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**MUTATION SCREENING OF THE *ENPP1* GENE AND ITS POSSIBLE
CONTRIBUTION TO THE DEVELOPMENT OF OBESITY/OVERWEIGHT AND
METABOLIC SYNDROME IN SOUTH AFRICAN CHILDREN**

By

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DECLARATION

I, Boitumelo Louisa Fanampe, declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

.....
Signed *Bfanampe*

.....
Date *28/08/09*

ABSTRACT

Epidemiological reports have shown that South Africa, whilst a developing country, its overweight and obesity prevalence rates in children is fast approaching those seen in the developed world. This country's population is unique in that it is made up of different ethnic groups with different socio-economic status, partly due to the past and present political environments in the country. South Africa, in particular, is faced with a rapid increasing childhood obesity of 10% among children under the age of two and 5-20% among those less than six 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. The Ectonucleotide Pyrophosphatase Phosphodiesterase 1 (*ENPP1*) gene is located on chromosome 6q22-q23; a locus linked to obesity and diabetes, spans 83 kb of genomic DNA and contains 25 exons. Studies in humans have shown a correlation between overexpression of *ENPP1* and insulin resistance, obesity, and type 2 diabetes. *ENPP1* has been implicated in up to 20% of Caucasian and 50% of Black communities suffering from obesity. The overall objective of the proposed study is to assess whether *ENPP1* polymorphisms contribute to childhood obesity/overweight, and their association with components of metabolic syndrome in a South African Coloured population.

Subjects for this study were identified through a screening program that aimed to determine the prevalence of obesity in learners between the ages of 8 – 18 years from the Western Cape Province, South Africa. The first phase of the project was to clearly differentiate between obese subjects and controls. The cut-off points for obesity established by Cole and co-workers in 2000, and adopted by the International Obesity Task Force (IOTF), were used to classify the obese subjects. The obese Coloured population that was used for this study had an obesity prevalence rate of 7.3%. Sixty four obese subjects and 64 controls were gender-age matched at a ratio of 1:1 for the second phase. The second phase of the project was to screen the *ENPP1* gene for the sequence variants by designing specific primers using a programme called Primer3plus, to amplify the regions of interest. Polymerase chain reaction (PCR) was performed on the DNA which was extracted from whole blood. PCR products were then purified and

automated sequencing was performed to identify the sequence variants. Three Single Nucleotide Polymorphisms (SNPs); rs997509, rs1044498 (K121Q) and rs9402349 were analysed. The genotype and allelic distribution of rs997509 (*C>T*) was significantly higher in obese subjects than in controls, $p < 0.05$. No significant differences were observed in the distribution of rs9402349 and rs1044498 (K121Q).

Lastly, the study investigated the association between genetic variants and metabolic syndrome components. The T allele of rs997509 was found to have a significant association with Body Mass Index (BMI), $p = 0.03$. The rs9402349 that has previously not been associated with type 2 diabetes, metabolic components or obesity was found to be associated with significant differences in diastolic blood pressure. No differences were found for rs1044498. The results of this study have formed a basis for further investigations as to how the *ENPP1*, particularly the T allele of rs997509 contribute to weight gain.

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- The financial assistance of the Cape Peninsula University of Technology towards this research is acknowledged. Opinions expressed in this thesis and the conclusions arrived at, are those of the author, and are not necessarily to be attributed to the Cape Peninsula University of Technology.

DEDICATION

To my late grandfather, Monnapule Sight Fanampe, who taught me the value of good education and would have been proud of this achievement.

LIST OF ABBREVIATIONS

<	Greater than
>	Smaller than
≥	Greater than or equal to
≤	Smaller than or equal
=	Equals
%	Percentage
°C	Degree Celsius
®	Registered trademark
™	Trademark
e.g.	Example
A	Adenine
ACDC	Adiponectin
ADA	American Diabetes Association
<i>AGRP</i>	Agouti-related peptide
<i>B3-AR</i>	β ₃ -adrenergic receptor
BLAST	Basic Length Alignment Search Tool
BMI	Body Mass Index
bp	Base pair
BP	Blood Pressure
BPB	Bromophenol blue
C	Cytosine
CDC	Center for Disease Control and Prevention
Central obesity	Abdominal obesity / truncal obesity. Obesity defined by an increased waist-to-hip ratio, waist-to-thigh ratio, waist circumference, and sagittal abdominal diameter, and linked to an increased risk of cardiovascular events.
cm	Centimetre
CVD	Cardiovascular Diseases
dH ₂ O	Distilled water

Disinhibited eating	The tendency to overeat in response to different stimuli, and can occur in a variety of circumstance such as when an individual is presented with an array of palatable foods or is under emotional distress.
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphate
DRD2	Dopamine D2 receptor
Dysmorphism	An abnormality in morphological development.
EDTA	Ethylenediamine Tetra-acetic acid
<i>ENPP1</i>	Ectonucleotide pyrophosphatase phosphodiesterase 1
EtBr	Ethidium Bromide
EtOH	Ethanol
FBG	Fasting blood glucose
g	Gram
G	Guanine
<i>GNB3</i>	G protein β_3 subunit gene
HDL-C	High density lipoprotein cholesterol
HIV/AIDS	Human Immunodeficiency Virus/ Acquired Immunodeficiency Syndrome
HPLC	High Performance Liquid Chromatography
HT2C	5-Hydroxytryptamine (serotonin) receptor 2C
IDF	International Diabetes Federation
IL6	Interleukin 6
IOTF	International Obesity Task Force
K121	A-allele at codon 121
K121Q	A-allele (adenine) at codon 121 changed to C-allele (cytosine)
kcal	Kilocalories
KCl	Potassium chloride
kg	Kilograms
kg/m ²	Kilograms per metres squared
KH ₂ PO ₄	Potassium dihydrogen phosphate
KHCO ₃	Potassium hydrogen bicarbonate
L	Litre

<i>LEP</i>	Leptin
<i>LEPR</i>	Leptin receptor
m	Meter
M	Molar (moles per litre)
m ²	Meters squared
<i>MC4R</i>	Melanocortin 4 receptor
MetS	Metabolic Syndrome
mg/ml	Milligrams per millilitre
min	Minute
Mixed Ancestry	Coloureds
ml	Millilitre
mm	Millimeter
mM	Millimolar
mmol/l	Millimoles per litre
Monogenic obesity	Obesity caused by a mutation in a single gene.
Monogenic syndromes	Disorders involving one gene, in which obesity is a clinical feature often associated with mental retardation, dysmorphic features, and organ-specific developmental abnormalities.
Morbidity	The condition of being diseased/ an illness or an abnormal condition.
NA ₂ HPO ₄	Di- sodium hydrogen phosphate
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information
NCEP ATP III	National Cholesterol Education Program Adult Treatment Panel III
ng/μl	Nanograms per microlitre
NH ₄ Cl	Ammonium Chloride
NHANES III	National Health and Nutrition Examination Survey
nm	Nanometer
NTPs	Nucleotide triphosphates
NTRK2	Neurotropic tyrosine kinase receptor type 2
<i>Ob gene</i>	Obese gene
PBS	Phosphate Buffered Saline

PC1	Plasma cell glycoprotein
<i>PC-1</i>	Prohormone convertase 1
PCR	Polymerase Chain Reaction
pH	Potential of Hydrogen
Pleiotropy	The control by a single gene of several distinct and seemingly unrelated phenotypic effects.
Polygenic obesity	Obesity caused by multiple genes.
<i>POMC</i>	Pro-opiomelanocortin
<i>PPARγ</i>	Peroxisome proliferator-activated receptor γ
PPi	Inorganic pyrophosphates
Q121/121Q	C-allele at codon 121
QTL	Quantitative Trait Loci
rpm	Revolutions per minute
SAP	Shrimp alkaline phosphatase
SB	Disodium Tetraborate decahydrate/ Sodium Borate
SDS	Sodium Dodecyl Sulphate
sec	Seconds
Sequel	Any disorder or pathological condition that results from a preceding disease.
<i>SIM 1</i>	Single-minded 1
SNP	Single Nucleotide Polymorphism
SOP	Standard Operating Procedures
T	Thymine
TE	Tris EDTA
TG	Triglycerides
<i>TNFα</i>	Tumour necrosis factor α
Tris	2- Amino-2-(hydroxymethyl)-1, 3-propanediol:C ₄ H ₁₁ NO ₃
TV	Television
<i>UCP1</i>	Uncoupling protein 1
<i>UCP2</i>	Uncoupling protein 2
<i>UCP3</i>	Uncoupling protein 3
UK	United Kingdom
μ l	Microlitre
μ M	Micromolar

US	United States
USA	United States of America
UV	Ultraviolet
UV	Ultraviolet
V	Volts
v/v	Volume per volume
w/v	Weight per volume
WC	Waist circumference
WHO	World Health Organization
β_2AR	β_2 -adrenergic receptor

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CHAPTER ONE
LITERATURE REVIEW

1.1 Introduction

Obesity is a state or condition where body fat content is increased to an extent that it poses a risk to cardiovascular, pulmonary (such as sleep apnoea), metabolic (diabetes and dyslipidaemia) and osteoarticular diseases, common forms of cancer (cervical, uterus, breast, ovarian and kidney), and serious psychological illnesses (learning difficulties, social isolation, poor self-image, depression ,anxiety) (Chen *et al.*, 1999; Challis *et al.*, 2002; Clement, 2006; Loktionov, 2003; O' Rahilly *et al.*, 2003). The global burden of overweight/obesity is both significant and rising, with most of the increase reported over the last decade (World Health Organization (WHO), 2000). While attention has previously been focused on under-nutrition in South African children, survey data suggests that up to 10% of children under the age of two years and 5-20% of children under the age of six years are overweight/obese (SASSO, 2001). Overweight/obese children tend to become overweight/obese adults, and are thus at an increased risk of developing insulin resistance (American Diabetes Association, 1998; Yensel *et al.*, 2004). Insulin resistance is considered to be the driving force of the metabolic syndrome (Reaven, 1988), a condition that predisposes to an increased risk for type 2 diabetes mellitus and coronary heart disease (Ogden *et al.*, 2007).

As with other health conditions, obesity results from an interaction between both environmental and genetic factors. Diet is the crucial environmental factor in the development of obesity. Overeating in combination with low physical activity is the main environmental cause of the obesity epidemic rapidly spreading in the modern world (Loktionov, 2003). Some studies assessing heritability within families have shown compelling evidence for the significant genetic influence on obesity. Genetic factors are currently estimated to account for approximately 40-70% of the variance in human adiposity (Chung & Liebel, 2005; Farooqi, 2005). The first evidence of the genetic contribution on obesity was from murine studies, which showed an association of obesity-related phenotypes with certain genetic loci (Collins *et al.*, 1993). A possible single genetic cause of human obesity only came into light when Zhang *et al* (1994) identified a mutation in an obese gene (*ob* gene) in mice, which results in obesity and type II diabetes as part of a syndrome that resembles morbid obesity in humans. Subsequently, several genes including the Ectonucleotide Pyrophosphatase/ Phosphodiesterase 1 (*ENPP1*) also called plasma cell glycoprotein 1 (*PC1*) have been implicated in the development of obesity and type 2 diabetes (Doty, 2005; Meyre *et al.*, 2005).

1.2 Diagnosis of Obesity and Overweight

Obesity and overweight in adults are measured by the body mass index (BMI), as [weight (kg)/height (m²)] with the internationally recognized cut-off points at 30 kg/m² and 25 kg/m² respectively (Mutch & Clement, 2006). However in children, the BMI changes substantially due to variability in hormonal levels, as a result, a statistical definition of overweight based on the 85th and 95th percentiles of sex-specific BMI-for-age in a specified reference population often is used in childhood (Cole *et al.*, 2000). The US data used by Cole was the same as those from which the 2000 CDC growth charts were derived. The references such as the WHO standards, the 1990 UK (British) reference and the 2000 CDC growth charts are intended for clinical use in monitoring children's growth. The IOTF on the other hand, were not intended as clinical definitions, but rather, they were developed to provide a common set of definitions that researchers and policy makers in different countries could use for descriptive and comparative purposes internationally (Ogden *et al.*, 2007).

1.3 Prevalence and Trends of Obesity and Overweight

1.3.1 Obesity and Overweight World-wide

The prevalence of obesity varies with higher rates being observed in developed countries such as the United States of America (USA) and the United Kingdom (UK), with females having lower rates of obesity than males. The WHO Global Comparable Estimates (2005) of adult overweight (BMI \geq 25 kg/m²) and obesity are depicted in Figure 1.1. With the exception of India and China, the prevalence of overweight in developing countries is approaching those in the developed countries with South African women having similar rates as the USA.

BMI / Overweight / Obesity - prevalence - BMI \geq 25 kg/m²
2005

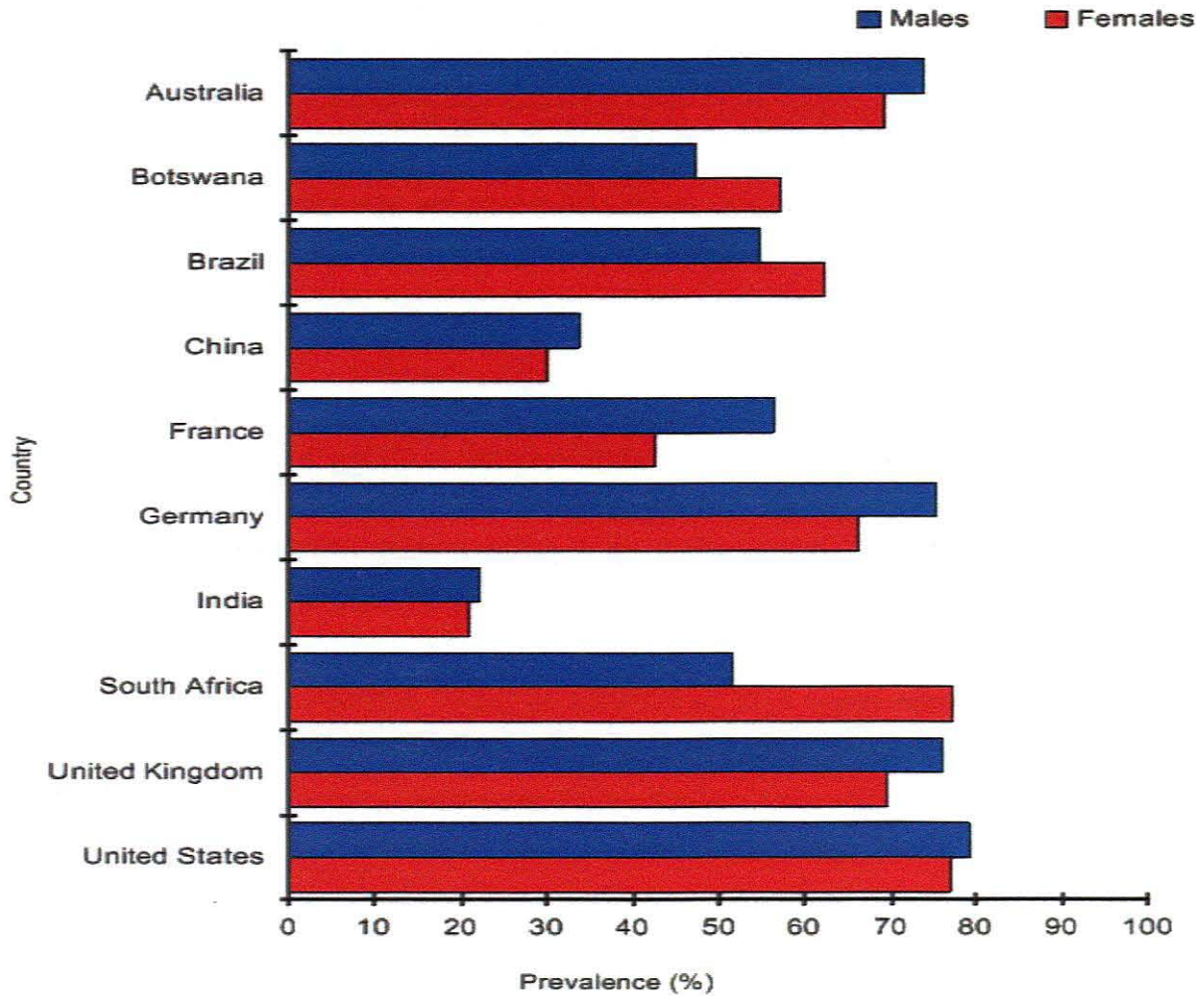


Figure 1.1: Prevalence of adult overweight worldwide (WHO Global Comparable Estimates, 2005)

Similar trends have also been observed in children (Ogden *et al.*, 2007). Data from the Health Survey for England, 2002 show that 7.2% of girls and 5.5% of boys aged 2-15 years are obese, and a further 16.3% and 20.3% are overweight (Jebb, 2004). In 1999, a cross-sectional study was done, which involved 2500 children and adolescents in Leipzig, Germany, with ages between 7 and 18 years, and 29% of the participants were overweight and 16% were found to be obese (Kiess *et al.*, 2006). The International Obesity Task Force (IOTF) has also examined the prevalence of obesity in children aged around ten years from data derived from 21 European countries between 1992 and 2001. They found variable levels of between 10% and 36%, with Italy, Malta, Greece and Croatia being the countries with the highest rate of childhood obesity (Bellizzi & Dietz, 1999). The occurrence of insulin resistance in children with obesity was first recognised in Pima Indians in 1963 and following this, in the Asian population (Sabin *et al.*,

2004). Data from the Center for Disease Control (CDC) in the USA demonstrate an increase in the prevalence of overweight children aged 6-19 years, increasing from 4-5% during 1963-1970 to 15% during 1990-2000 (Kuczmarski *et al.*, 2000). Using both the IOTF and the CDC cut-off values the prevalence of overweight and obesity in 11-15 years old Sicilian children was very high; nearly 40% at age 11 and, although it progressively decreased as age increased, 25% of the examined population was still considered obese at age 15. The prevalence of overweight and obesity in 11-15 year old Sicilian school children is one of the highest reported (Baratta *et al.*, 2006).

1.3.2 Obesity and Overweight in South Africa

According to the WHO Global Estimates (2005), South Africa has one of highest rates of obesity in Africa, with prevalence rates for males and females at 52% and 77%, respectively (Figure 1.2). Survey data has shown the overall prevalence of obesity and overweight in South Africa to be more than 29% in men and 56% in women who are classified as overweight or obese (Puoane *et al.*, 2002; Rocchini, 2002). In a sample of 7726 South African women aged 15-95 years old, black women had the highest prevalence of overweight and obesity (58.5%), followed by women of Mixed Ancestry (Coloureds) (52%), White women (49.2%) and then Indian women (48.9%). BMI was found to increase with age; urban women were found to have a significantly higher BMI than their rural counterparts. A different pattern was seen in men. In a sample of 5401 men aged 15-95 years, the prevalence of overweight and obesity was highest in White men (54.5%), followed by Indian men (32.7%), and men of Mixed Ancestry (31%), with the lowest prevalence in Black men (25%). Older men and those living in urban areas had significantly higher BMIs than younger men and those living in rural areas. Central obesity was found in 9.2% of men with higher levels, in older and in White men (Goedecke *et al.*, 2006).

