The association of ApoCIII, Beta-3 adrenergic receptor and TNF-alpha polymorphisms with Lipodystrophy in HIV Positive patients receiving antiretroviral therapy

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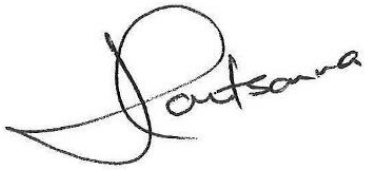
Declaration

I declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in the Faculty of Health Sciences at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.



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Abstract

Ever since the introduction of highly active antiretroviral therapy (HAART), body profile changes and metabolic anomalies have increasingly been witnessed in HIV-positive patients. Lipodystrophy which is characterized by lipoatrophy and lipohypertrophy is one of the most noticeable conditions. The aim of this study was to determine the possible association of ApoCIII, beta-3 adrenergic receptor and TNF-alpha gene polymorphisms with the presence of lipodystrophy in the HIV positive subjects on HAART.

HIV-positive subjects (*n*= 209) were recruited from Helen Joseph Hospital and Charlotte Maxeke Johannesburg Academic Hospital. Control group of HIV-negative subjects (*n*=100) were also recruited for the study. Lipodystrophy was identified through patient self-assessments on changes in body fat using a questionnaire. Anthropometric data was recorded and genomic DNA extracted. A PCR-based RFLP technique was used to screen for ApoCIII (-T455C and -C482T), beta-3 adrenegic (-T64A) and TNF-alpha (-G238A and -G308A) gene polymorphisms.

Lipodystrophy was detected in 27% of the HIV positive subjects and was characterized by lower mean weight (62.5 ± 11.1) compared to subjects without lipodystrophy (67.6 ± 12.9; $p<0.05$).The frequency for the TNF-alpha variant allele, –308A was significantly higher in individuals with lipodystrophy (25%) compared to those without lipodystrophy (13%; $p<0.05$). The allele frequencies for the ApoCIII, beta-3 adrenergic receptor and TNF alpha -238 polymorphisms were similar between patients with and without lipodystrophy. Lipodystrophy in this patient cohort is characterized by lipoatrophy and the presence of the variant A allele at the TNF alpha -308 locus.

Presentations

We presented the paper: The association of apoC-III, β-3 adrenergic receptor and TNF-α polymorphisms with Lipodystrophy in HIV-positive patients receiving Anti-retroviral therapy, by T.F Tlomatsana, N.H Naran, C. Julsing* and N.J Crowther; at the 45th meeting of the Society for Endocrinology, Metabolism and Diabetes of South Africa (SEDMSA), in April 2011.

We presented the paper: The association of apoC-III, β-3 adrenergic receptor and TNF-α polymorphisms with Lipodystrophy in HIV-positive patients receiving Anti-retroviral therapy, by T.F Tlomatsana, N.H Naran, C. Julsing* and N.J Crowther; at PathtechCongress 2011.

Published the Abstract: The association of apoC-III, β-3 adrenergic receptor and TNF-α polymorphisms with Lipodystrophy in HIV-positive patients receiving Anti-retroviral therapy, by T.F Tlomatsana, N.H Naran, C. Julsing* and N.J Crowther; in JEMDSA 2010 Volume 15 Number 1: P32.

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List of Abbreviations

ANOVA:	Analysis of Variaton
ARV:	Anti-Retroviral
ATP:	Adenosine Tri-phosphate
ATV:	Atazanavir
ApoCIII:	Apolipoprotein CIII
BIA:	Bioelectrical Impendence Analysis
β_3AR:	Beta-3 Adrenergic Receptor
BMI:	Body Mass Index
CT scan:	Computerized Axial Tomography scan
CAD:	Coronary Artery Disease
CoA:	Coenzyme A
DAD:	Data collection on Adverse events of anti-HIV Drugs
dNTPs:	Deoxyribonucleotide Triphosphate
DMSO:	Dimethyl Sulfoxide
DNA:	Deoxyribonucleic Acid
DEXA scan:	Dual Energy X-ray Absortiometry
EDTA:	Ethylenediaminetetraacetic Acid
FFA:	Free Fatty Acids
Ha:	Alternative Hypothesis
HAART:	Highly Active Antiretroviral Therapy
HAL:	HIV Associated Lipodystrophy
HADL:	HIV Associated Dyslipidemic Lipodystrophy
HDL:	High Density Lipoprotein
HWE:	Hardy-Weinberg Equilibrium
HOMA:	Homeostasis Model Assessment

HOPS:	HIV Outpatient Study
IFN:	Interferon
IL:	Inter-Leukin
IGT:	Impaired Glucose Tolerance
RNA:	Ribonucleic Acid
LBM:	Lean Body Mass
LDL:	Low Density Lipoproteins
LPV/r:	Kaletra
LDS:	Lipodystrophy Syndrome
NIH:	National Institutes of Health
NRTI:	Nucleoside reverse transcriptase inhibitors
NNRTI:	Non-nucleoside reverse transcriptase inhibitors
OGTT:	Oral Glucose Tolerance Test
PPAR:	Peroxisome Proliferator Activated Receptor
PI:	Protease inhibitors
PCR:	Polymerase Chain Reaction
RTV:	Ritonavir
SD:	Standard Deviation
TACE:	TNF-α converting enzyme
TBE:	Tris/Borate/EDTA
TNF-α:	Tumour Necrosis Factor-Alpha
TG:	Triglycerides
VLDL:	Very Low Density Lipoproteins
WHR:	Waist Hip Ratio
WHO:	World Health Organization
WC:	Waist Circumference

Chapter 1

Literature Review

1.1 Introduction

In 2013 it was estimated that the Human Immunodeficiency Virus (HIV) had infected approximately 35 million people worldwide (UNAIDS Gap Report 2014). Since the introduction of Highly Active Antiretroviral Therapy (HAART) life expectancy of HIV infected individuals has increased; however, a number of other complications have developed in its wake. One of the complications that has been described is Acquired Immune Deficiency Syndrome (AIDS) related metabolic syndrome, specifically lipodystrophy (Crum *et al*., 2006).

HIV/AIDS associated lipodystrophy is defined as the "redistribution" of body fat, which is characterized by loss of fat in the extremities and facial areas (known as lipoatrophy) and/or accumulation of fat in the neck and abdomen (defined as lipohypertrophy) (James *et al.*, 2006).

It has been shown that there are several factors, including dietary and metabolic, that may contribute to the development of lipodystrophy. Furthermore, genetic factors have been described to play a role in lipid metabolism including genes expressing Apolipoprotein C-III, βeta-3 Adrenergic Receptor and Tumour Necrosis Factor alpha genes. In addition, polymorphisms within these genes have been strongly linked with the development of lipodystrophy (Joy *et al*., 2008).

However, these gene polymorphism associations have not been studied in the South African HIV positive population. Furthermore, the rise in life expectancy of individuals with HIV on HAART makes the possible effect of lipodystrophy on the quality of life significant for further exploration (Mallon, 2007).

1.2 HIV/AIDS in South Africa

The Republic of South Africa had the highest number of people living with HIV and AIDS (an estimated 6.3 million) compared to any other country in 2013, (UNAIDS, 2013).

The Actuarial Society of South Africa released a model in 2005, shown in Table 1.1, which assisted in the estimation of AIDS infection, mortality and a variety of other indicators. This mathematical model was developed by the Actuarial Society of South Africa to assist the medical profession in assessing and addressing the impact of the HIV and AIDS epidemic in South Africa.

Table 1.1: AIDS infection and mortality projection model

Calendar Year starting 1 July	2006	2007	2010	2015
Total population	47,866,985	48,218,209	49,147,178	50,328,901
Total HIV infections	5,372,474	5,511,749	5,813,088	6,027,508
Total AIDS sick	599,298	633,931	701,508	797,003
Adults on ART	200,457	313,420	709,021	1,126,299
Children on ART	25,318	38,069	81,980	111,168
Total population	11.20%	11.40%	11.80%	12.00%

http://aids.actuarialsociety.org.za/Models-3145.htm

https://en.wikipedia.org/wiki/Actuarial_Society_of_South_Africa_HIV/AIDS_models

1.3 HIV treatment: Antiretroviral therapy

The availability of effective anti-retroviral (ARV) therapy, while incapable of curing the disease, has led to an improved life expectancy and has credibly been proved to reduce mortality (Behrens *et al.*, 2000).

1.3.1 Antiretroviral drug class classification and modes of action

There are currently five broad classes of ARVs being used, often in combination:

1. Protease inhibitors (PIs)

2. Nucleoside reverse transcriptase inhibitors (NRTIs)

3. Non-nucleoside reverse transcriptase inhibitors (NNRTIs)

4. Cell membrane fusion inhibitors

5. Integrase inhibitors

Protease inhibitors are believed to prevent viral replication by selectively binding to viral proteases (e.g. HIV-1 protease) and blocking proteolytic cleavage of protein precursors that are necessary for the production of infectious viral particles (Rang *et al*., 2007).

Both NRTIs and NNRTIs interfere with the process of RNA to DNA reverse transcription, but the mechanisms are entirely different. NRTIs inhibit this process by first being metabolized within the cells to be converted to their 5'triphosphate form, which then competes with the cells nucleotides for replication thereby inhibiting its DNA polymerization. Furthermore, NRTI's lack the 3'-OH group that is essential for replication thus acts as a replication terminator. Thus, NRTIs work by competitively inhibiting reverse-transcriptase. NNRTIs on the other hand bind directly to the reverse transcriptase enzyme. They are not nucleoside analogs and are not incorporated into the DNA strand.

NNRTIs work by non-competitive inhibition, by distorting the catalytic site from attaining the metal-binding conformation necessary for DNA replication (Lewis *et al.*, 2003).

Fusion inhibitors interfere with the binding, fusion and entry of an HIV virion into a human cell. By blocking this step in HIV's replication cycle, such agents slow the progression from HIV infection to AIDS (Biswas *et al*., 2007)

Integrase inhibitors are designed to block the action of integrase, a viral enzyme that inserts the viral genome into the DNA of the host cell. Since integration is a vital step in retroviral replication, blocking it can halt further spread of the virus (Steigbigel *et al*., 2008).

Highly Active Antiretroviral Therapy (HAART) is known as the most effective treatment for HIV and AIDS that combines protease inhibitors with reverse transcriptase inhibitors. Treatment with HAART reduces viral replication and the advancement to AIDS without eliminating the virus. The treatment objective with HAART is to maintain plasma viral load levels to < 50 copies/ml on a ultra-sensitive viral load assays (Justesen, 2006).

In the event that a patient develops resistance due to previous ARV exposure, another class of medication can be added (Miller *et al*., 2002). Therefore, to reduce the likelihood of resistance due to a viral strain to all the prescribed ARVs, the treatment plan must include drugs with diverse resistance profiles. Furthermore, rigorous patient adherence to the ARV cocktail is crucial in avoiding viral resistance to the selected HAART regimen (Bekker and Wood, 2011).

1.3.2 Effectiveness of HAART

One of the indicators to clinical treatment response is a decrease in the viral load with a concomitant rise in the CD4 count. If a patient adheres to the treatment regimen and there is suppression of viral load, drug resistance is lowered and treatment may be considered successful. However, a short duration of non-adherence to HAART, even if the patient had been on the treatment regimen for more than two years, will lead to a rapid rise in the viral load (Schulenburg and Le Roux, 2008).

In South Africa, the standardized national eligibility criteria for starting ART regimens for adults and adolescents is as follows (The South African Antiretroviral Treatment Guidelines, 2013);

1. Eligible to start ART
    - CD4 count ≤350 cells/mm^3 irrespective of World Health Organisation clinical stage (WHO, 2007)

OR

    - Irrespective of CD4 count

        - All types of Tuberculosis (TB) (In patients with TB/HIV drug resistant or sensitive TB, including extra pulmonary TB)

        - HIV positive women who are pregnant or breast feeding

OR

        - Patients with Cryptococcus meningitis or TB meningitis

- WHO stage 3 or 4 irrespective of CD4 count
    - WHO stage 3 is categorized by the following clinical manifestations, weight loss of greater than 10 percent of total body weight, prolonged (more than 1 month) unexplained diarrhoea, pulmonary tuberculosis, and severe systemic bacterial infections. (http://www.who.int/hiv/pub/guidelines/clinicalstaging.pdf)
    - The WHO clinical stage 4 (the severely symptomatic stage) designation includes all of the AIDS-defining illnesses. Clinical manifestations for stage 4 disease that allow presumptive diagnosis of AIDS to be made based on clinical findings alone are HIV wasting syndrome, Pneumocystis pneumonia (PCP), recurrent severe or radiological bacterial pneumonia, extrapulmonary tuberculosis, HIV encephalopathy, CNS toxoplasmosis, chronic (more than 1 month) or orolabial herpes simplex infection, esophageal candidiasis, and Kaposi's sarcoma (http://www.who.int/hiv/pub/guidelines/clinicalstaging.pdf)

2. Require fast track (i.e. ART initiation within 7 days of being eligible)
    - HIV positive women who are pregnant or breast feeding

OR

- Patients with low CD4 count <200 cells/mm^3

OR

- Patients with WHO Stage 4, irrespective of CD4 count

OR

- Patients with TB/HIV co-morbidity with CD4 count < 50 cells/mm^3

3. Patients with CD4 count above 350 cells/mm^3, not yet eligible for ART
    - Transfer to a wellness programme for regular follow-up and repeat CD4 testing 6-monthly.
    - Advise on how to avoid HIV transmission to sexual partners and children
    - Initiate INH prophylaxis if asymptomatic for TB
    - Provide counselling on nutrition and contraceptive use and perform annual pap smear

The introduction of HAART has not been without side effects and lipodystrophy has been shown to be associated with the use of HAART.

