

The effects of HIV protease inhibitors on the rat heart

By

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Declaration

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I dedicate this work to my parents. After recent occurrences I have come to realize how privileged I am to still have you in my life. Thank you for everything you have done for me. Without your love and support I would not be where I am today. Words cannot describe how grateful I am for you.

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Abstract

Since the early 1990's HIV/AIDS emerged as a global health pandemic, with sub-Saharan Africa the hardest hit. While the successful roll-out of antiretroviral (ARV) therapy provided significant relief to HIV-positive individuals, such treatment can also elicit damaging side-effects. Here especially HIV protease inhibitors (PIs) are implicated in the onset of cardiometabolic complications such as type-2 diabetes and acute myocardial infarction. As the underlying mechanisms driving PI-mediated side-effects remain unclear, this study set out to investigate this intriguing question by employing a rat model of chronic PI treatment (6 months). In addition, various co-treatments (Resveratrol, Aspirin, Vitamin C) were evaluated to ascertain whether such a therapeutic strategy may blunt PI-induced side-effects. Body weights and weight gains, blood metabolite levels, echocardiography and cardiac mitochondrial respiration were assessed. Our data reveal that after 2 months of PI treatment there were no significant changes for the various parameters evaluated in this study. However, after 4 months Vitamin C co-treatment ameliorated the PI-induced decrease in body weight, while it also blunted the PI-mediated inhibition of mitochondrial respiration. Aspirin co-treatment also improved mitochondrial respiration while this effect was not observed with Resveratrol. After 6 months of PI treatment all interventions prevented the PI-induced increase in body weights, while Aspirin and Vitamin C prevented the PI-induced increase in heart weight. At the later time point mitochondrial respiration was, unlike at the 4 month time point, not affected by PI treatment. However, Resveratrol co-treatment significantly increased mitochondrial respiration. This study demonstrates that early PI-mediated perturbations include alterations in body weights and inhibition of mitochondrial respiration. However, no significant changes were found for heart function or blood metabolites. Our findings show that the co-treatments triggered beneficial outcomes by reversing weight changes and enhancing mitochondrial respiratory function. As the co-treatments are already well-known and approved therapeutic agents, we are of the opinion that this study provides significant impetus to test such compounds to establish whether our basic findings can be successfully translated into the clinical setting to eventually improve the overall well-being of HIV-positive patients.

Abstrak

Sedert die vroeë 1990's het HIV/VIGS 'n globale pandemie geword, met Sub-Sahara Afrika veral ernstig getref. Alhoewel die suksesvolle uitreiking van antiretrovirale (ARV) terapie al talle HIV-positiewe individue gehelp het, kan hierdie middels skadelike nuwe-effekte veroorsaak. Meer spesifiek, HIV protease inhibitore (PIs) word geïmpliseer in die ontwikkeling van kardio-metaboliese komplikasies soos tipe-2 diabetes en miokardiale infarcties. Aangesien die meganismes van die komplikasies nie bekend is nie, is hierdie studie gemik daarop om hierdie meganismes te ondersoek. Dit is uitgevoer deur 'n rot-model van kroniese PI behandeling (6 maande). Addisioneel is verskeie intervensies (Resveratrol, Aspirien, Vitamien C) getoets om te ondersoek of sulke terapeutiese middels die PI-geïnduseerde nuwe-effekte kan beveg. Liggaamsgewig en gewigsvermeerdering, bloedmetaboliet vlakke, EKG en kardiale mitochondriale respirasie is geassesseer. Ons data wys na 2 maande van PI behandeling geen beduidende verskille in die getoetste parameters nie. Na 4 maande het mede-behandeling van Vitamien C egter die PI-geïnduseerde gewigsverlies voorkom, sowel as die PI-geïnduseerde inhibisie van mitochondriale respirasie. Aspirien mede-behandeling het ook mitochondriale respirasie verbeter, maar hierdie effek is nie waargeneem met Resveratrol mede-behandeling nie. Na 6 maande van PI behandeling het al die intervensies daarin geslaag om die PI geïnduseerde gewigsvermeerdering te voorkom. Aspirien en Vitamien C was ook suksesvol om die PI geïnduseerde gewigsvermeerdering te voorkom. Aspirien en Vitamien C het ook suksesvol die PI geïnduseerde hartgewig vermeerdering voorkom. Na 6 maande was mitochondriale respirasie nie deur PI behandeling geïmpak nie. Resveratrol mede-behandeling het wel mitochondriale respirasie beduidend verhoog na 6 maande van behandeling. Hierdie studie demonstreer dat veranderinge in liggaamsgewig en mitochondriale respirasie deel uitmaak van vroeë PI geïnduseerde nuwe-effekte. Daar is egter geen veranderinge in hart funksie of bloedmetaboliete waargeneem nie. Ons bevindings toon dat die mede-behandelinge voordelige resultate gelewer het deur liggaamsgewig veranderinge om te keer en mitochondriale respirasie te verbeter. Aangesien hierdie mede-behandelinge reeds welbekend en goedgekeurde terapeutiese middels is, glo ons dat hierdie studie goeie rede lewer om meer toetse op hierdie middels te doen. Dit kan gedoen word om vas te stel of ons basiese bevindings getransleer kan word in die kliniese omgewing om eventueel die gesondheid van HIV-pasiënte te verbeter.

Table of Contents

Declaration.....	1
Acknowledgements.....	2
Abstract.....	3
Abstrak.....	4
List of figures.....	8
List of Tables	11
List of abbreviations.....	12
Chapter 1.....	15
1.1. Introduction	15
1.1.1. HIV: a global health concern	15
1.1.2. Antiretroviral therapy: a lifeline for the HIV infected.....	16
1.1.3. South Africa severely burdened by HIV/AIDS	17
1.1.4. The HIV life cycle and its effects on health	18
1.1.5. Different classes of ARVs available	19
1.1.6. Treatment regimens employed in South Africa	20
1.1.7. The pros and cons of first-line therapy	20
1.1.8. Drug adherence affects treatment outcomes	22
1.1.9. Second-line therapy might be the answer in low-income regions	23
1.1.10. Possible mechanisms of Protease Inhibitor induced side-effects	24
1.1.11. Mitochondria: suppliers of life-sustaining energy and detrimental ROS.....	26
1.1.12. The importance and functioning of the electron transport chain	27
1.1.13. Mitochondrial proton gradient not only used for energy production.....	29
1.1.14. Mitochondrial morphology affects respiratory capacity	30
1.1.15. Decreases in mitochondrial number also implicated in type-2 diabetes	30
1.1.16. Modulators involved in regulation of mitochondria and metabolism.....	31
1.1.17. HIV and HAART can affect both regulators and flux of metabolism.....	32
1.1.18 PIs: where do we go from here?	34
1.1.19. Hypothesis.....	35
1.1.19. Aims.....	35
1.2. Materials and methods	36
1.2.1. Animal housing.....	36
1.2.2. Dosages and treatment preparation:	36
1.2.3. Treatment periods and data collection:	37
1.3. Results.....	41

1.3.1 Weight gain analysis.....	41
1.3.2 Analysis of triglyceride, cholesterol, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) blood levels	44
1.3.3 Analysis of mid-wall fractional shortening (MWFS) with the use of echocardiography.....	46
1.3.4 Organ harvesting for the determination of treatment-induced anatomical changes.....	48
1.3.5 Analysis of mitochondrial respiration	52
1.3.5.1 State III respiration of isolated sub-sarcolemmal mitochondria after 4 months of PI treatment.....	52
1.3.5.2 State III respiration of isolated sub-sarcolemmal mitochondria after 6 months of PI treatment.....	53
1.4. Discussion.....	54
Chapter 2.....	60
2.1. Natural interventions: in search of hope	60
2.2. Materials and methods	62
2.3. Results.....	62
2.3.1 Weight gain analysis.....	62
2.3.2 Analysis of triglyceride, cholesterol, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) blood levels	65
2.3.3 Analysis of mid-wall fractional shortening (MWFS) with the use of echocardiography.....	67
2.3.4 Organ harvesting for the determination of treatment-induced anatomical changes.....	69
2.3.5 Analysis of mitochondrial respiration	73
2.4. Discussion.....	75
Chapter 3.....	77
3.1. Inflammation as therapeutic target.....	77
3.2. Materials and Methods.....	79
3.3. Results.....	79
3.3.1. Weight gain analysis.....	79
3.3.2. Analysis of triglyceride, cholesterol, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) blood levels.	82
3.3.3. Analysis of mid-wall fractional shortening (MWFS) with the use of echocardiography.....	84
3.3.4. Organ harvesting for the determination of treatment-induced anatomical changes.....	85
3.3.5. Analysis of mitochondrial respiratory function	90
3.4. Discussion.....	92
Chapter 4.....	93
4.1. ROS as therapeutic target	93
4.2. Materials and Methods.....	94

4.3. Results.....	94
4.3.1. Weight gain analysis.....	94
4.3.2. Analysis of triglyceride, cholesterol, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) blood levels	97
4.3.3. Analysis of mid-wall fractional shortening (MWFS) with the use of echocardiography.....	99
4.3.4. Organ harvesting for the determination of treatment-induced anatomical changes.....	101
4.3.5. Analysis of mitochondrial respiration	105
4.4. Discussion.....	107
Conclusion.....	109
Limitations	109
References	111
Appendices.....	127
Mitochondrial Respiration Buffer Constituents and Preparation.....	127

List of figures

- Figure 1.1 HIV infection rate 2001-2010
- Figure 1.2 Simplified HIV life cycle
- Figure 1.3 Relative rates of myocardial infarction after exposure to PIs and NNRTIs
- Figure 1.4 Simplified electron transport chain
- Figure 3.1 Body weight and net weight gain in male Wistar rats following 2 months of treatment
- Figure 3.2 Body weight and net weight gain in male Wistar rats after 4 months of treatment
- Figure 3.3 Body weight and net weight gain in male Wistar rats after 6 months of treatment
- Figure 3.4 Triglyceride, cholesterol, LDL and HDL blood levels in male Wistar rats after 2 months of treatment
- Figure 3.5 Triglyceride, cholesterol, LDL and HDL blood levels in male Wistar rats after 4 months of treatment
- Figure 3.6 MWFS in male Wistar rats after 2 and 4 months of treatment
- Figure 3.7 Heart weights normalized to right tibia length in male Wistar rats on the day of culling after 2, 4 and 6 months of treatment
- Figure 3.8 Left and right ventricular weights normalized to total heart weight in male Wistar rats on the day of culling after 4 and 6 months of treatment
- Figure 3.9 Liver weights normalized to right tibia length in male Wistar rats on the day of culling after 2, 4 and 6 months of treatment
- Figure 3.10 Left and right ventricular weights normalized to heart weight in male Wistar rats on the day of culling after 4 and 6 months of treatment
- Figure 4.1 Body weight and net weight gain in male Wistar rats following 2 months of treatment
- Figure 4.2. Body weight and net weight gain in male Wistar rats after 4 months of treatment
- Figure 4.3 Body weight and net weight gain in male Wistar rats after 6 months of treatment
- Figure 4.4 Triglyceride, cholesterol, LDL and HDL blood levels in male Wistar rats after 2 months of treatment

- Figure 4.5 Triglyceride, cholesterol, LDL and HDL blood levels in male Wistar rats after 4 months of treatment
- Figure 4.6 MWFS in male Wistar rats after 2 and 4 months of treatment
- Figure 4.8 Heart weights normalized to right tibia length in male Wistar rats on the day of culling after 2, 4 and 6 months of treatment
- Figure 4.10 Left and right ventricular weights normalized to total heart weight in male Wistar rats on the day of culling after 4 and 6 months of treatment
- Figure 4.9 Liver weights normalized to right tibia length in male Wistar rats on the day of culling after 2, 4 and 6 months of treatment
- Figure 4.11 Left and right ventricular weights normalized to heart weight in male Wistar rats on the day of culling after 4 and 6 months of treatment
- Figure 5.1 Global projections for diabetes 2010-2030; Global HIV prevalence
- Figure 6.1 Body weight and net weight gain in male Wistar rats following 2 months of PI treatment \pm ASP
- Figure 6.2 Body weight and net weight gain in male Wistar rats after 4 months of treatment
- Figure 6.3 Body weight and net weight gain in male Wistar rats after 6 months of treatment
- Figure 6.4 Triglyceride, cholesterol, LDL and HDL blood levels in male Wistar rats after 2 months of treatment
- Figure 6.5 Triglyceride, cholesterol, LDL and HDL blood levels in male Wistar rats after 4 months of treatment
- Figure 6.6 MWFS in male Wistar rats after 2 and 4 months of treatment
- Figure 6.7 Heart weights normalized to tibia lengths in male Wistar rats after 2 and 4 months of treatment:
- Figure 6.8 Left and right ventricular weights normalized to total heart weight in male Wistar rats on the day of culling after 4 and 6 months of treatment
- Figure 6.9 Liver weights normalized to right tibia length in male Wistar rats on the day of culling after 2, 4 and 6 months of treatment
- Figure 6.10 Retroperitoneal fat mass normalized to tibia length in male Wistar rats on the day of culling after 4 and 6 months of treatment
- Figure 7.1 Body weight and net weight gain in male Wistar rats following 2 months of treatment
- Figure 7.2 Body weight and net weight gain in male Wistar rats after 4 months of treatment

- Figure 7.3 Body weight and net weight gain in male Wistar rats after 6 months of treatment
- Figure 7.4 Triglyceride, cholesterol, LDL and HDL blood levels in male Wistar rats after 2 months of treatment
- Figure 7.5 Triglyceride, cholesterol, LDL and HDL blood levels in male Wistar rats after 4 months of treatment
- Figure 7.6 MWFS in male Wistar rats after 2 and 4 months of treatment
- Figure 7.7 Heart weights normalized to right tibia length in male Wistar rats on the day of culling after 2, 4 and 6 months of treatment
- Figure 7.8 Left and right ventricular weights normalized to total heart weight in male Wistar rats on the day of culling after 4 and 6 months of treatment, respectively
- Figure 7.9 Liver weights normalized to right tibia length in male Wistar rats on the day of culling after 2, 4 and 6 months of treatment
- Figure 7.10 Left and right ventricular weights normalized to heart weight in male Wistar rats on the day of culling after 4 and 6 months of treatment

List of Tables

Table 1.1	Conversion of human equivalent dose (HED) to animal doses based on BSA
Table 3.1.A	Left ventricular parameters as measured with echocardiography after 2 months of PI treatment
Table 3.1.B	Left ventricular parameters as measured with echocardiography after 4 months of PI treatment
Table 3.2	Function of isolated left ventricular sub-sarcolemmal mitochondria after 4 months of PI treatment
Table 3.3	Function of isolated left ventricular sub-sarcolemmal mitochondria after 6 months of PI treatment
Table 4.1.A	Left ventricular parameters as measured with echocardiography after 2 months of treatment
Table 4.1.B	Left ventricular parameters as measured with echocardiography after 4 months of treatment
Table 4.2.A	Function of isolated left ventricular sub-sarcolemmal mitochondria after 4 months of PI treatment
Table 4.2.B	Function of isolated left ventricular sub-sarcolemmal mitochondria after 6 months of PI treatment
Table 6.1.A	Left ventricular parameters as evaluated by echocardiography after 2 months of PI treatment
Table 6.1.B	Left ventricular parameters as determined by echocardiography (4 months of PI treatment)
Table 6.2.A	Function of isolated left ventricular sub-sarcolemmal mitochondria after 4 months of PI treatment
Table 6.2.B	Function of isolated left ventricular sub-sarcolemmal mitochondria after 6 months of PI treatment
Table 7.1.A	Echocardiographic results after 2 months of treatment
Table 7.1.B	Echocardiographic results after 4 months of treatment
Table 7.2.A	Function of isolated left ventricular sub-sarcolemmal mitochondria after 4 months of PI treatment
Table 7.2.B	Function of isolated left ventricular sub-sarcolemmal mitochondria after 6 months of PI treatment

List of abbreviations

ABC	-	Abacavir
AIDS	-	Acquired Immunodeficiency Syndrome
AMPK	-	5' adenosine monophosphate-activated protein kinase
ART	-	Antiretroviral therapy
ARV	-	Antiretroviral
ATP	-	Adenosine Triphosphate
AZT	-	Zidovudine
BSA	-	Body Surface Area
CVD	-	Cardiovascular Disease
CYP2A	-	Cytochrome P450 2A Enzyme
CYP3A	-	Cytochrome P450 3A Enzyme (isoform)
D4T	-	Stavudine
ddl	-	Didanosine
DDT	-	Dichlorodiphenyltrichloroethane
dH ₂ O	-	Distilled Water
DNA	-	Deoxyribonucleic Acid
EFV	-	Efavirenz
ETC	-	Electron Transport Chain
FTC	-	Emitricitabine
HAART	-	Highly Active Antiretroviral Therapy
HDL	-	High-density Lipoprotein
HED	-	Human Equivalent Dose
HIV	-	Human Immunodeficiency Virus
HMG-CoA reductase	-	3-hydroxy-3-methyl-glutaryl-CoA reductase
IFM	-	Intermyofibrillar Mitochondria
IL-6	-	Interleukin-6
LCAD	-	Long-chain Acyl-coenzyme A Dehydrogenase

LDL	-	Low-density Lipoprotein
LVEDPWT	-	Left Ventricular End-Diastolic Posterior Wall Thickness
LVEDV	-	Left Ventricular End-Diastolic Diameter
LVESD	-	Left Ventricular End-Systolic Diameter
LVESPWT	-	Left Ventricular End-Systolic Posterior Wall Thickness
MCAD	-	Medium-chain Acyl-coenzyme A Dehydrogenase
MCP-1	-	Monocyte Chemoattractant Protein-1
MWFS	-	Mid-wall Fractional Shortening
NHLS	-	National Health Laboratory Services
NNRTI	-	Non-nucleoside Reverse Transcriptase Inhibitor
NOX	-	Nitricoxide Dismutase
NRTI	-	Nucleoside Reverse Transcriptase Inhibitor
NSAID	-	Non-steroidal anti-inflammatories
NVP	-	Nevirapine
PAT	-	Peripheral Adipose Tissue/Sub-cutaneous Adipose Tissue
PGC-1 α	-	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI	-	Protease Inhibitor
PMTCT	-	Prevention of mother-to-child transmission
PTFE	-	Polytetrafluoroethylene
QOL	-	Quality Of Life
RNA	-	Ribonucleic Acid
ROS	-	Reactive Oxygen Species
RSV	-	Resveratrol
RTE	-	Reverse Transcriptase Enzyme
SIRT-1	-	Silent mating type information regulation 2 homolog 1
SOD	-	Superoxide Dismutase
SSM	-	Sub-Sarcolemmal Mitochondria
TDF	-	Tenofovir

TNF- α	-	Tumour necrosis Factor Alpha
UCP	-	Uncoupling Protein
VAT	-	Visceral Adipose Tissue
VitC	-	Vitamin C
VL	-	Viral Load
WHO	-	World Health Organisation
3TC	-	Lamivudine

Chapter 1

1.1.Introduction

1.1.1. HIV: a global health concern

HIV has become a global pandemic over the last 30 years, affecting an estimated 34 million people at the end of 2010, of which 2.7 million were children under the age of 15 (WHO progress report, 2011). HIV began as a relatively insignificant disease in the 1980s, but escalated to become one of the leading causes of deaths and disease burdens worldwide (Ortblad *et al*, 2013; Rosas *et al*, 2013). The number of people newly infected every year remains remarkably high, with a staggering 2.2 million new HIV infections in 2010, and a particular concern is that 390 000 of newly infected individuals were children (WHO progress report, 2011).

During 2005, the annual global mortality rate from AIDS-related deaths peaked at ~ 2.2 million. Since then, this has stabilized and begun to decline. At the end of 2010 the number decreased to an estimated 1.8 million. There were also approximately 250 000 less AIDS-related deaths among children at the end of 2010, a 20% decrease since 2005 (WHO progress report, 2011).

The rate of new HIV infections is also declining; however, this trend is dependent on the region of focus. For example, in Sub-Saharan Africa an estimated 1.9 million people were newly infected in 2010. Although this appears relatively large it represents a decrease of 16% when compared to the 2.2 million new infections during 2001, and a 27% decrease when compared to 1996 and 1998 when the incidence of HIV peaked in the region of sub-Saharan Africa (WHO progress report, 2011).

As indicated, this trend of decreasing rate of infection is not universal, e.g. new HIV infections in the Middle East and Northern Africa increased by ~ 72% from 2001 to 2010. The same trend of increased number of new infections is occurring in Eastern Europe and Central Asia since 2008

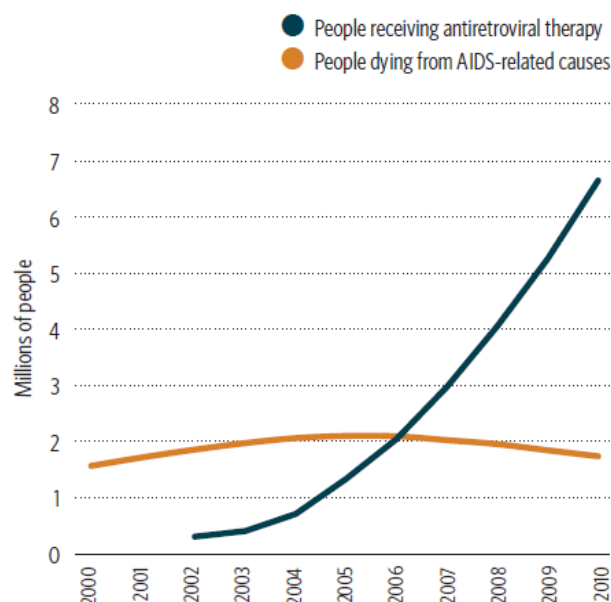


Figure 1.1. Graph depicts the declining number of HIV-infected individuals as ART roll-out increases worldwide (WHO Progress report 2011: Global HIV/AIDS response).

(UNAIDS and WHO, AIDS epidemic update, 2009). Such trends not only differ regarding new infections, but also in terms of AIDS-related deaths.

For example, in sub-Saharan Africa the number of AIDS-related deaths has stabilized, whereas for Eastern Europe and Central Africa it increased from ~ 7 800 in 2001 to ~ 90 000 in 2010, more than a 10-fold increase. The Middle East and Northern Africa follows a similar trend, with a 60% increase in AIDS-related deaths from 2001 to 2010 (WHO progress report, 2011).

Why are there differences in HIV infections and AIDS-related deaths between regions? This is possibly due to the availability of HIV testing, prevention methods and counselling in specific regions or countries, but more importantly it can be attributed to the supply and availability of antiretroviral (ARV) medicine, as demonstrated in Figure 1 (WHO progress report, 2011).

1.1.2. Antiretroviral therapy: a lifeline for the HIV infected

It is estimated that 1 million of the 6 million HIV-positive persons in South Africa received antiretroviral therapy (ART) by the end of 2009 (Epidemiological facts sheet on HIV and AIDS, 2008; STATSSA mid-year population estimates, 2012). The WHO estimates that this resulted in a 707 000 life years gained between 1996 and 2009 in this population (WHO progress report, 2011). The life years gained was achieved by the initiation of combination ARV treatment in 1996 which brought about a revolution in the treatment of HIV.

The above mentioned treatment regime changed the lives of those affected by the virus, but only in high-income countries. Even though 90% of the global HIV burden was concentrated in the low- and middle-income countries and regions, very few ARVs were available in such burdened areas. However, this problem of ARV availability changed in July 2000 at the XIII International AIDS Conference (Durban, South Africa) where activists, community leaders, scientists and health care providers joined forces to demand global access to treatment for those desperately in need of this life saving treatment (WHO progress report, 2011).

Within months the call was answered by some global initiatives, including the Global Fund to Fight AIDS and the United States President's Emergency Plan for AIDS Relief. ARV roll-out began to increase in the regions and countries most burdened by HIV (Goal 6: Combat HIV/AIDS, malaria and other diseases, 2000). By the end of 2003, 400 000 HIV-positive individuals in low- and middle-income class countries received ART. The number of individuals who received ART has since

increased exponentially with ~6.6 million HIV-positive persons now receiving the lifesaving ART globally. The increased availability of ART has led to a global decrease in AIDS-related deaths, and also significantly decreased the number of newly-infected cases, when compared to earlier predictions made (WHO progress report, 2011).

1.1.3. South Africa severely burdened by HIV/AIDS

In the same country where this unique feat of global health care was initiated, the HIV burden is particularly high. Since 1990 the prevalence of HIV in South Africa has steadily increased. During 2006 it was estimated that South Africa had just under 6 million HIV infected people; approximately 17% of the adult population, making it the country with the most HIV infected people in the world (STATSSA, mid-year population estimates, 2012; WHO progress report, 2011; USAID HIV/AIDS Health Profile, 2012). South Africa is also burdened with a staggering 350 000 AIDS-related deaths annually, placing a large strain on both economic and social growth (STATSSA, mid-year population estimates, 2012). Following world trends in general, however, there has been a decline in both the number of new infections and AIDS-related deaths since 2005 (STATSSA, mid-year population estimates, 2012). These statistics coincides with the nation-wide ARV roll-out that began in 2003.

Another success story in the fight against HIV in South Africa is the prevention of mother-to-child transmission (PMTCT) during birth and breast feeding. Since the start of this initiative in 2003, the number of infections transmitted in this manner has decreased to ~ 4%, which is encouraging (USAID HIV/AIDS Health Profile, 2012). The success can be largely attributed to the combination of HIV-testing of majority of pregnant women in clinics for HIV and more importantly, using ARV prophylaxis in new-borns (Kesho Bora study group, 2011), as well as treating an estimated 95% of pregnant women with HIV with ART (STATSSA, mid-year population estimates, 2012; USAID HIV/AIDS Health Profile, 2012).

According to the World Health Organization (WHO), only 36% of infected individuals are eligible for ART (based on the WHO guidelines) which will be briefly discussed later (WHO progress report, 2011). Although the coverage is very low, there are programs in place to increase the number of individuals receiving treatment, as well as improving the treatment regimens (National Strategic Plans for HIV, STIs and TB, 2011). Even with these programs in place, adherence to treatment regimens remains a major problem in the fight against HIV/AIDS. This, however, will be discussed at a later stage.

1.1.4. The HIV life cycle and its effects on health

To understand the treatment regimens and the improvements achieved over the years, the HIV life cycle (Fig. 2) will be briefly discussed as this will allow for a better understanding of the mechanisms and location of ARV functioning. The different classes of ARVs will then be discussed.

HIV is a retrovirus, consisting of a single strand RNA encapsulated in envelope proteins (Frankel and Young, 1998). The infective virion binds/fuses to a host cell and injects its genetic material into the host (Ferguson *et al*, 2002). Once inside the host cell, the RNA strand is reverse transcribed to double-stranded DNA, which becomes integrated into the host DNA, and subsequently transcribed (Ferguson *et al*, 2002; Sierra *et al*, 2005). The host cell then functions as a 'HIV factory', churning out virions by the millions. The HIV genome encodes for 8 proteins that are necessary for viral DNA replication, splicing and assembling of the newly formed viral proteins to produce new, infective virions (Teixeira *et al*, 2011).

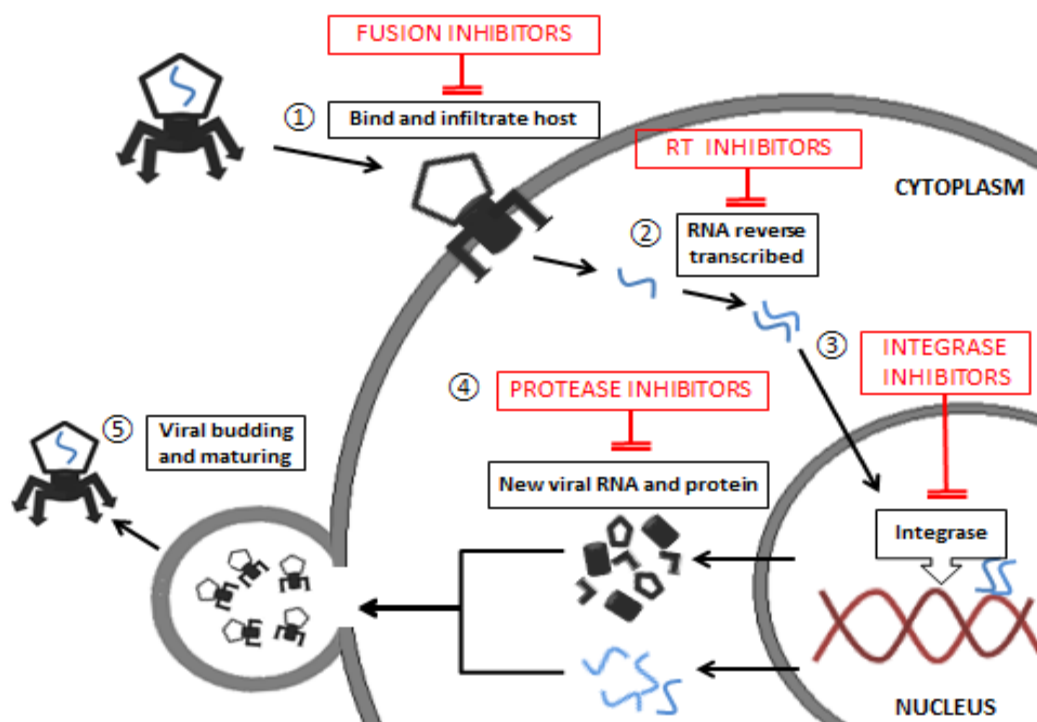


Figure 1.2. A simplified representation of the viral life cycle: ① Once the virus binds to the host cell, the viral RNA is injected into the cytoplasm. ② Here it is reverse transcribed into DNA through the viral enzyme, reverse transcriptase. ③ This viral DNA is then integrated into the host DNA and transcribed, producing new viral RNAs and proteins. ④ These get primed by the viral protease enzyme to produce mature proteins, ⑤ which are assembled to produce new, infective virions (Adapted from Sierra *et al*, 2005).

These proteins do, however, need to be primed by the viral protease enzyme before it can be used for the production of new virions (Frankel and Young, 1998). It is of interest to note that at this stage

some of these viral proteins can directly trigger serious detrimental effects on human health (Cummins and Badley, 2010). For example, Trans-activator of transcription (Tat) is implicated in many HIV-related symptoms, including from toxic effects on the cardiovascular, renal, hepatic and nervous systems (Pugliese *et al*, 2004; Johri *et al*, 2011). Envelope glycoprotein 120 (GP120) is another protein that can induce severe cardio-toxic effects *in vitro* (Coopusamy, 2010). This provides evidence that the virus is able to exert direct effects on cells and tissues through the proteins it produces, which results in various systemic complications (Cummins and Badley, 2010).

However, it is also postulated that the above-mentioned symptoms can occur or be exacerbated due to persistent immune activation and inflammation caused by the virus (Ho and Hsue, 2008). These findings can provide an explanation for the rapid progression of cardiovascular diseases (CVD) that can often present in HIV-positive patients. Such patients present with constant pro-atherogenic inflammation which is a well-known risk factor for development of CVD (Hober *et al*, 1989; Wolf *et al*, 2002; Stein *et al*, 2005, Ho and Hsue, 2008).

HIV thus seems to be causally associated with heart disease, even after controlling for age and traditional cardiovascular risk factors (Kaplan *et al*, 2008). It is important to note that the risk of developing cardiovascular complications is higher in untreated, compared to treated HIV-positive individuals (White, 2001; Wolf *et al*, 2002; Ho and Hsue, 2008). It is therefore evident that ART leads to increased life expectancy and decreased risk of developing CVD, and possibly hepatic, renal and nervous system complications as well (Vervoort *et al*, 2007; Tebas *et al*, 2008).

1.1.5. Different classes of ARVs available

ARVs all function to decrease the amount of infectious virions in the blood. There are different classes of the drugs which work at different stages of the viral lifecycle as seen in Figure 2. The classes of drugs are as follows: Fusion and entry inhibitors, reverse transcriptase inhibitors, integrase inhibitors, and protease inhibitors. Fusion and integrase inhibitors are not commonly used in low-income countries, thus we will only be discussing reverse transcriptase inhibitors (NRTIs and NNRTIs), and more of greater relevance to this study, protease inhibitors (PIs).

Currently these drugs are prescribed in combinations of two or three, and are referred to as 'highly active antiretroviral therapy' (HAART). The combined prescription allows for the drugs to work synergistically and inhibit the viral life cycle at different stages, thus not only rendering the

treatment more effective, but also decreasing the chances of developing drug resistance (Ammassari *et al*, 2002).

The choice of combination is based on various parameters which will not be discussed in detail here (WHO progress report, 2011). These parameters include patient treatment history, co-infections, response to drugs and ART availability. The general rule of thumb is if a patient is treatment-naïve, first line therapy is appointed. First line therapy generally consists of two nucleoside reverse-transcriptase inhibitors (NRTIs) and one non-nucleoside reverse-transcriptase inhibitor (NNRTI).

1.1.6. Treatment regimens employed in South Africa

The drugs currently available in South Africa are:

Zidovudine (AZT); Stavudine (D4T); Didanosine (ddl); Lamivudine (3TC); Abacavir (ABC); Efavirenz (EFV); Tenofovir (TDF) as NRTIs, with Nevirapine (NVP) and Efavirenz (EFV) as NNRTIs (The South African Antiviral National Department of Health, 2010).

Patients are started on first-line therapy according to the following WHO recommendations (WHO progress report, 2011):

- HIV-positive patients displaying a CD4+ count < 350 cells/mm³ irrespective of clinical symptoms
- CD4+ testing required to identify if patients with HIV and WHO clinical stage 1 or 2 diseases need to start antiretroviral treatment
- HIV-positive patients with HIV and WHO clinical stage 3 or 4, irrespective of CD4 count
- Pregnant women with a CD4+ count < 350 cells/mm³. If CD4+ counts > 350 cells/mm³, ARV prophylaxis is initiated after first trimester to avoid mother-to-child transmission

1.1.7. The pros and cons of first-line therapy

Thus first-line therapy is initiated when the host's CD4+ count drops below 350. The starting CD4+ value was initially < 250. It has since been determined that earlier initiation of HAART leads to better treatment outcome (both short- and long-term) (Jani *et al*, 2010; WHO progress report, 2011). Since the new recommendation has been adapted, a better outcome is expected for the treated

individuals, but it does raise a serious statistical and logistical problem: with the higher starting CD4+ value, the number of eligible individuals increased while ARV availability did not match this increase. These findings translate to a decrease in ART coverage, which greatly affects the efficacy of such treatment in the long-term battle against HIV/AIDS (UNAIDS South Africa: Country Situation, 2008; Colvin *et al*, 2010).

Although first-line therapy is very successful and is vital in improving the HIV/AIDS situation in many countries, its success however comes at a cost (Deeks and Phillips, 2009). We will initially discuss the NNRTIs to further elucidate this point. NNRTIs inhibit the viral reverse transcriptase enzyme (RTE) by binding and inactivating the enzyme. NNRTIs have high specificity and are excellent inhibitors of RTE activity, but are notorious for triggering rapid development of resistance (Nunberg *et al*, 1991; Richman *et al*, 1991; Mellors *et al*, 1992; Schinazi *et al*, 1997).

Such drugs, if used alone, or if drug adherence of triple therapy is not ideal, can lead to treatment failure in patients (discussed later) with detrimental consequences. It is thus important to monitor patient health over the course of treatment to ensure viral suppression is successful (WHO progress report, 2011).

NRTIs on the other hand operate by resembling natural nucleosides but do not contain free 3' hydroxyl groups (Wright and Brown, 1990; White, 2001). Once the NRTIs are added to the DNA chain the next nucleoside cannot bind due to the lacking 5'-3' phosphodiester bond needed to extend the DNA chain. Termination occurs and the viral proteins are rendered unusable (White, 2001). This termination causes the potential risk of inhibiting human DNA polymerase activity, leading to chain termination in host DNA.

Fortunately there are significant differences between the viral and host enzymes to ensure selective inhibition of the viral DNA polymerase (Wright and Brown, 1990; White, 2001). For example, DNA polymerase alpha, the main enzyme for DNA replication in humans, appears to be unaffected by the NRTI treatment (Lund *et al*, 2007). However, the activity of related isoforms, i.e. DNA polymerases beta and gamma decreased with NRTI treatment (Lund *et al*, 2007). DNA polymerase beta functions to synthesize short sections of DNA as part of a group of enzymes involved in DNA repair. The clinical significance of its down-regulation is still unclear. It should be noted that the HIV Tat protein induces the opposite effect and increases its expression (Cheng *et al*, 1990; Srivastava *et al*, 2001).

DNA polymerase gamma on the other hand is involved in mitochondrial DNA replication and if decreased this affects mitochondrial functioning and can lead to a variety of downstream effects

(Cheng *et al*, 1990). Such drugs are therefore notorious for inducing severe side-effects which are believed to be associated with impaired mitochondrial function, leading to insufficient energy production and resulting in subsequent cellular dysfunction (White, 2001; Gardner *et al*, 2013).

The side-effects of the older generation NRTI treatments were rather lethal, but have since been improved with newer drug class generations (Snedecor *et al*, 2013; Gupta *et al*, 2013). For example, earlier clinical studies showed patients exhibiting anemia and neutropenia when treated with NRTIs. In addition to these earlier clinical studies *in vitro* studies conducted on murine bone marrow progenitor cell lines confirmed toxic side-effects observed in patients (Luster *et al*, 1989). When neuronal cell models were later employed, ddI and d4T elicited toxicity, whereas AZT and 3TC did not - again echoing clinical findings (Cui *et al*, 1997). These *in vitro* and clinical studies are the reason why most patients are started on AZT and 3TC as NRTI backbones for HAART.

Although cell-based studies with AZT and 3TC did not induce toxicity it does not imply that there are no other side-effects involved. This claim is supported by clinical observations that range from simple nausea and vomiting, to more serious health issues such as liver cirrhosis and kidney damage (White, 2001). Such side-effects (both minor and major) are also part of the reason why many of ARV treatment regimens fail, since individuals feel too ill to continue treatment (Carr and Cooper, 2000; Hawkins, 2006).

1.1.8. Drug adherence affects treatment outcomes

The efficacy of ART is determined by circulating viral load (VL). Thus HIV-positive patients on ART require a VL test every 6 months to ensure the effectiveness of their treatment. If a patient's VL is persistently higher than 5000 copies/ml, it is considered as a treatment failure (WHO progress report, 2011). If VL is not available, immunological criteria are used to confirm clinical failure (WHO progress report, 2011).

Treatment failure can occur due to a drug being ineffective in an individual. Ineffective treatment usually occurs when drug adherence is low, since the drugs' steady state concentrations never reach their effective levels (Doyle and Geretti, 2012). Patients are therefore required to bring their ARVs with them when they visit clinics to evaluate drug adherence (National Department of Health, 2013).

Since many of the HIV-infected individuals in South Africa live in rural areas, far from clinics or ART distribution centres, drug adherence is consistently low (Epidemiological facts sheet on HIV and AIDS, 2008). Walking is often the only mode of transport for many in such regions and during winter

months or bad weather many simply do not go for their check-ups (Essop and Nell, unpublished data for Cape Winelands region).

The above mentioned logistical problems therefore pose a serious risk for patient care, both short and long-term. Also, ARV side-effects are another important reason for poor drug adherence. Once first-line treatment is considered 'failed' or the patient's drug adherence is < 90%, it is recommended that second-line therapy be initiated as soon as possible to avoid disease progression (WHO progress report, 2011).

1.1.9. Second-line therapy might be the answer in low-income regions

Second-line therapy consists of a boosted PI (explained later) plus two NRTIs. The drugs currently employed in South African clinics are Alluvia as a PI, and Zidovudine (AZT) and lamivudine (3TC) as the NRTI backbone (National Department of Health, 2010). The NRTI backbone is used to improve treatment efficacy as well as to ensure that the patient does not develop drug resistance. It has been found that boosted PI treatment has less chance for development of resistance in scenarios of low drug adherence (Paterson *et al*, 2000; Masquelier *et al*, 2005; Shuter, 2007).

Protease inhibitors function, as the name implies, by inhibiting the HIV protease enzyme (Fig. 2). The protease enzyme is essential in the viral life cycle since it is required for the cleaving of the newly formed proteins to create mature viral proteins (Frankel and Young, 1998). Once it is bound and inhibited by the drug it cannot function and thus the viral life cycle is halted because new virions cannot be assembled. Boosted PIs have become the choice of clinicians across the globe, since it is more effective at maintaining viral copies < 400 per millilitre than a single PI alone. The term 'boosted' merely implies that the PIs are given in combination, usually with Ritonavir.

Ritonavir is not the ideal PI for managing viral suppression alone. Ritonavir is however a potent inhibitor of the CYP3A enzyme. The latter enzyme is required for the metabolism of other PIs. The inhibition of CYP3A by Ritonavir allows more active PIs like Lopinavir to be available at higher circulating concentrations for longer durations. The use of boosted PIs has been demonstrated to be successful in a number of studies over the past decade (Zeldin, 2004; Bierman, 2010; Siripassorn, 2010).

In fact, boosted PI treatment is so effective that it can be used successfully as monotherapy. For example, in a 96-week analysis of Lopinavir-Ritonavir monotherapy versus normal firstline therapy

(two NRTIs + one NNRTI), PI monotherapy was not inferior to triple therapy (two NRTI's + one NNRTI). Interestingly enough it was observed that adverse events leading to discontinuation of treatment was significantly lower with PI monotherapy (Pulido *et al*, 2008; Arribas *et al*, 2009). In South African clinics boosted PIs also play an additional important role since it makes up part of first line therapy in pediatric regimens up to the age of three (National Department of Health, 2013).

Second-line therapies containing boosted PIs are therefore very important and beneficial treatment regimens. It is especially important in South Africa and other low-income countries because it provides long-lasting protection against HIV disease progression (Scherrer *et al*, 2013). Although second-line ART increased life expectancy and quality of life for many HIV-positive individuals, it does come at a cost. Side-effects occur with the use of boosted PIs just as they do with first-line therapy ARVs (White, 2001). For example, patients on PI-containing second-line ART can develop weight gain, lipodystrophy, metabolic syndrome, diabetes and CVD (Duong *et al*, 2001; Friis-Møller *et al*, 2007; Friis-Møller, 2010; Kumar, 2004; Lee *et al* 2004; Martinez and Gatell, 1998; Worm *et al*, 2010; Zeldin and Petruschke, 2004).