Despite being a developing country, overweight and obese prevalence rates in South African children are fast approaching to those in the developed world. In a study done on learners residing in the peri-urban areas of Cape Town, Western Cape Province, an overall prevalence of 15.7% for overweight and 6.2% for obesity was found. The highest prevalence rate (30.8%) for overweight was observed in Black females (Somers *et al.*, 2006). Similarly, Armstrong and co-workers (2006) reported increasing rates with increased age: the combined percentage of overweight and obese Black girls increased from 11.9% at age six years to 21.8% at 13 years. An opposite trend was evident among White girls (from 25.4% at six years to 14.5% at 13 years). Generally the rates are higher in girls (23%) than boys (15.2%) (Schutte *et al.*, 2003),

and among urban children and adolescents (20.1%), compared to those residing on farms (10.8%) (Steyn *et al.*, 2005).

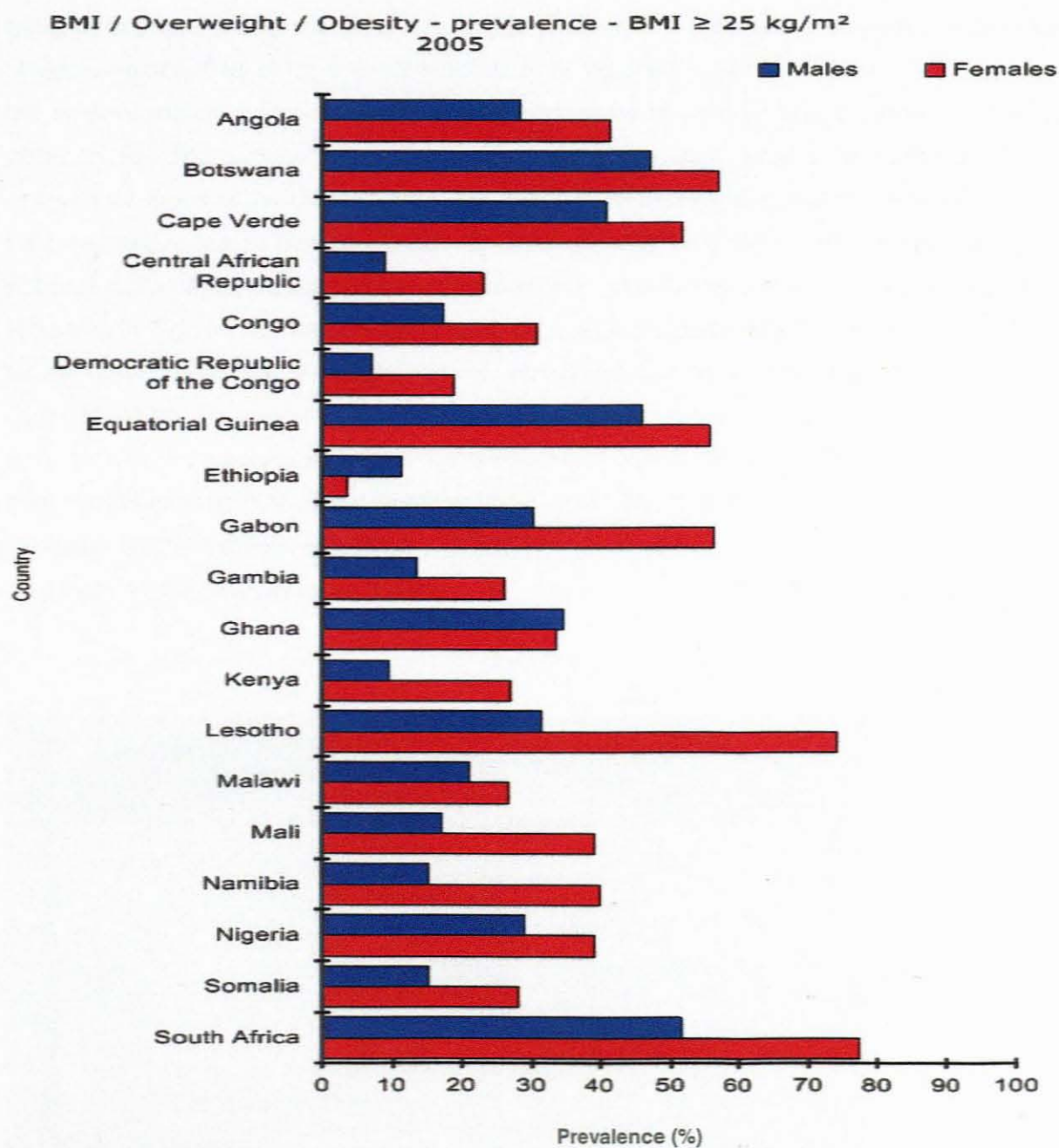


Figure 1.2: Prevalence of adult overweight in Africa. Overweight is considered as BMI > 25kg/m²

1.4. Aetiology of Obesity

At an individual level, the problem of obesity could be seen simply as a product of too much energy intake and not enough energy expended both under the control of the central nervous system. It is said that even a small daily energy imbalance eventually results in significant weight gain; for example, a daily excess of 100 kcal (equivalent to a chocolate bar) leads to an increase of approximately 5 kg of fat over 12 months, or 50 kg over 10 years (Wilding, 2006). However, the epidemiological evidence for the role of food intake has shown only a modest association between BMI and calories or fat consumed (Field *et al.*, 2004; Kant & Graubard, 2006). For example, in areas of the United States, there has been an overall decrease in the population's fat consumption but an increase in obesity rates (Heini & Weinsier, 1997). This has led to the notion that obesity develops from an intricate interaction between genes and the environment as illustrated in Figure 1.3. The condition arises when an individual's genetic makeup is susceptible to an environment that promotes energy consumption over energy expenditure (Mutch & Clément, 2006). There is strong evidence to suggest that like height, weight is a highly heritable trait (40-70% heritability), (Farooqi & O'Rahilly, 2005a; 2006), while cultural and societal factors may explain at least 30% of the variation (Marti *et al.*, 2004). The two major factors believed to contribute to the aetiology of obesity are diet, and physical inactivity. In turn, each is influenced by genetic traits (Weinsier *et al.*, 1998).

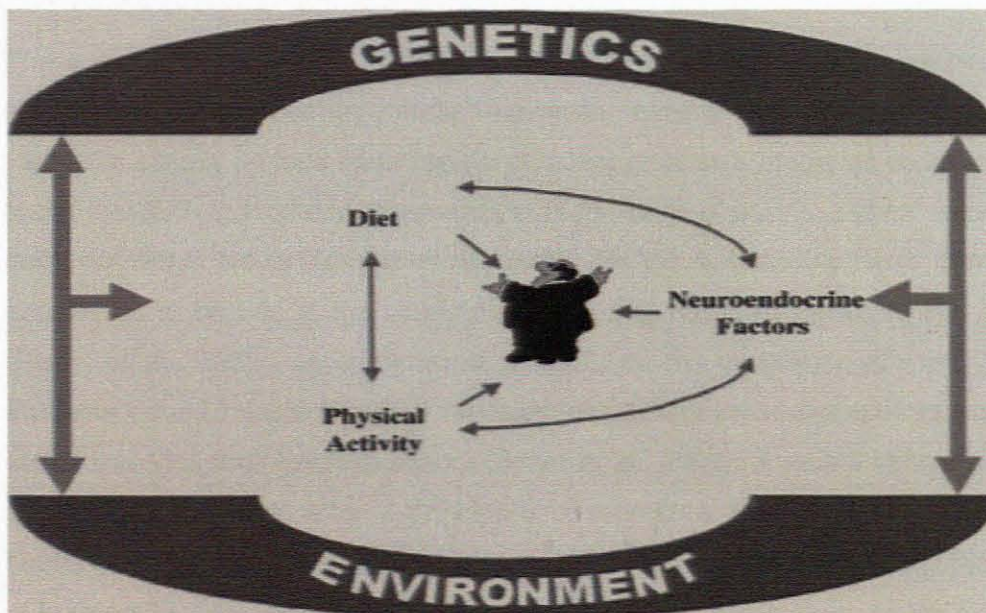


Figure 1.3: Gene-environment interaction on obesity risk (Marti *et al.*, 2004)

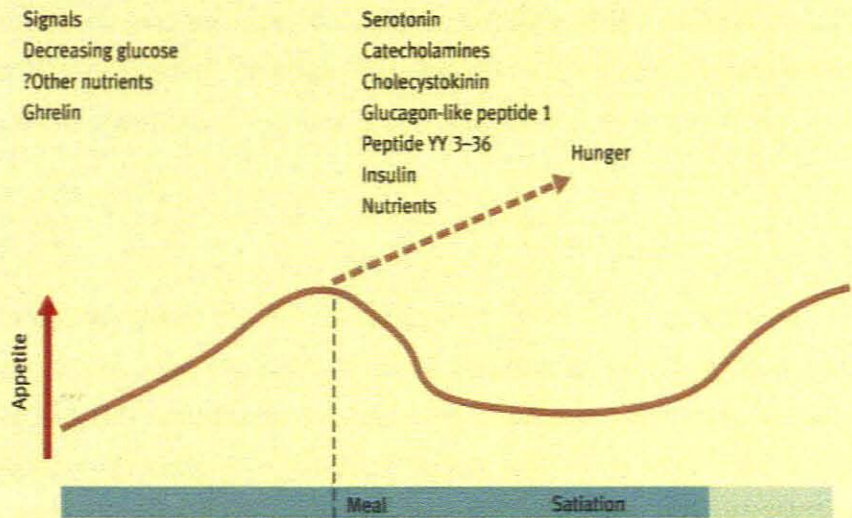
1.4.1 Diet and Physical Activity

Dietary habits in combination with low physical activity are the main causes of the obesity epidemic rapidly spreading in the modern world (Loktionov, 2003). Although excessive calorie intake is responsible for the development of obesity, it is the high-fat diets that promote obesity significantly more than high-carbohydrate diets. High-fat diets are preferred because most individuals find them to be more palatable and satisfying. Of greater importance is the effect of appetite regulation. During hunger the magnitude of response is strong whilst the response to energy excess is relatively weak (Figure 1.4) (Weinsier *et al.*, 1998; Wilding, 2006). High consumption of fatty foods is further compounded by the industry sector which has increased the availability of energy-dense meals (Temple *et al.*, 2006; Dennison & Edmunds, 2008). The role of diet in the development of obesity is further supported by the fact that healthy individuals who have lost significant amounts of weight usually achieve this only through restricting calories and/or increasing exercise suggesting a synergistic interaction between diet and exercise. Contributors to high calorie intake are influenced by lifestyle and cultural beliefs. In developed countries, eating out has an impact on the types and amounts of food consumed as it is convenient, affordable and fashionable. MacPhee (2008) observed that in the USA, when children eat out, the consumption of energy-dense foods is in greater amounts compared to when they eat at home. Hence, eating meals together as a family is recommended as it may decrease hours of TV viewing and increase nutritious food consumption.

There is strong evidence to suggest that health-related behaviours developed early in childhood are strongly influenced by media exposure, notably television viewing (Renzaho, 2004). Recently, a study on food advertisements during children's programs on television was done and found that 55% of food advertisements were for foods of poor nutritional value, and 42% were of good nutritional value (Temple *et al.*, 2008). By virtue of its economic growth, South Africa is considered to be one of the countries undergoing rapid demographic and nutritional transition (Steyn *et al.*, 2005). A study done in 1992, on the evaluation of food items consumed by students in the Limpopo Province revealed that 22% of the learners bought snacks at the school shop, and 10% took lunch to school (Steyn *et al.*, 2003). A recent study was done at a private school in Cape Town after a principal's observations and concern about the overall dietary habits of learners and the fact that 10% of them were obese. It was found that a large proportion of learners buy energy-dense foods that are high in fat and sugars, typically called "junk food" or "fast food" (Temple *et al.*, 2006).

Short-term regulation of appetite and satiety

Appetite signals increase progressively during the inter-meal interval. Once a meal is consumed, the process of satiation begins and appetite signals decline. However, satiety can be readily overcome by social and marketing influences. Without a meal, the physical sensation of hunger may develop.



Long-term regulation of energy balance

The hormone leptin is secreted by adipose tissue, and circulating levels reflect fat mass. Leptin receptors and downstream signalling pathways are in the hypothalamus. If leptin concentration decreases, secretion of neuropeptide Y is increased and there is a decrease in α -melanocyte-stimulating hormone, derived from the pro-opiomelanocortin (POMC) gene. Food intake is stimulated and thermogenesis is reduced. Genetic defects in leptin, its receptor, the POMC gene and the melanocortin-4 receptor have all been shown to result in obesity in humans.

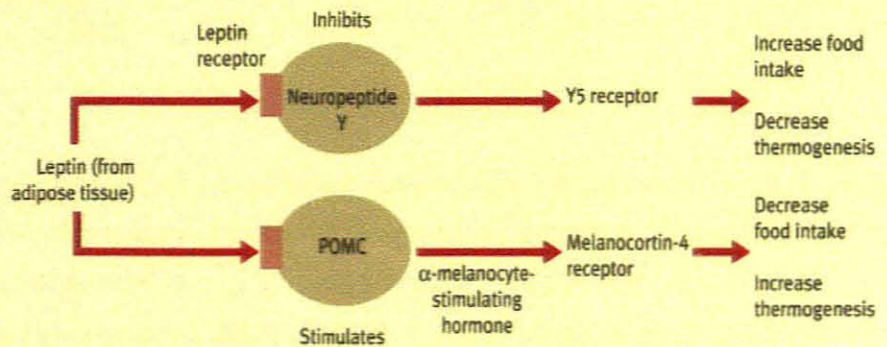


Figure 1.4: Short-term regulation of appetite and satiety and Long-term regulation of energy balance (Wilding, 2006)

In developing countries, cultural beliefs and practices related to food, though varying among ethnic groups, contribute to different patterns of obesity in children and youth (Kumanyika, 2008). In African societies, being overweight is associated with wealth and happiness; in contrast, White girls are influenced by the Western beauty ideal, which shuns fatness (Mvo *et al.*, 1999). In South Africa, urbanisation has a greater influence on the rates of obesity (Monyeki

et al., 1999; Goedecke *et al.*, 2006). Monyeki and co-workers (1999) reported lower obesity prevalence rates in rural children aged three to ten than their urban counterparts. Another factor that might be contributing to the increasing rates of obesity in South Africa as well as other African countries is the HIV/AIDS pandemic. The stigma associated with HIV/AIDS has resulted in obesity and overweight being evidence of not being affected by the disease (Clark *et al.*, 1999).

Parental eating attitudes are also linked to the development of obesity in children. The Framingham children's study, which was done in order to investigate the extent to which parents' degree of dietary self-control affects the development of excess body fat in the child, suggest that parents who display high levels of disinhibited eating, especially when coupled with high dietary restraints, may foster the development of excess body fat in their children (Hood *et al.*, 2000). Furthermore, the presence of an obese parent in a household increases the risk of having an obese child by 40% compared to 7% if parents are of normal weight. Similarly, children with physically active parents are usually far more active than children whose parents are inactive (Van Heerden, 1998).

A physically active lifestyle consistently performed has been shown to improve lipid profile, decrease body weight and percentage of body fat, lower blood pressure, and positively affect thromboembolic state and thus reduce overall cardiovascular disease risk (American Diabetes Association (ADA), 2004; Grundy *et al.*, 2002; Grundy, 2004; Haskell *et al.*, 2007; Waxman & Nesto, 2002). Other benefits of physical activity are the physiological changes that favorably affect muscle and liver insulin sensitivity, muscle glucose uptake and utilization, and overall glycaemic control (Goodyear & Kahn, 1998; Kelley *et al.*, 2002; Wasserman *et al.*, 2002; Peterson *et al.*, 2003; Sigal *et al.*, 2004; Goodpaster & Brown, 2005; Nair, 2005; Toledo *et al.*, 2007). Although physical activity is known to produce multiple health benefits, many people are considered to be relatively physically inactive. Less than half of American adults achieve activity levels recommended by the US Surgeon General (CDC, 2007). In children, sedentary lifestyle is facilitated by physically inactive pursuits, particularly television viewing (Marti *et al.*, 2004). Exercise (Janssen *et al.*, 2006) is associated with an inverse relationship between BMI and markers of socioeconomic status (Viner & Cole, 2006; Vieweg *et al.*, 2007; Ward *et al.*, 2007). Goedecke *et al.* (2006) reported that in persons of lower socio-economic status, television viewing was greater whilst opportunities for school-based or after-school sports and physical

activity were fewer. This is in part attributed to the lack of the facilities, equipment access, as well as peer influence (Maffeis, 2000).

1.4.2 Other Factors Associated with Obesity

Many drugs (notably centrally acting drugs) promote weight gain (Table 1.1). The mechanisms depend on the agent concerned and are not well understood, but may involve both central effects on appetite (as is thought to be the case with neuroleptics) and peripheral metabolic effects (as with oral hypoglycaemic drugs and protease inhibitors) (Haslam & James, 2005; Wilding, 2006).

Table 1.1: Drugs associated with weight gain (Wilding *et al.*, 2006)

Class	Examples
Anticonvulsants	Sodium vaporize, phenytoin, gabapentin
Antidepressants	Citalopram, mirtazepine
Antipsychotics	Chlorpromazine, risperidone, olanzepine
β -blockers	Atenolol
Corticosteroids	Prednisolone, dexamethasone
Insulin	All formulations
Migraine-relieving drugs (serotonin antagonists)	Pizotifen
Sex steroids	Medroxyprogesterone acetate, progesterone, combined oral contraceptives
Oral hypoglycaemic drugs	Glibenclamide, gliclazide, repaglinide, rosiglitazone, pioglitazone
Protease inhibitors	Indinavir, ritonavir

1.4.3 Genetic Factors

The growing prevalence of obesity around the world is mainly attributed to changes in lifestyle (increase of the consumption of high-energy-yielding foods enriched with carbohydrates and fats, and reduction of physical activity) that specifically may impact genetic susceptibility. Evidence of the genetic role in obesity is supported by the so-called “thrifty genes”. This theory purports that evolutionary pressure to preserve glucose for use by the brain during starvation led to a genetic propensity towards insulin resistance in peripheral tissue. It is believed that during

periods of prolonged famine that plagued our early ancestors, a survival advantage would have been conferred by genes favouring the economical use of stored energy. Thrifty genes are maladaptive in today's world, particularly where food is easily available and plentiful, consequently contributing to susceptibility for obesity and type 2 diabetes (Elbers *et al.*, 2006).

Familial studies have also repeatedly demonstrated that body mass index (BMI) is highly correlated among first-degree relatives. In these studies, obese parents produced the highest proportion of obese offspring. Stronger support for the role of genetics in BMI comes from twin studies (Koeppen-Schomerus *et al.*, 2001), in which BMI is consistently shown to be similar between twins, with the similarity being greater in monozygotic than dizygotic twins. Adoption studies (Sorensen *et al.*, 1992) have also shown evidence of the contribution of genetics to BMI. These studies have demonstrated significant correlations between the BMI of the biologic parents and the BMI of the adoptee in childhood and adulthood, but not between BMI of the adoptive parents and the BMI of the adoptee. These studies suggest a genetic influence on BMI in children and adults. (Naggert *et al.*, 1997; Hill *et al.*, 2000). Candidate genes and biomarkers for obesity are illustrated in Figure 1.5.

