Chronic stress-associated accelerated ageing: inflammation and oxidative stress treatment

by

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DECLARATION

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This dissertation includes two original papers published in peer-reviewed journals. The development and writing of the papers were the principal responsibility of myself.

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Abstract

In recent years, the incidence of non-communicable diseases (NCD) normally associated with advanced age has begun presenting in younger populations. This has resulted in a growing burden on global healthcare systems and decreasing quality of life in individuals. Cardiovascular diseases, cancers, chronic respiratory diseases, chronic inflammatory diseases and diabetes are some of the many NCD's and all these have two maladaptive characteristics in common, namely chronic low-grade inflammation and increased oxidative stress. The aim of this research was to identify a threshold prior to maladaptation in both redox and inflammatory status which can be targeted with preventative medicine strategies; in this way, we may identify suitable models which are sensitive enough to identify this threshold as well as show small effect sizes so that they can be used for drug screening of preventative medicine treatments.

In order to elucidate this threshold, two rodent models were employed to simulate a pre-onset and an early onset state. The pre-onset state was simulated by chronic D-galactose injections to mimic cumulative oxidative stress as is associated with chronological ageing. The early onset state was simulated with a collagen induced rheumatoid arthritis (RA) model. A grape seed polyphenol supplementation was employed to assess the sensitivity of the models. Comprehensive end-point analysis of the oxidative and inflammatory state of various compartments were performed. Analysis of parameters associated with ageing were also included as measure of relative ageing status in models.

The results of both studies indicated that the threshold or point of onset of accelerated ageing was indeed identified. In the D-galactose model, a novel finding was the compromised antioxidant capacity in plasma, even in the absence of experimentally elevated oxidative damage, observed as decreases in plasma FRAP. However, oxidative damage was observed in tissue specific investigations, such a morphological changes in the mesenteric lymph nodes. In the RA model, decreases in antioxidant capacity was noted along with oxidative damage in plasma, but not in all tissue types investigated - particularly the brain. This novel finding of pre-damage oxidative changes in the brain was indicated by decreases in MDA and increases in FRAP. This combined with a switch to a pro-inflammatory state within the circulation, confirms the early disease state within the RA model.

This investigation has elucidated the importance of monitoring the oxidative state within multiple compartments to identify the threshold at which disturbances to homeostasis turns into maladaptation and FRAP may be the most sensitive parameter to display this. The effect changes noted after supplementation with an antioxidant treatment also enhanced our knowledge of which parameters and tissue are susceptible to oxidative and inflammatory modulation to prevent maladaptations which may result in pathology.

Opsomming

Die insidensie van nie-aanmeldbare siektes (NAS) wat normaalweg met gevorderde ouderdom verbind word, het meer onlangs ook begin presenter in jonger populasies. Hierdie tendens veroorsaak ‘n groeiende las op die gesondheidstelsels en verlaag lewenskwaliteit. Kardiovaskulêre siekte, kanker, kroniese respiratoriese siekte en diabetes is van die NAS en het almal twee kenmerke van wanaanpassing in gemeen, naamlik laegraad inflammasie en verhoogde oksidatiewe stres. Die doel van hierdie navorsing was om die drumpel voor wanaanpassing te identifiseer, waar beide redoks en inflammatoriese status geteiken kan word met voorkomende medisyne strategieë; sodoende kan ons geskikte modelle identifiseer wat sensitief genoeg is om hierdie drumpel uit te wys en ook klein effekte op te tel sodat hierdie modelle gebruik kan word vir middeltoetsing van voorkomende medisynes.

Ten einde hierdie drumpel te belig, is twee knaagdiermodelle gebruik om wanaanpassingsgebeure voor siekte, asook vroeg in die proses, te simuleer. Die voor-siekte toestand is gesimuleer deur kroniese D-galaktose inspuitings om kumulatiewe oksidatiewe stress – en dus versnelde veroudering – te veroorsaak. Die vroeë siektetoestand is gesimuleer in ‘n kollageen-geïnduseerde model van rumatoïede artritis (RA). ‘n Druifpit-polifenool supplement is gebruik om die sensitiwiteit die model te toets. Omvattende eindpunt analise van oksidatiewe en inflammatoriese status in verskeie kompartemente is uitgevoer. ‘n Analise van parameters wat met veroudering verband hou, is ook ingesluit om die relatiewe toestand van veroudering te belig.

Resultate dui aan dat die drumpel waar versnelde veroudering begin, suksesvol aangedui is. ‘n Nuwe bevinding in die D-galaktose model, is die verswakte anti-oksidant kapasiteit selfs in die afwesigheid van eksperimentele verhoogde oksidatiewe skade, soos aangedui deur die verlaagde plasma FRAP. Oksidatiewe skade is egter wel opgemerk in weefsel-spesifieke ondersoeke, bv. die morfologiese veranderinge in die mesenteriese limfnodes. In die artritis model is verlaagde anti-oksidant kapasiteit saam met oksidatiewe skade in plasma opgemerk, maar nie in alle weefsels nie – veral die brein. Hierdie nuwe bevinding van redoks veranderinge voordat skade opgemerk word in die brein, word ondersteun deur verlaagde MDA en verhoodge FRAP vlakke, en saam met die skuif na ‘n pro-inflammatoriese status in sirkulasie, bevestig huidige data die vroeg-siekte status in die RA model.

Hierdie ondersoeke illustreer die belang van monitering van die oksidatiewe stress status in verskeie kompartemente, om die drumpel waar versteurings in homeostase na wanaanpassing verander, aan te dui. FRAP blyk die mees sensitiewe merker in hierdie verband te wees. Effekte van die supplement dra by tot ons kennis in terme van weefsel-verskille in terme van hul kwesbaarheid vir oksidatiewe en inflammatoriese modulasie, en waar terapie dus geteiken moet word om wanaanpassing te keer wat tot patologie kan lei.

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List of abbreviations

4-HNE - 4-hydroxy-2-nonenal

8-iso-PGF2α-8-iso-prostaglandin F2α

·OH - hydroxyl radical

·HO2 - hydroperoxyl

ABTS - 2,2'-azino-di-3-ethylbenzthialozine sulphonate

AChE - acetylcholine esterase

ADP- adenosine diphosphate

AMPK- adenosine monophosphate-activated protein kinase

ARC - antioxidant reducing capacity

CAT- catalase

CD - conjugated dienes

CIA - collagen-induced arthritis

CRP- C-reactive protein

COPD - chronic obstructive pulmonary disease

COX-2 - cyclooxygenase-2

Cu - copper

CVD - cardiovascular disease

DCFDA - 5-(and -6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate assay

DCF - 2',7'-dichlorofluorescein

DHE – dihydroethidium

DMPD- N,N-dimethyl-p-phen- ylenediamine,

DPPH - 2,2-diphenyl-1-picrylhydrazyl

DTNB - 5, 5'-dithio-bis (2-nitrobenzoic acid)

ESR- erythrocyte sedimentation rate

ETC - electron transport chain

Fe – iron

fMLP- N-formylmethionyl-leucyl-phenylalanine

FOXO - fork-head box protein O

FRAP - Ferric Reducing Ability of Plasma

GSH - Glutathione

GSSG - Glutathione disulphide

GSHx- glutathione peroxidase

GSSG:GSH- reduced glutathione/oxidized glutathione ratio,

GR- glutathione reductase

HAT – hydrogen atom transfer

H2O2 - hydrogen peroxide

HBSS – Hanks balanced salt solution

HOCL - hypochlorous acid

HPA – hypothalamic pituitary adrenal

HUVEC- human umbilical vein endothelial cells

hsCRP- High sensitivity C-reactive protein,

ICAM-1 -intercellular adhesion molecule-1

IGF-1 - insulin growth factor-1

IL - interleukin

IL- 1β – interleukin 1-beta

IL6 – interleukin 6

IL-17-interluekin-17

IL-10-interleukin-10

iNOS - inducible nitric oxide

JNK - c-Jun N-terminal kinase

KEAP1 - Kelch-like erythroid cell-derived protein with CNC homology-associated protein 1

LD – linear distance

LPS – lipopolysaccharides

LOOH - lipid hydroperoxides

MCP-1 - Monocyte chemoattractant protein-1

MDA – malondialdehyde

MFI – mean fluorescent intensity

Mn - magnesium

MPO – myeloperoxidase

NCD's – Non-communicable disease

NETS - neutrophil extracellular traps

NF-κB - nuclear factor-kappa beta

Nfkb1 - Nuclear Factor Kappa B Subunit 1

NLRP3 - nucleotide-binding domain leucine-rich repeat (NLR) and pyrin domain containing receptor 3

NO - nitric oxide

NOX - NADPH-oxidase

NRF2 - Nuclear factor erythroid 2-related factor 2

O2− superoxide

$O2^{\cdot-}$ - superoxide radical

ONO2− peroxynitrite

ORAC - oxygen radical absorbance capacity

PBMCs - peripheral blood mononuclear cells

PCO - proanthocyanidolic oligomer

PGE2 - prostaglandin E2

PI3K- Phosphoinositide 3-kinases

PTEN - Phosphatase and tensin homolog

RA – rheumatoid arthritis

RBC – red blood cell

RONS - reactive oxygen and nitrogen species

ROS - reactive oxygen species

SET – single electron transfer

SAPK - stress activated protein kinase

SD – standard deviation

SEM – Standard error of the mean

SLE - systemic lupus erythematosus

SOD - superoxide dismutase

TAS-total antioxidant status

TAC-total antioxidant capacity

TBARS - 2-Thiobarbituric Acid Reactive Substances

TD – total distance

TEAC - Trolox Equivalent Antioxidant Capacity

Terc - Telomerase RNA Component

TLR-4 - Toll-like receptors

TrxR- Thioredoxin reductase

TNF-α - Tumour necrosis factor-α

TNF-RI- Tumor necrosis factor receptor 1

WHO – World Health Organization

VEGF - vascular endothelial growth factor

Zn – zinc

Chapter 1: Background and rationale

1.1 Introduction

In recent years non-communicable disease has become an increasing burden on global healthcare systems. In 2015, the World Health Organisation predicated that most countries would fail to meet global targets on addressing non-communicable diseases (NCDs) – which were outlined by the second Global Status Report on Noncommunicable Diseases of 2014 – by the 2025 target (Mendis et al., 2015; Damasceno, 2016). Non-communicable diseases have become the leading causes of death and currently prescribed preventative strategies do not seem to be ameliorating the problem (Torabi et al., 2016).

The most common NCDs are cardiovascular diseases, cancers, chronic respiratory diseases, chronic inflammatory diseases and diabetes. One of the underlying causalities of many of these diseases is the interconnected and self-propagating cycle of sterile inflammation and oxidative stress, two systems activated and maintained by the modern lifestyle. Poor dietary choices, sedentary lifestyle, psychological stress and increased exposure to pollution has resulted in excessive stimulation of these two systems, resulting in a phenotype characterised by chronic low grade inflammation and poor redox status (Juster et al., 2010; Fernández-Sánchez et al., 2011; Kennedy et al., 2014). The increasing stressors of the modern lifestyle have caused a phenomenon that can be termed as accelerated ageing due to the fact that the above-mentioned diseases are occurring at earlier stages of life and affecting pre-geriatric populations (Smith, 2018). Investigating the role of inflammatory and oxidative stress driven maladaptation is imperative to enhancing our understanding of the gradual transition from allostatic load, to exhaustion of endogenous protective counter-regulatory systems and predisposition to clinical onset of chronic disease.

This gradual process, which presents as a phenotype resembling premature ageing – or accelerated ageing – is a particular focus of this thesis, as it represents the threshold between a normal and pathological phenotype. Thus, since it is my interest to prevent disease, the ability to move this threshold so that disease onset may be delayed or prevented, is a major focus in the studies presented in this thesis.

In terms of identifying this threshold, it is known that chronic low-grade inflammation and cumulative oxidative stress result from consistent load on the systems which are employed to regulate homeostasis (Valko et al., 2007; Pomatto and Davies, 2018). Unfortunately, the threshold at which these maladaptations become irreversible are unknown thus far. Identification, or characterisation of this threshold, is a major gap in existing literature which needs to be addressed, so that preventative strategies may be assessed in terms of their capacity to delay crossing of this “ageing-threshold”(Cohen, 2018).

Important requirements for such a tool, is that it should present a physiologically relevant simulation of pre-pathology states, while being sufficiently sensitive to detect the small effect size changes and maladaptations that are usually not clinically visible. In other words, research models capable of simulating maladaptive, pre-pathology changes which are still

reversible, will allow investigations into early maladaptive mechanisms to target even before overt signs of damage are observed. In the relevant literature to date, the majority of animal disease simulations are severe in order to increase the effect size measured and thus the statistical power, and thus cannot accurately simulate chronic, low grade accumulation of maladaptive changes. Of course, models of natural ageing have been employed, but the time and resources required impede the number and depth of studies.

In terms of preventative strategies, potential treatments should ideally be sufficiently potent to aid endogenous counter-systems, while being mild enough to not result in unanticipated, lasting changes which may have downstream effects on other systems (Nobili et al., 2009; Smith, 2018). In this niche, many phytomedicines are being investigated for efficacy as daily disease prevention (anti-ageing) supplements (Liu et al., 2018). For the purpose of this thesis, I have selected one such phytomedicine which is well-researched in this context, in order to assess the sensitivity of the models designed.

The layout of this thesis is as follows: in the next chapter, I present an overview of the most relevant published literature. In the next two chapters, I present data in the form of two published manuscripts, followed by a final chapter with concluding remarks and recommendations.

Chapter 2: Literature review

2.1 Introduction

The pressures of modern society increase the number of adverse stimuli that individuals are exposed to on a regular basis. These range from sedentary lifestyles and unhealthy diets to increasing psychological stress and anxiety, all of which lead to cumulative oxidative stress and persisting low grade inflammation (Petersen, 2016). This in turn results in a chronically maladapted phenotype characterised by increased oxidative damage and inefficient immune responses which are characteristically similar to a geriatric phenotype (Belsky, 2015; Benayoun, 2015). The occurrence of this status, first described at cellular level by Hayflick in 1965 (Hayflick, 1965), was therefore termed accelerated ageing. Senescent cellular phenotypes share many characteristics with cellular phenotypes identified in numerous diseases and conditions normally associated with advanced age, but which are now reported with increasing incidence in younger populations affected by certain conditions (Smith, 2009; Belsky, 2015; Liu, 2015; Mercado, 2015; Xu, 2018). In addition to predisposing individuals for pathologies such as cardiovascular disease, diabetes and even neuroinflammation (Karam, 2017; Powrie, 2018), emerging evidence suggests that this maladapted phenotype may be transferred epigenetically to subsequent generations (Moisiadis, 2017; Adams, 2019; Chang, 2019). This underlines the importance of developing preventative strategies with which to address the phenomenon of accelerated ageing.

This review provides evidence of an accelerated ageing phenotype across the majority of modern chronic diseases, followed by a critical assessment of the most pertinent obstacles preventing implementation of preventative medicine strategies, including those delaying preventative drug discovery research. In addition, an overview of cellular processes at play in promoting or delaying ageing via modulating redox and/or inflammatory status is provided, in order to elucidate cellular therapeutic targets of current supplementation strategies.

2.2 The accelerated ageing profile in clinical disease

The gradual development of an pro-inflammatory phenotype with advancing age, or as result of accelerated ageing in the aetiology of chronic disease as result of endocrine contributors – such as glucocorticoid resistance and decline of dehydroepiandrosterone (which has known antioxidant and anti-inflammatory effects) – was recently reviewed (Powrie and Smith 2018) and is not a focus in this review.

Rather, this review will focus on the allostatic effect of persistent activation of the inflammatory immune system and overabundance of free radicals, leading to unfavourable redox status, which appears omnipresent in modern chronic diseases and resembles a prematurely aged phenotype.

In this context, ample evidence for an aged phenotype in chronic disease has been presented in the published literature. In Table 2.1, using representative studies, we draw a parallel between a normal aged phenotype and those reported in chronologically younger populations with chronic disease. From this table, the reality of accelerated ageing in modern

chronic disease is clearly highlighted. Thus, the significant benefit to longer term clinical outcome via intervention to prevent redox and inflammatory dysregulation and thus delay acceleration of ageing, cannot be ignored.

Context	Population age	Tissue assessed	Redox status	Inflammation	Reference
Normal ageing					
Aged females	>65 years old	Whole blood	↑CD's,	↑MPO, ↑neutrophil CD66b	(Petersen, 2018)
Aged subjects	>65 years old	Neutrophils	-	↓ neutrophil chemokinesis, ↑ PI3K	(Sapey, 2014)
Aged males and females	>45 years old	Whole blood	↑MDA	↑IL-6	(Campesi, 2016)
Aged males and females	>60 years old	Whole blood	↑MDA, ↓TAS	-	(Narasimha Rai, 2013)
Aged males and females	72.7 ± 5.8 years old	Plasma	↑MDA, ↓FRAP, ↑PC, ↑DNA damage	-	(Mutlu-Türkoğlu, 2003)
Chronic conditions					
Rheumatoid arthritis	52.46 ± 7.39 years old	Whole blood	↑MDA, H_2O_2, $O2^-$, ↑SOD	↑NO, ↑CRP, ↑ESR	(Veselinovic, 2014)
Rheumatoid arthritis	53.4 ± 10.1 years old	Whole blood	↑MDA & DMPD	↑CRP	(Ozkan, 2007)
Rheumatoid arthritis	43.11 ± 10.86 years old	Whole blood	↑MDA, protein oxidation and DNA damage ↓FRAP, SOD, CAT	-	(Mateen, 2016)

Alzheimer's disease	>75 years old	Whole blood	↑protein carbonyls, ↓GSHx, TAS	↑TNF-α,IL-6, ADMA	(Gubandru, 2013)
Alzheimer's disease	69 ± 4 years old	Whole blood	↓FRAP, ↓TEAC	-	(Pulido, 2005)
Chronic psychological stress	Men: 47.4 ± 10.9 and women 44.4 ± 9.8 years old	Whole blood and saliva	↓Vit C	↑CRP	(Hapuarachchi, 2003)
Acute psychological stress	± 22 years	Lymphocytes	↓FRAP, ↑sensitivity to oxidation	-	(Sivoňová, 2004)
Anxiety	<19 years old	Blood	↓DHEA, ↑GR expression	↓ IL-10	(Viljoen, 2020)
Generalized anxiety disorder	46.4 ± 13.6 years old	Serum	↑MDA, ↓Vit E	-	(Bal, 2012)
Depression	45.2 ± 4.5 years old	Whole blood	↑MDA	↑IL-12, ↑PMN elastase, ↑MIP-1a	(Ogłodek, 2018)
Metabolic syndrome	44.0 ± 13.5 years old	Serum	↑MDA	↑TNF-α,↑CRP	(Guerrero-Romero, 2006)
Metabolic syndrome	47.3 ± 2.6 years old	Whole blood	↑MDA, ↓FRAP	-	(Bitla, 2012)
Obesity	48.71± 13.68 years old	Whole blood	↑free radicals, ↑MDA, ↑GSSG:GSH % ratio	↑hsCRP, ↑fibrinogen	(Skalicky, 2008)
Diabetes	Not disclosed	Serum	↑MDA, ↓thiol	↑IL-6 & TNF-α	(Neelofar, 2019)

			content, ↓SOD, GR,		
Diabetes	53.22 ± 10.04 years old	Whole blood	↓SOD and TAC, ↑MDA and 8-iso-PGF2α	↑TNF-RI, IL-1β, TNF-α and IL-6	(J. Li, 2014)
Cardiovascular disease	>65 years old	Serum	↑MDA	↑hs-CRP	(Abolhasani, 2019)
Hypertension	49.33 ± 10.01 years old	Serum	↑MDA	↑ hsCRP	(Nakkeeran, 2017)
Irritable bowel syndrome	37.66 ± 8.84 years old	Serum	↑MDA, ↓TAC	↑TNF-α, ↑IL-17, ↓IL-10	(Choghakhori, 2017)

Table 2.1. Representative studies, I draw a parallel between a normal aged phenotype and those reported in populations with chronic diseases. Note: CD's-conjugated dienes, MPO-myeloperoxidase , PI3K- Phosphoinositide 3-kinases, MDA-malondialdehyde, IL-6- interleukin 6, TAS-total antioxidant status, H_2O_2- hydrogen peroxide, $O2^-$ - superoxide radical, SOD- superoxide dismutase, NO-nitric oxide, CRP- C-reactive protein, ESR- erythrocyte sedimentation rate, DMPD- N,N-dimethyl-p-phenylenediamine, FRAP-ferric reducing antioxidant power, CAT- catalase, GSHx- glutathione peroxidase, hsCRP- High sensitivity C-reactive protein, GSSG:GSH- reduced glutathione/oxidized glutathione ratio, TNF-α- Tumor necrosis factor-alpha, GR- glutathione reductase, TAC-total antioxidant capacity, 8-iso-PGF2α-8-iso-prostaglandin F2α, TNF-RI- Tumor necrosis factor receptor 1, IL-1β-Interleukin 1 beta, IL-17-interluekin-17, IL-10-interleukin-10.

2.3 Why preventative medicine?

Many people do not realise that they have an underlying low-grade, but cumulative, inflammatory, or redox-related maladaptation until diagnosed with a disease. At that point, adaptation had already progressed to a stage of sufficient allostatic load for disease to manifest clinically and the body's endogenous antioxidant and/or anti-inflammatory mechanisms have been exhausted and are unable to effectively counter the disruption of homeostasis (Powrie, 2018). Given this relatively late stage at which patients usually present at medical practitioners, modern medicine is often based on symptomatic treatment which commences after clinical disease onset. While these therapeutic strategies provide symptomatic relief, very few succeed in correcting the dysregulation which resulted in disease onset. If the original aetiological factors such as oxidative stress and chronic inflammation are not addressed and allowed to maladapt irrevocably one becomes predisposed to developing a number of co-morbidities that share these aetiological factors. The addition of co-morbidities results in further complications for the primary pathology and long-term exposure to negative side effects associated with treating multiple conditions. The result is decreased quality as well as quantity of life of patients (Mikuls, 2003; Martin-Ruiz, 2014; Barnes, 2015; Scuric, 2017).

Furthermore, although medical practitioners do prescribe additional, vital strategies such as dramatic lifestyle changes and dietary improvements, which in fact could also have delayed disease onset if instituted timeously, compliance is generally relatively poor (Ganiyu, 2013; Abel Tibebu, Daniel Mengistu, 2017; Bhawana Sharma and Mukta Agrawal, 2017; Leung, 2017; Lee, 2018). Furthermore, the western lifestyle is associated with significant psychological stress - as evidenced by the increased incidence of modern chronic diseases as result of urbanisation (Ramachandran, 2008; Smith, 2009; Isaksson, 2015; González, 2017) - which contributes substantially to allostatic load. Given the significant occupation-associated pressure on especially economically active age groups, it is near impossible to avoid stress exposure. The recent COVID-19 pandemic highlighted an extreme form of uncontrollable psychological stress exposure, hitting home the requirement for external assistance to prevent unavoidable stressors from manifesting as accelerated ageing-associated pathology.

Given the relative non-compliance to current behaviour- or lifestyle changing strategies, it may be more realistic to rather aim applied health research to disease prevention using daily supplements to counter the maladaptation caused by unhealthy human behaviour. Indeed, there is a global trend towards increased preventative supplement use, in particular in the stress-relief and antioxidant niches (Y. J. Zhang, 2015). Unfortunately, the majority of supplement products on the shelves remain scientifically unvalidated, despite emerging evidence of significant toxicity risk especially in the context of antioxidant overdose (Rietjens, 2002; Hart, 2012; Cásedas, 2018). In particular in the niche of phytomedicine supplements, research is not always aimed at understanding mechanisms, but more often limited to superficial in vitro efficacy testing, so that little quality data is available on actual mechanisms or the cellular molecular targets that are most effectively targeted by efficient supplements

(Taylor, 2001). An unfortunate tendency in the phytomedicine literature is large scale *in vitro* screening and reporting of potential benefits of plant extracts, without follow-up research to advance to in vivo models, and a relative lack of publication of "negative data". This has resulted in the phytomedicine literature to be cluttered by seemingly positive *in vitro* results lacking *in vivo* confirmation/refutation.

For preventative medicine to succeed at ground level, a system is required which is driven by qualified personnel, such as primary care nurses or medical practitioners; of course, these individuals need to have access to up-to-date science-based support of in vivo efficacy for products across a variety of illnesses and chronic conditions. Without this information, it is difficult to advance preventative medicine to a formalised strategy within healthcare systems. In my opinion, this is a significant gap in especially the preventative phytomedicine niche which requires urgent attention, as relatively uninformed consumers are currently left to "fend for themselves", which of course poses a risk of unanticipated adverse effects, especially when supplement compounding is performed by the layperson, who operates under the assumption that anything "natural" is safe (Güney, 2019).

The key priority should be to establish suitable models with which to investigate mechanisms of maladaptation and thereby identify therapeutic targets. Importantly, in order to advance drug discovery in phytomedicine from bench to bedside, it is vital to simulate the turning point in the allostasis trajectory where adaptation changes into maladaptation and predisposition to disease. Only such models will enable accurate assessment of potential benefit or risk associated with new potential preventative medicines. Identifying changes in mechanisms may also elucidate accurate and feasible biomarkers which can both identify at risk persons and which are sensitive enough to reflect preventative treatment efficacy. Furthermore, given the omnipresence of inflammation and oxidative stress in modern chronic disease, characterisation of models in terms of inflammatory and redox profile will lend itself to broad application in the drug discovery niche.

2.4 A requirement for suitable pre-clinical models

As stated, an obstacle in the training of health care professionals in the preventative prescription and use of (antioxidant) phytomedicines, is probably the lack of definitive positive clinical data from human studies relative to the large and fast-growing body of evidence from cellular and animal studies (Steinhubl, 2008). One of the main reasons for many preventative and plant medicinal strategies not being successfully translated in human clinical trials, may be ascribed to the fact that the maladaptive "rate of departure" from normal during allostasis (McEwen, 1998) – and thus the measurable effect size in shorter term intervention studies – is relatively small when considered in conjunction with the relatively larger inter-individual and intra-individual variability expected in regulatory system parameters, such as the ones most relevant to accelerated ageing (Smith, 2018).

