Molecular investigation of genetic and environmental factors contributing to obesity in adolescent learners residing in the semi-urban/rural areas of the Western Cape Province, South Africa.

by

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DECLARATION

By submitting this thesis, I hereby declare that the entirety of the work contained therein is my own original work, that I am the owner of the copyright thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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ABSTRACT

Background/Aims: Obesity has increased rapidly in South African children and adolescents with significant variability observed among racial groups. Genes that regulate appetite have been studied in different populations worldwide, but their role in obesity among South African adolescents is unknown. The present study aimed at investigating the role of these genes, and their combined effect with physical activity in the development of obesity among South African adolescents.

Methods: A total of 1564 South African school learners of Caucasian (n= 146), Mixed Ancestry (n= 872) and Black African (n= 537) ethnic groups were recruited for a research project that aimed to elucidate diabetes and the metabolic syndrome in children and adolescents attending schools in peri-urban areas of the Western Cape. The present case-control study included 227 obese-overweight (115 Black Africans and 112 Mixed Ancestry), and 204 normal weight (94 Black Africans and 110 Mixed Ancestry) adolescents learners. The learners were genotyped for nine polymorphisms (*LEP*: 19G>A, Lys36Arg, Val94Met; *LEPR*: Lys109Arg; Gln223Arg, Lys656Asn; *CART*: c.160-33G>A, c.499delA, and c.517A>G; *GHRL*: Leu72Met; and *MC3R*: Thr6Lys, Val81Ile) using allele-specific restriction enzyme analysis and automated sequencing. Genotype and haplotype associations with anthropometric variables such as body mass index (BMI), waist, hip, and mid-upper-arm circumferences (WC, HC, MUAC), and metabolic traits (fasting blood glucose, high density lipoprotein-cholesterol, total cholesterol), and blood pressure were further conducted. Furthermore, the type and frequency of physical activity was assessed by means of structured questionnaires; and its effect on obesity-related variables investigated in learners that were genotyped for the *MC3R* Thr6Lys and Val81Ile polymorphisms.

Results: In a stepwise backward logistic regression analysis (containing age, gender, and *LEP*, *LEPR*, *CART* and *GHRL* polymorphisms), *CART* c.517A>G was independently significantly associated with obesity (OR= 5.98; 95%CI= 2.02, 21.27). *CART* c.517G carriers had higher MUAC (β coefficient= 1.88; 95%CI= 0.31, 3.44) while the *LEPR* 109Arg allele was significantly associated with decreased BMI (β coefficient = -2.36; 95%CI= -4.24, -0.47), WC (β coefficient = -5.66; 95%CI= -9.89, -1.44) and MUAC (β coefficient = -1.61; 95%CI= -3.00, -0.22); after adjusting for age, gender, and ethnicity. The haplotype containing the three *LEP* polymorphisms (A-A-A compared to the reference G-A-G haplotype) increased BMI (p= 0.0155), MUAC (p= 0.0146), and HC (p= 0.0128). The minor alleles of the *MC3R* polymorphisms decreased BMI, HC, WC, MUAC and TC; whilst only the Thr6Lys was associated with systolic and diastolic blood pressure (p= 0.0047 and 0.0027, respectively) in Mixed

Ancestry learners. Doing house chores was associated with lower total cholesterol, independently and in the presence of the 81Ile allele (β coefficient = -0.355; 95%CI= 0.148, 0.561).

Conclusion: To our knowledge, this is the first study that reports *CART* c.517A>G polymorphism as a risk factor for obesity in adolescents. Furthermore, the present study demonstrated that the *MC3R* polymorphisms had a positive effect on total cholesterol, which was further enhanced in physically active individuals. Similar to other studies, *LEPR* Lys109Arg and *LEP* polymorphisms were associated with variations in obesity-related variables among Black African and Mixed Ancestry South African learners.

OPSOMMING

Agtergrond/Doelwitte: Vetsug het drasties toegeneem in Suid-Afrikaanse kinders en adelossente met ‘n beduidende variasie opgemerk tussen verskillende rassegroepe. Gene verantwoordelik vir regulering van eetlus is reeds wêreldwyd in verskillende bevolkingsgroepe bestudeer, maar hul rol in oorgewig Suid-Afrikaanse adolessente is onbekend. Die huidige studie was daarop gerig om ondersoek in te stel na die rol van hierdie gene en hul gekombineerde effek met fisiese aktiwiteit in die ontwikkeling van vetsug onder Suid-Afrikaanse adolessente.

Metodes: ‘n Totaal van 1564 Suid-Afrikaanse leerders van Kaukasiese Afkoms (n=146), Gemengde Afkoms (n=872) en Swart Afkoms (n= 537) was gewerf in die navorsingsprojek wat ten doel gehad het om kinders en adolosente met diabetes en die metaboliese sindroom te identifiseer wat skole bygewoon het in semi-voorstedelike gebiede van die Wes-Kaap. Die huidige gevalle studie het 227 vetsugtige-oorgewig (115 Swart Afkoms en 110 Gemengde Afkoms) en 204 normale gewig (94 Swart Afkoms en 110 Gemengde Afkoms) leerders ingesluit. Die leerders was gegenotipeer vir nege polimorfismes (*LEP*: 19G>A, Lys36Arg, Val94Met; *LEPR*: Lys109Arg; Gln223Arg, Lys656Asn; *CART*: c.160-33G>A, c.499delA, and c.517A>G; *GHRL*: Leu72Met; and *MC3R*: Thr6Lys, Val81Ile) met die gebruik van alleel-spesifieke restriksie ensiem analises en geoutomatiseerde DNA volgorde bepalings tegnieke. Genotipiese en haplotipiese assosiasies met antropometriese veranderlikes soos liggaamsmassa indeks (BMI), middel-, heup- en mid-boarm omtrek (WC, HC, MUAC), metaboliese tendense (vastende bloed glukose, hoë-digtheid lipoproteïen-cholesterol, totale cholesterol) en bloeddruk was ook uitgevoer. Die tipe en frekwensie fisiese aktiwiteit was geassesseer deur middel van gestruktureerde vraelyste; en die uitwerking daarvan op vetsugverwante veranderlikes ondersoek in leerders wat vir die *MC3R* Thr6Lys en Val81Ile polimorfismes gegenotipeer was.

Resultate*:* Statistiese ontleding (“stepwise backward logistic regression analysis”), wat ouderdom, geslag en polimorfismes (*LEP*, *LEPR*, *CART GHRL)* ingesluit het, het getoon dat *CART* c.517A>G betekenisvol onafhanklik geassosiasieer was met vetsug (OR= 5.98; 95% CI= 2.02, 21.27). *CART* c.517G draers het ‘n hoër MUAC waarde gehad (β koeffisient = 1.88; 95%CI= 0.31, 3.44), terwyl die *LEPR* 109Arg alleel betekenisvol geassosieer was met verlaagde BMI ((β koeffisient = -2.36; 95%CI= -4.24, -0.47), WC (β koeffisient = -5.66; 95%CI= -9.89, -1.44) en MUAC (β koeffisient = -1.61; 95%CI= -3.00, -0.22) na die aanpassing van ouderdom, geslag en etnisiteit. Die haplotipe met die drie *LEP* polimorfismes (A-A-A teenoor die G-A-G verwysingshaplotipe) het die BMI (p= 0.0155), MUAC (p= 0.0146) en HC (p= 0.0128) verhoog. Die mindere allele van die *MC3R* polimorfismes het die BMI, HC, WC, MUAC en TC verlaag; terwyl slegs die Thr6Lys polymorfisme met sistolies en diastolies

bloeddruk (p= 0.0047 en p= 0.0027, onderskeidelik) geassosieer was in Gemengde Afkoms leerders. Die verrigting van algemene huistake was geassosieer met laer totale kolesterol vlakke, onafhanklik en in die teenwoordigheid van die 81Ile alleel (β koeffisient= -0.355; 95%CI= 0.148, 0.561).

***Gevolgtrekking**:* Na ons wete is hierdie die eerste studie wat die *CART* c.517A>G polimorfisme as 'n risikofaktor vir vetsug in adolessente aantoon. Die huidige studie toon ook dat die *MC3R* polimorfisme 'n positiewe effek op totale kolesterol gehad het, wat ook verder versterk was in fisiese aktiewe individue. Soortgelyk aan ander studies, was die *LEPR* Lys109Arg en *LEP* polimorfismes geassosieer met variasies in vetsug-verwante veranderlikes onder Suid-Afrikaanse Swart en Gemengde Afkoms leerders.

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LIST OF ABBREVIATIONS

$AgNO_3$: silver nitrate
ANGELO: analysis grid for environments linked to obesity
BMI: body mass index
BPB: bromophenol blue
CART: *c*ocaine- and amphetamine-related transcript gene
CDC: centres for disease control and prevention
cm: centimeter
dATP: deoxyadenosine triphosphate
DBP: diastolic blood pressure;
dCTP: deoxycytidine triphosphate
dGTP: deoxyguanosine triphosphate
DHS: department of health survey
dTTP: deoxythymidine triphosphate
EDTA: ethylenediamine tetra acetic acid
EtBr: ethidium bromide
FBG: fasting blood glucose;
GHRL: ghrelin gene
GIANT: genetic investigation of anthropometric traits
HBSC: health behaviour in school-aged children
HDL-C: high density lipoprotein-cholesterol
HP: hip circumference;
IDT: integrated DNA technology
IOTF: international obesity task force
KCl: potassium chloride
kg/m^2: kilogram per square meter
KH_2PO_4: potassium dihydrogen phosphate
$KHCO_3$: potassium bicarbonate
LD: Linkage disequilibrium
LEP: leptin gene
LEPR: leptin receptor gene
MAUC: midd-upper-arm circumference
MC3R: melanocortin 3 receptor gene

MC4R: melanocortin 4 receptor gene

NA_2HPO_4: disodium hydrogen phosphate

NaCl: sodium chloride

NaOH: sodium hydroxide

NCBI-BLAST: national centre for bioinformatics institute-basic alignment search tool

NFCS: national food consumption survey

NH_4Cl: ammonium chloride

NHANES: national health examination surveys

NIH: national institute of health

PBS: phosphate buffered saline

PCR: polymerase chain reaction

POMC: *P*ro-opiomelanocortin gene

SA YRBS: South African youth risk behaviour survey

SAVACG: South African vitamin A consultative group

SB: di-sodium borate

SBP: systolic blood pressure;

SDS: sodium dodecyl sulphate

SSCP: single strand conformation polymorphism

TBE: tris boric acid ethylenediamine tetra acetic acid buffer

TC: total cholesterol;

TE: tris ethylenediamine tetra acetic Acid

TEMED: Tetramethylethylenediamine

TG: triglycerides

TV: television

UYRBS: national youth risk behaviour survey

WC: waist circumference

WC: waist circumference

WHO: World Health Organisation

WHR: waist hip ratio

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CHAPTER 1

INTRODUCTION

Outline of thesis

The present study is of cross-sectional design, investigating factors contributing to the development of polygenic obesity in South African adolescent learners. Six genes of the leptin-melanocortin pathway that are known to regulate food intake and energy expenditure, and have been reported to contribute to the development of obesity, were investigated. In addition, the role of physical activity in obesity and its possible association with the melanocortin receptor-3 gene was investigated. Chapter 1 provides a brief insight on obesity and its contributing factors, presenting an overview of what is currently known from studies conducted globally. The relative contribution of environmental factors such as socio-economic status, sedentary lifestyle (consumption of high-calorie food and physical inactivity), urbanisation, and genetic predisposing variants are discussed. Due to the wide scope on genes contributing to the polygenic form of obesity, a brief overview of studies conducted on the subject (with special reference on monogenic obesity) is presented. A brief introduction of the six genes investigated in the present study follows in chapters 2 and 3. Literature published until January 2011 is included. Aims and objectives of the present study are presented as the last section of Chapter 1. Findings obtained from the present study are organised and described in detail in chapters 2 and 3, according to the manuscripts that were published. Each chapter consists of a brief introduction on the genes included, methods used for analyses, results and discussion. Finally, an integrated conclusion is presented in Chapter 4. References for all the chapters appear in the last section, followed by appendices.

1.1. Definition and diagnosis of obesity

1.1.1. Definition for adults

Obesity is listed as one of the ten leading risk factors for high mortality in both developed and developing countries as it is associated with potentially debilitating illnesses such as cardiovascular, pulmonary (such as sleep apnoea), metabolic (diabetes and dyslipidaemia) and osteoarticular diseases, common forms of cancer (cervical, uterus, breast, ovarian), and serious psychological illness (Berenson et al., 1993). Obesity together with insulin resistance, hypertension, type 2 diabetes, atherogenic dyslipidemia and hypertension are a group of metabolic components that constitute the metabolic syndrome. Physiologically, obesity is defined as an imbalance between energy intake and expenditure to such an extent that surplus energy is stored in fat cells (adipocytes), which expand and increase in number. Due to the difficulty of directly measuring body fat, obesity is often expressed as excess body weight rather than excess fat. As a quantitative entity, obesity is therefore defined on the basis of body mass index (BMI), which is calculated as weight in kilograms (kg) divided by height in metres squared (m^2). Although BMI is not a direct measure of body fat, it is a more accurate indicator of overweight excess weight and obesity compared to weight alone (NIH report, 1998). An expert panel convened by the National Heart, Lung and Blood Institute and the National Institute of Diabetes and Digestive and Kidney Diseases defined overweight as a BMI of 25 to 29.9 kg/m^2 and obesity as a BMI of 30.0 kg/m^2 (Table 1.1). Similar to the World Health Organisation (WHO) recommendations, the National Institute of Health (NIH) defined normal weight as a BMI of 18.50–24.9 kg/m^2.

Table 1.1. International Classification of adult underweight, overweight and obesity according to the body mass index as recommended by the National Institute of Health and World Health Organisation (WHO). *Adapted from www.who.int/bmi/index.jsp?introPage=intro_3.html*

Body weight category	Body mass index	Obesity class
Underweight	<18.5	
Normal	18.5–24.9 (NIH); 18.50 - 24.99 (WHO)	
Overweight	25.0–29.9 (NIH); 25.00 - 29.99 (WHO)	
Obesity	30.0–34.9 (NIH); 30.00 - 34.99 (WHO)	I
	35.0–39.9 (NIH); 35.00 - 39.99 (WHO)	II
Extreme obesity	>40.0 (NIH); ≥40.00 (WHO)	III

NIH, National Institute of Health; WHO, World Health Organisation

BMI is commonly used as a measure of obesity in clinical and research settings, however, alternative anthropometric measures such as waist circumference (WC), and waist-hip-ratio (WHR) that reflect abdominal adiposity have been suggested to be the most accurate determinants and predictors of cardiovascular diseases (CVD) and type 2 diabetes (Wei et al., 1997; Stevens et al., 2001; Janssen et al., 2004). This is based on the premise that increased visceral adipose tissue is associated with metabolic abnormalities such as decreased glucose tolerance, reduced insulin sensitivity and adverse lipid profiles, which are risk factors for type 2 diabetes and CVD. Several studies also suggest that coupling BMI with WC provides a stronger health risk indicator than using BMI alone (Chan et al., 1994; Rexrode et al., 1998; Janssen et al., 2002 ; Ardern et al., 2003; Bigaard et al., 2003; Huxley et al., 2010). In support of this, the NIH guidelines indicate that the risk of acquiring CVD and type 2 diabetes increases in a graded fashion when moving from normal-weight through obese BMI categories, and that within each BMI category men and women with high WC values are at a greater health risk than are those with normal WC values (Table 1.2[a]) (NIH, NHLBI, 1998). Vazquez and co-workers (2007), however, conducted a meta-analysis that was based on published studies on diabetes from 1966 to 2004, and concluded that there is a high statistically significant correlation between BMI and WC, suggestive of a similar ability of these tools to predict diabetes. Similarly, Højgaard and co-workers (2008) demonstrated that individuals with high BMI and/or high WC are more at risk of dying from CVD than those with lower cut-off points of these anthropometric measurements.

Table 1.2(a). Combined recommendations of body mass index and waist circumference cut-off points made for overweight or obesity classifications, and association with disease risk. *Taken from the WHO report, Geneva 2008.*

Body weight category	**Body mass index**	**Obesity class**	**Disease risk (relative to normal weight and waist circumference)**	
			Men < 102 cm Women < 88 cm	**Men >102 cm Women >88 cm**
Underweight	<18.5			
Normal	18.5–24.9			
Overweight	25.0–29.9		Increased	High
Obesity	30.0–34.9	I	High	Very high
	35.0–39.9	II	Very high	Very high
Extreme obesity	>40.0	III	Extremely high	Extremely high

The cut-off points of abdominal anthropometric measures that define the risk of CVD and type 2 diabetes varies among different populations (Table 1.2[b]) (Huxley et al., 2010). Studies conducted in Asian populations, for example, have demonstrated that these population groups have an increased metabolic risk at lower WC (85-90 cm and 80 cm for men and women, respectively) and WHR (0.90 and 0.80 for men and women, respectively) than Europeans (Diaz et al., 2007; Huxley et al., 2007; 2008). It has also been demonstrated that abdominal adiposity is a more consistent predictor of diabetes and CVD among Asians (Cassano et al., 1992; Boyko et al., 2000; Suka et al., 2011), while BMI was shown to be a more reliable tool for determining the risk of diabetes among American Caucasians (Spiegelman et al., 1992; Chan et al., 1994). The evidence is insufficient for WC and WHR specific cut-offs for African-American, Hispanic and Middle Eastern populations, although some studies suggest the use of current cut-offs for Europeans. The values for other ethnic groups are between those for Caucasians (WC and WHR= respectively, 97-99 cm and 0.90 for American and UK Caucasian men; and 85 cm and 0.83-0.85 for American and UK Caucasian women) and Asians, as reviewed by Qiao et al (2010). There is also very limited data for populations of African origin, and hence no cut-off points are recommended for sub-Saharan populations. The available data recommends WC cut-off points of 75.6 cm and 80.5 cm for men, and 71.5 cm and 81.5 cm for women of Nigerian and Cameroon origin, respectively, for the identification of hypertension (Okosun et al., 2000). It is, therefore, apparent that universal cut-off points of BMI and WC cannot be used globally. Other methods available to measure body composition in adults include dual energy X-ray absorptiometry, whole body plethysmography, and devices that measure body conductivity.

Table 1.2(b). World Health Organization cut-off points for waist circumference and waist-hip-ratio (WHR) and risk of metabolic complications. *Taken from the WHO report, Geneva 2008.*

Indicator	Cut-off points	Risk of metabolic complications
Waist circumference	94-102 cm (male); 80-88 cm (women)	Increased
	>88 cm (male); > 102 cm (women)	Substantially increased
Waist-hip ratio	0.90 cm (male); 0.85 cm (women)	Substantially increased

1.1.2. Definition for children and adolescents

Measuring obesity in children and adolescents is complex due to changes in body weight during developmental stages. The levels of growth hormone and insulin-like growth factor increase during puberty. Along with sex hormones, these peptides are responsible for the pubertal growth spurt (MacGillivray et al., 1998; Abbassi, 1998; Plant, 2001). As a result of variation in growth and sex

hormone during developmental stages, BMI in childhood changes substantially with age (Rolland-Cachera et al., 1982; Cole et al., 1995). At birth the BMI median is as low as 13 kg/m^2, increases to 17 kg/m^2 at age 1, decreases to 15.5 kg/m^2 at age 6, then increases to 21 kg/m^2 at age 20. It is for this reason that standardised cut-off points adjusted according to age and gender were developed. Definition of overweight and obesity in children and adolescents is based respectively, on the 85th and 95th percentiles of sex-specific BMI-for-age. These reference values were developed from reference populations specified for a given country. For example, the 1990 British growth reference was developed from a survey representative of English, Scotland and Wales (Cole et al., 1998). The United States of America developed its own reference values, the Centres for Disease Control and Prevention (CDC) 2000 growth charts, from five nationally representative surveys (the National Health Examination Surveys II and III in the 1960s, the National Health and Nutrition Examination Surveys [NHANES] I and II in the 1970s, and NHANES 1988-1994) (Kuczmarski et al., 2002). The 2000 CDC growth charts, which were adapted from the 1977 National Center for Health Statistics growth charts, included a sex-specific BMI-for-age growth chart for children 2 years and older. Overweight is defined as a BMI at or above the 95th percentile, and at risk for overweight as that between the 85th and 95th percentile. In these growth charts, the 'at risk' of overweight corresponds to the adult overweight and the child overweight to adult obesity. It is recommended that WHO growth charts be used to monitor growth for infants and children aged 0-2 years. The WHO also developed BMI-for-age growth charts for preschoolers from birth to 5 years of age (WHO Multicenter Growth Reference Study Group, 2006) that were based on height, weight, and age. These charts, as well as the 1990 UK reference, are intended primarily for clinical use in monitoring children's growth, other purposes such as definition and diagnosis of overweight and obesity are secondary.

Due to the non-representative nature of the CDC growth charts, the International Obesity Task Force (IOTF) developed BMI reference values that defined childhood obesity and overweight based on the data pooled from different countries such as Brazil, Britain, Hong Kong, Netherlands, Singapore, and the United States. The IOFT reference values were not developed to replace national reference values, rather to provide a common set of definitions that can be used in epidemiological and other related research studies, and by policy makers in different countries. These reference values were developed based on adult cut-off points that were linked to BMI centiles (85th and 95th percentiles) of children and adolescents (Cole et al., 2000). Comparing the prevalence of childhood obesity between and within countries using the IOTF reference values is still challenging due to the complexity of body composition among individuals of various racial groups.

Other anthropometric indicators have also been investigated as tools of determining weight status in children and adolescents. For example, a study by Chomtho et al (2006) found a strong correlation

between mid-upper-arm circumference (MAUC), triceps skinfold thickness, arm fat area; and total fat mass. In this study the authors observed that arm fat area, MAUC, and triceps skinfold thickness explained 67, 63, and 61% of variability in total fat mass in healthy children. Mazıcıoğlu and coworkers (2010) supported the use of more than one anthropometric measure by suggesting that the WC and MUAC be substituted for one another as an additional evaluation tool next to BMI in detecting overweight and obese children and adolescents. Regardless of these findings, it is recommended that reference values be developed for each population in order to properly identify children and adolescents at risk of developing cardiovascular diseases.

1.2. Prevalence of Childhood Obesity

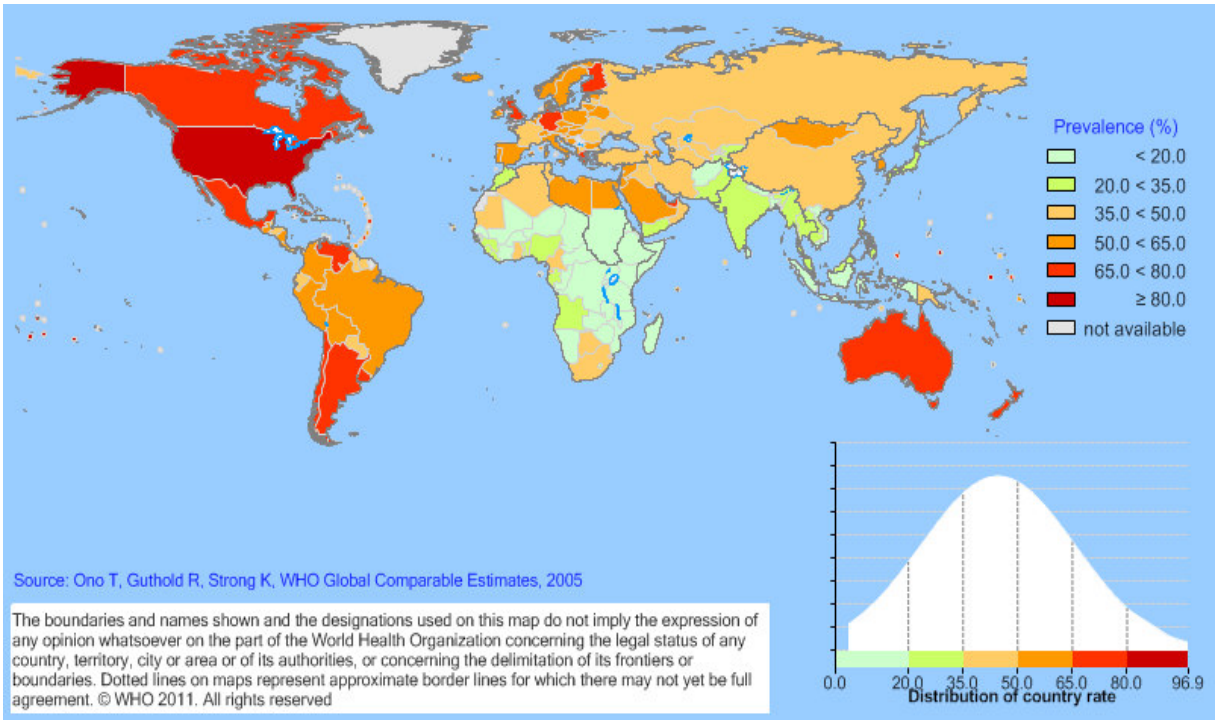
Obesity, initially thought to affect affluent countries, is now a global health problem estimated to affect more than 1 billion adults (http://www.who.int/mediacentre/factsheets/fs311/en/index.html). Obesity prevalence is distributed unevenly across countries ranging from below 5% in Japan, Republic of Korea, China (though in some cities rates are almost 20%) and Indonesia to 75% in urban Samoa. A similar uneven trend in overweight prevalence has been observed across countries worldwide, with Japan having a lower rate of 23.2% while in USA it was as high as 66.3% (Low et al., 2009). In developing countries, the prevalence of overweight ranges from 2.4% in Indonesia to 35.6% in Saudi Arabia.

South Africa is among the countries undergoing economic transition and as a result obesity and overweight prevalence are approaching those reported in developed countries (Puoane et al., 2002; Dept of Health & MRC 2008). In comparison to other African countries, South Africa is among (including Egypt, Libya, Cameroon, and Tunisia) those with the highest prevalence of obesity/overweight (WHO comparable estimates, 2005) (Fig. 1.1). Similar to other countries, gender differences are observed in African population groups with females more affected than males (Cameron and Getz, 1997; Wisniewski and Chernausek SD, 2009; Baalwa et al., 2010). In South Africa particularly, approximately 8.8% of men and 27.4% of women older than 15 years were found to be obese according to the second South African Demographic and Health Survey (SADHS) conducted in 2003 (Department of Health, 2007). Urban dwellers, particularly Black women, were more obese than their Mixed Ancestry and Caucasian counterparts, highlighting race and demography as other important contributing factors to the variable prevalence observed among the country's population (Steyn et al., 1998; Walker et al., 2001; Puoane et al., 2002). However, among males, the highest prevalence of overweight and obesity was found in Caucasians and lowest in Mixed Ancestry and Blacks.

Similar to adults, obesity is a health concern in children and adolescents as it persists into adulthood with 80% of obese children likely to become obese adults (Schonfeld-Warden and Warden, 1997). In some cases, many complications associated with obesity are already evident in childhood, necessitating implementation of health management strategies at an earlier age. According to the WHO report (http://www.who.int/mediacentre/factsheets/fs311/en/index.html), approximately 43 million children under the age of 5 years globally were overweight of which 35 million were in developing countries. Since the1960s, childhood obesity has increased rapidly in both industrialized countries and those undergoing economic transition. Between 2002 and 2005, up to 32% of the population in developed countries was overweight or obese (Wang and Lobstein, 2006). Similar rates were observed in developing countries such as India, Mexico, Nigeria, and Tunisia.

The prevalence of overweight (6.9% for boys and 24.5% for girls) and obesity (2.2% for boys and 5.3% for girls) in South African adolescents appear to be the highest among all African countries (Wang and Lobstein, 2006). The South African Youth Risk Behaviour Survey (SA YRBS) 2002 (Reddy et al., 2008) found 21% of adolescents aged 13-19 years to be obese/overweight, with gender differences ranging from 7% in boys to 25% in girls. However, a study conducted in a rural area of Mpumalanga province documented a lower prevalence of overweight/obesity of 10% in adolescents of the same age; 16% in girls and 7% in boys (Kimani-Murage et al., 2010). Regardless of the lower prevalence observed in this study, gender differences were noted similar to findings of the SA YRB survey. Gender differences may be due to higher energy requirement in physically active boys compared to girls, hormone variation, and body image preference in girls (particularly in Black Africans and African Americans) (Kruger et al., 2006). Obesity in Black African girls increases with age while an opposite pattern was observed in their Caucasian counterparts (Armstrong et al., 2006). The effect of age in the prevalence of overweight and obesity in youth was also noted in the study by Kimani-Murage et al. (2010). In this study, the prevalence of overweight and obesity in girls was moderate in early childhood and low in late childhood. However, the prevalence increased progressively from age 10 years. This paradigm shift of obesity as the population ages may be due to cultural pressures of appreciating full-figured females as opposed to thin females, particularly in Black Africans.

a)



b)

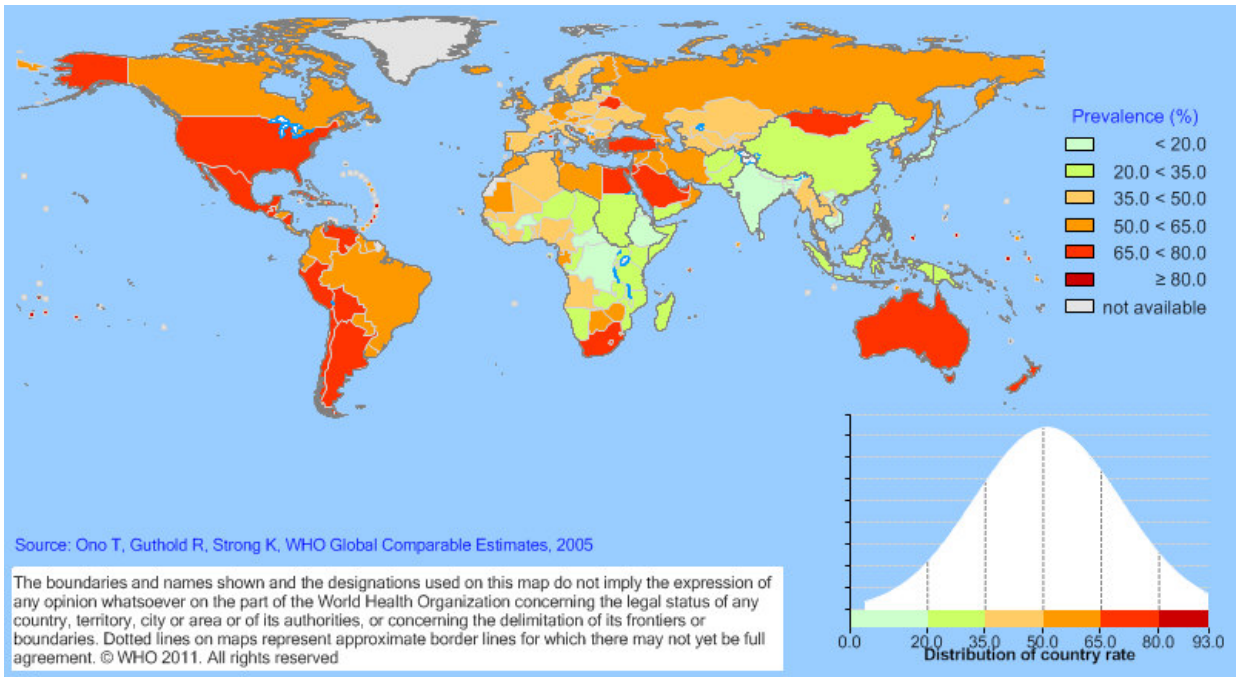


Figure 1.1. Global estimates of overweight and obesity (BMI ≥ 25 kg/m^2) prevalence, in a) males and b) females aged 15 years and above. *Accessed in World Health Organisation website in April 2011.*

As observed in other countries, obesity and overweight rates in South Africa also vary according to ethnicity and demographics. In all South African studies conducted thus far, urban children and adolescents are more overweight and obese than their rural counterparts, with the prevalence ranging from 4-13% (overweight) and 1-6% (obesity) (Table 1.3). These regional differences may be due to variations in physical activity levels and the type of food consumed. For example, rural dwellers are more physically active as they are likely to perform household and outdoor duties, and walk more frequently than their urban counterparts. They also consume less processed food with less fat content than urban dwellers (Vorster et al., 2005). Among obese/overweight children living in urban areas, Caucasians were mostly affected (Reddy et al., 2008). The higher prevalence observed in this population group may be due to cultural and socio-economic factors, which will be discussed in the following sub-section.

Table 1.3. Comparison of overweight and obesity in rural and urban dwellers in South African national studies. *Taken from Kimani-Murage et al. (2010).*

Study	Population (n)	Reference	Overweight		Obesity	
			Rural	Urban	Rural	Urban
NFCS (1999)	1-9 years (2894)	Labadarios et al., 2005	12	13	4	6
NFCS (2005)	1-9 years (2469)	Department of Health, Stellenbosch 2007	10	10	4	4
Agincourt (2007)	1-20 years (3489)	Kimani-Murage et al. (2010)	6		2	-
	1-4 years (671)		7	-	1	-
	5-9 years (970)		4	-	1	-
	10-14 years (944)		6	-	2	-
	15-20 years (904)		8	-	4	-

Abbreviations: DHS, Department of Health Survey; NFCS, National Food Consumption Survey; SAVACG, South African Vitamin A Consultative Group; UYRBS, National Youth Risk Behaviour Survey

1.3. Aetiology

The next section discusses the underlying causes of obesity. Although the causes apply in both children and adults, there are those that specifically affect the former group, and these will also be discussed.

1.3.1. Environmental factors

The aetiology of obesity is multifactorial in nature being a consequence of the interrelationship between increased food intake, physical inactivity, and an individual's genetic composition (Rosenbaum et al., 1997; Schwartz et al., 1999; Weber, 2003). However, bad dietary habits and

reduced physical activity or sedentary lifestyle are influenced or promoted by an obesogenic environment such as neighbourhoods with easily accessible transportation (Nestle and Jacobson, 2000; James, 2008) and high crime, social and cultural beliefs about the preferred body size, availability and marketing of energy-dense food (Drewnowski and Specter, 2004), and increased television viewing and TV or computer games. In children and adolescents, there are other contributing factors such as exposure to the intrauterine environment, infant feeding, and puberty that is marked by dynamic physiological and psychological transitions in both boys and girls. This sub-section describes environmental factors involved in the development of childhood obesity.

1.3.1.1. Socioeconomic and cultural factors

Cultural factors: Culture is defined as an integration of shared attitudes, values, and practices that characterise a group of people (or ethnicity), community, institution or organisation. It is learned and distributed within a group that does not necessarily possess the same knowledge or attitudes. Public knowledge about obesity is influenced by cultural beliefs and practices. For example, some socio-cultural environments among certain ethnic groups tend to favour the development of obesity as, to them, it is a sign of wealth rather than a risk factor of cardiovascular diseases, cancer and type 2 diabetes. These ethnic groups are usually characterized by low socio-economic status and accelerated weight gain during adulthood (Sobal and Stunkard, 1989; O'Dea, 2003). In USA the greatest increase in obesity prevalence is currently observed among African Americans, Hispanic, and Native American children (Crawford et al., 2001). There is evidence to suggest that African-American and Hispanic children are at greater risk for being overweight or obese, with 24% reported to be above the 95th percentile for body mass index (BMI) measurement. Data from African-American studies suggest that this population group has a preference for sweet and high-fat food, and as a result this type of food is highly consumed by this racial group (Bacon et al., 1994; Rocchini, 2002; Strauss, 2002; Centers for Disease Control and Prevention, 2003; Institute of Medicine, 2004; Larsen et al., 2006). Furthermore, a preference for a body image that favours obesity development has been documented, although in some communities this attitude co-exists with a negative perception about excess weight (Kumanyika et al., 1993; Flynn et al., 1998; Liburd et al., 1999; Jain et al., 2001). A 'heavy' body size particularly in females is considered attractive by African-Americans and does not necessarily have a negative effect on self-esteem, even among adolescent girls (Kimm et al., 1997). Being too thin on the other hand is linked to certain diseases such as cancer, tuberculosis or acquired immune deficiency syndrome, and drug abuse (Grosvenor et al., 1989; Ockenga J and Valentini L, 2005; Hira et al., 1998). Although these cultural beliefs are popular among adults, adolescents (from 13 to 18 years) are also affected as they are easily influenced by their environment.

Cultural differences were also observed in South African racial groups, and are believed to contribute to the variation in prevalence of overweight and obesity observed in Blacks, Mixed Ancestry and Caucasians. One of the cultural differences is in the perception of an acceptable 'body figure' among adolescent girls. As observed in African-Americans, it is desirable within the African culture for girls to be overweight as it is an indication of good health and happiness (Mvo et al., 1999; Mokhtar et al., 2001; Senekal et al., 2001; Puoane et al., 2005). In contrast, South African, American and Australian Caucasians negatively stigmatise obesity (Mvo et al., 1999; Crandall et al., 2001). These cultural groups are more discriminatory against obese people and perceive fitness and thinness as important determinants of interpersonal and economic success than Indian and African cultures. Men and adolescent boys, on the other hand, idealise large body size and muscularity; and would not view adiposity to be a problem.

Similarly, preferences for certain foods cooking methods may be culture specific with some cultures being traditionally inclined to have a higher fat intake, and other ethnic groups preferring healthy food. For example, Asian and Latino were known to consume higher fruit and vegetable and lower soda than Caucasians (Allen et al., 2007). However, recently the opposite is observed among Latinos while the intake of this type of food by Asians remained the same. Rural migration of other cultural groups, particularly in South Africa, has shifted the preference of food from traditional diets high in complex carbohydrates and fiber to highly processed and energy-dense diets (Bourne et al., 1994; Faber et al., 2005).

Socioeconomic status: Socioeconomic variables such as occupation, education, and household income are known to increase the risk of obesity in adulthood (Oh et al., 2011). The status of adults, however, directly affects that of children and adolescents as they are economically dependent on their parents. In a review done by Shrewsbury and Wardle (2008), the majority of studies conducted in developed countries found an inverse association between childhood obesity and socio-economic status (SES), similar to studies reviewed by Sobal and Stunkard (1989). Of the SES indicators investigated (such as age, gender, parental adiposity and marital status, maternal employment status, etc), parental education was more consistently inversely associated with adiposity in children (Shrewsbury and Wardle, 2008). It is suggested that the reason for this strong association may be that parent education is a more stable SES indicator than occupation and income. Additionally, education is suggested to influence knowledge and beliefs, which may be a key to healthy lifestyle more than access to resources is. Sex-specific differences in the relationship between SES and obesity were noted in adolescents, with the positive association identified in females but not in males (McLaren, 2007; Singh et al., 2008; Kautiainen et al., 2009; Oh et al., 2011). One of the suggested causes for

gender differences is attitude towards body image, with negative attitude towards obesity observed in girls (Yoon et al., 2006). Social and cultural perspective toward obesity is stricter in females than in males. Depending on cultural preferences, females quickly adjust their lifestyle to maintain a slimmer body shape. However, changes in lifestyle increase expenses and only individuals with high SES are able to maintain a healthy diet and engage in physical activities; hence the prevalence of obesity is higher in females with lower SES (Wardle et al., 2002; McLaren et al., 2007; Yoon et al., 2006; Wang and Beydoun, 2007).

In developing countries, on the other hand, a strong direct relationship between SES and childhood obesity was observed (Sobal and Stunkard, 1989). Developing countries are predominantly characterised by low SES with an abundance of affordable fast foods, snacks, and soft drinks, in addition to lack of facilities for physical activity (Darmon and Drewnowsk, 2008). Unhealthy food and lack of physical activity have been linked to rising obesity prevalence among children and youth. High-fat food and sweets cost only 30% more than 20 years ago while the cost of fresh produce has increased more than 100% (Monsivais and Drewnowski, 2007). Foods with the lowest energy density (mostly fresh vegetables and fruit) increased in price by almost 20% over 2 years, whereas the price of energy-dense foods high in sugar and fat remained constant. Thus, low-income household are restricted to buying unhealthy food, which in addition to the lack of physical activity facilities, results to uncontrolled weight gain. On the other hand, adequate calories may be available at low cost but requires an unrealistic investment in time. In most households in developing countries women are forced to work long hours in order to provide for their families, and as a result, there is a trade-off between money and time to prepare healthy food (Caprio et al., 2008). For example, it is reported that working women on average spend 6 hours per week preparing food while unemployed women spend 11 hours per week (Rose, 2003). In one South African study conducted in economically active individuals representing four different ethnic groups, factors such as Black ethnicity, low education and physical activity were associated with low SES. In addition to having one overweight parent, these factors were associated with higher BMI in children (Senekal et al., 2003).

1.3.1.2. Physical activity, sedentary lifestyle, and diet

Physical activity

Methods of measuring physical activity: Physical activity is an important aspect of life that must be practised to ensure good health as it prevents obesity and its associated co-morbidities (Scrutinio et al., 2005). The obesity epidemic is believed to be mainly due to a high dietary intake, particularly fat, and reduced physical activity. Participation in moderate physical activity for 45-60 minutes each day is required to prevent obesity (Gelaye et al., 2009; Department of Health, London 2004). The body achieves this by utilising and breaking down energy stored as fat through exercise. Physical activity can therefore be used as a tool to modulate the effect of fat intake in the body. It is for these reasons that many policies that are developed to combat obesity involve physical activity.

Several studies have been conducted to assess the magnitude of the effect of physical activity in obesity. It is however, a challenging endeavor as there are several methods used to define and measure physical activity. Physical activity can be defined as energy expenditure per body mass, physical activity level (daily energy expenditure as a multiple of resting energy expenditure), time spent in activities, etc. The validity of physical activity measurements depends on the methods used and the population under study. The doubly-labeled water test is the gold standard for measuring total daily energy expenditure under free-living conditions. This technique measures the average metabolic rate over a long period of time in subjects for whom other types of direct or indirect calorimetric measurements of metabolic rate would be difficult or impossible (Schoeller and van Santen, 1982). A minimum number of two samples (saliva, urine, or blood) are required to perform the method, an initial sample that is taken after the isotope has reached equilibrium, and a second sample taken some time later (24 hrs for small animals and 14 days for human adults). Although the method is considered a golden standard, it is expensive and therefore not suitable for large epidemiological studies (Goris and Westerterp, 2008). Physical activity questionnaires and diaries are more applicable in large epidemiological studies but, they provide less accurate estimates of physical activity level compared to more objective measures as the tool relies on self-reported (parental or child/adolescent) information (Bull et al., 2009). The questionnaires are a measure of physical activity variables such as games, sport, work, transportation, recreation, physical education, and planned exercise performed at home, school and communities. These variables are expressed in terms of duration (e.g., minutes), or frequency (e.g., times per week), or intensity, or a combination of these.

Pedometer is also one of the commonly used and inexpensive tools of monitoring and promoting physical activity in both adults and children. Given their low cost, pedometers are practical for use in large-scale epidemiological studies. It is an easy-to-use small device worn around the hip or waist that

is used to count the number of steps walked per day (Pedometers, 2004). A pedometer measures steps by using a spring-suspended mechanical lever that moves up and down in response to vertical displacement. Each of these movements is recorded and usually displayed digitally. Pedometers can also provide a number of derived output readings. These vary depending on the brand, and include distance traveled, calories expended and time spent at specific activity intensities (Tudor-Locke et al., 2009). However, these additional features are estimates and have not been validated among children. Although pedometers may be a good option to monitor walking in children, several studies have demonstrated that their accuracy and precision increases with speed, and tend to underestimate physical activity at a slower pace; and thus require discretion in their use (Schneider et al., 2003; Melanson et al., 2004; Beets et al., 2005; Crouter et al., 2005; Mitre et al., 2009). The use of pedometer to assess other movements such as cycling and swimming is another limitation that is not covered in the current literature, and this requires further investigation. Generally, the accuracy of a pedometer to assess physical activity was found to strongly correlate with accelerometer (r= 0.88) and lesser with doubly labeled water method (r= 0.67) (Ramirez-Marrero et al., 2005). The latter may be explained by the suitability of the doubly-labeled water method to measure energy expenditure than monitoring step counts, unlike the pedometer that only counts walking steps.

Built environment: Urbanisation has changed community structures, creating environments that promote obesity. An individual's external environment can be changed depending on the needs enforced by the changing lifestyle or modernisation. Such an environment is termed built environment and is defined as the range of structural elements in a residential setting: housing, roads, walkways, density, transportation networks, shops, parks, and public spaces (Weich et al., 2001; Handy et al., 2002). The built environment can either promote or hinder physical activity and healthy eating. This effect may vary according to gender, family structures, age groups and socio-economic status groups; depending on how individuals perceive and interact with the environment (Papas et al. 2007). For example, areas with few or lack of recreational facilities are characterised by high crime levels, uneven or hilly landscape, insufficient lightning and the presence of easily accessible public transport, and can therefore hinder physical activity (Booth et al., 2005). A significant association was observed between poor neighbourhoods with low-educated dwellers, the lack of physical activity facilities and subsequent disparities in health-related behaviour, and obesity measured at the individual level (Gordon-Larsen et al., 2006; Blomquist and Bergstrom, 2007; Vieweg et al., 2007). Children who spent more time outside the home were found to be more active, with farmland and grassland use in rural areas, and gardens and street environments in urban areas accounting for 40% of moderate to vigorous physical activity bouts (Jones et al., 2009). Furthermore, the presence of physical activity facilities in a particular area has been shown to be associated with an increased likelihood of engaging in 5 bouts of moderate-

vigorous physical activity per week and a decreased relative risk of overweight. However South African studies have reported the contrary: a higher prevalence of obesity was found among Caucasian children (14.2%) who lived near the central business district where physical activity facilities are expected to be found, compared to Blacks and Mixed Ancestry scholars who resided in areas characterized by low SES (Mvo et al., 1999). This may be explained, in part, by the presence of fast-food outlets and other highly available energy-dense food products in the central business district, near residential areas of Caucasian children. This picture however is beginning to change as obesity/overweight prevalence among Black African and Mixed Ancestry youth is approaching that of Caucasians due to the transitions in political environment and migration of the former population groups to urban areas (Bourne, 1994; Steyn et al., 1994; Steyn et al., 1996; Puoane et al., 2002).

