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Date of submission: February 2010

**Minor Dissertation**

**INVESTIGATION OF HIGH MOLECULAR WEIGHT  
ADIPONECTIN IN HIV-INFECTED PATIENTS ON  
ANTIRETROVIRAL THERAPY**

Submitted to the

**UNIVERSITY OF CAPE TOWN**

In partial fulfilment of the requirements for the degree

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## **ACKNOWLEDGEMENTS**

The following are gratefully acknowledged:

My family for making it possible for me to pursue this degree; Prof Tahir Pillay and Dr Judy King for their guidance and instruction; Prof Naomi Levitt and Dr Joel Dave for providing patient samples and data; Sacha West for helping with sample collection; Felicity Leisegang and Wan Iryani Wan Ismail for their instruction on Western blotting; Jenny Kannemeyer for her instruction on ELISAs, Dr Andrew Boule for help with statistical analysis and the National Health Laboratory Service for funding this project.

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## LIST OF ABBREVIATIONS

AdipoR1	Adiponectin receptor 1
AdipoR2	Adiponectin receptor 2
ADP	Adenosine diphosphate
AIDS	Acquired immunodeficiency syndrome
AMP	Adenosine monophosphate
AMPK	AMP-dependent protein kinase
Apo	Apolipoprotein
ARV	Antiretroviral
ATP	Adenosine triphosphate
AZT	Zidovudine
BMI	Body mass index
CAD	Coronary artery disease
CD4	Cluster of differentiation 4
CD36	Cluster of differentiation 36
C/EBP	CCAAT-enhancer-binding protein
CRABP-1	Cytoplasmic retinoic-acid binding protein-1
CRP	C-reactive protein
CTR1	c1q-tumour necrosis factor related protein
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FOXO1	Forkhead box O1
FPLC	Fast protein liquid chromatography
GLUT4	Glucose transporter 4
HAART	Highly active antiretroviral therapy
LDL	High density lipoprotein
HIV	Human immunodeficiency virus
HMW	High molecular weight
HOMA-IR	Homeostasis model assessment–insulin resistance
HPLC	High performance liquid chromatography
ICAM	Intracellular adhesion molecule

IκB	Inhibitor of Nuclear Factor-κB
IL	Interleukin
LDL	Low density lipoprotein
LMW	Low molecular weight
LRP	Low density lipoprotein receptor-related protein
Lp(a)	Lipoprotein (a)
MMW	Medium molecular weight
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial deoxyribonucleic acid
NRTI	Nucleotide or nucleoside reverse transcriptase inhibitor
NNRTI	Non-nucleotide reverse transcriptase inhibitor
PGC1	Peroxisome proliferator activated receptor coactivator 1
PI	Protease inhibitor
PPAR	Peroxisome proliferator activated receptor
PVDF	Polyvinylidene fluoride
QUICKI	Qualitative insulin-sensitivity check index
RIA	Radioimmunoassay
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RXR	Retinoid X receptor
SDS	Sodium dodecyl sulphate
SIRT	Sirtuin
SREBP-1c	Steroid response element binding protein -1c
TNF	Tumour necrosis factor
VCAM	Vascular cellular adhesion molecule
WHR	Waist: hip ratio

## A. RESEARCH PROTOCOL FOR MMED DISSERTATION

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<sup>1</sup> Dr King has subsequently become co-supervisor for this project, while Professor N Levitt and Dr J Dave (Division of Endocrinology, UCT & GSH) have become collaborators for this project.

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### ***A.1. TITLE***

Role of high molecular weight (HMW) multimeric adiponectin in the development of HIV-lipodystrophy syndrome following anti-retroviral therapy

### ***A.2. SHORT DESCRIPTION OF THE PROJECT***

This project aims to investigate the role of multimeric (high molecular weight) adiponectin in the development of metabolic disease resulting from anti-retroviral therapy. Specifically, the aim is to quantify the circulating levels of both total and high molecular weight (HMW) adiponectin and to establish whether a link exists between HMW adiponectin levels and susceptibility to HIV-induced lipodystrophy. Although total adiponectin levels have been shown to be significantly reduced in patients with HIV-induced lipodystrophy, there is no information on whether HMW adiponectin, which appears to be the most biologically active form of adiponectin, is altered in HAART-induced lipodystrophy, and whether patients with low levels of the HMW form are more susceptible to lipodystrophy.

### ***A.3. AIMS AND OBJECTIVES***

#### **A.3.1. Broad aims**

The project aims to better understand the molecular basis of the HIV-lipodystrophy syndrome.



### **A.3.2. Objectives**

- Quantify total and HMW adiponectin levels in patients with HIV-lipodystrophy syndrome<sup>2</sup>;
- Determine whether susceptibility to HIV- lipodystrophy is related to low circulating levels of the HMW multimeric form.

If indeed HMW adiponectin levels are implicated, then in the future there may be a possibility that HMW adiponectin infusions could be used to treat HAART-induced lipodystrophy and the associated metabolic disturbances. Recombinant adiponectin is already available for research purposes.

### **A.4. BACKGROUND**

Infection with HIV and consequent AIDS is a major public health problem in South Africa and the rest of the world. The prognosis of patients has been improved dramatically by the use of combination therapy based on HIV protease inhibitors and reverse transcriptase inhibitors. The use of highly active antiretroviral therapy (HAART) to suppress viral replication and restore immunity has improved prognosis of patients to such a degree that long-term complications are becoming more prevalent. One major complication is the HIV-lipodystrophy syndrome, which appears to have a metabolic and biochemical component. Initially these were observed with protease inhibitors, but they have also been seen with both non-nucleoside analogues and one nucleoside analogue (stavudine). The components of the syndrome include lipodystrophy, raised triglycerides and cholesterol, and insulin resistance, which may be associated with hyperglycaemia [1, 2]. Lipodystrophy refers to either loss of subcutaneous fat (peripheral fat wasting, termed lipoatrophy), fat tissue deposition subcutaneously or in visceral stores (fat accumulation, termed lipohypertrophy), or a

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<sup>2</sup> Due to low roll-out of anti-retroviral therapy at our clinics and therefore the low prevalence of lipodystrophy, we later amended this objective to quantifying Multimeric adiponectin levels in patients on HAART. See Appendix for amendments approved by the UCT Research Ethics Committee.

combination of the two. However, there are as yet no standardised criteria for diagnosing lipodystrophy clinically and metabolically. Diagnosis in a previous study was based on concordance between the opinions of subject and clinician[3].

The consequences of this syndrome are that patients on therapy are at increased risk for developing cardiovascular diseases as a result of HAART, and changes in body fat, manifested by the loss of facial and limb adipose tissue and central obesity as a result of lipodystrophy, may prompt patients to reject treatment. The body fat changes occur in about 40% of patients on HAART[2]. Lipid levels are affected particularly by Ritonavir, which increases LDL cholesterol, triglycerides and Lp(a). With lipodystrophy comes an increased risk of insulin resistance and diabetes[2].

The mechanisms underlying the development of lipodystrophy are poorly understood. It has been suggested that these metabolic abnormalities arise from hormonal perturbations, and disturbances in the regulation and secretion of adipokines such as adiponectin have been implicated.

Adiponectin is a 244-amino acid peptide hormone secreted by adipocytes [4], and circulating levels of adiponectin correlate with the amount of adipose tissue [5, 6]. Adiponectin circulates in the blood in a wide range of multimer complexes, existing in 3 major oligomeric forms viz. a low molecular weight trimer, a mid-molecular weight hexamer and a high molecular weight (HMW) multimeric form of 12 to 18 peptide molecules. Adiponectin binds to two seven-transmembrane domain-type receptors (AdipoR1 and -R2) [7], AdipoR1 is found mainly in muscle, whereas AdipoR2 predominates in the liver.

Adiponectin has several different physiological actions, such as protective activities against atherosclerosis, improvement of insulin sensitivity, and prevention of hepatic fibrosis. It enhances fatty acid oxidation in muscle [8] and enhances insulin action in the liver [9]. In mice with insulin resistance associated with lipodystrophy or obesity, adiponectin improves insulin action and reduces lipid levels [10]. Conversely, insulin

resistance and glucose intolerance, and vascular disease develop in adiponectin knockout mice [11]. Furthermore, type 2 diabetics have low adiponectin levels, especially in association with coronary artery disease [12], while improved insulin sensitivity in these patients, when treated with thiazolidinediones, is associated with increased expression of adiponectin [13]. Thiazolidinediones have also been shown to increase adiponectin expression in obese mice [14].

Recent studies indicate that the HMW multimeric form of adiponectin, rather than total adiponectin, has a significant predictive role in disease status [15, 16]. The individual structures of adiponectin multimers in human plasma have been elucidated, which has led to the discovery of an albumin-binding trimer, in addition to the already documented trimeric, hexameric and HMW forms [17]. Part of the multimeric form is selectively digested by certain proteases and it is now possible to introduce a pre-treatment step to selectively digest the low and mid- molecular weight forms, allowing measurement of the HMW form.

It is also possible to separate all the individual multimeric species using other techniques such as velocity sedimentation in sucrose gradients, HPLC, FPLC and immunoblotting, but these are not suitable for high throughput measurement involving many samples [17]. We do, however, plan to validate the results obtained, using immunoblotting.

A recent study has shown that plasma adiponectin levels have relatively low biovariability and that adiponectin can be sampled fasted or non-fasted due to only minor diurnal and post-prandial changes[18].

Plasma adiponectin levels vary minimally over 12 – 15 months, suggesting that a single adiponectin measurement may be adequate for risk assessment[18, 19]. Adiponectin has also been shown to be stable for 33 months (or 3 freeze-thaw cycles) when stored at -30°C[19].

Total adiponectin levels have been shown to be decreased in patients with HIV-lipodystrophy[18, 19]. However, the ratio of HMW adiponectin to the total adiponectin

levels in circulation has been shown to be a more accurate reflection of disease status. The relative levels in HIV-associated lipodystrophy are not known. This project aims to address this.

#### **A.5. RESEARCH PLAN**

This project will run over 12 to 18 months. The first 6 to 8 months will be dedicated to obtaining subject and control groups from the ARV clinics at Groote Schuur and Jooste Hospitals. Blood samples will be drawn from these patients and stored. Over the next 2 to 3 months, adiponectin analyses will be done by ELISA and Western blot<sup>3</sup>.

All equipment required is available in the C17 Core Laboratory, as well as in the Division of Chemical Pathology, UCT.

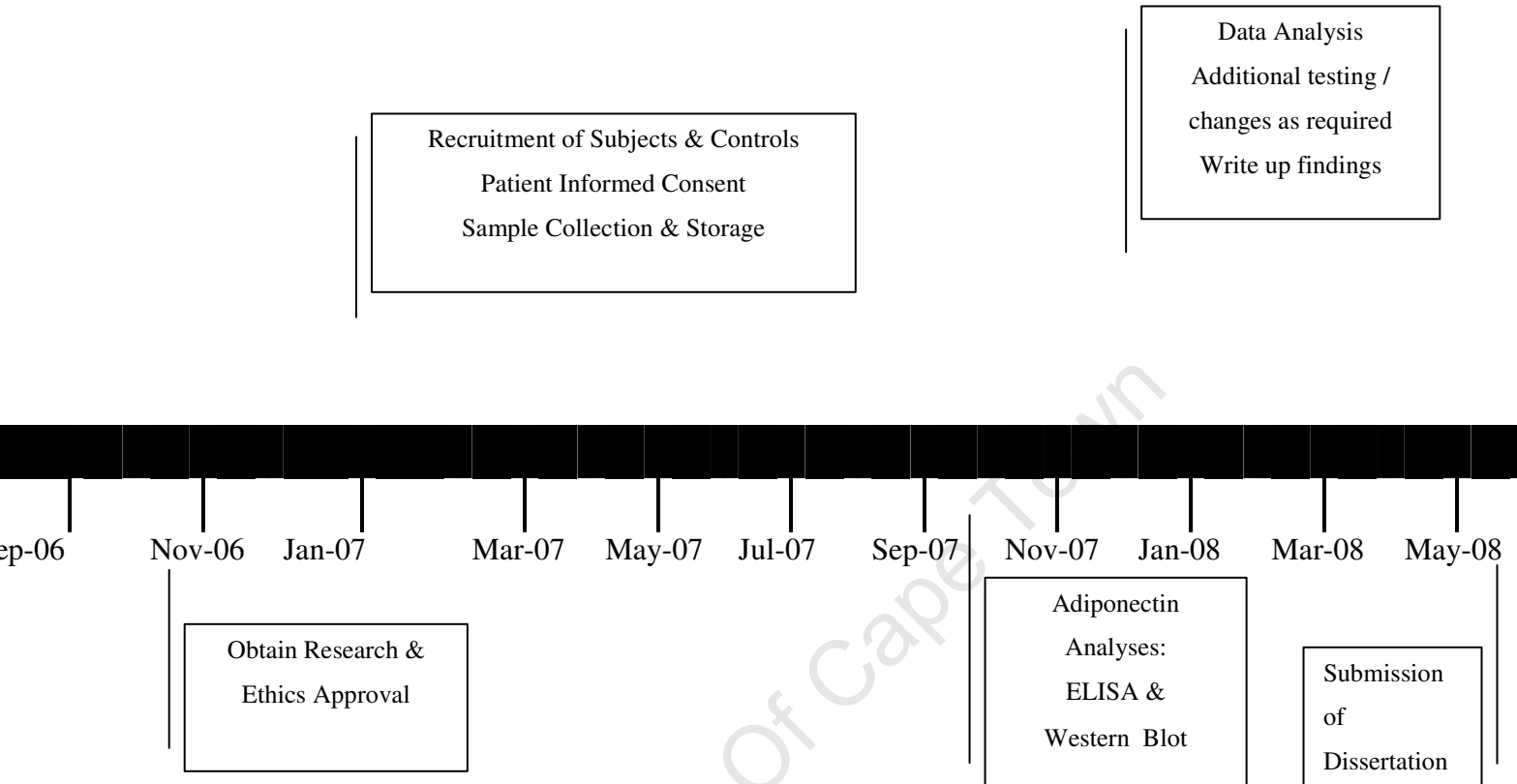
Once all data have been collected, data analysis will be done, conclusions drawn and any additional requirements attended to. This process should be completed within 3 to 6 months.

Dr Fierdoz Omar will be responsible for subject and control sample collection, as well as adiponectin analyses and data analysis<sup>4</sup>. This will be done in collaboration with Dr Judy King and under the supervision of Prof. Tahir Pillay.

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<sup>3</sup> Western blot analysis was subsequently not performed following work published by Liu et al. in Clinical Chemistry (Liu et al. Comparison of immunoassays for the selective measurement of human high-molecular weight adiponectin. Clin Chem, 2009. 55(3):568-72.), validating the multimeric adiponectin concentrations obtained by ELISA with Western blot analysis.

<sup>4</sup> Sample collection was performed by Dr Joel Dave – see footnote 7.



#### **A.6. DETAILED METHODOLOGY**

The project will be reviewed by the local ethics committee and approval obtained. Written informed consent (see attached consent form) will be obtained from test subjects and controls, and baseline blood samples obtained. Subjects will be given a questionnaire to assess the presence of lipodystrophy and this will be confirmed by clinical examination (concordance between the subject's opinion and that of the clinician, that at least moderate changes have occurred, will be considered as diagnostic of lipodystrophy).

Additional data will be obtained from the patients' routine management information and will include the following: demographic data, specific HAART therapy prescribed and biochemical tests, which may include lipid studies, glucose and insulin levels.

### **A.6.1. Study population**

The study population will consist of South African blacks and will be divided into 4 groups<sup>5</sup>:

One test group consisting of:

50 known HIV positive adult patients on antiretroviral treatment, with lipodystrophy

And four control groups:

- HIV positive patients on antiretroviral therapy but without lipodystrophy
- HIV positive patients with lipodystrophy but not on antiretroviral therapy
- HIV positive patients without lipodystrophy and not on antiretroviral therapy
- Non-diabetic healthy controls

The subjects and controls will be matched for age and gender. Persons with known diabetes mellitus or obesity (BMI>30) will be excluded<sup>6</sup>.

A total of 30 samples from each control group will be assayed. HIV patients will be sourced from HIV anti-retroviral roll-out clinics at Groote Schuur and Jooste Hospitals in the Western Cape<sup>7</sup>.

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<sup>5</sup> As mentioned earlier, patients with lipodystrophy were few in number and therefore the study population was changed to the following: 30 HIV-infected patients in each of the following groups: (a) treatment-naïve; (b) patients receiving HAART without protease inhibitors; (c) patients receiving HAART including a protease inhibitor; as well as 30 HIV-negative subjects. We were unable to obtain age- and BMI-matched HIV-negative subjects, and therefore the first three groups only were used in this study.

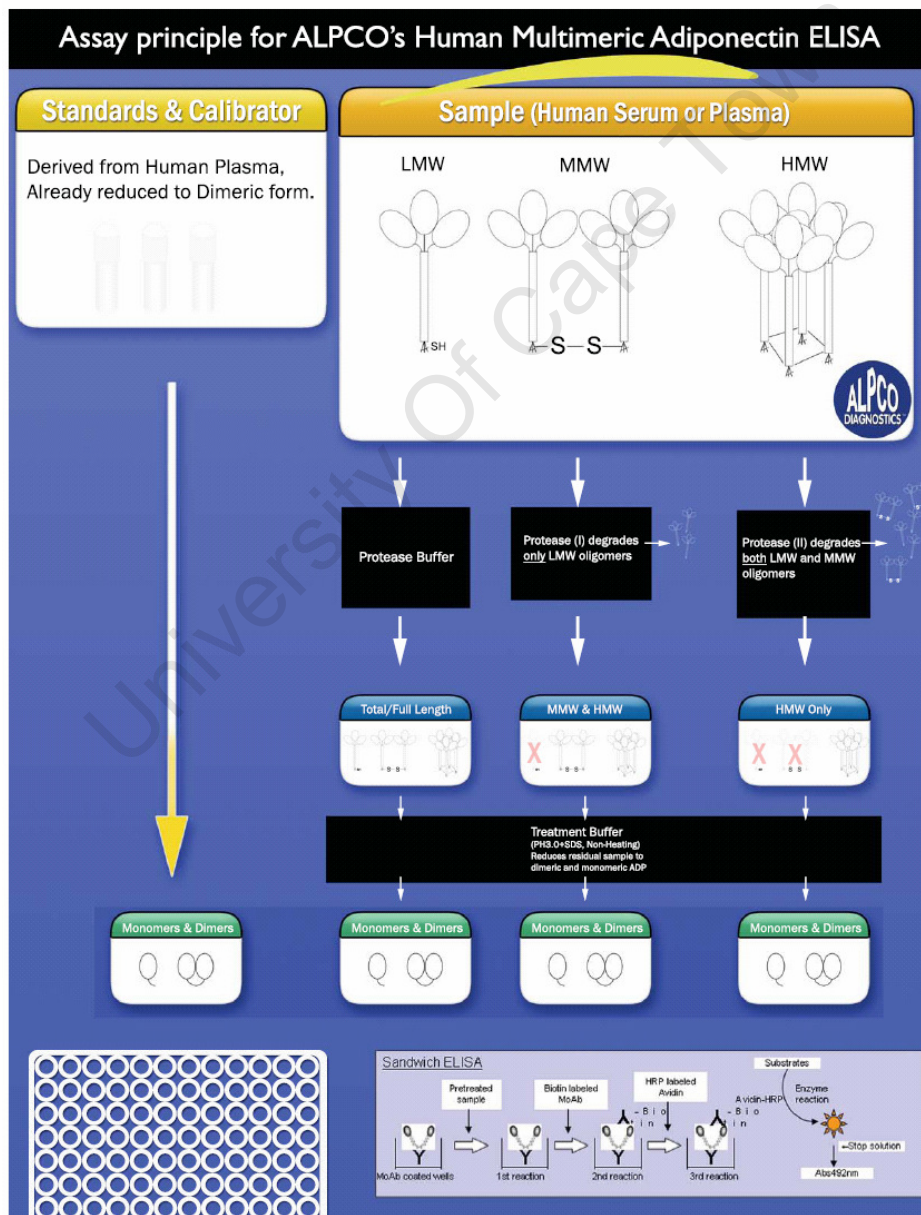
<sup>6</sup> Obese patients were subsequently included in the study.

<sup>7</sup> Subject and control sample collection, patient consent, clinical data and biochemical data (excluding adiponectin levels) were obtained by Dr Joel Dave (Division of Endocrinology, UCT & GSH) for a larger study involving a similar patient group. See Part D, D.3 & D.4 for patient consent form and information sheet.

## A.6.2. Laboratory assays

### A.6.2.1. Adiponectin assay

The protocol to be followed is as follows. A blood sample will be collected in an EDTA tube. The plasma will first undergo pre-treatment with proteases and SDS-buffer. The pre-treated specimen will then be assayed by enzyme linked immunosorbant assay (ELISA), using the Adiponectin (Multimeric) EIA kit marketed by ALPCO Diagnostics (Salem, New Hampshire, USA). The principle of the assay is illustrated in the figure.



This method allows for the quantitative and selective determination of HMW, MMW (mid-molecular weight), LMW (low molecular weight) and total adiponectin levels.

This new ELISA assay for multimeric forms of adiponectin will first be validated by correlation with the adiponectin RIA assay which is currently being used for quantification of total adiponectin.

#### ***A.6.2.2. Immunoblotting of adiponectin species<sup>8</sup>***

Approximately 30ul of serum will be used for immunoblotting under both native and denaturing conditions. Native gels will allow detection of the multimeric species prior to denaturation. The sample will be treated with electrophoresis sample buffer and then 15 ul will be loaded onto a 7.5% polyacrylamide gel (Biorad Miniprotean) and then subjected to electrophoresis at 150V for 1 hour. Following this, the gels will then be transferred to PVDF membranes at 250mA for 2 hours. The membranes will then be blocked with 5% Marvel and then probed with goat anti-adiponectin goat antibody (Santa Cruz Biotechnology). Bound antibody will be visualised using a second (rabbit) anti-goat antibody coupled to horse radish peroxidase, using chemiluminescence (SuperSignal, Pierce Chemical Company). The amount of chemiluminescence will then be quantified using an imager or by densitometry of X-ray films. Both facilities are available in the Division of Chemical Pathology at the University of Cape Town.

### ***A.7. ENVISAGED OUTPUTS/OUTCOMES***

It is envisaged that this study will provide more insight into the molecular basis for the development of lypodystrophy in HIV patients on antiretroviral treatment, and that this knowledge may lead to therapeutic improvement. The results from this study will be presented at the SA Society for Clinical Biochemistry meeting, the SEMSDA conference, the Association for Clinical Biochemistry conference (UK), and the American

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<sup>8</sup> As for footnote 3.



Association of Clinical Chemistry Annual conference<sup>9</sup>. It is hoped that the results will be published in a major international journal such as Clinical Chemistry or Journal of Clinical Endocrinology and Metabolism.

#### **A.8. IMPACT**

##### **Capacity development:**

Training of research workers:

Dr Omar will be the project leader. She is currently training as a Registrar in Chemical Pathology and is registered for the MMed Degree at UCT. The project will provide training in immunoassay techniques, including radioimmunoassay, ELISA and immunoblotting; and will be the basis for the dissertation towards the MMed degree.

The project will form an adjunct for a collaboration with the University of Gotheborg in Sweden and will therefore be important for enhancing institutional capacity.

The long-term complications of HIV/AIDS, and the impact thereof in the South African context requires research investment. This area has been poorly researched and has only emerged recently as larger numbers of patients are living longer as a result of HAART.

#### **A.9. INSTITUTIONAL APPROVAL**

This proposal has been submitted for ethics approval to the University of Cape Town Research Ethics Committee.

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<sup>9</sup> The findings of this research project were presented at the UCT Biomedical Research day, as well at a local and international conference. See Part D, D.5.

**A.10. BUDGET**

**Operating budget:**

<b>Consumable</b>	<b>Number of Units</b>	<b>Unit Price</b>	<b>Total Price</b>
Adiponectin ELISA kits from ALPCO	4	R10 000	R40 000
Adiponectin RIA kit from Linco	1	R7 000	R7 000
Adiponectin antibodies (Santa Cruz Biotechnology)	1	R20 000	R20 000
Reagents for gel electrophoresis and immunoblotting	1	R14 000	R14 000
		<b>TOTAL</b>	R81 000

**A.11. FUNDING**

Funding will be provided by the NHLS Research Trust Fund, subject to ethics approval.

Word count: 2204

**A.12. REFERENCES**

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## **B. LITERATURE REVIEW**

### ***B.1. OBJECTIVES***

The objectives of this literature review were to obtain the following background information:

- The characteristics associated with the HAART-induced HIV-associated lipodystrophy syndrome
- The mechanisms proposed for this lipodystrophy syndrome
- Adiponectin: regulation, mechanism of action, link to HAART-induced HIV-associated lipodystrophy

### ***B.2. SEARCH STRATEGY AND QUALITY CRITERIA***

The literature search was performed using the Pubmed Central digital archive. The following keywords were used for the Pubmed search: HIV, lipodystrophy, HAART, adiponectin. Further relevant papers were identified from reference lists. One hundred and four articles were identified and used in this review. Articles were numerically referenced, according to their appearance in the review.

### ***B.3. SUMMARY OF LITERATURE***

#### **B.3.1. Introduction**

The HIV epidemic is a major problem worldwide and more especially in sub-Saharan Africa. In South Africa, it was estimated that in 2008, 5.2 million (10.6%) South Africans were infected with HIV[1].

Currently, HIV therapy consists of a combination of highly active anti-retroviral therapy (HAART) drugs. These drugs can be divided into five classes, based on their mechanism of action, viz. nucleoside or nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleotide reverse transcriptase inhibitors (NNRTIs), protease inhibitors, integrase

inhibitors and entry/fusion inhibitors (Figure 1). Anti-retroviral drugs operate via interruption of the viral replication cycle. Reverse transcriptase inhibitors prevent DNA formation from RNA and protease inhibitors inhibit the protease required for cleavage of nascent HIV proteins, preventing maturation and replication of the HIV virus. Integrase inhibitors and entry inhibitors prevent integration of viral genome into the human genome and entry of HIV into host cell, respectively.

The South African National Department of Health has made available in the public sector, two HAART regimens. The first regimen consists of stavudine, lamivudine and either efavirenz or nevirapine i.e. a combination of two NRTIs and one NNRTI; while the second regimen consists of zidovudine (AZT), didanosine and lopinavir/ritonavir. AZT and didanosine are nucleotide- and nucleoside reverse transcriptase inhibitors, respectively, while liponavir/ritonavir is a protease inhibitor.