Side-effects such as mentioned above, occur at varying times in different patients. In some cases however, many of these side-effects (including heart diseases) have been observed to occur relatively early after initiating PI regimens (Friis-Møller *et al*, 2007). This early occurrence of side-effects does not correlate with the normal disease progression of HIV or CVD. These observations imply that PI treatment is implicated in the development of these serious side-effects. Since the emergence of such side-effects, studies attempted to elucidate the underlying mechanism whereby this occurs.

1.1.10. Possible mechanisms of Protease Inhibitor induced side-effects

Although the mechanisms are still relatively unclear, the duration of PI treatment plays an important role in its detrimental effects (Friis-Møller *et al*, 2007; Friis-Møller, 2010). The longer treatment on second-line therapy continues, the worse the clinical outcome of the patient becomes. For example, Friis-Møller and colleagues (2007) (Fig. 3), found that the risk for cardiovascular events increased several fold with longer PI treatment (Friis-Møller *et al*, 2007). The question this poses is, how can this drug lead to something as severe as CVD? Even though the underlying causes remain unclear, we have developed, with support from literature from the literature, some theories.

Our first theory is that the PIs, or its metabolites, directly affect specific tissue types by direct binding to heart cells or nuclei and thereby activating harmful pathways. Such pathways are then proposed

to cause decreased functioning or apoptosis, thereby leading to specific symptoms presenting in HIV-positive patients receiving ART. This theory is strengthened by work done on murine models that suggest the possibility of direct cellular mechanisms through which PI treatment might promote atherosclerosis (Jiang *et al*, 2006; Jiang *et al*, 2007, Wang *et al*, 2007).

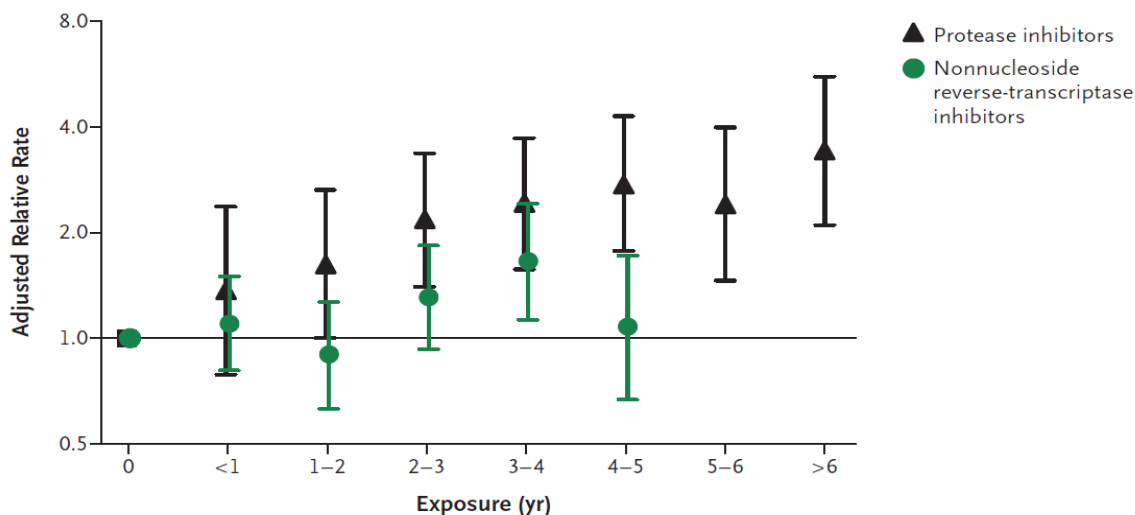


Figure 1.3. This graph depicts the adjusted relative rates of myocardial infarction according to the according to the cumulative exposure to PIs and NNRTIs. The estimates were based on Poisson regression models. The multivariable model was adjusted for sex, cohort, HIV transmission group, race or ethnic group, age, body-mass index, family history of cardiovascular diseases, smoking status, previous cardiovascular event, and calendar year (Friis-Møller *et al.*, 2007).

In addition Hong-Brown and colleagues (2008) showed that *in vitro* PI treatment can lead to the activation of 5' adenosine monophosphate-activated protein kinase (AMPK) through direct effects on AMPK downstream regulators (Hong-Brown *et al*, 2008). AMPK is an important cellular regulatory protein, involved in various pathways. It is catabolic by nature, allowing for increased energy availability. Activation of AMPK can thus lead to decreased protein synthesis (Hong-Brown *et al*, 2008). It is thus not farfetched to speculate that HIV PIs, or their metabolites, could potentially have direct effects on cells, tissues and their functioning through this and/or other pathways.

Our second theory is that the PIs (or its metabolites) lead to type-2 diabetes. It is commonly observed that patients on PI treatment develop altered blood profiles, presenting with hyperlipidemia, hypercholesterolemia and hyperglycemia (Carr, 2000; Stein, 2001; Tsiodras *et al*, 2000). How these metabolic disturbances occur is not known, but the above-mentioned symptoms are also common during the development of type 2 diabetes (Sivitz and Yorek, 2010). Type-2 diabetes is known to cause some severe health problems of its own if left untreated, e.g. kidney failure, blindness and of particular concern, CVD (Sivitz and Yorek, 2010).

Diabetes is also a known cause of mitochondrial dysfunction, which has been a suspected cause of some of the clinical symptoms. We therefore postulate that the PI treatment could cause type 2-diabetes through alterations in the blood profile, which eventually leads to mitochondrial dysfunction and heart disease. Thus the PI treatment does not directly cause CVD, but the latter is a secondary effect.

Our third and final theory is that PI treatment directly leads to mitochondrial dysfunction. In support, other studies also theorised that PIs or its metabolites may potentially affect mitochondria in a direct fashion (Phenix, 2001; White, 2001). We believe that such interaction(s) could affect mitochondrial function and energy production, for eg. directly binding or inhibiting electron transport chain (ETC) complexes. Mitochondrial dysfunction would subsequently lead to decreased energy production and increased reactive oxygen species (ROS) production potentially causing cell death. The findings by Hong-Brown and colleagues (2008) that PI activate AMPK *in vitro* strengthen this theory (Hong-Brown *et al*, 2008). If energy production by mitochondria are diminished the cells would respond by activating AMPK. The activation of AMPK would serve to increase energy production, but this will be discussed in detail at a later stage.

It is possible that the PIs directly affect the mitochondria by another means. It could be by adversely affecting metabolism of different substrates involved in mitochondrial energy production. Alterations of glucose and fatty acid metabolism, for instance, is believed to lead to an accumulation of such substrates in cells and possibly even within the circulation, leading to the presentation of the symptoms mentioned in the second theory. Eventually type-2 diabetes may develop as a secondary effect, which in turn can initiate cardiovascular complications that are often seen in these patients (Caballero, 2003; Sivitz and Yorek, 2010; Paneni, 2014).

To summarize our final theory we postulate that the PIs cause direct effects on mitochondria or their metabolism that lead to the eventual progression to diabetes, and ultimately CVD. This third and final theory that PIs directly leads to mitochondrial dysfunction has provided the platform for this study, and will be discussed in more detail from here on.

1.1.11. Mitochondria: suppliers of life-sustaining energy and detrimental ROS

Mitochondria function as the power stations of cells. Mitochondria are vital in a variety of cellular processes, with their primary function the production of adenosine triphosphate (ATP) by aerobic respiration (Slater, 2003). However, mitochondria have many other important cellular functions -

this will not be discussed at this time. For certain tissues that are continuously active (e.g. the heart and brain) ATP production is especially important (Ballinger, 2005). In these tissues mitochondria provide energy required for optimal functioning. If ATP production becomes impaired then the outcome is often pathological (Finsterer, 2004) – often referred to as “mitochondrial dysfunction” (Sivitz and Yorek, 2010).

There are a number of reasons why mitochondrial function may become impaired. Firstly, it may be due to mitochondrial enzymes that are less effective - due to decreased activity (Vondra *et al*, 1977; Finsterer, 2004). Although such enzymes technically do not affect mitochondria directly, they are vital for the normal process of respiration. Mitochondrial enzymes are often responsible for the splicing or priming of substrates for mitochondrial utilization. For example, medium-chain acyl-coenzyme A dehydrogenase (MCAD) and long-chain acyl-coenzyme A dehydrogenase (LCAD) (Wang *et al*, 1999; Zhang *et al*, 2007; Angelini *et al*, 2011) form part of the oxidoreductases family, and function to break down medium and long-chain fatty acids into acetyl-CoA, which can then be used in the Krebs cycle for the generation of the reducing agents, NADH and FADH₂ (Wang *et al*, 1999; White, 2001; Zhang *et al*, 2007, Sivitz and Yorek, 2010).

The reducing agents, NADH and FADH₂ are then used to produce ATP through the ETC which is located within the inner mitochondrial membrane. Inhibition of enzymes like LCAD can therefore lead to mitochondrial dysfunction since fatty acids such as palmitoyl-carnitine can accumulate and directly affect ETC efficiency (Bremer and Wojtczak, 1972; Bremer, 2001; Divakaruni *et al*, 2012). The accumulation of fatty acids, as described, would adversely affect energy production and hence cellular function. The ETC is an extremely complex system which will not be discussed in full detail, i.e. only details necessary for the understanding of the research question and study design of this project will be discussed.

1.1.12. The importance and functioning of the electron transport chain

The ETC is comprised of four protein-complexes spanning the inner-membrane of mitochondria (explained shortly) (White, 2001; Sivitz and Yorek, 2010). Mitochondria then utilize such complexes to generate energy as electrons are passed from donors at lower redox potentials, to acceptors at higher redox potentials (Sivitz and Yorek, 2010). In parallel, protons are pumped from the matrix to the inter-membrane space, leading to a potential difference across the inner-membrane (Scheffler, 1999).

The potential difference that is generated is then used to generate ATP through a fifth protein-complex spanning the inner-membrane known - ATP synthase [F_0F_1 ATPase] (Arechaga *et al*, 2001). A small portion of the proton gradient is dissipated in the form of heat as protons leak back into the matrix (Sivitz and Yorek, 2010). The majority of the electrons from the ETC eventually get transferred to oxygen, forming H_2O the well known by-product of respiration. A small amount of electrons do however leak during transport, leading to mitochondrial superoxide production, which also gives rise to additional free radical species (Sivitz and Yorek, 2010).

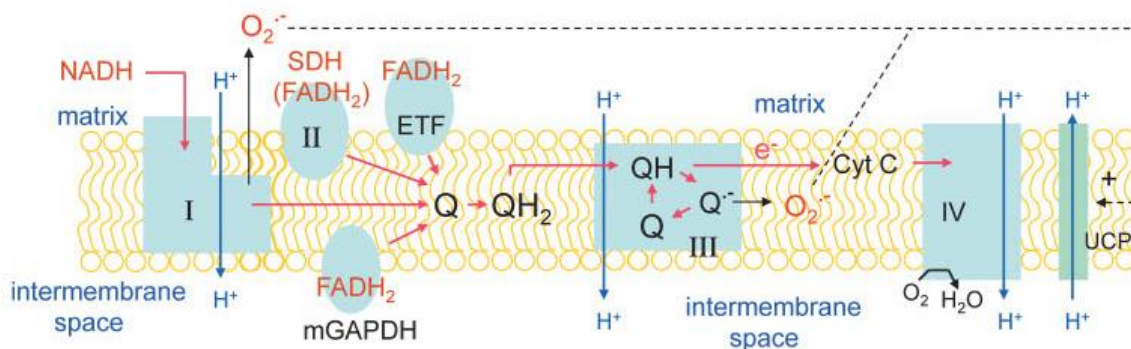


Figure 1.4. This figure depicts a simplified portrayal of the electron transport chain. It shows how reducing equivalents like NADH can enter the ETC through complex I, while $FADH_2$ enters through complex II. From here electrons are passed on to an intermediate ubiquinone, after which it is passed to complex III, whilst protons are moved across the inner-membrane of the mitochondrion, to produce a proton gradient. From complex III electrons are donated to intermediate cytochrome C, and passed on to complex IV, whilst more protons are moved across the membrane. Electrons are finally passed from complex IV to oxygen, to form metabolic water. The proton gradient that is produced through the movement of electrons is used to produce ATP through ATPase. It can however also be dispersed without production of ATP, through the uncoupling proteins (UCPs).

Although such ROS particles may have serious detrimental effects on cells, a small amount is necessary for normal cellular functioning (Finkel and Holbrook, 2000). ROS are important metabolic regulators, and can function as mitochondrial uncouplers as well as cellular signaling molecules (Dinakar *et al*, 2010, Ralph *et al*, 2010). However, when ROS are produced in higher amounts than is required for normal functioning it can trigger detrimental effects (Sivitz and Yorek, 2010). Such an excess of free radicals can wreak havoc, leading to the destruction of all biological molecules, including DNA, proteins and lipids (Finkel and Holbrook, 2000).

Cells possess antioxidant systems to help counter the above mentioned detrimental effects. These include systems such as superoxide dismutases (SOD) and NADPH Oxidase (NOX) (Förstermann, 2010; Buettner, 2011). Upregulation of systems like SOD and NOX can result in an adaptive response when needed; however with continuously high ROS production it cannot sustain its protective function and cellular damage occurs.

In severe cases the damage results in irreparable intracellular damage and cells can subsequently initiate apoptosis through pathways regulated by mitochondria (Green and Reed, 1998; Regula, 2003). It should be noted that the regulation of cell death is another key function of mitochondria (Green and Reed, 1998). It is therefore important that cells maintain “safe” ROS levels, since excess levels can lead to cell death, and in certain tissues (e.g. heart and brain) this can be seriously detrimental, if not lethal.

Under normal circumstances the reducing agents, NADH and FADH₂ enter the ETC through complex I (NADH ubiquinone reductase) and complex II (succinate dehydrogenase), respectively (Slater, 2003; Sivitz and Yorek, 2010). As highlighted in Figure 4, electrons are then transferred from the entry sites to complex III (ubiquinol-cytochrome c reductase) through a mobile intermediate ubiquinone (Scheffler, 1999; Slater, 2003).

At complex III ubiquinol becomes oxidized and electrons are transferred to another mobile intermediate known as cytochrome C (Slater, 2003). The electrons are then carried to complex IV (cytochrome C reductase), where cytochrome C becomes oxidized and electrons are passed to O₂, which leads to the production of metabolic H₂O (Scheffler, 1999). The proton gradient (membrane potential) is created by H⁺ ions that are continuously translocated to the intermembrane space through complex I, III and IV (Fig. 3). As mentioned previously, this proton gradient is then used to drive ATP production through complex V (ATPase).

1.1.13. Mitochondrial proton gradient not only used for energy production

Under certain circumstances the proton gradient is not coupled to ATP production (Nicholls, 2001). These circumstances where the proton gradient does not produce ATP occurs due to another class of protein complex known as uncoupling proteins (UCPs). UCPs span the mitochondrial membrane and cause electrons to leak back into the mitochondrial matrix without the production of ATP (Fig. 3) (Nicholls, 2001). Superoxide can activate UCPs, leading to less ATP formed per reducing agent (Fink *et al*, 2007). This process may appear as a detrimental effect but occurs under normal homeostatic conditions. For example, a well-known scenario is post-prandial heat production (Clarke *et al*, 2012), i.e. heat generation by mitochondrial UCPs explains why individuals feel warm after consuming a meal. Additionally, the heat generated by UCPs is vital for maintaining our constant body temperatures.

A more serious scenario occurs when one or more of the ETC complexes are inhibited. For example, compounds such as the toxin, cyanide, can inhibit complex IV (Zhang *et al*, 1990) and electrons transferred to O₂, inhibiting further flow of electrons through the ETC. This then causes dissipation of the proton gradient through UCPs with no further ATP formation. In addition to the inhibition of complex IV, superoxide is still produced by complexes I and III that further activates UCPs as mentioned before (Sivitz and Yorek, 2010).

Low doses of other types of compounds (e.g. fucoxanthin and caffeine) can also lead to uncoupling of mitochondria, but not to the extent that is fatal. It can instead increase superoxide production with mild UCP activation (Kogure *et al*, 2002; Maeda *et al*, 2005; Pang *et al*, 2008), resembling that of the post-prandial scenario. In light of these discussions, it seems plausible that PI treatment could potentially lead to mitochondrial uncoupling, causing decreased ETC efficiency, altered ATP production and increased ROS production.

1.1.14. Mitochondrial morphology affects respiratory capacity

Mitochondrial dysfunction may also be attributed to the morphology of these organelles (Sivitz and Yorek, 2010). Mitochondria are double-membraned, with the inner-membrane folded (cristae) extensively. Such folding leads to a dramatic increase of the mitochondrial surface area (Sivitz and Yorek, 2010). Since the ETC is situated in the inner-membrane, it is beneficial for its surface area to be as large as possible in order to house numerous ETC complexes.

Mitochondrial morphology can be dramatically altered with certain disease states. For example, type-2 diabetes is a well-known cause of mitochondrial dysfunction (Heilbronn *et al*, 2007; Ritov *et al*, 2010; Sivitz and Yorek, 2010) and can elicit morphological changes (e.g. enlarged with cristae almost absent) (Kelley *et al*, 2002). The altered mitochondrial morphology results in a decrease in surface area which in turn adversely affects aerobic metabolism. These effects on respiratory capacity are interesting since the prevalence of type-2 diabetes among patients receiving PI-treatment is relatively high (Capeau *et al*, 2012). The increased prevalence of type-2 diabetes among PI-treated individuals raises the question of whether mitochondrial dysfunction observed in such instances is causative or symptomatic of the diabetes. These findings form part of ongoing research endeavors world-wide.

1.1.15. Decreases in mitochondrial number also implicated in type-2 diabetes

Mitochondrial number is another determining factor in metabolism, e.g. lower numbers result in slower aerobic metabolism. There are two distinct populations of mitochondria in muscle tissue. The distinction between the two populations is based on their subcellular localization, but they also differ with regards to size, morphology and enzyme activities. These two populations are known as sub-sarcolemmal mitochondria (SSM) and intermyofibrillar mitochondria (IFM). The SSM are located just underneath the sarcolemma and have a large lamellar shape. The IFM are smaller in size, and located in between the contractile units.

Type-2 diabetes is implicated with decreased mitochondrial numbers, especially the sub-sarcolemmal (SSM) population (Kelley *et al*, 2002; Morino, *et al*, 2005; Ritov *et al*, 2005). Together with morphological and ETC alterations, this leads to metabolism being severely affected. Once mitochondria are damaged or display altered morphology, they are removed by a process known as autophagy. The process of removal occurs because damaged mitochondria (e.g. due to increased ROS) often leads to cell death signaling, and is therefore a preventative mechanism (Kim *et al*, 2007). The process of removing mitochondria through autophagy is termed mitophagy. However, in some instances mitophagy can be inhibited thereby leading to damaged mitochondria continuously producing ROS. The exact mechanism of such down-regulation is unclear (Kim *et al*, 2007). Nevertheless, mitochondrial number and efficacy is important for maintaining adequate metabolism and sustaining life.

1.1.16. Modulators involved in regulation of mitochondria and metabolism

Mitochondrial biogenesis have been found to be down-regulated in individuals with type-2 diabetes (Morino *et al*, 2005). Thus individuals suffering from type-2 diabetes can exhibit lower mitochondrial numbers and some degree of dysfunction. The exact mechanisms of such dysfunction are not known, but the answer may lie with regulators of mitochondrial biogenesis (Sivitz and Yorek, 2010). The three most important regulators are: AMPK, silent mating type information regulation 2 homolog 1 (SIRT-1), and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α). It is proposed that altered expression of such regulators are implicated in mitochondrial dysfunction (Sivitz and Yorek, 2010).

Such regulators can be activated or upregulated in different ways. However, once activated, they work synergistically to increase metabolism (Cantó *et al*, 2009; Ruderman *et al*, 2013). One way of achieving increased metabolism is by increasing mitochondrial biogenesis (Zong *et al*, 2002; Hardie *et al*, 2007). Mitochondrial biogenesis leads to increased mitochondrial numbers which in turn increases energy production. Secondly the regulators increase metabolism by increasing the number

of ETC complexes and ETC efficiency (Cantó *et al*, 2009). And lastly, the regulators cause increased metabolism through increased substrate availability and subsequent increases in metabolic flux (Cantó *et al*, 2009).

The regulators highlighted respond to a number of stimuli, e.g. insufficient intracellular ATP levels (Cantó *et al*, 2009; Zhang and Wu, 2009). When the ADP/ATP ratio increases, the cell senses an energy shortage which leads to activation of AMPK. The activation of AMPK increases metabolism and substrate utilization with the goal of producing more ATP. The increased ATP production by AMPK is achieved by increases in fatty acid uptake and oxidation, increased glycogen breakdown, increased gluconeogenesis, enhanced glucose uptake, upregulated ETC complex production, and a general increase in mitogenesis (Ruderman *et al*, 2013). In addition AMPK activation also leads to the stimulation of SIRT-1 and PGC-1 α . The importance of these two regulators will be explained in the following pages. It is important to note that AMPK activation has many other effects that are not the focus of this study.

These two regulators further increase mitochondrial numbers (Cantó *et al*, 2009; Ruderman *et al*, 2013). Together these effects can result in increased mitochondrial ATP production..

1.1.17. HIV and HAART can affect both regulators and flux of metabolism

Focusing on HIV-infected individuals (ARV-naïve), it has been discovered that cellular AMPK is down-regulated and thought to occur through the direct inhibition of SIRT-1 (Mankouri and Harris, 2011). This would be expected to elicit negative outcomes on the host's metabolism. This inhibition of SIRT-1 is thought to occur as a result of circulating HIV proteins e.g. TAT and GP120, both implicated in cardiotoxic effects of the virus itself (mentioned previously). Certain ARTs can trigger similar effects on the ETC, especially NRTIs (White, 2001) and this may occur due to a strong decrease in mitochondrial DNA. Here NRTIs are incorporated into the host DNA thereby preventing its replication (White, 2001). It therefore stands to reason that individuals suffering from HIV/AIDS and receiving ART, have increased chances of suffering from metabolic disturbances.

The regulator, SIRT-1, primarily comes into play during times of fasting/starvation. SIRT-1 is part of the sirtuin family, a group of deacetylases that regulate nutrient usage (Cantó *et al*, 2009). The sirtuins achieve this through their ability to modify gene expression as well as target histones, metabolic transcription factors and co-regulators, of importance in our setting is AMPK and PGC-1 α

(Amat *et al*, 2009; Chung *et al*, 2012). During fasting/starvation the body does not receive adequate calories through the diet, so alternative sources needs to be accessed (Chung *et al*, 2012).

The decreased energy production due to fasting/starvation is sensed on a cellular level due to an increase in the NAD⁺/NADH ratio (Cantó *et al*, 2009; Chung *et al*, 2012). SIRT-1 is up-regulated to correct this problem before the organism is detrimentally affected. As previously mentioned, activation of SIRT-1 leads to the co-activation of AMPK and PGC-1 α (Fullerton and Steinberg, 2010). These can then act in synchrony to increase availability of substrates for energy production. AMPK is catabolic, leading to the breakdown of all types of macromolecules in storage namely; protein, fat and carbohydrate. Thus supplying substrates for ATP production from sources stored in the body for exactly these scenarios. HIV has however been implicated in decreasing SIRT-1 and hence affecting metabolism (Zang and Wu, 2009).

PGC-1 α on the other hand, is a co-activator which serves as another master regulator of genes involved in metabolism (Mendelsohn and Larrick, 2011). It is thought to be the down-stream mediator of AMPK and SIRT-1 (Fullerton and Steinberg, 2010; Mendelsohn and Larrick, 2011). Its activity exerts different effects on metabolism in different tissues. In brown adipose tissue for example, it increases UCP1 expression and genes involved in mitochondrial oxidative pathways. In the liver PGC-1 α is responsible for inducing genes involved in gluconeogenesis. In skeletal muscle it is capable of increasing metabolic flux in response to exercise (Fullerton and Steinberg, 2010).

PGC-1 α is especially sensitive to acetylation/activation by SIRT-1 (Liang and Ward, 2006). Eventhough it has a range of effects in various tissues, all the effects are linked to increasing metabolism or efficacy thereof. As mentioned before HIV is suspected to lead to decreased activity of AMPK through the viral inhibition of SIRT-1 (Zhang and Wu, 2009). Thus implying that HIV might negatively affect PGC-1 α levels and activity as well. Given the major role that PGC-1 α plays in metabolism any alterations to its functioning could be detrimental to the organism, in our setting the HIV-positive patient receiving PIs.

Taken together, the three regulators of metabolism act in unison to; increase substrate availability, increase metabolic flux and efficacy, and ultimately increased energy production. These three regulators are all interlinked and if one is to be down-regulated it affects the other two (Canto *et al*, 2009). This would have undesirable effects on mitochondria and ultimately decrease energy production. As previously mentioned, it has been observed in certain studies that these regulators and subsequent metabolism can be negatively affected by certain compounds or viruses (Cant *et al*,

2009; Chung *et al*, 2012). It thus seems plausible to propose that PI treatment could have similar effects on one or more of these regulators.

It is important to note that there is another regulator of metabolism that has not been mentioned. It is known as thyroid hormone, and makes up part of the hypothalamic-pituitary-adrenal axis. This hormonal system controls reactions to stress and regulates many bodily processes (Bhangoo and Desai, 2013). Certainly one of the most important functions, with regards to this study, is energy storage and expenditure. Thyroid hormone can bind directly to the inner membrane of mitochondria and cause an increase in metabolic flux, mitochondrial oxygen consumption and ATP production (Meena *et al*, 2013).

Dysfunction or depletion of thyroid hormone can lead to serious metabolic abnormalities, for example hypothyroidism. In this disorder metabolism is impaired due to lowered levels of thyroid hormone (Mullur *et al*, 2014). Hypothyroidism causes patients to be chronically fatigued and gain weight (Carvalho *et al*, 2013; Mullur *et al*, 2014). In children this disorder can lead to developmental problems and cognitive impairment (Mullur *et al*, 2014). Clinically it has been observed that HIV and HAART treatment can affect thyroid hormone levels (Desforges *et al*, 1992; Bhangoo and Desai, 2013). The clinical presentation thereof does however vary dramatically, ranging from hypo- to hyperthyroidism, implying that there are various factors involved (Bhangoo and Desai, 2013).

The prevalence of hypothyroidism has however been found to be higher than that of hyperthyroidism (Bhangoo and Desai, 2013; Carvalho *et al*, 2013; Meena *et al*, 2013). The higher prevalence of hypothyroidism is of significance because it can contribute to the already diminished metabolic capacity of HIV-positive HAART treated individuals, worsening quality of life and clinical outcomes.

1.1.18 PIs: where do we go from here?

From the literature it is clear that the introduction of ARVs gave hope to patients infected with HIV. It significantly increased life expectancy and quality of life. However it became evident that some ARVs do have some detrimental side-effects. Some of these side-effects are so severe that it might cause patients to stop treatment. One important class of ARVs, PIs, has been known to cause some detrimental side-effects. As previously mentioned the mechanisms involved in these side-effects are not well understood.

With the brief background given on mitochondrial function it becomes evident that these organelles are likely candidates in the development of these side-effects. Further research is needed to identify whether the mitochondria are directly affected by the PIs or rather as result of type-2 diabetes and altered metabolism. Given PIs undeniable benefit to patients when used as part of combination therapy, gaining more insight into the mechanisms behind its side-effects could enable us to improve treatments. This could have great benefit to HIV-positive patients in poor regions globally.

1.1.19. Hypothesis

In light of this, we postulate that sole PI treatment in rodent experimental model would provide a unique opportunity to elucidate mechanisms underlying PI-mediated side-effects. We therefore hypothesize that PI treatment leads to direct detrimental effects on mitochondrial function. Furthermore, we propose that such detrimental effects lead to the presentation of metabolic dysfunction (e.g. hyperlipidemia, hypercholesterolemia, diabetes) and the eventual onset of CVD as secondary effect. Finally we postulate that various co-treatments with well-known drug therapies will blunt PI-mediated side-effects.

1.1.19. Aims

- To determine whether PI treatment results in direct detrimental effects on cardiac function.
- To assess whether PI treatments lead to alterations in blood profile and body composition.
- To evaluate whether PI treatment alters mitochondrial respiratory function.
- To assess whether duration of PI treatment affects any aforementioned aims.
- To determine whether co-treatments (Vitamin C, Resveratrol, Aspirin) can attenuate PI-mediated side-effects.

1.2. Materials and methods

1.2.1. Animal housing

All animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals of the National Academy of Sciences (NIH publication No. 85–23, revised 1996) and performed with the approval of the Animal Ethics Committee of Stellenbosch University (South Africa). Male Wistar rats were acquired at 150-180g body weight. Animals were randomly divided into 6 experimental groups consisting of 6 animals each. Animals were housed in standard-sized IVC rat cages (3 animals per cage), subjected to 12:12 h light-dark cycles with *ad libitum* food and water. Rats were fed standard rat chow. Animals were weighed every third day to assess weight gain and food was weighed daily. A week acclimatization period was permitted to allow animals to get used to handling, as well as acquiring the taste for the jelly and method of feeding.

1.2.2. Dosages and treatment preparation:

Experimental groups were as follows: Control, Jelly control, protease inhibitor (PI). Treatment dosages were calculated using body surface area (BSA) based on the following formula (Reagan-Shaw, S. 2008):

$$\text{Human Equivalent Dose} \left(\frac{mg}{kg} \right) = \text{Animal dose} \left(\frac{mg}{kg} \right) \times \frac{\text{Animal } K_m}{\text{Human } K_m}$$

Table 1.1. Conversion of human equivalent dose (HED) to animal doses based on BSA

Species	Weight	BSA (m ²)	K _m factor
Human			
Adult	60	1.6	37
Child	20	0.8	25
Baboon	12	0.6	20
Dog	10	0.5	20
Monkey	3	0.24	12
Rabbit	1.8	0.15	12
Guinea pig	0.4	0.05	8
Rat	0.15	0.025	6
Hamster	0.08	0.02	5
Mouse	0.02	0.007	3

Values based on data from FDA Draft Guidelines (Centre for Drug Evaluation and Research, Centre for Biologics Evaluation and Research. (2002) Estimating the safe starting dose in clinical trials for therapeutics in adult healthy volunteers, U.S. Food and Drug Administration, Rockville, Maryland, USA).

The HIV PI, trade name Alluvia™ (purchased from Neelsie pharmacy, Stellenbosch), containing 200mg Lopinavir/50mg Ritonavir per tablet, was used for the purposes of this experiment. For treatment purposes, all pills and capsules were first de-capsulated or de-filmed, crushed with mortar and pestle and weighed accordingly. Treatments were prepared and weighed out daily using a microscale (Shimadzu AW220) and appropriate weighing boats.

Raspberry flavoured gelatine (Tower Jelly) was combined with pure gelatine (Sheridans) in a ratio of 4:1. This ratio was determined during the optimisation period. Jelly blocks were prepared as follows: flavoured gelatine and pure gelatine were mixed in a predetermined amount of boiling tap water, and stirred until dissolved completely. Jelly solution was then directly pipetted into ice trays with volumes ranging from 1.5-2.5 ml, depending on the weights of the rats. Treatments were added to each 'well' of the ice tray once the jelly solution had cooled. Ice trays were placed in the fridge at 4 °C until set.

Treatments were administered daily between 16:00 – 18:00, which coincided with the start of the dark cycle. Jelly blocks were fed to rats individually in the same order, up until the day of culling.

1.2.3. Treatment periods and data collection:

Treatments were administered for 2, 4 and 6 months, and various parameters were analysed at the end of each treatment period:

1.2.3.1. Blood collection

Blood was collected at the end of 2 and 4 months of treatment. Rats were sedated using 2% isoflurane in oxygen. Adequacy of anaesthesia was monitored by lack of reflex response to a foot pinch, after which a total of 1 ml of blood was drawn from each rat's jugular vein with a 1 ml syringe and 18 gauge needles. Blood was then transferred into serum tubes (Lasec, 454098), and centrifuged at 2000 rpm at 4 °C for 20 min. After centrifugation, the plasma supernatant was aspirated and transferred into 500 µl Eppendorf tubes, and sent to the National Health Laboratory Services (NHLS – Tygerberg, Western Cape, South Africa) for analysis. Plasma levels of cholesterol, triglycerides and free fatty acids were analysed.

1.2.3.2. Echocardiography

Echocardiography was conducted at the end of 2 and 4 months of treatment. Rats were sedated as mentioned above, after which the fur on the chest was shaven. Data was recorded from the parasternal short-axis of the rat heart. The following parameters were measured and recorded for each rat: Left Ventricular End-Diastolic Diameter (LVEDV), Left Ventricular End-Systolic Diameter (LVESD), Left Ventricular End-Systolic Posterior Wall Thickness (LVESPWT), Left Ventricular End-Diastolic Posterior Wall Thickness (LVEDPWT) and the R-R interval. Fractional shortening was calculated as: $(\text{end-diastolic diameter} - \text{end-systolic diameter}) / \text{end-diastolic diameter}$.

1.2.3.3. Mitochondrial respiration

Mitochondrial respiration was measured using a DW1/AD oxygen electrode unit (Hansatech). The Oxygraph Plus System™ was used to record and analyse results (for buffer components, please refer to Appendix A).

1.2.3.4. Oxygraph assembly and calibration

Two hours before the culling of the rat, the water bath connected to the water jacket surrounding the oxygen electrode was turned on and set at 37 °C. All components of the oxygraph were thoroughly rinsed and cleaned with distilled water (dH₂O), and the metal elements of the electrodes were polished. The electrode dome was then covered with an oxygen permeable polytetrafluoroethylene (PTFE) membrane and the oxygraph chamber was then assembled. Respiration buffer (1.5 ml) was then pipetted into the chamber, and allowed to equilibrate at 37 °C for an hour. During this hour, all the necessary buffers were prepared.

In order to calibrate the oxygraph, a series of on-screen prompts were followed, requiring maximum (100%) and minimum (0%) oxygen levels. Maximum oxygen levels were achieved by air-saturation (vigorously shaking a tube containing respiration buffer before addition to the chamber), and to achieve minimum levels, 0.01 g sodium dithionate was added directly to the chamber.

1.2.3.4.1. Isolation of left ventricular sub-sarcolemmal mitochondria

Using 5% isoflurane as a sedative, rats were culled by exsanguination, after which the hearts were quickly removed and transferred into a 10 ml beaker containing ice-cold Buffer A. Hearts were trimmed of atria, connective tissue and fat and then washed once with 5ml of Buffer A. Left and right ventricles were weighed, after which the right ventricles were placed into 10% formal saline for histological analysis. The left ventricles were transferred into a different 10 ml beaker on ice, and chopped thoroughly into small pieces with a dissection scissor. Five millilitres of Buffer B was used to rinse the chopped heart, where after the buffer was discarded. The chopped heart was placed into 15 ml of Buffer B and homogenized with a polytron homogenizer (Ultr-Turax, tp 18-10) for three seconds. Homogenized hearts were then centrifuged (Eppendorf, 5804) at 580 x g for 7 min. The supernatant was then poured through mesh into a new centrifugation tube. The remaining pellet was subsequently resuspended in 7.5 ml of buffer B and centrifuged as before. The supernatant was then added to the supernatant of the previous step, also through the mesh, and then centrifuged once more at 580 x g for 7min to rinse. The remaining supernatant was subsequently transferred into a new centrifuge tube for a final centrifugation step at 3000 x g for 7 min where it was then discarded and the pellet containing the sub-sarcolemmal mitochondria was resuspended in 0.3 ml of KME buffer.

All of the steps involved were done either on ice, or at 4 °C, this includes centrifugation steps. Additionally, the liver, gastrocnemius muscle, retroperitoneal fat pad (RFD), testicular fat pad (TFP) and tibia were harvested from each rat for further analysis.

1.2.3.4.2 Mitochondrial oxygen consumption analysis

In order to ensure that an equal quantity of mitochondrial protein was utilized during analysis, the Bradford protein quantification method was employed (Bradford, 1976). Two-hundred-and-fifty micrograms of mitochondrial protein was added to the oxygraph chamber containing respiration buffer during each experiment, with a final volume of 1.5 ml, after which the stopper was put into place. Different combinations of substrates and inhibitors were added to the chamber to elucidate the functioning of different ETC complexes: glutamate (20mM) and malate (10mM), malate (10mM) and pyruvate (20mM), malate (10mM) and carnitine palmitoyl (40mM), succinate (20mM) and rotenone (7.5mM). Between the additions of each substrate combination, the chamber was thoroughly rinsed with 70% alcohol and dH₂O, after which new mitochondria were added (250 µg).

Furthermore, after the addition of each substrate combination, 10mM ADP was added. At the point where the graph reached a plateau, an additional 100mM of ADP was added. Once the slope of the line graph remained constant, 5mM Oligomycin-A was added in order to significantly reduce electron flow through the ETC. Mitochondria from each rat was analysed for all of the above mentioned substrate combinations.

Remaining mitochondria were snap-frozen in liquid N₂, and stored at -80 °C for future analysis in a cryoprotectant freezing buffer for further.

1.3. Results

1.3.1 Weight gain analysis

In order to establish the effects PI treatment on body weight and net weight gain, all experimental groups were weighed every third day (for 2, 4 and 6 month groups, respectively). The data revealed that all groups gained weight equally over the 2 month treatment period; no significant differences were observed in total body weights or net weight gain (Fig. 3.1).

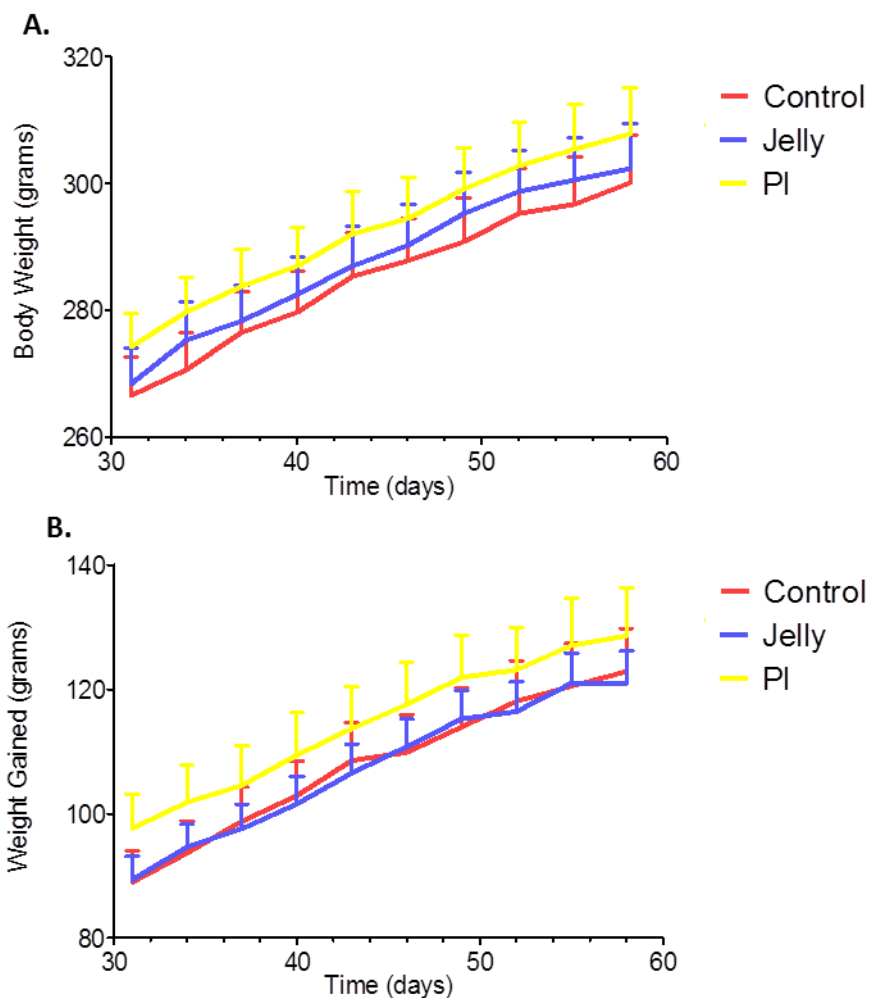


Figure 3.1. Body weight and net weight gain in male Wistar rats following 2 months of treatment. A) Total body weight during the second month of treatment, $n = 24$. **B)** Weight gained over the second month of treatment, as calculated from the starting weight, $n=24$. All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with a Bonferroni post-hoc test. PI – protease inhibitor.

After 4 months, PI-treated rats displayed decreased body weights compared to controls ($*p < 0.05$ vs. control; $@@@p < 0.001$ vs. Jelly, Fig 3.2.A). Similarly, net weight gain was decreased ($***p < 0.001$ vs. Control; $***p < 0.001$ vs. Jelly) compared to controls (Fig. 3.2.B).