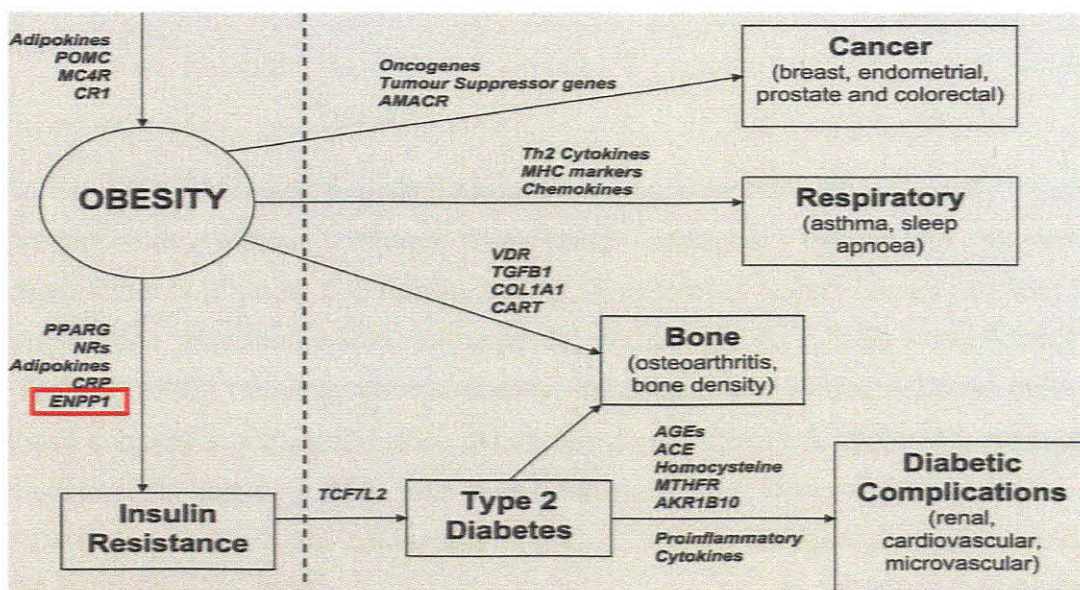


Figure 1.5: Examples of clinically relevant biomarkers for assessing the development of obesity and its sequelae. The dotted line represents the division between clinical health and disease. Key (in alphabetical order): ACE, angiotensin I-converting enzyme (peptidyl-dipeptidase A) 1; ages, advanced glycation end-products; AKR1B10, aldo-keto reductase family 1, member B10 (aldose reductase); AMACR, alpha-methylacetyl-coa racemase; CART, cocaine- and amphetamine-regulated transcript; COL1A1, collagen, type I, alpha 1; CR1, cannabinoid receptor 1; ENPP1, ectonucleotide pyrophosphatase/phosphodiesterase 1; MC4R, melanocortin-4 receptor; MTHFR, 5,10-methylenetetrahydrofolate reductase (NADPH); nrs, nuclear receptors; POMC, pro-opiomelanocortin; PPARG, peroxisome proliferative activated receptor, gamma; TCF7L2, transcription factor 7-like 2 (T-cell specific, HMG-box); TGFB1, transforming growth factor, beta 1; VDR, vitamin D (1,25-dihydroxyvitamin D3) receptor (Walley *et al.*, 2006)

Obesity exists as monogenic syndromes with or without dysmorphism and these include pleiotropic forms, in which affected individuals also exhibit mental retardation or other developmental and endocrine abnormalities (O’Rahilly *et al.*, 2003). In the majority of cases, however, obesity is a polygenic disorder that is not fully understood due to its complexity and the wide scope of implicated genetic loci and genes.

1.4.3.1 Monogenic Obesity

Monogenic obesity is divided into two groups according to the genes involved. The first group comprises very rare recessive forms of obesity that is associated with pituitary endocrine dysfunction, these being caused by mutations in the leptin (*LEP*), leptin receptor (*LEPR*) and Pro-opiomelanocortin (*POMC*) genes. Homozygous carriers consistently exhibit a phenotype of morbid obesity with an onset in the first weeks of life, of increased appetite and hyperphagia, and hypogonadotropic hypogonadism. Treatment with recombinant leptin is successful. The second group of monogenic forms of non-syndromic obesity is caused by numerous mutations in the Melanocortin 4 receptor (*MC4R*) gene. *MC4R* is the most prevalent obesity gene to date, affecting 2-4% of very obese cases depending on the population. *MC4R* mutations segregate in a family *via* an autosomal dominant mode of inheritance with variable penetrance (Boutin & Froguel, 2001).

To date, five monogenic obesity disorders have been reported where obesity in early childhood predominates without children developing additional behavioural abnormalities and dysmorphisms (Farooqi & O’Rahilly, 2005b). This form of obesity is very rare and is caused by single gene mutations within, for example, *MC4R*, (*LEP*), (*LEPR*) and (*POMC*) (Table 1.2) (Farooqi, 2005). Unlike syndromic obesity, the reason why excess body fat mass develops in these subjects is understood since genetic anomalies affect key factors related to the leptin and melanocortin pathways. Mutations have been identified in human genes coding for *LEP*, *LEPR*, *POMC* and Prohormone convertase 1 (*PC-1*), leading to obesity occurring soon after birth (Mutch & Clement, 2006).

1.4.3.2 Polygenic Obesity

Polygenic obesity is not fully understood due to its complexity and the wide scope of implicated genetic loci and genes. Factors that contribute to the complexity of the condition are; the inability to replicate some findings of genetic linkage studies, and the mode of others that aim to identify

these loci and genes. Studies have been conducted across populations and ethnic groups, and have demonstrated significant heritabilities (i.e. the portion of the phenotypic variability observed in a population that can be attributed to genetic effects) that are generally estimated to range from 30 to 70% (Comuzzie & Allison, 1998).

Due to the alarming increase of obesity in both adults and children, there is an increased interest in which the genetic complexity and the challenges in dissecting the perturbed biology underlying common obesity have been investigated. Currently, there are 22 gene associations supported by at least five positive studies and these are summarised in Tables 1.2 & 1.3. With the exception of *ENPP1*, these genes include members of the leptin-melanocortin pathway, pro-inflammatory cytokines and uncoupling proteins (Walley *et al.*, 2006). *ENPP1* on the other hand, has been shown to contribute directly to childhood obesity. A study conducted in France, strongly implicated the *ENPP1* gene to be the link between polygenic childhood obesity, adult obesity and type 2 diabetes (Meyre *et al.*, 2005).

Table 1.2: Rare monogenic forms of human obesity (Mutch & Clement, 2006)

Gene	References	Mutation type	Obesity	Associated phenotypes
Leptin (<i>LEP</i>)	Montague <i>et al.</i> , 1997 www.diogenes-eu.org	Homozygous mutation	Severe, from the first days of life	Gonadotropic and thyrotropic insufficiency
Leptin receptor (<i>LEPR</i>)	Clement <i>et al.</i> , 1998	Homozygous mutation	Severe, from the first days of life	Gonadotropic, thyrotropic and somatotropinsufficiency
Proopiomelanocortin (<i>POMC</i>)	Krude <i>et al.</i> , 2003 Ardlie <i>et al.</i> , 2002	Homozygous or compound heterozygous	Severe, from the first month of life	ACTH insufficiency Mild hypothyroidie and ginger hair if the mutation leads to the absence of POMC production
Proopiomelanocortin but in the β MSH coding region	Lee <i>et al.</i> , 2006 Bieberman <i>et al.</i> , 2006	Heterozygous non-synonymous mutations	Severe obesity occurring in childhood	Rapid size growth
Single-minded 1 (<i>SIM1</i>)	Holder <i>et al.</i> , 2000	Translocation between chromosomes 1p22.1 and 6q16.2 in the SIM 1 gene	Severe obesity occurring in childhood	–

Neurotrophic tyrosine kinase receptor type 2 (NTRK2)	Yeo <i>et al.</i> , 2004	De novo heterozygous mutation	Severe from the first months of life	Developmental delay Behavioural disturbance Blunted response to pain
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Table 1.3: Genes frequently associated with obesity phenotypes in humans (Mutch & Clement, 2006)

Genes (Code)	Locus	Animal model/phenotype	Human obesity locus (N)	Functional genetic variant
Food intake				
Leptin (LEP)	7q31	<i>ob/ob</i> mice/severe obesity	Yes	Variants associate with leptin serum levels
Leptin-R (LEPR)	1p31	<i>db/db</i> mice/severe obesity	Yes	No
Agouti-related peptide (AGRP)	16q22	Transgenic/obese	Yes	Modified promoter activity
Dopamine D2 receptor (DRD2)	11q23.2	KO/non-obese	No	Decrease receptor number
5-Hydroxytryptamine(serotonin) receptor 2C (HT2C)	Xq24	KO/late-onset obese	Yes	Diminished transcriptional activity
Energy metabolism				
Uncoupling protein 1 (UCP1)	4q28–q31	KO and transgenics/reduced adiposity	Yes	No
Uncoupling protein 2 (UCP2)	11q13	KO non-obese	Yes	Change in UCP2 mRNA
Uncoupling protein 3 (UCP3)	11q13	KO non-obese	Yes	No
β_3 -adrenergic receptor (B3-AR)	9p12	KO/increased adiposity	Yes	Decreased receptor activity
G protein β_3 subunit gene (GNB3)	12p13.3	No	Yes	Modified G-protein activation
Adipose tissue metabolism				
Adiponectin (ACDC)	3q27	KO diet-induced insulin resistance	Yes	Variants associate with adiponectin serum levels
Peroxisome proliferator-activated receptor γ (PPAR γ)	3p25	KO/decreased brown adipose tissue	Yes	Modified transcriptional activity
Tumour necrosis factor α (TNF α)	6p21.3	KO increased adiposity on high-fat diet	Yes	Modified transcriptional activity

β_2 -adrenergic receptor (β_2 AR)	5q31-q32	KO: increased adiposity on high-fat diet	Yes	Decreased receptor activity
Interleukin 6 (IL6)	7p21	KO/mature-onset obesity	Yes	Modified transcriptional activity
Ectonucleotide pyrophosphatase phosphodiesterase 1 (<i>ENPP1</i>)	6q22-q23	NA	Yes	K121Q with obesity, insulin resistance and type 2 diabetes

1.5 The *ENPP1* gene

Ectonucleotide Pyrophosphatase Phosphodiesterase 1 (*ENPP1*) (OMIM #173335, NCBI: NM_006208) was originally described as a plasma cell alloantigen and named membrane glycoprotein (*PC1*). It is a class II transmembrane glycoprotein with a short amino-terminal cytoplasmic tail, a single transmembrane domain, and a large extracellular carboxy-terminal domain that generates inorganic pyrophosphates (PPi) from nucleotide triphosphates (NTPs) and other PPi sources. *ENPP1* is widely expressed in the muscle, liver, renal tubules, salivary ducts epithelium, epididymis, adipose tissue, chondrocytes, pancreas, and capillary endothelium in the brain (Matsuoka *et al.*, 2006). This gene is located on chromosome 6q22-q23; a locus linked to obesity and diabetes, spans 83 kb of genomic DNA and contains 25 exons (Ruf *et al.*, 2005). *ENPP1* encodes ectonucleotide pyrophosphatase/ phosphodiesterase. This cell surface enzyme is a type II membrane glycoprotein comprising of identical disulfide-bonded subunits (Chen *et al.*, 2006).

ENPP1/PC-1 belongs to a family of enzymes (ENPPs) that are known to hydrolyze 5'-phosphodiesterase bonds in nucleotides (Figure 1.6). Evidence also suggests that ENPPs have multiple and related physiological roles, including nucleotide recycling, modulation of purinergic receptor signalling, regulation of extracellular pyrophosphatase levels, and regulation of insulin receptor signalling (Baba *et al.*, 2007). The latter is more specifically related to *ENPP1*, which could interact with insulin receptors and decrease the insulin-induced tyrosine phosphorylation of its intracytoplasmic domain. When over expressed, *ENPP1* has been shown to induce insulin resistance in cells, therefore appearing to be a physiologic modulator of insulin receptor function (Chandalia *et al.*, 2007).

Although the physiological function of *ENPP1* is not well characterised, it has been shown that *ENPP1* plays a role in the development of insulin resistance. *ENPP1* inhibits insulin signalling by direct interaction with the insulin receptor α subunit and reduces both insulin receptor function and subsequent downstream signalling. This glycoprotein is expressed in a wide range of tissue expression that includes skeletal muscle and the liver. Furthermore, protein levels of *ENPP1* are increased in insulin-resistant subjects and correlate with whole-body insulin resistance. In mice, over expression in the liver over induces insulin resistance and glucose intolerance, suggesting that this protein influences insulin sensitivity and thereby may affect the risk of developing type 2 diabetes (Barroso, 2005; Grarup *et al.*, 2006; Prudente & Trischitta, 2006; Chandalia *et al.*, 2007; Meyre *et al.*, 2007). A subsequent study in a Polish population confirmed that this genetic variant predicts diabetes in obese individuals (Bochenski *et al.*, 2006).

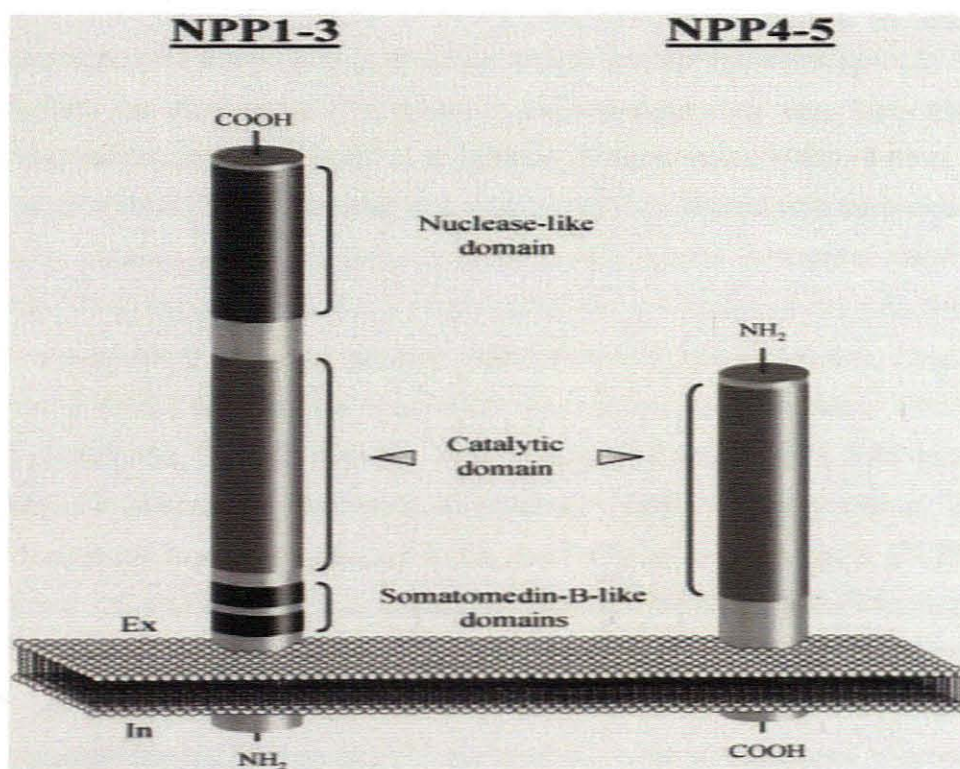


Figure 1.6: Domain structure and membrane orientation of NPP1-5(enpps1-5). In, intracellular; Ex, extracellular. For simplicity it is not indicated that NPP1-3 are actually homodimers (Bollen *et al.*, 2000)

Other studies in humans have shown a correlation between over expression of *ENPP1* and insulin resistance, obesity, and type 2 diabetes (Grarup *et al.*, 2006; Chandalia *et al.*, 2007), particularly the substitution of a lysine with a glutamine at codon 121 (K121Q). Furthermore, the minor Q allele of the K121Q variant of *ENPP1* has been shown to influence PC-1 protein

function by inhibiting insulin receptor function and insulin signalling more effectively than the major K allele (Maddux *et al.*, 2006). The mechanism through which *ENPP1* modulates BMI is unknown, however, it is hypothesized that the reported association with higher BMI is due to the fact that individuals carrying the Q121 variant develop insulin resistance in the brain, where insulin has potent anorectic action, and this in turn, increases appetite and eventually body weight (Pizzuti *et al.*, 1999; Constanzo *et al.*, 2001). Conversely, the reduced BMI in Q121 carriers might be due to the deleterious effect of this variant on peripheral insulin resistance, which, itself, has been reported in a prospective study to be a predictor of lower BMI, a possible consequence of impaired insulin-mediated lipid storage in adipocytes. Therefore, in some cases, genetic determinants improving insulin sensitivity have an opposite deleterious role on body weight and *vice versa* (Prudente & Trischitta, 2006).

The exonic K121Q amino acid substitution directly inhibits insulin receptor by a non-enzymatic mechanism; the other non-coding SNPs in the haplotype may have their effect by modifying gene expression, protein production, or splicing (Böttcher *et al.*, 2006). Indeed, the most potent effect of *ENPP1* on type 2 diabetes was found in French families with early-onset obesity and in Southern Indians, two populations showing severe insulin resistance (Meyre *et al.*, 2007). Barroso (2005) hypothesized that a single gene with pleiotropic effects was likely to predispose obese individuals to abnormal glucose metabolism and type 2 diabetes, based on findings by Meyre *et al.* (2005) and Maddux *et al.* (1995). Association studies between Q121 and features of insulin resistance, type 2 diabetes and obesity are inconsistent and have shown racial differences in susceptibility to increased adiposity (Table 1.4) (Meyre *et al.*, 2007). The K121 allele frequency has been reported to be much higher in Caucasians (79.9%) than African-Americans (25.4%) or Dominicans (45.8%). Although the K121 allele frequency was higher in Caucasians than African-Americans, the average BMI at each percentile was higher in African-Americans than in Caucasians. These racial differences suggest that other genetic and/or environmental factors beyond *ENPP1* are involved in the development of obesity (Matsouka *et al.*, 2006).

Other SNPs within *ENPP1*, namely rs997509 and rs9402349 located in the same haploblock as K121Q, have been investigated for their role in the development of obesity and/or type 2 diabetes. Co-existence of rs997509, located in the 3' end of intron 1 in a region that may contain a regulatory element, and K121Q was reported to be associated with type 2 diabetes among obese individuals (Bochenski *et al.*, 2006). The authors suggested that the polymorphic T-allele

of this SNP distinguishes one of the three 121Q-carrying haplotypes, which is strongly associated with type 2 diabetes among obese subjects and accounts for the association seen between the Q121 and type 2 diabetes among obese individuals. Recently it was reported that the rs997509 T-allele can predispose obese children to metabolic syndrome and impaired glucose tolerance (Santoro *et al.*, 2008). Thus far, no studies have found an association between the rs9402349 and obesity or type 2 diabetes (Bochenski *et al.*, 2006).