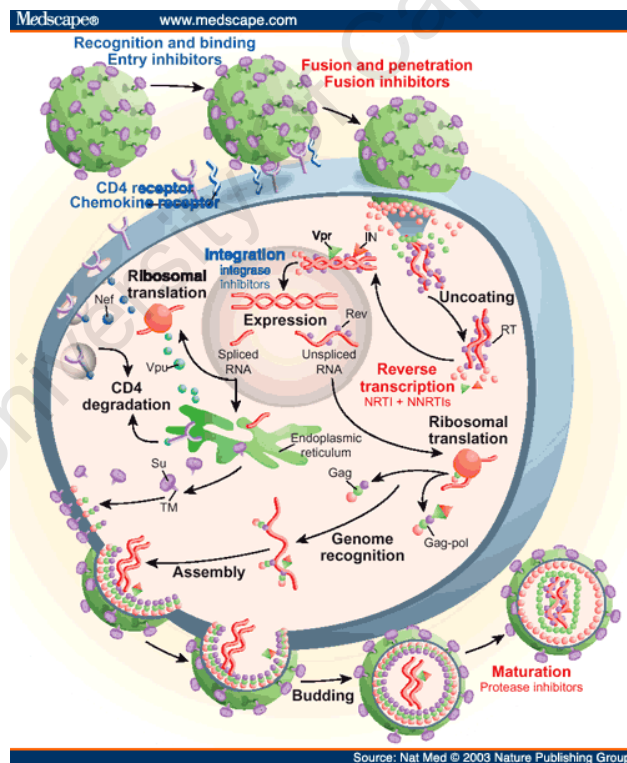


Figure 1: Replication of HIV and inhibition by antiretroviral drugs. Pomerantz et al. Twenty years of therapy for HIV-1 infection. Nat Med 2003; 9(7):867-873.

### **B.3.2. HIV-associated lipodystrophy**

The advent of HAART has dramatically increased the life expectancy in HIV-infected patients[2, 3]. However, this increased life span has come at a metabolic price. A HAART-associated syndrome, resembling rare congenital and acquired forms of lipodystrophy syndromes, was first described in the late 1990s[4] and is well documented[5-9]. This HIV-associated lipodystrophy syndrome comprises abnormal fat redistribution in the form of lipoatrophy (fat loss in the face, buttocks and extremities) and/or lipohypertrophy (excessive fat deposition in the abdominal (visceral fat accumulation), dorsocervical (buffalo hump) and breast regions and/or development of lipomata), as well as dyslipidaemia (hypertriglyceridaemia in particular, but also hypercholesterolaemia), insulin resistance (even type 2 diabetes mellitus) and lactic acidaemia[4, 7]. These patients therefore have increased visceral adipose tissue compared with subcutaneous adipose tissue, with insulin resistance related to the visceral and upper trunk adipose tissue[10]. The prevalence of lipodystrophy in HIV-infected individuals on HAART has been reported to be as high as 40% in developed countries[6, 8], with similar prevalences being reported in African studies - 30%[11], 34%[12] and 42.9%[13] in Tanzanian, Beninian, Rwandan and South African studies, respectively. In such patients, the prevalence of insulin resistance may be as high as 35%[14] compared with 12% in HIV-infected patients naïve of therapy[15]. A causative link may therefore exist between protease inhibitor therapy and the development of diabetes mellitus. In fact, there have been reports of patients presenting with diabetes mellitus after 2 – 52 weeks of protease inhibitor therapy, with normalisation of glycaemia after cessation of treatment[16, 17].

#### ***B.3.2.1. Risk factors for the development of lipodystrophy***

The lipodystrophy syndrome is predominantly associated with protease inhibitor therapy; however, treatment with thymidine analogues (stavudine and zidovudine) has also been implicated [18, 19]. Prolonged duration and combination-therapy with these drugs are associated with an increased risk for lipodystrophy, with clinical data suggesting that the protease inhibitor and NRTI groups of drugs act synergistically to cause the metabolic

disturbances associated with the lipodystrophy syndrome[4]. Other risk factors include female gender, age and stage of the HIV disease[9].

### ***B.3.2.2. Underlying mechanisms causing lipodystrophy***

The mechanisms underlying the development of the constellation of metabolic abnormalities associated with HAART are poorly understood. Several factors are at play, causing mitochondrial dysfunction, impaired lipid metabolism and impaired functioning of adipocytes and endothelial cells.

#### **Role of protease inhibitors in the pathogenesis of lipodystrophy**

Protease inhibitors exert their anti-HIV action by inhibiting an HIV protease necessary for its maturation. The catalytic region of this enzyme is homologous to that found in two human proteins, viz. cytoplasmic retinoic-acid binding protein-1 (CRABP-1) and low density lipoprotein receptor-related protein (LRP)[4, 20]. Protease inhibitors also inhibit cytochrome P450 3A. CRABP-1 and cytochrome P450 3A are necessary in the conversion of retinoic acid to cis-9-retinoic acid, which in turn binds retinoid X receptor (RXR). Protease inhibitors are thought to inhibit cis-9-retinoic acid formation and thereby, peroxisome proliferator activated receptor (PPAR)  $\gamma$  heterodimer formation with RXR. It is also thought to inhibit LRP, which normally, via the LRP-lipoprotein lipase complex, effects hepatic and endothelial clearance of triglycerides[20]. In addition, protease inhibitors inhibit steroid response element binding protein -1c (SREBP-1c), a key transcription factor and up-regulator of PPAR $\gamma$ [21]. The net effect is increased adipocyte apoptosis and reduced adipocyte differentiation, which in turn leads to decreased triglyceride storage, hyperlipidaemia and insulin resistance[4, 5, 20].

A genetic predisposition may play a role: An SREBP1c single nucleotide polymorphism has been shown to be predictive of HAART-associated hyperlipidaemia[22], with homozygosity for this polymorphism inversely associated with hyperinsulinism, hypercholesterolaemia and hyperglycaemia. Impaired adipocyte differentiation and adipocyte apoptosis caused by impairment of this gene product may be responsible for these metabolic abnormalities. Furthermore, the protease inhibitor-induced dyslipidaemia



(hypertriglyceridaemia and low level of high density lipoprotein (HDL)) may be linked to polymorphisms in the Apo CIII (inhibitor of lipoprotein lipase) gene[23].

Protease inhibitors have also been shown to decrease CD36, a protein involved in the uptake of modified lipoprotein by macrophages and the transport of fatty acids in tissues with high metabolic capacity (responsible for up to 80% of uptake in the myocardium, skeletal muscle and adipocytes)[24]. Patients deficient in CD36 present with hyperlipidaemia and insulin resistance while in CD36 knock-out rodents, CD36 administration improves the dyslipidaemia and insulin resistance.

Insulin resistance may be further explained by another mechanism viz. altered expression of adipocytokines in adipocytes, with increased inflammatory cytokines such as tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) and interleukin-6 (IL-6) (seen with specific protease inhibitors and with indinavir (a protease inhibitor), stavudine or zidovudine (both NRTIs)), and decreased adiponectin expression[21, 25]. Both TNF $\alpha$  and IL-6 have been causally linked to insulin resistance[26].

The role of protease inhibitors in the development of lipohypertrophy is not clearly understood. While the fat distribution is similar to that seen in patients with glucocorticoids excess, the hypothalamic-pituitary-adrenal axis in patients treated with protease inhibitors has not been shown to be disturbed[27]. Protease inhibitor-induced hyperinsulinism (by inhibition of insulin-degrading enzymes such as cathepsins) has been proposed as the initial trigger, leading to excess visceral adiposity, in turn leading to insulin resistance and hyperlipidaemia[27]. However, this hypothesis has yet to be proven experimentally.

### **Role of NRTIs/NNRTIs in the pathogenesis of lipodystrophy**

Certain NRTIs are associated with the development of lipoatrophy, with the duration of treatment on these specific drugs adding to the risk for development of this condition. The thymidine analogues AZT and stavudine have been especially implicated, with stavudine introducing a two-fold greater risk than AZT for developing lipoatrophy, while the non-thymidine NRTIs and NNRTIs are minimally (if at all) associated with lipoatrophy[28].

This lipoatrophy is thought to occur as a result of mitochondrial toxicity caused by the NRTI inhibition of mitochondrial polymerase  $\gamma$  (one of several enzymes and proteins essential in the replication of mitochondrial DNA (mtDNA)), leading to mitochondrial depletion (via decreased mtDNA content)[29]. mtDNA depletion has been demonstrated in subcutaneous adipose tissue in HIV-infected patients with lipodystrophy[30, 31]. This has been documented in particular with stavudine, correlating with the duration of exposure.

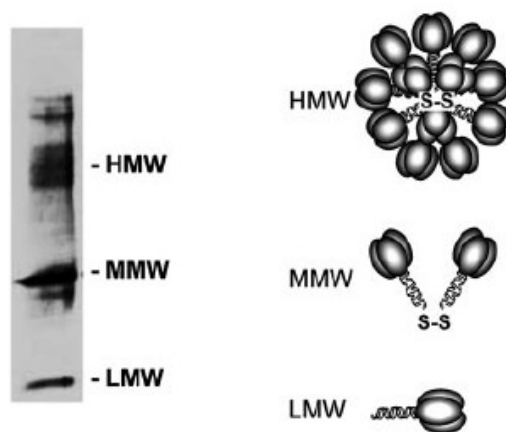
Non-polymerase  $\gamma$ -related mechanisms have also been described, with AZT treatment, causing impairment of adenylate kinase and ADP/ATP translocase, as well as physically interfering with the mitochondrial membrane[32]. AZT inhibits the pre-adipocyte proliferation rate and clonal expansion, leading to disturbed adipocyte homeostasis and impaired adipogenesis[33]. AZT has also been linked to the development of features reminiscent of mitochondrial diseases which include ragged red fibres, decreased mtDNA and impaired mtDNA replication[33]. Evidence suggests that mitochondrial dysfunction precedes the onset of type 2 diabetes mellitus in insulin-resistant patients. These drugs may, therefore, cause insulin resistance directly in skeletal muscle through mitochondrial dysfunction and indirectly through adipocyte loss [34, 35]. In these patients, insulin resistance correlates with lactate levels and duration of NRTI therapy[35]. In contrast to protease inhibitors, NRTIs (specifically AZT and stavudine) have not been shown to increase IL-6 and TNF $\alpha$  expression[25].

Nucleoside analogue reverse transcriptase inhibitors (stavudine more than zidovudine) are therefore associated with lipoatrophy while protease inhibitors are associated with metabolic complications such as dyslipidaemia and insulin resistance, having not been linked convincingly to the development of lipoatrophy[36].

### **B.3.3. Adiponectin**

As a consequence of the above perturbations in the adipocyte, patients with HIV-associated lipodystrophy have low levels of the adipokine, adiponectin. This 30kDa peptide hormone is secreted exclusively by adipose tissue and is found in high

concentrations in the circulation (0.5 – 30µg/ml)[37]. Adiponectin is structurally related to complement factor C1q. It also shares structural similarity with several other proteins, including TNFα and C1q-TNF-related protein (CTRP)[38]. The adiponectin monomer comprises three structural domains, viz. the N-terminal variable domain, the collagenous domain and a globular C-terminal domain [38]. Adiponectin may exist in its globular (proteolytically cleaved) form or as the full-length form, although only the full-length form is present in the circulation[39]. Three predominant adiponectin forms occur in the circulation: low molecular weight (LMW) trimer, medium molecular weight (MMW) hexamer and high molecular weight (HMW) 12-18mer (Figure 2)[38]. Of these, the HMW form (greater than 669kDa) is considered the bioactive form[40]. The globular form has also been shown to possess biological activity, but it is found in relatively low concentrations[37]. Trimerisation of adiponectin monomers takes place at the globular domain (by hydrophobic interactions), but interaction (disulfide bonds) between the collagenous domains is required for larger homomultimers to be formed (when multiples of trimers assemble)[38, 40]. In addition to the hydrophobic and disulfide bonds required for multimerisation, post translational modifications are critical for the formation and stabilisation of the HMW form. This process involves hydroxylation, followed by glycosylation of amino acid residues (lysine and proline) in the collagenous domain[40]. Impairment of this process has been demonstrated, both *in vitro* and *in vivo*, to lead to impaired intracellular assembly of HMW multimers and their secretion into the circulation[40]. This impairment translates into decreased adiponectin action, with low levels of glycosylated adiponectin demonstrated in type 2 diabetic patients compared with age and sex-matched controls[40]. Notably, these patients had decreased HMW to total adiponectin ratios.



**Figure 2: Multimeric forms of adiponectin; LMW, MMW and HMW are the low, medium and high molecular weight forms of adiponectin, respectively. Kadowaki et al. Adiponectin and adiponectin receptors. Endo Rev 2005; 26(3):439–451.**

### ***B.3.3.1. Regulation of adiponectin synthesis and secretion***

Adiponectin is secreted predominantly by mature adipocytes, its production being increased 100-fold during adipocyte differentiation[41]. However, unlike other adipokines such as TNF $\alpha$ , adiponectin levels are lower in obese patients[42], with visceral fat mass correlating more with adiponectin levels than subcutaneous fat[37]. Moreover, secretion of adiponectin is lower in large than in small adipocytes[37].

The adipocyte is also the major determinant of the relative proportions of each of the adiponectin complexes in the circulation. With reduced adiponectin production in adipocytes, assembly and secretion of the HMW multimer is reduced, with consequent low levels in the circulation[43].

Adiponectin production is upregulated during adipogenesis, being enhanced by adipogenic transcription factors such as PPAR $\gamma$ , CCAAT-enhancer-binding protein (C/EBP) $\alpha$ , C/EBP $\beta$ , Forkhead box O1 (FOXO1) and sirtuin (SIRT)1 (Figure 3)[37]. PPAR $\gamma$ , a nuclear receptor also involved in lipid and carbohydrate metabolism, binds a PPAR response element in the promoter of the adiponectin gene, causing upregulation of its secretion. Thus, PPAR $\gamma$  agonists, thiazolidinediones, upregulate adiponectin secretion, while PPAR $\gamma$ -knock-out mice have reduced adiponectin levels[44].

Additionally, adiponectin synthesis and secretion is inhibited by glucocorticoids, growth hormone, the pro-inflammatory factors TNF $\alpha$ , IL-6, C-reactive protein (CRP), activation of the sympathetic nervous system and hypoxia, and increased by inhibitors of the renin-

angiotensin system (angiotensin converting enzyme and angiotensin receptor antagonists) [37, 45, 46]. The effect of insulin on adiponectin secretion is poorly understood, with *in vitro* studies suggesting a positive relationship between adiponectin and insulin (thought to be via inhibition of the inhibitory effect of FOXO1 on PPAR $\gamma$  activation), while *in vivo* studies have shown a negative relationship between adiponectin and insulin[46]. Testosterone inhibits adiponectin secretion post translationally, and females therefore have higher levels of all fractions of adiponectin, in particular, the HMW form[40, 42]. Furthermore, HMW adiponectin levels increase with age[47]. Certain lifestyle factors may also influence adiponectin levels, including diet – soy protein, fish oils and linoleic acid increase adiponectin levels, while high carbohydrate diets are associated with lower adiponectin levels[48].

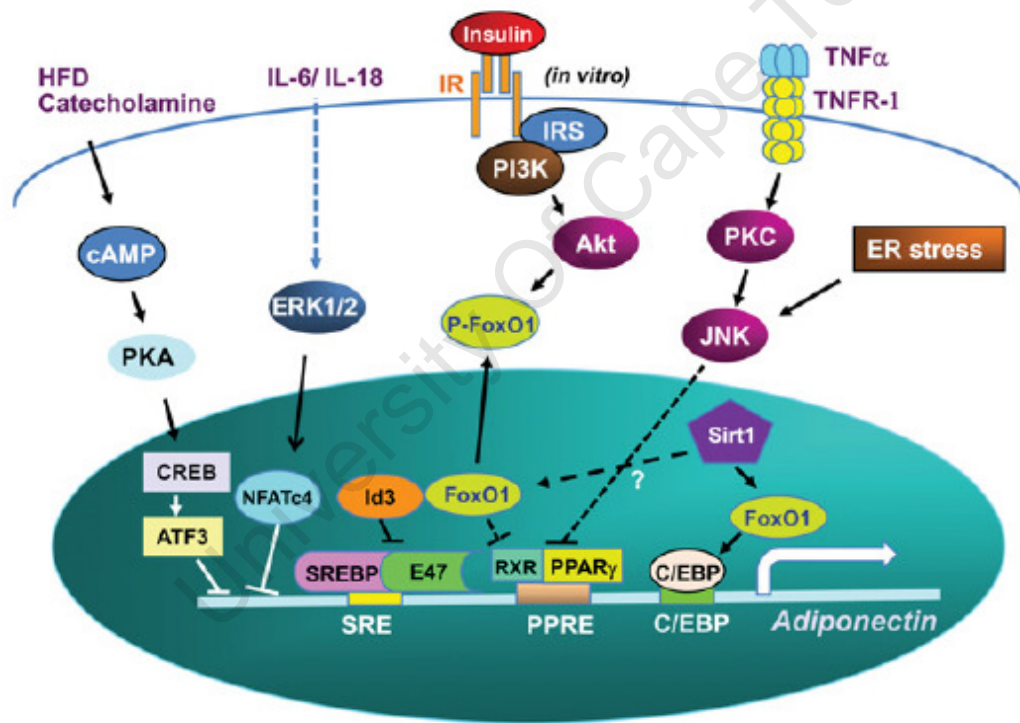


Figure 3: Diagram showing regulation of adiponectin at the gene level. Many factors stimulate or suppress adiponectin transcription, as is shown here. Liu et al. Transcriptional and post-translational regulation of adiponectin. *Biochem J* 2010; 425: 41–52.

### ***B.3.3.2. Metabolic and anti-inflammatory effects of adiponectin***

#### **Adiponectin receptors**

Adiponectin initiates its actions by binding to one of two adiponectin receptors, AdipoR1 and AdipoR2, integral membrane proteins that inversely resemble G-protein coupled receptors[49]. AdipoR1 is expressed ubiquitously (and is abundant in skeletal muscle), while AdipoR2 is predominantly expressed in the liver. While AdipoR1 has a high and low affinity for the globular and full-length adiponectin forms, respectively, AdipoR2 has intermediate affinity for both globular and full-length HMW adiponectin[50]. Moreover, AdipoR1 initiates the AMP kinase pathway (described later) and AdipoR2 the PPAR $\alpha$  pathway (also described later)[51]. These receptors are reduced in type 2 diabetic patients, and correlate with insulin sensitivity in non-diabetic individuals[48]. Their expression is increased during fasting, and decreased during refeeding, correlating with insulin levels. In fact, insulin administration to hepatocytes *in vitro* suppresses expression of adiponectin receptors (via the phosphatidylinositol 3-kinase branch of the insulin signalling pathway)[52]. A third receptor for adiponectin (specifically the HMW form), T-cadherin, is expressed in injured vascular endothelial and smooth muscle cells at sites of atherosclerosis, and may be linked to the anti-atherosclerotic actions of adiponectin[50]. The signalling pathways involved have yet to be elucidated.

#### **Insulin sensitising actions of adiponectin via AMP kinase**

Adiponectin is an insulin-sensitising hormone. In rodent models, both recombinant adiponectin administration and adiponectin over-expression lead to improved insulin sensitivity, lowering glucose levels[53, 54].

This insulin-sensitising action may occur via phosphorylation and activation of AMP-dependent protein kinase (AMPK). In skeletal muscle, apart from phosphorylating acetyl coenzyme A carboxylase and leading to fatty acid catabolism ( $\beta$  oxidation), this also increases glucose utilisation and glucose transporter 4 (GLUT4) translocation to the cell membrane, thereby increasing glucose uptake. In the liver, AMPK activation leads to a reduction in the stimulation of the gluconeogenic enzymes phosphoenolpyruvate carboxykinase and glucose-6-phosphatase, thus suppressing gluconeogenesis[39, 55].

**Insulin sensitising actions via activation of PPAR $\alpha$** 

Adiponectin was shown to activate PPAR $\alpha$ , leading to upregulation of PPAR $\alpha$  target genes (CD36, acyl-coenzyme A oxidase and uncoupling protein 2)[56]. CD36 and acyl-coenzyme A oxidase are involved in fatty acid transport and catabolism, while uncoupling protein 2 causes energy dissipation. Activation of PPAR $\alpha$  therefore leads to increased fatty-acid breakdown and energy consumption, leading to decreased triglyceride content in the liver and skeletal muscle[56]. As elevated tissue triglyceride content is known to cause insulin resistance by interfering with the insulin signalling cascade, this decrease leads to improved insulin sensitivity.

The insulin-sensitising action of adiponectin may also be due to its enhancement of the buffering capacity of subcutaneous adipose tissue, leading to decreased ectopic fat deposition and therefore decreased lipotoxicity. This has been shown in leptin-deficient mice, with adiponectin administration causing subcutaneous adipose tissue expansion while adiponectin deletion is associated with increased hepatic fat deposition [57, 58].

**Anti-atherosclerotic action of adiponectin**

Adiponectin exerts its cardio-protective action through its anti-atherosclerotic and anti-inflammatory actions. It has been shown to inhibit adhesion molecule expression, including E-selectin, vascular cellular adhesion molecule (VCAM)-1 and intracellular adhesion molecule (ICAM)-1. Adiponectin also inhibits inhibitor of nuclear factor- $\kappa$ B (I $\kappa$ B) phosphorylation, thereby inhibiting TNF $\alpha$ -induced nuclear factor- $\kappa$ B activation[39]. This may be the mechanism for preventing monocyte adhesion to endothelial cells. Furthermore, adiponectin suppresses the uptake of oxidised low density lipoprotein (LDL) by macrophages by inhibiting expression of the scavenger receptor class A-1 receptor, thereby inhibiting foam cell formation, and thus reducing vascular wall lipid accumulation. Moreover, adiponectin increases the HDL-mediated efflux of cholesterol from these cells[39, 59]. Adiponectin also inhibits platelet aggregation and thrombus formation[60]. In addition, by suppression of the production of certain growth factors (including heparin-binding epidermal growth factor-like growth factor, platelet-derived growth factor and basic fibroblast growth factor), adiponectin suppresses

proliferation and migration of vascular smooth muscle cells, suppressing neointimal thickening[61]. Furthermore, through AMPK signalling, adiponectin promotes angiogenesis in response to ischaemia-induced injury[62]. Finally, adiponectin, via AMPK activation, suppresses the oxidized LDL-induced excessive mitochondrial generation of reactive oxygen species (ROS), as well as hyperglycaemia-induced production of ROS[63]. Together, these actions inhibit atherosclerotic plaque formation.

#### ***B.3.3.3. Adiponectin in the clinical scenario***

In accordance with the mechanisms outlined above, adiponectin levels are reduced in patients with obesity (particularly visceral obesity), insulin resistance, metabolic syndrome, type 2 diabetes mellitus, dyslipidaemia, coronary artery disease (CAD) and hypertension[48]. In fact, low adiponectin levels may predict the development of insulin resistance and progression to type 2 diabetes mellitus[48] and hypertension[64], while high adiponectin levels are associated with a lower risk of myocardial infarction, independent of CRP[65]. Adiponectin levels are also elevated in type 1 diabetes patients, severely insulin-resistant patients and those with antibodies against the insulin receptor, once again suggesting that insulin and its signalling pathway influence adiponectin sensitivity and levels[66]. Furthermore, single nucleotide polymorphisms in the adiponectin gene are associated with the metabolic syndrome, type 2 diabetes mellitus, increased body mass index (BMI), dyslipidaemia, increased waist circumference, raised blood pressure and coronary artery disease[45].

Adiponectin levels correlate inversely with insulin resistance and CRP, apolipoprotein (apo) B, apo E and triglyceride levels, and positively with HDL and apo A levels[48, 67, 68]. Moreover, adiponectin correlates negatively with the pro-inflammatory marker TNF $\alpha$ .

#### ***B.3.3.4. HMW adiponectin***

The HMW form of adiponectin activates AMPK with a higher potency than the other adiponectin forms[69]. It has also been demonstrated to be superior to the measurement



of total adiponectin measurement as an indicator in the assessment of insulin sensitivity[70]. Mutations in the collagen domain, which prevent HMW formation, are associated with altered AMPK activation and diabetes[71], while administration of HMW adiponectin to insulin-resistant adiponectin-deficient mice is associated with a reduction in glucose levels[72]. Both total and HMW adiponectin forms (and the HMW to total adiponectin ratio) increase with weight reduction[73].

Both total and HMW adiponectin forms (and the ratio) correlate positively with HDL and fibrinogen and negatively with triglycerides, BMI, homeostasis model assessment of insulin resistance (HOMA-IR) and CRP[74]. HMW adiponectin and the ratio to total adiponectin are also lower in patients with hyperinsulinaemia, type 2 diabetes mellitus and CAD, correlating with the degree of CAD in men [74-77]. The association with type 2 diabetes mellitus is stronger with HMW than with total adiponectin[78]. Furthermore, both HMW-adiponectin and the ratio are independent risk factors for the development of type 2 diabetes mellitus[79].

Administration of thiazolidinediones causes increased HMW to adiponectin ratios, which correlate better with improved insulin sensitivity than do total adiponectin levels[80]. Furthermore, improvements in insulin sensitivity associated with gastric bypass surgery and weight loss also correlate better with HMW adiponectin than with total adiponectin[44, 73, 80]. The HMW to total adiponectin ratio (also called the adiponectin sensitivity index) was shown to be a better predictor for insulin resistance and the metabolic syndrome than total adiponectin[81, 82]. The ratio also correlates more significantly than total adiponectin with glucose and insulin levels[83].

Together, these findings suggest that measurement of HMW adiponectin (and its ratio to total adiponectin) may be more relevant in predicting insulin resistance.

#### ***B.3.3.5. Measurement of adiponectin isoforms***

Adiponectin isoform levels have been analysed using various cumbersome methods[84]. These include gel filtration chromatography, velocity gradient centrifugation, sodium dodecyl sulphate polyacrylamide (non-denaturing) gel electrophoresis and Western blot analysis. These methods are time-consuming and demand specific instrumentation and technical expertise, making implementation thereof in clinical laboratories difficult. A

recent, much simpler method involves the quantification of the different adiponectin isoforms by ELISA (using monoclonal antibodies), following a pre-treatment step with two proteases (protease A Amano digests LMW adiponectin alone, while protease K digests LMW and MMW adiponectin)[85]. This allows for the direct measurement of total and HMW adiponectin, and the indirect calculation of LMW and MMW adiponectin levels in approximately 4 hours[84].