1.4. Physical and Metabolic changes in HIV positive patients on HAART

1.4.1 Body Composition

Before the availability of HAART, the progression of HIV infection was often accompanied by body weight loss (Grinspoon *et al*., 2003). In its most severe form, loss of body weight results in HIV wasting syndrome, which is recognized as one of the AIDS-defining conditions (Robinson, 2004). The syndrome characterized by the loss of both fat mass and lean body mass is known as the wasting syndrome and is distinctly different from lipodystrophy, which may be characterized by changes in body fat distribution (Jain *et al.,* 2012).

1.4.2. HIV associated lipodystrophy

Lipodystrophy has broadly been classified as lipoatrophy and lipohypertrophy. Lipoatrophy is characterized by wasting of fat in the extremities and includes subcutaneous fat loss of the arms, legs, face and buttocks (Bonfanti *et al*., 2007), whereas lipohypertrophy is an ART linked adverse event which is characterized by fat accumulation in visceral areas and includes breast enlargement, buffalo hump, increase in neck circumference, dorsocervical fat accumulation and non-specific lipomatous growth (Safrin *et al.,* 1999). The increase in visceral circumference may be as a result of increased intra-abdominal fat. It is also known that lipohypertrophy promotes insulin resistance and has been documented to occur considerably earlier than lipoatrophy irrespective of the site.

Women have been shown to have a higher tendency to develop lipohypertrophy. Although the exact mechanism for the development of lipohypertrophy is not clear, the prolonged use of PIs has been implicated (Norris and Dreher, 2004).

Interestingly, the use of NRTIs, such as stavudine (d4T) and zidovudine (AZT), have been associated with lipoatrophy. Although in some of the middle to low income countries d4T has only recently been phased out, but AZT is still widely being used. (Abrahams *et al.* 2014).

The key clinical features of lipoatrophy involve the loss of subcutaneous adipose tissue from the face and extremities resulting in an overly muscular appearance with prominent veins and sunken facial features. In conjunction with the loss of peripheral

adipose tissue, patients also have excess accumulation of adipose tissue in the dorso-cervical spine (buffalo hump), intra-abdominal region and around the neck area (double chin) (Riddler *et al.,* 2003).

Lipodystrophy is defined as the loss of subcutaneous fat and accumulation of intra-abdominal fat (Lichtenstein *et al.*, 2001). Assessing the physical bodily composition is fairly complex and the characterization of lipodystrophy may require either abdominal CT scan, DEXA scan, anion gap in blood and measurement of serum lipids. These tests and scans may reach only 80% specificity and 79% sensitivity in identifying lipodystrophy (De Waal *et al*., 2013). A simpler method of assessing lipodystrophy which employs a self-assessing questionnaire based on body image giving reliable results has been described by Asensi *et.al*., 2006. (used in this study).

Although the physical changes observed are often associated with successful reduction of viral burden and elevated CD4 counts, they also carry a social stigma by presenting an outward sign of a patient's HIV status. Therefore, the appearance-related side effects have a profound psychological effect, such as anxiety, depression and low-self-esteem, on patients and have been shown to impact long-term compliance to treatment (Chironi *et al.,* 2003, Power *et al*., 2003, Asztalos *et al*., 2006).

HIV/HAART associated dyslipidemic lipodystrophy (HADL) is used to describe a complex assembly of physical and metabolic abnormalities directly associated with the use of HAART in the treatment of HIV infection (Puro *et al*., 2000). Frequently, HADL is indicated when HIV patients present with a combination of the following: elevated total cholesterol (> 11.0 mmol/L) combined with a decrease in high density lipoprotein (HDL) (<1.0 mmol/L) levels, increased triglycerides (> 1.7 mmol/L), insulin resistance and changes in body fat distribution with increased truncal obesity (fat accumulating around the abdomen and torso area) (Haerter *et al.,* 2004). It is the manifestation of HADL metabolic symptoms that are considered to be significant risk factors for emerging cardiovascular disease (Martinez *et al.,* 2001).

Multivariate analysis has shown that lipodystrophy and HAART to be independent risk factors for increased intima-media thickness (Mercie *et al*., 2003), resulting in an increased risk of atherosclerosis and coronary artery disease in HIV infected patients.

Associations between the use of HAART and the increased risk of cardiac events have been shown in a number of studies, however these associations have not been consistently observed. Data from studies of a large cohort type vary on the risk of cardiovascular events in patients treated with HAART. On a follow-up of 36 766 persons infected with HIV, the Veterans Affairs Cohort observed that there was a decrease in the amount of hospital admission for cardio- or cerebrovascular disease, related to the introduction of HAART (Bozzette *et al*., 2001). These patients however had been on a

combination of antiretroviral therapy for a fairly short duration: the NRTIs median time of exposure was for 17 months, 16 months for PIs and 9 months for NNRTIs (Bozzette *et al*., 2001). The occurrence of myocardial infarction in the HOPS cohort (HIV Outpatient Study) increased significantly after PIs were introduced and the use of PIs were strongly linked with the probability of having a myocardial infarction (Holmberg *et al.*, 2002).

1.4.3 Clinical and Metabolic characteristics of HIV Associated Lipodystrophy (HAL)

As the use of HAART increased, reports of HAART associated lipodystrophy, thinning of the thighs and buttocks, buffalo humps and hypertrophy of breasts were observed (Stone *et al*., 1999) followed by reports of lipoatrophy of the face and accumulation of fat in the intra-abdominal (Gerrior *et al*., 2001). Currently, HAL is characterized by these features. It is not only the physical, but metabolic disorders such as insulin resistance and hyperlipidemia have been described to be associated with patients with HAL (Grinspoon, 2003). In addition to the physical changes, metabolic disoreders such as hyperlipidaemia and insulin resistance have been associated with HAL.

1.4.4 HAART-associated Hyperlipidaemia and insulin resistance in HIV positive patients.

The advent of HAART has indeed modified the natural history and progression of patients infected with HIV. Abnormalities in the lipid metabolism are common in HIV infected patients but tend to be exaggerated in those receiving HAART. Although

metabolic effects of HIV infection such as increased serum triglyceride levels has been well documented in HAART naïve patients, studies have shown that the pattern of serum lipid profile changes following HAART (Singh et al., 2014); total cholesterol, triglyceride and low density lipoprotein levels increase following HAART initiation. It is thus consistent with the cardio-metabolic risk profile that defines hyperlipidaemia under normal conditions. However, interestingly the study also showed that there was a significant increase in high density lipoprotein (HDL) levels with the use of HAART. HDL is known as the good component in total cholesterol because of its role in reverse cholesterol transport. Whereas the role of LDL is to transport excess cholesterol and triglycerides from the liver to peripheral tissues, HDL transfers cholesterol from the peripheral tissues through to the liver for excretion as bile acids. This transport is accomplished through the exchange of cholesterol from cells to HDL through the interaction of ATP binding cassette A1 transporters (ABCA1) on cell surfaces and apolipoprotein-AI proteins within the HDL particle (Petit *et al*., 2002). Interestingly, even after arterial lesions have been established, increasing HDL levels have been shown to reduce the size and number of lesion in both mice and humans. Indicating that HDL levels may be more important in coronary artery disease (CAD) risk than total cholesterol and LDL levels (Das*,* 2005).

Furthermore, patients who develop insulin resistance often progress to lipodystrophy, which is manifested in turn by the central redistribution of adipose tissue (Tershakovec, 2004).

HIV associated with insulin resistance is indicative of the metabolic syndrome of diabesity (a form of diabetes that typically develops later in life and is associated with obesity) in other ways: increased plasminogen activator inhibitor-1 (PAI-1) levels and endothelial dysfunction is also evidently shown in these patients (Larranaga *et al*., 2004).

Therefore, it is not surprising that HAART associated metabolic syndrome increases cardiovascular risk (Sekhar *et al*., 2004) and perhaps may also be regulated by the same mechanisms as those assumed in diabesity.

Interestingly, the development of insulin resistance does not occur in all patients infected with HIV on HAART treatment suggesting that there may be a genetic predisposition that requires investigation (Penzak *et al.,* 2000). It is also possible that an increase in the number of HIV patients having insulin resistance will emerge due to HAART being readily accessible (Bonnet *et al*., 2004). Nevertheless, the standard insulin resistance syndrome treatment that integrates diabesity principle management through which patients respond to treatment includes PPAR agonists agents and metformin, that have been recommended (Hadigan *et al.,* 2000).

As treatments for insulin resistance and diabetes have been suggested and indicated, there are also treatments for hyperlipidaemia. Although the use of statins has been the standard treatment for most hyperlipidaemic patients, statins (with the exception of pravastatin) have been shown to have an unfavourable potential interaction with

HAART regimen due to them being metabolised by the hepatic cytochrome system. Therefore, the use of fibrates has been suggested to be more effective in treating dyslipidaemia in HIV patients on HAART. (Corsini *et al.,* 2010).

Although abnormalities in the metabolism of lipids during the progression of acquired immunodeficiency syndrome (AIDS) and HIV infection were detected earlier, before the initiation of new regimens based on PIs (Sellmeyer, 1996), the dyslipidaemia which was not related to HIV treatment presented with decreased LDL and HDL cholesterol plasma levels followed by an increase in triglyceride throughout the advanced stages of HIV disease. Such changes in the plasma lipid levels can be compared to those seen in chronic bacterial infections, some viruses and other parasites. These are linked to the elevation of the cytokine interferon-a (IFN-a) in the advanced phase of HIV infection that correlates with a decreased triglyceride production (Grunfeld *et al*., 1991).

Simultaneously, tumour necrosis factor (TNF) may be involved in HIV-associated dyslipidaemia. Whilst HIV infection may not give rise to the levels of TNF, TNF may rise due to opportunistic infections in patients who have AIDS. This elevation may aggravate a decline in serum concentrations of HDL and LDL cholesterol. Interestingly, the plasma levels of interleukin-1 (IL-1) are not detectable in subjects that present with AIDS and are not related to an increase of triglycerides or cholesterol in the blood stream (Constans *et al*., 1994).

The progression of the HIV infection may be monitored through hypertriglyceridaemia and hypocholesterolaemia and have been included as markers, with a reduced CD4 lymphocyte count being inversely related to both severity and frequency of dyslipidaemia (Grinspoon *et al.,* 2001, Dubé and Cadden, 2011). Predominantly, a CD4 cell count of lower than 200 lymphocytes/mm^3 in patients who have a significantly reduced total cholesterol concentration than the HIV-negative subjects and those below 400 lymphocytes/mm^3 have significantly greater triglyceride concentration, when they are compared to matched HIV-negative controls (Constans *et al*., 1994).

Hyperlipidaemia characterized by an increased concentration of serum total cholesterol, triglycerides, low density lipoprotein (LDL) cholesterol and low HDL cholesterol, occurs often in HIV positive patients who are on long-term antiretroviral therapy (ART) (Grinspoon *et al.,* 2001).

It has been well established that dyslipidemia and particularly increased LDL levels are a significant risk factor in the development of coronary artery disease (CAD) (Mallal *et al*., 2000). LDL particles are generated from very low density lipoproteins (VLDLs) which are secreted from the liver and carry excess hepatic cholesterol and triglycerides (TG) to peripheral organs for use as energy or for storage (Hui, 2003).

