intronic	No data	0.0002
rs1571151	1	*LEPR*	Intronic	5	0.0102
Male					
rs1805096	1	*LEPR*	Coding_S	No data	0.0123
rs1892534	1	*LEPR*	3' UTR	No data	0.017
rs6700896	1	*LEPR*	Intronic	3a	0.0088
rs8179183	1	*LEPR*	Coding_NS	No data	0.0406
rs1823718	15	AIM	Intergenic	6	0.0264

*Chr; Chromosome, coding_S; synonymous SNP within coding region, Coding_NS; Non-synonymous SNP within coding region, 3' UTR; Within 3' untranslated region, †RegulomeDB score means that a SNP overlaps with: 3a; a Transcription factor (TF) binding site and any motif and a DNase peak, 5; a TF binding site or DNase peak , 6; has other regulatory role.

a. All

b. Women

c. Men

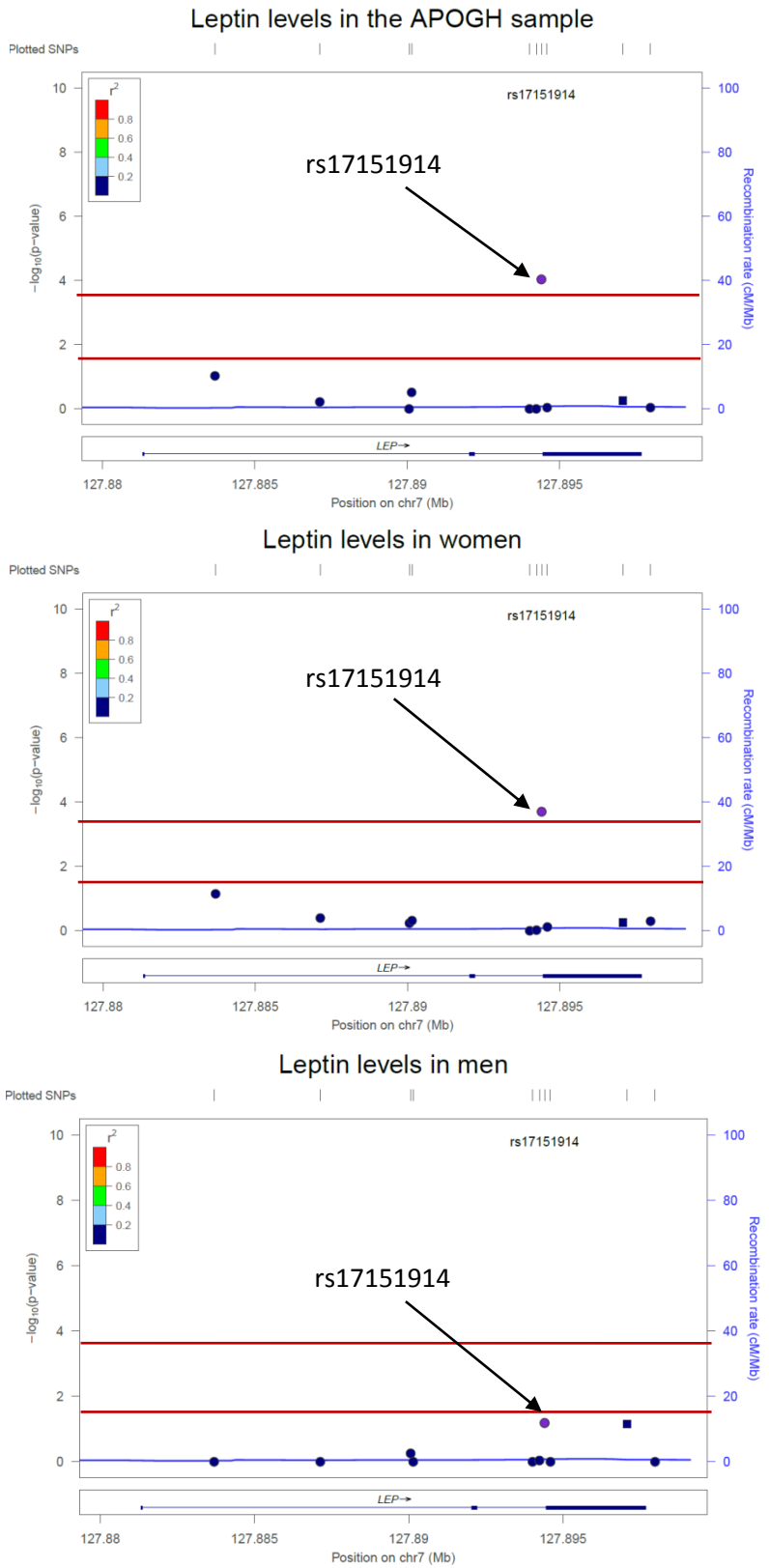
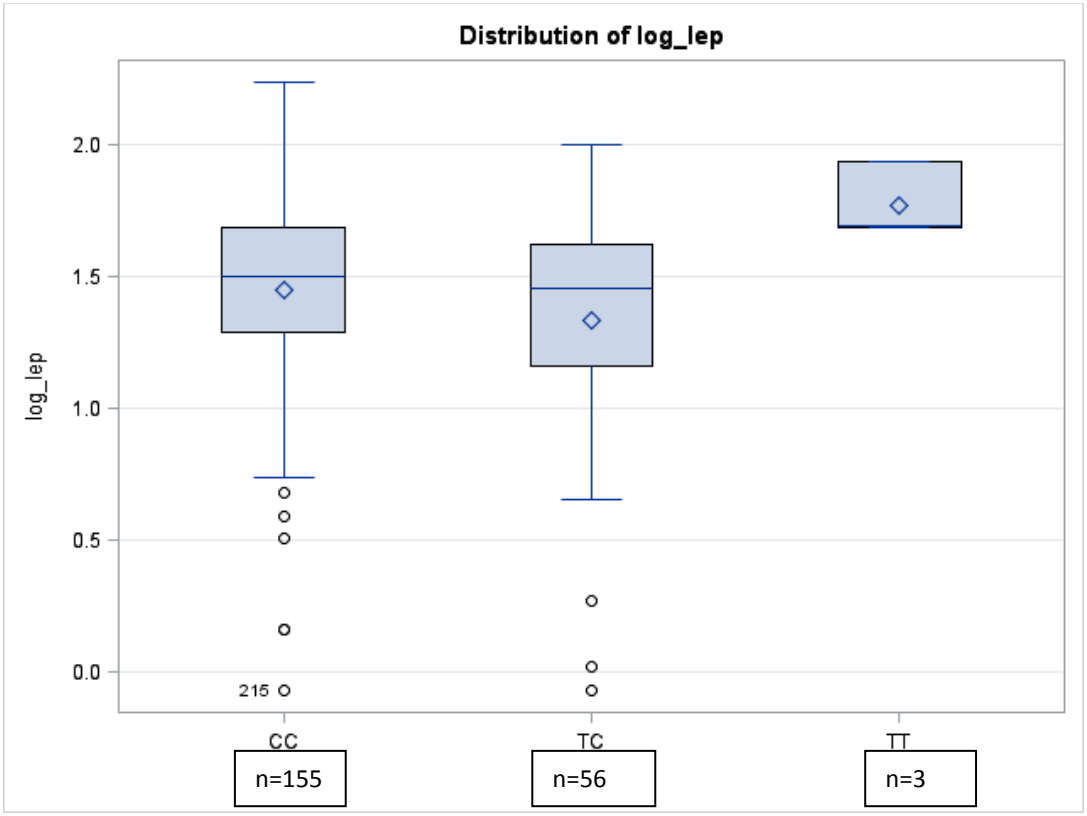


Figure 3.3: LocusZoom plots for *LEP* in leptin levels. rs17151914 showed a statistically significant association with leptin levels in the entire sample (a) and in women (b) but not in men (c) in men. Although the association of rs17151914 with leptin levels was not significant in men, rs17151914 showed the strongest association with leptin levels in men within the *LEP* gene. In the APOGH sample, rs17151914 was not in LD with any markers and this seems to be consistent in the general African population where the marker was not in LD with the other genotyped markers (navy circles; r^2 >0.2).

a. Women



b. Men

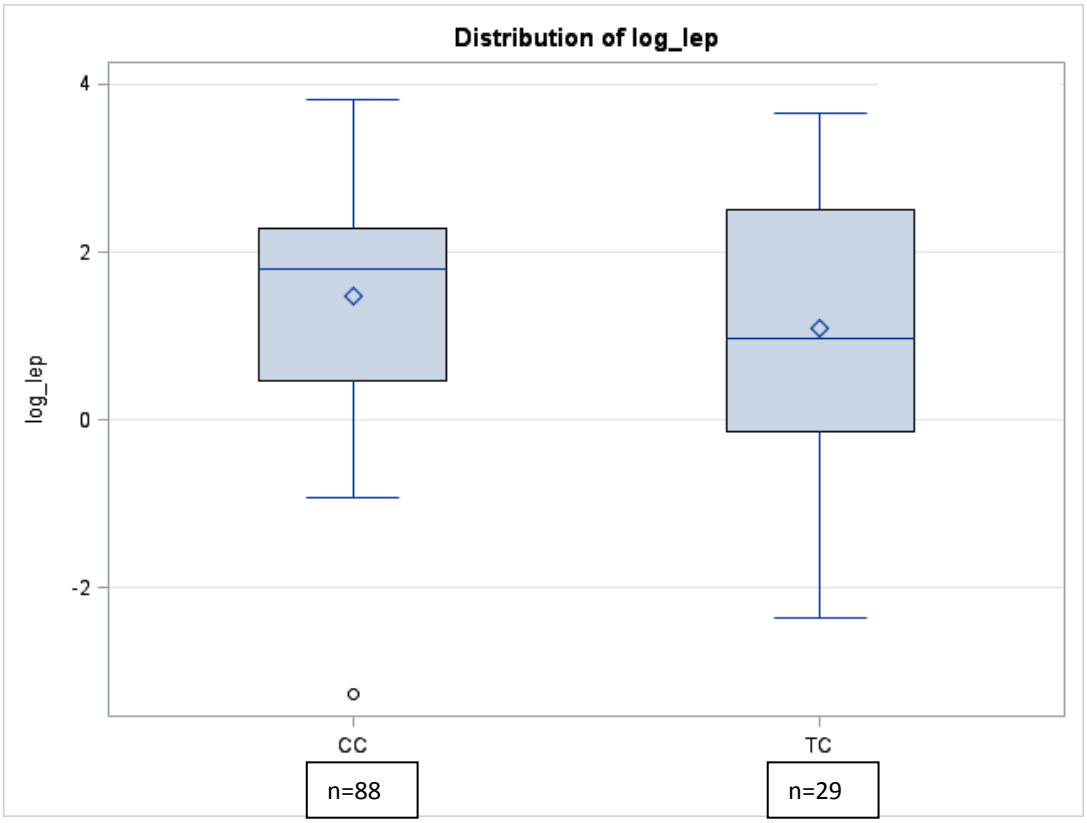


Figure 3.4: Distribution of rs17151914 genotypes in the APOGH sample with leptin level data. a: Distribution of rs17151914 genotypes in women. b: distribution of rs17151914 in men. The TT genotype was not observed in men with leptin level data

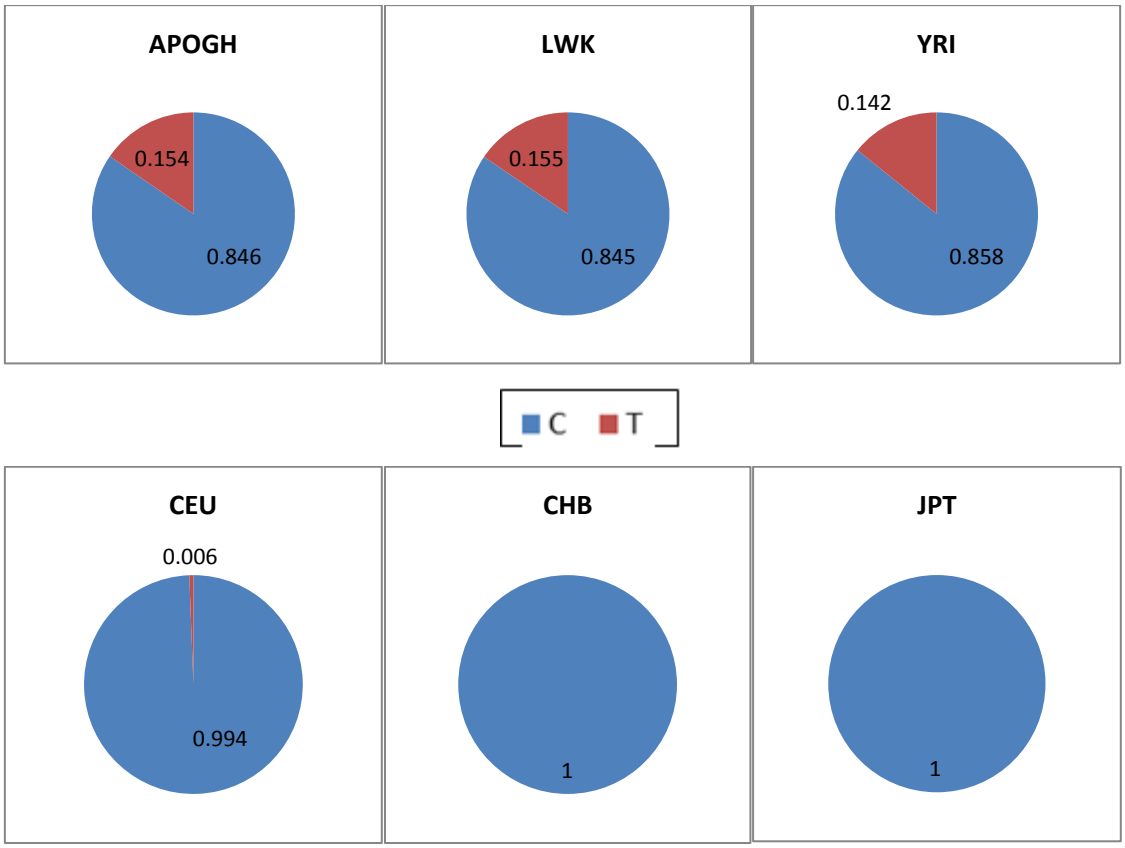


Figure 3.5: Allele frequencies of rs17151914 in APOGH and other populations. rs17151914 is only polymorphic in the African populations (LWK: Luhya in Webuye, Kenya and YRI: Yoruba in Ibadan, Nigeria) but not in non-African populations (CEU: CEPH (Utah residents with ancestry from northern and western Europe), CHB: Han Chinese in Beijing, China and JPT: Japanese in Tokyo, Japan). The T allele of rs17151914 occurs in similar frequencies across APOGH, LWK and YRI.

3.5.3. SNP association tests for blood pressure

LEPR rs6690661 was significantly associated with diastolic BP in women (P=0.0007) but not in men (Table 3.10 and Figure 3.6). The same SNP was nominally associated with diastolic BP in the entire group and since no association was observed in men, the nominal association in the entire sample was driven by the data for women. The distribution of rs6690661 genotypes for diastolic BP in men and women are shown in Figure 3.7. rs6690661 is an intronic SNP with no known regulatory function and is polymorphic in the APOGH sample and across other populations, but the C allele occurs at a higher frequency in African populations (Figure 3.8). rs6690661 showed no association with central systolic BP and systolic BP.

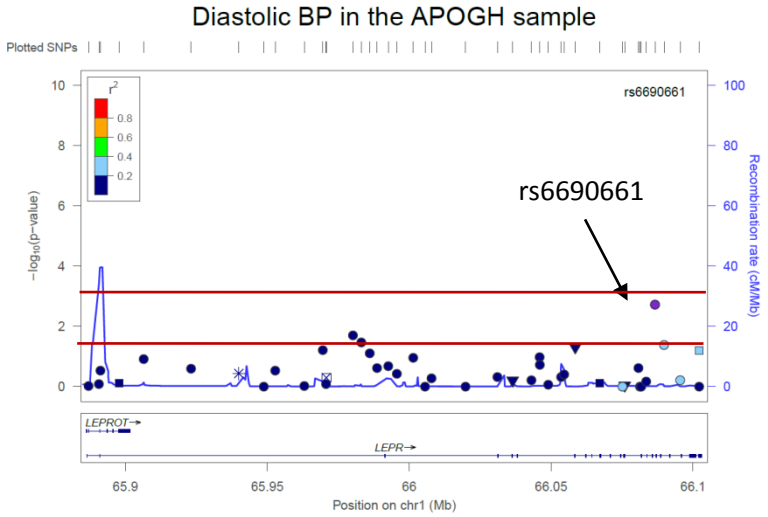
Many SNPs in both the *LEP* and *LEPR* genes showed nominal association with at least one BP measurement. *LEP* rs17151919 was nominally associated with central systolic BP in the entire sample and in men. No *LEPR* SNPs showed an association with diastolic BP and central systolic BP in men, but rs1137100 and rs1171261 were nominally associated with systolic BP in men. *LEPR* rs1137101 which has been shown to be associated with BP in other populations was nominally associated with diastolic BP in women. *LEPR* rs1805096 and rs6700896, which were nominally associated with leptin levels in men, were nominally associated with diastolic BP in women. AIMs rs1344870 and rs1363448 were both nominally associated with central systolic BP in the entire group whereas rs1344870 was also nominally associated with diastolic BP in women.

