Modulation of oxidative stress and inflammation using a grape seed derived polyphenol

by

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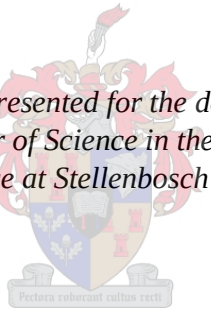

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Declaration

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Abstract

Oxidative stress and inflammation are intricately interlinked, especially in the context of ageing/accelerated ageing, as well as several chronic disease states, where they are implicated as aetiological factors. In the past decade polyphenols have been investigated for their potent anti-oxidant as well as anti-inflammatory properties. Previous research by our group has highlighted the potential of a grape seed-derived polyphenol as both anti-oxidant and anti-inflammatory modality. However, no data has been generated in human models and the mechanism(s) of action is still largely unknown. Thus the aim of this study was to more comprehensively investigate potential anti-oxidant or anti-inflammatory mechanisms of a grape seed-derived polyphenol (PCO) on acute (as found with exercise) and chronic (as in ageing) models of oxidative stress.

After obtaining ethical clearance, blood samples were obtained from healthy human subjects from two different groups (aged (n=7) and young (n=14). In the young group a further distinction was made between sedentary (n=8) and fit individuals (n=6) whom we exposed to an HIIT exercise intervention to induce acute oxidative stress. Oxidative status was assessed in plasma using validated photometric and colorimetric assays and plasma myeloperoxidase (MPO) assessed as baseline inflammatory indicator. Neutrophils were isolated at rest (and after exercise from young fit subjects) and treated with the polyphenol *in vitro* for 1 hour. The chemokinetic capacity of treated and control neutrophils in response to a chemotactic signal was then determined using a Dunn chamber and live cell imaging. In addition, neutrophils were analysed for the expression of functional capacity markers (intracellular MPO, Fcγ Receptor IIIb (CD16) and CEACAM 8 (CD66b)) via flow cytometry. Oxidative status of neutrophils were also performed on isolates.

The results indicate that the aged population had significantly worse oxidative and inflammatory profiles than young sedentary controls - this was evident in their conjugated dienes content as well as decreased ferric reducing ability of plasma (FRAP). In neutrophils, aged and young subjects had increased chemokinetic accuracy and capacity after 1 hour of *in vitro* polyphenol treatment. The treatment was also associated with a shedding of CD16 and increased expression of CD66b - both linked to improved neutrophil motility. The intracellular MPO content of neutrophils indicated that PCO had an anti-inflammatory effect across groups. The exercise intervention did not induce measurable changes in oxidative stress or inflammation.

We therefore concluded that grape seed-derived polyphenols modulate inflammation and oxidative responses by facilitating more efficient neutrophil motility. This decreases the

number of neutrophils required per response, effectively resulting in less secondary tissue damage, less oxidative stress and faster resolution of inflammation.

Uittreksel

Oksidatiewe stres en inflammasie is fyn verweef, veral in die konteks van veroudering en versnelde veroudering. Verder word beide prosesse geïmpliseer in die etiologie van veroudering en verskeie kroniese siektes. In die verlede is polifenole al ondersoek vir hul aansienlike anti-inflammatoriese en anti-oksidant effekte. Vorige navorsing deur ons groep het die potensiaal van ‘n druif-afgeleide polifenool al bewys in hierdie konteks. Daar is tans nog geen data uit mensmodelle gegenereer nie en die meganisme waardeur hierdie effekte uitgevoer word, is nog grootliks onbekend. Die doel van hierdie studie was dus om op ‘n meer omvattende manier, die moontlike anti-inflammatoriese en anti-oksidant megansismes van ‘n druif-afgeleide polifenool te ondersoek, deur modelle van akute (soos na harde oefening) en kroniese (soos met veroudering) oksidatiewe stres.

Nadat etiese klaring verkry is, is bloedmonsters verkry van gesonde vrywilligers in twee groepe (oud (n=7) en jonk (n=14)). In die jong groep is onderskeid ook getrek tussen fikse (n=6) en onfikse (n=8) individue. Die fikse groep is addisioneel onderwerp aan hoë intensiteit oefening om akute oksidatiewe stres te veroorsaak. Oksidatiewe status in plasma is bepaal duer erkende fotometriese en kolorimetriese toetse te gebruik, terwyl plasma MPO as indikasie van inflammasie getoets is. Neutrofiele is geïsoleer uit bloed wat in ‘n rustende toestand getrek is, asook na oefening in die fiske jong groep. Chemokinetiese kapasiteit van neutrofiele is voor en na 1 uur *in vitro* polifenool behandeling bepaal deur gebruik te maak van ‘n Dunn kamer en lewende sel mikroskopie. Neutrofiel uitdrukking van merkers vir funksionele kapasiteit (intrasellulêre MPO, Fcγ Receptor IIIb (CD16) and CEACAM 8 (CD66b)) is ook met vloeisitometrie bepaal. Oksidatiewe status merkers is ook op geïsoleerde neutrofiele bepaal.

Resultate dui aan dat die ouer populasie beduidend slegter oksidatiewe en inflammatoriese profiele gehad het as hulle jonger eweknieë – dit was bv. Die geval vir CD en FRAP in die plasma. In neutrofiele het beide ouer en jong vrywilligers verbeterde chemokinetiese kapasiteit en akkuraatheid getoon na polifenool blootstelling. Die polifenool blootstelling het ook verhoogde CD66b en verlaagde CD16 uitdrukking tot gevolg gehad, wat altwee met verbeterde neutrofiel beweeglikheid in verband gebring word. Verder het intrasellulêre MPO ook aangedui dat die polifenool ‘n anti-inflammatoriese effek in al die groepe gehad het deurdat selle minder MPO vrygestel het. Die oefening ingreep het nie beduidende verskille teweeggebring in die gekose merkers vir oksidatiewe stres of inflammasie nie.

Ons gevolgtrekking is dus dat druif-afgeleide polifenole die oksidatiewe en inflammatoriese response moduleer, ten minste gedeeltelik deurdat dit neutrofiele meer effektief maak. Dit het tot gevolg dat minder neutrofiele per respons nodig is, dat minder sekondêre skade aan weefsel aangerig word en dat inflammasie vinniger opklaar.

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Abbreviations

·OH - hydroxyl radical

·HO_2 - hydroperoxyl

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

ADP- adenosine diphosphate

AMPK- adenosine monophosphate-activated protein kinase

Baso - basophil

CAECAM 8- Carcinoembryonic antigen-related cell adhesion molecule 8

CCL3 - chemokine(C-C motif) ligand 3

CD - conjugated dienes

CINC -1 - cytokine-induced neutrophil chemoattractant1

COPD - chronic obstructive pulmonary disease

COX-2 - cyclooxygenase-2

CVD - cardiovascular disease

DHQ - dihydroquercetin

DOMS - delayed onset muscle soreness

DPPH - 2,2-diphenyl-1-picrylhydrazyl

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

Eos - eosinophil

fMLP- N-formylmethionyl-leucyl-phenylalanine

FOXO - fork-head box protein O

FRAP - Ferric Reducing Ability of Plasma

GSH - Glutathione

GSSG - Glutathione disulphide

HAT – hydrogen atom transfer

H_2O_2 - hydrogen peroxide

HBSS – Hanks balanced salt solution

HIIT – High intensity interval training

HOCL - hypochlorous acid

HPA – hypothalamic pituitary adrenal

HUVEC- human umbilical vein endothelial cells

ICAM-1 -intercellular adhesion molecule-1

IGF-1 - insulin growth factor-1

IL - interleukin

IL- 1β – interleukin 1-beta

IL6 – interleukin 6

iNOS - inducible nitric oxide

JNK - c-Jun N-terminal kinase

LD – linear distance

LPS - lipopolysaccharides

LRRFIP-1 - leucine-rich repeat in flii-interacting protein-1

Lymph – lymphocyte

M2VP - 1-methyl-2-vinyl-pyridinium triflouromethane sulfonate

MDA - malondialdehyde

MFI – mean fluorescent intensity

Mono – monocyte

MPO - myeloperoxidase

Neut – neutrophil

NF-κB - nuclear factor-kappa beta

NO - nitric oxide

NOX - NADPH-oxidase

O_2^- superoxide

ONO_2^- peroxynitrite

ORAC - oxygen radical absorbance capacity

ORAC - Oxygen radical anti-oxidant capacity

PBMCs - peripheral blood mononuclear cells

PCO - proanthocyanidolic oligomer

PECAM - Platelet endothelial adhesion molecule

PGC-1α - peroxisome proliferator activated receptor gamma co-activator 1-alpha

PGE_2 - prostaglandin E2

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

SOD - superoxide dismutase

TBARS - *2-Thiobarbituric Acid Reactive Substances*

TD – total distance

TrxR- Thioredoxin reductase

TNF-α - Tumour necrosis factor-α

VEGF - vascular endothelial growth factor

WBC – white blood cell

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Chapter 1

Introduction

There are various reasons why optimal bodily functions decline; disease, injury and lifestyle are just the broad categories which regulate this decline and are incidental when experienced during mid to early life. There is one factor which we cannot avoid and physiologically is the least understood phenomenon which causes the decline which ultimately leads to death that is ageing.

With ageing, the capacity of the body to function optimally progressively declines. There are a combination of genetic and lifestyle factors which may either accelerate or slow down the ageing process. A number of chronic diseases are associated with advanced age: including cardiovascular disease (CVD), diabetes, metabolic syndrome, and Alzheimer's disease to mention a few. The result is an exponential increase in the disease burden on modern society, relative to a few decades ago, due to longer life expectancy. The World Health Organisation states that from 2015 to 2050, the proportion of the world's population over 60 years will increase from 12% to 22% [1]. Taking into consideration that predominant research in the field of age-associated diseases considers the sixth decade of life to be a risk factor for the rapid progression and onset of age associated diseases, the disease burden will almost double in the next 35 years. It is thus vital not only to elucidate the causes and progression of these chronic conditions, but also to actively search and investigate potential preventative therapies that may slow the processes contributing to physiological ageing. Although the ageing-associated chronic disease states are each uniquely complex in terms of their aetiology, development, and progression, they do share common aetiologies which stem from two main entities, namely, cumulative oxidative stress and chronic inflammation.

Oxidative stress, briefly, results from oxidants which are produced by normal cell metabolism and various physiological responses. However, when the production of oxidants outweighs the capacity of endogenous antioxidant systems, oxidative stress is incurred. Furthermore, while inflammation is crucial for repair of tissue injury and primary defence against invading pathogens and chemicals, it also results in unintended detriment to previously uninjured cells due to the robust nature of the inflammatory mechanism. Although these are necessary systems in the body, both oxidative stress and the inflammatory response, if unchecked, can have detrimental consequences which have been linked to accelerated ageing and the progression of age-associated disease.

We postulated that the effects of the inflammatory immune system and oxidative stress on allostatic load are interlinked. This has led us to investigate the potential of antioxidants as

treatment options to attenuate the cumulative effects of both oxidative stress and chronic low grade inflammation. Given the modern consumer bias for natural medicines, we decided to focus on a group of plant medicines which are consistently associated with beneficial effects on these processes in the literature, namely grape-derived polyphenols.

Previous research done on a grape seed-derived proanthocyanidolic oligomer (PCO) by our group, coupled with anecdotal reporting on this supplement has led to research which elucidated potent anti-inflammatory mechanisms. This led me to investigate the links between oxidative stress, which can be alleviated by an anti-oxidant such as PCO, and inflammatory mechanisms to target. This investigation presents therapeutic possibilities for various diseases as described earlier which present with both cumulative oxidative stress and chronic low grade inflammation. The prospect of a preventative or adjuvant therapy in this regard could be beneficial not only to ageing but all states which have inflammatory and oxidative stress components.

Therefore we set out to investigate the mechanisms in the spectrum of oxidative stress and the inflammatory response affected by PCO. Firstly to address the potential ageing mechanisms of chronic cumulative oxidative stress and low grade inflammation and the effects PCO has on these. Secondly to elucidate the effect of PCO on a more acute state of oxidative stress and inflammation, such as induced by strenuous exercise. We believe this is the first step to elucidating the mechanisms of action of PCO. Once this is established, PCO can be investigated as an effective therapeutic strategy.

This thesis will begin with a review of the literature, giving an over view of research done thus far with regard to ageing. Added to the review is further literature on exercise and the mechanisms of oxidative stress and inflammatory induced. Thereafter a detailed methods section will explain the subject groupings, parameters chosen and methods used to assess the chosen parameters. The results section then presents all the data obtained and comparisons made as well as statistical analysis of the data. Lastly a discussion of the results will be presented and the interpretation of the data along with what was elucidated. Future recommendations will presented to indicate our next steps.

Chapter 2

Literature review

A truncated version of this review of the literature has been published in the journal of Oxidative Medicine and Cellular Longevity, under the title “Ageing-associated oxidative stress and inflammation: alleviated by products from grapes” (refer to appendix A for full text paper). In this chapter, I have added extra information pertaining to acute oxidative stress and experimental exercise protocols used in this context, to more fully encompass my whole thesis topic.

2.1 Introduction

In this review, we will provide a more in-depth review of interconnected molecular mechanisms of oxidative stress and inflammation in the physiological ageing process, before moving our focus to a discussion of the merits of these plant medicines as potential preventative therapies in this context.

2.2 Contribution of Oxidative Stress to premature ageing

In the context of ageing, reactive oxygen and nitrogen species (RONS) are generally the major molecules which contribute significantly to oxidative stress [2]. The most frequently studied free radicals are superoxide (O_2-), hydrogen peroxide (H_2O_2), hydroxylradical (·OH), peroxynitrite (ONO_2-), and nitric oxide (NO). The generation of these free radicals is necessary as they are essential for host defence: phagocytic cells use reactive oxygen species (ROS) to digest invading pathogens and debris. Furthermore, they act as signalling molecules, regulating cell growth and apoptosis, adhesion, and differentiation. More specifically, RONS are formed during processes such as the mitochondrial electron transport chain as well as enzyme systems such as cytochrome P450, lipoxygenase and cyclooxygenase, the NADPH-oxidase complex, xanthine oxidase, and peroxisomes [3]. In contrast to these roles in growth, repair, and immune functions which are all beneficial to the host, these molecules also have the ability to oxidise signalling molecules, DNA, macromolecules, and cell structures such as lipid membranes of healthy host cells, all of which are to the detriment of these cells. Usually, each cell has defence mechanisms to counteract the occurrence of oxidative stress. These endogenous enzymatic antioxidant defences include superoxide dismutase (SOD), glutathione peroxidase, catalases, glutathione, thioredoxin reductase (TrxR), and peroxiredoxins. An appropriately nutritious diet is vital to maintain these systems, as these natural antioxidants are supplemented or replenished by antioxidant constituents of various fruits and vegetables. It is only when the capacity of the body’s antioxidant defences is

outweighed by the rate of production of free radicals that oxidative stress is incurred by unquenched free radicals which can alter surrounding cell structures and environment. With advancing age, various factors, among these is a natural progressive decline in endogenous antioxidant capacity, because disruption in the balance between pro- and antioxidant mechanisms and RONS accumulate beyond the normal endogenous antioxidant system's quenching capacity, resulting in cumulative oxidative stress. Eventually this causes cell damage which cannot be repaired by internal mechanisms, leading to loss of organ mass and functionality, which ultimately culminates in system dysfunction [4]. Indeed, long-term oxidative stress states have been linked to various diseases associated with advanced age, most notably diabetes, chronic obstructive pulmonary disease (COPD), cardiovascular disease, cancer, diabetes, and asthma [5]. Many of these diseases are not only associated with ageing anymore, but also with the high-obesity, high stress, and sedentary modern lifestyle (albeit perhaps in milder form) [6]. It is thus vital to address the prevention of these detrimental long-term outcomes, as they affect not only the aged, but also relatively young populations, such as university students [7,8]. The aetiological mechanism(s) of the various chronic diseases mentioned are each different, as each disease has its own complexities. However, chronic cumulative oxidative stress is a common factor in these diseases, highlighting this system as a vital therapeutic and/or preventative medicine target [9]. Even in the absence of age-associated disease, various theories have linked oxidative stress directly to the normal ageing process. We briefly mention two here.

Firstly, the Free Radial Theory of Ageing proposes that the presence of free radicals and their effects on cells are one of the causes of cell ageing and subsequent cell death, which in turn lead to loss of organ mass and other features of whole organism ageing. This theory was first suggested by Harman in 1956 [10], who hypothesized that irradiation of cellular components resulting from the liberation of ·OH and $\cdot HO_2$ radicals would lead to dysfunction and mutations which cannot be reversed and thus result in ageing. This theory was later supported and expanded as research began elucidating the details of his proposed theory, for example, by describing the role of SOD [11]. Phenomenally, a very recent meta-analysis [12] confirmed the sustained validity of this theory, 60 years later. Of course, given the technological advances made over this period, it is no surprise that subsequent research has added more detail on mechanisms in support of this theory, although many unresolved questions remain [13].

Secondly, the Replicative Senescence Theory is based on the hypothesis that oxidative stress induces cell death and/or senescence, which necessitates an increase in the rate of cell replication. This in turn accelerates the detrimental effects associated with repeated cycles of mitosis. Telomere length and its accelerated shortening due to reparative replication is the

basis of this theory, which was first described by Hayflick in 1965, in response to the observation of a decline in functionality of cell cultures (fibroblasts) which had undergone numerous cycles of cell divisions [14]. This phenomenon was subsequently confirmed by experiments on primary peripheral blood mononuclear cells (PBMCs) and fibroblasts from a largely aged population with increased risk for vascular dementia [15]. In this report, decreased telomere length in both fibroblasts and PBMCs was correlated to risk for dementia induced by stroke. In addition, telomere shortening rate was reported to decrease with increasing antioxidant capacity in fibroblasts of the same population.

This theory, together with the subsequent data, presents evidence that, firstly, oxidative stress is responsible for the accelerated shortening of telomeres brought about by more frequent reparative replication and secondly, that an increase in antioxidant defence capacity could slow down the ageing process. Note at this point that we do not infer that telomere shortening results only from oxidative stress: other mechanisms such as chromosomal instability have indeed been linked to both ageing and pathological conditions such as cancer [16]. However, to remain focused on the main topic of this review, we have limited this discussion to accelerated telomere shortening in the context of cumulative oxidative stress. Holistic interpretation of the two theories introduced above implicates oxidative stress as major causative role player in the damage to cell constituents by oxidising membranes, molecules, and DNA. This initiates a cascade of events leading to the need for either cell growth and replication for repair, or death. This implicates ROS as the rate determining factor of cell lifespan due to the direct damage it inflicts. Furthermore, oxidative stress is implicated in causing irreversible damage to the mechanism of replication through accelerated telomere shortening and thus ultimately decreases the capacity of the cell to replicate optimally. With both repair and replication affected, cell senescence is encouraged. In keeping with the idea of a holistic approach, consideration of oxidative stress in isolation is insufficient. A basic but practical example of the interplay between the oxidative stress system and another system implicated in ageing, inflammation [17], is data available on detrimental effects of cigarette smoking. In this context, both acute smoking and long-term smoking were shown to overwhelm the glutathione antioxidant defence system within the lungs, which was associated with significant infiltration of inflammatory immune cells in the lungs [18]. This study clearly shows system interaction. Furthermore, in the same study, the severity of this maladaptation increased with duration of habitual smoking (in years) and was exacerbated by natural decreases in antioxidant capacity as experienced with ageing, resulting in increased oxidative stress, illustrating the significance of cumulative damage. However, before considering these interactions in more detail, the literature providing proof of a role for chronic inflammation in the process of ageing will be briefly reviewed.

2.3. Chronic low grade inflammation facilitates premature ageing

As is the case for oxidative stress, the inflammatory response is a system essential for normal body function. As component of the innate immune response, inflammation is a major first-line defence against infection and injury [19]. Apart from this largely independent, nonspecific role in immunity, inflammation is also vital for many specific immune responses to run its course [20]. However, in the process of repair and restoration after insult, the inflammatory response inadvertently disrupts cellular homeostasis of previously unharmed or unaffected cells [21,22].The injury repair cycle which inflammation regulates is an efficient system during youth, when optimal sensitivity and response to signalling molecules (such as cytokines, growth factors, prostaglandins, and peptides) maintain the general health of circulating immune cells and the tissue microenvironment, with minimum secondary damage. However, during natural chronological ageing, long-term, repeated stimulus response cycles change the receptor expression levels and thus sensitivity to these molecular stimuli [23]. This may necessitate relatively increased concentrations of any particular stimulus to maintain the required effect. Commonly reported characteristics of the natural ageing process include inflammation or oxidative stress-associated symptoms such as directionally inaccurate chemotaxis, premature or suboptimal respiratory burst, and increased pro-inflammatory signalling from immune cells, all of which form the basis of immunosenescence [24].

Immunosenescence is the term used to describe the ageing of immune cells and the functioning of the immune system as a whole. This occurs naturally with advancing chronological age or as result of lifestyle factors, as already mentioned. There is more than one way in which the immune system is compromised upon ageing. Firstly, immunocompetent cells are derived from hematopoietic stem cells. With ageing, a natural bias develops for stem cells to commit to expansion of the myeloid lineage at the cost of the lymphoid lineage [25]. This results in a shift in the balance of immune cells available to enter the circulation. Secondly, the chronic low grade inflammation associated with ageing recruits larger numbers of cells into circulation from the hematopoietic tissue. However, despite the higher circulating cell counts, phagocyte Toll-like receptor expression and phagocytic capacity are decreased in the aged [23], leaving the immune system with a lower capacity for becoming activated and for responding to a more acute insult, such as viral infection. These maladaptations, which together further predispose the individual to pro-inflammatory responses, are postulated to stem at least in part from alterations in hypothalamic pituitary adrenal (HPA)-axis signalling. The process of immunosenescence may be accelerated by unhealthy lifestyle, such as psychological stress and obesity. As mentioned before, lifestyle associated diseases share

clinical symptoms associated with normal ageing. Indeed rutin, a potent antioxidant, has been shown to protect against age-related metabolic dysfunction [26]. Thus, studies focused on these conditions may provide much insight in terms of ageing and the supplementation options to limit their progress. For example, in the context of obesity and/or inactivity, cytokine release from adipose tissue macrophages have been demonstrated by many researchers [27–30]. Tumour necrosis factor-α (TNF-α), interleukin- (IL-) 1β, and IL6 are among some of the cytokines shown to be released from resident macrophages in adipose tissue, resulting in a pro-inflammatory microenvironment [31]. Furthermore, chronic stress, and in particular psychological stress, is a generally accepted cause of chronic low grade inflammation. For example; in 38 medical students, psychological stress was associated with increased pro-inflammatory cytokine (TNFα, IL-6, IL-1Ra, and IFN-γ) levels, as well as decreased anti-inflammatory regulators (IL-10 and IL-4) [32]. Interestingly, these effects were exacerbated by high anxiety proneness as trait, again suggesting that a cumulative stimulus (in this case lifelong anxiety) further exacerbates the undesirable adaptation. Also in posttraumatic stress disorder (as extreme form of chronic psychological stress), an initial glucocorticoid hyper response is followed by glucocorticoid hypo-responsiveness, which is associated with a pro-inflammatory state. In this condition of continuous pro-inflammatory signalling, the feedback systems, which usually down regulate inflammation, adjust over time and result in maladaptations such as chronic but low grade upregulation of pro-inflammatory mediators (e.g.,IL-6,TNF-α,IL-1β,and prostaglandin E2) [33]. Incidentally, the secretion of the first three is mediated by the NFκB pathway, which is activated in response to cellular stress [34]. In addition to increased pro-inflammatory signalling, other more mechanical cellular mechanisms also seem to be compromised over time. For example, inappropriate and/or insufficient neutrophil responses result from its decreased phagocytic capacity, increased basal levels of intracellular calcium, and the resulting reduction in capacity for chemotaxis [35]. The result is a chronic low grade inflammatory status in relative absence of a specific threat which can persist for extended periods of time and cause harm and inefficiency of the system. Thus, although varied in specific causative mechanism, the outcome of all of these suboptimal life events or lifestyle habits, for example, chronic stress leading to glucocorticoid resistance and/or cardiovascular disease and high-calorie diets and inactivity (obesity) leading to insulin resistance and diabetes, is that of a chronic inflammatory state [17]. Of particular interest in the context of ageing is the fact that, apart from the now notorious low grade inflammation as the primary culprit, this maladaptation results in a compromised capacity to mount an efficient inflammatory response to acute insults. As overviewed in the review by Weinberger and colleagues [36], several reports from clinical literature support the notion that, in the aged individual, there is a significant increase in the convalescence period required for recovery from injury and pathogen clearance, as well as a decrease in the quality of repair, thus

favouring disease progression and morbidity due to injury. Very recently, Bäehl and colleagues (2015) demonstrated, in a longitudinal study of elderly patients, that the acute stress of a hip fracture had a negative effect on neutrophil function immediately after injury. While some neutrophil functions (chemotaxis, phagocytosis) were recovered over time, several others (superoxide production, complement C5A and CD11b receptor level, and cytokine secretory profile) were still impaired even six months after injury. From this, the authors concluded that the acute stress had a long-term negative effect on neutrophil responses, which negatively influenced clinical outcomes, such as the resolution of long-term inflammation, recovery, and susceptibility to opportunistic infections [37]. It is thus clear that ageing is an inflammatory-mediated process. However, from the inflammation/ageing literature, it is clear that inflammation and oxidative stress cannot be separated as causative factors in this context. For example, the ageing-related shift in balance between the glucocorticoid and inflammatory systems has been linked to increased ROS production, which in turn exacerbates low grade immune activation [38].