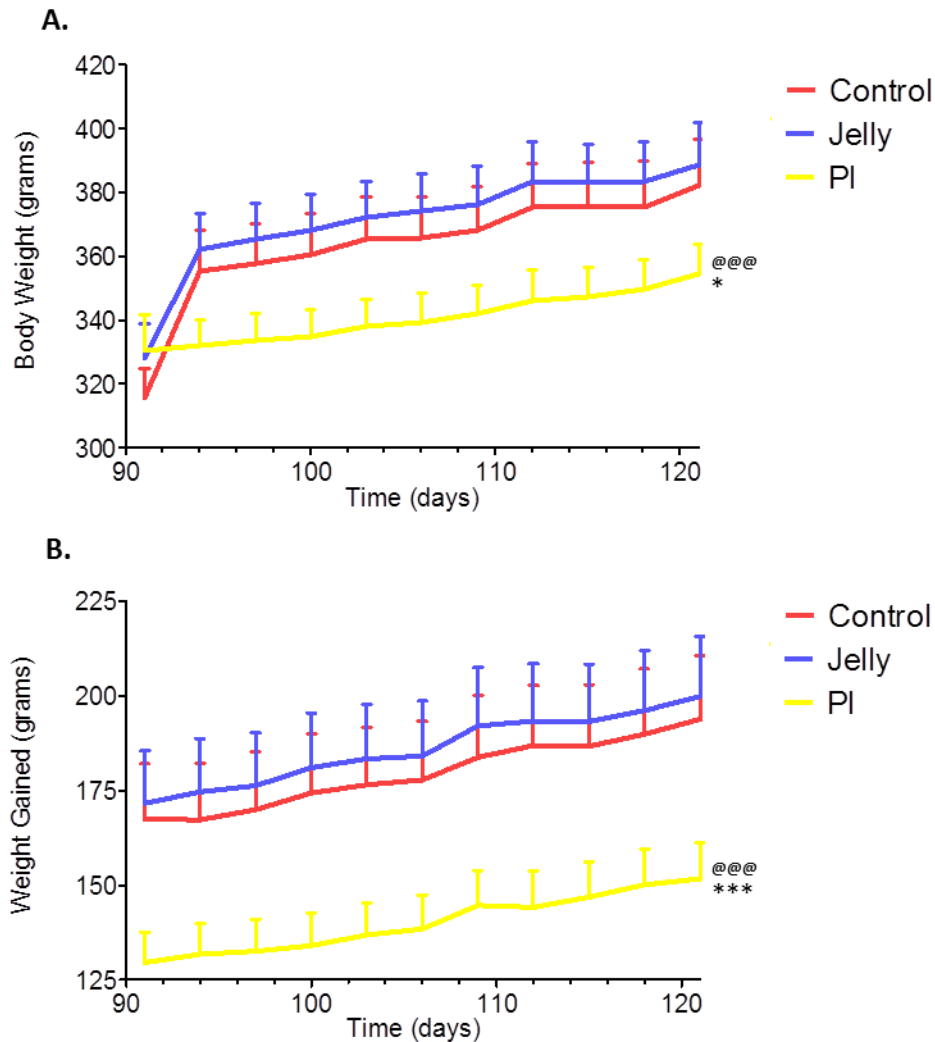


Figure 3.2. Body weight and net weight gain in male Wistar rats after 4 months of treatment. A) Total body weight over the fourth month of treatment: $*p < 0.05$ vs. control, $@@@p < 0.001$ vs. jelly, $n = 12$. **B)** Weight gained over the fourth month of treatment, as calculated from the starting weight: $***p < 0.001$ vs. control, $@@@p < 0.001$ vs. jelly $n=12$. All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with a Bonferroni post-hoc test. PI - protease inhibitor.

This pattern changed with prolonged treatment, i.e. 6 months PI treatment resulted in increased total body weight and net weight gain (** $p < 0.01$ vs. Jelly and *** $p < 0.001$ vs. Control) when compared to controls (Fig. 3.3).

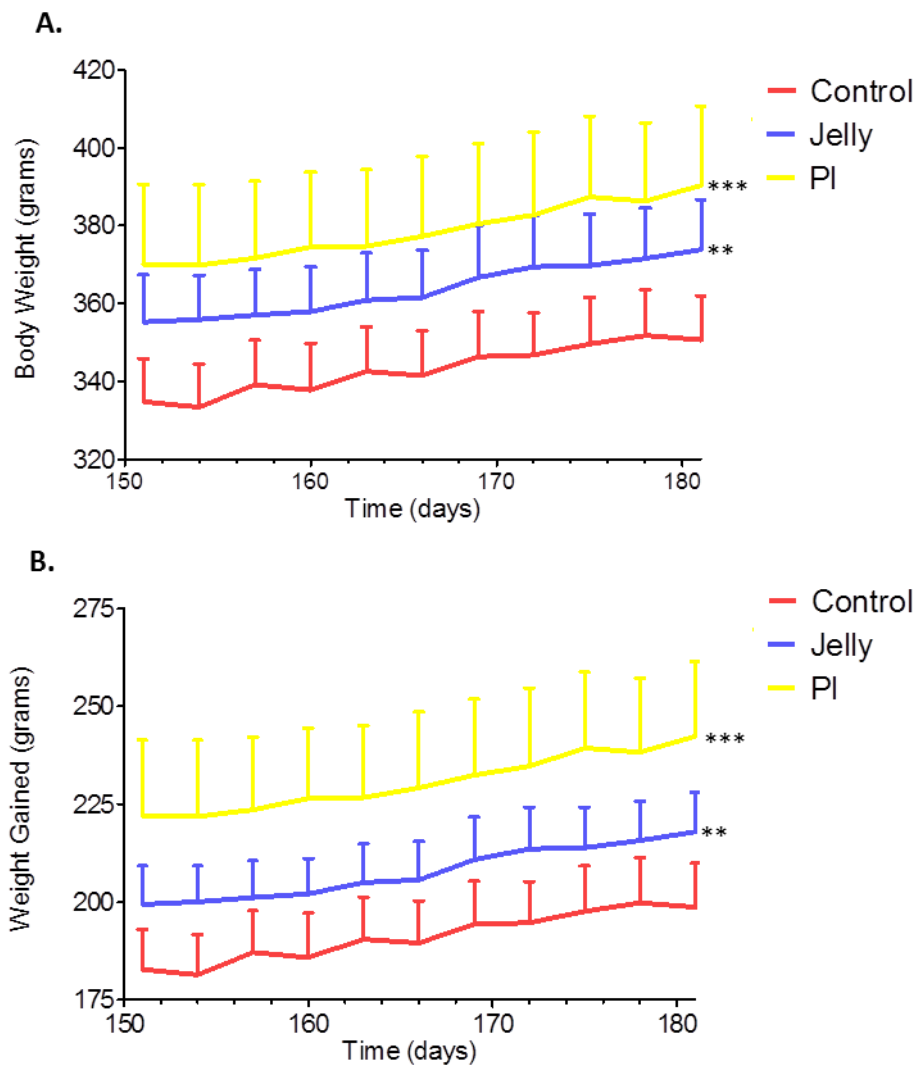


Figure 3.3. Body weight and net weight gain in male Wistar rats after 6 months of treatment. A) Total body weight over sixth month of treatment: *** $p < 0.01$ vs. control, ** $p < 0.01$ vs. control, $n = 6$. **B)** Weight gained over sixth month of treatment, as calculated from the starting weight, *** $p < 0.01$ vs. control, ** $p < 0.01$ vs. control, $n=6$. All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with a Bonferroni post-hoc test. PI - protease inhibitor.

1.3.2 Analysis of triglyceride, cholesterol, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) blood levels

In order to establish the effects of treatments on various markers that are commonly related to metabolic disturbances, blood samples were collected at the end of 2 and 4 months of treatment, respectively, for metabolite analysis. No changes were observed in any of the blood parameters after 2 months of PI treatment (Fig. 3.4).

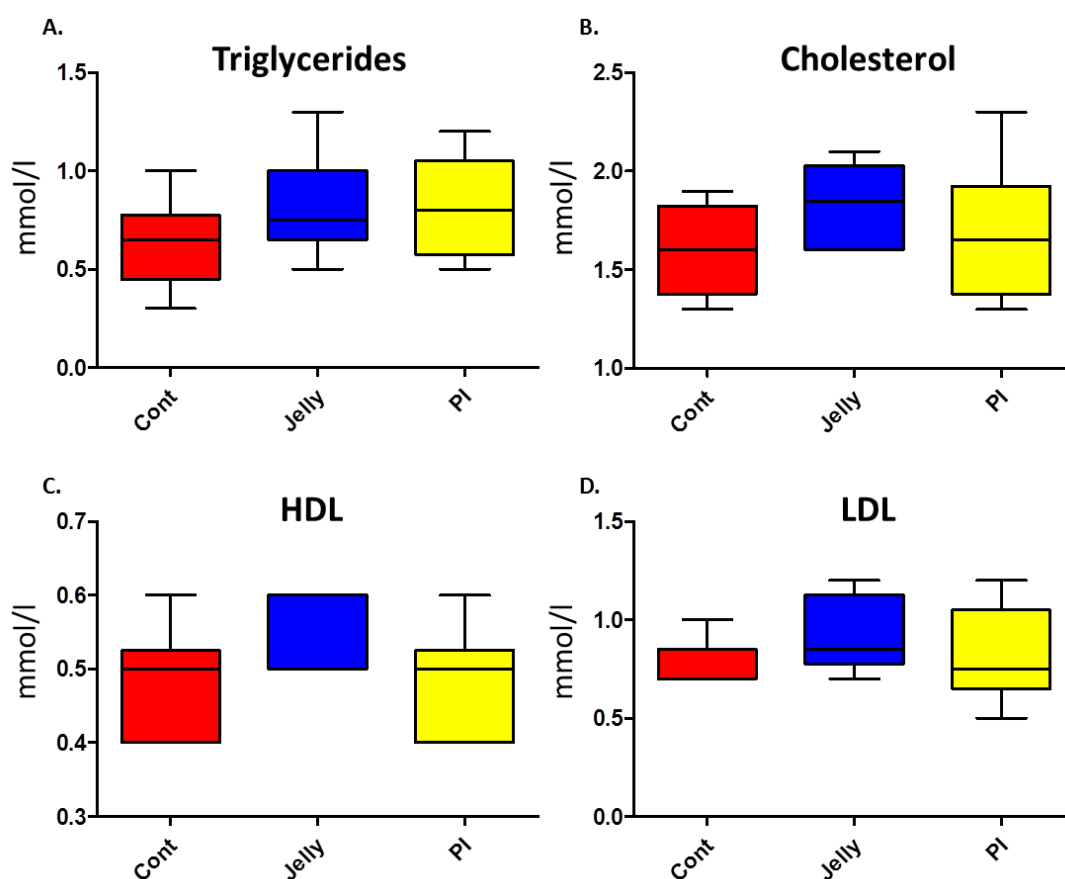


Figure 3.4. Triglyceride, cholesterol, LDL and HDL blood levels in male Wistar rats after 2 months of treatment. A) No changes were observed in triglyceride levels, n = 6. B) No changes were observed in cholesterol levels, n=6. C) No changes were observed in HDL levels, n=6. D) No changes were observed in the LDL levels, n=6. All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with Bonferroni post-hoc test. PI - protease inhibitor.

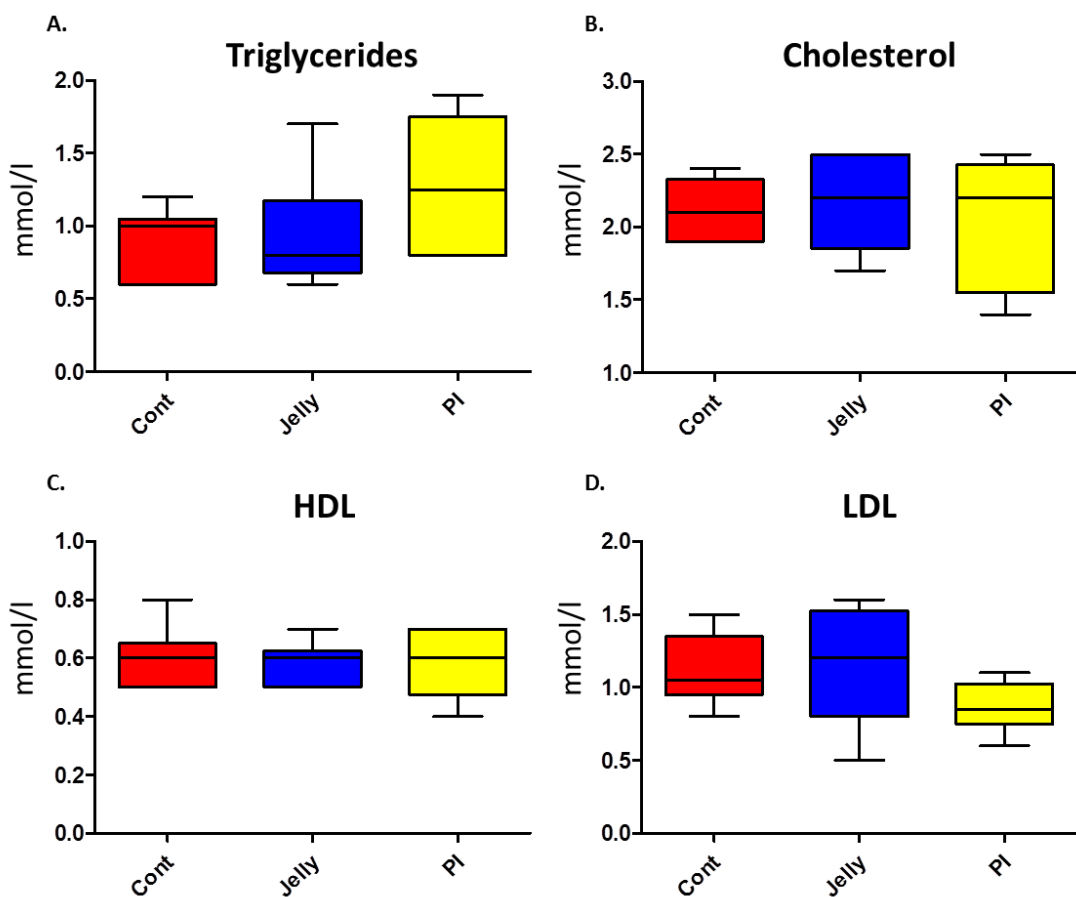


Figure 3.5. Triglyceride, cholesterol, LDL and HDL blood levels in male Wistar rats after 4 months of treatment. A) No changes were observed in triglyceride levels, $n=6$. **B)** No changes were observed in total cholesterol, $n=6$. **C)** No changes were observed in HDL, $n=6$. **D)** No changes were observed in LDL levels, $n=6$. All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with Bonferroni post-hoc test. PI - protease inhibitor.

1.3.3 Analysis of mid-wall fractional shortening (MWFS) with the use of echocardiography

In order to establish the effects of each treatment on systolic functioning, various functional parameters were assessed with echocardiography. These results were used to calculate mid-wall fractional shortening at the end of the 2 and 4 month treatment periods. We were unable to attain echocardiographical data at the 6 month time point due to logistical issues. No significant changes were observed in left ventricular functional parameters after 2 and 4 months of PI treatment, respectively (Table 3.1).

	Control	Jelly	PI
LVEDD	6.0 ± 0.13	6.0 ± 0.09	6.6 ± 0.08
LVESD	2.9 ± 0.14	3.3 ± 0.14	3.6 ± 0.11
LVESPWT	1.9 ± 0.05	1.8 ± 0.05	1.9 ± 0.08
LVEDPWT	2.6 ± 0.07	2.4 ± 0.09	2.6 ± 0.11
R-R Interval	0.16 ± 0.004	0.17 ± 0.005	0.16 ± 0.005

Table 3.1.A. Left ventricular parameters as measured with echocardiography after 2 months of PI treatment

LVEDD – Left Ventricular End Diastolic Diameter; LVESD – Left Ventricular End Systolic Diameter; LVESPWT – Left Ventricular End Systolic Posterior Wall Thickness; LVEDPWT – Left Ventricular End Diastolic Posterior Wall Thickness

	Control	Jelly	PI
LVEDD	6.4 ± 0.13	6.6 ± 0.16	6.4 ± 0.16
LVESD	3.6 ± 0.12	3.5 ± 0.15	3.8 ± 0.14
LVESPWT	2.1 ± 0.07	2.0 ± 0.09	1.9 ± 0.04
LVEDPWT	2.6 ± 0.1	2.6 ± 0.09	2.4 ± 0.09
R-R Interval	0.17 ± 0.004	0.18 ± 0.005	0.18 ± 0.003

Table 3.1.B. Left ventricular parameters as measured with echocardiography after 4 months of PI treatment

LVEDD – Left Ventricular End Diastolic Diameter; LVESD – Left Ventricular End Systolic Diameter; LVESPWT – Left Ventricular End Systolic Posterior Wall Thickness; LVEDPWT – Left Ventricular End Diastolic Posterior Wall Thickness

No significant changes were observed in MWFS in all groups after 2 and 4 months of treatments (Fig. 3.6).

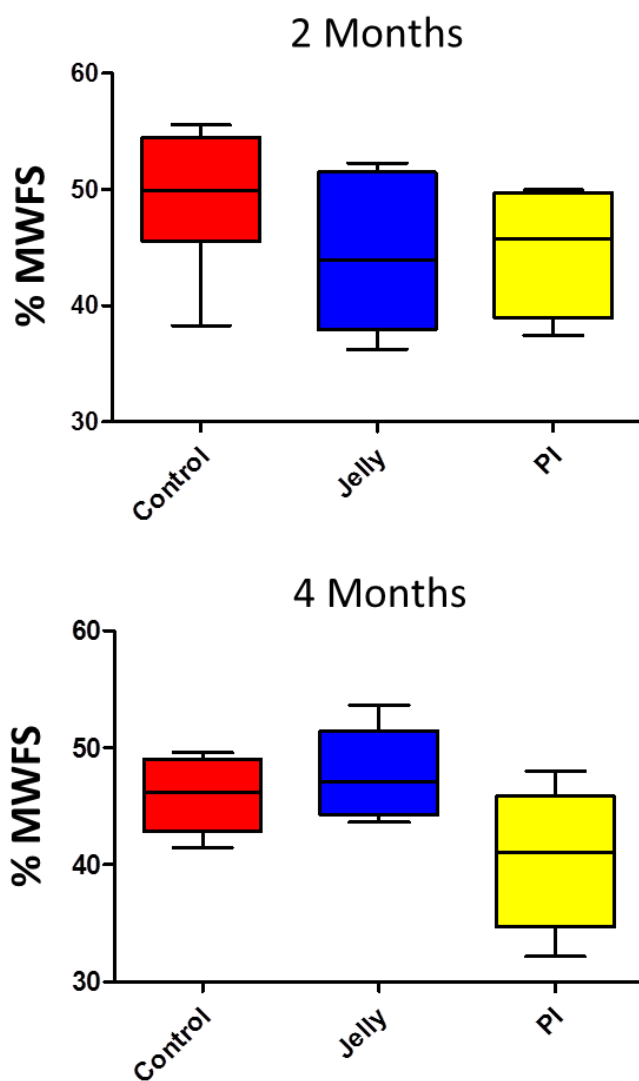


Figure 3.6. MWFS in male Wistar rats after 2 and 4 months of treatment. No significant changes were observed, n=6. All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with Bonferroni post-hoc test. PI - protease inhibitor.

1.3.4 Organ harvesting for the determination of treatment-induced anatomical changes

In order to determine whether the treatments induced any gross anatomical changes, body weight was determined together with various organ weights - normalized to tibial length. No significant changes were observed after 2 or 4 months of PI treatment (Fig. 3.7.A & B). After 6 months, PI-treated rats displayed increased heart weight ($*p < 0.05$ vs. control, $@p < 0.05$ vs. Jelly, Fig. 3.7.C).

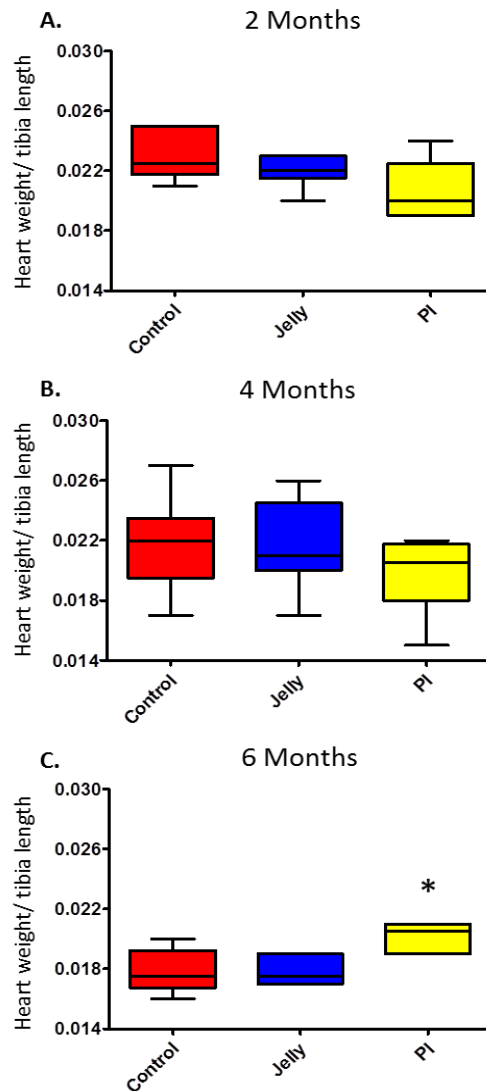


Figure 3.7. Heart weights normalized to right tibia length in male Wistar rats on the day of culling after 2, 4 and 6 months of treatment. 2 months: No significant changes were observed, $n=12$. **4 months:** No significant changes were observed, $n=12$. **6 months:** $*p < 0.05$ vs. control, $n=12$. All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with Bonferroni post-hoc test. PI - protease inhibitor.

Left ventricular weight increased in both Jelly and PI treated groups versus controls after 4 months of treatment ($*p < 0.05$ vs. control, Fig. 3.8A). In parallel, right ventricular weight decreased in both Jelly and PI treated groups after 4 month of treatment ($*p < 0.05$ vs. control, Fig. 3.8.C). No changes were observed after 6 months of PI treatment (Fig 3.8.C & D).

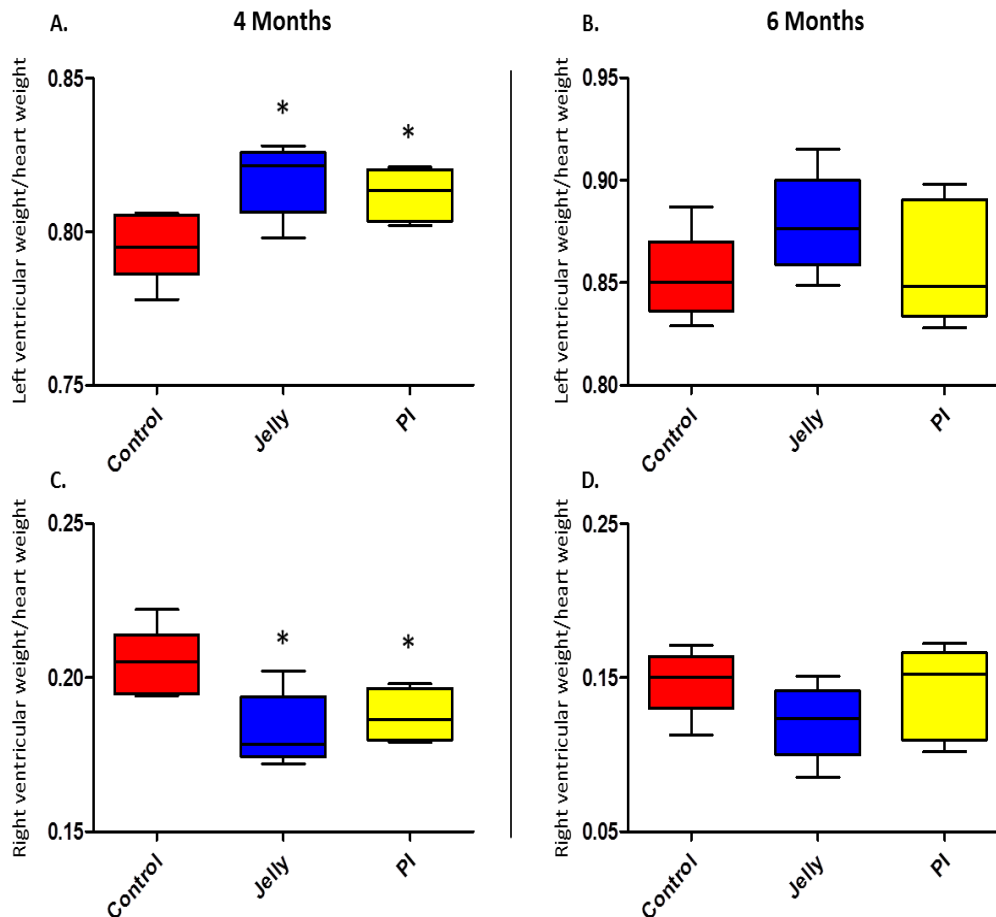


Figure 3.8. Left and right ventricular weights normalized to total heart weight in male Wistar rats on the day of culling after 4 and 6 months of treatment, respectively. **A) 4 months:** $*p < 0.05$ vs. control, $n=12$. **B) 6 months:** No significant differences were observed, $n=12$. All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with Bonferroni post-hoc test. PI - protease inhibitor.

Liver weight normalized to right tibia length remained unchanged for all groups after 2, 4 and 6 months of treatment (Fig. 3.9).

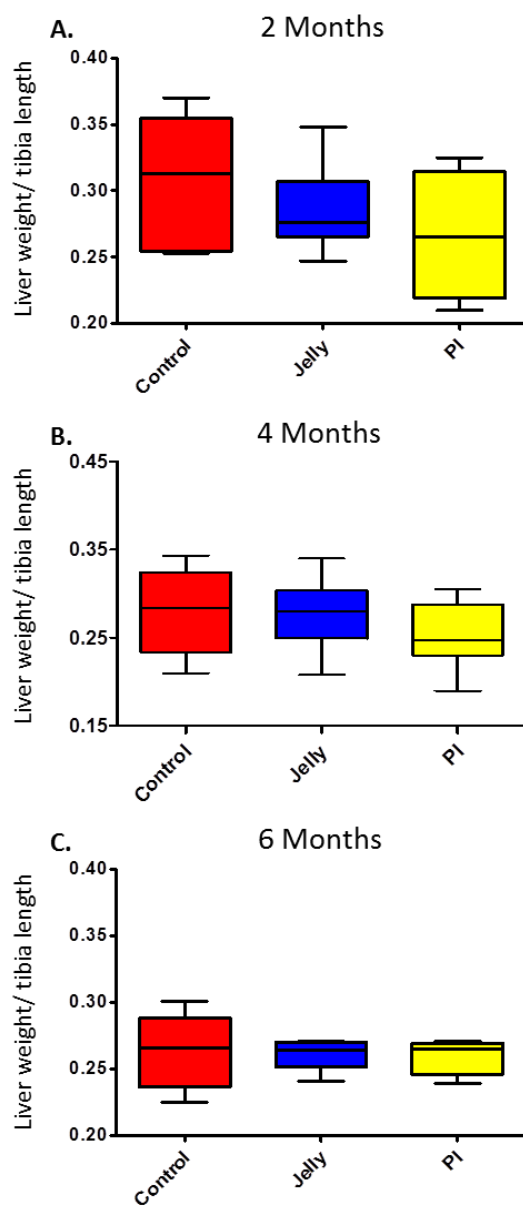


Figure 3.9. Liver weights normalized to right tibia length in male Wistar rats on the day of culling after 2, 4 and 6 months of treatment. 2 months: No significant changes were observed, $n=12$. **4 months:** No significant changes were observed, $n=12$. **6 months:** No significant changes were observed, $n=12$. All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with Bonferroni post-hoc test. PI - protease inhibitor.

For fat mass evaluations, no significant changes were observed after 4 months of PI treatment (Fig. 3.10.A). However, rats that were fed jelly cubes displayed increased retroperitoneal fat mass compared to controls after 6 months ($*p < 0.05$ vs. Control, Fig. 3.10.B).

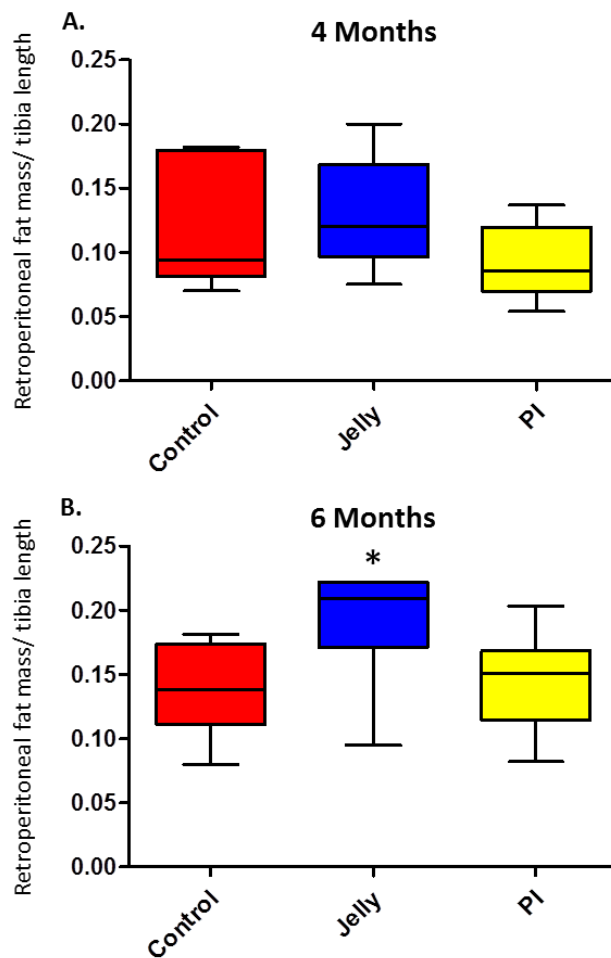


Figure 3.10. Left and right ventricular weights normalized to heart weight in male Wistar rats on the day of culling after 4 and 6 months of treatment. **A) 4 months:** No significant changes were observed, $n=12$. **B) 6 months:** $*p < 0.05$ vs. control, $n=12$. All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with Bonferroni post-hoc test. PI - protease inhibitor.

1.3.5 Analysis of mitochondrial respiration

1.3.5.1 State III respiration of isolated sub-sarcolemmal mitochondria after 4 months of PI treatment.

Mitochondrial oxygen consumption was assessed using oxygen electrodes (oxygraph) in sub-sarcolemmal mitochondria isolated from left ventricles after 4 and 6 months of PI treatment, respectively. After the 4 month treatment period, State III respiration was impaired in isolated mitochondria of PI-treated rats with glutamate and malate as substrates ($p < 0.05$ vs. control, Table 3.2.A). Although State III respiration was diminished with PI treatment, the ADP:O ratio was increased ($p < 0.05$ vs. control, Table 3.2.A).

	CONTROL	JELLY	PI
Glutamate + Malate State III	200 ± 12	248 ± 75	150 ± 13*
Glutamate + Malate State IV	56 ± 12	97 ± 26	64 ± 5
Glutamate + Malate RCR	2.53 ± 0.28	2.51 ± 0.21	2.37 ± 0.22
Glutamate + Malate ADP:O	2.0 ± 0.1	2.2 ± 0.4	2.7 ± 0.3*
Malate + Pyruvate State III	151 ± 19	164 ± 19	166 ± 22
Malate + Pyruvate State IV	67 ± 10	55 ± 4	63 ± 8
Malate + Pyruvate State RCR	2.4 ± 0.32	2.95 ± 0.2	2.87 ± 0.4
Malate + Pyruvate State ADP:O	2.78 ± 0.31	2.61 ± 0.30	2.58 ± .029
Palmitoyl – Carnitine + Malate State III	178 ± 31	303 ± 105	172 ± 17
Palmitoyl – Carnitine + Malate State IV	90 ± 4	93 ± 23	83 ± 9
Palmitoyl – Carnitine + Malate RCR	1.96 ± 0.3	3.14 ± 0.51	2.2 ± 0.34
Palmitoyl – Carnitine + Malate ADP:O	2.50 ± 0.39	1.95 ± 0.44	2.43 ± 0.21
Rotenone + Succinate State III	285 ± 53	287 ± 74	313 ± 71
Rotenone + Succinate State IV	146.7 ± 43	126 ± 26	193 ± 65
Rotenone + Succinate RCR	2.41 ± .59	2.29 ± 0.27	2.22 ± 0.78
Rotenone + Succinate ADP:O	1.78 ± 0.53	1.73 ± 0.28	1.62 ± 0.32

Table 3.2. Function of isolated left ventricular sub-sarcolemmal mitochondria after 4 months of PI treatment.

All results expressed as mean ± SEM, statistical analysis: one-way ANOVA with Bonferroni post-hoc test: * $p < 0.05$ vs. control, $n=6$. PI - protease inhibitor.

1.3.5.2 State III respiration of isolated sub-sarcolemmal mitochondria after 6 months of PI treatment.

No changes were observed in mitochondrial respiration following 6 months of PI treatment (Table 3.3).

	CONTROL	JELLY	PI
Glutamate + Malate State III	176 ± 10	167 ± 13	161 ± 16
Glutamate + Malate State IV	45 ± 5	48 ± 7	43 ± 4
Glutamate + Malate RCR	4.16 ± 0.46	3.7 ± 0.37	3.8 ± 0.28
Glutamate + Malate ADP:O	2.32 ± 0.13	2.47 ± 0.18	2.61 ± 0.26
Malate + Pyruvate State III	129 ± 24	157 ± 16	193 ± 28
Malate + Pyruvate State IV	38 ± 2	40 ± 6	49 ± 7
Malate + Pyruvate State RCR	3.97 ± 0.31	4.14 ± 0.28	3.99 ± 0.13
Malate + Pyruvate State ADP:O	2.73 ± 0.19	2.66 ± 0.21	2.26 ± 0.28
Palmityl – Carnitine + Malate State III	150 ± 15	156 ± 8	183 ± 8
Palmityl – Carnitine + Malate State IV	42 ± 6	44 ± 5	51 ± 1
Palmityl – Carnitine + Malate RCR	3.87 ± 0.52	3.71 ± 0.39	3.61 ± 0.25
Palmityl – Carnitine + Malate ADP:O	2.78 ± 0.24	2.6 ± .14	2.21 ± 0.1
Rotenone + Succinate State III	209 ± 47	285 ± 10	201 ± 65
Rotenone + Succinate State IV	51 ± 16	74 ± 4	51 ± 17
Rotenone + Succinate RCR	5.03 ± 1.1	3.93 ± 0.28	4.0 ± 0.33
Rotenone + Succinate ADP:O	1.69 ± 0.23	1.41 ± 0.05	1.33 ± 0.04

Table 3.3. Function of isolated left ventricular sub-sarcolemmal mitochondria after 6 months of PI treatment.

All results expressed as mean ± SEM, statistical analysis: one-way ANOVA with Bonferroni post-hoc test. PI - protease inhibitor, n=6.

1.4. Discussion

HIV is a major disease burden in South Africa and globally as well. It dramatically decreased quality of life (QOL) (Whoqol Hiv Group, 2004; Mannheimer et al, 2005) and is the leading cause of death in South Africa (Bradshaw et al, 2012). The introduction of HAART has not only slowed disease progression but also significantly increased QOL (Mannheimer et al, 2005). However, HAART can elicit side-effects that can threaten the well-being of treated patients and these include renal failure, hepatic steatosis, diabetes and CVD (Friis-Møller, 2010; Gupta et al, 2013).

The HIV protease inhibitors have especially been implicated in the risk of developing cardiovascular complications (Friis-Møller et al, 2007; Friis-Møller, 2010). For example, Friis-Møller and colleagues also observed a correlation between duration of protease inhibitor treatment and increased risk of cardiovascular events (Friis-Møller et al, 2007). There are various theories regarding the underlying mechanisms whereby protease inhibitors can trigger such side-effects, although compelling evidence is still lacking (Zha et al, 2011; Pérez-Matute et al, 2013). For example, it has been postulated that dyslipidemia may be the cause of cardiovascular complications in such individuals (Mallewa et al, 2008) as increased visceral and central fat have been implicated with risk of developing type-2 diabetes and CVD (Lafontan, 2013). In this theory, PI treatment leads to development of insulin resistance and type-2 diabetes, with CVD occurring only as a secondary treatment-induced effect. This concept is strengthened in the literature since insulin resistance and type-2 diabetes is a common occurrence in patients on HAART, especially regimens containing PIs (Rudich et al, 2005; Noor et al, 2006; Taylor et al, 2010; Capeau et al, 2012).

Others propose that the drug exerts its effects on the cardiovascular system due to mitochondrial dysfunction, ROS production, systemic inflammation and cellular apoptosis, leading to direct effects in terms of the onset of cardiovascular complications (Pérez-Matute et al, 2013; Reyskens and Essop, 2013). The schools of thought thus differ where one theory postulates that PI-treatment causes CVD directly with diabetes as an additional detrimental side-effect, while the other theory states that CVD are secondary due to the development of diabetes caused by the PI treatment. These theories thus cause a chicken-or-egg question; does one come before the other? In light of this, the current study was initiated to shed further light on this intriguing question.

The recorded bodyweights followed a surprising trend over the 6 months of treatment. Here after 2 months of PI treatment neither body weights nor weight gain differed between groups (Fig 3.1), while at 4 months however the PI-treated groups displayed the lowest body weight/weight gain (Fig. 3.2). However, after 6 months the PI-treated group exhibited the highest recorded body weights

and weight gains (Fig. 3.3). It is difficult to explain the initial trends in weight gain but we believe it follows the same pattern as clinical studies done by Nell and Essop (unpublished data) on HIV-positive patients receiving HAART. Such patients generally showed a period of stable body weight and body composition followed by wasting, i.e. a decline in body cell mass, lean body mass, free fat mass and peripheral adipose tissue were observed. As treatment continued, the pattern changed with body weight increasing along with muscle mass and body cell mass. This was accompanied by significant increases in visceral/central adipose tissue and waist-to-hip ratios, as confirmed in other studies (Mallewa et al, 2008; Pérez-Matute et al, 2013). However, the patients received combination ART of which most were on first-line therapy and not receiving PIs. Lee and colleagues (2004) found that HIV-negative men receiving clinical doses of PI monotherapy (ritonavir/lopinavir) twice daily for four weeks presented with metabolic disturbances and altered blood profile but no changes in body weight, body fat, or abdominal adipose tissue (Lee et al, 2004).

It thus begs the question; how does PI monotherapy in HIV-negative rats (as in our study), present with similar trends as that of clinical studies of HIV positive humans receiving HAART but differ from results from PI monotherapy in HIV-negative persons?

Firstly, the duration of the PI monotherapy administered by Lee and colleagues (2004) was only for a four-week period and this is a relatively short time (clinically speaking), considering that PI-mediated side-effects increase in severity with time (Lee *et al*, 2004; Friis-Møller, 2010). If treatment duration is not the reason for such discrepancies then another possible explanation may be that PI treatment of rats may resemble the effects caused by the combination of ART and HIV-infection in humans. There is no scientific support for this explanation; however, we postulate that this could occur due to side-effects occurring more rapidly in rats possibly due to faster metabolism. For instance, if rat mitochondria produce ROS at a much higher rate due to the drug effects then this should lead to systemic inflammation similar to that of an advanced-stage HIV-positive ARV-treated individual. In support of this idea, Gupta and colleagues (2012) found the manifestation of lipodystrophy, insulin resistance, and hyperlipidemia in mice after four weeks of treatment with ritonavir/lopinavir (Gupta et al, 2012). This study provides more evidence for the idea that four weeks of PI monotherapy is sufficient to induce clinically relevant alterations in body composition and blood profiles in small animal models. Such differences once again highlight the difficulty in translation from animal models to clinical studies, and vice versa.

To explain possible mechanism behind this pattern of weight gain observed in our study we propose changes in adipose tissue distribution to be the most likely cause. Lipodystrophy in HAART-treated HIV-positive individuals presents clinically as decreased peripheral adipose tissue and increases in

visceral/central adipose tissue (Freitas et al, 2011; Sacre et al, 2011). Lipodystrophy affects 50% or more of ARV treated individuals (Bacchetti et al, 2005) and drug-induced, site-specific lipoatrophy likely increases in severity as treatment duration increases (Freitas et al, 2011). Of note, although PI treatment is considered a primary causative factor in ARV-induced lipodystrophy there are studies that show that NRTIs are also implicated (Pérez-Matute et al, 2013). The alteration of fat distribution can also occur with a decrease in body weight and BMI and in certain cases this is attributed to only lipoatrophy occurring (Pérez-Matute et al, 2013). Moreover, certain ARV-treated individuals present with lipodystrophy but in the form of decreased general fat mass (both peripheral and visceral lipoatrophy) thus lowering body weight significantly. Others present with site-specific lipoatrophy (peripheral fat) and lipohypertrophy in other areas (visceral fat) with the net result meaning it has little or no impact on BMI (Pérez-Matute et al, 2013). In these cases of lipoatrophy in combination with lipohypertrophy BMI is not a reliable tool to assess/predict patient health and anthropometrical analysis would thus be a better option in this instance.

With this background in mind, we are of the opinion that lipodystrophy may be a possible and reasonable cause for the weight patterns here observed. However, we were unable to weigh fat pads/sites after 2 months of treatment as we postulated that this was not sufficient to induce significant changes. For 4 and 6 month treatment groups we could unfortunately not confirm the presence or absence of lipodystrophy since analysis of all fat sites was logistically not feasible at the time of sacrifice. This was due to the need to isolate mitochondria as rapidly as possible for respiration studies. However, we managed to harvest and weigh the retroperitoneal fat pads at both time points. Although data was not significant, there was a trend in the 4 month group that showed PI treatment alone decreased retroperitoneal fat pad mass in comparison to the other groups. This correlates with results from Gupta and colleagues (2012) who observed significantly lower retroperitoneal fat pad mass in mice treated with ritonavir/lopinavir (Gupta et al, 2012). Although we were not able to measure all fat sites we believe that this trend could have mimicked clinical findings by Nell and Essop (unpublished data). Thus the proposal is that 4 months of PI treatment may be responsible for the decreased general fat mass thereby matching the wasting period of HAART-treated patients. After 6 months the opposite was observed and PI treatment was associated with significantly increased body weights and weight gains. However, retroperitoneal fat mass weights at 6 months followed the same trend as after 4 months thus making it difficult to explain this finding. It is also possible that other fat pads/sites could have undergone lipohypertrophy and/or ectopic fat deposition to cause increases in body weight and weight gain and further studies would be required to test this interesting notion.

Alternatively, increased mitochondrial ROS production may also be a possible mechanism of PI-induced lipodystrophy as such drug treatment can lead to mitochondrial dysfunction (direct or indirect) which in turn causes increases in mitochondrial ROS production (Reyskens and Essop, 2013). This increase in ROS production could lead to serious cellular damage through oxidation of DNA, lipids and proteins, possibly even causing cell death through apoptosis (Pérez-Matute et al, 2013). Pérez-Matute and colleagues (2013) postulated that this could be a possible cause of lipodystrophy induced by PI treatment since peripheral adipose tissue and visceral adipose tissue possess differing mitochondrial numbers. It is proposed that peripheral fat (containing higher mitochondrial number) produce more ROS than visceral fat leading to more oxidative stress, inflammation, and apoptosis – can lead to decreasing peripheral fat mass (Zha et al, 2011). In support, others found decreased mitochondrial DNA in subcutaneous fat of HIV patients with ARV-induced peripheral lipodystrophy compared to HIV-positive controls (Shikuma et al, 2001). Decreased mitochondrial DNA may occur due to the effects of ROS on mitochondria or due to direct effects of PI treatment on mitochondria. Thus it is possible that ROS lead to damage and subsequent cell death of especially peripheral adipose tissue cells. However, further studies would be required to confirm whether this is the case in our experimental model.