Table 3.10: SNPs associated with different blood pressure measurements prior to correction for multiple testing

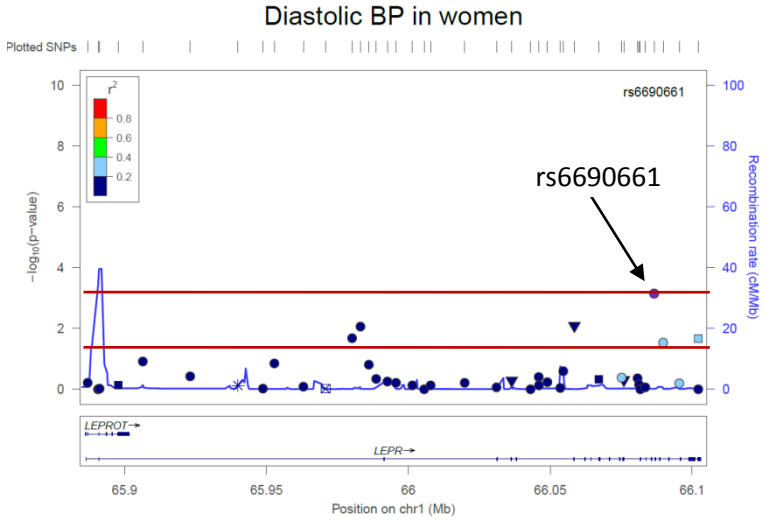
SNP	Chr*	Gene	Function	†RegulomeDB score	P-value		
					Central systolic BP	Systolic BP	Diastolic BP
All							
rs10249476	7	*LEP*	Regulatory	4	0.9013	0.0483	0.0442
rs17151919	7	*LEP*	Coding_NS	No data	0.0025	0.9667	0.09
rs6956510	7	*LEP*	Intronic	5	0.0375	0.4216	0.6301
rs11208656	1	*LEPR*	Intronic	6	0.028	0.673	0.5094
rs1137101	1	*LEPR*	Coding_NS	6	0.3224	0.6592	0.0495
rs1171261	1	*LEPR*	Intronic	No data	0.3276	0.0121	0.111
rs1171273	1	*LEPR*	Intronic	6	0.9861	0.0025	0.0196
rs1475397	1	*LEPR*	Intronic	5	0.2418	0.041	0.0331
rs6690661	1	*LEPR*	Intronic	No data	0.2706	0.1347	0.0019
rs6700896	1	*LEPR*	Intronic	3a	0.6366	0.3651	0.0413
rs1344870	3	AIM	Intergenic	No data	0.0285	0.8233	0.0609
rs1363448	5	AIM	Intronic	5	0.0353	0.8913	0.0553
Female							
rs10249476	7	*LEP*	Regulatory	4	0.7425	0.0169	0.0058
rs11761556	7	*LEP*	3' UTR	6	0.0443	0.0509	0.9984
rs1137101	1	*LEPR*	Coding_NS	6	0.1887	0.5198	0.0082
rs1171273	1	*LEPR*	Intronic	6	0.3308	0.0107	0.0207
rs1475397	1	*LEPR*	Intronic	5	0.5791	0.0233	0.0086
rs1805096	1	*LEPR*	Coding_S	No data	0.1743	0.5368	0.0216
rs6690661	1	*LEPR*	Intronic	No data	0.4545	0.0664	0.0007
rs6700896	1	*LEPR*	Intronic	3a	0.2582	0.4145	0.0289
rs1344870	3	AIM	Intergenic	No data	0.0833	0.6536	0.0463
rs1363448	5	AIM	Intronic	5	0.038	0.8919	0.1861
Male							
rs17151919	7	*LEP*	Coding_NS	No data	0.0072	0.9801	0.0534
rs1137100	1	*LEPR*	Coding_NS	No data	0.2181	0.015	0.5452
rs1171261	1	*LEPR*	Intronic	No data	0.9061	0.0076	0.311
rs679047	9	AIM	Upstream	No data	0.23	0.0296	0.2904

*Chr; Chromosome, Coding_S; synonymous SNP within coding region, Coding_NS; Non-synonymous SNP within coding region, 3' UTR; Within 3' untranslated region, †RegulomeDB score means that a SNP overlaps with: 5; a TF binding site or DNase peak , 6; has other regulatory role.

a. All



b. Women



c. Men

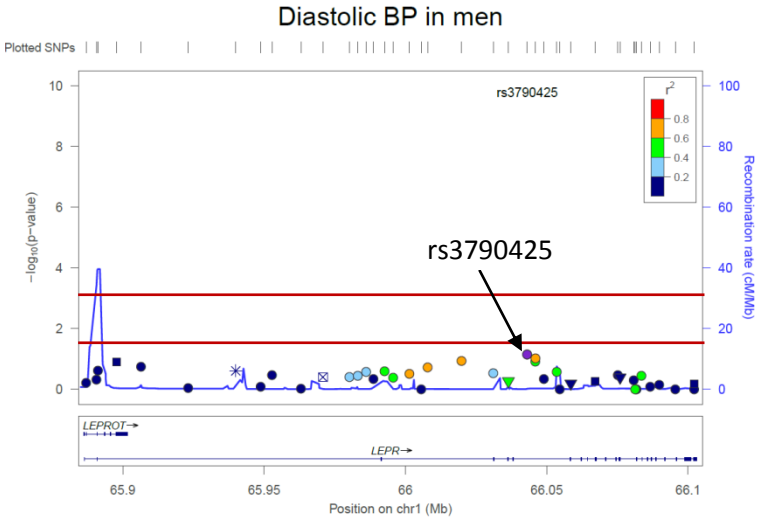
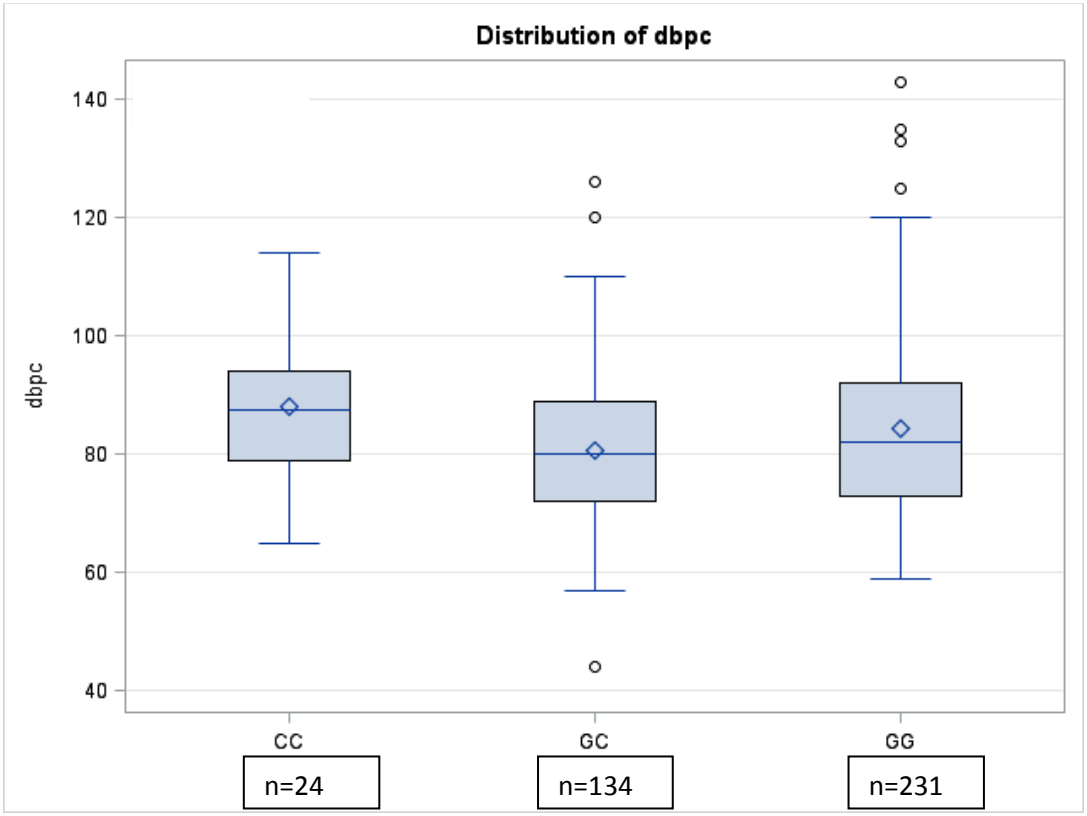


Figure 3.6: LocusZoom plots for *LEPR* in diastolic blood pressure. rs6690661 was nominally associated with diastolic BP in the entire sample (a) and significantly associated with diastolic BP in women (b) but not in men (c). rs3790425, although not significantly associated showed the strongest association with diastolic BP in men among the *LEPR* SNPs.

a. Women



b. Men

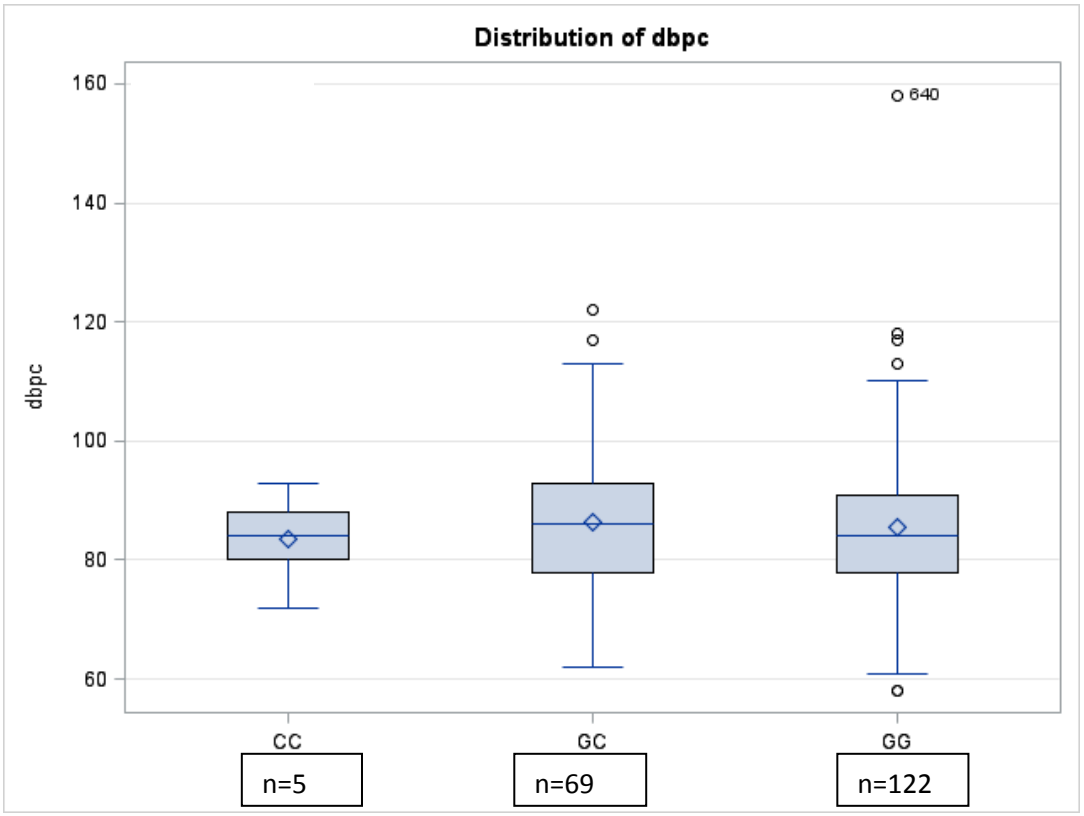


Figure 3.7: Distribution if rs6690661 genotypes in the APOGH sample with diastolic blood pressure (dbpc) data. a: Distribution of rs6690661 genotypes in women. b: distribution of rs6690661 in men.

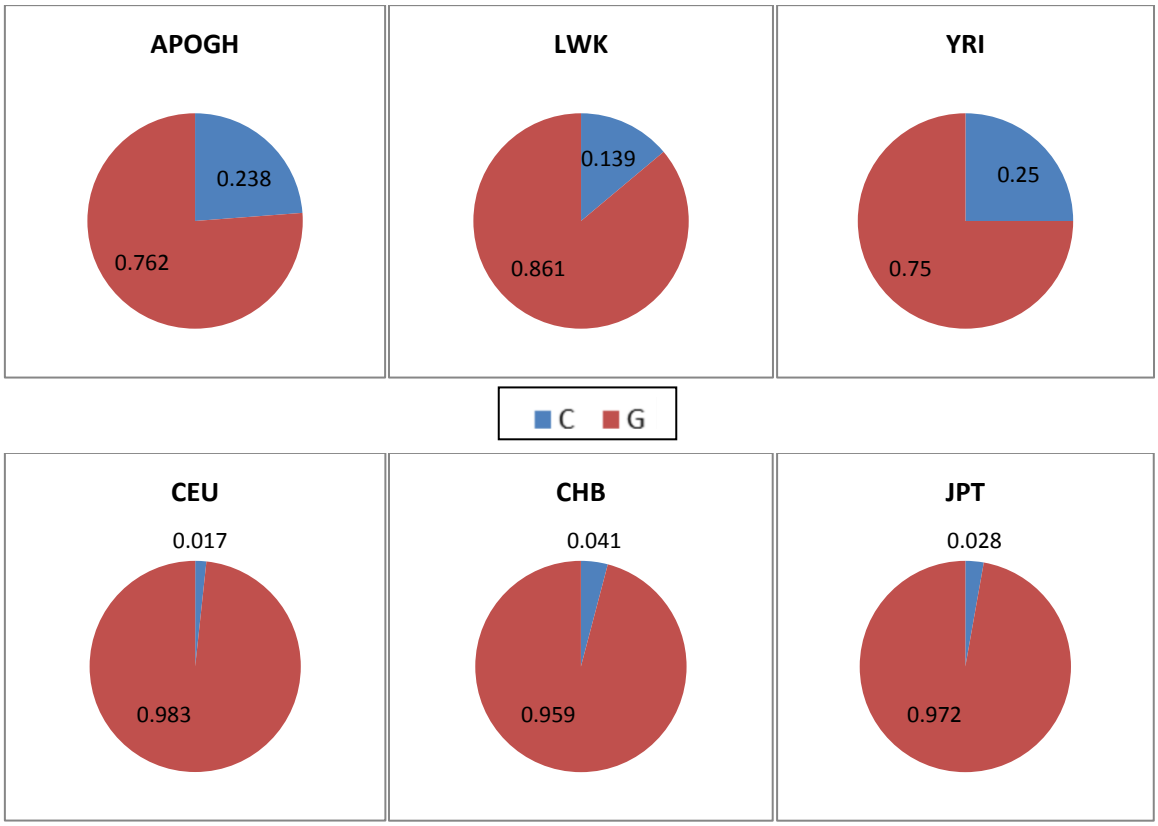


Figure 3.8: Allele frequencies of rs6690661 in African and non-African populations. rs6690661 is polymorphic African populations (LWK: Luhya in Webuye, Kenya and YRI: Yoruba in Ibadan, Nigeria) and non-African populations (CEU: CEPH (Utah residents with ancestry from northern and western Europe), CHB: Han Chinese in Beijing, China and JPT: Japanese in Tokyo, Japan) but the C allele is more common in African populations.

3.5.4. Summary of all significantly associated SNPs with different phenotypes

Table 3.11 summerises all the siginificant results for the SNP association tests. No SNPs showed a significant association with anthropometric measures, systolic BP and central systolic BP. LEP rs17151914 was significantly associated with leptin levels in the etire group and in women. Rs6690661 was significantly associated with diastolic BP in women but in in men.

Table 3.11: Significantly associated SNP after Bonferroni correction

Gene	SNP	Associated phenotype	Sex	P value (After Bonferoni correction)
LEP	rs17151914	Leptin levels	Combined group	0.0066
LEP	rs17151914	Leptin levels	Female	0.0132
LEPR	rs6690661	Diastolic BP	Female	0.0462

3.6. Linkage disequilibrium and haplotype association tests

Linkage disequilibrium (LD), also known as allelic association, within the *LEP* and the *LEPR* genes was determined using the Confidence Interval method (Gabriel et al., 2002) on HaploView. Trios (mother, father and child pedigrees, n= 35) along with singletons (not related to anyone in the sample, n= 228) were used to construct haplotypes but samples with one missing parent automatically were excluded.

Within the *LEP* gene, two SNPs, rs17151913 and rs6956510, were in LD, but the correlation between the SNPs was not very strong (correlation coefficient (r^2) = 0.22) (Figure 3.9). Twelve haplotype blocks with variable correlations were observed in the *LEPR* gene (Figure 3.10 and Table 3.12). The haplotype blocks were small as the largest haplotype block had four SNPs.

