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INYUVESI YAKWAZULU-NATALI

Current antiretroviral drugs- An investigation of metabolic syndrome promotion in HepG2 cells

By

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November 2022

DECLARATION

I, Jivanka Mohan, declare that:

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Miss Jivanka Mohan

DEDICATION

To my grandfather, Sunil nana, for constantly encouraging me to dream big and always believing in me. I further dedicate this dissertation to my loved ones who watch over me from heaven (Mia, Demon and Shivega) and influenced my journey along the way.

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Publications

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Abbreviations

3′-UTR	3’- untranslated region
3TC	Lamivudine
AKT	Protein kinase B
AMPK	AMP-activated protein kinase
ARVs	Antiretrovirals
ASC	Apoptosis-associated speck-like protein, including a caspase recruitment domain
ATP	Adenosine Triphosphate
BCA	Bicinchoninic acid
BSA	Bovine Serum Albumin
CAT	Catalase
C_{max}	Maximum (or peak) serum concentration
CVD	Cardiovascular Disease
DAMPs	Damage-associated molecular patterns
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DTG	Dolutegravir
EFV	Efavirenz
EMEM	Eagle's minimum essentials medium
ETC	Electron transport chain
FTC	Emtricitabine
GLUT2	Glucose transporter 2
GLUT4	Glucose transporter 4
gp120	Glycoprotein 120
h	Hour/hours
HAART	Highly active antiretroviral therapy
HepG2	Hepatocellular carcinoma cells
HIV	Human immunodeficiency virus
IKK	IκB kinase
IL	Interleukin
IL-6	Interleukin 6
IL-1β	Interleukin 1 beta
INSTI	Integrase Strand Transfer Inhibitor
IRS1	Insulin receptor substrate I

JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharide
MDA	Malondialdehyde
MetS	Metabolic syndrome
min	Minutes
miR-128a	Micro-RNA 128a
miRNA	Micro-RNA
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
NAD^+	Nicotinamide adenine dinucleotide
NAFLD	Non-alcoholic fatty liver disease
Nef	negative factor
NLRP3	(NOD-like) pyrin domain containing 3
NNRTI	Non-nucleoside reverse transcriptase inhibitors
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
NRTI	Nucleoside reverse transcriptase inhibitors
OXPHOS	Oxidative phosphorylation
p62	Ubiquitin-binding protein
PBS	Phosphate buffered saline
PDK1	Protein kinase-1
PDK2	Protein kinase-2
PI	Protease inhibitor
PI3K	phosphoinositide 3-kinase
PINK1	PTEN-induced kinase 1
PIP3	phosphatidylinositol (3,4,5)-triphosphate
p-IRS1	Phosphorylated IRS1
PLWH	People living with HIV
pNrf2	Phosphorylated (Ser40) Nrf2
Polymerase-γ	Polymerase gamma
PRR	Pattern recognition receptor
PTPC	Permeability transition pore complex
qPCR	Quantitative polymerase chain reaction
RLU	Relative light units
ROS	Relative band density
RT	Room temperature

SDS	Sodium dodecyl sulphate
SIRT3	Sirtuin 3
SOCS1	Suppressor of cytokine signalling 1
SOD2	Superoxide dismutase 2
T2DM	Type 2 diabetes mellitus
TBARS	Thiobarbituric acid reactive substances
TDF	Tenofovir disoproxil fumarate
TLRs	Toll-like receptors
TNF-α	Tumour necrosis factor alpha
TNXIP	the thioredoxin-interacting protein
TTBS	Tris buffered saline with Tween 20
UCP2	Uncoupling protein 2
UPR^{mt}	mitochondrial unfolded protein response
VLDLs	very low-density lipoproteins
VPR	Viral Protein R
WHO	World Health Organization
β-actin	Beta-actin

Abstract

Metabolic Syndrome (MetS) affects more than 20% of adults globally. Furthermore, the prevalence of MetS in HIV-infected patients on chronic antiretroviral (ARV) therapy continues to rise rapidly. This is alarming as a significant portion of people are HIV-infected worldwide, with the highest incidence experienced in Sub-Saharan Africa. An estimated 21% of people receiving ARV treatment display insulin resistance associated with mitochondrial dysfunction and inflammation. The current study aimed to determine the disruptions of metabolic processes associated with ARV use (Tenofovir disoproxil fumarate (TDF), Lamivudine (3TC) and Dolutegravir (DTG)) following a 120-h exposure period in HepG2 liver cells. Thereafter mitochondrial stress, inflammasome activation and insulin resistance promotion were assessed.

Following HepG2 cellular ARV exposure, it was found that mitochondrial stress proteins SIRT3 and UCP2 expressions were significantly suppressed. Due to these aberrations, endogenous cellular attempts to activate the antioxidant responses (pNrf2, SOD2, CAT) and mitochondrial maintenance systems (PINK1 and p62) in selected singular and combinational ARV treatments seemed insufficient. This resulted in lipid oxidative damage and reduced ATP production. These results indicate that ARVs induce mitochondrial dysfunction in liver cells.

Furthermore, it was deduced that combinational ARV exposure promoted inflammasome activation at a genomic level. This was seen in increased expression of *NLRP3* mRNA expression and caspase-1 activity with coinciding elevation in *IL-1β* in mRNA expression. Additionally, *JNK* expression was upregulated, with correlating increases in p-IRS1 protein expression and decreased *IRS1* mRNA expression being observed. Consequently, both *PI3K* and *AKT* mRNA expression was suppressed, whilst miR-128a expression was significantly upregulated.

It can be deduced that the combinational use of ARVs induced mitochondrial dysfunction and subsequently prompted inflammasome activation. This led to dysregulation of the IRS1/PI3K/AKT insulin signalling pathway and the initiation/promotion of insulin resistance. This is further supported through miRNA activation, suggesting possibilities for future studies on *in vivo* ARV use and related epigenetic changes.

CHAPTER 1

Introduction

Metabolic MetS is a non-communicable disease classified by having one or more metabolic irregulates associated with MetS. This includes insulin resistance, hypertension, dyslipidaemia, obesity and high cholesterol levels [1]. Such abnormalities lead to the development of diseases such as Type 2 Diabetes Mellitus (T2DM), cardiovascular diseases (CVD), strokes and Non-alcoholic fatty liver diseases (NAFLD) [1,2]. MetS affects an estimated 20-30% of adults worldwide, causing the World Health Organization (WHO) to declare the condition a global hazard [1,3].

HIV is an epidemic affecting over 38 million people globally, with more infections observed in marginalised groups [4]. An estimated 26 million PLWH receive highly active antiretroviral therapy (HAART), which significantly reduces mortality rates but may induce other complications such as MetS [5].

Interestingly, the occurrence of MetS in PLWH has grown significantly in the past decade. The highest percentage of HIV-infected individuals are in Sub-Saharan Africa, with 7.8 million infected individuals residing in South Africa [6]. The prevalence of MetS in the South African-infected population ranges from 24.1- 28.2% [7]. Despite the severity of MetS and the prevalence observed in PLWH taking ARVs, very few molecular or biochemical assessments exist studying relationships and possible mechanisms of MetS promotion [8].

Previous studies emphasise mitochondrial dysfunction and systemic inflammation as promoters of MetS. More commonly, both abnormalities promote insulin resistances that can lead to various metabolic complications, including T2DM and NAFLD [2]. Commonly induction of mitochondrial stress contributes to reactive oxygen species (ROS) production, oxidative stress, and inflammation which is strongly linked to MetS pathogenesis [9].

Excessive ROS production can cause serine phosphorylation of the insulin substrate receptor I (IRS1) through the upregulation of serine kinases such as c-Jun N-terminal kinases (JNK). Increases in phosphorylated IRS1 (p-IRS1) cause decreased expression of phosphoinositide 3-kinase (PI3K) and Protein kinase B (AKT), which prevent downstream signalling cascades that usually promote insulin sensitivity [10]. Aside from this, mitochondrial stress can increase the production of inflammatory cytokines and decrease the expression of IRS1 with similar consequences [9].

Furthermore, mitochondrial dysfunction is associated with the activation of the (NOD-like) pyrin domain containing 3 (NLRP3) inflammasomes and has been highlighted for its implications in

insulin resistance [11]. The inflammasome allows cleavage of pro- interleukin 1β to mature interleukin- 1β (IL-1β). The attire is responsible for the upregulation of serine kinase and decreased IRS1 expression [9]. The occurrence of the NLRP3 inflammasome in PLWH has been well studied; however, mechanisms surrounding combinational ARV usage and possible activation of inflammasomes and their' linkage to insulin resistance remain limited.

Several proteins and pathways regulate mitochondrial stress. The most common stress response is the antioxidant defence system, which reduces oxidative stress. Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is responsible for the transcription of several genes involved in reducing ROS [12]. These include superoxide dismutase 2 (SOD2) and catalase (CAT) [13,14]. However, Nrf2 further promotes the upregulation of PTEN-induced kinase 1 (PINK1) and ubiquitin-binding protein p62 (p62), which are synonymous with maintaining mitochondrial homeostasis [15,16].

Additionally, two essential proteins associated with mitochondrial stress maintenance are Sirtuin 3 (SIRT3) and Uncoupling protein 2 (UCP2) [17]. UCP2 is activated to increase NAD+/NADH ratios in cells to activate SIRT3 under oxidative stress conditions. [17]. SIRT3 deacetylates and activates other regulatory proteins, such as SOD2 and CAT [18]. Furthermore, UCP2 expression is synonymous with ROS reduction through an effect on oxidative phosphorylation (OXPHOS) and, thus, respiration regulation [19,20].

Additionally, insulin resistance can be regulated epigenetically through the expression of miRNAs [21]. MiRNAs function by inhibiting target gene translation through binding the 3' UTR regions [22,23]. Increased expression of specific miRNAs results in decreased expression of targets related to insulin resistance. In this study, we focus on miR-128a, which is known to negatively regulate the IRS1/AKT, thus promoting insulin resistance [24].

The use of the antiretrovirals 3TC, TDF, and DTG has been approved as a combinational treatment in the first line of therapy for HIV by WHO [25]. Studies often assess the side effects of these drugs in isolation, with a deficiency in studies evaluating biochemical mechanisms involved in their combinational usage [8]. This is imperative as most ARVs are consumed in a signal dose tablet containing all three drugs.

The liver is well known for metabolic function and first pass-hepatic metabolism of drugs. Furthermore, insulin resistance in the liver is associated with NAFLD and T2DM progression. HepG2 cells are commonly used for metabolism and toxicity studies due to their high metabolic activity [26]. Furthermore, they display similar genetic profiles to primary human hepatocytes and have been used in serval studies involving ARVs [26-29].

This study aimed to ascertain the relationship between the singular and combinational use of the drugs in mitochondrial- stress and -dysfunction and inflammasome activation *in vitro* as a possible method of MetS promotion. Furthermore, we propose possible miRNA regulation and its implications for the progression of insulin resistance.

We hypothesised that combinational ARV therapy would cause mitochondrial stress and promote activation of the NLRP3 inflammasome *in vitro*. As such, disarray in insulin signalling pathways would be observed, resulting in insulin resistance progression. An extensive literature review [8] was conducted to understand the relationship between HIV, MetS and ARV treatment. Research gaps were identified, and future aims, and objectives were developed based on previous studies.

The aim of the study was to ascertain the relationship between the singular and combinational use of ARVs in mitochondrial- stress and -dysfunction, inflammasome activation and insulin resistance promotion in HepG2 cells following a 120-h exposure period. The 3 main objectives were to determine the effects of ARVs in HepG2 cells after 120-h exposure:

1. Expression on mitochondrial stress markers, including SIRT3, UCP2, JNK, pNrf2, PINK1, p62, SOD2 and CAT. This was further complemented with an assessment of lipid peroxidation and ATP concentrations *in vitro*.
2. The expression of genes/markers related to the NLRP3 inflammasome (NLRP3, IL-1β and caspase-1)
3. Dysregulation of the IRS1/PI3K/AKT axis.

Ethical clearance for objectives using HepG2 *in vitro* models was obtained from the University of Kwazulu-Natal Biomedical Research Ethics Committee (Ethical approval number: BREC/00002256/2020) (Addendum C).

As per The University of KwaZulu-Natal's guidelines (Addendum B), this thesis is being submitted in the form of 3 manuscripts (one review paper and two experimental papers):

1. A review article has been published (Addendum A: *International Journal of Molecular Sciences*) and forms the basis of the study. A comprehensive Literature Review is presented in Chapter 2 with new information since the publication.
2. A research article outlining the effects of ARVs on mitochondrial stress/dysfunction in HepG2 cells submitted to *Biology.*
3. A research article describing the effects of ARVs on inflammasome activation and insulin resistance in HepG2 cells submitted to *Cells.*

CHAPTER 2

Literature Review

2.1 Human Immunodeficiency Virus

The first reported case of HIV was documented in 1981, with the infection reaching pandemic status by 2006 [30]. As of 2018, roughly 70 million people had acquired HIV since its discovery, with over 35 million deaths worldwide. Currently, an estimated 38 million people are HIV-infected, with approximately 1.7 million new infections per annum [4,31]. Of the total infected population, 26 million PLWH received ARV treatment by the end of 2019 [4]. HAART has saved 15.3 million lives and decreased the HIV-infected population's mortality [5].

Unfortunately, HIV disproportionately affects humans, with marginalised and disadvantaged groups experiencing higher infection rates. The most considerable portion of infected individuals exists in Sub-Saharan Africa, with 71% of infections occurring in adolescents [32]. In South Africa alone, there were 7.8 million PLWH in 2020, increasing the urgency for HIV research in the country [6].

HIV is associated with several comorbidities that result in severe adverse health outcomes and possibly death. At least 690,000 people died from HIV-related illnesses by the end of 2019. To effectively curb the pandemic, comorbidities need to be addressed. This necessitates studies surrounding immune activation, ARV toxicity and co-infections. PLWH are more susceptible to complications such as cancer, kidney failure, osteoporosis, and liver failure [33,34]. More recently, complications related to MetS have been highlighted, with more cases of cardiovascular diseases (CVD) and type 2 diabetes mellitus (T2DM) [35-37].

2.2 Human Immunodeficiency Virus and Metabolic Syndrome

Metabolic syndrome can be classified by having one or more metabolic irregularities, including insulin resistance, visceral obesity, dyslipidaemia and hypertension (Saklayen, 2018). Each pathology has risk factors that increase incidence rates, such as sedentary lifestyles, poor diet and age (Jaggers et al., 2014). In relation to HIV, MetS has a specific set of risk factors, including chronic inflammation and mitochondrial dysfunction, which promote atherosclerosis, CVD, and T2DM (Nguyen et al., 2016; Syed & Sani, 2013).

In the South African community, which has a significant percentage of PLWH, the prevalence of MetS ranges from 24.1- 28.2% [7]. Such statistics are substantial enough to warrant research surrounding the two conditions.

The biochemical basis of HIV-induced MetS remains ambiguous; however, commonly researched mechanisms include inflammatory activation, mitochondrial dysfunction and, ultimately, apoptosis (Figure 2.1). Initiation of any of the aforementioned pathways can induce more severe consequences, such as insulin resistance, central obesity and high blood pressure, among others [42].

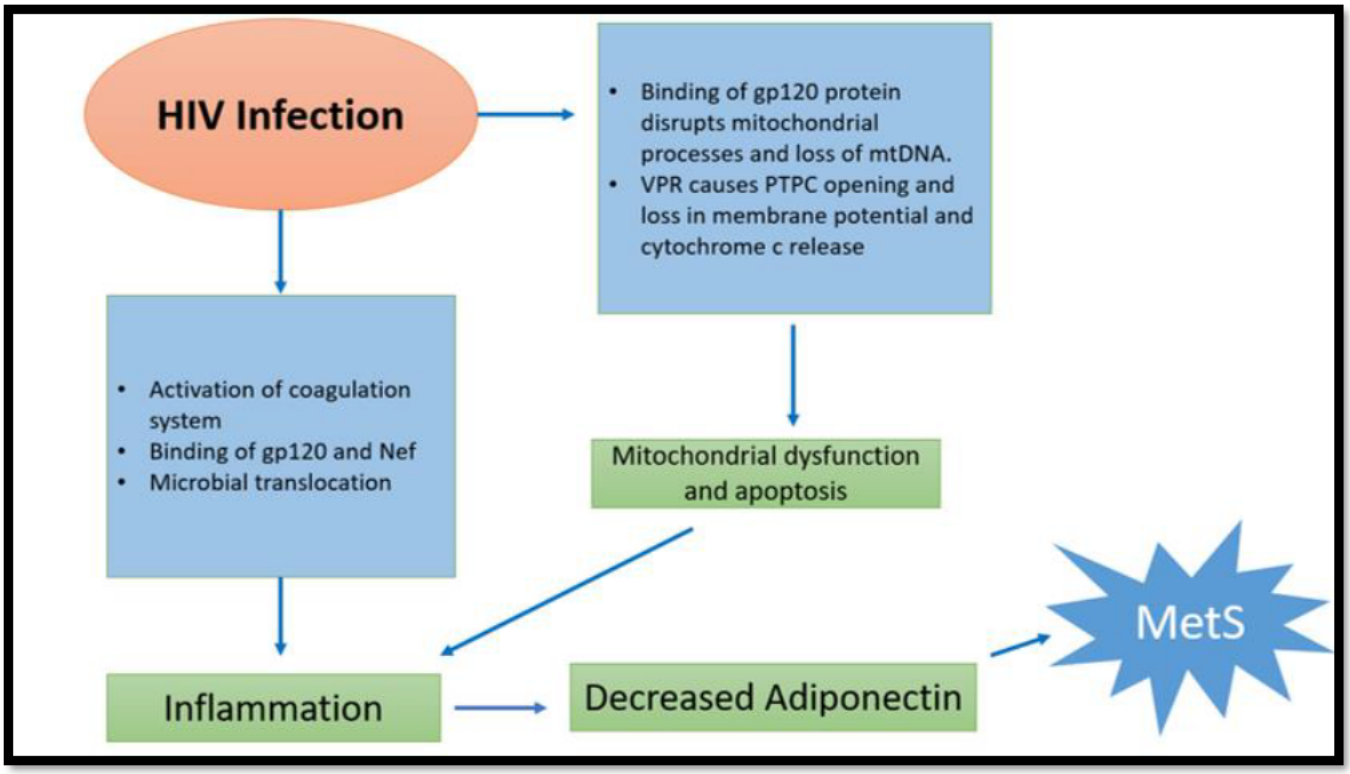


Figure 2.1: Summary of HIV-induced MetS processes (prepared by author). HIV can induce MetS in the human body through persistent inflammation and subsequent suppression in adiponectin. This is observed in multiple organs including the liver where NAFLD is promoted [43].

2.2.1 Human Immunodeficiency Virus and Inflammation

Human Immunodeficiency Virus and has been associated with persistent inflammatory status due to the activation of the coagulation system. This results in the production of pro-inflammation molecules, including cytokines and chemokines. Despite the use of HAART in certain patients, inflammation remains a problem through the production of C-reactive proteins, D-dimers and interleukins [44].

Gene products from the HIV infection can directly stimulate lymphocytes and macrophages, producing pro-inflammatory. More specifically, binding of the gp120 envelope protein promotes

immune activation. Aside from this, binding to CD4 receptors and co-receptors increases immune cells' susceptibility to activation [45-47]. Furthermore, lymphocyte activation may occur via the HIV accessory molecule Nef. Additionally, Nef can cause infection of macrophages resulting in indirect immune activation. [48-50].

Previous literature draws links between microbial translocation in HIV as a method for continual inflammation. More specifically, following infection, increased lipopolysaccharide (LPS) levels (an indicator of microbial translocation) are experienced [51]. Macrophages and dendritic cells are stimulated by increased LPS and initiate the production of inflammatory molecules such as IL-6, IL-1β, and tumour necrosis factor-alpha (TNF-α). This establishes the pro-inflammatory state commonly related to the virus [52]. More specifically, inflammatory conditions are initiated via inflammasome activation, which is strongly linked to the pathogenesis of MetS.

Chronic pro-inflammatory states result in the progression toward diseases such as T2DM and CVD [53-55]. Inflammation results in the functional inhibition of adiponectin. The latter is a vital protein hormone in fatty acid metabolism and glucose regulation. Therefore, the literature surrounding adiponectin suggests potent anti-inflammatory, anti-diabetic and anti-atherosclerotic functions that suppress MetS progression [56,57]. Inhibition associated with HIV-induced inflammation can result in insulin resistance and atherosclerosis which can progress to T2DM and CVD, respectively [58,59].

2.2.2 Human Immunodeficiency Virus and Mitochondrial Dysfunction

Mitochondrial dysfunction is one of the primary underlying mechanisms associated with MetS and is frequently observed in PLWH [60,61]. The binding of the gp120 protein during infection elicits pathogenic effects that can be seen through the loss of mitochondrial DNA (mtDNA), impaired calcium signalling and ultimate mitochondrial-mediated apoptosis [62]. Such mitochondrial dysfunction/dysregulation promotes the production of pro-inflammatory molecules and C-reactive protein [63,64].

In other instances, HIV induced mitochondrial membrane dysfunction through the accessory molecule Viral Protein R (VPR) [65,66]. This causes the opening of the mitochondrial permeability transition pore complex (PTPC) and loss of transmitochondrial potential [67]. Such effects disrupt mitochondrial processes and may initiate apoptosis by releasing cytochrome c and pro-caspase 9 [67,68]. More specifically, HIV can result in the uncontrolled release of cytochrome c, thus increasing apoptosis. Consequently, pro-inflammatory states are induced, resulting in adiponectin impaired function. This ultimately leads to MetS [63,69].

2.3 The history of ARVs and MetS

Highly active antiretroviral therapy has significantly reduced the severity of HIV and mortality rates in PLWH; however, its use is associated with several other complications [42,70]. Over time researchers have improved ARV formulations with reduced adversities. Older generations of ARVs resulted in severe adverse effects and many patients had to discontinue treatment or switch combinations of drugs to minimise problems [71].

Following several trials and testing, researchers developed singular and combinational ARVs with non-nucleoside reverse transcriptase inhibitors (NNRTIs) and Nucleoside reverse transcriptase inhibitors (NRTIs) being popular. However, first and second-generation ARVs were later associated with severe side effects and HIV drug resistance [72]. This was later improved to yield newer generations of drugs that induce fewer complications and adverse outcomes [73,74]. However, side effects remain a problem resulting in the WHO calling for more research to develop newer generations of ARVs, such as DTG, which is associated with less adverse outcomes [72].

Chronic use of certain ARVS is linked to the pathogenesis of MetS in PLWH [75]. This is observed through conditions such as mitochondrial dysfunction, inflammation, insulin resistance and dyslipidaemia (Figure 2.2). Metabolic complications associated with ARVs causes changes in glucose metabolism and fat distribution [76]. Several population studies report high prevalence's of MetS in PLWH on HAART. Promotion of MetS following HAART has been observed with protease inhibitors (PIs), NNRTIs, NRTIs, and integrase strand transfer inhibitors (INSTIs). The current literature indicates that PIs are most commonly implicated in MetS cases, and fewer studies show implications of newer generation ARVs in MetS progression [8].