Clinical reports on the prevalence of HADL are conflicting (Chene and Ducimetire, 2003). The percentage of patients presenting with symptoms associated with lipodystrophy and dyslipidemia range from 20% to as high as 80%. The lack of a

unifying definition of HADL combined with interpretational differences in the significance of changes in HADL syndrome markers is a likely reason for variability in the incidence of HADL in HIV positive patient populations. Despite these differences, it is certain that long-term HIV survivors will face additional health risks and quality of life issues associated with HAART (Chene and Ducimetire, 2003).

Antiretroviral agents have different effects on the hyperlipidaemia patterns and frequencies. PI induced hyperlipidaemia occurred in 47-75% of patients (Friis-Moller *et al.,* 2003). The maximum risk of elevation in LDL and TG occurs with the use of d4T amongst the NRTIs. NNRTIs users may have elevated HDL and TG levels. After commencing therapy, lipid profile disturbances are seen in weeks to months of starting therapy (Friis-Moller *et al.,* 2003).

Plasma lipid abnormalities appear to be common in patients receiving a PI treatment regimen. This is besides the fact that dyslipidaemia is sometimes associated with stavudine, lamivudine, zidovudine or non-nucleoside reverse transcriptase inhibitors (NNRTIs) (Carr and Amin, 2009).

As mentioned previously, the incidence of hyperlipidaemia ranges in between 28 to 80% in patients receiving PI as their treatment regimen. Hypertriglyceridaemia was present in most cases which may be followed by the development of hyperglycaemia and

hypercholesterolaemia (Roberts and Volberding, 1999). They also showed the fat redistribution syndrome prevalence ranges from 10 to 80%.

Dyslipidaemia is linked to the presence of lipodystrophy syndrome; however metabolic changes can also present without morphological changes that occur in patients who present with lipodystrophy. It has been observed that metabolic abnormalities usually precede the body fat redistribution (Mulligan *et al*., 2000).

Hypertriglyceridaemia is associated with the depletion of fat or lipoatrophy, whereas fat lipohypertrophy syndrome or accumulation of fat is accompanied by dyslipidaemia (Yanovski *et al.,* 1999). The following factors have been shown to be associated with hypertriglyceridaemia in several studies prior to the PI therapy initiation: heavier weight, male gender, older age, higher body mass index, homosexual orientation, AIDS diagnosis and higher levels of triglyceride with cholesterol plasma (Savès *et al.*, 2002).

However, studies show that these factors (gender, baseline lipid levels, HIV disease stage and weight of the body) may not be related with the incidence of hypertriglyceridaemia (Tsidras *et al.,* 2000). Nevertheless, the association of increased cholesterol concentration has been pointed out by some of the authors, in HIV patients having higher numbers of CD4 cell count, age and body mass index (Mooser *et al.,*

2001). Consequently, the hyperlipidaemia risk factors which are associated with PI treatment are still being investigated and hypothesized till today (Benson *et al*., 2002).

Patients who are treated with ritonavir frequently appear to have a mild to moderate increase in the levels of cholesterol when compared to patients on indinavir (Periard *et al.,* 1999). Indinavir, in a retrospective analysis was shown to be associated with a reduced risk of hypercholesterolaemia and hypertriglyceridaemia (Carr *et al*., 1998). However, it appears that it is the duration and dose that determines the progress of hyperlipidaemia throughout the administration of PIs. Serum lipid irregularities arise soon after commencing therapy, during the 3 to 12 months, although it may be earlier in subjects who are receiving the ritonavir-containing regimen (Manfredi *et al*., 2004).

The highest frequency and severity of elevations in lipid level is associated with Ritonavir on its own or in combination with lopinavir or saquinavir (Currier *et al*., 2000). However there are limitations on the availability of data and it remains inadequate to determine the real effect of indinavir, nelfinavir, saquinavir and amprenavir on plasma lipid concentrations (Currier *et al*., 2000).

Cumulative exposure of stavudine in some studies has been shown to be associated with hyperlipidaemia, even though the exact mechanism behind this effect on lipid metabolism is still being postulated (Mooser *et al*., 2001). Moreover, some studies have

shown that patients that have hyperglycaemia frequently present with hypertriglyceridaemia, while the progression of hypercholesterolaemia is inversely associated with chronic hepatitis C virus infection (Paparizos *et a*l., 2000).

Alterations in the metabolism of lipids have been frequently reported in paediatric HIV infected patients receiving HAART. In this study 70% of children who were on ritonavir treatment were found to have hyperlipidaemia whilst only 50% of those receiving nelfinavir were found to have hyperlipidaemia (Fiore *et al.,* 2000).

1.5. Potentially life-threatening and serious adverse events:

1.5.1. Mechanism of Insulin Resistance

The study reported by Perseghin *et al*. (2003) may be used to understand mechanisms responsible for insulin resistance, which may be related to the signaling pathway induced by acyl-CoA derivatives in the inhibition of insulin function. It is possible that excessive acyl-CoA inside the cytosol, exceeds mitochondrial acyl-CoA and peroxysomal degradation lead to the buildup of triglycerides in muscle and hepatocyte cells. This accumulation has been indicated in obesity, insulin resistance, metabolic syndrome and diabetes in several studies (Martins *et al*., 2012).

As reported by Myarcik *et al*., 2000, a strong association exists between the amount of fat accumulation in the liver and fat in the visceral area. Similarly, insulin resistance in diabetic and obese patients is related to the amount of fat intramyocellularly. This type of alterations leads to a condition referred to as lipotoxicity (Perseghin *et al*., 2003).

Recent studies have shown that adipose tissue location is important, suggesting that there is a proportional association between visceral fat and insulin resistance as measured by the Homeostasis Model Assessment (HOMA). A method used to quantify insulin resistance and beta-cell function (Myarcik *et al*., 2000).

In addition its effect may also be noted to its sensitivity to catecholamines and predisposition of free fatty acids (FFA) in the portal circulation. Whereas, subcutaneous fat releases lower levels of FFA with a priority towards peripheral tissues which is lipolysis resistant (Mulligan, 2001).

As with most inflammatory processes there is an alteration in the cytokines profile in the lipodystrophic adipose tissue including increased levels of interleukin (IL)-6 and tumor necrosis factor (TNF)-alpha. These cytokines secreted by adipocytes, are thought to play an important role in the development of insulin resistance, which results in FFA fluxes and increased lipolysis which induces insulin resistance. Additionally, adipose tissue which is lipodystrophic has a reduced adiponectin secretion, which could in turn

result in decreased lipid oxidation, glucose uptake by the muscles and consequently a rise of glucose production by the liver (Brown *et al*., 2005).

Therefore it is reasonable to suggest that lipodystrophic adipose tissue that is altered in function may aggravate insulin sensitivity and drug-induced alterations of metabolism in glucose. Additionally, it appears that it is possible that through this mechanism where protease inhibitors (PI) have a direct impact on lipid metabolism (Gougeon *et al*., 2004), may synergistically lead to the progression of metabolic disturbances.

However, due to the different methods used to measure glucose levels and diagnose of insulin resistance and diabetes it is difficult to compare the outcomes from different studies investigating the metabolism of glucose in patients with HAL. A study by Hadigen and colleagues, 2001, found that 35% of patients had impaired glucose tolerance (IGT) with HAL, and frank diabetes was observed in an additional 7%. Other studies have reported a 7 - 13% occurrence of diabetes measured by an OGTT, 17 - 46% frequency of IGT and peripheral insulin resistance incidence up to 61% measured with the use of intravenous insulin tolerance test in patients receiving PIs (Behrens *et al*., 1999). Furthermore, in one study that consisted of a 1011 patients who were on ARVs were followed for 289 days, showed 16 new cases of diabetes was diagnosed, suggesting that the possibility of developing diabetes was considerably higher in patients who were receiving stavudine or indinavir (Mallal *et al*., 2000).

However, diabetes was a rare occurrence in patients infected with HIV prior to the introduction of HAART. The disturbance in glucose metabolism by medications, such as megestrol acetate, pentamidine or corticosteroids was often hypothesized in HIV positive patients (Domingo *et al*., 2005). Prior to the introduction of HAART regimen, patients had increased serum concentrations of triglycerides but decreased concentrations of total, LDL and HDL cholesterol in the advanced stages of HIV infection (Grufeld, 2004).

Interestingly, pathways by which insulin resistance develops in HIV-infected individuals on HAART, with or without morphologic abnormalities, are believed to include impaired glucose uptake in skeletal muscle, impaired processing of pro-insulin to insulin and impaired beta cell function (Bodasing, 2003).

Before PI containing HAART regimes, patients with HIV were found to present with no substantial insulin resistance and also with normal or reduced glucose levels (Bodasing, 2003). Abnormality in homeostasis of glucose is suggested to be present in 20-60% of patients receiving PI therapy. These abnormalities are more likely to be present in lipodystrophy (Carr and Amin, 2009).

1.6 Assessment of HIV Lipodystrophy Syndrome (LDS)

Due to a lack of standardization, the assessment of HIV LDS varies widely among researchers (Norris and Dreher, 2004).

1.6.1 Methods for assessing HIV Lipodystrophy Syndrome

Methods that are used in HIV LDS research studies include subjective self-reporting, assessment by physicians, anthropometric measurements, bioelectrical impedance analysis (BIA), magnetic resonance imaging (MRI), computed tomography (CT) scan and dual energy X-ray absorptiometry (DEXA). These methods of assessing HIV LDS vary widely in objectivity, methodology, accuracy, cost and standardization of classification. A standard, universal acceptable case definition is needed to address the above problems. A case definition for HIV LDS should include all clinical components seen in HIV LDS, link abnormalities or pathophysiologic mechanisms and strengthen the association between abnormalities and specific HAART used (Carr *et al*., 2003).

1.6.1.1 Anthropometry

Inexpensive ways of assessing morphological changes are anthropometric measurements e.g. skinfolds and circumferences (Norris and Dreher, 2004). Anthropometric measurements are easy to perform, non-invasive, readily available and practical to use in a clinical environment. Accurate measurements are dependent on proper training and standardization of techniques as well as the prediction equation that is used (Gerrior *et al.*, 2001).

1.6.1.2 Weight

One of the simplest anthropometric methods used in HIV positive persons is monitoring weight. Many HIV infected individuals experience substantial weight loss in a short period of time before initiation of treatment. Earlier studies supported the idea that weight loss in untreated AIDS patients is mostly loss of body cell mass, but later studies showed an equal loss of both fat and lean body mass. However, interestingly it has been shown that malnourished women, in contrast to men, tend to lose more fat than lean body mass (Maia *et al*., 2005).

Once initiated on HAART individuals appear to regain their weight although it is mostly from the fat compartment. Even though body weight can be maintained throughout treatment on HAART, the loss of lean body mass (LBM) can still continue while visceral fat is accumulating (Jain *et al*., 2001). The manifestation of HIV wasting has changed in the era of HAART to body composition changes: LBM is wasted (lipoatrophy) and fat is centrally accumulated (lipohypertrophy), resulting in unchanged weight. The monitoring and interpretation of weight, in the face of HIV LDS, can thus be misleading and unreliable as a measure of fat-free-mass and changes in HIV infection (Gerrior *et al.*, 2001).

1.6.1.3 Waist and Hip circumferences

Abdominal obesity is highly correlated with the increase in visceral adipose tissue mass. Waist circumference (WC) has been shown to be more associated with visceral adipose tissue mass than Waist Hip Ratio (WHR) and Body Mass Index (BMI). Waist Circumference measurement is a practical way of evaluating the presence of regional fat depots. A WC of more than 102cm for men and 88cm for women is positively associated with fat depots (Dong and Henricks, 2005).

The most common sites for measuring WC are as follows:

- Immediately below the lowest area of the ribs
- At the narrowest waist (according to the Anthropometric Standardization Report Manual)
- Midpoint between lowest rib and iliac crest (WHO guidelines)
- Immediately above iliac crest (NIH guidelines) (Wang *et al*., 2003).

1.7 Association of ApoCIII, Beta-3 adrenegic receptor and Tumour Necrosis Factor-alpha gene polymorphisms with HAART associated lipodstrophy.

The development of lipodystrophy associated with genetic polymorphisms of, ApoCIII (Mallon*,* 2006), Beta 3 Adrenergic receptor (Okumura, 2003) and TNF alpha (Maher *et al.,* 2002), genes, has been documented as described, however, this association has not been shown in the South African HIV positive population.

1.7.1 ApoCIII in HAART associated Lipodystrophy

Apolipoprotein CIII (ApoCIII), an essential component of HDL and very low lipoprotein (VLDL), has been shown to be an important regulator of intravascular triglyceride metabolism, through the inhibition of lipoprotein lipase and interference with apoE mediated triglyceride–rich lipoprotein uptake by hepatic receptors (Onat *et al*, 2003).