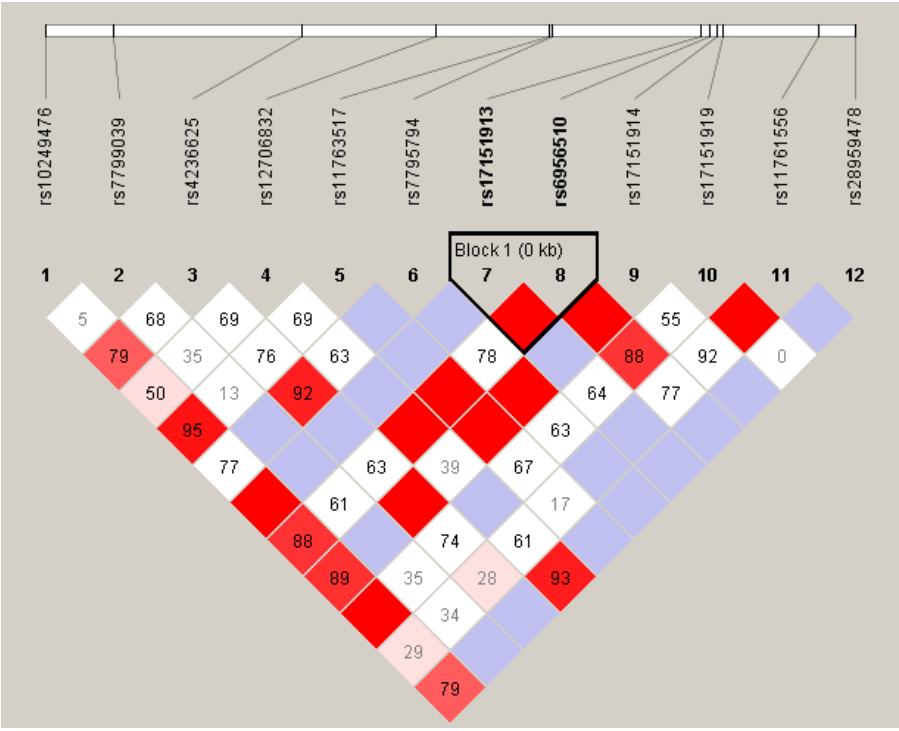


Figure 3.9: Linkage disequilibrium plot of the *LEP* gene. Markers 7 and 8 (in bold) form a haplotype block. Square colours represent the following D' and LOD values; White: D'<1 and LOD<2, blue: D'=1 and LOD<2, shades of pink/ red: D'<1 and LOD≥2 and bright red: D'=1 and LOD≥2. Numbers within squares represent D' and absence of numbers indicates that D'=1.

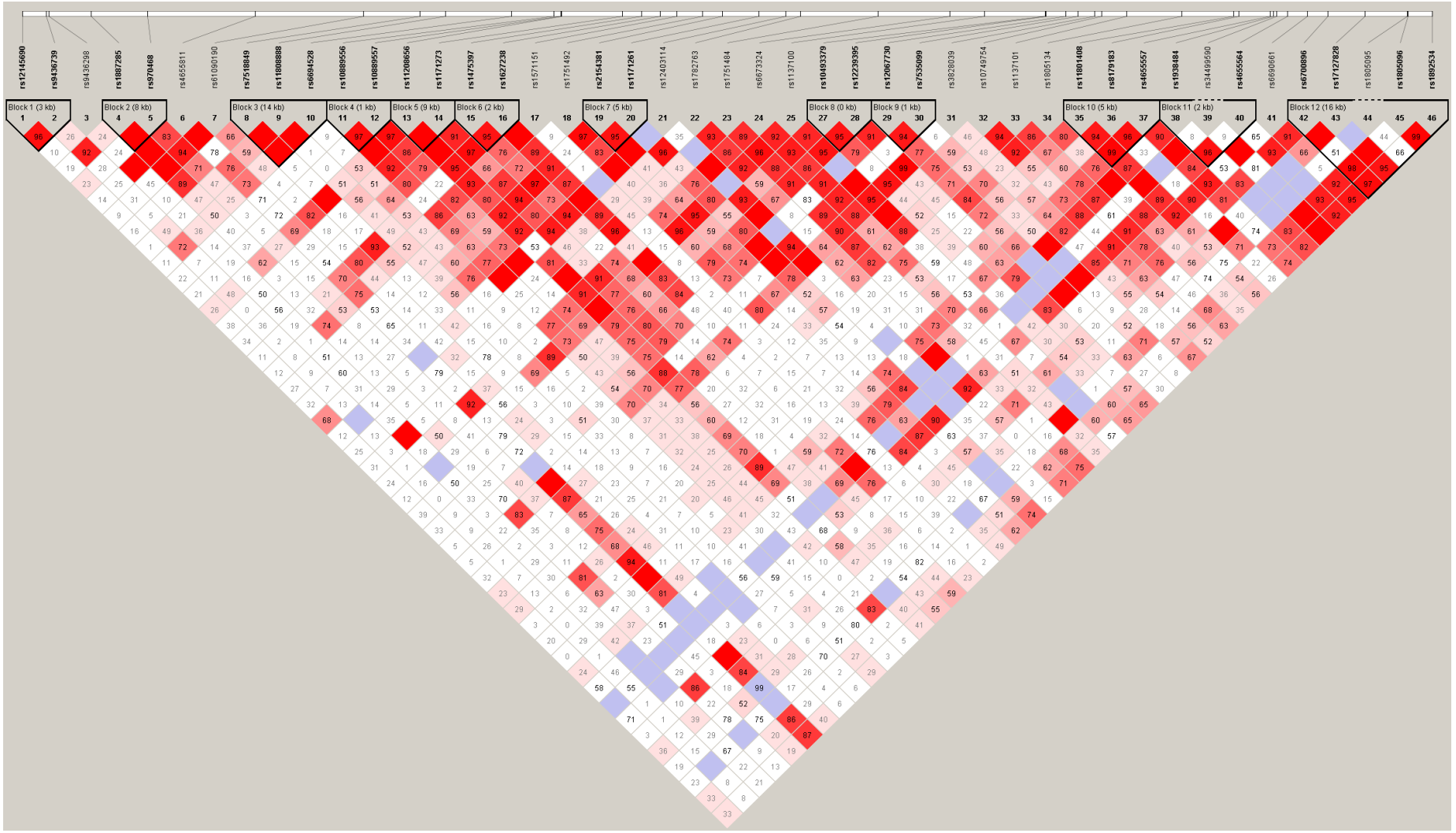


Figure 3.10: Linkage disequilibrium plot of the *LEPR* gene. Markers outlined within bold lines form a haplotype block. Square colours represent the following D' and LOD values; White: D'<1 and LOD<2, blue: D'=1 and LOD<2, shades of pink/ red: D'<1 and LOD≥2 and bright red: D'=1 and LOD≥2. Numbers within squares represent D' and absence of numbers indicates that D'=1

Table 3.12: Pairwise correlations between SNPs within the constructed haplotype blocks in the *LEPR* gene

Haplotype Block	SNP 1	SNP 2	D'	r^2
Block 1	rs12145690	rs9436739	0.965	0.144
Block 2	rs1887285	rs970468	1	0.219
Block 3	rs6694528	rs11808888	1	0.63
	rs7518849	rs6694528	1	0.195
	rs7518849	rs11808888	1	0.313
Block 4	rs10889556	rs10889557	0.974	0.949
Block 5	rs11208656	rs1171273	1	0.11
Block 6	rs1475397	rs1627238	0.956	0.3
Block 7	rs2154381	rs1171261	0.958	0.408
Block 8	rs10493379	rs12239395	0.957	0.798
Block 9	rs12067730	rs7535099	0.945	0.543
Block 10	rs11801408	rs4655557	0.991	0.767
	rs11801408	rs8179183	0.945	0.194
	rs4655557	rs8179183	0.966	0.157
Block 11	rs1938484	rs4655564	0.964	0.868
Block 12	rs17127828	rs1805096	1	0.139
	rs17127828	rs1892534	0.954	0.118
	rs1805096	rs1892534	0.992	0.91
	rs6700896	rs17127828	1	0.153
	rs6700896	rs1805096	0.985	0.895
	rs6700896	rs1892534	0.976	0.822

3.6.1. Haplotype analysis

Haplotype analysis was done on gPLINK using haplotypes constructed on Haploview (Figures 3.9 and 3.10). gPLINK haplotype analysis does not allow simultaneous correction for relatedness and multivariate models, therefore only unrelated individuals were used for the haplotype analysis (n=353).

Constructed haplotypes were tested for associations with anthropometric data, leptin levels and BP measurements in the entire sample (n=353) and in men (n=119) and women (n=234) separately. No haplotypes were significantly associated with anthropometric measures or leptin levels after correcting for multiple testing using the Bonferroni correction. The observed haplotype in *LEP* was significantly associated with central systolic BP in women and *LEPR* haplotype block 7 was significantly associated with systolic BP in men.

The *LEP* haplotype formed by rs17151913 and rs6956510 was significantly associated with central systolic BP in women but not in men (Table 3.13). The TG haplotype which remained statistically significant after correcting for multiple testing is associated with an increase in central systolic BP by 2.92 mm Hg. The TA haplotype was nominally associated with a decrease in central systolic BP (-3.01 mm Hg) but was no longer significant after correcting for multiple testing. rs6956510 appears to drive the association of the haplotype with central systolic BP. Firstly, for the TG and TA haplotypes which have opposite effects on central systolic BP, the T allele from rs17151913 is constant whereas when rs6956510 alleles are changed, central systolic BP either increases or decreases. Secondly, rs6956510 was nominally associated with central systolic BP in the entire sample suggesting that this SNP influences central systolic BP.

Table 3.13: rs17151913-rs6956510 haplotypes in *LEP* associated with central systolic blood pressure in women

Haplotype*	Frequency	Beta	P	P†
TG	0.8322	2.92	0.004	0.012
TA	0.1095	-3.01	0.023	0.069
AA	0.0583	-2.44	0.127	0.381

*Alleles listed in rs17151913-rs6956510 order, Beta; effect size, on central systolic blood pressure per haplotype, †P value after correcting for multiple testing using Bonferroni method using 3 tests/haplotypes.

LEPR haplotype block 7 (rs2154381 and rs1171261) was associated with systolic BP in men but not in women. The CT haplotype which remained statistically significant after correcting for multiple testing is associated with a significant decrease in systolic BP by 14.8 mm Hg. The TC haplotype was nominally associated with an increase in systolic BP (8.51 mm Hg) but was no longer significant after correcting for multiple testing. Table 3.14 shows the haplotypes in block 7 and their effect sizes in systolic BP (Beta).

Table 3.14: rs2154381-rs1171261 haplotypes in *LEPR* associated with systolic blood pressure in men

Haplotype*	Frequency	Beta	P	P†
CT	0.116	-14.8	0.0009	0.036
TC	0.759	8.51	0.013	0.505
CC	0.125	0.04	0.993	1

*Alleles listed in rs2154381-rs1171261 order, Beta; effect size, on systolic blood pressure per haplotype, †P value after correcting for multiple testing using Bonferroni method using 38 tests/haplotypes.

4. Discussion

Obesity and hypertension are common risk factors for cardiovascular disease. Both disorders have increased drastically in the past two decades, mainly as a result of a sedentary lifestyle and poor diet. Environmental factors play a critical role in the development and progression of both disorders but genetic variation also has an essential role in disease susceptibility. Leptin and its receptor are essential in appetite regulation and leptin has been shown to increase BP by activating the sympathetic nervous system and is therefore important in body composition and BP regulation (Ailhaud, 2006, Barba et al., 2003), making the *LEP* and *LEPR* genes which code for leptin and its receptor good functional candidate genes for obesity and hypertension. Single nucleotide polymorphisms (SNPs) in the *LEP* and *LEPR* genes have been shown to associate with obesity, hypertension and leptin levels in different populations (Ben Ali et al., 2008, Ma et al., 2009). Recent studies have shown that SNPs in the *LEP* gene influence BMI in black South African adolescents (Lombard et al., 2012) and *LEPR* SNPs influence BMI and WC in black and mixed-ancestry South African adolescents (Yako et al., 2011). Due to the compelling evidence that *LEP* and *LEPR* variants influence measures of obesity in black South African adolescents, we wanted to validate the role of these genes in obesity in a black adult South African sample.

The aims of this study were to determine the heritability estimates of anthropometric and BP measures and leptin levels; to identify additional informative SNPs in and around the *LEP* and *LEPR* genes; and to examine the potential relationships between these SNPs and measures of obesity, hypertension and leptin levels in a black South African cohort.

Overweight (24%), obesity (42%) and hypertension (46%) were highly prevalent in the sample (Table 3.1). Sex-specific differences were noted for all anthropometric measures and leptin levels with women having significantly higher measures than men. All tested anthropometric and BP measures

were significantly heritable but leptin levels were not heritable. No *LEP* SNPs showed significant associations with anthropometric measures and BP but rs17151914 was significantly associated with leptin levels in the entire sample and in women. A *LEP* haplotype (rs17151913T-rs6956510G) was significantly associated with an increased risk for elevated systolic BP in women. None of the *LEPR* SNPs were significantly associated with anthropometric measures, leptin levels and systolic and central systolic BP, but rs6690661 which was nominally associated with general obesity measures in men, was significantly associated with diastolic BP in women but not in men. A *LEPR* haplotype (rs2154381-rs1171261) was significantly associated with lower systolic BP in men but not in women. All associations were sex-specific, ie. only associated with men or women.

4.1. Prevalence of obesity and hypertension in the APOGH sample

In this study the prevalence of overweight (24%) and obesity (42%) was high in the entire sample with 67% of the entire sample being either overweight or obese (Table 3.1). A significant difference in the prevalence of overweight/obesity was noted between men and women (50.5% in men and 75% in women were overweight or obese, respectively (Table 3.1). The sex-specific differences concur with previously published data (Motala et al., 2011a) but the prevalence in the APOGH sample is significantly different from the prevalence observed in 949 individuals living in rural KwaZulu Natal (KZN) (Motala et al., 2011a). As expected when comparing overweight and obesity prevalence in urban against rural settings, the prevalence was much lower in the KZN rural sample (21.7% and 47.3% prevalence of overweight/obesity in men and women, respectively) (Motala et al., 2011a, Puoane et al., 2002). These sex-specific differences are more common in developing countries whereas the prevalence of overweight and obesity is similar in men and women of developed countries (Friedlander et al., 2010, Motala et al., 2011a). It is also interesting to note that when analysing overweight and obesity separately, obesity is less common in men whereas obesity is more common in women (Table 3.1).

Sex-specific differences were also observed for WC in the APOGH sample and other populations (Friedlander et al., 2010). On average, men had WC below the International Diabetes Federation (IDF) set abdominal obesity (a component of metabolic syndrome) cut-off values for diagnosing metabolic syndrome in Southern African men (≥94 cm) whereas females were well above the cut-off set for women (≥80 cm) (Alberti et al., 2009). The prevalence of overweight/obese (50.5% in men and 75% in women) was similar to the levels of elevated WC (55.2% in men and 70.4% in women) (Table 3.1). The trend in WC values observed in men and women in the APOGH samples was similar to results observed in rural black South Africans (from KZN)where the mean WC was lower than the set cut-off in men (83.1 cm) and the mean was above the cut-off in women (86 cm) (Motala et al., 2011a). However, the frequency of men above the IDF cut-off was significantly lower in Zulu men (18.5%) compared to the APOGH sample (55.2%), but the differences between women in the APOGH sample (70.4%) and the KZN women (63.3) were not significantly different (Motala et al., 2011a). Similar trends of sex-specific differences were also observed for other anthropometric measurements, leptin levels and glucose intolerance where values (with the exception of height) were significantly higher in women, correlating with larger body mass in women.

Hypertension has been reported to have the highest prevalence in the African region (according to the WHO) compared to the rest of the world. In South Africa; black individuals have the highest prevalence of hypertension compared to other race groups (Connor et al., 2005, Seedat, 1999, World Health Organisation, 2012). In this study, the prevalence of hypertension was 46% and there were significant differences between men and women (Table 3.1). The prevalence of hypertension in the APOGH sample (46%) is similar to the prevalence estimated by the WHO (2012) for 2008 (42.2%), but lower than the prevalence reported by Connor et al., (2005) in different South African races (59% in blacks, 55% in Coloured and Asians and 50% in whites) (Connor et al., 2005, World Health Organisation, 2011a). Seedat (1999) reported a prevalence that varied from 20-25% in blacks, which

was the highest when compared to other races (14-19% in Indians and in 17.2% whites). The variability in prevalence of hypertension in black South Africans may be due to differences in sample size and that some studies were conducted in rural areas whereas others were conducted in urban areas.

Overweight and obesity are major risk factors for hypertension and the prevalence of hypertension is doubled in obese individuals compared to non-obese individuals (Kotchen, 2008). In the APOGH sample the prevalence of joint occurrence of overweight/obesity and hypertension, also known as obesity-related hypertension, was high (38%) (Figure 3.1) (Kotchen, 2010), showing that a large number of these individuals have at least two risk factors for cardiovascular disease. The high prevalence of elevated WC and overweight and obesity is correlated with the high prevalence of hypertension in the APOGH sample. The high prevalence of joint occurrence of overweight/obesity and hypertension in the sample supports the notion that adiposity measures are highly correlated with BP (Bell et al., 2002, Warren et al., 2012).