2.4 Links between Inflammation and Oxidative stress in the Ageing Process: Identifying therapeutic targets

From the above sections it is evident that, during ageing, oxidative stress and inflammation are interdependent mechanisms. We postulate that the unravelling and understanding of these intricate links between the two responses hold the answer to identifying the major contributor(s) to allostatic load and maladaptation associated with ageing and age-related pathology. Generally, repeated exposure to RONS causes cell damage and thus a pro-inflammatory signalling response. For example, in aged mice, unquenched RONS act as pro-inflammatory signalling molecules and mediators of inflammation within the cell itself [39]. More specifically, oxidative damage to cells prompts the release of TNFα from these damaged cells [22]. Binding of TNF-α to cell surface TNF-α receptors activates the NF-κB inflammasome, which results in the further production of other pro-inflammatory cytokines, most notably IL-1β. Incidentally, TNFα specifically has also been implicated in ROS-mediated upregulation of adhesion molecules which facilitate the infiltration of immune cells into tissue [40], with more on this later. Upregulation of inflammation via the NF-κB inflammasome is probably the main aetiological mechanism for age-related chronic conditions with an inflammatory component. Indeed, TNF-α upregulation, which is a direct result of increased flux through the inflammasome, has been implicated as causative factor in cardiovascular disease [41]. Of particular relevance to the topic of ageing, the NF-κB inflammasome has regulatory roles in cell growth, survival, and proliferation. However, as recently reviewed [42], ROS production may have either inhibitory or stimulatory roles in the NF-κB pathway, suggesting a dose dependency in the effects of ROS. This unfortunately also means that

development of an intervention strategy/product to modulate this target mechanism is no simple feat and will have to be approached in a very tightly controlled “modification range”. From the literature consulted, it is clear that bidirectional communication is in place. For example, both neutrophils and macrophages are producers of oxidants via the NADPH oxidase system [2]. The NADPH-oxidase (NOX) proteins aid the transport of electrons across biological membranes and are found in all cells [43]. They are also one of the major generators of ROS in all cells. Particularly, these proteins are the predominant ROS producers in phagocytic cells, a process required for the normal respiratory burst that phagocytes use to kill pathogens and digest cell debris. Furthermore, activated neutrophils release myeloperoxidase (MPO), which contributes to the formation of hypochlorous acid (HOCL) by acting as a catalyst when reacting with hydrogen peroxide (H_2O_2). This directly increases the production of ROS [44]. The oxidative burst of neutrophils in itself releases oxidants such as H_2O_2, which are harmful to healthy cells and tissue [45]. Besides directly increasing the production of ROS which itself causes damage to surrounding cells, MPO specifically has been implicated as a risk factor for coronary artery disease, due to its capacity for oxidation of lipid membranes [46]. The increased oxidant concentration due to immune cell functions, as well as the resultant cell damage, results in increased metabolism in surrounding healthy tissue. Also at a gene level, role players have been elucidated in the context of an oxidative stress-inflammation link. For example, sirtuins are Class III histone deacetylases which are responsible for the deacetylation at N-epsilon lysine residues, a reaction which consumes NAD+. SIRT1 specifically, is a sirtuin commonly associated with antioxidant function [47]. Its regulation of oxidative stress is threefold: firstly, it stimulates the expression of antioxidants via the fork-head box protein O (FOXO) pathway. Secondly, it is involved in inhibiting the NF-κB signalling pathway by deacetylating the p65 subunit of the NF-κB complex which results in the inhibition of the NF-κB signaling. In contrast, however, excessive ROS can inhibit SIRT1 activity by oxidatively modifying its cysteine residues and thereby releasing its inhibition of the NF-κB pathway [48]. It is thus theoretically possible for cumulative stress to downregulate SIRT1 activity in the ageing process. Thirdly, SIRT1 has also been implicated in regulation of apoptosis by deacetylating p53 to inhibit p53 dependent transcription in models of cellular stress [48].This tripartite role defines SIRT1 as another important molecular target in the context of both normal and accelerated ageing. Apart from these targets related to cellular signalling, cell functional capacity should also be a focus. A striking example of the oxidative stress, inflammation link in ageing, is the decreased capacity for neutrophil chemokinesis reported in the elderly both in terms of motility and accuracy [49]. Normally, immune cells are attracted by chemotactic signals from injured tissue, to migrate to sites of injury or pathogen invasion. During this chemokinetic response, immune cells, typically neutrophils and classically activated macrophages, migrate through tissue toward the site of injury. This

movement is facilitated largely via adhesion molecules such as the beta-integrins and I-CAM1 in the case of neutrophils [23]. However, as mentioned earlier, expression of adhesion molecules on neutrophils increases with ageing [37], so that their movement is slowed. In addition, due to yet unclear mechanisms, but most probably due to adaptation of cellular "homing" molecules, directional accuracy of neutrophil migration is also compromised in the elderly. Sapey et al (2014) showed that inaccurate neutrophil migration was causally associated with increased constitutive phosphoinositide 3-kinase (PI3K) signalling. This results not only in inefficient inflammation due to prolonged response time, but the directional inaccuracy of movement also results in mechanical and oxidative damage to relatively more cells in the path of the migrating inflammatory cell [49]. It is clear that both oxidative stress and inflammation are able to induce and exacerbate one another (both indirectly and directly). Furthermore, regardless of the primary signal or which pathway was activated first, these interrelated processes form a vicious cycle which is difficult to target therapeutically because of its complexity. However, it is also this interrelated nature of the two systems that has led us to investigate the possibilities of antioxidants as treatment options to attenuate the cumulative effects of oxidative stress and in turn low grade chronic inflammation.

2.5 Practical considerations: information gained from acute oxidative stress protocols

Traditionally it was believed that long duration endurance exercise induced oxidative perturbations due to metabolic stressors, as well as inflammatory stimuli which occur over time [50]. This is supported by the notion that endurance exercise increases one's oxidative capacity [51]. The logic is that greater stressors over time elicit a greater response which results in adaptations. Recently though, acute protocols have been investigated as an effective stimulus of oxidative stress and inflammatory responses when the exercise intensity is high [52]. This poses as an appealing protocol as it can be used to elucidate the acute changes which occur and mimic other acute stressors such as injury and sickness, known to elicit higher responses in the more acute time frame. These protocols make use of what is known as high intensity interval training (HIIT) ,which consists of short bursts of high-maximal effort bouts of strenuous exercise with short rest periods in between and repeated cycles thereof [53]. This proposed method decreases protocol time, which enable the researchers to sample and elucidate data at earlier time points, capturing acute changes. It is also a protocol which is now popular amongst athletes and regularly fit persons making subject recruitment easier.

Some other methods of inducing oxidative stress include training in hypoxic conditions [54] and resistance training resulting in delayed onset muscle soreness (DOMS) [50]. These methods have been shown to be effective but are not ethically justifiable for research of this manner. These methods also do not affect the less injurious induction of oxidative stress and

inflammatory which this project aims to investigate and have various other undesirable side effects. It also would not be appropriate to compare this type of stressor with an aged population as ethically it would not be viable and there is no way of mimicking this in a human population.

Using a young population accompanied to exercise with an acute protocol thus seems as the most appropriate method to comprehensively assess these perturbations. A young fit population poses as a good group for two main reasons. Firstly a population accustomed to exercise means that the subjects will be able to complete the protocol safely and successfully [52], as HIIT protocols are being presented in training programmes as well as gym classes. We can thus be sure that the protocol will we completed. Secondly this population are expected to be free of confounding conditions or factors which would affect results, which is also why ethically this population is ideal.

Taking this into consideration an acute protocol seems to be appealing to elucidate the acute responses which are not overly injurious but demonstrate perturbations to compare the chronic effects of oxidative stress as well as low grade inflammation.

2.6 Are Grapes the Answer to Prevention of Ageing?

Despite the huge range of non-steroidal and natural anti-inflammatory products on the market today, the scientific literature shows a conspicuous lack of consistent support for any specific medication. This is perhaps at least in part due to the fact that researches investigating these products cannot keep up with the rate at which new ones are pushed onto the market. Hopefully new legislation on the control of these substances will affect this trend to the benefit of the consumer, by allowing for (or demanding) appropriate testing of these products. Nevertheless, antioxidants are being used almost routinely by many individuals who wish to supplement for enhancement of general health or as adjuvant therapy in conjunction with more mainstream, pharmaceutical medication. Although they are generally not regarded as a primary therapeutic option, antioxidants may hold particular potential in the realm of preventative medicine. The potential benefits of appropriate antioxidant supplementation are vast, especially when considering the connection between oxidative stress and inflammation. An antioxidant with the capacity to modulate inflammatory status can thus be beneficial to both normal ageing individuals and those suffering from lifestyle associated diseases. A comprehensive search of the scientific literature revealed that grape-derived antioxidants are consistently reported to have high benefit and low risk in the context of both oxidative stress and inflammation. These positive results are further strengthened by the fact that these consistent findings were reported across many different models and using a variety of different preparations, ranging from relatively crude extracts to highly purified ones. For example,

scientific literature and anecdotal reporting, highlights resveratrol ,a purified polyphenol present in grapes as well as other plants, as having anti-inflammatory [55] and antioxidant [56,57] action ,and thus by implication anti-ageing effects. Indeed, a recent paper [58] elucidated a role for resveratrol to protect against inflammatory damage via SIRT1 inhibition of the NFκB pathway (a mechanism discussed above in the context of ageing). In addition, more advanced studies have been undertaken on this polyphenol to better understand the relationship between the chemical structure of resveratrol and its biological activity, especially in terms of oxidant scavenging [59]. Also, pharmaceutical groups have been working on optimisation of delivery systems for resveratrol [60]. Such information may further advance the popularity of this very promising natural product with the pharmaceutical industry, to the benefit of consumers. The phenomenal frequency at which new papers on resveratrol appear, all providing evidence of positive effects in this context, suggests that this particular polyphenol should be investigated in the context of ageing as a matter of urgency. Even more promising than the many positive effects described for resveratrol above, is the fact that resveratrol is only one from a range of equally beneficial substances contained in grapes. The flavonoids quercetin and dihydroquercetin (DHQ), as well as proanthocyanidins and anthocyanins, all of which are present in grapes and a variety of other plant sources, have similarly been linked to both antioxidant and anti-inflammatory effects [55,56]. To date, despite appearance of a few very promising reports in this context, ageing specifically has not been the focus of many studies investigating these substances. Therefore, for the purpose of this review, we provide a comprehensive overview of the few existing ageing-related studies in this context that were available to us. Results from relevant papers that did not have ageing as a focus were also included, where those results contribute to our understanding of the role of grape derived polyphenols in oxidative stress and inflammation in the context of prevention or deceleration of the ageing process. When considering *in vivo* studies on ageing as a starting point, resveratrol (0.1 μM to 2.5 μM) exhibited a clear dose dependent effect on longevity in fish with known short lifespan: resveratrol supplemented fish almost doubled their expected 13-week lifespan and continued to produce healthy offspring long after all control fish had died. Even though resveratrol supplementation was only started in adulthood in this study (i.e., it compares to when humans might start to consider supplementation), it effectively delayed age dependent compromise of locomotor and cognitive performance and reduced expression of neurofibrillary degeneration in the brain [61]. This result of improved neural morphology was recently further substantiated in an aged rat model, where chronic resveratrol treatment prevented detrimental changes in dendritic morphology which is linked not only to ageing, but also to Alzheimer's disease [62]. Similarly, two months of ingesting polyphenols in the form of 10% grape juice resulted in enhanced potassium-evoked dopamine release and cognitive performance in aged rats [63].

A more recent review [64] provides more insight into the potential mechanisms by which age-related cognitive disorders may be curbed by grape polyphenols. Some of these mechanisms at first seem unrelated to the scope of this review, for example, preventing amyloid-beta deposition associated with Alzheimer's dementia [65]. However, recent research suggests a role for inflammation in the development of the disease [66], while natural antioxidants have been linked to prevention of amyloid-beta deposition [67]. Together, these data suggest that even these seemingly unrelated mechanisms may be interconnected to either inflammation, oxidative stress, or both. However, more clearly in context of this review, resveratrol was reported to increase NO production, resulting in vasodilation [64,68], which may play a role in the maintenance of central circulation and thus perhaps slower degenerative central processes, as has indeed been reported for resveratrol, as mentioned earlier. However, the role of NO in the context of antioxidant status is much more complex, so that this effect of grape polyphenols should probably receive more attention before it can be interpreted fully in terms of mechanism(s) involved. Furthermore, recently in a co-culture simulation of the human blood-brain barrier, another grape polyphenol, proanthocyanidin, was associated with significant inhibition of monocyte infiltration and proinflammatory cytokine secretion in HIV-associated neuroinflammation [69]. Such inhibition of neuroinflammation is associated with a better prognosis in terms of HIV-related neurodegeneration and dementia, further confirming the neuroprotective potential of grape-derived antioxidants. Taken together, these studies suggest that the neuroprotective effects of grape polyphenols involve both antioxidant and anti-inflammatory mechanisms, with the latter including not only modulated cytokine signalling, but also modulation of both motility and functional capacity of leukocytes, as previously illustrated by our group [69,70]. One may argue that both these results may be the result of decreased cell activation, perhaps as a result of the known altered cytokine environment. However, an age-associated lack of neutrophil chemokinetic accuracy in response to the chemotaxin fMLP has been reported [49], which suggests that the mechanism is probably related to age-induced compromise of specific cellular mechanisms, rather than activation. In addition, preliminary data from using Dunn chamber chemokinetic assays, suggest that grape polyphenols (specifically proanthocyanidin) may be able to correct this age-associated anomaly (unpublished data from my BSc Hons project). From Figure 2.1, which depicts typical digital images obtained for the path of individual neutrophils, it is clear that a more purposeful, directionally accurate movement was achieved in proanthocyanidin-treated neutrophils. This will ensure a more optimal inflammatory response (i.e., the response will be effective, resulting in relatively insignificant secondary damage, and be resolved in the minimum amount of time). In contrast the rather "aimless wander" of untreated neutrophils from aged individuals will result in not only ineffective immune cell infiltration to sites where they are required, but also relatively more secondary tissue damage and thus prolonged and exacerbated inflammation.

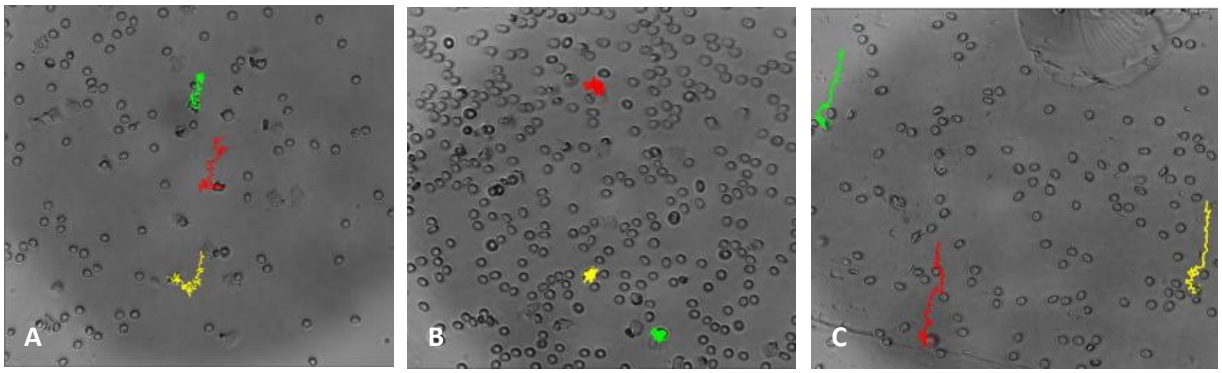

Figure 2.1: Typical neutrophil chemotaxis pathways for (a) a young participant (<25yr), (b) an aged participant (>65yr), and (c) an aged participant after acute in vitro treatment with grape-deed derived proanthocyanidin. The Olympus Cell system IX-81 inverted fluorescent microscope system with an F view cooled CCD camera (Soft Imaging Systems) at 20x magnification was used to capture these images, which was analyzed using Image J (Java software).

Turning attention to inflammation and oxidative stress again, the most probable targets for therapeutic intervention in the context of ageing have been described in Section 2.4. Since the ageing literature is relatively lacking in terms of papers on polyphenol intervention, we have tabulated effects of grape polyphenols reported for these identified targets in Table 2.1, citing relevant data that was mostly obtained in models other than ageing. The aim with this table was not to present all studies on grape-derived products; rather, it is an attempt to show the many models, species, and disease systems in which beneficial effects on oxidative stress and inflammatory status have consistently been reported. Also, although we have included doses used for *in vivo* studies for general comparison and again to illustrate the large variations in doses, these doses are product/extract specific and cannot be extrapolated across board. From Table 2.1 (page 15, legends on page 16), which is by no means a complete list of all studies reporting on grape-derived substances, grape derived products are undeniably beneficial in limiting the magnitude of the inflammatory response as well as to increase antioxidant activity and seem to have multiple targets.

Model	Treatment	Outcomes: inflammation	Outcomes: oxidative status	References
IN VITRO:				
Glucose and LPS-induced inflammation in HUVEC cells	Red grape polyphenols	↓IL-6, IL-8 and NF-κB at protein and mRNA levels ↓PECAM and ICAM-1 levels	↓ROS formation in dose-dependent manner	[71]
Primary human chondrocytes challenged with *E.coli* LPS (arthritis model)	Grape extract containing resveratrol, hopeaphenol and viniferin	↓PGE_2 production	↑scavenging of DPPH radicals	[72]
Osteoblast-like cells (MC3T3-Ei), treated with TGF-β to induce VEGF synthesis	Resveratrol	↓VEGF and VEGF mRNA, but no effect on p38 or SAPK/JNK, suggesting SIRT1 activation.	n.a.	[73]
Yeast models of sirtuin activation (*c.elegans, D. melanogaster)*	Resveratrol	↑sirtuin (SIRT1) activation	n.a.	[74,75]
Human adipose derived stem cells (hASCs)	Red grape (muscarine) grape seed oil, in comparison to rice bran and olive oils	↓adipogenetic factors (PPARγ and aP2) ↓IL-6 and IL-8 response to LPS ↓proinflammatory gene expression in adipocytes	Shown to be source of γ-tocopherol	[76]
High-glucose induced oxidative stress in porcine proximal tubule cells (LLC-PK_1)	Grape seed polyphenols	↓NF- κB pathway	↓intracellular ROS	[77]
IN VIVO ANIMAL:				
Rats exposed to localised bowel irradiation	Grape polyphenols OR pure quercitin 3-*O*-β-glucoside (10mg/ml, 7.14ml/kg body mass) by oral gavage for 5 days prior to irradiation	↓MPO activity ↓CINC-1 levels	↓SOD activity No change in glutathione peroxidase (GSHPx) activity No change in plasma malondialdehyde (MDA) concentration	[78]
Rats subjected to *E.coli*-induced septic shock	75 and 200 mg/kg/day grape seed procyanidin, by ip. injection for 15 days pre-*E.coli* challenge	↓IL-6 gene expression	↓NO in liver, spleen, plasma and RBCs ↓iNos gene expression ↓GSSG:total Glutathione ratio	[79]
Rat model of osteoarthritis	500mg/kg body mass of grape extract daily for 28 days	Prevented joint deterioration	n.a.	[72]
Rat model of skeletal muscle contusion injury	Acute OR 2-week supplementation, proanthocyanidins	↓pro-inflammatory cytokine signalling (TNF-α; IL-6) ↓neutrophil migration capacity Earlier macrophage switch from pro- to anti-inflammatory phenotype	↑ plasma and skeletal muscle ORAC	[55,80]
Rat model of ageing	Drinking water supplemented with 15g/l grape powder for 3 weeks	↓age-associated increase in corticosterone	↓plasma 8-isoprostane	[81]
Rat model of obesity	Grape procyanidin B2	↓IL-1β and NLRP3 levels in pancreas	n.a.	[82]
Middle-aged mice on high-calorie diet	Diet supplemented with 0.04% resveratrol	↓IGF-1	↑AMPK and PGC-1α activity ↑ mitochondrial number	[87]
Mouse model of pulmonary fibrosis	7-day oral resveratrol (50mg/kg/day) OR quercetin OR dihydroquercitin (both 10mg/kg/day)	↓neutrophil infiltration into lung tissue ↓inflammatory cells in bronchoalveolar lavage fluid ↓COX-2 and ↓ NF- κBp65 translocation	↓iNOS ↓oxidative lung damage (↓nitrotyrosine and poly-ADP-ribose polymerase levels)	[84]
Rabbit model of acute (*E.coli*) inflammatory arthritis	500 mg/kg body mass of extract acutely prior to *E.coli* challenge	↓PGE_2 production	n.a.	[72]
IN VIVO HUMAN:				
Non-diabetic haemodialysis patients	Grape powder (500mg polyphenols/day) for 5 weeks	Prevented increase in plasma CRP levels	↑glutathione peroxidase activity	[85]
Humans at risk for metabolic syndrome, aged 30-65	16-weeks of 20g wine grape pomice powder (822mg polyphenols) per day	n.a.	↑γ- and δ-tocopherol	[86]
Hypertensive, T2DM males, aged ≈55-65	8mg grape extract daily for 1 year	↓Alkaline phosphatase ↓TNF-α, IL-1β, IL-6 and CCL3 levels ↑transcriptional repressor LRRFIP-1 in PBMCs Modulation of miRNAs known to regulate inflammatory response	n.a.	[87]

Table 2.1. Representative reports on antioxidant and anti-inflammatory effects of grape-derived crude extracts and purified products.

Footnote to table 2.1:

Abbreviations: ADP, adenosine diphsophate; AMPK, adenosine monophosphate-activated protein kinase; CCL3, chemokine(C-C motif) ligand 3; CINC-1, cytokine-induced neutrophil chemoattractant1; COX-2, cyclooxygenase-2 ; DPPH, 2,2-diphenyl-1-picrylhydrazyl; HUVEC, human umbilical vein endothelial cells; iNOS, inducible nitric oxide; ICAM-1,intercellular adhesion molecule-1; IGF-1, insulin growth factor-1; IL, interleukin; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharides; LRRFIP-1, leucine-rich repeat in flii-interacting protein-1; NO, nitric oxide; NF-κB, nuclear factor-kappa beta; ORAC, oxygen radical absorbance capacity; PECAM, Platelet endothelial adhesion molecule; PGC-1α, peroxisome proliferator activated receptor gamma co-activator 1-alpha; PGE_2, prostaglandin E2; ROS, reactive oxygen species; SAPK, stress activated protein kinase; VEGF, vascular endothelial growth factor

Of course, when considering potential anti-ageing modalities, it is also of interest to include evaluation of changes in quality of life. Since ageing is associated with natural muscle wasting or sarcopenia [88], it is important to note that, in this context, supplementation with grape polyphenols (50mg/kg/day) for 4 weeks mitigated skeletal muscle atrophy in a mouse model of chronic inflammation [89]. This was achieved via modulation of two distinct pathways: one directly linked to inflammation (decreased NF-κB activation) and the other due to antioxidant function (limited ROS-associated mitochondrial damage and caspase-3 and -9 activation). Since caspase-3 activation is also a known pro-apoptotic signal [90], reduced activation and thus apoptosis may result in fewer mitotic cycles. This, in the context of the telomere hypothesis, may point to deceleration of ageing by the polyphenols. Very recently, grape proanthocyanidin treatment in rats was reported to have an anti-apoptotic effect which reduced damage after ischemia/reperfusion of the liver [91], which further substantiates this theory. Interestingly, a study in mice fed a high-fat diet indicated that grape polyphenols may modify gut microbial community structure to result in lower intestinal and systemic inflammation [92].This extraordinary result serves to remind us of the potential complexity of plant medicines and the requirement for comprehensive investigation of mechanisms of actions and interactions of any potential product via the traditional clinical trial process followed for new pharmaceutical drugs.

2.7 Summary

Ageing and accelerated ageing are not new concepts, but rather the norm in modern society. With a population that is growing relatively older due to advances in medicine, we are however pressed for answers on how to alleviate the symptoms or slow the progression of this

inevitable phenomenon. From the literature consulted, no negative effects of grape-derived products became evident, while beneficial effects in the context of oxidative stress and inflammation were consistently reported in the context of numerous cellular targets. Huge variation in product content and prescribed dosage complicates interpretation of the fast growing body of literature on this topic. A recommendation for future *in vivo* studies is the inclusion of more parameters per study, so that a more comprehensive interpretation of specific mechanisms becomes possible. Measurement of only basic indicators of either antioxidant status or inflammatory status, while providing proof of efficacy of the product, does not contribute much information on its mechanism of action.

Given the relative complexity of making firm conclusions with regard to mechanisms of action from *in vivo* data, the focus of this thesis was to further investigate mechanisms by which grape-derived PCO may achieve the beneficial effect reported from *in vivo* studies, using a human *in vitro* treatment intervention model.

2.8 Hypothesis and Aims

From the literature consulted, I have formulated the following hypothesis:

Firstly, I hypothesise that an aged sedentary population will have relatively higher levels of oxidative stress and a relatively worse pro-inflammatory profile than a younger sedentary population.

Secondly, I hypothesise that the grape seed-derived polyphenol (PCO) *in vitro* treatment will be beneficial to both populations, but more so in the aged population.

Thirdly, I hypothesise that in a normally fit young population, an acute bout of high-intensity exercise will effectively simulate acute inflammatory and oxidative stress, of higher magnitude than the cumulative stress elucidated in an aged population, which will also be alleviated by PCO.

Aims:

1. To characterise changes in our selected markers for inflammation and oxidative stress in an aged population.
2. To investigate whether fitness in a young fit population may result in a more favourable oxidative stress and/or inflammatory profile.
3. To assess the effect of an acute bout of high-intensity exercise on our chosen oxidative stress and inflammation markers.
4. To assess effects of *in vitro* PCO treatment on neutrophil functional capacity and oxidative stress parameters in all populations.

Objectives:

- Recruit healthy subjects in the following groups with the criteria;
    - Young (sedentary) between 18 and 25 years old healthy, not on medication or supplementation and does not partake in regular exercise. No chronic conditions
    - Aged over 65 years of age, healthy, not on medication or supplementation and does not have any chronic conditions.
    - Young (fit) between 18 and 25 years old healthy, not on medication or supplementation, partakes in habitual exercise at least 3 times per week and does not have any chronic conditions or injuries.
- Analysis of the oxidative status of plasma from all three groups at rest, by first measuring anti-oxidant capacity via ORAC, FRAP and TEAC as well as the GSH: GSSG ratio in whole blood. Then measure oxidative damage parameters, namely

conjugated dienes(an early marker of lipid peroxidation) and TBARS measurement of MDA (a late marker of lipid peroxidation)

- Isolate neutrophils and administer *in vitro* treatment of PCO (or placebo) for one hour, then track migratory capacity of neutrophils of all three groups at rest.
- Measure inflammatory markers expression of CD16 and CD66b on the surface of isolated neutrophils and MPO (intracellular) to attain neutrophil inflammatory functionality with and without *in vitro* treatment for all three groups at rest.
- Measure changes in neutrophil anti-oxidant capacity with ORAC and FRAP assays and changes in oxidative damage (CD) after *in vitro* treatment with PCO (or control placebo) for all three groups at rest.
- Induce acute oxidative stress with a HIIT exercise protocol in the young fit group and obtain blood samples before and after exercise.
- Perform above mentioned analysis on plasma from pre- and post-exercise samples.
- Isolate neutrophils from before and after exercise samples and perform above mentioned analyses.

