C·Z

Effect of Cannabinoid Receptor Antagonism on the Metabolism and Phenotype of Combined Anti-Retroviral Treated Sprague Dawley Rats

by

Brian Lishenga Makamu

H56/78839/09

A Thesis submitted in partial fulfillment of the conditions for the award of the degree of
Master of Science

in

Medical Physiology

of the

University of Nairobi

November, 2013

DECLARATION

This thesis is my original work and has not been presented for a degree at any other university.

Brian Lishenga Makamu, MB.ChB. (UON)

Department of Medical Physiology,

University of Nairobi,

P.O. BOX 30197-00100, NAIROBI, KENYA

Email: mlishenga@yahoo.com

TEL.: 0729930404

Signature _____ Date 24th NOV, 2013

This thesis is being submitted to the Board of Postgraduate Studies, University of Nairobi for examination with our approval as university supervisors:

DR. FREDRICK BUKACHI (MMED, PhD)

Department of Medical Physiology,

University of Nairobi,

P.O. BOX 30197-00100, NAIROBI, KENYA

Email: fbukachi@uonbi.ac.ke

TEL.: 0727804933

Signature _____ Date 25.11.13

DR. PETER WAWERU (PhD)

Department of Medical Physiology,

University of Nairobi,

P.O. BOX 30197-00100, NAIROBI, KENYA

Email: peterwaweru@uonbi.ac.ke

TEL.: 0713478659

Signature _____ Date 24/11/2013

ACKNOWLEDGEMENTS

This project would not have been possible without the support of the faculty and staff of the Department of Medical Physiology, University of Nairobi and I thank them all. I express my appreciation to the Chairman of Department, Professor Nilesh B. Patel for his patience with me and to Professor Owino Okong'o for navigating me through departmental proposal approval. I thank Mr. Jackson Mugweru (Dept. Vet Anatomy and Physiology) for help with that gavage and ELISA. I am grateful for Dr. Francis Babubora's (Rift Valley Provincial General Hospital) help donating the Anti-retroviral drugs. I am particularly indebted to the following; Dr. Frederick O. Bukachi, my Principal supervisor for keeping the faith in my work and giving me hope and Mr. David K. Wafula, who taught me everything I know about handling of laboratory animals and with whom we fruitfully spent many grueling weekdays and weekends. Lastly, my enduring gratitude to Dr. Peter Waweru Mwangi: For rekindling in me anew a passion for science; for showing me by example, the pursuit of our highest ideals.

ABSTRACT

Background: Treatment of Human Immunodeficiency Virus infection with protease inhibitors and/or nucleoside reverse transcriptase inhibitors often results in development of a lipodystrophic syndrome whose etiology, phenotype and metabolic derangements resemble that of general population patients with the metabolic syndrome. Significantly, in metabolic syndrome patients, an overactive endogenous cannabinoid system is observed and its antagonism improves physical and biochemical profiles. It can therefore be reasonably expected that the blockade of this system will prevent the derangements observed in patients with combined anti-retroviral therapy induced lipodystrophy.

Objective: To test the hypothesis that use of a cannabinoid type 1 receptor antagonist (rimonabant) with combined anti-retroviral therapy prevents the metabolic and phenotypic derangements associated with chronic combined anti-retroviral therapy in Sprague-Dawley rats.

Design: Randomized Block Design

Setting: Department of Medical Physiology, University of Nairobi

Materials and Methods: Thirty (30) adult male Sprague-Dawley rats housed under standard conditions were randomized into three groups namely negative control (0.5% starch solution), positive control (lopinavir/ritonavir + zidovudine) and test (lopinavir/ritonavir + zidovudine) + rimonabant). The rats received drugs or vehicle by oral gavage daily for 4 weeks. Body mass index were calculated at weekly intervals, Insulin tolerance test (ITT) was carried out at 2 week intervals while visceral, and subcutaneous fat depot weight, serum lipids and serum fatty acid binding protein type 4 were assessed after four weeks of treatment.

Results: The test group had better insulin tolerance than either positive controls or negative controls. Fasting blood sugar in the test group was 49.7% and 13.8% lower at 120 minutes (P =

0.002) than positive controls and negative controls respectively. Rimonabant treated rats had 39% less visceral adiposity ($P = 0.05$) than positive controls. Serum triglycerides were 57% and 33% higher in the test group vs. the positive controls and negative controls ($P = 0.002$) respectively. LDL cholesterol levels were 102% and 22% higher in the test group vs. positive controls and negative control rats respectively ($P = 0.001$). Rimonabant co-administration was associated with 60% lower HDL cholesterol levels vs. positive controls ($P = 0.009$). Serum fatty acid binding protein – type 4 levels were increased by 80% and 75 % in the test group vs. positive controls and control groups respectively but this was not statistically significant ($P = 0.23$).

Conclusions and Relevance: Concurrent treatment with the cannabinoid receptor type 1 antagonist rimonabant causes an increase in insulin sensitivity and reduces visceral fat accumulation. It paradoxically increases serum triglycerides and serum LDL cholesterol levels. These findings may limit the utility of this approach in addressing combined anti-retroviral therapy associated lipodystrophy. Further research is necessary to characterize the precise mechanisms that produced the dyslipidemia observed.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

2-AG: 2-arachidonylglycerol,

AC: Abdominal Circumference

ACC: Acetyl-CoA carboxylase

AEA: Anandamide, arachidonoyl ethanolamine

AM251: 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(1-piperidyl)pyrazole-3-carboxamide

AMPK: AMP dependent kinase

ANOVA: Analysis of Variance

ApoB: Apolipoprotein B

ART: Antiretroviral Therapy

BMI: body mass index

C/EBP-α: CCAAT-enhancer-binding protein-α

cART: combined Anti-retroviral Therapy

CB1: Cannabinoid Type 1 Receptor

CB2: Cannnabinoid Type 2 Receptor

CYP3A4: cytochrome P450 type 3A4

DAG: 1, 2-diacylglycerol

DIO: Diet Induced Obesity

DMSO: Dimethyl Sulphoxide

ELISA: Enzyme Linked Immunosorbent Assay

ER: Endoplasmic Reticulum

FAAH: Fatty Acid Amide Hydrolase

FABP4: Fatty Acid Binding Protein Type 4

FABP5: Fatty Acid Binding Protein Type 5

FABPs: Fatty Acid Binding Proteins

FAS: Fatty Acid Synthase

FLA: Facial Lipoatrophy

GH: Growth Hormone

Glut: Glucose Transporter,

GLUT4: Glucose Transporter Type

HAART: Highly Active Anti-Retroviral Therapy

HDL-C: High Density Lipoprotein - Cholesterol,

HIV: Human Immunodeficiency Virus

HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A reductase

HOMA-IR: Homeostatic Model Assessment of Insulin Resistance

IDV: Indinavir

IL-18: interleukin-18

LD: Lipodystropy, *31*

LDL-C: Low Density Lipoprotein-Cholesterol

***LPL*:** Lipoprotein Lipase

LPV/r: Lopinavir/ritonavir

MAGL: Monoacylglycerol Lipase

MS: Metabolic Syndrome

mtDNA: mitochondrial DNA

NDV: Nelfinavir

NNRTI: Non-Nucleoside Reverse Transcriptase Inhibitor

PI: Protease Inhibitor

PIs: Protease Inhibitors

PPAR – γ: Peroxisome Proliferator-Activated Receptor Γ

PPARα: Peroxisome Proliferator Activated Receptor A

PPARβ: Peroxisome Proliferator Activated Receptor β

RTV: Ritonavir

SAT: Subcutaneous Adipose Tissue

SD: Standard Deviation

SQVsgc: Saquinavir

SREBP-1c: Sterol Regulatory Element Binding Protein 1c

STI: Structured Treatment Interruptions

TC: Thoracic Circumference

TNF-α: Tumour Necrosis Factor Alpha

tNRTI: thymidine analog Nucleoside Reverse Transcriptase Inhibitor

UPR: Unfolded Protein Response

VAT: Visceral Adipose Tissue

VLDL: Very Low Density Lipoprotein

δ^9-THC: δ9-tetrahydocannabinol

CHAPTER 1

INTRODUCTION

Although the use HAART in HIV patients is associated with a large decrease in morbidity and mortality (Palella et al., 1998), treatment regimes that include a protease inhibitor (PI) and/or some thymidine analog Nucleoside Reverse Transcriptase Inhibitors (tNRTI) often produce a lipodystrophic syndrome (Carr et al., 1998; Safrin & Grunfeld, 1999; Saint-Marc, 1999).

Lipodystrophy in HIV patients is described as fat redistribution, including peripheral lipoatrophy, central fat accumulation, or lipomatosis (Carr & Law, 2003) and has a disease burden estimated in range of 20-80% (Carr & Cooper, 2000; Miller et al., 2003) depending upon the diagnostic criterion employed. The condition causes significant disfiguring and often leads to stigmatization, which in turn causes poor adherence to, and reduced effectiveness of anti-retroviral therapy (Duran et al., 2001; Ammassari et al., 2001).

Besides the disordered fat distribution, patients exhibit metabolic derangements similar to those seen in general population patients with the metabolic syndrome such as; hyperinsulinemia, hypertriglyceridemia, low HDL cholesterol level, low adiponectin levels, high TNF-α , hyperglycaemia and elevated fatty acid binding protein-type 4 (FABP4) which is a sensitive marker for the metabolic syndrome (Walli et al., 1998; Addy et al., 2003; (Escote' et al., 2011).

In recent years, evidence has accumulated demonstrating the key role played an overactive endogenous cannabinoid system (ECS) in the appearance of the metabolic syndrome within the general population (Di Marzo & Matias, 2005; Pagotto et al., 2006; Matias et al., 2006; Blüher et al., 2006; Cote et al., 2007). The ECS consists of the cannabinoid receptors CB1 and CB2 and

receptor ligands of which anandamide (*N*-arachidonoyl ethanolamide, AEA) and 2-arachidonylglycerol, 2-AG are the most widely expressed (Matsuda et al., 1990).

Pagano and co-workers found that both the subcutaneous and visceral abdominal fat of obese patients with metabolic syndrome exhibit high cannabinoid type 1 receptor expression and endocannabinoid turnover (Pagano et al. 2007). Significantly, the phenotypic and metabolic effects of the metabolic syndrome in both rodent and human studies are prevented or abolished by the use of CB1 antagonists such as Rimonabant or AM251 (Ravinet-Trillou et al., 2004).

A review of literature reveals that an overactive ECS makes use of molecular processes similar to those employed by PIs and tNRTIs to produce a shift towards visceral adiposity along with its associated metabolic derangements. Increased CB1 tone reduces circulating adiponectin levels, activates SREBP 1c in the liver and PPARγ in VAT and inhibits GLUT4 in subcutaneous adipocytes (Bensaid et al., 2003; Osei-Hyiaman et al., 2005; Laplante et al., 2006). These mechanisms are the same as those PIs and NRTIs utilize to produce HAART associated lipodystrophy (Caron et al., 2001; Bastard et al., 2002; Gelato et al., 2002; Noor et al., 2006)

These preceding observations imply that since mechanisms are shared, an increase in endogenous cannabinoid tone may accompany cART associated lipodystrophy. The present study therefore hypothesized that antagonism of cannabinoid type 1 (CB1) receptors using the selective antagonist rimonabant can prevent the phenotypic and metabolic derangements that occur with chronic use of certain cART regimes.

To test this hypothesis age and weight matched male Sprague Dawley rats were treated with a combination of ARTs (LPV/r + AZT) and rimonabant for four (4) weeks and then assessed for insulin sensitivity, fat distribution, dyslipidemia and levels of FABP4.

CHAPTER 2

LITERATURE REVIEW

2.1 HAART associated lipodystrophy

The introduction of Highly Active Anti-Retroviral Therapy (HAART) in the 1990's led to a dramatic decline in HIV associated morbidity and mortality (Palella et al., 1998). However, soon afterward, body composition changes in patients on HAART became noticeable, especially with treatment regimes based on protease inhibitors (PI's) and thymidine analog Nucleoside Reverse Transcriptase Inhibitors (tNRTIs)(Carr et al., 1998; Carr & Cooper, 2000). These body changes consist of a fat redistribution that favours both peripheral lipoatrophy and central lipohypertrophy (Lichtenstein, 2005) with a predilection for central adiposity.

In HIV-infected patients on PI or tNRTI based HAART, fat hypertrophy is observed in central depots such as the abdomen, trunk, breast (in women), face and neck (sometimes with dorso-cervical fat pad or buffalo hump) while peripheral lipoatrophy is observed in the face, arms, legs and buttocks. This pattern of body fat redistribution often impacts negatively on psychological and social domains of life. Owing to its physical manifestations HAART associated lipodystrophy was viewed as a visible marker of HIV disease.

At the level of social functioning, lipodystrophy leads to problems with personal and family relationships. Affected patients often report narrowing their social world along with varying degrees of social isolation (Power et al., 2003). The poor self image that results and the stigmatization that may follow frequently leads to adherence failure and poorer treatment outcomes.

The prevalence of HAART associated lipodystrophy is now estimated at 20-80%; the wide range being attributed to patient differences in terms of age, sex, pre-morbid energy balance, type and duration of HAART (Miller et al., 2003). It is worth noting that longitudinal studies of the association between HAART and adipose tissue alterations show that these alterations are more likely to occur in patients with good adherence at the start of anti-retroviral therapy and that in these patients the fat alterations develop sooner.

In addition to the body morphology changes, HAART induced lipodystrophy is frequently accompanied by overt features of the metabolic syndrome such as: inflammation (elevated plasma levels of soluble Tumour Necrosis Factor α, sTNF-α), high triglyceride levels, low high-density lipoprotein cholesterol (HDL-C) levels, low adiponectin levels, hyperglycaemia, and hyperinsulinemia (Mynarcik, 2000; Lihn, 2003). The insulin resistance and hyperlipidemia place affected patients at increased risk for the accelerated development of type 2 diabetes mellitus and coronary artery disease.

Current prevention and treatment strategies for HAART associated lipodystrophy produce only a modest effect on the condition. Switching from or avoidance of PIs or certain tNRTIs is the most widely employed strategy but this not always desirable. Replacing a PI is known to have positive effect on metabolic profiles; however, studies show that improvement in fat distribution is small or nil (John et al., 2003). Structured treatment interruptions (STI) or drug holidays are an attractive approach and in smaller studies patients have gained total and regional fat during consecutive cycles. However, STI is difficult to implement since it requires very close monitoring of patients and those with a preserved immune status. Treatment interruption is associated with a rebound in HIV viremia and increased HIV morbidity regardless of drug

combinations used. Thus, treatment interruption is not recommended in any patient with HIV infection, regardless of clinical status (Tebas et al., 2002; Nuesch et al., 2005; Ruiz et al., 2007)

The thiazolidinediones, pioglitazone and rosiglitazone, have produced mixed results as a therapeutic approach. In smaller uncontrolled studies rosiglitazone produced fat gain despite concurrent antiretroviral therapy (Gelato et al., 2002). However, randomized, placebo-controlled studies have shown that rosiglitazone does not improve fat mass in HIV lipoatrophy, and may even worsen dyslipidaemia despite improvement of insulin sensitivity (Carr et al., 2004). Pioglitazone has also shown promise in amelioration of the metabolic side effects of HAART in similar studies though these were limited by small sample size. Data on the effect of both drugs on body composition is limited (Gavrila et al., 2005).

Inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (statins) and fibric acid derivatives have also been used to treat hyperlipidemia caused by use of HAART. Statins, such as pravastatin, simvastatin and artovastatin reduce LDL cholesterol along with anti-inflammatory benefits; however, in HAART associated hyperlipidemia target LDL cholesterol or triglycerides levels are seldom achieved (Penzak et al., 2000). Most statins are metabolized by the cytochrome P4503A4/5 isozymes located within the liver and the gastrointestinal tract. These isozymes are also responsible for the metabolism of protease inhibitors (PI) and non-nucleoside reverse transcriptase inhibitors. In addition, all of the clinically available PIs are inhibitors of the cytochrome P4503A isozymes.