Due to the significant differences between men and women, SNP and haplotype analyses were done in the entire group and analysed in men and women separately. Although no significant inter-gender differences were noted for BP between men and women, sex-specific analyses were done for BP because BP is strongly influenced by anthropometric measures and all BP analyses were adjusted for WC (Majane et al., 2007).

4.2. Heritability

Obesity and hypertension are multifactorial disorders that are influenced by environmental and genetic factors. Anthropometric and blood pressure measures are heritable in African and non-African populations yet the heritability estimates vary widely in different populations (Elder et al.,

2009). Heritability estimates for anthropometric measures, BP and leptin levels have only been reported in a small number of African populations and even in these populations, not all measures such as skinfold thickness have heritability estimates. With the exception of height, parents had significantly higher anthropometric measures, leptin levels, BP and a higher prevalence of type II diabetes, overweight, obesity and hypertension than the children. In this study, after adjusting for various confounders (Table 2.8), height, body weight, BMI, skinfold thickness, waist and hip circumference, WHR, systolic and diastolic BP and central systolic BP were significantly heritable whereas leptin levels were not significantly heritable (Table 3.4).

Height has repeatedly been shown to be highly heritable in different populations and was highly heritable (76%) in the APOGH sample. Height heritability estimates were lower than those observed in Caucasian twins (91%) (Elder et al., 2009) and estimates similar to those in the APOGH sample were reported in Jamaicans (74%) (Luke et al., 2001). APOGH height estimates were higher than estimates observed in Nigerians (62%) and African Americans (43%) (Luke et al., 2001).

In this study, the heritability of body weight was 38% whereas the estimates were 62% in Caucasians (Elder et al., 2009), 56% in Nigerians and African Americans and 55% in Jamaicans (Luke et al., 2001). Although heritability estimates are similar in other populations of African descent (Nigerians, Jamaicans and African Americans), these estimates are much lower in the APOGH sample. BMI estimates were also much lower in the APOGH sample compared to other African populations. In the APOGH sample, the heritability of BMI was 26% whereas the estimates in Jamaicans were 58% (Luke et al., 2001) and varied from 48%-70% in Nigerians (Hicks et al., 2007, Luke et al., 2001). In African Americans, the BMI heritability estimates vary from and 45-64% (Hicks et al., 2007, Luke et al., 2001, Sale et al., 2005). BMI estimates in non-African populations were comparable with those in African populations. BMI heritability ranged from 40-50% in Caucasians (Elder et al., 2009, Pausova et al.,

2001) and was 55% in the Chinese (Luo et al., 2010). The heritability of BMI is much higher in other populations even when only age and sex are adjusted for as in other studies (See table in Appendix J). The heritability estimates may also be much lower in the APOGH sample because of the small sample size used in this study and the fact that in most studies, twins are used whereas in this study, parent-child pairs were used. Skinfold thickness was also heritable in this study but could not be compared with other populations because in this study, the heritability estimates for the average of the triceps and subscapular skinfold thickness were tested whereas many studies report the estimates for triceps and subscapular skinfolds separately.

The heritability of WC was 35% in the APOGH sample and was similar to estimates in French Canadians (38%) and smaller than those observed in the Chinese (42%) (Luo et al., 2010) and non-French Canadian Caucasians (41-76%) (Elder et al., 2009, Fox et al., 2004, Rose et al., 1998). Hip circumference (42%) and WHR (46%) had heritabilities similar to those observed in Caucasians (Hip circumference= 42% and WHR= 38-42%) (Pausova et al., 2001, Rose et al., 1998).

Leptin levels (15%, P=0.228) were not significantly heritable in the APOGH sample, in contrast to results observed in other populations. In Jamaicans, the heritability of leptin levels was 25% and was even higher in Nigerians (38%) and African Americans (43%) (Luke et al., 2001). A small number of individuals in the sample had leptin level data therefore the sample size used to obtain heritability estimates was very small (only 107 parent child pairs and 43 sibling pairs). Leptin levels appear to be heritable in African populations (Luke et al., 2001) and the lack of association in the APOGH sample may be a consequence of a small sample size.

Systolic BP (34%), diastolic BP (27%) and central systolic BP (33%) were all significantly heritable. The heritability estimates of systolic BP (34%) was higher than the estimates in Chinese (21%) (Luo et al.,

2010), but lower than estimates in Caucasians (45-64%) (Elder et al., 2009, Snieder et al., 2003, Tarnoki et al., 2012). Although the heritability estimates of systolic BP in African Americans (56%) (Snieder et al., 2003) were high, genetics appear to have smaller effects on systolic BP in non-admixed African populations. In Nigerians, the heritability of systolic BP was 34%, identical to the estimates observed in black South Africans (APOGH) (Adeyemo et al., 2002). Diastolic BP (27%) estimates were higher than estimates in Chinese (20%) (Luo et al., 2010) but lower than estimates observed in Caucasians (38-45%) (Elder et al., 2009, Snieder et al., 2003, Tarnoki et al., 2012). Like systolic BP, the estimates of diastolic BP were much higher in African Americans (58%) (Snieder et al., 2003), compared to non-admixed African populations. The estimates in Nigerians (30%), a non-admixed population, were similar to estimates observed in the APOGH sample (27%) (Adeyemo et al., 2002).

Central systolic BP, which is a better predictor of risk for target organ damage (Roman et al., 2010, Tarnoki et al., 2012) was significantly heritable in the APOGH sample. In this study the heritability estimates for central systolic BP was 33%. In Caucasian twins, the heritability was almost double (60%) (Tarnoki et al., 2012), but was much lower in Caucasian female twins (18%) (Snieder et al., 2000). The estimates in the female Caucasians were identical (18%) to the estimates observed in a larger APOGH sample (Redelinghuys et al., 2012, Snieder et al., 2000). Although the APOGH sample used by Redelinghuys et al. (2012) was from the same cohort, the sample sizes in the two studies were different. The difference in heritability estimates may be due to the difference in sample size and the variability in covariates that were adjusted for in the two studies. In this study, we added WC as an adiposity covariate instead of BMI (Redelinghuys et al., 2012) because Majane et al. (2007) showed that WC is a better predictor for cardiovascular disease in the APOGH sample.

To the best of our knowledge, this is the first study to describe the heritability estimates of the tested anthropometric measures, BP measures (except central systolic BP (Redelinghuys et al., 2012)) and leptin levels in a black South African sample. These results indicate that genetic factors are important susceptibility factors for obesity and hypertension in black South Africans. Because the heritability estimates tend to be lower, it may suggest that environmental factors which can often be controlled are more variable in this group and may have a disproportionate effect on obesity and hypertension in this cohort. The lower estimates in the APOGH sample may also in part, be due to the small sample size used for heritability portion of the study. Although environmental factors are large contributors to obesity and hypertension traits, environmental stimuli may activate genes in gene-environment interactions that result in obesity and hypertension, therefore understanding the genetic basis of obesity and hypertension related traits is still important. Some individuals are exposed to the same environmental risk factors for obesity and hypertension, yet some individuals never become overweight/obese or hypertensive whereas others do. Understanding which genetic factors protect against weight gain and hypertension even in the presence of detrimental environmental factors will help us understand pathways involved in adiposity and BP regulation and may provide target genes for targeted interventions to ameliorate the adverse effects of obesity and hypertension.

4.3. SNP and haplotype associations with obesity and hypertension traits

After adjusting for appropriate confounding factors (Table 2.8), many SNPs across the *LEP* and *LEPR* genes showed an association with anthropometry, leptin levels and BP, in a sex-specific manner. In accordance with previous studies in other populations, the association of many of the SNPs that showed nominal associations with the tested measures in the study were inconclusive. All the SNPs that had previously been shown to be associated with anthropometric measures, leptin levels or BP in other studies were either non-polymorphic or were not significantly associated with the measures tested in this study.

Since multiple SNPs were tested for association, correction for multiple comparisons/testing was done using the Bonferroni correction which is commonly used in association studies. This method addresses type 1 errors (false positives) (Gelman et al., 2012, Rothman, 1990) and the appropriate (significant) P value is based on the number of tests (i.e. alpha =0.05/number of tested SNPs). The Bonferroni correction is very stringent and corrects for type 1 errors at the cost of type 2 errors (false negatives) which may lead to significant associations being ignored (Gelman et al., 2012, Rothman, 1990). In this study, all associations with P≤0.05 were discussed. All associations that remained significant after Bonferroni correction (P≤0.00076) were regarded to be statistically significant and all associations that had 0.05≤P<0.00076 were considered as nominal associations. Significant and nominal SNP associations for each gene are discussed in sections 4.3.1 and 4.3.2.

4.3.1. *LEP*

4.3.1.1 *LEP* rs17151914 is significantly associated with leptin levels in women

rs17151914, an intronic SNP was significantly associated with leptin levels in females (P=0.0002) but not in males (P=0.064) (Table 3.9) and was not associated with any of the other tested phenotypes. In the combined sample of males and females, rs17151914 was significantly associated with leptin levels, but since no association was observed in males alone, the association observed in the combined group is driven by the females in the sample (n=220). rs17151914 has not been previously associated with any phenotypic data in any population.

rs17151914 is not polymorphic in non-African HapMap populations (CEU, CHB and JPT) but the MAF of rs17151914 in the APOGH sample is very similar to MAF observed in YRI and LWK population (Figure 3.5). Most association studies have been conducted in European and Asian populations and most chips used in GWAS are designed based on European SNP data therefore rs17151914 would be excluded from many commercial chips used for GWAS. rs17151914 is an intronic SNP and no data

were found on RegulomeDB to suggest that the SNP has a regulatory function so it may be in LD with a functional SNP that impacts leptin levels. In the combined LWK sample, rs17151914 was in strong LD with two intronic SNPs (rs69776701 and rs57191509) and a 3'UTR SNP (rs6966536). In the APOGH sample, rs17151914 may be in LD with rs6966536. Since 3' UTRs contain sequences involved in mRNA stability and post transcriptional regulation (Wilkie et al., 2003), rs6966536 may affect binding of regulatory proteins and miRNAs that would affect the levels of leptin mRNA to be translated. Since rs6966536 and rs17151914 (r^2=0.839) are in strong LD in the LWK and possibly the APOGH sample, the effects of rs6966536 on leptin levels may be reflected in the strong association of rs17151914 with leptin levels. Although the association between rs17151914 and leptin levels is very significant, the results should be analysed with caution because the power of this study is reduced because of the small number of individuals with leptin level data (n=218 and n=339 in women and the entire group, respectively).

4.3.1.1. Nominally associated SNPs within the *LEP* gene

rs7799039 which has been shown to be associated with measures of obesity, leptin levels and BP in other populations was non-polymorphic in the APOGH sample and could not be analysed any further (Ben Ali et al., 2008, Ben Ali et al., 2009b, Hinuy et al., 2010, Jiang et al., 2004, Ma et al., 2009, Wang et al., 2006). rs11763517 which was associated with BP and BMI in white women (Ma et al., 2009) was also not associated with any of the tested measures in the APOGH sample.

rs17151919, a non-synonymous (benign/tolerated) SNP, with no known regulatory functions, was nominally associated with central systolic BP in the entire sample and in men but not in women (Table 3.10), implying that the association observed in the entire sample is driven by the association observed in men (33% of the sample). rs17151919 was not associated with diastolic and systolic BP or any of the anthropometric measures. The lack of association between rs17151919 and

anthropometric data contradicts results reported in other African populations. In a study by Friedlander et al. (2010), the minor allele (A) of rs17151919 was associated with an increase in weight, BMI, WC and leptin levels in African-Americans but not in Caucasians (Friedlander et al., 2010). In a South African study by Yako et al. (2011), rs17151919 along with rs2167270 and rs111650308 formed a haplotype that was associated with BMI and hip circumference in black and mixed-ancestry adolescents. Although the APOGH sample and the sample used in the Yako study are both South African, results observed in the Yako study were not replicated in the APOGH sample possibly due the small size in the Yako paper (n=79 black Africans), or because rs17151919 associated as part of a haplotype in the Yako paper and because the two samples were not age matched (adolescents vs. adults).

rs10249476 was nominally associated with systolic BP and showed a stronger association with diastolic BP in the entire group and in women, but not in men (Table 3.10). Since the association was much stronger in women compared to the whole group and no association was seen in men, we can assume that the association in the entire sample is driven by the strong association in women. rs10249476 has previously been associated with obesity (Jiang et al., 2004) but was not associated with any measures of obesity or leptin levels in this study. rs10249476 falls within a regulatory region upstream of the *LEP* gene and falls within a transcription factor binding site and within a DNAse hypersensitivity peak and may therefore affect regulation of the *LEP* gene.

rs28959478, which is located downstream of the *LEP* gene and falls within a DNAse hypersensitivity region, was nominally associated with waist and hip circumference but not WHR in men (Table 3.8). The associations of rs28959478 with anthropometric measures, leptin levels and BP have not previously been studied. rs11761556, a 3' UTR SNP, showed a nominal association with central systolic BP in women (Table 3.10) but not with any other measurements. Lombard et al. (2012) also

found no association between rs11761556 and BMI in black South African adolescents. No studies have reported an association of rs11761556 with BP but rs11761556 was associated with type II diabetes and low-density lipoprotein levels (Han et al., 2008) and osteoarthritis in normal and overweight individuals but not in obese Korean women (Qin et al., 2010).

rs6956510 was nominally associated with WHR in women (Table 3.8), but not in men and was not associated with any of the other anthropometric measures. rs6956510 was also nominally associated with central systolic BP in the entire sample, but not with systolic or diastolic BP (Table 3.10). rs6956510 has not been previously studied for association with obesity, hypertension and leptin levels.

4.3.1.2. rs17151913-rs6956510 haplotype is significantly associated with central systolic BP in women

The only observed *LEP* haplotype block formed by markers rs17151913 and rs6956510 was significantly associated with central systolic BP in women (Table 3.13). The haplotype TG (rs17151913 T allele and rs6956510 G allele) was significantly associated with an increase in central systolic BP whereas the TA haplotype was nominally associated with decreased central systolic BP. rs6956510, but not rs17151913, was nominally associated with central systolic pressure in the entire sample (Table 3.10) and since only rs6956510 allele changes appear to affect central systolic BP, we can assume that the association of the haplotype is driven by rs6956510. We can also conclude that rs6956510 G allele is the risk allele for increased central systolic BP and expect a higher frequency of the G allele (frequency=0.8338) and TG haplotype (frequency=0.8322 in men) in this population due to the high prevalence of hypertension.

No previous association between these haplotypes and the SNPs forming the haplotype have been reported for central systolic BP or any BP measures. Interestingly, rs17151913 showed an association with longitudinal weight reduction in Caucasians but not in African Americans (Friedlander et al., 2010) and similar to the observation in African Americans, no association was observed with body weight or any anthropometric measures for the SNP or its haplotype measure in this study.

4.3.2. *LEPR*

4.3.2.1. *LEPR* rs6690661 is significantly associated with leptin levels in women

Of the *LEPR* SNPs tested, rs6690661 was the only SNP that met the set multiple testing threshold for diastolic BP in women (Table 3.10). Although not significantly associated, rs6690661 showed the most significance for body weight, BMI and skinfold thickness in men (Table 3.7). Anthropometric measures such as BMI and WC are known to be highly correlated with BP. It is therefore plausible that a SNP that showed a significant association with BP may also be associated with anthropometric measures; interestingly, the association with general obesity was in men, not in women. rs66990661 is a fairly newly discovered SNP (first described in the 1000 Genomes project), hence it was not included in previous studies and no association with rs6690661 has been published to date.

rs6690661 is an intronic SNP, in all *LEPR* transcripts and was not found to have any predicted regulatory functions on the RegulomeDB database. This SNP is polymorphic in all HapMap populations (Figure 3.8). Due to its genetic location within the gene and the lack of evidence for any regulatory functions, it is unclear how variation of rs6690661 affects diastolic BP and anthropometric measures in black South Africans. rs6690661 was in strong LD with rs72923286 in the combined LWK/YRI proxy populations, it is unlikely that rs72923286 affects BP as this is an intronic SNP, but rs6690661 may be in LD with a functional SNP that affects diastolic BP regulation.

4.3.2.2. Nominally associated SNPs within the *LEPR* gene

rs8179183, a non-synonymous (benign/tolerated) SNP was nominally associated with WC (Table 3.8) and leptin levels (Table 3.9) in men but not in women. No association with BMI was observed in the APOGH sample and in PIMA Indians and postmenopausal women of European ancestry (Gu et al., 2012, Traurig et al., 2012). rs8179183 also showed no association with metabolic abnormalities including insulin resistance, fasting glucose levels and hypertension in severely obese Brazilian children (Dias et al., 2012), but showed an association with overweight/obesity and BMI in Indian children (Tabassum et al., 2012). In a study by Wauters et al. (2001), rs8179183 was not associated with body weight, BMI, fat mass and leptin levels in pre- and postmenopausal women. In postmenopausal women, rs8179183 was associated with hip circumference, subcutaneous fat and total abdominal fat (Wauters et al., 2001). rs8179183 was also not associated with BMI in Korean women and Swedish men (Han et al., 2008, Rosmond et al., 2000).