Food intake is another important variable to take into account when investigating body weight and weight gain. We measured food intake daily but no differences were observed between the groups during any of the treatment periods (data not shown). The jelly control group showed significant increases in body weight and weight gain (Fig. 3.2 & 3.3). Since there were no significant changes in food intake this possibility can be ruled out. We therefore propose that this could be due to a slightly increased daily energy intake due to the caloric content of the jelly and/or due to increases in muscle mass because of the additional protein content of the gelatine. However, the results for body weights and weight gains for the other groups are likely to be due to PI treatment since the caloric content of jelly is very low per volume.

HIV infection and ART has a strong link to CVD which may (in part) be due to the pro-atherogenic lipid profile it encompasses. It is a common occurrence for patients on HAART (especially PI regimens) to develop a pro-atherogenic lipid profile (Stein et al, 2001; Badiou et al, 2003). This typically entails decreased HDL together with increased LDL and triglycerides levels (Stein et al, 2001; Badiou et al, 2003). However, for our study we found no changes in blood profiles of rats treated with PIs for 4 and 6 months, respectively (Figs. 3.4. & 3.5). This was surprising as it would be expected to accompany the increases in body weights here observed. There are several reasons why such changes may not have occurred in our model: a) the HIV infection itself has plays a significant role in the development of a pro-atherogenic blood profile, b) the PI steady-state

concentration was not high enough to elicit an altered blood profile. As the rats did not present with as pro-atherogenic blood profile we exclude it as an early mediator in direct PI-induced effects on the cardiovascular system in our setting.

Since the start of the HAART era, there has been an increased incidence of echocardiographic abnormalities and cardiovascular complications observed among HIV patients (Mondy et al, 2011). For example, Meng and colleagues (2002) found that PI-containing HAART regimens are associated with left ventricular hypertrophy and diastolic dysfunction (Meng et al, 2002). Moreover, HIV-positive children receiving HAART exhibited high incidences (18%) of mild ventricular dysfunction together with progressive increases in left ventricular weight (Fisher et al, 2005). The exact mechanisms of such PI-induced structural and functional abnormalities are elusive, but hypertension, vascular inflammation, endothelial dysfunction and cardiotoxicity have been proposed as potential mediators (Mondy et al, 2011). For the current study there were no changes in the functional parameters of the left ventricles (Table 3.1. A & B) nor did mid-wall fractional shortening show any differences between groups (Fig. 3.6). It remains unclear why no changes occurred in our model but we propose that a) the PI dosages may have been too low to cause echocardiographic outcomes in rats; b) it may be due to the lack of HIV infection in our experimental system; c) the drug on its own is unable to elicit this response and that a combination with HIV is required; and d) 4 month treatment duration may not have been sufficient to induce alterations and that the 6 month treatment may have been a better choice to perform the analysis, especially as heart weight was significantly increased. We were unable to perform these studies due to lack of sufficient animal numbers and resources at the time.

Since PI treatment can lead to anatomical disturbances in heart and adipose tissues, we also assessed organ weights that were normalized to tibia lengths since body weights fluctuate with aging in Wistar rats (Yin et al, 1982). Here PI treatment significantly increased heart weights after 6 months of treatment and this could be due to the initiation of a hypertrophic response (Fig. 3.7.C). Further studies are required to confirm this and whether this occurs due to pressure overload as a result of increased body size/obesity.

At 4 months we found a significant decrease in State III respiration with PI treatment together with a concomitant increase in the ADP:O ratio (Table 3.2). However, after 6 months no differences were found with PI treatment versus controls (Table 3.3). Thus these data suggest that at after 4 months there is an inhibition of complex I as malate/glutamate provide substrates for the citric acid cycle to generate NADH for NADH dehydrogenase (complex I). However, despite this PI-mediated damaging effect the ADP:O ratio was higher suggesting that there are some compensatory mechanisms that

are activated to elevate mitochondrial ATP generation. Further studies are required to investigate such possibilities. We are unclear why this effect was not observed after 6 months and speculate that it may have been due to the PI dosage not being high enough and/or physiological adaptation to long-term PIs availability. This theory of adaptation is strengthened by findings from Miro and colleagues (2004) who found up regulation of mitochondrial genes after ART thereby compensating for mitochondrial damage (Miro et al, 2004).

It is visible from these results that PI treatment has undesirable side-effects. Although the trends we observed are difficult to interpret and explain, they seem to implicate mitochondria as the mediator in our setting. Even though we could not confirm, we believe that adipose tissue is very much involved in the processes. It is clear that more research is required to explain the mechanisms behind these PI-induced side-effects. To further elucidate the possible mechanisms behind the PI-induced side-effects we thought it fitting to employ interventions with specific functions to test our theories behind PI-induced side-effects. These interventions will be the topic of discussion in the chapters to follow.

Chapter 2

2.1. Natural interventions: in search of hope

CVD related to HIV/AIDS generally occur late during disease progression (Friis-Møller *et al*, 2007; Friis-Møller, 2010). The life expectancy of HIV-positive individuals has increased dramatically since the introduction of ARVs. This has increased cardiovascular complications in this population group (Friis-Møller, 2010), although it remains unclear whether this is due to the virus and/or ARV treatment.

HIV infection is a known risk factor for CVD onset and this can occur by direct effects of viral proteins on the heart and vasculature (Cummings and Badley, 2010; Coopusamy, 2010; Johri *et al*, 2011). Thus the HIV-mediated proatherogenic state further increases CVD risk (Ho and Hsue, 2009; Porter and Sutliff, 2012). However, with initiation of ARV treatment HIV progression is halted and viral load lowered (Sierra *et al*, 2005). Despite such progress, ARV treatment is linked with cardiovascular complications (Friis-Møller *et al*, 2007; Friis-Møller, 2010). Here protease inhibitors (PI) and nucleoside reverse transcriptase inhibitors (NRTI) have been documented to cause metabolic disturbances (Friis-Møller *et al*, 2007; Friis-Møller, 2010), with PIs posing the greatest threat with prolonged treatment (Friis-Møller *et al*, 2007; Friis-Møller, 2010).

Although the mechanisms of ARV-induced cardiovascular side-effects are not entirely clear, mitochondrial dysfunction has been implicated in this process (Phenix *et al*, 2001; White, 2001). In fact, both HIV itself and ARVs are thought to directly affect these 'powerplants' of the cell, thus wreaking havoc with intracellular energy production and function (White, 2001). Mitochondrial energy production has to be kept in perfect balance since ROS generation is a by-product of this process (White, 2001; Hwang *et al*, 2008). For example, excess ROS are damaging to cell-membranes, proteins and DNA structures (Sivitz and Yorek, 2010). If left unchecked ROS can lead to inflammation, cell-death (apoptosis), mutations and dysfunctional proteins - all manifesting in HIV-positive patients receiving ARV treatments (Sivitz and Yorek, 2010; Porter and Sutliff, 2012).

The most important regulatory factors of mitochondrial energy production are AMPK, Sirt-1 and PGC-1 α (Sivitz and Yorek, 2010) that when activated can increase intracellular energy availability (Cantó *et al*, 2009; Ruderman *et al*, 2013). It has been clinically observed that AMPK levels are decreased in HIV-positive patients in the absence of ARV treatment (Mankouri and Harris, 2011). This could potentially disrupt the functioning of the downstream regulatory factors with adverse effects on intracellular metabolism (Cho and Lumeng, 2011). For example, attenuated intracellular

metabolism was implicated in cardiovascular complications onset found during latter stages of HIV infection (Porter and Sutliff, 2012).

ARVs have been implicated in the disruption of mitochondrial energy production, e.g. it can decrease mitochondrial DNA and disrupt mitochondrial function (Friis-Møller, 2010). In addition, Capeau and colleagues (2012) found that patients receiving HAART (containing PIs) displayed significantly higher than normal incidences of type-2 diabetes (2012), a known cause of mitochondrial dysfunction (Capeau *et al*, 2012). In support, Kelley *et al*. (2002) reported that mitochondrial numbers were significantly decreased and displayed altered morphology in individuals that were obese or diagnosed with type-2 diabetes (Kelley *et al*, 2002).

Compounding this bleak picture, lipodystrophy and weight gain are two common side-effects of PI treatment (Noor, 2007; Gupta *et al*, 2012) and both are known risk factors for developing type-2 diabetes and CVD (Noor, 2007; Capeau *et al*, 2012). Thus a rhetorical question emerges, i.e. are PIs directly responsible for causing mitochondrial dysfunction and CVD, or can it cause development of lipodystrophy and type-2 diabetes that eventually results in CVD onset? If the mitochondrial-based theory is valid this makes it a feasible therapeutic target to limit PI-mediated damaging effects on overall health and well-being. This chapter therefore focused on evaluating natural therapeutic compounds that may target the mitochondrion and could then be fairly rapidly translated into the clinical setting.

We focused on Resveratrol (RSV) that is enriched in the skin of red grapes (Edwards *et al*, 2011) and been identified as an activator of SIRT-1 activation (Edwards *et al*, 2011; Kitada *et al*, 2011). For example, RSV-mediated SIRT-1 activation triggered a protective effect against weight gain and metabolic syndrome in rats and mice fed a "Western" diet (high fat content) (Baur *et al*, 2006; Price *et al*, 2012). Although such promising effects were confirmed in a number of small animal models, its therapeutic impact in remains unclear (Poulsen *et al*, 2012). In light of this, we proposed RSV supplementation together with PI treatment may ameliorate the damaging side-effects usually elicited by PIs.

2.2. Materials and methods

RSV was purchased in the form of 500 mg capsules (Terraternal, USA). The dosage employed was 200 mg per kilogram body weight and all analyses/experiment performed were done as described in Chapter 1. Please refer to Chapter 1.2 for a full description of materials and methods.

2.3. Results

2.3.1 Weight gain analysis

In order to establish the effects of treatments on body weight and net weight gain, all experimental groups were weighed every third day (for the 2, 4 or 6 month experimental periods). The data revealed that all groups gained weight equally over the 2 month treatment period; no significant differences were observed (Fig. 4.1.).

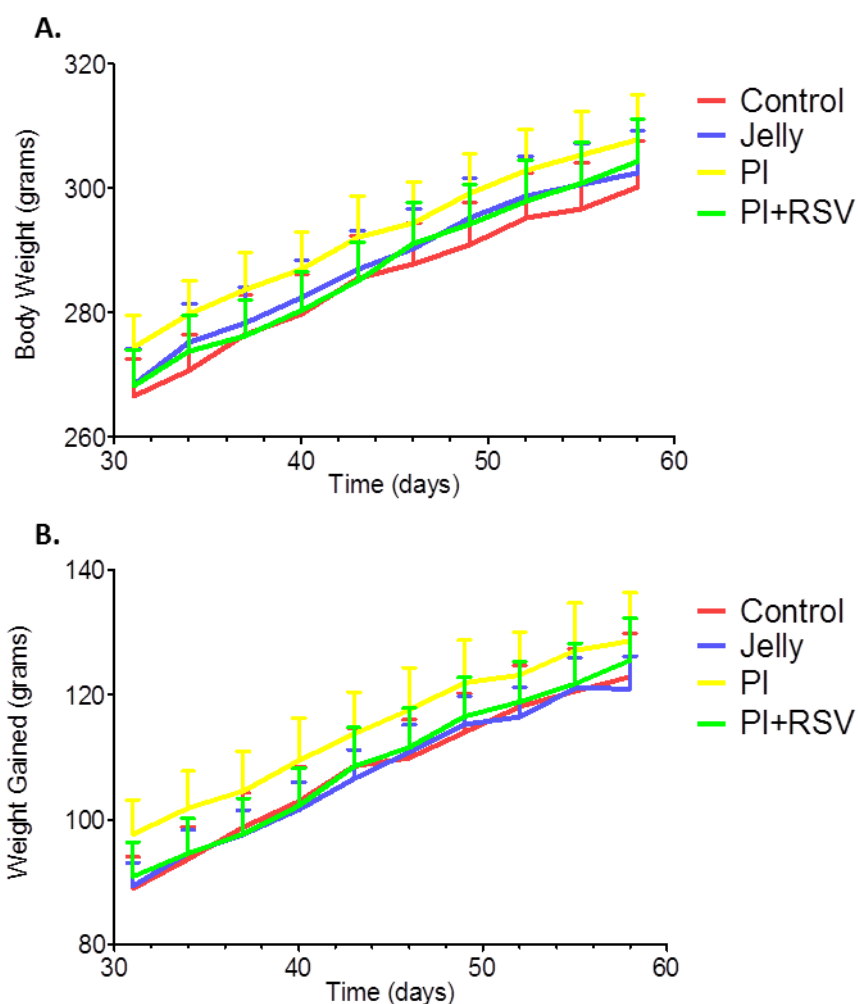


Figure 4.1. Body weight and net weight gain in male Wistar rats following 2 months of treatment. **A)** Total body weight during the second month of treatment, $n = 24$. **B)** Weight gained over the second month of treatment, as calculated from the starting weight, $n=24$. All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with a Bonferroni post-hoc test. PI – protease inhibitor, RSV - Resveratrol.

After 4 months, PI-treated rats displayed decreased body weights ($p < 0.05$ vs. control; $p < 0.001$ vs. Jelly, Fig. 4.2.A). In agreement, weight gain in the PI-treated group was also decreased compared to controls ($p < 0.001$ vs. Control; $p < 0.001$ vs. Jelly, Fig. 4.2.B). Rats receiving co-treatment of PI and RSV displayed increased weight gain when compared to PI-treated rats (Fig. 4.2.B).

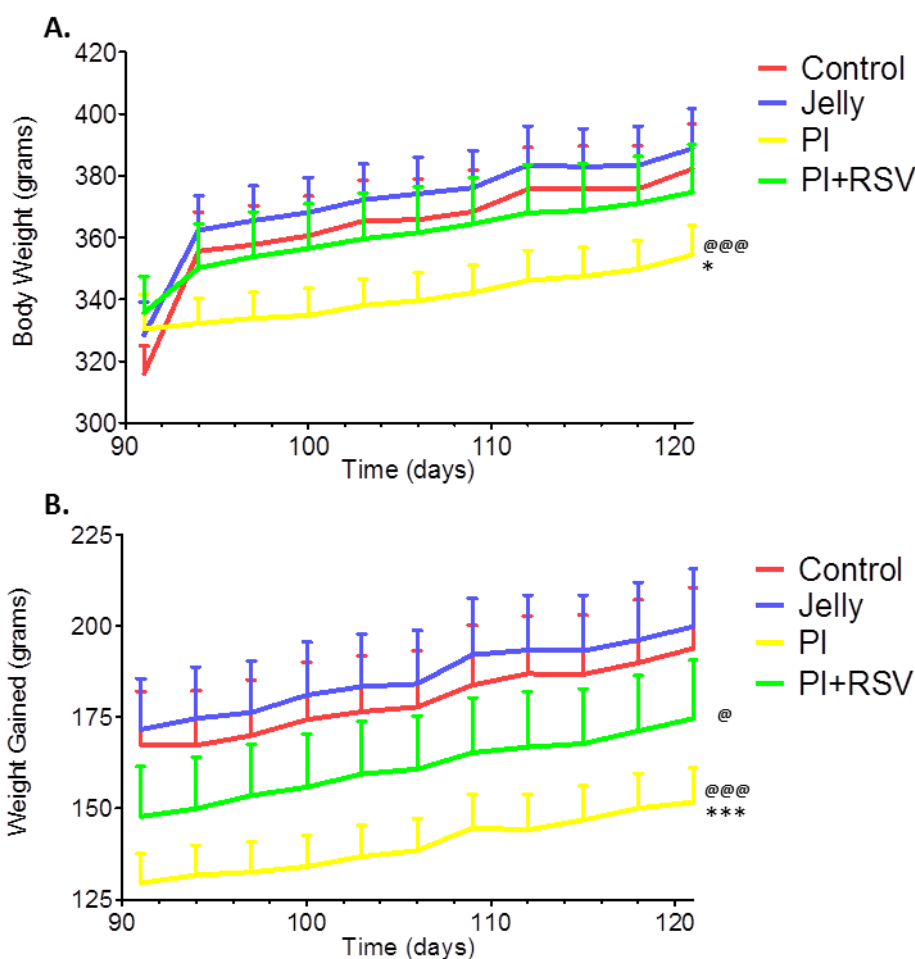


Figure 4.2. Body weight and net weight gain in male Wistar rats after 4 months of treatment. A) Total body weight over the fourth month of treatment: * $p < 0.05$ vs. control; @@@ < 0.001 vs. jelly, $n = 12$. **B)** Weight gained over the fourth month of treatment, as calculated from the starting weight: *** $p < 0.001$ vs. control, @@@ $p < 0.001$ vs. jelly, @ $p < 0.05$ vs. PI+RSV, $n=12$. All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with a Bonferroni post-hoc test. PI - protease inhibitor, RSV - Resveratrol.

This pattern changed with prolonged treatment, i.e. 6 months PI treatment resulted in increased body weight and weight gain (** $p < 0.001$) when compared to control rats (Fig. 4.3.A and B). Rats receiving only jelly also displayed increased body weights and weight gain (** $p < 0.01$) when compared to the control group (Fig. 4.3.A and B). The same trend was observed in the body weight of the rats receiving PI in combination with RSV ($^{ss}p < 0.01$) when compared to control rats. Of note, PI treatment in combination with RSV did not display the increased body weight found with PI-

treatment (Fig. 4.3. A). A similar result was found for weight gain evaluations ($^{$$$}p < 0.001$, Fig. 4.3. B).

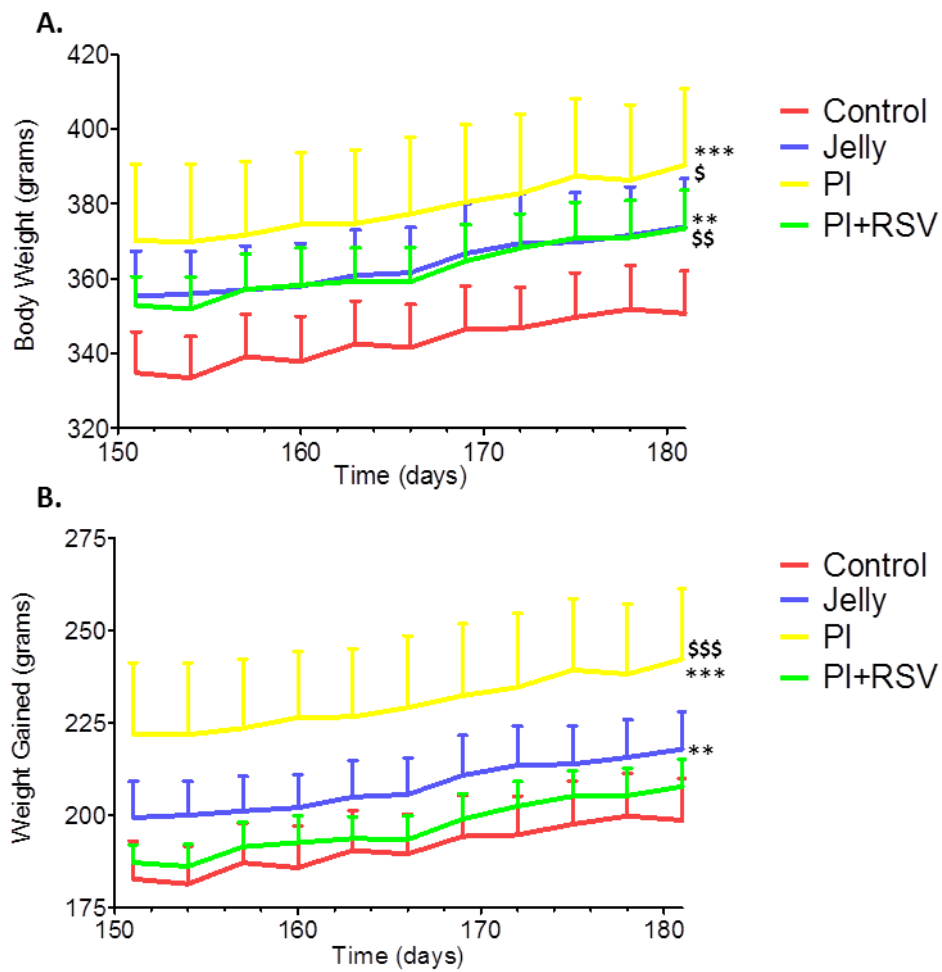


Figure 4.3. Body weight and net weight gain in male Wistar rats after 6 months of treatment. A) Total body weight over sixth month of treatment: $^{***}p < 0.001$ vs. control, $^{**}p < 0.01$ vs. control, $^{$$}p < 0.01$ vs. control, $^{}$}p < 0.05$ vs. PI+RSV, $n = 6$. **B)** Weight gained over 6 months of treatment, as calculated from the starting weigh: $^{***}p < 0.001$ vs. control, $^{$$$}p < 0.001$ vs. PI+RSV, $^{**}p < 0.01$ vs. control, $n=6$. All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with a Bonferroni post-hoc test. PI - protease inhibitor, RSV - Resveratrol.

2.3.2 Analysis of triglyceride, cholesterol, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) blood levels

In order to establish the effects of each treatment on various biochemical markers which are commonly related to metabolic disturbances, blood samples were collected at the end of 2 and 4 months of treatment for metabolite analysis. No changes were observed in any of the blood parameters after 2 and 4 months of treatment (Figs. 4.4 and 4.5).

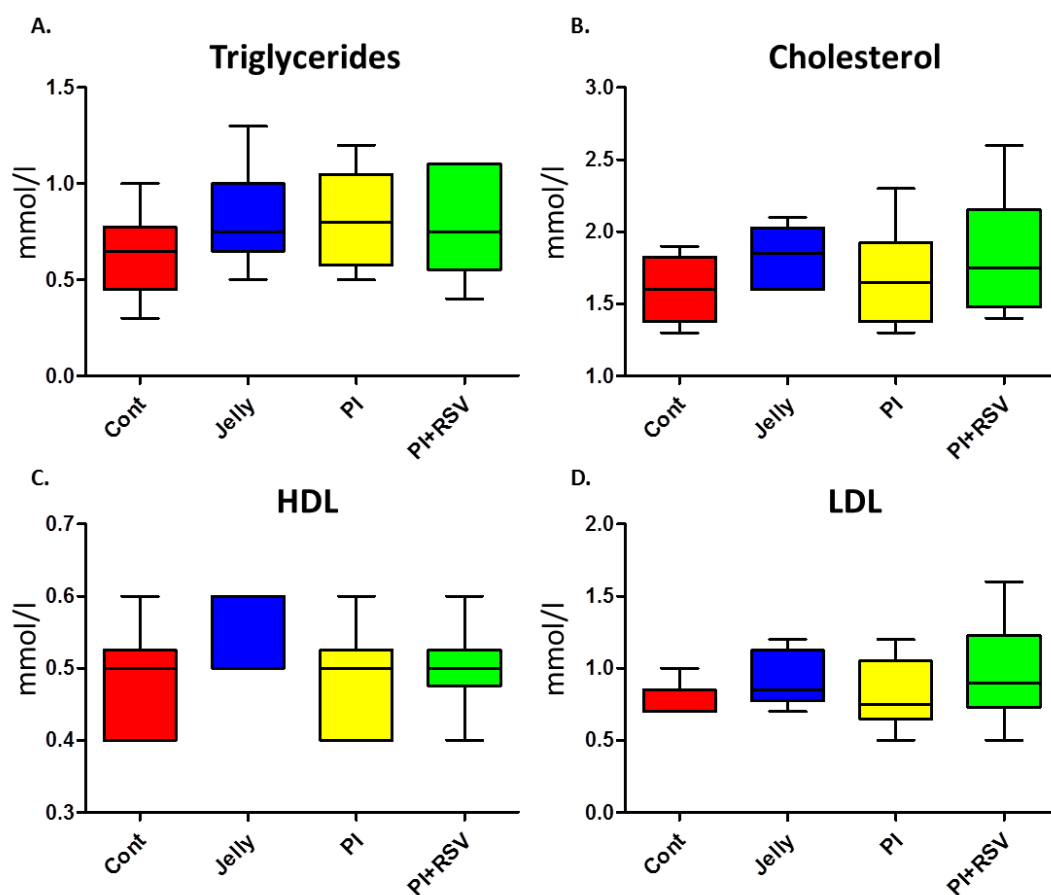


Figure 4.4. Triglyceride, cholesterol, LDL and HDL blood levels in male Wistar rats after 2 months of treatment. **A)** No changes were observed in triglyceride levels, $n = 6$. **B)** No changes were observed in total cholesterol levels, $n=6$. **C)** No changes were observed in HDL levels, $n=6$. **D)** No changes were observed in LDL levels, $n=6$. All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with Bonferroni post-hoc test. PI - protease inhibitor, RSV – resveratrol.

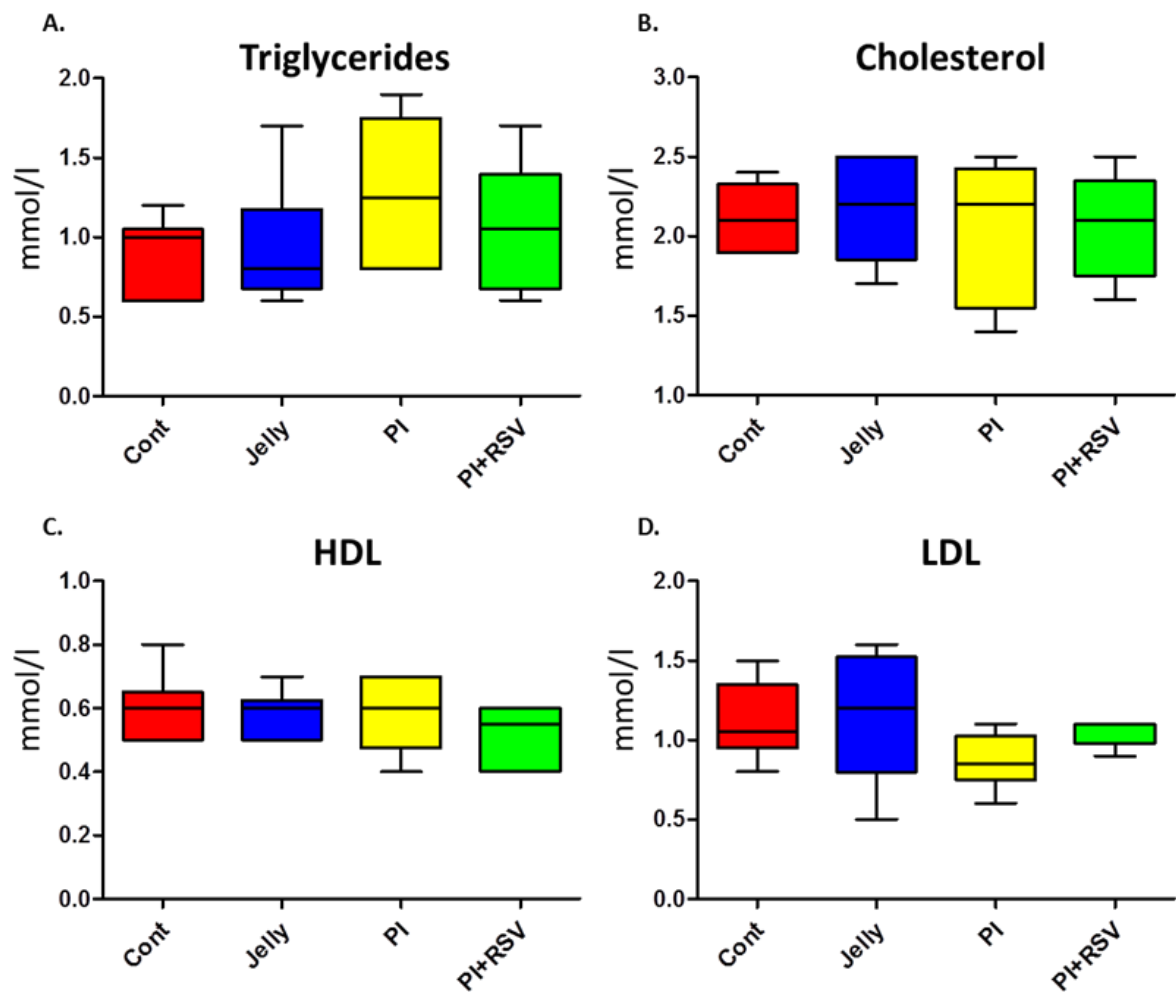


Figure 4.5. Triglyceride, cholesterol, LDL and HDL blood levels in male Wistar rats after 4 months of treatment. **A)** No changes were observed in triglyceride levels, $n = 6$. **B)** No changes were observed in total cholesterol, $n=6$. **C)** No changes were observed in HDL levels, $n=6$. **D)** No changes were observed in LDL levels, $n=6$. All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with Bonferroni post-hoc test. PI - protease inhibitor, RSV – Resveratrol.

2.3.3 Analysis of mid-wall fractional shortening (MWFS) with the use of echocardiography

In order to establish the effects of each treatment on systolic functioning, various functional parameters were assessed with echocardiography. These results were used to calculate mid-wall fractional shortening at the end of the 2 and 4 month treatment periods. We were not able to attain echocardiographical data at the 6 month time point due to logistical issues.

No significant changes were observed in left ventricular functional parameters after 2 and 4 months of treatment, respectively (Tables 4.1.A&B).

	Control	Jelly	PI	PI+RSV
LVEDD	6.0 ± 0.13	6.0 ± 0.09	6.6 ± 0.08	6.3 ± 0.11
LVESD	2.9 ± 0.14	3.3 ± 0.14	3.6 ± 0.11	3.6 ± 0.14
LVESPWT	1.9 ± 0.05	1.8 ± 0.05	1.9 ± 0.08	1.9 ± 0.08
LVEDPWT	2.6 ± 0.07	2.4 ± 0.09	2.6 ± 0.11	2.4 ± 0.11
R-R Interval	0.16 ± 0.004	0.17 ± 0.005	0.16 ± 0.005	0.17 ± 0.004

Table 4.1.A. Left ventricular parameters as measured with echocardiography after 2 months of treatment

LVEDD – Left Ventricular End Diastolic Diameter; LVESD – Left Ventricular End Systolic Diameter; LVESPWT – Left Ventricular End Systolic Posterior Wall Thickness; LVEDPWT – Left Ventricular End Diastolic Posterior Wall Thickness

	Control	Jelly	PI	PI+RSV
LVEDD	6.4 ± 0.13	6.6 ± 0.16	6.4 ± 0.16	6.8 ± 0.14
LVESD	3.6 ± 0.12	3.5 ± 0.15	3.8 ± 0.14	3.8 ± 0.14
LVESPWT	2.1 ± 0.07	2.0 ± 0.09	1.9 ± 0.04	1.9 ± 0.05
LVEDPWT	2.6 ± 0.1	2.6 ± 0.09	2.4 ± 0.09	2.6 ± 0.08
R-R Interval	0.17 ± 0.004	0.18 ± 0.005	0.18 ± 0.003	0.18 ± 0.004

Table 4.1.B. Left ventricular parameters as measured with echocardiography after 4 months of treatment

LVEDD – Left Ventricular End Diastolic Diameter; LVESD – Left Ventricular End Systolic Diameter; LVESPWT – Left Ventricular End Systolic Posterior Wall Thickness; LVEDPWT – Left Ventricular End Diastolic Posterior Wall Thickness

Furthermore, no significant changes were observed in MWFS in all groups after 2 and 4 months of treatment (Figs 4.6).

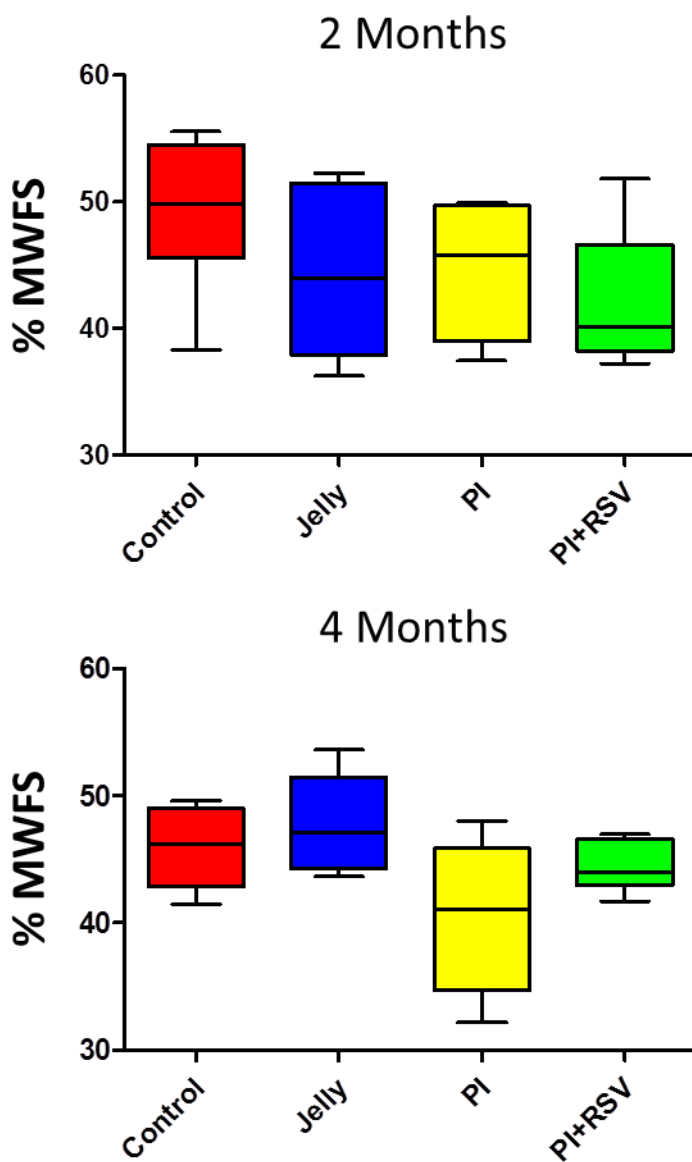


Figure 4.6. MWFS in male Wistar rats after 2 and 4 months of treatment. No significant changes were observed, n=6. All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with Bonferroni post-hoc test. PI - protease inhibitor, RSV – Resveratrol.

2.3.4 Organ harvesting for the determination of treatment-induced anatomical changes

In order to determine whether the treatments induced any gross anatomical changes, body weight was determined together with various harvested organs - normalized to tibial length.

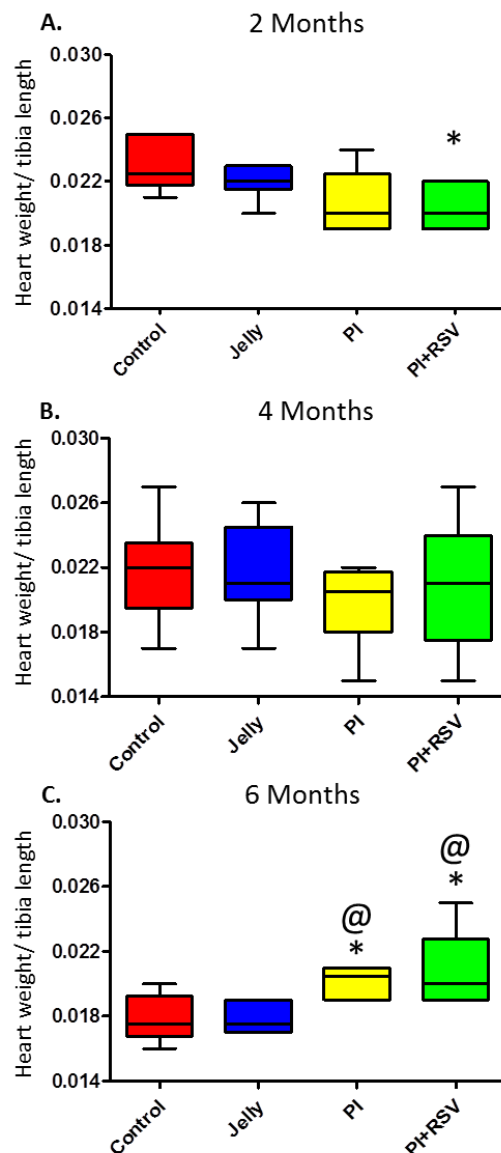


Figure 4.8. Heart weights normalized to right tibia length in male Wistar rats on the day of culling after 2, 4 and 6 months of treatment. 2 months: * $p < 0.05$ vs. control, $n=12$. **4 months,** $n=12$. **6 months:** * $p < 0.05$ vs. control, @ $p < 0.05$ vs. jelly, $n=12$. All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with Bonferroni post-hoc test. PI - protease inhibitor, RSV – Resveratrol.

After 2 months, rats treated with PI in combination with RSV displayed decreased heart weight ($p < 0.05$ vs. controls; Fig. 4.8.A). No significant changes were observed after 4 months of PI treatment (Fig. 4.8.B). After 6 months, PI-treated rats and rats co-treated with PI and RSV displayed increased heart weights ($p < 0.05$ vs. controls and Jelly; Fig. 4.8.C). Left ventricular weight is increased in all groups in comparison to the control after 4 months of treatment ($p < 0.05$ vs. control). Conversely, right ventricular weight decreased for all groups after 4 months of treatment versus controls ($p < 0.05$ vs. control, Fig. 4.10.A). At the 6 month time point RSV co-treatment resulted in a decrease in left ventricular weight ($p < 0.05$) and corresponding increase in right ventricular weight ($p < 0.05$) versus the jelly control (Fig. 4.10.B).

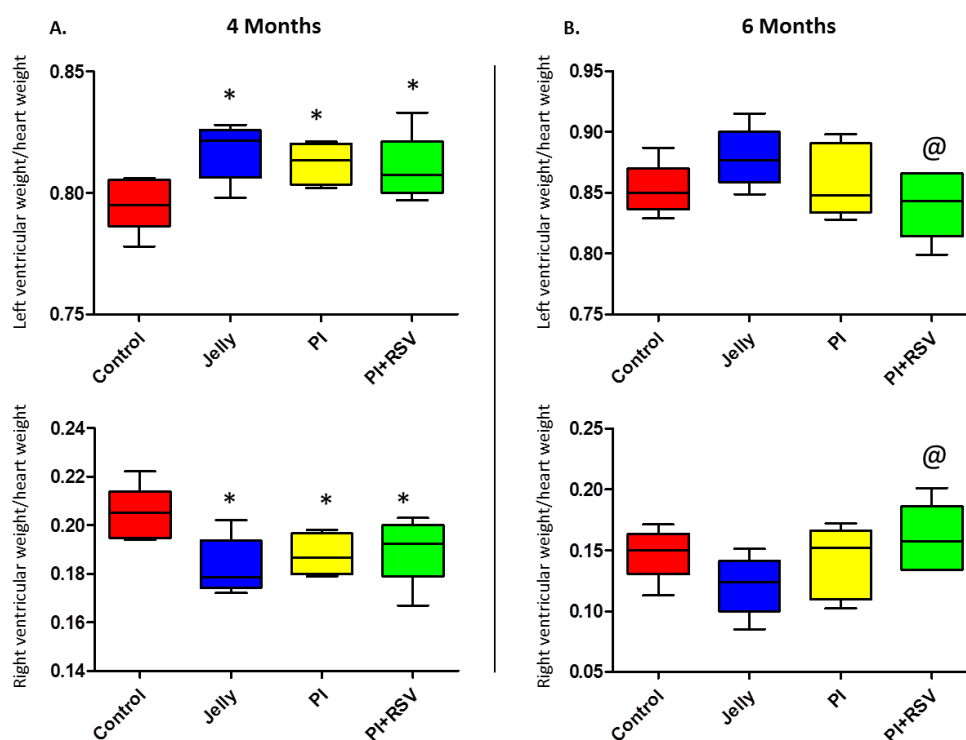


Figure 4.10. Left and right ventricular weights normalized to total heart weight in male Wistar rats on the day of culling after 4 and 6 months of treatment, respectively. A) 4 months: * $p < 0.05$ vs. control, $n=12$. B) 6 months: @ $p < 0.05$ vs. jelly, $n=12$. All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with Bonferroni post-hoc test. PI - protease inhibitor, RSV - Resveratrol.

Liver weight normalized to right tibia length remained unchanged for all groups after 2, 4 and 6 months of treatments (Fig. 4.9).

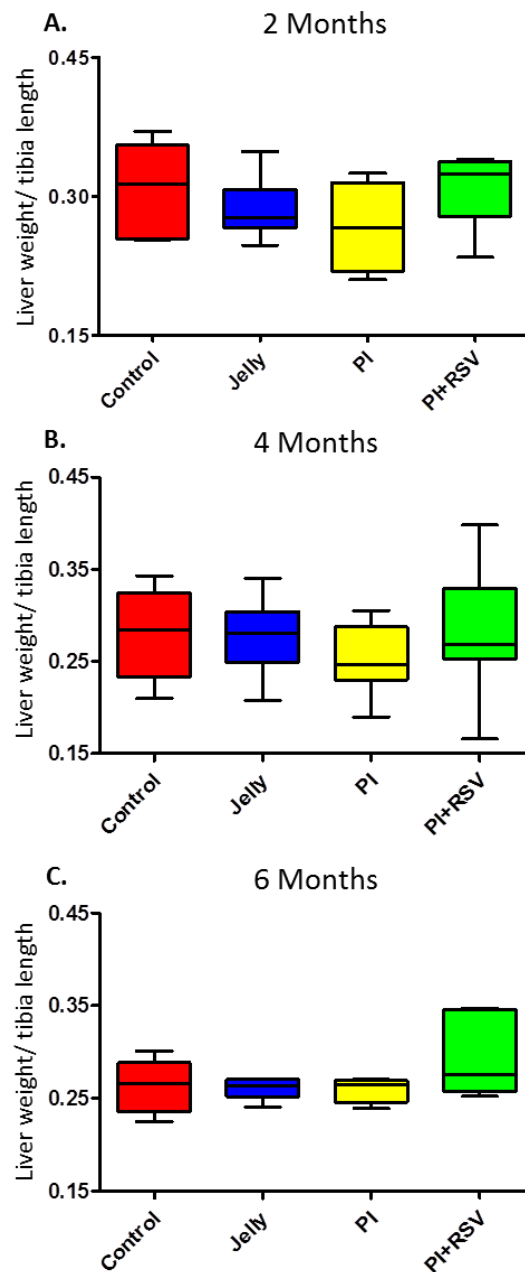


Figure 4.9. Liver weights normalized to right tibia length in male Wistar rats on the day of culling after 2, 4 and 6 months of treatment. **2 months:** No significant changes were observed, $n=12$. **4 months:** No significant changes were observed, $n=12$. **6 months:** No significant changes were observed, $n=12$. All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with Bonferroni post-hoc test PI - protease inhibitor, RSV - Resveratrol.