In-vitro and transgenic animal studies have demonstrated that over expression of apoCIII results in delayed clearance of triglyceride rich lipoproteins from plasma resulting in overt hypertriglyceridaemia. Furthermore, results of clinical studies have specified that apoCIII levels were better predictors of risk for the progression of CAD than traditionally measured serum triglyceride levels (Sacks *et al*., 2000). Recent interest has focused on the possible involvement of genetic variations in genes regulating lipid metabolism that may be responsible for hyperlipidaemia (Hegele*et al.,* 1997).

Various studies have shown that hyperlipidaemia and particularly hypertriglyceridaemia may have a genetic predisposition, but the gene responsible for it has not yet been fully elucidated (Talmud *et al.*, 2007). Olivieri and coworkers (2003) identified an apoCIII polymorphism that plays a role in the metabolism of circulating triglyceride rich lipoproteins.

The human Apolipoprotien CIII (apoCIII) gene has been mapped on the long arm of chromosome 11 and several variant alleles have been investigated as possible genetic markers of hypertriglyceridaemia. Two polymorphic nucleotides located at positions -455 (T to C) and -482 (C to T) in the apoC-III promoter region have been identified and shown to influence serum triglyceride levels (Mallon, 2006). Thus, there is a great interest in the role of apoCIII gene polymorphism on both the expression of apoCIII as well as on its effects on triglyceride metabolism particularly in HAART associated lipodystrophy.

1.7.2 Beta-3 Adrenergic receptor in HAART associated Lipodystrophy

The beta-3-adrenergic receptor (β_3AR) is found in visceral adipocytes linked to the receptor G-protein. In the visceral adipocytes it is involved in the regulation of thermogenesis and lipolysis. Stimulation of the receptor occurs by sympathetic nervous system activation and local secretion of norepinephrine. Enhancement of glucose uptake in rodent models of obesity, fat oxidation increase and energy expenditure improvement are pharmacological effects associated with stimulation the β_3AR (Moens, 2010).

The β_3AR gene is expressed in visceral fat and is a candidate gene for abdominal obesity. Polymorphism in the β3AR gene are closely related to insulin resistance and obesity of the abdomen that results in the replacement of tryptophan by arginine at position 64 (Trp64Arg) (Okumura, 2003).

To test the possibility that the development of lipodystrophy may be associated with β_3AR polymorphism, Vonkeman and colleagues (2000) investigated this possibility. From a cohort that consisted of 135 HIV patients on HAART, 39 HIV patients were chosen: 17 without lipodystrophy and 22 with lipodystrophy. The control group consisted of 200 non-HIV individuals with a body considered to be “normal” (non lipodystrophic). The HIV patient group with lipodystrophy having the characteristic features (LDS+, n=22), six patients were found to be heterozygous and none was homozygous for the Arg64 allele. In the HIV patients who were negative for LDS (LDS-, n=17) two were heterozygous, none homozygous. In the last group which were the control, 19 out of 200 individuals were heterozygous; one was homozygous for the 64Arg allele.

Their observation that β_3AR codon mutation in the Trp64Arg is a genetic risk factor for peripheral lipodystrophy associated with protease-inhibitor- may be novel finding. The abnormalities in β_3AR might lead to speculation that a reduction in lipolysis and thermogenesis in visceral adipocytes and thus result in the accumulation of fat in the abdominal region.

1.7.3 Tumour Necrosis Factor-Alpha in HAART associated Lipodystrophy

Tumour necrosis factor-alpha (TNF-α) is a multi-functional cytokine that is synthesized as a transmembrane monomer of 26-kDa (mTNF-α). This monomer is proteolytically cleaved by the TNF-α converting enzyme (TACE) to produce a soluble TNF-α molecule

that is 17-kD in size. Due to modulation of adipocytes differentiation and lipolysis adipose tissue expression of TNF-α has been implicated in lipoatrophy. The two cell surface receptors: tumour necrosis factor-alpha receptor 1 (TNFR1) and TNFR2 are used to facilitate TNF- biological effects on adipose tissue (Azmy *et al*., 2004).

As with TNF-α, the soluble form of the receptor (sTNFR) is released from proteolytic cleaving of TNFRs. The major effects on the function of adipose tissue are transduced by a signal from TNFR 1 to mediate TNF-α as suggested by Cawthorn and Co-workers. Their study showed that TNFR1 is essential for the inhibition of adipogenesis through TNFR1 and/or TNFR2-deficient preadipocytes. However, the activity of an intrinsic catalytic nature is not shown by both TNFRs. Thus, it is possible that they may employ intracellular proteins for signal transmissions, which interrelates with specific domains of the cytoplasmic portions of the receptors, thereby activating specific signals downstream. This promotes lipolysis and adipocyte differentiation (Cawthorn *et al.*, 2008).

TNF-α has actions similar to those seen in lipodystrophy; therefore Maher *et al* (2002), carried out an analysis, to determine whether the presence of lipodystrophy was associated with the polymorphisms in the promoter region of the TNF-α gene. The TNF-α gene was genotyped for -238A and -308A polymorphisms in all the individuals.

The polymorphism at position -238A in the promoter region of TNF-α has been associated with HAART treated patients with a likelihood of a more-rapid

commencement of lipoatrophy. This observation is confirmed by results in the above mentioned study. Therefore one can suggest that this polymorphism interferes with the ability of TNF-α in regulating lipolysis.

Whilst high levels of cholesterol, triglyceride and insulin resistance are common features of ApoCIII and β_3 adrenergic receptor polymorphisms, there is very sparse literature on the serum triglyceride and cholesterol levels associated with TNF-α polymorphism.

1.8 Rationale for the study.

Although extensive research on ApoC-III, β_3AR and TNF-α in lipodystrophic patients has been conducted in other countries, no such studies have been done in the South African population. Therefore we investigated the frequency / prevalence of these polymorphisms in the South African HIV positive population receiving HAART.

Chapter 2

Materials and Methods

2.1 Reagents and Chemicals

The following reagents listed in Table 2.1 below were used.

Table 2.1: List of Reagents

Reagents and Chemicals	**Source**
Agarose Tablets	Invisorb (Invitek, USA)
Apo CIII Primers	Roche Diagnostics
Beta-3 Adrenergic Receptor primers	Inqaba, Pretoria, South Africa
DNA (Deoxyribonucleic Acid) Extraction kit	Invisorb (Invitek, USA)
DMSO	Merck, NJ, USA
Ethidium Bromide	Roche Diagnostics, Indianapolis, USA
Molecular Marker V	Roche Diagnostics, Indianapolis, USA
PCR Master (Master Mix)	Roche Diagnostics, Indianapolis, USA
PCR Loading Buffer	Fermentas, Johannesburg, SA
Restriction Enzymes (*Msp I*, *Fok I* and *Nco I*)	Roche Diagnostics, Indianapolis, USA
TNF-alpha and ApoCIII primers	Roche Diagnostics, Indianapolis, USA

2.2 Collection of blood samples from subjects

A total of 209 HIV positive subjects were recruited from Helen Joseph and Charlotte Maxeke Academic Hospitals. Majority of these subjects, 65%, were on 1st line treatment (Lamivudine, Stavudine & Efivarenz) while 33% were on 2nd line treatment (Didanosine, Lopinavir & Zidovudine). The remaining 2% of subjects were on the combination of 1st and 2nd line treatments. 100 HIV negative subjects (assumed negative by personal verbal confirmation) were recruited from the University of the Witwatersrand Medical School and Charlotte Maxeke Academic Hospital. Ethics clearance was obtained from the Human Research Ethics Committee of the University of the Witwatersrand and Informed consent was obtained from all individuals. The information was coded to ensure privacy and confidentiality. Twenty millilitres of blood was collected from each subject, 10ml of clotted blood, 5ml for blood glucose in a tube containing oxaloacetate and a 5ml in a tube containing EDTA (for DNA extraction).

All the blood samples were centrifuged using the Beckman Centrifuge (Model TJ-6) for 15 minutes at 2000 rpm. Serum was stored in a -20ºC freezer for lipogram, Insulin and Glucose tests. The buffy coat region between plasma and packed red blood cells from EDTA containing tubes was extracted and also stored in a -20ºC freezer for DNA extraction.

2.3 Anthropometrical Measurements

The following anthropometric measurements were recorded; age, ethnicity, systolic and diastolic blood pressure, weight, hip, waist and height. Body mass index (BMI) was calculated by weight in kilograms (kg) divided by the square of the height in meters (Kg/m^2). The waist circumference was measured with a soft measuring tape to the nearest 0,5cm at the level of the smallest girth above the umbilicus in a standing position. The hip circumference was measured on the widest part of the gluteus region. Blood pressure was measured in a sitting position by using a mercury sphygmomanometer with a standard cuff.

2.4 Diagnosis of lipodystrophy

Lipodystrophy in HIV positive subjects was diagnosed using a questionnaire for assessing body fat re-distribution in HIV-positive patients receiving HAART in Appendix E (Asensi *et al*., 2006). The face, breast, belly, arms, legs and buttocks/hips were assessed for body fat changes (both losses and gains). Changes were rated as absent (score of 0), mild (score of 1; noticeable only on close inspection), moderate (score of 2; easily noticeable by patient and doctor) or severe (score of 3; easily noticeable by casual observer). These changes were reported by the patient and confirmed by the clinician. The scores for each of the 7 body areas were added together to diagnose lipodystrophy. Furthermore subjects with scores of 3 for any individual body area were also diagnosed with lipodystrophy.

2.5 Glucose and Lipid measurements

2.5.1 Glucose measurement

Fasting blood glucose levels were measured in the routine chemistry laboratory (NHLS) in Charlotte Maxeke Hospital by a glucose oxidase method (glucose GODPAP, Boehringer Mannheim) using the Hitachi 717 Autoanalyser. Subjects who at the time of the study had already been diagnosed as diabetics, were defined as diabetics irrespective of the levels obtained on the day. Hyperglycaemia was defined according to the WHO criteria. As per the World Health Organization people with fasting glucose levels from 6.1 to 6.9mmol/l (110 to 125mg/dl) are considered to have impaired fasting glucose. Subjects with plasma glucose at or above 7.8mmol/l (140mg/dl), but not over 11.1mmol/l (200mg/dl), two hours after a 75g oral glucose load are considered to have impaired glucose tolerance. Of these two pre-diabetic states, the latter in particular is a major risk factor for progression to full-blown diabetes mellitus, as well as cardiovascular disease.

2.5.2 Measurement of serum lipid levels

Lipogram measurements were done in the routine chemistry laboratory (NHLS) in Charlotte Maxeke Hospital using a Modular ISE 900 Autoanalyser (Roche Diagnostics, Mannheim, Germany).

LDL-cholesterol was calculated using triglyceride and cholesterol levels using the Friedewald formula as follows: LDL = (total cholesterol – HDL) – (triglycerides/2.22) (Friedwald, 1972). A value of between 4.1-4.9mmol/L was considered hypercholesterolaemia.

2.6 DNA Extraction

DNA (Deoxyribonucleic Acid) was extracted from the buffy coat of the blood, which is the leukocyte-enriched fraction of whole blood. The Invisorb spin blood mini kit (Invitek, USA) was used to extract DNA. The following protocol for DNA extraction was followed (supplied in the kit):

The required amount of elution buffer was transferred into a 2.0ml receiver tube and the tube placed in a Multi-Blok heater (Thermo Scientific, SA) at 56ºC.

- Lysis at 56 ºC for 10 min in a Thermomixer

Two hundred microlitres of buffy coat was placed into a 1.5ml tube, to which 200µl of lysis buffer and 20µl proteinase k was added. The tube vortexed and incubated in a Thermomixer at 56ºC for 10min.

- Realizing of optimal binding conditions

To the lysed sample 400µl of binding buffer was added, vortexed for 2-5 seconds and was carefully loaded onto the spin filter and incubated for 1min at room temperature. The sample was then centrifuged for 2min at 12 000rpm. The receiver tube was

discarded with the filtrate after centrifugation. The spin filter with the sample in it was placed in a new 2.0ml receiver tube.

- Washing I

To the spin filter 500µl wash buffer I (containing ethanol) was added and centrifuged for 1min at 12.000rpm. The filtrate was discarded and the spin filter was placed in the 2.0ml receiver tube.

- Washing II

To the spin filter 800µl wash buffer II was added and centrifuged for 1min at 12 000rpm. The filtrate was discarded then the spin filter was placed back into the 2.0ml receiver tube. To remove the wash buffer completely the receiver tube was centrifuged for 4min at 12 000rpm.