Chapter 3

Methods

3.1 Ethical considerations

Ethical clearance was obtained for this research from the Subcommittee C Health Research Ethics Committee 1 of Stellenbosch University prior to initiation of the study protocol (reference number N14/02/007). Research was conducted in accordance with South African Medical Research Council guidelines and the Declaration of Helsinki. All volunteers were informed about the study verbally and in writing. Informed consent was obtained from all subjects prior to their enrolment into the study, the informed consent form can be found in Appendix B.

3.2 Subject characteristics and experimental categories

Subjects were recruited from two age categories, namely young adult (18-25 yr) and geriatric (over the age of 65 yr, n=7). In the young adult category, a distinction was made between sedentary (n=8) and competitively active (n=6) individuals (more information on this group to follow). Subject inclusion criteria included a) non-smokers, b) no medication use, c) no nutritional supplements, including vitamins or anti-oxidants and d) no diagnosed inflammatory or other chronic disease. The young sedentary subjects were selected as a control population. The aged individuals were selected to simulate chronic oxidative stress, as the onset of this has been reported to occur at the 6^{th} decade of life [49]. The young fit population were selected to partake in an exercise intervention to simulate acute oxidative stress [50].

3.3. Experimental acute oxidative stress-inducing exercise protocol

Fit subjects were subjected to a running VO_2max treadmill (TechnoGym, Italy) test to establish the appropriate exercise intensity with which to induce acute oxidative stress. A pre-exercise lactate measurement was taken from a finger prick blood sample (Accucheck). A lactate reading of higher than 3 mmol/l required a 15 min rest period before re-testing to ensure the subject did not have residual lactate above normal resting level (0.5-1 mmol/l) due to caffeine or alcohol intake or prior exercise. Subjects were given 5 minutes to warm up on the treadmill at preferred speed (fast walking to slow jogging pace, ± 5km/hr) where after the Oxycon (Jaeger, Germany) mask was applied and the test was begun at 7 km/hr. The incremental test administered had incremental increases of 0,5 km/hr every 30 seconds until voluntary exhaustion, from which maximal oxygen uptake was measured. After maximum was reached, exercise intensity was established whilst subjects were given 3 minutes of active rest. Thereafter the subject was subjected to a high intensity interval exercise protocol consisting of one minute of treadmill running at 80% VO_2max, followed immediately by one minute of

treadmill running at 50% VO_2max. This cycle was repeated until voluntary exhaustion. Three minutes after reaching voluntary exhaustion, a post-exercise lactate measurement was taken following the same protocol (refer to Appendix C for the detailed procedure).

3.4 Sample collection

Phlebotomy. One blood sample was obtained from all subjects except the fit subjects who had a blood draw before as well as after the exercise intervention, i.e. 2 samples. For each sample, six ml of blood was drawn from antecubital area of the arm by an experienced phlebotomist, and collected into sterile Lithium Heparin- and EDTA-containing vacutainer tubes (Vacuette, Lasec).The tubes were gently inverted 8-10 times before specialised processing as required for each analysis.

For oxidative stress sample preparation, EDTA whole blood was aliquoted into three 50 µl Eppendorf tubes and frozen immediately at -80 °C. Three Eppendorf tubes of 100µl whole blood plus 10 µl M2VP was aliquoted and frozen immediately at -80 °C. The remaining EDTA sample was processed the same as the heparin sample for oxidative stress analysis on isolated neutrophils. Heparinised blood samples earmarked for neutrophil isolation, were kept at room temperature until isolation of neutrophils, which had to be performed within four hours from collection.

3.5 Neutrophil isolation

Neutrophils were isolated from blood using density centrifugation. Briefly, 2 ml of Histopaque1077 (Sigma) (at room temperature) was pipetted into a 15 ml falcon tube. A double volume (4 ml) heparin anti-coagulated whole blood was added by carefully pipetting it down the side of each tube onto the Histopaque layer. Tubes were transferred to the centrifuge machine with swinging rotor and spin at speed of 652xg for 30 minutes at 21 °C, 1800 rpm. The sediment containing the neutrophils and erythrocytes was taken by discarding the supernatant layers. Sediment was mixed with equal volume 6 % (room temperature) filtered dextran (in Normal Saline) and incubated at 37 °C for 45 minutes. Pellet containing the erythrocytes was discarded and the neutrophil rich supernatant was collected and mixed with 10 ml of PBS. Mixture was centrifuged at 290xg for 10 minutes at 4 °C, 1500 rpm. Neutrophil pellet was collected by removing the supernatant. The final neutrophil pellet was then re-suspended into 1 ml RPMI 1640 media to obtain an automated cell count (CellDyne 3700CS, Abbott Diagnostics, Germany). Neutrophils are re-suspended to a 2×10^{-6} cells per ml. (Please refer to Appendix D for a more detailed method.)

3.6 Grape seed-derived polyphenol

The grape seed-derived polyphenol used was analysed by an independent laboratory (LC- MS laboratory, Central Analytical Facility, Stellenbosch University) to be a proanthocyanidolic oligomer made up of catechin, epicatechin and proanthocyanidin B. This water soluble compound was dissolved in purified distilled water to make up a stock concentration of 1 mg/ml later diluted with RPMI 1640 to treat cells at a concentration of 100 µg/ml. According to the supplier's Certificate of Analysis, the PCO content of Oxiproven™ is 70 mg per capsule. The daily recommended dosage is 1-2 capsules with water after meals. Previous studies have elucidated the optimal dosage [69] via MTT-assay mitochondrial viability studies and concluded that 100 µg/ml is the highest dosage which does not result in a significant reduction in mitochondrial viability.

3.7 *In vitro* PCO treatment protocol

The neutrophil suspension was divided into two groups of 500 µl each. The control group had 500 µl RPMI 1640 media added. The treated group had 400 µl RPMI and 100 µl aqueous PCO at concentration 100 µg/ml added. Cells were incubated for one hour at 37 °C, 95% CO_2 and 5% N_2.

3.8 Neutrophil chemotaxis assay

Neutrophil migration is an indicator of its functional capacity, as aberrant neutrophil chemokinesis has been identified as a main contributing factor to secondary tissue damage [20]. This is characterised by damage done to surrounding healthy tissue by the release of elastins and oxidative molecules [93]. This secondary damage can lengthen the recovery time and perpetuate perturbations in homeostasis as is the nature of the robust inflammatory response. In the context of ageing this explanation of inefficient neutrophil function has been linked to various disease pathologies most notably arthritis [94].

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 (figure 3.1) 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 (100 µl solution with HBSS). A gradient was allowed to form for 20 minutes before imaging.

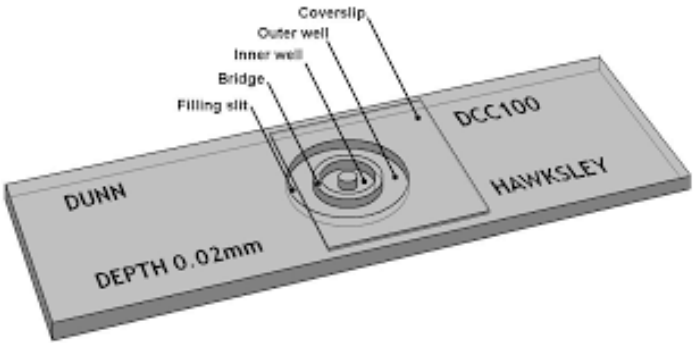

Figure 3.1: Dunn Chamber (Hawksley) which was used for visualisation of neutrophil migration

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 Figure 3.2.

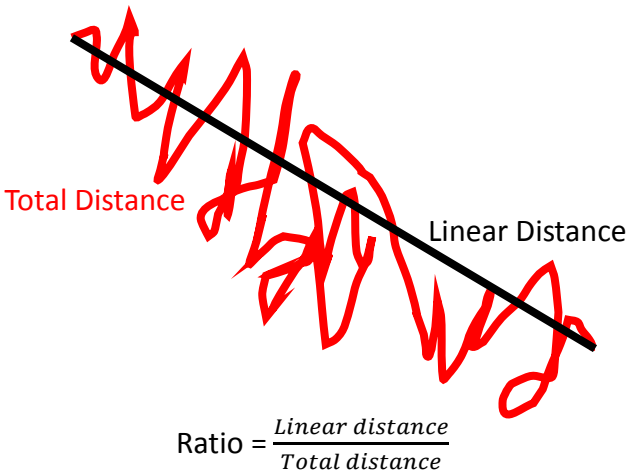

Figure 3.2: Illustrative neutrophil migratory track as obtained from live cell microscopy and cell tracking software. Actual cell path is presented in red, while the black line indicates the straight-line distance travelled from the starting point, in the direction of the chemotactic signal. The ratio of linear to total distance indicates accuracy of movement.

3.9 Neutrophil functional capacity

We chose two markers for assessment of neutrophil activation status, namely CD16 - a marker for the Fcγ Receptor IIIb (an IgG receptor), which is shed upon activation as described by Fossati et al. [116] – and CAECAM 8 (CD66b) – an adhesion molecule required for neutrophil extravasation and migration into tissue, which is exocytosed after activation, as described by Nair et al. [96]. In addition, intracellular MPO was assessed as marker of neutrophil functional capacity. After treatment; control and PCO-treated samples were prepared and stained with MPO (BD-Anti–Myeloperoxidase PE), FcγRIIIb (BD-Human CD16 ALEXA 647) and CEACAM 8 (BD-Human CD66b PERCP-CY5.5) antibodies after being permeabilised and fixed with CytoFix Cytoperm (BD Biosciences). The fluorescence was analysed on the BDFacs Aria Cell Sorter (Beckton Dickinson, San Jose, CA, USA) flow cytometer and analysed with BD FACSDiVa v6.1.3. Software to assess the expression of MPO, FcγRIIIb and CEACAM 8 expression on neutrophils treated with PCO versus control. Consult appendix E for detailed staining method and flow cytometry analysis guidelines.

3.10 Plasma Myeloperoxidase

Plasma myeloperoxidase was measured using an enzyme-linked immunoassays (Abcam Myeloperoxidase (MPO) human ELISA kit (ab119605, Abcam, ROW)) .The assay was performed on plasma samples separated from whole blood collected in EDTA vacutainer tubes. MPO was measured to assess inflammatory status as is reported in the literature. A detailed method of the ELISA can be found in appendix F.

3.11 Oxidative stress profile

Oxygen radical anti-oxidant capacity (ORAC) assay was performed on EDTA-anticoagulated blood plasma as well as isolated neutrophils after *in vitro* treatment with PCO or control. This assay is used to assess the peroxyl radical absorbance capacity (hydrogen atom transfer (HAT) mechanism of anti-oxidant action) of the endogenous antioxidant systems within plasma or cells in comparison to Trolox, the known anti-oxidant standard. It is measured via kinetic flourometric reading changes over time of β-phycoerythrin damage caused by peroxyl or hydroxyl radical attack which changes the absorbance of UV light. A detailed standard operating protocol can be found in appendix G.

Ferric Reducing Ability of Plasma Assay (FRAP) assay was performed on plasma separated from EDTA blood as well as isolated neutrophils treated *in vitro* with PCO and a control. This assay is used to assess the Fe^{3+} to Fe^{2+} reducing capacity (single electron transfer (SET) mechanism of anti-oxidant action) of a sample (plasma or cells) using colorimetric changes compared to Vitamin C. The full detailed protocol can be found in appendix H.

Conjugated dienes (early marker of oxidative damage) assay was used to assess the level of conjugated dienes present in samples (in plasma and cells).Conjugated dienes are formed as a product of the initial steps of lipid peroxidation. Conjugated dienes absorb light at 233 nm and this absorbance is measured compared to a cyclohexane standard. The full protocol for the assay with cells and plasma is detailed in appendix I

2-Thiobarbituric Acid Reactive Substances (late marker of oxidative damage) was used to assess the amount of malondialdehyde (MDA) present in plasma samples. Malondialdehyde is formed as an end product of lipid peroxidation, and can be detected spectrophotometrically at absorption of 532–572 nm. Thiobarbituric acid (TBA) is used in this assay to form a pink MDA-TBA complex which is then measured. The detailed protocol can be found in appendix J.

2,2'-azino-di-3-ethylbenzthialozine sulphonate (ABTS) Trolox equivalent anti-oxidant capacity (TEAC) assay was used to measure the total antioxidant status of plasma samples (combination of HAT and SET mechanisms of anti-oxidant action). This assay assesses the ability of $ABTS^{\bullet+}$ to scavenge and form a colour (green) of which absorbance is measured at 734 nm. The protocol can be found in appendix K.

Glutathione redox analysis (GSH/GSSG) measures the ratio of reduced (GSH) to oxidised (GSSG) glutathione as an indicator of oxidative stress status and DNA damage. Whole blood from subjects was used for this assay as well as whole blood treated with 1-methyl-2-vinyl-pyridinium triflouromethane sulfonate (M2VP) used to stop the formation of GSH and maintain GSSG. GSH is an antioxidant that provides reducing equivalents for the glutathione peroxidase. This reaction results in the formation of a disulfide bond between two GSH molecules to form GSSG. Exposure to increased levels of oxidative stress results in GSSG accumulating and the ratio of GSSG to GSH increases. An increased ratio of GSSG-to-GSH is an indication of oxidative stress. GSH is oxidised by the sulfhydryl reagent 5, 5′-dithio-bis (2-nitrobenzoic acid) (DTNB) to form the yellow which is measured at 412 nm for five minutes. GSSG formed can be recycled to GSH by glutathione reductase in the presence of NADPH which is added to stop the reaction. The ratio of GSH to GSSG is then measured by taking GSH_{total} (taken from first reading) and subtracting two multiplied by GSSG (taken from second reading) divided by GSSG reading (GSH/GSSG ratio = GSH_{total} – (2 x GSSG)). The full sample preparation and assay protocol can be found in appendix L.

All oxidative status analysis were performed at the Oxidative Stress Research Centre at the Cape Peninsula University of Technology under the supervision of Dr Fanie Rautenbach.

3.12 Statistical analysis

Statistical analysis was performed by an experienced biostatistician (detail) using the Statistica 64 v.13 (Stat Soft Software). Data were analysed for normalcy of distribution, where after main effects of treatments and age were assessed by parametric or non-parametric ANOVA and Post hoc Fisher's LSD tests. A $p \leq 0.05$ was considered significant. Effects of exercise was assessed using the student t-test.

Chapter 4

Results

Table 4.1 illustrates group averages for leukocyte counts performed to confirm apparent health of all volunteers. Counts were similar across all populations.

	WBC (10^9/l)	Neut (10^9/l)	Lymph (10^9/l)	Mono (10^9/l)	Eos (10^9/l)	Baso (10^9/l)	RBC (10^{12}/l)
Young sedentary	6.64(0.97)	3.91(0.72)	2.12(0.45)	0.48(0.08)	0.18(0.14)	0.10(0.04)	4.62(0.18)
Aged	6.13(2.74)	2.64(1.09)	1.79(0.38)	0.24(0.13)	0.10(0.04)	0.04(0.02)	5.36(0.67)
Young fit	6.69(1.83)	2.92(1.11)	2.42(1.05)	0.43(0.11)	0.27(0.12)	0.06(0.04)	5.45(0.80)

Table 4.1. Whole blood leukocyte counts at the point of sample collection. Values are means (SD).

4.1 Ageing and habitual strenuous exercise both increase oxidative stress and worsen the inflammatory profile

We set out to characterize our three populations and elucidate the baseline status of oxidative stress and inflammatory profile of groups. Firstly to establish whether our use of an aged population to simulate chronic oxidative stress was rational. Secondly we wanted to elucidate the oxidative stress status of the young fit population who are under the influence of habitual strenuous exercise as well as characterize their inflammatory profile. Both of these were done in comparison to the young sedentary group which we used as controls. The data presented in Table 4.2 displays the changes in anti-oxidant capacity as well as the level of level of oxidative damage present measured by content of lipid peroxidation by-products in the plasma. From the data we were able to establish that the aged population had a significantly increased level of conjugated dienes (an early marker of oxidative stress), while having no significant increases in anti-oxidant capacity. The fit population did not display any statistically significant increase in parameters when compared to the controls but displayed a tendency to have higher anti-oxidant capacity as well as conjugated dienes. Although the aged population displayed higher levels of oxidative stress, this was not matched to increased anti-oxidant capacity, which could indicate a relatively pro-oxidative stress state.

	ORAC (TE µmol/ml)	FRAP (VitC E µmol/ml)	TEAC (TE µmol/ml)	CD (nmol/ml)	TBARS(MDA) (µmol/ml)
Control: Young (sedentary)	1411(78)	429(28)	0.62(0.15)	0.006(0.001)	2.32(0.44)
Aged (sedentary)	1762(232)	411(28)	0.77(0.10)	0.021(0.007)*	0.47(0.44)**
Young (fit)	1943(895)	413(34)	1.00(0.34)	0.008(0.002)	1.98(0.14)

Table 4.2. Selected parameters indicative of oxidative stress status in plasma. ORAC, FRAP and TEAC are measures of anti-oxidant capacity and CD (an early marker) and TBARS (late marker) are markers of oxidative damage. Data is presented as group mean(SEM). Statistical analysis: * and **, indicates significant differences between young sedentary vs. aged sedentary, P<0.05 and P<0.001 respectively. Abbreviations: TE, Trolox equivalent; VitC E, Vit C equivalent; ORAC, oxygen radical absorbance capacity; FRAP, ferric radical absorbance capacity; TEAC, trolox equivalent absorbance capacity; CD, conjugated diene; TBARS(MDA), thiobarbituric acid reactive substances (malondialdehyde).

The ratio between GSH and GSSG was measured in whole blood to give an indication of glutathione content. A lower ratio indicates oxidative stress and has been linked to the presence of DNA damage [93]. In Figure 4.1, the ratios are displayed in a scatter plot to display the trends within and between groups. There was no significant difference between groups.

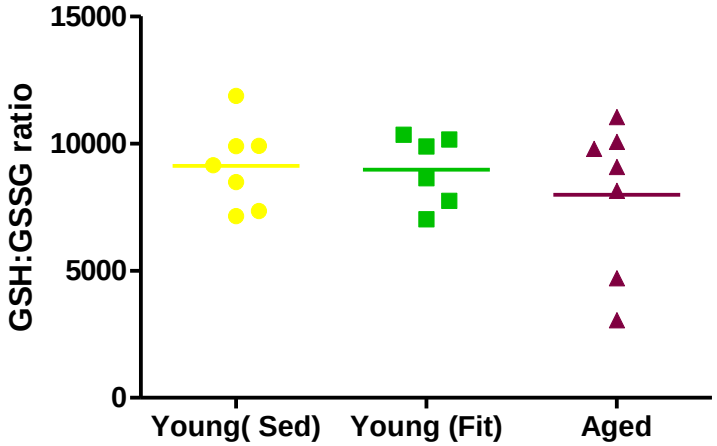

Figure 4.1. Whole blood ratio of GSH to GSSG content in the three study populations. Data for each subject is presented individually, with the group mean indicated by the corresponding group colour line.

To characterize baseline inflammatory profile plasma MPO was measured using an enzyme linked immunoassay. The measurement of MPO to characterize inflammatory profile is an established method in literature as described by Meuwese et al. [102]. In Figure 4.2 it is evident

that the aged population displayed a significantly higher plasma MPO content in comparison to the young sedentary controls. While the young fit population displayed a tendency to have increased MPO levels in comparison to the young sedentary controls this was not to the same extent as the aged population.

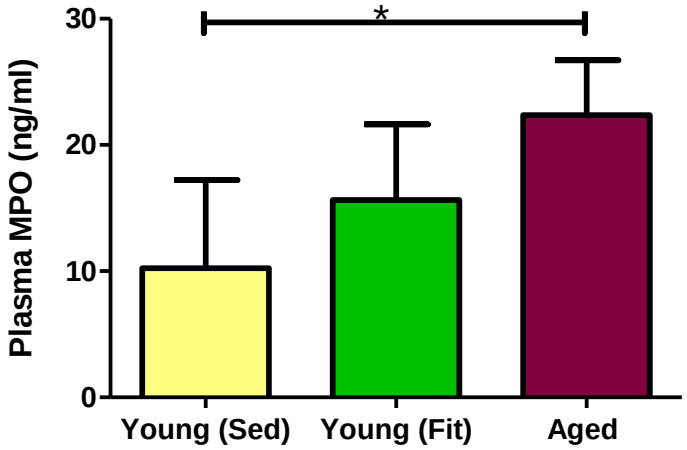

Figure 4.2. Plasma MPO content for the three populations. Values are means and error bars indicate SD. Statistical analysis: *, P<0.05.

The elevated plasma MPO levels in the aged population confirm that their inflammatory profile is worse than the young sedentary controls. Since baseline leukocytes did not suggest any evident inflammatory condition, we are confident that this is an ageing-associated result.

4.2 Acute high intensity exercise did not affect parameters of oxidative stress or neutrophil function significantly in the young fit population

The young fit group was exposed to an exercise intervention to induce acute oxidative stress. Anti-oxidant capacity, oxidative damage, inflammatory profile as well as neutrophil functional capacity was measured to assess the effect of the stimulus on this population and if oxidative stress was in fact induced.

Firstly we measured lactate as well as VO_2max to confirm the exercise intensity was in line with what the literature reports as evidence that exercise was sufficiently stressful to induce oxidative stress. We found that lactate was significantly increased after the exercise protocol, which suggests that oxidative stress was indeed successfully induced (Table 4.3).

Age	24(1.00)
VO_2max (ml.kg.min)	52.35 (9.18)
Lactate pre-exercise (mmol/l)	0.63(0.33)
Lactate post-exercise (mmol/)	6.1(3.16) *
Total duration of protocol (min)	19.31(3.12)

Table 4.3. Subject characteristics and parameters indicative of oxygen consumption and substrate utilisation. Data is presented as means (SD). Statistical analysis: *, pre- vs. post-exercise, P<0.005.

The results of characterization of the plasma from young, fit subjects before and after the exercise intervention with regard to anti-oxidant capacity (ORAC, FRAP, TEAC), oxidative damage (CD and TBARS) and inflammatory status (MPO). As displayed in the data in Table 4.4, no significant changes of the parameters were observed after exercise. Similarly, the ratio of GSH to GSSG in whole blood before and after exercise was unchanged (and similarly variable between individuals) after exercise (Figure 4.3).

	ORAC (TE µmol/ml)	FRAP (VitC E µmol/ml)	TEAC (TE µmol/ml)	CD (nmol/ml)	TBARS (µmol/ml)	Plasma MPO (ng/ml)
Pre-exercise	1943(895)	413(33.97)	1.01(0.34)	0.008(0.002)	1.98(0.14)	15.64(6.01)
Post-exercise	1904(915)	457(51.41)	0.94(0.34)	0.007(0.001)	2.13(0.17)	9.85(1.78)

Table 4.4. A summary of results for oxidative stress status (including anti-oxidant capacity and oxidative damage) and inflammatory profile of plasma samples of the fit young group before and after exercise. Data is presented as means (SEM).

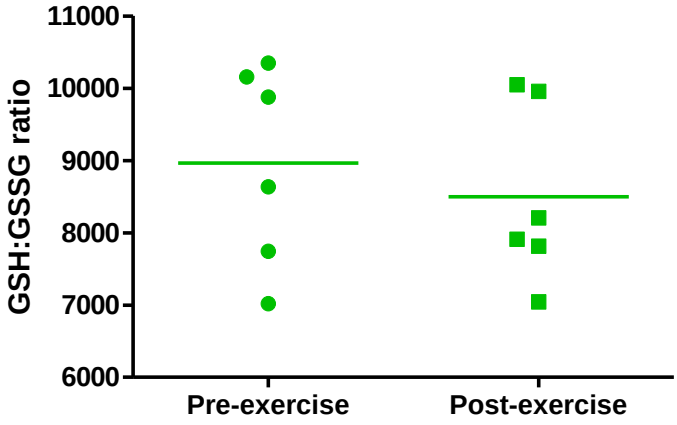

Figure 4.3: Ratio of GSH to GSSG content in whole blood of the subjects before and after exercise - each data point represents a subject, with the corresponding line indicating the mean.

Turning attention to cellular measures of oxidative stress within neutrophils specifically, results echo those of plasma measures, with no apparent effect of exercise (Table 4.5).

	ORAC (TE µmol/ml)	FRAP (VitCE µmol/ml)	CD (nmol/ml)
Pre-exercise	655(94.98)	146(24.52)	0.009(0.001)
Post-exercise	759(57.94)	155(22.21)	0.008(0.001)

Table 4.5. Neutrophil anti-oxidant capacity (ORAC and FRAP) and oxidative damage (CD) of the young fit group before and after exercise. Data is represented as the mean (SEM).

In terms of neutrophil functional capacity, intracellular MPO, FcγRIIIb (CD16) and CEACAM 8 (CD66b) expression indicated no effect of exercise (Table 4.6).

	MPO (MFI)	CD16 (MFI)	CD66b (MFI)
Pre exercise	361(61.62)	2955(575)	639(158)
Post exercise	339(48.89)	2849(482)	620(104)

Table 4.6. Functional parameters of isolated neutrophils before and after exercise. Data is presented as means (SEM).

In addition, the migratory capacity of neutrophils isolated from subjects was assessed using a Dunn migration chamber. In Table 4.7 the results indicate the total distance travelled, the linear distance travelled and the ratio of linear distance to total distance, a measure of directional accuracy of migration. No change was observed after the exercise intervention in any of the mentioned parameters.