Research has shown that advanced non-clinical analytical methodology is required in order to identify these small changes at cellular level. When following the traditional clinical trial route,

where testing is done in normal individuals changes are unlikely to show large effects in the preventative niche, simply because effect size is nearly non-existent (Smith, 2018). These types of studies are relatively sparse, which warrants the use of a somewhat more standardisable in vivo model.

Animal models in particular may be more feasible tools with which to investigate long-term outcome, as well as to elucidate the mechanisms of action and specific molecular targets affected by preventative treatments. Indeed, in more standardised systems such as animal and cell culture models, larger effect changes may be simulated and contribution of confounding factors – of which diet is a significant one – may be largely eliminated. An added advantage of these models is that they allow for more comprehensive investigations at tissue level than what is usually feasible in a human cohort, so that investigations may extend to more than just the circulatory compartment. Furthermore, given the relatively shorter total lifespan of small animals when compared to that of humans, longitudinal studies across a lifespan or substantial part (Leenaars, 2019) thereof – which is particularly relevant to the topic of accelerated ageing – are much more feasible in animals than humans (Holsapple, 2003; Sengupta, 2013). As such, animal models may provide important information that cannot be generated in humans within a reasonable time frame. In the context of inflammation and redox-associated research, animal models are particularly feasible, as the processes of inflammation and redox regulation are highly conserved across species (Barth, 2019; Patil, 2019).

Given these benefits, models of maladapted inflammatory and/or redox profiles are clearly most suitable as research tools in the context of accelerated ageing. However, before data generated from pre-clinical models can be evaluated in the context of prevention of accelerated ageing, and before a suitable model may be selected for drug testing, it is necessary to understand the nature and pattern of deviation from normal, of the most pertinent cellular and molecular pathways which determine nett redox and inflammatory status(Cohen, 2018). The following paragraphs will describe the gradual change from adaptation to pre-pathology which precedes several modern pathologies, which may be addressed by preventative approaches aimed at attenuating oxidative stress and/or chronic low-grade inflammation.

2.5 Accelerated ageing mechanisms

Oxidative stress is characterised by an excess of free radicals both within the cell and in the extracellular space (Valko, 2007). This is the result of not only production of higher levels of free radicals, but also down-regulation of the signalling systems initiating antioxidant responses (Aycicek, 2005).Especially in the modern, westernised society, many contributing factors jointly facilitate increased production of free radicals.

2.5.1 Increased levels of free radicals

2.5.1.1 Exogenous sources

In terms of exogenous contribution to oxidative stress, modern society unfortunately offers plenty of sources. Firstly, exposure to increasing amounts and variants of pollution may stimulate endogenous systems to create free radicals which cause cellular stress to the organism. Air pollution has been linked to increases in incidence of various diseases such as cancers, cardiovascular disease, Alzheimer's disease and chronic obstructive pulmonary disease, where onset and progression are attributed to chronic exposure to oxidative stress and inflammation caused by irritants from the air pollution (Autrup, 1999; Lodovici, 2011; Moulton, 2012). In line with this, the risks associated with smoking have been reported in literature and media for many years. Not only does the tar of cigarettes contain stable free radicals which upon inhalation produces O2·− and H_2O_2 in the aqueous environment but the presence of foreign particles in the alveoli of the lungs and epithelial of the mouth, throat and trachea activate an immune response which induces oxidative stress characterised by an increase in lipid peroxidation and DNA damage (van der Vaart et al., 2004; Valavanidis et al., 2009). Coupled with increased free radicals and chronically activated immune responses, smoking is also associated with relatively depleted antioxidant systems (e.g. decreased antioxidant capacity (TEAC), antioxidant reducing capacity (ARC) and lower GSSG ratio (Bloomer, 2007).

Poor nutrition has also been implicated as a causative factor in chronic disease (Shlisky, 2017). In the context of redox, the main concerns are excessive sugar, simple carbohydrates (Hu, 2006), saturated fats and processed meat products. This is due to the excess chemical additives as well as easily destabilised free radicals when these are digested, which have several downstream effects (Ma, 2019). Macronutrients in overabundance results in the saturation of metabolic processes and can disrupt the growth and maintenance of the gut microbiota(Brown, 2012). The resultant environment has an altered pH level, various reactive species and damaged molecules which exacerbate oxidative stress.

2.5.1.2 Endogenous sources

The endogenous sources, targets of and defensive systems against free radicals differ according to their biochemistry, location and function in the intra- and extracellular environment (Lü, 2010). Areas within the cell where free radicals are typically produced as part of normal metabolism include the mitochondria, peroxisomes, endoplasmic reticulum (ER) and nuclear membranes (Lushchak, 2014; Phaniendra, 2015). Free radicals can be classified as reactive oxygen species (ROS), nitrosative radicals (RNS) and reactive sulphur species (RSS). For the purpose of this thesis, the focus in the next sections will be on ROS/RNS only, as their dysregulation is most commonly associated with chronic disease. Although it has been suggested that reactive sulphur species should be considered (Giles, 2017), even the latest reviews (e.g. Olson, 2020) still seems highly speculative, with not much solid information in support of this notion. Given this relative lack of data, a discussion on RSS has not been included in this review.

The majority of reactive oxygen species (ROS) – which make up ≈90% of total free radicals present in the body – is generated from the unstable oxygen molecules formed by interactions with free electrons from the mitochondrial electron transport chain (ETC) (Lushchak, 2014). The ETC is responsible for the generation of ATP, but leaks electrons which then interact with surrounding oxygen to form free radicals. The main sites of leakage within the ETC are complexes I - the NADH-ubiquinone oxidoreductase - and III - cytochrome bc_I complex (Zhao, 2019). Escaped electrons are able to enter the intracellular space and interact with oxygen within the mitochondria to form superoxide (O2·–) or hydrogen peroxide (H_2O_2). Of these, H_2O_2 is more stable and therefore the parameter of choice for assessment of ROS levels.

Other significant inter- and intra- cellular sources of free radicals is the NADPH oxidase system, xanthine oxidases (XO) and endothelial nitric oxide synthase (eNOS), the latter of which forms part of RNS. NADPH oxidase (Nox), a cytoplasmic membrane enzyme, creates free radicals by transferring electrons from NADPH to oxygen (Magnani, 2019). There are two subunits which generates superoxide anion ($O^{2\cdot-}$) and hydrogen peroxide (H_2O_2) namely; gp91phox, responsible for sequestering of oxygen and p67phox which removes a electron from NADPH to NADP+ (Ozcan, 2015). This system of radical generation is particularly important within inflammation, specifically as a result of Nox activity on the membranes of activated neutrophils. The phagocytic capabilities of these and certain other immune cells depend on free radical generation via this pathway. Cytosolic enzymes xanthine oxidases and nitric oxide synthase works in a similar manner. Xanthine oxidase is an enzyme which functions to break down purine nucleic acids for redistribution and use within a cell. Xanthine oxidases, besides being able to generate O2·– and H_2O_2 , are the main contributors of uric acid formation (Battelli, 2016). Uric acid can exacerbate the intracellular prooxidant environment by providing metabolites for the further generation of free radicals. Similarly, nitric oxide synthase (NOS) - besides being donors of superoxide and hydrogen peroxide - is a key contributor for the production of the oxidant peroxynitrite (ONOO−)(Pole, 2016). NOS primarily uses L-arginine and oxygen as substrates and via reduced NADPH creates nitric oxide which has multiple functional roles within a cell (Förstermann, 2012). Nitric oxide has a multitude of functions in various cell types. In the central nervous system it aids synaptic plasticity, whereas in the periphery it functions to regulate blood pressure, smooth muscle relaxation and vasodilation (Levine et al., 2012).

It is important to note though that presence of free radicals are not always undesirable. It is important to note that free radicals play an important role in various mechanisms driving homeostasis. For example, free radical levels dictate proliferation and/or survival mechanisms within cells by activating or inactivating PI3 kinase, MAP kinases, PTEN, and protein tyrosine phosphatases, depending on their level present within the cell (Ray et al., 2012). Large increases in free radical production signals survival by upregulating antioxidant capacity whereas lower stable levels indicate a homeostatic state favouring proliferation. It is only when these systems which regulate proliferation and/or survival are overwhelmed that

cell death via apoptosis result. (Ray et al., 2012). These unstable radicals also play an integral part in the formation of digestive pathogen destruction capacities in immune cells such as neutrophils and macrophages (Kohchi, 2009). For example, bacterial endotoxin such as lipopolysaccharide (LPS), as well as pro-inflammatory cytokine (TNF-α) signalling, activate the production of ROS as a priming step for phagocytosis (Babior, 2000). These roles highlight the importance of sustaining a balance of free radicals within a cell instead of aiming to eradicate them altogether.

Under normal conditions, this balance is maintained by endogenous antioxidant defences. Only when these systems are overwhelmed, accelerated ageing will ensue.

2.5.2 Overwhelmed Antioxidant mechanisms

Turning attention to antioxidant mechanisms, the human body has many endogenous antioxidant defensive systems which under homeostatic conditions quench free radicals at approximately the same rate as they are produced. The free radical-activated translocation of NRF-2 from the cytoplasm to the nucleus where Kelch-like erythroid cell-derived protein with CNC homology-associated protein 1 (KEAP1)(Ma, 2013) to the nucleus, initiates the transcription of a number of antioxidant and anti-inflammatory factors, such as superoxide dismutase (SOD), glutathione (GSH), glutathione peroxidase (GPx) and peroxiredoxins (PRx) (Ray et al., 2012; Chen *et al.*, 2015; Zhang *et al.*, 2015). This finely regulated balance sustains a cellular environment which favours survival and optimal function of the cell. Most relevant to the topic of accelerated ageing, NRF2 signalling has been shown to be decreased in advanced age (Sykiotis, 2013). It has been speculated that this is mainly due to modification of NRF2 and Keap1 signalling. A similar trend was reported in diseases and genetic abnormalities (Zhang et al., 2016; Tu *et al.*, 2019). Signalling of these two factors changes due to cumulative stress or damage to DNA and post-translational machinery. (Sykiotis, 2013). The subsequent decrease in NRF2 signalling results in decreased antioxidant capacity, which leaves cells vulnerable to oxidative damage (discussed in more detail in section 2.5). The endogenous antioxidant systems downstream of NRF2 are adaptive to an extent and can be up or down regulated to cater to smaller increases or decreases in free radical production (Valko, 2007) to maintain homeostatic balance. There are various sites and types of antioxidant systems which target different free radicals based on location and type, to either quench free radicals, limit their production, or protect their targets against oxidative damage.

2.5.2.1 Enzymatic antioxidants

Enzymatic antioxidant defences include most prominently superoxide dismutase (SOD), glutathione peroxidase (GSHPx) and catalase (CAT), and peroxiredoxins (Prx–I). These antioxidant systems are present in the cytosol, mitochondria and peroxisomes, closest to the sources of free radicals. The main mechanism of action of this group of antioxidants is to catalyse the reduction of free radicals such as $O_2^{\cdot-}$ and H_2O_2 into stable molecules such as H_2O, O_2, GSSG, $Trx(SH)_2$ and H_2O_2 (Rahal, 2014). Depending on the location of these antioxidants, metal ions such as magnesium (Mn)(in the mitochondria), copper (Cu) and zinc (Zn) are used

to stabilise free radicals by donating ions (Nimse and Pal, 2015). Some of these enzymes are able to work in synergy to increase antioxidant capacity, especially during cellular stress. An example would be the interaction of the predominate enzymes SOD, CAT and GSHPx (Lushchak, 2014).

2.5.2.2 Non-enzymatic antioxidants

Non-enzymatic antioxidants such as ascorbic acid, α-tocopherol, glutathione (GSH), carotenoids and flavonoids supplement the predominating enzymatic antioxidant mechanisms(Powers, 2004). In this context in particular, preventative supplementation has become a focus, as many of these antioxidants may also be derived from food or plant extracts. Non-enzymatic antioxidants can be further divided into lipid soluble (vitamin A, α-tocopherol and carotenoids) and water soluble (ascorbic acid, vitamin B_6 and flavonoids) compounds and these properties dictate their location and mechanism of action(Lobo, 2010). Lipid soluble antioxidants are able to scavenge and disrupt peroxidation of lipids to prevent damage to cellular structures. Although under relatively stable conditions these molecules result in stable products under certain conditions of stress and excess $^{\cdot}OH$ they can result in unstable by-products which are reactive and add to the free radical burden.

Water soluble antioxidants, especially flavonoids which are predominately found in fruits and vegetables, function by donating electrons to free radicals and thereby stabilizing volatile molecules (Rietjens, 2002). Bioflavonoids include a variety of subcategories of antioxidants such as quercetin, taxifolin, catechin, anthocyanidin and daidzein to mention a few. Their structures determine their main mechanism of action, but their common function remains the quenching of radicals (Kandaswami, 1994). Due to the diversity of structures they are able to exert protective mechanisms on lipids, DNA and proteins depending on the number and location of phenolic rings and number of electrons available for donation, this includes the ability to chelate metal ions (Kandaswami, 1994).

Also, in this context, the modern lifestyle seems to have detrimental effects. In addition to the direct upregulation of free radicals due to poor diet, as already mentioned, the lower nutrient content of mass-produced food has resulted in a decrease in antioxidant intake by the general population (Hu, 2006; Cardoso, 2010). A relative lack of various antioxidants and components required to stimulate endogenous production of antioxidant systems was reported in mass-produced fresh produce (Wang, 2008; Fernandes, 2012). The result is decreased dietary intake of necessary macronutrients and antioxidants, decreasing both exogenous and stimulated endogenous antioxidant capacity.

Another significant role player contributing to the imbalance between free radical production and antioxidant counter mechanism activity, is persistent chronic low-grade inflammation, which is a widely accepted characteristic of modern chronic disease(Gill et al., 2010; Reuter *et al.*, 2010).

2.5.3 Low grade inflammation as confounding factor

While normal activation of neutrophil-associated ROS production via the NADPH oxidase pathway is desirable, persistent activation of inflammation – the so-called low-grade chronic inflammation – contributes significantly to accelerated ageing by dysregulation of the redox system.

The link between oxidative stress and inflammation is perhaps best demonstrated by using as example, the formation of neutrophil extracellular traps (NETS). The abilities of neutrophils range from antigen sensing to phagocytosis, and these cells are equipped with machinery which unchecked can cause a vast amount of secondary damage. NETS are complexes composed of DNA, histones, and granule proteins which form fibres designed to trap pathogens (Yang, 2016). Neutrophils create NETs when ligands bind to cell surface receptors and activate a cascade of events which are designed to use ROS production to digest the entrapped particles (Vorobjeva, 2014). There are varying degrees of NET formation which can either lead to the death of the neutrophil or structural changes, but both of these events use can lead to ROS increases (Driouich, 2019). Neutrophils use mainly NADPH, NOX and MPO to generate ROS after the influx of calcium in activated by the signalling of receptor binding (Papayannopoulos, 2018).

In the innate immune system unchecked or inefficient immune responses can cause severe secondary damage which perpetuates the inflammatory response and prolong it. Cells of the innate immune system can mount aggressive responses once activated and in doing so create a considerable amount of damage. Interestingly, the link between NET formation and ROS appears to be bidirectional: superoxide is also known to recruit neutrophils to sites of pathogenic inflammation, and recently it has been shown that superoxide is able to activate the neutrophil extracellular trap formation by activating the signalling cascade of Toll-like receptors (TLR-4) as well as NADPH oxidase (NOX) (Al-khafaji, 2016).

Furthermore, physical activity, both excessive exercise and lack thereof, has been identified as a promotor of inflammation and oxidative stress, and ultimately chronic disease. The sedentary lifestyle is characterised by lack of exercise, low muscle mass and decreased cardiovascular capacity (González, 2017). Lower muscle mass translates to decreased capacity to utilise glucose, especially when supplied in excess through dietary intake. This is due to fewer contractile muscle cells which utilise glucose as their main energy source (Davies, 2018). Besides decreased glucose uptake, lower muscle mass decreases insulin signalling, growth hormones and antioxidant capacity of surrounding tissue (Thyfault, 2020). This, combined with increased adipose deposition, is a predictor of chronic disease such as diabetes and insulin resistance (Hwang, 2016). In contrast, habitual mild exercise has been shown to increase antioxidant capacity and has anti-inflammatory properties (Wadley, 2016), even at low intensities and frequencies (Rabelo, 2017). Exercise has been prescribed to patients suffering with cardiovascular, metabolic and psychological diseases due to the positive results seen, mainly serum decreases in oxidative stress and inflammatory markers

(Luan, 2019). Unfortunately, the lack thereof in the daily routine, low adherence to exercise programs and lack of resources have made physical activity low priority to many in urbanized areas.

Finally, besides the dysfunction of the immune cells, organs and systems themselves, persistent low-grade inflammation has secondary effects on other organ systems. One such tissue is the adipose tissue, which is relatively well researched only in the context of diabetes and metabolic disease (Minamino, 2009; Ricordi, 2015; Nosalski, 2017), while data in the context of ageing is relatively lacking, especially when considering adipose tissue as a potential ageing accelerating tissue type. Adipose tissue is easily infiltrated by macrophages, T cells, regulatory cells, natural killer and granulocytes (Ahima, 2009), which results in a pro-inflammatory environment within the tissue specifically in obesity (Fernández-Sánchez, 2011). Coupled with the fact that adipocytes themselves produce high levels of ROS (Tchkonia, 2010; Engin, 2017), this tissue can become vulnerable to DNA damage due to relatively low levels of antioxidant defences (Fernández-Sánchez, 2011), again propagating the inflammatory damage-repair cycle. This condition is perpetuated by aged adipocytes via their increased production and secretion of pro-inflammatory cytokines (Tchkonia, 2010).

2.5.4 Oxidative damage

Excessive availability of free radicals alters the molecular make up and thereby damages cell constituents crucial for homeostatic functioning within the cell and in the surrounding cellular microenvironment. The extra-cellular environment is particularly vulnerable to leakage of damaged molecules and free radicals into the extracellular space, resulting in a stress signal to surrounding cells because there are no quenching mechanisms (Valko, 2007). Unstable molecules are thus able to act on circulating factors which has multiple downstream effects.

In terms of cellular oxidative damage, membrane lipids, cellular proteins and DNA are main targets affected by free radicals. The superoxide anion and the hydroxy radical are primarily responsible for damage to lipids, and in particular membrane lipids. The membranes affected are not only cellular membranes, but also organelle membranes within the cell, such as mitochondria (Miquel, 1980). Damaged membranes lead to exacerbated leakage of incomplete proteins, electrons and reactive molecules from cells (Avery, 2011).

Furthermore, DNA oxidation is initiated by hydroxy radicals and singlet oxygen, by exposing sections of DNA to iron (Fe) catalysation and ultimately chromosomal rearrangements. Any damage done to DNA is normally repaired as a result of repair mechanisms in place, such as Poly-ADP ribose polymerase 1 (PARP-1) (Houben, 2008). The combination of repair systems and telomeres at the ends of DNA work to protect DNA from damage, but their capacity is not unlimited, (Hayflick, 1965). This translates to a limited ability for cells to repair themselves after being damaged repeatedly. Telomeres are DNA repeats present at the both ends of chromosomes. Telomeres have a set length of kilobases per chromosome and these excess kilobases protect the DNA from being modified during replication and repair(Aubert, 2008).

Once the capacity of cells' innate repair systems such as telomeres or PARP-1 are depleted, the cell begins to senesce which causes downstream maladaptations.

Similarly, protein damage occurs when residues are exposed to oxygen radicals. This process is catalysed by Cu(II) or Fe(III), and results in the exposure of amino acids the ·OH which is formed in prior reactions with oxygen radicals (Cecarini, 2007). The free radical damage to proteins causes the dysregulation of signalling molecules which effects a plethora of intra- and intercellular cascades (Hawkins, 2019).

The combination of consistent damage repair cycles and overrun antioxidant mechanisms eventually lead to a state of maladaptation which when prolonged results in irreversible damage and disease. This is essentially how cumulative oxidative stress is postulated to lead to accelerated ageing.

2.6 Prevention of accelerated ageing

Over the past decade, a huge body of evidence has been established which suggests the benefit of preventative antioxidant supplementation in the context of accelerated ageing-associated chronic conditions, albeit not without limitations. Several animal models are used to study specific conditions. However, to my knowledge, no rodent model suitable for accurate simulation of pre-pathology events – i.e. *before onset* of accelerated ageing - has been described to date. There are a number of knockout models used to investigate mechanistic role players of the process of ageing such as Nuclear Factor Kappa B Subunit 1 (Nfkb1)−/− mice or Telomerase RNA Component (Terc)−/− mice (Kõks, 2016; Folgueras, 2018) but these are not a suitable simulation of gradual maladaptation pre-onset of accelerated ageing and its related pathologies. The maladaptations onset is immediate, and intervention is not possible.

There are animal models which demonstrate accelerated ageing diseases but are long in duration and induce severe pathologies which does not allow researchers to assess the threshold at which maladaptation becomes irreversible pathology (Yanar, 2011; Azman, 2019).

To design a suitably mild model for investigating delayed accelerated ageing, it is necessary to first understand the methodology used and parameters most commonly assessed in research related to decelerated ageing.

2.6.1 Assessing redox status

Redox status is most frequently assessed using a variety of tests which either measure the number of free radicals, the capacity of the antioxidant mechanisms to quench radicals, or the extent of cellular damage.

A number of assays are set up to measure the number of free radicals within a sample. One such assay is the 5-(and -6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (DCFDA) assay, a fluorescence based assay (Katerji, 2019). The DCFDA fluorescence probe can be used to

detect levels of H_2O_2, in a sample. This probe is able to diffuse into cells and reacts with esterases within the cell to form the hydrolyzed DCFH. H_2O_2 reacts with DCFH and results in the formation of 2',7'-dichlorofluorescein (DCF) which fluoresces at an excitation of 488 nm and emission of 530 nm. The intensity of the fluorescence is therefore indicative of the amount of H_2O_2 in the cell. In a similar mechanism, O_2^- reacts with dihydroethidium (DHE) to fluoresce red at an excitation of 488 nm and an emission of 585 nm. There are various probes used to target radicals in this way, and this has become the method of choice to quantify the radical load within cells or aqueous samples (Katerji, 2019).

Assays designed to test the antioxidant functional capacity of a sample are mainly based on the principle of introducing a standardised amount of free radicals and measuring the ability of the antioxidants within the sample to quench the new labelled radicals. Examples of these are ORAC (oxygen radical absorbance capacity), TEAC (Trolox equivalent antioxidant capacity) or FRAP (Ferric-reducing ability of plasma). The ORAC assay determines the loss of fluorescence based on the antioxidant ability of a sample to prevent the oxidation of the probe compared to a blank/control (Katerji, 2019). It does not give a clear indication of the specific antioxidant mechanisms involved but rather a total measure. The TEAC assay works on a similar principle, where the loss of colour is indicative of antioxidant power. TEAC makes use of the 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)(ABTS) oxidation to detect total antioxidant capabilities(Somogyi, 2007). FRAP measures the ability of a sample to reduce the ferrous–tripyridyltriazine complex indicated by a colour change (Benzie, 1996).

There are also assays assessing the level of specific endogenous antioxidant molecules such as SOD, CAT and the GSSG:GSH ratio (Katerji, 2019). Most of these methods are based on the ability of the antioxidant to inhibit the autoxidation of a colour changing or fluorescent probe or complex. The GSSG:GSH ratio is calculated from an assay which measures the amount of reduced GSH in comparison to oxidized GSH (GSSG). The ratio is an indicator of cellular health, as GSH is the most abundant antioxidant system within cells (Stevens, 2010).

Lastly, a number of tests are available to measure the amount of cellular damage, in three main categories, namely DNA, protein and lipid damage. Protein damage is evaluated by measurement of altered carbonyl groups on proteins. There are three types of damage proteins may incur; oxidation of specific amino acids, peptide cleavage due to free radical exposure and protein cross-linkage as a result of a reaction with lipid peroxidation products (Lobo, 2010).

Damaged lipids can be detected at various stages of oxidation: conjugated dienes are early markers of lipid damage whereas the TBARS (Thiobarbituric) assay measures late marker MDA (Malondialdehyde) (Lushchak, 2016). Conjugated dienes are the result of peroxyl free radicals adding to the carbon-carbon bonds of lipids. These complexes may be salvaged before further oxidation into MDA (Pisoschi, 2015). Metabolites at various stages of lipid peroxidation which are also measured include 8-iso- prostaglandin F2α (8-iso-PGF2α), 4-hydroxy-2-nonenal (4-HNE) and lipid hydroperoxides (LOOH) (Katerji, 2019). 8-iso-PGF2α is measured using rapid

ultra-high-performance liquid chromatography-tandem mass spectrometry whereas 4-HNE can be detected using immunohistochemistry or HPLC. CD's and MDA can both be detected using spectrophotometry using reasonably simple repeatable methods and equipment.

Then there are assays designed to measured DNA damage, namely 8-oxo-7,8-dihydroguanine (Collins, 2005) which detect the hydroxylation of the deoxyguanosine residues. This is mainly done using HPLC coupled with an electrochemical detector (ECD) (Katerji, 2019).

In order to form a holistic picture of redox status, assessments of redox parameters ideally should cover various steps within the cascade of free radical formation and antioxidant capacity increase signalling. Most commonly protein damage is reported in the literature because it is frequently detectable, and a stable metabolite of oxidative damage in various tissues and compartments. It is a good indication of the state of the oxidative balance at the specified time point but may not be the most sensitive parameter to measure to identify the threshold prior to maladaptation. Antioxidant capacity as measured by FRAP has shown to fluctuate according to the stressors placed on the system and may be a better early indication of changes to redox status. The antioxidant capacity upregulates when increased free radicals are sensed, but once damage has overrun compensatory mechanisms a decreased in FRAP has been reported, the sensitivity of this parameter may be a better indication of a shift towards maladaptation prior to pathology.

Preventative medicine aims to identify targets prior to such drastic shifts in cellular homeostasis. The majority of preventative antioxidant medicines investigated to date, are phytomedicines. In the next section, I provide an overview of relevant literature to demonstrate the mechanisms most commonly assessed and elucidated in this context.