- Elution of the DNA

The spin filter was then placed into a new 1.5ml receiver tube. To the spin filter 200µl of prewarmed (56ºC) elution buffer was added. The tubes were then centrifuged for 1min at 12000rpm. The filtered product contained DNA. The DNA was stored at -20ºC before use.

- DNA concentration

The concentration of the DNA was determined using a Nanodrop ND-1000 (Nanodrop Technologies, USA). DNA concentration was obtained at of 260nm wavelength and the purity of DNA assessed using the ratio of absorbance at 240/260 - 260/280nm was

used to assess the purity of DNA. A ratio of less than 2 indicates the presence of protein contamination.

2.7 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is a molecular technology used to amplify a single copy or a few copies of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence (Joshi *et al*., 2011). PCR consists of the following steps:

- *Initialization step*: This step consists of heating the reaction to a temperature of 94–96 °C for 1–9 minutes.
- *Denaturation step*: This step is the first step of the cycling event and consists of heating at 94–98 °C for 30-60 seconds resulting in the denaturation of the DNA template by disrupting the hydrogen bonds between matching bases, creating single-stranded DNA molecules.
- *Annealing step*: The reaction temperature is lowered to 50–65 °C for 30–60 seconds allowing annealing of the primers to the single-stranded DNA template. The annealing temperature is approximately 3–5 °C below the melting temperature (Tm) of the primers used.
- *Extension/elongation step*: The temperature at this step depends on the DNA polymerase used; *Taq* polymerase has its optimum activity temperature at 70–75 °C and commonly a temperature of 72 °C is used for 30-60 seconds. In

this phase the DNA polymerase synthesizes a new DNA strand complementary to the DNA template.

- *Final elongation*: This single step is frequently performed at a temperature of 70–74 °C for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.
- *Final hold*: This step at 4–15 °C for an unlimited time may be employed for short-term storage of the reaction.

PCR was done on the genomic DNA for the amplification of the specific genes (ApocIII, Beta-3 Adrenergic receptor & TNF Alpha) Primers specific for each gene were used table 2.2.

Table 2.2: Primer sequences for each gene

Gene	Forward primer sequence	Reverse primer sequence
ApoCIII455	5´-GGCTGTGAGACTCAGCCCT-3´	5´-TCACACTGGAATTTCAGGCC-3´´
ApoCIII 482	5´-GGCTGTGAGACTCAGCCCT-3´	5´-TCACACTGGAATTTCAGGCC-3´
β3- ADR	5´-CAATACCGCCAACACCAGTGGG-3´	5´-GGTCATGGTCTGGAGTCTCG-3´
TNF-α 238	5´- GAAGACCCCCCTCGGAACC -3´	5´- ATCTGGAGGAAGCGGTAGTG -3´
TNF-α 308	5´-GCAATAGGTTTTGAGGGCCATG -3´	5´- GGGACACACAAGCATCAAGGAT -3´

2.7.1 Preparation of the Master Mix

The master mix was prepared to ensure optimised concentrations of all reagents. The master mix contained distilled water, buffer, magnesium chloride, dNTPs, primers and *Taqpolymerase.*

The master mix was prepared as in Table 2.3 and DNA was added to each individual reaction. Primers specific for each gene were used for each master mix. A final total volume of 25μl PCR reaction was used.

Table 2.3: Volume and Concentration of the PCR reaction

Reagent	**Concentration**	**Volume**
Water	-	6.8 μl
Master mix (*Roche*) containing:		12.5μl
Taq	1 unit	
Mg++	1.5 mmol	
dNTP	3.2 mmol	
Forward primer	0.5 pmol	0.1μl
Reverse primer	0.5 pmol	0.1μl
DMSO	5%	0.5μl
Template DNA		5μl
Total Volume		25μl

2.7.2 PCR temperature cycles

The C1000 Thermal Cycler (Biorad-Laboratories) was programmed for PCR, conditions for each of ApoCIII, β3AR and TNF-α is shown in tables 2.4, 2.5, 2.6 and 2.7.

Table 2.4: PCR temperature conditions for *ApoCIII 455 and 482* genes.

Conditions	**Temperature**	**Time**	**No. of cycles**
Denaturation	95ºC	11 minutes	1 X
Denaturation	94ºC	45 seconds	35 X
Annealing	60ºC	30 seconds	
Extension	72ºC	45 seconds	
Final Extension	72ºC	10 minutes	1 X
Hold	4 ºC	∞	

Table2.5: PCR conditions for *Beta-3 adrenergic receptor* gene.

Conditions	**Temperature**	**Time**	**No. of cycles**
Denaturation	95ºC	11 minutes	1X
Denaturation	94ºC	45 seconds	30 X
Annealing	59ºC	2:30 minutes	
Extension	72ºC	45 seconds	
Final Extension	72ºC	10 minutes	1X
Hold	4 ºC	∞	

Table 2.6: PCR conditions for *TNF-α 238* gene.

Conditions	**Temperature**	**Time**	**No. of cycles**
Denaturation	95ºC	5 minutes	1X
Denaturation	94ºC	1 minute	35 X
Annealing	58ºC	1 minute	
Extension	72ºC	1 minute	
Final Extension	72ºC	7 minutes	1X
Hold	4 ºC	∞	

Table 2.7: PCR temperature conditions for *TNF-α 308* gene.

Conditions	**Temperature**	**Time**	**No. of cycles**
Denaturation	95ºC	11 minutes	1X
Denaturation	94ºC	45 seconds	35 X
Annealing	55ºC	30 seconds	
Extension	72ºC	45 seconds	
Final Extension	72ºC	10 minutes	1X
Hold	4 ºC	∞	

2.7.3 Gel electrophoresis and Single Nucleotide Polymorphism detection

- The amplified PCR products for each gene were digested with a specific restriction endonuclease. The ApoCIII 455 gene was digested with *Fok I* restriction enzyme and the ApoCIII482 with *Msp I* in two separate reaction tubes. The *β3*-ADRgene was digested with *Msp I* restriction enzyme *Msp I* and *Nco I* restriction enzymes were used to digest the TNF-α 238 and TNF-α 308 respectively. The digestion sites are also shown in Table 2.8.

- The total volume of the reaction mixture was 20μl (15μl of PCR product, 2μl of buffer, 2μl of water and 1μl of the restriction enzyme).

- The reaction was incubated at 37°C for 2hrs

Table 2.8: Cut site of each restriction endonuclease

Enzyme	**Cut Site**
Fok I (Roche Diagnostics)	5´- GGATG -3´ 3´- CCTAC -5´
Msp I (Roche Diagnostics)	5´-C CGG-3´ 3´-GGC C-5´
NcoI (Roche Diagnostics)	5´-C CATGG -3´ 3´- GGTAC C -5´

- The samples were incubated for 2 hours at 37°C
- After incubation the samples were prepared by mixing 20μl mIcrolitres of PCR product with 2μl the loading buffer and then loaded on a 2% agarose gel. Preparation of the loading buffer is described in Appendix A.
- After all the samples were loaded onto the electrophoresis gel, the voltage was set at 100V. The gel was run for 60 minutes.
- The resulting bands for each gene were visualized under UV light using the Bio-Rad Gel Doc. The expected bands for each polymorphism are shown in Table 2.9. A DNA base length Marker V (20-600bp) was included in all the gel runs and band lengths.

- **Table2.9**: Expected sizes of the different genotypes of each amplified product.

Gene	**Band size** **(Homozygote for the wildtype)**	**Band size** **(Heterozygote for variant)**	**Band size** **(Homozygote for the variant)**
Fok I (ApoCIII T455C)	129 bp + 133 bp TT	129 bp + 133 bp + 196 bp TC	196bp CC
Msp I (ApoCIII C482T)	143 bp CC	143 bp + 159 bp CT	159 bp TT
Msp I (β3-Adr T64A)	54 bp + 99 bp TT	54 bp + 70 bp + 99 bp TA	54 bp+70 bp AA
Msp I (TNF-α G238A)	132 bp GG	132 bp + 151 bp GA	151 bp AA
Nco I (TNF-α G308A)	126 bp GG	126 bp + 144 bp GA	144 bp AA

- The data in Table 2.9 was used to genotype an individual as a homozygous for the wildtype, heterozygous for variant or homozygous for variant.

2.8 Data Analysis

The data was analyzed using the Statistica program (version 8; Microsoft, USA). Distributions of different alleles were calculated by the Hardy-Weinberg equilibrium. Differences in prevalence of the different alleles between population groups were analyzed by using the Chi-Squared test. Differences in the anthropometric variables between the different genotypes and ethnic groups were analyzed using one way analysis of variance (ANOVA) which tests the differences between the means of two or more groups. The metabolic and anthropometric data is expressed in tables as mean ± SD.

2.8.1 Hardy-Weinberg Equilibrium (HWE)

Hardy-Weinberg Equilibrium (HWE) is the test comparisons of the observed and expected genotype frequencies calculated in an observed population. A population is said to be in HWE for a given locus if there is random mating with respect to the locus, no selection, no mutation, no gene flow and a population is big enough to avoid the random effect of genetic drifts. If a population is not in HWE, it might be due to one population with selection that causes it to be out of equilibrium. This may include non-random mating, gene selection and small population size.

If two populations display HWE, there is a possibility that difference in allele frequencies between the two populations is due to reproductive isolation. To determine if the population is in HWE, the equation $p^2+2pq+q^2=1$is used to determine expected genotype frequencies from allele frequencies where p and q represent homozygous allele frequencies.

2.8.2 Chi-Squared Goodness- of-Fit Test

This test is used to determine whether there is a significant difference between the observed and expected genotype frequencies. The Chi-Square (χ^2) is calculated by the following equation: $\chi^2 = \sum\frac{(\text{Observed-Expected})^2}{\text{Expected}}$

In the Chi-Square test, two hypotheses are tested. The first hypothesis is the Null Hypothesis (H_0), which states that there is no difference due to chance between the observed and expected values, therefore the population is in HWE. The second hypothesis is called Alternative Hypothesis (*Ha*), which states that the observed and expected values are significantly different due to other reasons other than chance. Thus the population is not in HWE.

Chapter 3

Results

3.1 Ethnicity, Gender and HIV status classification of study participants

Table 3.1 Subjects by ethnicity, gender and HIV status recruited for the study

	Africans		Indians		Whites	
	HIV positive	HIV negative	HIV positive	HIV negative	HIV positive	HIV negative
Males (*n*)	63	19	1	-	1	-
Females (*n*)	143	70	-	11	1	-

3.2 Anthropometric Measurements

The following anthropometric results were obtained from the subjects recruited in the study**;**

Table 3.2: Anthropometric results

	HIV positive (n=209)	HIV negative (n=100)	P-value (HIV positive vs HIV negative)
Males (n) (%)	65 (31%)	19 (19%)	-
Females (n) (%)	144 (69%)	81 (81%)	-
Age	39±8	36±11	<0.01**
Weight (kg)	67.5 ±14.0	74.0 ±18.3	<0.01**
Height (cm)	163.4 ±7.9	160.6±7.6	<0.01**
BMI (kg/m^2)	25.3 ±5.2	28.7 ±7.1	<0.01**
Waist (cm)	85.1 ±12.7	86.4 ± 14.8	0.43
Hip (cm)	98.5 ±12.1	106.7 ±14.2	<0.01**
Glucose (mmol/L)	5.2 ±1.3	4.8 ±0.6	<0.01**
Insulin (µU/mL)	16.1 ±17.4	11.0 ±8.9	<0.01**
Triglycerides (mmol/L)	1.8 ±1.2	1.1 ±0.6	<0.01**
Cholesterol (mmol/L)	4.6 ±1.2	4.6 ±1.0	0.81
HOMA	3.7 ± 5.8	2.3 ±2.2	<0.01**
HDL (mmol/L)	1.3 ±0.5	1.7 ±0.8	<0.01**
LDL (mmol/L)	2.6 ±1.0	2.5 ±0.9	0.32
Systolic (mmHg)	122.2 ±16.4	117.7 ±17.6	0.03**
Diastolic (mmHg)	78.9 ±10.5	78.4 ±10.7	0.68

Data shown as mean ±SD; **=statistically significant

The majority of HIV positive subjects were females with a 69% representation in this study. The HIV positive group had a significantly ($p<0.0.5$) lower weight compared to the control group (HIV negative). BMI (Body Mass Index) in the HIV positive group was significantly lower ($p<0.05$) in contrast to the control group. Hip measurements and HOMA revealed a significantly varied difference between the control and HIV positive group. The HIV positive group includes subjects which were both fasting and non-fasting, a comparison of glucose, cholesterol, insulin and lipid profile measurements with the control group may be distorted. However a direct comparison of fasting samples between the subject with and without lipoatrophy shown below in table 3.3.