After 4 months, retroperitoneal fat mass increased ($p < 0.05$) in rats co-treated with PI and RSV versus rats PI treated only (Fig. 4.11. 4 months). After 6 months, rats that received only jelly displayed increased retroperitoneal fat mass compared to control rats ($p < 0.05$, Fig. 3.11. 6 months).

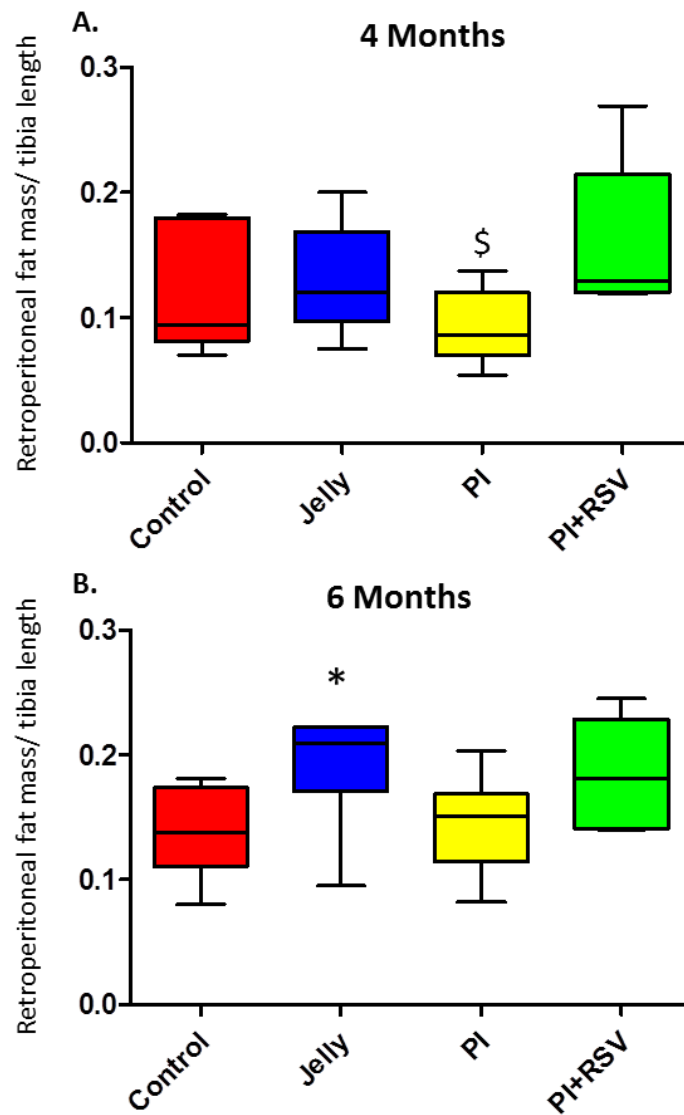


Figure 4.11. Left and right ventricular weights normalized to heart weight in male Wistar rats on the day of culling after 4 and 6 months of treatment. A) 4 months: $^{\$}p < 0.05$ vs. PI+RSV, $n=12$. B) 6 months: $^*p < 0.05$ vs. control, $n=12$. All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with Bonferroni post-hoc test. PI - protease inhibitor, RSV – Resveratrol.

2.3.5 Analysis of mitochondrial respiration

Mitochondrial oxygen consumption was assessed using oxygen electrodes (oxygraph) in sub-sarcolemmal mitochondria after 4 and 6 months of PI treatment, respectively. For the 4 month PI treatment period, State III respiration was impaired ($p < 0.05$ vs. control) in isolated mitochondria of PI-treated rats (glutamate and malate) (Table 4.2.A). Although State III respiration was diminished with PI treatment, the ADP:O ratio was increased indicating that it was respiring with greater efficiency. The same impairment was observed in rats receiving the PI in combination with RSV ($p < 0.05$ vs. control, Table 4.2.A)

	CONTROL	JELLY	PI	PI+RSV
Glutamate + Malate State III	200 ± 12	248 ± 75	150 ± 13*	154 ± 10*
Glutamate + Malate State IV	56 ± 12	97 ± 26	64 ± 5	65 ± 6
Glutamate + Malate RCR	2.53 ± 0.28	2.51 ± 0.21	2.37 ± 0.22	2.42 ± 0.19
Glutamate + Malate ADP:O	2.0 ± 0.1	2.2 ± 0.4	2.7 ± 0.3*	2.7 ± 0.2*
Malate + Pyruvate State III	151 ± 19	164 ± 19	166 ± 22	194 ± 17
Malate + Pyruvate State IV	67 ± 10	55 ± 4	63 ± 8	68 ± 3
Malate + Pyruvate State RCR	2.4 ± 0.32	2.95 ± 0.2	2.87 ± 0.4	2.92 ± 0.3
Malate + Pyruvate State ADP:O	2.78 ± 0.31	2.61 ± 0.30	2.58 ± .029	2.15 ± 0.21
Palmityl – Carnitine + Malate State III	178 ± 31	303 ± 105	172 ± 17	141 ± 7
Palmityl – Carnitine + Malate State IV	90 ± 4	93 ± 23	83 ± 9	66 ± 5*
Palmityl – Carnitine + Malate RCR	1.96 ± 0.3	3.14 ± 0.51	2.2 ± 0.34	2.3 ± 0.24
Palmityl – Carnitine + Malate ADP:O	2.50 ± 0.39	1.95 ± 0.44	2.43 ± 0.21	2.9 ± 0.14
Rotenone + Succinate State III	285 ± 53	287 ± 74	313 ± 71	388 ± 143
Rotenone + Succinate State IV	146.7 ± 43	126 ± 26	193 ± 65	95 ± 25
Rotenone + Succinate RCR	2.41 ± .59	2.29 ± 0.27	2.22 ± 0.78	2.69 ± 0.53
Rotenone + Succinate ADP:O	1.78 ± 0.53	1.73 ± 0.28	1.62 ± 0.32	1.45 ± 0.24

Table 4.2.A. Function of isolated left ventricular sub-sarcolemmal mitochondria after 4 months of PI treatment.

* $p < 0.05$ vs. control, $n=6$. All results expressed as mean ± SEM, statistical analysis: one-way ANOVA with Bonferroni post-hoc test. PI - protease inhibitor, RSV - Resveratrol.

No changes were observed in mitochondrial respiration following 6 months of PI treatment (Table 4.2.B). However, co-treatment of PI and RSV resulted in increased State III respiration ($p < 0.01$ vs. control) (glutamate and malate) and a simultaneous decrease in ADP:O ratio ($p < 0.001$ vs. control), indicating that respiration was impaired (Table 4.2.B). When rotenone and succinate were used as substrates, isolated mitochondria from rats co-treated with PI and RSV displayed increased rates of State III respiration ($p < 0.001$) compared to controls (Table 4.2.B). The ratio of ADP:O was however observed to be decreased ($p < 0.001$) when compared to controls, once again indicating impaired respiration (Table 4.2.B). Respiratory control ratios of isolated mitochondria from rats receiving PI in combination with RSV were increased ($p < 0.05$) when compared to rats receiving jelly and PI alone (Table 4.2.B).

	CONTROL	JELLY	PI	PI+RSV
Glutamate + Malate State III	176 ± 10	167 ± 13	161 ± 16	228 ± 17**
Glutamate + Malate State IV	45 ± 5	48 ± 7	43 ± 4	57 ± 7
Glutamate + Malate RCR	4.16 ± 0.46	3.7 ± 0.37	3.8 ± 0.28	4.2 ± 0.43
Glutamate + Malate ADP:O	2.32 ± 0.13	2.47 ± 0.18	2.61 ± 0.26	1.8 ± 0.13**
Malate + Pyruvate State III	129 ± 24	157 ± 16	193 ± 28	194 ± 8
Malate + Pyruvate State IV	38 ± 2	40 ± 6	49 ± 7	46 ± 3
Malate + Pyruvate State RCR	3.97 ± 0.31	4.14 ± 0.28	3.99 ± 0.13	4.25 ± 0.22
Malate + Pyruvate State ADP:O	2.73 ± 0.19	2.66 ± 0.21	2.26 ± 0.28	2.08 ± 0.09
Palmitoyl – Carnitine + Malate State III	150 ± 15	156 ± 8	183 ± 8	187 ± 18
Palmitoyl – Carnitine + Malate State IV	42 ± 6	44 ± 5	51 ± 1	56 ± 6
Palmitoyl – Carnitine + Malate RCR	3.87 ± 0.52	3.71 ± 0.39	3.61 ± 0.25	3.4 ± 0.15
Palmitoyl – Carnitine + Malate ADP:O	2.78 ± 0.24	2.6 ± .14	2.21 ± 0.1	2.29 ± 0.32
Rotenone + Succinate State III	209 ± 47	285 ± 10	201 ± 65	433 ± 16 **@
Rotenone + Succinate State IV	51 ± 16	74 ± 4	51 ± 17	87 ± 17
Rotenone + Succinate RCR	5.03 ± 1.1	3.93 ± 0.28	4.0 ± 0.33	5.08 ± 0.2 \$@
Rotenone + Succinate ADP:O	1.69 ± 0.23	1.41 ± 0.05	1.33 ± 0.04	0.93 ± 0.03 **@@

Table 4.2.B. Function of isolated left ventricular sub-sarcolemmal mitochondria after 6 months of PI treatment.

** $p < 0.01$ vs. Control; @ $p < 0.05$ and @@ $p < 0.01$ vs. Jelly; \$ $p < 0.05$ vs. PI, $n=6$. All results expressed as mean ± SEM, statistical analysis: one-way ANOVA with Bonferroni post-hoc test. PI - protease inhibitor, RSV - Resveratrol.

2.4. Discussion

After 2 months of PI and RSV treatment there were no significant differences in bodyweight (Fig. 4.1.A) or weight gains (Fig. 4.1.B) when compared to the control and PI groups, respectively. This is not surprising since we did not observe any differences with sole PI treatment. After 4 months, the addition of RSV lessened PI-mediated effects on body weight and weight gain, i.e. preventing the decrease in weights found with PIs (Fig. 4.2). After 6 months, RSV protected against PI-induced weight gain (Fig. 4.3). What is the basis for such effects? We are unclear regarding the precise mechanisms whereby RSV mediates its effects on weight gain, but the mitochondrion may indeed be a target. It is our opinion that RSV supplementation likely increased SIRT1 activation and downstream targets such as AMPK and PGC-1 α (refer Chapter 1) that can in turn increase mitochondrial number and mitochondrial respiration chain proteins to enhance intracellular metabolism (Ruderman *et al*, 2013). This would then be expected to lower ROS production and potentially inhibit inflammation e.g. in adipose tissues.

PPARs are a downstream target of AMPK and here PPAR γ is required for differentiation and maturation of adipose tissue (Fajas, 1999). Of note, PPAR γ levels were decreased in peripheral adipose tissue (PAT) of HAART treated individuals (Kannisto, 2003) and thus it is a possibility that RSV may have offered protection against PI-induced decreases in PAT. This may explain the effects of RSV after 4 months of treatment in our experimental model and is strengthened by the retroperitoneal fat pad weight data (Fig. 4.11). However, PPAR γ also affects visceral adipose tissue (VAT) and decreased levels can lead to VAT hypertrophy. This occurs through increased sterol synthesis and FFA uptake (Galescu *et al*, 2013). We thus propose that at the 6 month time point RSV acted largely on VAT by increasing regulatory proteins such as SIRT1 and AMPK. However, additional mechanistic studies are required to evaluate the proposals here put forward.

The levels of metabolites here studied did not significantly change after 2 and 4 months, respectively (Fig. 4.4 & 4.5). This was not surprising since we did not observe any changes in blood parameters with PI treatment alone. However, these data differ from Gupta and colleagues (2012) who found hyperlipidemia and insulin resistance after only four weeks of PI treatment (Gupta *et al*, 2012). We propose that such difference may be dependent on the nature of experimental models employed, drug dosages and length of treatments. Likewise, we did not find any significant differences for heart function by echocardiographic analysis (Table 4.1. A & B; Fig. 4.6). Of note, RSV had no effect on PI-mediated increase in heart weight after 6 months of treatment (Fig. 4.8). More studies are required to confirm whether there is a hypertrophic response after 6 months of PI treatment and also whether it is physiological or pathological in nature.

RSV elicited no significant effects on mitochondrial function following 4 months of PI treatment (Table 4.2.A). However, after 6 months State III respiration was higher with RSV treatment compared to sole PI-treatment (oxidative substrates: malate and glutamate; succinate and rotenone), with a concomitant decrease in ADP/O ratios (Table 4.2.B). These results indicate that RSV exerts its effects at both complex I and III of the mitochondrial phosphorylation chain are involved. These results are promising and confirm our earlier postulate that RSV is likely impacting on mitochondrial function to exert its beneficial effects in response to PI treatment.

Chapter 3

3.1. Inflammation as therapeutic target

Decreased physical activity levels together with Westernised diets has played a strong role in the surge in cardio-metabolic diseases (Hall *et al*, 2011; Cheen and Van Gaal, 2014), with both obesity and type-2 diabetes constituting recognized risk factors for CVD development (Chen *et al*, 2012). Developing countries are also at risk in this instance (Chen *et al*, 2012), e.g. the International Diabetes Federation estimates that type-2 diabetes will increase by 98% in sub-Saharan countries by 2025 (Sicree and Shaw, 2009; Chen *et al*, 2012) (Fig 5.1).

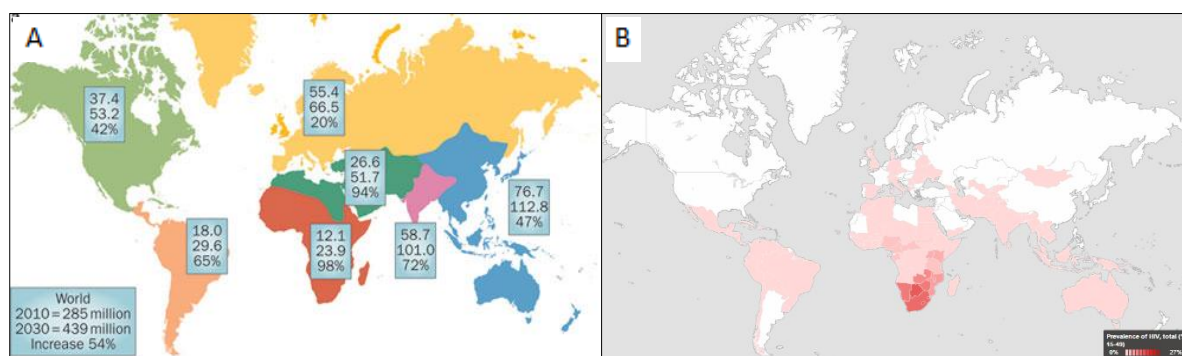


Fig. 5.1. A) Global projections for diabetes: 2010–2030 (Chen *et al*, 2012); B) Global prevalence of HIV (Epidemiological facts sheet on HIV and AIDS, 2008).

Moreover, this region contains roughly 90% of the HIV-infected individuals of the world (UNAIDS, 2010) thus further adding to the overall burden of disease. In addition, there is cross-talk between these conditions with HIV a risk factor for CVD onset (Stevenson, 2003) as discussed before. Here PIs are strongly implicated in the development of cardio-metabolic complications as highlighted earlier in this thesis (Friis-Møller, 2010; Duong *et al*, 2001; Stevenson, 2010). The underlying mechanisms whereby PIs exert damaging effects are not entirely clear, although mitochondria (Gardner *et al*, 2013; Perez-Matute *et al*, 2013), inflammation (Arenas-Pinto, *et al*, 2015) (Capeau *et al*, 2013; Gardner *et al*, 2013), and ROS (Hwang *et al*, 2008; Shadel and Horvath, 2015; Andreyev *et al*, 2005) have been identified as potential mediators.

Chronic inflammation in the vasculature is a risk factor and predictor of CVD (Arenas-Pinto *et al*, 2015) as it can lead to a pro-atherogenic state (Grundy, 2011). The atherogenic state can cause plaque build-up and can eventually lead to ischemic tissue damage, increasing the risk for myocardial infarction or strokes (Grundy, 2011). Inflammation is not the only cause for an atherogenic state and others include hyperglycemia, high blood pressure and elevated cholesterol

(Grundy, 2011). These generally work synergistically in ARV-treated HIV-positive patients and can negatively affect various tissues in the body (Zha *et al*, 2011).

Focusing on inflammation, adipose tissues have emerged as more than storage depots and acting more like an endocrine organ (Kershaw and Flier, 2004), producing various peptides, proteins and cytokines termed “adipokines” (Wozniak *et al*, 2009). Of note, adipose tissue is also greatly affected by and also involved in the inflammation process (Lafontan, 2013; Nakamura *et al*, 2014). It is important to note that white adipose tissue, depending on its location, responds differently to inflammation (Andrade-Oliveira *et al*, 2015). It is grossly divided into two categories based on location, i.e. PAT and VAT, respectively (Kershaw and Flier, 2004). VAT and PAT differ anatomically and also in terms of underlying physiology and molecular regulation. For example, VAT has larger adipocytes, contains more macrophages and is richer in blood supply and more innervated than PAT (Ibrahim 2010). VAT adipocytes also express higher levels of β_3 -adrenergic receptors than PAT and this is put forward as a reason why VAT is more sensitive to catecholamine-induced lipolysis (Arner *et al*, 1990; Helmer *et al*, 1992). The most important difference between PAT and VAT is, however, that the latter is associated with poor health outcomes (Nakamura *et al*, 2014) due to increased production of inflammatory markers. These markers include inflammatory cytokines such as Interleukin 6 (IL-6), Tumour Necrosis Factor alpha (TNF- α) and Monocyte Chemoattractant Protein-1 (MCP-1) (Lafontan, 2013; Kershaw and Flier, 2004) that are released into the circulation causing systemic inflammation. Chronically elevated levels of circulating inflammatory cytokines can lead to a pro-atherogenic state and CVD onset as discussed by Arenas-Pinto *et al* (2015).

If inflammation is indeed a primary cause of the increased occurrence of adverse cardiovascular events in the PI-treated patients, then anti-inflammatory medication could potentially ameliorate such effects. In light of this we explored Acetyl Salicylate (or better known as Aspirin) as an anti-inflammatory co-treatment option. It is the oldest pharmaceutical drug and is commonly used as an analgesic, anti-pyretic, anti-coagulant and part of the drug class termed non-steroidal anti-inflammatories (NSAID) (Leaberry, 2010). It is believed to inhibit inflammation by inhibiting NF- κ B, stopping the downstream events leading to inflammation. In light of this, we propose that PI-treatment together with Aspirin (Asp) supplementation can alleviate the adverse side-effects of PIs.

3.2. Materials and Methods

Asp was purchased in the form of Disprin™ tablets (Reckitt & Benckiser Healthcare) (300 mg/tablet). The dosage received was 300 mg per kilogram body weight per day and all analyses and experiment performed were done as described in Chapter 1. Please refer to Chapter 1.2 for full description of materials and methods.

3.3. Results

3.3.1. Weight gain analysis

In order to establish the effects of treatments on body weight and net weight gain, all experimental groups were weighed every third day (at 2, 4 and 6 months' time-points). Here our data reveal that no significant differences were found after 2 months of treatment (Fig. 6.1).

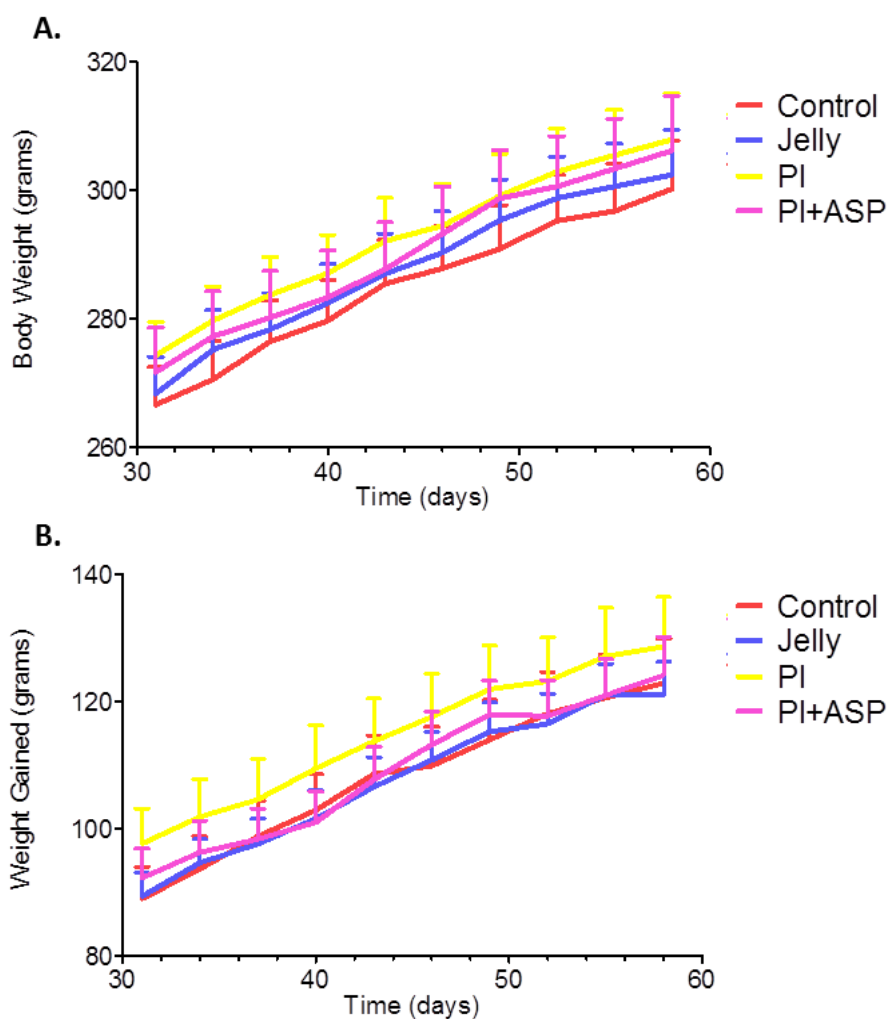


Figure 6.1. Body weight and net weight gain in male Wistar rats following 2 months of PI treatment \pm ASP. **A)** Total body weight during the second month of treatment ($n = 24$). **B)** Weight gained over the second month of treatment, as calculated from the starting weight ($n=24$). All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with a Bonferroni post-hoc test. PI – protease inhibitor, ASP – Aspirin.

However, after 4 months PI-treated rats displayed decreased body weights ($p < 0.05$ vs. control; $p < 0.001$ vs. Jelly, Fig. 6.2.A). Rats co-treated with PI and ASP also displayed decreased body weights ($p < 0.01$) compared to jelly controls (Fig. 6.2.A). Similarly, weight gain also decreased ($p < 0.001$ vs. Control; $p < 0.001$ vs. Jelly) compared to controls in rats receiving only PI, and PI in combination with ASP (Fig. 6.2.B).

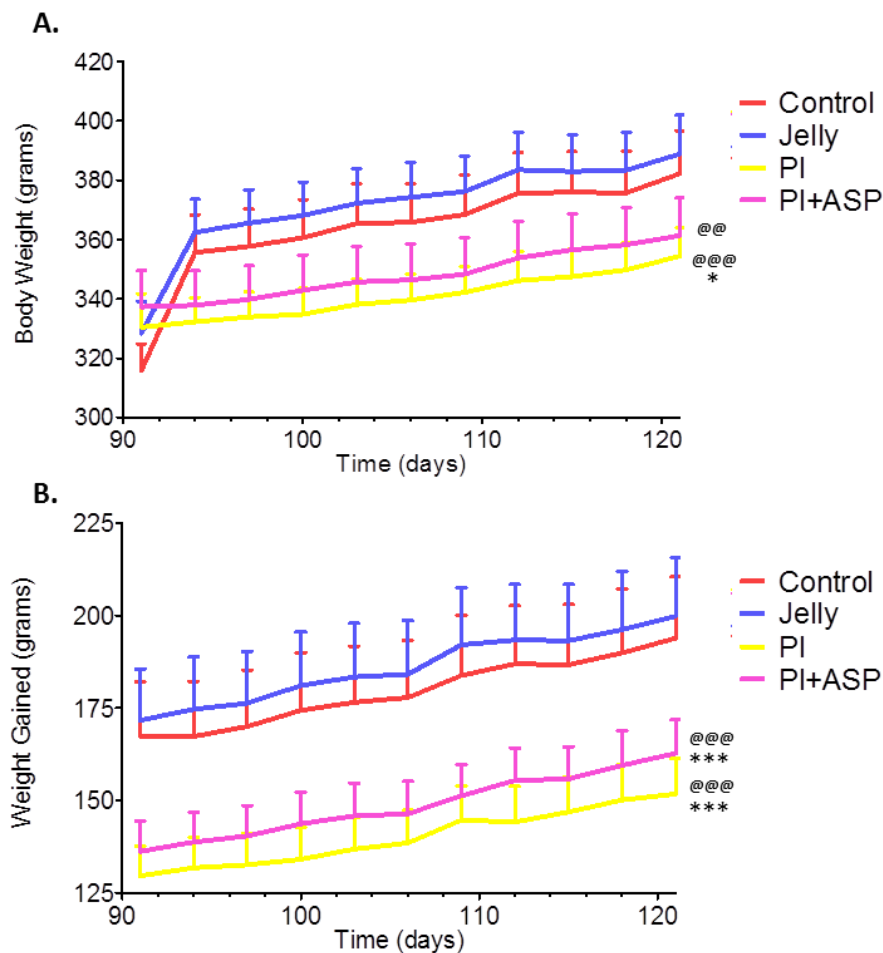


Figure 6.2. Body weight and net weight gain in male Wistar rats after 4 months of treatment. A) Total body weight during the fourth month of treatment: * $p < 0.05$ vs. control, @@@ $p < 0.001$ vs. jelly, @@ $p < 0.01$ vs. control, $n = 12$. **B)** Weight gained over the fourth month of treatment, as calculated from the starting weight: *** $p < 0.001$ vs. control, @@@ $p < 0.001$ vs. jelly, $n=12$. All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with a Bonferroni post-hoc test. PI - protease inhibitor, ASP – Aspirin.

This pattern changed with prolonged treatment, i.e. PI treatment resulted in increased body weight after 6 months when compared to control rats ($***p < 0.001$) (Fig. 6.3. A). However, co-treatment (PI + ASP) resulted in decreased body weights ($^{\$}p < 0.001$) when compared to rats receiving only PI (Fig. 6.3. A). Rats treated with jelly did not display any significant increases in body weight after the 6 month period ($**p < 0.01$) versus controls (Fig. 6.3. A). PI treatment led to significant increases in weight gain ($***p < 0.001$) when compared to control rats (Fig. 6.3. B). Interestingly, PI-treated rats receiving ASP prevented the PI-mediated increase in weight gain ($^{\$}\$p < 0.001$, Fig. 6.3. B). Rats receiving only jelly also displayed increased weight gain compared to control rats ($**p < 0.01$) and rats receiving co-treatment of PI and ASP ($^{\$}\$p < 0.01$, Fig. 6.3. B).

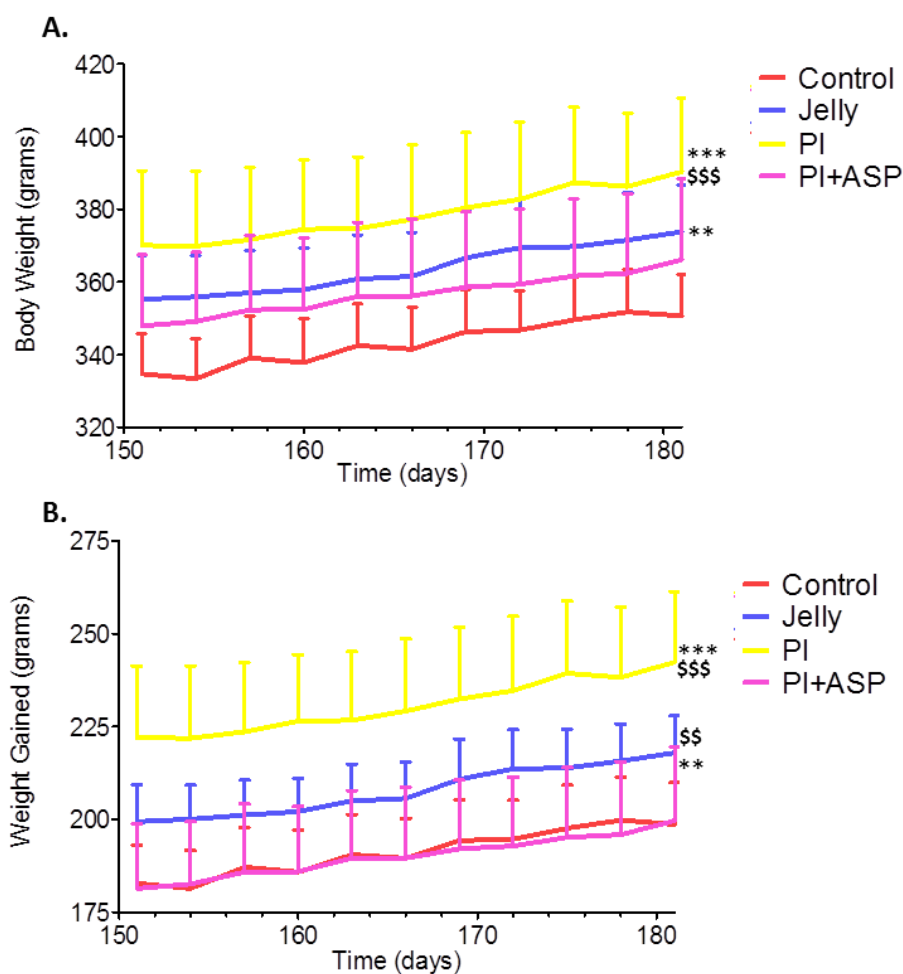


Figure 6.3. Body weight and net weight gain in male Wistar rats after 6 months of treatment. A) Total body weight during sixth months of treatment: $***p < 0.001$ vs. control, $**p < 0.01$ vs. control, $^{\$}\$p < 0.001$ vs. control, $n = 6$. **B)** Weight gained over 6 months of treatment, as calculated from the starting weight: $***p < 0.001$ vs. control, $^{\$}\$p < 0.001$ vs. PI+ASP, $**p < 0.01$ vs. control, $^{\$}\$p < 0.01$ vs. PI+ASP, $n=6$. All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with a Bonferroni post-hoc test. PI - protease inhibitor, ASP – Aspirin.

3.3.2. Analysis of triglyceride, cholesterol, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) blood levels.

In order to establish the effects of each treatment on various metabolites that are commonly related to metabolic disturbances, blood samples were collected at the end of 2 and 4 months of treatment for further analysis. Here no significant changes were observed after 2 months of treatment (Fig. 6.4).

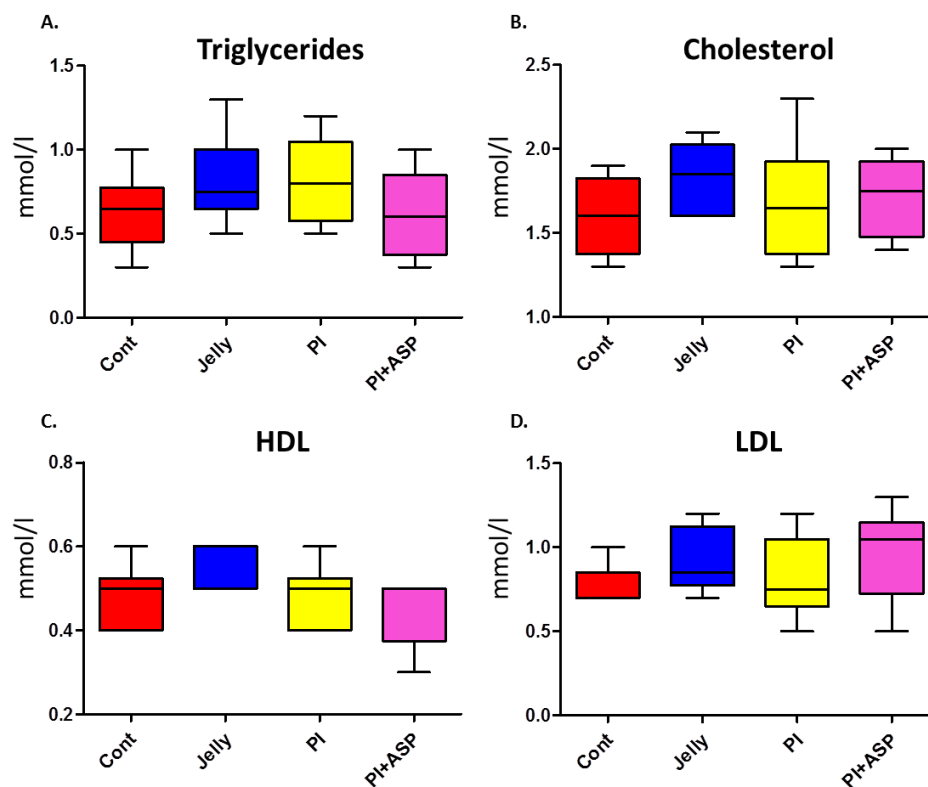


Figure 6.4. Triglyceride, cholesterol, LDL and HDL blood levels in male Wistar rats after 2 months of treatment. **A)** Triglyceride levels, $n = 6$. **B)** Total cholesterol levels, $n = 6$. **C)** HDL levels, $n=6$. **D)** LDL levels, $n=6$. All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with Bonferroni post-hoc test. PI - protease inhibitor, ASP – Aspirin.

Blood HDL, LDL and total cholesterol levels remained consistent for all groups after 4 months of treatment (Fig. 6.5. B, C and D). However, PI-treated rats receiving ASP exhibited an increase in circulating triglyceride blood levels ($*p < 0.05$) versus matched controls (Fig. 6.5. A). Due to a lack of sample material, we were unable to perform similar analyses at the 6 month time point.

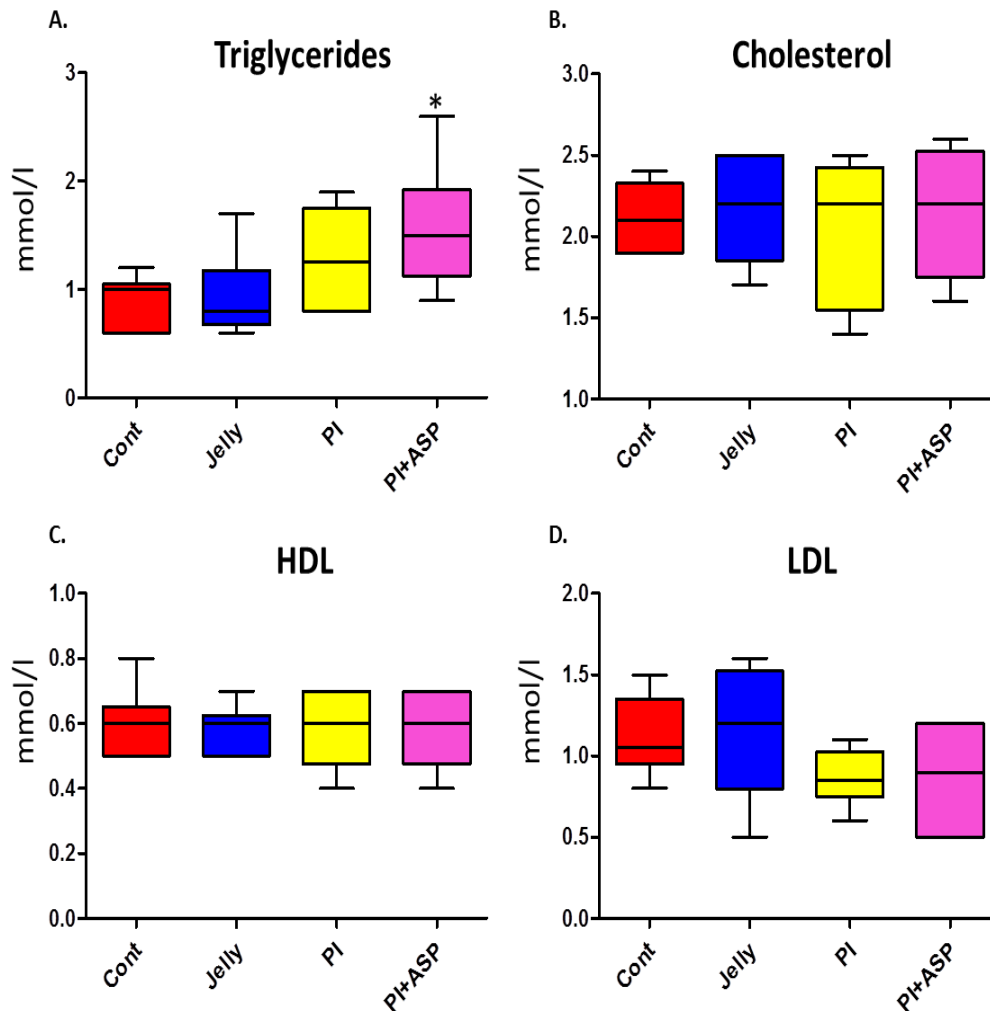


Figure 6.5. Triglyceride, cholesterol, LDL and HDL blood levels in male Wistar rats after 4 months of treatment. **A)** Triglyceride levels: $*p < 0.05$ vs. control, $n = 6$. **B)** Total cholesterol levels, $n=6$. **C)** HDL levels, $n=6$. **D)** HDL levels, $n=6$. All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with Bonferroni post-hoc test. PI - protease inhibitor, ASP – Aspirin.

3.3.3. Analysis of mid-wall fractional shortening (MWFS) with the use of echocardiography.

In order to establish the effects of each treatment on systolic functioning, various functional parameters were assessed with echocardiography. These results were used to calculate mid-wall fractional shortening at the end of the 2 and 4 month treatment periods, respectively. We were not able to attain echocardiographical data at the 6 month time point due to logistical issues and lack of animals. These data show that no significant changes were observed in left ventricular functional parameters after 2 and 4 months of treatment, respectively.

	Control	Jelly	PI	PI+ASP
LVEDD	6.0 ± 0.13	6.0 ± 0.09	6.6 ± 0.08	6.2 ± 0.12
LVESD	2.9 ± 0.14	3.3 ± 0.14	3.6 ± 0.11	3.1 ± 0.17
LVESPWT	1.9 ± 0.05	1.8 ± 0.05	1.9 ± 0.08	1.8 ± 0.08
LVEDPWT	2.6 ± 0.07	2.4 ± 0.09	2.6 ± 0.11	2.4 ± 0.1
R-R Interval	0.16 ± 0.004	0.17 ± 0.005	0.16 ± 0.005	0.17 ± 0.004

Table 6.1.A. Left ventricular parameters as evaluated by echocardiography after 2 months of PI treatment

LVEDD – Left Ventricular End Diastolic Diameter; LVESD – Left Ventricular End Systolic Diameter; LVESPWT – Left Ventricular End Systolic Posterior Wall Thickness; LVEDPWT – Left Ventricular End Diastolic Posterior Wall Thickness

	Control	Jelly	PI	PI+ASP
LVEDD	6.4 ± 0.13	6.6 ± 0.16	6.4 ± 0.16	7.1 ± 0.09
LVESD	3.6 ± 0.12	3.5 ± 0.15	3.8 ± 0.14	3.9 ± 0.12
LVESPWT	2.1 ± 0.07	2.0 ± 0.09	1.9 ± 0.04	2.0 ± 0.07
LVEDPWT	2.6 ± 0.1	2.6 ± 0.09	2.4 ± 0.09	2.6 ± 0.08
R-R Interval	0.17 ± 0.004	0.18 ± 0.005	0.18 ± 0.003	0.18 ± 0.004

Table 6.1.B. Left ventricular parameters as determined by echocardiography after 4 months of PI treatment

LVEDD – Left Ventricular End Diastolic Diameter; LVESD – Left Ventricular End Systolic Diameter; LVESPWT – Left Ventricular End Systolic Posterior Wall Thickness; LVEDPWT – Left Ventricular End Diastolic Posterior Wall Thickness

In addition, no significant changes were observed in MWFS in all groups after 2 and 4 months of treatments, respectively (Fig. 6.6).

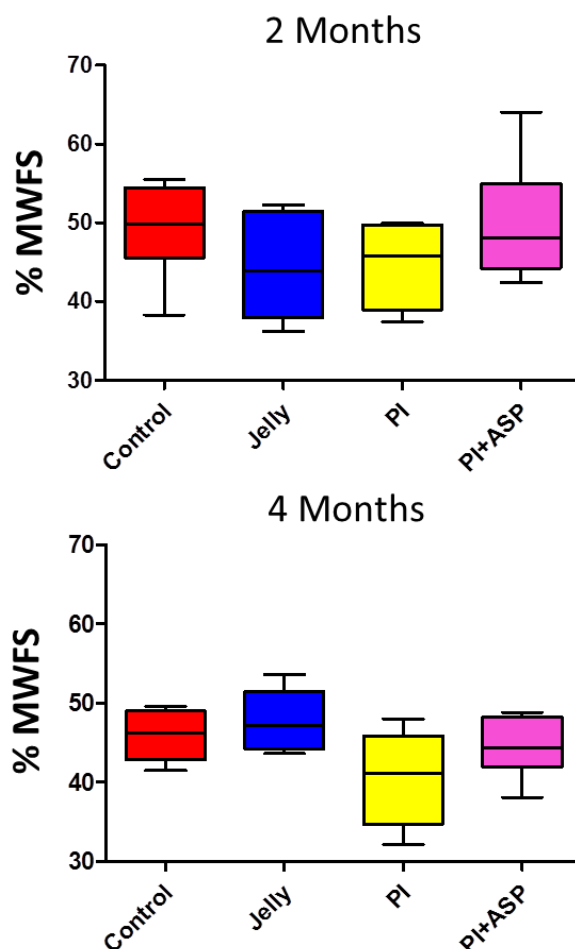


Figure 6.6. MWFS in male Wistar rats after 2 and 4 months of treatment. No significant changes were observed, n=6. All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with Bonferroni post-hoc test. PI - protease inhibitor, ASP – Aspirin.