Gender differences have also been observed in several studies. Boys have been found to be influenced to exercise when seeing their peers engaged in physical activity, if they reside in an area with roads and traffic calming measures (Carver et al., 2008). On the other hand, automobile transportation to school, length of annual school sports meetings, availability of game shops near the home and time spent playing video games were associated with boys' physical inactivity (Trang et al., 2009).

Sedentary lifestyle

There is evidence indicating that children and adolescents have become less physically active due to the decreased amount of time they spend outdoors (Andersen and van Mechelen, 2005; Dollman et al., 2005). It is reported that these age groups spend approximately 80% of their time engaged in minimum physical activities or sedentary activities such as television (TV) viewing, playing digital games and computer usage (Dietz Jr and Gortmaker 1985; Reilly et al., 2004; Rennie et al., 2005; Rivera et al., 2010). According to the WHO's Health Behaviour in School-aged children (HBSC) survey, the prevalence of TV viewing ranged from 26.5 to 49% (Currie et al., 2004). Dietz Jr and Gortmaker (1985) were the first to link high TV viewing with a higher risk of overweight and obesity among children. From this study it was also observed that among adolescents, for each additional hour of TV viewing per day, the prevalence of obesity increased by 2%. Since then, several studies have provided evidence supporting the relationship between TV viewing and obesity. A meta-analysis by Marshall and co-workers (2004), demonstrated a significant negative effect between TV viewing, video/computer game use and physical activity. Furthermore, a statistically significant association between TV viewing and body fatness was reported by Marshall and coworkers (2004), however, the effect size was small (0.066) implying that 99% of the variance in body fatness may be due to other factors. Other studies, however, reported a strong and conclusive relationship between TV viewing

and body fatness or obesity (Gortmaker et al., 1996; Beyerlein et al., 2011). For example, Gortmaker et at (1996) reported that 10-15 years old American adolescents that watched TV for more than 5 hours a day were 4.6 times more likely to be overweight than those watching TV for 2 hours or less per day. Similarly, a positive association between weekly hours spent sitting down and prevalence of obesity, and a negative correlation between obesity and leisure time physical activity was observed in a study conducted in males and females older than 15 years (Martinez-González et al., 1999). Potential determinants related to TV viewing, particularly in the home environment have been reported. It has been reported that children and adolescents having TV sets in their bedrooms spend more hours watching TV (van Zutphen et al., 2007; Delmas et al., 2007). However in few cases, children's desire to watch TV is counteracted by playing with their parents and the rules imposed on them about TV viewing, thereby minimising the time they spend watching TV (Salmon et al., 2006).

While the exact mechanism underlying the association of television viewing and obesity is unclear, it is evident that it occurs at the expense of physical activity. It has also been reported that television viewing is always accompanied by consumption of higher caloric food, and the lack of having healthy family meals (Epstein et al., 2005; Blass et al., 2006; Zutphen et al., 2007). Recently, in the California Health Interview Survey, Shi and Mao (2010) reported that adolescents who viewed more than three hours of television per weekday were 38% likely to consume fast food than those who did not view television as much. Additionally, the study reported an association between excessive weekday recreational computer use and consumption of sugary drinks more than once. Media advertising is believed to be promoting high caloric intake during TV viewing. According to Kunkel (2001), children spend an average of 5.5 hours per day using various media and are exposed to at least one food advertisement every 5 minutes (40 000 TV commercials annually). The majority of the adverts are for high-calorie food. Additionally, advertising campaigns of these types of food are linked to movies, cartoon characters, toys, video games, branded kids clubs, internet, and educational materials, features of which are enticing to children. Television viewing forms part of the family environment that promotes consumption of high caloric food, snacks and soft drinks, and therefore, influences eating behaviour of children.

Diet

In addition to TV viewing, Campbell and co-workers (2006) reported a high parental perception of a child's dietary adequacy to be associated with children's reduced consumption of vegetables and increased consumption of energy-dense savory and sweet snack foods. This association may stem from the fact that adults tend to perceive the aspects of their diet to be better than they actually are (Variyam et al., 2001; Lechner et al., 1997). Furthermore, parents perceive the cost of fruit and

vegetables to be high, hence the low preference and purchase of these products. Gillman and co-workers (2000) also showed evidence that eating a family dinner was linked cross-sectionally with health promoting dietary intake patterns, including the consumption of more fruit and vegetables. The reports presented here suggest that parents provide direct role models to children for the type of food they consume.

Fast-food, soft drink and candy (sweet) vendors are rapidly increasing in schools, further promoting consumption of such products by children and adolescents. On average, adolescents consume 11% of calories from soft drinks and twice as much from milk, putting them at a higher risk of becoming obese or overweight (Ludwig et al., 2001; Lim et al., 2009). Dietary intake behaviour or food intake is also affected by portion size, taste, and method of food preparation. There are also strong cultural influences on the type of food consumed with some racial groups having a preference for their traditional food. Often these types of food are enriched with carbohydrates, and are dominantly consumed in poorer countries, whilst a high fat diet enriched with saturated fatty acids is common in developed countries (Faber et al., 2001; Willett, 2002; Cordain et al., 2005). African countries, particularly, are characterised by the existence of nutrition transition and cultural-based nutritional differences, resulting in coexistence of under- and over-nutrition between and within populations and across all ages (Bourne et al., 1994; Steyn et al., 1994; Mvo, M Phil Thesis 1999; Savy et al., 2005; Bourne et al., 2007). Although overweight/obesity is generally associated with increased fat intake, Bray and Popkin (1998) reported a high prevalence of overweight on individuals consuming low-percentage energy from fat. Likewise, Kruger and co-workers (2002) found a weak correlation between dietary fat intake and BMI in the THUSA study. These studies suggest that a general assessment of diet is not an effective tool for investigating the association of nutrient intake and obesity. It is for this reason that several studies began to specifically investigate diet composition, instead of conducting a generalised assessment of diet and its effect on body weight changes.

Diet composition: There is substantial evidence showing that energy balance is achieved by separate regulation of carbohydrates, fats, and proteins. Insulin is an important regulator of glucose homeostasis, modulator of energy partitioning (Schenck et al., 2008), polyunsaturated fatty acid (PUFA) synthesis (Brenner, 2003; Wang et al., 2006), fatty acid transport (Ghebremeskel et al., 2004; Thomas et al., 2005), and hypothalamic action on the control of food intake (Bruning et al., 2000). In the postprandial state, insulin enhances fuel storage, inhibits lipolysis (Unger, 2003), and is involved in leptin release by adipocytes (Cheng et al., 2000). Any abnormalities related to dysregulation of carbohydrates, fats and protein can lead to cardio-metabolic disorders. For example, nutritional factors including energy excess, high energy density, increased consumption of carbohydrates and fat have

been reported to lead to obesity (Rolls et al., 2002; Astrup et al., 2004; Webber et al., 2005; Canete et al., 2007).

With specific reference to fat, it has also been proposed that dietary fat composition and the amount of energy intake may contribute to the development of obesity (Jones and Scholler, 1988; Khor, 2004). Fat composition refers to the structure of fatty acids, chain length, degree of unsaturation, position and configuration of the double bonds; which have been suggested to affect the metabolic fate of dietary fatty acids (DeLany et al., 2000; Madsen et al., 2005). Studies in animal models have demonstrated that body weight change may be responsive to different types of dietary fats (Lynn and Brown, 1959; Marette et al., 1990; Moussavi et al., 2008). However, as noted by Moussavi and coworkers (2008), the conditions and type of diet used in animal studies are not similar to those of human even though investigators tried to emulate them. Human diet contains diverse fatty acid profiles. The effect of different types of fats in human body weight seems to be complex as observed in both metabolic and epidemiological studies. Some studies have shown that consumption of a diet rich in medium-chain fatty acid could lead to a decrease in body weight (Kauntiz et al., 1958; Hainer et al., 1994; Dulloo et al., 1996; Tsuji et al., 2001; St-Onge et al., 2003).

The effect of a diet rich in monounsaturated fatty acids has also been investigated with the majority of studies reporting a positive association between this type of fatty acids and BMI (Williams et al., 2000; Brunner et al., 2001; Sanchez-Villegas et al., 2006). Several reasons for the conflicting outcomes of studies conducted were proposed (Moussavi et al., 2008). For example, in both clinical trials and epidemiological studies, physical activity which is the major component of the energy equation was not accounted for; the total energy intake in populations was either not adjusted for or it was estimated, impairing the power of detecting any effect present; different statistical methods were used.

As observed on fat diet, the composition of dietary carbohydrates as opposed to the total amount may potentially influence body weight and insulin sensitivity. Depending on chemical structure, carbohydrates are traditionally classified as simple or complex (polysaccharide) (Jenkins et al., 1981). The latter are considered to be a healthy alternative to dietary fats. However, many complex carbohydrates (eg, baked potatoes and white bread) produce even higher glycemic responses than do simple sugars (Ludwig, 2002). The quality of carbohydrates is determined by the amount and type of fiber, the extent of processing, and glycemic index and load (GI and GL). The GI of food is an area under the two hour blood glucose response curve measured two hours after ingesting 50 grams of a carbohydrate portion (Jenkins et al., 1981). GI values of individual food largely depend on the rate at which they are digested and the speed of carbohydrate absorption, making their physical forms an

important factor (Brouns et al., 2005). Typically, foods with more compact granules and high amounts of viscose soluble fiber (eg, barley, oats, and rye) are digested at a slower rate and have lower GI values than do highly processed carbohydrates (for example, white bread). These refined carbohydrates are more rapidly attacked by digestive enzymes due to grinding that reduces particle size and removes most of the bran and the germ. It has been shown that consumption of high-GI carbohydrates has less of an effect to suppress appetite and a diminished ability to induce satiation than foods with lower GI (Ludwig, 2000; Roberts, 2000). The long-term effect of consuming high-GI diets may lead to energy overconsumption and promote weight gain, creating a platform for the development of cardio-metabolic disorders. Refined carbohydrates are likely to cause even greater metabolic damage through increased GI and high insulin secretory responses than saturated fat in a predominantly sedentary and overweight population (Hu, 2010). This is despite any rigorous supportive evidence and convincing data to the contrary that central administration of insulin acts to suppress appetite and reduce energy intake in primates and rodents (Schwartz et al., 1992). The 2003 evidence-based report from the WHO found that the only convincing dietary factor protecting against weight gain and obesity was a high dietary fiber intake.

1.3.1.3. Intrauterine environment, and factors acting on early life and during puberty

Pre and post natal factors: The prenatal period, infancy, and early childhood may be stages of particular vulnerability to obesity development because they are unique periods for cellular differentiation and development. Maternal environment during fetal and infant development determines the future course of an individual from child- to adulthood (Lederman et al., 2004). For example, maternal malnutrition during fetal and infant development have been associated with permanent structural and functional changes of the body and later development of diseases of lifestyle such as type 2 diabetes, obesity, glucose intolerance, hyperlipidemia, insulin resistance, and hypertension (Gray et al., 2002). Both maternal malnutrition and postnatal overnutrition influence programming of obesity during fetal development through hormonal dysregulation of appetite (Remacle et al., 2004; Grattan et al., 2008). According to animal models the key player in this mechanism is leptin (LEP) (Bouret et al., 2004; McMillen et al., 2005; Chen et al., 2008). These studies have demonstrated that both maternal malnutrition and overnutrition may cause fetal programming through a common pathway that involves neuropeptide Y (NPY) and proopiomelanocortin (POMC), targets of leptin in the arcuate nucleus of the hypothalamus. Upon activation, neurons expressing NPY and POMC mature continuously for up to three weeks after birth in rodents, allowing permanent programming of appetite regulatory centers by maternal and fetal hormones (Grove et al., 2005; Melnick et al., 2007). Hyperphagia and leptin resistance are common phenotypes during pregnancy, leading to increased fat

accumulation in adipose tissue. In obese individuals, this may have a long-term effect on developing offspring while in lean females it is an adaptive response to hormonal changes associated with pregnancy so that the mothers can store energy in preparation for the high metabolic demands of the forthcoming lactation (Naef and Woodside, 2007; Augustine et al., 2008). One in five women in their reproductive age is obese with direct effects on maternal and fetal complications (Chu et al., 2008). Infant complications associated with maternal obesity include increased body fat mass and greater subcutaneous adipose tissue (Sewell et al., 2006; Forsum et al., 2006). It is also suggested that a higher maternal BMI during the first trimester of pregnancy is related to obesity in 3-6 months old infants (Rising and Lifshitz, 2008) and 2-4 year old children (Whitaker, 2004).

Insulin has also been implicated in mediating fetal programming that results in childhood and later adult metabolic complications. The insulin effect is a result of maternal diabetes during pregnancy. During pregnancy maternal glucose is transferred to the fetus, and the developing fetal pancreas respond to a glucose load by producing insulin, which acts as a fetal growth hormone in addition to glucose regulation (Freinkel, 1980). Increased insulin levels were observed in amniotic fluid reflecting fetal pancreatic insulin production, and this was correlated with obesity during adolescence (at ages 14 to 17 years) (Silverman et al., 1998). According to Silverman and co-workers (1991), obesity in children of diabetic mothers is apparent at birth, and only progresses after the age of 4 years. Not all studies, however, have found an association between gestational diabetes and risk of offspring obesity. Whitaker and co-workers (1998) found no increase in BMI among young adults of mothers with mild, diet-treated gestational diabetes. Another study found a moderate increase in risk of adolescent overweight among offspring of diabetic mothers, but the risk disappeared after adjustment for maternal BMI (Gillman et al., 2003). These studies suggest that severe fetal hyperinsulinemia may be required for the fetus to manifest programming for later obesity.

Intrauterine growth restriction possible due to maternal undernutrition affects approximately 30 million births per year, most of these occurring in developing countries: 75% in Asia, 20% in sub-Saharan Africa and 5% in Latin America and the Caribbean (de Onis et al., 1998). A meta-analysis that was based on cohorts in Brazilian, Guatemala, Indian, Philippines, and South African populations on long-term effects of maternal and child undernutrition observed the following outcomes related to cardiovascular diseases: undernutrition was associated with short adult height, and in women, with lower offspring birth-weight; increased size at birth and in childhood were positively associated with adult BMI (Victora et al., 2008). A baby of low birth-weight, who is stunted and underweight in infancy and rapidly gains weight in childhood and adult life upon exposure to obesigenic environment, can ultimately develop cardiovascular and metabolic disease, according to several studies (Bhargava et

al., 2004; Prentice and Moore, 2005; Barker et al., 2005; Lawlor et al., 2007). Rapid weight gain is relevant in low-income and middle-income countries that are undergoing rapid economic transition, and where there is co-existence of poverty and wealth. Indeed, a South African study conducted in rural villages of the Limpopo Province reported a high prevalence of stunting (48%) in 3-year old children of which 31% were overweight (Mamabolo et al., 2005). In Africa as a whole, 35% of children under 5 years of age are believed to be stunted (de Onis et al., 2000; Hautvast et al., 2000).

Birth-weight: The relationship between birth weight and obesity has also received great attention. As with maternal obesity, birth weight has been linked to various measures of abdominal adiposity in childhood and adulthood (Kuh et al., 2002). One of the studies that examined fetal origin of adult diseases reported evidence of association between obesity, accelerated growth velocity and low birth weight (Barker et al., 2002). Another study, however, suggests that being small for the gestational age and not a low birth weight alone is most likely to increase the risk for obesity in adulthood (Ong and Dunger, 2004). Other studies however, have reported an association between high birth weight and overweight and obesity in both children (Stettler et al., 2002; Dubois and Girard, 2006) and adults (Law, 2001; Freedman et al., 2005). Apart from maternal obesity, the role of small for gestational age (SGA) and birth weight in obesity is confounded by other factors such as preterm and term infancy, growth velocity, and maternal substance abuse (Gaskins et al., 2010). For example, preterm birth, low birth weight, and SGA have been associated with increased risk for adolescent overweight (Abe et al., 2007) but not associated with BMI among 8-year-old children (Casey et al., 2006). On the other hand, high birth weight was associated with overweight and obesity at 3 to 7 years in term children, (Hui et al., 1997; Mei et al., 2003; Zhang et al., 2009) and 11 years among children born preterm (Gaskins et al., 2010) but not in adolescents. However in one study, a 1 kg-increase in birth weight was associated with 30% increase in the risk of overweight during the ages of 11-14 years after adjusting for maternal BMI and other covariates (Gillman et al., 2003).

Growth velocity: There is substantial evidence supporting an association between rapid weight gain during early life and risk of obesity in adulthood (Monteiro and Victora, 2005). Rate of fetal growth is also determined by genetic factors. Increased weight gain velocity in childhood from 1 year and 9 months to 5 years was shown to be an independent predictor of adults BMI and waist circumference (McCarthy et al., 2007). Similarly, increased weight gain velocity between 0 and 6 months and between 2 and 5 years was found to be strongly associated with adult BMI among individuals of different ethnicities (Sachdev et al., 2005). This association was further supported in a South African study, which demonstrated that 20% of children sampled in the study with rapid weight gain were significantly lighter at birth and developed obesity throughout childhood (Cameron et al., 2003).

Crowther and co-workers (2008) also demonstrated that in South African children, increased growth velocity in early childhood at all ages lead to higher body weight at age 7 years. Increased growth velocity is often seen in children who are thought to have experienced intrauterine growth retardation, a phenomenon called catch-up growth. This process is called catch-up growth because growth rate increases as an effort to bring a child's height-for-age or weight-for-age status back toward the normal centile. Acceleration of growth velocity during the first 2 weeks of life has also been reported to increase insulin-related risk processes (McCarthy et al., 2007; Crowther et al., 2008).

Infant feeding: Infant feeding has been reported to play a major role in child's development. Several studies in developed countries suggest that breast-feeding reduces the risk of obesity during childhood and adolescence, with corresponding benefits on adult health (Hediger et al., 2001; Gillman et al., 2001; Owen et al., 2005). Some studies reviewed by Owen and co-workers (2005) suggested that the duration of breastfeeding is an important factor as it was shown to have a slightly stronger protective effect. How breastfeeding reduces the risk of obesity is unclear. It is suggested that the presence of leptin in breast milk protects breastfed babies against obesity later in life compared to those fed milk formula (Stocker and Cawthorne, 2008). Not all studies were able to demonstrate the reducing effect of breast milk on the risk of obesity (Butte et al., 2000; Hediger et al., 2001). The inconsistency observed in different studies may be due to the presence of confounding factors such as socioeconomic status, maternal obesity and smoking, and variation in the statistical power of difference studies. Formula feeding, on the other hand, has been associated with the development of atopy, diabetes mellitus, and childhood obesity (Gaynor, 2003; Wolf, 2003). Food and Drug Administration recommended that the following nutrients be present in all infant formulas: protein, fat, vitamins C, A, D, E, K, B1, B2, B6, and B12, niacin, folic acid, pantothenic acid, calcium, phosphorous, magnesium, iron, zinc, manganese, copper, iodine, sodium, potassium, and chloride (Stehlin, 1993). The amount of each formula nutrient varies significantly compared to breast milk, and their composition does not change as the infant ages. Formula is, thus, reported to be unresponsive to the growing infant's needs, making the digestive process more difficult (Lawrence, 1994). Differences in digestive and absorption of breast and formula milk have been implicated in the mechanism underlying poor child health outcomes. It is suggested that the mechanism involves increased insulin concentration in response to the presence of high protein and/or nitrogen in formula milk, which in turn stimulates fat deposition and early development of adipocytes (Lucas et al., 1980; 1981). Breast milk on the other hand, contains bioactive factors which may modulate epidermal growth factor and tumour necrosis factor α, both of which are known to inhibit adipocyte differentiation in vitro (Petruschke et al., 1994; Hauner et al., 1995).

Adiposity rebound: The adipose tissues undergoes several changes during human development (Häger, 1977; Knittle et al., 1979): during the first year adiposity increases due to increasing size of adipocytes, it decreases in the following year or two, and remains stable for several years. While adipose tissue growth is decreasing, body height continues to increase. During this first phase, the number of adipocytes remains stable. Adiposity rebound commences at about 6 years, and this is when both the number and size of adipocytes increases (Häger, 1977; Knittle et al., 1979). It has been demonstrated on several studies that there is an association between timing of adiposity rebound and later risk of obesity. Initial estimates of adiposity rebound were obtained using BMI, and it was uncertain whether early rebound reflects greater relative weight gain or reduced height velocity (Dietz, 2000). Only longitudinal studies could demonstrate that indeed early rebound is the result of higher rates of weight gain rather than slower average accrual of height, with the weight gain being predominantly due to a rapid increase in body fat (Taylor et al., 2004; Williams, 2005).

On investigating the relationship between timing of adiposity rebound, Cameron and Demerath (2002) observed that earlier adiposity rebound (that occurring before the age of 5–5.5 years) is associated with later obesity and adverse health outcomes including elevated blood pressure and diabetes. A study conducted in the US showed that each year decrease in age at adiposity rebound was associated with a 2.5 kg/m^2 increase in the predicted BMI level at age 19–23 years (Freedman et al., 2001). The clinical utility of adiposity rebound in identifying children at risk of developing obesity has been questioned by some authors (Williams et al., 1999; Cole, 2004), stating that BMI at age 7 has the same predictive value for later fatness as age at adiposity rebound, and measuring adiposity rebound requires a minimum of three serial BMI measurements. Taylor and co-workers (2005) further argued that if the timing of adiposity rebound can only be detected beyond the age of 7 years, those children showing a very early rebound would miss an opportunity to initiate interventions at an early stage. Other authors, however, have suggested that, compared to a high BMI that may only appear after a progressive increase over many years, an early adiposity rebound is a better indicator of the timing of the origin of obesity (Rolland-Cachera and Bellisle, 1990; Rolland-Cachera, 1993). These authors further suggest that very early adiposity rebound recorded in most obese subjects may imply that factors promoting obesity have operated very early in life and probably several years before the adiposity rebound. A reduced energy balance owing to high-protein, low-fat intake in early life is reported to be one of the possible factors (Rolland-Cachera et al., 2006). It is hypothesised that a relative energy deficit early in life may programme mechanisms of adaptative thermogenesis (Dulloo et al., 2002). According to Rolland-Cachera and co-workers (2006), adaptation to low fat intake in early life may have adverse effects later in life, when children are exposed to and eat a more abundant high-fat diet.

Physical activity has also been proposed to be one of the factors influencing early adiposity rebound. A retrospective analysis demonstrated that 10-year-old children who were more physically active underwent adiposity rebound at a later age than those who were less active (Deheeger et al., 1996). Watching television for more than 8 hours per week at the age of 3 years increased the risk of obesity at 7 years of age by 55%. In their study Janz and co-workers (2002) observed that inactive boys (measured using accelerometry) who watched a lot of television (as assessed by questionnaire) had 6.8% greater fatness than active boys who watched television the least. These authors also observed a similar trend in girls, although the differences were less (1.4 kg fat mass and 4.4% greater fatness).

Pubertal stage: This is a stage characterised by gender differences in adiposity, with females having a higher BMI than males (Del Parigi et al., 2002). Gender variation is more dramatic during adolescence, a transition period that begins with puberty and marked by profound physiological and psychological changes in boys and girls. The physiological and psychological changes, which are governed by hormonal levels, have been shown to influence the risk of obesity in females. Overeating among adolescents is associated with a variety of adverse behaviours and negative psychological experiences including low self-esteem and suicidal tendencies (Ackard et al., 2003). Some longitudinal studies identified depression as the major predisposing factor for late weight gain in adolescents (Tanofsky-Kraff et al., 2006). Depressed individuals tend to overeat leading to obesity, and affected individuals experience appearance-related teasing, which is associated with higher weight concerns, more loneliness, poor self-perception of physical appearance, higher preference for sedentary or isolated activities and lower preference for social activities (Hayden-Wade et al., 2005).

Physiologically, there are differences in fat distribution and changes in total and percentage body weight between girls and boys (Ogle et al., 1995; Taylor et al., 1997). According to Taylor and co-workers (1997) gender differences in percentage body fat are apparent even before puberty with boys having a lower fat content than girls, despite similarities in overall body weight, height and BMI. As a result, female adults have approximately 22% body fat while their male counterparts have an average of 15%. Furthermore, fat in females is usually deposited peripherally in breasts, hips, and buttocks while in males it is centralised in the abdomen (Deurenberg et al., 1990; Goodman-Gruen and Barrett-Connor, 1996). It is noteworthy that up to 80% of overweight adolescents are likely to become obese adults (Must and Strauss, 1999; Freedman et al., 2001).

1.3.2. Genetics of obesity

The complex nature of human obesity stems from the multiple interaction of several genes that control the physiology of food intake, energy expenditure, development of the body, and behavioral patterns towards food intake, and the environment (Tambs et al., 1992; Maes et al., 1997). According to twin, adoptees and family studies, genetic factors account for 40-70% of the variability observed in human adiposity (Stunkard et al., 1986; Bouchard et al., 1988; Tambs et al., 1992; Allison et al., 1996; Vogler et al., 1995; Maes et al., 1997). Twin studies have also demonstrated that the heritability of adiposity is higher than other quantitative traits. The heritability of obesity traits (Table 1.4) has been further supported by identification of quantitative trait loci (QTL) and genes through methods such as genome-wide scans (studies conducted on unrelated obese individuals), linkage analyses (conducted in families), and association studies (investigating the correlation between obesity and polymorphisms). The number of contributing genes, however, is still unknown.

Table 1.4. Heritability of obesity-related phenotypes as reported by several studies.

Obesity-related phenotypes	Heritability measure	Reference
Body mass index	0.50-0.70	Allison et al., 1996; Fabsitz et al., 1994
Total and regional fat	0.71-0.86	Malis et al., 2005
Skinfold thickness and waist circumference	0.72-0.82	Rose et al., 1998
Waist-hip-ratio	0.36-0.61	Rose et al., 1998
Cognitive to restraint to eating	0.59	Tholin et al., 2005
Uncontrolled eating	0.45	Tholin et al., 2005
Emotional eating	0.60	Tholin et al., 2005

Although research on the genetic basis of obesity has advanced, the mechanisms underlying the condition are still complex due to its heterogeneity even within families. Genetically, obesity can be classified into three forms: monogenic obesity caused by single gene defects, a syndromic obesity (Mendelian disorders for which obesity is a clinical manifestation but not a dominant feature), and common polygenic obesity that result from gene-gene and gene-environmental interaction. Genes of the leptin-melanocortin pathway that cause monogenic obesity only explains a small portion (1-5%) of

the condition. The polygenic form of obesity, on the other hand, is due to combined modest effects of many polymorphisms in several genes that interact with modifiable environmental factors (collectively termed obesogenic environment).

Animal models of obesity have been studied extensively in an effort to elucidate the mechanisms underlying human obesity, and currently there are several hundred genes that have demonstrated the heritability of body weight (Rankinen et al., 2006). Some of the popular genes implicated include leptin (*LEP*), leptin receptor (*LEPR*), agouti-related peptide (*AgRP*) and carboxypeptidase E genes. In addition to these genes, 408 QTL for obesity and body weight have been mapped in mice (Brockmann and Bevova, 2002; Rankinen et al., 2006). Identification of obesity genes in mice has paved a way of unraveling the complex nature of human obesity. This part of the literature review describes these genetic forms of obesity.

1.3.2.1. The Leptin-melanocortin system

Physiologically, obesity is characterised by excessive accumulation of fat in adipose tissue as a result of weight dysregulation (Campfield and Smith, 1999). Weight dysregulation involves an imbalance between energy intake and expenditure. The pathophysiology of obesity therefore requires an understanding of energy homeostasis, which is a complex system controlled through bidirectional communication between the periphery and brain (Stricker, 1990; Le Magnen, 1992). The periphery communicates with the brain relaying messages about the body's energy state through neural connections that are provided by the autonomic nervous system, hormones or metabolites. Of note is a number of circulating peptides and steroids that regulate food intake, which act mainly on the hypothalamus, brain stem, or afferent autonomic nerves (Table 1.5). These molecules are produced from at least three sits: gastrointestinal tract, adipocytes, and pancreas (beta cells).

Adipose tissue produces LEP (Zhang et al., 1994; Murakami and Shima, 1995; Maffei et al., 1995), while the pancreas secretes insulin (Steiner et al., 1967), and the gastrointestinal tract produces short-term meal-related signals such as peptide YY (Leiter et al., 1987), glucagon-like peptide-1 (Mojsov et al., 1986; Orskov et al., 1986), cholecystokinin (Friedman et al., 1985; Eysselein et al., 1986) and ghrelin (Kojima et al., 1999). In the brain, energy homeostasis is controlled by neural circuits that regulate feeding, mood, pleasure, pain, memories about the reward and punishment of behaviour, and cognitive functions such as liking, wanting, and decision-making for appetitive behaviours (Royall et al., 2002; Salamone and Correa, 2002; Berridge, 2003). There are three major centres in the brain that perform the above mentioned functions: the caudal brainstem, hypothalamus, and parts of the cortex and limbic system.

Table 1.5. Description of peptides and hormones involved in regulation of appetite. *Adapted from Macia et al., 2006*

Peptide	Main site of synthesis	Receptor	Site of action	Effect on food intake
BDNF	Hypothalamus and caudal brianstem	TrkB	Hypothalamus	↓
MCH	Hypothalamus	MCH1R	Hypothalamus	↑
OR	Hypothalamus, gut	OXR	Various brain regions and adrenal gland	↑
Drosophila Single-minded 1	Hypothalamus	Hypothalamus	-	↓
Serotonin (5-hydroxytrypamine)	Midbrain and hindbrain	$5HT_{2C}$ serotonin-receptor	Hypothalamus	↓
ADIPOQ	Adipocytes	AdipoR1	Hypothalamus	↑
Interleukin-1	Macrophages, keratinocytes, B lymphocytes, fibroblasts, and hypothalamus	Interleukin-1 type 1 receptor	Hypothalamus	↓
Interleukin-6	Macrophages, T lymphocytes, adipocytes	Interleukin-6-receptor	Hypothalamus	↓
Cholecystokinin	Gastro-intestinal tract	CCK_A	Hindbrain	↓
Peptide YY_{3-36}	Gastro-intestinal tract	neuropeptide Y receptor.	Hypothalamus	↓
Obestatin	Gastro-intestinal tract	GPR39	Hypothalamus	↓
GLP-1	Gastro-intestinal tract	GLP-1 receptor	Brainstem and hypothalamus	↓
Oxyntomodulin	Gastro-intestinal tract	GLP-1 receptor	Brainstem and hypothalamus	↓
Insulin	Pancreatic islets	INSR	Hypothalamus	↓
Pancreatic polypeptide	Pancreatic islets	Y4 and Y5 receptors	Medulla oblongata (area postrema)	↓
Glucocorticoids	Adrenal glands	Glucocorticoids receptors	Hypothalamus and brainstem	↑
Tri-iodothyronine	Thyroid gland	THRB*		↑
Estradiol	Gonads	ERα, and ERβ	Hypothalamus	↓

* thyroid hormone receptor α and *β* are alternately spliced to generate three major highly homologous nuclear receptor isoforms: TR*α*1, TR*β*1, and TR*β*2. The specific thyroid hormone receptor that mediates the effects of thyroid hormones on appetite is unknown. *Abbreviations:* ADIPOQ, Adiponectin; BDNF, Brain-derived neurotrophic factor; CCK_A, Cholecystokinin receptor subtype A; ERα, and ERβ, estradiol receptor alpha and beta; GLP-1, Glucagon-like peptide-1; GPR39, G protein-coupled receptor 39; 5-HT, 5-hydroxytrypamine; INSR, insulin receptor; MCH, melanin-concentrating hormone; OR, orexin; OXR, orexin receptor; PYY_{3-36}, Peptide YY_{3-36}; THRB, thyroid hormone receptor *β*; TrkB, Tyrosine kinase receptor; Sim1, Drosophila Single-minded 1; Y2R, neuropeptide Y receptor.

The hypothalamus in the central nervous system is the major centre that integrates signals about the nutritional status from the periphery. Within the hypothalamus, the arcuate nucleus (Fig. 1.2) in particular, is part of the network that controls appetite and energy expenditure by processing leptin and ghrelin signals, forming the leptin-melanocortin pathway. The arcuate nucleus contains two types

of cells that are responsive to leptin and ghrelin signals, namely, the appetite-stimulating (orexigens) and food-suppressing (anorexigens) neurons (Cone, 2005). The orexigens express two peptides, Agouti-related peptide (AgRP) and Neuropeptide Y (NPY), while the anorexigens produces peptides such as cocaine and amphetamine-related transcript (CART), and pro-opiomelanocortin (POMC). The action of leptin and ghrelin is reciprocal, resulting in opposite effects of energy regulation.

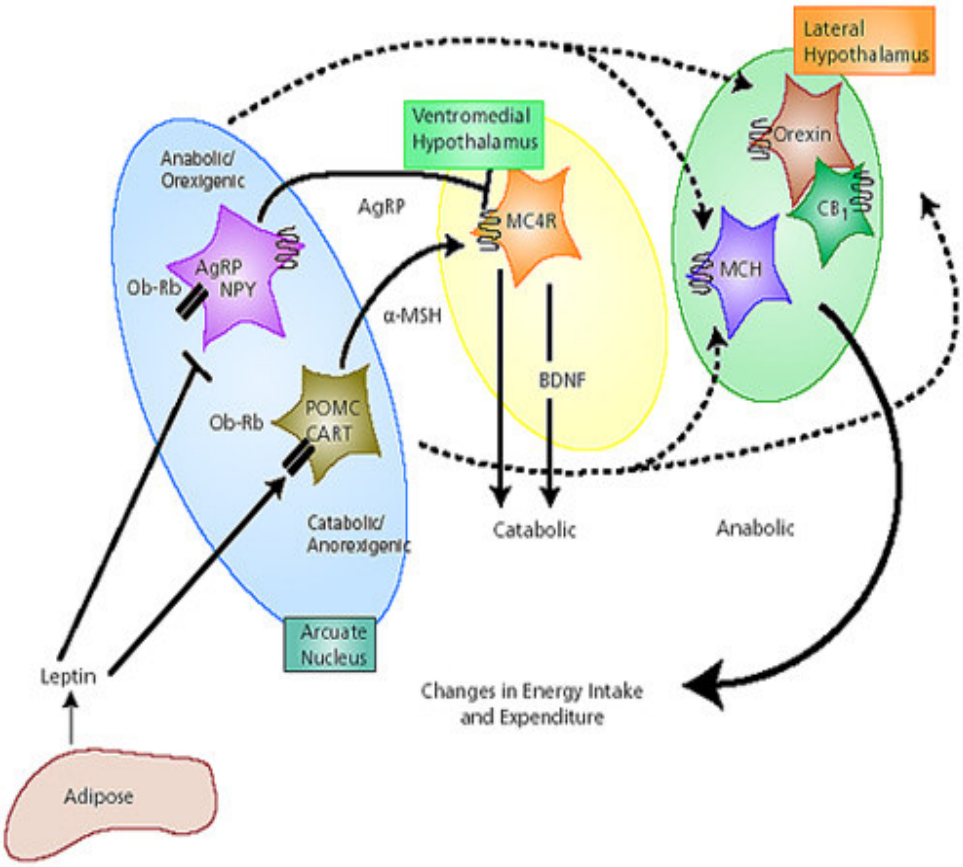


Figure 1.2. The leptin-melanocortin system. Leptin is secreted by the adipocytes and binds to hypothalamic leptin receptors, stimulating the expression of *POMC*-derived peptides and CART but inhibits the expression of NPY and AgRP. Neurons that express *POMC*-derived peptides and CART project to other sites of the hypothalamus that express MC4R and Y1R. Leptin also regulates BDNF–TrkB through an unknown pathway. *Abbreviations:* AgRP, Agouti-related peptide; BDNF, brain-derived neurotrophic factor; CB_1, CART, cocaine and amphetamine related transcript; MCH, melanin-concentrating hormone; MC3R, melanocortin 3 receptor; MC4R, melanocortin 4receptor; α-MSH, alpha melanocortin-stimulating hormone; NPY, neuropeptide Y; Ob-Rb, leptin receptor, b isoform; *POMC*, pro-opiomelanocortin gene. *Adapted from Flier, 2004.*

The level of leptin released by the adipocytes into the circulation is in proportion to body fat content. Upon release in response to fat storage, leptin crosses the blood-brain barrier to the hypothalamus where it binds to leptin receptors, thereby activating POMC/CART neurons and inhibiting NPY/AgRP neurons (Flier, 2004; Cone RD, 2005). Leptin acts via its receptors to also inhibit NPY/AgRP neurons thereby suppressing the expression and secretion of NPY and AgRP. Conversely, a decrease or

deficiency in leptin action stimulates appetite by the suppression of the synthesis of anorectic neuropeptides, and increased expression of orexigenic peptides. However, an absolute deficit of leptin does not underlie most cases of obesity. Instead, most obese individuals exhibit elevated circulating leptin levels (leptin resistance), which increase proportionally with their adipose mass (Considine et al., 1996; Maffei et al., 1996; Farooqi and O'Rahilly, 2005). Experiments revealed that naturally occurring obese (*ob*/*ob*) mice were deficient in leptin as a result of a loss-of-function mutation in the leptin gene, while diabetic (*db*/*db*) mice produced the circulating satiety factor in excess but lacked the ability to respond to it due to mutation in the leptin receptor gene (Zhang et al., 1994; Friedman and Halaas, 1998). These mutations, however, were not detected in obese humans, suggesting that different genetic defects may be responsible for this phenotype in humans. Leptin resistance was also observed in mice with diet-induced obesity, which were not responsive in peripheral leptin administration to reduce food intake and body fat (Van Heek et al., 1997).

A number of mechanisms have been proposed to explain leptin resistance, and these include the failure of circulating leptin to reach its target receptor within the brain, decreased expression of the leptin receptor within hypothalamic neurons, perturbations in developmental programming, etc (Bjørbaek and Kahn, 2004; Flier, 2004; Remacle et al., 2004). Another mechanism that has received attention is the attenuation of the intracellular leptin signaling cascade (Bjørbaek et al., 1998). The action of various cytokine receptors is negatively regulated by an intracellular protein, the suppressor of cytokine signaling-3 (Socs-3) (Starr et al., 1997). Leptin is structurally related to cytokines and acts via a receptor that belongs to the cytokine receptor family. It is, therefore believed that Socs-3 limits leptin signalling, and mediates leptin resistance (Kievit et al.; 2006). It has been demonstrated in mouse models that complete loss of *Socs-3* is embryonic lethal, and mice with only one functional copy of *Socs-3* or those with neuronal deletion of *Socs-3* are lean and leptin sensitive (Howard et al., 2004; Kievit et al., 2006; Mori et al., 2004). Regardless of the proposed mechanism, the question that still remains is whether leptin resistance reflects the underlying initiating mechanism of obesity, a consequence of obesity, or a combination of both.

Activation of NPY/AgRP neurons occurs via the release of ghrelin from the stomach, and its binding respective receptors in the arcuate nucleus when fasting ensues. Ghrelin has been shown to peak before meal initiation (Cummings et al., 2001) and to decrease postprandially in proportion to the caloric content of the meal (Tschöp et al., 2001; Callahan et al., 2004). This has also been observed in animal models, which demonstrated that ghrelin mRNA expression and secretion are increased by fasting, weight loss, and insulin-induced hypoglycaemia; and reduced by re-feeding and oral glucose administration (Tschöp et al., 2000). Ghrelin administration in rodents increased respiratory quotient

by stimulating glycolysis instead of fat oxidation, favouring fat deposition and explaining the increased weight gain and adiposity observed in these animals (Tschöp et al., 2000). In rats and humans ghrelin administration stimulated appetite and increased food intake, further supporting its role in energy regulation (Wren et al., 2000; 2001; Druce et al., 2004). In contrast, one study reported that the fasting plasma ghrelin concentration, independent of body weight, is a significant negative predictor of ad libitum energy intake (Salbe et al., 2004). Votruba and co-workers (2009), on the other hand, found no correlation between fasting ghrelin concentrations and food intake in a larger group. According to these authors, the possible explanation could be that ghrelin concentrations in obese individuals respond differently to meal challenges than in lean individuals. These conflicting findings may be due to the difference in the selection criteria of study groups: other studies have focused on such extremes, for example, many subjects with BMI <18.5 due to anorexia nervosa in one (Shiiya et al., 2002) and severely obese subjects with BMI 33–65 kg/m^2 in another (Holdstock et al., 2003).

Lack of NPY and AgRP had a modest effect on the pathophysiology of obesity in mice models, becoming modestly lean late in life (Wortley et al., 2005). In humans the effect varies according to age, with loss of NPY and AgRP leading to a severe life threatening hypophagia in adults while in newborn babies it results in mild food suppression and body weight loss (Flier, 2006). This suggests that neonates have a network-based compensation mechanism not present in adults. It is however, not known whether the suppressed food intake is due to the loss of NPY and AgRP or the absence of other neurotransmitters is involved.

The POMC is a polypeptide that is post-translationally cleaved in specific tissues by prohormone convertases 1 and 2 to produce molecules such as melanocortins α, β, and γ-melanocortin-stimulating hormones (MSH). One or more of the melanocortin peptides are involved in controlling food intake and energy expenditure by binding to and stimulating melanocortin-4-receptor (MC4R), and melanocortin-3-receptor (MC3R) (Fig 1.2). In addition, the melanocortins play significant roles in the cardiovascular system (Gruber and Callahan, 1989; Versteeg et al., 1998). They exert different effects by binding to 5 melanocortin receptors designated 1-5. These receptors are members of the G-protein-coupled family that are positively linked to adenylase cyclase, with cAMP as the intracellular second messenger (Chhajlani and Wikberg, 1992; Mountjoy et al., 1992; Tatro, 1996; Adan and Gispen, 1997). The γ-MSH has a high binding affinity for MC3R while α-MSH is the ligand for all the MCRs except MC2R, which mediates corticotrophin stimulation of steroidogenesis. Only the MC3R and MC4R play a role in body weight and blood pressure regulation as demonstrated in several studies (Coll et al., 2004; Greenfield et al., 2009; Ni et al., 2003).

Distal to the leptin-melanocortin pathway, there are other proteins that appear to contribute to the regulation of food intake and energy expenditure (Table 1.5). Of note is the brain-derived neurotrophic factor (BDNF), a neural growth factor that exerts its effect by binding to the kinase B receptor (TrkB). BDNF was known to play critical roles in the synaptic activity and plasticity in mature neurons (McAllister et al., 1999; Poo, 2001; Lu, 2003) until in 1992 when Lapchak and Hefti described that central infusion of BDNF attenuated weight gain in rats, suggesting another role in regulation of body weight. Subsequently, Pelleymounter and co-workers (1995) showed that infusion of BDNF in the lateral ventricule of adult rats induced severe, dose-dependent appetite suppression and weight. In humans, several genetic defects in BDNF gene or its receptor (TrkB) have also been linked with eating disorders accompanied by developmental delay, impaired short-term memory and unusually hyperactive behaviour (Yeo et al., 2004; Gray et al., 2006). Furthermore, individuals with Wilms' tumor, aniridia, genitor–urinary anomalies and mental retardation (WAGR) are characterized by gene defects and display obesity when deletions are extend into the *BDNF* locus (Kernie et al., 2000; Marlin et al., 1994; McGaughran et al., 1995). Four variants of the BDNF-encoding gene have been described in human patients with anorexia nervosa (AN) or bulimia nervosa (BN) (Ribases et al., 2003; Ribases et al., 2004). The most common polymorphism, *BDNF* Val66Met, has been shown to affect the sorting of BDNF into the nerve terminals and markedly reduces its activity-dependent secretion (Egan et al., 2003; Chen et al., 2004). Mutations in the gene encoding TrkB have also been linked to eating disorders. A de novo Y722C mutation was described in a young boy displaying severe obesity. This mutation was shown to considerably alter the signalling capabilities of the receptor TrkB (Yeo et al., 2004). Other mutations on the gene encoding TrkB have been linked to AN and BN, although the consequences of these mutations on the function of TrkB are currently unknown (Ribases et al., 2005).

BDNF has been proven to participate in the regulation of energy homeostasis downstream of leptin, with the ventromedial hypothalamus (VMH) identified as a key site for BDNF expression that is modulated by nutritional state (Xu et al., 2003). In this study, Xu and co-workers (2003) demonstrated that central infusion of BDNF in mice with abnormal *MC4R* suppressed hyperphagia and obesity. In addition, mice with a loss-of-function *trkb* mutation closely resembled those lacking *Mc4r* as they also developed hyperphagia and obesity, and increased linear growth when fed a high fat diet. To further support the role of BDNF in energy regulation, Wang and co-workers (2007) demonstrated that intraparenchymal injection of BDNF in the ventromedial hypothalamus reduced food intake and body weight gain, an effect that was abolished by preadministration of TrkB-Fc fusion protein, blocking binding between BDNF and its receptor TrkB. It has also been demonstrated that BDNF plays a role as a downstream effector of melanocortinergic signaling within the dorsal vagal complex (DVC), and

that its protein content within the DVC is under the control of brainstem melanocortin system (Bariohay et al., 2009). The DVC is located in the caudal brainstem and comprises three interconnected structures, the nucleus tractus solitarii (NTS), area postrema, and dorsal motor nucleus of the vagus nerve.

In the lateral hypothalamus, another orexigenic neuropeptide, melanin-concentrating hormone, is expressed and compelling evidence exists that demonstrates its role in energy balance (Flier, 2004). MCH knockout mice were hypophagic while those over-expressing the gene developed obesity and insulin resistance. In *ob/ob* mice, MCH was over-expressed while those lacking the gene had reduced body fat. In addition, Alon and Friedman (2006) showed that targeted ablation of MCH neurons caused hypophagia, increased energy expenditure and late-onset leanness.

1.3.2.2. Monogenic Obesity

Eleven genes have been implicated thus far in non-syndromic monogenic forms of obesity: corticotropin-releasing hormone receptor 1 (*CRHR1*); corticotropin-releasing hormone receptor 2 (*CRHR2*); G protein-coupled receptor 24 or melanin-concentrating hormone receptor 1 (*GPR24 or MCH1R*); leptin (*LEP*); leptin receptor (*LEPR*); melanocortin-3-receptor (*MC3R*)*;* melanocortin-4-receptor (*MC4R*); neurotrophic tyrosine kinase, receptor, type 2 (*NTRK2)*; proopiomelanocortin (*POMC*); prohormone convertases 1 (*PC1* or *PSCK1*); and single-minded homolog 1 (*SIM1*). This section will briefly describe a few genes due to their relevance to the present study.