#### **B.3.4. Adiponectin in HIV**

Due to adipose tissue redistribution and the associated metabolic disturbances characteristic of HIV-associated lipodystrophy, it is not surprising that there is a focus on adipocyte-secreted hormones such as adiponectin. In HIV-associated lipodystrophy, visceral adipose tissue is highly predictive of whole body glucose disposal, and this is possibly mediated by adiponectin as it correlates with both visceral adiposity and glucose disposal[86]. Adiponectin levels, both total and HMW, are decreased in such patients. In fact, total adiponectin levels in male patients with HIV-associated lipodystrophy are 50% that of patients without lipodystrophy and 25% of subjects without HIV[87]. Adiponectin in these patients correlates with measures of fat distribution, in line with its relationship to visceral adiposity[87]. In addition, it also correlates with insulin sensitivity (correlating positively with the qualitative insulin-sensitivity check index (QUICKI) and negatively with BMI, waist: hip ratio (WHR), fasting and 2h glucose and insulin, as well as triglycerides, apoB/A1 and CRP)[88]. Adiponectin levels are lower in HIV-infected patients who have high triglyceride levels than in those who have low triglyceride levels[89]. Adiponectin also correlates positively with HDL levels and negatively with C-peptide levels in HIV-associated lipodystrophy[90]. Patients on both stavudine (an NRTI) and protease inhibitors, have significantly lower adiponectin levels (and significantly lower insulin sensitivity parameters (QUICKI)) than those not treated with stavudine[88]. Both protease inhibitors and NRTIs cause a decrease in adiponectin expression in murine adipocytes, this decrease being most profound with the protease inhibitors, ritonavir and saquinavir[25].

In human subcutaneous adipocytes, adiponectin mRNA expression is significantly lower in patients with lipodystrophy than in those without lipodystrophy, correlating with serum

adiponectin levels[90]. This is also true for patients receiving protease inhibitors when compared to those not receiving protease inhibitors[91]. Adiponectin may play an instrumental role in the pathogenesis of this syndrome, since protease inhibitor administration to mice caused a dose- and drug-related reduction in adiponectin[92]. The resulting dyslipidaemia in these mice was ameliorated upon treatment with recombinant adiponectin[92].

The protease inhibitor ritonavir accumulates in adipocytes where it inhibits preadipocyte development into mature adipocytes, down-regulating expression of PPAR $\gamma$  and adiponectin, while up regulating TNF $\alpha$  and IL-6 expression[93].

Thiazolidinediones-induced improvement in insulin sensitivity in patients with HIV-induced lipodystrophy and insulin resistance is associated with a concomitant increase in adiponectin levels, in line with the increase in total body and subcutaneous fat also seen in these patients[94]. HMW adiponectin is significantly increased [95], with thiazolidinediones therapy increasing the HMW to total adiponectin ratio more significantly than total adiponectin, even before any significant change in body fat distribution is evident[96]. This treatment is also associated with increased expression, in subcutaneous fat, of adiponectin mRNA, PPAR $\gamma$  mRNA and PPAR $\gamma$  coactivator 1 (PGC1) mRNA, and decreased expression of IL-6 mRNA[97].

Furthermore, HMW adiponectin expression (as is the case with total adiponectin) is decreased in mature murine adipocytes treated with efavirenz (an NNRTI) or either of the protease inhibitors ritonavir or nelfinavir. Interestingly, this decrease is reversed by changing to atazanavir (a different type of protease inhibitor). HMW adiponectin is also significantly lower in HIV-infected patients after starting HAART compared with before HAART therapy (excluding atazanavir). This decrease is reduced upon the introduction of atazanavir to therapy, accompanied by an improvement of the dyslipidaemia[98] seen in these patients, suggesting that not all protease inhibitors induce the metabolic changes found in the lipodystrophy syndrome.

Although HMW (and its ratio to total) adiponectin is regarded as the most sensitive marker of insulin sensitivity, very little research has been done on the levels of this adiponectin form (and the ratio) in HIV-infected patients on HAART (specifically those

on combination therapy that include protease inhibitors compared to those not on protease inhibitors).

### **B.3.5. HIV-associated lipodystrophy and the risk for CAD**

Acute coronary syndromes are seen increasingly in HIV-infected patients receiving HAART (particularly protease inhibitors), the incidence of myocardial infarction related to longer exposure to combination drugs[99]. HIV itself may also contribute to cardiovascular risk, with one study demonstrating increased cardiovascular events in patients receiving episodic HAART (based on CD4 count) when compared with those receiving continuous therapy (hazard ratio 1.6,  $p=0.05$ )[100]. Apart from the decreased adiponectin levels (correlating with cardiac flow reserve), HIV-infected patients have other risk factors associated with cardiovascular disease [101, 102]. The presence of raised triglycerides and total cholesterol, as well as diabetes in these patients, is associated with a greater risk for presenting with myocardial infarction[99]. Furthermore, the decreased HDL levels, leading to decreased efflux of cholesterol from macrophages; and endothelial dysfunction seen in these patients, both promote atherosclerosis[103]. In fact, a large study reported a 16% relative increase in the incidence of myocardial infarction per year of HAART exposure, explained mainly by the hypercholesterolaemia, hypertriglyceridaemia and low HDL cholesterol[104].

### **B.3.6. Conclusion and needs for future research**

Insulin resistance is a prominent feature of the HIV-associated lipodystrophy syndrome. It is evident that decreased adiponectin, specifically its HMW form, is a major contributor to the development of insulin resistance, and may be partly responsible for the increased risk of developing CAD (through reduced anti-atherosclerotic actions), having been demonstrated to correlate with the extent of CAD. Furthermore, it has been shown to be a superior marker to total adiponectin for the detection and assessment of insulin resistance. Many studies have assessed total adiponectin levels in patients on HAART. However, very few studies have assessed the levels of the HMW form (and its ratio to total adiponectin) in such patients.

This project seeks to assess the levels of HMW adiponectin (and its ratio to total) in HIV-infected patients receiving HAART including protease inhibitors, comparing these levels to those in patients receiving HAART without protease inhibitors, and to those in patients not receiving HAART.

Word count: 4296

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## **C. MANUSCRIPT**

This manuscript was compiled in accordance with the author guidelines of Clinical Chemistry (Part D, Annexure).

### ***C.1. TITLE***

HMW: TOTAL ADIPONECTIN RATIOS ARE LOWER IN HIV-INFECTED WOMEN RECEIVING PROTEASE INHIBITORS

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### **KEY WORDS**

HMW adiponectin, lipodystrophy syndrome, HMW: adiponectin ratio, protease inhibitors, insulin resistance

### **JOURNAL CATEGORY**

Endocrinology and metabolism

Part of this work was presented at the American Association for Clinical Chemistry Annual Meeting and Laboratory Expo, July 2009, Chicago USA – POSTER PRESENTATION

University Of Cape Town

## **C.2. ABSTRACT**

### **Background**

Our current HIV-treatment regimens consist of highly active antiretroviral therapy (HAART) regimens 1 (nucleotide reverse transcriptase inhibitors (NRTIs) only), and 2 (protease inhibitors (PI) and NRTIs). HAART is associated with the lipodystrophy syndrome, insulin resistance and reduced total adiponectin (TA) levels. The high molecular weight (HMW):TA ratio is a superior marker of insulin resistance.

### **Objectives**

To establish whether HMW:TAs are lower in patients on PIs and whether findings correlate with insulin resistance and dyslipidaemia.

### **Methods**

66 HIV-infected females were recruited: 22 on regimen 2 (PI group), 22 on regimen 1 (non-PI) and 22 treatment naïve (TN), matched for BMI and age. Patients with a history of diabetes or impaired glucose tolerance were excluded. Serum adiponectin multimers were analysed using the Alpc<sup>TM</sup> Adiponectin (Multimeric) enzyme immunoassay. Lipodystrophy score, calf skin-fold thickness, waist hip ratios (WHR), lipids, glucose and insulin were assessed and HOMA-IR and QUICKI calculated. Data were analysed non-parametrically and multivariate analysis was performed.

### **Results**

TA and HMW levels were lower in the treatment groups than in the TN group. HMW:TA was lower in the PI than in the non-PI and TN groups, and in the non-PI than in the TN groups. HMW:TA correlated negatively with waist, insulin and HOMA-IR, and positively with QUICKI, independently of BMI. Triglycerides and cholesterol were significantly higher in the treatment groups than in the TN group.

### **Conclusion**

HMW:TA is significantly decreased with HAART (particularly with PIs) and may be linked to the development of lipodystrophy in these patients.

Word count: 245

### ***C.3. LIST OF ABBREVIATIONS***

**LMW**, low molecular weight; **MMW**, medium molecular weight; **HMW**, high molecular weight; **NFκB**, nuclear factor κB; **HIV**, human immunodeficiency virus; **HDL**, high density lipoprotein; **PI**, protease inhibitor; **d4T**, stavudine; **AZT**, zidovudine; **PPARγ**, peroxisomal proliferator activated receptor γ; **HAART**, highly active antiretroviral therapy; **3TC**, lamivudine; **EFV**, efavirenz; **ddI**, didanosine; **LPV/r**, liponavir/ritonavir; **TA**, total adiponectin; **ARV**, antiretroviral; **non-PI**, non-protease inhibitor; **BMI**, body mass index; **TN**, treatment naive; **WHR**, waist: hip ratio; **HOMA-IR**, homeostatic model assessment for insulin resistance; **QUICKI**, quantitative insulin-sensitivity check index; **CD4**, cluster of differentiation 4; **CV**, coefficient of variation; **IQR**, inter-quartile range; **KW**, Kruskal-Wallis

### ***C.4. BACKGROUND***

Adiponectin is an insulin-sensitising hormone[1, 2] found in three major multimeric forms in the circulation viz. the low molecular weight (LMW) trimer, the medium molecular weight (MMW) hexamer and the high molecular weight (HMW) 16-18mer (>400kDa). Adiponectin is present at relatively high concentrations in healthy individuals, making up approximately 0.05% of the total serum protein[3], with the HMW form the predominant and active form of adiponectin[4]. Sexual dimorphism exists, with females exhibiting higher circulating levels of adiponectin (total and HMW) than males[5-8].

Apart from its insulin-sensitising effects through increased fatty acid oxidation in muscle and liver and inhibition of gluconeogenesis [1, 2], adiponectin also exhibits anti-inflammatory and anti-atherosclerotic actions through inhibition of tumour necrosis factor-alpha (TNF α)-induced nuclear factor kappaB (NFκB) activation[9], adhesion molecule expression[10, 11], and class A scavenger receptor expression in macrophages, thereby inhibiting foam cell formation[12].

Although an adipokine, unlike other hormones secreted by adipocytes, adiponectin levels are reduced in patients with increased central body fat[13]. In fact, weight reduction has

been shown to increase total adiponectin, HMW adiponectin and the HMW to total adiponectin ratio. Low adiponectin levels are seen in patients with insulin resistance, type 2 diabetes mellitus and atherosclerosis as well as in patients with lipoatrophy and lipohypertrophy[13-15], with HMW adiponectin shown to correlate better with insulin sensitivity than total adiponectin[16] and the HMW: total adiponectin ratio to be a better predictor of coronary artery disease than total adiponectin[17]. This ratio has been shown to be suppressed in type 2 diabetes mellitus patients with coronary artery disease even when HMW and total adiponectin levels were unchanged[18].

In HIV-associated lipodystrophy, a syndrome consisting of fat redistribution (central hypertrophy and lipoatrophy of facial region and extremities), dyslipidaemia (particularly hypertriglyceridaemia) and insulin resistance, adiponectin levels are significantly lower, demonstrating a negative correlation with abdominal visceral fat mass, serum triglycerides and insulin resistance and a positive correlation with high density lipoprotein (HDL) and extremity fat[19, 20]. This syndrome is associated with antiretroviral (ARV) therapy, particularly protease inhibitors (PIs) (but also thymidine analogues such as stavudine (d4T) and zidovudine (AZT)) [21, 22]. In such patients, thiazolidinedione administration, via peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) activation, leads to improved insulin sensitivity[23] with upregulation of adiponectin levels, specifically the HMW form[24, 25], while in mice, adiponectin administration markedly ameliorates protease-induced dyslipidaemia, suggesting that hypoadiponectinaemia may be partially responsible for the metabolic derangements associated with PIs[26].

In South Africa, the National Department of Health made available to HIV-infected patients, two highly active antiretroviral therapy (HAART) regimens. The first regimen consists of d4T, lamivudine (3TC) and either efavirenz (EFV) or nevirapine i.e. a combination of two nucleotide reverse transcriptase inhibitors (NRTIs) and one non-nucleotide reverse transcriptase inhibitor (NNRTIs); while the second regimen consists of AZT, didanosine (ddI) and lopinavir/ritonavir (LPV/r). AZT and ddI are nucleotide- and nucleoside reverse transcriptase inhibitors, respectively, while LPV/r is a PI, inhibiting the protease required for cleavage of nascent HIV proteins and therefore maturation and replication of the HIV virus.

The purpose of this study was to establish whether the HMW: total adiponectin (TA) ratio is lower in HIV-infected patients receiving PI therapy compared with those not receiving PIs and whether these findings are associated with biochemical evidence of insulin resistance or dyslipidaemia in these patients.

### ***C.5. METHODS***

This study was performed in accordance with the Helsinki Declaration. A cross-sectional study was performed on subjects recruited from the ARV roll-out clinic at Groote Schuur Hospital, after departmental and ethics approval was obtained from the University of Cape Town. Sixty-six HIV-infected African females were enrolled into the study into three groups, viz. PI (Regimen 1 for at least six months), non-PI (Regimen 2 for at least six months) and treatment naïve (TN) groups, each consisting of 22 patients. The subjects in the PI group were recruited consecutively from the clinic, while the subjects in the other two groups were matched to the PI group for body mass index (BMI) and age. The TN group of patients were patients who had been referred to the ARV clinic for enrolment onto ARVs. Written informed consent was obtained from each patient and history taken regarding medical conditions, ARV therapy and other drug therapy. Bloods were drawn prior to their commencement on ARVs. Exclusion criteria included a history of impaired glucose tolerance or diabetes mellitus, active acute opportunistic infections, renal failure and pregnancy. Subjects were given a lipodystrophy questionnaire[27] relating to their perceived change of fat distribution in seven regions (face, arms, legs, buttocks, breasts, abdomen and neck) and were scored for atrophy or hypertrophy as follows: 0 = none, 1 = mild, 2= moderate and 3= severe. The total lipodystrophy score was then calculated as the sum of the individual scores for the seven regions. Body circumferences (waist and hip), weight, height and calf skin-fold thickness were measured as described previously[28] (briefly described in Supplemental Data), and the BMI and waist: hip ratio (WHR) were calculated. Fasting bloods were drawn for the measurement of total cholesterol, HDL, triglycerides, insulin and multimeric adiponectin in serum, as well as glucose levels in plasma. Samples were separated within 2 hours and serum / plasma stored at -70°C until analysis. The 2-h oral glucose tolerance test (75g

glucose) was performed and 2-h glucose measured. Thereafter, the homeostatic model assessment for insulin resistance (HOMA-IR) and quantitative insulin-sensitivity check index (QUICKI) were calculated. CD 4 count results were obtained from the hospital clinical records.

Adiponectin was analysed using the Alpc<sup>TM</sup> Adiponectin (Multimeric) enzyme immunoassay (sensitivity 0.04ng/mL and coefficient of variation (CV) <15%), insulin by Bayer ACS180 auto-analyser (CV 12 %), and the rest of the chemistry analytes using the Bayer Alera chemistry analyser.

### **Statistical analysis**

Results were analysed non-parametrically, using the Mann-Whitney U, Kruskal-Wallis and Spearman correlation tests. Multivariate analyses and power calculations were performed. A p-value of 0.05 was considered significant. Analyses were performed using Statistica 8 for Windows.

## **C.6. RESULTS**

### **Patient characteristics**

In the PI group, 21 patients were receiving LPV/r and one Atazanavir for at least six months. The median duration on PIs was 11.5 months, while the median duration on regimen 1 drugs prior to progression to regimen 2 was 15 months (Table 2). Four (18%) patients were receiving d4T and 13 (59%) AZT as part of their regimen. The median durations on these drugs were 15.5 and nine months, respectively. Twenty of the 22 patients were currently receiving PIs when the study was commenced, while two patients had been off PIs for 2 and 2.5 months, respectively. For these two patients the durations on PIs were 30 and 6 months, respectively. In the non-PI group, 21 of the 22 patients were receiving either AZT or d4T, with the median durations on d4T and AZT 10 and 12 months, respectively (Table 2). The patients in the treatment groups were following one

of the drug combinations shown in Table 1. None of the patients were receiving lipid-lowering therapy.

The CD 4 count was significantly higher in the PI than in the non-PI and TN groups (Table 2). It was also significantly higher in the non-PI groups than in the TN group (Table 2).

The median (inter-quartile range) age and BMI among the groups were 36 (29; 42) years and 27 (24; 30), respectively (medians and inter-quartile ranges for the individual groups are shown in Table 3). Waist measurement, calf skin-fold thickness and WHR did not differ among the groups (Table 3).

While neither the total lipodystrophy score nor its hypertrophy portion differed significantly among the groups, the atrophy portion was significantly higher in the PI group than in the non-PI group ( $p = 0.01134$ ) (Supplemental Data Table 1). However, it did not differ between the PI and TN or non-PI and TN groups.

In the PI group, 5 (23%) patients described facial, 5 (23%) arm and 8 (36%) leg atrophy, while no patients described fat deposition in the neck or dorso-cervical areas, although 5 (23%) patients described deposition in the abdomen, 2 (9%) in the buttocks, 6 (27%) in the breasts and 4 (18%) mentioned the presence of lipomata. In addition, 3 (14%) described fat deposition in the facial region. The lipodystrophy score distribution for the other groups can be seen in Supplemental Data Table 2.

### **Adiponectin levels in serum**

Total adiponectin levels were within the reference interval for women (3.5 – 22mg/L)[29] in all groups, however, they were significantly lower in both treatment groups than in the TN group ( $p = 0.0299$  and  $0.0290$ , respectively) (Figure 1 and Table 3). HMW adiponectin levels were also significantly lower in the treatment groups when compared with the TN group ( $p = 0.0005$  and  $p < 0.0001$ , respectively) (Figure 1 and Table 3). However, for both total and HMW adiponectin levels, no statistically significant difference was observed between the PI and non-PI groups (Figure 1 and Supplemental



Data Table 3). In contrast, the HMW: total adiponectin ratio differed significantly among the groups ( $p < 0.0001$ ) and was significantly lower in the PI group than in both the non-PI ( $p = 0.0019$ ) and the TN ( $p < 0.0001$ ) groups (Figure 1, Table 3 and Supplemental Data Table 3). The non-PI group also had significantly lower ratios than the TN group ( $p = 0.0167$ ).

While the LMW adiponectin fraction did not differ among the groups, its ratio to total adiponectin was significantly higher in both the PI and non-PI groups than in the TN group ( $p = 0.0004$  and  $0.0095$ , respectively) (Figure 1, Table 3 and Supplemental Data Table 3). The MMW adiponectin was similar among the groups (Supplemental Data Table 1).

### **Traditional markers of insulin resistance and dyslipidaemia**

There were no statistically significant differences among the groups for insulin, fasting and 2-h glucose or the derived parameters HOMA-IR and QUICKI (Table 3). However, in the entire group of HIV-infected patients, the 15 (23%) patients with HOMA-IR  $> 1.95$  had significantly lower ( $p = 0.0059$ ) HMW: total adiponectin ratios (median 0.31, IQR 0.22 and 0.47) than those with lower HOMA-IRs (median 0.50, IQR 0.40 and 0.57) (Figure 2). Similarly, 40 (60%) of the entire group of HIV-infected patients had a BMI  $\geq 25$ . These patients had significantly lower ( $p = 0.0475$ ) HMW: total adiponectin ratios (median 0.44; IQR 0.31 and 0.54) than those with lower BMIs (median 0.50, IQR 0.44 and 0.58).

Both fasting cholesterol and triglyceride levels were significantly higher in the PI and non-PI groups than in the TN group ( $p = 0.0072$  and  $0.0020$ , respectively, for cholesterol, and  $0.0037$  and  $0.0133$ , respectively, for fasting triglyceride levels) (Table 3 and Supplemental Data Table 3). However, both of these analytes were well within normal limits. In fact, triglyceride concentrations were low in the TN group. HDL and LDL cholesterol levels were also significantly higher in the non-PI group than in the TN groups, but did not differ significantly between either the PI and non-PI groups or between the PI and TN groups (Table 3 and Supplemental Data Table 3).

### **Correlates of adiponectin**

In the entire HIV- infected cohort (n = 66), the following relationships were found: HMW, total adiponectin, and their ratio had a significant negative correlation ( $p < 0.05$ ) with waist, BMI, fasting insulin, HOMA-IR, fasting triglycerides and CD 4 count, while they had a significant positive correlation ( $p < 0.05$ ) with QUICKI (Table 4). The same was true for MMW and the opposite true for the LMW: total adiponectin ratio (Table 4 and Supplemental Data Table 4). Interestingly, the LMW adiponectin correlated significantly negatively ( $p < 0.05$ ) with the 2-h glucose level and positively ( $p < 0.05$ ) with total and HMW adiponectin levels, individually. LMW adiponectin levels on their own did not correlate significantly with fasting insulin, fasting glucose, HOMA-IR, QUICKI, lipid levels, lactate or CD 4 count.

In addition to the above-mentioned findings, waist measurement correlated significantly positively ( $p < 0.05$ ) with calf skin-fold thickness, BMI, fasting insulin, HOMA-IR, fasting triglyceride and CD 4 count, and negatively ( $p < 0.05$ ) with QUICKI (Supplemental Data Table 4). Furthermore, fasting insulin and HOMA-IR correlated significantly positively ( $p < 0.05$ ) with BMI, while the opposite was true for QUICKI. A positive correlation ( $p < 0.05$ ) was also observed between fasting triglyceride and BMI, waist, 2-h glucose and fasting total cholesterol, and between calf skin-fold thickness and BMI. A negative (but not significant) correlation was noted between triglyceride and calf skin-fold thickness measurement. Total, HDL and LDL cholesterol showed no significant correlation with any of the adiponectin fractions or ratios.

The total lipodystrophy score correlated negatively ( $p < 0.05$ ) with BMI and calf measurement, however, no significant correlation was seen between this score and waist measurement, glucose, insulin, HOMA-IR, QUICKI, lipids or adiponectin fractions (Supplemental Data Table 4). The atrophy portion of the score correlated negatively with the BMI, LDL and HDL, and positively ( $p < 0.05$ ) with MMW: total adiponectin ratio. It had no significant correlation with calf skin-fold or waist measurements, nor with

glucose, insulin, HOMA-IR, QUICKI or other adiponectin fractions and ratios. The hypertrophy portion did not correlate significantly with any of the variables.

### **Multivariate analysis**

The negative correlations between total adiponectin, HMW: total adiponectin and HMW adiponectin, respectively, and waist, fasting insulin and HOMA-IR were still significant after multivariate analysis to adjust for BMI ( $p < 0.05$ ) (see Supplemental Data Tables 5-7). Furthermore, adjustment for BMI did not alter the significance of the difference between the groups for HMW: total adiponectin. Also, adjusting for BMI did not alter the significance of the positive correlation between HMW: total adiponectin and QUICKI, however, total and HMW adiponectin lost their significant correlation with QUICKI (Supplemental Data Table 8). The significant negative correlations between CD 4 count and total adiponectin, HMW: total adiponectin and HMW adiponectin were still evident after adjusting for duration of HAART (Supplemental Data Table 9), while the significance of the positive correlation initially seen between waist measurement and CD 4 count by univariate analysis, was attenuated when multivariate analysis was performed to adjust for duration of HAART (Supplemental Data Table 10).

The correlations seen in univariate analysis between LMW: total adiponectin and fasting insulin, HOMA-IR and QUICKI were still significant after multivariate analysis, adjusting for BMI; while the correlations between MMW adiponectin and fasting insulin, HOMA-IR and QUICKI, respectively, lost significance after adjusting for BMI (Supplemental Data Tables 11-13).

### **Power analysis for major variables**

#### *Adiponectin*

The study was sufficiently powered (83.4%) to detect a difference in HMW: total adiponectin of 0.10 (using 0.55 as the mean for patients not on HAART). [For a difference of 0.09, the power is 75.5%. A sample number of 30 would provide sufficient power (80%) to detect a difference of 0.09.] However, the study was not sufficiently powered to confirm that total (25.9%) and HMW (31%) adiponectin levels were not

significantly different among the treatment groups. A larger study number (at least n=100) would be needed to confirm these findings.

#### *Fasting insulin*

The study is also not sufficiently powered (8%) to confirm that fasting insulin levels were not significantly different among the groups. A much larger group would need to be assessed to make any real observations regarding insulin.

### **C.7. DISCUSSION**

The HIV-associated lipodystrophy syndrome is characterised by the presence of body fat redistribution (consisting of subcutaneous adipose tissue loss primarily from the facial region and extremities and / or deposition of excess adipose tissue around the neck, dorso-cervical spine, upper torso and intra-abdominal region), dyslipidaemia (which includes hypercholesterolaemia, hypertriglyceridaemia and low HDL cholesterol) and insulin resistance[21]. This syndrome is primarily seen in HIV-infected patients on HAART, with a 17% risk of developing lipodystrophy after the first year of HAART and each additional 6 month period associated with a 45% risk.[30]. These findings are particularly associated with PIs, but similar findings have been seen in some patients on thymidine analogues such as d4T and AZT[22].

In our patient samples, we did not demonstrate a difference in lipodystrophy score among the three treatment groups. This may be explained by the fact that the score was entirely subjectively provided by patients' impression of whether they had lost or gained fat tissue in the various body regions. However, both calf skin-fold thickness (used as a surrogate for lipoatrophy in the extremities) and waist measurements (used as a surrogate for abdominal weight gain) also did not differ among the groups, giving some credibility to the lipodystrophy scores derived. As we matched the non-PI and TN groups to the PI group for age and BMI, a difference in the waist measurement or calf skin-fold thickness could have been a useful indicator of lipoatrophy or lipohypertrophy. This may possibly be explained by the relatively short duration on HAART (median 11.5 months in the PI group). However, waist measurement correlated significantly negatively with adiponectin

concentrations (high, total and their ratio) independently of BMI (Supplemental Data Table 5), and therefore, by implication, independently of weight. This may indicate a connection between adiponectin concentrations (especially HMW: total adiponectin as the correlation is most significant for this form of adiponectin) and the development of lipodystrophy.

Furthermore, we demonstrated higher triglyceride and cholesterol concentrations in the treatment groups, independent of whether the patients were receiving PIs or not. Hypertriglyceridaemia is a recognized feature of the metabolic abnormalities associated with the lipodystrophy syndrome, and its inverse relationship with adiponectin is known[19]. However, in our study, fasting triglyceride levels were well within the reference interval, and may indicate improved health in response to HAART, rather than indicate progression to dyslipidaemia. In addition, we demonstrated a negative relationship between CD 4 count and adiponectin (HMW, total and their ratio), independent of the duration of HAART therapy (Supplemental Data Table 9), a relationship which requires further investigation before any inferences can be made. CD 4 counts were, as expected, higher in patients receiving both PIs and NRTIs than in patients receiving no PIs; and higher in these patients than in those not receiving HAART.