2.7. Antioxidant phytomedicines

From pre-clinical studies, there are multiple examples of preventative strategies which have proven effective in reducing oxidative stress – many of these phyto-extracts (Zittermann, 2003), grape derived polyphenols (Xiao-bin, 2011; Petersen, 2016), strawberries (Bialasiewicz, 2013), blueberries (Wu, 2018) and other herbal treatments (Stough, 2012). Plant-derived antioxidants may thus pose a compelling strategy to limit both oxidative stress and inflammation.

Several detailed reviews have been published on anti-inflammatory and/or antioxidant effects of various phytomedicines. In Table 2.2, I merely provide a representative summary to illustrate the wide variety of chronic conditions, as modelled in animal models, showing beneficial effects of phytomedicines.

Study model	Treatment	Antioxidant effects reported	Anti-inflammatory effects	Reference
Murine model of Crohn's disease	ellagic acid (EA)- enriched pomegranate extract		↓ MPO and TNF-α levels	(Rosillo, 2012)
Senescence-accelerated male mice	Resveratrol	↑SOD activity, glutathione peroxidase reaction restored to normal, ↓ MDA		(Liu, 2012)
Collagen-II Induced Arthritis (CIA) Rat Model	*Eucommia ulmoides* Oliv.		↓ RNA levels of IL-6 and IL-17	(Zhang, 2008)
Aging Model Induced by D-Galactose-Kunming mice	Medicinal Mushroom *Cordyceps taii*	↑superoxide dismutase, catalase, and glutathione Peroxidase, ↓ malondialdehyde production	↑phagocytic ability, ↑ spleen and thymus indices	(Xiao, 2012)
7,12-dimethylbenz(a)anthracene (DMBA) induced mammary carcinogenesis in female Sprague Dawley rats	Resveratrol		↓NF-κB activation, downregulation of cyclooxygenase-2, and matrix metalloprotease-9 expression.	(Banerjee, 2002)
Aluminium-Induced Alzheimer's Model in Rats	*Phyllanthus reticulatus* (PR) Poir.	↑SOD and CAT, ↓ MDA, ↓AChE activity		(Uddin, 2016)
Non-alcoholic Fatty Liver Disease Induced by High-fat Diet in Mice	Seed Coat of Euryale ferox Extract	↓malondialdehyde, ↑SOD activity		(Jian, 2019)

Table 2.2. Representative summary demonstrating various animal models which mimic chronic disease or accelerated ageing, and which have reported benefits of phytomedicine interventions in the context of redox and/or inflammatory profile

From this, clearly there is a place for phytomedicines in disease prevention.

Despite the small number of human studies available, data reported from clinical studies indeed support a role for antioxidants in the preventative medicine niche. For example, in a study conducted in elderly women who acutely consumed antioxidant-rich foods such as red wine, strawberries, vitamin C or spinach, marked increases in serum antioxidant capacity were reported four to 24 hours post-consumption, as reflected by increased Trolox equivalent antioxidant capacity (TEAC), oxygen radical absorbance capacity (ORAC) and ferric reducing ability (FRAP) (Cao, 1998). Also in a more chronic study design, benefits have been reported.

For example, rooibos tea (an aqueous extract of *Aspalathus linearis*) was also shown to have preventative potential in humans at risk for coronary heart disease (Schloms, 2014). In a study by (Macharia, 2008) volunteers exhibited increased plasma total polyphenol content after consumption of six cups of tea daily for a six-week period. Importantly there were significant decreases in markers of oxidative damage (conjugated dienes and malondialdehyde). This study demonstrated the potential of a plant derived antioxidant to decrease systemic markers of oxidative stress in at risk populations, but in the absence of clinical pathology.

Resveratrol has shown positive antioxidant modulatory capacity in multiple studies. In a study by Seyyedebrahimi and colleagues (2018), diabetic patients showed increased antioxidant capacity after a two-month supplementation period, combined with decreased levels of detectable damage and free radicals. The most interesting finding was the increased expression of Nrf2 encoding for various genes with products involved in antioxidant mechanisms (Seyyedebrahimi, 2018). Various studies, such as this have been reviewed by Singh et al. (2019) and consistently show antioxidant and anti-inflammatory modulation within various disease states (Singh, 2019). One of the most important findings is the upregulation of sirtuin following intake of resveratrol supplementation This protein is not only responsible for regulating antioxidant mechanisms but is also implicated in cellular senescence and ageing (McHugh, 2018; Singh, 2019). Sirtuin activation by the polyphenol resveratrol can mimic lifespan extending effects and alleviate metabolic diseases.

A significant gap in the literature in the context of prevention of accelerated ageing and associated chronic disease, is that very few studies assess and report on both oxidative and inflammatory status in the same study, while even fewer evaluate outcome in various tissue compartments in addition to circulatory measures. Also, for the purpose of elucidating the threshold at which endogenous counter mechanisms are exhausted and accelerated ageing ensues, clearly milder models and earlier time points should be assessed.

2.7.1 Grape-seed derived antioxidants

Of particular relevance to this thesis topic, a specific group of polyphenols derived from grapes has aroused much interest in the natural antioxidant and anti-inflammatory research niches. Grape derived polyphenols contain a variety of bioactive flavonoids which account for their wide array of actions (Xia, 2010; Abourashed, 2013). Most grape-derived polyphenols

contain mainly catechin, epicatechin, anthocyanidins and/or proanthocyanidins (Fernandes, 2016). The -OH groups of the phenolic rings of anthocyanidins are responsible for their ability to quench free radicals, as well as inhibiting oxidation of lipids by donating electrons and chelating unstable metal ions (Nimse, 2015a). Catechin and epicatechin work in a similar manner but require subsequent reactions to form stable products once -OH groups have been metabolised (Nimse, 2015b). Grape polyphenols' anti-inflammatory abilities stem from their ability to reduce ROS levels and thereby inhibit NADPH oxidase activation and NF-κB signalling, which are sensitive to increasing ROS level (Fernandes, 2016). Grape polyphenols have strong anti-inflammatory properties due to their additional metal chelating properties, removing copper and iron, which in inflammation are important signalling molecules in the inflammatory cascade (Nimse and Pal, 2015).

2.8 Potential risks involved in antioxidant supplementation

In terms of consumer safety, it is important to consider that – depending on the existing host redox status and of course antioxidant dose employed – antioxidants may have potent physiological effects. Thus, in order to prevent undesired effects or toxicity, especially when compounding different supplements, a clear understanding of the mechanism(s) being targeted by preventative strategies is vital, especially in the context of plant-based antioxidants because of the synergistic effects different active ingredients in plant-derived antioxidants may have (Li, 2016).

The risk for antioxidant toxicity has been illustrated in the literature (Rietjens, 2002). An example is ROS-induced MAPK signalling pathways, which can affect differentiation, growth and inflammatory signalling within a cell under normal homeostatic conditions. Under conditions of higher oxidative stress, antioxidant mechanisms are used to attenuate excessive free radicals before they affect signalling and the downstream biological functioning (Bhooshan Pandey, 2009). Upon adding an exogenous antioxidant or antioxidant stimulant, to the extent where free radicals are eradicated and the antioxidant itself becomes pro-oxidant, the beneficial effects of such supplement is outweighed by its cytotoxicity. Oversupply of small molecule antioxidants exhaust endogenous "stabilisers", so that unstable intermediaries are formed, which exert free radical-like damage themselves. A significant concern in the context of antioxidant supplementation is mega-dosing. Mega-dosing and compounding antioxidants can have toxic effects, and this is an issue which needs to be addressed because antioxidants are freely available to laypersons. Mega-dosing is the intake of larger than recommended dosages of a or multiple supplements with the intention of increasing the beneficial effects. Research is required to safely compound antioxidants or administer mega doses because as described above oversupply, especially in the absence of severe pathology, may lead to cytotoxicity (Powers, 2004; Cásedas, 2018).

2.9 Summary

In summary, the phenomenon of accelerated ageing clearly results from a plethora of different role players. An original visual summary of the interactions between free radical production and antioxidant mechanisms, as well as potential therapeutic sites targeted by

preventative phytomedicines, is presented in Figure 2.1. I have pointed out several gaps in our understanding of role players in accelerated ageing – the first being a lack of specific models with which we investigate the progression of accelerated ageing especially the threshold prior to the onset of irreversible pathology. This is imperative to identify potential parameters which we monitor to assess the progression of accelerated ageing and age-related pathologies as well as risk populations. This is important because it also allows researchers to identify possible therapeutic targets which may decelerate or attenuate the maladaptations which take place and lead to pathologies may give insight in not only treatment options but also preventative targets. Secondly the need to elucidate sensitive parameters which reflect small effect size changes before maladaptation takes place and shifts the homeostatic status quo. Considering the complex interplay between effectors of both oxidative stress and inflammation, it is clear that one cannot solely assess either one without the other. The gap in the literature is mainly in the identification of which combined parameters should be assessed prior to severe pathology and which could be targeted in the preventative medicine niche. It is also apparent that assessing a single compartment is insufficient as damage or maladaptation in one compartment may relate to a compensatory mechanism in another. Cross-sectional and comprehensive assessments are necessary to elucidate the whole-body status and this approach may be the key in identifying holistic treatment options.

Plant-derived antioxidants may significantly contribute to delaying this abnormal ageing towards disease, albeit not always without risk.

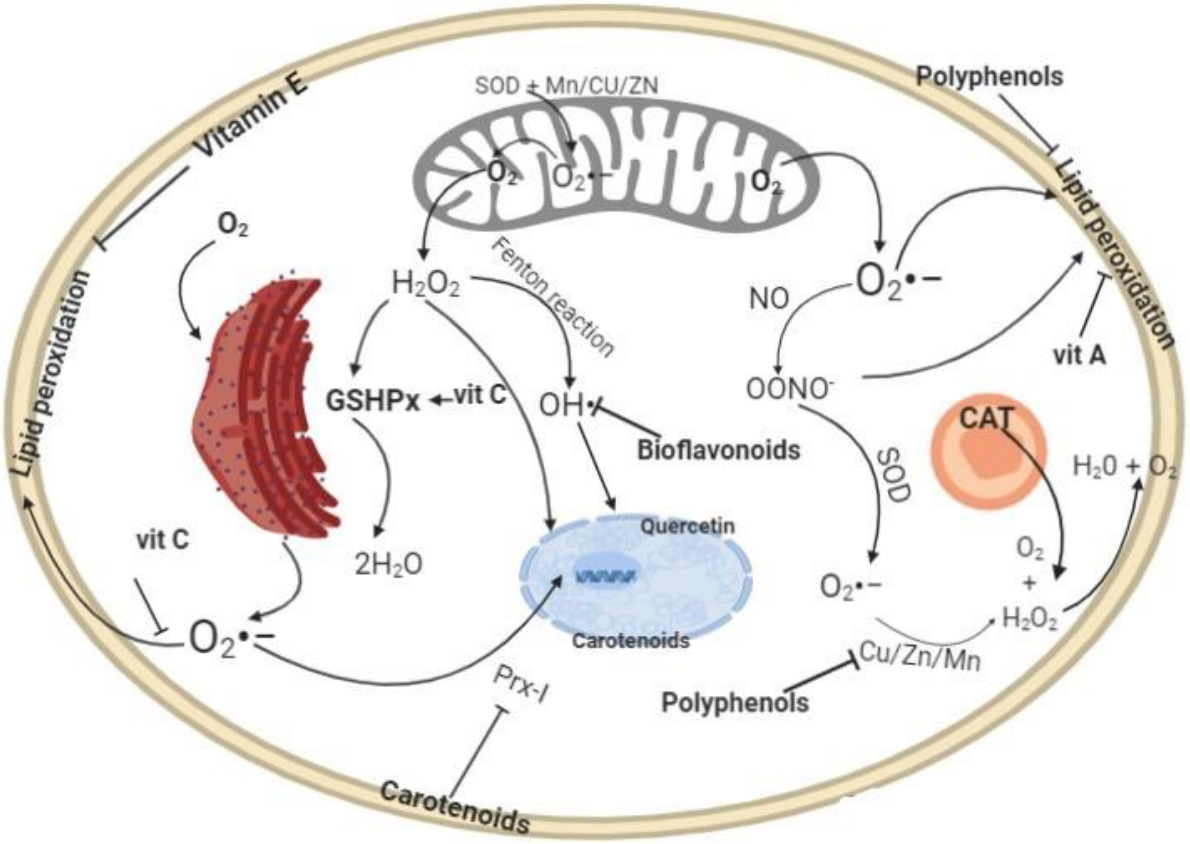

Figure 2.1: Various sites of free radical production and the main targets of different antioxidant defences, including sites of intervention by plant derived antioxidant treatments. Note: SOD- Superoxide dismutase, CAT- catalase, OONO·- peroxynitrite, Prx-1- peroxiredoxin-1, GSHPx- Glutathione Peroxidase, Cu- copper, Zn- zinc, Mn- manganese, NO- Nitric oxide, vit C/A- vitamin C/A, H_2O_2-hydrogen peroxide, OH·-hydroxyl radical, H_2O- water, O_2 -oxygen, O_2·- - superoxide anion

Considering the vast array of potential antioxidant and anti-inflammatory plant derived supplements, especially grape derived, a strategy which has modulatory potential but to a milder extent has the ability to enhance the preventative medicine niche.

This review has shown that a dysregulation of redox status results in oxidative damage, which causes inflammation and ultimately accelerated ageing. This phenomenon of accelerated ageing is valid for many different conditions, which are often associated with advancing age. Throughout this review it has also been highlighted that that suitable models for screening of preventative medicine within pre-pathology and specific disease contexts, are very scarce. The need is to identify the point at which adaptation becomes maladaptation, so that one is able to test the ability of supplements to prevent or delay a switch to pre-pathology. Enhancing the knowledge in this field is essential in order to responsibly prescribe antioxidant treatment in the preventative medicine niche, it is necessary to evaluate both redox pathways and inflammation comprehensively, as this is the only way in which therapeutic targets may be identified and effects (and side-effects) of supplements fully elucidated to begin targeting the burden of ageing and age-related diseases.

2.10 Hypothesis and aims

I hypothesised that it is possible to simulate pre-pathology accelerated ageing in rats, using either an oxidative stress insult or a trigger of chronic inflammation as primary stimulus.

Furthermore, I hypothesised that these models will be sufficiently sensitive to reflect benefits of antioxidant supplementation in the context of accelerated ageing.

In order to test these hypotheses, I aimed to simultaneously assess various redox and inflammatory parameters across various tissue compartments in both an inflammatory and a pro-oxidant model of accelerated aging in rats, in order to identify the most suitable therapeutic targets with which to assess potential efficacy of antioxidant preventative supplementation.

Specific objectives were formulated as follows:

- To modify a known model of D-galactose-induced neurodegeneration to simulate a pre-pathological state mimicking early stage accelerated ageing-associated maladaptation.
- To characterise the oxidative and inflammatory status in the D-galactose model in various compartments by assessing multiple tissue types for both oxidative and inflammatory parameters, as well as potential effects of a well-researched antioxidant
- To establish a model of accelerated ageing using induced rheumatoid arthritis in rodents to characterise the oxidative and inflammatory status in this immune response.
- To characterise the oxidative and inflammatory status in the induced rheumatoid arthritis in various compartments by assessing multiple tissue types for both oxidative and inflammatory parameters in the absence and presence of preventative antioxidant supplementation

Chapter 3

D-galactose model of oxidative stress-induced accelerated ageing in rats

A truncated version of this chapter has been published in Biogerontology (impact factor 3.667).

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3.1 Abstract

Considering that the phenomenon of accelerated ageing contributes to early onset of various chronic diseases, modelling of the relevant dysregulated systems or responses is vital for research aimed at identification of potential therapeutic targets. Here, we aimed to establish a model capable of simulating the redox and inflammatory changes of accelerated ageing – specifically, the aim was early phase accelerated ageing, which would allow therapeutic intervention in a preventative approach prior to clinical disease manifestation. A secondary aim was to evaluate the sensitivity of the model to reflect preventative treatment efficacy.

Daily D-galactose injections (250 mg/kg body mass/day) for 8 weeks in 9-week-old male Wistar rats induced a model of early accelerated ageing (decreased plasma FRAP; $P<0.05$ and altered inflammatory signalling) and an aged profile in lymph node ultrastructure, but did not yet result in telomere shortening. Preventative daily oral antioxidant administration (grape seed-derived polyphenol, 100 mg/kg body mass) prevented tissue ageing, beneficially modulated the inflammatory response, including neutrophil chemokinetic capacity, and tended to increase absolute telomere length.

Data suggests that using a mild model of D-galactose administration than those employed to induce neurodegeneration, simulated the point where oxidative stress starts to overwhelm the endogenous antioxidant response and where a pro-inflammatory phenotype switch manifests. Furthermore, despite the expected small effect size, the model was sufficiently sensitive to reflect benefits of preventative antioxidant treatment in the context of ageing. This model presents a practical model for use in drug discovery, particularly in the context of preventative medicine aimed at limiting oxidative stress-associated ageing. Since this starting point of accelerated ageing as illustrated by current data, is not expected to reflect major ageing-associated changes yet, we recommend that future preventative drug discovery studies employ a longitudinal study design in order to clearly demonstrate the delay of this starting point by preventative strategies.

3.2 Introduction

The concept of ageing is complex and insufficiently understood. There are many theories as to the causes of ageing; these range from the Free Radical theory of ageing (Harman, 2002) and the mitochondrial theory of ageing (Miquel, 1980) to the Replicative Senescence theory of ageing (Hayflick, 1965; Saretzki, 2002), as described in the literature review chapter. Each theory highlights a cellular event as the onset of cellular senescence, confounding factors and

possible treatments to “slow down” or prevent the ageing process. The one commonality across all theories however is the role of oxidative stress in the promotion of ageing.

Accelerated ageing is the presentation of a senescent phenotype at an earlier than expected chronological age and results from cumulative maladaptation and eventually pathologies which are characterised by oxidative stress and chronic inflammation (López-otín, 2013; von Kobbe, 2018). The current theories of accelerated ageing attributes this phenomenon to many factors induced by the modern lifestyle, such as increasing psychological stress, sedentary lifestyle, poor diet and exposure to environmental pollution (Benayoun, 2015; Pole, 2016; Ventura, 2017). Essentially these factors increase the exposure of the body to cellular oxidative damage and low-grade inflammation. Persistent activation of the immune system and imbalance of the redox state results in an increase of the allostatic load (Juster, 2010). The increased load leads to maladaptation of the systems normally in place to maintain homeostasis. Ultimately these persistent perturbations result in pathology which offsets disease and negative symptoms, clinical manifestations which normally are noted with aged populations. Although viewed as two separate mechanisms, oxidative stress and inflammation are linked by many signalling molecules and pathways. Propagation of either of these mechanisms can result in exacerbation and/or activation of the other (Reuter, 2010; Fernández-Sánchez, 2011). Thus, once started, this self-enforcing circle of oxidative damage and inflammation is hard to resolve.

Research into ameliorating accelerated ageing has now become a niche area, where the aim is to prevent disease onset and to address maladaptations which may ultimately lead to chronic disease and mortality (Petersen, 2016). Preventative medicine has recently gained momentum due to the growing burden that chronic non-communicable disease are placing on health care systems globally (Bauer, 2014), with relatively younger populations being diagnosed with conditions normally associated with advancing age (Reddy, 2005; Smith, 2009; Bauer, 2014).

In order to effectively identify therapeutic targets for preventative strategies, efficient models of accelerated ageing are required that would allow high throughput screening to investigate treatment options. One of the main challenges of studying the progression of ageing is developing suitable models that approach ageing-related research from a systems perspective (i.e. in vivo) but at the same time allow for targeted, in depth tissue analysis. Many models currently in use were designed to mimic specific age-related diseases at mid- to late stage and is therefore not appropriately to simulate the relatively gradual changes which occur over time with natural ageing, but which can lead to the onset of pathology once sufficiently cumulative (Ho, et al. 2003). This means that maladaptive changes occurring prior to clinical disease onset - which can be addressed with preventative treatments – cannot be accurately simulated. This limitation is a significant obstacle in the progress of drug discovery in the preventative medicine niche.

We have identified one model with potential to fill this gap in the preventative medicine research methodology. Originally intended (and still used) to simulate Alzheimer's disease-associated neurodegeneration (Ho, 2003; Shwe, 2018), this model warrants further assessment in the context of ageing, as it could address the logistical challenges which usually presents in ageing research models. The rationale behind the original design of the model was to induce excessive cumulative oxidative stress by daily subcutaneous injections of a high dose D-galactose solution. The excessive oxidative stress in the periphery would then induce oxidative stress centrally and offset neurodegeneration over an 8-10-week period.

Most relevant to the consideration of whether the model could be adapted as model for pre-pathology accelerated ageing, Alzheimer's disease is known to present with excessive free radical presence, oxidative damage and (at late stage) also decreased antioxidant capacity and protein aggregation (Valko, 2007; Uttara, 2009) centrally. While it is postulated that oxidative damage is an early hallmark of neurodegeneration, it is also associated with activating an inflammatory response which is more pronounced in mid to late stage disease (Calabrese, 2007; Moulton, 2012). Given these similarities between the model, natural ageing and the accelerated ageing profile reported in chronic diseases with an inflammatory component, we aimed to adapt and expand the use of the D-galactose model to a more general model of accelerated ageing, by characterising its systemic outcome in terms of ageing-related parameters associated with inflammation and/or oxidative stress.

A second aim was to assess the sensitivity of this model to reflect preventative efficacy of a grape-derived polyphenol antioxidant with demonstrated antioxidant and anti-inflammatory effect. (Bagchi *et al.*, 2000; Kruger and Smith, 2012; Myburgh *et al.*, 2012; Kruger *et al.*, 2014; Petersen *et al.*, 2018; Oyenihi *et al.*, 2019). Of particular reference to our topic of accelerated ageing, the ability to modulate oxidative stress and low-grade inflammation may present a broadly applicable preventative strategy for age-related disease and a novel application of this grape-derived polyphenol. Previous work done by myself demonstrated the potential of this grape-derived polyphenol to attenuate age-related detriments in the context of inflammation (Petersen et al., 2018). This study showed how neutrophils isolated from aged human donors, which displayed inefficient activation and chemotactic mobility, could be corrected to a more efficient "younger" phenotype after acute administration of the grape-derived polyphenol *in vitro*. Furthermore, a human clinical trial demonstrated molecular targets on neutrophils which may explain the more efficient movement achieved after polyphenol administration (Smith et al, 2018). An unavoidable limitation – given the normal phases in clinical trial methodology – of this first human study was that the study population was normally healthy, young individuals. Thus, given the expected small effect size possible in this relatively uncompromised population, in combination with the expected significant inter-individual variation, comprehensive redox analysis was not performed. Thus, an *in vivo* model of standardised experimental accelerated ageing may present a more feasible model with which to address these limitations. Here, we present data aiming to pinpoint the

"starting point" of accelerated ageing induced by D-galactose, by assessment of redox and inflammatory status in a variety of tissues.

3.3 Materials and Methods

3.3.1 Experimental animals and ethical considerations

All experimental procedures were conducted in accordance with ethical guidelines set out by the South African MRC. All protocols were ethically cleared by the Animal Research Ethics Committee of Stellenbosch University (ref#0146)(Appendix A).

Forty male Wistar rats were obtained from the Stellenbosch University Animal Facility at 6 weeks old (average body mass, 200 ± 46.66 gram). Rats were subjected to a 12:12 light-dark cycle (lights on at 6am) and housed in groups of 5 rats per cage in a temperature- and humidity-controlled room (23 ± 1°C, 40–60% humidity). Animals consumed standard rat chow and tap water ad libitum. Rats were matched according to body mass and divided into 4 groups; a normally ageing placebo group (NP, which received a placebo of the oral treatment), a normally ageing grape seed-derived proanthocyanidin treated group (NG, which received daily GSP treatment), a D-galactose-induced ageing group (AP, which received daily D-galactose injections only) and a group which received daily D-galactose injections and GSP supplement (AG). The experimental protocol is visually presented in Figure 3.1.

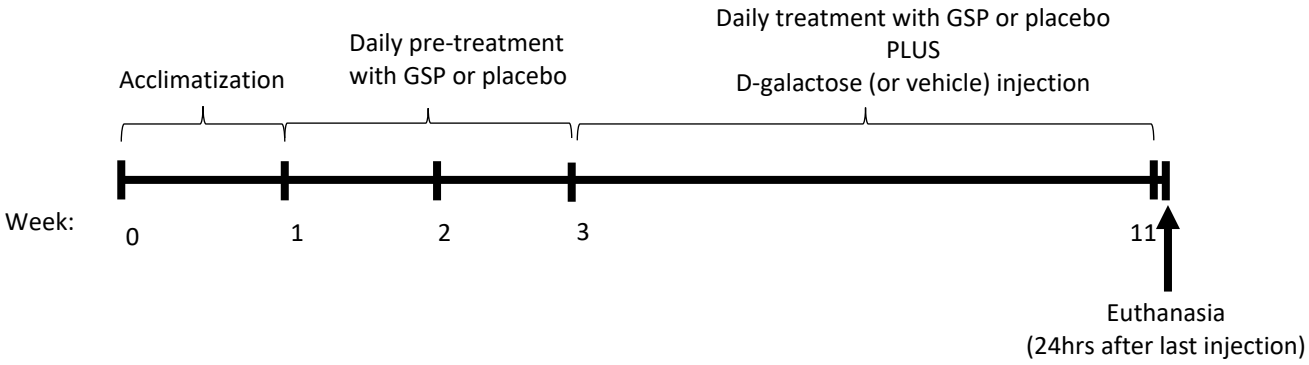

Figure 3.1: Visual presentation of experimental layout to induce accelerated ageing via daily injections of D-galactose.