Table 1.4: Studies done on the K121Q variant for associations with obesity, insulin resistance, type 2 diabetes and metabolic syndrome

FINDINGS	POPULATION	REFERENCE
K121Q strongly associated with insulin resistance in healthy non-obese, non-diabetics.	Sicilians	Pizzuti <i>et al.</i> , 1999
Obese children had a higher frequency of the Q121 allele than non-obese controls.	French	Meyre <i>et al.</i> , 2003
K121Q SNP is not associated with the insulin resistance or type 2 diabetes risk, but associated with obesity.	Danish Whites	Grarup <i>et al.</i> , 2006
No association of the K121Q variant with obesity represented by BMI.	Europeans and Americans	Lyon <i>et al.</i> , 2006
K121Q associated with increased BMI.	Caucasians and African-Americans	Matsouka <i>et al.</i>, 2006
K121Q not associated with type 2 diabetes mellitus or any features of metabolic syndrome.	Chinese	Chen <i>et al.</i> , 2006
Confirmed that the K121Q genetic variant predicts diabetes in obese individuals.	Polish	Bochenski <i>et al.</i>, 2006
K121Q variant related to insulin resistance and type 2 diabetes development in different adult populations was associated with childhood obesity.	German	Böttcher <i>et al.</i>, 2006
Does not support the role of K121Q in the development of obesity or type 2 diabetes.	French	Meyre <i>et al.</i> , 2007
Association of K121Q with diabetes	Hispanics, African-Americans, and non-Hispanic whites	Chandlia <i>et al.</i>, 2007
Variants in the K121Q were associated with insulin resistance and type 2 diabetes.	Finnish	Willer <i>et al.</i>, 2007
Demonstrated that the Q121 allele was the only non-synonymous coding single nucleotide polymorphism (SNP) in a comprehensive analysis of all SNPs within ENPP1 associated with 'diabesity' phenotypes.	Mexican-Americans	Jenkinsons <i>et al.</i>, 2008
The Q allele of the K121Q was associated with hyperglycaemia and insulin resistance. A stronger association of K121Q with diabetes related quantitative traits in people with a higher BMI was also found.	Whites	Stolerman <i>et al.</i>, 2008
No association was found between K121Q variant and obesity or type 2 diabetes.	Finnish	Valli-Jaakola <i>et al.</i> , 2008

Studies that showed an association are marked in bold.

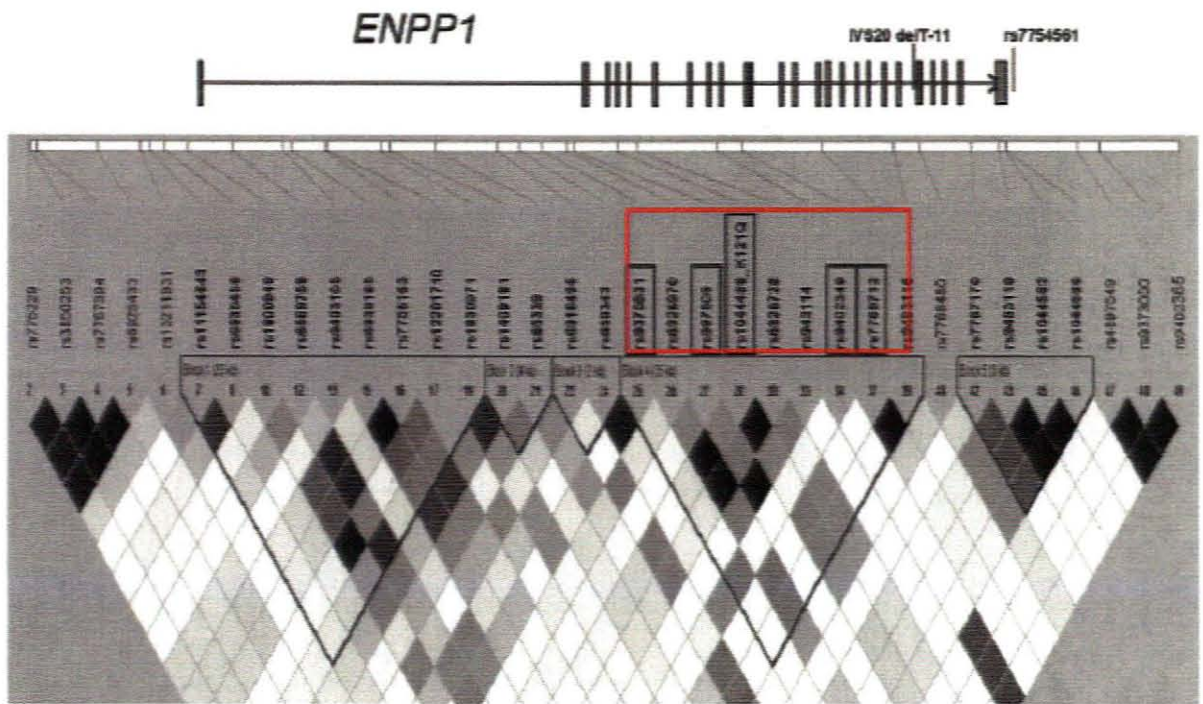


Figure 1.7: ENPP1 Haploview output. The haplotype block containing rs997509, rs9402349 and rs1044498 (K121Q) is highlighted in red (Bochenski *et al.*, 2006)

1.6 Complications of Obesity

Obesity affects almost every organ in the body. In public health terms however, the greatest burden of disease arises from obesity related morbidity (Jebb, 2004). Co-morbidity disorders, which arise from overweight and subsequent biochemical changes, actually predispose to additional co-morbidity such as cardiovascular diseases in early adulthood. A list of co-morbidities is depicted in Figure 1.8. Accumulation of fat in the abdominal area, particularly in the visceral fat compartment, is associated with increased risk of insulin resistance, diabetes, hypertension, dyslipaemia and atherosclerosis. Visceral adiposity is also the cornerstone of the metabolic syndrome, which is characterized by a clustering of cardiovascular risk factors (Goedecke *et al.*, 2006). Strong support for a causative role of visceral fat is associated with improved insulin sensitivity and delayed onset of diabetes when weight loss programme or treatment is initiated and successful (Gabriel *et al.*, 2002).

Though approximately 60-85% of obese children will stay obese in adulthood, childhood obesity, whether or not it persists in adulthood is thought to increase the risk of subsequent morbidity (Kiess *et al.*, 2006). Similar to the findings in adults, intra-abdominal adipose tissue in obese children has a significant relationship with adverse health conditions. Already in childhood about

60% of overweight 5-10 year old children were found to have at least one cardiovascular risk factor and 25% presenting with two or more risk factors (Goedecke *et al.*, 2006).

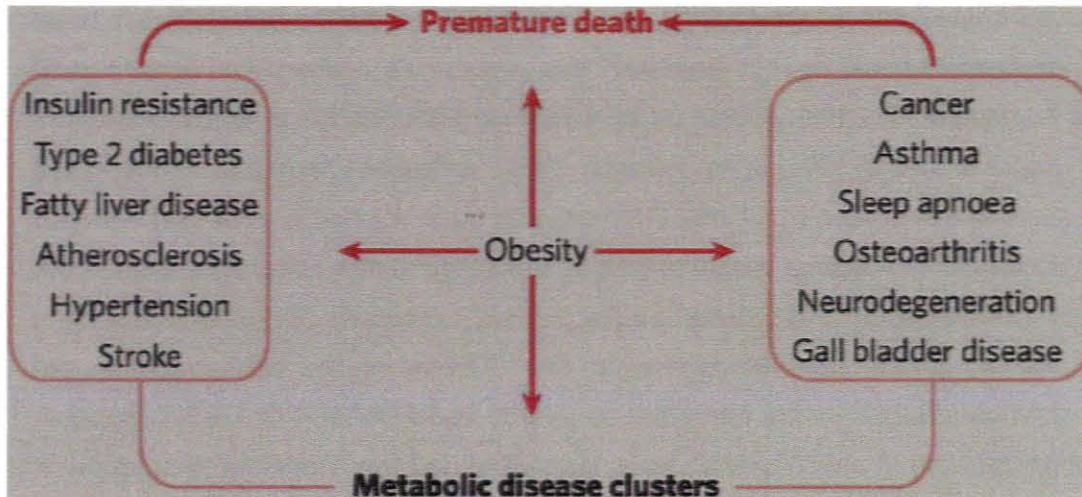


Figure 1.8: Clustering of metabolic diseases. Obesity is considered to be a central feature that increases the risk for a vast array of diseases, with significant morbidity and mortality. In general, the mechanistic basis of the link between obesity and the diseases listed on the right is poorly understood compared with that of those listed on the left (Hotamisligil, 2006)

1.7 Metabolic Syndrome

The Metabolic Syndrome (MetS) is a multiplex risk factor for cardiovascular diseases (CVD). Its prevalence is increasing rapidly in many countries. This increased occurrence is due in a large part to the “epidemic” of obesity in both developed and developing countries. Metabolic syndrome has been reported to predispose adults to an increased risk for type 2 diabetes mellitus and coronary heart disease, and this poses a serious problem as childhood MetS is thought to persist through to adulthood (Bao *et al.*, 1994; Isomaa *et al.*, 2001; Lakka *et al.*, 2002). Despite the observation of varying prevalence rates caused by the use of different definitions, there exists consensus that the metabolic syndrome develops in childhood and is highly prevalent in obese children (Cook *et al.*, 2003; Cruz & Goran, 2004) In the (National Health and Nutrition Examination Survey (NHANES) III study, the prevalence of the metabolic syndrome was 28.7% in overweight adolescents (BMI > 95th percentile) and 0.1% in those with a BMI below the 85th percentile (Cook *et al.*, 2003). In South Africa, obesity in children and adolescents has been shown to be increasing with rates up to 20% among 1-19 year olds (South African Society for the Study of Obesity, 2001). The obesity phenotype is however not similar in all ethnic groups. In South Asians, metabolic syndrome components have been observed at lower BMI and waist circumference levels considered to be normal (Whincup *et al.*, 2002). Black

African girls have been reported to be more vulnerable to overweight and obesity, but with a lower prevalence of the metabolic syndrome (Chen *et al.*, 1999; 2000; Cook *et al.*, 2003).

MetS definitions have been 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). An example of differences between two such definitions is summarised in Table 1.5. These levels of waist circumference are usually associated with a significant degree of insulin resistance. Elevations of triglycerides and reductions of HDL cholesterol are indicative of atherogenic dyslipidaemia. Most patients with higher triglycerides and lower HDL with or without elevated fasting glucose will be insulin resistant. NCEP ATP III identified obesity as the major driving force underlying the increasing prevalence of metabolic syndrome (Grundy *et al.*, 2004). There is growing evidence that both acquired and genetic factors contribute to metabolic syndrome, although others, such as, physical activity, also appear to be involved. As only about a third of people with obesity develop metabolic syndrome, it is likely that other factors, such as genetics, may also play a role (Hill *et al.*, 2000).

Table 1.5: Examples of Metabolic syndrome definitions used in children

<u>The IDF ages 10 – 16, (Zimmet <i>et al.</i>, 2007)</u>		<u>The NCEP ATP III, (Cook <i>et al.</i>, 2003)</u>
Central obesity (WC)	> 90th percentile	<u>Any 3 or more of the following:</u> > 90th percentile, age gender specific
<u>And any 2 or more of the following:</u>		
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)

1.8 Significance and Objectives of the Study

The global burden of overweight and obesity is both significant and rising, with most of the increase reported over the last decade. Childhood obesity has negative health implications with approximately 60% of overweight 5-10 year old children presenting at least one associated cardiovascular risk factor and 25% presenting two or more risk factors. While attention has previously been focused on under-nutrition in South African children, survey data suggests overweight and obese prevalence rates of South African children are fast approaching those in the developed world. In South Africa, several reports have demonstrated the relationship between obesity and environmental factors, yet no studies have reported on the genetic aetiology of obesity.

ENPP1 is a promising candidate gene for early onset obesity and related sequel. Children represent particularly an interesting study population for identifying primary genetic determinants involved in susceptibility to complex polygenic diseases such as obesity and related traits, because unlike adults, phenotypes are less influenced by co-morbidities and prolonged exposure to environmental factors. The overall objective of this study was to assess whether *ENPP1* polymorphisms contribute to childhood obesity/overweight and/or components of metabolic syndrome 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 can offer holistic opportunities for prevention and intervention programmes. The specific aims of this study were as follows:

- To screen obese and normal weight (controls) Mixed Ancestry children for the presence of **rs997509**, **rs1044498 (K121Q)** and **rs9402349** SNPs of the *ENPP1* gene by PCR amplification and semi-automated DNA sequencing analysis. Of the SNPs identified on the *ENPP1* gene, only the **rs997509** and **rs1044498 (K121Q)** have been shown to be associated with metabolic syndrome.
- To assess metabolic components of the study population, that is, fat distribution, blood pressure, lipid and fasting blood glucose levels.
- To investigate whether a relationship between metabolic components and *ENPP1* SNPs in both obese and control subjects existed.

CHAPTER TWO
MATERIALS AND METHODS

2.1 Ethical Approval

This study was conducted at Cape Peninsula University of Technology and Chemical Pathology Division at the University of Stellenbosch, Cape Town, South Africa. Ethical Approval was obtained from the Research Ethics Committee of the University of Stellenbosch (Project number: N07/07/160) and from the Faculty of Health and Wellness Science Ethics Committee, Cape Peninsula University of Technology (Project Reference number: CPUT/HAS-REC 0016).

2.1.1 Informed Consent

Written informed consents were obtained from the Western Cape Department of Education, School Governing Bodies and principals, and parents. Oral briefings were conducted with teachers, learners and their parents, to introduce the study. Written consents were obtained from parents of all subjects participating in the study. Oral assent from participants was compulsory on the day the sampling was done. Participants had a right to decide at any point to terminate their participation, refuse to give information, or to ask for clarification about the purpose of the study or the specific procedures to be performed. Therefore, participants had the right to voluntarily decide whether to participate in the study, without the risk of incurring penalties or prejudicial treatment. In light of this, a letter describing the study and requesting participation was sent to each potential participant and their parents. All consent forms were translated into Afrikaans and Xhosa, to ensure that all information was clearly understood by the subjects and their parents or guardians.

All individuals were given a written copy of their own clinical (weight status) results with an interpretation thereof. Individuals' results were confidential and personal information was only released with the participants' consent where the need arose. All subjects with abnormal results were offered referral to the appropriate centre for further evaluation. Progress reports and a final report were provided to the Western Cape Department of Education for their comment. Acknowledgements of all funding sources were included in all reports.

2.2 Research Design

The research design was quantitative. 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).

2.2.1 Study Population

The study population consisted of a total of 1564 (males = 621; females = 943) learners between the ages 8-18 years that 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 randomization took place at the learner and class levels. The study was limited to government funded schools, and a list of all the schools visited was obtained from the Western Cape Department of Education. The reason for choosing the specified areas was because the schools in these areas are economically and racially diverse, representing all socio-economic groups from low to high income communities. The age group was chosen for practical and physiological reasons. At age six the adiposity rebound occurs, and therefore obesity has a better predictive value in later childhood and adulthood (Rolland-Cachera *et al.*, 2002). Schools with a higher number of pupils were preferred.

2.2.2 Selection Criteria of the Study Population

2.2.2.1 Inclusion Criteria

- All consenting children were included in the study. The study population consisted of both male and female learners of different ethnicities aged 8- 18 years who consented to genetic analysis.
- Participants of Mixed Ancestry (Coloureds) were selected for genetic analysis due to the high number of obese individuals as compared to their White and Black counterparts.

2.2.2.2 Exclusion Criteria

- 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 group.
- Participants with other metabolic syndrome risk factors such as diabetes, hypertension, and high blood pressure were excluded as these might have interfered with analysis of results.
- Participants, who were found to have incomplete data.

2.2.3 Case Group

The study population was categorized into obese and overweight, based on the International References (gender-age specific cut-off points) provided by the International Obesity Task Force (IOTF) as developed by Cole and co-workers (2000). For the purpose of this study, only the obese group was used as cases for the analysis. The body mass index (BMI) in childhood is based on international data and linked to the widely accepted adult cut-off points of 25 and 30 kg/m² (Cole *et al.*, 2000). The method was developed in accordance to the changing BMI with age in childhood due to variability in hormonal levels. The BMI median in children and adolescents varies from 13 kg/m² (at birth) to 17 kg/m² (at age 1), and from 15.5 kg/m² (at the age of 6) to 21 kg/m² (at 21 years of age). In contrast to the Centers for Disease Control and Prevention (CDC) criteria, the IOTF cut off points were developed from an internationally representative survey of six countries, which included Brazil, Britain, Hong Kong, The Netherlands, Singapore and the USA (Cole *et al.*, 2000). The United States National Cholesterol Education Programs (NCEP) Adult Treatment Panel III (ATP III) criterion was used to diagnose metabolic syndrome. This proposed that for a diagnosis to be made, a person must have three of the five characteristics below:

- Abdominal obesity (elevated waist circumference)
- Serum triglycerides \geq 150 mg/dl
- Serum HDL cholesterol $<$ 40 mg/dl in men and $<$ 50 mg/dl in women
- Blood pressure \geq 130/85 mm.Hg
- Plasma glucose \geq 110 mg/dl

2.2.4 Control Group

The control group was comprised of individuals with normal BMI according to the IOTF criteria that were age-, gender-, and ethnic matched to the case group. The control group was selected from 1564 learners that were recruited as mentioned above.

2.2.5 Sample Size

The study population consisted of learners aged 8-18 years, who were randomly and proportionally recruited from primary and high schools in the northern suburbs of Cape Town and its adjacent rural areas. The 1:1 case: control ratio design gives adequate power ($>$ 80%) if the frequency of the minor allele in a general population is 10% and in a case group is 33%. This corresponds to an odd ratio of 4:4. The sample size for the proposed study was calculated based on the number of participants

that assented to participate, and the overall prevalence of obesity/overweight obtained in the study done by Somers (2004). According to this study the overall prevalence of overweight and obesity was 15.7% and 6.2%, respectively. The response rate was 65% and learners that declined to participate were approached twice personally. Of the 1564 learners sampled, 110 (whites =10; blacks =36; coloureds = 64) were found to be obese. The Coloured population was chosen for genetic analysis, reason being the high number of obese individuals within the population as compared to other population groups.

In order to successfully analyze association studies, the sample size and statistical power of the study need to be evaluated carefully. When designing these studies, the sample size needs to be sufficiently large to create enough statistical power to reduce the probability of generating false-positive associations. (Berry *et al.*, 1998)

2.3 Data Collection

One school was visited per week to collect research materials that included anthropometric measurements, biochemical analyses, and interviews about learner's lifestyles which were recorded in the form of questionnaires (Appendix C). Subjects were requested to fast for 10 hours prior to sampling.

2.3.1 Questionnaire Design

All questionnaires were developed in English and then translated into Afrikaans and Xhosa. They were designed to contain adequate indicators on the family history of diabetes, lifestyle, behaviour, dietary patterns, physical activity, leisure activities and exercise of participants. All questionnaires were based on existing ones (Somers, 2004), which were pre-tested in a pilot study with respect to the ability of respondents to comprehend and respond to questions well. Questionnaires were pre-tested in a random small sub-sample of learners. Learners chosen for this pre-test were the exact representation of the study population (Somers, 2004). To ensure the reliability of answers received from the questions asked in the family health-related history sections and Physical Activity & Energy Expenditure sections, questions were re-administered to the same learners a week after initial administration in a sub group. This was also used as a measure to further strengthen the study (Appendix C).

2.3.2 Measurements

Parameters of interest that were used included Anthropometric measurements (body weight and height; waist and hip circumference & waist-hip ratio and skinfold measurements), blood pressure, and biochemical analysis (blood glucose and lipid levels). Raw anthropometric data of body weight and height was used to calculate BMI.

2.3.3 Anthropometric Measurements

2.3.3.1 Weight and Height Measurements

- Weight

Weight measurements were done using a digital bathroom electronic scale. All heavy clothing and shoes were removed. The scale was calibrated and standardized 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. 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 erect position. The required accuracy was 0.1 cm (Martin *et al.*, 1988).