In the APOGH sample, rs1805096, a synonymous SNP was nominally associated with body weight, BMI and WC, leptin levels in men and diastolic BP in women (Tables 3.7-3.10). rs1805096 was nominally associated with BMI in Swiss women (Bochud et al., 2009), but was not associated with BMI in Pima Indians (Traurig et al., 2012), nor was it associated with insulin resistance, fasting glucose levels and hypertension in severely obese Brazilian children (Dias et al., 2012). The association of rs1805096 with leptin levels in the APOGH sample was also observed in Japanese children (Okada et al., 2010).

rs6700896, an intronic SNP, was nominally associated with body weight and leptin levels in men and diastolic BP in women and the entire group (Tables 3.7, 3.9 and 3.10). No previous studies have shown an association rs6700896 with BP, leptin levels or anthropometric measurements. rs1892534, a 3' UTR SNP in *LEPR* was nominally associated with leptin levels in men and the entire group (Table

3.9), but was not associated with any of the tested anthropometric or BP measures in both sexes. rs1892534 was also not associated with anthropometric measures and BP, the lack of association was also observed in Caucasians and Kosraens (Lowe et al., 2009).

rs1892534, rs6700896 and rs1805096 are in strong LD (Figure 3.10) and have all been shown to be associated with C-reactive protein (CRP) levels (risk factor for CVD) in other populations (Bochud et al., 2009, Elliott et al., 2009, Mahajan et al., 2011, Reiner et al., 2012, Ridker et al., 2008). It is interesting that the same SNPs show a trend towards an association with anthropometric and BP measurements as CRP levels have been shown to be correlated with BMI (Tchernof et al., 2002) and BP (Napoli and Papa, 2003).

rs1137100 and rs1137101 were nominally associated with systolic BP in women and diastolic BP in men, respectively (Table 3.10). rs1137101 showed a nominal association with diastolic BP in the entire sample and had a stronger association in women. rs1137100 and rs1137101 have been shown to be associated with both systolic and diastolic BP but no studies have been done with central systolic BP. In a study by Rosmond et al. (2000), both SNPs were associated with systolic and diastolic BP in men whereas no correlation was observed between the SNPs in hypertension in Brazilian children (Dias et al., 2012).

rs1137100 and rs1137101 showed no associations with anthropometric data, supporting and contradicting previous evidence that both SNPs were either associated or not associated with different anthropometric measures. Both SNPs have been shown to be associated with body weight in postmenopausal women (Wauters et al., 2001) and with BMI in PIMA Indians, Caucasian and Korean women but not associated with BMI in Japanese and American women (Gu et al., 2012, Han et al., 2008, Ogawa et al., 2004, Sun et al., 2010, Traurig et al., 2012, Wauters et al., 2001). rs1137101

was associated with obesity in Pacific Islanders (Furusawa et al., 2010) but not in Romanians (Constantin et al., 2010). In a study by Tabasassum et al. (2012), both SNPs were associated with BMI, WC, body weight and an increased risk for overweight/obesity in Indian children. rs1137101 and rs1137100 were not associated with BMI in South African adolescents living in Johannesburg (Lombard et al., 2012), but rs1137100 was associated with BMI and WC in black and mixed ancestry adolescents living in the Western Cape (Yako et al., 2011).

rs1137100 and rs1137101 showed no associations with leptin levels in the APOGH sample and in Caucasian women and Swedish men, whereas both SNPs were associated with leptin levels in Japanese women (Ogawa et al., 2004, Rosmond et al., 2000, Sun et al., 2010). rs1137101 was also reported to be associated with leptin levels in African Americans (Friedlander et al., 2010) and Tunisians (Ben Ali et al., 2009b) but not in Caucasians (Friedlander et al., 2010).

LEPR haplotype block 7 was significantly associated with systolic BP in men but not in women (Table 3.14). The CT haplotype (rs2154381 C allele and rs1171261 T allele) was significantly associated with a decrease in systolic BP whereas the TC haplotype was nominally associated with increased systolic BP and the CC haplotype has no effect on systolic BP. rs2154381 showed no association with systolic BP but rs1171261 was nominally associated with systolic BP in men and in the entire sample (Table 3.10). Since the T allele of rs1171261 is protective against increased systolic BP in the APOGH sample, which has a prevalence of more than 40% hypertensive, we would expect this allele (MAF=0.1239) and the CT haplotype (frequency=0.116) to be less common in the sample.

rs1171261 and rs2154381 have not been previously shown to be associated with systolic BP but both SNPs have been shown to be associated with soluble plasma receptor levels in Caucasian women (Sun et al., 2010). Leptin receptors are crucial in leptin signalling and activation of the sympathetic nervous

system. This haplotype may be affecting soluble leptin receptor levels which may hinder the sympathetic nervous system which will ultimately affect BP regulation (Kotsis et al., 2010, Yang and Barouch, 2007).

4.4. Regulatory roles of associated SNPs

Although both *LEP* rs17151914 and *LEPR* rs6690661 were significantly associated with leptin levels and diastolic BP in women, respectively; it is unclear how these SNPs play a role in their associated phenotypes. Both SNPs are intronic SNPs that were not within predicted regulatory regions and since these SNPs do not appear to have functional roles, it is unlikely that these SNPs are causal SNPs but may rather be in LD with causal SNPs.

All the nominally associated SNPs (except rs1171261 and rs1344870) were either potentially functional (in exons) or regulatory (DNAse hypersensitivity regions, 3' UTRs, upstream regulatory regions, transcription factor binding sites) SNPs. Functional and regulatory SNPs may change gene function, RNA splicing, protein translation and may alter gene expression (Savas et al., 2007).

Synonymous and non-synonymous SNPs were also nominally associated with tested measures. Synonymous SNPs do not change the resulting amino acid (Ramensky et al., 2002) and are therefore less likely to result in disease but may actually be in LD with causal variants. Non-synonymous SNPs such as *LEP* rs17151919 cause missense mutations which change the amino acid hence altering the resulting protein (Burke et al., 2007, Ramensky et al., 2002, Savas et al., 2007, Wang and Moult, 2001). Non-synonymous SNPs may likely affect gene structure and function (Savas et al., 2007) and bioinformatics tools like SIFT and PolyPhen are used to predict potential functional impact. None of the nominally associated non-synonymous SNPs were scored as deleterious in SIFT and PolyPhen and are therefore less likely to affect the structure and function of the protein. It is unclear how these

SNPs may affect pathways involved in adiposity, BP and leptin level regulation, but these SNPs may be in LD with functional SNPs that were not genotyped.

LEP rs11761556 and *LEPR* rs1892534 are 3' UTR SNPs and may affect post transcriptional regulation of their respective genes. Since 3' UTRs contains sequences involved in mRNA stability and post transcriptional regulatory sequences (Wilkie et al., 2003), rs11761556 and rs1892534 may affect binding of regulatory proteins and miRNAs that would affect the levels of leptin mRNA to be translated. The remainder of the regulatory SNPs overlapped with DNAse hypersensitivity sites which are nucleosome-free regions of the chromatin that are sensitive to DNAse I (Gross, 1988). These sites appear in and near genes and are present in cells where the gene is expressed (Gross, 1988). DNAse hypersensitive sites contain regulatory sequences such as enhancers, suppressors, upstream activation sites, promoters, replication origins and topoisomerase sites (Crawford et al., 2004, Gross, 1988). SNPs within DNAse hypersensitivity sites may affect regulation of other genes which may be genes regulating adiposity, leptin levels or BP pathways.

4.5. Population structure

Due to the unsuccessful genotyping of several (n=4) ancestry informative markers (AIMs), population structure could not be determined in the APOGH sample. The remaining 14 AIMs, along with the rest of the *LEP* and *LEPR* SNPs that overlap with other HapMap populations did not have enough power to accurately determine the presence of admixed individuals in the sample.

Interestingly, rs1823718 (an AIM located on chromosome 15) was nominally associated with body weight and skinfold thickness in women whereas rs1363448 (chromosome 5) and rs1344870 (chromosome 3) were nominally associated with central systolic BP in women. rs1823718 and rs1344870 are intergenic SNPs whereas rs1363448 overlaps with the PCDHGA genes. All SNPs overlap

with at least one regulatory region and may therefore regulate genes involved in body weight or skinfold thickness regulation or central systolic BP and diastolic BP regulation, though these SNPs were not selected as part of the study hypothesis. BP and anthropometric measures are known to be different in African populations compared to other populations and since these markers are used to differentiate people of African descent from other populations, their association with these phenotype measures may be indicating underlying stratification in the sample.

4.6. Role of *LEP* and *LEPR* in obesity, hypertension and leptin levels

SNPs in the *LEP* and *LEPR* genes have been shown to be associated with anthropometric measures, leptin levels and BP in many populations and in many of these studies, a sex-specific difference has been observed for the associations. Sex-specific associations may be due to the sex-specific regulation of adiposity, BP and leptin levels (Reckelhoff, 2001, Rosenbaum et al., 1996). Leptin levels have been shown to be gender specific with females having higher leptin levels in adult rats (Pinilla et al., 1999) and in adult humans (Rosenbaum et al., 1996). Leptin levels are higher in women even at birth (Tome et al., 1997) compared to their age and weight matched male counterparts, suggesting that leptin may be regulated or influenced by different factors, in addition to adiposity in males and females. In this study, although not significant, BP measures were higher in men compared to women. This is in line with published data that show that men have higher BP compared to their age matched female (pre-menopausal) counterparts (Reckelhoff, 2001). The presence of high levels of testosterone and low concentrations of oestrogen in men has been proposed to be correlated with higher BP in men (Reckelhoff, 2001).

In this study we were able to show that the *LEP* and *LEPR* genes are important genes for susceptibility to obesity and hypertension in a black, South African cohort. Lombard et al., (2012) showed the *LEP* gene is important in BMI in black adolescents and Yako et al., (2011) reported the *LEP* and the *LEPR*

SNPs were associated with BMI and WC in black and mixed-ancestry South African adolescents. Although SNPs in these genes were significantly associated with BMI and WC in adolescents, significant findings could not be replicated for obesity measures in this black adult sample (APOGH). SNPs in the *LEPR* gene showed nominal association with general obesity in men and with abdominal obesity in men and women suggesting that the gene may be important in adult obesity, but is likely to have small effects.

To our knowledge, no studies have shown an association with *LEP* and *LEPR* SNPs and leptin levels or BP in a black South African population. *LEPR* showed nominal associations with leptin levels and may therefore have very modest effects on leptin levels. The *LEP* and *LEPR* SNPs showed nominal association with different BP measures with most of the association being with diastolic BP in women or the entire sample. This suggests that the *LEP* and *LEPR* SNPs have small effects on BP and may be more important in diastolic BP regulation. In this study we were able to show a novel, significant association between a *LEP* SNP (rs17151914) with leptin levels and *LEPR* SNP (rs6690661) with diastolic BP in women. These SNPs have not been previously reported for any associations with any trait. rs17151914 is only polymorphic in African populations and rs6690661 is more common in African populations, and because of this, these SNPs may not have been included in studies because most studies are done in non-African and admixed (African Americans) populations. These novel finding emphasises the importance not only studying Caucasian, Asian and admixed populations, but also studying complex traits in African populations due to differences in LD in different populations (Tishkoff and Verrelli, 2003). African populations have the greatest level of genetic diversity and because of the low LD in African populations, significant SNPs may narrow down the regions of interest that contains the causal variant within a gene.

The sample sizes used in SNP analysis were small (n=713) and even smaller for leptin levels (n=339) and haplotype analysis (353 unrelated individuals); yet significant associations were observed for the *LEP* and *LEPR* genes even with stringency of the Bonferroni correction. In sex-specific analyses, the power was reduced even further yet we were able to show that rs17151914 is associated with leptin levels in 214 women and rs6690661 was associated with diastolic BP in 389 women. The sample sizes were much smaller for the sex-specific haplotype analysis but we were still able to show that a *LEP* haplotype (rs17151913T-rs6956510G) was significantly associated with central systolic BP in 232 women and that a *LEPR* haplotype (rs2154381 C allele and rs1171261 T allele) was significantly associated systolic BP in 119 men. The significant findings on such small sizes imply that the *LEP* and *LEPR* genes have large enough effects on their relevant phenotypes that can even be detected in small sample sizes.

4.8. Study Limitations

This study had several limitations. Firstly, the sample size was relatively small and as a result, the study may not have had enough power to detect the small effects that the SNPs may have on the phenotypes. As a result, true associations of the *LEP* or *LEPR* SNPs with various tested phenotypes may have been missed/not detected. Leptin level data was not collected for all individuals and the sample size for leptin levels was less than half the sample size of the entire cohort. This posed a particular problem in the sex-specific association tests because the sample was reduced even further. The sample comprised one third of men (33%) and due to the small sample size of males, true associations for SNPs with small effects may have been missed in men. With the exception of the *LEPR* haplotype that was significantly associated with systolic BP in men, all other significant results were noted in women, but not in men. This may have been because the SNPs have a true sex-specific effect or may be due to the small number of men tested compared to women. Age is a critical confounder for the tested traits, particularly for hypertension, as the risk of hypertension increases

with age. Although age was corrected for in all tests, it may have been more informative to divide the sample into age groups prior to testing for association; however this would have reduced sample size and power even further.

Tag SNPs were selected in an effort to capture most of the variability in each gene. Although this method is a cost effective way of doing association studies, it may be a limitation because a different but closely related population was used to select these SNPs (YRI and LWK were used as proxy populations for the South African population). Although the APOGH, YRI and LWK populations are all African populations, their genetic structure is different and tag SNPs that capture a set of SNPs in one population may not capture the same SNPs in the APOGH sample. Because of this, selected tag SNPs may have not necessarily captured all the variability within the *LEP* and *LEPR* genes. Another limitation of the study was that we only focused on SNPs and not other variants such as deletions/insertions and copy number variants which may contribute to phenotypes, but are not common in *LEP* but are common in the *LEPR* gene (Jeon et al., 2010). Also, not all the selected SNPs were successfully genotyped, including some of the ancestry informative markers (AIMs). Since some of the AIMs were not successfully genotyped, we did not have suitable data to test for population structure as a possible confounder.

4.9. Future Studies

In light of the results of this study, a number of suggestions for potential future research can be proposed. Two SNPs that have not been previously reported to be associated with leptin levels or diastolic BP in women have been found to be significantly associated with their respective phenotypes in this cohort. It would be ideal to replicate these findings for association with the tested traits in a much larger black South African sample. Although it may be very expensive, it may be

worthwhile to sequence the *LEP* and *LEPR* genes to uncover causal SNPs that may be in LD with the above mentioned significant SNPs.

Many of the SNPs that were nominally associated with the tested traits had regulatory roles. A systems biology approach investigating the epigenetic, gene-gene interactions and gene-environmental interactions may explain how these SNPs carry out their regulatory functions to affect anthropometry, leptin levels and BP. For future studies, testing associations of more obesity/hypertension related loci, including *LEP* and *LEPR*, within a cohort with comprehensive phenotype profiles (longitudinal data and more haemodynamic data such as renin and soluble leptin receptor levels may provide more insight into genes and pathways regulating adiposity and BP.

5. Conclusions

Height, body weight, BMI, skinfold thickness, waist and hip circumference, WHR, systolic BP, diastolic BP and central systolic BP were all significantly heritable in black South Africans, whereas leptin levels were not significantly heritable.

LEP SNPs showed no association with general obesity but were nominally associated with different anthropometric measures of abdominal obesity in men and women in a sex-specific manner. *LEP* rs17151914 was significantly associated with leptin levels in the entire sample and in women but not in men. rs17151914 is an intronic SNP and may therefore be in LD with a causal SNP that affects leptin levels. SNPs in the *LEP* gene were nominally but not significantly associated with systolic, diastolic and central systolic BP in a sex-specific manner.

LEPR SNPs were nominally associated with general obesity in men but not in women, with rs6690661 showing the strongest association with body weight, BMI and skinfold thickness in men. *LEPR* SNPs were also nominally associated with different anthropometric measures of abdominal obesity and leptin levels in men and women in a sex-specific manner. SNPs in the *LEPR* gene were nominally but not significantly associated with systolic, diastolic and central systolic BP in a sex-specific manner and rs6690661 was significantly associated with diastolic BP in women but not in men and nominally associated with diastolic BP in the entire sample. rs6690661 is an intronic SNP and may therefore be in LD with a causal SNP that affects diastolic BP in women.

LEP and *LEPR* SNPs may have small effects on anthropometric and BP measures and leptin levels which may not have been detected as significant associations due to a small sample size and sex-specific differences. This implies that the *LEP* and *LEPR* genes interact with hormonal genes and environmental stimuli that are specific to either men or women. Despite sample size and stated

limitations, enough data was generated in this study and other studies to warrant a more extensive investigation of these two genes in obesity and hypertension.