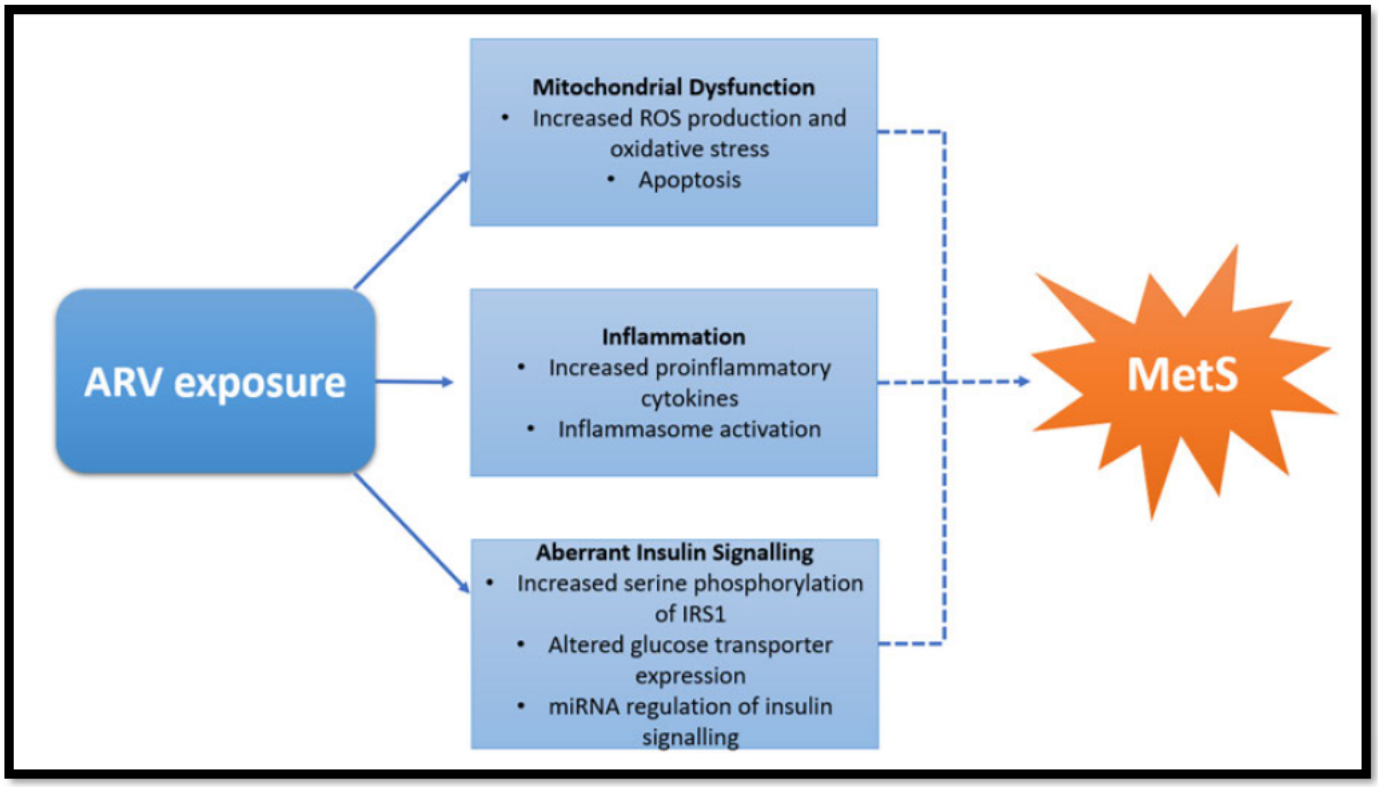


Figure 2.2: Summary of processes involved in ARV-induced MetS (prepared by author). ARV treatment promotes MetS in many organs through biochemical and epigenetic changes.

2.4 Current Antiretrovirals

In 2016, WHO proposed the usage of TDF, 3TC and emtricitabine (FTC)/efavirenz (EFV) as the preferred combinational treatment for HIV in young adolescents and adults. However, as research progressed, WHO updated its recommendations. By 2018, it was proposed that DTG and raltegravir be included in the first-line therapy of HIV. DTG gained significant popularity because of its supposed reduced side effects. This ultimately led to a combination of TDF, 3TC and DTG as the preferred first-line therapy for adolescents and adults [77]. Studies surrounding the combinational toxicity of the 3 ARVs remain limited.

2.5 Insulin Signalling

Insulin signalling has several different pathways; however, the IRS1/PI3K/AKT axis is the most well-studied (Figure 2.3). Binding of insulin to insulin receptors results in tyrosine phosphorylation of IRS1 [78]. Consequently, the recruitment and phosphorylation of other intermediates, including Shc and Grb-2-associated protein (Gab1). Such proteins provide docking sites for several downstream proteins that activate the PI3K/AKT signalling cascade [78,79].

Firstly, PI3K is activated, which causes the production of phosphatidylinositol (3,4,5)-triphosphate (PIP3), a second messenger activating 3-phosphoinositide dependent protein kinase-1 and -2 (PDK1 and PDK2). The latter activates AKT via phosphorylation at different residues [80]. AKT further activates several downstream targets, ultimately regulating glucose uptake (Figure 2.3), insulin secretion, and vasodilation. Therefore, aberrations in the IRS1/PI3K/AKT promote insulin resistance in cells [10,81].

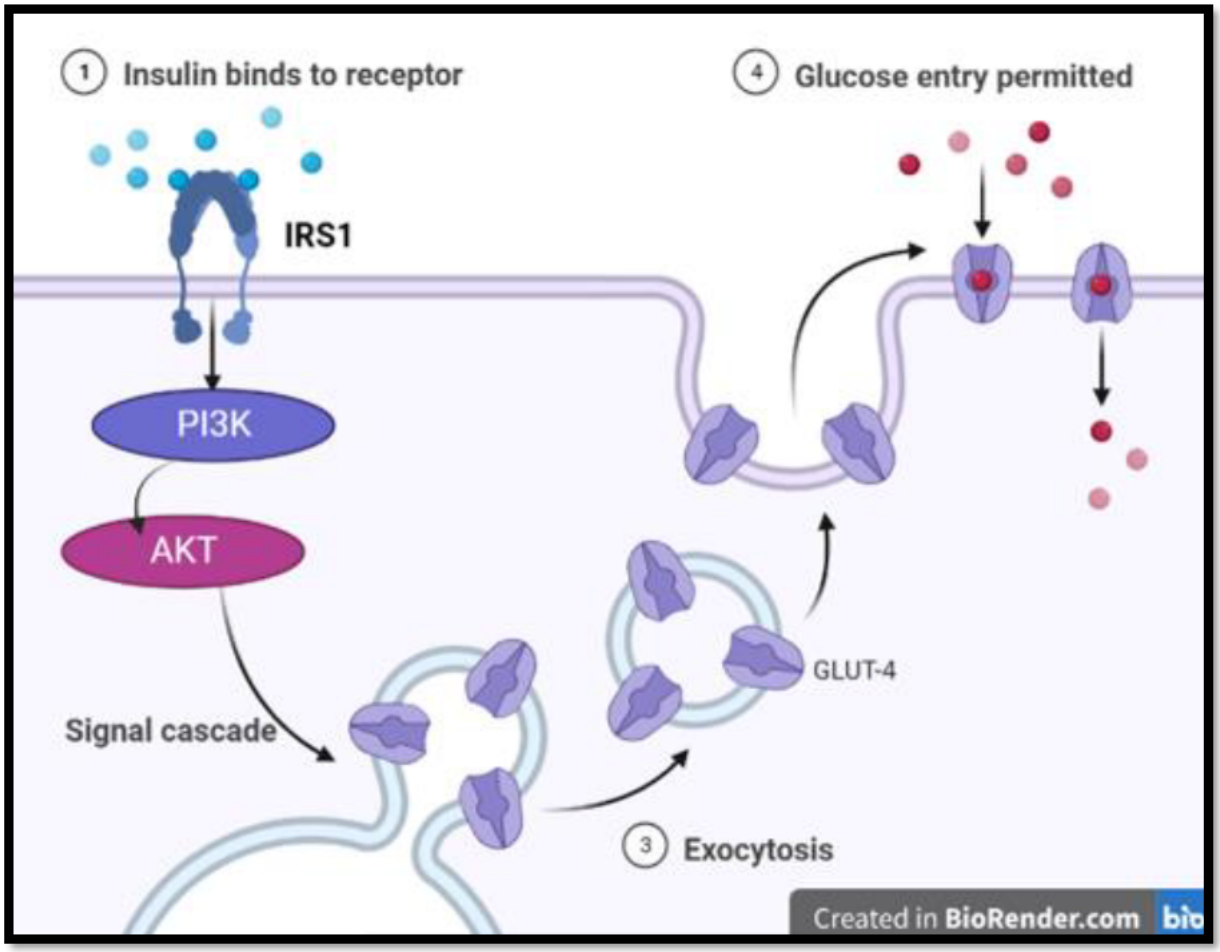


Figure 2.3: Simplified insulin signalling pathway (prepared by author). Insulin binding to receptors triggers downstream effects that promote glucose uptake among other processes.

2.5.1 Hepatic Insulin Signalling

As previously described, insulin signalling is imperative for glucose uptake in cells. In the liver, insulin is responsible for the initiation of fatty acid synthesis through the regulation of de novo lipogenesis. Consequently, β-oxidation of fatty acids in the mitochondria is inhibited. Lipogenesis

produces triglycerides that are transported via very low-density lipoproteins (VLDLs) to peripheral tissues for storage and usage [82].

However, impaired insulin signalling prevents such processes, and an accumulation of fatty acids in the liver occurs. The latter is described as NAFLD and is commonly seen in T2DM. Both conditions synergistically increase the risk of hepatic complications, with people experiencing NAFLD having a two-fold higher risk of developing T2DM [2]. This is mainly attributed to hepatic insulin resistance being the causative agent for impaired fasting glucose, leading to the development of T2DM [83].

2.6 Mitochondrial Dysfunction and Metabolic Syndrome

Metabolic syndrome is strongly associated with increased oxidative stress in the human body. Mitochondrial dysfunction results in ROS generation and release, which contributes to oxidative stress in case of insufficient antioxidant responses [1]. Furthermore, aberrant mitochondrial pathways promote systemic inflammation, which is synonymous with several pathologies in MetS, including T2DM, NAFLD and CVD [84-87]. Although the aforementioned has been described in several studies, specific pathways and mechanisms describing the mitochondria's role in the pathogenesis of MetS remain elusive [1].

Commonly, mitochondrial dysfunction is associated with insulin resistance. Aberrations in mitochondrial functions can promote insulin resistance through increased serine phosphorylation of IRS1. Elevated ROS levels in cells signal for the upregulation of serine kinases JNK and IκB kinase (IKK). Both kinases phosphorylate the receptor at serine residues leading to decreased metabolic signalling [10]. Aside from this, serine phosphorylation signals for the production of pro-inflammatory molecules, which directly interfere with insulin signalling [88,89].

Furthermore, mitochondrial ROS production can cause the release of damage-associated molecular patterns (DAMPs) which is instrumental in inflammasome activation [85]. Inflammasomes have been implicated in insulin resistance through their ability to upregulate serine kinases and interference with the IRS1/PI3K/AKT pathway [90,91]. Upregulation of mitochondrial stress responses has been associated with increased insulin sensitivity, and thus interference with stress responses may promote insulin resistance [10].

2.7 Mitochondrial Stress

To maintain mitochondrial integrity, several stress responses are upregulated during the detection of toxicity or endogenous irregularities. Such mechanisms are vital for reducing stress and preventing mitochondrial dysfunction [92]. Therefore, it can be concluded that aberrations in stress amelioration can promote the occurrence of MetS.

The most common mitochondrial stress response is the mediation of endogenous ROS production [93]. Although ROS are necessary for proper cellular function, including the synthesis of ATP in the mitochondria and oxygen-dependent death, excessive ROS (oxidative stress) can damage DNA, proteins, and lipids and disrupt cellular functions. Most cellular ROS are produced by the mitochondria, which increases the pace of oxidative injury within the organelle. Therefore, the antioxidant defence system provides a way for the mitochondria to minimise oxidative stress within the cell [94,95].

Another critical example is the mitochondrial unfolded protein response (UPRmt) which is initiated by the aggregation of unfolded proteins in the mitochondria [96]. These proteins are often oxidatively damaged. The organelle has a multitude of molecular chaperones and enzymes that regulate the correct folding of proteins. Furthermore, these are complemented with quality control enzymes/proteins to degrade misfolded proteins. The tightly regulated process is disturbed by excessive ROS production [96,97].

2.7.1 Mediators of Mitochondrial Stress

2.7.1.1 Sirtuin 3

Sirtuin 3 is endogenous to the mitochondria and results in the activation or inhibition of several proteins. SIRT3 is a nicotinamide adenine dinucleotide (NAD+) dependent protein belonging to a family of deacetylase enzymes that remove an acetyl group from the amino group of a lysine residue of target proteins [98,99] (Figure 2.4). This post-translation modification may increase the activity of the protein or cause suppression of activity. Specifically, SIRT3 has been described as a modulator for metabolism and ageing pathways [100,101].

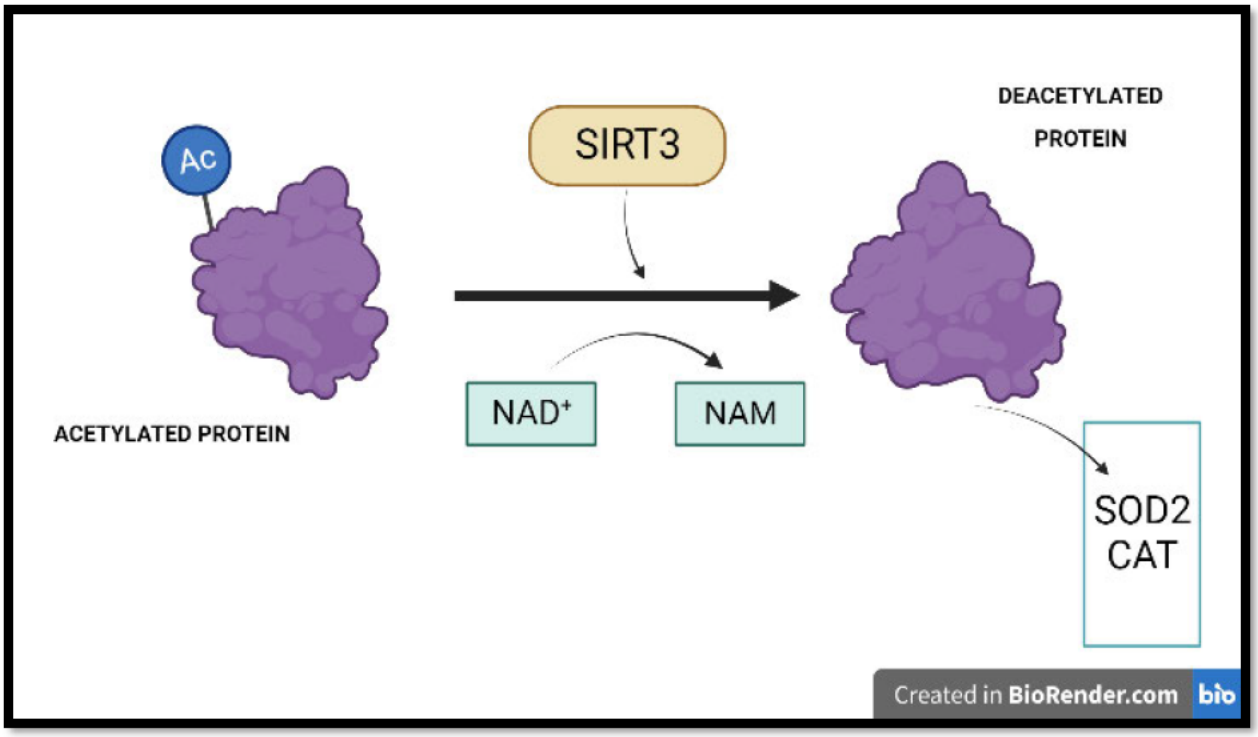


Figure 2.4: SIRT3 activates/suppresses target proteins (prepared by author). SIRT3 removes acyl groups of target protein resulting in activation/inhibition. During this process the cofactor NAD^+ is required.

SIRT3 indirectly regulates respiration by targeting isocitrate dehydrogenase 2 and aconitase in the citric acid cycle. Furthermore, SIRT3 increases mitochondrial OXPHOS and deacetylated complex I and II of the ETC, further modulating ATP production [102,103].

Aside from metabolic regulation, SIRT3 is essential for regulating mitochondrial ROS production. This is seen through the deacetylation of SOD2 and CAT, which is responsible for ROS scavenging, thus causing a reduction in oxidative stress [18,104].

Several studies have shown that SIRT3 protein and gene expression significantly increase in response to mitochondrial stress. SIRT3 inhibition prevents the amelioration of mitochondrial stress and can cause cell death [100,104]. The expression of the enzyme depends on the degree of stress present, and several proteins or enzymes can cause upregulation in SIRT3 concentrations [100].

2.7.1.2 Uncoupling protein 2

Reactive oxygen species production from the mitochondria is mainly driven through the proton gradient across the inner mitochondrial membrane. This gradient is regulated by a family of proteins, namely mitochondrial uncoupling proteins (UCP) [19].

Within this group, UCP2 has been highlighted for its role in reducing ROS production via the uncoupling of OXPHOS. Therefore, the expression of UCP2 is vital for mediating oxidative stress [19]. Aside from this, under oxidative stress, UCP2 expression is elevated to increase NAD+/NADH ratios in cells, thus promoting the activation of SIRT3 [17].

Furthermore, UCP2 has been shown to affect mitochondrial respiration. However, the effects on ATP production remain varied in different tissues. More specifically, UCP2 overexpression can decrease ATP production depending on the tissue, while in other cases, it may not affect respiration. This is attributed to increased protein expression causing changes in mitochondrial number and size at different magnitudes for specific cells [20].

2.7.1.3 Nuclear factor (erythroid-derived 2)-like 2

Nuclear factor (erythroid-derived 2)-like 2 is the master regulator of homeostatic responses in a cell. Upon detection of irregularities, the transcription factor induces first-line defences to maintain homeostasis in a cell. These include abnormalities such as oxidative stress and uncontrolled inflammation, which are synonymous with MetS [105]. Researchers have highlighted Nrf2 as a stress marker, and the transcription factor's expression can be manipulated during drug exposure [105,106].

Nrf2 is a cytoplasmic protein that remains bound to Kelch-like-ECH-associated protein (Keap1), which ensures it is constantly degraded. During stress conditions, kinases phosphorylate Nrf2 and triggers dissociation from KEAP1 via disruption of cysteine residues. This prevents degradation of Nrf2 and allows for nuclear translocation of phosphorylated (Ser40) Nrf2 (pNrf2). In the nucleus, pNrf2 can transcribe for various stress-relieving genes, including those involved in antioxidant and anti-inflammatory action [107] (Figure 2.5).

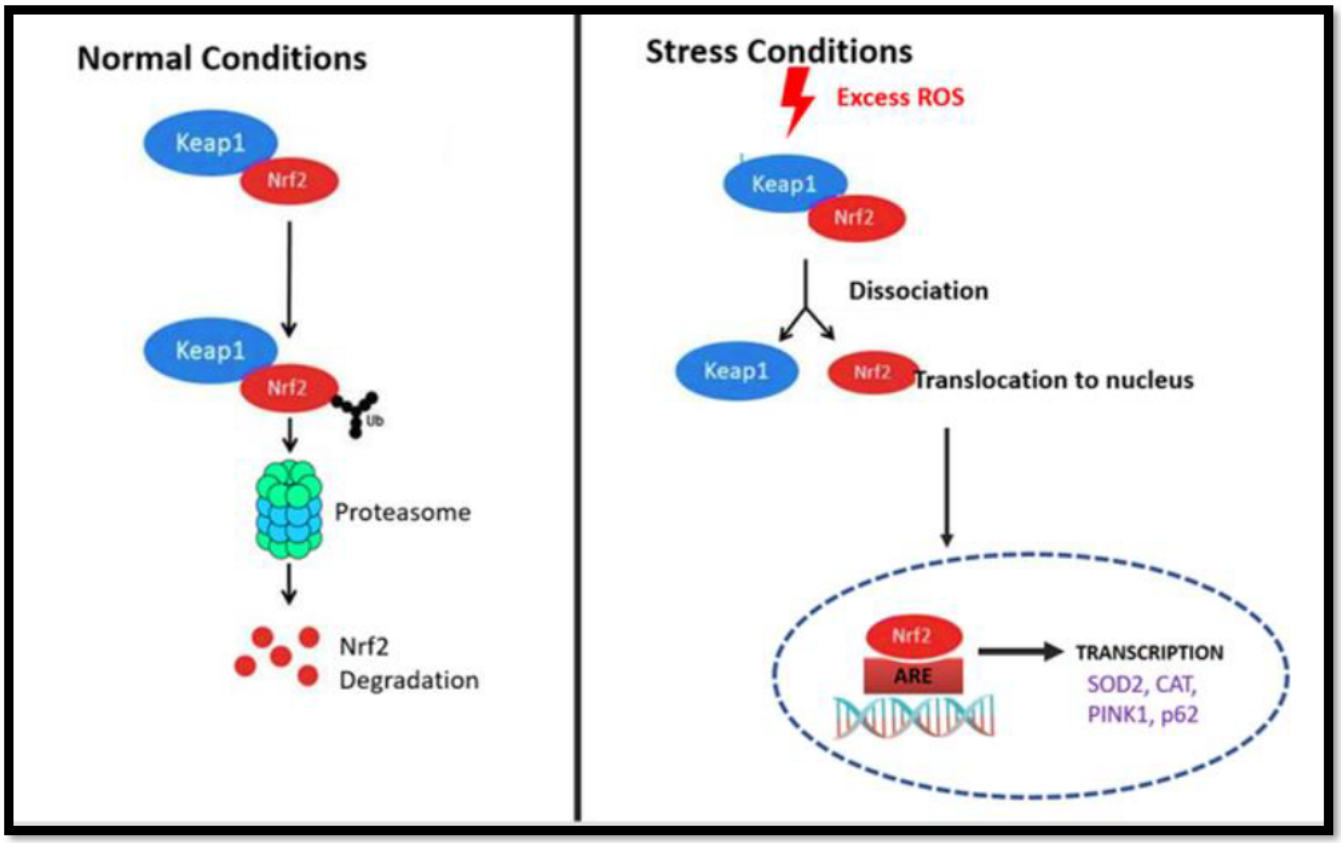


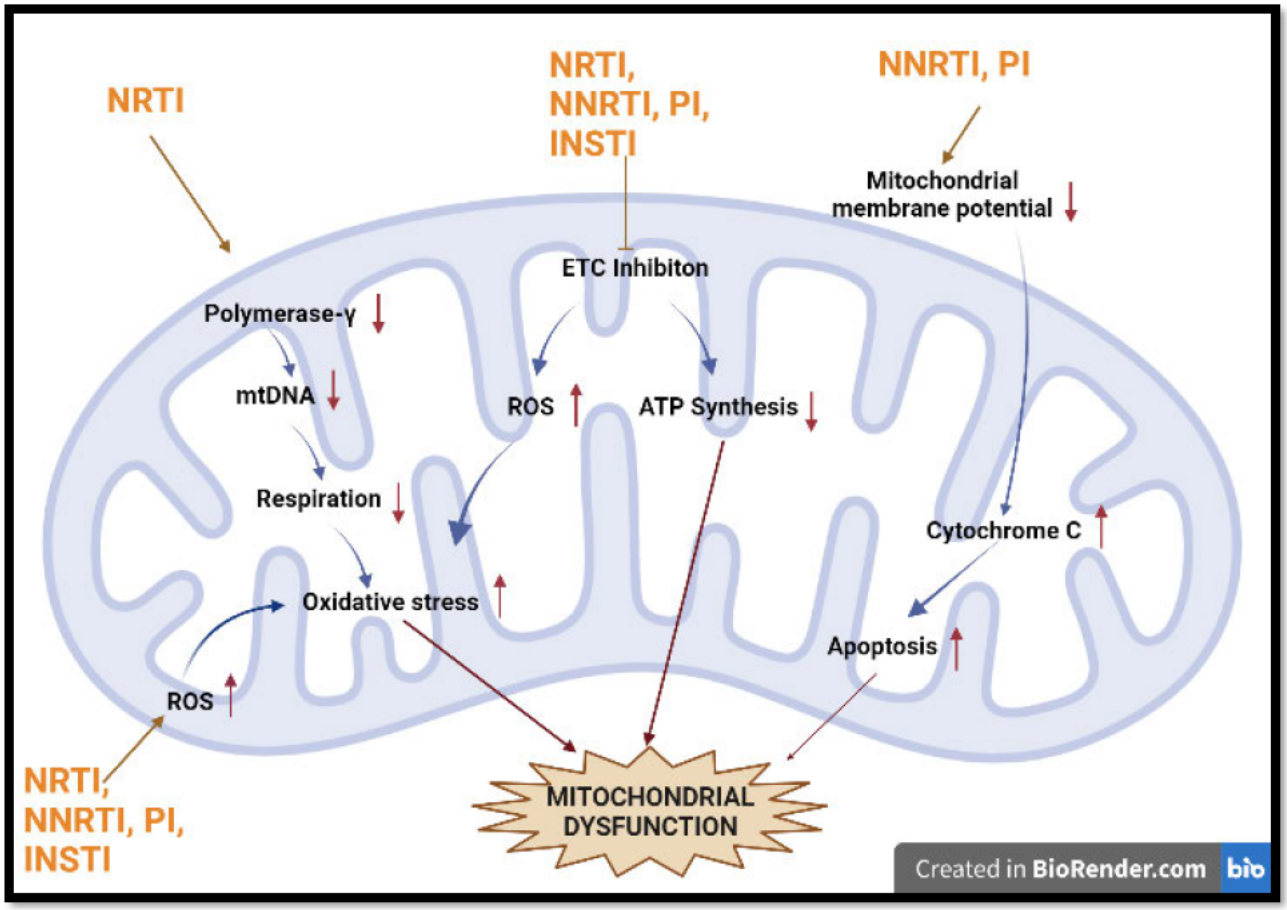
Figure 2.5: Nrf2 activation and mechanism of action under normal and stress conditions (prepared by author). Nrf2 activation has been observed in HepG2 cells following ARV exposure [27].

