Effect of *Aspalathus linearis* supplementation, during anti-retroviral treatment, on the heart and aortas of male Wistar rats and the effects of drinking rooibos on the cardiovascular profile of patients on ART.

By

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Declaration

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Emiliana Gomes Imperial

Abstract

Introduction

Improved survival rates in the human immunodeficiency virus (HIV) population, due to the use of antiretroviral therapy (ART), are associated with increased risk for cardiovascular disease (CVD). Although the cardiovascular effects of rooibos as an antioxidant have been well documented its nutraceutical properties have yet to be investigated as a possible supplement in ART.

AIM

To investigate the cardiovascular effects of *Aspalathus linearis*/ rooibos infusion supplementation during ART, on male Wistar rats and the effects of rooibos tea consumption on the cardiovascular profiles of patients on ART.

Methods

Male Wistar rats were randomly divided into groups and treated with a fixed dose combination (Efavirenz 600mg, emtricitabine 200mg, tenofovir 300mg) and a 2% (w/v) rooibos tea solution independently or in combination for 9 weeks. Fluid intake and weights were measured weekly. Hearts were perfused on the isolated working heart rig and subjected to 20min global or 35min regional ischemia. Functional parameters were recorded and percentage recovery and infarct size (IS) calculated. Using the aortic ring protocol for vascular reactivity, thoracic aortas (with and without perivascular adipose tissue (PVAT)) were subjected to phenylephrine-induced contraction as well as acetylcholine-induced relaxation. Rat serum was collected for biochemical analyses. Participants for the human study were recruited and blood samples collected by a phlebotomist. Blood pressure tests were conducted, anthropometric data recorded and samples prepared for biochemical analyses.

Results

ART increased IS compared to control [Control (% area at risk): 28.17±5.10 , ART: 50.56±4.08] however this was not seen in the Rooibos+ART group. In vascular reactivity experiments, without PVAT, ART significantly diminished relaxation compared to control ($p=0.03$) whilst this effect was not observed with Rooibos supplementation ($p=0.003$). Rooibos caused increased contraction compared to control ($p=0.01$), ART ($p=0.0457$) and the combination ($p=0.0468$). In the presence of PVAT all treatment groups relaxed significantly less than controls ($p<0.01$). Area under the curve (AUC): Control+PVAT, 0.00099; Rooibos+PVAT: 0.00079; ART+PVAT, 0.00091; ART+Rooibos+PVAT, 0.00088. Whilst Rooibos and ART showed significantly higher contraction than the control ($p=0.006$ and $p=0.03$ respectively).

AUC: Control+PVAT, 31.45; Rooibos+PVAT: 43.42; ART+PVAT, 36.32; ART+Rooibos+PVAT, 33.34. Overall the presence of PVAT caused a significant decrease in contraction compared to the absence thereof; Control+PVAT versus Control -PVAT group ($p<0.0001$); rooibos +PVAT versus rooibos -PVAT ($p<0.0001$), ART+PVAT versus ART -PVAT ($p<0.0001$) and Rooibos+ART +PVAT versus Rooibos+ART-PVAT ($p<0.0001$). Rooibos caused a significant reduction in total cholesterol (TC) levels ($p=0.048$) compared to all other groups. Epidemiological data revealed that drinking rooibos had no significant effects on the cardiovascular parameters of patients irrespective of HIV status or ART adherence.

Discussion and Conclusion

In aortic rings anti-vasodilatory effects of ART were not observed with rooibos supplementation. ART induced significant increase in IS was not observed in the Rooibos + ART treated group. Rooibos exerted pro-contractile effects whilst the presence of PVAT reduced contraction of the aortic rings. Deleterious effects by the current ART regimen were not observed in the epidemiological study and rooibos consumption had no significant effects on the assessed cardiovascular parameters of all patient groups. Further scientific investigation is required.

Abstrak

Inleiding

Antiretrovirale middels (ART) het gelei tot hoër lewensverwagtinge in die menslike immuniteitsgebreksvirus (MIV)-populasie, maar ongelukkig ook tot 'n verhoogde risiko vir kardiovaskulêre siektes (KVS). Alhoewel daar voldoende gepubliseer is oor die anti-oksidant effekte van rooibos op die kardiovaskulêre sisteem, is daar 'n tekort aan inligting oor die potensiaal van rooibos as aanvulling tydens ART gebruik.

Doelwit

Om in manlike Wistar rotte die kardiovaskulêre effekte van *Aspalathus linearis*/rooibos as aanvulling tydens ART te ondersoek. Om verder ook die effekte van rooibostee op die kardiovaskulêre profiel van pasiënte op ART te ondersoek.

Metodes

Rotte was ewekansig verdeel in groepe wat vir 9 weke met 'n vaste kombinasie dosis (Efavirenz 600mg, emtricitabine 200mg, tenofovir 300mg) en 'n 2% (w/v) rooibostee oplossing, alleen of in kombinasie, behandel was. Harte was geperfuseer middle van 'n geïsoleerde werkhart model, en was blootgestel aan 20 min globale-iskemie of 35 min streeks-iskemie. Funksionele veranderlikes was gemeet en die persentasie herstel asook infarktgrootte bereken. Vaskulêre reaktiwiteit was bepaal deur gebruik te maak van aorta ringe, waar die torakale aorta (met of sonder perivaskulêre vet (PVAT)) aan fenielefrien-geïnduseerde kontraksie en asetielkolien-geïnduseerde verslapping blootgestel was. Rot serum was vir biochemiese analises versamel. Deelnemers van die menslike studie was gewerf, waarna bloedmonsters deur 'n opgeleide verpleegster geneem is. Bloeddruk en antropometriese metings was bepaal, en bloedmonsters was vir biochemiese analise voorberei.

Resultate

ART behandeling het tot 'n vergrote infarkt grootte gelei [Kontrole (% risiko-area): 28.17±5.10, ART: 50.56±4.08). Hierdie resultaat was nie waargeneem in die Rooibos+ART groep nie. Aorta ring eksperimente sonder PVAT het getoon dat ART verslapping verminder het in vergelyking met kontrole diere ($p=0.03$), terwyl kombinasie behandeling met rooibos hierdie effek opgehef het ($p=0.003$). Rooibos behandeling het in vergelyking met kontrole ($p=0.01$), ART ($p=0.0457$) en die kombinasie ($p=0.0468$), tot verhoogde kontraksie van aortas gelei. In die teenwoordigheid van PVAT, h*et al* die groepe minder verslap as die Kontrole+PVAT groep; Rooibos+PVAT groep ($p<0.0001$), ART+PVAT ($p<0.01$) en Rooibos+ART+PVAT ($p<0.01$). Area onder die kurwe (AOK): Kontrole+PVAT, 0.00099;

Rooibos+PVAT: 0.00079; ART+PVAT, 0.00091; ART+Rooibos+PVAT, 0.00088. Rooibos en ART behandeling het tot verhoogde kontraksie in vergelyking met kontrole diere gelei (p=0.006 en p=0.03 onderskeidelik). AOK: Kontrole+PVAT, 31.45; Rooibos+PVAT: 43.42; ART+PVAT, 36.32; ART+Rooibos+PVAT, 33.34. Die teenwoordigheid van PVAT het oor die algemeen tot ‘n verlaging in kontraksie gelei [Kontrole+PVAT versus Kontrole-PVAT (p<0.0001); Rooibos+PVAT versus Rooibos-PVAT (p<0.0001); ART+PVAT versus ART-PVAT (p<0.0001); Rooibos+ART+PVAT versus Rooibos+ART-PVAT (p<0.0001)]. In vergelyking met ander groepe, het Rooibos behandeling tot ‘n betekenisvolle verlaging in totale cholesterol gelei (p=0.048). Epidemiologiese data het gewys dat ongeag MIV-status en ART behandeling, het rooibos geen effek op kardiovaskulêre parameters van pasiente nie.

Bespreking en gevolgtrekking

Die anti-verslappende effekte van ART was nie waargeneem in die teenwoordigheid van rooibos aanvulling nie. ART behandeling het tot vergrote infarkte gelei. Rooibos behandeling het tot ‘n pro-kontraktiele effek gelei, terwyl PVAT tot ‘n verlaging in kontraksie gelei het. Geen skadelike effekte geassosieer met die huidige ART regime was in die epidemiologiese studie waargeneem nie en verder het rooibos inname geen betekenisvolle effek op kardiovaskulêre parameters van pasiente gehad nie. Verdere navorsing word benodig.

Acknowledgments

Acts 17:28 "For in Him we live, move and have our being." To God be the glory first and foremost.

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List of Abbreviations

AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
AAR	Area at Risk
AC	Adenylate Cyclase
ACE	Angiotensin Converting Enzyme
ADRF	Adipocyte-Derived Relaxing Factor
AIDS	Acquired Immune Deficiency Syndrome
ANOVA	Analysis of Variance
ART	Antiretroviral Therapy
ARV's	Antiretrovirals
ASCVD	Atherosclerotic Cardiovascular Disease
Asp	Aspalathin
AZT	Azidothymidine
BAT	Brown Adipose Tissue
BMI	Body Mass Index
BP	Blood Pressure
cAMP	cyclic Adenosine Monophosphate
CAT	Catalase
CD's	Conjugated Dienes
cGMP	cyclic Guanosine-3,5-monophosphate
CMV	Cytomegalovirus
CO	Cardiac Output
CPUT	Cape Peninsula University of Technology
CRP	C-reactive protein

Cu-Zn-SOD	copper-zinc-superoxide dismutase
CVD	Cardiovascular Disease
DP	Diastolic Pressure
Ec-SOD	extracellular dismutase
ED	Endothelial Dysfunction
EDLVP	End Diastolic Left Ventricular Pressure
EFV	Efavirenz
ELISA	Enzyme-linked Immunosorbance Assays
eNOS	endothelial Nitric Oxide Synthase
ER	Endoplasmic Reticulum
ETC	Electron Transport Chain
FDC	Fixed Dose Combination
FTC/ 3TC	Emtricitabine 5'- triphosphate
G.I	Global Ischemia
GC	Guanylate Cyclase
GPx	Glutathione peroxidase
HAART	Highly Active Antiretroviral Therapy
HDL	High Density Lipoprotein
HIF- 1α	Hypoxia- Inducible Factor 1 Alpha
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
HR	Heart Rate
IFNγ	Interferon Gamma
IL-18	Interleukin-18
IL-1β	Interleukin-1 Beta

IL-6	Interleukin-6
iNOS	inducible Nitric Oxide Synthase
IS	Infarct Size
IsoPs	Isoprostanes
KHB	Krebs-Henseleit Buffer
KP	Kaiser Permanente
L	Langendorff
LAD	Left Anterior Descending
LBM	Lean Body Mass
LC-MS	Liquid Chromatography Mass Spectrometry
LDL	Low Density Lipoprotein
LPS	Lipopolysaccharide
LVDevP	Left Ventricular Developed Pressure
MDA	Malondialdhyde
MI	Myocardial Infarctions
Mn-SOD	Manganese superoxide dismutase
MS	Metabolic Syndrome
NF-κβ	Nuclear Factor Kappa Beta
NK	Natural Killer Cells
NLR's/NACHT	Nucleotide Oligomerization Domain-Like Receeptors
nNOS	neuronal Nitric Oxide Synthase
NNRTI	Non-nucleotide Reverse Transcriptase Inhibitor
NO	Nitric Oxide
Not	Nothofagin
Nox	NADPH Oxidase

NRTI	Nucleotide/ Nucleoside Reverse Transcriptase
Phe/Fe	Phenylephrine
PI	Protease Inhibitor
PL	Phospholipids
PON	Paraoxonase
PSP	Peak Systolic Pressure
PVAT	Perivascular Adipose Tissue
Qa/AO	Aortic Output
Qe/CF	Coronary Flow
R.I	Regional Ischemia
ROS	Reactive Oxygen Species
SEM	Standard Error of the Mean
sGC	soluble Guanylyl Cyclase
SOD	Superoxide Dismutase
SRC	Standard Rat Chow
STZ	streptizotocin
SV	Stroke Volume
T2DM	Type 2 diabetes Mellitus
TAC	Total Antioxidant Capacity
Tat	Trans-activator of transcription
TBA	Thiobarbituric Acid
TBARS	Thiobarbituric Acid Reactive Substances
TC	Total Cholesterol
TDF	Tenofovir Diphosphate
TNF-α	Tumor Necrosis Factor Alpha

TRIG	Triacylglycerols/Triglycerides
TTC	Triphenyltetrazoliumchloride
UCT	University of Cape Town
UPR	Unfolded Protein Response
US FDA	United States Food and Drug Administration
US	United States
VSMC	Vascular Smooth Muscle Cells
WAT	White Adipose Tissue
WH	Working Heart
Wt/TW	Total Work
XO	Xanthine Oxidase

UNITS OF MEASUREMENT

%	Percentage
°C	Degree Celsius
G	Gram
L	Litre
m	Meter
m^2	Square meter
mg	Milligram
min	Minute
ml	millilitre
mmHg	Pressure
mmol	Milimol
mol	Molar
mW	Miliwatt

nM	Nanomolar
v	Volume
µ	Micro
µl	Microliter
µmol	Micro molar

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Chapter 1 – Introduction

1.1. Background

1981 marked the year when mankind first became aware of the disease that would later be the heated topic of investigation amongst the medical and scientific community for more than 3 decades (Fauci 2003; Gresele *et al*. 2012). It was then, now thirty five years ago that *Acquired Immune Deficiency Syndrome* (AIDS) was first described and scientists embarked on the seemingly insurmountable task of understanding the disease and then later developing a treatment (Fauci 2003). Number of deaths attributable to AIDS quickly rose to devastating proportions. In 1996 the introduction of new drug combinations- *Highly Active Antiretroviral Therapy* (HAART), which targeted the replication and spread of the virus, caused a massive decline in deaths due to *Human Immunodeficiency Virus* (HIV)/AIDS (Lopez 2011).

This treatment gave HIV sufferers new found hope as their life expectancy was increased and the view of AIDS as a death sentence overthrown (Kallings 2008; Lopez 2011). The first few years of antiretroviral therapy (ART) involved infected patients taking as many as 30 tablets a day with large amounts of water and very unpleasant resultant side effects (Kallings 2008). Today, however, HIV sufferers need only take one tablet containing the combination of three or more drugs and therapy is much more effective (Kallings 2008; Schulenberg & Le Roux 2008).

Statistical figures clearly demonstrate a significant decrease in AIDS related deaths post 2005 when an increased rollout of antiretroviral (ARV) drugs occurred in South Africa, where 2005 had a 50.7% AIDS deaths (Statistics South Africa 2015). In mid-2015 the percentage of people dying due to AIDS was 30.5%. This is a positive sign but the 2015 report by Statistics South Africa further ascertains that an increased number of AIDS related deaths may be occurring 10 years post-ARV rollout (Statistics South Africa 2015).

Substantial amount of scientific evidence now shows that whilst ART allows for the chronic management of HIV infection, their prolonged use results in certain toxicities and negative impacts on the body. Amongst the reported cases are nephrotoxicity, development of hypersensitivity to certain ART classes and lipodystrophy (Margolis *et al*. 2014; Meintjes *et al*. 2014). Furthermore and perhaps of greater implications are the findings that long term ART is linked to myocardial infarctions (MI), and increased risk factors for non-HIV related diseases (Lopez 2011).

There are three emergent schools of thought regarding ART and cardiovascular health; 1) reactive oxygen species (ROS) driven toxic effects of long term ART results in pro-inflammatory effects on the endothelium and endothelial function thus increasing risk for cardiovascular disease (CVD) (Schulenburg & Le Roux 2008) as well as the metabolic abnormalities seen in long term users (Currier *et al*. 2008), 2) cardiovascular and metabolic complications arising in long term HIV sufferers are not due to possible toxic effects of treatment but rather to low level of ROS driven chronic inflammation caused by the HIV itself (Aounallah *et al*. 2016) and 3) interplay of both the HIV and toxicities due to ART leads to the altered risk for CVD in HIV positive patients (Currier *et al*. 2008).

Chronic inflammation is the principle driving force of cardiovascular disease in all these findings and although a consensus on the exact cause, whether long term ART, HIV or both, has yet to be made it is very clear that possible counter measures for ROS and thus inflammation have to be discovered and employed. For this reason it proves logical to investigate potent sources of antioxidants that may be able to intensify the body's antioxidant capacity and thus alleviate the oxidative effects of ROS and consequent inflammation.

Rooibos (*Aspalathus linearis*) is native to the Cederberg Mountains of the Western Cape where it grows as a shrub-like bush (McKay & Blumberg 2007). Apart from its many constituents rooibos contains vitamins, minerals and a high content of polyphenols which act as antioxidants (Standley *et al*. 2001; Barreira *et al*. 2013). It does not contain caffeine or other stimulants and has further been proven to have medicinal uses in the treatment of numerous ailments including HIV (Street & Prinsloo 2013). The health benefits of rooibos supplementation during ARV treatment, however, remains largely unsubstantiated by scientific research, creating a need for the present study.

1.2 Research Question

What are the cardiovascular effects of *Aspalathus Linearis*/ rooibos infusion supplementation during ART, on male Wistar rats and the effects of rooibos tea consumption on the cardiovascular profiles of patients on ART?

1.3 Research Aims

- Aim 1: To investigate the effects of rooibos tea supplementation, during first line fixed dose ART, on the isolated hearts and aortas of male Wistar rats.
- Aim 2: To determine the effects of rooibos tea consumption on the cardiovascular profiles of patients on ART.

1.4 Research Objectives

- **Aim 1 Objectives**

I. Treat rats for 9 weeks during which time the body weights and fluid intake are monitored by taking weekly measurements.
II. Examine the functionality of the hearts through isolated heart perfusions.
III. Conduct vascular reactivity studies to determine endothelial function and effect of perivascular adipose tissue (PVAT) on relaxation and contraction.
IV. Through biochemical analyses determine the lipid profiles of the rats and thiobarbituric acid reactive substances (TBARS) levels as an indicator of lipid peroxidation.

- **Aim 2 Objectives**

V. To perform descriptive statistics on the study population.
VI. Examine cardiovascular parameters and anthropometric parameters of patients.
VII. Through biochemical analysis of patient's serum determine lipid profiles and TBARS levels as an indicator of lipid peroxidation.

1.5 Significance of the research

Incidence of deaths attributable to HIV/AIDS is clearly increasing in the long term HIV positive population (Statistics South Africa 2015). Evidence shows that long term HIV/AIDS sufferers commonly develop CVD and complications (Currier *et al*. 2008; Aounallah *et al*. 2016). Few theories exist as to the exact causes, and even fewer theories postulating mechanisms leading to this increase in CVD. There is, therefore, a need for scientific studies that will examine possible relationships between ART,

HIV and CVD. The introduction of a new fixed dose combination (FDC), in SA in 2012, consisting of efavirenz, emtricitabine and tenofovir has further presented a need for research into the specific effects of this ART (Clinicians Society 2013). This study enables us to look at the direct effects of this drug combination on the heart and aorta of male Wistar rats in the absence of HIV and might shed some insight as to the effects of the same treatment in HIV positive patients.

Scientific studies have identified ROS as the key role player in the development of CVD in long term ART users (Currier *et al*. 2008; Aounallah *et al*. 2016). A plausible solution is to investigate possible co-treatments that may target the excessive ROS generation in this population. This research provides such an investigation using a drink that is affordable, with growing acclaim in the scientific community, is indigenous to SA and is a potent source of antioxidants: rooibos tea (Ajuwon *et al*. 2015). Albeit scientific studies with rooibos tea are plentiful, few have examined the qualities of this tea in the context of ART and HIV/AIDS (Marnewick *et al*. 2011).

1.6 Research Scope

Within the scope of this study are the isolated heart perfusions to determine functional recovery and the vascular reactivity experiments for the determination of endothelial function. These experiments are performed in our own laboratory and are therefore cost effective and feasible. The collection of blood samples for lipid profile determination and TBARS analyses allows us to gain as much information as possible from the same set of rats. The biochemical analyses are performed externally by a chemical pathologist to eliminate chances of error because the procurement of samples is a time consuming and costly process.

Objectives that fall outside of the scope of the study and thus represent limitations include determining the signaling pathways or molecules activated in the various treatment groups. This did not form part of the objectives of this study because of time constraints and the resultant work load would surpass that which is required for an MSc thesis. Examining other measures of oxidative stress also do not fall within the scope of this study because of monetary constraints.

The epidemiological study is a cross-sectional study focused on a study population from Durbanville, Fisantekraal, Elsies River, Uitsig, Bishop Lavis and Ravensmead. This study formed part of a parent study, termed “EndoAfrica”, which is coordinated by Prof Hans Strijdom and forms part of an international collaboration funded under the EU FP 7 programme. The study scope includes blood

serum analyses, determination of lipid profiles, recording of anthropometric measurements and determining the influence of life style choices on cardiovascular risk factors.

Chapter 2 – Literature Review

The purpose of this review of the literature is to examine the reported effects of HIV and ART on the cardiovascular system and particularly on endothelial function and to review the capacity of *Aspalathus linearis* to counter oxidative stress.

The review is structured so that the endothelium and the role of perivascular adipose tissue (PVAT) in the vasculature are discussed first. This is followed by the topic of HIV and CVD. ART is then discussed before entering into the topic of oxidative stress and *Aspalathus linearis* as a counter measure against ROS induced CVD.

2.1 The Endothelium

The endothelium is a thin layer of cells which forms the inner lining of blood vessels and plays an important role in regulating vascular function (Huang 2003; van Hinsbergh 2012). It performs an array of general and specific functions including preserving blood fluidity, controlling the recruitment of inflammatory cells and adhesion molecules, post inflammatory healing as well as regulation of vasodilation (van Hinsbergh 2012).

2.1.1 Endothelial Function

The endothelium achieves vasodilation through nitric oxide (NO) release in vascular smooth muscle cells (VSMC) (Sena *et al*. 2013). The enzyme endothelial nitric oxide synthase (eNOS) produces NO (Sena *et al*. 2013). eNOS is activated after increased levels of Ca^{2+} in the cytoplasm and facilitates binding of calmodulin to the enzyme (Sena *et al*. 2013). It produces NO by firstly adding a hydroxyl group to L-arginine to form N”-hydroxy-L-arginine, the second step involves the oxidation of N”-hydroxy-L-arginine by eNOS to produce L-citrulline and NO (Förstermann & Sessa 2012). NO activates soluble guanylyl cyclase (sGC) causing increased levels of cyclic guanosine-3,5-monophosphate (cGMP) (Sena *et al*. 2013). This in turn results in relaxation of VSMC through opening of K^+ channels and membrane repolarization (Sena *et al*. 2013).

NO is a signaling molecule that has many targets and is involved in a number of processes but with a very short half-life of about less than 4s before it is converted to nitrite (NO_2^-), then nitrate (NO_3^-) and ultimately excreted in the urine (Sena *et al*. 2013). It has anti-inflammatory and anti-atherosclerotic

effects through the inhibition of endothelial cell apoptosis (Förstermann & Sessa 2012). Other functions of NO include control of vascular smooth muscle proliferation (Förstermann & Sessa 2012).

2.1.2 Endothelial Function and Inflammation

Inflammation is a multifactorial response to a disturbance in tissue homeostasis resulting from either infection by microbes, injury or trauma or exposure to foreign particles, irritants or pollutants. The primary function of inflammation is to restore homeostasis by destroying or isolating the source of the disturbance. (Ashley *et al*. 2012)

Pro-inflammatory markers are produced in cases of infection, trauma or stress. Of particular importance is interleukin-6 (IL-6). IL-6 together with C-reactive protein (CRP) have been proven to act directly on NO decreasing its bioavailability and thus impairing endothelial vasodilation. CRP is an inflammatory marker that is produced by hepatocytes following stimulation by IL-6. It is used as a biomarker for endothelial dysfunction (ED) and can also be used as a cardiovascular risk factor (Lagrand *et al*. 1999). In pathophysiological states, characterised by increased inflammation, an increased synthesis of cells, cytokines and pro-inflammatory molecules ensues causing an increase in IL-6 and CRP production. This in turn feeds back to further exacerbate the inflammatory response thus creating an inflammatory cycle. (Teixeira *et al*. 2014)

Scientific studies have revealed a clear relationship between inflammation and endothelial dysfunction and inflammatory markers have been proven to directly cause a decrease in eNOS activity thus reducing NO bioavailability and catapulting the ED process (Teixeira *et al*. 2014).

2.1.3 Endothelial Dysfunction

ED occurs when there is an imbalance between the vasoconstricting and vasodilating factors of the vasculature. It is characterized, chiefly, by an impairment in NO bioavailability with, amongst other characteristics, lowered endothelium-mediated vasorelaxation and increased ROS leading to augmented oxidative stress environment with greater expression of inflammatory genes. (Sena *et al*. 2013)

The underlying mechanisms of ED include the trapping of NO by free radicals, chiefly via superoxide (O_2^-) binding with NO to form peroxynitrite ($ONOO^-$) (further discussed in section **2.6.1 Oxidative Stress: Definition and Causes**) and reduced eNOS activity. (Huang 2003)

2.2 Role of Perivascular Adipose Tissue (PVAT) in the Vasculature

Perivascular adipose tissue (PVAT) is the layer of adipose tissue surrounding most blood vessels. There are three different categories of PVAT that are classified in rodents according to the anatomical locations. The classifications are: brown PVAT located around the thoracic aorta and has the functions and characteristics of brown adipose tissue (BAT), white PVAT which is similar to white adipose tissue (WAT) and surrounds small arteries and lastly beige PVAT which is found around the abdominal aorta, this is a mixture of BAT and WAT. Scientific evidence points towards human PVAT having characteristics of both WAT and BAT. (Brown *et al*. 2014)

PVAT plays important roles in vascular function. The many functions of PVAT include offering mechanical protection to blood vessels by preventing contact induced friction with neighboring vessels, release of factors which cause vasodilation and influence VSMC contraction, proliferation, migration and thermoregulation. Furthermore, autocrine or paracrine effects through its release of several specific molecules and contractile effects (Brown *et al*. 2014). There are studies supporting both the contractile and vasodilator effects of PVAT (Rajsheker *et al*. 2010).

In healthy individuals and under normal physiological conditions, PVAT produces a factor called adipocyte-derived relaxing factor (ADRF). This factor has been proven to induce relaxation of the vasculature through its activation of VSMC K^+ channels thus inhibiting contraction. A study published in 2007 was able to demonstrate that ADRF can be transferred from one intact endothelium to the next and its induction not only dependent on an intact endothelium but also on endothelial nitric oxide release. Studies have further shown that the dilator effects of PVAT are lost under pathological conditions, most specifically in the presence of obesity. (Rajsheker *et al*. 2010)

PVAT dependent contraction in the vasculature has been demonstrated, through studies of PVAT in dogs, to be ROS mediated (Rajsheker *et al*. 2010). In a more recent study published in 2014 authors sought to determine the mechanism of PVAT regulation of vascular tone (Lee *et al*. 2014). They performed isometric tension studies on isolated thoracic aortas (with and without PVAT) of male

Wistar rats and found that the PVAT caused increased vasoconstriction and inhibited vasodilation by attenuating NO production (Lee *et al*. 2014).

2.3 HIV

2.3.1 Introduction

There are two different subtypes of HIV, HIV-1 and HIV-2 with HIV-1 being the most common type and HIV-2 being closely related but typically found in West Africa (Takhar & O'Laughlin 2014). This review will refer only to HIV-1 by simply using the term 'HIV'. HIV is transmitted through direct contact with either the blood stream or contact and attachment to the mucosal surface (Dau & Holodniy 2008). A mature HIV virion is composed of an inner core surrounded by a viral membrane and an outer envelope. The core contains two copies of RNA, the enzymes reverse transcriptase and integrase, and regulatory proteins. The viral membrane surrounding the core contains the glycoproteins gp41 and gp120 (**Fig. 2.1.**) (Takhar & O'Laughlin 2014).

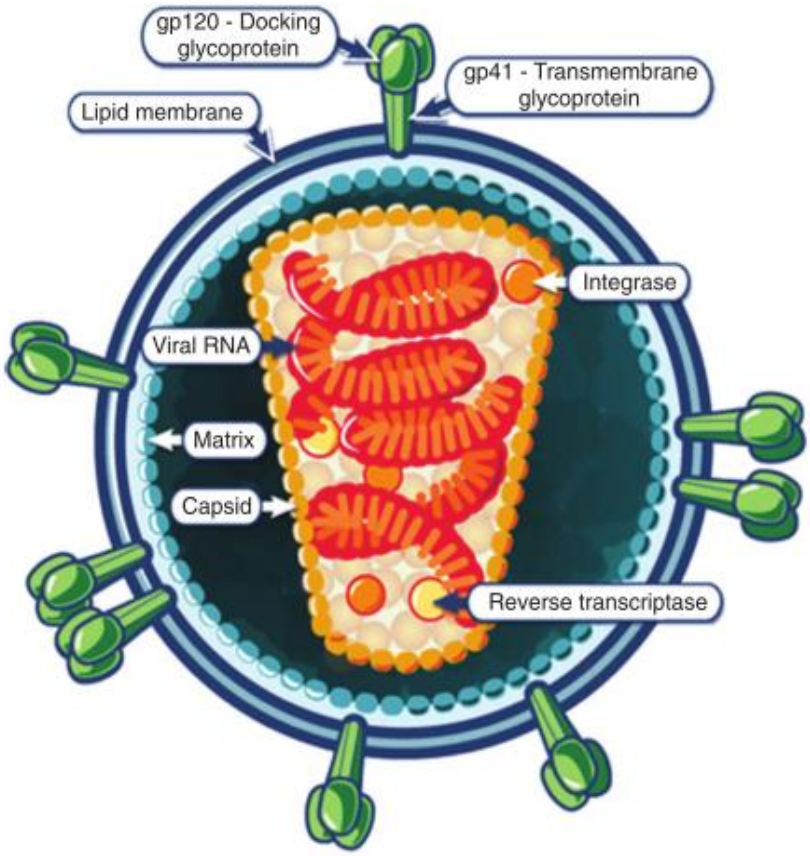


Fig. 2.1. *The HIV virion* (Takhar & O'Laughlin 2014).

The HIV envelope has an affinity for the CD4 receptor (Arts & Hazuda 2012). During infection the virus fuses to the target cell surface through attachment of its glycoproteins to $CD4^+$ glycoproteins and co-receptors. The outer envelope fuses with the target cell membrane thus facilitating the entry of the inner core into the host cell. Once inside, reverse transcriptase transcribes the viral RNA into DNA which is transported by integrase into the nucleus of the host cell. Viral DNA then integrates into the host DNA. New viral particles are created through the formation of viral polyproteins and RNA which are modified and spliced by the viral enzyme protease. The mature virus is thus formed and the cycle restarted. After infection the virus destroys host $CD4^+$ T-cell. The mechanism leading to this destruction is still unclear. (Takhar & O'Laughlin 2014)

2.3.2 Mechanisms of HIV Induced CVD

Once an individual is infected with a virus the body's immune system reacts as a defense against the disease. The innate immune system is the first line of defense and the cellular components thereof regulate transcription factors responsible for the production of interferons and cytokines (Thompson *et al*. 2011). Cytokines act as a cell stimulus which activate nuclear factor-kappa beta (NF-κβ) (Barnes & Karin 1997; Hoesel & Schmid 2013). The HIV protein Trans-Activator of Transcription (Tat) further stimulates the production of Tumor necrosis factor alpha (TNF-α) and NF-κβ (Gresele *et al*. 2012).

NF-κβ plays a crucial role in the pathogenesis of persistent inflammation and metabolic disorders in chronic disease states. It primes and causes the subsequent activation of a specific cellular component of the innate immune system termed the nucleotide oligomerization domain-like receptors (NLRs/NACHT). Once primed this cellular component then forms the NLRP3 inflammasome- a multi-domain complex chiefly responsible for initiating the maturation of the inflammatory markers interleukin 1β (IL-1β) and interleukin 18 (IL-18). This persistent production of IL-1β and IL-18 further stimulates NF-κβ resulting in a feedback system. This is the proposed mechanism by which chronic illness induced by viral infection, such as in the case of HIV infection, leads to tissue inflammation, damage and diseases including CVD. (Aounallah *et al*. 2016)

This link between HIV infection and CVD is reflected in the health records of HIV positive versus HIV negative individuals pertaining to the Kaiser Permanente (KP) data base, an electronic data base containing health records of more than 30 000 000 members in 8 regions of the United States (US). Records show a significantly higher hospitalisation rate for both coronary heart disease and

myocardial infarction in HIV positive members as opposed to HIV negative members. Cerrato *et al*. 2013 illustrate this link and demonstrate how persistent immune activation and endothelial dysfunction together with inflammation stimulate the pathogenesis of coronary artery disease in HIV positive patients (**Fig 2.2.**) (Cerrato *et al*. 2013).

It has been found that in the case of chronic inflammation, the metabolism of inflammatory immune cells is altered to one that is reliant on aerobic glycolysis as a response to their increased energy needs. This metabolic profile is characterised by a disturbed Krebs cycle that leads to a build-up of ROS, succinate and citrate. This further drives inflammation through the stabilisation of hypoxia-inducible factor 1α (HIF-1α) by mitochondrial succinate and increased NLRP3 inflammasome activation (Aounallah *et al*. 2016). ARTs are an effective treatment for $CD4^+$ T-cell recovery but they do not fully counteract inflammation (Aounallah *et al*. 2016). These findings highlight the importance of developing a supplement or co-treatment for HIV that will counter the persistent inflammation.

The study by Ross *et al*. 2008 had as part of its objectives to determine the role of HIV and ART in inflammation and subsequently endothelial activation. The study enrolled 182 patients and measured plasma levels of inflammatory cytokines and endothelial activation markers. Study groups were HIV+ on ART with HIV-1 RNA <1000 copies/mL with and without clinical lipoatrophy, HIV+ ART naive, and healthy controls. The results of this study suggested that endothelial dysfunction in the HIV positive group is driven by uncontrolled viremia and heightened inflammation. (Ross *et al*. 2008)

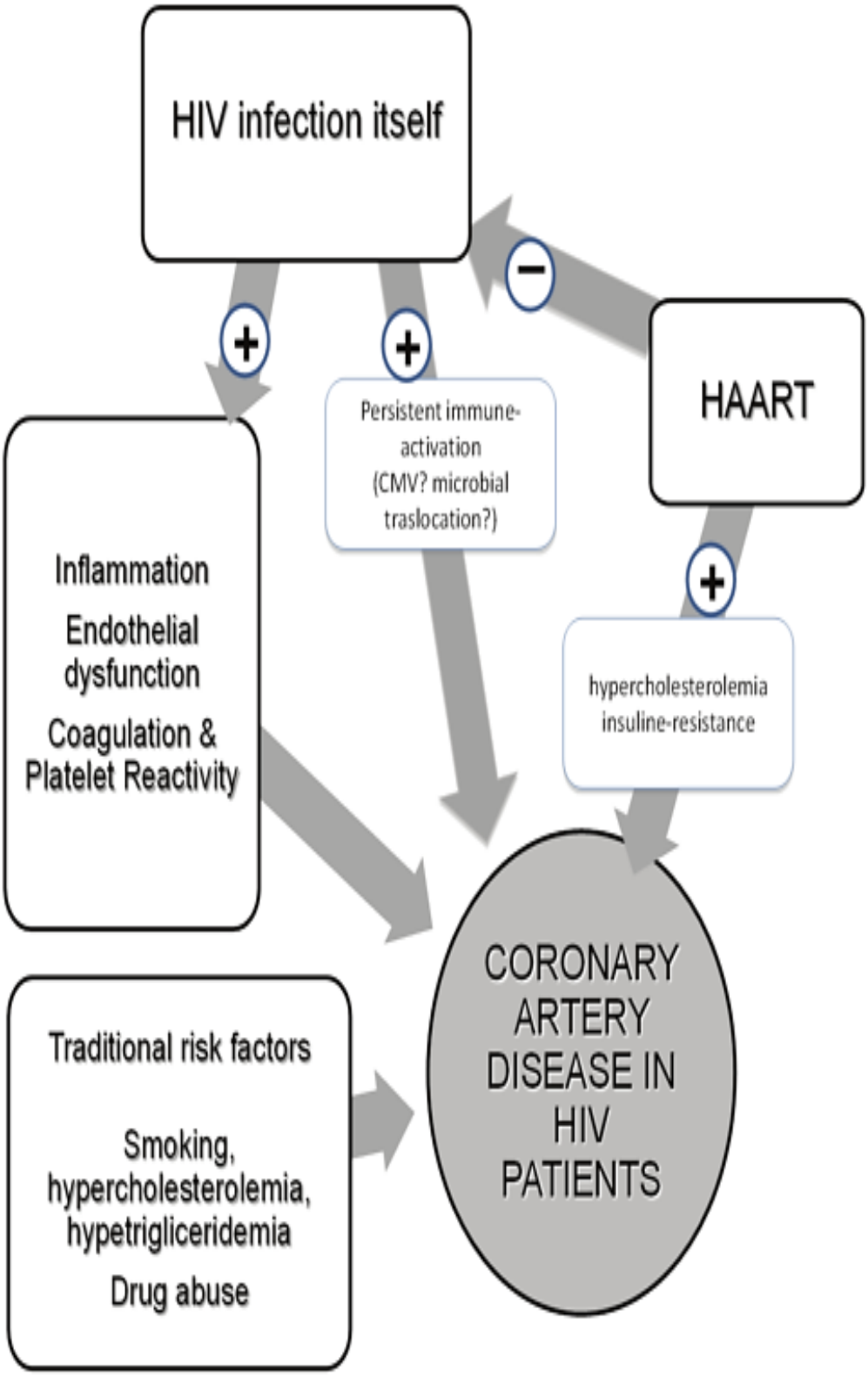


Fig. 2.2. *Pathogenesis of coronary artery disease in HIV patients*. **Abbreviations:** HIV: Human Immunodeficiency Virus; HAART: Highly Active Anti-retroviral Therapy; CMV: Cytomegalovirus (Dau & Holodniy 2008).