It is known that adiponectin levels in HIV-infected patients with HAART-associated lipodystrophy are substantially lower than in those without lipodystrophy or in uninfected patients[31]. In fact, adiponectin has been implicated in the pathogenesis of HIV-associated lipodystrophy[26]. PI administration produced dose-related reduction in adiponectin levels in mice, while administration of recombinant adiponectin ameliorated the associated dyslipidaemia in these mice. The subjects in our main study group have each been receiving PIs for at least six months, with the median duration on a PI drug being 11.5 months. In addition, some patients in this group have also been receiving either d4T or AZT (Table 2). It was, therefore, interesting to note that total adiponectin levels were well within the reference interval in all groups, however, they were significantly lower in HIV-infected patients on HAART therapy (both PIs and non-PIs) than in those not on treatment.

The active form of adiponectin is the HMW fraction, having been shown to correlate better with insulin sensitivity than total adiponectin[32]. As such, HMW adiponectin has been shown to be low in HIV-infected patients with insulin resistance[24]. Our study revealed the presence of significantly lower HMW adiponectin levels in patients receiving both PIs and non-PIs, but also in those receiving only non-PIs. This finding in the latter group, may be attributed to the use of d4T (16 (66%) patients) or AZT (5 (23%) patients), which have both been associated with lipodystrophy[33]. The HMW: total adiponectin ratio is a superior marker of insulin resistance, having been shown to be a better predictor of insulin resistance in patients with the metabolic syndrome than total and HMW adiponectin levels individually[34]. It is also an independent risk factor for coronary vascular disease (CVD)[35]. Previous studies have shown increased cardiovascular risk in HIV-infected patients, including those on HAART[36, 37]. We found this ratio to be significantly lower in the PI and less so (but nevertheless still significantly so) in the non-PI groups, implying an increased risk for CVD in patients on HAART (with the risk for CVD increased when both PIs and non-PIs are being used), in line with the findings of others. This significantly lower HMW: total adiponectin ratio in patients receiving both PIs and NRTI (with the majority, i.e. 17 of 22, of patients also receiving a thymidine analogue) confirms the findings of others that protease inhibitors and thymidine analogues induce metabolic complications synergistically[21].

Our patient groups were selected to exclude the presence of overt diabetes, and therefore it was not surprising that fasting glucose was not abnormal in any of the groups. However, we did not expect to find the median fasting insulin and 2-h glucose (post oral glucose tolerance test) levels to be normal as well (Table 3). These markers of insulin resistance, as well as HOMA-IR and QUICKI, did not differ among the groups in our study, despite the difference in total and HMW adiponectin, and their ratio, among the groups. However, a larger (sufficiently powered) study sample is required to verify this lack of significant differences among the groups for these variables. Notwithstanding, the relationship between insulin resistance markers and adiponectin, previously shown[19], was demonstrated here, with HOMA-IR correlating negatively with adiponectin levels (high, total and their ratio) and QUICKI correlating positively with the HMW: total adiponectin ratio, after adjusting for BMI. The HMW: total adiponectin ratio also

correlated negatively with WHR, fasting insulin levels, BMI and triglyceride levels (all markers of insulin resistance). Furthermore, the HMW: total adiponectin ratio was significantly lower in patients (in all groups) with HOMA-IR greater than 1.95 (cut-off for insulin resistance[38]) than in those with HOMA-IR less than or equal to 1.95 and also significantly lower in patients with BMI greater than or equal to 25 than in those with lower BMIs.

Interestingly, we also demonstrated that the LMW ratio to total adiponectin was significantly higher in patients receiving HAART (with patients on PI and NRTIs showing higher ratios than those not on PIs) than in treatment naïve HIV-infected patients. This increase may suggest that impaired multimerisation may be one of the mechanisms by which HAART therapy induces insulin resistance in HIV-associated lipodystrophy. Impaired multimerisation is known to be associated with decreased HMW: total adiponectin ratios, having been demonstrated in patients with type 2 diabetes mellitus[39].

### ***C.8. CONCLUSION***

These data demonstrate that both PI- and non-PI-containing HAART regimens significantly lower the HMW: total adiponectin ratio in HIV patients, with the ratio more significantly decreased in the PI-containing regimen, implying that PIs and NRTIs have an additive effect on the HMW: total adiponectin ratio. Although the HMW: total adiponectin ratio correlated negatively with indirect markers of insulin resistance, no overt insulin resistance was demonstrated. The HMW: total adiponectin ratio may therefore be an earlier or more sensitive marker of insulin resistance in HIV-infected patients on HAART. Furthermore, the increased LMW: total adiponectin ratios seen in these patients may imply that impaired multimerisation may be a mechanism by which the HMW adiponectin is lowered. Further studies are required to confirm this.

### **Limitations**

Due to funding restrictions, the sample size was limited to 22 in each group. The study was therefore not sufficiently powered to verify that insulin and total and HMW

adiponectin levels did not differ significantly among the various groups. Further studies are required in a larger study group to confirm that the findings for these variables are not subject to Type II statistical error. The study group was also limited to females as, in South Africa, females more frequently present for medical attention and therefore very few male patients were available for recruitment to the study. Assessment of lipodystrophy in this study was not reliable as this was a cross-sectional study and therefore an objective assessment by a clinician could not be performed. The score provided was determined solely by patient self-assessment. Drug compliance and dietary history in the different groups were not formally assessed, and may have had some influence on adiponectin levels.

**Acknowledgements:** The following are gratefully acknowledged for their part in this study: Sacha West for collecting the samples, Felicity Leisegang and Wan Iryani Wan Ismail for their assistance with assays, Jenny Kannemeyer for her assistance with the adiponectin ELISA, Andrew Boulle for his instruction on statistical analysis, and the National Health Laboratory Service for funding this project.

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**C.10. TABLES AND FIGURES**

<b>PI Group</b>	<b>non-PI Group</b>
LPV/r + ddI + AZT	AZT + 3TC + Nevirapine
LPV/r + 3TC + AZT	AZT + 3TC + EFV
LPV/r + EFV	d4T + 3TC + Nevirapine
LPV/r + Nevirapine	d4T + 3TC + EFV
LPV/r + Combivir	Nevirapine + Combivir
Atazanavir + 3TC + EFV	

**Table 1: Antiretroviral drug combinations in the two treatment groups. PI, protease inhibitor; non-PI, non-protease inhibitor; LPV/r, liponavir/ritonavir; ddI, didanosine; AZT, zidovudine; 3TC, lamivudine; EFV, efavirenz.**

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Group	Regimen 1	Regimen 2	d4T	AZT	CD 4 count
	median (IQR)	median (IQR)	median (IQR)	median (IQR)	median (IQR)
<b>PI</b>	15 (8.5 - 21.5)	11.5 (9.3 – 14.0)	15.5 (9.8 - 22.3)	9 (5 - 16.5)	465 (231 – 577)
					p = 0.0416*
<b>non-PI</b>	12 (9.5 – 20.0)		10 ( 8 - 17.5)	12 (11 – 15.0)	274 (186 – 352)
					p = 0.0128**
<b>TN</b>	-	-	-	-	171 (117 – 218)
					p = 0.0002***

**Table 2: Duration of therapy (months) and CD 4 counts (x 10<sup>6</sup> per litre) among the groups. PI, protease inhibitor group; non-PI, non- protease inhibitor group; d4T, stavudine; AZT, zidovudine; IQR, inter-quartile range; \*= p-value indicating significant difference between PIs and non-PIs; \*\*= p-value indicating significant different between non-PIs and TNs; \*\*\* = p-value indicating significant difference between PIs and TN.**

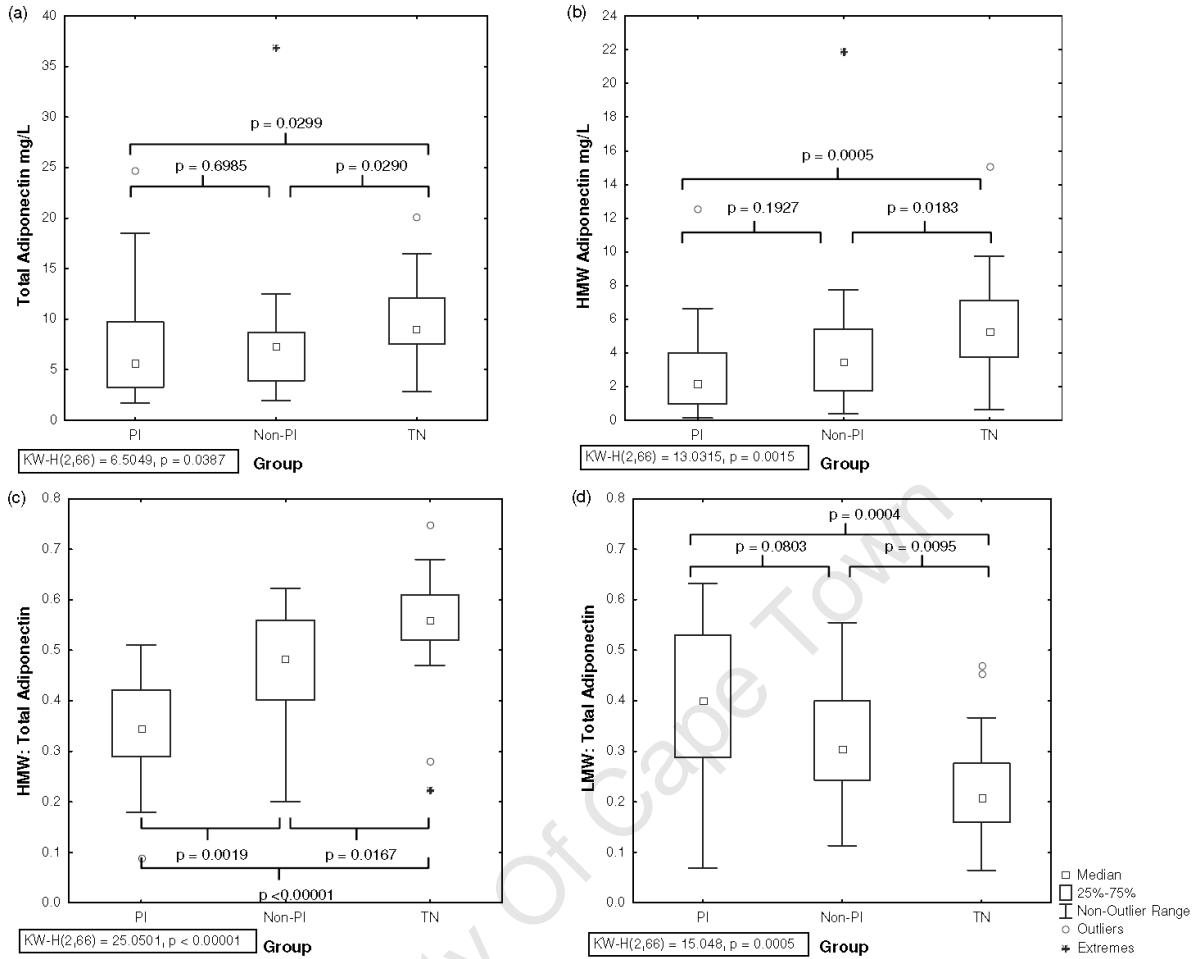
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	PI	non-PI	TN	Units	p-value KW
	Median	Median	Median		
	IQR	IQR	IQR		
<b>Age</b>	34.5	34.5	35.5	years	p = 0.9862
	(24.7 – 30.7)	(31 – 42)	28 – 42)		
<b>Weight</b>	69.6	65.3	60.9	kg	p = 0.2463
	(59.9-78.8)	(59.2 – 77.6)	(56.6 – 60.7)		
<b>BMI</b>	27.7	25	25.7		p = 0.5860
	(24.7 – 30.7)	(23.7 – 29.6)	(22.7 – 29.8)	-	
<b>WHR</b>	0.87	0.85	0.84	-	p = 0.3148
	0.82 – 0.93	0.78 -0.89	0.80- 0.90		
<b>Waist</b>	89.1	82.5	81.5	cm	p = 0.1944
	81.5 – 95.0	79.5 – 94.0	76.0 – 88.5		
<b>Calf skin-fold thickness</b>	16.5	15.3	17.5	mm	p = 0.5836
	8.9 – 21.2	10.8 – 19.1	11.2 – 22.6		
<b>Total adiponectin</b>	5.64	7.30	9.03	mg/L	p = 0.0387
	3.27 – 9.71	3.92 -8.70	7.58 – 12.12		
<b>HMW adiponectin</b>	2.23	3.49	5.29	mg/L	p = 0.0015
	0.99 – 4.01	1.77 -5.39	3.73 – 7.11		
<b>HMW: total adiponectin</b>	0.35	0.48	0.56	-	p < 0.0001
	0.29 – 0.42	0.40 -0.56	0.52 – 0.61		
<b>LMW adiponectin</b>	1.89	1.71	1.74	mg/L	p = 0.7523
	1.40 – 3.25	1.51 - 2.60	1.33 – 2.48		
<b>LMW: total adiponectin</b>	0.40	0.30	0.21	-	p = 0.0005
	0.28 – 0.53	0.24 - 0.40	0.16 – 0.28		
<b>Fasting insulin</b>	5.52	3.1	4.37	mIU/L	p= 0.3648
	2.62 – 10.12	2.1 -5.9	1.89 – 8.10		
<b>Fasting glucose</b>	4.8	5.1	5.1	mmol/L	p= 0.1452
	4.5 – 5.0	4.6 – 5.5	4.7 – 5.4		
<b>HOMA-IR</b>	1.18	0.72	1.05	-	p = 0.4788
	0.52 – 2.11	0.50 -1.19	0.39 – 1.75		
<b>QUICKI</b>	0.37	0.41	0.38	-	p = 0.2339
	0.33 – 0.42	0.37 – 0.433	0.35 – 0.46		
<b>2hr glucose</b>	5.8	5.2	5.2	mmol/L	p = 0.8765
	4.4 – 6.3	4.7 – 6.1	4.4 – 6.1		
<b>Fasting total cholesterol</b>	4.5	4.4	3.5	mmol/L	p = 0.0033
	3.8 – 5.4	4.2 – 5.3	3.0 – 4.4		
<b>Fasting triglycerides</b>	1.2	1.2	0.9	mmol/L	p = 0.0065
	0.9 – 1.8	0.9 – 1.4	0.7 – 1.1		
<b>HDL</b>	1.0	1.1	0.8	mmol/L	p = 0.0426
	0.8 – 1.2	0.9 – 1.4	0.6 – 1.1		
<b>LDL (calculated)</b>	2.5	2.8	2.2	mmol/L	p = 0.0223
	2.1 – 3.2	2.5 – 3.1	1.7 – 2.7		

Table 3: Median and inter-quartile ranges for the different variables in the three groups. P <0.05 demonstrates a significant difference for the variable among the groups. PI, protease inhibitor group; nonPI, non- protease inhibitor group; WHR, waist: hip ratio; HMW, high molecular weight; MMW, medium molecular weight; LMW, low molecular weight; HDL, high density lipoprotein, LDL, low density lipoprotein; KW, Kruskal-Wallis; IQR, inter-quartile range.

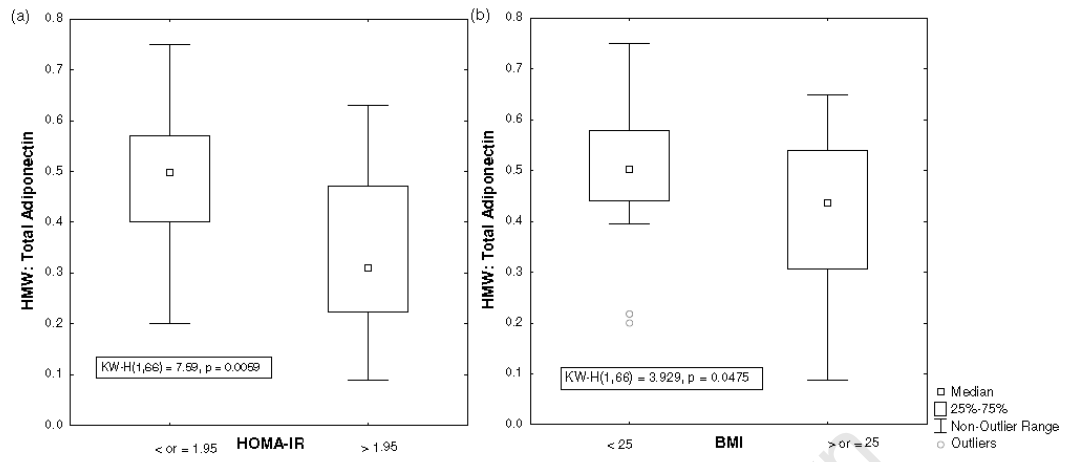
	Adiponectin fraction						
	Total	HMW	HMW: total	MMW	MMW: total	LMW	LMW : total
<b>Waist</b>	-0.5348	-0.5518	-0.4836	-0.5058	ns	ns	0.51486
<b>Calf</b>	ns	ns	ns	ns	ns	ns	ns
<b>BMI</b>	-0.3933	-0.3949	-0.3002	-0.4105	ns	ns	0.3801
<b>Fasting insulin</b>	-0.3745	-0.375	-0.3297	-0.4069	ns	ns	0.3556
<b>HOMA-IR</b>	-0.3768	-0.377	-0.3289	-0.4137	ns	ns	0.3514
<b>QUICKI</b>	0.4057	0.4224	0.3909	0.4509	ns	ns	-0.4272
<b>Fasting glucose</b>	ns	ns	ns	ns	ns	ns	ns
<b>2-h glucose</b>	ns	ns	ns	ns	ns	-0.3092	ns
<b>Fasting triglycerides</b>	-0.3984	-0.4513	-0.4579	-0.4907	ns	ns	0.4923

Table 4: Spearman correlation coefficient ( $r_s$ ) for significant correlations between adiponectin fractions and variables listed ( $p < 0.05$ ). BMI, body mass index; HMW, MMW and LMW, high, medium and low molecular weight, respectively; ns, not significant.



Manuscript Figure 1: Box and whisker plots showing distribution of (a)total adiponectin, (b)HMW adiponectin, (c)HMW: total adiponectin ratio and (d)LMW: total adiponectin ratio among the three groups. HMW, high molecular weight; LMW, low molecular weight; KW, Kruskal-Wallis; p <0.05 considered significant.





**Manuscript Figure 2: Distribution of HMW: total adiponectin ratio in patients with (a) HOMA-IR  $\leq$  or  $>$  1.95 and (b) BMI  $<$  25 or  $\geq$  25. HMW, high molecular weight; KW, Kruskal-Wallis;  $p < 0.05$  considered significant.**

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### ***C.11. SUPPLEMENTAL DATA***

#### **Anthropometry**

All measurements were performed with the participants standing, in duplicate, by trained examiners, and the mean used. Examiners were trained on proper performance of the various measurements. Measurements were cross-validated on a number of subjects until variability between duplicate measurements was low. Body weight was measured on a calibrated balance, with the participants wearing minimal clothing or an examination gown. A calibrated wall-mounted stadiometer was used to measure height. Body circumferences (waist and hip) were measured using measuring tape, as follows: waist circumference was measured at the highest point of the iliac crest, while the hip circumference was measured at the level of the greatest gluteal protuberance. Skin fold thickness at the calf was measured using calibrated skinfold calipers, medially at the level of largest circumference.

#### **PI patients**

Two of the 22 patients were subsequently discovered to have stopped PI therapy 2 and 2.5 months prior to recruitment to this study. Patient 1 had been receiving Atazanavir for 30 months before her PI was stopped. Patient 2 had been receiving LPV/r for 6 months. Her PI was stopped 2.5 months prior to recruitment. Despite being on Atazanavir, the HMW: total adiponectin ratio in this patient was 0.30 (lower than the median ratio for the PI group) while the ratio in Patient 2 was 0.35 (the median ratio in the PI group). Their data were therefore included in the analyses.

#### **Medium molecular weight adiponectin**

Using the multimeric adiponectin assay, we also assessed medium and low molecular weight (MMW and LMW, respectively) adiponectin. Supplemental Data Table 1 shows median (inter-quartile range (IQR)) for the MMW adiponectin form and its ratio to total adiponectin. It is evident that the MMW weight adiponectin is significantly lower in the PI and non-PI groups when compared to the TN group. However, as is the case with total and HMW adiponectin, there is not a statistical difference between the PI and non-PI groups (Supplemental Data Table 3). The MMW ratio to total adiponectin did not differ significantly among the groups.

	PI	non-PI	TN	KW p-value
	Median	Median	Median	
	<i>IQR</i>	<i>IQR</i>	<i>IQR</i>	
MMW adiponectin	1.27	1.53	1.87	p = 0.0688
	0.88 - 2.29	0.71 - 1.97	1.55 - 2.85	
MMW: total adiponectin	0.26	0.23	0.24	p = 0.1156
	0.21 - 0.34	0.16 - 0.27	0.17 - 0.29	
Lipodystrophy score - atrophy	2	0	0	p= 0.0479
	0 - 7	0 - 0	0 - 6	
Lipodystrophy score - hypertrophy	1	0	0	p = 0.311
	0-3	0-3	0-1	
Lipodystrophy score - total	4	0	2	p = 0.1485
	0 - 10	0 - 5	0 - 7	

**Supplemental Data Table 1: MMW (in ug/ml), its ratio to total adiponectin and the lipodystrophy scores: median (IQR). P<0.05 deemed a significant difference among the groups as analysed using the Kruskal-Wallis test.**

### Lipodystrophy findings

Supplemental Data Table 1 lists the median (IQR) scores for the lipodystrophy score and its lipoatrophy and -hypertrophy portions for each group. It is evident, as mentioned in the manuscript that the differences are not significant for the total score or the hypertrophy portion thereof. While the atrophy portion of the score shows a difference between the groups, it just barely reaches significance.

The lipodystrophy score comprised eight sites listed in Supplemental Data Table 2. Patients were asked to give a score of severity (0 to 3) if lipoatrophy or -hypertrophy was experienced in any of the sites. This table shows the number of patients affected in the different regions and the median (IQR) scores of severity reported in each region. It is evident that more patients in the PI group perceived atrophic changes in the 8 regions than did those in the non-PI group. This difference was confirmed using the KW test (Supplemental Data Table 1). Non-PI group had fewer patients reporting atrophic changes when compared to the TN group. However, no difference was evident between the PI and TN groups (Supplemental Data Table 3). Also, more patients in the PI and non-PI groups claimed to have experienced hypertrophy in the breasts, abdomen and buttocks, while only patients in the non-PI group complained about dorso-cervical spine

fat hypertrophy. Lipomata were present in all three groups. As this was a cross-sectional cohort study, this data was subjectively obtained from patients at one visit and were not compared to objective findings from a clinician. Therefore, the scores and therewith, the findings, may be questionable. However, as mentioned in the manuscript, the lack of obvious difference among the groups was confirmed by no difference in waist measurements of calf skinfold thickness measurements.

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	<i>PI Group</i>		<i>non-PI Group</i>		<i>TN Group</i>	
	n (%)	median (IQR)	n (%)	median (IQR)	n (%)	median (IQR)
<b>Lipoatrophy</b>						
Face	5 (23)	2 (1 - 3)	2 (9)	2 (1 - 2)	4 (18)	3 (2 - 3)
Neck	5 (23)	2 (1 - 2)	0 (0)	0 (0)	5 (23)	2 (2 - 3)
Dorso-cervical spine	4 (18)	2	0 (0)	0 (0)	5 (23)	2 (2 - 3)
Arms	5 (23)	3 (2 - 3)	1 (5)	3	8 (36)	2 (1 - 2)
Breasts	2 (9)	3 (2 - 3)	0 (0)	0 (0)	4 (18)	2
Abdomen	5 (23)	2 (1 - 3)	1 (5)	3	5 (23)	3 (2 - 3)
Buttocks	10 (45)	3 (2 - 3)	1 (5)	2	7 (32)	2 (2 - 3)
Legs	8 (36)	3 (2 - 3)	2 (9)	2 (1 - 2)	7 (32)	2 (2 - 3)
<b>Fat accumulation</b>						
Face	3 (14)	3	2 (9)	2 (2 - 3)	1 (5)	2
Neck	0 (0)	0 (0)	2 (9)	2 (2 - 3)	1 (5)	3
Dorso-cervical spine	0 (0)	0 (0)	2 (9)	2 (2 - 3)	0 (0)	0
Arms	2 (9)	2 (1 - 2)	3 (14)	1 (1 - 2)	1 (5)	2
Breasts	6 (27)	3 (2 - 3)	5 (23)	1 (1 - 2)	0 (0)	0
Abdomen	5 (23)	2 (1 - 2)	3 (14)	1	2 (9)	1
Buttocks	2 (9)	2 (1 - 2)	3 (14)	1 (1 - 2)	1 (5)	1
Legs	0 (0)	0 (0)	2 (9)	1	1 (5)	1
Lipomata	4 (18)	2 (1 - 3)	2 (9)	2 (2 - 3)	5 (23)	2

**Supplemental Data Table 2 : Distribution of lipodystrophy in HIV-infected patients in the different treatment groups.**

Supplemental Data Table 3 lists the variables that significantly different among the groups. Reference has been made to these analytes in the manuscript.