The most common stimulus of Nrf2 translocation is excessive ROS production. Nrf2 can transcribe for several genes including SOD2 and CAT which are directly involved in the breakdown of ROS [13,14].

Aside from its standard antioxidant transcription function, Nrf2 has been shown to transcriptionally activate the upregulation of PINK1 and p62 during oxidative stress conditions (Jain et al., 2010, Murata et al., 2015). PINK1 deficiency has been associated with excess ROS production and aberrant mitochondrial respiration [108]. On the other hand, p62 is upregulated in response to protein damage as a method of removal of dysfunctional proteins (part of the UPRmt) [109]. Nrf2 transcriptionally regulates PINK1 and p62 during oxidative stress [15,16]

2. 8 Antiretrovirals and Mitochondrial Dysfunction

Previous research extensively covers links between mitochondrial function/dysfunction and the pathogenesis of MetS. Aberrations in mitochondrial functions result in increased ROS production,



oxidative stress and inflammation strongly associated with MetS [1]. ARVs have been shown to induce disarray in mitochondria through several pathways (Figure 2.6).

Figure 2.6: Summary of pathways leading to ARV-induced mitochondrial dysfunction (prepared by author). Different classes of ARVs induce mitochondrial dysfunction through processes that contribute to decreased mtDNA, increased oxidative stress and apoptosis.

This was first observed with NRTIs that can inhibit polymerase-γ (responsible for mitochondrial DNA (mtDNA) replication). The effect is compromised mitochondrial integrity [110]. Decreased mitochondrial DNA initiates damage in oxidative phosphorylation (OXPHOS) proteins and ROS production. This results in the denaturation of mitochondrial protein and lipids, which causes aberration in other mitochondria processes [111].

Selected NRTIs can directly affect respiration and increases oxidative stress via inhibition of Complex IV and I of the electron transport chain (ETC) [112-114]. Inhibition of complexes in the ETC cause ROS leakage leading to the release of DAMPs and, thereafter, inflammatory activation [115].

More specifically, Tenofovir and TDF compromised mitochondrial function through increased superoxide production and oxygen depletion. As a result, mitochondrial membrane potential was altered, and oxidative stress ensued. Such effects have been implicated in T2DM and CVD pathogenesis [116]. TDF decreased the function of ETC complexes I, II, IV, and V, significantly reducing ATP production in rat kidneys [117]. Furthermore, usage of TDF and 3TC increased lipid peroxidation and depleted glutathione levels when paired with EFV in rat liver and kidneys [118].

On the other hand, the NNRTI, EFV, was found to alter mitochondrial mass and induce oxidative stress in hepatic cells. Furthermore, disruption in mitochondrial membrane potential promoted apoptosis through cytochrome c release [119,120]. Additionally, efavirenz inhibits complex I of the ETC, provoking ROS release and altering ATP production [121]. In neuronal cells, mitophagy (clearance of damaged mitochondria) was promoted through mitochondrial depolarisation [122].

Ritonavir (PI) increases ROS production and causes changes in mitochondrial membrane potential. Furthermore, it interferes with respiration by inhibiting steps in the ETC and OXPHOS [123]. Consequently, the drug initiates BAX translocation and cytochrome c release, allowing for apoptosis progression. Atazanavir (PI) causes superoxide production, leading to depolarised mitochondria and apoptosis [124,125].

DTG and other INSTIs can alter mtDNA copies and ROS production [123]. More specifically, DTG reduces respiration in CD4+ T cells through exacerbated mitochondrial ROS production. This leads to mitochondrial dysfunction, aberrant OXPHOS, and increased TNF-α responses, ultimately promoting MetS [123].

The wide array of research provides sufficient evidence of the effects of HAART on mitochondrial dysfunction. This gives plausible reasoning for targeting mitochondria to reduce side effects. However, most research asses the singular usage of ARVS and does not report combinational treatment. This is perplexing, as most infected individuals receive combinational treatment.

2.9 Inflammasomes and Metabolic Syndrome

As the prevalence of MetS has increased over the last decade, several studies highlight possible mechanisms promoting the condition [85]. Oxidative stress, advanced glycation end production and low-grade inflammation have been emphasised as contributing factors to MetS progression. More specifically, they collectively lead to metaflammation- an inflammatory state linked to the activation of innate immunity through assembling the multiprotein complexes- inflammasomes. Many inflammasomes exist; however, the NLRP3 inflammasome has been the most researched in terms of MetS pathogenesis [85].

Activation of inflammasomes is dependent on the stimulation or pattern recognition receptor (PRR), with the most common being toll-like receptors (TLRs) and nod-like receptors (NLRs). These can recognise external stimuli, namely pathogen-associated molecular patterns (PAMPs) or internal stimuli called damage-associated molecular patterns (DAMPs). DAMPs are released when damage occurs to cells [85,126]. Mitochondrial DAMPs include mitochondrial DNA and the thioredoxin-interacting protein (TNXIP) [127,128]. Specifically, once the NLRP3 PRR is stimulated, it binds to an apoptosis-associated speck-like protein, including a caspase recruitment domain (ASC) (Figure 2.7). This allows for the attachment of pro-caspase 1, which is cleaved to yield caspase 1. Caspase 1 is a well-known converter of IL-1β, i.e. converts pro-IL-β to mature IL-1β [126]. This is crucial for insulin resistance progression as IL-1β can activate the serine kinase JNK, increasing p-IRS1 expression. Additionally, it can directly reduce the expression of IRS1. Both these mechanisms are crucial for the progression of insulin resistance [10,90] (Figure 2.7).

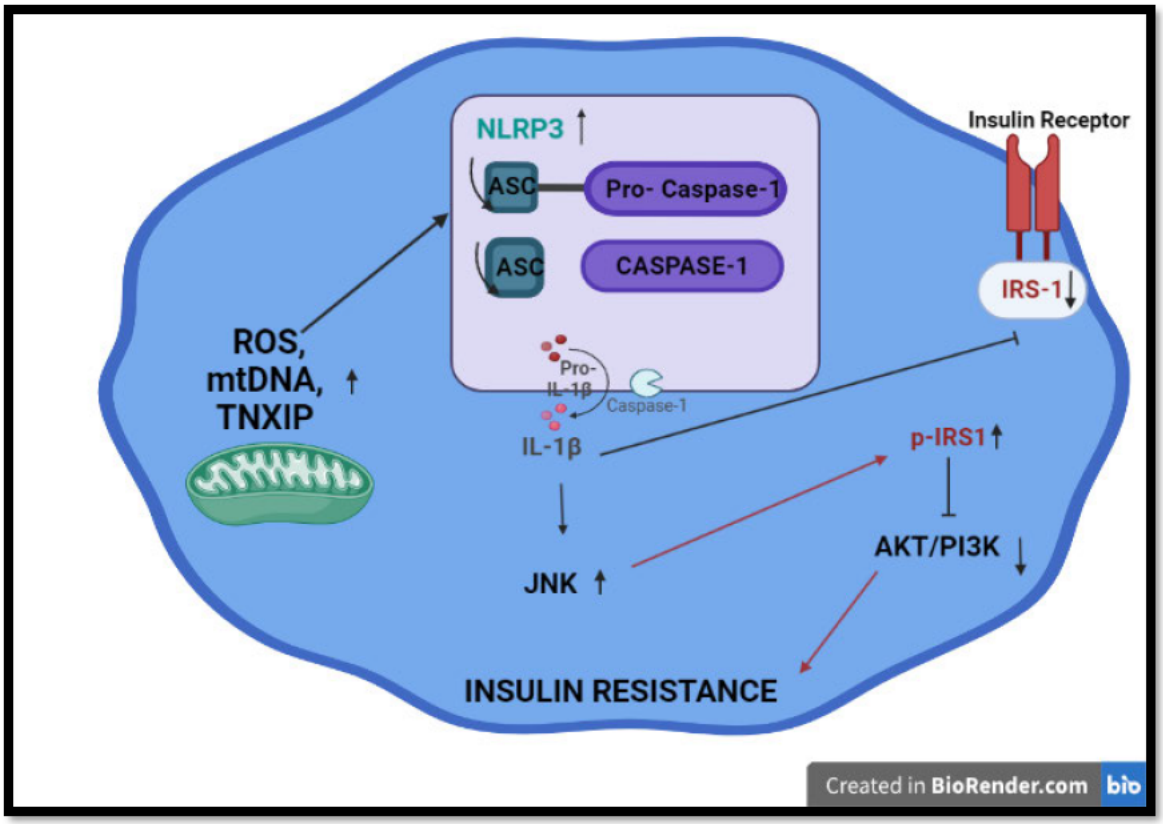


Figure 2.7: Inflammasome activation and progression to insulin resistance (prepared by author). HAART induces insulin resistance and aberrations in insulin signalling via promotion of mitochondrial toxicity, oxidative stress and inflammation resulting in dysregulation of IRS1/PI3K/Akt axis.

2.9.1 Antiretrovirals, Inflammation and Insulin Resistance

At least 21% of PLWH on ARVs have insulin resistance [129]. PIs are more frequently linked to insulin resistance than other ARVs through their effects on glucose transportation, mitochondrial stress and inflammation promotion.

PIs can induce mitochondrial dysfunction via numerous pathways, which signals an immune response via the action of the NALP3 inflammasome [130,131]. Inflammasomes are commonly linked to insulin resistance. Release of mitochondrial DAMPs upon mitochondrial damage initiate activation of inflammasomes. Upregulation of inflammasomes activates caspase 1, which stimulates pro-inflammatory cytokines (IL-1β and IL-18). IL-1β is strongly related to impaired insulin secretion and insulin resistance [132-134]. Aside from this, *in vitro* studies show that PIs

inhibit glucose 4 transporters (GLUT4) [135]. This impairs glucose tolerance and promotes peripheral insulin resistance [136].

Chronic use of PIs can reduce glucose uptake and impair insulin signalling [137]. Saquinavir increases the serine phosphorylation of IRS1 and thus affects insulin signalling. Furthermore, Indinavir alters TNF-α levels, activating JNK and IKK, which serine phosphorylates IRS1. The increased concentration of p-IRS1 inhibits the insulin receptor and affects downstream insulin signalling pathways [138,139].

NNRTIs exhibit pro-inflammatory promotion, which leads to reduced insulin sensitivity. They can increase the expression of TNF-α, IL-6, and IL-1β, which adversely affects adiponectin concentrations. The reduction in adiponectin allows insulin resistance progression [140]. NRTIs are not commonly implicated in insulin resistance; however, studies suggest they induce mitochondrial toxicity and thus affect insulin signalling [141].

Newer generations of ARVs, namely INSTIs, have been linked to insulin resistance. Mechanisms surrounding toxicity remain limited. DTG favours the onset of insulin resistance through increased oxidative stress and lipid accumulation. Additionally, a study revealed induction of mitochondrial dysfunction, which allowed authors to conclude that DTG promotes insulin resistance [142].

2.10 MicroRNAs

MiRNAs are small non-coding ribonucleotide acids (RNA) comprising ~ 22 nucleotides. Research has described the function of these molecules as gene expression modulators via protein translation. This has led to theories suggesting that miRNA can be used as biomarkers in disease and targets in therapeutic interventions [23,143].

MiRNAs regulate protein expression via binding to the 3'- untranslated region of mRNA. The interaction between the mRNA and miRNA inhibits protein translation (Figure 2.8). The function of miRNA allows for the regulation of various biological processes, including cell proliferation, cell death and metabolism [22,23].

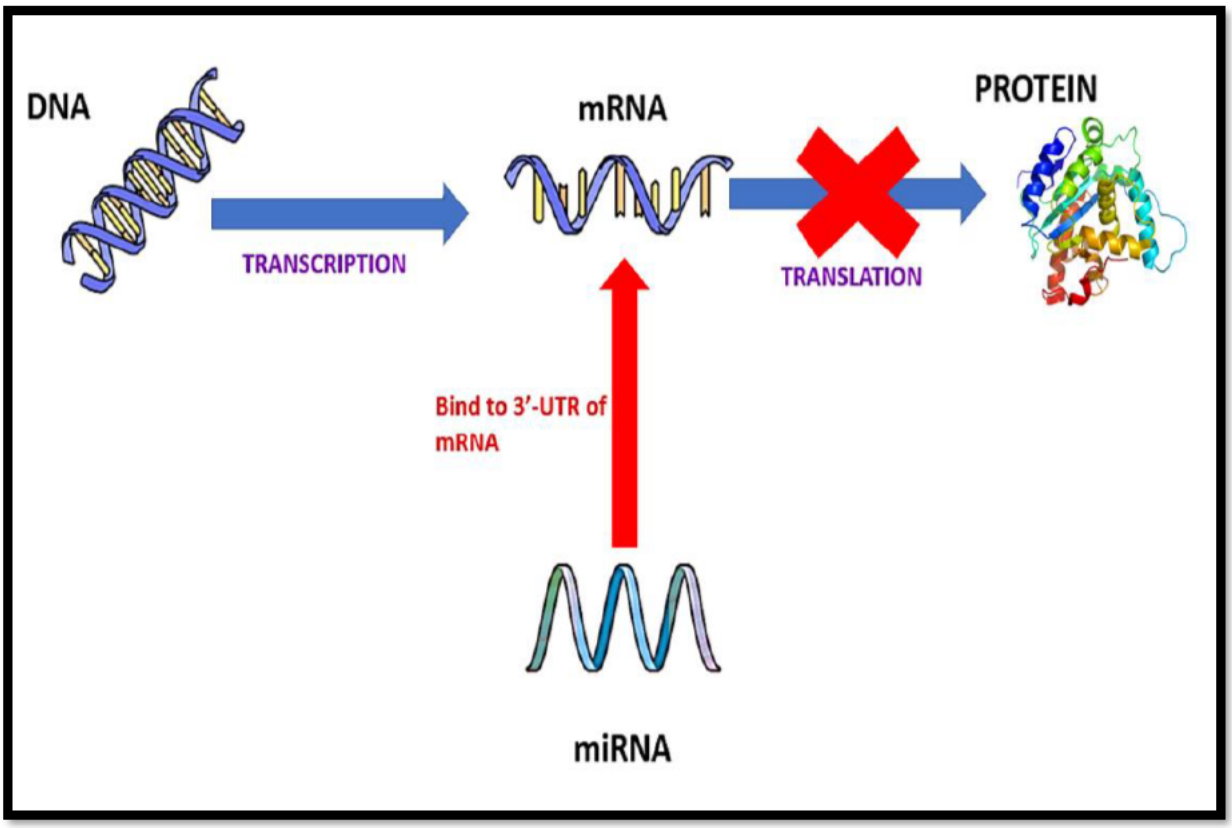


Figure 2.8: MiRNA mechanism of action (prepared by author). MiRNAs function by binding to 3'UTR of target genes and preventing translation.

2.10.1 Insulin signalling and miR-128a

The influence of miRNAs on metabolic syndrome has been covered extensively. In T2DM, miRNAs have been shown to affect insulin secretion and β-cell development [21,144]. More specifically, miR-128a expression has been shown to regulate the IRS1/PI3K/AKT pathway that was previously discussed. IRS1 expression is significantly reduced when overexpression of miR-128a is observed. Similar effects are experienced for AKT expression. Consequently, the insulin signalling pathway is disrupted, promoting insulin resistance and, ultimately, MetS [24].

2.10.2 ARVs and MicroRNAs- Emerging evidence

HAART-related epigenetic effects are observed through altered micro-RNA (miRNA) expression. Combinational ARV usage (varied combinations) caused suppression of miR-106a and miR-140 and increased miR-192 levels. Reduced expression of these miRNAs inhibited

CD4+ cell recovery, which is strongly connected to persistent inflammation and immune activation [145].

In other cases, Ritonavir (PI) increased levels of miR-28 which were inversely proportional to the expression of the GLUT-4 transporter. This suggests that the drug interferes with glucose metabolism [146]. Due to limited evidence, more research needs to be carried out to understand the role of ARV in promoting MetS through altered miRNA expression.

It is essential to note that HIV-infected individuals may have comorbidities that favour the onset of epigenetic modifications. Although pre-existing comorbidities can contribute to altered miRNA expression, there is substantial data to encourage future epigenetic studies related to ARV usage and possible implications in MetS.

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CHAPTER 3

Antiretrovirals promote metabolic syndrome through mitochondrial stress and dysfunction: an *in vitro* study

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Abstract

The prevalence of metabolic syndrome MetS in HIV-infected patients on chronic antiretroviral (ARV) therapy continues to rise rapidly, with an estimated 21% experiencing insulin resistance. The progression of insulin resistance is strongly related to mitochondrial stress and dysfunction. This study aimed to draw links between the singular and combinational use of Tenofovir disoproxil fumarate (TDF), Lamivudine (3TC), and Dolutegravir (DTG) on mitochondrial stress and dysfunction as an underlying mechanism for insulin resistance following a 120 h treatment period using an *in vitro* system- human liver cells (HepG2). The relative protein expressions of pNrf2, SOD2, CAT, PINK1, p62, SIRT3, and UCP2, was determined by Western blot. Transcript levels of *PINK1* and *p62* were assessed using quantitative PCR (qPCR). ATP concentrations were quantified using luminometry, and oxidative damage (malondialdehyde (MDA) concentration) was measured using spectrophotometry. The findings suggest that despite the activation of antioxidant responses (pNrf2, SOD2, CAT) and mitochondrial maintenance systems (PINK1 and p62) in selected singular and combinational treatments with ARVs, oxidative damage and reduced ATP production persisted. This was attributed to a significant suppression in mitochondrial stress responses SIRT3 and UCP2 for all treatments. Notable results were observed for combinational treatments with significant increases in pNrf2 (p=0,0090), SOD2 (p=0,0005), CAT (p=0,0002), PINK1 (p=0,0064), and p62 (p=0,0228); followed by significant decreases in SIRT3 (p=0,0003) and UCP2 (p=0,0119) protein expression. Overall there were elevated levels of MDA (p=0,0066) and decreased ATP production (p=0,0017). In conclusion, ARVs induce mitochondrial stress and dysfunction, which may be closely associated with the progression of insulin resistance.

Keywords: Metabolic syndrome, ARVs, mitochondrial stress, mitochondrial dysfunction, oxidative stress, insulin resistance.

Introduction

Metabolic syndrome (MetS) is a non-communicable disease affecting 20-30% of adults worldwide. Due to the increased incidence of MetS over the years, the World Health Organization (WHO) has classified the cluster of pathologies as a global hazard. The pathologies that can occur include hypertension, insulin resistance, and dyslipidaemia [1,2]. The occurrence of one or more of the pathologies can result in severe diseases such as cardiovascular diseases and Type 2 Diabetes Mellitus (T2DM).

The occurrence of MetS in people living with HIV (PLWH) has been described extensively in previous studies [3]. HIV affects the global population; however, the most severe effects and prevalence are observed in sub-Saharan Africa. By the end of 2019, roughly 38 million cases of HIV were reported worldwide, with 7.8 million cases being localised to South Africa [4,5].

Of the infected population, 26 million had access to ARV treatment [4]. Highly active antiretroviral therapy (HAART) has been associated with a significant decrease in the mortality rate in PLWH [6]. However, the other side effects remain numerous, with clinical studies showing a correlation between HAART usage and MetS. At least 21% of PLWH using HAART displayed insulin resistance [7-9]. Despite the severity and rising incidence of cases of MetS following HAART use, very few biochemical studies exist showing the mechanisms of action in MetS promotion following ARV usage.

One of the most common biochemical outcomes observed in MetS is mitochondrial dysfunction. It is well understood that mitochondrial stress contributes to reactive oxygen species (ROS) production, oxidative stress, and inflammation which are strongly associated with MetS [1]. Previous evidence links mitochondrial dysfunction and inflammation as an underlying process that can promote insulin resistance.

Several different pathways and proteins ameliorate mitochondrial stress. Two of the most common proteins involved in mitochondrial stress maintenance are Sirtuin 3 (SIRT3) and Uncoupling protein 2 (UCP2) [10]. Under oxidative stress conditions, UCP2 is activated to increase NAD+/NADH ratios in cells to lead to the activation of SIRT3 [10]. SIRT3 functions by deacetylating other regulatory proteins, such as superoxide dismutase 2 (SOD2) and catalase (CAT) [11]. However, the activation of such proteins is not exclusive to SIRT3 activity but may occur through upregulation of the transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) [12,13]. Nrf2 is responsible for the transcription of several genes involved in a cell's antioxidant response [14].

Additionally, Nrf2 has been highlighted in the activation of mitochondrial maintenance genes/proteins [15]. PTEN-induced kinase 1 (PINK1) and ubiquitin-binding protein p62 (p62) are both activated in response to oxidative stress. PINK1 deficiency has been associated with excess ROS production and aberrant mitochondrial respiration [16]. On the other hand, p62 is upregulated in response to protein damage as a method of removal of dysfunctional proteins [17]. Nrf2 transcriptionally regulates PINK1 and p62 during oxidative stress [18,19]. Mitochondrial dysfunction and subsequent excess ROS production promote insulin resistance by activating c-Jun N-terminal kinases (JNK) and NLR family pyrin domain containing 3 (NLRP3) inflammasome which leads to and deactivation of the IRS1/PI3K/AKT pathway and the progression of insulin resistance [20].

Insulin signalling is imperative for glucose uptake in cells however in the liver, insulin is responsible for the initiation of fatty acid synthesis through the regulation of de novo lipogenesis. Aberrations lead to fatty acid accumulation and progression of non-alcoholic fatty liver disease NAFLD [21]. The latter is closely linked with the progression in T2DM [22]

The use of the antiretrovirals 3TC, TDF, and DTG has been approved as a combinational treatment in the first line of therapy for HIV by WHO [23]. Studies often assess the side effects of these drugs in isolation, with very few studies evaluating biochemical mechanisms involved in their combinational usage [24]. This study aimed to ascertain the relationship between the singular and combinational use of the drugs in mitochondrial- stress and -dysfunction *in vitro* as a possible method of MetS promotion. In order to achieve the aims a HepG2 cell model was used for testing. These liver cells are commonly used for *in vitro* experiments involving drugs as they display similar physiological functions and genetic profiles as primary hepatocytes [25-27]. Evidence from this study can be used to develop therapies with reduced side effects related to MetS.

Materials and Methods

Antiretroviral drugs (3TC, TDF, and DTG) were sourced from the NIH AIDS reagents program. HepG2 cells were obtained from American Type Culture Collection (Johannesburg, South Africa). All media used for cell culture and supplements were obtained from Lonza (Basel, Switzerland). Luminometry kits (ATP) were obtained from Promega (Madison, Wisconsin, USA). Reagents used for Western Blots were acquired from Bio-Rad (Hercules, California, USA). All remaining reagents were obtained from Merck (Darmstadt, Germany) unless stated differently.

Cell culture and treatment

Culturing of HepG2 cells was carried out in 25 cm^3 cell culture flasks using CCM [Eagle's minimum essentials medium (EMEM) supplemented with 10% foetal calf serum, 1% pen-strep-fungizone, and 1% L-glutamine] and incubated in a humidified incubator (37 °C, 5% CO_2) until roughly 80% confluency was reached. Thereafter cells were treated with the physiological concentrations (C_{max}) of ARVs (3TC: 1.51µg/ml, TDF: 0.3µg/ml, DTG: 3.67 µg/ml) [19-21] for 120 hours (h) as per Nagiah et al., 2015 [26]. Fresh media and ARVs were replenished every 24 h. All subsequent assays were carried out following treatment as explained above.

ATP Quantification

Luminometry was used to quantify ATP concentration in HepG2 cells using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, #G7570). Following treatment, 20,000 cells/well in 0.1 M phosphate buffered saline (PBS) were seeded into an opaque 96-well microtitre plate in triplicate to ensure a final volume of 25 µL. The CellTiter-Glo® Reagent was reconstituted as per the manufacturer's instructions, and 25 µL of reagent was added to each well. Plates were incubated for 20 min at room temperature (RT) without light exposure. Thereafter, luminescence was measured using a Modulus™ Microplate Reader (Turner Biosystems, Sunnyvale, CA, USA). Results were expressed as relative light units (RLU).