	TD (µm)	LD (µm)	Ratio LD/TD
Pre-exercise	188(11.89)	16.15(4.87)	0.08(0.02)
Post-exercise	195(14.28)	25.07(8.82)	0.12(0.05)

Table 4.7. Measurements of total distance (µm), linear distance (µm) and the ratio of linear/total distance of neutrophil migration before and after exercise. Data is presented as means (SEM).

In summary, very few significant changes were noted in the parameters selected to indicate inflammation, oxidative stress or neutrophil functionality, from before to after the HIIT exercise protocol. This suggests that the exercise protocol did not induce perturbations sufficient to alter these parameters, or the sampling was done before these perturbations were physiological detectable.

4.3 PCO beneficially modulates oxidative stress and neutrophil function across groups

Previous research conducted in our group highlighted neutrophils as targets for investigation, as both *in vivo* (in a rodent model) and *in vitro* treatment with PCO had resulted in a number of beneficial effects in the context of inflammation. Thus we set out to elucidate the targets of PCO on various functional parameters of neutrophils, namely activation, migration and inflammatory capacity. Therefore we assessed the expression of Fcγ Receptor IIIb (CD16) – an IgG receptor responsible for activation and apoptosis of neutrophils (Figure 4.4) – as well as CEACAM 8 (CD66b), a transmembrane glycoprotein [95] (Figure 4.5).

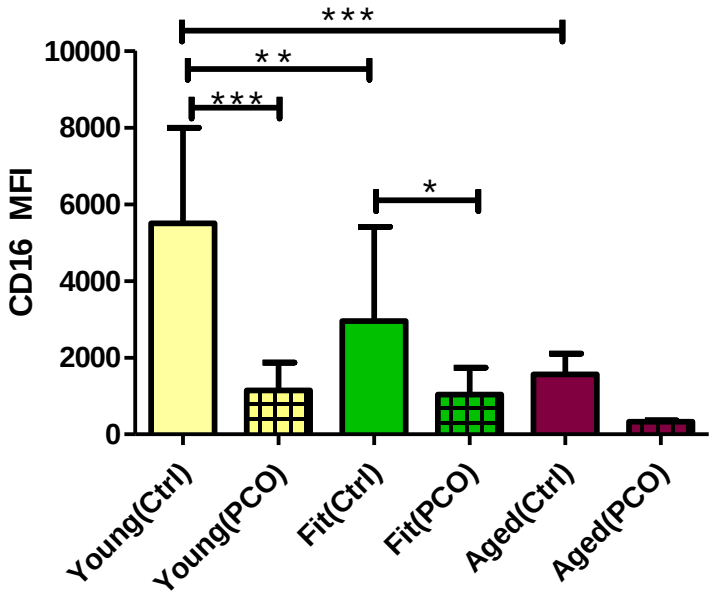

Figure 4.4 FcγRIIIb (CD16) expression on isolated control and PCO-treated neutrophils. Values are means ± standard error of the mean. Statistical analysis: * indicates treatment effect in young fit population, p<0.05. **indicates baseline difference between young controls and fit groups p<0.005. *** indicates treatment effect in young sedentary group p>0.001 and baseline difference between the young control group and aged group p>0.001. **Post hoc result indicates difference between groups under control conditions, P<0.00005 and p<0.005.**

Both age-related differences and a significant PCO treatment effect was recorded for both these parameters. There are marked decreases in all groups for CD16 after *in vitro* treatment with PCO, although not reaching statistical significance in the aged group. CEACAM 8 expression was higher in the aged population, but only significantly different from the young sedentary group, with expression increased after *in vitro* treatment with PCO in all three groups.

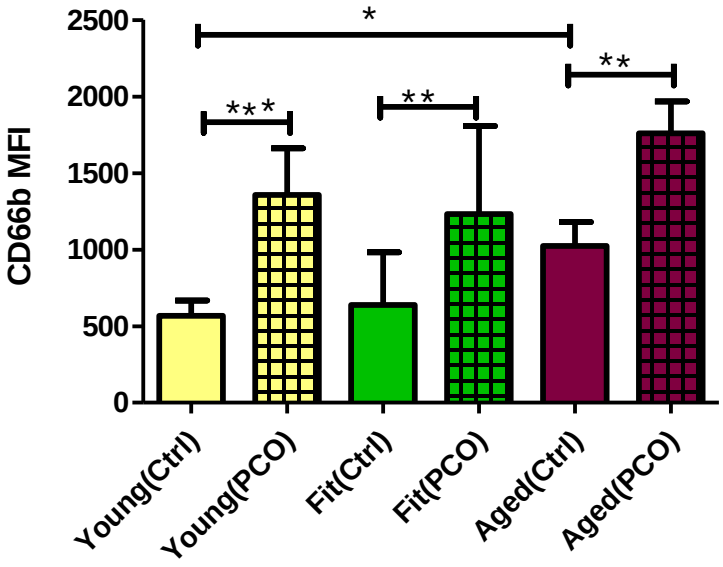

Figure 4.5: CD66b (CEACAM 8) expression on isolated control and PCO-treated neutrophils for all three groups. Statistical analysis: *** and ** indicate treatment effect of PCO of $p<0.0005$, $p<0.005$ respectively,*indicates significant difference between aged and young sedentary group under control conditions, $p < 0.05$.

4.4 Neutrophil migration is improved after *in vitro* treatment with PCO across all age groups.

Representative images for neutrophil chemokinesis (over a 20-minute period) are presented below (Figures 4.6, 4.7 and 4.8), for each subject group separately. There are clear differences in neutrophil migration patterns between the young and aged images (most notably when comparing the young fit and the aged populations), with less efficient migration in the aged. Also, both sedentary groups showed improved neutrophil migration after PCO treatment, while for the young fit group, no significant effect of PCO was observed.

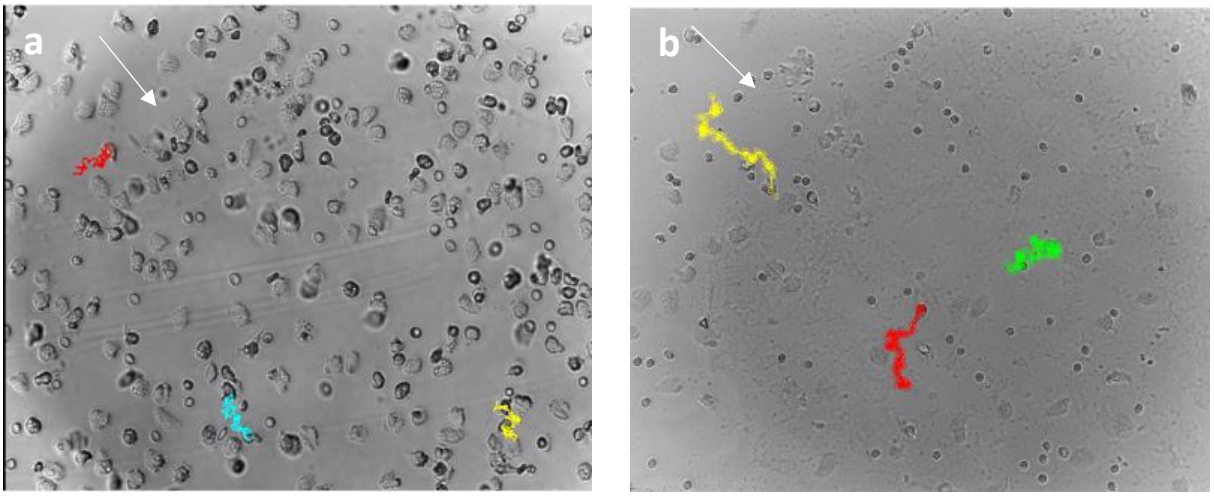

Figure 4.6. Representative images of neutrophil tracks, in a Dunn chamber, of a young sedentary subject treated with (a) placebo and (b) PCO. The Olympus Cell® system IX-81 inverted fluorescent microscope system with an F-view cooled CCD camera (Soft Imaging Systems) at 20x magnification was used to capture these images. White arrows indicate the direction of most accurate migration.

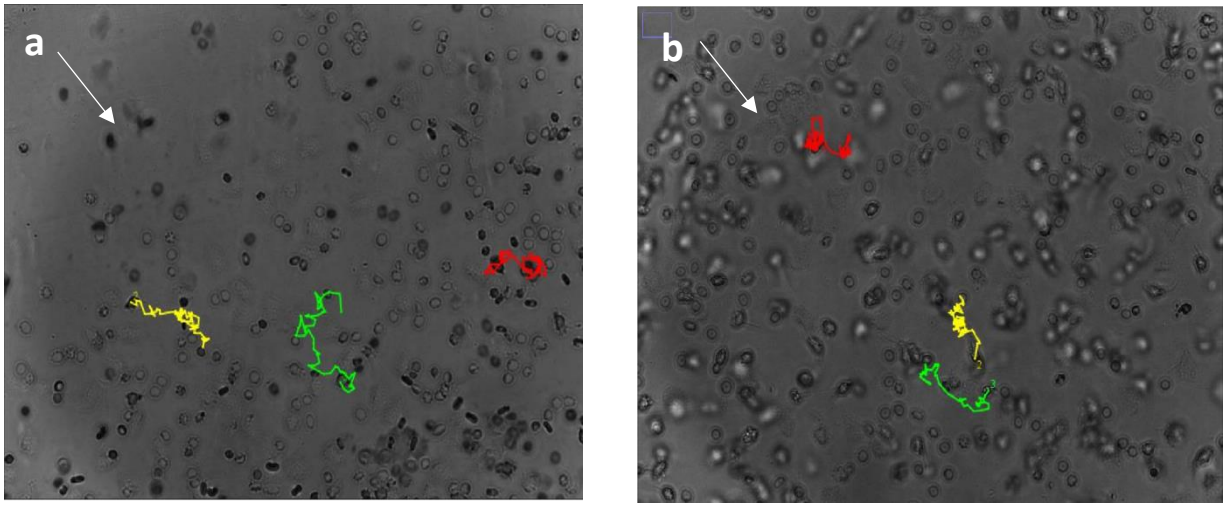

Figure 4.7. Representative images of neutrophil tracks, in a Dunn chamber, of a young fit subject treated with (a) placebo and (b) PCO. The Olympus Cell® system IX-81 inverted fluorescent microscope system with an F-view cooled CCD camera (Soft Imaging Systems) at 20x magnification was used to capture these images. White arrows indicate the direction of most accurate migration.

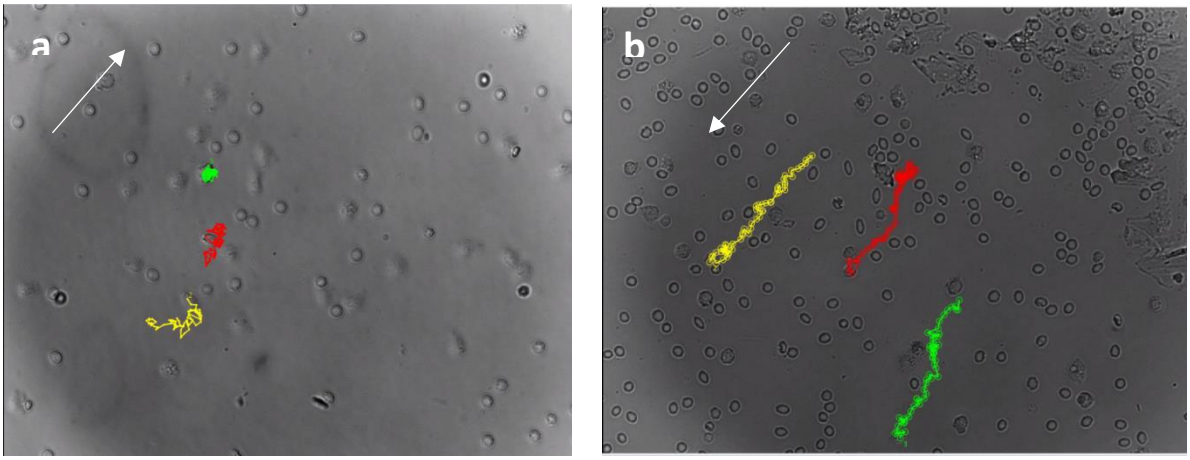

Figure 4.8: Representative images of neutrophil tracks, in a Dunn chamber, of an aged subject treated with (a) placebo and (b) PCO. The Olympus Cell® system IX-81 inverted fluorescent microscope system with an F-view cooled CCD camera (Soft Imaging Systems) at 20x magnification was used to capture these images. White arrows indicate the direction of most accurate migration.

Quantified results in support of the visual results are presented in Figures 4.9 and 4.10. In addition to observations above, statistical analysis revealed that both the fit population and the aged population had significantly increased control total distances when compared to the young sedentary controls. Although PCO treatment did not significantly affect total distance, this difference was not maintained after PCO treatment. The linear distances of the both young and aged sedentary populations showed significant improvements after treatment with PCO, which is in line with our observations above.

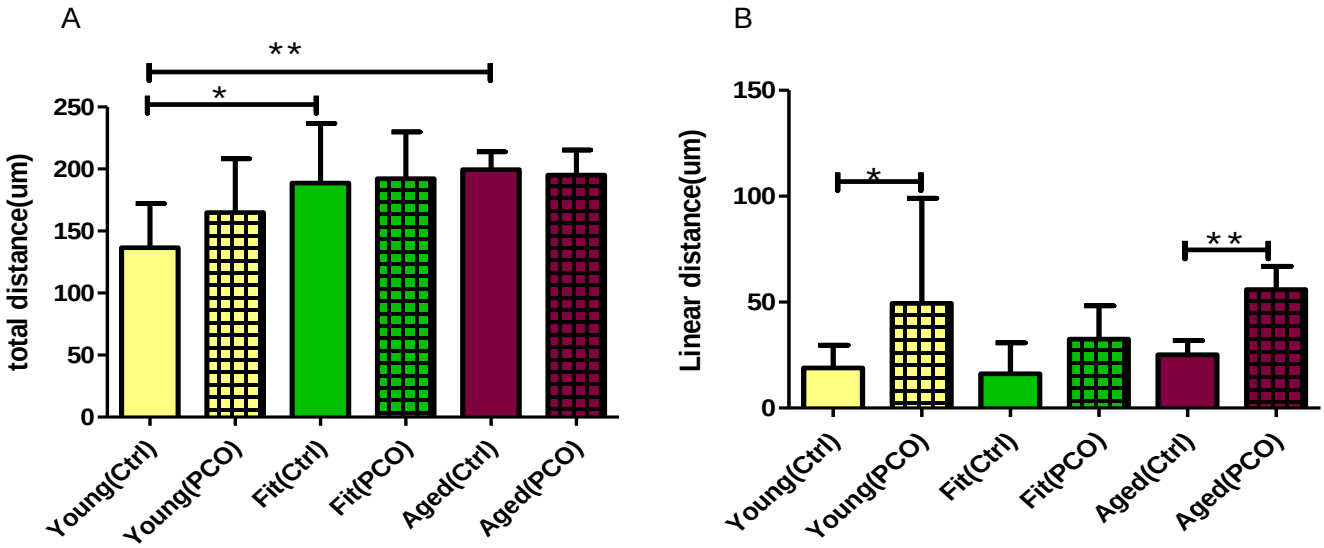

Figure 4.9. Total (a) and linear distances (b) travelled by neutrophils from the three groups before and after treatment with PCO. Data are means and error bars indicate SD. Statistical analysis: *, $p<0.05$; **, $p<0.05$.

When considering both linear and total distance as a ratio to indicate directional accuracy, the aged group exhibits a significant improvement after *in vitro* PCO treatment (Figure 4.10). A

similar effect was noted in the young sedentary group, although relatively higher variability in this group resulted in statistical analysis returning a tendency for PCO effect only (p=0.06).

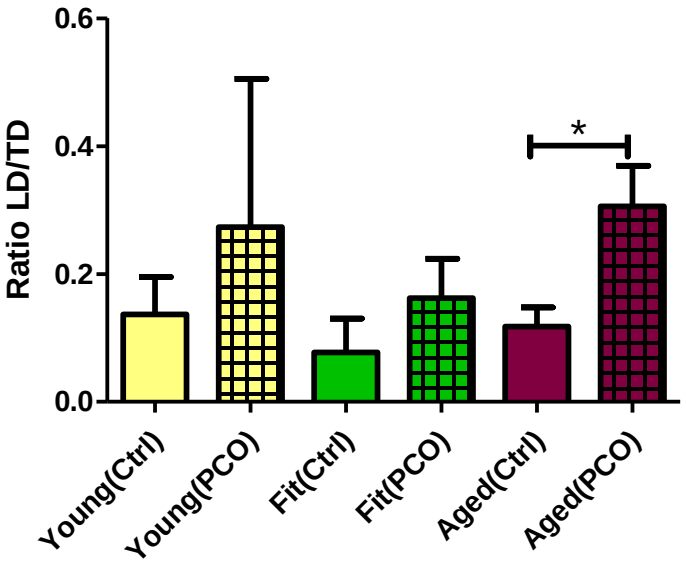

Figure 4.10. Ratio of linear distance/total distance, an indication of accuracy, travelled by neutrophils from the three groups before and after treatment with PCO. Data is presented as means and error bars indicate standard deviation. Statistical analysis: *, p<0.05.

Intracellular MPO, another indicator of neutrophil functional capacity, was lower in the fit young group when compared to the young sedentary group under control conditions (Figure 4.11), which may suggest either greater MPO release into the interstitial space which is pro-inflammatory, or less MPO production, an anti-inflammatory effect.

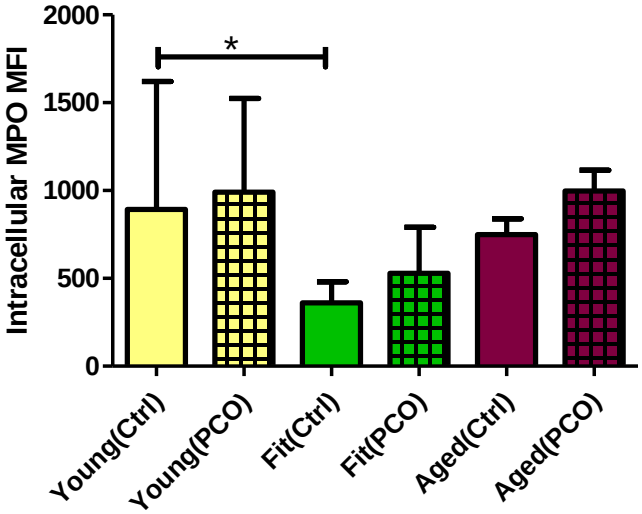

Figure 4.11. Intracellular MPO expression of neutrophils isolated from all three groups for control (placebo only) and *in vitro* PCO treated groups. Values are means and error bars SEM. Statistical analysis: *, p<0.05.

As seen with the results for total neutrophil migratory distance, this difference was no longer statistically significant after PCO treatment. The fact that intracellular MPO increased in all groups after PCO treatment (ANOVA main effect of treatment P<0.05), supports our first interpretation, that lower intracellular MPO indicates less release of MPO from cells – an anti-inflammatory effect.

4.5 Anti-oxidant capacity in neutrophils was improved with *in vitro* PCO treatment

Both ORAC and FRAP (Figure 4.12) indicated improvements in anti-oxidant capacity in neutrophils after PCO treatment, with significant effects recorded in the young fit group for both measures, and for ORAC only in the aged (tendency only for FRAP in this group, P=0.07).

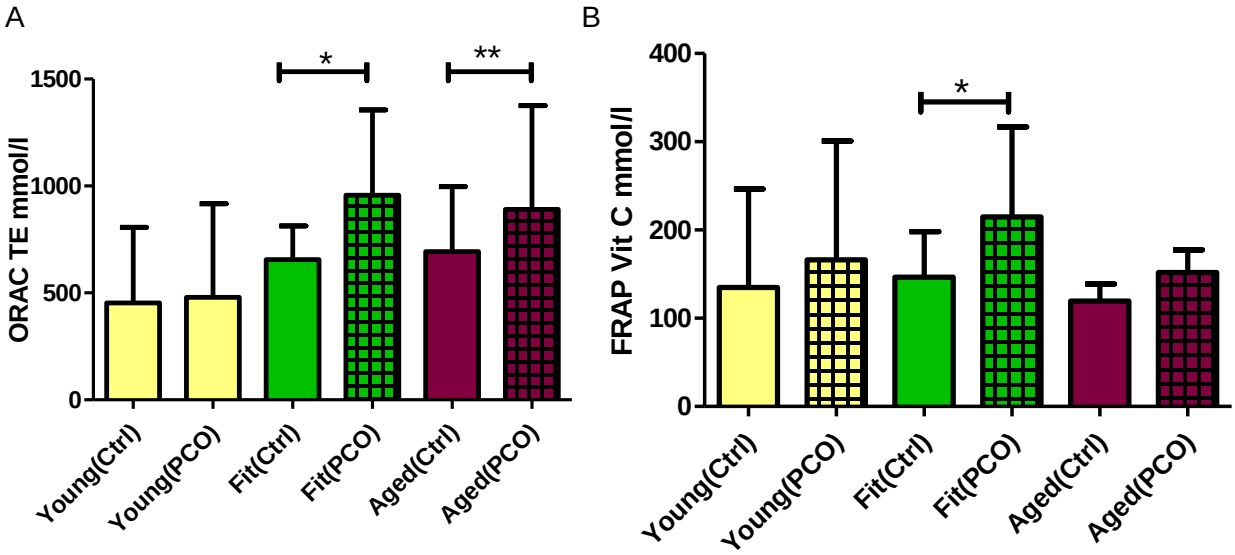

Figure 4.12. ORAC (a) and FRAP (b) for all three groups, both control (placebo) and after treatment with PCO. Values are means and error bars indicate SEM. Statistical analysis: *, P<0.05; **, p<0.005.

In terms of intracellular conjugated dienes, there was no significant differences between groups and no treatment effect, although the (placebo-treated) aged group had a tendency for lower intracellular conjugated dienes (p=0.064) than the young control group (Figure 4.13).

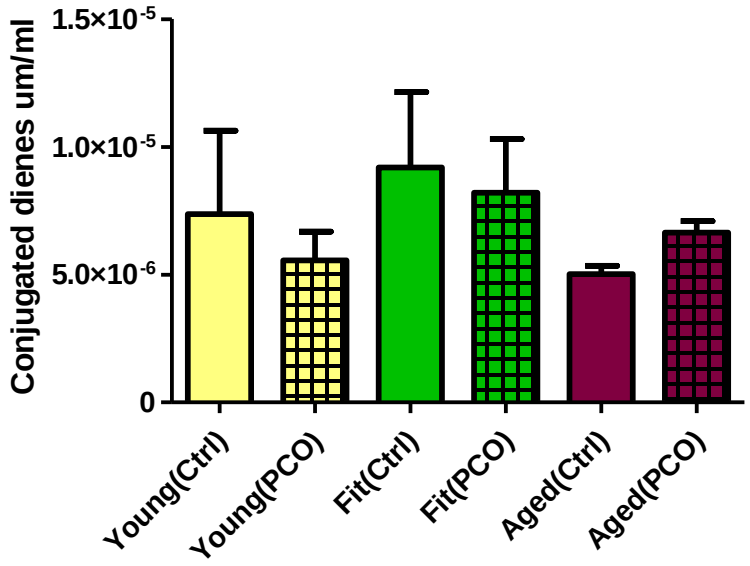

Figure 4.13. Conjugated dienes in neutrophils from subjects (for all groups) before and after treatment (with PCO). Values are means and error bars indicate SEM.

Chapter 5

Discussion

Our group has previously established that PCO has a potent anti-inflammatory potential [55, 69, 70,80], adding to the available literature which has already established grape-derived polyphenols as potent antioxidants. With this thesis, given the interrelated nature of oxidative stress and inflammation, we aimed to investigate the effects of *in vitro* PCO treatment on various oxidative and inflammatory parameters in models of chronic oxidative stress (as found in ageing) and acute oxidative stress (as found with exercise). In order to do this we first wanted to establish if our models of chronic oxidative stress and acute oxidative stress, did indeed differ in oxidative and inflammatory profile from a young healthy sedentary control population. We therefore analysed the effect of ageing and habitual strenuous exercise and how these affected oxidative and inflammatory profile. We then tried to induce an acute oxidative stress stimulus with exercise to elucidate acute responses. After this we investigated the effects of *in vitro* PCO treatment on isolated neutrophils to elucidate the mechanisms involved in the immune modulatory effects of PCO. Our main findings are discussed below, in terms of age-related changes seen, the success of inducing oxidative stress via exercise and the modulating effect of PCO.

5.1 Effects of ageing

Ageing has been characterised as having a generally less desirable oxidative stress profile such as decreased anti-oxidant capacity and increased free radical presence [2, 4]. The main reasons as summarized by MacNee et.al. [5] for this is the decline in anti-oxidant capacity, an increased exposure to stimulating stressors and repeated cell cycling – as explained by the Free Radical Theory of ageing [9]. In our study, aged individuals similarly had significantly worse outcome for several indicators of oxidative stress when compared to our younger population. For example, this is evident when looking at the content of conjugated dienes, which was significantly higher in the aged population (Table 4.2), indicating that the presence of (chronic) oxidative stress even at rest. This finding is in line with literature which shows that as one ages, oxidative stress is accumulated due to endogenous anti-oxidant saturation [96]. Coupled with this finding is the FRAP result. FRAP, another measure of anti-oxidant capacity, was lower in the aged population. Reports of decreased capacity with age is very broad and non-specific and thus it is not clear which mechanisms ageing affects and at what time frame this occurs. In our study, more pronounced effects of ageing was seen for plasma FRAP than for ORAC. A potential explanation for this is that the FRAP assay which measures the single electron transfer (SET) anti-oxidant mechanism may be more sensitive to ageing-associated

changes than the ORAC, which indicates hydrogen atom transfer (HAT) anti-oxidant function. Nevertheless, when taking these two parameters into account it does suggest that the aged population indeed had a worse oxidative status than the young (control) sedentary population.