	<i>PI vs non-PI</i>	<i>PI vs TN</i>	<i>non-PI vs TN</i>
Total adiponectin	0.6985	0.0299	0.0290
HMW adiponectin	0.1927	0.0005	0.0183
HMW: total adiponectin	0.0019	0.0000	0.0167
MMW adiponectin	0.8973	0.0845	0.0250
MMW: total adiponectin	0.0620	0.1392	0.3916
LMW adiponectin	0.7160	0.4455	0.7513
LMW: total adiponectin	0.0803	0.0004	0.0095
f- cholesterol	0.7513	0.0072	0.0020
f- triglycerides	0.5652	0.0037	0.0133
HDL	0.2649	0.0620	0.0290
LDL	0.3185	0.1242	0.0050
Lipodystrophy score - atrophy	0.0265	0.5732	0.1887
CD 4 count	0.0416	0.0001	0.0078

**Supplemental Data Table 3: Mann-Whitney U derived p-values for differences between the various groups. p <0.05 deemed significant difference.**

### Correlations

Supplemental Data Table 4 lists the correlation coefficients between the various variables, as determined by Spearman correlation test. The findings for most variables were discussed in the manuscript. Interestingly, as with total and HMW adiponectin, the MMW adiponectin form correlated positively with QUICKI and negatively with HOMA-IR, fasting cholesterol and fasting triglyceride levels. Furthermore, it correlated positively with total and HMW adiponectin and the HMW: total adiponectin ratio.

	age	bmi	whr	waist	calf	CD4	TA	HMW	HMW: TA	MMW	MMW: TA	LMW	LMW: TA
age	1.000	-0.077	0.231	0.140	-0.030	0.021	-0.016	-0.045	-0.135	-0.030	-0.005	0.078	0.132
bmi	-0.077	1.000	0.029	<b>0.834</b>	<b>0.431</b>	0.146	<b>-0.393</b>	<b>-0.395</b>	<b>-0.300</b>	<b>-0.410</b>	-0.071	-0.170	<b>0.380</b>
whr	0.231	0.029	1.000	<b>0.389</b>	-0.216	0.202	<b>-0.414</b>	<b>-0.425</b>	<b>-0.397</b>	<b>-0.387</b>	0.089	<b>-0.281</b>	<b>0.308</b>
waist	0.140	<b>0.834</b>	<b>0.389</b>	1.000	<b>0.315</b>	<b>0.325</b>	<b>-0.535</b>	<b>-0.552</b>	<b>-0.484</b>	<b>-0.506</b>	-0.018	-0.176	<b>0.515</b>
calf	-0.030	<b>0.431</b>	-0.216	<b>0.315</b>	1.000	0.031	0.048	0.043	0.060	0.094	0.099	0.010	-0.036
CD4	0.021	0.146	0.202	<b>0.325</b>	0.031	1.000	<b>-0.428</b>	<b>-0.494</b>	<b>-0.543</b>	<b>-0.298</b>	0.103	-0.099	<b>0.471</b>
TA	-0.016	<b>-0.393</b>	<b>-0.414</b>	<b>-0.535</b>	0.048	<b>-0.428</b>	1.000	<b>0.958</b>	<b>0.726</b>	<b>0.813</b>	-0.082	<b>0.530</b>	<b>-0.695</b>
HMW	-0.045	<b>-0.395</b>	<b>-0.425</b>	<b>-0.552</b>	0.043	<b>-0.494</b>	<b>0.958</b>	1.000	<b>0.870</b>	<b>0.771</b>	-0.135	<b>0.425</b>	<b>-0.784</b>
HMW: TA	-0.135	<b>-0.300</b>	<b>-0.397</b>	<b>-0.484</b>	0.060	<b>-0.543</b>	<b>0.726</b>	<b>0.870</b>	1.000	<b>0.578</b>	<b>-0.253</b>	0.152	<b>-0.826</b>
MMW	-0.030	<b>-0.410</b>	<b>-0.387</b>	<b>-0.506</b>	0.094	<b>-0.298</b>	<b>0.813</b>	<b>0.771</b>	<b>0.578</b>	1.000	<b>0.397</b>	0.185	<b>-0.832</b>
MMW: TA	-0.005	-0.071	0.089	-0.018	0.099	0.103	-0.082	-0.135	-0.253	<b>0.397</b>	1.000	<b>-0.495</b>	<b>-0.265</b>
LMW	0.078	-0.170	<b>-0.281</b>	-0.176	0.010	-0.099	<b>0.530</b>	<b>0.425</b>	<b>0.152</b>	0.185	<b>-0.495</b>	1.000	0.122
LMW: TA	0.132	<b>0.380</b>	<b>0.308</b>	<b>0.515</b>	-0.036	<b>0.471</b>	<b>-0.695</b>	<b>-0.784</b>	<b>-0.826</b>	<b>-0.832</b>	<b>-0.265</b>	0.122	1.000
f-ins	-0.030	<b>0.359</b>	0.221	<b>0.418</b>	0.127	0.116	<b>-0.375</b>	<b>-0.375</b>	<b>-0.330</b>	<b>-0.407</b>	0.073	-0.201	<b>0.356</b>
f-glu	0.220	0.025	0.218	0.146	0.021	0.144	-0.108	-0.094	-0.037	-0.135	-0.073	-0.165	0.034
2hr glu	0.226	0.117	<b>0.322</b>	0.115	-0.115	0.247	-0.184	-0.190	-0.188	-0.191	-0.037	<b>-0.309</b>	0.160
HOMA-IR	-0.032	<b>0.352</b>	0.236	<b>0.419</b>	0.127	0.136	<b>-0.377</b>	<b>-0.377</b>	<b>-0.329</b>	<b>-0.414</b>	0.066	-0.209	<b>0.351</b>
QUICKI	-0.022	<b>-0.355</b>	<b>-0.288</b>	<b>-0.451</b>	-0.128	-0.133	<b>0.406</b>	<b>0.422</b>	<b>0.391</b>	<b>0.451</b>	-0.019	0.142	<b>-0.427</b>
f-chol	0.140	0.148	0.072	0.210	-0.207	0.173	-0.004	-0.049	-0.171	-0.098	0.058	0.040	0.121
f-tg	<b>0.251</b>	<b>0.247</b>	<b>0.359</b>	<b>0.363</b>	-0.118	0.202	<b>-0.398</b>	<b>-0.451</b>	<b>-0.458</b>	<b>-0.491</b>	-0.094	-0.056	<b>0.492</b>
HDL	-0.053	0.097	-0.257	0.046	-0.144	0.099	0.159	0.159	0.080	0.118	0.030	0.075	-0.089
LDL	0.167	0.080	-0.013	0.109	-0.141	0.069	0.085	0.042	-0.069	0.000	0.045	0.088	0.016
hdl/ldl	-0.220	0.052	<b>-0.270</b>	-0.027	-0.023	0.043	0.014	0.060	0.127	0.080	-0.015	-0.039	-0.100
LS	-0.004	<b>-0.257</b>	0.021	-0.238	<b>-0.301</b>	-0.065	-0.038	-0.060	-0.137	0.098	0.242	-0.065	-0.065
LS-A	0.052	<b>-0.281</b>	0.124	-0.213	-0.238	0.054	-0.005	-0.049	-0.171	0.146	<b>0.287</b>	-0.037	-0.054
LS-H	-0.068	-0.017	-0.028	-0.017	-0.172	0.010	-0.111	-0.119	-0.100	-0.056	0.073	-0.140	0.008

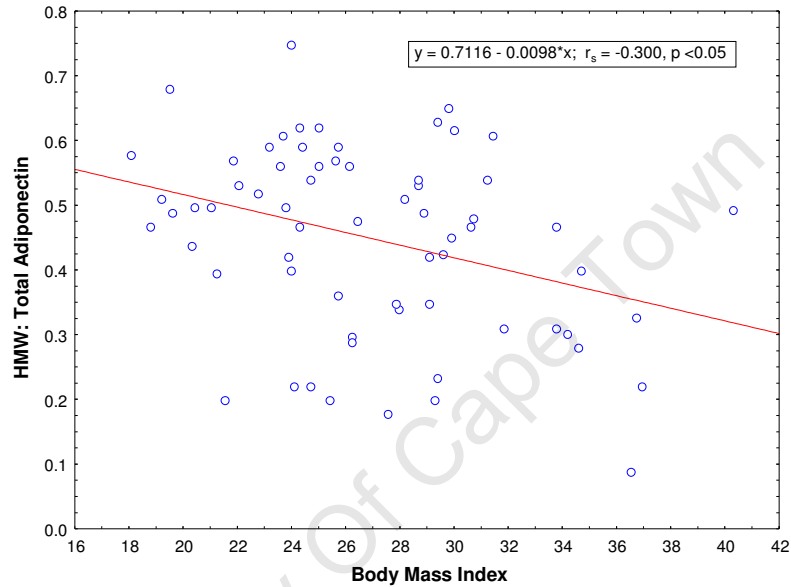
Supplemental Data Table 4: Spearman correlation coefficients (rs) showing the relationships between the different variables. Numbers in bold are significant with p<0.05.

	<i>f-Ins</i>	<i>f-glu</i>	<i>2hr glu</i>	<i>HOMA-IR</i>	<i>QUICKI</i>	<i>f-cho</i>	<i>f-tg</i>	<i>HDL</i>	<i>LDL</i>	<i>hdl/ldl</i>	<i>LS</i>	<i>LS-A</i>	<i>LS-H</i>
age	-0.030	0.220	0.226	-0.032	-0.022	0.140	<b>0.251</b>	-0.053	0.167	-0.220	-0.004	0.052	-0.068
bmi	<b>0.359</b>	0.025	0.117	<b>0.352</b>	<b>-0.355</b>	0.148	<b>0.247</b>	0.097	0.080	0.052	<b>-0.257</b>	<b>-0.281</b>	-0.017
whr	0.221	0.218	<b>0.322</b>	0.236	<b>-0.288</b>	0.072	<b>0.359</b>	<b>-0.257</b>	-0.013	<b>-0.270</b>	0.021	0.124	-0.028
waist	<b>0.418</b>	0.146	0.115	<b>0.419</b>	<b>-0.451</b>	0.210	<b>0.363</b>	0.046	0.109	-0.027	-0.238	-0.213	-0.017
calf	0.127	0.021	-0.115	0.127	-0.128	-0.207	-0.118	-0.144	-0.141	-0.023	<b>-0.301</b>	-0.238	-0.172
CD4	0.116	0.144	0.247	0.136	-0.133	0.173	0.202	0.099	0.069	0.043	-0.065	0.054	0.010
TA	<b>-0.375</b>	-0.108	-0.184	<b>-0.377</b>	<b>0.406</b>	-0.004	<b>-0.398</b>	0.159	0.085	0.014	-0.038	-0.005	-0.111
HMW	<b>-0.375</b>	-0.094	-0.190	<b>-0.377</b>	<b>0.422</b>	-0.049	<b>-0.451</b>	0.159	0.042	0.060	-0.060	-0.049	-0.119
HMW : TA	<b>-0.330</b>	-0.037	-0.188	<b>-0.329</b>	<b>0.391</b>	-0.171	<b>-0.458</b>	0.080	-0.069	0.127	-0.137	-0.171	-0.100
MMW	<b>-0.407</b>	-0.135	-0.191	<b>-0.414</b>	<b>0.451</b>	-0.098	<b>-0.491</b>	0.118	0.000	0.080	0.098	0.146	-0.056
MMW : TA	0.073	-0.073	-0.037	0.066	-0.019	0.058	-0.094	0.030	0.045	-0.015	0.242	<b>0.287</b>	0.073
LMW	-0.201	-0.165	<b>-0.309</b>	-0.209	0.142	0.040	-0.056	0.075	0.088	-0.039	-0.065	-0.037	-0.140
LMW: TA	<b>0.356</b>	0.034	0.160	<b>0.351</b>	<b>-0.427</b>	0.121	<b>0.492</b>	-0.089	0.016	-0.100	-0.065	-0.054	0.008
f-Ins	1.000	0.192	0.072	<b>0.993</b>	<b>-0.903</b>	0.069	0.188	-0.116	0.051	-0.205	-0.029	-0.065	0.071
f-glu	0.192	1.000	<b>0.408</b>	<b>0.285</b>	<b>-0.254</b>	0.225	0.093	0.094	0.239	-0.160	-0.158	-0.179	-0.016
2hr glu	0.072	<b>0.408</b>	1.000	0.099	-0.114	0.124	<b>0.268</b>	-0.021	0.031	-0.038	-0.162	-0.106	-0.024
HOMA-A-IR	<b>0.993</b>	<b>0.285</b>	0.099	1.000	<b>-0.910</b>	0.102	0.193	-0.085	0.081	-0.203	-0.046	-0.083	0.062
QUICKI	<b>-0.903</b>	<b>-0.254</b>	-0.114	<b>-0.910</b>	1.000	-0.113	-0.208	0.106	-0.108	0.232	0.099	0.124	-0.022
f-cho	0.069	0.225	0.124	0.102	-0.113	1.000	<b>0.390</b>	<b>0.658</b>	<b>0.851</b>	-0.071	-0.088	-0.228	0.124
f-tg	0.188	0.093	<b>0.268</b>	0.193	-0.208	<b>0.390</b>	1.000	0.033	0.134	-0.041	0.003	<b>0.073</b>	0.021
HDL	-0.116	0.094	-0.021	-0.085	0.106	<b>0.658</b>	0.033	1.000	<b>0.417</b>	<b>0.595</b>	-0.097	<b>-0.245</b>	0.158
LDL	0.051	0.239	0.031	0.081	-0.108	<b>0.851</b>	0.134	<b>0.417</b>	1.000	<b>-0.406</b>	-0.094	-0.246	0.063
hdl/ldl	-0.205	-0.160	-0.038	-0.203	0.232	-0.071	-0.041	<b>0.595</b>	<b>-0.406</b>	1.000	-0.076	-0.075	0.066
LS	-0.029	-0.158	-0.162	-0.046	0.099	-0.088	0.003	-0.097	-0.094	-0.076	1.000	<b>0.826</b>	<b>0.603</b>
LS-A	-0.065	-0.179	-0.106	-0.083	0.124	-0.228	0.073	<b>-0.245</b>	<b>-0.246</b>	-0.075	<b>0.826</b>	1.000	0.172
LS-H	0.071	-0.016	-0.024	0.062	-0.022	0.124	0.021	0.158	0.063	0.066	<b>0.603</b>	0.172	1.000

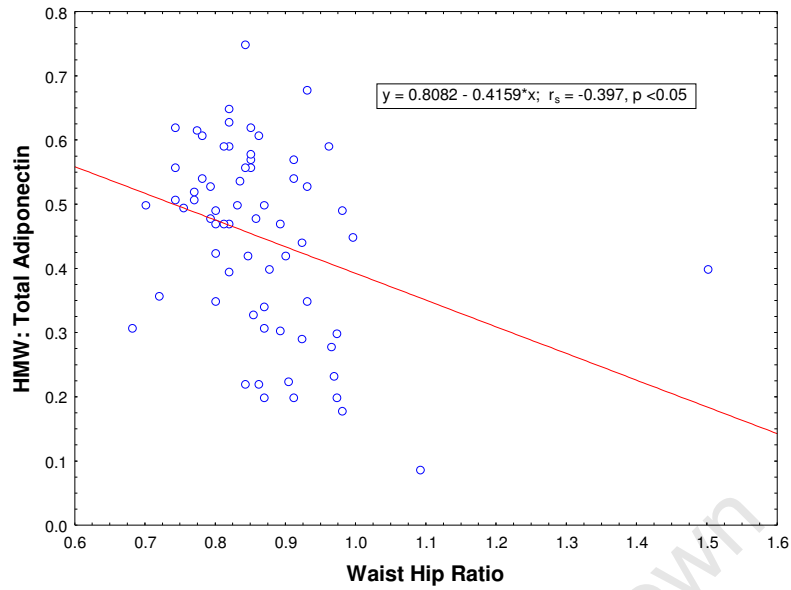
Supplemental Data Table 4: continued...



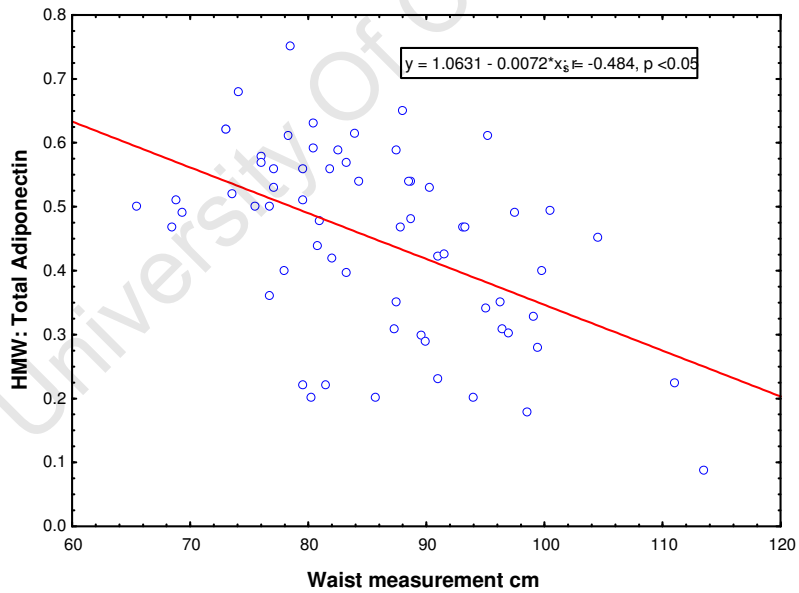
Supplemental Data Figures 1-8 are visual representations of correlation data for the HMW: total adiponectin ratio, already discussed in the manuscript. Notably, the ratio correlated negatively with established markers of insulin resistance (including BMI, HOMA-IR, WHR and triglycerides), but also with waist (which may be due to BMI increase) and positively with a known marker of insulin sensitivity (QUICKI). In addition, it also correlated negatively with the CD 4 count. As CD 4 count was highest in patients on protease inhibitor therapy, this may be the reason for the correlation.



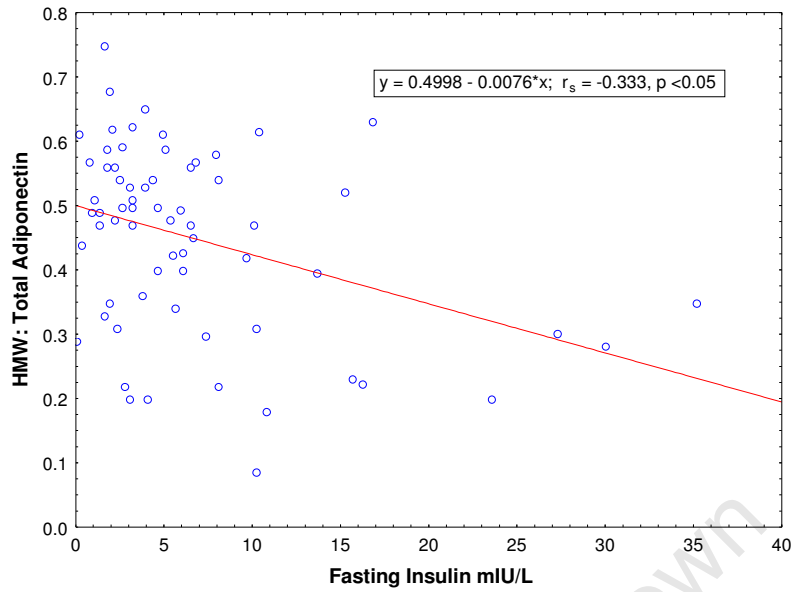
**Supplemental Data Figure 4: The relationship between HMW: total adiponectin ratios in serum and BMI.  $r_s$  is the Spearman correlation coefficient;  $p < 0.05$  deemed statistically significant.**



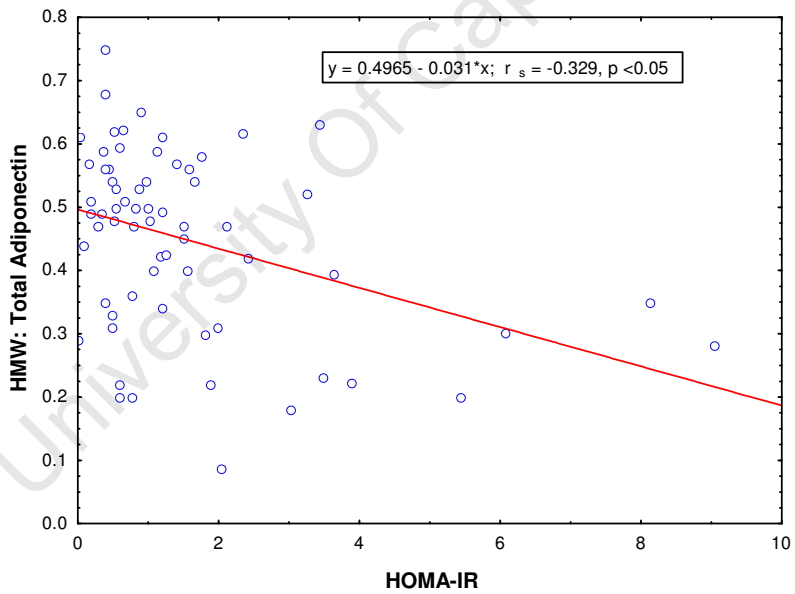
Supplemental Data Figure 5: Relationship between HMW: total adiponectin ratios in serum and WHR.  $r_s$  is the Spearman correlation coefficient;  $p < 0.05$  deemed statistically significant.



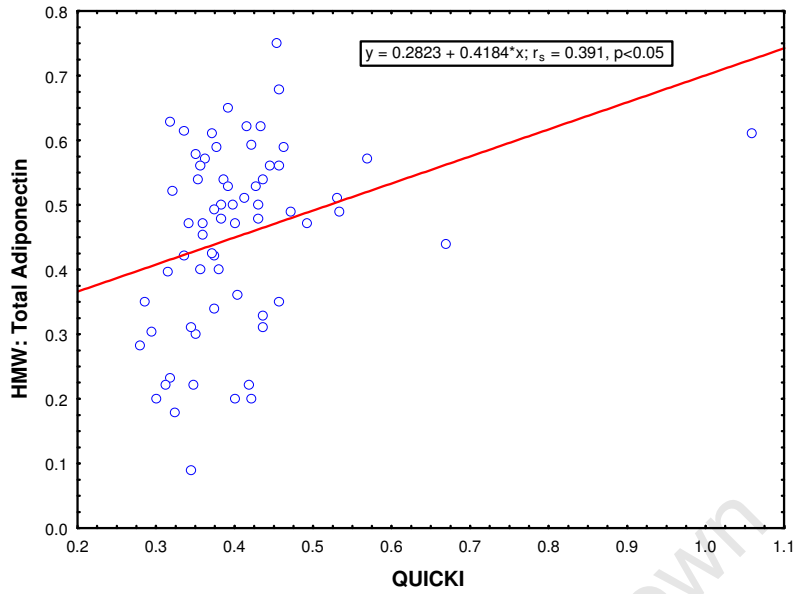
Supplemental Data Figure 6: Relationship between HMW: total adiponectin ratio and waist measurement.  $r_s$  is the Spearman correlation coefficient;  $p < 0.05$  deemed statistically significant.



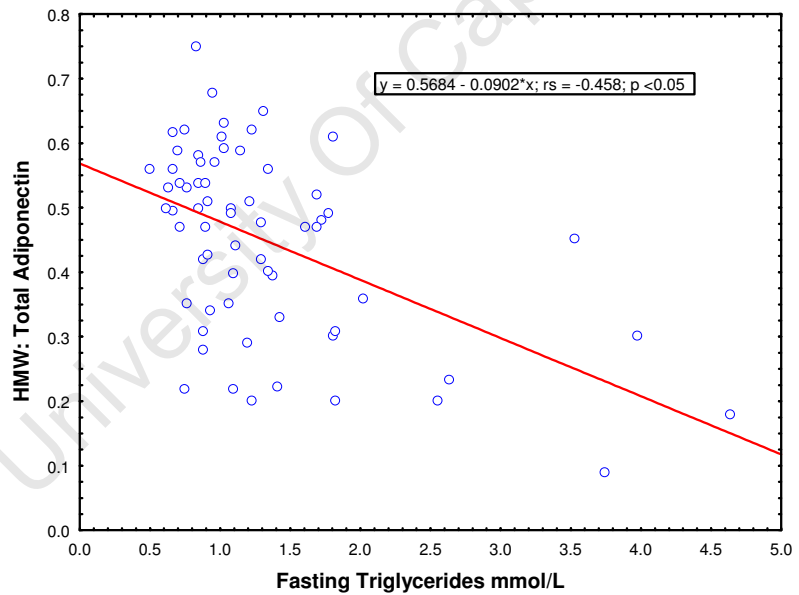
**Supplemental Data Figure 7: Relationship between HMW: total adiponectin and fasting insulin concentration in serum.  $r_s$  is the Spearman correlation coefficient;  $p < 0.05$  deemed statistically significant.**



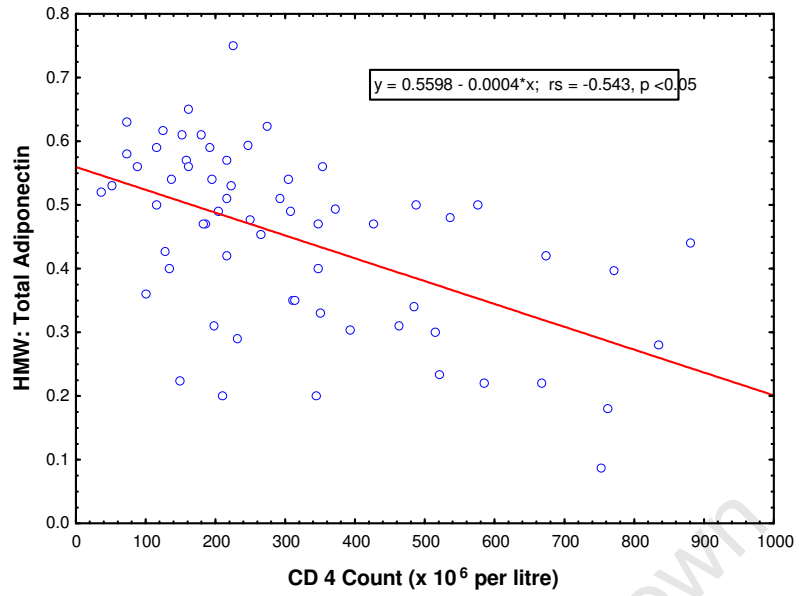
**Supplemental Data Figure 8: Relationship between HMW: total adiponectin ratio and HOMA-IR.  $r_s$  is the Spearman correlation coefficient;  $p < 0.05$  deemed statistically significant.**



Supplemental Data Figure 9: Relationship between HMW: total adiponectin ratio and QUICKI.  $r_s$  is the Spearman correlation coefficient;  $p < 0.05$  deemed statistically significant.



Supplemental Data Figure 10: Relationship between HMW: total adiponectin ratio and serum triglyceride concentration.  $r_s$  is the Spearman correlation coefficient;  $p < 0.05$  deemed statistically significant.



**Supplemental Data Figure 11: Relationship between CD 4 count and HMW: total adiponectin ratio in serum.**  
 $r_s$  is the Spearman correlation coefficient;  $p < 0.05$  deemed statistically significant.

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**Multivariate analyses**

Multivariate analysis was performed to assess the effect of possible confounders on the correlations described previously. The following tables (Supplemental Data Tables 5-13) represent these data. The findings have been described and discussed in the manuscript.