***LEP* mutations:** Zang and coworkers (1994) identified the first obesity-causing null mutation in *ob/ob* mice. Subsequently, inactivating homozygous mutations were reported in humans, which resulted in severe early-onset obesity accompanied by hyperphagia but with very low serum leptin levels (Montague et al., 1997; Strobel A et al., 1998).

***LEPR* mutations:** A short variant of lepr was identified in *db/db* mice (Chen et al., 1996; Lee et al., 1996) and Zucker *fa/fa* rats (Takaya et al. 1996), which, in both animal models resulted in severe obesity. Similarly, *LEPR* homozygous mutation that resulted in a truncated protein was reported in humans. This mutation was characterised by early-onset severe obesity, lack of pubertal development, and a reduced secretion level of both growth hormone and thyrotropin (Clément et al., 1998; Farooqi et al., 2007). Leptin receptor heterozygotes, similar to that of leptin, appeared to have a normal phenotype.

***POMC* mutations:** Homozygous and compound heterozygous mutations have been reported in *POMC* (Krude et al., 1998; Krude et al., 2003; Farooqi et al., 2006), which caused early-onset obesity accompanied by adrenal insufficiency and red hair pigmentation (Ichihara and Yamada, 2008). The reported mutations resulted in the loss of either one or two *POMC* products, or all of its derived peptides.

Prohormone convertase 1 gene (*PC1*) mutations: PC-1 defects are one of the rare causes of human monogenic obesity with currently two cases of compound heterozygous mutations reported. In one case, the two mutations resulted in a truncated immature and inactive PC1 that could not be transported outside the endoplasmic reticulum (Jackson et al., 1997). The affected adult female had early-onset morbid obesity that was associated with hypogonadotropic hypogonadism, abnormal glucose homeostasis, small bowel malabsorption, and elevated plasma concentration of proinsulin and POMC. The second case involved an individual with a missense and nonsense mutation characterised with severe refractory neonatal diarrhea due to absorptive dysfunction in the small intestine (Jackson et al., 2003).

***MC4R* mutations:** There is currently no known *MC4R* obesity-causing mutation in animal models reported, however, gene targeting in mice resulted in *Mc4r* activation and subsequently maturity-onset obesity syndrome associated with hyperphagia, hyperinsulinemia, and hyperglycemia (Huszar et al., 1997). In humans, *MC4R* defects are the most common causes of monogenic obesity with more than 100 mutations described in different ethnic groups, the majority of which are transmitted in an autosomal dominant manner (Yeo et al., 1998; Vaisse et al., 1998; Mergen et al., 2001; Kobayashi et al., 2002). These mutations are characterised by clinical heterogeneity, and sometimes incomplete disease penetrance.

***MC3R* mutations:** Unlike *MC4R*, *MC3R* is more a predisposing factor of polygenic obesity than a cause of monogenic obesity. It is only recently that 9 possible obesity-causing mutations were reported. Three mutations (Ile183Asn, Ala70Thr, and Met134Ile) were identified in 3 unrelated subjects but not in control subjects (Lee et al., 2007). Although functional studies revealed impaired MC3R signaling activity as a result of these mutations, in some cases the variants did not co-segregate with obesity in adults but interestingly did with childhood obesity. Individuals harbouring heterozygous mutations exhibited higher leptin levels and adiposity, and less hunger compared to obese controls, reminiscent of the *Mc3r* knockout mice. Three additional mutations (Ala293Thr, Ile335Ser and X361Ser), which co-segregated with obesity were identified in Italian obese subjects (Mencarelli et al., 2008). However, only one mutation caused a functional defect in *in vitro* expression

studies. Recently, three other heterozygous mutations (Asn128Ser, Val211Ile, Leu299Val) were identified in three unrelated children. Although no conclusive evidence for functional impairment of the Asn128Ser and Val211Ile mutated receptors could be established, the Leu299Val mutation negatively affected the function of the *MC3R* (Zegers et al., 2010).

Mutations in peptides that function downstream the melanocortin receptors: single-minded drosophila homolog 1 (*SIM1*) and neurotropic tyrosine kinase receptor type 2 (*NTRK2*) genes

Further downstream of the melanocortin receptors only two genes, single-minded drosophila homolog 1 (*SIM1*) and neurotropic tyrosine kinase receptor type 2 (*NTRK2*), are reported to be defective in children with severe obesity among other abnormalities. A *de novo* balanced translocation involving chromosome 1p22.1 and 6q16.2 was identified in a girl with early-onset obesity (Holder Jr et al., 2000). When these authors cloned and sequenced both translocation breakpoints, they observed that *SIM1* was disrupted on 6q, separating the 5-prime promoter region and the basic helix-loop-helix from the 3-prime PER-ARNT-SIM and putative transcriptional regulation domains. Holder and co-workers (2000) hypothesized that haploinsufficiency of SIM1, possibly acting upstream or downstream of MC4R in the PVN, was responsible for severe obesity in their patient. A deletion was also identified in chromosome 6q16.1-q21 in a patient with Prader Willi syndrome, resulting in the absence of SIM1 (Faivre et al., 2002). Although the transcriptional targets of Sim1 are unknown, it is expressed in the developing kidney and central nervous system, and is essential for formation of the supraoptic and paraventricular (PVN) nuclei of the hypothalamus (Holder et al., 2000). There is strong evidence suggesting that in the PVN neurons *SIM1* is coexpressed with *MC4R* (Balthasar et al., 2005; Kublaoui et al., 2006). Animal studies have also demonstrated that *SIM1* plays a role in regulation of appetite. As observed in a study by Holder and co-workers (2004), the absence of one copy of SIM1 in mice resulted in reduced food intake when these animals were switched from normal chow to a high-fat diet, suggesting that the gene is involved in regulation of appetite. Similar defects were observed in *Mc4r* knockout mice with defective melanocortin signaling (Huszar et al., 1997). Another *de novo* missense mutation, Tyr722Cys, was detected in *NTRK2* that caused a severe early-onset obesity and impaired memory, learning, and nociception. The *NTRK2* is a receptor of the BDNF, a peptide that regulates the development, survival, and differentiation of neurons (Xu et al., 2003).

1.3.2.3. Syndromic Obesity

Currently there are more than 25 human genetic syndromes associated with obesity and these include among others, Prader Willi, Alstrom, Bardet-Biedl, Cohen, Albright hereditary osteodystrophy, and Borjeson Forssman Lehmann, (Chung and Leibel, 2005). In these cases obesity occurs in association with other clinical phenotypes such as mental retardation, dysmorphic features, and organ-specific

developmental abnormalities. These syndromes are caused by genetic defects or chromosomal rearrangements, and can be transmitted either autosomal dominantly or X-linked.

Prader-Willi syndrome (PWS): The PWS is caused by defects in the inheritance of imprinted genes on chromosome 15q11.2-q12 (Horsthemke et al., 2003). The majority of cases (75%) arise from paternal deletions of this locus, 22% result from maternal uniparental disomy, less than 3% from imprinting errors caused by microdeletions of the imprinting center at the small nuclear ribonucleoprotein polypeptide N upstream reading frame locus or an abnormal imprint without a detectable microdeletion, and less than 1% from paternal translocations (Nicholls and Knepper, 2001). Other candidate genes in the affected interval include *NDN* (necdin-encoding gene, a growth suppressor present in all postmitotic neurons in the brain) (Jay et al., 1997), and three families of C/D-box small nucleolar RNA genes (HBII-13, HBII-52, HBII-85) (Runte et al., 2001). Individuals with PWS exhibit central obesity, neonatal hypotonia, hyperphagia, hypothalamic hypogonadism, and mild mental retardation with somatic abnormalities such as short stature, peculiar facial features and small hands (Gunay-Aygun et al., 2001). There may be other unknown genetic defects causing PWS because several patients with clinical features of the disease have normal chromosome 15 but with cytogenetic alterations of chromosome 6q (Gilhuis et al., 2000).

Bardet-Biedl syndrome (BBS): Unlike the PWS, BBS is genetically heterogeneous with more than two chromosomal loci reported, and these include chromosome 11q13, 16q21, 3p12-q13, 15q22.3-q23, 2q31, 20p12, 4q27, 14q32.1, 7p14, 12q21.2, 9q31-q34.1, and 4q27 (Beales et al., 1997; Stoetzel et al., 2006; Chiang et al., 2006; Stoetzel et al., 2007). As a result of these reported genetic loci, BBS cases were named BBS1-BBS12 in order of the identified chromosomal regions. Although several genetic loci have been reported, in approximately 50% of the cases of BBS the cause is unknown. The BBS was initially thought to be transmitted recessively and caused by homozygous mutations, but later an additional mutation was discovered in a second locus making the transmission triallelic (Katsanis et al., 2001). For example, a heterozygous mutation on chromosome 3p12-q13, which was found to modify the expression of the Met390Arg mutation on chromosome 11q13 (Fan et al., 2004). The Met390Arg mutation accounts for about 80% of all cases caused by defects found in chromosome 11q13 (Mykytyn et al., 2003). Clinically, BBS is characterised by early-onset obesity associated with progressive rod-cone dystrophy, morphological finger abnormalities, dyslexia, learning disabilities, and progressive renal disease (Beales et al., 1999).

Alstrom syndrome (ALMS): The ALMS is an autosomal recessive disorder but unlike BBS, it is genetically homogeneous caused by defect in the *ALMS1* that is located in chromosome 2p13

(Marshall et al., 1997). Defects of the *ALMS1* that cause ALMS include balanced translocations that disrupt the gene, and a few number of nonsense and frameshift mutations. ALMS is characterised by obesity associated with small stature, dilated cardiomyopathy, and type 2 diabetes. It is also associated with other clinical features of heterogeneous severity such as hyperthyroidism, retinal cone dystrophy, progressive sensorineural hearing loss, chronic nephropathy, and hepatic dysfunction.

Borjeson-Forssman-Lehmann syndrome (BFLS): The BFLS is an X-linked disorder caused by a zinc-finger plant homoedomain-like finger gene (*PHF6*), which plays a role in transcription. Eight different missense and truncation mutations have been detected in seven familial and sporadic cases of BFLS (Lower et al., 2002). Affected males exhibit clinical symptoms such as hypotonia, failure to thrive, big ears, small external genitalia as infants, moderate short stature with emerging truncal obesity, gynecomastia, macrocephaly, tapering fingers, and shortened toes (Dereymaeker et al., 1986). Females with heterozygous *PHF6* mutations have milder clinical features as a result of skewed X inactivation (Turner et al., 2004).

Cohen syndrome (COH1): This is an autosomal recessive disorder mostly found in Finnish populations (Kolehmainen et al., 2003). It is caused by mutations in the vascular protein sorting 13 yeast homolog of the B (*VPS13B*) gene. Several gene defects have been reported and include frameshift, nonsense and missense mutations. The *VPS13B* is thought to function in vesicle-mediated sorting and transportation of proteins within the cell. The COH1 syndrome is characterised by mild truncal obesity, thin extremities, and short stature. Other clinical features identified in affected Finnish individuals include non-progressive mild severe psychomotor retardation, motor clumsiness, microcephaly, characteristic facial features, hypotonia and joint laxity, progressive retinochoroidal dystrophy, myopia, intermittent isolated neutropenia and a cheerful disposition (Chandler et al., 2003).

1.3.2.4. Polygenic Obesity

Unlike monogenic obesity, the polygenic form is quite complex resulting from the effect of several polymorphisms in many genes, which only precipitate in *obesogenic* environments (Strauss and Knight 1999; Hebebrand et al., 2003; Hebebrand and Hinney, 2009). Completion of the Human Genome and HapMap projects have not only generated data but also provided tools to investigate the genetic basis of many diseases. As reviewed by Rankinen et al (2006), several genes implicated in common obesity have been detected through three approaches: linkage analysis, candidate gene and genome-wide association (GWA) studies. Employing these strategies however has its challenges as they are less effective in identifying polymorphisms with modest effect, and not replicable due to differences in study design or populations and insufficient power to detect significant association.

Responsible genes are identified through linkage analysis by either mapping genes or positional cloning, which is based on inheritance or segregation of genetic markers with the disease in affected multigeneration families or extensive groups of sib-pairs (McCarthy et al., 2008). Upon identification of chromosomal regions with positive linkage, candidate genes within that region are investigated further by association studies to identify polymorphisms and compare the frequencies of variant alleles between affected individuals and normal controls (Baron, 2001). Some of the genes identified through positional cloning based on linkage analysis include glutamate decarboxylase 2 (*GAD2*) (Boutin et al., 2003), ectonucleotide pyrophosphatase/phosphodiesterase 1 (*ENPP1*) (Meyre et al., 2005) and solute carrier family 6 (amino acid transporter), member 14 (*SLC6A14*) (Suviolahti et al., 2003; Durand et al., 2004). These genes are functionally related to obesity: *GAD2* catalyses the formation of the neurotransmitter γ-aminobutyric acid, which increases food intake; *ENPP1* inhibits insulin receptor activity, and *SlC6A14* is a tryptophan transporter. Apart from requiring families and the difficulty to replicate, the majority of linkage association studies of common complex disease can only identify polymorphisms with strong effects (Walley et al., 2009).

Candidate gene association studies have an advantage over linkage analysis in that their analysis involves fewer steps, are limited to functionally-related genes, and can be conducted in unrelated individuals who can be easily recruited. Several genes studied (Table 1.6) including *MC4R*, *BDNF*, *LEP and LEPR* were plausible candidates due to their role in monogenic and syndromic obesity. These genes are now known to predispose to polygenic obesity in both adults and children (Jiang et al*.,* 2004; Li et al., 1999; Chagnon et al., 2000; Roth et al., 1998). Candidate gene association studies have provided strong evidence for modest effects of genes on obesity, but replication of the results is also challenging.

Candidate gene and linkage association studies have been superseded by GWA studies, which enable identification of a wide range of genes including those with poorly understood functions (Walley et al., 2009). The GWA studies of complex diseases utilise gene variants data generated from the HapMap project, testing the correlation between polymorphisms across the entire human genome and traits variation in study populations. Large numbers of subjects can be analysed to increase the statistical power of identifying variants with smaller effects. From reported studies, GWA give higher significant effects ($p < 10^{-6}$) compared to linkage and candidate gene associations studies (Walley et al., 2009). Although the GWA approach has identified polymorphisms associated with complex diseases such as obesity, it is limited to common polymorphisms, and rare variants are often missed (Iyengar and Elston, 2007). To date, 253 QTL for human obesity have been detected from 61 genome-wide scans in different racial groups (Rankinen et al., 2006) of which only approximately 21%

of these associations have been replicated in two or more studies. Novel genes such as insulin-induced gene 2 (*INSIG2*) (Herbert et al., 2006), Niemann-Pick disease type C1 (*NPC1*), the proto-oncogene (*MAF*) and phosphotriesterase related (*PTER*) (Meyre et al., 2008), catenin beta-like 1 (*CTNNBL1*) (Liu et al., 2008) have been identified through GWA studies. Table 1.7 summarises published findings on polymorphisms that have been found to be associated with obesity and BMI through GWA studies. Fat mass and obesity-associated (*FTO*) is the first gene with replicated association with BMI, followed by *MC4R*. However, both genes only explain approximately 0.84% of the total variance in BMI (Frayling et al., 2007; Loos et al., 2008; Willer et al., 2009; Beckers et al., 2011). The Genetic Investigation of ANthropometric Traits (GIANT) consortium study identified four known obesity-associated genes (*FTO*, *MC4R*, *BDNF* and *SH2B1*) in addition to seven new chromosomal regions (*TMEM18, KCTD15, NEGR1, SEC16B, ETV5*, *BCDIN3D)* that influence BMI (Thorleifsson et al., 2009). Furthermore, Naukkarinen and co-workers (2010) recently conducted a genome-wide transcript profiling of adipose tissue samples and GWA in Finnish and European Caucasian populations, respectively, identifying thirteen genes harboring twenty three SNPs that were slightly significantly associated with BMI. Seven SNPs identified in the coagulation factor XIII A1 subunit gene (*F13A1*) showed association with BMI. The GWA identified the intronic rs714408 polymorphism in *F13A1* as showing the strongest association with BMI.

Apart from SNPs, other sequence aberrations such as copy number variations (CNVs) and DNA methylation have been implicated as risk factors for obesity. Studies have demonstrated association of common CNVs, a deletion near the neuronal growth regulator 1 gene and a duplication of a region encompassing the neuropeptide Y receptor, with BMI (Willer et al., 2009; Sha et al., 2009; Jarick et al., 2011). The NPY receptor is directly involved in food intake and a key regulator of energy homeostasis with potential role in body weight variation (Kamiji and Inui, 2007), while the neuronal growth regulator 1 is thought to affect neuronal outgrowth (Schäfer et al., 2005). Other studies reported rare, large CNVs, which were initially implicated to cause complex disorders (autism and mental retardation) with obesity but were later associated with severe obesity (Bochukova et al., 2010; Walters et al., 2010; Wang et al., 2010). Recently, Jarick and coworkers (2011) reported a CNV region on chromosome 11q11, which covers sequences of three olfactory receptor genes (*OR4P4*, *OR4S2* and *OR4C6*). A possible link between obesity and the olfactory receptor genes is suggested to be based on altered olfactory acuity in morbidly obese individuals.

Table 1.6. Candidate genes for polygenic obesity studied from 2005 to 2009. *Adapted from Walley et al (2009).*

Gene	Number of subjects	Phenotype	P value	Odds ratio
ENPP1	6147	Obesity	6×10^{-3} (adults)	1.50 (adults)
			6×10^{-4} (children)	1.69 (children)
PCSK1	13659	Obesity	7×10^{-8} (adults)	1.34 (adults)
			2×10^{-12} (children)	1.22 (children)
NAMPT	4559	Severe obesity	8×10^{-15} (adults)	Not applicable
			6×10^{-9} (children)	Not applicable
LMNA	4559	Obesity	1×10^{-2}	1.25
	5693	Waist circumference	3×10^{-13}	1.14
GHSR	2513	Obesity	7×10^{-4}	2.74
SOCS1	8108	Obesity	4.7×10^{-2}	Not applicable
SOCS3	1425	Body mass index	3×10^{-3}	Not applicable
		Waist-hip ratio	2×10^{-4}	
KLF7	14818	Obesity	1×10^{-3}	0.90
MTMR9	3220	Body mass index	5×10^{-4}	1.40
DLK1	1025 trios	Obesity	2×10^{-3}	1.34
CNR1	5750	Obesity	1.1×10^{-6} (adults)	1.85 (adults)
			3×10^{-5} (children)	1.52 (children)
TBC1D1	9 multiplex pedigree, 423 population controls	Obesity	7×10^{-6}	Not applicable
NPY2R	3096	Obesity	2×10^{-3} (adults)	1.40 (adults)
			2×10^{-2}	1.20 (children)

Abbreviations: *ENPP1*, Ectonucleotide pyrophosphatase/phosphodiesterase 1; *PCSK1*, Proprotein convertase subtilisin/kexin type 1; *NAMPT*, Nicotinamide phosphoribosyltransferase; *LMNA*, Lamin A/C; *GHSR*, Growth hormone secretagogue receptor; *SOCS1,* Suppressor of cytokine signalling 1; *SOCS3,* Suppressor of cytokine signalling 3; *KLF7,* Krüppel-like factor 7; *MTMR9,* Myotubularin related protein 9; *DLK1*, Delta-like homologue 1; *CNR1*, Cannabinoid type 1 receptor; *TBC1D1*, TBC1 (tre-2/USP6, BUB2, cdc16) domain family, member 1; *NPY2R*, Neuropeptide Y receptor Y2.

Table 1.7. Polymorphisms associated with obesity and body mass index (BMI) in genome-wide association studies. *Adapted from Walley et al (2009).*

SNP rs number	Gene	Number of subjects	Phenotype	P value	Odds ratio
rs9939609	*FTO*	28,587 adults	BMI	3×10^{-15}	1.67
		10,172 children		7×10^{-9}	1.27
rs9930506	*FTO*	6148 subjects	BMI	8.6×10^{-7}	Not applicable
rs17782313	*MC4R*	77,228 adults	BMI	2.8×10^{-15}	1.12
		10,583 children	BMI	1.5×10^{-8}	1.30
rs10508503	*PTER*	8,128 adults	BMI	8.7×10^{-5}	0.68
		8,855 children	BMI	1.9×10^{-4}	0.64
rs1805081	*NPC1*	8,128 adults	BMI	7.7×10^{-8}	0.75
		8,855 children	BMI	2.1×10^{-2}	0.75
rs1424233	*MAF*	8,128 adults	BMI	1.9×10^{-8}	1.39
		8,855 children	BMI	1.6×10^{-6}	1.12
rs6548238	*TMEM18*	84,823 adults	BMI	1.4×10^{-18}	1.19
		9,320 children	BMI	3.4×10^{-5}	1.41
rs7561317	*TMEM18*	69,593 adults	BMI	4.2×10^{-17}	1.20
rs11084753	*KCTD15*	71,706 adults	BMI	2.3×10^{-8}	1.04
		9,156 children	BMI	9.7×10^{-4}	0.96
rs29941	*KCTD15*	69,593 adults	BMI	7.3×10^{-12}	1.10
rs7498665	*SH2B1*	86,677 adults	BMI	5.1×10^{-11}	1.11
		69,593 adults	BMI	3.2×10^{-10}	1.08
rs10838738	*MTCH2*	80,917 adults	BMI	4.6×10^{-9}	1.03
rs10938397	*GNPDA2*	81,758 adults	BMI	3.4×10^{-16}	1.12
		9,309 children	BMI	2.0×10^{-2}	1.20
rs2815752	*NEGR1*	83,499 adults	BMI	6.0×10^{-8}	1.05
rs2568958	*NEGR1*	69,593 adults	BMI	1.2×10^{-11}	1.07
rs6013029	*CTNNBL1*	1,000 adults	BMI	2.69×10^{-7}	1.42
		3,812 adults	Obesity	7.8×10^{-4}	1.42
rs10913469	*SEC16B*	69,593 adults	BMI	6.2×10^{-8}	1.11
rs7647305	*ETV5*	75,043 adults	BMI	7.2×10^{-11}	1.11
rs925946	*BDNF*	69,593 adults	BMI	8.5×10^{-10}	1.11
rs7138803	*BCDIN3D*	69,593 adults	BMI	1.2×10^{-7}	1.14

Abbreviations: BMI, body mass index; *TMEM18,* transmembrane protein 18*; BCDIN3D,* BCDIN3 domain-containing protein*; BDNF,* brain-derived neurotrophic factor*; CTNNBL1,* catenin beta like 1*; ETV5,* ets variant 5*; FTO; GNPDA2,* glucosamine-6-phosphate deaminase 2*; KCTD15,* potassium channel tetramerisation domain containing 15*; MAF,* v-maf musculoaponeurotic fibrosarcoma oncogene homolog*; MC4R,* melanocortin 4 receptor*; MTCH2,* mitochondrial carrier homolog 2*; NEGR1,* neuronal growth regulator 1*; NPC1,* Niemann-Pick disease, type C1*; PTER,* phosphotriesterase-related protein*; SEC16B,* SEC16 homolog B (S. cerevisiae)*; SH2B1,* SH2B adaptor protein 1.

1.3.2.4.1. Gene-environmental interaction

As noted earlier in the current review, both the individual's genetic background and obesogenic environment play a significant role in polygenic obesity. The concept of gene-environment interaction refers to a situation where the precipitation of a disease (in this case, obesity) depends on the exposure of a certain genotype to a specific environment. In obesity, these genotypes must be susceptible to alterations in energy balance. Both human (Bouchard et al., 1990; Bouchard C et al., 1990; Bouchard et al., 1994) and animal models (West et al., 1992; West et al., 1995; Prpic et al., 2002) have provided strong evidence demonstrating that genes influence a substantial percentage of responses to nutrition and exercise. The existing literature on animal studies focuses on finding specific genes that respond to weight gain rather than investigating the allele contribution in response to diet changes. Although extensive research has been conducted, the knowledge of which specific genes, diets and exercise, and their interaction contribute to the majority of human obesity risk is largely unknown. The reported findings from these studies are generally in an exploratory phase and require further investigation. Below are some of the findings obtained on such studies.

In the context of gene-diet interactions, *PPARG2* Pro12Ala is the most widely studied polymorphisms, particularly in Caucasians of different countries such as Spain, USA, Britain, Canada and Germany. The majority of the studies have focused on the interaction between fat intake and *PPARG2* Pro12Ala (Kadowaki et al., 2002; Memisoglu et al., 2003; Robitaille et al., 2003), probable due to the role of *PPARG* in adipocyte differentiation, lipid storage, and insulin sensitivity (Spiegelman, 1998). The relationship between the variant and carbohydrate intake or alcohol consumption has also been investigated in different studies (Marti et al., 2002). In relation to fat intake, a Quebec Family study reported an interaction between the Ala12 variant and total fat intake and its association with higher BMI and waist circumference (Robitaille et al., 2003). In a study by Memisoglu and co-workers (2003), a significant interaction between total fat intake and BMI was found: a highest quintile of total dietary fat was associated with increased risk of obesity among Pro12Pro subjects. A PREDIMED intervention trial found that a Mediterranean diet rich in virgin olive oil or nuts was able to reverse the negative effect that the 12Ala allele had on waist circumference (Razquin et al., 2009). Interestingly, 12Ala carriers that followed a conventional low fat diet had a significantly higher waist circumference compared to individuals carrying the Pro12 allele. The interaction of the *PPARG2* Pro12Ala polymorphisms with diet was further supported by a trial conducted on animal models, in which the beneficial effect of the 12 Ala allele was diminished when carriers were introduced into high-fat diet instead of a chow diet (Heikkinen et al., 2009).

The *FTO* rs9939609 polymorphism has also been widely studied in relation to its interaction with fat intake and the effect on obesity. In a cross-sectional study, Sonestedt and co-workers (2009) identified a significant interaction between fat intake and *FTO* genotype and also between carbohydrate intake and *FTO* genotype on BMI. An increase in BMI across all genotypes of the rs9939609 polymrphisms (*TT* and *AA* homozygotes and *TA* heterozygotes) was restricted to those who reported intake of high-fat diet, whereas in those consuming lower fat diet the polymorphism was not associated with BMI. Similarly, individuals who reported lower intake of carbohydrates had lower BMI compared to those who consumed a carbohydrate-rich diet. Intervention studies based on the presence of the *FTO* rs9939609 (T>A) polymorphism were also conducted, however, most of them were related to both dietary changes and physical activity. In one longitudinal study conducted in children (who were followed from the age of 7 months until 15 years old), the polymorphism was associated with increased BMI in children older than 7 years even after being assigned to lifestyle intervention (parents told to reduce the exposure of children to environmental risk factors of coronary heart diseases) (Hakanen et al., 2009). According to these authors, these results suggested that the intervention was not effective enough to cancel the effect of the *FTO* polymorphism. Similarly, other studies did not find any modulating effect of the *FTO* polymorphism on BMI after lifestyle modification (Lappalainen et al., 2009; Reinehr et al., 2009). However, in one study carriers of the *FTO* rs9939609 *A* allele had lower body weight gain after 3 years of nutritional intervention with a Mediterranean-style-diet but no weight loss was observed in all individuals included in the study (Razquin et al., 2010). The findings of this study suggest that the magnitude of the effect of the *FTO* polymorphism on BMI may be dependent on the type of diet consumed by individuals, particularly the *A* allele carriers.

Most studies investigating the interaction of genetic polymorphisms with physical activity examined the effectiveness of exercise regimes on individuals with certain genotypes, many of which have not been replicated (Rankinen et al., 2006). Some of these studies were selected to understand the energy intake pathways that influence energy balance (*LEPR, AGRP, MC4R,* and *GHRL)* (Rankinen et al., 2006; Loos et al., 2005). One example is the Finnish Diabetes Prevention study which demonstrated that the *GHRL* Leu72Met and -501A/C polymorphisms modified the effect of moderate-to-vigorous physical activity on changes in weight and waist circumference, and high-density lipoprotein cholesterol (Kilpeläinen et al., 2008). The authors further showed that the *LEPR* Lys109Arg polymorphism in combination with physical activity was associated with changes in blood pressure. Elsewhere, an interaction between the *FTO*-rs9939609 genotype and physical activity was observed, where physically inactive homozygous risk *A*-allele carriers had a 1.95 +/- 0.3 kg/m^2 higher BMI compared with homozygous *T*-allele carriers (Andreasen et al., 2008).

1.3.2.4.2. Epigenetics

Apart from the interaction of SNPs and environmental stimuli, modified gene expression levels in response to external stimuli and epigenetic factors such as DNA methylation and histone modification have been proposed as other mechanisms underlying the complexity of common obesity (Franks and Ling, 2010). Epigenetics is a phenomenon of reversible genetic modifications that occur irrespective of nucleotide sequence changes but affect regulation of genomic function. These changes can either be inherited or can be a response to nutrient availability, physical exercise and aging, among other factors. Early-life nutrition is one good example of a stimulus that can trigger epigenetics, affecting an individual's response to metabolic load and disease susceptibility in adulthood. Several animal studies have supported this hypothesis. In a study conducted in rodents, unbalanced prenatal nutrition (through protein restriction) induced hypomethylation that increased the expression levels of glucocorticoid receptor (GR) and peroxisomal proliferator-activated receptor alpha (PPARα) genes in offspring (Lillycrop et al., 2005). These authors further demonstrated that induced methylation changes of individual CpG dinucleotides in the *PPARα* promoter in juvenile rats persisted into adulthood, and that such changes may consequently affect the capacity of the tissue to respond to a metabolic challenge (Lillycrop et al., 2008).

The effect of caloric restriction in DNA methylation was also demonstrated in human studies (wang et al., 2010; Bouchard et al., 2010). One example of modified gene expression level is explained by the effect of aerobic exercise on a subset of genes, particularly those involved in oxidative energy metabolism, which are upregulated upon exposure to the stimulus (Franks and Loos, 2006). Recently, in a weight-loss intervention study, Bouchard et al. (2010) identified 644 genes that showed differential transcript levels between low and high responders to calorie restriction after intervention. Two of the genes that were differentially methylated encode a potassium channel (KCNA3) and the nuclear factor I/X (NFIX), and were implicated in weight control as demonstrated by knockout mouse models (Driller et al., 2007). The third gene, ectodermal-neural cortex 1 (*ENC1)*, is required for adipocyte differentiation making it a plausible candidate obesity predisposition (Zhao et al., 2000). DNA methylation of the peroxisome proliferator-activated receptor-γ coactivator 1-α gene in response to a high-fat diet was also reported in human studies (Brøns et al., 2010). Furthermore, an association between methylation in the *POMC* promoter and early life overnutrition and obesity was observed in a study by Plagemann and coworkers (2009). A direct link between DNA methylation and obesity phenotype was reported by Wang and co-workers (2010). In this study, two genes that were differentially methylated between obese and normal-weight individuals were identified: the ubiquitin-associated and SH3 domain-containing A gene was highly methylated than the tripartite motif-containing 3 gene in obese compared to the normal-weight group. Both these genes are involved in

obesity-induced immune responses. A possible role of DNA methylation in the development of obesity was also reported in a study by Bell and co-workers (2010). This study identified a genotype-epigenotype interaction in the FTO gene: the *FTO* rs8050136 risk allele A homozygotes were shown to have a higher average level of methylation, followed by heterozygotes at intermediate level, and carriers of the homozygous C allele with the lowest level of methylation. The SNP is within a linkage disequilibrium block that contains polymorphisms shown to be associated with BMI in several studies. The association identified in this study, however, was with the individual's genotype not their body weight status. While these studies have demonstrated a relationship between DNA methylation and obesity, they did not establish whether changes in methylation are a cause or consequence of the weight gain. Epigenetic studies are limited by high costs of methylation chips, hypothesis tests performed in multiplex experiments and corresponding procedures to correct for type 1 errors, and the source of genetic material used.

1.4 Complications of obesity

Obesity is considered to be a risk factor for several diseases with significant morbidity and mortality. Obesity as measured by BMI should be treated with caution as BMI measures both muscle and fat mass. Accumulation of fat to the abdomen, particularly, is the cornerstone of the metabolic disorders as it is associated with increased risk of insulin resistance, diabetes, hypertension, dyslipidemia and atherosclerosis. Together with dyslipidemia, hypertension, and insulin resistance, visceral fat accumulation (referred to as central obesity) constitute the Metabolic Syndrome (MetS) (Reaven, 1988). Combined, these metabolic disorders pose a risk of developing diabetes, cardiovascular diseases, polycystic ovary syndrome, some forms of cancer, and liver diseases. MetS can also occur in non-obese individuals as previously observed in the National Health and Nutrition Examination Survey (NHANES) III in 5% to 10% of the population with BMIs ranging from 20 to 25 kg/m^2 (Cook *et al.*, 2003). In this study, people with MetS were not obese but had substantial visceral fat.

Although MetS is well recognized, its definition and diagnosis across population is problematic as there are currently more than two definitions used with revised cut-off points for several components. Although it was initially thought to affect adults, MetS presents early in life and progresses with age and puberty (Chen et al., 1999; Maffeis, 2002; Goedecke et al., 2006; Zachurzok-Buczyńska et al., 2011; Kassi et al., 2011). This necessitated early diagnosis of MetS with possible identification of adolescents at increased and premature cardiovascular risk. Adult definitions could not be applied due to age- and sex- dependent changes in normal body proportion and other components during pubertal development, which may vary among individuals of different ethnic groups. Thus, adult MetS

definitions were modified for children, by the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults Adult Treatment Panel III (NCEP ATP III) (Cook et al., 2003), WHO (Goodman et al., 2004), and International Diabetes Federation (IDF) (Zimmet *et al*., 2007). Table 1.8 summarises two of the commonly used criteria to identify the MetS in children. The IDF and the American Heart Association/National Heart, Lung and Blood Institute (AHA/NHLBI) released a consensus definition for Mets, the Joint Interim Statement (JIS), whereby abdominal obesity was removed as a prerequisite for the diagnosis of metabolic syndrome (Alberti et al., 2009). Consequently, the JIS definition classifies an individual as having MetS if an individual has three of either, raised blood pressure, elevated triglycerides, low high-density lipoprotein cholesterol levels, central obesity or hyperglycaemia. A variety of studies using three or four criteria reported diverse prevalence of MetS in children and adolescents (Kassi et al., 2011). It is however, evident that the condition is prevalent in obese children, and if they stay obese in adulthood, this will further increase the risk of subsequent related disorders (Kiess et al., 2006). The existence of MetS, regardless of the definition used, was also evident in a South African study, which reported MetS in two thirds of obese or overweight learners aged 10-16 years (Matsha et al., 2009). According to the recent review by Kass and co-workers (2011), the main concern of clinicians is the extrapolation of adult MetS definitions to children and adolescents. The hypothesis that MetS in childhood is related to MetS in adulthood has not been fully tested, and there is a limited number of large longitudinal studies linking pediatric MetS with adult cardiovascular disease.

Table 1.8. Examples of Metabolic Syndrome definitions used in children

The IDF ages 10 – 16, (Zimmet *et al.,* 2007)		**The NCEP ATP III,** (Cook *et al*., 2003)
		Any 3 or more of the following:
Central obesity (WC)	> 90th percentile	> 90th percentile, age gender specific
And any 2 or more of the following:		
FBG	≥ 5.6 mmol/L (100mg/dL) or known type 2 diabetes	≥ 5.6 mmol/L (100mg/dL)
Hypertension	SBP ≥130 or DBP ≥ 85 mmHg	≥ 90th percentile for age, sex & height
TG	≥ 1.7 mmol/L (150mg/dL)	≥ 1.24 mmol/L (110mg/dL)
HDL-C	< 1.03 mmol/L (40mg/dL)	≤ 1.04 mmol/L (40mg/dL)

1.5. Aims and objectives of the present study

Childhood obesity is one of the most serious public health problems facing the developed, and increasingly the developing world. South Africa, in particular, is faced with a rapid increase in childhood obesity of 10% among children under the age of 2 and 5-20% among those less than 6 years of age. The prevalence of obesity is increasing in children of all ages and represents the complex integration of genetic, behavioural and environmental influences. Genetic factors are currently estimated to account for approximately 40-70% of the variance in human adiposity. Although intensive investigation has been conducted in other countries including Nigeria and Ghana (Kramer et al., 2005; Bonilla et al., 2006), to our knowledge, there is limited data reported on the molecular aetiology of obesity (both monogenic and polygenic forms) in South African populations. It is only recently (in 2011) that Dr Zane Lombard of the National Health Laboratory services reported a significant finding on *LEP* risk alleles, which increased the risk of obesity in adolescents residing in Soweto (Johannesburg).

The present investigation is a case-control study aimed at assessing the contribution of genetic factors, together with environmental factors, to childhood obesity/overweight in South African adolescents as observed in other populations. A better understanding of the factors that contribute to the increasing incidence of childhood obesity/overweight could offer holistic opportunities for prevention and intervention programmes. **The hypothesis of the present study is that genetic variations, particularly those of the leptin-melanocortin pathway in association with environmental factors may promote early onset obesity.** The hypothesis is based on the following observations: **Firstly,** the leptin-melanocortin system regulates food intake and energy homeostasis in humans. **Secondly,** since the discovery of the rare monogenic forms of obesity, several genes that are part of the leptin-melanocortin axis have been reported as causing this condition. To date, five monogenic obesity disorders have been reported where obesity in early childhood predominates without children developing behavioural abnormalities and dysmorphisms. However, recently, genetic variations in this pathway have also been linked with polygenic forms of obesity. **Thirdly,** environmental factors, particularly physical inactivity due to a sedentary lifestyle has been shown to play a major role in the development of obesity.

Thus the aims of this project are as follows:

1. **Identify sequence variants in six genes involved in the leptin-melanocortin pathway that may play a role in childhood obesity in adolescents residing in the semi-urban/rural and urban areas of the Western Cape Province, South Africa.** There is currently no data showing the contribution of genetic factors to obesity in South African children and adolescents. This will be a

case-control study involving obese/overweight adolescents of Black and Mixed Ancestry ethnic groups (both males and females) who consent to genetic analysis.

2. **To assess the role of identified mutations/sequence variations in obesity/overweight and its related traits such as blood pressure, blood glucose and lipid levels, and anthropometric parameters, and their relationship with physical activity.**

CHAPTER 2

MC4R, MC3R AND PHYSICAL ACTIVITY

2.1. BACKGROUND

The melanocortin receptors belong to a family of rhodopsin 7-transmembrane, G-protein coupled receptors that signal through intracellular cyclic adenosine monophosphate (Gantz and Fong, 2003; Cone et al., 2005). Their structure is composed of a single polypeptide of seven α-helical transmembrane domains, an extracellular amino (N)-terminus, and an intracellular carboxyl (C)-terminus. Although melanocortin receptors share many structural features conserved in other G protein-coupled receptors, they are the smallest receptors in this group, with short N- and C-terminal ends, and small second extracellular loops (Fig 2.1). All human melanocortin receptors contain conserved amino acids such as the aspartic acid-arginine-tyrosine motif at the junction of the transmembrane 3 domain, and cysteine residues at the C-terminus and extracellular loop. Functional studies indicate that the cysteine residues in the extracellular loop form a disulfide bond that is essential for the receptor function while those in the C-terminus may have different roles in receptor expression, ligand binding and receptor activation (Yang et al., 2007).

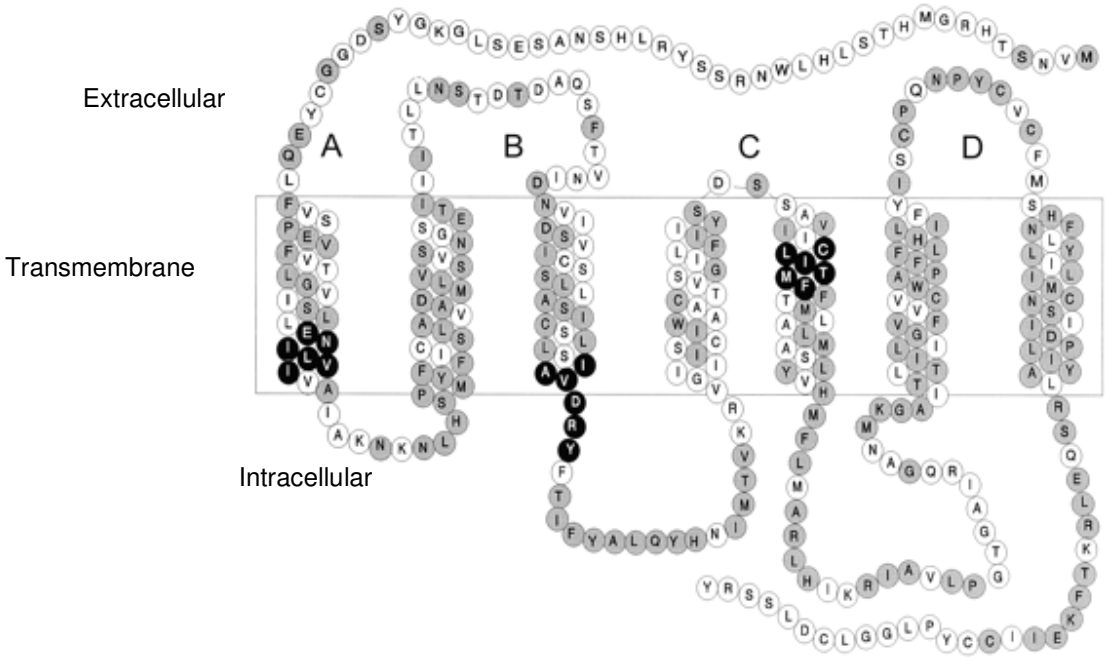


Figure 2.1. Model of human MC4R, comparison to the rat Mc3r. ***Gray circles*** indicate residues identical in the rat Mc3r and the human MC4R. ***Black circles*** indicate regions with complete homology. *Abbreviations*: MC3R, melanocortin 3 receptor; MC4R, melanocortin 4 receptors. *Adapted from Oosterom et al., 1999.*

MC3R and *MC4R* are single-exon genes encoding 360- and 333-amino acid proteins, respectively (Gantz et al., 2003). They have been mapped, respectively, on chromosomes 20q13.2 and 18q22 (Gantz et al., 1993; Gantz et al., 1993). The two receptors are widely distributed in the brain and

central nervous system, mainly in the paraventricular, dorsomedial and lateral nuclei of the hypothalamus (Mountjoy et al., 1994; Cone, 1999; Gantz et al., 2003). Melanocortin receptors, particularly the *MC4R*, have been reported to play a major role in the development of both monogenic and polygenic obesity (Farooqi et al., 2003; Carroll et al., 2005). Mutations in *MC4R* are the most common cause of early onset obesity, accounting for up to 6% of the condition, particularly in European populations (Farooqi et al., 2003). Furthermore, more than 50 additional *MC4R* single nucleotide polymorphisms (SNPs) have been reported in obese and control individuals with different effects depending on the population and family studied (Carroll et al., 2005). On the other hand, it is only recently that obesity-associated SNPs were identified in the *MC3R* (Tao et al., 2007; Lee et al., 2007; Mencarelli et al., 2008; Mencarelli et al., 2011; Zegers et al., 2011). Similar to *MC4R* polymorphisms, common variants in *MC3R* show significant frequency variation, with nonreplicable association tests with obesity-related variables among different populations (Table 2.1). Although *MC3R* have been associated with morbid (Ala293Thr, Ile335Ser, Ter361Ser, Ile183Asn, Ala70Thr, and Met134Ile) (Lee et al., 2007; Mencarelli et al., 2008) and mild obesity, the effect of reported polymorphisms has not been linked to physical activity.

Physical activity plays an important role in maintaining the balance between energy intake and expenditure. It is therefore acknowledged that physical inactivity and predisposing genetic factors are major determinants of obesity in both adults and children despite the small number of South African studies demonstrating their association with obesity (Kruger et al., 2002; 2003; Senekal et al., 2003; McVeigh et al., 2004; Mamabolo et al., 2007). A combination of poor environment with lack of exercise facilities, high crime rate, and attitudes towards slim body figures seem to contribute to low levels of physical activity among South Africans (Reddy et al., 2002; Kruger et al., 2003). Urbanisation in South African children has introduced sedentary lifestyle, replacing physical activity with watching television, playing computer games, and use of transport.

It is widely accepted that weight gain can be reversed by increasing energy expenditure and reducing energy intake. Physical activity, therefore, has been identified as one of the interventions for obesity management in both adults and children, and several studies have reported the effectiveness of physical activity in reducing weight and associated cardiovascular risk factor (Klem et al., 1997; Skerrett and Manson, 2002; Wing, 1999; Paterson and Warburton, 2010; Janssen and LeBlanc, 2010). However, there is interindividual variation on the effect of both inactivity and activity on weight and associated cardiovascular risk factor that may be caused, in part, by the presence of susceptibility polymorphisms. Among the genes that mediate the effect of physical activities on metabolic diseases are *MC4R*, leptin receptor and ghrelin genes (Lakka et al., 2004; Loos et al., 2005; Kilpeläinen et al.,

2005; Rankinen et al., 2006). For example, The Finnish Diabetes Prevention study demonstrated that the Leu72Met and -501A/C polymorphisms in the ghrelin gene modified the effect of moderate-to-vigorous physical activity on weight and waist circumference, and high-density lipoprotein cholesterol; while the leptin receptor Lys109Arg polymorphism mediated the effect of physical activity in blood pressure (Kilpeläinen et al., 2005). Recent studies have consistently reported significant associations between body mass index (BMI) and fat mass and obesity-associated (FTO) gene polymorphisms and the modified effects of the gene when food intake and physical activity or inactivity are accounted for (Lee et al., 2010; Sonestedt et al., 2010; Ahmad et al., 2011). For example, Ahmad et al. (2011) reported an additive effect of the *FTO* rs8050136 risk allele among women who were both inactive and consuming large amounts of food. Although the *FTO* is predicted to have slight DNA demethylase (Gerken et al., 2007) and non-heme dioxygenase activities (Sanchez-Pulido et al., 2007), the reported association studies are suggestive of its role in energy intake and expenditure. Energy intake and expenditure are mainly regulated by the leptin-melanocortin pathway. The pathway also plays a role in glucose homeostasis, and insulin secretion. Neuropeptides in the melanocortin system regulate food intake and energy balance by binding to melanocortin receptors, particularly *MC4R*, and to a lesser extent *MC3R.*

Although both *MC3R* and *MC4R* have been extensively studied and shown to presdispose to obesity in various population groups globally, to our knowledge, no studies have been published on the contribution of the two genes in obesity among South African ethnic groups. The present chapter therefore, was aimed at analysing *MC3R* and *MC4R* for identifying polymorphisms that may predispose to obesity in South African Black and Mixed Ancestry adolescents. Furthermore, this chapter focused at establishing whether or not the association of *MC3R* polymorphisms with obesity-related traits could be influenced by physical activity.