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7. Appendices

Appendix A: Ethics clearance certificates

M110464

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Miss Thandiswa Ngcungcu

CLEARANCE CERTIFICATE	**M110464**
PROJECT	Exploring the Role of Genetic Variation at the Leptin and the Leptin Receptor Genes (LEP and
LEPR)	in Obesity and hypertension in a Black South African Cohort
INVESTIGATORS	Miss Thandiswa Ngcungcu.
DEPARTMENT	School of Pathology/Div Human Genetics
DATE CONSIDERED	06/05/2011
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 06/05/2011 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Professor Angela Woodiwss

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**
PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

Appendix B: DNA extraction protocol

By Higuchi, 1989

Isolation of white blood cells

Whole blood (1.5 ml) was mixed with an equal volume of lysis buffer [Sucrose (0.32M), MgCl2 (5mM), Triton X-100 (1%) in Tris-HCl buffer (10mM; pH 7.5)] and centrifuged at 3000xg for 1 minute. The supernatant was carefully decanted and the pellet was resuspended in lysis buffer (1ml). The centrifugation/decantation and resuspension steps were repeated until the white cell pellet turned from white to colourless.

Lysis of white blood cells

The resulting pellet was suspended in a PCR buffer (0.5ml) with non-ionic detergents [KCL (50mM), $MgCl^2$ (2.5mM), gelatine (0.1 mg/ml), Nonidet P40 (0.45%m/v), Tween 20 (0.45%m/v) in Tris-HCl buffer (10mM; pH 8.3)] containing proteinase K (6 µg/100 µl)]. After incubation for 4-6 hours at 60°C, the DNA suspension was transferred into tubes as 10 and 50 µl aliquots and stored frozen at -20°C until required. The suspension was heated for 10 minutes in a thermocycler at 95°C to inactivate the proteinase K. The resulting suspension contained approximately 4 µg genomic DNA per 100 µl of this lysate.

Appendix C: SNP Selection

Table C1: Literature SNPs

Gene	Literature	No genotype data/not in database/Included	360	340
LEP	rs13245201	Force include		
	rs4728096	Force include		
	rs10244329	Force include		
	rs11763517	Force include		
	rs2167270	Force include		
	rs12535747	Force include		
	rs10249476	Force include		
	rs12536535	X		
	rs11770725	X		
	rs12535708	X		
	rs1349419	X		
	rs7799039	Alternative genotyping	X	
LEPR	rs1137100	Force include		
	rs1137101	Force include		
	rs1805096	Force include		
	rs1805134	Force include		

Table C2: Potentially functional SNPs

Gene	Coding	No genotype data/not in database/Included	360	340	
LEP	rs13306517	Force include			
	rs1800583	Force include			
	rs17151919	Force include			
	rs28954113	Force exclude		X	
	rs76529182	X			Low MAF in African populations<0.02/ No genotype data
	rs111650508	X			
	rs104894023	X			
	rs1800564	X			
	rs75506045	X			
LEPR	rs113353179	Force exclude			
	rs11811070	Force exclude			
	rs13306523	Force exclude			
	rs13306528	Force exclude			
	rs13306526	Force exclude			
	rs1805095	Force include			
	rs1805096	Force include			
	rs1805134	Force include			
	rs1137100	Force include			
	rs1137101	Force include			
	rs8179183	Force include			
	rs1751492	Force include			
LEPR	rs6700896	Force include			
	rs1892534	Force Include			
	rs34499590	Force Include			
	rs2228301	X			No genotype data
	rs34130975	X			
	rs57218441	X			
	rs61781316	X			
	rs112236672	X			
	rs113213085	X			
	rs113550476	X			
	rs35573508	X			
	rs56117117	X			
	rs6413506	Exclude, close to NS		X	

Table C3: Ancestry informative markers

AIM	Position	Chr	Alleles
rs723854	192511012	1	C/G
rs1876482	17362568	2	A/G
rs952718	215888624	2	G/T
rs1344870	21307401	3	G/T
rs720096	179551071	4	C/G
rs1363448	140783596	5	C/T
rs217538	108483470	6	C/G
rs65264	28545611	7	C/T
rs679047	12883664	9	A/T
rs2077559	36014850	9	A/G
rs714857	15974389	11	A/G
rs953386	110943692	13	C/T
rs722869	97277005	14	C/G
rs735612	34076642	15	G/T
rs2089740	36310531	15	A/C
rs1823718	74147244	15	A/G
rs1858465	51142920	17	A/T
rs2112527	9603751	19	A/G

Appendix D: Desalting and concentrating DNA solutions

Samples were purified using the QIAEX II Gel Extraction Kit (500). The protocol was modified for the amount of DNA (≥10 kb). All highlighted sections have been altered from the original protocol.

Important points before starting

■ The yellow colour of Buffer QXI indicates a pH 7.5.

■ Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).

■ All centrifugation steps are at maximum speed (10,000 x g, ~13,000 rpm) in a conventional table-top micro centrifuge.

■ For DNA fragments larger than 10 kb, mix by gently flicking the tube to avoid shearing the DNA. Do not vortex the tube.

Procedure

1. Transfer the sample to a colourless tube. Add 3 volumes of Buffer QX1 and 2 volumes of ddH2O to 1 volume of sample.
2. Check that the colour of the sample mixture is yellow.
   - If the colour of the mixture is orange or purple, add 10 µl 3 M sodium acetate, pH 5.0and mix. The colour should now be yellow. The adsorption of DNA to QIAEX II particles is only efficient at pH 7.5. Buffer QX1 now contains a pH indicator which is yellow at pH 7.5 and orange or violet at higher pH, allowing easy determination of the optimal pH for DNA-binding.
3. Resuspend QIAEX II by vortexing 30 s.
4. Add 10 µl of QIAEX II per 5 µg of DNA and mix by gently flicking the tube. Incubate at room temperature for 10 min. Mix every 2 min to keep QIAEX II in suspension.
5. Centrifuge the sample for 30 s and remove supernatant.
6. Wash the pellet twice with 500 µl of Buffer PE.
7. Dry the pellet using a table top incubator set at 25°C for 10-15 min or until the pellet becomes white.
   - Do not vacuum dry, as this may cause over drying. Over drying the QIAEX II pellet may result in decreased elution efficiency.
8. To elute DNA, add 20 µl of 10 mM Tris·Cl, pH 8.5 and resuspend the pellet by vortexing briefly.
9. Incubate at room temperature for 5 min.
10. Centrifuge at 3000 rpm for 30s and carefully remove supernatant (purified DNA) and place in a clean tube.
11. Repeat steps 8-10 once and place supernatants of the same sample in the same tube.
    - A second elution step will increase the yield by approximately 10–15%.

Appendix E: rs7799039 Flanking sequence and *in-silico* PCR

1. rs7799039 flanking sequence.

- SNP flanking sequences obtained from Ensembl release 62. The SNP is indicated by the red colour.

GAATTAGCTGCAAGGGGAACTAGGAAAAGCTTCTTTAAGGATGGAGAGGCCCTAGTGGAATGGGGAGATTCT
TCCGGGAGAAGYGATGGATGCRCAGTTGGGCATCCCCACAGACRGACTGGAAAGAAAAAAGGCCTGGARGAA
TCAATGTGCAAYGYATGTGTGTTCCCTGGTTCAAGGGCTGGGAACTTTCTCTAMAGGGCCAGGTAGAAAACAT
TTTAGGCTTTCTAAGCCAAGGCAAAATTGAGGATATTACRTGGGTASTTATACAACAARAATAAACAATTTACAC
AATTTTTTGTTGACAGAATTCAAAACTTTATAGACACAGAAATGCAAATTTCCTGTAATTTTYCCRTGAGAACTAT
TCTTCTTTTGTTTTGTTTTGCGACAGGGTTGY[R]CTGATCCTCYYGCCTCAGTCTCCCTAAGTGCTGAGATGTTGC
AGGAAGTCAGGGACCCCGAACAGAGAGATMRGCTGGAGCCRTGGCAGAGGAACATAAATTTTGAAGATKTCA
TTTTAATATGGACACTTWTCAGTTCCCAAATAATAYTTTTATAATTTTTTATGCCTGTCTTTGCTTTAATCTCTTAA
TCCTGTTATCTYCATAAGCTAAGGATGTACRTCACCTCAGGACCACTGTGATAATTGTGTTAACTGTACAGATTG
AYTGCAAAACATGTGTGTTTGAACAATATGAAATCAGTGCACCTTGAAAAAGAGCAGAATAACAGCAATTTTTA
GGGAACAAGGGAAGACAACTATAAGGTCTGACTGCCTGCRGGGTCGGGCAAAGGGAGCCA

2. UCSC in-silico PCR product

Forward and reverse primers highlighted in yellow and blue respectively

>chr7:127878613+127878862 250bp AGCCAAGGCAAAATTGAGG TCCAGCCGATCTCTCTGTTC
AGCCAAGGCAAAATTGAGGatattacatgggtacttatacaacaagaata
aacaatttacacaatttttttgttgacagaattcaaaactttatagacaca
gaaatgcaaatttcctgtaattttcccatgagaactattcttcttttgtt
ttgttttgcgacagggttgcgctgatcctcccgcctcagtctccctaagt
gctgagatgttgcaggaagtcagggacccGAACAGAGAGATCGGCTGGA

Primer melting temperatures

Forward: 60.2 C agccaaggcaaaattgagg

Reverse: 60.5 C tccagccgatctctctgttc

Appendix F: Reagents and solutions

Primer Dilutions

ddH_2O	90 µl
100 µM/primer	10 µl
	100 µl of 10 µM of primer

dNTP mix

dATP (100 mM)	12.5 µl
dATP (100 mM)	12.5 µl
dATP (100 mM)	12.5 µl
dATP (100 mM)	12.5 µl
	1000 µl of 5mM dNTPs

10X TBE buffer

Tris	108 g
Boric Acid	55 g
EDTA	7.44 g

Filled solution up to 1L, brought buffer to pH 8.0 and autoclaved it.

3% Agarose Gel

10X TBE buffer	40 ml
ddH_2O	360 ml
Agarose	12 g

Added 12µl (3 µl/ 100 ml of gel) EtBr when agarose had melted.

Appendix G: Post PCR Sequencing protocol

1. **Nucleospin: From PCR product** (If there are no spurious bands)

- Adjust PCR product's volume to 50 μl with TE buffer (pH 7.5).
- Mix 2 volumes of NT buffer with 1 volume PCR product:

  ∴ 100 μl NT + 50 μl PCR product
- Place column in collection tube and load sample onto column.
- Centrifuge: 1 min @ 14 000 rpm.
- Discard flow-through and place column back in collection tube.
- Pipette 600 μl NT3 buffer onto column.
- Centrifuge: 1 min @ 14 000 rpm.
- Discard flow-through and place column back in collection tube.
- Centrifuge: 2 min @ 14 000 rpm.
- Incubate samples for 2-5 min @ 70ºC (to evaporate ETOH).
- Discard flow-through and place column into a clean Eppendorf tube.
- Pipette 15-50 μl ddH_2O (volume equal to starting volume)**.**
- Incubate samples for 1 min @ RT (to increase yield).
- Centrifuge: 1 min @ 14 000 rpm.
- Discard column.

2. Cycle sequencing

- Set up the cycle sequence reaction.
  - One tube for forward and one tube for reverse for each PCR product.
  - Remember product is light sensitive
- Cycle sequencing PCR mix (1/8th reaction)

2 ml	Cleaned PCR product
1 ml	Bigdye v3.1 (in -20°C freezer with Taq)
1.5 ml	5x Bigdye buffer
1 ml	Primer (forward OR reverse)
4.5 ml	ddH2O
10ml	

NB: If sequencing reaction fails either dilute PCR product 1/10 or add 1u PCR product and 2 μl Big Dye.

- Cycle sequencing PCR program:

30 sec	@	96ºC	} 25 cycles
15 se	@	50ºC	
4 min	@	60ºC	
Hold	@	24ºC	

3. Cycle sequencing product clean

- ***QIAGEN DyeEX***

(NB: Switch the cooling unit of the vacuum system on ½ hour before use)

- Loosen column cap by ½ turn vortex to resuspend resin then snap of the bottom of column tube.
- Place column in a collection tube.
- Centrifuge: 3 min @ 3000 rpm.
- Discard eluent and place column in a clean collection tube.
- Transfer column to a clean tube.
- Carefully apply the sequencing reaction to the gel bed in the centre without piercing the column
- Centrifuge: 3 min @ 3000 rpm.
- Remove the column from the tube - the elutant contains the purified DNA.
- Dry (concentrate) samples under vacuum without heat, for approximately 1 hour with sample lids open.
- Remove dried samples and store in the dark at 4ºC (Remember product is light sensitive).
- Add 10µl HiDye (aliquots in the -20ºC chest freezer) to each sample no more than an hour before sequencing, making sure the dried product is re-dissolved in the dye.
- Denature samples for 2 min at 94ºC and keep on ice until loaded on the capillary sequencer

4. Sequencing

- **Set up run**
  - Expand “Foundation data collection” window on bottom of screen
  - Select “Plate manager” and Click NEW
    - Give plate a name without any spaces
    - Leave description blank
    - Application – Sequence analysis
    - Plate type – 96-well
    - Owner – sero
    - Operator – Own name
  - Click OK
  - Sequencing analysis plate editor window will open
  - Enter your sample ID’s in the first column of the window
  - Fill in the blanks so you have complete runs which are 2 columns/runs
  - Fill in the other columns:
    - Priority – 100
    - Results group – “sequencing”
    - Instrument protocols – Z_Seq POP7-36 Ultra (for shorter sequences 400-500 bp) or Rapid (for longer sequences 700-900bp)
    - Analysis protocol – POP7_BDTv3 kb
  - Click OK. Go to Plate view and hit “Find All” then look for your plate ID
  - Select your plate then right click on the appropriate plate on the display of the two plates in the sequencer to link your data to your plate

- **Loading sample**
  - After denaturing and chilling plate, place special sequencing cover on plate with black mark on lid aligned with the notch on the plate.
  - Snap the plate into the sequencing adaptor with the notches on plate and black base aligned. Place the white adaptor lid on so the A on the lid aligns with notch on base.
  - If plate is clipped on properly then the round spaces should all be black when you look through the assembly.

- Make sure the green light on the sequencer is not blinking indicating that no runs are in progress
- Press “Tray” button and wait until the tray moves forward.
- Open the door
- Place the plate assembly into position with notch towards the back of machine ensuring that the tit lies flush with the buffer chambers in the front of the machine.
- Check the buffer levels in the chambers which should be on or above the black lines.
- Open the door on the right side of the sequencer and check the probe in the feeder bottle for bubbles. If there is a bubble remove the bottle gently and replace – bubble should disappear. Close both doors of the sequencer

- **Starting run**
  - On plate view, find and select your plate then right click on the yellow box indicating the filled plate positions in the sequencer. The plate should turn green
  - Click the green button on the top panel for the window to start the run
  - On instrument status on the menu on the right it’ll show what the machine is doing
  - Leave the computer on **INSTRUMENT SETTING** for the duration of the run.
- **Viewing Sequences**
  - On desktop, click “shortcut to data”
  - Open the folder you saved the run under - “sequencing” to view the raw data
  - To analyse the samples, Open sequence analysis on the desktop
  - ***User***: 3130x1 seq ***Password*** – Sequencer
  - Under File select “Add samples”
  - Under “sequencing” open your run, select all, add selected samples and Press OK. NB: in the results under the column SPACING the value should be 12-14 if sequence runs worked well
  - Click PLAY button to analyse the samples (base call)
  - In SAMPLE MANAGER view you can select SHOW to view individual sequences or select “P” box to print individual sequences. Push PLAY to print the selected sequences.
  - After checking all sequences close window and Save “Yes to all”
  - Open shortcut to data and select your folder. All three sequence files will be shown and you can select and move to a flash drive.