Variable	<i>Univariable</i>			<i>Multivariable</i>		
	Coefficient	95% CI	p-value	Coefficient	95% CI	p-value
<b><u>TOTAL ADIPONECTIN</u></b>						
ARV treatment						
None(ref)	1			1		
Yes, without PI's	-1.7530	(-5.1842,-0.0024)	0.3205	-1.3005	(-4.4220,1.8211)	0.4174
Yes, with PI's	-2.3200	(-5.7512,-0.5693)	0.1899	-1.0230	(-4.2154,2.1694)	0.5323
BMI (per unit increase)	-0.4485	(-0.7145,-0.3127)	0.0016*	0.0340	(-0.4507,0.5186)	0.8912
Waist (per unit increase)	-0.2657	(-0.3895,-0.2026)	0.0001*	-0.2726	(-0.5114,-0.0339)	0.0289*
<b><u>HMW:TOTAL ADIPONECTIN</u></b>						
ARV treatment						
None(ref)	1			1		
Yes, without PI's	-0.0825	(-0.1527,-0.0467)	0.0244*	-0.0720	(-0.1336,-0.0105)	0.0253*
Yes, with PI's	-0.2052	(-0.2754,-0.1694)	<0.0001*	-0.1729	(-0.2358,-0.1099)	<0.0001*
BMI (per unit increase)	-0.0098	(-0.0165,-0.0063)	0.0062*	0.0066	(-0.0030,0.0161)	0.1830
Waist (per unit increase)	-0.0072	(-0.0102,-0.0056)	<0.0001*	-0.0084	(-0.0131,-0.0037)	0.0008*
<b><u>HMW ADIPONECTIN</u></b>						
ARV treatment						
None(ref)	1			1		
Yes, without PI's	-1.4482	(-3.5402,-0.3809)	0.1797	-1.1789	(-3.0928,0.7351)	0.2320
Yes, with PI's	-2.8091	(-4.9010,-1.7418)	0.0107*	-2.0387	(-3.9961,-0.0812)	0.0456*
BMI (per unit increase)	-0.2824	(-0.4509,-0.1965)	0.0017*	0.0165	(-0.2807,0.3136)	0.9138
Waist (per unit increase)	-0.1704	(-0.2484,-0.1306)	0.0001*	-0.1609	(-0.3073,-0.0145)	0.0352*

**Supplemental Data Table 5: : Multivariate analysis adjusting for BMI, showing the correlations between waist measurement and total and HMW (high molecular weight) adiponectin and ratio, respectively.\* = significant p<0.05.**

Variable	<i>Univariable</i>			<i>Multivariable</i>		
	Coefficient	95% CI	p-value	Coefficient	95% CI	p-value
<b><u>TOTAL ADIPONECTIN</u></b>						
ARV treatment						
None(ref)	1			1		
Yes, without PI's	-1.7530	(-5.1842,-0.0024)	0.3205	-1.9878	(-5.1402,1.1646)	0.2212
Yes, with PI's	-2.3200	(-5.7512,-0.5693)	0.1899	-1.6013	(-4.7402,1.5376)	0.3213
BMI (per unit increase)	-0.4485	(-0.7145,-0.3127)	0.0016*	-0.3423	(-0.6155,-0.0691)	0.0169*
Fasting insulin (per unit increase)	-0.2795	(-0.4683,-0.1831)	0.0051*	-0.2176	(-0.4104,-0.0249)	0.0307*
<b><u>HMW:TOTAL ADIPONECTIN</u></b>						
ARV treatment						
None(ref)	1			1		
Yes, without PI's	-0.0825	(-0.1527,-0.0467)	0.0244*	-0.0910	(-0.1549,-0.0271)	0.0070*
Yes, with PI's	-0.2052	(-0.2754,-0.1694)	<0.0001*	-0.1913	(-0.2549,-0.1276)	<0.0001*
BMI (per unit increase)	-0.0098	(-0.0165,-0.0063)	0.0062*	-0.0055	(-0.0110,0.0001)	0.0572
Fasting insulin (per unit increase)	-0.0076	(-0.0123,-0.0053)	0.0020*	-0.0058	(-0.0097,-0.0018)	0.0054*
<b><u>HMW ADIPONECTIN</u></b>						
ARV treatment						
None(ref)	1			1		
Yes, without PI's	-1.4482	(-3.5402,-0.3809)	0.1797	-1.5841	(-1.8064,-1.3618)	0.1134
Yes, with PI's	-2.8091	(-4.9010,-1.7418)	0.0107*	-2.3801	(-2.4160,-2.3441)	0.0183*
BMI (per unit increase)	-0.2824	(-0.4509,-0.1965)	0.0017*	-0.2057	(-0.2432,-0.1681)	0.0192*
Fasting insulin (per unit increase)	-0.1744	(-0.2941,-0.1133)	0.0058*	-0.1282	(-0.2017,-0.0547)	0.0375*

**Supplemental Data Table 6: Multivariate analyses adjusting for BMI showing the correlations between fasting insulin concentration and total and HMW (high molecular weight) adiponectin and ratio, respectively. \*= significant p<0.05.**

Variable	<i>Univariable</i>			<i>Multivariable</i>		
	Coefficient	95% CI	p-value	Coefficient	95% CI	p-value
<b><u>TOTAL ADIPONECTIN</u></b>						
ARV treatment						
None(ref)	1			1		
Yes, without PI's	-1.7530	(-5.1842,-0.0024)	0.3205	-1.9957	(-5.1502,1.1587)	0.2197
Yes, with PI's	-2.3200	(-5.7512,-0.5693)	0.1899	-1.7071	(-4.8452,1.4311)	0.2905
BMI (per unit increase)	-0.4485	(-0.7145,-0.3127)	0.0016*	-0.3455	(-0.6180,-0.0729)	0.0157*
HOMA-IR (per unit increase)	-1.1053	(-1.8677,-0.7163)	0.0060*	-0.8682	(-1.6398,-0.0965)	0.0312*
<b><u>HMW:TOTAL ADIPONECTIN</u></b>						
ARV treatment						
None(ref)	1			1		
Yes, without PI's	-0.0825	(-0.1527,-0.0467)	0.0244*	-0.0924	(-0.1556,-0.0293)	0.0057*
Yes, with PI's	-0.2052	(-0.2754,-0.1694)	<0.0001*	-0.1940	(-0.2569,-0.1312)	<0.0001*
BMI (per unit increase)	-0.0098	(-0.0165,-0.0063)	0.0062*	-0.0054	(-0.0108,0.0001)	0.0589
HOMA-IR (per unit increase)	-0.0310	(-0.0497,-0.0215)	0.0018*	-0.0250	(-0.0404,-0.0095)	0.0024*
<b><u>HMW ADIPONECTIN</u></b>						
ARV treatment						
None(ref)	1			1		
Yes, without PI's	-1.4482	(-3.5402,-0.3809)	0.1797	-1.5972	(-3.5274,0.3331)	0.1100
Yes, with PI's	-2.8091	(-4.9010,-1.7418)	0.0107*	-2.4423	(-4.3626,-0.5220)	0.0154*
BMI (per unit increase)	-0.2824	(-0.4509,-0.1965)	0.0017*	-0.2061	(-0.3729,-0.0394)	0.0184*
HOMA-IR (per unit increase)	-0.6922	(-1.1753,-0.4457)	0.0066*	-0.5257	(-0.9979,-0.0536)	0.0329*

**Supplemental Data Table 7: Multivariate analysis, adjusting for BMI, showing the correlations between HOMA-IR and total and HMW (high molecular weight) adiponectin and ratio, respectively. \*= significant p<0.05.**



Variable	<i>Univariable</i>			<i>Multivariable</i>		
	Coefficient	95% CI	p-value	Coefficient	95% CI	p-value
<b><u>TOTAL ADIPONECTIN</u></b>						
ARV treatment						
None(ref)	1			1		
Yes, without PI's	-1.7530	(-5.1842,-0.0024)	0.3205	-1.7981	(-5.0211,1.4249)	0.2785
Yes, with PI's	-2.3200	(-5.7512,-0.5693)	0.1899	-1.5902	(-4.8045,1.6241)	0.3360
BMI (per unit increase)	-0.4485	(-0.7145,-0.3127)	0.0016*	-0.3748	(-0.6540,-0.0956)	0.0108*
QUICKI (per unit increase)	12.7802	(1.1205,18.7290)	0.0355*	8.4803	(-3.3898,20.3504)	0.1665
<b><u>HMW:TOTAL ADIPONECTIN</u></b>						
ARV treatment						
None(ref)	1			1		
Yes, without PI's	-0.0825	(-0.1527,-0.0467)	0.0244	-0.0881	(-0.1535,-0.0228)	0.0104
Yes, with PI's	-0.2052	(-0.2754,-0.1694)	<0.0001	-0.1901	(-0.2553,-0.1250)	<0.0001
BMI (per unit increase)	-0.0098	(-0.0165,-0.0063)	0.0062*	-0.0059	(-0.0116,-0.0003)	0.0436*
QUICKI (per unit increase)	0.4150	(0.1320,0.5593)	0.0055*	0.2817	(0.0411,0.5222)	0.0252*
<b><u>HMW ADIPONECTIN</u></b>						
ARV treatment						
None(ref)	1			1		
Yes, without PI's	-1.4482	(-3.5402,-0.3809)	0.1797	-1.4989	(-3.4629,0.4651)	0.1398
Yes, with PI's	-2.8091	(-4.9010,-1.7418)	0.0107*	-2.3633	(-4.3219,-0.4046)	0.0212*
BMI (per unit increase)	-0.2824	(-0.4509,-0.1965)	0.0017*	-0.2200	(-0.3901,-0.0499)	0.0138*
QUICKI (per unit increase)	8.8494	(1.5237,12.5870)	0.0209*	5.7069	(-1.5263,12.9402)	0.1272

**Supplemental Data Table 8: Multivariate analysis, adjusting for BMI, showing the correlations between QUICKI and total and HMW (high molecular weight) adiponectin and ratio, respectively. \*= significant p<0.05.**

Variable	Univariable			Multivariable		
	Coefficient	95% CI	p-value	Coefficient	95% CI	p-value
<b><u>HMW:TOTAL ADIPONECTIN</u></b>						
ARV treatment						
None(ref)	1			1		
Yes, without PI's	-0.0825	(-0.1527,-0.0467)	0.0244*	-0.0277	(-0.1139,0.0585)	0.5308
Yes, with PI's	-0.2052	(-0.2754,-0.1694)	<0.0001*	-0.0864	(-0.2170,0.0441)	0.1992
CD4 count (per unit increase)	-0.0004	(-0.0005,-0.0003)	<0.0001*	-0.0002	(-0.0004,-0.0001)	0.0056*
Duration HAART (per unit increase)	-0.0060	(-0.0081,-0.0049)	<0.0001*	-0.0023	(-0.0061,0.0016)	0.2492
<b><u>TOTAL ADIPONECTIN</u></b>						
ARV treatment						
None(ref)	1			1		
Yes, without PI's	-1.7530	(-5.1842,-0.0024)	0.3205	-3.1000	(-7.3679,1.1678)	0.1596
Yes, with PI's	-2.3200	(-5.7512,-0.5693)	0.1899	-4.6426	(-11.1050,1.8199)	0.1642
CD4 count (per unit increase)	-0.0091	(-0.0157,-0.0058)	0.0082*	-0.0096	(-0.0171,-0.0021)	0.0149*
Duration HAART (per unit increase)	-0.0211	(-0.1249,0.0318)	0.6910	0.1633	(-0.0271,0.3537)	0.0980
<b><u>HMW</u></b>						
ARV treatment						
None(ref)	1			1		
Yes, without PI's	-1.4482	(-3.5402,-0.3809)	0.1797	-1.9612	(-4.6029,0.6804)	0.1508
Yes, with PI's	-2.8091	(-4.9010,-1.7418)	0.0107*	-3.6227	(-7.6226,0.3773)	0.0809
CD4 count (per unit increase)	-0.0067	(-0.0108,-0.0046)	0.0019*	-0.0055	(-0.0101,-0.0008)	0.0250*
Duration HAART (per unit month increase)	-0.0552	(-0.1195,-0.0223)	0.0978	0.0749	(-0.0429,0.1928)	0.2175

**Supplemental Data Table 9: Multivariate analysis, adjusting for duration of HAART, showing the correlation between CD 4 count and total, HMW (high molecular weight) adiponectin and ratio, respectively. \*= significant p<0.05.**

Variable	<i>Univariable</i>			<i>Multivariable</i>		
	Coefficient	95% CI	p-value	Coefficient	95% CI	p-value
<b><u>WAIST</u></b>						
ARV treatment						
None(ref)	1			1		
Yes, without PI's	1.7386	(-4.2359,4.7869)	0.5704	1.3819	(-6.3591,9.1229)	0.7276
Yes, with PI's	4.9318	(-1.0427,7.9800)	0.1107	3.7401	(-7.9813,15.4616)	0.5340
CD 4 count (per unit increase)	0.0139	(0.0023,0.0198)	0.0223*	0.0120	(-0.0016,0.0257)	0.0887
Duration HAART (per unit month increase)	0.1173	(-0.0624,0.2090)	0.2055	-0.0604	(-0.4058,0.2850)	0.7330

**Supplemental Data Table 10: Multivariate analysis, adjusting for duration of HAART, showing the relationship between waist measurement and CD 4 count. \*=significant p<0.05.**

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Variable	<i>Univariate</i>			<i>Multivariate</i>		
	Coefficient	95% CI	p-value	Coefficient	95% CI	p-value
<b><u>MMW ADIPONECTIN</u></b>						
ARV treatment						
None(ref)	1			1		
Yes, without PI's	-0.3973	(-1.4546,0.6600)	0.4641	-0.4528	(-1.4413,0.0516)	0.3729
Yes, with PI's	-0.0458	(-1.1031,1.0116)	0.9327	0.1624	(-0.8219,0.6646)	0.7475
BMI (per unit increase)	-0.1254	(-0.2077,-0.0431)	0.0040*	-0.1030	(-0.1886,-0.0592)	0.0217*
Fasting insulin (per unit increase)	-0.0723	(-0.1310,-0.0136)	0.0186*	-0.0581	(-0.1186,-0.0273)	0.0642
<b><u>LMW:TOTAL ADIPONECTIN</u></b>						
ARV treatment						
None(ref)	1			1		
Yes, without PI's	0.0951	(0.0179,0.1722)	0.0186*	0.1026	(0.0326,0.1383)	0.0056*
Yes, with PI's	0.1655	(0.0883,0.2426)	0.0001*	0.1491	(-0.0697,0.0355)	0.0001*
BMI (per unit increase)	0.0110	(0.0043,0.0177)	0.0021*	0.0071	(0.0010,0.0102)	0.0249*
Fasting insulin (per unit increase)	0.0075	(0.0028,0.0122)	0.0027*	0.0058	(0.0015,0.0080)	0.0101*

**Supplemental Data Table 11: Multivariate analysis adjusting for BMI, showing the correlations between fasting insulin concentration and MMW (medium molecular weight) adiponectin and the LMW (low molecular weight): total adiponectin ratio, respectively. \*= significant p<0.05.**

Variable	<i>Univariate</i>			<i>Multivariate</i>		
	Coefficient	95% CI	p-value	Coefficient	95% CI	p-value
<b><u>MMW ADIPONECTIN</u></b>						
ARV treatment						
None(ref)	1			1		
Yes, without PI's	-0.3973	(-1.4546,0.6600)	0.4641	-0.4511	(-1.4418,0.0543)	0.3756
Yes, with PI's	-0.0458	(-1.1031,1.0116)	0.9327	0.1341	(-0.8514,0.6370)	0.7906
BMI (per unit increase)	-0.1254	(-0.2077,-0.0431)	0.0040*	-0.1045	(-0.1900,-0.0608)	0.0199*
HOMA-IR (per unit increase)	-0.2844	(-0.5212,-0.0475)	0.0217*	-0.2256	(-0.4679,-0.1019)	0.0730
<b><u>LMW:TOTAL ADIPONECTIN</u></b>						
ARV treatment						
None(ref)	1			1		
Yes, without PI's	0.0951	(0.0179,0.1722)	0.0186*	0.1035	(0.0339,0.1390)	0.0050*
Yes, with PI's	0.1655	(0.0883,0.2426)	0.0001*	0.1519	(-0.0692,0.0353)	0.0001*
BMI (per unit increase)	0.0110	(0.0043,0.0177)	0.0021*	0.0071	(0.0011,0.0101)	0.0244*
HOMA-IR (per unit increase)	0.0300	(0.0110,0.0489)	0.0029*	0.0243	(0.0073,0.0330)	0.0068*

**Supplemental Data Table 12: Multivariate analysis adjusting for BMI, showing the correlations between HOMA-IR and MMW (medium molecular weight) adiponectin and the LMW (low molecular weight): total adiponectin ratio, respectively. \*= significant p<0.05.**

Variable	<i>Univariate</i>			<i>Multivariate</i>		
	Coefficient	95% CI	p-value	Coefficient	95% CI	p-value
<b><u>MMW ADIPONECTIN</u></b>						
ARV treatment						
None(ref)	1			1		
Yes, without PI's	-0.3973	(-1.4546,0.6600)	0.4641	-0.4494	(-1.4368,0.0544)	0.3759
Yes, with PI's	-0.0458	(-1.1031,1.0116)	0.9327	0.1836	(-0.8011,0.6861)	0.7160
BMI (per unit increase)	-0.1254	(-0.2077,-0.0431)	0.0040*	-0.1031	(-0.1887,-0.0595)	0.0213*
QUICKI (per unit increase)	4.3327	(0.8050,7.8604)	0.0190*	3.5268	(-0.1100,5.3822)	0.0621
<b><u>LMW:TOTAL ADIPONECTIN</u></b>						
ARV treatment						
None(ref)	1			1		
Yes, without PI's	0.0951	(0.0179,0.1722)	0.0186*	0.1044	(0.0361,0.1393)	0.0040*
Yes, with PI's	0.1655	(0.0883,0.2426)	0.0001*	0.1462	(-0.0682,0.0348)	0.0001*
BMI (per unit increase)	0.0110	(0.0043,0.0177)	0.0021*	0.0067	(0.0008,0.0098)	0.0296*
QUICKI (per unit increase)	-0.5124	(-0.7880,-0.2368)	0.0005*	-0.4105	(-0.6623,-0.2821)	0.0022*

**Supplemental Data Table 13: Multivariate analysis adjusting for BMI, showing the correlations between QUICKI and MMW (medium molecular weight) adiponectin and the LMW (low molecular weight): total adiponectin ratio, respectively. \*= significant p<0.05.**

## D. SUPPORTING DOCUMENTS

### D.1. ETHICS APPROVAL



UNIVERSITY OF CAPE TOWN

Health Sciences Faculty  
Research Ethics Committee  
Room E52-24 Groote Schuur Hospital Old Main Building  
Observatory 7925  
Telephone [021] 406 6338 • Facsimile [021] 406 6411  
e-mail: preaward@curie.uct.ac.za

12 January 2007

REC REF: 381/2006

Prof TS Pillay  
Chemical Pathology

Dear Prof Pillay

**PROJECT TITLE: ROLE OF HIGH MOLECULAR WEIGHT MULTIMERIC ADIPONECTIN  
IN THE DEVELOPMENT OF HIV LIPODYSTROPHY SYNDROME FOLLOWING ANTI-  
RETROVIRAL THERAPY**

Thank you for submitting your study to the Research Ethics Committee for review.

It is a pleasure to inform you that the Ethics Committee has **formally approved** the above-mentioned study.

Please add the Research Ethics Committee contact details to the Patient Information Sheet.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

**Please quote the REC. REF in all your correspondence.**

Yours sincerely

A/PROF. M. BLOCKMAN  
CHAIRPERSON, HSF HUMAN ETHICS

**D.2. COVER LETTER AND ETHICS APPROVAL FOR AMENDMENT  
OF RESEARCH PROTOCOL**

Chemical Pathology  
C20  
Groote Schuur Hospital  
Anzio Road  
Observatory  
7925  
Tel: 404 4135  
Fierdoz.omar@uct.ac.za  
9 October 2007

**REF: 450/2006**

Chairperson  
Research Ethics Committee  
Health Sciences Faculty

Dear Sir/Madame

**PROJECT TITLE: ROLE OF HIGH MOLECULAR WEIGHT MULTIMERIC  
ADIPONECTIN IN THE DEVELOPMENT OF HIV LIPODYSTROPHY SYNDROME  
FOLLOWING ANTI-RETROVIRAL THERAPY**

Last year ethics approval was granted for the above-mentioned research project.

Due to the fact that there are too few HIV patients on anti retroviral therapy that have already been diagnosed with lipodystrophy receiving treatment at the ARV clinic at Groote Schuur Hospital, we have decided to alter our subject and control groups slightly to the following 4 groups:

Test group:

1. 30 known HIV positive adult patients on Protease inhibitor anti retroviral treatment for 6 months and longer.

Control groups:

2. 30 known HIV positive adult patients on non-protease inhibitor anti retroviral treatment for 6 months and longer.
3. 30 known HIV positive adult patients, anti retroviral treatment naïve.
4. 30 known HIV negative healthy subjects.

Also, we have been advised by Prof Gary Maartens to collaborate with Dr Joel Dave's research group as they are also looking into lipodystrophy in the above-mentioned groups, and are obtaining the same data that we were looking to obtain (apart from adiponectin levels). We will now be obtaining this data as well as blood samples from stored samples obtained by them from the above-mentioned subject / control groups. This blood will be used for measurement of the different adiponectin levels by Elisa as well as by Western blot. Attached, please find their consent form and patient information sheet.

I trust that this meets with your approval.

Yours sincerely

Dr Fierdoz Omar  
Chemical Pathology Registrar



## ETHICS APPROVAL FOR AMENDMENT TO PROTOCOL



UNIVERSITY OF CAPE TOWN

Health Sciences Faculty  
Research Ethics Committee  
Room E52-24 Groote Schuur Hospital Old Main Building  
Observatory 7925  
Telephone [021] 406 6338 • Facsimile [021] 406 6411  
e-mail: lamees.emjedi@uct.ac.za

11 October 2007

REC REF: 450/2006

Dr Fierdoz Omar & Prof TS Pillay  
Chemical Pathology  
C17  
GSH

Dear Dr Omar & Prof Pillay

**PROJECT TITLE: ROLE OF HIGH MOLECULAR WEIGHT MULTIMERIC ADIPONECTIN  
IN THE DEVELOPMENT OF HIV LIPODYSTROPHY SYNDROME FOLLOWING ANTI-  
RETROVIRAL THERAPY**

Thank you for your letter to the Research Ethics Committee dated 09 October 2007.

It is a pleasure to inform you that the Ethics Committee has approved the amendments to the above-mentioned study.

We note and approved the following:

1. Cover letter, documenting the changes made.
2. Amended Research Protocol.
3. Dr Joel Dave's Patient Information Sheet and Consent Form (REC REF 226/2006).

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

**Please quote the REC. REF in all your correspondence.**

Yours sincerely

**A/PROF. M. BLOCKMAN**  
**CHAIRPERSON, HSF HUMAN ETHICS**

lemjedi

**D.3. PATIENT CONSENT FORM (FROM DR JOEL DAVE'S STUDY)**

**Written informed consent for patient**

**Study number :** \_\_\_\_\_

**Patient initials :** \_\_\_\_\_

I, \_\_\_\_\_

(Name of patient in block letters)

have read and understood all the information given to me about my participation in this study and I have been given the opportunity to discuss it and ask questions. I voluntarily agree to take part in this study and have received an information sheet outlining the details of this study. I understand that I am able to withdraw from this study at anytime.

\_\_\_\_\_  
Signature of patient

\_\_\_\_\_  
Date

\_\_\_\_\_  
Printed name of patient

I have explained the nature and purpose of this study to the patient named above.

\_\_\_\_\_  
Signature of doctor

\_\_\_\_\_  
Printed name of doctor

#### ***D.4. PATIENT INFORMATION SHEET (FROM DR JOEL DAVE'S STUDY)***

##### **Patient information sheet for the HAART Study**

You are invited to participate in a research project.

Before you agree to take part you need to understand the following:

Many HIV-positive patients around the world are treated with special medications collectively called highly active antiretroviral therapy (HAART). Although HAART cannot cure HIV/AIDS it has helped to fight the human immunodeficiency virus and has helped patients feel a lot better. However, HAART also has side-effects. Some of these side-effects have included the development of diabetes, cholesterol problems and changes in body shape (usually either increased or decreased fat in certain areas of the body). Very little is known about these problems in the HIV-positive patients in South Africa. We therefore want to monitor the effects of HAART in patients in Cape Town. This would help the doctors and patients to understand the side-effects of HAART, how commonly they occur and how they can best be treated.

##### **WHAT WILL HAPPEN IF I AGREE TO PARTICIPATE?**

We will ask you not to eat or drink anything from 10 pm the night before your appointment however you must take your morning doses of antiretroviral drugs with a full glass of water. At your appointment you will be required to complete a questionnaire and give some basic medical information about yourself. Following this the doctor will examine you and will then insert a drip cannula (a small plastic device that stays inside the vein allowing all further blood samples to be taken from it thus avoiding any further need for needle pricks). Using a syringe a sample of blood (~ 25 ml, 1 tablespoon) will be taken from the cannula. After taking the blood sample, you will be asked to drink a glass of water with glucose, a form of sugar. Further blood samples will be taken 30 min and 120 min after completing this drink. The blood will be sent to a laboratory and tested for glucose, insulin, cholesterol, lactate and levels of the antiretroviral drugs. Some of the blood will be frozen and kept for testing at a later stage. Whilst waiting for your blood samples to be taken the study staff will do the following: a) using a tape measure they will measure certain areas of your body including your waist, your hips, your height, your upper arm and your thigh b) take blood pressure measurements c) measure your body weight using a scale. At another appointment special X-rays will be organised, called DEXA and CT scans. These scans are done to examine the amount of fat in various areas of your body. All transport to and from the X-rays will be provided. Each test takes less than 30 minutes to complete. All transport expenses will be re-imbursed and participation in the study will cost you nothing. The study staff will also review all previous clinic records including all previous blood test results (including HIV status, viral load, CD4 count, liver function, kidney function, etc)

**WHAT ARE THE POSSIBLE DISCOMFORTS OF PARTICIPATING IN THE STUDY?**

Having blood taken will be the only discomfort in this study. Risk of infection will be minimized by using sterile procedures, and all blood samples will be taken by suitably qualified persons.

**WHAT ARE THE POSSIBLE BENEFITS?**

If you are found to have any of the above-mentioned side-effects of HAART then arrangements will be made for you to be seen at a hospital where doctors specially trained in looking after these problems will look after you. This study will help doctors find these problems early therefore the required investigation and treatment can be done before any further problems arise.

**DO I HAVE TO PARTICIPATE?**

You do not have to participate in this study. Your participation is voluntary and if you agree to participate then you will be required to sign a form. You can withdraw from the study at anytime and this will in no way affect your treatment in the future.

**WILL THE INFORMATION REMAIN CONFIDENTIAL?**

Your records will only be viewed by your doctors and people involved in this study. Your details will not be made available to anybody not involved in this study. Although absolute confidentiality cannot be guaranteed the staff involved in this study will strive to keep your records as confidential as possible.

**CONTACT DETAILS OF STUDY STAFF**

Should you have any questions then please contact

## ***D.5. PROCEDURE FOR MULTIMERIC ADIPONECTIN ANALYSIS (FROM PACKAGE INSERT)***

### **ALPCO™ Adiponectin (Multimeric) EIA**

For the quantitative, selective determination of High Molecular Weight (HMW), Mid-Molecular Weight (MMW) and Total Adiponectin in human serum or plasma.

For Research Use Only. Not For Use In Diagnostic Procedures.

Catalog Number: 47-ADPH-9755

Size: 96 Wells

Storage: 2-10°C

Version: 03/15/06 – ALPCO04/19/06

#### **APPLICATION**

This assay is intended for the quantitative, selective determination of High Molecular Weight (HMW), Mid-Molecular Weight (MMW) and Total Adiponectin in human serum or plasma.