A model of D-galactose-induced ageing was executed in 9-week-old male Wistar rats by daily subcutaneous injections of D-galactose (G0750-50G, Sigma) dissolved in saline at 250 mg /kg body/ day for 8 weeks. Injections sites were altered on a weekly basis from the nape of the neck to mid posterior of the thoracic region of the animal to prevent discomfort and localized inflammation. The final injection was delivered 24 hours prior to killing for endpoint sample collection. The method was adapted from Stojkovski, Mitrov, & Mladenov (2015), in that the dosage used was decreased from 300 mg/kg/day to 250 mg/kg/day, to achieve a somewhat less severe level of chronic oxidative stress. The period of induction was also reduced to 8 weeks instead of 10 weeks to assess an earlier time point, which would be prior to the previously reported onset of overt pathology (neurodegeneration) (Stojkovski, 2015).

3.3.2 Antioxidant supplement

The grape seed polyphenol (GSP) supplement used in this study (Oxiprovin, Brenn-o-kem Pty Ltd, Tulbagh, South Africa) was derived from grape seed of *Vitis vinifera* and consists of 45% proanthocyanidin B and less than 5% monomers, with the residue made up by long-chain sugars and glycosides attached to the polyphenolic oligomers such as catechin and epicatechin. GSP was dissolved in distilled water at a concentration of 100 mg/ml and added to a gelatin and raspberry jelly mixture (1:10) to form a solid block and fed directly to the animal. GSP-supplemented rats were supplemented with 100 mg/kg GSP once daily for the duration of the 6-week protocol. All placebo animals received a jelly block with added water instead of supplement solution. For rats also treated with D-galactose, a wash-in period of 2 weeks was allowed for GSP before D-galactose injections were initiated. The final supplementation dose was administered 24 hours prior to the protocol endpoint.

3.3.3 Sample collection and initial processing

Body mass and overall wellness were assessed daily. After 8 weeks of D-galactose (or vehicle) injections (i.e. at the end of week 11), rats were killed by inhalation of isoflurane at 4%. Aortic puncture was performed to extract blood into EDTA and heparin anticoagulated Vacutainer® tubes (BD Systems, Plymouth, UK). Tissue samples were excised, weighed and stored for further analysis. EDTA anticoagulated whole blood was centrifuged at 3000 rpm for 10 minutes where after plasma and buffy coats were collected and stored at -80˚C. Neutrophils, peripheral blood mononuclear cells (PBMCs) and plasma were isolated from heparin anticoagulated whole blood using density centrifugation with Histopaque®-1077 (Cat # 10771-100ML, Sigma-Aldrich) where after they were separated from erythrocytes using 5% (w/v) Dextran(Sigma) sedimentation for 45 minutes. Neutrophils were washed with cold sterile PBS and resuspended at $2x10^6$ cells/ml in RPMI 1640 (with glutamate and phenol red, Sigma) media for chemokinesis assessment. PBMCs and plasma were stored at -80˚C (Appendix B).

The whole brain, whole liver, visceral adipose tissue and mesenteric lymph nodes were excised. Samples were snap frozen using isopentane and immediately submerged in liquid nitrogen, where after they were stored at -80˚C. Samples intended for histological analysis were stored in 4% buffered formalin solution.

Tissue samples collected for oxidative stress analysis were weighed and homogenized in cold 10mM phosphate buffered saline (pH 7.2) at appropriate ratios: for liver 100 mg/mL and for adipose tissue 500 mg/mL. Brain tissue was homogenized in cold 50mM Tris–HCl buffer (pH 7.4) in a 100 mg/mL ratio. Tissue was mechanically homogenized and centrifuged at 10,000 x g for 15 min at 4°C (Appendix F). Supernatants were aliquoted and stored at -80˚C until assayed.

3.3.4 Oxidative stress analysis

Oxidative stress status was assessed by various assays measuring free radicals, oxidative damage and antioxidant capacity in plasma. Hydrogen peroxide levels were assessed as representative indicator of free radicals present in the sample, using an OxiSelect™ In Vitro

ROS/RNS Assay Kit (Cell BioLabs) and following the manufacturer's protocol (Appendix J). Oxidative damage was measured using the Thiobarbituric acid reactive substances (TBARS) assay which measures the lipid peroxidation product, malondialdehyde (MDA). The method used was an adapted method as used by Varshney and Kale (1990) (Appendix G). Briefly, the adaptation entails the addition of butanol and saturated sodium chloride, as the MDA-TBA complex formed performs best under acidic conditions. Absorbance was read at 532 nm (with a reference wavelength of 572 nm) using a SPECTROstar Nano® absorbance plate reader (BMG Labtech, Ortenberg, Germany). Ferric Reducing Antioxidant Power (FRAP) was assessed using the method as previously described (Benzie, 1996) (Appendix H). The method used to measure total antioxidant capacity was the Trolox Equivalent Antioxidant Capacity (TEAC) assay (Miller, 1993) (Appendix I).

3.3.5 Evaluation of inflammatory profile

The inflammatory profile of plasma was expressed in terms of inflammatory cytokine profile, as assessed using a Milliplex Rat Expanded Cytokine Magnetic Bead panel (RECYTMAG-65K-05; Merck Millipore; Darmstadt, Germany). MCP-1, IL-4, IL-1β and IL-10 levels were assessed following the manufacturers protocol, with the following CV% for each analyte: MCP-1, 2.3%; IL-4, 3.1%; IL-1β, 3.6% and IL-10, 3.8%. Assay kit lower limits for the linear standard curve was 29.3, 4.9, 2.4 and 7.3pg/ml for MCP-1, IL-4, IL-1β and IL-10 respectively.

The chemokinetic efficacy of neutrophils was assessed by tracking movement of cells along a chemotactic gradient in a Dunn chamber for 30 minutes, using fMLP (N-FORMYL-MET-LEU-PHE F3506, Sigma, USA) as chemotaxin. The method first described (Sapey *et al.*, 2014), was adapted in our laboratory previously (Petersen *et al.*, 2018) and was reproduced for the results reported here. Live cell microscopy was performed and recorded on the Olympus Cell system IX-81 inverted fluorescent microscope system, with F-view cooled CCD camera and using Soft Imaging Systems software. Images were then analysed using Image J (Java Software) with the Mtrack J plugin (Appendix C).

3.3.6 Adipose inflammatory status

Adipose tissue inflammatory status was assessed by measuring levels of myeloperoxidase (MPO) and two adipokines, namely resistin and visfatin, using commercially available rat resistin (RTEN) ELISA kits (E-EL-R0614, Elabscience), rat visfatin (VF) ELISA kits (E-EL-R1067, Elabscience) and a MPO colorimetric assay kit (E-BC-K074, Elabscience) (Appendix D). Adipose tissue was mechanically ground after being submerged momentarily in liquid nitrogen. Thereafter samples were homogenized with ice cold PBS in a 550 mg:1000 ml ratio. Adipose tissue homogenates were centrifuged at 10,000 x g for 15 min at 4˚C where after supernatants were collected and stored at -80˚C. Protein concentration was measured using a Direct Detect® Infrared Spectrometer (Merck, Germany) and the Direct Detect assay-free cards. Homogenate MPO and adipokine levels were assessed using the manufacturer's protocols for each assay and absorbances read on SPECTROstar Nano® absorbance plate reader (BMG Labtech, Ortenberg, Germany) at 450 nm for ELISA assays and 460 nm for the MPO assay. Results were expressed as nanograms of protein per gram of tissue.

3.3.7 Histological analysis

As mentioned, excised organs were fixed in 4% buffered formalin for a minimum of 3 days, before they were impregnated with and embedded into paraffin wax using an automated carousel system which dehydrates tissue with descending grades of alcohol, thereafter clearing the tissue with xylene and impregnating tissue with molten paraffin wax. Tissue was sectioned on a microtome into 5 nm slices and floated onto glass slides. Hematoxylin and Eosin (H&E) stains were performed on mesenteric lymph nodes using standard procedures. Slides were mounted using mounting media (DPX, 06522, Sigma-Aldrich, USA). Tissue sections were visualized at 100x magnification using a bright-field microscope (Nikon Eclipse E400) and images captured with a mounted camera (Nikon DS-Fi2) fitted with a digital sight processor (DS-U3, Nikon, Japan).

3.3.8 Absolute PBMC telomere length analysis

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood anticoagulated with EDTA. PBMCs were resuspended in phosphate buffered saline and a DNeasy blood and tissue kit (Qiagen) was used to isolate DNA from cells according to manufacturer guidelines with slight modifications. Cells were lysed in the lysing buffer overnight in a water bath at 56˚C before being washed and eluted. Thereafter isolated DNA was resuspended in 50ml elution buffer. DNA integrity and concentration was quantified using the Xpose™ Touch & Go reader (Trinean, Belguim) and Drop Sense 16 software (Appendix E). DNA (20ng/µl) was amplified using KAPA SYBR® FAST qPCR Master Mix (2X) Kit (Merck, Darmstadt, Germany), using the primers presented in Table 3.1.

Real time qPCR was used to measure absolute telomere length using the StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA). The method first described by O'Callaghan and Fenech (2011) was modified for rat PBMCs by using 2 x10^6 cells/ml. Cycling conditions for the reaction on StepOnePlus™ Real-Time PCR machine were as follows: pre-incubation of 10 min at 95°C, followed by 40 cycles of 95°C for 15 sec, 60°C for 15 sec and 72°C for 10 sec (Appendix K).

Standard curves were set up using telomere and 36B4 (reference gene) standards and primers, as presented in the table below. All samples were amplified in triplicate and mean cycle time (Ct) values were used in subsequent calculations. All standards and primers were acquired from Integrated DNA Technologies (USA).

	OLIGOMER NAME	SEQUENCE (5'-3')	AMPLICON SIZE
STANDARDS			
	Telomere standard	(TTA GGG)14	84
	36B4 standard	CAG CAA GTG GGA AGG TGT AAT CCG TCT CCA CAG ACA AGG CCA GGA CTC GTT TGT ACC CGT TGA TGA TAG AAT GGG	75
PRIMERS			
	Telomere forward	CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT	39
	Telomere reverse	GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT	39
	36B4 forward	CAG CAA GTG GGA AGG TGT AAT CC	23
	36B4 reverse	CCC ATT CTA TCA TCA ACG GGT ACA A	25

Table 3.1. Primers and standards utilised to determine absolute telomere length of PBMCs.

3.3.9 Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 8.0.2). The normality of data sets was assessed using the Shapiro-Wilk test. Normally distributed data sets were compared using two-way ANOVA and Fishers LSD post hoc tests. Non-parametric data sets were compared using Kruskal-Wallis and Dunn's post hoc tests. The level of statistical significance was set at $P<0.05$.

3.4 Results

3.4.1 Body and organ mass

All experimental groups on average gained body mass at a similar rate over the course of the study protocol, with no apparent effect of either GSP or D-galactose treatments (Figure 3.2).

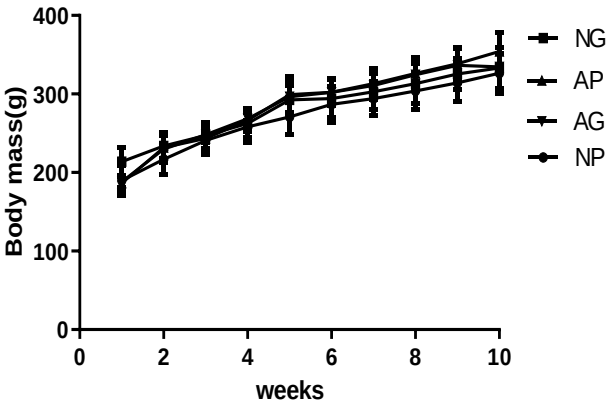

Figure 3.2: Change in average body mass gained over the ten-week duration of the study. Data is presented as means for body mass per group (n=10 each) at weekly intervals. Error bars indicate standard deviations. NP – normal placebo, NG – normal grapeseed, AP – ageing placebo, AG – ageing grapeseed.

Organ masses for all animals were recorded at the end of the study protocol and after dissection. There were no significant differences in organ masses between experimental groups (Table 3.2).

	NP	NG	AP	AG
	mean (SD)	mean (SD)	mean (SD)	mean (SD)
BRAIN	1,87 (±0,07)	1,90 (±0,07)	1,91 (±0,09)	1,90 (±0,09)
NECK FAT PAD	0,81 (±0,29)	0,83 (±0,27)	0,77 (±0,27)	0,77 (±0,21)
HEART	0,90 (±0,10)	0,95 (±0,07)	0,98 (±0,08)	0,98 (±0,08)
SPLEEN	0,60 (±0,06)	0,69 (±0,09)	0,71 (±0,09)	0,70 (±0,08)
LIVER	10,73(±1,05)	10,82 (±0,73)	11,47 (±0,83)	10,87 (±0,97)
VISERCAL FAT	1,73(±1,06)	2,19 (±0,71)	2,36 (±0,54)	2,60 (±0,69)
IN. LYMPHS	1,09(±0,39)	0,77 (±0,33)	1,32 (±0,86)	1,06 (±0,49)
VASTUS	2,12(±0,25)	2,58 (±0,25)	2,58 (±0,26)	2,38 (±0,23)
GASTROC	1,40 (±0,16)	1,58 (±0,21)	1,64 (±0,18)	1,57 (±0,15)
SOLEUS	0,23 (±0,05)	0,26 (±0,03)	0,27 (±0,03)	0,26 (±0,03)
EDL	0,12 (±0,05)	0,11 (±0,02)	0,13 (±0,01)	0,12 (±0,01)
KIDNEY(L)	1,11 (±0,12)	1,12 (±0,09)	1,18 (±0,15)	1,16 (±0,14)
KIDNEY(R)	1,11 (±0,12)	1,17 (±0,07)	1,15 (±0,17)	1,18 (±0,08)
ADRENAL(L)	0,04 (±0,01)	0,05 (±0,01)	0,05 (±0,01)	0,05 (±0,01)
ADRENAL(R)	0,04 (±0,01)	0,05 (±0,01)	0,05 (±0,01)	0,04 (±0,02)

Table 3.2: Average organ masses at protocol endpoint measured in grams (n=10 per group). Data is presented as means and standard deviations NP – normal placebo, NG – normal grapeseed, AP – ageing placebo, AG – ageing grapeseed, IN. LYMPHS -intestinal lymph nodes, GASTROC – gastrocnemius, EDL - Extensor digitorum longus, (L) – left, (R) – right.

3.4.2 Qualitative observations

The frailty index assessment tool recently described (Yorke et al., 2017) is a comprehensive way of quantifying clinical ageing. Although the time point of sample collection was too early in the ageing process to make full use of this tool, some qualitative assessments in line with this tool were recorded. For example, all D-galactose-treated placebo animals (but not those also treated with GSP) exhibited poorer coat quality and mild piloerection at the end of the protocol, when compared to control animals. This clinical indication of early ageing was also reflected at tissue level. Representative H&E images of mesenteric lymph nodes (Figure 3.3 A-D) depict the changes in ultrastructure with D-galactose-induced ageing, as well as the preventative effect of GSP. In frame A, an image of a mesenteric lymph from a control rat exhibits a homogenous ultrastructure with clear, circular follicular formation, minimal

subcapsular space and few darkly purple stained cells (leukocytes) in the peripheral sinuses. A similarly normal picture was evident in groups treated with GSP only (frame B). In contrast, in frame C, the mesenteric lymph node of an animal from the D-galactose-induced ageing group showed extensive fibrosis disrupting the interior cortical ultrastructure, areas of extended subcapsular space and an increased number of leukocytes within the peripheral sinuses. In frame D, a mesenteric lymph node from an animal subjected to both D-galactose-induced ageing as well as GSP supplementation, exhibits general ultrastructure that is very similar to that of the control animals, suggesting preventative efficacy of the antioxidant treatment.

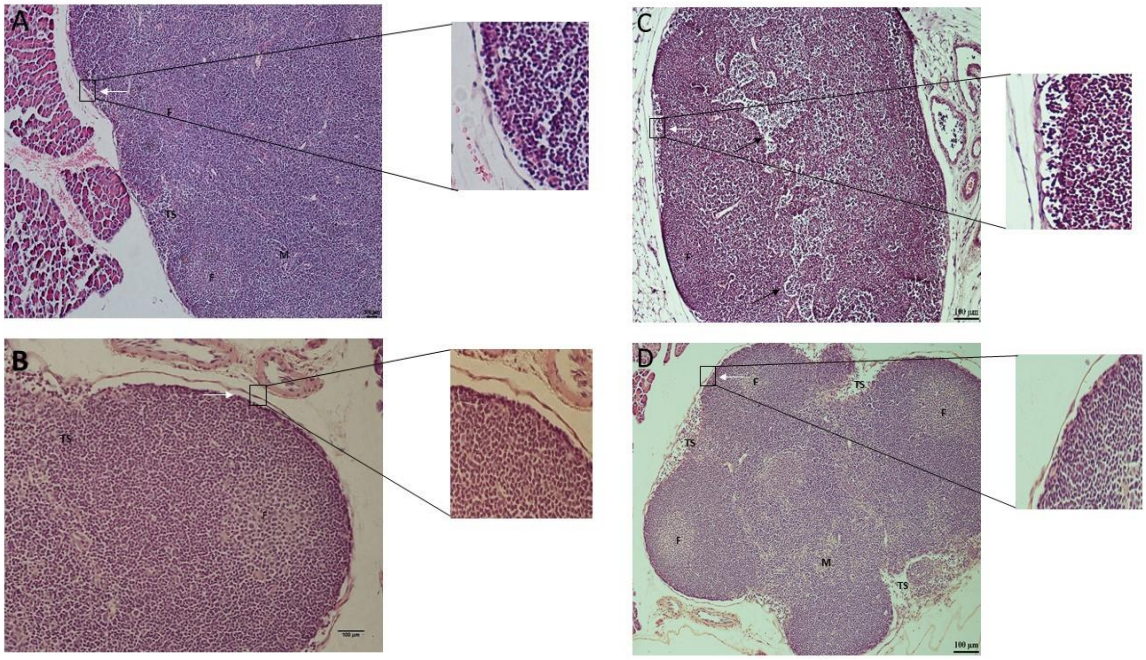

Figure 3.3: Representative histological images of the mesenteric lymph nodes from A) a control animal, showing very few immune cells in the subcapsular space (white arrows), B) a control animal treated with GSP, C) an animal treated with D-galactose, illustrated by abundant cells in the subcapsular space (white arrows) and diffuse fibrosis (black arrows) and D) an animal administered both GSP and D-galactose, where normal histology with clear follicles are visible. TS indicates the transverse sinuses located between follicles of mesenteric lymph nodes. Magnification of 20x was used to capture the images on the left.

3.4.3 Oxidative stress status

Considering plasma data firstly, there were no significant changes in lipid oxidative damage marker MDA (Figure 3.4A) or ROS content (H_2O_2; refer to supplementary material) in groups subjected to either D-galactose administration and/or GSP supplementation. However, the

plasma ascorbic acid equivalent ferric iron reducing capacity (FRAP) was decreased significantly in the D-galactose-treated group when compared to the normal control, while GSP supplementation increased the antioxidant capacity in the D-galactose-treated group towards normal when compared to AP (Figure 3.4B). The GSP supplement tended to increase total antioxidant capacity in plasma in both treated groups (NG and AG) when compared to their respective controls (Figure 3.4C), with the increase in TEAC for AG reaching statistical significance when compared to NP.

MDA, FRAP and TEAC assays were also conducted on brain and liver tissue samples. No significant changes were evident in response to either D-galactose- or GSP treatment (Figure 3.4 D-I), with the exception of brain TEAC, which seemed slightly higher in both D-galactose-treated groups, although this effect did not reach statistical significance due to high variability in especially AG.

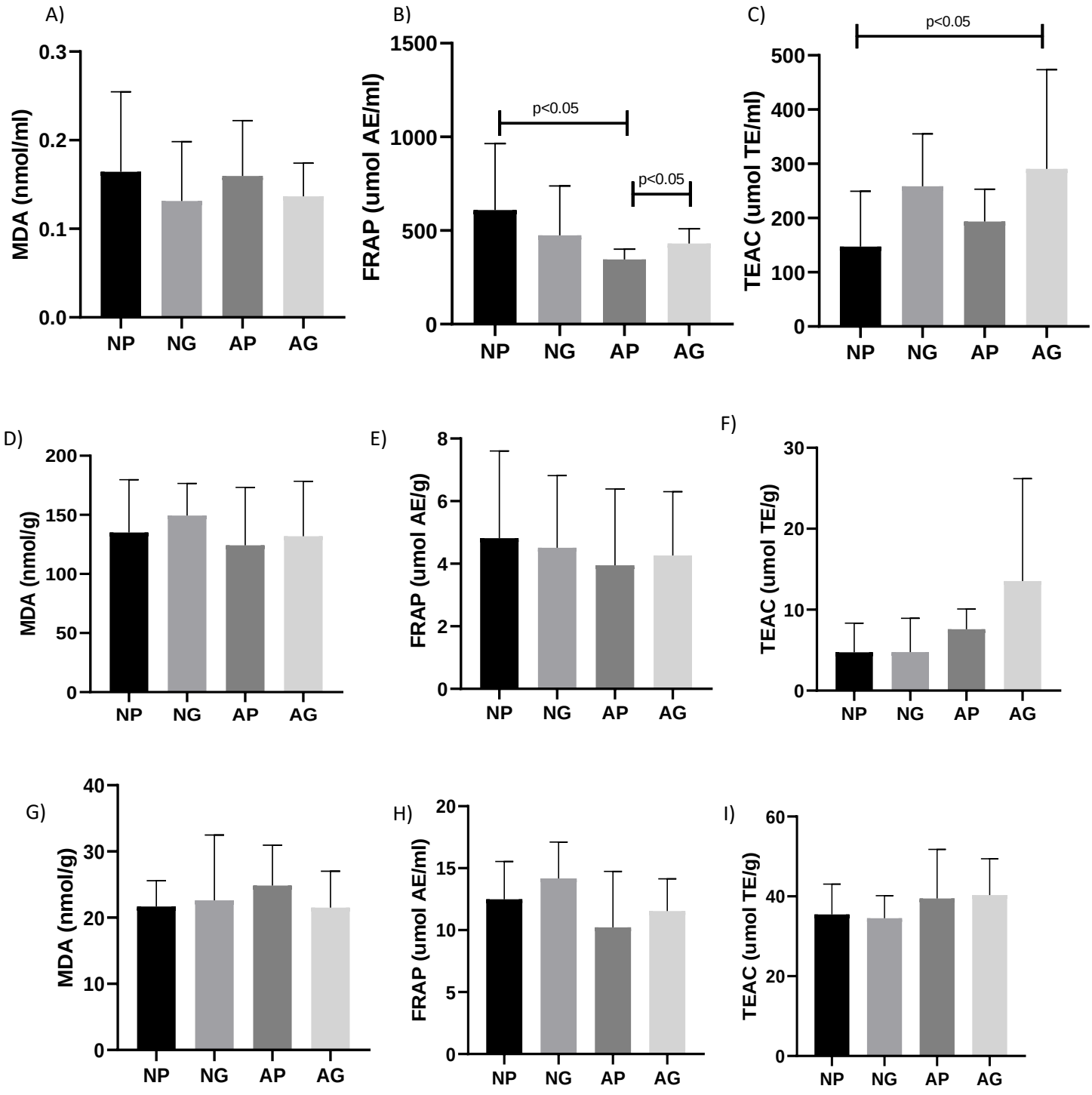

Figure 3.4: Plasma levels of A) TBARS assay measuring MDA, B) Ferric iron reducing power (FRAP) and C) Trolox equivalent antioxidant capacity (TEAC). Similar analysis were also performed for brain (D-F) and liver (G-I) tissue. Data shown are group means and standard deviations. NP – normal placebo, NG – normal grapeseed, AP – ageing placebo, AG – ageing grapeseed.

3.4.4 Inflammatory status

The accelerated ageing protocol resulted in significant pro-inflammatory (IL-1β) signalling, which was reversed in the presence of GSP, with a striking 50% increase in plasma IL-1β levels between normal placebo and aged placebo groups (Figure 3.5A). In contrast, plasma MCP-1 concentration was significantly decreased in response to both D-galactose and/or GSP treatment, when compared to the control (Figure 3.5B). IL-10 was significantly decreased with D-galactose-induced ageing, and lower in the combined treatment group (Figure 3.5C). IL-4 levels decreased with D-galactose ageing but seemed partially prevented by GSP treatment (Figure 3.5D).