Fichtenbaum and collegues demonstrated significant drug –to- drug interactions between some of the statins and a PI combination of ritonavir with saquinavir (RTV/SQVsgc). Simvastatin acid concentrations increased 30-fold in persons taking RTV/SQVsgc. Atorvastatin and its active

metabolite concentrations were also increased though to a lesser degree than simvastatin acid. Conversely, pravastatin levels declined in subjects taking RTV/SQVsgc (Fichtenbaum et al., 2002). Increased serum concentrations of statins place patients at increased risk for rhabdomyolysis. These findings led to the recommedation that simvastatin should be avoided in patients taking PIs, artovastatin should be used with caution as a second line therapy; and that pravastatin was largely safe.

Fibric acid derivatives such as bezafibrate, gemfibrozil and fenofibrate are effective for lowering serum triglycerides in patients with HAART associated lipodystrophy but have more nuanced effect on LDL cholesterol levels (Calza et al., 2003). Studies using gemfibrozil showed that although it reduced loss of limb fat in comparison to placebo patients still lost fat in the course of concurrent treatment with HAART. This observation has led to lower recommendation for use of fibrates in treatment or prevention of HAART associated lipoatrophy (Martínez et al., 2003).

Growth Hormone Replacement Therapy has been used with unsatisfactory results. In patients with HIV and abdominal fat accumulation Growth Hormone (GH) secretion is reduced and many have a relative GH deficiency (Koutkia et al., 2005). Clinical trials for treatment with supraphysiologic doses of growth hormone obtained reduction in visceral fat accumulation but were associated with more GH related adverse effects such as peripheral edema, arthralgias, and increased blood glucose (Kotler et al., 2004).

Administration of low-dose physiological growth hormone in patients with HIV and abdominal fat accumulation also produced mixed results. Significant reductions were observed in visceral fat and truncal obesity, triglycerides, and diastolic BP; however, 2-hour glucose levels on glucose tolerance testing were higher. The implication as concluded by the study is that the

therapeutic window to achieve an optimal risk-benefit ratio of GH in individuals with HIV, abdominal fat accumulation, and insulin resistance may be very narrow and difficult to achieve (Lo et al., 2008).

Due to the unreliable results obtained from pharmacological interventions, cosmetic surgery remains the only viable option available for the patients with facial lipoatrophy (FLA) and cervical fat accumulation (buffalo hump). These two are often the most disfiguring effects of HAART associated lipodystrophy. Surgical approaches for management of FLA include autologous fat transplantation from dorso-cervical fat pad, and implantation of facial fillers that are either non-permanent or permanent (Guaraldi et al., 2011). Non permanent fillers such as hyaluronic acid, poly-L-lactic acid [PLA] and hydroxyapatites require multiple injections and gradually degenerate over time. Permanent fillers such as Bio-Alcamid® while reducing the need for retreatment, are associated with a higher long term risk of infections often presenting years after treatment (Nadarajah et al., 2012).

Although the probability of developing HIV associated lipodystrophy is falling in Western nations due to better selection of HAART regimes (Nguyen, 2008), in developing nations choice is limited and stavudine, the tNRTI with the severest lipodystrophy effect, has until recently been widely used as first-line treatment.

Developing nations are also home to a large population of adolescents or young adults perinatally infected with HIV and exposed to long term HAART who face increased risk of developing HAART associated lipodystrophy.

The challenge of addressing the devastating phenotypic and metabolic effects of HAART-lipodystrophy is real and immediate and the search for effective interventions still inspires scientific inquiry.

2.2 Protease inhibitors and HAART lipodystrophy

Evidence linking PIs to the metabolic and phenotypic derangements that accompany HAART lipodystrophy has accumulated since 1996 (Carr et al., 1998). Current knowledge suggests multiple mechanisms are involved in the pathogenesis of these PI induced changes.

2.2.1 Protease inhibitor effects on Adipose Tissue and Lipid Metabolism

Protease inhibitors such as nelfinavir (NDV), ritonavir (RTV) and saquinavir (SQV) are known to inhibit the proteosome subunit 20S (Pajonk et al., 2002). Proteasomes are large multi-subunit proteases that are found in the cytosol, either attached to the endoplasmic reticulum or free, they are also found in the nucleus of eukaryotic cells. Proteasomes recognize, unfold, and digest protein substrates that have been marked for degradation by the attachment of a ubiquitin moiety (Bochtler et al., 1999). Proteasomes are therefore regulators of cellular homeostasis; by inactivating these proteins they prevent the development of endoplasmic reticulum (ER) stress known as the unfolded protein response (UPR).

Inhibition of proteosome subunit 20S by NDV, RTV and SQV causes increased concentrations of unfolded proteins in the ER. UPR is responsible for inhibition of cellular energy synthesis and apoptosis during states of unbalanced energy homeostasis or oxidative stress (Kaufman, 2002).

To remedy this situation cells have evolved a homeostatic mechanism. This process involves slowing down of translation of mRNA, up-regulation of ER chaperones and degradation of

unfolded proteins. Although these pathways are aimed at restoring homeostasis, in the extreme, they ultimately result in apoptosis (Panjok, 2002; Parker, 2005; Flint et al., 2009).

Inhibition of proteasomal chymotryptic and tryptic activities by protease inhibitors affects lipid metabolism in several ways. Human plasma lipid levels, especially for low density lipoprotein (LDL) cholesterol and chylomicrons are determined by apolipoprotein B (ApoB). The synthesis and secretion of ApoB in turn is regulated by proteasomal degradation (Fisher et al., 1997). PI-inhibition of pre-secretory ApoB degradation at the proteasome increases the assembly and secretion of very low density (VLDL) and low-density lipoproteins (LDL) and is a physiologically relevant mechanism by which PIs produce hyperlipidemia (Liang et al., 2001).

The inhibition of proteosomes also leads to an extension of the life of sterol regulatory element binding protein 1c (SREBP-1c). SREBP-1c is an important pro-lipogenic transcription factor especially active in hepatocytes. SREBPs regulate the expression of genes involved in cholesterol synthesis, transport, and triglyceride and fatty acid biogenesis (Flint et al., 2009). The PI Ritonavir is known to block the nuclear turnover of SREBP in the liver and adipose tissue (Riddle et al., 2001).

Other PIs, Indinavir, nelfinavir and amprenavir have been shown to cause mislocalization of SREBP-1. PI treated adipocytes demonstrated SREBP-1 accumulation in the nuclear periphery rather than intra-nuclear. Affected adipocytes failed to build up lipid stores and this observation may explain the subcutaneous lipoatrophy seen in patients on HAART regimes that include PIs (Caron et al., 2001; Bastard et al., 2002). In fact, the effects of PIs on hepatocytes and adipocytes differ in a striking manner. In hepatocytes PIs induce multiple genes involved in gluconeogenesis and lipogenesis. The result is increased triglyceride synthesis in hepatocytes that contributes a

great deal to the dyslipidemia occasioned by PIs (Parker, 2005; Lum et al., 2007)..Meanwhile in adipocytes there is suppression of glucose transport and ultimately of triglyceride synthesis. Transcription factors involved in lipogenesis such as SREBP-1, CCAAT-enhancer-binding protein-α (C/EBP-α), and PPAR-γ/β are all down regulated. Loss of PPAR-γ activity in the liver of lipoatrophic mice leads to hepatic steatosis, hypertriglyceridemia, and muscle insulin resistance, all features seen in PI-treated HIV patients with lipodystrophy (Gelato et al., 2002).

The net effect of these differences in lipid and glucose handling by the two tissues is the appearance of hyperlipidemia that is hepatic in origin alongside fat loss that is most pronounced in subcutaneous depots.

2.2.2 Protease inhibitors effects on Glucose Metabolism

Protease inhibitor treatment has long been associated with hyperglycaemia, impaired insulin tolerance and overt diabetes (Dubé et al., 1997; Dever et al., 2000). In several longitudinal and cross sectional studies the prevalence of overt diabetes in PI treated patient is a modest 1-7% while insulin resistance and impaired glucose tolerance is reported at a much higher 16-48% (Behrens et al., 1999; Carr et al., 1999). The fixed-combination PI lopinavir/ritonavir (LPV/r) is known to induce insulin resistance, as assessed by oral glucose tolerance testing. Studies with Indinavir (IDV) show that the earliest effects of PIs on glucose metabolism result from altered glucose uptake by tissues that begins even after a single dose of a PI (Noor et al., 2002). The PIs LPV, RTV and IDV have the strongest antagonistic effect on insulin-mediated glucose transport whereas amprenavir, saquinavir and atazanavir have weak effects.

In subcutaneous adipocytes, PIs inhibit glucose uptake by binding to glucose transporter type 4 (GLUT4) (Noor et al., 2006). A study by Murata et al on indinavir-mediated suppression of

glucose uptake using 3T3-L1 fibroblasts, 3T3-L1 adipocytes, and primary rat adipocytes demonstrated IDV to be a relatively selective inhibitor of the Glut4 isoform at physiologic dose levels. Among Glut isoforms the sensitivity to inhibition by IDV was as follows in decreasing order: GLUT4 >> GLUT2 > GLUT3 > GLUT1 (Murata et al., 2002). The GLUT4 isoform is the major glucose transporter involved in whole body glucose disposal; its inhibition disrupts glucose homeostasis (Mueckler, 1995). Transgenic animal models of diabetes mellitus in which GLUT4 is over expressed show improved glucose handling (Hansen et al., 1995). PIs inhibit GLUT4 in a non-competitive dose dependent fashion. PI inhibition of GLUT4 appears to be due the core aspartyl peptidomimetic structure these compounds possess that allows them to directly bind to the transport protein (Hertel et al., 2004).

In addition to their effects on glucose transport, protease inhibitors produce pancreatic β-cell dysfunction that undermines glucose stimulated insulin release. In a study by Koster et al, IDV significantly inhibited insulin release from MIN6 insulinoma cells and rodent islets without impairing glucose utilization. This suggests that PI induced insulin resistance acts at multiple tissue targets much like the multi-factorial and polygenic nature of type II DM in the general population (Koster et al., 2003). Subsequent to the absence of glucose transport into adipose tissue, a decrease in triglyceride synthesis follows leading to shrinkage of adipocytes and peripheral lipoatrophy

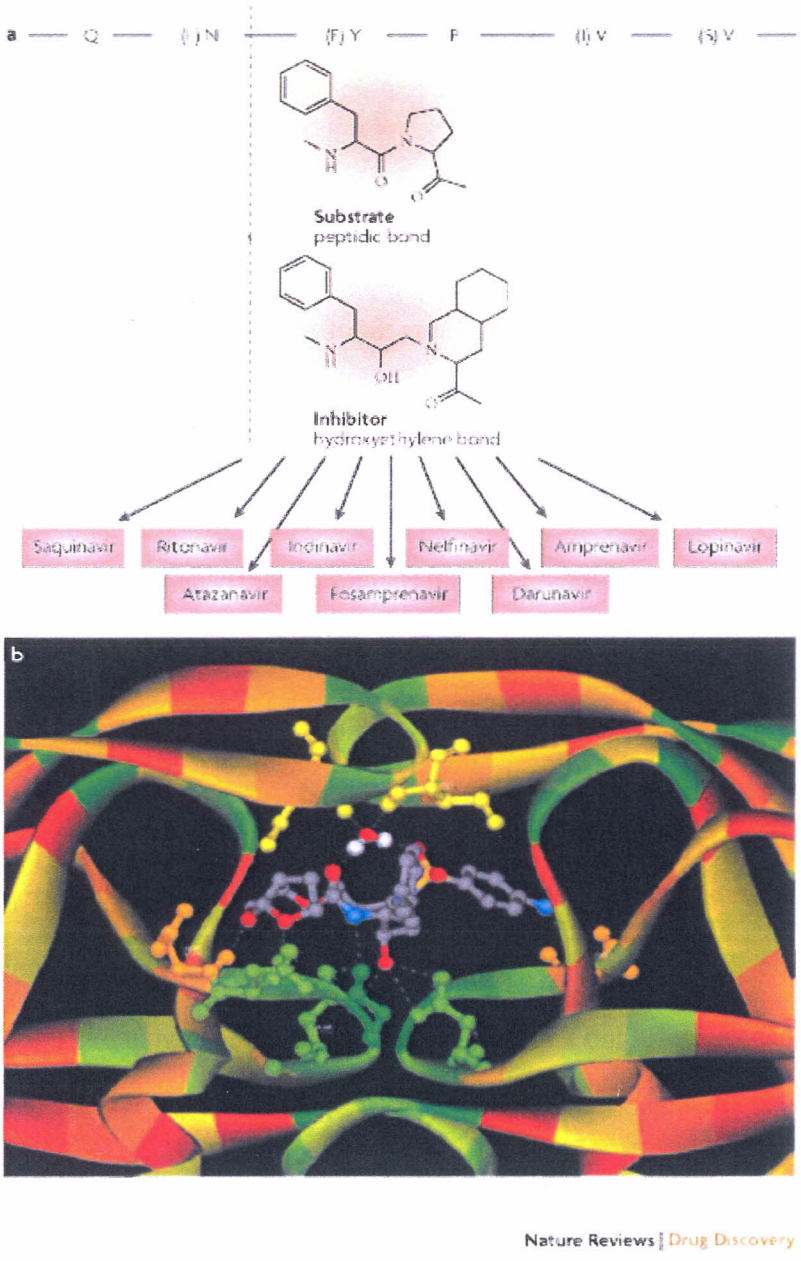


Figure 1 HIV protease structure with darunavir **(Clercq, 2007)**

2.3 Thymidine analog Nucleoside Reverse Transcriptase Inhibitors (tNRTIs)

Thymidine analog NRTIs are a major class of HAART drugs. They have been associated with the some of the severest forms of dyslipidemia seen with HAART therapy (Saint-Marc, 1999; Joly, 2002). Numerous clinical studies have reported a link between the tNRTI stavudine and the

development of lipoatrophy, which led to the removal of this drug from first-line antiretroviral therapy (ART) in western countries, developing countries with resource constraints are now only beginning to follow suit.

Zidovudine, a tNRTI which is still very much a part of first line therapy in developing countries is associated with lipodystrophy (Blümer, 2008; van Vonderen, 2009).

2.3.1 NRTIs and mitochondrial toxicity

The pathophysiological mechanism by which tNRTIs cause lipodystrophy is mitochondrial toxicity (Lewis, 1995; Brinkman, 1998). In much the same way that tNRTIs inhibit HIV reverse transcriptase, they inhibit also mitochondrial DNA (mtDNA) polymerase.

In rodent studies, chronic oral administration of 3′-azido-3′-deoxythymidine (AZT) altered mitochondrial function differentially depending upon the anatomic site. In inguinal fat (a surrogate for visceral adipose tissue), AZT significantly reduced mitochondrial DNA (mtDNA) content and cytochrome *c* oxidase activity, whereas the activity of citrate synthase, a mitochondrial protein exclusively encoded by the nucleus, was not affected (Deveaud, 2005).

Following treatment with NRTI dual therapy (zidovudine/lamivudine or stavudine/lamivudine) for 6 weeks, subcutaneous adipose tissue in healthy volunteers showed a significant reduction in mtRNA (with normal mtDNA levels), down regulation of the pro-lipogenic nuclear factor *PPARG* and up regulation of nuclear genes involved in oxidation of fatty acids (*PPARA* and *LPL)* (Mallon et al., 2005).

The resultant mitochondrial dysfunction leads to the formation of reactive oxygen species (Skuta, 1999) that ultimately alter the expression of various adipogenic genes and inhibit PPAR – γ expression in subcutaneous adipose tissue (SAT) explaining NRTI induced lipoatrophy.

Zidovudine and Stavudine alter lipid disposal in differentiating adipocytes. They decrease adipocyte lipid content and the expression of markers involved in lipid metabolism, namely C/EBPα, peroxisome proliferator-activated receptor γ, adipocyte lipid binding protein 2, fatty acid synthase and acetyl-coenzyme A carboxylase (Caron et al., 2004). However, in visceral adipose tissue (VAT), six months of treatment with zidovudine increases visceral adipose tissue as a percentage of body fat; it also increases the markers of adipocyte differentiation and lipid accumulation, probably resulting in fat hyperplasia and hypertrophy (Boothby, 2009).

Animal studies reveal that SAT is more sensitive to zidovudine induced mitochondrial dysfunction than VAT. This is thought to be due to the lesser mitochondrial content of SAT that lends it to easy oxidative stress. VAT on the other hand has a larger number of mitochondria; it is therefore more metabolically active and less prone to tNRTI induced lipoatrophy. Instead VAT shows no impairment of adipogenic gene expression in comparison with SAT (Deveaud, 2005).