Lipid Peroxidation- TBARS assay

Oxidative damage was assessed using the thiobarbituric acid reactive substances (TBARS) assay, which quantifies the malondialdehyde (MDA) levels, a by-product of lipid peroxidation. MDA concentration is proportional to ROS production in cells. Following treatment, the supernatant was collected from flasks and added to test tubes (200 µL). This was supplemented with 2% H_3PO_4 (200 µL), 7% H_3PO_4 (200 µL), and thiobarbituric acid/butylated hydroxytoluene solution (400 µL). The pH of all samples was adjusted to 1.5 and then boiled for 15 min. After cooling, samples were supplemented with 1.5 mL of butanol and vortexed for separation into distinct phases. Following separation, 100 µL of the upper phase of each sample was dispensed into a 96-well microtitre plate in triplicates. The optical density was measured on a spectrophotometer at 532 nm with a reference wavelength of 600 nm. The average optical density was calculated and divided by the absorption coefficient (156 mM^{-1}). Results were represented as MDA concentration (µM).

Western Blots

Following 120 h treatment, cells were incubated for 30 min with 150 µL Cytobuster™ Reagent (Novagen, San Diego, CA, USA, catalogue no. 71009). Cells were dislodged and lysed with a cell scraper, and contents were transferred to 2 mL micro-centrifuge tubes. Samples were centrifuged (400× g, 10 min, 4 °C), and crude protein isolates (supernatants) were removed and transferred to fresh microcentrifuge tubes. The bicinchoninic acid assay (BCA) was used to assess protein concentration, and samples were standardised to a concentration of 1 mg/mL. Following standardisation, samples were boiling (5 min, 100 °C) in Laemmli Buffer (distilled water, glycerol, 10% SDS, β-mercaptoethanol, 0.5 M Tris-HCl (pH 6.8), 1% bromophenol blue and glycerol) in preparation for SDS-PAGE.

A Bio-Rad compact supply was used to separate proteins. 20 µL of each sample was transferred to sodium dodecyl sulphate (SDS) polyacrylamide gels (4% stacking, 10% resolving) and electrophoresed (1 h, 150 V). The Bio-Rad Trans-Blot® Turbo Transfer was used to transfer separated proteins to nitrocellulose membranes. Membranes were blocked for 1 h at RT with 5% Bovine Serum Albumin (BSA) in Tween 20-Tris buffer saline (TTBS: 150 mM NaCl, 3 mM KCl, 25 mM Tris, 0.05% Tween 20, dH2O, pH 7.5)

Following blocking, membranes were immuno-probed with the required primary antibody (1: 1000) (Table 3.1) for 1 h at RT and thereafter 16 h at 4° C. Membranes were washed using 5 mL TTBS (5x 10 min). HRP-conjugated secondary antibodies were added to membranes for 1 h at RT (Cell signalling Technology; anti-rabbit (#7074S); anti-mouse (#7076S) 1:5000 in 5% BSA). Following incubation, membranes were washed (5x 10 min in TTBS) and rinsed with distilled water. Protein detection was carried out using the Clarity Western ECL Substrate detection reagent (400 µL) (Bio-Rad, Hercules, CA, US)), and images were captured using the Bio-Rad ChemiDoc™ XRS+ Imaging System.

Following detection, membranes were stripped using 5% hydrogen peroxide for 30 min at 37 °C. Thereafter, 5% BSA was used for blocking, followed by incubation with HRP-conjugated antibody for β-actin (A3854, Sigma-Aldrich). β-actin is a housekeeping protein expressed evenly across cells. Image Lab™ Software v6.0 (Bio-Rad, Hercules, CA, USA) was used to analyse the results. The relative band density of protein was calculated by normalising results against β-actin.

Table 3.1: Antibodies used for immunoprobing

Antibody	Company	Catalogue number
Anti-PINK1 antibody [N4/15]	Abcam	ab186303
Anti-SIRT3	Abcam	ab264041
Recombinant Anti-Nrf2 (phospho S40) antibody [EP1809Y]	Abcam	ab76026
Anti-SQSTM1 / p62 antibody [2C11] - BSA and Azide free	Abcam	ab56416
SOD2 (D9V9C) Rabbit mAb	Cell Signalling	13194S
Catalase (D4P7B) Rabbit mAb	Cell Signalling	12980S
UCP2 (D1O5V) Rabbit mAb	Cell Signalling	89326S

Quantitative PCR

RNA Isolation and Quantification

Following treatment, cells were incubated with 500 µL Trizol and 500 µL PBS (5 min, RT). Cells were dislodged and lysed with a cell scraper content was transferred to 2 mL micro-centrifuge tubes and stored (24 h, −80 °C). Samples were then thawed and supplemented with 100 µL chloroform. This was followed by centrifugation (12,000× g, 10 min, 4 °C). Supernatants were aspirated and transferred to 2 mL micro-centrifuge tubes containing 250 µL isopropanol. Samples were stored overnight at −80 °C. Following incubation, samples were thawed and centrifuged (12,000× g, 20 min, 4 °C). Supernatants were removed and discarded, and the remaining pellet was washed in 500 µL of 75 % cold ethanol. Thereafter, centrifugation (7400× g, 15 min, 4 °C) was carried out. Ethanol was removed, and the resulting RNA pellets were air dried (1 h, 24 °C) and re-suspended in 15 µL nuclease-free water. RNA was quantified using the Nanodrop2000 spectrophotometer (Thermo-Fisher Scientific, Waltham, MA, USA). RNA quality was determined using the A_{260}/A_{280} ratio. All RNA samples were standardised to 1000 ng/µL.

Quantification of mRNA Expression

Following standardisation, cDNA was synthesised using the iScript™ cDNA Synthesis kit as per manufactures instructions (Bio-Rad, 107-8890, Hercules, CA, USA).

Transcript levels of relevant genes (Table 3.2) were assessed using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, 1725270). The thermo-cycler conditions for each gene were as follows: initial denaturation (8 min, 95 °C), followed by 40 cycles of denaturation (15 s, 95 °C), annealing (40 s, Table 3.1), and extension (30 s, 72 °C). GAPDH is evenly expressed across cells and was used for normalisation. Results were calculated using the Livak and Schmittgen (2001) method and were represented as fold change relative to the control cells ($2^{-\Delta\Delta CT}$) [28].

Table 3.2: Primer sequences with respective annealing temperatures for genes assessed

Gene		**Sequence (5′-3′)**	**Annealing Temperature (°C)**
PINK1	Forward	GGAGGAGTATCTGATAGGGCAG	57
	Reverse	AACCCGGTGCTCTTTGTCAC	
p62	Forward	CAGAGAAGCCCATGGACAG	60
	Reverse	AGCTGCCTTGTACCCACATC	
GAPDH	Forward	TCCACCACCCTGTTGCTGTA	---
	Reverse	ACCACAGTCCATGCCATCAC	

Statistical analysis

GraphPad Prism version 5.0 (GraphPad Software Inc., California) was used to perform all statistical analyses. Data were analysed using an unpaired t-test (control vs treatment) and represented as the mean ± standard deviation unless otherwise stated. A value of $p<0.05$ was considered statistically significant.

Results

Antiretrovirals activated antioxidant responses in HepG2 cells.

Phosphorylated Nrf2 (Ser40) (pNrf2) is the active form of Nrf2 that can translocate to the nucleus and transcribe for proteins. It is activated under oxidative stress conditions. Following treatment with ARVs, only DTG (p=0,0014) and combinational treatments (p=0,0090) showed significant increases in pNrf2 expression (Figure 3.1A). Significant increases in protein expression of SOD2 were only observed for 3TC (p=0,0029), DTG (p=0,0006), and combinational usage (p=0,0005) (Figure 3.1B). All treatments were able to increase the protein expression of CAT (TDF; (p=0,0003), 3TC; (p=0,0025), DTG; (p=0,0002), combinational use; (p=0,0002)) (Figure 3.1C).

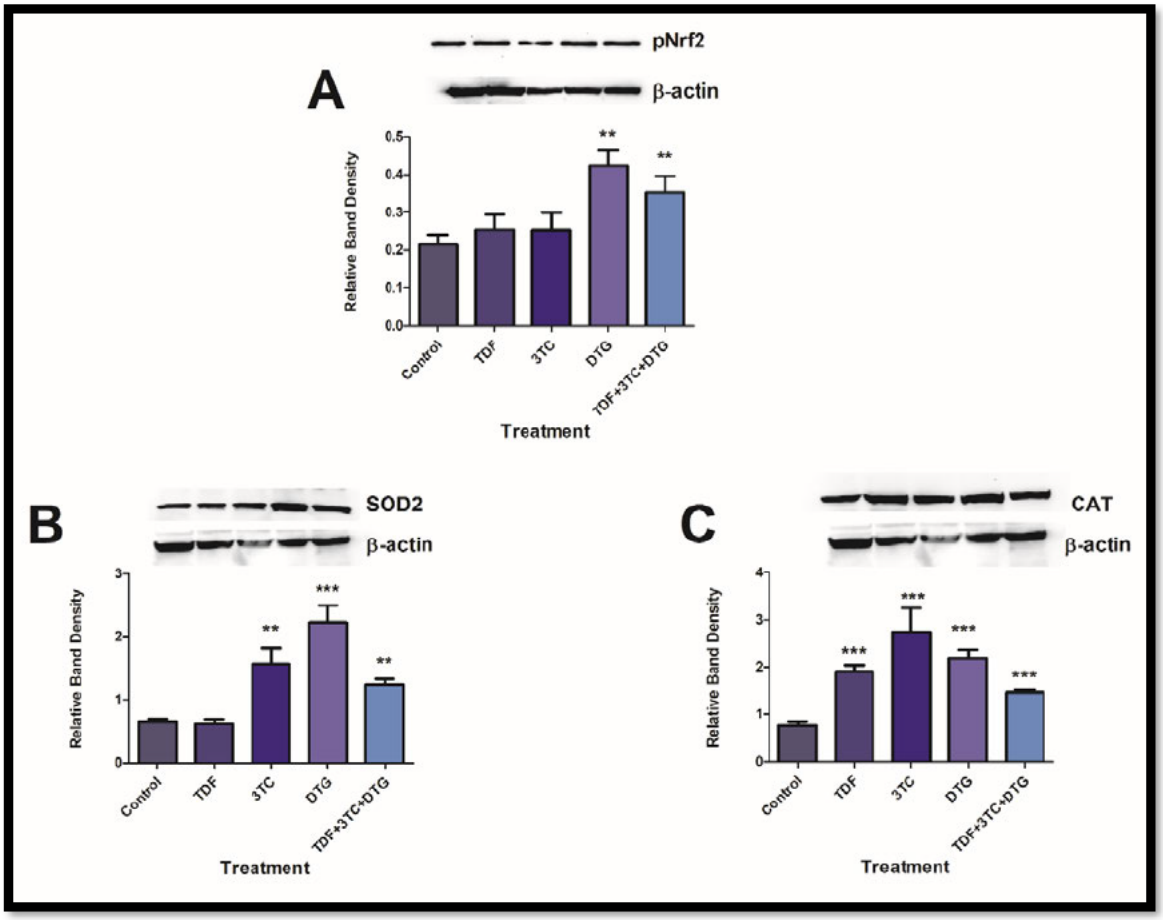

Figure 3.1: Antiretrovirals activated antioxidant responses. Protein expression of pNrf2 (A) and SOD2 (B) was significantly increased in selected treatments, whereas all treatments increased protein expression of CAT (C). ***p<0.005, ***p<0.0001*

Antiretrovirals activated mitochondrial maintenance intermediates.

PINK1 gene expression (Figure 3.2A) increased significantly by 3TC (p=0,0002) and combinational treatments (p=0,0383); however, protein expression was elevated by 3TC (p=0,0323), DTG (p=0,0042), and combinational treatments (p=0,0064) (Figure 3.2C). Interestingly *p62* mRNA expression (Figure 3.2B) was elevated for all treatments (TDF; (p=0,0298), 3TC; (p=0,0259), DTG; (p=0,0012), combinational use; (p=0,0006)) however protein expression only showed significant elevations in DTG (p=0,0073) and combinational usage treatments (p=0,0228) (Figure 3.2D).

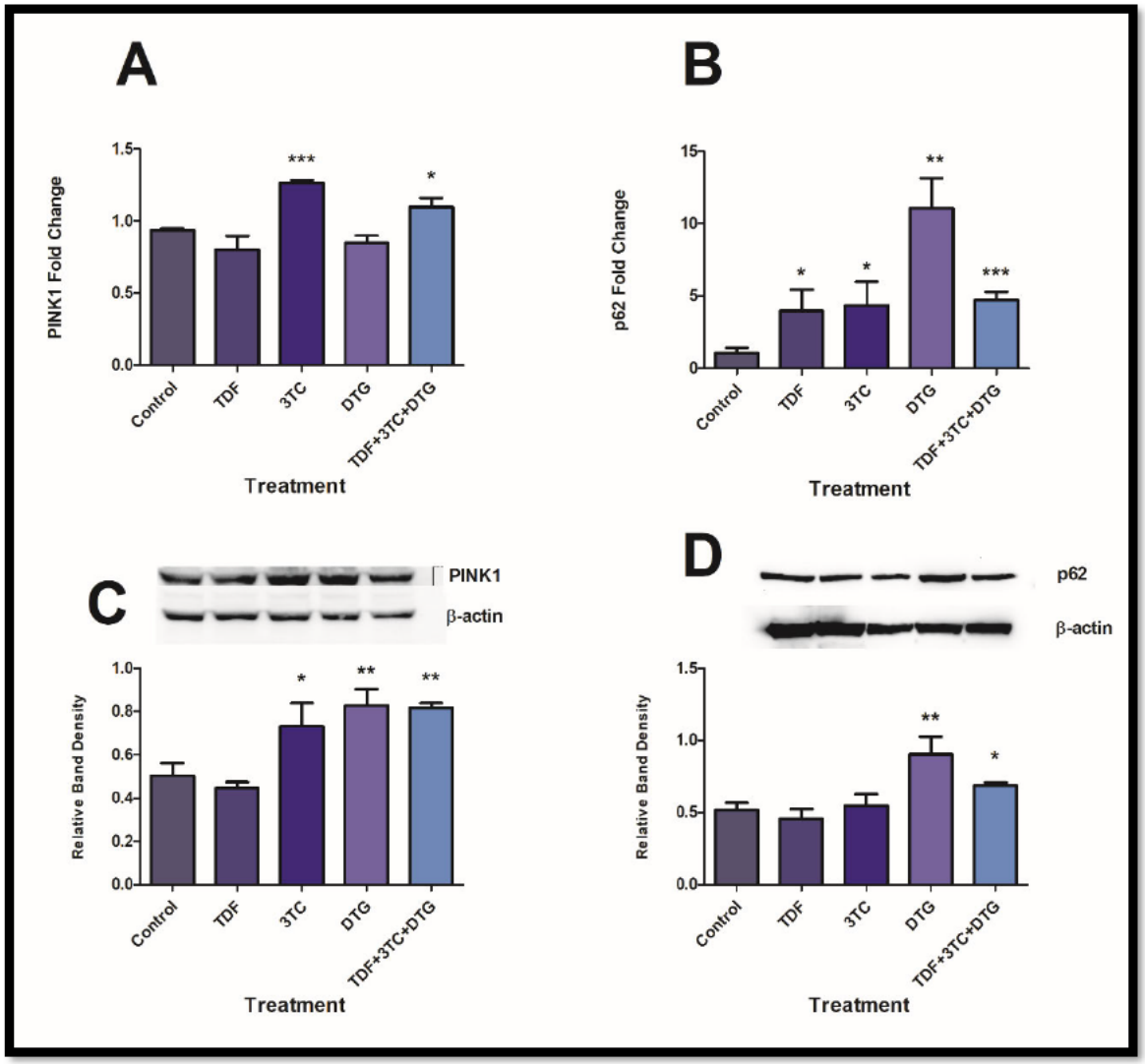

Figure 3.2: Transcript and protein expression of mitochondrial maintenance components. *PINK1* mRNA expression (A) and protein expression (C) as elevated for selected ARV treatments. Significant increases in *p62* mRNA expression were observed for all treatments (B); however,

only DTG and combinational usage showed considerable increases in p62 protein expression (D). *$p<0.05$, **$p<0.005$, ***$p<0.0001$*

Antiretrovirals suppress essential mitochondrial stress proteins.

SIRT3 is a crucial regulator of mitochondrial stress, and significant decreases in protein expression were observed for all treatments (TDF; (p=0,0039), 3TC; (p=0,0008), DTG; (p=0,0029), combinational use; (p=0,0003)) (Figure 3.3A). This was followed by coinciding decreases in UCP2 protein expression (TDF; (p=0,0056), 3TC; (p=0,0039), DTG; (p=0,0024), combinational use; (p=0,0119) (Figure 3.3B) indicating a suppression in mitochondrial stress responses.

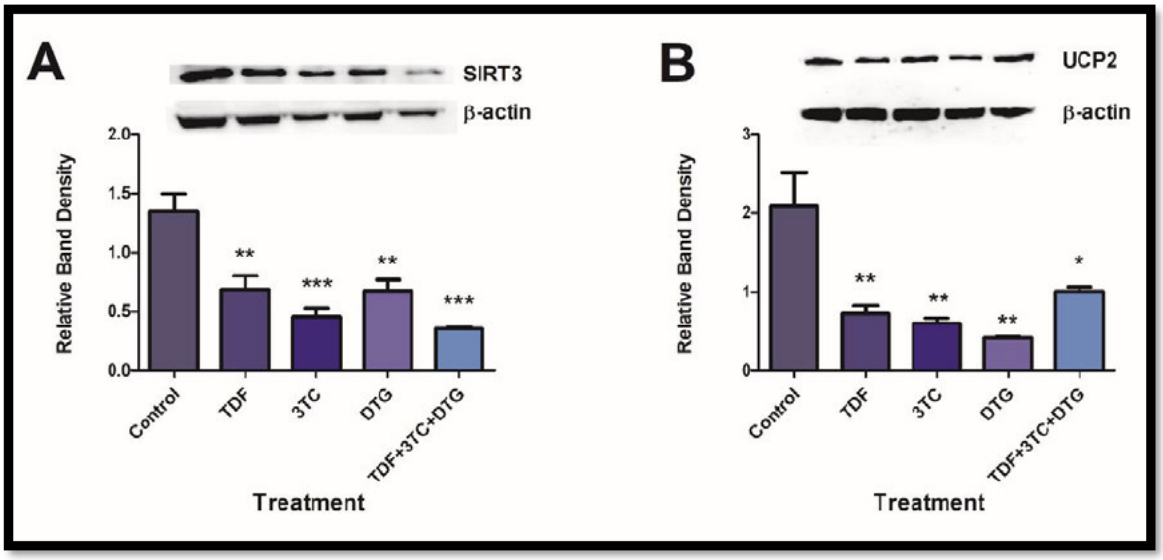

Figure 3.3: ARVs suppressed mitochondrial stress responses. SIRT3 protein expression (A) was significantly decreased with corresponding decreases in UCP2 protein expression (B). *$p<0.05$, **$p<0.005$, ***$p<0.0001$*

Antiretrovirals increased lipid peroxidation and decreased ATP concentrations.

MDA concentration is indicative of lipid peroxidation in cells and is proportional to ROS formation in cells. Singular treatments, TDF (p<0.0001), 3TC (p<0.0001), and DTG (p<0.0001), showed significant increases in MDA concentration (Figure 3.4A). This was further observed in the combinational treatment (p=0,0066) (Figure 3.4A). ATP concentrations are used to assess

mitochondrial function where decreases indicate compromised mitochondrial function. All singular treatments, TDF (p=0,0065), 3TC (p=0,0299), and DTG (p=0,0003), showed significant reductions in ATP concentration with similar results observed for combinational treatment (p=0,0017) (Figure 3.4B).

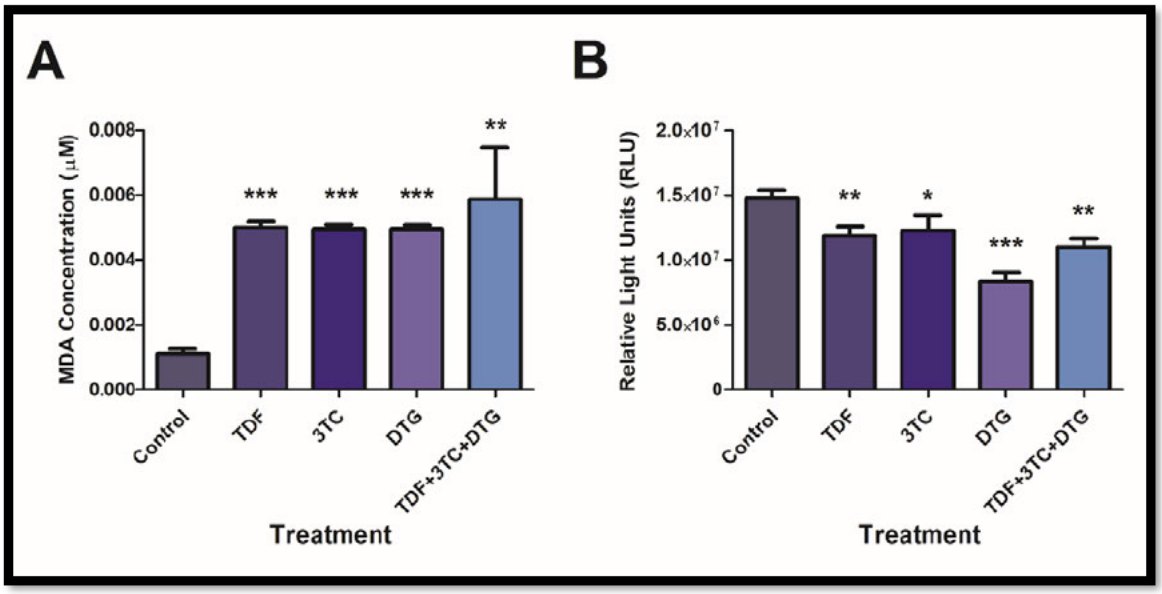

Figure 3.4: ARVs increased MDA concentration (A) and decreased ATP concentration (B). Considerable increases in MDA were observed for all ARV treatments (A), with significant decreases in ATP concentration (B). *$p<0.05$, **$p<0.005$, ***$p<0.0001$

Discussion

MetS from chronic drug use is induced when one or more metabolic irregularities occur in the human body. One of the most common pathologies associated with MetS is insulin resistance which can lead to the pathogenesis of T2DM if not controlled [29,30]. Several different biochemical abnormalities may cause insulin resistance; however, mitochondrial stress and dysfunction have been highlighted as one of the most frequent causes of insulin resistance [31]. Mitochondrial stress leads to aberrations in the electron transport chain (ETC), resulting in decreased ATP production and leakage of ROS [31]. Increased ROS production and leakage are responsible for activating the NLRP3 inflammasome [32,33] and the upregulation of JNK [20]. These processes result in increased phosphorylation of the insulin receptor substrate 1 (IRS1) and subsequent decreased activity of Protein kinase B (AKT) and phosphoinositide 3-kinase (PI3K). Consequently, reduced glucose uptake, vasodilation, and insulin secretion are experienced, resulting in the progression of insulin resistance [20].

Alarmingly, the prevalence of MetS in PLWH and ARV usage is increasing rapidly, with at least 21% experiencing insulin resistance [7-9]. Newer generation ARVs are associated with fewer side effects than older generations; however, metabolic complications persist with usage [6,23]. This study determined the impact of singular and combinational use of ARVs on mitochondrial stress as an underlying mechanism for insulin resistance promotion.

Nrf2 has been described extensively for its endogenous role in antioxidant responses. It allows for the transcriptional activation of several genes required to ameliorate oxidative stress [14]. Nrf2 can lead to the activation of SOD2 and CAT; however, the latter two enzymes can be independently activated in response to high levels of ROS and inflammation [34,35]. SOD2 is located in the mitochondrial matrix, making it a suitable indicator for mitochondrial stress [36]. In the present study, only DTG and combinational usage significantly increased the expression of pNrf2 (Figure 3.1A). However, it is important to note that the remaining treatments did not decrease pNrf2 expression but rather showed no significant changes. Previous studies in HepG2 cells using older generation ARVs showed selective upregulation of Nrf2 [26] and supports the results from the current study. The significant upregulation of pNrf2 following combinational usage indicates possible oxidative stress.