Appendix H: Company names of machines, consumables and software

Table H1: Company names of Machines and Consumables

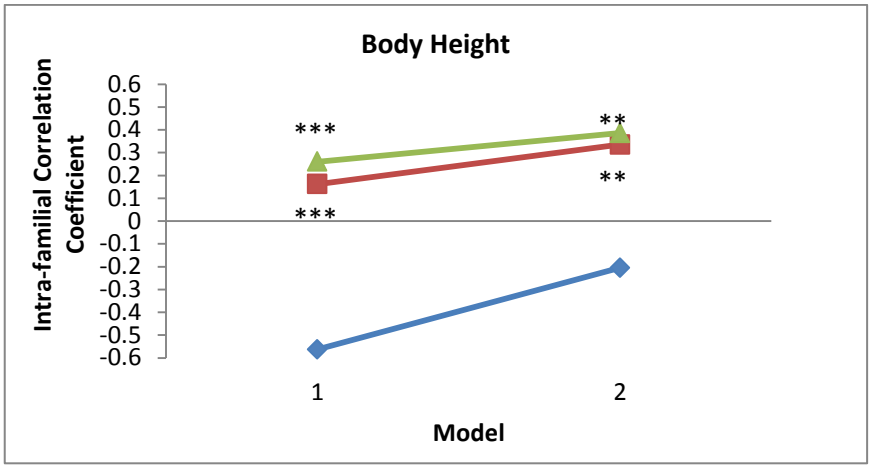
Reagent/Machine	Manufacturer	Country
10 X VeraCode Read Buffer	Illumina	USA
10% Ficoll	Promega	USA
10X buffer	Applied Biosystems (Roche)	USA
192-plex BeadXpress Kit	Illumina	USA
2720 Thermal Cycler	Applied Biosystems	USA
3130xl Genetic Analyzer	Applied Biosystems	USA
5810 Centrifuge 5810	Eppendorf	Germany
7900HT Fast Real-Time PCR System	Applied Biosystems	USA
Allegra® X-15R Centrifuge	Beckman Coulter	USA
AmpliTaq Gold with GeneAmp™	Applied Biosystems (Roche)	USA
BeadXpress® Reader	Illumina	USA
BigDye® Terminator v3.1 Cycle Sequencing Kit	Applied Biosystems	USA
C1000 Thermal Cycler	BioRad	USA
Cycler Gradient Eppendorf	Merck	Germany
DNAse/RNAse free Agarose	Bioline	UK
dNTPs	Bioline	UK
DyeEx™ 2.0 Spin Kit	QIAGEN	Germany
Elisa kit	BioVendor	Czech Republic
Health o meter Professional Electronic scale	Welch Allyn	USA
Ethidium Bromide	Sigma-Aldrich	USA
G-Box and GeneSnap®	Syngene	England
Gene Ruler™ 50bp DNA ladder	Fermentas	Germany
Heat Sealer	Eppendorf	Germany
Hybex® Microsample Incubator	SciGene	USA
Labnet AccuBLOCK™	Labnet	USA
Labnet orbit 1000	Labnet	USA
MgCl2	Applied Biosystems (Roche)	USA

Reagent/Machine	Manufacturer	Country
MicroAmp™ Optical 384-well reaction plates with barcode	Applied Biosystems	USA
MicroAmp™ Optical 96-well reaction plates	Applied Biosystems	USA
MillivacTM Maxi Vacuum Pump	Millipore	USA
MixMate®	Eppendorf	Germany
NanoDop ND 100 Spectrophotometer	NanoDrop Technologies	USA
Nucleospin® Extract II Kit	MACHEREY-NAGEL GmbH & Co. KG	Germany
Primers	Whitehead Scientific	RSA
QIAEX II Gel Extraction Kit (500)	QIAGEN	Germany
Quantifiler™ Human DNA Quantification Kit	Applied Biosystems	USA
Skinfold calliper	Harpenden	England
SPC micromanometer	Miller Instrument Inc.	USA
Stadiometer	Panamedic Corp	Korea
TaqMan®® Genotyping Master Mix	Applied Biosystems	USA
TaqMan®® Pre-designed SNP genotyping assay	Applied Biosystems	USA
TITANIUM™ Taq DNA polymerase	Clontech	USA
Vortemp™ 56 shaking incubator	Labnet	USA

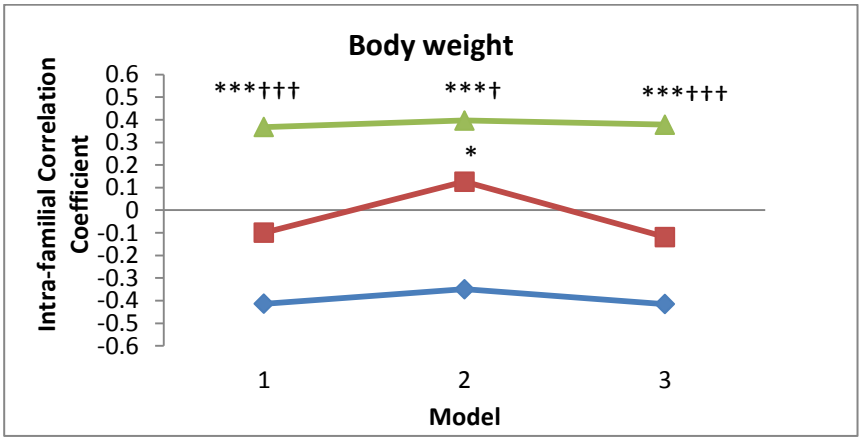
Table H2: Software

Developer	Product	Version	Country
Applied Biosystems	SDS Software	2	USA
Gauderman and Morrison, 2006	QUANTO	1.2	-
Illumina	Beadstudio	3.1.3.0	USA
Illumina	Beadstudio Genotyping Module	3.2.32	USA
Pruim et al., 2010	LocusZoom	1.1	-
Purcell, S, 2007	gPLINK	2.05	-
Department of Epidemiology and Statistics, Case Western Reserve, University of Cleveland	S.A.G.E	6.0.1	USA
SAS Institute Inc.	SAS Enterprise Guide	5.1	USA
SAS Institute Inc.	SAS	9.3	USA
AtCor Medical Pty. Ltd.	SphygmoCor software	6.21	Australia

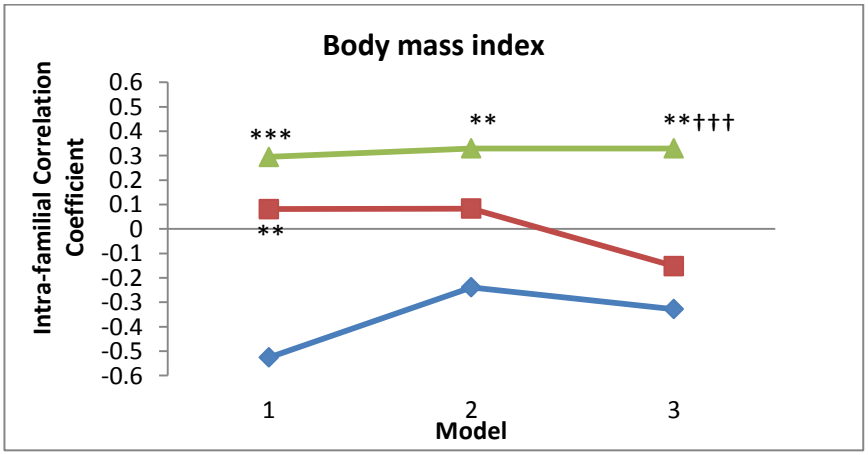
Appendix I: Familial Aggregation Results



A.

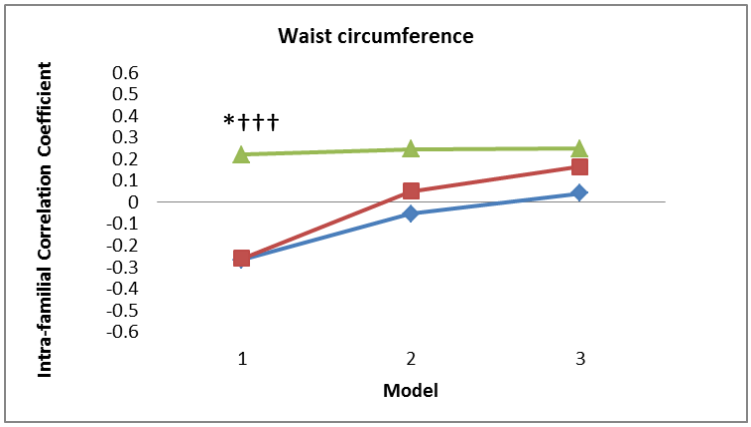


B.

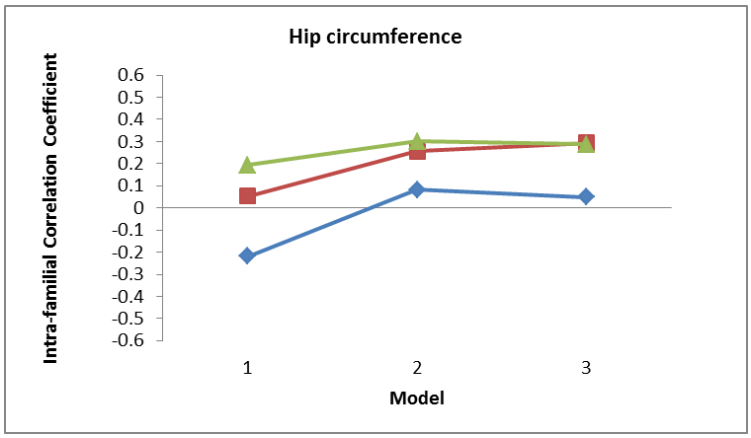


C.

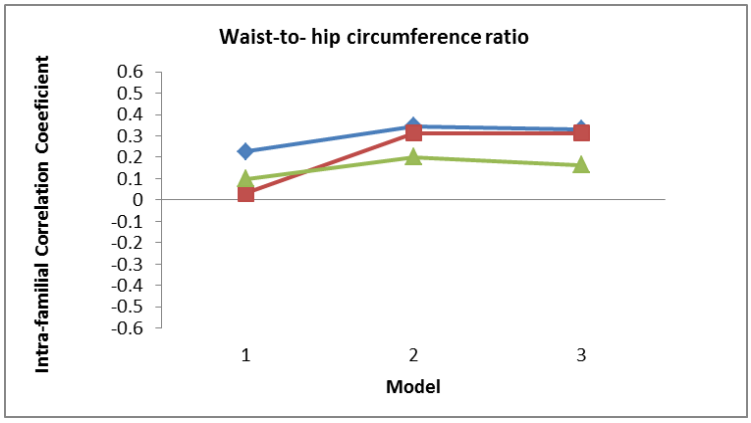
Figure I1: Intra-familial correlation of body weight and height indices. Figures 3.1. A, B and C are intra-familial correlation graph for height, weight and body mass index(BMI), respectively. In all graphs, Each model is adjusted for different confounding factors as follows; Model 1 is unadjusted, model 2: age and sex, model 3: age, sex, tobacco and alcohol consumption. Partial R values (intra-familial correlation coefficient) were compiled using 29 father-mother pairs (blue diamonds), 184 parent child pairs (red squares) and 82 sibling-sibling pairs (green triangles). **/††; 0.01≥P>0.001***/†††;0.001>P≥0.0001.



a.



b.



c.

Figure I2: Intra-familial correlation of waist and hip circumference. Figures 3.4. a, b and c are intra-familial correlation graph for waist circumference, hip circumference and waist and hip ratio, respectively. In all graphs, each model is adjusted for different confounding factors as follows; Model 1 is unadjusted, model 2: age and sex, model 3: age, sex, tobacco and alcohol consumption. */†; 0.05>P≥0.01***/†††;0.001>P≥0.0001.

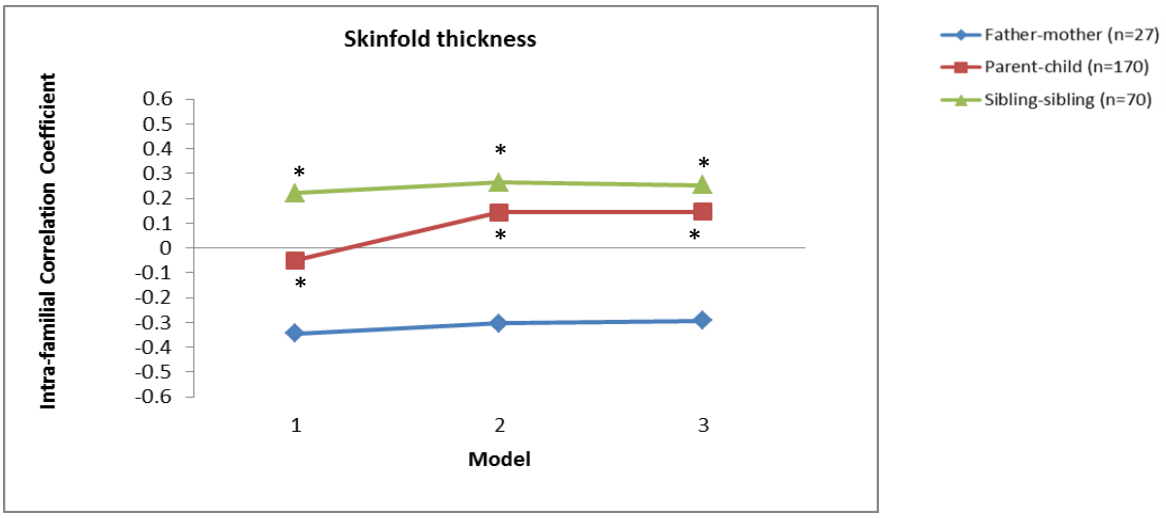


Figure I3: Intra-familial correlation of skinfold thickness. Each model is adjusted for different confounding factors as follows; model 1 is unadjusted, model 2: age and sex, model 3: age, sex, tobacco and alcohol consumption. *; 0.05>P≥0.01.

Table I1: Anthropometric data P values for determining if intra-familial correlation coefficient is significantly different from zero

Pair	P1	P2	P3
Body Height			
Father-mother	0.0004	0.2759	-
Parent-child	0.0269	0.0101	-
Sibling-sibling	0.0162	0.0002	-
Body mass index			
Father-mother	0.0014	0.2011	0.0716
Parent-child	0.2714	0.258	0.0387
Sibling-sibling	0.0058	0.0018	0.0018
Body weight			
Father-mother	0.0182	0.0527	0.0177
Parent-child	0.1763	0.0863	0.1073
Sibling-sibling	0.0004	0.0001	0.0003
Hip circumference			
Father-mother	0.2924	0.7019	0.8187
Parent-child	0.5233	0.0015	0.0002
Sibling-sibling	0.1024	0.0095	0.0133
Waist circumference			
Father-mother	0.1939	0.798	0.8451
Parent-child	0.0012	0.5403	0.0443
Sibling-sibling	0.0626	0.0368	0.0353
Waist to hip ratio			
Father-mother	0.2427	0.066	0.0788
Parent-child	0.6885	<0.0001	<0.0001
Sibling-sibling	0.4149	0.0894	0.1733
Skinfold thickness			
Father-mother	0.0659	0.1099	0.1263
Parent-child	0.5076	0.0591	0.0563
Sibling-sibling	0.0609	0.024	0.0312

*P1, P2 and P3 are P values corresponding to the intra-familial correlation coefficients models 1-3 shown in Figures I1-I3 to determine whether the intra-familial correlation coefficients at each model is significantly different from zero.

Table I2: Comparison of anthropometric intra-familial correlation coefficients from different pairs

Phenotype	Model	P[1]		
		F-M vs P-C[2]	F-M vs S-S[2]	S-S vs P-C[2]
Height	1	0.0001	0.0001	0.4469
	2	0.0079	0.0065	0.6662
	3	-	-	-
Weight	1	0.1046	0.0003	0.0003
	2	0.019	0.0005	0.0299
	3	0.1232	0.0002	0.0001
Body mass index	1	0.0015	0.0001	0.0982
	2	0.1184	0.0095	0.0548
	3	0.3711	0.0025	0.0002
Hip circumference	1	0.2379	0.0934	0.3305
	2	0.44	0.3611	0.7454
	3	0.2769	0.3225	0.9647
Waist circumference	1	0.975	0.0466	0.0009
	2	0.6519	0.3246	0.1763
	3	0.5933	0.3987	0.5594
Waist-to-hip ratio	1	0.3582	0.5763	0.6399
	2	0.8631	0.5136	0.4161
	3	0.9227	0.448	0.2711
Skinfold thickness	1	0.1568	0.0138	0.0557
	2	0.0351	0.0139	0.3859
	3	0.04	0.0186	0.4396

[1]P value for differences in intra-familial correlation coefficients between different pairs in models 1-3 in Figures I1-3,
[2]F-M: father-mother pairs, P-C: Parent-child pairs, S-S: sibling-sibling pairs.

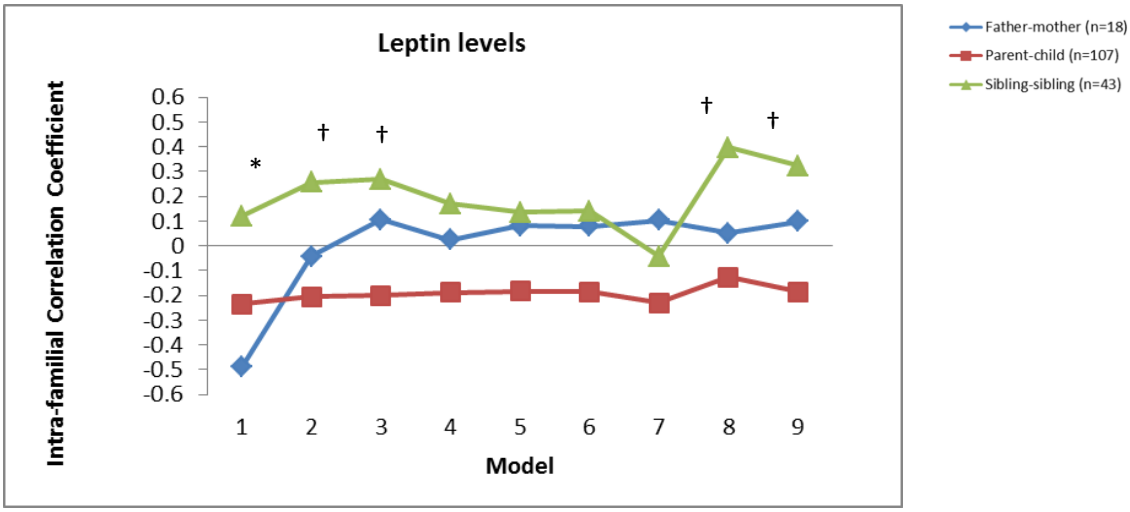


Figure I4. Intra-familial correlation of leptin levels. Each model is adjusted for different confounding factors as shown in the table below. */†; 0.05>P≥0.01

Model	Adjusters for leptin levels
1	None
2	Age, sex
3	Age, sex, tobacco and alcohol consumption
4	Model 3 and BMI
5	Model 3 and body weight
6	Model 3 and weight and height
7	Model 3 and waist circumference
8	Model 3 and skinfold thickness
9	Model 3 and hip circumference

Table I3: Anthropometric data P values for determining if intra-familial correlation coefficient is significantly different from zero

Pair	P1	P2	P3	P4	P5	P6	P7	P8	P9
Father-mother	0.0258	0.8644	0.672	0.9254	0.7476	0.7548	0.6806	0.8381	0.6957
Parent-child	0.0136	0.0308	0.0366	0.0485	0.056	0.0523	0.0155	0.1944	0.0521
Sibling-sibling	0.4399	0.0886	0.0746	0.2721	0.3846	0.3616	0.7796	0.0056	0.0286

*P1-P9 are P values corresponding to the intra-familial correlation coefficients models 1-9 shown in Figure I4 to determine whether the intra-familial correlation coefficients at each model is significantly different from zero.