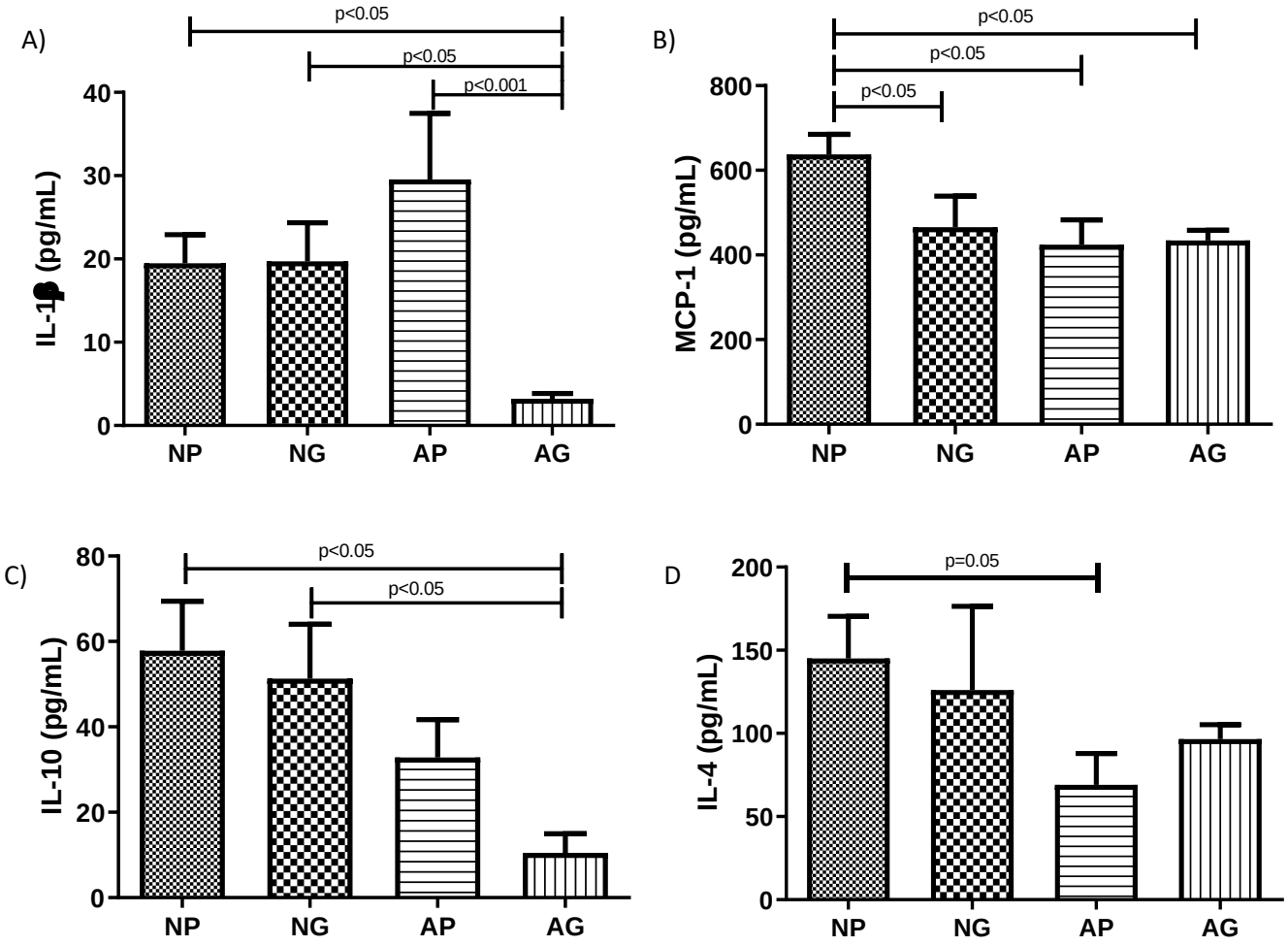

Figure 3.5: Effect of D-galactose-induced ageing and preventative GSP supplementation on plasma cytokine concentrations: a) MCP-1, b) IL-4, c) IL-1β and d) IL-10. Graphs depicted show the group means and standard deviations. NP – normal placebo, NG – normal grapeseed, AP – ageing placebo, AG – ageing grapeseed.

In terms of neutrophil chemotactic capacity (Figure 3.6), the total and linear distances travelled towards a chemotactic signal did not seem to change significantly in response to either D-galactose or GSP administration. However, the fact that linear distance decreased by a third in the group treated with D-galactose may have clinical significance despite the lack of statistical significance. In line with this linear distance as well as total distance was significantly shorter in the group treated with both treatments (Figure 3.6).

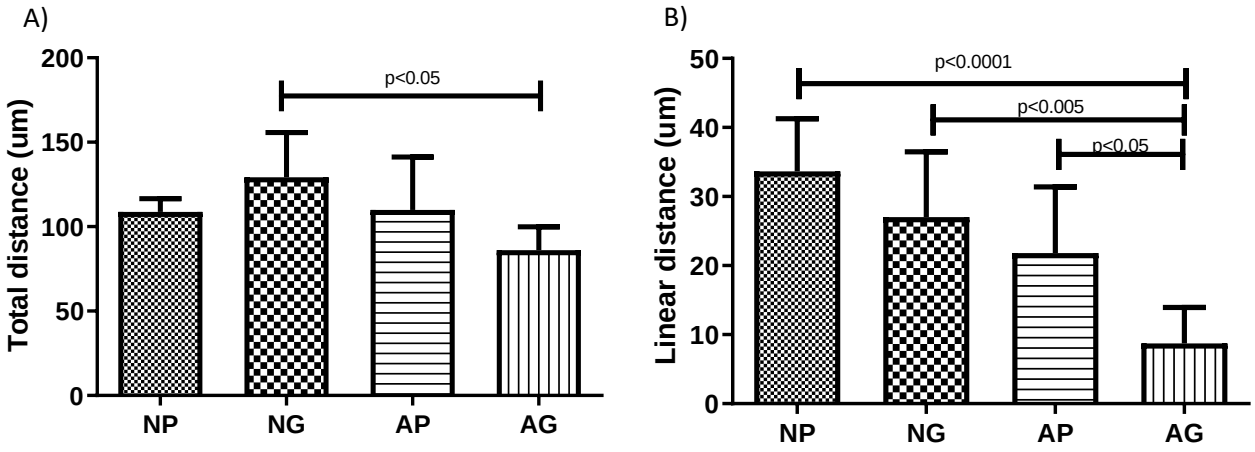

Figure 3.6: Neutrophils chemokinetic ability was measured using live cell imaging to track neutrophil movement towards fMLP in a Dunn chamber. A) the total distance travelled by neutrophils. B) the linear distance covered by neutrophils from starting position to the final position and. Data shown is the group means and standard deviations. NP – normal placebo, NG – normal grapeseed, AP – ageing placebo, AG – ageing grapeseed.

In terms of absolute PBMC telomere length (Figure 3.7), there were no statistically significant changes in absolute telomere length following either treatment. However, there was a trend for increased telomere length after supplementation with GSP (ANOVA main effect of treatment, p = 0.08).

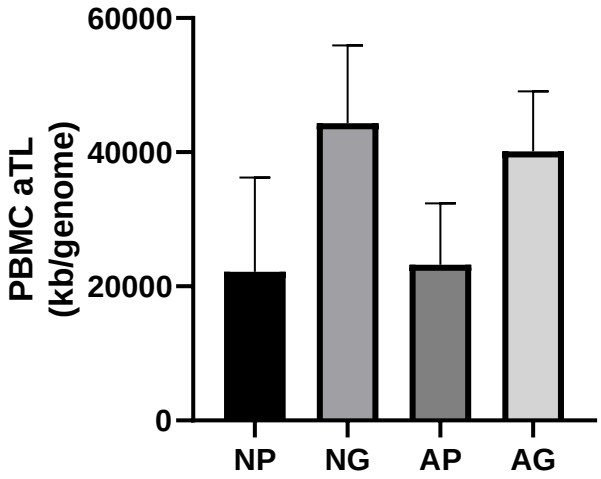

Figure.3.7: PBMCs were isolated from whole blood and DNA extracted which was used to measure absolute telomere length using real-time qPCR. Data is represented as kilo bases per genome in box and whisker diagrams, data is presented as group average (bars) with standard deviation. NP – normal placebo, NG – normal grapeseed, AP – ageing placebo, AG – ageing grapeseed.

A significant decrease in adipose tissue levels of resistin was observed in animals subjected to D-galactose treatment (AP), with a partial preventative effect of GSP (AG) (Figure 3.8A). No statistically significant changes were seen in the concentration of visfatin in response to D-galactose (Figure 3.8B), but in the absence of D-galactose, GSP supplementation tended to increase visfatin when compared to the normal placebo (NP) group. No significant differences were noted in the adipose tissue levels of MPO between experimental groups, although AG appeared to reflect more inter-individual variation (Figure 3.8C).

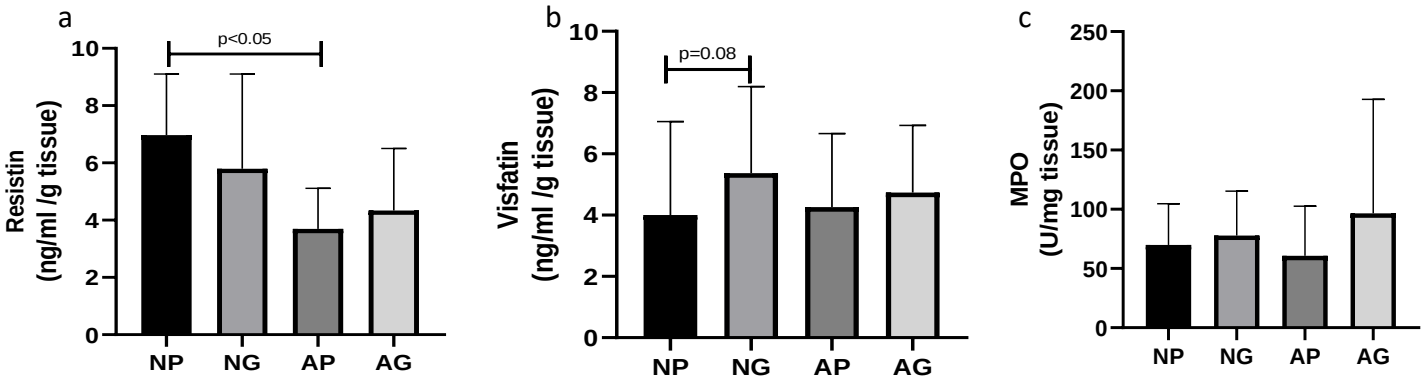

Figure 3.8: The inflammatory status of visceral adipose tissue was assessed using ELISA kits; for resisitn and visfatin, and a colorimetric assay to measure MPO. a) shows the resistin concentration of visceral adipose tissue. Graph b) shows the visfatin levels of adipose tissue. Graph c) shows the MPO

levels of visceral adipose tissue. Graphs depicted show the group means and standard deviations. NP – normal placebo, NG – normal grapeseed, AP – ageing placebo, AG – ageing grapeseed.

3.4 Discussion

The main aim of this study was to establish a model of early transition to accelerated ageing that is induced by excessive oxidative stress, using daily injections of D-galactose. We aimed to assess both suitability and sensitivity of this model in the preventative medicine sphere, for the assessment of efficacy of daily supplements in the prevention of oxidative stress-associated accelerated ageing.

The results obtained in the current study reflects the complexity of simulating accelerated ageing. In particular, early stage ageing, where dysregulation may still be countered by antioxidant treatment as a preventative strategy, is extremely difficult to simulate convincingly, given the small effect sizes that is to be expected. However, given our comprehensive approach in terms of analytical parameters, we are confident that our interpretation of results is accurate.

Firstly, considering the ability to induce systemic accelerated ageing using a somewhat milder model than that used to induce Alzheimer-like neurodegeneration (Stojkovski et al., 2015; El-Baz *et al.*, 2018; Hor *et al.*, 2018; Baradaran et al., 2019), the mesenteric lymph nodes clearly reflect an aged histological phenotype in line with a previous report of tissue damage associated with ageing, depicted in the mesenteric lymph node using H&E stains (Zolla, 2015). Current data suggest that even the milder D-galactose intervention (lower dose and shorter duration) already resulted in significant damage at tissue level. The significant fibrosis observed in the current study – a hallmark of ageing tissue – suggests that a chronic inflammatory response had been activated, as lymph node fibrosis is associated with a pro-inflammatory immune profile (Thompson et al., 2017; Magnuson *et al.*, 2019).

The ageing literature suggests the changes in ultrastructure to modulate the maturation of various immune cells and their function (Thompson et al., 2017; Turner and Neil, 2017). Although immune cell function was not comprehensively assessed in the current study, cytokine data is in line with an interpretation of a relatively aged lymph node after D-galactose treatment. Indeed, the plasma cytokine profile (increased IL-1β and decreased IL-4 and IL-10) is suggestive of chronic low grade inflammation, in line with reports on hyperactivity of the inflammasome in advancing age, as noted in the brain and in circulation previously (Lee, 2017;

Li, 2018; Nam, 2019). In addition, the neutrophil chemotactic activity assay illustrates a more than 30% decrease in neutrophil linear distance despite unchanged total distance travelled in response to a chemotactic stimulus. This is suggestive of decreased neutrophil directional accuracy, as previously reported in neutrophils of aged individuals (Sapey *et al.*, 2014; Petersen et al., 2018). These small but statistically significant changes in the inflammatory profile is suggestive of the gradual, cumulative maladaptive inflammatory changes resulting from chronic oxidative damage and which eventually leads to immunosenescence and a compromised aged phenotype (McEwen, 1998). However, given the relatively early endpoint for sample collection in the current study, a longer duration longitudinal study is required to fully substantiate this interpretation. Nevertheless, the cytokine profile is in line with a switch to an early pro-inflammatory phenotype.

Our data showing no effect on MCP-1 levels further supports our interpretation of an early pro-inflammatory phase of maladaptation. Increased plasma MCP-1 levels are commonly reported in chronic low grade inflammation in obesity, where it is secreted by resident macrophages within the adipose tissue (Ahima, 2009; Tchkonia, 2010) , and in models of advanced inflammation-associated neurodegeneration (Powrie, 2018). The fact that MCP-1 levels were not yet increased in the current study suggests that the pro-inflammatory shift has not yet resulted in the significant neuroinflammation characteristic of the more severe D-galactose model, or sufficient macrophage infiltration into adipose tissue – the latter is further supported by our analysis of adipose chemokines, which reflects no detrimental changes in either resistin, visfatin or myeloperoxidase. Together, this picture is in line with an early maladaptive state.

The changes in inflammatory status in the D-galactose model has been demonstrated to be the result of oxidative damage (Sadigh-eteghad, 2017), as already mentioned. However, given the picture of very early pro-inflammatory changes in the current study and the existence of several endogenous antioxidant systems, it is realistic to expect that not all redox assays will reflect damage at this early stage, as at least some antioxidant counter mechanisms should still be sufficiently functional to neutralise oxidative stressors to an extent.

This was indeed the case. Previous reports of redox changes in more severe D-galactose models include significant oxidative stress (increased ROS, MDA and and Acetylcholinesterase (AChE) activity) and compromised antioxidant capacity (decreased superoxidae dismutase

(SOD), FRAP, glutathione (GSH) levels) in brain tissue of experimentally aged (500 mg D-galactose/kg body mass for 6 weeks) rats when compared to age-matched controls (Garg, 2016). In a similar study by a different research group, circulatory measures demonstrated similar changes in redox status in serum and liver, as reflected by increased oxidative damage (MDA and H_2O_2) and decreased antioxidant activity (SOD, catalase (CAT) and glutathione reductase (GR)) in rats after administration of 150 mg/kg body mass D-galactose per day for 10 weeks (Y. Li, 2014). These studies demonstrate that in more severe models, significantly compromised redox status reflected in multiple parameters is consistently evident. In contrast, in the current, more moderate model, only plasma FRAP decreased significantly in response to D-galactose treatment, suggesting that other systems, such as the GSH and SOD systems, may still be sufficiently functional to prevent large scale cellular oxidative damage (e.g. as would be reflected by an increased MDA). We have recently described a similar profile in the context of rheumatoid arthritis-associated early accelerated ageing, where plasma FRAP (followed by liver FRAP) was again the first indicator of dysregulation (Ross and Smith, 2020). These data are in line with the literature, where it is commonly accepted that cellular damage is only detected after exhaustion of endogenous antioxidant systems (Tan et al., 2018).

Lastly in terms of accelerated ageing, the assessment of absolute telomere length in peripheral blood mononuclear cells was a particularly novel aspect of this study. Telomere length has been proposed as a biomarker of ageing as it dictates the lifespan of a cell (Saretzki, 2002; López-otín, 2013; Xi, 2013) - once cells reach a crucial shortened telomere length due to repeated damage repair cycles, the cell switches to a senescent phenotype which can cause cellular stress to surrounding cells and the microenvironment. The results from the real time qPCR assessment of absolute telomere length suggests that the extent of accelerated ageing has not yet resulted in detectable change to the telomere length of PBMC's, which is in line with the MDA data. While this result indicates that telomere shortening is probably not an accurate diagnostic biomarker in the early phases of ageing, it supports our model as a simulation of early ageing-associated physiological changes.

One of the aims of this study was to complete comprehensive whole-body investigation of different compartments, which are not normally examined, to elucidate changes which may affect the central and peripheral compartments. A particularly novel aspect of the current

study was the analysis of adipokines in adipose tissue. We report D-galactose-associated maladaptation in adipose *in the absence* of obesity, in the form of decreased levels resistin when compared to controls. Resistin is normally responsible for opposing the effects of insulin in peripheral tissue, thus promoting chronic disease development (Maebuchi, 2003; Guzik, 2017). This data has shown that lower levels of resistin are present as well as decreased circulating MCP-1. A possible explanation for this may be lower levels of activated circulating immune cells, which translates to a change in inflammatory signalling (Guzik, 2017).

Turning attention now to the specificity of the model to reflect preventative treatment efficacy, it is important to firstly understand the effect anticipated for the chosen treatment – in the current study, a grape-derived antioxidant. According to several studies, grape seed-derived supplements can reduce oxidative stress by reducing free radicals through its ability to donate a hydrogen proton as well as quenching singlet oxygen via it's multiple -OH groups (Y. J. Zhang, 2015; Fernandes, 2016) – this would directly reduce ROS levels and reduce demands on antioxidant systems (e.g. as indicated by FRAP and TEAC). Coupled with its ability to reduce free radical levels, grape seed antioxidants also have significant metal chelating properties which serve to reduce lipid peroxidation (Shi *et al.*, 2003; Xia *et al.*, 2010; Aybast et al., 2018), thus preventing MDA formation. The grape seed polyphenol investigated in this study has also previously been studied and shown to have significant antioxidant and anti-inflammatory properties by increased oxygen radical absorption capacity (ORAC) - indicative of an ascorbic acid-like quenching of hydroxyl ions by donation of hydrogen protons - in a rodent model of muscle damage (Kruger, 2007; Kruger and Smith, 2012; Myburgh et al., 2012; Kruger et al., 2014).

Indeed, plasma FRAP - the only redox parameter in the current study to be significantly compromised after D-galactose treatment – was significantly higher in D-galactose-treated animals treated with GSP when compared to D-galactose placebo-treated animals, in line with literature on similar supplements (Cao, 1998; Fernandes, 2016). In addition, although not statistically significant, GSP treatment in both control and D-galactose animals showed a consistent tendency to increase plasma TEAC and decrease MDA levels, in line with conferred antioxidant economy and decreased cellular damage. Of further significance, again independent of D-galactose, GSP treatment also showed a tendency to increase absolute telomere length. This is in line with the anti-ageing effect described for grape-derived

polyphenols in different models (Liu, 2012; Petersen, 2016). Furthermore, this result suggests that absolute PBMC telomere length - although not accurately reflecting gradual allostatic effects of ageing *per se* – is an accurate indicator of antioxidant efficacy. This is in line with reports of improved telomerase activity after treatment with other antioxidant phytomedicines (Xiao-bin, 2011; Nisticò, 2015; Thi, 2019). These antioxidant effects were also evidenced in lymph node histology, which in GSP-treated D-galactose-exposed animals resembled a normal phenotype. However, in contrast to the data on tissue ultrastructure and redox changes, the inflammation data is more complex to interpret.

Combined with the direct improvements in redox status already discussed, GSP supplementation also improved certain inflammatory parameters. The ultrastructure of the mesenteric lymph nodes displayed limited fibrosis and maintained follicle integrity in the D-galactose exposed group which were supplemented with GSP, resulting in a similar histological profile to that of the normal placebo group. Related to this, the ageing-associated decrease in IL-4 levels was prevented by GSP treatment. Together, this suggests beneficial outcome in terms of adaptive immune function. When considering the inflammatory system, both the decreased linear distance travelled by neutrophils in vitro in response to fMLP and the decreased IL-1β levels suggest an anti-inflammatory response of GSP, in line with other studies of polyphenols (Liu *et al.*, 2017; Petersen et al., 2018; Ueda-wakagi *et al.*, 2019). In an anti-inflammatory scenario, one would potentially expect to see an increase in circulating levels of IL-10 – however, in the current study, IL-10 levels seem to decrease cumulatively in response to induced ageing and GSP treatment. These decreases in IL-10 should not be interpreted as pro-inflammatory though; NLRP3 inflammasome activation – which leads to IL-1β production – is known to activate IL-10 as counter mechanism (Ip, 2018), thus the significant GSP-associated downregulation of IL-1β production likely also limited the activation of IL-10 production as counter mechanism. Of further interest, a recent paper suggested a more direct antioxidant effect of IL-10 (Gao, 2020), which in the current study was simply not necessary yet at this early stage of dysregulation. Furthermore, GSP was recently reported to reduce the requirement for IL-10 signalling in IL-10 knockout mice (Yang et al., 2014), which further supports our interpretation. We acknowledge however that a more comprehensive assessment of the inflammasome as well as anti-inflammatory signalling is required to confirm our interpretation.

Going forward, we have identified a few more parameters which may be sufficiently sensitive to reflect the early stages of ageing. For example, very recently, primary murine tissue cultured in media containing D-galactose showed dose-dependent mitochondrial dysfunctional changes after only 48 hours (Gao et al., 2020). Thus, assessment of mitochondrial measures such as mitochondrial ROS levels or membrane potential may accurately reflect damage at cellular levels already at an early time point in the D-galactose ageing model. Similarly, another recently published study illustrated mitochondrial damage in murine tissue after 6 weeks of *in vivo* D-galactose treatment, which was reflected by significant decreases (≈25%) in superoxide dismutase (SOD) levels and significantly increased H_2O_2 (indicative of ROS) and 8-hydroxy-2-deoxyguanosine (indicative of DNA damage) levels (Du et al., 2020). Although the lowest dose employed in the latter study was already double the dose employed in the current study, these parameters should be assessed as potential sensitive early indicators in the context of early accelerated ageing. Furthermore, in order to elucidate potential mechanisms of ageing modulated beneficially by experimental treatments, antioxidant mechanisms could be elucidated in more detail, e.g. by assessment of changes in SOD and glutathione peroxidase activity. Together, these recent publications indicate not only the fact that mitochondrial markers are sensitive early markers in the current context, but also that there currently is an increased interest in the use of D-galactose models in research pertaining to all stages of ageing and across a wide variety of specific foci.

To conclude, current data illustrate the milder D-galactose model to be a suitable pre-pathology model exhibiting characteristics of early ageing, oxidative stress and inflammation. In addition, this model seems suitably sensitive to reflect preventative effects of an antioxidant treatment, including potential beneficial effects on absolute telomere length. In the context of preventative medicine, we would further recommend that a longitudinal study design is applied, so that definitive proof of a delayed onset of the starting point of ageing may be generated for new drugs specifically aimed at the preventative medicine niche. Extension of the study protocol to include more time points will additionally enable the assessment of known age-related deficits such as parameters indicative of central amyloid β deposition reported in Alzheimer's disease (Nakamura et al., 2018) and the frailty index developed for ageing studies in rodents (Yorke et al., 2017), to allow for a more comprehensive contextualisation relative to existing ageing literature.

3.5 Acknowledgements

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Chapter 4: Collagen-induced rheumatoid arthritis-associated accelerated ageing in rats

A truncated version of this chapter has been published in Inflammopharmacology (impact factor 3.450).

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4.1 Abstract

Rheumatoid arthritis is prevalent in more than 1% of the global population, with highest occurrence between ages 35-50, which places a huge burden on the economy. Drug discovery for prevention of this chronic disease is therefore a priority. It is known that subclinical progression of many chronic non-communicable diseases are exacerbated via accelerated ageing, a pro-inflammatory phenotype shift. However, since rheumatoid arthritis additionally has significant humoral immune activation, inflammatory signalling – and thus the accelerated ageing profile – may differ from other chronic inflammatory diseases. The current study simulated rheumatoid arthritis onset in a collagen-induced arthritis (CIA) rodent model, to characterise the redox and inflammatory profile at onset of clinical symptoms, in different tissues, in the presence and absence of preventative antioxidant treatment. Data illustrates that an increased free radical level are evident already very early on in RA disease progression. Furthermore, oxidative stress seems to somewhat precede a significant pro-inflammatory state, perhaps due to humoral immune activation. Our data across different compartments further suggest that the compensatory increase in endogenous antioxidant activity is gradually exhausted at a different pace, with the liver showing the first signs of oxidant damage, even before significant evidence exist in circulation. Current data further suggest that preventative antioxidant intervention may have a sparing effect on endogenous antioxidant mechanisms and preserve telomere length to delay disease progression - or at least the accelerated ageing known to exacerbate RA symptoms – although it did not seem to have a significant direct effect on auto-immune activity.

4.2 Introduction

Rheumatoid arthritis (RA) is a growing disease burden and the incidence is expected to grow over the next ten years because of the increasing size of the global elderly population (Van Onna, 2016). With a largely unknown aetiology and robust progression, this autoimmune disease is characterised by a complex model of pathology, such as immune cell infiltration and proliferation of synovium activated by an auto-immune response, resulting in chronic inflammation in the synovial fluid within joints causing pain, stiffness, degradation and weakness. Furthermore, whole body effects such as in fatigue, malaise, cachexia, and loss of appetite, are also commonly reported.

Chronic inflammatory diseases in general have many hallmarks in common with ageing and are linked to exacerbated symptoms in co-morbid diseases also associated with advanced age (Mikuls, 2003), such as dysregulation of redox and inflammatory processes which favour oxidative stress (and damage) and a pro-inflammatory phenotype. Given the still poor understanding of disease aetiology and the complexity of management of auto-immune diseases such as RA and systemic lupus erythematosus (SLE), it is imperative to identify preventative strategies by which clinical manifestation of these conditions may be delayed or prevented altogether. However, RA is more complex than other chronic inflammatory diseases as it directly results from abnormal humoral immune activation, so that the clinical phenotype presents with both chronically activated antibody-mediated immunity and chronic inflammation. This is an important consideration in RA-related research, as the changes in accelerated ageing profile may not follow the same trajectory as in chronic inflammatory diseases where antibody production is not chronically activated.

In recent years, RA sufferers have increasingly turned to plant-derived adjuvants to alleviate disease symptoms without the side effects of conventional medication (Dragos, 2017). Many plant-derived supplements display potent antioxidant potential and moderate anti-inflammatory capabilities, making them ideal for chronic supplementation strategies in the context of RA. The World Health Organisation (WHO) Traditional Medicine Strategy 2014–2023 indeed also outlines the priority that should be placed on research to elucidate best practices in this context.

Many studies on resveratrol (Correâ, 2018; Yang, 2018; Wang, 2019; El-ghazaly, 2020) and other phytomedicines (Saleem, 2020) consistently report beneficial clinical outcome in terms of RA in the collagen-induced arthritis (CIA) model in rodents, which uses immunisation with type II bovine collagen and incomplete Freund's adjuvant to trigger an auto-immune response (Umar, 2012), resulting in inflammatory polyarthritis which has both clinical, immunological and pathological features similar to that of human RA.