Dau and Holodniy in their 2008 publication "*The Relationship Between HIV Infection and Cardiovascular Disease*" explore the direct effects of HIV on the endothelium. The reported effects include that of secreted HIV protein Tat and envelope glycoprotein gp120. Tat has been found to weaken vasorelaxation in porcine coronary arteries by significantly reducing eNOS mRNA whilst gp120 causes endothelial damage by increasing the production of NO in activated macrophages and directly stimulating endothelial cells resulting in more leukocytes binding to the endothelium (Dau & Holodniy 2008). Tat also causes the production of TNF-α and NF-κβ by mononuclear cells, this is reported to initiate an inflammatory response (Gresele *et al*. 2012).

2.3.3 The Inflammatory Cycle in HIV Infection

IL-1β and IL-18 play important roles in initiating the body's second line of defence: the adaptive immune system (Thompson *et al*. 2011). IL-18 causes increased production of natural killer cells (NK) by interferon gamma (IFNγ) and plays a role in recruiting and activating neutrophils while IL-1β plays a role in activation of T-lymphocytes, recruitment of innate immunity cells and initiating a fever (Thompson *et al*. 2011). Studies have proven a direct correlation between $CD8^+$ activation and HIV disease progression, furthermore, markers of activation and/or apoptosis of $CD8^+$, $CD4^+$, T cells, B cells and NK cells and monocytes are elevated in HIV infected individuals (Appay & Sauce 2008).

Lymphocyte activation leads to the production of pro-inflammatory cytokines and chemokines and directly increases intracellular levels of NF-κβ. NF-κβ promotes HIV-1 replication by inducing increased viral transcription which in turn exacerbates immune activation, feeding back to increased viral transcription- a cycle is thus created (**Fig. 2.3.**). (Appay & Sauce 2008)

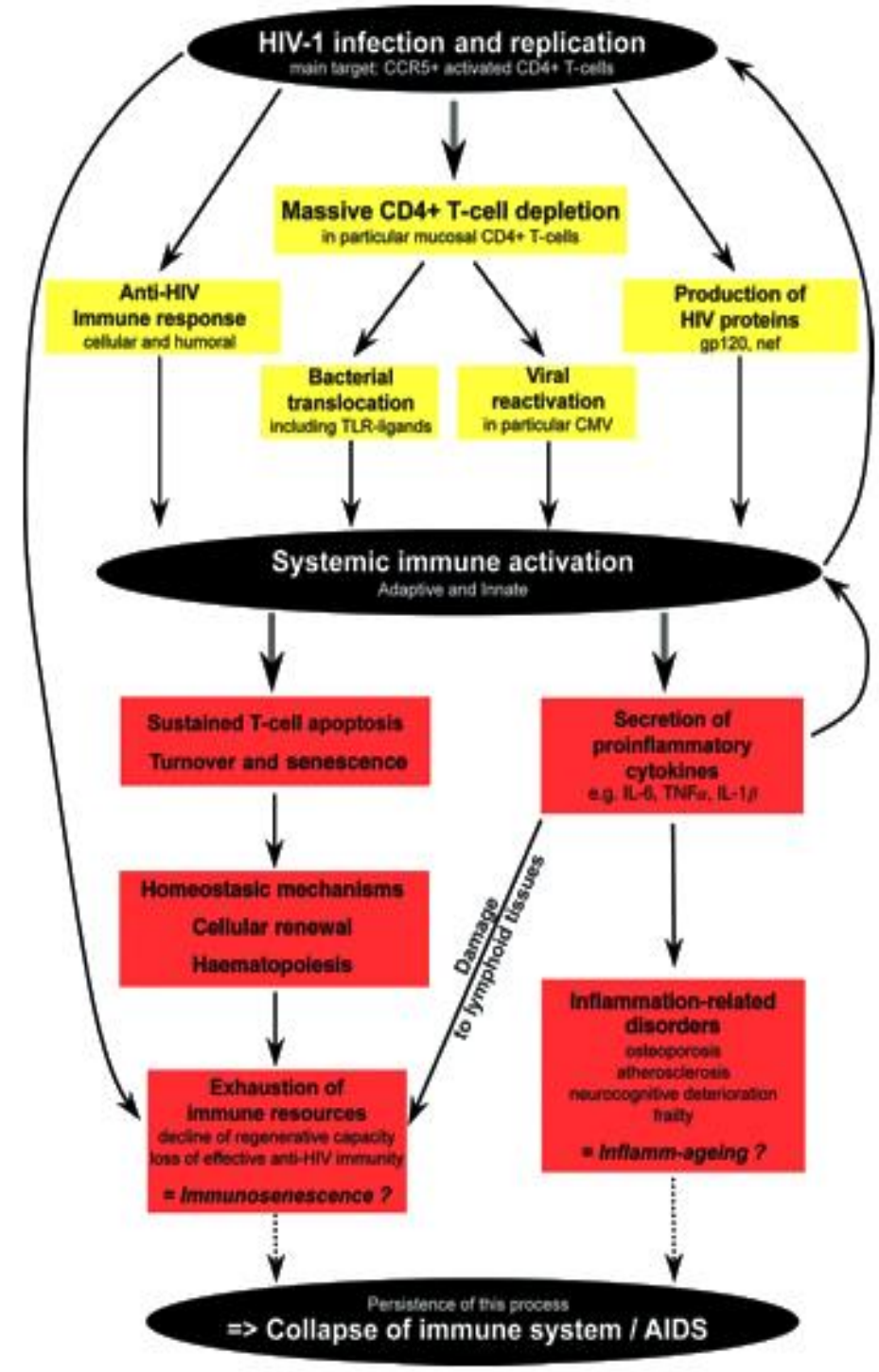


Fig. 2.3. *The causes and consequences of immune activation in HIV progression* (Appay & Sauce 2008).

2.4 ART

2.4.1 The Different Classes of ART

ART drugs have been developed to target different parts of the HIV life cycle and are divided into classes dependent on the step that they target (Arts & Hazuda 2012). There are five classes available in Southern Africa:

- Entry inhibitors/ cell membrane fusion inhibitors- block viral entry into host cells
- Nucleoside and non-nucleotide reverse transcriptase inhibitors (NRTI & NNRTI)- target the enzyme reverse transcriptase
- Protease inhibitors (PI) - block proteolysis of the viral polyprotein inhibiting the production of viral particles.
- Integrase inhibitors- inhibit the integration of viral DNA into the cellular DNA. (Arts & Hazuda 2012; Meintjes *et al*. 2014)

PIs are the most potent agents available and regimens with PI contain a fourth drug that serves as a "boosting" agent to inhibit their metabolism and boost drug levels (Arts & Hazuda 2012). HIV treatment is divided into three specific lines each characterized by different drug combinations (Meintjes *et al*. 2014).

2.4.2 Different Lines of Therapy

Initially commencement with first line ART treatment was dependent on the patients CD4 count, viral load, and willingness to commit to lifelong treatment (Dau & Holodniy 2008). According to WHO guidelines, however, initiation of first line of treatment is now recommended among all HIV positive adults regardless of CD4 cell count (World Health Organization 2015). This criteria may be changed at a later stage.

The first line ART regimen is made up of two NRTI's and one NNRTI with the preferred drugs being tenofovir diphosphate (TDF) + emtricitabine 5'-triphosphate (FTC or 3TC) + efavirenz (EFV) (Meintjes *et al*. 2014). These drugs are available as a FDC in a once daily, single tablet formulation (Deeks & Perry 2010) and in 2012 was rolled out as the first line regimen for South Africa by the SA Department of Health (Clinicians Society 2013). The doses in this FDC tablet are efavirenz/emtricitabine/tenofovir: 600mg/200mg/300 mg respectively (Deeks & Perry 2010).

Efavirenz (EFV): NNRTI that is a noncompetitive inhibitor of HIV-1.

Emtricitabine (FTC or 3TC): NRTI that is a competitive inhibitor of HIV-1, stops viral DNA chain growth

Tenofovir (TDF): NRTI competitively inhibits reverse transcriptase of HIV-1 stopping viral DNA chain growth. (Deeks & Perry 2010)

The recommended second line of ART treatment are 2 NRTI's and a ritonavir boosted PI and the change from first line to second line regimen only occurs after a long period of virological failure. The change to third line of therapy only occurs if there is virological failure caused by NRT and NNRTI or resistance to PI. ART's have been developed with the purpose of reducing morbidity and mortality caused by HIV, to suppress viral load for as long and as much as possible and to further improve the quality of life of infected individuals by re-instating and maintaining immune function. (Meintjes *et al*. 2014)

2.4.3 Reported Effects of ART

Human studies have proven the efficacy of ART but reported long term effects are a cause for concern. Reported side effects of ART range from effects on the central nervous system such as hallucinations, insomnia and drowsiness caused by efavirenz, renal dysfunction and acute renal failure caused by tenofovir as well as hyperlipidemia being a causative effect of PI's (Dau & Holodniy 2008). More specifically the effects on the cardiovascular system have been investigated.

HIV is reported to have pro-inflammatory effects which leads to endothelial activation. ART, in general, is reported to lower this endothelial activation, but this is still an active area of research (Dau & Holodniy 2008). Metabolic syndrome (MS) is used as a marker to identify individuals who are at risk of CVD and type 2 diabetes mellitus (T2DM) (Wand *et al*. 2007). Evidence shows MS to become significantly progressive within 3 years of ART, specifically with the NRTI's didanosine and stavudine together with the NNRTI efavirenz or nelfinavir (Wand *et al*. 2007). The observed increased incidences of MS and obesity evident in the HIV infected population lacks clear evidences of whether it is as a result of the medication or the disease itself (Lopez 2011). The study by Ross *et al*. 2008, previously referred to in the section **2.3.2 Mechanism of HIV Induced CVD**, found that although ART decreased markers of inflammation patients remained with heightened inflammation compared to controls. ART, particularly PI's and NRTI's, has been linked to myocardial infarctions and have been shown to increase risk factors for non HIV related diseases (Lopez 2011).

The study by Bastard *et al*. 2014 which sought to investigate the roles of personal factors and HIV infection severity on the inflammatory profile of long-term ART controlled patients found markers of inflammation and immune activation to be elevated in the long-term treatment controlled HIV-infected patients compared to healthy controls. Surprisingly this elevation was related to demographic and behavioral factors and had no correlation with markers of severity of HIV infection. (Bastard *et al*. 2014)

The *Cinical Trials Group Study 5152s* aimed to evaluate the effects of three class-sparing ART regimens on endothelial function in HIV-infected patients in a randomized trial. Patients were randomly assigned to groups of either a PI-sparing regimen of NRTIs+ efavirenz, a NNRTI-sparing regimen of NRTIs+ lopinavir/ ritonavir or a NRTI-sparing regimen of efavirenz+ lopinavir/ ritonavir (Torriani *et al*. 2008). Endothelial function was determined by measuring the brachial artery flow-mediated dilation (FMD) before starting ART and at 4 and 24 weeks of treatment. The study employed 82 patients and found that all three ART regimens improved endothelial function.

It has, however, been reported that commencing HAART treatment increases endothelial activation and dysfunction, this was at a mean duration of 9 months. Whilst patients on HAART for longer periods showed signs of endothelial dysfunction. More evidence exists on the effects of PI exposures causing endothelial dysfunction, overall damage to the vasculature by its increase of ROS formation and metabolic abnormalities than any other class of ART. (Haser & Sumpio 2016)

There is some scientific evidence for NRTI's contributing to increased cardiovascular disease risk. A recent review on the effects of HAART and HIV on the heart described the possible mechanisms by which HAART causes atherosclerotic cardiovascular disease (ASCVD). These mechanisms are through lipodystrophy, dyslipidemia (which both cause an increase in circulating triglycerides and lipid derangements), through direct damage of mitochondrial DNA and insulin resistance. (Lambert *et al*. 2016)

It is currently difficult to find studies that have examined the cardiovascular effects of long term tenofovir-based first line ART. A particular study conducted in western Kenya has examined the efficacy of treatment and incidence of drug resistance in this therapy. The tenofovir-based treatment group was compared against a non-tenofovir-based group and a tenofovir-only group. The study found the tenofovir-based ART to be most efficient in viral suppression compared to the non-

tenofovir-based group and have lower incidences of resistance compared to the tenofovir-only group. (Brooks *et al*. 2016)

2.4.4 Mechanism of ART induced CVD

Although the mechanisms by which ART causes increased risk for CVD and toxicities such as hepatotoxicity are largely unclear, recent evidences have begun to shed some light on this topic. The growing body of evidence linking ART to CVD demonstrate the central role of the endoplasmic reticulum (ER) in the underlying pathophysiological mechanisms.

The ER is found in all cell types and is the organelle responsible for protein synthesis, translation, folding and trafficking. Proper functioning of the ER demands a strict control over the intraluminal oxidative environment and the high calcium concentration. Disturbances to the environment of this organelle occur through, amongst other factors, viral infections, oxidative stress, energy deprivation, calcium depletion and elevated protein trafficking. Disturbances to the ER lead to the accumulation of unfolded proteins within the ER - a biological state termed “ER stress”. In ER stress the cell activates a complex signalling network, which acts as a protective mechanism, known as the unfolded protein response (UPR). While this response seeks to restore ER homeostasis by increasing transcription of ER-chaperones, triggering the breakdown of misfolded proteins and decreasing protein translation, prolonged action triggers the activation of inflammatory pathways and ultimately leads to cell death by apoptosis. (Apostolova *et al*. 2013; Galán *et al*. 2014; Cimellaro *et al*. 2016)

ER stress has, over the recent years, been recognised as a common feature in the inflammatory response underlying cardiovascular disease. Chronic ER stress also leads to the accumulation of ROS and plays a crucial role in inducing endothelial dysfunction. This mechanism is well depicted by Cimellaro *et al* (**Fig 2.4.**). (Cimellaro *et al*. 2016)

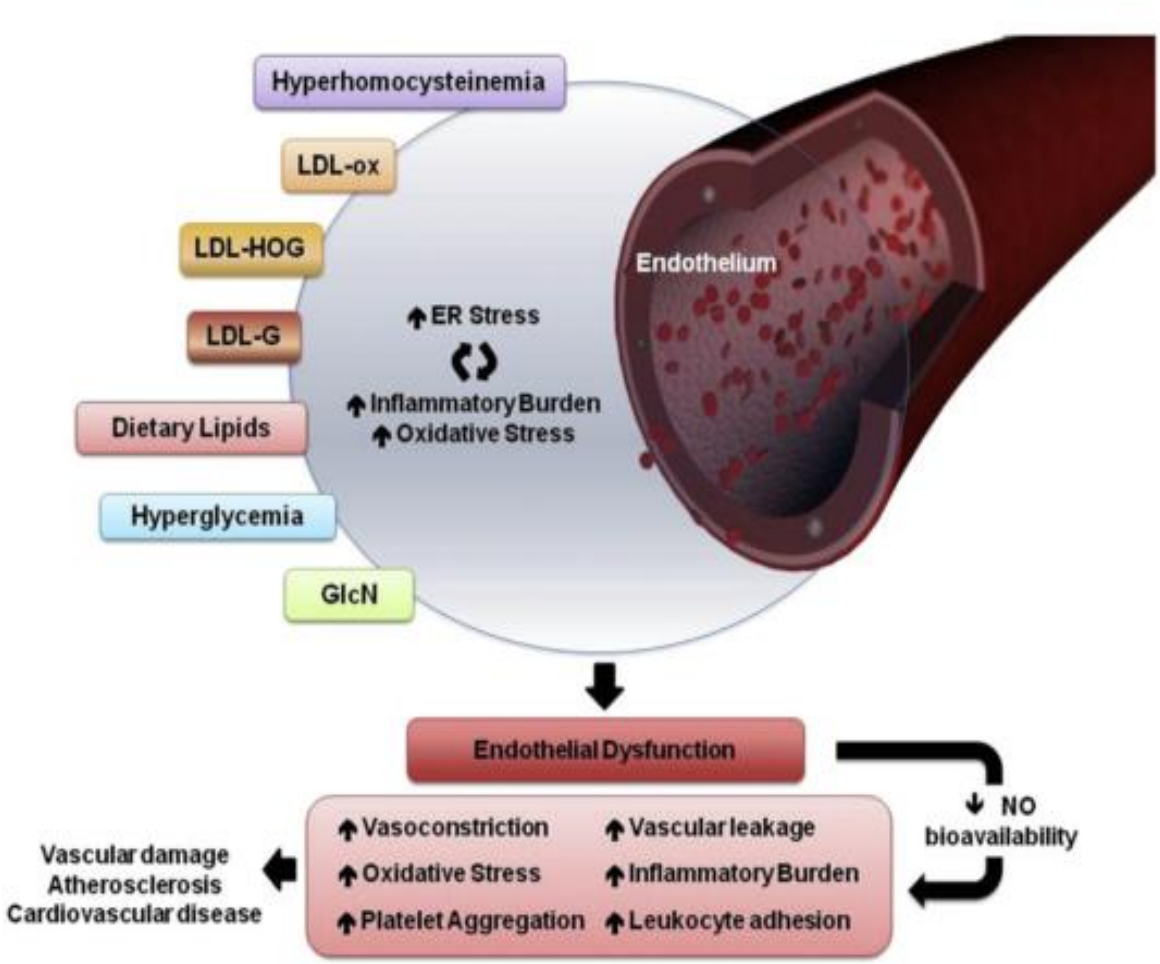


Fig. 2.4. *Pathogenesis of ER stress mediated endothelial dysfunction (ED) and cardiovascular disease* (Cimellaro et al. 2016).

ER stress is both the cause and the effect of chronic inflammation and oxidative stress found in endothelial dysfunction. Metabolic risk factors also play a role in ED. ER: Endoplasmic Reticulum; GlcN: Glucosamine; LDL-G: Glycated Low-density lipoprotein; LDL-ox: Oxidized Low-density lipoprotein; LDL-HOG: Oxidized and Glycated Low-density lipoprotein; NO: Nitric Oxide. (Cimellaro *et al*. 2016)

This mechanism of ER stress mediated ED has been described in drug-induced toxicities and more specifically in ART induced toxicity (Cimellaro *et al*. 2016). EFV, being the NNRTI that is most widely used and a constituent of the once daily FDC tablet, was investigated in the induction of ER stress in human hepatic cells (Apostolova *et al*. 2013). The study treated primary human hepatocytes with clinically relevant concentrations of EFV and found ER stress markers to be elevated in the cells treated with EFV (Apostolova *et al*. 2013). Another study observed the same findings when exposing human hepatic cells to EFV and further linked it to mitochondrial dysfunction (Alegre *et al*. 2013). A study in

the UK exposed the rat heart H9C2 cell line to three different concentrations of EFV for 24 or 48 hours and observed a concentration dependant loss in cell viability and EFV induced apoptosis and necrosis (Bergin *et al*. 2015).

2.5 Oxidative Stress

2.5.1 Definition and Causes

Free radicals are chemical species that contain an unpaired electron in their outer orbital. These chemicals are very unstable and react with larger molecules thus oxidizing them and forming other free radicals. They are formed from four elements; oxygen to form reactive oxygen species (ROS), nitrogen, sulphur and chlorine to form reactive nitrogen species, reactive sulphur species and reactive chlorine species respectively. Of the four types ROS and RNS are the most relevant to cellular biology, with ROS being of greatest significance. (Ajuwon *et al*. 2015)

Reactive oxygen species (ROS) are free oxygen radicals, oxygen ions and peroxides. At moderate levels ROS are important in maintaining tissue homeostasis. These signaling molecules play key roles in vascular tone, oxygen sensing, cell growth and proliferation, apoptosis and inflammatory response. There are a few sources of ROS in the vasculature and of major importance are the following enzyme systems: NADPH oxidase (Nox), xanthine oxidase (XO), enzymes of the mitochondrial respiratory chain, and irregular endothelial nitric oxide synthase (eNOS) activity (Li *et al*. 2014). When ROS derived oxidation outweighs antioxidant capacity oxidative stress occurs (Birben *et al*. 2012).

Nox is made up of many proteins which assemble in the cell membrane to make up the active form of the enzyme (Li *et al*. 2013). It is expressed in various cell types including endothelial cells, smooth muscle cells and fibroblasts (Li *et al*. 2013). Of the existent isoforms of the enzyme Nox2 and Nox4 are mostly found in endothelial cells (Li *et al*. 2014). NADPH is used as an electron donor which transfers an electron to molecular oxygen thus producing superoxide (O_2^-) (Li *et al*. 2014). XO is expressed both in endothelium and in plasma and it too donates electrons to molecular oxygen producing O_2^- and hydrogen peroxide (H_2O_2) (Li *et al*. 2013). The liver synthesizes xanthine dehydrogenase which is converted to XO by proteolysis(Li *et al*. 2013). Evidence shows XO is expressed by endothelial cells and is up regulated with increased activity of Nox (Li *et al*. 2013).

The mitochondria produces ROS via the mitochondrial electron transport chain (ETC). Although ROS is produced by other electron complexes and mitochondrial enzymes (Ajuwon *et al*. 2015) the chief emitters of ROS are complexes I and III. Both complexes emit ROS in the form of O_2^- into the mitochondrial intermembrane space during energy transduction. ROS production by the mitochondria varies depending on a number of factors including cardiovascular risk factors, electron donor availability and uncoupling proteins. (Li *et al*. 2013)

NOS proteins have three different isoforms, namely neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) all of which are homodimers (Förstermann & Sessa 2012). Under normal physiological conditions eNOS produces NO. In conditions of oxidative stress, however, eNOS is converted from an enzyme that produces NO to one that produces O_2^- (Förstermann & Sessa 2012). The process of its conversion is termed "NOS uncoupling" and to date quite a few mechanisms for this process have been described (Förstermann & Sessa 2012).

In essence the process occurs through the oxidation of NO in the presence of excess O_2^- to produce peroxynitrite ($ONOO^-$) (Li *et al*. 2013). One of the mechanisms by which $ONOO^-$ can lead to eNOS uncoupling is through the oxidation of the essential cofactor BH_4 rendering it biologically inactive (Förstermann & Sessa 2012). NO bioavailability is further reduced in that ROS accelerate the breakdown of NO (Sena *et al*. 2013).

2.5.2 Biomarkers of Oxidative stress

There are two ways in which oxidative stress can be quantified biologically; one is by measuring molecules that are modified through interaction with ROS in the microenvironment (DNA, lipids, proteins and carbohydrates) and the second is by measuring the alteration in molecules of the antioxidant system as they respond to increased redox stress. One such change in molecules reacting with ROS occurs in lipids and is known as lipid peroxidation. In this process lipids are readily oxidized by free radicals because of the large presence of reactive double bonds in their molecular structure. (Ho *et al*. 2013)

The most well-known markers of lipid peroxidation are isoprostanes (IsoPs) and malondialdehyde (MDA). IsoPs are products of arachidonic acid, a fatty acid found in the phospholipids of cell membranes, peroxidation. They are released into circulation by phospholipases and can thus be quantified in tissues, urine and blood samples. Such quantification can be achieved via mass

spectrometry, enzyme-linked immunosorbance assays (ELISA) and radioimmunoassay in plasma and urine samples. Both animal and human studies have validated this correlation between IsoPs levels in urine and plasma with in vivo oxidative stress levels. (Ho *et al*. 2013)

MDA is the product of polyunsaturated fatty acid peroxidation. It can react with a number of proteins. The most significant reaction of MDA in the quantification of oxidative stress is its reaction with thiobarbituric acid (TBA) generating TBA reacting substances (TBARS). TBARS can be quantified in plasma through colorimetric assay and MDA can be detected with the use of high-performance liquid chromatography (HPLC) as well as a number of ELISA kits. (Ho *et al*. 2013)

2.5.3 Antioxidants

The counter mechanisms employed against ROS are antioxidants. There are endogenous and exogenous sources of antioxidants (Ajuwon *et al*. 2015). The body produces a number of different antioxidant enzymes, these are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and paraoxonase (PON). There are three different isoforms of SOD depending on the transition metal found in the active site; SOD1 or copper-zinc-SOD (Cu-Zn-SOD) is found in the cytoplasm and in the mitochondrial intermembrane space, SOD2 or manganese-SOD (Mn-SOD) is located in the mitochondrial matrix and SOD3 or extracellular-SOD (EC-SOD) is mainly expressed in the extracellular matrix. All superoxide dismutases catalyse the conversion of O_2^- to O_2 and H_2O_2. (Li *et al*. 2013; Li *et al*. 2014)

Catalases catalyse the degradation of H_2O_2 to O_2 and H_2O (Ajuwon *et al*. 2015). Catalases have a low affinity for hydrogen peroxide and thus are most efficient when there is an accumulation of the peroxide (Ajuwon *et al*. 2015). GPx degrade free H_2O_2 to water and lipid hydro peroxides to their alcohol derivative (Li *et al*. 2013; Li *et al*. 2014). GPx uses GSH and other thiols to reduce H_2O_2 (Ajuwon *et al*. 2015). This decomposition of H_2O_2 requires two GSH molecules and produces GSSG (Ajuwon *et al*. 2015). The GSH:GSSG ratio is used as an indicator of redox activity (Ajuwon *et al*. 2015). GSSG can be reduced back to GSH by the enzyme glutathione reductase (Ajuwon *et al*. 2015).

The localisation and actions of the three different forms of PONS differs. PON1 is predominant in high density lipoproteins (HDLs) where it acts as a defence against the peroxidation of HDL and low density lipoproteins (LDLs) by breaking down cholesteryl esters and phospholipids in oxidized lipoproteins (Li *et al*. 2013; Li *et al*. 2014).

The endogenous sources of antioxidants may not be sufficient to counter elevated levels of ROS formation, especially in disease states. It is, therefore, important to consume exogenous/dietary antioxidants. The most well-known sources of diet-derived antioxidants are vitamins C (ascorbate), E, A and carotenoids. Vitamin C scavenges free radicals by donating one of its electrons and thus becoming oxidised to an ascorbate radical ($Asc^{\cdot}$). NADH- and NADPH-dependent dehydroascorbate reductase reduces $Asc^{\cdot}$ back to ascorbate. Vitamin E contains an –OH group which it uses to reduce lipid peroxyl groups to hydro peroxides. (Ajuwon *et al*. 2015)

An additional exogenous source of antioxidants that has proven to have potent health promoting and antioxidant effects over the years are plant derived phenolics and polyphenols. Of particular interest and with great health implications are polyphenols derived from Rooibos (*Aspalathus linearis*). (Ajuwon *et al*. 2015)

2.6 Aspalathus Linearis

2.6.1 Introduction

Aspalathus linearis is a plant that is indigenous to South Africa and from which the renowned Rooibos infusion is produced. In its natural/unfermented state it is a green infusion and the traditional red-brown color is obtained through the process of fermentation (Villaño *et al*. 2010). Albeit its agricultural value was discovered in 1930 this infusion is now famous for its taste, antioxidant, vitamin and mineral content as well as its lack of caffeine and other stimulants (Street & Prinsloo 2013).

Components of rooibos that are responsible for its anti-oxidant capacity are known as polyphenolic compounds or polyphenols (Villaño *et al*. 2010). Their chemical structure contains one aromatic ring attached to one or more hydroxyl groups (Del Rio *et al*. 2013). Phenolic compounds are subdivided into flavonoids and non-flavonoids (Del Rio *et al*. 2013). The principle flavonoids present in Rooibos infusion are aspalathin, nothofagin, C-linked glycosides of the flavones, epigenin and luteoilin and four eriodictyol-C-glycoside isomers (Villaño *et al*. 2010).

2.6.2 Processing

Processing of rooibos involves first shredding of the plant shoots into 3-4mm lengths which are then placed in a fermentation heap where the inner and outer structures of the leaves are broken down

through “bruising”. This takes place in the late afternoon. Water is added and more bruising takes place in order to speed up the fermentation process which already begins with the shredding of the leaves. Fermentation occurs during the night and in the morning the infusion is left to dry in the sun. The fermentation process can last from 8-24 hours. (Joubert & Schultz 2012)
Although studies have proven that the unfermented rooibos infusion has a more potent antioxidant capacity than the fermented variant both the fermented and the unfermented variant have proven antioxidant effects in vivo (Villaño *et al*. 2010).

The most active compounds in Rooibos are aspalathin (Asp) and nothofagin (Not). The process of fermentation extensively oxidizes aspalathin whilst the nothofagin is affected to a lesser degree. (Ajuwon *et al*. 2015)

2.6.3 Bioavailability and Antioxidant Potential After Consumption

Absorption of some polyphenol metabolites into the circulation occurs in the small intestine. Initial metabolism takes place in the brush border of the small intestine, thereafter metabolites enter the circulation and quickly reach the liver where they are further metabolised. Even with absorption occurring in the small intestine most of the polyphenols pass through to the large intestine where they are cleaved into smaller molecules by colonic microbiota. Further metabolism then takes place in the liver before the metabolites are excreted in the urine. (Del Rio *et al*. 2013)

2.6.4 Anti-inflammatory Capacity

A study by Lee & Bae 2015 tested the anti-inflammatory effects of both these compounds in vivo- on mice and in vitro on LPS- activated human umbilical vein endothelial cells (HUVECs). Studies have found that Asp and Not inhibited LPS-induced hyper permeability thus favoring endothelial integrity and tissue homeostasis, suppressed leukocyte migration and inhibited the activation of TNF-α or interleukin (IL)-6 and NF-kβ or ERK. The authors concluded that Asp and Not can be used as a therapy for vascular inflammatory diseases. (Lee & Bae 2015)

Although a substantial amount of research still needs to be done in order to investigate the anti-inflammatory properties of herbal teas using animal models the mechanism by which tea polyphenols inhibit NF-κβ activation and thus inflammation has been explored (Gonzalez De Mejia *et al*. 2013). Gonzalez De Mejia *et al*. in their 2013 publication, *Tea and Inflammation*, illustrate how tea

polyphenols inhibit markers of inflammation related to NF-κβ. The mechanism involves the inhibition of ROS, IL-1β and TNF-α by tea polyphenols. The inhibition of these substances inhibits NF-κβ activation and translocation into the nucleus thus preventing the transcription of inflammatory genes. As a consequence of this repression the production of cytokines and adhesion molecules is reduced and thus inflammation repressed as well (**Fig. 2.5**). (Gonzalez De Mejia *et al*. 2013)

2.6.5 Effects on oxidative stress and ischemia

Aqueous extract of fermented rooibos infusion has been tested in the context of diabetic cardiomyopathy (DCM) (Dludla *et al*. 2014). The study treated cultured cardiomyocytes, isolated from adult Wistar rats that had been previously injected with streptozotocin (STZ) to induce diabetes, with an aqueous extract of fermented rooibos. Cardiomyocytes were then exposed to either hydrogen peroxide (H_2O_2) or an ischemic solution. The results showed that the aqueous extract of fermented rooibos protected the cardiomyocytes against oxidative stress and ischemia. (Dludla *et al*. 2014)

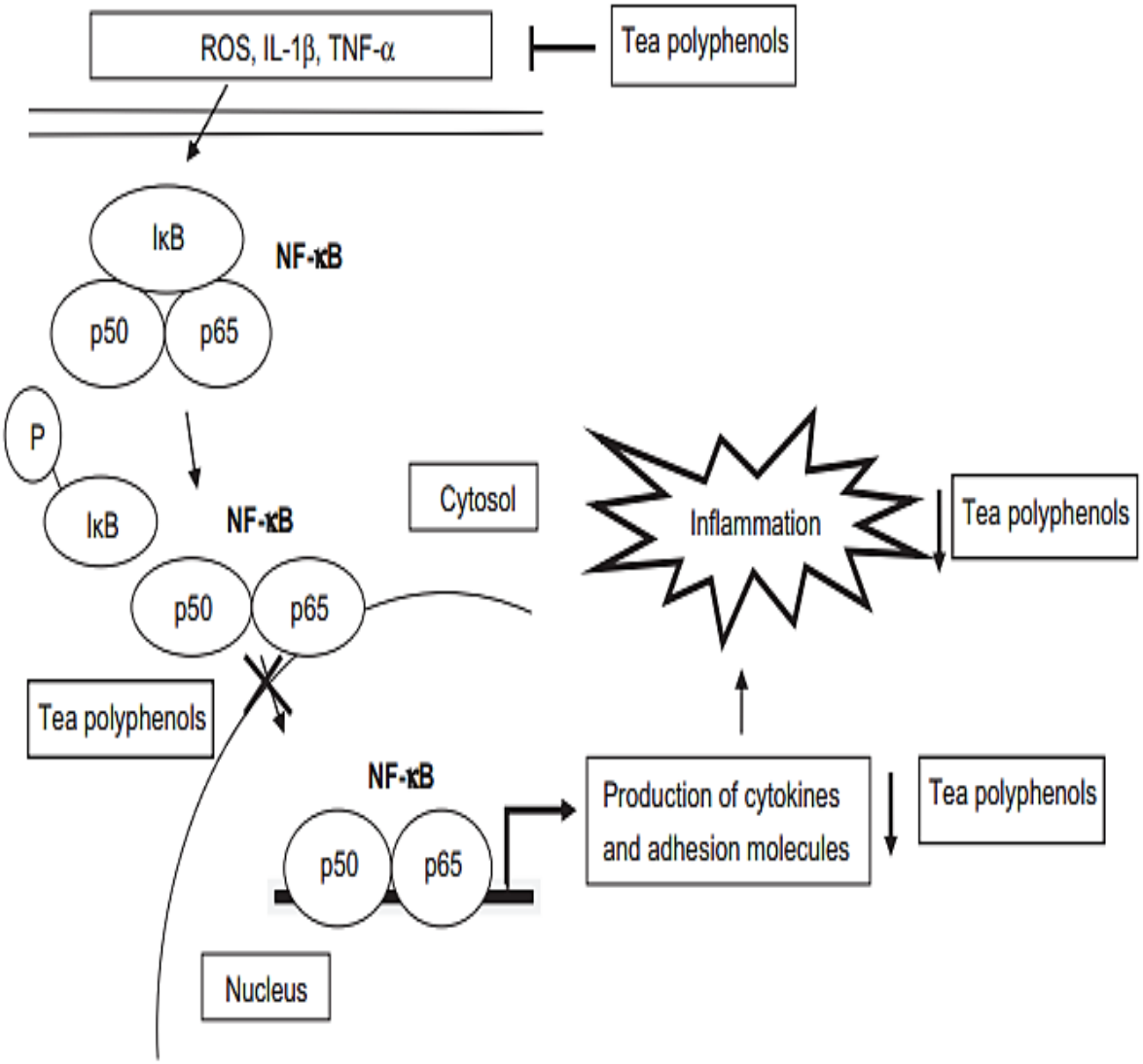


Fig. 2.5. *Mechanisms by which tea polyphenols can inhibit NF-κB driven inflammation* (Gonzalez De Mejia *et al*. 2013).