Interestingly, SOD2 protein expression was increased by 3TC, DTG, and combinational usage (Figure 3.1B), suggesting that high levels of ROS were present in the mitochondria following exposure. This was followed by increase in CAT expression (Figure 3.1C). The results correlate with ambient and upregulated pNrf2 expression. Previous studies have shown strong links between DTG usage and increased ROS production following the deregulation of Ca^{2+} signalling [37]. Increased ROS potential via DTG exposure coincides with upregulated responses in this study.

Although only selected singular treatments were able to upregulate antioxidant responses, the significant increase in combinational treatment indicates synergistic stress induced by combining different ARVs. This is critical information as ARVs are rarely ingested individually but in a single dose tablet that contains all three drugs.

PINK1 and p62 expressions were next analysed due to the prevailing oxidative stress environment. PINK1 and p62 are mitochondrial maintenance mediators that can be activated by Nrf2 [18,19]. PINK1 is upregulated to ensure mitochondrial respiration occurs and ROS production is reduced [16]. Although only 3TC and combinational treatments increased *PINK1* mRNA levels (Figure 3.2A), the protein expression of PINK1 for 3TC, DTG, and combinational drug treatments was upregulated (Figure 3.2C). Following treatment with these ARVs, PINK1

was possibly post-transcriptionally upregulated in response to aberrations in mitochondrial function. The results agree with increases in pNrf2 expression for selected treatments.

Conversely, we observed significant elevations in transcript levels of *p62,* but only significant increases were noted for DTG and combinational ARV usage of p62 protein expression (Figure 3.2B; D). Literature indicates that p62 is upregulated in response to protein damage as a method for the clearance of oxidatively damaged proteins [17]. This suggests that DTG and combinational ARV treatments induced damage of proteins, thus eliciting significant upregulation in p62 expression. Additionally, p62 can activate Nrf2 in a positive feedback loop in response to oxidative stress in cells [18,38]. The considerable upregulation in pNrf2 and p62 for DTG and combinational ARV treatment suggest that the positive feedback loop was activated in response to stress in the HepG2 cells. Moreover, no significant changes in pNrf2 and p62 protein expression were observed for TDF and 3TC treatments, suggesting possible absence of the loop.

All treatments with ARVs showed significant downregulation in SIRT3 and UCP2 expression (Figure 3.3). Both proteins play a role in the amelioration of mitochondrial stress. More specifically, suppression of SIRT3 has strongly been associated with mitotoxicity due to inadequate stress relief. UCP2 is known to activate SIRT3, among other stress responses in cells which are imperative to mitochondrial integrity and function [10]. Furthermore, SIRT3 is responsible for the deacetylation of complexes in the ETC, which maintains ATP production, while UCP2 is responsible for oxygen consumption in ATP synthesis [39]. Aside from this, UCP2 has been associated with reductions in mitochondrial oxidative stress, and dysfunction in the protein has been linked to cardiovascular diseases [40]. It can be deduced that aberrations in mitochondrial stress responses were experienced following exposure to all drugs. Additionally, SOD2 and CAT were activated independently of SIRT3 activity.

Following exposure to ARVs, oxidative damage occurred in the form of lipid peroxidation, which has previously been associated with ROS production [41]. MDA concentration (Figure 3.4A) was significantly upregulated despite the activation of antioxidant responses and mitochondrial maintenance in selected treatments. Older generation ARVs showed increased MDA levels in HepG2 cells [23], indicating that newer generation ARVs have similar adverse effects. Furthermore, usage of TDF and 3TC increased lipid peroxidation and depleted glutathione levels when paired with Efavirenz in rat liver and kidneys [42]. The increase in oxidative damage indicates that the cells had insufficient antioxidant responses.

Significant reductions in ATP production for all treatments was observed (Figure 3.4B). The considerable suppression in mitochondrial stress proteins SIRT3 and UCP2 (Figure 3.3) provides reasoning for decreased ATP production despite PINK1 upregulation (Figure 3.2C). Reduced

ATP production is indicative of dysfunctional mitochondrial activity [43]. Previous studies have shown that DTG and TDF decreased mitochondrial ATP production via action on the ETC [44,45], supporting the findings in the present study. We further observed increased depolarisation of mitochondrial membranes which is indicative of dysfunction however results were not significant (Supplementary Figure 3.1)

Increased oxidative damage is a sign of excess ROS production, which coincides with decreased ATP production. The current study provides evidence that ROS production increased despite an attempt in liver cells to upregulate antioxidant responses. This was mainly attributed to suppressed mitochondrial stress responses. Elevations in mitochondrial ROS have been shown to activate JNK and the NLRP3 inflammasome, both of which are responsible for the phosphorylation of IRS1 through several intermediates. Subsequently, insulin resistance can be promoted through decreased action of the IRS1/PI3K/AKT pathway [20]. The present study, therefore, provides evidence that combinational usage of ARVs can lead to mitochondrial dysfunction that can promote insulin resistance. This information is crucial as HAART is popularly consumed in combination.

Future recommendations and limitations

The present study was an *in vitro* study, which has limitations in terms of application to humans. Future studies should assess similar markers in an *in vivo* humanised HIV^+ mouse model to fully understand the mechanism of ARV induction of MetS. Aside from this, different exposure periods should be considered.

Author Contributions

Conceptualisation, J.M., and T.G.; investigation, J.M.; writing—original draft preparation, J.M.; writing—review and editing, T.G., T.S and A.A.C.; supervision, T.G, and A.A.C. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Biomedical Research Ethics Committee (protocol code: BREC/00002256/2020 and date of approval: 15 April 2021).

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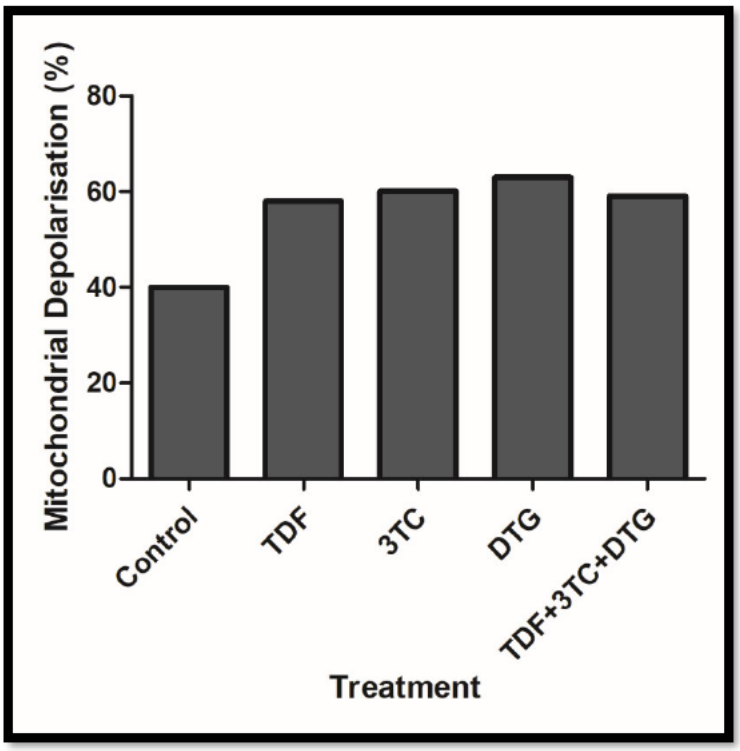
Appendix A: Mitochondrial Depolarisation



Supplementary Figure 3.1: Percentage of mitochondrial depolarisation following treatment. All treatments showed an increase in depolarisation however values were not significant.

CHAPTER 4

Antiretrovirals promote insulin resistance in HepG2 liver cells through miRNA regulation and activation of the NLRP3 inflammasome.

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Abstract

Metabolic syndrome (MetS) is a non-communicable disease characterised by a cluster of metabolic irregularities. Alarmingly, the prevalence of MetS in people living with Human Immunodeficiency Virus (HIV) and antiretroviral (ARV) usage is increasing rapidly. Insulin resistance is a common characteristic of MetS that leads to the development of Type 2 diabetes mellitus (T2DM). The progression of insulin resistance is strongly linked to inflammasome activation. This study aimed to draw links between the combinational use of Tenofovir disoproxil fumarate (TDF), Lamivudine (3TC), and Dolutegravir (DTG), inflammasome activation and subsequent promotion of insulin resistance following a 120 h treatment period. Furthermore, we assess microRNA (miR-128a) expression as a negative regulator IRS1/AKT signalling pathway. The relative expression of phosphorylated IRS1 was determined by Western blot. Transcript levels of *NLRP3*, *IL-1β, JNK, IRS1, AKT, PI3K*, and miR-128a were assessed using quantitative PCR (qPCR). Caspase-1 activity was measured using luminometry. Following exposure to ARVs for 120 h, *NLRP3* mRNA expression (p= 0,0500) and caspase-1 activity (p<0.0001) significantly increased. This was followed by a significant elevation in *IL-1β* in mRNA expression (p= 0,0015). Additionally, *JNK* expression (p= 0,0093) was upregulated with coinciding increases in p-IRS1 protein expression (p< 0.0001) and decreased *IRS1* mRNA expression (p=0,0004). Consequently, decreased *AKT* (p= 0,0005) and *PI3K* expressions (p= 0,0007) were observed. Interestingly miR-128a expression was significantly upregulated. The results indicate that combinational use of ARVs upregulates inflammasome activation and promotes insulin resistance through dysregulation of the IRS1/PI3K/AKT insulin signalling pathway.

Keywords: Antiretrovirals, metabolic syndrome, insulin resistance, inflammasome, miR-128a

Introduction

MetS is classified as a global hazard that affects 20-30% of adults by the World Health Organization (WHO). The non-communicable diseases can be classified by several different pathologies, including insulin resistance, obesity, hypertension, and dyslipidaemia [1,2]. These isolated pathologies may contribute to the development of more severe conditions such as Type 2 Diabetes Mellitus (T2DM) and cardiovascular diseases (CVD) [2].

Literature indicates a strong correlation between the occurrence of MetS in people living with HIV (PLWH) [3]. HIV affects severely affects a considerable portion of the global population, with greater specificity in Sub-Saharan Africa. By the end of 2019, roughly 38 million people worldwide lived with HIV, with 1.7 million new infections for the year [4]. Of these statistics, 7.8 million PLWH were localised to South Africa [5].

Of the total infected global population, 26 million had access to antiretroviral (ARV) treatment at the end of 2019 [4]. Highly active antiretroviral therapy (HAART) has significantly decreased the HIV-infected population's mortality [6]. However, clinical studies have indicated that the usage of HAART promotes MetS in PLWH, with at least 21% displaying insulin resistance [7-9]. Despite these findings, proper mechanisms of action surrounding the combinational usage of HAART remain elusive.

It is well understood that inflammation is strongly linked to the occurrence of insulin resistance [10]. The upregulation of inflammatory genes and proteins leads to the serine phosphorylation of the insulin receptor substrate I (IRS1), which has several downstream targets that reduce insulin sensitivity [11]. More specifically, increased phosphorylated IRS1 (p-IRS1) causes decreased expression of Protein kinase B (AKT), and phosphoinositide 3-kinase (PI3K) allow for the progression of insulin resistance [12].

Aside from the more common pro-inflammatory cytokines, the (NOD-like) pyrin domain containing 3 (NLRP3) inflammasome has gained popularity for its implications in insulin resistance [13]. This is observed through the cleavage of pro- interleukin 1β to interleukin- 1β (IL-1β), which allows for the serine phosphorylation of IRS1 both directly and indirectly [11]. The occurrence of the NLRP3 inflammasome in PLWH has been well studied; however, mechanisms surrounding combinational ARV usage and possible activation of inflammasomes and its' linkage to insulin resistance remain limited [14].

Additionally, insulin resistance can be regulated epigenetically through the expression of miRNAs [15]. Increased expression of specific miRNAs results in decreased expression of targets

related to insulin resistance. In this study, we focus on miR-128a, which is known to negatively regulate the IRS1/AKT, thus promoting insulin resistance [16].

Insulin resistance in the liver contributes to reduced lipogenesis and consequently causes an accumulation of fats. This eventually leads to the pathogenesis of non-alcoholic fatty liver disease (NAFLD) [17]. Literature indicates that the risk of T2DM is significantly increased when NAFLD occurs [18].

3TC, TDF, and DTG have been proposed as a combinational treatment in the first line of therapy for HIV by WHO [19]. Studies often assess the side effects of these drugs in isolation, with very few studies evaluating biochemical mechanisms involved in their combinational usage [20]. This study aimed to understand the relationship between the combinational use of TDF, 3TC and DTG, inflammasome activation and its promotion of insulin resistance in liver cells following prolonged *in vitro* exposure. HepG2 liver cells were chosen as they exhibit similar functions as primary hepatocytes and have been used in several ARV studies [21-23]. Furthermore, we highlight miRNA regulation and its possible implications for the progression of insulin resistance. Evidence from this study can be used to develop therapies with reduced side effects related to MetS.

Materials and Methods

Materials

Antiretroviral drugs were obtained from the NIH AIDS reagents program. HepG2 cells were purchased from American Type Culture Collection (Johannesburg, South Africa). Cell culture media and supplements were purchased from Lonza (Basel, Switzerland). Luminometry kits were obtained from Promega (Madison, Wisconsin, USA). Western Blot reagents were purchased from Bio-Rad (Hercules, California, USA). Unless otherwise stated, all remaining reagents were obtained from Merck (Darmstadt, Germany).

Cell culture and treatment

HepG2 cells were cultured in 25 cm^3 cell culture flasks using CCM [Eagle's minimum essentials medium (EMEM) supplemented with, 10% foetal calf serum, 1% pen-strep-fungizone, and 1% L-glutamine] and maintained in a humidified incubator (37 °C, 5% CO_2) until approximately 70% confluent. Cells were then exposed to physiological concentrations (C_{max}) of ARVs (3TC: 1.51µg/ml, TDF: 0.3µg/ml, DTG: 3.67 µg/ml) [24-26] for 120 hours (h) as per Nagiah et al., 2015

[22]. Cells were washed every 24 h with 0.1 M phosphate buffered saline (PBS) and fresh CCM with ARVs were added to flasks. Further assays were carried out following treatment as explained above.

Caspase-1 Detection

Caspase-1 activity was measured using the Caspase-Glo® 1 Inflammasome Assay (G9951, Promega, Madison, USA). Following incubation cells with treatment, 50 µL of cells suspension (20 000 cells/well in 0.1 M PBS) was added into an opaque microtitre plate in triplicate. The Caspase-Glo® 1 reagents were reconstituted as per manufacturer's guidelines and 50 µL was added to each well containing cells. Plates were then incubated (dark, 1 h, RT). Luminescence was measured using a Modulus™ Microplate Reader (Turner Biosystems, Sunnyvale, CA, USA). Results were expressed as relative light units (RLU).

Western Blot

Following 120 h treatment of HepG2 cells with ARVs, cells were washed with 1 M PBS. Thereafter, 150 µL Cytobuster™ Reagent was added to each flask (Novagen, San Diego, CA, USA, catalogue no. 71009) and incubated on ice for 30 minutes (min). Mechanical lysis of cells was performed using a cell scraper and contents were transferred to 1.5 mL micro-centrifuge tubes followed by centrifugation (400× g, 10 min, 4 °C). The supernatant containing crude protein isolates were removed and transferred to fresh microcentrifuge tubes and protein concentration was quantified. The bicinchoninic acid assay (BCA) was used to quantify proteins, and samples were standardised to a concentration of 1.5 mg/mL. Protein samples were prepared for further usage by boiling (5 min, 100 °C) in Laemmli Buffer (distilled water, glycerol, 10% SDS, β-mercaptoethanol, 0.5 M Tris-HCl (pH 6.8), 1% bromophenol blue and glycerol).

A Bio-Rad compact supply was used to electrophorese 20 µL samples (1 h, 150 V) in sodium dodecyl sulphate (SDS) polyacrylamide gels (4% stacking, 10% resolving). Separated proteins were transferred onto nitrocellulose membranes using the Bio-Rad Trans-Blot® Turbo Transfer. Blocking of membranes were carried out using 5% Bovine Serum Albumin (BSA) in Tween 20-Tris buffer saline (TTBS: 150 mM NaCl, 3 mM KCl, 25 mM Tris, 0.05% Tween 20, dH2O, pH 7.5) for 1 h at room temperature (RT).

Membranes were then immuno-probed with the requisite primary antibody (Cell signalling Technology; Phospho-IRS1 (Ser1101) Antibody (#2385T) 1:1000 dilution in 5% BSA) for 1 h at

RT and overnight at 4 °C. Thereafter, membranes were washed 5 times for 10 min using 5 mL TTBS. Membranes were then incubated in HRP-conjugated secondary antibodies (Cell signalling Technology; anti-rabbit (#7074S) 1:5000 in 5% BSA) for 1 h at RT. Following incubation membranes were washed (5x 10 min in TTBS) and rinsed with distilled water. Proteins were detected following the addition of Clarity Western ECL Substrate detection reagent (400 µL) (Bio-Rad, Hercules, CA, US)), and images were captured using the Bio-Rad ChemiDoc™ XRS+ Imaging System.

Membranes were quenched using 5% hydrogen peroxide for 30 min at 37 °C, blocked using 5% BSA and incubated in HRP-conjugated antibody for β-actin (A3854, Sigma-Aldrich). β-actin is a housekeeping protein expressed evenly across cells. Image Lab™ Software v6.0 (Bio-Rad, Hercules, CA, USA) was used for analysis of results. Relative band density of protein was calculated by normalising results against β-actin.

Quantitative PCR

RNA Isolation and Quantification

Following treatment cells were washed using 1 M PBS and incubated with a mixture of 500 µL Trizol and 500 µL PBS (5 min, RT). Mechanical lysis of cells was performed using a cell scraper and contents was transferred to 1.5 mL micro-centrifuge tubes and stored (24 h, −80 °C). Thereafter samples were thawed and 100 µL chloroform was added to each tube followed by centrifugation (12,000× g, 10 min, 4 °C). Supernatants were removed and transferred to 1.5 mL micro-centrifuge tubes containing 250 µL. Tubes were incubated overnight at −80 °C. Following incubation, samples were thawed and centrifuged (12,000× g, 20 min, 4 °C). Supernatants were aspirated and discarded, and the remaining pellet was washed in 500 µL of 75 % cold ethanol followed by centrifuged (7400× g, 15 min, 4 °C). RNA pellets were air dried (30 min, 24 °C) and re-suspended in 15 µL nuclease-free water. RNA quantification was carried out using Nanodrop2000 spectrophotometer (Thermo-Fisher Scientific, Waltham, MA, USA). RNA quality was determined using the A_{260}/A_{280} ratio. All RNA samples were standardised to 1000 ng/µL.

Quantification of mRNA Expression

The cDNA was synthesised from the standardised RNA samples using the iScript™ cDNA Synthesis kit as per manufactures instructions (Bio-Rad, 107-8890, Hercules, CA, USA).

Transcript levels relevant genes (Table 4.1) were assessed using the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, 1725270) and the CFX96 Touch™ Real-Time PCR

Detection System (Bio-Rad, Hercules, CA, USA). The thermo-cycler conditions for each gene were as follows: initial denaturation (8 min, 95 °C), followed by 40 cycles of denaturation (15 s, 95 °C), annealing (40 s, Table 4.1), and extension (30 s, 72 °C). Data were normalised against the housekeeping gene, GAPDH which is evenly expressed across cells. Results were calculated using the Livak and Schmittgen (2001) method and was represented as fold change relative to the control cells ($2^{-\Delta\Delta CT}$) [27].

Table 4.1: Primer sequences with respective annealing temperatures for genes assessed

Gene		**Sequence (5′-3′)**	**Annealing Temperature (°C)**
NLRP3	Forward	CAGGTGTTGGAATTAGACAAC	60
	Reverse	TTCAGACAACCCCAGGTTCT	
IL-1β	Forward	ACGAATCTCCGACCACCACTAC	60
	Reverse	TCCATGGCCACAACAACTGACG	
AKT	Forward	TGGACTACCTGCACTCGGAGAA	59
	Reverse	GTGCCGCAAAAGGTCTTCATGG	
PI3K	Forward	GAAGCACCTGAATAGGCAAGTCG	59
	Reverse	GAGCATCCATGAAATCTGGTCGC	
IRS1	Forward	AGTCTGTCGTCCAGTAGCACCA	59
	Reverse	ACTGGAGCCATACTCATCCGAG	
JNK	Forward	GACGCCTTATGTAGTGACTCGC	59
	Reverse	TCCTGGAAAGAGGATTTTGTGGC	
GAPDH	Forward	TCCACCACCCTGTTGCTGTA	---
	Reverse	ACCACAGTCCATGCCATCAC	

Quantification of miR-128a expression

As per the manufacturer's instructions, cDNA was synthesised using standardised RNA using the miScript II RT kit (Qiagen, 218161, Hilden, Germany)). The expression of miR-128a was assessed using the miScript SYBR Green PCR Kit (Qiagen, 218073, Hilden, Germany)) and CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The thermo-cycler conditions were as follows: initial denaturation (15 min, 95 °C), followed by 40 cycles of

denaturation (15 s, 94 °C), annealing (30 s; 55 °C) and extension (30 s; 70 °C). Data were normalised against the housekeeping gene, GAPDH which is evenly expressed across cells. Results were calculated using the Livak and Schmittgen (2001) method and was represented as fold change relative to the control cells ($2^{-\Delta\Delta CT}$) [27]

Statistical Analysis

GraphPad Prism version 5.0 (GraphPad Prism Software Inc.) was used to perform all statistical analyses. Data were analysed using an unpaired t-test with data having $p < 0.05$ considered to be significant.

Results

Combinational usage of ARVs results in the upregulation of key components of the inflammasome pathway

The main components of the inflammasome pathway were assessed to determine activation following prolonged exposure. NLRP3 results in several downstream actions that cleave procaspase-1 to caspase-1. This ultimately leads to the activation of IL-1β from pro-IL-1β. Following exposure to ARVS, *NLRP3* mRNA expression was significantly increased (Figure 4.1A; p= 0,0500) with resulting increases in caspase-1 activity (Figure 4.1B; p= < 0.0001). This was accompanied by increased expression of *IL-1β* mRNA (Figure 4.1C; p= 0,0015).

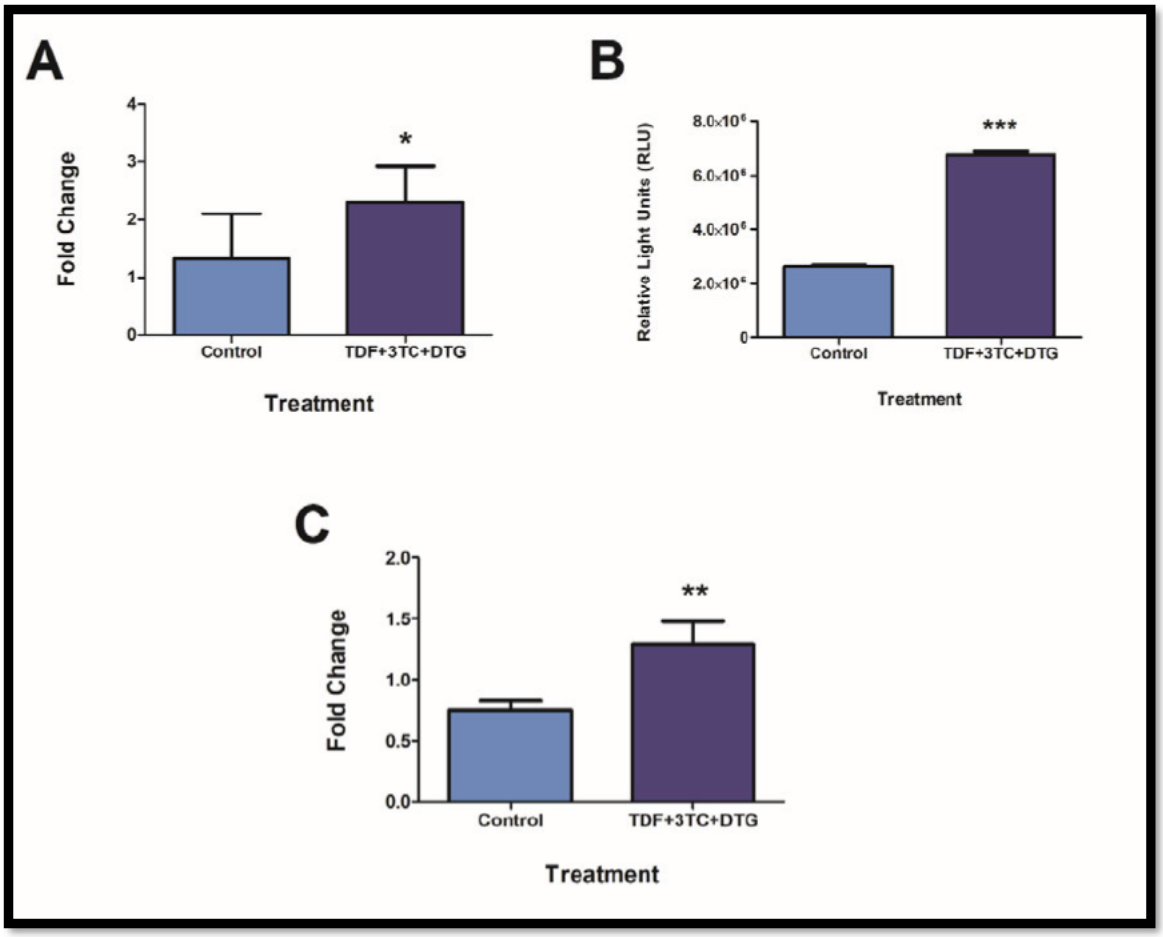

Figure 4.1: ARVs increase the expression and activity of components in the NLRP3 inflammasome. *NLRP3* mRNA expression was significantly increased (A; * $p < 0.05$). Additionally, caspase-1 activity showed significant upregulation following exposure (B; *** $p < 0.0001$). *IL-1β* mRNA showed significant elevations in expression (C; ** $p < 0.005$).