#### **INTRODUCTION**

Adiponectin is a 244 amino acid protein (one of several known adipocytokines) secreted by the adipocyte. It has been reported that adiponectin circulates in the blood in various oligomeric complexes consisting of multiple proteins bound together. These complexes range from dimeric forms to a “bouquet” structure of 9 or more proteins.<sup>1-3</sup> Adiponectin has been reported to have several physiological actions, such as protective activities against atherosclerosis, improvement of insulin sensitivity, and prevention of hepatic fibrosis. In recent years, the relationship of these physiological actions with the circulating multimer structure of adiponectin has been attracting wide attention. It has been reported recently that the ratio of high-molecular weight adiponectin in circulation to total adiponectin level of the subject reflects the condition of disease more clearly than total adiponectin levels alone.<sup>4-6</sup> The structure of adiponectin multimers was elucidated recently by selectively separating each adiponectin multimer from human plasma. This led to the discovery of an albumin-binding trimer, in addition to the already documented trimer, hexamer and HMW forms. Following a further report that part of the multimer form is able to be digested selectively by a certain protease, this kit utilizes a method of measurement incorporating pretreatment with proteases for selective measurement of human multimeric adiponectin.<sup>7</sup> In addition, in order to provide a reference for total adiponectin levels, a new, simple pretreatment method is utilized. By using this method, multimeric adiponectin in the serum is converted mainly to a dimer via the addition of an SDS-containing acid buffer (without boiling step).<sup>8</sup>

#### **ASSAY PRINCIPLES**

This kit operates on the principle of a “sandwich” format enzyme-linked immunosorbent assay (ELISA). The specific antibodies used in the kit are anti-human adiponectin monoclonal antibodies (MoAbs) directed to two independent epitopes. The specimens are pre-treated as described below, and total adiponectin and individual multimers of adiponectin are determined selectively, directly or indirectly. Multimers of adiponectin are classified into four fractions with this kit:

- 1) Total adiponectin fraction: “Total-Ad”-assayed directly on the plate
- 2) High-molecular adiponectin fraction (equivalent of dodecamer -octodecamer): “HMW-Ad”-assayed directly on the plate

3) Middle-molecular adiponectin fraction (equivalent of hexamer): “MMW-Ad”-inferred value obtained by subtracting the concentration of HMW-Ad from the combined concentration of MMW-Ad + HMW-Ad

4) Low-molecular adiponectin fraction (equivalent of trimer including albumin-binding adiponectin): “LMWAd”- inferred value obtained by subtracting the combined concentration of MMW-Ad + HMW-Ad from the total concentration of Ad.

The microtiter plate wells have been coated with an anti-human adiponectin monoclonal antibody. Adiponectin in the standards and pretreated specimens are captured by the antibody during the first incubation. Afterwards, a wash step removes all unbound material. Subsequently, an anti-human adiponectin antibody that has been biotin-labeled is added and binds to the immobilized adiponectin in the wells. After the second incubation and subsequent wash step, HRP-labeled streptavidin is added. After the third incubation and subsequent wash step, substrate solution is added.

Finally, stop reagent is added after allowing the color to develop. The intensity of the color development is read by a microplate reader. The absorbance value reported by the plate reader is proportional to the concentration of adiponectin in the sample.

In this kit, pre-treated normal human serum by sample pre-treatment buffer is used as the Calibrator. The sample Pre- Treatment procedure used in this assay is very important for good results. It is outlined here briefly, and explained further in the Assay Procedure section of this insert.

1) “Total-Ad” assay: Specimens are treated with SDS-containing acid buffer to convert multimeric adiponectin mainly to a dimer form.

2) For “HMW-Ad” assay: The protease that selectively digests LMW-Ad and MMW-Ad acts on specimens, and remaining MW-Ad fraction is treated with SDS-containing acid buffer to convert it to a dimer form. Upon addition of the SDS-buffer, the digestive reaction of protease is stopped.

3) For combined “MMW-Ad” and “HMW-Ad” assay: The protease that selectively digests only LMW-Ad acts on specimens, and remaining MMW-Ad and HMW-Ad are treated with SDS - containing acid buffer to convert these fractions to a dimer form. Upon addition of the SDS-buffer, the digestive reaction of protease is stopped.

#### **KIT COMPONENTS**

Reagent Composition Amount Part Number

Wash buffer concentrate Phosphate buffer (pH 7.2) 100 ml x 1 vial 975513-001

Sample pre-treatment buffer

Citrate buffer (pH 3.0) containing SDS 50 ml x 1 vial 975520-001

Dilution buffer Phosphate buffer (pH 7.2) containing BSA 100 ml x 1 vial 975537-001

Monoclonal Ab coated plate

Anti-human Adiponectin mouse monoclonal Ab coated 96-well 1 plate 975544-001

Calibrator Human serum stablized in sample pre-treatment buffer 0.25 ml x 1 vial 975551-001

Biotin Labeled MoAb Biotin Conjugated anti-human adiponectin 6.0 mL x 1 vial 975568-001

Enzyme-labeled streptavidin

Horse-radish peroxidase (HRP)- labeled streptavidin 6.0 ml x 1 vial 975575-001

Substrate (lyophilized) O-phenylenediamine 2 vials 975582-001

Substrate buffer Citrate buffer (pH 5.0) containing H<sub>2</sub>O<sub>2</sub> 15 ml x 1 vial 975599-001

Stop reagent 7.7% H<sub>2</sub>SO<sub>4</sub> 15 ml x 1 vial 975605-001

Protease I (lyophilized) Protease 1 vial 975612-001

Protease II (lyophilized) Protease 1 vial 975629-001

Protease buffer Tris buffer (pH 8.0) 50 ml x 1 vial 975636-001

#### **REAGENT PREPARATION AND STORAGE**

All reagents must be allowed to reach room temperature prior to use.

##### **a) Wash buffer**

Dilute Wash buffer concentrate with 900mLof purified water. Working Wash buffer should be stored at 2-10 °C.

**b) Sample pre-treatment buffer**

A white precipitate may form in this vial. This precipitate will be completely dissolved by warming the solution to room temperature and thoroughly stirring it before use.

**c) Dilution buffer**

Dilution buffer is supplied ready-to-use.

**d) Monoclonal-antibody coated wells**

MoAb coated wells are supplied ready-to-use. The unused strips should be returned to the laminate bag and stored at 2-10 °C.

**e) Working Calibrator**

**Note:** There may be a precipitate in the tube. Allow the solution to stand at room temperature and stir thoroughly before using. Avoid foaming.

Just prior to use, dilute the Calibrator 1:101 with Dilution buffer and further the serial dilution to create a standard curve:

\*\*Standard number 1 is the Stock calibration material included with the kit, diluted 1:101. Actual concentration (diluted 1:101) is found on the vial label; subsequent standards must be calculated from this value.

Standard number:	Calibration material to add:	Diluent to add:
1	150 µl of stock solution	0 µl
2	150 µl of standard 1	150 µl
3	150 µl of standard 2	150 µl
4	150 µl of standard 3	150 µl
5	150 µl of standard 4	150 µl
6	150 µl of standard 5	150 µl
7	150 µl of standard 6	150 µl
8	0	150 µl

\* The remaining Calibrator should be stored at 2-10 °C and the same procedure should be repeated when it is to be used again.

\* Since precipitation is temporarily caused when Calibrator is added to Dilution buffer, stir it thoroughly.

\* The working calibrator should be prepared at the same time as the specimen treatment and dilution. Add the treated specimen and the working calibrator to the MoAb coated wells in succession.

**f) Biotin labeled-MoAb**

Biotin labeled-MoAb is supplied ready-to-use.

**g) Enzyme labeled streptavidin**

Enzyme labeled streptavidin solution is supplied ready-to-use.

**h) Substrate solution**

Just prior to use, reconstitute the Substrate (lyophilized) by adding 6 mL of substrate buffer to the substrate vial. The substrate solution should be used immediately after reconstitution and the remaining solution should be discarded.

**i) Stop reagent**

Stop reagent is ready-to-use.

**j) Protease solution I**

Reconstitute Protease I (lyophilized) by adding 10 mL of protease buffer to the vial and dissolve completely by mixing at room temperature for 15-30min. Protease I is stable for 2 days at 2-10 °C. Freeze below -30 °C for extended storage.

**k) Protease solution II**

Reconstitute Protease II (lyophilized) by adding 10 mL of protease buffer to the vial and dissolve completely by mixing at room temperature for 15-30 min. Protease II is stable for 2 days at 2-10 °C. Freeze below -30 °C for extended storage.

**ASSAY PROCEDURE**

### 1) Pretreatment of specimens

Each specimen may be treated by any combination of the pretreatment options below, but separate aliquots must be used for each option. For example, if HMW and total adiponectin concentrations are being quantified on the same sample, treat two aliquots of the sample: one by the “total-Ad” procedure and one by the “HMW-Ad” procedure.

•**Pre-treatment option 1: For “Total-Ad” assay**

Add **100 µl** of Protease Buffer (Tris buffer, pH 8.0) and **400 µL** of Sample Pre-treatment Buffer to **10 µL** of serum or plasma. Stir thoroughly (sample dilution = 1:51).

•**Pre-treatment option 2: For combined “MMW-Ad” and “HMW-Ad” assay**

Add **100 µL** of Protease Solution I to **10 µL** of serum or plasma and incubate for 20 min at 37°C. Immediately add **400 µL** of Sample Pre-Treatment Buffer. Stir thoroughly (sample dilution = 1:51).

•**Pre-treatment option 3: For “HMW-Ad” assay**

Add **100 µL** of the Protease Solution II to **10 µL** of serum or plasma and incubate for 20 min at 37°C. Immediately add **400 µL** of the Sample Pre-Treatment Buffer. Stir thoroughly (sample dilution = 1:51).

### 2) Dilution of Pretreated specimens

Further dilute pre-treated specimens 1:101 as follows:

Add **10 µL** of the pre-treated specimen obtained in Steps 1 to 3 (see “Pretreatment of Specimens”) to 1.0 ml Dilution Buffer (Phosphate Buffer + BSA, pH 7.2). **FINAL Sample dilution = 1:5151.**

\* **Since precipitation is temporarily caused when a pretreated specimen is added to Dilution buffer, stir thoroughly.**

### 3) Assay Method

- 1) Plan your plate configuration. Determine how many strips will be needed and remove the excess strips from the microtiter plate. Store the remaining strips in the laminate bag.
- 2) Add 50 µL each of the working calibrator and diluted samples to the appropriate wells, according to the plate configuration.
- 3) Cover the plate with a plate sealer and incubate for 1 hour at room temperature (20-30 °C).
- 4) Decant the plate and strike the plate against absorbent towels to remove any excess liquid. Do not introduce absorbent materials into the wells! Wash by adding 350-400 µL of Wash Buffer to each well (using a laboratory squeeze bottle, wash manifold, or automated plate washer); decant wash buffer and strike plate against absorbent towels to remove residual liquid. Repeat this cycle twice, for a total of 3 washes.
- 5) Add 50 µL of Biotin-labeled monoclonal antibodies to each well. Cover the plate with a plate sealer and incubate for 1 hour at room temperature (20-30 °C).
- 6) Repeat wash step as described in Step 4.
- 7) Add 50 µL of the Enzyme-labeled streptavidin to each well. Cover the plate with a plate sealer and incubate for 30 min at room temperature (20-30 °C).
- 8) Repeat wash step as described in Step 4.
- 9) Add 50 µL of the Substrate solution to each well. Protect the plate from light and incubate for 10 min at room temperature (20-30 °C).
- 10) Add 50 µL of the Stop solution to each well.
- 11) Using a microtiter plate reader set to 492 nm, read the absorbance of each well. Use a reference wavelength of 600-700 nm if desired.

### 4) Calculations

Calculate the  $\Delta$  absorbance by subtracting the absorbance of the 0 ng/mL calibrator from those of other calibrators and diluted samples. Plot the  $\Delta$  absorbance of calibrators against the calibrator concentration on log-log or semi-log graph paper. Draw a smooth curve through these points to construct the calibration curve. Read the concentrations for the Diluted samples from the calibration curve. Calculate the concentration for the Diluted samples by multiplying by dilution factor (1:5151). Each fraction of Adiponectin multimers are calculates as follows.

-----



- “Total-Ad”: Concentration of the specimen prepared by Pre-treatment option 1.
- “HMW-Ad”: Concentration of the specimen prepared by Pre-treatment option 3.
- “MMW-Ad”: Concentration of the specimen prepared by Pre-treatment option 2 (HMW + MMW Ad) minus the concentration of the specimen prepared by Pre-treatment option 3 (HMW-Ad)
- “LMW-Ad”: Concentration of the specimen prepared by Pre-treatment option 1 (Total-Ad) minus the concentration of the specimen prepared by Pre-treatment option 2 (HMW + MMW Ad).

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### PROCEDURAL NOTES

1. This kit has been validated for analyzing human serum, EDTA-plasma or heparinized plasma. Citrated plasma must not be used due to unqualified lower results.
2. Measurements of different adiponectin species of the same serum or plasma samples **MUST** be in the same plate. **DO NOT** calculate results between different plates.
3. A calibration curve must be run with each assay. Calibrators and samples should be assayed in duplicate.
4. If the concentration of adiponectin in a specimen exceeds the highest point of the calibration curve range, dilute pretreated specimen further with Dilution Buffer and re-assay.
5. Observe all specified reaction times and temperatures outlined in this manual. These parameters are especially important while pre-treating samples with proteases.
6. If the kit is not used entirely during the premier run, the remaining reagents may be stored as directed in the package insert and used one subsequent time before the expiration date.
7. Samples must be run promptly after the addition of the Sample pre-treatment buffer and added to the MoAb coated wells.
8. Remove all residual liquid completely after each step of the wash procedure.
9. Do not allow the wells to dry out or be damaged during the washing procedure.
10. Avoid carrying out this procedure in direct sunlight.

### WARNINGS AND PRECAUTIONS

1. The human serum contained in the calibrator was tested and found negative for presence of the HBs antigen, HIV antibody, and HCV antibody, however all specimens should be handled carefully as though capable of transmitting infection.
2. Stop reagent (7.7% H<sub>2</sub>SO<sub>4</sub>) is hazardous and can cause severe burns. In case of eye contact, rinse immediately with plenty of water, and seek medical advice. In case of contact with skin or clothing, rinse immediately with plenty of water.

### STORAGE OF REAGENTS

The kit reagents should be stored at 2-10°C. **DO NOT FREEZE.**

### **D.6. OUTCOMES ACHIEVED**

**Findings or parts thereof were presented at the following:**

- 1. American Association for Clinical Chemistry Annual Meeting and Lab Expo, July 2009; Chicago USA – POSTER PRESENTATION\* (Abstract B)**
- 2. University of Cape Town School of Biomedical Sciences 3<sup>rd</sup> Research Day – POSTER PRESENTATION, September 2008; POSTER PRESENTATION\*\* (Abstract A)**
- 3. Congress of the Federation for South Societies of Pathology – Update 48, July 2008; Cape Town, South Africa – ORAL PRESENTATION\*\*\* (Abstract A)**

**Abstracts included below.**

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**\*Travel award won for presentation of this work.**

**\*\*Best poster award won**

**\*\*\*Awarded the Beckman Coulter Postgraduate Bursary Award won for this work.**

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#### **ABSTRACT A**

Antiretroviral protease inhibitors decrease high molecular weight / total adiponectin ratios in HIV positive patients

Omar F<sup>1</sup>, Dave JA<sup>2</sup>, King JA<sup>1</sup>, Levitt NS<sup>2</sup>, Pillay TS<sup>1</sup>

<sup>1</sup>Division Chemical Pathology, UCT, NHLS & Groote Schuur Hospital, <sup>2</sup>Division Endocrinology, Department of Medicine, UCT

Introduction: Antiretroviral therapy in HIV patients (especially protease inhibitors (PIs)) is associated with the lipodystrophy syndrome, with low total adiponectin (TA) levels reported. The high molecular weight (HMW):TA ratio is a better marker of insulin resistance. Aim: To establish whether HMW:TA ratios were lower in HIV positive patients receiving PIs compared with those not on PIs. Methodology: 66 HIV positive Xhosa female patients were recruited: 22 on PIs for at least 6 months, 22 on non-PIs (NPIs) for the same period, and 22 treatment naïve (TN). The NPI and TN groups were BMI- and age-matched to the PI group. Adiponectin levels (TA, HMW, medium molecular weight (MMW) and low molecular weight (LMW)) were analysed on serum

samples using the Alpco Adiponectin (Multimeric) enzyme immunoassay. Data were non-parametrically analysed. **Results:** The PI and NPI groups had significantly lower HMW adiponectin levels than the TN group (median 2.23 and 3.49 vs 5.29mg/l;  $p<0.005$ ). Furthermore, the TA was statistically lower in HIV patients in both treatment groups compared with the TN group ( $p<0.05$ ). HMW:TA ratios were significantly lower in the PI group than in both the NPI ( $p<0.05$ ) and TN ( $p<0.0001$ ) groups. **Conclusion:** Low HMW:TA ratios in HIV positive patients on PIs may be associated with the development of lipodystrophy in these patients.

### **ABSTRACT B**

High molecular weight / total adiponectin ratios are decreased in HIV-infected women receiving protease inhibitors

Omar F<sup>1</sup>, Dave JA<sup>2</sup>, King JA<sup>1</sup>, Levitt NS<sup>2</sup>, Pillay TS<sup>1</sup>

<sup>1</sup>Division of Chemical Pathology, University of Cape Town, National Health Laboratory Service (NHLS) & Groote Schuur Hospital, <sup>2</sup>Division of Endocrinology, University of Cape Town

**Relevance:** We use two Highly Active Anti-Retroviral Therapy (HAART) regimens for the treatment of HIV. Regimen 1 contains 2 nucleotide reverse transcriptase inhibitors (NRTIs) (stavudine and lamivudine) and a non-NRTI (efavirenz), while regimen 2 contains a protease inhibitor (PI) (liponavir/ritonavir) and 2 NRTIs (zidovudine and didanosine). HAART (including PIs and NRTIs) is associated with the lipodystrophy syndrome, with insulin resistance as an important feature. Furthermore, total adiponectin (TA), an insulin sensitizing hormone is also decreased in these patients. Recently, the high molecular weight (HMW) form of adiponectin has been shown to be the active form and the HMW:TA ratio is known to be a better marker of insulin resistance than either form individually.

**Objectives:** To establish whether HMW:TA ratios are lower in HIV patients receiving PIs, compared with those not on PIs and to establish whether the HMW:TA correlates with other markers of insulin resistance in these patients.

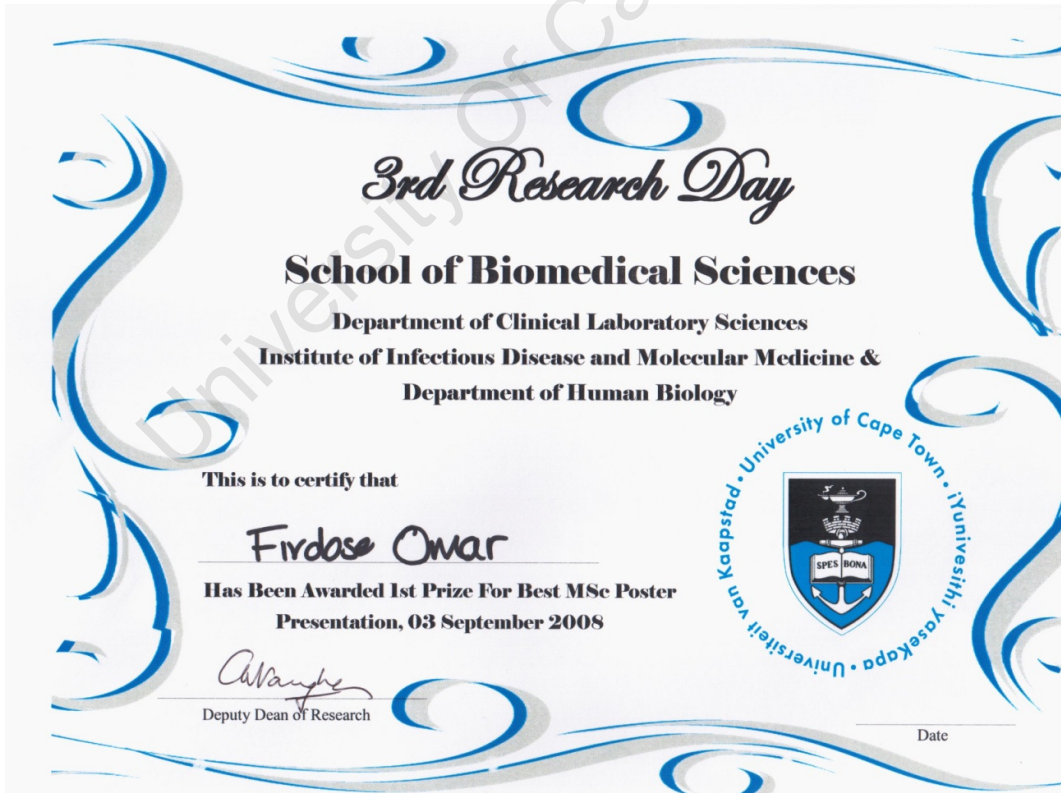
**Methodology:** 66 HIV-infected African females were recruited: 22 on regimen 2 (PI group) for at least 6 months, 22 on regimen 1 (non-PI group) for the same period and 22 treatment naïve (TN). All the groups were matched for BMI and age. Patients with overt diabetes, renal failure or cardiovascular disease were excluded. Waist hip ratios (WHR)

were measured. Adiponectin levels (TA and HMW) were analysed on fasted serum samples using the Alpco Diagnostics<sup>TM</sup> Adiponectin (Multimeric) enzyme immunoassay. Glucose (fasting and 2h post 75g oral glucose) and fasting insulin were measured and mathematical models of insulin resistance (HOMA-IR, HOMA-β% and QUICKI)

calculated. Data were analysed non-parametrically.

Results: The PI and non-PI groups had significantly lower HMW adiponectin levels than the TN group (median 2.23 and 3.49, respectively, vs 5.29mg/l;  $p<0.005$ ), but did not differ significantly from each other. Similarly, TA was significantly lower in both treatment groups compared with the TN group (median 5.64 and 7.3, respectively, vs 9.03mg/L;  $p<0.05$ ), but did not differ significantly between the PI and non-PI groups. In contrast, the HMW:TA ratio was significantly lower in the PI group than in both the NPI ( $p<0.05$ ) and TN ( $p<0.0001$ ) groups, and was also lower in the non-PI than in the TN group ( $p<0.05$ ). Insulin, glucose, HOMA-IR, HOMA-β% and QUICKI did not differ significantly amongst the groups. The HMW:TA ratio correlated negatively with WHR ( $p<0.005$ ) and fasting insulin ( $p<0.005$ ). A negative, though not statistically significant, correlation was also found between HMW:TA ratio and fasting and 2h glucose levels, as well as age and BMI.

Conclusion: These data demonstrate that both PI- and non-PI containing HAART regimens significantly lower the HMW:TA ratio in HIV patients, with the ratio more significantly decreased in the PI-containing regimen, implying that PIs and NRTIs have an additive effect on the HMW:TA ratio. Although the HMW:TA ratio correlated negatively with indirect markers of insulin resistance, no overt insulin resistance was demonstrated. HMW:TA ratio may therefore be a more sensitive marker of insulin resistance in these patients. (Funding was provided by the NHLS, UCT and NRF.)



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48<sup>th</sup> Annual Congress of the Federation of the South African Societies of Pathology



University Of Cape Town

## ***D.7. CLINICAL CHEMISTRY GUIDELINES FOR SUBMISSION OF MANUSCRIPT***

This is an abridged version of the guidelines. The full version may be found at the following web address:

[http://www.clinchem.org/info\\_ar/info\\_authors.shtml](http://www.clinchem.org/info_ar/info_authors.shtml)

### **Clinical Chemistry**



#### **Information for Authors**

*Revised January 2010*

#### **Overview**

*Clinical Chemistry*, issued monthly, is published in print and electronically by the American Association for Clinical Chemistry. The journal welcomes contributions, either experimental or theoretical, in the field of laboratory medicine. It is the leading forum for peer-reviewed, original research on innovative practices in today's clinical laboratory. In addition to being the most cited journal in the field, *Clinical Chemistry* has the highest Impact Factor among journals of clinical chemistry, clinical (or anatomic) pathology, analytical chemistry, and the subspecialties, such as transfusion medicine and clinical microbiology.

Submissions of the following nature are welcomed:

- Basic materials or principles
- Analytical techniques
- Molecular diagnostics
- Test utilization or testing-related health or financial outcomes
- Instrumentation
- Data processing
- Statistical analyses of data
- Clinical investigations in which laboratory testing has played a major role

- Laboratory animal studies of chemically-oriented problems of human disease

Contributions should consist of subject matter that is original and significantly advances the state of knowledge of clinical chemistry, and conclusions that are justified from the design of the experiments and the data presented. The information must be sufficiently detailed to permit replication of the work by a competent worker in the field. Lastly, the writing must be clear, concise and grammatically correct.

Equal consideration is given to original manuscripts in English from any country, regardless of membership in the Association. It is, however, advised that all non-English speaking authors enlist the aid of a native-English speaking colleague to correct English language usage before submission. Submissions must adhere to the “Uniform Requirements for Manuscripts Submitted to Biomedical Journals” (1).

Submissions are accepted via the manuscript tracking system at <http://submit.clinchem.org>. The “Information for Authors” will offer assistance with journal style and requirements. Please contact the Editorial Office via e-mail should you have any questions or need assistance: [clinchemed@clinchem.aacc.org](mailto:clinchemed@clinchem.aacc.org)

#### References:

1. International Committee of Medical Journal Editors. Uniform requirements for manuscripts submitted to biomedical journals. *Ann Intern Med* 1997;126:36-47. [\[Full Text\]](#)

#### ***Standards for Reporting Scientific Data***

- [Statistics](#)
- [Studies with Human Subjects](#)

#### ***Statistics***

Describe statistical methods with enough detail to enable a knowledgeable reader with access to the original data to verify the reported results.

When possible, quantify findings and present them with appropriate indicators of measurement error or uncertainty.



Avoid sole reliance on statistical hypothesis testing, such as the use of P values, which fails to convey important quantitative information.

When appropriate, confidence intervals should be presented.

### ***Studies with Human Subjects***

Authors are responsible for ensuring compliance of human studies with the Helsinki Declaration of 1975 as revised in 1996:

[http://www.wma.net/e/humanrights/policy\\_meetings.htm](http://www.wma.net/e/humanrights/policy_meetings.htm). Approval by the appropriate institutional committee on human research (Institutional Review Board) must be documented in the manuscript and, unless excepted by that committee, informed consent of all participants studied for the report must be included.

### ***Article***

Research or scientific articles are submitted directly from authors. There are no restrictions on topics that are considered for publication, as long as the subject matter is original and relates experimentally or theoretically to the field of laboratory medicine. The information must be sufficiently detailed so that readers not only can understand and appreciate the material presented, but also permit replication of the work by other scientists in the field.

Articles should consist of a structured abstract limited to 250 words while the main text should not exceed 3,500 words. The manuscript should have no more than 40 references and a total of 6 tables and/or figures. Supplemental data are permitted for Articles.