Interestingly, not all selected parameters gave unequivocal evidence of undesired outcome with ageing. For example, the aged population had significantly lower levels of MDA (a late marker of oxidative stress) and better ORAC and TEAC capacity in comparison to the young sedentary controls in their plasma. TEAC is a measure of the combination of both HAT and SET mechanisms, and is often used as total antioxidant measure [98]. Since it is highly unlikely that ageing results in improved anti-oxidant capacity, especially given the CD and FRAP results, a more feasible explanation is that the young control population in our study may have been making relatively poorer lifestyle choices than the older population, which may have negatively affected their MDA result. This phenomenon of poor lifestyle choices in the majority of a population similar to the one used in this study has indeed been reported previously [6]. The selection criteria – which included no use of chronic medication – likely caused a natural selection of an aged population at the higher end of the scale in terms of general health status relative to the total aged population, which translated to less pronounced age-related inflammatory and oxidative stress perturbations, as assessed by MDA, ORAC and TEAC.

This interpretation is supported by relevant literature. Our inclusion criteria was sound, as similar criteria have been used in other ageing studies [22, 36, and 99]. Also, healthy lifestyle choices (diet and activity) in the aged has been directly linked to longevity [5, 100,101]. Although these lifestyle habits were not recorded quantitatively, they were informally communicated to the researcher by subjects. Thus, in studies of this nature, caution should be exercised when interpreting data – it is my opinion that the physiological profile reported here, may actually be significantly less healthy across the total population. This should be kept in mind when recommendations are made.

In terms of inflammation, plasma MPO was measured to give a general indication of the inflammatory status at baseline of subjects (Figure 4.2). Although not ideal, assessment of plasma MPO as an inflammatory marker is standard practise in literature which do not have detailed immunological assessment as primary focus [102], which allows contextualisation of results from our current study. The aged population had a significantly higher content of plasma MPO in comparison to the young sedentary control group, indicating a higher baseline inflammatory profile, which supports our hypothesis and previous reports in literature. The literature reports that aged individuals display elevated levels of MPO years before onset of disease (or cardio vascular events) and thus it is used to predict clinical outcomes [102,103]. Interestingly, increased MPO is also often reported during chronic disease, such as

cardiovascular disease, diabetes and metabolic syndrome, reporting levels as high as 704 pmol/L (≈102 ng/ml) in a population which is above 65 years of age. [102]. This is significantly higher than the ≈20 mg/ml measured in our older population, suggesting that the MPO in the current study indeed indicates ageing-related changes, as subjects clearly do not have high enough levels to suggest presence of illness. This data also therefore indicates that even in a healthy population, the 6th decade of life shows an enhanced inflammatory profile, which can be accurately reflected by plasma MPO in the absence of illness.

The intracellular MPO seen in neutrophils indicate that the aged population had similar levels to the young sedentary controls as there was no statistical difference between these two groups. This result is different from the plasma MPO, which showed clear age-related differences. Most likely, this suggests that under control conditions, neutrophils do not sequester MPO intracellularly, or that ageing in itself does not modify this capacity. Simultaneous assessment of intracellular and plasma MPO would provide more information here, but the current study design did not allow for matching time points, so that a more definite conclusion cannot be made with the present data.

In our investigations of immunosenescence, with focus on neutrophil functional ability, we observed results similar to that of the plasma. Immunosenescence has been identified as a key factor in the progression of age-related diseases and correlated with high mortality rates of aged persons experiencing bacterial infections [104, 105], with neutrophil dysfunction implicated as a main contributing factor [106]. We therefore investigated neutrophil functional capacity to elucidate the effects in these primary responders of innate immunity. In isolated neutrophils, ORAC capacity was once again higher in the aged population, but not so with FRAP, which again echo's the plasma result. This indicates clear changes between our aged populations in comparison to the young controls, and shows how population selection can alter the outcome of a study. One should thus be careful to consider these confounders in data interpretation in order to derive the correct conclusions. Also, this result indicates that relatively more acute factors, such as dietary choices, may affect plasma MDA, TEAC and ORAC, while other factors such as plasma FRAP and CD may be more indicative of chronic adaptation.

Despite higher plasma CD in the aged population, neutrophil conjugated diene content was similar in the aged and young control group. Since plasma CD may originate from all cell types, this result points to a more systemic oxidative stress state rather than a primary specific response mounted by neutrophils. To our knowledge, this study is the first to assess CD content in neutrophils, so that a more informed interpretation is not possible at this point.

Although neutrophil CD content does not suggest excessive oxidative processes in this cell type when considering measures of inflammatory status, significant ageing-associated

changes indeed indicate a relatively more pro-inflammatory state within neutrophils. For example, our results on neutrophil FcγRIIIb (Figure 4.4), CEACAM 8 (Figure 4.5), intracellular MPO (Figure 4.11) and chemokinesis (Figure 4.9 and 4.10) confirms that ageing is indeed a pro-inflammatory phenomenon, as also supported by the ageing-literature [95,106]. In terms of CD16 and CAECAM 8, our results is in agreement with the literature, which report that CD16 expression decreases and CAECAM 8 increases with age and that there is an association between these changes and age-associated pathology [95,107,108,109]. Given the similarities between ageing and accelerated ageing as outlined in the literature review, it may be useful to monitor CD16 decline and CAECAM 8 increase over time in apparently healthy younger populations, in order to identify people at risk of accelerated ageing and its associated pathologies, so that preventative treatment can be timeously implemented. This warrants further investigation.

Since CAECAM 8 is an important signalling molecule of the integrin cascade within neutrophils which dictate migration directionality as well as accuracy [96], our data indicating maladaptation with ageing suggested that neutrophil chemokinetic capacity may also be aberrant.

Indeed, the accuracy of neutrophils of aged subjects show little to no directional activity and in fact appeared to be “stuck” in one spot in the Dunn chambers – a result in line with the increased surface expression of CAECAM8.This results together suggests that in the aged, neutrophils would be exerting pro-oxidant inflammatory effects in otherwise healthy tissue.

From these results, we can conclude that in our study population, ageing-associated adaptations in terms of cumulative oxidative stress and inflammation is probably best reflected by CD, FRAP, CD16, CAECAM 8, MPO and neutrophil chemokinetic capacity.

5.2 Acute oxidative stress simulation by exercise

Our aim was to induce a relatively more acute oxidative stress insult, to facilitate investigations into acute (exercise-induced) versus chronic (ageing-associated) responses/adaptation to oxidative stress. The significant increase in lactate post-exercise (Table 4.3) is in line with reports which indicate that exercise induces an oxidative stress response. Lactate is produced when metabolic demand is increased during exercise and has previously been used to assess exercise intensity and metabolic response [110]. Our lactate response confirms that oxidative stress was achieved.

Despite the significant increase in lactate – suggestive of anaerobic energy production - there were no significant changes in anti-oxidant capacity before and after exercise, nor in markers

of oxidative damage. The exercise protocol used was adapted from the exercise science literature [52, 53] which reported that HIIT induces oxidative stress and an inflammatory response sufficiently severe to result in muscle damage and to prolong recovery time. We therefore combined the VO_2max testing with a HIIT protocol. Severe acute intensity exercise was reported by [52] to increase oxidative after 30 minutes and included incremental exercise similar to that of a VO_2max and HIIT protocol. We anticipated that the combined time of the VO_2max and HIIT would exceed this time point, but the subjects did not respond as anticipated, with total time to fatigue much shorter than the expected 30 minutes. It is possible that the exercise protocol was too severe for this population. However, their VO_2max results suggest that they are of sufficient standard to be able to perform such an exercise and that lack of motivation may have confounded the outcome.

This may have resulted in blood being sampled too soon after initiation of exercise, as previous work reports that MPO release occurs ≈4 hours post cessation of exercise [80], a time point that was not feasible in this study – as neutrophil migration was a focus here, it was imperative to sample and conduct analysis as soon as possible after cessation of exercise as this is the time of neutrophil extravasation and infiltration into damaged tissue. Furthermore, previous studies such as Syu et. al. [52] and Quindry et. al. [53] assessed multiple time points after exercise and their protocol was repeated on more than one occasion. We deviated from this for two reasons: firstly the cost of multiple time points was not feasible, and secondly, we did not want the training effect potentially achieved by multiple bouts. A future recommendation would be to elucidate training techniques of subjects and take this into consideration before prior to exercise stressor to ensure the subject can successfully complete protocol, or to offer incentives to elicit maximal effort and thus ensure longer time to voluntary fatigue.

The changes normally observed in MPO and neutrophil MPO levels were thus not observed in this study. We therefore recommend that blood sampling should be postponed to a time point at least 2-4 hours post exercise as reported by Quindry et al. [53] and Myburgh et al. [80] to investigate these parameters in particular. Future considerations would be an exercise protocol of longer duration as well as sampling at various time points taken hours after exercise intervention. The effect of habitual strenuous exercise we observed gave us more insight into the effect acute bouts of exercise over a period of time in the young population which was interesting. We therefore looked at the young fit group in comparison to the sedentary control and aged populations to ascertain their baseline status and found interesting results.

The young fit group displayed a tendency to have higher TEAC and ORAC readings (Table 4.2), measures of anti-oxidant capacity of their plasma. Fitness is known to increase the endogenous anti-oxidant capabilities of persons [50] and this was seen here. The results

though were not statistically significant which we attribute to a small subject group number allows for larger variation, which we observed. Future considerations would be to have larger subject recruitment as well as stricter assessment of subjects. This is warranted as well to exclude overtraining and injury as these factors would affect oxidative stress and therefore anti-oxidant capacity [50] which could account for the variations we observed.

With regard to oxidative damage we noted that both conjugated dienes and MDA content of the plasma in the young fit group did not differ from the young sedentary (control) group, which indicates that this population has effective anti-oxidant capacity. We did however observe a slightly increased inflammatory state of the fit population relative to the sedentary group, as they exhibited higher levels of plasma MPO, although once again with large inter-individual variation. We postulate that this may be due to overtraining or insufficient rest and recovery time, as has been observed with competitive athletes.

In the neutrophils of young fit subjects we noted that intracellular MPO was significantly decreased while their plasma MPO had a tendency to be increased. Considering once again that these assessments were done in unstimulated neutrophils this indicates a more pro-inflammatory state, as this indicates MPO release. When taken into account with the trends seen in the CEACAM 8 and FcγRIIIb, the basal state of neutrophils in this population indicate a pro-inflammatory state similar to the aged population but not to the same extent. FcγRIIIb was decreased in the fit population, who considering are in the same age group as sedentary controls indicates that they are engaging in an inflammatory stimulating activity. CEACAM 8 expression is in line with the young controls, indicating that their activation status is stable, which is favourable.

The fit population displayed a tendency to have less accurate neutrophil migration when compared to the young sedentary group which ties in with the differences in FcγRIIIb expression and highlights the possible mechanism which affects migration. Overall this group displays a tendency have a worse inflammatory profile than the young sedentary group. Overtraining, insufficient recovery time and recurring injuries can all attribute to this. Unfortunately injury history and training routine were not taken into account when subjects were recruited, by majority of the subjects within this group are competitive athletes. This warrants further investigations on the inflammatory profiles of competitive athletes.

To assess comprehensively and for completeness, assessment of all parameters previously measured within neutrophils of both pre- and post-exercise neutrophils with *in vitro* PCO treatment and control conditions was done. This data can be found in appendix M.

5.3 Modulatory effects of PCO

Polyphenols have been identified to have potent anti-ageing effects [111] and ageing is associated with various disease states. Thus we set out to elucidate the extent of effect our extract has and if this differs from relatively acute to more chronic exposure to oxidative stress. For this project we investigated the *in vitro* effects on neutrophils only, supplementation of subjects will be carried out in future studies. We observed that *in* vitro treatment with PCO had an effect on both the aged as well as fit groups in more than one parameter on isolated neutrophils.

We saw that PCO treatment significantly increased ORAC across age groups. This suggests that there is room for improvement in both the fit and aged populations, even though large differences were not initially noted before treatment in the case of neutrophils, which we already identified as cells which are victim to perturbations. The same trend was observed in the fit group in the FRAP assay, which we noted showed the most change for this group for assays which assessed anti-oxidant capacity. This highlights FRAP as a parameters to further investigate for this group.

I postulate that an increasing anti-oxidant capacity observed in these groups are indicative of the need to increase endogenous capacity to facilitate the larger or continued exposure to oxidative stress incurred with ageing and strenuous exercise. The young sedentary group did not display significant changes, suggesting that they did not have a significant need for an exogenous anti-oxidant. This result also confirms that our control group was a suitable control for our experiment. Overall we can conclude that PCO can improve anti-oxidant abilities in aged and young populations. This is in line with related relevant literature [85, 86, 87], but importantly our data also adds to existing knowledge by showing neutrophil-specific anti-oxidant outcome, which has not been done before in the context of ageing an/or accelerated ageing.

Also in terms of oxidative damage indicators, PCO treatment was associated with better outcome, with the aged populations' neutrophil intracellular conjugated dienes increasing. This supports our earlier assumption that CD are released into plasma, so that intracellular sequestration of CD would have a desired clinical outcome. This assumption is made with the knowledge that intracellular conjugated dienes are able to be recycled [98] but once excreted into the plasma it is more likely to have negative effects as a free radical. This mechanism could thus be investigated as a possible target of PCO or other antioxidants, by which to alleviate oxidative damage.

The young fit group on the other hand had higher levels of conjugated dienes in the neutrophil when compared to the young sedentary groups. Their presence at an increased level in the cells from this population may indicate that their immune cells are also under oxidative stress, as observed in the plasma. Furthermore, as described above, endogenous systems are capable of recycling thus sequestering conjugated dienes in the cells [112] and thus hinder their release. After treatment with PCO the young sedentary and young fit conjugated dienes both decreased – considering that PCO is an anti-oxidant with radical scavenging capabilities, suggests that PCO is effective in affecting the lipid peroxidation cycling. Considering that this was an unstimulated assessment, this is a likely explanation. The exact mechanisms involved is however yet to be elucidated in future studies.

With regard to inflammation, our group has previously done work on macrophages and cytokine levels regarding PCO and this project was motivated by the very significant effect of PCO on the progression and resolution of inflammation, which was characterised by very limited neutrophil infiltration into tissue [70]. The hypothesis of the authors were that this outcome resulted from more efficient neutrophil function, which necessitated fewer neutrophils to achieve the required effect and which would have limited neutrophil-associated secondary damage [20] and result in faster resolution of inflammation, which was also reported in rodent models [80]. In the current thesis, rather than performing comprehensive cytokine analyses – since our previous studies have already shown an anti-inflammatory outcome in this context [70, 80] – neutrophil functional parameters were assessed in order to elucidate mechanisms by which these desired effects are achieved.

Firstly, MPO is one of the enzymes released from a neutrophil's azurophilic granules upon activation and is a key catalysing enzyme in the formation of HOCL [113]. MPO mediates neutrophil activation by association with CD11bCD18 integrin's [108]. MPO-derived oxidants in low concentrations have been implicated in the modulation of signalling pathways such as activation of the mitogen-activated protein kinases (MAPK), the induction of translocation to the nucleus of transcription factors, the regulation of cell growth via the activation of tumor-suppressor proteins as well as the modulation of metalloproteinases activity. Neutrophil intracellular MPO was significantly lower in the young fit population compared to the young sedentary controls, whereas the aged population was not different from the young sedentary controls. The fact that the young fit groups' plasma MPO had a tendency to be higher than sedentary controls (albeit not significantly) suggests that the young fit group had a higher baseline inflammatory profile due to residual inflammatory stimuli, such as strenuous exercise in the 24 hours prior to sampling, which we explored above. A factor to consider is that these neutrophils are unstimulated and thus the release of MPO is not likely. Therefore one has to consider that the decrease observed in intracellular MPO is due to decreased production. The

same observation can be made in the aged group, who displayed a tendency (p = 0,062) for lowered MPO after treatment with PCO. Overexpression of MPO has been associated with neutrophil dysfunction and is a key regulator of secondary damage [114].

Secondly, PCO treatment significantly decreased expression of FcγRIIIb across groups. This acute decreased in expression which is due to shedding of this receptor from the surface of neutrophils is an initial step in activation of neutrophils and necessary for their effective functioning, the acute shedding of FcγRIIIb is replaced via intracellular stores which maintain levels after the initial activation and allow the neutrophil to complete its other actions for example phagocytosis [115, 116]. The decrease seen resulted in all groups showing an expression similar to the young sedentary controls, which we assumed was the optimum level. Unlike the aged population whose basal level is decreased which indicates a pathological chronic lack or efficient replenishment of this receptor on the surface. This data facilitates the improvement in migration of neutrophils after treatment.

Thirdly, CEACAM 8 expression was increased after *in vitro* PCO treatment. Considering that this marker is often used as a marker of activation we can assume that the post-treatment level reached is the optimal expression, as both the aged and fit group showed similar expression when compared to the young sedentary controls. CEACAM 8 is used as a marker of activation because its expression is the initiating step of neutrophils binding to the endothelial tissue for extravasation, and thereafter facilitates the migration through tissue. CEACAM 8 presence on the tissue chronically is indicative of constant activation as it is only exocytosed upon activation – this is what we see in the aged population – whereas an acute increase indicates activation to facilitate migration. Both these results aid the improvement of migration seen with treatment. Interestingly, PI3K is found as an important molecule in both FcγRIIIb and CAECAM 8 mechanisms. PI3K has been demonstrated in the literature to be a molecule of investigation with regard to polyphenol action [49]. Therefore I think that this is the key effector to target for further investigation. The fact that both CEACAM 8 (CD66b) and FcγRIIIb (CD16) expression has been investigated in the context of arthritis [95,116], supports the importance of these markers as good indicators for studying the mechanism of pathological neutrophil function.

Finally, neutrophil migration was improved across groups after *in vitro* treatment with PCO. These changes are most notably in the aged population who as described above showed very little directional accuracy, while the young sedentary group exhibited a statistical tendency for improvement as well. This ties in with the improvements in CEACAM 8 and FcγRIIIb expressions already discussed. Also, with migrational accuracy being linked to the limitation of secondary damage, the improved migration efficacy reported here, can then account for

previously reported improvements in recovery of muscle damage [55], and the data suggests this that improvement is facilitated by PCO-modulated CEACAM 8 and FcγRIIIb expression.

It is clear that PCO *in vitro* treatment effects improvements across groups, but these changes are different for each. This highlights that this extract has multiple mechanisms of action to affect changes in both the chronic and acute models. Further investigations are warranted, in my opinion in an *in vivo* models to definitively elucidate these mechanisms.

5.4 Broader applicability of results

Given the consistency of positive results observed here with an anti-oxidant, anti-inflammatory intervention, we are confident that current results may be extrapolated to include broader application to spheres other than ageing. For example, it is possible to draw comparisons between ageing and chronic diseases and the possibility that PCO (or a similar anti-inflammatory/anti-oxidant treatment) can alleviate the progression of both. With regard to oxidative status, it is reported that oxidative stress is increased by the presence of free radicals which exert damage [38]. In chronic disease this is caused by increased metabolism, excessive inflammatory action and ineffective/insufficient anti-oxidant capacity. We see with this research that PCO is capable of increasing anti-oxidant capacity. The use of anti-oxidant to treatment ischemic injury [117] displays the potential of a polyphenol anti-oxidant to be beneficial for chronic disease.

Secondly, with chronic diseases there is a chronic low grade inflammation present which results in maladaptation facilitating disease progression. The effect on intracellular MPO already gives us an indication of improved inflammatory response of neutrophils. Comprehensive further investigations are warranted to elucidate the exact flux and secretion of MPO from neutrophils but this data suggests that this is an important mechanism.

5.5 Conclusions

This thesis, while elucidating potential mechanisms that could account for the desirable effects of PCO previously reported, also identified potential therapeutic targets to consider for the alleviation or limitation of ageing and accelerating ageing.

In terms of the extract employed here, and considering how closely linked oxidative stress and inflammation are, PCO displays therapeutic potential for many diseases with an inflammatory or oxidative stress component in their aetiology.

In addition, plasma MPO was highlighted as an accurate indicator of ageing-associated inflammation in the absence of other illness, while plasma CD and FRAP best indicated chronic oxidative stress. Our data on CAECAM8 and FcγRIIIb in the context of ageing adds to the

available literature on these parameters, and suggests that these mechanisms in the neutrophil migratory response should be further investigated as targets for therapeutic interventions aiming to alleviate ageing, accelerated ageing and inflammatory disease in general.

5.6. Future studies

Given the focus of the thesis and the fact that an *in vitro* model was used in the current study, there was limitations with regard to timing of samplings and parameters that could be assessed – as discussed above. An *in vivo* study is thus necessary to further elucidate mechanisms and investigate some of the data which was not conclusive in this study. In terms of investigations related to inflammatory disease, I believe that we have now generated sufficient data on PCO to warrant a human *in vivo* study. However, given the logistical difficulty of ageing studies, I propose that an animal model would be best to comprehensively assess systemic and compartment effects of PCO in context of oxidative stress as well as inflammation, and in particular the gradual maladaptation over time.

Chapter 7

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Appendices

Appendix A – Literature Review

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Review Article

Ageing-Associated Oxidative Stress and Inflammation Are Alleviated by Products from Grapes

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Advanced age is associated with increased incidence of a variety of chronic disease states which share oxidative stress and inflammation as causative role players. Furthermore, data point to a role for both cumulative oxidative stress and low grade inflammation in the normal ageing process, independently of disease. Therefore, arguably the best route with which to address premature ageing, as well as age-associated diseases such as diabetes, cardiovascular disease, and dementia, is preventative medicine aimed at modulation of these two responses, which are intricately interlinked. In this review, we provide a detailed account of the literature on the communication of these systems in the context of ageing, but with inclusion of relevant data obtained in other models. In doing so, we attempted to more clearly elucidate or identify the most probable cellular or molecular targets for preventative intervention. In addition, given the absence of a clear pharmaceutical solution in this context, together with the everincreasing consumer bias for natural medicine, we provide an overview of the literature on grape (*Vitis vinifera*) derived products, for which beneficial effects are consistently reported in the context of both oxidative stress and inflammation.

1. Introduction

With ageing, the capacity of the body to function optimally declines. There is a combination of genetic and lifestyle factors which may either accelerate or slow down the ageing process. A number of chronic diseases are associated with advanced age: these include cardiovascular disease (CVD), diabetes, metabolic syndrome, and Alzheimer's disease. This results in an exponential increase in the disease burden on modern society, relative to a few decades ago, due to longer life expectancy. The World Health Organisation states that from 2015 to 2050, the proportion of the world's population over 60 years will increase from 12% to 22% [1]. Taking into consideration that predominant research in the field of age-associated diseases considers the sixth decade of life to be a risk factor for the rapid progression and onset of age-associated diseases, the disease burden will almost double in the next 35 years. It is thus vital not only to elucidate the causes and progression of these chronic conditions, but also to actively search and investigate potential preventative therapies that may slow the processes contributing to physiological ageing.

Although the ageing-associated chronic disease states are each uniquely complex in terms of their aetiology, development, and progression, they do share common aetiologies which stem from two main entities, namely, cumulative oxidative stress and chronic inflammation. Briefly, oxidants are produced by normal cell metabolism and various physiological responses. However, when the production of oxidants outweighs the capacity of endogenous antioxidant systems, oxidative stress is incurred. Furthermore, while inflammation is crucial for repair of tissue injury and primary defence against invading pathogens and chemicals, it also results in unintended detriment to previously uninjured cells. Although these are necessary systems in the body, both oxidative stress and the inflammatory response, if unchecked, can have detrimental consequences which have been linked to accelerated ageing and the progression of age-associated disease. We postulated that the effects of the inflammatory immune system and oxidative stress on allostatic load are interlinked. This has led us to investigate the potential of antioxidants as treatment options to attenuate the cumulative effects of both oxidative stress and chronic low grade inflammation. Given the modern consumer bias for natural medicines, we focused on a group of plant medicines which are consistently associated with beneficial effects on these processes in the literature, grape-derived polyphenols. In this review, we will provide a more in-depth review of interconnected molecular mechanisms of oxidative stress and inflammation in the physiological ageing process, before moving our focus to a discussion of the merits of these plant medicines as potential preventative therapy in this context.

2. Contribution of Oxidative Stress to Premature Ageing

In the context of ageing, reactive oxygen and nitrogen species (RONS) are generally the major molecules which contribute significantly to oxidative stress. The most frequently studied free radicals are superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$), peroxynitrite (ONO_2^-), and nitric oxide (NO). The generation of these free radicals is necessary as they are essential for host defence: phagocytic cells use ROS to digest invading pathogens and debris. Furthermore, they act as signalling molecules, regulating cell growth and apoptosis, adhesion, and differentiation. More specifically, RONS are formed during processes such as the mitochondrial electron transport chain as well as enzyme systems such as cytochrome P450, lipoxygenase and cyclooxygenase, the NADPH-oxidase complex, xanthine oxidase, and peroxisomes [2]. In contrast to these roles in growth, repair, and immune functions which are all beneficial to the host, these molecules also have the ability to oxidise signalling molecules, DNA, macromolecules, and cell structures such as lipid membranes of healthy host cells, all of which are to the detriment of these cells. Usually, each cell has defence mechanisms to counteract the occurrence of oxidative stress. These endogenous enzymatic antioxidant defences include

superoxide dismutase (SOD), glutathione peroxidase, catalases, glutathione/TrxR, and peroxiredoxins. An appropriately nutritious diet is vital to maintain these systems, as these natural antioxidants are supplemented or replenished by antioxidant constituents of various fruits and vegetables. It is only when the capacity of the body's antioxidant defences is outweighed by the rate of production of free radicals that oxidative stress is incurred by unquenched free radicals which can alter surrounding cell structures and environment.

With advancing age, various factors, among these is a natural progressive decline in endogenous antioxidant capacity, cause disruption in the balance between pro- and antioxidant mechanisms and RONS accumulate beyond the normal endogenous antioxidant system's quenching capacity, resulting in cumulative oxidative stress. Eventually this causes cell damage which cannot be repaired by internal mechanisms, leading to loss of organ mass and functionality, which ultimately culminates in system dysfunction [3]. Indeed, long-term oxidative stress states have been linked to various diseases associated with advanced age, most notably diabetes, chronic obstructive pulmonary disease (COPD), cardiovascular disease, cancer, diabetes, and asthma [4]. Many of these diseases are not only associated with ageing anymore, but also with the high-obesity, high-stress, and sedentary modern lifestyle (albeit perhaps in milder form) [5]. It is thus vital to address the prevention of these detrimental long-term outcomes, as they are afflicting not only the aged, but also relatively young populations, such as university students [6, 7].