Table I4: Comparison of anthropometric intra-familial correlation coefficients from different pairs

Model	P[1]		
	F-M vs P-C[2]	F-M vs S-S[2]	S-S vs P-C[2]
1	0.2877	0.0311	0.0535
2	0.5461	0.3125	0.0111
3	0.2641	0.5757	0.0102
4	0.4365	0.6265	0.0515
5	0.3355	0.8557	1.9156
6	0.3348	0.8325	0.0757
7	0.2223	0.6286	0.3056
8	0.5206	0.222	0.0033
9	0.3	0.4314	0.0048

[1]P value for differences in intra-familial correlation coefficients between different pairs in different models in Figure I4, [2]F-M: father-mother pairs, P-C: Parent-child pairs, S-S: sibling-sibling pairs.

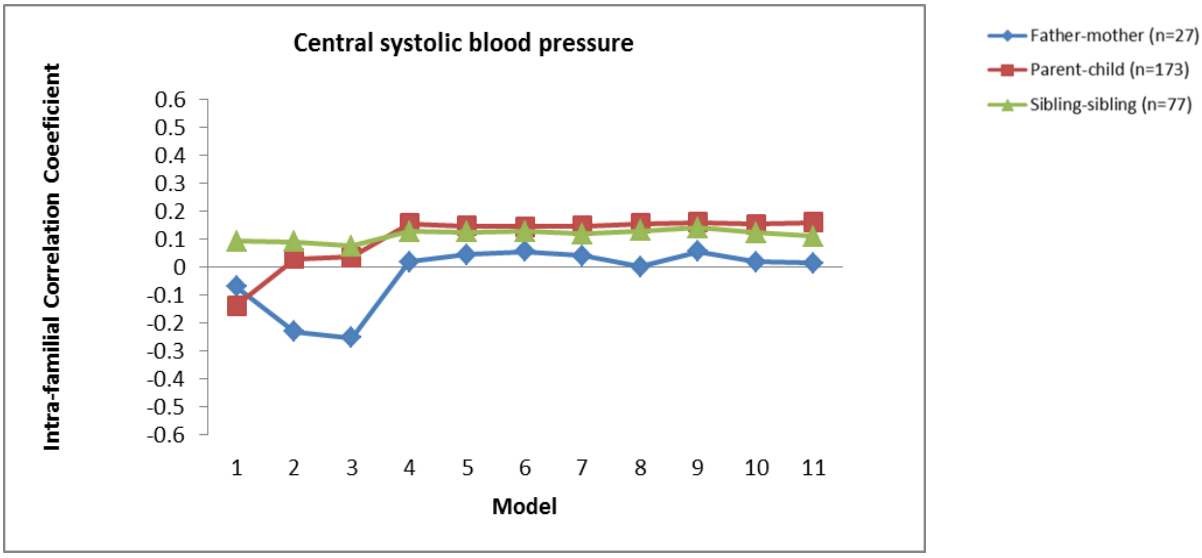
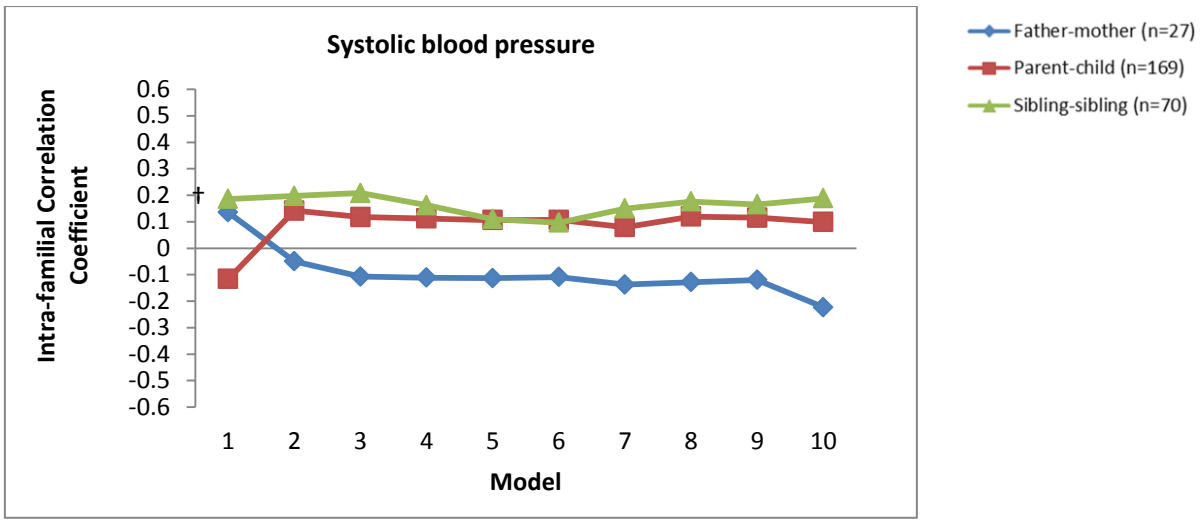
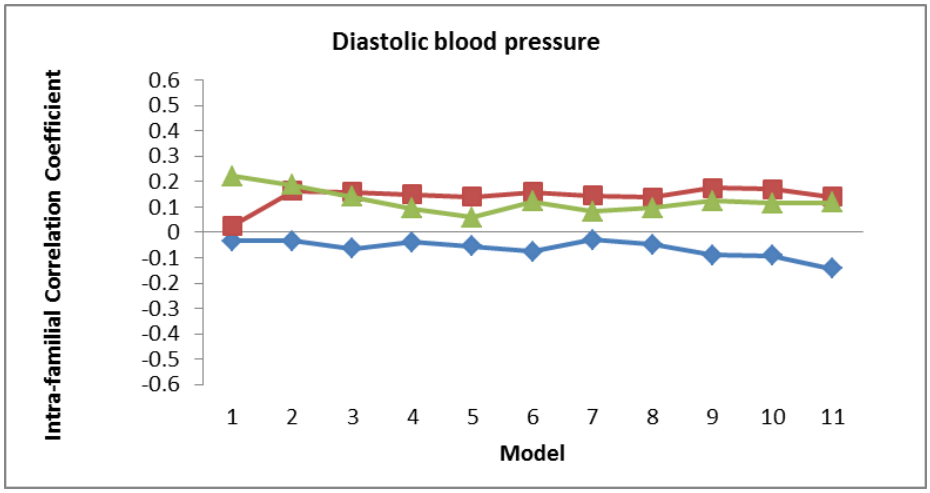


Figure I5. Intra-familial correlation of brachial blood pressure. Each model is adjusted for different confounding factors as shown in the table below.

Model	Adjusters
1	None
2	Age, sex
3	Age, sex, tobacco and alcohol consumption, treatment for hypertension and glucose intolerance
4	Model 3, heart rate and mean arterial pressure
5	Model 4 and BMI
6	Model 4, Body weight and height
7	Model 4 and Body weight
8	Model 4 and waist circumference
9	Model 4 and skinfold thickness
10	Model 4 and hip circumference
11	Model 4 and waist -to-hip ratio



a.



b.

Figure I6: Intra-familial correlation of brachial blood pressure. Figure 3.8 a and b show intra-familial correlation for brachial systolic and diastolic blood pressure measurements, respectively. In both graphs, each model is adjusted for different confounding factors as shown in the table below.

Model	Adjusters
1	None
2	Age, sex
3	Age, sex, tobacco and alcohol consumption, treatment for hypertension and glucose intolerance
4	Model 4 and BMI
5	Model 4, Body weight and height
6	Model 4 and Body weight
7	Model 4 and waist circumference
8	Model 4 and skinfold thickness
9	Model 4 and hip circumference
10	Model 4 and waist -to-hip ratio

Table I5: Anthropometric data P values for determining if intra-familial correlation coefficient is significantly different from zero

Model	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11
Systolic blood pressure											
Father-mother	0.4949	0.8041	0.5896	0.5749	0.5693	0.5829	0.489	0.5185	0.5457	0.2527	-
Parent-child	0.1315	0.0642	0.1258	0.144	0.169	0.1657	0.3031	0.1207	0.1349	0.2003	-
Sibling-sibling	0.1192	0.097	0.0791	0.1758	0.362	0.4236	0.2133	0.1397	0.1674	0.1147	-
Diastolic blood pressure											
	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11
Father-mother	0.8658	0.8659	0.7458	0.8457	0.7862	0.8809	0.8124	0.6494	0.6482	0.4683	-
Parent-child	0.7367	0.0327	0.0367	0.05	0.0657	0.0583	0.0726	0.0214	0.0254	0.0681	-
Sibling-sibling	0.0618	0.1187	0.2394	0.4368	0.6234	0.4926	0.425	0.3061	0.337	0.3311	-
Central systolic blood pressure											
	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11
Father-mother	0.7234	0.2358	0.1899	0.9255	0.8236	0.7835	0.9943	0.7836	0.9265	0.9443	0.9443
Parent-child	0.0703	0.7035	0.6354	0.0397	0.0517	0.056	0.0396	0.0342	0.0437	0.0332	0.0332
Sibling-sibling	0.4166	0.4362	0.5131	0.2658	0.2782	0.265	0.259	0.221	0.2841	0.3425	0.3425

*P1-P11 are P values corresponding to the intra-familial correlation coefficients models 1-11 shown in Figures I1-I3 to determine whether the intra-familial correlation coefficients at each model is significantly different from zero.

Table I6: Comparison of anthropometric intra-familial correlation coefficients from different pairs

Phenotype	Model	P_F-MvsP-C	P-F-MvsS-S	P_S-SvsP-C
Systolic blood pressure	1	0.2473	0.8275	0.0354
	2	0.3782	0.294	0.6929
	3	0.3008	0.1797	0.5194
	4	0.3032	0.2468	0.7263
	5	0.314	0.3465	1.0223
	6	0.321	0.3853	0.9438
	7	0.3191	0.2254	0.6248
	8	0.2548	0.1968	0.6866
	9	0.2797	0.2272	0.7235
	10	0.1356	0.0795	0.5283
Diastolic blood pressure	1	0.7842	0.277	0.1696
	2	0.3635	0.3508	0.8709
	3	0.301	0.3841	0.8965
	4	0.3839	0.5758	0.6934
	5	0.3687	0.6325	0.5686
	6	0.42	0.6345	0.6634
	7	0.3943	0.5447	0.7719
	8	0.2195	0.3669	0.7116
	9	0.2277	0.3834	0.6985
	10	0.1912	0.2703	0.8727
Central systolic blood pressure	1	0.7575	0.4839	0.0961
	2	0.2258	0.1667	0.6629
	3	0.1752	0.154	0.7783
	4	0.5266	0.6411	0.8374
	5	0.6341	0.7323	0.8664
	6	0.6772	0.7545	0.901
	7	0.6251	0.7413	0.835
	8	0.476	0.5841	0.8471
	9	0.6253	0.7142	0.8832
	10	0.5348	0.655	0.8273
	11	0.4964	0.6845	0.7042

[1]P value for differences in intra-familial correlation coefficients between different pairs in models 1-11 in Figure I5-I6, [2]F-M: father-mother pairs, P-C: Parent-child pairs, S-S: sibling-sibling pairs.

Appendix J

Table J1: Heritability estimates after adjusting for various confounding factors

Confounding factors that were adjusted for	h^2	S.E.	P-value
Height			
No adjusters	0.604	0.094	<0.0001
Age, sex	0.764	0.078	<0.0001
Body weight			
None	0.194	0.097	0.023
Age, sex	0.414	0.095	<0.0001
Age, sex, drinking, smoking	0.383	0.098	<0.0001
BMI			
None	0.034	0.106	0.373
Age, sex	0.28	0.102	0.003
Age, sex, drinking, smoking	0.257	0.103	0.006
Waist circumference			
None	0.047	0.103	0.326
Age, sex	0.377	0.103	0.00013
Age, sex, drinking, smoking	0.351	0.104	0.00038
Hip circumference			
None	0.285	0.106	0.003
Age, sex	0.442	0.096	<0.0001
Age, sex, drinking, smoking	0.418	0.098	<0.0001
WHR			
None	0.205	0.098	0.018
Age, sex	0.468	0.098	<0.0001
Age, sex, drinking, smoking	0.461	0.098	<0.0001
Central systolic BP			
None			
Age, sex	0.221	0.096	0.0104
Age, sex, drinking, smoking, hypertension treatment, poor glucose control or HbA1C>6.1%,	0.232	0.094	0.007
Age, sex, drinking, smoking, hypertension treatment, poor glucose control or HbA1C>6.1%, , heart rate, mean arterial pressure	0.261	0.129	0.0211
Age, sex, drinking, smoking, hypertension treatment, poor glucose control or HbA1C>6.1%, , heart rate, mean arterial pressure, BMI	0.253	0.127	0.0229
Age, sex, drinking, smoking, hypertension treatment, poor glucose control or HbA1C>6.1%, , heart rate, mean	0.26	0.127	0.02

arterial pressure, body weight,body height			
Age, sex, drinking, smoking, hypertension treatment, poor glucose control or HbA1C>6.1%, , heart rate, mean arterial pressure, body weight	0.257	0.127	0.0209
Age, sex, drinking, smoking, hypertension treatment, poor glucose control or HbA1C>6.1%, , heart rate, mean arterial pressure, WC	0.332	0.132	0.006
Age, sex, drinking, smoking, hypertension treatment, poor glucose control or HbA1C>6.1%, , heart rate, mean arterial pressure, SKF	0.332	0.133	0.008
Age, sex, drinking, smoking, hypertension treatment, poor glucose control or HbA1C>6.1%, , heart rate, mean arterial pressure, HC	0.298	0.132	0.012
Age, sex, drinking, smoking, hypertension treatment, poor glucose control or HbA1C>6.1%, , heart rate, mean arterial pressure,Wheart rate	0.323	0.133	0.0077
Systolic BP			
None			
Age, sex	0.372	0.113	0.00049
Age, sex, drinking, smoking, hypertension treatment, poor glucose control or HbA1C>6.1%,	0.367	0.111	0.00047
Age, sex, drinking, smoking, hypertension treatment, poor glucose control or HbA1C>6.1%, , BMI	0.382	0.114	0.00039
Age, sex, drinking, smoking, hypertension treatment, poor glucose control or HbA1C>6.1%, , body weight, body height	0.390	0.113	0.00027
Age, sex, drinking, smoking, hypertension treatment, poor glucose control or HbA1C>6.1%, , body weight	0.371	0.113	0.00052
Age, sex, drinking, smoking, hypertension treatment, poor glucose control or HbA1C>6.1%, , WC	0.342	0.115	0.00141
Age, sex, drinking, smoking, hypertension treatment, poor glucose control or HbA1C>6.1%, , SKF	0.337	0.114	0.00148
Age, sex, drinking, smoking, hypertension treatment, poor glucose control or HbA1C>6.1%, , HC	0.347	0.115	0.00131
Age, sex, drinking, smoking, hypertension treatment, poor glucose control or HbA1C>6.1%, , ,WHR	0.332	0.114	0.00183
Diastolic BP			
None	0.146	0.092	0.057017
Age, sex	0.270	0.099	0.003343
Age, sex, drinking, smoking, hypertension treatment, poor glucose control or HbA1C>6.1%,	0.266	0.099	0.003579
Age, sex, drinking, smoking, hypertension treatment, poor glucose control or HbA1C>6.1%, , BMI	0.281	0.101	0.002663

Age, sex, drinking, smoking, hypertension treatment, poor glucose control or HbA1C>6.1%, , body weight, body height	0.288	0.101	0.002186
Age, sex, drinking, smoking, hypertension treatment, poor glucose control or HbA1C>6.1%, , body weight	0.371	0.113	0.000519
Age, sex, drinking, smoking, hypertension treatment, poor glucose control or HbA1C>6.1%, , WC	0.269	0.103	0.004458
Age, sex, drinking, smoking, hypertension treatment, poor glucose control or HbA1C>6.1%, , SKF	0.267	0.102	0.004347
Age, sex, drinking, smoking, hypertension treatment, poor glucose control or HbA1C>6.1%, , HC	0.276	0.104	0.003853
Age, sex, drinking, smoking, hypertension treatment, poor glucose control or HbA1C>6.1%, , WHR	0.236	0.103	0.010931