### ***Manuscript Preparation***

- [Author Contribution Requirements](#)
- [General Guidelines](#)
- [Journal Categories](#)

- [Title Page](#)
- [Abstract](#)
- [Text](#)
- [Disclosures/Conflict of Interest](#)
- [Acknowledgements](#)
- [References](#)
- [Tables](#)
- [Figure Captions](#)
- [Figures](#)
- [Color](#)
- [Supplemental Data](#)

### ***Author Contribution Requirements***

Manuscripts are considered for publication with the understanding of the following:

1. Each author has participated significantly in the work in a substantive way and is prepared to take public responsibility for its content;
2. Each listed author must have
3. participated in conception, design, analysis, or interpretation;
4. drafted or critically revised the manuscript; and
5. read and approved the final submitted manuscript and revisions.

Any change in authors and/or contributors after initial submission must be approved by all authors. This applies to additions, deletions, change of order to the authors, or contributions being attributed differently.

Any alterations made to the manuscript after submission must be approved by the editor. Authors may upload the request letter to the online submission system as a supplemental file or send the letter via e-mail to the *Clinical Chemistry* editorial office; [clinchemed@clinchem.aacc.org](mailto:clinchemed@clinchem.aacc.org). The editor may contact any of the authors and/or contributors to ascertain whether they have agreed to any alteration.

1. The International Committee of Medical Journal Editors (ICMJE) Uniform Guidelines for Manuscripts Submitted to Biomedical Journals (1) specifically state that "all contributors who do not meet the criteria for authorship, such as a person who . . . provided purely writing assistance" be named in the acknowledgments.
2. Important contributions to an article should be recognized and appropriately attributed in that article.

Good medical writers and editors can make valuable contributions to the publication process, often improving the clarity of the communication, broadening the scope of literature review, providing an extra level of data review, adding balance and objectivity, and shortening the time needed for manuscript development.

The American Medical Writers Association (AMWA) <http://www.amwa.org> believes that these important contributions deserve recognition.

3. Readers benefit from knowing about the involvement of professional writers and editors.

Disclosing the editorial contribution and the source of funding of the writer and editor allows the reader to make informed judgments about the objectivity of the article.

Note that the AMWA position statement recommends acknowledgment of pertinent professional or financial relationships as well as acknowledgment of the contributions of writers and editors.

It also recommends that the person being acknowledged be given the opportunity to grant or refuse permission for the acknowledgment.

#### References:

1. International Committee of Medical Journal Editors. Uniform requirements for manuscripts submitted to biomedical journals. *Ann Intern Med* 1997;126:36-47. [\[Full Text\]](#)

#### ***Manuscript Guidelines***

- MS Word document (.doc) is required for all submissions.
- All figures must be uploaded separately as Image Files in Tagged Image File Format (.tiff), Encapsulated Postscript (.eps) or PowerPoint (.ppt) with embedded fonts.
- All submissions must be double-spaced, 1 inch margin, twelve-point font size in Arial, Helvetica, Times New Roman and Symbol font (for non-text characters).
- All submissions must be page numbered.
- Do not use headers or footers.
- Use [standard abbreviations](#) and define all nonstandard abbreviations.
- All submissions require a title page.
- SI units must be used throughout your submission. Conventional units may only be added as additional information in parentheses after the SI units. SI units are available at [Bureau International des Poids et Mesures](#).
- Supplemental Data are accepted for online publication only and is limited by submission types (See [Types of Submissions](#) for details).

- Follow the guidelines for length restrictions, abstract, reference, table and figure, and supplemental data limits as outlined in the chart below:

Type of Submission	Word Limit*	Structured** (S) or Unstructured (U) Abstract:Word Limit	Maximum Number of References	Total Number of Tables/Figures	Supplemental Data Permitted
<b>Article</b>	<b>3,500</b>	<b>S: 250</b>	<b>40</b>	<b>6</b>	<b>Yes</b>
Bookshelf	500	Non Applicable	5	1	No
Brief Communication	1,500	S: 250	20	1 each***	Yes
Citation Classics	700	Non Applicable	6	Non Applicable	No
Clinical Case Studies (Case description) w/ 3-5 questions and up to 5 points to remember	1,000 (500)	Non Applicable	10	2	No
Commentary	300	Non Applicable	Non Applicable	Non Applicable	No
Editorial	1,500	Non Applicable	15	Non Applicable	No
Letter to the Editor / Reply	750	Non Applicable	5	1***	No
Mini-Review	3,500	S: 250	40	4	Yes
Obituary	600	Non Applicable	Non Applicable	1	No
Opinion	1,500	Non Applicable	15	1	No
Perspective	1,500	Non Applicable	5	1	No

Point/Counterpoint	1,500	Non Applicable	15	1	No
Reflection	2,000	Non Applicable	20	1	No
Review	5,000	S: 250	75	6	Yes
Special Report	5,000	S or U: 250	40	4	Yes

\*Word limit consists of the body of the manuscript only; it does not encompass the title page, abstract, acknowledgments, references, tables, figure legends, figures, or Clinical Case descriptions, questions, and points to remember.

\*\*Structured abstracts contain the headings (1)BACKGROUND, (2)METHODS, (3)RESULTS, (4)CONCLUSIONS for all applicable article types except for Reviews and Mini-Reviews. Abstracts for Reviews and Mini-Reviews contain the headings (1)BACKGROUND, (2)CONTENT, (3)SUMMARY.

\*\*\*If a figure accompanies the paper, the image should not be multipart (i.e., Fig. 1A, 1B, 1C, Part 1, Part 2).

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### ***Journal Categories***

Articles are grouped in the journal according to subject. Upon submission, authors are required to select the journal category that best describes their manuscript from the list indicated below:

- Molecular Diagnostics and Genetics (MDG)
- Evidence-Based Laboratory Medicine and Test Utilization (TUO)
- Hemostasis and Thrombosis (HAT)
- Proteomics and Protein Markers (PPM)
- Cancer Diagnostics
- Lipids, Lipoproteins, and Cardiovascular Risk Factors (LLP)
- Drug Monitoring and Toxicology (DMT)
- Hematology (HEM)
- Endocrinology and Metabolism (END)
- Point-of-Care Testing
- Automation and Analytical Techniques (AAT)
- Informatics and Statistics
- Laboratory Management (LMA)
- General Clinical Chemistry (GCC)
- Animal Clinical Chemistry (ANI)
- Clinical Immunology (CLI)

- Pediatric Clinical Chemistry (PED)
- Nutrition (NUT)
- Infectious Disease
- Other Areas of Clinical Chemistry (OTH)

### ***Title page***

The first page of the manuscript should include the following information: (1) full title of submission, which should include only generic, not trade, names when describing a test, assay, etc.; (2) running head of fewer than 65 characters (including spaces); (3) list of all authors (first name, middle initial, last name in that order); (4) names of each author's institution and an indication of each author's affiliation; (5) name, address, telephone and fax number, and e-mail address of the corresponding author; (6) keywords; (7) list any previous presentation of the manuscript; (8) list abbreviations, in order cited; (9) list any "Human Genes" discussed in the paper. For each gene, indicate the gene symbol and gene name approved by the [HUGO Gene Nomenclature Committee](#). Include other name(s) that are used in the paper or are widely used in the literature for the gene.

### ***Abstract*** (Structured and Unstructured)

Structured abstracts should be formatted to include separate headings of: (1) BACKGROUND, (2) METHODS, (3) RESULTS, and (4) CONCLUSION. For Mini-Review and Review articles the headings should be: (1)BACKGROUND, (2) CONTENT, and (3) SUMMARY.

Unstructured abstracts do not require separate headings.

### ***Text***

The body of the manuscript should be written as concisely as possible and must not exceed the manuscript category word limits described herein. All pages must be double-spaced and all lines numbered. The body of the paper should include: Introduction, Materials and Methods, Results and Discussion.

- Introduction - why was the study undertaken?

- Materials and Methods - how was the study done?
- Results - what did the study find?
- Discussion - what might it mean, why does it matter, what next?

Full corporate names of manufacturers of materials should be utilized and should include the city, state, and e-mail or website of the company.

SI units are required; however, conventional units may follow in parenthesis.

Use of human subjects requires a statement in the text indicating whether the procedures followed were approved by your institution's responsible committee or were in accordance with the current revision of the Helsinki Declaration and whether subjects gave informed consent.

### ***Disclosures/Conflict of Interest***

All authors are required to complete a full disclosure form upon submission. The disclosure form is electronic and completed during the submission process within the Bench>Press submission system. Complete disclosures must be submitted electronically, and should not be included within the submitted manuscript. Failure to adhere to this rule may result in a return of the submission to the author for correction.

In order to complete disclosures, registration with *Clinical Chemistry* is required for all authors.

The American Medical Writers Association (AMWA) recognizes the valuable contributions of biomedical communicators to the publication team. Biomedical communicators who contribute substantially to the writing or editing of a manuscript should be acknowledged with their permission and with disclosure of any pertinent professional or financial relationships. In all aspects of the publication process, biomedical communicators should adhere to the AMWA code of ethics at <http://www.amwa.org/default.asp?>.

### ***Acknowledgments***

Acknowledgments are limited to 60 words and should follow the main text of the

manuscript directly above the reference section in a separate paragraph heading labeled “Acknowledgments.” They should not appear as footnotes.

### *References*

References should appear in a separate section directly following the body of the manuscript. The section must be labeled “References” with no additional punctuation.

- Italics or boldface type is prohibited in the referenced citations.
- List and number the references in the order that they appear in the text.
- Do not use the MS Word document (.doc) numbering tool. Number each reference manually with the numeral, a period, followed by a space.
- For articles with more than seven authors, list the first six authors followed by "et al." For seven or fewer, list all authors.
- Authors’ names are inverted (last name, first/second initial). Do not add periods or commas within an individual author name; however, separate author names with a comma and end the author list with a period (Smith J, Doe JJ, Adams B.).
- Capitalize only the first word of the title or subtitle, and any proper names that are part of the title. The title should end with a period.
- The Journal names should be abbreviated as indicated at PubMed. For a list of journal abbreviations, please visit: [LinkOut Journals](#).
- Do not add a period after the journal abbreviation, but continue with a space followed by the year.
- The year should be followed by a semicolon and then the volume number which is followed by a colon and then the page numbers; delete redundant numbers, for example 1998;12:231-45.
- Do not include the months in parentheses, this information is not needed.
- Use inclusive page numbers for articles and book chapters.

Abstract and supplement numbers should be provided, if applicable. Citations of unpublished abstract books, manuscripts in preparation or under review, personal communications, and manufacturers’ information should only be cited in the text and should not appear in the reference list. Personal communications should also be listed parenthetically and should contain the first initial and last name of the contact as well as the month and year of the communication. Published manuscripts and manuscripts that have been accepted and are pending publication should be cited in the reference list.

In press references cited in the reference list must be accompanied by a copy of the cited manuscript and a letter of acceptance, or a complete author proof from the publisher. These resources should be uploaded as supplemental data along with the manuscript and other print materials.



The submission system will extract the references from the submitted MS Word document (.doc) to display in a hyperlinked HTML format as an aid for reviewers and editors. This linking option allows for the checking of the correct formatting and the accuracy of the citations.

Authors must check the linking of their references to PubMed during the “Ready for You to Proof” stage of submission. Correct linking of the references depends on strict adherence to Journal style as indicated.

### Reference Style

- *Journal article with seven or fewer authors:*
  1. Vermeersch P, Mariën G, Bossuyt X. A case of pseudoparaproteinemia on capillary zone electrophoresis caused by geloplasma. Clin Chem 2006;52:2309-11.
- *Journal article with more than seven authors:*
  2. Fiechtner M, Ramp J, England B, Knudson MA, Little RR, England JD, et al. Affinity binding assay of glycohemoglobin by two-dimensional centrifugation referenced to hemoglobin Alc. Clin Chem 1992;38:2372-9.
- *Abstract:*
  3. Hortin GL, King C, Kopp J. Quantification of rhesus monkey albumin with assays for human microalbumin [Abstract]. Clin Chem 2000;46:A140-1.
- *Editorial:*
  4. Demers LM. New biochemical marker for bone disease: is it a breakthrough? [Editorial]. Clin Chem 1992;38:2169-70.
- *Letter to the Editor:*
  5. Davey L, Naidoo L. Urinary screen for acetaminophen (paracetamol) in the presence of N-acetylcysteine [Letter]. Clin Chem 1993;39:2348-9.
- *Book Chapter:*
  6. Rifai N, Warnick GR. Lipids, lipoproteins, apolipoproteins, and other cardiovascular risk factors. In: Burtis CA, Ashwood ER, Bruns DE, eds. Tietz textbook of clinical chemistry and molecular diagnostics. 4th Ed. St. Louis: Elsevier Saunders 2006:903-81.
- *Thesis:*
  7. Houghton MA. Immunonephelometric measurement of vitamin D binding protein [MAppSci thesis]. Sydney, Australia: University of Technology, 1989:87pp.

- *Books and Monographs (serial volumes should be treated as journals):*
  8. Harrell FE Jr. Regression modeling strategies. New York: Springer; 2001.
  9. Bailar JC III, Mosteller F, eds. Medical uses of statistics, 2nd Ed. Boston: NEJM Books; 1992:449pp.
- *Publish-Ahead of Print:*
  10. Milbury CA, Li J, Makrigiorgos GM. PCR-based methods for the enrichment of minority alleles and mutations. [Epub ahead of print] Clin Chem February 6, 2009 as doi:10.1373/clinchem.2008.113035.
- *Supplement:*
  11. Castelli WP. Lipids, risk factors and ischaemic heart disease. Atherosclerosis 1996;124 Suppl:S1-9.
- *Internet Source:*
  12. American Association for Clinical Chemistry. AACC continuing education. <http://www.aacc.org/services/default.stm> (Accessed November 1998).

### **Tables**

Tables are considered text. They should appear embedded as part of the submission directly following the reference section. Each table should appear on an individual page. Manuscripts will be returned to authors if the tables are uploaded as separate files.

Each table should be clearly labeled and numbered consecutively with Arabic numerals (Table 1, Table 2, etc.). The abbreviations used must be defined. As with the text, SI units must be used; however, conventional units may be added in parentheses. Conversion factors may be added as footnotes. Indicate explanatory footnotes with superscript lowercase italic letters in alphabetical order.

Tables should not include parts. Tables with parts (Table 1A, Table 1B, etc.) will be returned to authors for correction.

Table captions should be limited to 60 words.

Each column of an individual table must have a heading and be clearly defined. Indicate explanatory footnotes with superscript lowercase italic letters in alphabetical order.

Color tables will be considered for publication. As in the case with color figures, the costs of publishing color illustrations will be borne by the author. Costs are \$1500 for the

first color table and \$500 for each subsequent color table, or part thereof. Please note the author agreement to pay in the cover letter.

Large, complex, or tables that include graphic elements should be submitted as figures or as supplemental data for online publication only. The Editor may choose to make any table supplemental as he/she deems necessary.

### ***Figure Captions***

Figure captions and sub-captions must be listed together on an individual page directly following the tables or the references if submission does not include tables. Each figure requires a figure caption and should be clearly labeled (Figure 1, Figure 2, etc.). Multi-panel figures; figures with parts (a, b, c, etc.), should be distinctively labeled (Figure 1A, Figure 1B, Figure 2A, etc.).

Figure captions should be limited to 60 words.

If a figure has been published previously, acknowledge the original source in the figure caption and submit written permission from the copyright holder to reproduce the figure as supplemental data.

### ***Figures***

All figures must be uploaded separately as image files in Tagged Image File Format (.TIFF) or Encapsulated Postscript (.EPS). Microsoft Power Point (.PPT) is also acceptable; however, the graphics must contain embedded fonts with one image per slide, one slide per file. Each image should have a resolution of 600 dots-per-inch (dpi). More detailed specifications can be found on our print publication vendor's page at [CADMUS Digital Art Support](#).

The submission system is designed to retrieve the manuscript file and separate image file(s) and combine or merge the files into an individual Portable Document Format (.PDF) for reviewing purposes. It is the responsibility of the author to verify the quality of the image file(s) remains after the conversion to PDF. It may be necessary to alter the individual image file(s) to ensure the content is not lost or misconstrued in the merged PDF.

Submissions will be returned to authors if the figures are embedded within the manuscript file and not uploaded separately, or the figures have become eligible in the merged PDF.

Do not place unnecessary graphics, such as borders, in or around your figure. Pay particular attention to the quality of the lines, symbols, and patterns. Published figures are reduced to 1 column (85mm) or 2 columns (176 mm) in width. If, at that width, the figure symbols or lettering are not clear, you will need to increase the font size. Letters should be 8/10 points when reduced, subscripts no less than 6 point. Initial capitals and lower case letters and a medium (not bold) sans serif font is required. European PI, Helvetica, Mathematical PI, Times Roman, and Symbol fonts are Type 1 PostScript fonts supported by our printer for the creation of digital art figures.

If publication quality electronic figure files cannot be provided, acceptable publication prints are:

- Halftone: glossy prints
- Line drawing: glossy prints, laser prints on coated (nonabsorbent) laser-printer paper, or drawings in black India ink on tracing paper.
- Color: glossy prints, laser prints on coated (nonabsorbent) laser-printer paper

[Halftone: Image with blocks or portions that are gray opaque, such as a photograph; Line drawing: Image is in black and white only, such as a line graph; Color: Images where color is necessary to the meaning of the image]

The x-axis and y-axis should be clearly labeled, and the units of measurements given. This includes using decimal points instead of commas, italicizing species or gene symbols, and using U.S. English.

### ***Color***

The costs of publishing color illustrations in print will be borne by the author. As with tables, costs are \$1500 for the first figure and \$500 for each subsequent figure, or parts of figures. Upon acceptance, authors will be billed for the color costs unless a request to print figures in black and white is received via cover letter or e-mail. Color versions of figures may be provided for publication in the on-line Journal at no extra charge. Please

note your color preference and/or your agreement to pay for color printing in your cover letter.

### ***Supplemental Data***

Figures or tables too large for print, manuscript material that exceeds the limitation for the specific submission type, or appendices should be submitted for online publication only. These files should be marked and uploaded separately as supplemental files during submission, and should be referenced within the text as supplemental data (i.e., "See Supplemental Data Table 1", "See Supplemental Data Figure 1", etc.). Supplemental files must be labeled consecutively (i.e., Supplemental Data Table 1, Supplemental Data Table 2, Supplemental Data Figure 1, Supplemental Data Figure 2, etc.).

Captions for the supplemental data should be uploaded as a separate supplemental file or embedded within the supplemental data/table/figure file. Do not include the supplemental captions in your manuscript file.

Please be advised, supplemental data are permitted for Article, Brief Communication, Inspiring Minds, Mini-Review, Review, and Special Report submissions only. Manuscripts of all other types with supplemental data will be returned to authors.

Supplemental data will be reviewed as part of the manuscript and will be evaluated for its importance and relevance; however, it should not appear in the "Peer-reviewed PDF" file created by the submission system.

If accepted and posted online, the supplemental data will be referenced in the text of the print article directing readers to the URL of the Web site.

Authors are expected to identify to the Editor any other manuscripts (in print, accepted, have been submitted, in preparation) that relates to the submitted manuscript, especially if any patients or results are described in both.

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### ***D.8. UNIVERSITY OF CAPE TOWN DISSERTATION GUIDELINES***

#### **Minimum Requirements for Dissertations for MMed and MPhil for Subspecialities Degrees**

Following extensive discussion with Heads of Divisions, Dr S Kalula and Prof S Kidson recommend the following minimum criteria for dissertations for MMed and MPhil (subspeciality) degrees:

The MMed minor dissertation (or the MPhil dissertation in the case of sub-specialities) is one of three examination components of the MMed/MPhil degree. This minor dissertation carries one third of the weight of a full master's dissertation in terms of its credit weighting.

The dissertation must be a study containing the results of an analytical, quantitative, or epidemiological study carried out by the candidate (for certain disciplines, the candidate may choose instead to do a qualitative study, an audit cycle or a formal systemic review). A case report is not acceptable for the dissertation.

The dissertation must be the result of independent work of the candidate conducted under the guidance and direction of a supervisor(s) and should demonstrate evidence of an ability to undertake research, to adequately interpret results and to comprehensively and critically review the relevant literature. Although the findings of the research need not necessarily be original, they must be seen to advance scientific understanding. The topic and scope of research will depend on the particular disciplines and must be agreed upon in consultation with the supervisor(s).

#### **Research protocol**

Candidates intending to register for the MMed/MPhil Part III are required to submit a full research protocol for approval to their respective Departmental Research Committee (DRC). The candidate must also obtain FHS UCT Ethics approval prior to conducting their research. This full research protocol (together with a copy of the ethics approval letter) must be submitted to the postgraduate administration for approval by the Board of the Faculty of Health Sciences, prior to commencement of the research. For most disciplines, submission of the research protocol should be made no later than the end of year 2.

The research protocol should outline the scope and content of the dissertation and must include the title of the proposed dissertation, name of the supervisor(s) and their brief curriculum vitae.

### **Submission of dissertations**

On completion, the dissertation should be submitted to the Faculty Postgraduate Officer. The candidate should inform the Faculty Officer one month in advance of the intention to submit.

Submission deadlines:

1. March 15<sup>th</sup> for June graduation
2. August 15<sup>th</sup> for December graduation

Supervisors will be requested by the Faculty Postgraduate Officer to submit a letter supporting submission. This letter should be supplied by the primary supervisor. If this supervisor is external, the internal supervisor must be kept informed at every stage of the process. Specific submission requirements may be set by individual disciplines.<sup>2</sup>

*Note on fees:* To avoid attracting fees, dissertations need to be submitted before the beginning of the first quarter (first day of academic year), and before the start of the second semester (mid July) to qualify for a 50% fee rebate.

### **Supervisors**

One cannot overemphasize the importance of identifying a dissertation supervisor as early as possible. The supervisor should be an individual who can relate to the candidate's research project, be available for frequent and regular discussion and advice, and someone with whom the candidate can develop a good working relationship. Where specialised equipment and/or laboratory work is required for the study, the supervisor should assist in facilitating such access to such facilities. Supervisors may assist candidates in developing scientific communication skills but they are not required to do detailed editing or correction of spelling, grammar, or style. They may refer candidates to the UCT Writing Centre for this purpose.

The primary supervisor may be based outside the candidate's home department, faculty or university. In such a case, an internal (or secondary) supervisor will be required in addition to the primary supervisor, to serve as a guide and link to discipline-specific procedures. Primary supervisors retain responsibilities to the candidate and the university until the dissertation process is complete.

Please note: in order to assist a candidate with a master's research topic the supervisor needs to hold a master's degree or higher, or have relevant research experience. If the



primary supervisor does not hold a higher degree or equivalent (such as a Fellowship of The College of Medicine of South Africa), then a secondary supervisor who has a higher degree will need to be appointed in addition to the primary supervisor.

Candidates are strongly encouraged to publish the study with the supervisor(s) as co-author(s). This may require work beyond the graduation date. Such arrangements should be discussed and documented in advance.

2 For Public Health Medicine and Occupational Medicine the dissertation must be submitted for examination at least *4 months* prior to the deadline for registration for the examinations of the relevant College. This is in order to ensure that a final examination mark for the dissertation can be submitted by the candidate to the College of Medicine of South Africa (CMSA) at the time of registration as required by CMSA examination regulations.

### **The dissertation**

Submission of the dissertation should satisfy the following criteria:

1. The title page should contain the candidate's name, dissertation title and the name of the university.

It must also state the degree, e.g. Master of Medicine (MMed) in Public Health Medicine, Occupational Medicine, Family Medicine, Surgery, etc. The title page should also include a statement to the effect that the research report is based on independent work performed by the candidate and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree to any other university. It must also state that this work has not been published *prior to registration* for the abovementioned degree.

2. The body of the dissertation, which must be structured in 4 parts, should include the following:

**Part A:** The *protocol* (as approved by the Departmental Research Committee and Faculty Research Ethics Committee). The protocol should not exceed 4000 words.

**Part B:** A *structured literature review* appropriate to the subject matter and methods of the dissertation. The literature review must, amongst other things, show that the student is sufficiently acquainted with the relevant literature and is able to perform a critical appraisal and, if appropriate for the topic, show a good understanding of evidence-based medicine. The review should be between 3 000 and 4 000 words.

A suggested structure for the literature review is as follows:

- a) Objectives of literature review
- b) Literature search strategy, including inclusion and exclusion criteria
- c) Quality criteria - some leeway will be allowed here, as candidates will vary in their ability to appraise studies. This will also vary with the nature of the dissertation.
- d) Summary or interpretation of literature
- e) Identification of gaps or needs for further research
- f) References (which will overlap with but will not be the same lists as in the journal article and protocol)

Part C: The results of the study must be presented in the form of a *manuscript* of an article for a named peer reviewed journal, meeting all the requirements set out in the “Instructions for Authors” of that journal, including the word count and referencing style. (Unless specially motivated, the journal chosen will need to allow for *at least* 3000 words excluding abstract, tables, figures and references). The “Instructions to Authors” of the journal must be appended. The journal chosen for publication must be appropriate to the subject matter of the dissertation and accredited by the Department of Education or listed in the citation index of the Institute for Scientific Information (ISI).

Important note: the candidate need not have submitted the article, not is the acceptance of the article and requirement for passing the degree. The norm of practise is to publish the study with the supervisor(s) as co-author(s) and candidates are strongly encouraged to submit their manuscript either before or after examination of the mini-dissertation.

Part D: All *supporting documents* including:

- Questionnaire/data capture instrument
- Consent forms and any related participant information sheets
- Technical appendices, including, if considered necessary, any additional tables not included in the main manuscript for the examiner to have available. These should be accompanied by a brief narrative.
- Official Ethics approval letter from the Faculty Research Ethics Committee

3. The article does *not* have to be submitted to the journal in order to meet academic requirements.

4. A candidate must submit 2 copies of the dissertation in temporary binding, and an electronic copy oncompact disc in a universally readable format (e.g. pdf).

### **Examiners**

The full dissertation will be submitted for examination through the Postgraduate office of our Faculty to two external examiners (nominated by the supervisors and HOD). Three examiners will be nominated, two of which are invited to examine, and one held as an alternate. All examiners must be external to UCT. These nominations are circulated to the Faculty Dissertation Committee. It is the *supervisor's (or co-supervisor's)* responsibility to submit names of potential examiners to the Faculty Officer when the candidate is ready to submit.

The examiners will be well briefed regarding the specific requirements and criteria for submission and examination of the mini-dissertation. Such criteria will clearly explain the difference between the minidissertation and a Master's degree by dissertation alone.

Details required for each examiner are: academic qualifications, postal and/or physical address, telephone and fax numbers and e-mail address, and one paragraph description of their standing in the relevant field (drawn from their CV if need be.)

*The candidate may not be informed of the identity of the examiners.* After the outcome of the minidissertation has been finalised, the examiners' identities are made known if the examiners have indicated that they do not object to this.