Table 2.1. Association studies for melanocortin 3 receptor gene polymorphisms and metabolic parameters in different population groups, worldwide.

Polymorphism	Population group	Phenotype	Association	Reference
-201C>G, -239 A>G, -762A>T, -769T>C; Val81Ile	209, adults	Obesity	No	Li WD et al., 2000
Thr6Lys and Val81Ile	French (n=526)	Type 2 diabetes, body mass index	No	Hani et al., 2001
+2138InsCAGACC	Québec, adults (n=812)	Fat mass, percent body fat, and total abdominal fat	Yes	Boucher et al., 2002
Ile183Asn	Asian (n= 162)	Percent body fat	Yes	Lee et al., 2002
Thr6Lys and Val81Ile	Finnish (n= 300)	Increased insulin-glucose ratio; hyperleptinemia; body mass index	Yes	Schalin-Jantti et al., 2003
Val81Ile	Greek, adults (n=228)	Hyperinsulinemia; hyperleptinemia; body mass index and fat mass	Yes	Yiannakouris et al., 2004
Thr6Lys and Val81Ile	African and Caucasian American children (n=350)	Fat mass and leptin	Yes	Feng et al., 2005
Thr6Lys and Val81Ile	Finnish, adults (n= 216)	Lipid and glucose oxidation	Yes	Rutanen et al., 2006
Thr6Lys and Val81Ile	Asian children (n= 389)	Hyperleptinemia, percentage body fat, insulin sensitivity	Yes	Lee et al., 2007
Thr6Lys and Val81Ile	Italian children (n= 368)	Weight loss	Yes	Santoro et al., 2007
Ala293Thr, Ile335Ser and Stop361Ser	Italian, adults (n= 505)	Obesity	Yes	Mencarelli et al., 2008
Thr6Lys and Val81Ile	African and Caucasian American children and adolescents (n= 416)	body mass index and fat mass	Yes	Savastano et al., 2009;
Arg257Ser, Thr6Lys/Val81Ile	Canadian Caucasians, adults (n=1821)	Obesity	No	Calton et al., 2009
Leu299Val	Caucasian 448 children and 236 adults	Obesity	Yes	Zegers et al., 2011

rs3746619	Caucasians, adults (n=1321)	Body mass index	Yes	Zegers et al., 2010

2.2. MATERIALS AND METHODS

2.2.1 Ethical approval, Consent and Confidentiality

The present study was accepted and registered by the Research Ethics Committee of the University of Stellenbosch (Project number: N07/07/160, Appendix A). Permission to conduct the 'parent' research project (Reference number: CPUT/HAS-REC 0016, Appendix A) was granted by the Faculty of Health and Wellness Science Ethics Committee, Cape Peninsula University of Technology, while the study was conducted according to the Code of Ethics of the World Medical Association (Declaration of Helsinki). Permission to perform the study was also obtained from the Western Cape Department of Education, school governing bodies and principals. Written informed consent from parents or guardians and oral assent from learners was obtained after all the procedures had been fully explained. All recruited participants had a right to decide voluntarily whether to participate in the study or to withdraw without the risk of incurring penalties or prejudicial treatment. They also had a right to refuse to give information and ask clarity about the objectives of the study or specific procedures.

2.2.2 Study design

The study was a cross-sectional study in which the dependent and independent variables were measured simultaneously. The school setting provided an ideal social context and almost ready-made sampling frame (gender, age, education level, geographical area, etc.) to obtain information, making it the most appropriate sampling frame for this study (Retzlaff et al., 1997).

2.2.3 Study population

The present study was part of an investigation that aimed to elucidate obesity, diabetes, hypertension and the metabolic syndrome in 8-18 year old learners residing in the peri-urban communities of the Western Cape. The study population of the 'parent' research project consisted of a total of 1564 (males = 621; females = 943) learners between the ages 8-18 years that were recruited as previously described (Matsha *et al.*, 2009). Briefly, participants were recruited through a proportionally stratified multistage random sampling technique from government funded primary and secondary schools using a list of 107 schools obtained from the Western Cape Education Department. As private schools represented less than 2% of the total number of schools they were excluded from the sampling frame. Fourteen schools participated in the study and randomisation took place at the learner and class levels. The response rate was 65% and learners that declined to participate were approached twice personally.

Study group

- Learners were classified according to their weight status as obese, overweight and normal using the International Obesity Task Force criteria as reported by Cole et al (2000). The parent study population comprised of learners of Black African, Caucasian and Mixed Ancestry racial groups of South Africa. However, for this project only the Black African and Mixed Ancestry subjects were included due to the limited number of Caucasians from the parent study. A total of 431 (227 obese-overweight, and 204 normal) individuals between 11-16 years of age were selected for this study. The case group (227 obese-overweight learners) was made up of 115 Black Africans and 112 Mixed Ancestry learners whilst the age-, gender-, and ethnic-matched normal weight learners consisted of 94 Black Africans and 110 Mixed Ancestry learners. Participants who chose not to give blood for DNA analysis because of various personal reasons such as low blood pressure, anxiety problems related to the sight of blood and fear of surgical needles, and those not consented by parents were excluded from the study.

Inclusion Criteria

- The study groups were selected from learners who consented to participate and gave blood for genetic analysis.
- Black African and Mixed Ancestry learners aged 11-16 years were selected from the parent group as these racial groups were well represented in the parent study (Matsha et al., 2009).
- **Case group**: This consisted of 227 (115 Black Africans and 112 Mixed Ancestry) obese and overweight learners, males and females.
- **Control group**: Age-, gender-, and ethnic-matched normal weight learners (94 Black Africans and 110 Mixed Ancestry) formed a control group.

2.2.4 Sample size

The main research project consisted of 1564 learners from which the study population of the present project was selected. The study reported an overweight and obesity prevalence of 15.5% and 7.2%, respectively (Matsha et al., 2009). To obtain statistically significant observations on allelic distributions and adequate power for association tests between obesity-related traits and genotypes given the reported prevalence, the number of a control group was chosen based on a 1:1 case-control ratio. In order to successfully analyse association studies, the sample size must be sufficiently large to create enough statistical power to reduce the probability of generating false-positive associations (Berry *et al*., 1998). The following guided our genotype sample size calculation: If the “risk” allele frequency in

the general population (controls) is 40%, then a study with 227 cases and 204 controls have 80% power to detect, at a 5% significance level, a “risk” allele frequency of 57% in cases.

2.2.5. Data collection

A detailed protocol describing data collection procedures (questionnaires and physical examination) was developed. Team members consisting of professional nurses and community health care workers were trained, and a pilot study consisting of 22 learners was performed to validate the questionnaires and synergise the workflow. A supervisor was allocated for each team who monitored the performance of the personnel and was responsible for calibrating equipment according to a standard protocol. In addition, weekly meetings were held to assess progress and solve encountered problems. The school premises were used as points of data collection and temporary screens were constructed to serve as examination rooms in order to maintain the privacy of participants. The questionnaires were completed confidentially under the supervision of a professional nurse or learner educator. One school was visited per week to collect research materials such as personal details of learners, anthropometric measurements, biochemical analyses and lifestyle indicators by professional nurses and trained field-workers. This information was recorded in the form of questionnaires (Appendix B). Learners were requested to fast for at least 8 hours prior to sampling. On the day of sampling, venous blood was collected from learners who consented for genetic analysis.

2.2.6. Physical activity measurement

The Physical Activity and Energy expenditure was assessed in the form of questionnaires that were developed based on the validated questionnaires designed by Arvidsson and coworkers (2005). This set of questionnaire contained indicators of sporting as well as leisure activities, and assessed the frequency and type of activities learners participated in. All questionnaires were developed in English, and then translated into Afrikaans and Xhosa. A pilot study was conducted among 22 school learners that were randomly selected from a school with similar characteristics as the present study population. The questionnaire was further used in a research study that aimed at investigating the prevalence of obesity amongst learners attending schools in Belhar, Delf, and Mfuleni in the Western Cape Province (Somers, MTech Thesis, 2004). On the sampling day, each learner was asked about the frequency at which they performed activities during the week such as walking, sport, cycling, household chores, and time spent on watching television. Furthermore, learners were asked if their schools offered physical education, and if yes, the number of days per week they attended the session. Each activity was then categorized according to the frequency of performing each activity as follows: not at all or physically inactive (0 days per week), occasional (1-2 days per week), and often (3-5 days per week).

The frequency of performing these activities was used as an indirect measure of learner's physical fitness.

2.2.7. Obesity-related anthropometric and metabolic variables

Anthropometric and biochemical parameters of interest that were measured are as follows: body weight and height; waist and hip circumference & waist-hip ratio and skinfold measurements, blood pressure, blood glucose and lipid levels. Body weight and height measurements were used to calculate the BMI. Professional nurses and field workers who were trained in the use of prescribed standardised techniques conducted anthropometric measurements and biochemical analyses. These standardised techniques and associated data-collection methods were piloted, and used in a research project that aimed at investigating the prevalence of obesity amongst learners attending schools in Belhar, Delf, and Mfuleni in the Western Cape Province (Somers, MTech Thesis, 2004). In a pilot study, careful selection of instruments with adequate detection limits and sensitivity was done to enhance the accuracy and validity of results. Statistical measures in the form of repeated measures were used to ensure inter- and intra-validity. Measurements and analyses were performed as follows:

A. Anthropometric measurements

Weight and height measurements

- Weight

Weight measurements were done using a digital bathroom electronic scale (Fuzhou Sunny Electronic Co., Ltd., China). All heavy clothing and shoes were removed. The scale was calibrated and standardised using a weight of known mass. The subject was asked to stand in the middle of the scale platform after the scale had been zeroed. It was then ensured that the subject's weight was evenly distributed with the arms hanging relaxed along the sides. Readings were taken to the nearest 0.1 kg.

- Height

The height of each participant was measured using a Stadiometer (Anand Medical Exports, India). The subject was asked to stand on a flat surface that is at 90° angle to the vertical lever/ board of the Stadiometer. The subject's weight must be evenly distributed on both feet. The scapula and the buttock had to be in contact with the wall/ board, with the buttock and the heel in the same vertical line. The subject was then asked to take a deep breath in and maintain a fully upright position. The required accuracy was 0.1 cm (Martin *et al*., 1988).

- Body mass index (BMI)

The BMI was calculated for each subject as weight (kg) ÷ [height (m)]^2, and used for classifying learners according to their weight status using the International reference gender-and age-based cut-off points provided by the IOTF as developed by Cole and co-workers (2000).

Circumference measurements

- Mid-upper-arm

The measurement was done on the arm mid-way between the acromion and radial points, with the arm relaxed and hanging by the sides. The required accuracy was 2 mm.

- Waist circumference

The waist measurement was taken with the subject in an erect position, abdomen relaxed, arms at the sides and feet together. Measurements were performed facing the subject, in a horizontal plane, with a non-elastic tape measure placed at the level of the natural waist. The natural waist is defined as the narrowest part of the torso as seen from the anterior view. In obese subjects it may be difficult to see the waist narrowing, therefore the smallest circumference measured in the area between the ribs and the iliac crest was taken. The measurement was taken three times at the end of normal expiration and average recorded. The accuracy was 0.1 cm.

- Hip circumference

Hip circumference was measured as the maximal circumference over the buttocks using a non-elastic tape. The field worker had to squat beside the subject so that the maximum extension of the buttock in the horizontal plane at this level could be taken without compressing the skin. The measurements were taken three times and the average of the measurements recorded. The required accuracy was 0.1 cm.

- Waist-hip Ratio

This was calculated as the average of the waist circumference divided by the average of the hip circumference. This was recorded to four decimal places.

Skinfold measurements

The skinfold measurement is a measurement of the compressed thickness of a double layer of skin and the underlying subcutaneous adipose (fat) tissue. The 4-site methods used in this study were: triceps, biceps, sub-scapula and supra-iliac skinfold. All measurements were taken on the right side of the subject (Harrison *et al*., 1988). In some obese subjects it may be impossible to elevate a skinfold

with parallel sides. In these circumstances the measurement was not taken, instead the two-handed technique was used. In this technique, the skinfold is lifted using two hands and measured.

Sites of measurements

- Triceps

The triceps were measured from the back on the posterior surface of the arm, mid-way between the top of the shoulder (acromion process) and the posterior aspect of the elbow (olecranon process). It was ensured that the upper limb hung loosely by the subject's side with the subject in the standing position.

- Biceps

The biceps were measured on the anterior (front) surface of the arm, mid-way between the top of the shoulder (acromion process) and the front of the elbow (anterior surface of the cubital fossa). The subject remained in the same position as for the triceps measurement.

- Sub-scapula

The measurement was taken about 20 mm just below the inferior (lower) angle of the scapula, with the fold in an oblique plane descending outwards and downwards at an angle of approximately 45° to the horizontal.

- Supra-illiac

The measurement was taken about 20 mm above the iliac crest, in the axillary line, with the fold in the oblique plane, descending medially and downwards at an angle of 45˚ to the horizontal. The subject remained erect with the upper limbs by the side and the abdominal muscles relaxed.

B. Biochemical analysis

Blood glucose and lipid measurements

Fasting blood samples obtained through finger pricking was used for glucose and lipid measurements. Prior to screening, the fasting state was determined by interview on the morning of examination. Blood glucose levels were measured using the Accutrend GCT glucometer (Stellenbosch Medical Supplies CC, South Africa). Capillary glucose measurements are as suitable as venous glucose measurements in the diagnosis and detection of type 2 diabetes mellitus in epidemiological studies (Solnica et al., 2003; Kruijshoop et al., 2004). The commercial glucometer used in this study had a mean imprecision

of < 5%, with a range of 1.1– 33.3 mmol/L on capillary whole blood. Total cholesterol, HDL-cholesterol, and triglycerides were measured using CardioCheck^{TM} P.A analyzer (Polymer Technology Systems, Inc. USA) according to the manufacturer's instructions. CardioChek PA complied with the National Cholesterol Education Program Expert Panel guidelines for total cholesterol (TC) and high density lipoprotein-cholesterol (HDL-C) as tested in a study conducted by Panz and coworkers (2005). According to this study, lipid levels measured by the CardioChek PA analyser agreed satisfactorily with the laboratory results, and instrument therefore could be used for screening purposes. For quality control purposes, one control sample was included everyday during sample collection.

C. Blood pressure

Qualified healthcare professionals performed all the clinical examinations. Blood pressure measurements were performed according the WHO guidelines (1999). Measurements were performed using a semi-automatic digital blood pressure monitor (Rossmax PA, USA) on the right arm in sitting and relaxed position with the learner not having ingested coffee or smoked for 30 minutes before measurement. The cuff was placed at a point midway between the olecranon and acromion to ensure accurate measurement. After a 10 minute rest period, three readings were taken at 5 minutes interval and the lowest of the three readings was taken as the blood pressure.

2.2.8. Data management

Each participant was assigned a unique code that was used for confidentiality purposes and all data collection documents (questionnaires, Appendix C) and biological specimen containers reflected this code. Data was captured on an Excel Spreadsheet at the end of each day of sampling. For quality control purposes, data captured was randomly checked by an independent person. Data was extensively cleaned and analysed using various options available in statistical package STATA version 7. (STATA Copyright 1984-1999 Stata Corporation. Texas, version 7.0). All consent forms (Appendix B) with the same code as the questionnaire were attached together. Both consent forms and questionnaires were then stored in confidential files and securely locked away for follow-up studies.

2.2.9. Genetic analyses

2.2.9.1. DNA extraction

A. Extraction from whole blood

Venous blood samples were collected from consenting learners (learners ≥15 years old) by professional medical nurses into specimen tubes containing ethylenediamine tetra acetic acid (EDTA). Samples were transported to the laboratory at room temperature, and stored at -20°C when immediate extraction was not possible. Genomic DNA was extracted from venous blood samples using a modified salting out procedure (Miller et al., 1988). A volume ranging from 5-8 ml of whole blood was transferred from an EDTA tube into a 50 ml Falcon tube. Cold lysis buffer (Appendix D) was adjusted according to the volume of blood used (according to the procedure 30 ml of cold lysis buffer for every 10 ml of blood) and added to a final volume of 40 ml. All reagents used in subsequent steps were adjusted accordingly. The mixture was then placed on ice for 15 minutes and inverted every five minutes. This mixture was then centrifuged at 1500 rpm (400 x g) for 10 minutes at 4°C (J-6M/E centrifuge, Beckman, United Kingdom). The supernatant was carefully discarded and the pellet (white blood cells) resuspended in 0.9% phosphate buffered saline (PBS) (Appendix D). This was followed by centrifugation at 1500 rpm for 10 minutes after which the supernatant was discarded and the pellet resuspended in nuclear lysis buffer (Appendix D), 0.3 mg/ml Proteinase K and 1% (w/v) sodium dodecyl sulphate (SDS) (Appendix D). The contents were mixed well and incubated at 55°C overnight. Thereafter, 6 M NaCl (Appendix C) was added to the solution and the tubes shaken vigorously for 1 minute. The mixture was centrifuged at 2500 rpm (1500 x g) for 30 minutes. The supernatant containing the DNA was subsequently transferred to a clean Falcon tube, and the pellet discarded. The supernatant was vortexed for 15 seconds, followed by centrifugation at 2500 rpm (1500 x g) for 15 minutes. The supernatant was transferred to a clean Falcon tube without the foam or the pellet. Two volumes of cold 99.9% (v/v) ethanol (EtOH) was added to each tube and agitated to precipitate the DNA. The DNA was pulled out using a sterile pipette tip, placed in a clean 1.5 ml eppendorf tube, and washed with cold 70% (v/v) ethanol. The tubes were centrifuged using a benchtop microcentrifuge (Microcentrifuge® Lite, Beckman Coulter™) at 8000 rpm (6000 x g) for 2 minutes. The 'washing step' was repeated until the pellet was clear. The ethanol was discarded, tubes left at room temperature to dry. Depending on the size of the pellet, 200-800 µl of 1X tris ethylenediamine tetra acetic acid (TE) buffer (Appendix D) was added to dissolve the DNA. To ensure a homogenous solution, the DNA was dissolved by shaking the tubes using a rotator at room temperature. Alternatively, a TE buffer heated at 56°C can be used to dissolve the DNA pellet.

DNA concentration and purity were determined using the Nanodrop® ND-100 Spectrophotometer v3.0.1 (NanoDrop Technologies Inc, DE, and USA). The NanoDrop® employed UV/VIS spectrophotometer to accurately determine nucleic acid concentration in a sample which is recorded in nanograms per microlitre (ng/µl). DNA samples, where possible, were diluted to obtain a final concentration of 200 ng/µl. The quality and purity of the DNA was determined by measuring the ratio of absorbance at 260 nm and 280 nm, whereby a ratio of approximately 1.8 was considered to be of good quality

B. Extraction from Whatman FTA® cards

This method was used for obtaining DNA samples from children less than 15 years old. The use of only a minute amount of whole peripheral blood (~80 µl) allows collection of blood by heel or finger prick, requiring less skill and fewer supplies. Whole blood specimens provide high quality DNA yields, however, venipuncture is an invasive procedure that is not always practical for infants and children; requires specialist collection, processing, and storage facilities; and can necessitate the added expense and inconvenience of travel for families, often resulting in low participation rates (Dlugos et al., 2005). Whatman FTA® cards are convenient and safe for collecting the required amount of blood and obtaining DNA samples from children. FTA Cards are impregnated with a patented chemical formula that lyses cell membranes and denatures proteins on contact. Nucleic acids are physically entrapped, immobilised and stabilised for storage at room temperature. Capillary blood obtained from a finger prick was collected on circles of Whatman FTA cards (Sigma Aldrich, Canada), each circle assigned to a particular participant. A minimum of six spots of blood were applied on each circle. Sample cards were allowed to dry at room temperature for at least one hour before being stored in Ziploc plastic bags. DNA was extracted according to the manufacturer's instructions as follows: from each sample spot a disc was cut using a Uni-Core Punch (varies according to size, 1.2 or 2 mm Uni-Core Punch can be used depending on the PCR reaction volume to be prepared) and placed in a labeled 0.2-ml PCR tube. The sample disc was washed 3 times by a FTA purification reagent, discarding the used reagent after a 5 minute incubation period at room temperature. The disc was then washed with a TE buffer (10 mM Tris-HCl and 0.1 mM EDTA, pH 8.0) 2 times discarding the used TE buffer after a 5 minute incubation period at room temperature. The disc was allowed to dry at room temperature for about 1 hour or at 56℃ for 10 minutes.

2.2.9.2. Identification of sequence variants in melanocortin 3 and 4 receptor genes (*MC3R* and *MC4R*)

Possible sequence variants in *MC3R* and *MC4R* were detected using a polymerase chain reaction-based single strand conformation polymorphism (SSCP) analysis. Seventy six samples from obese-overweight individuals were analysed using SSCP, and those with different mobility shifts were sequenced. The remaining individuals that were included in the study were genotyped for the two *MC3R* polymorphisms that were detected using allele-specific restriction enzyme (ASREA) analysis. Below is the description of the techniques used in the present study.

Polymerase chain reaction (PCR)*: MC3R* and *MC4R* are single-exon genes of 1084 and 1438 base-pairs (bp) long, respectively. The coding regions of both genes are 972 and 999 bp long. Due to the recommended optimum detection limit of 150 to 200 bp fragment size for the SSCP technique, *MC3R* and *MC4R* coding regions were divided into 5 fragments, resulting in 5 sets of oligonucleotide primers. Five previously reported primer pairs (Vaisse et al., 2000) were used to amplify the *MC4R* coding region as follows: a total volume of 50 microliters (µl) reaction mixture contained 0.25 micrograms (µg)/µl DNA template, 0.24 micromolar (µM) of both forward and reverse primers, 1x buffer, 1.0 millimolar (mM) magnesium chloride ($MgCl_2$), 0.3 mM dNTP, 0.4 units DNA polymerase, and distilled water (dH_2O) added to a final volume. *MC4R* was amplified in a Perkin Elmer 2720 thermal cycler (Applied Biosystems, USA) as follows: initial denaturation at 95°C for 3 minutes, 35 cycles of denaturation at 95°C for 30 seconds, annealing at 54°C and 55°C (depending on the primer set) for 45 seconds, extension at 72°C for 45 seconds, and final extension at 72°C for 5 minutes. Amplicons, which ranged from 246-273 bp, were electrophoresed on 2% agarose gels and visualized in a GelDoc system (BioRad, GmbH).

Five *MC3R* fragments were amplified using previously reported primers (Schalin-Jantti et al., 2003) and BIO-X-ACT short PCR kit (Bioline, USA) as follows: a total volume of 50 µl reaction mixture contained 0.25 µg/µl DNA template, 0.24 µM of both forward and reverse primers, 1x Opti buffer, 2.5 mM $MgCl_2$, 0.3 mM dNTP (Promega, USA), 2.5 units DNA polymerase, and dH_2O was added to a final volume. Amplification was carried in a Perkin Elmer 2720 thermal cycler (Applied Biosystem) as follows: initial denaturation at 94°C for minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 45 seconds and extension at 72°C for 5 minutes, and final extension at 72°C for 5 minutes. All amplicons were electrophoresed on a 2% agarose gel and visualized in a GelDoc system (BioRad, GmbH). Amplicons generated ranged from 245-300 bp.

SSCP: SSCP is a molecular technique that is used to detect possible different mobility shifts due to nucleotide changes in amplified DNA sequences (Orita *et al*., 1989). This technique is based on the principle that single stranded DNA molecules form secondary structures under certain conditions in a solution. The secondary structures formed depend on the base composition of the sequence and DNA fragments with as small as one nucleotide difference. SSCP analysis involves denaturing double stranded DNA and then rapidly cooling it so that the single stranded DNA form secondary structures to create a unique banding pattern when subjected to electrophoresis on a polyacrylamide gel. Certain conditions may be altered in order to obtain optimal results. In this study, one such optimisation was employed by varying the percentage of polyacrylamide and urea in the gel. Under optimal conditions (fragment size, 150 to 200 bp), 80–98% of potential nucleotide changes are detectable by SSCP.

The samples were run on both 8% and 10% mildly-denaturing polyacrylamide gels containing 5% glycerol and 15% urea that were prepared as illustrated in appendix D. The SSCP gel was prepared as follows: gel was developed between a back and front glass plates. The two plates were first cleaned with 70% ethanol, and the gel bond placed with its hydrophobic side on the back plate. The gel bond (provides support for the gel so that it does not break during staining) was attached to the plate using ethanol, making sure to remove air bubbles that may be formed by pushing them out of the glass. Spacers were put on left and right sides of the back plate to create space for the well comb. The spacers and the well comb must be of the same thickness. The front plate was placed over the gel bond and two plates sealed with a yellow tape and gasket to prevent leaking of the gel solution. The SSCP solution was poured and a well comb inserted immediately. The gel was allowed to polymerise for 2 hours. Once polymerised, the plates with a gel were mounted onto a vertical SSCP tank with a built-in fan heater sensor (Cleaver Scientific Ltd, UK), and its reservoirs filled with 0.5x Tris/Borate/EDTA (TBE) buffer. A total volume of 5 µl PCR product were mixed with equal volume of SSCP loading dye (Appendix), and denatured at 95℃ for 5 minutes, followed immediately by snap-chilling on ice to prevent reannealing of single-stranded DNA. Denatured amplicons were loaded into the gel and electrophoresed at room temperature overnight at 22 Watts overnight with the cooling system of the tank switched on to avoid overheating of the gel. For every gene region amplified a test sample was sequenced, and these were included in respective SSCP electrophoresis. Subsequently, DNA bands were visualised by silver staining (Appendix D). Samples with different mobility band patterns were sequenced to determine possible sequence variations.

Purification of PCR products: For sequencing, DNA was purified using *Exonuclease I* and Shrimp Alkaline Phosphatase (Inqaba Biotechnologies, SA) according to manufacturer's instructions. 5U of Shrimp Alkaline Phosphatase and 5U of *Exonuclease I* was added to 5µl of PCR product. The mixture was then incubated at 37ºC for 15 minutes followed by 80ºC for 15 minutes to deactivate the

enzymes. The concentration and purity of the PCR products were checked using the Nanodrop® according to manufacturer's specifications. Where necessary, samples were diluted down to the desired concentration using nuclease-free water.

Automated sequencing: Purified amplicons were sequenced using a BigDye terminator version 3.1 Cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions, and resolved on an ABI 3130X ® Genetic Analyzer (Applied Biosystems, USA) at the Central Analytical Facility, University of Stellenbosch. The sequencing data was analysed using the Geospiza's FinchTV version 1.4 software programs.

Genotyping of case and control groups: Obese, overweight and normal individuals were genotyped for the *MC3R* Thr6Lys and Val81Ile polymorphisms by digesting the amplicons with *Mae* III and *Bse*D 1, respectively (Schalin-Jäntti et al., 2003). The Thr6Lys and Val81Ile polymorphisms were detected in fragments 1 and 2 of *MC3R*, and the PCR protocol for amplifying these fragments to be digested is described above. The restriction enzyme digest mixture used was prepared as follows: 0.35 U of enzyme was mixed with 2 µl of 10x buffer, 7.3 µl of nuclease-free water, and 10 µl of amplicon. Restriction digest mixtures for cutting fragment 1 and 2 amplicons were then incubated overnight at 65℃ and 55℃, respectively. Digested amplicons were separated along with a negative control (undigested amplicon) and a 100-bp ladder in 3% agarose gels and visualised with GelRed under ultraviolet light.

2.2.10. Statistical analysis

All statistical analyses were conducted by Prof Lize van der Merwe, a Biostatistician at the Medical Research Council of South Africa. Data was analysed using the freely available programming language R (R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0; URL: www.r-project.org). In order to test association between relevant clinical variables and genetic markers (genotype and allelic), and to determine linkage disequilibrium (LD) and Hardy-Weinberg equilibrium (HWE), specific packages DGC-genetics was used.

A. Statistical analyses of clinical data

Clinical variables such as anthropometric measures, fasting blood glucose, blood pressure, and lipid profiles were tabulated for all learners included in the study. These main variables were compared between obese-overweight and normal, and genders. Variables are summarised as mean±SD unless indicated otherwise, and p-values are for joint model, each adjusted for the other. Triglycerides,

Diastolic and Systolic blood pressure were summarised as median, (interquartile range) and were log-transformed to improve normality**.**

B. Statistical analyses of genetic data

The aim of the study was to determine whether in the selected genetic polymorphisms, there are differences in allelic or genotype distribution between Mixed Ancestry and Black African learners, and between obese-overweight (case group) and normal weight (control group) individuals. Increased allele or genotype frequencies in the case group compared to controls may indicate that carriers of that specific allele or genotype are susceptible to obesity, or the polymorphism is in linkage disequilibrium with such a risk variant.

Comparing allele frequencies between cases and controls is not statistically valid without prior assumption of independent segregation of alleles within each individual. For this to be true, the population under investigation should obey the Hardy-Weinberg law, which states that in a large randomly mating population, the allele and genotype frequencies remain constant from one generation to the next. Consequently, for a locus consisting of alleles *A* and *a*, with population frequencies p and q respectively, the genotype frequencies within a population in HWE will be equivalent to p^2 (*AA*), 2pq (*Aa*) and q^2 (*aa*). P-values for a HWE were calculated using the R package DGC-genetics.

Pairwise LD was analysed using the *LD* function from R package DGC-genetics. Values of D'> 0.33 (Moffat et al., 2000; Kruglyak, 1999), and r^2> 0.1 (Ardlie et al., 2002; Tiret et al., 2002) were selected as useful LD. More than one polymorphism were identified per gene, and haplotypes were constructed and analysed using "haplo.stats" (version 1.2.0). This program assigns the probability for the occurrence of each haplotype in each individual, and then directly models an individual's phenotype as a function of the inferred haplotype, weighted by their estimated probability to account for haplotype ambiguity. Global and haplotype-specific scores with associated p-values are then generated (Schaid et al., 2002). A global haplotype is obtained by applying a global test for association on H-1 df (where H is the number of haplotypes for the data available). A haplotype-specific score refer to a test for association for that particular haplotype. The number of simulations for the empirical global and specific p-values was set at 1000. We inferred haplotypes from the genotypes of the two variants and compared between obese and control subjects. All models were adjusted for age and gender, and replicated measurements were also controlled for as random effects.

Logistic regression was used to assess case-control association with genotype and ethnicity in joint models. Allelic distributions differed highly significantly between ethnic groups. The differences were

of such a nature, that adjusting trait-gene association analyses for ethnicity would not be valid. All analyses were therefore stratified by ethnicity. Linear models were used to test association between quantitative traits and genotypes, alleles (additive) and haplotypes. Triglycerides, diastolic and systolic blood pressure were log-transformed prior to analysis, because of skewed distributions. Genotype-association (2 degrees of freedom) was tested by coding genotype as a categorical factor as described by Cordell and Clayton (2005) and additive allelic association (1 degree of freedom) with a numerical variable, counting the number of minor alleles. Haplotype analysis was done using methods described by Schaid and co-workers (2002).

Association between MC3R genotypes and physical activity: Linear models were also used to assess the effect of the *MC3R* genotypes on anthropometric and metabolic parameters while adjusting for frequency of participation in each activity (walking, sport, cycling, and household chores), age, ethnicity and gender. Finally linear models were used to assess the interaction between the *MC3R* genotypes and frequency of participation in various physical activities (such as walking, sport, cycling, household chores) on anthropometric and metabolic parameters while adjusting for age, ethnicity and gender.

Multiple comparison consideration: P-values obtained from all analyses were not corrected for multiple testing because it has been suggested that correction is too conservative when several associations are tested in the same group of individuals (Nyholt, 2004), and might not be appropriate in a situation such as this, where there is prior evidence that such effects exist (Perneger, 1998). Our tests are not of independent null hypotheses as required by Benjamini, Yoav; Hochberg, Yosef (1995), as the SNPs are in tight LD with each other, and we explored different (genotype and additive allelic) models with individual SNPs, combinations of genotypes and also combinations of alleles from the same SNPs. The statistical significance of each association can only be validated when their biological meaning have been identified, and replicated in multiple studies. In all analyses conducted, results with p-values below 5% are described as significant. The effect sizes and 95% confidence intervals reported in the results and tables were calculated from each specific model.

2.3. RESULTS

Clinical characteristics of participants according to ethnicity are summarized in Table 2.2. By selection, anthropometric obesity variables (weight, BMI, WC, hip and MUAC) and blood pressure were significantly higher in obese-overweight learners. However, there was no significant interaction between obesity susceptibly and ethnicity on anthropometric obesity variables. The fasting blood glucose (FBG) was significantly higher in Mixed Ancestry learners ($p = 0.0088$) irrespective of BMI categories (obese-overweight and normal weight status).

SSCP and subsequent sequencing of samples that showed different mobility shifts failed to detect sequence variants in *MC4R* (Figure 2.2), whilst the different mobility shifts detected on *MC3R* SSCP analysis represented two common polymorphisms upon sequencing. Different mobility shifts were detected in fragments 1 and 2 of *MC3R* (Figure 2.3). Nucleotide sequences of *MC3R* fragment 1 revealed a C>A nucleotide substitutions at position 618 (g.618C>A) whilst that of fragment 2 showed G>A nucleotide change at position 842 of the genomic sequence (g.842G>A). The sequences were further translated to determine the effect of the substitutions in the amino acid sequence. The C>A variant resulted in a threonine to lysine substitution at position 6 (Thr6Lys or T6K) (rs3746619), and G>A to a valine to isoleucine amino acid substitution at position 81 (Val81Ile or V81I) (rs3827103) of the protein sequence. The Thr6Lys and Val81Ile polymorphisms are located, respectively, in the amino-terminal extracellular region and the first transmembrane helix of the MC3R protein. The nucleotide substitutions identified in *MC3R* affect restriction enzyme recognition sites: the C>A abolishes a *Mae* II recognition site and the G>A abolishes a *BSeD* 1 site. Seventy six samples underwent SSCP analysis, however, the entire study population was genotyped for the two *MC3R* polymorphisms using ASREA (Figure 2.4).

Table 2.2. Anthropometric and metabolic variables of obese-overweight and normal Black African and Mixed Ancestry South African learners.

	Obese and overweight		Normal weight		P-value		
	Black Africans	**Mixed ancestry**	**Black Africans**	**Mixed ancestry**	**Obesity**	**Race**	**Interaction**
n	115	112	94	110			
Age (years)	13.2±2.0	13.4±2.0	13.3±2.0	13.6±2.1	0.5897	0.3293	0.8795
Gender, male (%)	21 (25)	26 (31)	16 (19)	20 (24)	0.8150	0.8280	0.6530
Weight (kg)	62.7±12.5	65.4±13.9	46.3±8.5	45±9.6	**<0.0001**	0.4273	0.0730
Height (cm)	153.3±8.1	156.3±8.7	153.5±9.3	153.3±10.8	0.8901	0.8533	0.0732
BMI (kg/m^2)	26.5±3.6	26.6±4.1	19.5±2.1	19±2.5	**<0.0001**	0.2278	0.3050
FBG (mmol/L)	3.9±0.8	4.2±0.8	3.8±0.8	4.1±0.7	0.1545	**0.0088**	0.9134
TC (mmol/L)	3.8±1	3.7±0.8	3.7±0.8	3.7±0.8	0.3645	0.9612	0.5332
TG (mmol/L)*	0.73 (0.57, 0.95)	0.65 (0.57, 0.99)	0.57 (0.57, 0.79)	0.57 (0.57, 0.80)	0.2736	0.1621	0.4489
HDL-C (mmol/L)	1.1±0.4	0.9±0.3	1.2±0.4	1.1±0.4	0.0650	0.2048	0.1473
MUAC (cm)	27.5±3.4	27.7±3.2	22.7±2.5	22.1±2.3	**<0.0001**	0.1011	0.1134
Waist (cm)	79.4±8.8	80.4±10.1	66.4±6.6	64.9±6.7	**<0.0002**	0.1934	0.1183
Hip (cm)	99.1±9.5	100.9±10.1	84.7±8.3	84.6±9.1	**<0.0003**	0.9871	0.3119
SBP (mm Hg) *	114 (104, 123)	111 (105, 117)	103 (97, 115)	104 (95, 116)	**<0.0004**	0.8680	0.3440
DBP (mm Hg)*	69 (63, 78)	69 (64, 73)	64 (59, 71)	66 (59, 72)	**0.0002**	0.6880	0.3889

Summary statistics are mean±SD unless indicated otherwise. P-values are for joint model, so each is adjusted for the other. *Triglycerides, Diastolic and Systolic blood pressure were summarised as median, (interquartile range) and were log-transformed to symmetry for tests**.**
Abbreviations: BMI, body mass index; FBG, fasting blood glucose; DBP, diastolic blood pressure; HDL-C, high density lipoprotein-cholesterol; MUAC, mid-upper-arm circumference; SBP, systolic blood pressure; TC, total cholesterol; TG, triglycerides; WC, waist circumference.

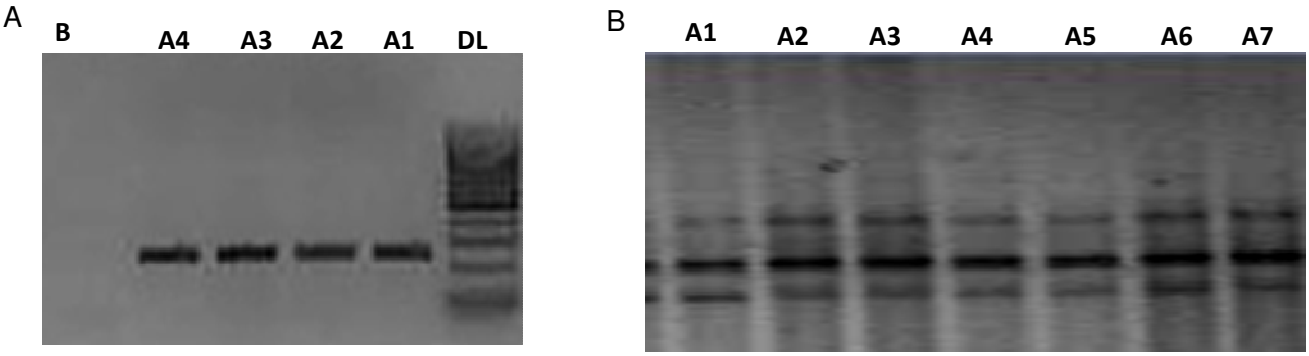


Figure 2.2. Single strand conformation polymorphism analysis of *MC4R*. A) 2% agarose gel showing 5 amplified fragments (A1-4), a 100-bp ladder (DL) and a blank (B). B) 10% polyacrylamide SSCP gel showing 7 amplicons with similar mobility shifts.

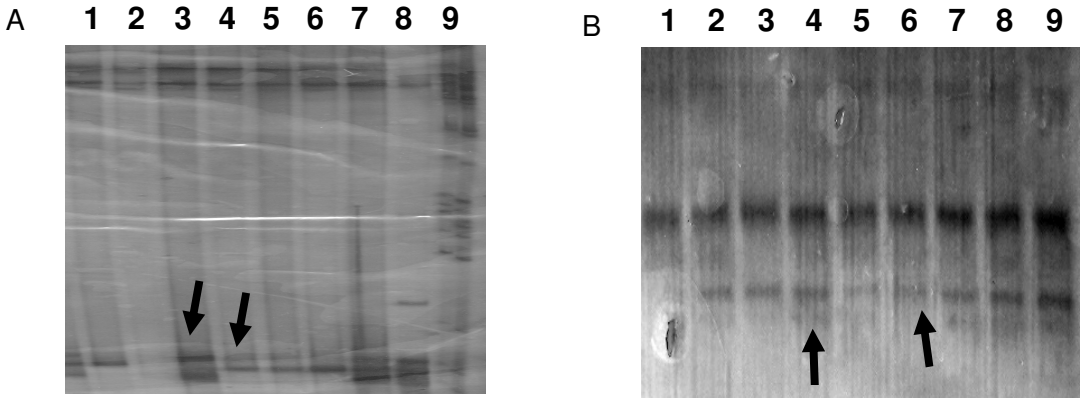


Figure 2.3. Single strand conformation polymorphism analysis of *MC3R.* (A, fragment 1, and B, fragments 2) A: Fragment 1- two different band patterns (forming heteroduplexes) were identified as indicated by arrows (lanes 3 and 4). From left: lanes 1-7, amplicons; lane 8, undenatured amplicon; lane 9, lambda Pst 1 DNA ladder. B: Fragment 2- two different band patterns were identified as indicated by arrows. From left: lanes 1-9, amplicons.

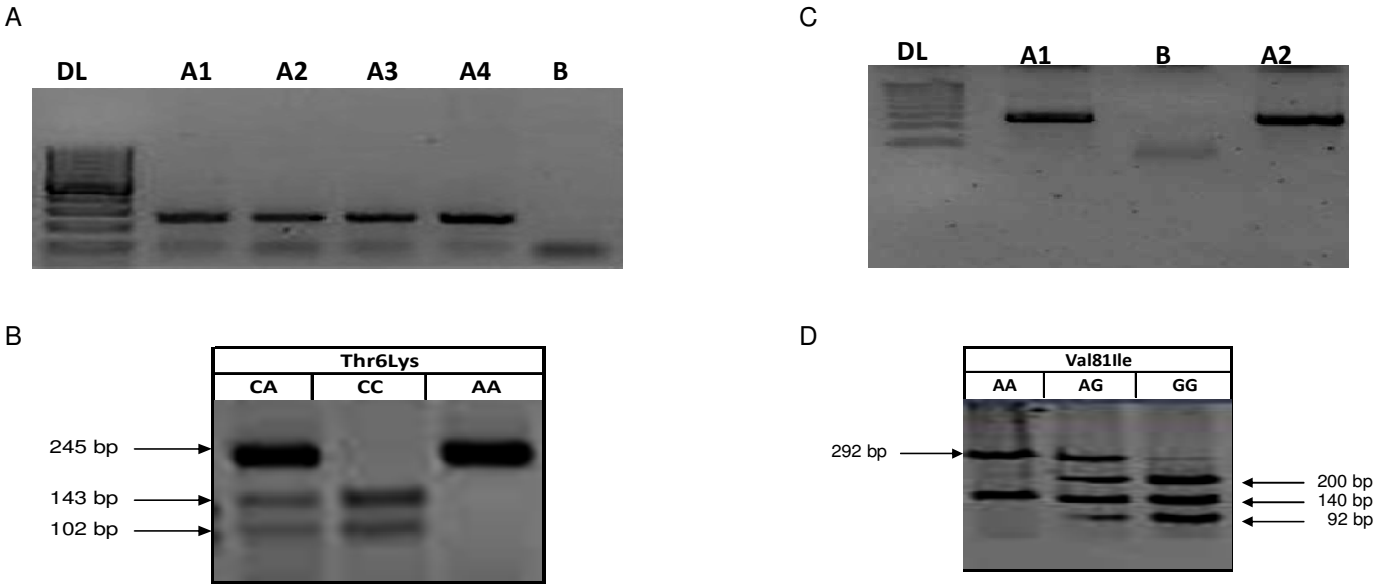


Figure 2.4. Polymerase chain reaction-based allele-specific restriction enzyme analysis of *MC3R*, fragments 1 and 2 that contained Thr6Lys and Val81Ile, respectively. The two fragments were amplified successfully resulting in a 245 bp amplicon (A, fragment 1) and a 292 bp amplicon (C, fragment 2) as indicated. Lanes from left to right: A) DL= 100-bp ladder, A1-4= amplicons, B= blank (PCR solution without a DNA template). B: DL= 100-bp ladder, A1 and A2= amplicons, B= blank. Three genotypes were identified for each polymorphism as indicated.

Thr6Lys and Val81Ile were in strong linkage disequilibrium in all groups, ranging from D'= 0.7437 in Mixed Ancestry normal weight learners to D'=0.9264 in the Black African obese learners. The frequency distributions, both genotype and allele, differed significantly between the racial groups, after adjusting for case-control status. The differences were of such a nature that potential effects would be cancelled out if the genotype association test was adjusted for ethnicity. Therefore all subsequent analyses were stratified according to race. Only the distribution of the Val81Ile *A* allele was significantly different between Mixed Ancestry obese/overweight and normal weight learners, respectively 28% and 40%, p = 0.0144 (Table 2.3). Table 2.4a shows P-values from individual genotype and allele (additive) association tests with anthropometric and metabolic obesity traits stratified by race, adjusted for gender and age for both polymorphisms. Both Thr6Lys and Val81Ile showed associations with obesity anthropometric traits (body weight, BMI, and hip and waist circumferences, and mid-upper-arm) and total cholesterol, whilst only the Thr6Lys was associated with blood pressure in Mixed Ancestry learners. For example, the estimated effect of the Thr6Lys *A* allele was a decrease of 0.814 cm in mid-upper-arm circumference, 2.873 cm in hip circumference, 3.225 kg in weight and a corresponding 0.959 kg/m^2 reduction in BMI (Table 2.4b). Total cholesterol was significantly decreased, respectively, by 0.170 and 0.162 mmol/L in Lys6Lys and Ile81Ile carriers compared to the respective wild type homozygotes. Learner homozygous for the 6Lys allele had approximately 2.4 mm Hg less blood pressure than those carrying the wildtype allele.

Table 2.3. Allelic and genotype counts (n), frequencies (f) and p-values for tests of association with obesity status, stratified by race** and genotype association with race, adjusting for case-control status*.