3.3.4. Organ harvesting for the determination of treatment-induced anatomical changes

In order to determine whether the treatments induced any gross anatomical changes, body weight and various organs were harvested, weighed and normalized to tibial length.

No significant changes were observed for total heart weight after 2 and 4 months of PI treatment (Figs. 6.8.A and 6.8.B). However, after 6 months treatment the PI-treated rats displayed increased heart weights ($p < 0.05$ vs. controls and Jelly; Fig. 6.8.C). Of note, this effect was lost with ASP treatment.

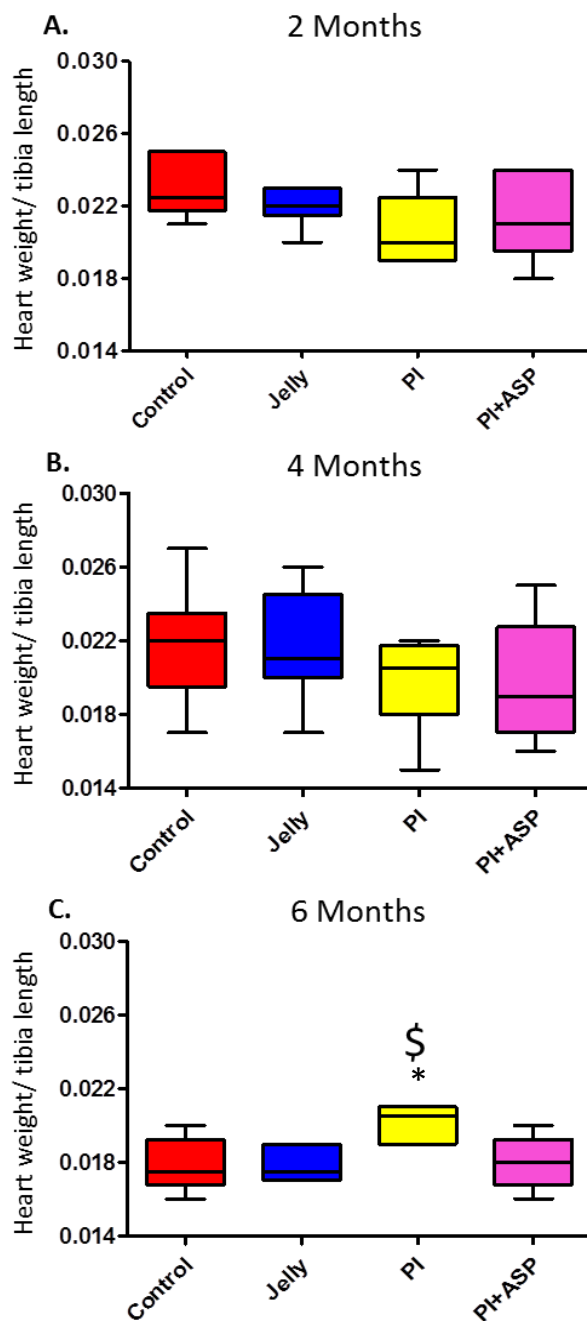


Figure 6.7. Heart weights normalized to tibia lengths in male Wistar rats after 2 and 4 months of treatment: * $p < 0.05$ vs. control, $^{\$}p < 0.05$ vs. jelly, $n=6$. All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with Bonferroni post-hoc test. PI - protease inhibitor, ASP – Aspirin.

To gain additional insights, we also separately evaluated left and right ventricular weights. Here left ventricular weights were higher for all groups versus controls after 4 months of treatment ($p < 0.05$ and $p < 0.01$ vs. control, Fig 6.10.A). Conversely, right ventricular weight decreased for all groups after 4 month of treatment ($p < 0.05$ and $p < 0.01$ vs. control, Fig. 6.10.A). No changes were observed in left and right ventricle weights after 6 months of treatment.

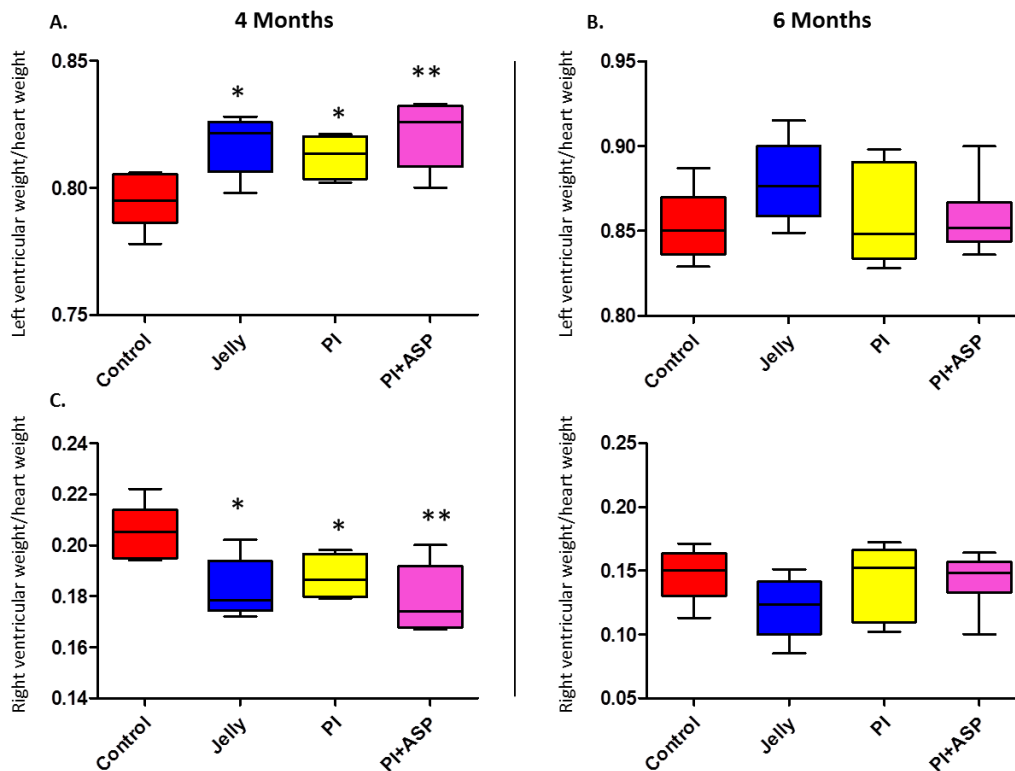


Figure 6.8. Left and right ventricular weights normalized to total heart weight in male Wistar rats on the day of culling after 4 and 6 months of treatment, respectively. A) 4 months: * $p < 0.05$ and ** $p < 0.01$ vs. control, $n=12$. B) 6 months: No significant changes were observed, $n=12$. All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with Bonferroni post-hoc test. PI - protease inhibitor, ASP - Aspirin.

Liver weight normalized to right tibia length remained unchanged for all groups after 2, 4 and 6 months of treatment (Fig. 6.9).

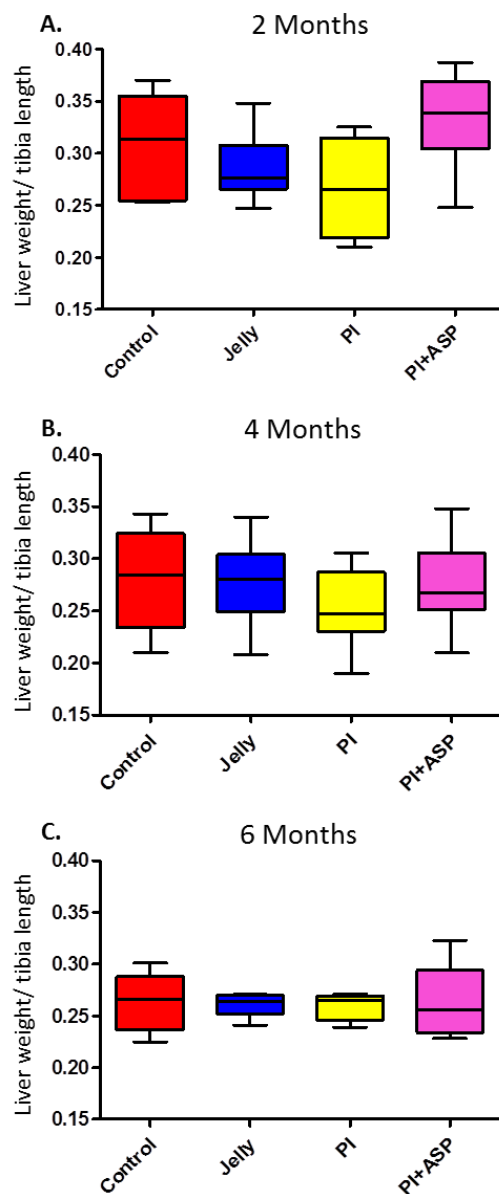


Figure 6.9. Liver weights normalized to right tibia length in male Wistar rats on the day of culling after 2, 4 and 6 months of treatment. 2 months: No significant changes were observed, $n=12$. 4 months: No significant changes were observed, $n=12$. 6 months: No significant changes were observed, $n=12$. All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with Bonferroni post-hoc test. PI - protease inhibitor, ASP – Aspirin.

No changes were observed in retroperitoneal fat mass after 4 months of treatment (Fig. 6.11. 4 months). However, rats fed with jelly cubes displayed increased retroperitoneal fat mass compared to control rats after 6 months ($p < 0.05$, Fig. 6.11. 6 months).

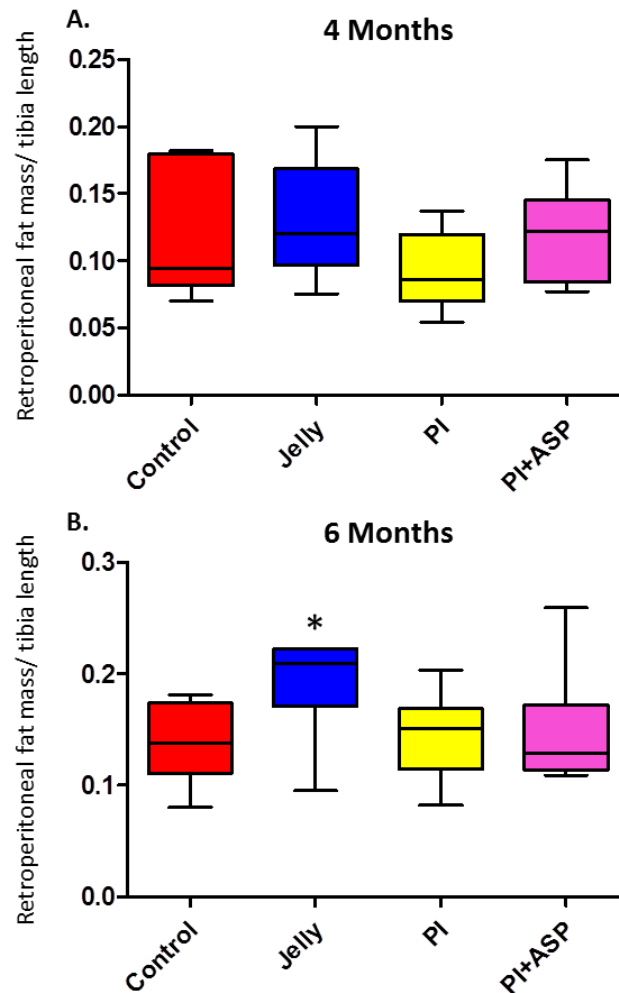


Figure 6.10. Retroperitoneal fat mass normalized to tibia length in male Wistar rats on the day of culling after 4 and 6 months of treatment. A) 4 months, $n=12$. B) 6 months: * $p < 0.05$ vs. control, $n=12$. All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with Bonferroni post-hoc test. PI - protease inhibitor, ASP - Aspirin.

3.3.5. Analysis of mitochondrial respiratory function

We next determined mitochondrial (sub-sarcolemmal) oxygen consumption by employing oxygen electrodes (oxygraph) after 4 and 6 months of treatment, respectively.

	CONTROL	JELLY	PI	PI+ASP
Glutamate + Malate State III	200 ± 12	248 ± 75	150 ± 13*	230 ± 38 [§]
Glutamate + Malate State IV	56 ± 12	97 ± 26	64 ± 5	71 ± 10
Glutamate + Malate RCR	2.53 ± 0.28	2.51 ± 0.21	2.37 ± 0.22	3.35 ± 0.5
Glutamate + Malate ADP:O	2.0 ± 0.1	2.2 ± 0.4	2.7 ± 0.3*	1.9 ± 0.2
Malate + Pyruvate State III	151 ± 19	164 ± 19	166 ± 22	161 ± 15
Malate + Pyruvate State IV	67 ± 10	55 ± 4	63 ± 8	58 ± 4
Malate + Pyruvate State RCR	2.4 ± 0.32	2.95 ± 0.2	2.87 ± 0.4	2.79 ± 0.17
Malate + Pyruvate State ADP:O	2.78 ± 0.31	2.61 ± 0.30	2.58 ± .029	2.59 ± 0.24
Palmitoyl – Carnitine + Malate State III	178 ± 31	303 ± 105	172 ± 17	184 ± 27
Palmitoyl – Carnitine + Malate State IV	90 ± 4	93 ± 23	83 ± 9	71 ± 10
Palmitoyl – Carnitine + Malate RCR	1.96 ± 0.3	3.14 ± 0.51	2.2 ± 0.34	2.71 ± 0.37
Palmitoyl – Carnitine + Malate ADP:O	2.50 ± 0.39	1.95 ± 0.44	2.43 ± 0.21	2.37 ± 0.28
Rotenone + Succinate State III	285 ± 53	287 ± 74	313 ± 71	267 ± 6.61
Rotenone + Succinate State IV	146.7 ± 43	126 ± 26	193 ± 65	163 ± 31
Rotenone + Succinate RCR	2.41 ± .59	2.29 ± 0.27	2.22 ± 0.78	1.86 ± 0.26
Rotenone + Succinate ADP:O	1.78 ± 0.53	1.73 ± 0.28	1.62 ± 0.32	1.51 ± 0.03

Table 6.2.A. Function of isolated left ventricular sub-sarcolemmal mitochondria after 4 months of PI treatment.

* $p < 0.05$ vs. control; [§] $p < 0.05$ vs. PI, $n=6$. All results expressed as mean ± SEM, statistical analysis: one-way ANOVA with Bonferroni post-hoc test. PI - protease inhibitor, ASP – Aspirin.

For the 4 month PI treatment period, state III respiration was impaired ($p < 0.05$ vs. control, Table 6.2.A) in isolated mitochondria of PI-treated rats with glutamate and malate as substrates. Although state III respiration was diminished with PI treatment, the ADP:O ratio was increased indicating that it was respiring with greater efficiency. With the addition of ASP to the PI treatment regime the opposite effect was observed. After 4 months, state III respiration in isolated mitochondria increased ($p < 0.05$ vs. control, Table 6.2.A) with glutamate and malate as substrates, although no difference in the ADP:O ratio was observed.

No major changes were observed following 6 months of PI treatment (Table 6.2.B). However, with the addition of ASP to the PI treatment regime, state IV respiration increased ($p < 0.05$ vs. PI, Table 6.2.B) with glutamate and malate as substrates. State IV respiration also increased ($p < 0.05$ vs. control, Table 6.2.B) with palmitoyl-carnitine and malate as substrates.

	CONTROL	JELLY	PI	PI+ASP
Glutamate + Malate State III	176 ± 10	167 ± 13	161 ± 16	246 ± 44
Glutamate + Malate State IV	45 ± 5	48 ± 7	43 ± 4	94 ± 12 [§]
Glutamate + Malate RCR	4.16 ± 0.46	3.7 ± 0.37	3.8 ± 0.28	2.95 ± 0.76
Glutamate + Malate ADP:O	2.32 ± 0.13	2.47 ± 0.18	2.61 ± 0.26	1.83 ± 0.24
Malate + Pyruvate State III	129 ± 24	157 ± 16	193 ± 28	179 ± 34
Malate + Pyruvate State IV	38 ± 2	40 ± 6	49 ± 7	66 ± 25
Malate + Pyruvate State RCR	3.97 ± 0.31	4.14 ± 0.28	3.99 ± 0.13	3.6 ± .91
Malate + Pyruvate State ADP:O	2.73 ± 0.19	2.66 ± 0.21	2.26 ± 0.28	2.56 ± 0.51
Palmitoyl – Carnitine + Malate State III	150 ± 15	156 ± 8	183 ± 8	211 ± 29
Palmitoyl – Carnitine + Malate State IV	42 ± 6	44 ± 5	51 ± 1	111 ± 25*
Palmitoyl – Carnitine + Malate RCR	3.87 ± 0.52	3.71 ± 0.39	3.61 ± 0.25	2.4 ± 0.53
Palmitoyl – Carnitine + Malate ADP:O	2.78 ± 0.24	2.6 ± .14	2.21 ± 0.1	2.1 ± 0.27
Rotenone + Succinate State III	209 ± 47	285 ± 10	201 ± 65	287 ± 15
Rotenone + Succinate State IV	51 ± 16	74 ± 4	51 ± 17	76 ± 6
Rotenone + Succinate RCR	5.03 ± 1.1	3.93 ± 0.28	4.0 ± 0.33	3.8 ± 0.23
Rotenone + Succinate ADP:O	1.69 ± 0.23	1.41 ± 0.05	1.33 ± 0.04	1.4 ± 0.07

Table 6.2.B. Function of isolated left ventricular sub-sarcolemmal mitochondria after 6 months of PI treatment.

* $p < 0.05$ vs. Control; [§] $p < 0.05$ vs. PI, $n=6$. All results expressed as mean ± SEM, statistical analysis: one-way ANOVA with Bonferroni post-hoc test. PI - protease inhibitor, ASP – Aspirin.

3.4. Discussion

PIs are known to cause serious side-effects such as CVD onset (Friis moller *et al*, 2010). As mitochondrial dysregulation and inflammation are implicated in this process, this study evaluated whether Asp could ameliorate PI-induced side-effects. Our data reveal that after 4 months PI treatment, Asp co-treatment was unable to inhibit weight loss as was the case for RSV (Fig. 6.2). This suggests that the mitochondrion rather than inflammation is implicated in PI-induced wasting at this time point. However, at the 6 month time point ASP (like RSV) protected against the PI-induced weight gain suggesting that both mitochondria and systemic inflammation may be involved attenuating PI-induced weight gain (Fig. 6.3). More studies are, however, required to confirm these claims.

Blood metabolite profiles (Fig. 6.4 & 6.5) and echocardiographical analyses (Table 6.1. A & B; Fig. 6.6) once again did not show any differences between groups, which remains difficult to explain. Heart weights displayed a strange trend, i.e. all treatment groups exhibited increased left ventricular weights/heart weight and decreased right ventricular weights/heart weight ratios after 4 months versus the control group (Fig. 6.8.A). This is difficult to explain especially since the body weights of these groups differed significantly. After 6 months ASP co-treatment was effective at blunting the PI-mediated increase in heart weight (Fig. 6.7). These results indicate that inflammation may play a role to limit the hypertrophic response in our experimental system, although more research is required to confirm this.

After 4 months of co-treatment with ASP, State III respiration was significantly increased when compared to sole PI treatment and resulted in no alterations in the ADP:O ratio (Table 6.2.A). After 6 months State IV respiration was increased in the ASP-treated group when glutamate + malate and palmitoyl-carnitine + malate were used as substrates, respectively (Table 6.2.B). Thus these data suggest that ASP may have detrimental effects after 6 months by increasing proton leak. Alternatively, higher proton leak may be an adaptive step to decrease ROS generation as discussed before (Miro *et al*, 2004)). However, more detailed research is required to further investigate these interesting possibilities.

Chapter 4

4.1. ROS as therapeutic target

The development of CVD in ARV-treated patients is multifactorial (Chastain *et al*, 2015) as discussed in the previous chapters of this thesis (Friis moller *et al*, 2010). PIs in particular can elicit damaging effects that typically include metabolic disturbances, lipodystrophy and the development of type-2 diabetes (Friis moller *et al*, 2010; Worm *et al*, 2010). Such side-effects are of course well-described risk factors for CVD onset (Friis moller *et al*, 2010). AS the focus of this chapter is on a strategy against oxidative stress, it is crucial for cells to initiate protective mechanisms against the damaging effects of ROS. This is usually achieved by antioxidant surveillance systems, e.g. superoxide dismutase systems (SOD) and glutathione (Alleman *et al*, 2014). However, if ROS generating capacity exceeds the antioxidant protective systems this will result in excess ROS with detrimental outcomes (Reiche and Kaminami, 2014).

It has been proposed that PIs can act on mitochondrial to increase ROS production (Reyskens and Essop, 2013). However, it remains unclear exactly how this occurs, although increased ROS levels could eventually lead to systemic inflammation, increased lipodystrophy and a higher risk for CVD onset. Thus a useful therapeutic strategy would be to supplement PI treatment with dietary antioxidants to potentially combat increased ROS levels. For our study, we focused on ascorbic acid, or better known as Vitamin C (VitC), a common naturally occurring organic compound with antioxidant properties (Iqbal *et al*, 2004). VitC reacts with and reduces all physiologically relevant radicals and oxidants (Carr and Frei, 1999) making it an excellent antioxidant for our experimental setting. Moreover, HIV-positive patients display decreased VitC levels making our choice even more meaningful (Stephensen *et al*, 2006). In addition, it is relatively cheap and easy to access and would therefore be a perfect therapeutic option to employ in resource-deprived settings. In light of this, we propose VitC supplementation may ameliorate PI-induced cardio-metabolic complications in our experimental model.

4.2. Materials and Methods

VitC was purchased in the form of powdered sodium ascorbate (Enhance, Compli-Med) and the dosage employed was 300 mg per kilogram body weight per day. All analyses and experiments were performed as described in Chapter 1. Please refer to Chapter 1.2 for full description of materials and methods.

4.3. Results

4.3.1. Weight gain analysis

In order to establish the effects treatment on body weight and net weight gain, all experimental groups were weighed every third day (for 2, 4 and 6 months treatment, respectively). The data revealed that all groups gained weight equally over the 2 month treatment period; no significant differences were observed (Fig. 7.1).

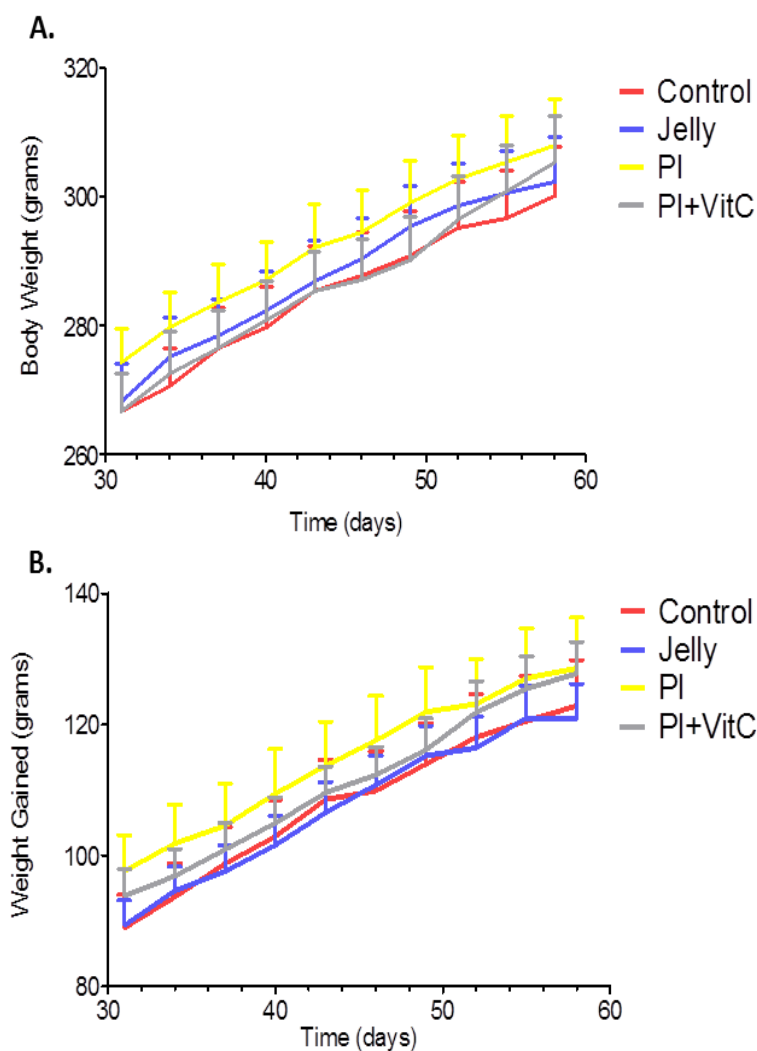


Figure 7.1. Body weight and net weight gain in male Wistar rats following 2 months of treatment. **A)** Total body weight during the second month of treatment, $n = 24$. **B)** Weight gained over the second month of treatment, as calculated from the starting weight, $n=24$. All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with a Bonferroni post-hoc test. PI – protease inhibitor. VitC – vitamin C.

After 4 months, PI-treated rats displayed decreased body weights compared to controls ($p < 0.05$ vs. control; $p < 0.001$ vs. Jelly; Fig. 7.2.A). Similarly, weight gain was also decreased compared to controls ($p < 0.001$ vs. Control; $p < 0.001$ vs. Jelly; Fig. 7.2.B). The addition of VitC to the PI treatment normalized body weights ($p < 0.001$) and weight gains ($p < 0.01$) when compared to the rats receiving PI treatment alone (Fig. 7.2).

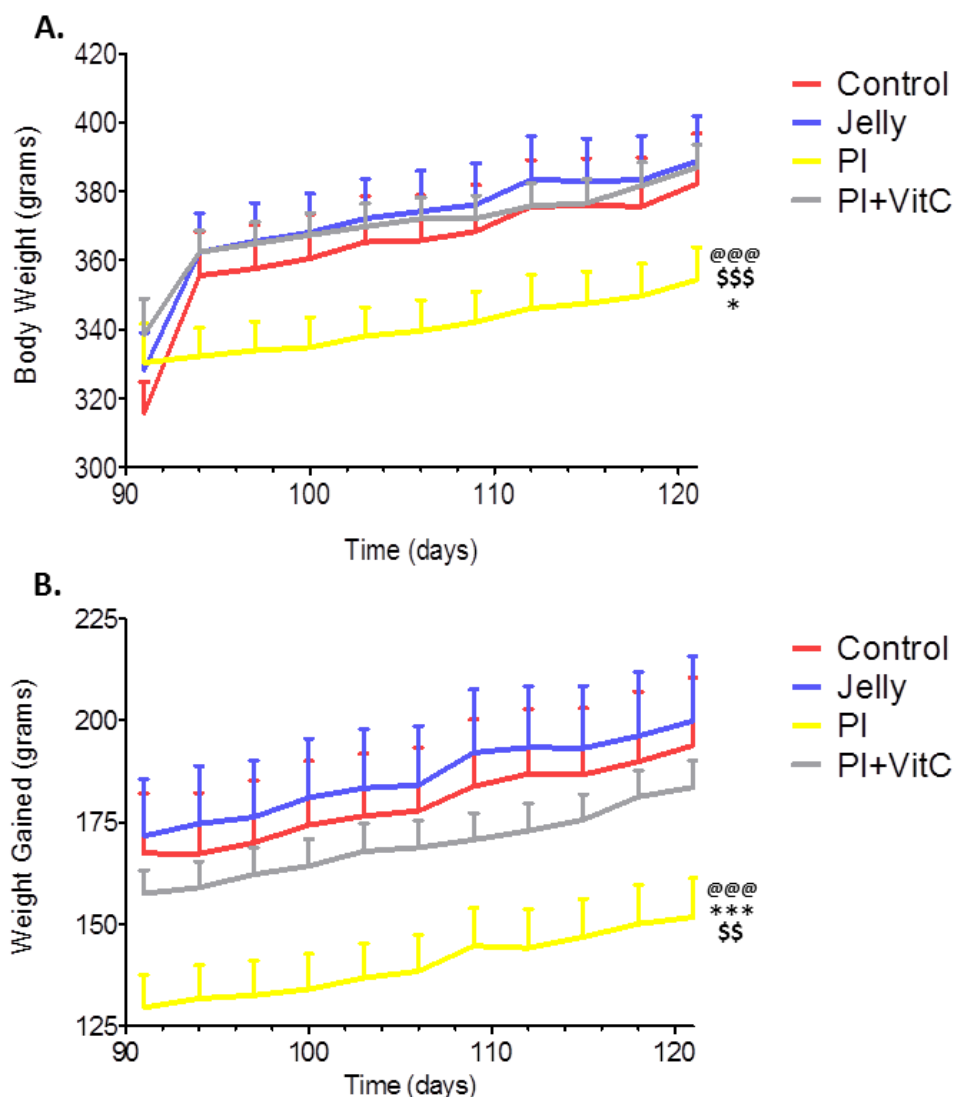


Figure 7. 2. Body weight and net weight gain in male Wistar rats after 4 months of treatment. A) Total body weight over the fourth month treatment period: * $p < 0.05$ vs. control, @@@ $p < 0.001$ vs. jelly, \$\$\$ $p < 0.001$ vs. PI+VitC, $n = 12$. **B)** Weight gained over the fourth month treatment period, as calculated from the starting weight: *** $p < 0.001$ vs. control; @@@ $p < 0.001$ vs. jelly, \$\$ $p < 0.01$ vs. PI+VitC, $n=12$. All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with a Bonferroni post-hoc test. PI - protease inhibitor, VitC - vitamin C.

However, this pattern changed with prolonged treatment with increased body weight found after 6 months ($***p < 0.001$) with protease inhibitors only when compared to control rats (Fig. 7.3.A). Rats receiving jelly also displayed increased body weights ($**p < 0.01$) when compared to control rats (Fig. 7.3.A). Rats receiving VitC in their PI treatment regime also exhibited increased body weights ($^{SS}p < 0.01$) when compared to controls (Fig. 7.3.A), but did not display decreased weight gain ($p < 0.05$) compared to rats receiving only PI (Fig. 7.3.B)

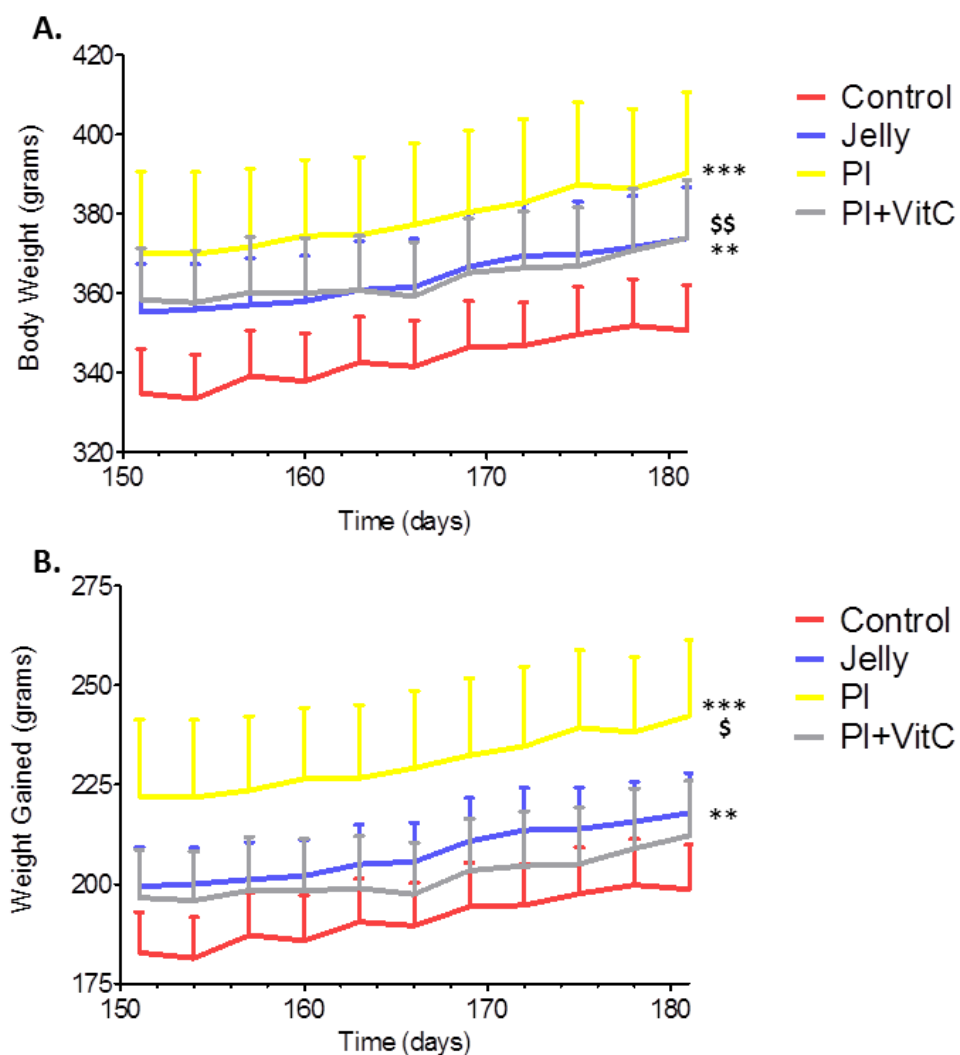


Figure 7.3. Body weight and net weight gain in male Wistar rats after 6 months of treatment. A) Total body weight over sixth months of treatment: $**p < 0.01$ vs. control, $^{SS}p < 0.01$ PI, $***p < 0.001$ vs. control, $n = 6$. **B)** Weight gained over 6 months of treatment, as calculated from the starting weight: $**p < 0.01$ vs. jelly, $***p < 0.001$ vs. control, $^Sp < 0.05$ vs. PI+VitC, $n=6$. All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with a Bonferroni post-hoc test. PI - protease inhibitor, VitC – vitamin C.

4.3.2. Analysis of triglyceride, cholesterol, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) blood levels

In order to establish the effects of treatments on various markers that are commonly related to metabolic disturbances, blood samples were collected at the end of 2 and 4 months of treatment, respectively, for metabolite analysis. Due to lack of samples, these assays were not performed for the 6 month treatment group. After 2 months, no changes were observed in circulating triglyceride and HDL blood levels for all treatment groups (Fig. 7.4. A and C). However, PI-treated rats also receiving VitC displayed increased total cholesterol ($p < 0.05$) and LDL ($p < 0.01$) levels when compared to controls (Fig. 7.4. B and D).

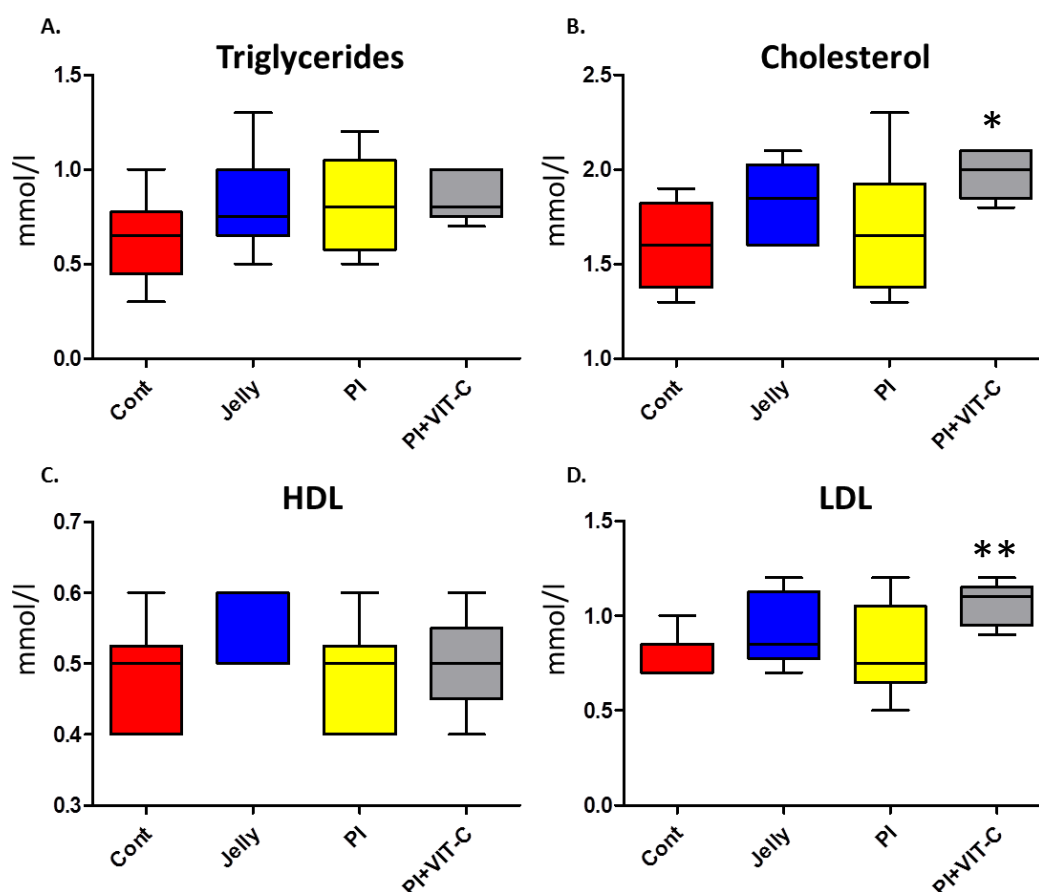


Figure 7.4. Triglyceride, cholesterol, LDL and HDL blood levels in male Wistar rats after 2 months of treatment. **A)** No changes were observed in triglyceride levels in any of the treatment groups, $n = 6$. **B)** Total cholesterol: * $p < 0.05$ vs. control, $n=6$. **C)** No changes were observed in the HDL levels, $n=6$. **D)** LDL: ** $p < 0.01$ vs. control, $n=6$. All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with Bonferroni post-hoc test. PI - protease inhibitor, VitC – vitamin C.

HDL, triglycerides and total cholesterol blood levels remained consistent for all groups after 4 months of PI treatment (Fig. 7.5. A, B and C). However, LDL levels were increased ($p < 0.05$) in PI-treated rats receiving VitC compared to rats receiving PI treatment alone (Fig. 7.5. D).

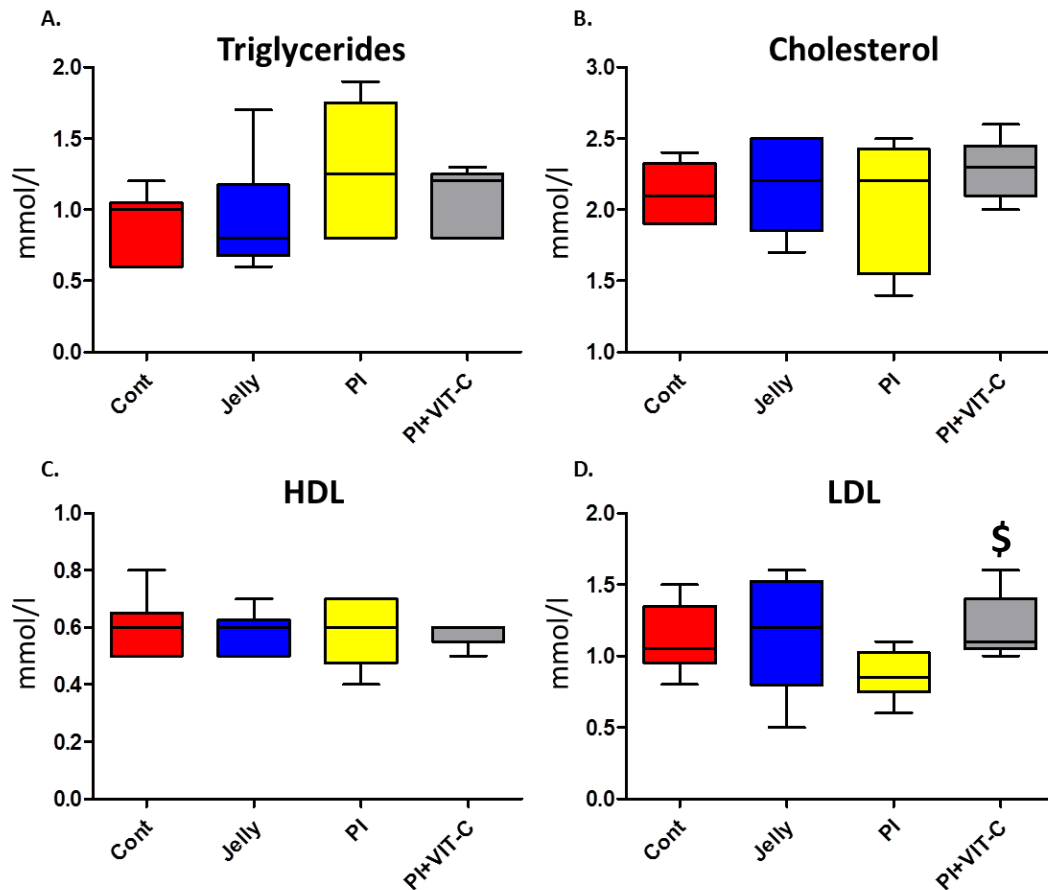


Figure 7.5. Triglyceride, cholesterol, LDL and HDL blood levels in male Wistar rats after 4 months of treatment. A) No changes were observed in triglyceride levels, $n = 6$. **B)** No changes were observed in total cholesterol, $n=6$. **C)** No changes were observed in HDL levels in any of the treatment groups, $n=6$. **D)** LDL: $*p < 0.05$ vs. PI, $n=6$. All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with Bonferroni post-hoc test. PI - protease inhibitor, VitC – vitamin C.

4.3.3. Analysis of mid-wall fractional shortening (MWFS) with the use of echocardiography

In order to establish the effects of each treatment on systolic functioning, various functional parameters were assessed with echocardiography. These results were used to calculate mid-wall fractional shortening at the end of the 2 and 4 month treatment periods. We were not able to attain echocardiographical data at the 6 month time point due to logistical issues.

No significant changes were observed in left ventricular functional parameters after 2 and 4 months of treatment, respectively.