NRTIs also alter insulin mediated glucose disposal although the exact mechanisms have not been elucidated. Three month treatment of HIV-1-positive ART-naive men taking lopinavir/ritonavir with either a NRTI-containing regimen (zidovudine/lamivudine) or a NRTI-sparing regimen (nevirapine) resulted in a 25% decrease in insulin-mediated glucose disposal and a 22% increase in fasting lipolysis in the NRTI arm but no changes in the NNRTI arm (Blümer, 2008).

2.4 The Endogenous Cannabinoid System (ECS)

Cannabis sativa and its major psychoactive component δ^9-tetrahydocannabinol (δ^9-THC) have been extensively utilized for recreational purposes, and one of its widely known effects is the onset of a ravenous appetite and eating behaviour (Kirkham, 2001; Cota, 2003).

The discovery of an endogenous cannabinoid system and the cloning of its receptors have kindled interest in the extensive physiologic effects of the ECS. The ECS consists of the cannabinoid receptors CB1 and CB2, the endogenous cannabinoid receptor ligands of which anandamide (*N*-arachidonoyl ethanolamine, AEA) and 2-AG (2-arachidonylglycerol) are the most widely expressed.

The synthesis of anandamide follows the following pathway (Sugiura, 2002; Di Marzo, 2004).

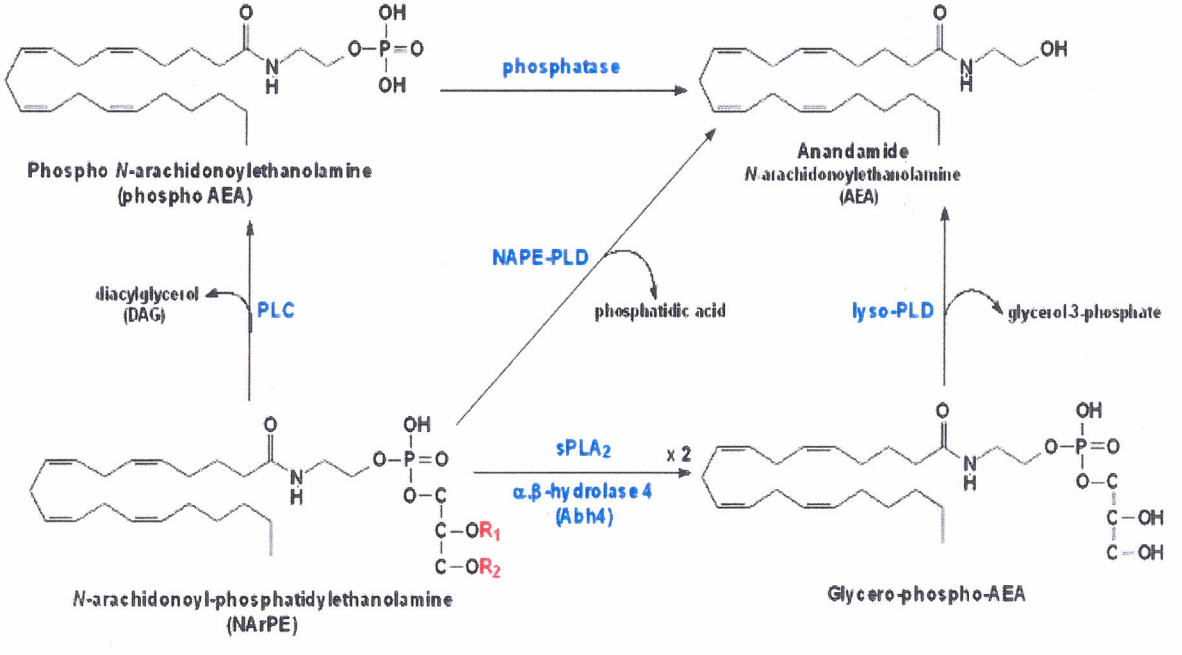


Figure 2 Pathways for the synthesis of N-arachidonoylethanolamine (AEA, anandamide) **(King, 1996-2013)**

2-AG biosynthesis occurs in response to a rise in intracellular Ca^{2+} and is formed by the hydrolysis of 1, 2-diacylglycerol (DAG) by DAG lipase (DAGL) (Sugiura, 1995).

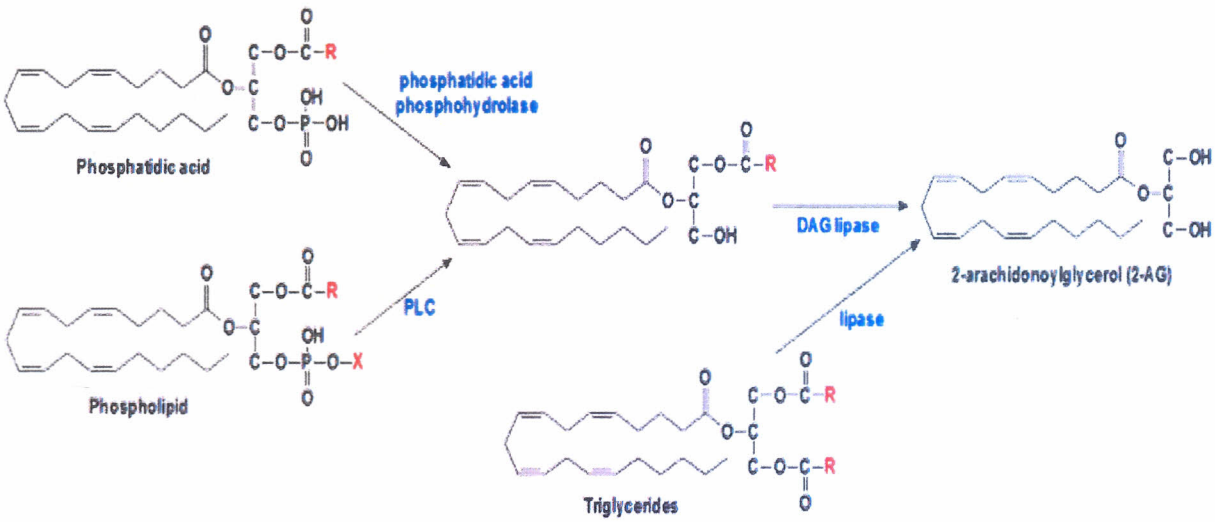


Figure 3 Pathways for the synthesis of 2-arachidonoylglycerol (2-AG) **(King, 1996-2013)**

Anandamide and 2-AG are removed from extracellular compartments by a carrier-mediated re-uptake process, and once within the cell degradation of AEA is by fatty acid amide hydrolase (FAAH) which cleaves anandamide into arachidonic acid and ethanolamine, while 2-AG hydrolysis is by monoacylglycerol lipase (MAGL) to arachidonic acid and glycerol (Di Marzo, 2007).

Human subcutaneous and visceral adipocytes express CB1 and CB2 receptors (Roche et al., 2006) and it is now recognized that overactivity of the EC system in adipose tissue, skeletal muscle and in the pancreas contributes to the disordered energy homeostasis seen the metabolic syndrome (Blüher et al., 2006; Cote et al., 2007). In overweight or obese patients with mild hyperglycaemia, an almost 2-fold higher level of 2-AG has been found in visceral adipose tissue as compared to normal weight controls (Matias et al., 2006).

Of note is the fact that the only significant difference in the fat redistribution that is observed in patients with HAART lipodystrophy as compared general population patients with metabolic syndrome is that the total body fat and visceral fat increase uniformly in metabolic syndrome while in HAART induced lipodystrophy though there may be a decrease in total body fat but almost invariably visceral fat increases as a percentage of total and peripheral fat (Safrin & Grunfeld, 1999).

2.4.1 Endogenous cannabinoids effects on Adipose Tissue and Lipid Metabolism

To study the effects of the ECS on adipocytes and adipogenesis three approaches are frequently employed: first, use of CB receptor antagonists such rimonabant and AM251, second, the use of CB receptor agonists such as HU-210 and WIN-55212 and third use of CB1receptor knockout rodents. CB1 receptor activation promotes de novo lipogenesis by dramatically increasing the expression of genes involved in adipocyte differentiation (PPAR-γ, aP2, C/EBP-α) and lipogenesis (SREBP-1c, ACC, FAS) (Muccioli et al., 2010). CB1 −/− (knockout) mice have lower body weight due to a reduction of fat mass during a period of 12 weeks after birth accompanied by a later increase in lean mass(Cota et al., 2003).

The CB1 agonist WIN-55212 has been shown to up-regulate lipoprotein lipase (LPL) in a primary epidydimal-derived adipocyte cell line 3T3F442 (representative of white adipose tissue). LPL is an enzyme involved in the hydrolysis of the triglycerides and makes fatty acids available for storage in adipose tissues leading to fat accumulation (Cota et al., 2003).

Cannabinoid receptor activation increases expression of the pro-adipogenic nuclear factor PPAR-γ. Chronic treatment of Mouse 3T3-F442A adipocytes with the potent CB1/CB2 agonist HU-210 stimulates the expression of PPAR-γ (Matias et al., 2006). The PPAR family of nuclear receptor

transcription factors plays a key role in lipid metabolism, glucose disposal and inflammatory response. The members of this family are PPARα, PPARβ, and PPARγ. Besides stimulating expression of these transcription factors, cannabinoids are now recognized agonists at these receptors. The endogenous cannabinoid N-acyl ethanolamine OEA which is structurally related to anandamide regulates feeding and body weight via a PPARα-dependent mechanism whereas THC is a known activator of PPARγ.

Bouaboula et al investigated the effects of anandamide on peroxisome proliferator-activated receptor γ (PPARγ) activity. They demonstrated that anandamide bound directly to PPAR-γ ligand binding domain and induced its transcriptional activation in a dose dependent manner resulting in 3T3-L1 fibroblast differentiation into adipocytes. The effects of anandamide on this cell line were inhibited by the PPAR-γ antagonist GW9662 (Bouaboulaa et al., 2005) (Bouaboulaa et al., 2005).

Stimulation of CB1 receptors inhibits formation of cAMP in adipocytes; without cAMP, AMP dependent kinase (AMPK) is not activated (Salt et al., 1998). Under usual circumstances AMPK stimulates catabolism (lipolysis and β-oxidation) and inhibits fatty acid synthesis by blocking the enzyme Acetyl-CoA carboxylase (ACC). ACC catalyzes the irreversible carboxylation of acetyl-CoA to produce malonyl-CoA, the main substrate for fatty acid synthesis. Therefore by inhibiting the formation of AMPK endogenous cannabinoids promote lipogenesis an action that is effectively prevented by the CB1 receptor antagonist rimonabant.

Similar to the mechanisms of protease inhibitor induced lipodystrophy, the activation of CB1 receptors especially in the liver stimulates the expression of the pro-lipogenic transcription factor sterol regulatory element binding protein (SREBP-1 c) and its targets, acetyl coenzyme A

carboxylase 1 (ACC1) and fatty acid synthase (FAS) (Osei-Hyiaman et al., 2005). This is a crucial step in pathophysiology of hepatic steatosis. Furthermore, Gary-Bobo et al have demonstrated that CB1antagonism prevents development of hepatic steatosis both in obese fa/fa rats treated with the CB1 antagonist rimonabant and in CB1 knock-out mice fed a high-fat diet (Gary-Bobo et al., 2007).

In addition to the direct effects of cannabinoids on lipogenesis, stimulation of the ECS promotes lipogenesis by decreasing levels of adiponectin the major adipocyte-derived hormone (Matias et al., 2006). Adiponectin is an abundant adipocytokine in plasma. It increases fatty acid oxidation by stimulating AMP dependent kinase (Kubota et al., 2007); it also improves insulin sensitivity and facilitates insulin mediated suppression of hepatic glucose production (Kadowaki et al., 2006). Adiponectin drives fat metabolism and storage by reducing the expression of enzymes involved in lipogenesis and increasing expression of those associated with associated with b-oxidation and energy dissipation, such as acyl-CoA oxidase and uncoupling protein-2 (Yamauchi et al., 2001). Activation of CB1 receptors by the agonists HU-210 and WIN55212 inhibits adiponectin expression in mature/hypertrophic adipocytes (Perwitz et al., 2006). Blockage of CB1 receptors by the antagonist rimonabant leads to an increase of adiponectin expression in the adipose tissue of obese zucker (fa/fa) rats (Bensaid et al., 2003). Notably, in similar fashion to the CB1 receptor antagonist SR141716A (rimonabant), PPAR-agonists such as rosiglitazone increase circulating adiponectin level and this is associated with improved insulin sensitivity (Yang et al., 2002).

It is interesting that low plasma adiponectin concentration and the associated insulin resistance/impaired glucose tolerance are features of the lipodystrophy seen in HIV-infected or HIV-free men receiving antiretroviral therapy (Addy et al., 2003).

In general CB1receptor expression is up-regulated in metabolic disorders and CB receptor activation has been associated with a wide range of disorders such as obesity, metabolic syndrome, type 2 diabetes and cardiovascular disease. Studies involving the CB1 antagonist rimonabant have demonstrated the importance of the CB1 receptor in these disorders. Rimonabant was used in a diet induced obesity (DIO) rat model and produced a persistent fall in energy intake and a reduction in fat content after 5-weeks treatment (Ravinet-Trillou et al., 2003). The reduction in food intake was temporary; however, the weight loss observed outlived the initial weight loss due to reduced food intake.

In other studies involving a DIO rat model, it was observed that the metabolic effects of obesity such as hyperinsulinemia, hypertriglyceridemia, low HDL cholesterol level, low adiponectin levels and hyperglycaemia were prevented by the congenital knockout of CB1 receptor expression or by the use of a CB1 antagonist such as rimonabant. These effects were independent of food intake (Ravinet-Trillou et al., 2004).

The phenomena of unbalanced ECS activity in visceral versus subcutaneous fat depots when coupled with the pro-lipogenic effects of the ECS eventually results in more fat being stored in abdominal depots and less in subcutaneous depots, with potential harmful consequences on cardio-metabolic risk factors. From the foregoing, these findings concerning ECS actions concur with the shift in fat depots that is seen with initiation of PI and/or tNRTI based HAART. It can be hypothesized that changes in ECS activity preclude the derangements lipid metabolism common with HAART-lipodystrophy.

2.4.2 Endogenous cannabinoid effects on Glucose Metabolism

The Endogenous cannabinoids anandamide and 2-AG are often elevated in obese patients with type 2 diabetes. This occurs along with increased expression of CB1 receptors and down regulation of the main degrading enzyme, fatty acid amide hydrolase (FAAH) in adipose tissue (Engeli et al., 2005).

Administration of either the endogenous cannabinoid anandamide or the CB1 receptor agonist Arachidonyl-2′-chloroethylamide (ACEA) results in glucose intolerance after a glucose load (Bermúdez-Sivaa et al., 2006). In rat insulinoma RIN-m5 F β-cells stimulation by the CB receptor agonist HU-210 increases insulin secretion and this effect is completely attenuated by the CB1 antagonist rimonabant. Overactivity of the ECS has been observed in hyperglycaemic and obese states. In mice models of DIO the expression of cannabinoid biosynthetic enzymes NAPE-PLD and DAGL-α inside the pancreatic islets increased while levels of FAAH, the degrading enzyme, reduced. Elevation of endogenous cannabinoids therefore contributes to hyperinsulinemia which in turn increases adipogenesis causing hypoadiponectinemia (Matias et al., 2006).

In a randomized, double-blind, placebo-controlled trial of 3045 obese (body mass index ≥30) or overweight (body mass index >27 patients with treated or untreated hypertension or dyslipidemia, rimonabant at 20mg oral daily produced favourable changes in levels of HDL cholesterol, triglycerides, fasting insulin and in HOMA-IR (Pi-Sunyer et al., 2006).

Pagano and co-workers have shown that pre-adipocytes differentiated in the presence of the insulin sensitizing PPARγ agonist rosiglitazone have a down-regulation of CB1 receptor expression along with increased expression of the cannabinoid degrading enzyme, FAAH. They

found that CB1 receptor activation has an insulin-like effect; it induces GLUT4 translocation to the plasma membrane of adipocytes and an over 50% increase in glucose uptake. This effect was blocked by the CB1 antagonist SR141716A (rimonabant) (Pagano et al., 2007).