The aetiological mechanism(s) of the various chronic diseases mentioned are each different, as each disease has its own complexities. However, chronic cumulative oxidative stress is a common factor in these diseases, highlighting this system as a vital therapeutic and/or preventative medicine target [8].

Even in the absence of age-associated disease, various theories have linked oxidative stress directly to the normal ageing process. We briefly mention two here. Firstly, the Free Radial Theory of Ageing proposes that the presence of free radicals and their effects on cells are one of the causes of cell ageing and subsequent cell death, which in turn lead to loss of organ mass and other features of whole organism ageing. This theory was first suggested by Harman in 1956 [9], who hypothesized that irradiation of cellular components resulting from the liberation of $^{\cdot}OH$ and $^{\cdot}HO_2$ radicals would lead to dysfunction and mutations which cannot be reversed and thus result in ageing. This theory was later supported and expanded as research began elucidating the details of his proposed theory, for example, by describing the role of SOD [10]. Phenomenally, a very recent meta-analysis [11] confirmed the sustained validity of this theory, 60 years later. Of course, given the technological advances made over this period, it is no surprise that subsequent research has added more detail on mechanisms in support of this theory, although many unresolved questions remain [12].

Secondly, the Replicative Senescence Theory is based on the hypothesis that oxidative stress induces cell death and/or senescence, which necessitates an increase in the rate of cell replication. This in turn accelerates the detrimental effects associated with repeated cycles of mitosis. Telomere length and its accelerated shortening due to reparative replication is the basis of this theory, which was first described by Hayflick in 1965, in response to the observation of a decline in functionality of cell cultures (fibroblasts) which had undergone numerous cell divisions [13]. This phenomenon was subsequently confirmed by experiments on primary peripheral blood mononuclear cells (PBMCs) and fibroblasts from a largely aged population with increased risk for vascular dementia [14]. In this report, decreased telomere length in both fibroblasts and PBMCs was correlated to risk for dementia induced by stroke. In addition, telomere shortening rate was reported to decrease with increasing antioxidant capacity in fibroblasts of the same population.

This theory, together with the subsequent data, presents evidence that, firstly, oxidative stress is responsible for the accelerated shortening of telomeres brought about by more frequent

reparative replication and, secondly, that an increase in antioxidant defence capacity could slow down the ageing process. Note at this point that we do not infer that telomere shortening results only from oxidative stress: other mechanisms such as chromosomal instability have indeed been linked to both ageing and pathological conditions such as cancer [15]. However, to remain focused on the main topic of this review, we have limited this discussion to *accelerated* telomere shortening in the context of cumulative oxidative stress.

Holistic interpretation of the two theories introduced above implicates oxidative stress as major causative role player in the damage to cell constituents by oxidising membranes, molecules, and DNA. This initiates a cascade of events leading to the need for either cell growth and replication for repair, or death. This implicates ROS as rate determining factor of cell lifespan due to the direct damage it inflicts. Furthermore, oxidative stress is implicated in causing irreversible damage to the mechanism of replication through accelerated telomere shortening and thus ultimately decreases the capacity of the cell to replicate optimally, or at all. With both repair and replication affected, cell senescence is encouraged.

In keeping with the idea of a holistic approach, consideration of oxidative stress in isolation is insufficient. A basic but practical example of the interplay between the oxidative stress system and another system implicated in ageing, inflammation [16], is data available on detrimental effects of cigarette smoking. In this context, both acute smoking and long-term smoking were shown to overwhelm the glutathione antioxidant defence system within the lungs, which was associated with significant infiltration of inflammatory immune cells in the lungs [17]. This study clearly shows system interaction. Furthermore, in the same study, the severity of this maladaptation increased with duration of habitual smoking (in years) and was exacerbated by natural decreases in antioxidant capacity as experienced with ageing, resulting in increased oxidative stress, illustrating the significance of cumulative damage.

However, before considering these interactions in more detail, the literature providing proof of a role for chronic inflammation in the process of ageing will be briefly reviewed.

3. Chronic Low Grade Inflammation Facilitates Premature Ageing

As is the case for oxidative stress, the inflammatory response is a system essential for normal body function [18]. As component of the innate immune response, inflammation is a major first-line defence against infection and injury [18]. Apart from this largely independent, nonspecific role in immunity, inflammation is also vital for many specific immune responses to run its course [19]. However, in the process of repair and restoration after insult, the inflammatory response inadvertently disrupts cellular homeostasis of previously unharmed or unaffected cells [20, 21]. The injury-repair cycle which inflammation regulates is an efficient system during youth, when optimal sensitivity and response to signalling molecules (such as cytokines, growth factors, prostaglandins, and peptides) maintain the general health of circulating immune cells and the tissue microenvironment, with minimum secondary damage. However, during natural chronological ageing, long-term, repeated stimulus-response cycles change the receptor expression levels and thus sensitivity to these molecular stimuli [22]. This may necessitate relatively increased concentrations of any particular stimulus to maintain the required effect. Commonly reported characteristics of the natural ageing process include inflammation or oxidative stress-associated symptoms such as directionally inaccurate chemotaxis, premature or suboptimal respiratory burst, and increased pro-inflammatory signalling from immune cells, all of which form the basis of immunosenescence [23].

Immunosenescence is the term used to describe ageing of immune cells and the functioning of the immune system as a whole. This occurs naturally with advancing chronological age or as result of lifestyle factors, as already mentioned. There is more than

one way in which the immune system is compromised upon ageing. Firstly, immunocompetent cells are derived from hematopoietic stem cells. With ageing, a natural bias develops for stem cells to commit to expansion of the myeloid lineage at the cost of the lymphoid lineage [24]. This results in a shift in the balance of immune cells available to enter the circulation. Secondly, the chronic low grade inflammation associated with ageing recruits larger numbers of cells into circulation from the hematopoietic tissue. However, despite the higher circulating cell counts, phagocyte Toll-like receptor expression and phagocytic capacity are decreased in the aged [22], leaving the immune system with a lower capacity for becoming activated and for responding to a more acute insult, such as viral infection. These maladaptations, which together further predispose the individual to pro-inflammatory responses, are postulated to stem at least in part from alterations in HPA-axis signalling.

The process of immunosenescence may be accelerated by unhealthy lifestyle, such as psychological stress and obesity. As mentioned before, lifestyle-associated diseases share clinical symptoms associated with normal ageing. Indeed rutin, a potent antioxidant, has been shown to protect against ageing-related metabolic dysfunction [25]. Thus, studies focused on those conditions may provide much insight in terms of ageing and the supplementation options to limit its progress.

For example, in the context of obesity and/or inactivity, cytokines release from adipose tissue macrophages has been demonstrated by many researchers [26–29]. Tumour necrosis factor-α (TNF-α), interleukin- (IL-) 1β, and IL6 are among some of the cytokines shown to be released from resident macrophages in adipose tissue, resulting in a pro-inflammatory microenvironment [30]. Furthermore, chronic stress, and in particular psychological stress, is a generally accepted cause of chronic low grade inflammation. For example, in 38 medical students, psychological stress was associated with increased pro-inflammatory cytokine (TNF-α, IL-6, IL-1Ra, and IFN-γ) levels, as well as decreased anti-inflammatory regulators (IL-10 and IL-4) [31]. Interestingly, these effects were exacerbated by high anxiety proneness as trait, again suggesting that a cumulative stimulus (in this case lifelong anxiety) further exacerbates the undesirable adaptation.

Also in posttraumatic stress disorder (as extreme form of chronic psychological stress), an initial glucocorticoid hyper-response is followed by glucocorticoid hyporesponsiveness, which is associated by a relatively pro-inflammatory state. In this condition of continuous pro-inflammatory signalling, the feedback systems, which usually downregulate inflammation, adjust overtime and result in maladaptations such as chronic but low grade upregulation of pro-inflammatory mediators (e.g., IL-6, TNF-α, IL-1β, and prostaglandin E_2) [32]. Incidentally, the secretion of the first three is mediated by the NF-κB pathway, which is activated in response to cellular stress
[33].

In addition to increased pro-inflammatory signalling, other more mechanical cellular mechanisms also seem to be compromised over time. For example, inappropriate and/or insufficient neutrophil responses result from its decreased phagocytic capacity, increased basal levels of intracellular calcium, and the resulting reduction in capacity for chemotaxis [34]. The result is a chronic low grade inflammatory status in relative absence of a specific threat which can persist for extended periods of time and cause harm and inefficiency of the system. Thus, although varied in specific causative mechanism, the outcome of all of these suboptimal life events or lifestyle habits, for example, chronic stress leading to glucocorticoid resistance and/or cardiovascular disease and high-calorie diets and inactivity (obesity) leading to insulin resistance and diabetes, is that of a chronic inflammatory state
[16].

Of particular interest in the context of ageing is the fact that, apart from the now notorious low grade inflammation as primary culprit, this maladaptation results in a compromised capacity to mount an efficient inflammatory response to acute insults. As

overviewed in the review by Weinberger and colleagues [35], several reports from clinical literature support the notion that, in the aged individual, that there is a significant increase in the convalescence period required for recovery from injury and pathogen clearance, as well as a decrease in the quality of repair, thus favouring disease progression and morbidity due to injury. Very recently, Baehl¨ and colleagues (2015) demonstrated, in a longitudinal study of elderly patients, that the acute stress of a hip fracture had a negative effect on neutrophil function immediately after injury. While some neutrophil functions (chemotaxis, phagocytosis) were recovered over time, several others (superoxide production, complement C5A and CD11b receptor level, and cytokine secretory profile) were still impaired even 6 months after injury. From this, the authors concluded that the acute stress had a long-term negative effect on neutrophil responses, which negatively influenced clinical outcomes, such as the resolution of long-term inflammation, recovery, and susceptibility to opportunistic infections [36].

It is thus clear that ageing is an inflammatory-mediated process. However, from the inflammation/ageing literature, it is clear that inflammation and oxidative stress cannot be separated as causative factors in this context. For example, the ageing-related shift in balance between the glucocorticoid and inflammatory systems has been linked to increased ROS production, which in turn exacerbates low grade immune activation [37].

4. Links between Inflammation and Oxidative Stress in the Ageing Process:

Identifying Therapeutic Targets

From the above sections it is evident that, in ageing, oxidative stress and inflammation are interdependent mechanisms. We postulate that unravelling and understanding of these intricate links between the two responses hold the answer to identification of the major contributor(s) to allostatic load and maladaptation associated with ageing and age-related pathology.

Generally, repeated exposure to reactive oxygen and nitrogen species causes cell damage and thus a pro-inflammatory signalling response. For example, in aged mice, unquenched reactive oxygen and nitrogen species act as pro-inflammatory signalling molecules and mediators of inflammation within the cell itself [38]. More specifically, oxidative damage to cells prompts the release of TNF-α from these damaged cells [21]. Binding of TNF-α to cell surface TNF-α receptors activates the NF-κB inflammasome, which results in the further production of other pro-inflammatory cytokines, most notably IL-1β. Incidentally, TNF-α specifically has also been implicated in ROS-mediated upregulation of adhesion molecules which facilitate the infiltration of immune cells into tissue [39], with more on this later. Upregulation of inflammation via the NF-κB inflammasome is probably the main aetiological mechanism for age-related chronic conditions with an inflammatory component. Indeed, TNF-α upregulation, which is a direct result of increased flux through the inflammasome, has been implicated as causative factor in cardiovascular disease [40]. Of particular relevance to the topic of ageing, the NF-αB inflammasome has regulatory roles in cell growth, survival, and proliferation. However, as recently reviewed [41], ROS production may have either inhibitory or stimulatory roles in the NF-□B pathway, suggesting a dose dependency of the effects of ROS. This unfortunately also means that development of an intervention strategy/product to modulate this target mechanism is no simple feat and will have to be approached in a very tightly controlled "modification range."

From the literature consulted, it is clear that bidirectional communication is in place. For example, both neutrophils and macrophages are producers of oxidants *via* the NADPH-oxidase system [42]. The NADPH-oxidase (NOX) proteins aid the transport of electrons across biological membranes and are found in all cells [43]. They are also one of the major generators of ROS in all cells. Particularly, these proteins are the predominant ROS producers in

phagocytic cells, a process required for the normal respiratory burst that phagocytes use to kill pathogens and digest cell debris.

Furthermore, activated neutrophils release myeloperoxidase (MPO), which contributes to the formation of hypochlorous acid (HOCL) by acting as a catalyst when reacting with hydrogen peroxide (H_2O_2). This directly increases the production of ROS [44]. The oxidative burst of neutrophils in itself releases oxidants such as H_2O_2, which are harmful to healthy cells and tissue [45]. Besides directly increasing the production of reactive oxygen species itself which causes damage to surrounding cells, MPO specifically has been implicated as a risk factor for coronary artery disease, due to its capacity for oxidation of lipid membranes [46]. The increased oxidant concentration due to immune cell functions, as well as the resultant cell damage, results in increased metabolism in surrounding healthy tissue.

Also at gene level, role players have been elucidated in the context of a ROS-inflammation link. For example, sirtuins are Class III histone deacetylases which are responsible for the deacetylation at N-epsilon lysine residues, a reaction which consumes NAD+. SIRT1 specifically is a sirtuin commonly associated with antioxidant function [47]. Its regulation of oxidative stress is threefold: firstly, it stimulates the expression of antioxidants *via* the fork-head box protein O (FOXO) pathway. Secondly, it is involved in inhibiting the NF-κB signalling pathway. In contrast, however, excessive ROS can inhibit SIRT1 activity by oxidatively modifying its cysteine residues and thereby releasing its inhibition of the NF-κB pathway [47]. It is thus theoretically possible for cumulative stress to downregulate SIRT1 activity in the ageing process. Thirdly, SIRT1 has also been implicated in regulation of apoptosis by deacetylating p53 to inhibit p53dependent transcription in models of cellular stress [48]. This tripartite role defines SIRT1 as another important molecular target in the context of both normal and accelerated ageing.

Apart from these targets related to cellular signalling, cell functional capacity should also be a focus. A striking example of the oxidative stress, inflammation link in ageing, is the decreased capacity for neutrophil chemokinesis reported in the elderly both in terms of motility and accuracy [49]. Normally, immune cells are attracted by chemotactic signals from injured tissue, to migrate to sites of injury or pathogen invasion. During this chemokinetic response, immune cells, typically neutrophils and classically activated macrophages, migrate through tissue toward the site of injury. This movement is facilitated largely *via* adhesion molecules such as the beta-integrins and I-CAM1 in the case of neutrophils [21]. However, as mentioned earlier, expression of adhesion molecules on neutrophils increases with ageing [39], so that their movement is slowed. In addition, due to yet unclear mechanisms, but most probably due to adaptation of cellular “homing” molecules, directional accuracy of neutrophil migration is also compromised in the elderly. Sapey et al. showed that inaccurate neutrophil migration was causally associated with increased constitutive phosphoinositide 3-kinase (PI3K) signalling [49]. This results not only in inefficient inflammation due to prolonged response time, but the directional inaccuracy of movement also results in mechanical and oxidative damage to relatively more cells in the path of the migrating inflammatory cell [49].

It is clear that both oxidative stress and inflammation are able to induce and exacerbate one another (both indirectly and directly). Furthermore, regardless of the primary signal or which pathway was activated first, these interrelated processes form a vicious cycle which is difficult to target therapeutically because of its complexity. However, it is also this interrelated nature of the two systems that has led us to investigate the possibilities of antioxidants as treatment options to attenuate the cumulative effects of oxidative stress and in turn low grade chronic inflammation.

5. Are Grapes the Answer to Prevention of Ageing?

Despite the huge range of nonsteroidal and natural product anti-inflammatories on the market today, the

scientific literature shows a conspicuous lack of consistent support for any specific medication. This is perhaps at least in part due to the fact that researches investigating these products cannot keep up with the rate at which new ones are pushed onto the market. Hopefully new legislation on the control of these substances will affect this trend to the benefit of the consumer, by allowing for (or demanding) appropriate testing of these products.

Nevertheless, antioxidants are being used almost routinely by many individuals who wish to supplement for enhancement of general health or as adjuvant therapy in conjunction with more mainstream, pharmaceutical medication. Although they are generally not regarded as a primary therapeutic option, antioxidants may hold particular potential in the realm of preventative medicine. The potential benefits of appropriate antioxidant supplementation are vast, especially when considering the connection between oxidative stress and inflammation. An antioxidant with the capacity to modulate inflammatory status can thus be beneficial to both normal ageing individuals and those suffering from lifestyle-associated diseases.

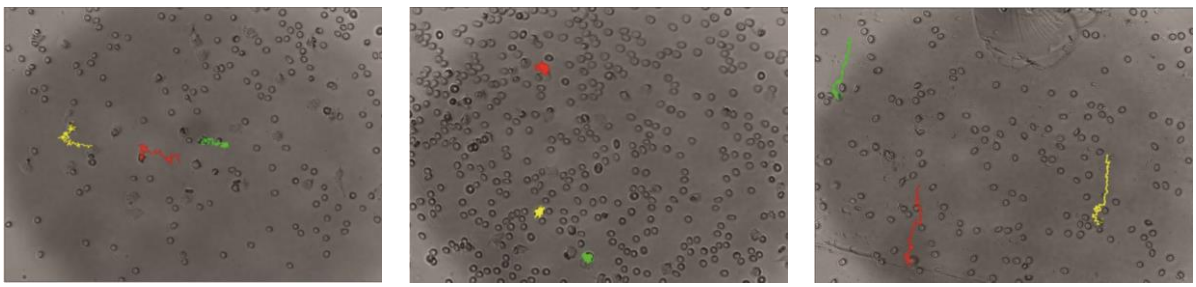
A comprehensive search of the scientific literature revealed that grape-derived antioxidants are consistently reported to have high benefit and low risk in the context of both oxidative stress and inflammation. These positive results are further strengthened by the fact that these consistent findings were reported across many different models and using a variety of different preparations, ranging from relatively crude extracts to highly purified ones. For example, in terms of purified polyphenols, resveratrol, one of the best-known polyphenols present in grapes as well as other plants, is commonly linked to anti-inflammatory [50], antioxidant [51, 52], and thus by implication anti-ageing effects in the scientific literature, as well as anecdotally. Indeed, a recent paper [53] elucidated a role for resveratrol to protect against inflammatory damage via SIRT1 inhibition of the NF-κB pathway (a mechanism discussed above in the context of ageing). In addition, more advanced studies have been undertaken on this polyphenol to better understand the relationship between the chemical structure of resveratrol and its biological activity, especially in terms of oxidant scavenging [54]. Also, pharmaceutical groups have been working on optimisation of delivery systems for resveratrol [55]. Such information may further advance the popularity of this very promising natural product with the pharmaceutical industry, to the benefit of consumers. The phenomenal frequency at which new papers on resveratrol appear, all providing evidence of positive effects in this context, suggests that this particular polyphenol should be investigated in the context of ageing as a matter of urgency.

Even more promising than the many positive effects described for resveratrol above is the fact that resveratrol is only one from a range of equally beneficial substances contained in grapes. The flavonoids quercetin and dihydroquercetin (DHQ), as well as proanthocyanidins and anthocyanins, all of which are present in grapes and a variety of other plant sources, have similarly been linked to both antioxidant and anti-inflammatory effects [50, 52]. To date, despite appearance of a few very promising reports in this context, ageing specifically has not been the focus of many studies investigating these substances. Therefore, for the purpose of this review, we provide a comprehensive overview of the few existing ageing-related studies in this context that were available to us. Results from relevant papers that did not have ageing as a focus were also included, where those results contribute to our understanding of the role of grape derived polyphenols in the oxidative stress, inflammation link in the context of prevention or deceleration of the ageing process.

When considering *in vivo* studies on ageing as a starting point, resveratrol (0.1 μM to 2.5 μM) exhibited a clear dose-dependent effect on longevity in fish with known short lifespan: resveratrol supplemented fish almost doubled their expected 13-week lifespan and continued to produce healthy offspring long after all control fish had died. Even though resveratrol supplementation was only started

in adulthood in this study (i.e., it compares to when humans might start to consider supplementation), it effectively delayed age-dependent compromise of locomotor and cognitive performance and reduced expression of neurofibrillary degeneration in the brain [56]. This result of improved neural morphology was recently further substantiated in an aged rat model, where chronic resveratrol treatment prevented detrimental changes in dendritic morphology which is linked not only to ageing, but also to Alzheimer's disease [57]. Similarly, 2 months of ingesting polyphenols in the form of 10% grape juice resulted in enhanced potassium-evoked dopamine release and cognitive performance in aged rats
[58].

A more recent review [59] provides more insight into the potential mechanisms by which age-related cognitive disorders may be curbed by grape polyphenols. Some of these mechanisms at first seem unrelated to the scope of this review, for example, preventing of amyloid-beta deposition associated with Alzheimer's dementia [60]. However, recent research suggests a role for inflammation in the development of the disease [61], while natural antioxidants have been linked to prevention of amyloid-beta deposition [62]. Together, these data suggest that even these seemingly unrelated mechanisms may be interconnected to either inflammation, or oxidative stress, or both. However, more clearly in context of this review, resveratrol was reported to increase NO production, resulting in vasodilation [59, 63], which may play a role in the maintenance of central circulation and thus perhaps slower degenerative central processes, as has indeed been reported for resveratrol, as mentioned earlier. However, the role of NO in the context of antioxidant status is much more complex, so that this effect of grape polyphenols should probably receive more attention before it can be interpreted fully in terms of mechanism(s) involved. Furthermore, recently in a co-culture simulation of the human blood-brain barrier, another grape polyphenol, proanthocyanidin, was associated with significant inhibition of monocyte infiltration and pro-inflammatory cytokine secretion in HIV-associated neuroinflammation [64]. Such inhibition of neuroinflammation is associated with a better prognosis in terms of HIV-related neurodegeneration and dementia, further confirming the neuroprotective potential of grape-derived antioxidants. Taken together, these studies suggest that the neuroprotective effects of grape polyphenols involve both antioxidant and anti-inflammatory mechanisms, with the latter including not only modulated cytokine signalling, but also modulation of both motility and functional capacity of leukocytes, as previously illustrated by our group [64, 65].
One may argue that both these results may be the result of decreased cell activation, perhaps as a result of the known altered cytokine environment.
However, an age-associated lack of neutrophil chemokinetic accuracy in response to the chemotaxin fMLP has been reported [49], which suggests that the mechanism is probably related to age-induced compromise of specific cellular mechanisms, rather than activation. In addition, in an ongoing study by our group, we have been able to illustrate by using Dunn chamber chemokinetic assays that grape polyphenols (specifically proanthocyanidin) are able to correct this age-associated anomaly (unpublished data). From Figure 1, which depicts typical digital images obtained for the path of individual neutrophils, it is clear that a more purposeful, directionally accurate movement was achieved in proanthocyanidin-treated neutrophils. This will ensure a more optimal inflammatory response (i.e., the response will be effective, result in relatively insignificant secondary damage, and be resolved in the minimum amount of time). In contrast the rather "aimless wander" of untreated neutrophils from aged individuals will result in not only ineffective immune cell infiltration to sites where they are required, but also relatively more secondary tissue damage and thus prolonged and exacerbated inflammation.



(a) (b) (c)

Figure 1: Typical neutrophil chemotaxis pathways for (a) a young participant (<25 yr), (b) an aged participant (>65 yr), and (c) an aged participant after acute *in vitro* treatment with grape-deed derived proanthocyanidin. The Olympus Cell system IX-81 inverted fluorescent microscope system with an F-view cooled CCD camera (Soft Imaging Systems) at 20x magnification was used to capture these images, which was analysed using Image J (Java software).

Turning attention to inflammation and oxidative stress again, the most probable targets for therapeutic intervention in the context of ageing have been described in Section 4. Since the ageing literature is relatively lacking in terms of papers on polyphenol intervention, we have tabulated effects of grape polyphenols reported for these identified targets in Table 1, citing relevant data that was mostly obtained in models other than ageing. The aim with this table was not to present all studies on grape-derived products; rather, it is an attempt to show the many models, species, and disease systems in which beneficial effects on oxidative stress and inflammatory status have consistently been reported. Also, although we have included doses used for *in vivo* studies for general comparison and again to illustrate the large variations in doses, these doses are product/extract specific and cannot be extrapolated across board.

From Table 1, which is by no means a complete list of all studies reporting on grape-derived substances, grape derived products are undeniably beneficial in limiting the magnitude of the inflammatory response as well as to increase antioxidant activity and seem to have multiple targets.

Of course, when considering potential anti-ageing modalities, it is also of interest to include evaluation of changes in quality of life. Since ageing is associated with natural muscle wasting or sarcopenia [83], it is important to note that, in this context, supplementation with grape polyphenols (50 mg/kg/day) for 4 weeks mitigated skeletal muscle atrophy in a mouse model of chronic inflammation [84]. This was achieved via modulation of two distinct pathways: one directly linked to inflammation (decreased NF-κB activation) and the other due to antioxidant function (limited ROS-associated mitochondrial damage and caspase-3 and -9 activation). Since caspase-3 activation is also a known pro-apoptotic signal [85], reduced activation and thus apoptosis may result in fewer mitotic cycles. This, in the context of the telomere hypothesis, may point to deceleration of ageing by the polyphenols. Very recently, grape proanthocyanidin treatment in rats was reported to have an anti-apoptotic effect which reduced damage after ischemia/reperfusion of the liver [86], which further substantiates this theory.

Interestingly, a study in mice fed a high-fat diet indicated that grape polyphenols may modify gut microbial community structure to result in lower intestinal and systemic inflammation [87]. This extraordinary result serves to remind us of the potential complexity of plant medicines and the requirement for comprehensive investigation of mechanisms of actions and interactions of any potential product *via* the traditional clinical trial process followed for new pharmaceutical drugs.