- Body Mass Index (BMI)

The BMI was calculated for each subject as:-

$$[\text{Weight (kg)/height (m}^2\text{)}]$$

The weight status of each subjects was determined by the BMI, and individuals were classified using International Reference (gender-age cut-offs) provided by the International Obesity Task Force (IOTF) as developed by Cole and co-workers (2000).

2.3.3.2 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 the erect position, abdomen relaxed, arms at the sides and feet together. The measurer performed the measurements facing the subject, and in the horizontal plane, placed the tape measure 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 measurer 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 was 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.

2.3.3.3 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 the case of the obese, it may be impossible to elevate a skinfold with parallel sides. Thus in these circumstances, the measurement was not taken or the two-handed technique was used. In this technique one measurer lifts the skinfold using two hands and another measures the skinfold.

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 tricep 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-iliac**

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. (Barlow and Dietz, 1998).

2.3.4 Blood Pressure (BP) Measurements

Blood pressure measurements were performed using a semi-automatic digital blood pressure monitor (Rossmax PA, USA) on the right arm in sitting and ambulatory position. After a 10 minute rest period, three readings were taken at five minute intervals and the lowest of the three readings was recorded and taken as the blood pressure.

2.3.5 Biochemical Analysis

- **Glucose Measurements**

Blood glucose was analyzed using the Accutrend® GCT meter (Roche, South Africa). Capillary blood was obtained through finger pricking, using a new lancet for each subject. Blood drops

were applied to the test strips outside the meter. The meter was disinfected with alcohol after each subject was tested. This was done to avoid contamination and infection.

The impaired fasting glucose was determined using the normal threshold values ranging from 5-7 mmol/l that were set according to the American Diabetes Association (ADA) criteria. The values greater than 7 mmol/l, was diagnostic of diabetes.

- **Lipid Measurements**

Lipid measurements were analyzed using Cardiocheck™ PA point of care analyzer according to the manufacture's instructions. The accuracy and precision of Cardiocheck™ PA Analyzer had been found to be reliable and comparable to laboratory based methods for the measurement of lipids (Vanessa *et al.*, 2005).

2.3.6 Data Management

Each participant was assigned a unique code that was used for confidentiality purposes and all data collection documents (questionnaires) and biological specimen containers reflected this code. Data was captured on an Excel Spreadsheet at the end of each day of sampling. All consent forms with the same code as the questionnaire were attached together to minimize errors that may arise. Both consent forms and questionnaires were then stored in confidential files and securely locked away for follow-up studies. The entire database, questionnaires and laboratory test results were entered into statistical package STATA version 7. (STATA 7.0 Copyright 1984-1999 Stata Corporation. Texas USA 800-STATA-PC, Serial number: 1960514508).

2.4 DNA Extraction

Genomic DNA was extracted from whole blood contained in vacutainer Ethylenediamine Tetra-acetic Acid (EDTA) tubes, and from Whatman FTA® Cards (Merck Laboratories, United Kingdom) containing capillary blood.

2.4.1 DNA Extraction from Whole Blood

2.4.1.1 Blood Collection

Specimen collection was done according to Standard Operating Procedures (SOP). Subjects were requested to sit and relax for 15 minutes prior to specimen collection. Venous blood was drawn from the cubital vein with the arm at an angle of $\pm 45^\circ$ downwards from the horizontal plane. Subjects were requested not to assist in blood flow promotion, i.e. pumping of the muscles of the hand. An alcohol swab was used to disinfect the skin, and then the skin was allowed to dry before venesection. Following this, a tourniquet was applied to the arm for no longer than a minute to limit variation in the concentration of the blood. All blood samples were collected in Ethylenediamine Tetra acetic Acid (EDTA) tubes and then mixed with the additive by inverting the tube a few times. Samples were transported routinely at room temperature and stored at -20°C when immediate extraction was not possible.

2.4.1.2 Extraction of DNA

DNA was extracted using a modified salting out procedure (Miller *et al.*, 1988). A volume of 5-8 ml of whole blood contained in the Ethylenediamine Tetra-acetic acid (EDTA) tube was emptied into a 50 ml Falcon tube. Cold lysis buffer (Appendix A) 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 for ten minutes at 4°C (J-6M/E centrifuge, Beckman, United Kingdom). The supernatant was carefully discarded and the pellet (white blood cells) washed with 0.9% Phosphate Buffered Saline (PBS) (Appendix A). This solution was then centrifuged at 1500 rpm for ten minutes after which the supernatant was discarded and the pellet resuspended in nuclear lysis buffer (Appendix A), to break the nuclear membrane; 0.3 mg/ml Proteinase K for proteolysis, and 1% (w/v) Sodium Dodecyl Sulphate (SDS) (Appendix A), for breaking down the lipid layer of the cell membrane. The contents were then mixed well and incubated overnight, in a water bath at 55°C . Thereafter, 6 M NaCl (Appendix A) was added to the solution and the tubes shaken vigorously for 1 minute. The mixture was centrifuged at 2500 rpm for 30 minutes. At this point, the salt binds to the proteins and other cellular debris and precipitates to form a white pellet. The supernatant now contains the DNA which was subsequently transferred to a clean Falcon tube, and the pellet discarded. The supernatant was then vortexed for 15 seconds followed by a centrifugation of 15 minutes at 2500 rpm. 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 is insoluble in ethanol; as a result all the strands will be distributed throughout the ethanol. The DNA was "spooled" or "fished-out" using a sterile pipette tip, placed in a clean 1.5 ml eppendorf tube, and washed with cold 70% (v/v) ethanol. The 30% water component within the 70% ethanol will remove the excess salt and ethanol will preserve the DNA. The tubes were centrifuged using a benchtop microcentrifuge (Microcentrifuge® Lite, Beckman Coulter™). The 'washing step' was repeated until the pellet was clear. The ethanol was discarded, tubes covered with pierced parafilm, and left at room temperature to dry. Depending on the size of the pellet, 200-800 µl of 1X TE buffer (Appendix A) 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 warm (55°C) TE buffer can be used to dissolve the DNA pellet. In cases where the spooling procedure did not produce a good yield, the DNA from the sample was precipitated overnight at -20°C. After centrifugation at full speed for 1 hour, the supernatant was discarded and the pellet was dissolved in 1X TE buffer.

2.4.2. DNA Extraction from Whatman FTA card

Capillary blood from a finger prick was collected on circles of Whatman FTA cards, 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.

A Uni-Core punch was used to remove one sample disc from desired sample spots. The sample disc was then placed in a flat-cap PCR amplification tube. Two hundred microlitres of Whatman FTA purification reagent as per manufacturer's instructions was added to the PCR tube containing the Whatman FTA disc. The tube was incubated for five minutes at room temperature, while being given moderate manual mixing. The FTA purification reagent was then discarded by pipeting it out using sterile tips. This washing step was repeated twice for a total of three washes. As an additional washing step, cold 1X TE buffer (Appendix A) was added to the tube. The solution was incubated for five minutes at room temperature, followed by discarding of the TE buffer as explained previously. This step was repeated at least once for a total of two washes. Discs in the PCR tube were allowed to dry at room temperature for about an hour. The washed and dried FTA discs were used as templates in a PCR for amplifying *ENPP1* fragments. The manufacturer recommended a 1.2 mm disc for use in a 25 µl PCR reaction mixture and a 2.0 mm disc in a 50 µl reaction mixture.

2.5 DNA Quantification

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 ng/ µl. The DNA samples, where possible, were diluted to obtain a final concentration of not more than 200 ng/ µl. Also measured was the quality and purity of a nucleic acid sample by measuring the ratio of absorbance at 260 nm and 280 nm. The 260/280 ratio for good quality, purified DNA is ~1.8. A deviation in this ratio is normally indicative of contaminants, such as salts, in a sample.

2.6 Molecular Analysis of Single Nucleotide Polymorphisms (SNP) in the Ectonucleotide Phosphatase Phosphodiesterase 1 (*ENPP1*) gene

2.6.1 Oligonucleotide Primers

All primers (Table 2.1) were designed using Primer3plus (<http://www.primer3plus.com>). Also linked to Primer3 is the National Centre for Biotechnology Information (NCBI) and Basic Length Alignment Search Tool (BLAST) that can be used to assess possible hybridization of primers to other regions in the human genome. The oligonucleotide primers were synthesized and purified with HPLC at the Department of Molecular and Cell Biology, University of Cape Town (UCT), South Africa.

Table 2.1: Oligonucleotide primer sequences in the five prime (5') to three prime (3') direction

Primer name	Primer Sequence (5'-3')	Length (Bases)	Annealing Temperature	Product size
rs997509				
ENPP1 – Forward	TCC TTC AGT GTA TAA CAG TCT TTG C	25	49,2°C	249 bp
ENPP1 – Reverse	CCC ATT CTC CAC TCT TCT GG	20		
rs1044498				
ENPP1 – F	ACT TTG GAC ATG TTG AAT TTG AGA C	25	54°C	179 bp
ENPP1 – R	ACA CAC AGA ACT GTA GTT GAT GCAG	25		
rs9402349				
ENPP1 – F	TTC CTC TGG ACA CAG GCT TT	20	59,9°C	248 bp
ENPP1 – R	GAG GTG GAG ATT GCA GTG AA	20		

2.6.2 Polymerase Chain Reaction (PCR)

The PCR mixture for the Single Nucleotide Polymorphisms (SNPs) rs997509 and rs9402349 was prepared using the Sureband PCR Optimizing Kit (Bio-X-ACT™ Short DNA Polymerase, Bionline Ltd. London, England) as illustrated in the PCR protocols following. The Sureband PCR Optimizing Kit contains 12 different buffers (labelled A-L) and a shortband *Taq* polymerase. Of the 12 buffers, only one (Buffer G), successfully amplified the two regions of interest. The buffer used for amplifying each target region of the gene contained: Magnesium chloride (MgCl), dNTPs and some additives, of which concentrations are only known to the manufacturer (Bionline Ltd. London, England). A standard primer concentration of 0.24 μ M was used for all reactions that were carried out.

Cycling conditions used for amplifying all 3 fragments using the Perkin Elmer thermal cycler, GeneAmp® 2720 (Applied Biosystems) varied between SNPs. The correct buffer (G) was used to amplify the target region of the two SNPs (rs997509 and rs9402349). The rs1044498 was amplified using the BIOLINE PCR kit. Tables below illustrate the PCR conditions used for amplifying each fragment.

2.6.2.1 PCR set-up for rs997509

Reaction mixture

Forward primer	0.24 μ M
Reverse primer	0.24 μ M
Template (Whatman FTA disc)	1.2 mm disc
Genome blood	200 ng/ μ l
Shortband <i>Taq</i> Polymerase	0.6 units
Buffer (G)	10X
Sterile distilled water	-
Total volume	25μl

Primer pair	PCR conditions	Product size	
F: 5'- TCC TTC AGT GTA TAA CAG TCT TTG C- 3'	94 °C: 5 min (initial denaturation)	249 bp	
R: 5'- CCC ATT CTC CAC TCT TCT GG- 3'	94 °C: 30 sec		45 cycles
	49, 2 °C: 30 sec		
	72 °C: 30 sec		
	72 °C: 7 min (final extension)		

F= forward primer; R= reverse primer

2.6.2.2 PCR set-up for rs1044498

Reaction mixture

Forward primer	0.24 µM
Reverse primer	0.24 µM
MgCl ₂	2.5 mM
dNTPs	10 mM
Buffer	10X
Taq DNA polymerase	0.6 units
Template (Whatman FTA disc)	1.2 mm disc
Genome blood	200 ng/ µl
Sterile distilled water	-
Total volume	50µl

Primer pair	PCR conditions	Product size	
F: 5'- ACTTTGGACATGT TGAATTTGAGAC- 3'	94 °C: 5 min (initial denaturation)	179 bp	
R: 5'- ACACACAGAACTG TAGTTGATGCAG- 3'	94 °C: 30 sec		35 cycles
	54 °C: 30sec		
	72 °C: 30 sec		
	72 °C: 7 min (final extension)		

2.6.2.3 PCR set-up for rs9402349

Reaction mixture

Forward primer	0.24 μ M)
Reverse primer	0.24 μ M)
Template (Whatman FTA disc)	1.2 mm disc
Genome blood	200 ng/ μ l
Shortband Taq Polymerase	0.6 units
Buffer (G)	10X
Sterile distilled water	-
Total volume	25μl

Primer pair	PCR conditions	Product size
F: 5'- TTC CTC TGG ACA CAG GCT TT- 3'	94 °C: 5 min (initial denaturation) 94 °C: 30 sec 59, 9 °C: 30 sec 72 °C: 30 sec 72 °C: 7 min (final extension)	35 cycles 248 bp
R: 5'- GAG GTG GAG ATT GCA GTG AA -3'		

2.7 Agarose Gel Electrophoresis

Two percent (2%) agarose gels were used for visualizing and sizing the PCR products accordingly. The gel was prepared by weighing 1 g of agarose powder (Whitehead Scientific, South Africa) and mixing/dissolving it in 50 ml 1x Sodium Borate (SB) buffer (Appendix A). The solution was heated in a microwave oven for approximately 1 minute, to dissolve the powder, and thereafter, allowed to cool to approximately 60°C. The agarose solution was then poured in a 7x15 cm-casting tray (Bio-Rad Laboratories, South Africa) containing a sample comb and allowed to set at room temperature. (Sambrook & Russel, 2001).

After the agarose gel had set and still in its casting tray, the comb was carefully removed and the gel inserted into a horizontal electrophoresis tank containing the running buffer (1x SB

buffer) that is enough to cover the gel. PCR products (8 µl) were mixed with 2 µl loading dye (Appendix A) and pipetted into the wells, lids and electrodes connected to the tank, and a current applied (a voltage of 160V). (Davis, 1991). The SB buffer has a low conductivity; therefore, a much higher voltage could be used allowing for shorter electrophoresis time.

Following electrophoresis, amplicons were visualized by staining the gel with ethidium bromide solution (Appendix A) for 10-15 minutes and exposed to the ultraviolet (UV) transilluminator GelDoc™ XR System (Bio-Rad Laboratories). The ethidium bromide will intercalate into the DNA and fluoresce as reddish-orange and distinct bands of DNA will become visible. A gel photograph was taken using a digital camera incorporated in the GelDoc System.

2.8 PCR Product Purification

Purification of the PCR product is necessary to ensure good template quality for sequencing to be carried out. The purification step removes all excess debris (primers, dNTPs, etc) that were used during the PCR process. There are many techniques that can be used to purify the PCR product. In the present study the Shrimp alkaline phosphatase (SAP) - exonuclease purification method was used to purify the PCR products as described by (Nordström *et al.*, 2000). Briefly, the product is purified of excess dNTPs and primer, as these may interfere with the sequencing process. Shrimp alkaline phosphatase (SAP) and Exonuclease I enzymes remove these materials. Both are active in the PCR buffer and are, thus, added directly to the PCR products. Exonuclease I removes residual primer and any extraneous single-stranded DNA. The SAP removes the remaining dNTPs.

2.9 DNA Sequencing and Sequence Analysis

Sequencing was done using the Big-Dye Terminator Sequence Ready Reaction Kit version 3.1 (Applied Biosystems) and products electrophoretically separated on a 3130xl Genetic Analyzer (Applied Biosystems) at Inqaba Biotechnology. The sequencing data was analysed using the Sequencing Analysis version 5.2 (Applied Biosystems) and Finch 4.0 software programs. (Inqaba Biotechnical Industries, South Africa).

2.10 Statistical Analysis

An Excel spreadsheet (Excel 2000, Microsoft Corp) and a statistics programme, STATISTICA (STATISTICA 7, StatSoft Inc 1984 – 2004) were used to perform all statistical analyses. Descriptive data are presented as Means and Standard Error. For categorical data the One-way ANOVA -Bonferroni test was used to analyse subjects with a single categorical independent variable. Results were considered significant if p -values were less than 0.05.

All single nucleotide polymorphisms (SNPs) used in the present investigation were analysed for deviation from Hardy-Weinberg equilibrium using *Haploview version 4.1* (www.hapmap.org). Haploview 4.1 was also used to construct linkage disequilibrium plots for the investigated SNPs. The possible association between the respective markers and each of the quantitative traits investigated were scrutinized using *PowerMarker version 3.25* (www.powermarker.net). This software package was also used to carry out genotype and allelic association analyses.

2.11 Quality Control and Reliability of Results

To ensure reliability and validity of data, and also to ensure the safety of participants, all field workers and health professionals received intensive training prior to any data collection. Trained health professionals assisted with all measurements using standardized techniques. Standard protocols for the sampling of specimens were provided to all field workers. Calibration of instruments was performed on a regular basis and recorded as a measure of enhancing the quality of the study. All the digital instruments were calibrated every morning of each sampling day. In addition to standard quality control procedures, samples were randomly selected for laboratory analyses to confirm the accuracy of results obtained using the point of care instruments. All abnormal results were repeated and further confirmed in a laboratory (Chemical Pathology, National Health Laboratory Services, Tygerberg Hospital).

With respect to DNA isolation, standard control measures to avoid false positive results were applied strictly. To prevent the occurrence of carryover contaminations in the preamplification stage (DNA extraction from FTA cards), the puncher was washed thoroughly with 70% ethanol and a blank disc punched in between samples. Pipette tips were changed for every sample and every wash step. When all molecular analysis (PCR) was performed, a negative control was run with every batch. A negative control (PCR mixture with distilled water used as a template) was

included in every PCR run. PCR reactions and sequencing was done in batches of 10 to minimize errors. Both reverse and forward sequencing were done.

CHAPTER THREE

RESULTS

3.1 Clinical Characteristics of Participants

The study population was randomly selected from another study which had 1564 participants (males = 621; females = 943) learners between the ages of 8-18 residing in the northern suburbs and its adjacent rural areas of Cape Town. Obesity was assessed using gender-age specific cut-off points international references provided by the International Task Force as developed by Cole and his co-workers (2000). The overall prevalence of obesity in the study population was found to be 7% and was higher in females than males, 9.2% and 4.5%, respectively. Obesity was highest amongst the Coloured population 7.3% (64), followed by Whites 6.8% (10), then by Blacks 6.7% (36). In the present study all 64 obese Coloureds were selected for genetic analysis. Sixty four age and gender matched controls at a 1:1 ratio were also included.

The clinical characteristics of participants are summarised in Table 3.1 and significant differences between cases and controls are illustrated in Figures 3.1a-g. As expected the anthropometric measurements were significantly higher in obese subjects. Significant differences were also noted for fasting blood glucose, systolic and diastolic blood pressures. Lipid levels did not differ significantly; however, both female cases and controls had higher HDL-cholesterol whilst triglycerides of male controls were higher than those of the females.