Paradoxically CB1 receptor antagonism with the selective blocker rimonabant ameliorates insulin resistance linked to obesity. The mechanisms involved appear to be both adiponectin dependent and independent pathways. In a study involving ob/ob and *adipo*(-/-)ob/ob mice (adiponectin receptor knockout mice), significant improvement of insulin resistance, accompanied by an increase in adiponectin level, was observed in the ob/ob mice following rimonabant treatment. Although, *adipo*(-/-)ob/ob mice also showed improved insulin sensitivity when treated with rimonabant, the degree of improvement was much lower (Watanabe et al., 2009).

2.5 Fatty Acid Binding Proteins (FABPs)

Discovered first in 1972, FABPs are small 14-15KDa proteins that are ubiquitous to many organs (Furuhashi & Hotamisligil, 2008). The expression of FABPs in a given cell type reflects its lipid-metabolizing capacity (Veerkamp & van Moerkerk, 1993). In hepatocytes, adipocytes and cardiac myocytes, where fatty acids are prominent substrates for lipid biosynthesis, storage or breakdown, the respective FABPs make up between 1 and 5% of all soluble cytosolic proteins (Haunerland & Spener, 2004).

FABPs control intracellular trafficking (as chaperons), storage, export and metabolism of lipids. As such they facilitate the transport of hydrophobic lipids such as saturated and unsaturated long chain fatty acids, eicosanoids and other lipids to specific intracellular compartments such as mitochondria, lipid droplets and the nucleus. Amounts of tissue specific FABPs can increase

dramatically during periods of large lipid influx or fatty acid exposure (Veerkamp & van Moerkerk, 1993). The levels can also be altered by endurance exercise training or in chronic conditions like diabetes or obesity (Zanotti, 1999). Thus FABPs function as adaptive sensors for lipid homeostasis reflecting the nutrient status of tissues. Due to this observation FABPs (adipocyte-FABP or FABP4 in particular) are now considered attractive targets for drugs targeting metabolic disorders like diabetes, obesity and atherosclerosis.

Adipocyte specific FABP (FABP4), a 15KDa member of this family of proteins is a particularly attractive therapeutic target. The only cells that co-express FABP4 are adipocytes and macrophages (Makowski et al., 2001); this unique position allows FABP4 to operate at the interface of metabolism and inflammation.

FABP4 is a known marker of obesity and the metabolic syndrome (MS), levels in plasma correlate positively with a risk for metabolic syndrome even in those without overt clinical symptoms of MS (Xu et al., 2006). In addition, plasma concentrations of FABP4 are increased in type II diabetics but especially in those with the metabolic syndrome. FABP4 concentrations correlate with triglycerides, markers of oxidation and inflammation, and systolic blood pressure (Cabré et al., 2007). Mice deficient for FABP4 exhibit reduced hyperinsulinemia and insulin resistance in the context of dietary and genetic obesity (Uysal et al., 2000).

In macrophages, FABP4 regulates inflammatory responses and cholesterol ester accumulation (Makowski et al., 2005). The overexpression of aP2 (FABP4) in human macrophage cell lines drives the accumulation of cholesterol esters and foam cell formation while macrophages deficient of FABP4 have increased free fatty acids and decreased cholesterol and cholesterol esters (Fu et al., 2002).

The presence of FABP4 in macrophages is increased by lipopolysaccharide/Toll receptor activation, oxidized LDL, and PPAR-γ agonists and decreased by treatment with cholesterol lowering statin (Makowski et al., 2005). Total FABP4 deficiency confers dramatic protection against atherosclerosis in apolipoprotein e (ApoE)-deficient mice with or without the additional challenge of high-cholesterol-containing diet implying the involvement of inflammatory pathways in atherosclerosis (Makowski et al., 2001).

Maeda and co workers developed a mouse model with targeted mutations in two related FABPs, adipocyte FABP4 (aP2) and FABP5 (mal1) to determine their role in systemic lipid, glucose, and energy metabolism. They demonstrated that not only were these mutations protective against atherosclerosis as previously described, but the mice developed enhanced insulin receptor signalling, enhanced muscle AMP-activated kinase (AMP-K) activity, and dramatically reduced liver stearoyl-CoA desaturase-1 (SCD-1) activity. DEXA analysis of adiposity demonstrated significantly reduced total body adipose mass in aP2-mal1$^{-/-}$ mice both on regular and high-fat diet compared to weight matched controls. This study demonstrates that FABP4 (ap2) and FABP5 (mal1)-combined deficiency has a dramatic attenuating effect on all aspects of the metabolic syndrome (Maeda et al., 2005).

2.5.1 FABPs and HIV Lipodystrophy

Systemic overproduction of FABP4 in ART treated HIV-1 infected patients with lipodystrophy has been documented. FABP4 levels correlate positively with degree of lipodystrophy, body mass index, (BMI), insulin resistance, total cholesterol, triglycerides, and the inflammation markers sTNF-R1, leptin and IL-18. High FABP4 levels also correlate with reduced expression of PPARγ in subcutaneous adipose tissue following treatment with NRTIs (Escote' et al., 2011).

In a study of 183 HIV infected patients Coll et al., established that FABP4 concentration was significantly higher in those patients with either metabolic syndrome (MS) or lipodystropy (LD) criteria than those without any metabolic disturbance. Similarly, FABP4 concentration significantly increased with an increase of MS features and was strongly correlated with body-mass index, triglycerides, HDL-cholesterol concentrations, insulin and blood pressure. Patients in the highest quartile of FABP4 presented a six-fold increased odds ratio for MS and a three-fold increased odds for LD, adjusted by age, sex, body-mass index and the antiretroviral therapy. They concluded that FABP4 was a strong plasma marker of metabolic disturbances in HIV-infected patients, and therefore, could serve to guide therapeutic intervention in this group of patients (Coll et al., 2008)

Escote' and collegues on the other hand used a cohort of HIV-1-infected patients treated with combination antiretroviral therapy (cART) and to investigate the relationships between FABP-4 levels and insulin resistance, dyslipidaemia, lipodystrophy and levels of proinflammatory adipocytokines. They established that plasma FABP4 concentration was significantly higher in patients with lipodystrophy than in those without and that FABP4 showed a negative correlation with high-density lipoprotein (HDL) cholesterol and adiponectin concentrations. These patients had increased plasma levels of proinflammatory cytokines and insulin resistance as determined by HOMA-IR method. They concluded that cART-treated HIV-1-infected patients with lipodystrophy have a systemic overproduction of FABP-4, which is closely linked to insulin resistance and inflammatory markers in subcutaneous adipose tissue (Escote' et al., 2011).

2.5.2 FABPs and Endogenous Cannabinoids

Endogenous cannabinoids are lipid ligands of both extracellular (CB1 and CB2) and intracellular receptors (PPARγ and PPARα) (Felder et al., 1993; O'Sullivan, 2007). In order to activate

intracellular receptors or to terminate action at extracellular receptors, endocannabinoids are accumulated intracellularly (Hillard & Jarrahian, 2003); a proposed mechanism through which this is achieved is the intracellular sequestration of endocannabinoids by FABPs. Increased uptake of AEA has been observed in N18TG2 neuroblastoma cells that over-express FABP5 or FABP7 (Kaczocha et al., 2009) and these are currently being studied as pharmacological targets (Berger et al., 2012). It can be hypothesized that increased synthesis of endogenous cannabinoids as is observed in the metabolic syndrome, some of which are proven transport ligands for FABPs may also be accompanied by a facilitatory on-demand increase in tissue specific FABP protein.

CHAPTER 3

MATERIALS AND METHODS

3.1.1 Null Hypothesis:

Concurrent treatment with rimonabant does not prevent metabolic and phenotypic derangements associated with chronic combined anti-retroviral therapy in Sprague-Dawley rats.

3.1.2 Alternate Hypothesis

Concurrent treatment with rimonabant prevents metabolic and phenotypic derangements associated with chronic combined anti-retroviral therapy in Sprague-Dawley rats.

3.2 Overall Objective

To determine the effects of the CB1 receptor antagonist rimonabant on the metabolic and phenotypic characteristics of Sprague – Dawley rats treated chronically with LPV/r and Zidovudine.

3.3 Specific Objectives

1. Determine the effect of rimonabant on insulin tolerance in LPV/r and AZT treated Sprague-Dawley rats.
2. Determine the effect of rimonabant on fat distribution in LPV/r and AZT treated Sprague-Dawley rats.
3. Determine the effect of rimonabant on total cholesterol, LDL-C, HDL-C and triglycerides in LPV/r and AZT treated Sprague-Dawley rats.

4. Determine the effect of rimonabant on FABP4 levels in LPV/r and AZT treated Sprague-Dawley rats.

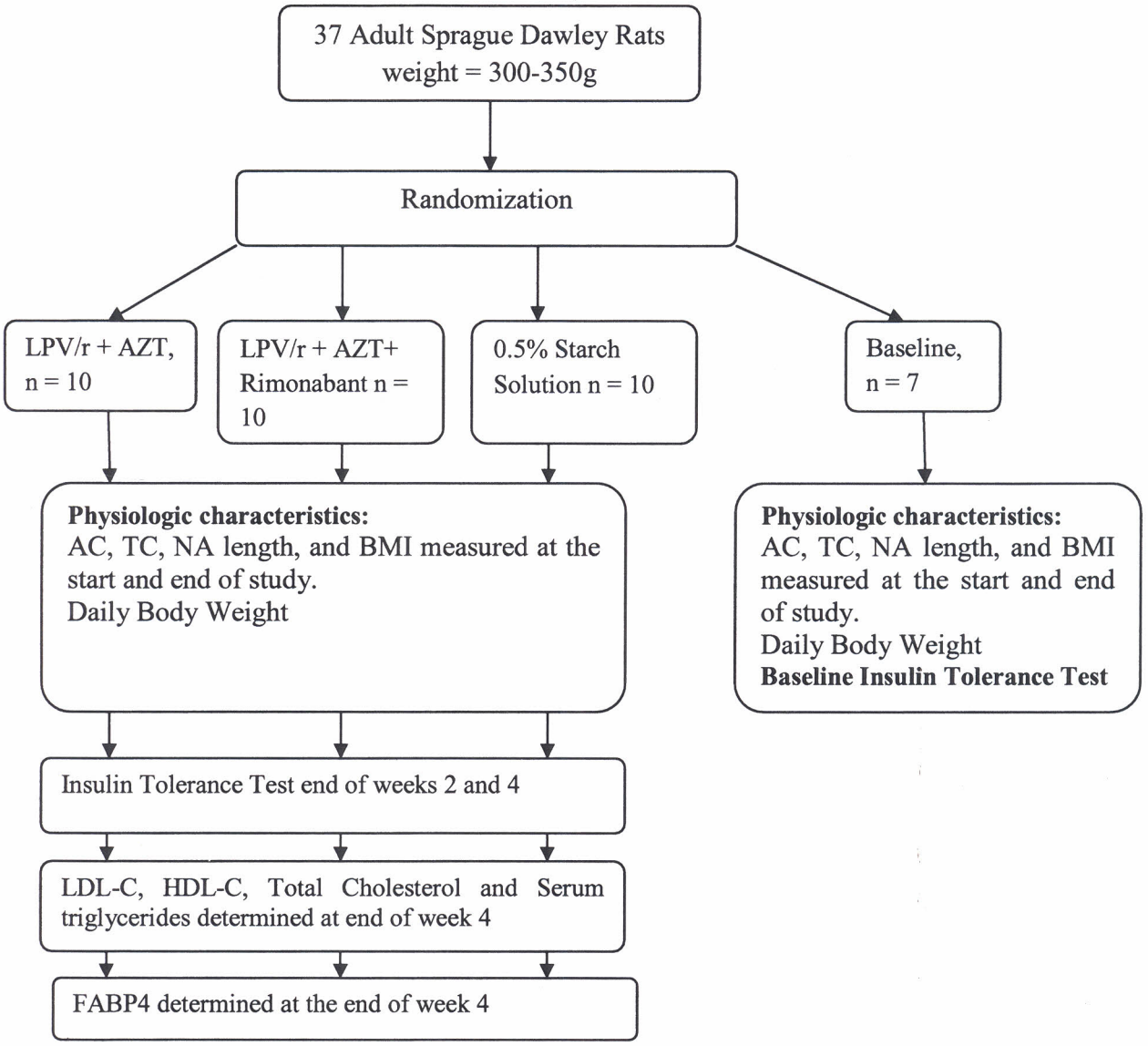
3.4 Experimental Animals

Thirty-seven (37) adult, male, Sprague- Dawley rats weighing between 300 and 350 g at the beginning of the study were purchased from the Department of Biochemistry, University of Nairobi and housed in communal cages at the Department of Human Physiology, University of Nairobi.

The rats were provided with standard rat chow (Unga Feeds, Nairobi, Kenya) and drinking water *ad libitum*. Rats were habituated to handling and testing procedures under a 12:12-h light–dark cycle (lights off at 1800 h) for two (2) weeks prior to testing. Dry wood shavings were used as beddings and changed daily. At the end of the habituation period, seven (7) randomly selected rats were sacrificed for a baseline assessment of insulin tolerance and fat depots. The remaining 30 rats were randomized into 3 groups of 10 rats each, namely; negative control, positive control (LPV/r + AZT) and the test group (LPV/r + AZT + rimonabant). The randomization scheme was generated by the web site Randomization.com (http://www.randomization.com) using the method of randomly permuted blocks. The rats were also randomly selected for drug administration and experimental procedures. Drug administration continued over a period of 4 weeks a duration equivalent to 2.8 – 3 human years (Andreollo et al., 2012).

The study protocol was approved by the Post graduate Research Committee, Department of Medical Physiology, School of Medicine, University of Nairobi. Rats were handled in accordance with the U.S National Research Council *Guide for the Care and Use of Laboratory Animals* (NRC, 1996). Sprague -Dawley rats were selected because of their suitability for hormone profile studies and ease of handling.

Figure 4 Experimental Design and Groups



3.5 Drugs and Chemicals

The PI drugs used in the study were Lopinavir and Ritonavir (Mylan laboratories Ltd., Maharashtra, India). These drugs are supplied in a fixed dose combination of Lopinavir/ritonavir of ratio 4:1 respectively which is approved for use as part of HAART regimens in Kenyan ART guidelines (Ministry of Medical Services, 2011). The tNRTI used was Zidovudine (Hetero drugs Ltd., Hyderabad, India). Both anti-retroviral drugs were donated by the office of the District Aids and STI Coordinator (DASCO), Nakuru, Kenya.

Drug dosages were calculated based on dosing guidelines for daily oral Lopinavir/ritonavir and Zidovudine in adult HIV patients; 800/200 total mg or 13.33/3.33mg/kg/day and 600mg total mg or 10mg/kg/day respectively, assuming a body weight of 60kg; Using body surface area normalization this translated to a dosage of approximately 80mg/20mg/kg/day of LPV/r and 60mg/kg/day of AZT in rats (Reagan-Shaw et al., 2007).

To reconstitute the PIs, a single tablet of LPV/r (200mg/50mg) was completely dissolved in 10ml of drinking water to yield a concentration of 20/5mg/ml. To reconstitute the tNRTI for administration, a single tablet was completely dissolved in 30mls of drinking water to yield a solution concentration of 10mg/ml. The drugs were administered daily between 0900h and 1100h by oral gavage daily for a period of 4 weeks. Individualized dosages were determined based on the rats weight. The animals were weighed daily before drug administration.

Rimonabant Hydrochloride also known as SR141716 (99% purity, Clearsynth Labs Limited., Mumbai, India) was dissolved in dimethyl sulphoxide (DMSO) by gentle sonication before being diluted with Tween 20 and saline (2% DMSO, 1% Tween 20, 97% saline) to final concentrations of 1 mg ml^{-1}. This was delivered by oral gavage at doses of 3mg/kg/day. This dose was based on

dose response curves developed previously (Vickers et al., 2003). Control rats received a weight matched volume of the drug vehicle, 0.5% pharmaceutical grade starch solution. Starch was purchased from F&S scientific LTD (Shamneel Court, Parklands Road, Westlands, Nairobi, Kenya). Mouse FABP4 ELISA kit with proven rat serum cross reactivity was purchased from BioVendor Laboratory Medicine, (Palackeho, Czech Republic).