Rooibos has further been tested against ischemia reperfusion injury on hearts of male Wistar rats. A study by Pantsi *et al*. treated male Wistar rats with a 2% (2g/100ml) rooibos tea solution, by putting it in place of their drinking water, for seven weeks. The study tested four groups; a control- that received tap water, unfermented/"green" rooibos, fermented rooibos and green tea group with an n value of 10 hearts per group. Hearts were perfused with Krebs-Henseleit buffer (KHB) and after stabilization exposed to 15 minutes of global ischemia. Aortic output recovery (AO) was measured as the functional parameter. The aortic recovery of hearts supplemented with both the unfermented and the fermented rooibos tea variants was significantly higher compared to that of the controls. (Pantsi *et al*. 2011)

The effects of a similar study were reported in 2003 where rats were fed a 2% (w/v) aqueous extract of the fermented and unfermented rooibos for 10 weeks. Although no effects were observed in the antioxidant capacity of the liver both forms improved the redox status of the liver by significantly increasing the GSH:GSSG ratio. Effects of rooibos treatment in rats has also been tested against shorter treatment periods. One particular study pre-fed fermented rooibos to rats for 4 weeks and observed an inhibition in the formation of plasma and hepatic malondialdehyde (MDA). Postulated mechanisms by which rooibos counters oxidative stress is through the direct scavenging of ROS by polyphenols or their inhibition of lipid peroxidation. (Ajuwon *et al*. 2015)

Ajuwon *et al* in their 2014 publication "*Amelioration of lipopolysaccharide-induced liver injury by aqueous rooibos (Aspalathus linearis) extract via inhibition of pro-inflammatory cytokines and oxidative stress*" reported findings that a 4 week supplementation of male Wistar rats with fermented rooibos extract produced an anti-inflammatory response countering the inflammation induced by lipopolysaccharide injection. This supplementation of a 2% (w/v) rooibos infusion effectively inhibited the increased production of pro-inflammatory cytokines, TNF-α and IL-6 but increased plasma levels of anti-inflammatory cytokine IL-10. (Ajuwon *et al*. 2014)

There is a sparse body of studies that have investigated the health benefits of rooibos in humans and even fewer studies in the clinical setting of oxidative stress and cardiovascular disease risk. One such study has investigated the effects of 8 weeks of unfermented rooibos tea consumption, as an intervention, by workers who were occupationally exposed to lead. Investigators found an improvement in the workers antioxidant status, this was evident in an increase in SOD activity and GSH levels and decrease in MDA levels. (Nikolova *et al*. 2007)

In another study, Marnewick *et al*. tested the effects of consumption of fermented rooibos on people who were at risk for cardiovascular disease. To achieve their aim they had 40 volunteers consume 6 cups of rooibos daily for 6 weeks and after a control period they tested the blood biochemical parameters indicative of antioxidant activity, lipid peroxidation and lipid profile. Results showed that the plasma antioxidant capacity did not change but total polyphenol levels in plasma was increased. Furthermore there was a significant decrease in markers of lipid peroxidation mainly conjugated dienes (CD's) and TBARS. Rooibos consumption also significantly reduced serum levels of low density lipoprotein (LDL) - cholesterol and triacylglycerols (TG) whilst significantly increasing high density lipoproteins (HDL) - cholesterol. (Marnewick *et al*. 2011)

2.7 Conclusion

HIV infection causes an underlying low level of chronic inflammation which is not countered by ART (Dau & Holodniy 2008). ART, although being able to block viral replication and thus increase CD4 cell counts its long term use leads to an inflammatory burden and increased ROS production through ER stress induced mechanisms (Cimellaro *et al*. 2016). This stress manifests itself through ED ultimately being the chief cause of CVD in long term ART users (Cimellaro *et al*. 2016). Natural sources of anti-oxidants as a supplement to ART still require considerable investigation within the laboratory setting. Of particular note the use of Rooibos has not been scientifically investigated in the settings of ART thus further highlighting the need for the present study. Rooibos polyphenols are able to counter inflammation and directly scavenge ROS and may, therefore, be able to prevent the pathogenesis of ED in long term ART users (Gonzalez De Mejia *et al*. 2013).

Therefore, rooibos as a supplement during ART may provide the counter measure for oxidative stress induced ED thus improving antioxidant capacity of the organism and overall functioning of the heart.

Chapter 3 – Materials and Methods

3.1 Materials

Reagent/ Material	**Supplier**
2,3,5-Triphenyltetrazolium chloride (TTC; $C_{19}H_{15}ClN_4$) Calcium chloride dihydrate ($CaCl_2.2H_2O$) D(+)-Glucose anhydrous ($C_6H_{12}O_{16}$) Formaldehyde (CH_2O) Sodium chloride(NaCl); Sodium bicarbinate ($NaHCO_3$); Potassium chloride(KCl); Potassium dihydrogen phosphate(KH_2PO_4); Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$); Sodium sulphate ($NaSO_4$)	*Merck (Pty) Ltd.* (Darmstadt, Germany)
Acetylcholine Epinephrine Evans blue	*Sigma* (St. Louis, MO, USA)
Aspalathus linearis (Fermented Rooibos)	*Carmien Tea (Pty) Ltd.* (Brakfontein Estate, Citrusdal, SA)
Blood collection tubes Storage eppendorfs	*The Scientific Group (Pty) Ltd.* (Milnerton, Western Cape, RSA)
Consumables used for sample collection (tornequet, vacuette collection set, needles, gauze, plasters)	*Pharmacy City*
Ethicon Sutures Mersilk	*Johnson & Johnson*
Eutha-naze (Sodium pentobarbital; $C_{11}H_{17}N_2NaO_3$)	*Bayer (Pty) Ltd.* (Isando, Gauteng, RSA)
Odimune Tablets	*Cipla MedPro (Pty) Ltd.* (Bellville, Western Cape, SA)
OMRON M6 automatic digital blood pressure monitor	*Omron Healthcare* (Kyoto, Japan)
Pathology transport box and pod	*Lab Specialist Services*

3.2 Methods

3.2.1 Determining the effects of *Aspalathus linearis* supplementation during first line fixed dose ART on the isolated hearts and aortas of male Wistar rats.

3.2.1.1 Ethics Clearance

Approval for the use of male Wistar rats for this study was obtained from the Research Ethics Committee for Animal Care and Use, Stellenbosch University (Protocol #: SU-ACUD14-00021).

3.2.1.2 Animal Husbandry

The rats were housed in a facility that is Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited and their handling was in accordance with the institutional ethical guidelines. Rats were treated from 4 weeks of age for 9 weeks during which time they had free access to food and water or rooibos tea, according to the treatment groups. All rats received normal rat chow. Rats were weighed and monitored for hair loss and other markers of stress weekly. The fluid intake was measured weekly.

3.2.1.3 Odimune Preparation

Odimune tablets contain the recommended daily dose of active ingredients (600 mg EFV, 200 mg FTC, and 300 mg TDF) for an average human (70kg). The specific dose for the rats was calculated and prepared weekly, according to the average total body mass of the rats per cage, using the USA FDA guidelines (Center for Drug Evaluation and Research 2005) (**Appendix A:** Odimune Dose Calculations). Each tablet was first crushed individually, the exact mass needed was determined based on the average mass per cage. The determined amount of powder was suspended in 1ml water/rat/day and vortexed thoroughly. The drug preparations were kept in a -4^{o}C fridge and administered, via oral gavage, to the rats daily by a laboratory animal husbandry professional.

3.2.1.4 Rooibos Tea Preparation

Fermented rooibos leaves were stored in a light proof sealed container at room temperature and under dry conditions. Rooibos infusion was either prepared weekly or two weeks in advance in which case it would be stored at -20°C. A fresh batch of rooibos infusion was allocated to the relevant treatment groups weekly.

Aqueous extract of rooibos was made up using an already established method of preparation with a concentration that is customarily used for tea making purposes. A 2% (w/v) tea solution was made up each time by steeping 20g of the tea leaves into 1L of boiling tap water for 30 minutes. After steeping, the tea was filtered through a layer of cheese cloth, no.4 filter paper and a no.1 filter paper sequentially and then placed into light proof bottles. (Marnewick *et al*. 2003)

Liquid chromatography mass spectrometry (LC-MS) analysis was conducted on the rooibos infusions after applying the storage methods used (fresh, fridge for 1 week or frozen at -20°C for more than 1 week) showed no differences in polyphenols levels and the composition was comparable to that of rooibos infusions used in previously published data (**Appendix B: LC-MS of Rooibos**) (Beelders *et al*. 2012).

3.2.1.5 Animal Supplementation and Treatment

The water or tea was poured into the rats drinking bottles (one bottle per cage) and bottles with the tea were covered with tin foil and masking tape in order to lightproof them. Bottles were filled with 800-1000ml of water or tea, and fluid consumption was measured before the fluid was replaced weekly.

ART was administered every morning by oral gavage. This procedure was performed by a professional and involves the delivery of the compound straight to the stomach by inserting a bulb tipped gastric gavage needle along the side of the mouth, into the esophagus and toward the stomach. Non-ART treated rats received oral gavage with tap water only. ART and rooibos supplementation were administered for 9 weeks and correlates with time periods used in several studies that investigated the effects of chronic supplementation of rooibos tea and obtained positive results (Ajuwon *et al*. 2015).

3.2.1.6 Experimental Design

The study made use of a total of 100 rats. Experiments were performed in 3 separate periods during 1 year. Period 1: 36 rats, period 2: 32 rats, period 3: 32 rats. Rats were randomly assigned into four treatment groups (**Fig 3.1**):

- Control (25 rats): gavaged with tap water and received tap water to drink
- Rooibos (25 rats): gavaged with tap water and received rooibos to drink
- ART Control (22 rats): gavaged with ART and received tap water to drink
- ART+Rooibos (24 rats): gavaged with ART and received rooibos to drink

A total of 65 rats were used for perfusions, where 34 were used to determine functional recovery by the induction of regional ischemia and 31 by inducing global ischemia. Twenty two rats were fasted overnight and blood was collected from the thoracic cavity after sacrifice. The sum of 4 rats were lost to gavage during the different treatment periods.

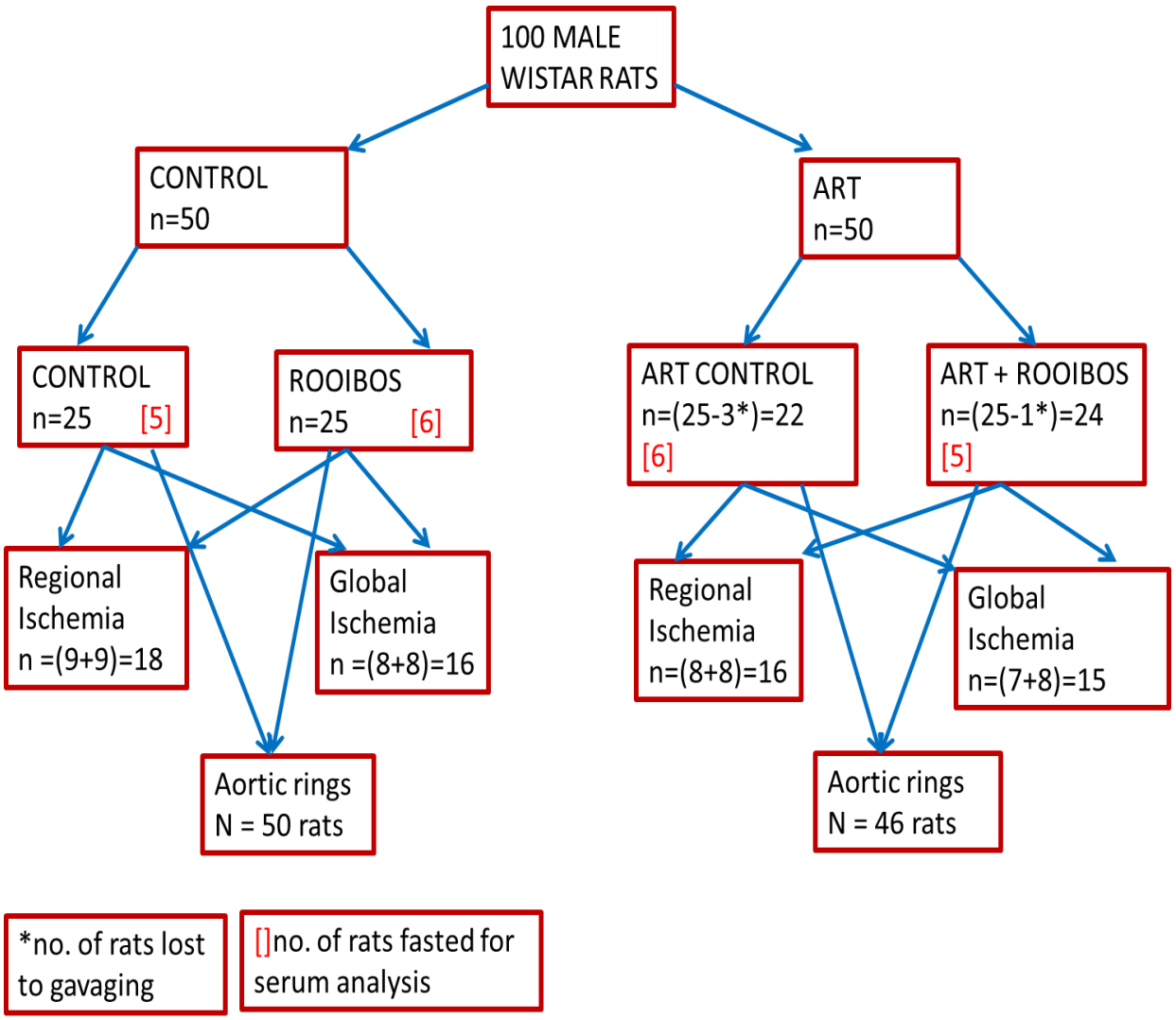


Fig. 3.1. *Experimental groups and sample sizes.* * indicate number of animals lost to oral gavage whilst [] indicate the number of rats fasted overnight for serum collection and analysis. **Abbreviations:** ART: Antiretroviral Therapy; n: sample size.

3.2.1.7 Euthanasia of Rats

Rats were put to sleep with an injection of pentobarbital of 1ml/kg body weight concentration. The injection was done intraperitoneally and performed by an authorized professional of the South African Veterinary Council (SAVC) for paravetiranary procedures. To ascertain that rats were deep enough in sleep the toe pinch method was used. Once no reflexes were observed hearts were removed by thoracotomy and rats sacrificed by exsanguination.

3.2.1.8 Perfusion Technique and Protocols

Hearts were excised rapidly and arrested in ice cold Krebs-Henseleit buffer (KHB) before mounting onto the working heart perfusion system (Neely *et al*. 1967). The hearts were mounted by insertion of the aortic cannula into the aorta allowing for a retrograde perfusion in Langendorf mode at a constant pressure (100cm H_2O) for 10min whilst insertion of the second cannula into the left atrium occurred (Zimmer 1998). This was followed by 20min of working heart at a preload of 15cm H_2O and an afterload of 100cm H_2O.

Mechanical function was noted through the recording of haemodynamic data and calculating of specific parameters. Coronary (Qe/CF) and aortic (Qa/AO) flow rates in ml/minute were measured manually whilst the peak systolic and diastolic pressures (PSP & DP) and the heart rate (HR) were recorded with a Gentronics chart recorder v3.00. The cardiac output (CO) (ml/min) = (Qa + Qe) and Stroke volume (SV) (ml/min) = (CO / HR). Total work (TW) was measured as a function of the cardiac output and systolic pressure: (TW= COxSPx0.0022).

Hearts were subjected to either a 20min global ischemia period, where perfusion flow to the heart was blocked completely, or 35min of regional ischemia by occlusion of the coronary artery. Ischemia was succeeded by reperfusion as per the time periods indicated below (**Fig. 3.2.** and **Fig. 3.3.**). Haemodynamic data was recorded and parameters listed above calculated at each time period.

3.2.1.8.1 Regional Ischemia

In the infarct size protocols hearts were subjected to 35min of regional ischemia by occlusion of the coronary artery and succeeded by a reperfusion period of 60min (Bell *et al*. 2011). The induction of regional ischemia occurred through the insertion of a silk suture underneath and around the proximal left anterior (LAD) coronary artery. Tightening of the suture resulted in an occlusion of the coronary artery and subsequent cessation of myocardial perfusion in the surrounding region. During this period the temperature was monitored and kept at a constant 36.5^{o}C. Effective occlusion of the vessel resulted in a 33% reduction in total coronary flow and cyanosis of the region. As per protocol, hearts were then re-perfused for 60min.

Measurement of risk zone and infarct size after regional ischemia

The suture around the coronary artery was tied off and a 0.5% Evan's Blue solution infused through the aorta to stain the viable tissue. The hearts were frozen overnight, sliced into 2mm thick slices and incubated in a 1% w/v triphenyltetrazoliumchloride (TTC) in phosphate buffer solution for 15 minutes(pH 7.4) before being placed in 10% formalin. Heart segments were placed between two glass plates. The left ventricle area at risk (R) and the area of infarct (I) tissue were determined using computerised planimetry (UTHSCSA Image Tool program, developed at the University of Texas Health Science Center at San Antonio, Texas). Infarct size was expressed as a percentage of the area at risk (%AAR).

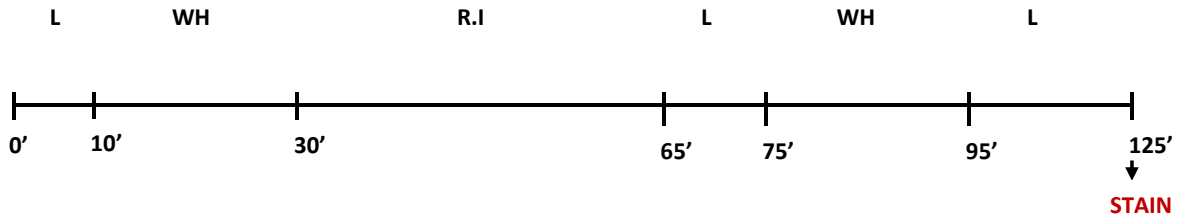


Fig. 3.2. *Experimental protocol of regional ischemia.* **Abbreviations:** L: Langendorff; WH: Working Heart; R.I: Regional Ischemia

3.2.1.8.2 Global Ischemia

For the determination of functional recovery hearts were subjected to a 20min global ischemia period where perfusion flow to the heart was blocked completely. A 30min reperfusion period then took place before freeze clamping.

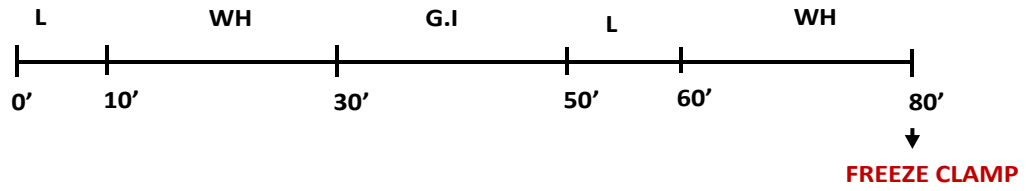


Fig. 3.3. *Experimental protocol of global ischemia.* **Abbreviations:** L: Langendorff; WH: Working Heart; G.I: Global Ischemia

3.2.1.9 Aortic Ring Technique and Protocol

For vascular reactivity studies the protocol was adapted from Privett *et al*., in order to determine NO dependent vasodilation (Privett *et al*. 2004). This method of determining vascular response to contraction and relaxation is well established (Li *et al*. 2004; Ma *et al*. 2010; Rodrigues *et al*. 2014). Phenylephrine and acetylcholine were dissolved in 0.9% saline solution to make up the stock solutions. The thoracic aorta was carefully dissected and placed in a petri dish with ice cold KHB containing in mM/L: NaCl 119; $NaHCO_3$ 24.9; KCl 4.74; KH_2PO_4 1.19; $MgSO_4$ 0.6; $NaSO_4$ 0.59; $CaCl_2$ 1.25; glucose 10.

To determine the role of perivascular adipose tissue (PVAT) the aortas of group one were sectioned in half. The one half was cleaned, under a magnifying glass, by the removal of PVAT and connective tissue whilst the other was left with part of the surrounding PVAT (**Fig. 3.4A.**). A 3-4mm segment from each half was cut for the experiment. Both the clean aorta and the ring containing the surrounding PVAT were mounted on two separate already calibrated aortic ring systems.

Mounting involves placing the ring between two steal hooks and then lowering it into the organ bath (AD Instruments, Bella Vista, New South Wales, Australia) with KHB at 36.5-37°C gassed with 95% O_2 and 5% CO_2 (**Fig. 3.4B.**). Aortic ring tension was recorded with an isometric force transducer (TRI202PAD, Panlab, ICornellà, BCN, Spain). The protocol (**Fig. 3.5**) begins with a 30 min stabilization period during which the tension was gradually increased to a resting tension of 1.5g and the KHB replaced with warmed buffer at 10 and 20 minutes. After stabilization the buffer in the organ bath was set at exactly 25ml and 2.5 µL of a 1mM Phenylephrine (Phe) stock, with a final concentration of 100nM, was added to the organ bath. Once contraction reached a plateau 25µL of a 10nM Ach stock, with a final concentration of 10µM, was added and likewise relaxation was left to reach a plateau. The organ bath was flushed and rinsed with clean KHB three times, followed by another stabilization period of 30 min. The buffer was changed at 10 and 20 minutes and the tension maintained at a steady 1.5g. After this second stabilization period, a cumulative contraction was induced by five consecutive additions of Phe with the concentrations; 100nM, 300nM, 500nM, 800nM and 1µM. This was followed by a cumulative relaxation period with five consecutive additions of Ach with concentrations; 30nM, 100nM, 300nM, 1µM and 10µM. Results were analysed using LabChart Reader v8.1.2 (Dunedin, New Zealand) and GraphPad Prism 6.

Fig. 3.4. *Images of aortic ring after cleaning and sectioning*. **A.** Two 3-4mm aortic ring sections, one with surrounding PVAT and the other without. **B.** Aortic ring mounted between the two steel hooks just before its submergence into the organ bath.

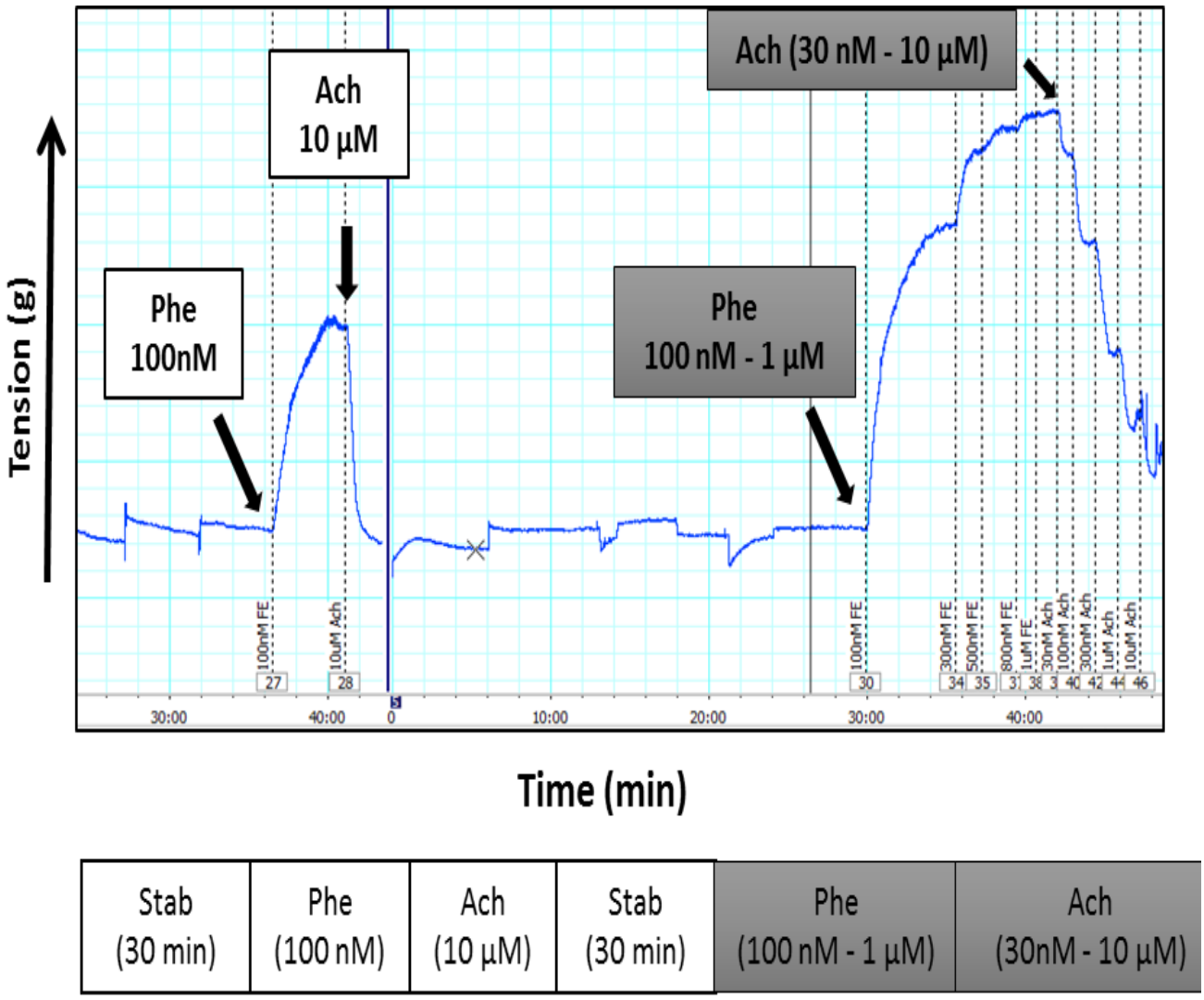


Fig. 3.5. *Depiction of the aortic ring protocol.* After mounting, protocol begins with a 30min stabilization period which is followed by a 100nM addition of Phe and 10µM Ach once a plateau is reached. Another stabilization period of 30min ensues followed by a cumulative period of Phe addition of concentration ranging from 100nM-1µM and a cumulative period of Ach addition of a contraction range of 30nM-10µM. Abbreviations: Ach: Acetylcholine; Phe/Fe: Phenylephrine; Stab: Stabilization. (Westcott, C., 2015. Investigating the cholesterol-independent (pleiotropic) effects of selected hypolipidaemic agents in functional and dysfunctional endothelial cells. PhD thesis, University of Stellenbosch.)

3.2.1.10 Blood Sample Collection, Preparation and Storage

Blood samples were collected from the thoracic cavity of the rats and placed in serum separation tubes as follows:

- 1x3ml purple top EDTA tube (with anticoagulating factors): delivered to UCT for thiobarbituric acid reactive substances (TBARS) analysis
- 1x3ml red top Serum tube (with clot activating silicone): delivered to UCT for a phospholipid test

The samples were then centrifuged in an IEC Model Clini-cool refrigerated centrifuge (4^{o}C) for 15 minutes at 3000rpm. Supernatants (plasma from EDTA tube and serum from red top Serum tube,) were stored at -80^{o}C before being delivered to Division of Chemical Pathology at University of Cape Town (UCT) where the serum triglycerides (TRIG), phospholipids (PL) and TBARS concentrations were determined.

3.2.1.11 Triglycerides, Phospholipids and TBARS

Serum TRIG and PL concentrations were determined using enzymatic colorimetric kits and a SPECTRA-maxPLUS-384 spectrophotometer with SoftMax pro 4.8 data acquisition and analysis software. In principle, performing the assay involved pipetting the TRIG standard and samples into the microwell plate, adding a diluted enzyme buffer solution to each well and incubating at room temperature before reading the absorbance.

TBARS were measured according to the previously published method of Jentzsch *et al*. In this method plasma samples (200 µL) are mixed with 10 µL of butylated hydroxytoluene and vortexed for 10 seconds. Thiobarbituric acid reagent is added before vortexing once again and then incubating in a water bath at 90^{o}C for 45 minutes. Tubes are placed on ice to terminate the reaction, TBARS are extracted, NaCl added and the mixture is centrifuged at 10 000 g for 1 minute (Jentzsch *et al*. 1996). The absorbance was read at 532 nm and values were expressed in micro molar serum TBARS.

3.2.2 Determining the effects of rooibos tea consumption on the cardiovascular profiles of patients on first line fixed dose ART compared to controls

The human study formed part of a parent study called EndoAfrica (project coordinator Prof Hans Strijdom), which is an international collaboration funded under the EU FP 7 programme. It enlisted a total of 105 participants of which 23 were HIV negative, 14 HIV positive ART naïve and 68 were HIV positive and on ART. Participants were recruited from Durbanville, Fisantekraal, Elsies River, Uitsig, Bishop Lavis and Ravensmead by a professional nurse. The nurse obtained written consent from the participants before enrolling them in the study. All participants were given study identities (study I.D). Anthropometric measurements, that is; height, weight, body mass index (BMI) and hip waist circumference as well as the cardiovascular parameters; diastolic, systolic pressure and heart rates were determined and recorded. Lifestyle indices relative to rooibos tea consumption and smoking were obtained via a questionnaire. Participant data was stored on an online data base called *RedCap*. Ethical approval was obtained from the Health Research Ethics Committee (HREC) (Reference number: N13/05/064).

3.2.2.1 Inclusion and Exclusion Criteria

The study excluded individuals who were less than 18 years of age, pregnant women and women who were less than 3 months post-partem. HIV positive and negative patients who did not fit this criteria were all included.

3.2.2.2 Biological Sample Collection

Participants fasted overnight. Blood samples were collected by a phlebotomist. The samples were collected and tubed as follows:

- 2x5ml purple top EDTA tube: sent to the National Health Laboratory Service (NHLS) to test haemoglobin, PLG CD4, HBA1C, HIV viral load
- 1x7ml yellow top Serum tube: sent to NHLS for fasting lipogram, creatinine and GGT test
- 1x5ml grey top: sent to NHLS for fasting glucose test
- 1x10ml purple top EDTA tube (with anticoagulating factors): sent to Department of Chemical Pathology, UCT for TBARS analysis

- 1x3ml red top Serum tube (with clot activating silicone): sent to Department of Chemical Pathology, UCT for a phospholipid test

Tubes were inverted 8 times before being placed in a plastic bag with an absorbent strip. The bag was sealed and placed in a hard cover pod which was then placed in a pre-cooled pathology transport box containing two ice packs. Samples were then driven, together with the participant, from the local clinic to Tygerberg Medical Campus, Division of Medical Physiology. At Tygerberg all samples were labelled with the study I.D, barcoded and logged onto a computerized data base called *REDCap*. After sample logging the purple, yellow and grey top sample tubes were delivered to NHLS for testing as per the above indications

3.2.2.3 Sample Preparation and Storage

The 1x10ml purple top EDTA tube and the 1x3ml red top Serum tube were centrifuged for 10 minutes at 2000g at room temperature. Each of the samples were then aliquoted into 4x1.5ml eppendorffs which were barcoded and logged onto RedCap as derivatives. These were all stored at -80^{o}C and some of the samples, selected at random, were at a later stage delivered to UCT for analysis (see **section 2.2.1.11.**). TBARS analysis was done on supernatant from centrifuged purple top EDTA samples and a phospholipid test on supernatant from the red top Serum tube.

3.2.2.4 Blood Pressure Measurement

Once in the exam room and explanations of the procedures were made participants were seated in an upright, relaxed position with the right arm supported at heart level and legs uncrossed. Confirmation that the participant was indeed fasted, had not smoked prior to the exam nor exercised or even climbed stairs in the 15-30min prior to the exam, was obtained. Participant was kept calm for at least 10 minutes. After properly fixing the cuff the OMRON M6 blood pressure monitor was used to record the blood pressure and heart rate measurements. Measurements were taken three times on the upper left arm with a 2-5 min interval between them. The systolic, diastolic pressures and heart rate was recorded.

3.2.3 Statistics

The animal data was analyzed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA) where the analysis of variance (ANOVA) was utilised for multiple comparisons testing. Post-hoc testing for differences between selected groups was determined by using Bonferroni's method and a p-value of <0.05 was considered significant.

A biostatistician at Stellenbosch University, Faculty of Medicine and Health Sciences, analysed the human data with SPSS. In the descriptive statistics normally distributed data were analysed using one-way ANOVA and Bonferroni post hoc test was used to determine significant differences between groups. For categorical data Pearson's Chi square analysis. Non-parametric data was analysed using a one way Kruskal-Wallis ANOVA which is a pairwise comparisons test. Forward stepwise regression analysis was conducted in order to determine independent predictors and associations between the groups. Normally distributed/ parametric data are expressed as mean ± standard deviation, non-parametric data is expressed as median with 25th and 75th percentiles.

Chapter 4 – Results

The results are listed in order according to the aims and the objectives of the study.

4.1 Aim 1: Effects of *Aspalathus linearis* supplementation during first line fixed dose ART on isolated hearts and aortas of male Wistar rats.

4.1.1 Body Weight Gained and Fluid Consumption

Of particular interest in weighing the rats and measuring their fluid consumption weekly was to observe potential differences in these parameters between the groups. The average weight gained per group from commencement of treatment until day of sacrifice is depicted and the fluid consumption expressed as the average amount of fluid consumed by the group weekly.

4.1.1.1 Body Weight Gained

No significant differences were found in the average weight gained between the groups. Control: 118.9±7.09 (g); Rooibos: 124.1±7.11 (g); ART: 108.4±7.72 (g); ART+Rooibos: 119±8.77 (g). N=22-25

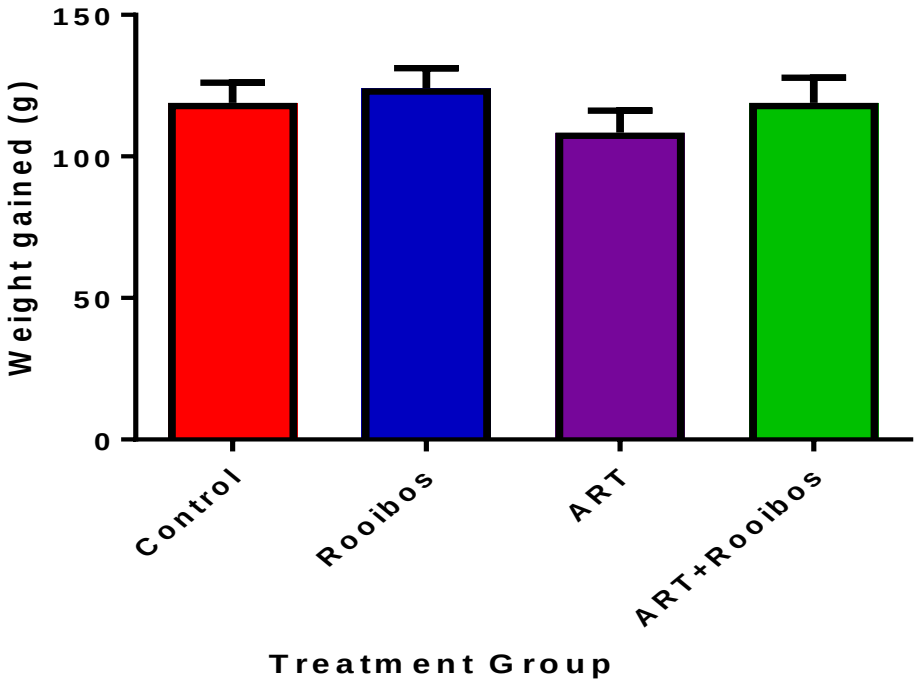


Fig. 4.1. *Average weight gained/group during treatment period*. Data expressed as mean ± SEM/group.

4.1.1.2 Fluid Consumption

No differences were observed between groups in the average weekly fluid intake. Control: 599.5±80.94 (ml/week); Rooibos: 620±88.17 (ml/week); ART: 598±60.09 (ml/week); ART+Rooibos: 643.1±44.46 (ml/week). N=22-25

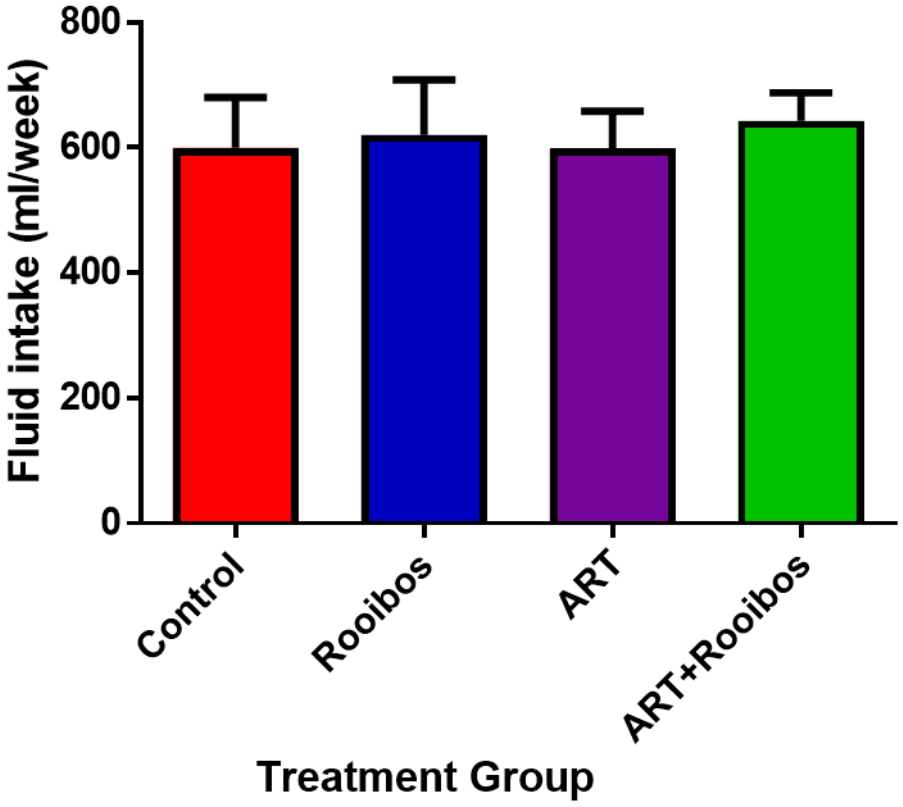


Fig. 4.2. *Average weekly fluid intake between groups.*

4.1.2 Isolated Heart Perfusions

In both the regional and global ischemia protocols the cardiac output (CO), aortic output (AO), coronary flow (CF), peak systolic pressure (PSP), diastolic pressure (DP), heart rate (HR) and total work (Wt) were the end points calculated and recorded during the period before the ischemic event, herein indicated by the term “baseline”, and after the ischemic event at reperfusion, indicated by the term “recovery”. Also illustrated below is the % AO recovery, % CO recovery and the % Wt recovery. These were calculated by dividing the recovery values by the baseline values and multiplying by 100. The end points were calculated at certain intervals of the protocols shown in **fig. 3.2** and **fig. 3.3** in section **3.2.1.8**. Baseline data for the global ischemia and regional ischemia were pooled to collectively investigate significant differences but because none were found all global and regional ischemia data are presented separately in this thesis.