A lipodystrophy questionnaire was used in HIV positive subjects to identify the presence of lipodystrophy. The questionnaire identified 78 subjects with lipodystrophy. Lipoatrophy was particularly present in 21 of the 78 subjects. The anthropometric and biochemical results for subjects with and without lipodystrophy are shown in Table 3.3 below.

Table 3.3: Anthropometric results for subjects with and without lipoatrophy

	HIV(+ve) with Lipoatrophy fasting (n=21)	HIV(+ve) without Lipoatrophy fasting (n=57)	P-value
Age	42 ±8	38±7	0.05*
Weight (kg)	62.5±11.1	67.6±12.9	0.09*
Height (cm)	162.7±8.9	164.1±8.0	0.54*
BMI (kg/m^2)	23.7±4.5	25.1±4.6	0.23*
Waist (cm)	82.5±9.9	85.9±11.9	0.21*
Hip (cm)	94.6±10.4	98.2±10.7	0.18*
Glucose (mmol/L)	5.5±1.8	4.8±0.6	0.11*
Insulin (µU/mL)	19.0±36.6	15.3±14.2	0.68*
Triglycerides (mmol/L)	1.9±1.3	1.9±1.6	0.92*
Cholesterol(mmol/L)	4.7±1.4	4.7±1.1	0.90*
Homa (Molar Units)	3.7±5.8	3.6±3.9	0.40*
HDL(mmol/L)	1.2±0.3	1.2±0.4	0.86*
LDL(mmol/L)	2.6±1.3	2.7±0.9	0.78*
Systolic (mmHg)	118.0±8.5	121.1±17.1	0.28*
Diastolic (mmHg)	76.8±5.5	76.7±12.8	0.98*

Data shown as mean ±SD; *=statistically not significant

Fasting subject's results within the HIV positive group were used in table 3.3. Thirteen (62%) of HIV positive with lipoatrophy were females. As expected the weight and BMI of the HIV positive group with lipoatrophy was lower than the group without lipoatrophy, although not statistically significant. The waist and hip sizes for subjects with lipoatrophy was insignificantly smaller than subjects without lipoatrophy. Glucose, HOMA, cholesterol, triglycerides, insulin and lipid profile measurements were not significantly different between patients with and without lipoatrophy.

Table 3.4 Gender and ethnicity comparison in subjects with and without lipodystrophy

		HIV+ with Lipoatrophy fasting (n=21)	HIV+ without Lipoatrophy fasting (n=57)
Males (n) (%)		8 (38%)	16 (28%)
Females (n) (%)		13 (62%)	41 (72%)
Ethnicity	African	20 (95%)	56 (98%)
	Indian	1 (5%)	-
	White	-	1 (2%)

Data shown as actual n (%)

The above results further indicates that females contribute the highest percentage (62%) in the subject group of HIV + with Lipatrophy.

3.3 PCR and single nucleotide polymorphism (SNPs)

3.3.1ApoCIII T455C and C482T genotyping

PCR products were run on 2% agarose gel to confirm amplification of DNA. Restriction enzyme *Fok I* was added into the PCR product to identify the T455C polymorphism and *Msp I* to identify the C482T polymorphism in two separate reactions.

Figure 3.1 and **3.2** show visualized results of ApoCIII T455C and C482T under UV light respectively.

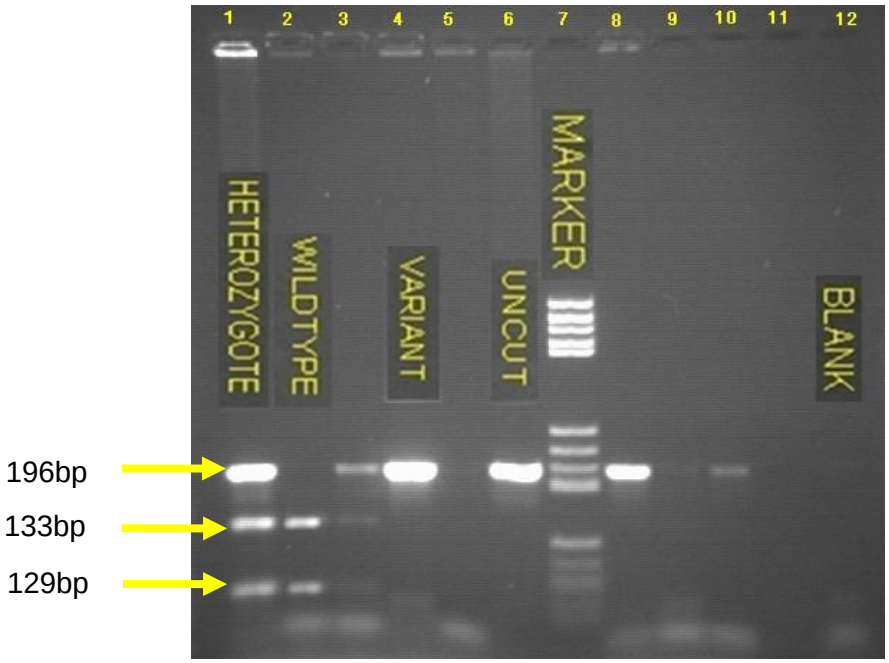

Figure 3.1: Gel image of PCR products for the ApoCIII T455C polymorphism. Lane 1= Heterozygote for the variant TC (196bp+133bp+129bp), Lane 2= Homozygote for the wildtype TT (133bp+129bp), Lane 4= Homozygote for the variant CC (196bp), Lane 6= Undigested PCR product (Control), Lane 7= Molecular Marker V and Lane 12= Blank (Control).

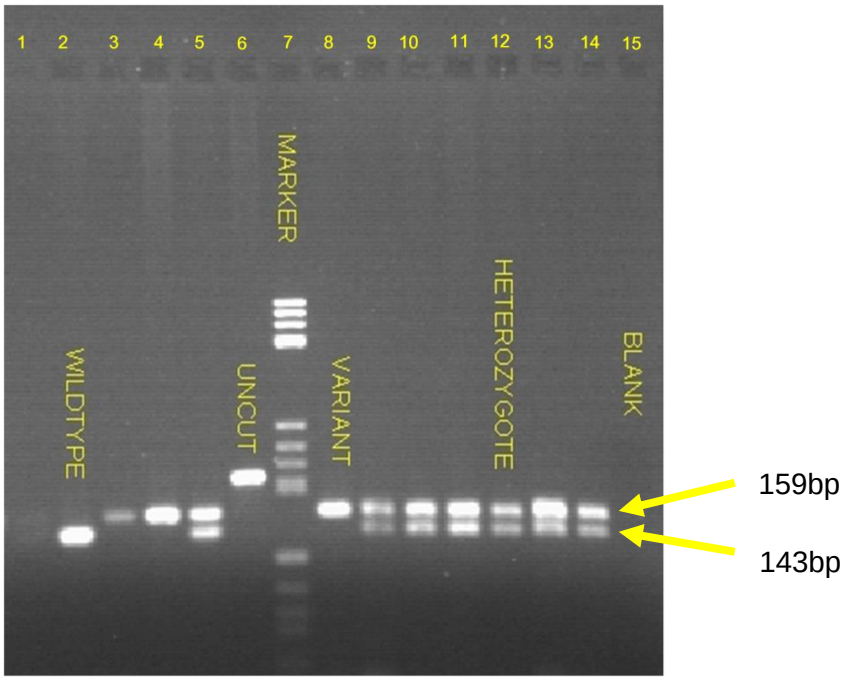

Figure 3.2: Gel image of PCR products for the ApoCIII C482T polymorphism. Lane 2= Homozygote for the wildtype CC (143bp), Lane 6= Undigested PCR product (Control), Lane 7= Molecular Marker V, Lane 8= Homozygote for the variant TT (159bp), Lane 12= Heterozygote for the variant CT (159bp+143bp) and Lane 15= Blank (Control).

3.3.2 TNF-alpha G238A and G308A genotyping

Restriction enzymes *Msp I* and *Nco I* were was added into the PCR product to identify polymorphism G238A and G308A respectively. Figure 3.3 and 3.4 show visualized results of TNF-alpha G238A and G308A respectively.

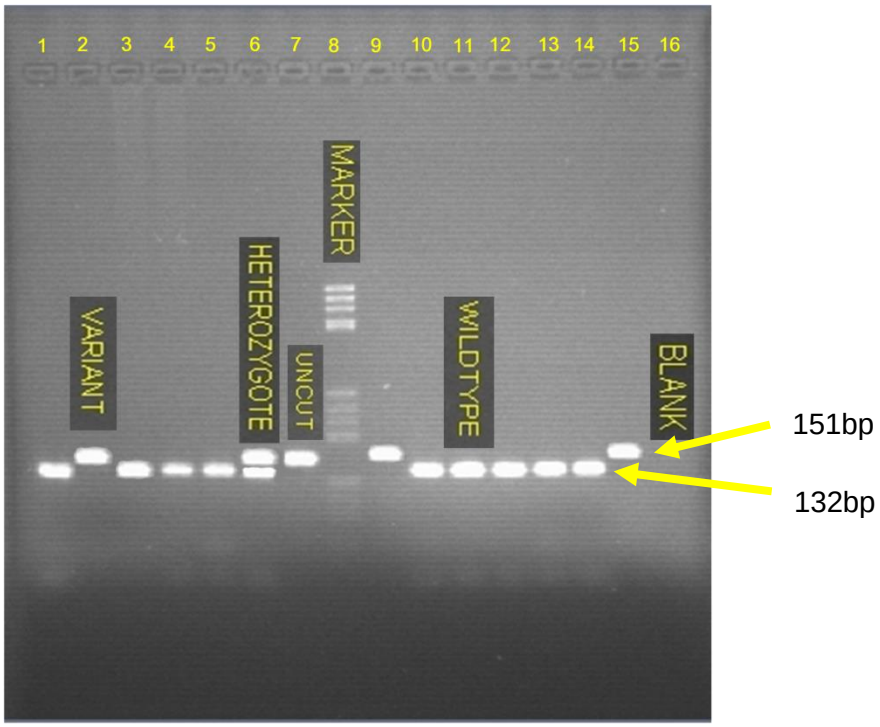

Figure 3.3: Gel image of PCR products for the TNF-alpha G283A polymorphism (Digested with *Msp I* enzyme). Lane 2= Homozygote for the variant AA (151bp), Lane 6= Heterozygote for the variant GA (151bp+132bp), Lane 7= Undigested PCR product (Control), Lane 8= Molecular Marker V, Lane 11= Homozygote for the wiltype GG (132bp) and Lane 16= Blank (Control).

:

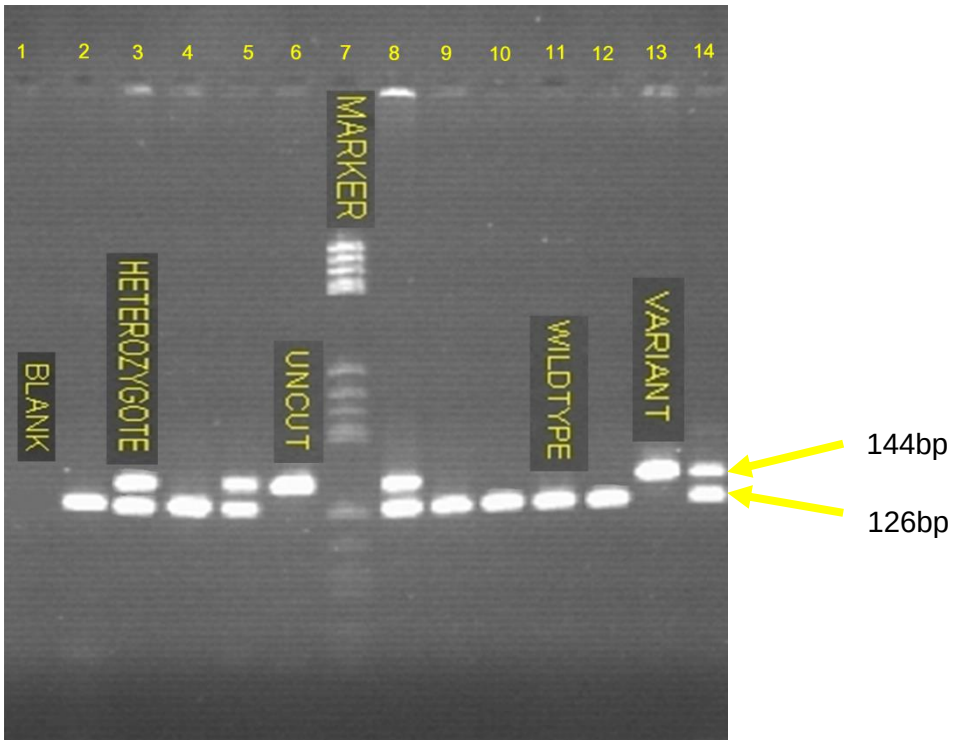

Figure 3.4: Gel image of PCR products for the TNF-alpha G308A polymorphism (Digested with *Nco I* enzyme). Lane 1= Blank (Control), Lane 3= Heterozygote for the variant GA (144bp+126bp), Lane 6= Undigested PCR product (Control), Lane 7= Molecular Marker V, Lane 11= Homozygote for the wildtype GG (126bp) and Lane 13=Homozygote for the variant AA (144bp).