Table 3.1: Clinical characteristics of participants

	Controls		Cases	
	Female	Male	Female	Male
Participant numbers	48	16	48	16
Age	12.92 ± 2.74	13.19 ± 2.66	13.21 ± 2.66	13.19 ± 2.59
Cholesterol	3.77 ± 1.16	3.39 ± 0.85	3.70 ± 0.71	3.42 ± 0.67
Triglycerides	0.80 ± 0.53	0.77 ± 0.25	0.80 ± 0.31	0.92 ± 0.43
HDL cholesterol	1.14 ± 0.38	0.98 ± 0.32	0.93 ± 0.33	0.72 ± 0.20

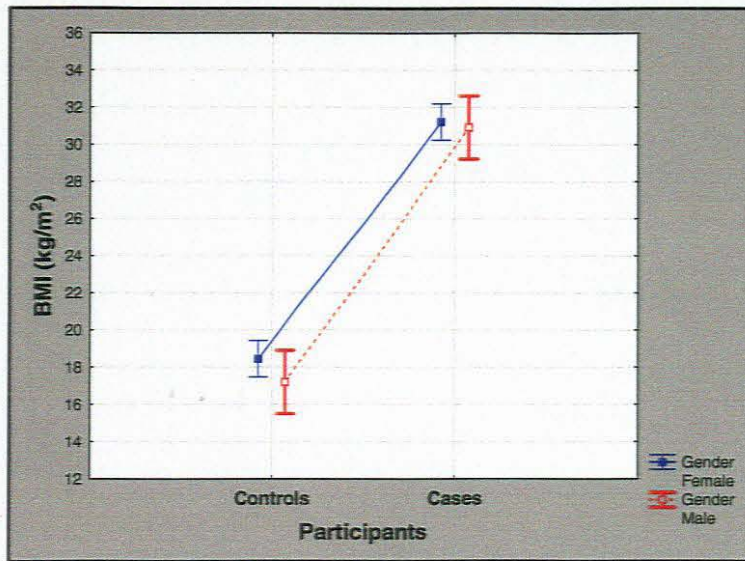


Figure 3.1a: BMI. In both females and males cases, BMI was significantly higher than controls, $p = 0.01$

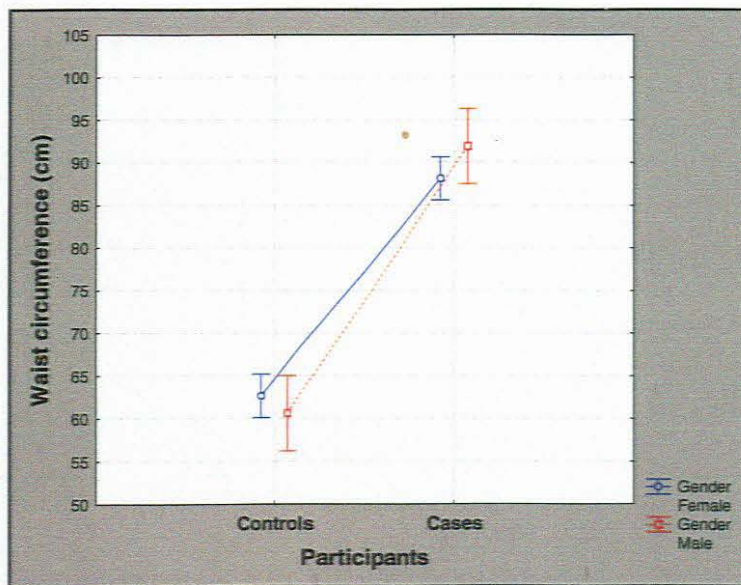


Figure 3.1b: Waist circumference. Significant differences between female controls and cases, $p = 0.01$, as well as between male controls and cases are shown, $p = 0.01$

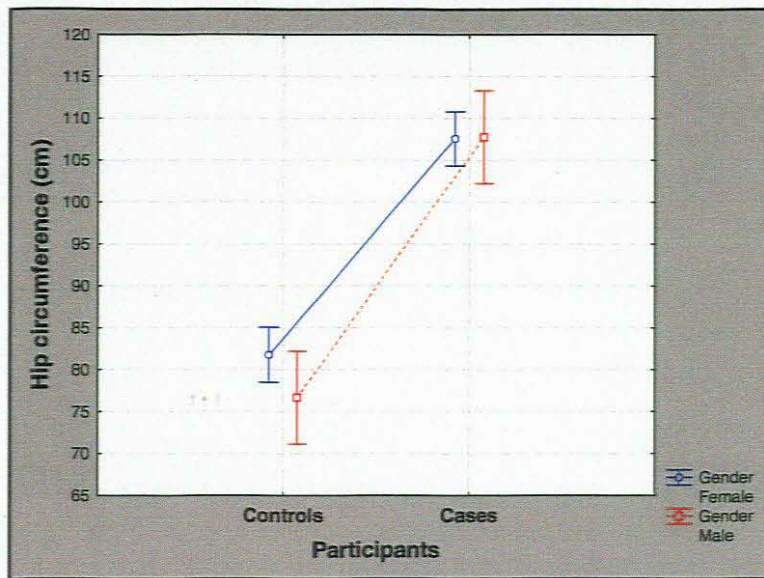


Figure 3.1c: Hip circumference. Significant differences between female controls and cases $p = 0.01$, as well as between male controls and cases are shown, $p = 0.01$

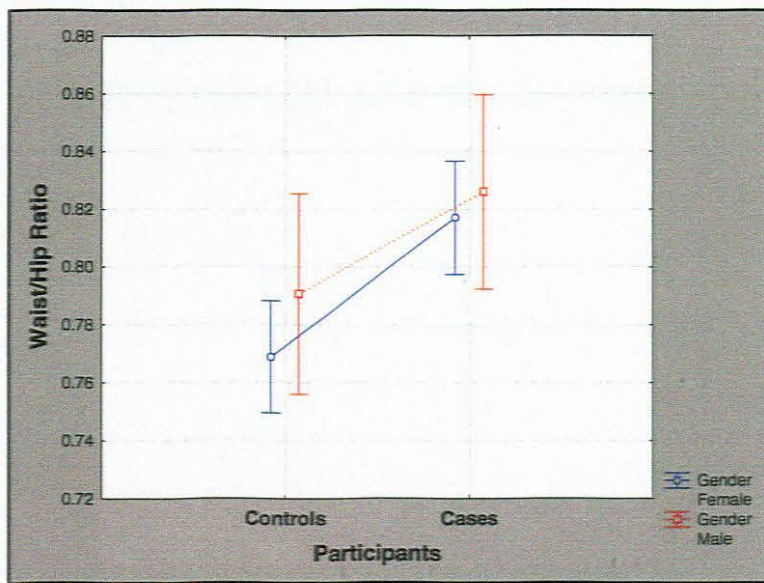


Figure 3.1d: Waist/Hip Ratio. Significant differences between female controls and cases were found $p = 0.01$

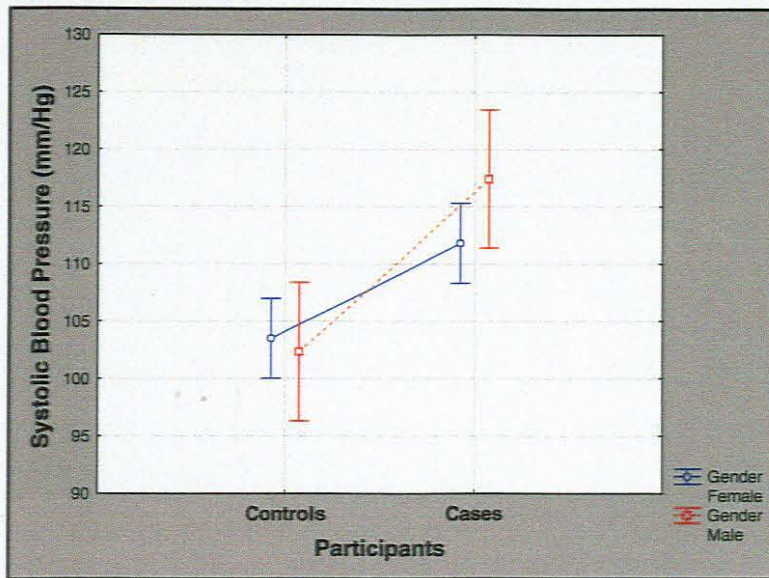


Figure 3.1e: Systolic Blood Pressure. Significant differences between female controls with both female and male cases $p = 0.01$, significant difference between male controls and female and male cases are shown, $p = 0.01$

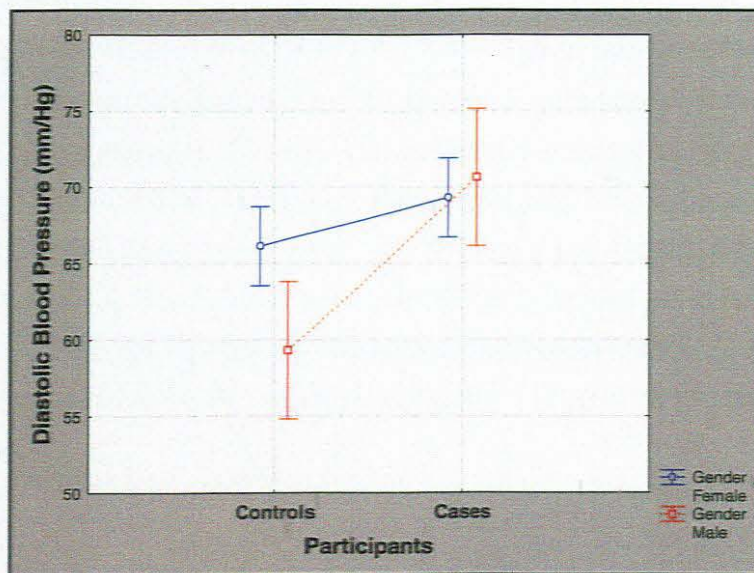


Figure 3.1f: Diastolic Blood Pressure. Male controls are significantly lower than male cases $p = 0.01$, and also with female controls and cases; $p = 0.06$

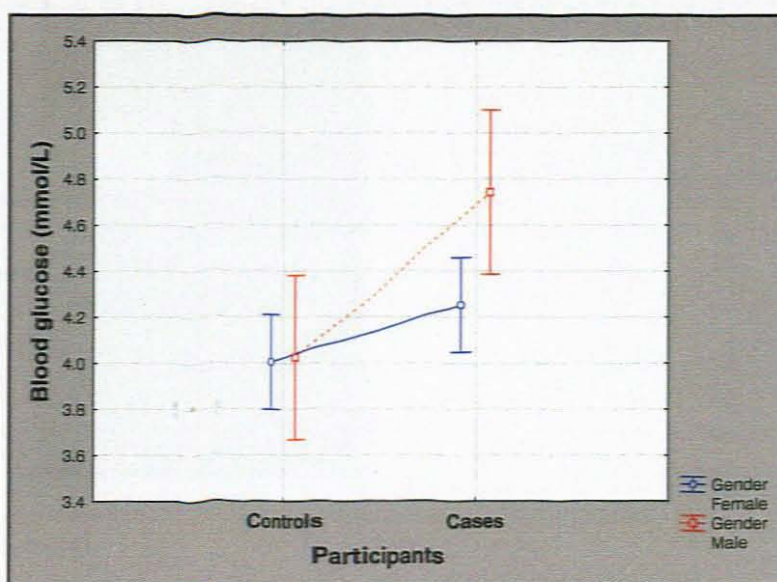


Figure 3.1g: Fasting Blood Glucose. Blood glucose levels in male cases was higher than in male controls
 $p = 0.03$

3.2 *ENPP1* Genotyping

3.2.1 *ENPP1* Variants

SNPs rs997509, rs1044498 and rs9402349 of the *ENPP1* gene were amplified by PCR and separated on a 2% agarose gel (Figure 3.2 a-c). Sequence variant analysis was determined by semi-automated DNA sequencing analysis. The following chromatographs in Figures 3.3-3.5 illustrate the homozygous normal (wildtype), heterozygous and homozygous variant T-allele for each SNP. The rs997509 variant lies in the 3' end of intron 1 and causes a cytosine (C) to thymine (T) transition at nucleotide position (c.258-939C>T), rs1044498 is located on exon 4 causing an adenine (A) to cytosine (C) transversion at nucleotide number (c.534A>C) and the variant rs9402349 lies in intron 9 and causes a thymine (T) to guanine (G) transversion (c.56487-538T>G).

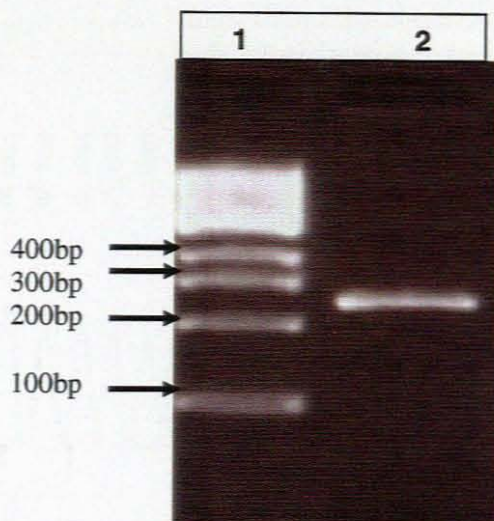


Figure 3.2a: PCR amplification on 2% agarose gel. Lane 1: 100 bp marker; Lane 2: rs997509 (249 bp)

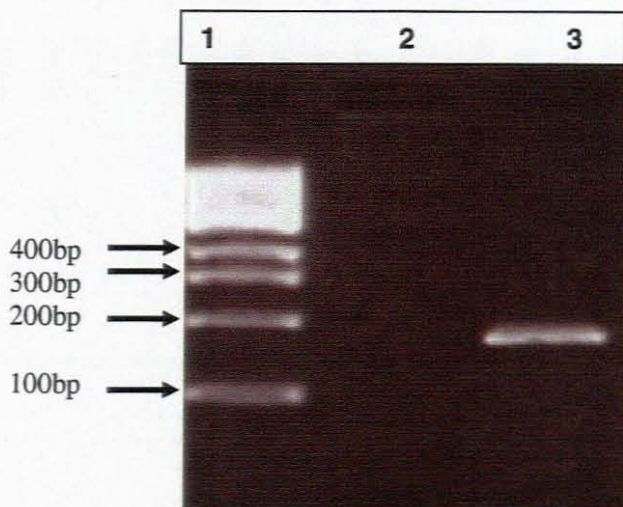


Figure 3.2b: PCR amplification on 2% agarose gel. Lane 1: 100 bp marker; Lane 2: negative control; Lane 3: rs1044498 (179 bp)



Figure 3.2c: PCR amplification on 2% agarose gel. Lane 1: 100 bp marker; Lane 2: rs9402349 (248 bp)

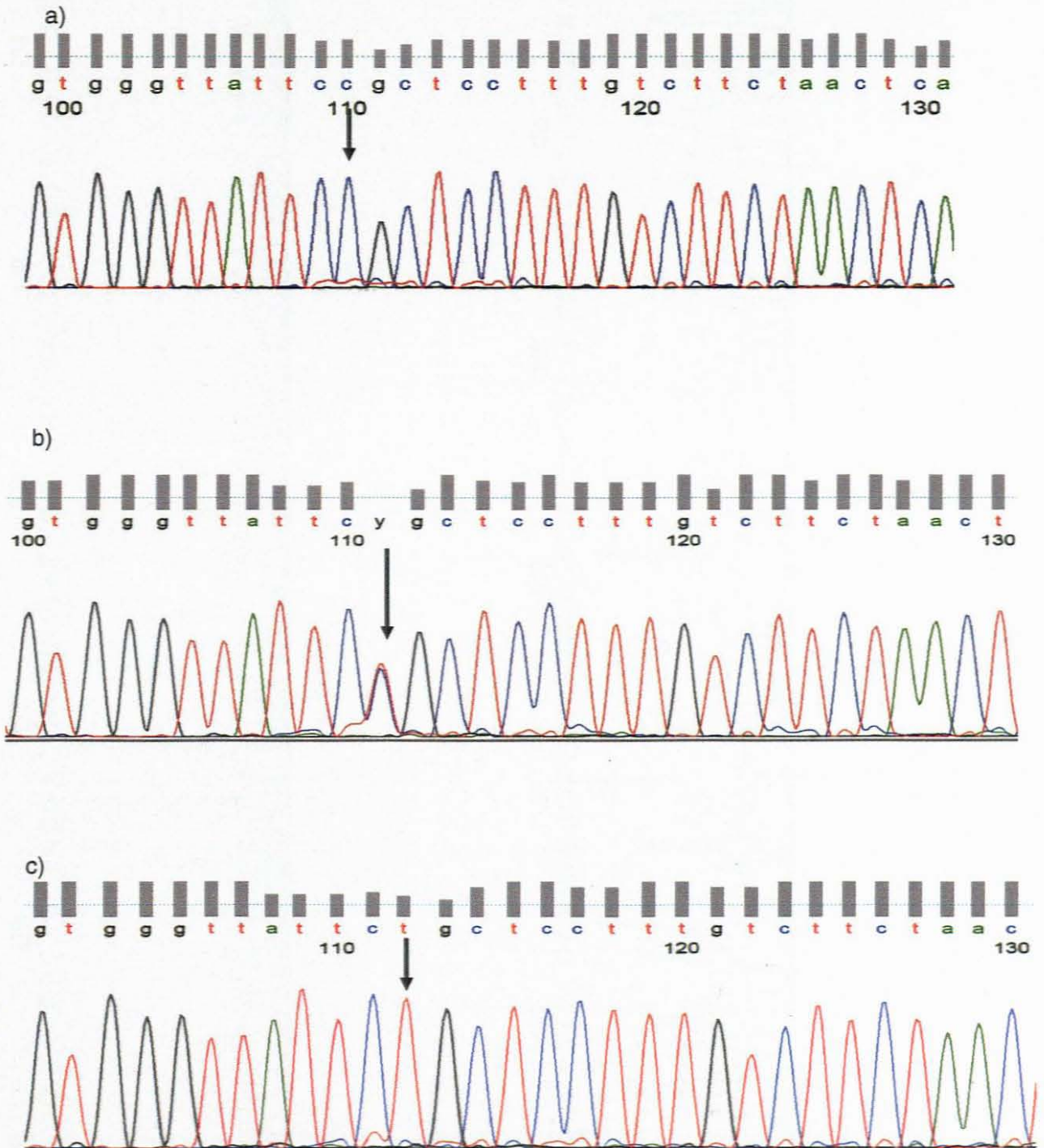
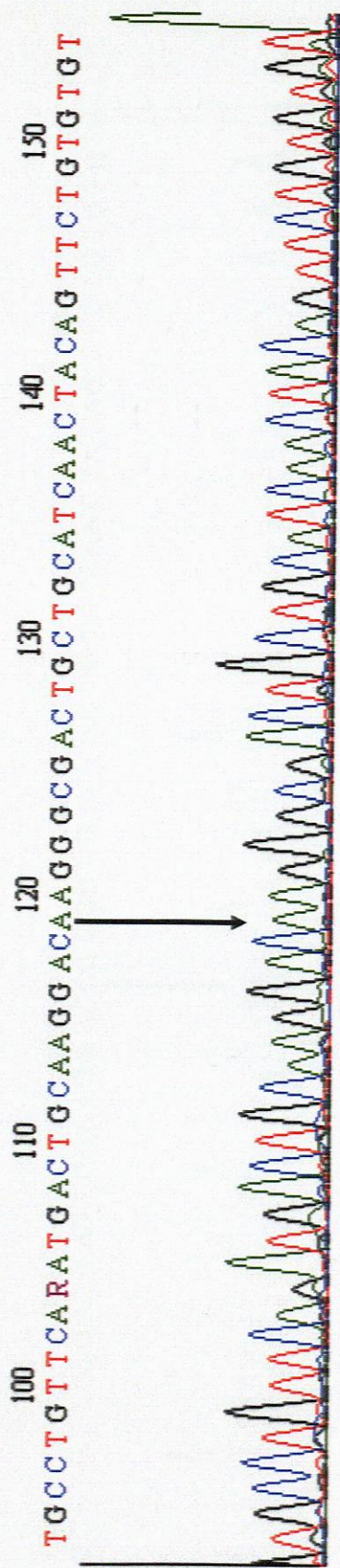
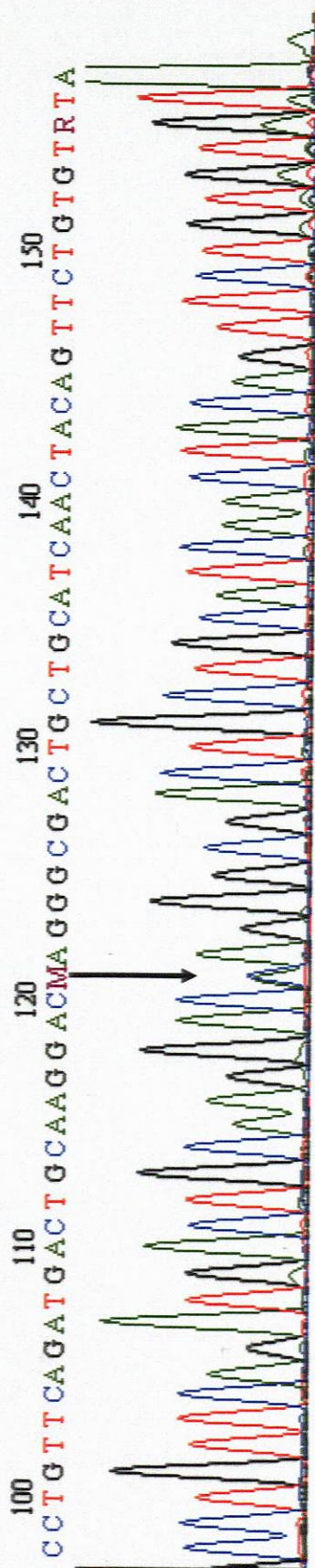