An additional end-point in the regional ischemia protocol is infarct size which is expressed as a percentage of the area at risk.

4.1.2.1 Regional Ischemia

Regional ischemia was established via occlusion of the left anterior descending coronary artery with a silk suture. This cessation of myocardial perfusion to the region surrounding the left coronary artery occurred for 35 min and a 60 min reperfusion period ensued thereafter.

4.1.2.1.1 AO

No significant differences were observed in the baseline; recovery AO as well as the percentage AO recovery. AO baseline; Control: 42±4.07 (ml/min), ART: 36.86±1.84 (ml/min), Rooibos: 40±2.10 (ml/min), ART+ Rooibos: 38.5±1.12 (ml/min). AO recovery; Control: 9.75±3.61 (ml/min), Rooibos: 5±2.23 (ml/min), ART: 9.57±2.49 (ml/min), ART+ Rooibos: 13.75±3.88 (ml/min). AO recovery %; Control: 19.85±6.70 (ml/min), ART: 24.52±5.62 (ml/min), Rooibos: 11.53±4.80 (ml/min), ART+ Rooibos: 17.39±3.98 (ml/min). N=7-8

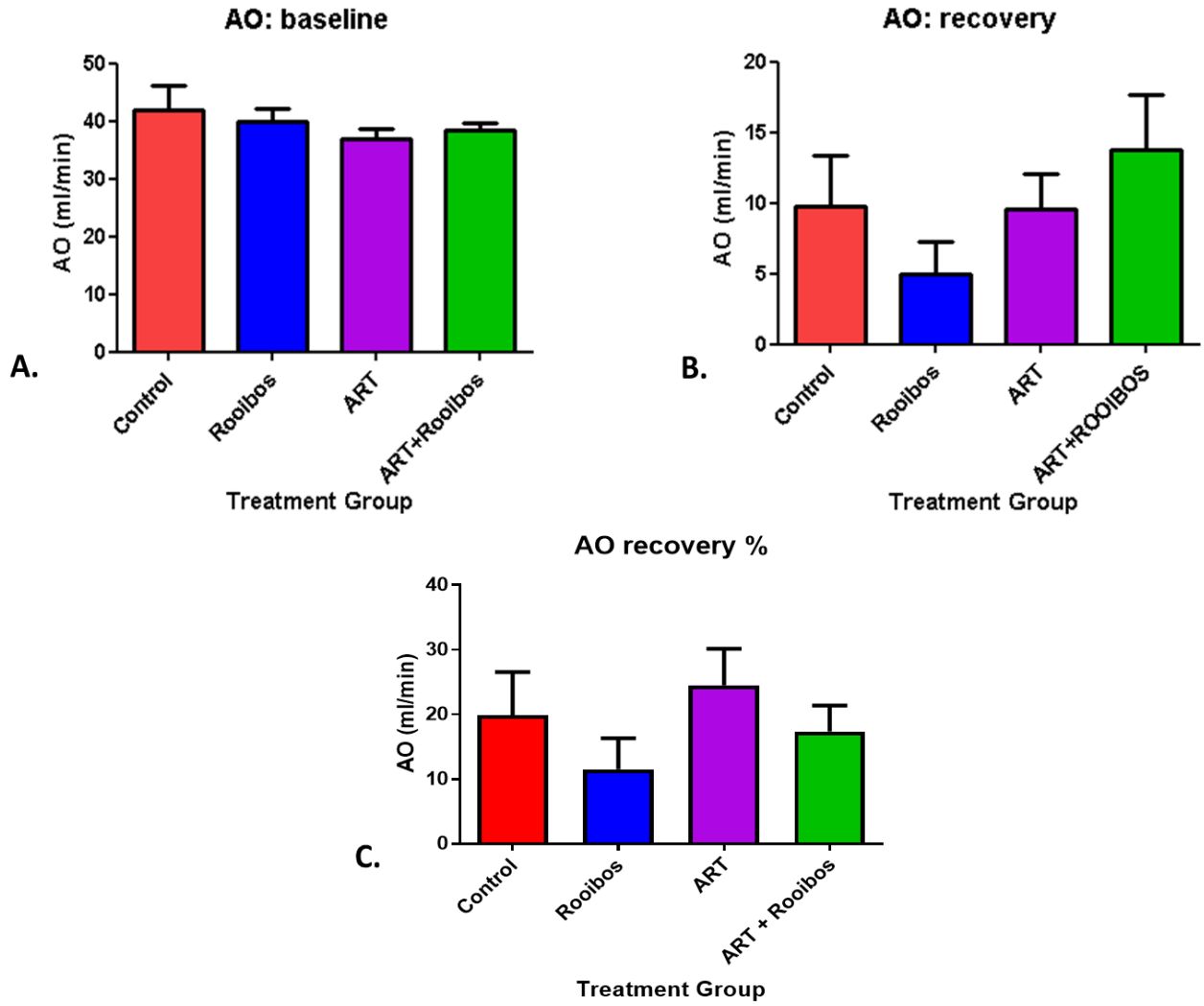


***Fig. 4.3**. AO at baseline, recovery and the % recovery. (R.I).* **A.** AO baseline. **B.** AO recovery. **C.** AO recovery %

4.1.2.1.2 CF

CF did not differ between groups. **A.** CF baseline; Control: 16.63±0.78 (ml/min), Rooibos: 14.56±1.03 (ml/min), ART: 16.29±0.52 (ml/min), ART+ Rooibos: 15.63±0.79 (ml/min). **B.** CF recovery; Control: 13.31±2.91 (ml/min), Rooibos: 13.69±1.38 (ml/min), ART: 14.29±1.03 (ml/min), ART+ Rooibos: 14.19±0.85 (ml/min). **C.** CF recovery %; Control: 78.86±17.31 (ml/min), Rooibos: 93.59±5.28 (ml/min), ART: 88.2±6.67 (ml/min), ART+ Rooibos: 92.6±7.12 (ml/min). N=7-8

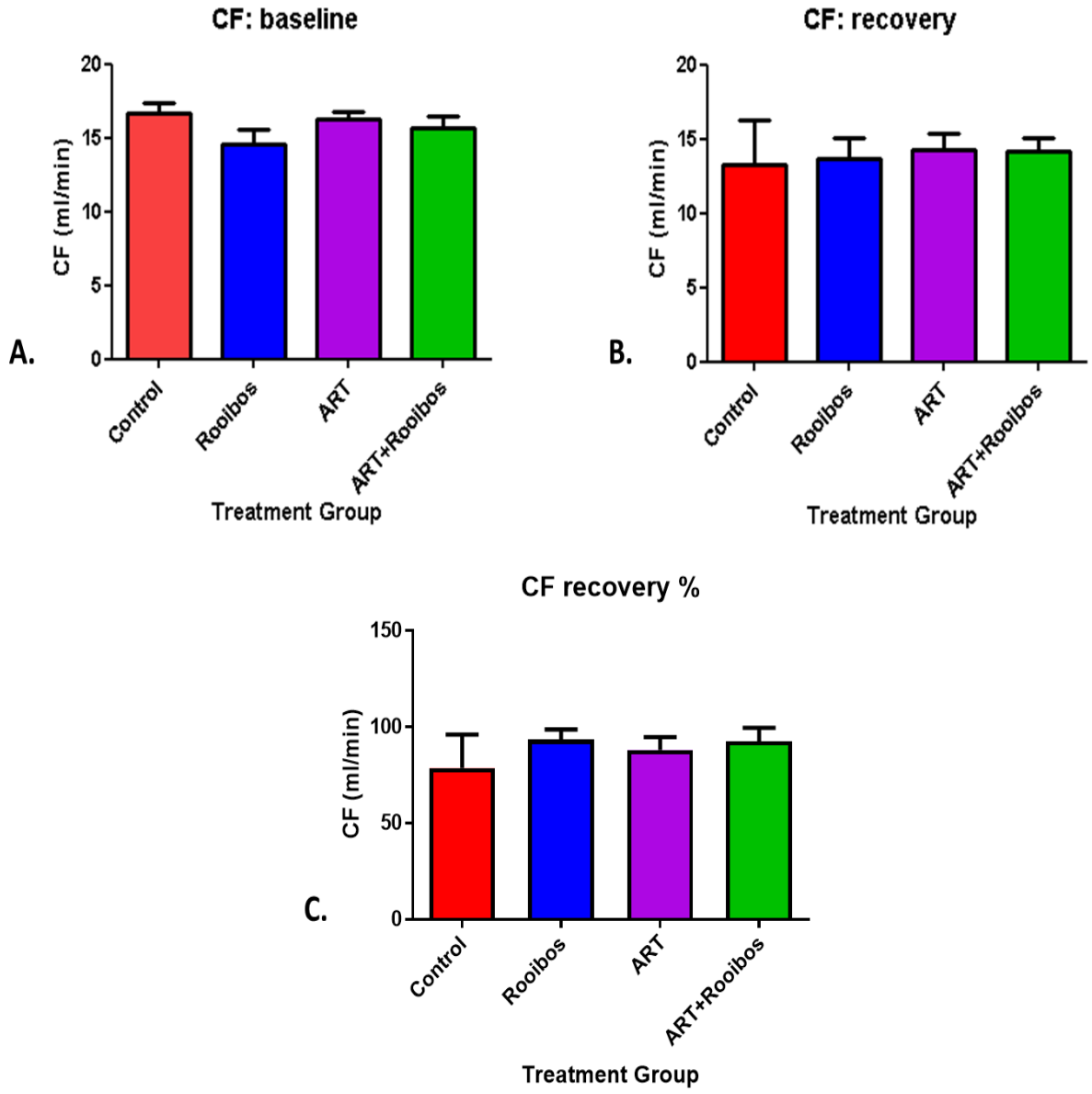


Fig. 4.4. *CF at baseline, recovery and as recovery %. (R.I).* **A.** CF baseline. **B.** CF recovery. **C.** CF recovery %

4.1.2.1.3 CO

CO did not differ between groups. **A.** CO baseline; Control: 58.63±3.87 (ml/min), Rooibos: 54.56±2.84 (ml/min), ART: 53.14±2.22 (ml/min), ART+ Rooibos: 54.13±1.63 (ml/min). **B.** CO recovery; Control: 23.06±5.83 (ml/min), Rooibos: 18.69±2.34 (ml/min), ART: 23.86±2.46 (ml/min), ART+ Rooibos: 20.84±2.18 (ml/min). **C.** CO recovery %; Control: 36.61±8.60 (ml/min), ART: 38.81±7.13 (ml/min), Rooibos: 33.5±3.10 (ml/min), ART+ Rooibos: 38.97±4.21 (ml/min). N=7-8

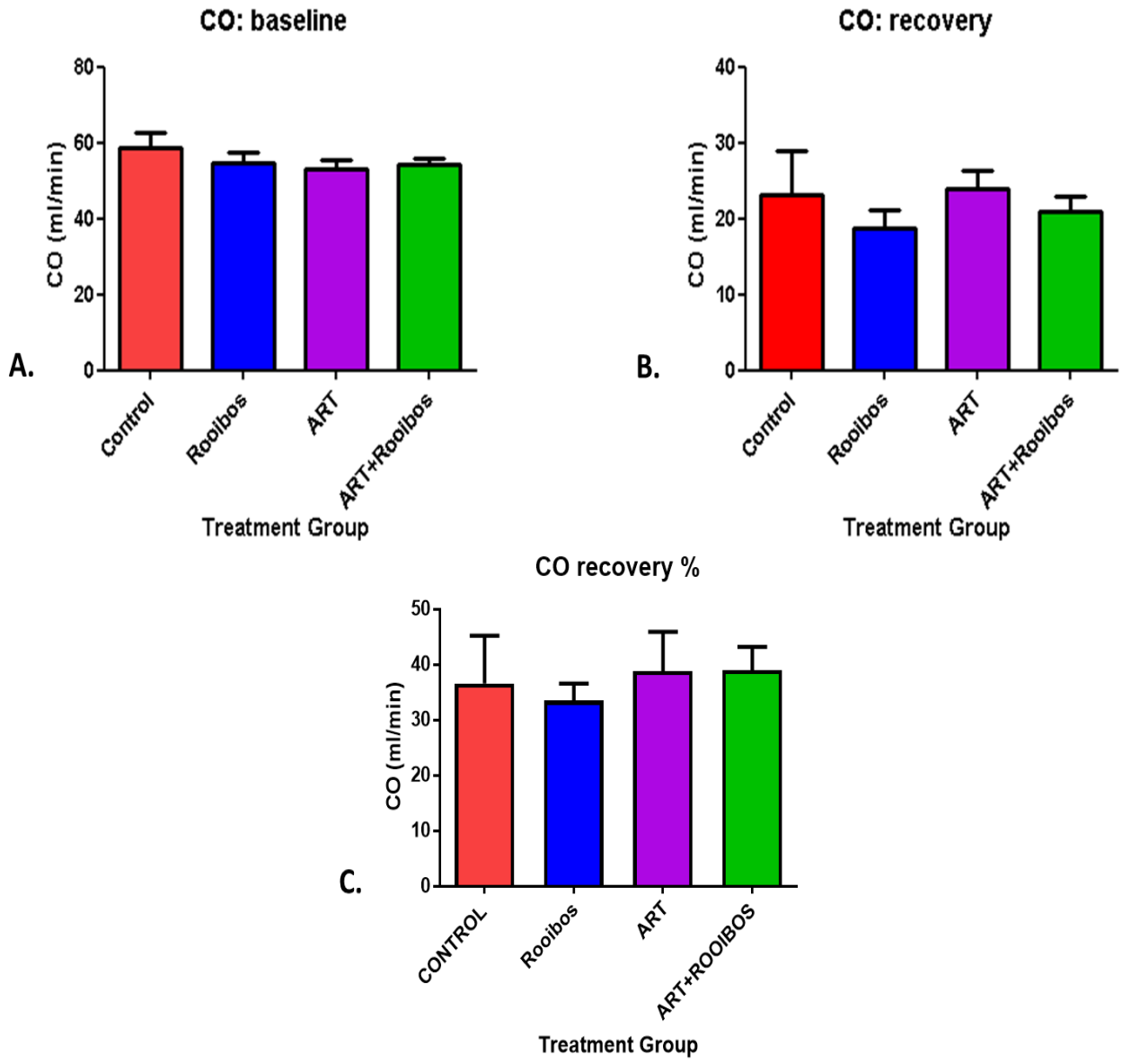


Fig. 4.5. *Cardiac output at baseline, recovery and % cardiac output recovery. (R.I).* **A.** CO baseline. **B.** CO recovery. **C.** CO recovery %

4.1.2.1.4 PSP

No differences were observed, between groups, in the peak systolic pressure. **A.** PSP baseline; Control: 92±2.14 (mmHg), Rooibos: 90.25±1.28 (mmHg), ART: 89.86±0.633 (mmHg), ART+ Rooibos: 90.38±1.15 (mmHg). **B.** PSP recovery; Control: 61.13±13.44 (mmHg), Rooibos: 79.75±2.01 (mmHg), ART: 82.14±0.46 (mmHg), ART+ Rooibos: 78.63±3.70 (mmHg). **C.** PSP recovery %; Control: 66.56±14.57 (mmHg), Rooibos: 88.54±2.67 (mmHg), ART: 91.44±0.87 (mmHg), ART+ Rooibos: 87.2.±4.42 (mmHg). N=7-8

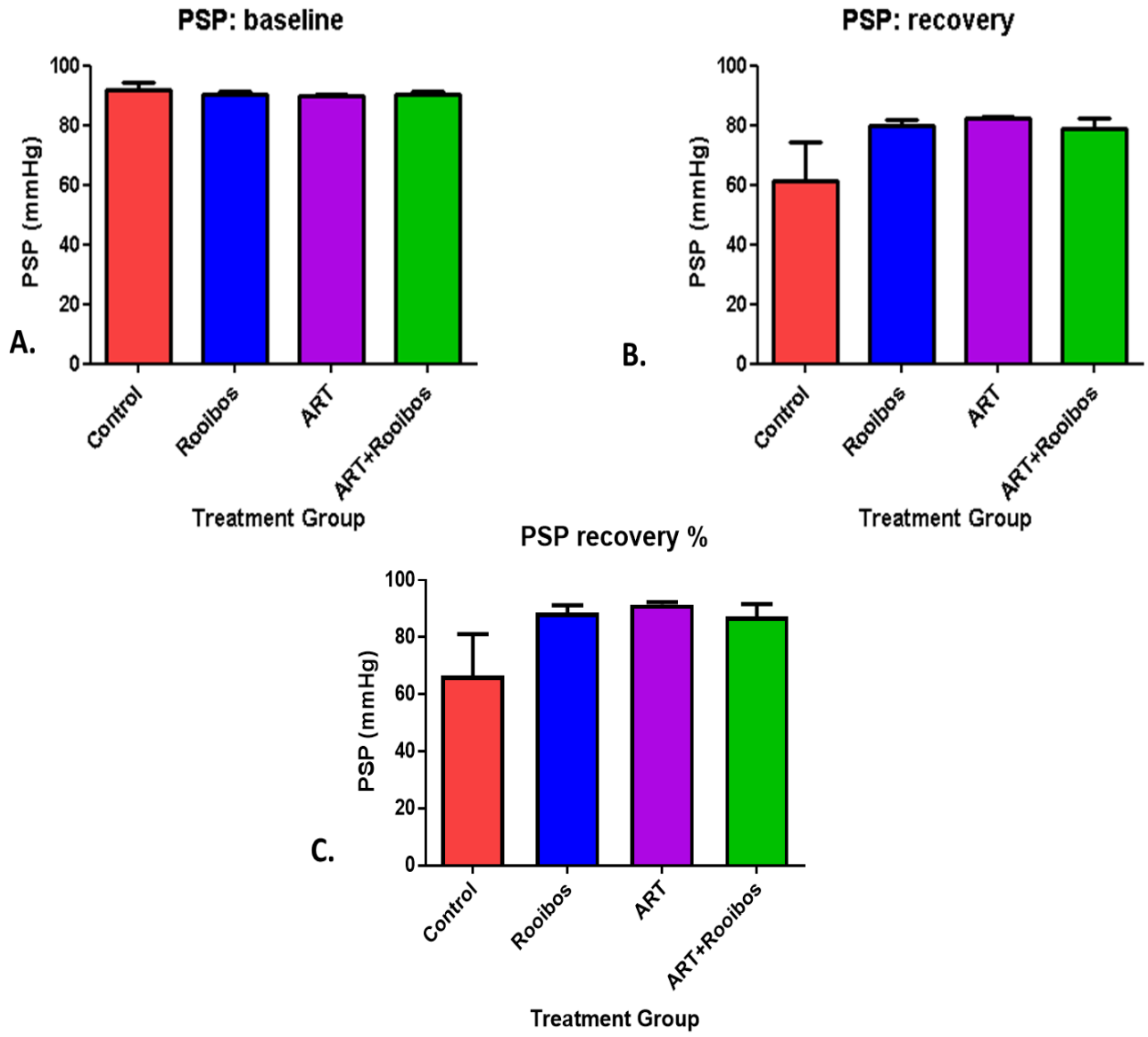


Fig. 4.6. *Peak systolic pressure at baseline, recovery and the recovery as a %.* (R.I). **A.** PSP baseline. **B.** PSP recovery. **C.** PSP recovery %

4.1.2.1.5 DP

DP showed no differences between groups. **A.** DP baseline; Control: 67.75±2.25 (mmHg), Rooibos: 66±0.85 (mmHg), ART: 67.14±0.63 (mmHg), ART+ Rooibos: 66.5±1.03 (mmHg). **B.** DP recovery; Control: 49.75±10.91 (mmHg), Rooibos: 67.25±1.065 (mmHg), ART: 67.71±0.47 (mmHg), ART+ Rooibos: 65.88±2.57 (mmHg). **C.** DP recovery %; Control: 72.45±15.98 (mmHg), Rooibos: 101.9±1.3 (mmHg), ART: 100.9.±0.65 (mmHg), ART+ Rooibos: 99.41±4.68 (mmHg). N=7-8

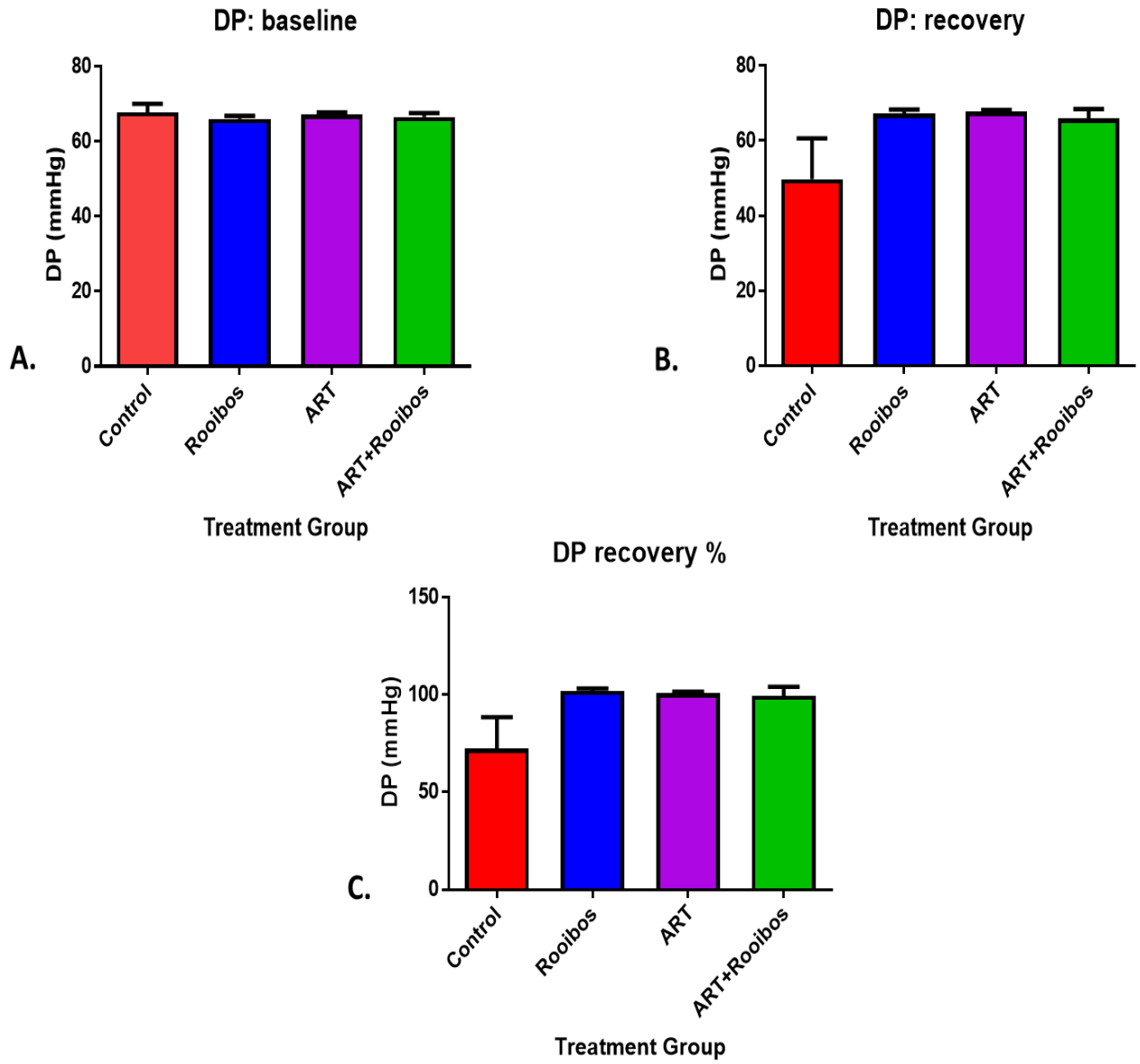


Fig. 4.7. *DP at baseline, recovery and % recovery. (R.I).* **A.** DP baseline. **B.** DP recovery. **C.** DP recovery %

4.1.2.1.6 HR

No differences were found in the HR pre and post ischemia. **A.** HR baseline; Control: 260.1±15.66 (BPM), Rooibos: 270.9±8.31 (BPM), ART: 259.3±15.59 (BPM), ART+ Rooibos: 278±9.14 (BPM). **B.** HR recovery; Control: 206±47.89 (BPM), Rooibos: 258.6±6.35 (BPM), ART: 246.6±15 (BPM), ART+ Rooibos: 264.6±11.39 (BPM). **C.** HR recovery %; Control: 75.28±16.88 (BPM), Rooibos: 95.68.6±1.56 (BPM), ART: 96.01±5.82 (BPM), ART+ Rooibos: 95.31±3.06 (BPM). N=7-8

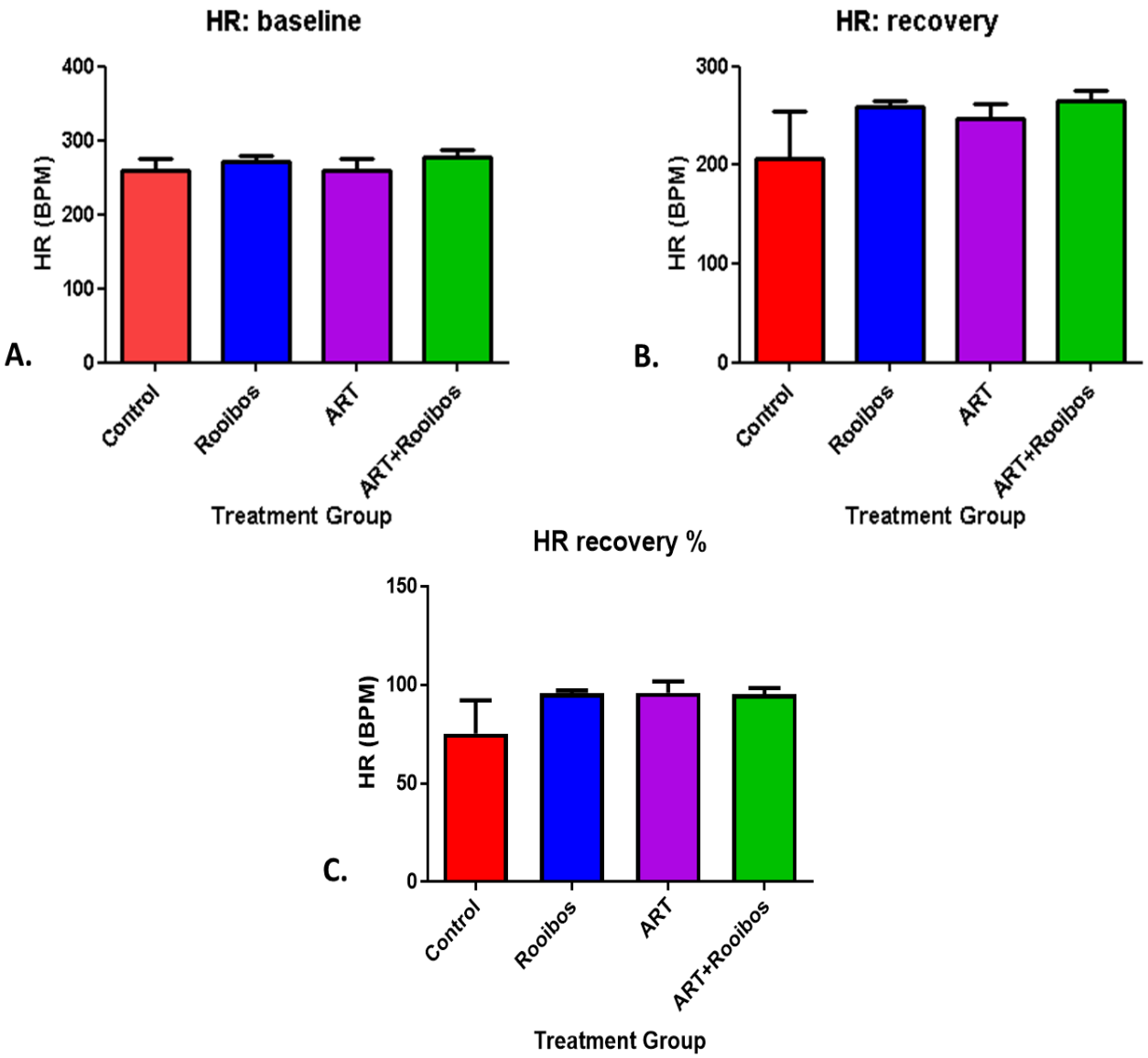


Fig. 4.8. *HR at baseline, recovery and % recovery. (R.I).* **A.** HR baseline. **B.** HR recovery. **C.** HR recovery %

4.1.2.1.7 Wt

Wt was similar between groups before and after ischemia and as a percentage of the recovery. **A.** Wt baseline; Control: 11.95±0.98 (mW), Rooibos: 10.88±0.68 (mW), ART: 10.52±0.48 (mW), ART+Rooibos: 10.75±0.32 (mW). **B.** Wt recovery; Control: 4.19±1.10 (mW), Rooibos: 3.31±0.47 (mW), ART: 4.31±0.46 (mW), ART+ Rooibos: 3.71±0.48 (mW). **C.** Wt recovery %; Control: 32.64±7.71 (mW), ART: 40.53±2.82 (mW), Rooibos: 33.5±3.10 (mW), ART+ Rooibos: 35.03±4.42 (mW). N=7-8

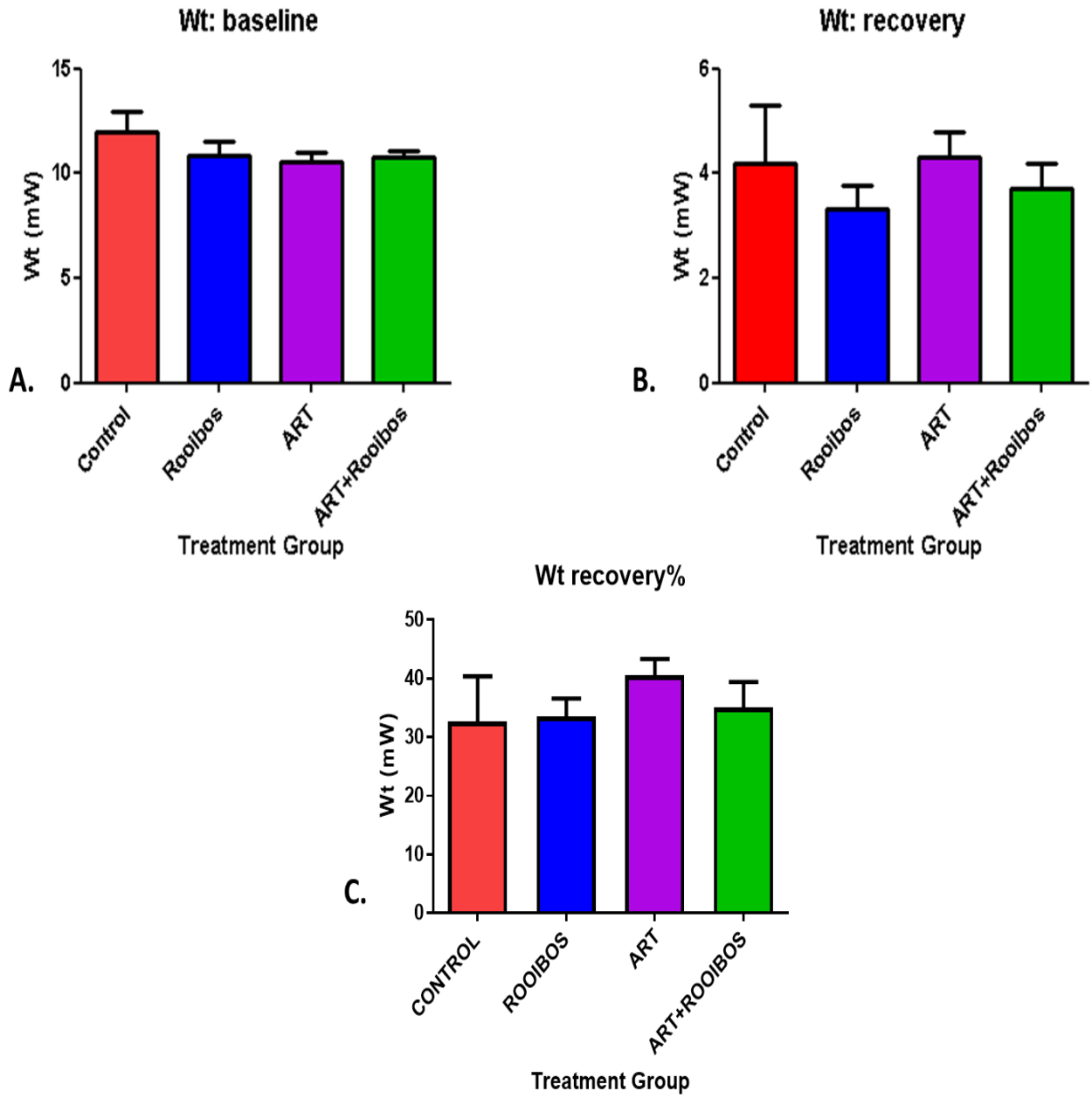


Fig. 4.9. *Wt at baseline, recovery and % Wt recovery. (R.I).* **A.** Wt baseline. **B.** Wt recovery. **C.** Wt recovery %

4.1.2.1.8 IS

The ART group showed significantly greater % area of infarct than the control group (Control: 28.17±5.10 (%AAR), ART: 50.56±4.08 (%AAR)). No significant differences were observed between the other groups. Control: 28.17±5.10 (%AAR), ART: 50.56±4.08 (%AAR), Rooibos: 39.37±5.83 (%AAR), ART+ Rooibos: 46.75±4.72 (%AAR). N=7-8