3.3.3 Beta-3 Adrenergic receptor genotyping

Restriction enzyme *Msp I* was added into the PCR product and incubated for two hours in at 37ºC. Figure 3.5 shows visualized results of Beta-3 Adrenergic receptor T64A under UV light.

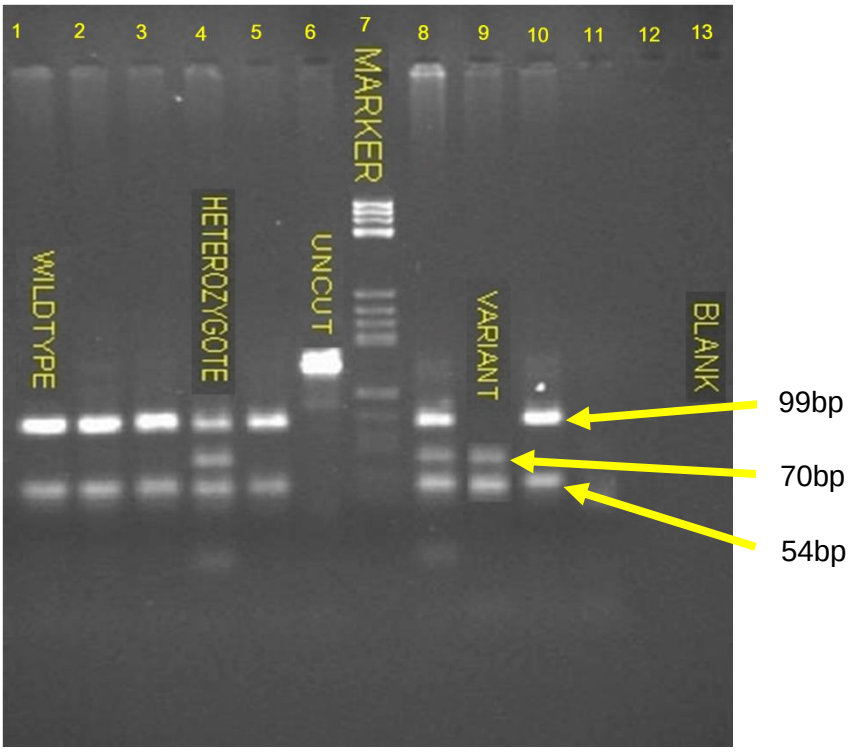

Figure 3.5: Gel image of PCR products for theBeta-3 Adrenergic receptor T64A polymorphism (Digested with *Msp I* enzyme). Lane 1= Homozygote for the wildtype TT (99bp+54bp), Lane 4= Heterozygote for the variant TA (99bp+70bp+54bp), Lane 6= Undigested PCR product (Control), Lane 7= Molecular Marker V, Lane 9= Homozygote for the variant AA (70bp+54bp) and Lane 13= Blank (Control).

3.4 Genotyping results

Table 3.5 and Table 3.6 represent the frequency distribution of genotype across each subject group. Distribution for all the frequency polymorphisms was within the Hardy-Weinberg Equilibrium.

Table 3.5: Comparison of genotype distribution between HIV negative and HIV positive subjects.

		HIV Positive (n=209)	HIV Negative (n=100)
ApoCIII	T455C	T= 0.26	T= 0.23
		C= 0.74	C= 0.77
	C482T	C= 0.28	C= 0.23
		T= 0.72	T= 0.77
β3-Adrenergic receptor	T64A	T= 0.88	T= 0.87
		A= 0.12	A= 0.13
TNF alpha	G238A	G= 0.90	G= 0.92
		A= 0.10	A= 0.08
	G308A	G= 0.83	G= 0.84
		A= 0.17	A= 0.16

Genotyping results for ApoCIII, both T455C and C482T, revealed no significant allele frequency difference between the HIV positive and HIV negative groups. There was similar finding with genotyping results for the ApoCIII C482T.

The lipodystrophy questionnaire was used to identify subjects with and without lipoatrophy. Table 3.6 represents those findings across each genotype.

Table 3.6: Comparison of genotype distribution between subjects with and without Lipoatrophy

		With Lipoatrophy(*n*=71)	**Without** Lipoatrophy(*n*=138)
ApoCIII	T455C	T= 0.29	T= 0.28
		C= 0.71	C= 0.72
	C482T	C= 0.25	C= 0.27
		T= 0.75	T= 0.73
β3-Adrenergic receptor	T64A	T= 0.94	T= 0.87
		A= 0.06	A= 0.13
TNF alpha	G238A	G= 0.94	G= 0.93
		A= 0.06	A= 0.07
	G308A	G= 0.75	G= 0.87
		A= 0.25*	A= 0.13*

*TNF-A (variant) 308: 0.25 vs 0.13; $p<0.05$

The difference in frequency of the T and A alleles in the β3-Adrenergic receptor polymorphism between subject with and without lipoatrophy was not significant. The major finding showed that the frequency of the A allele in the TNF alpha 308 polymorphism was significantly higher in subjects with lipoatrophy. The result suggests that lipoatrophy is associated with the presence of the variant allele A at the TNF alpha 308 locus.

3.5 HIV treatment results

The results of patients with and without lipoatrophy were further classified into the treatment type these subjects were on. The treatment duration was also considered in this classification. The results are represented in table 3.7.

Table 3.7: Comparison of treatment duration between patients with and without Lipodystrophy

	Patients with Lipoatrophy (*n*=71)	**Patients without Lipoatrophy (*n*=138)**	**P-value**
Treatment duration (months)	39 months ± 20	32 months ± 18	0.02
1st Line treatment (%)	85% (*n*=60)	57% (*n*=79)	
2nd Line treatment (%)	15% (*n*=11)	43% (*n*=59)	

1st Line treatment = Lamivudine, Stavudine & Efivarenz
2nd Line treatment= Didanosine, Lopinavir & Zidovudine

Results in table 3.7 indicate that a higher percentage (85%) of subjects with lipoatrophy is on the 1st line treatment of HIV. There was also a significant difference in the treatment duration between subjects with and without lipoatrophy. This finding indicates that the longer subjects are on treatment the higher the possibility of developing lipoatrophy.

Chapter 4

Discussion & Conclusion

Discussion

The introduction of highly active antiretroviral therapy has been accompanied by body profile changes and metabolic anomalies in HIV-positive patients. Lipodystrophy is one of the most noticeable condition which is characterized by lipoatrophy and lipohypertrophy. The main objective of this study was to determine the possible association of ApoCIII, beta-3 adrenergic receptor and TNF-alpha gene polymorphisms with the presence of lipodystrophy in the South African population.

Anthropometric Measurements

Majority of the HIV positive subjects (*n*=209) recruited in this study were females (69%) compared to the 31% of males. HIV positive subjects had significantly lower weight (67.5 ±14.0 vs. 74.0 ± 18.3; $p< 0.05$) and BMI (25.3 ±5.2 vs 28.7 ± 7.1; $p< 0.05$) as compared to the HIV negative control subjects. Although there was no significant differences in the total serum cholesterol and LDL concentrations between the HIV positive subjects and the HIV negative control subjects, serum triglyceride concentrations were significantly higher in the HIV positive subjects (1.8±1.2 vs 1.1±0.6; $p<0.05$) and HDL was significantly (1.3 vs 1.7mmol/l) lower in the HIV positive group. There have been theories as to why lipid abnormalities occur in the HIV-infected population, including those implicating both the viral infection as well as the antiretroviral therapy (Carr *et al.,* 1998). A number of studies have shown hyperlipidemia to be a complication in HIV-infected patients receiving antiretroviral therapy, especially protease inhibitors and stavudine. The incidence of hyperlipidemia in HIV-infected patients taking protease inhibitors has extended from 8% to as high as 66% (Kaul *et al.,* 1999).

In addition the glucose concentrations (5.2mmol/l vs 4.8mmol/l) in the HIV positive subjects was significantly ($p<0.05$) higher than the HIV negative subjects. Insulin concentrations were also significantly higher ($p<0.05$) in the HIV positive subjects (16.1µU/mL) as compared to the HIV negative subjects 11.0µU/mL), thereby indicating that the HIV positive subjects may be at risk of developing insulin resistance as indicated by a higher HOMA index of 3.7 vs 2.3 for the HIV positive subjects as compared to the HIV negative subjects.

The HIV positive subjects were further stratified into two groups comprising of subjects with lipoatrophy and without lipoatrophy. The HIV positive group with lipoatrophy had an *n* number of 21(27%) whilst the group without lipoatrophy had an *n* of 57 (73%). The anthropometric results showed that patients with lipoatrophy had a trend of lower weight and BMI although the difference was not statistically significant. The waist and hip measurements were also smaller in subjects with lipoatrophy although the difference was not significant. Furthermore, there was no significant difference in the biochemical parameters, glucose, cholesterol, triglycerides, insulin and lipogram between the two groups. These results were similar to the cross sectional study conducted by Innes *et al.* 2012, on the high prevalence of lipoatrophy in pre-pubertal South African children on antiretroviral therapy that showed similar findings. In their study among 100 subjects that were recruited, the prevalence of visually obvious lipoatrophy was 36%. Overall, children with and without lipoatrophy had similar weight-for-age Z-score, height-for-age Z-score, gender distribution, ethnic distribution, WHO clinical stage, viral load and mean CD4.

Interestingly, although there were more female patients in the present study which could be viewed as a biased sampling, the results further indicated that 62% of the 21 subjects that presented with lipoatrophy were females and 95% were of the African ethnicity. These results indicate that females may be at higher risk of lipoatrophy than males, similar results were also shown by Sorli *et al.*2007, who reported that women were at a greater risk of lipodystrophy compared to men. A plausible explanation for this is that females are more likely to report fat accumulation in the abdomen and breasts and hypertriglyceridemia, whereas men are more likely to describe fat depletion from the face and extremities, along with hypertension and hypercholesterolemia.

Indeed there have been studies reporting on various genetic factors and polymorphisms implicated in the regulation of lipid metabolism and development of lipodystrophy including polymorphisms in, Apo CIII, Beta 3 Adrenergic receptor and TNF alpha genes. However, only a few studies reported on their impact on HIV and specifically on HIV positive patients receiving HAART. Bonnet (2004), reported elevated concentration of triglycerides in HIV patients having the ApoCIII polymorphisms with the frequency of .04 for the -455C allele and 0.31 for the -482T allele. However, in the present study ApoCIII genotype results for both the T455C and C482T polymorphisms were not significantly different between the HIV positive and negative group. Furthermore, the allelic and genotypic frequencies at the polymorphic -482 and -455 loci of the apoCIII were similar to the findings by *Naran et al.,* 2009, which was investigated in the South African populations. Although ApoCIII has been implicated in the development of hypertriglyceridaemia the results of this study indicates that the Apo CIII polymorphisms

may play only a limited role if any in the development of lipodystrophy and hypertriglyceridaemia in HIV positive subjects on HAART. A possible explanation for the differences between this study and that of Bonnet (2004) is that the prevalence of gene polymorphism have been shown to differ between different ethnic groups.

Additionally, there was no significant difference in the TNF-alpha (G238A & G308A) and Beta-3 adrenergic receptor (T64A) genotyping allele frequencies between the 2 sample populations (HIV positive and HIV negative). The genotyping results for these polymorphisms are shown in table 3.5 across the 2 populations.

The HIV positive population were further classified into subjects with and without lipoatrophy based on the self-assessment lipodystrophy questionnaire. Lipoatrophy was present in 34% (n=71) of the subjects. Zinn and co-workers (2013) in South Africa found the prevalence of HIV-associated lipodystrophy in their study to be 11.7%. Nearly 90% of those patients were on stavudine (d4T). HIV patients in the present study majority (65%) of the subjects had been on stavudine hence the prevalence of lipodystrophy was higher.

Similar to the above finding, the frequency of ApoCIII C455T and T482C revealed no significant differences in the allele frequencies between the group with and without lipoatrophy. The difference in frequency of the T and A alleles in the β3-Adrenergic

receptor polymorphism between subject with and without lipoatrophy was not significant either.