3.6 Experimental Procedures

The insulin tolerance test was conducted at 2-week intervals and carried out as previously described (Heikkinen et al., 2007). Briefly, after a 2-h fast, the tip of the tail was nicked with a scalpel blade, a drop of blood expressed on a blood glucose test strip, and glucose values measured with a glucose meter (Prestige Smart System; Walgreens). Once fasting glucose had been determined, rats were injected subcutaneously at the back of the neck with 0.5 units/kg of Actrapid® insulin (Novo Nordisk; U.S.A) and blood glucose levels measured after 30 minutes at times, 30, 45, 60 and 120 minutes.

To determine lipid profiles colorimetric methods using commercially available kits (cholesterol, triglycerides, HDL-C and LDL-C, Chronolab, Switzerland) were utilized as previously described (McGowan et al., 1983). Briefly, blood samples (5-8mls) for lipid profile were collected by cardiocentesis in anaesthetized animals (Ketamine 80-100 mg/kg and Xylazine 8-10 mg/kg i.p.) and stored in EDTA containing vacutainer tubes. The sample tubes were inverted 8-10 times to mix blood with anti-coagulant and kept at room temperature until centrifugation. Samples underwent centrifugation at 2000G for 10 minutes to separate into three layers. The supernatant was aspirated and pooled into centrifuge tubes. Aliquots of plasma that were not assayed for lipids immediately were stored at -80^{0}C. These tests were also conducted after a 4 weeks interval.

Plasma FABP4 concentrations were determined as per ELISA kit instructions (Mouse FABP4 ELISA, BioVendor Laboratory Medicine Inc., Palackeho, Czech Republic) using a semi automated ELISA analyzer system (HumaReader HS, HUMAN GmbH, Wiesbaden, Germany). Briefly, standard and samples were incubated at room temperature for 2 hours in microplate wells pre-coated with polyclonal anti-mouse FABP4 antibody and shaken at ca. 300 rpm on an orbital microplate shaker. After two hours of incubation and washing, biotin labelled polyclonal anti-mouse FABP4 antibody was added and incubated with captured FABP4 for one hour. After another washing, streptavidin-HRP conjugate was added.

After further 30 minutes incubation at room temperature and the last washing step, the remaining conjugate was allowed to react with the substrate solution containing tetramethylbenzidine (TMB). The reaction was stopped by addition of acidic solution and absorbance of the resulting yellow product is measured. The absorbance is proportional to the concentration of FABP4. A standard curve was constructed by plotting absorbance values against concentrations of standards, and concentrations of unknown samples determined using this standard curve by linear intra-polation. Blood samples (5-10mls) for FABP4 Elisa were collected by cardiocentesis in anaesthetized animals (Ketamine 80-100 mg/kg and Xylazine 8-10 mg/kg i.p.).

To determine changes in fat distribution, subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) depots were carefully excised and weighed from all sacrificed rats. Interscapular brown adipose tissue (IBAT) was representative of SAT while inguinal and epididymal white adipose tissue represented VAT.

The abdominal circumference (AC) (immediately anterior to the forefoot), thoracic circumference (immediately behind the foreleg), body length (nose-to-anus or nose–anus length) were measured in all rats at 2-week intervals. The measurements were made in anaesthetized rats

(Ketamine 80-100 mg/kg and Xylazine 8-10 mg/kg i.p.). The body weight and body length were used to deter mine the Body mass index by the formula; {BMI = ¼ body weight (g)/length^2 (cm^2)}.

3.7 Data Analysis

All results are expressed as mean ± SD. Differences between the positive control and the test groups have been calculated using the unpaired Student's *t* test. To test for differences between the three groups, one way analysis of variance (ANOVA) was used. Differences were considered significant at $P < 0.05$. Tukey's HSD (honestly significant difference) post hoc test was carried out in case of significance

CHAPTER 4

RESULTS

4.1 Body Mass Index and Weight

There were no differences in weight (353 ± 50 vs. 338 ± 42 vs. 346 ± 20 g, F (2, 27) = 0.37, P = 0.69), BMI (0.163 ± 0.01 vs. 0.162 ± 0.02 vs. 0.168 ± 0.01 g/cm^2, F (2, 27) = 0.35, P = 0.71), Abdominal Circumference (17.7 ± 1.40 vs. 18.0 ± 1.30 vs. 17.5 ± 0.20 cm, F (2, 27) = 0.40, P = 0.67) and Thoracic circumference (15.5 ± 0.90 vs. 15.5 ± 1.30 vs. 15.3 ± 0.40 cm, F (2, 27) = 0.17, P = 0.85) at the beginning of the study (Table 1).

Table 1. Anthropometric characteristics at the beginning of the study

Groups	Number of rats (n = 10)	Weight (g)	BMI [a] (g/cm^2)	AC [b] (cm)	TC [c] (cm)
Positive Controls		353 ± 50**	0.163 ± 0.01*	17.7 ± 1.40¶	15.5 ± 0.90§
Test		338 ± 42**	0.162 ± 0.02*	18.0 ± 1.30¶	15.5 ± 1.30§
Negative Control		346 ± 20	0.168 ± 0.01*	17.5 ± 0.20	15.3 ± 0.40

[a]AC = abdominal circumference: [b]BMI = body mass index;; [c]TC = thoracic circumference.

Values represented as mean ± SD

** P = 0.69; * P = 0.71; ¶ P = 0.67; § P = 0.85

Further analysis of weekly BMIs by one way ANOVA showed significant difference (F (2, 12) = 9.43, P = 0.003). Post hoc analyses by Tukey's HSD criterion for significance indicated that weekly BMIs were lower in the test group than both positive control and negative control groups (0.163 ± 0,00 vs. 0.167 ± 0.00 vs. 0.172 ± 0.00 g/cm^2, F(2, 12) = 9.43, P = 0.003). Similarly, average weekly weights were lower in the test group vs. positive controls and negative controls (338 ± 2.53 vs. 360.78 ± 7.00 vs. 353.94 ± 8.35g, (F (2, 12) = 14.93, P <0.001) (Table 2).

Table 2. Average Weekly Weight and Body Mass Index

Groups	Number of rats (n = 10)	Average Weekly Weight (g)	Average Weekly BMI [a] (g/cm^2)
Test		338.96 ± 2.53*	0.163 ± 0.00**
Positive Controls		360.78 ± 7.00*	0.167 ± 0.00**
Negative Controls		353.94 ± 8.35	0.172 ± 0.00

[a]AC = abdominal circumference: [b]BMI = body mass index;; [c]TC = thoracic circumference.
Values represented as mean ± SD
** P = 0.003; * P = 0.0005

There were no significant differences observed in weight (364 ± 51 vs. 337 ± 36 vs. 357 ± 24 g, F (2, 27) = 1.32, P = 0.28), BMI (0.169 ± 0.01vs. 0.162 ± 0.02 vs. 0.173 ± 0.01 g/cm^2, F (2, 27) = 2.01, P =0.15), Abdominal Circumference (16.5 ± 1.00 vs. 16.1 ± 0.90 vs. 16.40 ± 0.7 cm, F (2, 27) = 0.38, P = 0.69) or Thoracic circumference (14.8 ± 0.90 vs. 14.3 ± 0.60 vs. 14.8 ± 0.40 cm, F (2, 27) = 2.02, P = 0.15) at the end of the study (Table 3).

Table 3. Anthropometric characteristics at the end of the study

Groups	Number of rats (n = 10 per group)	Weight (g)	BMI [a] (g/cm^2)	AC [b] (cm)	TC [c] (cm)
Positive Controls		364 ± 51**	0.169 ± 0.01*	16.5 ± 1.00¶	14.8 ± 0.90§
Test		337 ± 36**	0.162 ± 0.02*	16.1 ± 0.90¶	14.3 ± 0.60§
Negative Controls		357 ± 24	0.173 ± 0.01	16.4 ± 0.70	14.8 ± 0.40

[a]AC = abdominal circumference: [b]BMI = body mass index;; [c]TC = thoracic circumference.
Values represented as mean ± SD
** P = 0.28; * P = 0.09; ¶ P = 0.69; § P = 0.15

.2 CB1 antagonism decreases visceral fat accumulation

There was a marginal difference in the weight of visceral fat pads between the groups on one way ANOVA (4.93 ± 2.09 vs. 7.33 ± 2.88 vs. 6.27 ± 1.92 g, F (2, 27) = 2.65, P =0.09). The mean weight of visceral fat pads was 39% and 23.9% lower in the test group than in positive controls and negative controls respectively.

Table 4. Weights of inguinal, epididymal and Interscapular fat pads

Groups	Number of rats (n = 10 per group)	Inguinal and epididymal fat pads weight (grams)	Interscapular brown adipos tissue weight (grams)
Test group		4.93 ± 2.09*	0.71 ± 0.37
Positive Controls		7.33 ± 2.88*	0.76 ± 0.23
Negative Controls		6.27 ± 1.92	0.77 ± 0.31

Values represented as mean ± SD

* $P = 0.09$.

Analysis of variance found no differences between the groups in weight of subcutaneous fat pads (0.76 ± 0.23 vs. 0.71 ± 0.37 vs. 0.77 ± 0.31 g, F (2, 27) = 0.12, P = 0.86).

Table 5. Weights of inguinal, epididymal and Interscapular fat pads for baseline rats

Group	Number of rats (n = 7)	Inguinal and epididymal fat pads weight (grams)	Interscapular brown adipose tissue weight (grams)
Baseline Rats		4.53 ± 1.21	0.71 ± 0.15

4.3 Concurrent Rimonabant with cART administration and insulin sensitivity

The use of Rimonabant with cART increased insulin sensitivity as determined by a two hour ITT. Fasting blood sugar was 49.7% lower in the test group compared to the positive controls and 13.8% lower than negative controls. On one way ANOVA, fasting blood sugar differed significantly between the groups (2.3 ± 0.65 vs. 3.82 ± 0.61 vs. 2.64 ± 0.34 mmols/L, F (2, 12) = 3.89, P = 0.002). On post hoc analyses, test animals had significantly lower FBS than positive

controls (2.3 ± 0.65 vs. 3.82 ± 0.61, F (2, 12) = 3.89, P = 0.002) 120 minutes after a loading dose of insulin (Table 6).

Table 6. Fasting blood sugar levels on insulin tolerance test

		Time 0	Time 30	Time 45	Time 60	Time 120
				(minutes)		
Groups	Number of rats (n)	Fasting Blood Sugar (mmols/L)				
Positive Controls	5	4.76 ± 0.68	3.00 ± 0.39	2.78 ± 0.08**	2.78 ± 0.35**	3.82±0.61*
Test	5	4.66 ± 0.7	3.38 ± 0.53	2.7 ± 0.71	2.3 ± 0.82	2.30±0.65*
Negative Controls	5	4.06±0.19	2.78 ± 0.2	2.36 ± 0.22	2.2 ± 0.24	2.64±0.34

Fasting blood sugar was measured following a 5hour fast (Time 0), 0.5 IU/kg of Actrapid insulin were then injected subcutaneously and blood sugar levels measured at 30, 45, 60 and 120 minutes after the injection.

Values represented as mean ± SD

**$P < 0.05$; *$P = 0.002$

There were no differences in FBS values among the groups at Time 0 (4.66 ± 0.7 vs. 4.76 ± 0.68 vs. 4.06±0.19 mmols/L, $P = 0.16$) (Table 6).

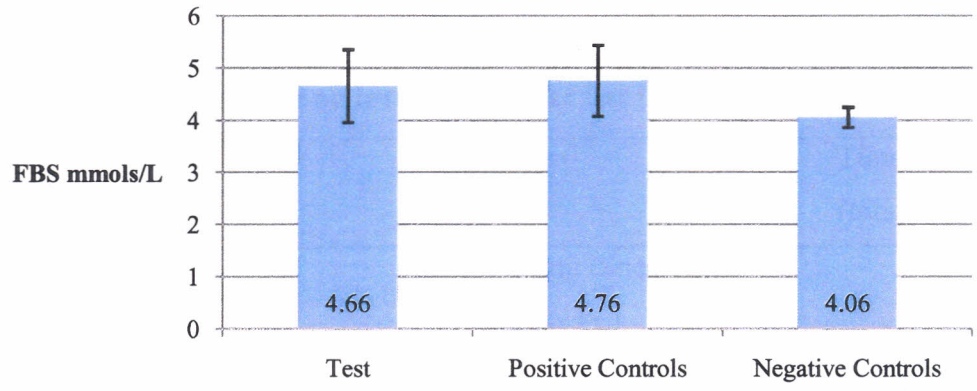


Figure 5. Fasting Blood Sugar levels obtained at Time 0 prior to insulin loading.
Bars represent means ± SD of 5 samples.

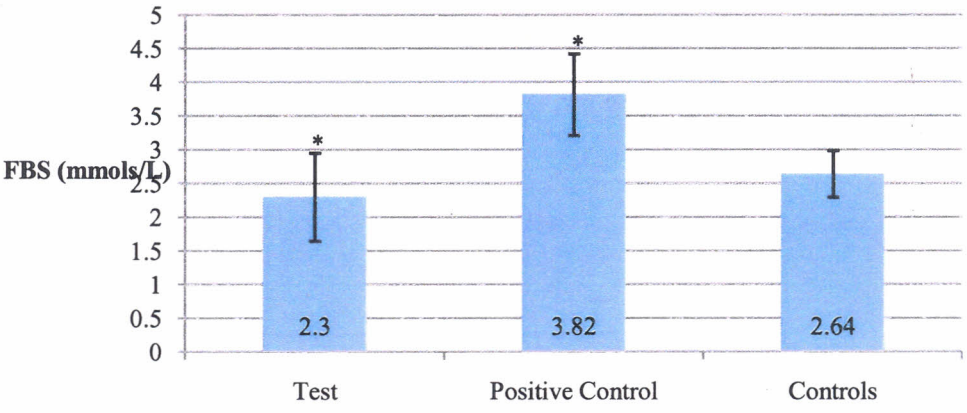


Figure 6. Fasting Blood Sugar obtained 120 minutes after a 0.5 IU kg^{-1} subcutaneous dose of insulin.
Bars represent means ± SD of 5 samples.
*$P = 0.002$

Results of baseline assessment of insulin sensitivity are shown in Table 7 below

Table 7. Fasting blood sugar on insulin tolerance test for baseline rats

		Time 0	Time 30	Time 45 (minutes)	Time 60	Time 120
Groups	Number of rats (n)	Fasting Blood Sugar (mmols/L)				
Baseline Rats	7	4.49±1.21	1.69 ± 0.47	1.16±0.34	_[a]	2.09±1.0

[a]Not detectable. Sixty (60) minutes after insulin injection all rats used in baseline study had FBS readings below the lower limit of detection (1.1 mmols L^{-1}) by the glucometer used.
All values represented as mean ± SD.

4.4 Concurrent Rimonabant with cART administration and serum lipids

Serum triglycerides were higher by 57% and 33% in test animals versus positive controls and controls respectively. These differences were significant by one way analyses of variance (1.86 ± 0.63 vs.1.04 ± 0.35 vs.1.33 ± 0.4 mmols/L, $F(2,27) = 7.67$, $P = 0.002$,) However, significance was not maintained on post hoc analyses.

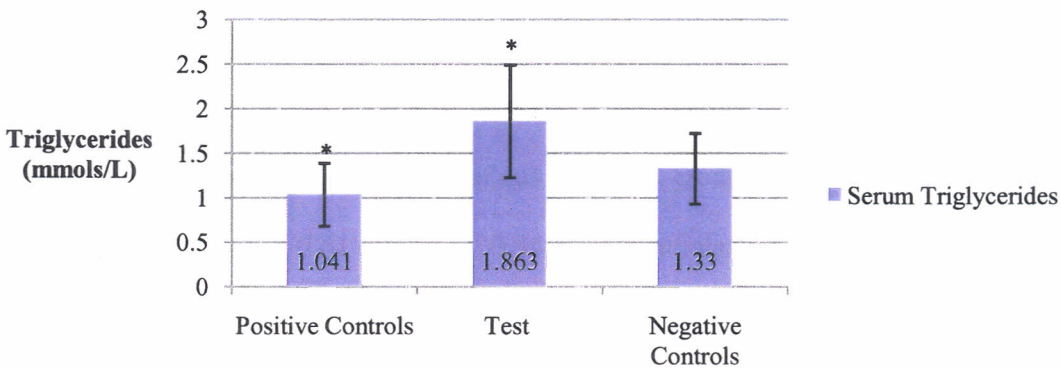


Figure 7. Serum triglyceride concentrations

Bars represent means ± SD

*P = 0.002

Similarly, significant differences occurred in LDL-C concentrations (1.64 ± 0.85 vs. 0.53 vs. ± 0.41vs. 1.31 ± 0.58 mmols/L, $F(2,27) = 7.89, P = 0.002$) between groups with one way ANOVA, In terms of magnitude, LDL-C concentrations were 102% higher (1.64 ± 0.85 vs. 0.53 vs. ± 0.41mmols/L) in the test group than positive controls and 20% higher vs. controls (1.64 ± 0.85 vs. 1.31 ± 0.58 mmols/L). The level of significance was not maintained by post-hoc analysis.