	Obese (n = 227)				Normal (n = 204)				p-value		
	Black Africans		Mixed ancestry		Black Africans		Mixed ancestry		Black Africans*	Mixed ancestry*	Race**
	n	f	n	f	n	f	n	f			
Thr6Lys											
typed	115		112		94		110				
C	104	0.45	159	0.71	99	0.53	137	0.62			
A	126	0.55	65	0.29	89	0.47	83	0.38	0.1055	0.0589	**<0.0001**
C/C	21	0.18	59	0.53	22	0.23	43	0.39			
C/A	62	0.54	41	0.37	55	0.59	51	0.46			
A/A	32	0.28	12	0.11	17	0.18	16	0.15	0.2237	0.1246	**<0.0001**
Val81Ile											
typed	115		112		94		110				
G	106	0.46	162	0.72	96	0.51	133	0.60			
A	124	0.54	62	0.28	92	0.49	87	0.40	0.2873	**0.0144**	**<0.0001**
G/G	22	0.19	61	0.54	22	0.23	46	0.42			
G/A	62	0.54	40	0.36	52	0.55	41	0.37			
A/A	31	0.27	11	0.1	20	0.21	23	0.21	0.561	**0.0401**	**<0.0001**

P-values are for tests of difference in allele and genotype distribution between the case and control groups within each racial group; adjusting for age and gender; and between the two racial groups, adjusted for case-control status, age and gender.

Table 2.4a. P-values from tests of association between clinical variables and polymorphisms, both genotype and allelic, stratified by race, adjusted for age and gender.

	Black African				Mixed Ancestry			
	Thr6Lys		Val81Ile		Thr6Lys		Val81Ile	
Clinical variable	genotype	allelic	genotype	allelic	genotype	allelic	genotype	allelic
Weight (kg)	0.2944	0.1246	0.3871	0.1768	0.0668	**0.0216**	**0.0172**	**0.0074**
Height (cm)	0.9729	0.8388	0.5729	0.4938	0.2508	0.1462	0.3128	0.2287
BMI (kg/m^2)	0.2664	0.1203	0.3861	0.2388	0.1270	**0.0476**	**0.0160**	**0.0110**
FBG (mmol/L)	0.4337	0.7875	0.1725	0.7528	0.7216	0.4218	0.5231	0.2862
TC (mmol/L)	0.2166	0.1404	0.0726	0.1218	0.0625	**0.0315**	**0.0499**	**0.0307**
TG (mmol/L)*	0.0974	0.0404	0.4328	0.2065	0.2912	0.2183	0.1355	0.0910
HDL-C (mmol/L)	0.3268	0.6687	0.1033	0.8736	0.6723	0.8861	0.9766	0.8283
Midupperarm (cm)	0.5468	0.5133	0.4606	0.7714	0.0825	**0.0268**	**0.0195**	**0.0119**
Waist (cm)	0.1386	0.2799	0.3112	0.6478	0.1867	0.0668	**0.0451**	**0.0362**
Hip (cm)	0.3901	0.1738	0.4176	0.2155	**0.0338**	**0.0099**	**0.0136**	**0.0071**
SBP (mm Hg) *	0.8249	0.5382	0.4505	0.7829	**0.0311**	**0.0467**	0.1468	0.5359
DBP (mm Hg)*	0.9976	0.9599	0.4667	0.7060	**0.0083**	**0.0213**	0.2810	0.3798

*Triglycerides, Diastolic and Systolic blood pressure were log-transformed to symmetry for tests.

Table 2.4b. Estimated effect and standard error of the *A* allele of each polymorphism, corresponding to allelic p-values in Table 2.4a, on clinical variables, adjusted for age and gender. A negative effect indicates that individuals with the *A* allele have lower values for the trait than those with the wild type allele.

	Black African				Mixed Ancestry			
	Thr6Lys		Val81Ile		Thr6Lys		Val81Ile	
Clinical variable	**Effect**	**SE**	**Effect**	**SE**	**Effect**	**SE**	**Effect**	**SE**
Weight (kg)	1.941	1.259	1.686	1.244	**-3.225**	**1.394**	**-3.575**	**1.321**
Height (cm)	0.152	0.748	0.505	0.737	-1.171	0.803	-0.924	0.765
BMI (kg/m^2)	0.725	0.465	0.543	0.460	**-0.959**	**0.481**	**-1.168**	**0.456**
FBG (mmol/L)	-0.022	0.081	-0.025	0.079	0.060	0.074	0.075	0.070
TC (mmol/L)	-0.137	0.093	-0.142	0.091	**-0.170**	**0.079**	**-0.162**	**0.075**
TG (mmol/L)*	**-0.107**	**0.052**	-0.065	0.052	-0.050	0.040	-0.065	0.038
HDL-C (mmol/L)	-0.017	0.039	0.006	0.039	0.005	0.034	-0.007	0.032
Midupperarm (cm)	0.245	0.374	0.108	0.370	**-0.814**	**0.365**	**-0.878**	**0.347**
Waist (cm)	1.096	1.011	0.458	1.000	-1.971	1.070	**-2.141**	**1.016**
Hip (cm)	1.490	1.092	1.339	1.078	**-2.873**	**1.105**	**-2.852**	**1.050**
SBP (mm Hg)*	0.893	1.449	0.395	1.431	**-2.498**	**1.249**	-0.743	1.198
DBP (mm Hg)*	-0.061	1.210	-0.451	1.194	**-2.087**	**0.899**	-0.761	0.865

*Triglycerides, Diastolic and Systolic blood pressure were not log-transformed for estimation.

There was no statistically significant variation in the distribution of the Thr6Lys-Val81Ile haplotype between Black African obese/overweight and normal weight learners. However, the A-A haplotype was the most common in the former group, 52% vs 44%, p = 0.0749. In Mixed Ancestry learners, the haplotype distributions differed significantly between obese/overweight and normal weight learners (global *P*= 0.0185): C-G was more prevalent in obese/overweight learners (69% versus 55%, *P*=0.0063) whereas C-A was more frequent in normal weight learners (8% vs 2%, *P*=0.0142) (Table 2.5). Table 2.6 shows the Thr6Lys-Val81Ile haplotype association tests with anthropometric and metabolic obesity traits and their corresponding estimated effect size. No significant associations were observed between the Thr6Lys-Val81Ile haplotype and anthropometric and metabolic obesity traits in Black African learners. On the other hand, in Mixed Ancestry learners, the C-A haplotype was associated with a decrease of 2.4 kg in body weight compared to C-G carriers; while those carrying the A-A haplotype weighed 3.3 kg less. The systolic as well as diastolic blood pressures were decreased by 10 and 7.5 mmHg, respectively in A-G haplotype compared to the C-G haplotype carriers.

Table 2.5. Inferred Thr6Lys-Val81Ile haplotype frequencies in normal and overweight-obese children, and joint individual p-values for test of association with overweight, stratified by race. Global p-values are given in the heading.

	Black Africans (p = 0.1089)			**Mixed Ancestry (p = 0.0185)**		
Haplotype	**Obese and overweight**	**Normal**	**P-value**	**Obese and overweight**	**Normal**	**P-value**
C-A	0.02	0.05	0.0688	0.02	0.08	**0.0142**
C-G	0.43	0.48	0.3541	0.69	0.55	**0.0063**
A-G	0.03	0.03	0.7389	0.04	0.06	0.3496
A-A	0.52	0.44	0.0749	0.25	0.32	0.1172

Table 2.6. P-values for Thr6Lys-Val81Ile haplotype (global) association with clinical traits, stratified by race and adjusted for age and gender. For significant effects in mixed ancestry children: estimated effect [and standard error of estimate (SE)] of specific Thr6Lys-Val81Ile haplotype compared to C-G (wild types), adjusted for age and gender.

	P-value		Mixed Ancestry		
Clinical variable	**Black African**	**Mixed ancestry**	**Haplotype**	**Effect**	**SE**
Weight (kg)	0.4062	**0.0338**	A-A vs C-G	-3.3	1.5
Height (cm)	0.7946	0.5443			
BMI (kg/m^2)	0.3542	**0.0351**	C-A vs C-G	-2.4	1.1
FBG (mmol/L)	0.9761	0.7336			
TC (mmol/L)	0.3791	0.1070			
TG (mmol/L)*	0.2409	0.2619			
HDL-C (mmol/L)	0.7290	0.7252			
Midupperarm (cm)	0.8572	**0.0278**	C-A vs C-G	-1.7	0.8
			A-A vs C-G	-0.8	0.4
Waist (cm)	0.3871	0.0993			
Hip (cm)	0.4666	0.077			
SBP (mm Hg) *	0.8694	**0.0047**	A-G vs C-G	-10.0	2.8
DBP (mm Hg)*	0.8363	**0.0027**	A-G vs C-G	-7.5	2.0

*Triglycerides, Diastolic and Systolic blood pressure were log-transformed to symmetry for tests. Effect sizes are not transformed.

When physical activity was incorporated into the regression analyses, only total cholesterol showed a significant association with *MC3R* genotypes when adjusted for the frequency of each physical activity, age, gender and race. These effects are summarised in table 2.7. Total cholesterol was significantly decreased in learners who were homozygous for the Thr6Lys and Val81Ile minor alleles compared to the respective wild type homozygotes. Furthermore, the only activity by genotype interactions we detected on total cholesterol. They were between walking and both polymorphisms (Figure 2.5), and between doing house chores and Val181Ie (Figure 2.6). The walking-by-genotype interaction on total cholesterol did not follow a defined pattern and therefore could not be interpreted. The interaction between frequency of performing house chores and Val81Ie can be summarised as on total cholesterol was as follows: learners who did house chores often and carried *AA* or *AG* genotypes, had 0.355 mmol/L (95% CI: 0.148, 0.561) reduction in total cholesterol compared to *GG* homozygotes.

Table 2.7. P-values and effect sizes for the association between *MC3R* genotypes and clinical variables; adjusted for age, gender, race, and the frequency of each physical activity category.

		Thr6Lys (N= 431)			Val81Ile (N= 431)		
Clinical variable	**Activity**	**P-value**	**β coefficient (95% CI)**		**P-value**	**β coefficient (95% CI)**	
			CA vs CC	**AA vs CC**		**GA vs GG**	**AA vs GG**
Weight (kg)							
	walking	0.5861	-1.52 (-4.44,1.40)	-1.18 (-5.00,2.63)	0.3843	-1.35 (-4.29,1.59)	-2.51 (-6.15,1.13)
	sport	0.4366	-1.90 (-4.85,1.05)	-1.60 (-5.42,2.21)	0.3343	-1.74 (-4.71,1.23)	-2.56 (-6.23,1.11)
	TV viewing	0.6948	-1.20 (-4.11,1.71)	-1.19 (-4.95,2.58)	0.4292	-1.20 (-4.12,1.72)	-2.36 (-5.97,1.25)
	cycling	0.5339	-1.63 (-4.55,1.29)	-1.42 (-5.21,2.38)	0.3886	-1.38 (-4.33,1.56)	-2.48 (-6.13,1.16)
	House chores	0.5325	-1.62 (-4.55,1.31)	-1.51 (-5.31,2.29)	0.3703	-1.47 (-4.42,1.48)	-2.53 (-6.19,1.12)
Height (cm)							
	walking	0.4700	-1.03 (-2.72,0.66)	-0.94 (-3.15,1.27)	0.7494	-0.62 (-2.33,1.08)	-0.61 (-2.72,1.50)
	sport	0.3357	-1.26 (-2.97,0.46)	-1.13 (-3.35,1.10)	0.6831	-0.73 (-2.46,1.01)	-0.71 (-2.85,1.43)
	TV viewing	0.5327	-0.93 (-2.63,0.77)	-0.89 (-3.09,1.31)	0.8346	-0.48 (-2.19,1.23)	-0.51 (-2.63,1.61)
	cycling	0.4156	-1.13 (-2.83,0.57)	-0.94 (-3.15,1.27)	0.7670	-0.62 (-2.34,1.10)	-0.53 (-2.66,1.60)
	House chores	0.4074	-1.14 (-2.84,0.56)	-0.98 (-3.19,1.23)	0.7531	-0.63 (-2.35,1.08)	-0.57 (-2.69,1.56)
BMI (kg/m^2)							
	walking	0.6783	-0.45 (-1.49,0.58)	-0.17 (-1.53,1.18)	0.4616	-0.44 (-1.49,0.61)	-0.80 (-2.09,0.50)
	sport	0.5859	-0.55 (-1.59,0.50)	-0.28 (-1.63,1.07)	0.4230	-0.57 (-1.62,0.48)	-0.78 (-2.08,0.51)
	TV viewing	0.7967	-0.35 (-1.39,0.68)	-0.19 (-1.53,1.15)	0.4889	-0.41 (-1.45,0.63)	-0.77 (-2.05,0.52)
	cycling	0.6703	-0.47 (-1.51,0.57)	-0.26 (-1.60,1.09)	0.4525	-0.45 (-1.49,0.60)	-0.81 (-2.10,0.49)
	House chores	0.6826	-0.46 (-1.50,0.58)	-0.29 (-1.64,1.06)	0.4381	-0.48 (-1.53,0.57)	-0.82 (-2.11,0.48)

Table 2.7. *continues*

Clinical variable		Thr6Lys (N= 431)			Val81Ile (N= 431)		
	Activity	P-value	β coefficient (95% CI)		P-value	β coefficient (95% CI)	
			CA vs CC	AA vs CC		GA vs GG	AA vs GG
WC (cm)							
	walking	0.0807	-2.39 (-4.60,-0.17)	-0.52 (-3.41,2.36)	0.2349	-1.74 (-3.97,0.50)	-1.98 (-4.74,0.79)
	sport	0.0627	-2.57 (-4.80,-0.35)	-0.77 (-3.65,2.10)	0.1800	-2.00 (-4.25,0.25)	-1.95 (-4.73,0.82)
	TV viewing	0.1506	-2.10 (-4.31,0.11)	-0.61 (-3.47,2.24)	0.2564	-1.64 (-3.86,0.58)	-1.94 (-4.69,0.80)
	cycling	0.0872	-2.40 (-4.61,-0.19)	-0.75 (-3.61,2.12)	0.2192	-1.78 (-4.01,0.45)	-2.01 (-4.77,0.75)
	House chores	0.1033	-2.33 (-4.54,-0.12)	-0.80 (-3.67,2.06)	0.2054	-1.80 (-4.03,0.43)	-2.07 (-4.84,0.69)
Hip (cm)							
	walking	0.3464	-1.83 (-4.31,0.66)	-0.87 (-4.12,2.38)	0.3815	-1.36 (-3.87,1.16)	-2.07 (-5.18,1.05)
	sport	0.2600	-2.09 (-4.59,0.41)	-1.26 (-4.49,1.97)	0.3044	-1.64 (-4.16,0.88)	-2.20 (-5.31,0.91)
	TV viewing	0.4550	-1.57 (-4.04,0.90)	-0.79 (-3.99,2.41)	0.4378	-1.21 (-3.70,1.28)	-1.92 (-5.00,1.17)
	cycling	0.3300	-1.88 (-4.37,0.60)	-1.08 (-4.31,2.15)	0.3840	-1.37 (-3.88,1.15)	-2.05 (-5.16,1.06)
	House chores	0.3450	-1.85 (-4.33,0.64)	-1.07 (-4.30,2.15)	0.3710	-1.39 (-3.90,1.11)	-2.08 (-5.19,1.03)
WHR (cm)							
	walking	0.1289	-0.009 (-0.022,0.004)	0.006 (-0.011,0.023)	0.3110	-0.01 (-0.02,0.00)	0.00 (-0.02,0.01)
	sport	0.1693	-0.009 (-0.022,0.005)	0.005 (-0.012,0.022)	0.2898	-0.011 (-0.024,0.003)	-0.004 (-0.021,0.012)
	TV viewing	0.2069	-0.008 (-0.021,0.005)	0.005 (-0.012,0.022)	0.3105	-0.010 (-0.024,0.003)	-0.005 (-0.021,0.011)
	cycling	0.1660	-0.009 (-0.022,0.004)	0.005 (-0.012,0.022)	0.3024	-0.010 (-0.024,0.003)	-0.005 (-0.021,0.011)
	House chores	0.1876	-0.009 (-0.022,0.004)	0.004 (-0.013,0.021)	0.2814	-0.011 (-0.024,0.003)	-0.005 (-0.022,0.011)

Table 2.7. *continues*

Clinical variable			Thr6Lys (N= 431)			Val81Ile (N= 431)	
	Activity	P-value	β coefficient (95% CI)		P-value	β coefficient (95% CI)	
			CA vs CC	AA vs CC		GA vs GG	AA vs GG
SBP (mm Hg)							
	walking	0.1525	-2.78 (-5.70,0.14)	-0.84 (-4.66,2.97)	0.8690	-0.778 (-3.738,2.182)	-0.271 (-3.935,3.392)
	sport	0.1044	-3.10 (-6.05,-0.15)	-1.04 (-4.89,2.80)	0.7438	-1.13 (-4.12,1.86)	-0.34 (-4.03,3.35)
	TV viewing	0.1907	-2.68 (-5.63,0.27)	-1.05 (-4.89,2.79)	0.8852	-0.74 (-3.72,2.23)	-0.36 (-4.04,3.32)
	cycling	0.1715	-2.73 (-5.66,0.21)	-0.95 (-4.78,2.88)	0.8796	-0.76 (-3.73,2.22)	-0.31 (-3.99,3.36)
	House chores	0.1781	-2.71 (-5.65,0.22)	-1.00 (-4.83,2.83)	0.8733	-0.78 (-3.76,2.19)	-0.36 (-4.04,3.32)
DBP (mm Hg)							
	walking	0.1246	-2.40 (-4.71,-0.09)	-1.55 (-4.57,1.47)	0.7365	-0.50 (-2.84,1.84)	-1.15 (-4.04,1.75)
	sport	0.0789	-2.67 (-5.00,-0.34)	-1.98 (-5.00,1.04)	0.6478	-0.85 (-3.20,1.51)	-1.30 (-4.21,1.62)
	TV viewing	0.1600	-2.23 (-4.55,0.09)	-1.82 (-4.83,1.18)	0.7137	-0.49 (-2.83,1.84)	-1.21 (-4.10,1.68)
	cycling	0.1214	-2.40 (-4.71,-0.09)	-1.82 (-4.82,1.18)	0.7093	-0.61 (-2.95,1.73)	-1.21 (-4.10,1.69)
	House chores	0.1270	-2.37 (-4.69,-0.06)	-1.85 (-4.86,1.15)	0.7033	-0.59 (-2.93,1.75)	-1.23 (-4.13,1.67)
FBG (mmol/L)							
	walking	0.6556	-0.030 (-0.198,0.138)	0.065 (-0.155,0.284)	0.2821	-0.07 (-0.24,0.10)	0.09 (-0.12,0.30)
	sport	0.7437	0.002 (-0.167,0.171)	0.076 (-0.142,0.295)	0.3441	-0.042 (-0.211,0.128)	0.104 (-0.105,0.314)
	TV viewing	0.7661	-0.023 (-0.192,0.147)	0.052 (-0.167,0.271)	0.3161	-0.066 (-0.236,0.103)	0.083 (-0.127,0.293)
	cycling	0.7163	-0.024 (-0.192,0.144)	0.060 (-0.159,0.278)	0.3058	-0.065 (-0.234,0.104)	0.087 (-0.123,0.296)
	House chores	0.7491	-0.022 (-0.191,0.146)	0.056 (-0.163,0.274)	0.3118	-0.067 (-0.236,0.103)	0.084 (-0.126,0.293)

Table 2.7. *continues*

Clinical variable		Thr6Lys (N= 431)			Val81Ile (N= 431)		
	Activity	**P-value**	**β coefficient (95% CI)**		**P-value**	**β coefficient (95% CI)**	
			CA vs CC	**AA vs CC**		**GA vs GG**	**AA vs GG**
TC (mmol/L)							
	walking	**0.0292**	-0.170 (-0.353,0.012)	-0.312 (-0.550,-0.074)	**0.0261**	-0.133 (-0.317,0.051)	-0.313 (-0.541,-0.086)
	sport	**0.0207**	-0.201 (-0.385,-0.017)	-0.316 (-0.555,-0.078)	**0.0264**	-0.156 (-0.341,0.030)	-0.312 (-0.541,-0.083)
	TV viewing	**0.0388**	-0.175 (-0.358,0.007)	-0.292 (-0.529,-0.055)	**0.0354**	-0.134 (-0.317,0.050)	-0.299 (-0.526,-0.072)
	cycling	**0.0325**	-0.177 (-0.359,0.006)	-0.302 (-0.538,-0.065)	**0.0304**	-0.136 (-0.319,0.048)	-0.306 (-0.534,-0.079)
	House chores	**0.0265**	-0.179 (-0.360,0.002)	-0.310 (-0.545,-0.074)	**0.0245**	-0.139 (-0.322,0.043)	-0.314 (-0.540,-0.088)
TG (mmol/L)							
	walking	0.0586	-0.085 (-0.185,0.016)	-0.155 (-0.287,-0.023)	0.1072	-0.065 (-0.167,0.037)	-0.135 (-0.261,-0.009)
	sport	0.0705	-0.089 (-0.190,0.013)	-0.147 (-0.278,-0.015)	0.1351	-0.066 (-0.169,0.036)	-0.127 (-0.254,0.000)
	TV viewing	0.0680	-0.085 (-0.186,0.016)	-0.149 (-0.280,-0.018)	0.1161	-0.064 (-0.165,0.038)	-0.132 (-0.259,-0.006)
	cycling	0.0581	-0.085 (-0.186,0.015)	-0.153 (-0.284,-0.023)	0.1048	-0.067 (-0.169,0.034)	-0.135 (-0.260,-0.009)
	House chores	0.0601	-0.086 (-0.186,0.015)	-0.151 (-0.282,-0.021)	0.1006	-0.066 (-0.167,0.036)	-0.136 (-0.261,-0.011)
HDL-C (mmol/L)							
	walking	0.2593	0.047 (-0.033,0.127)	-0.025 (-0.129,0.079)	0.2945	0.051 (-0.029,0.131)	-0.012 (-0.111,0.088)
	sport	0.3181	0.042 (-0.038,0.122)	-0.024 (-0.128,0.079)	0.3825	0.046 (-0.034,0.127)	-0.008 (-0.108,0.091)
	TV viewing	0.2716	0.046 (-0.034,0.125)	-0.025 (-0.128,0.078)	0.3276	0.049 (-0.032,0.129)	-0.011 (-0.110,0.088)
	cycling	0.2444	0.048 (-0.032,0.127)	-0.025 (-0.128,0.078)	0.2971	0.050 (-0.030,0.130)	-0.012 (-0.111,0.087)
	House chores	0.2403	0.047 (-0.033,0.126)	-0.027 (-0.130,0.076)	0.3051	0.049 (-0.031,0.129)	-0.013 (-0.112,0.086)

Table 2.7. *continues*

Clinical variable		Thr6Lys (N= 431)			Val81Ile (N= 431)		
	Activity	P-value	β coefficient (95% CI)		P-value	β coefficient (95% CI)	
			CA vs CC	AA vs CC		GA vs GG	AA vs GG
LDL-C (mmol/L)							
	walking	0.2525	-0.069 (-0.289,0.151)	-0.249 (-0.544,0.046)	0.1177	-0.080 (-0.306,0.146)	-0.288 (-0.563,-0.012)
	sport	0.2647	-0.088 (-0.308,0.133)	-0.241 (-0.533,0.050)	0.1669	-0.097 (-0.323,0.129)	-0.267 (-0.544,0.010)
	TV viewing	0.3127	-0.093 (-0.311,0.126)	-0.224 (-0.515,0.067)	0.1759	-0.100 (-0.324,0.123)	-0.261 (-0.535,0.014)
	cycling	0.2216	-0.083 (-0.302,0.136)	-0.256 (-0.546,0.034)	0.1181	-0.110 (-0.334,0.114)	-0.289 (-0.563,-0.015)
	House chores	0.2384	-0.091 (-0.310,0.128)	-0.251 (-0.542,0.040)	0.1338	-0.103 (-0.328,0.123)	-0.281 (-0.556,-0.006)

Estimated effects and 95% CI of the A allele of each polymorphism. A negative effect indicates that individuals with stated level have lower values for the trait than those with the reference level.
Abbreviations: 95% CI: 95% confidence interval; BMI, body mass index ; DBP, diastolic blood pressure; FBG, fasting blood glucose; HDL-C, high density lipoprotein-cholesterol; HP, hip circumference; SBP, systolic blood pressure; TC, total cholesterol; TG, triglycerides; TV, television; WC, waist circumference; WHR, waist hip ratio.

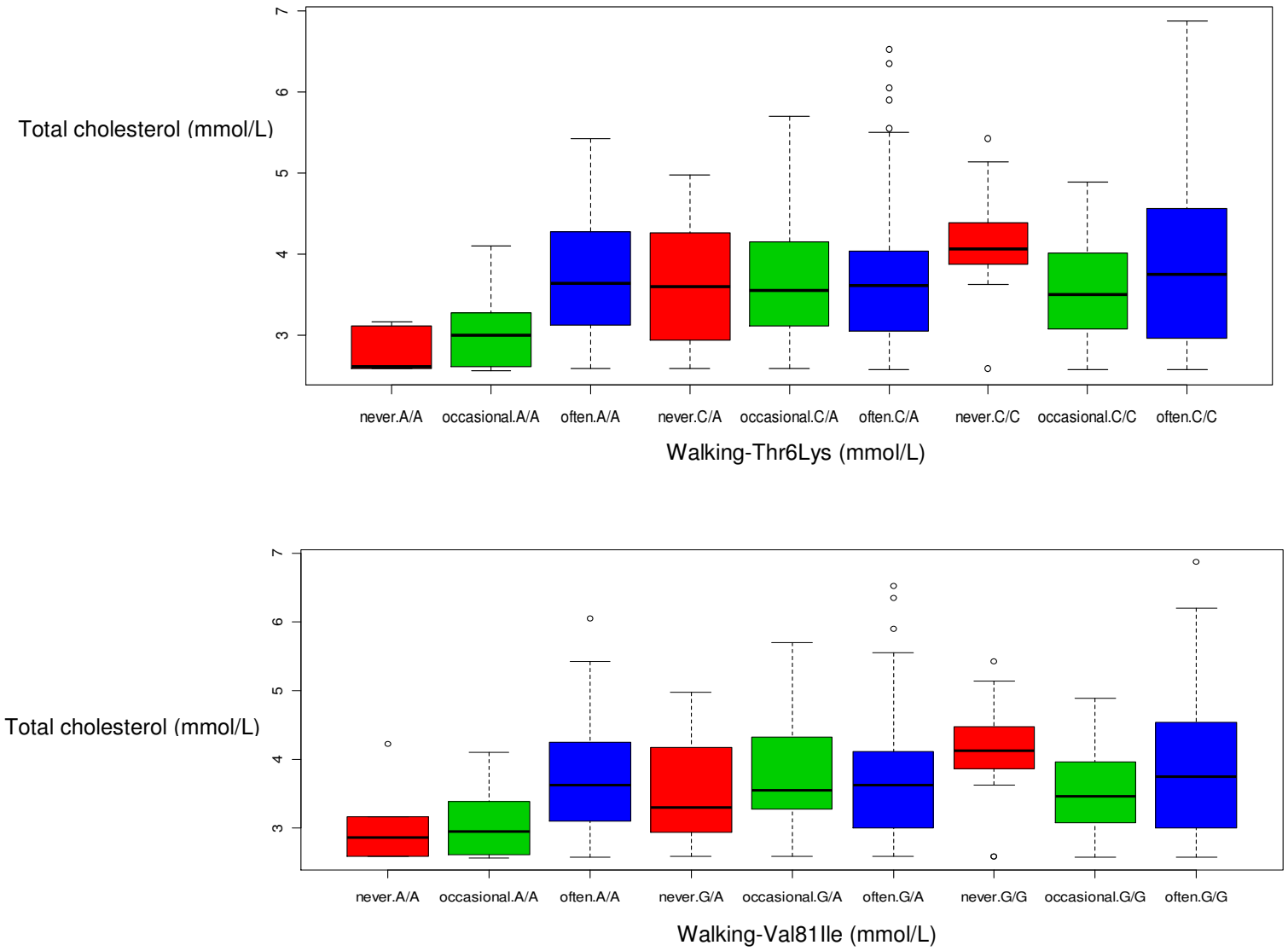


Figure 2.5. A box plot of total cholesterol against the frequency of walking (categorized as never, occasional and often). The plot shows an interaction between the two variables in learners with different *MC3R* Val81Ile and Thr6Lys genotypes.

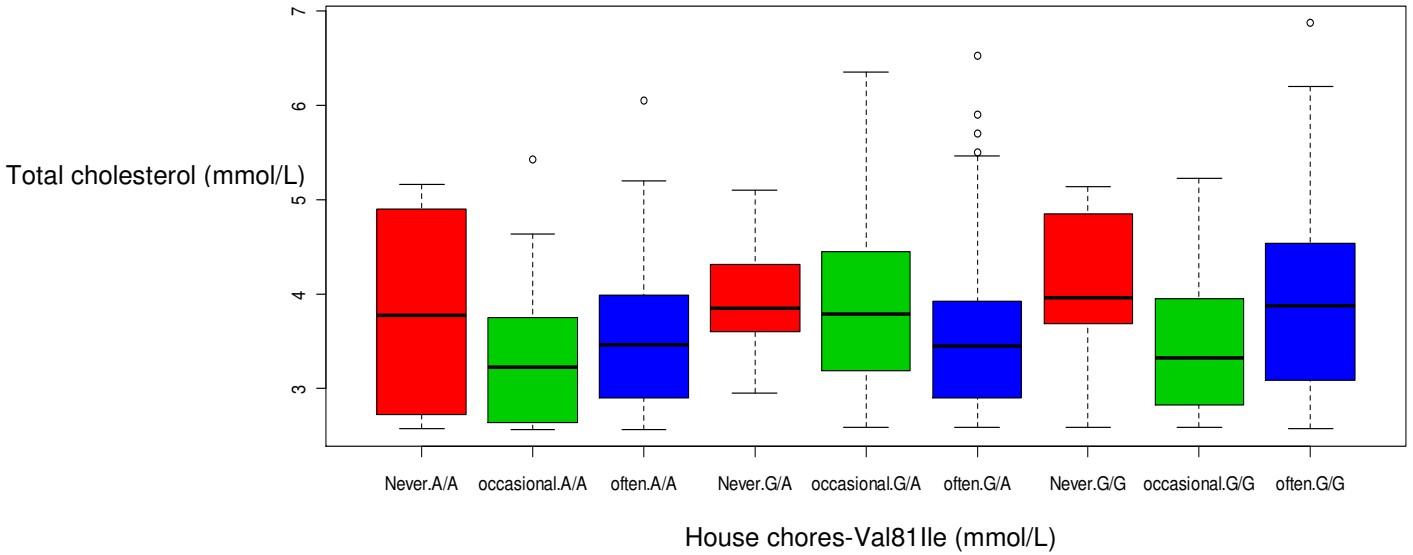


Figure 2.6. A box plot of total cholesterol against the frequency of doing house chores (categorized as never, occasional and often). The shows an interaction between the two variables in learners with different *MC3R* Val81Ile genotypes genotypes.

2.4. DISCUSSION

In this study, *MC4R* and *MC3R* were analysed for sequence variants that may contribute to polygenic obesity in two South African racial groups, namely, Mixed Ancestry and Black Africans. Sequence analysis of *MC3R* in our study groups detected two common polymorphisms that showed variability in allele and corresponding genotype frequencies between the Mixed Ancestry and Black African learners.

The minor *A* allele frequency of both polymorphisms were higher in Black African learners compared to Mixed Ancestry individuals, independent of their obesity status. Similarly, differences in the presence of the homozygous state of both Thr6Lys and Val81Ile polymorphisms have been observed in different populations elsewhere. In comparison to American Caucasians (Feng et al., 2005; Santoro et al., 2007), the homozygosity of these variants in Mixed Ancestry learners was not as low. Feng and co-workers (2005) reported a prevalence of 15.8% of the *MCR3* homozygote variant allele in African-Americans compared to 24% found in Black Africans in the present study. Interestingly, the two polymorphisms were associated with lower anthropometric and metabolic variables in Mixed Ancestry learners.

The genotype and allele association was confirmed by haplotype analysis, demonstrating a negative association of Thr6Lys-Val81Ile haplotype (A-A and C-A compared to the C-G haplotype) with anthropometric variables, while the A-G (compared to the C-G haplotype) was negatively correlated with blood pressure in Mixed Ancestry learners. These results suggest a possible protection of minor-allele carriers from obesity and hypertension. The effect posed by the C-A haplotype should, however, be analysed with caution and investigated further as there was a small number (2% in obese and 8% in normal) of learners harbouring this haplotype. Although the *A* allele was more prevalent in Black African learners, neither allele nor haplotype association tests demonstrated correlation with anthropometric and metabolic obesity traits in this ethnic group. Thr6Lys and Val81Ile are found, respectively, in the NH_2-terminal extracellular part and the first transmembrane helix of MC3R protein. Mutations in either location are predicted to affect melanocortin receptor function, especially the first transmembrane region that is believed to be involved in the binding of melanocortin peptides. Co-occurrence of *MC3R* Thr6Lys and Val81Ile was associated with impaired cAMP generation in vitro (Wong et al., 2002; Tao and Segaloff, 2004) and with greater BMI, greater body fat mass, and higher plasma levels of insulin and leptin in African-American and Caucasian children (Hani et al., 2001; Wong et al., 2002; Schalin-Jantti et al., 2003; Li et al., 2004). Similar to our findings in Black African learners, Li and co-authors (2000) failed to demonstrate an association between these polymorphisms and body weight in African-Americans. Results observed in Mixed Ancestry learners are in contrast

with findings from other studies that demonstrated an association between double homozygosity of these variants and high body weight: minor allele carriers had greater body fat mass than those carrying the major alleles (Savastano et al., 2009; Feng et al., 2005). It has also been demonstrated that *MC3R*-knockout mice have a characteristic reduced lean body mass and increased fat mass and are slightly hypophagic relative to wildtype controls (Butler et al., 2000; Chen et al., 2000; Savastano et al., 2009). Despite the increased adiposity seen in the *MC3R*-knockout mice, the animals did not exhibit increased food intake or weight gain, even on a high fat chow. The authors speculated that the obese phenotype of the *MC3R*-knockout mouse may be explained by defects in fatty acid oxidation and decreases in home-cage activity (Butler et al., 2000; Chen et al., 2000). The impaired MSH-stimulated cAMP production may be due to the reduced total ligand binding capacity that is introduced by these two polymorphisms as suggested by Feng and coworkers (2005). This study also suggested that the mechanism of the lower activity of the double *MC3R* mutant may involve less receptor protein expression due to posttranslational events rather than reduced messenger RNA levels. Apart from brain areas, *MC3R* is expressed in peripheral tissues such as skeletal muscles, adipose tissue, kidney, stomach, and pancreas (Chhajlani, 1996). It is possible that the negative association of the *MC3R* polymorphisms with BMI and other anthropometric variables, total cholesterol and blood pressure observed in the present study may be due to the interaction of this defective gene with other genes in a tissue-specific manner. This may also explain the different effects obtained in different studies. Our study did not specifically measure fat mass, rather reported values of generalized anthropometric measures. We cannot rule out the possibility that the association was related to lean mass instead of fat mass. It is also theoretically possible that the observed association could have occurred by chance.

The two *MC3R* polymorphisms were also associated with variable measures of total cholesterol. Total cholesterol was decreased in learners who carried Thr6Lys and Val81Ile minor *A* alleles after adjusting for each physical activity category. In the homozygous state, the Val81Ile minor *A* allele doubled the reduction of total cholesterol (0.299 mmol/L) compared to one *A* allele which reduced cholesterol by 0.134 mmol/L. Doing house chore further reduced total cholesterol in *A* allele carriers from 0.0.162 mmol/L to 0.355 mmol/L compared to wild type homozygotes. The type of house chores performed by adolescent learners in our study was not specified to explain the reduced levels of total cholesterol observed in the presence of *MC3R* genotypes. In general, several physical activity intervention studies done on school children and adolescents have reported statistically significant positive effects on mean blood total cholesterol (Alexandrov et al., 1988; Lionis et al., 1991; Vasconcelos et al., 2008), consistent with findings reported in adults (Scheers et al., 2008; Guo et al., 2011). There is limited evidence for the direct effect of *MC3R* on lipid metabolism. However, Rutanen et al (2007) demonstrated that the *MC3R* Thr6 and Val81 alleles are associated with low lipid

oxidation, findings that were in line with those obtained from a *MC3R*-knockout mice study (Butler et al., 2000). Animal studies have also demonstrated the role of *MC3R* in energy expenditure, in which *MC3R* knockout mice exhibited diminished physical activity (Chen and Marsh, 2000; Butler et al., 2000). Our findings, therefore, provides more evidence for the role of *MC3R* on energy expenditure and lipid metabolism. Surprisingly, HDL-C which provides soluble means of transporting cholesterol was not associated with *MC3R* polymorphisms and physical activity. The concentration of HDL-C is determined by a balance between the dietary fuel mix (with fat and alcohol increasing, and carbohydrates reducing HDL), the extent of caloric excess, the accompanying degree of obesity and lack of physical activity (Weggemans et al., 2001; Durrington, 2003), the effect of polymorphisms on response to dietary cholesterol and exercise, and clearance of HDL-C from plasma (Bernard et al., 1998; Davignon et al., 1999). It has been suggested that which of these factors prevail may differ from one study to another, and thus balancing each other out so that no differences in HDL-C concentration are seen in individuals studied (Knuiman et al., 1987). Low density lipoprotein-cholesterol measurements of learners were not available to determine the effect of *MC3R* polymorphisms and physical activity.

The association of Thr6Lys with blood pressure is of interest as this further supports the role of *MC3R* in blood pressure regulation (Butler and Cone, 2002). Although both human and animal studies have supported the role of *MC3R* in the development of obesity, only animal models have demonstrated the contribution of this gene in blood pressure regulation. In a study by Ni et al (2003), mice lacking *Mc3r* had elevated levels of γ-MSH when on both low- and high-salt diet but developed hypertension when on a high-salt diet. This study suggests that the role of *MC3R* in blood pressure may only be apparent in the presence of high salt concentration. However, in the present study this association was not investigated.

The *MC4R* has also been implicated in blood pressure regulation. Both animal and human studies have demonstrated that mutant *MC4R* confers a relative protective role on the expected increase of blood pressure in obese subjects (Ni et al., 2006; Greenfield et al., 2009). Intracerebroventricular injection of α-MSH elevated blood pressure and heart rate in wild-type mice but not in obese mice lacking *Mc4r* (Ni et al., 2006). Obesity is known to increase blood pressure, but in a study by Greenfield and co-workers (2009) humans with *MC4R*-deficiency had lower blood pressure than the control group. Blood pressure of control subjects was similar to that of overweight and obese subject with *MC4R* defects, suggesting that *MC4R*-deficient subjects were relatively protected from the expected relationship between overweight and raised blood pressure. In the present study, no sequence variants were identified in the *MC4R*. The absence of *MC4R* variants in our study

population is not surprising due to rarity of polymorphisms implicated in mild obesity in this gene (Carroll et al., 2005). Similar to our findings some previous studies have also failed to detect sequence variants in *MC4R* (Ohshiro et al., 1999; Jacobson et al., 2002; Tao et al., 2004; Feng et al., 2005). According to Jacobson and co-workers, *MC4R* mutations are likely to occur in population groups exhibiting severe or early-onset obesity, and therefore, are not a common cause of obesity in the general population (Jacobson et al., 2002).

Blood pressure, central obesity (measured by the waist circumference) and elevated lipid profile are metabolic components that are used to identify subjects with the metabolic syndrome (Reaven, 1988). Metabolic syndrome is a multiplex risk factor for cardiovascular diseases and type 2 diabetes. Individuals with lower waist circumference and blood pressure are known to be at a lower risk of developing cardiovascular diseases (Seidell et al., 2001). Although the *MC3R* polymorphism showed a negative effect on blood pressure, total cholesterol and weight gain (as measured by BMI and waist circumference) among Mixed Ancestry learners, the prevalence of metabolic syndrome between Black African and Mixed Ancestry learners was not significantly different in the cohort from which the sample for this study was obtained (Matsha et al., 2009). It is, therefore, unlikely that this association translates to the occurrence of metabolic syndrome in these two ethnic groups. Be it the case maybe, these findings are important as they suggest that carriers of the Thr6Lys and Val81I minor allele are at a lower risk of developing metabolic syndrome and type 2 diabetes. It is also important to note the significant association was detected in a South African ethnic group (Mixed Ancestry) that is understudied and with a unique genetic make-up. Mixed Ancestry are predominantly of Khoi and San (African) origin, with genetic contributions from European settlers (predominantly Dutch, German and French) and Asian (Indonesian and Madagascan) who migrated to the Western Cape Province of South Africa in the late 1600s (Nurse et al., 1985).

Even when analysed individually, there was no significant variability in the prevalence of individual components of metabolic syndrome in these two ethnic groups, except the FBG. FBG was significantly higher in Mixed Ancestry learners, and this may be related to the prevalence of type 2 diabetes in this ethnic group. One of the early studies conducted on a sample of five ethnic groups (Cape Malay, Caucasians, Bantu-speaking Africans, and Mixed Ancestry) living in Cape Town, and (East) Indians in Durban reported higher blood glucose levels and diabetes among Indians and Mixed Ancestry individuals, and least among Caucasians and Bantu-speaking, each of the latter having a diabetes prevalence of approximately 3·5% over the age 15 years (Jackson, 1972). It is noted that the Mixed Ancestry ethnic group have more diabetes than any of the constituent groups from which they originated (Levitt et al., 1999). Several risk factors for the development of diabetes have been identified, and the most consistent significant independent risk factor is the blood glucose

concentration, both fasting and 2-hour post load plasma glucose; and less consistent BMI (Knowler et al., 1990; 1993). Longitudinal studies are required to determine the long-time effects of the higher fasting blood glucose level in the presence of obesity in Mixed Ancestry learners considering that this ethnic group has been previously reported to have a higher prevalence of type 2 diabetes.

Obesity is known to increase blood pressure. However, multiple factors underlie blood pressure responses, and these include significant genetic and ethnic components, and environmental effects. In the present study, there were no ethnic differences observed in blood pressure, but higher values were found in obese-overweight learners. The increased blood pressure in adolescents in the presence of obesity may be a reason that 25.2% of South African adults were reported to be hypertensive in 1998 (Steyn et al., 2001). This is worrying considering that hypertension is the major risk factor for stroke (MacMahon et al., 1990). A positive association between obesity and blood pressure has been reported elsewhere. For example, in the Bogalusa Heart study overweight children were 4.5 times more likely to have elevated SBP (Freedman et al., 1999). A study conducted in rural Canadian children also reported a significant positive association between obesity and elevated blood pressure, independent of family history of hypertension and kidney disease (Salvadori et al., 2008). Because weight gain has been reported to be a key contributor to the development of increasing blood pressure, and ultimately, hypertension and a degree of metabolic risk factor clustering; further studies are required in this study population to determine the association between adiposity and blood pressure.

A limitation of our study is its cross-sectional design. Cross sectional studies have been reported to be ineffective in demonstrating the relationship between physical activity and obesity or other metabolic parameters (Davison et al., 2006). Additionally, the use of questionnaires to measure physical activity may have introduced bias. Physical activity questionnaires and diaries are more applicable in large epidemiological studies, but they provide less accurate estimates of physical activity level compared to more objective measures (such as doubly-labeled water, indirect calorimetry, or heart rate calibration equations) as the tool relies on (parental or child/adolescent) self-reported information (Bull et al., 2009). The type of house chores performed by adolescent learners was not specified in order to estimate the intensity of activity involved. Due to the mixed ancestry origin of the “Coloured” (Mixed Ancestry) ethnic group, associations identified in the present study may be due to population stratification, which was not adjusted for. Population stratification is one of the confounding factors that can lead to false positives, if the study group is heterogeneous as it is the case with the Mixed Ancestry ethnic group. Apart from ethnic differences in genetic background, it has been reported that puberty and sex hormones affect lipid profile, decreasing HDL-C, low density lipoprotein-cholesterol (LDL-C), and increasing TG levels (Codoñer-Franch et al., 2010). Serum lipid levels are also

influenced by sex hormones in children and adolescents, with lower HDL-C and LDL-C levels associated with increased testosterone in boys and increased estradiol in girls (Morrison et al., 2003). It has also been reported that growth hormone therapy causes a decline in LDL-C and HDL-C levels (Hilczer et al., 2008). These factors were also not adjusted for in the present study, and may have influenced the association obtained.

In summary, there was a significant difference in frequency distribution of the *MC3R* Thr6Lys and Val81Ile between Black Africans and Mixed Ancestry ethnic groups. The two polymorphisms were associated with reduced anthropometric, blood pressure, and total cholesterol in Mixed Ancestry learners. The two *MC3R* polymorphisms have been extensively studied elsewhere and shown to be associated with different metabolic traits such as increased body fat, insulin resistance, high glucose oxidation and low lipid oxidation in different population groups. Further analysis is required to confirm the validity of the association observed in the present study, adjusting for population stratification, puberty and blood sex hormone levels.

CHAPTER 3

LEP, *LEPR*, *GHRL*, AND *CART*

3.1. BACKGROUND

Leptin (LEP) and its receptor (LEPR), and the cocaine- and amphetamine-regulated transcript (CART) are among the peptides of the leptin-melanocortin pathway that are involved in energy homeostasis. The actions of LEP and ghrelin (GHRL) are reciprocal, resulting in opposite effects of energy regulation. Genetic disruption of molecules in the leptin-melanocortin system results in obesity in rodents and humans (Farooqi and O'Rahilly, 2005).

Leptin and leptin receptor

Leptin and its receptor were first discovered in obese (*ob/ob*) and diabetic (*db/db*) mice (Zhang et al., 1994; Tartaglia et al., 1995). According to these animal models, *ob/ob* mice failed to produce a circulating factor from adipose tissue but their brains could respond to it when injected and would reduce food intake, while the *db/db* mice could not respond even when the peptide was produced by adipose tissue. It was later discovered that the rodent models had mutations in the *ob* (leptin) and *ob-r* (leptin receptor) genes (Vaisse et al., 1996).