Exposure to ARVs alters JNK and Insulin Receptor Expressions

The expression of IRS1 and intermediates was assessed after confirmation of inflammasome activity. IL-1β can lead to JNK activation and a decrease in IRS1 expression. JNK allows for the serine phosphorylation of present IRS1 to yield p-IRS1. The latter is responsible for the progression of insulin resistance. After exposure to ARVs, *JNK* mRNA expression significantly increased (Figure 4.2A; p= 0,0093) with resulting decreases in *IRS1* mRNA expression (Figure 4.2B; p=0,0004). Furthermore, p-IRS1 showed increases protein expression (Figure 4.2C: p< 0.0001).

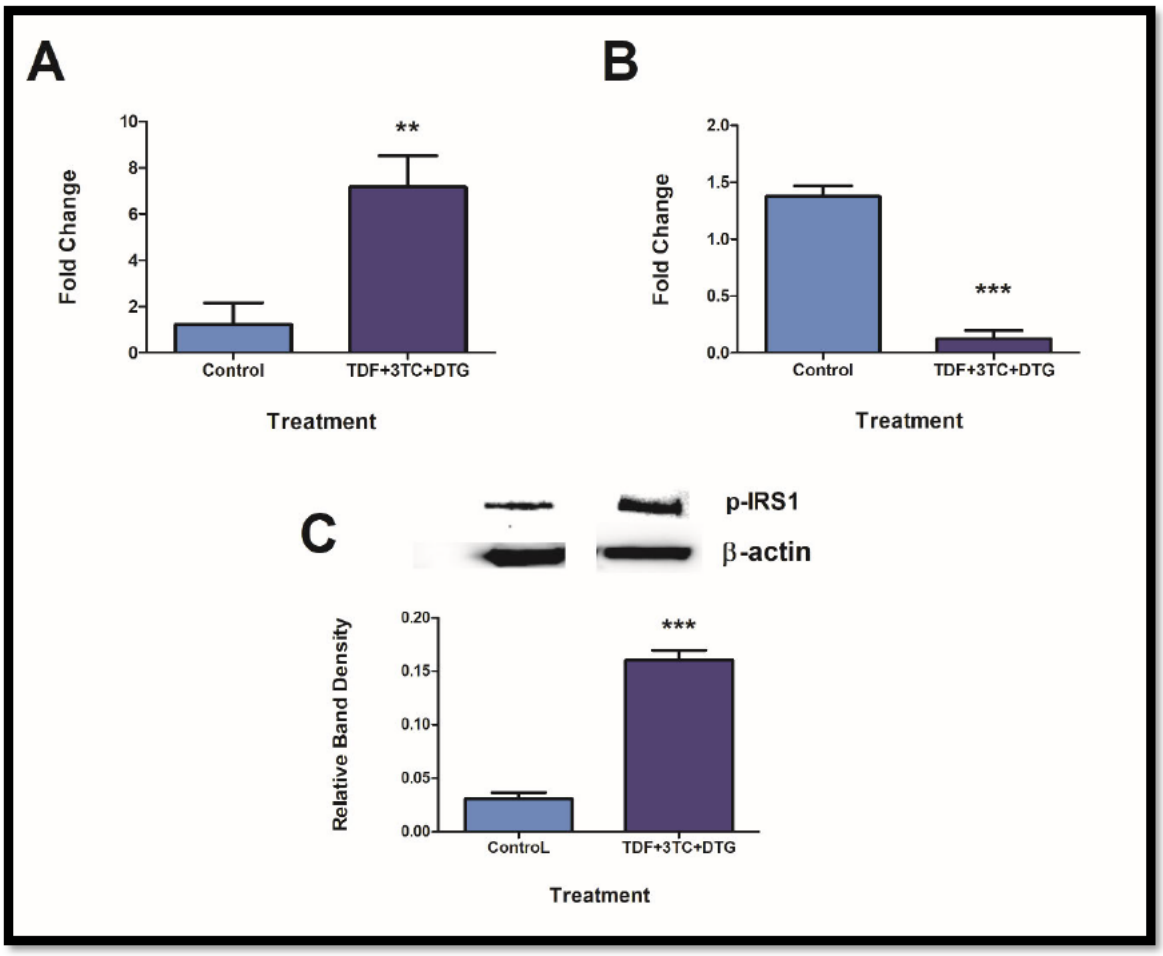

Figure 4.2: ARVs interfere with Insulin receptor expressions via JNK. *JNK* expression was significantly increased (A; ** $p < 0.005$) whereas *IRS1* expressions showed suppression (B; *** $p < 0.0001$). Furthermore, phosphorylated *IRS1* protein expression showed significant elevations (C; *** $p < 0.0001$).

Prolonged exposure to ARVs resulted in disruption of the PI3K/AKT pathway via inflammasome activation and miR-128a expression.

Elevation in p-IRS1 expressions coincide with decreased *AKT* and *PI3K* expression. Such effects result in insulin resistance promotion. Furthermore, miR-128a is known to regulate the expression of AKT and IRS1 negatively. Following exposure both *AKT* (Figure 4.3A; p= 0,0005) and *PI3K*

(Figure 4.3B; p= 0,0007) were significantly reduced whereas the expression of miR-128a showed significant elevation (Figure 4.3C; p= 0,0002).

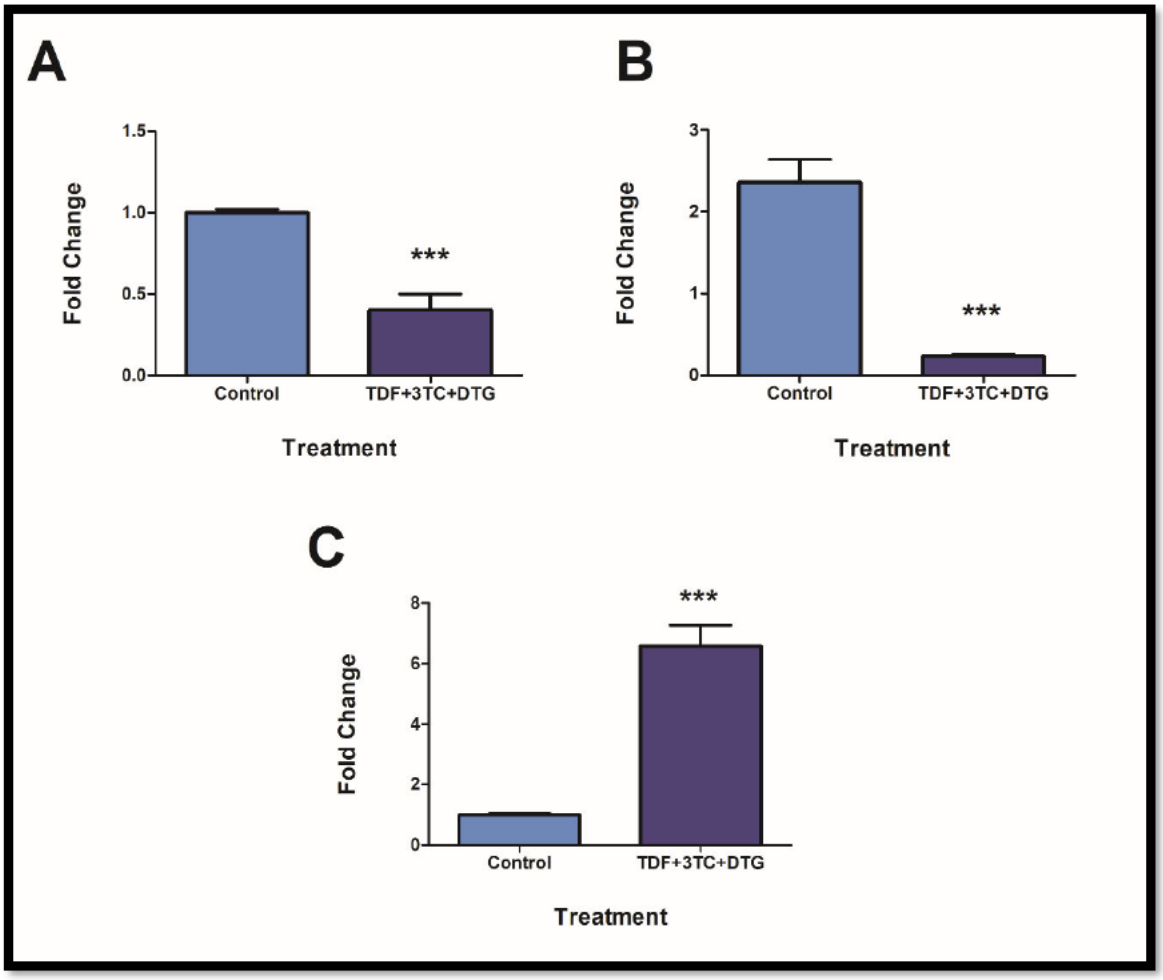

Figure 4.3: PI3K/AKT axis was disrupted through inflammasome activation and miRNA regulation. *AKT* (A; *** $p < 0.0001$) and *PI3K* (B; *** $p < 0.0001$) mRNA expression showed significant decreases. Conversely, miR-128a was elevated (C; *** $p < 0.0001$).

Discussion

MetS is a non-communicable disease that is diagnosed by having one or more of a cluster of metabolic irregularities. Alarmingly, the prevalence of MetS in PLWH and ARV usage is increasing rapidly. Older generations of ARVS were associated with severe side effects that resulted in patients discontinuing usage [20,22]. Fortunately, newer generations of drugs have fewer side effects but are still associated with metabolic complications. WHO has stressed the

need for the development of new ARVs and phasing in off newer generations of ARVs to ensure side effects are manageable and reduce HIV drug resistance [6,19]. This study aimed to look at biochemical mechanisms and epigenetic modifications associated with ARVs and MetS in liver cells which originate from the metabolic hub of the human body. Evidence from this study will aid in understanding possible mechanisms associated with ARV usage and insulin resistance. More specifically, we highlight the role of the NLRP3 inflammasome in the progression of insulin resistance and miRNA regulation of targets associated with insulin resistance/ sensitivity.

Previous evidence has shown links between inflammasome activation, and the constant pro-inflammatory states associated with the HIV infection [14]. Little evidence exists to show the combinational use of ARVs and inflammasome activation. Inflammasomes are multimeric protein complexes that assemble in response to different stressors. Several different types of inflammasomes exists with similar functions and different response stimuli [28]. The NLRP3 inflammasome is mostly activated in response to mitochondrial stress. Upon stimulation the NLRP3 proteins bind to the ASC proteins via pyridinoline interactions [29,30]. Pro-caspase-1 then interacts with the ASC protein via CARD domains which ultimately leads to the auto-proteolytic maturation of pro-caspase-1 into active caspase-1. The latter allows for pro-inflammatory cytokines into their bioactive forms. More specifically pro-IL-1β is cleaved to IL-1β and can then perform inflammatory functions [28] (Figure 4.4).

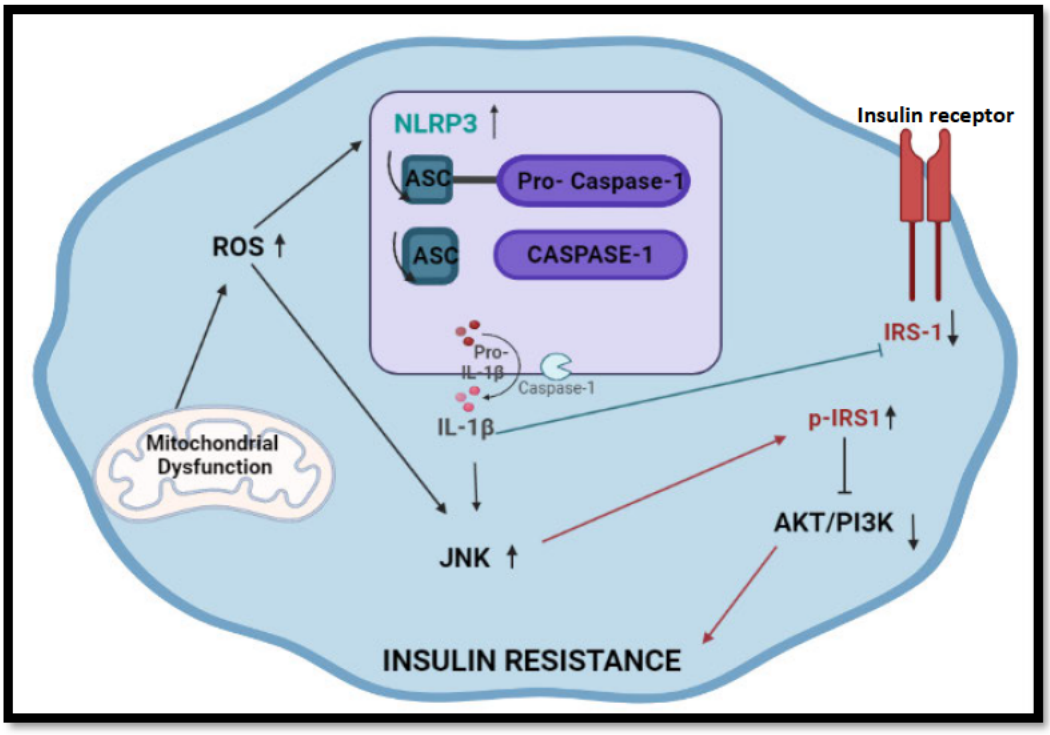

Figure 4.4: Inflammasome activation and subsequent occurrence of insulin resistance. Following activation of inflammasomes via stress, IL-1β allows for JNK activation leading to increases in p-IRS1 and aberration in AKT/PI3K signalling. Furthermore, decreased expression of IRS1 occurs.

The current study shows significant increases in *NLRP3* gene expression and caspase-1 activity (Figure 4.1A, B). Additionally, *IL-1β* mRNA expression showed substantial elevations (Figure 4.1C). The evidence presented coincides with the upregulation of inflammasome at a transcriptional level. The ARVs tested have previously been associated with increased ROS production and mitochondrial stress. More specifically, DTG has been implicated in the rise in ROS production via the dysregulation of Ca^{2+} signalling [31]. Aside from this, studies in rats' liver and kidneys showed that using TDF and 3TC increased lipid peroxidation (a consequence of ROS production) and depleted glutathione levels when paired with Efavirenz [32]. These are common markers for mitochondrial dysfunction. The ability of the ARVs tested to induce ROS production provides plausible reasoning for the increase of genes and components related to the NLRP3 inflammasome. Despite the individual ARVs being unable to cause a significant increase in *NLRP3* gene expression (Supplementary Figure 4.1A), combinational usage caused considerable elevation. This is possibly attributed to synergistic effects in mitochondrial dysfunction observed in previous studies [31,32].

In *in vitro* work, IL-1β suppresses insulin sensitivity by increasing JNK-dependent serine phosphorylation of IRS1. Subsequently, increased p-IRS1 causes aberrations in insulin-induced PI3K/Akt signalling in cells [11]. Aside from activation via IL-1β, JNK can be upregulated by detecting excessive ROS production and mitochondrial dysfunction [12]. The present study shows a significant increase in *JNK* expression following exposure (Figure 4.2A). Furthermore, p-IRS1 protein expression increased, coinciding with the increases in *JNK* (Figure 4.2C). Aside from this, previous studies show correlations between increased *IL-1β* expression and decreased IRS1 expression [11], correlating with data from the present study (Figure 4.2A, B). Similarly, singular ARV treatment produced no significant change in *JNK* expression (Supplementary Figure 4.2A); however, combinational usage prompted responses. Studies have shown that DTG can reduce mitochondrial ATP production and redox activity in murine cells, further providing reasoning for JNK activation [33]. In similar studies using rat kidneys, TDF was found to reduce ATP production via action on electron transport chain complexes signalling for aberrant mitochondrial metabolism [34]. The individual capacity of these drugs to initiate mitochondrial dysfunction [34,35] provides reasoning for their synergistic activation of related targets such as JNK and, subsequently, *IRS1* gene expression.

Under typical conditions, IRS1 allows for the activation of PI3K and AKT. The latter intermediates allow for an increase in glucose uptake, vasodilation, and insulin secretion in cells. However, elevated expression of p-IRS1 as a consequence of serine phosphorylation by JNK causes a decrease in *PI3K* and *AKT* expression (Figure 4.3), thus promoting the occurrence of insulin resistance and, ultimately, T2DM if not controlled [12]. The current study showed significant decreases in *AKT* and *PI3K* expression (Figure 4.3A, B) coinciding with the observed elevation of p-IRS1 expression. Previous literature indicates that DTG can promote insulin resistance in adipose tissue through the induction of oxidative stress; however, mechanisms remained unclear [23]. In earlier studies, using 3TC with other ARVs, caused disturbances in glucose metabolism with a significant decrease in insulin-mediated glucose disposal, thus showing the promotion of insulin resistance. However, no biochemical mechanism of action was established [36]. The current study provides possible mechanisms for the occurrence of insulin resistance at a genomic and protein level following combinational usage.

Aside from this, the evidence suggests that the tested ARVS can promote insulin resistance through epigenetic changes. It is well known that miRNAs can negatively regulate the expression of specific targets [37]. We assessed the expression of miR-128a which was found to negatively regulate IRS1/AKT signalling in previous studies [16]. This occurs when miR-128a binds to the 3′-untranslated region (3′-UTR) of target mRNA [16]. Following exposure in the current studies,

miR-128a was significantly increased while *AKT* and *IRS1* showed correlating decreases (Figure 4.3C). The data suggests that ARVs can promote insulin resistance through the upregulation of miRNA expression. This has implications for future studies that are imperative to understanding epigenetic changes induced by ARV exposure. At present, studies showing combinational use and epigenetic modifications remain limited.

Overall, this study provides insights into the possible mechanism of insulin resistance through inflammasome activation. Furthermore, we highlight epigenetic changes that coincided with insulin resistance promotion in cells. Combinational usage showed an increase in inflammasome-related genes and enzymes, resulting in reduced IRS1 signalling and, subsequently, promotion in insulin resistance despite the drugs not achieving the same result during individual exposure. This was further promoted through miRNA expression.

Future recommendations and limitations

The present study was carried out using HepG2 cells. Future studies should assess similar markers in an *in vivo* HIV^+ model at different exposure periods to fully understand mechanisms. The present study quantifies expression of miR-128a as a regulator of the IRS1/AKT pathway, however, future studies need to include more markers and experiments that can contribute to understanding epigenetic modifications associated with ARV use.

Author Contributions

Conceptualisation, J.M. and T.G.; investigation, J.M.; writing—original draft preparation, J.M.; writing—review and editing, T.G., M.S.M and A.A.C.; supervision, T.G and A.A.C. All authors have read and agreed to the published version of the manuscript.

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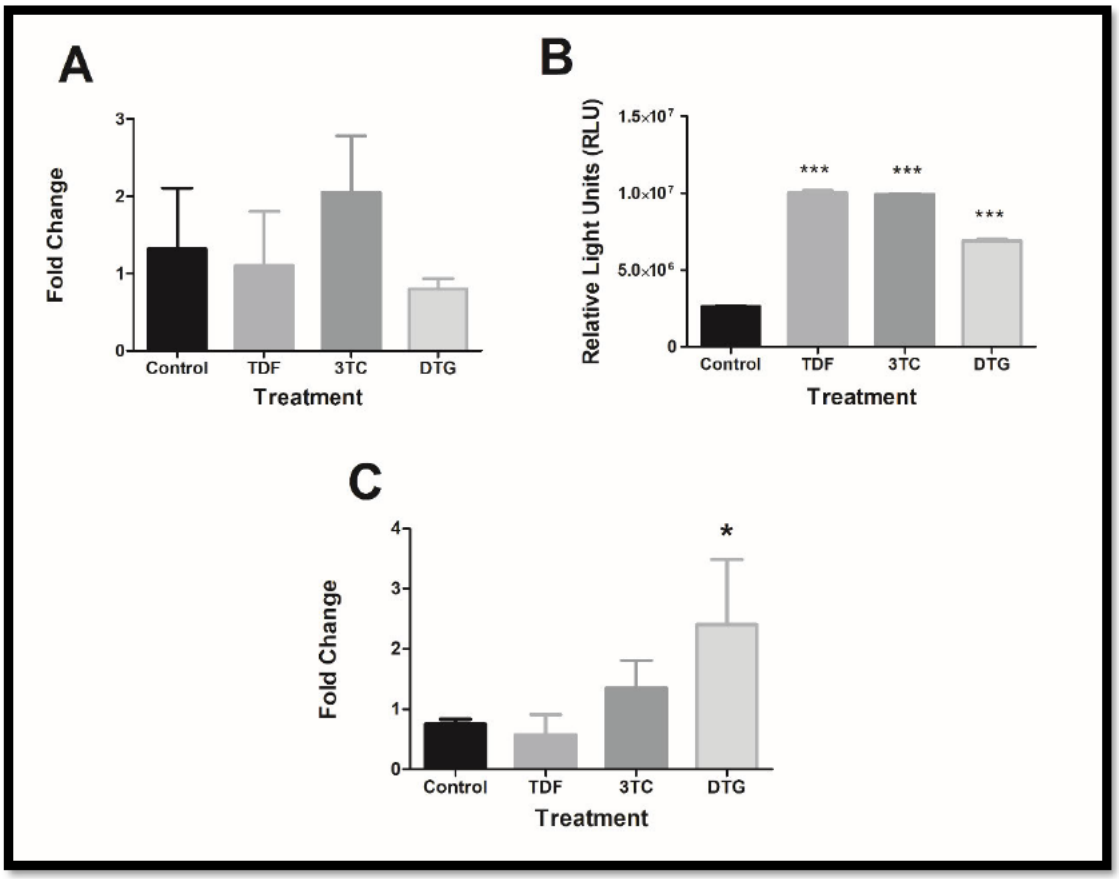
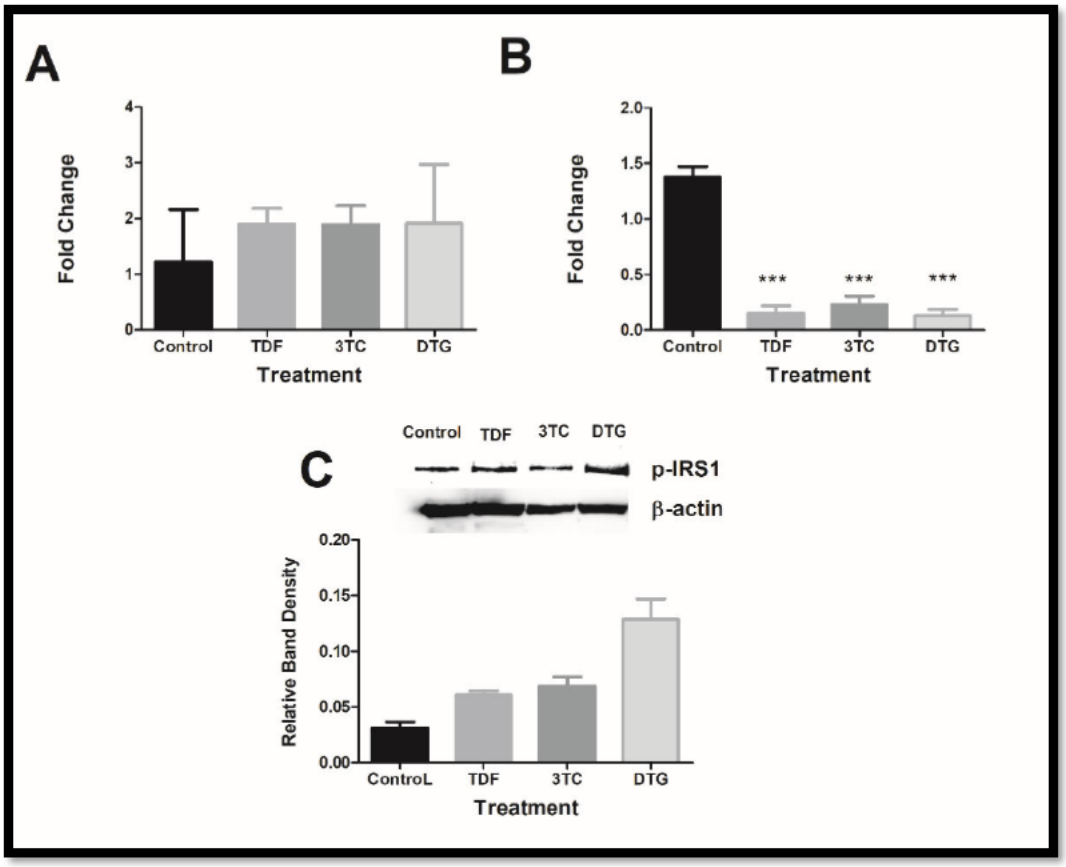
Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of Biomedical Research Ethics Committee (protocol code: BREC/00002256/2020 and date of approval: 15 April 2021).