6. Conclusion

Ageing and accelerated ageing are not new concepts, but rather the norm in modern society. With a population that is growing relatively older due to advances in medicine, we are however pressed for answers on how to alleviate the symptoms or slow the progression of this inevitable phenomenon. From the literature consulted, no negative effects of grape-derived products became evident, while beneficial effects in the context of oxidative stress and inflammation were consistently reported in the context of numerous cellular targets. Huge variation in product content and prescribed dosage complicates interpretation of the fast growing body of literature on this topic. A recommendation for future studies is the inclusion of more parameters per study, so that a more comprehensive interpretation of specific mechanisms becomes possible. Measurement of only basic indicators of either antioxidant status or inflammatory status, while providing proof of efficacy of the product, does not contribute much information on its mechanism of action. Despite this shortcoming, the literature clearly indicates that grape-derived products are undeniably a therapeutic force to be reckoned in the combat of ageing and age-associated conditions.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Table1:Representativereportsonantioxidantandanti-inflammatoryeffectsofgrape-derivedcrudeextractsandpurifiedproducts.

ModelTreatmentOutcomes:inflammationOutcomes:oxidativestatus				References
Invitro:				
GlucoseandLPS-inducedinflammation inHUVECcells	Redgrapepolyphenols	↓IL-6,IL-8,andNF- Batprotein andmRNAlevels ↓PECAMandICAM-1levels	↓ROSformationindose-dependent manner	[66]
Primaryhumanchondrocyteschallenged with *E.coli* LPS(arthritismodel)	Grapeextractcontainingresveratrol, hopeaphenol,andviniferin	↓PGE $_2$ production	↗scavengingofDPPHradicals	[67]
Osteoblast-likecells(MC3T3-Ei),treated withTGF- toinduceVEGFsynthesis	Resveratrol	↓VEGFandVEGFmRNA,but noeffectonp38orSAPK/JNK, suggestingSIRT1activation	n.a.	[68]
Yeastmodelsofsirtuinactivation(*c. elegans*; *D.melanogaster)*	Resveratrol	↗sirtuin(SIRT1)activationn.a.		[69,70]
Humanadiposederivedstemcells hASCs	Redgrape(muscarine)grapeseedoil,in comparisontoricebranandoliveoils	↓adipogeneticfactors(PPAR and aP2) ↓IL-6andIL-8responsetoLPS ↓proinflammatorygeneexpression inadipocytes	Shown tobesourceof tocopherol	[71]
High-glucoseinducedoxidativestressin porcineproximaltubulecells(LLC-PK $_1$)	Grapeseedpolyphenols	↓NF- Bpathway	↓intracellularROS	[72]
Invivo animal:				
Ratsexposedtolocalisedbowel irradiation	GrapepolyphenolsORpurequercetin 3-*O*- glucoside(10mg/mL,7.14mL/kg bodymass)byoralgavagefor5dayspriror toirradiation	↓MPOactivity ↓CINC-1levels	↓SODactivity Nochangeinglutathione peroxidase(GSHPx)activity Nochangeinplasma malondialdehyde(MDA) concentration	[73]
Ratssubjectedto *E.coli* inducedseptic shock	and200mg/kg/daygrapeseed procyanidin,byip.injectionfor15days pre-*E.coli* challenge	↓IL-6geneexpression	↓NOinliver,spleen,plasma,and RBCs ↓iNosgeneexpression ↓GSSG:totalglutathioneratio	[74]
Ratmodelofosteoarthritis	mg/kgbodymassofgrapeextract dailyfor28days	Preventedjointdeteriorationn.a.		[67]

71

75

500

Table1:Continued.

Model	Treatment	Outcomes:inflammation	Outcomes:oxidativestatus	References
Ratmodelofskeletalmusclecontusion injury	AcuteOR2-weeksupplementation, proanthocyanidins	↓proinflammatorycytokine signalling(TNF-α;IL-6) ↓neutrophilmigrationcapacity Earliermacrophageswitchfrom pro-toanti-inflammatory phenotype	↗plasmaandskeletalmuscleORAC	[50,75]
Ratmodelofageing	Drinkingwatersupplementedwith15g/L grapepowderfor3weeks	↓age-associatedincreasein corticosterone	↓plasma8-isoprostane	[76]
RatmodelofobesityGrapeprocyanidinB2		↓IL-1α andNLRP3levelsin pancreas	n.a.	[77]
Middle-agedmiceonhigh-caloriedietDietsupplementedwith0.04%resveratrol		↓IGF-1	↗AMPKandPGC-1α activity ↗mitochondrialnumber	[78]
Mousemodelofpulmonaryfibrosis	dayorallresveratrol(50mg/kg/day)OR quercetinORdihydroquercetin(both mg/kg/day	↓neutrophilinfiltrationintolung tissue ↓inflammatorycellsin bronchoalveolarlavagefluid ↓COX-2 ↓NF-κBp65translocation	↓iNOS ↓oxidativelungdamage (↓nitrotyrosineand poly-ADP-ribosepolymeraselevels)	[79]
Rabbitmodelofacute(*E.coli*) inflammatoryarthritis	10 mg/kgbodymassofextractacutely priorto *E.coli* challenge	↓PGE$_2$ productionn.a.		[67]
Invivo human: Nondiabetichaemodialysispatients	7- Grapepowder(500mgpolyphenols/day) for5weeks	PreventedincreaseinplasmaCRP levels	↗glutathioneperoxidaseactivity	[80]
Humansatriskformetabolicsyndrome, aged30–65	500 weeksof20gwinegrapepomace powder(822mgpolyphenols)perday	n.a.	↗α and γ tocopherol	[81]
Hypertensive,T2DMmales,aged	10 ~ –658mggrapeextractdailyfor1year	↓alkalinephosphatase ↓TNF-α,IL-1α,IL-6,andCCL3 levels ↗transcriptionalrepressorLRRFIP-1 inPBMCs Modulationofexpressionofgroup ofmiRNAsknowntoregulate inflammatoryresponse	n.a.	[82]

ADP,adenosinediphosphate;AMPK,adenosinemonophosphate-activatedpr oteinkinase;CCL3,chemokine(C-Cmotif)ligand3;CINC-1,cytokine-ind ucedneutrophilchemoattractant-1;COX-2,cyclooxygenase-2;DPPH,2,2-diphenyl-1-picrylhydrazyl;HUVEC,humanumbilicalveinendothelialcells;iNOS,induciblenitricoxide;ICAM-1,intercellularadh esionmolecule-1;IGF-1,insulingrowthfactor-1;IL,interleukin;JNK,c-JunN-terminalkinase;LPS,lipopolysaccharides;LRRFIP-1,leucine-richrepeatinflii-interactingprotein-1;NO,nitricoxide;NF-κB,nuclearfactor-kappabeta;ORAC,oxygenradicalabsorbancecapacity;PECAM,plateletendothelialadhesionmolecule;PGC-1α,peroxisomeproliferatoractivatedreceptorgammacoactivator1-alpha;PGE$_2$,prostaglandinE$_2$;ROS,reactiveoxygenspecies;SAPK,stressactivated proteinkinase;VEGF,vascularendothelialgrowthfactor.

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Appendix B – Consent form

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

TITLE OF THE RESEARCH PROJECT: Modulation of neutrophil migration patterns by in vitro treatment with grape seed-derived proanthocyanidolic oligomers

REFERENCE NUMBER:N14/02/007

PRINCIPAL INVESTIGATOR: Prof Carine Smith

You are being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part.

This study has been approved by the **Health Research Ethics Committee at Stellenbosch University** and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

What is this research study all about?

- *The study will be conducted at the Dept. Physiological Sciences at Stellenbosch University. We aim to recruit a total of 24 individuals for this study, of which sixteen will be 18-25 years old and eight 65-75 years old.*
- *It is known that certain types of stress, as well as ageing, can cause immune cells to have trouble locating problem areas in body tissue, so that they take longer to get there to solve to problem, and they may damage healthy cells in the process. All of this exacerbates inflammation. We would like to take some blood and test a new anti-inflammatory treatment in a test tube, to see how it produces it anti-inflammatory effect.*
- *If you are within the older age group, we will need only two teaspoons of blood from you while you are completely rested. An experienced person will draw it from a forearm vein only once. If you are in the younger group, we will need a blood sample in a rested condition as for the older group, but then we will ask you to run on a treadmill until you decide you are too tired to continue, and then we will take another identical sample, so two teaspoons of blood in total.*
- *You will not be required to take any medication, as we will be testing it in a test tube only. However, to make sure that no supplement that you are taking interferes with our test, you will be required to not take anti-oxidants or vitamins for two weeks before we take the blood sample. If you are not sure about supplements or medicine that you are taking, please ask the researcher if you should continue or stop using it.*

Why have you been invited to participate?

- *You have been invited because you fall within the specified age groups, are normally fit and healthy and do not smoke.*

What will your responsibilities be?

- *As explained above, you will have to sign the consent form if you agree, then stop taking supplements as specified for a period of two weeks .Then you will visit our laboratory once only, after a day in which you did not do any exercise. We will take a blood sample from you. If you are in the older group, that is the end of your direct involvement in the study. If you are in the younger group, you will also be asked to run on a treadmill until you are too tired to continue, and then we will take another blood sample from you, before you go home.*

Will you benefit from taking part in this research?

- *If we find any abnormal result – i.e. if your result deviate drastically from the others in your group – we will inform you. Although you will not get an immediate benefit, you will be contributing to our knowledge on how to limit the undesired effects of inflammation.*

Are there in risks involved in your taking part in this research?

- *Taking a blood sample carries a small risk of bruising or infection, but we will only use very experienced staff to draw blood, to minimise the risk to you.*
- *If you are not used to running on a treadmill (younger participants only), there is the risk of falling off and hurting yourself. However, if you have not run on a treadmill before, we will show you how to do this at low speed when you start running, and we will have experienced staff to supervise the whole procedure.*

If you do not agree to take part, what alternatives do you have?

- *Your participation is voluntary and you may decide to take part, or not, with no consequence.*

Who will have access to your medical records?

- *We will not access your medical records. We will also label your blood with a code only, so that only the researchers will know your identity.*

What will happen in the unlikely event of some form injury occurring as a direct result of your taking part in this research study?

- *We have an emergency team on standby during the procedures, should you be injured. In the unlikely event that you sustain any form of injury as a result of your participation in this study Stellenbosch University has insurance to cover medical expenses. Reasonable and appropriate medical treatment will then be provided to you without cost.*

Will you be paid to take part in this study and are there any costs involved?

No, you will not be paid to take part in the study but your transport and meal costs will be covered for each study visit. There will be no costs involved for you, if you do take part.

Is there any thing else that you should know or do?

- You can contact Prof Carine Smith at 082 821 3600 if you have any further queries or encounter any problems.
- You can contact the Health Research Ethics Committee at 021-938 9207 if you have any concerns or complaints that have not been adequately addressed by your study doctor.
- You will receive a copy of this information and consent form for your own records.

Declaration by participant

By signing below, I …….…….. agree to take part in a research study entitled *(insert title of study)*.

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.

- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be penalised or prejudiced in any way.
- I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (*place*) .....…….. on (*date*) ……...…….. 2016.

…… ……

Signature of participant **Signature of witness**

Declaration by investigator

I *(name)* ……..…… declare that:

- I explained the information in this document to ……..
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I did/did not use a interpreter. (*If a interpreter is used then the interpreter must sign the declaration below.*

Signed at (*place*) .....…….. on (*date*) ……...…….. 2016.

…… ……

Signature of investigator **Signature of witness**

Declaration by interpreter

I *(name)* ……..…… declare that:

- I assisted the investigator (*name*) ……. to explain the information in this document to (*name of participant*) ……..…….. using the language medium of Afrikaans/Xhosa.
- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (*place*) .....…..…….. on (*date*) ……...……2016

…… ……

Signature of interpreter **Signature of witness**

Appendix C- Exercise Protocol

1. **Take lactate measurement by finger prick**
    - Clean finger with alcohol swab.
    - Prick fingertip with lancet.
    - Switch on accucheck device and insert lactate measuring strip.
    - Place droplet of blood on lactate strip.
    - Close accucheck, wait 60 seconds and then read result.
2. **Pre-test blood draw**
    - 8ml of blood samples were drawn from antecubital area of the arm with sterile 10 ml needle,1x Heparin vacutainer tube and 1x EDTA vacutainer tube
    - Label and place blood in white foam bucket
3. **VO_2Max Test**:
    - Place subject on treadmill and attach Oxycon as well as Heart rate monitor
    - Warm up of 8 minutes at (self-selected pace)
    - Initial speed of 7km/h for 30 seconds at 0% gradient
    - After 30 seconds increase speed by 0.5km/h
    - 7.5km/h for 30 seconds
    - Repeat increase by 0.5km/h for 30 second periods until exhaustion.

Read VO_2max, calculate 80% and 50% of VO_2max and the corresponding speed at which VO_2 was 80% and 50%.

4. **HIIT Exercise Protocol**
    - 3 mins : 50% (walking pace ±6km/hr)
    - 1 min : 80 % VO_2max
    - 1 min : 50% VO_2max
    - 1 min: 80% VO_2max
    - Repeat until exhaustion
5. **Post-test blood draw**
    - 8ml of blood samples were drawn from antecubital area of the arm with sterile 10 ml needle,1x Heparin vacutainer tube and 1x EDTA vacutainer tube
    - Label and place blood in white foam bucket
6. **3 minutes post-test lactate measurement by finger prick**

Appendix D-Neutrophil Isolation

Whole Blood Count

- Aliquot 300µl of whole blood into Eppendorf tube
- Calibrate Cell Dyne 3700 machine
- Enter sample for whole blood count
- Read and print result

Neutrophil Isolation

- 2ml Histopaque1077(room temperature) is pipette into a 15ml falcon tube and then double volume (4ml) of Heparin anticoagulant added blood was carefully poured onto the Histopaque layer down the side of each tube.
- Tubes are transferred to the centrifuge machine with swinging rotar 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 is taken by aliquoting out the supernatant layer containing lymphocytes, monocytes and plasma layer on top respectively.
- Plasma is aliquoted into eppi's and frozen at -80°C, aliquot PBMC's into eppi's 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 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.
- The pellet containing the neutrophils is taken by removing the supernatant.
- 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 eppi
- Run sample on the CellDyne 3700 to determine neutrophil counts.

- Adjust to count of $2x10^{-6}$ cells/ml

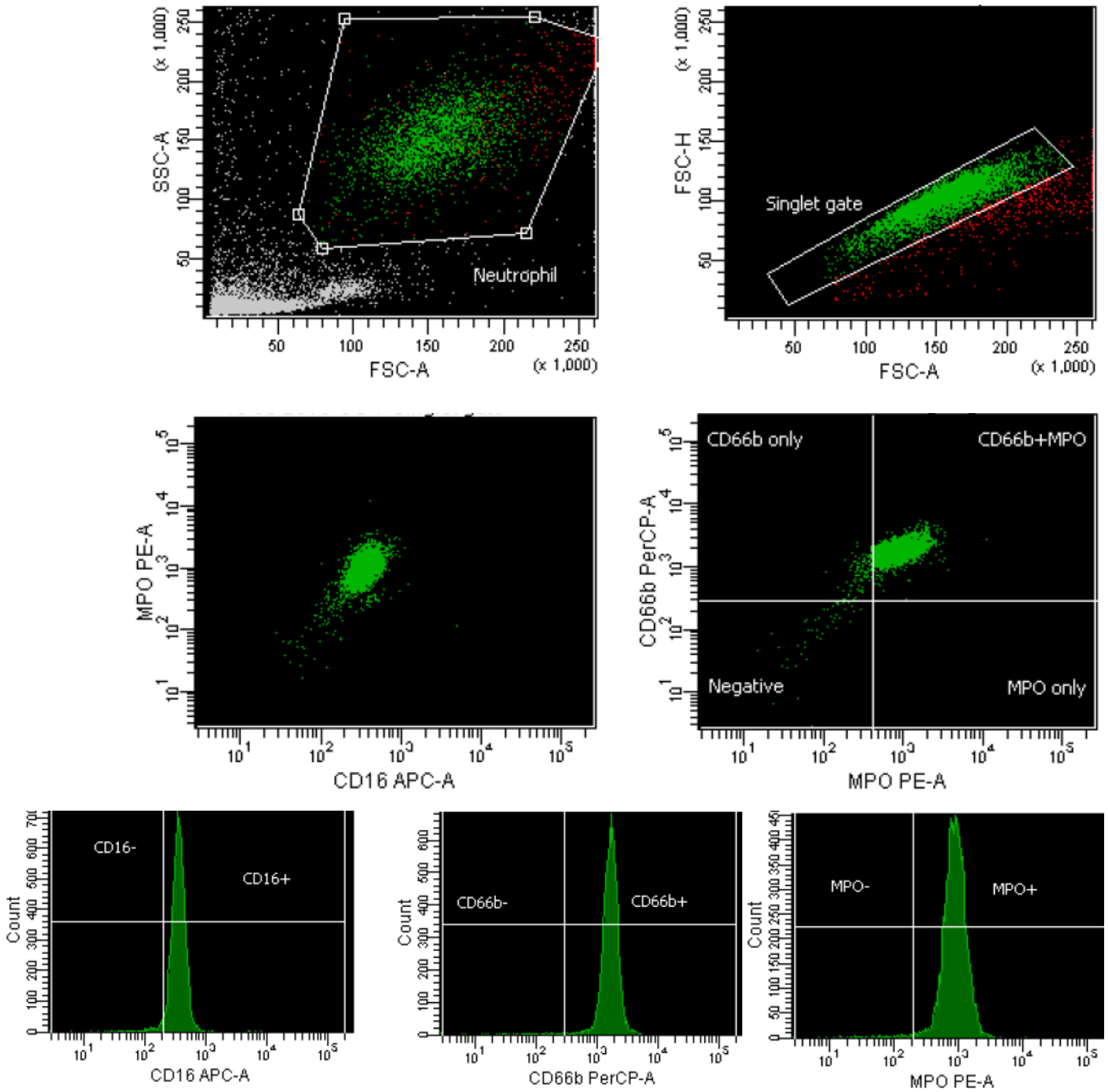
Appendix D- Staining Protocol and Flow Analysis Guidelines

Preparation for flow cytometry

1. Spin down remaining neutrophil isolate (after chemotaxis assay sample has been aliquoted out)
2. Remove supernatant
3. Resuspend pellet in PBS
4. Do cell count on Cell Dyne 3700 (background count)
5. Aliquot 100 µl with $1x10^{-6}$ cells conc.
6. Add surface markers antibodies : 5µl CD16 and 5µl CD66b
7. Leave for 30 min at 4°C (fridge)
8. Add 500µl PBS
9. Vortex gently
10. Centrifuge at 300g for 6 min
11. Remove supernatant
12. Resuspend in 250µl cytofix cytoperm
13. Mix gently
14. Leave for 25 min at 4°C
15. Add 500µl BD perm wash
16. Vortex gently
17. Centrifuge at 300g for 6 min
18. Remove supernatant
19. Add 20µl MPO antibody with 80 µl PBS
20. Leave for 30 at 4 °C
21. Repeat wash with 250µl BD perm wash
22. Vortex gently
23. Centrifuge at 300g for 6 min
24. Remove supernatant
25. Resuspend in 500 µl 4% paraformaldehyde
26. Leave in fridge at 4°C, covered in foil
27. Perform flow cytometry analysis.

Flow cytometry analysis guidelines

FACSDiva Version 6.1.3



Appendix F – ELISA Protocol

1. Preparation of reagents

1X Biotinylated anti-Human Myeloperoxidase

- Add 1 µL Biotinylated anti-Human Myeloperoxidase antibody to 99 µL antibody Diluent buffer, mis thoroughly. Total volume should be 100 µL /well (± 200µl for pipetting error).

1X Avidin-Biotin-Peroxidase Complex

- Add 1 µL Avidin-Biotin-Peroxidase Complex to 99 µL ABC Diluent Buffer, mix thoroughly. Make up to end volume of 100 µL/well (± 200µl for pipetting error).

0.01M TBS

- Add 1.2 g Tris, 8.5 g NaCl and 700 µL of concentrated hydrochloric acid to distilled water. Adjust pH to 7.2 - 7.6. Adjust volume to 1 L with distilled water.

2. Standards preparation
    1. Reconstituting the Myeloperoxidase standard with addition of 1 mL Sample Diluent Buffer, this makes standard no. 1 of concentration 10,000 pg/ml (Store at room temperature for 10 minutes).
    2. Label tubes #2 – 8, and add 300 µL Sample Diluent Buffer into tubes.
    3. Make up standard #2 by transferring 300 µL from Standard #1 to tube #2. Mix thoroughly, final concentration 5,000 pg/ml.
    4. Make up standard #3 by transferring 300 µL from Standard #2 to tube #3, mix thoroughly, 2,500 pg/ml final concentration
    5. Make up standard #4 by transferring 300 µL from Standard #3 to tube #4, mix thoroughly, 1,250 pg/ml final concentration.
    6. Make up standard #5 by transferring 300 µL from Standard #4 to tube #5, mix thoroughly, 625 pg/ml final concentration.
    7. Make up standard #6 by transferring 300 µL from Standard #5 to tube #6, mix thoroughly, 312 pg/ml final concentration.
    8. Tube #8 is the blank control and contains no protein, only 300 µl of sample diluent buffer.

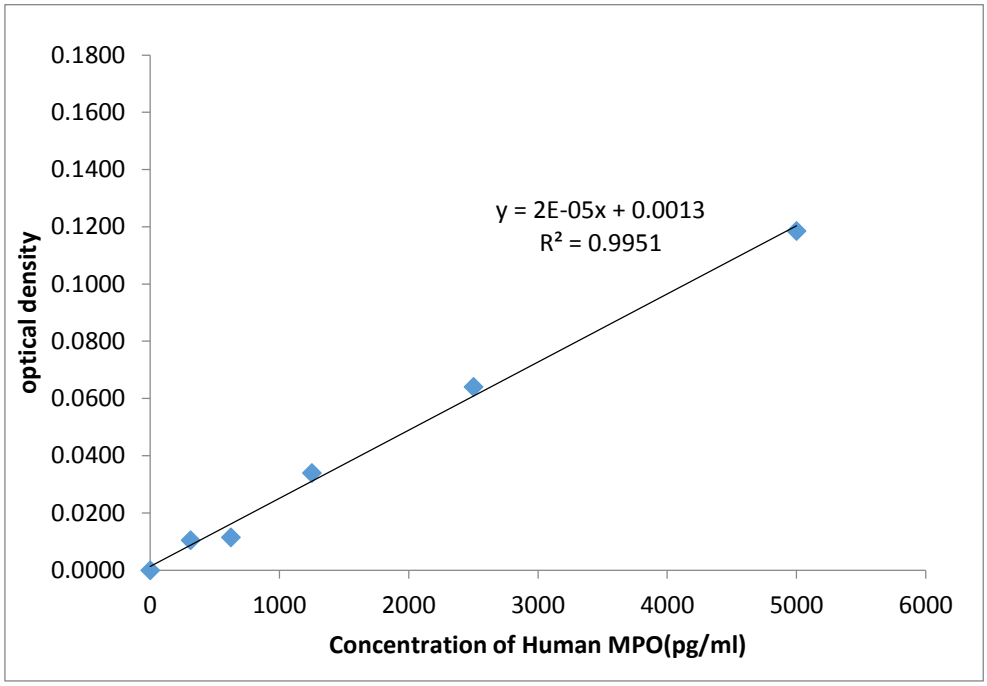
3. Sample preparation

- Plasma was collected from heparin containing Vacutaniners. Once spun down plasma was aliquoted and stored at -20°C.
- MPO concentration is human plasma ranges from 20 - 200 ng/mL. Therefore a working dilution of 1:10was used. 10 µL of plasma was aliquoted into 90 µL of the sample diluent buffer.

4. ELISA procedure

1. 100 µL of prepared standards and diluted samples to was added to appropriate wells in duplicate.
2. Plate was sealed and incubate at 37°C for 90 minutes.
3. After 90 minutes, contents of each well was discarded and plate was blotted onto paper towel.
4. 100 µL of 1X Biotinylated anti-Human Myeloperoxidase antibody was added to each well, and plate was incubated at 37°C for 60 minutes.
5. Plate was washed three times with 300 µL 0.01 M TBS, with each wash washing buffer was left in plate for 1 minute. Then washing buffer was discarded plate was blotted onto paper towels.
6. 100 µL of 1X Avidin-Biotin-Peroxidase Complex working solution was added into each well and plate was incubated at 37°C for 30 minutes.
7. Plate was washed five times with 0.01M TBs leaving the washing buffer in wells for one minute each time and whereafter washing buffer was discarded and plate was blotted onto paper towels.
8. 90 µL of prepared TMB color developing agent was added into each well and plate was incubated at 37°C wrapped in foil in the dark for 25 - 30 minutes.
9. 100µL of prepared TMB Stop Solution was added to each well (the colour should change into yellow immediately).
10. Read the optical density absorbance at 450 nm in a microplate reader within 30 minutes after adding the stop solution.

5. The standard curve from the results of the ELISA



6. Concentration calculations

Relative O.D. = (the O.D.450 of each well) – (the O.D.450 of Zero well).

Concentration = the absorbance (relative O.D.0) – slope (0, 0013) ÷ y-intercept (0, 00002)

Final MPO concentration of sample = concentration x dilution factor (10)

Appendix G – ORAC assay

This protocol was developed by Dr F Rautenbach at the Oxidative Stress Research Centre at Cape Peninsula University of Technology.

1. Regents preparation

- Phosphate (ORAC) buffer (75mM, pH 7.4)
  - Weigh 1.035 g sodium di-hydrogen orthophosphate-1-hydrate ($NaH_2PO_4.H_2O$) in a 100 mL media bottle, add 100 mL ddH_2O and mix until dissolved.
  - Weigh 1.335 g di-sodium hydrogen orthophosphate dehydrate ($Na_2HPO_4.2H_2O$ Merck Cat nr. 5822880EM) in a 100 mL media bottle, add 100 mL ddH_2O and mix until dissolved.
  - Mix 18 mL of 1st solution with 82 mL of 2nd solution.
  - Check pH and adjust with either phosphate buffer if required. Store @ 4°C. Recheck pH before each assay.