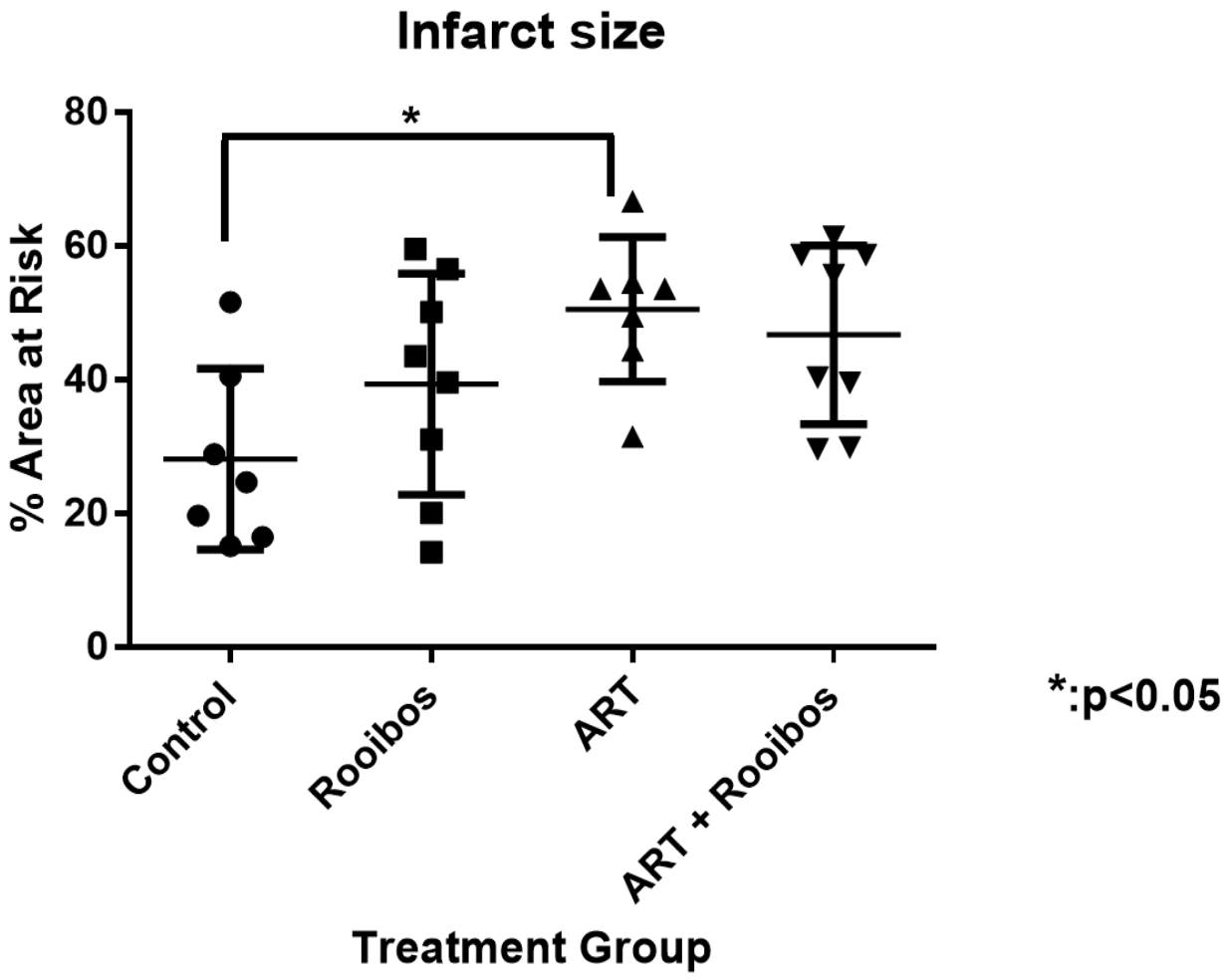


Fig. 4.10. *IS expressed as a percentage of the area at risk (AAR).*

4.1.2.2 Global Ischemia

4.1.2.2.1 AO

No significant differences were found in the AO baseline, recovery and percentage recovery between groups. **A.** AO baseline; Control: 37.78±1.87 (ml/min), Rooibos: 38±1.7 (ml/min), ART: 33±5.85 (ml/min), ART+ Rooibos: 40.5±1.80 (ml/min). **B.** AO recovery; Control: 28.3±7.81 (ml/min), Rooibos: 47.36±5.88 (ml/min), ART: 31.08±8.63 (ml/min), ART+ Rooibos: 32.63±8.82 (ml/min). **C.** AO recovery %; Control: 28.33±7.81 (ml/min), Rooibos: 47.36±5.88 (ml/min), ART: 31.08±8.63 (ml/min), ART+ Rooibos: 32.63±8.82 (ml/min). N=8-9

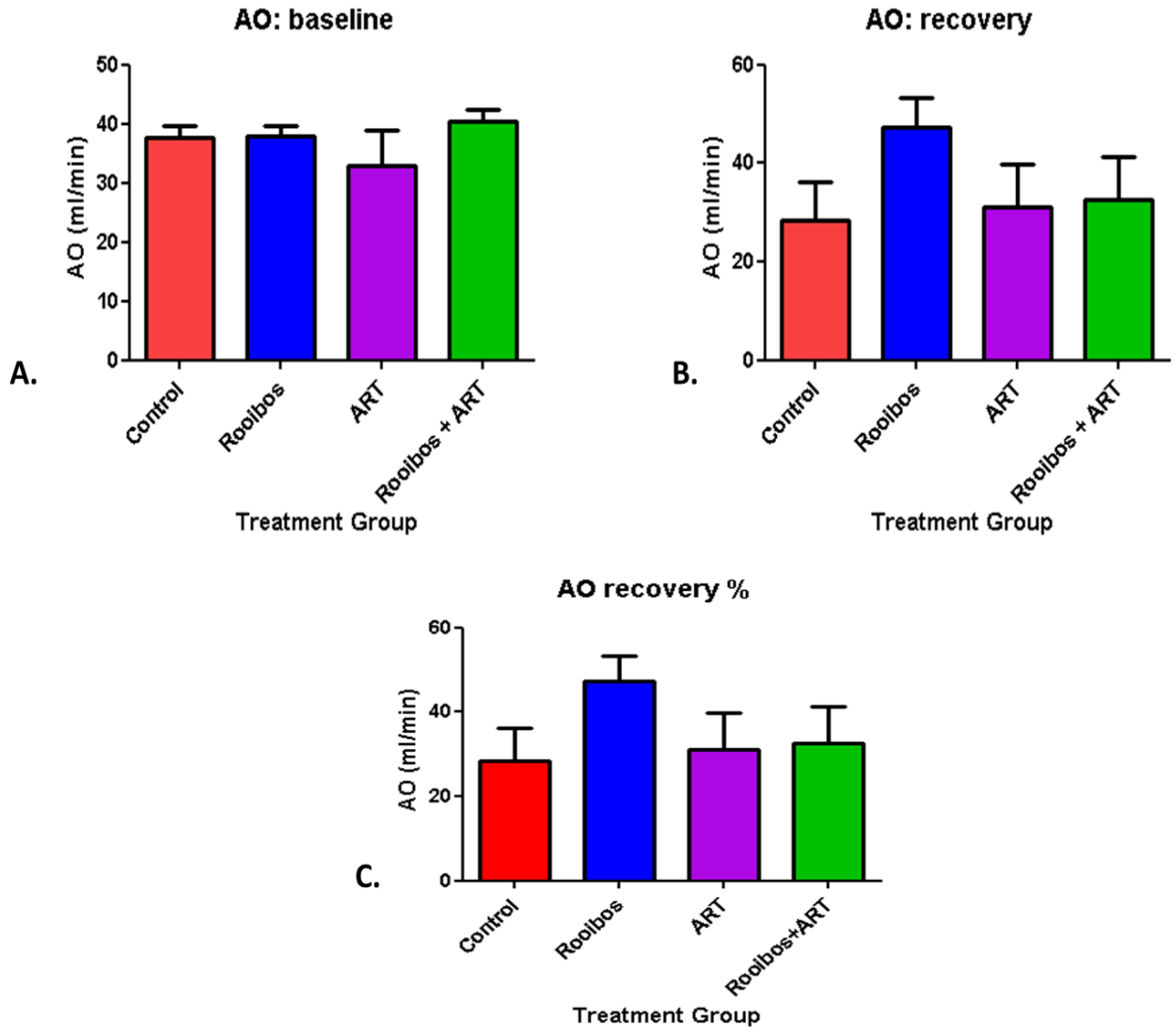


Fig. 4.11. *AO at baseline, recovery and the % recovery. (G.I).* **A.** AO baseline. **B.** AO recovery. **C.** AO recovery %

4.1.2.2.2 CF

A significant difference was found in the CF at baseline between the ART (11.63±0.91 (ml/min)) and ART+Rooibos (15.06±0.72 (ml/min)) group. No significant differences were observed between all other groups at recovery and the percentage recovery. **A.** CF baseline; Control: 13.94±0.77 (ml/min), Rooibos: 12.83±0.75 (ml/min), ART: 11.63±0.91 (ml/min), ART+ Rooibos: 15.06±0.72 (ml/min). **B.** CF recovery; Control: 12.06±0.52 (ml/min), Rooibos: 11.44±1.01 (ml/min), ART: 9±2.035 (ml/min), ART+ Rooibos: 11.75±1.33 (ml/min). **C.** CF recovery %; Control: 87.87±5.00 (ml/min), Rooibos: 89.56±6.28 (ml/min), ART: 76.8±17.29 (ml/min), ART+ Rooibos: 78.51±7.96 (ml/min). N=8-9

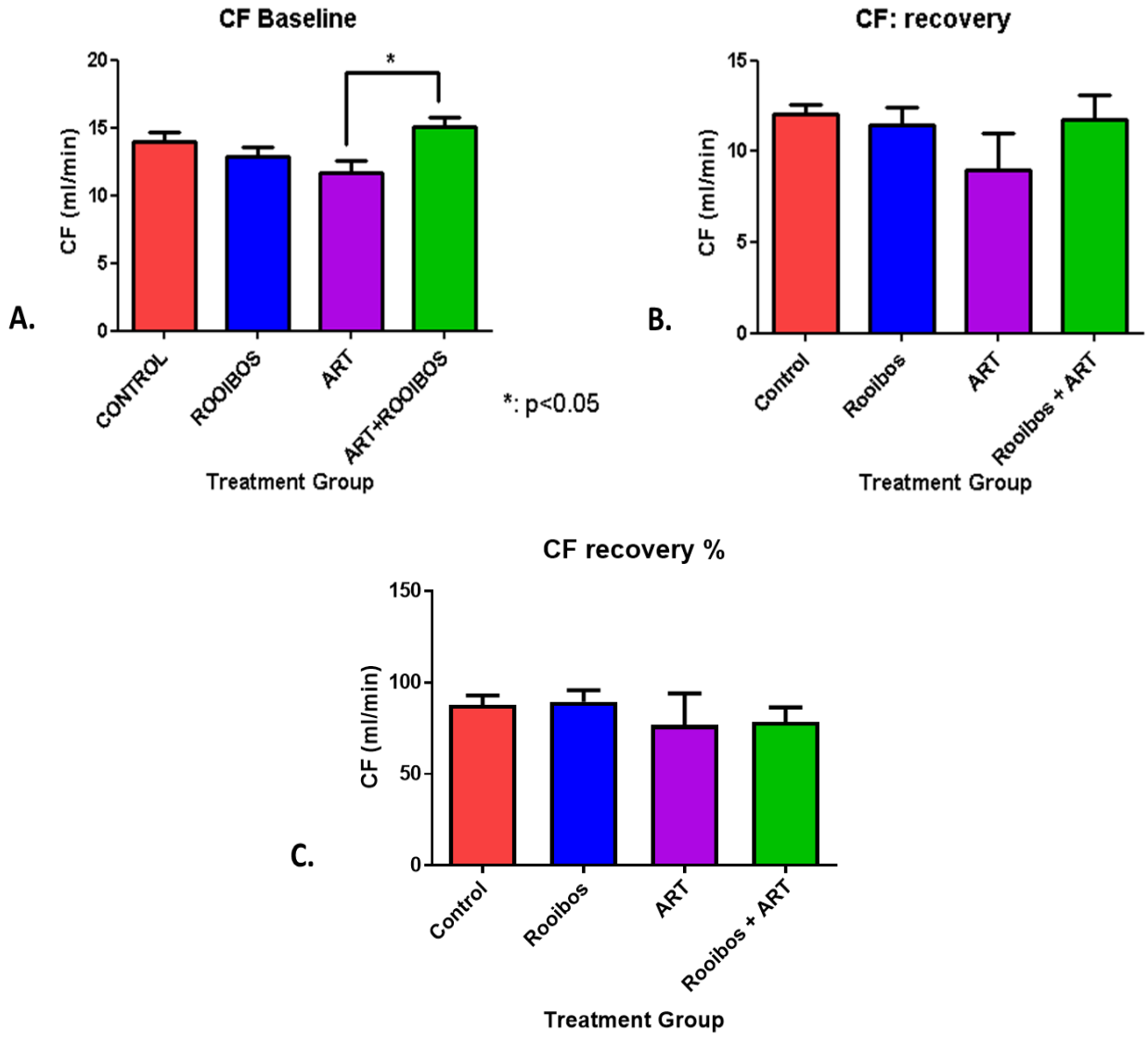


Fig. 4.12. *CF at baseline, recovery and recovery as a %. (G.I).* **A.** CF baseline. **B.** CF recovery. **C.** CF recovery %

4.1.2.2.3 CO

CO at baseline, recovery and the % recovery did not differ significantly between groups. **A.** CO baseline; Control: 51.72±2.37 (ml/min), Rooibos: 50.83±2.26 (ml/min), ART: 44.63±6.61 (ml/min), Rooibos+ART: 55.56±2.10 (ml/min). **B.** CO recovery; Control: 22.94±3.22 (ml/min), Rooibos: 30.11±3.79 (ml/min), ART: 21.44±5.51 (ml/min)**,** Rooibos+ART: 25.5±4.46 (ml/min). **C.** CO recovery %; Control: 45.89±6.71 (ml/min), Rooibos: 57.87±5.43 (ml/min), ART: 44.8±10.3 (ml/min), ART+ Rooibos: 45.35±7.48 (ml/min). N=8-9

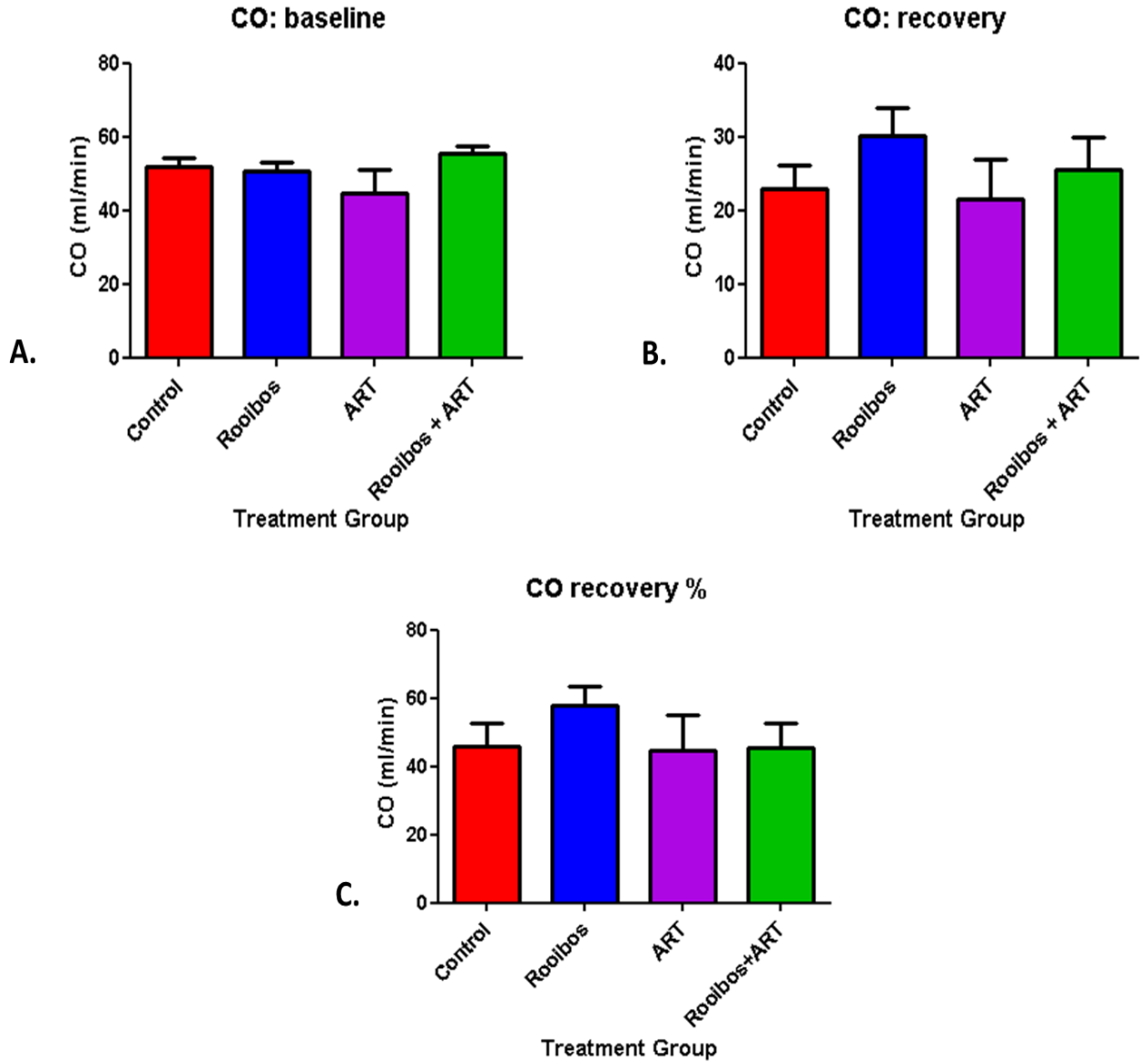


Fig. 4.13. *Cardiac output at baseline, recovery and % cardiac output recovery. (G.I).* **A.** CO baseline. **B.** CO recovery. **C.** CO recovery %

4.1.2.2.4 PSP

PSP showed no significant differences between groups at baseline, recovery and recovery %. **A.** PSP baseline; Control: 88.44±0.58 (mmHg), Rooibos: 89.22±1.11 (mmHg), ART: 88.13±2.12 (mmHg), Rooibos+ART: 90.38±0.98 (mmHg). **B.** PSP recovery; Control: 80.89±2.32 (mmHg), Rooibos: 84.78±1.33 (mmHg), ART: 71.71±12 (mmHg), ART+ Rooibos: 75.75±9.54 (mmHg). **C.** PSP recovery %; Control: 91.52±2.80 (mmHg), Rooibos: 95±0.60 (mmHg), ART: 69.98±15.31 (mmHg), ART+ Rooibos: 83.75±10.42 (mmHg). N=8-9

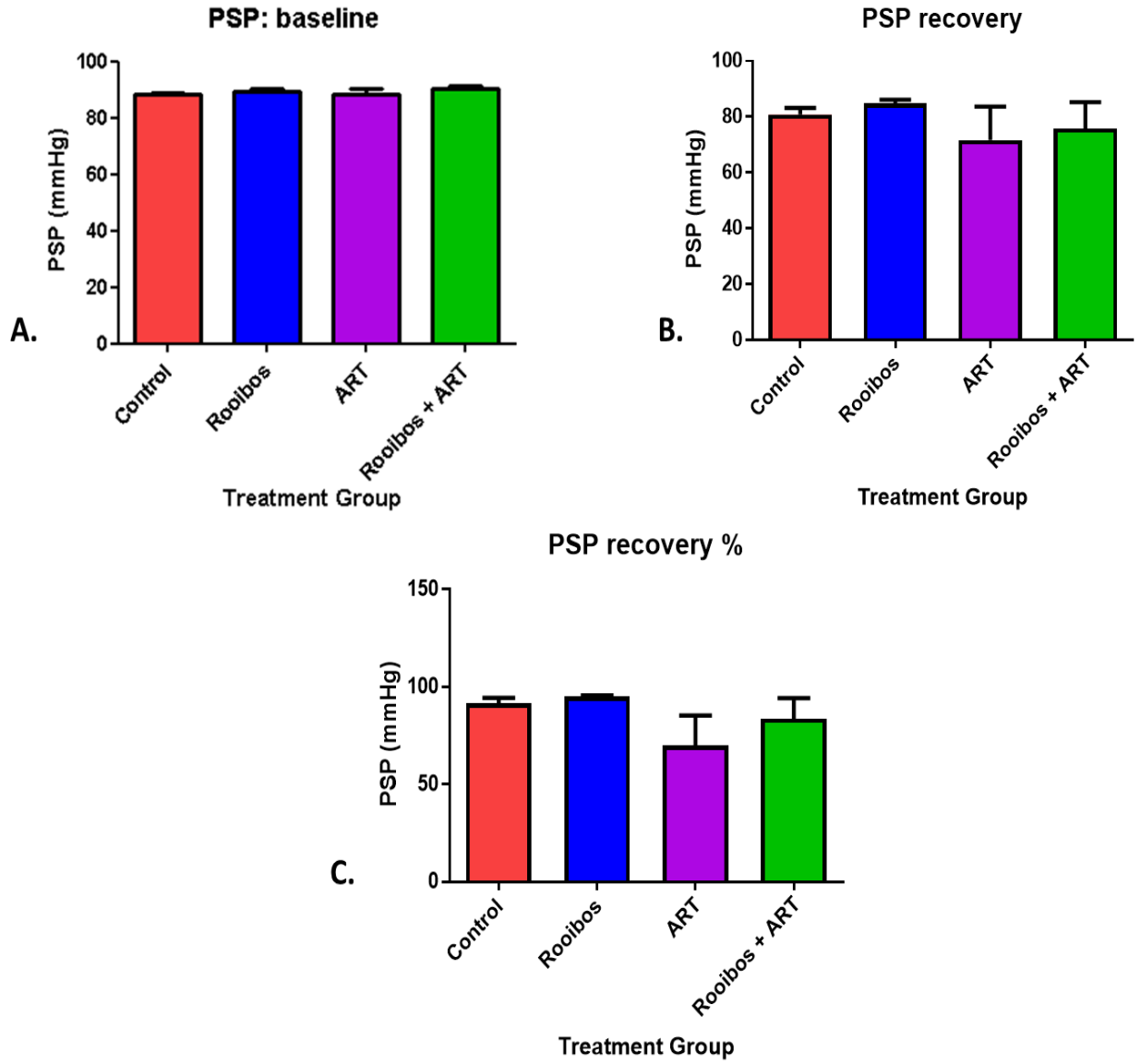


Fig. 4.14. *PSP at baseline, recovery and the recovery %. (G.I).* **A.** PSP baseline. **B.** PSP recovery. **C.** PSP recovery %

4.1.2.2.5 DP

No significant differences were found in the DP baseline, recovery and recovery % values between groups. **A.** DP baseline; Control: 65.56±0.80 (mmHg), Rooibos: 64.44±1.09 (mmHg), ART: 66.2±0.79 (mmHg), Rooibos+ART: 65.13±0.91 (mmHg). **B.** DP recovery; Control: 64.78±2.07 (mmHg), Rooibos: 66.33±0.96 (mmHg), ART: 49.75±10.93 (mmHg), Rooibos+ART: 58.63±8.39 (mmHg). **C.** DP recovery %; Control: 98.93±3.28 (mmHg), Rooibos: 103.2±2.31 (mmHg), ART: 75.36±16.5 (mmHg), Rooibos+ART: 91.02±12.62 (mmHg). N=8-9

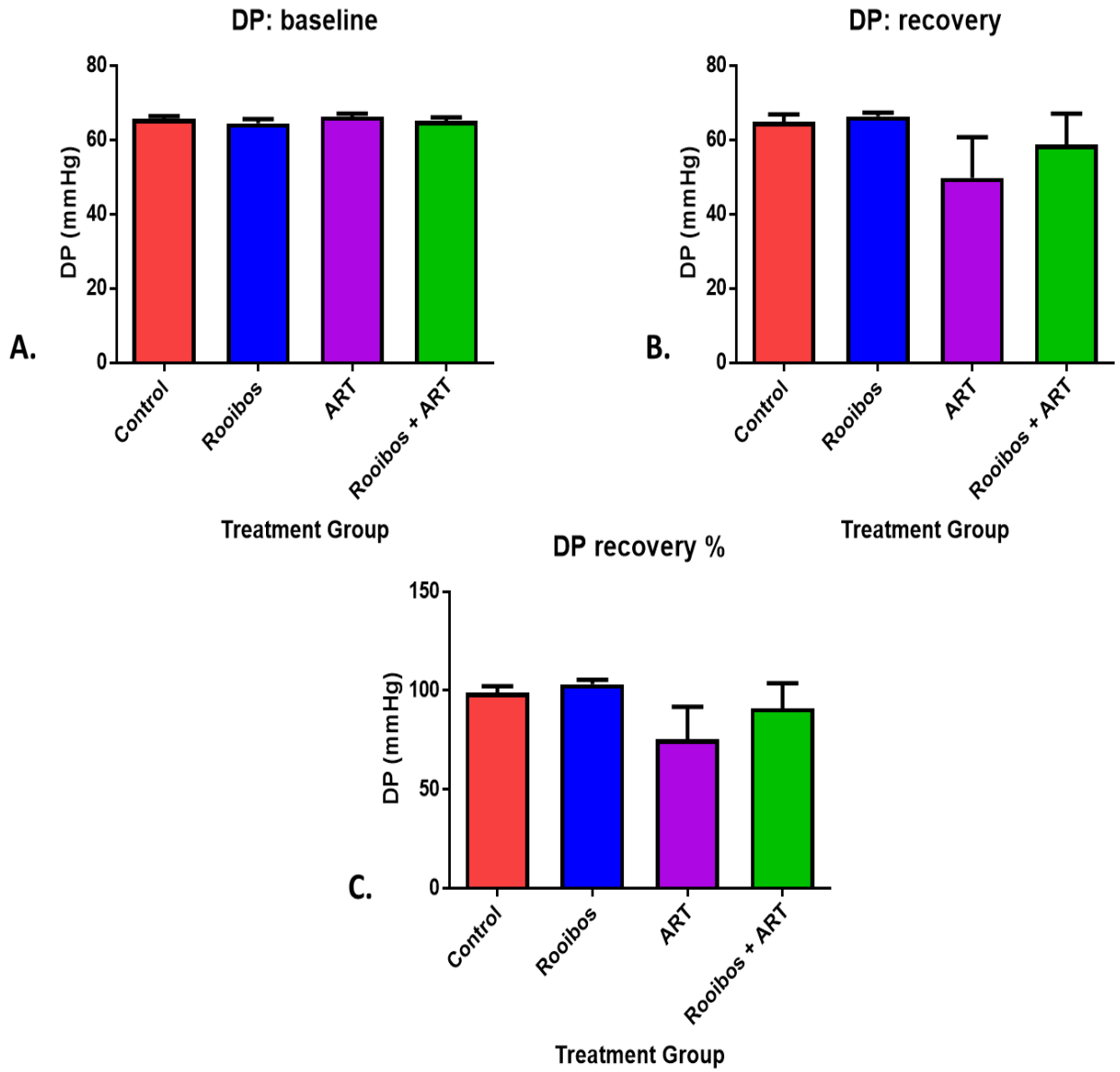


Fig. 4.15. *DP at baseline, recovery and recovery %. (G.I).* **A.** DP baseline. **B.** DP recovery. **C.** DP recovery %

4.1.2.2.6 HR

HR at baseline, recovery and the recovery % were not significantly different between groups. **A.** HR baseline; Control: 280.2±13.76 (BPM), Rooibos: 268.2±15.71 (BPM), ART: 237.9.3±5.81 (BPM), Rooibos+ART: 263.5±6.30 (BPM). **B.** HR recovery; Control: 260.8±8.67 (BPM), Rooibos: 229.3±11.57 (BPM), ART: 187.8±41.69 (BPM), Rooibos+ART: 232.6±34.34 (BPM). **C.** HR recovery %; Control: 94.27±4.30 (BPM), Rooibos: 87.01±4.90 (BPM), ART: 81.26±18.05 (BPM), Rooibos+ART: 88.44±12.93 (BPM). N=8-9

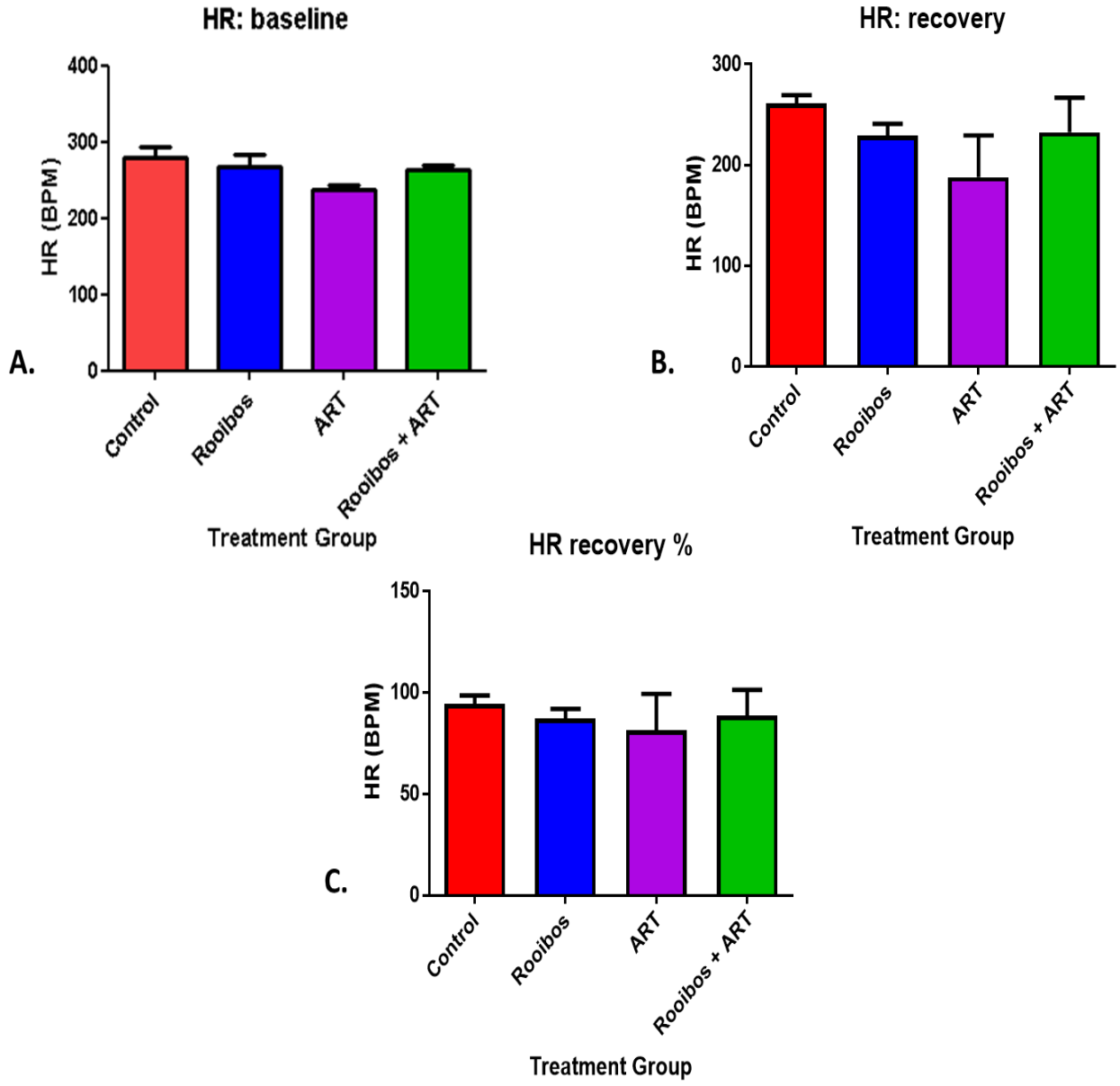


Fig. 4.16. *HR at baseline, recovery and recovery %. (G.I).* **A.** HR baseline. **B.** HR recovery. **C.** HR recovery %

4.1.2.2.7 Wt

There were no significant differences between groups in the Wt baseline, recovery and percentage recovery. **A.** Wt baseline; Control: 10.08±0.52 (mW), Rooibos: 10.01±0.54 (mW), ART: 8.85±1.45 (mW), Rooibos+ART: 11.05±0.47 (mW). **B.** Wt recovery; Control: 4.17±0.66 (mW), Rooibos: 5.68±0.77 (mW), ART: 3.98±1.04 (mW), Rooibos+ART: 4.76±0.97 (mW). **C.** Wt recovery %; Control: 41.54±6.47 (mW), Rooibos: 55.17±5.36 (mW), ART: 41.96±9.77 (mW), Rooibos+ART: 41.99±7.91 (mW). N=8-9

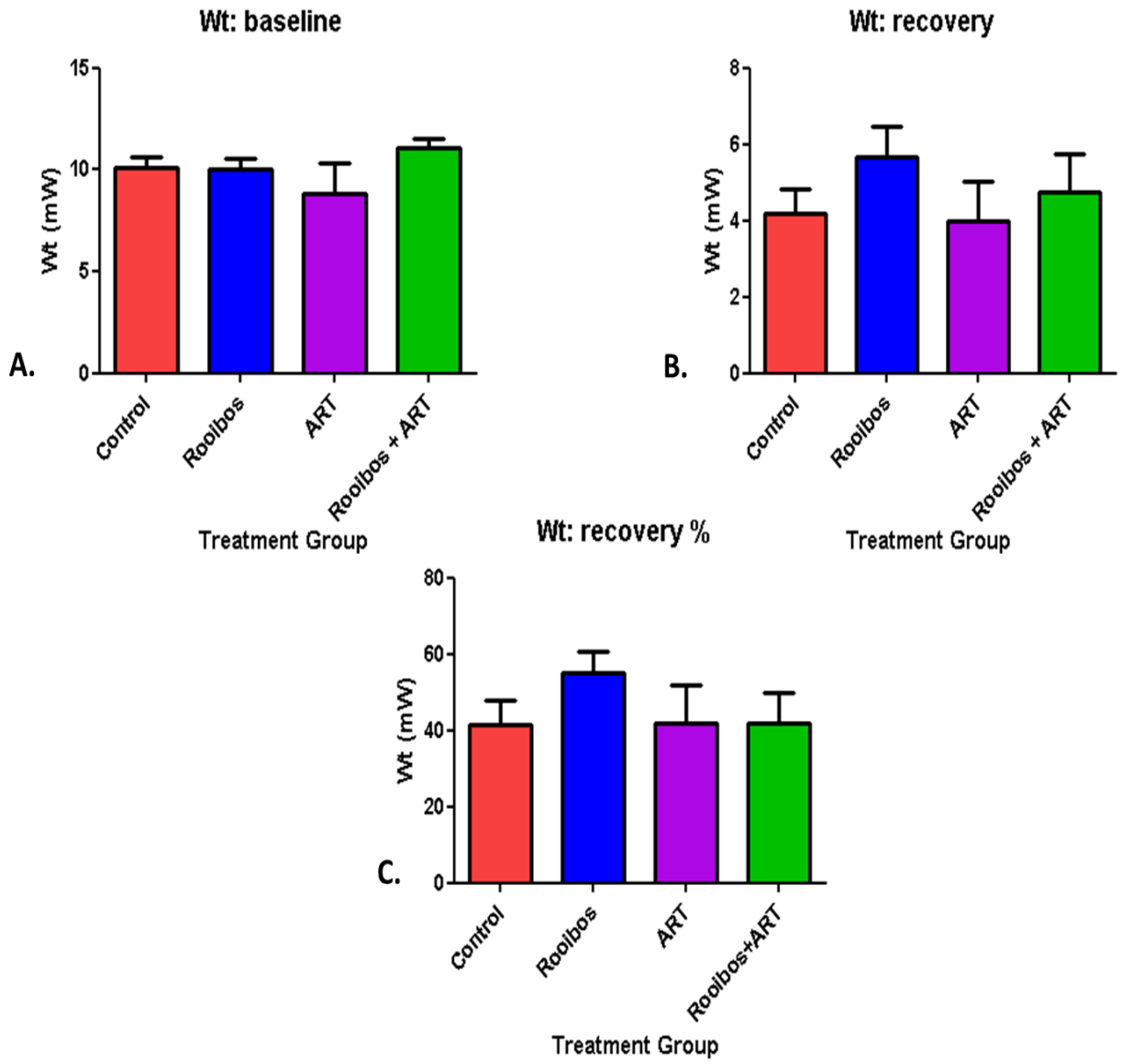


Fig. 4.17*. Wt at baseline, recovery and % Wt recovery. (G.I).* **A.** Wt baseline. **B.** Wt recovery. **C.** Wt recovery %

4.1.3 Vascular Reactivity Experiments

The vascular reactivity experiments demonstrate the endothelial response to relaxation and contractility and are indicative of endothelial function. Significance was examined by a two-way ANOVA and determining the area under the curve (AUC) values.

4.1.3.1 Vascular Reactivity Experiments without PVAT

This first section depicts the vascular response to relaxation and contraction in the absence of perivascular adipose tissue (PVAT).

4.1.3.1.1 Vascular response to acetylcholine (Ach): Cumulative relaxation

The ART treated group relaxed significantly less than the ART+Rooibos treated group (p=0.003) and the control group (p=0.03). AUC: Control, 0.00094; Rooibos, 0.00093; ART, 0.00089; ART+Rooibos, 0.00097.