Perhaps of note in this study is the finding in the TNF alpha G308A allele frequency differences. The frequency of the variant allele -308A was significantly higher (0.25 vs. 0.13; $p<0.05$) in subjects with lipoatrophy as compared to those without lipoatrophy. The result suggested that lipoatrophy was associated with the presence of the variant allele A at the TNF alpha 308 locus. Mahajan and coworkers (2014) showed a different finding in the Indian population, that the presence of the TNF-α SNPs especially, 238G/A promoter polymorphism was found to be associated with lipodystrophy. It was anticipated that the TNF-α SNP may be associated with HIV-1 lipodystrophy progression and could therefore be used as clinical prognostic marker to predict the development of HIV-1 associated lipodystrophy. The pathogenic mechanisms underlying HIV-1 lipodystrophy progression are unknown, but several studies suggest that TNF- α can alter the normal metabolic state and contribute to the progression of lipodystrophy (Mekinian *et al*., 2011). Again it is possible that ethnic difference may play a role in the different allelic distribution seen between different studies.

HIV Treatment Results

The results of patients with and without lipoatrophy were further classified into the treatment type the subjects were on. The treatment duration was also considered in this classification. The results indicated that a higher percentage (85%) of subjects with

lipoatrophy were on the 1st line treatment of HIV. There was also a significant difference in the treatment duration between subjects with and without lipoatrophy. This finding indicated that the longer subjects were on treatment the higher the possibility of developing lipoatrophy. This finding confirms what had been shown in the South African population by Innes and his co-workers (2012) that the overall time on ART, period on standard dose stavudine, increasing lamivudine exposure and cumulative efavirenz exposure were associated with visually obvious lipoatrophy.

4.2 Conclusion

The association of TNF-alpha (G308A) polymorphism with lipodystrophy in HIV positive patients receiving antiretroviral therapy was shown in this study. The presence of the variant allele of TNF-alpha (G308A) was significantly higher in subjects with lipoatrophy, however the lipid profile of the subjects with lipoatrophy was not affected by the presence of this variant allele. Ethnic differences might have contributed to lipoatrophy being associated with the variant allele of TNF-alpha 308 compared to other studies that found association with TNF-alpha 238.

Furthermore, a higher percentage of subjects with lipoatrophy were on 1st line treatment of antiretroviral therapy ($p=0.02$) and had been on treatment for a longer duration. The presence of Stavudine in 1st line treatment may have contributed to patients developing of lipoatrophy. This correlation has been shown in other studies as well.

4.3 Limitations of the Study

The 100 subjects used as controls for the study were assumed HIV negative. There were no actual tests performed to confirm that they were HIV negative. This was a cross-sectional study and no baseline data was available which meant that changes in anthropometric measurements since starting HAART could not be noted.

The access to body imaging techniques is very limited and expensive in South Africa. Hence morphological differences between HIV positive with LDS and without LDS could not be compared.

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Appendix A: Loading buffer and gel electrophoresis preparation

- 1X TBE was prepared by dissolving 10.8g Tris (hydroxymethylaminomethane) (Merck, Germany),5.5g boric acid (Merck, Germany) and 4ml of 0.5M EDTA (Sigma, Germany) in distilled water. The distilled water was added up to 1000 ml. 0.5M EDTA (ethylene diaminetetraacetic acid) was prepared by dissolving 9.3g EDTA in 50ml distilled water and adjsted to pH 8.0.
- 2% (w/v) agarose solution was prepared by dissolving 1g agarose tablets in 50ml 1X TBE (running buffer)and heating in a microwave for 1 minute.
- 5µl ethidium bromide was added.
- Gel was prepared for electrophoresis using a gel comb & casting tray
- The gel was allowed to set for 30min before electrophoresis

Appendix B: Subject information sheet for participant group

Subject information for participant group

Hello, my name is Francis Tlomatsana and I am from the Department of Chemical Pathology at the University of the Witwatersrand. We are studying genetic changes (polymorphisms) of Apolipoprotein C-III, Beta-3 adrenergic receptor and Tumour necrosis factor alpha, which play important roles in the breakdown of fat in the body. HIV positive individuals sometimes lose fat in the legs, arms, face or build-up fat in the stomach and neck area which may be due to the HIV treatment they receive. Therefore we are interested in studying the control of fats in HIV positive individuals receiving HIV treatment. The fat that these individuals lose or build-up is normally controlled by Apolipoprotein C-III, Beta-3 adrenergic receptor and Tumour necrosis factor alpha in the body. Any changes in the genes of ApoC-III may play an important role in the regulation of triglyceride, whereas changes in TNF alpha and β adrenergic receptor genes may lead to loss of breakdown and redistribution of body fats. Therefore we need to compare the distribution of fat and the role of genetic changes in HIV positive individuals who have and those who do not have the loss or build-up of fat in their bodies. A group of HIV negative individuals will be compared to the HIV positive group as references to see fat levels, distribution and any genetic changes.

We are inviting you to take part in this study as part of the participant group. If you agree to take part, you will be asked to come to the Day Wad between 8:00am-9:00pm, after an overnight fast from 9:00pm the previous night, i.e. no cigarette smoking, no food or drink (only water is allowed). A reference number will be given to you, thereby strict confidentiality will be applied at all times. You will be checked for possible signs of loss or build-up of fat by clinicians through measurements of your waist and hip circumference and answering of a form with questions (questionnaire). Only 30ml (6 teaspoons) of blood will be taken by a qualified nursing sister to measure your blood sugar and blood fats. Blood will also be used to check for differences in genetic (DNA) features between the participants and controls.

You will not be asked to take any drugs or medications other than ones you are taking. There are no risks to you other than the discomfort during donating the blood. Participation in this study is voluntary and you are free to refuse to participate in the study at anytime and will NOT affect your treatment in anyway.

If you have questions regarding the study you are to ask at anytime. Contact details:
Francis Tlomatsana
University of the Witwatersrand
Department of Chemical Pathology
Medical School 3rd floor
Cell: (083) 348 3886
Email:

Appendix C: Informed consent form

Informed Consent Form

I .... have been fully informed as to the procedures and the purpose of the study. In signing this consent form I agree to participate in the study and I also understand that I am free to refuse to participate or withdraw my consent and discontinue my participation in this study at any time. I understand that if I have any questions pertaining to the study at any time they will be answered.

.... , ...

Signature Date

Appendix D: Data collection form

Date: .....

Name: .....

Tel number.....

Lab Number.....

Ethnic group.....

Age.....

Weight: ....

Height: .....

BMI.....

Waist .....

Hip .....

Blood pressure..... .....

Smoking history .....

Exercise.....

Alcohol .....

Occupation .....

Medical history

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Appendix E: Lipodystrophy assessment questionnaire

QUESTIONNAIRE

Code:

SECTION B: INDICATE THE CHANGES YOU HAVE NOTICED ON YOUR BODY

(These changes should have occurred after starting the HIV drugs)

1) Have you noticed/ observed any unusual physical changes related to increasing body fat or decreasing body fat on your body (*when you start to take drugs*)?

Yes ☐

No ☐

2) Please indicate the part of the body where you noticed/ observed these changes

(If more than one, please tick all relevant body parts affected)

	Yes	No
▪ Face		
▪ Neck		
▪ Arms		
▪ Legs		
▪ Breasts		
▪ Abdomen		
▪ Buttocks		

3) Please indicate the type of change (s) you have noticed

(If more than one, please tick all relevant changes)

- **Decrease of fat** on some body parts ☐
- **Increase of fat** on some body parts ☐
- **Few decrease and increase** of fats in some body parts ☐
- **Both increase/ decrease** of fat on some body parts ☐

1

- **No body changes** observed at all

4) How can you rate these body changes in **general** on a scale of 0 to 3 (3 being the most severe change)?

- **Absent** (0) ☐
- **Mild** (*noticeable on close inspection*) (1) ☐
- **Moderate** (*readily noticeable by you or your medical practitioner*) (2) ☐
- **Severe** (*readily noticeable to anyone like a casual observer*) (3) ☐

PLEASE COMPLETE THESE SPECIFIC QUESTIONS.

In case you get difficulties to answer these questions, please ask for help from the person who gave you these questions

5) Has there been any change in the amount of fat in your **cheeks**, just next to your **nose and mouth?**

No ☐
yes ☐
Do not know (not sure) ☐

6) **IF YES,** what type of change?

Severely Increased	Moderately Increased	Mildly Increased	Mildly Decreased	Moderately Decreased	Severely Decreased
1. ☐	2. ☐	3. ☐	4. ☐	5. ☐	6. ☐

7) **When** did you first notice the change? Period: ..... **(month)**/..... **(year)**

8) Has there been a change in the amount of fat **behind your neck?**

No ☐
yes ☐
Do not know (not sure) ☐

9) **IF YES,** what type of change?

Severely Increased	Moderately Increased	Mildly Increased	Mildly Decreased	Moderately Decreased	Severely Decreased
1. ☐	2. ☐	3. ☐	4. ☐	5. ☐	6. ☐

10) When did you first notice the change? Period: ..... **(month)**/..... **(year)**

11) Has there been a change in the amount of fat **along all your arms?**

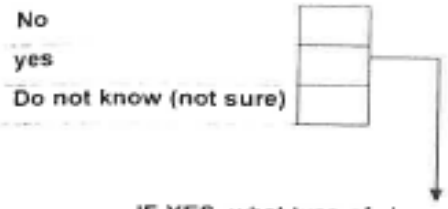
No ☐
yes ☐
Do not know (not sure) ☐

12) **IF YES,** what type of change?

Severely Increased	Moderately Increased	Mildly Increased	Mildly Decreased	Moderately Decreased	Severely Decreased
1. ☐	2. ☐	3. ☐	4. ☐	5. ☐	6. ☐

13) **When** did you first notice the change above? Period: ..... **(month)**/..... **(year)**

14) Has there been a change in the amount of fat **along all your legs?**

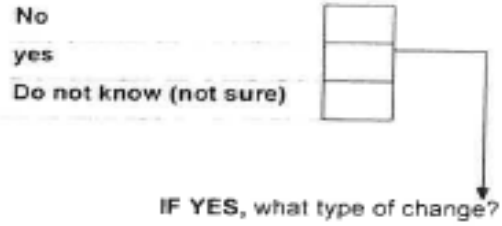


15) **IF YES,** what type of change?

Severely Increased	Moderately Increased	Mildly Increased	Mildly Decreased	Moderately Decreased	Severely Decreased
1. ☐	2. ☐	3. ☐	4. ☐	5. ☐	6. ☐

16) **When** did you first notice the change above? Period: ..... **(month)**/..... **(year)**

17) Has there been a change in the amount of fat **in your breasts?**



18) **IF YES,** what type of change?

Severely Increased	Moderately Increased	Mildly Increased	Mildly Decreased	Moderately Decreased	Severely Decreased
1. ☐	2. ☐	3. ☐	4. ☐	5. ☐	6. ☐

19) **When** did you first notice the change? Period: ..... **(month)**/..... **(year)**

20) Has there been a change in the amount of fat **for your abdomen**?

No ☐
yes ☐
Do **not know (not sure)** ☐

21) **IF YES,** what type of change?

Severely Increased	Moderately Increased	Mildly Increased	Mildly Decreased	Moderately Decreased	Severely Decreased
1. ☐	2. ☐	3. ☐	4. ☐	5. ☐	6. ☐

22) **When** did you first notice the change? Period: ..... **(month)**/..... **(year)**

23) Has there been a change in the amount of fat **for your buttocks**?

No ☐
yes ☐
Do not know (not sure) ☐

24) **IF YES,** what type of change?

Severely Increased	Moderately Increased	Mildly Increased	Mildly Decreased	Moderately Decreased	Severely Decreased
1. ☐	2. ☐	3. ☐	4. ☐	5. ☐	6. ☐

25) **When** did you first notice the change? Period: ..... **(month)**/..... **(year)**

Thank you for your participation!

Appendix F: Ethics clearance

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Tlomatsana

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M080905**
PROJECT	The Association of Apo CIII, B-3 Adrenergic Receptor and TNF-a polymorphisms with Lipodystrophy in HIV Positive Patients Receiving Antiretroviral Therapy
INVESTIGATORS	Mr T Tlomatsana
DEPARTMENT	Chemical pathology
DATE CONSIDERED	08.09.26
DECISION OF THE COMMITTEE*	Approved unconditionally

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

DATE 08.11.26 **CHAIRPERSON** .....
(Professor P E Cleaton Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Dr N Naran

DECLARATION OF INVESTIGATOR(S)

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

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...