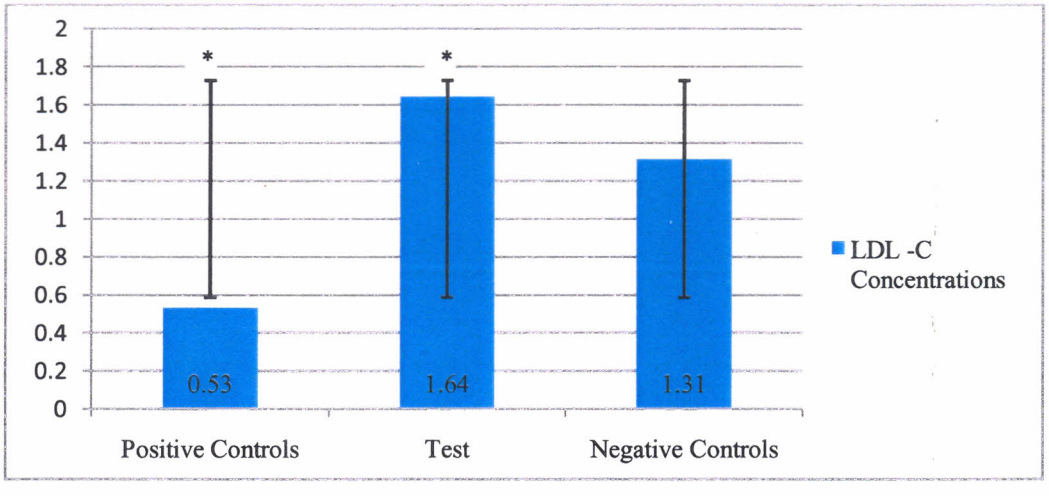


Figure 8, Low Density lipoprotein – cholesterol levels

Bars represent means ± SD

*P = 0.002

Differences in serum HDL-C concentrations between groups were also observed by one way ANOVA (0.33 ± 0.16 vs. 0.61 ± 0.34 vs. 0.34 ± 0.16 mmols/L, $F(2, 27) = 4.5, P = 0.02$). HDL-C was 60% lower (0.33 ± 0.16 vs. 0.61 ± 0.34 mmols/L) in the test group compared to positive controls. The level of significance was not maintained on post-hoc analysis.

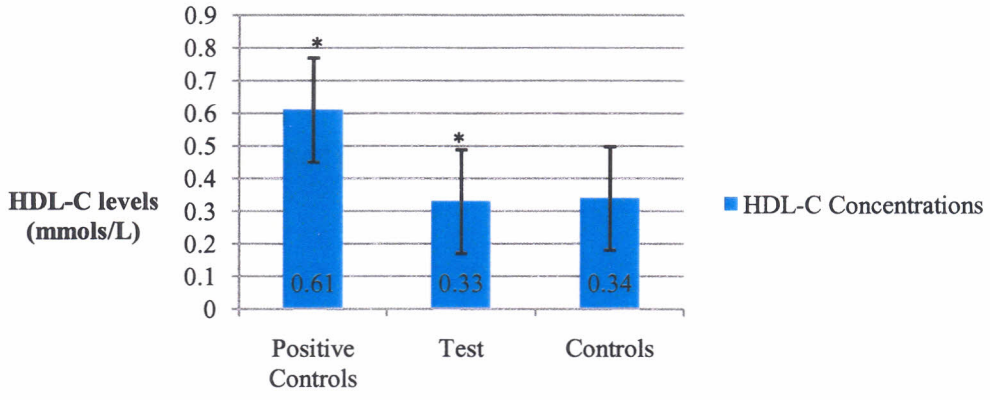


Figure 9. High density lipoprotein – cholesterol levels

Bars represent means ± SD; HDL-C, high density lipoprotein –cholesterol.

*$P = 0.02$

Table 8. Lipid profile differences between groups

Groups	Number of rats (n = 10)	Triglycerides (mmols/L)	LDL-C (mmols/L)	HDL –C (mmols/L)
Test		1.86 ± 0.63*	1.64 ± 0.85*	0.33 ± 0.16**
Positive Controls		1.04 ± 0.35*	0.53 ± 0.41*	0.61 ± 0.34**
Controls		1.33 ± 0.40	1.31 ± 0.58	0.34 ± 0.16

HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol;

Values are means ± SD.

*$P = 0.002$; **$P = 0.02$

4.5 Concurrent Rimonabant and cART administration and FABP4 concentrations

Serum FAPB4 concentrations were 80% and 75% higher in the cART + Rimonabant group compared to cART-only and controls respectively (19.1 ± 18.3 vs. 8.1 ± 9.0 vs. 8.6 ± 7.6 mg/dL, $F(2.27) = 1.53$ $P = 0.23$)

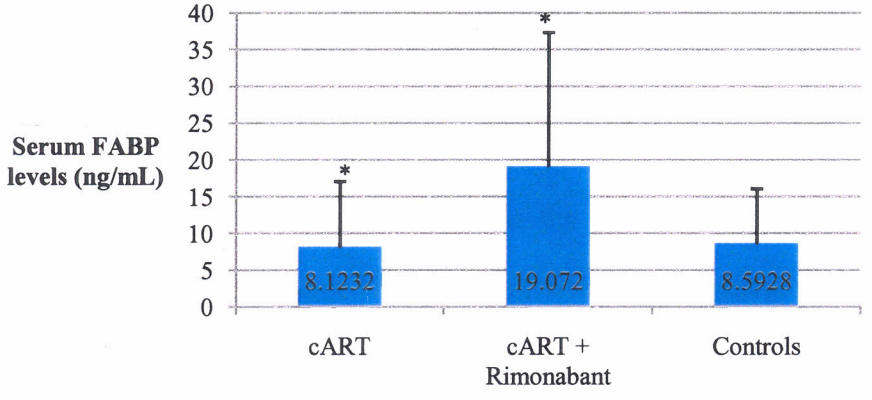


Figure 10. Serum Fatty Acid Binding Protein – type 4 concentrations. Bars represent means ± SD cART, combined Anti-Retroviral Therapy; FABP4, Fatty Acid Binding Protein–type 4 *$P = 0.23$.

CHAPTER 5

DISCUSSION

Metabolic syndromes comprise another category, albeit hitherto unappreciated, of HIV-1-associated diseases. It is now a documented fact that HIV-1-infected patients have up to two times the risk, when compared to the uninfected population, of developing cardiovascular disease (CVD), atherosclerosis, dyslipidemia and/or insulin resistance (Klein et al., 2002; Currier et al., 2008; Hsue et al., 2009).This increased risk has been ascribed to both the drugs used in antiretroviral therapy as well as the HIV infection itself (Lamers et al., 2012).

HIV-associated lipodystrophy manifests as the abnormal accumulation of fat at central sites (lipohypertrophy) accompanied by loss of fat tissue at peripheral sites (lipoatrophy) (Mallewa et al., 2008). It is often co-morbid to and precedes the metabolic syndrome. It also occurs in both treated and untreated patients but its incidence is increased in patients on protease inhibitor containing antiretroviral drug treatment regimens (Grinspoon & Carr, 2005).

Current strategies to address cART associated lipodystrophy that include switching from culpable cART drugs or drug holidays, use of thiazolidinediones, statins and fibric acid derivatives, Growth Hormone Replacement Therapy and cosmetic surgery produce only a modest effect on the condition. Effective interventions are therefore required to address this growing concern.

Antagonism of the CB1 receptor is known to improve insulin sensitivity, lipid profiles and fat distribution in rodent models of obesity (Ravinet-Trillou et al., 2003; Gary-Bobo et al., 2007) and in patients with the metabolic syndrome (Pi-Sunyer et al., 2006) however the potential

benefits of CB1 antagonism have not been explored in the context of cART induced derangements of metabolism and phenotype.

In the present study, the use of the CB1 antagonist rimonabant with cART improved insulin sensitivity significantly resulting in FBS levels that were 39% and 14% lower in test animals at 120 minutes than in cART-only and control rats respectively. The better insulin sensitivity suggests that activation of the endogenous cannabinoid system is involved in the etiology of insulin resistance that accompanies use of LPV/r and AZT containing cART regimens.

The use of rimonabant alongside cART produced a leaner rat phenotype by reducing accumulation of visceral fat by 39% and 24% when compared positive and control rats respectively. These results suggest that the endogenous cannabinoid system is a driver of adipogenesis in the cART induced metabolic disorder. Additionally, the findings concur with previous work showing that reduction in size of visceral fat fads (epidydimal, perirenal, and lumbar tissues) following treatment with rimonabant was accompanied with improved insulin sensitivity (Ravinet-Trillou et al., 2003).

Serum triglycerides and LDL-C increased while serum HDL-C concentration decreased significantly in test animals. The results were obtained at a dose of rimonabant (3mg/kg) that is in the lowest range of doses used in similarly designed studies (Colombo et al., 1998; Bensaid et al., 2003). The findings differ with other studies of CB1 antagonism which have shown that treatment with rimonabant reduces serum triglycerides and LDL-C while modestly increasing HDL-C levels in both rodent and human studies (Di Marzo, 2004; Ravinet-Trillou et al., 2004; Poirier et al., 2005; Després et al., 2009)..

It is possible that rimonabant produced a boosting effect on the serum concentrations of the anti-retroviral drugs particularly the protease inhibitor Lopinavir. Lopinavir is effectively and rapidly metabolized by hepatic cytochrome CYP3A4 leading to sub-therapeutic concentrations when the drug is administered alone. Ritonavir potently inhibits CYP3A4 resulting in a positive drug-drug interaction when the two drugs are co-administered. Recently, rimonabant has also been found to irreversibly inhibit CYP3A4 in a time-dependent manner (Bergström et al., 2011). CYP3A4 metabolizes rimonabant to produce an iminium ion metabolite as its major by-product. This metabolite is a potent cytotoxic that binds to and inhibits cellular microsomes including CYP3A4 (Foster et al., 2013).In the present study, irreversible covalent inhibition of CYP3A4 by this iminium ion metabolite may have increased the serum concentrations of lopinavir beyond the initial boost provided by ritonavir into a toxic range that resulted in dyslipidemia.

In addition, Foster and co-workers (Foster et al., 2013) demonstrated that ritonavir can block the metabolism of rimonabant thereby increasing its duration of action but also abolishing the cytotoxic effects of its iminium ion metabolite. From their result one can deduce that the use of the LPV/r combination should be protective against rimonabant induced cellular injury, however that study was not designed to explore what effect the interaction of ritonavir and rimonabant would have on cellular lipid synthesis. The present study provides some insight into the possible effects of a ritonavir-rimonabant interaction on lipid synthesis. That interaction appears to produce dyslipidemia and points to advanced boosting of serum lopinavir concentrations as being possibly responsible even though this was not directly assessed.

Treatment with rimonabant increased serum concentrations of fatty acid binding protein – type 4 (FABP4) by 75 and 80% compared to controls and cART-only rats respectively. This result is consistent with the increased serum lipids in the rimonabant group since serum FABP4 levels

have been shown to correlate positively with serum triglycerides and LDL-C levels and negatively with HDL-C levels both in rodent studies and in evaluation of HIV infected patients with cART associated lipodystrophy (Makowski et al., 2001; Coll et al., 2008; Escote' et al., 2011). There however appears to be a conflict in the relationship between increased FABP4 levels and better insulin sensitivity in cART + Rimonabant rats. An increase in serum FABP4 is usually accompanied by insulin resistance which is driven by cytokines TNFα, IL-6, IL-18 and leptin resistance (Kirchgessner et al., 1997; Mynarcik, 2000; Kern et al., 2001). On the other hand, rimonabant is known to reduce expression of pro-inflammatory cytokines particularly TNFα and to improve leptin sensitivity (Gary-Bobo et al., 2007; Osei-Hyiaman et al., 2008). It is possible that these anti-inflammatory properties of rimonabant undermined the development of insulin resistance in spite of the higher serum FABP4 levels observed.

As far as we can determine this is the first study to explore the possible link between the endogenous cannabinoid system and cART associated metabolic and phenotypic disturbances. The assay of cannabinoid biosynthetic enzymes DAGL and NAPE PLD and measurement of plasma levels of their products would be a more direct assessment of ECS tone. We also note that it would have been more informative to include an additional experimental group treated with rimonabant only. In spite of these limitations the study's main objective was to determine the effects of the CB1 receptor antagonism on the metabolic and phenotypic characteristics of rats treated concurrently with LPV/r and AZT. As such the study sheds light on the utility of treatment strategies that target the ECS tone in mitigating the unwanted effects of certain HAART regimes.

In conclusion this study of CB1 antagonism with concurrent cART in rats suggests that, although insulin sensitivity improves in rats given cART with rimonabant, the possible benefits of using

rimonabant are lessened by the increase in serum triglycerides, LDL-C and FABP4 and these inappropriately raise the risk for atherosclerosis and cardiovascular morbidity (Makowski et al., 2005). It is also likely that even though visceral adiposity was diminished by using rimonabant along with cART, translation of this regimen would not improve peripheral fat loss given that rimonabant treated rats showed 8% less interscapular brown adipose tissue when compared to both cART-only and control rats. Future work can shed light on the exact drug-drug interactions that produced the dyslipidemic profile and the effects of such treatment on expression of specific inflammatory markers and pro-adipogenic transcription factors in various tissues.

CHAPTER 6

BIBLIOGRAPHY

1. Addy, C.L. et al., 2003. Hypoadiponectinemia Is Associated with Insulin Resistance, Hypertriglyceridemia, and Fat Redistribution in Human Immunodeficiency Virus-Infected Patients Treated with Highly Active Antiretroviral Therapy. *J Clin Endocrinol Metab*, 2, pp.627-36.

2. AIDSmeds.com, 2011. *www.aidsmeds.com*. [Online] Available at: http://www.aidsmeds.com/articles/Lipodystrophy_10732.shtml [Accessed 7 August 2012].

3. Ammassari, A. et al., 2002. Relationship between HAART adherence and adipose tissue alterations. *J Acquir Immune Defic Syndr*, 31(3), pp.140-44.

4. Ammassari, A. et al., 2001. Self-Reported Symptoms and Medication Side Effects Influence Adherence to Highly Active Antiretroviral Therapy in Persons With HIV Infection. *J Acquir Immune Defic Syndr*, 28(5).

5. Andreollo, N., Santos, E., Araújo, M. & Lopes, L., 2012. Rat's age versus human's age: what is the relationship? *Arq Bras Cir Dig.*, 25(1), pp.49-51.

6. Bastard, J.-P. et al., 2002. Association between altered expression of adipogenic factor SREBP1 in lipoatrophic adipose tissue from HIV-1-infected patients and abnormal adipocyte differentiation and insulin resistance. *The Lancet*, 359(9311), pp.1026-31.

7. Behrens, G. et al., 1999. Impaired glucose tolerance, beta cell function and lipid metabolism in HIV patients under treatment with protease inhibitors. *AIDS*, 13(10), pp.F63-70.

8. Bensaid, M. et al., 2003. The cannabinoid CB1 receptor antagonist SR141716 increases Acrp30 mRNA expression in adipose tissue of obese fa/fa rats and in cultured adipocyte cells. *Molecular Pharmacology*, 63(4), pp.908-14.

9. Berger, W. et al., 2012. Targeting fatty acid binding protein (FABP) anandamide transporters - a novel strategy for development of anti-inflammatory and anti-nociceptive drugs. *PLoS One*, 7(12).