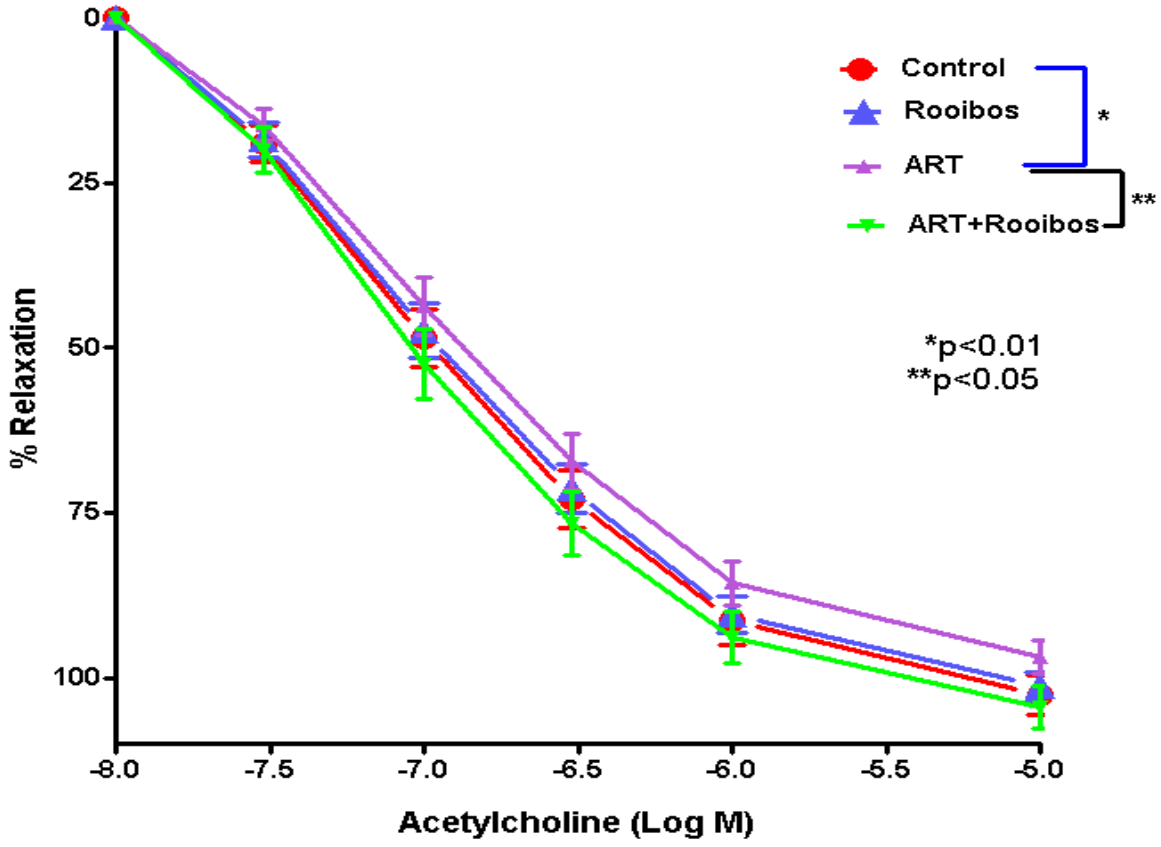


Fig. 4.18. *Ach induced relaxation of the vasculature without PVAT.* ART treated group relaxed significantly less than the ART+Rooibos (p=0.003) and the control (p=0.03) groups. N=20-25/group

4.1.3.1.2 Vascular response to phenylephrine (Phe): Cumulative contraction

In the contraction response significant differences were observed between groups. The rooibos group contracted significantly more than the controls (p=0.0149), ART (p=0.0457) and ART+Rooibos groups (p=0.0468). AUC: Control, 53.26; Rooibos, 60.66; ART, 54.63; ART+Rooibos, 54.27.

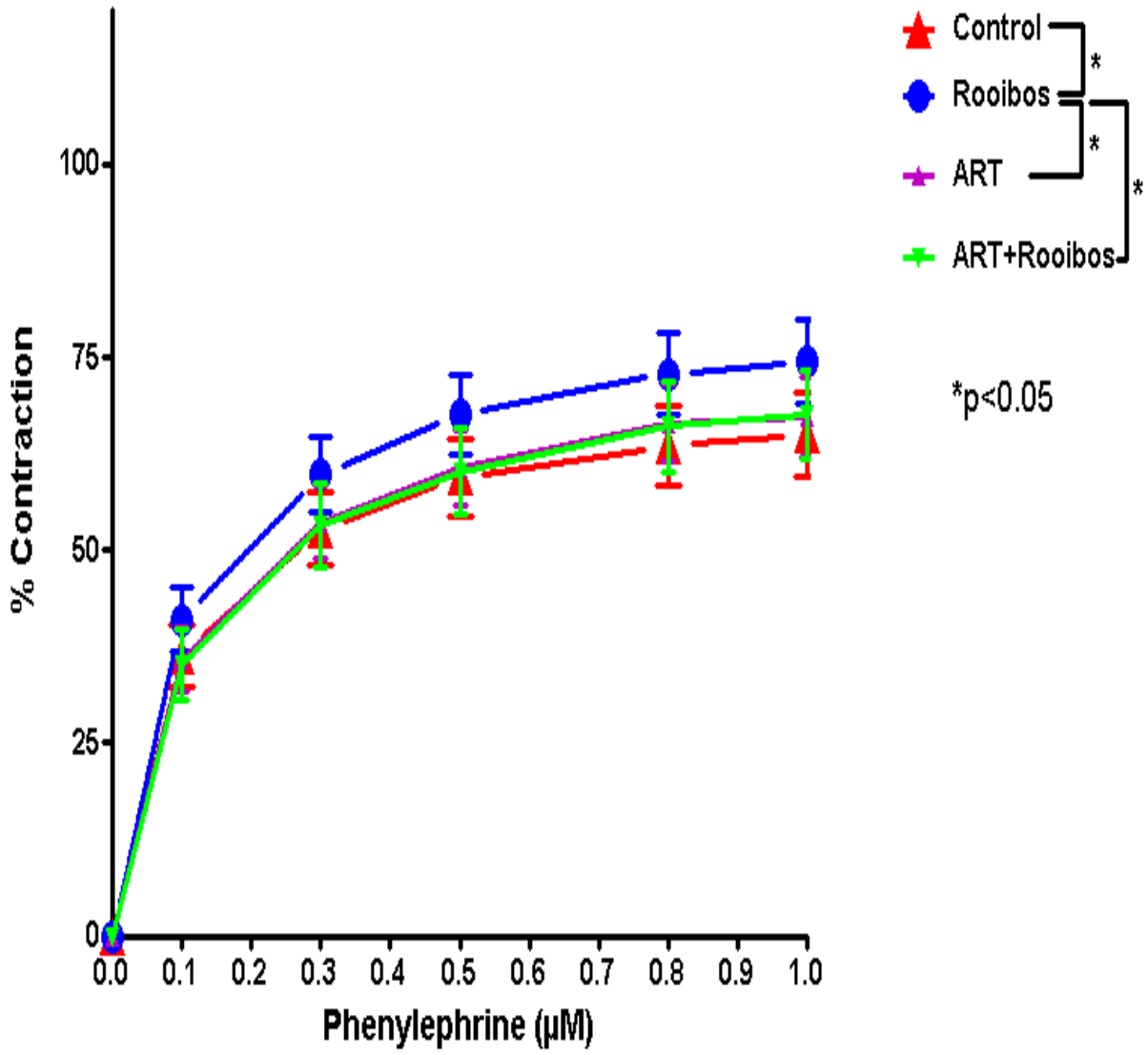


***Fig. 4.19**. Phe induced contraction of the vasculature without PVAT.* **Abbreviations**; ART: antiretroviral therapy. The rooibos group contracted significantly more than the controls (p=0.0149), ART (p=0.0457) and the ART+Rooibos (p=0.0468). N=20-25/group

4.1.3.2 Vascular Reactivity Experiments with PVAT

The following section depicts the contraction and relaxation responses of aortic rings in the presence of PVAT.

4.1.3.2.1 Vascular response, with PVAT, to Ach: Cumulative relaxation

In the presence of PVAT significant differences were found between the Control+PVAT group versus the Rooibos+PVAT group ($p<0.0001$), Control+PVAT versus ART+PVAT ($p<0.01$) and Control+PVAT versus Rooibos+ART+PVAT ($p<0.01$) in which case all of the treatment groups relaxed less than control. AUC: Control+PVAT, 0.00099; Rooibos+PVAT: 0.00079; ART+PVAT, 0.00091; ART+Rooibos+PVAT, 0.00088.

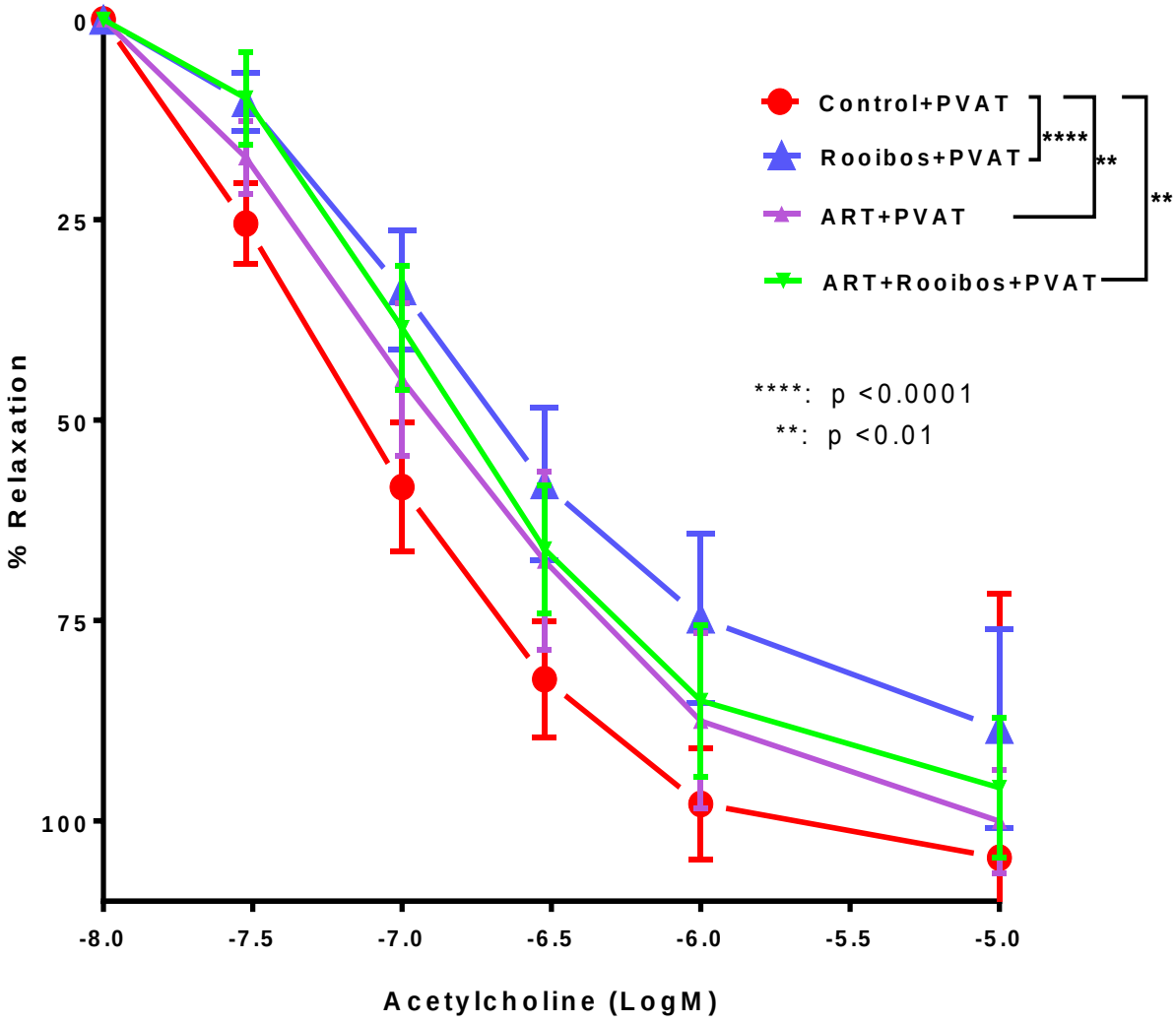


Fig. 4.20*. Ach induced relaxation of the vasculature with PVAT.* **Abbreviations**; PVAT: Perivascular adventitial tissue, ART: antiretroviral therapy. All treated groups relaxed significantly less than controls. Control+PVAT group versus the Rooibos+PVAT group ($p<0.0001$), Control+PVAT versus ART+PVAT ($p<0.01$) and Control+PVAT versus ART+Rooibos+PVAT ($p<0.01$). N=6-9/group

4.1.3.2.2 General effect of PVAT on Ach induced relaxation

No significant differences were found in the relaxation between the rings with PVAT and the rings without PVAT.

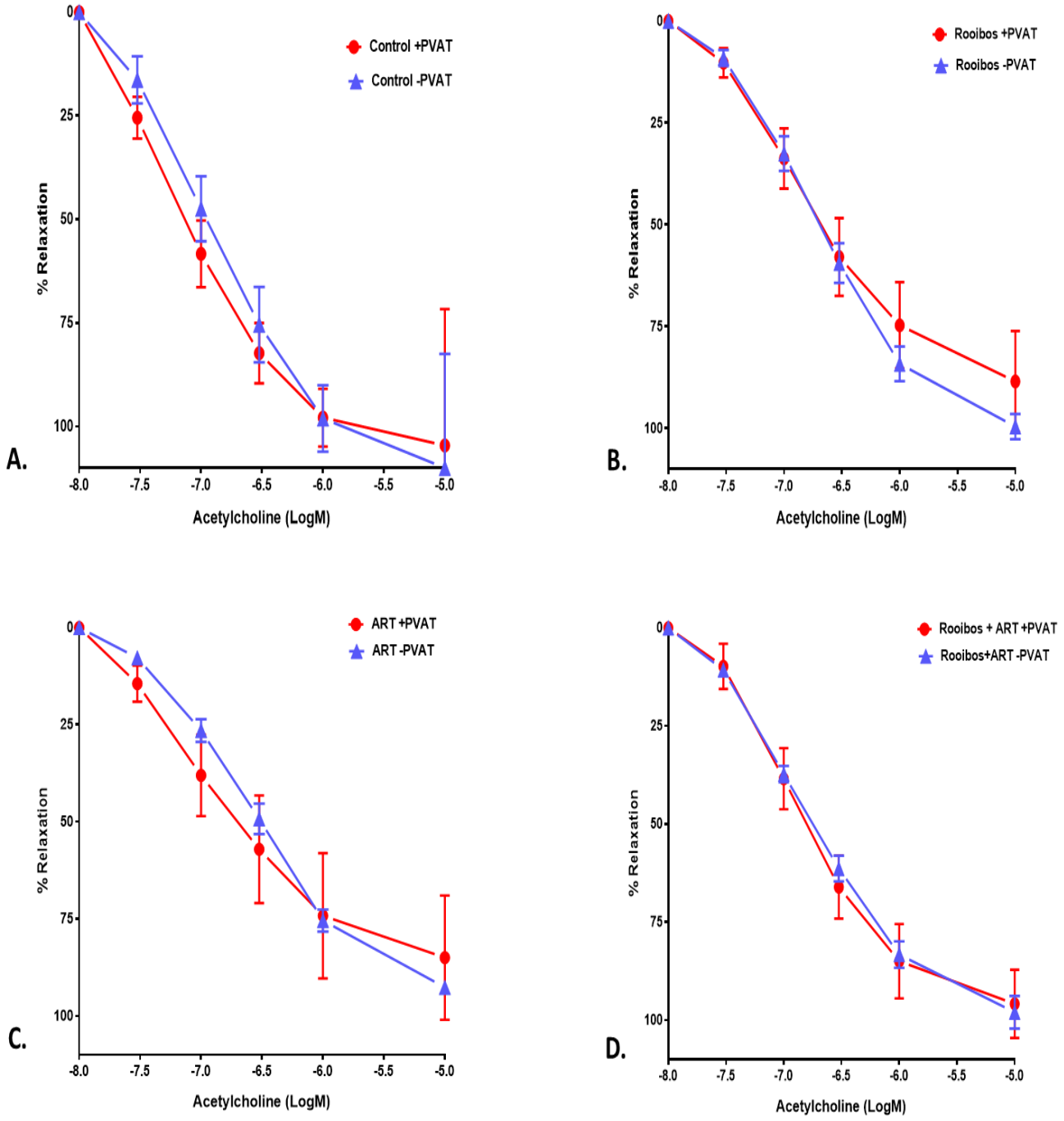


***Fig. 4.21**. General effect of PVAT on Ach induced relaxation.* **Abbreviations**; ART: antiretroviral therapy. The presence of PVAT had no significant effect on Ach induced relaxation of the rings. **A.** Control +PVAT versus Control –PVAT. **B.** Rooibos +PVAT versus rooibos –PVAT. **C.** ART +PVAT versus ART –PVAT. **D.** Rooibos +ART +PVAT versus Rooibos +ART –PVAT. N=6-9/group

4.1.3.2.3 Vascular response, with PVAT, to Phe induced contraction: Cumulative contraction

In the presence of PVAT, Phe induced contraction showed significant differences between the treatment groups. Rooibos+PVAT contracted significantly more than Control+PVAT ($p<0.01$), ART+PVAT group also contracted significantly more than the Control+PVAT group ($p<0.05$) and the combination treatment of ART+Rooibos+PVAT contracted significantly less than the Rooibos+PVAT group ($p<0.05$). AUC: Control+PVAT, 31.45; Rooibos+PVAT: 43.42; ART+PVAT, 36.32; ART+Rooibos+PVAT, 33.34.

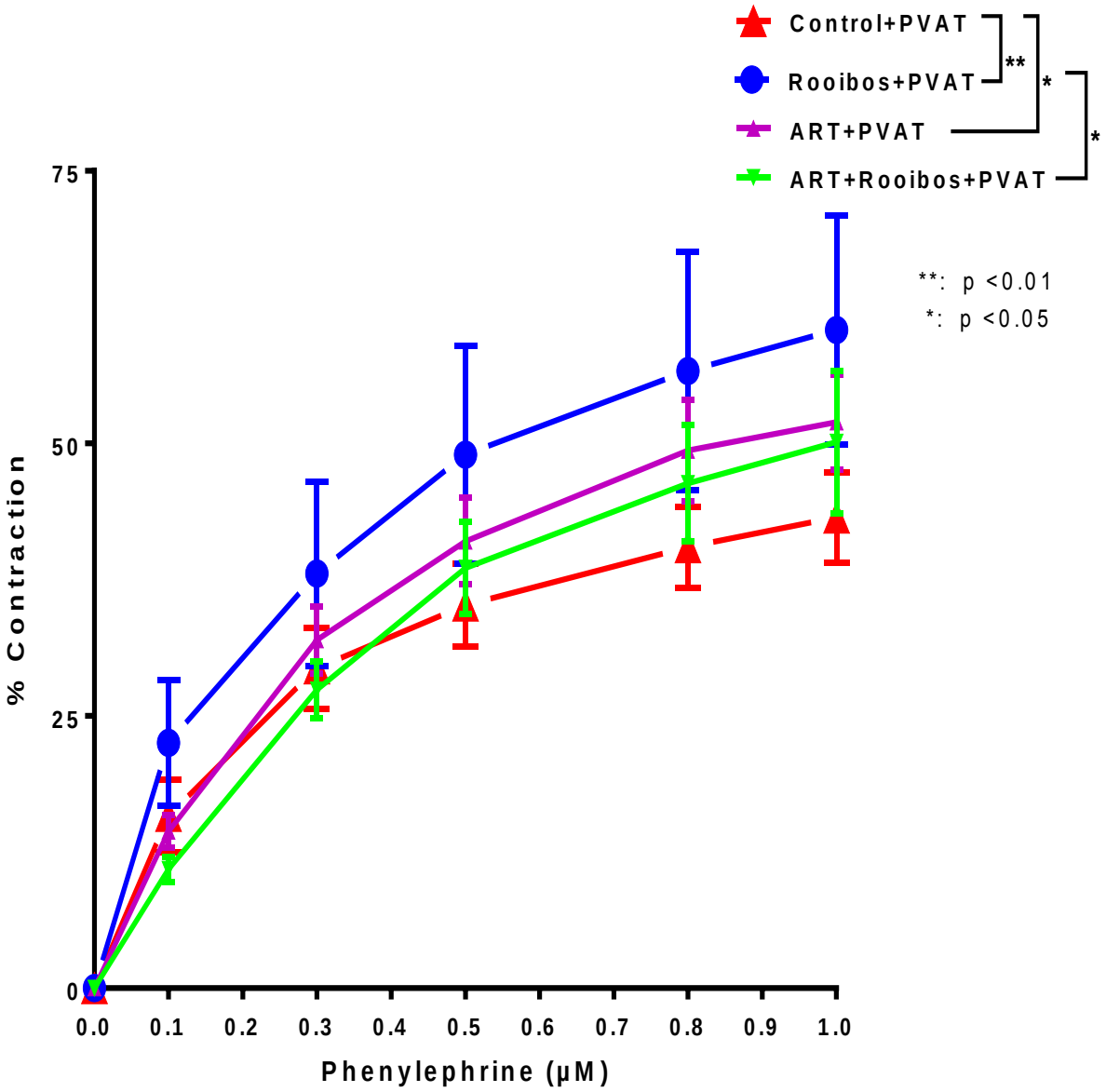


Fig. 4.22*. Phe induced contraction of the vasculature with PVAT.* **Abbreviations**; PVAT: Perivascular adventitial tissue, ART: antiretroviral therapy. Rooibos+PVAT and ART+PVAT contracted significantly more than Control+PVAT ($p<0.01$ and $p<0.05$ respectively). The combination treatment of ART+Rooibos+PVAT contracted significantly less than the Rooibos+PVAT group ($p<0.05$). N=6-9/group

4.1.3.2.4 General effect of PVAT on Phe induced contraction

Significant differences were found in the contraction between aortic rings with PVAT and those without PVAT in all of the treatment groups. PVAT was anticontractile in all instances: Control +PVAT versus Control -PVAT group ($p<0.0001$); Rooibos +PVAT versus Rooibos -PVAT ($p<0.0001$). The ART +PVAT versus ART -PVAT ($p<0.0001$) had significant differences in contraction at all concentrations (100nM, 300nM, 500nM, 800nM and 1µM) of Phe ($p<0.01$ and $p<0.001$, where indicated) and Rooibos +ART +PVAT versus Rooibos +ART -PVAT ($p<0.0001$) with significant differences at 500nM, 800nM and 1µM Phe concentrations.

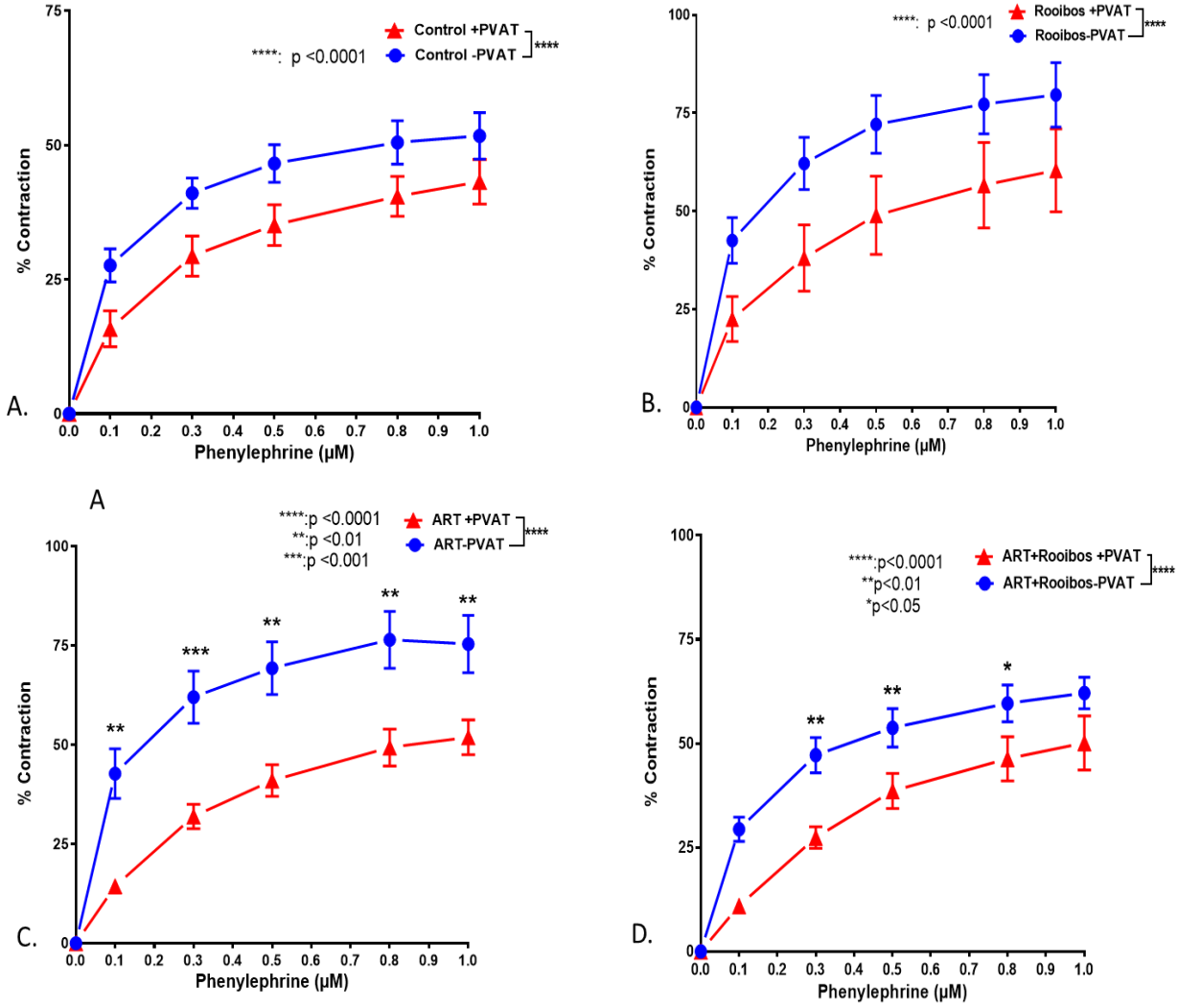


***Fig. 4.23**. General effect of PVAT on Phe induced contraction*. **Abbreviations**; ART: antiretroviral therapy. The presence of PVAT significantly reduced Phe induced contraction in all groups. **A.** control +PVAT versus control -PVAT ($p<0.0001$). **B.** rooibos +PVAT versus rooibos -PVAT ($p<0.0001$). **C.** ART +PVAT versus ART -PVAT ($p<0.0001$) and $p<0.01$ at points 2,4,5,6 of Phe addition and $p<0.001$ at point 3. **D.** rooibos +ART +PVAT versus rooibos +ART -PVAT ($p<0.0001$), $p<0.01$ at points 3, 4 and $p<0.05$ at point 5. N=6-9/group

4.1.4 Lipid profiles and TBARS levels

The total cholesterol (TC); triglycerides (TG) and phospholipid (PL) levels are a representation of the lipid profiles and the TBARS levels an indicator of lipid peroxidation. A total of 22 samples were used for these analyses.

4.1.4.1 Lipid profiles

TC level in the rooibos group was significantly different to that of controls. TG and PL levels were not significantly different between the treatment groups.

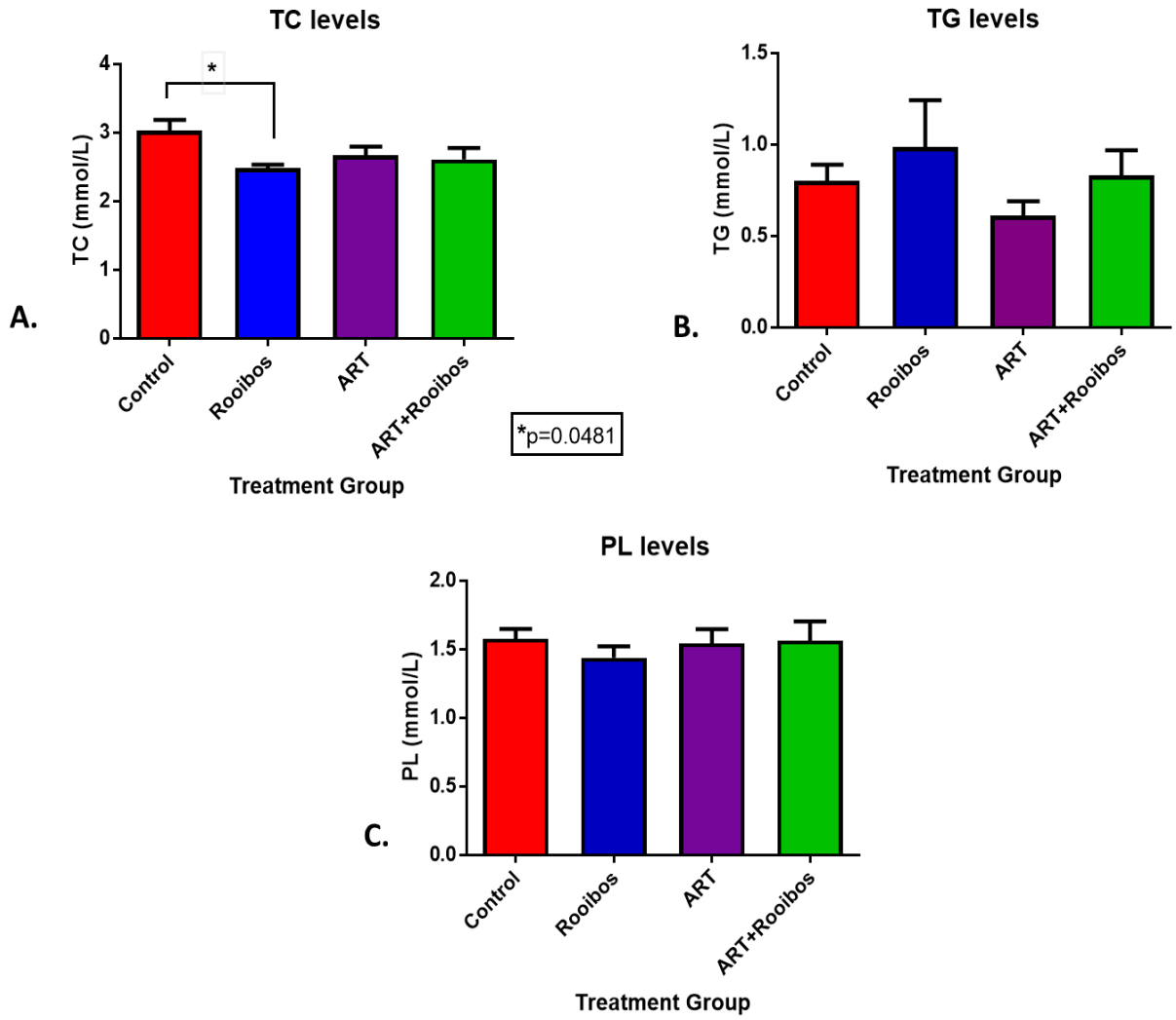


Fig. 4.24. *Figure representing lipid levels.* **A.** TC levels. Significant difference between rooibos and control (p=0.0481). Control:3.03±0.16 (mmol/L); Rooibos:2.48±0.05 (mmol/L); ART:2.67±0.13 (mmol/L); ART+Rooibos: 2.61±0.16 (mmol/L). **B.** TG levels. Control:0.8±0.09 (mmol/L); Rooibos:0.99±0.26 (mmol/L); ART:0.61±0.08 (mmol/L); ART+Rooibos:0.83±0.14 (mmol/L). **C.** PL levels. Control:1.57±0.07 (mmol/L); Rooibos:1.44±0.08 (mmol/L); ART:1.54±0.10 (mmol/L); ART+Rooibos:1.56±0.14 (mmol/L). N=5-6/ group

4.1.4.2 TBARS Levels

No significant differences were found in the TBARS levels between the groups.

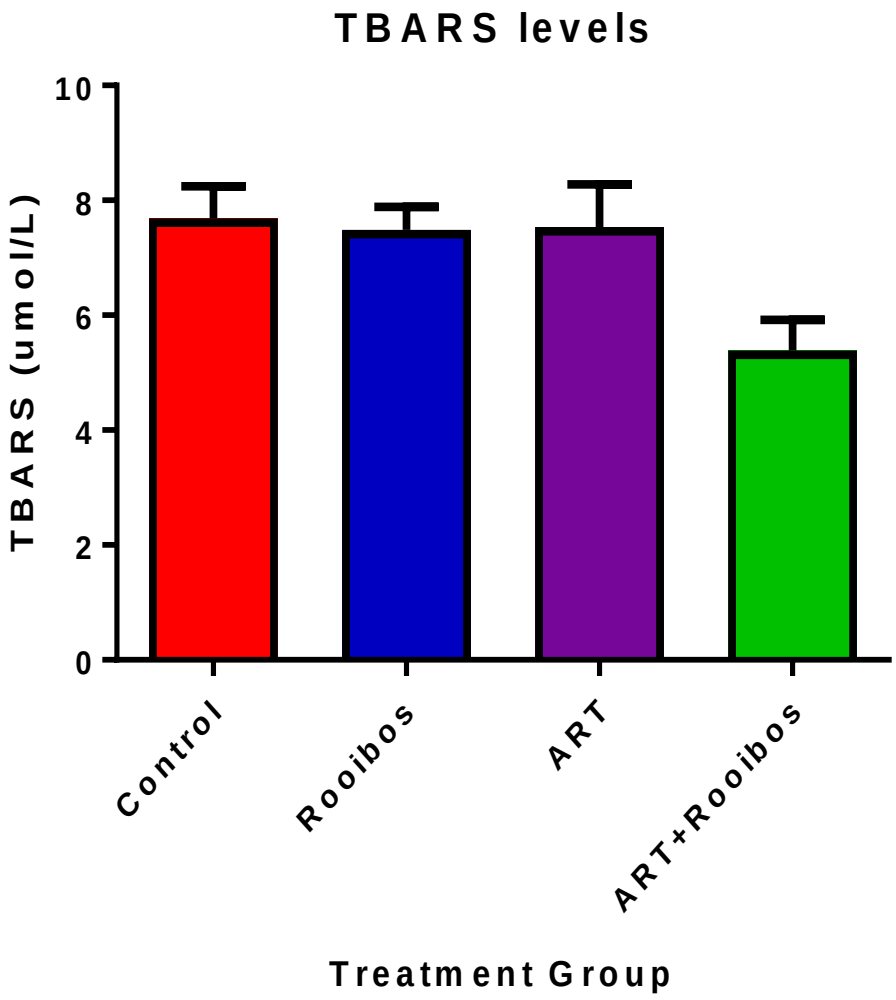


Fig. 4.25*. TBARS levels in µmol/L.* No significant differences were found between groups. Control:7.69±0.55 (µmol/*L*); Rooibos:7.49±0.39 (µmol/*L*); ART:7.54±0.74 (µmol/*L*); ART+Rooibos:5.39±0.53 (µmol/*L*). N=5-6/group

4.2 Aim 2: Effects of rooibos tea consumption on the cardiovascular profiles of patients on first line fixed dose ART compared to controls

4.2.1 Descriptive analyses of study population

The study population comprising of 105 study participants from Durbanville, Fisantekraal, Elsies River, Uitsig, Bishop Lavis and Ravensmead consisted of 23 HIV negative, 14 HIV positive participants who were not on ART and 68 HIV positive who were on ART. The data is first described in **Table 4.1** with the anthropometric data (ages, BMI, waist to hip ratio), cardiovascular outcomes and biochemical analyses (DP, SP, TC, HDL, LDL, TG, CRP and TBARS). **Table 4.2** describes the population according to smokers versus non-smokers and **Table 4.3** on the basis of gender. Percentage of participants who drank rooibos versus those who didn't is described in **Table 4.4**.

Table 4.1. *Description of study population with parametric data expressed as mean ± standard deviation and CRP (non-parametric) expressed as median with 25th and 75th percentiles.*

n=105	**HIV negative n=23**	**HIV positive no ART n=14**	**HIV positive on ART n=68**
Outcomes/Variables	**Mean ± Standard Deviation**		
Age	39.10±13.00	36.60±10.2	39.90±7.80
BMI (kg/m^2)	28.90±9.11	23.54±9.06	24.25±6.05
Waist to hip ratio	0.91±0.084	0.86±0.07	0.87±0.81
Diastolic Pressure (mmHg)	93.80±15.20	80.10±8.90	83.50±9.90
Systolic Pressure (mmHg)	132.20±23.60	114.90±13.30	120.40±15.00
Total Cholesterol	4.27±0.84	4.29±1.30	4.34±0.84
HDL	1.23±0.39	1.22±0.51	1.30±0.39
LDL	2.52±0.75	2.58±0.94	2.50±0.70
Triglycerides	1.09±0.46	1.08±0.67	1.25±1.23
TBARS	0.67±0.26	0.60±0.27	0.63±0.30
	Median (Minimum;Maximum)		
CRP*	3.80(0.20;25.00)	2.05(0.20;59.70)	6.35(0.20;120.00)

Abbreviations: HDL, high density lipoproteins; LDL, low density lipoproteins; TBARS, thiobarbituric acid reactive substances; CRP, C-reactive protein. *Non-parametric data expressed as median (minimum;maximum).