Described in this dissertation is a protocol for consistently inducing arthritis in female Lewis rats by immunizing them with bovine type II collagen (CII) emulsified in complete Freund's adjuvant which has been modified to induce an early stage of disease progression in male

Wistar rats. This model is of value not only in defining the underlying pathogenesis of RA, but also as a tool for evaluating pharmacological strategies for treating this condition.

Our group have previously shown that a grape-derived proanthocyanidin extract may be used as preventative strategy to delay oxidative stress-related accelerated ageing (manuscript under review), due to its anti-inflammatory (Kruger, 2012; Myburgh, 2012; Cásedas, 2017; Petersen, 2018) and antioxidant properties (Bagchi, 2000; Pajuelo, 2011; Chedea, 2014).

Therefore, the aim of this study was firstly to characterise the established rodent model of rheumatoid arthritis (Bendele, 1999; Umar, 2012; Oyenihi, 2019) in terms of redox status, cytokine signalling and ageing profile (absolute telomere length) - including both tissue and circulatory compartments of the body, to comprehensively elucidate sites of maladaptation – and secondly, to evaluate the efficacy of a grape seed-derived proanthocyanidin as preventative strategy in the context of RA-associated accelerated ageing.

4.3 Methods and Materials

4.3.1 Ethical considerations

All experimental procedures were conducted in accordance with ethical guidelines set out by the Animal Research Ethics Committee of Stellenbosch University with ethical clearance (reference number 0146) (Appendix A).

4.3.2 Experimental groups and housing

Forty male Wistar rats were obtained from the Stellenbosch University Animal Facility at 6 weeks old and weighing 200 ± 46.66 gram. Rats were subjected to a 12:12 light dark cycle and housed under normal conditions. Animals consumed standard chow and water ad libitum. Rats were randomly divided into 4 groups; a normal control group (NP, placebo-treated), a normal group which received daily grape seed proanthocyanidin treatment (NG), a CIA group treated with placebo (AP) and a CIA group which received daily grape seed proanthocyanidin treatment (AG) (n=10 each).

4.3.3 Collagen-Induced Rheumatoid Arthritis Model

The bovine collagen-induced rheumatoid arthritis (CIA) method described by Bendele and colleagues (1999) as used to induced rheumatoid arthritis in male wistar rats. Bovine collagen type II, incomplete Freund's adjuvant were obtained from Chondrex Inc., WA, USA. In brief, bovine heterologous type II collagen was dissolved in 0.01N glacial acetic acid (2 mg/ml) where after an emulsion was prepared using an equal volume of incomplete Freund's adjuvant as per manufacturer instructions. The bovine collagen/Freund's incomplete adjuvant emulsion was gradually injected intradermally at two sites above the tail region of each rat. Rats were placed under isoflurane (3%) anaesthesia to conduct injections. Initial injections were done on the first day of week 7 and a second booster shot was administered 7 days later in the same procedure with the exception of only one injection at a site slightly above the previous sites. The onset of swelling in rat paws was observed to confirm clinical symptomatic onset of disease approximately 2-3 days after the booster injection was administered.

4.3.4 Antioxidant treatment

Male wistar rats were pre-treated with a grape seed-derived proanthocyanidin (GSP) for 6 weeks before rheumatoid arthritis was induced and throughout the 3-week protocol. The grape seed polyphenol (GSP) supplement (Oxiprovin, Brenn-o-kem Pty Ltd, Tulbagh, South Africa) was produced from grape seed of *Vitis vinifera* and consists of 45% proanthocyanidin B and less than 5% monomers. The remaining residue is made up by long-chain sugars and glycosides attached to the polyphenolic oligomers; catechin and epicatechin. The GSP supplement was dissolved in distilled water at a concentration of 100 mg/ml and added to a gelatin and raspberry jelly mixture (1:10) to form a solid block. The blocks were fed directly to the individual animal at a dose of 100 mg/kg GSP, once daily for the duration of the protocol of 8 weeks. All placebo animals received a jelly block with added water instead of supplement solution. The final supplementation dose was administered 24 hours prior to the protocol endpoint.

4.3.5 Sample collection

Body weights and overall wellness were assessed daily. After week 10 rats were euthanised with inhalation of isoflurane at 4% inhalation. Aortic puncture was done to extract blood into vacutainer® EDTA and Heparin anticoagulated tubes. Tissue samples were excised, weighed and either stored in 4% formalin or for further analysis. Blood drawn was centrifuged and separation in plasma, buffy coats and neutrophil isolations. Brain and liver tissues were snap frozen and stored at -80°C.

Tissue samples used for oxidative stress analysis were weighted and homogenized in cold 10mM phosphate buffered saline (pH 7.2) for liver (100 mg/1mL) and adipose tissue (500 mg/1 mL). Brain tissue was homogenized in cold 50mM Tris–HCl buffer (pH 7.4) in a 100 mg/1 mL ratio. Tissue was mechanically homogenized and centrifuged at 10,000xg for 15 min at 4°C to obtain the supernatants used for the analyses. Supernatants were stored at -80°C until assayed (Appendix F).

4.3.6 Rheumatoid arthritis confirmation

To confirm that rheumatoid arthritis was induced, plasma anti-collagen II IgG titre was assessed by ELISA kit (Chondrex Inc., WA, USA). The manufacturer's protocol was followed precisely. Joint swelling was monitored as described previously (Oyenihi, 2019).

4.3.7 Oxidative stress analysis

Redox status was evaluated using five assays to measure free radicals, oxidative damage, and antioxidant capacity in plasma as well as tissue. Hydrogen peroxide free radical levels were measured using an OxiSelect™ *in vitro* reactive oxygen and nitrogen species (ROS/RNS) assay kit (Cell BioLabs) following the manufacturer's protocol (Appendix J). Oxidative damage was assessed with the thiobarbituric acid reactive substances assay (TBARS), which measures lipid peroxidation product malondialdehyde (MDA). The method used was according to the adapted protocol of (Varshney, 1990)(Appendix G). Absorbance was read at 532 nm (with a reference wavelength of 572 nm) using a SPECTROstar Nano® absorbance plate reader (BMG Labtech, Ortenberg, Germany). Antioxidant capacity was evaluated using two assays namely;

Ferric iron reducing antioxidant power (FRAP) and total antioxidant capacity equivalent to trolox antioxidant capacity (TEAC) assay. FRAP was assessed using the same method as described in the paper by (Benzie, 1996) (Appendix H) and TEAC was performed using previously established methods (Miller et al., 1993) (Appendix I).

4.3.8 Inflammatory status

The inflammatory status as reflected in rat plasma was assessed using a Milliplex rat expanded cytokine magnetic bead panel ELISA-based assay kit (RECYTMAG-65K-05; Merck Millipore, Darmstadt, Germany). Monocyte chemoattractant protein-1 (MCP-1), interleukin (IL)-10, IL-4 and IL-1β levels were assessed following the manufacturer's protocol, with the following coefficient of variation (CV) for each analyte: MCP-1, 2.3%; IL-4, 3.1%; IL-1β, 3.6% and IL-10, 3.8%. Assay kit lower limits for the linear standard curve was 29.3, 4.9, 2.4 and 7.3pg/ml for MCP-1, IL-4, IL-1β and IL-10 respectively.

The chemokinetic efficacy of neutrophils was assessed by tracking movement of cells in a Dunn chamber, after being stimulated with fMLP (Sigma) for 30 minutes. This method was adapted from an earlier method (Sapey, 2014), as previously reported (Petersen, 2018). Live cell microscopy was recorded on the Olympus Cell system IX-81 inverted fluorescent microscope system with F-view cooled CCD camera using the Soft Imaging Systems software. Images were then analysed using Image J (Java Software) with the Mtrack J plugin (Appendix C).

4.3.9 Absolute PBMC telomere length analysis

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood anticoagulated with EDTA. PBMCs were resuspended in phosphate buffered saline and a DNeasy blood and tissue kit (Qiagen) was used to isolate DNA from cells according to manufacturer guidelines with slight modifications. Cells were lysed in the lysing buffer overnight in a water bath at 56˚C before being washed and eluted. Thereafter isolated DNA was resuspended in 50ml elution buffer. DNA integrity and concentration was quantified using the Xpose™ Touch & Go reader (Trinean, Belguim) and Drop Sense 16 software (Appendix E). DNA (20ng/µl) was amplified using KAPA SYBR® FAST quantitative polymerase chain reaction (qPCR) Master Mix (2X) Kit (Merck, Darmstadt, Germany), using the primers presented in Table 4.1.

Real time qPCR was used to measure absolute telomere length using the StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA). The method first described by O'Callaghan and Fenech (2011) was modified for rat PBMCs by using 2 x10^6 cells/ml. Cycling conditions for the reaction on StepOnePlus™ Real-Time PCR machine were as follows: pre-incubation of 10 min at 95°C, followed by 40 cycles of 95°C for 15 sec, 60°C for 15 sec and 72°C for 10 sec (Appendix K).

Standard curves were set up using telomere and 36B4 (reference gene) standards and primers, as presented in the table below. All samples were amplified in triplicate and mean cycle time (Ct) values were used in subsequent calculations. All standards and primers were acquired from Integrated DNA Technologies (USA).

	OLIGOMER NAME	SEQUENCE (5'-3')	AMPLICON SIZE
STANDARDS			
	Telomere standard	(TTA GGG)14	84
	36B4 standard	CAG CAA GTG GGA AGG TGT AAT CCG TCT CCA CAG ACA AGG CCA GGA CTC GTT TGT ACC CGT TGA TGA TAG AAT GGG	75
PRIMERS			
	Telomere forward	CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT	39
	Telomere reverse	GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT	39
	36B4 forward	CAG CAA GTG GGA AGG TGT AAT CC	23
	36B4 reverse	CCC ATT CTA TCA TCA ACG GGT ACA A	25

Table 4.1.: Primers used for determination of absolute telomere length.

4.4 Results

Animals were monitored over the ten-week period and all groups gained body mass at a similar rate for the duration of the study (data not shown). Organ masses for all groups were recorded at the end of the protocol. There were several significant differences in organ masses across groups with the induced rheumatoid arthritis and GSP treated group (AG). The induction of RA caused increases in visceral adipose and mesenteric lymph node mass in comparison to the normal placebo group. Whereas GSP treatment combined with RA induction resulted in decreases in subcutaneous adipose (in comparison to both the NP and AP groups), liver, visceral adipose and mesenteric lymph node mass in comparison to the NP group. GSP with RA resulted in increased spleen mass. These changes depicting the effect of the inflammatory disease and of the disease in combination with the supplement (Table 4.2).

	NP		NG		AP		AG	
	Mean(mg)	std. dev	Mean(mg)	std. dev	Mean(mg)	std. dev	Mean(mg)	std. dev
BRAIN	1,87	±0,07	1,9	±0,07	1,82	±0,08	1,86	±0,08
NECK FATPAD	0,81	±0,29	0,83	±0,27	0,98	±0,33	0, 54$^{*,\#}$	±0,17
HEART	0,90	±0,10	0,95	±0,07	0,92	±0,13	0,91	±0,07
SPLEEN	0,60	±0,06	0,69	±0,09	0,70	±0,14	0,74*	±0,09
LIVER	10,73	±1,05	10,82	±0,73	9,87	±1,38	8,87*	±1,20
VISERCAL FAT	1,73	±1,06	2,19	±0,71	2,36*	±0,65	1,19*	±0,38
IN. LYMPHS	1,09	±0,39	0,77	±0,33	1,35*	±0,43	0,74*	±0,48
KIDNEY(L)	1,11	±0,12	1,12	±0,09	1,06	±0,11	1,07	±0,08
KIDNEY(R)	1,11	±0,12	1,17	±0,07	1,07	±0,11	1,05	±0,12
ADRENAL(L)	0,04	±0,01	0,05	±0,01	0,04	±0,01	0,05	±0,01
ADRENAL(R)	0,04	±0,01	0,05	±0,01	0,04	±0,01	0,05	±0,01

Table 4.2: Average organ mass in rats after 10 weeks of CIA with and without GSP treatment. The data is represented as means and standard deviations (n=10 per group). Statistical analysis: *, statistically different from NP p<0.05; and #, statistically different from AP, p<0.05. IN. LYMPHS -intestinal lymph nodes, GASTROC – gastrocnemius, EDL - Extensor digitorum longus, (L) – left, (R) – right.

4.4.1 Rheumatoid arthritis confirmation

Rheumatoid arthritis onset was confirmed in both collagen-treated groups, by significant increases in anti-collagen IgG titre when compared to controls (Figure 4.1).

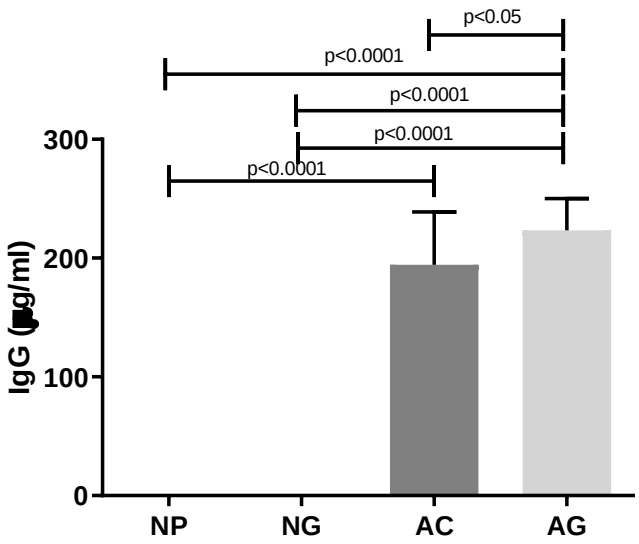

Figure 4.1: Plasma anti-collagen IgG titre in CIA rats with and without GSP preventative treatment. Graphs are means and standard deviations (n=10 per group)

Representative images of the hind paws of rats after the induction of collagen-induced rheumatoid arthritis illustrate significant joint swelling and erythema in CIA (Figure 4.2).

A B

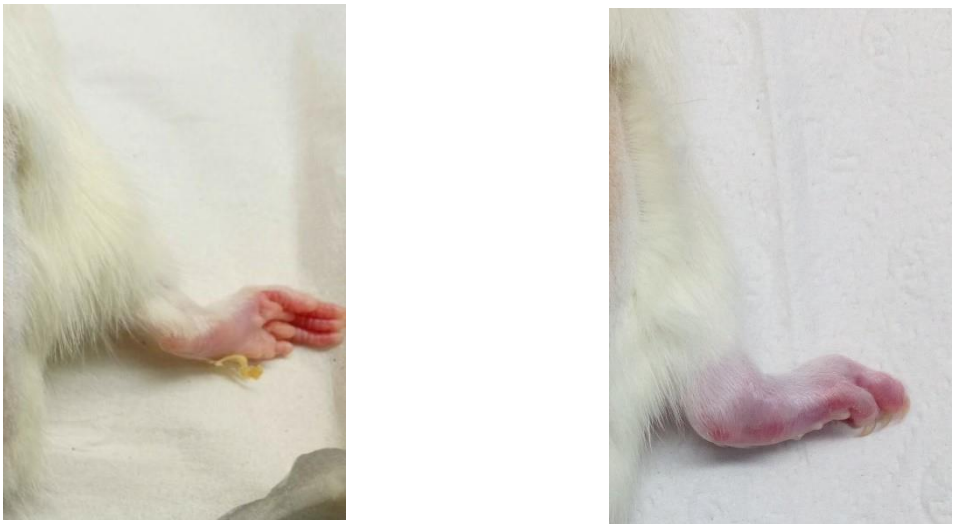

Figure 4.2: Representative images of right hind paws of rats a) before and b) after induction of collagen induced rheumatoid arthritis.

4.4.2 Oxidative stress analysis

In terms of redox status as reflected in the circulation, there was a significant increase H_2O_2 in both RA groups as shown in Figure 4.3. This increase was exacerbated by the supplementation of GSP.

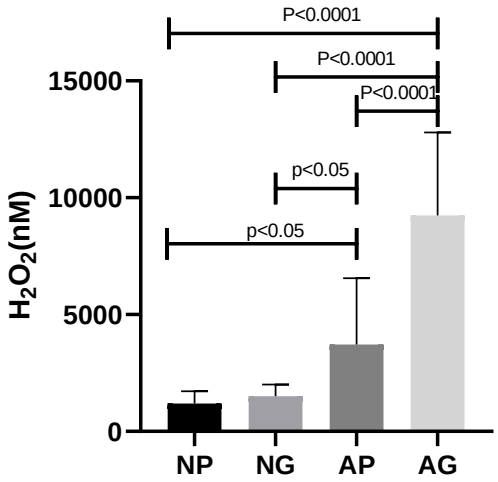

Figure 4.3: graph showing results of the OxiSelect assay which measured the levels of H_2O_2 radicals in the plasma for all groups. Graphs depicted show the group means and standard deviations.

There was a significant decrease in plasma lipid peroxidation marker MDA (Figure 4.4a). In terms of plasma antioxidant capacity (Figure 4.4b&c), only FRAP seemed to be affected by RA, although the decrease only reached statistical significance in AG (Figure 4.4b), suggesting a cumulative effect of RA and GSP.

In the liver, MDA was significantly higher in AC, but not AG, when compared to control (Figure 4.4d). Both FRAP and TEAC seemed slightly reduced in AC, and at least in part corrected by GSP, although not all differences reached statistical significance (Figure 4.4e&f). In the brain itself, there were significant changes in brain tissue redox status. In line with results in circulation, MDA was again decreased in both RA groups when compared to the control. In terms of antioxidant capacity, in contrast to findings in circulation, both FRAP, and TEAC were increased in both RA groups, independent of GSP, when compared to the normal control group (Figure 4.4g-i).

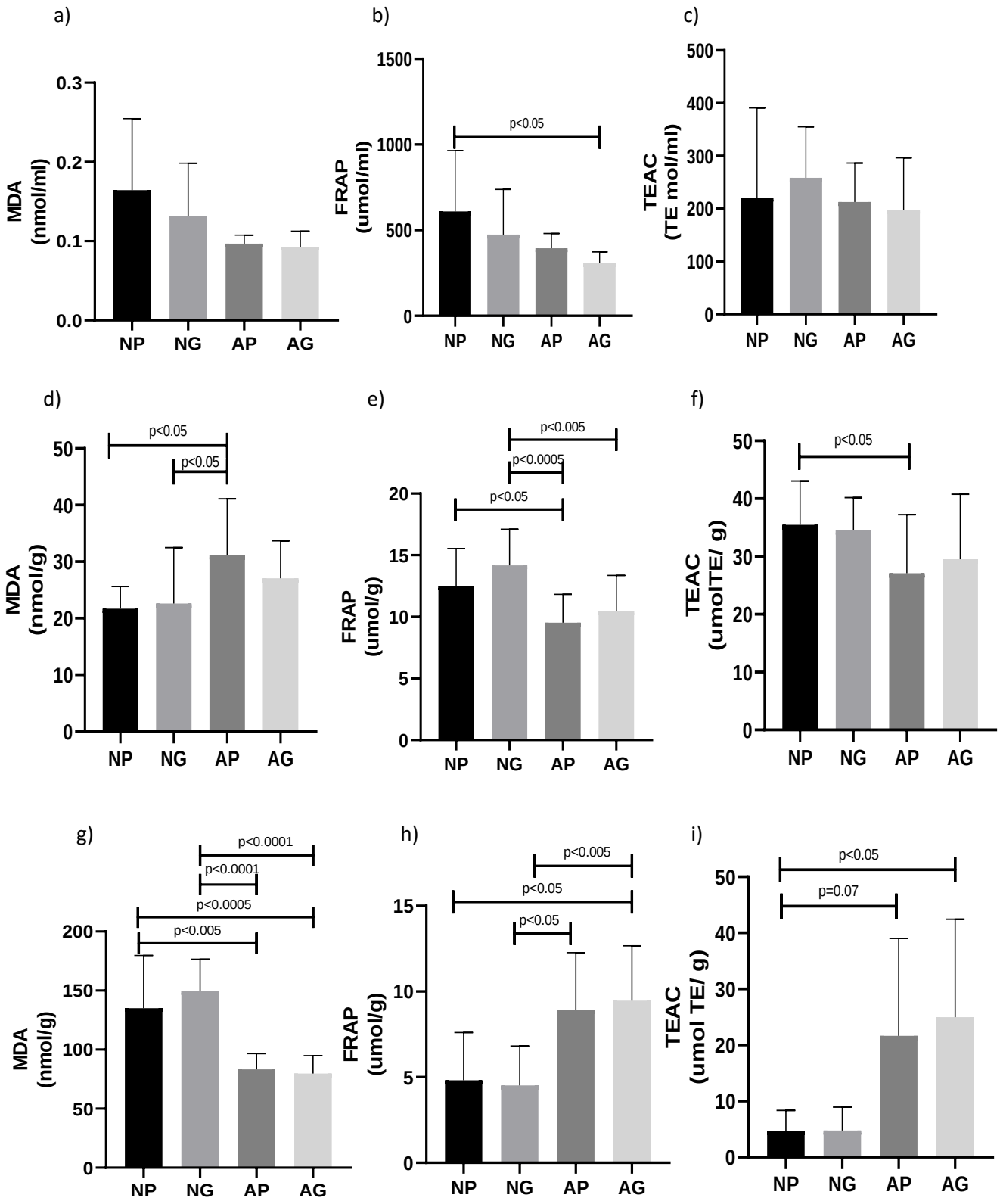

Figure 4.4: a) TBARS assay measuring MDA, b) Ferric iron reducing power (FRAP) and c) Trolox equivalent antioxidant capacity (TEAC) of plasma comparing all groups. Images d-f) MDA, FRAP and

TEAC of liver tissue. Graphs g-i) MDA, FRAP and TEAC of brain tissue. Data shown are the group means and standard deviations.

4.4.3 Inflammatory status

In terms of neutrophil chemotactic capacity, the total distance travelled towards a chemotactic signal was significantly increased in response to rheumatoid arthritis induction, which was partly attenuated by GSP supplementation (Figure 4.5a). Linear distance followed a similar pattern, although the GSP effect did not reach statistical significance (Figure 4.5b).

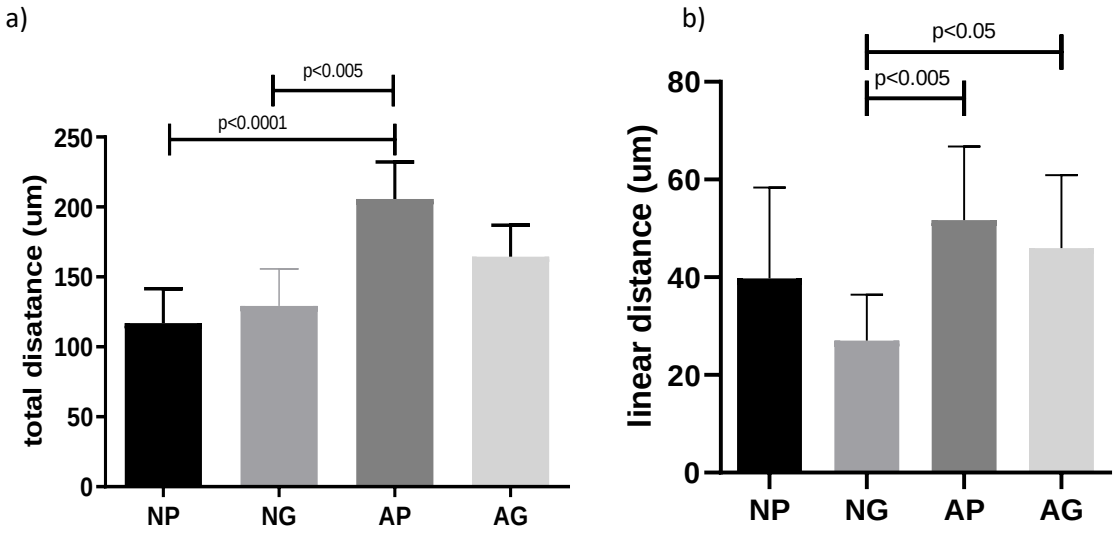

Figure 4.5: Neutrophils chemokinetic ability was measured using live cell imaging to track neutrophil movement towards fMLP in a Dunn chamber. The graphs depict a) the total distance travelled by neutrophils. b) the linear distance covered by neutrophils from starting position to the final position. Data is displayed as average micrometer per group and standard deviation.

When investigating the circulating inflammatory cytokines, we found that although there were no statistical differences in IL-1β, there was a tendency for this pro-inflammatory cytokine to increase when RA was induced (Figure 4.6a). While MCP-1 did not seem to respond to grapeseed supplementation or the induction of rheumatoid arthritis (Figure 4.6b). IL-10 plasma concentration was significantly increased when rheumatoid arthritis was induced in animals when compared to normal (NP) and grapeseed control (NG). GSP supplement while rheumatoid arthritis was induced decreased the level of Il-10 back to a concentration similar to control (NP and NG) levels (Figure 4.6c). IL-4 is significantly decreased in both collagen rheumatoid arthritis groups (AC and AG) when compared to normal controls (Figure 4.6d).