	Control	Jelly	PI	PI+VitC
LVEDD	6.0 ± 0.13	6.0 ± 0.09	6.6 ± 0.08	6.2 ± .13
LVESD	2.9 ± 0.14	3.3 ± 0.14	3.6 ± 0.11	3.5 ± 0.16
LVESPWT	1.9 ± 0.05	1.8 ± 0.05	1.9 ± 0.08	1.8 ± 0.05
LVEDPWT	2.6 ± 0.07	2.4 ± 0.09	2.6 ± 0.11	2.3 ± 0.06
R-R Interval	0.16 ± 0.004	0.17 ± 0.005	0.16 ± 0.005	0.16 ± 0.004

Table 7.1.A. Echocardiographic results after 2 months of treatment

LVEDD – Left Ventricular End Diastolic Diameter; LVESD – Left Ventricular End Systolic Diameter; LVESPWT – Left Ventricular End Systolic Posterior Wall Thickness; LVEDPWT – Left Ventricular End Diastolic Posterior Wall Thickness

	Control	Jelly	PI	PI+VitC
LVEDD	6.4 ± 0.13	6.6 ± 0.16	6.4 ± 0.16	6.8 ± 0.11
LVESD	3.6 ± 0.12	3.5 ± 0.15	3.8 ± 0.14	3.8 ± 0.15
LVESPWT	2.1 ± 0.07	2.0 ± 0.09	1.9 ± 0.04	2.0 ± 0.06
LVEDPWT	2.6 ± 0.1	2.6 ± 0.09	2.4 ± 0.09	2.5 ± 0.07
R-R Interval	0.17 ± 0.004	0.18 ± 0.005	0.18 ± 0.003	0.18 ± 0.003

Table 7.1.B. Echocardiographic results after 4 months of treatment

LVEDD – Left Ventricular End Diastolic Diameter; LVESD – Left Ventricular End Systolic Diameter; LVESPWT – Left Ventricular End Systolic Posterior Wall Thickness; LVEDPWT – Left Ventricular End Diastolic Posterior Wall Thickness

Moreover, no significant changes were observed in MWFS in all groups after 2 and 4 months of treatments, respectively (Fig. 7.6).

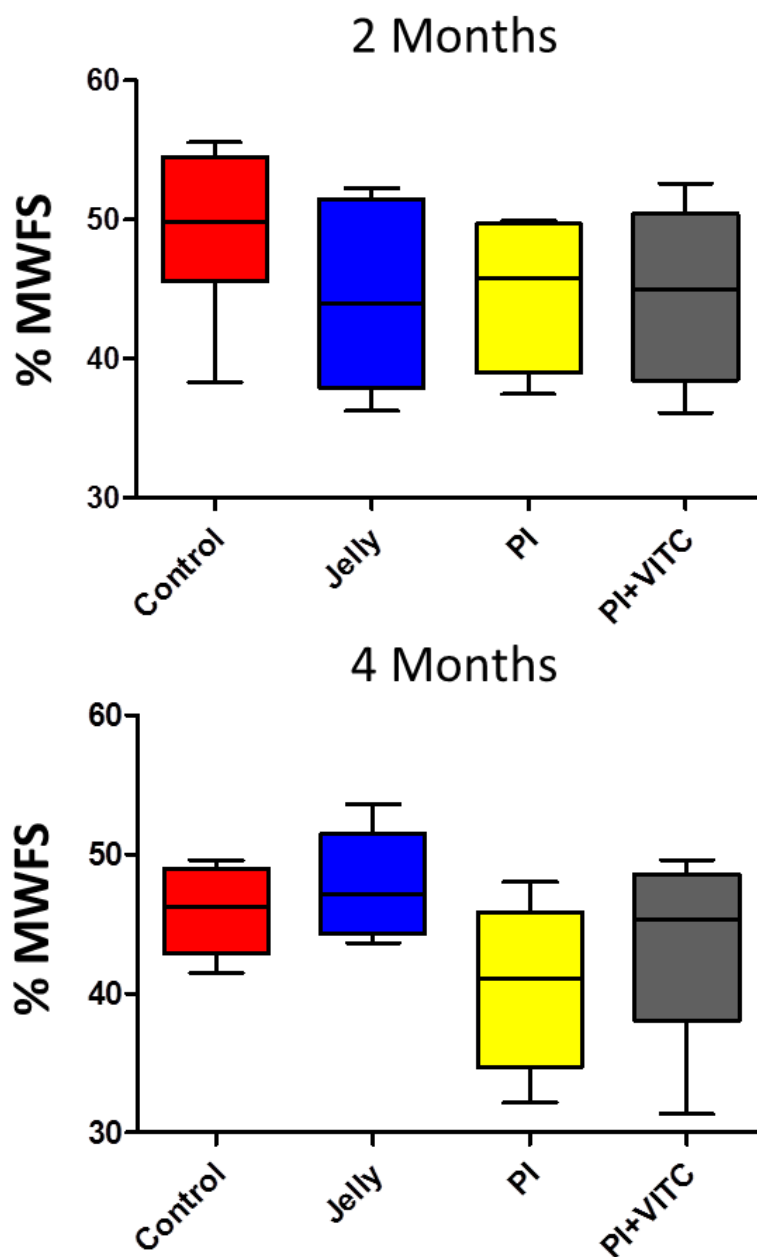


Figure 7.6. MWFS in male Wistar rats after 2 and 4 months of treatment. No significant changes were observed, $n=6$. All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with Bonferroni post-hoc test. PI - protease inhibitor, VitC – vitamin C.

4.3.4. Organ harvesting for the determination of treatment-induced anatomical changes

In order to determine whether the treatments induced any gross anatomical changes, body weight was determined as well as weights of various organs harvested - normalized to tibial length. No significant changes were observed after 2 months of PI treatment (Fig. 7.8.A). However, rats receiving PI in combination with VitC did display decreased heart weights ($p < 0.05$) compared to control rats (Fig. 7.8.A). No significant changes were observed after 4 months of treatment (Fig. 7.8.B), while PI-treated rats displayed increased heart weights after 6 months ($p < 0.05$ vs. controls and Jelly; Fig. 7.8.C).

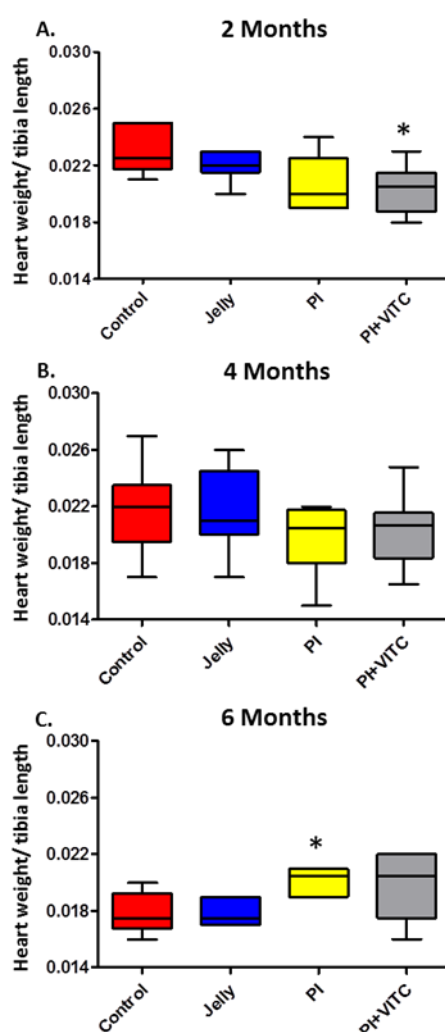


Figure 7.7. Heart weights normalized to right tibia length in male Wistar rats on the day of culling after 2, 4 and 6 months of treatment. 2 months: * $p < 0.05$ vs. control, $n=12$. 4 months: No significant changes were observed, $n=12$. 6 months: * $p < 0.05$ vs. control, $n=12$. All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with Bonferroni post-hoc test. PI - protease inhibitor, VitC – vitamin C.

After 4 months, right ventricular weight was lower in all groups versus the control (Fig. 7.10.A), while left ventricular weight increased in parallel for all groups ($p < 0.05$ and $p < 0.01$ vs. control, Fig. 7.10.A). No changes were observed after 6 months of treatment (Fig. 7.10.B).

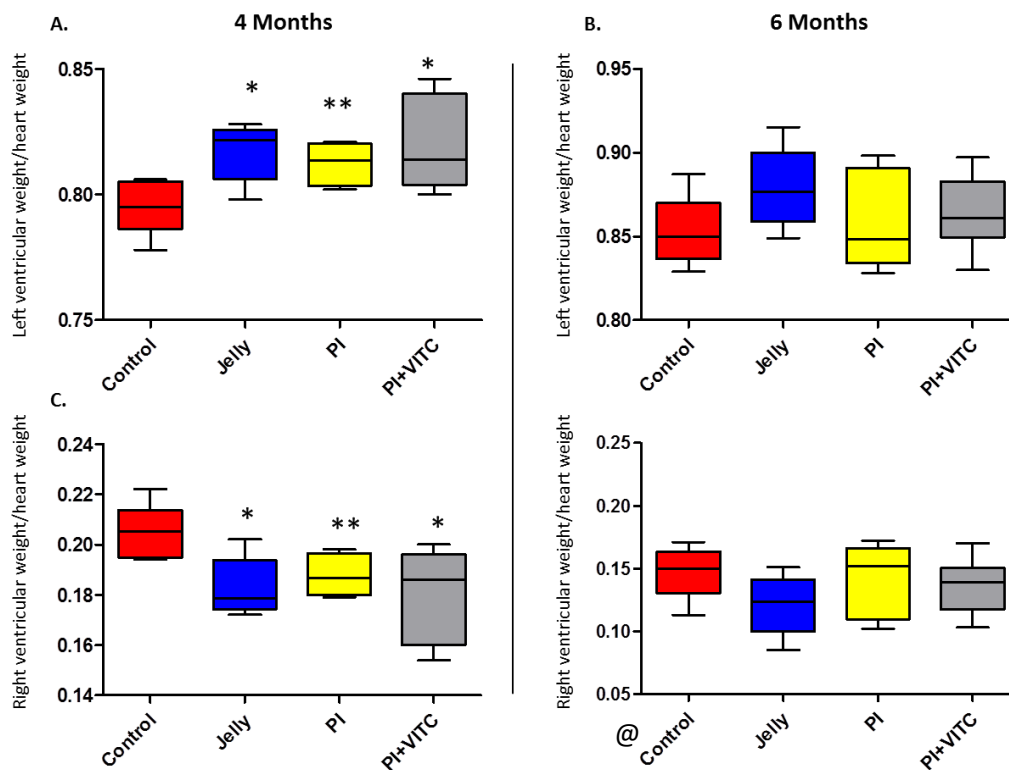


Figure 7.8. Left and right ventricular weights normalized to total heart weight in male Wistar rats on the day of culling after 4 and 6 months of treatment, respectively. **A) 4 months:** * $p < 0.05$ and ** $p < 0.01$ vs. control, $n=12$. **B) 6 months:** No changes were observed in ventricular weights, $n=12$. All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with Bonferroni post-hoc test. PI - protease inhibitor, VitC – vitamin C.

We next evaluated liver weights and retroperitoneal fat mass. Here liver weights remained unchanged for all groups after 2, 4 and 6 months of PI treatment (Fig. 7.9).

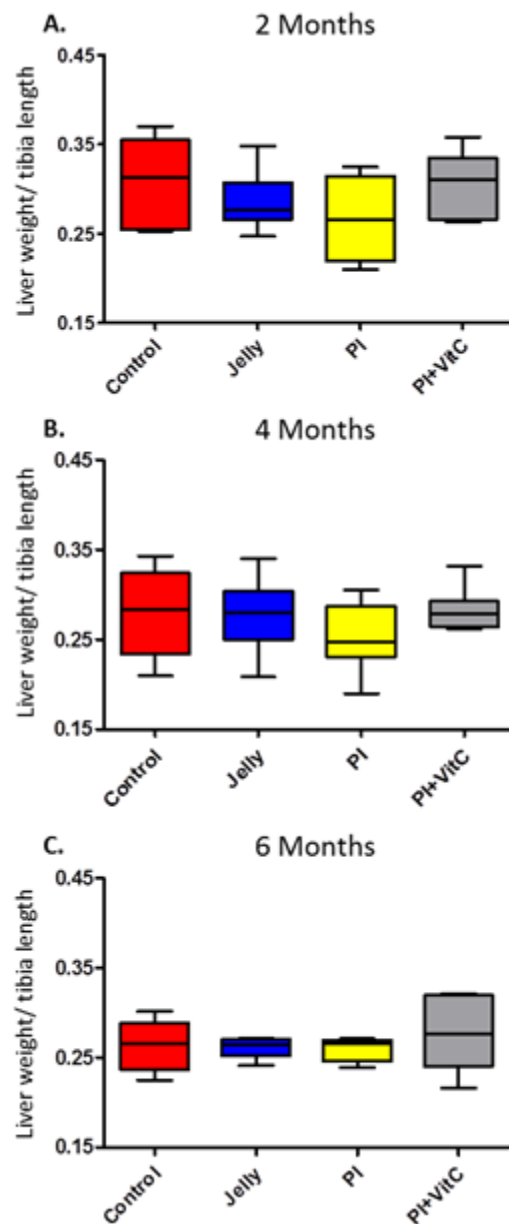


Figure 7.9. Liver weights normalized to right tibia length in male Wistar rats on the day of culling after 2, 4 and 6 months of treatment. **2 months:** No significant changes were observed, $n=12$. **4 months:** PI vs. PI+VitC, $^{\$}p < 0.05$, $n=12$. **6 months:** No significant changes were observed, $n=12$. All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with Bonferroni post-hoc test. PI - protease inhibitor, VitC - vitamin C.

For fat mass assessments, no significant changes were observed after 4 months of PI treatment (Fig. 7.10.A). However, rats that were fed jelly cubes displayed increased retroperitoneal fat mass after 6 months (* $p < 0.05$) compared to control rats (Fig. 7.10.B).

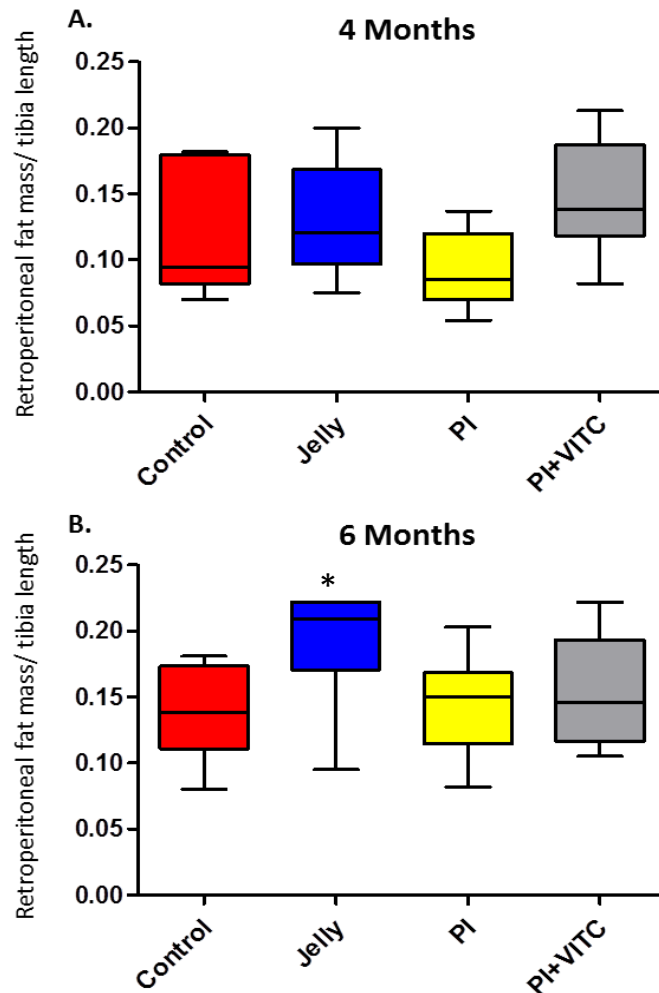


Figure 7.10. Left and right ventricular weights normalized to heart weight in male Wistar rats on the day of culling after 4 and 6 months of treatment. **A) 4 month:** No changes were observed, $n=12$. **B) 6 months:** * $p < 0.05$ v. jelly, $n=12$. All results expressed as mean \pm SEM, statistical analysis: one-way ANOVA with Bonferroni post-hoc test. PI - protease inhibitor, VitC – vitamin C.

4.3.5. Analysis of mitochondrial respiration

Mitochondrial oxygen consumption was next assessed using oxygen electrodes (oxygraph) in sub-sarcolemmal mitochondria after 4 and 6 months of PI treatment, respectively.

For the 4 month treatment period, state III respiration was impaired in isolated mitochondria of PI-treated rats with glutamate and malate as substrates ($p < 0.05$ vs. control, Table 7.2.A). Although state III respiration was diminished with PI treatment, the ADP:O ratio was increased ($p < 0.05$ vs control, Table 7.2.A) indicating that it was respiring with greater efficiency. When malate and pyruvate served as substrates, isolated mitochondria from rats receiving PI+VitC displayed higher respiratory control ratios ($p < 0.05$ vs. jelly, Table 7.2.A) when compared to rats receiving only jelly.

	CONTROL	JELLY	PI	PI+VitC
Glutamate + Malate State III	200 ± 12	248 ± 75	150 ± 13*	180 ± 22
Glutamate + Malate State IV	56 ± 12	97 ± 26	64 ± 5	72 ± 8
Glutamate + Malate RCR	2.53 ± 0.28	2.51 ± 0.21	2.37 ± 0.22	2.52 ± 0.21
Glutamate + Malate ADP:O	2.0 ± 0.1	2.2 ± 0.4	2.7 ± 0.3*	1.8 ± 0.3
Malate + Pyruvate State III	151 ± 19	164 ± 19	166 ± 22	137 ± 11
Malate + Pyruvate State IV	67 ± 10	55 ± 4	63 ± 8	59 ± 5
Malate + Pyruvate State RCR	2.4 ± 0.32	2.95 ± 0.2	2.87 ± 0.4	2.37 ± 0.15@
Malate + Pyruvate State ADP:O	2.78 ± 0.31	2.61 ± 0.30	2.58 ± .029	3.0 ± 0.23
Palmityl – Carnitine + Malate State III	178 ± 31	303 ± 105	172 ± 17	162.7 ± 19
Palmityl – Carnitine + Malate State IV	90 ± 4	93 ± 23	83 ± 9	69 ± 11
Palmityl – Carnitine + Malate RCR	1.96 ± 0.3	3.14 ± 0.51	2.2 ± 0.34	2.58 ± 0.5
Palmityl – Carnitine + Malate ADP:O	2.50 ± 0.39	1.95 ± 0.44	2.43 ± 0.21	2.61 ± 0.33
Rotenone + Succinate State III	285 ± 53	287 ± 74	313 ± 71	254 ± 22
Rotenone + Succinate State IV	146.7 ± 43	126 ± 26	193 ± 65	204 ± 26
Rotenone + Succinate RCR	2.41 ± .59	2.29 ± 0.27	2.22 ± 0.78	1.32 ± 0.19
Rotenone + Succinate ADP:O	1.78 ± 0.53	1.73 ± 0.28	1.62 ± 0.32	1.64 ± 0.14

Table 7.2.A. Function of isolated left ventricular sub-sarcolemmal mitochondria after 4 months of PI treatment.

* $p < 0.05$ vs. control; @ $p < 0.05$ vs. jelly, $n=6$. All results expressed as mean ± SEM, statistical analysis: one-way ANOVA with Bonferroni post-hoc test. PI - protease inhibitor.

For the 6 month time point, State III respiration increased (malate and pyruvate as substrates) ($p < 0.05$, Table 7.2.B) for PI-treated rats that received VitC when compared to controls.

	CONTROL	JELLY	PI	PI+VitC
Glutamate + Malate State III	176 ± 10	167 ± 13	161 ± 16	204 ± 13
Glutamate + Malate State IV	45 ± 5	48 ± 7	43 ± 4	54 ± 6
Glutamate + Malate RCR	4.16 ± 0.46	3.7 ± 0.37	3.8 ± 0.28	3.94 ± 0.44
Glutamate + Malate ADP:O	2.32 ± 0.13	2.47 ± 0.18	2.61 ± 0.26	2.0 ± 0.14
Malate + Pyruvate State III	129 ± 24	157 ± 16	193 ± 28	218 ± 18*
Malate + Pyruvate State IV	38 ± 2	40 ± 6	49 ± 7	54 ± 9
Malate + Pyruvate State RCR	3.97 ± 0.31	4.14 ± 0.28	3.99 ± 0.13	4.5 ± 0.74
Malate + Pyruvate State ADP:O	2.73 ± 0.19	2.66 ± 0.21	2.26 ± 0.28	1.89 ± 0.17
Palmitoyl – Carnitine + Malate State III	150 ± 15	156 ± 8	183 ± 8	173 ± 28
Palmitoyl – Carnitine + Malate State IV	42 ± 6	44 ± 5	51 ± 1	61 ± 13
Palmitoyl – Carnitine + Malate RCR	3.87 ± 0.52	3.71 ± 0.39	3.61 ± 0.25	3.0 ± 0.36
Palmitoyl – Carnitine + Malate ADP:O	2.78 ± 0.24	2.6 ± .14	2.21 ± 0.1	2.48 ± 0.36
Rotenone + Succinate State III	209 ± 47	285 ± 10	201 ± 65	235 ± 22
Rotenone + Succinate State IV	51 ± 16	74 ± 4	51 ± 17	64 ± 5
Rotenone + Succinate RCR	5.03 ± 1.1	3.93 ± 0.28	4.0 ± 0.33	3.7 ± 0.07
Rotenone + Succinate ADP:O	1.69 ± 0.23	1.41 ± 0.05	1.33 ± 0.04	1.72 ± 0.16

Table 7.2.B. Function of isolated left ventricular sub-sarcolemmal mitochondria after 6 months of PI treatment.

* $p < 0.05$ vs. control, $n=6$. All results expressed as mean ± SEM, statistical analysis: one-way ANOVA with Bonferroni post-hoc test. PI - protease inhibitor.

4.4. Discussion

HIV/AIDS has become a global health concern over the past decade. Although HAART is successfully being rolled out, it can also trigger unwanted side-effects such as cardio-metabolic complications (Friis-Møller, 2010; Miro *et al*, 2010). As ROS are implicated in this process, we employed VitC as an antioxidant in our experimental setting. After 4 months of treatment, VitC significantly ameliorated the effects of sole PI treatment on body weight and weight gain (Fig. 7.2. A & B). This may be explained as we have done previously, i.e. the initial weight loss occurring due to increased ROS and that although VitC possesses additional metabolic benefits (Levine *et al*, 1999), most of its benefits accrue due to antioxidant capacity, e.g. decreasing oxidative DNA and/or protein damage, LDL oxidation and lipid peroxidation (Levine *et al*, 1999).

It is our postulate that VitC protected against PI-mediated mitochondrial ROS production. This is in keeping with the notion that PI treatment can lead to mitochondrial dysfunction (direct or indirect) that in turn enhances mitochondrial ROS production (Reyskens and Essop, 2013). Increased ROS production can lead to serious intracellular damaging effects as previously discussed. In addition, Pérez-Matute and colleagues (2013) postulated that this may cause PI-mediated lipodystrophy since PAT and VAT possess different amounts of mitochondria (Pérez-Matute *et al*, 2013). They propose that PAT (contains more mitochondria) can produce more ROS than VAT thereby leading to greater oxidative stress, inflammation, and apoptosis to decrease peripheral fat mass (Zha *et al*, 2011). Mitochondrial dysfunction seem like a plausible cause since Shikuma and colleagues (2001) also found decreased mitochondrial DNA in subcutaneous fat of HIV patients with ARV-induced peripheral lipodystrophy, when compared to HIV positive controls (Shikuma *et al*, 2001). This could possibly occur due to the effects of ROS on the mitochondria or due to direct effects of PI treatment on mitochondria. We therefore postulate that excess ROS lead to damage and subsequent cell death in especially peripheral adipose tissues and that VitC inhibited this from occurring through its oxidant scavenging potential.

After 6 months of treatment the weight results resembled that of the other interventions, i.e. lowering PI-mediated body weight and weight gains (Fig. 7.3). This suggests that VitC may be effective against both the early weight loss (4 month time point) and following weight gain (6 month time point). An alternative mechanism whereby VitC may achieve its effects could be by increasing thyroid hormone production. In support, Feuring and colleagues (2011) fed guinea-pigs a diet containing low or VitC doses for 6-8 weeks (Feuring *et al*, 2011). Here they found a significant increase in body weight with high dose supplementation together with a significant increase in liver mitochondrial number. Moreover, these mitochondria were also morphologically smaller compared

to the low VitC dose group (Feuring et al, 2011). VitC supplementation can also lead to increased serum thyroxine levels (Degkwitz and Bodeker, 1993) that can increase mitochondrial oxygen consumption (Gross, 1971) and ATP production (Seitz et al, 1985). This can occur directly via its binding to target sites on the mitochondrial inner-membrane (Sterling et al, 1978; Azimova et al, 1984). Moreover, relatively low clinical levels of thyroid hormone have been found in HIV-positive patients on HAART (Madeddu et al, 2006). Thus these data support a wider role for VitC than a mere antioxidant, although more clinical studies are required to test the veracity of such early findings.

Organ weights followed a similar trend as with RSV and ASP treatments, i.e. after 2 months PI + VitC total heart weights were lower versus the control group (Fig. 7.7.A). No differences in total heart weights were observed after 4 months of treatment (Fig. 7.7.B). However, the addition of VitC to PI treatment was effective at inhibiting the PI-mediated increase in heart weight after 6 months (Fig. 7.7.C).

After 4 months of VitC treatment, cholesterol and LDL levels were increased versus controls (Fig. 7.4), while after 6 months only LDL levels remained elevated (Fig. 7.5). This finding is difficult to explain as it is contradictory to what was expected, e.g. VitC supplementation has been implicated in decreasing plasma LDL levels (Ginter et al, 1969; Ginter et al, 1970; Ginter et al, 1973). There is no information in the literature to support our findings and we therefore speculate the involvement of reverse feedback regulation of sterol synthesis. Sterol synthesis is regulated via 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) (Brown and Goldstein, 1980) and the latter catalyzes the rate-limiting step in the mevalonate pathway that regulates cholesterol synthesis. We postulate that this pathway is likely to be activated in our model, i.e. rats treated with PI + VitC. However, further studies are required to investigate such proposals. In addition, blood metabolites could also be analyzed at more regular time points during the treatment regimen to assess whether cholesterol levels fluctuate as would be expected when feedback regulation is involved.

After 4 months of treatment, VitC ameliorated the PI-mediated decrease in State III respiration when glutamate and malate was used as substrates, implying that PI-induced effects may occur due to excessive ROS production (Table 7.2.A). VitC could thus have normalized mitochondrial respiration by scavenging ROS, suggesting that our findings RSV and ASP were not due to antioxidant effects. However, further studies are required to investigate these options.

Conclusion

Our study reveals that PI treatment triggers detrimental effects on body weight gain and mitochondrial respiration function. However, we did not observe significant changes in terms of dyslipidemia and heart function. Thus our newly established in vivo model of PI treatment recapitulates a moderate, and early stage in the progression of PI-mediated cardio-metabolic complications. These data therefore identify the earlier changes in this timeline thus offering attractive therapeutic targets, especially at the mitochondrial level. The interventions employed generally showed promise in this regard by targeting body weight changes and attenuating mitochondrial dysfunction. VitC treatment resulted in altered lipid levels that may prove damaging in the long run and therefore RSV and ASP emerge as co-treatments that offer promise for HIV-positive patients.

Limitations

There are species-specific differences in terms of metabolic pathways, e.g. the enzyme responsible for acetyl salicylate metabolism where humans use Cytochrome P450 2A (CYP2A) while rats rely on Cytochrome P450 3A (CYP3A). This is problematic as CYP3A is also used for the metabolism of both lopinavir and ritonavir and potentially result in drug-drug interactions that would not be the case in humans. In addition, as rats here employed were not HIV positive, we were unable to study the effects of the virus in combination with the PI treatment.

The number of rats per cage could also have a dramatic effect on weight gain, where the strict hierarchy could result in differences between weight gains. A further potential limiting factor is that steady state concentrations of PIs were not tested in this study. This would have been a good method to assess whether dosages reached the same plasma levels as for HIV-positive individuals on Alluvia™ treatment, while also assessing the accuracy of the BSA dose translation formula in our setting. Our initial plan was to use liquid chromatography/mass spectrometry to assess circulating lopinavir levels but we required purified compound for this purposes. We attempted to source this from local pharmaceutical companies but did not receive any cooperation and were thus unsuccessful in this regard.

The fact that we could perform all the techniques at all of the experimental time points is a limitation. However, we are of the opinion that this is not detrimental in proving our hypothesis for the following reasons: a) as the echocardiography data show no differences at 2 or 4 months

treatment indicates that PIs did not affect heart function during the early stages of this experiment; and b) recording the echocardiography data together with blood metabolite levels confirmed the lack of risk for metabolic syndrome after 2 and 4 months, respectively.

References

- Allard, J.P. 1998. Effects of Vitamin E and C Supplementation on Oxidative Stress and Viral Load in HIV-Infected Subjects. *Aids*, 12(13). :1653-1659.
- Amat, R., Planavila, A., Chen, S.L., Iglesias, R., Giralt, M. & Villarroya, F. 2009. SIRT1 Controls the Transcription of the Peroxisome Proliferator-Activated Receptor-Gamma Co-Activator-1alpha (PGC-1alpha) Gene in Skeletal Muscle through the PGC-1alpha Autoregulatory Loop and Interaction with MyoD. *The Journal of biological chemistry*, 284(33). :21872.
- Ammassari, A. 2002. Correlates and Predictors of Adherence to Highly Active Antiretroviral Therapy: Overview of Published Literature. *Journal of acquired immune deficiency syndromes (1999)*, 31:S123-7.
- Andrade-Oliveira, V., Câmara, N.O. & Moraes-Vieira, P.M. 2015. Adipokines as Drug Targets in Diabetes and Underlying Disturbances. *Journal of diabetes research*, 2015
- Andreyev, A.Y., Kushnareva, Y.E. & Starkov, A. 2005. Mitochondrial Metabolism of Reactive Oxygen Species. *Biochemistry (Moscow)*, 70(2). :200-214.
- Angelini, C., Federico, A., Reichmann, H., Lombes, A., Chinnery, P. & Turnbull, D. 2011. Fatty Acid Mitochondrial Disorders. *European Handbook of Neurological Management, Second Edition, Volume 1, Second Edition*, :501-511.
- Arechaga, I., Ledesma, A. & Rial, E. 2001. The Mitochondrial Uncoupling Protein UCP1: A Gated Pore. *IUBMB life*, 52(3-5). :165-173.
- Arenas-Pinto, A., Milinkovic, A., Peppas, D., McKendry, A., Maini, M. & Gilson, R. 2015. Systemic Inflammation and Residual Viraemia in HIV-Positive Adults on Protease Inhibitor Monotherapy: A Cross-Sectional Study. *BMC infectious diseases*, 15(1). :138.
- Arner, P., Hellstrom, L., Wahrenberg, H. & Bronnegard, M. 1990. Beta-Adrenoceptor Expression in Human Fat Cells from Different Regions. *The Journal of clinical investigation*, 86(5). :1595-1600.
- Arribas, J.R. 2009. Lopinavir-Ritonavir Monotherapy Versus Lopinavir-Ritonavir and 2 Nucleosides for Maintenance Therapy of HIV: 96-Week Analysis. *JAIDS Journal of Acquired Immune Deficiency Syndromes*, 51(2). :147.
- Arribas, J.R. 2005. Lopinavir/ritonavir as Single-Drug Therapy for Maintenance of HIV-1 Viral Suppression: 48-Week Results of a Randomized, Controlled, Open-Label, Proof-of-Concept Pilot Clinical Trial (OK Study). *JAIDS Journal of Acquired Immune Deficiency Syndromes*, 40(3). :280.
- Azimova, S., Umarova, G.D., Petrova, O.S., Tukhtaev, K.R. & Abdugarimov, A. 1984. The Nature of Thyroid Hormone Receptors. Translocation of Thyroid Hormones through Plasma Membranes. *Biokhimiia (Moscow, Russia)*, 49(8). :1350-1356.
- Bacchetti, P. 2005. Fat Distribution in Men with HIV Infection. *Journal of acquired immune deficiency syndromes*, 40(2). :121-131.

- Badiou, S., Merle De Boever, C., Dupuy, A., Baillat, V., Cristol, J. & Reynes, J. 2003. Decrease in LDL Size in HIV-Positive Adults before and After lopinavir/ritonavir-Containing Regimen: An Index of Atherogenicity? *Atherosclerosis*, 168(1). :107-113.
- Ballinger, S.W. 2005. Mitochondrial Dysfunction in Cardiovascular Disease. *Free Radical Biology and Medicine*, 38(10). :1278-1295.
- Bastard, J. 2002. Association between Altered Expression of Adipogenic Factor SREBP1 in Lipoatrophic Adipose Tissue from HIV-1-Infected Patients and Abnormal Adipocyte Differentiation and Insulin Resistance. *The Lancet*, 359(9311). :1026-1031.
- Baur, J.A. 2006. Resveratrol Improves Health and Survival of Mice on a High-Calorie Diet. *Nature*, 444(7117). :337-342.
- Bhangoo, A. & Desai, N. 2013. Endocrinopathies in HIV, AIDS and HAART. *Reviews in Endocrine and Metabolic Disorders*, 14(2). :101-103.
- Bierman, W.F., Humphreys, E.H., van Agtmael, M.A., Boucher, C. & Rutherford, G.W. 2010. Ritonavir-Boosted Protease Inhibitor Monotherapy is 6% Less Effective than Combination Antiretroviral Therapy in a Meta-Analysis. *Journal of the International AIDS Society*, 13(Suppl 4). :O18.
- Bradford, M.M. 1976. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Analytical Biochemistry*, 72(1). :248-254.
- Bradshaw, D. 2012. Cause of Death Statistics for South Africa: Challenges and Possibilities for Improvement. *Cape Town, SA: Medical Research Council*, :18-20.
- Braidy, N. Age Related Changes in NAD⁺ Metabolism Oxidative Stress and Sirt1 Activity in Wistar Rats. *PLoS ONE*, 6(4).
- Bremer, J. 2001. The Biochemistry of Hypo-and Hyperlipidemic Fatty Acid Derivatives: Metabolism and Metabolic Effects. *Progress in lipid research*, 40(4). :231-268.
- Bremer, J. & Wojtczak, A.B. 1972. Factors Controlling the Rate of Fatty Acid β -Oxidation in Rat Liver Mitochondria. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism*, 280(4). :515-530.
- Brown, M.S. & Goldstein, J.L. 1980. Multivalent Feedback Regulation of HMG CoA Reductase, a Control Mechanism Coordinating Isoprenoid Synthesis and Cell Growth. *Journal of lipid research*, 21(5). :505-517.
- Buettner, G.R. 2011. Superoxide Dismutase in Redox Biology: The Roles of Superoxide and Hydrogen Peroxide. *Anti-cancer agents in medicinal chemistry*, 11(4). :341.
- Caballero, A.E. 2003. Endothelial Dysfunction in Obesity and Insulin Resistance: A Road to Diabetes and Heart Disease. *Obesity research*, 11(11). :1278-1289.
- Calza, L., Manfredi, R. & Verucchi, G. 2010. Myocardial Infarction Risk in HIV-Infected Patients: Epidemiology, Pathogenesis, and Clinical Management. *Aids*, 24(6). :789.
- Cantó, C. & Auwerx, J. 2009. PGC-1 [Alpha], SIRT1 and AMPK, an Energy Sensing Network that Controls Energy Expenditure. *Current opinion in lipidology*, 20(2). :98.

- Capeau, J. 2005. Pathogenesis of HAART-associated metabolic syndrome, *Cardiovascular disease in AIDS*. Springer. : 17-32.
- Capeau, J. 2012. Ten-Year Diabetes Incidence in 1046 HIV-Infected Patients Started on a Combination Antiretroviral Treatment. *AIDS (London, England)*, 26(3). :303-314.
- Caron-Debarle, M., Lagathu, C., Boccara, F., Vigouroux, C. & Capeau, J. 2010. HIV-Associated Lipodystrophy: From Fat Injury to Premature Aging. *Trends in molecular medicine*, 16(5). :218-229.
- Carr, A.C. & Frei, B. 1999. Toward a New Recommended Dietary Allowance for Vitamin C Based on Antioxidant and Health Effects in Humans. *The American Journal of Clinical Nutrition*, 69(6). :1086-1107.
- Carr, A. & Cooper, D.A. 2000. Adverse Effects of Antiretroviral Therapy. *The Lancet*, 356(9239). :1423-1430.
- Carvalho, L.G.d. 2013. Evaluation of Thyroid Function and Autoimmunity in HIV-Infected Women. *Arquivos Brasileiros de Endocrinologia & Metabologia*, 57(6). :450-456.
- Chang, T.K.H. & Yeung, R.K.Y. 2001. Effect of Trans-Resveratrol on 7-Benzyloxy-4-Trifluoromethylcoumarin O-Dealkylation Catalyzed by Human Recombinant CYP3A4 and CYP3A5. *Canadian journal of physiology and pharmacology*, 79(3). :220-226.
- Chastain, D.B., Henderson, H. & Stover, K.R. 2015. Epidemiology and Management of Antiretroviral-Associated Cardiovascular Disease. *The open AIDS journal*, 9:23.
- Chauvin, B., Drouot, S., Barrail-Tran, A. & Taburet, A. 2013. Drug–Drug Interactions between HMG-CoA Reductase Inhibitors (Statins) and Antiviral Protease Inhibitors. *Clinical pharmacokinetics*, :1-17.
- Chen, L., Magliano, D.J. & Zimmet, P.Z. 2012. The Worldwide Epidemiology of Type 2 Diabetes mellitus—present and Future Perspectives. *Nature Reviews Endocrinology*, 8(4). :228-236.
- Cheng, Y., Gao, W., Chen, C., Vazquez-padua, M. & Starnes, M.C. 1990. DNA Polymerases Versus HIV Reverse Transcriptase in AIDS Therapy. *Annals of the New York Academy of Sciences*, 616(1). :217-223.
- Cho, K.W. & Lumeng, C.N. SirT1: A Guardian at the Gates of Adipose Tissue Inflammation.(COMMENTARY). *Diabetes*, 60(12). :3100.
- Chung, J.H., Manganiello, V. & Dyck, J.R. 2012. Resveratrol as a Calorie Restriction Mimetic: Therapeutic Implications. *Trends in cell biology*, 22(10). :546-554.
- Clarke, S.D. 2012. Postprandial Heat Production in Skeletal Muscle is Associated with Altered Mitochondrial Function and Altered Futile Calcium Cycling. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 303(10). :1071-1079.
- Clay Montier, L.L., Deng, J.J. & Bai, Y. 2009. Number Matters: Control of Mammalian Mitochondrial DNA Copy Number. *Journal of genetics and genomics*, 36(3). :125-131.
- Colvin, C.J. 2010. Expanding Access to ART in South Africa: The Role of Nurse Initiated Treatment. *SAMJ: South African Medical Journal*, 100(4). :210-212.

- Coopusamy, D. 2010. The HIV-1 glycoprotein gp120 elevates NF-kB levels in human cardiomyocytes which may be reversed with the treatment of a sesquiterpene lactone isolated from *Vernonia staeheleinoides*, *mobile.wiredspace.wits.ac.za*.
- Coyne-Meyers, K. & Trombley, L.E. 2004. A Review of Nutrition in Human Immunodeficiency Virus Infection in the Era of Highly Active Antiretroviral Therapy. *Nutrition in clinical practice*, 19(4). :340-355.
- Cui, L., Locatelli, L., Xie, M. & Sommadossi, J. 1997. Effect of Nucleoside Analogs on Neurite Regeneration and Mitochondrial DNA Synthesis in PC-12 Cells. *Journal of Pharmacology and Experimental Therapeutics*, 280(3). :1228-1234.
- Cummins, N. & Badley, A. 2010. Mechanisms of HIV-Associated Lymphocyte Apoptosis: 2010. *Cell death & disease*, 1(11). :e99.
- De Bruto, P.,C. 2006. *ART-related body composition changes in adult women in a semi-rural south african context*. Unpublished thesis. Assignment (MPhil)--University of Stellenbosch, 2006.
- Deeks, S.G. & Phillips, A.N. 2009. CLINICAL REVIEW-HIV Infection, Antiretroviral Treatment, Ageing, and Non-AIDS Related Morbidity. *BMJ (CR)-print*, 338(7689).
- Degkwitz, E. & Bodeker, R.H. 1993. Characterization of Guinea Pigs After Adaptation to Different High Vitamin C Supplies. 3. Microsomal Cytochromes in the Liver and Kidney. *Zeitschrift fur Ernahrungswissenschaft*, 32(3). :198-208.
- Deng, W., Baki, L., Yin, J., Zhou, H. & Baumgarten, C.M. 2010. HIV Protease Inhibitors Elicit Volume-Sensitive Cl⁻ Current in Cardiac Myocytes Via Mitochondrial ROS. *Journal of Molecular and Cellular Cardiology*, 49(5). :746-752.
- Denu, J.M. 2012. Fortifying the Link between SIRT1, Resveratrol, and Mitochondrial Function. *Cell metabolism*, 15(5). :566-567.
- Desforges, J.F., Grinspoon, S.K. & Bilezikian, J.P. 1992. HIV Disease and the Endocrine System. *New England Journal of Medicine*, 327(19). :1360-1365.
- Dinakar, C., Abhaypratap, V., Yearla, S.R., Raghavendra, A.S. & Padmasree, K. 2010. Importance of ROS and Antioxidant System during the Beneficial Interactions of Mitochondrial Metabolism with Photosynthetic Carbon Assimilation. *Planta*, 231(2). :461-474.
- Divakaruni, A.S., Humphrey, D.M. & Brand, M.D. 2012. Fatty Acids Change the Conformation of Uncoupling Protein 1 (UCP1). *Journal of Biological Chemistry*, 287(44). :36845-36853.
- Doyle, T. & Geretti, A.M. 2012. Low-Level Viraemia on HAART: Significance and Management. *Current opinion in infectious diseases*, 25(1). :17.
- Dragović, G. 2015. The Role of Adipokines in HIV Lipodystrophy. *Germs*, 5(3). :64.
- Duong, M. 2001. Coronary Heart Disease Associated with the use of Human Immunodeficiency Virus (HTV)-1 Protease Inhibitors: Report of Four Cases and Review. *Clinical cardiology*, 24(10). :690-694.