Figure 3.3: rs997509 SNP. Sequencing chromatographs indicating a) homozygous normal (wildtype); b) C>T variant in the heterozygous state; c) homozygous variant T-allele

a)



b)



c)

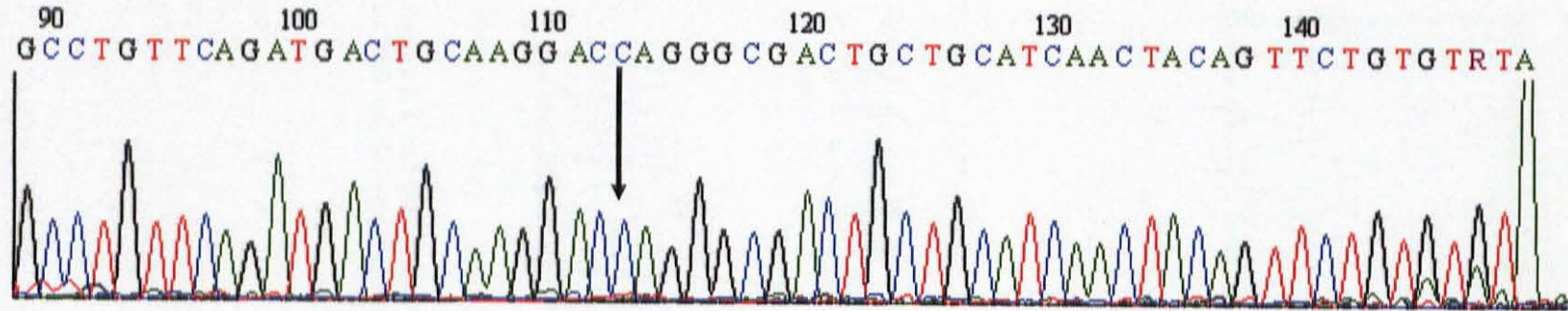


Figure 3.4: rs1044498 SNP. Sequencing chromatographs indicating a) homozygous normal (wildtype); b) **A>C** variant in the heterozygous state; c) homozygous variant-allele

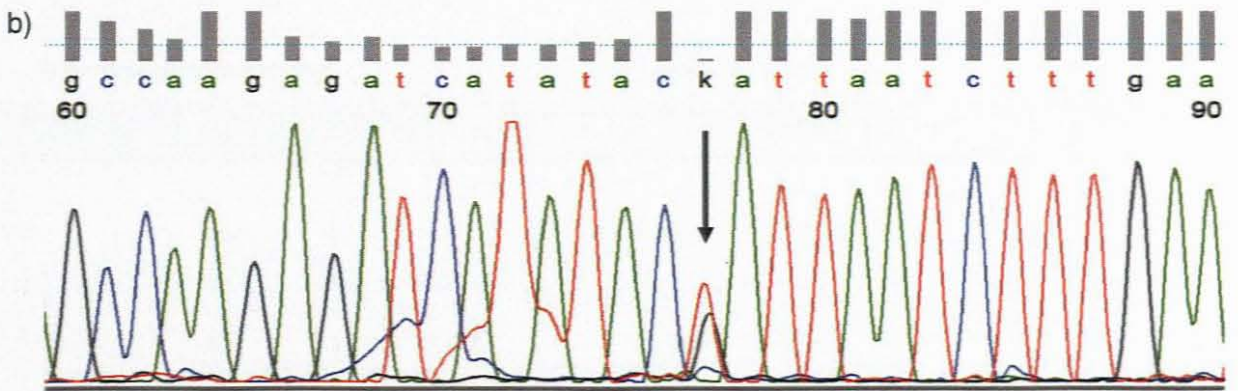
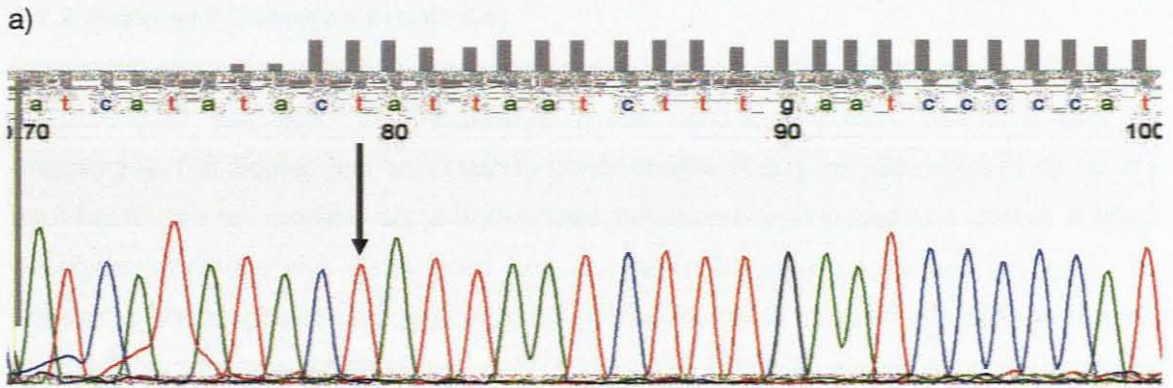


Figure 3.5: rs9402349 SNP. Sequencing chromatographs indicating a) homozygous normal (wildtype); b) *T>G* variant in the heterozygous state. No homozygous variant allele was found/present.

3.2.2 Allele and Genotype Frequency

PowerMarker version 3.25 (www.powermarker.net) was used in the present study to determine both the allele and genotype frequencies. It was used to calculate both allelic and genotype frequencies. Chi Square test was used to verify whether the genotypes were in Hardy-Weinberg equilibrium and to compare allele frequencies between obese cases and control subjects. The genotype frequency was determined and compared between cases and controls. Table 3.2 shows the prevalence of each genotype as well as the allele frequency in both case and control groups. The genotype distribution of rs997509 (c.258-939C>T) was significantly higher in obese than normal weight learners, $p = 0.031$ and allelic prevalence significantly differed between cases and controls, $p = 0.01$. The T-allele of the rs997509 was present in 23% of obese and 7% of controls, $p < 0.05$.

Table 3.2: Allele frequencies and genotype distribution of variants analysed (Significant difference is indicated in red)

Genotype distribution (%)					Allele frequency (%)				
<i>rs997509</i>	Case	Control	χ^2	p-value	<i>rs997509</i>	Case	Control	χ^2	p-value
CC	58	88			C-allele	(77%)	(93%)		
CT	38	12	6.906	0.031				6.910	0.008
TT	4	0			T-allele	(23%)	(7%)		
<i>rs1044498</i>					<i>rs1044498</i>				
Case	Control		p-value		Case	Control		p-value	
AA	28	16			A-allele	(47%)	(40%)		
AC	39	49	2.637	0.267				1.236	0.266
CC	33	35			C-allele	(53%)	(60%)		
<i>rs9402349</i>					<i>rs9402349</i>				
Case	Control		p-value		Case	Control		p-value	
TT	85	81			T-allele	(92%)	(90%)		
TG	15	19	0.252	0.615				0.229	0.632
GG	0	0			G-allele	(7.6%)	(9.5%)		

3.3. Association Analysis

The association analysis between the variants and BMI and metabolic components was done using *Haploview version 4.1* (www.hapmap.org). The p-values of the different parameters with the specified polymorphism are shown in Table 3.3. Only one polymorphism, rs997509 was significantly associated with BMI, as shown below. None of the other variants were associated with the metabolic components.

Table 3.3: Association of parameters with the different polymorphisms (Significant difference is indicated in red and near significant in bold)

	rs997509	rs1044498	rs9402349
BMI	0.030176208	0.670389283	0.330635567
Waist circumference	0.082148288	0.552625742	0.627088893
Hip circumference	0.137155445	0.379539797	0.213680304
Waist/Hip Ratio	0.270268172	0.595793167	0.167392172
Systolic Blood Pressure	0.800170236	0.602850632	0.758220713
Diastolic Blood Pressure	0.675500756	0.702585523	0.663417111
Blood Glucose	0.19261932	0.850716211	0.26606174
Cholesterol	0.50329353	0.479407144	0.35998039
Triglycerides	0.842812821	0.389704896	0.444247697
HDL-cholesterol	0.94399692	0.211674119	0.360955352

3.4 ENPP1 Variants and Metabolic Components

ANOVA and *posthoc* Bonferroni were used to assess the effect of genotypes in metabolic components in all individuals that were screened in the study. Control subject carriers' of the rs997509 CT had a significantly higher waist hip ratio than wild type CC carriers' p-value 0.03. This difference was not observed in obese subjects, with p-values >0.05 (Table 3.5). No significant differences were observed in both cases and controls with regards to rs1044498 (Table 3.6). When rs9402349 was analysed, near significant differences were found in waist/hip ratios of carrier cases than non-carriers, (respectively, 0.87 ± 0.07 , 0.81 ± 0.09 , $p = 0.07$), whilst in control subject the diastolic blood pressure was significantly lower in rs9402349 T>G carriers (Table 3.7).

Table 3.4: Genetic variants of the rs997509 with metabolic components (Significant difference is indicated in red)

	Obese subjects				Control subjects			
	Homozygous normal	Heterozygous	Homozygous abnormal		Homozygous normal	Heterozygous	Homozygous abnormal	
	Mean ± STD				Mean ± STD			
rs997509	CC	CT	TT	P-value	CC	CT	TT	P-value
Age	13.36 ± 2.87	13.11 ± 3.02	11.00	0.71	13.07 ± 2.80	15.25 ± 2.63	NA	0.15
BMI	30.03 ± 4.02	30.39 ± 2.83	25.99	0.96	18.09 ± 2.91	20.54 ± 2.17	NA	0.12
Waist circumference	88.38 ± 9.98	89.59 ± 8.53	81.40	0.92	62.86 ± 7.58	63.45 ± 7.58	NA	0.89
Hip circumference	108.6 ± 12.3	106.2 ± 12.1	95.00	0.49	81.25 ± 10.74	88.39 ± 10.85	NA	0.22
Waist/hip ratio	0.80 ± 0.05	0.85 ± 0.08	0.86	0.10	0.78 ± 0.04	0.72 ± 0.06	NA	0.03
Systolic Blood Pressure	114.05 ± 9.96	107.9 ± 12.1	102.67	0.14	102.3 ± 13.7	110.6 ± 18.2	NA	0.28
Diastolic Blood Pressure	77.07 ± 11.56	71.56 ± 6.04	66.00	0.14	62.41 ± 7.89	66.75 ± 4.79	NA	0.30
Blood glucose	4.16 ± 0.80	4.47 ± 0.69	5.30	0.23	4.03 ± 0.76	4.10 ± 1.26	NA	0.88
Cholesterol	3.33 ± 0.47	3.56 ± 0.60	3.01	0.45	3.39 ± 0.79	3.66 ± 0.53	NA	0.52
Triglycerides	0.72 ± 0.26	0.81 ± 0.36	0.57	0.61	0.73 ± 0.28	0.58 ± 0.02	NA	0.32
HDL cholesterol	0.76 ± 0.19	0.79 ± 0.22	0.95	0.57	1.02 ± 0.31	1.17 ± 0.30	NA	0.40

Table 3.5: Genetic variants of the rs1044498 with metabolic components

	Obese subjects				Control subjects			
	Homozygous normal	Heterozygous	Homozygous abnormal		Homozygous normal	Heterozygous	Homozygous abnormal	
	Mean ± STD				Mean ± STD			
rs1044498	AA	AC	CC	P-value	AA	AC	CC	P-value
Age	13.00 ± 2.52	14.04 ± 2.60	12.35 ± 2.46	0.76	12.67 ± 3.04	12.93 ± 2.65	13.15 ± 3.01	0.73
BMI	31.07 ± 4.85	31.85 ± 3.86	30.61 ± 3.70	0.84	16.51 ± 2.62	18.21 ± 2.67	18.57 ± 2.71	0.33
Waist circumference	89.83 ± 9.97	90.72 ± 11.46	86.39 ± 10.15	0.95	58.55 ± 6.50	62.18 ± 7.02	63.07 ± 7.06	0.79
Hip circumference	108.9 ± 12.7	110.4 ± 10.7	103.0 ± 12.9	0.48	76.30 ± 11.26	80.3 ± 10.07	81.17 ± 10.19	0.78
Waist/hip ratio	0.80 ± 0.13	0.82 ± 0.06	0.83 ± 0.07	0.73	0.77 ± 0.05	0.77 ± 0.04	0.78 ± 0.05	0.89
Systolic Blood Pressure	115.6 ± 11.7	111.33 ± 8.48	113.8 ± 15.5	0.47	100.7 ± 12.60	103.6 ± 14.2	102.1 ± 10.6	0.83
Diastolic Blood Pressure	75.00 ± 6.98	74.13 ± 3.84	73.60 ± 12.68	0.78	58.33 ± 7.65	61.18 ± 6.98	62.15 ± 7.54	0.79
Blood glucose	4.10 ± 0.66	4.45 ± 0.79	4.51 ± 0.68	0.35	4.13 ± 0.96	4.10 ± 0.69	3.89 ± 0.69	0.86
Cholesterol	3.52 ± 0.67	3.52 ± 0.62	3.85 ± 0.78	0.47	3.74 ± 0.94	3.56 ± 0.66	3.70 ± 1.64	0.50
Triglycerides	0.79 ± 0.29	0.76 ± 0.27	0.98 ± 0.43	0.36	1.03 ± 0.99	0.76 ± 0.37	0.73 ± 0.29	0.39
HDL cholesterol	0.81 ± 0.22	0.85 ± 0.28	1.00 ± 0.41	0.39	1.07 ± 0.40	1.05 ± 0.37	1.10 ± 0.29	0.83

Table 3.6: Genetic variants of the rs9402349 with metabolic components ((Significant difference is indicated in red and near significant in blue)

	Obese subjects				Control subjects			
	Homozygous normal	Heterozygous	Homozygous abnormal		Homozygous normal	Heterozygous	Homozygous abnormal	
	Mean ± STD				Mean ± STD			
rs9402349	TT	TG	GG	P-value	TT	TG	GG	P-value
Age	13.44 ± 2.55	12.67 ± 2.74	NA	0.41	12.79 ± 2.63	11.75 ± 2.38	NA	0.31
BMI	31.32 ± 3.85	30.32 ± 4.19	NA	0.48	18.14 ± 2.81	17.01 ± 2.80	NA	0.31
Waist circumference	88.44 ± 10.07	90.64 ± 9.08	NA	0.54	62.21 ± 7.52	58.93 ± 7.67	NA	0.28
Hip circumference	108.3 ± 11.4	103.9 ± 14.4	NA	0.32	80.32 ± 10.12	73.73 ± 8.75	NA	0.12
Waist/hip ratio	0.81 ± 0.09	0.87 ± 0.07	NA	0.07	0.78 ± 0.04	0.78 ± 0.04	NA	0.88
Systolic Blood Pressure	112.8 ± 12.7	116.78 ± 8.07	NA	0.37	104.3 ± 12.7	98.64 ± 16.17	NA	0.29
Diastolic Blood Pressure	73.54 ± 6.94	78.11 ± 14.11	NA	0.14	62.62 ± 6.82	56.50 ± 6.65	NA	0.03
Blood glucose	4.30 ± 0.75	4.53 ± 0.47	NA	0.38	3.73 ± 0.72	4.04 ± 0.72	NA	0.29
Cholesterol	3.67 ± 0.70	3.30 ± 0.68	NA	0.14	3.81 ± 1.31	3.72 ± 0.73	NA	0.85
Triglycerides	0.80 ± 0.30	0.92 ± 0.50	NA	0.33	0.70 ± 0.30	0.72 ± 0.39	NA	0.86
HDL cholesterol	0.91 ± 0.33	0.74 ± 0.25	NA	0.15	1.11 ± 0.37	1.09 ± 0.40	NA	0.88

CHAPTER FOUR

DISCUSSION AND CONCLUSION

4.1 Discussion

The participants in this project were selected from a study population comprising of three ethnic groups from South Africa; Blacks, Coloureds and Whites. The prevalence of obesity in that study was lower in males compared to their female counterparts. The age of the study population ranged between 8-18 years. Therefore these findings could be influenced by puberty though similar trends are also observed in adults. For normal development, particularly establishment of a normal menstrual cycle, a critical weight of 48 kg, or body fat level of 22% is recommended in order for puberty to progress (Frisch & Reyelle, 1971). Obesity was more prevalent amongst the Coloureds compared to their Black and White counterparts. These results are almost similar to those reported by Somers *et al* (2006). The authors reported 6.2% obesity prevalence among learners in an urban area of Cape Town. These results provide evidence that obesity rates in South Africa are fast approaching those seen in developed countries. The IOTF inspected the prevalence of obesity aged around ten years from data derived from 21 European countries between 1992 and 2001, and found levels of vary between 10% and 36%, with Italy, Malta, Greece and Croatia being the countries with the highest rate of childhood obesity (Sabin, 2004).

One of the major contributors to obesity in South Africa is urbanisation, which is associated with better socio-economic conditions due to employment opportunities being more available in urban areas. By virtue of its economic growth, South Africa is considered to be one of the countries that are undergoing rapid demographic and nutritional transition (Steyn, 2005). As a result, there is an adoption of a more Westernised diet that is higher in fat and has fewer carbohydrates (Goedecke *et al.*, 2006). Unlike their Black counterparts, the Coloured population had enjoyed a higher socio-economic status, therefore earlier exposure to the Western lifestyle. During the apartheid era, South African populations were artificially divided into four groups: African or Black, Mixed Race or "Coloured", Asian or Indian, and White and these groups enjoyed different standards of health and quality of life; from smallest to largest, was Black rural, Black urban, Coloured urban, Indian urban, White urban (Cameron, 2003).