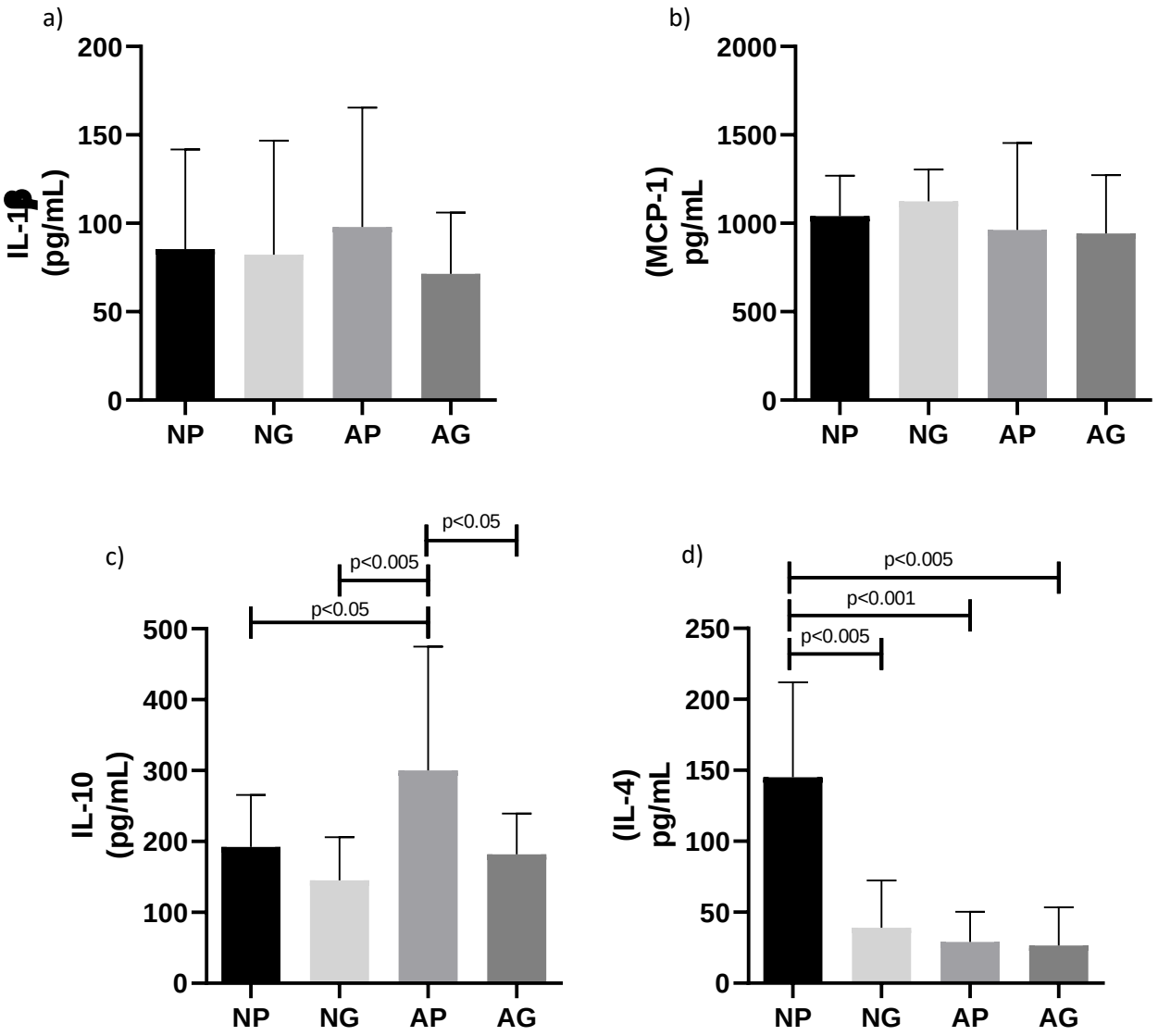

Figure 4.6: Inflammatory cytokines were assessed using a Multiplex assay measuring plasma concentrations of the following cytokines: a) IL-1β, b) MCP-1, c) IL-10 and d) IL-4. Graphs depicted show the group means and standard deviations.

Grapeseed supplementation combined with the induction of rheumatoid arthritis significantly increased telomere length in comparison to the normal placebo control (Figure 4.7). There were no notable differences between arthritis induced groups, but the telomere length was conserved to a similar extent as that observed after grapeseed supplementation under normal conditions.

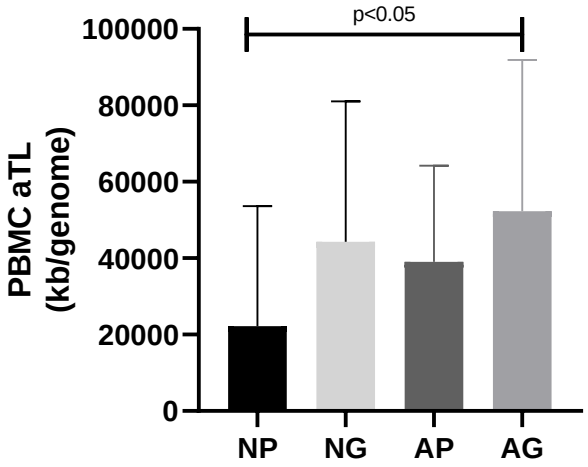

Figure 4.7: PBMCs were isolated from whole blood and DNA extracted which was used to measure absolute telomere length using real-time qPCR. Data is represented as kilo bases per genome as mean and standard deviation.

4.5 Discussion

Rheumatoid arthritis activates both the innate and humoral immune systems and is characterised by significant oxidative damage. The major aim in the current study was to investigating the redox and inflammatory profile of this model of early RA development in order to elucidate preventative strategy targets specific to RA. A secondary aim was to evaluate potential modulatory effects of a grape seed-derived polyphenol supplement as a preventative modality in rheumatoid arthritis.

Successful experimental induction of RA was confirmed in rats by observation of clinical symptoms such as joint swelling and erythema, as well as significantly increased plasma levels of rat anti-collagen IgG antibodies, in line with other studies using this model (Bendele, 1999; Oyenihi, 2019).

Several studies in animal models of RA have reported altered redox status (Oyenihi et al., 2019; Stamp et al., 2012; Vasanthi et al., 2009) that is characterised by increased free radicals, increased oxidative damage markers and decreased antioxidant capacity. In the current study, we expand on the literature by assessments of these parameters in both the circulatory compartment and muscle tissue. Furthermore, we employed a time point early in disease development and induced RA only after animals have reached adulthood. By taking this approach, we were able to demonstrate a protocol ideally suited for the study of preventative strategies, as we were able to perform sample collection at a time point in disease development that was early enough to reflect activity of endogenous compensatory mechanisms, and only early markers of damage that were compartment specific.

Our model differs from results reported in more severe, or longer duration, protocols. For example, we report significantly decreased levels of plasma MDA in animals with induced RA

when compared to controls. Two factors potentially contributed to this finding. Firstly, we have chosen a relatively early sampling time point, at 21 days after experimental induction of RA. Although other groups have reported increased oxidative damage at this early time point, they used different strains of rodents and females, who have been reported to be more sensitive to RA induction (Ibraheem, 2013; Wilson-Gerwing, 2013). Several studies using a longer experimental protocol have reported increased MDA (Choi, 2006; Vasanthi, 2009; Umar, 2013) suggestive of established and chronic oxidative damage, although more comprehensive redox status assessments yield varied results in the hands of different research groups (Choi, 2006; Zhang, 2008; Arntz, 2015; Oyenihi, 2019). Together, this data indicates the importance of comprehensive model characterisation and optimisation, as well as the importance of inclusion of both genders in drug discovery investigations. Secondly, the age of the animals was likely another contributing factor in the milder disease outcome in the current study. We chose to induce RA in animals at 12 weeks of age, to allow for rats to reach adulthood and develop fully functional immune systems and endogenous antioxidant defences (Holsapple et al., 2003). In our opinion, this is a more accurate choice for simulation of RA that other studies who commonly induce RA at ages from 6-8 weeks (Choi, 2006; Zhang *et al.*, 2008; Ibraheem et al., 2013), when rats have barely reached adolescence (Sengupta, 2013). Given the generally poor translation of results from rodent models – and in particular in the preventative medicine niche - into human ones (Smith, 2018), it is imperative that animal simulations of disease mimic patient demographics as closely as possible, to maximise accuracy of results obtained.

Using adult animals and a time point early after clinical onset of RA symptoms, oxidative damage was not yet detectable in circulation, although oxidative stress was indeed clearly present, as is evidenced by the increased plasma hydrogen peroxide levels. Oxidative damage was at this stage likely prevented due to endogenous antioxidant systems being able to attenuate the damage caused by free radicals in specific tissue compartments. This interpretation is supported by the increased antioxidant capacity (TEAC), suggesting that an upregulation of endogenous antioxidant activity was still effectively ameliorating lipid peroxidation. This is in line with a report in human patients, where significant increases in antioxidant capacity was reported in combination with significantly increased levels of hydrogen peroxide (Veselinovic, 2014). Interestingly, increases in circulatory hydrogen peroxide levels has been noted in ageing literature as well (Giorgio, 2007), where this increase in oxidative stress is known to be a by-product of the inflammatory process. This suggests that already this early in RA disease development, accelerated ageing has a significant role, suggesting the potential of an antioxidant strategy in this context. Of further interest, in the current study a decreased MDA and somewhat increased antioxidant capacity (FRAP and TEAC) was reported in the brain, while an opposite profile was present in the liver. This suggests on the one hand that early on in disease, the brain is exhibiting superior anti-inflammatory capacity, and on the other, that the liver is a sensitive early tissue indicator of oxidative damage (or prevention thereof) and thus most useful as investigative target for RA preventative drug discovery research. On a more clinical note, these results, as well as the

beneficial effects of the polyphenol supplement, is in line with previous research which proposes, that the significant risk for dementia in RA patients (Ungprasert, 2016) may be able to be delayed by antioxidant intervention starting very early in disease progression.

Turning attention now to the inflammatory signalling in RA, concentrations of a variety of cytokines were evaluated to gain insight into different branches and functions of the immune system. Although significantly high levels of the macrophage chemoattractant MCP-1 are present in the synovium of RA patients (Szekanecz, 1998), and IL-1β is released as pro-inflammatory chemoattractant from damaged tissues, both plasma MCP-1 and IL-1β levels were similar to controls in the current study, suggesting that oxidative damage precedes a significant inflammatory response in RA aetiology, or that pro-inflammatory secretion may occur only localised in affected tissue at this point.

In terms of cytokines directly linked to RA progression, increased levels of IL-10 and IL-4 are implicated in the disease progression (Vervoordeldonk, 2002). Although IL-10 is commonly accepted to have anti-inflammatory effect, such as facilitating downregulation of TNF-α secretion, it has also been shown to activate the inflammasome and exacerbate rheumatoid arthritis (Greenhill, 2014). This is in line with current data, which reflects an increased IL-10 in RA animals. Significantly, this increase was prevented by polyphenol treatment, which has been reported to downregulate the action of the inflammasome (Liu, 2017). IL-4 is vitally important to stimulate the production of antibodies and its presence in synovial fluid has been linked to protection of cartilage against degradation (Van Roon et al., 1996; Park et al., 2017). Plasma IL-4 was significantly decreased in RA groups, which may suggest that IL-4 secretion has not yet been significantly upregulated despite the significant presence of auto-immune antibodies detected in circulation. It is also possible that IL-4 production at this point may be localised in the synovium, but this remains to be confirmed, as synovial fluid was not analysed in the current study.

In terms of the intervention treatment, GSP supplementation did not affect IL-4 levels, but did improve neutrophil chemokinetic mobility. Neutrophils are implicated as one of the major role players in the pathogenesis of rheumatoid arthritis (Wright, 2014). In the ageing literature, diminished neutrophil capacity to move directly toward a chemotactic signal with as little as possible disturbance to surrounding tissue, is implicated as major cause of inflammatory tissue damage(Sapey *et al.*, 2014; Petersen et al., 2018) and resulting telomere shortening (Petersen, 2016). In the current study, neutrophil chemokinetic accuracy was compromised in RA animals (increased total distance and decreased linear distance) – however, GSP preventative treatment prevented this detrimental phenomenon. In line with this, GSP treatment was associated with a tendency for somewhat longer telomere length. Together, these findings suggest specificity of the GSP treatment as anti-inflammatory and antioxidant, while it seemed to have little direct effect on antibody production. This suggests that although GSP may have significant benefit early in disease to limit oxidant and inflammatory damage, it does not directly affect auto-immune progression.

It is evident that there are several similarities between the profile seen in early RA and that of oxidative stress-driven accelerated ageing (Van Onna, 2016). The oxidative stress data elucidated in this study shows that increased free radicals are evident already very early on in RA disease progression. Oxidative stress seems to slightly precede a significant pro-inflammatory state, although some pro-inflammatory changes are evident as well. Our data across different compartments further suggest that the compensatory increase in endogenous antioxidant activity (Burton, 2018) is gradually exhausted, with the liver showing the first signs of oxidant damage, even before significant evidence exists in plasma. Current data further suggest that antioxidant intervention at this point may have a sparing effect on endogenous antioxidant mechanisms to delay disease progression - or at least the accelerated ageing known to exacerbate RA symptoms.

In conclusion, current data show that even at the early time point assessed, significant redox and inflammatory dysregulation has occurred. Furthermore, a grape seed-derived proanthocyanidin antioxidant extract was shown to have preventative benefit in this context, although it did not directly modulate the auto-immune response itself. Given the positive effects of early antioxidant treatment, it is recommended that RA high risk populations – as well as patients with early clinical disease onset – should consider preventative antioxidant supplementation to delay disease progression.

Chapter 5
Conclusion

Chronic disease has become a significant and growing burden on global health systems, and the situation is further exacerbated by ever increasing incidence of co-morbidities. This thesis highlights the need for suitable models with which to investigate the onset of accelerated ageing, i.e. to elucidate the threshold at which homeostatic disturbances caused by stressors result in maladaptation leading to clinical disease onset. Using these tools, candidate preventative medicine strategies may be more accurately evaluated.

Oxidative stress and inflammation have been identified as the main aetiological factors which display maladaptation in many chronic diseases and as a result accelerated ageing. It therefore seems imperative to investigate a threshold prior to irreversible dysregulation which can be targeted with preventative strategies to delay or prevent the onset of accelerated ageing and disease. As mentioned, the difficulty of investigating such a threshold is that there are not any suitable models which mimic the gradual cumulative maladaptation which precedes clinical disease onset via accelerated ageing. Natural (chronological) ageing models are resource intensive, and in human studies have multiple uncontrollable confounding factors. A second consideration is the ability to do comprehensive multi-compartment investigations to elucidate tissue specific changes.

This thesis aimed to investigate the potential of two models to mimic a state of early accelerated ageing and to characterise the *earliest* dysregulatory changes noticeable in oxidative stress and inflammation parameters, both before and shortly after clinical disease onset. The rationale for this was to identify the most vulnerable mechanisms contributing to an unfavourable redox status, which may be targeted for preventative strategies.

In addition, the simultaneous execution of these studies ensured rodent conditions were consistent and eliminated confounding factors, enabling direct comparison of results obtained and a comprehensive investigation of inflammatory and oxidative status.

Data from the D-galactose study showed an altered redox status in animals exposed to D-galactose, when compared to normal controls. A novel finding was the decreased FRAP that was evident in the absence of detectable damage, confirming that exhaustion of this compensatory antioxidant mechanism precedes other signs of dysregulation. This was particularly evident in the plasma in the D-galactose study as depicted in the graph below (Figure 5.1).

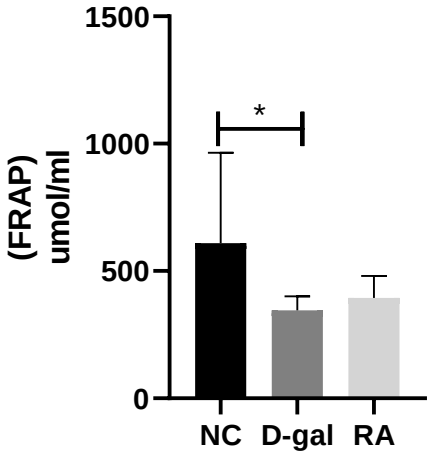

Figure 5.1: FRAP assay measuring antioxidant capacity in the plasma, Data shown are the group means and standard deviations. Significant statistical difference of >0.05 is represented by *.

From current data, other antioxidant mechanisms such as represented by TEAC in this data set seem to have sufficient capacity to upregulate quenching activity. The TEAC assay measures the ability of both water and lipid soluble antioxidants to scavenge the stable radical cation $ABTS^{\bullet}$ via donation of an electron or by hydrogen atom donation (Santos-Sánchez, 2019). FRAP on the other hand measures the capacity of electron-donating antioxidants to reduce ferric iron (Fe3+) to its ferrous form (Fe2+). This means FRAP is a more specific measure of antioxidants such as CAT and vitamin E, while TEAC takes into account SOD and glutathione (Kurutas, 2016). Within the context of this study, this alteration in the capacity of FRAP affects the presence of electron unstable radicals which are more prone to damage protein and DNA if unquenched. This may suggest that in future studies, parameters which measure protein and DNA damage markers may be more useful to detect oxidative stress.

While the decrease in FRAP alone has been reported previously, it is usually in combination with decreases in other antioxidant measures and increases in oxidative damage markers such as MDA (Mutlu-Türkoğlu, 2003; Cebe, 2014). These studies, however, make use of more severe disease states or advanced chronological age.

The animals which received RA intervention had decreased FRAP capacity combined with increases in damage markers such as MDA and H_2O_2, when compared to the normal controls. this indicates that decreases in FRAP may an appropriate parameter to assess both pre- and early onset maladaptation, even in the absence of damage.

FRAP may thus be one of the more sensitive, early marker of dysregulation of redox status and can potentially be used as a parameter to monitor longitudinally to assess changes in redox balance and for intervention, this is especially true if assessed in the appropriate compartments. In the more severe rheumatoid arthritis study, the decrease in FRAP was

significantly detected in specific compartments opposed to the plasma, showing significant decreases in the liver, combined with increased damage marker MDA. In a previous study done on skeletal muscle, similar decreases in FRAP and increases in MDA were noted (Oyenihi, 2019). This tells us that redox status varies in different compartments and monitoring only plasma status may be misleading.

In the RA model, redox status in the brain was opposite to what was observed in the liver; a significant increase in FRAP and decrease in MDA. This was the second novel finding, as previous studies have not comprehensively assessed antioxidant status within the brain of RA induced animals. While previous research has postulated about rheumatoid arthritis associated neurodegeneration, the link between damage and oxidative status has not been fully elucidated especially within the context of this model. The risk of RA associated neurodegeneration has been reviewed previously (Ungprasert, 2016) and this finding supports the concept that this may be a later onset secondary pathology which at the stage of progression investigated in this study is susceptible to preventative intervention.

These changes were not observed in the D-galactose model, although upon further investigation a second novel finding may give further insight into the compartment specific changes which take place prior to detectable damage in the circulatory system. This was the damage noted in the mesenteric lymph node morphology as noted in animals subjected to D-galactose intervention. Ultrastructure changes within the mesenteric lymph nodes of D-galactose induced subjects even at a pre-pathology onset state indicated that although circulatory measures did not display a severe pro-inflammatory state tissue specific analysis suggested that changes had already begun occurring which may lead to consistent low grade inflammation. An interesting further direction would be investigating the morphological changes which occur in the brain and liver considering the changes seen in these organs in both models regardless of circulatory status.

The importance of compartment specific investigations is highlighted here as the varying redox balance indicates the resistance to damage different compartments possess which may elucidate the progression of secondary pathologies associated with different disease states.

There were similarities in findings across models for various parameters which are traditionally measured in ageing studies as well as age-related diseases. One of these were telomere length which in both studies did not show statistically significant changes, indicative of a pre-accelerated ageing phenotype. Changes in telomere length has been noted in studies such as Liu et al. (2015) who showed decreased leukocyte telomere length in HIV positive patients or Gamal et al. (2018) who showed telomere shortening in RA patients and correlated the shortening with disease severity (Liu, 2015; Gamal, 2018). Both these studies measured this parameter at a further progression of maladaptation, as to what the aim of this body of work was investigating. The measurement of telomere length has however been shown to reflect efficacy of antioxidant supplementation and poses as a tool in the context of preventative drug discovery and screening. Another study by Richards et al. (2007) shows that

this finding confirms this finding, wherein women supplemented with vitamin D showed longer leukocyte telomere(Richards, 2007).

When investigating the inflammatory status of both models, there was evidence of a low grade pro-inflammatory status within animals subjected to either intervention (D-galactose and RA) when compared to normal controls. In the RA model, although clinical observations and IgG titre confirmed RA onset, the early timepoint indicated that a severe pro-inflammatory state had not yet been established. In both models the compensatory mechanisms within the inflammatory system, such as inflammasome activation indicated by IL-10, have changed status when compared to normal. In the D-galactose model IL-10 is decreased whereas in the RA model it is increased. In the RA this was an expected result as inflammasome activation is exacerbated in rheumatoid arthritis, while in the D-galactose model this was not noted suggesting this type of activation of the immune system has not taken place as yet.

Collectively the results of both studies suggest that the circulatory measures for detection of oxidative stress and inflammation may not be sensitive enough to detect pre-pathology or early pathology changes. This highlights the need for tissue-specific analysis, as displayed in the RA model, where oxidative damage and changes in antioxidant capacity was noted in the brain and liver before it was evident in circulation. In the D-galactose model changes were noted in the lymph node which were not obvious in circulation.

Further studies are definitely required to further analyse different tissues and time points within the progression of cumulative damage to identify the threshold at which compensatory mechanisms and acute changes become permanent maladaptations which lead to pathology. A longitudinal study which measures redox and inflammatory status at multiple time points during progression would be recommended. Parameters to include would be comprehensive measures of antioxidant and oxidative damage such FRAP, SOD and the upstream Nrf2 gene expression, while damage markers such as protein carbonyls and 8-oxo-2'-deoxyguanosine should be included to assess the entire cellular state. Additional inflammatory markers which should be assessed should include the activation markers within immune cell types to observe the efficacy of cells as well as telomere and telomerase measurements of leukocytes.

This study also assessed the potential of a grape seed-derived proanthocyanidin (GSP) to attenuate the changes in redox and inflammatory status which precedes pathology, to elucidate if these models were sensitive enough to reflect these small effect sizes changes of preventative treatments.. The GSP used in this research has previously been shown to have antioxidant and anti-inflammatory modulatory properties.

In both studies the GSP increased antioxidant capacity which showed more effect in certain tissue types and compartments. This highlights the importance of these measures in preventative medicine research as the small effect changes noted may be beneficial when screening drug efficacy not only in the circulatory compartment. Similar modulatory effects were noted in inflammatory status, especially the ability of the GSP to return cytokine levels

to normal after supplementation and counteract the disturbances to the allostatic load caused by the intervention.

The one result which highlights this is the correction of ultrastructure of the mesenteric lymph nodes after supplementation, a finding that would not have been noted unless tissue specific analysis was done in the D-galactose model. In the RA study, GSP supplementation resulted in longer telomere length, a novel finding which warrants further investigation such as the measurement of telomerase, this has been postulated in previous research and would enhance the preventative medicine field greatly.

This study had various limitations which have been important in elucidating requirements for future studies. Namely the duration and sampling times; this study measured the progression of the D-galactose and RA model at one time point, and while these results have enhanced our understanding of the threshold prior to maladaptation, it would be beneficial to have an earlier as well as later time point to comprehensively assess the progression. Secondly it would beneficial to this research if more tissue types and compartments are investigated, much is still unknown regarding the gastrointestinal track, bone, cardiovascular and respiratory systems in the context of the D-galactose and RA models, especially at this pre-onset stage.

Ideally a longitudinal study approach will be beneficial for future studies, but the research presented in these studies has illustrated the potential benefits which are to be gained from using these models in preventative medicine research development.

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Appendices

Appendix A: Ethics Approval

UNIVERSITEIT
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UNIVERSITY

Final Report Approval

Date: 15 August 2019

PI Name: Mrs Kelly Ross

Protocol #: ACU-2019-0146

Title: Assessing detriments of d-galactose induced ageing and rheumatoid arthritis in rats, and the potential modulatory effect of a grape seed-derived proanthocyanidin.

Dear Kelly Ross ,

Your final report was reviewed on 14 August 2019 by the Research Ethics Committee: Animal Care and Use via committee review procedures and was approved.

Approval Date: 14 August 2019

Applicants are reminded that they are expected to comply with accepted standards for the use of animals in research and teaching as reflected in the South African National Standards 10386: 2008. The SANS 10386: 2008 document is available on the Division for Research Developments website www.sun.ac.za/research.

As provided for in the Veterinary and Para-Veterinary Professions Act, 1982. It is the principal investigator's responsibility to ensure that all study participants are registered with or have been authorised by the South African Veterinary Council (SAVC) to perform the procedures on animals, or will be performing the procedures under the direct and continuous supervision of a SAVC-registered veterinary professional or SAVC-registered para-veterinary professional, who are acting within the scope of practice for their profession.

Please remember to use your protocol number 0146 on any documents or correspondence with the REC: ACU concerning your research protocol.

Please note that the REC: ACU has the prerogative and authority to ask further questions, seek additional information, require further modifications or monitor the conduct of your research.

Any event not consistent with routine expected outcomes that results in any unexpected animal welfare issue (death, disease, or prolonged distress) or human health risks (zoonotic disease or exposure, injuries) must be reported to the committee, by creating an Adverse Event submission within the system.

We wish you the best as you conduct your research.

If you have any questions or need further help, please contact the REC: ACU Secretariat at wabeukes@sun.ac.za or 021 808 9003.

Sincerely,

Winston Beukes

REC: ACU Secretariat

Research Ethics Committee: Animal Care and Use

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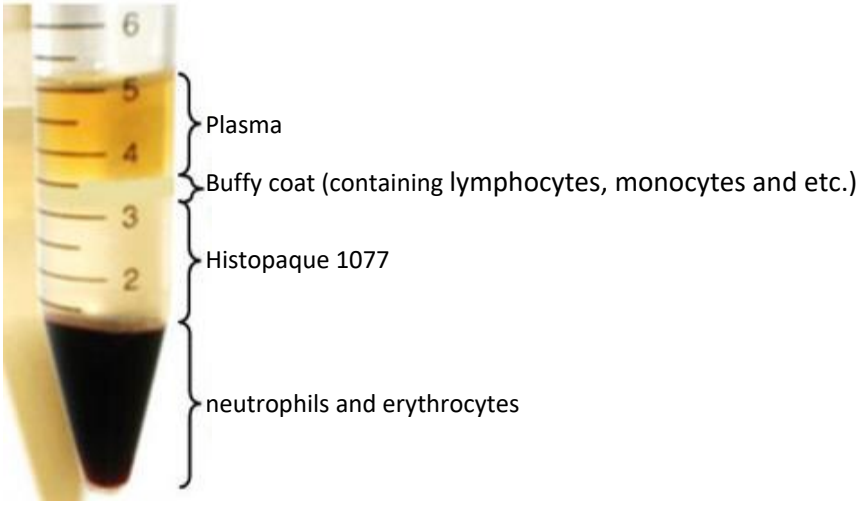
Appendix B: Neutrophil Isolation protocol

- 2ml Histopaque1077(room temperature) is pipette into a 15ml falcon tube and then double volume (4ml) of blood obtained by aortic puncture collected in heparin anticoagulated Vacutainer® tubes (BD Systems, Plymouth, UK) was carefully poured onto the Histopaque layer down the side of each tube.
- Tubes are transferred to the centrifuge machine with swinging rotor (Eppendorf,5804R, Germany) and are spun at speed of 652xg for 30 minutes at 21°C, 1800rpm.