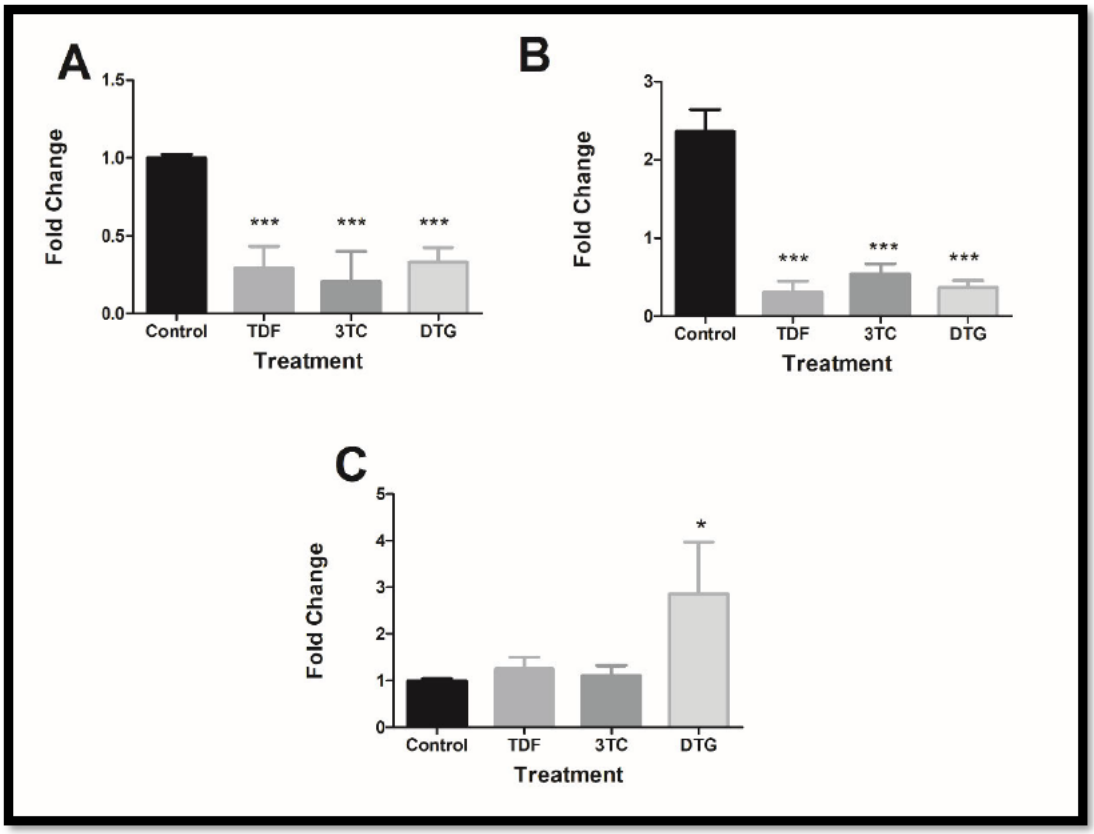
Supplementary data



Supplementary Figure 4.1: Individual exposure for tested ARVs. A: *NLRP3* expression; B: Caspase-1 activity; C: *IL-1β* expression. *(p: *** $p < 0.0001$; * $p < 0.05$).*



Supplementary Figure 4.2: Individual exposure for tested ARVs. A: *JNK* expression; B: *IRS1* expression; C: p-IRS1 protein expression. *(p: *** $p < 0.0001$)*



Supplementary Figure 4.3: Individual exposure for tested ARVs. A: *AKT* expression; B: *PI3K* expression; C: miR-128a miRNA expression. *(p: *** $p < 0.0001$; * $p < 0.05$).*

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CHAPTER 5

Conclusion

Over the past 30 years, the prevalence of MetS has increased dramatically. Changes in the environment, diet and lack of exercise have increased the risk of developing pathologies associated with the syndrome. These include insulin resistance, visceral obesity, prothrombotic, hypercholesterolemia and hyperglycaemia [1-3]. The pathogenesis of these conditions is initiated and significantly promoted by mitochondrial dysfunction and inflammation. Additionally, the occurrence of one or more risk factors increases the chance of developing T2DM and CVD by 5-fold and 2-fold, respectively [4].

HIV infection affects a significant portion of the global population. PLWH experience mitochondrial dysfunction and persistent inflammation, which can be exacerbated by ARV therapy. Following the extensive review of biochemical and epigenetic pathways involved in HAART, we found that ARVs drastically increased the lifespan of PLWH. In spite of this increased lifespan, side effects remain severe and may manifest as metabolic complications [5].

Thereafter, the effects of singular and combinational usage of approved ARVs on mitochondrial stress and dysfunction in HepG2 cells was assessed. This in vitro model is useful for determining toxicity as it mimics primary hepatocytes with similar physiological and genetic profiles in terms of drug metabolism [6,7]. The result indicated severe disarray of mitochondrial stress responses required for mitochondrial homeostasis. This was complemented by increased ROS and reduced ATP production, indicating possible mitochondrial dysfunction.

Thereafter, effects on the NLRP3 inflammasome were assessed at transcript levels which showed significant upregulation following combinational exposure. Downstream effects included aberrations in the IRS1/PI3K/AKT pathways seen through significantly decreased gene expression. This was further accompanied by increased phosphorylation of the insulin receptor substrates, confirming possible serine phosphorylation.

Overall, it can be concluded from the HepG2 in vitro model that combinational ARV use induces mitochondrial dysfunction and subsequently promotes the activation of the inflammasome. Consequently, insulin signalling cascades are disrupted, promoting insulin resistance in liver cells and, ultimately, MetS progression. This result is imperative for future studies aiming to reduce the side effects of ARVs and possibly prevent MetS in PLWH. Furthermore, findings are significant for South Africa, where a high prevalence of MetS is observed in PLWH.

CHAPTER 6

Limitations and Recommendations

These findings provide further insight into ARV-induced toxicity that can lead to MetS, albeit in an *in vitro* model. The HepG2 liver cell line is a transformed cancer cell line with similar physiological profiles to primary hepatocytes; however, certain genetic components may be lacking. Therefore, it is recommended that primary hepatocytes be utilised to verify the HepG2 cell data. Aside from this, it is recommended that future studies include multiple cell line models possible derived from muscle and cardiac tissue to strengthen the current findings.

In general, *in vitro* monoculture models have limitations due to the lack of a fully functional system of organs and limited time exposure. It is recommended that future studies use *in vivo* animal models; these models would allow a greater degree of complexity and multicellularity, and results can be extrapolated to humans. It would, be interesting to test the same parameters using samples from HIV-infected individuals that display insulin resistance and compare it with healthy non-infected individuals and HIV positive patients without insulin resistance. Data from these studies with aid in solidifying the result in the present study.

Currently, there are limited studies on the role of epigenetic modifications and HAART-induced MetS. A future study on DNA methylation, histone modifications and miRNA regulation in HAART-induced MetS is recommended.

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Addendum A: Review Article

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Review

A Critical Review of the Biochemical Mechanisms and Epigenetic Modifications in HIV- and Antiretroviral-Induced Metabolic Syndrome

Jivanka Mohan, Terisha Ghazi and Anil A. Chuturgoon

MDPI

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Review

A Critical Review of the Biochemical Mechanisms and Epigenetic Modifications in HIV- and Antiretroviral-Induced Metabolic Syndrome

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Abstract: Metabolic syndrome (MetS) is a non-communicable disease characterised by a cluster of metabolic irregularities. Alarmingly, the prevalence of MetS in people living with Human Immunodeficiency Virus (HIV) and antiretroviral (ARV) usage is increasing rapidly. This study aimed to look at biochemical mechanisms and epigenetic modifications associated with HIV, ARVs, and MetS. More specifically, emphasis was placed on mitochondrial dysfunction, insulin resistance, inflammation, lipodystrophy, and dyslipidaemia. We found that mitochondrial dysfunction was the most common mechanism that induced metabolic complications. Our findings suggest that protease inhibitors (PIs) are more commonly implicated in MetS-related effects than other classes of ARVs. Furthermore, we highlight epigenetic studies linking HIV and ARV usage to MetS and stress the need for more studies, as the current literature remains limited despite the advancement in and popularity of epigenetics.

Keywords: metabolic syndrome; HIV; ARVs; mitochondrial dysfunction; inflammation; epigenetics

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1. Introduction

The World Health Organisation (WHO) describes MetS as a pathological condition characterised by a cluster of metabolic irregularities, including abdominal obesity, insulin resistance, atherogenic dyslipidaemia, and hypertension [1]. The syndrome is diagnosed by having at least three of the following symptoms: central obesity, elevated triglycerides, reduced high-density lipoprotein cholesterol levels, high blood pressure, and elevated fasting glucose levels [2–4].

MetS is considered a global health hazard, with an estimated 20–30% of adults being affected [5]. It contributes to the development of other pathological conditions such as Type 2 Diabetes Mellitus (T2DM), cardiovascular diseases (CVD), and hypertension [1]. Therefore, MetS has been implicated in widespread morbidity and mortality [5].

The biochemistry of MetS has been linked to chronic systemic inflammation and oxidative stress [6,7]. Consequently, the literature concludes that the disease is strongly associated with processes that contribute to the excess production of reactive oxygen species (ROS) and cytokine activation, such as mitochondrial dysfunction and inflammatory pathways [5]. More recent advancements in science have highlighted the role of epigenetics in MetS progression. This field of study is novel and remains elusive; however, promising studies are emerging with data that provide mechanistic insights into epigenetic modifications and MetS occurrence [8].

Interestingly, there is an increase in the occurrence of MetS in HIV-infected individuals [9]. HIV affects a significant percentage of the global population. By the end of 2019, roughly 38 million people worldwide lived with HIV, with 1.7 million new infections for the year [10]. In 2020, the South African government reported that 7.8 million people were infected with HIV [11]. The high prevalence of HIV, especially in South Africa, emphasises

the need for studies on co-morbidities such as MetS. Research on the subject may provide insight for possible treatments to reduce the prevalence of MetS associated with HIV.

Furthermore, 26 million HIV-infected individuals had access to ARV treatment at the end of 2019 [10]. The use of highly active antiretroviral therapy (HAART) in HIV-infected patients has decreased the HIV-infected population's mortality, with 15.3 million lives saved in 2019 [12]. However, the use of HAART promotes metabolic complications that resemble MetS in people living with HIV (PLWH) [13,14]. The prevalence of MetS following ARV treatment varies over different studies, with some exceeding 30%, further highlighting the need for future studies [15].

Considering the high incidence of HIV and MetS, it is vital to understand the relationship between the two conditions and the use of antiretroviral drugs (ARVs). This study aimed to explore mechanisms used by (a) the HIV infection and (b) different classes of HAART to induce metabolic irregularities. This review establishes the biochemical mechanisms of HIV and ARVs in MetS-related problems such as mitochondrial dysfunction, insulin resistance, inflammation, dyslipidaemia, and lipodystrophy. Furthermore, we look at epigenetic modifications induced by ARVs and HIV that result in metabolic complications. More specifically, the review highlights gaps in current knowledge and addresses the need for further studies to reduce inconsistencies in research.

2. HIV and MetS

Many risk factors contribute to the incidence of MetS, including an unhealthy diet, lack of exercise, and age [16]. However, research has established a unique set of risk factors associated with HIV infection [17]. The common risk factors highlighted in HIV infection include chronic inflammation and immune dysfunction, which promotes atherosclerosis, dyslipidaemia, and T2DM [18].

At least 690,000 people had died from HIV-related illness (including metabolic complications) by the end of 2019. The majority of the global HIV-infected population (20%) resides in South Africa [10]. In multiple South African community studies, it was determined that the prevalence of MetS ranges from 24.1 up to 60.6%, with mostly females being affected [19–21]. Furthermore, following four cross-sectional studies in sub-Saharan Africa, a prevalence of 21.5% MetS in PLWH was found, whereas un-infected individuals had a 12% prevalence [9]. Therefore, the incidence of MetS in HIV is significant enough to warrant extensive research.

The biochemical basis of HIV-induced MetS remains elusive; however, research has established a few common factors. HIV can induce MetS via several mechanisms (Figure 1). The most common is via the activation of inflammatory responses, cellular apoptosis, and mitochondrial dysfunction. However, epigenetic modifications are emerging in recent research surrounding HIV and inflammation. The ability to induce the aforementioned pathways and changes leads to more severe consequences, such as insulin resistance, dyslipidaemia, and obesity [2].

We discuss the most common mechanisms used by HIV to induce MetS in PLWH.

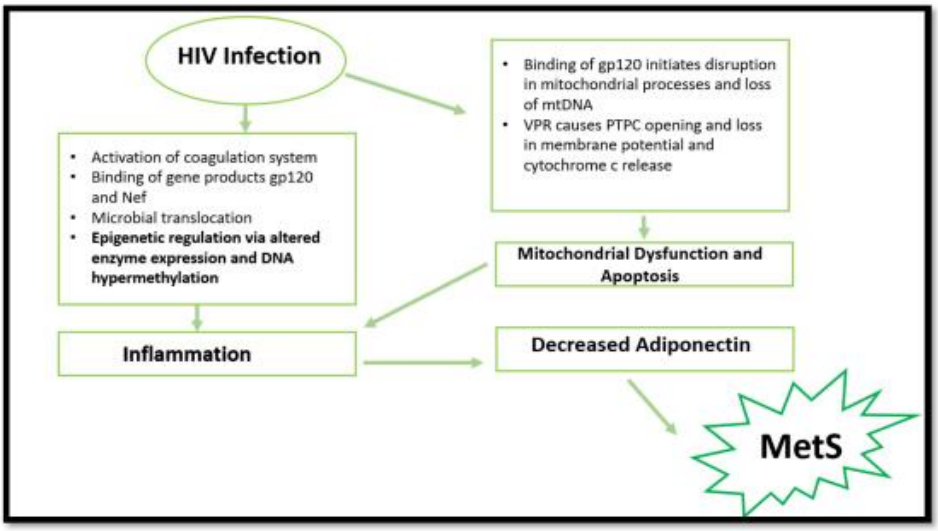

Figure 1. Summary of processes involved in MetS promotion via HIV infection. HIV infection can cause mitochondrial dysfunction, apoptosis, epigenetic changes, and inflammation, resulting in decreased adiponectin expression. Consequently, MetS initiation occurs. (gp120—envelope glycoprotein GP120; Nef—negative factor; DNA—deoxyribonucleotide acid; mtDNA—mitochondrial DNA; VPR—viral protein R; PTPC—permeability transition pore complex).

3. HIV and Inflammation

Untreated HIV infection has been linked to the activation of the coagulation system, which results in the production of pro-inflammatory molecules, including cytokines and chemokines. Following treatment of HIV with HAART, inflammation persists through high levels of interleukin IL-6, C-reactive proteins, and D-dimers [22].

Early research shows that HIV gene products can directly trigger lymphocyte and macrophage activity and promote the production of pro-inflammatory cytokines and chemokines. The HIV envelope protein gp120 directly activates immune cells or increases their susceptibility to activation by binding to CD4 receptors and co-receptors [23–25]. Furthermore, the HIV accessory molecule Nef can activate lymphocytes or cause indirect activation via infection of macrophages [26–28].

The literature describes HIV-associated microbial translocation as a tool for persistent inflammation. HIV is linked to increased lipopolysaccharide (LPS) levels (an indicator of microbial translocation) [29]. Increased LPS concentrations stimulate macrophage and dendritic cells to produce inflammatory molecules, including tumour necrosis factor alpha (TNF-α), IL-6, and IL-1β. This establishes the pro-inflammatory state commonly related to the virus [30]. More specifically, inflammatory states are initiated via the activation of inflammasomes. Upon stimulation by LPS, Toll-like receptors signal for NLRP3 inflammasome activation. These protein complexes ultimately allow for the autoproteolytic activation of pro-caspase-1 to yield caspase-1. The latter is responsible for the conversion of pro-IL-1β to IL-1β, which creates inflammatory conditions [31]. The activation of inflammasomes in HIV infection encourages the occurrence of metabolic disorders [32].

Chronic pro-inflammatory environments promote conditions such as T2DM and CVD [33,34]. The increase in inflammation causes an inhibition in the function of adiponectin—a protein hormone involved in glucose regulation and fatty acid breakdown. Adiponectin is associated with anti-diabetic, anti-atherosclerotic, and anti-inflammatory functions that suppress MetS progression. Therefore, inhibition results in enhanced insulin resistance and atherosclerosis, further manifesting into T2DM and CVD, respectively [35].

Although there are multiple adipocytokines involved in the prevention of the pathogenesis of MetS, adiponectin had been the most commonly described in the literature. There are several possibilities for adiponectin to reduce insulin resistance; however, this review describes the most common mechanism. In summary, adiponectin binds to the appropriate receptor and activates various intracellular pathways. More frequently, it allows for the activation of the AMP-activated protein kinase (AMPK) pathway, which results in AMPK phosphorylation [36]. The latter promotes glucose utilization which increases fatty acid oxidation. Additionally, it encourages glucose uptake in muscle cells through increased glucose transport 4 (GLUT4) translocation and reduces gluconeogenesis in the liver [37,38]. AMPK is associated with increased insulin sensitivity and a reduction in glucotoxicity oxidative stress in target cells/organs [38]. Therefore, lower adiponectin concentrations or inhibition of its function promotes the occurrence of insulin resistance and fat accumulation that can lead to other clinical outcomes of MetS.

4. HIV, Mitochondrial Dysfunction, and Cell Apoptosis

Mitochondrial dysfunction is commonly observed in PLWH. As previously mentioned, the gp120 protein binds to the CD4 receptor and co-receptors to elicit infection. Such binding initiates pathogenic effects, including mitochondria-mediated apoptosis, loss of mitochondrial DNA (mtDNA), and impaired calcium signalling [39]. It is well understood that dysregulation of mitochondrial function results in the production of inflammatory cytokines, including TNF-α, interleukins, and C-reactive proteins [40].

In other instances, HIV may induce MetS by inducing mitochondrial membrane dysfunction [41]. Viral protein R (VPR), an HIV accessory molecule, can cause mitochondrial permeability transition pore complex (PTPC) opening and loss of transmitochondrial potential upon mitochondrial exposure [42]. The opening of the PTPC disrupts mitochondrial processes and releases proapoptotic factors such as cytochrome c and procaspase 9 [42,43]. HIV promotes the uncontrolled release of cytochrome c, thus increasing apoptosis. Consequently, a pro-inflammatory state is favoured. Inflammatory cytokines suppress adiponectin function and impair insulin function in muscles. This ultimately leads to MetS [40,44].

5. HIV and Epigenetic Modifications

Mechanisms surrounding epigenetic modifications remain elusive; however, some research has highlighted possible linkages. This provides motivation for further extensive research to be carried out to fully explain the processes involved in the area.

Aside from the common mechanisms, more recent research has suggested that HIV-1 infection can cause epigenetic changes when exposed to *Mycobacterium tuberculosis*. The latter results in altered monocyte function and dysregulation in pro-inflammatory cytokine production. The same study suggested that a decrease in global DNA methylation occurred in HIV-infected individuals. This was mainly attributed to the downregulation of DNA methyltransferases and the upregulation of methyl-CpG-binding proteins. Consequently, the reduction in global DNA methylation caused an increased activation status of monocytes. This result was accompanied by increased production of pro-inflammatory cytokines [45]. These findings are significant, considering the vulnerability to *Mycobacterium tuberculosis* in developing countries with high HIV prevalence, such as South Africa. The study provides plausible cause to initiate future in vivo research that will aid in understanding epigenetic changes associated with HIV and MetS.

More recent findings report DNA hypermethylation in HIV-infected patients. These epigenetic modifications result in cell dysfunction and decreased cytokine production, thus increasing CVD and T2DM risk [46]. More specifically, the HIV-1 infection causes epigenetic changes in the T-cell population, resulting in aberrant expression of pro-inflammatory cytokines and immune-related genes [47]. This is caused by the virus inducing DNA methylation changes in essential genes (IL-2, PD-1 and FOXP3) in T-cells which initiates cell dysfunction [48–50].

Furthermore, single-nucleotide polymorphisms in IL-6 and IL-10 were associated with accelerated ageing and the dysregulation of inflammatory responses in PLWH [46]. However, the lack of studies on related polymorphisms causes gaps in knowledge regarding the mechanisms.

The overall evidence strongly suggests that HIV can induce various epigenetic modifications that may lead to disarray in inflammatory responses. The sparse literature on HIV and epigenetics emphasises the necessity for more research on the subject, to further underline the virus's role in MetS progression.

6. The Evolution of ARVs and Implications in MetS

The use of ARVs provides temporary relief for HIV-induced effects but has induced various complications over time (Masuku et al., 2019). Side effects associated with the use of ARVs have improved following extensive research. Previously, adverse outcomes seen with ARV usage resulted in patients discontinuing treatment or switching combinations of drugs until the side effects were manageable. This was mainly observed in first- and second-generation treatment (O'Brien et al., 2003). Initially, the discovery of ARVs led to various trials of singular and combinational usage, with NNRTIs and NRTIs being popular. However, the usage of the earlier generations of ARVs were later associated with adversity and HIV drug resistance (WHO, 2020). Fortunately, the newer generation of ARVs are linked with fewer complications and adversity (Barnhart and Shelton, 2015; Rai et al., 2018). More specifically, the World Health Organization has highlighted the need to move to newer generations of ARVs such as dolutegravir to prevent the HIV drug resistance associated with former generations and reduce side effects (WHO, 2020). However, some problems still arise following the usage of the current generation of ARVS.

Long-term use of ARVs is associated with the development of MetS through induction of dyslipidaemia, lipodystrophy, mitochondrial dysfunction, and insulin resistance [51]. Over time, metabolic dysregulation occurs, initiating changes in fat distribution and glucose homeostasis [52]. The consensus in population studies indicates a high prevalence of MetS in PLWH receiving ARV treatment [9,53,54]. This review looks at the effects of nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), and integrase strand transfer inhibitors (INSTIs) on mitochondrial dysfunction, insulin resistance, inflammation, and lipodystrophy/dyslipidaemia which are common markers and outcomes of MetS (Figure 2). Currently, the literature indicates that PIs are most commonly implicated in MetS cases.

The role of the mitochondria in the pathogenesis of MetS is not fully understood. However, it is well noted that mitochondrial dysfunction contributes to inflammation, ROS production, and oxidative stress, which is strongly associated with MetS [5]. More specifically, mitochondrial dysfunction and inflammation are considered to be the underlying processes that lead to insulin resistance. In other instances, it was found that a side effect of ARV usage was the induction of dyslipidaemia and lipodystrophy—a clinical outcome of MetS. Below, we look at the different classes of ARVs and their role in the initiation or promotion of MetS.