The LEP gene (*LEP*) exists as a single copy with three exons (Isse et al., 1995). The gene covers 20 kb of chromosome 7q31.3. It is exclusively expressed in adipose tissue (Kline et al., 1997) and placenta (Gong et al., 1996) under hormonal and metabolic control. Studies in rodents and humans have demonstrated that hyperinsulinemia increases leptin levels (Cusin et al., 1995; Saladin et al., 1995; Utriainen et al., 1996; Vidal et al., 1996) but there is no evidence that shows the role of insulin in regulation of leptin secretion (Slieker et al., 1996). In addition, *LEP* expression is regulated by cortisol, cAMP, and thiazolidinediones (De Vos et al., 1995; Slieker et al., 1996). *LEP* encodes a highly conserved 167-amino acid polypeptide, with the first 21 amino acids cleaved as a signal peptide (John and Bart, 1998). The remaining 146 residues form a protein of four antiparallel α-helical and β-sheet turns, with each helix 5-6 turns long (Fig 3.1). The helices are arranged in a left-handed helical bundle, and connected to each other by long crossover links: helix A connected to B, C to D; and B to C by a short loop (Fruhbeck, 2001). The helices lie between residues 3–26, 51–67, 71–94 and 121–143, while the four β turns lie between residues 39–42, 44–47, 104–107 and 106–119.

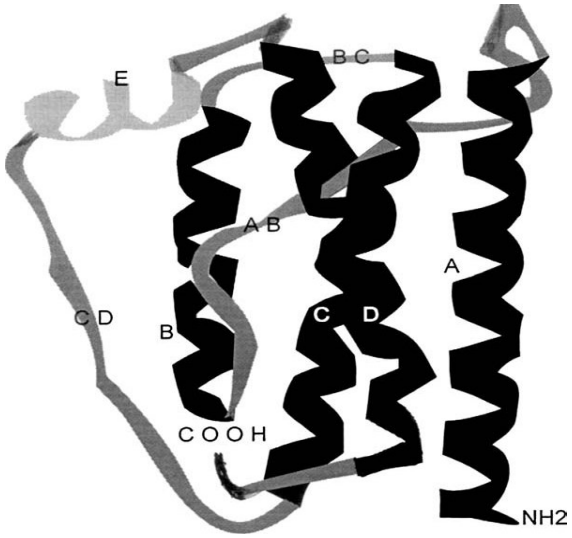


Figure 3.1. The helical loop of leptin. Letters A-E indicate helices joined by crossover links and a short loop. *Adapted from Anubhuti and Arora, 2008.*

Several splice variants of *LEPR* exists, with the longest form consisting of 18 exons. Chung et al (1996) mapped the gene at chromosome 1p31. Unlike its ligand, the short forms of LEPR are ubiquitously expressed while the long splice variant is predominantly found in the hypothalamus (Mercer et al., 1996b; Schwartz et al., 1996c). The long splice variant encodes a 1165-amino acid membrane-spanning glycoprotein that consists of a signal sequence, two immunoglobulin domains, two cytokine receptor homology (CRH) domains each containing a Trp-Ser-X-Trp-Ser motif, fibronectin type III domains, a transmembrane region, and an intracellular domain (Fig 3.2) (White and Tartaglia,1996).

LEP is secreted by the adipose tissue in response to increased fat storage (Masuzaki et al., 1995). Upon its release into the blood stream, LEP circulates and through its receptors, activates hypothalamic neurons expressing pro-opiomelanocortin (POMC) and CART located in the arcuate nucleus. It has been proposed that some of the LEPR short isoforms are involved in transportation of leptin in blood and its crossing of the blood-brain barrier (Devos et al., 1996) to sites of action. LEP's physiological role is to suppress food intake and increasing energy expenditure. Due to the wide distribution of the *LEPR* in many tissues in its alternatively spliced forms, LEP has a variety of functions including regulation of angiogenesis and formation of new blood cells (Bouloumié et al., 1998; Sierra-Honigmann et al., 1998), wound healing (Klingbeil et al., 1991; Frank et al., 2000), and the immune and inflammatory response (Loffreda et al., 1998; Maya-Monteiro et al., 2008).

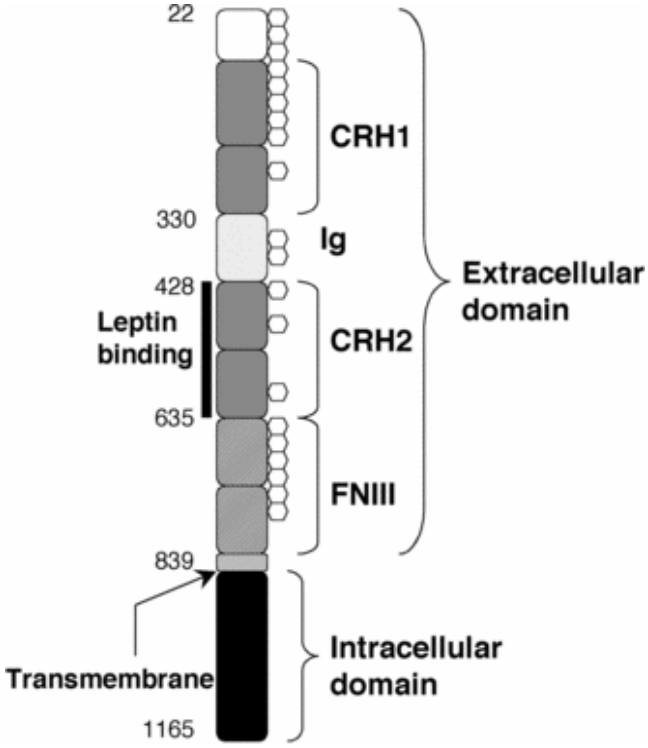


Figure 3.2. Schematic representation of the human LEPR (Ob-R). *Taken from Kamikubo et al., 2008*.

Ghrelin

The ghrelin gene (*GHRL*) was mapped to chromosome 3p25–26, and was initially thought to contain four preproghrelin-coding exons (Kanamoto et al., 2004), but in 2007 Seim and Collet reported additional upstream exons. Human ghrelin is now reported to contain multiple first exons and is extensively spliced. Prepro-des-Gln^{14}-ghrelin is a splice variant which is produced by employing an alternative splice site in exon 2, resulting in a 116 amino acid preproghrelin peptide (lacking one glutamine residue) that is likely to be processed and function in the same manner as the wild-type preproghrelin transcript (Hosoda et al., 2000; 2003). Another transcript with a deleted exon 3 exists that encodes a 91-amino acid preprohormone and lacks obestatin, and due to a change in reading frame, generates a novel 16 amino acid C-terminal sequence. GHRL and obestatin are derived from a 117-amino acid precursor (proghrelin). Protease cleavage and acyl-modification at Ser-3 of the ghrelin precursor result in the production of a 28-amino-acid-long active acyl-modified ghrelin peptide. *n*-Octanoylation at Ser-3 is essential for GHRL activity (Fig. 3.3). Ghrelin exists in four different forms due to acylation at Ser-3: nonacylated, octanoylated (C8:0), decanoylated (C10:0), and decenoylated (C10:1). These forms are either 27- or 28-amino acid long with the former lacking Arg28 at the C-terminus. GHRL was discovered in the stomach where it is predominantly expressed as an endogenous ligand for the growth hormone secretagogue receptor (Kojima et al., 1999; Date et al.,

2000; Ariyasu et al., 2001; Kojima M et al., 2001). *GHRL*-expressing cells were also detected in the duodenum, jejunum, ileum, and colon (Date et al., 2000). As in the stomach, the main molecular forms of intestinal ghrelin are *n*-octanoyl and des-acyl ghrelin. Pancreas also contains GHRL-producing cells, although it is not yet clear in which type of pancreatic cells (α, β, or ϵ cells) *GHRL* is expressed (Wierup et al., 2002; Date et al., 2002; Wierup et al., 2004; Prado et al., 2004).

Apart from regulating growth by stimulating secretion of growth hormone (Nakazato et al., 2001), GHRL has been shown to play a role in energy homeostasis as an orexigen. Ghrelin stimulates appetite through the melanocortin system, activating neuropeptide Y/agouti-related protein, and inhibiting POMC and CART neurons (Cowley et al., 2003; Korbonits et al., 2004). Several studies have supported the role of GHRL and its receptor in weight regulation, demonstrating that deficiency of one of these proteins results in resistance to diet-induced obesity (Asakawa et al., 2003; Wortley et al., 2005; Zigman et al., 2005; De Smet et al., 2006; Shearman et al., 2006; Vizcarra et al., 2007).

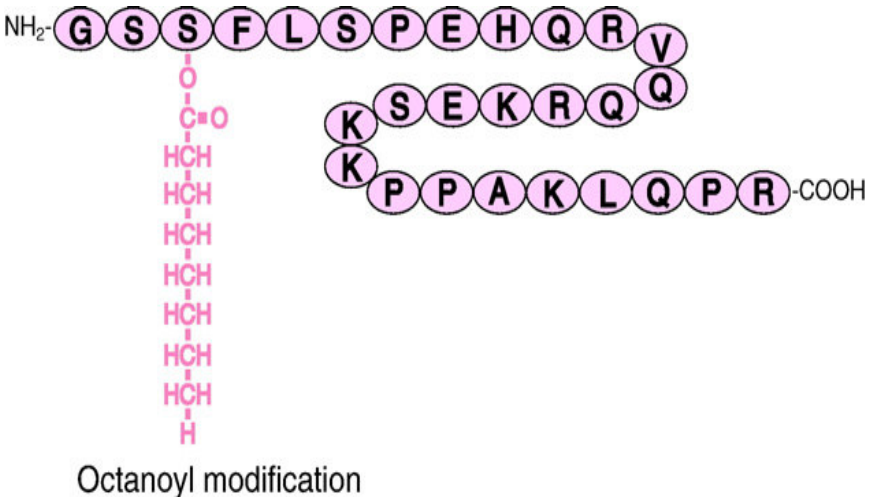


Figure 3.3. Structure of human ghrelin. Ghrelin is a 28-amino acid peptide that is acylated at the third serine residue. *Adapted from Kojima M et al., 2008*.

In addition to the mature ghrelin, a 23-amino acid peptide is derived from proghrelin. This peptide, which was named obestatin, is conserved in 11 mammalian species and is flanked by potential convertase cleavage sites (Zhang et al., 2005). The amidated obestatin was initially demonstrated in mice models to suppress food intake. However, a number of studies failed to reproduce obestatin's anorexigenic effect upon acute administration, and the reduction of body weight gain after chronic treatment (Gourcerol et al., 2007). According to Gourcerol and co-workers, this discrepancy between

the initial and subsequent studies was unlikely to be due to different experimental protocols, species or origin of the peptide used. For example, the function of obestatin was tested under conditions in which anorexigenic control substances such as cholecystokinin-8 (CCK-8), melanocortin-receptor agonist, MT-II, exendin, sibutramine or dexfenfluramine could produce the expected satiety effect or body weight reduction, or test control orexigenic peptide, such as ghrelin, increased food intake and body weight gain (Gourcerol et al., 2006; Holst et al., 2007; Nogueiras et al., 2007). Furthermore, it is recognised that peptides that regulate meal initiation, meal-ending satiation, and inter-meal satiety modulate each other's activities. Interaction of these peptides results in a more effective satiety response than when acting alone, or one peptide can counteract the effect of the other. For example, studies have reported synergistic interactions between CCK and LEP at the level of capsaicin-sensitive vagal afferents, which resulted in enhanced satiety response (Barrachina et al., 1997; Wang et al., 2000; Peters et al., 2006). Studies, however, have failed to demonstrate the interaction of ghrelin with obestatin as Zhang and co-workers (2005) initially reported. According to Zhang et al (2005), injections of obestatin prevented the body weight gain induced by peripheral injection of ghrelin in lean mice over a 1-week period. Subsequent reports using central or peripheral injections of obestatin-ghrelin, however, failed to confirm such an interaction (Seoane et al., 2006; Nogueiras et al., 2007). From these studies, it is clear that the role of obestatin in conveying a satiety signal and inhibiting upper gastrointestinal tract motility under basal- and ghrelin-stimulated conditions remains unconfirmed, instead a new central role has been proposed in regulation drinking behavior and sleep (Samson et al., 2007; Szentirmai and Krueger, 2006).

Cocaine- and amphetamine-regulated transcript

CART was first identified in rodents (rats) as a transcript regulated by cocaine and amphetamine (Douglass et al., 1996). Since the discovery of the rat transcript, CART genes (*CARTPT*) for human, mouse, bovine, and goldfish have been reported. The human *CART* covers approximately 2.0 kilobases of chromosome 5q13-14 and consists of three exons (Douglass et al., 1996). *CART* is expressed in higher levels in the hypothalamus, frontal cortex, and midbrain. In addition to leptin, CART mRNA is regulated by lipopolysaccharides (Sergeyev et al., 2001; Volkoff et al., 2004), corticosterone (Hunter et al., 2005), and environmental toxins such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (anorexia inducer) (Fetissov et al., 2004). Unlike the rodent *Cart*, human *CART* is transcribed into proCART resulting in a 116-amino acid protein (Douglass et al., 1996). In humans the polypeptide is post-translationally processed at Lys 40-Arg 41 in a tissue-specific manner into an N-terminal peptide of 39 amino acids (CART1-39) and a C-terminal 48 residues (CART42-89) (Kuhar and Yoho, 1999). A sequence variant that affects post-translational processing has been reported to have a possible role in obesity (Dominguez et al., 2004). CART as a protein consists mainly of turns and

loops spanned by a few stretches of antiparallel β-sheets (Fig 3. 4). The C-terminal part contains three disulfides bridges and is the biologically active part of the molecule (Ludvigsen et al., 2001).

CART is a satiety factor that is closely associated with LEP and neuropeptide Y as demonstrated in animal studies (Kristensen et al., 1998). Animals deprived of food exhibited a significant decrease in the expression level of Cart messenger RNA in the arcuate nucleus. In obese animal models with disrupted Lep signaling, Cart mRNA was not detected. When recombinant Cart peptide was injected into animals, normal and starvation-induced feeding was inhibited, as well as feeding response induced by neuropeptide Y.

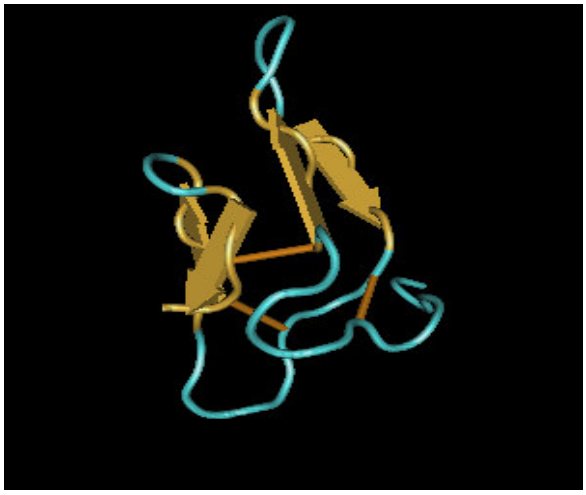


Figure 3.4. Schematic representation of the human CART protein. Three turns could be identified: type I turn (residues 56-59), type II' turn (residues 65-68), and type II turn (residues 76-79). Short antiparallel β-sheet is the most prominent secondary structural element, residues 62-64 and 69-71. *Adapted from Wang Y et al., 2007*

Association with obesity and metabolic traits

Polymorphisms in *LEP* and *LEPR* are known to confer susceptibility to polygenic obesity in both adults and children as demonstrated in several studies (Tables 3.1 and 3.2). However, replication of their association with obesity across different racial groups is still challenging. Three *LEPR* polymorphisms (Lys109Arg, Arg223Gln, Lys656Asn) have been extensively studied, but have not been replicated in their association with obesity and its related traits. Those studies showing a positive association reflect the distribution of *LEPR* in different tissues emphasizing the role of LEP in regulation of energy and the cardiovascular system. In *LEP* the most studied polymorphisms are located in the promoter region, and similar to *LEPR*, the association reported is related to energy homeostasis and the cardiovascular system. Polymorphisms in *GHRL* not only predispose to polygenic obesity (Ando et al., 2007; Zwirska-Korczala et al., 2007), but have been associated with other components of metabolic syndrome (higher fasting glucose, lower high density lipoprotein, and higher triglyceride levels) and type 2

diabetes in both adults and children (Table 3.3). Only two *GHRL* polymorphisms have been extensively studied, and are located in the protein coding region. Unlike *LEP*, *LEPR*, and *GHRL*, few studies have investigated the role of *CART* in polygenic obesity. Only 6 polymorphisms have been studied in association with obesity and its related metabolic parameters (Table 3.4).

It is evident from these association studies that polygenic obesity is due to a composite of polymorphisms in different genes, each polymorphism having variable physiological effects in various population groups depending on its interaction with background sequence variants. Variable physiological effects of polymorphisms may be a reflection of varying genotype and allele frequencies between populations from different ethnicities or geographical regions (Tiwari et al. 2008). It is therefore important to account for population stratification when association studies are conducted to eliminate its possible effect. Over- or -underestimation and a lack of association may also result from heterogeneity of the study group (Lewis 2002; Andersson et al. 2009). However, there are also confounding factors that may affect the outcome of association studies resulting in nonreplicable findings even among study groups of the same racial populations. One of the major contributing factors is the study design, for example cross-sectional and longitudinal studies may have different outcomes (Rey-Lopez et al., 2008). A good example is the study conducted by Davison et al. (2006), which did not observe any correlation between TV viewing and obesity when done cross-sectionally, but showed a positive association when it was done longitudinally. Other aspects of study design include sample size, power estimates, genome wide association vs. candidate/pathway approach, Hardy Weinberg Equilibrium, and genotyping success and errors (Andersson et al. 2009).

Extensive investigation has been conducted on the contribution of environmental factors on obesity among South African ethnic population groups. However, there is limited data on genes and corresponding polymorphisms that predispose to obesity in both children and adults. The present chapter therefore, was aimed at analysing *LEP*, *LEPR*, *GHRL*, and *CART* for identifying polymorphisms and their possible association with obesity-related traits in two South African ethnic groups.

Table 3.1. Association studies for leptin gene (*LEP*) polymorphisms and metabolic parameters in different population groups, worldwide.

Polymorphism	Population group	Phenotype	Association	Reference
Gln25Gln	Japanese (n= 64)	Obesity	No	Shigemoto et al., 1997
-2549C>A, -1817C>T	Caucasians (n=117)	Leptin levels	Yes	Mammes et al., 1998
-2548G/A	Caucasian (n=423)	Obesity, leptin levels	Yes	Mammes et al., 2000
TTTC repeat in the promoter region	American Samoan, adults (n= ?)	BMI	Yes	McGarvey et al., 2002
-2549C>A	French, Caucasian, girls (n=233)	Leptin level and body fat	Yes	Le Stunff et al., 2000
Gln25Gln	Japanese (n=185)	Obesity	Yes	Ohshiro et al., 2000
–2548 G>A	European Caucasians, adults (n=308)	Diet-related obesity risk	Yes	Nieters et al., 2002
19A>G	Brazilian, adults (n=336)	BMI	No	Mattevi et al., 2002
Tetranucleotide repeat in the 3' UTR	Japanese, adults (n=205)	Hypertension	Yes	Shintani et al., 2002
19A>G	Italian, adults (n=205)	Obesity-related traits	No	Lucantoni et al., 2000
-2548G>A	Chinese, adults (n=128)	Obesity, Fat distribution	Yes	Zhang et al., 2003
19A>G + Lys109Arg	Caucasian and African Americans, adults (n=540)	PA, insulin sensitivity	Yes	Lakka et al., 2004
-2549C>A	Chinese, adults (n= 269)	Fasting leptin and type 2 diabetes	Yes	Ren et al., 2004
Tetranucleotide repeat in the 3' UTR	Italian, adults (n= 218)	Leptin levels	No	Porreca et al., 2005
19A>G	French, adults (n= 1195)	MetS components	No	Meirhaeghe et al., 2005
19A>G, -2548G>C	French, adults (n=65)	Weight loss, leptin levels	Yes	Poitou et al., 2005
19A>G + Lys656Asn	Caucasians	respiratory quotient	Yes	Loos et al., 2005
TTTC repeat in the promoter region	Italian, adults (n= 325)	Hypertension	No	Maestrini et al., 2006
-2548G>A	Spanish, adults (n = 909)	Obesity	No	Portoles et al., 2006

Table 3.1. *continues*

Polymorphism	Population group	Phenotype	Association	Reference
3'HVR	Brazilian, adults (n = 210)	BMI and WC, leptin levels	Yes	Hinuy et al., 2006
-2548G>A	Taiwanese aborigines (n = 448)	Morbid obesity	Yes	Wang et al., 2006
19A>G	African- and Caucasian American (n= 13 405)	BMI, body weight, leptin levels	Yes	Hart Sailors et al., 2007
-2548G>A	Chinese (n= 102)	BMI	Yes	Zhang et al., 2007
g.65875652A>G, g.65881417C>G, g.65868975G>A	European Caucasian, adults (n = 17,357)	Snacking behaviour	Yes	de Krom et al., 2007
+4950G>A, +4998A>C	Korean, adults (n= 1284)	SBP and LDL-C	Yes	Han et al., 2008
Tetranucleotide repeat in the 3' UTR	n=75	Obesity	Yes	Gardezi et al., 2008
19A>G	Japanese (n= 3,653)	Sweet preference	Yes	Mizuta et al., 2008
c.-29+711G>C	Indian (n=154)	Drug-induced weight gain	Yes	Srivastava et al., 2008
–2548G>A	Finnish, adults (n=78)	Overweight and obesity	Yes	Iciek et al., 2008
–2548G>A	Finnish, adults (n= 1284)	Obesity	Yes	Drews et al., 2008
19A>G +Arg223Gln	European Caucasians, adults (n= 628)	HDL-C	Yes	Souren et al., 2008
17SNPs	Caucasian American, (n= 695)	Hypertension	Yes	Ma et al., 2009
–2548 G>A	Tunisian (n=480)	SBP	Yes	Ben Ali et al., 2008
rs791601	Caucasian (n-=10260)	BMI	Yes	Chung et al. 2009
–2548 G>A	European Caucasians, adults (n=200)	Obesity	No	Gregoor et al., 2009

Table 3.1. *continues*

Polymorphism	Population group	Phenotype	Association	Reference
–2548 G>A	Brazilian, adults (n= 140)	Blood pressure, high insulin levels	Yes	Genelhu et al., 2009
–2548 G>A	Austronesian (n= 745)	Weight, BMI	No	Furusawa et al., 2009
Telomere	Indian (n= 93)	Hypertension	Yes	Das et al., 2009
–2548 G>A	European Caucasian (n= 103)	Hunger and satiety	No	den Hoed et al., 2009
-2548G>A	Not available	TC/HDL-C ratio	Yes	Gregoor et al., 2010
-2548G>A	Finnish, adults (n= 48)	Body fat, BMI, PA	No	Huuskonen et al., 2010
19A>G	Czech Caucasian (n= 140)	BMI, percentage body fat, skinfold thickness, and eating pattern	Yes	Bienertova-Vasku et al., 2010
–2548 G>A	Romanian (n=202)	Leptin levels	Yes	Constantin et al., 2010

Abbreviations: BMI, body mass index; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; MetS, metabolic syndrome; PA, physical activity; SBP, systolic blood pressure; TC/HDL-C ratio, total cholesterol/ high density lipoprotein cholesterol ratio

Table 3.2. Association studies for leptin receptor gene (*LEPR*) polymorphisms and metabolic parameters in different population groups, worldwide.

Polymorphism	Population group	Phenotype	Association	Reference
Lys109Arg	Caucasian(n= 361)	BMI	No	Echwald et al., 1997
Lys109Arg	Asian (n= 115)	BMI	No	Matsuoka et al., 1997
Lys109Arg	Pima Indians (n= 20)	percentage body fat	No	Thompson et al., 1997
Arg223Gln	British Caucasians (n= 322)	BMI	No	Gotoda et al., 1997
Arg223Gln	Pima Indians (n= 20)	percentage body fat	No	Thompson et al., 1997
Arg223Gln	Caucasian(n= 361)	BMI	No	Echwald et al., 1997
Arg223Gln	Asian (n= 115)	BMI	No	Matsuoka et al., 1997
Lys656Asn	British Caucasians (n= 322)	BMI	Yes	Gotoda et al., 1997
Lys656Asn	Caucasian(n= 361)	BMI	No	Echwald et al., 1997
Lys656Asn	Asian (n= 115)	BMI	No	Matsuoka et al., 1997
Lys109Arg	British Caucasians (n= 322)	BMI	No	Gotoda et al., 1997
Arg223Gln	Asian(n= 553)	Obesity	No	Endo et al. 2000
Lys109Arg	Swedish, adults(n= 284)	BMI	Yes	Rosmond et al., 2000
Arg223Gln	Swedish, adults(n= 284)	Blood pressure	Yes	Rosmond et al., 2000
Lys109Arg	Greek, adults (n= 118)	BMI, percentage fat mass	No	Yiannakouris et al., 2001
Lys109Arg	Caucasians, adults (n= 358)	Insulin, glucose tolerance	Yes	Wauters et al., 2001
Arg223Gln	Greek, adults (n= 118)	BMI, percentage fat mass	Yes	Yiannakouris et al., 2001
Lys656Asn	Greek, adults (n= 118)	BMI, percentage fat mass	No	Yiannakouris et al., 2001
Lys656Asn	Caucasians, adults (n= 358)	Insulin, glucose tolerance	Yes	Wauters et al., 2001
Arg223Gln	Brazilian (n= 335)	BMI	Yes	Mattevi et al., 2002

Table 3.2. *continues*

Polymorphism	Population group	Phenotype	Association	Reference
Lys656Asn	Caucasians	RQ	Yes	Loos et al., 2005
Lys656Asn	French, adults (n= 13000)	Insulin resistance	No	Phillips et al., 2010
Lys656Asn + 19A>G	Caucasians	RMR	Yes	Loos et al., 2005
Arg223Gln	Spanish, adults (n = 909)	Obesity	Yes	Portoles et al., 2006
Lys109Arg	Korean (n= 1463)	BMI	Yes	Park et al., 2006
Lys109Arg	Finnish (n= 487)	PA, blood pressure	Yes	Kilpeläinen et al., 2008
Arg223Gln+ 19G>A	European Caucasians, adults (n= 628)	Birth weight, HDL-C	Yes	Souren et al., 2008
Arg223Gln	Caucasians, adults (n= 7596)	BMI change	Yes	Gallicchio et al., 2009
Arg223Gln	Tunisian (n= 692)	Leptin level, BMI	Yes	Ben et al., 2009
Arg223Gln	Caucasians, adult (n= 372)	Chronic heart failure	Yes	Bienertová-Vasků et al., 2009
Arg223Gln	European Caucasians, adults (n= 200)	Obesity	Yes	Gregoor et al., 2009
Lys109Arg	167 African and 184 Caucasian Americans	Fat	Yes	Iuliano et al., 2009
Lys109Arg	Finnish, adults (n= 526)	BMI, TC	Yes	Saukko et al., 2010
Lys109Arg	Austronesian (n= 745)	BMI	No	Furusawa et al., 2010
Lys109Arg	European Caucasians, adults (n= 2379)	Leptin levels	Yes	Sun et al., 2010
Arg223Gln	Finnish, adults (n= 526)	SBP	Yes	Saukko et al., 2010
Arg223Gln	European Caucasians, adults (n= 2379)	Leptin levels	Yes	Sun et al., 2010
Arg223Gln	Japanese, adults (n= 4,193)	*LEPR*+*ADRB2*, obesity	Yes	Pereira et al., 2011

Abbreviations: *ADRB2,* adrenergic beta-2-receptor gene; BMI, body mass index; *LEPR*, leptin receptor gene; RMR, resting metabolic rate; RQ, respiratory quotient; TC, total cholesterol
WC, waist circumference

Table 3.3. Association studies for ghrelin gene (*GHRL*) polymorphisms and metabolic parameters in different population groups, worldwide.

Polymorphism	Population group	Phenotype	Association	Reference
Arg51Gln, Leu72Met	Swedish, adults (n= 219)	BMI	Yes	Ukkola et al., 2001
Arg51Gln, Leu72Met	European Caucasian (n= 308)	Obesity	No	Hinney et al., 2002
Leu72Met	British Caucasian, children (n= 70)	BMI, insulin sensitivity	Yes	Korbonits et al., 2002
Arg51Gln	Finnish (n=1045)	Type 2 diabetes, hypertension	Yes	Poykko et al., 2003
Leu72Met	Finnish (n=780)	Type 2 diabetes	No	Ukkola and Kesäniemi, 2003
Leu72Met	Italian (n= 249)	Larger gestational weight, and lower BMI in controls	Yes	Vivenza et al., 2004
Arg51Gln, Leu72Met	Italian children and adolescents (n= 600)	Early age of obesity onset	Yes	Miraglia del Giudice et al., 2004
Leu72Met	Caucasians (n= 2413)	MetS	No	Bing et al., 2005
6 SNPs	Caucasians (n= 1347)	Type 2 diabetes, obesity	No	Larsen et al., 2005
Arg51Gln, Leu72Met	Amish (n= 856)	MetS	Yes	Steinle et al., 2005
Leu72Met	Korean, children (n= 222)	Obesity, Other metabolic parameters	No	Jo et al., 2005
Arg51Gln, Leu72Met	Caucasian, adults (n= 292)	Anorexia nervosa, bulimia nervosa	No	Monteleone et al., 2006
Leu72Met , 3056 T > C	Caucasian, adults (n= 636)	Eating disorders	Yes	Ando et al., 2006
Leu72Met	Korean, children (n= 132)	Diabetic nephropathy, TC	Yes	Lee et al., 2006
Leu72Met	Japanese (n= 2238)	BMI, WC, WHR	Yes	Kuzuya et al., 2006
g.-1062G>C	Korean (n= 1401)	HDL-C	Yes	Choi et al., 2006
Leu72Met	French (n= 114)	Anorexia nervosa	Yes	Dardennes et al., 2006
Leu72Met	Finnish (n= 1029)	Type 2 diabetes	Yes	Mager et al., 2006
-501A>C	Finnish (n= 1045)	BMI, WC	Yes	Vartiainen et al., 2006

Table 3.3. *continues*

Polymorphism	Population group	Phenotype	Association	Reference
5 SNPs	Caucasian adults (n= 1377)	Height	No	Gueorguiev et al., 2007
3056 T>C	Japanese (n= 264)	BMI, fat mass, WC, skinfold thickness, HDL-C	Yes	Ando et al., 2007
8 SNPs	European Caucasian (n= 3380)	MI, CAD	No	Baessler et al., 2007
Leu72Met	Finnish (n= 487)	PA, weight, WC	Yes	Kilpeläinen et al., 2008
-501A/C	Finnish (n= 487)	PA, HDL-C	Yes	Kilpeläinen et al., 2008
Arg51Gln	Chinese (n= 225)	HDL-C	Yes	Xuan Xie et al., 2008
Leu72Met	Chinese (n= 854)	MetS	Yes	Xu et al., 2008
g.10850C>T, g.11382T>G	European Americans (n= 1,982)	BMI	Yes	Chung et al., 2009
Leu72Met	European and African American (n= 351)	Fat accumulation in the liver	No	Iuliano et al., 2009
5 SNPs	British Caucasian (n= 610)	Type 2 diabetes	No	Garcia et al., 2009
Leu72Met	European Caucasian (n= 850)	Type 2 diabetes	Yes	Berthold et al., 2009
g.8113G>A, g.7267A>C	European Caucasian (n= 2632)	Hypertension	Yes	Berthold et al., 2010

Abbreviations: BMI, body mass index; CAD, coronary artery disease HDL-C, high density lipoprotein cholesterol; MetS, metabolic syndrome; MI, myocardial infarction; PA, physical activity; TC, total cholesterol; WC, waist circumference; WHR, waist-hip-ratio;

Table 3.4. Association studies for cocaine- and amphetamine-regulated transcript gene (*CART*) polymorphisms and metabolic parameters in different population groups, worldwide.

Polymorphism	Population group	Phenotype	Association	Reference
c.499delA, g.1475A>G	Danish Caucasian (n= 191)	Obesity	No	Challis et al., 2000
c.499delA, g.1475A>G	Italian, children and adolescents (n= 130)	Obesity	No	del Giudice et al., 2001
Leu34Phe	Italian, children and adolescents (n= 130)	Obesity	Yes	del Giudice et al., 2001
c.499delA	Chinese (n= 401)	TC	Yes	Fu et al., 2002
-156T>C	Japanese (n= 528)	BMI	Yes	Yamada et al., 2002
-1475A>G + -2745C>T	Caucasian (n= 669)	BMI, PA	Yes	Loos et al., 2004
-A1475A>G		PA	Yes	Loos et al., 2004
-3608C>T	French (n= 840)	LDL-C, LDL/HDL ratio	Yes	Vasseur et al., 2006
-3608T>C	French (n= 660)	Obesity	Yes	Guerardel et al., 2005

Abbreviations: BMI, body mass index; LDL-C/HDL-C ratio, low density lipoprotein cholesterol/high density lipoprotein cholesterol; PA, physical activity

3.2. MATERIALS AND METHODS

3.2.1. Study group

The study group for this part of the study was selected as described in Chapter 2. Briefly, learners were classified according to their weight status as obese, overweight and normal using the International Obesity Task Force criteria as reported by Cole et al (2000). Due to the limited number of samples obtained for genetic analyses, 189 (89 obese-overweight, and 100 normal) individuals between 13-17 years of age were selected for this study. The case group (89 obese-overweight learners) was made up of 37 Black Africans and 52 Mixed Ancestry learners whilst the age-, gender-, and race-matched normal weight learners consisted of 42 Black Africans and 58 Mixed Ancestry learners. Due to the limited number of participants who chose to give venous blood for DNA analysis, the sample size for this part of the study decreased from the original number (431 learners) that was obtained and genotyped in chapter 2. For this analysis the sample size was calculated as follows: if a risk allele frequency of a specific polymorphism (e.g. c.517A>G) in a general population is 4%, then we had 80% power to detect, at a 5% significant level, a risk allele frequency of 16% in cases.

3.2.2. Data collection

Anthropometric measurements (body weight and height; waist and hip circumference; waist-hip ratio and skinfold thickness measurements etc), were performed on all learners as described in Chapter 2. Body Mass Index (BMI) was calculated as weight per square meter (kg/m^2). Three readings were taken for blood pressure, waist and hip circumferences. Skinfold thickness was measured at three different body sites, namely subscapular, supra-ileac and upper arm. Blood glucose, lipid levels and blood pressure have been previously described (please refer to chapter 2).

3.2.3. Genetic analyses

The coding regions (including splice junctions) of CART, *GHRL*, *LEP*, and three exons (exons 2, 4, and 12) of *LEPR* were amplified from genomic DNA extracted from either whole blood collected in vacutainer Ethylenediamine Tetra-acetic Acid (EDTA) tubes or capillary blood collected onto Whatman FTA® Cards (Merck Laboratories, United Kingdom) as described in chapter 2.

3.2.3.1. Identification of sequence variants in leptin and leptin receptor genes (*LEP* and *LEPR*)

***Polymerase chain reaction (PCR)*:** *LEP* consists of three exons that form a transcript of 3420 bp long, but with only 504 bp that is translated into a protein sequence. Oligonucleotide primers (Table

3.5) were designed for amplifying the protein coding region that spans exon 1, 2 and part of exon 3 (including splice junctions) using Primer3Plus and Integrated DNA Technology (IDT) freely available software. The longest form of *LEPR* consists of 18 exons. Due to the large number of polymorphisms identified across the entire LEPR gene, we selected three single nucleotide polymorphisms (SNP) (Arg109Lys, Arg223Gln, and Lys656Asn) that have been clinically associated with obesity and its related metabolic traits. Arg109Lys, Arg223Gln, and Lys656Asn are respectively located in exons 2, 4, and 12. The three *LEPR* exons were amplified using oligonucleotide primers (Table 3.6) designed by the Primer3Plus and Integrated DNA Technology (IDT) freely available software (with the exception of *LEPR* exon 4 primers that were taken from Farooqi et al., 2007), and Bioline Taq DNA polymerase PCR kit. Designed primers were checked for occurrence of dimers and hairpin loops using the Integrated DNA Technology (IDT) freely available software. Furthermore, primers were submitted to the National Centre for Bioinformatics Institute (NCBI) primer Basic Alignment Search Tool (BLAST) for possible non-specific binding. Primer sequences were analysed for the presence or absence of polymorphisms that may inhibit amplification of corresponding gene regions. The PCR protocol was prepared as follows: a total volume of 50 µl reaction mixture contained 0.25 µg/µl DNA template, 0.24 µM of both forward and reverse primers, 1x buffer, 1.0 mM $MgCl_2$, 0.3 mM dNTP, 0.4 units DNA polymerase, and distilled H_2O added to a final volume. *LEP* and *LEPR* gene regions were amplified in a Perkin Elmer 2720 thermal cycler (Applied Biosystems) as follows: initial denaturation at 94℃ for 3 minutes, 35 cycles of denaturation at 94℃ for 30 seconds, annealing at 50-53℃ for 45 seconds, extension at 72℃ for 45 seconds, and final extension at 72℃ for 5 minutes. All amplicons were electrophoresed on a 2% agarose gel and visualised in a GelDoc system (BioRad).

Table 3.5. Oligonucleotide primers and their characteristics for amplifying leptin gene (*LEP*)

Primer Name	Sequence (5'-3')	GC content (%)	Tm (℃)	Ta (℃)	Product size (bp)
LEP_1F	CGGGAGCTGGCGCTAGAAAT	60.0	59.7	53	206
LEP_1R	CGGGATCCAGAGTTGTGTGGG	61.9	59.7		
LEP_2F	GATGGGTGTATTCTGAGATACCG	47.8	59.7	53	291
LEP_2R	GCCACTAGGAGCCAGTGCT	63.2	60.6		
LEP_3F	GATTCCTCCCACATGCTGA	52.6	59.6	53	496
LEP_3R	TGCAATGCTCTTCAATCCTG	45.0	59.9		

Table 3.6. Oligonucleotide primers and their characteristics for amplifying the leptin receptor gene (*LEPR*)

Primer Name	Sequence (5'-3'	GC content	Tm ℃	Ta ℃	Product size (bp)
LEPR_SNP2F	CATGCCACCAAATTCAACCT	45.5	60.8	52	346
LEPR_SNP2R	TCATAGCCATAAGACATCTATTTCA	32.0	57.1		
LEPR_4BF	GTGTTCATGAATGTTGTGAAT	33.3	57.2	50	226
LEPR_4BR	AGCTAGCAAATATTTTTGTAAGCAAT	26.9	60.8		
LEPR_SNP12F	AGGACCTGAATTTTGGAGAA	40.0	56.3	52	176
LEPR_SNP12R	ATTGTTGAGCTTTCCGAAGA	40.0	57.5		

Genotyping of case and control groups: Participating learners were genotyped for the *LEPR*-Arg223Gln and *LEP*-19G>A by cutting the amplicons with *Msp* I (*Hae* III) and *Msp*A1 I (Promega, USA), respectively (Gotoda et al., 1997). The *LEPR*-exon 4 fragment containing the Arg223Gln polymorphisms is 226-bp long. The A to G substitution created a *Hae* III recognition site, and digesting exon 4 fragment with *Hae* III produced two fragments of 125 and 101 bp long. The restriction enzyme digest was performed in a volume of 20 µl that contained 0.5 µl (10 U/µl) of *Msp* I, 2 µl of restriction enzyme 10x buffer, 1 µl of bovine serum albumin (1 µg/µl), 10 µl of amplicon, and nuclease-free water to a required total volume. The reaction mixture was incubated at 37℃ for 4 hours. Similarly, the A to G substitution in *LEP* exon 1 created a recognition site for *Msp*A1 I, producing two fragments of 114- and 92-bp long when the amplicon (206-bp long) was digested with the restriction enzyme. The restriction enzyme digest was performed in a volume of 20 µl as described above, and the reaction mixture incubated overnight at 37℃. Digested fragments were mixed with a bromophenol blue (appendix D) loading dye and GelRed™ staining solution (Inqaba Biotechnology), and separated electrophoretically in 3% agarose gels. Amplicons with each genotype were verified by sequencing. Amplicons of the remaining exons were sequenced as described in section 2.6.2.1.

3.2.3.2. Identification of sequence variants in cocaine- and amphetamine-related transcript, and ghrelin genes (*CART* and *GHRL*)

PCR: *CART* and *GHRL* exons (3 of each gene) were analysed by PCR, and subsequently underwent automated sequencing. The CART gene consists of three exons that form a transcript of 915 bp long, but with only 351 bp that is translated into a protein sequence. Oligonucleotide primers (Table 3.7) were designed for amplifying the protein coding region that spans exon 1, 2 and part of exon 3 using the Primer3Plus freely available software. The full-length preproghrelin gene contains four exons, with exon 2 spliced to generate a 117 amino acid preproghrelin peptide. Oligonucleotide primers were

designed for amplifying exon 2-4 (Table 3.8). Designed primers were checked for occurrence of hetero- and homodimers, and hairpin loops using the IDT software. Furthermore, primers were submitted to the NCBI-primer BLAST to check for non-specific binding. The two genes were amplified using GoTaq Flexi PCR kit (Promega, USA) as follows: a total volume of 50 µl reaction mixture contained 0.25 µg/µl DNA template, 0.24 µM of both forward and reverse primers, 1x buffer, 1.0 mM $MgCl_2$, 0.3 mM dNTP, 1.25 units DNA polymerase, and dH_2O added to a final volume. The two genes were amplified in a Perkin Elmer 2720 thermal cycler (Applied Biosystems) as follows: initial denaturation at 95℃ for 3 minutes, 35 cycles of denaturation at 95℃ for 30 seconds, annealing at 54℃ and 55℃ (depending on the primer set) for 45 seconds, extension at 72℃ for 45 seconds, and final extension at 72℃ for 5 minutes. All amplicons were electrophoresed on a 2% agarose gel and visualised in a GelDoc system (BioRad).

Genotyping of case and control groups: Participating individuals were genotyped for *GHRL*-Leu72Met polymorphism by digesting amplicons with *Bse*N I (*Bsr* I) restriction enzymes (Fermentas) according to the manufacturer's instructions. The C to A substitution abolished a *Bse*N I recognition site, and when the 598-bp amplicon with the wildtype allele was digested, two fragments of 501- and 97-bp long were obtained while the minor allele was uncut. The protocol for digestion of amplicons was as follows: 16 µl of nuclease-free water was mixed with 2 µl of 10x restriction enzyme buffer, 0.17 units of *Bse*N I, and 10 µl of amplicon. After mixing, the reaction mixture was incubated at 65℃ overnight. Digested fragments were mixed with a bromophenol blue (appendix) loading dye and GelRed™ staining solution (Inqaba Biotechnology), and separated electrophoretically in 3% agarose gels. Amplicons with each genotype were verified by sequencing. Amplicons of the remaining exons were sequenced as described in section 2.6.2.1.

Table 3.7. Oligonucleotide primers and their characteristics for amplifying the cocaine- and amphetamine related transcript gene (*CART*).

Primer Name	Sequence (5'-3'	GC content	Tm (℃)	Ta (℃)	Product size (bp)
CART1F	CTGTTCTCTGCGCTCTAGCC	60.0	60.4	55.0	494
CART1R	CCCTGACTCTGGGAATAGGA	55.0	59.1		
CART2F	GTCCGGGGCTCCTTATAACT	55.0	59.4	54.0	242
CART2R	ACGATTCAAGGCGGTGTACT	50.0	59.6		
CART3F	GAGACTTGCCTGTTGGGAAC	55.0	59.7	54.0	247
CART3R	AACTCCAGGGAGGAAGGTGT	55.0	60.0		

Table 3.8. Oligonucleotide primers and their characteristics for amplifying the ghrelin gene (*GHRL*).

Primer Name	Sequence (5'-3')	GC content	Tm (°C)	Ta (°C)	Product size (bp)
GHRL_2F	GAGCAAGCTCAGAGGCACAT	55.0	60.7	55	598
GHRL_2R	CTGTTCACTGCCACCTCTCC	60.0	60.9		
GHRL_3F	CAGAGCCCCCTTTGAGATG	57.9	60.7	55	247
GHRL_3R	CCACAGAGTGAACTCCCATGT	52.4	60.0		
GHRL_4F	CCACCATTACATCCCACCTC	55.0	60.0	55	380
GHRL_4R	TGGCCTTGCTACTTGTCCTT	50.0	59.9		

3.2.4. Statistical analyses

All statistical analyses were executed by Prof Lize van der Merwe, a Biostatistician at the Medical Research Council of South Africa. Data was analysed using the freely available programming language R (R Foundation for Statistical Computing, Vienna, Australia. ISBN 3-900051-07-0; URL: www.r-project.org). In order to test association between relevant clinical variables and genetic markers (genotype and allelic), and to determine linkage disequilibrium (LD) and Hardy-Weinberg equilibrium (HWE), specific packages DGC-genetics was used as described in chapter 2.

Statistical analyses of genetic data: Logistic regression was used to model obesity category. The models were used to assess the obesity-genotype and -ethnic associations in joint models for each polymorphism; as well as to select factors included in an optimal model for obesity susceptibility. Logistic regression was also used to model the ethnic group and test its association with each polymorphism, while adjusting for obesity status.

An optimal model for obesity susceptibility was selected using the following backwards selection procedure: obesity susceptibility was modeled as a function of all the polymorphisms, age, gender and ethnicity. Predictors which contributed least to the model, judging by Akaiki's Information Criterion, were removed and the model tested again. Candidates were discarded one-by-one, until the model could not be improved. Linear models were used to test association between quantitative traits and genotypes, alleles (additive) and inferred haplotypes. We inferred haplotypes from 2, 3 and 4 polymorphisms adjacent to each other inside each gene for analysis. Genotype-association (2 degrees of freedom) was tested by coding genotype as a categorical factor as described by Cordell

and Clayton (2005) and additive allelic association (1 degree of freedom) with a numerical variable, counting the number of minor alleles. Haplotype analysis was done using methods described by Schaid and co-workers (2002). All models were adjusted for ethnicity, age and gender, where possible and replicated measurements were controlled for as random effects. All effect sizes and p-values are derived from the models described here.

Multiple comparison consideration: P-values obtained from all analyses were not corrected for multiple testing because it has been suggested that correction is too conservative when several associations are tested in the same group of individuals (Nyholt, 2004), and might not be appropriate in a situation such as this, where there is prior evidence that such effects exist (Perneger, 1998). Our tests are not of independent null hypotheses as required by Benjamini, Yoav; Hochberg, Yosef (1995), as the polymorphisms are in tight LD with each other, and we explored different (genotype and additive allelic) models with individual variants, combinations of genotypes, and combination of alleles from the same polymorphisms. P-values below 5% are described as statistically significant. The effect sizes and 95% confidence intervals reported in the results and tables were calculated from each specific model.