- Fluorescein sodium salt (Sigma Cat nr.: F6377):
  - Stock solution: Dissolve 0.0225 g $C_{20}H_{10}Na_2O_5$ in 50 mL Phosphate buffer. Store @ 4 °C in dark container.
  - When used in assay add 10 µl fluorescein stock solution in 2 mL ORAC buffer and then dilute 240 µl of this solution in 15 mL ORAC buffer.

- Peroxyl radical: AAPH (2, 2'-Azobis (2-methylpropionamidine) dihydrochloride (Aldrich Cat nr. 440914): 25 mg/mL.
  - Weigh 150 mg (0.150g) of AAPH (Reagent rack) into a 15 mL screw cap tube. Fresh prepared by adding solution when samples are placed in wells.
  - When used for assay add 6 mL of the ORAC buffer, mix until dissolved.

- PCA (70% Perchloric acid, SAARCHEM Cat nr.: 494612): 0.5M
  - In a 250 mL Media bottle add 195 mL distilled water and 15 mL 70% perchloric acid (store at room temperature).

- Trolox (standard): 500 µM Stock solution.
  - Weigh 0.00625 g 6-Hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid (Aldrich Cat nr.: 238831) (Reagent rack) in 50 mL screw cap tube, add 50 mL

phosphate buffer and mix until dissolved. Dilute 2x when using to make up standards.

Rooibos Tea was used as control sample

2. Sample preparation
   - Plasma - 100µl of PCA was added to 100 µl of plasma and centrifuged. Supernatant was aliquoted out into eppi's and pellet discarded. (2 x dilution). 100µl of supernatant was added to 400µl of ORAC buffer (5x dilution). The resultant dilution factor was 10.
   - Neutrophils – cell suspension was centrifuged and 20 µl of supernatant was added to 80µl of ORAC buffer (5x dilution).
3. Standard preparation

   Standards were made up according to the table below in 6 Eppendorf tubes.

Tube	Standard concentration (µM)	Trolox stock solution (µL)	Phosphate Buffer (µL)	Well number
A(Blank)	0	0	750	A1-A3
B #1	83	125	625	A4-A6
C #2	167	250	500	A7-A9
D #3	250	375	375	A10-A12
E #4	333	500	250	B1-3
F #5	417	625	125	B4-6

4. Assay procedure
   - 12 µl of blank and standards #1-5 was added to the first 18 wells (in triplicate) of a black plate.
   - 12 µl of the control (Rooibos) was added to the wells (B7-B12).
   - 12 µl of sample (in triplicate) was added to the wells (C1-H12).
   - Add 138 µl of flourescein solution (with a multichannel pipette) into each well of plate.
   - Place in plate reader and let incubate for 5 minutes
   - Add 50 µl Peroxyl radical: AAPH solution to each well.
   - Start reading on fluorescence plate reader (reads every 1 minute).

5. ORAC calculations

- The ORAC values are calculated using a regression equation ($Y = a + bX + cX^2$) between Trolox concentration (Y) (μM) and the net area under the fluorescence decay curve (X).
- Data is expressed as micromoles of Trolox equivalents (TE) per liter or per milligram of sample.
- The area under the curve (AUC) is calculated as:

  AUC = (0.5 + f2/f1 + f3/f1 + f4f1 + … + fi/f1) x CT

  Where f1 = initial fluorescence reading at cycle 1,

  fi = fluorescence reading at cycle i, and CT = cycle time in minutes.

Appendix H – FRAP assay

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 eppendorf 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 265nm.)

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 6 Eppendorf tubes.

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

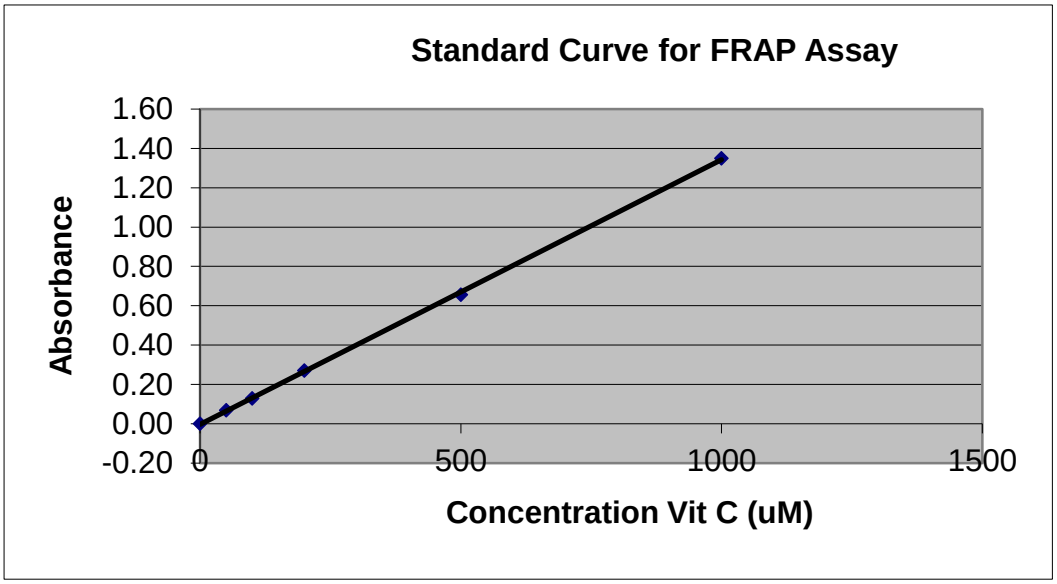
4. Assay procedure

- 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-Conjugated dienes assay

1. Sample preparation

- Plasma was aliquoted after EDTA anti-coagulated whole blood was centrifuged and stored at -80°C.
- Neutrophils were isolated (via protocol above) and stored in PBS at 80°C.

2. Reagents preparation

- Chloroform and methanol were mixed in a 2:1 ratio
- Cyclohexane was used as a blank

3. Assay procedure

- After thawing plasma (or neutrophils) samples were treated with 667ml of chloroform:methanol mixture and vortexed.
- Mixture was centrifuged at 4800 rpm for 1 minute.
- The upper layer was discarded along with protein pellet while 200µl of the lower chloroform layer was aliquoted into another eppi and left overnight at room temperature to dry out.
- The residue was dissolved in 700µl cyclohexane
- Cycloheaxne blank is added in triplicate to a clear 96-well plate.
- 200µl samples are added in triplicate to plate.
- Plate is read in multiskan at absorbance of 233nm.

4. Concentration calculation

Beer Lamberts Law

$A = \varepsilon \times C$

$C = A/\varepsilon$

With the extinction co-effienct (ε) being 29500

Appendix J – TBARS (MDA) Assay

1. Sample preparation

- Plasma was aliquoted after EDTA anti-coagulated whole blood was centrifuged and stored at -80°C.

2. Assay Procedure

- In 2 ml Eppendorf 50µl of plasma is added to 6.25µl of 4 mM of cold BHT/ethanol and 50µl 0.2M ortho-phosphoric acid
- Vortex for 10 seconds
- Add 6.25 µl TBA reagent (0.11M in 0.1M NaOH)
- Vortex for 10 seconds
- Heat in warm bath to 90°C for 45 minutes
- Then put on ice for 2 minutes
- Let stabilise at room temperature for 5 minutes
- Add 500µl n-butanol and 50µl saturated NaCL
- Vortex for 10 seconds
- Centrifuge for 2 minutes at 4°C at 4800 rpm
- Aliquot 300 µl from top butanol layer into clear 96-well plate wells (in triplicate)
- Use just butanol as blank in first three wells.
- Read on multiskan at absorbance at 535

3. Concentration calculation

Beer Lamberts Law

$A = \varepsilon \times C$

$C = A/\varepsilon$

With the extinction co-effienct (ε) being 240.98

Appendix K – ABTS (TEAC) Assay

1. Sample preparation

- Plasma was collected from whole blood (with EDTA) after centrifuging and stored at -80°C.

2. Reagents preparation (all reagents expect ABTS mix must be prepared fresh on day of assay)

- ABTS (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) Diammonium salt:7mM (Sigma Cat nr.: A1888)
    - Weigh out 0.0192 g of ABTS into a 15ml falcon tube and add 5 mL distilled water. Mix until dissolve.

- Potassium-peroxodisulphate 140mM (Merck Cat Nr.: 105091)
    - Weigh out 0.1892 g $K_2S_2O_8$ into a 15 mL falcon tube and add 5 mL distilled water. Mix until dissolve.

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

- Trolox Standard Stock 1.0mM (Aldrich Cat nr.: 238831)
    - Weigh 0.0125 g Trolox in a 50 mL falcon tube and add 50 mL of Ethanol (Saarchem Cat Nr: 2233540LP). Mix until dissolved.

3. Standards preparation

In 6 Eppendorf tubes prepare the standards and blank according to the table below.

Tube	Trolox standard (µl)	Ethanol (µl)	Trolox conc. (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	150	850	150	A10-A12
E #4	250	750	250	B1-3
F #5	500	500	500	B4-6

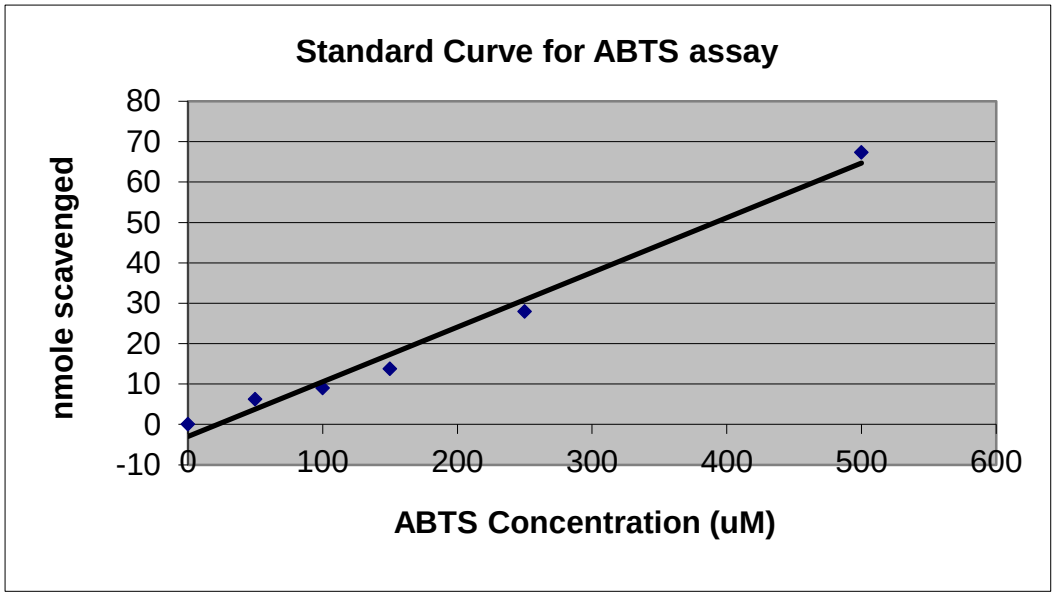
4. Assay procedure

- Add 25 µl of blank (in triplicate) into first 3 wells in 96 well clear plate.
- Add 25 µl of standards #1-5 (tubes A-E) in triplicate in the designated wells in a clear 96-well plate.
- Samples:
    - Pipette 100µl plasma into eppi and add 100 µl PCA (x2 dilution)
    - Centrifuge
    - Aliquot 25 µl supernatant (in triplicate) in clear 96 well plate
- Add 275µl of ABTS mix to each well using a multichannel pipette.
- Leave plate at room temperature for 30 minutes
- Read on multiskan plate reader 734nm

5. Concentration calculation

A standard curve is used to calculate the concentration, with data expressed as µmole Trolox equivalents per litre of sample.

An example of the standard curve used



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 L – Glutathione Redox Analysis assay

1. Sample preparation
    - 3 x 50 µl whole blood (with EDTA) is aliquoted into eppi's.
    - 3 X 100µl whole blood (with EDTA) is alquoted into eppi's and 10µl M2VP is added.
    - Samples are stored at -80°C
2. Reagents required
    - Buffer A -500mM $NaPO_4$ and 1mM EDTA of pH7.5)
    - M2VP (1-methyl-2-vinyl-pyridinium trifluoromethane sulfonate)solution of 30mM mixed with 0.1N HCl
    - NADPH(1mM, 0.83mg/ml) in buffer A
    - DTNB (5,5' Dithiobis-(2-nitrobenzoic acid) in 0.3mM(0.12mg/ml) mixed in buffer A
    - Glutathione reductase (GR) solution 0.02U/µl in buffer A. Dilute in buffer A from 0.61U/µl stock solution(Roche) to 0.2U/µl so that 50µl contains 1U. eg for 1ml use 16µl enzyme in 90.4 µl buffer A
    - Metaphosphoric acid (MPA) 5% 16.6g in 100ml
3. Standard preparation

    In 6 eppendorf tubes prepare standards and blank according to the table below.

Tube	Buffer volume (µl)	GSH (µl)
1 (Blank)	1000	0
2 #1	833	167
3 #2	667	333
4 #3	500	500
5 #4	333	667
6 #5	167	833

4. Assay procedure
    - Sample treatment

      GSH samples

      350µl MPA add to 50µl of whole blood. Centrifuge at 4800 rpm for 1 minute. Take 10 µl supernatant and add 600 µl of buffer A

      GSSG samples

      290µl MPA add to 110 µl of whole blood + M2VP. Centrifuge at 4800 rpm for 1 minute. Take 25µl of supernatant and add 350 µl buffer A
    - For GSH assay
        - Add 50µl of blank (in triplicate) to first three wells of 96 well clear plate
        - Add 50µl of standards (in triplicate) to wells (following blank)

- Add 50µl of GSH samples (in triplicate) to wells (following standards)
- With multichannel pipette add 50µl DTNB to each well
- Prepare GR by adding 80µl GR stock to 4920µl buffer A
- With multichannel pipette add 50µl GR solution
- Take to machine
- Before starting assay add 50µl NADPH
- Read absorbance at 412nm for 5 mins (taking reading at every 30 seconds)

- For GSSG assay
  - Add 50µl of blank (in triplicate) to first three wells of 96 well clear plate
  - Add 50µl of standards (in triplicate) to wells (following blank)
  - Add 50µl of GSSG samples (in triplicate) to wells (following standards)
  - With multichannel pipette add 50µl DTNB to each well
  - Prepare GR by adding 80µl GR stock to 4920µl buffer A
  - With multichannel pipette add 50µl GR solution
  - Take to machine
  - Before starting assay add 50µl NADPH
  - Read absorbance at 412nm for 5 mins (taking reading at every 30 seconds)

5. Concentration calculation

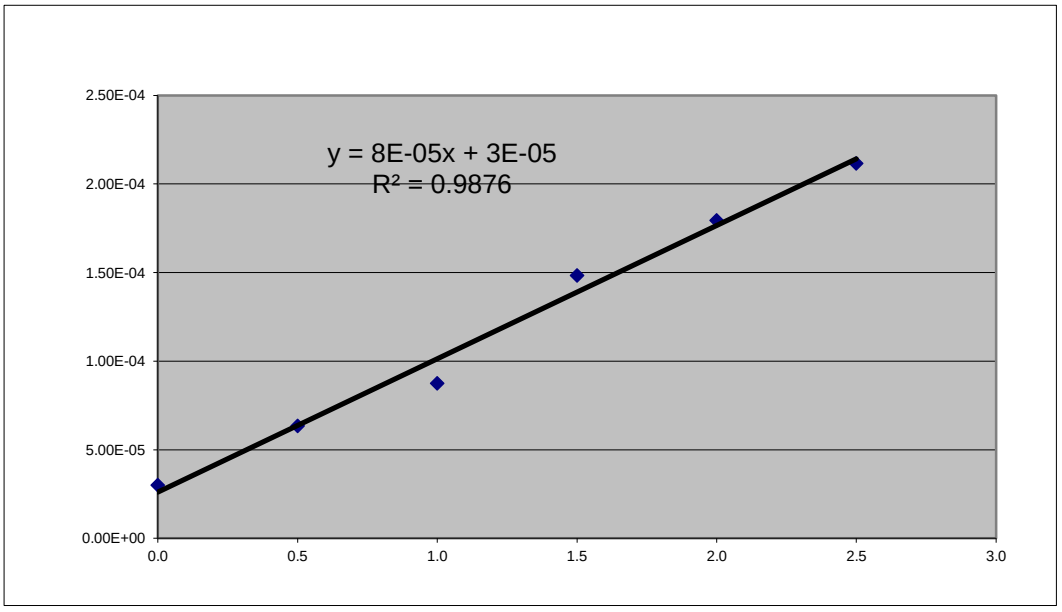
Samples are interpreted at ratio of GSH to GSSG using a standard curve to calculate GSH or GSSG concentration. Dilution factor for GSH is 488 and GSSG is 60

An example

Conc. std	Slope	Intercept	RSQ
0,0	3,01E-05	0,056	0,940
0,5	6,34E-05	0,059	0,998
1,0	8,75E-05	0,059	1,000
1,5	1,48E-04	0,064	0,999
2,0	1,79E-04	0,062	0,999
2,5	2,12E-04	0,065	1,000
Slope	0,00008		
Intercept	2,6E-05		
RSQ	0,988		

The slope of decline for each sample of over time is calculated and plugged compared to the standards slope. As example below:

	Sample 1	Sample 2	Sample 3	Sample 4
Slope	0,000	0,000	0,000	0,000
Intercept	0,062	0,065	0,064	0,062
RSQ	0,999	1,000	1,000	0,999
GSH	2,039	2,467	2,146	1,404
DF			60	60
GSHt			**129**	**84**
Sample nr			1	2



The ratio is calculated at GSH_{total} – 2GSSG/GSSG

Appendix M – Supplementary data

Neutrophil functional measures of pre- and post-exercise with *in vitro* PCO treatment and control conditions, for the young fit group.

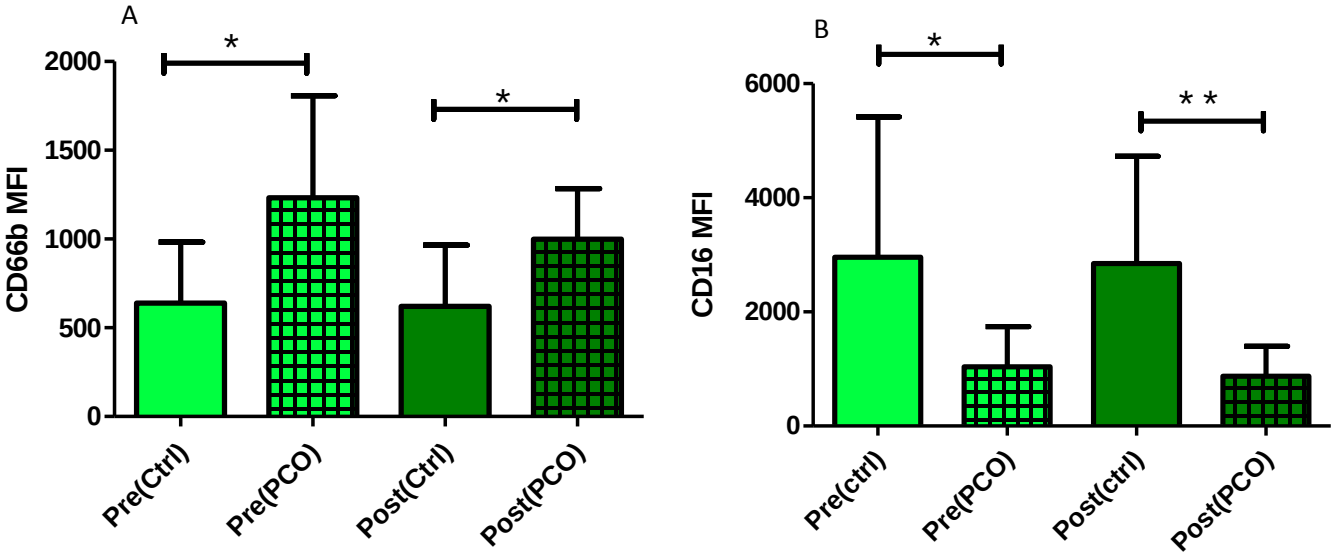


Figure M1.A & B CD66b and CD16for the young fit group pre- and post-exercise with PCO *in vitro* treatment and control neutrophils. Values are means and error bars indicate SEM. Statistical analysis: * and ** represent p<0.05 and 0.005 respectively.

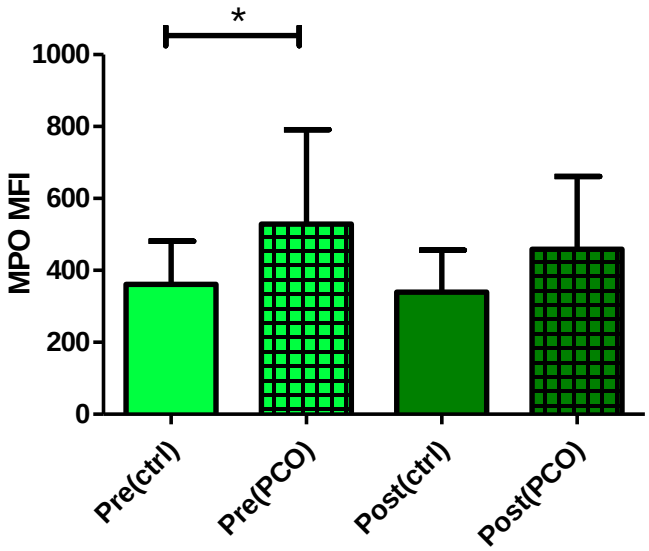


Figure M2 Intracellular MPO for the young fit group pre- and post-exercise with PCO *in vitro* treatment and control neutrophils. Values are means and error bars indicate SEM. Statistical analysis: * p<0.05.

Neutrophil chemokinesis data of young fit subjects before and after exercise intervention, with and without *in vitro* PCO treatment

	TD (μm)	LD (μm)	Ratio LD/TD
Pre-exercise(Ctrl)	188(47.96)	16.15(14.71)	0.08(0.05)
Pre-exercise(PCO)	192(37.77)	32.53(15.81)	0.16(0.06)
Post-exercise(Ctrl)	195(59.81)	25.07(16.14)	0.12(0.06)
Post-exercise(PCO)	178(40.22)	46.54(39.54)	0.27(0.22)

Table M1. Measures of total distance (TD), linear distance(LD) and the ratio of linear distance to total distance for the young fit group pre- and post-exercise with *in vitro* PCO treatment and under control conditions. Values are presented as mean(SD).

Neutrophil anti-oxidant and oxidative stress measures of pre- and post-exercise with *in vitro* PCO treatment and control conditions, for the young fit group.

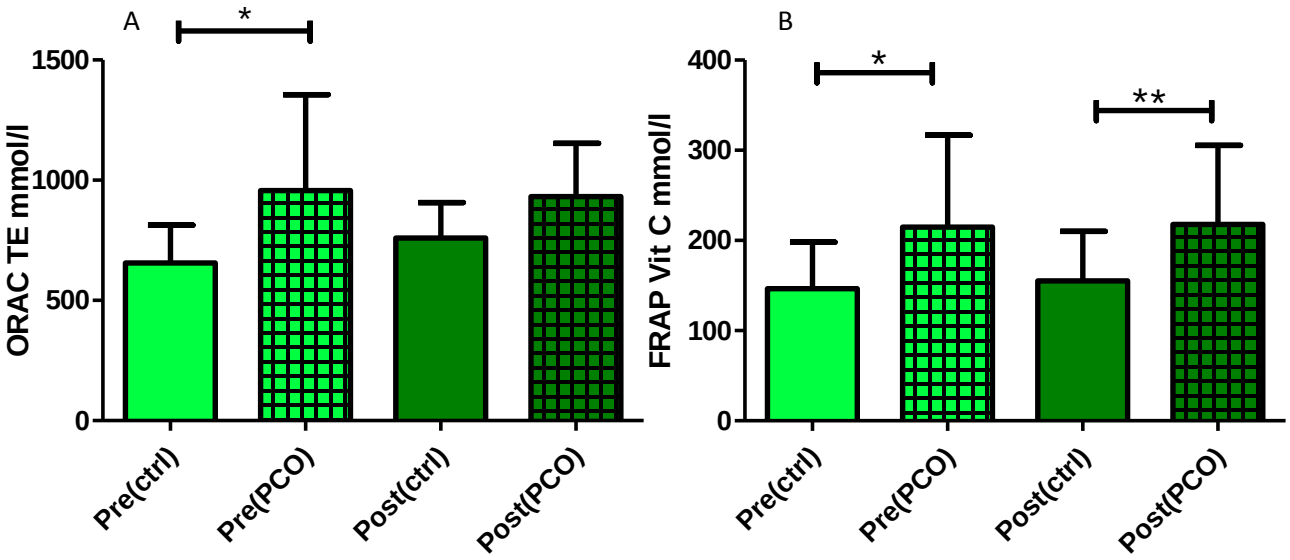


Figure M3.A & B ORAC and FRAP for the young fit group pre- and post-exercise with PCO *in vitro* treatment and control neutrophils. Values are means and error bars indicate SEM. Statistical analysis: * and ** represent $p<0.05$ and 0.005 respectively.

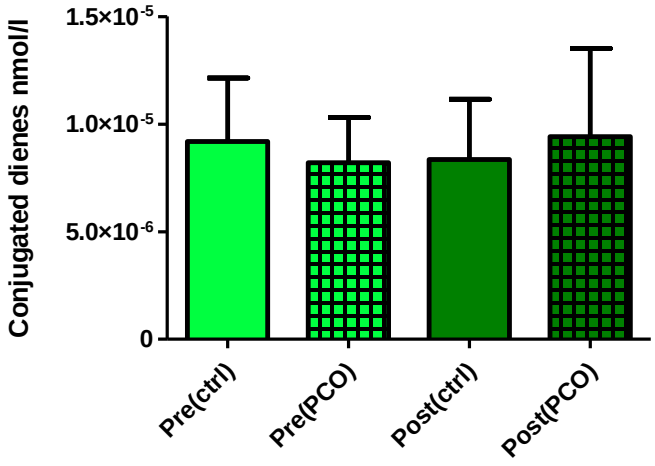


Figure M4 Neutrophil conjugated diene content for the young fit group pre- and post-exercise with PCO *in vitro* treatment and control neutrophils. Values are means and error bars indicate SEM.