Table 4.2. *Number of people in the study population that smoke versus non-smokers.*

			GROUP			Total
			HIV negative	HIV positive no ART	HIV positive on ART	
smoke	**No**	Count	6	4	26	36
	Yes	Count	17	10	42	69

Table 4.3. *Description of study population in terms of gender.*

			GROUP			
			HIV negative	HIV positive no ART	HIV positive on ART	Total
Gender	**M**	Count	2	2	24	28
		% within group	8.7%	14.3%	35.3%	26.7%
	F	Count	21	12	44	77
		% within group	91.3%	85.7%	64.7%	73.3%

Abbreviations: M, Male; F, Female

Table 4.4. *Percentage of study population that drink rooibos.*

			HIV negative	HIV positive no ART	HIV positive on ART	
Drink Rooibos?	0	Count	10	6	34	50
0 = no 1 = yes		% within group	43.5%	42.9%	50.7%	48.1%
	1	Count	13	8	33	54
		% within group	56.5%	57.1%	49.3%	51.9%
Total		Count	23	14	67	104
		% within group	100.0%	100.0%	100.0%	100.0%

4.2.2 Multiple comparisons test: one way ANOVA with Post Hoc test

The multiple comparisons test, as represented in **Table 4.2**, compared the "(I) group" to the "(j) groups" of each variable. This revealed significant differences in the BMI, DP and SP levels. The HIV negative group (28.90±9.11 (kg/m^2)) had a significantly higher BMI compared to the HIV positive no ART (23.54±9.06 (kg/m^2)) group. Blood pressure (DP and SP) was significantly higher in the HIV negative group (93.80±15.20 (mmHg)) compared to the HIV positive no ART (10.327±2.689 (mmHg)) and HIV positive on ART (83.50±9.90 (mmHg)).

Table 4.5. *Results of multiple comparisons analysis- One way ANOVA.*

Dependent Variable	(I) group	(J) groups	Sig.	Mean Difference(confidence interval)
Age	HIV negative	HIV positive no ART	1.00	2.49(-5.33;10.31)
		HIV positive on ART	1.00	-0.72(-6.29;4.84)
	HIV positive no ART	HIV negative	1.00	-2.49(-10.31;5.33)
		HIV positive on ART	.75	-3.21(-9.98;3.56)
BMI	HIV negative	HIV positive no ART	.09	5.36(-0.62;11.33)
		HIV positive on ART	**.027**	4.65(0.40;8.90)
	HIV positive no ART	HIV negative	.09	-5.36(-11.33;0.61)
		HIV positive on ART	1.00	-0.71(-5.88;4.46)
Wasit to hip ratio	HIV negative	HIV positive no ART	.23	0.05(-0.02;0.11)
		HIV positive on ART	.08	0.04(-0.00;0.09)
	HIV positive no ART	HIV negative	.23	-0.05(-0.11;0.02)
		HIV positive on ART	1.00	-0.00(-0.06;0.05)
DP	HIV negative	HIV positive no ART	**.001**	13.71(4.51;22.91)
		HIV positive on ART	**.001**	10.33(3.78;16.87)
	HIV positive no ART	HIV negative	**.001**	-13.71(-22.91;-4.51)
		HIV positive on ART	.91	-3.38(-11.35;4.58)
SP	HIV negative	HIV positive no ART	**.01**	17.29(3.24;31.33)
		HIV positive on ART	**.01**	11.82(1.83;21.81)

	HIV positive no ART	HIV negative	**.01**	-17.29(-31.33;-3.24)
		HIV positive on ART	.83	-5.47(-17.63;6.67)
Total cholesterol	HIV negative	HIV positive no ART	1.00	-0.02(-0.78;0.74)
		HIV positive on ART	1.00	-0.07(-0.62;0.47)
	HIV positive no ART	HIV negative	1.00	-0.02(-0.74;0.78
		HIV positive on ART	1.00	-0.05(-0.71;0.60)
HDL	HIV negative	HIV positive no ART	1.00	0.01(-0.33;0.35)
		HIV positive on ART	1.00	-0.07(-0.32;0.17)
	HIV positive no ART	HIV negative	1.00	-0.01(-0.35;0.33)
		HIV positive on ART	1.00	-0.09(-0.38;0.20)
LDL	HIV negative	HIV positive no ART	1.00	-0.06(-0.68;0.56)
		HIV positive on ART	1.00	0.02(-0.42;0.47)
	HIV positive no ART	HIV negative	1.00	0.06(-0.56;0.68)
		HIV positive on ART	1.00	0.08(-0.45;0.62)
Triglycerides	HIV negative	HIV positive no ART	1.00	0.01(-0.86;0.89)
		HIV positive on ART	1.00	-0.16(-0.79;0.47)
	HIV positive no ART	HIV negative	1.00	-0.01(-0.89;0.86)
		HIV positive on ART	1.00	-0.17(-0.92;0.58)
CRP	HIV negative	HIV positive no ART	1.00	-5.59(-20.03;8.84)
		HIV positive on ART	.49	-5.90(-16.18;4.37)
	HIV positive no ART	HIV negative	1.00	5.59(-8.84;20.03)
		HIV positive on ART	1.00	-0.04(-0.18;0.26)
TBARS µmol/mmol	HIV negative	HIV positive no ART	1.00	0.07(-0.23;0.37)
		HIV positive on ART	1.00	0.04(-0.18;0.26)
	HIV positive no ART	HIV negative	1.00	-0.07(-0.37;0.23)
		HIV positive on ART	1.00	-0.03(-0.18;0.26)

Bold results indicate statistically significant results (P value <0.05)

Abbreviations: DP, diastolic pressure; SP, systolic pressure; HDL, high density lipoproteins; LDL, low density lipoproteins; CRP, C-reactive protein; TBARS, thiobarbituric acid reactive substances

4.2.3 Regression analysis to determine effects of drinking rooibos

So as to determine the effects of rooibos in the presence and absence of ART, regression analysis was conducted only on HIV positive participants. The results therefore compare the groups no Rooibos on ART, Rooibos no ART and Rooibos on ART, relatively to the baseline group of no Rooibos no ART. The outcomes/dependent variables analysed were: DP, SP, HDL, LDL, Trigs, TC, CRP and TBARS. ART status and rooibos drinking were considered as independent variables and BMI, waist to hip ratio, age, gender and smoking were all controlled for. No significance was found in any of the comparisons.

Table 4.6. *Regression analysis on rooibos drinking data.*

	No Rooibos no ART		**Rooibos no ART**		**Rooibos on ART**	
Outcomes	**β coefficient(confidence interval)**	**P value**	**β coefficient(confidence interval)**	**P value**	**β coefficient(confidence interval)**	**P value**
DP	-1.55(-10.06,6.97)	0.72	-6.93(-17.43,3.56)	0.19	0.05(-17.43, 3.56)	0.99
SP	-3.02(-14.85, 8.81)	0.61	-8.46(-23.04, 6.12)	0.25	-0.85(-12.68, 10.99)	0.89
TC	-0.31(-1.11, 0.49)	0.44	-0.89(-1.88, 0.10)	0.08	-0.73(-1.54, 0.07)	0.07
HDL	-0.10(-0.47, 0.28)	0.60	-0.35(-0.81, 0.11)	0.13	-0.11(-0.48, 0.27)	0.57
LDL	-0.26(-0.94, 0.42)	0.44	-0.52(-1.38, 0.34)	0.23	-0.60(-1.29, 0.08)	0.08
TG	0.42(-0.55, 1.40)	0.38	0.50(-0.73, 1.74)	0.42	0.18(-0.81, 1.16)	0.72
TBARS	-0.03(-0.40, 0.32)	0.83	-0.18(-0.62, 0.25)	0.40	-0.14(-0.49,0.21)	0.44
CRP	0.40(-0.17, 0.98)	0.16	0.25(-0.48, 0.98)	0.50	0.43(-0.15,1.01)	0.15

Abbreviations: DP, diastolic pressure; SP, systolic pressure; TC, total cholesterol; HDL, high density lipoproteins; LDL, low density lipoproteins; CRP, C-reactive protein; TBARS, thiobarbituric acid reactive substances

Chapter 5 – Discussion

5.1 Effects of *Aspalathus linearis* supplementation during ART on isolated hearts and aortas of male Wistar rats.

In order to avoid repetition and promote congruency the BMI, lipid profile and TBARS results of the human study are also discussed in this section, below the respective titles. **Section 5.*2*** "Effects of rooibos consumption on the cardiovascular profiles of patients on ART" contains the discussion of all other findings of the epidemiological study starting with a description of the study population and ending with the effects of drinking rooibos.

5.1.1 Body Weights and Fluid Consumption

The body weight gained over the 9 week treatment period and the weekly fluid intake did not differ significantly between the groups. Thus the ART, rooibos and combination had no significant effect on the weights and fluid consumption of the rats. In the epidemiological aspect of the present study the HIV negative group had a significantly higher BMI (28.90±9.11 (kg/m^2)) compared to the HIV positive on ART (24.25±6.05 kg/m^2) group. Extensively described in literature are the metabolic effects of HAART. The reported metabolic effects of the efavirenz/emtricitabine/tenofovir single dose formulation are not very consistent. Some studies have found no changes in body weight, body mass index (BMI) and whole body fat after a switch from a previous ART combination to the once-daily fixed dose efavirenz/emtricitabine/tenofovir combination (Deeks & Perry 2010). An earlier study conducted during 1997-2000 specifically looked at the body composition of 224 antiretroviral-naïve and antiretroviral-experienced human participants. The study found median increases in body weight and lean body mass (LBM) occurred after 16 weeks of therapy but significantly greater median increases in body weights was found in individuals who had achieved virological suppression. Participants who were antiretroviral naïve at baseline gained more weight and LBM after 16 weeks of treatment than participants who were previously on ART. (Shikuma *et al*. 2004)

The study by Akinboro *et al*. investigated the effects of first line HAART on body weight and demonstrated that HAART represses weight loss along with CD4 cell depletion. They examined the body weights of newly diagnosed HIV patients upon diagnosis and 48 weeks post HAART and found a significant increase in the mean body weight post HAART. It is important to note, however, that these

are results obtained within the context of HIV, that is, in a pathological condition. These results therefore support and explain the epidemiological findings of the present study, where HIV infection without ART inevitably induces weight loss. HIV without HAART has been found to decrease body weight by inducing oxidative stress which activates NF-κB leading to apoptosis of T-lymphocytes and increased HIV replication. HAART induces viral suppression and in this way also suppresses weight loss- hence the observed changes in weight once HAART is commenced. (Akinboro *et al*. 2013)

One could, therefore, reason that in the absence of HIV, as in the context of the animal part of this study, the previously observed effects of HAART on body weight would be nonexistent. Another possible reason for ART having no effect on body weight in the present study could be the duration of the treatment period. Perhaps with a treatment period that is considerably longer than 9 weeks one would observe significant differences in body weights.

Rooibos infusion having no significant effect on body weight and fluid consumption is not a novel finding (Katengua-Thamahane *et al*. 2014). When investigating the anti-inflammatory effects of rooibos infusion in rats, Baba *et al*. treated two groups for four weeks. One was the control group, that received water to drink and the other was given unfermented rooibos infusion. After four weeks of treatment no significant differences were found in the body weight gained, appetite or fluid consumption between the groups (Baba *et al*. 2009). Another study that tested the ability of an aqueous fermented rooibos extract to ameliorate lipopolysaccharide-induced liver injury also treated rats with the infusion for 4 weeks (Ajuwon *et al*. 2014). They found no significant differences in body weight gained and fluid consumption between the groups (Ajuwon *et al*. 2014).

The results of a few studies performed on cell cultures, animals and human studies suggest that dietary polyphenols have the potential to facilitate weight loss or inhibit weight gain (Meydani & Hasan 2010). These studies, however, investigated only specific polyphenols none of which being the ones that are predominantly found in rooibos infusion, that is, aspalathin and nothofagin (Meydani & Hasan 2010). Polyphenol rich foods remain a dietary recommendation especially for preserving an ideal body weight (Meydani & Hasan 2010).

5.1.2 Isolated Heart Perfusions: Functional Recovery and Infarct Size (IS)

The results of the global and regional ischemia protocols showed no significant differences between groups in all examined end points except IS and coronary flow (CF) at baseline in the global ischemia protocol. IS was significantly increased in the ART treated group compared to the controls and the ART+Rooibos treated group had a significantly increased CF at baseline compared to the ART group. It was observed that the AO and CO at recovery and the respective recovery percentages of the rooibos treated group had lower output values in the regional ischemia protocol but higher output values in the global ischemia protocol compared to the control, ART and combination groups. These were, however, not significant. The effects of rooibos infusion on cardiovascular function in a perfusion setting have not been well explored thus studies pertaining to this topic are very limited.

A 2011 publication by Pantsi *et al*. investigated the ability of rooibos to offer cardiac protection against ischemia reperfusion injury. In this study male Wistar rats received rooibos (fermented or unfermented) or water to drink and standard rat chow (SRC) for 7 weeks. The concentration of rooibos used was a 2% (w/v) infusion. The investigators documented mechanical function by measuring the AO before and after a 15 minute global ischemia insult. AO recovery was the only functional parameter measured. Groups that were treated with rooibos (fermented and unfermented) had significantly increased AO recoveries compared to controls. (Pantsi *et al*. 2011)

Another study investigated the anti-inflammatory effects of red palm oil and rooibos on rats. The rats were divided into different treatment groups and received either a 2% (w/v) rooibos formulation, red palm oil or the combination for 4 weeks and there after certain groups were given a lipopolysaccharide (LPS) injection 18 hours before sacrifice in order to induce acute systemic inflammation. The hearts were mounted and perfused on the Langendorff system for 15minutes. The parameters measured were, amongst others, coronary flow (CF), heart rate (HR), left ventricular developed pressure (LVDevP) and end diastolic left ventricular pressure (EDLVP). The authors observed no significant differences in any of these cardiac function parameters. (Katengua-Thamahane *et al*. 2014)

The differences between these two studies and the present study are worth mentioning. Although Katengua-Thamahane *et al*. found no significant differences at baseline in cardiac function parameters their perfusion protocol consisted solely of Langendorff perfusion and no ischemia was induced, furthermore, the treatment period was significantly shorter. The study by Pantsi *et al*. had a more similar perfusion protocol and treatment period but found significant differences in the AO. The lack

of significant differences in cardiac parameters between groups in the present study, when compared to that of Pantsi *et al*, could be due to a longer ischemic event in this study which could have caused increased post-ischemic contractile abnormalities and thus reduced contractile performance at recovery (Bolli & Marban 1999). This phenomenon is termed myocardial stunning (Bolli & Marban 1999).

IS was significantly increased in the ART group compared to the control whereas this significant increase in IS was not observed in the rooibos nor the Rooibos+ART treated groups when compared to controls. These results indicate that ART could cause decreased cell viability. The possible mechanism by which this single dose ART formulation could have resulted in increased IS is through its effects on ER stress. EFV has been demonstrated to induce ER stress and ER stress induced apoptosis and therefore decreased cell viability- **section 2.4.4 "Mechanism of ART Induced CVD"** (Bergin *et al*. 2015). The effect of ART on IS is a novel finding and suggests that the mechanism by which this FDC causes CVD is through ER stress induced apoptosis.

Treatment with rooibos showed no change in IS when compared to control. Rooibos infusion as a potent antioxidant should theoretically be able to increase an organism's antioxidant capacity. One of the known causes and, simultaneously, an effect of ER stress is oxidative stress. In ameliorating the anti-oxidant capacity, supplementation with rooibos should result in a decrease in oxidative stress and thus downstream alleviation of inflammation and endothelial dysfunction. This effect was, however, not observed.

Supplementation of ART treated rats with rooibos did not show the significantly increased IS above control that ART treatment alone displayed, in fact the IS in the ART + Rooibos group was no different to control values. While the difference in IS between ART (50.56±4.08 (%AAR)) and ART + Rooibos (46.75±4.72 (%AAR)) was not significant, perhaps having pro-longed the treatment period, increased the rooibos infusion concentration or even orally administered set quantities of the rooibos could have translated to significant effects on cell viability.

5.1.3 Vascular Reactivity Studies

5.1.3.1 Endothelial Response without PVAT

The aortic ring studies demonstrated that ART significantly reduced Ach induced relaxation compared to both the control and the Rooibos + ART treated groups. The downstream effects of ER stress are increased oxidative stress leading to endothelial dysfunction and reduced NO bioavailability (**Fig 2.5**). The significant reduction in relaxation in the ART treated group is a clear indication of impaired endothelial function which may be attributed to ER stress signaling or ROS. Supplementation with rooibos infusion effectively changed this outcome causing the combination treated group (Rooibos + ART) to relax similarly to the control group and significantly more than the ART group.

The body of laboratory studies that have investigated the direct links of ART on the endothelium are sparse. A 2006 publication report investigated effects of azidothymidine (AZT), an NRTI, and indinavir, a PI, on endothelial function. They treated male Sprague-Dawley rats with clinically relevant doses of AZT, indinavir or the combination for 30 days through their drinking water. Endothelial function was assessed through isometric force measurements in aortic rings. Results showed that endothelial relaxation was dramatically reduced in AZT and AZT + indinavir treated rats. Surprisingly, indinavir treatment alone showed no effects on endothelial function (Jiang *et al*. 2006). AZT, like emtricitabine and tenofovir, is a NRTI and thus have the same mechanism of action.

Scientific studies have begun to unravel the mechanisms by which flavonoids induce vasodilation apart from scavenging ROS, promoting proper endothelial functioning and thus indirectly promoting vasodilation. The proposed direct mechanisms are through opening of potassium (K^+) channels and inhibition of vascular calcium current (Scholz *et al*. 2010; Gilani *et al*. 2006). In vascular smooth muscle cells contraction is achieved when the cell membrane is depolarized through an influx of calcium (Ca^{2+}) stimulating the release of calcium from intracellular stores (Consigny 1991). Flavonoids are proposed to induce relaxation by activating the enzyme adenylate cyclase (AC) or guanylate cyclase (GC) resulting in the elevation of cyclic adenosine monophosphate (cAMP) or cyclic guanosine monophosphate (cGMP) respectively (Consigny 1991; Ledoux *et al*. 2006). Once these are activated either nucleotide can cause the opening of potassium channels leading to K^+ efflux, repolarization of the membrane and consequently relaxation (Consigny 1991). Gilani *et al*, through their work on rabbit jejunum, have shown that the vasodilatory effects of rooibos could be due to an interplay of K^+ channel activators and low level Ca^{2+} antagonistic activity (Gilani *et al*. 2006).

The rooibos treatment did not significantly affect relaxation compared to the other groups and interestingly this treatment, in the absence of ART, resulted in increased contraction compared to all the other treatment groups. There is evidence to suggest that polyphenol supplementation, in high or excessive dose, is detrimental to the health (Martin 2009). The plausible mechanism behind this deleterious effect lies in the ability of phenolic antioxidants to auto-oxidate and thus behave like pro-oxidants at high concentrations (Martin 2009). Villanueva and Kross in the review "*Antioxidant-induced stress*" present the hypothesis that an excessive amount of antioxidants can overwhelm the body's free radicals, disrupting the endogenous balance between antioxidants and reactive species thus giving rise to antioxidant-induced stress (Bouayed & Bohn 2010; Villanueva & Kross 2012). The review cites a number of studies that have found antioxidant supplementation to have harmful effects (Albanes *et al*. 1996; Challem 1997; Cheung *et al*. 2001; Villanueva & Kross 2012). The results of the present study propose that rooibos supplementation is beneficial under certain stress conditions whereas under normal physiological conditions it could lead to certain abnormalities.

The studies on antioxidant supplementation have focused solely on the ingestion of antioxidants in the form of supplements and not as a food source, unlike the present study. Ingesting antioxidants from natural sources, as in rooibos, allows for synergistic activity by the anti-oxidants, limiting their chance of auto-oxidating and consequently resulting in more beneficial effects (Villanueva & Kross 2012). However, the fact that an excessive amount of antioxidants not only has the potential of auto-oxidating but can also overwhelm the body's free radicals might explain the surprising results in the rooibos treated group. Although not given in the form of a supplement, the rooibos infusion was the only fluid this particular group had to drink. In the absence of a pathological condition, this may have overwhelmed the body's essential basal level of free radicals thus causing abnormalities or more specifically speaking, increased contractile response of the aorta.

No studies could be found that investigated possible toxicity of rooibos ingestion in the clinical or laboratory setting. Determining the quantities or upper limit that may be detrimental to a healthy person is, therefore, of utmost significance.

5.1.3.2 Endothelial Response with PVAT: Effect of PVAT

Whilst no considerable effect was found of the PVAT surrounding the aorta on ach induced relaxation, contraction graphs showed that PVAT considerably decreased contraction in all treatment groups. An investigation by Lee *et al*. (2014) sought to determine the mechanism by which PVAT regulates vascular tone by conducting isometric tension studies on isolated thoracic aortas from Wistar rats. The study used adult rats that had free access to water and standard rat chow (Lee *et al*. 2014). Unlike the present study their results revealed that PVAT significantly increases vasoconstriction and inhibits vasodilation (Lee *et al*. 2014). Through western blot analyses they were able to conclude that this resulted from decreased aortic NO production without any effect on the expression and activity of eNOS (Lee *et al*. 2014). These authors were able to prove that PVAT induced contractility of the vasculature results from PVAT causing over expression of caveolin-1 (CAV-1). CAV-1 is a negative regulator of eNOS and may have been a key role player in the PVAT elicited contractility observed in the present study.

Some studies have however, provided evidence that PVAT exerts anti-contractile effects by releasing an endothelium dependent relaxing factor (Gao *et al*. 2009). Also termed adipocyte-derived relaxing factor (ADRF), this factor has been proven to cause relaxation of the vasculature by activating K^+ channels found in vascular smooth muscle cells (VSMC) (Rajsheker *et al*. 2010). In contrast, other studies have found the pro-contractile effect of PVAT to be related to an obese phenotype either via oxidative stress (Ketonen *et al*. 2010) or obesity induced alterations in the PVAT proteome (Owen *et al*. 2013). Because the results of the present study showed decreased contractility for all groups with PVAT, it would appear that PVAT elicits anti-contractile effects irrespective of the phenotype. The discrepancies between the results of the present study and studies that have proven pro-contractile effects can be explained by PVAT'S ability to elicit both relaxation and contraction of the vasculature (Brown *et al*. 2014). Also, a key point to remember is that the rats in the present study were not obese. The exact conditions under which these effects are manifested have yet to be fully elucidated.

5.1.4 Biochemical Analyses

5.1.4.1 Lipids

In the animal study no significant differences were found in the phospholipid (PL) and triglyceride levels between groups but total cholesterol (TC) levels decreased significantly in the rooibos treated

group compared to the control group. The human study showed no significant differences in lipid profiles between the groups. A study conducted at the Cape Peninsula University of Technology (CPUT) tested the effects of different concentrations of rooibos extract on lipid profiles, antioxidant status, and liver histopathology in male Wistar rats that had been orally administered the extract. A 2, 4 and 6% aqueous rooibos extracts were administered orally for 7 weeks. The authors found no significant differences in lipid profiles between the groups and rooibos in all concentrations led to improved antioxidant capacity. (Ayeleso *et al*. 2013)

Several studies do, however, support the lipid and particularly cholesterol lowering ability of dietary polyphenols in health and disease state (Marnewick *et al*. 2011; Bahadoran *et al*. 2013; Zanotti *et al*. 2015). One such study entailed hyperlipemic and control male mice being given free access to either rooibos (10g/l), or water and food for 14 weeks (Beltrán-Debón *et al*. 2011). The treatment resulted in significant reductions in serum cholesterol and TRIG levels but only in the hyperlipemic mice and no effects were observed in the control mice, that is, the mice without metabolic disturbances (Beltrán-Debón *et al*. 2011). In a small epidemiological study rooibos consumption was found to decrease LDL-cholesterol and TRIG levels whilst increasing HDL- cholesterol (Marnewick *et al*. 2011). The study involved a cohort of 40 volunteers who were at risk for cardiovascular disease (Marnewick *et al*. 2011). The volunteers drank 6 cups of traditional fermented rooibos daily for 6 weeks. Thus the controversy surrounding the effects of dietary polyphenols on lipid metabolism studies have evidenced the cholesterol lowering ability of dietary polyphenols to result from inhibition of cholesterol synthesis (Zanotti *et al*. 2015).

The lack of significant findings in the present study comparative to the epidemiological study by Marnewick *et al* could be mainly due to the different natures of the two studies. The present study was an observational study and relied on the accurate reporting of rooibos consumption by the participants. The current study might have observed differences had a set amount of rooibos been given to a certain group as an intervention for a pre-determined period of time.

The results in the ART group were not as expected. In the present study ART had no significant effects on the lipid profiles of the rats whereas it is well established that ART causes dyslipidemia in the long term (Valle *et al*. 2013; Nduka *et al*. 2015). The absence of significant differences in the lipid profiles of the patients in the human study was also surprising. Studies have found patients on ART, particularly the broad spectrum of HAART including PI's, to have significantly higher total cholesterol levels and triglycerides compared with HIV positive ART naïve patients (Valle *et al*. 2013; Nduka *et al*. 2015). A

study conducted in America investigating certain effects of HIV disease in an ART-naïve cohort found advanced HIV disease to be associated with decreased HDL and LDL cholesterol. The study population consisted of 419 patients of which the average age was 38.2 years (El-Sadr *et al*. 2005).

A little closer to SA, a cross-sectional study conducted in Cameroon between November 2009 and January 2010 investigated the lipid profiles of 138 HIV positive ART-naïve patients and 138 HIV positive patients who were on first line NNRTI based ART regimens (Pefura Yone *et al*. 2011). The study found the NNRTI based ART regimen to be significantly and positively associated with raised TC and LDL-cholesterol (Pefura Yone *et al*. 2011). A more recent study has investigated the prevalence of dyslipidemia in HIV infected patients in South Africa. Once again the two groups were HIV infected ART naïve patients (406 patients) and HIV infected receiving either a NNRTI- based regimen or a PI-based regimen (551 patients) (Dave *et al*. 2016). The study found patients on ART to have significantly higher TRIG, TC, LDL-cholesterol and HDL- cholesterol, than the ART naïve patients, irrespective of the ART regimen (Dave *et al*. 2016). Interestingly, although these levels were significantly increased compared to the ART naïve patients, levels of lipid abnormalities requiring evaluation and treatment were uncommon (Dave *et al*. 2016). Most studies have found PI's to be associated with remarkable dyslipidemia and NNRTI's to improve plasma lipid levels (Fisher *et al*. 2006). Taking this fact into account, the fact that the present study is one of the first to investigate not only this specific FDC but to further do so on a SA cohort and the limited number of participants recruited for the study sheds some understanding as to the lack of similarity to findings of other studies. Furthermore, the absence of a significant difference in lipids between ART naïve and ART compliant individuals may be reflective of a possible positive aspect of the current FDC.

Whereas the studies that have found significant results investigated population sizes with an n-value of no less than 400. The present study had a population size of 105 participants which was further sub-divided into HIV negative, n=23; HIV positive no ART, n=14 and HIV positive on ART, n=68. The sample size of the present study was, thus, considerably smaller. Perhaps with a longer period of ART the present study may have found significant changes in the lipid profiles of the ART group in the animal study.

5.1.4.2 TBARS

Surprisingly in both the animal and human study, no significant differences were evidenced in the TBARS levels between the groups. As TBARS level is a marker of lipid peroxidation and ROS indicator it can thus be used as an indirect marker of antioxidant capacity. Ideally we expected to observe, in the animal study, a significant increase in TBARS levels in the ART group compared to all the other groups and reduced TBARS levels in the rooibos and Rooibos + ART treated groups. Scientific reports that have investigated ART induced toxicity have found different ART combinations to significantly reduce antioxidants and antioxidant capacity while causing an increase in lipid oxidation and oxidative damage (Valle *et al*. 2013).

The novelty of this study and furthermore the novelty of these results, although not significant, provides insight on the effects of the efavirenz/emtricitabine/tenofovir FDC. We can deduce from these results that this specific FDC possibly does not cause ROS as extensively as other previously investigated combinations or that it would need a longer period than 9 weeks to cause observable changes in lipid peroxidation or antioxidant levels.

A number of studies have demonstrated an improved anti-oxidant capacity in rats supplemented with fermented rooibos extracts for up to 10 weeks (Marnewick *et al*. 2003; Kucharská *et al*. 2004; Ajuwon *et al*. 2013). Furthermore, several studies have confirmed the antioxidant and lipid peroxidation inhibiting properties of rooibos (Standley *et al*. 2001; Krafczyk *et al*. 2009; Ajuwon *et al*. 2015). The proposed mechanism by which rooibos polyphenols improve oxidative status is through direct scavenging of ROS and free radicals (Steenkamp *et al*. 2004; Ajuwon *et al*. 2015). The above mentioned studies that have examined the antioxidant and lipid peroxidation inhibiting properties of rooibos, have used more than one technique to determine the level of lipid peroxidation and or anti-oxidant capacity. Perhaps therein lies a possible short fall in the present study, for both the animal and the human aspect, which resulted from monetary constraints. Significant differences in lipid peroxidation products and anti-oxidant capacity between groups may have been observed had we complemented the TBARS test with a test for conjugated dienes or total antioxidant capacity (TAC).

5.2 Effects of rooibos consumption on the cardiovascular profiles of patients on ART.

Of the 105 study participants there were 23 HIV negative, 14 HIV positive who were not on ART and 68 HIV positive who were on ART. Females accounted for 73% (n=77) of the population. 65% percent of the population were smokers and 51.9% indicated that they drink rooibos tea. The average age of the total population was 39.3 years.

Multiple comparisons test on the whole population revealed significant differences in blood pressure (BP) and no differences in the CRP levels between groups. The HIV negative patients were found to have significantly higher BP (DP and SP) than the HIV positive no ART and the HIV positive on ART. Literature on the effects of HIV and ART on BP is somewhat controversial. A relatively small British study that investigated the effect of ART on BP in 95 patients who were previously ART naïve found the BP levels to increase significantly after 48 weeks of ART (Palacios *et al*. 2006).

A larger cross-sectional survey conducted on a population of 612 adults aimed to determine the associations between BP, HIV status and ART with and without NNRTI's (Wilson *et al*. 2009). The study found NNRTI's to be associated with increased BP (Wilson *et al*. 2009). These studies were, however, conducted in Britain where the population is considerably different to South Africa's mixed ancestry and lower socio economic population (Western Cape Government 2015). Data on the effects of HIV and ART on SBP in SA can be found in at least one study conducted in Kwazulu-Natal (Feigl *et al*. 2016). The study evaluated the SBP of 505 individuals in the groups HIV negative, seroconverters, HIV positive not on ART and HIV positive on ART (Feigl *et al*. 2016). SBP readings were taken in 2003 and in 2010 and it was found that SBP dropped significantly in the HIV positive no ART group and in the HIV positive+ ART group (Feigl *et al*. 2016). These decreases were further investigated relative to the HIV negative group and were found to still be significant (Feigl *et al*. 2016). Similar results were obtained in an earlier Sub-Saharan Africa study of the association of HIV and ART with cardiometabolic traits where HIV was found to be associated with decreased BP (Dillon *et al*. 2013).

Regression analysis on consumption of rooibos showed no significant differences in BP, lipid profiles, CRP and TBARS between patients who indicated to be rooibos drinkers and those who didn't. In scientific investigations tea polyphenols, and specifically rooibos has been established to lower BP via inhibition of the angiotensin converting enzyme (ACE) (Persson *et al*. 2006; Persson 2012). These investigations were conducted in vitro on endothelial cells from human umbilical veins (HUVEC's) and

on incubated serum of healthy volunteers (Persson *et al*. 2006; Persson 2012). The present study differs in that it examined possible effects of rooibos drinking on BP in vivo. Moreover, participants possibly were not consistent in drinking the tea daily and amounts consumed might not have been enough to translate to observable changes in BP and other parameters.

CRP is a well-established inflammatory marker and, therefore, increases in the presence of inflammation (Teixeira *et al*. 2014). It is also used as a biomarker for ED (Teixeira *et al*. 2014). According to literature tea polyphenols are able to directly inhibit inflammation, **Fig 2.5** section **2.6.5** (Gonzalez De Mejia *et al*. 2013). The results for the second aim of the present study are most important and significant in the study of rooibos as a nutraceutical product and the safety of the current ART regimen. The current ART regimen showed no significant inflammatory effects. This may be why rooibos consumption did not result in any observed ameliorating effects. Results of the animal aspect of the study have indicated the possible beneficial effects of rooibos to be most effectively harnessed under stress conditions.

The variances and lack of significant results in the investigation of the second aim are possibly due to an interplay of the following factors:

1) A small sample size which was further subdivided into groups (no Rooibos no ART, Rooibos no ART and Rooibos on ART).
2) Limited biochemical tests due to monetary and logistical constraints. Perhaps with the investigation of TAC or the use of a supplementary antioxidant tests, e.g conjugated dienes, significant differences might have been observed.
3) Observational study which, for rooibos drinking, relied on accurate reporting by the participants. Maybe if the rooibos had been given as an intervention, in set quantities and for a certain period of time significant changes may have resulted.
4) The specific FDC investigated may not, until a certain period, have deleterious effects on the cardiovascular system.

Chapter 6 – Summary and Final Conclusion

6.1 Summary

With the number of deaths attributable to AIDS clearly rising in the long term HIV positive population and the 2012 roll out of the new first line ART regimen in the form of a FDC of efavirenz/emtricitabine/tenofovir (Clinicians Society 2013; Statistics South Africa 2015), there is a clear gap in the literature which investigates the effects of this FDC in SA and possible co-therapies. With long term ART induced toxicities being linked to chronic inflammation (Currier *et al*. 2008; Schulenberg & Le Roux 2008; Aounallah *et al*. 2016) the present study proposed the use of *Aspalathus linearis/*rooibos, a plant that is indigenous to SA soil and a potent source of antioxidants that have led to its use as a nutraceutical product in many scientific studies (Ajuwon *et al*. 2015). This study thus aimed to elucidate the cardiovascular effects of rooibos infusion supplementation during ART, on male Wistar rats and the effects of rooibos tea consumption on the cardiovascular profiles of patients on ART.

To achieve these aims the study included a laboratory and an epidemiological investigation. In the laboratory study we treated 100 male Wistar rats with the FDC ART (600 mg EFV, 200 mg FTC, and 300 mg TDF) according to the weekly average weights of the rats and supplied them with a weekly 2% (w/v) rooibos infusion as a supplement. Treatment lasted 9 weeks and thereafter working heart perfusions and isometric tension studies were conducted in order to elucidate the cardiovascular and endothelial effects of the drug and rooibos infusion. In the epidemiological study the effects of ART and rooibos consumption on cardiovascular profile were investigated in 105 participants of whom 23 were HIV negative, 14 HIV positive ART naïve and 68 HIV positive and on ART. Blood samples were collected by a phlebotomist, BP was measured and anthropometric data recorded. Biochemical analyses of serum was conducted and TBARS test done in both studies to determine lipid peroxidation levels.

No significant differences were observed in the body weights and fluid consumption of the rats between the groups. In the human study the the HIV negative group was found to have a significantly higher BMI (28.90±9.11 (kg/m^2)) compared to the HIV positive on ART (24.25±6.05 (kg/m^2)) group. In the perfusion data IS was significantly increased in the ART treated group compared to the controls and the Rooibos+ART treated group had a significantly increased CF at baseline compared to the ART

group. In the vascular reactivity/ isometric tension studies ART was found to significantly reduce Ach induced relaxation compared to both the control and the Rooibos + ART treated groups. Rooibos treatment without ART significantly increased contraction when compared to all other groups. PVAT reduced contraction in all treatment groups and did not significantly affect relaxation. TC levels were found to decrease significantly in the rooibos treated group whilst PL and TRIG and TBARS levels were similar between groups. The human study showed no significant differences in lipid profiles, TBARS and CRP levels between the groups, however, BP in HIV negative patients was found to be significantly higher than in the HIV positive no ART and the HIV positive on ART.