3.3. RESULTS

Clinical characteristics of participants according to race are summarized in Table 3.9. By selection, anthropometric obesity traits were significantly higher in obese-overweight learners. There were significant interactions between obesity susceptibly and ethnicity on weight, WC and MUAC. For WC and MUAC but not weight, the difference between obese and normal was larger in Mixed Ancestry than in Black African learners.

Selected regions of *LEP*, *LEPR*, *CART*, and *GHRL* were successful amplified, and expected fragment size was obtained for each primer set (Figure 3.5-3.8). Sequence analysis of amplicons generated for selected regions detected the following polymorphisms: *LEP* (19A>G: rs2167270, Lys36Arg: rs111650508, Val94Met: rs17151919); *GHRL* (Leu72Met: rs696217); *CART* (c.160-33G>A: rs16871443, c.499delA: rs5868607, and c.517A>G: rs41271753) (Table 3.10). Different techniques such as ASREA (Figure 3.9) and sequencing (example of genotypes in Figures 3.10-3.12) were used to genotype the selected study groups. In addition to the above mentioned polymorphisms, the study groups were genotyped for the *LEPR* polymorphisms, namely, Arg109Lys, Arg223Gln, and Lys656Asn.

Table 3.9. Anthropometric characteristics and metabolic outcomes of obese-overweight and normal Black African and Mixed Ancestry South African learners.

Variables	Obese-overweight		Normal weight		P-values		
	Black Africans	**Mixed ancestry**	**Black Africans**	**Mixed ancestry**	**Obesity**	**Race**	**Interaction**
n	37	52	42	58			
Age	15.1 ± 1.5	15.3 ± 1.5	15.0 ± 1.7	14.7 ± 1.5	0.6530	0.4980	0.3890
Gender, male (%)	5 (14)	9 (17)	8 (19)	14 (24)	0.5098	0.5450	0.9904
Weight (m)	68.9 ± 8.5	72.3 ± 13.7	52.4 ± 6.7	49.2 ± 8.0	**< 0.0001**	0.1176	**0.0253**
BMI (kg)	27.6 ± 3.1	28.4 ± 4.4	20.7 ± 1.9	19.9 ± 2.3	**< 0.0001**	0.1854	0.0789
WC (cm)	82.8 ± 7.9	85.7 ± 10.3	70.1 ± 5.5	67.0 ± 5.6	**< 0.0001**	**0.0308**	**0.0062**
Hip (cm)	103.2 ± 6.6	106.4 ± 8.7	89.6 ± 5.6	88.8 ± 7.6	**< 0.0001**	0.5880	0.0920
Midupperarm (cm)	28.8 ± 2.5	29.1 ± 3.1	24.0 ± 2.1	23.0 ± 2.1	**< 0.0001**	**0.0187**	**0.0474**
FBG(mmol/L)	3.91 ± 0.65	4.14 ± 0.75	3.85 ± 0.84	3.96 ± 0.74	0.6865	0.4491	0.6264
TC (mmol/L)	3.68 ± 1.08	3.73 ± 0.81	3.55 ± 0.77	3.73 ± 0.84	0.5074	0.3106	0.6204
TG (mmol/L)*	0.78 (0.57, 1.11)	0.65 (0.57, 1.00)	0.66 (0.57, 0.95)	0.62 (0.57, 0.81)	0.5413	0.0579	0.9504
HDL-C (mmol/L)	1.01 ± 0.33	0.89 ± 0.25	1.14 ± 0.38	1.06 ± 0.35	0.0816	0.2016	0.7062
SBP (mm Hg)*	114 (106, 123)	115 (106, 120)	106 (95, 118)	108 (94, 117)	**0.0110**	0.9540	0.8314
DBP (mm Hg)*	68 (61, 76)	69 (64, 73)	64 (56, 74)	65 (59, 73)	**0.0399**	0.5182	0.6899

Summary statistics are mean±SD unless indicated otherwise. P-values are for joint model, so each is adjusted for the other.
*Triglycerides, Diastolic and Systolic blood pressure were summarised as median, (interquartile range) and were log-transformed to symmetry for tests**.** *Abbreviations*: BMI, body mass index; FBI, fasting blood glucose; DBP, diastolic blood pressure; HDL-C, high density lipoprotein-cholesterol; SBP, systolic blood pressure; TC, total cholesterol; TG, triglycerides; WC, waist circumference.

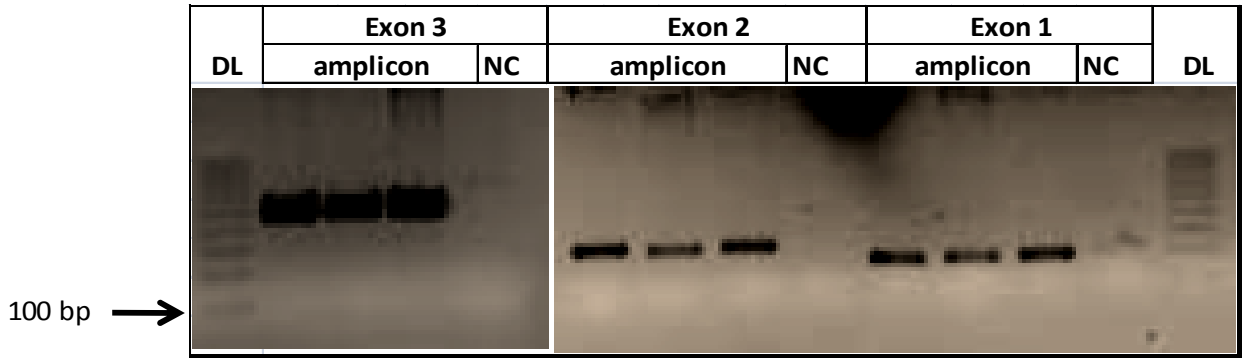


Figure 3.5. A representative 2% agarose gel of *LEP* exons 1-3. The product size for each exon was as follows: exon 1= 206 bp, exon 2= 291 bp, exon 3= 496 bp. A 100-bp DNA ladder (DL) was loaded alongside the amplicons to confirm their size. A negative control (NC) (PCR solution with water as a template) was included in every polymerase chain reaction.

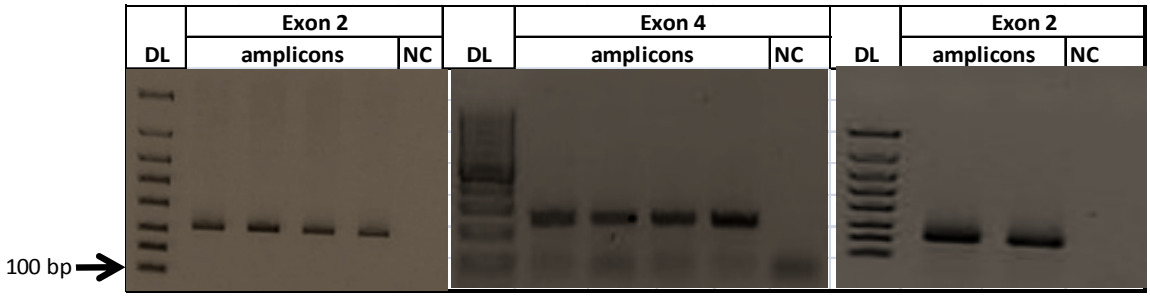


Figure 3.6. Representative 2% and 3% (first and third pictures from left) agarose gels of *LEPR* exons 2, 4, and 12. The gels show polymerase chain reaction products yielded from the sets of primers used to amplify selected regions of *LEPR* exons 2 (246 bp), 4 (226 bp), and 12 (176 bp). The lane marked DL contains a 100bp DNA ladder to confirm sizes of amplicons. A negative control (NC) (PCR solution with water as a template) was included in every polymerase chain reaction.

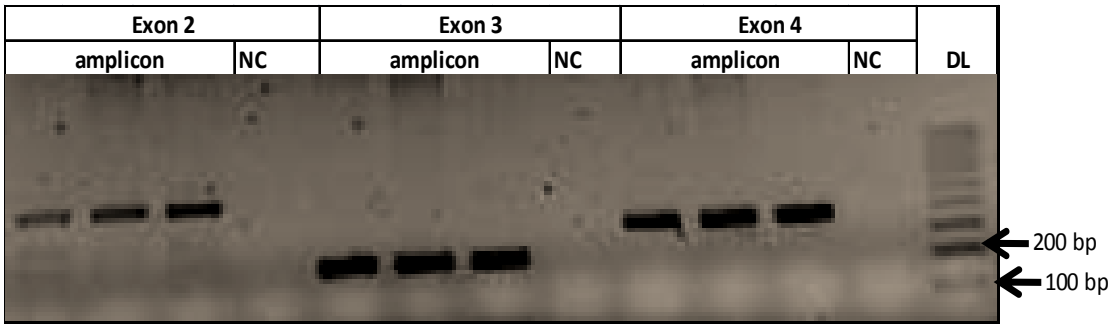


Figure 3.7. A representative 2% agarose of *GHRL* exons 2-4. The gel shows polymerase chain reaction products yielded from the sets of primers used to amplify selected regions of *GHRL* exons 2-4. The product size for each exon was as follows: exon 2= 598 bp, exon 3= 247 bp, exon 4= 380 bp.The lane marked DL contains a 100bp DNA ladder. A negative control (NC) (PCR solution with water as a template) was included in every polymerase chain reaction.

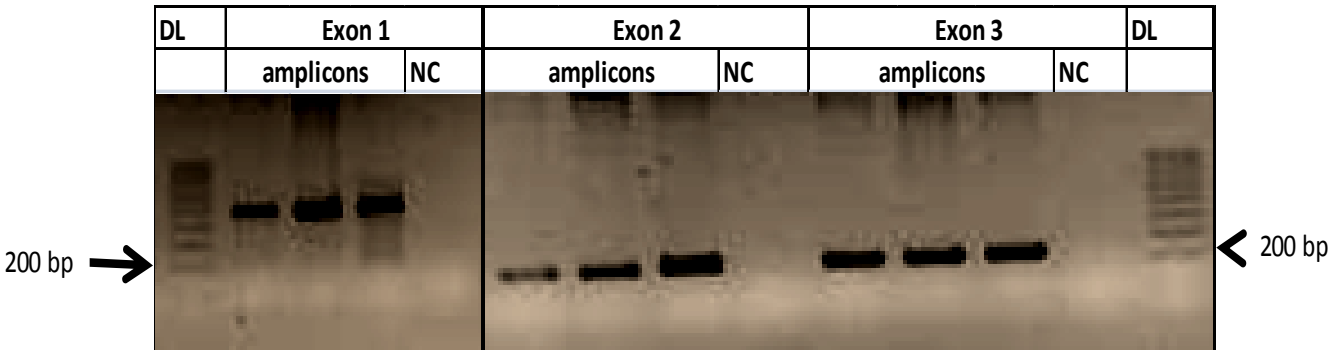


Figure 3.8. Representative 2% agarose gels of *CART* exons 1-3. The gel shows the polymerase chain reaction-based amplification of *CART* exons 1-3. A 100-bp DNA ladder (DL) was used for confirming the PCR product size. The product size for each exon was as follows: exon 1= 494 bp, exon 2= 242 bp, exon 3= 247 bp. A negative control (NC) (PCR solution with water as a template) was included in every PCR.

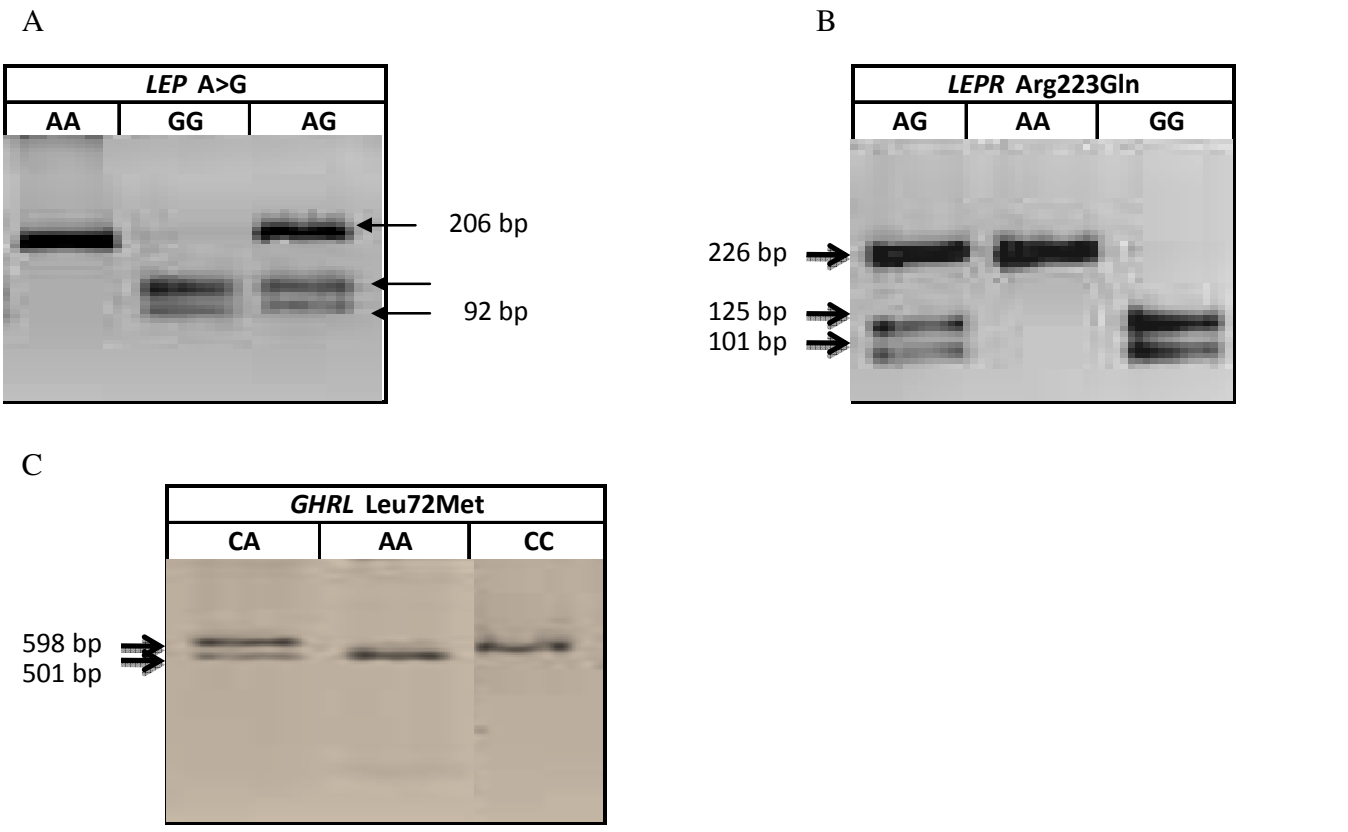


Figure 3.9. Representative 3% agarose gels showing the allele-specific restriction enzyme analysis of *LEP* 19A>G (A), *LEPR* Arg223Gln (B), and *GHRL* Leu72Met (C). *LEP*-exon 1, *LEPR*-exon 4, and *GHRL* exon 3 were amplified and subjected to restriction enzyme digest with *Bse*N I, *Msp* I and *Msp*A1 I, respectively. Three different genotypes were generated as indicated.

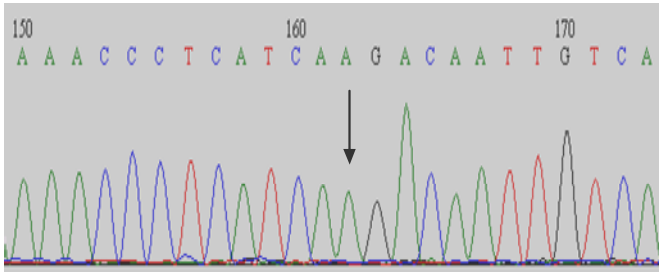
Table 3.10. Genes analysed in the present study and respective polymorphisms identified with their corresponding positions.

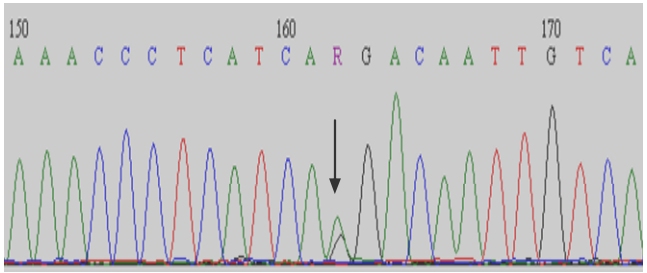
Gene	Sequence variants	SNP identification number	Gene region
LEP	c.19G>A	rs2167270	Exon 1
	Lys36Arg (c.164A>G)	rs111650508	Exon 2
	Val94Met (c.385G>A)	rs17151919	Exon 3
GHRL	Lue72Met (c. 214C>A)	rs696217	Exon 3
CART	c.160-33G>A	rs16871443	Intron 1
	c.499delA	rs5868607	Exon 2
	c.517A>G	rs41271753	3'UTR

Abbreviations: *GHRL*, Ghrelin; *CART*, cocaine- and amphetamine-regulated transcript; *LEP*, leptin.

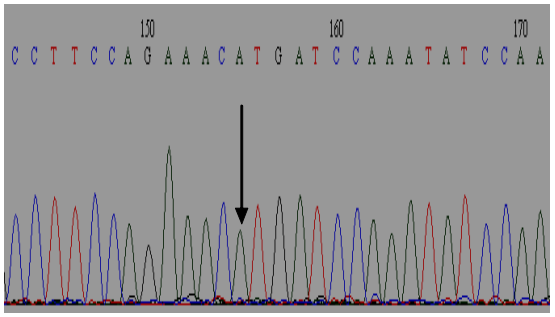
Exact HWE were performed on case (obese-overweight) and control (normal weight) individuals separately for each bi-allelic polymorphism, and were found to obey HWE in both case and control groups; except for Lys109Arg and Arg223Gln (Table 3.11[a]). LD analyses were performed, and table 3.11(b) depicts the D' values obtained. A high degree of LD was observed between the following polymorphisms: *LEP* 19A>G and Val94Met, Lys656Asn, *CART* c.517A>G; *LEP* Lys36Arg and *LEPR* Lys109Arg; *LEP* Val94Met and *CART* c.160-33G>A; *LEPR* Arg223Gln and *CART* c.160-33G>A. Very little pairwise LD was observed between *LEP* 19A>G and Lys36Arg although they are within a relatively short distance to each other. Table 3.11(c) shows the genotype and allelic distributions of the polymorphisms in overweight/obese and normal weight learners within each racial group, and between the two racial groups (adjusted for obesity status), adjusted for age and gender. The allele and/or genotype distributions differed between the racial groups, adjusted for obesity status, in Lys109Arg, Arg223Gln, c.160-33G>A, c.499delA, Leu72Met, and Val94Met polymorphisms. In the Mixed Ancestry learners, the distribution of alleles and genotypes differed between case (overweight/obese) and control (normal weight) groups in Lys109Arg, Lys656Asn, c.517A>G, and Lys36Arg polymorphisms. No differences in allele/genotype frequency were detected for any of the polymorphisms between Black African overweight/obese and normal weight learners.

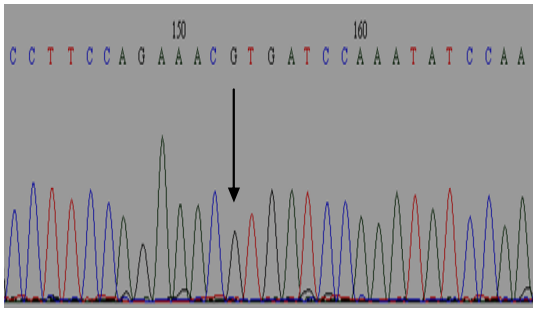
A





B





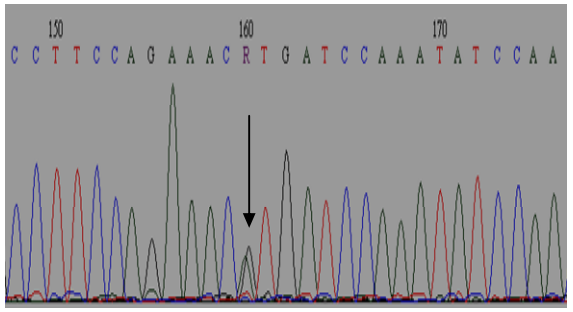
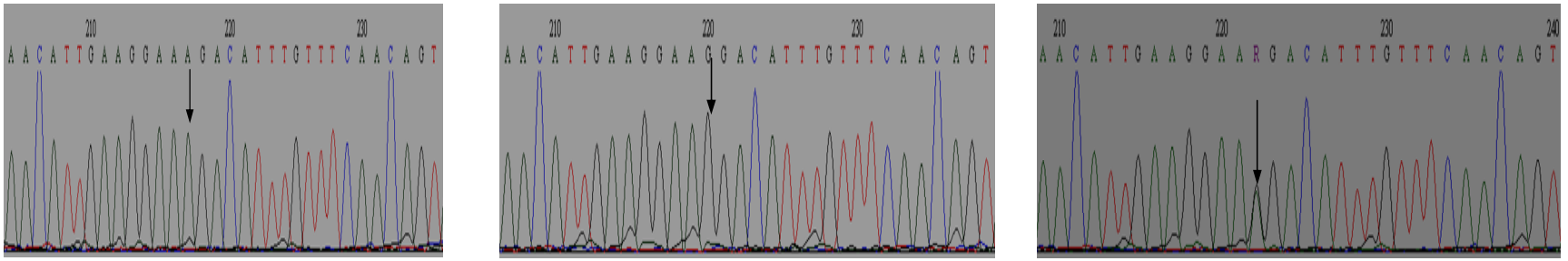


Figure 3.10. Chromatograph depicting polymorphisms identified in *LEP*. A) A>G substitution at position 164 of the cDNA that resulted in Lys36Arg amino acid change. B) G>A substitution at position 337 of the cDNA that resulted in Val94Met amino acid change. The substituted nucleotides are indicated by arrows.

A



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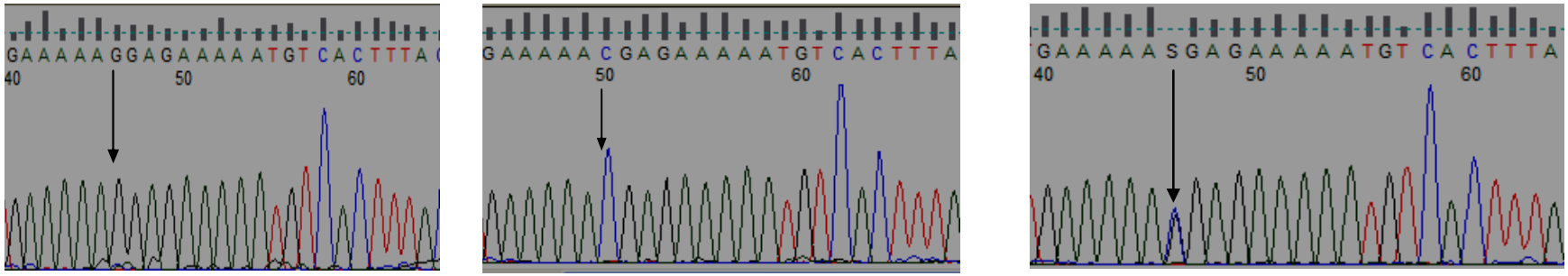

Figure 3.11. Chromatographs depicting polymorphisms identified in *LEPR*. A) A>G substitution at position 519 of the cDNA that resulted in Lys109Arg amino acid change. B) G>C substitution at position 2161 of the cDNA that resulted in Lys656Asn amino acid change. The substituted nucleotides are indicated by arrows.

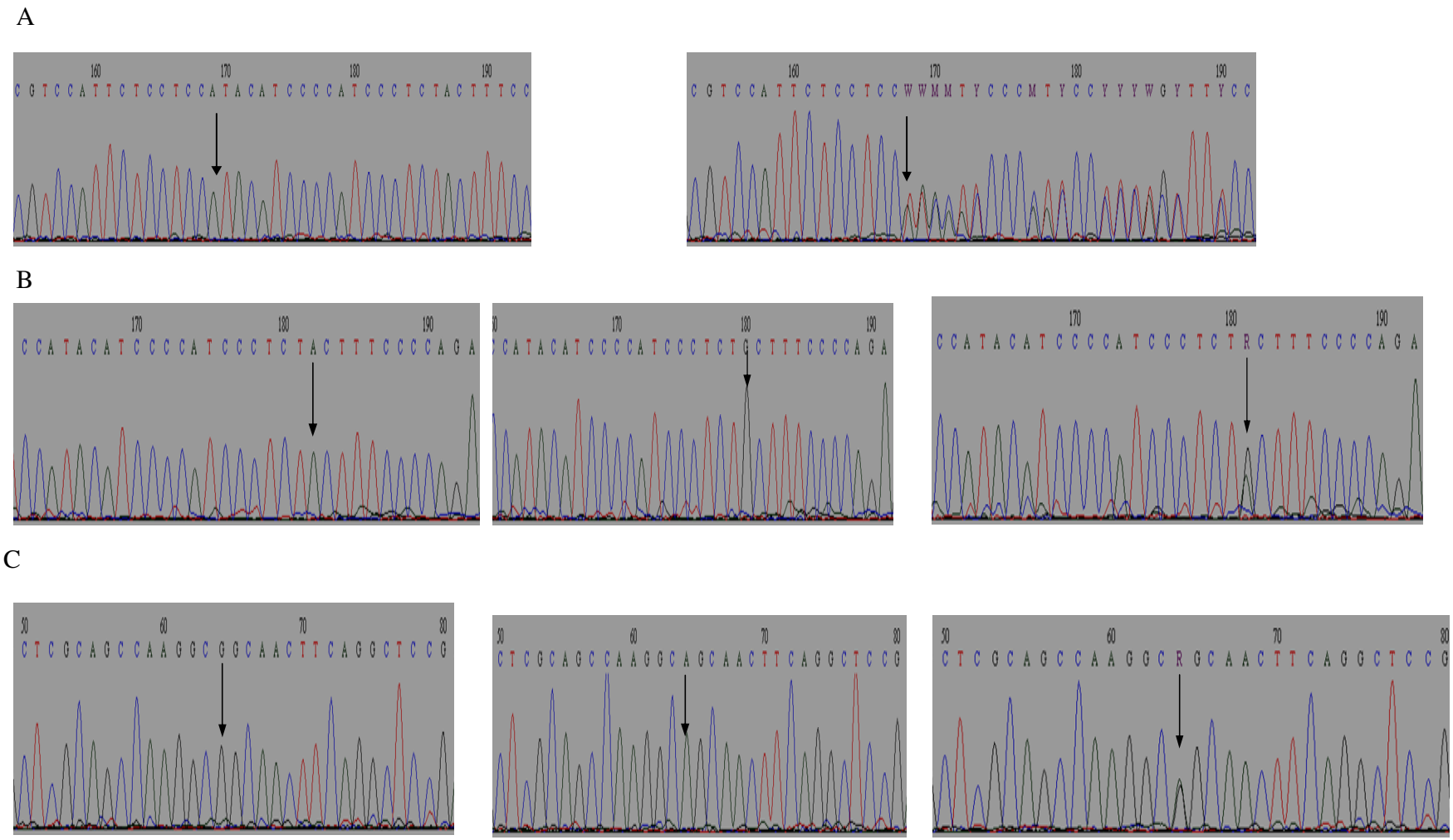

Figure 3.12. Chromatographs depicting polymorphisms identified in *CART*. A) an *A* deletion identified at position 499 of the cDNA. B) an A>G substitution of the cDNA at position 519. C) G>A substitution detected at position 160-33 of the cDNA. Arrows indicate the substituted nucleotides. All genotypes that were identified for each polymorphism in the study group are presented.

Table 3.11(a). Exact Hardy-Weinberg equilibrium p-values for the obese-overweight and normal weight learners, and heterozygosity statistics for the bi-allelic candidate loci.

Polymorphism	Black Africans		Mixed Ancestry	
	Normal	Obese/ Overweight	Normal	Obese/ Overweight
LEP				
19G>A	1.0000	0.4897	0.2834	1.0000
Lys36Arg	1.0000	1.0000	1.0000	1.0000
Val94Met	0.5382	0.3156	1.0000	1.0000
LERP				
Lys109Arg	1.0000	0.0839	0.1814	0.4062
Arg223Gln	0.0289	1.0000	0.3955	0.5275
Lys656Asn	0.5643	1.0000	1.0000	0.1814
GHRL				
Lue72Met	1.0000	1.0000	1.0000	1.0000
CART				
c.160-33G>A	0.1965	1.0000	0.3450	1.0000
c.517A>G	1.0000	1.0000	0.0858	0.2246

Abbreviations: *GHRL*, Ghrelin; *CART*, cocaine- and amphetamine-regulated transcript; *LEP*, leptin; *LEPR*, leptin receptor.

Table 3.11(b). Pairwise LD (D') values for polymorphisms occurring within the same gene for the whole dataset in the present chapter.

Polymorphism	Lys36Arg	Val94Met	Lys109Arg	Arg223Gln	Lys656Asn	Lue72Met	c.160-33G>A	c.517A>G
19G>A	0.8079740*	0.2948910	0.0615479	0.0935268	0.3711410	0.0966840	0.1207813	0.0366715
Lys36Arg	-	0.9830961	0.0356369	0.1787810	0.3517405*	0.9713226	0.9898887	0.0871564
Val94Met	-	-	0.9900789	0.1553333	0.2066776	0.6852888	0.2090884*	0.9914644
Lys109Arg	-	-	-	0.9981495*	0.9951822*	0.1174697	0.9959783*	0.1419448*
Arg223Gln	-	-	-	-	0.1438931	0.2089118	0.1741305	0.6700476*
Lys656Asn	-	-	-	-	-	0.0548696	0.3852756	0.0989520
Lue72Met	-	-	-	-	-	-	0.9857407	0.4827273
c.160-33G>A	-	-	-	-	-	-	-	0.9947736

* Nonsignificant LD test (P>0.05). *Abbreviations*: *GHRL*, Ghrelin; *CART*, cocaine- and amphetamine-regulated transcript; *LEP*, leptin; *LEPR*, leptin receptor.

Table 3.11(c). The frequency distribution of polymorphisms between obese-overweight and normal weight learners in the Black African and Mixed Ancestry groups.

	Obese-overweight		Normal		P-value		
					Separate tests on obesity susceptibility		
	Blacks	**Mixed Ancestry**	**Blacks**	**Mixed Ancestry**	**Blacks**	**Mixed Ancestry**	**Race difference**
LEPR							
Lys109Arg							
Typed	36	48	41	52			
Allelic					0.3784	0.0955	**0.0005**
A	68 (0.94)	86 (0.9)	80 (0.98)	85 (0.82)			
G	4 (0.06)	10 (0.1)	2 (0.02)	19 (0.18)			
Genotype					0.5392	**0.0457**	**0.0001**
A/A	33 (0.92)	39 (0.81)	39 (0.95)	33 (0.63)			
A/G	2 (0.06)	8 (0.17)	2 (0.05)	19 (0.37)			
G/G	1 (0.02)	1 (0.02)	0 (0)	0 (0)			
Arg223Gln							
Typed	35	50	42	56			
Allelic					0.1489	0.5305	0.1308
G	43 (0.61)	66 (0.66)	43 (0.51)	69 (0.62)			
A	27 (0.39)	34 (0.34)	41 (0.49)	43 (0.38)			
Genotype					0.1081	0.8180	**0.0265**
G/G	13 (0.37)	23 (0.46)	7 (0.17)	23 (0.41)			
G/A	17 (0.49)	20 (0.4)	29 (0.69)	23 (0.41)			
A/A	5 (0.14)	7 (0.14)	6 (0.14)	10 (0.18)			
Lys656Asn							
Typed	36	52	40	56			
Allelic					0.5822	**0.0278**	0.2106
G	58 (0.81)	85 (0.82)	67 (0.84)	102 (0.91)			
C	14 (0.19)	19 (0.18)	13 (0.16)	10 (0.09)			
Genotype					0.7405	**0.0278**	0.2736
G/G	23 (0.64)	33 (0.63)	27 (0.68)	46 (0.82)			
G/C	12 (0.33)	19 (0.37)	13 (0.32)	10 (0.18)			
C/C	1 (0.03)	(0)	0 (0)	0 (0)			

Table 3.11(c).*continues*

	Obese-overweight		Normal		P-value		
					Separate tests on obesity susceptibility		
	Blacks	**Mixed Ancestry**	**Blacks**	**Mixed Ancestry**	**Blacks**	**Mixed Ancestry**	**Race difference**
CART							
c.160-33G>A							
Typed	30	52	39	58			
Allelic					0.4986	0.8047	**0.0118**
G	48 (0.8)	96 (0.92)	66 (0.85)	106 (0.91)			
A	12 (0.2)	8 (0.08)	12 (0.15)	10 (0.09)			
Genotype					0.4762	0.5148	**0.0417**
GG	19 (0.63)	44 (0.85)	29 (0.74)	49 (0.84)			
GA	10 (0.33)	8 (0.15)	8 (0.21)	8 (0.14)			
AA	1 (0.03)	0 (0)	2 (0.05)	1 (0.02)			
c.499delA							
Typed	29	50	36	58			
Allelic					**1.0000**	**0.5613**	**0.0031**
A	58 (1)	95 (0.95)	72 (1)	112 (0.97)			
-A	0 (0)	5 (0.05)	0 (0)	4 (0.03)			
Genotype					**1.0000**	**0.5613**	**0.0031**
A/A	29 (1)	45 (0.90)	36 (1)	54 (0.93)			
-A/A	0 (0)	5 (0.10)	0 (0)	4 (0.07)			
c.517A>G							
Typed	32	50	36	58			
Allelic					**0.3112**	**0.0218**	**0.1254**
A	60 (0.94)	86 (0.86)	70 (0.97)	111 (0.96)			
G	4 (0.06)	14 (0.14)	2 (0.03)	5 (0.04)			
Genotype					**1.0000**	**0.0115**	**0.2158**
A/A	28 (88)	38 (0.76)	34 (0.94)	54 (0.93)			
A/G	4 (12)	10 (0.20)	2 (0.06)	3 (0.05)			
G/G	0 (0)	2 (0.04)	0 (0)	1 (0.02)			
GHRL							
Leu72Met							
Typed	34	50	37	55			
Allelic					**1.0000**	**0.8741**	**0.0010**
C	68 (1)	95 (0.95)	74 (1)	105 (0.95)			
A	0 (0)	5 (0.05)	0 (0)	5 (0.05)			
Genotype					**1.0000**	**0.8741**	**0.0010**
C/C	34 (1)	45 (0.90)	37 (1)	50 (0.91)			
C/A	0 (0)	5 (0.10)	0 (0)	5 (0.09)			

Table 3.11(c). *continues*

	Obese-overweight		Normal		P-value		
					Separate tests on obesity susceptibility		
	Blacks	**Mixed Ancestry**	**Blacks**	**Mixed Ancestry**	**Blacks**	**Mixed Ancestry**	**Race difference**
LEP							
19A>G							
Typed	34	51	37	58			
Allelic					0.9761	0.2114	0.5259
G	32 (0.47)	49 (0.48)	35 (0.47)	66 (0.57)			
A	36 (0.53)	53 (0.52)	39 (0.53)	50 (0.43)			
Genotype					0.8154	0.3511	0.2524
G/G	6 (0.18)	12 (0.24)	8 (0.22)	21 (0.36)			
G/A	20 (0.59)	25 (0.49)	19 (0.51)	24 (0.41)			
A/A	8 (0.24)	14 (0.27)	10 (0.27)	13 (0.22)			
Lys36Arg							
Typed	34	51	37	58			
Allelic					0.7786	**0.0457**	0.3103
A	65 (0.96)	92 (0.90)	70 (0.95)	112 (0.97)			
G	3 (0.04)	10 (0.10)	4 (0.05)	4 (0.03)			
Genotype					0.7786	**0.0457**	0.5259
A/A	31 (0.91)	41 (0.80)	33 (0.89)	54 (0.93)			
A/G	3 (0.09)	10 (0.20)	4 (0.11)	4 (0.07)			
Val94Met							
Typed	31	49	34	57			
Allelic					0.5842	0.1627	**0.0011**
G	55 (0.89)	93 (0.95)	58 (0.85)	112 (0.98)			
A	7 (0.11)	5 (0.05)	10 (0.15)	2 (0.02)			
Genotype					0.7558	0.1627	**0.0039**
G/G	25 (0.81)	44 (0.9)	25 (0.74)	55 (0.96)			
G/A	5 (0.16)	5 (0.10)	8 (0.24)	2 (0.04)			
A/A	1 (0.03)	0 (0)	1 (0.03)	0 (0)			

P-values are for tests of difference in allele and genotype distribution between the case and control groups within each racial group; adjusting for age and gender; and between the two racial groups, adjusted for case-control status, age and gender. *Abbreviations*: *GHRL*, Ghrelin; *CART*, cocaine- and amphetamine-regulated transcript; *LEP*, leptin; *LEPR*, leptin receptor.

An optimal model for obesity susceptibility was selected from the following variables: age, gender, ethnicity, and all polymorphisms. Some genotypes (Lys109Arg, Lys656Asn, c.517A>G) were dichotomized (minor homozygotes combined with the heterozygotes) due to a small number or absence of the minor homozygotes. The optimal model (p=0.0018) contained the following predictors: gender and Lys109Arg, Lys656Asn, c.517A>G polymorphisms. Of the selected predictors, the c.517A>G polymorphism was independently significantly associated with obesity susceptibility, with the *AG* and *GG* carriers six times (OR= 5.98; 95%CI= 2.02, 21.27) likely to be obese than the *AA*

homozygotes, after adjusting for the other predictors. Even when not adjusted, the c.517A>G remained significantly associated with obesity (OR= 3.56; 95%CI= 1.38, 10.36). All subsequent analyses were adjusted for age, gender and ethnicity. From individual polymorphisms-phenotype association test, only Lys109Arg and c.517A>G polymorphisms were individually significantly associated with anthropometric obesity variables (Table 3.12). At least one 109Arg allele (*AG* and *GG* genotypes compared to *AA*) was associated with an average reduction of 2.36 kg/m^2 in BMI, 5.66 cm in WC, and 1.61 cm in MUAC. At least one c.517*G* allele (A*G* and *GG* genotypes compared to *AA*) increased the MUAC by 1.88 cm on average.

Table 3.12. Summary of significant associations between *LEPR, LEP* and *CART* polymorphisms and anthropometric and metabolic obesity variables; adjusted for age, gender, and ethnicity.

Variables	SNP	Specific genotype	Reference genotype	P- value	Beta coefficient (95% CI)
BMI (kg/m^2)	Lys109Arg	AG and GG	AA	0.0148	-2.36 (-4.24, -0.47)
MUAC (cm)	Lys109Arg	AG and GG	AA	0.0238	-1.61 (-3.00, -0.22)
WC (cm)	Lys109Arg	AG and GG	AA	0.0089	-5.66 (-9.89, -1.44)
MUAC (cm)	c.517A>G	AG and GG	AA	0.0192	1.88 (0.31, 3.44)

A negative effect indicates that individuals with stated level have lower values for the trait than those with the reference level. Allelic effect is the estimated difference when adding another allele.
Abbreviations: BMI, body mass index; WC, waist circumference

No significant effects of individual *LEP* polymorphisms on anthropometric obesity and metabolic traits were detected, but the haplotype inferred from the three *LEP* polymorphisms, 19A>G, Lys36Arg and Val94Met, was significantly associated with the BMI, MUAC and hip circumference (Table 3.13). The A-A-A haplotype increased BMI by 17.4 kg/m^2, MUAC by 2.3 cm, and hip circumference by 8.0 cm, compared to the reference haplotype G-A-G. A significant interaction was observed between the three *LEP* polymorphisms and Lys109Arg (19A>G+Lys36Arg+Val94Met+Lys109Arg) for weight, BMI, MUAC and WC. Carriers of the A-A-A-A haplotype-allele combination of 19A>G, Lys36Arg, Val94Met and Lys109Arg had significantly increased weight, BMI, MUAC and WC compared to G-A-G-A carriers. Interestingly, individuals carrying the *LEP* haplotype G-A-G and the 109Arg allele had decreased anthropometric obesity phenotype indicators compared to those with the *A* allele (Table 3.13). The effect of the Arg223Gln-Lys656Asn haplotype (A-C compared to the G-G) was an increase of 2.6 cm in MUAC. Combinations of the Arg223Gln-Lys656Asn haplotype with other polymorphisms (Lys109Arg and Leu72Met) were also associated with an increase in MUAC.

Table 3.13. Summaries of significant haplotype or allelic combination effects on anthropometric obesity variables (adjusted for age, gender, and ethnicity), and their frequency in the study group.

Variables	Haplotype or allelic combination	p-value	Specific haplotype	Haplotype frequency	Reference haplotype	Beta coefficient
Weight (cm)	19A>G + Lys36Arg + Val94Met + Lys109Arg	0.0168	AAAA	0.050	GAGA	11.5
	19A>G + Lys36Arg + Val94Met + Lys109Arg		GAGG	0.058	GAGA	-6.9
BMI (kg/m^2)	19A>G + Lys36Arg + Val94Met	0.0155	AAA	0.047	GAG	17.4
	19A>G + Lys36Arg + Val94Met + Lys109Arg	0.0408	AAAA	0.051	GAGA	4.2
	19A>G + Lys36Arg + Val94Met + Lys109Arg		GAGG	0.059	GAGA	-2.8
MUAC(cm)	19A>G + Lys36Arg + Val94Met	0.0146	AAA	0.047	GAG	2.3
	19A>G + Lys36Arg + Val94Met + Lys109Arg	0.0411	AAAA	0.049	GAGA	2.5
	19A>G + Lys36Arg + Val94Met + Lys109Arg		GAGG	0.059	GAGA	-1.6
	Arg223Gln + Lys656Asn	0.0377	AC	0.052	GG	2.6
	Lys109Arg + Arg223Gln + Lys656Asn	0.0429	AAC	0.049	AGG	2.2
	Arg223Gln + Lys656Asn + Leu72Met	0.0029	ACC	0.048	GGC	1.9
WC (cm)	19A>G + Lys36Arg + Val94Met + Lys109Arg	0.0168	AAAA	0.049	GAGA	7.7
	19A>G + Lys36Arg + Val94Met + Lys109Arg		GAGG	0.059	GAGA	-7.3
Hip (cm)	19A>G + Lys36Arg + Val94Met	0.0128	AAA	0.045	GAG	

A negative effect indicates that individuals with stated haplotype have lower values for the trait than those with the reference haplotype. Only haplotypes with frequencies >0.05 are listed. *Abbreviations*: BMI, body mass index; MUAC, mid-upper-arm circumference; WC, waist circumference.

3.4. DISCUSSION

Several polymorphisms were identified with significantly different distributions between the Mixed Ancestry and Black African population groups, as well as between cases and controls (obesity susceptibility) within the Mixed Ancestry ethnic group. The genotype frequency distributions of both Arg223Gln and Lys109Arg polymorphisms were different between the two ethnic groups. For the Arg223Gln polymorphisms, Black African learners had a higher heterozygote frequency, while the Mixed Ancestry group had higher frequencies of *GG* homozygotes; hence no significant difference in allelic frequencies. When compared to other population groups, the frequency of the *G* allele in Mixed Ancestry learners was higher than that reported in Caucasians (approximately 40%) but lower than that observed in Asians (85%) (Paracchini et al., 2005). A study by Ragin and co-workers (2009) reported a *GG* homozygous frequency of 24.73% in Africans, similar to that observed in the Black African ethnic group (26%). According to a meta-analysis done by Paracchini et al (2005), generally allelic frequency of the Arg223Gln polymorphisms is variable across different ethnic groups (in Pima Indians the *G* allele frequency was 32%). When comparing the genotype distribution of the Lys109Arg polymorphisms, the *A* allele was more prevalent in the Black African ethnic group (96% vs 86%). Similarly, Paracchini et al (2005) reported a higher frequency of the *A* allele in Caucasians while it was lower in Asians. The HapMap data also reported a higher frequency of the *A* allele in Sub-Saharan Africans and African Americans.

Ethnic variation in the distribution of the Val94Met polymorphism was observed between Mixed Ancestry and Black African learners. Significant differences in the distribution of c.160-33G>A, c.499delA, Val94Met and Leu72Met between the two ethnic groups were also observed. Minor alleles of *CART* c.499delA and *GHRL* Leu72Met polymorphisms were only found in Mixed Ancestry learners but at low frequencies (up to 10%), similar to other population groups such as Finnish, Chinese, African-Americans and Caucasians (Ukkola and Kesäniemi, 2003; Bing et al., 2005 ; Xu et al., 2008; Friedlander et al., 2010). The *A* allele of the Val94Met polymorphisms was also found at a lower frequency in both ethnic groups (10% in Blacks and 3% in Mixed Ancestry). Similarly, African-Americans had a lower frequency of the *A* allele (8.7%) while it was not found in Caucasians. The delA allele was found in Mixed Ancestry learners at a lower frequency (4.2%) compared to that observed by other authors (Challis et al., 2000; Guérardel et al., 2005) in Caucasians (7.3-10% and 8.7%).

The difference in genotype and/or allelic distribution of the polymorphisms observed between the two ethnic groups may be a reflection of a genetic admixture in Mixed Ancestry. The Mixed Ancestry

population (commonly known as Cape Coloured) has the highest levels of mixed ancestry in the world, representing a complex genetic background. The first generation of this racial group originated from an admixture of Hottentot and European racial groups (Desmore, 1937). Another racial mixture has occurred over the years between Europeans, Indians, Malays, various Bantu tribes, and other African groups along with indigenous Khoi and San, resulting in a heterogeneous genetic background (The Coloureds of Southern Africa, MixedFolks.com. Retrieved on 23/10/2009).