- Edwards, J., Beck, M., Riegger, C. & Bausch, J. 2011. Safety of Resveratrol with Examples for High Purity, trans-resveratrol, resVida>>. *Annals of the New York Academy of Sciences*, 1215(1). :131-137.
- Fajas, L. 1999. Regulation of Peroxisome Proliferator-Activated Receptor γ Expression by Adipocyte Differentiation and Determination Factor 1/sterol Regulatory Element Binding Protein 1: Implications for Adipocyte Differentiation and Metabolism. *Molecular and cellular biology*, 19(8). :5495-5503.
- Fauci, A.S. 2003. HIV and AIDS: 20 Years of Science. *Nature medicine*, 9(7). :839-843.
- Feinberg, J., Bernstein, B. and King, M. 2002. Once Daily Vs. Twice Daily Kaletra (lopinavir/ritonavir) in Antiretroviral-Naïve HIV Patients: 72-Week Follow-Up. Paper presented at XIV International AIDS Conference, Barcelona, Spain.
- Ferguson, M.R., Rojo, D.R., von Lindern, J.J. & O'Brien, W.A. 2002. HIV-1 Replication Cycle. *Clinics in laboratory medicine*, 22(3). :611-636.
- Feuring, M., Schultz, A. & Hersemeyer, K. 2011. Long-Term, High Intake of Vitamin C Decreases Size and Increases Quantity of Liver Mitochondria in Guinea-Pigs. *Journal of International Medical Research*, 39(6). :2330-2334.
- Fink, B.D., Herlein, J.A., Almind, K., Cinti, S., Kahn, C.R. & Sivitz, W.I. 2007. Mitochondrial Proton Leak in Obesity-Resistant and Obesity-Prone Mice. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 293(5). :R1773-R1780.
- Finkel, T. & Holbrook, N.J. 2000. Oxidants, Oxidative Stress and the Biology of Ageing. *Nature*, 408(6809). :239-247.
- Finsterer, J. 2004. Mitochondriopathies. *European Journal of Neurology*, 11(3). :163-186.
- Fisher, S.D. 2005. Mild Dilated Cardiomyopathy and Increased Left Ventricular Mass Predict Mortality: The Prospective P 2 C 2 HIV Multicenter Study. *American Heart Journal*, 150(3). :439-447.
- Flint, O.P. 2009. The Role of Protease Inhibitors in the Pathogenesis of HIV-Associated Lipodystrophy: Cellular Mechanisms and Clinical Implications. *Toxicologic pathology*, 37(1). :65-77.
- Förstermann, U. 2010. Nitric Oxide and Oxidative Stress in Vascular Disease. *Pflügers Archiv-European Journal of Physiology*, 459(6). :923-939.
- Frankel, A.D. & Young, J.A.T. 1998. HIV-1: Fifteen Proteins and an RNA. *Annual Review of Biochemistry*, 67(1). :1-25.
- Freitas, P. 2011. Impact of Lipodystrophy on the Prevalence and Components of Metabolic Syndrome in HIV-Infected Patients. *BMC infectious diseases*, 11(1). :246.
- Friis-Møller, N. 2010. Predicting the Risk of Cardiovascular Disease in HIV-Infected Patients: The Data Collection on Adverse Effects of Anti-HIV Drugs Study. *European Journal of Cardiovascular Prevention & Rehabilitation*, 17(5). :491-501.

- Fullerton, M.D. & Steinberg, G.R. 2010. SIRT1 Takes a Backseat to AMPK in the Regulation of Insulin Sensitivity by Resveratrol. *Diabetes*, 59(3). :551-553.
- Gardner, K., Hall, P.A., Chinnery, P.F. & Payne, B.A. 2013. HIV Treatment and Associated Mitochondrial Pathology: Review of 25 Years of in Vitro, Animal, and Human Studies. *Toxicologic pathology*.
- Garrabou, G. 2008. Mitochondrial Toxicity of Antiretrovirals in Non-HIV-Infected Patients. *Journal of the International AIDS Society*, 11:P146.
- Gehm, B.D., McAndrews, J.M., Chien, P.Y. & Jameson, J.L. 1997. Resveratrol, a Polyphenolic Compound found in Grapes and Wine, is an Agonist for the Estrogen Receptor. *Proceedings of the National Academy of Sciences*, 94(25). :14138.
- Ghosn, J. 2010. Long-term (96-week) follow-up of antiretroviral-naïve HIV-infected Patients Treated with first-line lopinavir/ritonavir Monotherapy in the MONARK Trial*. *HIV medicine*, 11(2). :137-142.
- Ginter, E., Babala, J. & Červeň, J. 1969. The Effect of Chronic Hypovitaminosis C on the Metabolism of Cholesterol and Atherogenesis in Guinea Pigs. *Journal of Atherosclerosis Research*, 10(3). :341-352.
- Ginter, E., Kajaba, I. & Nizner, O. 1970. The Effect of Ascorbic Acid on Cholesterolemia in Healthy Subjects with Seasonal Deficit of Vitamin C. *Annals of Nutrition and Metabolism*, 12(2). :76-86.
- Ginter, E. 1973. Cholesterol: Vitamin C Controls its Transformation to Bile Acids. *Science*, 179(4074). :702-704.
- Green, D.R. & Reed, J.C. 1998. Mitochondria and Apoptosis. *Science-AAAS-Weekly Paper Edition*, 281(5381). :1309-1311.
- Gross, N.J. 1971. Control of Mitochondrial Turnover Under the Influence of Thyroid Hormone. *The Journal of cell biology*, 48(1). :29-40.
- Grundy, S.M. 2011. *Atlas of atherosclerosis and metabolic syndrome*. Springer Science & Business Media
- Gupta, R.K. 2013. Oral Antiretroviral Drugs as Public Health Tools for HIV Prevention: Global Implications for Adherence, Drug Resistance, and the Success of HIV Treatment Programs. *Journal of Infectious Diseases*, 207(suppl 2). :S101-S106.
- Gupta, S., Knight, A.G., Losso, B.Y., Ingram, D.K., Keller, J.N. & Bruce-Keller, A.J. 2012. Brain Injury Caused by HIV Protease Inhibitors: Role of Lipodystrophy and Insulin Resistance. *Antiviral Research*, 95(1). :19-29.
- Hall, K.D. 2011. Quantification of the Effect of Energy Imbalance on Bodyweight. *The Lancet*, 378(9793). :826-837.
- Hansen, J.B., Zhang, H., Rasmussen, T.H., Petersen, R.K., Flindt, E.N. & Kristiansen, K. 2001. Peroxisome Proliferator-Activated Receptor δ (PPAR δ)-Mediated Regulation of Preadipocyte Proliferation and Gene Expression is Dependent on cAMP Signaling. *Journal of Biological Chemistry*, 276(5). :3175-3182.

- Hardie, D.G. 2007. AMP-activated/SNF1 Protein Kinases: Conserved Guardians of Cellular Energy. *Nature Reviews Molecular Cell Biology*, 8(10). :774-785.
- Hawkins, T. 2006. Appearance-Related Side Effects of HIV-1 Treatment. *AIDS Patient Care & STDs*, 20(1). :6-18.
- Hawley, S.A. 2012. The Ancient Drug Salicylate Directly Activates AMP-Activated Protein Kinase. *Science*.
- Heilbronn, L.K., Gan, S.K., Turner, N., Campbell, L.V. & Chisholm, D.J. 2007. Markers of Mitochondrial Biogenesis and Metabolism are Lower in Overweight and Obese Insulin-Resistant Subjects. *Journal of Clinical Endocrinology & Metabolism*, 92(4). :1467-1473.
- Hellmer, J., Marcus, C., Sonnenfeld, T. & Arner, P. 1992. Mechanisms for Differences in Lipolysis between Human Subcutaneous and Omental Fat Cells. *The Journal of clinical endocrinology and metabolism*, 75(1). :15-20.
- Ho, J.E. & Hsue, P.Y. 2009. Cardiovascular Manifestations of HIV Infection. *Heart*, 95(14). :1193-1202.
- Hober, D., Haque, A., Wattre, P., Beaucaire, G., Mouton, Y. & Capron, A. 1989. Production of Tumour Necrosis Factor-Alpha (TNF-Alpha) and Interleukin-1 (IL-1) in Patients with AIDS. Enhanced Level of TNF-Alpha is Related to a Higher Cytotoxic Activity. *Clinical and experimental immunology*, 78(3). :329.
- Hock, M.B. & Kralli, A. 2009. Transcriptional Control of Mitochondrial Biogenesis and Function. *Annual Review of Physiology*, 71:177-203.
- Hong-Brown, L.Q., Brown, C.R., Huber, D.S. & Lang, C.H. 2008. Lopinavir Impairs Protein Synthesis and Induces eEF2 Phosphorylation Via the Activation of AMP-activated Protein Kinase. *Journal of cellular biochemistry*, 105(3). :814-823.
- Hossain, P., Kavar, B. & El Nahas, M. 2007. Obesity and Diabetes in the Developing world—a Growing Challenge. *New England Journal of Medicine*, 356(3). :213-215.
- Hwang, J.T., Kwon, D.Y., Park, O.J. & Kim, M.S. 2008. Resveratrol Protects ROS-Induced Cell Death by Activating AMPK in H9c2 Cardiac Muscle Cells. *Genes & nutrition*, 2(4). :323-326.
- Ibrahim, M.M. 2010. Subcutaneous and Visceral Adipose Tissue: Structural and Functional Differences. *Obesity reviews*, 11(1). :11-18.
- Imbeault, P., Couillard, C., Tremblay, A., Despres, J.P. & Mauriege, P. 2000. Reduced Alpha(2)-Adrenergic Sensitivity of Subcutaneous Abdominal Adipocytes as a Modulator of Fasting and Postprandial Triglyceride Levels in Men. *Journal of lipid research*, 41(9). :1367-1375.
- Ingster, L.M. & Feinleib, M. 1998. Salicylate Intake and Cardiovascular Disease: Ingster and Feinleib Respond to Hu and Willett. *American Journal of Public Health*, 88(8). :1268-1269.
- Iqbal, K., Khan, A. & Khattak, Muhammad Muzaffar Ali Khan. 2004. Biological Significance of Ascorbic Acid (Vitamin C) in Human health—a Review. *Pakistan Journal of Nutrition*, 3(1). :5-13.
- Jani, I. 2010. Point-of-Care CD4 Improves Patient Retention and Time-to-Initiation of ART in Mozambique. Paper presented at XVIII International AIDS Conference.

- Jiang, B., Hebert, V.Y., Zavec, J.H. & Dugas, T.R. 2007. Antiretrovirals Induce Direct Endothelial Dysfunction in Vivo. *JAIDS Journal of Acquired Immune Deficiency Syndromes*, 42(4). :391-395.
- Jin, X. 2014. Protective Efficacy of Vitamins C and E on p, p'-DDT-Induced Cytotoxicity Via the ROS-Mediated Mitochondrial Pathway and NF- κ B/FasL Pathway. *PloS one*, 9(12). :e113257.
- Johri, M.K., Mishra, R., Chhatbar, C., Unni, S.K. & Singh, S.K. 2011. Tits and Bits of HIV Tat Protein. *Expert opinion on biological therapy*, 11(3). :269-283.
- Kahn, B.B., Alquier, T., Carling, D. & Hardie, D.G. 2005. AMP-Activated Protein Kinase: Ancient Energy Gauge Provides Clues to Modern Understanding of Metabolism. *Cell metabolism*, 1(1). :15-25.
- Kannisto, K. 2003. Expression of Adipogenic Transcription Factors, Peroxisome Proliferator-Activated Receptor Gamma Co-Activator 1, IL-6 and CD45 in Subcutaneous Adipose Tissue in Lipodystrophy Associated with Highly Active Antiretroviral Therapy. *Aids*, 17(12). :1753-1762.
- Kaplan, R.C. 2008. Low CD4 T Cell Count as a Major Atherosclerosis Risk Factor in HIV-Infected Women and Men. *AIDS (London, England)*, 22(13). :1615.
- Kelley, D.E., He, J., Menshikova, E.V. & Ritov, V.B. 2002. Dysfunction of Mitochondria in Human Skeletal Muscle in Type 2 Diabetes. *Diabetes*, 51(10). :2944-2950.
- Kershaw, E.E. & Flier, J.S. 2004. Adipose Tissue as an Endocrine Organ. *The Journal of Clinical Endocrinology & Metabolism*, 89(6). :2548-2556.
- Kesho Bora Study Group & de Vincenzi, I. 2011. Triple Antiretroviral Compared with Zidovudine and Single-Dose Nevirapine Prophylaxis during Pregnancy and Breastfeeding for Prevention of Mother-to-Child Transmission of HIV-1 (Kesho Bora Study): A Randomised Controlled Trial. *The Lancet infectious diseases*, 11(3). :171-180.
- Kim, I., Rodriguez-Enriquez, S. & Lemasters, J.J. 2007. Selective Degradation of Mitochondria by Mitophagy. *Archives of Biochemistry and Biophysics*, 462(2). :245-253.
- Kitada, M., Kume, S., Imaizumi, N. & Koya, D. 2011. Resveratrol Improves Oxidative Stress and Protects Against Diabetic Nephropathy through Normalization of Mn-SOD Dysfunction in AMPK/SIRT1-Independent Pathway. *Diabetes*, 60(2). :634-643.
- Koczor, C., Kohler, J. & Lewis, W. 2010. Transgenic Mouse Models of Mitochondrial Toxicity Associated with HIV/AIDS and Antiretrovirals. *Methods*, 51(4). :399.
- Kogure, A. 2002. Effects of Caffeine on the Uncoupling Protein Family in Obese Yellow KK Mice. *Clinical and experimental pharmacology and physiology*, 29(5-6). :391-394.
- Kopp, E. & Ghosh, S. 1994. Inhibition of NF-Kappa B by Sodium Salicylate and Aspirin. *Science*, 265(5174). :956-959.
- Krief, S. 1993. Tissue Distribution of Beta 3-Adrenergic Receptor mRNA in Man. *The Journal of clinical investigation*, 91(1). :344-349.
- Kumar, G.N. 2004. Metabolism and Disposition of the HIV-1 Protease Inhibitor Lopinavir (ABT-378) Given in Combination with Ritonavir in Rats, Dogs, and Humans. *Pharmaceutical research*, 21(9). :1622-1630.

- Kwon, H.S. & Ott, M. 2008. The Ups and Downs of SIRT1. *Trends in biochemical sciences*, 33(11). :517-525.
- Lafontan, M. 2013. Fat Mass Expansion, Fatty Acids and Adipokines: Metabolic Markers and Risk Factors for Cardiovascular Pathologies. Paper presented at Annales pharmaceutiques francaises.
- Lagouge, M. 2006. Resveratrol Improves Mitochondrial Function and Protects Against Metabolic Disease by Activating SIRT1 and PGC-1 [Alpha]. *Cell*, 127(6). :1109-1122.
- Leaberry, B.A. 2010. Aspirin for the Prevention of Cardiovascular Disease: Systematic Review. *Journal of nursing care quality*, 25(1). :17.
- Lee, G.A. 2004. The Metabolic Effects of lopinavir/ritonavir in HIV-Negative Men. *AIDS (London, England)*, 18(4). :641.
- Levine, M., Rumsey, S.C., Daruwala, R., Park, J.B. & Wang, Y. 1999. Criteria and Recommendations for Vitamin C Intake. *JAMA: the journal of the American Medical Association*, 281(15). :1415-1423.
- Li, H., Xia, N. & Förstermann, U. 2012. Cardiovascular Effects and Molecular Targets of Resveratrol. *Nitric Oxide*.
- Li, H., Xu, M., Lee, J., He, C. & Xie, Z. 2012. Leucine Supplementation Increases SIRT1 Expression and Prevents Mitochondrial Dysfunction and Metabolic Disorders in High-Fat Diet-Induced Obese Mice. *American Journal of Physiology-Endocrinology And Metabolism*, 303(10). :E1234-E1244.
- Liang, H. & Ward, W.F. 2006. PGC-1alpha: A Key Regulator of Energy Metabolism. *Advances in Physiology Education*, 30(4). :145-151.
- Lowell, B.B. 1999. An Essential Regulator of Adipogenesis and Modulator of Fat Cell Function: PPAR γ . *Cell*, 99(3). :239-242.
- Lowell, B.B. & Shulman, G.I. 2005. Mitochondrial Dysfunction and Type 2 Diabetes. *Science*, 307(5708). :384-387.
- Lund, K.C., Peterson, L.L. & Wallace, K.B. 2007. Absence of a Universal Mechanism of Mitochondrial Toxicity by Nucleoside Analogs. *Antimicrobial Agents and Chemotherapy*, 51(7). :2531-2539.
- Luster, M.I. 1989. A Comparison of Three Nucleoside Analogs with Anti-Retroviral Activity on Immune and Hematopoietic Functions in Mice: *in vitro* Toxicity to Precursor Cells and Microstromal Environment. *Toxicology and applied pharmacology*, 101(2). :328-339.
- Madeddu, G. 2006. Thyroid Function in Human Immunodeficiency Virus Patients Treated with Highly Active Antiretroviral Therapy (HAART): A Longitudinal Study. *Clinical endocrinology*, 64(4). :375-383.
- Maeda, H., Hosokawa, M., Sashima, T., Funayama, K. & Miyashita, K. 2005. Fucoxanthin from Edible Seaweed, *Undaria Pinnatifida*, shows Antiobesity Effect through UCP1 Expression in White Adipose Tissues. *Biochemical and biophysical research communications*, 332(2). :392-397.
- Malaty, L.I. & Kuper, J.J. 1999. Drug Interactions of HIV Protease Inhibitors. *Drug Safety*, 20(2). :147-169.

- Mankouri, J. & Harris, M. 2011. Viruses and the Fuel Sensor: The Emerging Link between AMPK and Virus Replication. *Reviews in medical virology*.
- Mannheimer, S. 2005. Quality of Life in HIV-Infected Individuals Receiving Antiretroviral Therapy is Related to Adherence. *AIDS Care*, 17(1). :10-22.
- Martinez, E. & Gatell, J. 1998. Metabolic Abnormalities and use of HIV-1 Protease Inhibitors. *The Lancet*, 352(9130). :821-822.
- Masquelier, B. 2005. Intermittent Viremia during First-Line, Protease Inhibitors-Containing Therapy: Significance and Relationship with Drug Resistance. *Journal of clinical virology*, 33(1). :75-78.
- Mayosi, B.M., Lawn, J.E., van Niekerk, A., Bradshaw, D., Abdool Karim, S.S. & Coovadia, H.M. 2012. Health in South Africa: Changes and Challenges since 2009. *The Lancet*.
- McBride, H.M., Neuspiel, M. & Wasiak, S. 2006. Mitochondria: More than just a Powerhouse. *Current Biology*, 16(14). :R551-R560.
- Meena, L. 2011. Endocrine Changes in Male HIV Patients. *Journal of the Association of Physicians of India*, 59(6). :365-366.
- Mehellou, Y. & De Clercq, E. 2010. Twenty-Six Years of Anti-HIV Drug Discovery: Where do we Stand and Where do we Go. *J Med Chem*, 53(2). :521-538.
- Mellors, J., Dutschman, G., Im, G., Tramontano, E., Winkler, S.R. & Cheng, Y. 1992. In Vitro Selection and Molecular Characterization of Human Immunodeficiency Virus-1 Resistant to Non-Nucleoside Inhibitors of Reverse Transcriptase. *Molecular pharmacology*, 41(3). :446-451.
- Mencarelli, A. 2012. Ritonavir—induced Lipoatrophy and Dyslipidaemia is Reversed by the Anti-Inflammatory Drug Leflunomide in a PPAR- γ -Dependent Manner. *Antiviral Therapy*, 17(4). :669-678.
- Mendelsohn, A. & Larrick, J.W. 2011. Master Switch of Mitochondrial Biogenesis: A Clinical Target for Health Span Enhancement? *Rejuvenation research*, 14(2). :223-226.
- Meng, Q. 2002. Use of HIV Protease Inhibitors is Associated with Left Ventricular Morphologic Changes and Diastolic Dysfunction. *JAIDS Journal of Acquired Immune Deficiency Syndromes*, 30(3). :306-310.
- Merenstein, D. 2012. An Investigation of the Possible Interaction between the use of Vitamin C and Highly Active Antiretroviral Therapy (HAART) Adherence and Effectiveness in Treated HIV Women. *Complementary therapies in medicine*, 20(4). :222-227.
- Miró, Ò. 2003. Mitochondrial DNA Depletion and Respiratory Chain Enzyme Deficiencies are Present in Peripheral Blood Mononuclear Cells of HIV-Infected Patients with HAART-Related Lipodystrophy. *Antiviral Therapy*, 8(4). :333-338.
- Mitobe, Y., Hiraishi, H., Sasai, T., Shimada, T. & Terano, A. 2000. The Effects of Aspirin on Antioxidant Defences of Cultured Rat Gastric Mucosal Cells. *Alimentary Pharmacology & Therapeutics*, 14:10-17.
- Mondy, K.E. 2011. High Prevalence of Echocardiographic Abnormalities among HIV-Infected Persons in the Era of Highly Active Antiretroviral Therapy. *Clinical Infectious Diseases*, 52(3). :378-386.

- Morino, K. 2005. Reduced Mitochondrial Density and Increased IRS-1 Serine Phosphorylation in Muscle of Insulin-Resistant Offspring of Type 2 Diabetic Parents. *Journal of Clinical Investigation*, 115(12). :3587-3593.
- Mullur, R., Liu, Y.Y. & Brent, G.A. 2014. Thyroid Hormone Regulation of Metabolism. *Physiological Reviews*, 94(2). :355-382.
- Nakamura, K., Fuster, J.J. & Walsh, K. 2014. Adipokines: A Link between Obesity and Cardiovascular Disease. *Journal of cardiology*, 63(4). :250-259.
- Nicholls, D. 2001. A History of UCP1. *Biochemical Society transactions*, 29(Pt 6). :751-755.
- Nisoli, E., Clementi, E., Carruba, M.O. & Moncada, S. 2007. Defective Mitochondrial Biogenesis. *Circulation research*, 100(6). :795-806.
- Noor, M.A., Flint, O.P., Maa, J. & Parker, R.A. 2006. Effects of atazanavir/ritonavir and lopinavir/ritonavir on Glucose Uptake and Insulin Sensitivity: Demonstrable Differences in Vitro and Clinically. *Aids*, 20(14). :1813-1821.
- Nunberg, J.H. 1991. Viral Resistance to Human Immunodeficiency Virus Type 1-Specific Pyridinone Reverse Transcriptase Inhibitors. *Journal of virology*, 65(9). :4887-4892.
- Ortblad, K.F., Lozano, R. & Murray, C.J. 2013. The Burden of HIV: Insights from the Global Burden of Disease (GBD) Study 2010. *The Lancet*, 381:S103.
- Ou, H.C., Lee, W.J., Wu, C.M., Chen, J.F.M. & Sheu, W.H.H. 2012. Aspirin Prevents Resistin-Induced Endothelial Dysfunction by Modulating AMPK, ROS, and Akt/eNOS Signaling. *Journal of Vascular Surgery*.
- Paneni, F. 2014. 2013 ESC/EASD Guidelines on the Management of Diabetes and Cardiovascular Disease: Established Knowledge and Evidence Gaps. *Diabetes & vascular disease research : official journal of the International Society of Diabetes and Vascular Disease*, 11(1). :5-10.
- Pang, J., Choi, Y. & Park, T. 2008. Ilex paraguariensis Extract Ameliorates Obesity Induced by High-Fat Diet: Potential Role of AMPK in the Visceral Adipose Tissue. *Archives of Biochemistry and Biophysics*, 476(2). :178-185.
- Paterson, D.L. 2000. Adherence to Protease Inhibitor Therapy and Outcomes in Patients with HIV Infection. *Annals of Internal Medicine*, 133(1). :21-30.
- Pérez-Matute, P., Pérez-Martínez, L., Blanco, J. & Oteo, J. 2013. Role of Mitochondria in HIV Infection and Associated Metabolic Disorders: Focus on Nonalcoholic Fatty Liver Disease and Lipodystrophy Syndrome. *Oxidative medicine and cellular longevity*, 2013
- Phenix, B.N., Lum, J.J., Nie, Z., Sanchez-Dardon, J. & Badley, A.D. 2001. Antiapoptotic Mechanism of HIV Protease Inhibitors: Preventing Mitochondrial Transmembrane Potential Loss. *Blood*, 98(4). :1078-1085.
- Picard, M. 2011. Mitochondrial Structure and Function are Disrupted by Standard Isolation Methods. *PLoS ONE*, 6(3).
- Pignone, M. 2010. Aspirin for Primary Prevention of Cardiovascular Events in People with Diabetes. *Circulation*, 121(24). :2694-2701.

- Porter, K.M. & Sutliff, R.L. 2012. HIV-1, Reactive Oxygen Species and Vascular Complications. *Free Radical Biology and Medicine*.
- Poulsen, M.M. 2013. High-Dose Resveratrol Supplementation in Obese Men: An Investigator-Initiated, Randomized, Placebo-Controlled Clinical Trial of Substrate Metabolism, Insulin Sensitivity, and Body Composition. *Diabetes*, 62(4). :1186-1195.
- Price, N.L. 2012. SIRT1 is Required for AMPK Activation and the Beneficial Effects of Resveratrol on Mitochondrial Function. *Cell metabolism*, 15(5). :675-690.
- Pugliese, A., Vidotto, V., Beltramo, T., Petrini, S. & Torre, D. 2004. A Review of HIV-1 Tat Protein Biological Effects. *Cell biochemistry and function*, 23(4). :223-227.
- Pulido, F. 2008. Lopinavir-Ritonavir Monotherapy Versus Lopinavir-Ritonavir and Two Nucleosides for Maintenance Therapy of HIV. *Aids*, 22(2). :F1.
- Ralph, S.J., Rodríguez-Enríquez, S., Neuzil, J. & Moreno-Sánchez, R. 2010. Bioenergetic Pathways in Tumor Mitochondria as Targets for Cancer Therapy and the Importance of the ROS-Induced Apoptotic Trigger. *Molecular aspects of medicine*, 31(1). :29-59.
- Reagan-Shaw, S., Nihal, M. & Ahmad, N. 2008. Dose Translation from Animal to Human Studies Revisited. *The FASEB Journal*, 22(3). :659-661.
- Redfield, R.R. 2011. Targeting Cell Cycle to Enhance the Potency of Antiretroviral Therapeutic Agents. *JAIDS Journal of Acquired Immune Deficiency Syndromes*, 56:74.
- Regula, K.M., Ens, K. & Kirshenbaum, L.A. 2003. Mitochondria-Assisted Cell Suicide: A License to Kill. *Journal of Molecular and Cellular Cardiology*, 35(6). :559-567.
- Reiche, E.M.V. & Kaminami, H. 2014. Oxidative Stress and Human Immunodeficiency Virus Type 1 (HIV-1) Infection. *Role of Oxidative Stress in Chronic Diseases*, :45.
- Reyskens, K.M. & Essop, M.F. 2014. HIV Protease Inhibitors and Onset of Cardiovascular Diseases: A Central Role for Oxidative Stress and Dysregulation of the ubiquitin–proteasome System. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, 1842(2). :256-268.
- Richman, D. 1991. Human Immunodeficiency Virus Type 1 Mutants Resistant to Nonnucleoside Inhibitors of Reverse Transcriptase Arise in Tissue Culture. *Proceedings of the National Academy of Sciences*, 88(24). :11241-11245.
- Ritov, V.B. 2010. Deficiency of Electron Transport Chain in Human Skeletal Muscle Mitochondria in Type 2 Diabetes Mellitus and Obesity. *American Journal of Physiology-Endocrinology And Metabolism*, 298(1). :E49-E58.
- Ritov, V.B., Menshikova, E.V., He, J., Ferrell, R.E., Goodpaster, B.H. & Kelley, D.E. 2005. Deficiency of Subsarcolemmal Mitochondria in Obesity and Type 2 Diabetes. *Diabetes*, 54(1). :8-14.
- Rodgers, J.T., Lerin, C., Gerhart-Hines, Z. & Puigserver, P. 2008. Metabolic Adaptations through the PGC-1 [Alpha] and SIRT1 Pathways. *FEBS letters*, 582(1). :46-53.
- Rosas, S. 2013. High Clustering Rates of Multidrug-Resistant Mycobacterium Tuberculosis Genotypes in Panama. *BMC infectious diseases*, 13(1). :442.

- Rosen, E.D. 1999. PPAR γ is Required for the Differentiation of Adipose Tissue in Vivo and in Vitro. *Molecular cell*, 4(4). :611-617.
- Ruderman, N.B., Carling, D., Prentki, M. & Cacicedo, J.M. 2013. AMPK, Insulin Resistance, and the Metabolic Syndrome. *The Journal of clinical investigation*, 123(7). :2764-2772.
- Rudich, A., Ben-Romano, R., Etzion, S. & Bashan, N. 2005. Cellular Mechanisms of Insulin Resistance, Lipodystrophy and Atherosclerosis Induced by HIV Protease Inhibitors. *Acta Physiologica Scandinavica*, 183(1). :75-88.
- Sacre, H. 2011. Clinic-Based Anthropometric Measurements of Lipodystrophy and Associations with Antiretroviral Therapy in HIV-Infected Adolescents.
- Scheen, A.J. & Van Gaal, L.F. 2014. Combating the dual burden: Therapeutic Targeting of Common Pathways in Obesity and Type 2 Diabetes. *The Lancet Diabetes & Endocrinology*, 2(11). :911-922.
- Scheffler, I.E. 1999. Mitochondrial Electron Transport and Oxidative Phosphorylation. *Mitochondria*, :141-245.
- Scherrer, A.U. 2013. Correction: Long-Lasting Protection of Activity of Nucleoside Reverse Transcriptase Inhibitors and Protease Inhibitors (PIs) by Boosted PI Containing Regimens. *PLoS one*, 8(7).
- Schinazi, R., Larder, B. & Mellors, J. 1997. Mutations in Retroviral Genes Associated with Drug Resistance. *International Antiviral News*, 5:129-142.
- Schleinitz, D., Böttcher, Y., Blüher, M. & Kovacs, P. 2014. The Genetics of Fat Distribution. *Diabetologia*, 57(7). :1276-1286.
- Seitz, H., Muller, M. & Soboll, S. 1985. Rapid Thyroid-Hormone Effect on Mitochondrial and Cytosolic ATP/ADP Ratios in the Intact Liver Cell. *Biochem.J.*, 227:149-153.
- Shadel, G.S. & Horvath, T.L. 2015. Mitochondrial ROS Signaling in Organismal Homeostasis. *Cell*, 163(3). :560-569.
- Shi, X. 1999. Antioxidant Properties of Aspirin: Characterization of the Ability of Aspirin to Inhibit Silica-Induced Lipid Peroxidation, DNA Damage, NF- κ B Activation, and TNF- α Production. *Molecular and cellular biochemistry*, 199(1-2). :93-102.
- Shikuma, C.M. 2001. Mitochondrial DNA Decrease in Subcutaneous Adipose Tissue of HIV-Infected Individuals with Peripheral Lipoatrophy. *Aids*, 15(14). :1801-1809.
- Shuter, J., Sarlo, J.A., Kanmaz, T.J., Rode, R.A. & Zingman, B.S. 2007. HIV-Infected Patients Receiving lopinavir/ritonavir-Based Antiretroviral Therapy Achieve High Rates of Virologic Suppression Despite Adherence Rates Less than 95%. *JAIDS Journal of Acquired Immune Deficiency Syndromes*, 45(1). :4-8.
- Sicree, R. & Shaw, J. 2009. Zimmet: The Global Burden: Diabetes and Impaired Glucose Tolerance. *Diabetes Atlas, IDF*, 4
- Sierra, S., Kupfer, B. & Kaiser, R. 2005. Basics of the Virology of HIV-1 and its Replication. *Journal of Clinical Virology*, 34(4). :233-244.

- Siripassorn, K. 2010. Effectiveness of Boosted Protease Inhibitor-Based Regimens in HIV Type 1-Infected Patients Who Experienced Virological Failure with NNRTI-Based Antiretroviral Therapy in a Resource-Limited Setting. *AIDS Research and Human Retroviruses*, 26(2). :139-148.
- Sivitz, W.I. & Yorek, M.A. 2010. Mitochondrial Dysfunction in Diabetes: From Molecular Mechanisms to Functional Significance and Therapeutic Opportunities.(Report). *Antioxidants & Redox Signaling*, 12(4). :537.
- Slater, E. 2003. Keilin, Cytochrome, and the Respiratory Chain. *Journal of Biological Chemistry*, 278(19). :16455-16461.
- Snedecor, S.J. 2013. The Prevalence of Transmitted Resistance to First-Generation Non-Nucleoside Reverse Transcriptase Inhibitors and its Potential Economic Impact in HIV-Infected Patients. *PLoS one*, 8(8). :e72784.
- Srivastava, D.K., Tendler, C.L., Milani, D., English, M.A., Licht, J.D. & Wilson, S.H. 2001. The HIV-1 Transactivator Protein Tat is a Potent Inducer of the Human DNA Repair Enzyme [Beta]-Polymerase. *Aids*, 15(4). :433-440.
- Stein, J.H. 2001. Use of Human Immunodeficiency Virus-1 Protease Inhibitors is Associated with Atherogenic Lipoprotein Changes and Endothelial Dysfunction. *Circulation*, 104(3). :257-262.
- Stein, J. 2005. Antiretroviral Therapy Improves Endothelial Function in Individuals with Human Immunodeficiency Virus Infection: A Prospective, Randomized Multicenter Trial (Adult AIDS Clinical Trials Group Study A5152s. Paper presented at Circulation.
- Stephensen, C.B., Marquis, G.S., Jacob, R.A., Kruzich, L.A., Douglas, S.D. & Wilson, C.M. 2006. Vitamins C and E in Adolescents and Young Adults with HIV Infection. *The American Journal of Clinical Nutrition*, 83(4). :870-879.
- Sterling, K., Lazarus, J.H., Milch, P.O., Sakurada, T. & Brenner, M.A. 1978. Mitochondrial Thyroid Hormone Receptor: Localization and Physiological Significance. *Science*, 201(4361). :1126-1129.
- Stevenson, M. 2003. HIV-1 Pathogenesis. *Nature medicine*, 9(7). :853-860.
- Taylor, S.A. 2010. Boosting-Dose Ritonavir does Not Alter Peripheral Insulin Sensitivity in Healthy, HIV Seronegative Volunteers. *Journal of acquired immune deficiency syndromes (1999)*, 55(3). :361.
- Tchkonia, T. 2013. Mechanisms and Metabolic Implications of Regional Differences among Fat Depots. *Cell metabolism*, 17(5). :644-656.
- Tebas, P. 2008. Metabolic and Immune Activation Effects of Treatment Interruption in Chronic HIV-1 Infection: Implications for Cardiovascular Risk. *PLoS One*, 3(4). :e2021.
- Teixeira, C., Gomes, J.R.B., Gomes, P. & Maurel, F. 2011. Viral Surface Glycoproteins, gp120 and gp41, as Potential Drug Targets Against HIV-1: Brief Overview One Quarter of a Century Past the Approval of Zidovudine, the First Anti-Retroviral Drug. *European journal of medicinal chemistry*, 46(4). :979-992.
- Touzet, O. & Philips, A. 2010. Resveratrol Protects Against Protease Inhibitor-Induced Reactive Oxygen Species Production, Reticulum Stress and Lipid Raft Perturbation. *Aids*, 24(10). :1437-1447.

- Tsiodras, S., Mantzoros, C., Hammer, S. & Samore, M. 2000. Effects of Protease Inhibitors on Hyperglycemia, Hyperlipidemia, and Lipodystrophy: A 5-Year Cohort Study. *Archives of Internal Medicine*, 160(13). :2050.
- Van Berendoncks, A.M., Garnier, A., Ventura-Clapier, R. & Conraads, V.M. 2013. Adiponectin: Key Role and Potential Target to Reverse Energy Wasting in Chronic Heart Failure. *Heart failure reviews*, 18(5). :557-566.
- Vervoort, S.C., Borleffs, J.C., Hoepelman, A.I. & Grypdonck, M.H. 2007. Adherence in Antiretroviral Therapy: A Review of Qualitative Studies. *Aids*, 21(3). :271-281.
- Vondra, K., Rath, R., Bass, A., Slabochova, Z., Teisinger, J. & Vitek, V. 1977. Enzyme Activities in Quadriceps Femoris Muscle of Obese Diabetic Male Patients. *Diabetologia*, 13(5). :527-529.
- Wadden, T.A., Brownell, K.D. & Foster, G.D. 2002. Obesity: Responding to the Global Epidemic. *Journal of consulting and clinical psychology*, 70(3). :510-525.
- Wang, K. & Klionsky, D.J. 2011. Mitochondria Removal by Autophagy. *Autophagy*, 7(3). :297-300.
- Wang, S.S., Fernhoff, P.M., Harnnon, W.H. & Khoury, M.J. 1999. Medium Chain acyl‐ CoA Dehydrogenase Deficiency: Human Genome Epidemiology Review. *Genetics in Medicine*, 1(7). :332-339.
- Wang, X., Chai, H., Yao, Q. & Chen, C. 2007. Molecular Mechanisms of HIV Protease Inhibitor-Induced Endothelial Dysfunction. *JAIDS Journal of Acquired Immune Deficiency Syndromes*, 44(5). :493-499.
- White, A.J. 2001. Mitochondrial Toxicity and HIV Therapy. *Sexually transmitted infections*, 77(3). :158-173.
- WHO, U. & UNICEF. 2008. Epidemiological Facts Sheet on HIV and AIDS. *Core data on epidemiology and response. Columbia*.
- Whoqol Hiv Group. 2004. WHOQOL-HIV for Quality of Life Assessment among People Living with HIV and AIDS: Results from the Field Test. *AIDS Care*, 16(7). :882-889.
- Wolf, K., Tsakiris, D.A., Weber, R., Erb, P. & Battegay, M. 2002. Antiretroviral Therapy Reduces Markers of Endothelial and Coagulation Activation in Patients Infected with Human Immunodeficiency Virus Type 1. *Journal of Infectious Diseases*, 185(4). :456-462.
- Worm, S.W. 2010. Risk of Myocardial Infarction in Patients with HIV Infection Exposed to Specific Individual Antiretroviral Drugs from the 3 Major Drug Classes: The Data Collection on Adverse Events of Anti-HIV Drugs (D: A: D) Study. *Journal of Infectious Diseases*, 201(3). :318-330.
- Wozniak, S.E., Gee, L.L., Wachtel, M.S. & Frezza, E.E. 2009. Adipose Tissue: The New Endocrine Organ? A Review Article. *Digestive diseases and sciences*, 54(9). :1847-1856.
- Wright, G.E. & Brown, N.C. 1990. Deoxyribonucleotide Analogs as Inhibitors and Substrates of DNA Polymerases. *Pharmacology & therapeutics*, 47(3). :447-497.
- Yin, F., Spurgeon, H.A., Rakusan, K., Weisfeldt, M.L. & Lakatta, E.G. 1982. Use of Tibial Length to Quantify Cardiac Hypertrophy: Application in the Aging Rat. *American Journal of Physiology-Heart and Circulatory Physiology*, 243(6). :H941-H947.

- Yin, M.J., Yamamoto, Y. & Gaynor, R.B. 1998. The Anti-Inflammatory Agents Aspirin and Salicylate Inhibit the Activity of I κ B Kinase- β . *Nature*, 396(6706). :77-80.
- Young, T.S., Young, D.D., Ahmad, I., Louis, J.M., Benkovic, S.J. & Schultz, P.G. 1998. Evolution of Cyclic Peptide Protease Inhibitors.(BIOCHEMISTRY)(Author Abstract). *Proceedings of the National Academy of Sciences of the United States*, 108(27). :11052.
- Zeldin, R.K. & Petruschke, R.A. 2004. Pharmacological and Therapeutic Properties of Ritonavir-Boosted Protease Inhibitor Therapy in HIV-Infected Patients. *Journal of antimicrobial chemotherapy*, 53(1). :4-9.
- Zha, B.S., Studer, E.J., Zha, W., Hylemon, P.B., Pandak, W. & Zhou, H. 2011. Highly Active Antiretroviral Therapy (HAART) and Metabolic Complications.
- Zhang, D. 2007. Mitochondrial Dysfunction due to Long-Chain Acyl-CoA Dehydrogenase Deficiency Causes Hepatic Steatosis and Hepatic Insulin Resistance. *Proceedings of the National Academy of Sciences*, 104(43). :17075-17080.
- Zhang, H.S. & Wu, M.R. 2009. SIRT1 Regulates Tat-Induced HIV-1 Transactivation through Activating AMP-Activated Protein Kinase. *Virus research*, 146(1-2). :51-57.
- Zhang, Y., Marcillat, O., Giulivi, C., Ernster, L. & Davies, K. 1990. The Oxidative Inactivation of Mitochondrial Electron Transport Chain Components and ATPase. *Journal of Biological Chemistry*, 265(27). :16330-16336.
- Zong, H. 2002. AMP Kinase is Required for Mitochondrial Biogenesis in Skeletal Muscle in Response to Chronic Energy Deprivation. *Proceedings of the National Academy of Sciences*, 99(25). :15983-15987.

Appendices

Mitochondrial Respiration Buffer Constituents and Preparation

1. Buffer A

Constituents: 100 mM KCl, 50 mM mops, 5.0 mM MgSO₄, 1.0 mM EGTA, and 1.0 mM ATP

To prepare:

15 ml 2.0 M KCl

15 ml 1.0 M mops

15 ml 0.1 M MgSO₄·7H₂O

3 ml 0.1 M EGTA

The solution was then made up to 300 ml with ddH₂O where after 0.183 g ATP (Sigma, A7699) was added. The final solution was made up to pH 7.4 with 10 N KOH.

2. Buffer B

Buffer A with 2 mg/ml BSA (fatty-acid free)

3. Respiration Buffer

Constituents: 2.0 M KCl, 1.0 M mops, 0.1 M MgSO₄·7H₂O, 0.1 M EGTA, 2xKM*,
100 mM KH₂PO₄, 5% BSA

*200mM KCl and 100 mM mops

To prepare:

250 ml 2xKM

25 ml 0.1 M EGTA

10 ml 5% BSA (fatty acid free)

Solution was adjusted to pH 7.4 with 10 N KOH and made up to 500 ml with ddH₂O.

4. KME Buffer

To prepare:

5 ml EGTA was added to 495 ml KM buffer (EGTA final volume – 1mM)