As with other health conditions, obesity results from an interaction between both environmental and genetic factors. Diet is the crucial environmental factor in the development of obesity. Overeating in combination with low physical activity is the main environmental cause of the obesity epidemic rapidly spreading in the modern world (Loktionov, 2003). Some studies assessing heritability within families have shown compelling evidence for the significant genetic influence on obesity. Genetic factors are currently estimated to account for approximately 40-

70% of the variance in human adiposity (Farooqi, 2005). This study investigated the role of Ectonucleotide Pyrophosphatase Phosphodiesterase 1 (*ENPP1*) SNPs in obesity. Single nucleotide polymorphisms (SNP) is a name given to a single base substitution and is the most common type of genetic variation that occurs at a frequency of 1% or more in a general population. SNPs are often found to play a role in the aetiology of many human diseases. SNP analysis is one of the most common methods in genetic association studies and is employed in the study of genetic variation. In such studies SNPs are frequently used as markers to identify genes and quantitative trait loci (QTL) that confer susceptibility to genetic diseases (Rapley & Harbron, 2004).

ENPP1 has been shown to play a role in the development of insulin resistance, type 2 diabetes and obesity, particularly the substitution of a lysine with a glutamine at codon 121 (K121Q) (Grarup *et al.*, 2006; Matsouka *et al.*, 2006; Chandalia *et al.*, 2007). Recently, other SNPs within *ENPP1* located in the same haploblock as K121Q, namely rs997509 and rs9402349 have been investigated for their role in the development of obesity and/or type 2 diabetes. The rs997509 was demonstrated to be a risk factor for the development of type 2 diabetes among obese individuals, whilst no significant associations were observed between rs9402349 and type 2 diabetes or obesity (Bochenski *et al.*, 2006). The minor T-allele of rs997509 distinguishes one of the three 121Q-carrying haplotypes, and is strongly associated with type 2 diabetes. Bochenski and co-workers (2006) hypothesized that the 121Q variant is just a silent marker of the T-allele of rs997509 while the latter is functional and fully responsible for the effect of the risk haplotype. In addition, Santoro *et al* (2008) reported an association between the T-allele and impaired glucose tolerance (IGT). Contrary to previous reports, this study provides evidence that the T-allele of rs997509 is strongly associated with obesity in Mixed Ancestry children from the Western Cape, South Africa. This is a novel finding in this study. Furthermore, the T-allele was nearly significantly associated with an increased waist circumference. The waist circumference is commonly used as an indicator of central obesity, and its metabolic consequences, insulin resistance and type 2 diabetes (Alberti *et al.*, 2005). Recognition of gender and ethnic differences in body fat distribution have led to the use of different waist circumference cut-off points in the classification of central obesity (Executive summary of the clinical guidelines on the identification, evaluation and treatment of overweight and obesity in adults, 1998; Alberti *et al.*, 2005;). The mixed ancestry population of South Africa is a combination of European settlers and the indigenous Africans. Though these associations may be unique to this population group, they buttress the effect of rs997509 on obesity and indirectly on metabolic syndrome traits.

The mechanism through which *ENPP1* modulates BMI is unknown. It is hypothesized that the reported association with higher BMI is due to the fact that individuals carrying the K121Q variant develop insulin resistance in the brain, where insulin has potent anorectic actions, and this, in turn, increases appetite and eventually body weight. In contrast, Pizzuti *et al* (1999), in a prospective study had shown the K121Q to be a predictor of lower BMI. The authors suggested that this might be due to the deleterious effect of this variant on peripheral insulin resistance, a possible consequence of impaired insulin-mediated lipid storage in adipocytes. Therefore, in some cases, genetic determinants improving insulin sensitivity have an opposite deleterious role on body weight and *vice versa*. In the present study, the Q121 (CC) was similar in obese individuals and controls and found to be 33% and 35%, respectively, and was not associated with an increased BMI. Similar to observations reported in African-Americans, the *ENPP1* 121Q allele frequency was high in this study (Lyon *et al.*, 2006; Chandalia *et al.*, 2007).

The 121Q allele frequency has been shown to differ greatly between ethnic groups with the least number of carriers amongst Caucasians and higher numbers in Asian Indians and African-Americans (Abate *et al.*, 2003; 2005; Lyon *et al.*, 2006; Chandalia *et al.*, 2007). Matsouka *et al* (2006), found the 121QQ (CC) and K121Q (AC) to be present in 50.9% and 39.6% of obese African-Americans. In this study the KK121 (AA) individuals (9.5% obese or 5.9% lean) had a higher BMI. In total, the KK121 was present in 21% (28% obese and 16% normal weight) of the Coloured population investigated in this study but no difference in BMI was observed. Though the African-Americans have been shown to have the highest 121Q allele frequency, no associations with BMI or obesity were demonstrated in previous studies (Lyon *et al.*, 2006; Chandalia *et al.*, 2007). Likewise, in the present study the frequency of K121Q and obesity was found not to be associated.

Obesity in childhood is strongly associated with metabolic syndrome (MetS) a condition that predisposes to an increased risk for type 2 diabetes mellitus and coronary heart disease in adults. Metabolic syndrome is defined as a constellation of risk factors in a person that includes obesity, insulin resistance, hypertension and other metabolic abnormalities (Grundy, 2004). Differences in the prevalence rates of MetS in children based on various definitions have been reported (Cruz & Goran, 2004; Goodman *et al.*, 2004; Kelishadi *et al.*, 2006). Irrespective of the definition used to define MetS in children, investigators have shown the presence of MetS in early childhood and an increased prevalence in overweight and obese individuals, and this poses a serious problem as childhood MetS is thought to persist through adulthood (De Ferranti

& Osganian, 2007). Until recently (2009), in Africa, there was virtually no study on the prevalence of Mets in childhood, but the few studies from other developing countries have shown that MetS in childhood is on the increase correlating with the increasing obesity observed in these countries (Kelishadi, 2007). Recent survey data indicate that South Africa is faced with a rapid increase in childhood obesity of up to 20% among children and adolescents between the ages of 1-19 years old (South African Society for the Study of Obesity, 2001; Medical Research Council, 2002; Steyn *et al.*, 2005). A national survey conducted in 10 195 South African 6-13 year old primary school learners reported an overweight prevalence of 10.8% and 13.0% in boys and girls respectively (Armstrong *et al.*, 2006). Overweight children tend to become overweight adults, and are thus at an increased risk of developing insulin resistance (American Diabetes Association, 1998; Yensel *et al.*, 2004), which together with obesity are considered to be the driving force of metabolic syndrome (Reaven, 1988; Eckel *et al.*, 2005). Recently, in a study of learners aged 10-16 years, Matsha *et al.* (2009) showed that the metabolic syndrome was more prevalent in obese subjects using either the NCEP ATP III or IDF definitions of metabolic syndrome. In this study, MetS was not determined; however, differences in components of MetS were investigated. Significant differences were observed between cases and controls with respect to their fasting blood glucose and systolic or diastolic blood pressure. High levels of blood pressure and heart rates were significantly different between the obese children and their lean counterparts. These results further support the notion that metabolic syndrome traits develop in childhood and are highly prevalent in obese children (Cook *et al.*, 2003; Cruz & Goran, 2004). Recently, Longo-Mbenza and co-workers investigated MetS components in 1535 Congolese children and found higher blood pressure and heart rate in obese children (Longo-Mbenza, 2007).

This study also investigated the relationship between *ENPP1* SNPs, that is, rs997509 (c.258-939C>T), rs1044498 (c.534A>C) and, rs9402349 (c.56487-538T>G) and components of MetS. The rs997509 and rs9402349 variants showed significant differences with MetS components between carrier and non carriers in both cases and controls, namely waist/hip ratio and diastolic blood pressure. The study conducted by Bochenski *et al.* (2006) found no association between rs9402349 T>G and either type 2 diabetes or obesity. In the present study, lean individuals with the polymorphism had significantly lower diastolic blood pressure. Similarly only in controls, the waist hip ratio of the T-allele of rs997509 was significantly lower than their C-allele counterpart. It is unclear whether the low waist/hip ratio observed is the direct result of the T-allele or the larger hip circumference seen in these individuals. The waist circumference and/or waist-to-hip (WHR) ratio are commonly used as indicators of fat distribution and are consequently a measurement

for the risk of MetS. However, differences in body structure of various ethnic groups have been observed. For example, in Caucasians a larger hip circumference is dissociated with MetS (Snijder *et al.*, 2004). Although no significant differences were observed, the blood glucose levels of obese individuals with the T-allele were slightly higher, supporting the findings by Bochenski and co-workers (2006).

The K121Q (AC) or 121QQ (CC) allele has been reported to be associated with insulin resistance and type 2 diabetes (Goldfine *et al.*, 2008), however reports have been inconsistent. Lyon *et al* (2005) failed to find an association between K121Q and diabetes. Similarly, we also did not find any association between 121Q or 121QQ and fasting blood glucose levels though the fasting blood glucose levels of obese K121Q (AC) or 121QQ (CC) were higher than KK121 (AA) but not in control subjects. Based on these results, perhaps other genetic and/or environmental factors in synergy with K121Q increase the risk of developing type 2 diabetes or obesity, further strengthening the notion that obesity is a multifactorial condition.

4.2 Conclusion

Several studies have reported on the role of genetics in obesity. This study complements reports that genetic factors together with environmental factors contribute to the development of obesity. The association between T-allele of rs997509 and BMI is a novel finding in this study and concur with previous reports that *ENPP1* variants contribute to obesity. The findings of this study suggest that *ENPP1* polymorphisms may contribute to different metabolic traits, all of which are associated with obesity and its metabolic consequences, insulin resistance and type 2 diabetes. For example, the *ENPP1* K121Q polymorphism that has been strongly associated with metabolic traits has also shown different outcomes regarding its effect on insulin resistance, type 2 diabetes and obesity in different populations. This variant was associated with type 2 diabetes in Caucasians living in the United States (Abate *et al.*, 2003; 2005) and Finland (Kubaszek *et al.*, 2004), but not in Swedish Caucasians (Gu *et al.*, 2000).

In conclusion, this study has enriched our understanding of the *ENPP1* gene's involvement in obesity. It has clearly demonstrated that the T-allele of rs997509 may be a factor in the development of obesity in Mixed Ancestry population of South Africa.

4.3 Limitations of this Study

- Children may not have provided accurate information regarding their fasting state, which was important to measure the fasting blood glucose.
- Only one population (Coloureds) was studied, as a result no comparisons could be made with other population groups (Blacks and Whites) within the same study.
- Because this study was done on children, not enough blood was obtained for genetic analysis, as a result, only a limited DNA was available. The capillary blood collected on Whatman FTA cards was also very minimal.
- The study was limited to government funded or public schools situated in the communities of the Western Cape Province, South Africa.
- The study population consisted of children and adolescents aged 8-18. This group is broad and would probably influence the statistical analysis. Bearing in mind that, puberty and sexual maturation, differ significantly between individuals.
- The SNPs could not be genotyped in all individuals due to the limited amount of DNA obtained from the FTA cards as venous blood was only collected in learners aged ≥ 16 years. Therefore statistical analysis of haplotypes e.g. haplotype associations could not be done.

4.4 Future Work

- This study should be repeated and other population groups included. It would be interesting to see what the allele frequencies of the different SNPs in the other local population groups (Blacks and Whites) would be as *ENPP1* polymorphisms, particularly K121Q has been shown to predispose individuals to different metabolic syndrome traits according to population groups.
- Although the components of metabolic syndrome were looked at in this present study, it would particularly be interesting to investigate these SNPs in children with metabolic syndrome and those with type 2 diabetes or Impaired glucose tolerance (IGT) as recent reports suggest that the T-allele of rs997509 predisposes obese children to metabolic syndrome and IGT.

- Future research aimed at understanding the mechanisms regulating *ENPP1* expression and function promises to yield important information for translational human investigation. This will provide a new target, in combination with other genetic and non-genetic information, for identification of individuals prone to insulin resistance, obesity, type 2 diabetes, and related cardiovascular diseases and possible novel common avenues for treatment of these diseases.

CHAPTER FIVE

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APPENDICES

APPENDIX A: 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)

100 μl 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 dH₂O 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 dH₂O.

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 dH₂O

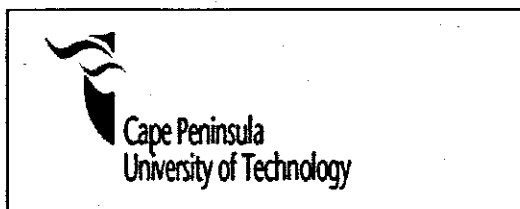
20X Sodium Borate (SB) Buffer

Dissolve 38.137 g of SB powder in dH₂O and add up to 1 L.

1X Sodium Borate Buffer

Dilute 100 ml of 20xSB buffer in 1900 ml of dH₂O

APPENDIX B: 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 NUMBER: N06/03/059

PRINCIPAL INVESTIGATOR:

Dr T Matsha
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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).

Article I. 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 analyze 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.

Section 1.01 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 magnitudes 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.

Section 1.02 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 counseling 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

(a)

(b)

(c) 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 pressurized 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

(d) 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

Section 1.03 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 C: QUESTIONNAIRE

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?

2 What is your gender?

 0 Male

 1 Female

3 In which grade are you?

 0

 4

 8

 1

 5

 9

 2

 6

 10

 3

 7

4 How long have you been attending this school?

 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)

 a) Black
1

 b) White
2

 c) Coloured
3

 d) Asian
4

 e) Other
5

 If other, Please clarify
6

6a How would you describe your(Select one)
Mother?

 a) Black
1

 b) White
2

 c) Coloured
3

 d) Asian
4

 e) Other
5

Father?

 a) Black
1

 b) White
2

 c) Coloured
3

 d) Asian
4

 e) Other
5

6b Are any of your grandparents of a different description as you and your parents?
If yes, please state, who, of which description they are, & how they are related to you

Mom's mother Father's mother
Mom's dad Father's father

7 Are you related to someone also participating in this project? a) Yes b) No
If yes, please state who and how you are related?

8 How many brothers and sisters do you have?

a) Brothers b) Sisters Total

FAMILY HEALTH HISTORY

9 Have you ever been told that you have diabetes (sugar levels)?
a) Yes b) No

10 Does your natural mother, father, brother or sister have diabetes?
a) Yes b) No

11 Has anyone in your extended family (Aunts, Uncles, Grandparents) ever suffer from diabetes?
a) Yes b) No

12 At which age did you have your first menstrual period?
 Tanner Stage

LIFESTYLE

13a Do you smoke?
a) Yes b) No

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

14 Do you consume any alcoholic beverages?
a) Yes b) No

SOCO-ECONOMIC DETAILS

15 What type of house do you live in?

- a) House 1 b) Flat 2 c) Back Room 3
 d) Hostels 4 e) Shack 5 f) Bungalow/
 wendy house 6
 g) Other 7

2

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

- a) In-house Flush system 1 b) Out-door Flush system 2
 c) In-house Bucket system 3 d) Out-door Bucket system 4

2

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

2

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

Mother	<input type="text"/> Yes / No	Father	<input type="text"/> Yes / No
	<input type="text"/>		<input type="text"/>

2

2

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

- a) Yes 1 b) No 2
 Who?

2

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

SECTION B

FOOD CONSUMPTION SURVEY

Name & Reference Number

--

Please complete the following by making a tick (✓) next to the appropriate answer:

Yesterday, did you eat:

	Yes	No
Breakfast?	<input type="checkbox"/>	<input type="checkbox"/>
during the rest of the morning?	<input type="checkbox"/>	<input type="checkbox"/>
Lunch?	<input type="checkbox"/>	<input type="checkbox"/>
during the rest of the afternoon?	<input type="checkbox"/>	<input type="checkbox"/>
Supper	<input type="checkbox"/>	<input type="checkbox"/>
After supper or during the night?	<input type="checkbox"/>	<input type="checkbox"/>

Now I would like to ask you about the types of foods that you ate yesterday during the day and night. Did you eat..... yesterday? Please tick off yes even if you only ate one of the foods mentioned in the group.

	FOOD GROUP	EXAMPLE	Ate it yesterday		
			Yes	No	Score
A	Cereals, roots & tubers	Mielie-meal porridge (stiff or soft), Mabela, Maghewu, Sorghum Fermented/sour porridge, Morvite, Samp, Mielie rice, corn-on-cob, Wheat, Bread, Home-made bread, Dumplings, Vetkoek, Rice, Pasta, Oats, Breakfast cereals, Potato, Potato salad, Sweet Potato	1	2	score
B	Vitamin A-rich Fruit & vegetables	Yellow/Orange Coloured: Mango, Paw-paw, Yellow peach, Butternut, Carrot, Pumpkin, Dark-green Leafy: Pumpkin leaves, Beetroot leaves, spinach, Marogo, Dried green Cowpea leaves.	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
C	Other Fruit	Apples, Apricot, Banana, Grapes, Grapefruit, Guava, Lemon, Lime, Marula fruit, Naarjie, Orange, Peach, Pear, Plum, Pinapple, Prickly pear, Raspberries, Strawberries, Wild berrie, Watermelon, Wild fruit, Any dried fruit, Any canned fruit	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
D	Other Vegetables	Beetroot, Broccoli, Cabbage, Cauliflower, Chickpeas, Green Beans, Green peas, Green pepper, Lettuce, Mielie (corn-on-the -cob), Mushrooms, Onions, Tomato	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
E	Eggs	Eggs	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

For Office Use

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F	Meat, Poultry & Fish	Beef, Pork, Lamb, Goat, Mutton, Sausage, Chicken, Chicken feet, Chicken heads Chicken Giblets, Chicken stew, Stew with any meat, polony, vienna, canned beef Ham, venison, locust, rabbit, dove, pigeon, hare, sparrow, birds, snails, tripe, grass-hoppers, offal, intestines, animal feet, kidney, heart, lung, fried fish, canned fish, frozen fish, fish cakes, fish fingers	Yes	No	Score
G	Legumes, Nuts & Seeds	Dried Beans, Sugar beans, Baked beans, Lentils, Dried Peas, Cowpeas, Split Peas, Peanuts, Nuts, Sunflower seeds, Pumpkin seeds	Yes	No	Score
H	Dairy	Milk, Amasi / Maas. Yogurt, Condensed Milk, Milk Powder, Cheese,	Yes	No	Score
I	Oils & Fats	Any food made with oil, margarine, butter, holsun	Yes	No	Score
J	Sugars	Sugar, Syrup, Sweets, Honey, Chocolate	Yes	No	Score
K	Beverages	Tea, Coffee, cooldrinks, fruit juice, beer, home-made-beer, non-dairy creamers (eg cremora)	Yes	No	Score

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 state 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
Watching TV					
Computer Games					
Cycling, Dancing, Swimming					
Household Chores					
Other					

Thank you for participating in our survey

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)					
Body Weight (kg)					
Body Height (cm)					
Body Mass Index (BMI) (m/kg ²)					

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)					

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4

Waist Circumference 1 (cm)					
Waist Circumference 2 (cm)					
Waist Circumference 3 (cm)					
Waist Circumference (cm)					

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Waist hip ratio 1					
Waist hip ratio 2					
Waist hip ratio 3					
Waist Hip Ratio					

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SKINFOLD MEASUREMENTS

5

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

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

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Sub-scapular skinfold 1 (cm)

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

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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			

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

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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)			
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URINALYSIS

Glucose mmol/L (N=Negative, P=Positive)	P	N
Protein mmol/L (N=Negative, P=Positive)	P	N
Microalbumin μ mol/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 performed to ensure accuracy and reliability? Y N

Controlled and Signed by _____

Date: _____