The c.517A>G polymorphism was selected as the best predictors of obesity in an optimal model. Additionally, the c.517A>G *G* allele (*GG* and *AG* genotypes, compared to *AA*) was associated with increasing MUAC, when the regression analysis was adjusted for age, gender, and race. The importance of this finding is in line with the proposed role of the mid-upper-arm as another indicator of obesity (Chomtho et al., 2006), suggesting that the c.517A>G polymorphisms may be a predisposing factor for obesity. In their study consisting of healthy boys aged 4.4-13.9 years, Chomtho et al (2006) found a strong correlation between MUAC, triceps skinfold thickness, arm fat area and total fat mass. From their findings Mazıcıoğlu and coworkers (2010) suggested substitution of the WC and MUAC for one another as an additional evaluation tool next to BMI in detecting overweight and obese children and adolescents. There is currently no known clinical association of the c.517A>G polymorphism with obesity. c.517A is located in the 3 prime untranslated region of the *CART* gene, and the A>G substitution has no known effect on the function of CART or the expression level of its gene. It is likely that c.517A>G is in linkage disequilibrium with a functional casual polymorphism. There is currently very limited data on the role of *CART* polymorphisms (Leu34Phe, 1475A>G, -156T>C, and -3608T>C) in the development of obesity (Miraglia del Giudice et al., 2001; Yamada et al., 2002; Guérardel et al., 2005; Yanik et al., 2006; Rigoli et al., 2010).

The *LEPR* polymorphism (Lys109Arg) decreased BMI (as a measure of obesity), waist circumference and mid-upper-arm, as a single locus and in a haplotype with *LEP* variants. Interestingly, other haplotypes containing the *A* allele of the Lys109Arg polymorphism had an opposite effect, increasing obesity indices, suggesting that only the minor allele protected the carriers from obesity. Similarly, Rosmond and co-workers (2000) found Arg109 homozygotes to have lower BMI in addition to lower abdominal sagittal diameter and blood pressure. In addition, the Lys109Arg variant was found to be marginally associated with BMI in a gene dose-dependent manner (Park et al., 2006). The *C* allele of the Lys656Asn variant was associated with increased obesity, consistent with the haplotype analysis that demonstrated an association between the A-A-*C*, A-C-C, A-C (haplotype containing the *C* allele of the Lys656Asn variant) and increasing obesity indices. Our findings are similar to those reported by Masuo and co-workers (2008), which showed Caucasian male subjects carrying the Arg223

homozygous or the Asn656 allele to have higher BMI, waist circumference, and waist-to-hip ratio in addition to increased levels of plasma leptin. Furthermore, other studies found weak associations between the Lys656Asn and either BMI or free fat mass in subgroups of lean British subjects (Gotoda et al., 1997) and/or overweight Caucasian females (Chagnon et al., 1999), respectively.

Leptin polymorphisms as single loci were not associated with any variability in anthropometric and metabolic obesity-related traits. Instead haplotypes formed by these polymorphisms were associated with variation in BMI, MAUC, and hip circumference. The 19A>G-Lys36Arg-Val94Met haplotype (A-A-A compared to the G-A-G) increased BMI, MUAC and hip circumference. The Lys109Arg + Arg223Gln + Lys656Asn haplotypes (A-A-C and A-C compared to A-G-G and G-G, respectively), on the other hand, increased only the MUAC. When the Lys109Arg polymorphisms were analysed as a single locus, its minor allele was significantly associated with anthropometric obesity traits; decreasing BMI, MUAC and WC. Interestingly the A-A-C haplotype contains the Lys109Arg *A* allele, which on its own did not show any significant association with anthropometric obesity traits. The Arg223Gln-Lys656Asn haplotype of minor alleles had a greater effect on MUAC compared to the haplotype containing all three *LEPR* polymorphisms, suggesting that the A-C haplotype on its own is sufficient as an obesity risk factor compared to G-G. *LEP-LEPR* joint effects were also observed. When the Lys109 allele was combined with the *LEP* haplotypes (A-A-A-A versus G-A-G-A), the synergistic effect of the *LEP* polymorphisms was modulated, decreasing the effect on BMI from 17.4 to 4.2 kg/m^2 while the effect on MUAC was increased from 2.3 to 2.5 cm. When the Lys109Arg *G* allele interacted with the *LEP* G-A-G haplotype, (G-A-G-G versus G-A-G-A), this combination decreased the anthropometric obesity phenotype indicators (BMI, weight, MUAC, waist and hip circumferences), further strengthening evidence for the protective role of the Lys109Arg minor allele. Due to the small sample size of the present study and the heterogenous nature of the study population, the association observed should be analysed with caution and further studies conducted in a larger homogenous sample. Of note and a surprising finding is the effect of the A-A-A-A and A-A-A haplotypes, increasing weight and BMI, respectively. The small sample size of the present study could be the reason some polymorphisms as a single loci (for example, *LEP* polymorphisms) were not associated with obesity anthropometric variables. It is possible that individual polymorphisms have a small effect that may only be detected at a population level or in combination with other variants (as observed with haplotype analyses). The overestimated increase in weight and BMI could be due to population stratification as it is known that the Mixed Ancestry population has the highest levels of mixed ancestry, representing a complex genetic background. According to Andersson and co-workers (2009), if population stratification is not accounted for, as it is the case in the present study, over or underestimation of associations can occur.

Although genes involved in regulation of food intake and energy expenditure have been extensively studied, replicating the association of polymorphisms with obesity across populations has been challenging. The *LEPR* polymorphisms (Lys109rg, Arg223Gln and Lysn656Asn) have been found to be either associated with (Quinton et al., 2001; Park et al., 2006; Han et al., 2008) or not have any effect (Heo et al., 2001) on obesity or its anthropometric traits. Most of the *LEP* and *LEPR* polymorphisms investigated in the present study produce amino acid changes and may thus have functional consequences, but there is no true evidence of their functionality. Two of the *LEPR* polymorphisms, Arg223Gln and Lys656Asn, result in nonconservative changes and are therefore the most likely to have functional consequences (Thompson et al., 1997; Chung et al., 1997). However, the exact functional consequence of these polymorphisms on the *LEPR* mRNA and protein is unknown. The Lys109Arg polymorphism is a conservative change and may not have functional implications.

The 19A>G polymorphism located in the 5' UTR of the LEP gene has also been associated with lower levels of leptin concentration (Hager et al., 1998; Karvonen et al., 1998; Li et al., 1999; Hart Sailors et al., 2007), while other studies failed to detect this association (Lucantoni et al., 2000; Jiang et al., 2004; Meirhaeghe et al., 2005). The Lys36Arg polymorphism lies within the first short loop that connects helix A to B, while Val94Met is the last residue of the third helix. These two polymorphisms have not been associated with any metabolic phenotypes, but their location suggests that they may also impair LEP physiological activity. Leptin levels were not measured in the present study, it is therefore not known whether or not the *LEP* and *LEPR* polymorphisms have an effect on leptin concentration our study groups.

It is evident from several studies that *LEP* and *LEPR* polymorphisms have different effect in various population groups. Linkage disequilibrium between the genotyped polymorphism and the causal one is reported to be one of the possible reasons for between-study heterogeneity. Another reason could be the modulating effect of interacting polymorphisms in the background of the investigated variant or marker, thereby neutralising or enhancing its association with the phenotype. The joint effect of polymorphisms is suggested to be the plausible model of inheritance for several obesity-related disorders. Although obtaining supporting evidence has been challenging, a few studies in human obesity have supported this hypothesis. For example, a significant interaction between *LEP* 19A>G and *LEPR* Lysn656Asn polymorphisms for respiratory quotient was observed in a study by Loos et al (2006). Souren et al (2008) observed a joint effect of the Lys109rg and Arg223Gln polymorphisms on birth weight, in which carriers of the Lys109Arg/Gln223Gln genotypes had significantly lower birth weight compared to carriers of other genotypes. The relevance of these findings stems from the association of low birth weight with an increased risk of obesity and type 2 diabetes. Another example

of the gene-gene interaction is found in a study by Lakka and coworkers (2004), which reported an association of the A19A/Arg109Arg and A19A/Lys109Arg genotypes with exercise-induced change in fasting insulin. *LEPR* polymorphisms also interact with other genes, as noted in a study by Angeli et al (2011), which reported a joint effect of Arg223Gln and β_2-adrenergic receptor in the risk of overweight/obesity. A combined effect of different polymorphisms on BMI was also observed in a meta analysis by Willer et al (2009) in which six variants accounted for 0.4% of variance in BMI, and in conjunction with melanocortin-4-receptor and fat mass and obesity associated genes (*MC4R* and *FTO*), the association accounted for 0.84% of variability. Our findings, therefore, further supports the joint effect of *LEP* and *LEPR* on obesity and its related phenotypes, and provides a foundation for investigating interaction of polymorphisms in addition to single locus associations.

Apart from genetic factors, ethnicity also contributes to the variability in anthropometric measurements as observed in the present study. Generally, anthropometric measurements were significantly higher in obese-overweight learners. When ethnicity was considered, anthropometric obesity indices were higher in obese Mixed Ancestry learners but only WC and MUAC were significantly different between the two ethnic groups. It is possible that the difference observed is due to variable pubertal stages of learners studied. Physiologically, there are differences in fat distribution and changes in total and percentage body weight between girls and boys (Ogle et al., 1995; Taylor et al., 1997). During adolescence, on average girls tend to have increased fat and fat-free mass, while boys gain more muscle mass (Deurenberg et al., 1990; Goodman-Gruen and Barrett-Connor, 1996). As a result, female adults have approximately 22% body fat while their male counterparts have an average of 15%. Furthermore, fat in girls is usually deposited peripherally in breasts, hips, and buttocks while in boys it is centralised in the abdomen. There was a positive correlation between obesity and WC and MUAC based on ethnicity. However, the question that still remains in the South African context is whether or not other measurements such as WC and MUAC can be used as additional evaluation tools next to BMI in detecting overweight and obese children and adolescents. It has been recommended that WC and MUAC; which are the leading indirect methods of assessing fat mass, be used as an additional tool to screen children and adolescent for obesity as they show a good level of correlation with body mass (Jelliffe and Jelliffe, 1968; Cook et al., 2003; Mazıcıoğlu et al., 2010). However, systematic monitoring of WC and MUAC is not a commonly performed method in pediatric studies in many countries and internationally accepted cut–off values are also not yet established for different population groups.

CHAPTER 4

CONCLUSION AND RECOMMENDATIONS

4.1. Summary of findings

To our knowledge, few obesity genetic studies have been conducted in South African populations. In March 2011 at the South African Society for Human genetics conference held in Cape Town, Dr Zane Lombard of the National Health Laboratory services reported a significant finding on leptin risk alleles, which increased the risk of obesity in adolescents residing in Soweto (Johannesburg). To add to this important finding, the present study investigated the contribution of six genes (*LEP*, *LEPR*, *GHRL*, *CART*, *MC3R*, and *MC4R*) of the leptin-melanocortin pathway in polygenic obesity in South African learners of Black African and Mixed Ancestry residing in Cape Town northern surbubs. The following polymorphisms were identified:

Gene	Polymorphism	SNP identification number	Gene region
MC4R	-	-	-
MC3R	Thr6Lys	rs3746619	Exon 1
	Val81Ile or Val44Ile	rs3827103	
LEP	c.19G>A	rs2167270	Exon 1
	Lys36Arg	rs111650508	Exon 2
	Val94Met	rs17151919	Exon 3
GHRL	Lue72Met	rs696217	Exon 3
CART	c.160-33G>A	rs16871443	Intron 1
	c.499delA	rs5868607	Exon 2
	c.517A>G	rs41271753	3'UTR

Association analysis of the polymorphisms with obesity-related quantitative traits identified the following:

Gene	Polymorphism	Association	Clinical variable
MC4R	-	-	-
MC3R	Thr6Lys	Yes	Lower BMI, weight, MAUC, hip, TC, BP in MA
	Val81Ile	Yes	Lower BMI, weight, MAUC, hip, TC, BP in MA
MC3R + House chores	Val81Ile	Yes	TC
LEP	c.19G>A	No	
	Lys36Arg		
	Val94Met		
GHRL	Lue72Met+Gln223Arg + Lys656Asn	No	
CART	c.160-33G>A	No	
	c.499delA	No	
	c.517A>G	Yes	Higher MUAC, obesity risk
LEPR	Lys109Arg	Yes	Lower BMI, MUAC, WC
	Gln223Arg	No	
	Lys656Asn	No	
LEP	Haplotype:c.19G>A+Lys36Arg+ Val94Met (AAA)	Yes	Higher BMI, MUAC, hip
LEPR	Haplotypes: AC and AAC	Yes	Higher MUAC
GHRL+LEPR	Haplotype: Lue72Met+ Gln223Arg + Lys656Asn (CCA)	Yes	Higher MUAC
LEP+LEPR	Haplotype: 19G>A+Lys36Arg+ Val94Met+ Lys109Arg (AAAA)	Yes	Higher BMI, weight, WC, MUAC
LEP+LEPR	Haplotype:19G>A+Lys36Arg+ Val94Met+ Lys109Arg (GAGG)	Yes	Lower BMI, weight, WC, MUAC

Abbreviations: BMI, body mass index; BP, blood pressure; MA, Mixed Ancestry; MUAC, mid-upper-arm circumference TC, total cholesterol; WC, waist circumference.

4.2. Conclusion

The *CART* c.517A>G polymorphism was identified as a predisposing factor for obesity in adolescent learners: learners harbouring the minor allele were more likely to be obese and had higher MUAC compared to those carrying the *A* allele. However, it is not known whether or not this risk allele acts synergistically with other polymorphisms and/or in response to obesogenic environment. *MC3R* and *LEPR* polymorphisms were associated with lower values of anthropometric obesity traits (BMI, MUAC, hip and waist circumference), blood pressure and total cholesterol, suggesting a protective role against Metabolic Syndrome (MetS); which is defined as a cluster of metabolic disorders such as dyslipidemia, hypertension, insulin resistance, and visceral fat accumulation (referred to as central obesity). The effect of *MC3R* Val81Ile polymorphism on total cholesterol was enhanced in learners who reported to be physically active. The present study also demonstrated that more than one polymorphisms act together to elicit a specific phenotype. For example, genotype combination of *LEP* and *LEPR* polymorphisms displayed different effects on anthropometric obesity traits, depending on which alleles interacted. Individual *LEP* polymorphisms did not have an effect on obesity-related traits. Further studies are required to investigate whether or not findings of the present study translates to the lower prevalence of MetS previously identified in Mixed Ancestry learners (Matsha et al., 2009).
Although the present study identified only polymorphisms with larger effects due to smaller sample size, it served as an example of the potential benefits of considering a combined effect of polymorphisms from one or more genes. At this point the association of polymorphisms with obesity-related traits that is reported in the present study has no clinical implications. Replication of these findings is critical and needs to be based on multiple independent samples of substantial sizes. Once replicated, clinical trials will be required to assess the effectiveness of genotype-based intervention programs against obesity and/or cardiovascular diseases. It will also be critically important to understand how the public (individuals at risk and those with protective allele) will perceive individualized genetic results. Predictive genetic testing may raise issues such as labeling a family at risk, inducing feelings of blame, or anxiety associated with knowledge of affected relatives. Moreover, some people may be falsely reassured if they are told that they have no genetic risk, and engage in unhealthy behaviour. On the other hand, results may have positive outcome, significantly improving patient outcomes and motivate people to engage in preventative behaviour to reduce their disease risk.

The novel findings of the present study should thus be viewed as “hypotheses generating” for further studies. Once proven to be applicable in clinical practice, our findings may help to identify individuals at high risk for developing obesity, cardiovascular diseases or type 2 diabetes without the knowledge of family history. So far, most predictive genetic tests are used for identifying predispositions for

monogenic diseases with a strong genetic influence such as hereditary forms of cardiac diseases and cancers. Genotype-based management or prevention of obesity and related cardiovascular diseases such as hypercholesterolemia and hypertension can be subsequently developed for those individuals found to have risk alleles.

Overall, our findings are based on international reference cut-off points (as developed by Cole and co-workers) that were developed using data from other countries. Although these reference cut-off points are recommended for research settings, whether or not they are related to the development of cardiovascular diseases and MetS in South African ethnic groups remains to be proven through longitudinal studies. The use of these international reference cut-off points in South African population groups may have serious health-related implications if these findings are not investigated using a well-defined study design.

4.3. Limitations

Single strand conformation polymorphisms (SSCP) and allele-specific restriction enzyme analysis (ASREA) are some of the molecular techniques that were used in the present study to detect possible sequence variants and genotype the selected study group, respectively; and this might have contributed to the detection rate of sequence variants. These techniques are subject to human error regarding interpretation of mobility shifts and genotypes, and hence other sequence variants could have been missed or genotyping error introduced. SSCP has been reported to detect up to 98% (addition of 15% urea in both 8% and 10% polyacrylamide gels increases the detection limit from 90 to 98%) of nucleotide changes (IFCC Scientific Division, Committee on Molecular Biology Techniques). Genotyping errors can be suspected when frequencies are not in Hardy-Weinberg Equilibrium (HWE). However, deviations from HWE can also indicate that the sample consists of a heterogeneous population or in cases it can indicate a true genetic effect or strong association with the disease state. Therefore, the genotype frequencies of control or population-based samples (if selected from large populations where random mating is assumed) should be in HWE, whereas samples consisting of only cases may deviate from HWE (Lewis, 2002; Andersson et al. 2009).

The use of questionnaires to measure physical activity may have introduced bias. Physical activity questionnaires and diaries are more applicable in large epidemiological studies, but they provide less accurate estimates of physical activity level compared to more objective measures as the tool relies on self-reported (parental or child/adolescent) information (Bull et al., 2009). The type of house chores performed by learners was not specified (i.e., heavy or light duties) in order to estimate the intensity of

activity involved and make assumptions about the observed association between house chores, total cholesterol, and *MCR3* genotypes.

The study population consisted of adolescents, who at the time of the study, might have been at different developmental stages. Puberty, a stage characterized by physiological changes in girls and boys, was not accounted for during the analyses due to the small sample size. Although the statistical analyses were adjusted for age and gender, puberty is another confounding factor that may introduce spurious positive or negative association. This developmental stage has been reported to affect lipid profile and body composition. With specific reference to lipid profile, puberty has been shown to decrease high and low density lipoprotein-cholesterol (HDL-C and LDL-C), and increase triglycerides (TG) levels (Codoñer-Franch et al., 2010). Serum lipid levels peak at age 9 to 10 years, followed by a decrease, and then begin to increase again in adolescence (Stozický et al., 1991; Labarthe et al., 1997; Freedman et al., 2001). Changes in TC have also been observed, decreasing between the ages of 10 and 16 years in boys and 9 and 14 years in girls (Berenson et al., 1981). TC and HDL-C are associated with growth (age and height) in pubertal children (Kouda et al., 2003; Fujita et al., 2011). It has been reported that pubertal children who experience a large increase in height tended to show a decrease in serum lipids (TC and HDL-C), and children who experience a small increase in height tended to show an increase in serum lipids. Serum lipid profile is also affected by the levels of sex hormones in children and adolescents, with lower HDL-C and LDL-C levels associated with increased testosterone in boys and increased estradiol in girls (Morrison et al., 2003). Furthermore, growth hormone therapy has also been reported to cause a decline in LDL-C and HDL-C levels (Hilczer et al., 2008).

In addition to hormonal fluctuations, rapid growth in body size accompanied by marked changes in body composition is observed during pubertal stage. When considering gender and puberty, differences in fat distribution and changes in total and percentage body weight can be observed between girls and boys who are at this stage of development. During adolescence, on average girls tend to have increased fat and fat-free mass, while boys gain more muscle mass (Naumova et al., 2001). Furthermore, fat in girls is usually deposited peripherally in breasts, hips, and buttocks while in boys it is centralised in the abdomen. It is therefore possible that the observed association between *MC3R* and *LEPR* polymorphisms anthropometric variables (BMI, MUAC, hip and waist circumference) is confounded by puberty. Different age of puberty onset may also contribute to the difference in lipid profile and body composition observed among children and adolescents of the same gender and age group, and thus affecting statistical analyses if not accounted for. Some individuals can experience precocious or delayed puberty (Herman-Giddens et al., 1997; Grumbach and Styne, 1998). Precocious puberty is defined as the development of secondary sexual characteristics before the age

of 8 years in girls and boys. Delayed puberty is the absence of breast development by 13 years of age in girls or testicular volume <4 mL by 14 years of age in boys.

Due to the mixed ancestry origin of the “Coloured” (Mixed Ancestry) ethnic group, associations identified in the present study may be due to population stratification, which was not adjusted for. Potential population stratification in unrelated sample may cause spurious positive or negative associations in population-based association studies (Deng, 2001). Several statistical methods have been proposed to reduce the effect of population stratification on population-based association analyses, and the three major categories include structured association (SA) (Pritchard et al., 2000; 2000), genomic control (GC) (Devlin et al., 1999), and principal components analysis (PCA) (Price et al., 2006). SA uses a set of ancestral informative markers (AIMs) to estimate population structure and individual ancestries (Pritchard et al., 2000; 2000). The model used in the analysis then accounts for the correlations between linked loci that arise in admixed populations. In the GC method, the effect of population stratification can be assessed using a set of disease-unlinked marker loci, providing a correction factor that can then be applied to adjust for statistical bias at candidate loci (Devlin and Roeder, 1999). For PCA method, classical principal components analysis is first applied to genotype data to model ancestral differences between cases and controls, which are then used to correct allele frequency variations at candidate loci across ancestral populations (Price et al., 2006). When Zhang and co-workers (2008) compared these methods, SA and PCA performed better than the GC only if 120 or more AIMs were used. Using a small set of AIMs in the SA methods may result in high type 1 error rate in populations with high stratification level.

Structure analysis conducted in the South African Coloured population revealed that its origin is predominantly Khoesan (32-43%), Bantu-speaking African (20-36%), European (21-28%), and a small proportion Asian (9-11%) (de Wit et al., 2010). This analysis, however, was based on the Affymetrix 500k SNP chip containing markers primarily designed for use in Europeans. According to de Wit and coworkers, the use of European markers in their study may have lead to ascertainment bias that might have influenced the quantitative details of the analyses. Additional markers of Malaysian, Indonesian, San (which are not publicly available), and appropriate tags of Bantu-speaking ethnic groups are needed to improve resolution of ancestral contribution in the Mixed Ancestry group. Appropriate markers that can be used to map disease genes or correct for population stratification in the Mixed Ancestry are not available as yet. Performing this analysis to correct for population stratification in the present study was beyond the ethical scope of the proposed study. We recommend that either SA (when appropriate markers are available) or PCA be conducted to correct for population stratification if further studies are to be conducted to confirm the association detected in the present study.

4.4. Recommendations for further studies

Population-specific and standardised reference cut-off points are required for accurate classification of South African children and adolescents, and adults according to weight status or BMI. The response rate of the parent study was 65%, and thus affected the sample size of the present study. The response rate obtained is worrying as this may confound conducting further confirmatory studies. Be it the case may be, a larger sample size may be required to identify polymorphisms with small effects that might have been missed in the present study, and to replicate the observed associations with confidence taking into account confounding factors such as pubertal stage, diet, alcohol consumption, and physical activity. Larger sample size generally improves the power of detecting polymorphisms with smaller effect sizes (Andersson et al. 2009, De Krom et al. 2009). An improved study design is therefore recommended, which can also incorporate measurements of leptin that can be correlated with genotypes of the genes investigated in the present study. Further studies can be conducted in one properly selected South African racial group to eliminate the possible effect of population stratification. Genotype and allele frequencies may vary between populations from different ethnicities or geographical regions, resulting in over or underestimation of associations if the study group is not properly selected. Our study consisted of two racial groups, which although different, are primarily of African origin and have received little attention in population genetics of complex disorders, particularly obesity.

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URL for websites used

Primer3plus: http://www.primer3plus.com
Integrated DNA Technology: http://eu.idtdna.com/site
National Center for Bioinformatics Institute: http://www.ncbi.nlm.nih.gov
World Health Organisation: http://www.who.int/mediacentre/factsheets/fs311/en/index.html

APPENDICES

APPENDIX A: ETHICAL CLEARANCE

27 November 2007

Prof RT Erasmus
Division of Chemical Pathology
Dept of Pathology

Dear Prof Erasmus

RESEARCH PROJECT : "MOLECULAR INVESTIGATION OF GENETIC FACTORS CONTRIBUTING TO OBESITY IN ADOLESCENT LEARNERS RESIDING IN THE SEMI- URBAN/RURAL AREAS OF THE WESTERN PROVINCE, SOUTH AFRICA"
PROJECT NUMBER : N07/07/160

At a meeting of the Committee for Human Research that was held on 1 August 2007 the above project was approved on condition that further information that was required, be submitted.

This information was supplied and the project was finally approved on 27 November 2007 **for a period of one year from this date**. This project is therefore now registered and you can proceed with the work. Please quote the above-mentioned project number in all further correspondence.

Please note that a progress report (obtainable on the website of our Division) should be submitted to the Committee before the year has expired. The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly and subjected to an external audit.

Patients participating in a research project in Tygerberg Hospital will not be treated free of charge as the Provincial Government of the Western Cape does not support research financially.

Due to heavy workload the nursing corps of the Tygerberg Hospital cannot offer comprehensive nursing care in research projects. It may therefore be expected of a research worker to arrange for private nursing care.

Yours faithfully

CJ VAN TONDER
RESEARCH DEVELOPMENT AND SUPPORT (TYGERBERG)
Tel: +27 21 938 9207 / E-mail: cjvt@sun.ac.za

CJVT/pm

15 October 2009

MAILED

Prof RT Erasmus
Department of Pathology
Division Chemical Pathology
9th Floor
Tygerberg Hospital

Dear Prof Erasmus

"Molecular investigation of genetic factors contributing to obesity in adolescent learners residing in the semi-urban/rural areas of the Western Province,South Africa,"

ETHICS REFERENCE NO: N07/07/160

RE : PROGRESS REPORT

At a meeting of the Health Research Ethics Committee that was held on 14 October 2009, the progress report for the abovementioned project has been approved and the study has been granted an extension for a period of one year from this date.

Please remember to submit progress reports in good time for annual renewal in the standard HREC format.

Yours faithfully

MRS ETIENNE VAN HAND
RESEARCH DEVELOPMENT AND SUPPORT
Tel: 021 9389677 / E-mail: elr@sun.ac.za
Fax: 021 931 3352

Verbind tot Optimale Gesondheid . Committed to Optimal Health
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06 April 2011

MAILED

Prof RT Erasmus
Department of Pathology
Division Chemical Pathology
9th Floor
Tygerberg Hospital

Dear Prof Erasmus

"Molecular investigation of genetic factors contributing to obesity in adolescent learners residing in the semi-urban/rural areas of the Western Province,South Africa."

ETHICS REFERENCE NO: N07/07/160

RE : PROGRESS REPORT

At a meeting of the Health Research Ethics Committee that was held on 17 November 2010, the progress report for the abovementioned project has been approved and the study has been granted an extension for a period of one year from this date.

Please remember to submit progress reports in good time for annual renewal in the standard HREC format.

Approval Date: 17 November 2010

Expiry Date: 17 November 2011

Yours faithfully

MRS MERRUOE DAVIDS
RESEARCH DEVELOPMENT AND SUPPORT
Tel: 021 9389207 / E-mail: mertrude@sun.ac.za
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APPENDIX B: QUESTIONNAIRE

UNIVERSITY OF STELLENBOSCH AND CAPE PENINSULA UNIVERSITY OF TECHNOLOGY

SECTION A **FAMILY HEALTH HISTORY AND LIFESTYLE SURVEY**

Name and Reference Number

PERSONAL & BIOGRAPHICAL DATA

Age in Months

1 **What is your date of birth?** ____ Age in Months ____ **1**

2 **What is your gender?** 0 Male 1 Female **2**

3 **In which grade are you?** 0 1 2 3 4 5 6 7 8 9 10 **3**

4 **How long have you been attending this school?** **4**

a) < 6 Months 1 b) < 1 Year 2 c) 1-5 years 3 d) 6-10 years 4

5 **How would you describe yourself? (select one response)** **5**

a) Black 1 b) White 2 c) Coloured 3 d) Asian 4 e) Other 5 If other, Please clarify 6 **6**

6a **How would you describe your ……..(Select one)** **7**

	Mother?		**Father?**		
a) Black	1	a) Black	1	**8**
b) White	2	b) White	2	

c) Coloured	3	c) Coloured	3
d) Asian	4	d) Asian	4
e) Other	5	e) Other	5

6b **Are any of your grandparents of a different description as you and your parents?** 9

If yes, please state, who, of which description they are, & how they are related to you

Mom's mother	1	Father's mother	3
Mom's dad	2	Father's father	4

7 **Are you related to someone also participating in this project?** a) Yes ☐ b) No ☐ 10

If yes, please state who and how you are related?

8 **How many brothers and sisters do you have?** 11

a) Brothers ☐ b) Sisters ☐ **Total** ☐

FAMILY HEALTH HISTORY

9 **Have you ever been told that you have diabetes (sugar levels)?** 12

a) Yes 1 b) No 2

10 **Does your natural mother, father, brother or sister have diabetes?** 13

a) Yes 1 b) No 2

11 **Has anyone in your extended family (Aunts, Uncles, Grandparents) ever suffer from diabetes?** 14

a) Yes 1 b) No 2

12 **At which age did you have your first menstrual period?** 15 16

☐ **Tanner Stage** ☐

LIFESTYLE

13a **Do you smoke?** 17

a) Yes [1] b) No [2]

13b If you answered yes, how many cigarettes do you smoke per day? [ ] 18

14 Do you consume any alcoholic beverages? 19

a) Yes [1] b) No [2]

SOCO-ECONOMIC DETAILS

15 What type of house do you live in? 20

a) House [1] b) Flat [2] c) Back Room [3]

d) Hostels [4] e) Shack [5] f) Bungalow/ wendy house [6]

g) Other [7]

16 What type of toilet facility do you have at your house? 21

a) In-house Flush system [1] b) Out-door Flush system [2]

c) In-house Bucket system [3] d) Out-door Bucket system [4]

17 How many people, including your Mom and Dad, live in your house? [ ] 22

18 Are your parents working? If so, what do they do for a living? 23

Mother [Yes / No] [ ] Father [Yes / No] [ ] 24

19 Is anyone else (brother, sister, cousin, etc) in your house working? Who? 25

a) Yes [1] b) No [2]

Who? [ ] 26

HABITUAL PHYSICAL AND LEISURE TIME ACTIVITY SURVEY

Name & Reference Number ☐

1 How many days per week do you walk for at least 10 minutes (for recreation, pleasure or exercise)? ☐ 1

a) 0 days ☐ 1 b) 1-2 days ☐ 2 c) 3-5 days ☐ 3 d) more ☐ 4

---

2 How many times per week do you participate in after-school sports activities (extramural Activities)? ☐ 2

a) 0 days ☐ 1 b) 1-2 days ☐ 2 c) 3-5 days ☐ 3 d) more ☐ 4

---

3 If you never participate in sports activities, please stat the reason for this non-participation. ☐ 3

---

4 In which sports do you participate? ☐ 4

a) Chess ☐ 1 b) Tennis ☐ 2 c) Rugby ☐ 3 d)Swimming ☐ 4

e) Netball ☐ 5 f) Cricket ☐ 6 g) Soccer ☐ 7 h) Other ☐ 8

If other, please specify ☐

---

5 If you play after school in the afternoon, which games do you usually play? ☐ 5

a) Dolls ☐ 1 b) Tennis ☐ 2 c) Rugby ☐ 3 d)Swimming ☐ 4

e)Board games ☐ 5 f) Cricket ☐ 6 g) Soccer ☐ 7 h) Other ☐ 8

---

6 Where do you normally play? ☐ 6

a) Street ☐ 1 b) Backyard ☐ 2 c) In house ☐ 3 d)Other ☐ 4

---

7 Does your school offer physical education as a school subject? ☐ 7

a) Yes ☐ 1 b) No ☐ 2

---

8 If you answered yes at (7), how many days do you have physical education? ☐ 8

a) 0 days ☐ 1 b) 1-2 days ☐ 2 c) 3-5 days ☐ 3 d) more ☐ 4

---

9 How many days per week do you spend watching TV or playing Computer games? ☐ 9

a) 0 days [1] b) 1-2 days [2] c) 3-5 days [3] d) more [4]

10 How often do you participate in the following activities? [10]

ACTIVITY	Never	Sometimes	Often	Very often	Always
Waching TV					
Computer Games					
Cycling, Dancing, Swimming					
Household Chores					
Other					

Thank you for participating in our survey

UNIVERSITY OF STELLENBOSCH AND CAPE PENINSULA UNIVERSITY OF TECHNOLOGY

BODY MEASUREMENTS DATA COLLECTION SHEET

Reference Number _ _ _ _

Date of Interview 0 _ _ _ _ _ _ (y y m m d d)

1 Did Subject eat this morning? [1]

Age of Participant (years) [2]

2	Birth weight (kg)	[3]
	Body Weight (kg)	[4]
	Body Height (cm)	[5]
	Body Mass Index [BM] (m/kg^2)	[6]

CIRCUMFERENCE MEASUREMENT

3	Mid-upper arm Circumference 1 (cm)	
	Mid-upper arm Circumference 2 (cm)	
	Mid-upper arm Circumference 3 (cm)	
	Mid-upper arm Circumference (cm)	
4	Waist Circumference 1 (cm)	
	Waist Circumference 2 (cm)	
	Waist Circumference 3(cm)	
	Waist Circumference (cm)	
	Waist hip ratio 1	

Waist hip ratio 2	
Waist hip ratio 3	
Waist Hip Ratio	

SKINFOLD MEASUREMENTS

5

Triceps skinfold 1 (cm)	
Triceps skinfold 2 (cm)	
Triceps skinfold 3 (cm)	
Triceps skinfold (cm)	

Biceps skinfold 1 (cm)	
Biceps skinfold 2 (cm)	
Biceps skinfold 3 (cm)	
Biceps Skinfold (cm)	

Sub-scapular skinfold 1 (cm)	
Sub-scapular skinfold 2 (cm)	
Sub-scapular skinfold 3 (cm)	
Sub-scapular skinfold (cm)	

Supra-iliac skinfold 1 (cm)	
Supra-iliac skinfold 2 (cm)	
Supra-iliac skinfold 3 (cm)	
Supra-iliac skinfold (cm)	
SUM OF SKINFOLDS	

BLOOD PRESSURE MEASUREMENTS

Systolic Pressure 1 (mmHg)	
Systolic Pressure 2 (mmHg)	
Systolic Pressure 3 (mmHg)	
Systolic Pressure	

Diastolic Pressure 1 ((mmHg)	
Diastolic Pressure 2 ((mmHg)	
Diastolic Pressure 3 ((mmHg)	
Diastolic Pressure	

Pulse 1 (Beats per minute)	
Pulse 2 (Beats per minute)	
Pulse 3 (Beats per minute)	
Mean Pulse	

Systolic Pressure (mmHg)	
Diastolic Pressure (mmHg)	

BLOOD GLUCOMETER ANALYSES

Glucose (mmol/L)	

URINALYSIS

Glucose mmol/L (N=Negative, P=Positive)	**P**	**N**

Protein mmol/L (N=Negative, P=Positive)		P	N	
Microalbumin µmol/l				

CARDIOCHEK ANALYSIS

Cholesterol			
Triglycerides			
HDL			
LDL			

BLOOD ANALYSIS

Microalbumin				
C-reactive Protein (CRP)				

For Official Use Only

Were any measurements repeated **Y** **N**

If yes, which one(s) and what was the repeated measurement?

_____				
_____				
_____				
_____				

Were all QC procedures perfomed to ensure accuracy and reliability? **Y** **N**

Controlled and Signed by _____ Date: _____

APPENDIX C: CONSENT FORM

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM FOR USE BY PARENTS/LEGAL GUARDIANS ON RESEARCH INVOLVING GENETIC STUDIES

TITLE OF RESEARCH PROJECT:

1. *Identification of the* ENPP1 *three-allele risk haplotype and its possible contribution to the development of obesity and insulin resistance in 8–18 year old learners in communities of the Western Cape*
2. ***Molecular investigation of genetic factors contributing to obesity in adolescent learners residing in the semi-urban/rural areas of the Western Province, South Africa.***
3. *The role of environmental and the* ENPP1 *gene in obesity and insulin resistance in South African children*

REFERENCE NUMBERS: N06/03/059; N07/07/160

PRINCIPAL INVESTIGATORS:
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ADDRESS:
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CONTACT NUMBER:
Department of Chemical Pathology
Dr Mariza Hoffmann - Tel: 021 938 4174
Cape Peninsula University of Technology
Dr Tandi Matsha - Tel: 021 460 3209

We would like to invite your child to participate in a research study that involves DNA (genetic) analysis and possible long-term storage of blood or tissue specimens. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied and clearly understand what this research entails and how your child could be involved. Also, your child's participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you or your child negatively in any way whatsoever. You are also free to withdraw him/her from the study at any point, even if you do initially agree to let him/her take part.

This research study has been approved by the ethics **Committee for Human Research at Stellenbosch University** and it will be conducted according to international and locally accepted ethical guidelines for research, namely the Declaration of Helsinki, Guidelines on Ethics for Medical and Genetic Research of the Medical Research Council of South Africa (MRC).

What is DNA Analysis or Genetic research?

Genetic material, also called DNA, is usually obtained from a small blood sample. Occasionally other tissues may be used. DNA consists on numerous genes, strung together in long strands and found in every cell in the human body. Genes are the "blueprint" that determines who we are, what we look like and sometimes what kind of diseases we may be susceptible to. Worldwide research in this field is continuously discovering new information that may be of great benefit to future generations and also that may benefit people today, who suffer from particular diseases or conditions.

What does this particular research study involve?

Worldwide new causes of certain diseases or conditions are continuously being discovered by research on the cells and molecules of the body. This project aims to find out if certain genes may be one of the factors for the high incidence of obesity, overweight and diabetes.
Additionally, this project aims to collect genetic material from blood samples to analyse for certain changes that may be linked to overweight and obesity, and to store excess material for future research. When a large group of patients with similar diseases has been collected, meaningful research into the disease processes may become possible.

Why has your child been invited to participate?

Obesity amongst young children is currently a problem world wide, including South Africa. In order to asses the magnitude of the problem, local schools have been approached to participate in this project to determine the incidence of obesity amongst our children.

Your child has randomly been selected by means of a computer program to participate in the above-mentioned study. Children of all races, gender, age (between ages 8 and 18 years) and weight will be approached as subjects.

What procedures will be involved in this research?

Venous blood will be drawn from your child by a professional nurse for DNA isolation and my finger pricked to collect blood for biochemical analysis. **A maximum of 2-5 ml of venous blood** will be collected for DNA isolation and a finger prick done for biochemical analysis. Additionally, your child will be requested to provide information about his/her medical history, family history and information on eating, drinking and smoking habits. Completion of the questionnaire will take no longer than 10 minutes.

Are there any risks involved in your child taking part in this genetic research?

The child may experience minor pain or bruising at the site where blood is taken.

Are there any benefits to your child taking part in this study and will you get told your results?

Although there may not be any direct benefits to the participant by participating at this stage, family members and future generations may benefit if the researchers succeed in scientifically delineating the specific genes involved. Thereby the rational approach to the clinical diagnosis and therapy of its manifestations may be facilitated. The identification and location of the genes involved in such disorders could in the end lead to the development of methods for prevention and to forms of new treatment aimed at curing or alleviating these conditions. Additionally, **depending on the outcome of the research** your child may undergo genetic counselling that will advise on changing his/her lifestyle (for example; eating habits, exercising, introduction of any ways of reducing body weight).

In the unlikely event that the research may lead to the development of commercial applications, the participant or the participant's heirs will not receive any compensation, but profits will be reinvested into supporting the cause of further research which may bring benefits to the participant's family and community, such as health screening, medical treatment, educational promotions, etc.

How long will your blood be stored and where will it be stored?

The DNA may be stored for as long as it is needed for this research study and at the research institution where the study will be conducted.

If your blood is to be stored is there a chance that it will be used for other research?

Your blood will only be used for genetic research that is directly related to
obesity. If the researchers wish to use your stored blood for **additional research in this field** they will be required to apply for permission to do so from the Human Research Ethics Committee at Stellenbosch University.

If you do not wish your blood specimen to be stored after this research study is completed you will have an opportunity to request that it be discarded when you sign the consent form.
Your blood will only be used for genetic research that is directly related to

If you do not wish your blood specimen to be stored after this research study is completed you will have an opportunity to request that it be discarded when you sign the consent form.

How will your confidentiality be protected?

The participant's identity will be kept confidential throughout. Information will not be associated with the participant's name. The research staff will use only a coded number, access will be limited to authorized scientists and any scientific publications, lectures or reports resulting from the study will not identify you or your child.

Some insurance companies may mistakenly assume that taking part in genetic research indicates a higher risk for disease. Thus no information about you or your family will be shared with such companies.

Will you, your child, or the researchers benefit financially from this research?

You will not be paid to take part in this study although your travel expenses may be reimbursed. There will be no costs involved for you if your child does take part. In the unlikely event that the research leads to the development of a commercial application or patent you or your family will not receive any profits or royalties. However profits will be reinvested to supporting the cause of further research, which may bring benefits to your family or community in the future.

Assent of minor

I (*Name of Child/Minor*)..... have been invited to take part in the above research project.

- **The study doctor/nurse and my parents have explained the details of the study to me and I understand what they have said to me.**
- **They have also explained that this study will involve collection of blood by a professional nurse for DNA isolation and biochemical analysis.**
- **I also know that I am free to withdraw from the study at any time if I am unhappy.**
- **By writing my name below, I voluntary agree to take part in this research project. I confirm that I have not been forced either by my parents or doctor to take part.**

....

Signature of participant Signature of witness

Declaration by parent/legal guardian

By signing below, I …….…….. agree to allow my child (name of child) ……..…. who is ……. years old, to take part in a research study entitled:

- *Identification of the* ENPP1 *three-allele risk haplotype and its possible contribution to the development of obesity and insulin resistance in 8–18 year old learners in communities of the Western Cape*
- *Molecular investigation of genetic factors contributing to obesity in adolescent learners residing in the semi-urban/rural areas of the Western Province, South Africa.*
- *The role of environmental and the ENPP1 gene in obesity and insulin resistance in South African children*

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that my child's participation in this study is voluntary and I or my child have not been pressurised to take part.

☐ I agree that my child's blood or tissue sample can be stored, but I can choose to request at any time that the stored sample be destroyed. I have the right to receive confirmation that my request has been carried out.

OR

☐ Please destroy my blood sample as soon as the current research project has been completed. **(Tick the option you choose**)

Signed at (*place*) ..... on (*date*) .....

…… ……

Signature of parent/legal guardian Signature of witness

Declaration by investigator

I *(name)* …… declare that:

- I explained the information in this document to ……..……...
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research as discussed above.
- I did/did not use a interpreter. (*If a interpreter is used then the interpreter must sign the declaration below.*

Signed at (*place*) .....…..…….. on (*date*) ……..…….. 2005.

…… ……

Signature of investigator Signature of witness

Declaration by Interpreter

I *(name)* …… declare that:

- I assisted the investigator (*name*) ……. to explain the information in this document to (*name of participant*) …….. using the language medium of Afrikaans/Xhosa.
- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (*place*) ..... On (*date*) ……..…….. 2005.

…… ……

Signature of interpreter Signature of witness

APPENDIX D: SOLUTIONS

Phosphate Buffered Saline (PBS)- pH 7.4

0.2 g KCl

8.0 g NaCl

0.2 g KH_2PO_4

1.15 g NA_2HPO_4

Add all components, one at a time to 900 ml of dH_2O, then dissolve by adding dH_2O to 1 L.

6 M Sodium Chloride (NaCl)

350.64 g of NaCl was dissolved in 800 ml of dH_2O and then the volume was adjusted to 1 L with dH_2O.

Nuclear Lysis Buffer (500 ml)- pH 8.2

11.5 g NaCl

10 ml Tris (1M stock)

10 ml EDTA (10mM stock).

Lysis Buffer- pH 7.4

31 ml from NH_4Cl (1M stock)

1 ml of $KHCO_3$ (1M stock)

100µl of EDTA (100mM stock)

1X Tris EDTA (TE) Buffer

10 mM Tris (10 ml 1 mM stock)

1 mM EDTA (2 ml of 0.5M stock)

Made up to 1 L with dH_2O

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

1 g EtBr in 100 ml dH_2O was added together and stored in a dark bottle

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

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

The solution was heated and stirred with a magnet stirrer to assist dissolution.

Loading Buffer (Bromophenol blue)

0.2 g (2%) BPB powder

1 ml (10 mM) of 1M Tris stock (pH 8.0)

50 ml (50%) Glycerol

49 ml dH_2O

20X Sodium Borate (SB) Buffer

Dissolve 38.137 g of SB powder in dH_2O and add up to 1 L.

SSCP Gel preparation

Components	8%	10%
Urea	24 g	24 g
distilled H_2O	91.8 ml	84 ml
10XTBE	8ml	8ml
Glycerol	8ml	8ml
10% APS	1.6ml	1.6ml
TEMED	160µl	160µl

SSCP Loading dye

- 95% formamide
- 20 mM EDTA
- 0.01% Bromophenol Blue
- 0.05% of Xylene cyanol.

Method:

Glass plates and tanks were cleaned and prepared. A sheet of Gelbond® PAG film (Cambrex Bio science, Rockland, USA), was placed between the glass plates where it covalently bonded to the acrylamide gel during polymerization, thus providing support. Urea, sterile distilled H2O, 10X TBE and glycerol was added to a sterile beaker and mixed by stirring. 10% APS and TEMED were added last (Table A). The solution was then immediately poured into the cast that had previously been prepared. The gels were kept in the cast until set. Meanwhile, 0.5X TBE running buffer was poured into the tank. The samples were prepared by combining equal volumes of SSCP loading dye to the samples and denatured at 94.5ºC for 5 minutes. The samples were incubated on ice for 5 minutes or until just before loading. The gels were run at 23 Watts at 4ºC overnight.

Silver staining of SSCP gels

Reagents:

- 1% $AgNO_3$ (Solution B) :
- 1g $AgNO_3$ was dissolved in distilled H_2O to make up 1L.
- Developing Solution (Solution C):
- 15 g NaOH; 0.1g of NaBH4 and 4ml of Formaldehyde was dissolved in sterile
- Distilled H_2O to make up 1L.

Method:

500ml Solution B was added to a tray containing the SSCP gel .The gel was stained for 10 minutes on a shaker. Solution B was discarded and the gel was rinsed with distilled H_2O. 500ml Solution C was then added to the tray containing the gel and placed on a shaker for a further 15 minutes or until the bands were clearly visible. The solution was discarded and the gel was once again rinsed with distilled H_2O. The gels were then left to dry and sealed in plastic.