10. Bergström, A.M., Isin, E.M., Castagnoli Jr, N. & Milne, C.E., 2011. Bioactivation Pathways of the Cannabinoid Receptor 1 Antagonist Rimonabant. *Drug Metab Dispos*, 39(10), pp.1823-32.

11. Bermúdez-Sivaa, F.J. et al., 2006. Activation of cannabinoid CB1 receptors induces glucose intolerance in rats. *Eur J Pharmacol*, 531(1-3), p.282–284.

12. Blüher, M. et al., 2006. Dysregulation of the Peripheral and Adipose Tissue Endocannabinoid System in Human Abdominal Obesity. *Diabetes*, 55(11), pp.3053-60.

13. Blümer, R.M., 2008. Zidovudine/lamivudine contributes to insulin resistance within 3 months of starting combination antiretroviral therapy. *AIDS*, 22(2), pp.227-36.

14. Bochtler, M. et al., 1999. The Proteasome. *Annu Rev Biophys Biomol Struct*, 28, pp.295-317.

15. Boothby, M., 2009. Adipocyte differentiation, mitochondrial gene expression and fat distribution: differences between zidovudine and tenofovir after 6 months. *Antiviral Therapy*, 14, pp.1089-100.

16. Bouaboulaa, M. et al., 2005. Anandamide induced PPARgamma transcriptional activation and 3T3-L1 preadipocyte differentiation. *Eur J Pharmacol*, 517(3), p.174–181.

17. Brinkman, K., 1998. Adverse effects of reverse transcriptase inhibitors: mitochondrial toxicity as common pathway. *AIDS*, 12, p.1735–1744.

18. Cabré, A. et al., 2007. Fatty acid binding protein 4 is increased in metabolic syndrome and with thiazolidinedione treatment in diabetic patients. *Atherosclerosis*, 195(1), pp.e150-158.

19. Calza, L., Manfredi, R. & Chiodo, F., 2003. Statins and fibrates for the treatment of hyperlipidaemia in HIV-infected patients receiving HAART. *AIDS*, 17(6), pp.851-59.

20. Caron, M. et al., 2004. The HIV-1 nucleoside reverse transcriptase inhibitors stavudine and zidovudine alter adipocyte functions in vitro. *AIDS*, 18(16), pp.2127-36.

21. Caron, M. et al., 2001. The HIV Protease Inhibitor Indinavir Impairs Sterol Regulatory Element-Binding Protein-1 Intranuclear Localization, Inhibits Preadipocyte Differentiation, and Induces Insulin Resistance. *Diabetes*, 50(6), pp.1378-88.

22. Carr, A. & Cooper, D.A., 2000. Adverse effects of antiretroviral therapy. *Lancet*, 356(9239), p.1423–1430.

23. Carr, A. & Law, M., 2003. An Objective Lipodystrophy Severity Grading Scale Derived From the Lipodystrophy Case Definition Score. *JAIDS*, 33(5), pp.551-657.

24. Carr, A. et al., 1998. A syndrome of peripheral lipodystrophy, hyperlipidaemia and insulin resistance in patients receiving HIV protease inhibitors. *AIDS*, 12(7).

25. Carr, A. et al., 1999. Diagnosis, prediction, and natural course of HIV-1 protease-inhibitor-associated lipodystrophy, hyperlipidaemia, and diabetes mellitus: a cohort study. *LANCET*, 353(9170), pp.2093-2099.

26. Carr, A. et al., 2004. No effect of rosiglitazone for treatment of HIV-1 lipoatrophy: randomised, double-blind, placebo-controlled trial. *Lancet*, 363, pp.429-38.

27. Clercq, E.D., 2007. The design of drugs for HIV and HCV. *Nat Rev Drug Discov*, 6, pp.1001-08.

28. Coe, N.R. & Bernlohr, D.A., 1998. Physiological properties and functions of intracellular fatty acid-binding proteins. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism*, 1391(3), p.287–306.

29. Coll, B. et al., 2008. The fatty acid binding protein-4 (FABP4) is a strong biomarker of metabolic syndrome and lipodystrophy in HIV-infected patients. *Atherosclerosis*, 199(1).

30. Colombo, G. et al., 1998. Appetite suppression and weight loss after the cannabinoid antagonist SR 141716. *Life Sci.*, 63(8), p.PL113–PL117.

31. Cota, D., 2003. Endogenous cannabinoid system as a modulator of food intake. *Int J Obes Relat Metab Disord*, 27(3), p.289–301.

32. Cota, D. et al., 2003. The endogenous cannabinoid system affects energy balance via central orexigenic drive and peripheral lipogenesis. *J Clin Invest*, 112(3), p.423–431.

33. Cote, M. et al., 2007. Circulating endocannabinoid levels, abdominal adiposity and related cardiometabolic risk factors in obese men. *Int J Obes*, 31, p.692–699.

34. Currier, J.S. et al., 2008. Epidemiological Evidence for Cardiovascular Disease in HIV-Infected Patients and Relationship to Highly Active Antiretroviral Therapy. *Circulation*, 118, pp.e29-e35.

35. Dallal, G.E., 2008. *Randomization.com.* [Online] Available at: http://www.randomization.com/ [Accessed 7 August 2012].

36. Despres, J.-P. & Di Marzo, V., 2009. Endocannabinoid Dysregulation In Abdominal Obesity And Hyperglycemia: Human Studies. In J.-P. Despres & V. Di Marzo, eds. *Abdominal Obesity and the Endocannabinoid System*. New York, NY 10017: Informa Healthcare USA, Inc. pp.205-06.

37. Després, J.-P. & Di Marzo, V., 2009. ENDOCANNABINOID DYSREGULATION IN ABDOMINAL OBESITY AND HYPERGLYCEMIA: HUMAN STUDIES. In J.-P. Després & V. Di Marzo, eds. *Abdominal Obesity and the Endocannabinoid System*. New York, NY 10017: Informa Healthcare USA, Inc. pp.205-06.

38. Després, J.-P. et al., 2009. Effect of Rimonabant on the High-Triglyceride/ Low–HDL-Cholesterol Dyslipidemia, Intraabdominal Adiposity, and Liver Fat. *Arterioscler Thromb Vasc Biol*, 29, pp.416-23.

39. Deveaud, C., 2005. Site specific alterations of adipose tissue mitochondria in 3'-azido-3'-deoxythymidine (AZT)-treated rats: an early stage in lipodystrophy? *Biochemical Pharmacology*, 70, pp.90-101.

40. Dever, L. et al., 2000. Hyperglycemia associated with protease inhibitors in an urban HIV-infected minority patient population. *Ann Pharmacother*, 34(5), pp.580-84.

41. Di Marzo, V., 2004. The endocannabinoid system and its therapeutic exploitation. *Nat Rev Drug Discov*, 3(9), pp.771-84.

42. Di Marzo, V., 2007. Endocannabinoids and the regulation of their levels in health and disease. *Curr Opin Lipidol*, 18, pp.129-40.

43. Dubé, M.P., Johnson, D.L., Currier, J.S. & Leedom, J.M., 1997. Protease inhibitor-associated hyperglycaemia. *Lancet*, 350(9079), pp.713-14.

44. Duran, S., Savs, M., Spire, B. & etal, 2001. Failure to maintain long-term adherence to highly active antiretroviral therapy: the role of lipodystrophy. *AIDS*, 15, pp.2141-44.

45. Engeli, S. et al., 2005. Activation of the Peripheral Endocannabinoid System in Human Obesity. *Diabetes* , 54(10), pp.2838-2843.

46. Escote', X. et al., 2011. A study of fatty acid binding protein 4 in HIV-1 infection and in combination antiretroviral therapy-related metabolic disturbances and lipodystrophy. *HIV Medicine*, 12, pp.428-37.

47. Felder, C. et al., 1993. Anandamide, an endogenous cannabimimetic eicosanoid, binds to the cloned human cannabinoid receptor and stimulates receptor-mediated signal transduction. *Proceedings of the National Academy of Sciences USA*, 90.

48. Fichtenbaum, C.J. et al., 2002. Pharmacokinetic interactions between protease inhibitors and statins in HIV seronegative volunteers: ACTG Study A5047. *AIDS*, 16(4), pp.569-77.

49. Fiorenza, C.G., Chou, S.H. & Mantzoros, C.S., 2011. Lipodystrophy: pathophysiology and advances in treatment. *Nat Rev Endocrinol*, 7, pp.137-50.

50. Fisher, E.A. et al., 1997. The Degradation of Apolipoprotein B100 Is Mediated by the Ubiquitin-proteasome Pathway and Involves Heat Shock Protein 70. *J. Biol. Chem.*, 272, pp.20427-34.

51. Flint, O.P. et al., 2009. The Role of Protease Inhibitors in the Pathogenesis of HIV-Associated Lipodystrophy: Cellular Mechanisms and Clinical Implications. *Toxicol Pathol*, 37(1), pp.65-77.

52. Foster, A.J. et al., 2013. Bioactivation of the Cannabinoid Receptor Antagonist Rimonabant to a Cytotoxic Iminium Ion Metabolite. *Chem. Res. Toxicol.*, 26(1), p.124–135.

53. Fu, Y., Luo, N., Lopes-Virella, M. & Garvey, W., 2002. The adipocyte lipid binding protein (ALBP/aP2) gene facilitates foam cell formation in human THP-1 macrophages. *Atherosclerosis*, 165(2), pp.259-69.

54. Furuhashi, M. & Hotamisligil, G.S., 2008. Fatty acid-binding proteins: role in metabolic diseases and potential as drug targets. *Nature Reviews Drug Discovery*, 7.

55. Gary-Bobo, M. et al., 2007. Rimonabant reduces obesity-associated hepatic steatosis and features of metabolic syndrome in obese Zucker fa/fa rats. *Hepatology*, 46(1), p.122–129.

56. Gavrila, A. et al., 2005. Improvement in Highly Active Antiretroviral Therapy—Induced Metabolic Syndrome by Treatment with Pioglitazone but Not with Fenofibrate: A 2 × 2 Factorial, Randomized, Double-Blinded, Placebo-Controlled Trial. *Clin Infect Dis*, 40(5), pp.745-49.

57. Gelato, M.C. et al., 2002. Improved Insulin Sensitivity and Body Fat Distribution in HIV-Infected Patients Treated With Rosiglitazone: A Pilot Study. *J Acquir Immune Defic Syndr*, 31(2), pp.163-67.

58. Grinspoon, S. & Carr, A., 2005. Cardiovascular Risk and Body-Fat Abnormalities in HIV-Infected Adults. *N Engl J Med* , 352, pp.48-62.

59. Guaraldi, G. et al., 2011. Surgical correction of HIV-associated facial lipoatrophy. *AIDS*, 25(1), pp.1-12.

60. Hansen, P.A. et al., 1995. Skeletal Muscle Glucose Transport and Metabolism Are Enhanced in Transgenic Mice Overexpressing the Glut4 Glucose Transporter. *J. Biol. Chem.*, 270, pp.1679-84.

61. Haunerland, N.H. & Spener, F., 2004. Fatty acid-binding proteins – insights from genetic manipulations. *Progress in Lipid Research*, 43(4), p.328–349.

62. Heikkinen, S., Argmann, C.A., Champy, M.-F. & Auwerx, J., 2007. Evaluation of Glucose Homeostasis. *Curr Protoc Mol Biol.*, p.Chapter 29:Unit 29B.3.

63. Hertel, J., Struthers, H., Horj, C.B. & Hruz, P.W., 2004. A Structural Basis for the Acute Effects of HIV Protease Inhibitors on GLUT4 Intrinsic Activity. *J. Biol. Chem.*, 279(53), pp.55147-52.

64. Hillard, C.J. & Jarrahian, A., 2003. Cellular accumulation of anandamide: consensus and controversy. Br J Pharmacol., 140(5).

65. Hotamisligil, G.S., 2006. Inflammation and metabolic disorders. *Nature*, 444.

66. Hotamisligil, G.S. et al., 1996. Uncoupling of obesity from insulin resistance through a targeted mutation in aP2, the adipocyte fatty acid binding protein. *Science*, 274(5296), pp.1377-79.

67. Hsue, P.Y. et al., 2009. Role of Viral Replication, Antiretroviral Therapy, and Immunodeficiency in HIV- Associated Atherosclerosis. *AIDS.*, 23(9), p.1059–1067..

68. Huang, S. & Czech, M.P., 2007. The GLUT4 Glucose Transporter. *Cell Metab*, 5(4), p.237–252.

69. Jeong, W.-i. et al., 2008. Paracrine Activation of Hepatic CB1 Receptors by Stellate Cell-Derived Endocannabinoids Mediates Alcoholic Fatty Liver. *Cell Metab*, 7(3), p.227–235.

70. John, M. et al., 2003. Randomized, controlled, 48-week study of switching stavudine and/or protease inhibitors to combivir/abacavir to prevent or reverse lipoatrophy in HIV-infected patients. *J Acquir Immune Defic Syndr*, 33(1), pp.29-33.

71. Joly, V., 2002. Increased risk of lipoatrophy under stavudine in HIV-1-infected patients: results of a substudy from a comparative trial. *AIDS*, 16, p.2447–2454.

72. Kaczocha, M., Glaser, S. & Deutsch, D., 2009. Identification of intracellular carriers for the endocannabinoid anandamide. *PNAS,* 106(15).

73. Kadowaki, T., Yamauchi, T., Kubota, N. & al, e., 2006. Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome. *J Clin Invest*, 116, p.1784–1792.

74. Kaufman, R.J., 2002. The unfolded protein response in nutrient sensing and differentiation. *Nat Rev - Mol Cell Biol*, 3, pp.411-21.

75. Kern, P.A. et al., 2001. Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance. *AJP - Endo*, 280(5), pp. E745-E751.

76. King, M.W., 1996-2013. *Endocannabinoids in Feeding Behavior and Energy Homeostasis*. [Online] themedicalbiochemistrypage.org, LLC Available at: http://themedicalbiochemistrypage.org/endocannabinoids.php#metabolism [Accessed 22 November 2013].

77. Kirchgessner, K.G. et al., 1997. Tumor necrosis factor-alpha contributes to obesity-related hyperleptinemia by regulating leptin release from adipocytes. *J. Clin. Invest.*, 100(11), p.2777–2782.

78. Kirkham, T., 2001. Synergistic effects of opioid and cannabinoid antagonists on food intake. *Psychopharmacology (Berlin)*, 153(2), p.267–270.

79. Klein, D., Hurley, L., Quesenberry, C. & Jr, S.S., 2002. Do protease inhibitors increase the risk for coronary heart disease in patients with HIV-1 infection? *J Acquir Immune Defic Syndr*, 30(5), pp.:471-477.

80. Koster, J.C. et al., 2003. HIV Protease Inhibitors Acutely Impair Glucose-Stimulated Insulin Release. *Diabetes*, 52(7), pp.1695-1700.

81. Kotler, D.P. et al., 2004. Effects of Growth Hormone on Abnormal Visceral Adipose Tissue Accumulation and Dyslipidemia in HIV-Infected Patients. *J Acquir Immune Defic Syndr*, 35(3), pp.239-52.

82. Koutkia, P., Canavan, B., Breu, J. & Grinspoon, S., 2005. Growth hormone (GH) responses to GH-releasing hormone-arginine testing in human immunodeficiency virus lipodystrophy. *J Clin Endocrinol Metab*, 90(1), pp.32-38.

83. Kubota, N. et al., 2007. Adiponectin Stimulates AMP-Activated Protein Kinase in the Hypothalamus and Increases Food Intake. *Cell Metab*, 6(1), p.55–68.

84. Lamers, S.L. et al., 2012. HIV-1 Nef in Macrophage-Mediated Disease Pathogenesis. *Int Rev Immunol*, 31(6), pp.432-50.

85. Laplante, M. et al., 2006. Mechanisms of the Depot Specificity of Peroxisome Proliferator–Activated Receptor γ Action on Adipose Tissue Metabolism.. *Diabetes*, 55(10), p.2771–2778.

86. Lehtonen, M. et al., 2011. Determination of endocannabinoids in nematodes and human brain tissue by liquid chromatography electrospray ionization tandem mass spectrometry. *Journal of Chromatography B*, 879, p.677–694.