The significantly higher BMI in the HIV negative group compared to the HIV positive on ART is supported in literature by HIV's documented induction of weight loss through viral replication (Akinboro *et al*. 2013). The ART regimen used clearly demonstrated the potential to induce stress over long term through increased infarct size and reduced Ach induced relaxation in the animal study. This observation is supported by literature to be due to increased ROS induced stress (Bergin *et al*. 2015). The Rooibos+ ART treated group did not show the same deleterious effects as ART did on its own. Rooibos as a form of supplement requires further investigation so that medical science may be able to fully harness the health benefits of this natural plant. BP results in the current study, although not consistent with some international data, data from Sub-Saharan Africa supports a decreased BP in the HIV positive population when compared to the HIV negative population (Palacios *et al*. 2006; Wilson *et al*. 2009; Feigl *et al*. 2016). The current first line ART showed no deleterious effects in the human study and further long term studies where rooibos tea is used as an intervention are required.

6.2 Final Conclusion

When considering the research question posed in the introductory section of the study: "What are the cardiovascular effects of *Aspalathus linearis*/ rooibos infusion supplementation during ART, on male Wistar rats and the effects of rooibos tea consumption on the cardiovascular profiles of patients on ART?" the present study has successfully met the aims and thus also answered the research question. This study was able to elucidate effects of the FDC of efavirenz/emtricitabine/tenofovir not only in the laboratory but clinically as well.

Given the laboratory results it may be concluded that the current ART regimen can potentially cause deleterious health effects in the long term- mechanism still illusive. Rooibos may be used as a possible supplement but further studies are required to fully unravel the conditions in which rooibos'

nutraceutical properties can be completely harnessed. Because no result is a bad result, results of the epidemiological study showed no detrimental effects of the FDC comprising of 1 NNRTI and 2 NRTI's and thus may be the safest or otherwise least detrimental combination to use as a first line therapy at present.

Limitations of the study

- The biochemical tests conducted to determine the antioxidant capacity of participants and rats. This was limiting because conducting different antioxidant tests such as a test for total antioxidant capacity (TAC) or conjugated diene's may have better shed a light on the ROS induced effects of the ART used or antioxidant effects of rooibos consumption.
- The epidemiological study being an observational study was limited to reliance on the accurate reporting of rooibos consumption by the participants.
- A relatively small sample size for an epidemiology study.

Recommendations for future research

For future studies it would be useful to use more than one biochemical test to determine antioxidant capacity. Having an increased sample size and giving a specific amount of rooibos tea for participants to drink over a set period may also prove to be most beneficial. More significant changes may be observed if participants are followed up on over a course of perhaps 10 years, and in the laboratory study, a longer treatment period for the rats.

Further studies on rooibos in different conditions of physiological stress and health need to be conducted. These studies should include analyses of signaling pathways in order to fully elucidate the mechanisms of action of rooibos and the conditions under which its synergistic effects are most pronounced.

APPENDIX A: Odimune Dose Calculations

Contents of one FDC tablet:

Efavirenz (EFV) - 600 mg

Emtricitabine (FTC) - 200 mg

Tenofovir (TDF) - 300 mg

Dose for an average 70 kg mature human

Let EFV 600 mg= x

Therefore human dose (x_H) in mg/kg/day = x/70 kg

x_H (EFV)= 600 mg/70kg= 8.6mg/ kg/ day

x_H (FTC)= 2.9 mg/kg/day

x_H (TDF)= 4.3 mg/kg/day

Human to rat conversion

Rat dose (x_{R1}) = x_H *6

x_{R1} (EFV)= 8.6*6 = 51.6 mg/kg/day

x_{R1} (FTC)= 17.4 mg/kg/day

x_{R1} (TDF)= 25.8 mg/kg/day

For a rat weighing 250 g:

x_{R2} (EFV): 12.9 mg (x_{R1} * 0.250) (This is adjusted according to the weekly weights of the rats)

x_{R2} (FTC): 4.35 mg

x_{R2} (TDF): 6.45 mg

To determine weight of the actual tablet allocated per rat:

1. Weighing the tablet

Take human dose (x) / specific dose of rat (x_{R2})

i.e for EFV: 600 mg / 12.9 mg = 46.51 = x_{R3}

Crush tablet with mortar and pestle until completely fine.

Therefore amount of crushed tablet to be dissolved in 1ml water for EFV

= weight of crushed tablet / x_{R3}

2. Preparing the drug and water mixture

- For a 4 day preparation for an entire cage, we first determine the average weight of the rats per cage.
- If average weight of cage is 352 g, thus 0.352 kg:
- Weight of tablet after crushing = 1.6 g (1600 mg)
- Active ingredients (AI) = 600 +200 +300 = 1100mg
- Actual percentage of AI = 1100 / 1600 = 67.75%
- Average weight of rats in cage = 352 g (0.352 kg)
- Dose per rat = EFV: 51.6 mg * 0.352 mg = 18.163 mg

  FTC: 17.4 mg * 0.352 mg = 6.125 mg

  TDF: 25.8 mg * 0.352 mg = 9.082 mg

  EFV: 600 / 18.16 = (33 * 68 %) / 100 = y

  FTC: 200/ 6.125 = (33* 68 %) / 100 = y

  TDF: 300/ 9.082 = (33 * 68 %) / 100 = y

- Final dose of tablet per rat: weight of tablet (x) / y

EFV = 1600 mg/ 22.78 = 70. 22 mg

- Powder of tablet in 1ml water = 0.07 g
- For a 4 day preparation with 5 rats in cage = 4 * 5= 20 ml water

Therefore final dose = 0.070 g * 20 = 1.40 g/ 20 ml water

APPENDIX B: LC- MS of Rooibos

LC-MS conducted by the Central Analytical Facility (CAF) of Stellenbosch

Phenolics method

A Waters Synapt G2 quadrupole time-of-flight mass spectrometer was used for LC-MS analysis. It was fitted with a Waters Ultra pressure liquid chromatograph and photo diode array detection. Separation was achieved on a Waters HSS T3, 2.1x150 mm column with 1.7 um particles. A gradient was applied using 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B). The gradient started at 100% solvent A for 1 minute and changed to 28 % B over 22 minutes in a linear way. It then went to 40% B over 50 seconds and a wash step of 1.5 minutes at 100% B, followed by re-equilibration to initial conditions for 4 minutes. The flow rate was 0.3 ml/min and the column was kept at 55 ºC. The injection volume was 1 µL.

Data was acquired in MS^E mode which consisted of a low collision energy scan (6V) from *m/z* 150 to 1500 and a high collision energy scan from *m/z* 40 to 1500. The high collision energy scan was done using a collision energy ramp of 30-60V. The photo diode array detector was set to scan from 220-500 nm.

The mass spectrometer was optimized for best sensitivity, a cone voltage of 15 V, desolvation gas was nitrogen at 650 L/hr and desolvation temperature 275 ºC. The instrument was operated with an electrospray ionization probe in the negative mode. Sodium formate was used for calibration and leucine encephalin was infused in the background as lock mass for accurate mass determinations.

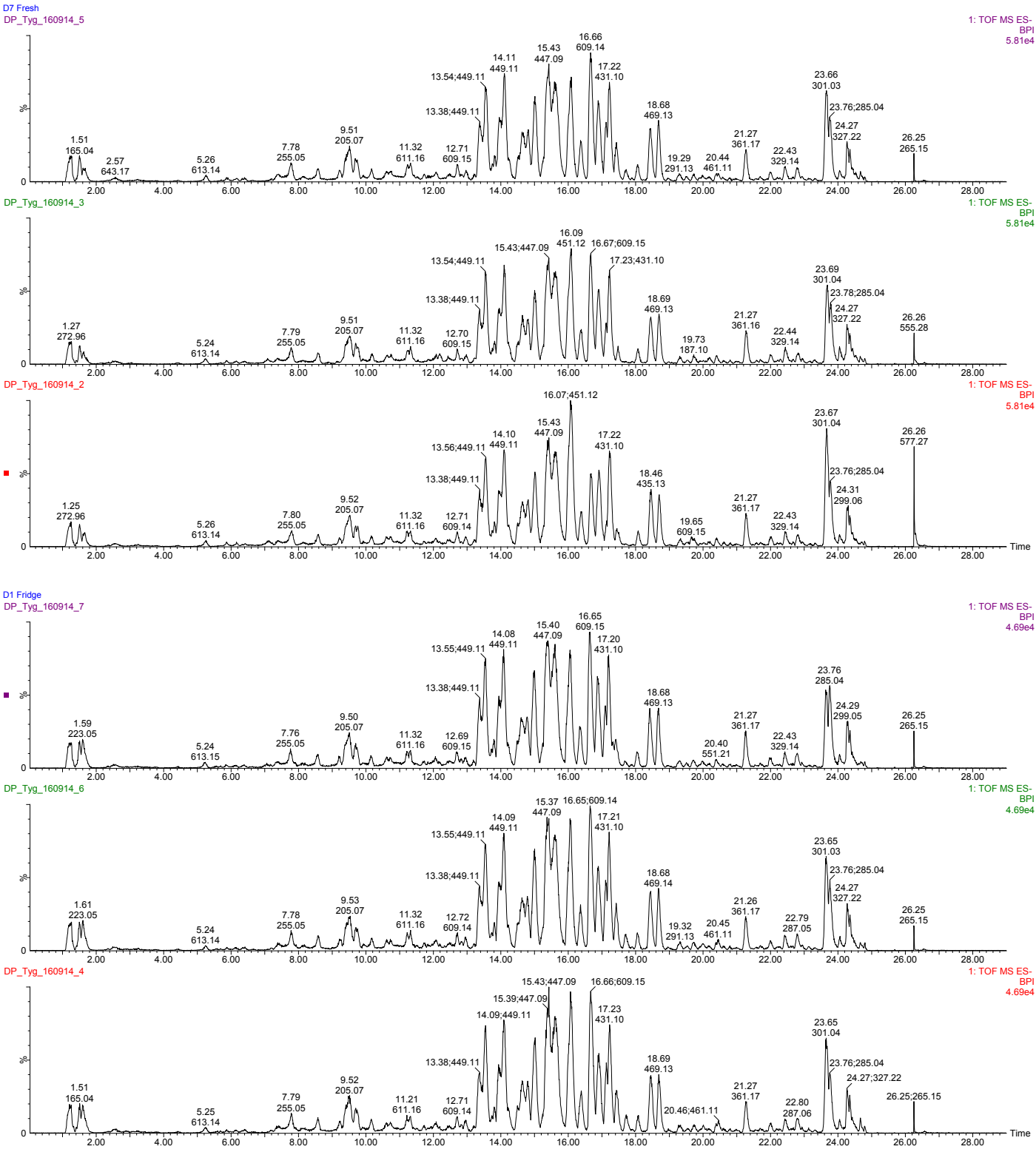
Sample names:

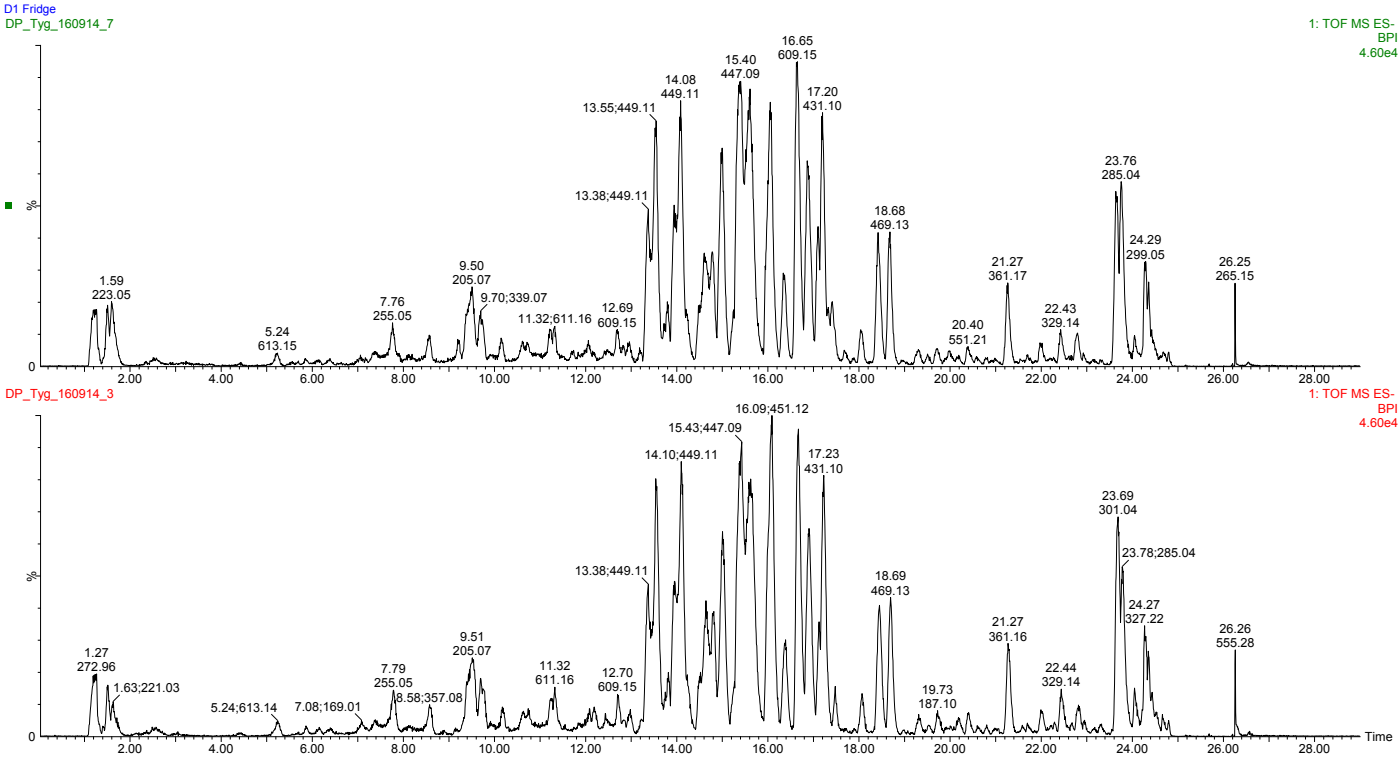
DP_Tyg_160914_2	D7 Fresh
DP_Tyg_160914_3	D7 Fridge
DP_Tyg_160914_4	D1 Fresh

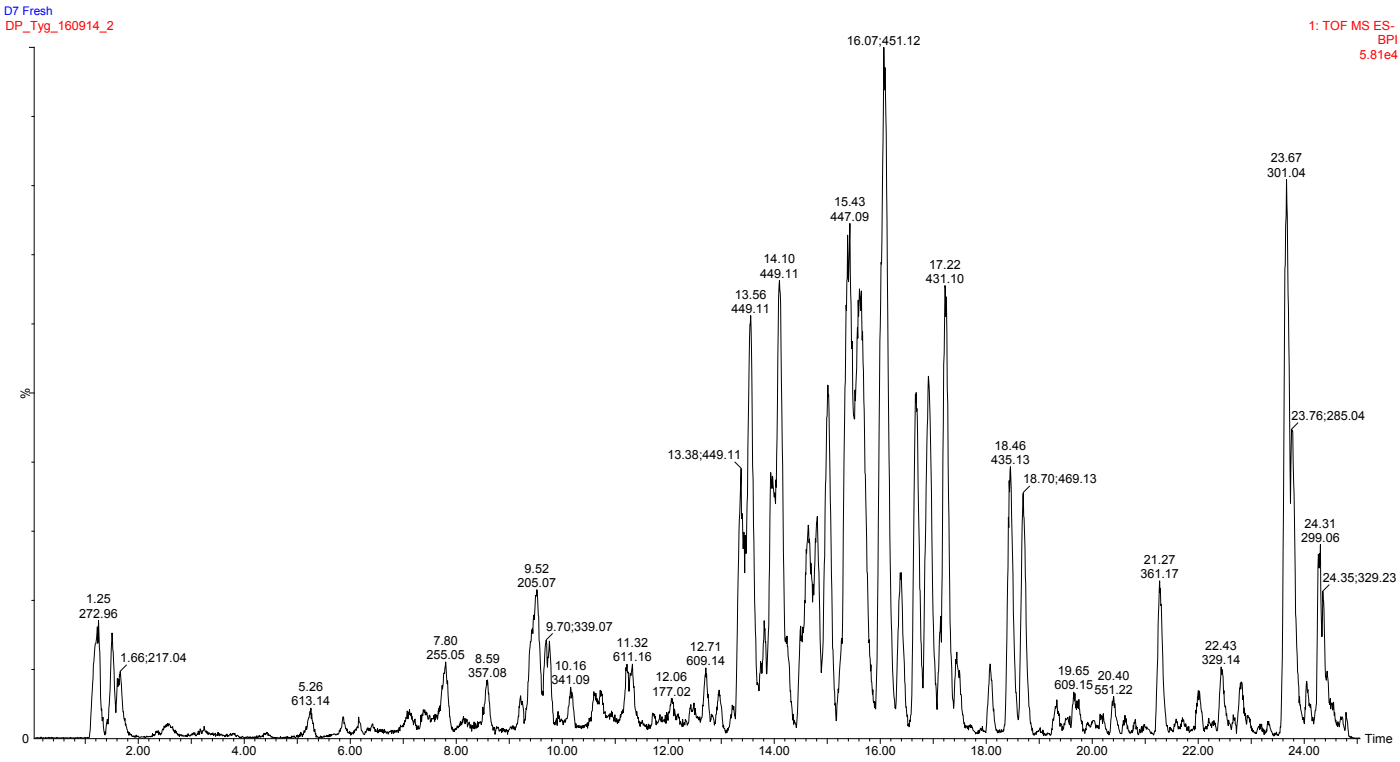
DP_Tyg_160914_5 D7 Frozen

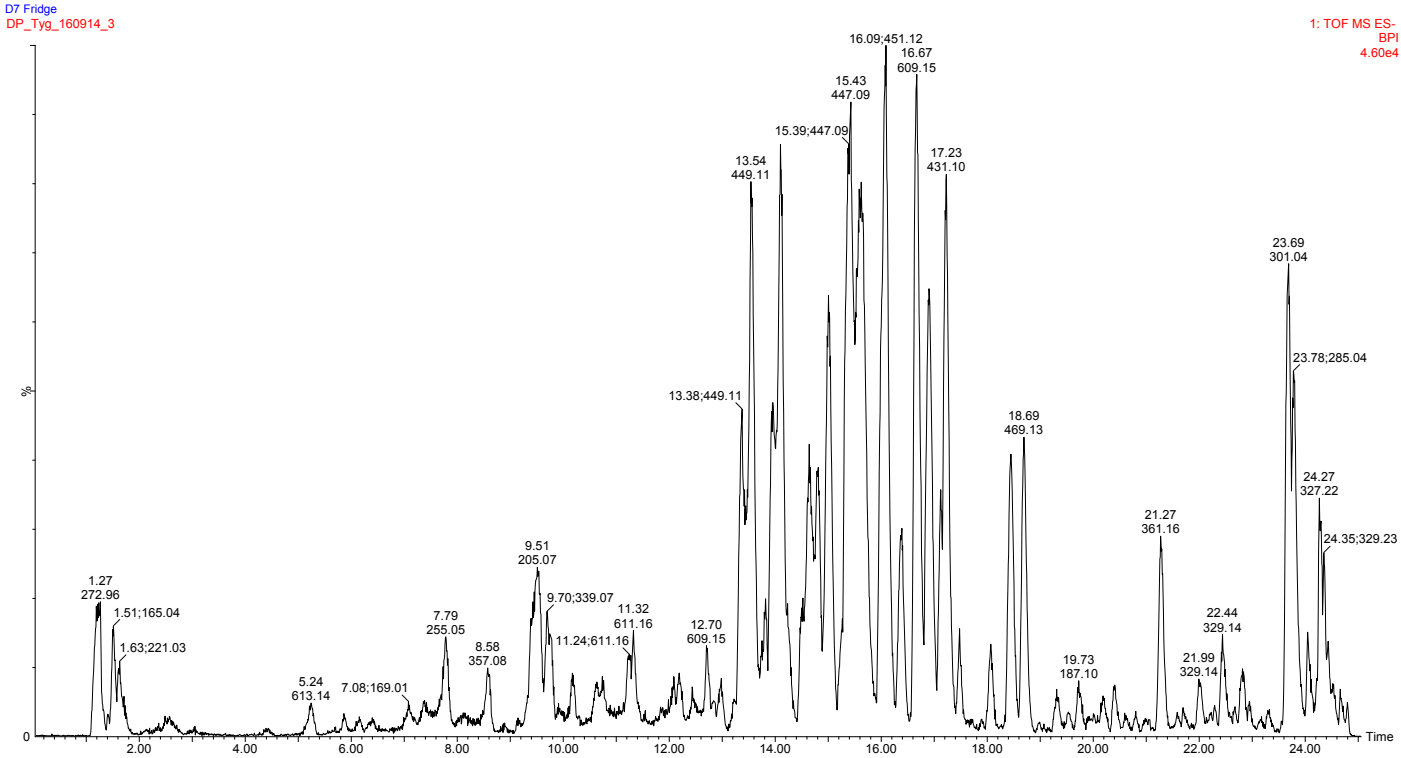
DP_Tyg_160914_6 D1 Frozen

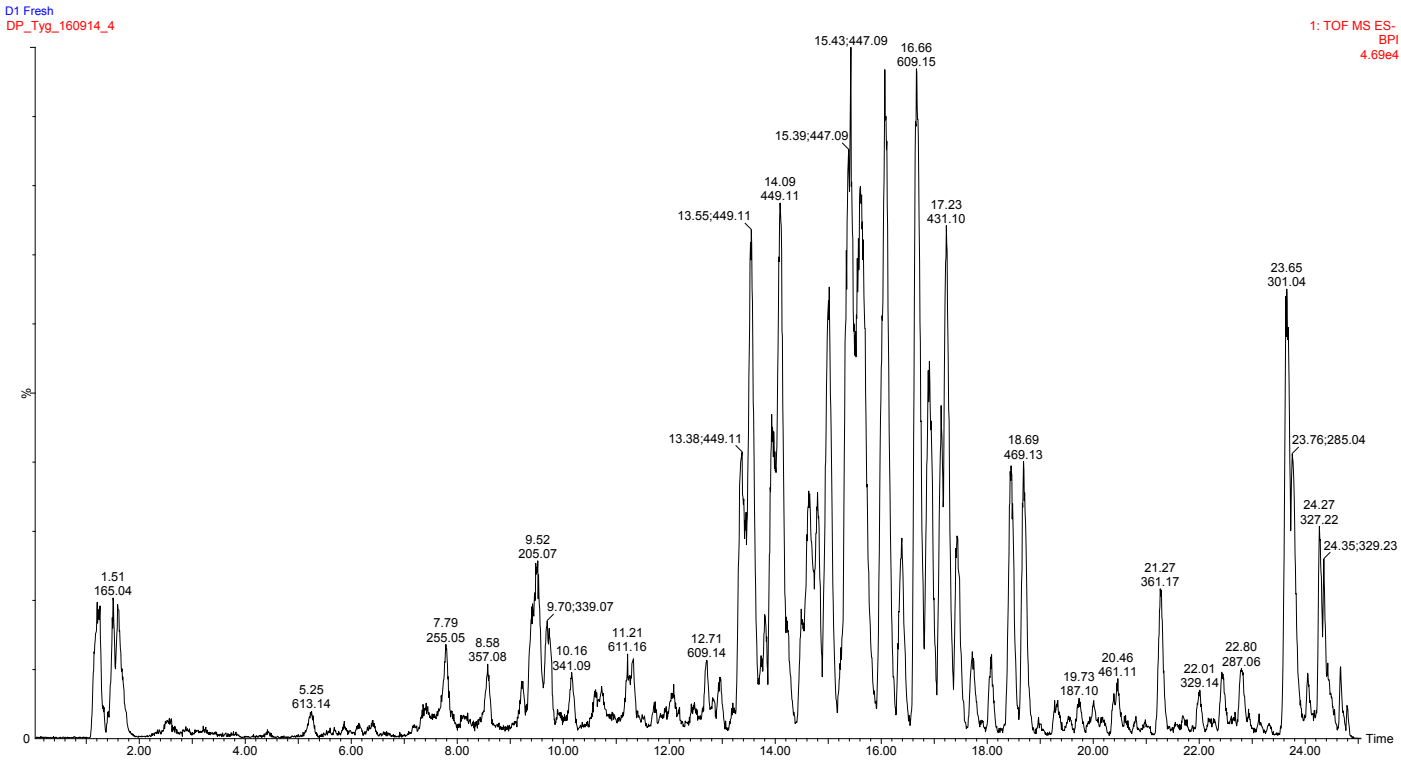
DP_Tyg_160914_7 D1 Fridge

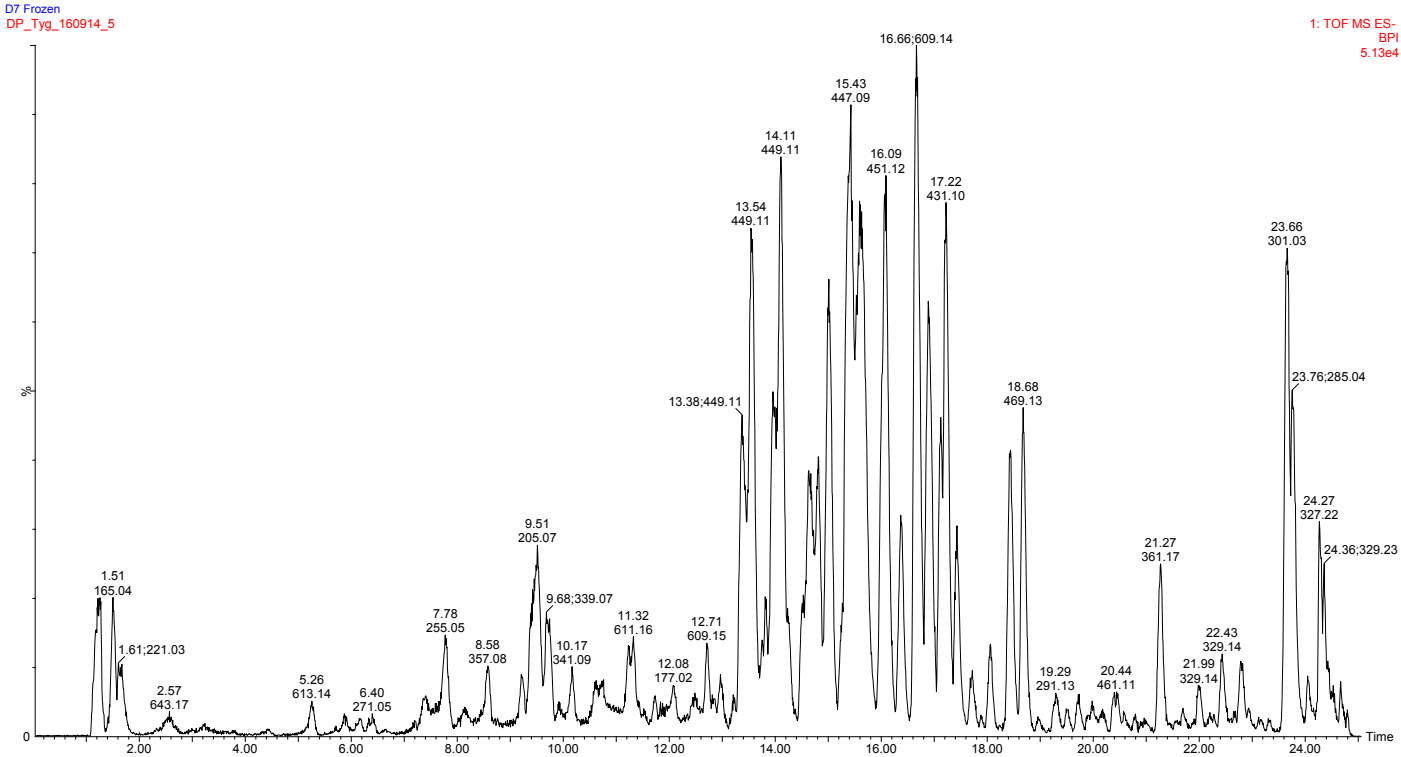


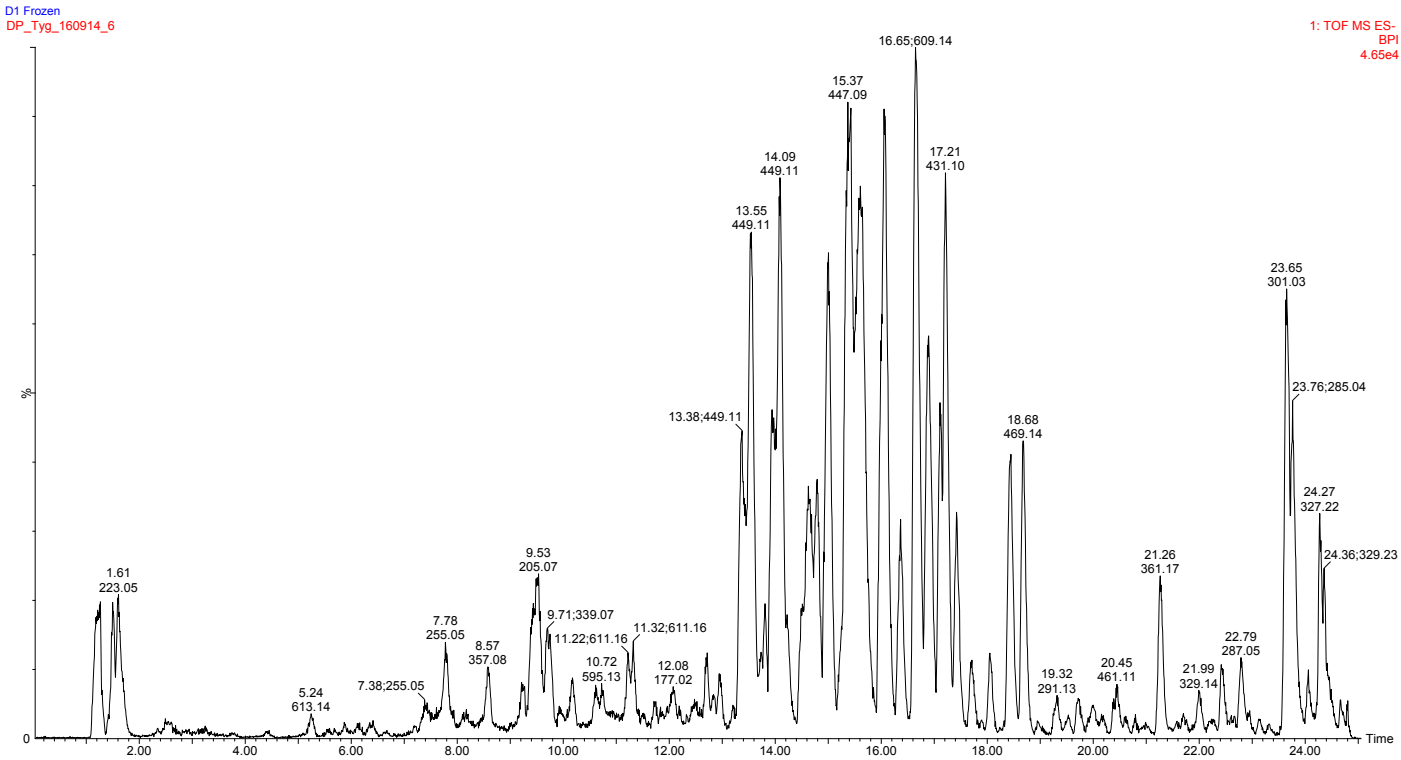


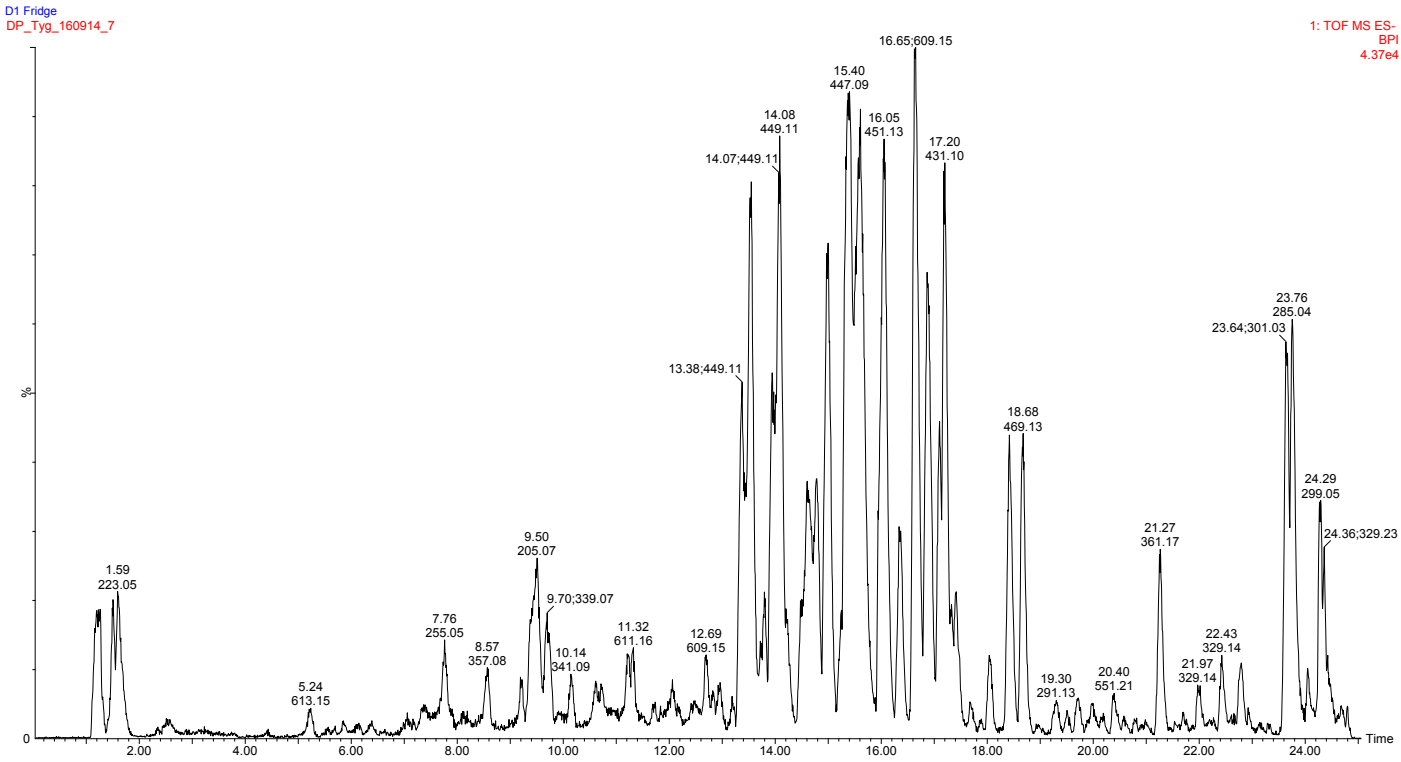












Helpful hints in interpreting MS results from Masslynx data

1. The ionization mode is indicated in the top right hand corner of spectra:

ESMS and LCMS lab (more suitable for non-volatile compounds):

ES+ = Electrospray positive: Typically a M+H or M+Na ion is observed

ES-= Electrospray negative: Only used for molecules that can be negatively charged like phenols and carboxylic acids, typically a M-H or M+Cl is observed

APCI = Atmospheric pressure chemical ionization: A softer technique for non-polar molecules – rarely used

GCMS (more suitable for smaller and volatile compounds):

EI = Electron impact, this is a harsher ionization technique. A fragmentation pattern and M^+ is sometimes observed

2. The retention time is given on the top left hand corner of a spectrum after the file name in brackets for GCMS and LCMS results.

3. Only the TOF instruments (GCT and Synapt) are capable of High resolution, samples should be pure and free of salts and buffers. Most journals require that the ppm deviation from the theoretical mass should be less than 5 ppm or in the case of very small molecules it should differ by less than 2 mDa. The iFit Confidence % gives an indication of how well the isotope pattern correlates to the theoretical pattern.

4. Advanced MS analysis techniques (MSMS, ion mobility and high resolution LCMS and LCMSMS analysis) require day bookings.

5. Mass spectrometry is not a universal technique, especially the atmospheric ionization techniques (ESI and APCI) give no representation of the purity of compounds: different compounds have different ionization potentials under different conditions.

6. ASAP probe: This is a solids technique that uses APCI to ionize molecules from the tip of a melting point tube. This technique is labour intensive and time consuming and will only be used as last resort for insoluble and unstable molecules. The cost is the same as for LCMS analysis.

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1. Ownership of the data and/or samples provided by the client shall remain so vested.
2. All data and/or samples provided by the Client will be treated as confidential.
3. The Analysis Report prepared by SU shall become the property of the Client after payment.
4. Although the greatest care is taken by SU during analysis, SU accepts no responsibility for the loss of any work, samples or data provided by the Client.
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6. (Please advise the laboratory staff within one week after results have been received if any additional analysis or processing of data is required. It remains the responsibility of our Clients to make proper backups of data.)

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