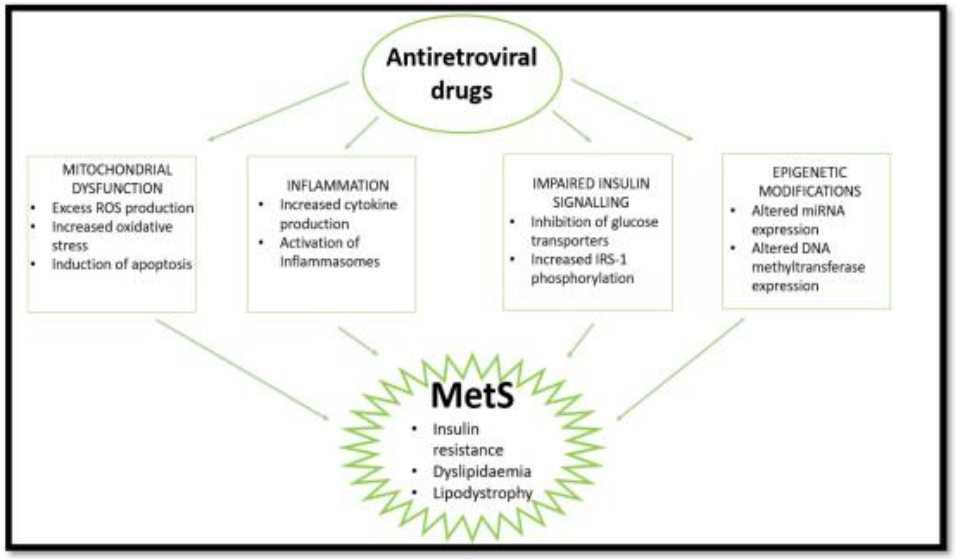

Figure 2. HAART induces MetS via several pathways. HAART causes mitochondrial dysfunction, inflammation, and impaired insulin signalling and epigenetic changes, leading to insulin resistance, lipodystrophy, and dyslipidaemia. (ROS—reactive oxygen species; IRS-1—insulin receptor substrate 1; miRNA—micro ribonucleotide acid).

7. Non-Nucleoside Reverse Transcriptase

NNRTIs were the first ARVs to receive regulatory approval for treatment of HIV. The class consists of drugs that have diverse drug backgrounds but similar mechanisms of action. They work by binding to the HIV-1 reverse transcriptase and inducing conformational changes that reduce the function of the enzyme [55]. However, NNRTIs were found to be problematic in several studies.

The most common NNRTI is efavirenz. Efavirenz is associated with increases in apoptosis, increased mitochondrial mass, and oxidative stress in hepatic cells (Figure 3). In hepatocytes, disruption in mitochondrial membrane potential was observed, promoting cytochrome c release and apoptosis [56,57]. Additionally, efavirenz inhibits complex I of the ETC, stimulating ROS production and decreases in ATP [58]. In neuronal cells, the drug caused increased mitochondrial depolarisation, altered mitochondrial morphology, and ultimately led to mitophagy (clearance of damaged mitochondria) [59]. Mitochondrial dysfunction is considered to promote the progression of MetS by encouraging the occurrence of inflammation and oxidative stress.

Efavirenz causes a decrease in ATP concentration and increases ROS production, which leads to an elevation in the lipid content of hepatic cells [58]. Furthermore, studies have shown that efavirenz acts as a pregnane X receptor agonist, which promotes the occurrence of dyslipidaemia and hypercholesterolemia [60].

In terms of insulin resistance, NNRTIs were found to reduce insulin sensitivity through its pro-inflammatory effects (Figure 4). Previous studies show that NNRTIs increase the expression of TNF-α, IL-6, and IL-1β, which results in decreased adiponectin concentrations. The reduction in the insulin sensitivity modulator allows for progression to insulin resistance, as previously discussed [61].

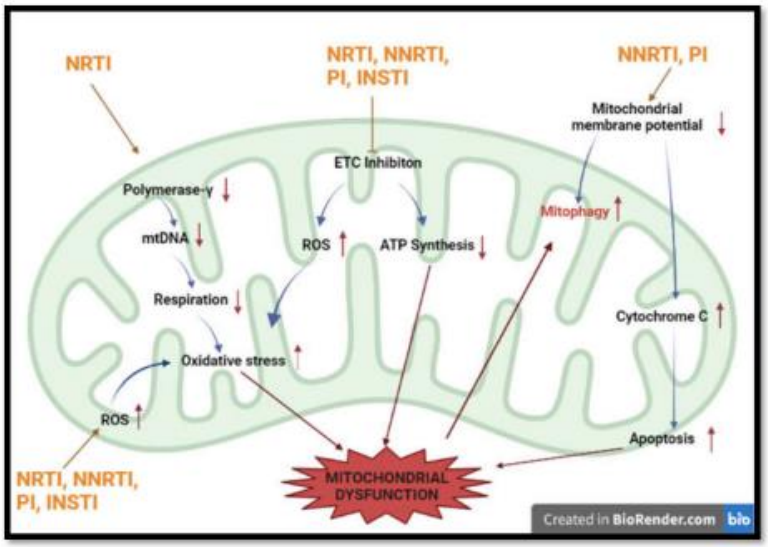

Figure 3. Summary of most common methods implicated in HAART-induced mitochondrial dysfunction. HAART induces mitochondrial toxicity via inhibition of respiration, production of ROS, induction of apoptosis and interference with mtDNA numbers. (Polymerase-γ —Polymerase gamma; ETC—electron transport chain; ATP— adenosine triphosphate; mtDNA-mitochondrial DNA).

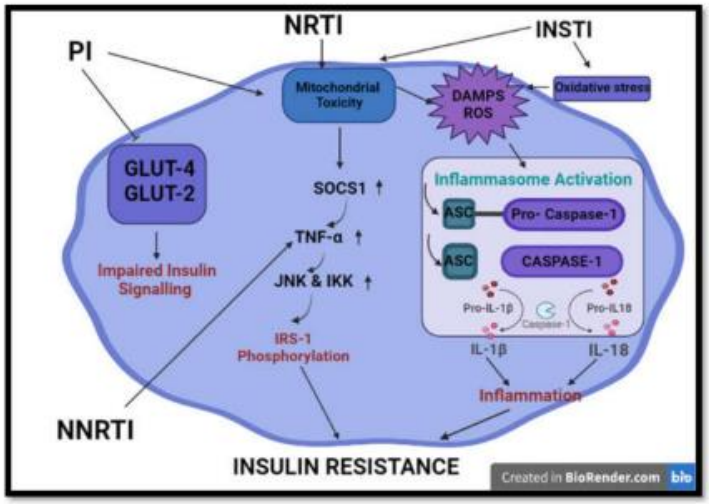

Figure 4. Summary of common processes implicated in HAART-induced insulin resistance and impaired insulin signalling. HAART induces insulin resistance and aberrations in insulin signalling via initiation of mitochondrial toxicity, oxidative stress, inflammation and inhibition of glucose transporters. (GLUT2/4- glucose transporter 2/4; IRS-1 -Insulin receptor 1; TNF-α—tumour nerosis factor alpha; SOCS1—suppressor of cytokine signaling 1; JNK—Jun N-terminal kinase; IKK-IκB kinase; ASC—Apoptosis-associated speck-like protein containing a CARD; IL-1β/18—interlukin 1 beta/ 18; DAMPS- damage activated molecular patterns).

Research indicates that earlier NNRTIs were problematic and newer drugs in the class are seen to have greater resistance and fewer adversities [55].

8. Protease Inhibitors

Protease inhibitors are described as the most problematic ARVs in several studies. PIs work by inhibiting the function of HIV protease. In doing so, PIs prevent the cleavage of the precursor polyprotein needed to produce mature viral proteins necessary for infection [62]. Although this class of drugs is widely used in the treatment of HIV, the adverse outcomes following treatment are severe and are commonly associated with MetS.

Firstly, PIs are associated with mitochondrial dysfunction (Figure 3). The protease inhibitor (PI) ritonavir increases mtDNA copy number, increases ROS production, disrupts mitochondrial membrane potential, and interferes with respiration via its action on the ETC and OXPHOS [63]. Furthermore, it causes increases in oxidative stress and decreases in ATP synthesis [64]. Ritonavir promotes mitochondrial membrane potential changes, which initiates BAX translocation and cytochrome c release, allowing for apoptosis progression (Figure 3). Another PI, atazanavir, causes superoxide production in the mitochondria, depolarisation of the mitochondrial membrane, and apoptosis [65].

Furthermore, PIs are the most common ARVs associated with insulin resistance. PIs may induce insulin resistance through their effects on mitochondrial dysfunction, glucose transport, and inflammation induction. Upon mitochondrial damage or dysfunction, an increase in ROS is observed. This signals for an immune response via the action of the NALP3 inflammasome [66,67] (Figure 4). Inflammasomes are responsible for the clearance of pathogens and damaged cells. Furthermore, mitochondrial DAMPs such as mtDNA and DRP-1 may be released upon mitochondrial damage and are associated with inflammasome activation. The activation of inflammasomes promotes the release of caspase 1, which activates pro-inflammatory cytokines (IL-1β and IL-18). IL-1β was strongly linked with insulin resistance and impaired insulin secretion [68–70].

Previous in vitro studies showed that PIs cause the inhibition of the glucose 4 transporters (GLUT-4) [71]. This promotes impaired glucose tolerance and peripheral insulin resistance [72]. In liver cells, insulin signalling has been observed via action on GLUT-2. This transporter is responsible for hepatoportal glucose sensor function [71]. Furthermore, in therapeutic doses, PIs induce insulin resistance and beta pancreas cell damage in mice [72].

Prolonged exposure to PIs can affect insulin signalling and directly affect glucose uptake [73]. Saquinavir alters insulin signalling and IRS-1 phosphorylation. Indinavir activates the suppressor of cytokine signalling-1 (SOCS1), which causes an elevation in TNF-α levels [74]. TNF-α affects the IRS proteins that lead to insulin resistance. The cytokine induces activation of serine kinases such as JNK and IKK, which serine phosphorylates IRS-1. The increased concentration of phosphorylated IRS-1 inhibits the insulin receptor, thus causing insulin resistance [75].

In other studies, PIs are implicated in adipocyte differentiation inhibition via their action on the sterol regulatory element-binding protein-1 and peroxisome proliferator-activated receptor gamma (PPAR-γ). The inhibition in adipocyte differentiation alters lipid metabolism, leading to lipodystrophy and dyslipidaemia [76]. Furthermore, indinavir (PI) is associated with non-oxidative insulin-stimulated glucose disposal. This results in insulin resistance and dysregulation in lipid metabolism [77].

PI-mediated lipodystrophy is associated with irregular fat distribution and the accumulation of fat in the subcutaneous region [78,79]. The accumulation of fat causes an elevation in cholesterol and triglyceride levels, which leads to dyslipidaemia [80]. Furthermore, the use of PIs was associated with increased endoplasmic reticulum stress and the inhibition of proteasome action [79]. This results in autophagy inhibition, which is essential in regulating hepatic lipid metabolism and adipocyte lipid storage. The overall disruption leads to dyslipidaemia [81].

The plethora of evidence highlights the problems associated with PI usage and its implication in MetS progression. This has caused the PI class of drugs to be less favoured in recent years while newer drugs are being developed.

9. Nucleoside Reverse Transcriptase

NRTIs are one of the most common classes of ARVs. Drugs of this class are able to act as nucleoside analogues of HIV reverse transcriptase, thus terminating viral DNA synthesis in HIV [82]. Although NRTIs are popular in research, the plethora of studies indicate several adversities and side effects that allude to the progression of MetS.

The first observed effects of ARVs on the mitochondria were seen with NRTI use (Figure 3). NRTIs inhibit polymerase-γ, which has a role in mitochondrial DNA replication, thus compromising mitochondrial integrity [83]. Furthermore, NRTIs prevent ATP/ADP translocation. This causes impairment in respiration and ATP synthesis [84,85]. Studies show that decreases in mitochondrial DNA (mtDNA) content were linked to changes in respiration. The reduction in mtDNA initiates impairment in oxidative phosphorylation (OXPHOS) proteins and increases oxidative stress in mitochondria. This results in damage to mitochondrial proteins and lipids, further impairing mitochondrial function [86].

Furthermore, they cause alterations in nucleotide phosphorylation and directly affect mitochondrial respiration and ATP production [87,88]. Selected NRTIs reduce respiration and increase ROS via the inhibition of complex IV and I of the electron transport chain (ETC) [89–91] (Figure 3). This action has implications for mitochondrial dysfunction. ROS causes dysregulation of pathways leading to the release of damage-associated molecular patterns (DAMPS) and, thereafter, inflammatory activation [92].

In other instances, it was shown that tenofovir and tenofovir disoproxil fumarate (NRTI) were able to accumulate in renal cells, altering their viability and proliferation. This compromised mitochondrial function. The latter caused an increase in superoxide production and depletion of oxygen. Furthermore, a decrease in mitochondrial membrane potential was observed (Figure 3). The resultant effect was an increase in oxidative stress, which is hazardous to cells and implicated in T2DM and CVD pathogenesis [50]. Tenofovir disoproxil fumarate was found to decrease the function of ETC complexes I, II, IV, V, leading to reduced ATP production and proximal tubular damage in rat kidneys [93].

Mitochondrial dysfunction affects the occurrence of lipodystrophy. The generation of excessive ROS at sites of the ETC causes disruptions in adipocyte differentiation and induces apoptosis. At low concentrations, ROS may initiate lipogenesis and adipogenesis; however, at high concentrations, it may inhibit differentiation through the suppression of PPAR-γ [94]. The decrease in adipogenesis and induction of apoptosis leads to the development of lipodystrophy. NRTIs have been implicated in lipodystrophy development through its effects on oxidative stress and mitochondrial dysfunction [95,96].

10. Integrase Strand Transfer Inhibitors

INSTIs are a relatively new class of ARVs that are gaining popularity due to their reduced side effects. Their novel mechanism of action includes preventing HIV integrase from incorporating pro-viral DNA into their host cell. This inhibits the HIV-catalysed strand transfer step. INSTIs are incredibly useful as this step has no known human analogue, increasing the specificity of the drug and reducing the toxicity [97]. However, research remains unable to fully explain the adversities associated with the usage of this class of drugs. Current research does implicate INSTIs in mitochondrial dysfunction, inflammation, and linkage to insulin resistance [63,98].

Dolutegravir and other INSTIs initiate increases in mtDNA number and ROS production [63]. Dolutegravir specifically is associated with decreased respiration of $CD4^+$ T cells, excessive mitochondrial ROS production, and increased mitochondrial mass. This leads to mitochondrial dysfunction, slower cell proliferation rates, lower OXPHOS, and increased TNF-α responses [63].

More recently, INSTIs have been linked to insulin resistance, but literature remains limited (Figure 4). Dolutegravir was found to increase oxidative stress and promote lipid accumulation, favouring the onset of insulin resistance in adipose tissue and adipocytes. Furthermore, mitochondrial dysfunction was observed, which promotes insulin resistance. The results allowed the authors to infer that dolutegravir contributes to insulin resistance [98].

Considering the reduced side effects of the drugs and approval by WHO for usage in combinational treatment of HIV [99], research needs to be expanded to fully elucidate the mechanisms of toxicity of INSTIs.

11. ARVs and Epigenetic Modifications—Emerging Evidence

Studies regarding ARV usage and epigenetic modifications remain limited. Furthermore, studies that exist do not directly link MetS to ARV-induced epigenetic change, but rather provide evidence for strong possibilities and indirect links. This highlights the novelty of the concept and provides cause for extensive research to be undertaken in the subject area.

Unlike the epigenetic modifications induced by HIV, HAART-related epigenetic effects are mostly associated with altered micro-RNA (miRNA) expression. Interestingly, it was found that combinational ARV usage resulted in decreased expression of miR-106a and miR-140 and increased miR-192 levels. Lower levels of these miRNAs were seen to reduce CD4+ cell recovery, which is strongly connected to persistent inflammation and immune activation [100].

Recent epigenetic studies suggest that ritonavir (PI) alters miRNA expression. The increased levels of miR-28 were inversely proportional to the expression of the GLUT-4 transporter, implying that the drug alters glucose metabolism [101]. However, more studies are required to understand the epigenetic regulation of insulin resistance by PIs and establish a mechanism of action.

Besides altered miRNA expression, previous studies provide evidence that illustrates the role of NRTIs in causing the overexpression of DNA methyltransferase 1 (DNMT1). This was coupled with mtDNA hypermethylation in Hepatitis B infection [102]. Increases in DNMT1 are synonymous with decreased PPAR-γ expression and, thus, increased pro-inflammatory cytokine production in atherosclerosis patients' blood monocytes [103]. Furthermore, increased DNMT1 in the adipocytes of obese mice correlates with hypermethylated adiponectin, which decreases the expression of vital energy homeostasis regulators [104].

Although pre-existing conditions may influence these changes, there are substantial data to encourage future epigenetic studies related to ARV usage and MetS. Furthermore, it is essential to note that HIV-infected individuals may have co-morbidities that favour the onset of epigenetic modifications

12. Conclusions and Recommendations

This study provides an overview of the metabolic irregularities induced by HIV and HAART. We deduce that mitochondrial dysfunction is the most common underlying mechanism used by HIV and most ARVs to cause inflammation, insulin resistance, dyslipidaemia, and lipodystrophy. This information is vital as drugs used to treat HIV should be designed with less mitochondrial toxicity. Additionally, we found that most studies describe mechanisms of action following the use of ARVs individually. This is alarming as most ARV therapy is combinational, highlighting the need for mechanistic research into combinational ARV usage.

Considering the evidence, we can conclude that PIs are the most dangerous of ARVs due to their ability to initiate many toxicities leading to MetS. Furthermore, this paper addresses the urgency for more epigenetic experimentation to link HIV, HAART, and MetS. This novel approach may help fill current knowledge gaps and help prevent the side effects

of the drugs. This is vital considering the global burden of HIV and deaths caused by HIV-related illness.

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Addendum B: Guidelines for Thesis

GUIDELINES FOR PRESENTATION OF MASTERS AND PHD DISSERTATIONS/THESES BY RESEARCH

1. Purpose The purpose of this document is to provide guidance to students and supervisors on how to prepare a dissertation/thesis for Masters by Research and PhD degrees using the manuscript or publication format..

2. Introduction These guidelines must be read together with the College of Health Sciences (CHS) Handbook as well as the Jacobs documents on examination policies and procedures for PhD degrees. The rules on thesis format are based on modification of point 1 of the definition of terms section in the Jacobs document. In this section a thesis is defined as "the supervised research component of all PhD degrees, whether by supervised research only, or coursework and research, or by papers that are either published or in manuscript form (the supervised research component of the PhD degree by paper(s) comprises the introduction, literature review, account of the methodology, selection of manuscripts, and conclusion)." A dissertation is defined as "the supervised research component of all Masters degrees, whether by supervised research only, or coursework and research, or by papers that are either published or in manuscript form (the supervised research component of the Masters degree by paper(s) comprises the introduction, literature review, account of the methodology, selection of manuscripts, and conclusion)."

2.1 PhD thesis In the CHS Handbook the rules for a PhD thesis are not in one place; they are stated in DR8 a i & ii, DR9 c and CHS 16. DR8 a i & ii and direct that a thesis be presented in the standard format together with one published paper or an unpublished manuscript that has been submitted to an accredited journal, arising from the doctoral research. CHS16 (thesis by publications states that the thesis may comprise of at least three published papers or in press in accredited journals; such papers must have the student as the prime author. The same CHS16 provides for a thesis by manuscripts that may have at least 3 papers with the student as the prime author that have not yet been published but are in the form of manuscripts; at least two of such papers must constitute original research. In both cases (thesis by publications and manuscripts), there must be introductory and concluding integrative material sections.

The standard type thesis is being phased out in many African countries in favour of the other options that originate from the Scandinavian countries. While this format ensures that all details of the work done for the doctoral degree are captured and thoroughly interrogated, they often remain as grey literature which is mainly useful to other students, usually within the same university, although with digitization of theses, such work may become more accessible beyond the source university. Apart from the risk of losing good work because of it not being on the public domain, as students rarely publish such work after graduating, this approach denies the college additional productivity units (PUs) emanating from publications.

The thesis by publication encourages students to publish key aspects of their doctoral research as they will not graduate if the papers are not published or in press. This approach ensures that the work of the student enters the public domain before the thesis is examined, providing the examiner

with some assurance of prior peer review. The thesis must constitute a full study of the magnitude expected of a PhD with the papers providing a sound thread or storyline. Furthermore, the college maximizes the students' work as PUs are awarded for the papers as well as for graduating. However, this approach may negatively affect throughput and frustrate students as 2 they cannot graduate unless all the papers are published or in press, in addition to the synthesis chapter demonstrating the story line of the thesis.

The option of a thesis by manuscripts ensures that students make efforts to start publishing. The risk of not passing because of failure to publish all papers (as in the thesis by publication) does not exist under this option. However, the PUs emanating from publications from the doctoral work are not guaranteed as the submitted papers may eventually be rejected. Thus there is a possibility of the doctoral work remaining on the university library shelves as is the case for the standard thesis format. The standard thesis does have the advantage that more details of the doctoral work are usually included.

In view of the above, the best option for the college is that of a thesis by publication. However, in the interim, the attractive option is that of thesis by manuscripts, as it provides the possibility of publication without putting the student at risk of delayed graduation when some of the manuscripts are not published/accepted, which also disadvantages the college in terms of PU earnings. The standard thesis option should ultimately be phased out for the stated reasons and students are not encouraged to present their theses in that format. Consequently this document does not describe the standard thesis.

Addendum C: Ethical Clearance

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

15 February 2021

Miss Jivanka Mohan (215024251)
School of Lab Med & Medical Sc
Westville

Dear Miss Mohan,

Protocol reference number: BREC/00002256/2020
Project title: Current anti-retroviral drugs- An investigation of metabolic syndrome initiation and promotion in HepG2 liver cells
Degree Purposes: PhD

EXPEDITED APPLICATION: APPROVAL LETTER

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application.

The conditions have been met and the study is given full ethics approval and may begin as from 15 February 2021. Please ensure that outstanding site permissions are obtained and forwarded to BREC for approval before commencing research at a site.

This approval is subject to national and UKZN lockdown regulations, see (http://research.ukzn.ac.za/Libraries/BREC/BREC_Lockdown_Level_1_Guidelines.sflb.ashx). Based on feedback from some sites, we urge PIs to show sensitivity and exercise appropriate consideration at sites where personnel and service users appear stressed or overloaded.

This approval is valid for one year from 15 February 2021. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be noted by a full Committee at its next meeting taking place on 09 March 2021.

Yours sincerely,

[redacted]

Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

Biomedical Research Ethics Committee
Chair: Professor D R Wassenaar
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Addendum D: Standard Curve

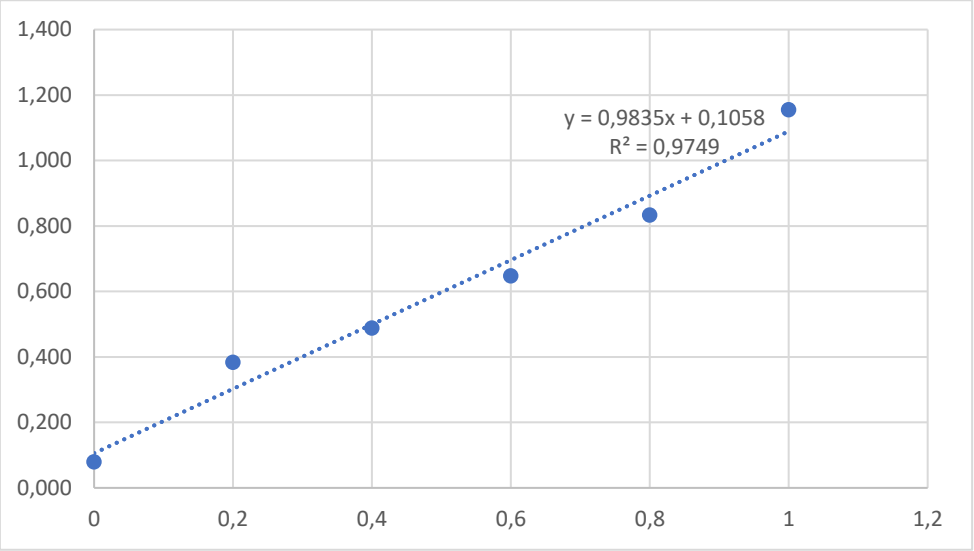

Figure A1: Standard curve used to determine protein concentrations for Western Blots