87. Lemoine, M. et al., 2006. Altered hepatic expression of SREBP-1 and PPAR[gamma] is associated with liver injury in insulin-resistant lipodystrophic HIV-infected patients. *AIDS*, 20 (3), pp.387-95.

88. Lewis, W., 1995. Mitochondrial toxicity of antiviral drugs. *Nat Med*, 1, pp.417-22.

89. Liang, J.-S. et al., 2001. HIV protease inhibitors protect apolipoprotein B from degradation by the proteasome: A potential mechanism for protease inhibitor-induced hyperlipidemia. *Nat Med*, 7(12), pp.1201-327.

90. Lichtenstein, K.A., 2005. Redefining lipodystrophy syndrome:risks and impact on clinical decision making. *J Acquir Immune Defic Syndr*, 39(4), p.395–400.

91. Lichtenstein, K.A., 2005. Redefining lipodystrophy syndrome:risks and impact on clinical decision making. *J Acquir Immune Defic Syndr*, 39(4), pp.395-400.

92. Lihn, A.S., 2003. Increased expression of TNF-alpha, IL-6, and IL-8 in HALS: implications for reduced adiponectin expression and plasma levels. *Am J Physiol Endocrinol Metab*, 285(5), pp.E1072-E1080.

93. Lo, J. et al., 2008. Low-Dose Physiological Growth Hormone in Patients With HIV and Abdominal Fat Accumulation: A Randomized Controlled Trial. *JAMA*, 300(5), pp.509-19.

94. Lum, P. et al., 2007. Gene expression profiling of rat liver reveals a mechanistic basis for ritonavir-induced hyperlipidemia. *Genomics*, 90, pp.464-73.

95. Maeda, K. et al., 2005. Adipocyte/macrophage fatty acid binding proteins control integrated metabolic responses in obesity and diabetes. *Cell Metab*, 1(2), p.107–119.

96. Makowski, L. et al., 2001. Lack of macrophage fatty-acid-binding protein aP2 protects mice deficient in apolipoprotein E against atherosclerosis. *Nature Medicine*, 7.

97. Makowski, L. et al., 2005. The fatty acid-binding protein, aP2, coordinates macrophage cholesterol trafficking and inflammatory activity. Macrophage expression of aP2 impacts peroxisome proliferator-activated receptor gamma and IkappaB kinase activities. *Journal of biological chemistry*, 280(13), pp.12888-95.

98. Makowski, L. & Hotamisligil, G.S., 2004. Fatty Acid Binding Proteins—The Evolutionary Crossroads of Inflammatory and Metabolic Responses. *J. Nutr.*, 134(9), pp.2464S-8S.

99. Makowski, L. & Hotamisligil, G.S., 2005. The role of fatty acid binding proteins in metabolic syndrome and atherosclerosis. *Current opinion in lipidology*, 16(5), pp.543-48.

100. Mallewa, J.E. et al., 2008. HIV-associated lipodystrophy: a review of underlying mechanisms and therapeutic options. *J. Antimicrob. Chemother.*, 62(4), pp.648-60.

101. Mallon, P.W. et al., 2005. In Vivo, Nucleoside Reverse-Transcriptase Inhibitors Alter Expression of Both Mitochondrial and Lipid Metabolism Genes in the Absence of Depletion of Mitochondrial DNA. *J Infect Dis*, 191(10), pp.1686-1696.

102. Martínez, E. et al., 2003. Effects of metformin or gemfibrozil on the lipodystrophy of HIV-infected patients receiving protease inhibitors. *Antivir Ther.*, 8(5), pp.403-10.

103. Matias, I. et al., 2006. Regulation, Function, and Dysregulation of Endocannabinoids in Models of Adipose and β-Pancreatic Cells and in Obesity and Hyperglycemia. *J Clin Endocrinol Metab*, 91(8), pp.3171-80.

104. Matsuda, L.A. et al., 1990. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature*, 346(6284), p.561–564.

105. McGowan, M.W., Artiss, J.D., Strandbergh, D.R. & Zak, B., 1983. A peroxidase-coupled method for the colorimetric determination of serum triglycerides. *Clin Chem*, 29(3), pp.538-42.

106. Miller, J. et al., 2003. HIV lipodystrophy: prevalence, severity and correlates of risk in Australia. *HIV Med*, 4(3), p.293–301.

107. Ministry of Medical Services, R.o.K., 2011. *Guidelines for anti-retroviral therapy in Kenya*. 4th ed. National AIDS/STI Control Program (NASCOP).

108. Muccioli, G.G. et al., 2010. The endocannabinoid system links gut microbiota to adipogenesis. *Mol Syst Biol*, 6(392), pp.1-15.

109. Mueckler, M., 1995. Glucose Transport and Glucose Homeostasis: New Insights From Transgenic Mice. *Physiology*, 10(1), pp.22-29.

110. Murata, H., Hruz, P.W. & Mueckler, M., 2002. Indinavir inhibits the glucose transporter isoform Glut4 at physiologic concentrations. *AIDS*, 16(6), pp. 859-863.

111. Mutimura, E., Stewart, A. & Crowther, N.J., 2007. Assessment of quality of life in HAART-treated HIV-positive subjects with body fat redistribution in Rwanda. *AIDS Res Ther*, 4(19), pp.1-8.

112. Mynarcik, D.C., 2000. Association of Severe Insulin Resistance With Both Loss of Limb Fat and Elevated Serum Tumor Necrosis Factor Receptor Levels in HIV Lipodystrophy. *J Acquir Immune Defic Syndr*, 25, pp.312-21.

113. Nadarajah, J.T. et al., 2012. Infectious Complications of Bio-Alcamid Filler Used for HIV-Related Facial Lipoatrophy. *Clin Infect Dis*, 55(11), pp.1568-74.

114. Nguyen, A., 2008. Lipodystrophy and weight changes: data from the Swiss HIV C ohort Study, 2000-2006. *HIV Medicine*, 9, p.142 – 150.

115. Noor, M.A., Flint, O.P., Maa, J.-F. & Parker, R.A., 2006. Effects of atazanavir/ritonavir and lopinavir/ritonavir on glucose uptake and insulin sensitivity: demonstrable differences in vitro and clinically. *AIDS*, 20(14), pp.1813-16.

116. Noor, M.A. et al., 2002. Indinavir acutely inhibits insulin-stimulated glucose disposal in humans: A randomized, placebo-controlled study. *AIDS*, 15(5), pp.F1-F8.

117. NRC, 1996. *Guide for the Care and Use of Laboratory Animals*. 7th ed. Washington, DC: National Academy Press.

118. Nuesch, R. et al., 2005. Development of HIV with drug resistance after CD4 cell count-guided structured treatment interruptions in patients treated with highly active antiretroviral therapy after dual-nucleoside analogue treatment. *Clin Infect Dis.*, 40(5), p.728.

119. O'Sullivan, S., 2007. Cannabinoids Go Nuclear: Evidence for activation of peroxisome proliferator-activated receptors. *British Journal of Pharmacology*, 152(5).

120. Osei-Hyiaman, D. et al., 2005. Endocannabinoid activation at hepatic CB1 receptors stimulates fatty acid synthesis and contributes to diet-induced obesity. *J. Clin. Invest.*, 115(5), p.1298– 1305.

121. Osei-Hyiaman, D. et al., 2005. Endocannabinoid activation at hepatic CB1 receptors stimulates fatty acid synthesis and contributes to diet-induced obesity. *J Clin Invest*, 115(5), p.1298– 1305.

122. Osei-Hyiaman, D. et al., 2008. Hepatic CB1 receptor is required for development of diet-induced steatosis, dyslipidemia, and insulin and leptin resistance in mice. *J. Clin. Invest.*, 118(9), p.3160–3169.

123. Pagano, C. et al., 2007. The Endogenous Cannabinoid System Stimulates Glucose Uptake in Human Fat Cells via Phosphatidylinositol 3-Kinase and Calcium-Dependent Mechanisms. *J Clin Endocrinol Metab*, 92(12), pp.4810-19.

124. Pagotto, U. et al., 2006. The Emerging Role of the Endocannabinoid System in Endocrine Regulation and Energy Balance. *Endocr Rev*, 27(1), pp.73-100.

125. Pajonk, F. et al., 2002. The Human Immunodeficiency Virus (HIV)-1 Protease Inhibitor Saquinavir Inhibits Proteasome Function and Causes Apoptosis and Radiosensitization in Non-HIV-associated Human Cancer Cells. *Cancer Res*, 62, pp.5230-35.

126. Palandra, J. et al., 2009. Endogenous ethanolamide analysis in human plasma using HPLC tandem MS with electrospray ionization. *Journal of Chromatography B*, 877 , p.2052–2060.

127. Palella, F.J. et al., 1998. Declining Morbidity and Mortality among Patients with Advanced Human Immunodeficiency Virus Infection. *N. Engl. J. Med.*, 339, pp.405-06.

128. Panjok, F., 2002. The human immunodeficiency virus (HIV)-1 protease inhibitor saquinavir inhibits proteasome function and causes apoptosis and radiosensitization in non-HIV-associated human cancer cells. *Cancer Research*, 62, pp.5230-35.

129. Parker, R.A., 2005. Endoplasmic reticulum stress links dyslipidemia to inhibition of proteasome activity and glucose transport by HIV protease inhibitors. *Molecular Pharmacology*, 67, pp.1909-19.

130. Penzak, S.R., Chuck, S.K. & Stajich, G.V., 2000. Safety and Efficacy of HMG-CoA Reductase Inhibitors for Treatment of Hyperlipidemia in Patients with HIV Infection. *PHARMACOTHERAPY*, 20(9), pp.1066-71.

131. Perwitz, N., Fasshauer, M. & Klein, J., 2006. Cannabinoid receptor signalling directly inhibits thermogenesis and alters expression of adiponectin and visfatin. *Hom Metab Res*, 38, p.356–358.

132. Pi-Sunyer, F. et al., 2006. Effect of Rimonabant, a Cannabinoid-1 Receptor Blocker, on Weight and Cardiometabolic Risk Factors in Overweight or Obese PatientsRIO-North America: A Randomized Controlled Trial. *JAMA*, 95(7), pp.761-75.

133. Poirier, B. et al., 2005. The anti-obesity effect of rimonabant is associated with an improved serum lipid profile. *Diabetes Obes Metab*, 7(1), pp.65-72.

134. Power, R., Tate, H., McGill, S. & Taylor, C., 2003. A qualitative study of the psychosocial implications of lipodystrophy syndrome on HIV positive individuals. *Sex Transm Infect* , 79, pp.137-41.

135. Ravinet-Trillou, C. et al., 2003. Anti-obesity effect of SR141716, a CB1 receptor antagonist, in diet-induced obese mice. *AJP - Regu Physiol*, 284(2), pp.R345-53.

136. Ravinet-Trillou, C. et al., 2004. CB1 cannabinoid receptor knockout in mice leads to leanness, resistance to diet-induced obesity and enhanced leptin sensitivity. *Int J Obes*, 28, p.640–648.

137. Reagan-Shaw, S., Nihal, M. & Ahmad, N., 2007. Dose translation from animal to human studies revisited. *The Faseb Journal*, 22(3), pp.659-661.

138. Riddle, T. et al., 2001. HIV protease inhibitor induces fatty acid and sterol biosynthesis in liver and adipose tissues due to the accumulation of activated sterol regulatory element-binding proteins in the nucleus. *J. Biol. Chem.*, 276, p.37514–37519.

139. Roche, R. et al., 2006. Presence of the cannabinoid receptors, CB1 and CB2, in human omental and subcutaneous adipocytes. *Histochemistry and Cell Biology*, 126(2), pp.177-87.

140. Ruiz, L. et al., 2007. Antiretroviral therapy interruption guided by CD4 cell counts and plasma HIV-1 RNA levels in chronically HIV-1-infected patients. *AIDS*, 21(2), p.169.

141. Safrin, S. & Grunfeld, C., 1999. Fat distribution and metabolic changes in patients with HIV infection. *AIDS*, 13, p.2493–2505.

142. Saint-Marc, T., 1999. A syndrome of peripheral fat wasting (lipodystrophy) in patients receiving long-term nucleo side analogue therapy. *AIDS*, 13, p.1659 – 1667.

143. Salt, I.P., Gabrielle, J., Ashcroft, S.J.H. & Hardie, G., 1998. AMP-activated protein kinase is activated by low glucose in cell lines derived from pancreatic B cells, and may regulate insulin release. *Biochem. J*, 335, pp. 533-539.

144. Ségolène, D. et al., 2001. Failure to maintain long-term adherence to highly active antiretroviral therapy: the role of lipodystrophy. *AIDS*, 15(18), pp.2441-44.

145. Sharon, S. & Carl, G., 1999. Fat distribution and metabolic changes in patients with HIV infection. *AIDS*, 13(18), pp.2493-505.

146. Skuta, G., 1999. Molecular mechanism of the short-term cardiotoxicity caused by 2',3 '-dideoxycytidine (ddC): modulation of reactive oxygen species levels and ADP ribosylation reactions. *Biochemical Pharmacology*, 58, p.1915–1925.

147. Sugiura, T., 1995. 2-Arachidonoylglycerol: A possible endogenous cannabinoid receptor ligand in brain. *Biochem Biophys Res Commun*, 215(1), p.89–97.

148. Sugiura, T., 2002. Biosynthesis and degradation of anandamide and 2-arachidonoylglycerol and their possible physiological significance. *Prostaglandins Leukot Essent Fatty Acids*, 66(2-3), pp.173-92.

149. Tebas, P. et al., 2002. Effect of prolonged discontinuation of successful antiretroviral therapy on CD4+ T cell decline in human immunodeficiency virus-infected patients: implications for intermittent therapeutic strategies. *J Infect Dis*, 186(6), p.851.

150. Terra, X. et al., 2011. FABP 4 is associated with inflammatory markers and metabolic syndrome in morbidly obese women. *European Journal of Endocrinology*, (164), p.539–547.

151. Thierry, S.M. et al., 1999. A syndrome of peripheral fat wasting (lipodystrophy) in patients receiving long-term nucleoside analogue therapy. *AIDS*, 13(13), pp.1659-67.

152. Uysal, K.T. et al., 2000. Improved Glucose and Lipid Metabolism in Genetically Obese Mice Lacking aP2. *Endocrinology*, 141 (9), pp.3388-3396.

153. van Vonderen, M., 2009. Zidovudine/Lamivudine for HIV-1 Infection Contributes to Limb Fat Loss. *PLoS ONE*, 4(5), p.e5647.

154. Veerkamp, J.H. & van Moerkerk, H.T., 1993. Fatty acid-binding protein and its relation to fatty acid oxidation. *Molecular and Cellular Biochemistry*, 123.

155. Vickers, S.P. et al., 2003. Preferential effects of the cannabinoid CB1 receptor antagonist, SR 141716, on food intake and body weight gain of obese (fa/fa) compared to lean Zucker rats. *Psychopharmacology (Berl)*, 167(1), pp.103-11.

156. Vincenzo, D.M. & Matias, I., 2005. Endocannabinoid control of food intake and energy balance. *Nat Neurosci*, 8, pp.585-89.

157. Walli, R. et al., 1998. Treatment with protease inhibitors associated with peripheral insulin resistance and impaired oral glucose tolerance in HIV-1-infected patients. *J Acquir Immune Defic Syndr*, 12(15), p.F167–F173.

158. Watanabe, T. et al., 2009. Rimonabant Ameliorates Insulin Resistance via both Adiponectin-dependent and Adiponectin-independent Pathways. *J. Biol. Chem.*, 284, pp.1803-12.

159. Xu, A. et al., 2006. Adipocyte Fatty Acid–Binding Protein Is a Plasma Biomarker Closely Associated with Obesity and Metabolic Syndrome. *Clin Chem*, 52(3), pp.405-13.

160. Yamauchi, T. et al., 2001. The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nat Med*, 7, pp.941 - 946.

161. Yang, W.S. et al., 2002. Synthetic Peroxisome Proliferator-Activated Receptor-γ Agonist, Rosiglitazone, Increases Plasma Levels of Adiponectin in Type 2 Diabetic Patients. *Diabetes Care*, 25(2), pp.376-80.

162. Zanotti, G., 1999. Muscle fatty acid-binding protein. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, 1441(2-3).