* Four different layers of blood are formed*

- Sediment containing neutrophils and erythrocytes (bottom layer) is separated by aliquoting out the plasma layer on top thereafter the second layer containing lymphocytes, monocytes and third layer containing residual histopaque (as seen in image).



- Plasma is aliquoted into 2 ml sterile capped microtubes and frozen at -80°C, aliquot PBMC's into 2 ml sterile capped microtubes and freeze.
- Sediment is mixed with equal volume of 6%(room temperature) filtered dextran (in Normal Saline) and incubated at 37°C for 45 minutes to allow the sedimentation by gravity of erythrocytes.

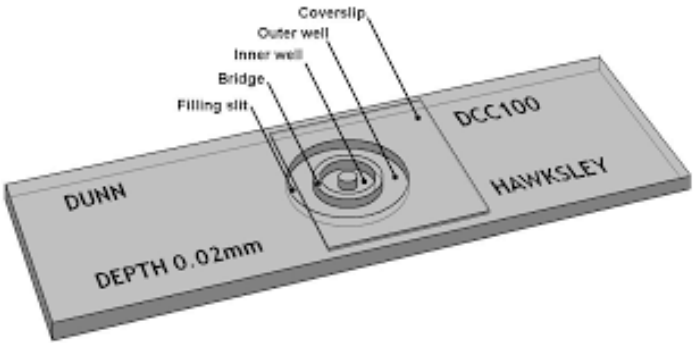
- Pellet containing erythrocytes is discarded and the neutrophils rich supernatant is collected and mixed with 10ml of PBS.
- Neutrophil PBS mixture is centrifuged at 290xg for 10 minutes at 4°C, 1500rpm.
- Supernatant was removed from tube leaving the pellet containing the neutrophils in the tube.
- Washing with 1ml PBS was repeated once or twice depending on the amount of erythrocytes present as a contaminant.
- The final neutrophils pellet was resuspended into desired volume of media to achieve the appropriate cell concentration usually $2x10^{-6}$ cells/ml.

Neutrophil count

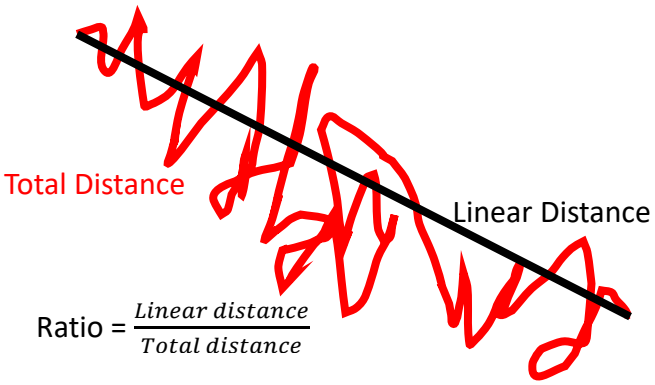
- Aliquot 200µl in 2 ml sterile capped microtubes
- Run sample on the CellDyne 3700 to determine neutrophil counts.
- Adjust to count of $2x10^{-6}$ cells/ml

Appendix C: Neutrophil chemotaxis assay Protocol

- A Dunn chamber was used to assess the migratory capacity of neutrophils (in terms of distance and directional accuracy) toward a chemotaxin.
- 10 µl of neutrophils in suspension were seeded on coverslip and left to adhere for one minute. Their migratory capacity up a chemotactic gradient was then assessed by filling both annular wells of the Dunn migration Chamber with media (RPMI 1640).



- The coverslip with cells was then placed inverted onto chamber and sealed with hot wax mixture (Vaseline: Paraffin: beeswax, 1:1:1).
- The media was then drained out of the outer well using filter paper.
- The outer well was and filled with the chemoattractant fMLP (N-FORMYL-MET-LEU-PHE F3506, Sigma, USA (100 µl solution with HBSS). A gradient was allowed to form for 20 minutes before imaging.
- Neutrophil migratory paths were visualized by combining time lapse images taken at 20X magnification using an Olympus Cell® system IX-81 inverted fluorescent microscope system with an F-view cooled CCD camera (Soft Imaging Systems).
- Images were acquired every 20 seconds for a total time of 20 minutes. Image J (Java Software) was used to track the movement of neutrophils and, measure distance covered as well as distance from start using the Mtrack J plugin.
- Neutrophil tracks were quantified by measuring the total distance they moved (from point to point) and the linear distance they covered (a straight path from start to finish) as depicted in



Ratio = $\frac{Linear\ distance}{Total\ distance}$

Appendix D: Myeloperoxidase (MPO) Colorimetric Assay Kit Protocol

Catalog No: E-BC-K074

Method: Colorimetric method

Specification: 100 Assays

Kit components

	Component	Specification	Storage
Reagent 1	Buffer solution	35 mL × 1 vial	2-8℃, 6 months
Preparation of **Reagent 1 application solution**: Prepare the required amount according to the ratio of Reagent 1: double distilled water =1:9. The prepared solution can be stored at 2-8℃ for 1 month.			
Reagent 2	Powder A	2 vials	2-8℃, 6 months
Preparation of **Reagent 2 application solution:** Dissolve a vial of Powder A with 60 mL **Reagent 1 application solution** before use, or heat at 37℃ to dissolve, store at 2-8℃ for 2 weeks.			
Reagent 3	Powder B	3 vials	2-8℃, 6 months
Reagent 4	Saline solution	10 mL × 3 vials	2-8℃, 6 months
Preparation of **Reagent 3 application solution:** Add 1 vial of Powder B into 1 vial of Reagent 4 to dissolve completely before use. The prepared solution can be store at 2-8℃ for 2 weeks.			
Reagent 5	Clarificant	24 mL × 1 vial	2-8℃, 6 months
It will freeze in cold condition, shake in 37℃ water bath to dissolve fully (transparent) before use.			
Reagent 6	Powder C	2 vials	2-8℃, 6 months, shading light
Reagent 7	Substrate	0.5 mL × 1vial	2-8℃, 6 months
Preparation of chromogenic agent: dissolve a vial of Powder C with 100 mL **Reagent 1 application solution** fully, then add 0.1 ml **Reagent 7**, mix fully and store at 2-8℃ with shading light.			
Reagent 8	Acid reagent	6 mL × 1 vial	2-8℃, 6 months

Sample preparation:

5% tissue homogenate sample:

Weigh tissue and add reagent 2 application solution to ratio weight(g): volume(mL) = 1:9

Mechanical homogenate the sample in ice water bath. (*don't centrifuge*) preserve on ice until detection. Store at -80℃ for detection on another day.

Sample treatment:

Take 0.9mL of sample and add 0.1mL of Reagent 3 application solution. Mix fully and incubate at 37℃ for 15 mins.

Original Protocol:

Note: The following operating table could be as a reference.

	Control tube	Sample tube
Double distilled water (mL)	3	
Sample (mL)	0.2	0.2
Reagent 5 (mL)	0.2	0.2
Chromogenic agent (mL)		3
Mix thoroughly, incubate at 37℃ water bath for 30 min.		
Reagent 8 (mL)	0.05	0.05
Mix thoroughly and incubate at 60℃ water bath for 10 min. Centrifuge the tubes at 2325 × g for 10 min and take the supernatant. Set the spectrophotometer to zero with double distilled water and measure the OD value of each tube at 460 nm with 1.0 cm optical path cuvette immediately.		

1) 3mL ddH_2O + 0.2mL sample + 0.2mL reagent 5 = 3.4mL
2) 3.4mL 1) + 0.05mL reagent 8 = 3.45mL

1)15:1 ratio

2) 68:1

Adapted protocol:

1) 1mL ddH_2O + 0.066mL sample + 0.066mL reagent 5 = 1.132 mL
2) 1.132 mL 1) + 0.0166 mL reagent 8 = mL

Appendix E: DNA Extraction Protocol: Qiagen DNeasy Blood and Tissue Kit Protocol

Before starting:

- Preheat waterbath to 56℃
- Defrost sample to room temperature

1. Transfer sample to 1.5 ml sterile capped microtubes.

2. Centrifuge sample at 8000 rpm in Eppendorf 5804R (Germany) for 5 mins to form cell pellet.

3. Remove supernatant (first with p1000 tip, then with p200 tip, leaving small amount so as to not disturb pellet) and resuspend pellet in 200 ul PBS.

4. Pipette 20 ul proteinase K into sample and add 200ul buffer AL.

5. Mix thoroughly by vortexing.

6. Incubate sample in water bath (in floatie) at 56℃ overnight.

7. Add 200 ul cold absolute ethanol (96%-10%), mix thoroughly by vortexing.

8. Pipette mixture into a spin column placed in a 2 ml collection tube.

9. Centrifuge at 6000xg (8000 rpm) for 1 minute.

10. Discard flow through and collection tube.

11. Place spin column in a new 2ml collection tube and add 500ul buffer AW1.

12. Centrifuge at 6000 x g (8000 rpm) for I minute.

13. Discard flow through and collection tube.

14. Place spin column in new 2 ml collection tube and add 500ul buffer AW2.

15. Centrifuge at 20 000 x g (14 000 rpm) for 3 minutes.

16. Discard flow through and collection tube.

17. Transfer spin column to new 1.5 ml sterile capped microtubes.

18. Elute DNA by adding 50ul buffer AE to the centre of spin column (be sure to pipette elution buffer onto entire surface of spin column filter).

19. Incubate for 2 minutes at room temperature (15-25 ℃).

20. Centrifuge at 14 000 rpm (max) for 1 minute.

21. Repeat elution (steps 18-20) with eluant from step 20.

22. Measure DNA yield by pipetting 2ul onto 200 (green) slide and reading on expose.

Appendix F: Protocol: Homogenization of tissue

Brain homogenates Tissue homogenates:

50mM Tris–HCl buffer (pH 7.4) containing 1.15% potassium chloride

Tris-HCL buffer (50mM pH7.4):

- 7.80g of Tris + 11.5g KCl dissolved in 800mL dH_2O
- pH to 7.4 with HCL
- Fill to 1 L with dH_2O

100mg of tissue in 1ml of buffer

1. 20ml buffer with tissue, homogenize. Add remaining buffer
2. The homogenates were centrifuged at 10000 x g for 15mins at 4 degrees.
3. Supernatants were kept at -80 for the assays.

Tissue homogenates:

1x PBS (pH 7.2) for tissue homogenization

To make 1L of 1xPBS:

- 8g of NaCl + 0.2g of KCl + 1.44g Na2HPO4 + 0.24g KH2PO4
- Add 800mL dH_2O
- pH 7.2. with HCL
- Fill to 1L

100mg of tissue was placed in 1ml of PBS and homogenized

1. 20ml buffer with tissue, homogenize. Add remaining buffer
2. The homogenates were centrifuged at 12000rpm for 15mins at 4 degrees.
3. Supernatants were kept at -80 for the assays.

Appendix G: TBARS(MDA) Assay protocol

Reagents:

1. Absolute ethanol
2. 2mM MDA standard stock (Must be made fresh*):
   - 0.0125408g (12.5408mg) MDA in 20ml 40% ethanol.
3. 4mM BHT (butylated hydroxytoluene) (Must be made fresh*):
   - 0.008814g (8.814mg) BHT in 10ml abs ethanol.
4. 0.2M ortho-phosphoric acid (OPA)
5. 0.1M NaOH
6. 0.11M TBA (2-thiobarbituric acid) (must be made fresh*):
   - 0.159g (158.565mg) in 10 mL 0.1M NaOH
7. N-butanol
8. NaCl (saturated)

Standards preparation:

	MDA (ul of stock)	dH_2O (ul)	Conc
Std A	1000ul	0 ul	2mM
Std B	500ul	500 ul	1mM
Std C	250ul	750 ul	0.5mM
Std D	125ul	875 ul	0.25mM
Std E	62.5ul	937.5 ul	0.125mM
Std F	31.25ul	968.75 ul	0.0625mM
Std G	15.625ul	984.375 ul	0.03125mM
Std H	7.8125ul	992.1875 ul	0.015625mM
Blank	0	1000 ul	0 mM

Assay Protocol:

- Add 100µl of sample/stdin into 2 ml sterile capped microtubes
- Add 12.5µl of 4mM BHT (reagent 4)
- Add 100 µl of 0.2M OPA (reagent 5)
- Vortex for 10 secs
- Add 12.5 µl TBA (reagent 7)
- Heat at 90°C for 45 mins
- Put on ice for 2 mins
- Keep at room temperature for 5 minutes
- Add 1mL n-butanol (reagent 8)
- Add 100 µl saturated NaCl (reagent 9)
- Vortex for 10 seconds
- Centrifuge at 6000rpm for 5 mins at 4°C
- Aliquot 300 µl of supernatant (top layer only) into 96 well plate in triplicate
- Read at 532-572 nm

Reagents:

- Malondialdehyde tetrabutylammonium salt (MDA)
  - Molecular Weight: 313.52
  - 2 mM Stock
    = 0.0125408g (12.5408 mg) MDA in 20 ml abs ethanol.
- (butylated hydroxytoluene) BHT
  - MW: 220.35
  - 4 mM stock
    = 0.008814 g (8.814 mg) BHT in 10 ml absolute ethanol.
- ortho-phosphoric acid (OPA)
  - MW: 97.99
  - 0.2M stock
    =9.799 g OPA in 500 ml dH_2O
- Sodium hydroxide (NaOH)
  - MW: 40
  - 0.1M stock
    = 2g NaOH in 500 ml dH_2O
- 2-thiobarbituric acid (TBA)
  - MW: 144.15
  - 0.11M TBA stock
    = 0.158565 g (158.565mg) in 10 mL 0.1M NaOH

Appendix H: FRAP assay Protocol

1. Reagents preparation

- Acetate Buffer (300 mM, pH 3.6)
    - In a 1 L media bottle add 1.627 g Sodium acetate, 16 mL Glacial acetic acid (SAARCHEM Cat Nr.: 1021000) and distilled water up to 1 L.
- HCl (0.1M) (SAARCHEM Cat Nr.: 100319 LP)
    - In a 1 L media bottle add 1.46 mL concentrated HCl (32% HCl) and add distilled water to final volume of 1L.
- TPTZ (2,4,6-tri[2-pyridyl]-s-triazine) (10 mM (Sigma Cat Nr.: T1253))
    - In a 15 mL conical tube, add 0.0093g TPTZ to 3 mL of 0.1 mM HCl (must be prepared fresh for on day of assay).
- Iron (III) chloride hexahydrate (20 mM (F2877))
    - In a 15 mL conical tube, add 0.054 g $FeCl_3.6H_2O$ to 10 mL distilled water (must be prepared fresh for on day of assay).
- L-Ascorbic acid (Sigma Cat Nr.: A5960) Standard (1.0 mM) (Vitamin C)
    - Weigh 0.0088 g Ascorbic acid in a 50 mL centrifuge tube and add 50 mL distilled water. Mix until dissolved. (Stock solution)

  (Check: In an 2 ml sterile capped microtubes tube, dilute 125 µl of this stock solution with 1375 µl water to obtain a concentration of 83 µM. This solution should give an absorbance of 0.830 ±0.010 at 265 nm.)

Before starting the analysis the FRAP reagent needs to prepared

- In a 50 mL conical tube add 30 mL Acetate buffer, 3 mL TPTZ solution, 3 mL FeCl3 solution and 6.6 mL Distilled water. The solution should be straw colored, if not discard and prepare fresh solution.

2. Sample preparation

- Plasma was collected from whole blood (with EDTA anti-coagulant) after centrifugation and stored at -80.

3. Standard preparation

Standards were made up according to the table below in 6x2 ml sterile capped microtubes.

Tube	**Ascorbic acid stock solution µL**	**Distilled water µL**	**Standard concentration µM**	**Well number**
A (Blank)	0	1000	0	A1-A3
B #1	50	950	50	A4-A6
C #2	100	900	100	A7-A9
D #3	200	800	200	A10-A12
E #4	500	500	500	B1-3
F #5	1000	0	1000	B4-6

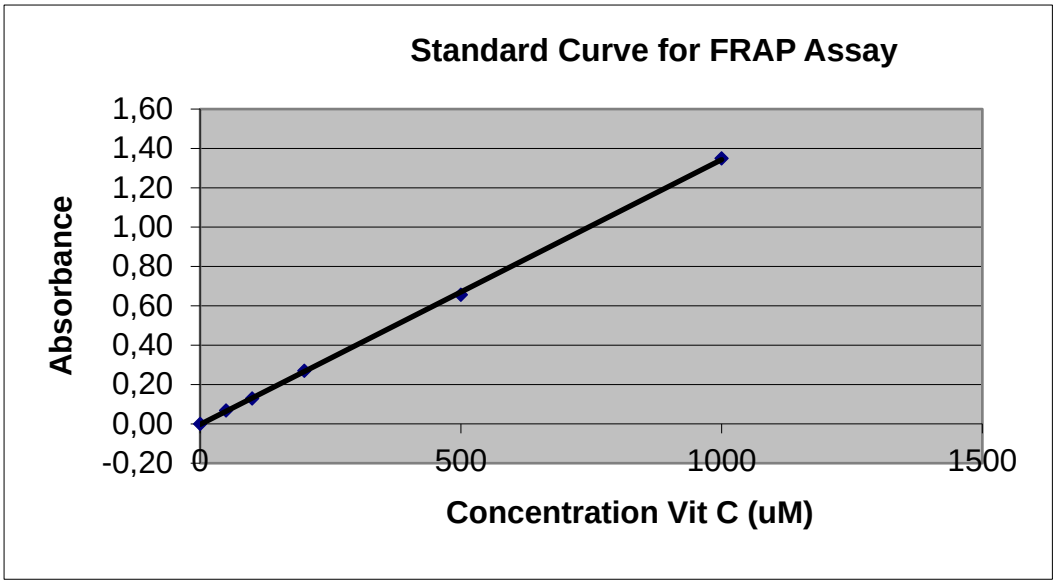
4. Assay procedure

- In a clear 96-well plate add 10 µl of the blank to wells A1-3.
- Add ascorbic acid standards #1-5 (tubes B-E) per well in the designated wells (as directed in the table above).
- Add 10 µl of samples (in triplicate) to the wells (B7-H12).
- Add 300µ of the FRAP reagent to each well with a multichannel pipette.
- Incubate the plate for 30 minutes in the incubating oven set at 37°C.
- Run plate on Multiskan plate reader

5. FRAP Calculations

Data is expressed as micromoles of Vitamin C equivalents per liter of sample and calculated from a standard curve.

Below is an example of one of the standard curves used for this data set.



Concentration is then calculated as:

Conc = (average absobance of sample – average absorbance of blank) - the y intercept / the slope of the line of best fit.

Appendix I: ABTS (TEAC) ASSAY Protocol

Reagents:

1. ABTS (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) Diammonium salt: 7mM (Sigma Cat nr.: A1888).
    a. Weigh 0.0192 g of ABTS (Fridge) in a 15 mL screw cap tube and add 5 mL distilled water. Mix until dissolve. Prepare fresh.

2. Potassium-peroxodisulphate: 140mM (Merck Cat Nr.: 105091)
    a. Weigh 0.1892 g K2S2O8 (Reagent rack) in a 15 mL screw cap tube and add 5 mL distilled water. Mix until dissolve. Prepare fresh.

3. ABTS mix (This must be done 24 hours before starting the assay):
    a. Add 88µl of the potassium-peroxodisulphate solution to 5 mL of the ABTS solution in a 15 mL screw cap tube. Mix well. Leave in the dark at room temperature for 24 hours before use.

4. Standard (Trolox also known as 6-Hydrox-2,5,7,8-tetramethylchroman-2-carboxylic acid):1.0mM (Aldrich Cat nr.: 238831)
    a. Weigh 0.0125 g Trolox in a 50 mL screw cap tube and add 50 mL of Ethanol (Saarchem Cat Nr: 2233540LP). Mix until dissolved. Prepare fresh. Use this solution as the stock standard. (Check: When diluted 5x with ethanol this solution should give an absorbance of 0.650 ±0.015 at 289 nm.)

Protocol:

a) Ensure that the plate reader is set to read at 734 nm and the temperature is set at 25°C.

b) Preparation of standard series – Take 6 2 ml sterile capped microtubes and mark them A-F. Add the amount of standard stock solution and diluents to each tube as described in the table below. Dilute the stock solution as follows to make a series of standards:

Tube	**Trolox standard µl**	**Ethanol µl**	**Trolox conc. µM**	**Well number**
A	0	1000	0	A1-A3
B	50	950	50	A4-A6
C	100	900	100	A7-A9
D	150	850	150	A10-A12

E	250	750	250	B1-3
F	500	500	500	B4-6

c) Trolox standard wells – add 25 µl of standard (tubes A-E) per well in the designated wells in a clear well plate.

d) Control wells – add 25 µl of the control to the wells (B7-B12).

e) Sample wells – add 25 µl of sample IN TRIPLICATE to the wells (C1-H12).

f) Dilute the ABTS mix solution with ethanol to read an absorbance of approximately 2 (±0.1) (Approximately 1ml ABTS mix and 20mL EtOH). Add 300µl of this ABTS mix to each well using a multichannel pipette.

g) Leave the plate for 30 minutes at room temperature before taking a reading.

h) Run plate

Appendix J: OxiSelect ROS/RNS kit Protocol

Reagents:

1. Priming agent: 250µl
2. Stabilization solution: 1.5mL
3. Catalyst (250X): 20µl
4. DCF-DiOxyQ: 50µl
5. DCF standard: 100µl
6. H_2O_2 : 100µl (8.821M)

Preparation of reagents:

2. Stabilization solution:
    - 1:10 dilution - 1.5mL stabilization solution in 13.5mL of dH_2O
    - Vortex, store at 4°C.
3. Catalyst:
    - 1:250 dilution - 10µl catalyst in 2.49mL PBS
    - Vortex, use immediately.
4. DCFH solution:
    - 1:5 dilution - 25µl DCF-DiOxyQ in 100µl priming agent (reagent 1)
    - Vortex, incubate for 30 mins at room temperature.
    - Dilute solution (DCF-DiOxyQ + priming agent) 1:40 - 125µl DCFH in 4.875mL Stabilization solution(solution 2).
    - Vortex, store at -20°C for up to 1 week in the dark!

Preparation of DCF standard curve:

1:10 dilution series of DCF standard in PBS

Range: 0µm - 10µm

Standard tubes	DCF standard (µl)	PBS (µl)	DCF conc(nM)
Blank	0	1000	0
1	10	990	10000
2	100 of tube 1	900	1000
3	100 of tube 2	900	100
4	100 of tube 3	900	10
5	100 of tube 4	900	1
6	100 of tube 5	900	0.1

Standard curve protocol:

- Pipette 200µl of each standard into 96 well plate in duplicate.

- Read relative fluorescence at 480 nm excitation and 530 nm emission.

Assay protocol:

- Pipette 50µl of sample/ standard/ control to wells (in duplicate).
- Add 50µl of catalyst to each well.
- Mix.
- Incubate 5 mins at room temperature.
- Add 100µl of DCFH solution (solution 4) to each well.
- Cover 96-well plate (to protect from light)
- Incubate at room temperature for 30 mins.
- Read fluorescence with fluorescence plate reader at 480 nm excitation and 530 nm emission.

Appendix K: Real time qPCR Protocol

- PBMC's harvested from neutrophil isolation Appendix B and frozen down at a density of 2 x10^6 cells/ml.
- DNA used from extraction as detailed in Appendix E
- DNA concentrations were measured on Xpose™ Touch & Go reader (Trinean, Belguim) and Drop Sense 16 software.
- DNA (20ng/µl) was amplified using KAPA SYBR® FAST quantitative polymerase chain reaction (qPCR) Master Mix (2X) Kit (Merck, Darmstadt, Germany), using the primers presented in Table 1.

	OLIGOMER NAME	**SEQUENCE (5'-3')**	**AMPLICON SIZE**
STANDARDS			
	Telomere standard	(TTA GGG)14	84
	36B4 standard	CAG CAA GTG GGA AGG TGT AAT CCG TCT CCA CAG ACA AGG CCA GGA CTC GTT TGT ACC CGT TGA TGA TAG AAT GGG	75
PRIMERS			
	Telomere forward	CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT	39
	Telomere reverse	GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT	39
	36B4 forward	CAG CAA GTG GGA AGG TGT AAT CC	23
	36B4 reverse	CCC ATT CTA TCA TCA ACG GGT ACA A	25

- Standard curves were set up using telomere and 36B4 (reference gene) standards and primers
- Stock solutions for telomere std:
  Stock= 0.06ng/ul (1.18x10^8 tel std repeats)
  1:10 dilution series =10ul stock +90µl TE buffer
- Stock solutions for 36B4 std:
  Stock= 0.02ng/ul (2.63x10^9 tel std repeats)
  1:10 dilution series =10ul stock +90µl TE buffer
- Stock solutions for tel std forward and reverse primers:
  F=MW: 7.137 made up to 100µM stock
  Working solution =30µl in 240µl H_20
  R=MW:7.570 made up to 100µM
  Working solution =30µl in 240µl H_20

- Stock solutions for 38B4 forward and reverse primers:
  F=MW: 7.137 made up to 100µM stock
  Working solution =30µl in 240µl H20
  R=MW:7.570 made up to 100µM
  Working solution =30µl in 240µl H20
- Cycling conditions:

  StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA)

  1. pre-incubation of 10 min at 95°C,
  2. 40 cycles of 95°C for 15 sec
  3. 60°C for 15 sec
  4. 72°C for 10 sec.
- Reaction make up:

KAPA mastermix	5µl
Forward primer	1 µl
Reverse primer	1 µl
Standard/sample	1 µl
H_2O	2 µl

- All samples were amplified in triplicate and mean cycle time (Ct) values were used in subsequent calculations.